User Guide

ZEN 2.5 (blue edition)

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1 About this Help

1.1 Text Conventions

The following text formats are used in this documentation:

Text format	Description
Format "**bold**"	The format "Bold" within text is used for ■ Clickable user interface elements, e.g. buttons and icons **Example**: **Save** icon ■ Hardware buttons on the microscope **Example**: **On/Off** button ■ Non-clickable user interface elements, e.g. name of a dialog **Example: Image** panel
Format "**italic**"	Mostly used for keys or keyboard shortcuts **Example:** *Ctrl* key
Text in brackets	Text to be entered by the user **Example**: Input text
Format for Links (internal or web links)	Links to further information internally or in the web. **Example**: *Link* [▶ 22]
Procedural Instruction	A numbered procedural instruction is used for actions which are performed by the user. The steps must be performed in the given order. Optionally there are pre-requisites which have to be fulfilled in advance. At the end of the instruction normally the result of the procedure is presented. **Example:** 1 Switch on the microscope and computer. 2 Start the software and log in with your user name.

Additional information is indicated as follows:

INFO

Helpful additional information, e.g. about necessary additional actions.

1.2 Safety Notes and Safety Labels

The display of safety notes in the documentation and software follows a system of risk levels, that are defined as follows:

WARNING

Risk of personal injury

WARNING indicates a potentially hazardous situation which, if not avoided, could result in major personal injury or death.

CAUTION

Risk of personal injury

CAUTION indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate personal injury.

NOTICE

Risk of property damage

NOTICE indicates a property damage message. In addition, NOTICE is used for data loss or corrupt data as well.

The safety icons / labels on the device or in the documentation refer to potential dangers or information that are defined as follows:

Icon / Label	Name	Description
	**Crushing Fingers**	This icon warns you of a potential risk of crushing fingers.

1.3 Legal Notes

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Safety

Refer to the safety notes and instructions in the documentations of all necessary devices (e.g. microscope peripherals, cameras, computers, computer accessories, etc.) before installing and using the software.

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Notice of the Producer

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The fields of application of the Software are common tasks and applications in microscopy respectively imaging (so called "Off-The-Shelf Software"). Though the user acknowledges that in any kind of use the end user of the software is responsible for the validation of the Software for the end user's dedicated intend of use considering all requirements of law and standards (e.g. FDA/21 CFR part 11, IvDD, etc.). If necessary the end user has to establish, to document, to implement and to maintain a special process to fulfill all the requirements to be conform with the validate rules of law and standards. It is pointed out that displayed measure values (e.g. length measurement) may not be used directly as analytical values for diagnostic results.

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2 Basic Concepts

2.1 Introduction

ZEN (blue edition) is a modular image acquisition, processing and analysis software for digital microscopy. The abbreviation ZEN stands for ZEISS Efficient Navigation and points out that the software can be used to control all microscopes and imaging systems by ZEISS.

In addition to basic functionality for image acquisition, elementary image processing and annotations, image analysis and reporting functions a lot of optional modules for specific microscopy tasks are available.

With ZEN lite the basic version of the software is available for free. Starting from a basic functionality for image acquisition, simple image processing, image analysis and documentation a lot of optional modules are available for ZEN lite as well. More detailed information is available in the product brochure.

2.2 Image Acquisition

The software completes all microscopes and cameras from ZEISS to efficient and tailor-made imaging systems. With little training you will interactively control the entire workflow from image acquisition, processing and analysis.

Depending on the system you can capture single images, multi-channel fluorescence images or video sequences with up to 16-bit per channel image information. The software contains the so called 'Smart Setup' which automatically delivers several proposals for the optimal dye and wavelength combinations for an experiment.

A wide range of different cameras can be used, starting from simple consumer cameras through to high-resolution and high-sensitivity microscope cameras. The seamless integration of cameras into the software allows you to acquire complex images and image sequences by one mouse click. For best results we recommend to use ZEISS Axiocam microscope cameras.

2.3 Image Processing

After acquiring an image it is immediately displayed on your screen. It can then be optimized using a wide range of techniques:

- Contrast, brightness and color adjustment
- Noise suppression, smoothing and contour enhancement
- Sharpness enhancement/emphasizing of details
- Correction of illumination influences and white balance

The software can also be used to add any annotations that you may require to the images. All elements, from scale bars and colored markings through to text and graphics, have been integrated into the program.

2.4 Image Analysis

Even with **ZEN lite** you are able to perform simple interactive measurements. The measured values (e.g. lengths, areas and perimeters) are made available in a data table, and can be processed further using spreadsheet programs.

With the optional modules **Image Analysis** and **Measurement** you can perform professional analysis tasks like generating automatic measurement procedures or measuring microscopic structures interactively.

2.5 File Format

For the ZEN software we developed a special file format called ***.czi** (Carl Zeiss Image). Besides the image data itself, the image format saves a lot of additional data, for example the date of acquisition, microscope settings, exposure values, size and scale details, contrast procedures which were used. Also all annotations and measured values are saved with the file.

To learn more about the ZEISS image file format we recommend to visit the ZEISS Microscopy Community forum in the internet (http://forums.zeiss.com/microscopy/community/forum.php). There you can join interesting discussions or download the detailed documentation of the file format.

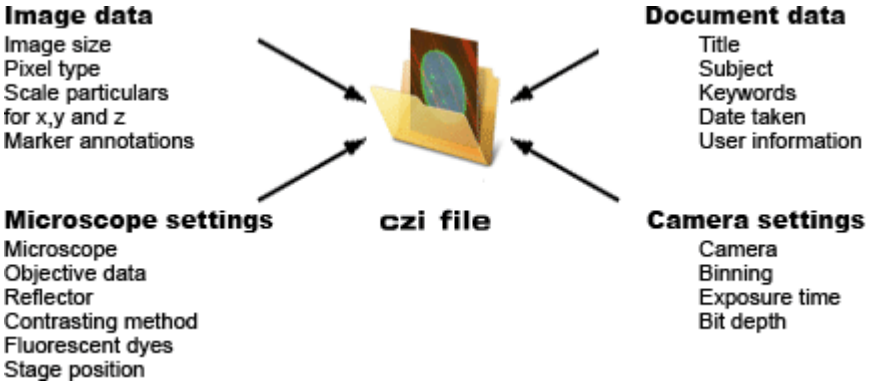


Fig. 2.1: CZI file format

2.6 Extensions

The extensions concept allows you to extend ZEN dynamically in its functionality. From a technical point of view the concept is comparable with plug-in`s or add-on`s. For the extensions we reserved a special area (*Extensions tab* [▶ 641]) within the software so that you can find all loaded extensions at a glance.

3 First Steps

3.1 Starting the Software

Prerequisites

- You have installed **ZEN (blue edition)** on your computer.

Procedure

1 Double click on the program icon on your desktop.

2 Alternatively click on **Start** | **All Programs** | **Carl Zeiss Microscopy** | **ZEN** | **ZEN (blue edition)** entry (blue icon).

The software starts. After a while you see the login screen.

3 Click on the button of the application you want to work with. The available applications depend on your licenses and system (e.g. if you work with an LSM only **ZEN system** and **Image Processing** is available). Make sure that the hardware components you use are switched on and are ready for operation.

The software starts. During the program start the hardware settings will be initialized.

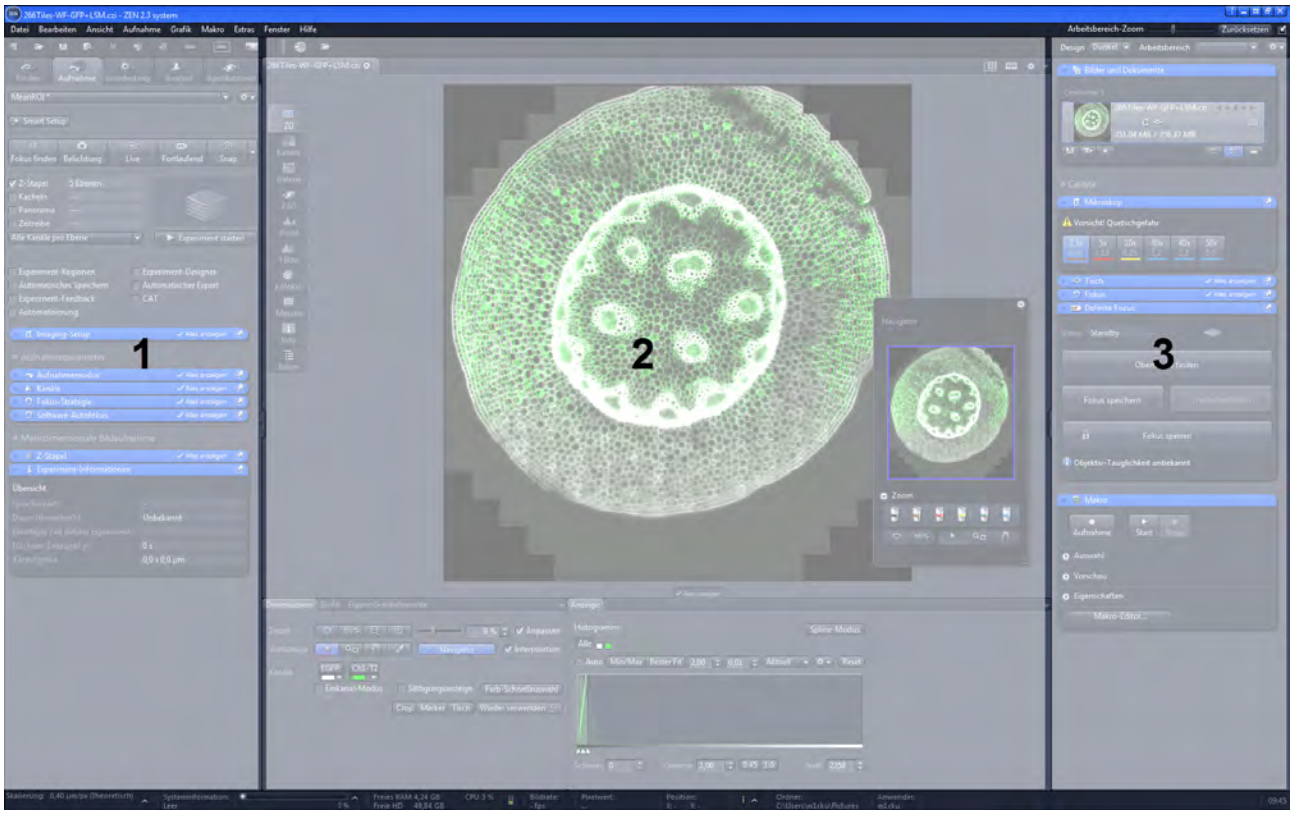
You successfully started the software.

INFO

For using pre-recorded images when starting the software, in the menu **Tools** | **Options** | **Startup**, the **Reload Last Used Documents** checkbox must be activated.

3.2 User Interface

The software user interface is divided into three main areas. Via the tabs in the **Left Tool Area (1)** you can access all the main tools for microscope control (**Locate** tab), acquisition (**Acquisition** tab), image processing (**Processing** tab) and image analysis (**Analysis** tab). The **Center Screen Area (2)** is used to display your images with several image views available. In the **Right Tool Area (3)** you find the Images and Documents Gallery, the Objective Selection and the Stage and Focus controls. Additionally system specific tools can be available here (e.g. **Definite Focus.2** controls).



3.2.1 Title bar

Parameter	Description
Help icon	Activates the "drag & drop“ help function. A question mark appears beside the mouse pointer. Move the mouse pointer to a place in the software where you need help. Left-click on the desired location. The online help opens.
Minimize	Minimizes the program window.

Parameter	Description
Maximize Over 2 Screens	Maximizes the program window across 2 screens if available. This option is only possible if you are working with 2 screens with the same resolution.
Maximize	Maximizes the program window to the main screen.
Restore Down	Reduces the program window to any selected size.
Close	Closes the program window.

3.2.2 Workspace Configuration

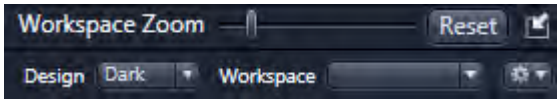


Fig. 3.1: Workspace Configuration

Here you find settings to adjust your workspace. Select **Light/Dark Design** of the user interface or enlarge the screen with **Workspace Zoom** slider. You can also save and reload all personal settings in a workspace configuration. With the **Dock all tool windows** button  in the top right corner you can easily dock all undocked tools by one click.

3.2.3 Menu bar

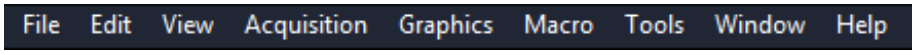


Fig. 3.2: Menu bar

The menu bar contains all the menus you need to manage, edit and view your projects, see *Menus* [▶ 586].

3.2.4 Tool bar

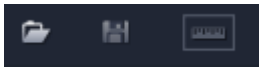


Fig. 3.3: Tool Bar

Here you gain quick access to important functions, e.g. saving or opening files. Further right you find more workspace settings, e.g. **Design** and **Workspace** selection. Read how to customize the Tool bar in chapter *Customizing Toolbar* [▶ 287].

3.2.5 Left Tool Area

Here you find the main tabs for microscope and camera settings (**Locate** tab), image acquisition (**Acquisition** tab), image processing (**Processing** tab), image analysis (**Analysis** tab) and reporting (**Reporting** tab).The main tabs are organized in an order which follows the typical workflow of experiments in bioscience or material science.

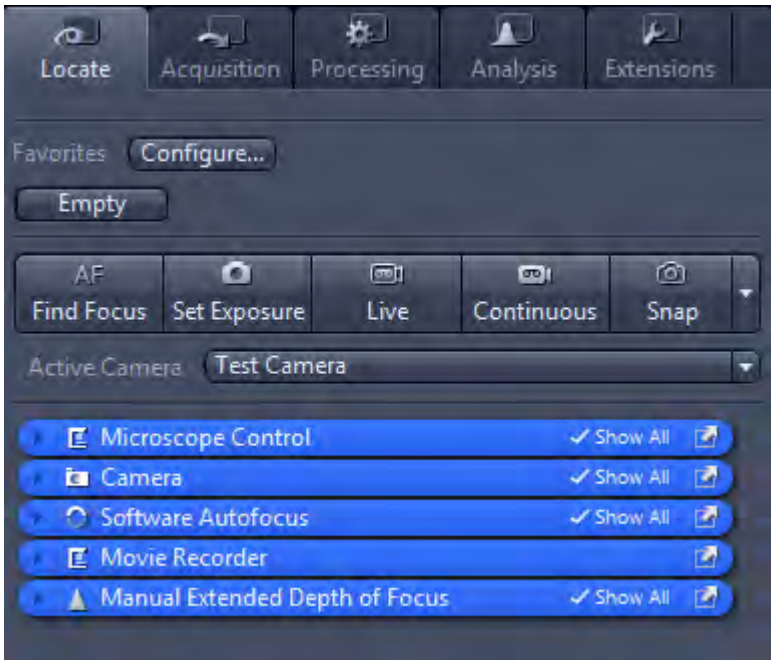


Fig. 3.4: Left Tool Area

3.2.6 Center Screen Area

The Center Screen Area is structured in 4 areas. The **Document bar (1)** is on top. Down the left side of the displayed image you find the tabs for the general and specific **Image Views (2)**. In the middle of Center Screen Area is the **Image Area (3)**, images, reports and tables were shown here. Under the image area you find the **General -** and **Specific View Options (4)** organized on tabs. View specific control tabs are flagged with a blue corner.

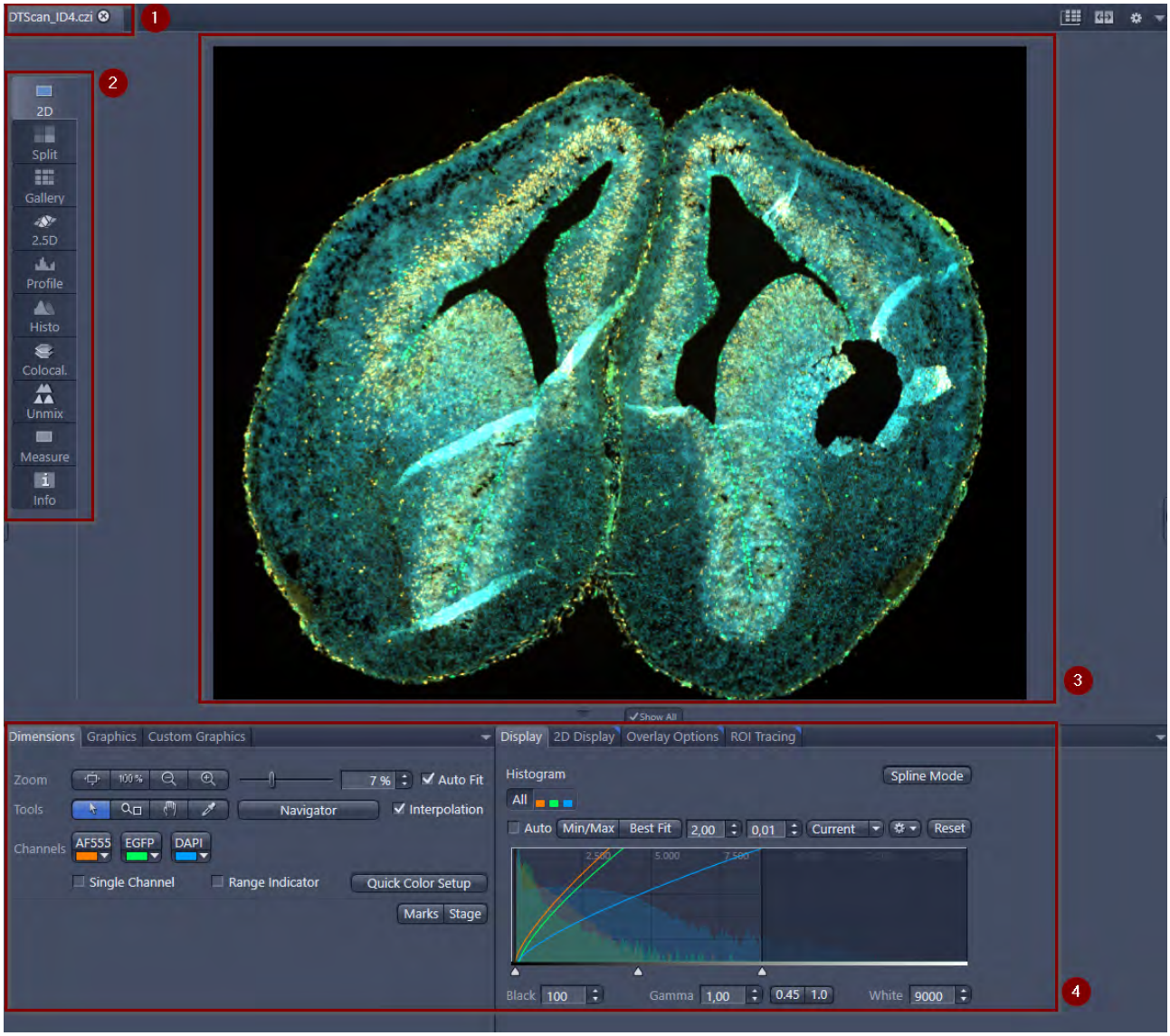


Fig. 3.5: Center Screen Area

1 Document bar, *learn more* [▶ 33].
2 Image Views (organized on tabs), *learn more* [▶ 778].
3 Image Area
4 General and specific view options, *learn more* [▶ 844].

3.2.7 Right Tool Area

Here you find the **Images and Documents** gallery, **Project/Layers**, the **Microscope**, **Stage**, **Focus** and **Definite Focus** control tools and the **Macro** tool (depending on module/license options).

3.2.8 Document bar

Fig. 3.6: Document bar

Here you see tabs of all opend documents. Click on a tab to view the image/ document. On the right end of document bar you find buttons to switch view mode from **Expose** to **Splitter** mode and further view options (**View** menu).

INFO

A **asterisk** (*) next to an image/document title indicates that unsaved changes have been made to this document. Save your pictures/documents from time to time in order to avoid data loss.

3.2.9 Status bar

Here you will see important information on the system status:

Parameter	Description
Scaling	Displays which lateral scaling is currently being used. If you click on the arrow, the *Scaling dialog* [▶ 604] will be opened. There you have access to advanced scaling settings and the scaling wizard.
System Information	Always shows the latest, currently active process that the system is performing.
Progress bar	Displays the progress of the currently active process. Each new process added supersedes older still active processes. If you click on the arrow button, a window opens with a list of all processes in chronological order. You can stop a process that is running using the **Stop** button.
Performance indicators	In this group you will see an overview of the performance of individual computer components: ■ **Free RAM** indicates how much physical memory is still available. ■ **Free HD** indicates how much space is still available on the hard drive onto which the next image is to be acquired (see Extras/Options/Save). ■ **CPU** indicates the usage of the Central Processing Unit.

Parameter	Description
	■ The small **status bar** provides an overall assessment of the system usage. **Info**: Double-clicking in the Performance Indicators area opens the Windows Task Manager.
Frame Rate	Indicates the current frame rate in frames per second (fps) used by the active camera for producing new images. Please note in most cases that at speeds greater than 100 frames per second, this value cannot always be accurately determined.
Pixel Value	Displays the gray value to the image at the current position of the mouse pointer. In the case of multichannel images the gray value/channel is displayed for up to 4 channels.
Position	Displays the X/Y position (in pixel coordinates) of the mouse pointer in the image.
Information (i)	If you click on the icon, a window opens with a *System Messages* [▶ 34].
Storage Folder	Displays the location where new images are automatically saved. This path can be changed in the menu **Tools** | **Options** | **Saving**.
User	Shows the Windows user name of the logged in user.
Time	Shows the current Windows system time.

3.2.9.1 System Messages

If you right click on a system message the **Copy** button will appear. Left click on **Copy** button to copy the message to clipboard. Then paste it into a text file or an E-Mail. The idea behind is that you can easily send error messages to your support team for example. This copy/paste function works for all upcoming system messages or error messages within the application as well.

Parameter	Description
 **Information**	System information that arises during normal operation. This system information does not lead to an interruption of the workflow. The information window is not displayed automatically.
 **Warnings**	Information that requires input from the user, e.g. a prompt to change a manual microscope component. This information leads to the information window being shown briefly. However, it closes again after a few seconds.

Parameter	Description
 **Errors**	Error messages indicate a malfunction by the system. In this case the information window opens and remains open. The system requires input from the user in order to continue.

INFO

Hundreds of messages can accumulate in the course of a session. A maximum of 300 messages are displayed. To display messages for a certain category, activate or deactivate the corresponding checkboxes.

3.3 Setting the User Language

Prerequisites

- You have successfully started the application.

Procedure

1. Click on menu **Tools** | **Options**.

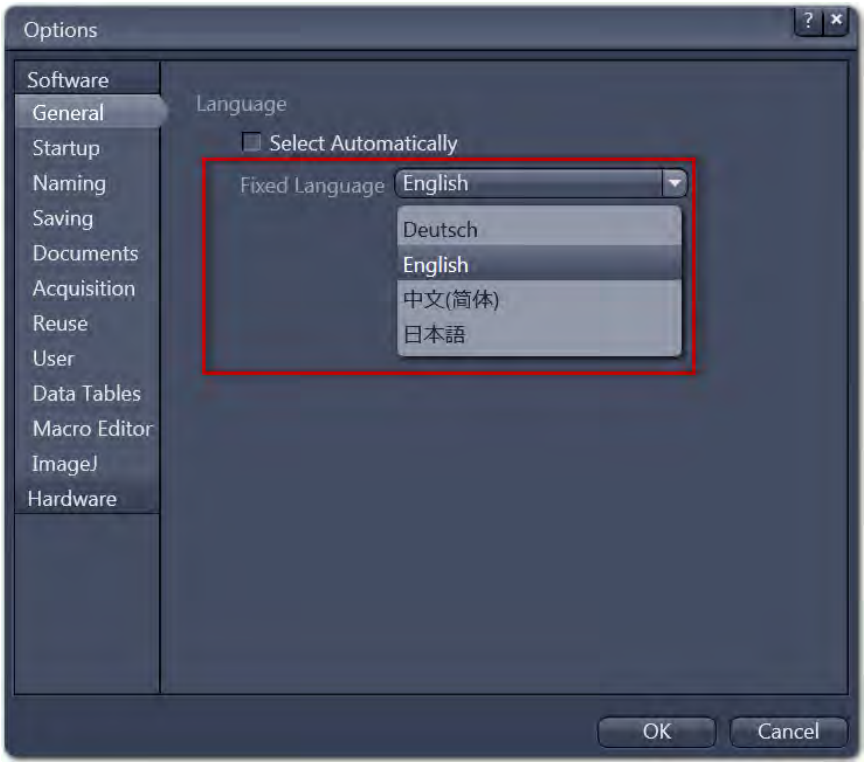
   The **Options** dialog opens. The **General** entry in the **Software** group is selected.

2. Deactivate the **Select Automatically** checkbox if you want to set the language manually.

INFO

If the **Select Automatically** checkbox is activated the software uses the language which is set in the system settings of your computer. This is the default setting.

3 Select user language from the **Fixed Language** dropdown list.



A message appears to restart the application.

Note, that the availability of additional languages can differ between software versions.

4 Click on **OK**.

The **Options** dialog closes.

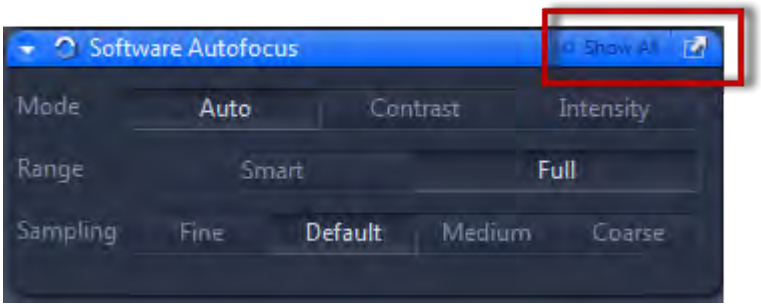
5 Exit and restart software.

You have successfully set the user language.

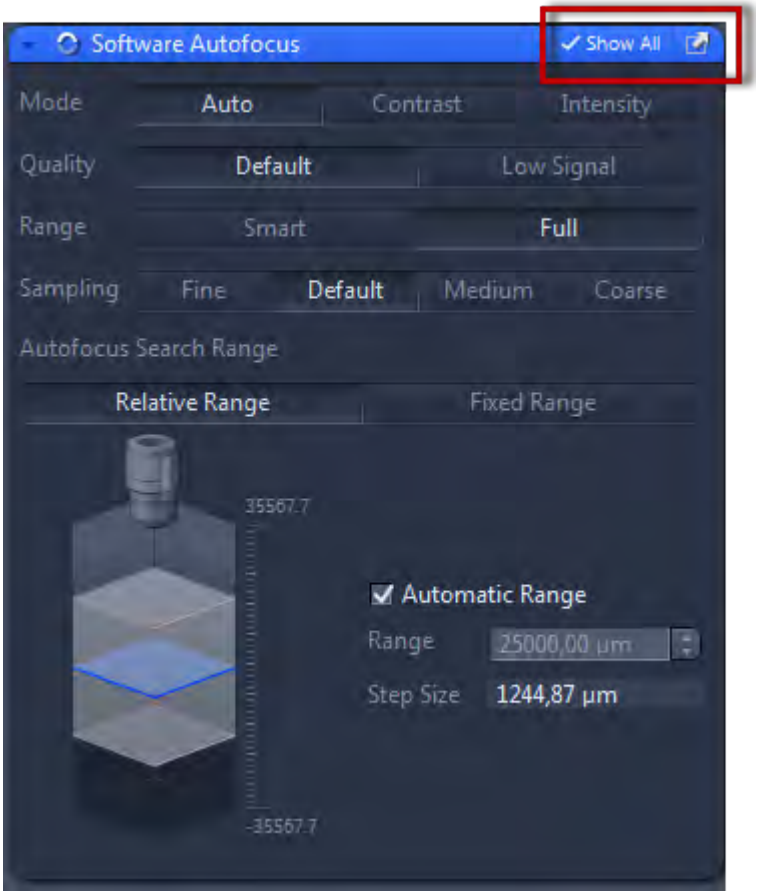
3.4 Acitvating the Show All Mode

Procedure

1 With the **Show All** mode deactivated (default setting), only the basic functions of tool windows or view options are shown.



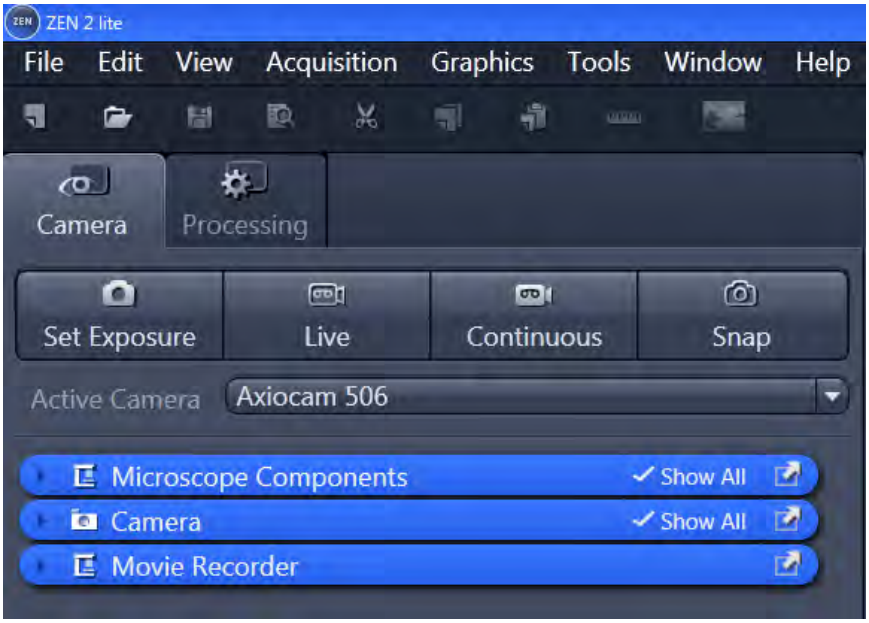
2 To show the advanced settings or expert functions of tool windows or view options, click on the **Show All** button.



3.5 Configuring Microscope Components

This chapter refers to the manual configuration of the microscope components in **ZEN lite**. All microscope components definitions will be stored in the meta data of the acquired image.

Prerequisites ■ You have selected the **Camera** tab.



Procedure **1** Click to the blue header of the **Microscope Components** tool.

The tool will open. Consider that the button **Show all** is activated.

2 Under **Objective** select that objective you will use for your acquisitions.

3 Select all other microscope components you eventually will use (i.e. Optovar, Reflector, etc.).

INFO

If you have activated the **Select automatically** button in the status bar under **Scaling** (standard settings), the scaling will be calculated on the basis of your definitions. If you want to perform a manual scaling, read the chapter *Creating a Manual Scaling* [▶ 43].

You have successfully configured your microscope components.

3.6 Acquiring a First Camera Image

This topic guides you through acquiring your first camera image with **ZEN (blue edition)** software.

Prerequisites

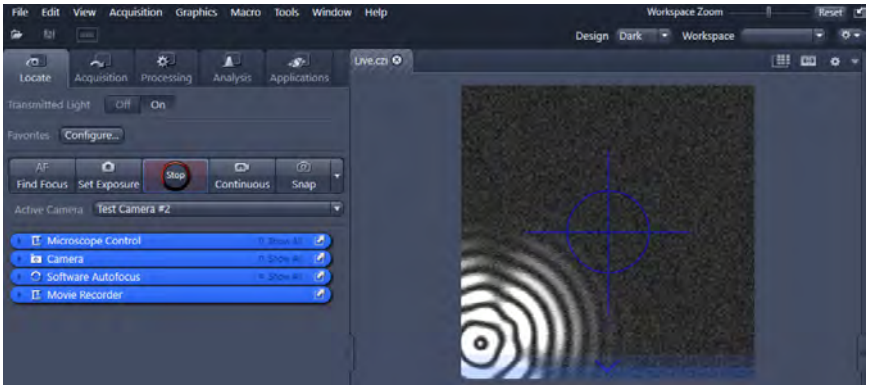
- You have connected and configured a microscope camera (i.e. AxioCam MR) to your system.
- You have started the software.

- You have configured the microscope components (e.g. objective, camera adapter) und you are using the automatic or manual scaling.
- You are on the **Camera** (ZEN light only) or **Locate** tab.
- You see your microscope camera available in the **Active Camera** section. If not, select the camera from the list.

Procedure

1. Position your sample on the microscope and adjust the microscope to see a focused image through the eyepieces.
2. Adjust the tube slider of the microscope to divert the image to the camera (e.g. **50% camera** and **50% eyepieces**).
3. Click on **Live** button.

   The **Live Mode** will be activated. In the **Center Screen Area** you will see the camera live image. By default the live image shows a cross hair helping to navigate on the specimen. In the chapter *Adjusting Live Image Settings* [▶ 42] you will learn how to optimize live image display.



4. Click on **Set Exposure** button.

   The exposure time will be automatically determined and set.

INFO

If you do not see a focused image please refocus the specimen on the microscope. You may activate the focus bar as an additional aid. Right click in the **Center Screen Area** to open the context menu. Select the entry **Focus Bar**. The focus bar will be shown.

5 Click on **Snap** button.

You successfully acquired your first image. Save the image in the file system via the **File** menu | **Save as**.

3.7 Adding Annotations to an Image

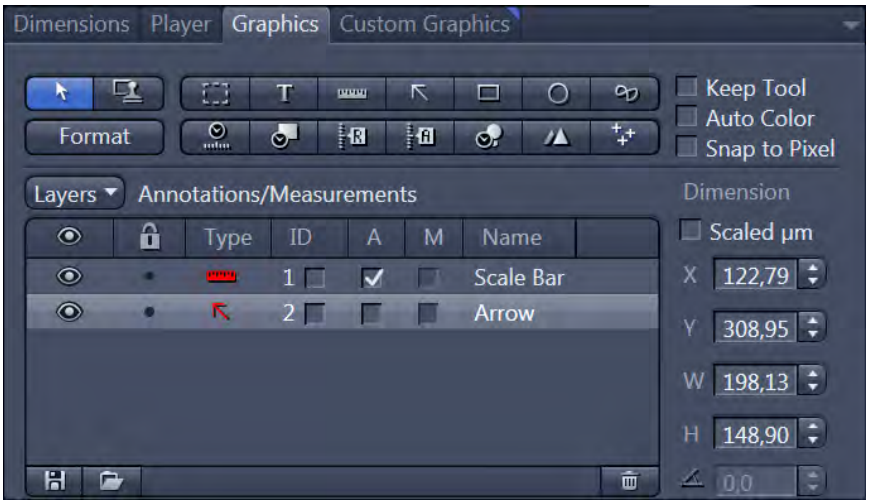
Annotations are the generic term for all the graphics (e.g. rectangle, arrow, scale), measurements, texts or other metadata (e.g. recording time) that you can add to your image.

Prerequisites

- You have acquired or loaded an image.

Procedure

1 In the **Center Screen Area** select the **Graphics** tab.



2 Click on the  **Scale Bar** button.

The scale bar will appear directly in the image.

INFO

Right click on any requested annotation in the image to edit this annotation (e.g. color, line width). This will open the context menu. Select the entry **Format Graphical Element**. In this dialog you have numerous formatting possibilities.

3 Click on the  **Draw Arrow** button.

The button will turn into blue to indicate its activation. Now you may draw an arrow into your image.

You added the annotations **Scale Bar** and **Arrow** from the toolbar to your image.

3.8 Adjusting Live Image Settings

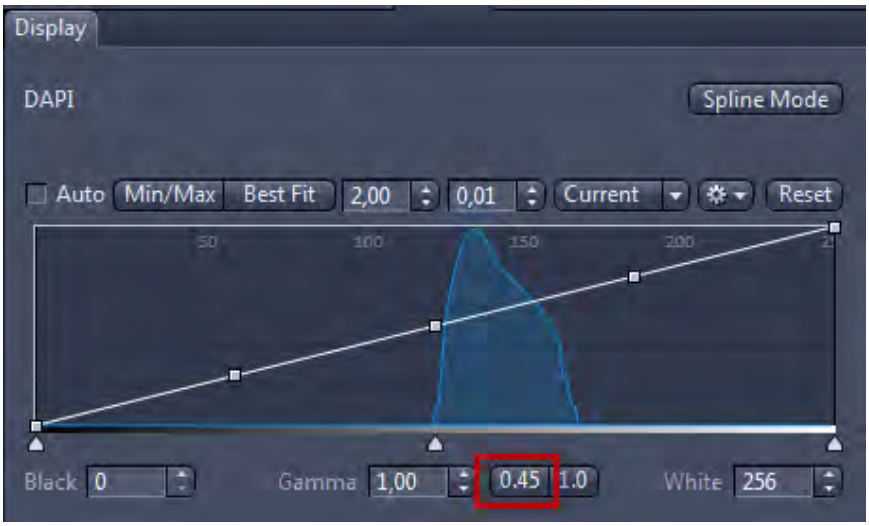
Prerequisites

- You have started the **Live** mode via the **Live** button and see the camera's live image in the **Center Screen Area**.
- Under the image area you see the general view options on **Dimensions** tab, **Graphics** tab and **Display** tab.

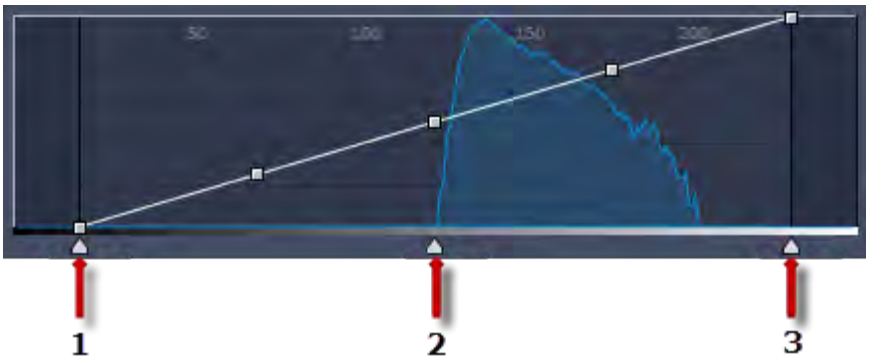
Procedure

1 In the **Dimensions** tab activate the **Range Indicator** checkbox. This will mark overexposed (too bright) areas in the live image in red and underexposed (too dark) areas in blue.

2 On the **Display** tab click the **0.45** button. The display curve will be adapted to a gamma value of 0.45. This will set the optimum color presentation. If you do not see this button, activate the **Show all** mode.



3 Move the controls under the display curve left and right in order to directly adjust the values for **Brightness** (**White**), **Gamma**, and **Contrast** (**Black**) in the live image.



1 Contrast (black point) control

2 Gamma control

3 Brightness (white point) control

INFO

With the settings above the display of the live image will be adapted. These settings will also be transferred to your acquired image. This will not change the camera settings.

3.9 Creating a Manual Scaling

Prerequisites

- You have an object micrometer oriented horizontally on the microscope stage.
- You have selected all definitions for your microscope correctly in the **Microscope Components** tool (ZEN lite only). In our example we use an objective with a 10x magnification.

Procedure

1 Acquire an image (see *Acquiring a First Camera Image* [▶ 39]) of the scale in your object micrometer using the objective to be scaled manually.

2 In the bottom status bar click on the arrow in the **Scaling** area. In the **Scaling** dialog deactivate the **Select Automatically** checkbox.

3 In the **Create new scaling** section, click on the **Interactive Calibration...** button.

The calibration wizard will appear in the image area.

4 Click on single **Reference Line** button (selected as default) and activate the **Automatic Line Detection** button (activated as default).

INFO

The function **Automatic Line Detection** calculates the theoretical maximum of the reference line's both end points to the closest scale lines in the image. Thus the distance will be calculated with sub-pixel accuracy.

5 Draw in the reference line along the scale.

6 Enter the true distance between both scale lines in the calibration wizard. In our example this is 500 micrometer.

7 Enter a name for the scaling (i.e. Obj 10x) and click the **Save Scaling** button.

You performed a manual scaling for your objective. Repeat this sequence for all objectives you will need a manual scaling for. Always ensure that you did select the correct objective in the tool **Microscope Components** and for this performed and selected the matching scaling in the status bar.

INFO

If you defined manual scalings for your available objectives, and you click in the status bar in the **Scaling** area to open the **Scaling** dialog and to activate the checkbox **Select Automatically** again, the system will use the measured scalings instead of the theoretic ones. You will recognize this via the label "**Measured**" instead of "**Theoretic**" beside the pixel size.

3.10 Closing the Software

Procedure

1 Click on **File** | **Exit**. Alternatively you can use the short cut *ALT+F4* or click on the **Close** icon in the program bar.

INFO

If you haven't saved your files the **Save Documents** dialog will open before the program closes. Select files you want to save or unselect files you don't want to save.

4 Image Acquisition

4.1 Acquiring Multi-Channel Images

In the following chapters you will learn how to set-up and run multi-channel experiments quick and easy.

i INFO

Make sure that you work with a fully motorized microscope system. In advance all microscope components (e.g. objectives, filters, etc.) must be configured correctly in the MicroToolBox (MTB) software.

In principle there are two variants for setting up multi-channel experiments. The first variant uses **Smart Setup**, while the second variant uses the **Channels** tool. Both variants have similarities and differences, which are presented in the following overview:

Commonalities

- Fluorescent dyes and transmitted light techniques can be selected from a database.
- Hardware settings for motorized microscopes, which take the properties of the selected dye and the available microscope hardware into account, can be created automatically.
- Bases for experiments can be created using both variants and experienced users can optimize settings further.

Differences

Smart Setup	Channels tool
A maximum of 4 camera channels, 8 confocal channels and 1 transmitted light channel are available	No restriction on the number and type of channels
Offers up to 3 proposals of variants of the experiment (depending on the selected combination of dyes and available hardware)	-
Offers more optimization of experiment settings by using the Motif buttons	-

Smart Setup	Channels tool
Graphic overview of the expected signal strength for the selected dyes	-
Graphic overview of the expected spectral crosstalk with the selected dye combinations	-
Display of the excitation and emission spectra of the selected dyes	-
-	Channels can be configured for dyes that are not supported (or not supported sufficiently well) by the available hardware

4.1.1 Set up a new experiment

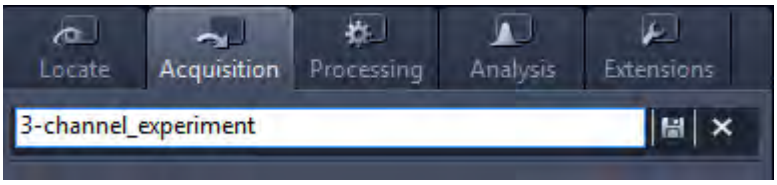
Prerequisites

- You have switched on and configured your microscope system and all components.
- You have successfully started the software.

Procedure

1. In the **Left Tool Area** click on the **Acquisition** tab.
2. In the **Experiment Manager** click on the **Options** button.

   The **Options** dropdown list opens.
3. To create a new, "empty" experiment, click on the **New** entry.
4. Enter a name for the experiment, e.g. "3-channel_NEW".



5. To create the experiment, click on the **Save** button.

You have created a new, blank experiment. All other settings are now stored in this experiment. If you make changes to the experiment, an asterisk (*) after the file name appears. This means that the experiment was modified and not saved. Save your experiments from time to time to ensure that your settings are not lost.

4.1.2 Variant 1: Smart Setup

Procedure

1. Click on the **Smart Setup** button on the **Acquisition** tab.

   The **Smart Setup** dialog opens.
2. Select the **WF** button on top of the dialog.

3 To add a channel, click on the  **Add** button in the **Configure your Experiment** section.

The **Add Dye or Contrasting Method** dialog opens.

4 Select the desired dye or contrast method.

5 Click on the **Add** button. Alternatively you can double-click on the entry in the dye database. The dye is then adopted directly into the experiment.

You have added a channel to your experiment. To add further channels, repeat the last 2 steps.

INFO

If you see the error message "**Smart Setup calculation failed**", it was not possible for Smart Setup to calculate any proposal. This may be because the filters and light sources available on the system do not allow an image of the dye to be acquired with a good signal strength or with little crosstalk. The channel for this dye or the contrast method cannot therefore be created. In this case, try selecting another, similar dye.

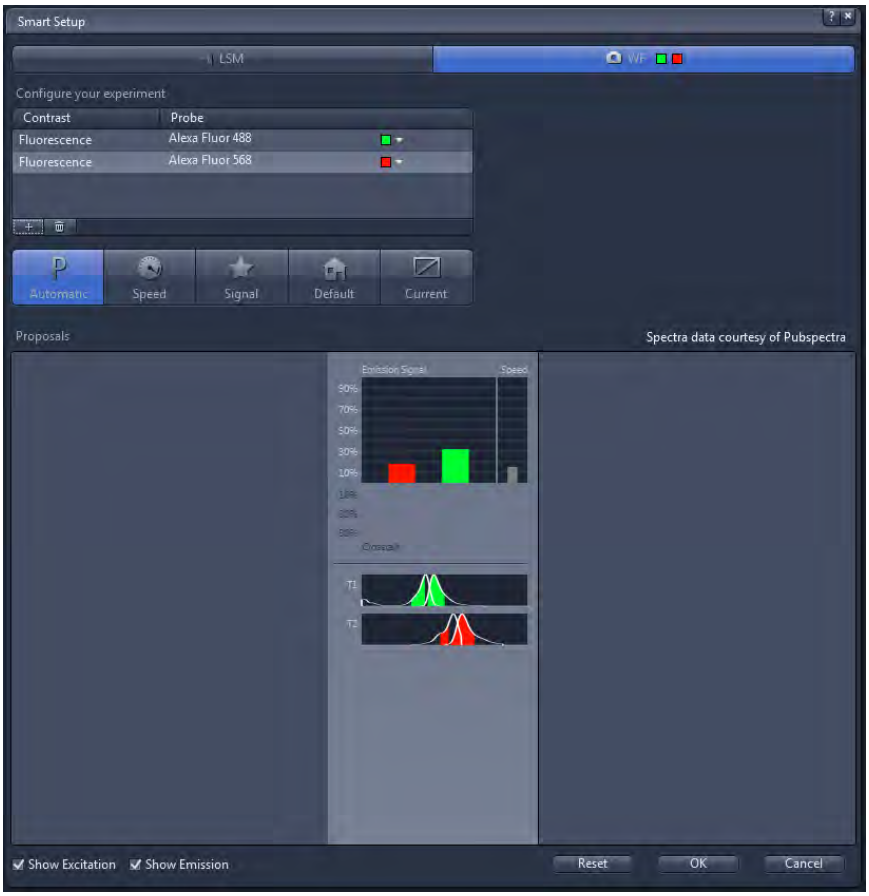
Should the error message be displayed for all dyes that you select, this may be due to one of the following causes:

- no light source has been configured or the light source is switched off

- no camera has been configured on the system, the camera is not connected or (on some models) has been switched off.

6 To return to **Smart Setup**, click on the **Close** button.

You will now see a graphic overview in the **Proposals** section. This displays the spectra of the dyes, the expected signal strengths per dye and the spectral crosstalk schematically.



INFO

Depending on which dye you have selected and the microscope hardware available, up to three different proposals (Best Signal, Fastest, Best Compromise) are displayed. These differ in terms of signal strength, crosstalk and speed. Select the proposal that best meets the needs of your experiment.

7 To select a proposal (if there's more than one), for all active configured channels, activate the proposal.

8 To optimize experiment settings additionally, click on a **Motif** button. The **Automatic** button is set as default.

INFO

By the **Motif** buttons you can optimize acquisition parameters and camera settings automatically either for a high quality (**Quality** button) image or a faster acquisition but reduced image quality (**Speed** button). Find a more detailed description of **Motif** buttons in Smart Setup dialog.

9 To optimize experiment settings, adopt the suggestion and leave **Smart Setup**, click on the **OK** button.

The added channels are adopted automatically into the **Channels** tool.

10 Click on the **Set Exposure** button in the **Action buttons** bar on top of the **Acquisition** tab.

The exposure time is now measured for all three channels one after the other. This is adopted into the settings for the channels. Following the measurement of the exposure time, the multi-channel image is acquired automatically and displayed in the **Center Screen Area**.

11 To save the experiment together with all the settings, click in the **Experiment Manager** on the **Options** button .

12 In the **Experiment Manager** click on the **Save** entry in the dropdown list.

You have set up the multichannel experiment using **Smart Setup**, executed it and then saved the configuration. This means that you can repeat the experiment as often as you like using the same settings.

4.1.3 Variant 2: Channels Tool

Procedure

1 Open the **Channels** tool in the **Acquisition Parameter** group.

2 From the drop down list, select the channel.

The **Add Dye or Contrast Method** dialog opens.

3 Select the desired dye or contrast method. You can search for a dye by entering its name (or starting letter) in the **Search** input field.

4 Click on the **Add** button at the bottom of the dialog or simply double click on an entry.

The channel will be added to your experiment. To add more channels, repeat the last 2 steps.

5 Click on the **Close** button.

You will see the added channels in the **Channels** tool.

6 Click on the **Set Exposure** button in the main buttons bar on top of the **Acquisition** tab.

The exposure time is now measured for all active configured channels one after the other. It will be also adopted into the settings for the channels. To set the exposure time channel specific, use the **Set Exposure** button in the channel settings of the channels tool.

7 To save the experiment together with all the settings, click in the **Experiment Manager** on the **Options** button.

8 Click on the **Save** entry in the dropdown list.

You have set up the multichannel experiment using the **Channels** tool, executed it and then saved the configuration. This means that you can repeat the experiment as often as you like using the same settings.

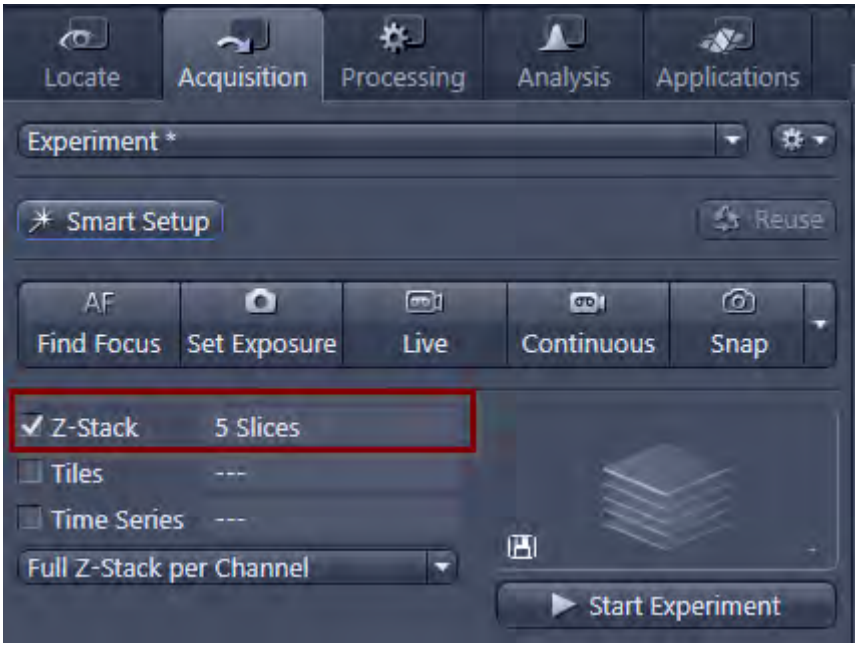
4.2 Acquiring Z-Stack Images

Prerequisites

- You have licensed **ZEN celldiscoverer**, **ZEN slidescan**, **ZEN system**, or **ZEN pro** and additionally licensed the **Z-Stack** module.
- You have switched on and configured your microscope system inclusive all components.
- You have *set up a new experiment* [▶ 46], at least defined one channel and adjusted focus and exposure time correctly.
- You are on the **Acquisition** tab.

Procedure

1 In the **Acquisition dimensions** section activate the **Z-Stack** checkbox.



The **Z-Stack tool** appears in the **Multidimensional Acquisition** tool group.

2 Simply click on the blue bar to open the tool.

You have successfully completed the general preparations. You can now set up Z-Stack experiments automatically or manually.

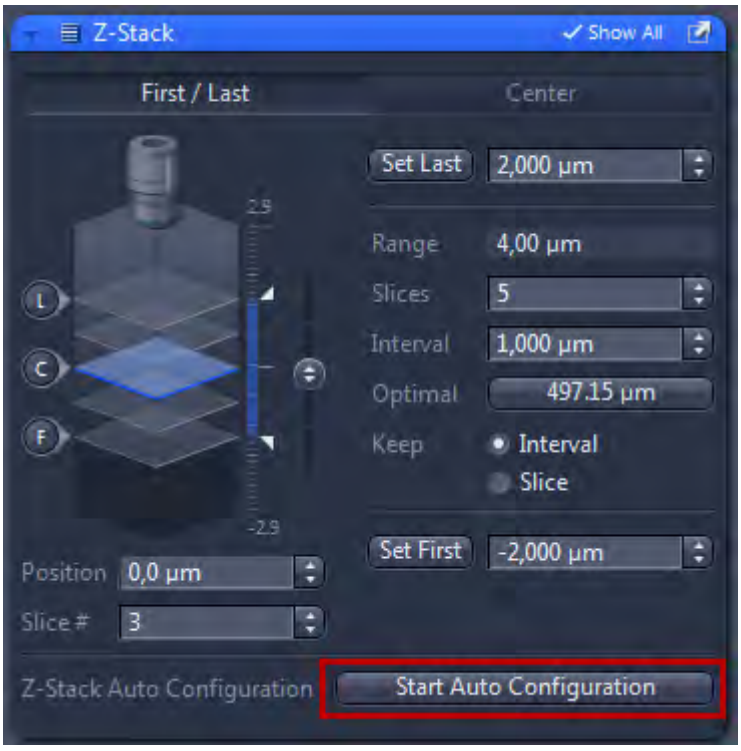
4.2.1 Configuring a Z-Stack automatically

INFO

Note that the automatic Z-Stack configuration will only work, if no LSM tracks are added in the **Channels** tool.

Procedure

1. Make sure that you have placed a sample in the visual field of the camera and that the sample is roughly in focus. Set the exposure time of the camera fair enough for receiving a good signal.
2. Click on the **Start Auto Configuration** button.



Confirm the system message by clicking on **OK**. The automatic configuration will be started.

INFO

The auto configuration can last for a few seconds up to half a minute depending on the acquisition settings. You can check the configuration status on **Progress bar** in the **Status bar**.

The auto configuration sets the focus position for the first, last and center slice of the Z-Stack, the number of slices and the interval automatically. The Z-Stack experiment is set up successfully now.

3 Click on the **Start Experiment** button to start the experiment.

You have successfully set up and performed an Z-Stack experiment.

INFO

You can change the area of the sample (Z direction in %) covered by the Z-Stack auto configuration under **Tools** | **Options** | **Acquisition** | **Z-Stack** section. Smaller values will enlarge the Z-Stack. Bigger values will make the Z-Stacks smaller.

4.2.2 Configuring a Z-Stack manually (First/Last Mode)

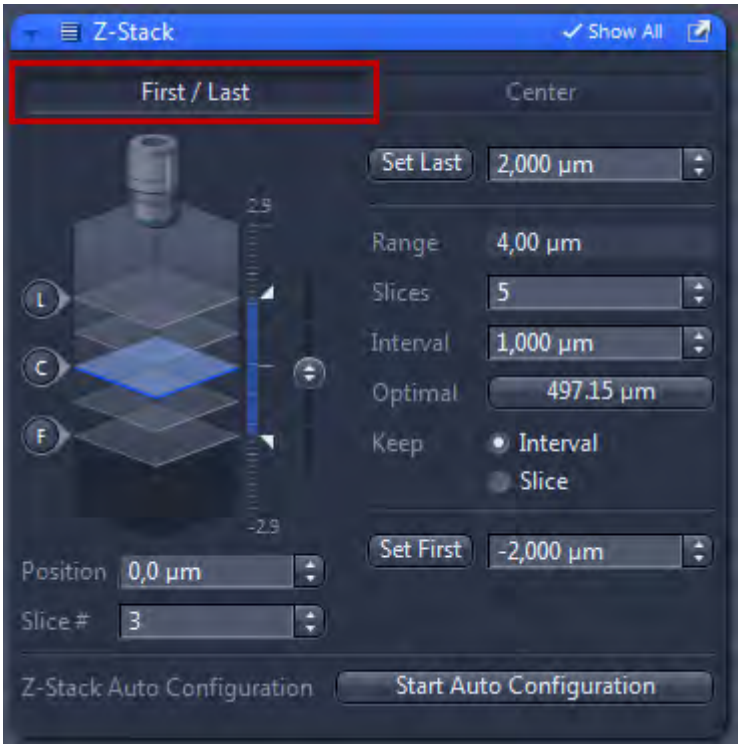
Using this mode you set the first and the last plane of the Z-Stack. This mode is suitable if you don't know the thickness of your sample exactly.

Prerequisites

- You are in the **Z-Stack tool**.

Procedure

1 Activate the **First /Last** mode by clicking on the **First /Last** button. This button is selected by default.



2 In the **Live** mode adjust the Z-drive until you have reached the upper plane of the Z-Stack. The blue plane in the illustration shows the actual focus plane.

3 Click on **Set First** button to set the adjusted Z-Position as first position of the Z-Stack.

4 In the **Live** mode adjust the Z-drive until you have reached the lower plane of the Z-Stack.

5 Click on **Set Last** button to set the adjusted Z-Position as last position of the Z-Stack.

You have set the upper and lower boundaries of the Z-Stack.

6 Click on the **Optimal** button. This will adjust the number of slices and the best interval according to the Nyquist criteria. Alternatively you can set the desired interval and number of slices in the input fields manually.

INFO

Depending on which option is selected in the **Keep** section either the **Interval** or the **Slices** will be held constant.

7 Click on **Start Experiment** button to start the experiment.

You have successfully set up and performed an Z-Stack experiment using **First/Last** mode.

4.2.3 Configuring a Z-Stack manually (Center Mode)

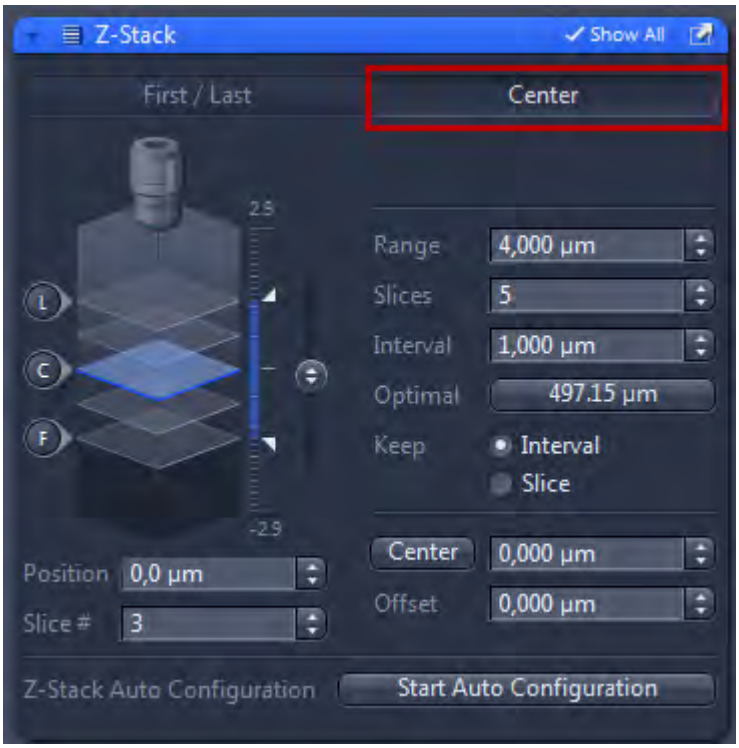
Using this mode you set the center plane of the Z-Stack. This mode is suitable if you know the thickness of your sample. It will be the fastest method to set up a Z-Stack in this case.

Prerequisites

- You are in the **Z-Stack tool**.

Procedure

1 Activate the **Center** mode by clicking on the **Center** button on top of the tool.



2 In the **Live** mode adjust the Z-drive until you have focused the center of the sample exactly. The blue plane in the illustration shows the actual focus plane.

3 Click on the **Center** button under the settings section to set the actual focus position as center of the Z-Stack.

4 Click on the **Optimal** button. This will adjust the number of slices and the best interval according to the Nyquist criteria. Alternatively you can set the desired interval and number of slices in the input fields manually.

INFO

Depending on which option is selected in the **Keep** section either the **Interval** or the **Slices** will be held constant.

5 Click on **Start Experiment** button to start the experiment.

6 You have successfully set up and performed a Z-Stack experiment using the **Center** mode.

4.3 Acquiring Time Series Images

Prerequisites

- To set up **Time Series** experiments you need to license the **Time Series** module.
- You have set up a *new experiment* [▶ 46], at least *defined one channel* [▶ 45] and adjusted focus and exposure time correctly.
- You are on **Acquisition** tab.

Procedure

1 Activate the **Time Series** tool by activating the **Time Series** checkbox in the **Acquisition Dimensions** section.

The **Time Series** tool appears in the **Left Tool Area**.

2 Open the **Time Series** tool.

3 Set length of your time series by the **Duration** slider. You are able to select an interval (days, hours, minutes, seconds, milliseconds) or the cycles (1-n) e.g. 10 cycles.

4 Set interval of your time series by the **Interval** slider, e.g. 5 s.

5 Click on **Start Experiment** button.

The time series experiment will be started. You`ve successfully learned the basics of how to set up time series experiments. In our example in 10 cycles after each 5 seconds an image is acquired. The Time Series image also contains 10 single images.

INFO

You can display the individual images via the **Time** slider on the **Dimensions** tab.

4.4 Acquiring Confocal Images

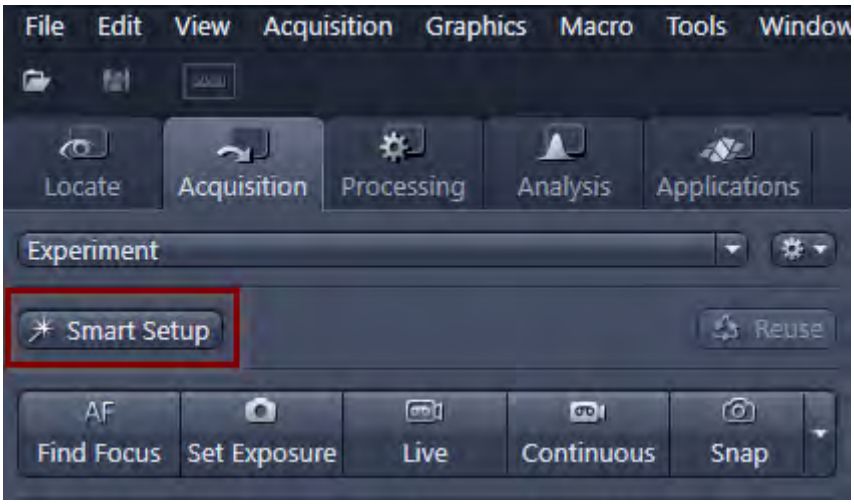
To acquire confocal images you first have to setup the acquisition parameters and configure your experiment. Therefore we recommend to use **Smart Setup** as this will automatically give you certain suggestions for the experiment configuration, e.g. Airyscan acquisition or camera based acquisition. In the following guide you will learn how to use Smart Setup and acquire a first confocal image. Because there`s a huge variety of samples (and suitable experiment configurations) this guide shows the necessary basics only.

Prerequisites

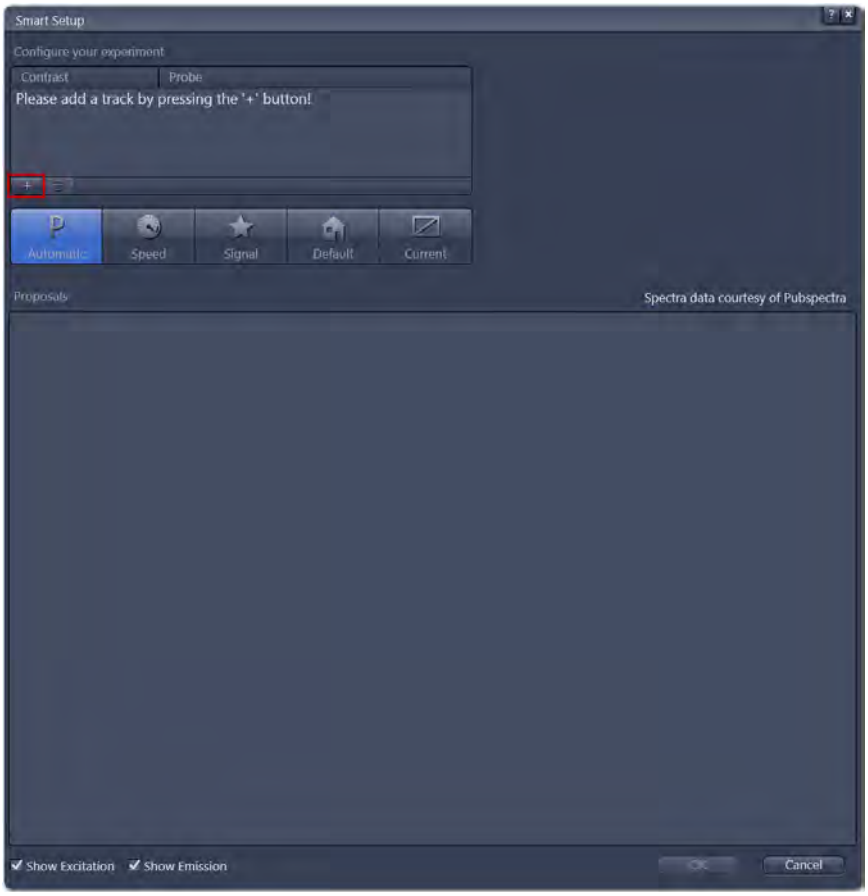
- You are on the **Acquisition** tab.

Procedure

1. Click on **Smart Setup**.

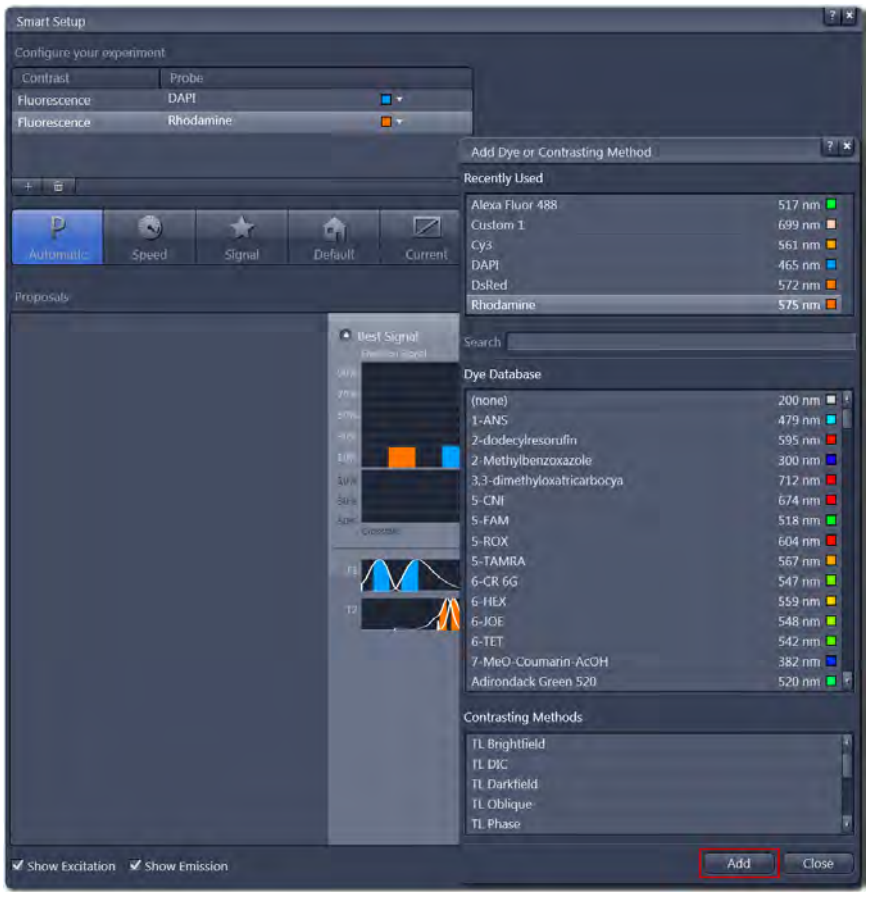


2 Click on **Add**.



The **Add Dye** dialog opens.

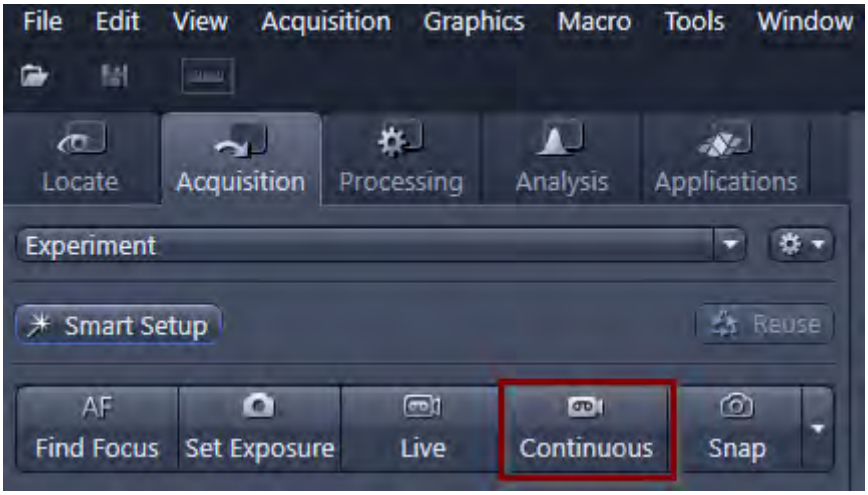
3 Select the dyes used in your experiment. Double click on a dye entry to add it to the list.



4 Click on **Ok** to close Smart Setup.

5 Make sure that the cover glass of you sample points towards the objective. Use the appropriate immersion medium for the objective.

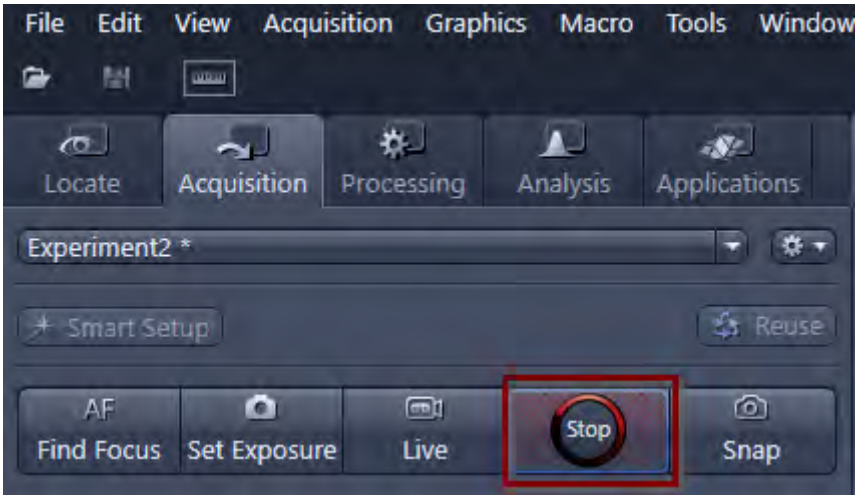
6 Click on **Continuous** to start a continuous scan.



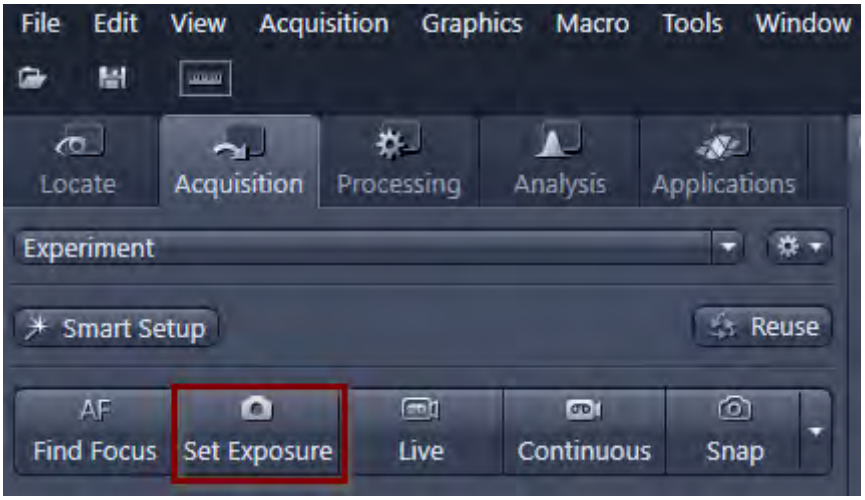
You will see the image of your sample in the center screen area.

7 Search the desired sample area and focus with the joystick. Alternatively use the mouse wheel while pressing the *Ctrl* key.

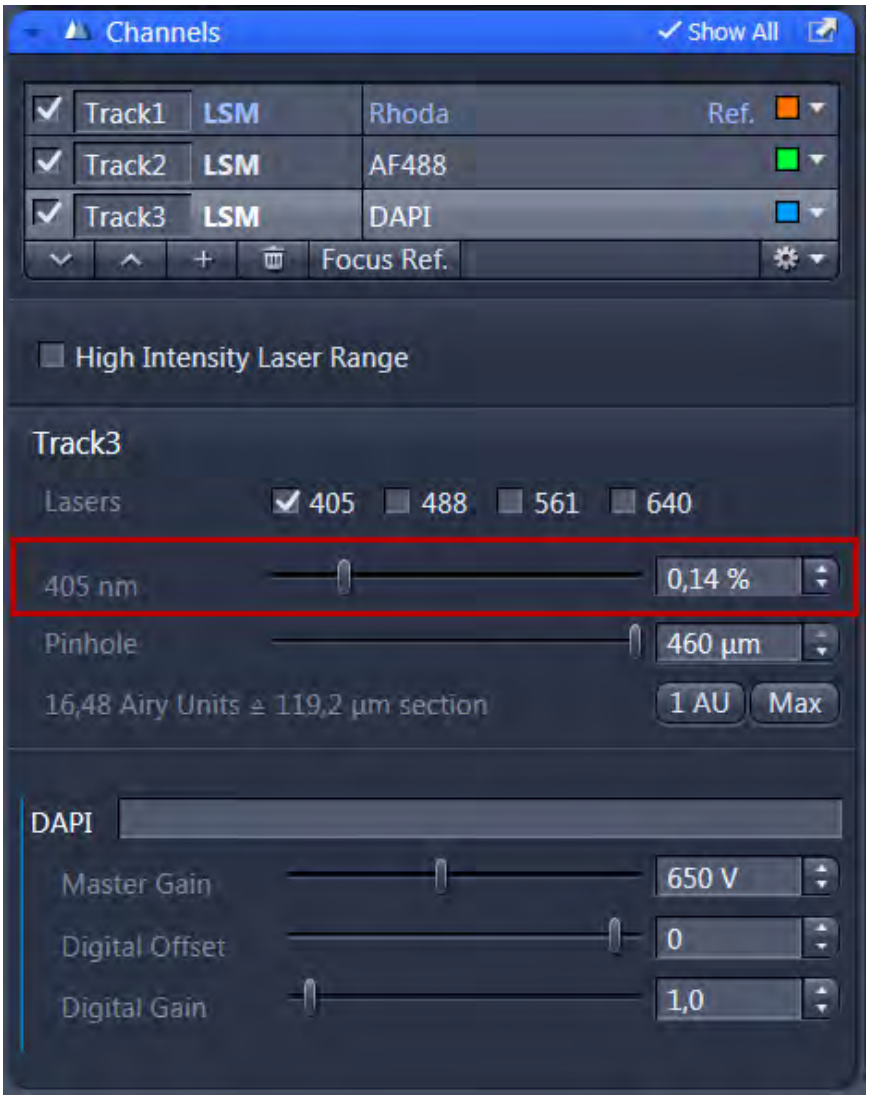
8 Click on **Stop** to stop the continuous scan.



9 Click on **Set Exposure** to automatically adopt the sensitivity of the detector to the sample brightness.

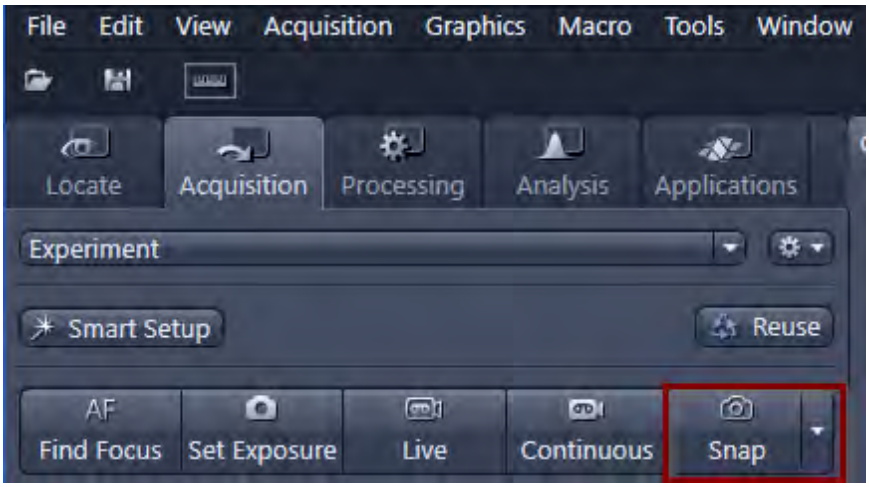


10 **Optional:** If you work with weakly stained samples you can try to increase the laser power under **Channels** | **Lasers**. Then repeat the last step by clicking on **Set Exposure**.



The sample is adjusted correctly.

11 Click on **Snap** to acquire a single confocal image.



You have successfully acquired a confocal image. Save the image in the **Images and Documents** tool or under **File** | **Save**.

4.5 Acquiring EDF Images with ZEN lite

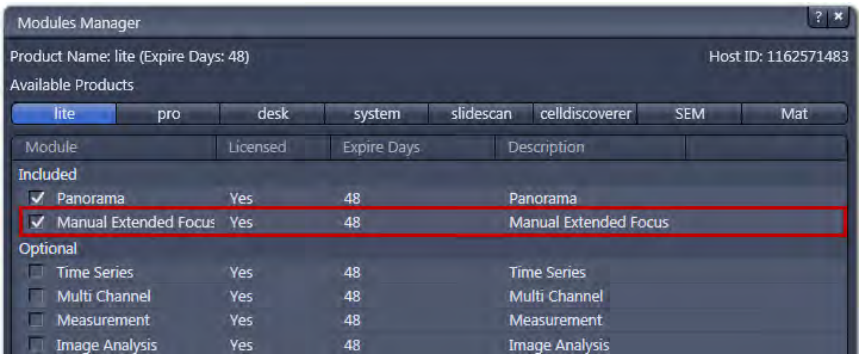
4.5.1 Introduction

The module **Manual Extended Focus** for **ZEN lite** allows to create one single image, out of several acquisitions with different focus positions. The sharp areas of all acquisitions are combined to one consistently sharp image, the so called EDF image (EDF = Extended Depth of Focus).

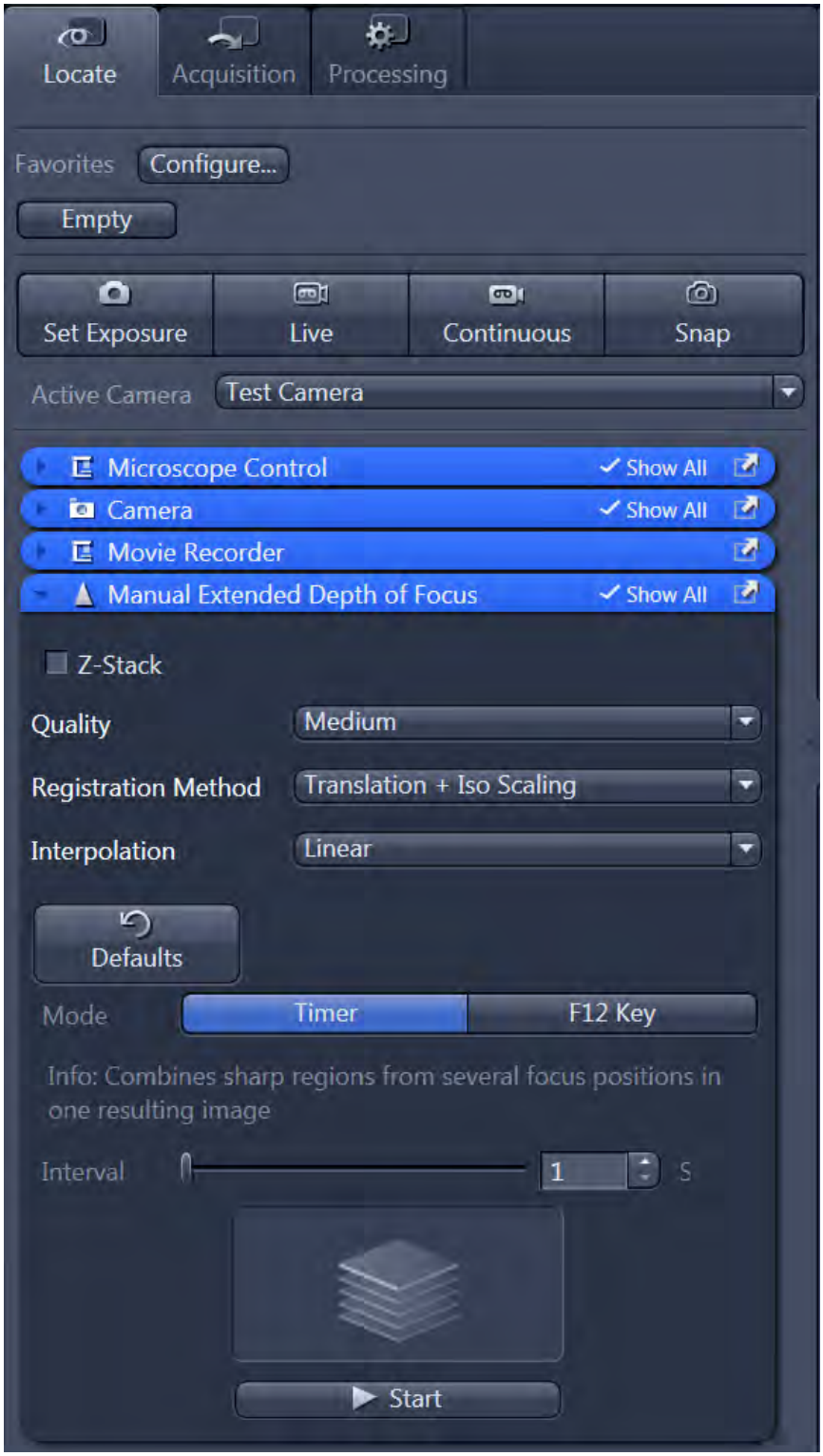
Preconditions

To work with the module **Manual Extended Depth of Focus**, the following conditions must be fulfilled.

- You have licensed your module and ensured, that in the menu **Tools** | **Modules Manager** the function **Manual Extended Focus** is activated via the checkbox.



- The tool **Manual Extended Depth of Focus** now will be shown on the **Locate** tab.



4.5.2 Prerequisites

Prerequisites

- You are on the **Locate** tab.

Procedure **1** Open the live image via the **Live** button.

The **Live** button changes to a **Stop** button during a live image.

2 For the extended focus acquisition look for a suitable position on the sample.

INFO

As long as the live image is active you cannot start the **Manual Extended Depth of Focus** acquisition.

3 Click on the **Stop** button to switch off the live image.

You have made all necessary arrangements. For EDF image acquisition you have now three modes available. These will be revealed in the following chapters.

4.5.3 Acquisition with Timer mode

In this mode, after a freely selectable interval, a new picture will be automatically added. In the period between the acquisitions you can set a different focus position. After each recording a new image with an extended depth of focus is immediately calculated. When you finish the acquisition you will get the image which was calculated last.

Prerequisites ■ You are in the tool **Manual Extended Depth of Focus.**

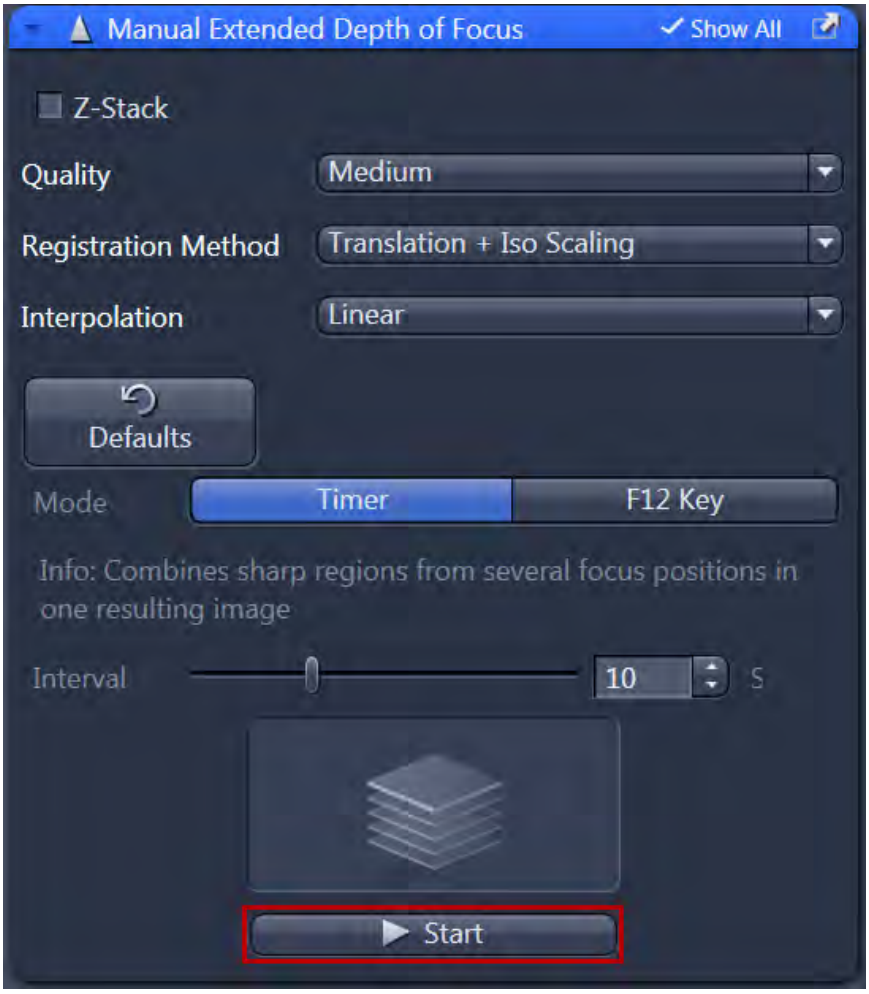
Procedure **1** Select the **Timer** mode.

2 Adjust the length of your interval by the time-slider. After lapse of time, an image of your actual position will be automatically acquired.

INFO

Set the interval long enough, to move the specimen comfortably to a new focus position.

3 Click on the **Start** button, to start the acquisition. The Start button will only be active when the live image is closed.



The **Central Screen Area** will be split into two parts. Right you will always see the current live image, left you will see after each new acquisition the actual calculated extended focus image.

4 Set a new focus position after each acquisition. Repeat this procedure until you are satisfied with the result.

5 To finish your acquisition, click on the **Stop** button. If you click on the **Pause** button you are able to pause the acquisition.

You have successfully acquired an image, showing the extended depth of focus, with the **Timer** mode.

4.5.4 Acquisition with F12 Key mode

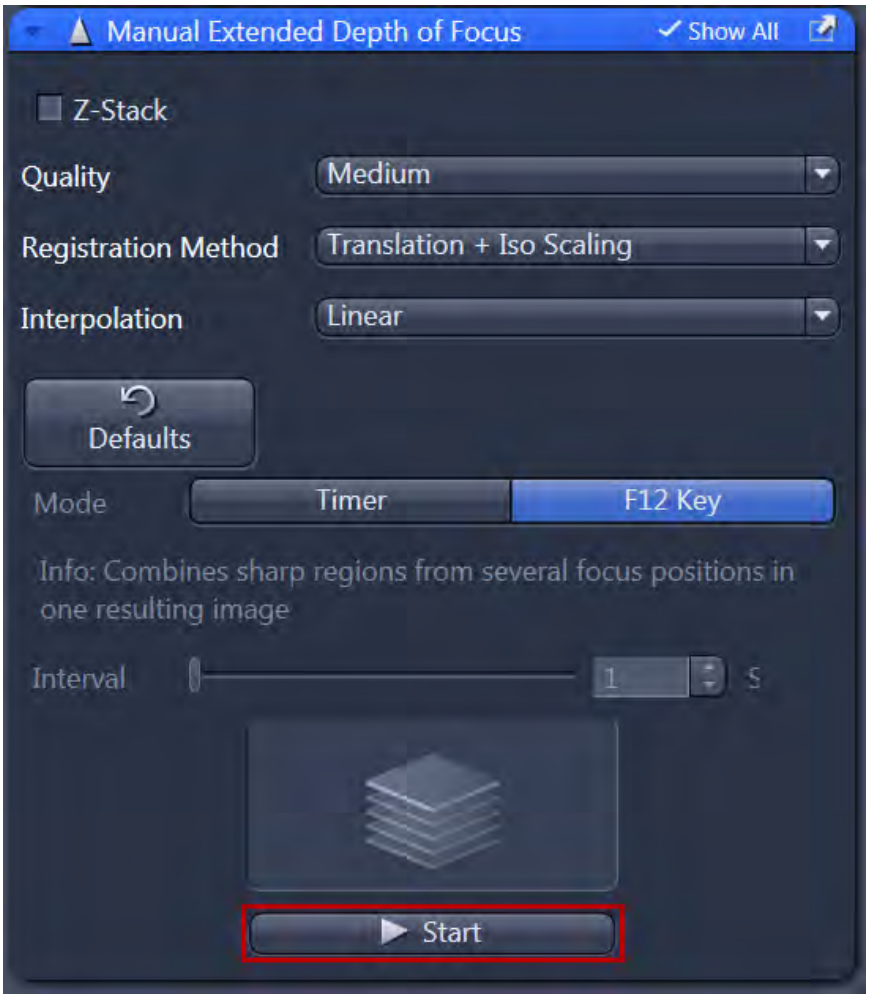
In this mode you can achieve different time intervals between the individual focus steps. By pressing the F12 key on your keyboard, you can manually record a new image. Between the acquisitions, you can change the focus position.

Prerequisites

- You are in the tool **Extended Depth of Focus.**

Procedure

1. Select the **F12 Key** mode.



2. Click on the **Start** button. The Start button will only be active when the live image is closed.
3. Press the *F12* key on your keyboard to initiate manually, each acquisition with a different time step.
4. Select manually a new focus position.

   Repeat the last two steps until you have sharp images from all desired areas of your sample.
5. To finish the acquisition, click on the **Stop** button.

You have successfully acquired an image, showing the extended focus, with the *F12* key mode.

4.5.5 Acquisition with Z-Stack mode

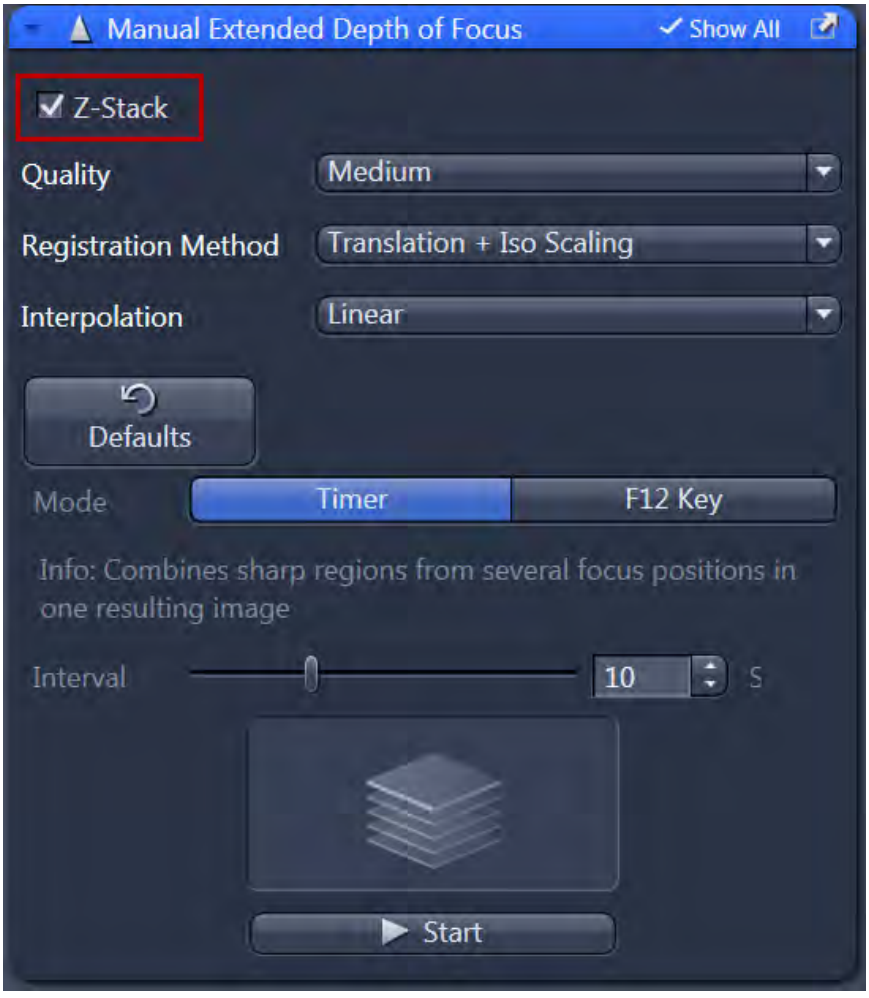
In this mode, you can create an extended depth of focus image using a Z-Stack. This solution is suitable, if you have already acquired a Z-Stack. You can also acquire a Z-Stack in this mode and let it calculate the image with extended depth of focus. Both possibilities are described in the following chapters.

4.5.5.1 Processing of an Extended Depth Focus with Z-Stack

In the following section you will learn, how to create an image with extended depth of focus out of an existing Z-Stack image.

Procedure

1. Open the Z-Stack image.
2. Activate the **Z-Stack** checkbox.
3. Click the **Start** button.



The image with extended depth of focus will be calculated out of the already existing Z-Stack images and will be opened in a new image container.

You have successfully created an image, showing the extended focus, out of a Z-Stack.

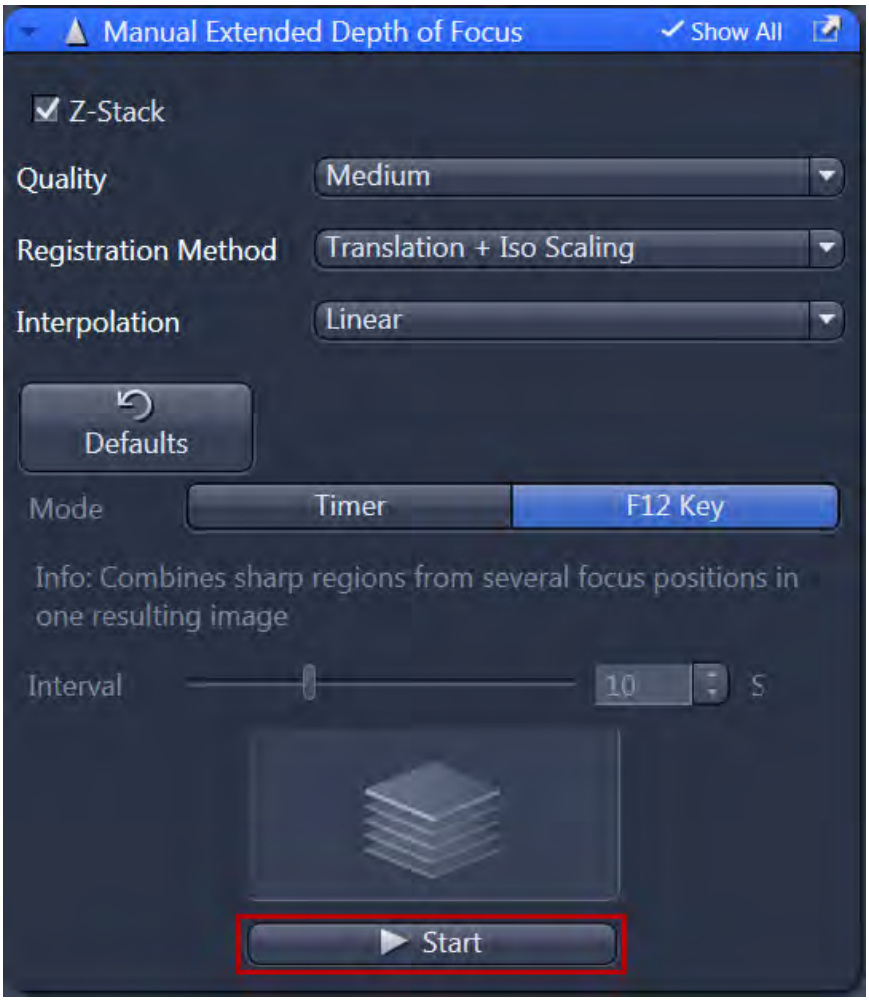
4.5.5.2 Create a Z-Stack manually

> **INFO**
>
> Try to acquire all Z-Stack images in nearly identical focus distances, and only in one not changing direction.

To create a **Z-Stack** manually, perform the following steps:

Procedure

1. Activate the **Z-Stack** checkbox. The **Start** button will only be active when the live image is closed.
2. Select the **F12 Key** mode.
3. Click on the **Start** button.



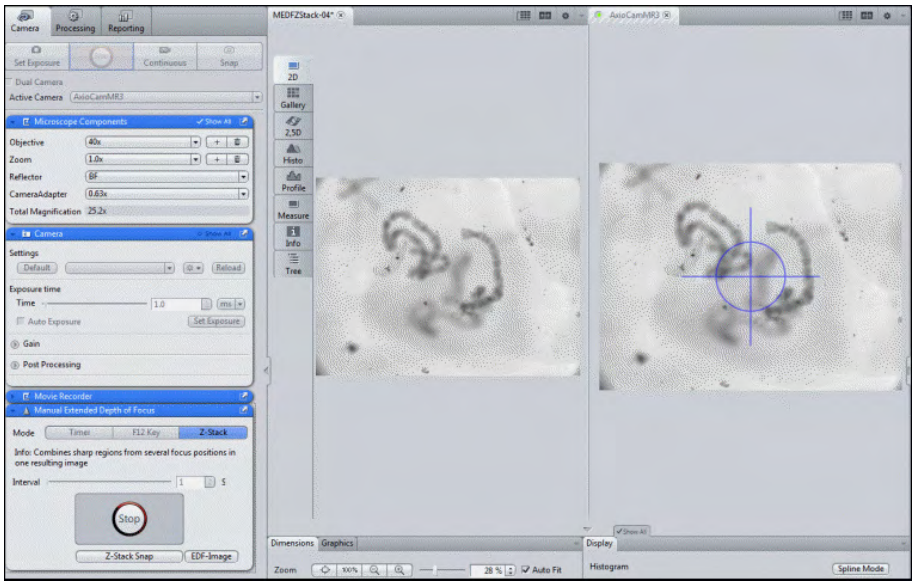
4. Press the *F12* key to add new image planes to the Z-Stack. Adjust a new focus position during the acquisitions.

5 Finish the acquisition after the last image of the Z-Stack, via the **Stop** button.

You have successfully acquired a Z-Stack. All single image planes of the Z-Stack you may check in the **Gallery** view. An EDF image will be calculated out of your Z-Stack image.

4.5.5.3 Z-Stack (using a stereo microscope)

Producing Z-Stack images with a stereo-microscope is a special case. The image planes will be shifted in the X-direction during the focusing. This is a result of the tilted light path in these microscopes. While you move the specimen through the focus, the specimen will move off horizontally from image plane to image plane. Depending on the number of steps in the Z-direction during the acquisition the offset in the X-direction may be huge.



You will also recognize this offset from image plane to image plane in the Gallery view of the Z-Stack image.

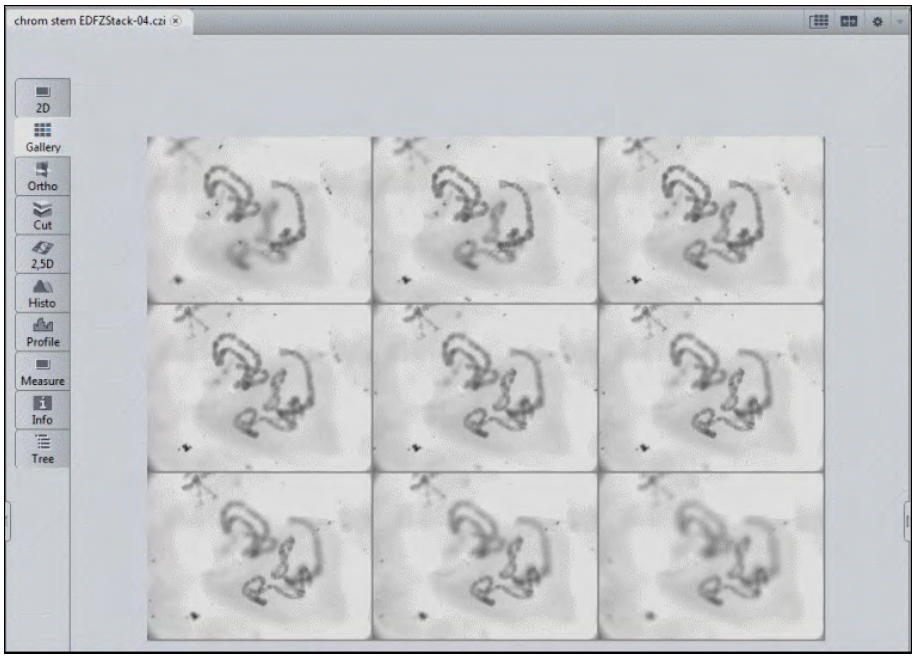


Fig. 4.1: Offset in the X-direction.

INFO

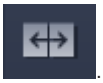
The function used for the calculation of the extended depth focus, is also using an optimal function for the correction of the horizontal offset between the Z-Stack planes.

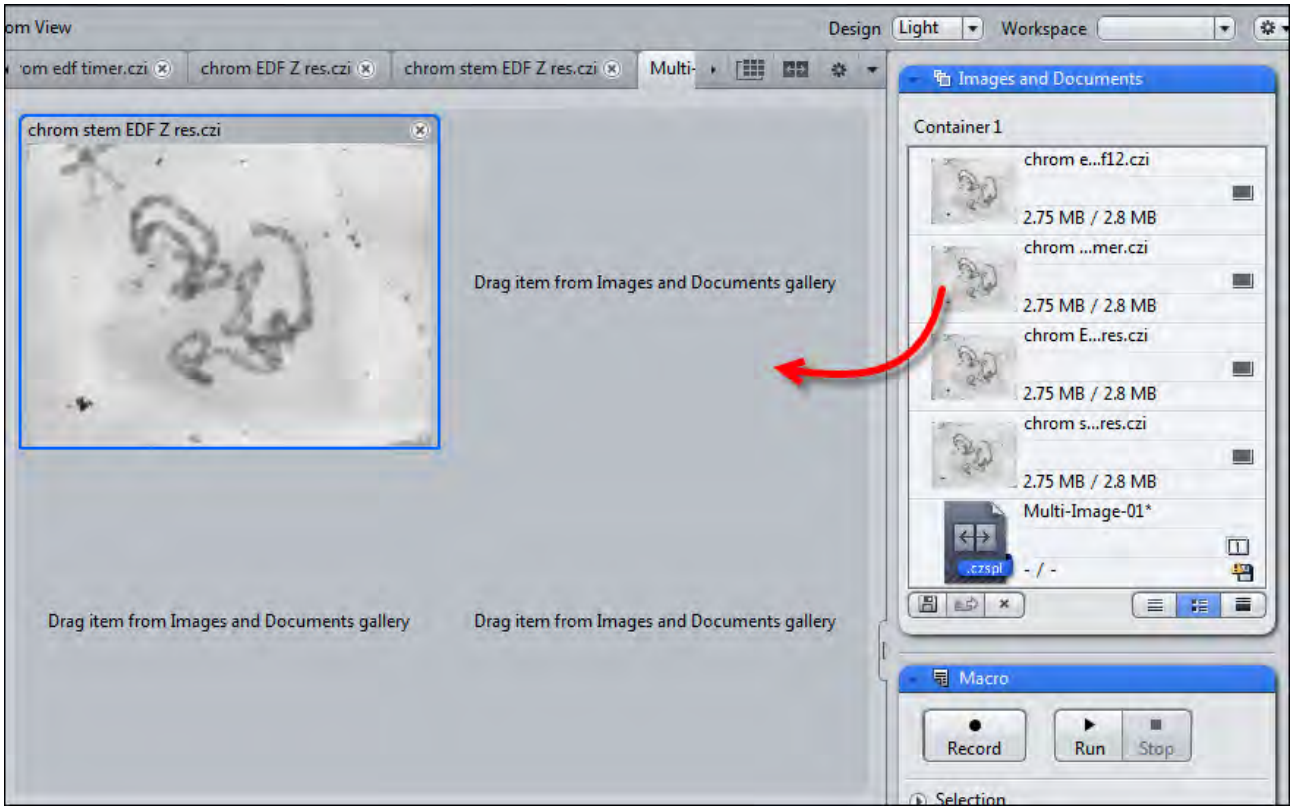
4.5.6 Compare Images using Split Display

Prerequisites

- You have already acquired images with extended depth of focus which you want compare to each other.

Procedure

1. To compare images click on the **Create new multiple image** button  .
2. In the **view options** on the **Split Display** tab define an arrangement of 2x2 images to be shown aside .
3. Move the images via Drag & Drop from the **Image and Documents** gallery in the right tool area, to the **Center Screen Area**. If necessary, click the **show/hide** button to show the **Image and Documents** gallery.



The comparison of all 4 methods used (via Timer, F12-key, Z-stack without shift, Z-stack with shift) shows identical resulting images.

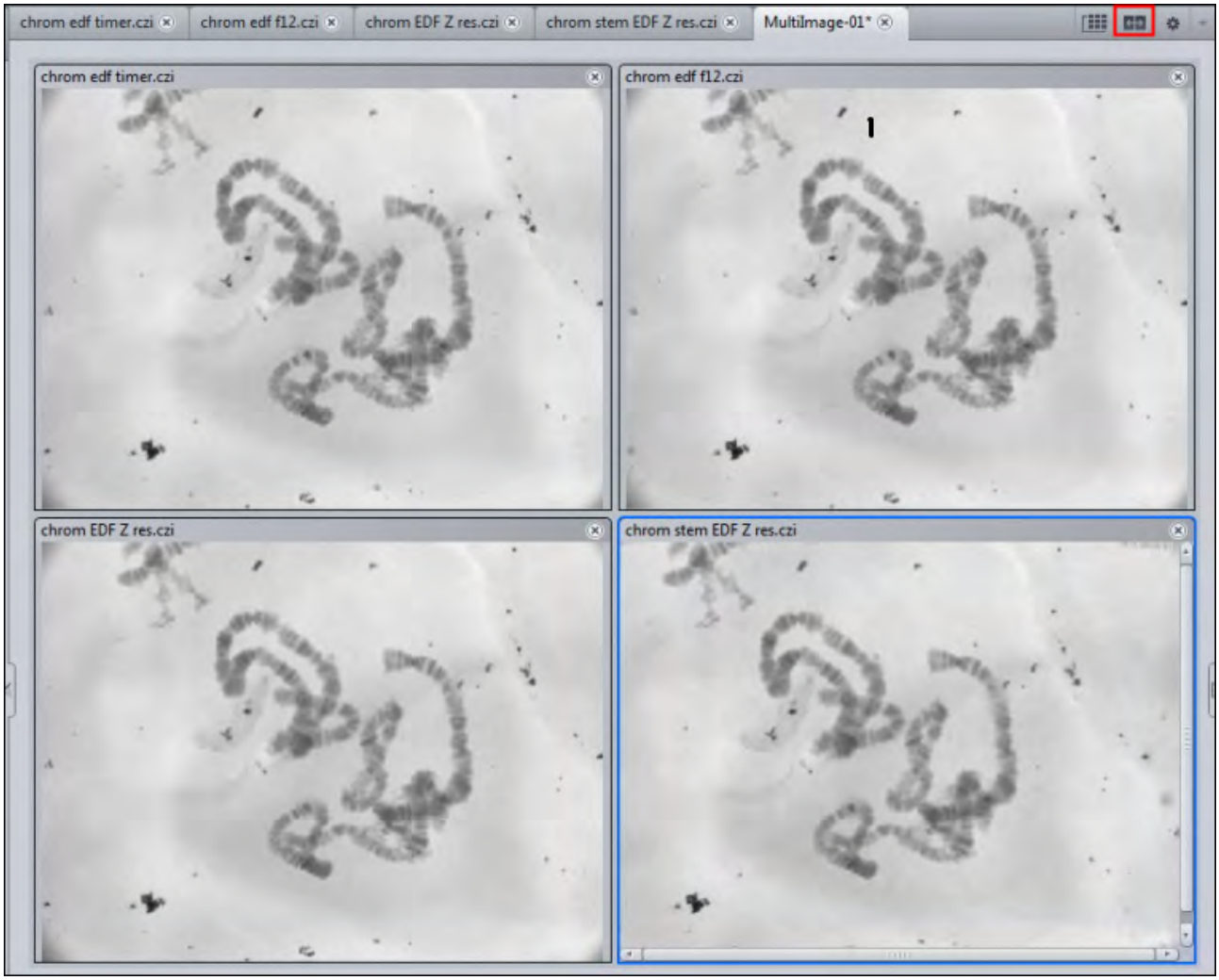


Fig. 4.2: Image comparison with multi image

4.6 Working with Focus Strategies

4.6.1 Introduction

If you want to work with focus strategies you have to use the **Focus Strategy** tool. There you can select the suitable strategy and adjust the corresponding settings, e.g. defining Z-positions manually or automatically and update these during the experiment. Note that the availability of certain focus strategies depends on your system and available components (e.g. Definite Focus.2).

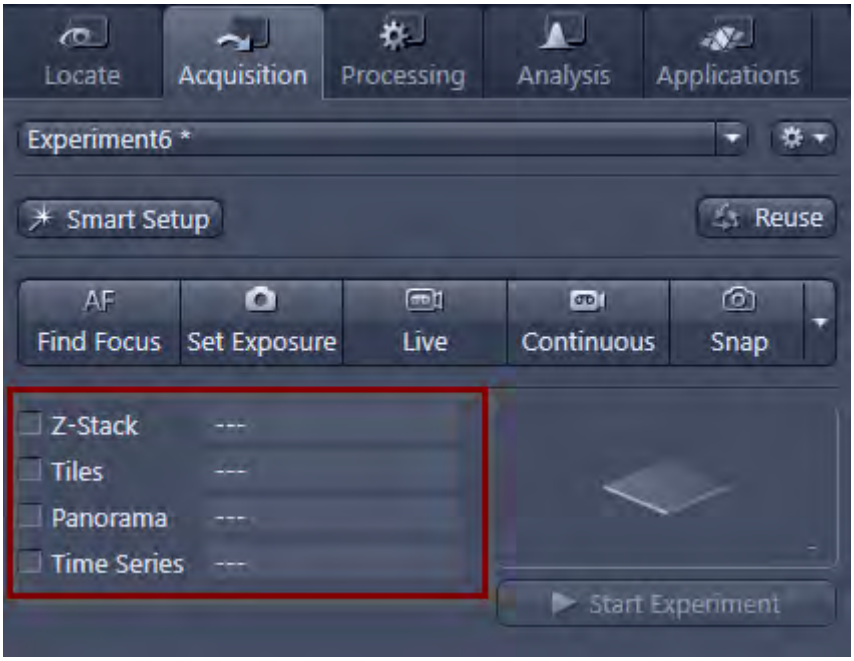
General Preparations

Prerequisites

- To use focus strategies, you will need a motorized focus drive/Z-drive.
- You are in the **Left Tool Area** on the **Acquisition** tab.
- You have *created a new experiment* [▶ 46], *defined at least one channel* [▶ 45] and adjusted the focus and exposure time.

Procedure

1 Activate the acquisition dimensions (e. g. Tiles, Time Series) that you want to use for your experiment.



2 Open the **Focus Strategy** tool.

The available focus strategies are displayed in the dropdown list.

INFO

The number of focus strategies available depends on the activated acquisition dimensions (e.g. tiles, time series), the available hardware devices (e.g. definite focus) present and software license (e.g. **Software Autofocus** and **Tiles** module allow additional focus strategies).

3 Decide which strategy is best suited to your experiment. In case of a **Tiles** experiment the software will automatically select the most appropriate focus strategy if you have not previous selected one. For a detailed description of all strategies, read the chapter *Focus Strategy Tool* [▶ 699].

You have successfully completed the general preparations. Now follow one of the following sets of instructions for specific focus strategies.

4.6.2 Using Software Autofocus

Select this focus strategy to automate the focusing of your specimen before and during acquisition with the help of the **Software Autofocus**. This is particularly useful for **Time Series** or **Tiles** experiments.

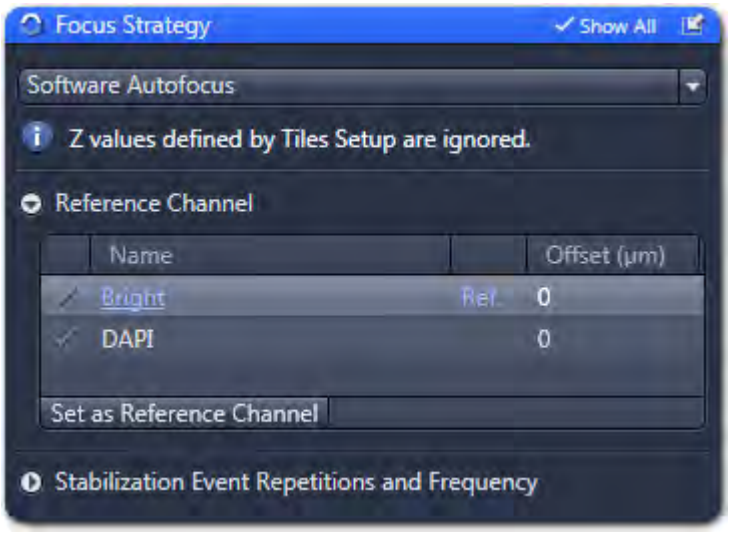
Prerequisites

- To use the **Software Autofocus** focus strategy, you will need the **Autofocus** module.

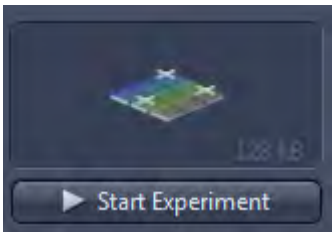
- You have completed the *general preparations* [▶ 71] for using focus strategies (experiment created, at least one channel defined, acquisition dimensions activated).
- You are on the **Acquisition** tab in the **Focus Strategy** tool.

Procedure

1. Select the **Software Autofocus** entry from the dropdown list. Note that the Z values defined by **Tiles Setup** are ignored.



2. In the **Reference Channel** section select the channel that you want to use for the focus action from the list. Expand the section if you don`t see it in full.
3. Click on the **Set as Reference Channel** button.
4. In the **Time Series Loop** and/or **Tiles Loop** sections of the **Focus Strategy** tool you can define when focus actions should be performed during the course of the experiment.
5. Open the **Software Autofocus** tool.
6. Adjust the autofocus settings (e.g. **Quality**, **Sampling**, etc.) to your experiment conditions or use the default settings first.
7. Set up your tile and/or time series experiment.
8. To start the experiment, click on the **Start Experiment** button.



You have successfully used the Software Autofocus to bring images into focus automatically during the experiment.

4.6.3 Using Definite Focus in Time Series Experiments

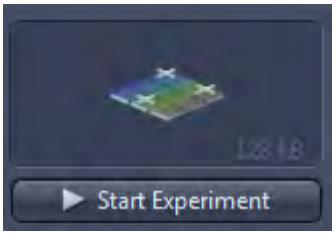
Select this focus strategy to use the definite focus device to stabilize the focus in the event of temperature fluctuations during your **Time Series** experiments.

Prerequisites

- To use the **Definite Focus** focus strategy, you will need the **Definite Focus** hardware device.
- You have completed the *general preparations* [▶ 71] for using focus strategies.
- You are on the **Acquisition** tab in the **Focus Strategy** tool.

Procedure

1. Select **Definite Focus** as focus strategy from the dropdown list. Note that Z values defined by **Tiles Setup** are ignored.
2. In the **Stabilization Event Repetitions and Frequency** section select **Standard** mode. This mode will use our recommend default settings for stabilization. When selecting the **Expert** mode you can adjust all settings according to your needs.
3. Set up a **Time Series** experiment, see *Acquiring Time Series Images* [▶ 54].
4. Use the **Live** mode to set the focus position using the focus drive.
5. To start the experiment, click on **Start Experiment**.



Definite Focus is initialized at the start of the experiment at the current focus position. The focus is then stabilized in accordance with your settings during the time series experiment. You will be reminded to set the focus accordingly prior to the experiment starting. You can do this by navigating to a suitable location (position or Tile region) and starting live or continuous. You can then continue with the experiment or cancel it.

You have successfully used the **Definite Focus** to stabilize the focus during a Time Series experiment.

4.6.4 Using Local or Global Focus Surfaces

For Local or Global Focus Surfaces you need to select the focus strategy **Use Focus Surface/Z Values Defined by Tiles Setup**. This strategy is selected by default, if you have licensed the **Tiles** module. Then you can acquire tiles images along local or global focus surfaces (tile region specific/position specific) and use the focus strategy for optimal image results.

A local/global focus surface ensures that all tiles are in focus on tilted or irregular specimens. Local focus surfaces for tile regions are interpolated on the basis of the focus positions of support points. Positions automatically have a horizontal focus area with the Z-value of the position.

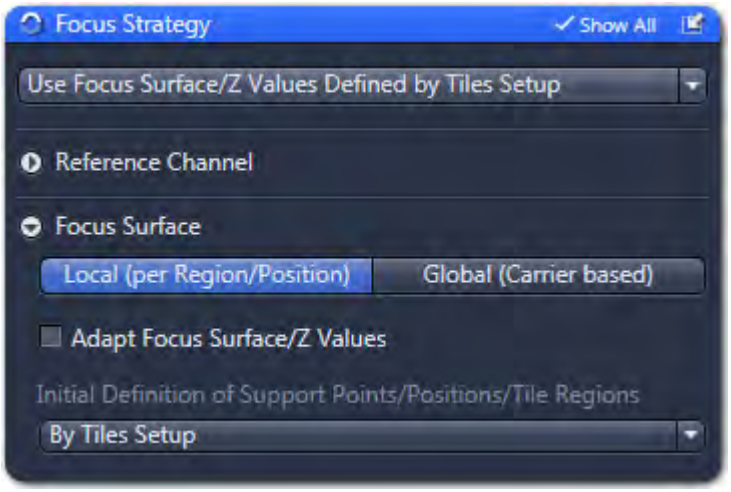
The following guide explains how to use the focus strategy for local focus surfaces.

Prerequisites

- To use the **Use Focus Surface/Z-Values Defined by Tiles Setup** focus strategy, you need a licence of the **Tiles** module.
- You have read *Introduction* [▶ 71] for using focus strategies (experiment created, at least one channel defined, acquisition dimensions activated).
- You are on the **Acquisition** tab in the **Focus Strategy** tool.

Procedure

1. Select the **Use Focus Surface/Z-Values Defined by Tiles Setup** entry from the dropdown list (if not selected by default).
2. In the **Focus Surface** section select **Local (per Region/Position)**.
3. Under **Initial Definition of Z-Values for Support Points/Positions** select **By Tiles Setup** entry from the dropdown list.



INFO

Alternatively, you can select the options **By Software Autofocus** or **By Definite Focus : Recall Focus** if you have licensed the **Software Autofocus** module and/or use **Definite Focus.2** hardware device. This allows you to specify that the Z-positions of the support points should be updated by the software autofocus / Definite Focus.2 immediately before acquisition.

4. Set up a **Tiles** experiment. For more information, see *Setting Up a Simple Tiles Experiment* [▶ 302].
5. Create a **local focus surface** for the tile regions, see *Creating a Local Focus Surface* [▶ 320].

6 To start the experiment, click on the **Start Experiment** button.

The tiles of tile regions and positions are acquired using the local focus surfaces.

If you are performing a **Time Series** experiment we recommend to use the **Adapt Focus Surface/ Z Values** functions, see *Focus Strategy Tool* [▶ 699]. By these you can correct the focus for small drifts that occur in long multidimensional time series experiments with living cells, for example.

4.7 Using the Dye Editor

4.7.1 Introduction

The ZEN software is delivered with a large number of preset dyes. Dyes and it's parameter are stored in the dye database. In case you have created a custom made filter cube, you may need additional dyes.
You can create new dyes and a custom dye database with the **Dye Editor** in the **Tools** menu.

4.7.2 Dye Editor Dialog

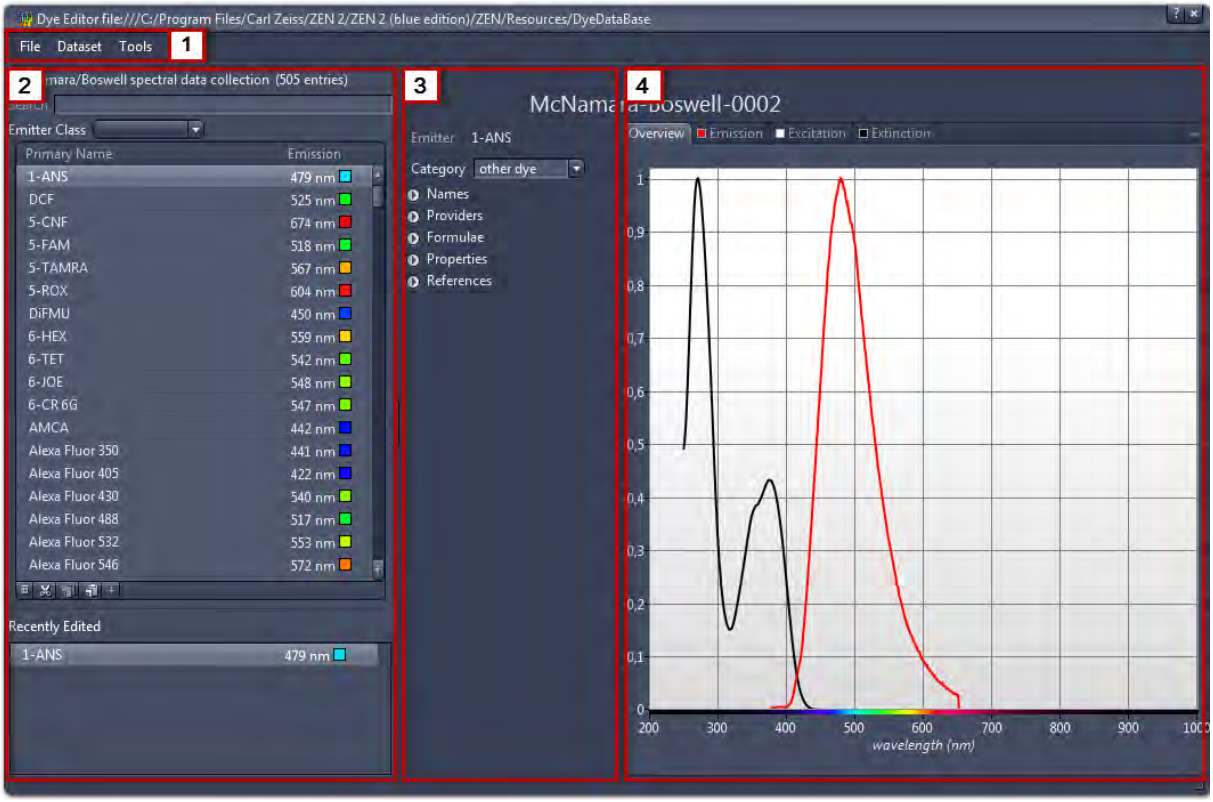


Fig. 4.3: Dye Editor Dialog

No.	Parameter	Description
1	**Menu**	Here you can create new data sets and import available dye databases. A detailed description can be found under *Dye Editor Menus* [▶ 78].
2	**Dye search & database**	In this list you see the dyes that are available in the open database. You can search for dyes and see which dyes you edited last.
3	**Dye information area**	Here you see all the additional information about a selected dye.
4	**Dye spectra**	Here you see the available dye spectra. Click on the relevant tabs to display the **emission**, **excitation**, or **extinction** spectra. On the **Overview** tab you can see all spectra at a glance.

4.7.3 Dye Editor Menus

File menu

Menu item	Description	Short cut
New	Creates a new ExEml file in which you can create dye data sets.	
Open file...	Opens a single ExEml file.	
Open folder...	Opens several ExEml files that have been saved together in the same folder.	*Ctrl+S*
Import file...	Imports an ExEml file.	
Save...	Saves the open ExEml file.	
Save As...	Saves the open ExEml file under a new name.	
Save As Folder...	Saves all open ExEml files in a folder.	
Close	Closes the Dye Editor.	*Ctrl+F4*

Dataset menu

Menu item	Description	Short cut
Add new...	Creates a new, empty data set.	
Copy	Copies the selected data set to the clipboard.	*Ctrl+C, Ctrl+Ins*
Cut	Cuts the selected data set and copies it to the clipboard.	*Ctrl+X, Shift+Del*
Delete	Deletes the selected data set.	
Paste	Pastes a data set from the clipboard.	*Ctrl+V, Shift+Ins*
Paste Part	Pastes a range of a data set from the clipboard.	

Tools menu

Menu item	Description
Generate short names...	Creates abbreviations for names of dyes. This helps to avoid duplication.
Update max. wavelength ermitter properties	Calculates the main emission wavelength of the selected dye from spectral data that has either been copied or entered manually.

4.7.4 Creating a Custom Dye

If non of the preset dyes matches your requirements, you can create a new custom dye.

Procedure

1 Open the **Dye Editor** via **Tools** | **Dye Editor...**.

The **Dye Editor** dialog opens.

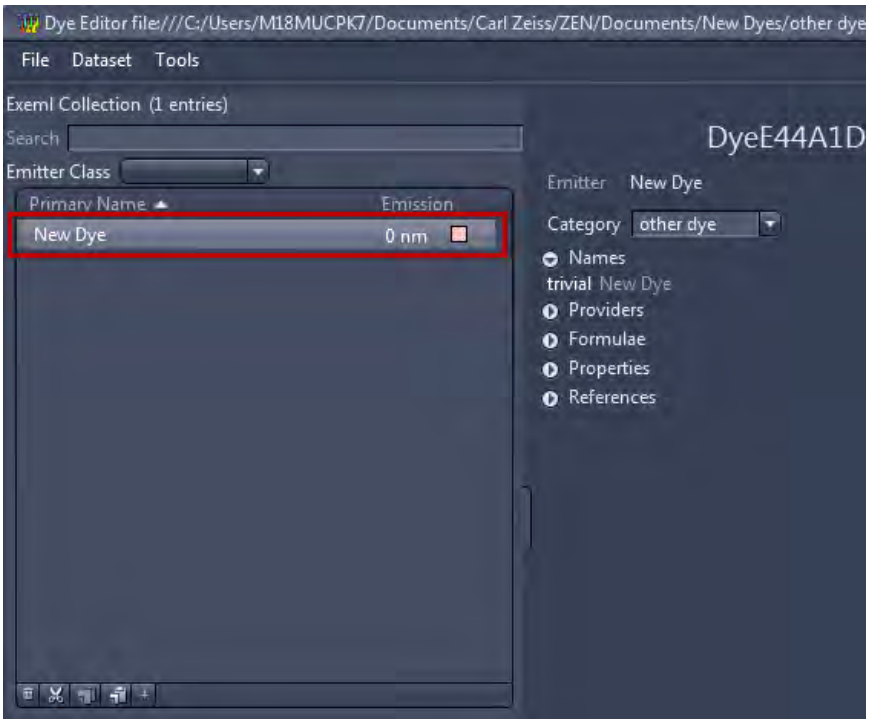
2 In the **File** menu, click the **Save As Folder...** entry.

3 In the Windows dialog, create a new folder to save your custom dye database, e.g. C:/User/Documents/Carl Zeiss/ZEN/Documents/**New Folder**.
Name the new folder, e.g. New Dyes.

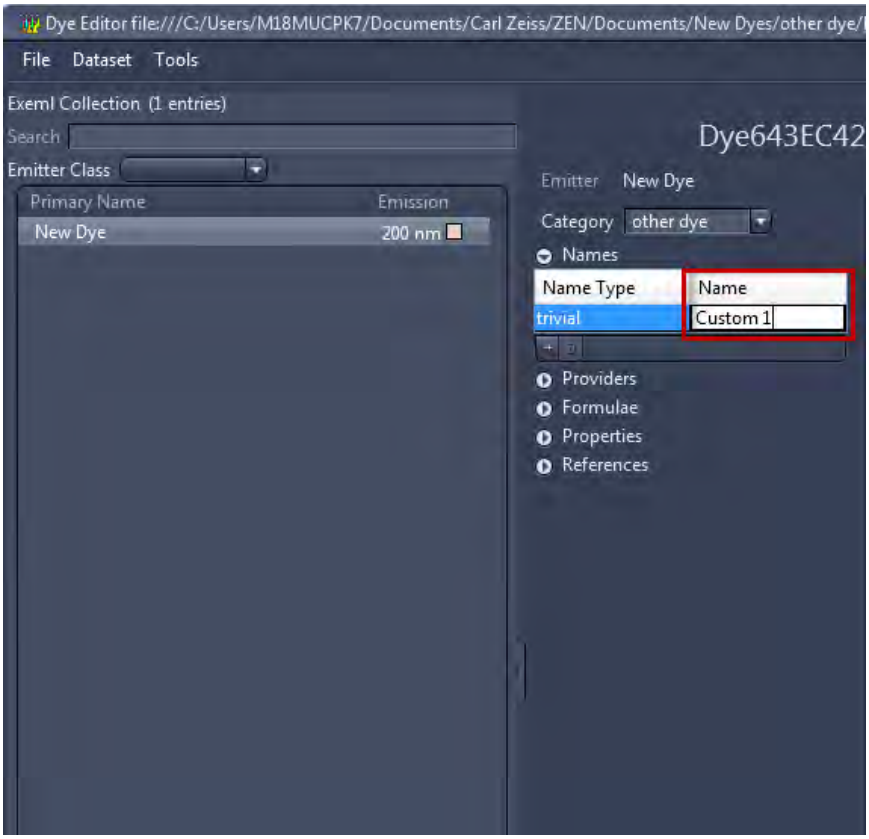
You have created a custom dye database to which all new dyes are stored.

4 Click on the **New Dye** entry in the **Exeml Collection** list.

The properties section opens.



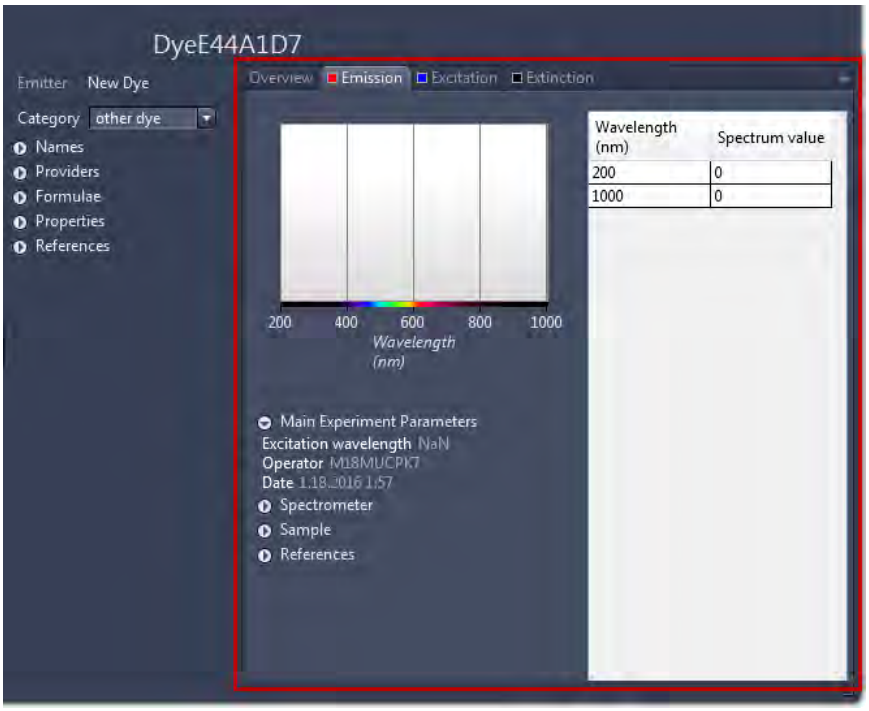
5 In the **Names** section, click in the **Name** input field to change the name of the custom dye.



The name enables you to find your custom dye in the **Smart Setup** or **Channels** tool.

6 Fill in other known parameters in the following sections, e.g. **Providers**, **Properties**, **References**.

7 Change to the **Emission** tab.



8 To fill in **Wavelength (nm)** and **Spectrum value** to the table on the right, you have 3 options:

- *Type in data manually.* [▶ 81]
- *Copy and paste data from e.g. an Excel sheet.* [▶ 82]
- *Copy and paste data from a preset dye.* [▶ 83]

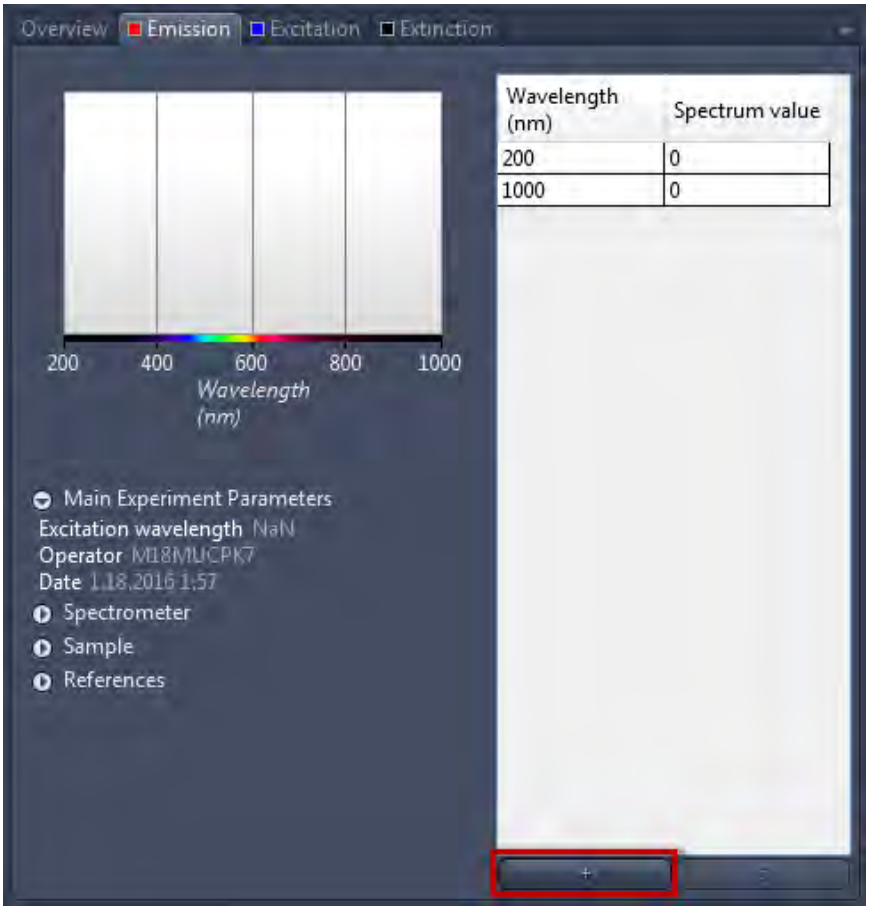
4.7.4.1 Type in Data Manually

Prerequisites

- You have created a new dye in the **Dye Editor** dialog.
- Your are on the **Emission** tab.

Procedure

1 In the **Wavelenth (nm) | Spectrum value** table, click on the **Plus** icon.
Add 10 rows, by pressing *Shift* and the **Plus** icon.
Add 100 rows, by pressing *Alt+Shift* and the **Plus** icon.



2 Type in the corresponding **Spectrum Value** in the input fields.

The emission graph is displayed next to table.

3 Save the dye via **File** | **Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

4.7.4.2 Copy Data from an Other Source

If you have recorded **Wavelength (nm)** and **Spectrum value** of your custom dye in an other source e.g. an Excel sheet, you can copy the data and paste it directly into the **Dye Editor** dialog.

Prerequisites

- You have created a new dye in the **Dye Editor** dialog.
- You have opened your source, e.g. an Excel sheet with the emission data.

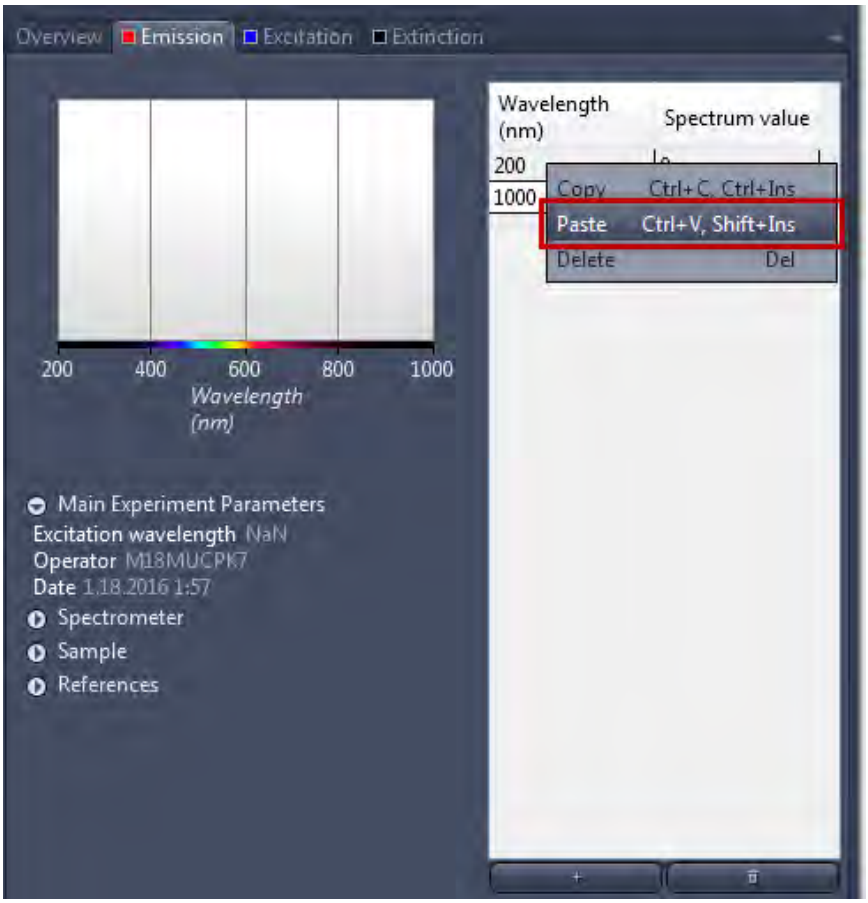
Procedure

1 Arrange the data in two columns like on the **Emission** tab.

2 Mark the data and copy it via *Ctrl+C*. Alternatively, right-click on the marked area to open the shortcut menu and select **Copy**.

3 Change to the **Dye Editor** dialog.

4 On the **Emission** tab, click in the first row of the **Wavelength (nm) | Spectrum value** table an press *Ctrl+V*. Alternatively, right-click in the first row to open the shortcut menu and select **Paste**.



The emission data is inserted in the table and the emission graph is displayed next to the table.

5 Save the dye via **File | Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

4.7.4.3 Copy Data from a Preset Dye

If the data of a preset dye, e.g. the emission data of the DAPI dye resembles your custom dye, you can copy the data from the preset dye into a new custom dye.

NOTICE

Do not edit preset dyes

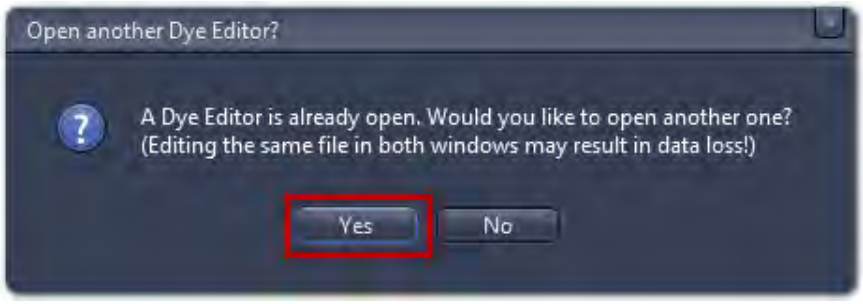
Editing preset dyes leads to irreversible data loss.
Note that you should therefore only copy the data set of a preset dye.

Prerequisites

- You have created a new dye in the **Dye Editor** dialog.

Procedure

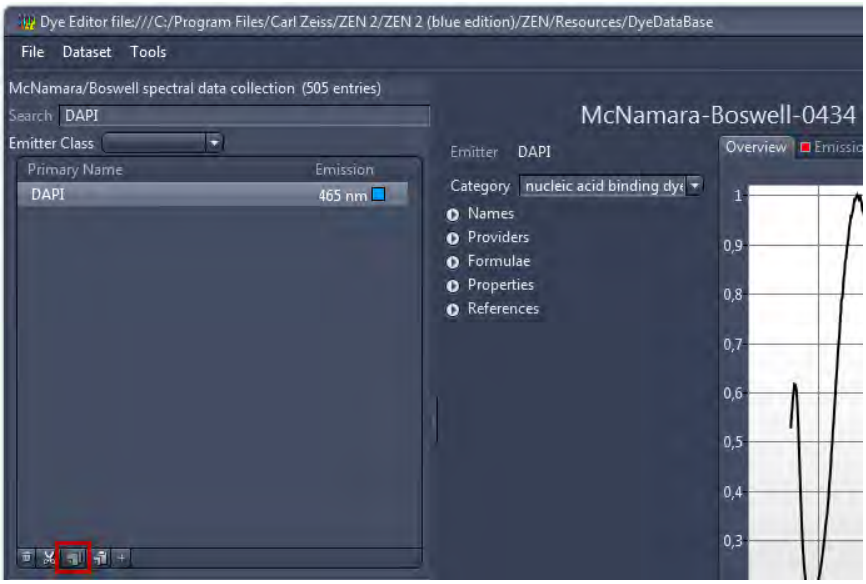
1. Open a second **Dye Editor** dialog via **Tools** | **Dye Editor...**.
2. Confirm the occurring dialog with **Yes**.



Perform the following steps to open the **ZEN Dye Database** in the second **Dye Editor** dialog:

3. In the **File** menu, click on the **Open folder...** entry.
4. In the Windows dialog, open the **DyeDataBase** with all preset dyes by following the file path:
   C:/Program Files/Carl Zeiss/ZEN/ZEN 2 (blue edition)/ZEN/Resources/DyeDataBase

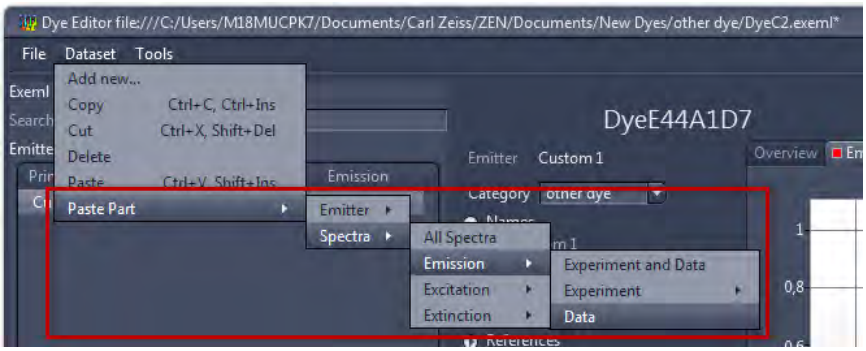
   All preset dyes are displayed in the second **Dye Editor** dialog.
5. Use the **Search** input field, to quickly find a preset dye with similar values, e.g. DAPI.
6. Select the desired dye.
7. Click on the **Copy the current dataset as XML** button. Alternatively, click **Dataset** | **Copy**.



The emission data is copied to the clipboard.

8. Change to the first **Dye Editor** dialog, to edit your custom dye.

9 In the **Dataset** menu select **Paste Part** | **Spectra** | **Emission** | **Data**.



The emission data from the preset dye is copied into your custom dye.

10 Edit the custom dye emission data, until it matches your requirements.

The emission graph is displayed next to the table.

11 Save the dye via **File** | **Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

INFO

You can paste other properties of preset dyes just as the emission data, e.g. **All Spectra** data or **Emitter Properties**.

4.7.5 Adding a New Dye to the Data Set

Prerequisites

- You have successfully created a first custom dye and a custom dye database, see *Creating a Custom Dye* [▶ 79].
- You have opened the **Dye Editor** dialog.

Procedure

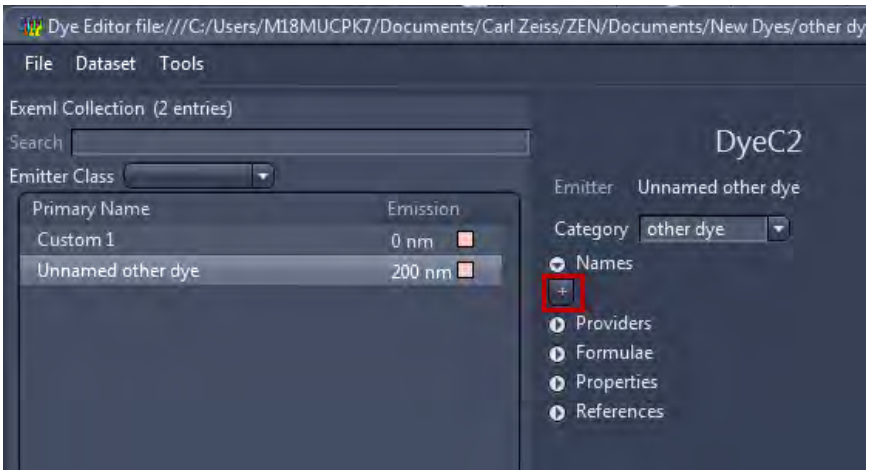
1 In the **Dataset** menu select **New...**.

The **Create new dataset** dialog opens.

2 Enter an ID for your new dye, e.g. DyeC2 and confirm with **OK**.

The entry **Unnamed other dye** appears in the **Exeml Collection** list.

3 Add a name for the new dye by clicking on the **Plus** icon in the **Names** section.



4 Enter a name in the **Name** input field.

5 Edit the properties of the new dye, see
Type in Data Manually [▶ 81] or
Copy Data from an Other Source [▶ 82] or
Copy Data from a Preset Dye [▶ 83].

4.8 Displaying and adapting a grid in the image area

In this section you will find out how to display a grid in your images and how to adapt it.

INFO

A grid can only be displayed in an acquired image or in a live image in **Continuous** mode.

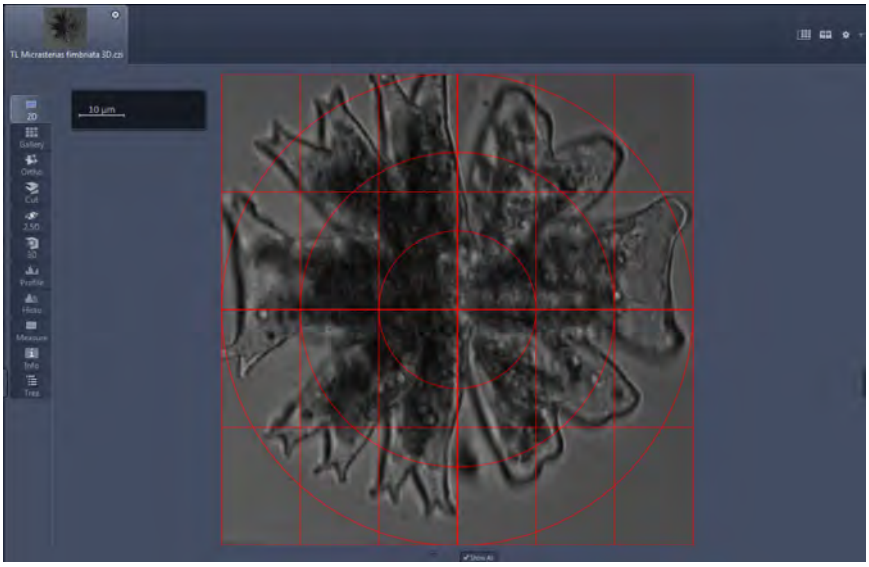
Prerequisites

- You have opened an image in which you want to display a grid.

Procedure

1 Click on the **Grid** entry on the **Graphics** menu.

The grid is displayed in the image.



2 Right-click precisely on a grid line.

The shortcut menu opens.

3 Click on the **Format Graphical Elements** entry in the shortcut menu.

The **Format Grid** dialog is displayed.

4 Activate the **Synchronize** checkbox. This function means that any changes made, e.g. to the number of columns, are adopted simultaneously for the number of rows. The grid therefore remains square.

5 Set a higher number of columns using the **Columns** slider. Alternatively, you can enter a value in the input field.

The changes are displayed directly in the image.

6 Click on the **Save** button to save the grid settings.

The Windows dialog for saving settings is opened.

7 Enter a name and click on **Save**.

8 Close the **Format Grid** dialog.

You have inserted a grid into your image, edited the grid and saved your grid settings.

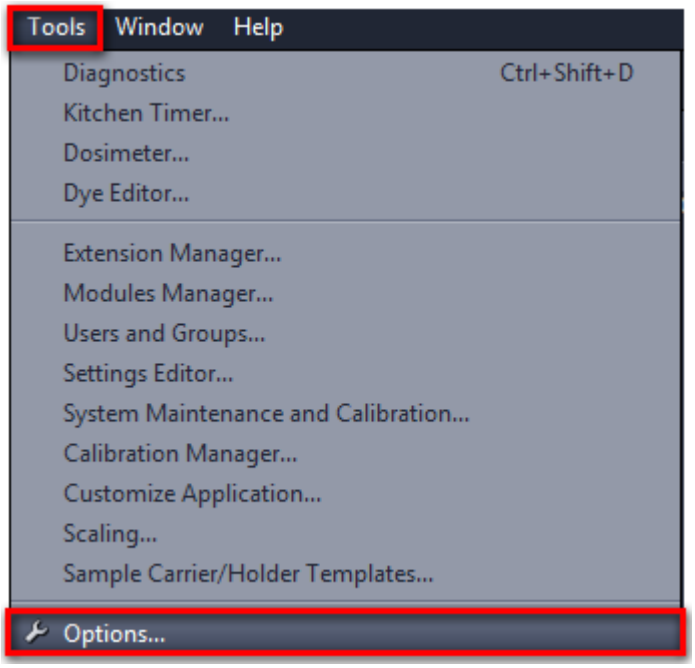
4.9 Setting an experiment as startup default

Any experiment can be set as a startup default indicated by a symbol.

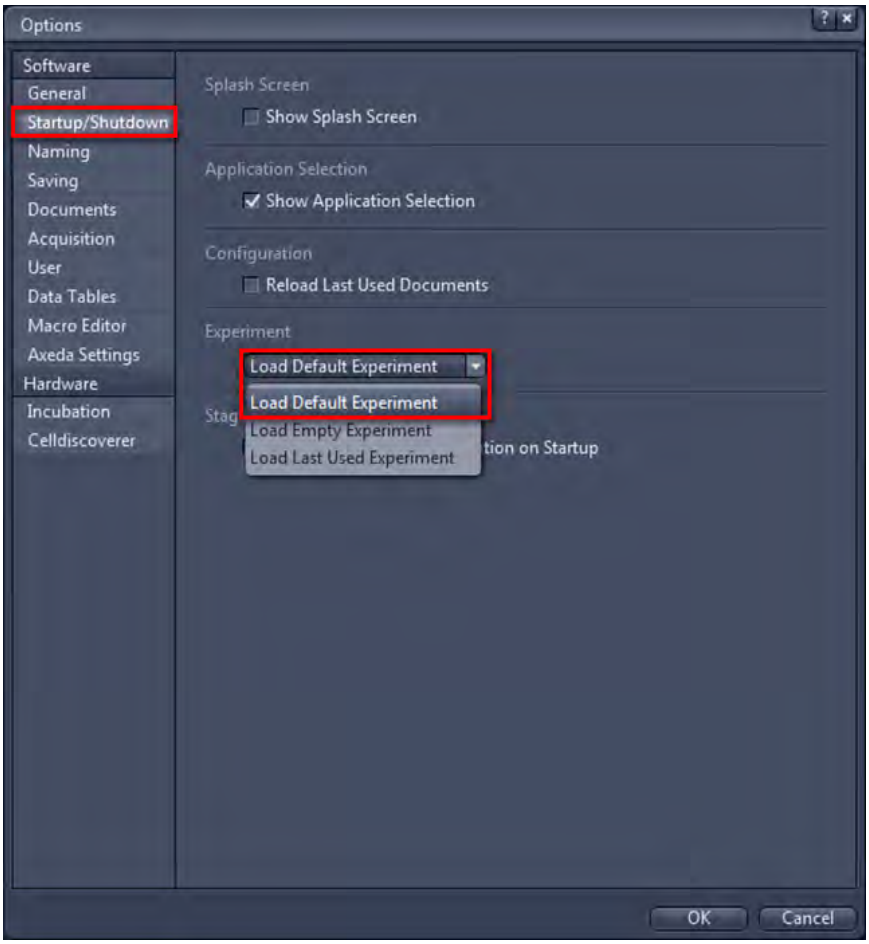
If you want to set an experiment as startup default, the following steps are necessary.

Procedure

1 In the **menu bar** | **Tools** select **Options...**.



2 Click on **Options** and select **Startup/Shutdown**.



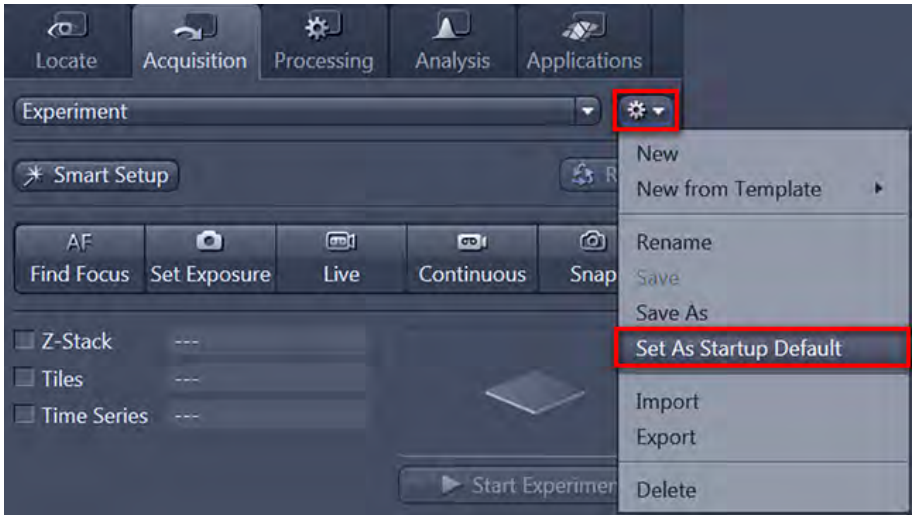
3 Open the **Experiment** selection box and select **Load Default Experiment**.

4 Click on **OK**.

The **Options** dialog closes.

5 Configure your experiment for startup default or select an existing experiment.

6 Click on the options symbol and select **Set As Start up Default**.



The actice experiment is set as startup default now and marked with the  symbol.

This experiment is loaded when starting the ZEN software.

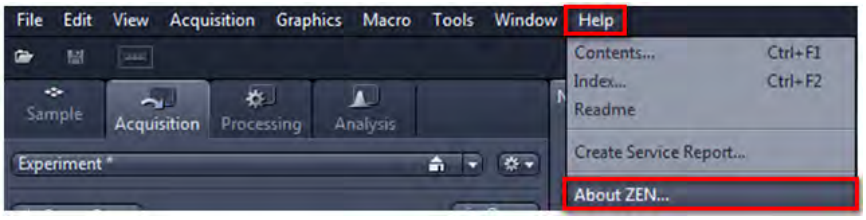
4.10 Creating default experiments as templates automatically

It is possible to generate an experiment template. This cannot be modified but used as a starting point for your acquisition.

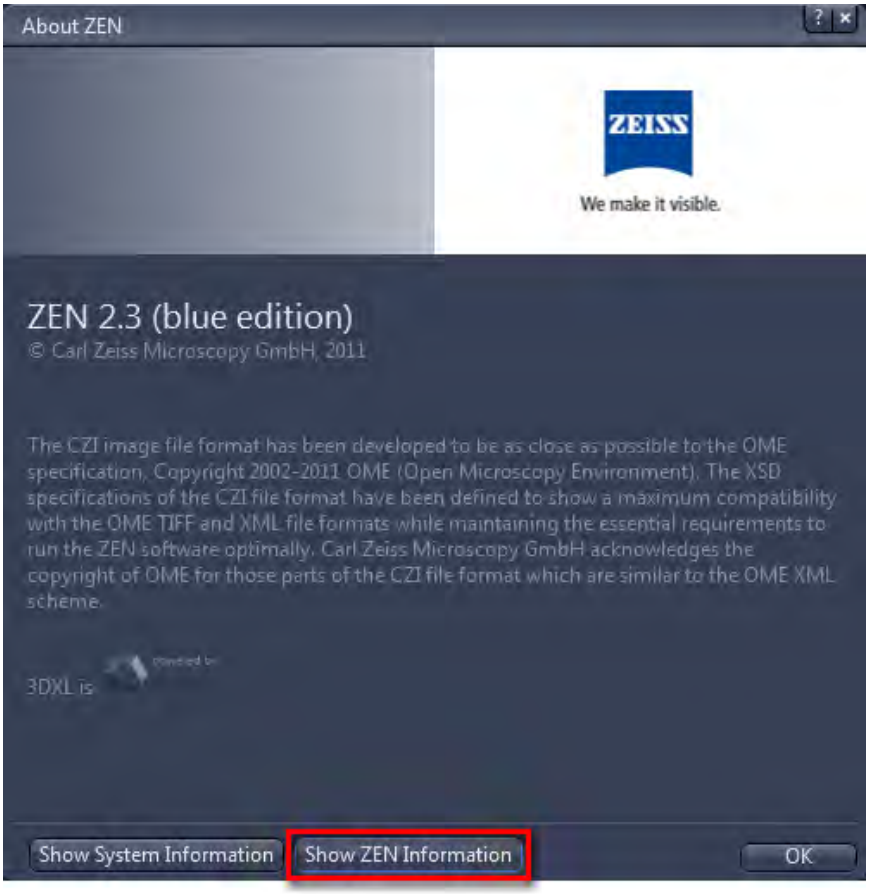
If you want to generate an experiment template, the following steps are necessary.

Procedure

1. Create an experiment.
2. In the **menu bar** | **Help** select **About ZEN...**

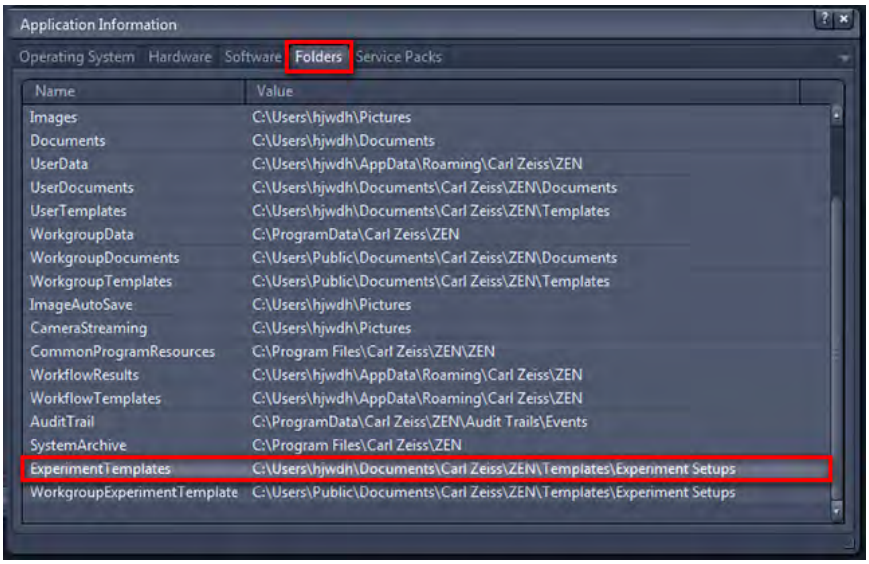


The **About ZEN** dialog appears.



3 Click on **Show ZEN Information**.

The **Application Information** dialog appears.



4 Select the **Folders** tab, scoll to the **Experiment Templates** entry and double click on it.

The folder **Experiment Setups** will open.

5 Export the experiment generated before into the **Carl Zeiss\ZEN\Templates \Experiment Setups** folder.

6 Create a descriptive name for the experiment template.

You have successfully created an experiment template.

It is possible to generate and save multiple templates.

To apply an experiment template, see chapter *Applying an experiment template* [▶ 92].

4.11 Applying an experiment template

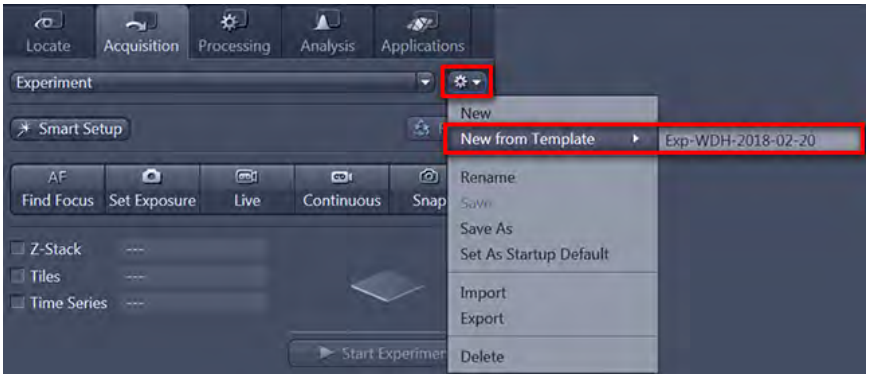
If you want to apply an experiment template as a starting point for your acquisition, the following steps are necessary.

Prerequisites

- The experiment template to be used has been created before, see chapter *Creating default experiments as templates automatically* [▶ 90].

Procedure

1 Click on the options symbol and select **New from Template**.



The available experiment templates will be shown on the right side.

2 Click on the name of the appropriate template (i.e. **Exp-WDH-2018-02-20**).

The selected experiment template will be loaded as starting point for acquisition.

5 Image Processing

5.1 Image Processing Workflow

On the **Processing** tab you apply image processing functions (IP functions) to acquired or loaded images. The basic workflow is quite simple:

- Acquire or open an image that you want to process.
- Open the **Processing** tab.

  When you open the tab, the last active image (e.g from Acquisition tab) will be used as the input image for the processing function.

  If you want to select an other input load the new image under **Image Parameters** | **Input**. Note that the image must be opened in ZEN before.
- Select the desired processing function under **Method**, e.g. **Color Balance**. You can search for processing functions in the **Search** field. Therefore just enter the initials of the functions you want to search.
- Set the parameters of the function under **Parameters**.
  If you need help for a specific function and its parameters press the F1 key. You will find detailed descriptions for each functions in the online help.
- To see how the functions works you can click on the **Preview** button under **Image Parameters** | **Output**.
- Click on the **Apply** button to apply the processing functions to the image. This will create a new image in a new image container. The original image will not be changed.

5.2 General Settings

Save / Load Settings

In the **Settings** section on top of a function`s **Parameters** tool you are able to save and reload the adjusted settings. If you have adjusted the parameters for a function, simply click on **Options** |**New** to save your setting under a new name.

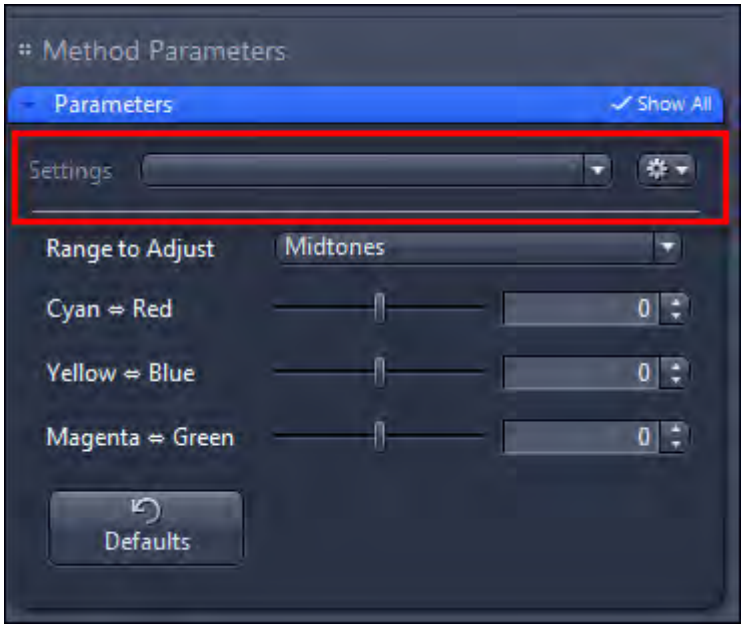


Fig. 5.1: Settings section

Adjust Settings per Channel

If your input image is a multichannel image, all settings can be adjusted individually for each channel. Therefore the checkbox **Adjust per Channel** must be activated.

Third Dimension Settings

If there are more dimensions in the input image and/or the **Show all** mode is activated the **Third Dimension** dropdown list becomes visible. There you can select to which dimension you want to apply the function additionally. Several choices are available, depending on your input image:

Choice	Function
2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
Z, T or C	Here you can select to which additional dimension the functions should be applied to: ■ Z = Z-Stack ■ T = Time ■ C = Channels

Reset Settings to Default

You can reset the settings for a function to default by clicking on the **Defaults** button.

5.3 Performing Deconvolution Using Default Values

Successful deconvolution depends mainly on good image quality, knowledge about the optical parameters of the sample and detailed knowledge about the type of instrument used for image acquisition. While information about the used instrument type can be easily extracted from the image metadata, optical parameters of the sample might not be known and the image quality can vary widely. Many parameters are available for deconvolution which allow you to make corrections to the image quality and adjust the algorithms to match the various optical condiditions such as coverslip type or the medium, in which the sample is embedded. This wide range of parameters can be overwhelming.

With the **Deconvolution (defaults)** method, good initial result are achieved by using a carefully preselected set of default parameters. The parameters are automatically adapted to the following instrument types: widefield, confocal, lightsheet and ApoTome.

While these parameters usually give nice results, there are cases, where further parameter changes will be necessary, e.g., activating and using spherical aberration correction. In such cases, you should use the **Deconvolution (adjustable)** method.

Prerequisites

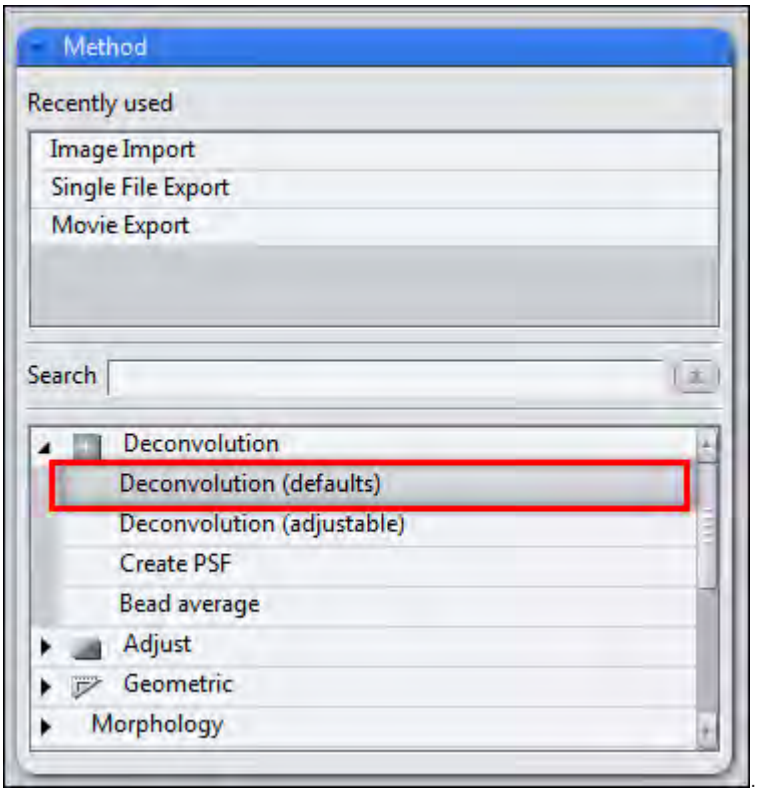
- You are on the **Processing** tab.
- You have acquired or opened a fluorescence image on which you wish to perform deconvolution.
- All tools are in **Show All** mode.

Procedure

1. Open the **Method** tool under **Method Selection.**
2. Click on the **Deconvolution** group.

   You will see the methods **Deconvolution (defaults)** and **Deconvolution (adjustable)**.

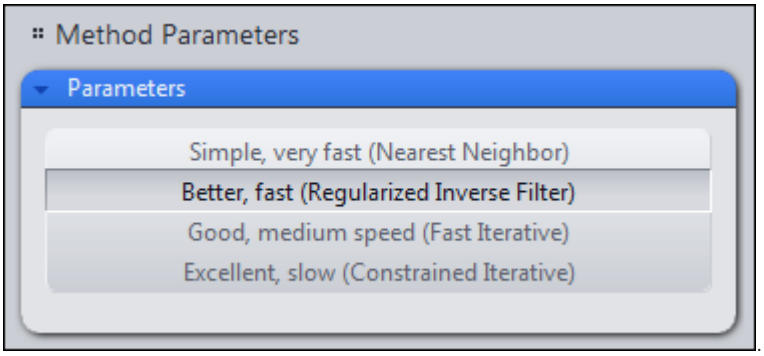
3 Click on **Deconvolution (defaults).**



4 Open the **Parameters** tool under **Method Parameters**.

Here you will see 4 different algorithms for deconvolution (Nearest Neighbor, Inverse Filter, Fast Iterative, Iterative), which you can apply to your image automatically.

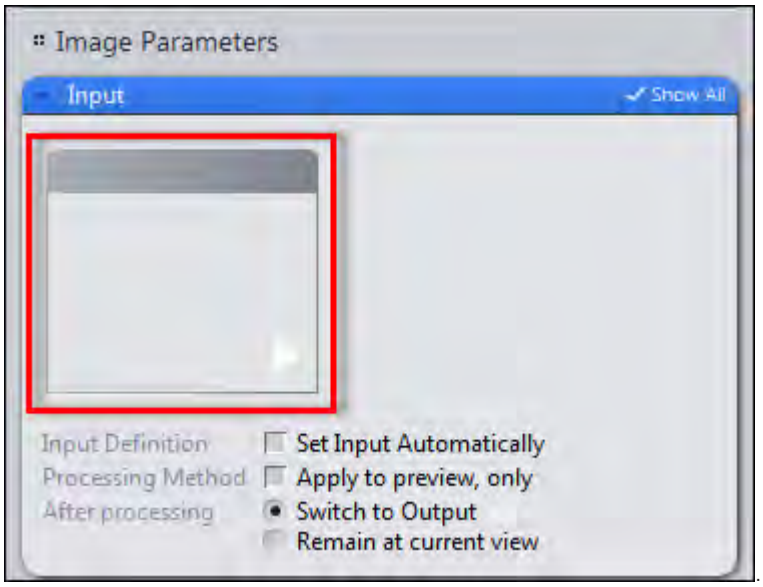
5 To select an algorithm, click on the relevant entry



6 Open the **Input** tool under **Image Parameters.**

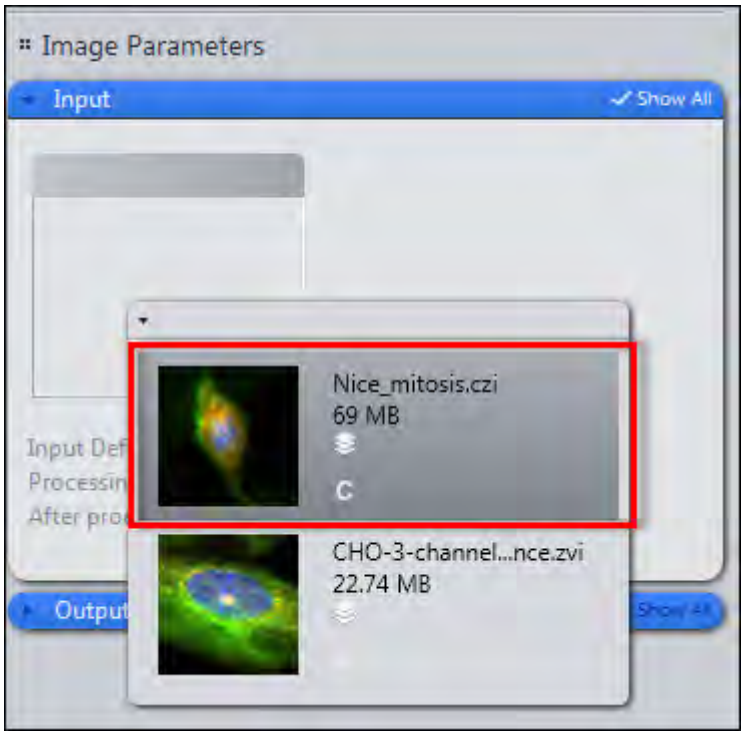
You will see the **Input** tool. If the **Set Input Automatically** checkbox is checked, the currently active image has been loaded as input image automatically. If the checkbox is unchecked, the container for the input image is empty. In this case continue with step 7.

7 Click on the empty image container.



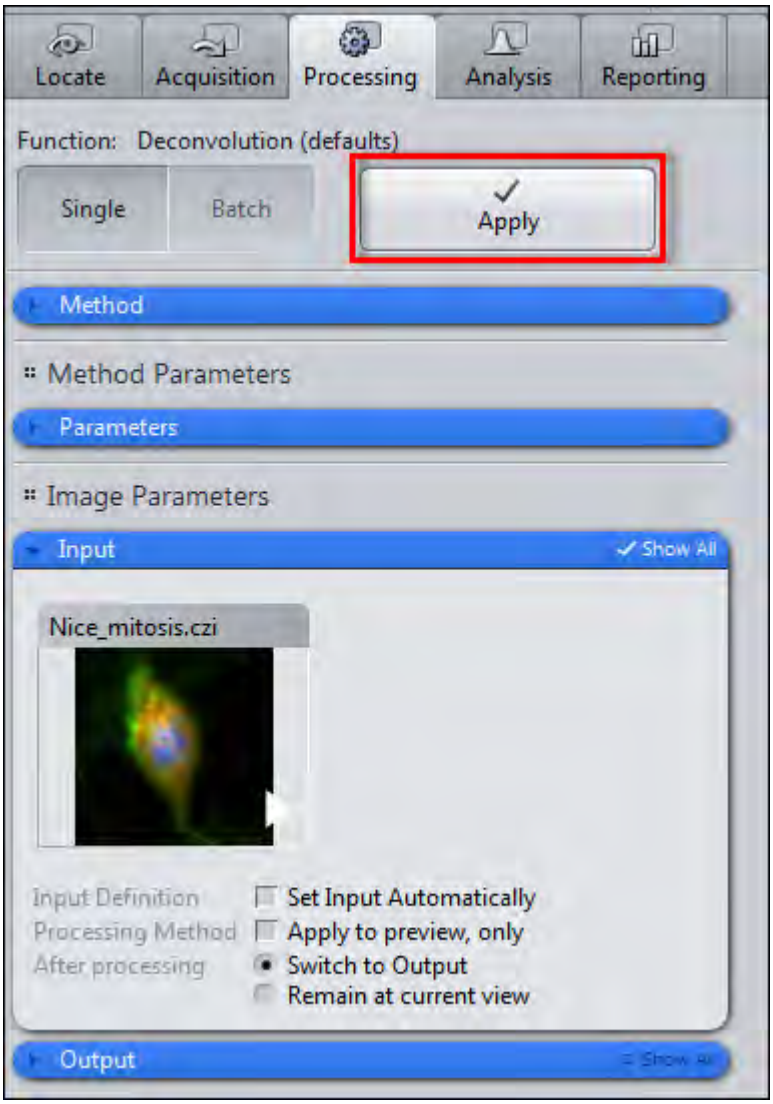
A list opens containing preview images of all images currently open.

8 Click on the image to which you want to apply deconvolution.



The image can now be found in the image container and will be used as the input image for processing.

9 To perform deconvolution, click on the **Apply** button.



Deconvolution is performed. A new image file is generated and is opened automatically in the center screen area after processing. If you are satisfied with the result, save the processed image. Repeat deconvolution using the other default values to obtain different results. If you have expert knowledge, you can configure all the deconvolution settings yourself using the **Deconvolution (adjustable)** method.

5.4 Performing Configurable Deconvolution

These instructions explain how to deconvolve a Z-stack image correctly step by step.

We will use the best method **Constrained Iterative** and a **theoretical PSF**.

Preparation

To follow these instructions you will need a Z-stack image of your sample. You have opened the software and no images are loaded.

Overview

The following steps are described in these instructions.

Prerequisites

- You are on the **Processing** tab.
- You have acquired or opened a fluorescence image on which you wish to perform deconvolution.
- All tools are in **Show All** mode.

Steps

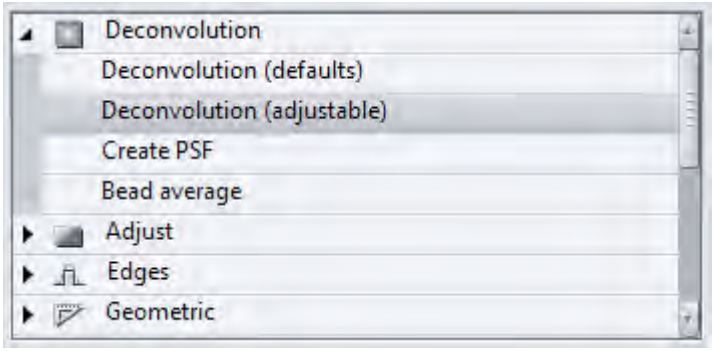
- **Step 1: Load input image**
  In this section you will find out how to load an input image in Deconvolution (Configurable).
- **Step 2: Set parameters**
- In this section you will find out how to set the parameters.
- **Step 3: Process image**
  In this section you will find out how to process the image and compare it with the input image.

5.4.1 Step 1: Load input image for deconvolution

In this step you will select the image to be processed and load it as an input image for deconvolution.

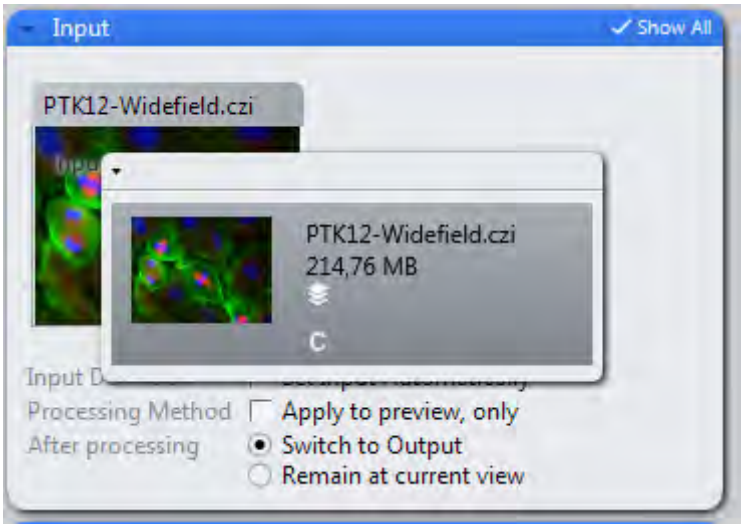
Procedure

1 On the **Processing** tab **| Method** tool in the **Deconvolution** group select the **Deconvolution (adjustable)**method



2 In the **Input** tool select the image that you want to deconvolve. If the **Set Input Automatically** checkbox is checked, the currently active image has been loaded as input image automatically. Since this means, that the output image is

then also automatically selected as input, it is recommended to uncheck this checkbox. Select the desired input image.



INFO

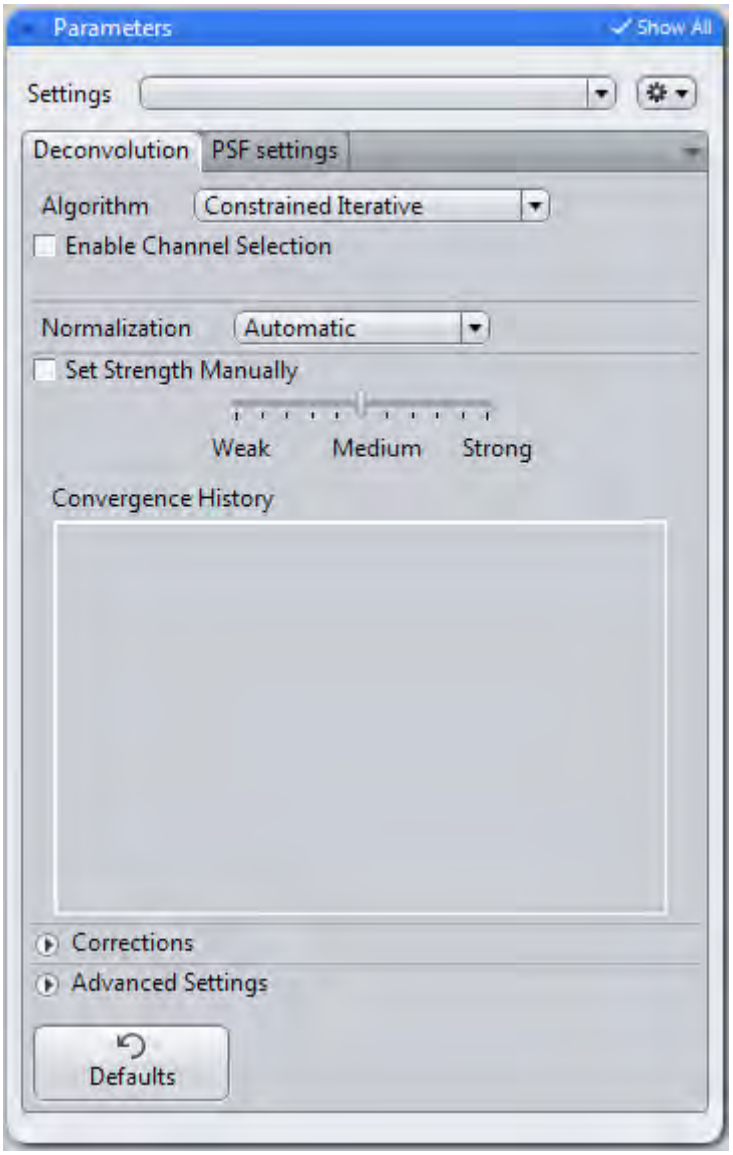
If a warning appears at this point, it is likely that parameters required for deconvolution are missing from the image. You can subsequently enter or change these values in the **Parameters** tool | **PSF settings** tab | **Microscope** parameters.

5.4.2 Step 2: Set parameters for deconvolution

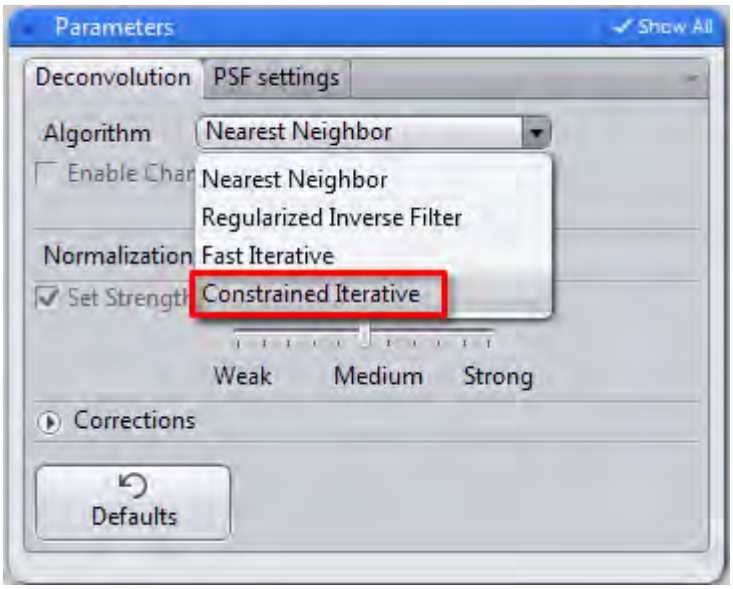
In this step you will choose the desired algorithm and the associated method parameters. Then you will perform deconvolution and save the result.

Prerequisites

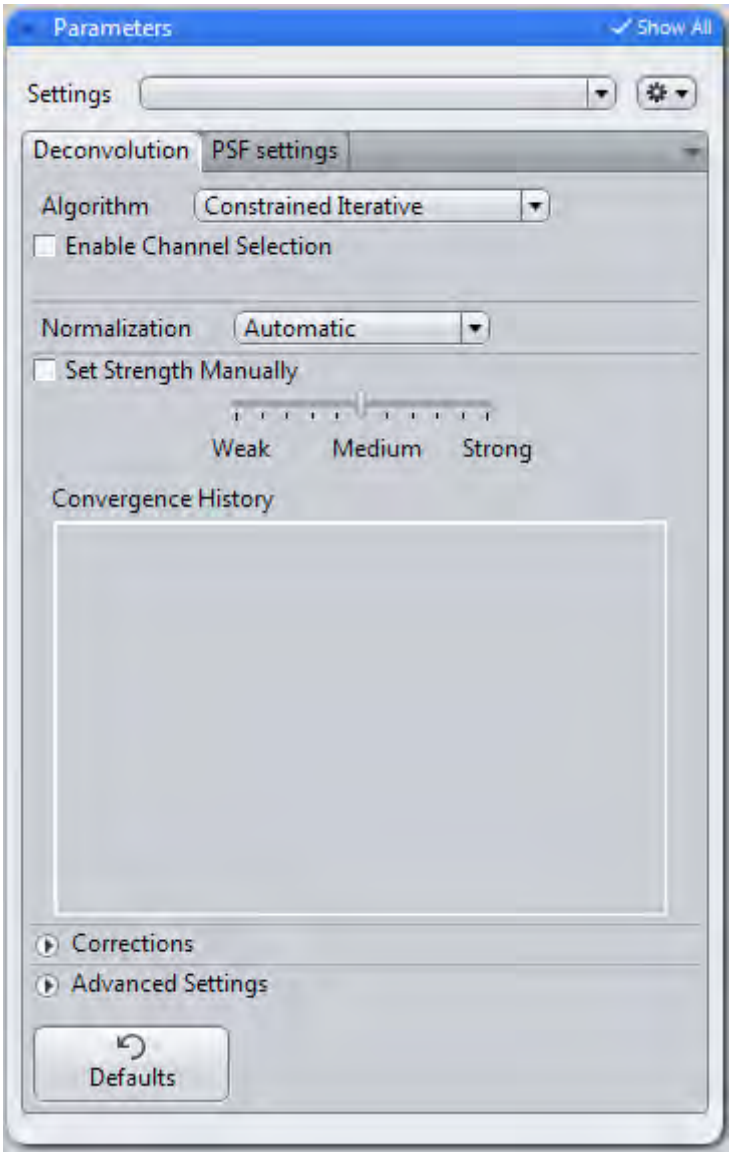
- On the **Processing** tab, in the **Method Parameters** group, you have opened the **Parameters** tool in the **Show All** mode. You can usually leave these parameters alone as they are automatically set to give you a good result.



Procedure **1** On the **Deconvolution** tab first set the desired algorithm. In our example we are using the Constrained Iterative algorithm, which is the most complex algorithm, but normally the best one to use.



You will now see a number of additional parameters for the Constrained Iterative method appear on the tab (**Show All** mode).



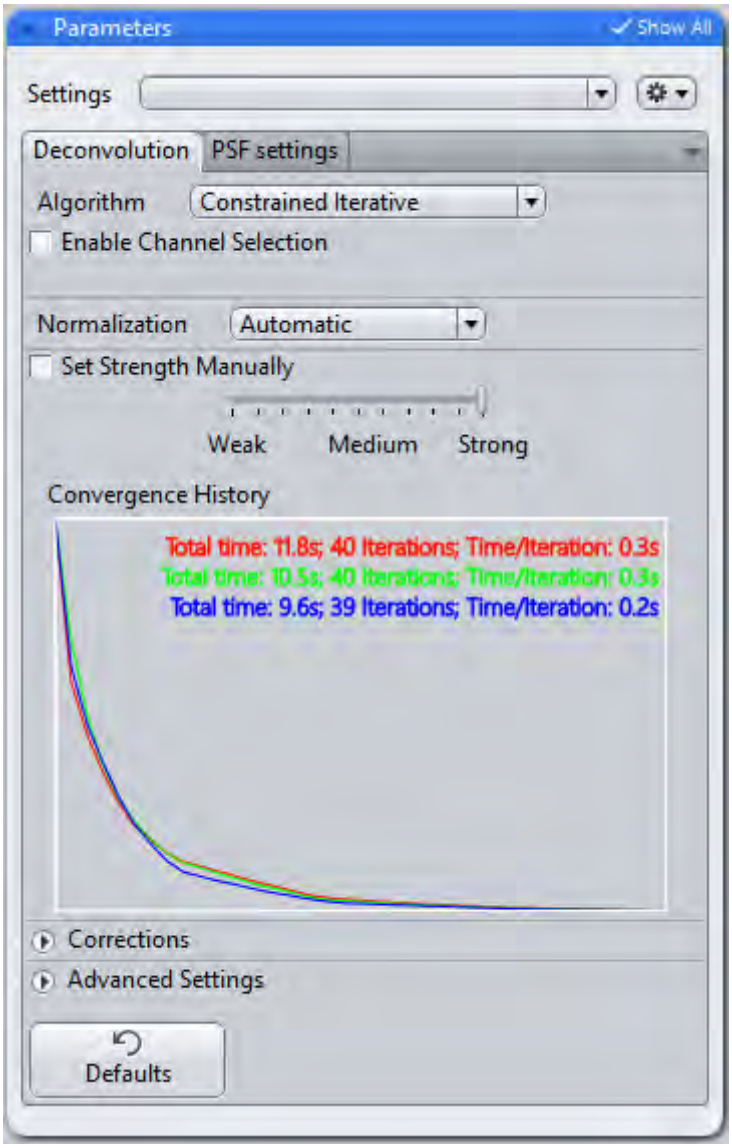
5.4.3 Step 3: Perform deconvolution

In the last step you will perform deconvolution. The resulting image will be compared with the input image and details relating to the processing procedure will be observed.

Procedure

1. Go to the **Deconvolution** tab in the **Parameters** tool to keep the **Diagnosis** section in the foreground.
2. Click on the **Apply** button.

You can monitor the progress in the **Convergence History** graph, in which the gradual improvement is plotted against the number of iterations.

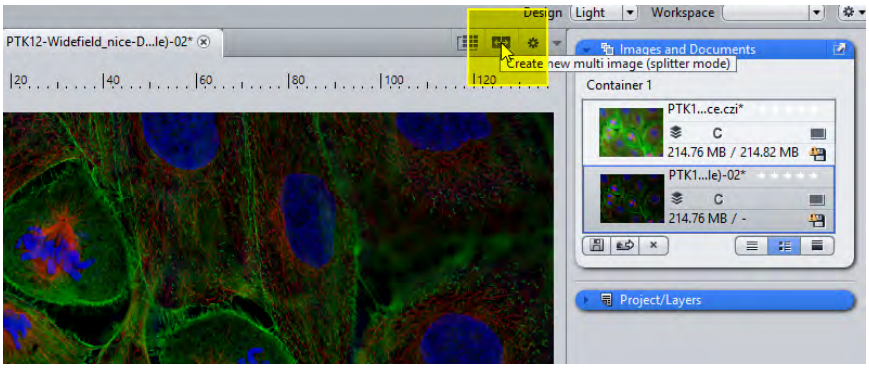


INFO

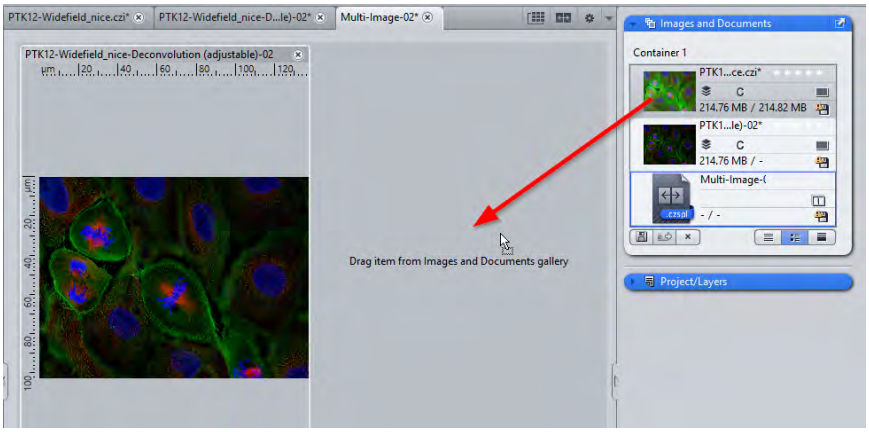
The processing of large Z-stack images or long time series can take some time. During processing we recommend that you do not perform any other complex actions in ZEN or in other programs on the computer, to avoid increasing the processing time unnecessarily.

3 Save the resulting image under a meaningful name.

4 Click on the **Split Mode** button in the document bar. This creates a multi image (split image).



5 The currently active image is loaded automatically into the multi image. Now drag the input image from the document gallery in the Right Tool Area into the split document.



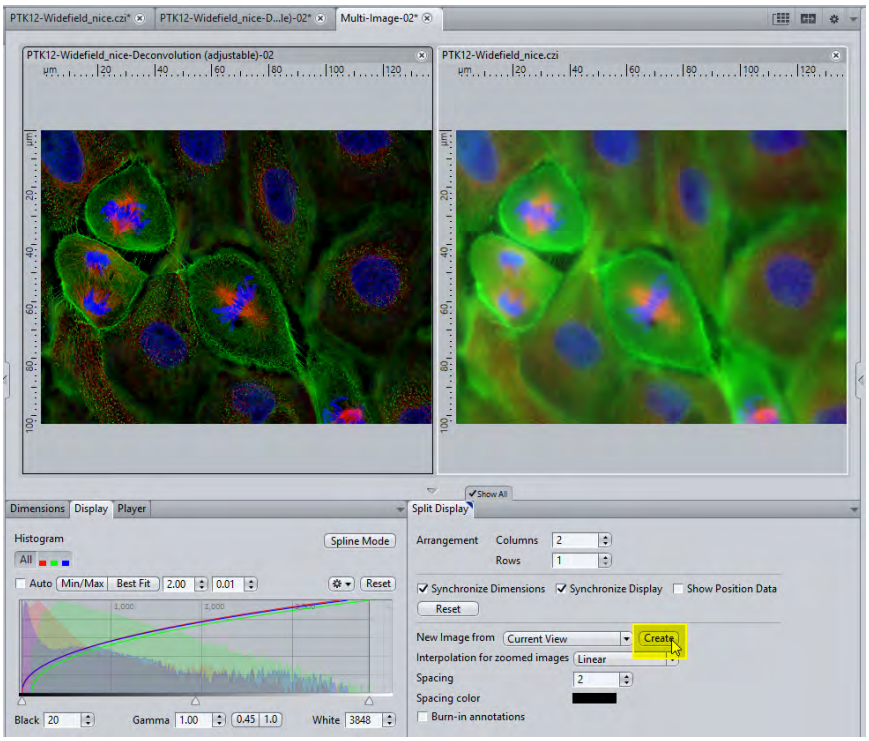
6 On the **Display** tab, change and adjust the display as desired, e.g. **Best Fit** with 0.01 and for black and white value with a **Gamma** of 0.8.

Both images are adjusted simultaneously.

7 If on the **Split Display** tab the **Synchronize Dimensions** checkbox is activated, you can now zoom synchronously into the images (mouse wheel) and, with the mouse wheel held down, move the image content as desired to focus on the regions of interest.

8 To create an image of the desired view click on **New Image from** | **Current View** | **Create**.

This creates a new output image document with both images shown side by side.



You have successfully performed deconvolution, observed the processing procedure and created an output image to compare the deconvolved with the input image.

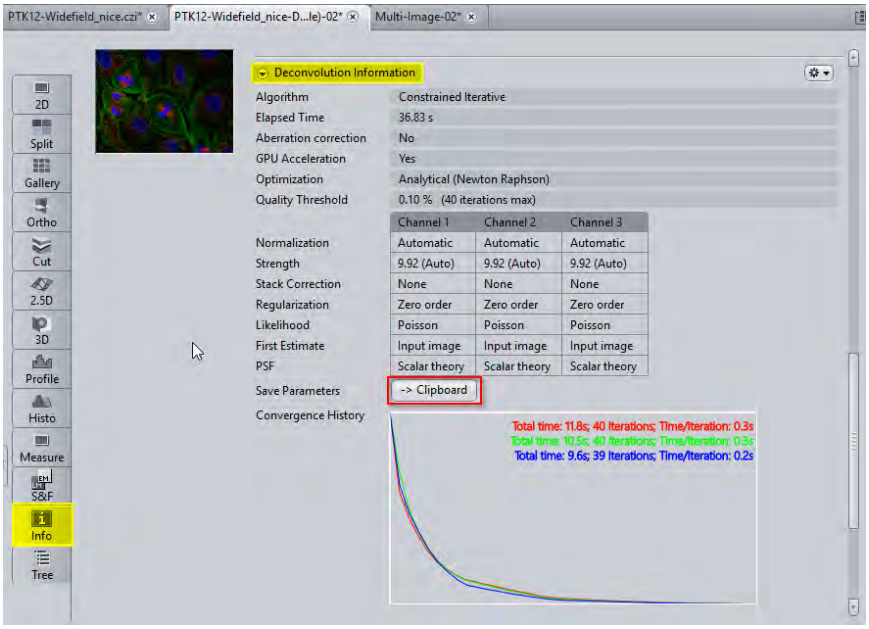
5.4.4 Step 4: Info View and re-using Deconvolution parameters from a processed image

The **Info View** contains a section **Deconvolution Information**, which shows a summary of the parameters used for deconvolution of the image. It also contains the **Convergence History** graph displaying the time it took to get this image processed.

If you like to use the same settings in order to process another image, the following steps show how to do this:

Procedure **1** Select the processed image from the previous steps.

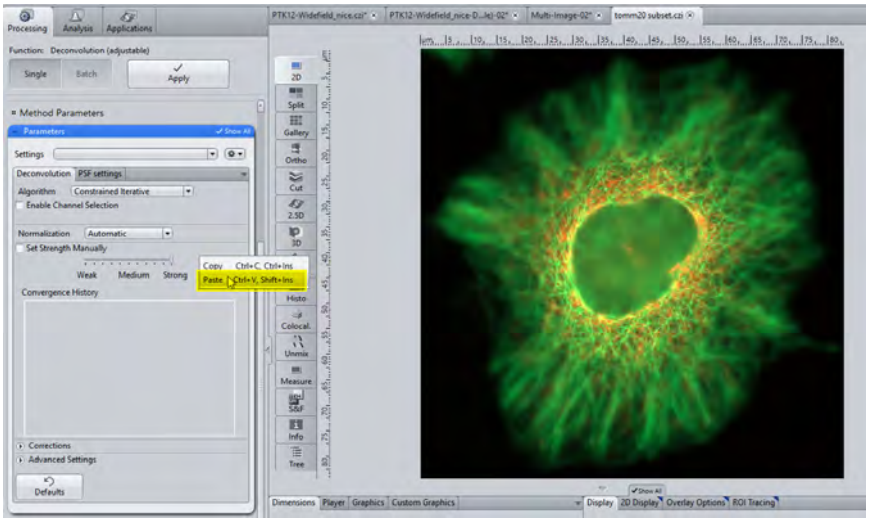
2 Select the **Info View** and scroll down to the **Deconvolution Information** group:



3 Press the **Clipboard** button.

All deconvolution parameters are copied to the clipboard.

4 Open the new image you want to deconvolve and select it as input in the **Deconvolution (adjustable)** function. Right mouse-click and select **Copy** and then right mouse-click and select **Paste** to copy the parameters into Deconvolution.



5 Press the **Apply** button to run Deconvolution with the identical function parameters as used for the previous image.

5.5 Measuring the PSF using subresolution beads

When measuring the PSF (point spread function) you perform the following process. Note the prerequisite preparation and hints.

Preparation

Procedure

1. The surface of coverslips is hydrophobic which means, liquid droplets do not spread out easily and beads tend to aggregate at the edges. For PSF measurements you want individually spread out beads.
2. Bath the coverslips for 10 minutes in 100% ethanol.
3. Use forceps to remove the coverslip. Shake off excess liquid and run through bunsen burner flame.

   This makes the surface slightly hydrophilic which means, the droplets and beads spread out easier.

i INFO

Ideally, use ZEISS coverslips with a defined thickness of 170 µm. However, coverslips and mounting media for bead measurements must be identical to the ones used for the sample, the image of which shall be deconvolved. Beads should have a diameter below the resolution limit of the objective, e.g. 0.175 µm. Smaller diameters are better, but smaller beads are dimmer and can therefore be difficult to locate on the cover slip.

Tetraspeck beads from Thermo Fisher Scientific have the advantage of covering four colors which are frequently used in imaging research, but some batches can show rapid loss of fluorescence. Single color beads are typically brighter.

4. Break up agglomerates by sonicating stock suspension in a waterbath for 20 minutes. Stocks suspensions are way too dense, so dilute 1:100 with 70% ethanol.
5. Create further dilutions of 1:1.000 and 1:10.000 by adding 100 µl to 900µl 70% ethanol. Mix well using a Vortex mixer.
6. Put one 5 µl drop for each dilution on a cover slip using a 20 µl Eppendorf pipette
7. Let dry. This should take less than 5 minutes. You can speed it up by putting the cover slip on a warm surface.
8. Put 10-20 µl mounting medium on the coverslip. For aqueous mounting media seal edges of coverslip with valap (1:1:1 mixture of vaseline, lanolin, paraffin), nailpolish or paraffin.

In the next step, you acquire an image.

Imaging

Procedure

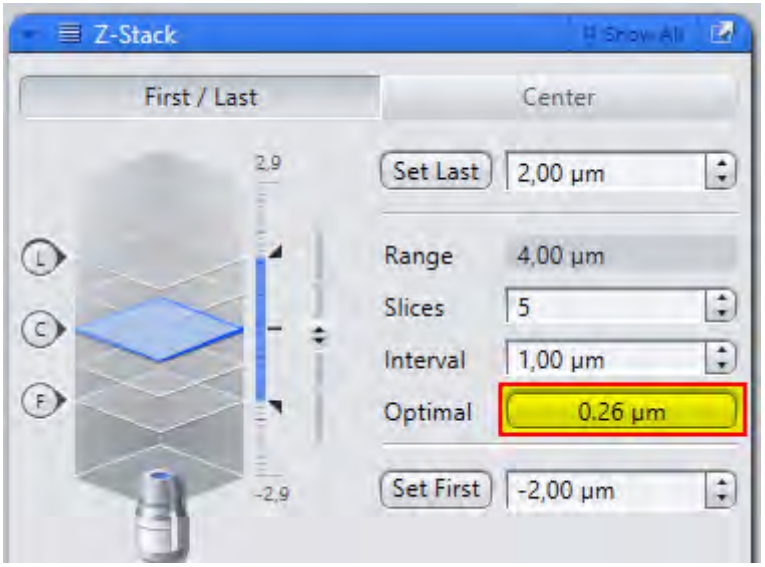
1. Locate the beads on the microscope (e.g. use a 20 x lens first, then move to 63x oil). Start at the 1:100 spot which should have tons of beads and be easy to find.
2. When found, move to a sparser spot and try to find an area with a couple of single beads in the FOV. This can be tricky, but usually you will be able to find good areas if you keep looking around.
3. Acquire a Z-stack of a suitable area, observing the following rules.

   No saturation. This can be tricky with LSM's. Use 12-bit mode at least.

   Only fill the dynamic range in the histogram to about 80%.

   Make sure to focus up and down when setting up the exposure times to measure the exposure suitable for the bright bead center to avoid the risk of saturation.

4. Set up the z-stack as follows: in the Z-stack tool, click on the **Optimal** button.



   This sets the distance according to Nyquist. For bead measurments further reduce the slice distance to about half of what **Optimal** suggests.

5. Also, define the top and bottom of the stack in such a way that the airy disc of the beads cannot be distinguished any longer.
6. When ready, save and name the image properly.

Look at the result in **OrthoView**: Do you see spherical aberrations? Are the beads symmetrical? Are there enough individual beads in the stack? Is the background low enough?

Processing in ZEN Blue

Procedure

1 Use the PSF wizard (Processing/Deconvolution) to extract the PSF from the bead-z-stack. For more information, see *Creating a PSF - With Wizard and Without* [▶ 140].

The PSF wizard guides you step by step through the necessary procedure.

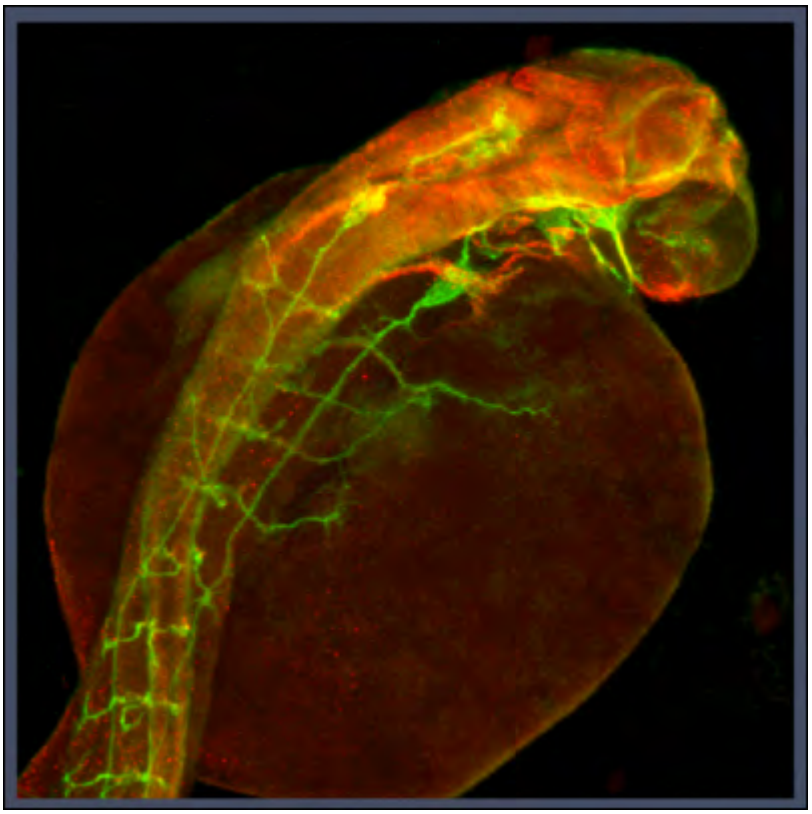
The result of the wizard is a PSF file which you use in deconvolution for images acquired under the same conditions.

5.6 Extracting Individual Fluorescence Images of a Multichannel Image

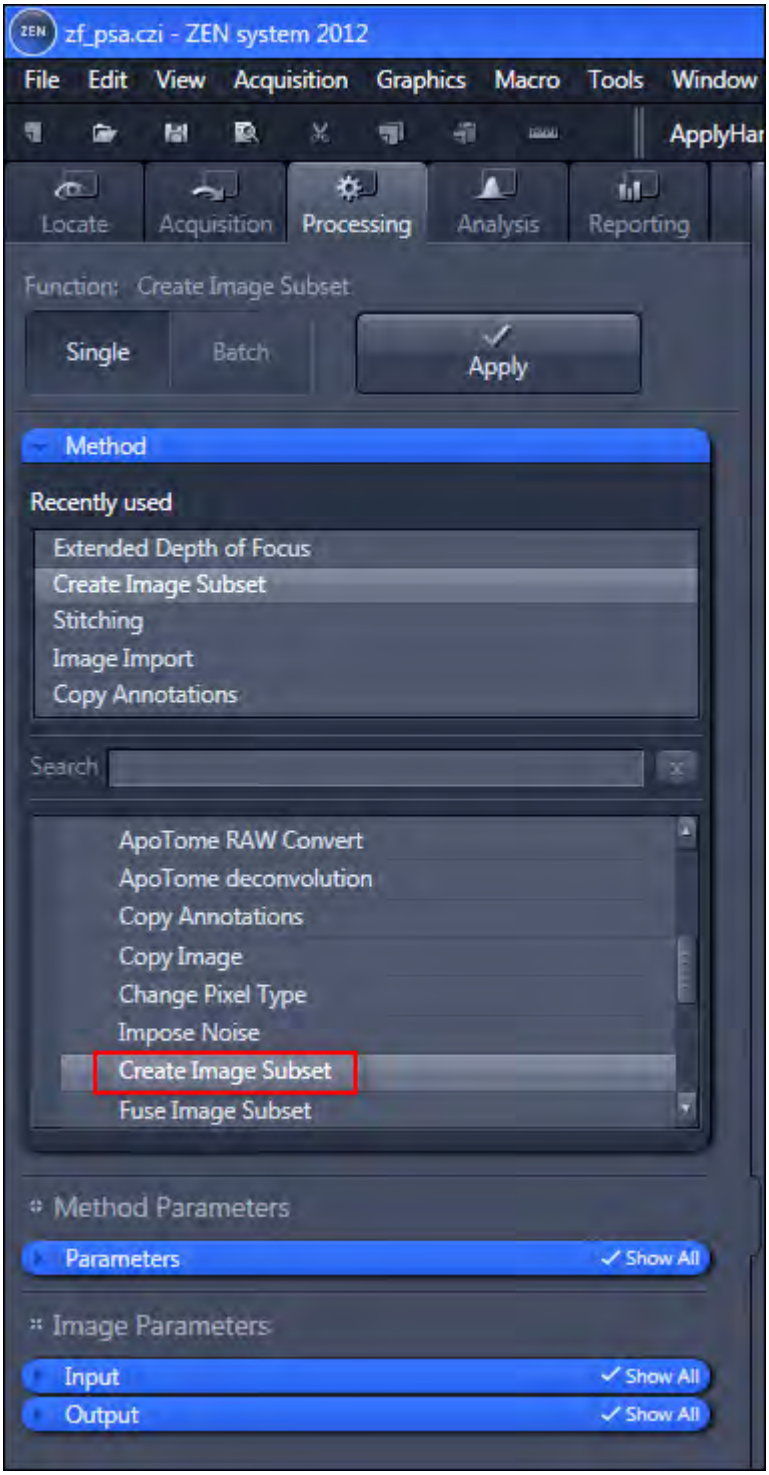
In this topic we will show you how to extract individual fluorescence channel images of a multichannel image.

Prerequisites

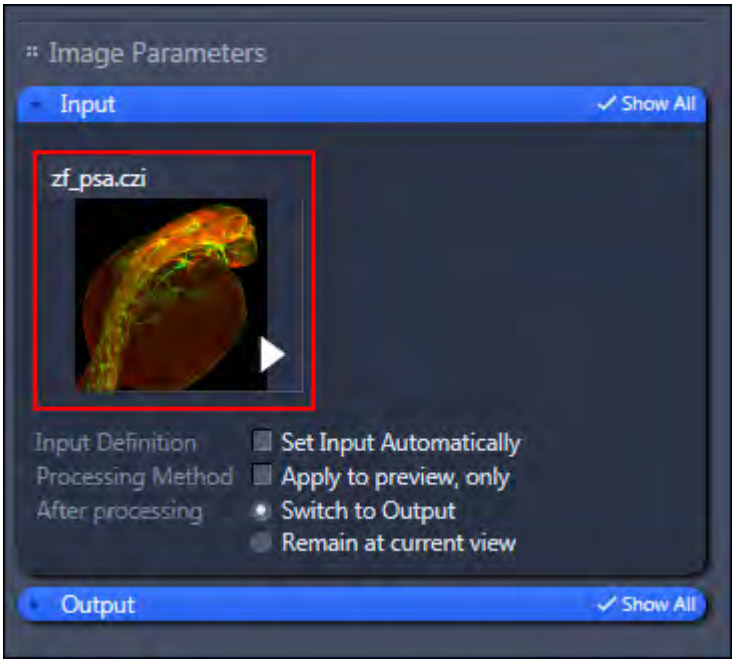
- You have acquired or opened a multichannel image (for example product DVD 40 (09/2011)-(ZEN)\Example Image Databases\zf_psa.czi).



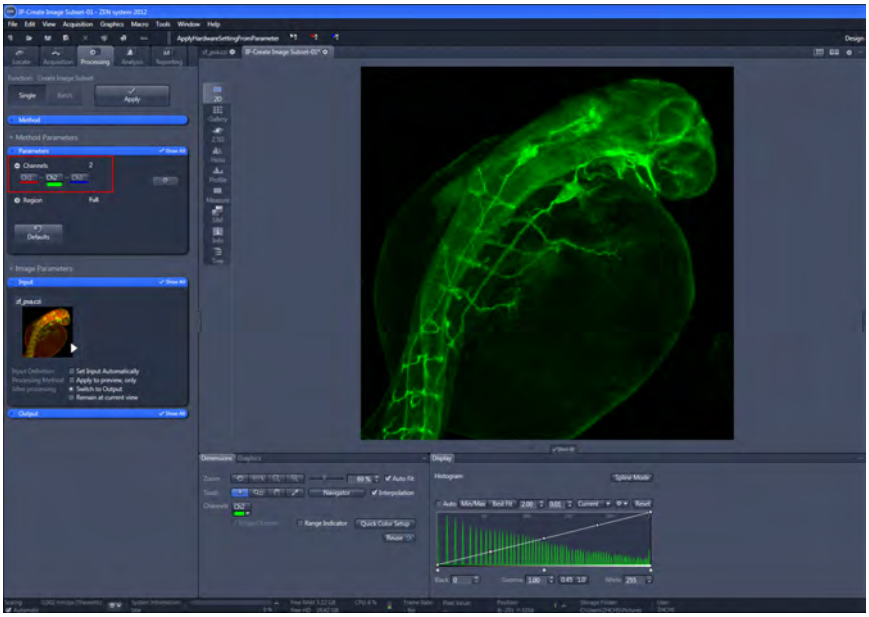
Procedure **1** Select the **Processing** tab. Open the **Method** tool and select under **Utilities** the entry **Create Image Subset.**



2 In the section **Image Parameters** open the **Input** tool and select the multichannel image as Input image.



3 In the section **Method Parameters** open the **Parameter** tool and the select the entry **Channels**. Deactivate the red and the blue fluorescence channel. Now only the green fluorescence image is selected.



4 Click on the **Apply** button.

You have the green fluorescence channel image extracted of the Multi-Channel image. Activate the corresponding channel and deactivate the other channels to extract further individual fluorescence images. Save the extracted sub images as separate files.

5.7 Applying Batch Processing

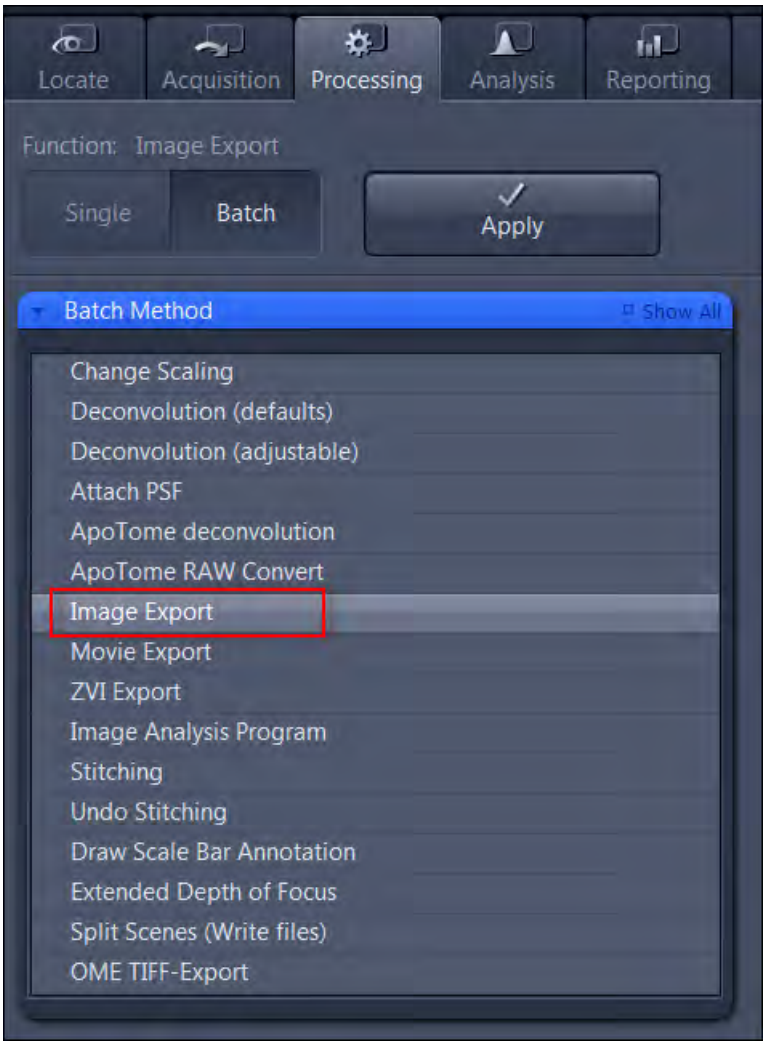
In this topic we will show you how to export all images of a folder as batch. For each image you can use identical or different export settings.

Prerequisites

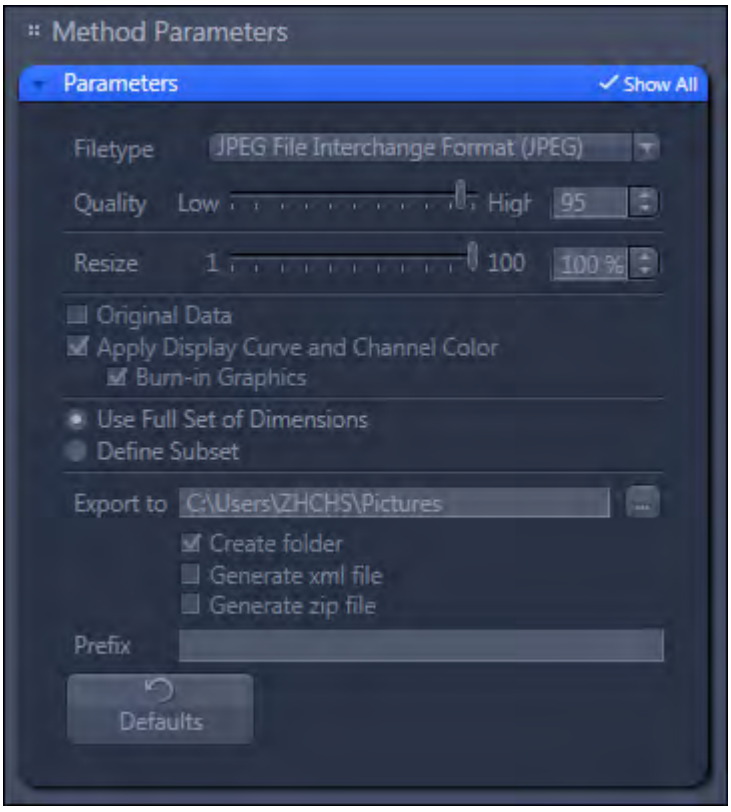
- You have a folder with several CZI-images to be exported in a new image type (ie. TIFF, BMP, JPEG). For example two 2channel-Timelapse images, one 2channel-Z-stack image, one 3Channel image of the product DVD 40 (09/2011)-(ZEN)\Example Image Databases\CaRationSeries.czi, kaede5lsm.czi, ratbrainstack.czi, zf_psa.czi).

Procedure

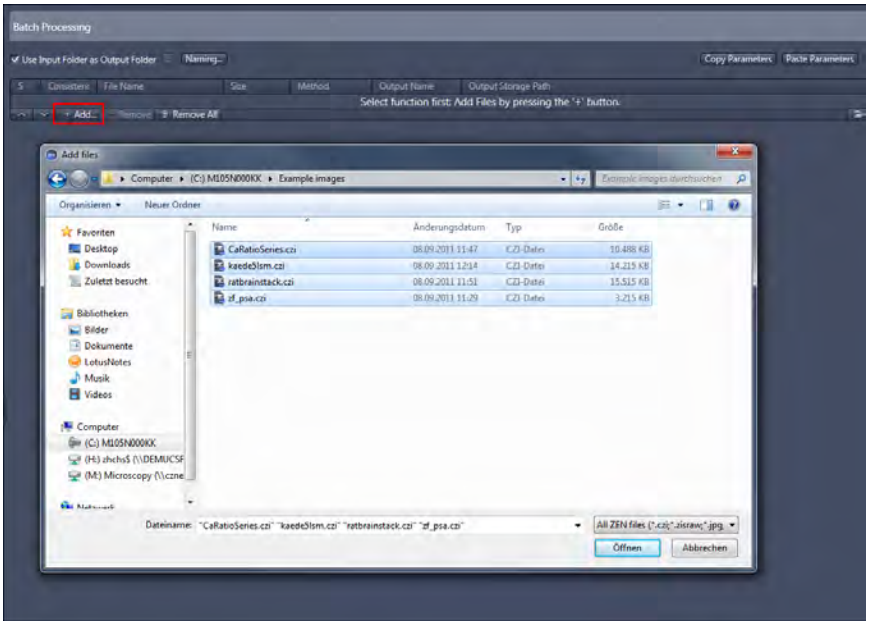
1. Select the **Processing** tab. Click on the **Batch** button.
2. Open the **Method** tool and select the entry **Image Export**.



3 Open in the **Method Parameters** section the **Parameters** tool.

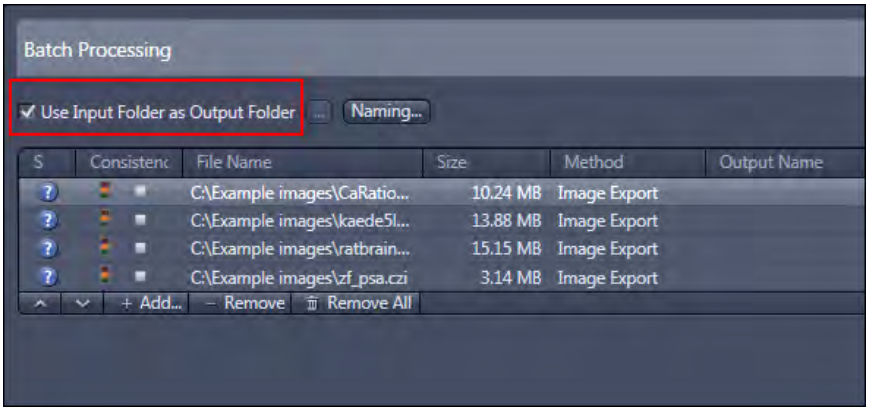


4 Click in the tool **Batch Processing** on the **Add** button. Select the folder and marked all images to be exported and click on the **Open** button.



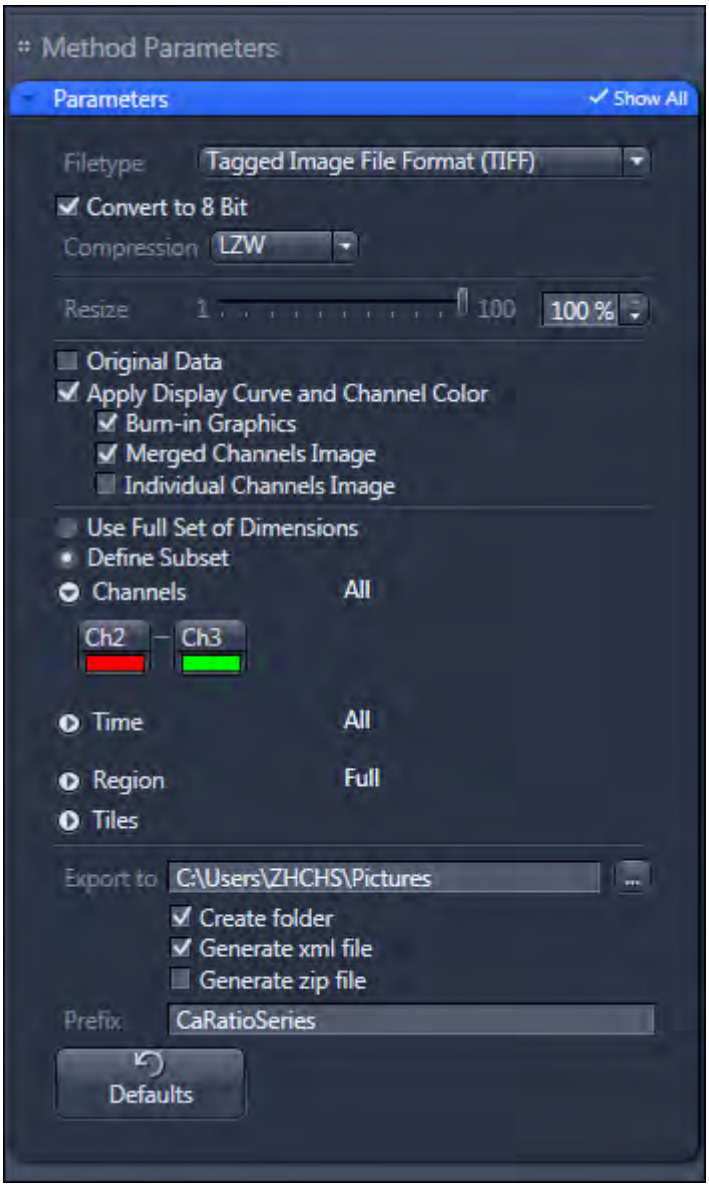
The list of images is displayed in the **Batch Processing.**

5 Activate the checkbox **Use Input Folder as Output Folder** to save the exported images in the folder of the original images.



6 Click on one image in the list and do the export settings in the **Parameters** tool. In the example the TIFF format will be used for all time points and channels. This setting is only valid for the selected image, but can be copied to

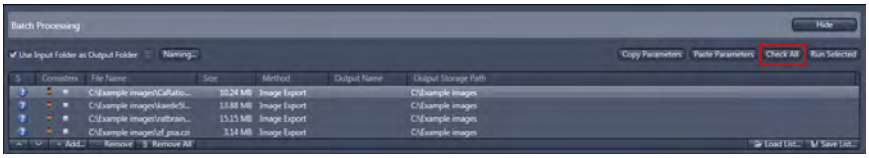
further images in the list with identical dimensions (i.e. 2channel Time-Series image).



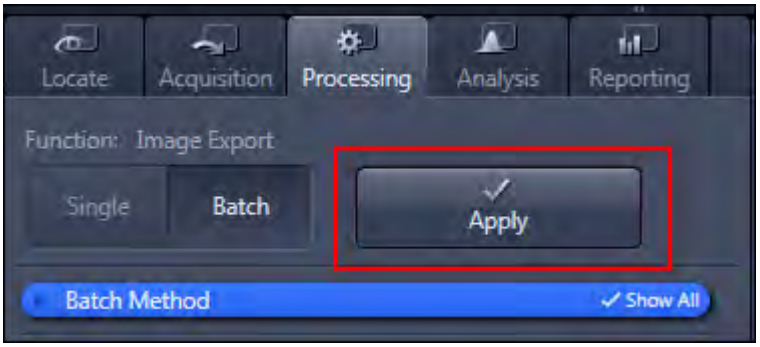
7 Click on the **Copy Parameters** Copy Parameters button. Select the image with identical dimensions in the list and click on the **Paste Parameters** Paste Parameters button.

8 Continue with the other images of the list. Use **Copy Parameters** and **Paste Parameters** or define the export settings for each image individually in the **Parameters** tool.

9 Click on the **Check All** button. All images in the list will be tagged with a green marker, if they have the correct setting.



10 Click on the **Apply** button.



All exported images in the list will be checked and are exported in separate folders in the Input folder.

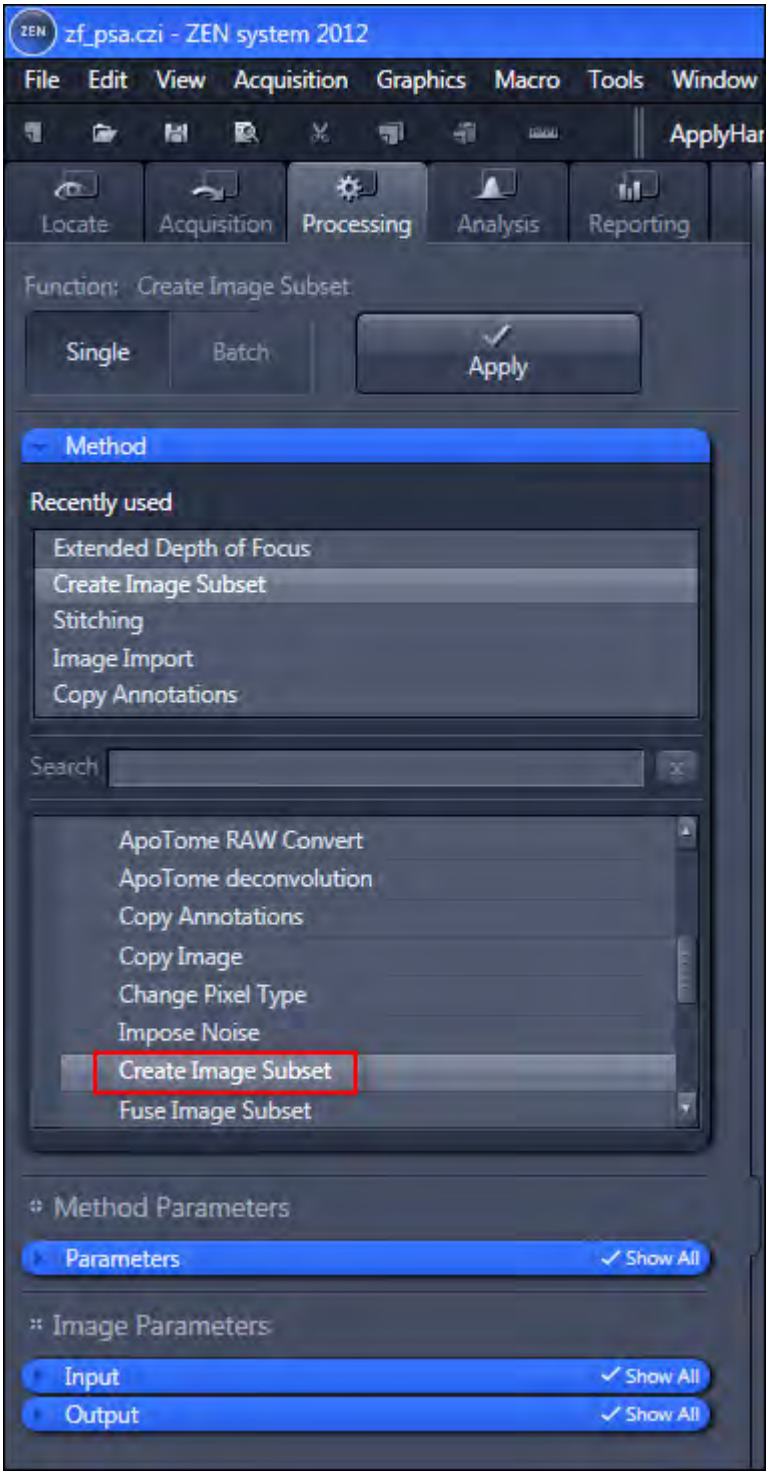
5.8 Cropping a ROI

In this topic we will show you how to crop a region of interest (ROI) of a image.

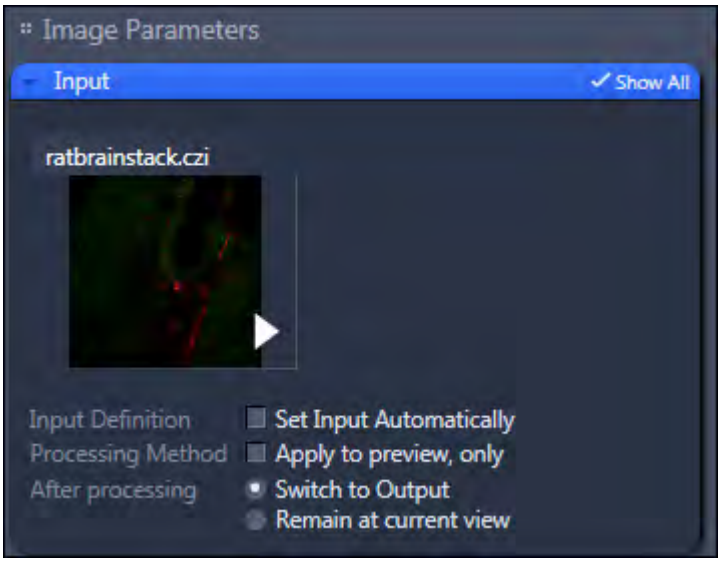
Prerequisites

- You have acquired or opened a multichannel image.

Procedure **1** Select the **Processing** tab. Open the **Method** tool and select under **Utilities** the entry **Create Image Subset**.



2 In the **Image Parameters** section open the **Input** tool and select the image.

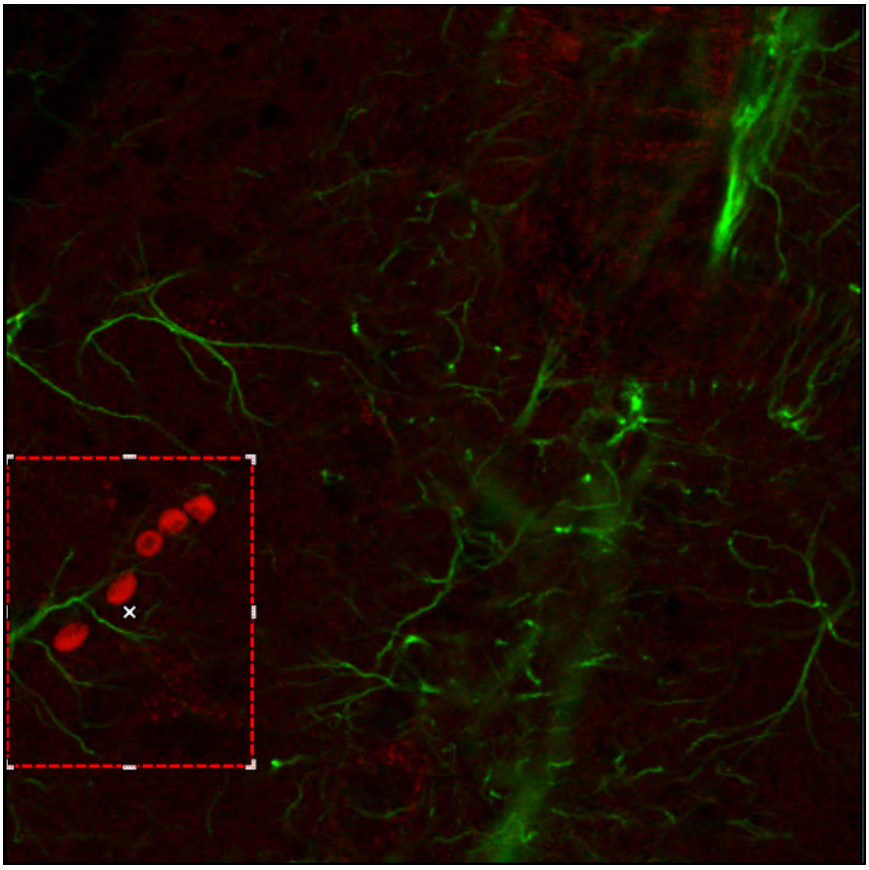


3 In the **Method Parameters** section open the **Parameters** tool.

4 Open the **Z-position** dimension setting and select **Extract Range** of the dropdown list. Set the start position to **11** and the end position to **20** using the slider.

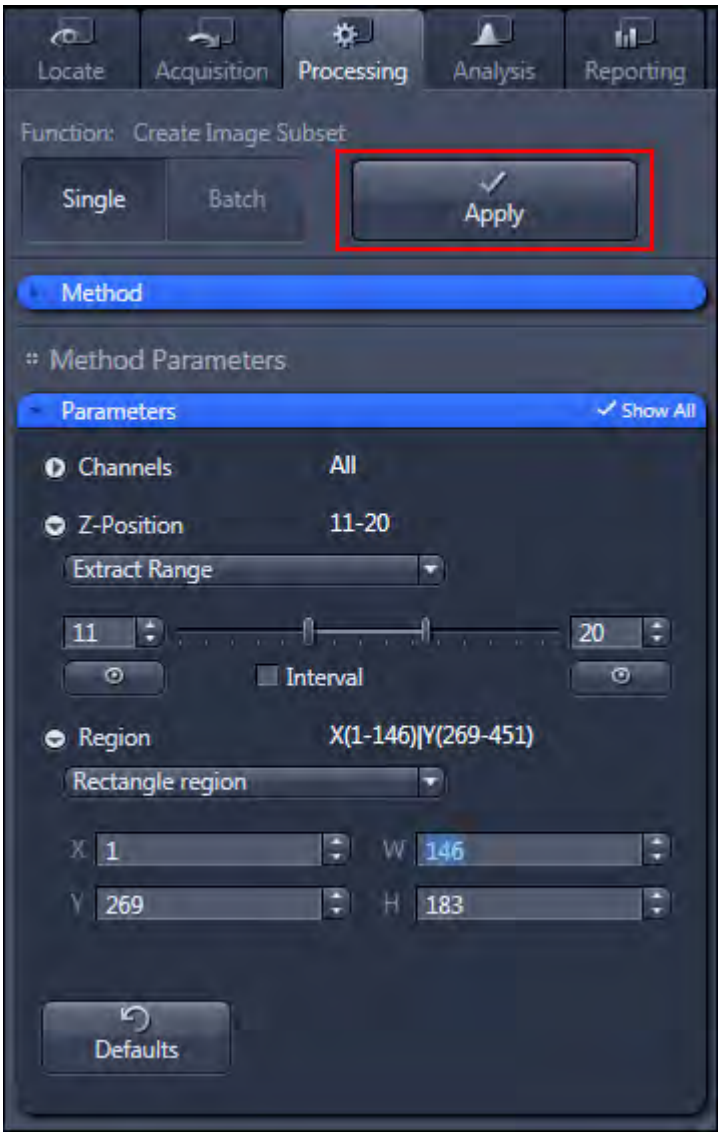
5 Open the **Region** setting and select **Rectangular Region** of the dropdown list.

6 Click in the image on the start position of the ROI and drag out a rectangular region.



You have marked the ROI which should be cropped.

7 Click on the **Apply** button.



The marked ROI with the defined dimensions is extracted of the image. You can now save or process this image.

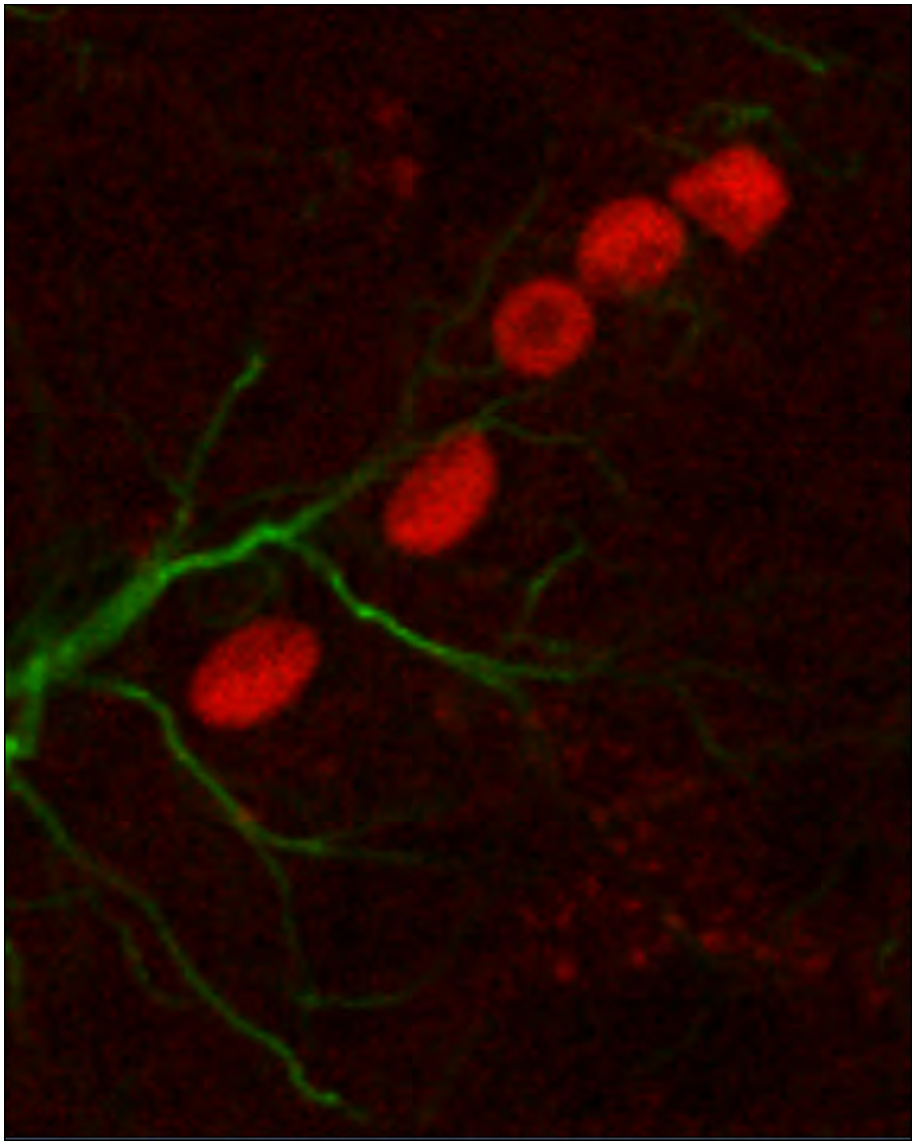


*Fig. 5.2: Cropped ROI with the dimensions **All channels** and the **Z-Postion 11-20**.*

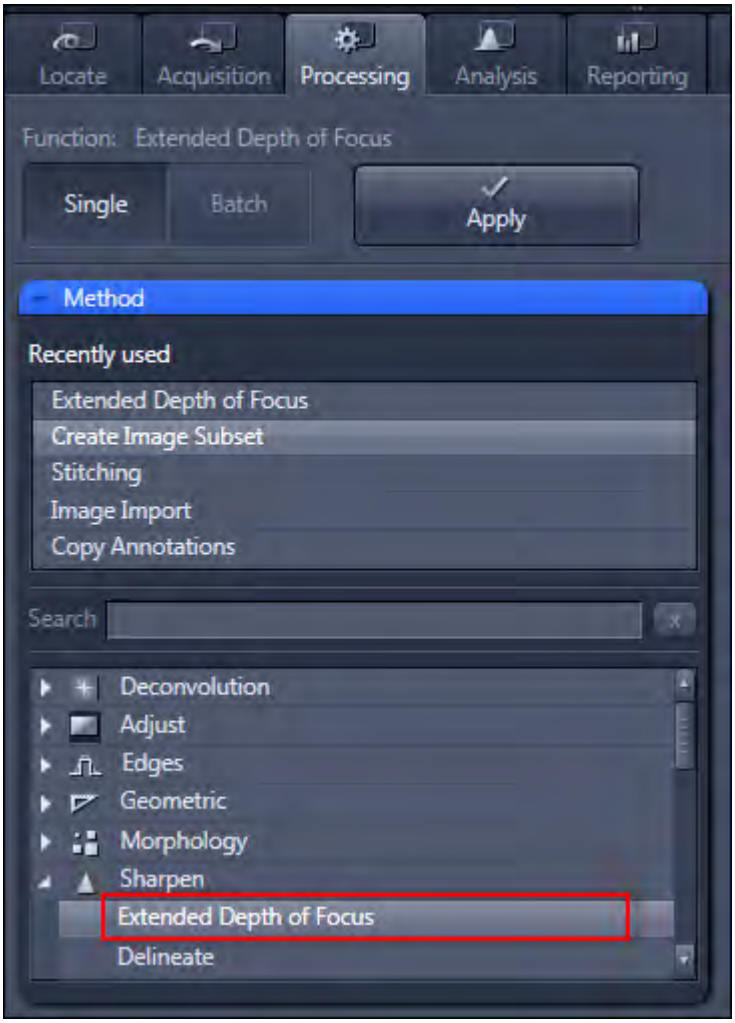
5.9 Creating an EDF Image

In this topic we will show you how to create an extended depth of focus (EDF) image of a Z-Stack image. The focus planes of all the z-positions will be calculated to one EDF image.

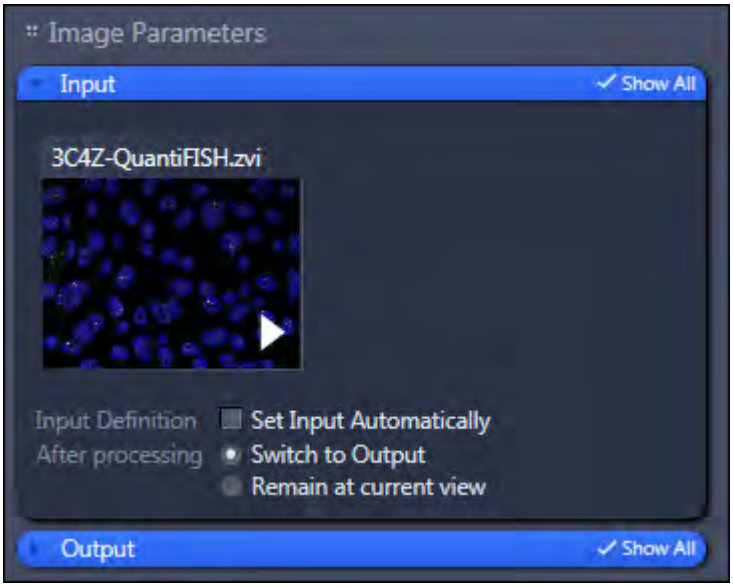
Prerequisites

- You have acquired or opened a z-stack image.

Procedure 1 Select the **Processing** tab. Open the **Method** tool and select under **Sharpen** the entry **Extended Depth of Focus**.



2 In the **Image Parameters** section open the **Input** tool and select the Z-Stack image as Input image.

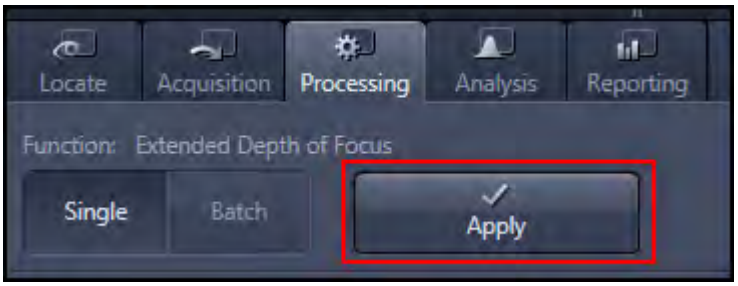


3 In the **Method Parameters** section open the **Parameters** tool.

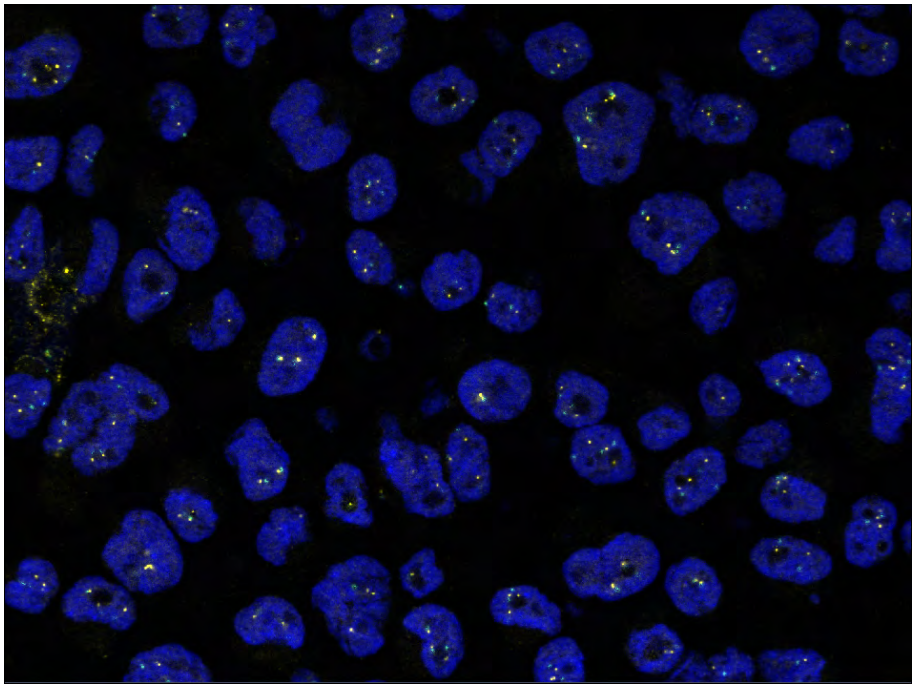
4 Select **Wavelets** of the **Method** dropdown list.

5 Select **High** of the **Z-Stack Alignment** dropdown list.

6 Click on the **Apply** button.



All focus planes of the Z-Stack image are now calculated to one EDF image.



5.10 Image Processing Functions

5.10.1 Deconvolution

5.10.1.1 Introduction

Microscopy creates images of objects which should represent the nature of the object as well as possible. Fluorescent light, which emanates from the object, passes through the various optical elements of the beampath and eventually gets collected by the detector. Unfortunately, on the way to the detector the signal is changed in such a way, that the quality of the resulting image suffers. As a consequence, the image is never a 100% correct representation of the object. This effect is strongest seen in classical widefield fluorescence microscopy which does in fact not offer any optical sectioning capability, but also exists to a different degree in optical sectioning microscope systems, e.g. Confocal, Lightsheet or ApoTome.

Fortunately, the dominant function which has this deleterious effect on the image, is based on the optical design principles of the light microscope and therefore well understood. We call this the point spread function (PSF) of the microscope system.

Deconvolution is a mathematical method which can reverse the effect of the PSF on the image and can therefore to a large extent restore the image to better represent the object. In the case of widefield imaging, Deconvolution can even convey optical sectioning properties to the result image allowing true three-dimensional restoration.

The following improvements can be obtained by using deconvolution:

- Denoising
- Removal of out of focus light -> deblurring, improved contrast
- Increasing signal to noise ratio by reassigning photons
- Restoration of sparsely sampled data
- Increase of resolution in X, Y and Z

As the object and the way, it was prepared, becomes part of the optical system during imaging, the largest variable to consider when doing deconvolution is the sample itself. Since the sample conditions can vary widely, information about the sample needs to be provided to the deconvolution function. The better the sample conditions are known, the better the outcome will be.

Deconvolution in ZEN offers a wide range of parameters, which allow us to adapt the deconvolution to the sample conditions. The downside of this fact is, that using these parameters can be a challenge.

The following functions are available for deconvolution in ZEN and are described in the following chapters:

- **Deconvolution (defaults):** Four methods are available and are automatically adapted to the type of instrument, which was used to acquire the image. This is the easiest to use deconvolution function
- **Deconvolution (adjustable):** Offers access to all available function parameters and therefore provides the necessary flexibility for demanding samples and sample conditions.
- **PSF Wizard:** this function offers a wizard which guides the user through a series of steps to create a PSF from a z-stack image of multiple fluorescent beads. This function is recommended to create experimentally measured PSF's.

5.10.1.2 Deconvolution (defaults)

This method allows you to use 4 different algorithms for deconvolution, without any further settings.
The following algorithms are provided in the **Parameter** tool:

Parameter	Description
Simple, very fast (Nearest Neighbor)	Executes the fast Nearest Neighbor method using default parameters.
Better, fast (Regularized Inverse Filter)	Executes the Regularized Inverse Filter algorithm for image enhancement.
Good, medium speed (Fast Iterative)	Executes the Fast Iterative restoration method.
Excellent, slow (Constrained Iterative)	Executes the Constrained Iterative quantitative restoration method.

5.10.1.3 Deconvolution (adjustable)

Deconvolution can be done either by using the computers CPU or by using a graphics card. Using a graphics card can speed up deconvolution processing quite dramatically. A NVIDIA graphics card is required which supports the CUDA processing library. Contact your sales representative for further details about supported graphic card models.

For a detailed overview of all deconvolution methods (and its combinations) see *Deconvolution Methods in ZEN* [▶ 142]. This method allows you to use and individually configure 4 different algorithms for deconvolution (short DCV). Under **Parameters** two tabs are available for detailed configuration:

- On the **Deconvolution** tab, you can select the desired algorithm and define the precise settings for it, see *Deconvolution tab* [▶ 127].
- On the **PSF Settings** tab, you can see and change all key parameters for either generating a theoretically calculated PSF, or selecting an experimentally measured PSF, see *PSF Settings tab* [▶ 134].

5.10.1.3.1 Deconvolution tab

INFO

Expert knowledge is required for some of the settings. If you are in doubt, leave the settings unchanged.

Algorithm dropdown list

Here you can select the algorithm that is used. The following algorithms are available:

- Nearest Neighbor
- Regularized Inverse Filter
- Fast Iterative
- Constrained Iterative

Enable Channel Selection

Activated: Applies the settings on a channel-specific basis. This allows you to set parameters for each channel individually. You will see a separate, colored tab for each of the channels.

Deactivated: Applies the same settings to all channels of a multichannel image.

5.10.1.3.1.1 Normalization section

Here you can specify how the data of the resulting image are handled:

Parameter	Description
Clip	Sets negative values to 0 (black). If the values exceed the maximum possible gray value of 65636 when the calculation is performed, they are limited to 65636 (pixel is 100% white). Results from different input images can be quantitatively compared with each other.
Automatic	Normalizes the output image automatically.

Parameter	Description
	In this case the lowest value is 0 and the highest value is the maximum possible gray value in the image (gray value of 65636). The maximum available gray value range is always utilized fully in the resulting image. Results from different input images cannot directly be compared quantitatively with each other.

5.10.1.3.1.2 Set Strength Manually section

If you have selected the **Nearest Neighbor** algorithm, the checkbox is always activated.

INFO

If you have selected the **Fast Iterative** algorithm, the checkbox is also always activated. Using the slider you can then enter the number of iterations used directly, as, in contrast to the other methods, no regularization is performed.

Activated: Enter the desired degree of restoration using the slider.

To achieve strong restoration and best contrast, move the slider towards **Strong**.

To achieve lower restoration but smoother results, move the slider towards **Weak**.

If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear.

Deactivated: Determines the restoration strength for optimum image quality automatically. This is recommended for widefield and confocal images and is therefore deactivated by default.

The restoration strength is inversely proportional to the strength of so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

5.10.1.3.1.3 Convergence History

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Fast Iterative** or **Constrained Iterative** algorithm.

The progress of the calculation is displayed here as line graph. Several quality parameters are measured for each iteration and once either an optimum or the maximum allowed number of iterations is reached, the processing is stopped. This display allows you to observe directly how the iterative method affects the available data. It also shows how many iterations have been used and how much time is being used per iterations.

5.10.1.3.1.4 Corrections section

To show the section in full, click on the **arrow** button  .

Parameter	Description
Lamp Flicker	**Activated:** Analyzes the total brightness of each Z-plane. In the event of non-constant deviations in the total brightness between neighboring planes, a compensation factor is taken into account. Activate this function if you have acquired your images using an old fluorescent lamp that exhibits strong fluctuations/flickering in brightness.
Fluorescent Decay	**Activated:** Corrects bleaching of the sample during acquisition of the Z-stack. This function should only be activated for widefield images. Use it if your sample undergoes strong bleaching during acquisition.
Background	**Activated:** Analyzes the background component in the image and removes it before the DCV calculation. This can prevent background noise being intensified during DCV.
Bad Pixel Correction	**Activated:** Employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the deconvolution result. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is recommended to use this parameter only, if stuck pixels are observed in the input image.

5.10.1.3.1.5 Advanced Settings section

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Inverse Filter**, **Iterative (Fast)** or **Iterative** algorithm.

To show the section in full, click on the **arrow** button  .

Depending on which algorithm you have selected, different advanced setting options are available. The relevant settings are described in the following chapters for each algorithm:

5.10.1.3.1.6 Advanced settings (Regularized Inverse Filter)

Regularization

Here you can select which frequencies in the image are taken into account during regularization:

Parameter	Description
Regularization	
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. May be better suited to the processing of confocal data sets.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.

5.10.1.3.1.7 Advanced settings (Fast Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson (Meinel)	The calculation according to Meinel works with one convolution per iteration and converges very fast, normally in 4-5 iterations. This method can also produce artifacts, however.
- Poisson (Richardson-Lucy)	The calculation according to Richardson-Lucy, on the other hand, normally requires hundreds of iterations and therefore takes considerably longer. This method is, however, somewhat more robust producing less artifacts. Precondition is however, that the PSF is known very well.
Regularization	For the **Poisson (Meinel)** calculation it is also possible to perform **zero order** (G-difference) regularization here as an option. This means, however, that the calculation will take considerably longer and the main advantage of the greater speed of Meinel is lost.
- None	No regularization is performed.

Parameter	Description
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- Total Variation	Total Variation regularization denoises the input data but protects the edges of structures. This can improve results for noisy data but increases the computation time quite significantly.
Optimization	
- Numerical Gradient	If selected, an attempt is made to determine the trend of the iterations in advance and extrapolate this to the entire calculation. This can significantly speed up the calculation.
First Estimate	
- Input Image	The input image is used as the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made, the mean gray level of the input image is being used. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time will increase, but missing information can be recovered from the PSF.
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want. In the case of Richardson-Lucy, you should allow significantly more iterations here.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. 1% is the default value. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.
GPU Tiling	Only available for very large images which exceed the available graphic card memory.

Parameter	Description
	With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result. If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

5.10.1.3.1.8 Advanced settings (Constrained Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson	Calculation according to Poisson, this is normally the correct noise model for microscopic images.
- Gauss	Calculation according to Gauss. If detector noise is dominant over sample noise, using a Gaussian noise model can be advantageous, however, this is rarely the case with modern microscopy systems.
Regularization	Here you can enter which frequencies in the image are taken into account during regularization:
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. This regularization can sometimes produce better results for the processing of confocal data sets.
- Second Order	Regularization according to Tikhonov-Miller. Here higher frequencies are penalized more than in the case of Good's roughness. Results have a tendency to become overly smoothed.
Optimization	

Parameter	Description
- Analytical (Newton Raphson)	Here an attempt is made to optimize the iterations analytically. This option is usually faster but may also be somewhat less precise.
- Line Search	Searches rigorously and comprehensively for the minimum. It is therefore more robust, but the calculation takes longer. Line search is recommended for confocal data sets especially, if they are noisy as this can enforce convergence even for noisy and sparsely sampled data.
First Estimate	
- Input Image	The input image is used for the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made for the next iteration. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time increases increase, but missing information can be recovered from the PSF.
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.
GPU Tiling	Only available for very large images which exceed the available graphic card memory. With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result.

Parameter	Description
	If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

5.10.1.3.2 PSF Settings tab

All key parameters for generating a theoretically calculated Point Spread Function ("PSF") are displayed here.

i INFO

Ordinarily, images that have been acquired using **ZEN** (of the *.czi type) automatically contain all microscope parameters, meaning that you do not have to configure any settings on this page. Most parameters are therefore grayed out in the display. It is possible, however, that as a result of an incorrect microscope configuration values may not be present or may be incorrect. You can change these here. The correction of spherical aberration can also be set here.

5.10.1.3.2.1 Microscope parameters section

The most important microscope parameters for PSF generation that are not channel-specific are displayed in this section.

NOTICE

If you enter incorrect values, this can lead to incorrect calculations. If the values here are obviously wrong or values are missing, check the configuration of your microscope system.

Parameter	Description
Microscope dropdown list	Displays which type of microscope has been used. There are two main options: conventional microscope (also known as a widefield microscope) and confocal microscope, for which the additional pinhole diameter parameter applies.
NA Objective	Displays the numerical aperture of the objective.

Parameter	Description
Immersion	Displays the refractive index of the immersion medium. Please note that this can never be smaller than the numerical aperture of the objective. You can make a selection from typical immersion media in the dropdown list next to the input field.
Scale lateral	Displays the geometric scaling in the X/Y direction.
Scale axial	Displays the geometric scaling in the Z direction.
Override button	To change the input fields that are normally grayed out, click on the button. The input fields and dropdown lists are now active. The text on the button then changes to **Reset**. To restore the original values saved in the image, click on the **Reset** button.
Master Reset	Resets the metadata to the values which were originally stored in the image at time of acquisition. It reverts any changes made by pressing the **Override** button.

5.10.1.3.2.2 Advanced section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button  .

Phase Ring dropdown list

If you have acquired a fluorescence image using a phase contrast objective, the phase ring present in the objective is entered here. This setting has significant effects on the theoretical Point Spread Function ("PSF").

PSF generation dropdown list

There are two models for calculating the PSF:

Parameter	Description
Scalar Theory	The wave vectors of the light are interpreted as electrical field = intensity and simply added. This method is fast and is sufficient in most cases (default setting).
Vectorial Theory	The wave vectors are added geometrically. However, the calculation takes considerably longer.

Z-Stack

This field can only be changed if it was not possible to define this parameter during acquisition, e.g. because the microscope type was unknown. It describes the direction in which the Z-stack was acquired. Note that this setting is only relevant, if your are using the spherical aberration correction.

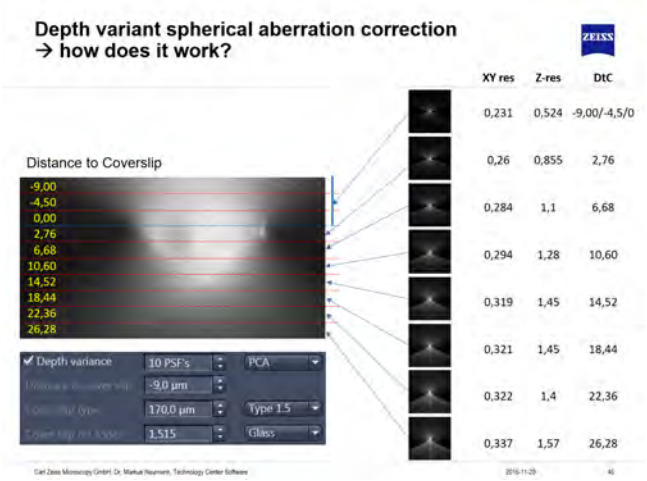
Parameter	Description
Descending	The Z-Stack descends away from the objective.
Ascending	The Z-Stack ascends towards the objective.

5.10.1.3.2.3 Aberration correction section

Only visible if the **Show All** mode is activated.

Here you can select whether you want spherical aberration to be taken into account and corrected during the calculation of the PSF. As with the other PSF parameters, most values are extracted automatically from the information about the microscope that is saved with the image during acquisition. The input option is therefore inactive.

Parameter	Description
Enable Correction	**Activated:** Uses the correction function. All options are active and can be edited.
Embedding medium	Here you can select the embedding medium used from the list.
Refractive index	Displays the refractive index of the selected embedding medium. Enter the appropriate refractive index if you are using a different embedding medium.
Manufacturer	Displays the manufacturer, if known.
Depth variance	When Aberration correction is activated, it is also possible to enable the creation of depth variant PSF's. This method allows for dramatic improvements in image restoration of thicker samples by creating axially variant theoretical PSF's as a function of the distance to the coverslip and the refractive index of the mounting medium. To use depth variant aberration correction activate the checkbox. In the spin box edit field you can define how many PSF's should be generated. The more PSF's you create, the better the results, but choosing many PSF's will increase the processing time. You should choose at least 3 PSF's.

Parameter	Description
	From the dropdown list you can choose between the PCA method (Primary Component Analysis, M. Arigovindan et al., 2005, IEEE Transactions on Image Processing 14. nr. 4 p.450ff) which is best suited for constrained iterative and fast iterative method and the Strata method (Myneni and Preza, Frontiers in Optics 2009, Optical Society of America, paper CThC4.), which is best for regularized inverse filter and Richardson Lucy iterative deconvolution.
Distance to cover slip	Displays the distance of the acquired structure from the side of the cover slip facing the embedding medium. Half the height of the Z-stack is assumed as the initial value for the distance from the cover slip. The value can be corrected if this distance is known. If possible, this distance should be measured. Note: Use **Ortho View** and the **Distance Measurement** option to define the distance of the sample to the coverslip. It is also important to estimate the position of the glass/embedding medium interface as precise as possible. If the z-stack extends into the coverslip, the determined range of the stack which reaches into the glass should be entered as a negative value. Example: Z-stack is 26 µm thick, glass/medium interface is positioned at 9 µm distance from the first plane of the stack. Resulting value for Distance to cover slip: - 9.0 µm. 
Cover slip type (Thickness and type)	Commercially available cover slips are divided into different groups depending on their thickness (0, 1, 1.5 and 2), which you can select from the dropdown list. Cover slips of the 1.5 type have an average thickness of 170 µm. In some cases, however, the actual values can vary greatly depending on the manufacturer. For best results the use of

Parameter	Description
	cover slips with a guaranteed thickness of 170 µm is recommended. Values that deviate from this can be entered directly in the **input field**.
Cover slip ref. index	Select the material that the cover slip is made of from the dropdown list. The corresponding refractive index is displayed in the input field next to it.
Working distance	Displays the working distance of the objective (i.e. the distance between the front lens and the side of the cover slip facing the objective). The working distance of the objective is determined automatically from the objective information, provided that the objective was selected correctly in the MTB 2004 Configuration program. You can, however, also enter the value manually.
Override	Only active if the **Enable correction** checkbox is activated. To reset the values, click on the **Reset** button.

5.10.1.3.2.4 Channel specific section

In this section you will find all settings that are channel-specific. This means that they may be configured differently for each channel.

Parameter	Description
Use external PSF checkbox	**Activated:** Uses an external measured PSF. You'll find an additional input window under **Image Parameters**	**Input** where you can choose the external PSF file. The software will check if the PSFs microscope parameters match with the input image. Deviations (10nm deviation in wavelength will be accepted) will make the software use a theoretical PSF.
Attach to input button	If an external PSF was selected you can attach the file to the input image. The saved input image will then contain the correct measured PSF. Usage of a theoretical PSF is possible as well for such an image. Just deactivate the **Use external PSF** checkbox.
Illumination display field	Displays the excitation wavelength for the channel dye [in nm] by using the peak value of the emission spectrum. The color field corresponds to the wavelength (as far as possible).

Parameter	Description
Detection display field	Displays the peak value of the emission wavelength for the channel dye. The color corresponds to the wavelength (as far as possible).
Sampling lateral display field	Depends on the geometric pixel scaling in the X/Y direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be close to 2 or greater in order to achieve good results during DCV. As, in the case of widefield microscopes, this value is generally determined by the objective, the camera adapter used and the camera itself, it can only be influenced by the use of an Optovar. With confocal systems, the zoom can be set to match this criterion.
Sampling axial display field	Depends on the geometric pixel scaling in the Z direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be at least 2 or greater in order to achieve good results during DCV. This value is determined by the increment of the focus drive during acquisition of Z-stacks and can therefore be changed easily.
Pinhole display field	Only available if a confocal microscope has been entered under the microscope parameters. Displays the size of the confocal pinhole in Airy units (AU).
NA Cond. display field	Only visible if the microscope is a **Conventional Microscope** and **Transmitted Light** has been selected as the illumination type. Displays the numerical aperture of the condenser with which transmitted-light acquisition was performed.

5.10.1.3.2.4.1 Microscope info section

Displays advanced microscope information that influences the form of the PSF in a channel-dependent way:

Parameter	Description
Illumination dropdown list	Here you can select the illumination method with which the data set has been acquired. In the event that a **Conventional Microscope** has been entered under the microscope parameters, the following options are available here: **Epifluorescence**, **Multiphoton Excitation** and **Transmitted Light**. In the case of confocal microscopes, **Epifluorescence** is the only option.

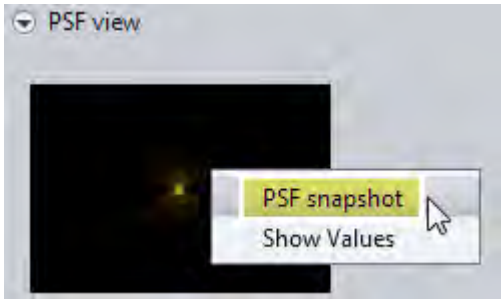
Parameter	Description
Image Formation	Displays whether the imaging was incoherent (**Conventional Microscope**) or coherent (**Laser Scanning Microscope**).
Lateral Resolution	Displays the lateral resolution of the calculated PSF.
Axial FWHM	Displays the FWHM (Full Width Half Maximum) as a measure of the axial resolution of the PSF.

5.10.1.3.2.4.2 PSF view section

This tool shows you the PSF that is calculated for a channel based on the current settings. If you select the **Auto Update** checkbox, all changes made to the PSF parameters are applied immediately to the PSF view. This makes it possible to check quickly whether the settings made meet your expectations.

Procedure

1 To extract the PSF from the image, right mouse-click and select **PSF snapshot**.



The result is a new PSF document opened in the center screen area.

5.10.1.4 Creating a PSF - With Wizard and Without

The PSF Wizard combines two steps which are necessary for extracting experimental point spread functions (PSF) from Z-Stacks of subresolution fluorescent beads:

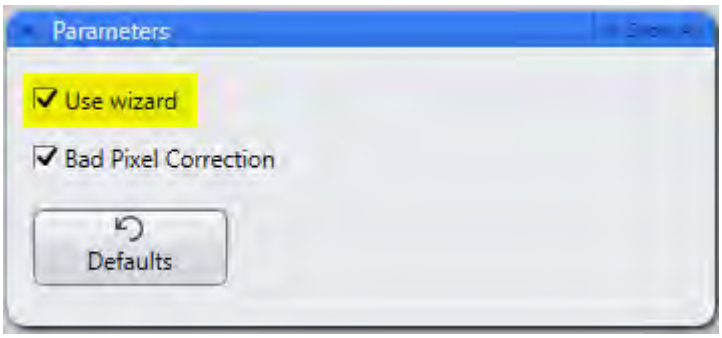
- A bead averaging step finds individual beads, presents them for inspection, allows you to select the ones you like and then creates an averaged combination of all selected beads. This stack shows a single bead which is, as a consequence of the averaging function, fairly free of noise.
- The averaged bead stack is then run through the **Create PSF** function which removes background and residual noise, correctly scales the PSF and also converts the stack into a 32-bit floating point format which is better suitable for the mathematical procedures used in deconvolution.

Prerequisites

- You have acquired a Z-Stack image. For more information, see *Measuring the PSF using subresolution beads* [▶ 108].
- The use of the PSF wizard is activated.

Procedure **1** Select the PFAD to open the **Parameters** dialog box.

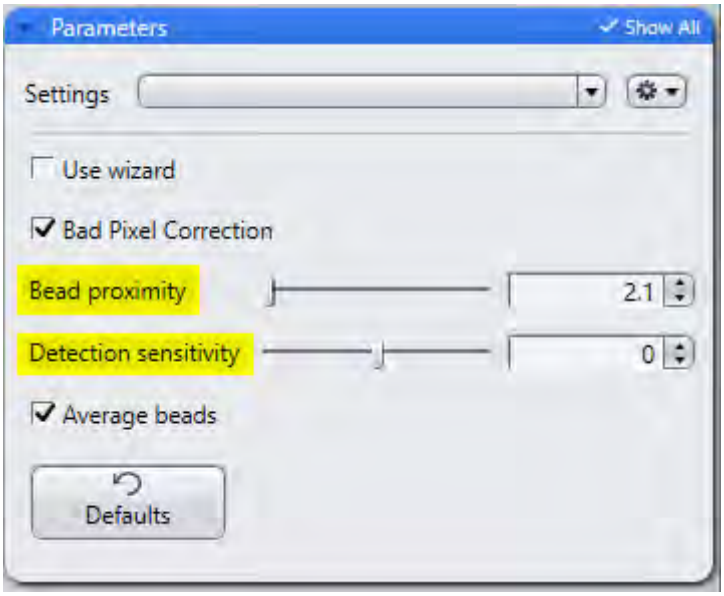
The functions **Use wizard** and **Bad Pixel Correction** are activated by default.



2 On the **Processing** tab, press the **Apply** button.

The PSF wizard opens and guides you throught the creation of the PSF.

If the **Use wizard** checkbox is not activated, the function shows the parameters for **Bead averaging**. Note that these parameters are only available in **Show all** mode. We recommend using the PSF wizard. The result of the wizard is a PSF file which you use in deconvolution for images acquired under the same conditions.



This method determines the position of fluorescent beads in a Z-stack image. If these beads are too close to one another they are excluded from the calculation. Beads which are far enough apart from one another are combined into a single bead, from which it is then possible to calculate a PSF using the **Create PSF** function (Processing / Utilities).

Description of the algorithm

This function consists of a series of steps before and after processing. The aim is to find beads that are far enough apart from one another. The processing steps are as follows:

- Select input image
- Image smoothing
- Segmentation
- Alignment of the center of the found beads
- Averaging of the beads

Parameters

- **Bead proximity**: Defines the distance between two neighboring beads in µm. A bead is excluded from the averaging if the distance to a neighboring bead is greater than the minimum distance set. A smaller value leads to the detection off fewer beads, albeit ones that are further apart, while a larger value leads to the detection of more beads, but with the risk that beads will partially overlap. Range between 2.1 and 20 µm.
- **Detection sensitivity**: determines the sensitivity with which beads are being detected. Range is between -5 to 5. Smaller Values lower the sensitivity excluding beads with weaker staining and lower signal to noise ratio, higher values include also beads with weaker staining.
- **Average Beads**: If activated, an image of an individual, averaged bead is produced. If deactivated, an image is produced in which each found bead is centered, but saved in the R dimension. A slider for Rotation (R dimension) appears on the **Dimensions** tab.

Bad Pixel Correction

This parameter employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the PSF extraction procedure. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is usually recommended to leave this parameter active.

5.10.1.5 Deconvolution Methods in ZEN

Method (common name)	Reference	Settings	Comments
Nearest Neighbor	K. Castleman, "Digital image processing", Prentice Hall 1997	Algorithm (default): **Nearest Neighbor**	Ad-hoc "2D de-blurring algorithm" focuses on subtraction of out of focus blur.

Method (common name)	Reference	Settings	Comments
Regularized Inverse also known as: Linear Least Squares	For zero order g-difference: Schaefer et al. (2001)	Algorithm (default): **Regularized Inverse Filter** Advanced settings | Regularization: **Zero order**	Uses difference of observation and estimate as regularization term.
Regularized Inverse also known as: Linear Least Squares	For first order regularization, or *Good's roughness*: Verveer et al. (1997)	Algorithm: **Regularized Inverse** Advanced settings | Regularization: **First order**	Uses *Good's roughness* first derivative of estimate as regularization term.
Regularized Inverse also known as: Linear Least Squares In conjunction with structured illumination microscopy (ApoTome)	Schaefer et al. (2006) Schaefer et al. (tbs)	Algorithm: **Regularized Inverse** Advanced settings | Regularization: **Zero / First order**	Patented method for maximum exploitation of ApoTome raw images
Fast Iterative Also known as: Meinel Algorithm Gold Meinel	Meinel (1986)	Algorithm (default): **Fast Iterative** Advanced settings | Likelihood: **Poisson (Meinel)**	Classic, non-regularized Meinel algorithm.
Fast Iterative	Meinel (1986),	Algorithm: **Fast Iterative**	Regularized Meinel algorithm using g-

Method (common name)	Reference	Settings	Comments
Meinel Algorithm + Regularization:	For zero order g-difference: Schaefer et al. (2001)	Advanced settings: - Likelihood: **Poisson (Meinel)** - Regularization: **Zero order**	difference (difference of observation and estimate) term.
Fast Iterative Meinel Algorithm + Optimization	Meinel (1986), Biggs (1998)	Algorithm: **Fast Iterative** Advanced settings: - Likelihood: **Poisson (Meinel)** - Regularization: **None / Zero order** - Optimization: **Numerical Gradient**	Meinel algorithm using a numerical gradient estimator as proposed by D. Biggs.
Fast Iterative Also known as: Richardson Lucy (RL) Algorithm	Richardson (1972) Lucy (1974)	Algorithm: **Fast Iterative** Advanced settings | Likelihood: **Poisson (Richardson, Lucy)**	Classic, original non-regularized Richardson Lucy algorithm. May need many more iterations than any other algorithm.
Fast Iterative Also known as: Richardson Lucy Algorithm + Optimization	Richardson (1972) Lucy (1974) Biggs (1998)	Algorithm: **Fast Iterative** Advanced settings: - Likelihood:	Classic, original non-regularized Richardson Lucy algorithm. Improved rate of convergence. About a factor of

Method (common name)	Reference	Settings	Comments
		Poisson (Richardson, Lucy) - Optimization: **Numerical Gradient**	10 faster than RL using a numerical gradient estimator as proposed by D. Biggs.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm (default): **Constrained Iterative** Advanced settings: - Likelihood: **Poisson** - Regularization: **Zero order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses difference of observation and estimate as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson** - Regularization: **First order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Good's roughness* derivative operator as regularization term.
Constrained Iterative	Tikhonov (1977) Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Tikhonov Miller Phillips* second

Method (common name)	Reference	Settings	Comments
		- Regularization: **Second orde**r	derivative operator as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **Zero order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses difference of observation and estimate as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **First order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Good's roughness* derivative operator as regularization term.
Constrained Iterative Also known as: ICTM Iterative Constrained Tikhonov Miller	van der Voort et al. (1995) Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **Second order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Tikhonov Miller Phillips* second derivative operator as regularization term.

Method (common name)	Reference	Settings	Comments
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson/Gauss** - Regularization: **0/1/2nd order** - Optimization: **Line search / Analytical**	Generic conjugate gradient restoration using squared estimate to impose positivity. **Default for optimization** is the fast analytical (Newton Raphson) method. Line search may be more accurate but is also much slower

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5.10.1.6 Table of Default Parameter for Deconvolution

The following table lists the parameters which are used by default for widefield, confocal, lightsheet and ApoTome images.

Microscope Type	Widefield	Confocal	Lightsheet	ApoTome
General Deconvolution Parameter Defaults				
Normali-zation	Auto	Auto	Auto	Auto
Backgound Correction	Off	Off	Off	Off
Flicker Correction	Off	Off	Off	Off
Decay Correction	Off	Off	Off	Off
Hot Pixel Correction	Off	Off	Off	Off
Constrained Iterative Specific Defaults				
Strength (automatic, manual; range 0..10)	Auto	Auto	Manual (str 5)	Auto
Likelihood	Poisson	Poisson	Poisson	—
Regulari-zation	ZeroOrder	FirstOrder	ZeroOrder	—
Optimization	Analytical	LineSearch	Analytical	—
First Estimate	Input	Mean	Input	—
Maximum Number Of Iterations	40	7	40	—
Auto Stop Percentage	0.1	0.1	0.1	—
Fast Iterative Specific Defaults				

Microscope Type	Widefield	Confocal	Lightsheet	ApoTome
Method	Poisson / Meinel	Poisson / Richardson Lucy	Poisson / Meinel	—
Regularization	None	None	None	—
Optimization	None	None	None	—
FirstEstimate	Input	Mean	Input	—
Maximum Number Of Iterations	15	50	15	—
AutoStop Percentage	0.1	0.1	0.1	—
Regularized Inverse Specific Defaults				
Regularization	Zero Order	Zero Order	Zero Order	First Order

5.10.2 Adjust

5.10.2.1 Color Balance

This method allows you to adjust the weighting of the individual color channels of a true color image.

Parameters

Parameter	Description
Range to Adjust	Here you can select the adjustment range for the color balance. There are 3 ranges available:
- Shadows	The settings relate to tones in the dark color range.
- Midtones	The settings relate to tones in the mid color range.
- Lights	The settings relate to tones in the light color range.
Cyan - Red	Adjust the desired color balance using the slider or input field.
Yellow - Blue	Adjust the desired color balance using the slider or input field.

Parameter	Description
Magenta - Green	Adjust the desired color balance using the slider or input field.

5.10.2.2 Hue/Saturation/Lightness

This method allows you to adjust the hue, saturation and brightness of a true color image.

Parameters

Parameter	Description
Hue	The value of the shift represents an angle on the color wheel. The values -180 and +180 therefore have an identical effect. Negative angles shift the color tone towards blue and positive ones shift it towards red. Adjust the desired shift in the color tone using the slider or input field.
Saturation	Saturation describes how intense the color of a pixel is. "Chromatic" is the maximum saturation, while "achromatic" describes colors that do not leave a color impression. Adjust the desired saturation using the slider or input field.
Lightness	Lightness describes how light or dark a pixel appears. The greatest difference is between black and white or between violet and yellow. Adjust the desired brightness using the slider or input field.

5.10.2.3 White Balance

This method allows you to adjust the white balance of an image.

Parameters

Parameter	Description
Automatic	**Activated**: The white spot is calculated automatically from the image data.
White Spot	Only visible if the **Automatic** checkbox is deactivated.

Parameter	Description
	Define the white spot by clicking on the  **Pick..** button. The mouse pointer then changes to a pipette symbol. Use it to click on a white region of your input image. The coordinates and color values of the selected white spot are displayed next to the button.
Temperature Delta	Adjust the delta that will be added on to the newly calculated color values. Negative values reduce the color temperature, while positive values increase it. A value of 1 corresponds to 10 Kelvin.

5.10.2.4 Color Temperature

This method allows you to adjust the color temperature of a true color image. Therefore use the **Temperature Delta** slider. A description of the slider can be found under *White Balance* [▶ 150].

5.10.2.5 Brightness/Contrast/Gamma

This method allows you to adjust the brightness, contrast and gamma value of an image.

i INFO

Unlike the adjustments that can be made on the **Display** tab, here the pixel values of the image are changed.

Parameters

Parameter	Description
Brightness	Adjust the desired brightness using the slider or input field. Changing the brightness means that each gray or color value is increased or decreased by the same value. The difference between the biggest and smallest gray or color value in the image remains the same, however.
Contrast	Adjust the desired contrast using the slider or input field. Changing the contrast means that the gray or color values are multiplied by a factor. The difference between the biggest and smallest gray or color value changes.
Gamma	Adjust the desired gamma value using the slider or input field.

Parameter	Description
	Changing the gamma value means that the gray or color values are multiplied by individual factors.

5.10.2.6 Stack Correction

This method allows you to improve the quality of Z-Stack images that have been affected by bleaching effects during acquisition.

Parameters

Under **Correction** you can select the desired correction mode or a combination of the modes.

Parameter	Description
Decay	This mode compensates the bleaching effect.
Flicker	This mode compensates the flicker of the lamp voltage.
Background	This mode reduces background noise.

5.10.2.7 Shading Correction

This method allows you to improve images in which the quality has been impaired by uneven illumination or vignetting.

If you want to perform shading correction before an experiment (recommended) you have to use the shading correction function in the **Camera** tool in the *Post-Processing section* [▶ 649].

Parameters

Parameter	Description
Shading Mode	
- Camera shading	Applies the Shading correction to each tile of a tile image.
- Global shading	Applies the Shading correction to the whole tile image. Requires a shading reference image with the same size as the tile image.
Automatic	**Activated**: The function automatically calculates the reference image for shading correction from the input image.
Display Mode	

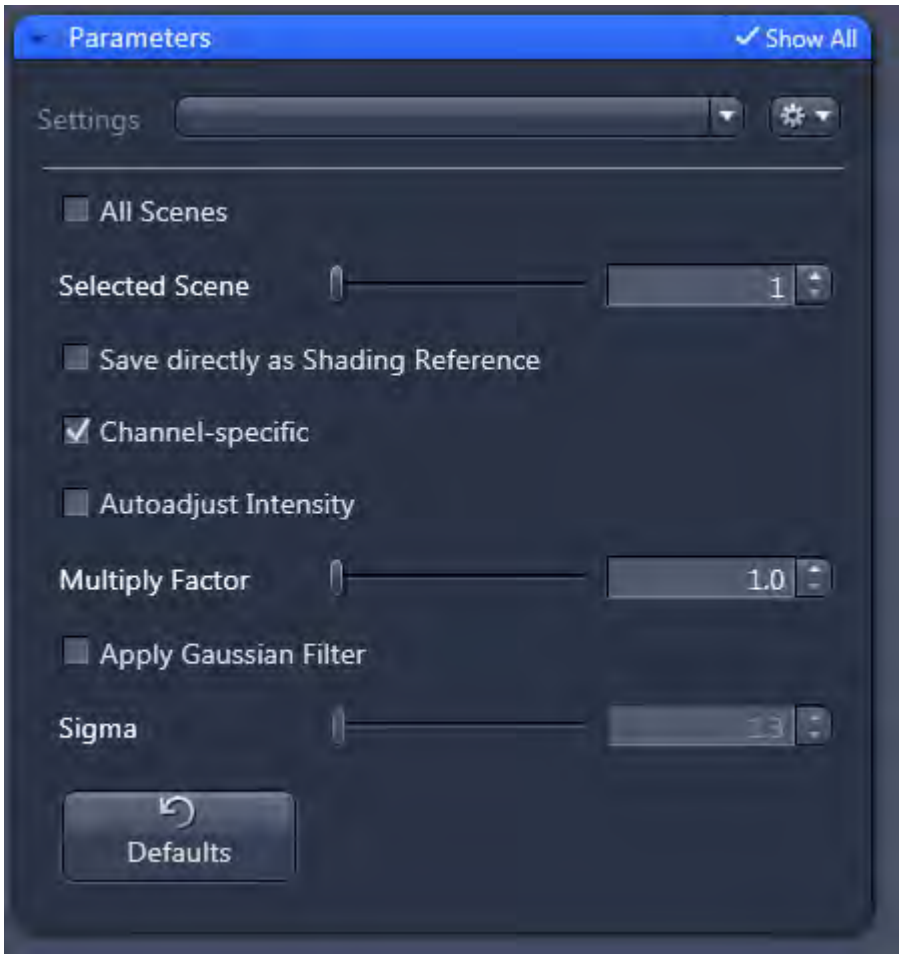
Parameter	Description
- Additive	In this mode the normalized reference image is subtracted from each camera frame. This influences the brightness of the image.
- Multiplicative	In this mode each camera frame is divided by the normalized reference image. This influences the contrast of the image. This is the default setting. The simulated/auto reference image is created by averaging up to 20 camera frames in the input image and running a lowpass filter on them.
Offset	Adjust the gray value that will be added on to the newly calculated gray values using the slider or input field. If this results in negative values, these are set to 0. Values that exceed the maximum gray value are set to the maximum gray value.

5.10.2.8 Shading Reference from Tile Image

With this method you can create shading reference images for multi-channel tile images.

Parameters (Brightfield Image)

For a **Brightfield** image you can select following parameters:



Note that the parameter **Selected Scene** and the checkbox **All Scenes** are only available if you process a multi-scene image.

Parameters	Description
Save directly as Shading Reference	**Activated**: The software creates the shading reference image and stores it directly to the Calibration Manager (Shading Reference). The software provides no possibility to check the image before they will be saved, thus it is recommended to deactivate the checkbox and execute this function. The system will create the reference images and present it to you. If the images have a good quality, you can activate the checkbox and run the function again. Note that, if you activate this option it is important that you check afterwards the system messages (the "i" in the lower part of the screen). If e.g. the resulting shading reference image is too dim the system will not use it as valid shading reference image, this will be

Parameters	Description
	shown under the system messages. Also if the import of the reference database were successful this will be shown!
Channel-specific	**Activated**: The software performs channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. The following components will be considered: Contrasting method and condenser, fluorescence filter, magnification: Objective and Optovar; camera bit depth and RGB/BW mode, camera type and port position. **Deactivated**: The system creates an **All Channel** calibration and perform an objective specific shading correction. The following components will be considered: magnification (Objective and Optovar); Camera bit depth and RGB/BW mode and camera type and port position
Multiply Factor	Here you can apply a multiply factor, thus the software will multiply the pixel intensity for each pixel of the shading reference image by this value. If you use an own sample it is mostly the case that the images are very dim and the intensity does not reach the value needed to be used within the shading reference calibration manager, thus it will be rejected.
Auto Adjust Intensity	If activated, this option calculates automatically the multiply factor based on the gray values of the image and the needed gray values for using it in the shading reference calibration manager. If activated, the setting for **Multiply Factor** has no influence anymore on the image generation.
Apply Gaussian Filter	If activated, a Gaussian filter is applied after the averaging of the field of views from a tiled image is done. This enables to smooth the shading reference image. The **Sigma** factor defines the strength of the smoothing. Use this filter and the Sigma factor very carefully as it could remove also features which are real shading structures. This feature could be recommend if the number of tiles in the scanned image is low and cannot be increased for certain reasons

Parameters (Multichannel Image)

For a multichannel image (e.g. fluorescence image) all parameters (description see above) can be adjusted channel specific.

Note that the parameter **Selected Scene** and the checkbox **All Scenes** are only available if you process a multi-scene image.

Parameters	Description
Adjust per Channel	If activated, you can adjust the settings for every channel separately. To use a channel select **Process Channel** or select **Skip Channel** if you don`t want a channel to be processed.

Parameters	Description
	The settings **Save directly as Shading Reference** and **Channel-specific** are applied separately for each channel. If you want to use the same settings for all channels deactivate the checkbox.

5.10.2.9 Background Subtraction

With this function you remove smooth background or correct uneven illumination. The implementation is adapted from the corresponding function in ImageJ and based on the "rolling ball" algorithm (S. Sternberg, "Biomedical Image Processing", IEEE Computer, January 1983).

Parameters

Parameter	Description
Radius	Here you adjust the radius of the rolling ball in pixels. This value should be at least as large as the radius of the largest object in the image that is not part of the background. Larger values will also work unless the background of the image is too uneven.
Create Background only	If activated, an image which contains the detected background only is created. Use this image to subsequently perform manual corrections of the image background, e.g. using the image calculator function.
Do smoothing beforehand	If activated, a 3x3 pixel averaging is performed before analyzing the background. Use this option to ensure that the image data after subtraction will not be below the background.
Light Background	Use this option if your image contains bright background and dark objects.

5.10.2.10 Histogram Equalization

This function enhances the contrast by linearizing the histogram of the image to equal area fractions in the histogram. The areas (pixel count multiplied by gray value range) of all gray values in the histogram of the result image are the same.

Parameter	Description
All Z	If activated, the function is applied to all Z planes.
All T	If activated, the function is applied to all time points.

Parameter	Description
High Threshold	The fraction of pixels that will be mapped to the highest gray value of the output image.
Lower Threshold	The fraction of pixels that will be mapped to gray value 0.

5.10.3 Edges

5.10.3.1 Highpass

This method performs high-pass filtering. The high pass filter is defined as the difference between the original image and the low-pass filtered original.

Parameter

Parameter	Description
Normalization	Depending on the IP function you have selected not all choices are available in the list.
- Clip	Gray levels that exceed or fall below the specified gray value range are automatically set to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
- Automatic	Automatic normalization of gray values to the available gray value range.
- Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.
- Shift	Normalizes the output to the value gray value + max. gray value/2.
- Absolute	Values are used positive only.
Count	Here you set the number of repetitions. I.e. the number of times the function is applied sequentially to the respective result of the filtering. The effect is increased correspondingly.
Kernel Size	You can set the filter size in the x-, y-and z-direction, symmetrically around the subject pixel. This should be the size of the transition region between objects and background match.

5.10.3.2 Roberts

This method calculates a gradient image using the Roberts filter matrix. Large gray value differences between neighbors are shown as light gray values. No changes are indicated by a value of 0 (black). Edges are thinner than with the **Sobel** method.

5.10.3.3 Gradient Max

This method performs a gradient filtering. Based on the sum of a 2x2 matrix in the X-and Y-direction, a gradient image is calculated and using the larger of the two components. The edges are darker than that of the method **Gradient Sum**.

5.10.3.4 Gradient Sum

This method performs a gradient filtering. Based on the sum of a 2x2 matrix in the X-and Y-direction, a gradient image is calculated. The edges are brighter than that of the method **Gradient Max**.

5.10.3.5 Sobel

Sobel calculates a gradient image using a Sobel filter.

This method indicates gray value changes in the image. Large differences between neighbors are displayed as bright gray values, no changes are indicated by a value of 0 (zero). The pixels in the output image are calculated with the Sobel differential operator on the basis of a 3x3x3 fold of the input image.

5.10.3.6 Laplace

This function performs a Laplace highpass filter on an image.

The calculation is based on a 3x3x3 Laplace operator in all directions. The function does not show smooth gray value changes very well.

5.10.3.7 Local variance

This method is an edge filter, which calculates the variance of each pixel with its neighboring pixels by the lateral filter size.

Parameter

Kernel Size in X/Y

Here you set the matrix size in X / Y symmetrically around the pixel. This determines the degree of smoothing effect in the X / Y direction.

5.10.4 Geometric

5.10.4.1 Channel Alignment

Using this method it is possible to automatically align the individual channels of a multi-channel image correctly to one another.

Parameters

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

5.10.4.2 Z-Stack Alignment

This method allows you to bring the individual planes of a Z-stack image into line if these are not positioned precisely one above the other. This is the case, for example, when you acquire Z-stacks at an oblique angle using a stereo microscope.

Parameters

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.

Parameter	Description
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

5.10.4.3 Stitching

This method allows you to align the individual tiles of a tile image with one another automatically and correctly.

Parameters

Parameter	Description
Inplace	The stitching is applied to the original image.
New Output	A new image is generated as a result of the stitching process. The original image is not modified.
Fuse Tiles	Only visible when the button **New Output** is selected. **Activated**: All individual tile images are fused together after alignment. **Deactivated**: The individual tile images are aligned but not fused.
Correct shading	Only visible when the button **New Output** is selected. **Activated:** Applies a shading correction (Multiplicative mode, see *Shading Correction* [▶ 152]) to each image of prior to stitching. Select from the dropdown list which reference should be used for shading correction:
- Automatic	The function automatically calculates a reference image from the input image.
- Reference	The function uses an existing reference image. This must be selected in addition to the input image in the input tool of the image parameters section.

Select dimension references for stitching

Only visible for multidimensional input images.

Select here a reference dimension (one channel, one z-position, one time point) from your multidimensional data set. This reference dimension is either stitched exclusively (no other planes of the dimensions are stitched) or serves as reference when stitching all planes of the dimensions.

Parameter	Description
Get all dimensions from 2d view	Reads the current planes of the dimensions from the 2D view.
Z-Position	Select here a z-position for the 2D image.
Time	Select here a time point for the 2D image.
Channels	Select here a channel for the 2D image.

Stitch multiple dimensions section

Only visible for multidimensional input images. Under the respective dimension you can select which planes of the dimensions should be considered for stitching.

Parameter	Description
All by reference	All planes of this dimension are stitched and appear in the output image. The stitch is calculated individually for each plane.
Reference only	Only the reference plane (2D image) for this dimension is taken into consideration for calculating the stitch. All other planes are stitched accordingly and appear in the output image.
All individually	Only the selected reference plane (2D image) of this dimension is stitched. No other planes appear in the output image.

Parameters

Parameter	Description
Edge Detector	
- Yes	An edge detector is applied to localize image edges. This may improve the stitching result.
- No	No edge detector is applied.
Minimal Overlap	Sets the extent of the area of minimal overlap (in %) of the individual tiles
Max Shift	Sets the maximal extent of the shift (in %) of the individual tiles to one another.
Comparer	Here you can select how the conformance of the tiles in the overlapping regions is evaluated.

Parameter	Description
- Basic	Basic comparison (faster).
- Best	Elaborate comparison (slower).
- Optimized	Optimized comparison.
Global Optimizer	Select here which tile overlaps are evaluated.
- Basic	Only one overlap per tile is evaluated.
- Best	All overlaps of a tile are evaluated.

5.10.4.4 Image Overlay

With this function you can align (or overlay) two images that are displaced in relation to each other. It is also possible to make the individual planes of a z-stack image congruent, in the event that they are not lying exactly on top of one another.

You can define 3 related points (**Input Pixel**) in both the input image and in the reference image (**Reference Pixel**) that is displaced in relation to it. Therefore click interactively on conspicuous points, which are present in both images. If you click **Apply** the function calculates the Output image, in which the new fitting points have the same coordinates as in the Input image.

Parameters

Parameter	Description
Input Pixel 1 - 3	If you click on the corresponding buttons you can define the 3 input pixel points. The selected point is shown in the graphics plane. This serves as an aid to orientation when you are clicking on the reference points.
Reference Pixel 1 - 3	If you click on the corresponding buttons you can define the 3 reference pixel points.
Interpolation	Here you can specify how the rotation influences the neighboring pixels.
- Linear	The rotated pixel is given the gray value calculated from the linear combination of the gray values of the pixel closest to it and this pixel's nearest neighbor.
- Cubic	The rotated pixel is given the gray value resulting from a polynomial function of the pixel that is closest to it.

5.10.4.5 Rotate

With this method you can rotate images by defined angles. This function was especially developed for rotating complex (multi-dimensional) images in the available image dimensions. Therefore the function can be a little bit slower but offers more settings for the rotation. For simple, 2-dimensional rotations we recommend to use the **Rotate 2D** function which is usually lots of faster.

Parameter

Parameter	Description
Third Dimension	Only visible, if there is a third dimension in the input image and/or **Show all** mode is activated. Here you can select how you want the function to work in the case of multidimensional images.
- 2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
- Z, T or C	Here you can select to which additional dimension the functions should be applied to.
Adapt sizes	**Activated:** The size of the output image is adjusted in accordance with the settings for the scaling. **Deactivated:** The output image has the same size as the input image. Depending on the image size and rotation angle, partial areas of the input image may not be visible in the output image.
Adjust per Channel	Only visible if your input image is a multi-channel image. **Activated:** You can adjust the parameters for each channel individually.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

Parameter	Description
Angle	Enter the angle by which you want the input image to be rotated around using the slider or input field. Positive angles rotate the images clockwise.
Angle X	Enter the angle by which you want the input image to be rotated on the X axis using the slider or input field.
Angle Y	Enter the angle by which you want the input image to be rotated on the Y axis using the slider or input field.
Angle Z	Enter the angle by which you want the input image to be rotated on the Z axis using the slider or input field.
The following parameters are only visible if the **Adapt sizes** checkbox is deactivated:	
Center X	Enter the X coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted to the left in relation to the image's center point. Positive values shift the center to the right.
Center Y	Enter the Y coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted downwards in relation to the image's center point. Positive values shift the center upwards.
Center Z	Enter the Z coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted forwards in relation to the image's center point. Positive values shift the center backwards.

5.10.4.6 Rotate 2D

With this method you can easily rotate an image clockwise around its center axis. Simply set the desired angle with the slider. Of course you can enter the angle value in the input field directly. To perform the rotation click on the **Apply** button on top of the **Processing** tab.

5.10.4.7 Mirror

This method allows you to flip an image horizontally or vertically. In the case of multidimensional images, such as Z-stack or time lapse images, you can also use the mirror method to reverse the sequence of the relevant dimension.

Parameters

Parameter		Description
Display Mode		
-	Horizontal	Flips the image horizontal
-	Vertical	Flips the image vertical
-	T/ Z	Only visible if input image is a multichannel image. Reverses sequence of the sections (Z) or time points (T)

5.10.4.8 Change Orientation

With this function you can easily change the image orientation.

Parameters

Parameter		Description
Orientation		
-	Flip Horizontally	Flips the image horizontally.
-	Flip Vertically	Flips the image vertically .
-	Rotate 90 CW	Rotates the image by 45 degrees clockwise (CW).
-	Rotate 90 CCW	Rotates the image by 45 degrees counter clockwise (CCW).
-	Rotate 180	Rotates the image by 180 degrees.
-	Mirror at +45 Diagonal	Mirrors the image at +45 degrees diagonal.
-	MIrror at -45 Diagonal	Mirrors the image at -45 degrees diagonal.

5.10.4.9 Resample

This method allows you to change the size of an image in every dimension. You can either enlarge or reduce the image size.

Parameters

Parameter	Description
Third Dimension	Only visible, if there is a third dimension in the input image and/or **Show all** mode is activated. Here you can select how you want the function to work in the case of multidimensional images.
- 2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
- Z, T or C	Here you can select to which additional dimension the functions should be applied to.
Adapt sizes	**Activated:** The size of the output image is adjusted in accordance with the settings for the scaling. **Deactivated:** The output image has the same size as the input image. Depending on the image size and rotation angle, partial areas of the input image may not be visible in the output image.
Adjust per Channel	Only visible if your input image is a multi-channel image. **Activated:** You can adjust the parameters for each channel individually.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

Controls

Parameter	Description
Scaling in X	Adjust the desired scaling for X using the slider or input field.
Scaling in Y	Adjust the desired scaling for Y using the slider or input field.
Scaling in Z	Adjust the desired scaling for Z using the slider or input field.
The following parameters are only visible if the **Adapt sizes** checkbox is deactivated:	
Shift in X	Enter the shift in the X direction using the slider or input field.
Shift in Y	Enter the shift in the Y direction using the slider or input field.
Shift in Z	Enter the shift in the Z direction using the slider or input field.

5.10.4.10 Orthogonal Projection

With this method you can extract specific parts of the image of three-dimensional images. This is accomplished with three alternative projection planes, frontal in the XY direction, sagittal in YZ direction or transverse in XZ direction as seen from the observer of the image. You can choose between different projection methods, all have in common is that the pixels are analyzed by the observer along an imaginary projection beam. You can also determine the thickness of the projection planes, and thus the projection depth.

Parameters

Parameter	Description
Projection Plane	Here you choose the type of the projection plane (Frontal X/Y, Transverse (X/Z), Sagittal (Y/Z).
Method	
- Maximum	Uses the brightest pixel along the projection beam.
- Minimum	Uses the darkest pixel along the projection beam.
- Average	Calculates the average of all pixel along the projection beam.

Parameter	Description
- Weighted average	This method is related to the calculation of the extended depth of focus. It prefers structures with more lateral sharpness along the projection beam. The output image contains more significant details.
- Standard deviation	Calculates the standard deviation of pixel grey values along the projection beam.
Start position	Here you adjust the starting position of the project plane (in pixel units or z-stack positions depending on the chosen projection plane). The maximum range results automatically of the size of the input image.
Thickness	Here you adjust the thickness of the cutting plane (in pixel or z-stacks depending on the chosen projection plane). The maximum range results automatically of the size of the input image.

5.10.4.11 Shift

This method allows you to shift the content of an image in the direction of the 3 axes X, Y and Z. To adjust the shift use the respective sliders in under **Parameters.**

5.10.4.12 Color-coded Projection

The Color-coded projection function generates a maximum intensity projection image along the z-, or time-dimension of a multidimensional data set. Instead of using the colors assigned to the channels, it displays the position in z or in time with a color gradient.

Parameters

Parameter	Description
Palette	Her you select the color-pattern for the projection.
ROI	Here you specify the range of the dimension (chosen below), which will be used for the projection.
Dimension	Here you select wether to perform the projection along the time (**T**) or along the z-axis (**Z**). Only dimensions which are available in your data set are shown.

5.10.4.13 Sample Down

With this function you can reduce the size of an image in a flexible way. The reduction is performed along with an averaging of the respective dimension. If the parameters are set to 1, the corresponding dimension is not modified.

Parameters

Parameter	Description
Average Pixels X	Here you adjust how many pixels are averaged in the lateral X dimension to calculate the output image. The size of the output will be smaller by this factor.
Average Pixels Y	Here you adjust how many pixels are averaged in the lateral Y dimension to calculate the output image. The size of the output will be smaller by this factor.
Third Dimension	If your input image has a third dimension you can select it here for re-sampling. ■ **Z** (sections of a Z-Stack) ■ **T** (time points of a time series) ■ **C** (Channels) or ■ **H** (Phases). ■ If **2D Slices** is chosen, the third dimension will not be re-sampled.

5.10.4.14 Channel Alignment (Extended)

Using this method it is possible to automatically align the individual channels of a multi-channel image correctly to one another.

Parameters

Parameter	Description
Load transformation	If activated, the result of a previous transformation can be loaded. Click on  to select an according ***.xml** file.
Save transformation	If activated, the result of the transformation process is saved in an ***.xml** file for later use.

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.

Parameter		Description
-	Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
-	Iso Scaling	The magnification is adjusted from section to section.
-	Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
-	Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter		Description
Quality		Here you can select the quality level that you want the function to work with.
-	Low	Highest speed with low image quality.
-	Medium	High speed with medium image quality.
-	High	Low speed with high image quality.
-	Highest	Lowest speed with highest image quality.
Interpolation		Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
-	Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
-	Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
-	Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

5.10.5 Morphology

Morphology functions apply structure elements to images. A structure element is like a stencil with holes. When the stencil is placed on an image then only some pixels are visible through the holes. The gray values of these pixels are collected and their external gray value (minimum or maximum) is computed.

This external gray value is assigned to that pixel of the resulting image which corresponds to the place of the origin of the stencil on the input image. When the stencil is placed at all positions of the input image, all pixels of the resulting image

are thus assigned. When bigger structure elements are required than those which are provided, these can be achieved by iterating the small elements using the **Count** parameter.

The following functions are available:

Function	Description
Erode	Shrinks bright structures on a darker background in the input image. Thin connections between structures and small structures itself will disappear.
Dilate	Expands bright structures on a darker background in the input image. Small gaps between structures are filled and those structures become connected.
Open	First erodes (Erode class) the bright structures on a darker background in the input image, then it dilates (Dilate class) the result by the same number of steps. Thus it separates bright structures on a darker background, but approximately keeps the size of the structures.
Close	First dilates (Dilate class) the bright structures on a darker background in the input image, then it erodes (Erode class) the result by the same number of steps. Thus it connects bright structures on a darker background, but approximately keeps the size of the structures.
Top Hat (White)	Computes the difference between the original image and the image produced by an open operation (Open class). Bright structures which were flattened by the opening are strengthened in the result. This is like putting a top hat with the size of the open operation upon the structure and keep only the part inside the hat.
Top Hat (Black)	Computes the difference between the original image and the image produced by a close operation (Close class). Dark structures which were flattened by the closing are strengthened in the result. This is like lifting a top hat with the size of the close operation beneath the structure from the dark side and keep only the part inside the hat.
Gradient	Computes the difference between the dilated image and the eroded image (Dilate and Erode class). Since a point in the dilated image has the maximum gray value and the corresponding point in the eroded image has the minimum gray value within the structure element the difference is zero for regions of constant gray values and gets bigger for steeper gray value ramps or edges.

Function	Description
Watersheds	Computes the barriers between catchment basins of local minima in the gray valued input image. A local minimum is a connected plateau of points from which it is impossible to reach a point of lower gray value without first climbing up to higher gray values. A catchment basin of a local minimum is a connected component which contains that minimum and all downstream points to it. A downstream is a path of points along which gray values are monotonically descending. Thus all catchment basins of local minima are expanded until they collide with another catchment basins. At those point barriers (watersheds) are built up. The output image is binary and contains all watersheds. If the 'Basins' flag is set, the catchment basins themselves are in the output image as uniquely labeled connected components without any border lines.
Grey Reconstruction	Works mainly as an iterated dilation (Dilate class) of the image, but with a constraint image as a second input image. After every dilation step the pixel wise minimum of the dilated image and the constraint image is computed and gives the next image to be dilated. The computation stops automatically when all the just dilated pixels are bigger than the corresponding ones in the constraint image.

Parameters

Parameter	Description
Structure Element	Here you select the desired structure element. Following elements are available: Horizontal, Diagonal 45°, Vertical, Diagonal 135°, Cross, Square, Octagon
Count	Here you can adjust the number of repetitions to define the size of the structure element.
Binary	Only available for **Erode**, **Dilate**, **Open** and **Close** function. **Activated:** Creates a binary image. The calculation will be faster.

5.10.6 Sharpen

5.10.6.1 Extended Depth of Focus

Using this method you can combine the sharp regions from the individual sections of a Z-stack image to form a single image. This enables you to display a considerably larger depth of field than is possible on a microscope.

Parameters

Parameter	Description
Method	
- Wavelets	A wavelet transform is used to detect the sharpest areas in the images.
- Contrast	For the this method the value is the difference between the brightest and the darkest pixel value within the "Kernel".
- Maximum Projection	Images with the brightest and darkest pixels are generated first. Of these images the image with the higher variance is used as the resulting image.
- Variance	For this method the variance of the pixel values is calculated within the "Kernel".
Z-Stack Alignment	Here you can select whether you want the Z-stack image to be aligned before the calculation and with what quality level. If you want to acquire images with a stereo microscope, the images are displaced against each other. This displacement can be corrected. The higher the quality of alignment is selected, the longer is the calculation. Select **No Alignment**, if you want to acquire images with a compound microscope.
- No Alignment	The Z-stack image is not aligned before the calculation. You should select this setting if the Z-stack image has not been acquired using a stereo microscope.
- Normal	High speed with normal image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with best image quality.

5.10.6.2 Delineate

This method enhances the edges of individual regions in an image. It corrects the halo effect and only affects edges.

Parameters

Parameter	Description
Threshold	Enter the threshold value for edge detection using the slider or spin box/input field. The threshold value should correspond roughly to the gray value difference between objects and the background.
Size	Enter the size of the edge detection filter using the slider or spin box/input field. The value should correspond to the size of the transition area between objects and the background.

5.10.6.3 Enhance Contour

This method allows you to enhance contours in an image and emphasize regions in which gray values change. The function is suitable for visually emphasizing fine structures in an image.

Parameters

Parameter	Description
Strength	Here you can adjust the factor for increasing edge enhancement.
Normalization	Here you can select how the gray/color values that exceed or fall short of the value range should be dealt with.
- Clip	Automatically sets the gray levels that exceed or fall short of the predefined gray value range to the lowest or highest gray value (black or white). The effect corresponds to underexposure or overexposure. In certain circumstances some information may therefore be lost.
- Automatic	Normalizes the gray values automatically to the available gray value range.
- Wrap	If the result is larger than the maximum gray value of the image, the maximum gray value + 1 is deducted from this value.
- Shift	Normalizes the output to the value "gray value + maximum gray value/2".
- Absolute	Converts negative gray levels into positive values.

5.10.6.4 Unsharp Mask

Using this method you can increase the impression of sharpness in an image and consequently obtain an image display that is richer in detail. The function allows contrasts at small structures and edges to be enhanced in a targeted way.

Parameters

Parameter	Description
Strength	Enter the strength of the Unsharp Masking using the slider or spin box/input field. The higher the value selected, the greater the extent to which small structures are enhanced.
Radius slider	Enter the radius using the slider or spin box/input field. **Radius** defines the width of structures that you want to appear sharper. A small radius enhances smaller details. If the radius is too big, halo effects can occur at edges.
Color Mode	Select the desired color mode from the dropdown list.
- RGB	Calculates the sharpness for each color channel individually. The color saturation and the color of structures may be changed and color noise may occur.
- Luminance	Only calculates the sharpness on the basis of the brightness signal detected. This mode does not show any color noise and changes the color saturation accordingly.
Threshold Mode	Here you can select a setting from the dropdown list for calculating the boundary between the sharpened image regions. It is only effective if the value for the **Lower Threshold Value** parameter is not equal to 0 or the value for the **Upper Threshold Value** parameter is not equal to 100.
- None	No adjustment takes place.
- Binary	The transition follows the threshold values.
- Linear	Calculates a linear course.
Threshold Low	Enter the lower threshold value using the slider or spin box/input field. This determines the lower limit from which existing contrast structures are changed.
Threshold High	Enter the upper threshold value using the slider or spin box/input field. This prevents the existing strong contrasts in the image from being increased further unnecessarily.

Parameter	Description
Clip To Valid Bits	**Activated:** The value range of the gray/color values of the output image is adjusted to the value range of the input image.

5.10.7 Smooth

5.10.7.1 Median

This method allows you to reduce noise in an image. Each pixel is replaced by the median of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix. The median is the middle value of the gray values of the pixel and its neighbors sorted in ascending order.

Parameter

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

5.10.7.2 Sigma

This method allows you to reduce noise in an image. Each pixel is replaced by the average of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix. To calculate the average, only the gray values that lie within a defined range (+/- sigma) around the gray value of the central pixel are taken into consideration. As a result, fine object structures are not blurred; only the gray levels in image regions that belong together are adjusted.

Parameter

Parameter	Description
Sigma	Enter the sigma value using the slider or input field.

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

5.10.7.3 Lowpass

This method allows you to reduce noise in an image. Each pixel is replaced by the average of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix.

Parameter

Parameter	Description
Count	Enter the number of repetitions using the slider or input field. The function can be applied several times in succession to the result of the filtering. This intensifies the effect accordingly.

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

5.10.7.4 Binomial Filter

This method allows you to reduce noise in an image. Each pixel is replaced by a weighted average of its neighbors. The weighting factors are calculated from the binomial coefficients in accordance with the filter size. The binomial filter is very similar to a Gaussian filter in its effect.

Parameter

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

5.10.7.5 Gauss

This method allows you to reduce noise in an image. Each pixel is replaced by a weighted average of its neighbors. The neighboring pixels are weighted in accordance with a two-dimensional Gauss bell curve.

Parameter

Parameter	Description
Sigma	Here you can adjust the sigma value. If the **Show All** mode is activated you can adjust the values in X, Y and Z direction individually.

5.10.7.6 Single Pixel Filter

With this function you can remove single pixel phenomena, such as those that occur in the case of clocking induced charge with EMCCDs and as radio telegraph signal noise with CMOS sensors. It is a filter which analyzes the input image and removes pixels, whose intensity value diverges strongly from the median intensity of its neighboring pixels.

The filter analyzes the input image and removes pixels that are "much" larger than the median of their neighbors. The algorithm works as follows:

1. Sort all 9 pixel in a 3x3 neighborhood.
2. Determine the median intensity value of the sorted pixels.
3. Multiply the median by the threshold factor to get the limit.
4. If the center pixel intensity is larger than the limit, replace the pixel with the median.

Larger values of the threshold factor increase the value of the intensity limit and decrease the number of pixels that are replaced. The default value of 1.5 is arbitrary, but seems to remove charge induced noise from images acquired using cameras with EM gain capabilities. This filter can also be used to remove hot pixels from images.

Parameter

Parameter	Description
Threshold	Here you adjust the threshold value.

5.10.7.7 Rank

This method performs a rank order filtering. The gray levels of the resulting image is determined by calculating the ranking within the matrix of the **filter size** in the **X** and **Y** directions. Even numbers are automatically set to the next odd number. A low value for the **rank value** enlarges dark areas, a higher value will increase bright areas of the image.

5.10.7.8 Denoise

This method removes noise from images using a real or a complex wavelet transformations. The process of denoising an image can be broken down into the following three parts:

- Calculate the wavelet transform of the noisy image.
  The wavelet transformation can be calculated by the method **Real Wavelets** and **Complex Wavelets**.
- Modify the noisy wavelet coefficients.
  This is done by using bivariate shrinkage with local variance estimation (thresholding). [Bivariate Shrinkage with Local Variance Estimator, Levent Sendur and Ivan W. Selesnick, IEEE Signal Processing Letters, Vol. 9, No. 12, December 2002]
- Compute the inverse transform using the thresholded coefficients.

Parameters

Parameter		Description
Method		
-	Complex wavelets	The Dual Tree Complex Wavelet transform provides better results due to the fact that it is nearly direction invariant and makes more directional sub bands available. The results will be less prone to block-artefacts. However, this method is computationally more intense and therefore takes longer.
-	Real wavelets	The real wavelet transform only considers three sides (XYZ) and is therefore faster. However, the result can show block artefacts.
Strength		Here you adjust the strength with which the function is applied.

5.10.8 Time Series

5.10.8.1 Time Alignment

Using this function you can automatically align individual time points in order to compensate for shifts between time points.

INFO

For the alignment function to work the presence of immobile and clearly distinguishable object structures in the time series is required. Also, when aligning z-stacks over time, you should always use the z dimension from the Third Dimension drop down list. Otherwise each z-plane would be aligned over time potentially leading to z-stack artefacts.

Parameter

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.

Parameter		Description
-	Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
-	Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
-	Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

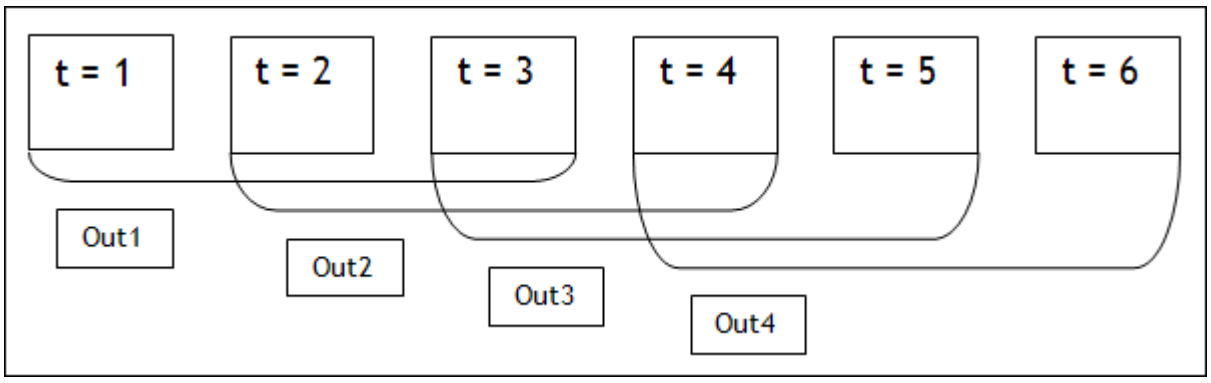
5.10.8.2 Gliding Average

With this function a smoothing effect can be achieved due to averaging out noise. It therefore calculates the gliding average of a time series image, taking into account the defined number of time points according to the following schematic:

Input Image: SizeT = 6

Averaging Length: AvL = 3

Output image: SizeT(output) = SizeT – AvL + 1 = 6 -3 +1 = 4



Parameter

Parameter	Description
Average Length	Specifies the number of images used to determine the mean value. The maximum value correlates with the number of time points.
Scaling Factor	The preset value is 1. Values > 1 can be applied for images with low intensity. In this case all pixel values are multiplied by the specified factor.

5.10.8.3 Time Differential

This function calculates the first and second order differential of a time lapse image according to the following formula and schematic:

First Order Differential:

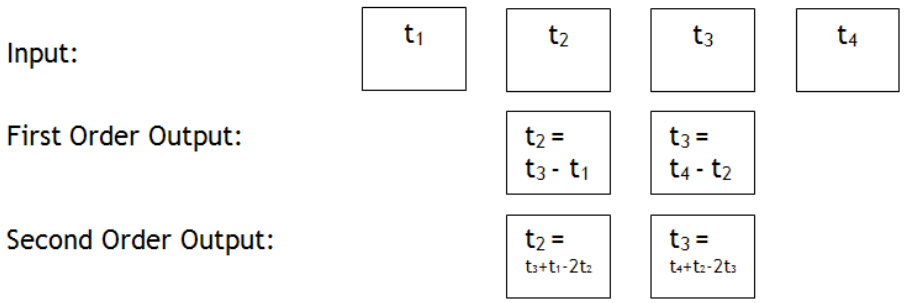
Output[t] = Input[t+1] – Input[t-1]

-> the difference between consecutive pixels is not calculated so that the output is not directional. The first order differential represents the **Speed**.

Second Order Differential:

Output[t] = Input[t-1] + Input[t+1] – 2 x Input[t]

->Second order differential is also known as the "Laplacian" and represents the **Acceleration**. It enhances the fine details in the image (including noise). The smoothing kernel helps reduce this noise.



Parameter

Parameter	Description
Derivative	Here you can select whether to calculate the first (speed) or second (acceleration) order differential.
Smoothing	Indicates the iterative, binominal smoothing filter. This reduces noise in the differential images, whilst retaining maximums and minimums. Value range: 0 – 50
Normalization	Defines what to do with negative values resulting from the calculation. ■ **Clip:** negative values are set to 0. ■ **Absolute:** negative values are used positively.

5.10.8.4 Time Concatenation

This function joins two images to form a new time series image. Select the desired images in the **Input** tool and click **Apply**.

INFO

Images with varying dimensions will be put into different blocks by this function. The resulting image document shows a **Block** slider in the **Dimensions** view block.

5.10.8.5 Kymograph

This method creates a Kymograph. The input image has to be a time series image containing a graphical element (e.g. a line, arrow, curve or polygon) which is not locked.

Parameter

Parameter	Description
Graphic tool	Here you see all graphic tools which can be used. Choose the desired graphic tool from the list. Note that the tool must be selected in the image as well.
Width	Here you adjust the graphic tools width (in pixel). This determines which pixels are used to calculate the average gray value along the width.

5.10.8.6 Time Stitching

This function stitches heterogeneous **CZI** images together to create a new, single homogeneous time series containing all dimensions and time points in their proper order. This differs from the **Time Concatenate** function, which simply pastes one time lapse series to the end of another without regard for the proper time order or channel content.

Missing images can either be filled with copies of the previous valid image in the series or filled with black images.

When combining Z-stack time series with non-Z-stack time series a choice can be made between either using only the center plane of the Z-stack or creating an extended focus projection of the Z-stack before stitching the images together.

Parameter

Parameter		Description
Fill Missing with		
-	Previous	Fills a missing dimension index with a copy of the last existing image from that index.
-	Black	Fills a missing dimension index with a black image.

Parameter		Description
Z-Stacks		
-	Collapse (EDF)	Reduces a z-stack with an extended focus function to a single plane image which is then added to the output.
-	Collapse (Center Plane)	Only uses the center plane from a z-stack for the output image.
-	Expand	Copies the z-stack to the output unchanged, fills the missing indices according to the setting in the **Fill Missing with** dropdown list.

5.10.9 Arithmetics

5.10.9.1 Add

This function adds the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image. Note that a resulting gray value may be greater than the maximum gray value of the image.

Parameter

Parameter		Description
Normalization		Depending on the IP function you have selected not all choices are available in the list.
-	Automatic	Automatic normalization of gray values to the available gray value range.
-	Clip	Automatically sets the gray levels that exceed or fall below the specified gray value range to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
-	Shift	Normalizes the output to the value gray value + max. gray value/2.
-	Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.

5.10.9.2 Add Constant

This function adds the factor **Addend** to each pixel of the **Input** image and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

Parameter

Parameter	Description
Addend	Here you adjust the addend.

5.10.9.3 Subtract

This function subtracts the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image. Note that a resulting gray value may be less than 0.

Parameter

Parameter		Description
Normalization		Depending on the IP function you have selected not all choices are available in the list.
-	Automatic	Automatic normalization of gray values to the available gray value range.
-	Clip	Automatically sets the gray levels that exceed or fall below the specified gray value range to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
-	Shift	Normalizes the output to the value gray value + max. gray value/2.
-	Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.

5.10.9.4 Multiply

This function multiplies the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image.

Parameter

Parameter	Description
Factor	Here you adjust the scaling factor by which the result of the multiplication is divided. Using this factor it is possible to keep the gray values of the **Output** image within the range of 0 to the maximum gray value. Values that are greater than the maximum gray value are in any case limited to the maximum gray value. Negative values are set to 0. **Value range**: -20,000 ... +20,000

5.10.9.5 Multiply Constant

This function multiplies each pixel of the **Input** image with a adjustable **Factor** and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

Parameter

Parameter	Description
Factor	Here you adjust the factor to be multiplied.

5.10.9.6 Divide

This function divides the images **Input1** by **Input2** pixel by pixel and generates the **Output** image.

Parameter

Parameter	Description
Factor	Here you adjust the scaling factor by which the result of the division is multiplied. Using this factor it is possible to keep the gray values of the output image within the range of 0 to the maximum gray value. Values that are greater than the maximum gray value are in any case limited to the maximum gray value. Negative values are set to 0. **Value range**: -20.000 ... +20.000

5.10.9.7 Square

This function calculates the square of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

5.10.9.8 Square Root

This function calculates the square root of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

5.10.9.9 Logarithm

This function calculates the logarithm of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

5.10.9.10 Exponential

This function calculates the exponential function of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

5.10.9.11 Invert

This function additively inverts the gray values of the input image into the output image. Bright pixels will become darker and vice versa. To adjust the output range the parameter **Operand** is used. The actual mathematical operation is then: output-gray value = constant - input-gray value. Negative results are clipped to 0 and overflow results are clipped to the maximum possible gray value.

5.10.9.12 Reciprocal

This function computes the reciprocals of the gray values in the input image into the output image. Bright pixels will become darker and vice versa. To adjust the output range the parameter "factor" is used. The actual mathematical operation is then: output-gray value = factor / input-gray value. Negative results are clipped to 0 and overflow results are clipped to the maximum possible gray value.

5.10.9.13 Average

The function calculates the average of the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image.

5.10.9.14 Combine

This function calculates the linear combination of two images on a pixel basis.

Both **Input** images are first multiplied by the specified factor and then added together. The brightness of the **Output** image can then be adjusted. The combination of two images can be used to reduce noise, for example. This is achieved by acquiring several images of the same scene and subsequently combining them.

Parameters

Parameter	Description
Factor 1	Weighting factor for input image 1. **Value range**: -1,00 ... +1,00
Factor 2	Weighting factor for input image 2. **Value range**: -1,00 ... +1,00
Offset	Offset between input images 1 and 2.

5.10.9.15 Minimum

The function calculates the maximum value of the two images **Input1** and **Input2** pixel by pixel.

5.10.9.16 Maximum

The function calculates the minimum values of the two images **Input1** and **Input2** pixel by pixel.

5.10.10 Segmentation

5.10.10.1 Threshold

This function performs a segmentation based on the definition of a brightness range (separated according to color channels (red, green, blue)) for the regions to be segmented. All pixels whose color values lie within the defined color range are marked as region pixels in the resulting image. All the pixels whose color values lie outside the defined color range are marked as background pixels (black).

In the resulting image, the color values of the region pixels can either be set permanently to white or adopted unchanged. If you set the region pixels permanently to white, the result is a binary image, which can then be used as a mask image for a subsequent automatic measurement.

Parameters

Parameter	Description
Level Low	Determines the lower brightness threshold for the regions to be segmented. All the pixels whose gray values lie below this threshold value are marked as background pixels (black).
Level High	Determines the upper brightness threshold for the regions to be segmented. All the pixels whose gray values lie above this threshold value are marked as background pixels (black).

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

5.10.10.2 Threshold (auto)

This method performs an automatic gray value segmentation. The function calculates the two minimums in the individual channels in the gray value histogram of the input image (**Input**) and uses these for the segmentation.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Method	
- Otsu	
- Maximum Peak	
- Iso Data	
- Triangle Threshold	
- Three Sigma Threshold	
Process tiles separately	

Parameters

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

5.10.10.3 Threshold (dynamic)

This method performs an adaptive gray value segmentation. This procedure is particularly well suited to the segmentation of small structures against a varying background.

The function initially applies a low pass filter and then subtracts this low-pass-filtered image from the input image. The effect of this function mainly depends on the size of the filter matrix: Select a low value for **Size** to segment small regions or regions with low gray value contrast from the background. Select a higher value for **Size** to segment larger regions from the background.

Parameters

Parameter	Description
Kernel Size	Matrix size of the low pass filter in x- and y-direction symmetrically around the pixel in question. Determines the extent of the smoothing effect. As the affected pixel is at the center, the edge length of the filter matrix is always an odd number. If an even number is entered via the keyboard, the value is always set to the next highest odd number.
Threshold	This value defines the gray value difference between the regions to be detected and the background. Segmented pixels are set to the maximum gray value (white), whilst other pixels are set to the gray value 0.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

5.10.10.4 Valleys

This method detects dark lines (gray value valleys) on a bright background and contours between bright regions.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of valleys. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of valleys are detected. Fewer valleys are detected with a high value.
Threshold	Curvature at the valley bottom high/low. Depending on the setting made, weakly pronounced valleys are also detected, or only ones that are strongly pronounced.

5.10.10.5 Canny

Canny detects edges in an image. This function detects relatively thick contours at the edge of bright regions.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of edges. A Gauss filter is used as a smoothing function. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of edges are detected. Fewer edges are detected with a high value. If the value 0 is set, no smoothing is performed.
Threshold	Steepness of the edges to be detected. Low values mean "flat" edges with a wide transition area between two regions. In this case lots of edges are detected. If high values are used, fewer edges are detected, as only steep transition areas are interpreted as edges.

5.10.10.6 Marr

This mehtod detects edges or regions in an image. In contrast to **Valleys** and **Canny**, here a Laplace filter is calculated, followed by smoothing using a **Gauss** filter, and the edges (Display Mode | Edges) or regions (Display Mode | Regions) are detected.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of edges or regions. A Gauss filter is used as a smoothing function. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of edges are detected. Fewer edges are detected with a high value. If the value 0 is set, no smoothing is performed.
Display Mode	
- Edges	Region edges are detected.
- Regions	The regions, not just their edges, are detected.

5.10.11 Binary

5.10.11.1 Fill Holes

This method fills holes in regions. Holes are structures that have the gray scale value 0, and is completely surrounded by pixels with a gray value equal to 0. Of regions outside of the image, it is assumed that they are black. Black areas that touch the edge of the image are preserved, therefore, even if they are surrounded by a contour.

5.10.11.2 Scrap

This method removes regions within a certain area.

Parameters

Parameter	Description
Minimum Area	Here you adjust the minimum area in Px.
Maximum Area	Here you adjust the maximum area in Px.
Select in Range	

5.10.11.3 Mark Regions

This function marks binary regions of the input image. For each region in the input image, a check is performed to establish whether a pixel has been set in the marker image.

Parameters

Parameter	Description
Select Marked	**Activated:** Copies the marked region into the output image. **Deactivated:** Copies the unmarked region into the output image.

5.10.11.4 Apply Mask

This tool enables you to isolate features in an image and to suppress image areas not of interest using a mask image.

Parameter	Description
Input	The input image from which you wish to isolate features or suppress areas not of interest
Mask	The mask image that is applied to the input image The mask is laid on top of the input image. Image regions of the input image **in1** where the mask is white remain unchanged, image regions where the mask is black are blacked out and suppressed. Both images are aligned at the upper left corner. If the mask image is smaller than the input image **in1**, the mask is applied only to part of the input image, beginning at the upper left corner. The rest of the input image remains unchanged.

5.10.11.5 And

This method performs a bit-by-bit AND calculation for the input images (**Input1** and **Input2**). This function is particularly useful for the masking of images. All the pixels that are white in input image 1 AND input image 2 are set to white in the resulting image. Pixels that are white in only one of the two input images become black.

5.10.11.6 Or

This method performs a bit-by-bit OR calculation for the **Input1** and **Input2** images. This function can be used to combine binary masks or regions. All the pixels that are white in input image 1 OR input image 2 are set to white in the resulting image. This means that all the white pixels in the two input images are white in the resulting image.

5.10.11.7 Xor

This method performs a bit-by-bit Xor calculation for the **Input1** and **Input2** images. This function can be used to combine binary masks or regions. All the pixels that are white in input image 1 or input image 2 are set to white in the resulting image. Pixels that are white in both input images are set to black.

5.10.11.8 Not

This function performs a binary "not" operation on all bits of the binary representation of an input pixel's gray value. A 0-bit in the input pixel results in an 1-bit in the corresponding output pixel an 1-bit in the input gets a 0-bit in the output. For integral image types the resulting output gray value is the difference of the maximum possible gray value minus the input gray value, but for float image type the results are strange due to the inhomogeneous float format.

5.10.11.9 Ultimate Erode

This function works in the same way as normal erosion. Structures in the input image are reduced. Thin connections between regions are separated. The difference between this function and normal erosion is that structures are eroded until they would be deleted by the next erosion step. With erosion, the pixel in question is set to the gray value 0 (black) in the resulting image. For regions (pixels) at the image edge, the assumption is that the pixels outside the image are white.

Parameters

Parameter	Description
Structure Element	Here you select the preferred direction of morphological change (e.g. Cross, Diagonal).
Count	Here you set the number of repetitions. This means that the function is applied a number of times in succession to the filtering result. This increases the effect accordingly.
Converge	If activated, the function is automatically repeated until all regions will be deleted by the next erosion step.

5.10.11.10 Distance

This method creates a distance-transformed image (distance map, distance image) from a binary image. The Euclidean distance to the next background pixel (gray value 0) is calculated for each pixel within the white regions of the binary image (input image), and coded as a gray value. Bright pixels (high gray values) indicate a long distance to the background.

5.10.11.11 Thinning

This method thins objects to a line of single pixel thickness.

Parameters

Parameter	Description
Thinning Element	Select the desired thinning method here.
- Arcelli	Applies thinning in accordance with the Arcelli method.
- Levialdi	Applies thinning in accordance with the Levialdi method.
Count	Sets the number of repetitions. This means that the function is applied a number of times in succession to the filtering result. This increases the effect accordingly. If the number of repetitions is set to 0, the function is automatically repeated until all regions have been thinned as far as possible. The value range is from 0...256.
Prune	Cuts off the ends of the thinned lines.
Converge	Stops thinning if no further changes can be achieved.
AxioVision Compatibility	Performs the function exactly like in AxioVision to achieve identical results.

5.10.11.12 Exoskeleton

This method generates an image with the skeleton of the influence zone of regions. The background in the **Input** image is analyzed, and the skeleton of the influence zones of the objects is determined. This is then saved as a binary image in the **Output** image.

5.10.11.13 Separation

Using this function you can attempt to separate objects that are touching (and that you have been unable to separate using segmentation) automatically.

Parameters

Parameter	Description
Separation Mode	
- Morphology	
- Watersheds	
Count	

5.10.11.14 Label Image

Assigns a gray value to each object in a binary image.

Parameters

Parameter	Description
Label Background	**Activated:** Assigns gray values to the background objects with connectivity 4. **Deactivated:** Assigns gray values to the background objects with Connectivity 8.

5.10.12 Utilities

5.10.12.1 ApoTome RAW convert

This method accepts ApoTome raw data only.

You can configure the same settings like you have on **ApoTome** tab (view option for ApoTome images).

Parameter

Find the description of the parameters here: *ApoTome tab* [▶ 866]. For ApoTome raw data you`re able to apply following correction methods:

- No correction
- Local bleaching
- Global bleaching
- Phase errors
- Phase errors and global bleaching
- Phase errors and local bleaching

The function is available for batch processing as well. This makes it easy to convert a series of ApoTome RAW data images into deconvolved images.

5.10.12.2 ApoTome deconvolution

This method accepts **ApoTome** raw data only.

It was derived from the **Deconvoltuion** modul and is available in every licensed version of **ZEN (blue edition)**. It contains settings and parameters which make sense for an Apotome deconvolution only.

Parameter

Find the description of the parameters under: Deconvolution (adjustable) parameters . This method is available for batch processing as well.

5.10.12.3 Copy Annotations

This method copies the annotations of one image into another image.

5.10.12.4 Copy Image

This method creates a copy of an image.

5.10.12.5 Change Pixel Type

This method allows you to change the pixel type of an image. This can be useful if you want to compare or combine images that have different pixel types.

Parameters

Parameter	Description
Pixel Format	Select the desired pixel format from the dropdown list.
- 8 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 255.
- 16 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 65535.
- 32 Bit B/W Float	The output image is a monochrome image with real numbers as pixel values.
- 2x32 Bit Complex	The output image is a monochrome image with complex numbers (real part and imaginary part) as pixel values. Such images are generally created by means of transformation into the Fourier space.
- 24 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 255.
- 48 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 65535.
- 2x32 Bit RGB Float	The output image is a color image with real numbers as color values in the red, green and blue channels.
- 3x64 Bit RGB Complex	The output image is a color image with complex numbers (real part and imaginary part) in the red, green and blue channels. Such images are generally created by means of transformation into the Fourier space.

5.10.12.6 Impose Noise

This function imposes an image with a defined noise for testing purposes.

Parameter	Description
Signal to Noise Ratio	Adjusts the signal to noise ratio. Range 0.10 - 100.00.
Distribution	
- Poisson	Imposes a Poisson distributed noise.
- Gauss	Imposes a Gauss distributed noise.

5.10.12.7 Create Image Subset

This method allows you to extract parts from one image and use these to create a new image. You can select these parts freely from the individual dimensions of the image.

i INFO

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections.

Parameter		Description
		Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
-	Get current position	Adopts the position from the current display in the image area.
-	Interval	**Activated:** Interval mode is active. The Interval spin box/ input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region		Here you can select if you want to use the entire image or just a region (ROI) of the input image.
-	Full	If selected this option, the full image is used for the new image.
-	Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
-	Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

5.10.12.8 Fuse Image Subset

This method allows you to insert an image subset back into the original image. Its contents are replaced by the contents of the image subset. Using this method you can process a previously created image subset using image processing functions and copy the result back into the original image.

Parameters

Parameter	Description
In Place	
Subset	Contains the description of how the input image was created as a subset. Shows which areas have been selected in generating the subset image for each dimension (channels, Z-stack, time series), as well as for the defined image section. **Example:** The entry "Z (1-8: 2) | T (2-7)" means that the sub-image consists of sections 1,3,5,7 at the intervals of 2 to 7 of the input image.

5.10.12.9 Image Generator

This function creates a synthetic image where the dimensions can be defined.

Parameter	Description
Width	Width in x of the image
Height	Height in y of the image
Z Slices	Number of z slices of the image. If the value is > 1, it will become a Z-stack image.
Channels	number of channels of the image, if value is > 1, it will become a multi channel image
Time Slices	Number of time slices, if value is > 1, it will become a time series image.
Min. Gray Value	Minimum Gray Value for generation.
Max Gray Value	Maximum Gray Value for generation.
Pixel Type	Specifies the pixel type of the image.
Pattern	
- Uniform	all pixels of the image have identical Min. Gray Value
- 2D Gray Scale Vertical	the image shows a gray scale with values between **Min. Gray Value** and **Max. Gray Value** from top to bottom.
- 2D Gray Scale Horizontal	the image shows a gray scale with values between **Min. Gray Value** and **Max. Gray Value** from left to right.

Parameter		Description
-	Ramp	the image shows a ramp with values between **Min. Gray Value** and **Max. Gray Value** starting from each corner of the image to the center.
-	Gaussian	the image shows a Gaussian shaped grayscale with values between **Min. Gray Value** and **Max. Gray Value** starting from the borders of the image to the center.
-	Checkerboard	the image shows a checkerboard where the "dark" fields have **Min. Gray Value** and the "bright" fields have **Max. Gray Value**.
-	Cosine Checkerboard	the image shows a checkerboard where the "dark" fields have **Min. Gray Value** and the "bright" fields have **Max. Gray Value** overlaid with a cosine modulation.
-	Chirp Cosine	the image shows a cosine pattern where the "dark" fields have **Min. Gray Value** and the "bright" fields have **Max. Gray Value** overlaid with a chirp modulation.
-	Chirp Checker	the image shows a checkerboard where the "dark" fields have **Min. Gray Value** and the "bright" fields have **Max. Gray Value** overlaid with a chirp modulation.
-	Random Spheres	a 3D (Z stack) image is created which contains **Number of Spheres** spheres with **Sphere Diameter** diameter which are randomly distributed in the image.
-	Sphere Array	a 3D (Z stack) image is created which contains **Number of Spheres** spheres with **Sphere Diameter** diameter which are equally distributed in the image.
-	Single Sphere	a 3D (Z stack) image is created which contains a single sphere with **Sphere Diameter** diameter which is positioned in the center of the image.
Sphere Diameter		Diameter of the created spheres.
Number of Spheres		Number of spheres which are generated in the 3D image.

5.10.12.10 Create Gray Scale Image

This method allows you to create a gray scale image.

Parameters

Parameter	Description
Pattern	Select the desired pattern for the gray scale image here.
- Uniform	All pixels have an identical gray/color value.
- 2D Gray Scale Vertical	The gray scale runs from top to bottom, starting with the gray value selected in parameter **Min. Gray Value**.
- 2D Gray Scale Horizontal	The gray scale runs from left to right, starting with the gray value selected in parameter **Min. Gray Value**.
Width	Set the desired width of the output image in pixels using the slider or the input field.
Height	Set the desired height of the output image in pixels using the slider or the input field.
Min. Gray Value	Set the minimum gray value of the gray scale using the slider or input field.
Max. Gray Value	Set the maximum gray value of the gray scale using the slider or input field.
Pixel Type	Select the desired pixel type here.
- 8 Bit B/W	The output image is a monochrome image whose integer gray values can be in the range of 0 to 255.
- 16 Bit B/W	The output image is a monochrome image whose integer gray values can be in the range of 0 to 65535.
- 24 Bit RGB	The output image is a color image whose integer color values in the channels Red, Green, Blue can be in the range of 0 to 255.
- 48 Bit RGB	The output image is a color image, with integer color values in the color channels Red, Green, Blue can be in the range of 0 to 65535.

5.10.12.11 Image Calculator

This method allows you to apply arithmetic operations to images in the form of a calculator.

You can process a single image or combine two images.

All operations are performed pixel by pixel.

Parameters

Parameter	Description
Channel Input 1	Here you can select whether you want to use an individual channel or all channels of the first input image for the calculation.
Channel Input 2	Here you can select whether you want to use an individual channel or all channels of the second input image for the calculation.
First Images	**Activated:** For the second input image uses only the first time points of a time lapse image for the calculation. This allows you, for example, to normalize a time lapse image to the intensity values of the first time points. Enter the number of images that you want to be used for the calculation using the input field.
Formula	Enter the calculation formula here using the keyboard and numeric keypad. Use "S1" as a placeholder for the first input image and "S2" for the second input image.
Input 1	Inserts the placeholder for the first input image into the **Formula** input field at the current cursor position.
Input 2	Inserts the placeholder for the second input image into the **Formula** input field at the current cursor position.
Absolute Intensities	Activate this radio button if input image 1 and input image 2 have the same pixel type.
Normalize Intensities 0..1	Activate this radio button if input image 1 and input image 2 have different pixel types. To allow such images to be combined, the intensity values of the two images are normalized to the value range from 0 to 1 before the calculation.
Operators...	Opens a list of all available operators. Here you can select the operator that you want. If you double-click on a list entry, it is inserted into the **Formula** input field at the current cursor position.
Delete	Deletes the contents of the **Formula** input field.
Undo	Undoes the last entry in the **Formula** input field.

5.10.12.12 Add Channels

This method allows you to combine two input images that have different channels but otherwise have the same dimension (Z-stack, time series, tile, scene). An image is produced that contains all the channels of the input images.

If the two input images differ from one another in the dimensions Z-stack, time series, tiles or scene, input image 1 and input image 2 are copied into the output image as two separate blocks.

5.10.12.13 Generate Image Pyramid

This method allows you to calculate a resolution pyramid for an image. Using the resolution pyramid you can navigate extremely efficiently even in very large tile images and display individual regions in the image window.

Parameters

Option	Description
Background	This option will influence how the background of the image pyramid will look like.
– Auto	The region outside the scenes keeps the grey value of the image, i.e. white for brightfield images and black for fluorescence images.
– Black	The region outside the scenes will be displayed black.
– White	The region outside the scenes will be displayed white.

5.10.12.14 Split into RGB

This method generates the individual color extractions for red, green and blue from the RGB input image. The resulting images for red, green and blue take the form of gray images.

Parameters

Parameter	Description
Output Pixel type	Here you choose the desired output image format, e.g. 8 Bit B/W.

5.10.12.15 Combine RGB

With this method a color image can be generated out of three input images of the single color extractions **Red**, **Green** and **Blue**.

Parameters

Parameter	Description
Output Pixel type	Here you choose the desired output image format, e.g. 24 Bit RGB.

5.10.12.16 Split into HLS

This method generates the individual color extractions for a HLS input image. The resulting images for hue, lightness and saturation take the form of gray images.

5.10.12.17 Combine HLS

With this method a HLS image can be generated of the single color extractions H, L, S.

5.10.12.18 Calculate Histogram

This method calculates a histogram distribution for selected measurement parameters of a measurement data table.

Parameters

Parameter	Description
Columns	Define the measurement parameters for classification by entering the column numbers freely, e.g. 1,3,5, or 1-6 or 1,3-7,8. Clicking on the  button to open the **Select columns** dialog. Here the column names of the data can be activated or deactivated by clicking on the relevant checkbox.
Class Boundaries	Select here, how you want the class boundaries of the calculated histogram to be determined.
- >=,...,<	A numerical value falls into the histogram class if it is greater than or equal to the lower class boundary and less than the upper class boundary.
- >,...,=<	A numerical value falls into the histogram class if it is greater than the lower class boundary and less than or equal to the upper class boundary.
Automatic Classification	**Activated**: The class boundaries are calculated automatically from the data. The value range from the lowest to the highest data value is divided into as many classes of equal width as you have set in the **Class Number** input field. Example: Minimum value is 0 Maximum value is 10000 Range is 10000 units Class Count is 4 Then the class boundaries are as follows:

Parameter	Description
	Class 1: 0 .. 2500 Class 2: 2501 .. 5000 Class 3 : 5001 .. 7500 Class 4: 7501 .. 10000
Logarithmic	Only active, if the **Automatic Classification** checkbox is activated. **Activated**: The class boundaries are scaled logarithmic. Example: Minimum value is 0 Maximum value is 10000 Range is 10000 units Number of classes is 4 Then the class boundaries are as follows: Class 1: 0 .. 10 Class 2: 11 .. 100 Class 3: 101 .. 1000 Class 4: 1001 .. 10000
Class Count	Specifies the number of classes that shall be created.
Display Mode	Select here, how you want the values of the histogram to be calculated.
- Count	The histogram indicates how many data sets fall into the relevant class, it contains the frequency of the values in the class concerned.
- Count Cumulative	The histogram cumulates the counts of values in each class. Class 1 contains the number of values for class 1, class 2 contains the sum of the values from class 1 and class 2, class 3 contains the sum of the values from class 2 and class 3, etc.
- Percentage	The histogram indicates what percentage of the data sets fall into the relevant class, it therefore contains the percentage share of the values in the class concerned.
- Percentage Cumulative	The histogram cumulates the percentage of the count of values in each class. Class 1 contains the percentage for class 1, class 2 contains the sum of the percentages from class 1 and class 2, class 3 contains the sum of the percentages from class 2 and class 3, etc. The last class therefore contains 100%.

Parameter	Description
- Sum	The histogram contains the sum of the numerical values of the data sets that fall into the relevant class, the values of the data sets that fall into the class concerned are therefore added together.
- Sum Cumulative	The histogram cumulates the sums of the values in each class. Class 1 contains the sum of the numerical values from class 1, class 2 contains the sum of the numerical values from class 1 and class 2, class 3 contains the sum of the numerical values from class 2 and class 3, etc. The last class therefore contains the sum of all individual values.
- Percentage Sum	The histogram indicates the percentage share of the total numerical values in the relevant class.
- Percentage Sum Cumulative	The histogram cumulates the percentage of the sums of values of all data points which belong to the class. Class 1 contains the percentage of the total numerical values from class 1, class 2 contains the sum of the percentages of the total numerical values from class 1 and class 2, class 3 contains the sum of the percentages of the total numerical values from class 2 and class 3, etc. The last class therefore contains 100%.

5.10.12.19 Split Scenes (Write files)

This method saves the single scenes (tiles or positions) of a multiscene image (i.e. image of a multiwell plate) as single images in a folder in **CZI** format.

Parameter

Parameter	Description
Display field	The path of the destination folder is displayed automatically in the display field. To change the folder, click on the  button to the right of the display field.
Include Scene Information in Generated File Name	**Activated:** Includes the scene information in the file name of the separate image.
Overwrite existing files	**Activated:** All files in the target folder will be deleted, if the function is applied again.
Compression	Choose the type of compression here, eg. **Original** (no compression) or **Compression (JPEG XR)**.

5.10.12.20 Split Scenes

This method separates scenes from a tiles or positions image. The individual images are displayed in the **Center Screen Area**. Note that the images in this method, in contrast to the method **Split Scenes (write files)**, are not automatically stored in a folder.

5.10.12.21 Split Multiblock Image

This method saves the single blocks/dimensions (Tiles or Positions) of a multiblock image (i.e. image of an inhomogeneous experiment) in a folder in **.CZI** format.

Parameter

Parameter	Description
Split Mode	Choose the mode how to split the multiblock image.
- Homogeneous groups	Splits the multiblock image into the single dimensions. The blocks will remain.
- Single blocks	Splits the multiblock image into single blocks.
Display field	The path of the destination folder is displayed automatically in the display field. To change the folder, click on the  button to the right of the display field.

5.10.12.22 Airyscan Processing

With this function you can access the superresolution data in images acquired with Airyscan.

NOTICE

Please note that starting with ZEN 2.5 blue edition, the black border of the processed image is automatically removed. Hence the resulting image will be smaller by 24 pixels in X and Y dimension.

Parameters

Parameter	Description
3D Processing	This option is only available for images with 5 or more z-positions. If activated, this option improves the resolution in axial and lateral direction. The data set needs to have at least 5 z-sections acquired with an optimal step size. 3D Processing

Parameter	Description
	is slower than 2D Processing. For 3D Processing, the whole z-stack (single channel and time point) needs to fit into the physical memory.
Auto Filter	If activated, a suitable **Super Resolution** parameter for the Airyscan processing is automatically determined for the selected data set. To manually adjust the **Super Resolution** parameter, deactivate the checkbox. Then determine suitable values by using the corresponding function in the Airyscan viewer in the **Airyscan** view. Note that the preview is only suitable for 2D Airyscan processing. A preview for 3D Airyscan processing is not available. For adjusting 3D processing parameters, you should first process your data set once using the Auto Filter and then check the value that was actually applied by the Airyscan processing function. This value is stored in the metadata of the processed image and can be accessed using the **Info** view.
Adjust per Channel	Only visible, when the **Auto Filter** is deactivated. Only available for images with two or more Airyscan channels. If activated, you can manually set channel-specific Airyscan processing parameters.
Strength	Use this option for an increased (high) or decreased (low) strength of the automatically assigned filter value. This is especially useful for 3D processing, as the 2D preview of the processing filter value in the Airyscan viewer does not allow to conclude the result after a 3D data processing. The increment of this parameter is ± 0.4 compared to the standard auto Airyscan processing. This setting is not available when manual processing strength is selected.

5.10.12.23 Convert To Lambda

With this function you can convert Lambda stacks which were acquired with **LSM 800** into a file with the same appearance as inside the **Lambda** view. In contrast to the generic raw data format of the Lambda stacks, these files can be opened and analyzed normally in **ZEN (black edition)**.

5.10.12.24 Linear Unmixing

With this function you can extract the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in multi-channel images or Lambda stacks (only available in LSM imaging mode).

With the knowledge of the spectral characteristic of individual components within a multi-component sample, even heavily overlapping individual spectral characteristics can be mathematically extracted from an experimental multi-channel data set. This method is a strictly pixel-by-pixel image analysis procedure. Experimentally, fluorescence spectra of mono-labeled samples are acquired and stored in the Spectra Database as an external reference. Then a multi-channel image or Lambda stack from the multi-labeled sample is acquired. The individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be used in the least-square-fit based algorithm to produce a 10-channel output image without any partial overlap between the channels.

Avoid saturation of fluorescence signal in the data set to be unmixed. Saturation will generate a high signal in the residual channel.

If mono-labeled samples are not available, the references can be obtained by the following methods:

- Interactively by user-selection of regions in the image where only one fluorescence dye is present (only available in the **Unmixing** view).
- Automatically by software analyses of what the individual spectral signatures are. This processing function is called Automatic Component Extraction (ACE).

Note that in some cases, spectrally acquired images are not appropriate for ACE and linear unmixing can lead to wrong results.

Parameters

Parameter	Description
Components	Here you adjust the number of spectrally distinguishable fluorescent components within the imaged sample can be selected. The number of extractable components cannot be higher than the number of acquired channels. The maximum possible value is 10 components.
Autoscale	Balances the intensity of the unmixed channels to equal levels.
Calculate Residuals	Generates an additional channel in which the intensity values represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed. The higher the intensity in this additional channel, the worse is the fit of the spectra to the data set.

5.10.12.25 Correlation

With this function you can, in conjunction with confocal data sets, display the spatial or temporal correlation of an image or image stack. You can select which kind of correlation you want to perform by activating the corresponding checkboxes.

Parameters

Parameter	Description
Cross Correlation	If activated, you can to correlate two images with each other. Note that the second input image needs to have the same dimensionality and size.
X, Y	Correlates the signal in the X or Y direction.
Z	Correlates the signal in Z. Only available for data sets containing Z-sections.
Time	Correlates the signal in time. Only available for time series data sets.

5.10.12.26 VivaTome RAW Convert

5.10.12.27 Create PSF

For creating experimental point spread functions from a Z-stack of subresolution fluorescent beads please use the function **PSF Wizard** which is available together with the **Deconvolution** module and offers a guided procedure starting with a stack of many beads and includes the **Create PSF** functionality.

Prerequisite for the **Create PSF** function here is, that bead averaging has already been done. It is available only for legacy reasons.

This function creates a PSF (Point Spread Function) image from a Z-stack image of a bead acquired for PSF measurement. Please observe the instructions for optimal acquisition here: Using beads for PSF measurement.

The result is a so-called PSF image. For advanced settings and options, please use the specific control elements on the *PSF Display* [▶ 864] tab.

Parameters

Parameter	Description
Z-Stack Correction	**Activated:** Performs background correction of the Z-stack before the processing.

Parameter	Description
Circular Average	**Activated:**Forces a PSF with lateral symmetry. This option should not usually be activated as lateral asymmetries correspond better to the real situation. Circular averaging is only recommended when a measured PSF is used with the Fast Iterative method.
Threshold Cropping	**Activated:** The PSF is restricted to gray value ranges up to 0.25% of the brightest voxel present. If the value is reduced or the option is deactivated, the PSF may be larger. This increases the calculation time. However, it is also possible to achieve slightly better results in this case. This option is deactivated by default.
Threshold	Using this slider and input field you can set the percentage from which the PSF is clipped if the Volume Clipping option has been selected.
Iterative Restoration	**Activated:** If Z-stack images of beads with diameters greater than the microscope's resolution limit are used to generate the PSF, this option must be selected. The bead diameter used can be entered using the slider and input field.

5.10.13 Export/Import

5.10.13.1 Image Export

Using this method you can export single images into various file types so that you can continue to use them in other programs. Multidimensional images (multichannel, Z-stack, time lapse, tile images) are exported as individual images.

Parameters

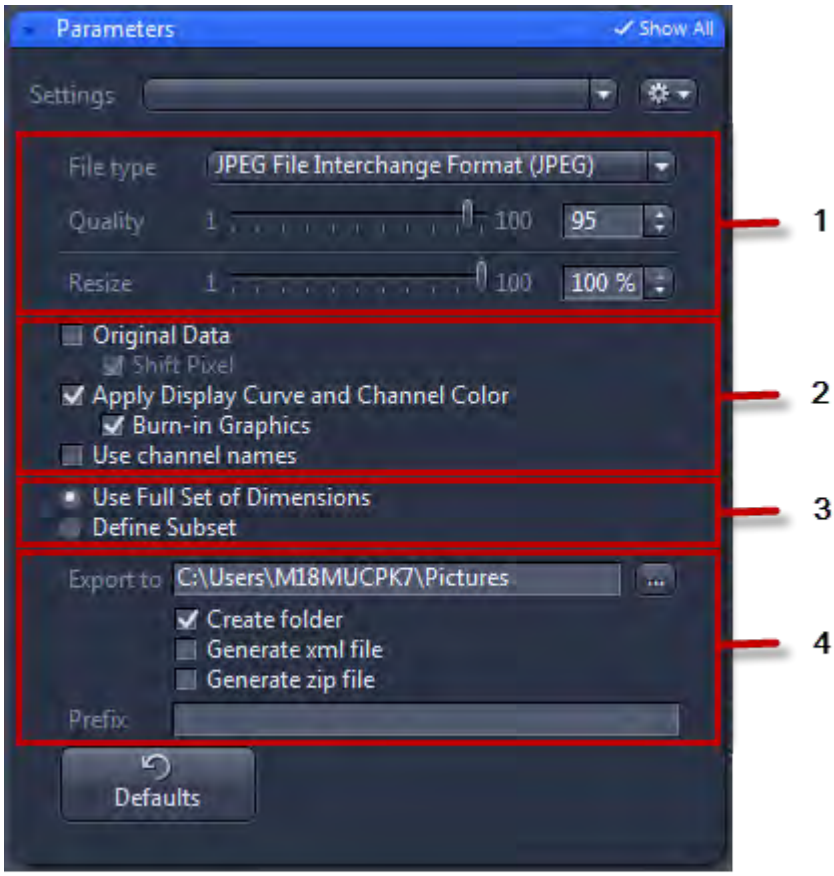


Fig. 5.3: Parameter Image Export

No.	Description
1	Here you define the settings for the file type, e.g. format or compression. An exact description of the functions, see *File Type section* [▶ 217]. Depending on the file type you selected for the image export, the settings of the other parameters change
2	Here you define, how to deal with the image data during the export, e.g. whether you want to burn graphics firmly in the picture. An exact description of the functions, see *Image Data section* [▶ 218].
3	Here you specify, how to proceed with the dimensions, e.g. whether you want to export all the dimensions of an image, or only certain. An exact description of the functions, see *Dimensions section* [▶ 219].
4	Here you define other export settings, e.g. the export folder. An exact description of the functions, see *Export to section* [▶ 221].

5.10.13.1.1 File Type section

Name	Function
File Type	Select the desired file type from the dropdown list: ■ **JPEG** (JPEG File Interchange Format) ■ **BMP** (Windows Bitmap) ■ **TIFF** (Tagged Image File Format) ■ **Big TIFF** Tiff Format (64 bit) ■ **PNG** (Portable Network Graphics) ■ **WDP** (JPEG XR) Note that various options are available depending on the file type you have selected.
Quality	Only available for the file types **JPEG** and **JPEG XR**. Enter the image quality using the slider or input field to influence the size of the file. Although low values result in very small files, image quality may be considerably reduced.
Resize	Adjust the image size in percent using the slider or input field.
Convert to 8 Bit	Only available for the file types **TIFF**, **BigTIFF**, **PNG** and **JPEG XR**. **Activated:** Converts a 16 bit gray level image into an 8 bit gray level image, or a 48 bit color image into a 24 bit color image.
Compression	Only available for the file type **TIFF** and **BigTIFF**. Select the compression method for reducing the data volume here:
- None	Retains the data volume of the original image. No compression is performed.
- LZW	Only available for the file type **TIFF**. Performs lossless compression in accordance with the Lempel-Ziv-Welch algorithm (LZW).
- ZIP	Only available for the file type **TIFF.** Performs lossless compression in accordance with the ZIP method.
- Loss less	Only available for the file type **BigTIFF.** Performs lossless compression in accordance with the Lempel-Ziv-Welch algorithm (LZW).

Name	Function
- Lossy	Only available for the file type **BigTIFF.** Performs lossy compression in accordance with the JPEG XR (extended range) method.
BigTIFF	Only available for the file type **BigTIFF**. **Activated:** Generates a BigTIFF image. The maximum image size is larger than 4GByte. **Deactivated:** Generates a TIFF image with maximum size of 4GByte.
TIFF Tiles	Only available for the file type **BigTIFF**. **Activated:** Generates new rectangle tiles for internal data handling. **Deactivated:** Combines tiles as stripes for internal data handling..
Pyramid	Only available for the file type **BigTIFF**. **Activated:** Calculates an image pyramid.
Merge All Scenes	Only available for the file type **BigTIFF**. **Activated:** Generates one image including all scenes. Single scene images will be generated, if the checkbox is deactivated.

5.10.13.1.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Original Data	**Activated:** Exports the image with the original channel colors and the original display characteristic curve.
Convert to 8 Bit	**Activated:** Converts the image to 8 Bit.
Apply Display Curve and Channel Color	**Activated:** Exports the image with the changed channel color and display characteristic curve settings. These settings are applied to the pixel values of the exported images. They are particularly important if you want to use dark images with a dynamic range of more than 8 bits in other programs.

Parameter	Description
Burn In Graphic	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.
Merged channels image	Only visible for multichannel images. **Activated:** Exports the pseudo color image of all selected channels.
Individual Channels image	Only visible for multichannel images. **Activated**: Exports the individual colored image of all selected channels.

i INFO

At least one of the three checkboxes must be activated. If the **Merged channels Image** and **Individual Channel image** checkboxes are activated, you can export the individual colored images and the pseudo color images in a single step.

Parameter	Description
Use channel names	Only visible for multi-channel images. **Activated:** Integrates the channel name in the name of the exported image.

5.10.13.1.3 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

i INFO

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/ input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image.

Parameter	Description
	If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

5.10.13.1.4 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the  button to the right of the display field.

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Create folder	**Activated:** Creates a separate folder with the name of the input image.
Create xml file	**Activated:** Creates two XML files containing the meta information relating to the input image. ■ **Meta.xml** contains all additional information relating to the input image (e.g. hardware settings, dimensions). ■ **Info.xml** only contains additional information relating to the exported individual images (e.g. names, dimensions, sizes).
Generate zip file	**Activated**: Creates a ZIP file in which all exported individual images, including the XML files, are saved.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

5.10.13.2 Movie Export

Using this function you can export multidimensional images (e.g. Time Series or Z-Stack images) into various file types in the form of film sequences so that you can continue to use them in other programs.

i INFO

If you want to export **MOV** files (H264 or MPEG4 codec) successfully, download the application **FFmpeg** (e.g. on http://www.ffmpeg.org/ oder hier http://ffmpeg.zeranoe.com/blog/?p=178). Copy **ffmpeg.exe** in to the same folder where **ZEN.exe** is located.

5.10.13.2.1 File Type section

i INFO

AVI (MS-Video1) mode is available for 32-bit Windows operating systems only.

Parameter	Description
Mode	Select the desired mode here. The following formats are available for the movie export. ■ AVI (M-JPEG compression) ■ AVI (uncompressed) ■ AVI (DV) ■ WMF (WindowsMedia) ■ MOV (H.264) ■ MOV (MPEG4) ■ AVI (MS-Video1)
Format	Select the desired format here.
- Original Size	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the height and width of the input image and sets the frame rate to 5 frames per second.
- User-Defined	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Enter the values in the Width, Height and Frame Rate input fields.

Parameter	Description
- 720x576/25fps (PAL 576p/25)	Uses the PAL (Phase Alternating Line) video resolution with 25 frames per second.
- 720x480/29.97fps (NTSC)	Uses the NTSC (National Television Systems Committee) video resolution with 29.97 frames per second.
- 1280x720/50fps (HD 720p/50)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 720) video resolution with 25 frames per second.
- 1920x1080/25fps (HD 1080p/25)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 1080) video resolution with 25 frames per second.
- 1920x1080/29.97fps (HD 1080p/29.97)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 1080) video resolution with 29.97 frames per second.
Width	Only active if you have selected **User-Defined** in the **Format** dropdown list. Here you can enter the width of the image in pixels (px).
Height	Only active if you have selected **User-Defined** in the **Format** dropdown list. Here you can enter the height of the image in pixels (px).
Frame Rate	Only active if you have selected **User Defined** in the **Format** dropdown list. Here you can enter the frame rate in frames per second (fps).
Quality	Only visible if you have selected **AVI (M-JPEG compression)** or **MOV** in the **Mode** dropdown list. Here you can set the image quality using the slider or spin box/input field. This influences the size of the file. Although low values result in very small files, image quality may be considerably reduced.

5.10.13.2.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Burn In Graphic	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.
Merged channels image	Only visible for multichannel images. **Activated:** Exports the pseudo color image of all selected channels.
Individual Channels image	Only visible for multichannel images. **Activated**: Exports the individual colored image of all selected channels.

i INFO

At least one of the three checkboxes must be activated. If the **Merged channels Image** and **Individual Channel image** checkboxes are activated, you can export the individual colored images and the pseudo color images in a single step.

5.10.13.2.3 Fitting section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Fitting	Select the desired type of fitting here.
- Fit All (Uniform)	Fits the image to the selected resolution. The original aspect ratio is retained.
- Fit and Crop (Uniform to Fill)	Fits the image to the selected resolution and clips it. The original aspect ratio is not retained.
- Fit and Stretch (Fill)	Stretches the image to the selected resolution. The original aspect ratio is not retained.
- Crop (None)	Crops the image to the selected resolution. The original aspect ratio is retained.

5.10.13.2.4 Sequence section

The following functions are only visible if the **Show All** mode is activated:

Change the sequence of the dimensions in which you want the movie to be created.

button

Shifts the selected dimension up a line.

button

Shifts the selected dimension down a line.

5.10.13.2.5 Mapping section

Parameter	Description
Mapping	Select how you want the images to be assigned.
- Fixed Duration	Enter the time per image in seconds using the spin box/ input field. The total length is displayed in the **Final Movie Length** display field.
- 1 Frame per Image	Assigns one frame per image.
Image count	Indicates the number of images in the input image.
Final Movie Length	Indicates the total length of the resulting movie, depending on the selected image sequence and the time.

5.10.13.2.6 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

INFO

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/ input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image.

Parameter	Description
	If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

5.10.13.2.7 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the  button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

5.10.13.3 OME TIFF Export

Using the **OME TIFF Export** function you can export your images into OME (Open Microscopy Environment) TIFF format so that you can continue to use them in other programs. The images are then available as a multipage TIFF file.

5.10.13.3.1 Image Format section

Parameter	Description
Resize	Adjust the image size in percent using the slider or spin box/input field.
The following parameters are only visible, if **Show All** is activated.	
BigTIFF	**Activated:** Creates a BigTIFF image that can be bigger than 4 gigabytes and uses 64-bit offset format.
Compress	**Activated:** Performs internal tif compression.
Use Tiles	**Activated:** Performs an internal tiling.

Parameter	Description
	Note that this option is recommend for large images as some tif readers do not support large images.
Shift Pixel	**Activated:** Shifts the grey value of a 10-bit or 12-bit image to 16-bit.
Merge All Scenes	**Activated:** Generates one image including all scenes. Single scene images will be generated, if the checkbox is deactivated.

5.10.13.3.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Original Data	**Activated:** Exports the image with the original channel colors and the original display characteristic curve.
Apply Display Curve and Channel Color	**Activated:** Exports the image with the changed channel color and display characteristic curve settings. These settings are applied to the pixel values of the exported images. They are particularly important if you want to use dark images with a dynamic range of more than 8 bits in other programs.
Burn In Graphics	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.

5.10.13.3.3 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

i INFO

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/ input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image.

Parameter	Description
	If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

5.10.13.3.4 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the  button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

5.10.13.4 ZVI Export

Using the **ZVI Export** function you can export your images into ZVI format so that you can continue to use them in AxioVision.

5.10.13.4.1 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the  button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

5.10.13.5 Image Import

Using the **Image Import** function you can create a multidimensional image (multichannel, Z-stack, time lapse, tile, position image) from individual images. The individual images may be in any of the external formats supported by ZEN (see below). The resulting image can then be saved in **CZI** format and processed further using the functions available in ZEN.

Supported file types

- JPG images
- BMP
- TIFF
- PNG
- GIF
- DeltaVision images
- MetaFluor images
- Multi page images

Parameters

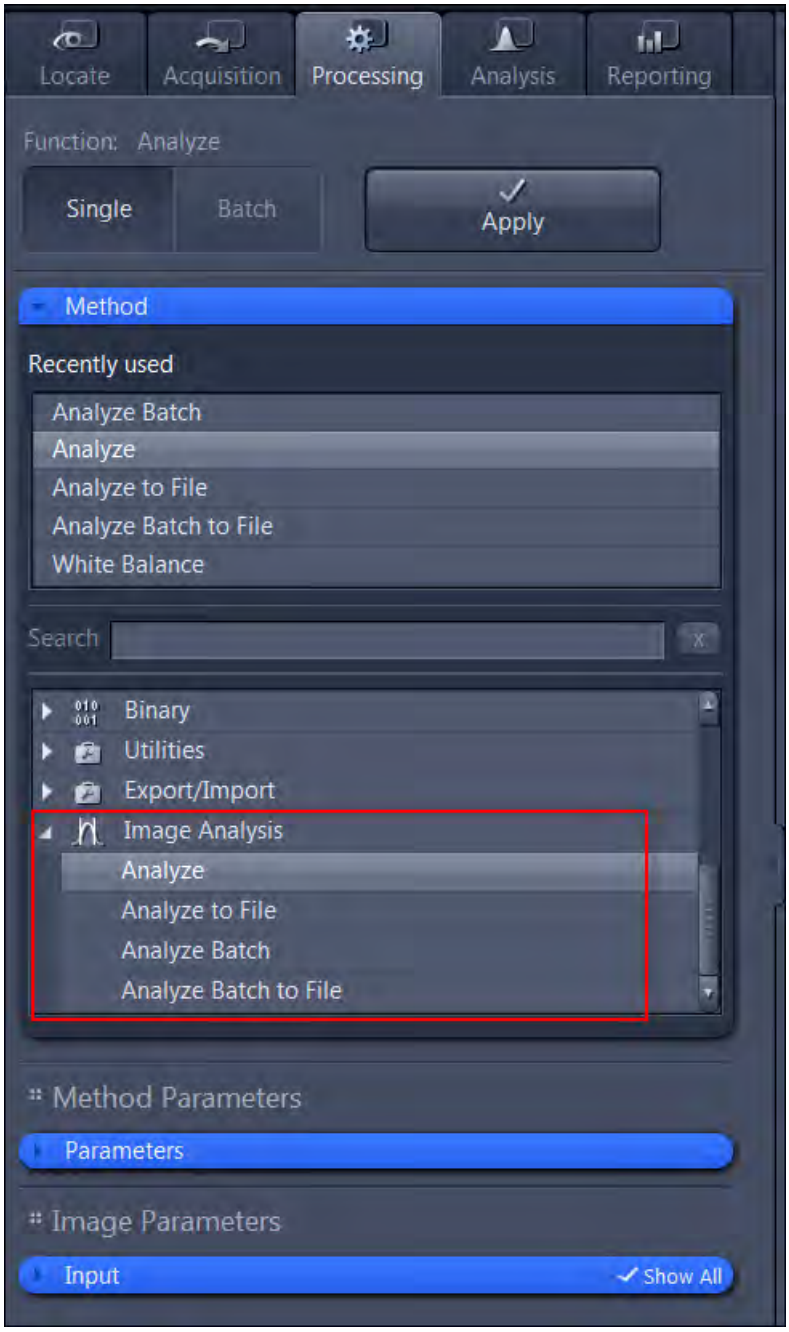
Parameter	Description
Multichannel	**Activated:** Activates the settings to import multichannel images. Adjust the settings for the multichannel image to be imported in the list below.
- Use Channel Name as Identifier	**Activated:** Uses the name specified in the **Name** column to identify the channel. The channel name will appear in the **Preview** display field in the **Specify the Identifiers** section. **Deactivated:** Activates the **Identifier**, **Start Index** and **Interval** columns in the **Specify the Identifiers** section.
Z-Stack	**Activated:** Activates the settings to import Z-Stack images. Adjust the settings for the Z-stack image to be imported in the list below.

Parameter		Description
-	Interval	Here you enter the value in µm for the distance between the individual slices. The total height of the Z-stack is calculated automatically from this value and the number of slices.
-	Slices	Here you enter the number of slices of the Z-Stack image.
-	Range	Here you enter the total height (range) of the Z-stack in µm. The distance between the individual Z-stacks is calculated automatically from this value and the number of slices and displayed in the **Interval** display field.
-	Extended Microscope Parameters	Activates additional parameters that are necessary for further processing of the imported image (e.g. for deconvolution), like: ■ **Magnification:** Select the objective magnification that was used for acquisition here. ■ **Aperture:** Here you can enter the value of the numerical aperture of the objective that was used for acquisition. ■ **Immersion:** Select the immersion medium that was used for acquisition here.
Time Series		**Activated:** Activates the settings to import time series images.
-	Interval	Here you enter the value for the interval between the individual time points. Select the unit of time from the dropdown list to the right of the input field.
-	Cycles	Here you enter the cycles of the time series. The entered value will affect the duration/the interval depending on which value you have selected.
-	Duration	Here you can enter the value for the duration of the entire time series. Select the unit of time from the dropdown list to the right of the input field. Enter the number of time points in the **Time Points** input field.
Tiles		**Activated:** Activates the settings to import tile images.
-	Columns	Here you can enter the number of columns of the tile image.

Parameter	Description
- Overlap	Here you can enter the percentage by which the tiles do overlap.
- Rows	Here you can enter the number of rows of the tile image.
- Meander	Select this option, if the images to be imported were acquired in the Meander acquisition/travel mode.
- Comb	Select this option if the images to be imported were acquired in the Comb acquisition/travel mode.
Positions	**Activated:** Activates the settings to import images of individual positions.
Scalings	To show the section in full, click on the arrow button 
- Use Current Scaling	Uses the geometric scaling currently selected and displays the values for **Scale (X)** and **Scale (Y)** with the corresponding unit in the relevant display field.
- Define scaling	Enter the desired values in the **Scale (X)** input field and in the **Scale (Y)** spin box/input field. Select the unit for the scaling value from the dropdown list to the right of each input field.
Automatic	If selected, all images that are available in an import folder are imported automatically.
- Import From	Here you can select the import folder. To select a folder, click on the folder button  to the right of the display field. The names of the images are displayed in the **File Name** list below the display field.
Sequential	If selected, an image sequence in a certain order (e.g. image dimensions that are encoded by means of numbers in the image name) can be imported.
- Import From	Here you can import images according to a **File List** or a **Multi page image**. Click on the folder button  to the right of the display field. The names of the images are displayed in the **File Name** list below the display field.
Specifiy the Identifiers	Here you can enter and check all the settings you need to identify your images.

5.10.14 Image Analysis

The Group **Image Analysis** provides four different options to analyze images in single or batch mode. The measurement data can be embedded in the image or save in a ***csv**-datalist.



Analyze

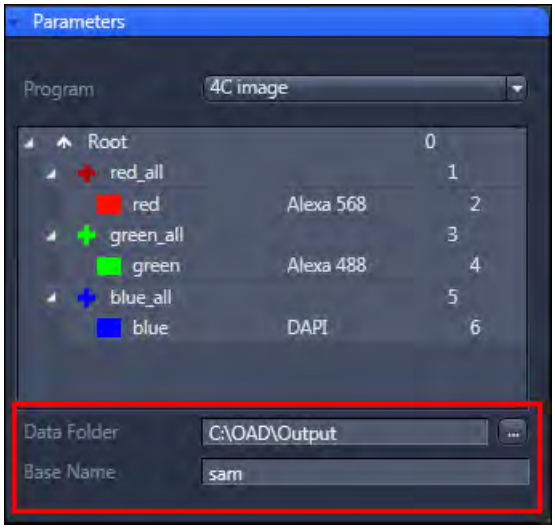
This function is applicable only for one picture: This must be selected in the **Input** tool. The measurement data are embedded in the image.

Analyze to File

This function is applicable only for one picture: This must be selected in the **Input** tool. The measurement data is stored in a ***csv** list and not embedded into the image.

The following file types are supported:

- CZI
- ZVI
- BMP
- TIF
- JPG



Parameter	Description
Data Folder	Folder where the ***csv**-data lists will be stored.
Base Name	Name of the data list.

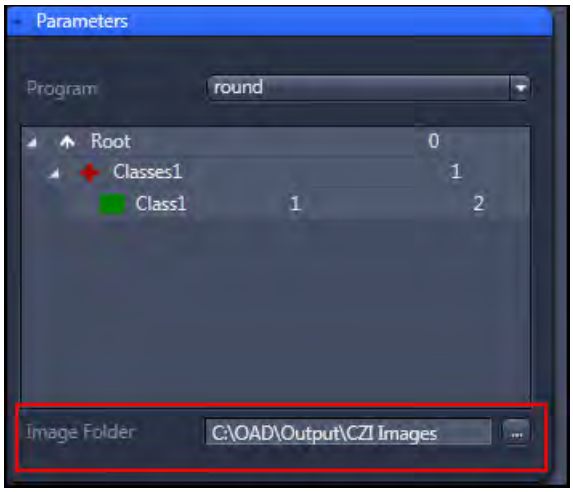
Analyze to Batch

This function allows the analysis of all images in a folder.

INFO

The function **Analyze Batch** can only be applied to images with the file type ***czi**.

The measured data are embedded in each original image.



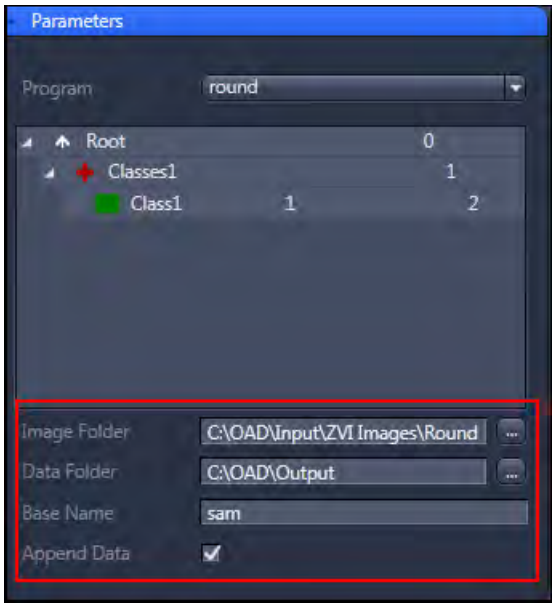
Parameter	Description
Image Folder	Folder of the ***czi** images to be measured.

Analyze Batch to File

This function allows an analysis of all images in a folder. The measured data are stored in a ***csv** list and not embedded into the image.

The following file types are supported:

- CZI
- ZVI
- BMP
- TIF
- JPG



Parameter	Description
Image Folder	Folder of the images to be measured.
Data Folder	Folder where the ***csv**-data lists will be stored.
Base Name	Name of the data list.
Append Data	**Activated**: For each class, an accumulated csv-data list is stored with the measurement data of all images. **Deactivated**: For each image, one data list is stored per class.

6 Image Analysis

6.1 Introduction

With the **ZEN (blue edition)** Image Analysis module you can create automatic measurement routines very easily. The Image Analysis wizard guides you through the steps to create an automatic measurement program. It allows you to set up even complex measurement tasks easily. The steps of the wizard include image segmentation, object separation and measurement of geometrical or intensity features. After you have completed the setup you can apply these settings to the data to be analyzed and obtain precise measurement results. You can display the results in table and list form and export them to csv-format.

For more information, see the following examples:

- *Measuring Fluorescence Intensity in a Multichannel Image* [▶ 241]
- *Counting Number of Fluorescence Signals per Nuclei* [▶ 248]
- *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256]
- *Counting the number of Objects in a Ring around the Nucleus* [▶ 263]

See also the following description:

- *Image Analysis Tool* [▶ 741]
- *Image Analysis Wizard* [▶ 742]

6.2 Creating a new image analysis setting

When creating a new analysis setting for your images, you can select the following segmentation methods:

- **Segment region classes independently**: This method allows you to define several classes and subclasses. With this method, you can define the segmentation algorithm for each class independently.
- **ZOI (Zones of Influence)**: This method constructs a zone of influence (ZOIs) and a ring around each primary object. The primary objects are generated by segmenting the selected image channel with the selected class segmenter. The ring is defined by its width and distance from the primary object. The distance from the ZOI border from the ring can be specified. The ZOI area also incorporates the primary object and ring area.
- **Segment binary images**: This method allows you to segment binary images. With this method, you can define several classes and subclasses. The step **Automatic Segmentation** uses the defined binary image channels for each class to segment the image.

- **Whole image**: This segmenter uses the whole image as a region. You can use this to perform e.g. intensity measurements on the whole image without detecting objects. Therefore it is not possible to create more classes or subclasses.
- **Interactive Segmentation**: This method allows you to define multiple classes and subclasses. Unlike the other methods, it does not include an automatic segmentation step but uses only the interactive segmentation step. That means, for each class all regions must be drawn manually.

Procedure

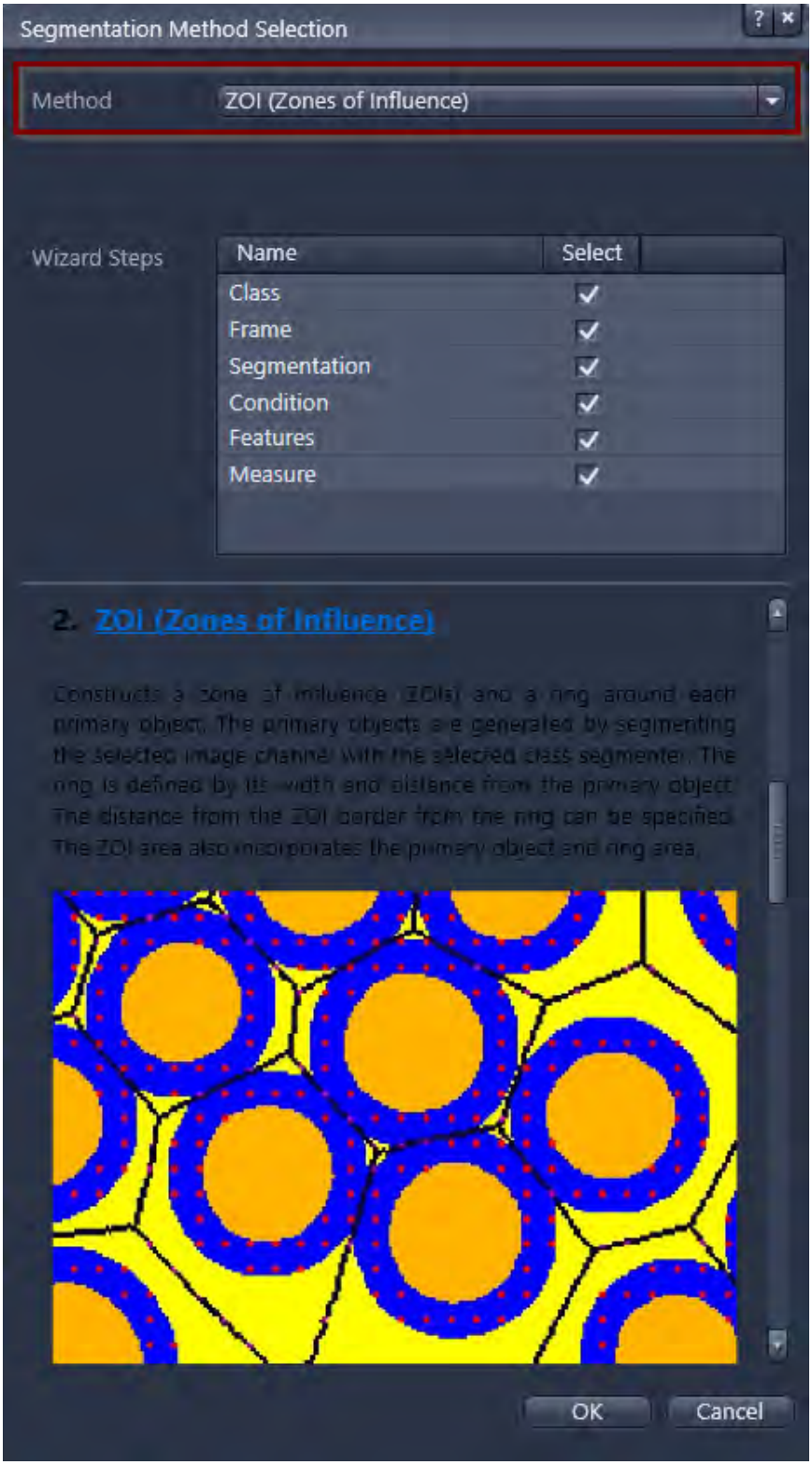
1 Select **Analysis tab | Image Analysis**, and click  **Options | New**.

2 In the **Settings** field, enter a file name for your measurement program. Note that the file name must be at least one character long. Click **Save**.

You have created a file for your measurement program.

3 Click  **Options | Edit**.

The **Segmentation Method Selection** dialog opens.



4 From the **Method** drop down menu, select a method and click **OK**.

You have created a new image analysis setting using the method of your choice.

5 In the **Image Analysis** Tool, press the **Setup Image Analysis** button.

The **Image Analysis Wizard** opens with the **Classes** step and includes already a predefined set of classes, depending on the selected method. Follow the steps in the wizard to define your image analysis. Each method comes with a predefined set of steps which allows you to make all necessary settings for image analysis.

- **Classes:** Allows you to add classes and subclasses.
- **Frame:** Allows you to define a measurement frame. Only the area of the frame will be analyzed.
- **Region Filter:** Allows you to define simple or complex conditions to filter the detected objects according to their parameters.
- **Automatic Segmentation:** Allows you to set the parameters for the automatic segmentation.
- **Interactive Segmentation:** Allows you to modify the results of the automatic segmentation or draw/delete objects. Note: this step only generates relevant results in **Analyze Interactively** run.
- **Features:** Allows you to select measurement features from an extensive list and to define measurement features for classes and subclasses independently.
- **Results Preview:** Shows a preview of your measurement results for the current view port.

For more information, see the following examples:

- *Measuring Fluorescence Intensity in a Multichannel Image* [▶ 241]
- *Counting Number of Fluorescence Signals per Nuclei* [▶ 248]
- *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256]
- *Counting the number of Objects in a Ring around the Nucleus* [▶ 263]

6.3 Measuring Fluorescence Intensity in a Multichannel Image

This topic will show you how to set-up a measurement program using the Image Analysis Wizard. After the setup is successfully completed, the program will be used to measure fluorescence intensity in a multichannel image.

In this example we are using a multichannel image with 2 channels (1st channel blue, (DAPI), 2nd channel red (mRFP1)) of fluorescence-stained cells. First we detect the blue-stained cell nuclei in the first channel. Then we measure the fluorescence intensity for both channels for the detected nuclei.

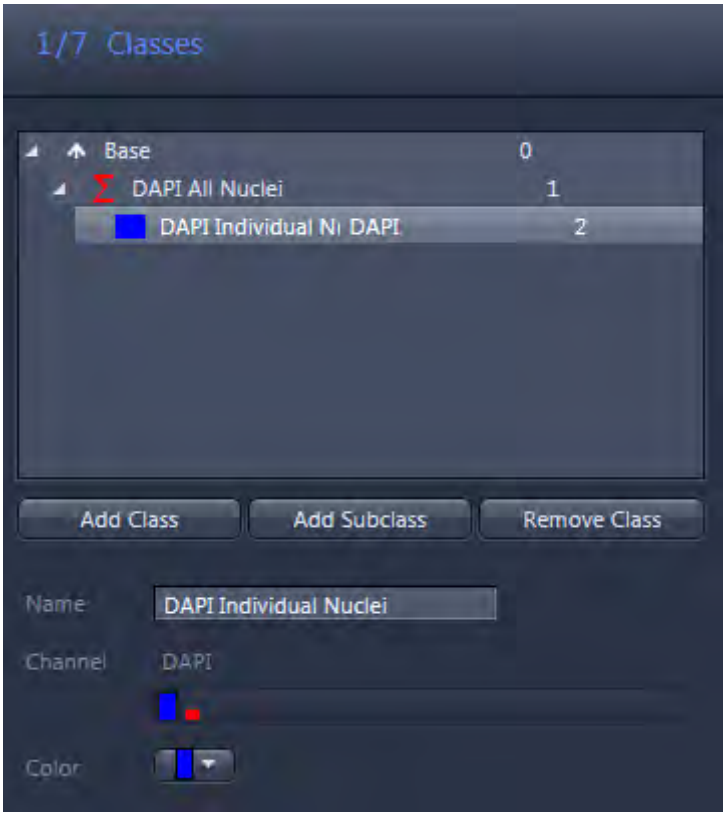
Prerequisites

- You have created a new image analysis setting with the **Segment region classes independently** method.

- You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting* [▶ 238].

Procedure

1. In the **Image Analysis Wizard 1/7 Classes**, click on **Class1** in the list and enter **DAPI Individual Nuclei** in the **Name** input field.
2. Click on **Classes1** in the list and enter **DAPI All Nuclei** in the **Name** input field.



3. Click on **Next**.

Step 2: Measurement frame

Procedure

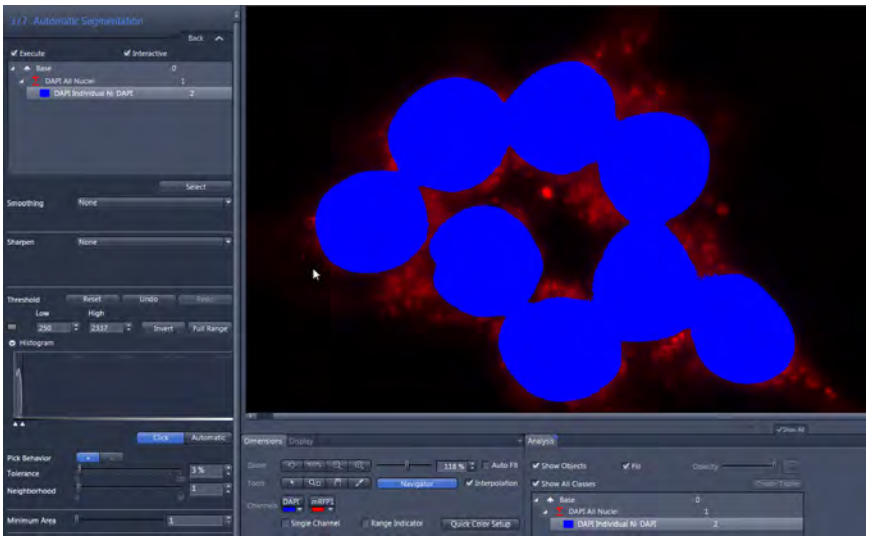
1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

Step 3: Automatic segmentation

Procedure

1. Click on **DAPI Individual Nuclei** entry in the list.
2. In the **Threshold** section set the **Tolerance** parameter to 1%.
3. Click in the image on the blue-stained cell nuclei.

The detected nuclei are overlaid in blue. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.



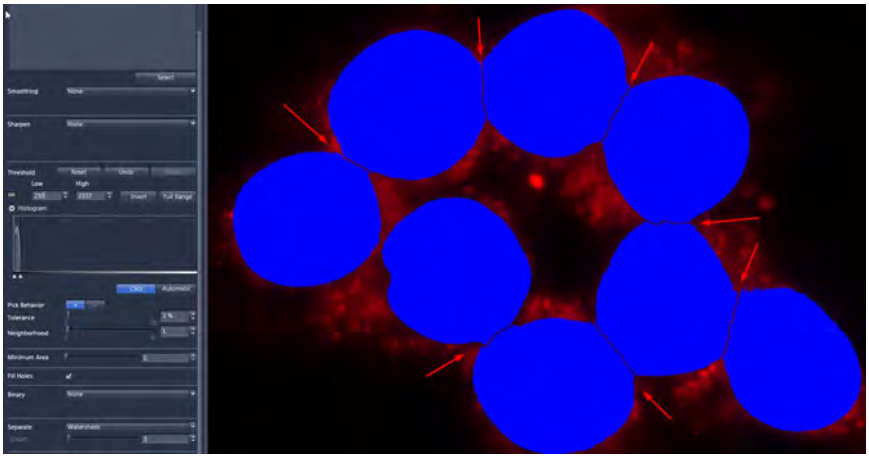
4 Click on the areas of the blue cell nuclei that have not yet been detected until these have been completely overlaid.

5 Activate the **Fill Holes** checkbox.

This fills any holes in the detected cell nuclei.

6 Select the **Watersheds** entry from the dropdown list in the **Separate** section and set the number to 3.

Clear separation lines are now visible between the cell nuclei.



7 Click on **Next**.

Step 4: Region Filter

Procedure

1 Deactivate the **Execute** checkbox.

2 Click on **Next**.

Step 5: Interactive segmentation

Procedure

1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

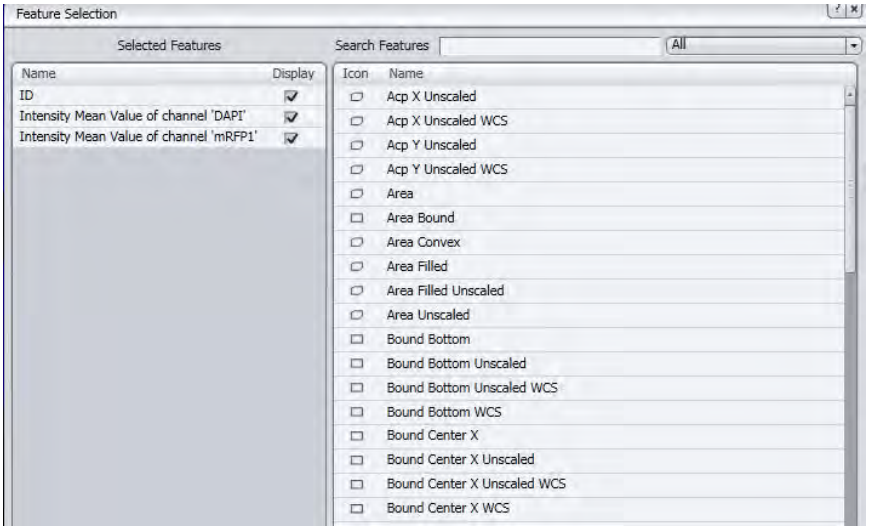
Step 6: Features

Procedure

1. Click on the **DAPI Individual Nuclei** entry in the list.
2. Click on the **Edit** button in the **Region Features** section.

   The **Feature Selection** dialog is opened.
3. Double-click on **Intensity Mean Value of channel 'DAPI'** and **Intensity Mean Value of channel 'mRFP1'** features, one after another.

   The features are displayed in the **Selected Features** list on the left.
4. Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the  **Delete** button .
5. Activate the **Display** checkbox for the features.



6. Click on **OK**.

   The selected features are displayed in the **Region Features** section.
7. In the section **Annotation Options**, activate the checkbox **Color**.
8. Select **Yellow** from the drop-down list.
9. Click on **Next**.

Step 7: Results Preview

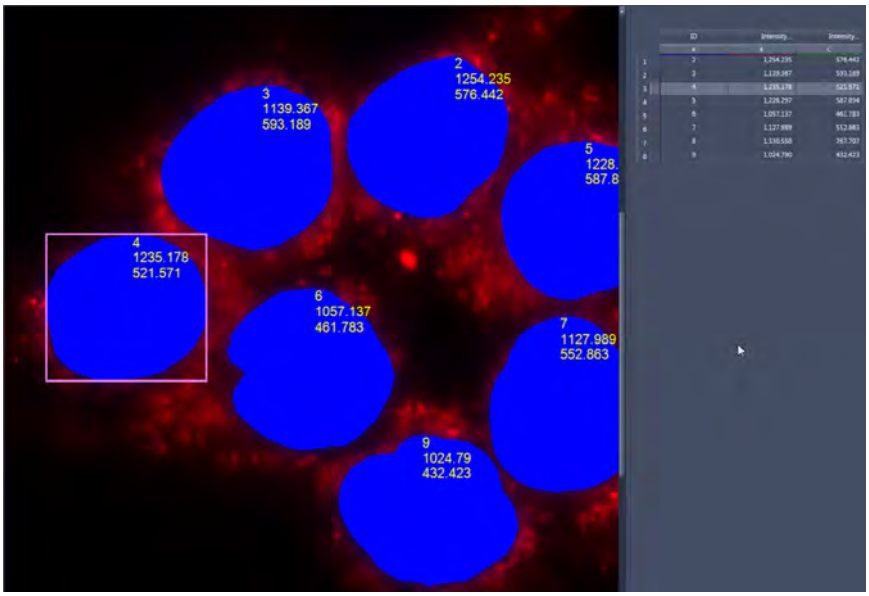
Procedure

1. Click on **DAPI All Nuclei** in the list.

   The number of measured cell nuclei is displayed in the data table to the right of the image.

2 Click on **DAPI Individual Nuclei** in the list.

The object ID and the values for the average fluorescence intensities per channel are displayed in the image at the cell nuclei in question and in the data list to the right of the image.



3 Click on the **Finish** button.

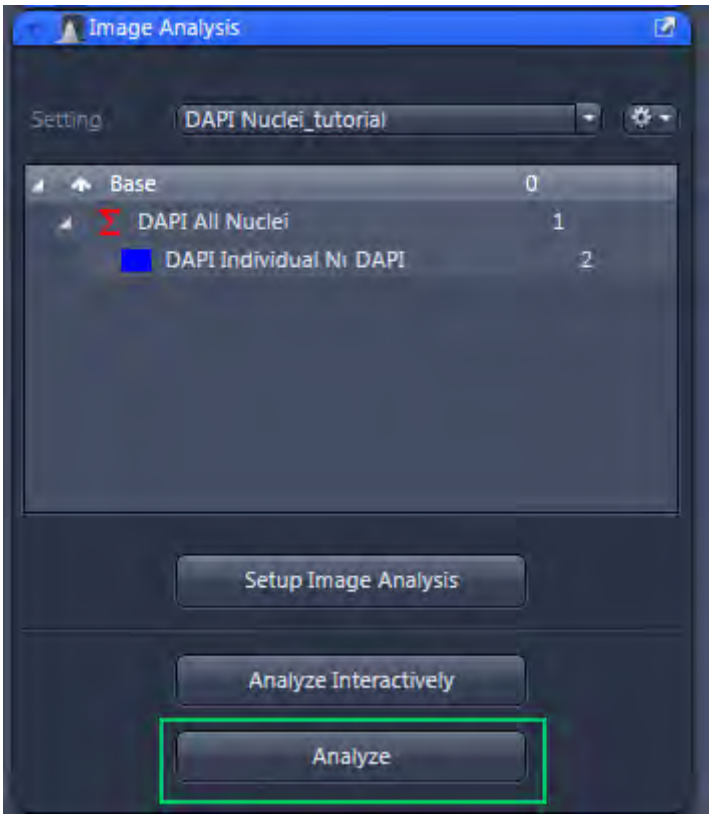
This saves the measurement program.

Executing the measurement program

Prerequisites

- You are in the **Image Analysis** tool.
- You have loaded the measurement program that you have generated.

Procedure **1** Click on the **Analyze** button.



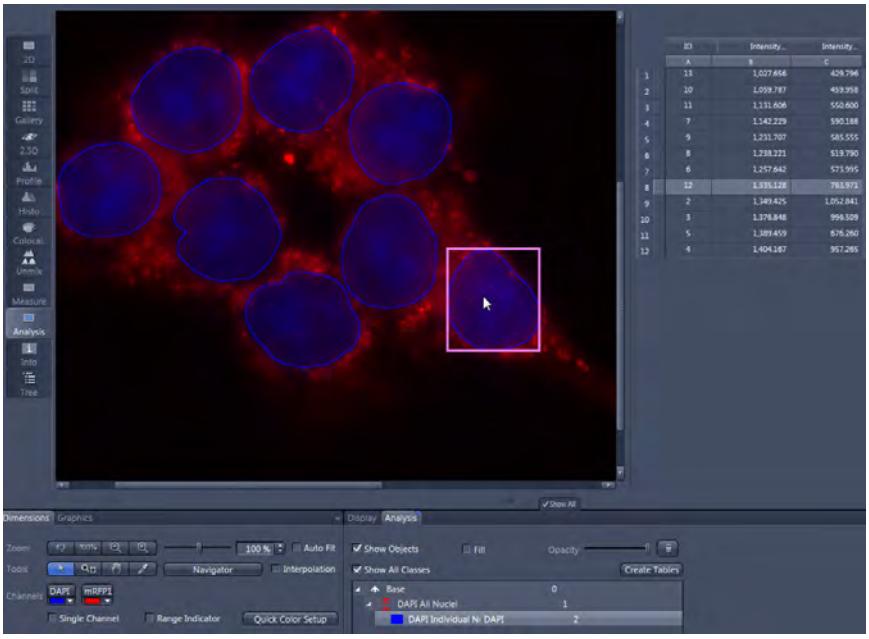
The measurement program is applied to the image.

The **Analysis View** now also appears in the **Center Screen Area**.

2 In the **Analysis View** you will see your image with the measured cell nuclei overlaid in blue and, to the right of this, the data list containing the individual measurements.

3 Deactivate the **Fill** checkbox in the **Analysis** control element.

The selected cell nuclei are displayed as contours.



4 Click on a row in the data list or alternatively on a cell nucleus in the image.

The row in the data list containing the measurement values is highlighted. The associated cell nucleus is surrounded by a red rectangle.

INFO

There is a direct link between the measured cell nuclei in the image and the measured values in the data table. You can either click on a measured cell nucleus in the image or on a row in the data table.

Creating a measurement data table

Procedure

1 Click on the **Create Measurement Data Table** button on the **Analysis** tab.

The two data lists are now separate documents.

2 Save each of the data lists via the **File** menu | **Save As**. Allocate a name and select .**csv** as the file type.

The measurement data tables are saved in **CSV** format and can therefore be opened directly in Excel.

3 Click on the image and save it via the **File** menu | **Save As**. Allocate a name and select .**czi** as the file type.

The image is saved with the measurement results. If you open the image, the measurement results can be viewed in the **Analysis View**.

6.4 Counting Number of Fluorescence Signals per Nuclei

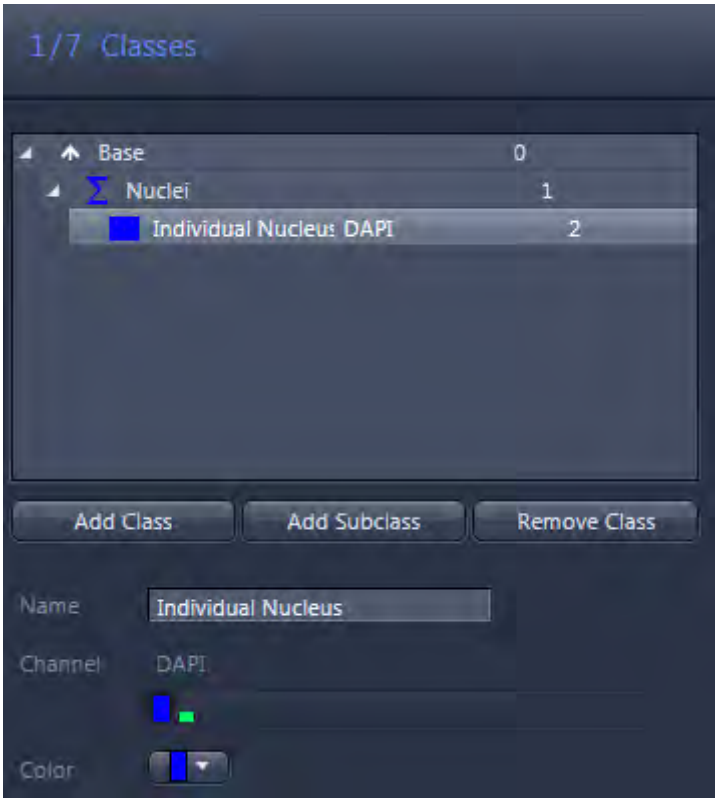
This topic will show you how to set-up a measurement program using the Image Analysis Wizard. After this the program will be used to count the number of fluorescence spots in a multichannel image.
In this example we are using a multichannel image with 2 channels (1st channel blue (DAPI), 2nd channel green (GFP)) of fluorescence-stained cell nuclei. First we detect the blue-stained cell nuclei in the first channel and then the green stained signals in the second channel. Then we measure the number of green fluorescence signals per nucleus.

Prerequisites

- You have created a new image analysis setting with the **Segment region classes independently** method.
- You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting* [▶ 238].

Procedure

1. In the**1/7 Classes**, click on **Classes1** in the list and enter **Nuclei** in the **Name** input field.
2. Select a blue color from the dropdown list in the **Color section**.
3. Click on the **Class1** entry in the list and enter **Individual Nucleus** in the **Name** input field.
4. Click on the blue channel icon in the **Channel** section .



You have now setup a class pair for the nuclei.

Procedure

1. Click on the **Add Subclass** button .

2 Click on **Classes3** in the list and enter **Signals** in the **Name** input field.

3 Select a green color from the dropdown list in the **Color** section

4 Click on the **Class3** entry in the list and enter **Individual Signal** in the **Name** input field.

5 Click on the green channel icon in the **Channel** section.

You have now setup subclass for the signals inside the Individual Nucleus class (parent class).

Procedure 1 Click on **Next**.

Step 2: Measurement frame

Procedure 1 Deactivate the **Interactive** checkbox.

2 Click on **Next**.

Step 3: Automatic Segmentation

Procedure 1 Click on the **Individual Nucleus** entry in the list.

The segmentation parameters (**Smooth**, **Sharpen**, **Minimum Area**, etc.) are displayed below the list.

2 In the **Smooth** section select **Gauss** from the dropdown list and set the parameter **Sigma** to **1.5**.

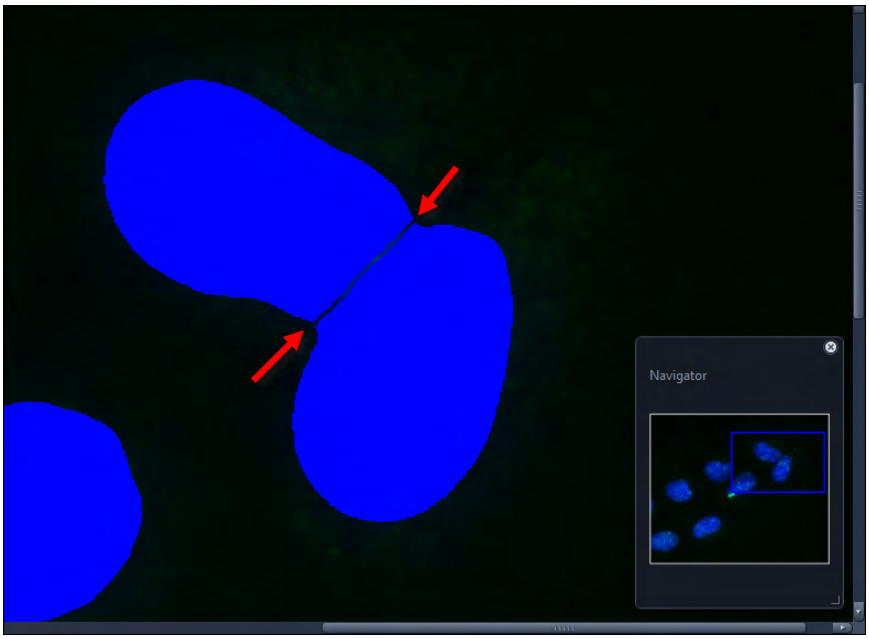
3 Click on the blue-stained cell nuclei in the image.

The detected nuclei are overlaid in blue. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.

4 Click on the areas of the blue cell nuclei that have not yet been detected until these have been completely overlaid.

5 Select the **Watersheds** entry from the dropdown list in the **Separate** section and set the number to **17**.

Clear separation lines are now visible between the cell nuclei.



6 Click on the **Individual Signal** entry in the list.

The segmentation parameters (**Smooth**, **Image Sharpness**, **Minimum Area**, etc.) are displayed below the list.

7 In the **Smooth** section select **Gauss** from the dropdown list and set the parameter **Sigma** to **1.5**.

8 Click in the image on the green-stained signals.

The detected signals are overlaid in green. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.

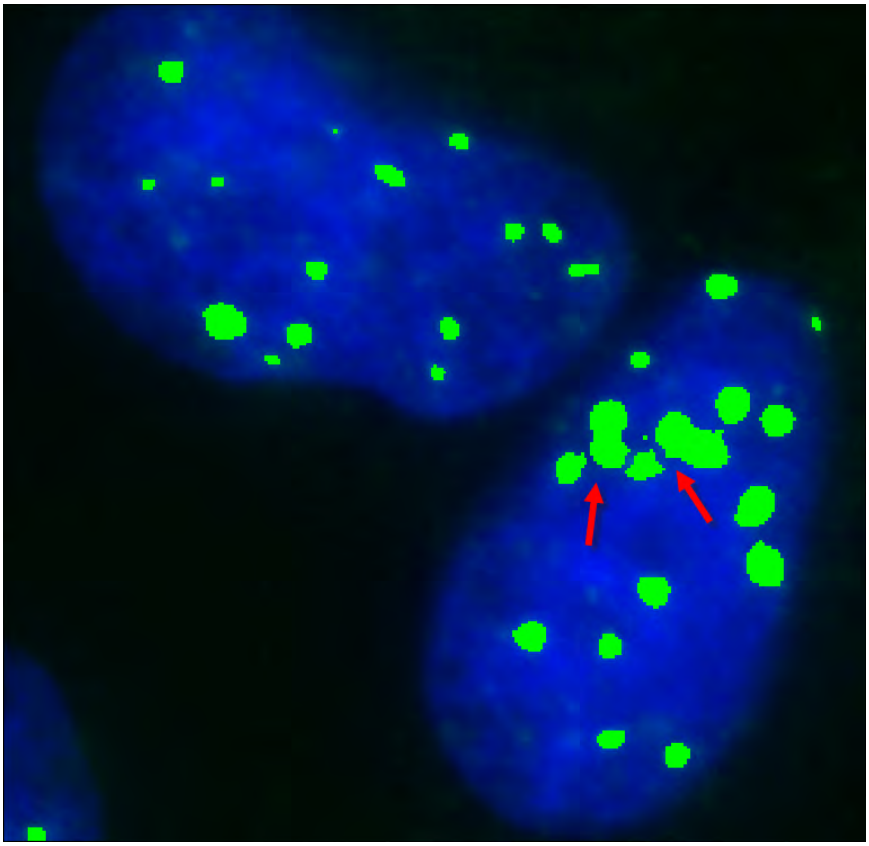
9 Click on the areas of the green signals that have not yet been detected until these have been completely overlaid.

10 Activate the **Fill Holes** checkbox.

11 This fills any holes in the detected signals.

12 In the **Separate** section select the **Watersheds** entry from the dropdown list and set the number to **17**.

Clear separation lines are now visible between the signals.



13 Click on **Next**.

Step 4: Region Filter

Procedure

1 Deactivate the **Execute** checkbox.

2 Click on **Next**.

Step 5: Interactive Segmentation

Procedure

1 Deactivate the **Interactive** checkbox.

2 Click on **Next**.

Step 6: Features

Procedure

1 Click on the **Nuclei** entry in the list.

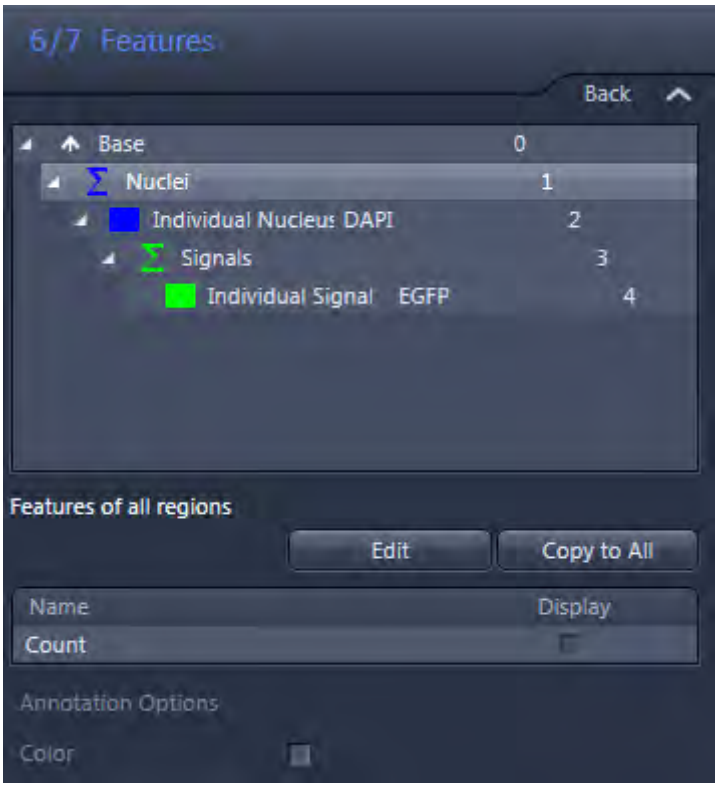
2 Click on the **Edit** button in the **Regions Features** section.

The **Feature Selection** dialog is opened.

3 Double-click in the right-hand list on the **ID**.

The features are displayed in the **Selected Features** list on the left.

4 Remove superfluous features from the list. Select the feature and click on the  **Delete** button .



5 Click on the **Individual Nucleus** entry in the list.

6 Click on the **Select** button in the **Region Features** section.

The **Feature Selection** dialog opens.

7 Double-click in the right-hand list on the **ID of the parent**.

The features are displayed in the **Selected Features** list on the left.

8 Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the  **Delete** button.

9 Click on the **Signals** entry in the list.

10 Click on the **Edit** button in the **Regions Features** section.

The **Feature Selection** dialog opens.

11 Double-click in the right-hand list on the **ID, Count**.

The features are displayed in the **Selected Features** list on the left.

12 Remove superfluous features from the list. Select the feature and click on the  **Delete** button.

13 Click on the **Individual Signal** entry in the list.

14 Click on the **Select** button in the **Region Features** section.

The **Feature Selection** dialog opens.

15 Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the  **Delete** button.

16 Click on the **OK** button.

The selected features are displayed in the **Regions Features** section.

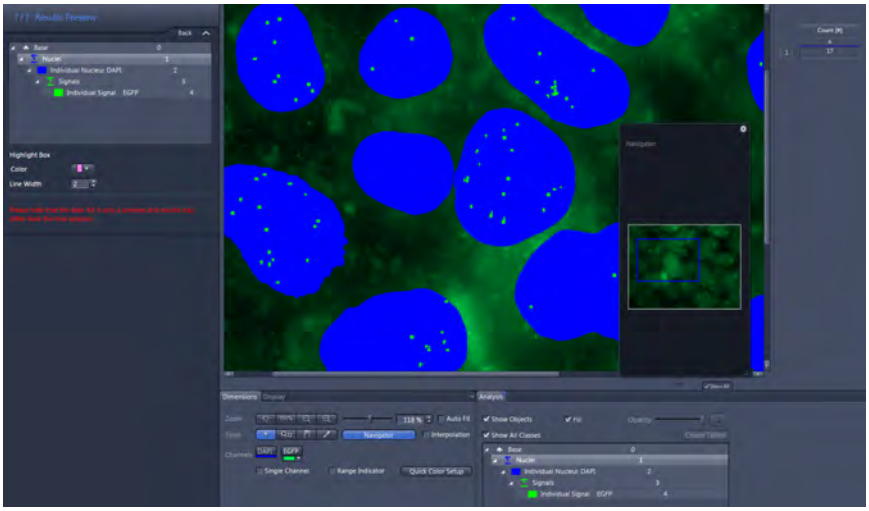
17 Click on **Next**.

Step 7: Results Preview

Procedure

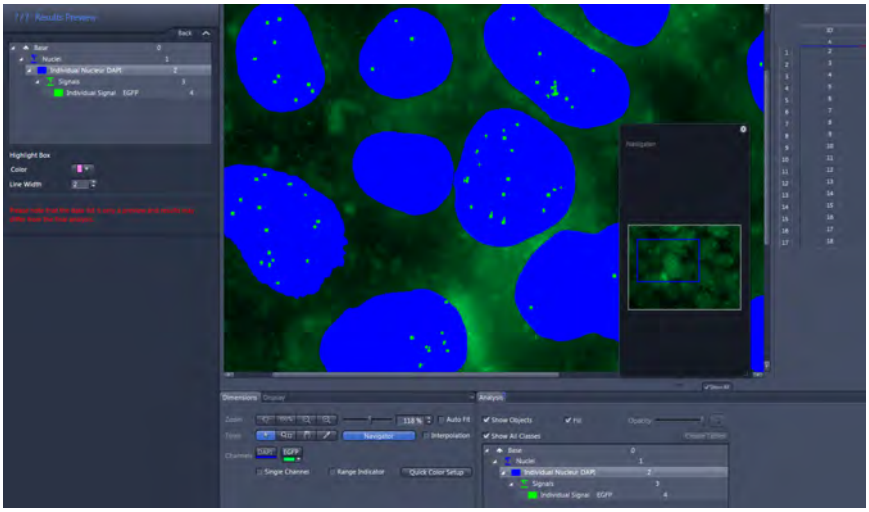
1 Click on **Nuclei** in the list.

The number of measured nuclei is displayed in the data table to the right of the image.



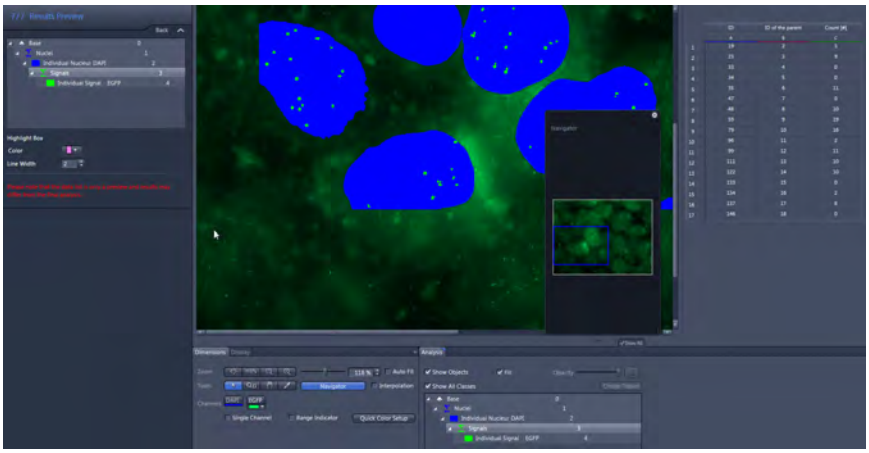
2 Click on **Individual Nucleus** in the list.

The object ID of the measured nuclei is displayed in the data list to the right of the image.



3 Click on **Signals** in the list.

The **ID of the parent** (corresponds to the ID of the nucleus) and the number of measured signals are displayed in the data table to the right of the image.



4 Click on the **Finish** button.

This saves the measurement program.

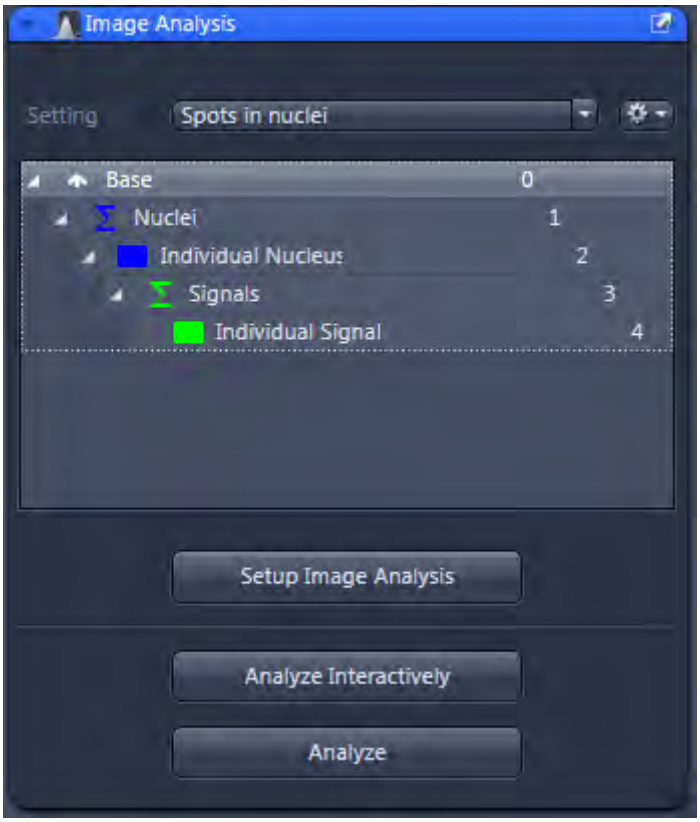
Executing the measurement program

Prerequisites

- You are in the **Image Analysis** tool.
- You have loaded the measurement program that you have generated.

Procedure

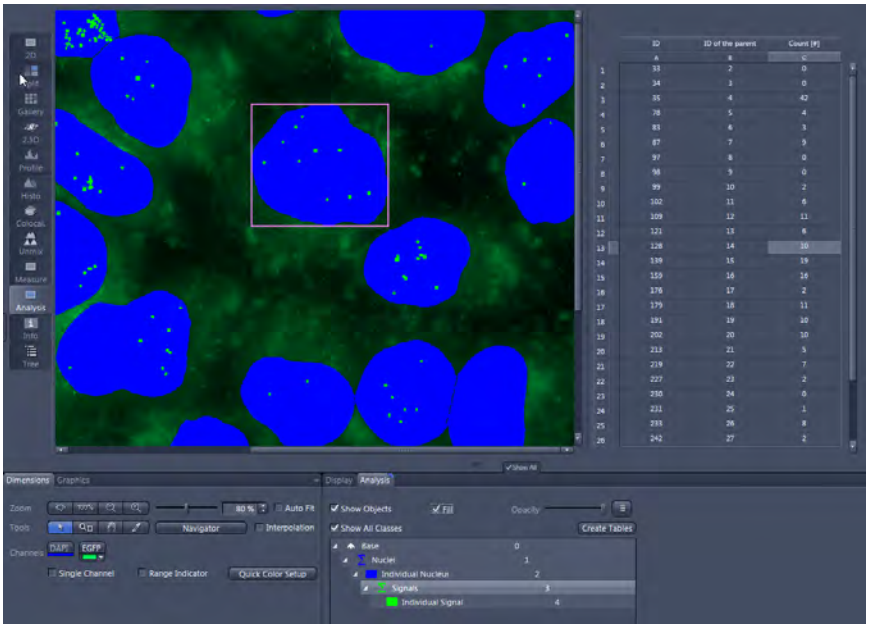
1 Click on the **Analyze** button.



The measurement program is applied to the image.

The **Analysis View** now also appears in the **Center Screen Area**.

In the **Analysis View** you will see your image with the measured cell nuclei overlaid in blue and the signals overlaid in green. Right of this, the data list containing the number of signals per nucleus.



Only the number of signals of measured nuclei is displayed. Nuclei touching the frame are not taken into account.

Creating a measurement data table

Procedure

1. Click on the **Create Measurement Data Table** button on the **Analysis** tab.

   The two data lists are now separate documents and have been removed from the image.

2. Save each of the data lists via the **File** menu | **Save As**. Allocate a name and select .**csv** as the file type.

   The measurement data tables are saved in **CSV** format and can therefore be opened directly in Excel.

3. Click on the image and save it via the **File** menu | **Save As**. Allocate a name and select .**czi** as the file type.

The image is saved with the measurement results. If you open the image, the measurement results can be viewed in the **Analysis View**.

6.5 Measuring Mean Fluorescence Intensity on a Ring around the Primary Object

The following example shows how to use the Zone of Influence (ZOI) method to measure intensities within a ring that is associated to the main object, e.g. the cell nucleus. An application example are transport assays where the intensities of a certain fluorescent marker in the cytoplasm are compared to the intensities within the nucleus.

In this example we use a multichannel image of fluorescence-stained cells. The cell nuclei are stained with AF568 and the mitochondria are stained with AF488. First, we detect the nuclei in the AF568-channel as primary object. A zone of influence is generated around each detected primary object. In this area, we can define a ring and specify its thickness and distance from the main object. You can use this ring to measure intensities or to detect further sub-objects on it. For more information, see *Counting the number of Objects in a Ring around the Nucleus* [▶ 263].

Prerequisites

- You have set up the image analysis setting with the method **ZOI (Zones of Influence)**. This has created the classes **ZOIs/ZOI**, **Primary Objects/Primary Object**, and **Rings/Ring** by default.
- You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting* [▶ 238].

Procedure

1. If you want to extend the predefined list of classes, in the **Image Analysis Wizard 1/6 Classes**, click **Add Subclass**.

   You can find an example how to detect objects on the ring in the second part of this section. For more information, see *Counting the number of Objects in a Ring around the Nucleus* [▶ 263].

2. If neccessary, click **Add Class** to extend the predefined list by another independent class of objects.
   Note that you cannot add further rings.

3 Select the image channel which you want to use for object detection. In this example, the primary objects (the nuclei) are in the AF568 channel. Therefore, click on the class **Primary Object** and select the channel containing the nuclei.

Parameter	Description
ZOIs	Class of all zone of influences
ZOI	Individual zone of influence
Primary Objects	Class of all primary objects
Primary Objects	Individual primary object
Rings	Individual ring
Ring	Parts of a ring (mostly only one)
Add Class	Adds a new independent class (under base).
Add Subclass	Adds a new subclass to primary object, ring or another class.
Remove Class	Removes the selected class or subclass.
Name	Name of the classes. You can rename them, if necessary.
Channel	Select the channel to be used for object detection
Color	Changes the color how the classes will be displayed.

Step 2: Frame

Optionally, you can define the area to be analyzed of each image. In case there are shading effects or other reasons that make you want to include only a certain area of each image for analysis, you can define a frame (rectangle, circle or polygon). Only the area within this frame will be further analyzed.

With the **Mode** parameter, you can furthermore choose how the analysis treats objects that are cut by the border of the image or the frame:

- **Cut at frame**: Cuts the objects at the frame.
- **Inside only**: Discards all objects that are cut by the frame.

Note that the whole ZOI is taken into account for the decision if the object is inside the frame or not (not only the primary object). This means there are cases where the primary object might lie completely within the image / frame, but the Ring or ZOI is cut by the border. If you select **Inside only** these cells will be discarded.

Comparison of image analysis results for **Cut at frame** compared to **Inside only**:

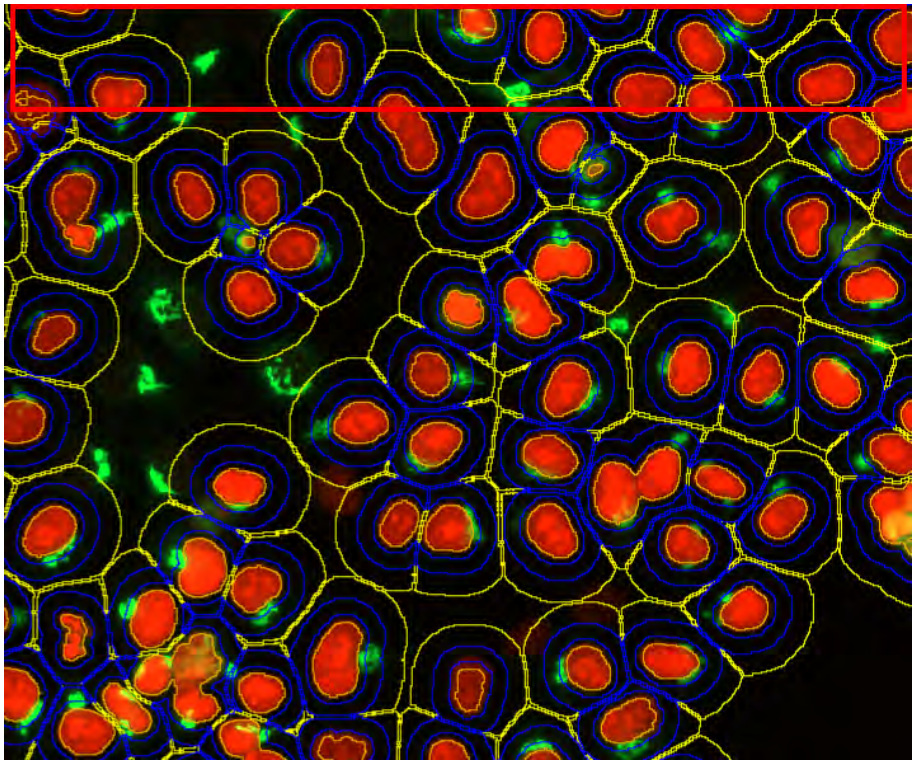


Fig. 6.1: Cut at frame

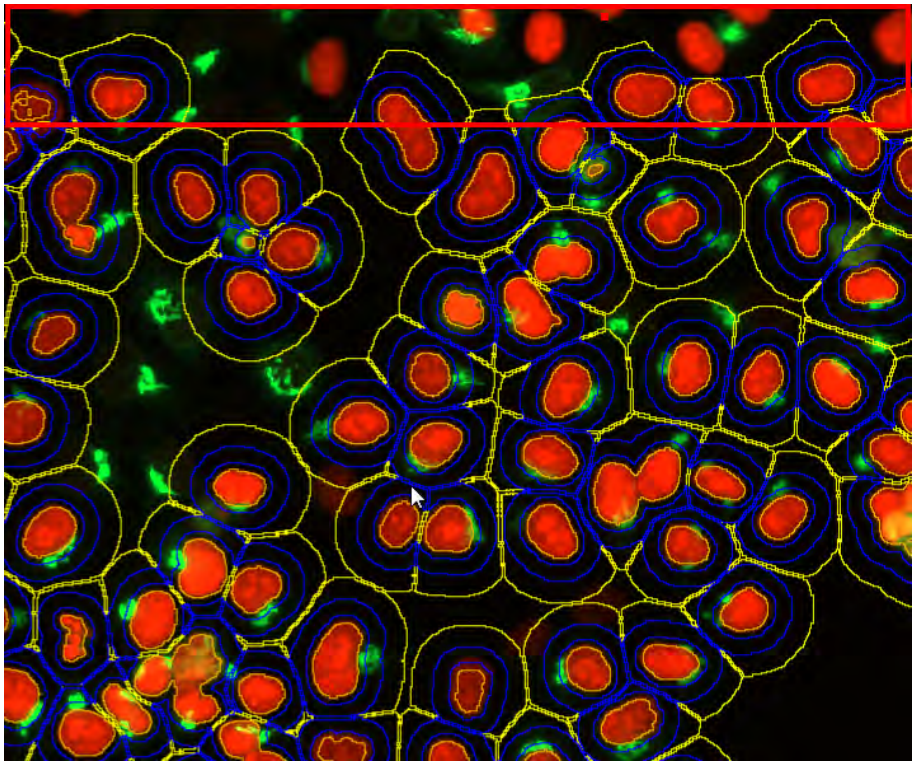


Fig. 6.2: Inside only

Step 3: Automatic Segmentation

Procedure

1 Select **Primary Object** and set up suitable parameters to detect the objects, i.e. threshold, area, separation.

As soon as objects are detected, the ZOI and Ring are automatically created around each primary object with the preset parameters.

2 To modify **Ring Distance** and **Width**, select the **Ring Element** class.

Now, you can define the location and dimension of the ring very flexible. You can set it just outside or inside the main object, and also at an arbitrary distance.

3 Define the following parameters:

Ring Distance: Distance from surface of the primary object. Negative values means that the ring starts at the defined distance within the primary object.
Ring Width: Defines the width of the ring.

The ZOI is automatically adapted to exceed the class with the larger diameter, i.e. either ring or primary object, by at least 3 pixel (default setting).

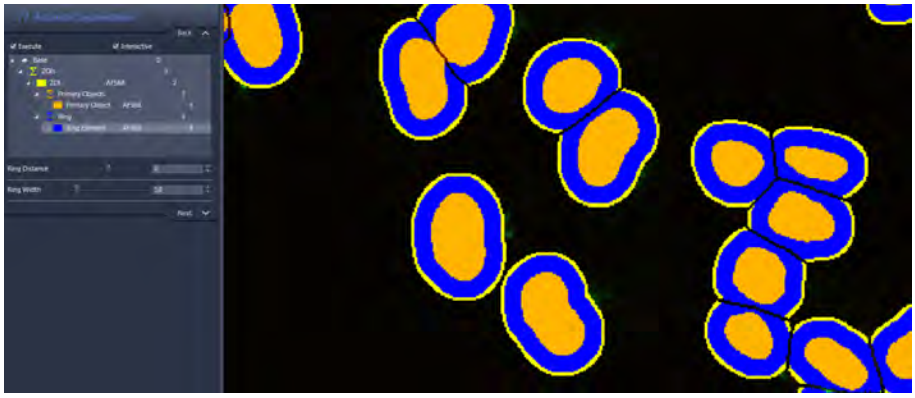


Fig. 6.3: Ring distance: 0 pixel; Ring width: 10 pixel

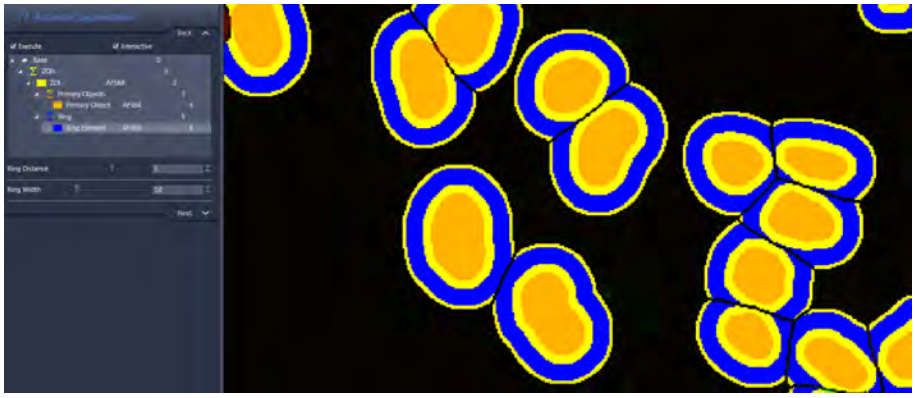


Fig. 6.4: Ring distance: 5 pixel; Ring width: 10 pixel

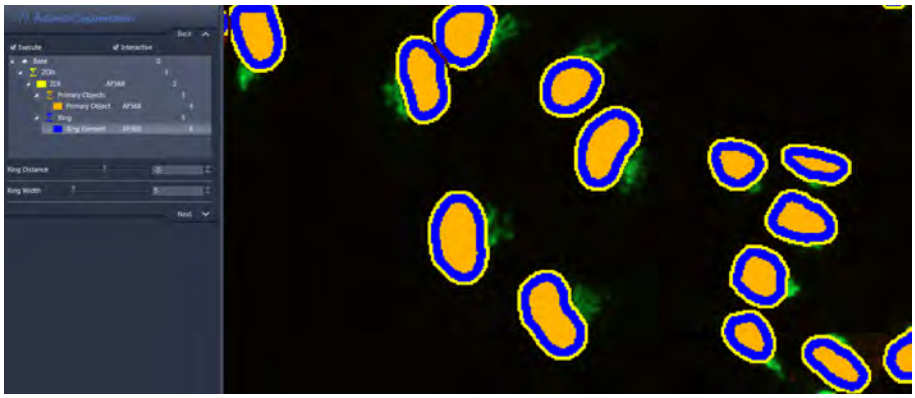


Fig. 6.5: Ring distance: -5 pixel; Ring width: 5 pixel

Modifying the ZOI Width

Procedure

1. Select **ZOI-class**, and with the **ZOI Width** slider, set the distance. You can set the distance between the outer border of the ZOI and the outer border of either ring or primary object, respectively. The **ZOI Width** is at least 3 pixel from the border of the ring or the primary object, whichever is larger. The ZOI area incorporates also the area of the ring and the primary object, and thus can serve for example for measuring features over the complete cell.

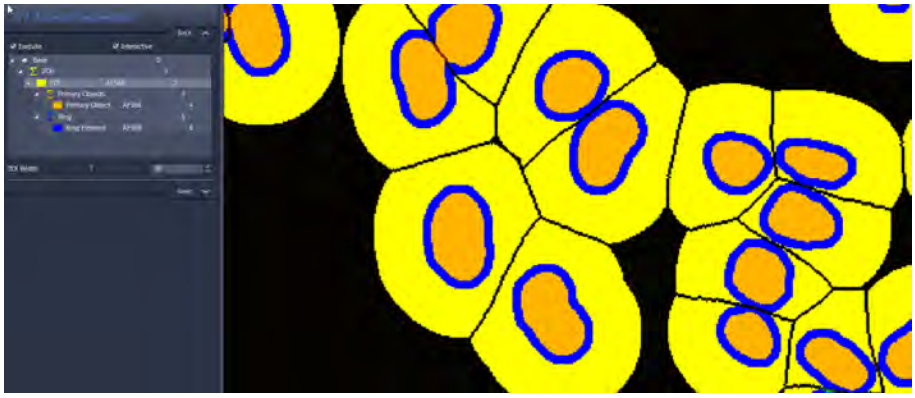


Fig. 6.6: ZOI Width set to 30 pixel

Step 4: Region Filter

You can define conditions for the primary objects (and additionally defined subclasses) to be measured, e.g. include only objects of a certain size, shape, intensity or other parameters. You can define suitable parameters for each of the defined objects.

Procedure

1. Select the **Primary Object** and click **Edit**. From the list of features on the right, you can add features to build the condition by a double-click on the feature. Once you have added all desired features, click **OK**.

   The selected conditions appear in the left tool area.

2. Set the minimum and maximum values by clicking on the objects with the desired features, or by entering the numbers directly.

The following figure is an example and shows the result if a certain condition of the circularity of each primary object needs to be fulfilled.

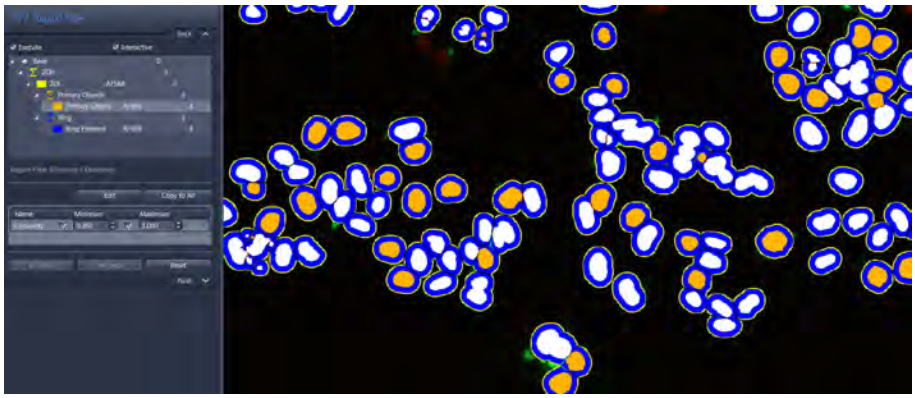


Fig. 6.7: Region Filter based on the circularity of the primary object

Step 5: Features

You can define individual measurement features for each class. You can copy the measurement features defined for one class to the other classes via **Copy to all**.

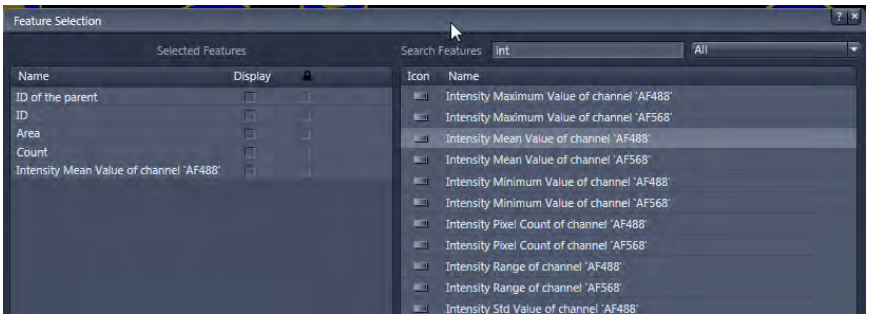
Procedure

1 Select the class for which you want to define measurement features, and click **Edit**. From the list of features on the right you can add features to the selected features list on the left.

These features are automatically calculated for every object during image analysis. All classes have the ID of the parent and ID as default features. With these IDs you can later group the associated parameters from the result excel lists, if necessary.

The class **Ring** additionally has **Area** and **Count** as default parameters. From these features, the class **Primary Object** derives the features **Ring Area** and **Ring Event Count**. With these you can attribute these features of the ring directly to the **Primary Object**.

2 To attribute the mean intensity for channel AF488 (the mitochondria) measured on the **Ring** to the **Primary Object**, first select the feature **Intensity Mean Value of channel 'AF488'** for the **Ring** class.



3 Click on **Primary Object** and edit the feature list.

The feature **Ring Mean Intensity 1** gives you the mean intensity of the first Intensity **Mean Value** feature defined for the Ring. In this case, it gives you the **Intensity Mean Value of channel 'AF488'** for the **Ring** class as feature of the **Primary Object**. Therefore, when you execute the image analysis, these features will also appear in the results list of the **Primary Object** .

Step 6: Result Preview

In this step you see a preliminary result of the image analysis.

Procedure

1 Click on the different objects in the **Analysis** tab to get the preliminary measurement result for all objects.

2 Click **Finish**, to save the analysis settings and close the wizard.

The wizard closes. The analysis settings are saved.

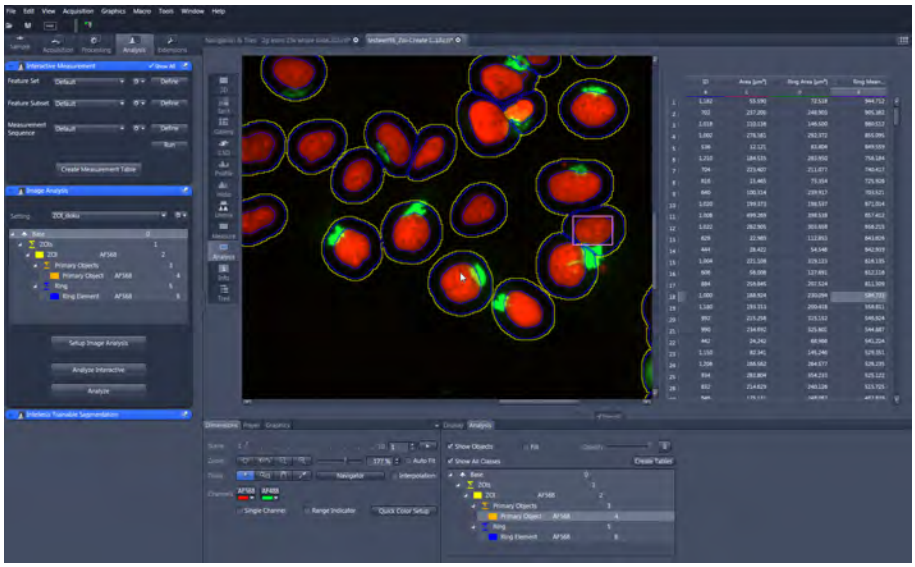
Executing the measurement program

You have the following options to run a predefined image analysis setting on your data set:

- **Analyze Interactively**: Analyze interactively with all steps that have been selected with the checkbox **Interactive** during setup of the image analysis.
- **Analyze**: Runs the image analysis setting without dialog.

When the analysis is finished, the main view switches to the **Analysis** tab and displays the segmented image along with the results of the analysis.

Select the different objects to display the corresponding measurement tables. The data in the tables and the regions in the image are interlinked. A click on the object in the image highlights the corresponding line in the data table and vice versa.



6.6 Counting the number of Objects in a Ring around the Nucleus

This example is similar to *Measuring Mean Fluorescence Intensity on a Ring around the primary Object* [▶ 256] and also uses the same data. This example shows how to count the number of objects on a ring that is associated with the main object, e.g. the cell nucleus. The images are taken from AF568-labeled nuclei. The mitochondria are stained with AF488. The channel of the nuclei is used for image segmentation. The ZOI-segmentation method attributes a zone of influence (ZOI) and a ring to each detected nucleus. This area is used as a search range to detect subobjects, in this case the number of mitochondria.

Prerequisites

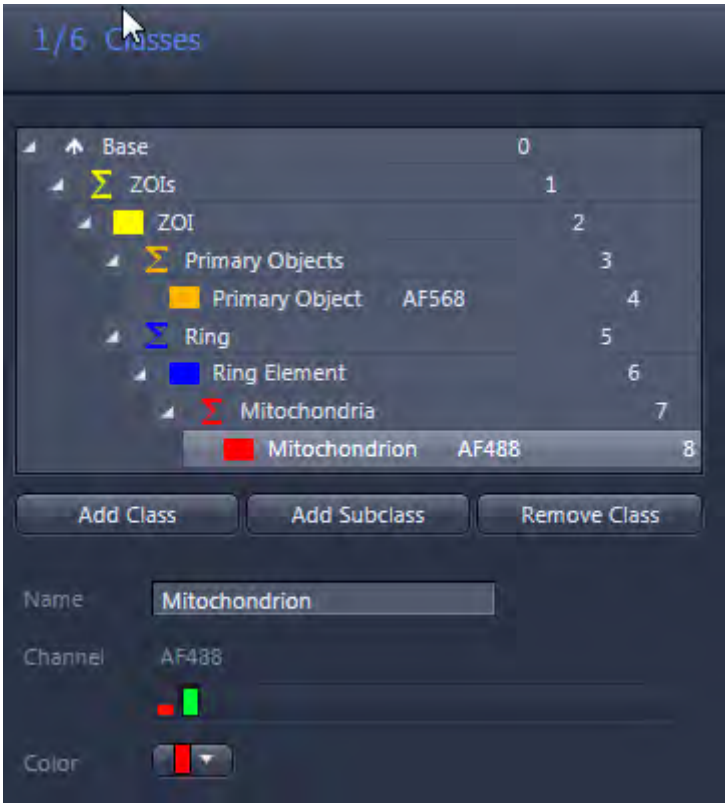
- You have created a new image analysis setting using the ZOI method.
- You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting* [▶ 238].

Procedure

1 In the **Image Analysis Wizard 1/6 Classes**, select Ring or Ring Element and click **Add Subclass** to extend the predefined set of classes with a subclass of the Ring Element.

2 Another class below the Ring Element is added. Give this class a meaningful name, e.g. Mitochondria / Mitochondrion.

3 The cell nuclei (primary objects) are labeled with AF568 (red channel), therefore you need to select this channel to segment the cell nuclei. Select **Primary Object** and for **Channel** select **AF568**.



4 Click on **Mitochondrion** and in the field **Channel** select **AF488**.

The mitochondria are labeled with **AF488**, therefore you need to use this channel for image segmentation.

Step 2: Frame

For more information, see **Step 2: Frame** in *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256] .

Step 3: Automatic Segmentation

Prerequisites

- For more information, see **Step 3: Automatic Segmentation** in *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256].

Procedure

1 Additionally, you need to set the segmentation parameters for the object **Mitochondrion**. Choose suitable parameters to segment the objects.

You have successfully detected the mitochondria on the ring.

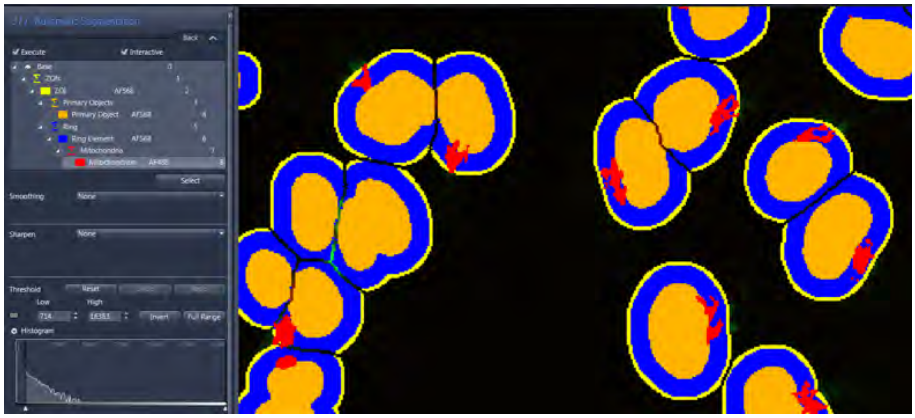


Fig. 6.8: Result of the Automatic Segmentation step

Step 4: Region Filter

For more information, see **Step 3: Automatic Segmentation** in *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256].

Step 5: Features

You can define individual measurement features for each class. The measurement features defined for one class you can copy to the other classes via **Copy to all**.

Procedure

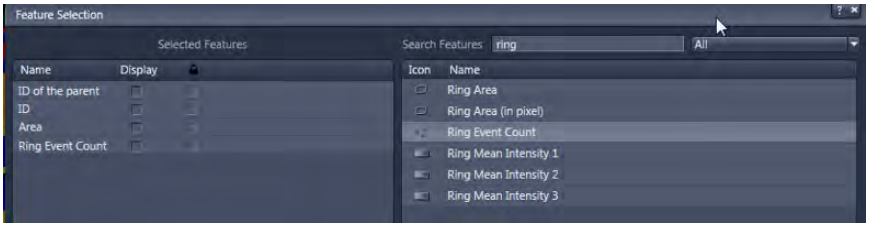
1. Select the class for which you want to define measurement features, and click **Edit**. From the list of features on the right you can add features to the selected features list on the left.

   These features are automatically derived for every object during image analysis. All classes have **ID of the parent** and **ID** as default features. This allows you to later to group the associated parameters from the excel lists, if necessary.

   The class **Ring** additionally has **Area** and **Count** as default parameters. From these features, the class **Primary Object** derives the features **Ring Area** and **Ring Event Count**. With these you can attribute these features of the ring directly to the **Primary Object**.

2. In this example, we use the parameter **Ring Event Count** to count the number of objects (the number of mitochondria-parts) within each ring. Select the **Primary Object** and click **Edit**.

   The **Feature Selection** dialog opens.



3. In the parameter list on the right, select the feature **Ring Event Count**.

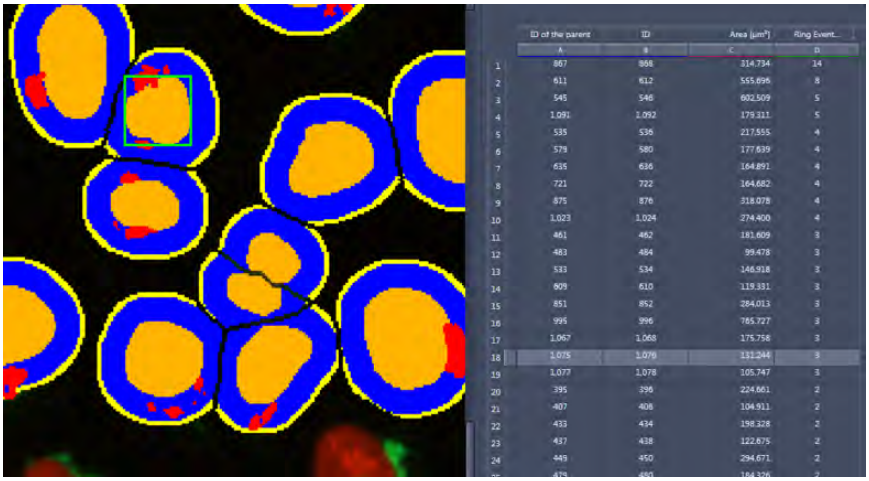
   The feature is added to the **Selected Features** list on the left.

Step 6: Preview Result

In this step you see a preliminary result of the image analysis.

Procedure

1. Click on the different objects in the **Analysis** tab to get the preliminary measurement result for all objects.
2. Select the **Primary Object** to get a list with preliminary measurement results with the features ID of the parent, ID, Area, and Ring Event Counts. Note that the results are preliminary and only include the part of the image you see in the viewport.



The result of the image analysis shows **Ring Event Counts** as a feature of **Primary Object**.

3. In the table, click on column **Ring Event Counts** to sort the entries in increasing or decreasing order.
4. Click on **Finish** to save the analysis settings and to close the wizard.

You can now run the analysis as described in *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object* [▶ 256].

7 Importing/ Exporting Images

7.1 Workflow Export/Import

This example describes the workflow for the **Image Export**. The typical workflow is the same for both export and import of images.

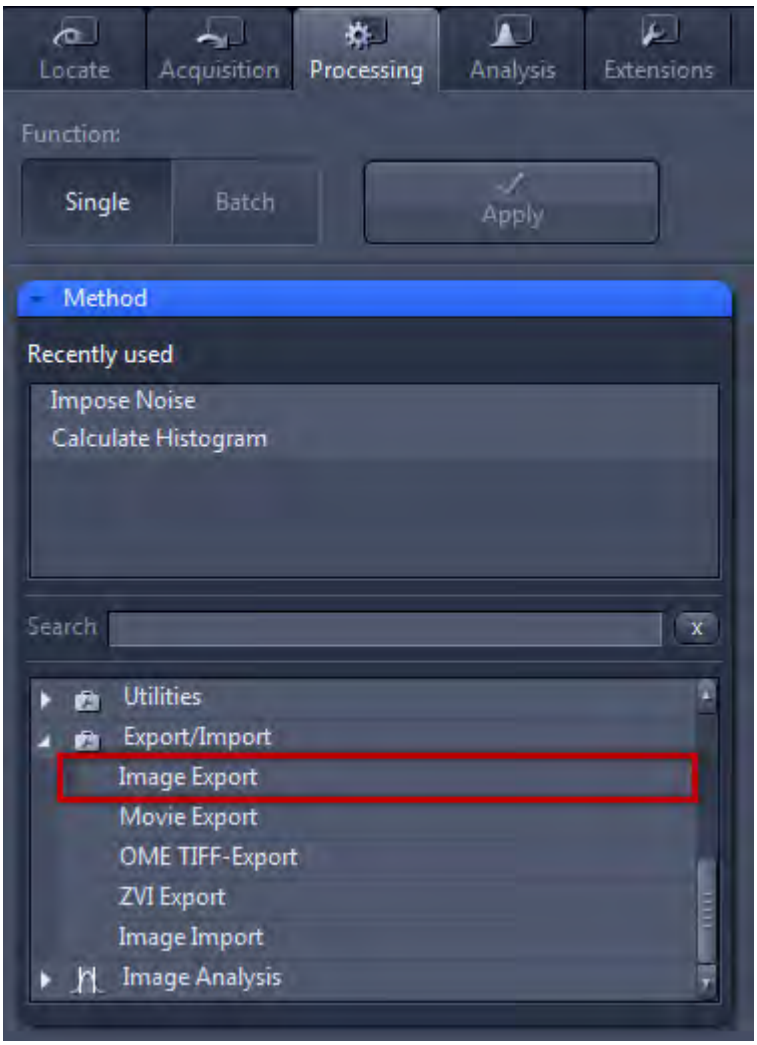
Prerequisites

- You have selected the **Processing** tab.

Procedure

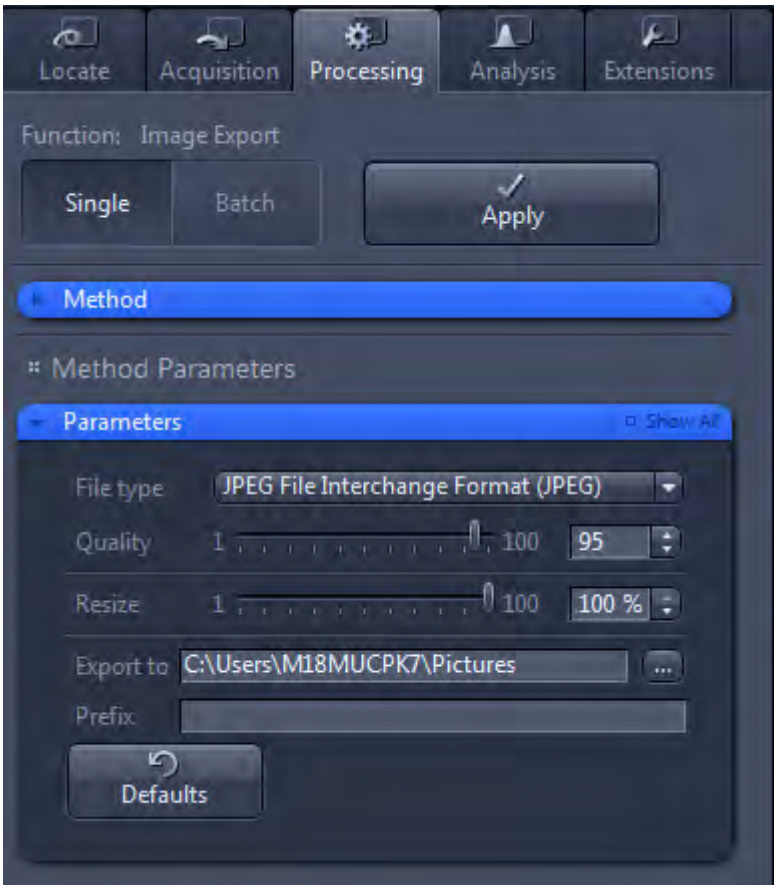
1 **Select a method**

In the **Method** tool open the **Export/Import** group and select the **Image Export** method.



2 Set method parameters

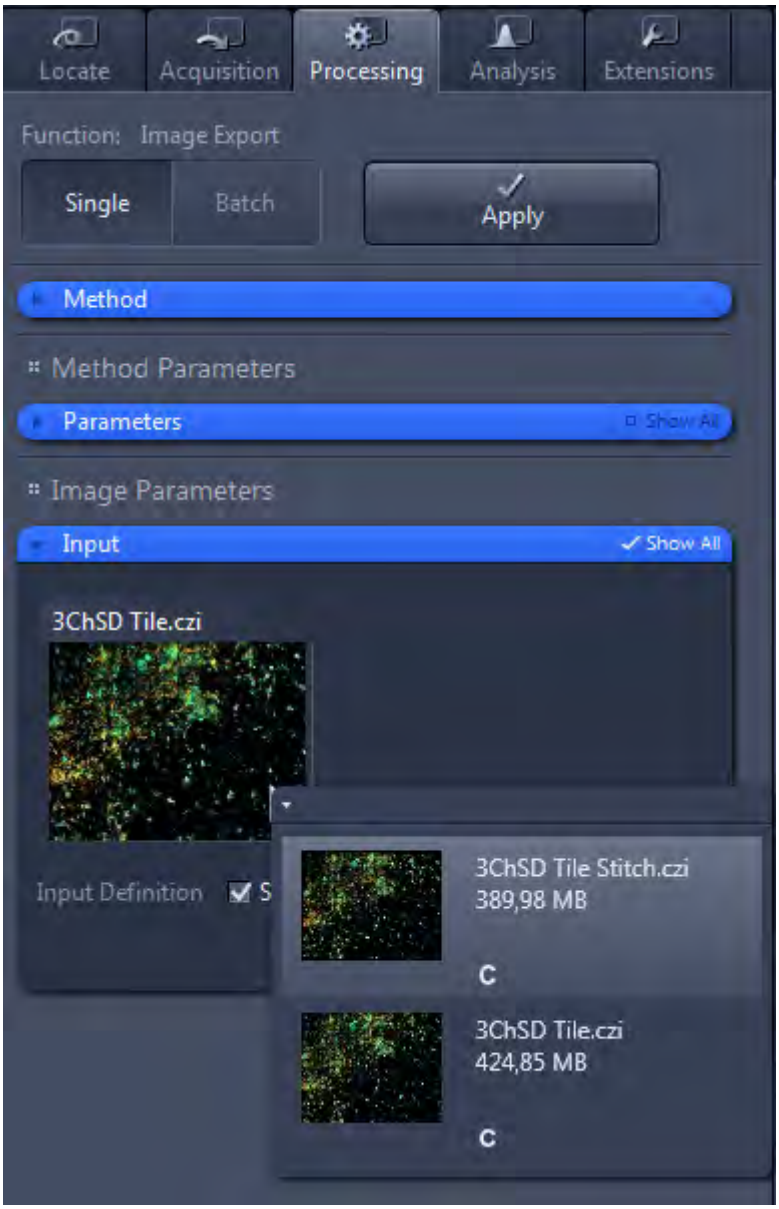
Under **Method Parameters** | **Parameters**, set the desired export settings, e.g. file type, quality, export folder, etc..



3 Select the image to export

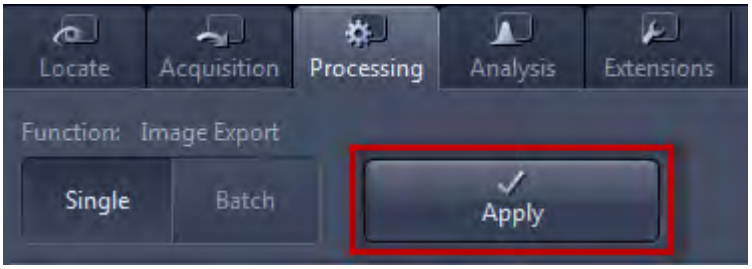
Under **Image Parameters** | **Input**, select the image you want to export. To do this, click on the small preview image within the **Input** tool. You will see a preview of all the open images. To select an image, click on the image that you want to use. This is only necessary if you have several images open

simultaneously. By default, the image currently selected is always used as the input image.



4 Export the image

On the top part of the **Processing** tab, click on the **Apply** button.



You have successfully exported the selected image.

7.2 Exporting images

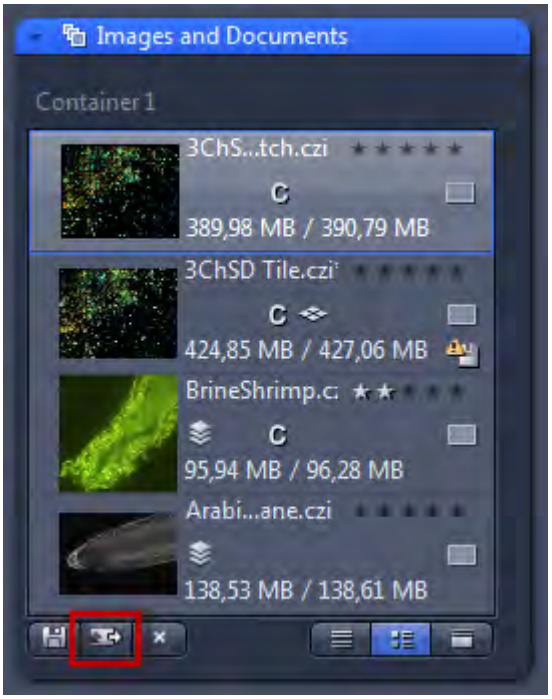
Using the **Quick Export** function you can export images automatically with a single click of the mouse, without setting the method parameters.

Prerequisites

- You have acquired or opened an image.

Procedure

1 In the **Right Tool Area** in the **Images and Documents** tool, click on the **Quick Export** button at the bottom of the tools window.



Alternatively, you can click on the **Quick Export** entry via the **File** menu | **Export/Import**.

The selected image is automatically exported with the default settings of the **Image Export** method (JPEG, quality 95%, size 100%). The image can then be found in a subfolder within the Windows image folder (**.../User/My Pictures**).

INFO

Type and source of risk

If you export a time lapse image using the Quick Export function, a movie is automatically generated using the default values of the **Movie Export** method.

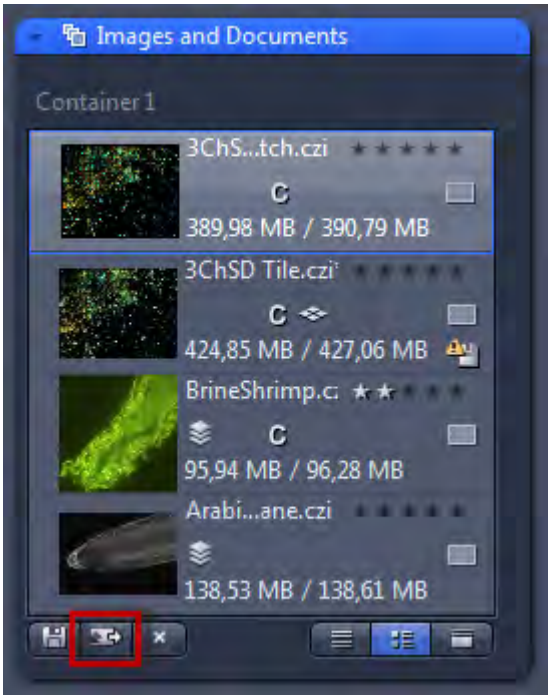
7.3 Exporting movies

Using the **Quick Export** function you can export movies automatically with a single click of the mouse, without setting the method parameters.

Prerequisites

- You have acquired or opened an image from a Time Series or a Z-stack image.

Procedure **1** In the **Right Tool Area** in the **Images and Documents** tool, click on the **Quick Export** button at the bottom of the tools window.



Alternatively, you can click on the **Quick Export** entry on the **File** menu | **Export/Import**.

The selected experiment is automatically exported with the default settings of the **Movie Export** method (AVI, original size). The movie can then be found in a subfolder within the Windows image folder (**.../User/My Videos**).

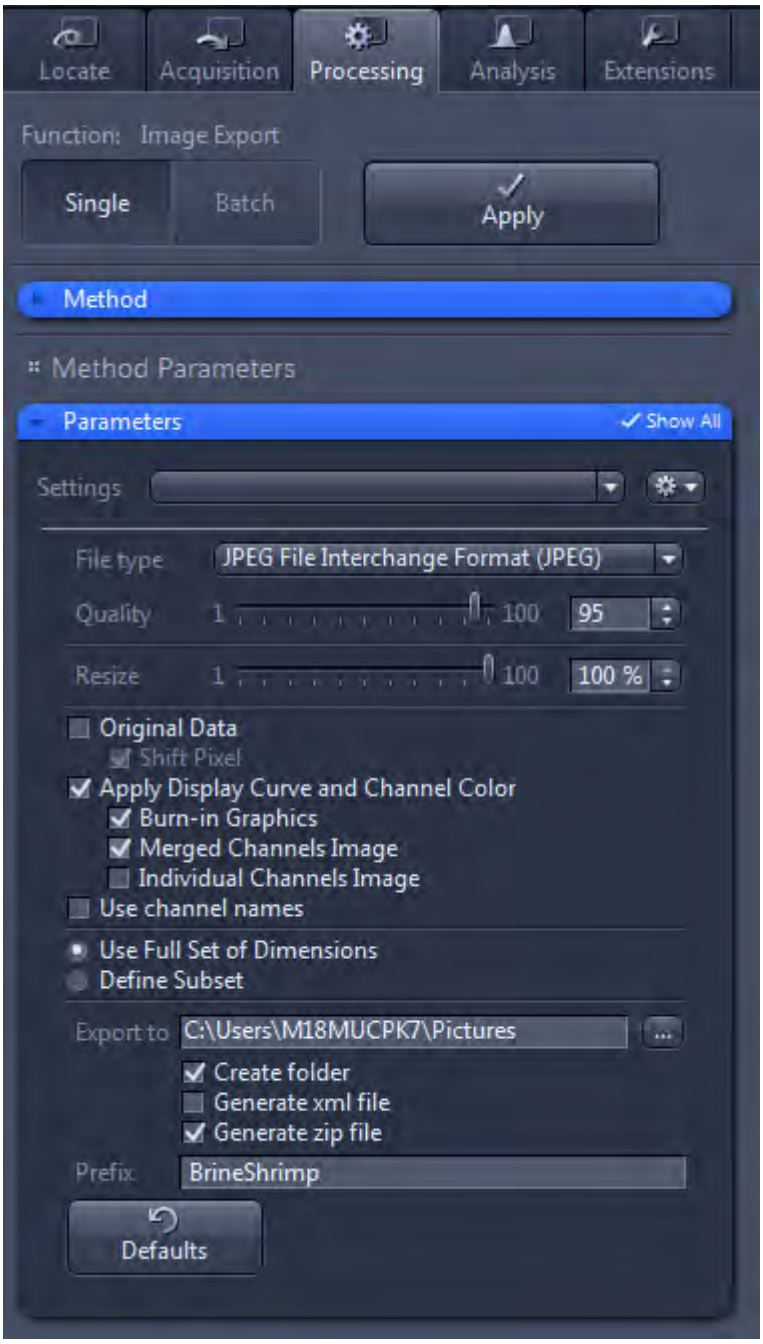
7.4 Exporting Multichannel Images

Here you will find out how to export individual images from a multichannel image with three channels and save them automatically in a **ZIP** archive. You will also discover how to export the whole multichannel image (pseudo color image) as an individual image.

Prerequisites ■ You have acquired or opened a multichannel image.

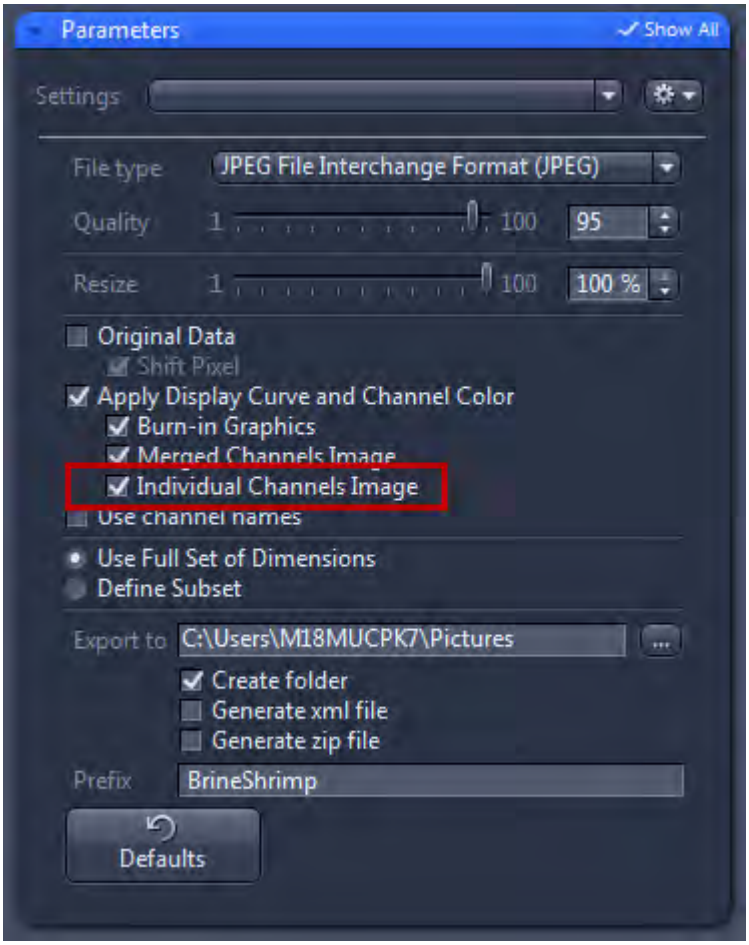
Procedure **1** On the **Processing** tab, open the parameters for **Image Export** (*Ctrl+6* or via the **File** menu | **Export/Import** | **Export**).

You will see the default settings of the parameters for image export. Make sure that the **Show All** mode has been activated.



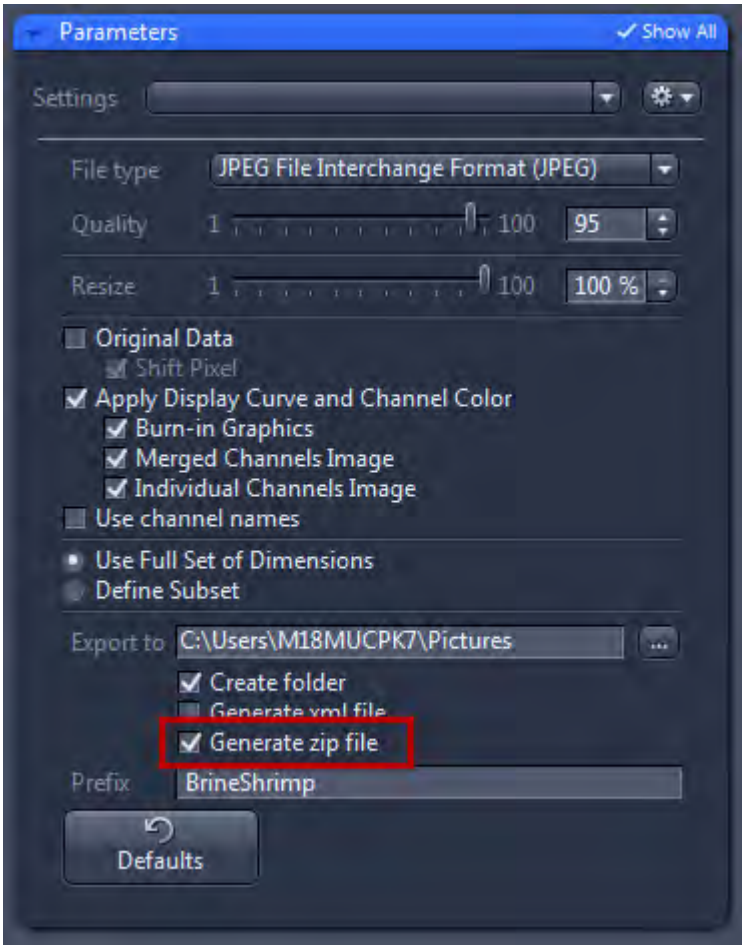
2 Activate the **Individual Channels Image** checkbox.

An image will be exported from each channel. The **Multichannel Image** checkbox is activated by default. This means that the pseudo color image (mixed color image from all channels) will also be exported as an individual image.

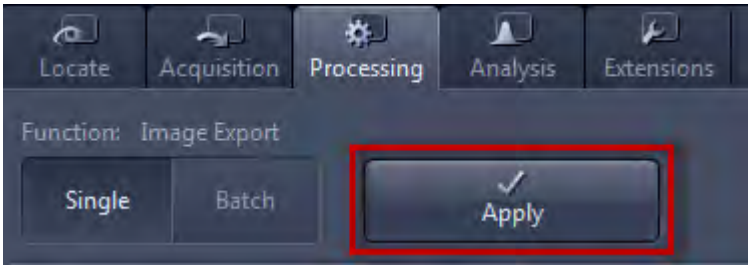


3 Activate the **Generate zip file** checkbox under **Export to**. Activated the **Create folder** checkbox, to create a subfolder with the name of the prefix.

The ZIP archive is saved in the subfolder.



4 Click on the **Apply** button at the top of the **Processing** tab.



You have exported the images of the individual channels and the pseudo color image of your multichannel image and automatically saved them in a ZIP archive. The ZIP archive containing the 4 images can be found in the export folder indicated.

7.5 Importing Z-Stack images

Prerequisites

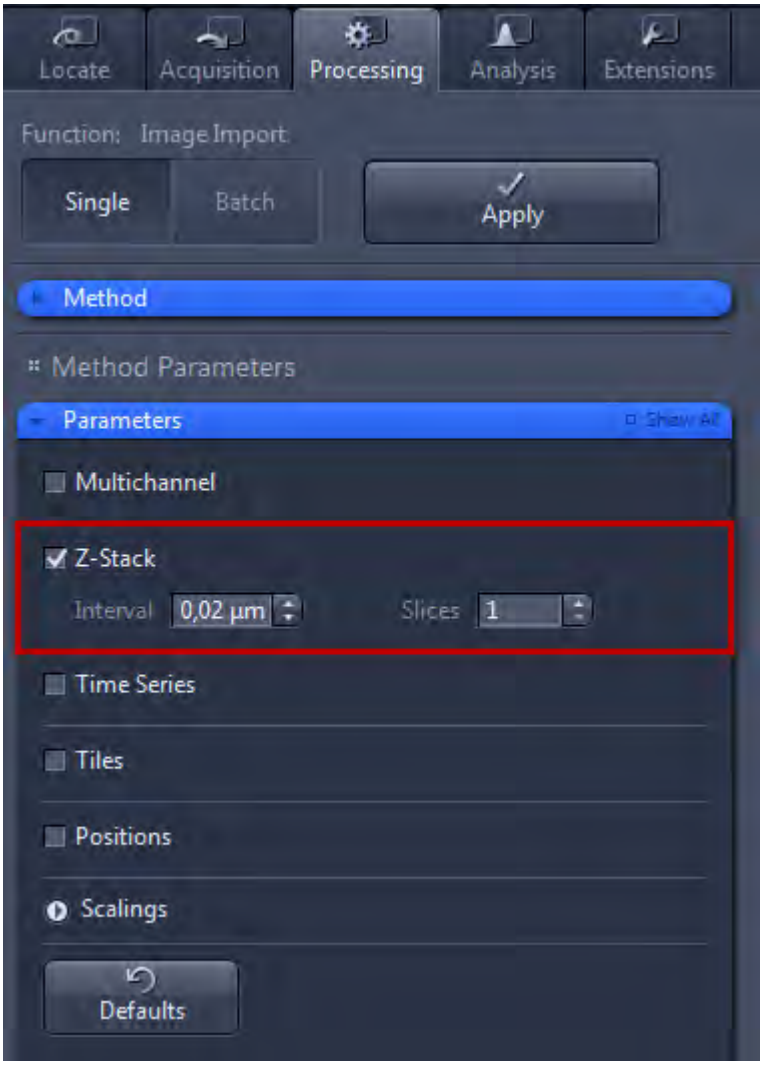
- You have saved the individual images of a Z-stack in a folder on your computer. The images have been named systematically, e.g. Image_Z0, Image_Z01, etc...

Procedure

1 On the **Processing** tab, open the parameters for **Image Import** (or via the **File** menu | **Export/Import** | **Import**).

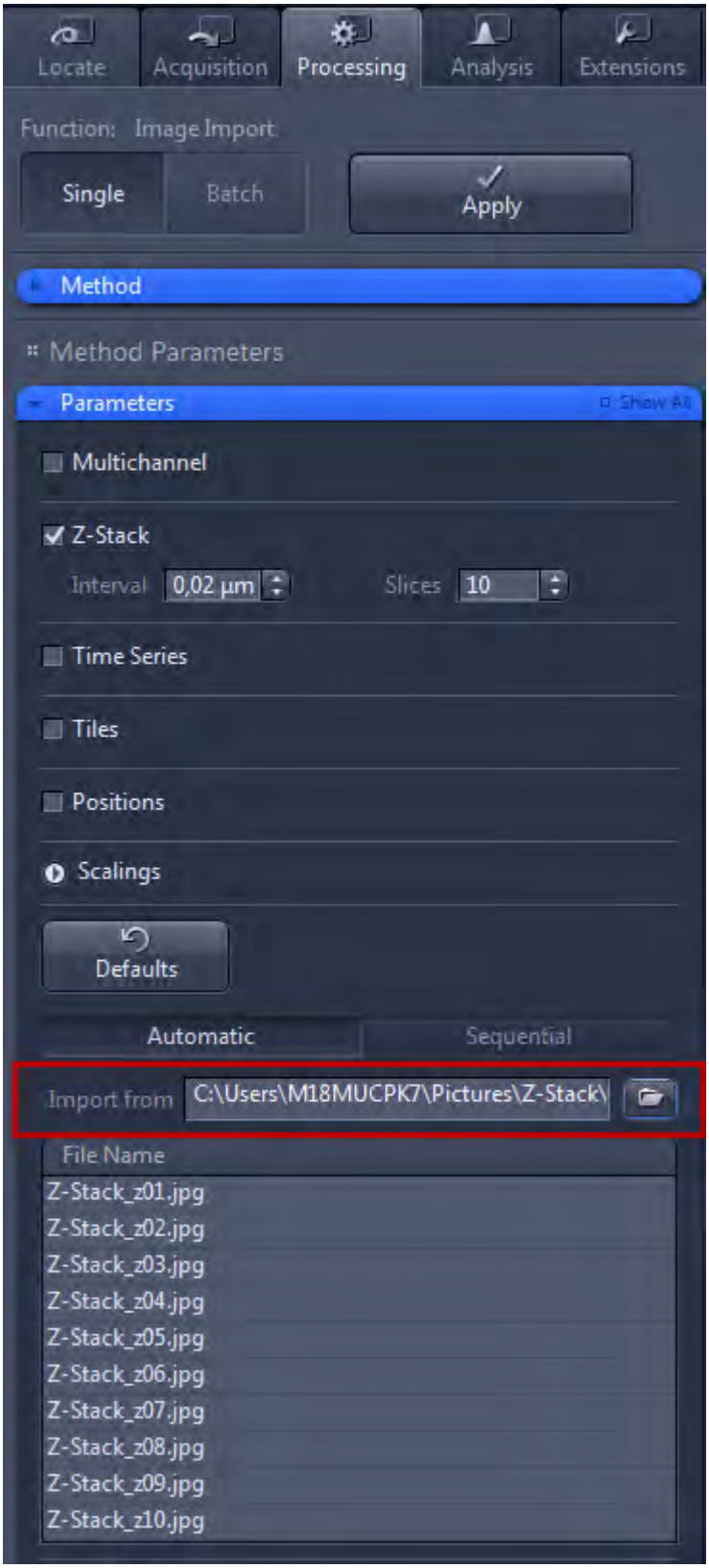
You will see the default settings of the parameters for **Image Import**.

2 Activate the **Z-stack** checkbox. Deactivate all the other dimensions.



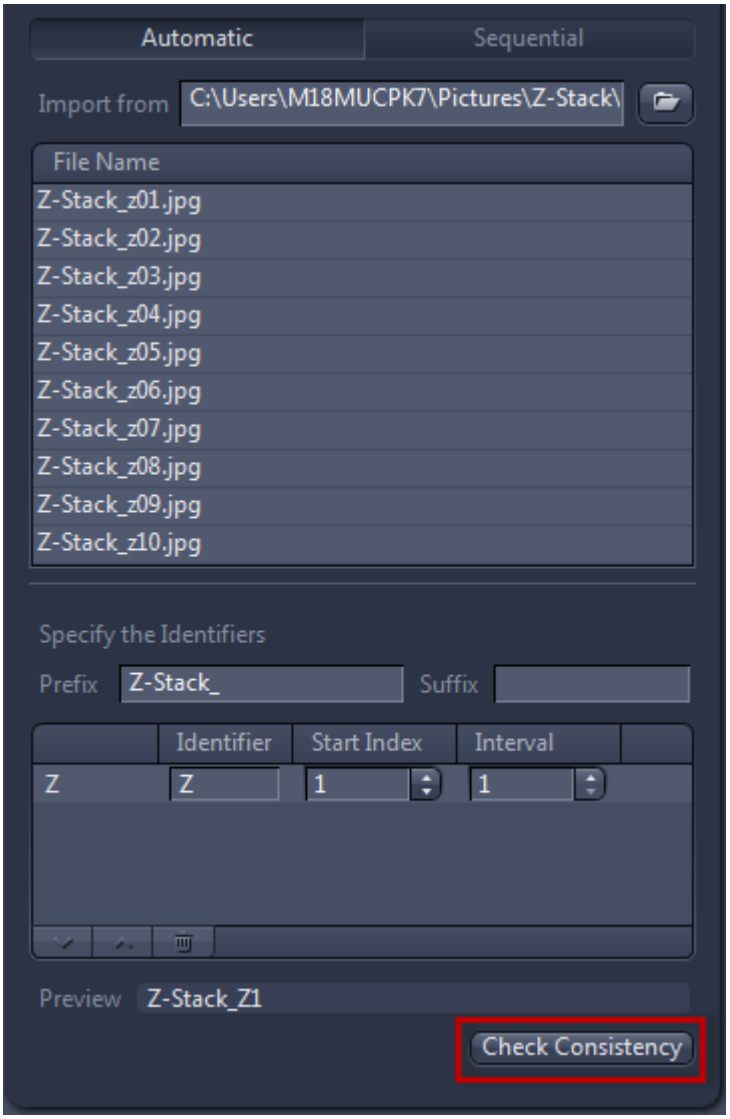
3 Enter the interval for the Z-stack. The number of planes is set automatically if the images have been named systematically.

4 In the **Import from** section, select the folder that contains the individual images of your Z-stack image.

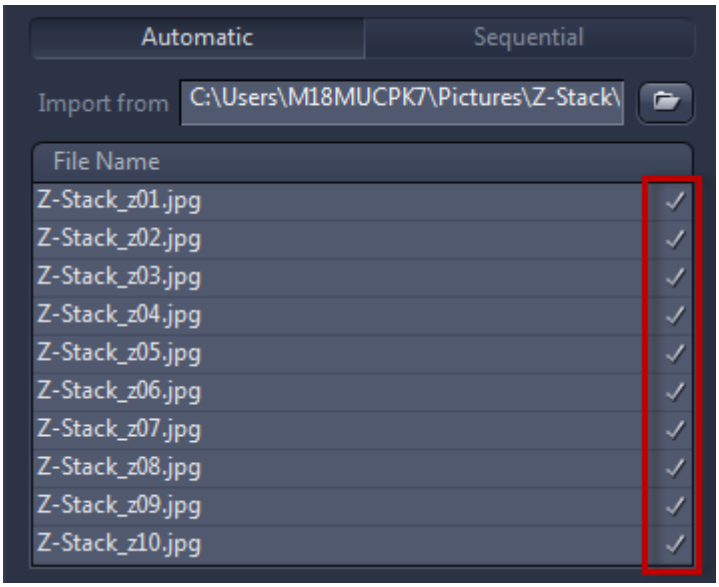


The individual images are displayed automatically in the list under the import directory.

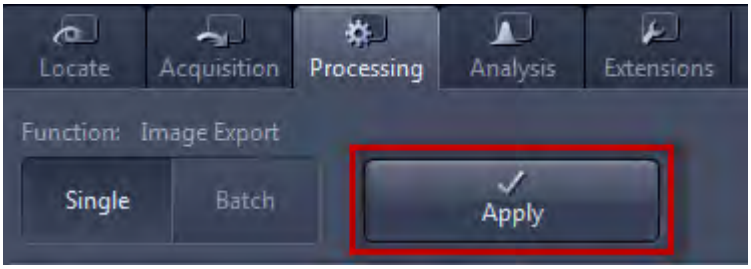
5 Click on the **Check Consistency** button. This allows you to check whether the images can be imported correctly.



A check mark appears after each file name in the list. You can import the individual images.



6 Click on the **Apply** button at the top of the **Processing** tab.



The individual images are imported and combined to form a Z-stack image. You have successfully imported a Z-stack image from individual images.

8 Managing Users and Groups

8.1 Introduction

The software can be used with or without user management.

Without user management

User management is disabled by default. This means that every user has the same rights. No username or password is required and there are no user roles within the software (i.e. the user can perform any action).

With user management

If user management is enabled, each user has an account which is used to log into the software. Each user account is assigned to one or more user groups.

User groups define the privileges (actions the user can perform in the software) for the users assigned to the group. Groups typically correspond to the roles in the software (e.g. Administrator, User). However, you can also create new user groups if required.

Typically, one user is assigned to one group, but can be assigned to multiple user groups if required. Users have the sum of all permissions of the groups to which they are assigned.

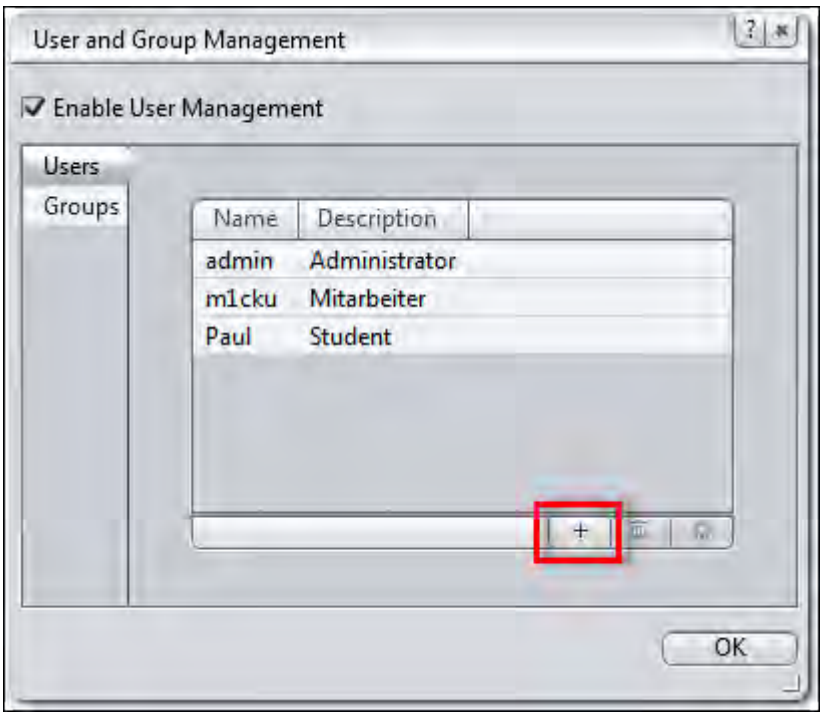
8.2 Creating a new user

Procedure

1. In the menu **Tools | Users and Groups...** open the **User and Group Management** dialog.
2. Activate **Enable User Management** checkbox.

   As the user management is enabled now all settings will be effective by the next start of the software. Make sure that you remember password, username, etc.

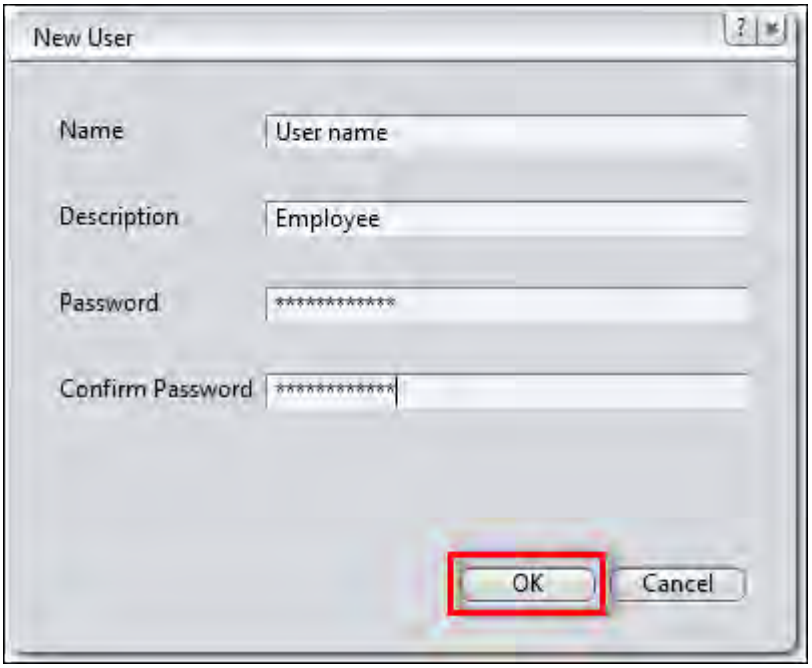
3 In the **Users** tab, click on the **Add** button.



The **New User** dialog opens.

4 Enter a **user name**.
Optional: Enter a **description** and/or enter and confirm a **password**.

5 Click on the **OK** button.



You have successfully created a new user. Now you can add the user to a specific user group.

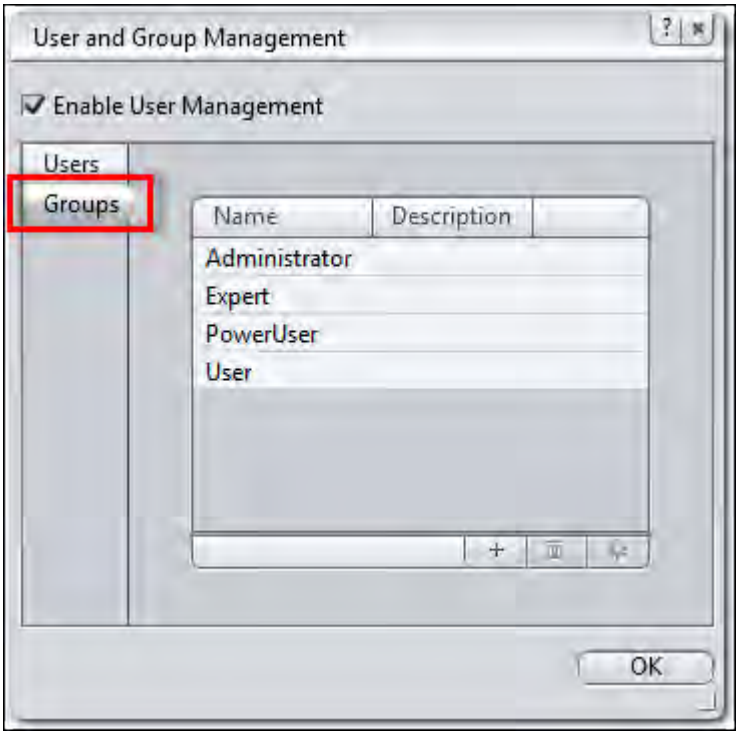
8.3 Adding users to a group

Prerequisites

- You are in the **Tools** menu | **User And Group Management** dialog.
- **Enable User Management** is activated.

Procedure

1 Click on the **Groups** tab.



By default you will find 4 groups (**Admin**, **Expert**, **PowerUser**, **User**) with each group having the same rights. Learn how you can manage access rights for user groups under *Managing access rights for user groups* [▶ 282].

i INFO

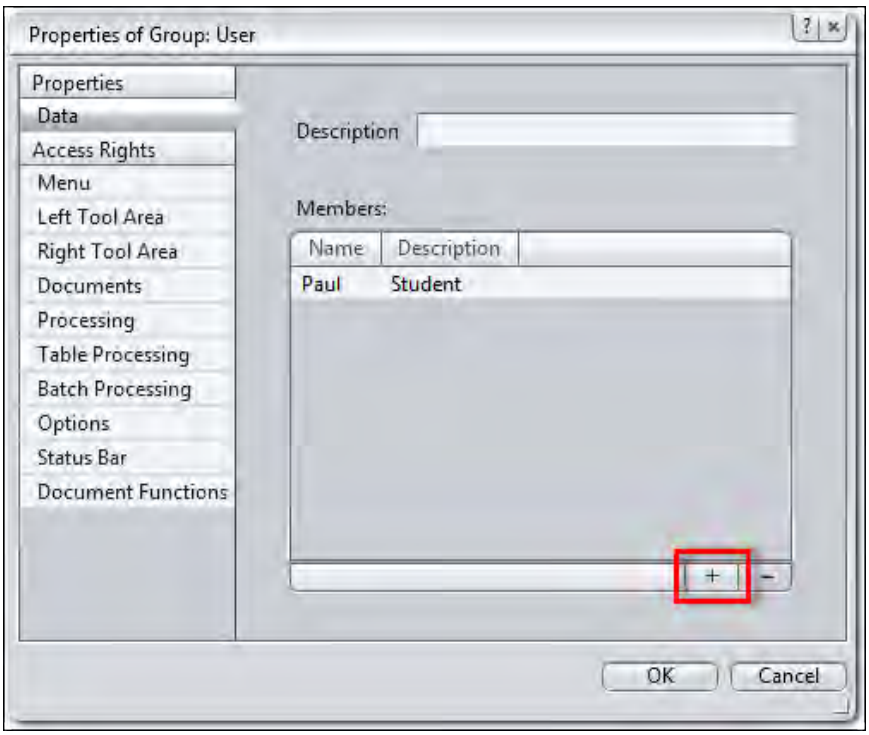
A member of the group **User** can't access the **User and Group Management** dialog. The menu entry is not visible at all.

2 Select the group you want to add a user to, e.g. **User**.

3 Click on the  **Group Properties** button in the list.

The **Group Properties** dialog opens. Under **Data** | **Members** you see the members list.

4 Click on **Add** button.



The **Select User** dialog opens.

5 Select the user you want to add to the group and click **OK** button.

You have successfully added a user to a user group.

8.4 Managing access rights for user groups

Prerequisites

- You are in the **Tools** menu | **User and Group Management** dialog.

Procedure

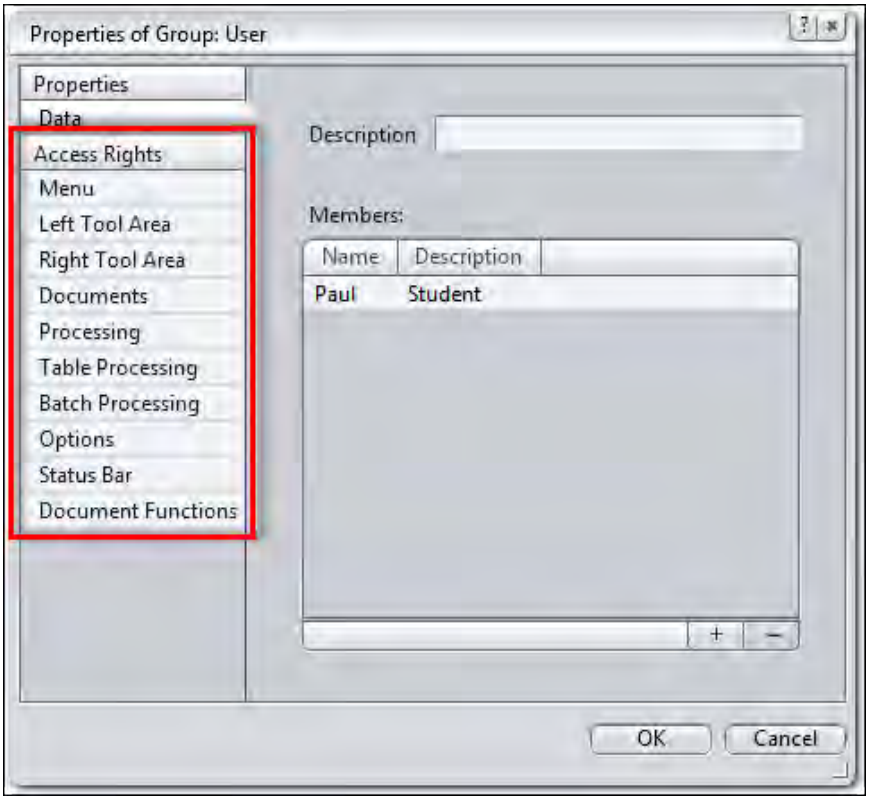
1 Click on the **Groups** tab.

By default you will find 4 groups with each group having the same rights.

2 Select the group you want to manage access rights for.

3 Click on the **Group Properties** button .

The **Group Properties** dialog opens.



i INFO

In the left column under **Access Rights** you will find all areas which you can configure access right for (e.g. **Menu**, **Processing**). As an example we continue with denying the selected group access right for **Processing** tab in the **Left Tool Area**. The tab is not visible to the group members when they login to the software at all.

4 Under **Access Rights** click on **Left Tool Area** tab.

You see a list with elements of the **Left Tool Area**.

5 Click on the **Check mark** button in front of the entry **Processing**.

The button changes to an **Minus** icon. The access to the **Processing** tab is restricted for all members of this group by now.

6 Click on **OK** button and close the dialog.

7 Restart the software for the changes to be effective.

You have successfully changed access rights within a user group.

8.5 Options

The options apply to all users, regardless of the user groups to which the user is assigned.

Parameter	Description
Check the following rules for a password	Here you can specify certain rules or criteria for a password that is created. If the checkbox is activated, the rules must be fulfilled when a new password is created. The following rules can be adjusted:

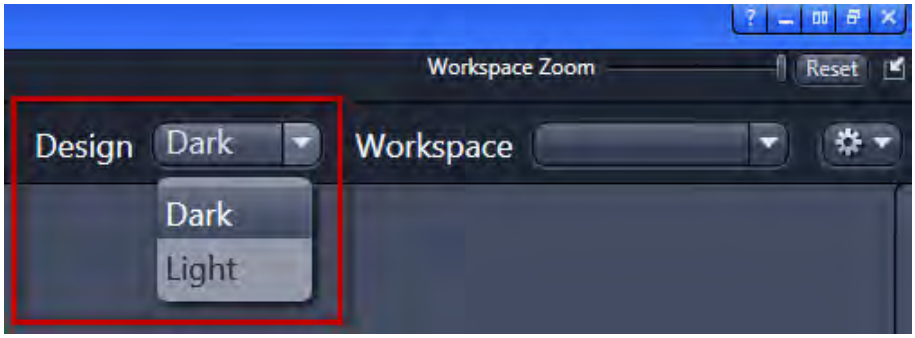
Parameter	Description
	■ Minimal number of lower case characters (e.g. when you set "2", the password must contain at least two lower case characters e.g. "e" and "f") ■ Minimal number of upper case characters (e.g. when you set "2", the password must contain at least two upper case characters e.g. "C" and "G") ■ Minimal number of digit characters (e.g. when you set "3", the password must contain at least 3 digits (from 0 - 9), e.g. "5", "6", "7") ■ Minimal number of special characters (e.g. when you set "1", the password must contain at least one special character, e.g. "&") ■ Minimum length (e.g. when you enter "9", the password must consist of at least 9 characters (any from above).
Do not allow Windows or ZEN user name as password	If activated, it is not allowed to use an existing user name from Windows or ZEN installation as password for the software.
Disable the reuse of last used passwords	If activated, you can enter a certain number of passwords which can`t be reused after each other. E.g. if you enter the number '**3**' you have to assign 3 different passwords one after another before you can use (reuse) an old password.
Disable the use of common passwords	If activated, you can create and edit a list which contains passwords which you can lock for usage. E.g. if you add the entry '123456789Password' this password can not be assigned from a user.
Force users to change password after period of time	**Activated**: The user must change his password after the specified period of time elapses. **Deactivated**: The password never expires.
– Days before expiry	Specifies the period of time after which the password expires.
Lock user after wrong password entries	If activated, you can determine the number of attempts the user has if he enters a wrong password. E.g. if you enter '**3**' , the user can enter a wrong password for 3 times before his user account is locked.

Parameter	Description
Lock screen after certain time span	**Activated**: After a period of inactivity the screen is locked and the user must enter his/her password to continue working. **Deactivated**: The password never expires.
– Minutes until screen lock	Specifies the time span after which the screen is locked.
Enable Auto-Login	■ Activated: No password is required The user is logged in automatically based on the Windows username. Create a user group in the software that is based on Windows Active Directory (**Type = AD**) and ensure that all relevant Windows users are present in the group and that the group has sufficient privileges in the software. ■ Deactivated: Each user has to log in with their own password.
Export/Import user database	Enables you to export or import the user database, including all user groups and privilege sets, for example to exchange it with another system.
– Export...	Specify the location on the file system where the database should be exported
– Import...	Select the database location on the file system

9 Customizing the Application

9.1 Selecting a Screen Design

In the upper right corner of the program window under **Design** you can select a **Light** or **Dark** screen layout .



9.2 Customizing Toolbar

Prerequisites

- You are in the **Tools** menu | **Customize Application** dialog.
- The **Toolbar** tab is selected by default.

Procedure

1. Click on an entry in the **Available Toolbar Items** list.

   You will see a list of all available items in this group.

2. Double-click on an item.

   The item will be added to the **Selected Toolbar Items** list and does appear in the **Toolbar** within the application. Alternatively you can add the items per Drag&Drop.

3. In order to change the order of symbols in the toolbar use the **Up**/**Down** buttons.

4. If you want to delete an Icon from the toolbar, click on the  **Delete** button.

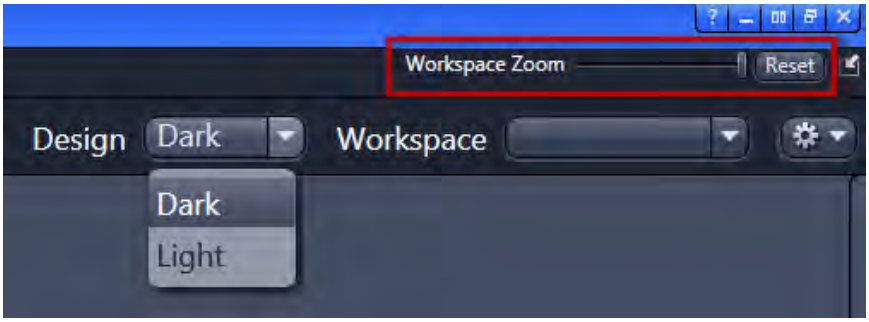
5. Click on **Close** button to close the dialog. The changes will be effective right now.

You have successfully customized the **Toolbar**.

9.3 Adjusting the Workspace Zoom

Procedure

1. To zoom in or out of the workspace move the slider left or right.

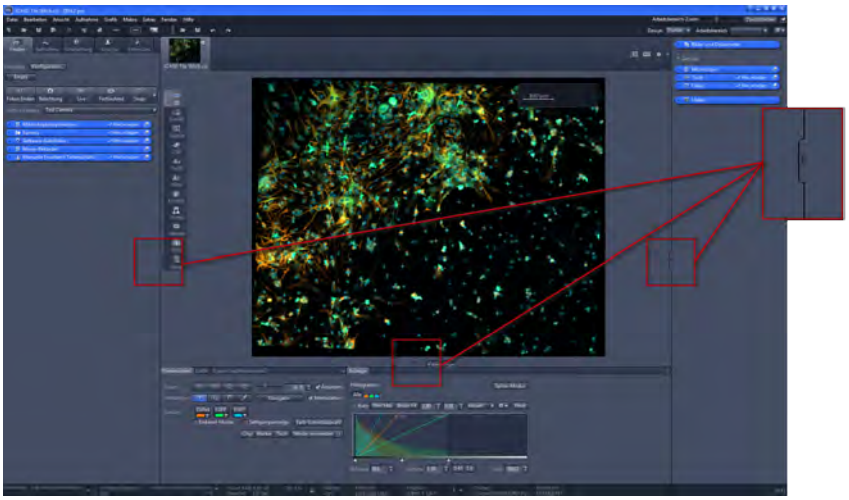


2. To reset workspace zoom to default click on **Reset** button.

9.4 Showing/Hiding Workspace Areas

Procedure

1. Click on **show**/**hide** buttons to show or hide areas.

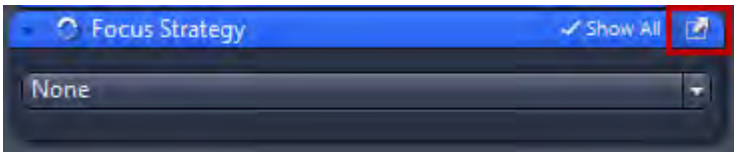


9.5 Undocking/Docking Tool Windows

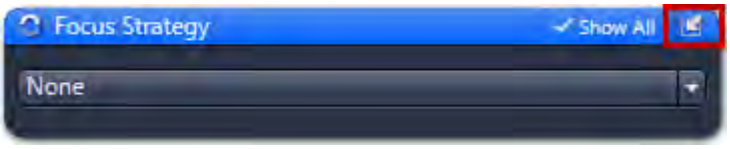
This function allows you to undock/dock a tool window. An undocked tool window can be positioned anywhere on the screen.

Procedure

1. Click the **Undock** button to undock a tool window. Once undocked, the tool window can be moved around by clicking and dragging it on the blue bar.



2 Click the **Dock** button to dock a tool window back to its place in the left tool area.



INFO

With the **dock all tools** function in the Workspace Configuration you can globally attach all undocked tool windows back to the **Left Tool Area**.

10 Open Application Development (OAD)

10.1 Introduction

The acronym OAD for Open Application Development is a term describing both the OAD platform on ZEN as well as the process of developing applications on it. The platform has been made available for our customers to enhance the functionality of ZEN in a flexible way. With OAD typical microscopy workflows can be integrated into the ZEN software. A short list of OAD highlights: Macro Interface to access the major functionality of ZEN and its objects and the access to external libraries like the .Net Framework to significantly enlarge the field of application.

The software offers the following components which we regard as main parts for Open Application Development (OAD):

- Macro Runtime Environment (integrated)
- Macro Recorder
- Macro Editor
- Macro Debugger
- Macro Interface (Object Library)
- ImageJ Extension

Basic functionality

All ZEN Products (ZEN lite excluded) come with a basic macro functionality which allows to play existing macros within the software (**Macro tool**).

i INFO

Within ZEN you can only run **.czmac** macro files which are aquired or saved in the ZEN macro environment. To run your macros they must be located in the folder: …/**User/Documents/Carl Zeiss/ZEN/Documents/Macros**.

Advanced functionality

The Macro Recorder, Editor and Debugger form the Integrated Development Environment (IDE) comes with the ZEN module **Macro Environment**. The IDE for the Macro Environment consists of two parts. There is a reduced IDE in the **Right Tool Area** which you find within the *Macro tool* [▶ 773]. The full blown IDE is available in the Macro Editor dialog and allows users to generate and work with macros similar to Excel/Word macros. The Macro Interface is built into the software and therefore not a separate product. The **ImageJ** Extension is the first extension for ZEN and will be free of charge. It will not be available in ZEN lite.

User forum

A user forum was established to allow users to exchange macros and to discuss solutions. You will find a lot of example macros and further documentation there. The user forum can be reached under www.zeiss.com/ZEN-OAD.

10.2 Running an existing macro

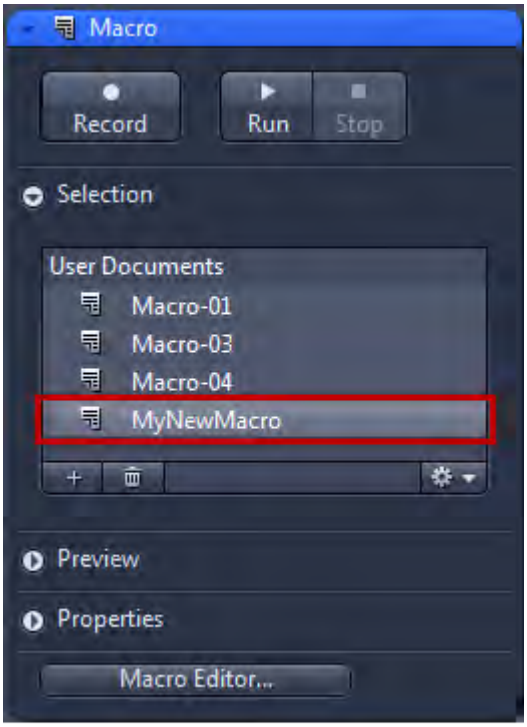
Prerequisites

- You work with a licensed version of **ZEN (blue edition)**, e.g. **ZEN pro**, **desk** or **system**. Note that the macro environment for ZEN lite is not available.
- You have not licensed the **Macro Environment** module.
- You have a macro file available that you want to play in ZEN.

Procedure

1. Copy your macro file in the following folder: **.../User/My Documents/Carl Zeiss/ZEN/Documents/Macros**.
2. Start the software.
3. In the **Right Tool Area** open the **Macro** tool.

   You see your macro in the list under **User Documents**.



4. Select your macro.
5. Click on the **Run** button.

Your macro is executed. You have successfully played a macro in ZEN.

10.3 Recording a macro

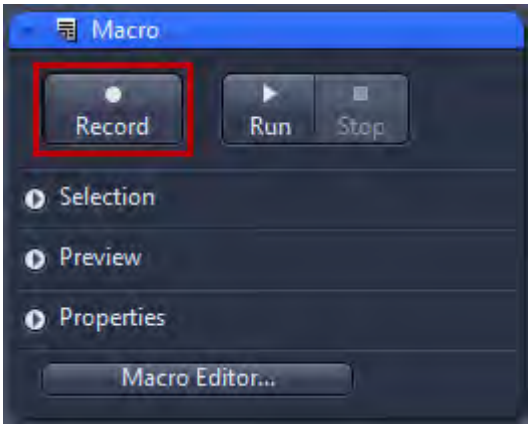
This guide shows how to record a macro of a simple processing workflow.

Prerequisites

- You have licensed the **Macro Environment** module.
- You are in the **Right Tool Area** | **Macro** tool**.**

Procedure

1 Click on the **Record** button.



2 Load a color image via menu **File** | **Open**…

3 Go to the **Processing** tab.

4 Under **Method** select **Edges** | **Sobel**.

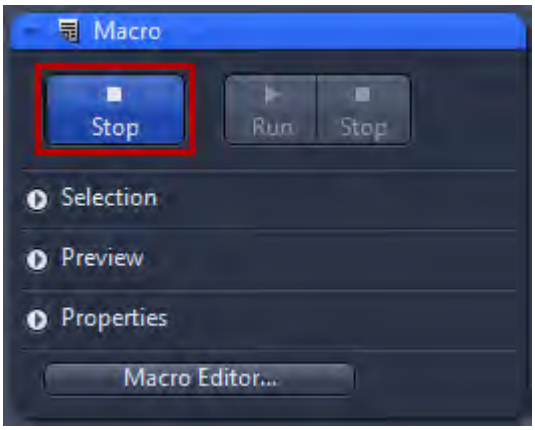
5 Under **Method Parameters** | **Normalization** select the entry **Clip.**

6 Under **Image Parameters** set your color image as **Input** Image.

7 On top of the **Processing** tab click on the **Apply** button.

The Sobel method will be applied to your image. The output image will be generated and opened in a new image container.

8 In the **Macro** tool click on the **Stop** button.



You have successfully recorded a macro for a simple processing workflow. The workflow can now be repeated automatically just by playing the recorded macro file.

11 Experiment Feedback

11.1 Introduction

The experiment feedback (conditional or adaptive experiments) functionality of ZEN allows the definition of specific rules and actions to be performed during an experiment. E.g. changing the course of an experiment depending on the current system status or the nature of the acquired data on runtime. Moreover, it is possible to integrate certain tasks like data logging or starting an external application, directly into the imaging experiment. Typically, but not exclusively, such an experiment connects the image pickup with an automatic online image analysis.

Feedback experiments can be set up and controlled by the help of the *Experiment Feedback Tool* [▶ 722] and the *Script Editor* [▶ 723]. Please note that experiment feedback functionality is a part of the **Advanced Processing** module. An example workflow for experiment feedback is described in the chapter *Workflow Experiment Feedback* [▶ 293].

Please note that we will not describe experiment feedback in detail here, as you will find a detailed instruction on how to perform feedback experiments and a lot of tutorials on the latest ZEN DVD in the folder ....**...Manuals/ZEN(blue edition)/OAD Content/Experiment Feedback**.

11.2 Workflow Experiment Feedback

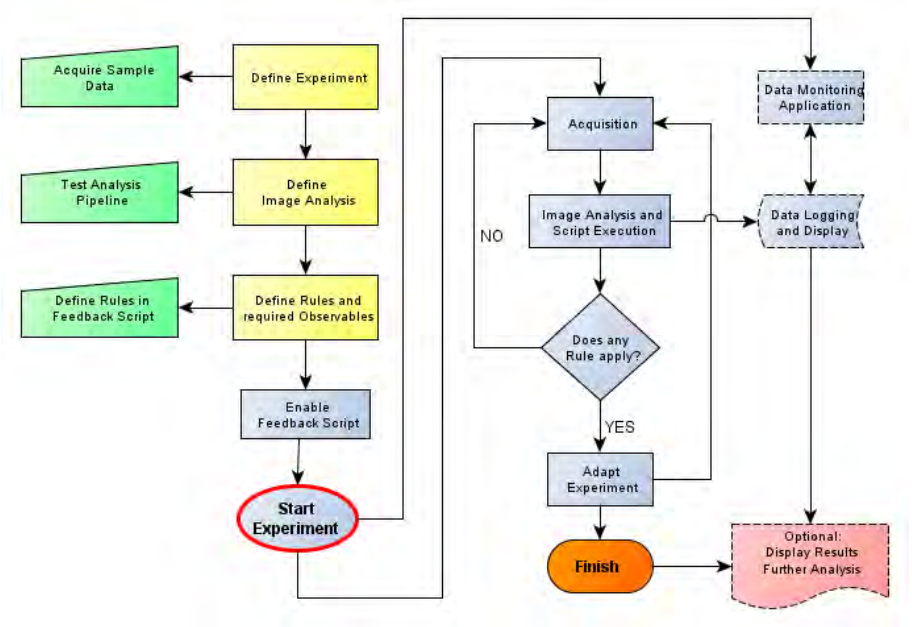


Fig. 11.1: Workflow Experiment Feedback

This diagram is one possibility to illustrate the typical workflow of feedback experiments. It contains the most important steps:

Step	Description
Define Experiment	The experiment should be well planned including the right parameters (Time Series, Z-stack, Multi-Channel, etc.), that are required to the get the correct data in a study once defined it is recommended to **Acquire Sample Data**, which can be used later on to test the image analysis.
Define Image Analysis	This step is using the image analysis wizard in order to create and image analysis pipeline for the latter use inside the feedback script only the specified parameters can be accessed from with the script later and it is strongly recommended to **Test Image Analysis Pipeline** to ensure the created results are meaningful. Optionally it is also possible to create an image analysis setting programmatically via an OAD macro.
Define Rules and Observables	This step is all about making up one´s mind on how the script should actually work. What must be observed and how should the experiment react upon a certain event once the main idea becomes clear one can start to **Define Rules in Feedback Script**.
Start Experiment	At this point one can start the experiment and watch the output. The general concept behind this workflow can be described as a loop, which is the actual acquisition. Every time “something”, usually an image acquisition took place the script will be executed. The rules will be checked and if required, certain tasks will be carried out. Additionally it is possible to export or log data into a text file and/or start an external application at any time point during the experiment. The best time point for those actions depends on the type of application.

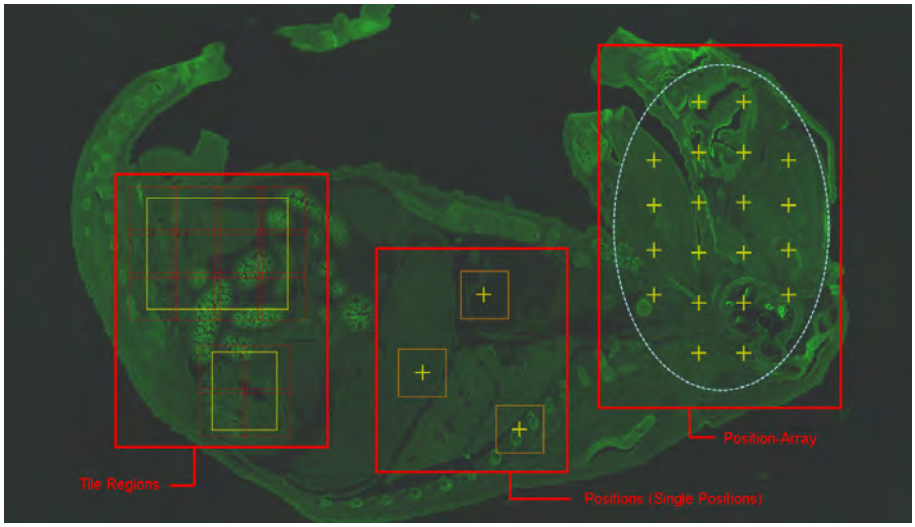
12 Module Tiles & Positions

12.1 Introduction

With the **Tiles** module you can acquire images that are made up of a number of individual images (tiles). To do this, it is possible to define tile regions and positions.

If you have licenced the **Tiles** module the basic functions of the **Tiles** tool will be supplemented by the **Advanced Setup** functionality. This will allow you to set up **Tiles** experiments more easily and also to use sample carriers and focus surfaces.

Note that when you activate the **Tiles** tool the software will automatically select the suitable focus strategy **Use Focus Surface/Z-values Defined by Tiles Setup**. This focus strategy also is only available if you have licensed the **Tiles**module.



INFO

If you want to acquire tile regions or positions with different Z-positions, you need to use a suitable focus strategy. To find out more read the chapters *Using focus strategies* [▶ 71] in the ZEN Online Help.

12.2 General Preparations

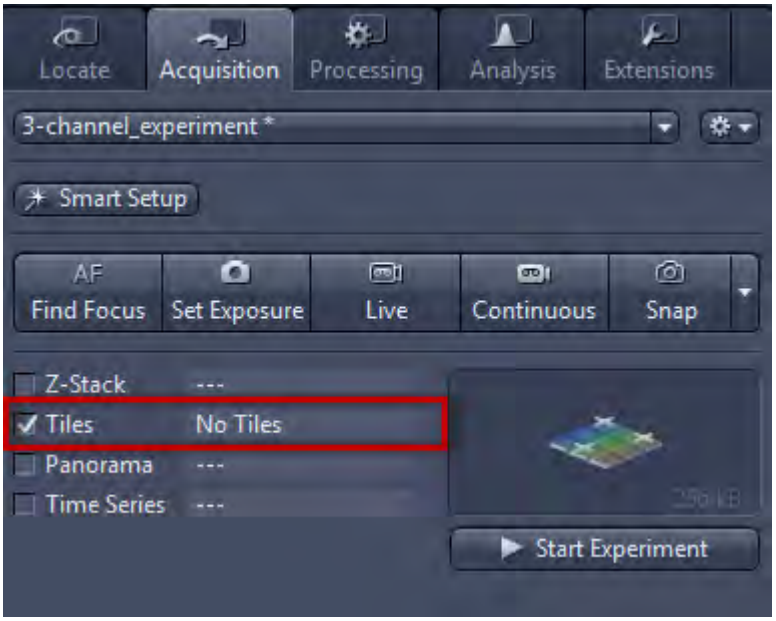
Prerequisites

- The Tiles module is activated under **Tools** menu | **Modules Manager...** | **Tiles & Positions**.
- To set up **Tiles** experiments, you require a motorized stage. This must be configured and calibrated correctly in accordance with the camera orientation. For more information read *Calibrating Stage and Selecting Channel* [▶ 296].

- You have *created a new experiment* [▶ 46], *defined at least one channel* [▶ 45] and correctly set the focus and exposure time.
- You are on the **Acquisition** tab.

Procedure

1 Activate the **Tiles** tool by activating the **Tiles** checkbox in the **Acquisition Dimensions** section.



In the **Left Tool Area** the **Tiles** tool appears in the **Multidimensional Acquisition** tool group.

You have successfully completed the general preparations. You can now continue with the next steps of this guide.

12.3 Calibrating Stage and Selecting Channel

On start up of a system with motorized stage and/or focus a request will appear asking if the components should be driven to the end switches and calibrated. This ensures that you begin working with absolute coordinates in this session with the microscope. If the microscope power is cycled then this process should be repeated. This function is of particular use if you continually work with a sample carrier e.g. 96 well plate, of the same format mounted in the same manner repeatedly with a given experiment template. If you perform a carrier calibration (Sample Carrier section of the Tiles tool. Note these features are only availble if you have a Tiles and postions licence) once with a calibrated stage then the carrier calibration is essentially always valid.

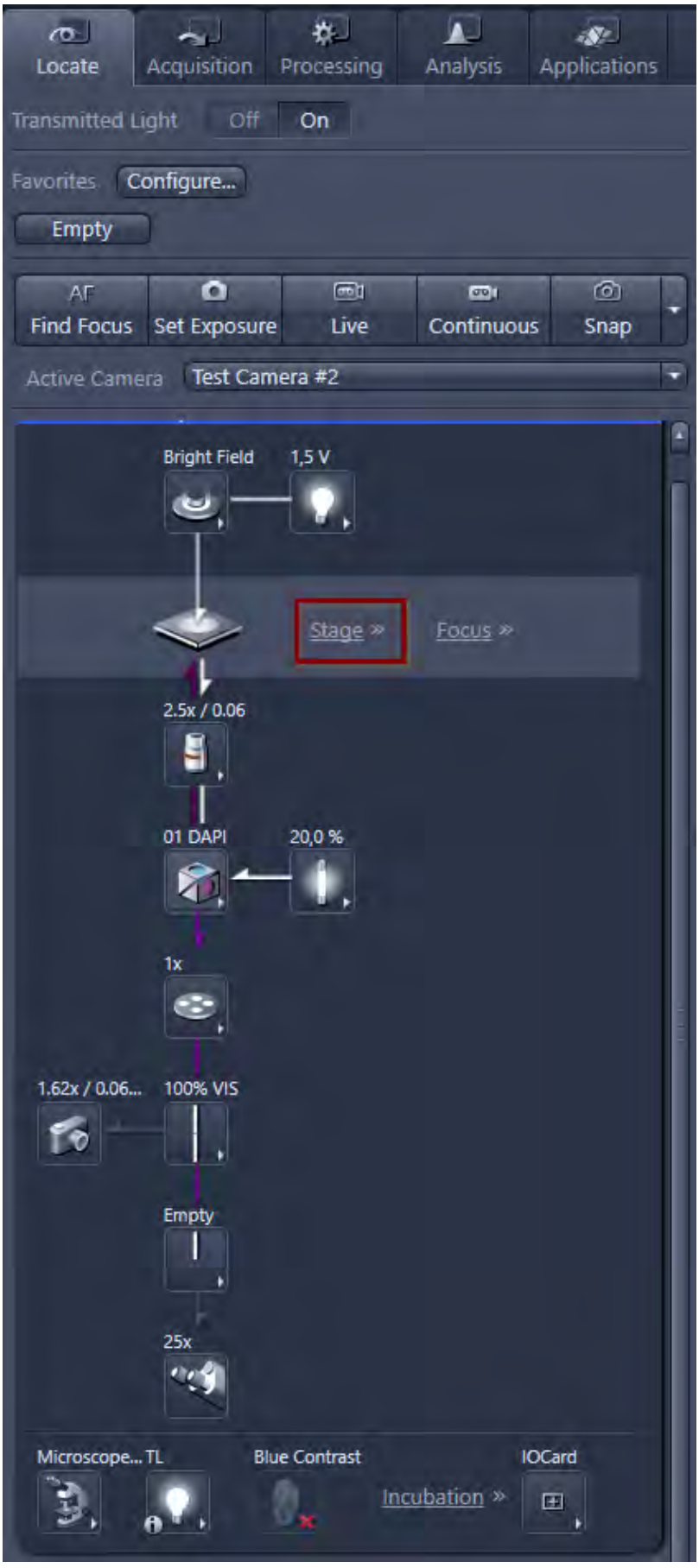
i INFO

The request to calibrate stage and focus on Startup can be activated/deactivated under **Options** | **Startup** | **Stage/Focus Calibration**.

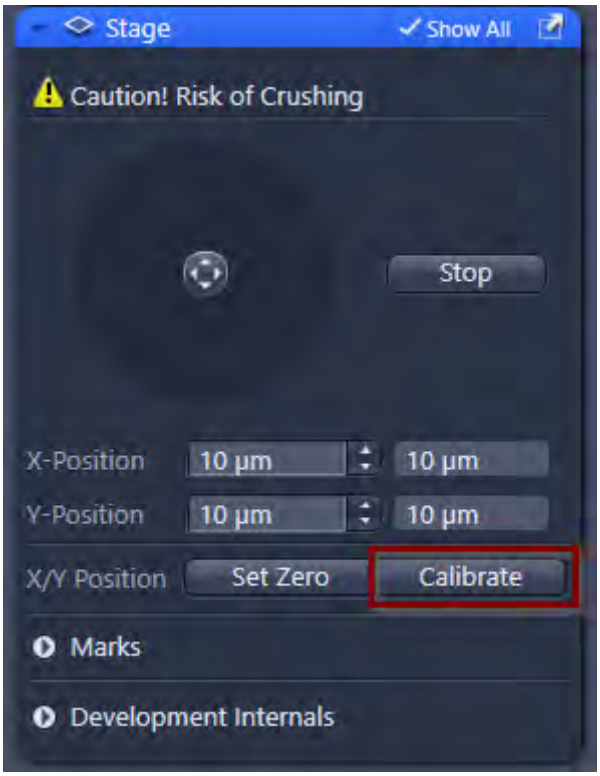
Procedure

1. Put your Sample Carrier on the stage.
2. Go to the **Locate** tab.
3. Choose a low magnification objective (e.g. 10x).
4. Click on the **Live** button and find your focus area either using transmission or fluorescence light.

5 In the **Microscope Control** tool click on the **Stage** button.



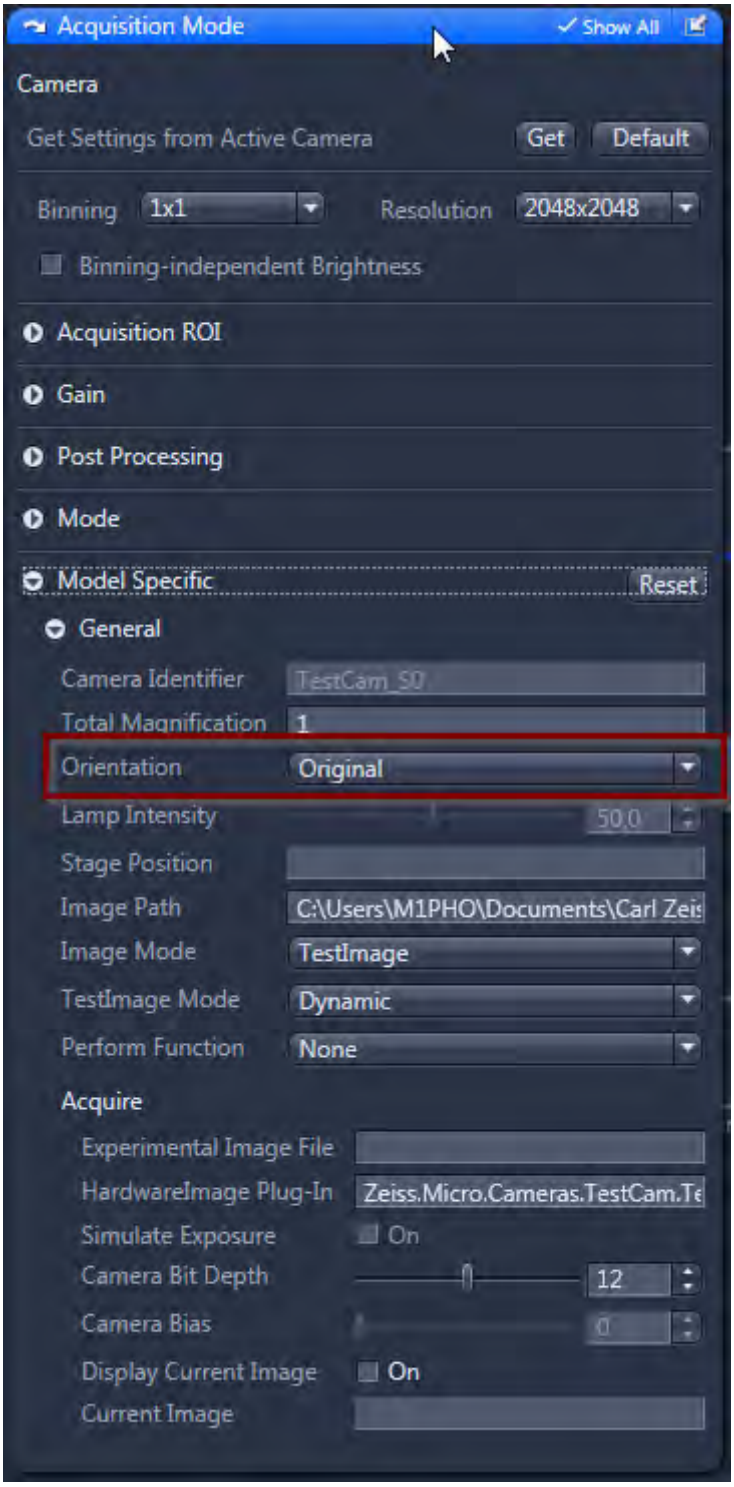
6 Activate the **Show all** mode and then click on the **Calibrate** button.



7 Check if the alignment of your camera and joystick is correct by dragging the software joystick up, down, left and right and observe whether the movement of your image corresponds to movement of the circle. In addition, check whether the image movement also corresponds accordingly when you move the joystick.

8 If the alignment is incorrect, go to the **Camera** tab activate the **Show all** mode and click **Model Specific**.

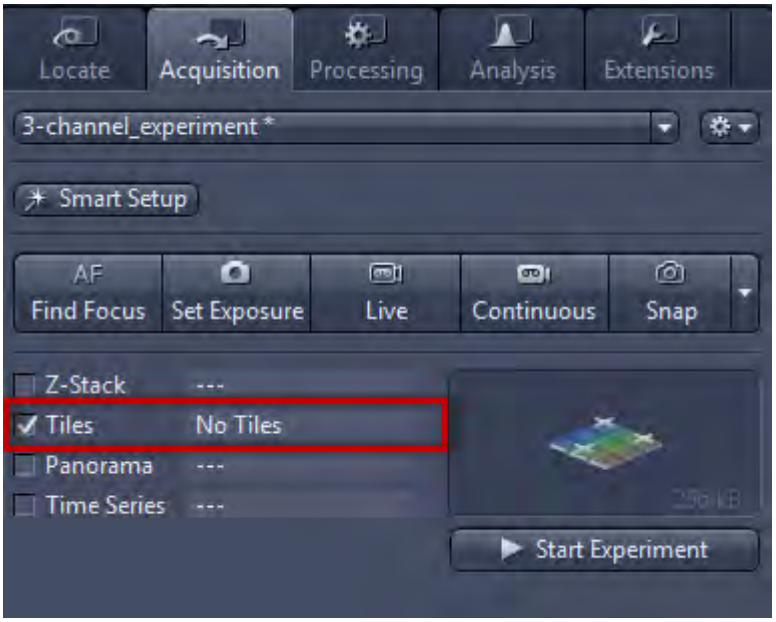
9 In **Orientation** you can now adjust the camera orientation to the joystick orientation. Alternatively, you can and may also need to invert the X- and Y-axis of your stage in the **MTB** in order to align the joystick and the software-controlled stage movement.



10 Go to the **Acquisition tab**.

11 Do all the prerequisites (e.g. channel and camera settings) for a **Tiles & Positions experiment** on your sample. For that use Smart Setup and, if needed, Experiment Designer (not advisable for beginners).

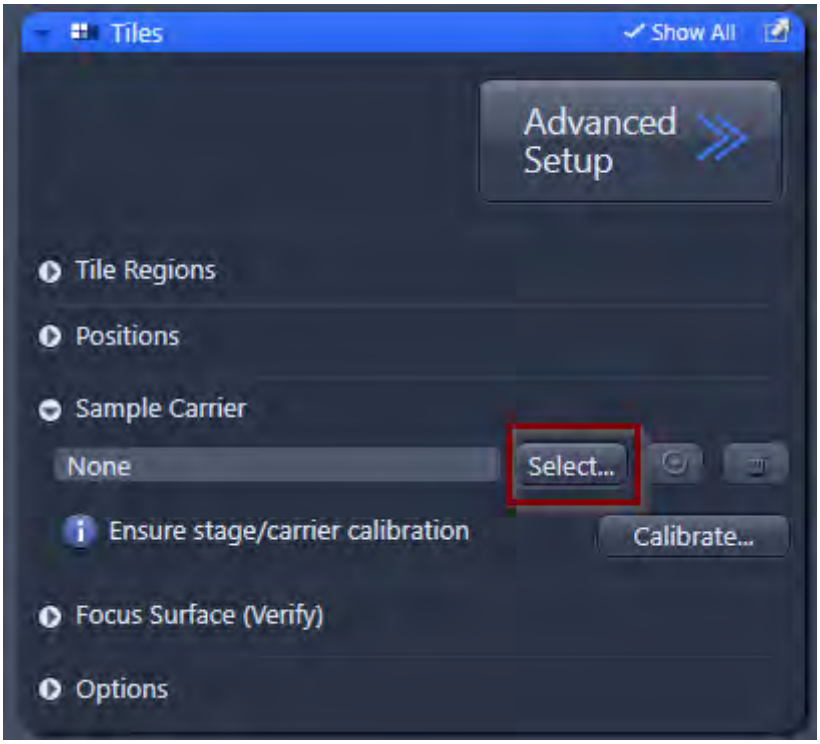
12 After you have defined at least one channel (e.g. EGFP), activate the **Tiles** module checkbox.



13 Open the **Tiles** tool in the **Multidimensional Acquisition** module and activate the **Show all** mode.

14 In the **Tiles** tool open the **Sample Carrier** section.

15 Click on the **Select...** button.



16 Choose a predefined **Sample Carrier template** and click **OK**.

12.4 Setting Up a Simple Tiles Experiment

Prerequisites

- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1 Start the **Live** mode to use the stage to locate a point that you want to be at the center of your tile region.

2 Bring the specimen into focus using the focus drive.

3 Open the **Tile Regions** section.

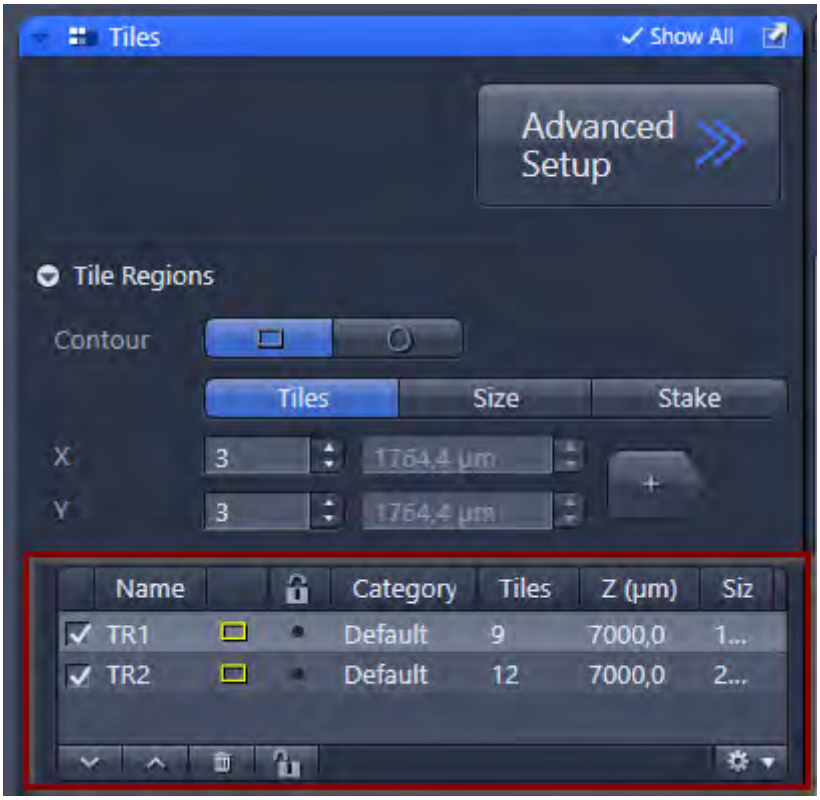
4 The **Tiles** mode is activated by default. In this mode enter the number of tiles you want in the **X** and **Y** input fields, e.g. **X** = 3, **Y** = 3 equals a tiles region containing 9 tiles.
Alternatively, you can enter the size of the tile region that you want to add. To do this, activate the **Size** mode.

5 Click on the **Add** button .

The tile region is added to your experiment. The current stage position determines the center and the Z-position of the tile region.

6 To add further tile regions, move the stage to another position on the sample and repeat the previous steps.

The added tile regions (**TR1**, **TR2**, etc.) are displayed in the tile regions list.



If you scroll to the right in the table, you can read the Size of the tile regions.

INFO

To ensure that the individual Z values of the tile regions are taken into account ZEN automatically selects the most appropriate *focus strategy* [▶ 71] when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all tile regions at the same Z-position then you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual Z-positions are then ignored and the current Z-position at the time the experiment is started is used for all tile regions.

7 Save the experiment. To do this, in the **Experiment Manager** click on the  **Options** button and select **Save As**. Enter a name for the experiment in the input field (e.g. Simple Tile Experiment).

8 Click on the **Start Experiment** button.

The **Tile Region** experiment is acquired.

The individual tile regions are displayed in the acquired file as scenes and can be selected using the **Scene** slider on the **Dimensions** tab. If you deactivate the **Scene** checkbox, all tile regions are displayed as an overview.

You have successfully set up and acquired a simple **Tile Region** experiment.

12.5 Setting Up a Simple Positions Experiment

Prerequisites

- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1 Open the **Positions** section.

2 Start the **Live** mode to use the stage to locate a position that you want to acquire.

The X and Y coordinates of the current position are displayed in the **Current X/Y** display fields.

3 Bring the specimen into focus using the focus drive.

4 Click on the  **Add** button.

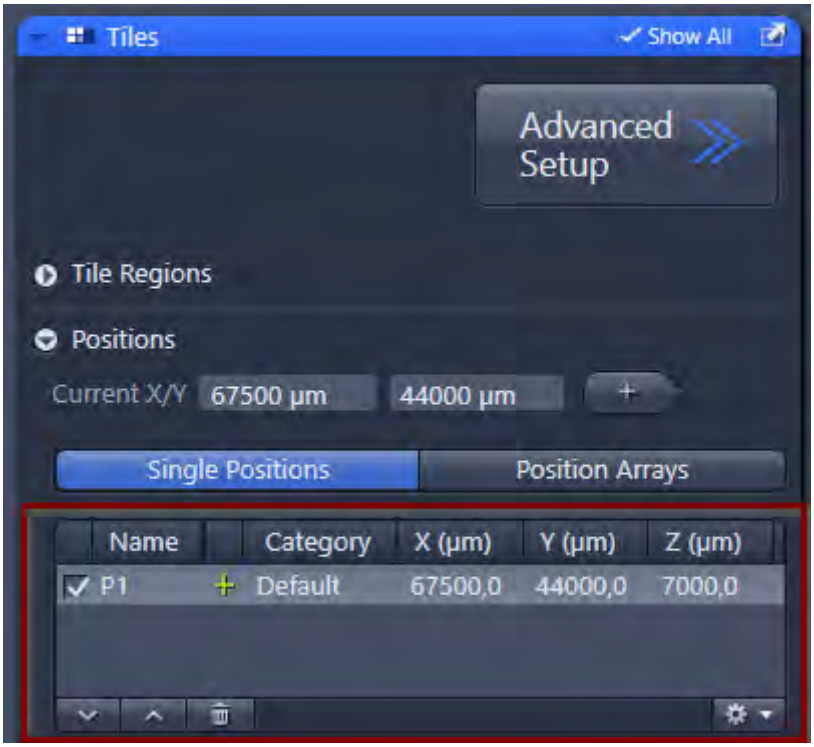
The current position is added to your experiment.

INFO

If you are close to a position that you added previously the software will ask you if you really want to add another position at this location (the threshold for this lies within a circle whose radius is less than half the approximate width of the cameras visible field).

5 To add further positions, move the stage to another position on the sample and repeat the previous steps.

The added positions are shown in the list in the **Single Positions** section with their X, Y and Z-coordinates.



INFO

To ensure that the individual Z values of the positions are taken into account the software automatically selects the most appropriate *focus strategy* [▶ 71] when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all positions at the same Z-position then you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual Z-positions are then ignored and the current Z-position at the time the experiment is started is used for all positions.

6 Save the experiment. To do this, in the **Experiment Manager** click on the **Options** button and select the **Save As** entry. Enter a name for the experiment in the input field (e.g. Simple Tile Experiment).

7 Click on the **Start Experiment** button.

The Positions experiment is acquired.

The individual positions are displayed in the acquired file as scenes and can be selected using the **Scene** slider on the **Dimensions** tab. If you deactivate the **Scene** checkbox, all positions are displayed simultaneously as an overview.

You have successfully set up and acquired a Positions experiment.

12.6 Tiles & Positions with Advanced Setup

Advanced Setup makes it easier for you to create tile regions and positions by displaying the distribution and dimensions of tile regions and positions in the travel range of the stage. You can generate a **Preview Scan** and drawn in tile regions or positions precisely on the basis of this template. For the preview scan you have the option of using an objective with a lower magnification and/or a different channel (e.g. transmitted light).

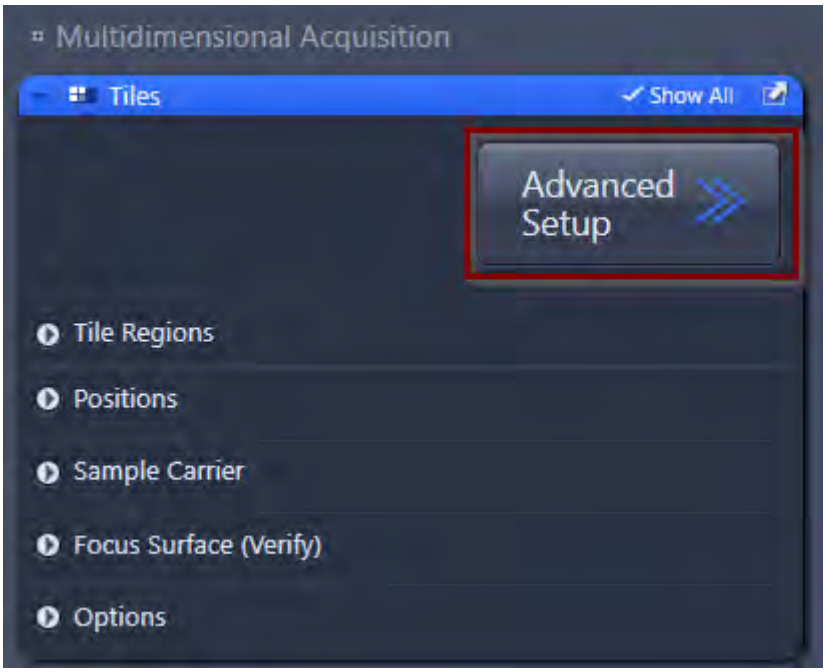
INFO

To ensure that the individual Z values of the tile regions and/or positions are taken into account ZEN automatically selects the most appropriate *focus strategy* [▶ 71] when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all tile regions at the same Z-position then you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual Z-positions are then ignored and the current Z-position at the time the experiment is started is used for all tile regions.

Prerequisites

- To set up tiles experiments in **Advanced Setup**, you need the **Tiles** module.
- You have read the chapter Introduction.
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure 1 Click on the **Advanced Setup** button.



The **Advanced Tiles Setup** view opens.

The Live mode is activated automatically. Deactivate the live mode if you do not need it to prevent bleaching of the sample. To do this, click on the active **Stop** button in the **Left Tool Area**.

12.6.1 Generating a Preview Scan

Prerequisites ■ You are in **Advanced Setup** in the **Tiles** tool.

Procedure 1 In the **Specific View options** area open the **Preview Scan** tab.

2 Select an objective with a relatively low magnification.

3 In the **Preview Scan** tool, uncheck **Use Existing Experiment Settings** and select/ unselect the channels that you want to use for the preview scan.

4 If necessary, use the Live mode to adjust the focus area and exposure following a change of objective or channel.

5 To obtain a better overview, zoom out of the **Advanced Setup** view slightly.

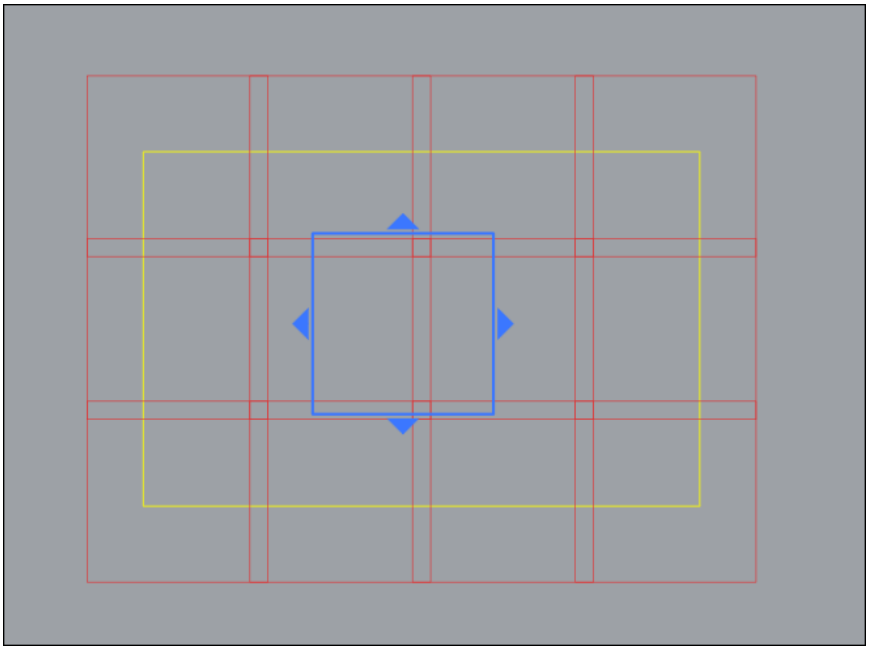
6 Start the **Live** mode to use the stage to locate approximately the center of the region for which you want to generate a preview scan.

7 Select the **Tile Region Setup** tab from the **Advanced Setup** view options.

8 Under **Setup by** click on the **Contour** button.

9 Under **Contour** select the **Rectangular Contour** tool.

10 In the stage view, use the tool to drag out a rectangle that approximately encloses the region for which you want to generate a preview scan.

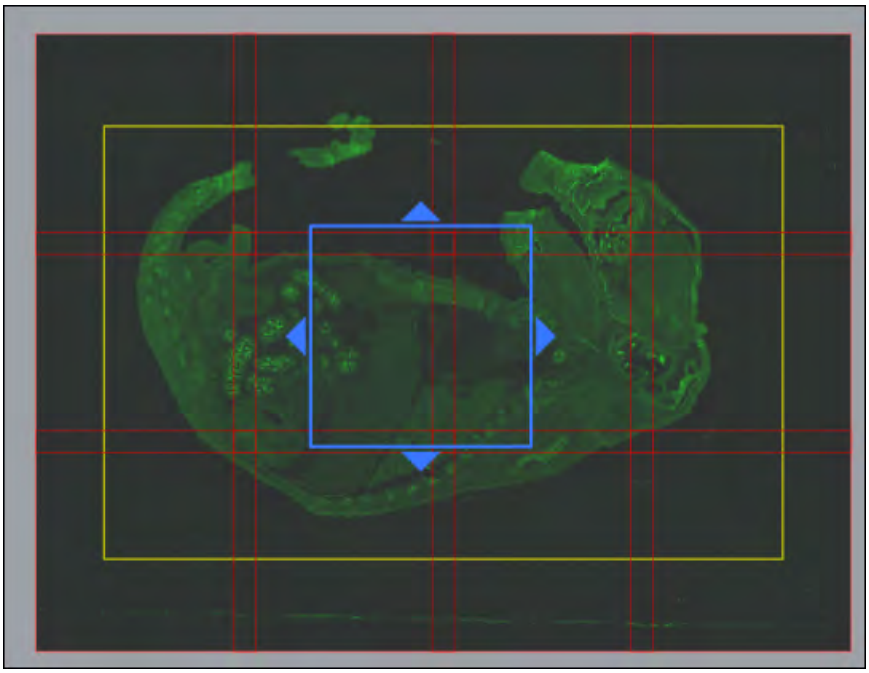


A tile region is created for the marked region and displayed in the list in the **Tile Regions** section of the **Tiles** tool.

11 With the help of the **Live** mode, check whether the desired image region is covered by the tile region. To do this, use the stage to locate the corners and edges of the tile region and increase or reduce the yellow selection frame as necessary.

12 In the **Preview Scan** tab click on the **Start Preview Scan** button.

A series of snap images is acquired to generate a preview of the marked region.

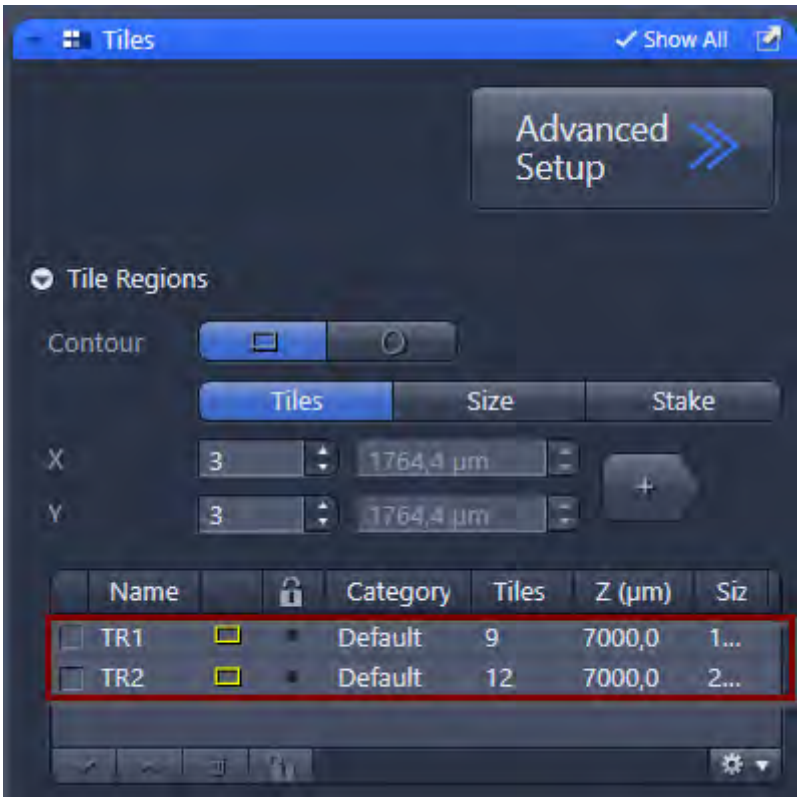


You have successfully generated a preview scan.

Before you continue with the actual experiment, carry out the following steps:

Procedure

1. In the **Tiles Regions** section deactivate the preview tile region (TR 1) by deactivating the checkbox of the corresponding list entry. This prevents the acquisition of the preview tile region during the actual experiment.



2. In the **Preview Scan** tool or the **Microscope Control** tool in the Right Tool Area (ZEN 2.3 and higher) select the objective you want to use for final acquisition.
3. Use the **Live** mode to adjust the focus area and exposure accordingly.

You can now continue setting up the tile experiment.

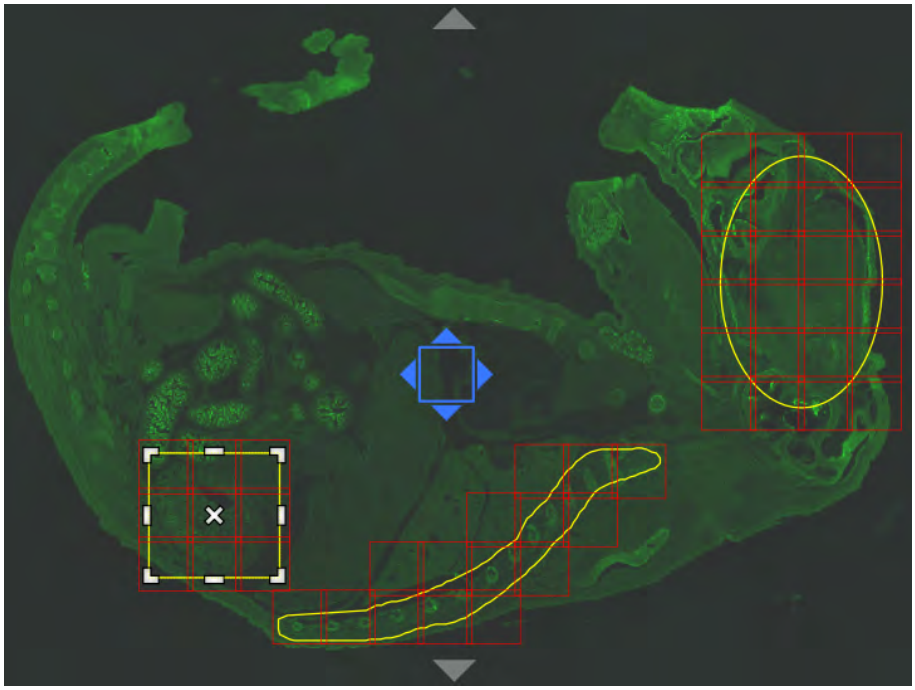
12.6.2 Creating Tile Regions by Contour

Prerequisites

- You have generated a *preview scan* [▶ 306] that will help you to position the tile regions more easily.

Procedure

1. Select the **Tile Region Setup** tab from the **Advanced Setup** view options.
2. Under **Setup by** select **Contour**.
3. In the **Contour** section select the desired contour tool.
4. Use the **Contour** tool in the stage view to draw in the tile regions you want to acquire.



Tile regions are created for each marked region. They are added to the list in the **Tile Regions** section of the **Tiles** tool.

You have successfully created tile regions in **Advanced Setup**.

12.6.3 Creating Tile Regions by Predefined

Prerequisites

- You are in **Advanced Setup** in the **Tiles** tool.
- You have selected and calibrated a sample carrier with one or more wells/ containers.

Procedure

1 In the **Carrier** tab (lower left side, below the Center Screen area) select the well(s) of interest by holding **Ctrl-key** and clicking on the desired wells.

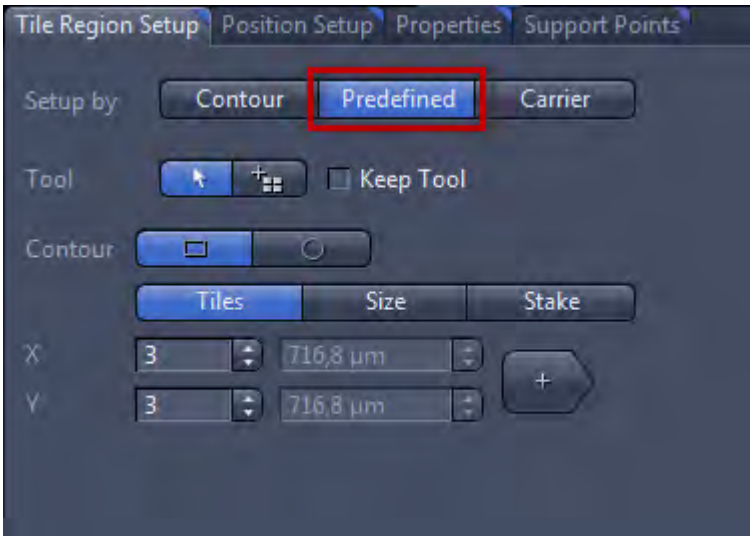
The selected wells are now bordered by a blue circle.

INFO

If only one well is double-clicked, the stage will move to the center of that well.

2 Open the **Tile Region Setup** tab below the Center Screen Area and activate the **Show All** mode.

3 In the **Tile Region Setup** tab under **Setup by** select **Predefined**.



4 Choose how many tiles in x and y dimension you want to add and click on the **Add** button to confirm.

12.6.4 Creating Tile Regions by Carrier

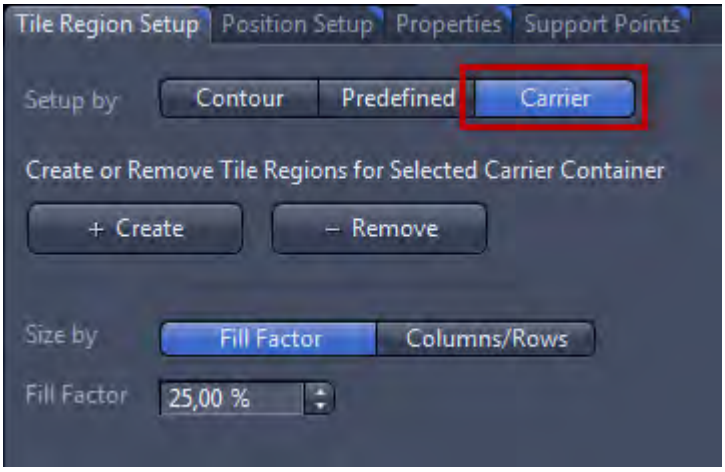
Prerequisites

- You are in **Advanced Setup** in the **Tiles** tool.
- You have selected and calibrated a sample carrier with on or more wells/ containers.

Procedure

1 Open the **Tile Region Setup** tab below the Center Screen Area and activate the **Show All** mode.

2 In the **Tile Region Setup** under **Setup by** select **Carrier**.



3 In the **Carrier** tab select the individual wells for which you want to create Tile regions by holding *Ctrl*-key and clicking the desired wells.

4 Under **Size by** select **Fill Factor** and enter the desired value in the **Fill Factor** input field.

5 Click on the **+ Create** button.

According to the selected **Fill Factor**, the wells will be filled with a calculated number of tiles that are located around the center. To create a given size of tile region use the **Columns/Rows** function in a similar manner.

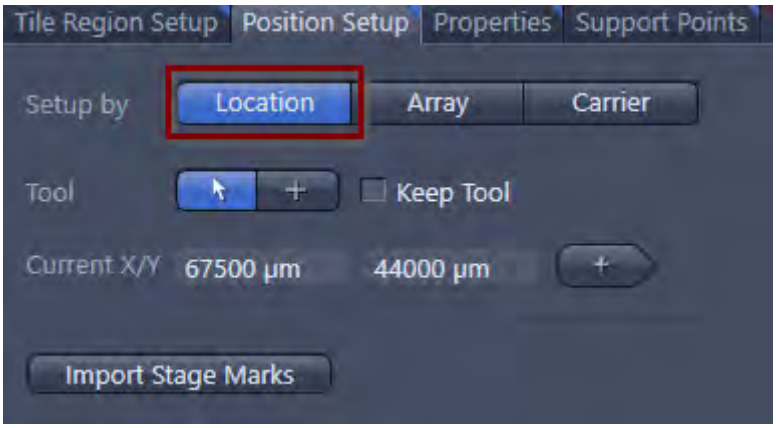
12.6.5 Creating Positions by Location

Prerequisites

- You are in **Advanced Setup** in the **Tiles** tool.

Procedure

1. Select the **Position Setup** tab from the **Advanced Setup** view options.
2. Under **Setup by** click on the **Location** button.



3. In the **Tool** section, select the  **Add** tool**.**
4. In the stage view click on the location at which you want to add a position.

   The added positions are displayed in the **Single Positions** list in the **Positions** section of the **Tiles** tool.

You have successfully created positions in **Advanced Setup**.

12.6.6 Creating Positions by Array

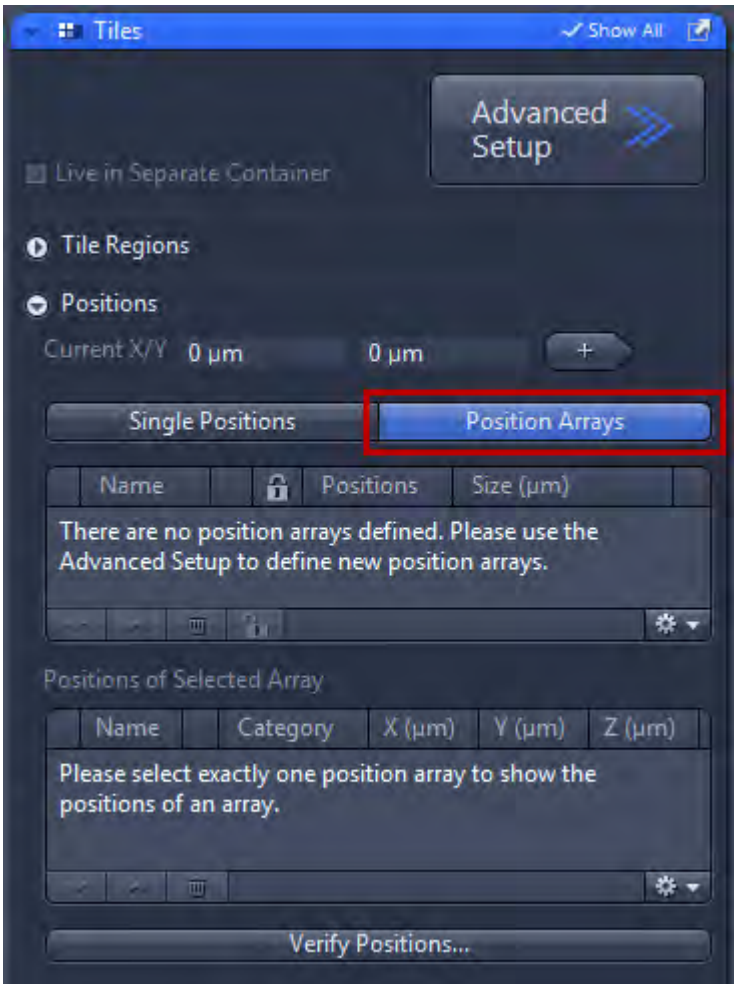
Prerequisites

- You are in **Advanced Setup** in the **Tiles** tool.

Procedure

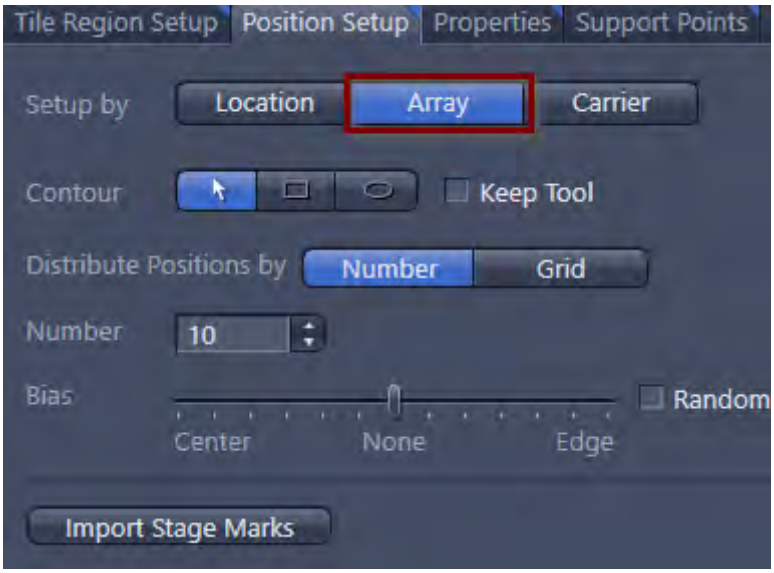
1. In the **Tiles** module go to the **Positions** section.

2 Select **Position Arrays**.



3 Go to the **Position Setup** tab (below the Center Screen Area) and activate the **Show All** mode.

4 Under **Setup by** click on **Array**.



5 Either choose the rectangular or circular **Contour**, adjust the **Number** of required positions and the **Bias** where the positions should be located.

> **INFO**
>
> If the **Random** checkbox is activated the chosen number of positions for the array will be determined randomly within the arrays space.

6 Subsequently, mark the interesting area of the carrier in the Center Screen Area by keeping the left mouse button clicked.

The positions will be automatically generated.

12.6.7 Creating Positions by Carrier

Prerequisites

- You have selected and calibrated a *sample carrier template* [▶ 339].
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1 Click on the **Advanced Setup** button.

Advanced setup is opened.

2 To obtain a complete overview of the sample carrier, zoom out of the view (**Crtl** + Mouse wheel).

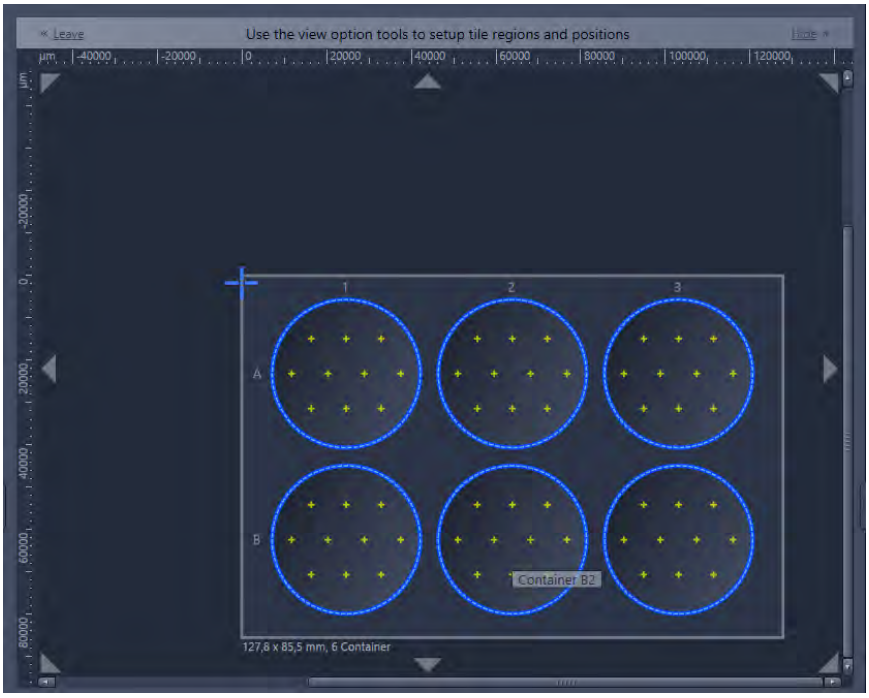
3 In the view options, select the **Position Setup** tab**.**

4 In the **Setup by section** click on the **Carrier** button.

5 Select the **containers** in which you want to distribute **positions** by holding down the *Ctrl* key and clicking on the relevant containers.

6 Click on the **Create** button.

The selected **containers** are each filled with a **Position Array** (group of positions).



In the **Positions** section of the **Tiles** tool the **Position Arrays** are displayed in the **Position Arrays** list.

You have successfully used a **sample carrier** and the **Setup by Carrier** to create positions.

INFO

Analogous to the **Position Arrays** tile regions can also be created on the**Tile Region Setup** tab by using the **Carrier** button. In both cases, you can use the additional functions of the carrier setups to make other useful settings. For example, the patch surface of containers or the number and distribution of positions can be set, see *Setup by carrier (tile region)* [▶ 367] and *Setup by carrier (position)* [▶ 372].

12.7 Copying a Tile Region or Position

When you want to copy and paste a Tile Region or Position setting (e.g. a certain arrangement of tiles, positions or local support points) from one well to other wells or even to all containers of a carrier, apply the following workflow.

Procedure

1 Select the well from where the Tile Region/ Position setting should be copied.

The selected well is now highlighted by a blue border.

2 Right click within the selected well in the Center Screen Area (outside the tile region) to open the context menu.

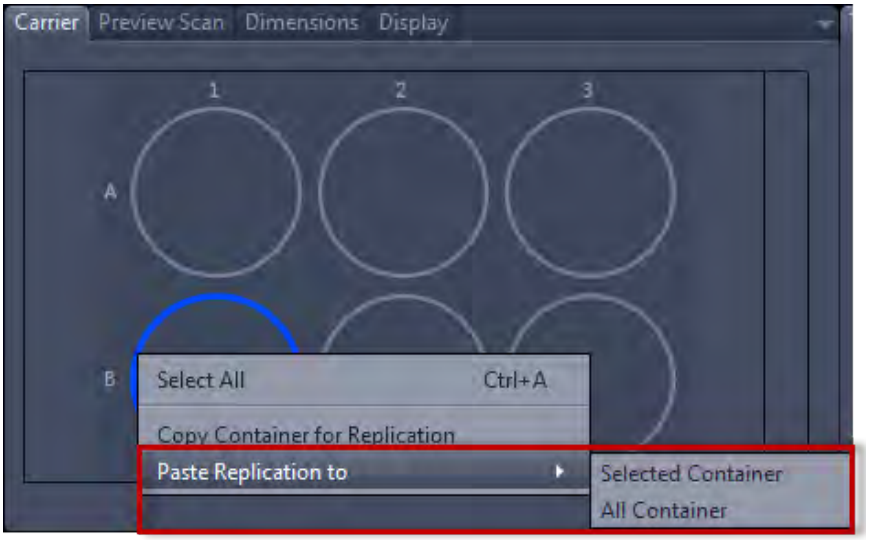
3 Select **Copy Container for replication**.

4 If you want to choose specific wells use the left mouse button to select the wells into which you want to paste the copied Tile Region/ Position setting.

INFO

You can select multiple wells in combination with the **Ctrl**-key.

5 Right click in the Center Screen Area and select the context menu entry **Paste Replication to** and either choose **Selected Container** or **All Container**.



The copied Tile Region/ Position setting is pasted into the selected wells or all the wells of the carrier with the same relative coordinates to the center of each well.

12.8 Adjusting Z-Values

If you add positions or tile regions, the current Z-value is automatically adopted for the tile region or position.

- Learn about how to adjust and verify the Z-values of positions in the chapter *Adjusting Z-Values of Positions* [▶ 317].
- Learn about how to adjust and verify the Z-values of tile regions in the chapter *Adjusting Z-Values of Tile Regions* [▶ 317]. Note that the Z-values defined here are valid for all tiles in the tile region in question.

INFO

To ensure that the individual Z-values of the tile regions are taken into account the software automatically selects the most appropriate *focus strategy* [▶ 71] when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all tile regions at the same Z-value you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual Z-values are then ignored and the current Z-value at the time the experiment is started is used for all tile regions.

12.8.1 Adjusting Z-Values of Tile Regions

Prerequisites

- You have set up a **Tiles** experiment with at least one tile region.

Procedure

1. To check the Z-value of tile regions, open the **Tile Regions** section in the **Tiles** tool.

   The Z-values of the tile regions are displayed in the **Z** column of the list.

2. Double-click on the list entry of the tile region that you want to check.

   The stage automatically locates the center of the tile region and the associated Z-position.

3. Use the **Live** mode to check the Z-value of the tile region.

4. To adjust the Z-value, set the new Z-position using the focus tool.

5. In the **Tile Regions** list click in the bottom right on the **Options** button  and select **Set Current Z For Selected Tile Regions**.

6. To check further tile regions, repeat steps 2 to 4.

7. To check and adjust large number of Tile Regions, click on the **Verify Tile Regions** button.

   The **Verify Tile Region** dialog opens. There you have a interface for the verification process of each tile region.

8. Click on **Close** after you have verified all tile regions.

You have successfully checked and adjusted the individual Z-values for the tile regions.

12.8.2 Adjusting Z-Values of Positions

Prerequisites

- You have set up a tile experiment with at least one position.
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1. To check and adjust the Z-value of positions, open the **Positions** section.

   The Z-values are displayed in the **Z** column of the **Single Positions** list.

2. Double-click on the list entry of the position that you want to check.

The stage automatically locates the position.

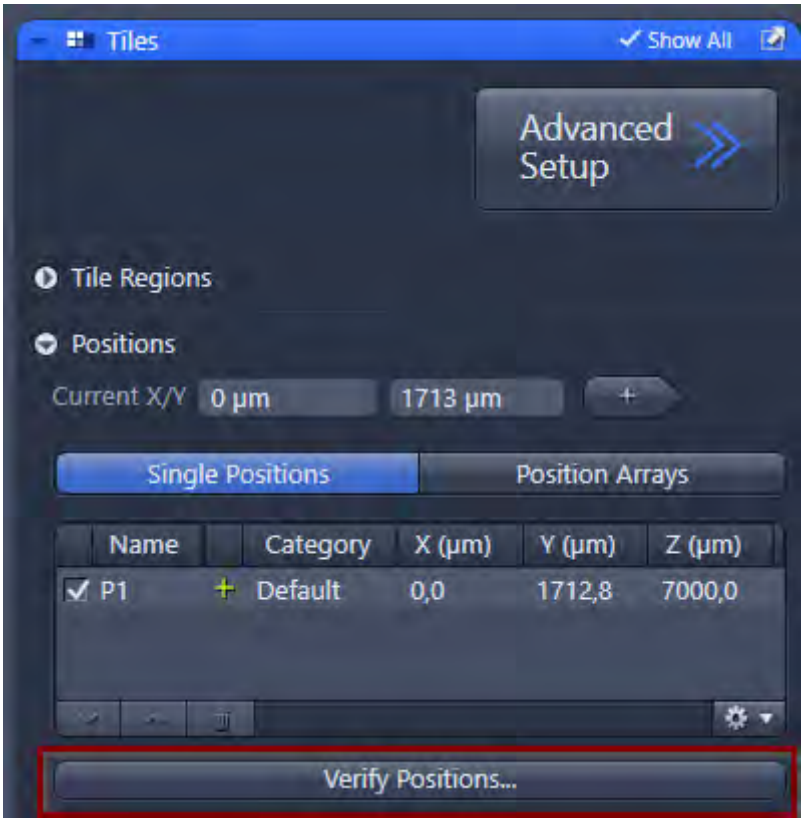
3 Use the **Live** mode to check the Z-position of the position.

4 To adjust the Z-value, set the desired position using the focus drive.

5 In the **Single Positions** list click in the bottom right on the **Options** button and select **Set Current Z For Selected Positions**.

6 To check and adjust a large number of **positions**, use the **Verify Positions** dialog.

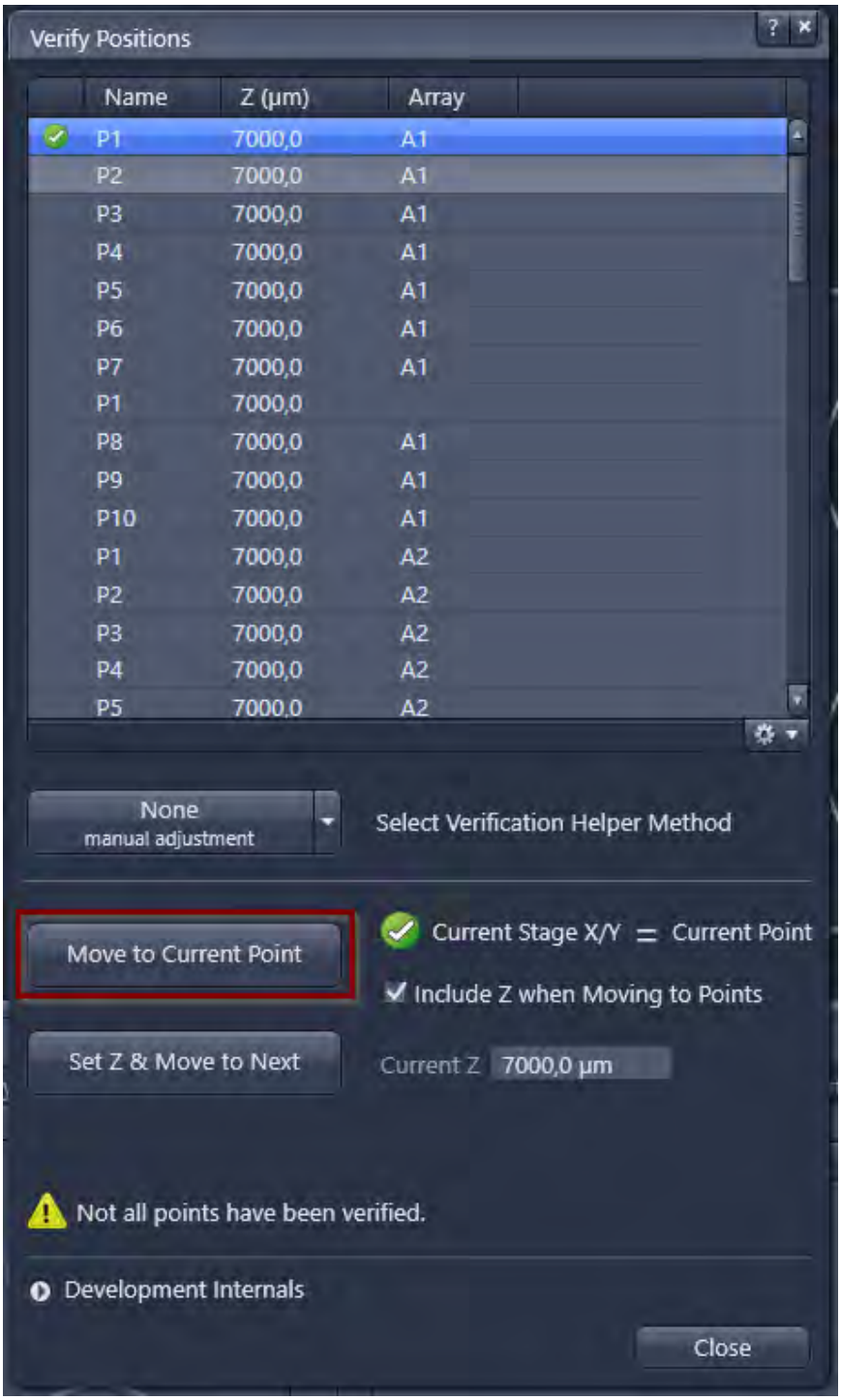
7 To do this, click on the **Verify Positions…** button in the **Positions** section.



The **Verify Positions** dialog opens.

8 Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are Autofocus (AF) and Definite Focus (DF). If you have neither then you can only adjust z-values manually.

9 Click on the **Move to Current Point** button.



The stage moves automatically to the position in the list that is highlighted in blue. Alternatively, you can double-click on the position in the list that you want to check.

10 In the **Live** mode use the **Focus** tool (or SW Autofocus) to adjust the desired Z-value.

11 Click on the **Set Z and Move to Next** button.

The position is marked with a check mark.

The stage moves automatically to the next position in the list.

12 Repeat the last 3 steps until you have checked all the points in the list.

The message **All points have been verified** appears.

13 Close the **Verify Positions** dialog.

You have successfully verified and adjusted the individual Z-values for positions.

12.9 Local and Global Focus Surfaces

12.9.1 Introduction

Local Focus Surface

To acquire large tile regions on tilted or uneven specimens, you need to assign individual Z-values to each tile of a tile region. Therefor you need to create a **Local Focus Surface**. Note that a local focus surface is always associated with precisely one tile region. You therefore need to create a focus area separately for each tile region.

Global Focus Surface

To create a focus area covering the entire sample, you need to create a **Global Focus Surface**. Global focus surfaces are based on a sample carrier template (e.g. for slides or multiwell plates) and result in a focus surface that is valid for the entire sample carrier and therefore for all the tile regions and positions it contains. This allows you to compensate for any tilting and bending of the sample carrier.

12.9.2 Creating a Local Focus Surface

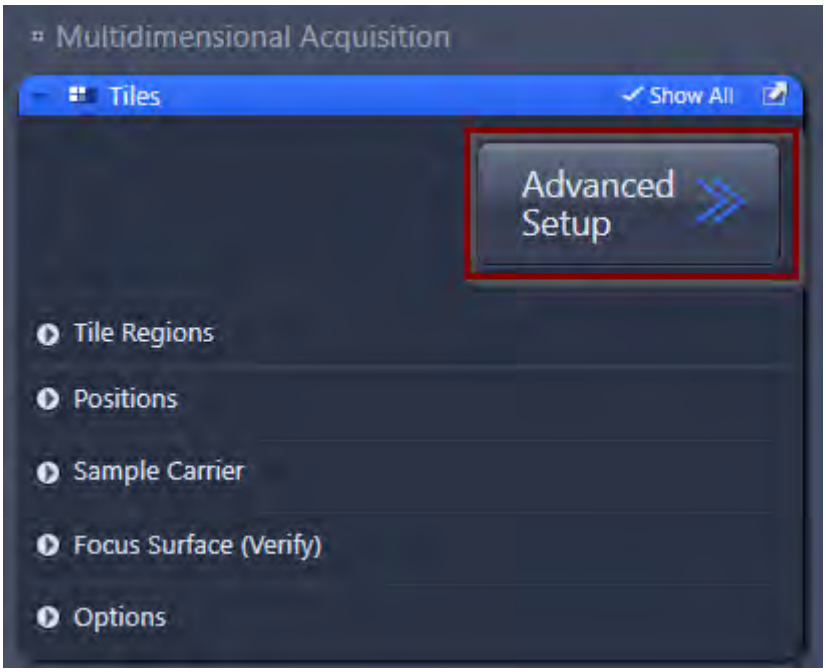
To create local focus surfaces, you must distribute support points across your tile regions and assign their focus position. Tile-region-specific focus areas are then interpolated from the values of these support points.

12.9.2.1 Distributing Support Points

Prerequisites

- To create a local focus surface you will need the **Tiles** module.
- You have set up a **Tiles** experiment with at least one tile region. For more information on this, see *Setting Up a Simple Tiles Experiment* [▶ 302].
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure **1** Click on the **Advanced Setup** button.



Advanced tile setup is opened.

2 Select a tile region for which you want to create support points. To do this, click on the corresponding tile region in the list in the **Tile Regions** section of the **Tiles** tool.

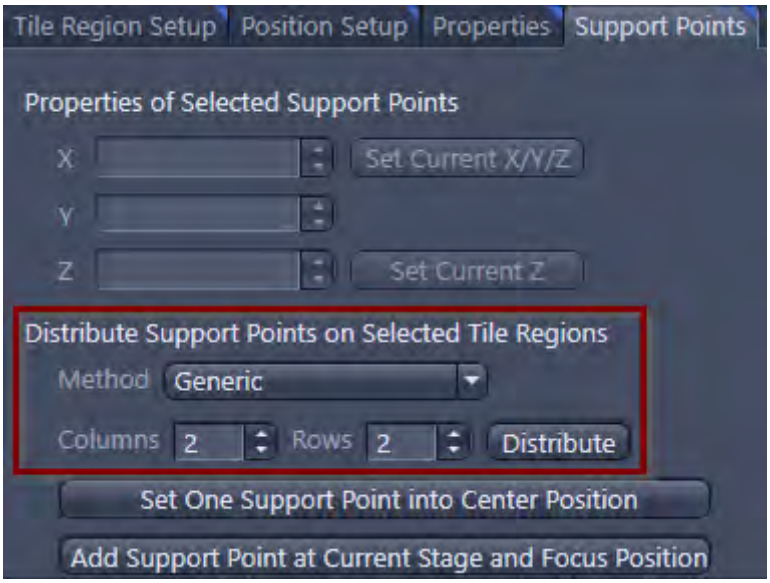
INFO

Alternatively, you can select tile regions by clicking directly on the desired tile region in the **Advanced Setup** view. Both methods allow you to select several tile regions simultaneously by holding down the **Ctrl** key.

3 Select the **Support Points** tab from the **Tiles - Advanced Setup** view options.

4 Under **Distribute Support Points on Selected Tile Regions**, indicate the number of columns and rows for the distribution of the reference points. Alternatively for larger Tile regions (>200 Tiles) you can use the distribution method Onion Skin. Depending on the total size and shape you might need to adjust the density parameter and/ or the maximum number of support points

to optimize the result. Typically, this method works best with large irregular or rounded Tile Regions.



5 Click on the **Distribute** button.

The support points are distributed within the tile region selected and shown as yellow points in the  stage view.

The support points of the selected tile region are displayed with their coordinates in the **Local (per Tile Region)** list in the **Focus Surface** section of the **Tiles** tool.

6 If necessary, you can adjust the distribution of the support points manually in the **Advanced Setup**. You can change the position of the support points using drag & drop.

7 Additional, individual support points can be added by using the stage to locate the desired position and clicking on the **Add Support Points At Current Stage and Focus Position** button on the **Support Points** tab.

i INFO

Distribute the support points evenly across your **tile region**. The more irregular the surface of your specimen, the more reference points you should set. An even but tilted surface requires at least 4 reference points for a solid calculation, while a simple saddle surface requires at least 9 reference points. A high reference-point density leads to a more precise result, although the maximum useful density is one reference point per tile. The generic method follows a simple grid pattern to place the support points. It works well on regular rectangular tile regions smaller in size. For larger (>200 tiles) tile regions the Onion skin will likely provide better results. In some cases some trial and error might be needed to optimize the parameters.

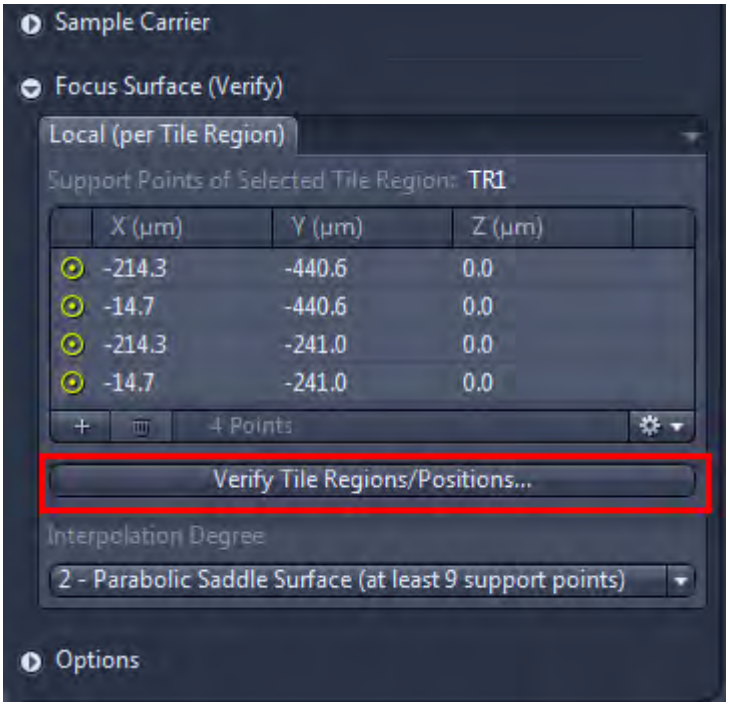
8 Repeat steps 2 to 6 until you have distributed reference points across all desired tile regions.

You have successfully distributed support points across the tile regions.

12.9.2.2 Verifying Z-Values of Support Points

Procedure

1 Click on the **Verify Tile Regions/Positions...** button in the **Focus Surface (Verify)** section of the **Tiles** tool.



The **Verify Tile Regions / Positions** dialog opens.

2 Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are Autofocus (AF) and Definite Focus (DF). If you have neither then you can only adjust z-values manually.

3 Click on the **Move To Current Point** button.

The stage moves automatically to the support point that is highlighted in blue in the reference point list. Alternatively, you can also double-click on the support point in the list that you want to check.

4 In the **Live** mode use the **Focus** tool to adjust the Z-value (or SW Autofocus).

5 Click on the **Set Z and Move to Next** button.

The checked reference point is marked with a green check mark.

The stage moves automatically to the next support point in the list.

6 Repeat the last 3 steps until you have checked all the support points.

The message **All points have been verified** appears.

7 Close the **Verify Tile Regions/Positions** dialog.

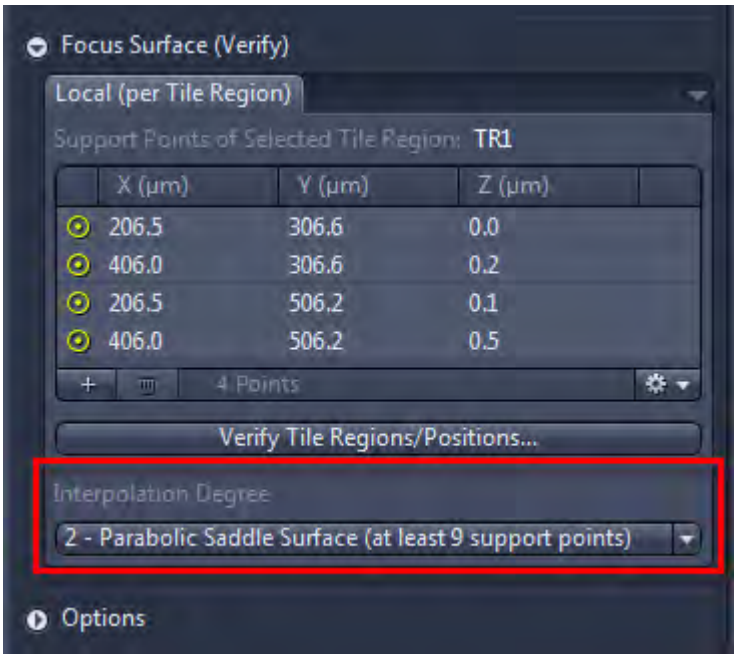
You have adjusted and verified the Z-values of all support points.

INFO

Positions always have a horizontal local focus surface, which is determined by the Z-value of the position. If you use positions in addition to tile regions, you can verify the Z-values of the positions with the help of a similar dialog. Open this dialog by clicking on the **Verify Positions...** button in the **Positions** section of the **Tiles** tool.

12.9.2.3 Selecting Interpolation Degree

Procedure **1** Select the interpolation level in the **Interpolation Degree** dropdown list in the **Focus Surface** section.



INFO

The minimum number of support points necessary per tile region is indicated in the **Interpolation Degree** dropdown list for each entry. The calculation is more solid if the number of support points exceeds this minimum number. We therefore recommend that you only increase the interpolation degree as far as the surface of the sample demands, even if you have set more support points. If the number of support points does not correspond to the minimum number for the selected interpolation degree, the interpolation degree will be reduced automatically.

You have successfully created a local focus surface. You can now start the experiment. To ensure that the tiles are acquired along the focus surface the software automatically selects the most appropriate focus strategy. For more information on focus strategies read the chapter *Working with Focus Strategies* [▶ 71] in the ZEN Online Help.

12.9.3 Creating a Global Focus Surface

To create a global focus surface, you must distribute support points across your sample carrier and indicate their focus position. A focus area across the sample carrier is then interpolated from the values of these reference points.

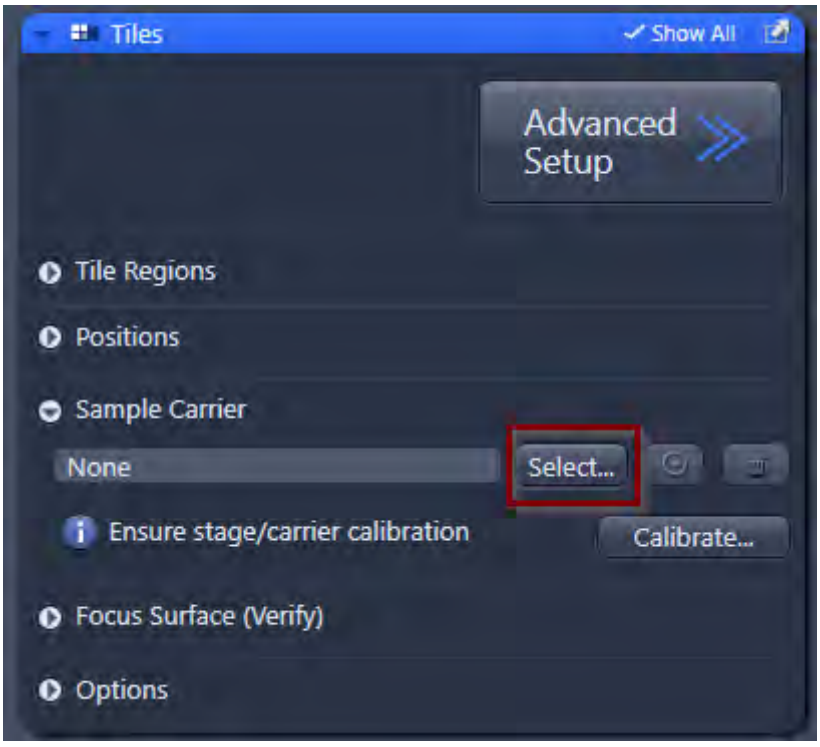
12.9.3.1 Distributing Support Points

Prerequisites

- You have configured the general settings for setting up a tile experiment (experiment created, at least one channel defined, Tiles dimension activated).
- To create a global focus surface, you will need the **Tiles** module.
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1. Open the **Sample Carrier** section.
2. Click on the **Select...** button.

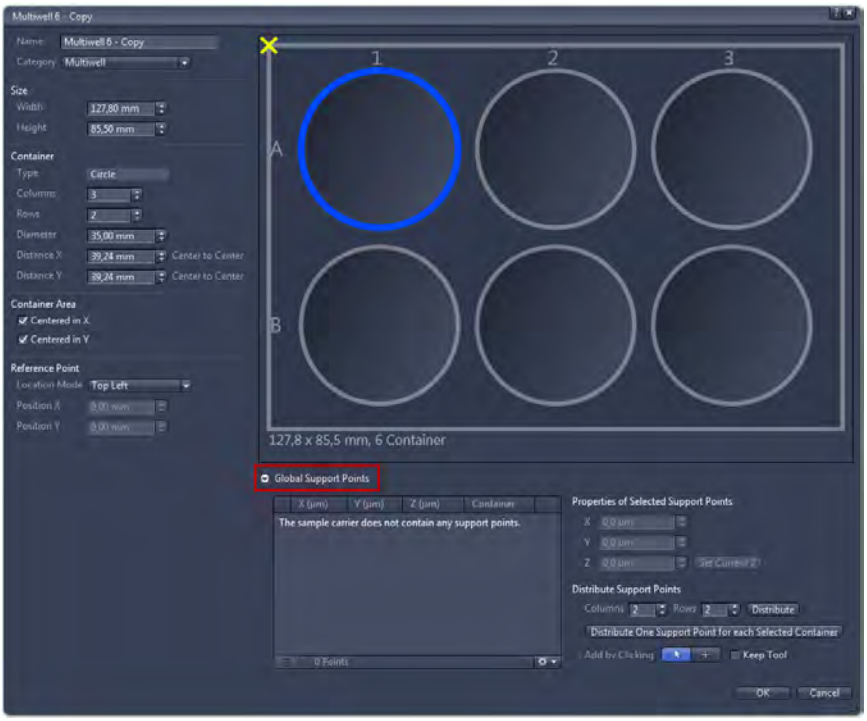


   The **Select Template** dialog opens.

3. Select the sample carrier template that you want to use.
4. Click on the **Options** button and select the **Copy And Edit...** entry.

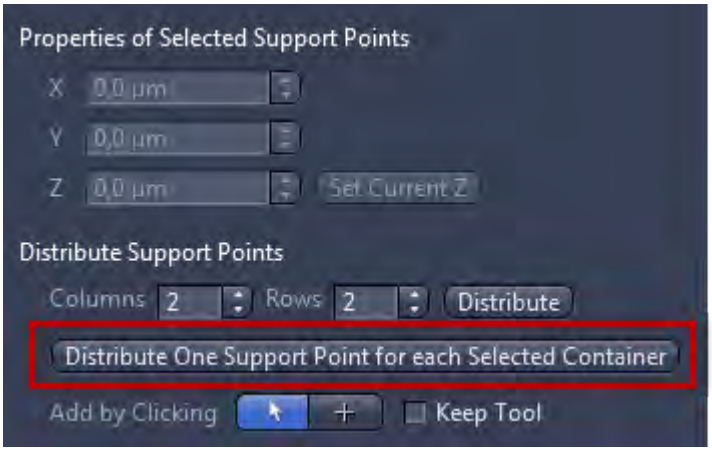
A copy of the existing template is generated and opened in the Sample Carrier Editor.

5 To distribute support points across the sample carrier template, open the **Global Support Points** section.



6 Select the containers in which you wish to create global support points. To do this, hold down the **Ctrl** key and click on the containers.

7 Click on the **Distribute One Support Point For Each Selected Container** button.



One support point is assigned to each container selected.

i INFO

If you use a sample carrier without containers (e.g. slide), use the **Distribute** button instead, to distribute support points on the basis of columns and rows.

The support points are distributed automatically across the sample carrier.

You can add further support points manually using the **Add** button  .

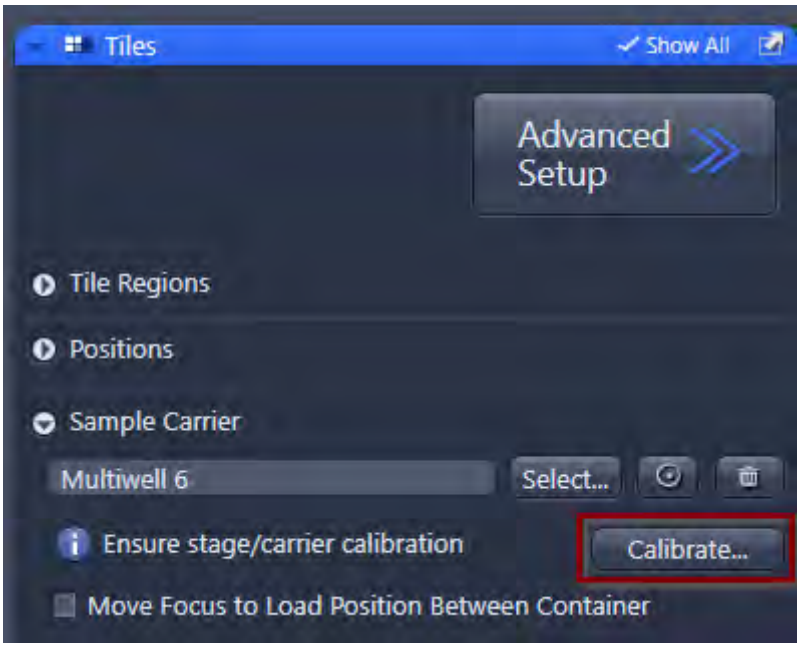
> **INFO**
>
> Only create support points where you can bring the sample into focus (within the containers). This is the only way that you can determine and setup the Z-values of the support points later. The assignment of container-based global support points to the center of the container is fixed i.e. they cannot be moved. If the surface of your sample carrier is tilted but even, you will need at least 4 support points for a solid calculation. The more irregular the surface, the more support points you should distribute.

8 To close the **Editor** window, click on the **OK** button.

9 To select the edited sample carrier template, click on the **OK** button.

If you wish to re-edit the global support points at any time click on the **Edit** icon  to open the sample carrier editor again.

10 Calibrate the sample carrier by clicking on the **Calibrate…** button and following the wizard.



You have successfully distributed support points across a sample carrier template and have selected and calibrated it.

12.9.3.2 Verifying Z-Value of Support Points

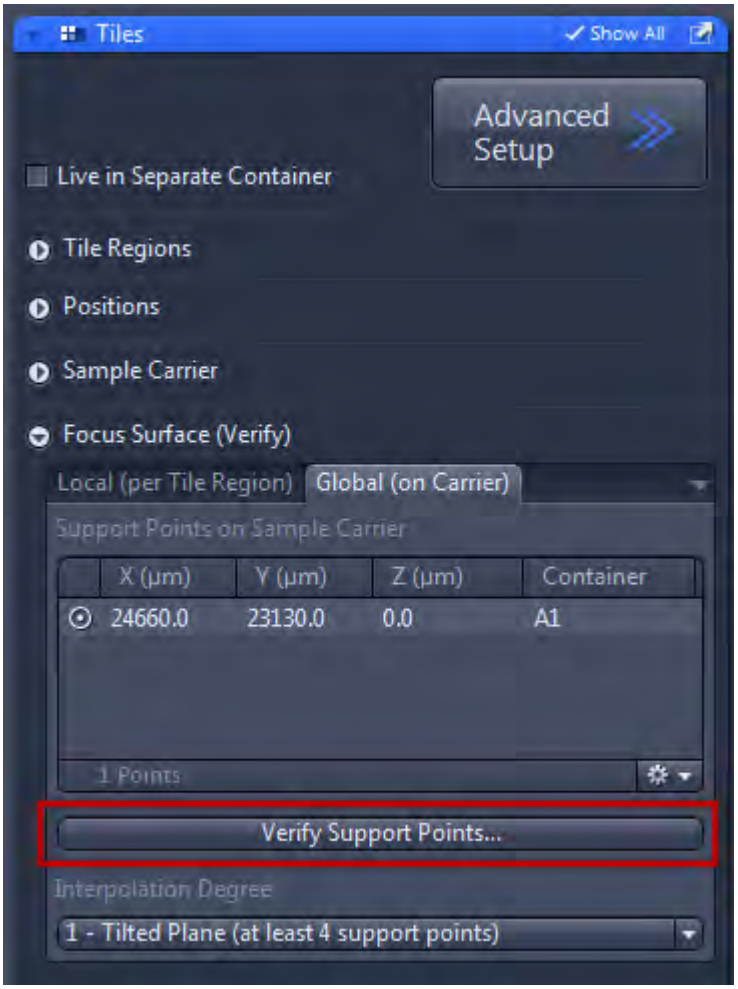
Procedure

1 In the **Tiles** tool open the **Focus Surface** section.

2 Go to the **Global (on Carrier)** tab.

All the support points of the selected sample carrier template are displayed in the **Support Points on Sample Carrier** list.

3 Click on the **Verify Support Points…** button.



The **Verify Global Support Points** dialog opens.

4 Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are Autofocus (AF) and Definite Focus (DF). If you have neither then you can only adjust z-values manually.

5 Click on the **Move To Current Point** button.

The stage moves automatically to the support point that is highlighted in blue in the list. Alternatively, you can also double-click on the support point in the list that you want to check.

6 In the **Live** mode use the **Focus** tool (or SW Autofocus) to set the Z-value.

7 Click on the **Set Z and Move to Next** button.

The support point is marked with a check mark.

The stage moves automatically to the next support point in the list.

8 Repeat the last 3 steps until you have checked all the support points.

The message **All points have been verified** appears.

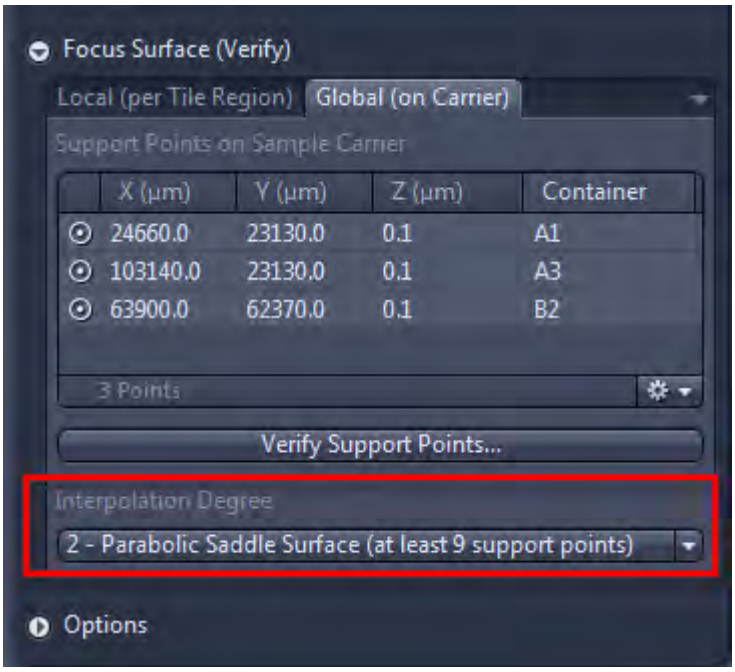
9 Close the **Verify Global Support Points** dialog.

You have adjusted and verified the Z-values of all support points.

12.9.3.3 Selecting Interpolation Degree

Procedure

1 Select the interpolation degree in the **Interpolation Degree** dropdown list in the **Focus Surface (Verify)** section.



INFO

The minimum number of support points necessary is indicated in the **Interpolation Degree** dropdown list for each entry. The calculation is more solid if the number of support points exceeds this minimum number. We therefore recommend that you only increase the interpolation degree as far as the surface of the carrier demands, even if you have created more support points. If the number of support points does not correspond to the minimum number for the selected interpolation degree, the interpolation degree will be reduced automatically. Interpolation degree **1 – Tilted Plane (at least 4 support points)** is sufficient to compensate for any tilting of the sample carrier.

You have successfully created a global focus surface.

You can now set up your tile experiment using the sample carrier. Further information on this can be found under: *Using Sample Carriers* [▶ 339]. To ensure the tiles are acquired along the focus surface during the experiment the software automatically selects the most appropriate focus strategy in the **Focus Strategy** tool. For information on focus strategies read the chapter *Working with Focus Strategies* [▶ 71] in the ZEN Online Help.

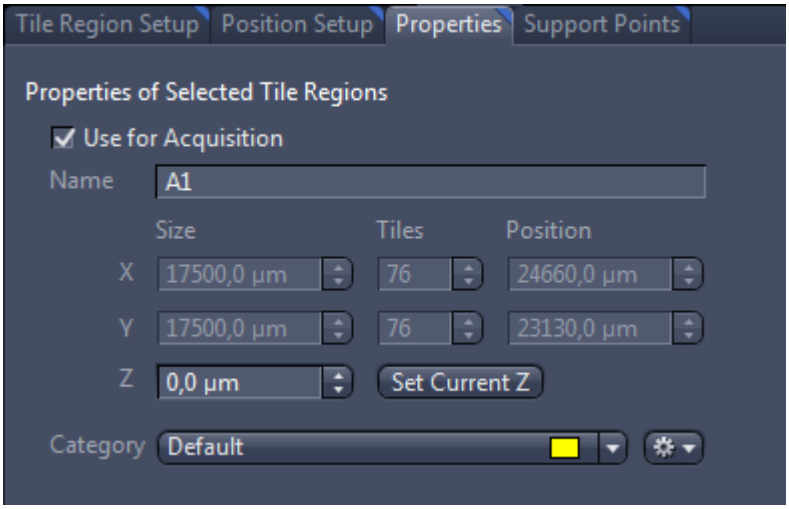
12.10 Assigning Categories to Tile Regions and Positions

In some cases it can be helpful to not only display the well number together with the acquired images (Path: **Graphics | Frequent Annotations | Carrier Container Name**) but also to create certain additional annotations for different tile regions or positions, e.g. "control condition" or "experimental condition 1".
For that purpose, the software allows you to add and edit names and categories to the different Tiles Regions/ Positions that have been generated.

Procedure

1 In order to assign individual names to different individual positions and/or tile regions in a well plate experiment, click on the respective Tile Region or Position and open the **Properties** tab in the Center Screen Area (Tiles – Advanced Setup).

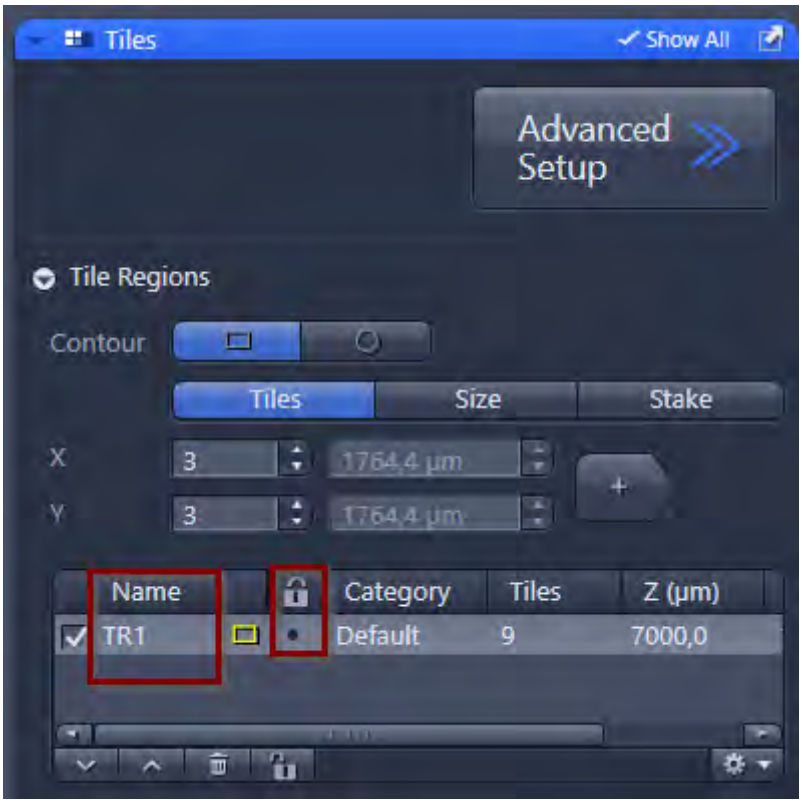
2 Activate the **Use for Acquisition** checkbox.



3 Edit the **Name** for your selected Tile Region/ Position.

4 Alternatively, edit the **Name** of a Tile Region/ Position by clicking in **Tiles** module on the respective name under **Tile Regions** or **Positions**. The lock is used for tiles and positions that are created in the carrier mode. It prevents

them from being edited if one switches the carrier mode off, and can then be toggled by the users as needed.



5 Repeat this step to rename different Tile Regions or Positions.

INFO

To display the name of your Tile Region/ Position later in your acquired image(s), go to **Graphics** | **Frequent Annotations** | **More...** and select **Image.Scene.Name** from the Metadata list.

6 To assign or edit categories of your Tile Regions/ Positions, first activate the checkbox of all desired Tile Regions/ Positions that should be grouped in the same category.

7 In the **Properties** tab click under **Category** on the **Options** button.

8 Select **New...** from the dropdown list

The **New Category** window opens.

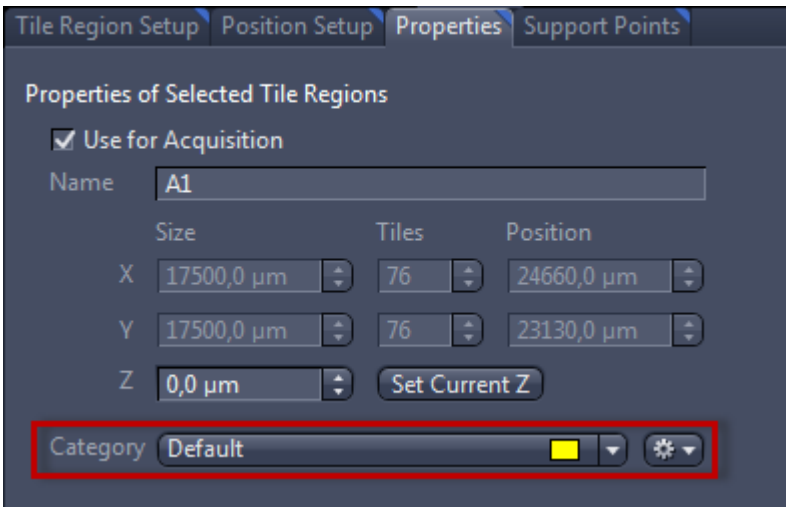
9 Enter a **Name** and add a **Description** for the selected Tile Regions/ Positions.

10 Assign a **Color** for the new category by clicking on the color bar and choosing a preferred color.

11 Click on **OK** to create the new category.

The **New Category** window closes and the new category is created.

12 Under **Category** choose the desired category for the selected Tile regions/ Positions from the drop down list.



The chosen category is now assigned to the selected Tile Regions/ Positions.

INFO

Note that a predefined category can also be applied to a differentiated selection of Tile Regions/ Positions from more than one well.
Note also, that the assigned color is only used as a feature in the Tiles tab (Left Tool Bar Area).

INFO

To display a Tile Region/ Position Category feature (**Name** and/or **Description**) in your acquired image, you go to **Graphics** | **Frequent Annotations** | **More…**.

Type "category" in the search bar and select the desired feature to be displayed. (Although the option "Color" is given, no reasonable element will be displayed by the software)

INFO

To adjust parameters of your annotations (e.g. font size), right-click on it and go to **Format** | **Graphical Elements**.

Displaying categories in the Tiles/ Positions List (Left Tool Area)

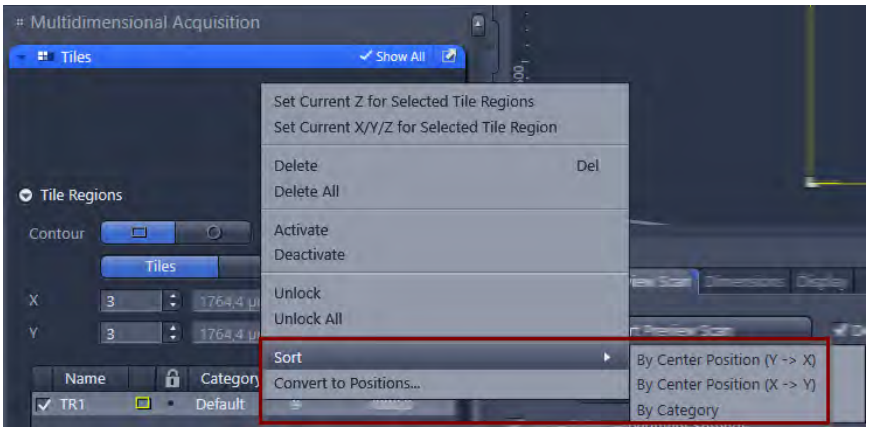
Prerequisites

- You have selected several different positions or tile regions and assigned different categories.

Procedure

1 Under **Positions** or **Tiles** of the Tiles module (Left Tool Area) select a position or tile region.

2 Right-click on the selected position/ tile, choose **Sort** and select **By Category**



The positions/ tiles will be sorted alphabetically according to the assigned categories.

12.11 Re-positioning Sample Carrier after Incubation

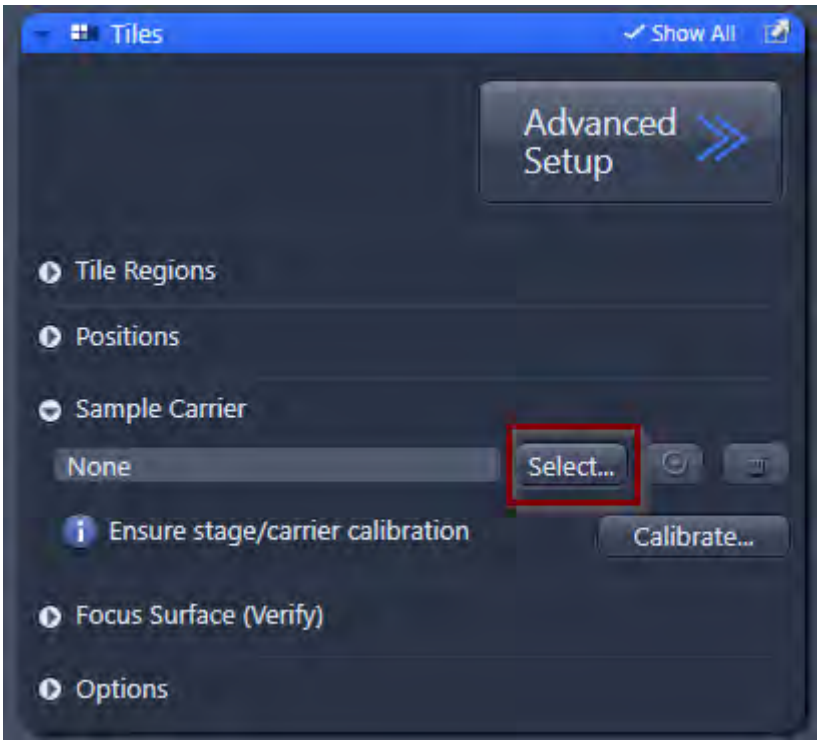
When you want to take images of positions/ tile regions on a sample carrier, that has to be taken off the stage, e.g. for incubation purposes or changes of the immersion medium, proceed as follows to re-position your sample carrier.

Starting the Experiment

Prerequisites

- You have run the stage calibration and have located your sample, see chapter *Calibrating Stage and Selecting Channel* [▶ 296].
- You have set up at least one channel and adjusted the light/ camera exposure time.
- You have activated the **Tiles** checkbox and the **Show All** mode

Procedure **1** Click on **Sample Carrier** and then click **Select**.



The **Select Template** dialog opens.

2 Select the template of choice.

INFO

For demo purposes, select a standard slide that can mimic your test sample.

INFO

Regarding calibration of your template, you can customize your own carrier see the chapter *Customizing a Sample Carrier Template* [▶ 341], but for slides with one coverslip or well, there is only the option for Single Reference Point Calibration. For Multi-well plates, you will have the option for 7-point, 4-point, 3-point or 1-point calibration. This becomes important for adjusting for the rotation of the sample.

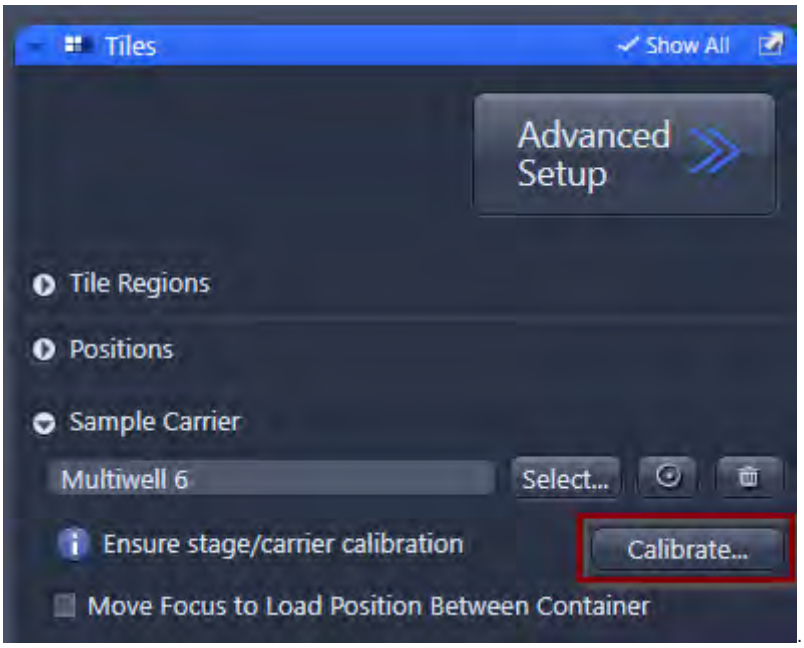
3 Adjust the surface of the sample carrier. Refer to the chapter *Creating a Global Focus Surface* [▶ 325].

The **Select Template** dialog closes.

INFO

In the following it is assumed that you just use a conventional glass slide with some cells or tissue that is positioned in the center

4 Press **Calibrate**

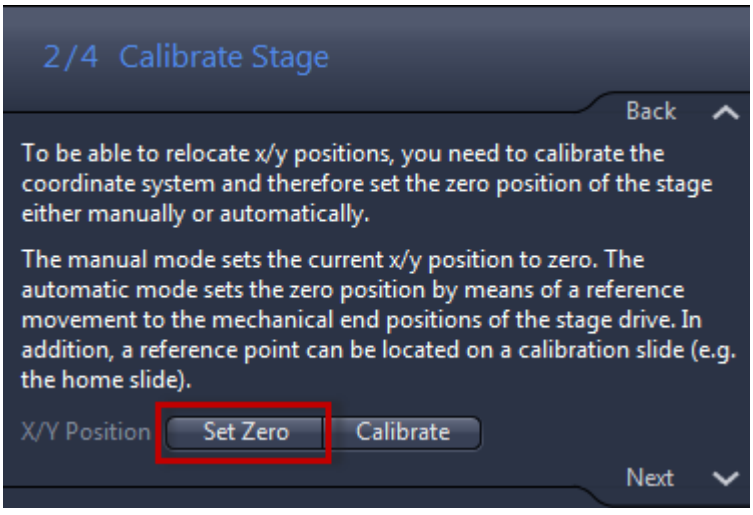


The **Sample Carrier Calibration Wizard** opens.

5 Move a sample reference point into the middle of the crosshairs. This reference point can be any unique identifiable point on the slide and does not have to in the middle of the slide.

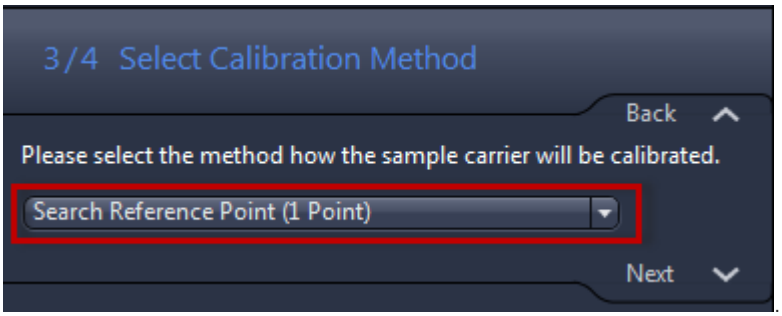
6 Click on **Next**.

7 Under **X/Y Position** click on **Set Zero**.



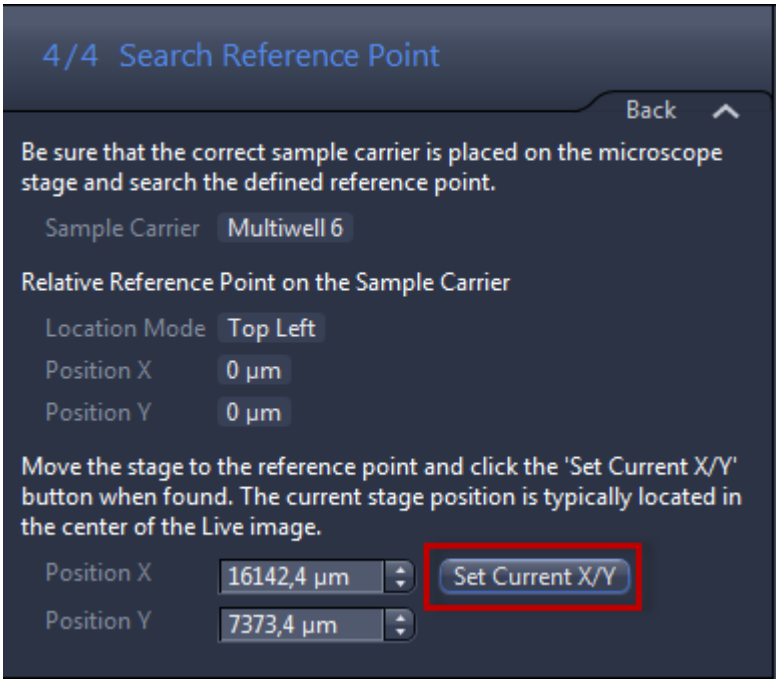
8 Click on **Next**.

9 As Calibration Method, select **Search Reference Point**



10 Click on **Next**.

11 Click on **Set Current X/Y**.



12 In the **Tiles** tool click on **Advanced Setup** and add positions/tile regions at your locations of interest.

i INFO

You can zoom in and out using the mouse scroller, and move the stage in the Center Screen Area to a point of interest with a double-click on the sample carrier.

13 Once you have defined all your positions/tile regions, click the  **Options** button on the **Acquisition** tab to in the **Experiment Manager**.

14 Save your experimental settings, including the lists of positions/tile regions, by selecting either the **Save As** or **Export** entry.

INFO

With **Save As** the settings will be saved directly in the Experiment Manager. With **Export** the settings will be saved in a folder of your choice.

15 Start your experiment and record images from your selected positions/ tile regions.

16 Remove your sample off the stage and e.g. put it back into the incubation chamber.

17 Close the software.

You have done all settings for a successful re-positioning of your sample carrier after the experiment.

Re-Positioning of the Sample Carrier after the experiment

INFO

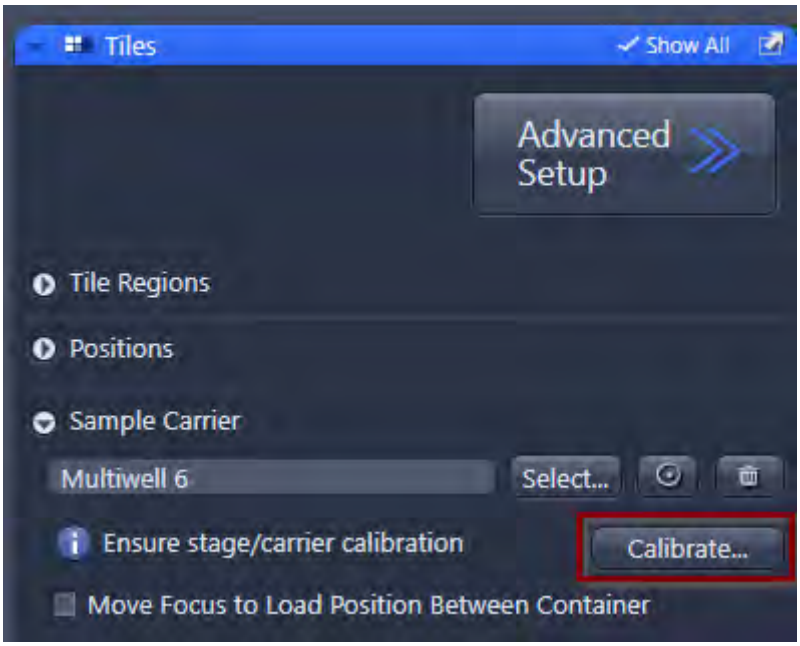
If you cycle the power on the microscope the software will prompt you to calibrate the stage and/or focus drives. Thus, if the calibration of the multi-well plate was performed under the same conditions then the sample carrier calibration will still be valid. You must however, ensure that other parameters like plate orientation and placement on the microscope have not changed.

Procedure

1 Restart the software.

2 In the **Acquisition** tab go to the **Experiment Manager** and **Reload** or **Import** your experimental settings including your list of positions/tile regions.

3 In **Tiles** tool under **Sample Carrier** click on the **Calibrate** button.

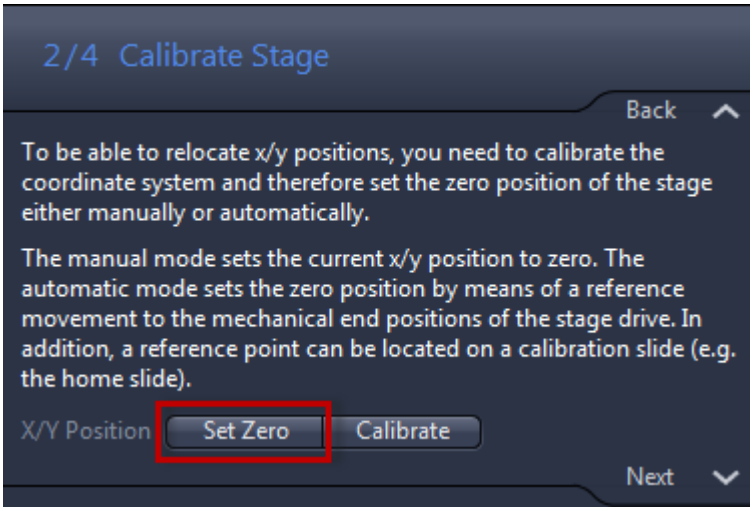


The **Sample Carrier Calibration Wizard** opens.

4 Move your previously chosen sample reference point into the middle of the crosshairs.

5 Click on **Next**.

6 Under **X/Y Position** click on **Set Zero**.

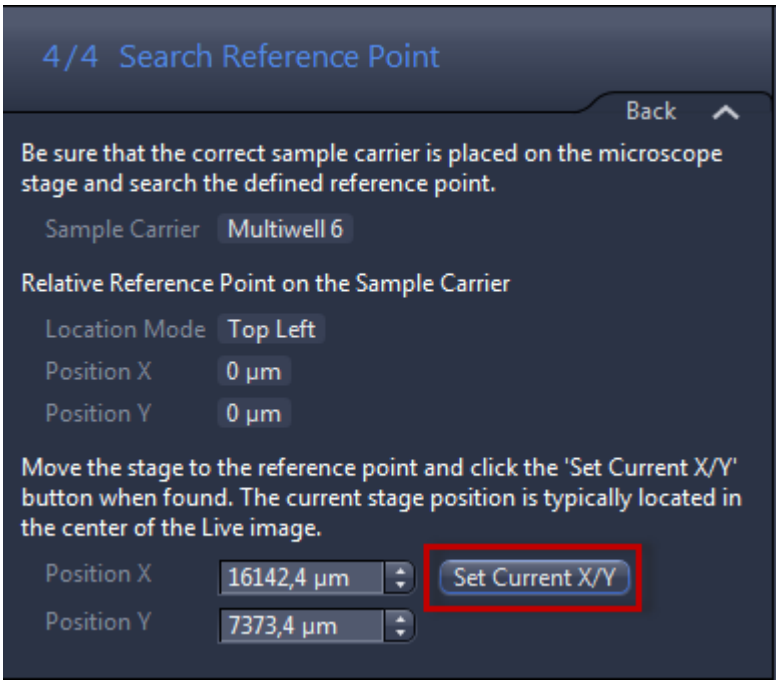


7 Click on **Next**.

8 As Calibration Method, select **Search Reference Point**.

9 Click on **Next**.

10 Click on **Set Current X/Y**.



11 Now, you still need to verify the Z-offset of your positions. Therefore, follow the corresponding instructions given in the chapters *Adjusting Z-Values of Tile Regions* [▶ 317] and *Adjusting Z-Values of Positions* [▶ 317].

All of your selected positions/tile regions are now re-assigned to the correct X/Y/Z-values in relation to your (unique identifiable) reference point.

You can re-start your experiment and record images from your selected positions/tile regions.

12.12 Using Sample Carriers

Use a sample carrier template to display the size and appearance of your sample carrier (e.g. slide or multiwell plate) in **Advanced Setup**. This allows you to distribute tile regions or positions easily across your sample carrier.

12.12.1 Selecting a Sample Carrier Template

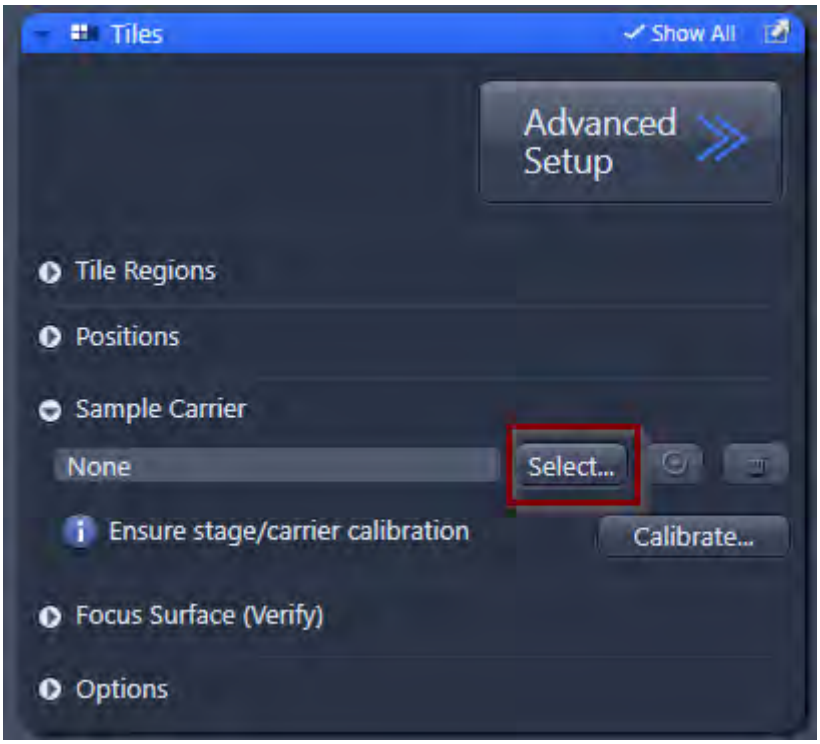
Prerequisites

- You have configured the general settings for setting up a tile experiment (experiment created, at least one channel defined, Tiles dimension activated).
- You are on the **Acquisition** tab in the **Tiles** tool.

Procedure

1 Open the **Sample Carrier** section.

2 Click on the **Select...** button.



The **Select Sample Carrier Template** dialog opens.

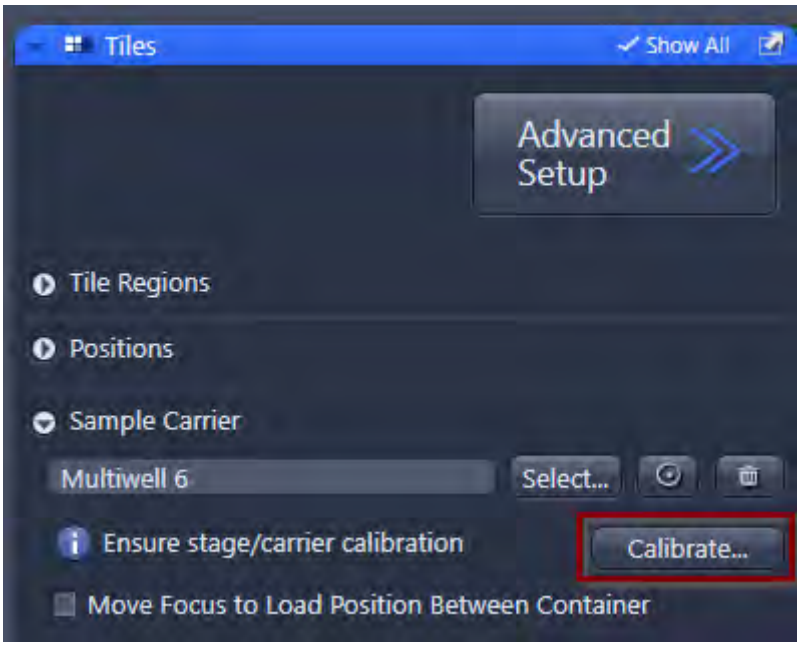
3 Select an existing **sample carrier template** or generate a new template by clicking on the + button.

INFO

You can display or edit the **user templates** by clicking on the button and selecting the **Display/Edit…** entry. **Zeiss templates** cannot be edited. To create an editable user copy, select the **Copy And Edit…** entry.

4 To close the dialog, click on the **OK** button.

5 Calibrate the sample carrier by clicking on the **Calibrate...** button.



The **Sample Carrier Calibration Wizard** opens.

6 Follow the wizard until you have fully calibrated the sample carrier.

The information **The sample carrier is calibrated** appears in the **Sample Carrier** section.

INFO

Note that the calibration values are stored in the experiment and can be re-used if you work with absolute coordinates by calibrating the end stops of the stage and focus drives and no other changes are made to the hardware set-up. This can save considerable time when you want to work repeatedly with the same sample carrier model and acquisition regime.

You have successfully selected a **sample carrier**.

12.12.2 Customizing a Sample Carrier Template

If you want work with a sample carrier that is not listed in the template database, you will need to apply the following workflow in order to create a new template.

Prerequisites

- You have done all prerequisites for a Tiles & Positions experiment
- You have defined at least one channel.
- You have activated the **Tiles** checkbox.

Procedure

1 Go to the **Acquisition** tab.

2 Open the **Tiles** tool and activate the **Show All** mode.

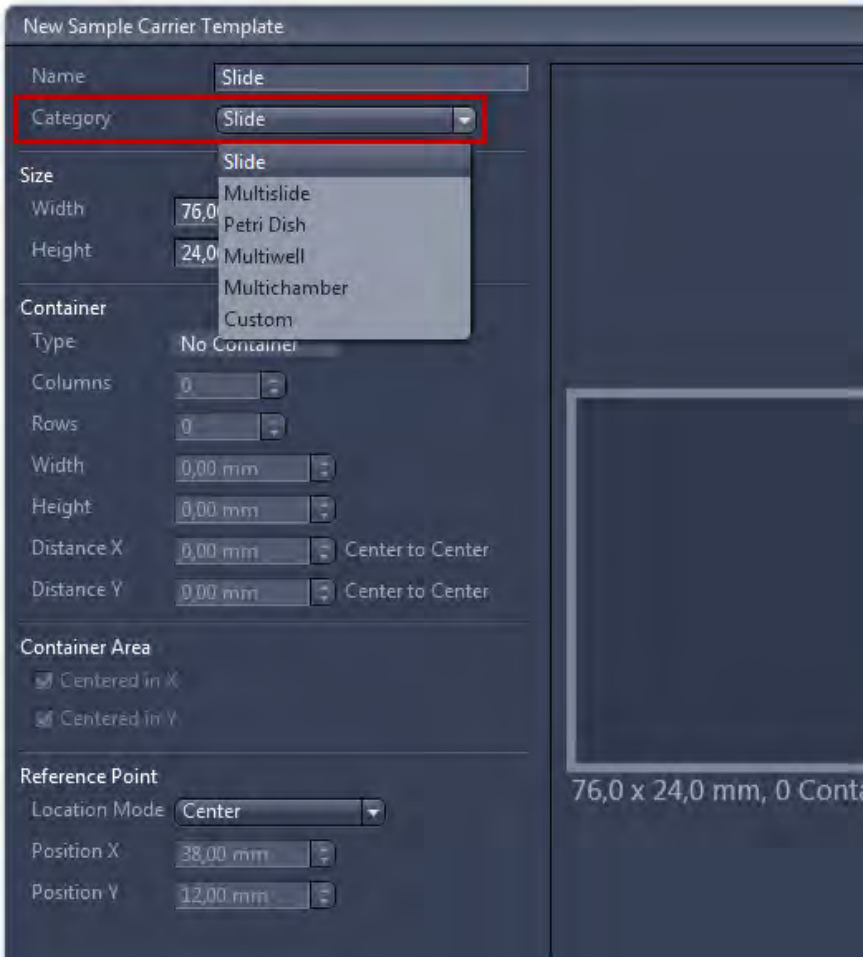
3 Open the **Sample Carrier** section and click on the **Select…** button.

The **Select Template** dialog opens.

4 Click on the **Options** button and choose **New Template…**.

The **New Sample Carrier Template** dialog opens.

5 Choose a **Category** that corresponds to the type of your carrier and assign a **Name** to your template.



INFO

Corresponding to the **Category** you can choose different parameters for the template.

6 For example if you select **Slide**, you can now configure the **Width**and **Height** of the slide and adjust the location of a **Reference Point**.

7 If you select a **Multislide**, **Petri Dish**, **Multiwell** or **Multichamber** template, you can configure and adjust additional parameters of your carrier.

8 In case you need to modify one of the above depicted templates even further, first select the **Category** that appears closest to your carrier, go again to the **Category** tab and then choose **Custom**.

i INFO

If you select **Custom** additional predefined options of the template will be made accessible for further modification.

You have customized a sample carrier template or set up a custom one.

12.13 Exporting Tile Images

Prerequisites

- You have acquired or opened a tile image.

Procedure

1 On the **Processing** tab, open the parameters for **Image Export** (*Ctrl+6* or via the **File** menu | **Export/Import** | **Export**).

You will see the settings of the parameters for **Image Export**. Make sure that the **Show All** mode has been activated.

2 Select the file type that you want to use. We recommend the **PNG** format, as this is a format that offers lossless compression with an acceptable file size.

i INFO

Type and source of risk

In the case of particularly large tile images, we recommend that you reduce the size of the images you want to export. To do this, set a percentage in the **Resize** slider by which you want the images for export to be reduced, e.g. by 25%.

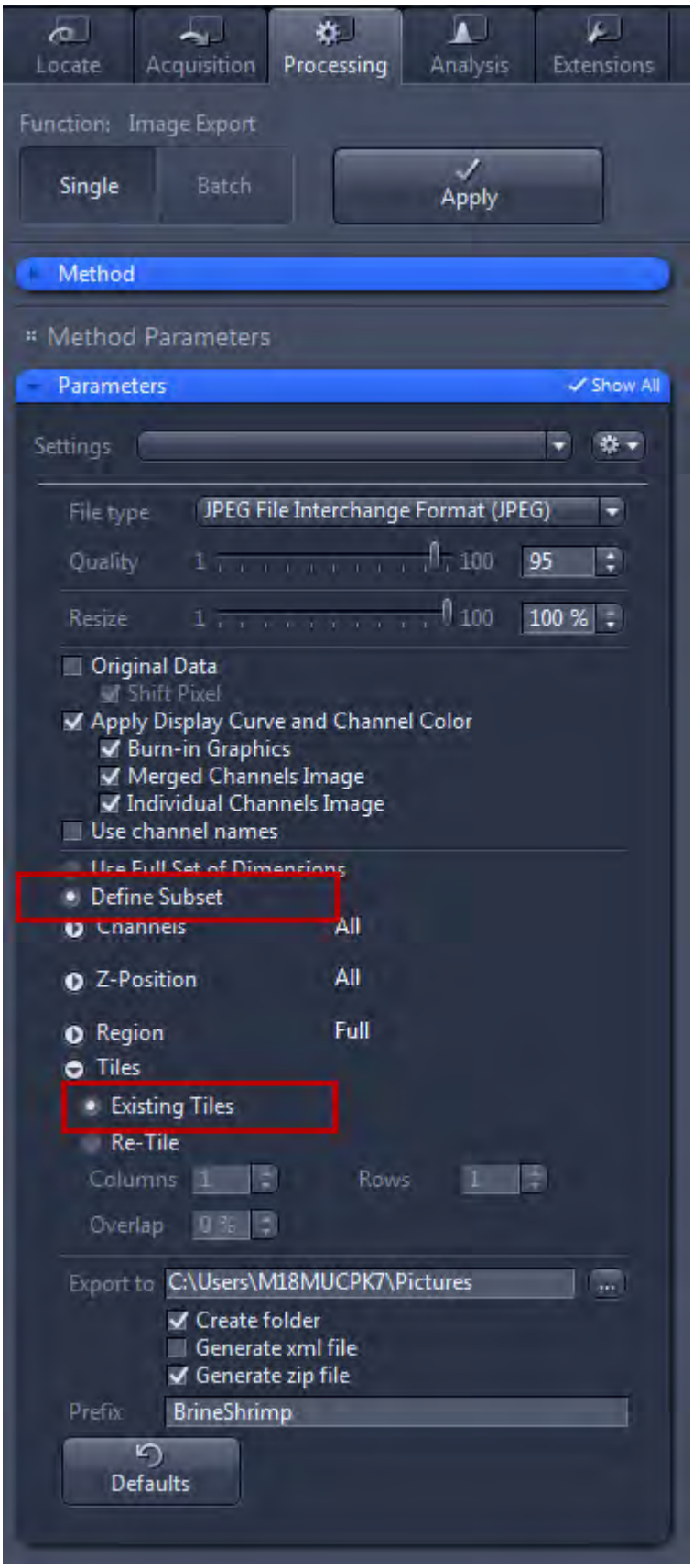
3 Activate the **Apply Display Curve and Channel Color** checkbox. This means that the images will be exported with the settings you have made, e.g. tonal value corrections or contrast. If you activate the **Original Data** checkbox, the images are exported unchanged. In this case, the settings from the display curve, e.g. tonal value corrections and contrast, are not adopted.

4 Select the **Define Subset** radio button.

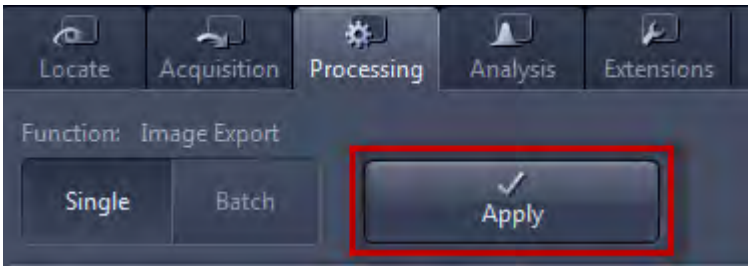
The settings for the available dimensions open.

5 Open the settings for the **Tiles** dimension.

6 Select the **Existing Tiles** radio button.



7 Click on the **Apply** button at the top of the **Processing** tab.



You have exported the individual tiles from a tile image. The files can be found in the export folder indicated.

12.14 Functions and Reference

12.14.1 Tiles Tool

INFO

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**.

In the **Tiles** tool you configure the acquisition of images that consist of several image fields. Therefore you define Tile Regions or Positions. In addition you can set up Focus Surfaces and Sample Carrier Templates here.

The Tiles tool is located in the **Left Tool Area** under **Multidimensional Acquisition**.

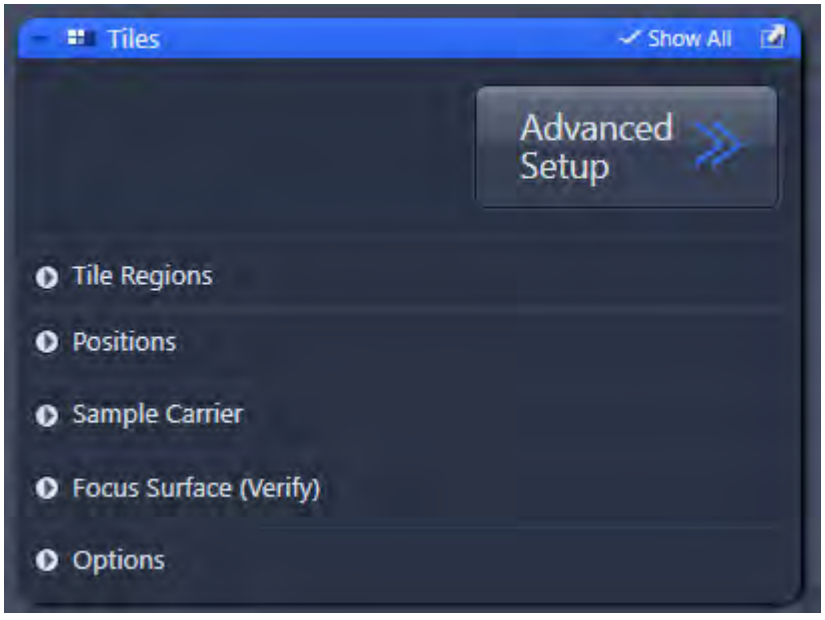


Fig. 12.1: Tiles Tool

Parameter	Description
Advanced Setup	Opens the *Tiles Advanced Setup* [▶ 359] view in the **Center Screen Area**.

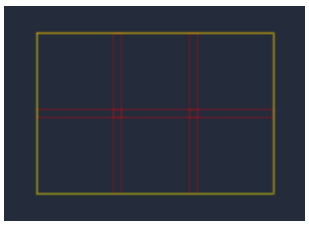
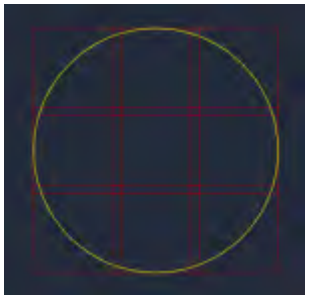
INFO

The **Sample Carrier**, **Focus Surface** and **Options** sections are only visible if the **Show All** mode is activated.
If you have no license for the **Tiles** module you will only find **Tile Regions**, **Positions** and **Options** sections here.

The different sections of the tool are described in the next chapters.

12.14.1.1 Tile Regions Section

Here you can define the desired tile regions and add it to the image.

Parameter	Description
Contour	This parameter is only visible if the **Show All** is activated. Here you select the shape or contour of the tile region that you are adding. Simply click on the corresponding button to select the desired contour. The selected contour is highlighted in blue color.
- Rectangle	If selected, you can create rectangular tile regions. 
- Circle	When selected, you can create circular tile regions. 
Mode	

Parameter		Description
-	Tiles	If selected, you have to enter the number of tiles as a reference for the size of the tile region. Enter the number of tiles in the **X/Y** input fields. If you are adding a circular tile region, enter the number of tiles for the diameter in the **Diameter** input field.
-	Size	If selected, you have to enter the size as a reference for the size of the tile region. Enter the size of the tile region in the **X/Y** input fields. If you are adding a circular tile region, enter the diameter of the tile region in the **Diameter** input field.
-	Stake	If selected, you can define a tile region by the placement of at least two markers (user defined X/Y stage coordinates). If you want to modify the tile region (expand/reduce) you have to adjust the tile region to the desired size. To complete the tile region press **Done**. Circular or rectangular tile region can be created in this manner by selection of the appropriate contour.
-	Add	Adds the tile region to the image. The added tile region will also appear in the **Tile Regions List** and is activated for acquisition. Added tile regions are displayed in the form of red grids in the stage view of the **Advanced Tiles Setup**.
Tile Regions List		Displays the added tile regions. The list contains the following columns:
-	Checkbox	Activates the relevant list entry for acquisition.
-	Name	Here you can edit the name of the tile region.
-	Contour	Displays the contour of the tile region.
-	Category	Displays the category of the tile region. Categories can be defined in the view options of the advanced tiles setup on the properties tab.
-	Tiles	Displays the number of tiles of the tile region.
-	Z	Displays the Z-position of the tile region.
-	Size	Displays the size of the tile region.
Verify Tile Regions...		Opens the **Verify Tile Regions** dialog. There you can verify each point of the tile region according focus und position.

Tile Region List Options

Parameter	Description
Up  and **Down** 	With the Up / Down buttons you can shift the selected list entry one position up or down in the tile regions list. This allows you to modify the acquisition order.
Delete 	Deletes the selected list entry.
Lock 	Unlocks the selected list entries to allow editing. The tile regions or positions are only locked if created in carrier mode.
Options 	If you click on the button, you see the following options:
- Set Current Z for Selected Tile Regions	Sets the current Z-Position for all selected tile regions.
- Set Current X/Y/Z for Selected Tile Regions	Sets the current X/Y/Z-Position for all selected tile regions.
- Delete	Deletes the current tile region.
- Delete All	Deletes all tile regions.
- Activate	Activates the current tile region for acquisition.
- Deactivate	Deactivates the current tile region for acquisition.
- Unlock	Unlocks the current tile region. The tile regions or positions are only locked if created in carrier mode.
- Unlock All	Unlocks all locked tile region.
- Sort	**By Center Position (Y -> X)** sorts all tile regions according to their overall Y position. **By Center Position (X -> Y)** sorts all tile regions according to their overall X position. **By Category** sorts all tile regions according to their category.
- Convert to Positions…	Converts a selected tile region into Positions or a Position Array.

12.14.1.2 Positions Section

Current Position section

Displays the current stage position.

Parameter	Description
X Position	Displays the X coordinate of the current position.
Y Position	Displays the Y coordinate of the current position.
 **Add**	Adds the current position to the **Positions List** and activates it for acquisition.

Display mode section

Parameter	Description
Single Positions	Shows the **Single Positions List**. To learn more about single positions see glossary "Position".
Position Arrays	Shows the **Position Arrays List** and the **Positions of selected Array List**, that shows a full **Single Positions List** for the selected position array. To learn more about position arrays see glossary "Position".

Single Position List

Displays the added positions. The list contains the following columns and buttons:

Parameter	Description
Checkbox	Activates the relevant list entry for acquisition.
Name	Here you can edit the name of the single position.
Category	Displays the category of the single position. Categories can be defined in the view options of the advanced tiles setup on the properties tab.
X	Displays the X-position of the single position.

Parameter	Description
Y	Displays the Y-position of the single position.
Z	Displays the Z-position of the single position.
List Navigation  and 	With the buttons you can shift selected list entry one position up or down in the tile regions list. This allows you to modify the acquisition order. Note that the **Tile Regions/Positions** checkbox has to be deactivated *Tiles Options* [▶ 380].
 **Delete**	Deletes the selected list entry.
 **Lock**	Unlocks the selected list entries to allow editing.
 **Options**	Opens the *Options for editing Single Positions* [▶ 351].

Position Array List

Displays the added position arrays. The list contains the following columns and buttons:

Parameter	Description
Checkbox	Activates the relevant list entry for acquisition.
Name	Here you can edit the name of the tile region.
Contour	Displays the contour of the position array.
Positions	Displays the number of positions of the position array.
Size	Displays the size of the position array.
List Navigation  and 	With the buttons you can shift selected list entry one position up or down in the tile regions list. This allows you to modify the acquisition order.
 **Delete**	Deletes the selected list entry.

Parameter	Description
 **Lock**	Unlocks the selected list entries to allow editing.
 **Options**	Opens the *Options for editing Position Arrays* [▶ 352].

Verify Positions section

Parameter	Description
Verify Positions...	Opens the *Verify Tile Regions or Verify Positions Dialog* [▶ 356].

12.14.1.2.1 Options for editing Single Positions

Parameter	Description
Set Current Z for Selected Positions	Sets the current Z-Position for all selected positions.
Set Current XYZ for Selected Position	Sets the current X-Y-Z-Position for the selected position.
Delete	Deletes the current position.
Delete All	Deletes all positions.
Activate	Activates the current position for acquisition.
Deactivate	Deactivates the current position for acquisition.
Sort	Sorts the list entries according to the chosen parameter.
- By Center Position (Y -> X)	Sorts all positions according to their overall Y position.
- By Center Position (X -> Y)	Sorts all positions according to their overall X position.
- By Category	Sorts all positions according to their category.

12.14.1.2.2 Options for editing Position Arrays

Parameter	Description
Delete	Deletes the current position array.
Delete All	Deletes all position arrays.
Activate	Activates the current position array for acquisition.
Deactivate	Deactivates the current position array for acquisition.
Unlock	Unlocks the current position array.
Unlock All	Unlocks all locked position arrays.
Sort	Sorts the list entries according to the chosen parameter.
- By Center Position (Y -> X)	Sorts all positions according to their overall Y position.
- By Center Position (X -> Y)	Sorts all positions according to their overall X position.

12.14.1.3 Sample Carrier Section

Only visible if the **Show All** mode is activated.

Parameter	Description
Sample Carrier	Displays the selected sample carrier template. If no template is selected it will display **None**.
Select...	Opens the *Select Template Dialog* [▶ 358]. Here you can select the sample carrier template.
 (Edit Support Points)	Opens the sample carrier selection / editor dialog. There you can edit and add global support points to the selected sample carrier.
 **Delete**	Deletes the selected sample carrier from the sample carrier field. The template will still be available in the **Select Sample Carrier Template** dialog.
Calibrate...	Opens the **Sample Carrier Calibration Wizard**. Here you will be guided through the sample carrier calibration.

Parameter	Description
Move Focus Drive to Load Position Between Containers	**Activated:** Moves the focus drive to the loading position during the movement of the stage to another container of the sample carrier (e.g. a well or slide). This prevents possible damage.

12.14.1.4 Focus Surface (Verify) Section

Only visible if the **Show All** mode is activated.

Local (per Tile Region) Tab

Displays the added local support points of a selected tile region. These can be edited in the view options of the advanced tiles setup on the support points tab. The list contains the following columns and buttons:

Parameter	Description
X	Displays the X coordinate of the focus reference point.
Y	Displays the Y coordinate of the focus reference point.
Z	Displays the Z coordinate of the focus reference point.
 **Add**	Adds a new support point to the selected tile region at the current stage and focus position.
 **Delete**	Deletes the selected list entry.
 **Options**	Opens the Options for editing Support Points.

Global (on Carrier) Support Tab

Displays the added global support points of the selected sample carrier. These can be edited in the Select Sample Carrier Template dialog.
As the list does not differ much from the **Local Support Points List** only the additional columns are explained in the following:

Parameter	Description
Container	Allows you to sort the global support points according to their container on the sample carrier.

Parameter	Description
Verify Support Points...	Opens the Verify Z Position dialog.

Parameter	Description
Interpolation Degree	Shows the selected degree of interpolation. Select an other degree of interpolation from the dropdown list.

INFO

The more variable the surface of your specimen the higher you should choose the interpolation degree. For higher degrees you will need more support points. The minimum number of support points for each interpolation degree is given in the dropdown list. As an overachievement of this minimum number ensures a solid calculation, we recommend minimizing the interpolation degree even if you added more support points. Increase the interpolation degree only so far as the surface condition of your specimen demands.

If the number of support points is too low for the selected interpolation degree, the next lower level for which the minimum is forefilled will be used.

12.14.1.5 Options Section

Only visible if the **Show All** mode is activated.

Here you can set options like acquisition and stage travel behavior during the experiment. Changes in this section of the tool affect all elements, tile acquisitions, positions and position arrays.

Parameter	Description
Tile Overlap	Defines the overlap in percent of individual tiles of the tile regions here. The value is set to 10 % by default. Note that lower overlap might cause artifacts. No overlap will not allow the images to be stitched correctly.
Stage Travel Optimization	In this section you can adjust settings for stage traveling during an experiment.
Travel in Tile Regions	

Parameter	Description
- Meander	Acquires tile regions following a meander pattern – alternately from both travel directions (left -> right; right -> left). This scan movement is faster.
- Comb	Acquires tile regions following a comb pattern – always from one travel direction only (left -> right). This scan movement is more precise.
- Spiral	Acquires tile regions following a spiral pattern – from the center of the region to the outer bounds in a clockwise motion. This mode works only for regions with rectangular or elliptical contours.
Tile Regions/ Positions	**Activated**: Individual positions and tile regions are not acquired in the sequence in which they are defined in the **Tile Regions** list. The stage movement will be automatically adapted to the location of the individual tile regions and positions. If you add or remove tile regions or positions, the sequence of acquisition therefore also changes.
- Sort by X, then Y	The tile regions and positions are sorted by their absolute position (first x, then y).
- Sort by Y, then X	The tile regions and positions are sorted by their absolute position (first y, then x).
Carrier Wells/ Container	**Activated**: Applies the selected travel patterns (see description above) when acquiring tiles using wells/ containers.
Use Stage Speed from Stage Tool	**Activated:** The software uses the stage travel speed which is set in the **Stage** tool (Right Tool Area).
Stage and Focus Backlash Correction	**Activated**: Stage and focus positioning is done with a backlash correction which is more precise but slightly slower.
Keep Number of Tiles Constant on Re-scaling	**Activated**: The number of tiles (columns and rows) remains constant when the scaling changes, e.g. due to an objective change. When this option is activated, the scanned area on the stage does not remain fixed. It depends on the current tile size.
Split Scenes into Separate Files	**Activated**: The scenes (e.g. tile regions and positions) are stored into separate physical files. They are still combined into one logical image file.

Parameter	Description
Stitching During Acquisition	**Activated**: Stitching of tiles is done during the acquisition.
Image pyramid during acquisition	**Activated**: An image pyramid is generated during the acquisition. This optimizes the image for fast display. If this option is not activated, the acquired image will not be shown and updated in the document area while the acquisition is running.

12.14.1.6 Verify Tile Regions or Verify Positions Dialog

Note that the Z values of positions, local support points and global support points are verified in a separate dialog accessed via the corresponding sections. As the dialog contains the same items and options for verifying the Z values it is described here once.

Parameter	Description
Tile Regions/ Positions List	Displays the drawn in tile regions (TR), positions (P) or Support Points (SP). The list contains the following columns and buttons:
- Status	Here you can see if the Z-Position is already verified. Then a green checkmark will appear in the corresponding row.
- Name	Displays the name of the selected support point.
- Z (µm)	Displays the Z-position of tile region or position.
- Tile Region	Shows the tile region of the local support point.
- Array	Shows if the position is part of an Position Array.
-  Options	Opens the options menu for verifying tile regions / positions, see description below.
Verification Helper Method	
- **None** (manual adjustment)	If selected, you need to manually adjust the focus for each point to be verified.
- Autofocus (AF)	Only available if your system has a motorized focus drive (z-axis). If selected, you can use the software autofocus for adjusting the focus for each point to be verified. The corresponding buttons will then appear in the dialog.

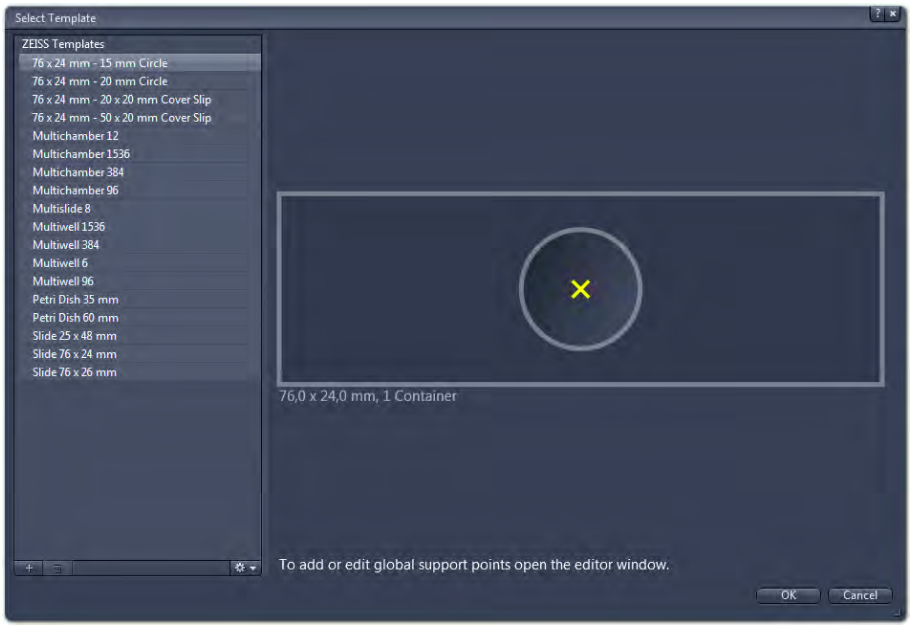
Parameter	Description
- Definite Focus (AF)	Only available if your system has a **Definite Focus** device. If selected, you can use the definite focus for adjusting the focus for each point to be verified. The corresponding buttons will then appear in the dialog further below.
Move to Current Point	Moves the stage to the selected point.
Include Z when Moving to Points	**Activated:** Runs the software autofocus and sets the determined z value for the support point.
Set Z & Move to Next	Sets the current z value for the selected support point and sets the status to verified. Then the software moves the stage to the next support point.
Run AF (or DF) and Set Z	This button is only visible if you have selected **Autofocus (AF)** or **Definite Focus (DF)** as Helper Method. Runs the software autofocus / definite focus and sets the current z position to verified.
Use AF (or DF) to Verify the Remaining	This button is only visible if you have selected **Autofocus (AF)** or **Definite Focus (DF)** as Helper Method. Automatically moves to the remaining points and determines the z value with the software autofocus for them.

Options for verifying tile regions / positions

Parameter	Description
Current Point Verified	Here you can change the status of the selected support point from verified to unverified (or vice versa).
Set all Points as Verified	Changes the status of all points to verified.
Reset Verification State of all Points	Changes the status of all points to unverified.
Set Current Z for Selected Points	The current z value is set for all selected points.
Set Current Z for all Points	The current z value is set for all points.

Parameter	Description
Apply Z-Offset...	Opens al dialog to apply a z-offset for all or the selected points.

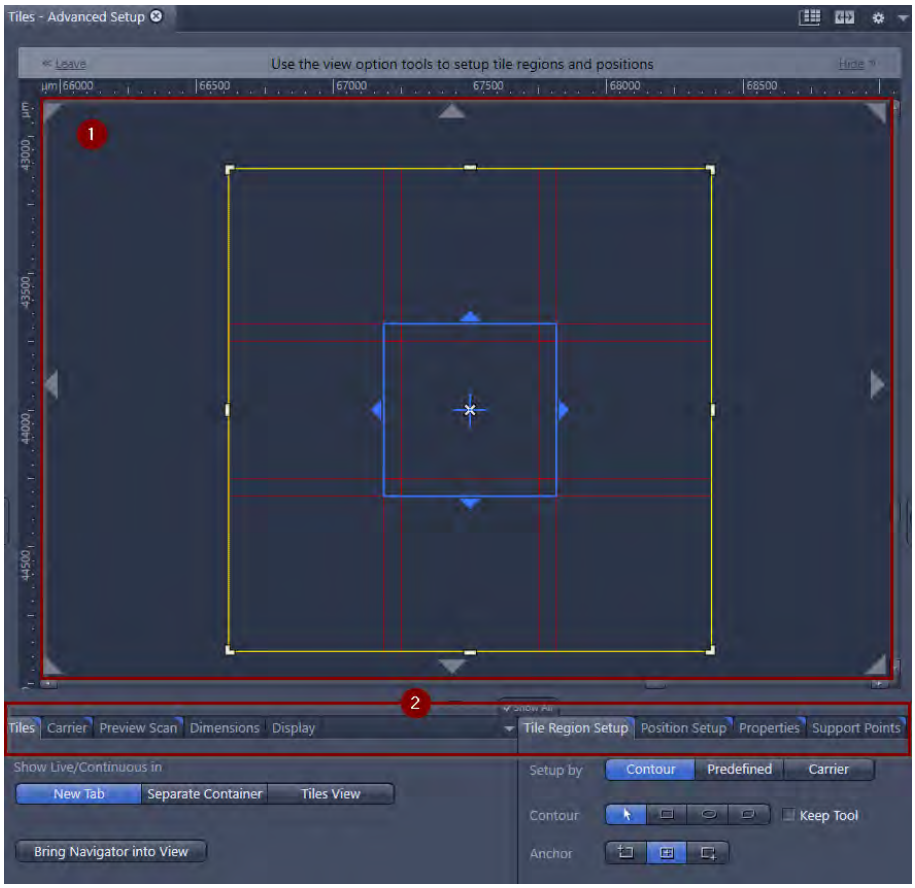
12.14.1.7 Select Template Dialog



Parameter	Description
User Templates	Shows all custom sample carrier templates.
ZEISS Templates	Shows predefined ZEISS sample carrier templates for several sample carriers.
 **Add**	Opens the Edit Sample Carrier Template dialog to create a new template.
 **Delete**	Deletes the selected sample carrier template.
 **Option**	Opens the Options for editing sample carrier templates.
Preview	Shows a preview of the selected sample carrier template.

12.14.2 Tiles Advanced Setup

Here you can configure advanced settings and plan your Tiles and Positions experiment. In the **Center Screen Area** you can see the *Stage view* [▶ 360]. When the **Advanced Tiles Setup (ATS)** opens, the stage view is "zoomed" to a predefined factor. You can change the Zoom in the **Dimensions** tab | **Zoom**, or by scrolling the mouse wheel.
To navigate around you have the following options:
In each corner and along each edge with the arrowheads, you can click to move the view in this direction. Additional settings and tools relating to tile regions or positions can be found in the specific view options.



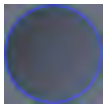
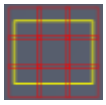
1 *Stage View* [▶ 360]

2 **Specific View Options**:
Tiles Tab
Carrier Tab [▶ 361]
Preview Scan Tab [▶ 363]
Dimensions Tab. For more information, see *Dimensions Tab* [▶ 844].
Display Tab. For more information, see *Display Tab* [▶ 861].
Tile Region Setup Tab [▶ 364]
Position Setup Tab [▶ 368]
Properties Tab [▶ 374]
Support Points Tab [▶ 377]

Note that the functionalities between activated and unactivated **Show All** layout might differ.

12.14.3 Stage View

The **Center Screen Area** shows the full travel range of the microscope stage, along with the current stage position, the graphical display of sample carriers and your acquired mosaic images. You can control the stage view using the arrow icons at the edges of the image area. The view can be enlarged, reduced or moved using the general control elements.

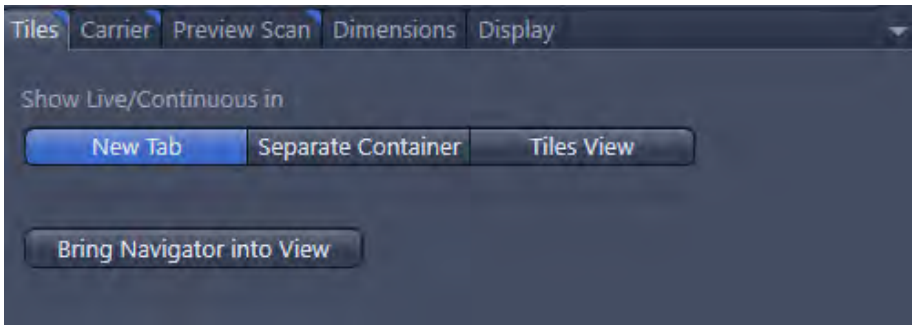
Symbol	Parameter	Description
 / 	**Selected/Active**	Represents the **Selected/Active** container/well by a blue border.
	**Live Navigator**	Shows the current stage position including the live image as a frame outlined in blue. To move the frame, double-click on the position to which you want to move it. Alternatively hold the left mouse button on the live navigator tool while dragging the mouse. The frame can also be used to control acquisition. If you click on one of the frame's blue arrow icons, an image is acquired. The Live Navigator tool is moved one frame width in the relevant direction. You can create tile images of your sample easily in this way.
	**Tile Region**	Represents the tile regions in the stage view by a red grid.
	**Positions**	Represents positions in the stage view by a yellow plus symbol.
	**Position Array**	Represents the Position Arrays in the stage view by the corresponding position symbols surrounded by a dashed line.
	**Local Support Point**	Represents local Support Points in the stage view by a yellow circle with a dot in the middle.

Symbol	Parameter	Description
	**Global Support Point**	Represents global Support Points in the stage view by a white circle with a dot in the middle.

12.14.4 Specific View Options

12.14.4.1 Tiles Tab

You can define how the software behaves when the live image is started and the **Advanced Setup** is open.



Parameter	Description
New Tab	Opens the live or continous image into a new image container.
Separate Container	Opens a live or continous image into a separate container. Both the **Advanced Setup** and the live or continous images are visible in parallel. We recommend to activate this checkbox, especially if you use two separate monitors.
Tiles View	Displays the live or continous image in place in the **Stage View** of **Advanced Setup**.
Bring Navigator into View	You can use this function if you need quickly to re-find your current location. Re-centers the **Stage View** onto the current stage position coordinates at the preset zoom level. You can also activate this function with *Ctrl* + *B*. You can call the function to focus the image with the mouse wheel by pressing and holding the *Ctrl* key when the mouse cursor is over the stage view.

12.14.4.2 Carrier Tab

Only visible if a sample carrier was selected.

Here you can see a graphical preview of the sample carrier being used.

INFO

Only the containers / wells whose tile regions and positions were set up with the *Setup by Carrier* [▶ 367] of the **Tile Regions Setup** tab or the *Setup by Carrier* [▶ 372] of the **Positions Setup** tab will be taken into account.

Please note the following features of the display:

Symbol	Description
/	**Empty** containers / wells, meaning that no tile regions or positions were set up with the Carrier option, are represented by a grey conatiner / well.
/	The currently **Active** container / well is represented by a blue border.
/	A container / well only filled with **Tile Regions** is represented by a yellow filled conatiner / well.
/	A conatiner / well only filled with **Positions** is represented by a orange filled conatiner / well.
/	A container / well filled with **Tile Regions** and **Positions** is represented ba a half yellow, half orange filled conatiner / well.

INFO

Right click opens a small context menu. Here you can copy the contents of the selected well, or paste the contents to the selected or all wells.

12.14.4.3 Preview Scan Tab

Here you can define the settings for a preview scan. Typically a low magnification objective and a channel which protects your sample (e.g. transmitted light) is used. This will give you a low resolution overview of the sample to mark tile regions and/ or positions.

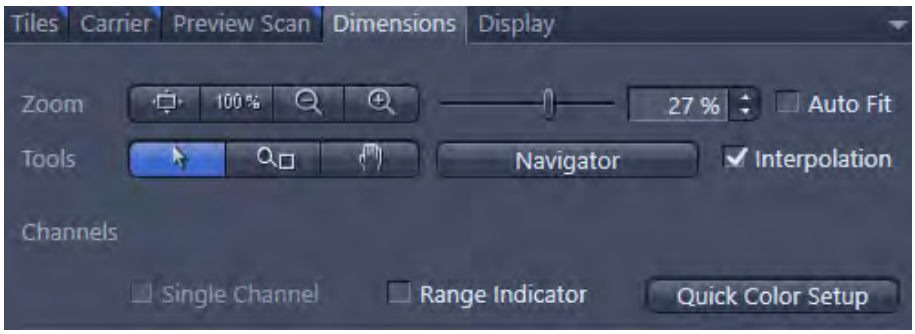
INFO

Note that the selected objective for the **Preview Scan** is connected to the experiment settings. If you change the objective, it also changes in the settings on **Locate** or **Acquisition** tab. Thus, you must actively change the objective after the preview scan if you want to use another objective for your experiment.

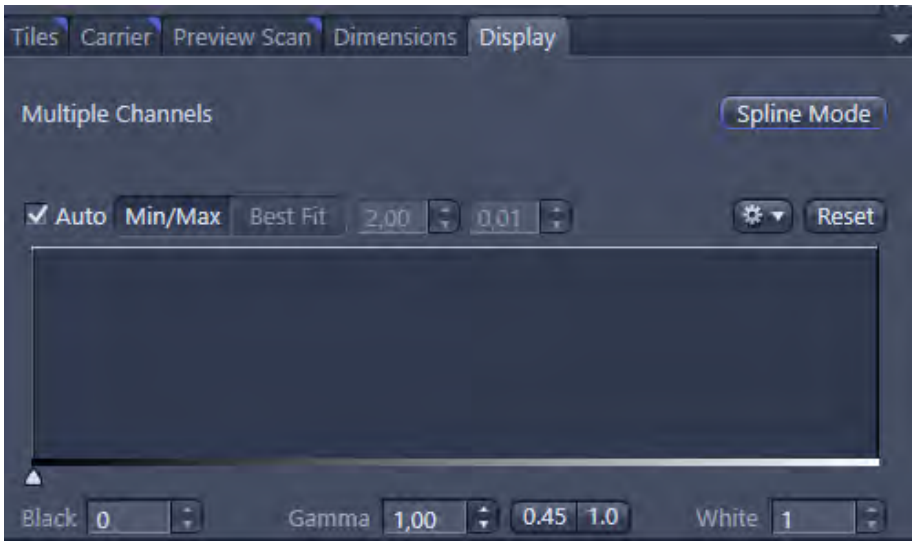
Parameter	Description
Start Preview Scan	Starts the preview scan to acquire the overview images.
Delete Existing Preview Images	**Activated:** Deletes all existing preview images when the next preview is acquired.
Objective/Optovar	Here you can select the objective and Optovar which will be used for the preview scan. To acquire an overview of all active tiles and positions images, switch to an objective with a low magnification. Set the channel exposure time and start the acquisition of the overview image.
Use Existing Experiment Settings	**Activated:** Uses the existing experiment Settings. That is the default setting. **Deactivated:** The additional options **Camera** and **Channels** appear. These allow independent activation/ deactivation of channels. **Use Binning from Experiment** will allow you to define a binning setting independent from that used in the experiment.

Parameter	Description
	If binning is used then the exposure time is automatically compensated to avoid saturation. Changing these parameters does not effect the settings that will be used for the experiment. Note that if necessary you can modify the the compensation factor in **Tools | Options | Acquisition |Tiles & Positions**. Normally, the default parameters should be sufficient.

12.14.4.4 Dimensions Tab



12.14.4.5 Display Tab



12.14.4.6 Tile Region Setup Tab

Here you can select which setup you want to be used for the settings of the tile regions. Three setups with different setting options are available:

12.14.4.6.1 Setup by Contour

Here you can define the tile regions by means of the contour.

Contour section

Here you can select the contour of your tile region. The following tools are available:

Parameter	Description
Selection Mode 	With this tool you can select an already created tile region by clicking on it to move or edit it.
Rectangle 	With this tool you can draw a rectangle tile region.
Ellipse 	With this tool you can draw a elliptical tile region.
Polygon 	With this tool you can draw a polygonal tile region.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.

Parameter	Description
Top left anchor position 	The anchor of the defined shape is at the top left.
Center anchor position 	The anchor of the defined shape is centered.
Bottom right anchor position 	The anchor of the defined shap is bottom right.

12.14.4.6.2 Setup by Predefined

Here you can define the tile regions by means of the number or size.

Tool section

Here you can select a tool to work with. The following tools are available:

Parameter	Description
 **Selection**	Select an element in the stage view to edit or move it.
 **Add Tile Region**	Adds the current tile definition in the image area.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.

Contour section

Only visible if the **Show All** mode is activated.

Here you can select the contour of your tile region. The following tools are available:

Parameter	Description
 **Rectangle**	Adds a rectangular tile region.
 **Circle**	Adds a circular tile region

Mode section

Parameter	Description
Tiles	Using this mode you have to enter the number of tiles as a reference for the size of the tile region. Enter the number of tiles in the **X / Y** input fields. If you are adding a circular tile region, enter the number of tiles for the diameter in the **Diameter** input field.
Size	Using this mode you have to enter the size as a reference for the size of the tile region. Enter the size of the tile region in the **X / Y** input fields. If you are adding a circular tile region, enter the diameter of the tile region in the **Diameter** input field.

Parameter	Description
Stake	This mode allows the definition of a tile region by the placement of at least two markers (user defined X/Y stage coordinates). If you want to modify the tile region (expand/ reduce) you have to adjust the tile region to the desired size. To complete the tile region press **Done**. Circular or rectangular tile region can be created in this manner by selection of the appropriate contour.
Add	Adds the tile region to the **Tile Regions List** and activates it for acquisition. Added tile regions are displayed in the form of red grids in the stage view of the **Advanced Tiles Setup**.

Anchor Section

The **Anchor Section** is only visible in **Show All** mode.

Parameter	Description
 **Top left anchor position**	The anchor of the defined shape is at the top left.
 **Center anchor position**	The anchor of the defined shape is centered.
 **Bottom right anchor position**	The anchor of the defined shap is bottom right.

12.14.4.6.3 Setup by Carrier

Here you can define the tile regions automatically by means of the fill factor of the sample carrier.

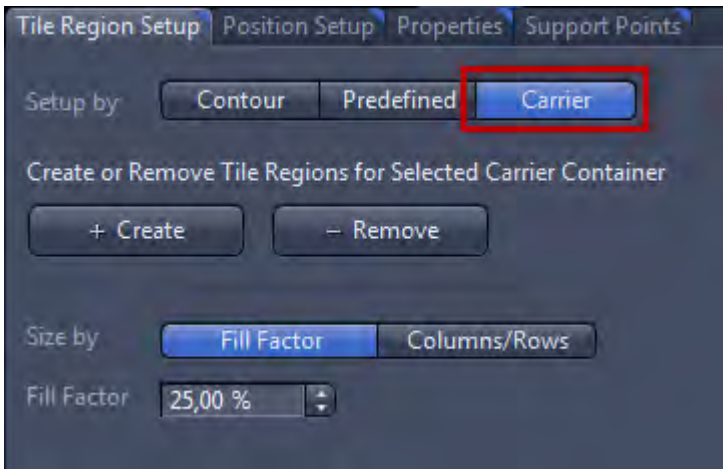
i INFO

A sample carrier must have been selected in the *Sample Carrier* [▶ 352] section of the **Tiles** tool.

INFO

Manually created tile regions and positions (setup by **Contour** and setup by **Predefined**) will be deleted, if you switch to the setup by **Carrier**. If you want to combine manual and automatic setup, first use setup by **Carrier** and then switch to a manual setup.

Tile regions that are created automatically by setup by **Carrier**, are defined to a container and permanently assigned and locked by default, against manual editing. You can unlock the tile regions in the **Tiles** tool by selecting the desired tile region and click on the unlock button.



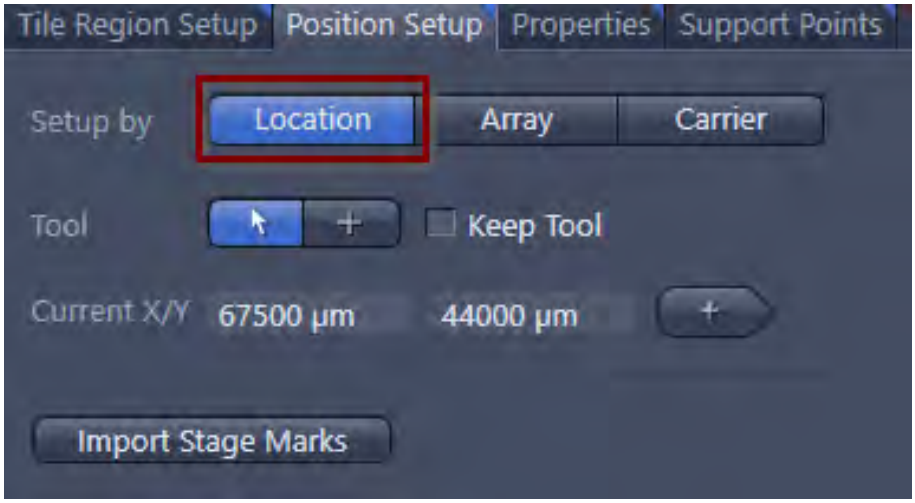
Parameter	Description
Create	Only active if you have selected a container on the *Carrier Tab* [▶ 361] or in the *Stage View* [▶ 360]. Automatically creates the tile regions with the set fill factor in the selected container of the sample carrier.
Remove	Removes all tile regions in the selected container.
Fill Factor	Here you can enter the fill factor used to fill the selected container.
Columns/Rows	Here you can add single tile regions to a container by defining the number of columns and rows of the tile. The tile region is always placed at the center of the well container.

12.14.4.7 Position Setup Tab

Here you can select which setup you want to be used for the settings of the positions. Three setups with different setting options are available:

12.14.4.7.1 Setup by Location

Here, you can define the positions by means of the location. You can add various positions in the **Stage View** using the mouse.



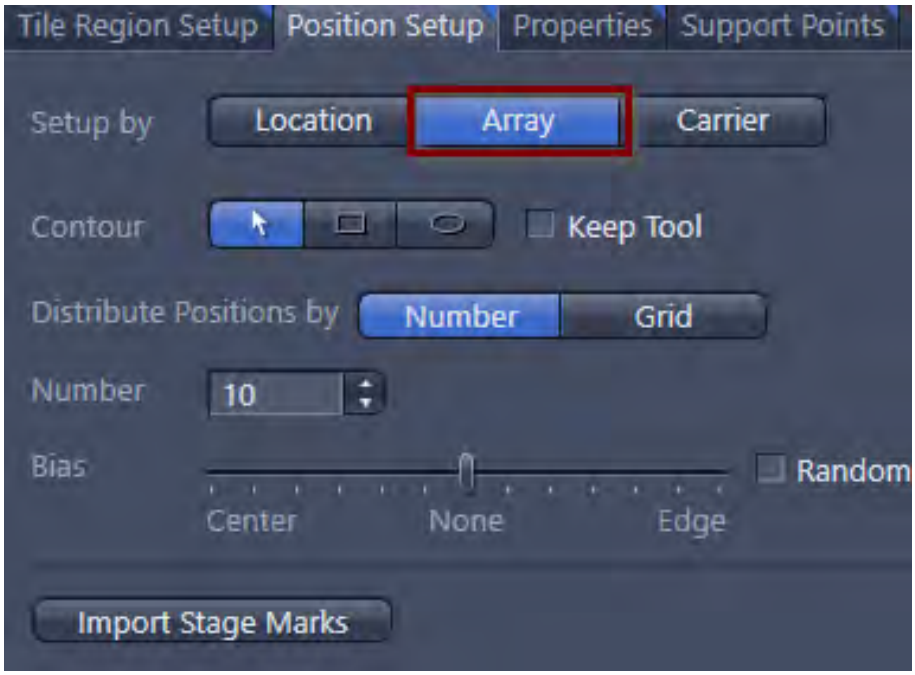
Parameter	Description
Selection	Select an element in the stage view to edit or move it.
Add	Add a new position on the stage view.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.
X Position/Y Position	Displays the current stage position (X/Y).
Add	Adds a new position at the current stage position.
Import Stage Marks	Imports marks (X/Y positions with optional z-values) that are stored in the stage tool.

12.14.4.7.2 Setup by Array

Here you can define the positions by means of position arrays. You can add various contours for position arrays in the stage view.

INFO

Position arrays are groups made up of a number of individual positions. Typically, position arrays contain several hundred individual positions. They make your work easier if you work with regular or evenly distributed samples.



Contour section

Here you can select the contour of your tile region. The following tools are available:

Parameter	Description
Selection 	With this tool you can select an already created position array by clicking on it to move or edit it.
Rectangle 	With this tool you can draw a rectangle position array.
Ellipse 	With this tool you can draw a elliptical position array.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.

Distribute Positions by section

Parameter	Description
Number	Activates **Number** and **Bias**.
Grid	Activates **Overlap**.

Number section

Parameter	Description
Number	Shows the current number of positions that are distributed to newly created position array. Change the number to increase or decrease the number of single positions obtained by a position array.

Bias section

Only visible if the **Show All** mode is activated.

Here you can set the distribution bias of the single positions created for a new position array.

Parameter	Description
Bias	Adjusts the overall position of the single positions in the position array.
- None	The single positions of the position array will be distributed evenly within the array.
- Center	The single positions of the position array will mainly be distributed near to the center of the position array. Less positions will be at the edges of the array.
- Edge	The positions of the position array will be distributed to the edges of the array. Less positions will be in the center of the array.
Random	**Activated:** The single positions will be distributed randomly within the position array. The overall bias will still be taken into account.

Overlap section

Parameter	Description
Overlap	Shows the current degree of overlap in % of each position in the array relative to its neighbours. Both positive and negative values are possible.

Import Stage Marks

Parameter	Description
Import Stage Marks	Imports marks (X/Y positions with optional z-values) that are stored in the stage tool.

12.14.4.7.3 Setup by Carrier

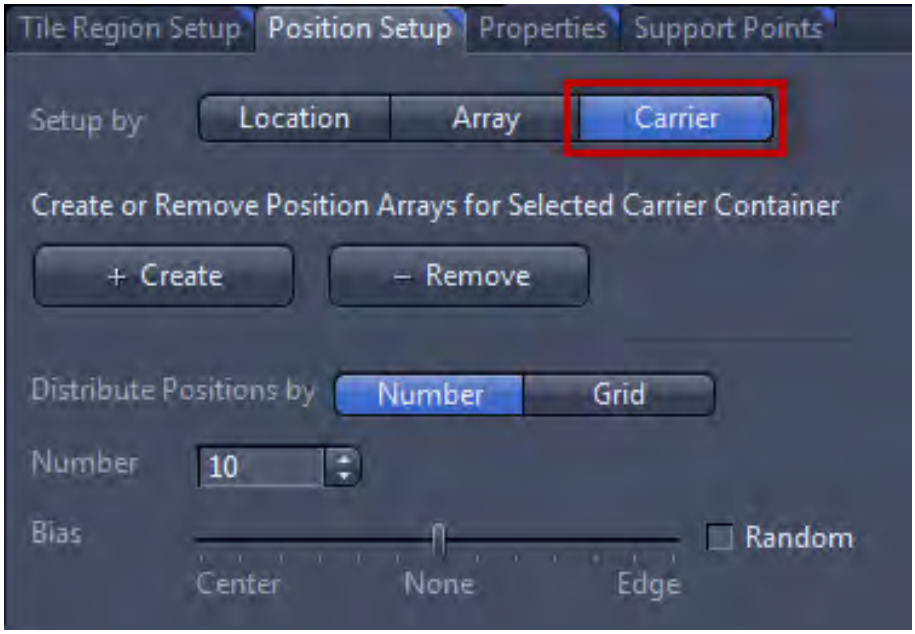
Here you can define the positions automatically by means of the relevant sample carrier.

INFO

A sample carrier must have been selected in the Sample Carrier section of the **Tiles** tool.

INFO

Manually created tile regions and positions (**Setup by Contour** and **Setup by Predefined**) will be deleted, if you switch to the setup by **Carrier**. If you want to combine manual and automatic setup, first use **Setup by Carrier** and then switch to a manual setup.



Parameter	Description
Create	Only active if you have selected a container on the *Carrier Tab* [▶ 361] or in the *Stage View* [▶ 360]. Automatically creates the tile regions with the set fill factor in the selected container of the sample carrier.
Remove	Removes all tile regions in the selected container.

Number section

Parameter	Description
Number	Shows the current number of positions that are distributed to newly created position array. Change the number to increase or decrease the number of single positions obtained by a position array.

Bias section

Only visible if the **Show All** mode is activated.

Here you can set the distribution bias of the single positions created for a new position array.

Parameter		Description
Bias		Adjusts the overall position of the single positions in the position array.
-	None	The single positions of the position array will be distributed evenly within the array.
-	Center	The single positions of the position array will mainly be distributed near to the center of the position array. Less positions will be at the edges of the array.
-	Edge	The positions of the position array will be distributed to the edges of the array. Less positions will be in the center of the array.
Random		**Activated:** The single positions will be distributed randomly within the position array. The overall bias will still be taken into account.

12.14.4.8 Properties Tab

Here you can adjust the properties of a selected tile region or position.

INFO

You have to select a tile region or single position to see the parameters available on this tab. It is not possible to see and adjust the parameters for a whole position array.

12.14.4.8.1 Properties of selected Tile Regions

Here you can adjust the properties of the selected tile region.

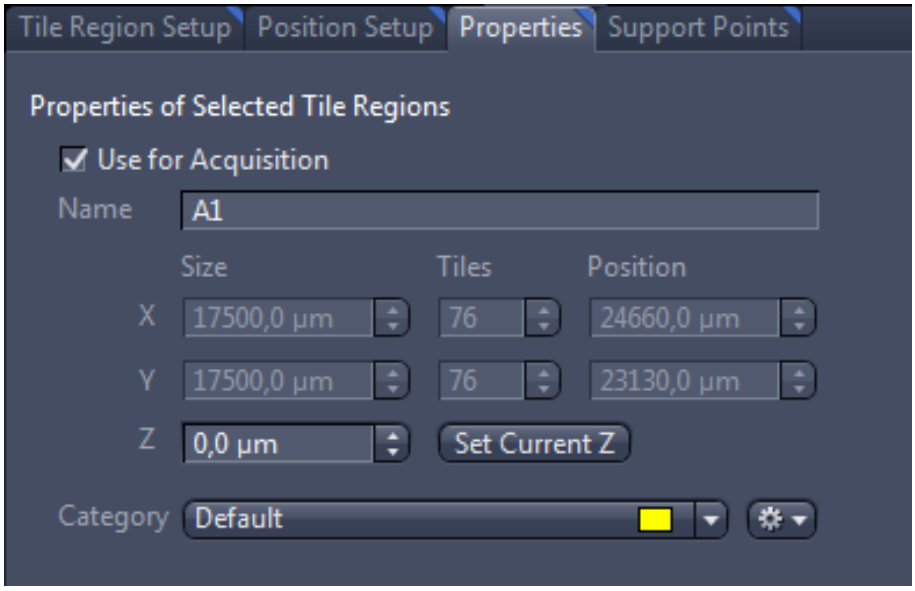


*Fig. 12.2: Properties Tab (**Show All** mode)*

Parameter	Description
Use for Acquisition	**Activated:** Uses the selected tile region for acquisition.
Name	Here you can enter a name for the selected tile region.
Size	Here you can see and edit the size of the tile region in the **X/Y/Z** dimensions. The **X/Y** dimensions of tile regions created with the *Setup by Carrier* [▶ 367] can not be edited as they are fixed by the container/well size.
Tiles	Here you can see the number of tiles in the **X**/Y dimensions. You can not edit the number of tiles as it is fixed by the size of the tile region.
Position	Here you can enter the position of the selected tile region in **X/Y** dimensions. The position of tile regions created with the *Setup by Carrier* [▶ 367] can not be edited as they are fixed by the container/well.
Set Current Z	Sets the **Z** dimension at the current **Z** position of the focus drive.

Only visible if the **Show All** mode is activated.

Here you can assign categories to tile regions. Category definitions will be displayed in the appropriate column of the table in the Tiles tool. This value is also written in the image meta data. Thus, well definition patterns or variables can be created and stored as part of a experiment template.

Parameter	Description
Category	Shows the currently assigned category of the selected tile region. The **Default** category is set for all new tile regions.
 **Options**	Opens the options for editing and creating categories.
- New...	Opens the **New Category** dialog to create a new category.
- Edit…	Opens the **Edit Category** dialog to edit the selected category.
- Delete	Deletes the selected category and sets the category of the tile region to **Default**.

12.14.4.8.2 Properties of selected Positions

Here you can adjust the properties of the selected position.

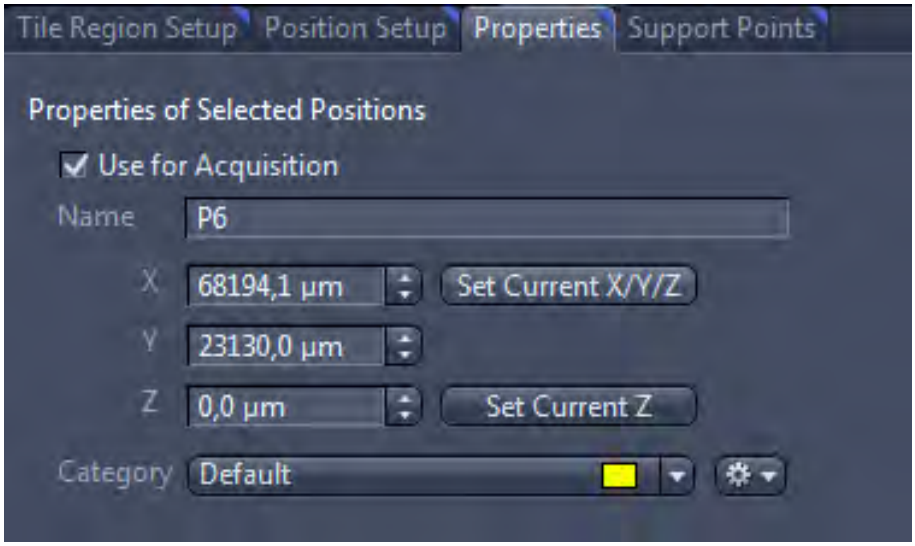


*Fig. 12.3: Properties Tab (**Show All** mode)*

Parameter	Description
Use for Acquisition	**Activated:** Uses the selected position for acquisition.

Parameter	Description
Name	Here you can enter a name for the selected position.
X/Y/Z	Here you can see and edit the position of the selected position on the stage in **X/Y/Z** dimensions.
Set Current X/Y/Z	Sets the **X/Y/Z** dimension at the current **X/Y/Z** position of the stage / focus drive.
Set Current Z	Sets the **Z** dimension at the current **Z** position of the focus drive.

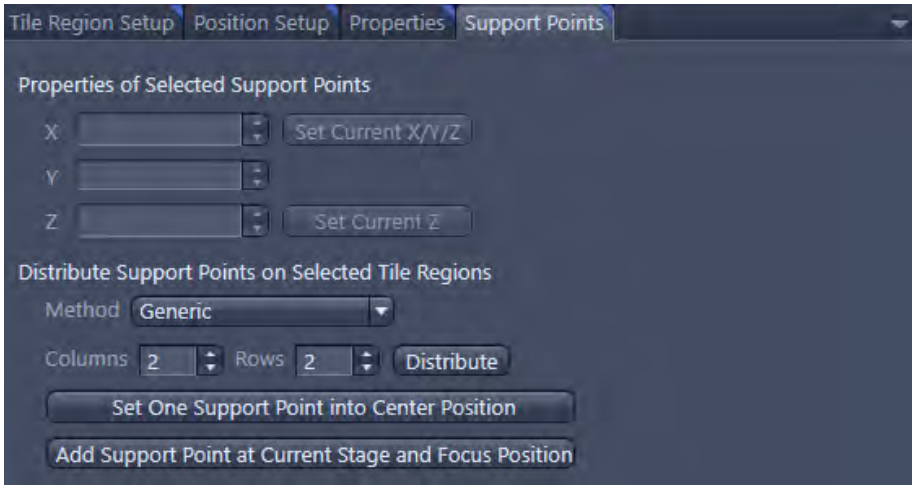
Only visible if the **Show All** mode is activated.

Here you can assign categories to tile regions. Category definitions will be displayed in the appropriate column of the table in the Tiles tool. This value is also written in the image meta data. Thus, well definition patterns or variables can be created and stored as part of a experiment template.

Parameter	Description
Category	Shows the currently assigned category of the selected tile region. The **Default** category is set for all new tile regions.
 **Options**	Opens the options for editing and creating categories.
- New...	Opens the **New Category** dialog to create a new category.
- Edit…	Opens the **Edit Category** dialog to edit the selected category.
- Delete	Deletes the selected category and sets the category of the tile region to **Default**.

12.14.4.9 Support Points Tab

Here you can create, distribute and adjust the properties of selected local and global support points.



Properties of Selected Support Point section

The properties of selected support point section contains the following columns and buttons:

Parameter	Description
Position	**X/Y** dimensions are only visible if the **Show All** mode is activated. Here you can see and edit the position of the selected position on the stage in **X/Y/Z** dimensions.
Set Current X/Y/Z	Only visible if the **Show All** mode is activated. Sets the **X/Y/Z** dimension at the current **X/Y/Z** position of the stage / focus drive.
Set Current Z	Sets the **Z** dimension at the current **Z** position of the focus drive.

i INFO

Properties of Global Support Points

The properties of a selected global support point slightly differ from those of a local one as you can not edit the **X/Y** dimensions because they are fixed by the sample carrier template you have selected. Therefore there is no **Set Current X/Y/Z** button for global support points. If you want to edit the number and XY dimension of your global support points this can be done directly via the **Sample Carrier** section of the Tiles tool.
Activating / Deactivating the **Show All** mode will not show / hide any additional options for global support points not even the options that are shown / hidden for local support points.

Distribute Support Points on Selected Tile Regions section

INFO

You can see this section only if you have selected one or more tile regions or a local support point of a tile region. Selecting a local support point will disable the functions of the distribute support points section.

The distribute support points on selected tile regions section contains the following input fields and buttons:

Parameter	Description
Method	
- **Generic**	Distribution method with a simple column and row approach. ZEISS recommends using this method for smaller tile regions (<200 tiles) of a regular shape, e.g. quatratic, rectangular, and circular.
- **Onion Skin**	Distribution method for mid- or larger tile regions (>200 tiles) of an irregular shape like you might use to image large area tissue specimens, e.g. brain slices.
Columns	Only available with **Generic**. Sets the number of columns of support points within the selected tile region.
Rows	Sets the number of rows of support points within the selected tile region.
Density	Only available with **Onion Skin**. Determines the number of Support points (up to a preset maximum number) that are used to cover the tile region. Example: A rectangular or square tile region with 400 tiles has 20 support points with the default parameters. The resulting focus points are displayed as layers of onion skin, providing an even distribution and also ensuring that the tile border in particular has enough focus points. The parameter density is set with a standard value of 0.05.
Max No.	Only available with **Onion Skin**. Determines the maximum number of support points for a tile region. As the density parameter is set to 5%, this would necesitate for very large specimens that the number can get very large. But a larger number of focus points does not always mean a better quality calculation of the focus surface. For this reason, you can define a maximum number of focus points in the range of 24 to 36 points.

Parameter	Description
Distribute	Distributes the entered number of support points defined in the column and row input fields within the tile region. Previously defined support points will be deleted.
Set One Support Point into Center Position	Only visible if the **Show All** mode is activated. Only available with **Generic**. Sets one support point in the center of the selected tile region. Previously defined support points will be deleted.
Add Support Point at Current Stage and Focus Position	Only visible if the **Show All** mode is activated. Adds a support point at the current stage and focus position. Does not affect previously defined support points.

12.14.5 Tiles Options

The additional options for the tiles module allow to set up several options for image acquisition and additional information. The tiles options dialog can be found in the menu bar under **Tools** | **Options...** | **Acquisition** | **Tiles**.

Parameter	Description
Automatically Start Live Mode in the Advanced Setup View	**Activated:** Automatically starts the **Live** mode in the **Center Screen Area** if you klick in the **Acquisition** tab in the **Tiles** tool on the **Advanced Setup** button. Uncheck this option to prevent unnecessary specimen bleaching. The default is not activated.
Show Information Title in the Advanced Setup View	**Activated:** Displays a bar above the Advanced Setup view containing additional information, if you klick in the **Acquisition** tab in the **Tiles** tool on the **Advanced Setup** button.
Show Snap Animation	**Activated:** Shows the snap animated when snapping a new image in Advanced Setup.
Automatic Snap by Clicking the Live Navigator Buttons	**Activated:** Takes a snap in the **Tiles-Advanced Setup** tab every time the live navigator tool is moved with its navigation arrows.
Enable Stage Moving with Live Navigator Handle	In the Live navigator tool the current stage position including the live image is shown as a frame outlined in blue. To move the frame,

Parameter	Description
	double-click on the position to which you want to move it. The frame can also be used to control acquisition. **Activated:** Acquires an image if you click on one of the frame's blue arrow icons. The Live Navigator tool moves one frame width in the relevant direction. You can create tile images of your sample easily in this way.
Show Label on Sample Carrier Container	**Activated:** Shows a label on every container / well of a selected sample carrier.
Show Tool Tip on Sample Carrier Container	**Activated:** Shows a tool tip with the name of the container / well when the mouse is over it in the **Carrier** tab.
Show Stage and Focus Backlash Correction Setting in the Options	**Activated:**In the Tiles option, the setting to switch the backlash correction on or off is shown.
Delimiter for CSV Export/ Import	Specifies the delimiter for a CSV export or import. Select **Comma** (default), **Semicolon** or **Tab**.
Ask Whether Support Points/Positions Should be Overwritten	When the support points and/or positions are determined by a software autofocus run the existing points can be overwritten with the new **Z** values. **Activated:** Shows a message box asking if the points should be overwritten if there is a autofocus **Z** value.
Enable Removing of Focus Surface Outlier	**Activated:** Ignores support points that are significantly outside the interpolated focus surface. You have the following setting options available:
- Maximum Interpolation Degree for Outlier Detection	This value can be 0 or 1. If 1 then a linear fit is used to detect the outlier support points. This is the default. If 0 a simple average value is used to detect outliers.
- Threshold in Terms of the Standard Deviation (Sigma)	This parameter defines a threshold value to determine which of the support points are outliers from the fitting process. This is defined by the standard deviation (sigma value) set in

Parameter	Description
	the spin box. Support points not meeting this criteria are subsequently ignored when the focus surface is determined.
Activate Stitching During Acquisition for New Experiments	**Activated:** Activates stitching during acquisition as default for all new experiments. This value is overwritten by the corresponding option in the **Tiles** setup for a new experiment.
Use Local Focus Surface for Preview Scans	**Activated:** Uses local focus surface values (z-values of positions, tile regions and if defined interpolated focal surfaces defined by support points) for the acquisition of preview scan images. Note that on activation of the Tiles dimension the appropriate strategy **Use Focus Surface Defined by Tiles Setup** is pre-selected.
Binning Compensation of Exposure Time in Preview Scans	Defines the power to which the binning ratio is modified to automatically determine the exposure time value used for a preview scan were the binning setting between the experiment and preview scan differs. The default value is 2.0 i.e. quadratic. Thus, for example the exposure time would be reduced by a factor of four if the experiment binning is 1x1 and the preview scan binning is 2x2. The value can be varied between 1.0 and 2.0 in steps of 0.1.
Live Image in Sample Carrier Calibration Wizard (relevant only for systems with camera)	
- Use Imaging Device from Selected Channel with "Acquisition" Settings	**Activated:** Default setting for the live image that allows navigation and focus interaction during the carrier calibration wizard.
- Use Active Camera with "Locate" Settings	This option is only relevant for systems with a wide field (camera based) detector. **Activated:** Allows you to alternatively apply locate camera settings for use in the carrier calibration wizard (live image). By default the experiment settings for the currently selected channel/track will be used.

13 Module Dynamics

13.1 Working with MeanROI View (offline)

13.1.1 Introduction

Using the **MeanROI** offline functions you can specify user-defined measurement regions (ROIs) after acquisition of your time lapse experiment and analyze their time-dependent changes in intensity. You can display the intensity curves in diagrams or export the values in the form of tables. This basic functionality is available for time series images opend in **ZEN (blue edition)** (excluded ZEN lite).

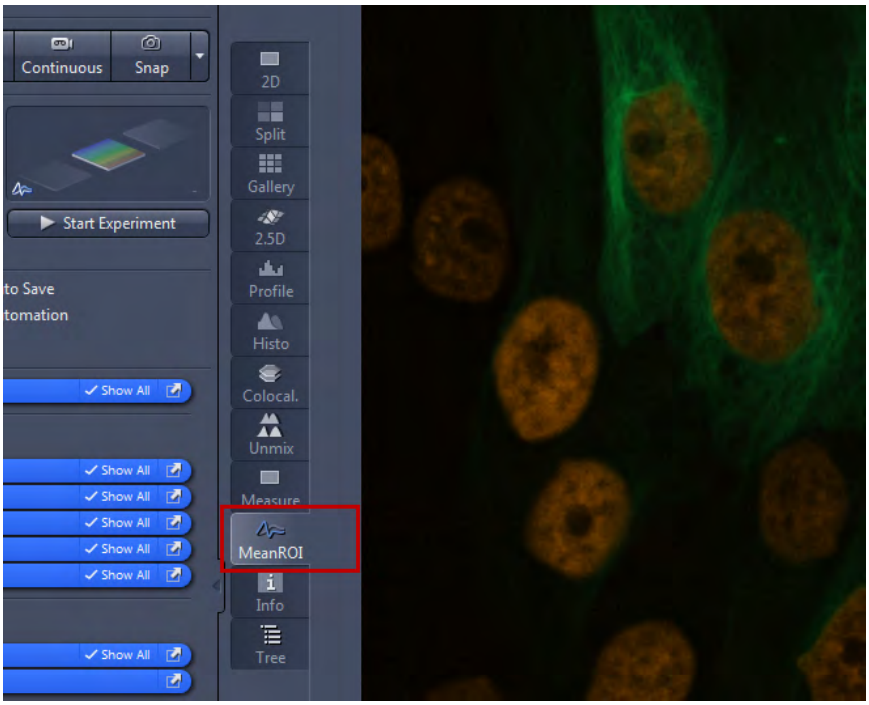
The **Dynamics** module expands the MeanROI offline functions to give you the option of calculating online/offline (during/post aquisition) ratios and makes additional display layouts, and analysis functions available (**Timeline** view, ROI tracing etc.).

Prerequisites

- You have acquired a time series experiment. The experiment is open and the first time point is displayed in the **2D view**.

Procedure

1 Select the **MeanROI** tab from the image view tabs in the **Center Screen Area**.



The **MeanROI** view opens.

You`re now prepared to start working with the **MeanROI** view. The following chapters will show you the first steps.

13.1.2 Drawing in and Adjusting ROIs

Here you will find out how to draw in and edit measurement regions (ROIs) for intensity measurements and how to adjust them for individual time points.

INFO

If you save the experiment, the **ROIs** are automatically saved with the experiment. They are available to you once again in the **MeanROI View** the next time the file is opened. Click on the **Recalculate** button on the **ROI Tools** tab to perform and display the intensity calculations again for the saved ROIs.

13.1.2.1 Drawing in ROIs

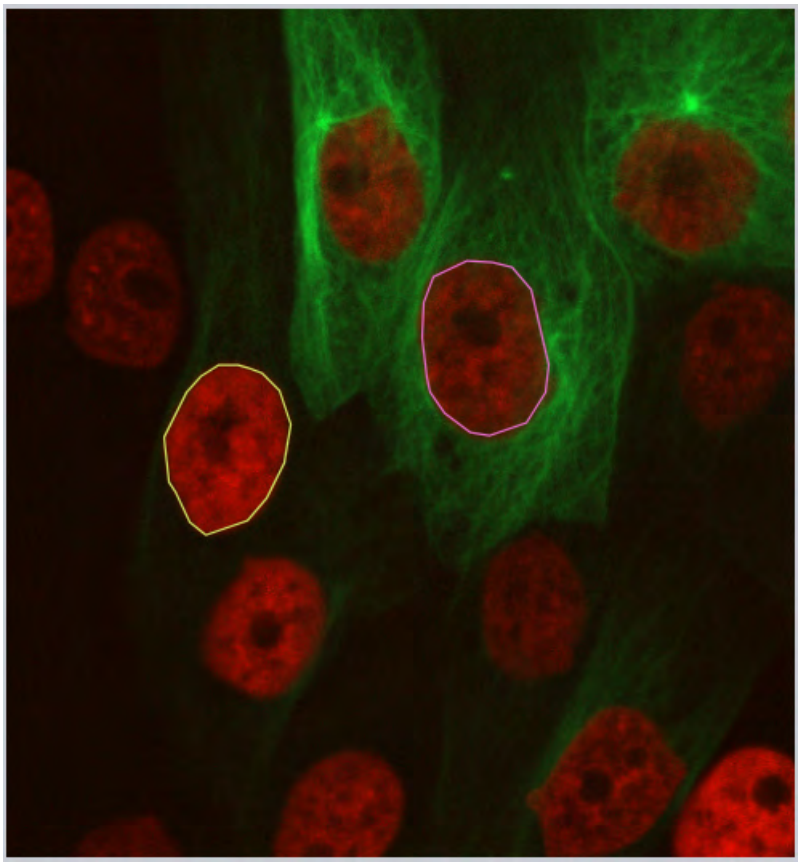
Prerequisites

- You are in the **MeanROI View** or in the **MeanROI Setup** on **Acquisition** tab.

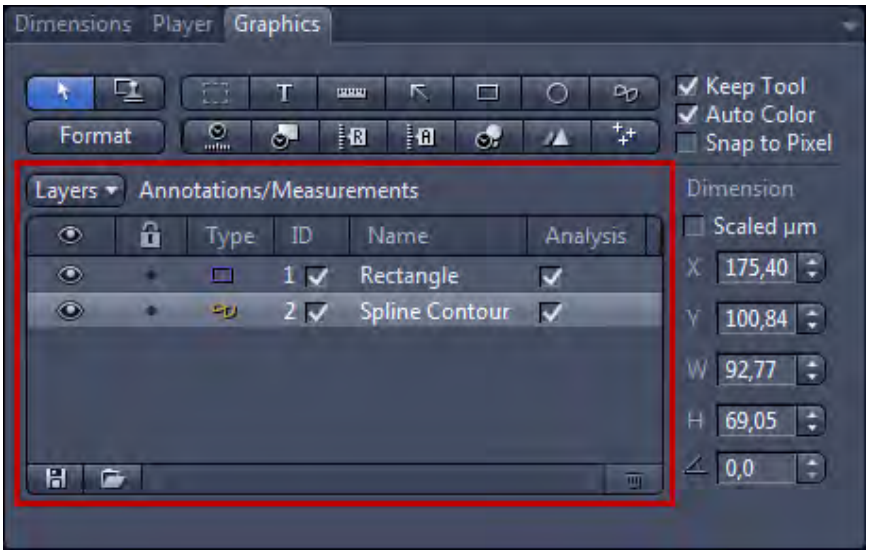
Procedure

1. Go to the **Graphics** tab in the **View Options**.
2. Select a tool for drawing in **ROIs**, e.g. the **Polygon** tool.
3. Activate the **Keep tool** checkbox.

   The selected tool remains active after you have drawn in an **ROI**. This means you can draw in several ROIs without having to re-select the tool.
4. Using the selected tool, in the image view draw in the objects or regions (ROIs) for which intensity measurements are required.



The **ROIs** are displayed in the list (Annotations/ Measurements Layer) on the **Graphics** tab.



Intensity measurements are performed for each **ROI** and displayed in the diagram area to the right of the image view.

You have successfully defined measurement regions for the intensity measurement.

13.1.2.2 Adjusting ROIs for Time Points

If objects move laterally in the course of the time series, you can adjust the **ROIs** at each **Time Point** in order to follow the objects.

Prerequisites

- You have defined at least one ROI.
- You are in the **MeanROI** view.

Procedure

1. Open the **Dimensions** tab in the general view options.
2. Use the **Time slider** to scroll through the time points. Stop at the first time point at which you want to adjust a ROI**.**
3. Adjust the position of the ROI using drag & drop. To do this, select the ROI in the image area by left-clicking and hold the mouse button down. Then move the ROI to the new position and release the mouse button.
4. Open the **ROI Tracing** tab and activate the **Enable ROI Tracing** checkbox. To edit the position of a single **Key frame** change the **Key Frame Edit Mode** to single mode  . Note that you can only select the key frame edit mode if the frame number is set to a value >1.
5. If necessary, you can change the shape of a ROI, by right-clicking on an ROI and select the **Edit Points** entry (e.g. for polygon contours).
   Note that if the area of the ROI changes the Mean intensity value will change. You might want to change to the measurement **Intergral intensity** on **MeanROI** tab.
6. Adjust the shape of the ROI, by drag & drop the contour points.

Changes to the position and shape of the ROIs are adopted for all subsequent time points.

7 Repeat the previous steps for all other time points for which you want to adjust an ROI.
For a selected ROI you can see a list of the time points in which its position/ shape was modified. As the distance (in frames) between each key frame can vary, a linear interpolation is used to smoothly progress the ROI through the time points. Alternatively, deactivate this (constant) or set it to a spline method that may describe the progression of the object you are tracing.

You have successfully adjusted the measurement regions to the course of the experiment.

13.1.3 Adjusting the Display

Here you will find out how to adjust the display of the measured intensity values in diagrams and tables according to your wishes.

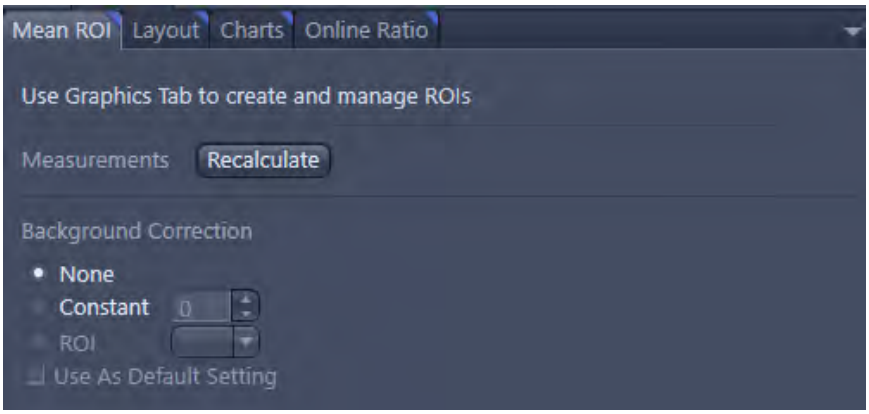
Prerequisites

- You are in the **MeanROI** view or **MeanROI Setup**.
- You have defined at least one ROI.

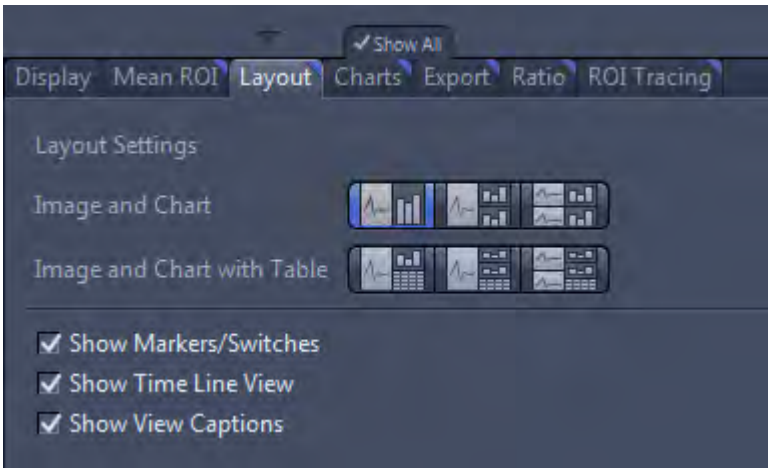
Procedure

1 Select the **MeanROI** tab in the view options.

2 If the number of images in your data set exceeds a given number, in the **Measurements** section, press **Recalculate**.



3 Select the **Layout** tab in the view options.

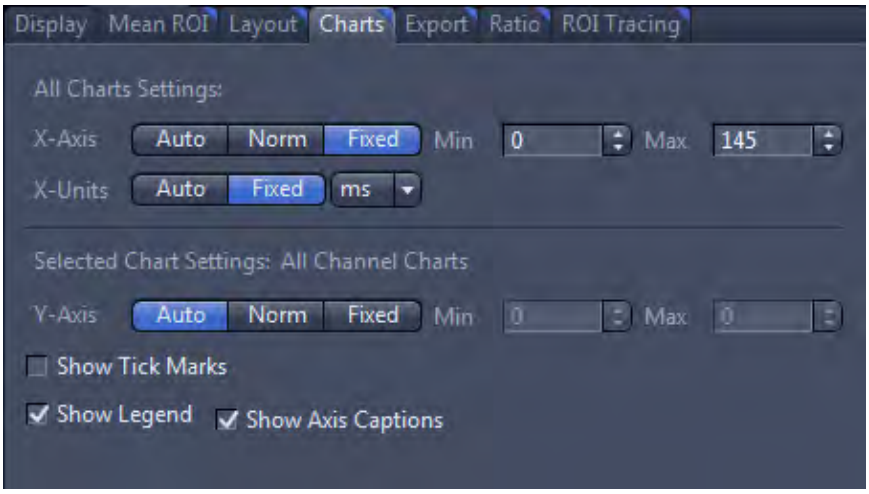


4 To adjust the layout of the image and diagram display, select the desired display mode under **Image and Chart**.

5 If you also want your data to be displayed in table form, select the desired display mode under **Image and Chart with Table**. Note that when the number of times points is >2000, the **Image and Chart** layout is not available. Export your data or, on the **Export** tab, create a separate data table.

6 For **Offline Analysis** only: Select a suitable layout for the image, diagram and table display.

7 If you want to adjust the axis scaling, switch to the **Charts** tab in the view options.



8 To define the minimum and maximum values of the axes manually, click on the **Fixed** button under **X-/Y-Axis**.

The **Min** and **Max** input fields for the axis are activated.

9 Enter the desired values first into the **Max** and then the **Min** input fields.

The minimum and maximum axis values of the diagrams are adjusted.

10 To change the unit of the X axis, click on the **Fixed** button under **X Units**.

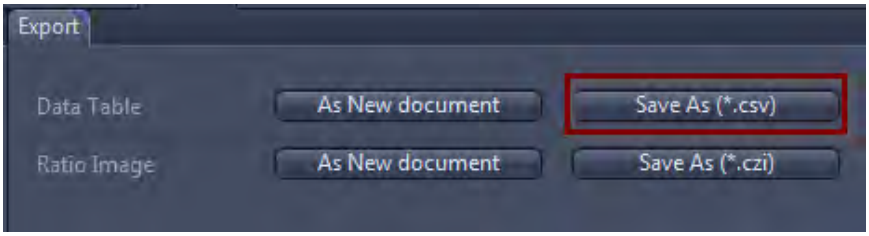
The dropdown menu for the units is activated. You can now select the desired unit.

You have successfully adjusted the display of the intensity values.

13.1.4 Exporting a Data Table

Prerequisites
- You are in the **MeanROI** view.
- You have defined at least one ROI.

Procedure
1. Select the **Export** tab in the View Options.
2. In the **Data Table** section click on the **Save As (*.csv)** button.



The **Save As** dialog opens.

3. Enter a suitable file name, navigate to the desired folder and click on **Save**.

All the measurement data are saved as comma-separated values in a csv file. This contains the time information, the area of the ROIs and the values for three types of intensity measurements (Mean, Intergral, Maximum) for each channel and each ROI.

13.1.5 Sample Experiment

13.1.5.1 Step 1: Creating channels

Prerequisites
- To perform the experiment, you need the **Dynamics** module.
- You have a **Sutter DG4/5** with appropriate excitation filters for **Fura-2** and a **Fura-2 filter set** in the microscope's reflector wheel.
- You are on the **Acquisition** tab.

Procedure
1. Create a new experiment in the **Experiment Manager**, e.g. "**Physiology Fura-2**".
2. Add the channel Fura-2 using **Smart Setup**.
3. Activate the **Time Series** checkbox in the acquisition dimensions.
4. Open the **Channels** tool.
5. Select the Fura-2 channel from the list.
6. Click on the **Options** button and select the **Duplicate** entry.
7. Select the first Fura-2 channel from the list.

8 Click on the **Options** button  and select the **Rename** entry.

You can now rename the channel, e.g. **Fura-2 340 nm**.

9 Repeat steps 7 and 8 to rename the second channel, e.g. **Fura-2 380 nm**.

10 Select the **Fura-2 380 nm** channel.

11 Select another LUT from the dropdown list, e.g. red.

12 Select the entry **21 HE Ex. FURA 380** from the Excitation dropdown list.

The excitation filter is used for this channel.

13 Adjust the exposure time and focus for both channels.

You have created the channels for your experiment.

13.1.5.2 Step 2: Setting up a time series and creating switches

Procedure

1 Open the **Time Series** tool.

2 Using the **Duration** slider and the dropdown list for the unit, specify the duration of the experiment, e.g. 10 min.

3 Using the **Interval** slider and the dropdown list for the unit, specify the length of the interval between acquisitions, e.g. 1 second.

4 To create interactive switches open the **Interactive Switches** section in the **Time Series** tool. This section is visible only if the **Show All** mode is activated.

5 Click on the **Add** button  .

A new switch is added.

6 Edit the switch by clicking on the black arrow to the right of the switch.

The switch properties are visible.

7 Enter a name, e.g. **Fast**. Activate the **Color** checkbox and select a color, e.g. blue. Define an action to be performed when you activate the button, e.g. **As fast as possible**.

You have successfully set up a time series and created a switch.

13.1.5.3 Step 3: Setting up an online ratio

Procedure

1 Open the **MeanROI setup** tool.

Snaps of the configured channels are acquired automatically and displayed in the Center Screen Area. The diagrams for each image are displayed to the right of this.

2 Select the **Ratio** tab from the view options and activate live ratio generation.

3 Under **Method** select the **Dual Wavelength** entry from the dropdown list.

4 Under **Calculation** select the **Fura-2 340 nm** entry from the dropdown list in the numerator of the formula.

5 Under **Calculation** select the **Fura-2 380 nm** entry from the dropdown list in the denominator of the formula.

A preview of the ratio image, which is calculated according to the ratio settings, is displayed.

You have successfully activated the ratio functions and specified the calculation of the ratio.

13.1.5.4 Step 4: MeanROI Setup

Procedure

1 In the **Dynamics** tool click on the **MeanROI Setup** button.

2 On the **Graphics** tab, select a tool for drawing in ROIs, e.g. Circle.

3 Activate the **Keep Tool** checkbox.

4 Draw your ROIs into one of the images.

5 Deactivate the **Keep Tool** checkbox and select the selection tool (**arrow**) again.

6 Under **Measure** on the **Mean ROI** tab select the type of intensity measurement to be displayed, e.g. Mean Intensity.

7 On the **Layouts** tab select a layout for the image and diagram display, e.g. Multi-Image Multi-Chart.

8 Go to **Charts** tab and click on the **Fixed** button under **X Units** and select a unit from the dropdown list, e.g. seconds.

9 Click on **Exit** at the top left of **MeanROI Setup**.

You have successfully configured and adjusted the MeanROI Setup.

13.1.5.5 Step 5: Starting, analyzing and influencing an experiment

Procedure

1 Start the experiment by clicking on the **Start Experiment** button.

The experiment is started. In our example an image is acquired every second for a period of 10 minutes. The experiment opens in the online mode of the MeanROI View, which displays the current images and measurements.

2 Activate the created switch at the desired time point. To do this, open the **Switches** section in the **Time Series** tool. Click on a switch as soon as you want its action to be performed, e.g. click on the "**Fast**" switch to acquire the subsequent images as quickly as possible one after the other. A marker will mark the time point at which the switch was activated on the X axis in the color of the switch (e.g. blue).

3 Once the time series has been completed you can analyze the experiment in the offline mode of the **MeanROI** view, process it and export its values.

You have successfully performed the experiment.

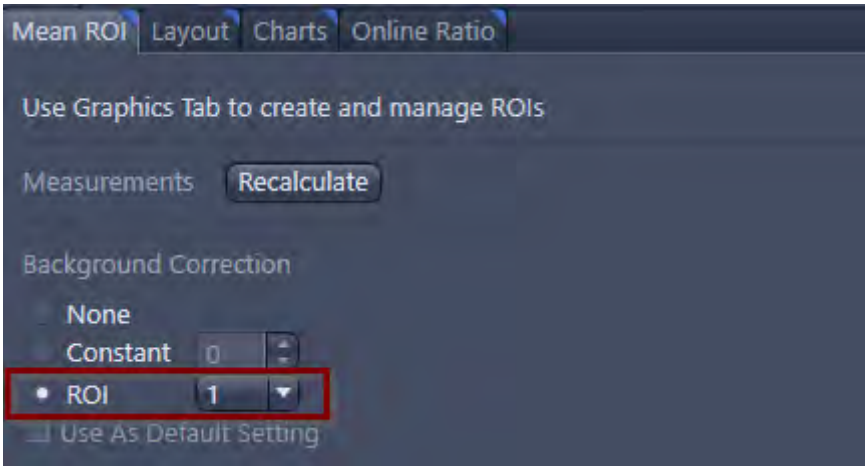
13.1.6 Using Background Correction

Use this function to subtract background values from the measurement values. A background correction will allow you to make a better comparison of the magnitude of any fluorescent intensity changes observed over the time course of an experiment. Determine the background value with the help of a Background ROI or define a fixed value.

Defining a Background ROI

Prerequisites
- You are in the **MeanROI** View or **MeanROI** Setup.

Procedure
1. At the first time point of the time series draw an **ROI** into a region of the image that contains only background signal in all channels.
2. Go to the **MeanROI** tab | **Background Correction** section and activate the radio button **ROI**.
3. Select the ROI-ID of the ROI that you defined in the first step.



4. To edit the **Background ROI**, simply draw a new ROI and select it from the dropdown list.

You have successfully defined a **Background ROI**. The mean intensity of the background ROI is subtracted from the measured values of the **ROIs** in a channel- and time-point-specific way. The corrected values are adopted into all diagrams and tables.

Defining a fixed background value

Prerequisites
- You are in the **MeanROI** View or **MeanROI** Setup.

Procedure
1. On the **MeanROI** tab in the **Background Correction** section select the **Constant** option.

   The associated input field is activated.
2. Enter a fixed background value into the **Constant** input field.
3. Press *Enter* to update the measurements.

The defined background value is subtracted from all measured values of the **ROIs**.

13.1.7 Calculating Ratios

13.1.7.1 Calculating a Ratio for One Wavelength

Prerequisites

- To calculate ratios (quotient of two fluorescence intensities) and display ratio images, you need the **Dynamics** module.
- You have a suitable image date set open.
- You are in the **MeanROI View** on the **Ratio** tab (view option).

Procedure

1. In the **Method** dropdown list select the **Single Wavelength (F/F_0)** entry.
2. In the **Calculation** dropdown list select the channel for calculating the ratio.
3. In the **Reference image (Ft_0) Set-up**, define the frames of the time series image from which you want the reference value Ft_0 to be calculated.
4. Click on the **Recalculate** button.

   The ratio values are calculated. The ratio image and a diagram for the ratio values are displayed in the MeanROI View.

You have successfully calculated a ratio for a single wavelength dye such as Fluo-4.

13.1.7.2 Calculating a Ratio for Two Wavelengths

Prerequisites

- To calculate ratios (quotient of two fluorescence intensities) and display ratio images, you need the **Dynamics** module.
- You have a suitable image date set open.
- You are in the **MeanROI View** on the **Ratio** tab (view option).

Procedure

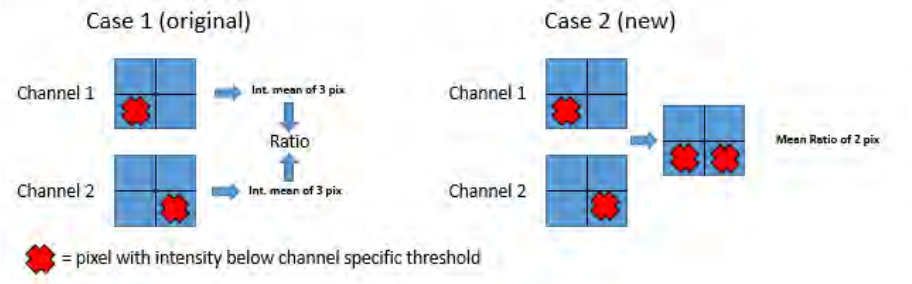
1. In the **Method** dropdown list select the **Dual Wavelength** entry.
2. In the **Calculation** dropdown lists select the channels for calculating the ratio.
3. Click on the **Recalculate** button.

   The ratio values are calculated. The ratio image and a ratio diagram are added to the MeanROI View.

You have successfully calculated a ratio for a dual wavelength dye such as Fura-2..

13.2 Calculation of ratio values

ZEN (blue edition) supports two approaches for the calculation of ratio values depending on how thresholded pixel values are handled. Here, Case 1 and Case 2. The illustration shows how these approaches differ for a dual wavelength ratiometic experiment.



The following example shows how these two approaches influence the ratio value. Generally, the second method gives more realiable results if objects move outside the ROI or if the ROI is drawn larger than the object. Hence, case 2 is a good choice if your objects move a laterally during the experiment or if size fluctuations are expected, e.g. contraction.

Consider a region of interest that is 6 pixels wide by 1 pixel high. The pixels of the region in the Wavelength 1 image are as follows:
[50, 75, 100, 125, 150, 175].

For the purpose of the example, assume the threshold = 60

ZEN thresholds Wavelength 1 to obtain:
[--, 75, 100, 125, 150, 175].

ZEN averages all of the valid (not thresholded away) pixels to produce the average intensity of Wavelength 1. In this case, the valid pixels are 75+100+125+150 +175, divided by a count of 5 valid pixels, results in the Average for this region in Wavelength 1 of 125.

The pixels of the region in the Wavelength 2 image are as follows:
[25, 25, 25, 25, 100, 100].

For the purpose of the example, assume the threshold = 50. ZEN thresholds Wavelength 2 to obtain:
[---, ---, --- , ---, 100, 100].

ZEN averages all of the valid (not thresholded away) pixels to produce the average intensity of Wavelength 2. In this case, the valid pixels are 100 + 100, divided by a count of 2 valid pixels, results in the average for this region in Wavelength 2 of 100.

ZEN compute the Ratio by only rationing the averaged values for the valid pixels in each individual wavelength. To recap, the pixels for each wavelength were:

[--, 75, 100, 125, 150, 175] (Wavelength 1)
[---, ---, --- , ---, 100, 100] (Wavelength 2)

The ratio is equal to 125/100 = 1.25. This is case 1.

If ratio values are needed in which the area is corrected based on the valid pixel found from both wavelengths, a slightly different result is given as follows. Using the same threshold values as above:

The pixels that are rationed are:
[--, --, --, --,150/100, 175/100].

Which gives a ratio of 1.625. This is case 2.

In order to make use of Case 1 or 2, follow these steps:

Case1 follows the original methods described in the user manual (f1: help file)

Once the ratio calculation has been done, a data table can be created that contains the mean intensities from each channel corrected by the threshold. For each time point, a ratio value is given calculated as described (case 1)*.

Case 2 requires some additional steps described below:

Procedure

1. After calculating a ratio in meanROI view using the desired thresholding values, background correction etc., select the **Export** tab below the images.
2. Use the function Ratio image **As new document** by clicking on the button.

   A copy of the ratio image for all time points (ratio pairs) using the current settings/parameters previously defined is extracted. A new image container opens.
3. If necessary, delete the ROI used for background correction as it will no longer be needed.
4. Switch to **MeanROI** view. In the **Export** tab, click on the function data table **Create**.

A data table opens that includes for each ROI the following information measurements:

- Geometric area of ROI (constant)
- Threshold (wavelength 1 & 2) corrected area (variable from time point to time point)
- Ratio values per time point calculated according to case 2*

* In both instances, press the **Save As (*.csv)** button on **Export** tab, alternatively to export the data as a comma separated values (*.csv) file.

13.3 Workflow Dynamics Experiments

13.3.1 Introduction

Using the **Dynamics** tool **MeanROI** setup, you can specify user-defined measurement regions (ROIs) before the acquisition of your time lapse experiment and analyze their time-dependent changes in intensity online during acquisition. Ratios can also be calculated and displayed online - these are the typical functions used in physiology experiments.

Before the experiment

A precondition for a physiology experiment is a **Time Series experiment,** which is set up in the **Time Series** tool. The tool contains the button for opening **MeanROI Setup**. There you can draw in ROIs and adjust the display layout of the measurement results. When Setup is opened a snap is automatically acquired, on the basis of which you can configure the settings for the subsequent experiment. The structure of MeanROI Setup is based on the MeanROI View making it easier to learn.

During the experiment

After being started, physiology experiments are displayed in the online mode of the **MeanROI View**. This allows you to analyze and follow the experiment during acquisition. The structure and options largely correspond to the offline mode of the **MeanROI View**. We therefore recommend that you familiarize yourself with the MeanROI View (offline) before performing your Physiology experiment.

After the experiment

After you have performed your Physiology experiment the data are displayed in the offline mode of the MeanROI View and can be analyzed, processed and exported there. Further information on this can be found under: Use MeanROI View (offline).

Prerequisites

- To perform physiology experiments, you need the **Dynamics** module.
- You have *created a new experiment* [▶ 46], *defined at least one channel* [▶ 45] and adjusted the focus and exposure time.
- You are on the **Acquisition** tab.

Procedure

1. Activate the **Time Series** tool in the **Acquisition Dimensions** section.

   The **Time Series** tool appears in the **Left Tool Area** under **Multidimensional Acquisition**.

2. Enable the **Dynamics** tool in the experiment manager.

   The dynamics tool now appears in the in the **Left Tool Area** under **Applications**.

> **INFO**
>
> The **Dynamics** tool is not available if the **Tiles** or **Panorama** dimensions are activated. Deactivate these dimensions to make the **Dynamics** tool available.

3. Set up a *time series experiment* [▶ 54].
4. Open the **Dynamics** tool.
5. Click on the **MeanROI Setup** button.

You have completed the general prerequisites for dynamics experiments.

13.3.2 Setting up the Online Ratio Calculation

Prerequisites

- You have read the *Introduction* [▶ 394] chapter.

Procedure

1. In the **MeanROI** setup, select the **Online Ratio** tab in the view options .
2. Activate the **Activate Live Ratio Generation** checkbox.
3. In the **Method** dropdown list select a method for the ratio calculation.

> **INFO**
>
> If you select the **Single wavelength (F/F0)** method for the calculation of the online ratio, you need to define a reference image. To define a reference image, indicate the number of images from which the reference image should be averaged in the input field of **Reference Image Set-up** in the **Online Ratio** tab. Then click on the **Define** button to acquire the images and calculate a reference image from them.

4. If you want to use background correction for the calculation of the online ratio, activate the desired entry under **Background Correction.**

   To allow you to indicate a constant background value (**Constant** entry), an input field, in which you can enter the desired value, appears under **Calculate** in the formula for the ratio calculation.

   The **ROI** entry can only be selected once you have defined a ROI in your image.
5. Under **Calculate** complete the formula for calculating the online ratio by selecting the desired entries from the dropdown lists and indicating values in the input fields.
6. Activate the **Threshold** checkbox, if you want to set a threshold in your experiment.

> **INFO**
>
> In ZEN the calculation of the ratio value R of an individual pixel xy in case of a dual wavelength dye is determined as followed:
>
> $$Rxy = \frac{Ch1xy}{Ch2xy}$$
>
> where:
> Ch1xy = 0 **if** (Ch1xy - background of Ch1) < Threshold of Ch1
> Ch1xy = Ch1xy - background of Ch1 **if** (Ch1xy - background of Ch1) > Threshold of Ch1
> The same is true for Ch2xy using the corresponding values for background and threshold.

7 You can adjust the settings for the ratio calculation as required and the preview image on the MeanROI setup is adapted accordingly. Press the **Snap** button if you need to update the images.

You have successfully activated/adjusted the calculation of the online ratio.

13.3.3 Setting up an Experiment in MeanROI Setup

Prerequisites

- You have read the *Introduction* [▶ 394] chapter.

Procedure

1 Activate the Dynamics checkbox in the Experiment Manager.

2 In the **Dynamics** tool, click on the **MeanROI Setup** button.

MeanROI Setup is opened.

An image is acquired automatically, on the basis of which you can configure your settings. You can click on **Snap** at any time to update the image.

13.3.3.1 Drawing in ROIs

Prerequisites

- You are in the **MeanROI View** or in the **MeanROI Setup** on **Acquisition** tab.

Procedure

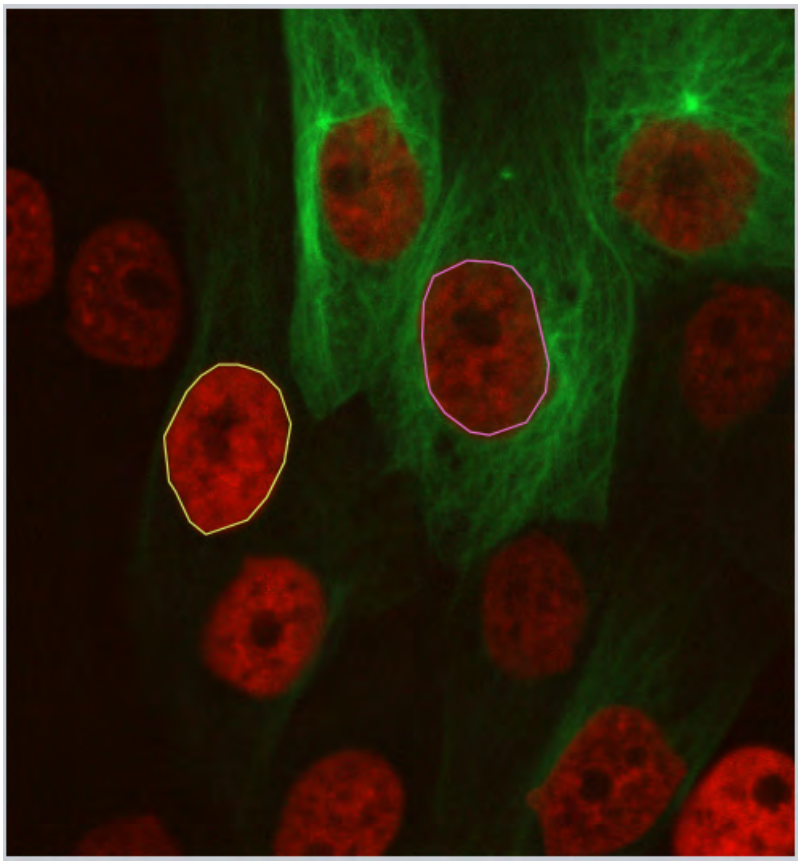
1 Go to the **Graphics** tab in the **View Options**.

2 Select a tool for drawing in **ROIs**, e.g. the **Polygon** tool.

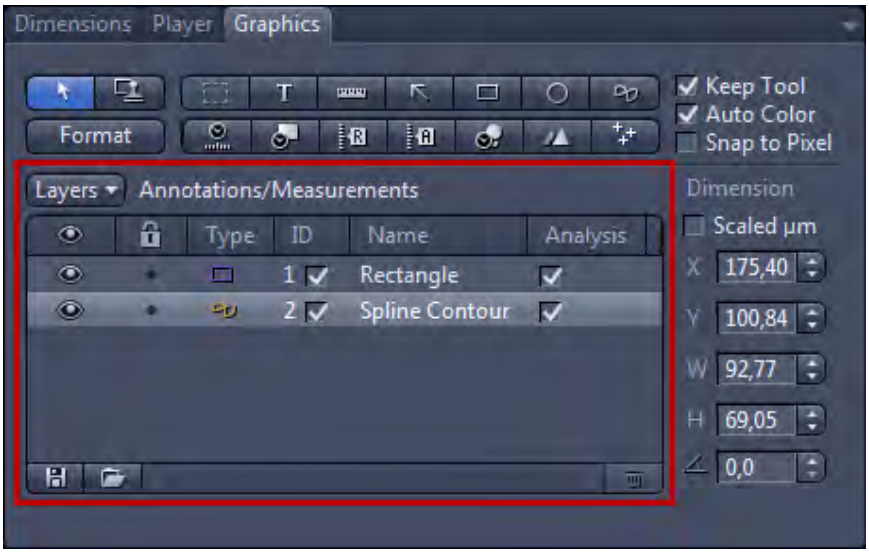
3 Activate the **Keep tool** checkbox.

The selected tool remains active after you have drawn in an **ROI**. This means you can draw in several ROIs without having to re-select the tool.

4 Using the selected tool, in the image view draw in the objects or regions (ROIs) for which intensity measurements are required.



The **ROIs** are displayed in the list (Annotations/ Measurements Layer) on the **Graphics** tab.



Intensity measurements are performed for each **ROI** and displayed in the diagram area to the right of the image view.

You have successfully defined measurement regions for the intensity measurement.

13.3.3.2 Adjusting the Display

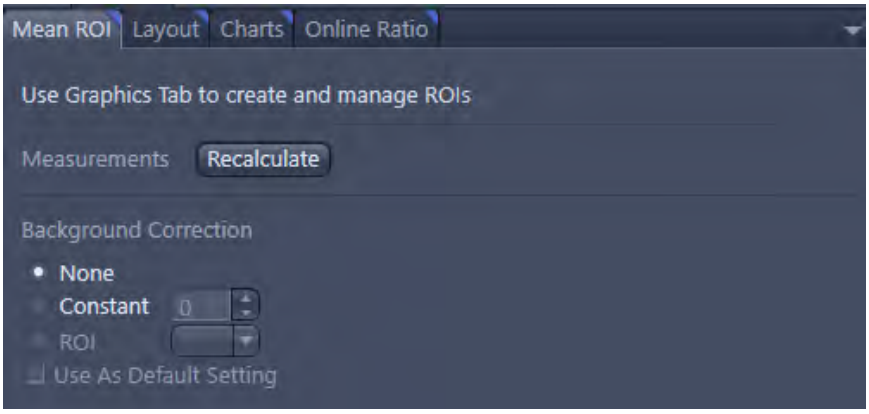
Here you will find out how to adjust the display of the measured intensity values in diagrams and tables according to your wishes.

Prerequisites

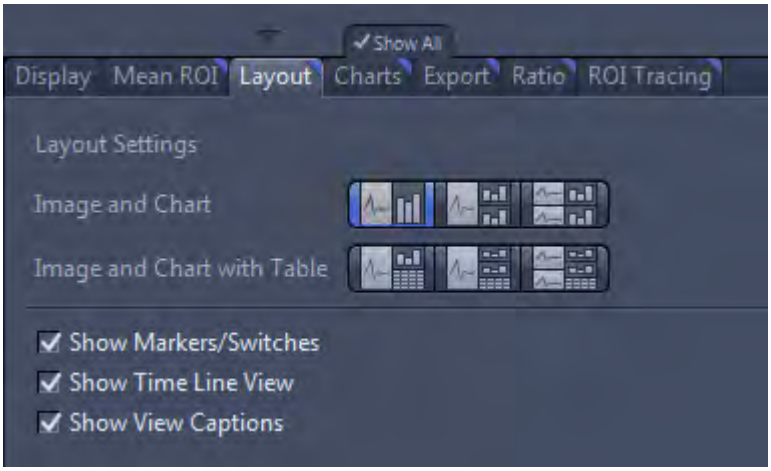
- You are in the **MeanROI** view or **MeanROI Setup**.
- You have defined at least one ROI.

Procedure

1. Select the **MeanROI** tab in the view options.
2. If the number of images in your data set exceeds a given number, in the **Measurements** section, press **Recalculate**.

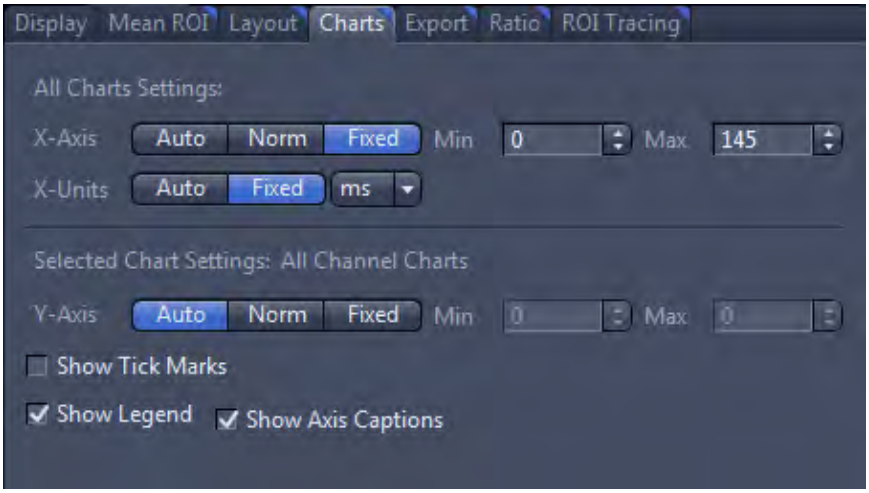


3. Select the **Layout** tab in the view options.



4. To adjust the layout of the image and diagram display, select the desired display mode under **Image and Chart**.
5. If you also want your data to be displayed in table form, select the desired display mode under **Image and Chart with Table**. Note that when the number of times points is >2000, the **Image and Chart** layout is not available. Export your data or, on the **Export** tab, create a separate data table.
6. For **Offline Analysis** only: Select a suitable layout for the image, diagram and table display.

7 If you want to adjust the axis scaling, switch to the **Charts** tab in the view options.



8 To define the minimum and maximum values of the axes manually, click on the **Fixed** button under **X-/Y-Axis**.

The **Min** and **Max** input fields for the axis are activated.

9 Enter the desired values first into the **Max** and then the **Min** input fields.

The minimum and maximum axis values of the diagrams are adjusted.

10 To change the unit of the X axis, click on the **Fixed** button under **X Units**.

The dropdown menu for the units is activated. You can now select the desired unit.

You have successfully adjusted the display of the intensity values.

13.3.3.3 Using Background Correction

Use this function to subtract background values from the measurement values. A background correction will allow you to make a better comparison of the magnitude of any fluorescent intensity changes observed over the time course of an experiment. Determine the background value with the help of a Background ROI or define a fixed value.

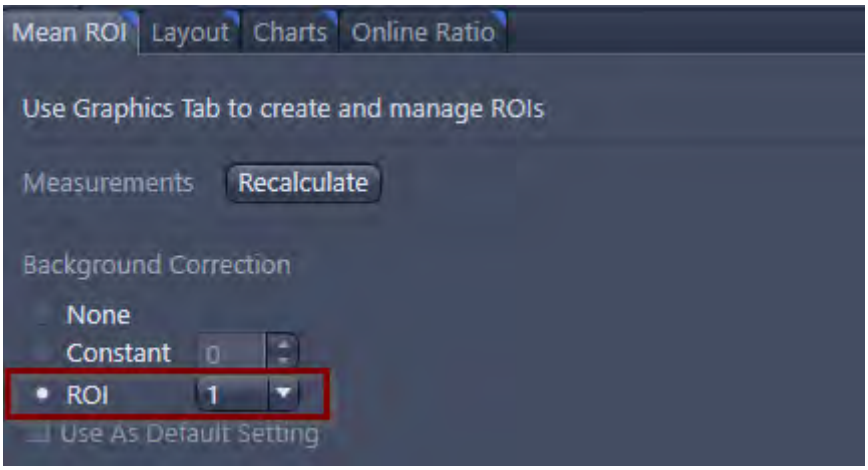
Defining a Background ROI

Prerequisites
- You are in the **MeanROI** View or **MeanROI** Setup.

Procedure
1 At the first time point of the time series draw an **ROI** into a region of the image that contains only background signal in all channels.

2 Go to the **MeanROI** tab | **Background Correction** section and activate the radio button **ROI**.

3 Select the ROI-ID of the ROI that you defined in the first step.



4 To edit the **Background ROI**, simply draw a new ROI and select it from the dropdown list.

You have successfully defined a **Background ROI**. The mean intensity of the background ROI is subtracted from the measured values of the **ROIs** in a channel- and time-point-specific way. The corrected values are adopted into all diagrams and tables.

Defining a fixed background value

Prerequisites
- You are in the **MeanROI** View or **MeanROI** Setup.

Procedure
1 On the **MeanROI** tab in the **Background Correction** section select the **Constant** option.

The associated input field is activated.

2 Enter a fixed background value into the **Constant** input field.

3 Press *Enter* to update the measurements.

The defined background value is subtracted from all measured values of the **ROIs**.

13.3.4 Starting and Influencing an Experiment

Prerequisites
- You have read the *Introduction* [▶ 394] chapter and set up an experiment in **MeanROI Setup**.
- You are on the **Acquisition** tab.

Procedure
1 Start your Physiology experiment by clicking on the **Start Experiment** button.

The time series experiment is started. The MeanROI View (online) opens and displays the current images and the intensity curves for each ROI measured online. The intensity curves are displayed in the Time Line View and in the diagrams.

INFO

Depending on your system performance there will be a short delay between the appearance of the MeanROI view and the start of image acquisition on the order of 2-3 seconds. This does not affect the image acquisition. Thus, this display delay should fall into the typically base line of this type of experiments.

2 You can pause the experiment at any time by clicking on the **Pause Experiment** button and continue it again by clicking on the **Continue Experiment** button.

3 The **Focus** can be adjusted during the experiment. To prevent images that are not sharp being acquired, pause your experiment and use the **Live** acquisition button to adjust the focus. Then continue the experiment. Note that using the Live view only works with experiments run in interactive mode.

4 Adjust the display of the intensity values during the experiment by changing the settings on the **Layout or Charts** tab. The unit of the X-axis cannot be changed during the experiment.

5 You can move and change ROIs during acquisition. The changes are adopted for all time points, see *Drawing in and Adjusting ROIs* [▶ 384]. Note that ROI tracing functions are only available after an acquisition.

6 Activate **Switches** in the **Time Series** tool during the experiment to perform the corresponding actions.

INFO

Various events, such as the activation of switches or the pausing of the experiment, are labeled in the Time Line view by markers.

7 On the **Dimensions** tab deactivate the **Follow Acquisition** checkbox to analyze the data acquired up to that point. To do this, select the corresponding time points using the **Time** slider, the diagram sliders or the Time Line view slider in the **MeanROI** view.

8 Change the size of the area marked in blue in the Time Line View to adjust the section displayed in the diagrams.

You have successfully started the experiment, analyzed it online and influenced it.

13.3.4.1 Adjusting ROIs during experiments

If objects move laterally in the course of the experiment, you can adjust the **ROIs** at any time during the experiment in order to follow the objects.

Prerequisites
- You have defined at least one ROI.
- You have started your Physiology experiment.

Procedure
1 In the **Experiment Manger** click on **Pause experiment** button.

2 Adjust the position of the **ROI** using drag & drop. To do this, select the ROI in the **image area** by left-clicking and hold the mouse button down. Then move the ROI to the new position and release the mouse button.

3 To change the shape of an **ROI** left click on an ROI and drag the bounds to adjust the size.

Changes to the position and shape of the **ROIs** are adopted for all time points.

4 Repeat the previous steps for all subsequent ROIs that you wish to adjust. Note that you can select multiple ROIs and adjust all the positions simultaneously.

You have successfully adjusted the measurement regions (ROIs) to the course of the experiment.

13.3.5 Sample Experiment Fura-2 with DG4/5

13.3.5.1 Step 1: Creating channels

Prerequisites

- To perform the experiment, you need the **Dynamics** module.
- You have a **Sutter DG4/5** with appropriate excitation filters for **Fura-2** and a **Fura-2 filter set** in the microscope's reflector wheel.
- You are on the **Acquisition** tab.

Procedure

1 Create a new experiment in the **Experiment Manager**, e.g. "**Physiology Fura-2**".

2 Add the channel Fura-2 using **Smart Setup**.

3 Activate the **Time Series** checkbox in the acquisition dimensions.

4 Open the **Channels** tool.

5 Select the Fura-2 channel from the list.

6 Click on the **Options** button  and select the **Duplicate** entry.

7 Select the first Fura-2 channel from the list.

8 Click on the **Options** button  and select the **Rename** entry.

You can now rename the channel, e.g. **Fura-2 340 nm**.

9 Repeat steps 7 and 8 to rename the second channel, e.g. **Fura-2 380 nm**.

10 Select the **Fura-2 380 nm** channel.

11 Select another LUT from the dropdown list, e.g. red.

12 Select the entry **21 HE Ex. FURA 380** from the Excitation dropdown list.

The excitation filter is used for this channel.

13 Adjust the exposure time and focus for both channels.

You have created the channels for your experiment.

13.3.5.2 Step 2: Setting up a time series and creating switches

Procedure

1. Open the **Time Series** tool.
2. Using the **Duration** slider and the dropdown list for the unit, specify the duration of the experiment, e.g. 10 min.
3. Using the **Interval** slider and the dropdown list for the unit, specify the length of the interval between acquisitions, e.g. 1 second.
4. To create interactive switches open the **Interactive Switches** section in the **Time Series** tool. This section is visible only if the **Show All** mode is activated.
5. Click on the **Add** button  .

   A new switch is added.
6. Edit the switch by clicking on the black arrow to the right of the switch.

   The switch properties are visible.
7. Enter a name, e.g. **Fast**. Activate the **Color** checkbox and select a color, e.g. blue. Define an action to be performed when you activate the button, e.g. **As fast as possible**.

You have successfully set up a time series and created a switch.

13.3.5.3 Step 3: Setting up an online ratio

Procedure

1. Open the **MeanROI setup** tool.

   Snaps of the configured channels are acquired automatically and displayed in the Center Screen Area. The diagrams for each image are displayed to the right of this.
2. Select the **Ratio** tab from the view options and activate live ratio generation.
3. Under **Method** select the **Dual Wavelength** entry from the dropdown list.
4. Under **Calculation** select the **Fura-2 340 nm** entry from the dropdown list in the numerator of the formula.
5. Under **Calculation** select the **Fura-2 380 nm** entry from the dropdown list in the denominator of the formula.

   A preview of the ratio image, which is calculated according to the ratio settings, is displayed.

You have successfully activated the ratio functions and specified the calculation of the ratio.

13.3.5.4 Step 4: MeanROI Setup

Procedure

1. In the **Dynamics** tool click on the **MeanROI Setup** button.
2. On the **Graphics** tab, select a tool for drawing in ROIs, e.g. Circle.
3. Activate the **Keep Tool** checkbox.

4 Draw your ROIs into one of the images.

5 Deactivate the **Keep Tool** checkbox and select the selection tool (**arrow**) again.

6 Under **Measure** on the **Mean ROI** tab select the type of intensity measurement to be displayed, e.g. Mean Intensity.

7 On the **Layouts** tab select a layout for the image and diagram display, e.g. Multi-Image Multi-Chart.

8 Go to **Charts** tab and click on the **Fixed** button under **X Units** and select a unit from the dropdown list, e.g. seconds.

9 Click on **Exit** at the top left of **MeanROI Setup**.

You have successfully configured and adjusted the MeanROI Setup.

13.3.5.5 Step 5: Starting, analyzing and influencing an experiment

Procedure

1 Start the experiment by clicking on the **Start Experiment** button.

The experiment is started. In our example an image is acquired every second for a period of 10 minutes. The experiment opens in the online mode of the MeanROI View, which displays the current images and measurements.

2 Activate the created switch at the desired time point. To do this, open the **Switches** section in the **Time Series** tool. Click on a switch as soon as you want its action to be performed, e.g. click on the "**Fast**" switch to acquire the subsequent images as quickly as possible one after the other. A marker will mark the time point at which the switch was activated on the X axis in the color of the switch (e.g. blue).

3 Once the time series has been completed you can analyze the experiment in the offline mode of the **MeanROI** view, process it and export its values.

You have successfully performed the experiment.

14 Module Shuttle & Find

14.1 Introduction

Fig. 14.1: SEM / LM system for correlative microscopy

The **Shuttle & Find** module is used for the relocation of sample positions in two different microscopes, e.g. a light microscope and a scanning electron microscope (SEM), and the correlation of two images to one merged image. This technique is called correlative microscopy or just "CorrMic". It is used to combine the two worlds of scanning electron microscopy and light microscopy and brings it together in one image.

The samples can be mounted in special designed correlative holder systems (with three correlative calibration markers) from ZEISS. Also user-defined holder systems with three calibration markers can be used. Biological samples are mainly deposited on cover glasses or on TEM grids. In contrast to biological samples, the shape and size of material samples vary strongly. In respect to these requirements, the correlative holders were designed accordingly.

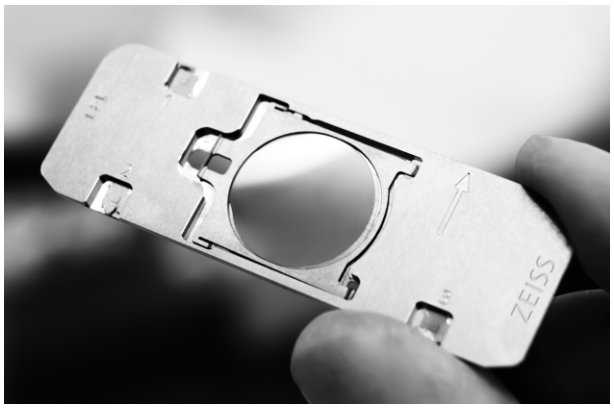


Fig. 14.2: Example of a correlative ZEISS sample holder

14.2 Workflow Shuttle & Find

14.2.1 Settings and Image Acquisition with the Light Microscope

Before acquiring an image with the light microscope and using it for correlative microscopy, it is necessary to make general settings e.g. stage calibration, camera orientation, calibrating objectives and setting the correct scaling. Please notice that we do not describe all theses topics within this guide as we focus on the Shuttle & Find workflow only.

Furthermore we will not describe basic functionality of the software in this guide, like program layout or general image acquisition topics.

14.2.1.1 Mounting the Sample Holder to the LM

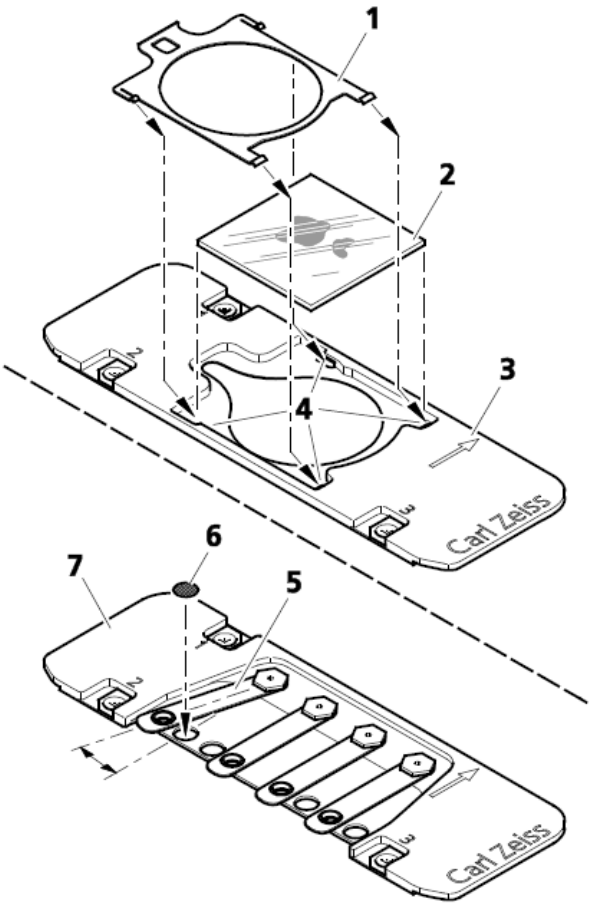


Fig. 14.3: Mounting of cover glasses or TEM grids

1 Place the cover glass **(2)** in the suitable sample holder and fix it.
In case of using the holder **Life Science Cover Glass 22x22**:

- Remove the clamping frame **(1)** using tweezers.
- Insert the cover glass **(2)** in the sample holder **(3)**.

– Slide in the clamping frame into the sample holder until the clamps are clicking into place **(4)**.

In case of using the holder **Life Science for TEM grids**:

– Lift the spring of the appropriate position and turn it sidewards **(5)**.

– Insert the TEM grid **(6)** into the provided holding spot of the holder and fix it with the spring **(7)**.

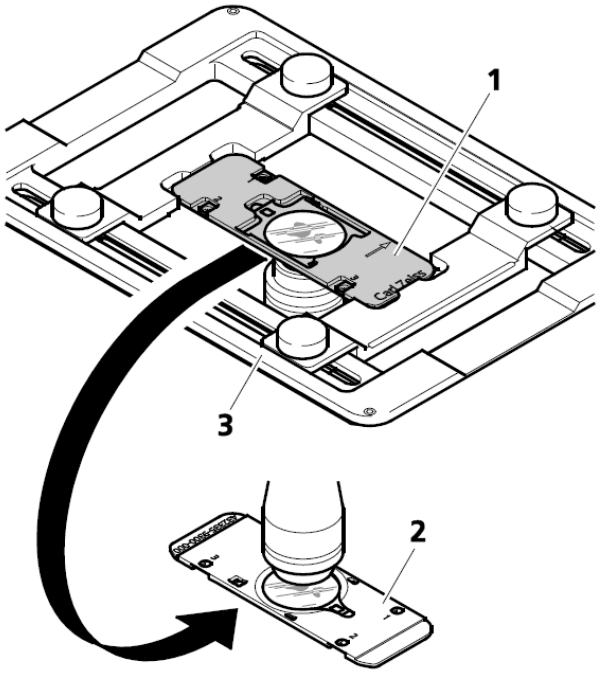


Fig. 14.4: Inserting a sample holder

2 Insert the sample holder **(1)** into the mounting frame of the microscope stage in the following way:

– For inverted stands, see **(3)**.

– For upright stands, see **(2)**.

14.2.1.2 Starting the LM Software

For correlative microscopy with light microscopes **ZEN (blue edition)** software has to be installed. In addition you need to licence the **Shuttle & Find** modul.

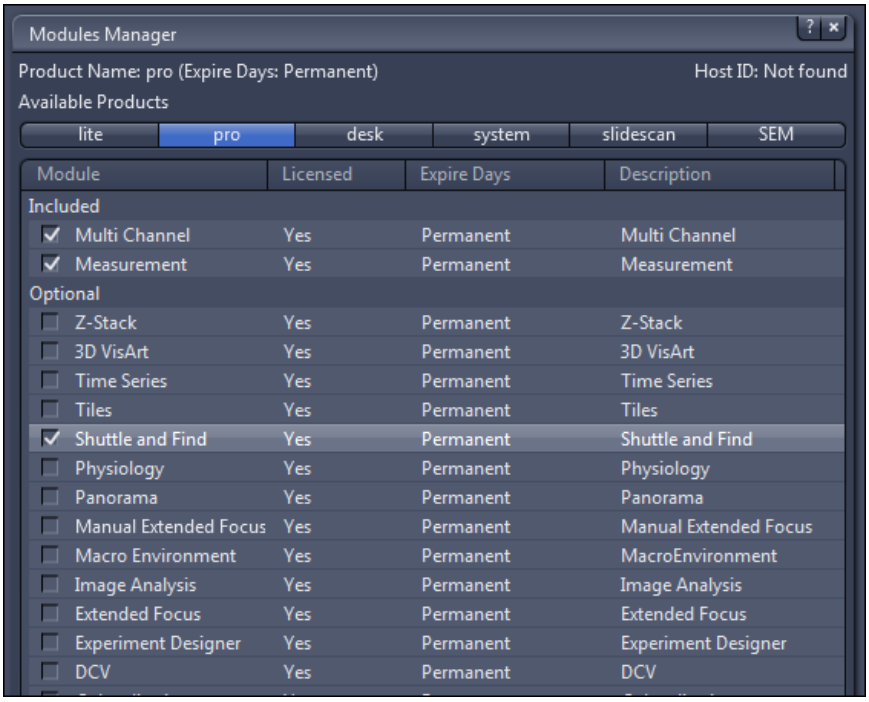
Procedure

1 To start the software click on the corresponding program icon on your desktop.

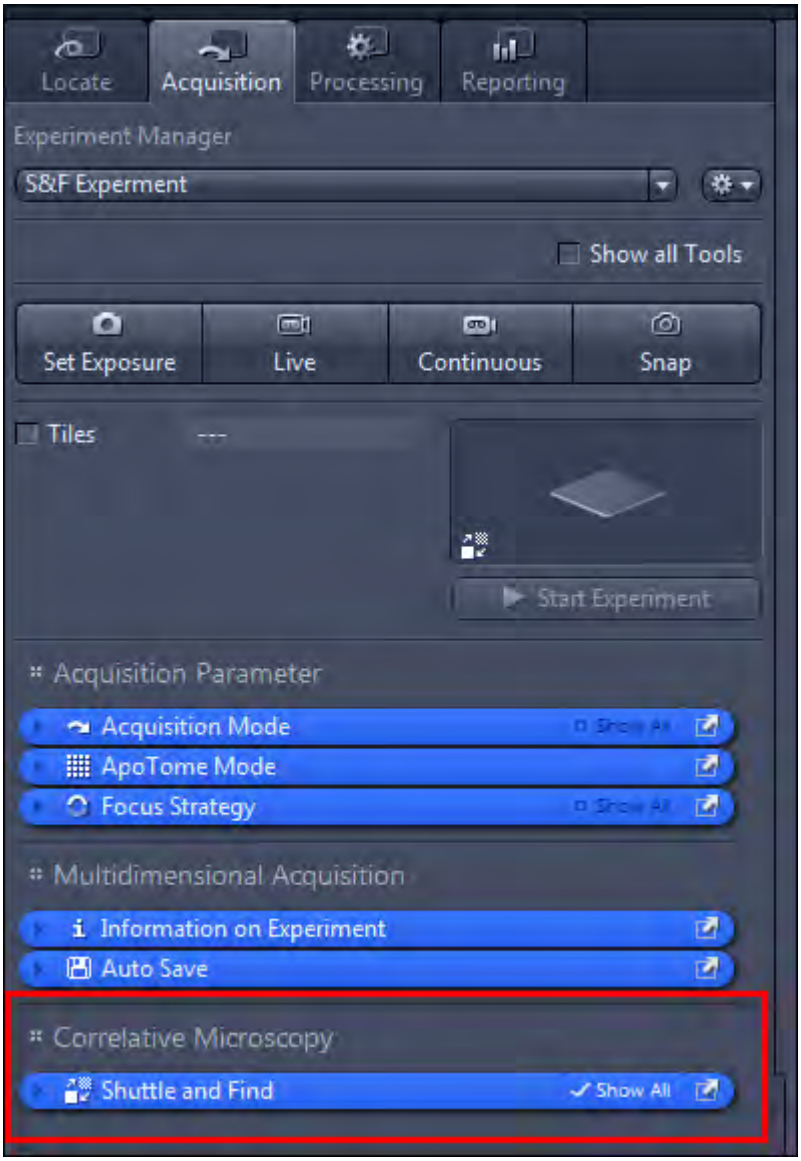
Following window will appear.

2 Click on **ZEN pro** or **ZEN system** to start the desired application.

The software will start now. Make sure that you have activated the **Shuttle & Find** module in the menu **Tools** | **Modules Manager ...**



3 In the **Left Tool Area** switch to **Acquisition** tab and open the **Shuttle & Find** tool.



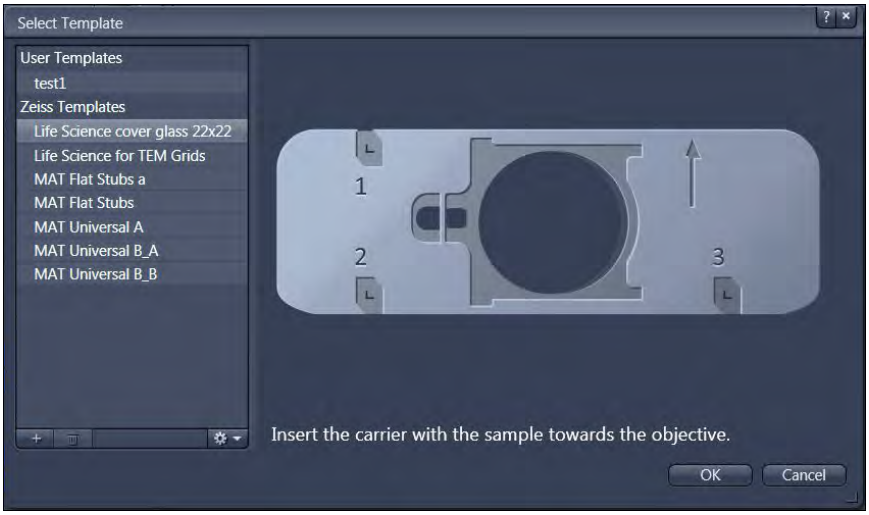
You have successfully started the software. Now you can start working with the **Shuttle & Find** module.

14.2.1.3 Selecting the Sample Holder

Prerequisites
- You have activated **Shuttle & Find** in the **Experiment Manager**.
- You are in the **Shuttle & Find** tool.

Procedure **1** Click on the **Select...** button to open the **Select Template** dialog and to choose the correlative holder you want to use. Different types of correlative holders are available, see Appendix Correlative Sample Holders

2 In the **Select Template** dialog select the correlative holder you want to work with. If you want use your own sample holders, click on the **+ (Add)** button below the list and follow the instructions in the chapter *Defining a new sample holder template* [▶ 411].



3 Click on the **Ok** button to close the dialog.

You can now continue with the calibration of the sample holder, like it is shown in the chapter *Calibrating the sample holder* [▶ 413]. The calibration of the sample holder is mandatory to acquire images.

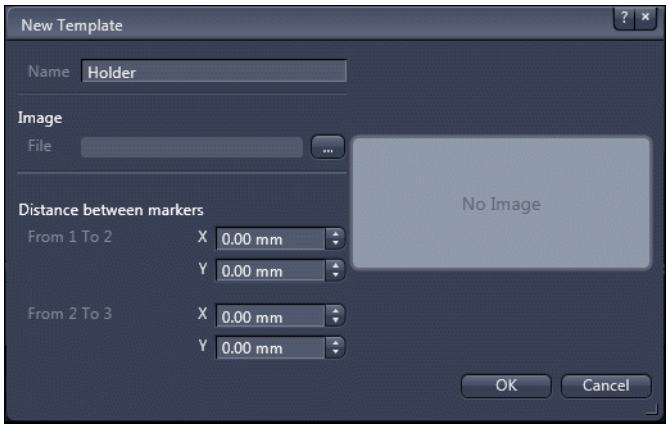
14.2.1.4 Defining a New Sample Holder Template

With this dialog you can define new correlative holders in addition to the existing holder templates. It is not mandatory to use correlative holders from ZEISS. User-defined correlative holders with 3 fiducial markers can be used as well.

Procedure

1 To open the dialog click on  **Add** in the **Select Template** dialog. This dialog can be opened via the **Shuttle & Find** tool.

The **New Template** dialog opens.



2 Type in a name for the new holder / sample carrier. An image of the new holder can be loaded as well.

3 Insert the distances (in millimeters) between the first and the second marker and between the second and third marker.

The distances can be determined using the **Stage Control** dialog accessible via the **Light Path** tool in **Right Tool Area** tab. We recommend to do this before you start the New template dialog. Write down the distances to be prepared to enter them within the New Template dialog.

1 Activate the live view in the Center Screen Area by clicking on the **Live** button in the Locate tab.

2 Navigate the stage manually to the calibration marker on the sample holder by means of the joystick and note the x/y-coordinates of the marker.

3 Repeat this procedure for all three markers and calculate the distances between marker 1 and marker 2 and between marker 2 and marker 3, respectively.

Fig. 14.5: Stage Tool

14.2.1.5 Calibrating the Sample Holder

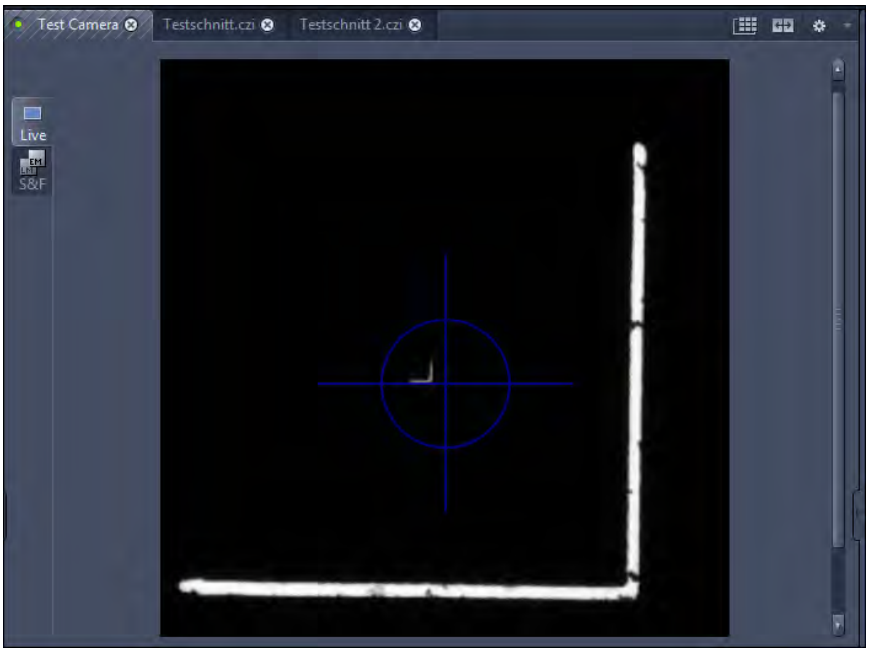
Correlative sample holders have three fiducial markers enabling a three point calibration (signed with the numbers 1-2-3) The calibration markers consist of one small (length 50 µm) and a large L-shape marker (length 1 mm). The bigger marker is used for coarse orientation, whereas the smaller marker is used for the calibration.

14.2.1.5.1 Preparing Calibration

Procedure

1 Click on **Live** in the **Acquisition** tab to activate the live view in the **Center Screen Area**.

2 Navigate the stage manually to the first calibration marker on the sample holder (marked with No. 1) by means of the joystick. It is enough if you move the stage to the larger L-shaped calibration marker. The smaller marker will be detected automatically within the **Sample Holder Calibration Wizard**.To locate the marker positions we recommend to use a dry objective with low magnification (5x – 20x).



3 Open the **Shuttle & Find** tool.

4 Click on **Calibrate…** to open the **Sample Holder Calibration Wizard.**

14.2.1.5.2 Setting Calibration Options

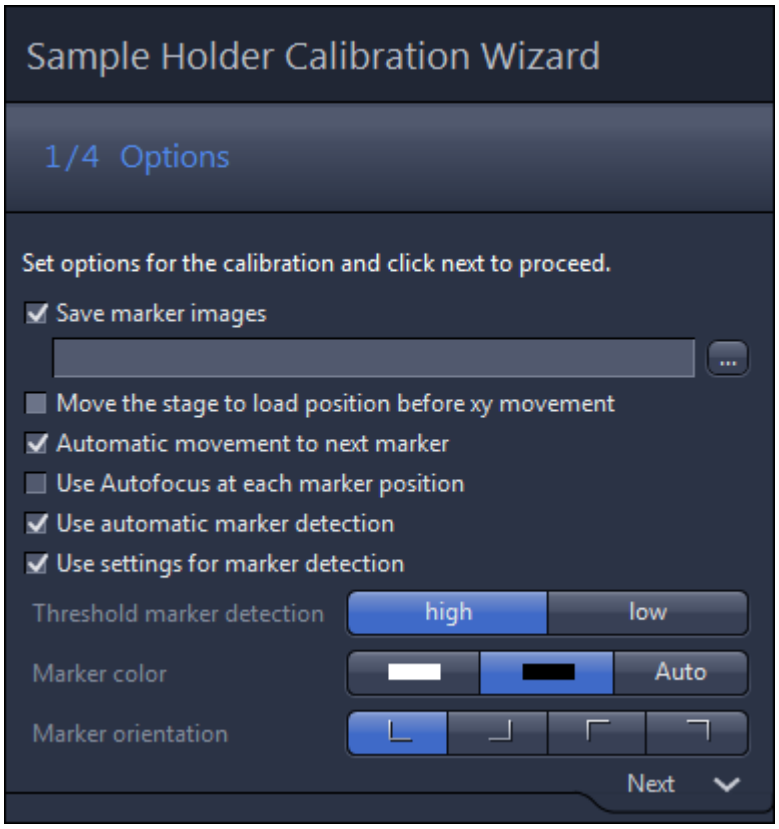


Fig. 14.6: Sample Holder Calibration Wizard Options

In step 1 of the wizard, the following options should be activated to follow our recommended workflow:

Procedure

1. Check if the **Automatic movement to next marker** checkbox is activated.

   This will automatically move the stage to the next marker position after you have confirmed the position of the marker and clicked on **Next**.

2. Check if the **Use automatic marker detection** checkbox is activated.

   The software will try to find the correct positions of each marker automatically.

3. If you need to change the marker color, or check if the marker orientation is set correctly, activate the **Use settings for marker detection** checkbox to access these functions.

4. Click on **Next** to move to the next wizard step.

14.2.1.5.3 Performing Calibration

Procedure

1. Click on **Set** to detect the first marker position.

   An automatic stage calibration will be performed. After the stage calibration, the system will try to detect the marker position of the small marker automatically.

   A message appears which asks if the marker was detected correctly.

2 Click on **Yes** to confirm the message.

INFO

If the marker was not detected correctly, you have the possibility to set the marker position manually. Therefore simply left-click on the intersection of the L marker in the image and the position will be adapted. Pay attention that you select always the same calibration point on each L-marker.

3 Click on **Next** to move to the next step of the wizard.

The stage will automatically move to the next (coarse) marker position. If the stage moves into the wrong direction you can use the **invert X** / **invert Y** buttons to correct the movement direction.

4 Repeat the previous steps and set marker position 2 and 3 accordingly.

After setting marker position 3 you will find a green check mark icon which shows that the calibration was successful.

5 Click on **Finish** to save the calibration and close the wizard.

To check if the calibration was successful acquire an image and open the **Tree** view in the Center Screen Area. There you should see the correlative calibration data in the list. If the Tree view is not visible go to **Tools** | **Options** | **Documents** and activate the **Enable Tree View** checkbox.

14.2.1.6 Acquiring the LM Image

Basically image acquisition is performed as you are used to do it within ZEN software. The file format for Shuttle & Find data is the common ***.czi** file format. Saved images can be loaded in ZEN via the menu **File** | **Open**.

After image acquisition the next step in the correlative workflow is to define / draw in ROIs / POIs in your image. Therefore you can use the **Region** tools on the **S&F** tab, see *Regions, Find and Dimensions* [▶ 428].

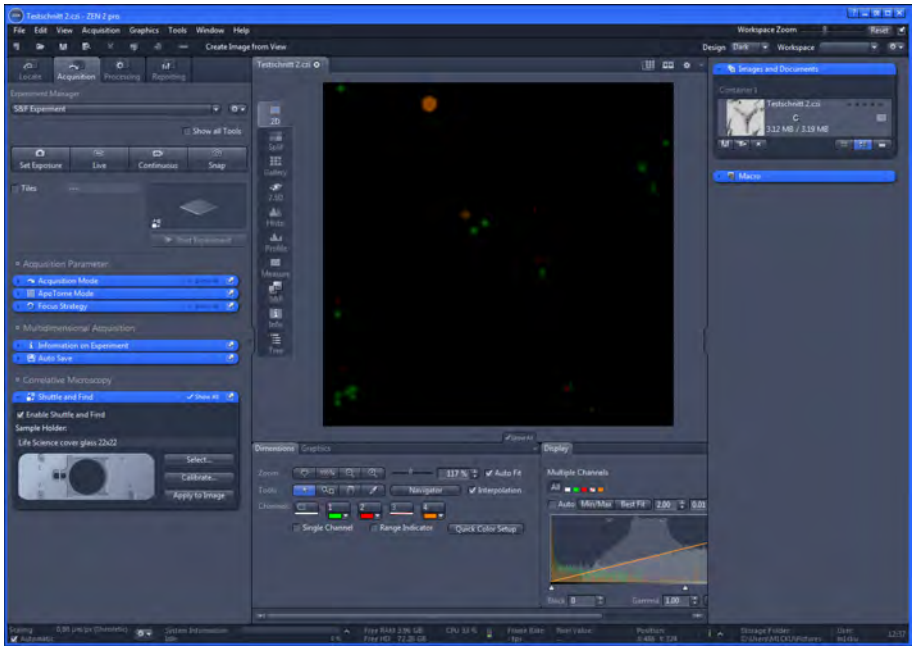


Fig. 14.7: LM image

14.2.2 Shuttle & Find Sample Positions at the Electron Microscope

Now you can transfer (Shuttle) the sample and the LM (Light Microscope) image file (**.czi**) to the SEM (Scanning Electron Microscope). There you can easily relocate (Find) the same sample positions and acquire a corresponding image within the ZEN **SEM** software. Therefore exactly the same steps have to be done as for the light microscope.

14.2.2.1 Mounting the Sample Holder to the SEM

For imaging your sample in the SEM, insert the sample holder **(2)** in the special SEM adapter **(1)** and mount it to the SEM.

i INFO

The arrow of the sample holder has to face the arrow of the SEM adapter.

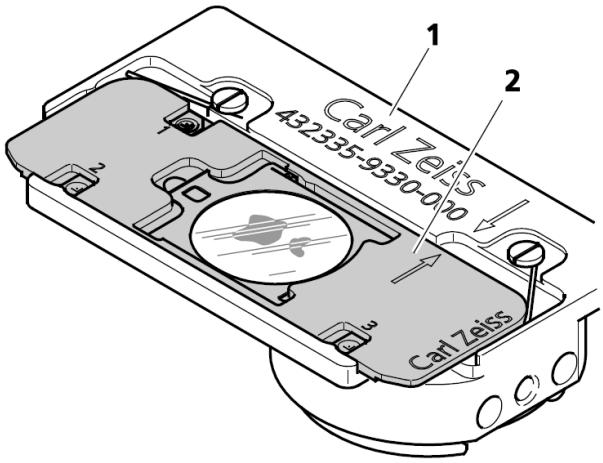


Fig. 14.8: Sample holder mounted in SEM adapter

14.2.2.2 Starting the ZEN SEM Software

For correlative microscopy with scanning electron microscopes **SmartSEM** and **ZEN (blue edition)** SEM have to be installed. SmartSEM is still the control software of the scanning electron microscope. **ZEN (blue edition) SEM** comes as an add-on for SmartSEM to perform correlative microscopy and using Shuttle & Find on a SEM.

Prerequisites

- You have started SmartSEM.

Procedure

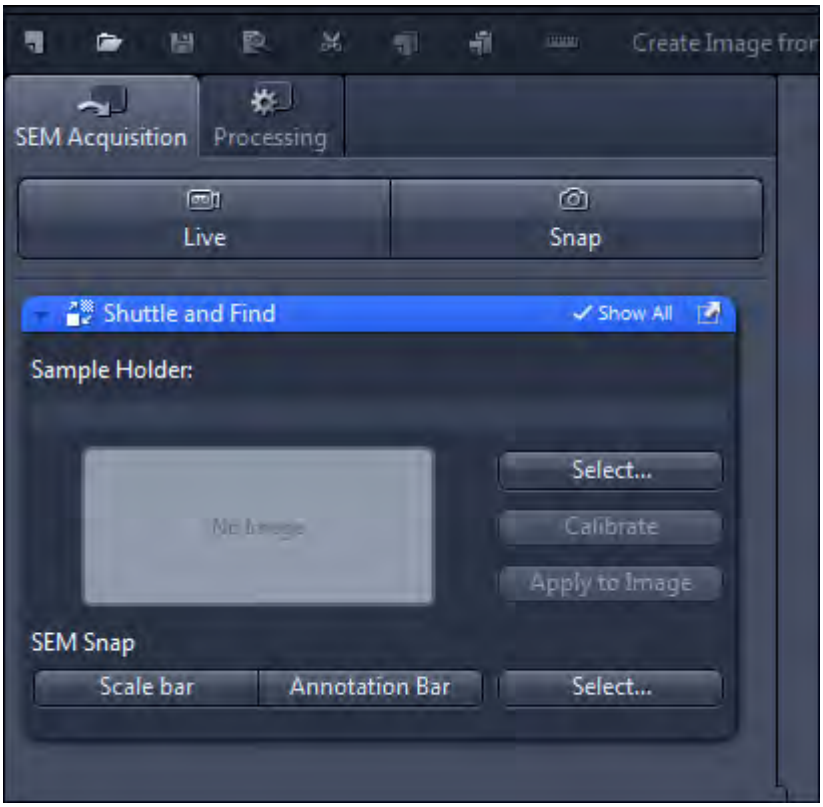
1. Start the **ZEN (blue edition)** software by clicking on the program icon on your desktop.

   Following window will appear.

2. Click on the **SEM** button to start.

   You will see the program interface with a reduced user interface comparing to **ZEN (blue edition)**. In the Left Tool Area the **SEM Acquisition** tab and the **Processing** tab are available only. On the SEM Acquisition tab you will find the

Shuttle & Find tool which has 3 additional buttons at the lower part of the tool.



14.2.2.3 Selecting the Sample Holder

This step is exactly the same step like for the light microscope, so please read the chapter *Selecting the Sample Holder* [▶ 410] if you want to know the exact steps which you have to perform.

14.2.2.4 Calibrating the Sample Holder

Like the step before this step is exactly the same like for the light microscopy, so please refer to the chapter *Calibrating the sample holder* [▶ 413] for details.

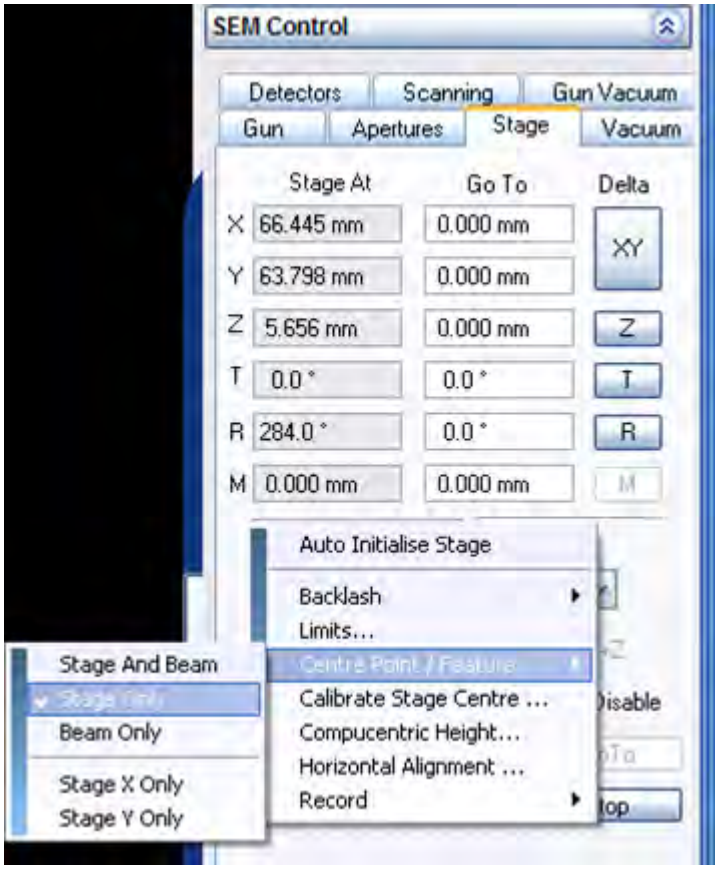
i INFO

The calibration of the sample holder has to be done on both systems the LM and the SEM. Otherwise the relocation of your sample positions or ROIs / POIs stored in the image won`t be successful.

INFO

Note that for Shuttle & Find the beam shift must be switched off. The beam shift is deactivated in **SmartSEM** as follows:

- Call up the shortcut menu **Center Point / Feature** by right-clicking on the **Stage** property page.
- Select **Center Point / Feature** and select **Stage only**.



14.2.2.5 Acquiring an EM Image

Procedure

1. Load your LM image to ZEN SEM (.**czi**).

   The image will be displayed in the center screen area.

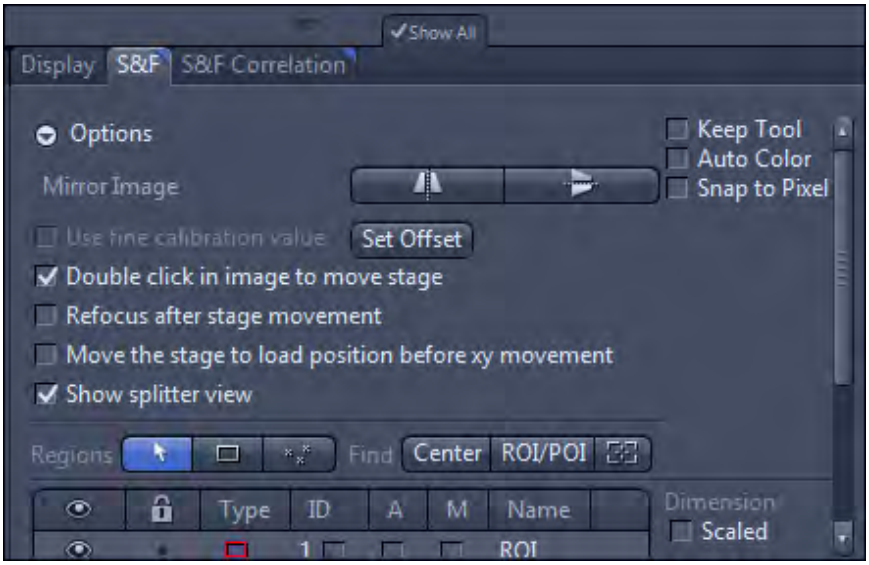
2. Activate the **Live** mode.

   You will see the Live image from the SEM. Notice that all settings for the SEM image have to be done within the SmartSEM software.

3. Activate the **S&F View** in Center Screen Area.

4. Go to the **S&F** tab.

5 Check if the **Double click in image to move stage** and **Show splitter view** checkboxes are activated (default setting).



In the left image container you see the live image from the SEM. The right image container is empty.

6 Drag the loaded LM image from the **Images and Documents** gallery into the empty image container.

Now you can easily relocate sample positions by double clicking within the image or on the ROI/POI button (if ROI / POI are drawn in and selected) on S&F tab.

For image acquisition you have to use the **Snap** button within ZEN SEM. Notice that we will not describe setup and image acquisition with the SEM. Please read the online help or user guide for the SEM software.

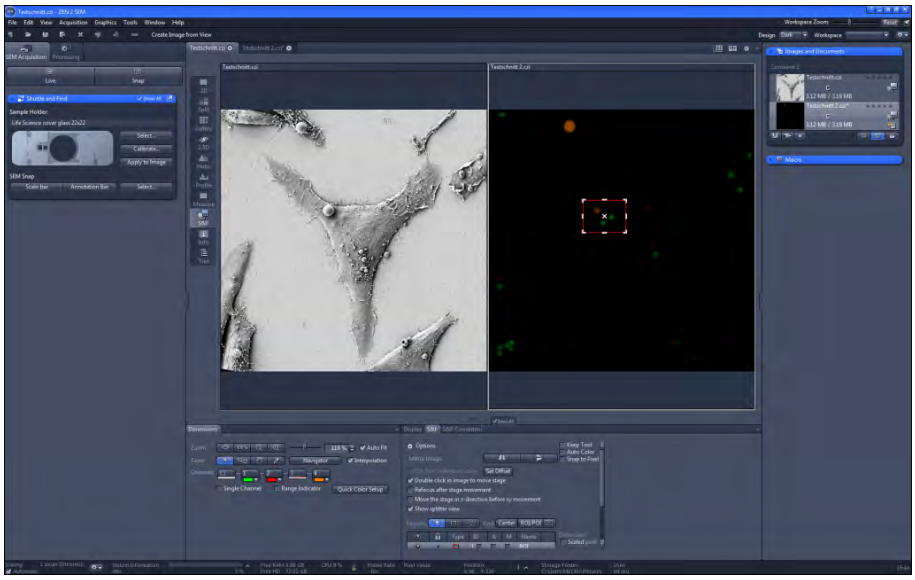


Fig. 14.9: SEM and LM image

14.2.2.6 Fine Calibration of the Sample Holder

The precision of relocation can be improved by determination of an offset value. This value describes the position offset between the loaded image and the live image. The defined offset value is only valid for the loaded image. If another image is loaded or if you close the dialogue, the offset value will be deleted.

Prerequisites

- An offset is visible when you try to relocate marker positions on the live image comparing to the LM image.

Procedure

1. Click on the **Set Offset** button.

   The stage moves to the selected marker position. Then a message appears which asks you to move the stage to the correct position.

2. Move the stage manually to the correct position by using the joystick.
3. Confirm the message by clicking on the **OK** button.

Now you can repeat the relocation. The positions should be identical now.

14.2.3 Image Correlation

14.2.3.1 Correlating Two Loaded Images

Prerequisites

- You have acquired and loaded two images containing S&F calibration data (e.g. LM / SEM) to be correlated. If the images are not oriented identically you can use the **Mirror Image** buttons under **Options** on the **S&F Correlation** tab.
- You see the two images next to each other (splitter view) in the center screen area. If not, drag your images from the **Images and Documents** gallery into the center screen area.

Procedure

1. Click on the **Set correlation points** button in the **S&F Correlation** tab.

   The cursor will change to a pipette symbol.

2. Click in the left image to set a correlation point. Set all 3 marker points in the left image first, before you set the corresponding 3 markers in the right image. If a correlation point is set, a check mark icon will appear in front of the corresponding point.

   Make sure that the positions in both images are identical. After you have set all 6 points the cursor will be changed backwards from the pipette to the arrow.

3. Click on the **Create Correlation** button.

The correlated image will be generated and opened in a new image container.

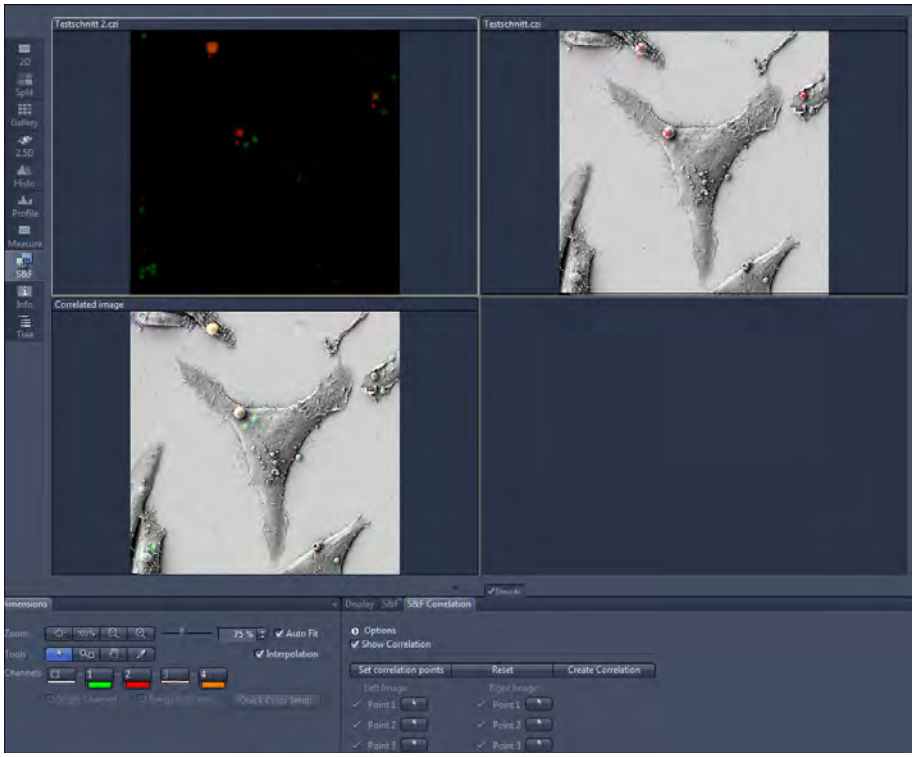


Fig. 14.10: Correlated image

Tips & Tricks

- It is also possible to set each correlation point individually. Therefore under **Left Image** / **Right Image** click on the **Arrow** button behind a point (e.g. **Point 1**). Then click on the desired position within the image.
- To improve the accuracy of the identification you can zoom into the images by using the mouse wheel.
- To edit/move a point, click on the point you would like to move. When the point is marked with a dashed rectangle you are able to move the point by holding the left mouse button. Alternatively, below **Left Image** / **Right Image** click on the points **Arrow** button you want to move and click on a new position within the image.

14.2.3.2 Correlation of Live Image and Loaded Image

Prerequisites

- You have activated the **Live** mode.

Procedure

1. Select the **S&F** view in the **Center Screen Area** and click on the **S&F Correlation** tab.

   The splitter view will become visible in the Center Screen Area. In the left image container you see the live image.

2. Drag the corresponding LM image from the **Documents and Images Gallery** into the Center Screen Area.

3 Click on **Set correlation points** button to set the correlation points. Always start with setting 3 points in the left (live) image, then continue with setting the identical points (in the same order) in the loaded image.

4 After setting all 6 correlation points the image correlation will be performed automatically.

The correlated image will be visible in a third image container below the live image and the loaded image.

14.3 Functions and Reference

14.3.1 User Interface

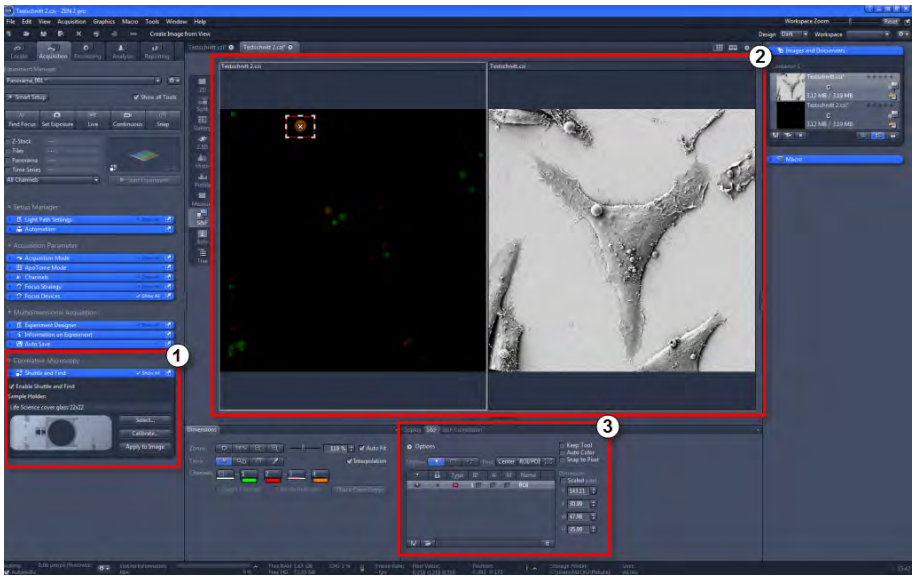


Fig. 14.11: User Interface

- **Shuttle & Find tool** in the Left Tool Area **(1)**
- **S&F view** in the Center Screen Area **(2)**
- **S&F** and **S&F Correlation view options** below the Center Screen Area **(3)**

14.3.2 Shuttle & Find Tool

Here you choose and calibrate your sample holders. The tool is visible only if you have activated the **Shuttle & Find** checkbox in the **Experiment Manager**.

Fig. 14.12: Shuttle & Find Tool

Parameter	Description
Sample holder	Here you see the name and preview of the selected sample holder.
Select...	Opens the **Select Template** dialog. There you select the preferred sample holder or define new holder templates, see *Selecting the Sample Holder* [▶ 410].
Calibrate...	Opens the *Sample Holder Calibration Wizard* [▶ 431]. There you can calibrate the selected sample holder.
Apply to Image	Only visible if the **Show All** mode is activated. **Use this button only, when you forgot to calibrate the holder before you acquire the image.** Applies a calibration to an acquired image. Do not remove the sample out of the correlative holder between image acquisition and calibration.

Shuttle & Find tool for SEM

Only visible if you have started the ZEN SEM software.

The tool window is adapted to the requirements of the correlative workflow on a SEM. Therefore three additional buttons are available.

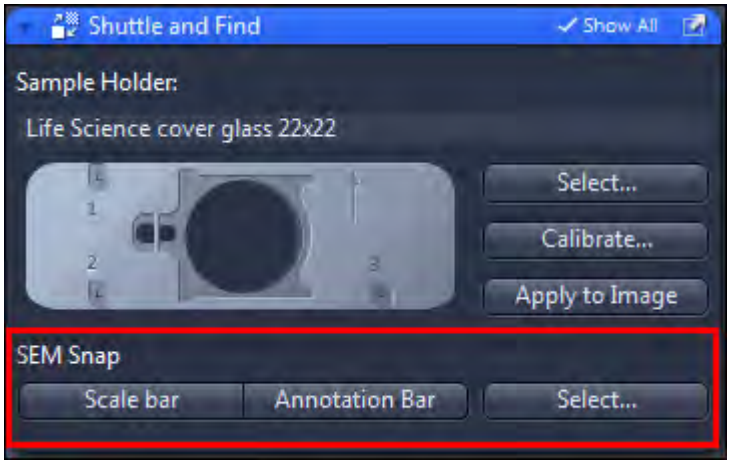


Fig. 14.13: Shuttle & Find tool in ZEN SEM software

Parameter	Function
Scale bar	Adds a scale bar to the snapped (acquired) image.
Annotation bar	Adds an annotation bar to the snapped (acquired) image.
Select...	By clicking on this button a dialog opens to select parameters for the annotation bar. You can select max. 9 parameters for the annotation bar.

14.3.3 Shuttle & Find View

Besides the **Shuttle & Find** tool in the **Left Tool Area**, the **S&F (Shuttle & Find)** view is visible in the **Center Screen Area** of the ZEN software. If the S&F view is selected, the **S&F** tab and **S&F Correlation** tab will appear as specific view options under the image area.

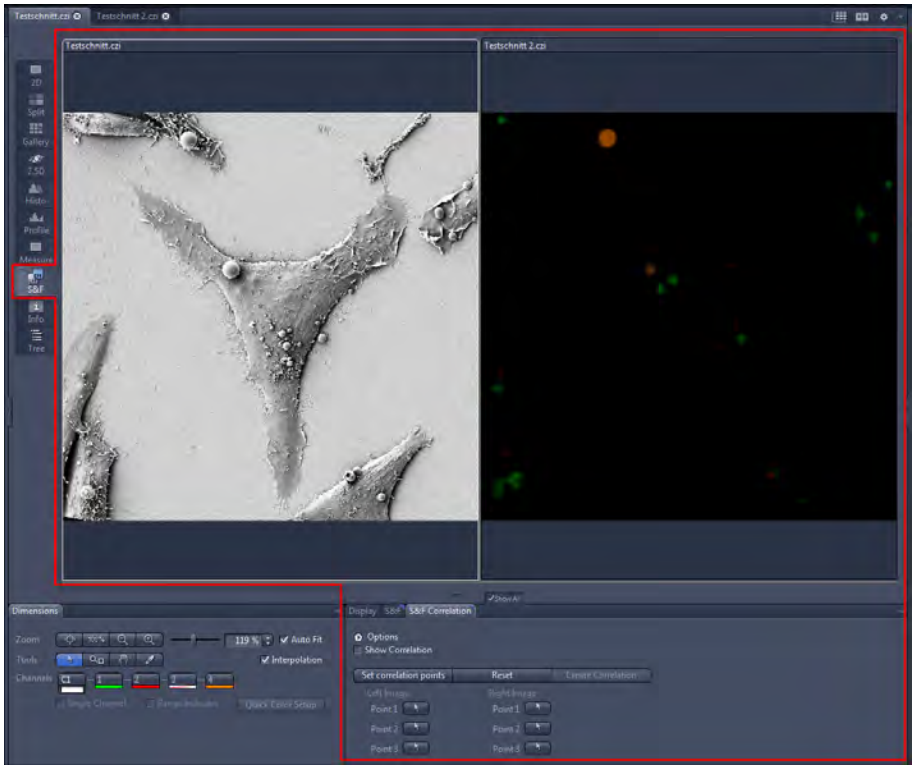


Fig. 14.14: Shuttle & Find View

14.3.3.1 S&F Tab

Here you find helpful options and tools to draw in and relocate regions of interests (ROIs) or points of interest (POIs) within the sample image.

14.3.3.1.1 Options

Parameter	Description
Mirror Image	Here you can mirror the image **horizontally** or **vertically** by using the two buttons at the right. The alignment of the images depends on the microscope (upright/inverted) and orientation of the sample holder.
Keep tool	**Activated:** Keeps the current tool active. That's helpful if you want to draw in more than one ROI/ POI.
Auto color	**Activated:** Uses a new color for each new element which is drawn in.
Snap to Pixel	**Activated:** Draws in graphical elements using the pixel grid.
Use fine calibration value	**Activated:** Uses the measured fine calibration.

Parameter	Description
	The precision of relocation and therefore the quality of the overlay image can be improved by determination of an offset value. This value describes the offset between the loaded image and the live image. The defined offset value is only valid for the loaded image which you can see in the container. If another image is loaded or if you close the dialogue, the offset value will be deleted. Determine the offset by identification of a POI (Point Of Interest) within the snapped image. To identify a POI use the buttons in the **Regions** section. By clicking on the **Set Offset** button, the stage moves to the supposed sample position. Compare the sample position within the live image with the set POI and correct the stage in that way that both shown positions are identically. Confirm the fine calibration with the **OK** button. Now the fine calibration is measured and the checkbox is activated. More information, see *Fine Calibration of the Sample Holder* [▶ 421].
Double click in image to move stage	**Activated:** Moves the stage to the position you have double clicked on.
Refocus after stage movement	**Activated:** Adjusts the focus automatically after the stage has moved.
Move stage in z-direction before x/y movement	**Activated:** Moves the stage to the load position before it moves to the next correlative calibration marker.
Show splitter view	**Activated:** Activates **Splitter** Mode in the **Center Screen Area**.

14.3.3.1.2 Regions, Find and Dimensions

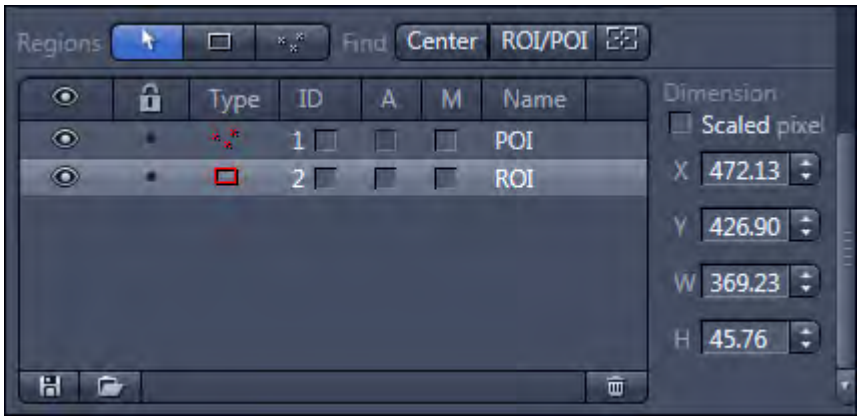


Fig. 14.15: Regions, Find, Dimension

Regions and Find tool bar

Parameter	Description
Selection mode	Selects the ROIs or POIs in the image area. If you are currently in another mode, you can switch back to the **Selection** mode using this button.
Draw rectangle	Draws in a rectangle (**Region of Interest (ROI)**) that is always parallel to the edges of the image.
Draw marker	Draws in a marker point (**Point of Interest (POI)**).
Center	Moves the stage to the center of the opened image.
ROI / POI	Moves the stage to the selected **ROI** / **POI**.
Show stage position	Shows the current stage position as a rectangle in the image.

Dimension section

Here you see coordinates and dimensions of the selected graphical element in the list. If the **Scaled** checkbox is activated, the unit is µm, otherwise Pixel.

- Parameter **X**: Shows the horizontal position (x coordinate) of the center of the graphical element.
- Parameter **Y**: Shows the vertical position (v coordinate) of the center of the graphical element.
- Parameter **W**: Shows the width of the graphical element.
- Parameter **H**: Shows the height of the graphical element.

Graphical elements list

Here you see the list of all ROI / POI which are drawn in.

Parameter	Description
Eye symbol	Shows or hides the ROI / POI in the image.
Lock symbol	Locks a ROI / POI to prevent changes.
Type	Displays the icon for the tool type (ROI/POI). To format a graphic element, double-click on the icon. The **Format Graphic Elements** dialog opens.
ID	Only visible if the **Show All** mode is activated. Displays the ID for the graphic element. To do this, activate the checkbox at the corresponding list entry.
A	Only visible if the **Show All** mode is activated. Displays annotations for a graphic element (ROI). To do this, activate the checkbox at the corresponding list entry. Then double click on the checkbox. The **Format Graphic Elements** dialog opens. Choose an annotation you want to have displayed within the image from the **Annotation** dropdown list.
M	Only visible if the **Show All** mode is activated. Displays measurement data for a graphic element. To do this, activate the checkbox at the corresponding list entry.
Name	Displays the name of the graphic element. To change the name, double-click in the Name field. Then enter the text of your choice.

14.3.3.2 S&F Correlation Tab

Here you find all functions to overlay (correlate) two images.

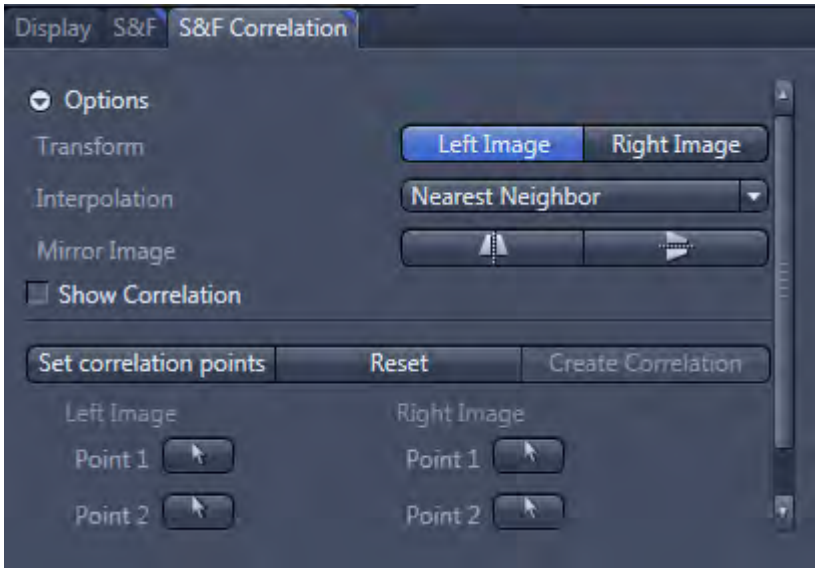


Fig. 14.16: S&F Correlation tab

Parameter	Description
Transform	Here you select which image will be transformed. Choose via the **Left Image**/**Right Image** buttons, which image should be transformed in the other. During transformation a pixel in the overlay image is calculated by using pixels of the two original images that shall be overlaid / merged.
Interpolation	Here you can select one of the following interpolation methods:
- Nearest Neighbor	The gray value of the resulting pixel in the overlay image is made of a pixel which is located next. This interpolation method is very fast.
- Linear	The resulting or calculated pixel in the overlay image is assigned to a gray value, which is the result of a linear combination of gray values derive from pixels located nearby (in the original image).
- Cubic	The calculated pixel in the overlay image is assigned to a gray value, which is calculated by means of a polynomial function using gray values of pixels in the original images; these pixels are located nearby the calculated pixel.
Mirror image	Here you can mirror the image horizontally or vertically. Therefore simply click on the corresponding button. Mirroring an image is necessary, when the loaded image shows a different orientation than the live image.
Show Correlation	**Activated:** Opens the correlated image in a new image document / new container.

Parameter	Description
Set correlation points	Enables you to set 6 points (3 points in each image) as correlation markers in a row, see *Correlating Two Loaded Images* [▶ 421].
Reset	Deletes all correlation points in the images.
Create Correlation	Active only, if all correlation points are set in both images. Creates a correlative overlay image. A third image container with the correlated image will be opened in the **Center Screen Area** and the **Show Correlation** checkbox will be activated automatically.

14.3.4 Sample Holder Calibration Wizard

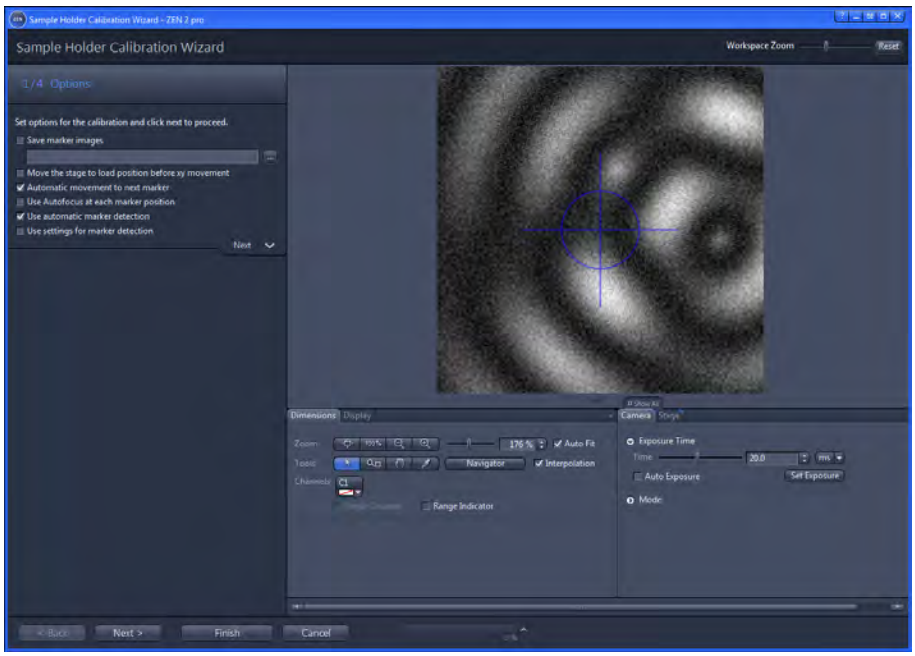


Fig. 14.17: Sample Holder Calibration Wizard

With the Sample Holder Calibration Wizard you calibrate your selected correlative sample holder. The wizard is opened via the **Shuttle and Find** tool. Make sure that you have activated the Shuttle and Find tool and selected a sample holder, see *Selecting the Sample Holder* [▶ 410].

14.3.4.1 Step 1: Options

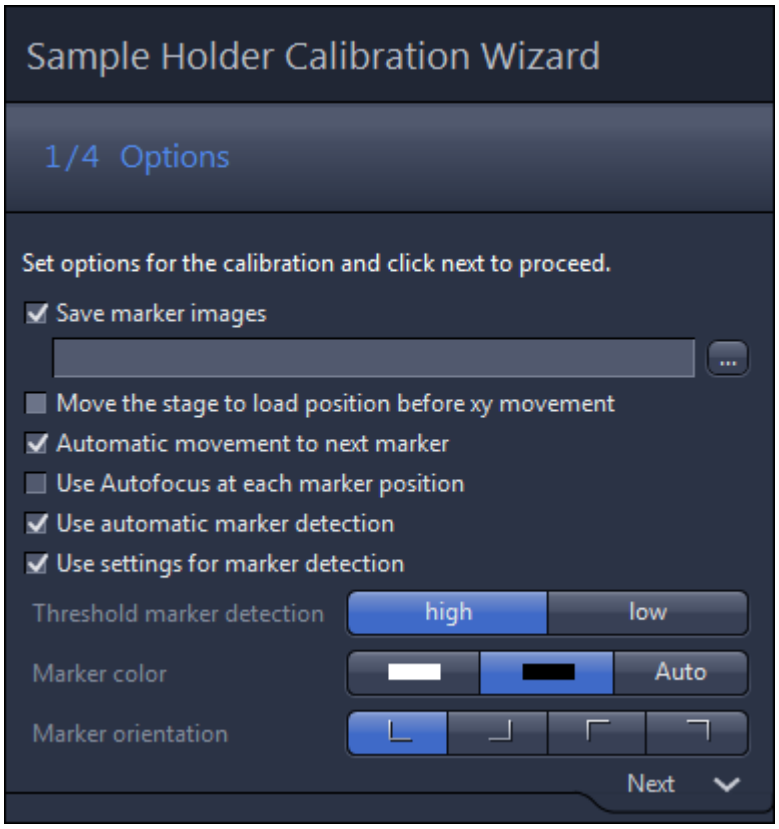


Fig. 14.18: Sample Holder Calibration Wizard Options

Option	Description
Save marker images	**Activated:** the marker images are saved during the calibration. The images can be used to check the calibration afterwards. Click on the **Select Folder (...)** button to select a storage folder.
Move the stage to load position before x/y movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Automatic movement to next marker	**Activated:** By clicking on the **Next** button within the wizard the stage moves automatically to the next calibration marker.
Use Autofocus at each marker position	This option is active only if the **Automatic movement to next marker position** checkbox is activated. **Activated:** the focus is adjusted automatically after moving to the next marker position.

Option	Description
Use automatic marker detection	**Activated:** The software will try to detect the small calibration marker automatically.
Use settings for marker detection	This option is active only if the **Use automatic marker detection** checkbox is activated. **Activated:** shows settings for marker detection (see description below). Here you select the properties of the calibration markers.

Settings for marker detection

Only visible if the **Use settings for marker detection** checkbox is activated.

Option	Description
Threshold marker detection: high – low	A low threshold for marker detection is used when the dimensions of the correlative L markers cannot be recognized precisely, e.g. when the sample holder is slightly filthy.
Marker color	Here you select the color of the markers displayed in the live image. **White:** the marker is displayed white on a dark background. **Black:** the marker is displayed dark on light background. **Auto:** the marker color is set automatically.
Marker orientation	Here you need to set the orientation of the L-markers on your sample holder. Click on the corresponding button to select the orientation of the calibration marker which you can see in the live image

If you click on the **Next** button you will move to the next step of the wizard.

14.3.4.2 Step 2-4: Calibration

In steps 2-4 of the wizard you will be guided through the calibration procedure.

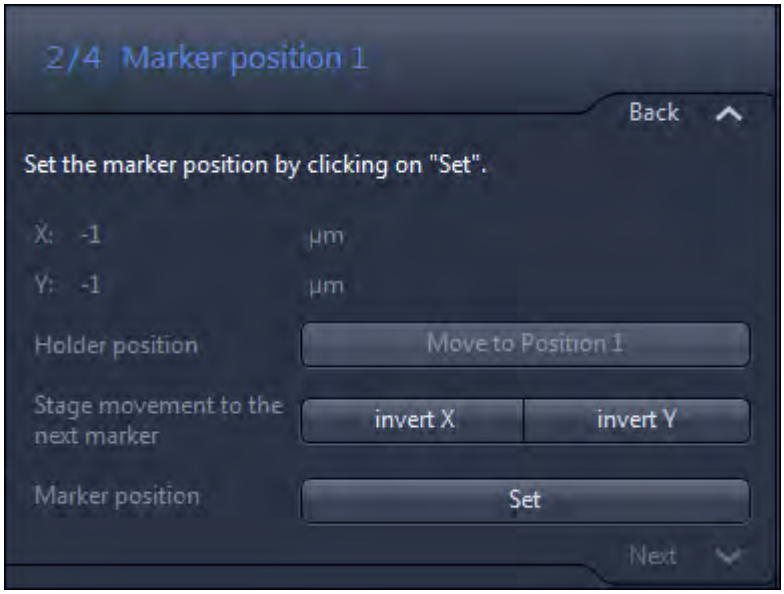


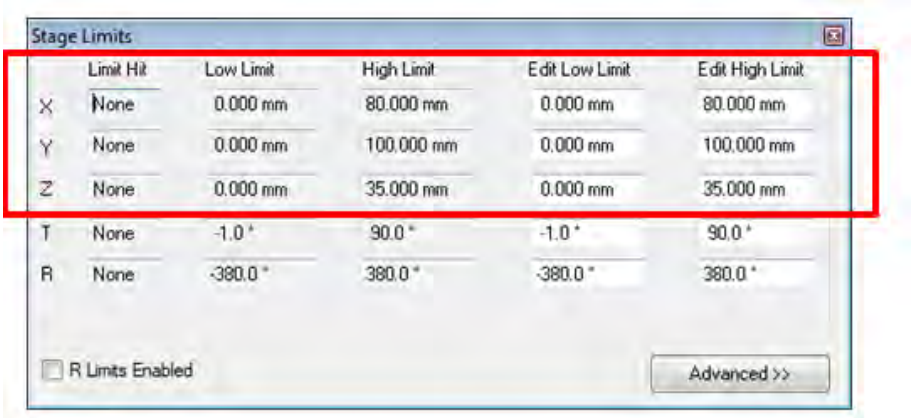
Fig. 14.19: Sample Holder Calibration Wizard

Option	Function
Holder position	**Move to Position 1** button Moves the stage to marker position 1. This is possible only if the first position was set before and x/y coordinates are given. **Current** button Only visible for marker position 2 and 3. Moves the stage to the current marker position. This is possible only if the current position was set before and x/y coordinates are given.
Stage movement to the next marker	Here you can change the movement of the stage in x or y direction. This is necessary if during calibration the stage moves in the wrong direction.
Marker position	By clicking on the **Set** button, the actual marker position will be confirmed.

14.4 Appendix

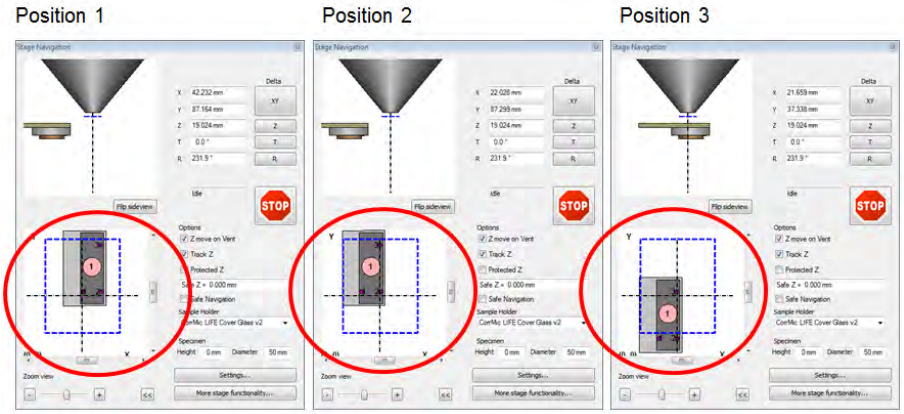
14.4.1 Shuttle & Find with an EVO 10

To use Shuttle & Find (SW and correlative holders) with an EVO 10 make sure that the stage limits (for x, y and z) are set as follows:



Holder Positions

The holder positions must be oriented like shown in the images

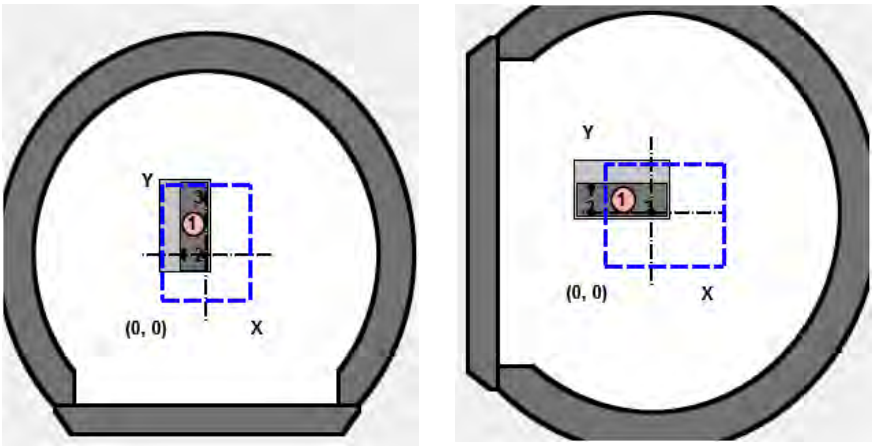


NOTICE

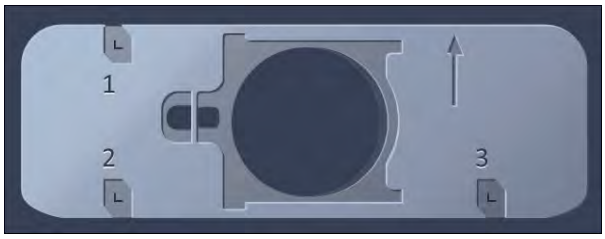
If you set a wrong orientation the stage cannot be moved to all correlative markers because of the stage limits for the EVO 10.

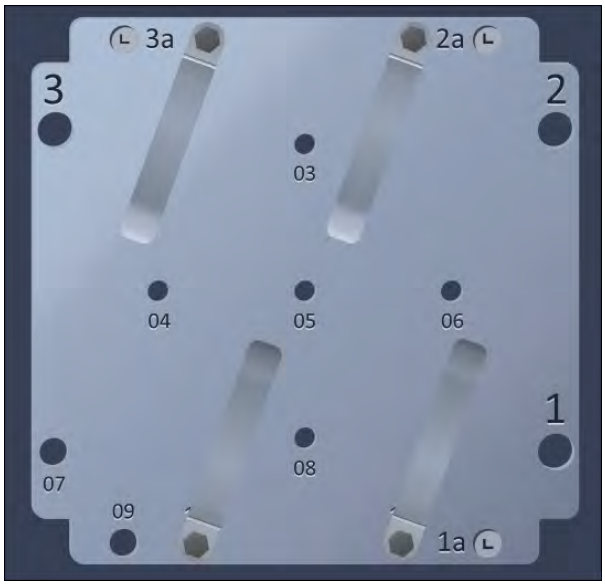
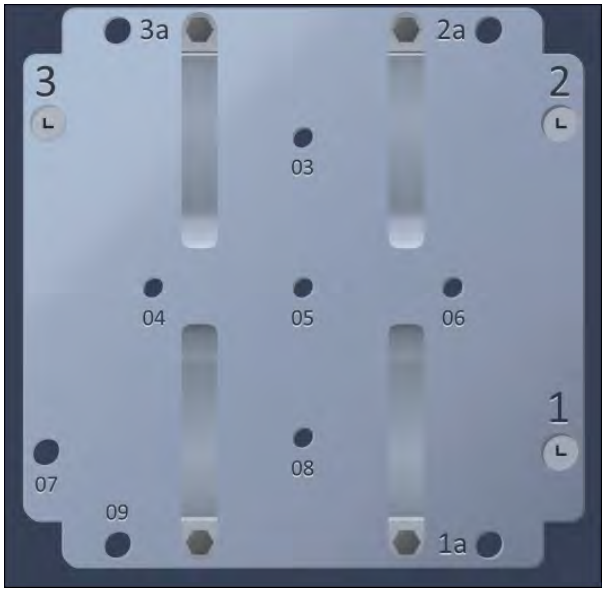
- The holder has to be mounted into the EVO in that the way that the correlative markers (**1**) and (**2**) have to be near the chamber door whereas marker (**3**) is located furthest from the chamber door (see **Mounting A/B**).
- If necessary, the SEM image can be rotated according to the LM image using the option **Scan Rotate** in SmartSEM.

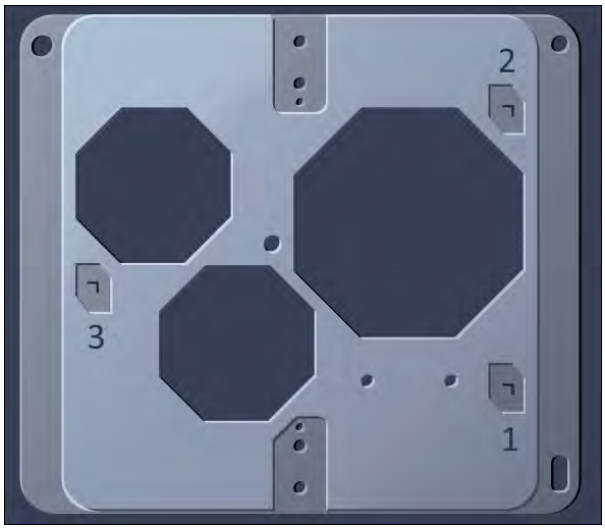
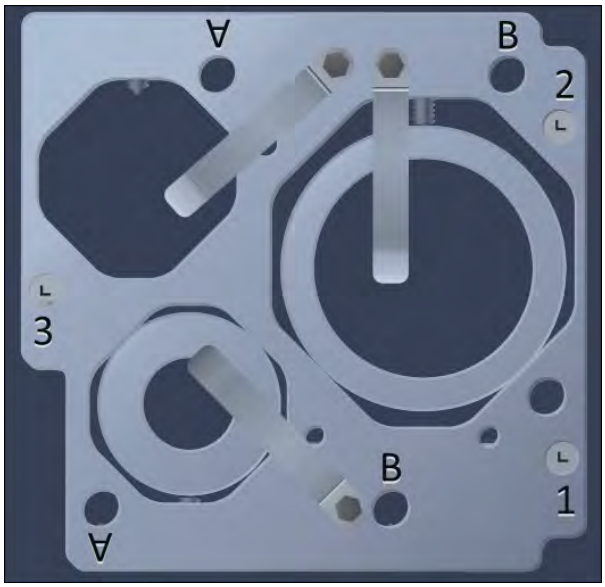
Mounting A: **Mounting B:**

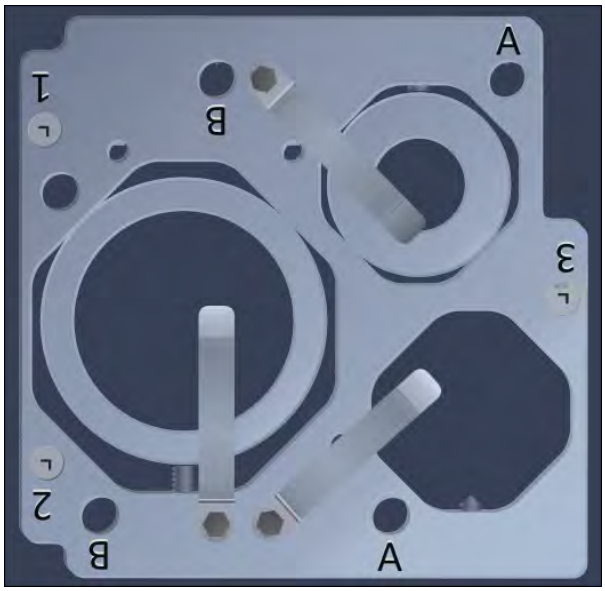


14.4.2 Correlative Sample Holders

Name	Image
Life Science cover glass 22x22	
Life Science for TEM Grids	
Cover glass with fiducials 22 x 22	

Name	Image
MAT Flat Stubs A	
MAT Flat Stubs	

Name	Image
MAT Universal A	
MAT Universal B_A	

Name	Image
MAT Universal B_B	

15 Module CAT

15.1 Introduction

Array Tomography is a volumetric microscopy method employed to visualize and reconstruct 3D images of serial sections. Tissue samples or cells embedded in resin are cut into consecutive sections with an ultramicrotome and collected onto a sample carrier (e.g. cover glass). The sequence of the sections determines the z-position and allows the reconstruction of the 3rd dimension. Therefore the z-resolution of the resulting 3D data set is determined by the thickness of the section.

The correlation of scanning electron microscope (SEM) data especially with an image acquired using a fluorescence light microscope (LM), enables the visualization of fluorescently labeled biological structures in their ultrastructural context not only in 2D but now in 3D with the ZEN Correlative Array Tomography module.

ZEN Correlative Array Tomography (CAT)

The software module ZEN Correlative Array Tomography (CAT) enables automated imaging of ultra-thin serial sections (ribbons) using the light- and scanning electron microscope. After calibration of the sample carrier and detection of the sections, regions of interest can be defined manually in a single section that will be automatically propagated to all sections. The selected regions of interest can then be imaged with different contrast methods and magnifications using the LM.

In the SEM the previously defined regions of interest will then be imaged automatically after loading the image previously acquired at the LM. The corresponding 2D image sequences recorded by the LM and SEM are aligned into a 3D Z-Stack using the integrated alignment and correlation algorithms of the ZEN Correlative Array Tomography module. This process results in a correlative 3D data set combining LM and SEM information into one image volume.

For the correlative workflow, one CAT module has to be installed on the widefield system, a second module hast to be installed on the SEM. A detailed how-to guide of the workflow can be found in the chapter *The CAT Workflow* [▶ 440].

The software module can be used with ZEISS widefield microscopes as well as with ZEISS scanning electron microscopes. In addition to the CAT tool the module offers four wizards. Detailed descriptions of the functions of the tools and wizards can be found in the linked chapters.

- **Correlative Array Tomography** tool, see *CAT Tool* [▶ 479].
- **Calibration Wizard**, see *Sample Holder Calibration Wizard* [▶ 483].
- **Acquisition Wizard**, see *Acquisition Wizard* [▶ 486].
- **Z-Stack Alignment Wizard**, see *Z-Stack Alignment Wizard* [▶ 495].
- **Correlation Wizard**, see *Correlation Wizard* [▶ 497].

15.2 General Preparations

15.2.1 Sample Preparation

Type of Sample Carrier / Cover Glasses

We recommend cover glasses coated with Indium tin oxide and fiducials. Indium tin oxide minimizes charging effects in the scanning electron microscope. Cover glasses with fiducials enables additional preparation steps after imaging the sample with the light microscopes and before imaging with a scanning electron microscope.

Deposition of Serial Sections on the Cover Glass

- **Sequence of serial sections**
  During serial sectioning, make sure that you know the sequence of the ribbons as well as the start and the end point of the ribbons.
- **Positioning of serial sections on a cover glass**
  It is important to position the serial sections in the center of the cover glass. If the sections are too close to the edge of the cover glass, it might happen that the objective touches the sample carrier during the image acquisition. This might happen particularly for immersion objectives. The consequence will be that the focus map is not calculated in the correct way or images are out of focus.
- **More than one ribbon (serial section) on a cover glass**
  Take care that the single serial sections are not in close contact to each other, this might confuse the numbering algorithm of the software and creates a wrong numbering.

15.2.2 Pre-Settings (Light Microscope)

Before you can start working with the CAT module, you have to check the following settings on the light microscope system (hardware and software settings). In general the system is calibrated by a service technician but we recommend to check the settings again especially when you have changed components e.g. objectives or filter cubes. As these general settings are not described here in detail please ask your service technician or read the ZEN Online Help.

- **Check Parcentricity and Parfocality**
  Note that the calibration of parcentricity and parfocality has to be done at the TFT display of the microscope.
- **Check Camera Orientation and Stage Movement**
  Before checking the stage movement the correct camera orientation always has to be set first. The camera orientation should match your view through the ocular of the microscope. If this is not the case you can change the camera orientation in the **Camera** tool.
  To check the stage movement we recommend to acquire a tiles image and

check if the tiles are put together correctly. If this is not the case you have to close ZEN software and open the **MTB 2011** software. There you must change the stage inversion in the configuration list under **Motorized Stage**.

- **Perform Shading Correction**
  Before starting the CAT workflow a shading correction has to be performed in ZEN software. Please read the corresponding chapter in the ZEN Online Help.

15.2.3 Experiment Settings

For working with the CAT module you need to set up an experiment in the ZEN software first. As this is already described in the ZEN Online Help, we will focus here on the most important settings which are essential for the CAT workflow:

- In the menu **Tools** | **Options** | **Acquisition** | **Acquisition Tab** the checkbox **Enable Advanced Imaging Setup** must be activated.
- In the **Imaging Setup** tool **Show All** must be activated.
- In the **Imaging Setup** tool **Advanced Imaging Setup** must be selected.
- In the **Imaging Setup** tool in the lightpath display the following settings have to be adjusted:
  - The **Microscope Manager** must be excluded from all settings. To exclude a setting, click on the icon of the component / setting. You will see a menu where you can activate/deactivate the checkbox **Include in this Setting**. If the checkbox is not activated, the component/setting is excluded from the selected setting. Note that you have to select each Before/After setting and check if the components are excluded or included and adjust it accordingly.
  - The **TL / RL Switch** must be excluded from all settings.
  - All **TL / RL Shutters** in the light path must be included in all settings. Note that icons of included settings are highlighted in blue color.
  - All **light sources** (TL / RL) within the light path must be included in all settings.
  - The light intensity of the light sources for all settings must be the same (e.g. 2V).
- In general for the **Before Experiment** and **After Experiment** settings adjust the following:
  - All **Shutters** must be closed.
- Example for Phase Contrast settings:
  - For the **Before TL Phase** settings adjust the following:
    - The **TL Shutter** must be opened
    - The **RL Shutter** must be closed
  - For the **After TL Phase** adjust the following:
    - The **TL Shutter** must be closed

- The **RL Shutter** must be closed

- Example for one Fluorescence Channel (here DAPI) settings:
  - For the **Before DAPI** settings adjust the following:
    - The **TL Shutter** must be closed.
    - The **RL Shutter** must be opened
  - For the **After DAPI** adjust the following:
    - The **TL Shutter** must be closed.
    - The **RL Shutter** must be closed

15.3 Workflow CAT

15.3.1 Introduction

In this chapter you will find how-to guides describing the typical CAT workflow. The chapter is for users who search for an introduction to the CAT module and workflow. Starting from general preparations to the acquisition on the LM (light microscope), we will also explain how to acquire images with the SEM (Scanning Electron Microscope). After the image acquisition we will focus on the image alignment and correlation.

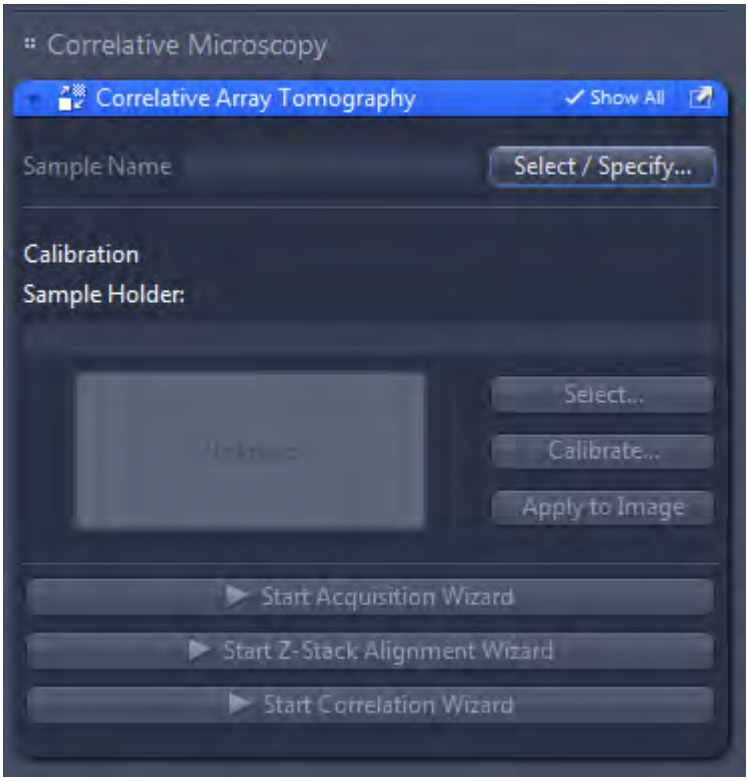
Please note that we will not explain how to set up an experiment in detail as this step is beyond the scope of this guide which is focused mainly on the CAT workflow. Instead of that please read the chapter *General Preparations* [▶ 441], where we describe the most important pre-requisites for a CAT experiment. We will not take a look at the further processing of the resulting images as well.

15.3.2 Creating a new sample

If you have configured your experiment in ZEN (e.g. a multi-channel experiment) the next step is to create and select a sample. When you work with the software for the first time you have to create a new sample first.

Prerequisites

- You are in the **Correlative Array Tomography** tool.



Procedure

1. Click on **Select** / **Specify**.

   The **Select Sample** dialog opens.

2. Click on the  **Add** button under the **List of specified samples**.

   The **New Sample** dialog opens.

3. Enter the necessary sample information: Name, Description, Number of sample carriers, Type of sample carrier and Section thickness.

> **INFO**
>
> Note that specifying the correct number of sample carriers is important for the numbering of the ribbons/sections afterwards. The sample information will be stored within the image data and will be used for further image processing and data management.

4. Click on **OK**.

   The dialog closes. You will see the new sample in the **List of specified samples**.

5. Select the new sample from the list and click on **OK**.

You have created and selected a new sample.

15.3.3 Selecting the Sample Holder

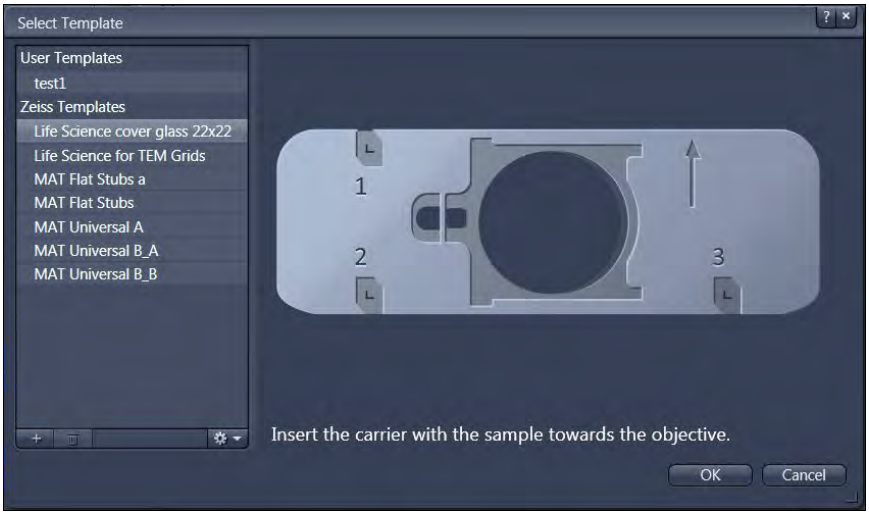
Prerequisites

- You are in the **CAT** tool.

Procedure

1. In the **Sample Holder** section click on **Select…** to open the **Select Template** dialog and to choose the correlative sample holder you want to use. Different types of correlative holders are available, see Appendix *Correlative Sample Holders* [▶ 500].

2. In the **Select Template** dialog select the correlative holder you want to use. If you want to use your own sample holders, click on the **Add** button below the list and follow the instructions in the chapter *Defining new sample holder templates* [▶ 445].



3. Click on **OK** to close the dialog.

You can now continue with the calibration of the sample holder, as described in the chapter *Calibrating the sample holder* [▶ 447]. Note that the calibration of the sample holder is mandatory to acquire images.

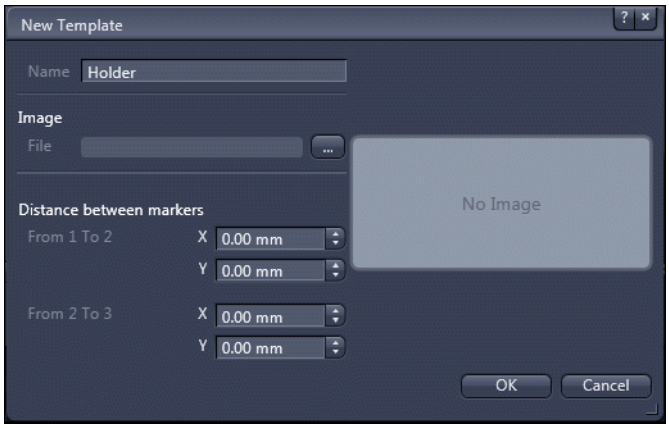
15.3.4 Defining a New Sample Holder Template

With this dialog you can define new correlative holders in addition to the existing holder templates. It is not mandatory to use correlative holders from ZEISS. User-defined correlative holders with 3 fiducial markers can be used as well.

Procedure

1 To open the dialog click on  **Add** in the **Select Template** dialog. This dialog can be opened via the **Shuttle & Find** tool.

The **New Template** dialog opens.



2 Type in a name for the new holder / sample carrier. An image of the new holder can be loaded as well.

3 Insert the distances (in millimeters) between the first and the second marker and between the second and third marker.

The distances can be determined using the **Stage Control** dialog accessible via the **Light Path** tool in **Right Tool Area** tab. We recommend to do this before you start the New template dialog. Write down the distances to be prepared to enter them within the New Template dialog.

1 Activate the live view in the Center Screen Area by clicking on the **Live** button in the Locate tab.

2 Navigate the stage manually to the calibration marker on the sample holder by means of the joystick and note the x/y-coordinates of the marker.

3 Repeat this procedure for all three markers and calculate the distances between marker 1 and marker 2 and between marker 2 and marker 3, respectively.

Fig. 15.1: Stage Tool

15.3.5 Calibrating the Sample Holder

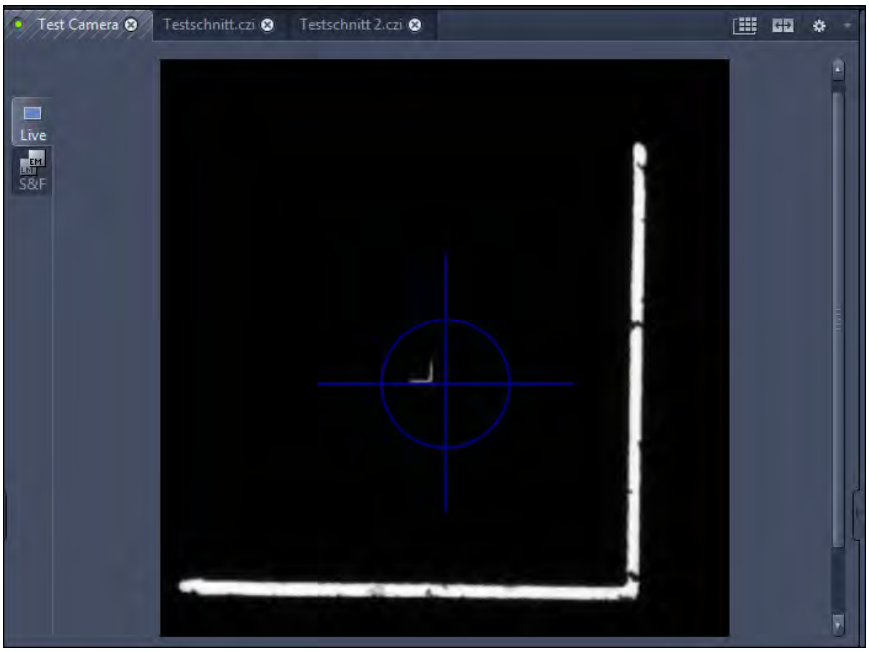
Correlative sample holders have three fiducial markers enabling a three point calibration (signed with the numbers 1-2-3) The calibration markers consist of one small (length 50 µm) and a large L-shape marker (length 1 mm). The bigger marker is used for coarse orientation, whereas the smaller marker is used for the calibration.

15.3.5.1 Preparing Calibration

Procedure

1 Click on **Live** in the **Acquisition** tab to activate the live view in the **Center Screen Area**.

2 Navigate the stage manually to the first calibration marker on the sample holder (marked with No. 1) by means of the joystick. It is enough if you move the stage to the larger L-shaped calibration marker. The smaller marker will be detected automatically within the **Sample Holder Calibration Wizard**.To locate the marker positions we recommend to use a dry objective with low magnification (5x – 20x).



3 Open the **CAT** tool.

4 Click on **Calibrate…** to open the **Sample Holder Calibration Wizard.**

15.3.5.2 Setting Calibration Options

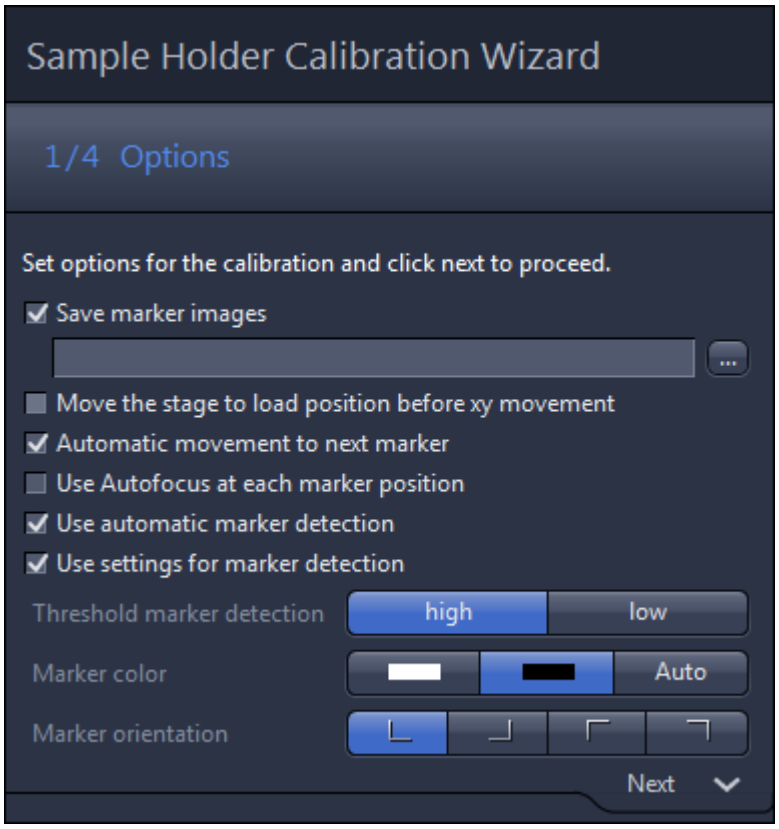


Fig. 15.2: Sample Holder Calibration Wizard Options

In step 1 of the wizard, the following options should be activated to follow our recommended workflow:

Procedure

1. Check if the **Automatic movement to next marker** checkbox is activated.

   This will automatically move the stage to the next marker position after you have confirmed the position of the marker and clicked on **Next**.

2. Check if the **Use automatic marker detection** checkbox is activated.

   The software will try to find the correct positions of each marker automatically.

3. If you need to change the marker color, or check if the marker orientation is set correctly, activate the **Use settings for marker detection** checkbox to access these functions.

4. Click on **Next** to move to the next wizard step.

15.3.5.3 Performing Calibration

Procedure

1. Click on **Set** to detect the first marker position.

   An automatic stage calibration will be performed. After the stage calibration, the system will try to detect the marker position of the small marker automatically.

   A message appears which asks if the marker was detected correctly.

2 Click on **Yes** to confirm the message.

INFO

If the marker was not detected correctly, you have the possibility to set the marker position manually. Therefore simply left-click on the intersection of the L marker in the image and the position will be adapted. Pay attention that you select always the same calibration point on each L-marker.

3 Click on **Next** to move to the next step of the wizard.

The stage will automatically move to the next (coarse) marker position. If the stage moves into the wrong direction you can use the **invert X** / **invert Y** buttons to correct the movement direction.

4 Repeat the previous steps and set marker position 2 and 3 accordingly.

After setting marker position 3 you will find a green check mark icon which shows that the calibration was successful.

5 Click on **Finish** to save the calibration and close the wizard.

To check if the calibration was successful acquire an image and open the **Tree** view in the Center Screen Area. There you should see the correlative calibration data in the list. If the Tree view is not visible go to **Tools** | **Options** | **Documents** and activate the **Enable Tree View** checkbox.

15.3.6 Acquiring LM Images

15.3.6.1 Introduction

The image acquisition will be performed by the help of the Acquisition Wizard which is opened via the CAT tool.

The wizard contains the following 7 steps:

- *Overview Imaging* [▶ 450]
- *Ribbon Definition (optional)* [▶ 452]
- *Ribbon Imaging (optional)* [▶ 453]
- *Section Specification* [▶ 455]
- *ROI Specification* [▶ 459]
- *ROI Imaging* [▶ 461]
- *Re-Shoot (optional)* [▶ 462]

15.3.6.2 Acquiring the overview image

Prerequisites

- You have started the **Acquisition Wizard** via the **CAT** tool.
- You are in step **1/7 Overview Imaging**.

Procedure

1. Check if **Image Acquisition** mode is selected. This is the default setting when entering the wizard.
2. From the **Experiment** dropdown list select the experiment that you have prepared in advance.

3 From the **Objective** list select an objective with a low magnification, e.g. 5x.

4 Select the **Channel** and the **Light Source** you want to use for acquiring the overview image. For the overview image we recommend to select **Phase contrast** as channel mode.

INFO

Take care that the **Auto** checkbox on the **Dimensions** tab is deactivated.

5 Move the stage to the upper left corner of your sample.

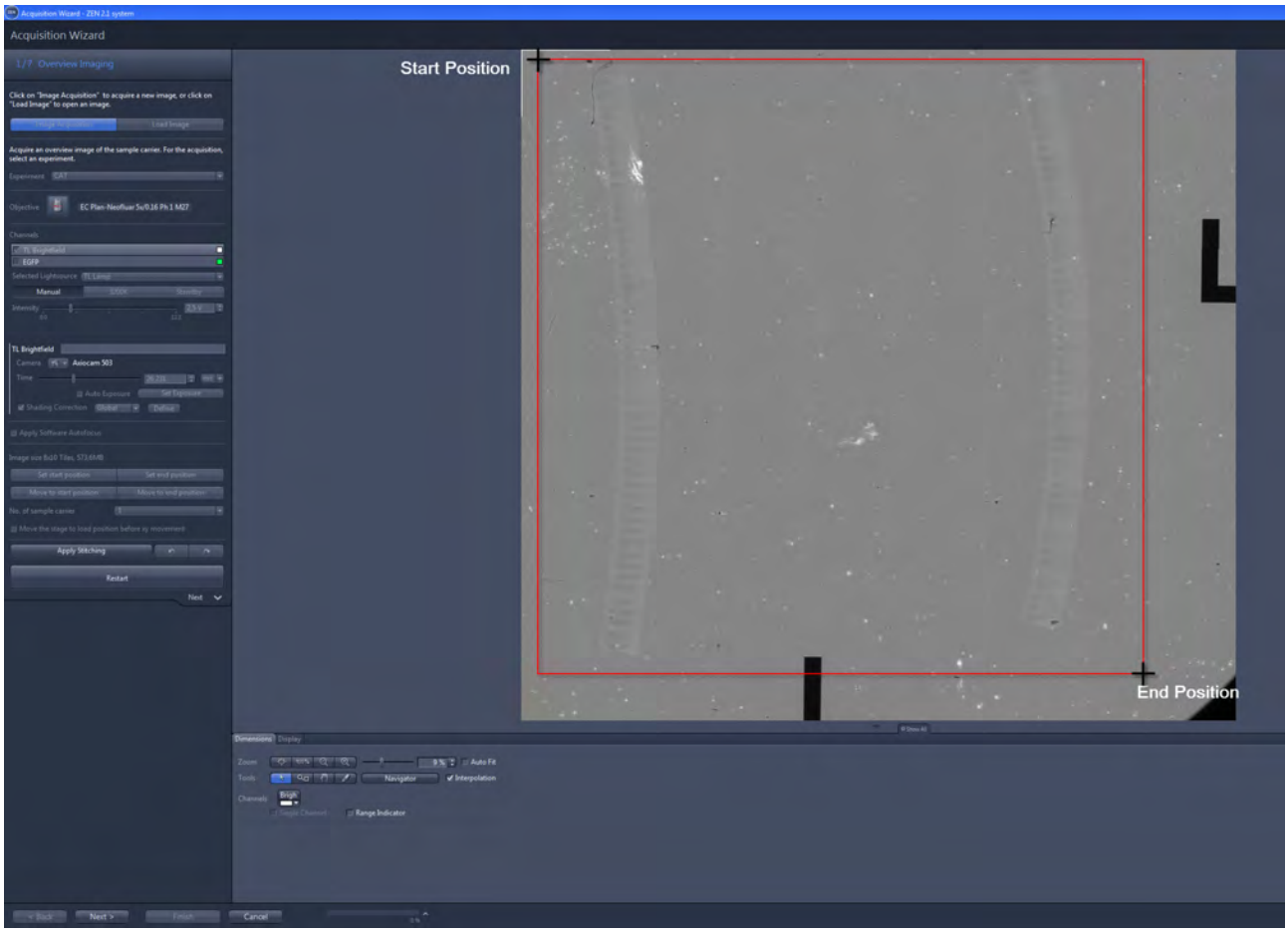
6 Click on **Set start position** to define the starting position of the overview image.

7 Move the stage to the bottom right corner of your sample.

8 Click on **Set end position** to define the end position of the overview image.

9 Click on **Acquire Overview Image**.

The overview image will be acquired. Then you should see the complete sample showing all ribbons you want to image.



10 Click on **Apply Stitching** to remove the offset between the single tile images.

You have successfully acquired the overview image. You can now continue with the next step by clicking on **Next**.

15.3.6.3 Defining the ribbons

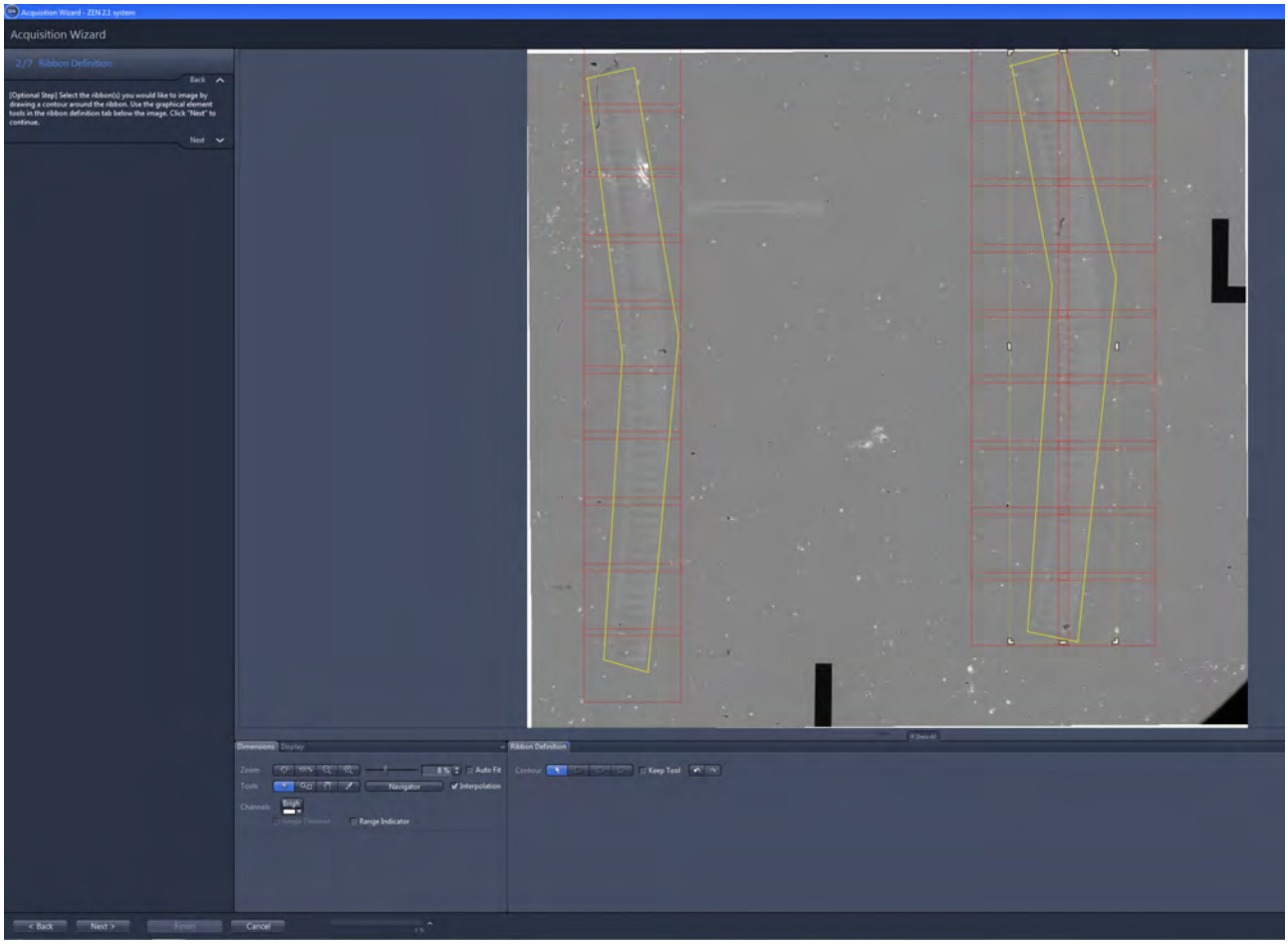
> **INFO**
>
> When no detailed sample information is necessary to identify regions of interest within the sections, you can skip this step and the following step 3 Ribbon imaging as well. You can then go on with the wizard step 4 *Section specification* [▶ 455].

Prerequisites

- You are in step **2/7 Ribbon Definition** of the Acquisition Wizard.

Procedure

1. Use the tools on the **Ribbon Definition** tab to mark the contour lines of the ribbons which should be imaged. The contour lines are displayed in yellow color.



The software will automatically create as many tiles as necessary for imaging the ribbons. The number of the tiles depend on the selected objective. The frames of the tiles will be displayed in red color. When you have marked the contours, click **Next**.

15.3.6.4 Imaging the ribbons

Please note that this is an optional step and must be performed only when you have defined ribbons as described in step 2. In summary, you have to perform the same actions mentioned in step 1 but you should use an objective with higher magnification and apply the **Global** focus strategy under **Focus Surface**.

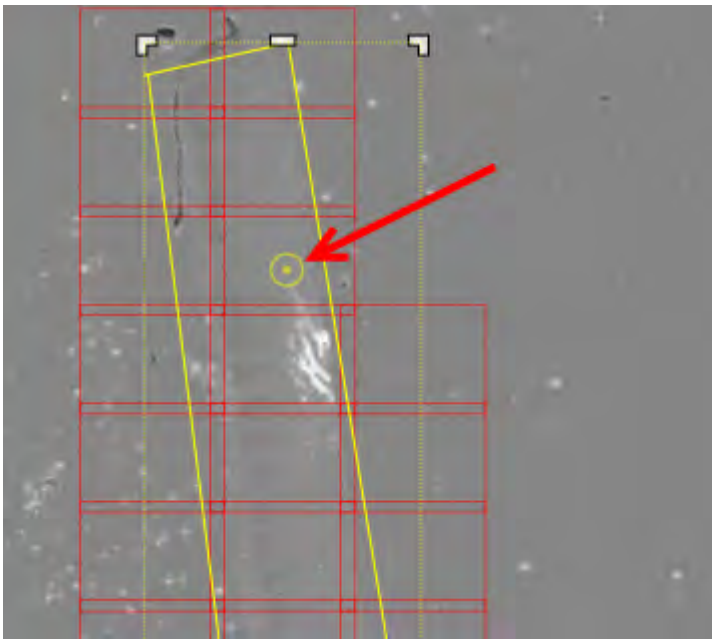
Prerequisites

- You are in step **3/7 Ribbon Imaging**.

Procedure

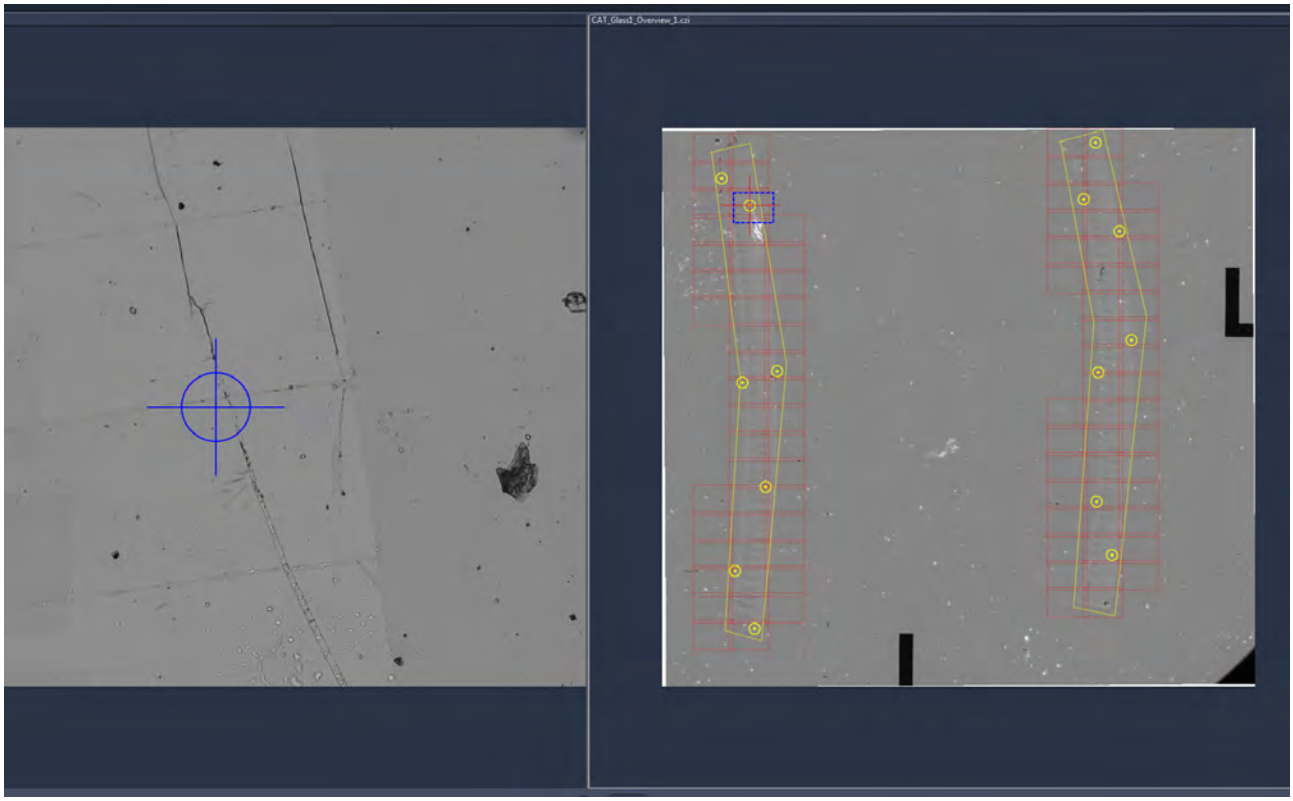
1. Under **Objective** select an objective with a higher magnification than for the overview image, e.g. 10x. The objective should enable you to clearly recognize the structures of interest on your sample.
2. Under **Focus Surface** select **Global (all Regions/ Ribbons)**.
3. Click on **Distribute Support Points...**.

   The support points will be distributed automatically over the ribbons. They are displayed as yellow circles with a point in the middle.



4. You can add further support points if necessary by using the **+** (add) button below the **Distribute Support Points** button. Simply click on the image at the position where you would like to add another support point.
5. Click on **Verify Support Points**.

   Now you can check if each support point is in focus. You will see the overview image in the right image container and the detail image in the left image container. The verification process will start with the first support point which was set. The current support point is marked with a red crosshair. When you activate **Show stage position within the image** on the **Ribbon Definition** tab below the **Center Screen Area** you will see the current position of the stage in the image as a rectangle with a blue dashed frame.



6 Hold *CTRL* key and use the mouse wheel to adjust the focus for the corresponding support point.

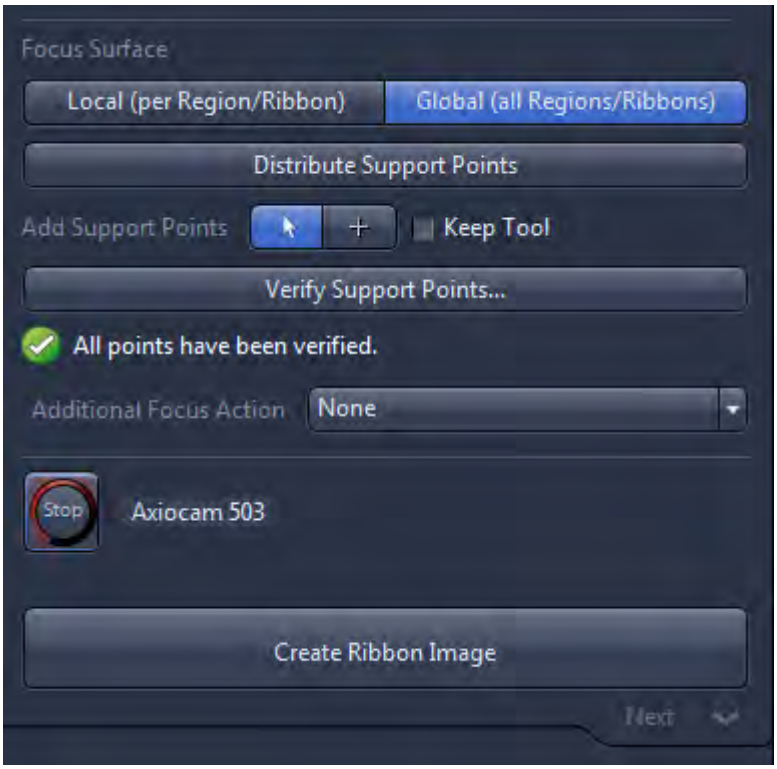
INFO

Adjust the focus only by using the mouse wheel. Using the mouse wheel considers the backlash correction. Do not use the focus wheel at the microscope stand.

7 When the support point is in focus click on **Confirm**.

The software will automatically move to the next support point.

8 Repeat the last two steps until you have corrected and verified all support points. At the end of the process you will see the message **All points have been verified**.



9 Click on **Create Ribbon Image**.

The ribbon image will be generated. Note that each ribbon will be displayed as one scene.

10 Again we recommend to click on **Apply Stitching** to remove the offset between the single tile images.

You have successfully acquired the ribbon image and can now continue to the next step by clicking on **Next**.

15.3.6.5 Specifying the sections

In this step all sections will be identified by using a section detection algorithm. In summary you have to mark the outline of at least one section on each ribbon. Then the section detection algorithm will detect the sections of the ribbon automatically. If the automatic section detection does not work properly or if not all sections are detected you can stamp in the missing sections. It is also possible to edit the shape, location and orientation of the section frames afterwards.

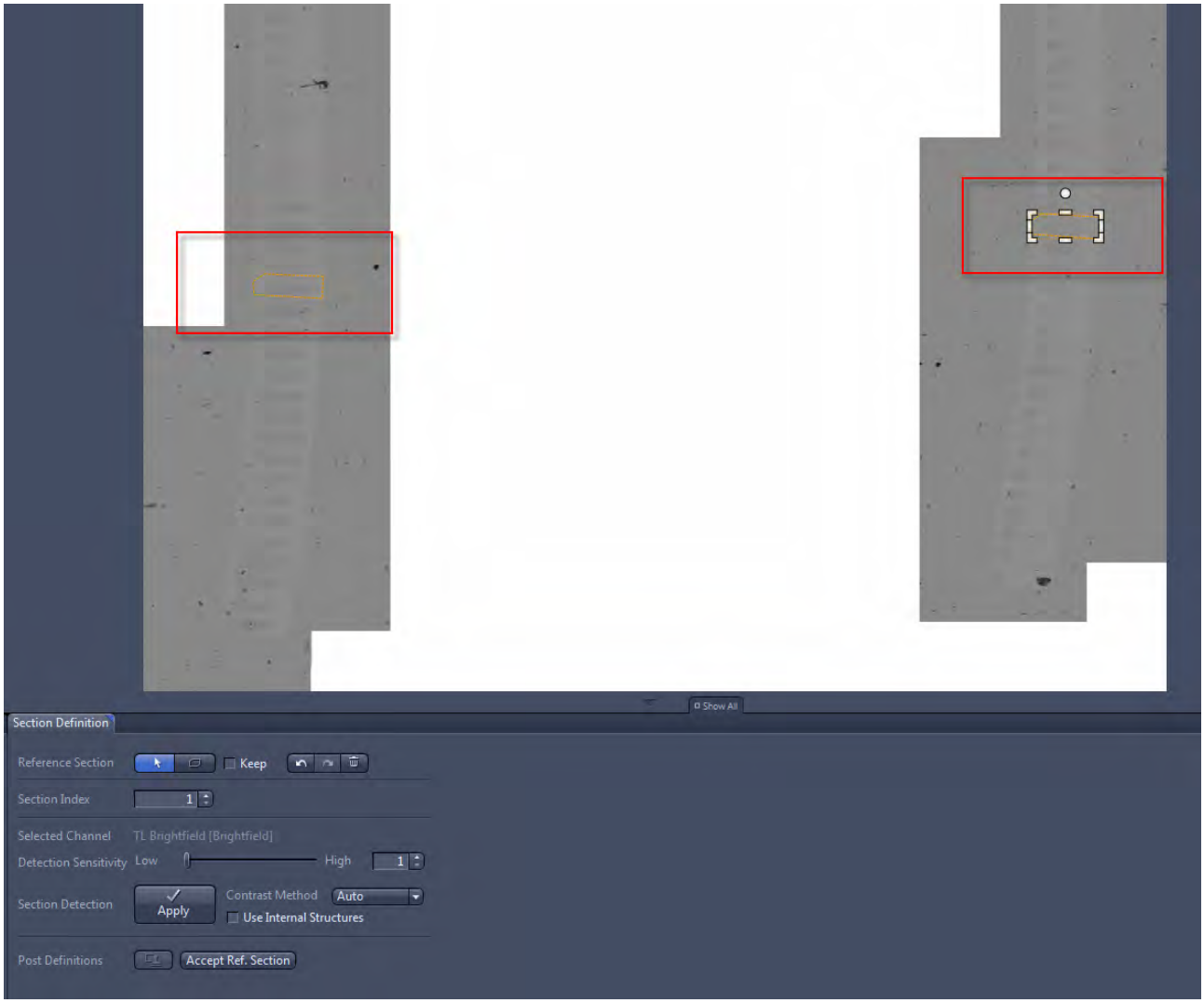
Prerequisites

- You are in step **4/7 Section Specification**.

Procedure

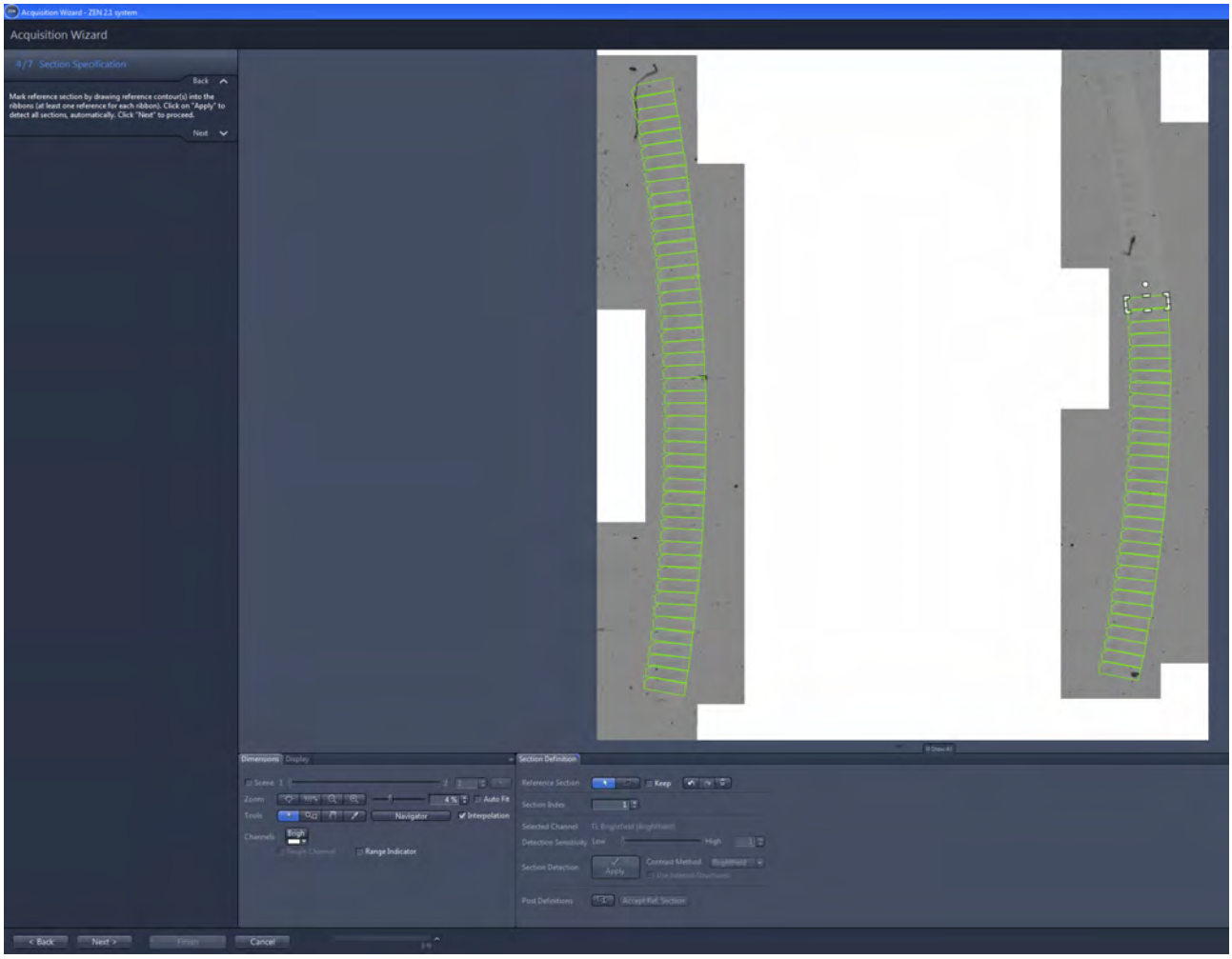
1 On the **Section Definition** tab select the **Polygon** tool.

2 Mark the outline of one section in each ribbon. The outlines of these reference contours are displayed in orange color.

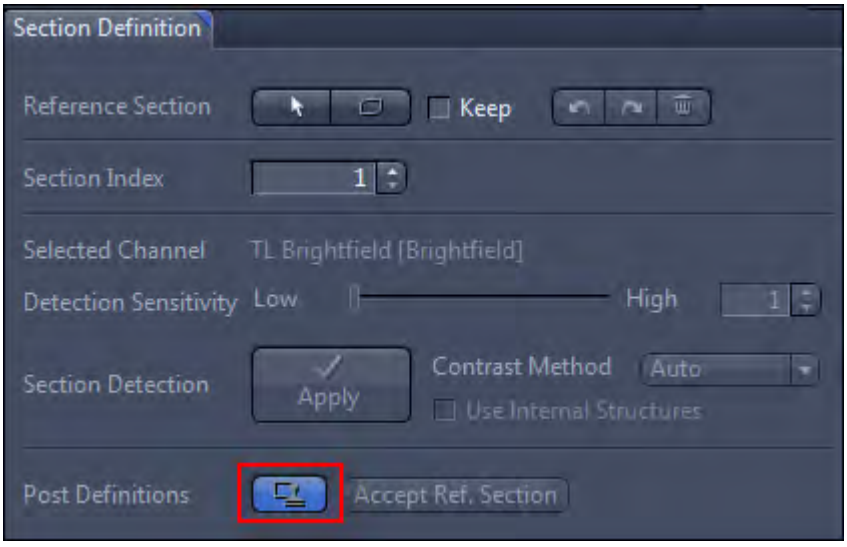


3 Click on **Apply**.

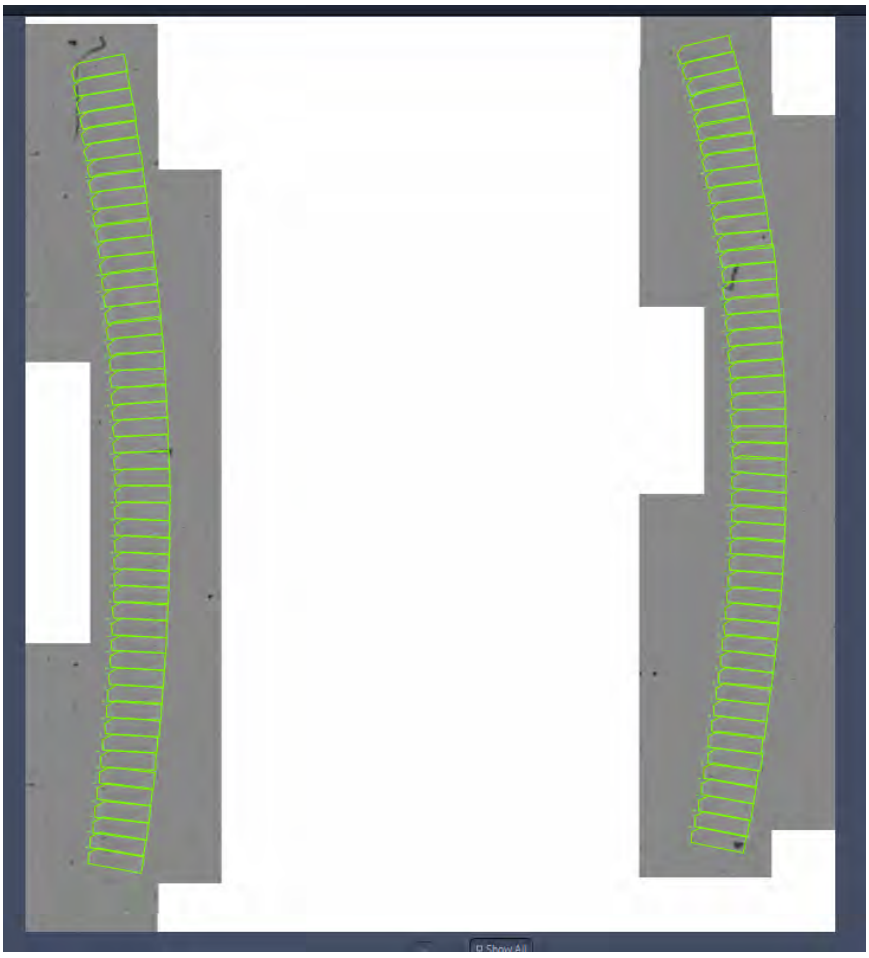
The software will try to detect the remaining sections automatically. When finished the detected sections appear in green color.



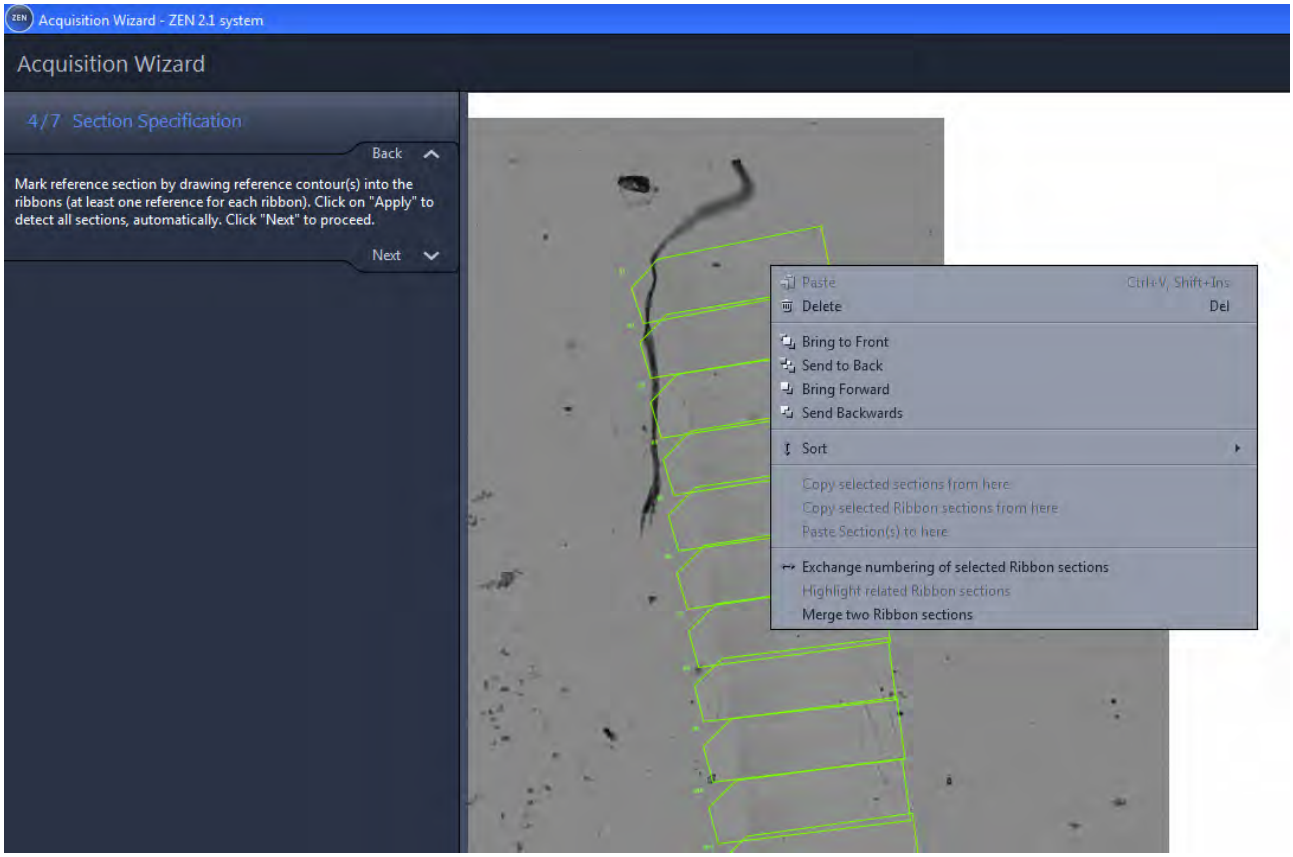
4 If not all sections can be detected, mark the last section which was detected and activate the **Stamp** tool in the **Section Definition** tab.



5 Stamp in the missing sections so that each section is marked.



Please take your time to check the numbering carefully. A correct numbering is prerequisite for a successful alignment of the sections, afterwards. The numbering of the ribbons depends on how you deposited the ribbons during the cutting. To adjust the numbering you have several options available in the context menu. To open the context menu move the cursor over a section and right click with the mouse.



If the numbering is correct proceed to the next wizard step by clicking on **Next**.

15.3.6.6 Specifying the ROIs

Prerequisites

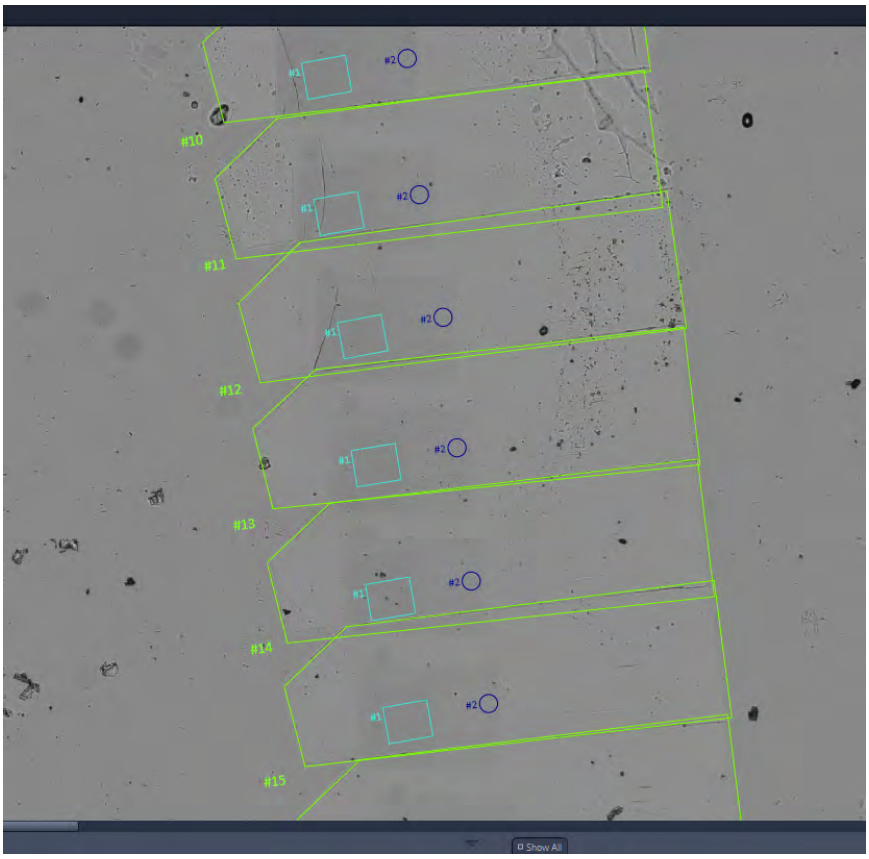
- You are in step **5/7 ROI Specification**.

Procedure

1. On the **ROI Definition** tab select the desired tool for marking a ROI, e.g. **Rectangle** or **Circle**.
2. Mark the desired ROIs in one section. Marked ROIs will be displayed in purple color.

3 Click on **Apply**.

The software will position the defined region of interests in each section according to section contours. The detected ROIs then appear on each section of the ribbons.



If the ROIs are detected correctly proceed with the next wizard step by clicking on **Next.**

15.3.6.7 Imaging the ROIs

In this step we will image the ROIs using a high magnification objective and apply a local focus strategy. This will result in very detailed and sharp images of the ROIs which are used for the further processing (e.g. creating Z-Stacks and image correlation with SEM images).

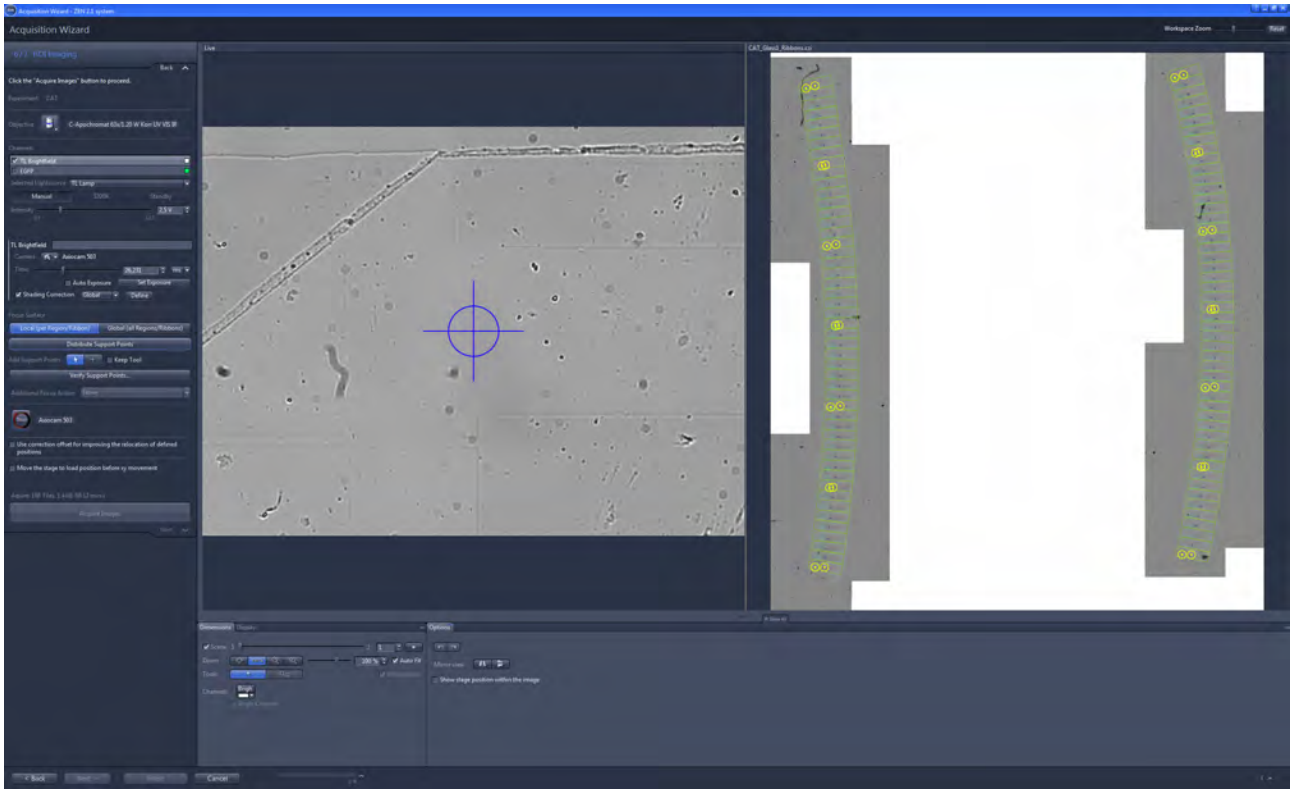
Prerequisites

- You are in step **6/7 ROI Imaging**.

Procedure

1. Under **Objective** select an objective with a high magnification, e.g. 63x.
2. If you are using fluorescence samples you can now activate the corresponding fluorescence channel.
3. Under **Focus Surface** select **Local (per Region / Ribbon)**.
4. Click on **Distribute Support Points**.

   According to the step **3 Ribbon Imaging** the support points will be distributed automatically. The support points are distributed alternately outside and inside a ROI to guarantee best focusing results.



5 Click on **Verify Support Points**.

The software will guide you through the process in the same way you were been guided in step **3 Ribbon Imaging**.

If you have verified all existing support points the message **All points have been verified** will appear.

6 Click on **Acquire Images**.

The ROIs will be imaged now. You can use the next step to check each image of the ROIs and re-acquire (re-shoot) images from ROIs which do not fit your expectations. To proceed with the next step click on **Next**.

15.3.6.8 Re-Shooting ROIs

This step is basically used for re-acquiring images from ROIs that are out of focus.

Prerequisites

- You are in step **7/7 Reshoot**.

Procedure

1 Check if the **Select Tiles** mode is activated. This mode should be selected by default when entering this step.

2 You can use the arrows on each side of the image to navigate through the ROI series or the **Z-Position** slider on **Dimensions** tab.

3 Select an image you want to re-acquire by clicking on it with the left mouse.

The image will be displayed with a dashed green frame.

4 Continue with checking the images and selecting the images you want to re-acquire.

5 When you have selected all images to re-acquire click on **Acquire**.

The software will move to the first image which you have selected.

6 Adjust the focus and click on **Snap**.

7 Click on **Replace** to replace the old image with the new one.

The software will automatically move to the next selected image to be re-acquired.

8 Continue until you have re-acquired all selected images.

9 Click on **Finish**.

You have successfully completed the Acquisition Wizard for the light microscope images of your ribbons. Continue with the process described in the next chapter of this guide.

15.3.7 Acquiring SEM Images

15.3.7.1 General Preparations

For acquiring the SEM image the following general preparations have to be done.

Prerequisites

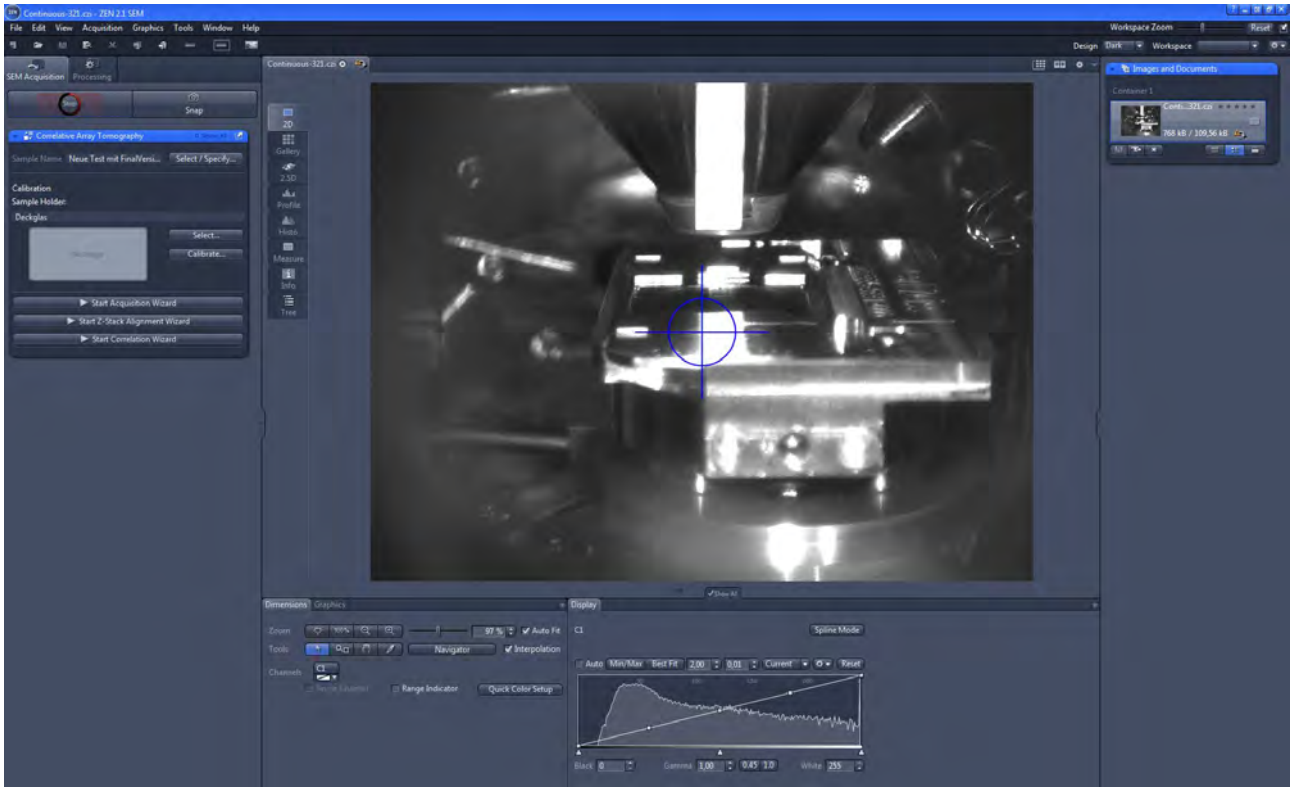
- You have acquired the LM image according to the instructions in the chapter *Acquiring the LM image* [▶ 450].
- You have copied/transferred the image data of the light microscope to the SEM PC.

Procedure

1 Start the **Smart SEM** software. Note that the SEM software is used for setting up the acquisition parameters, e.g. detector settings, magnification, display settings and scan speed.

2 Start the **ZEN SEM** software. Please take care that before you start ZEN , SmartSEM was started.

You will see the **SEM Acquisition** tab and the **Correlative Array Tomography** (CAT) tool.



3 In the CAT tool click on **Select / Specify ...**.

4 Select the sample file which you have created during the LM acquisition. It should be located in the LM folder which was generated automatically.

5 Select the sample holder you are using for your sample.

6 Calibrate the sample holder like described in the chapter Calibration.

You have successfully finished the general preparations. You can now continue with the acquisition of the SEM image.

15.3.7.2 Acquiring the SEM image

Prerequisites

- You have done the general preparations, see *General Preparations* [▶ 463].

Procedure

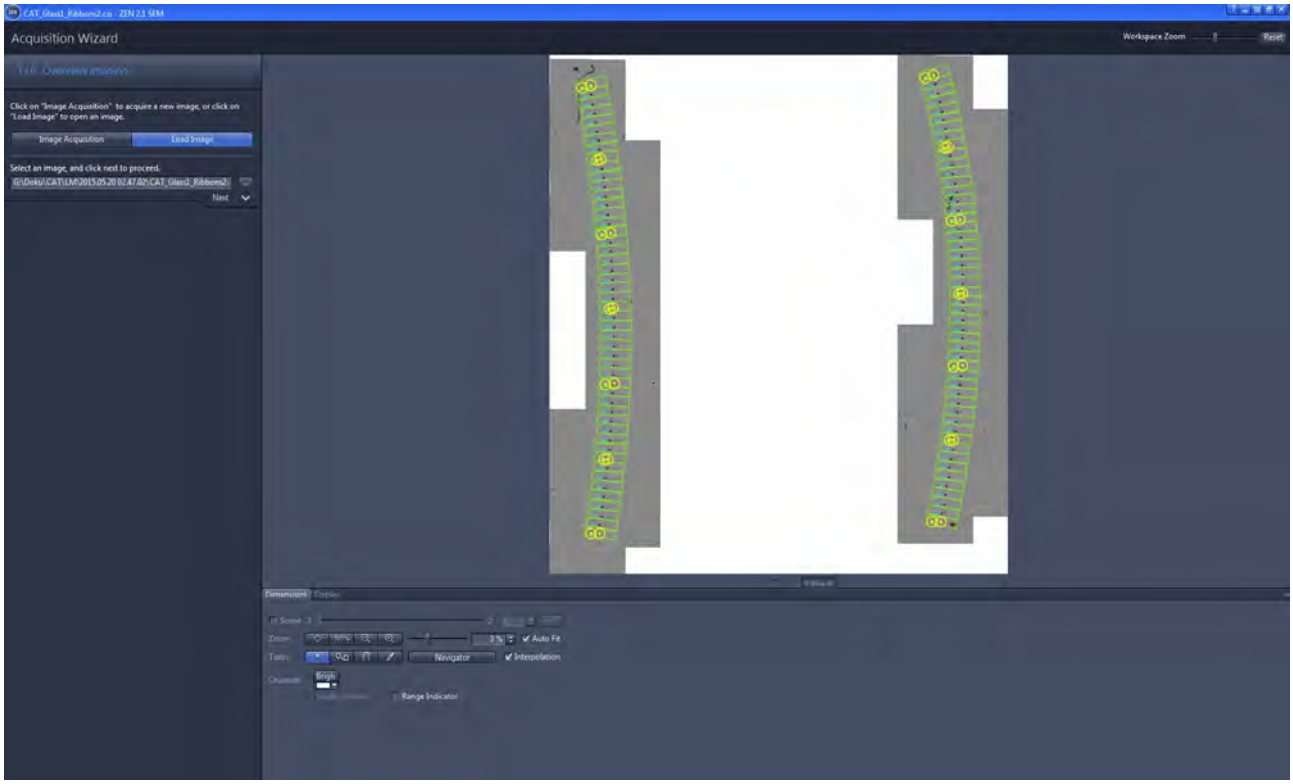
1 In the **CAT** tool click on **Start Acquisition Wizard**.

You will see the first step **Overview Imaging** of the wizard.

2 Click on **Load Image**.

3 Select the image file containing the ROIs from the CAT / LM folder on your file system.

You will see the image containing the ROIs.



4 Click on **Next**.

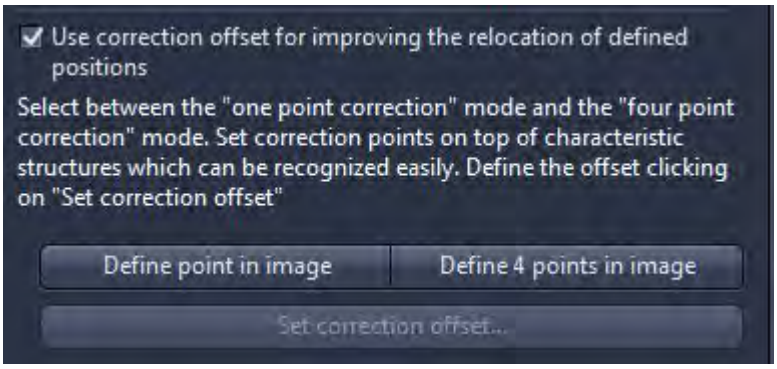
The wizard will now jump directly to step **6/7 ROI Imaging** as the software recognizes the marked ROIs in the image file.

Before starting to acquire the ROIs we recommend to perform the offset correction. Therefore proceed as follows:

5 In the ROI image click on a position with a prominent structure.

The stage will move to that position automatically. Now you may recognize a difference between the position you have clicked on and the actual position. This is the offset we want to correct now.

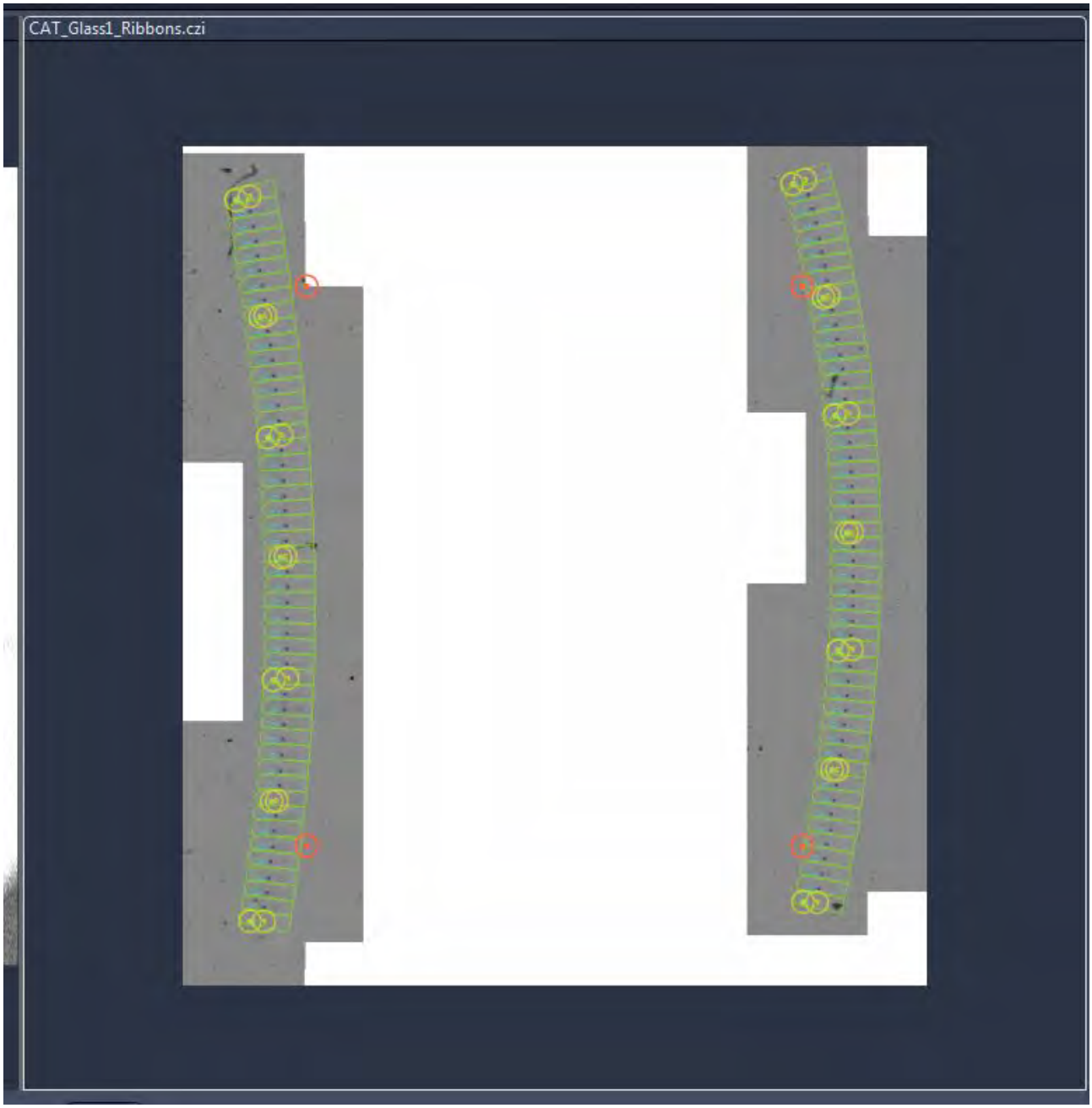
6 Activate the offset correction checkbox.



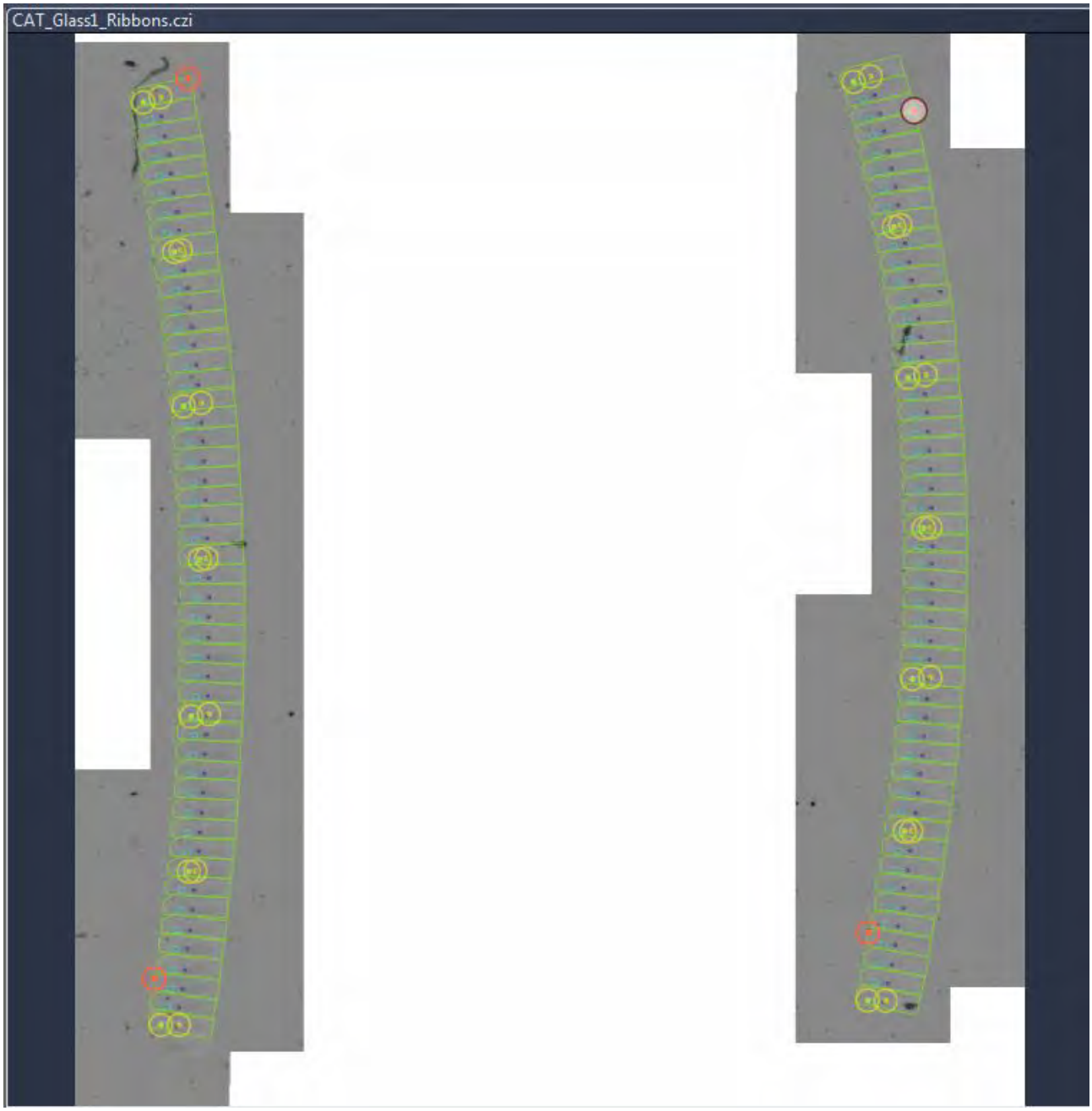
The controls for the offset correction will appear.

7 Click on **Define 4 points in image**.

The 4 correction points are distributed automatically within the image. The correction points look like the support points (red outline with a red dot in the middle).



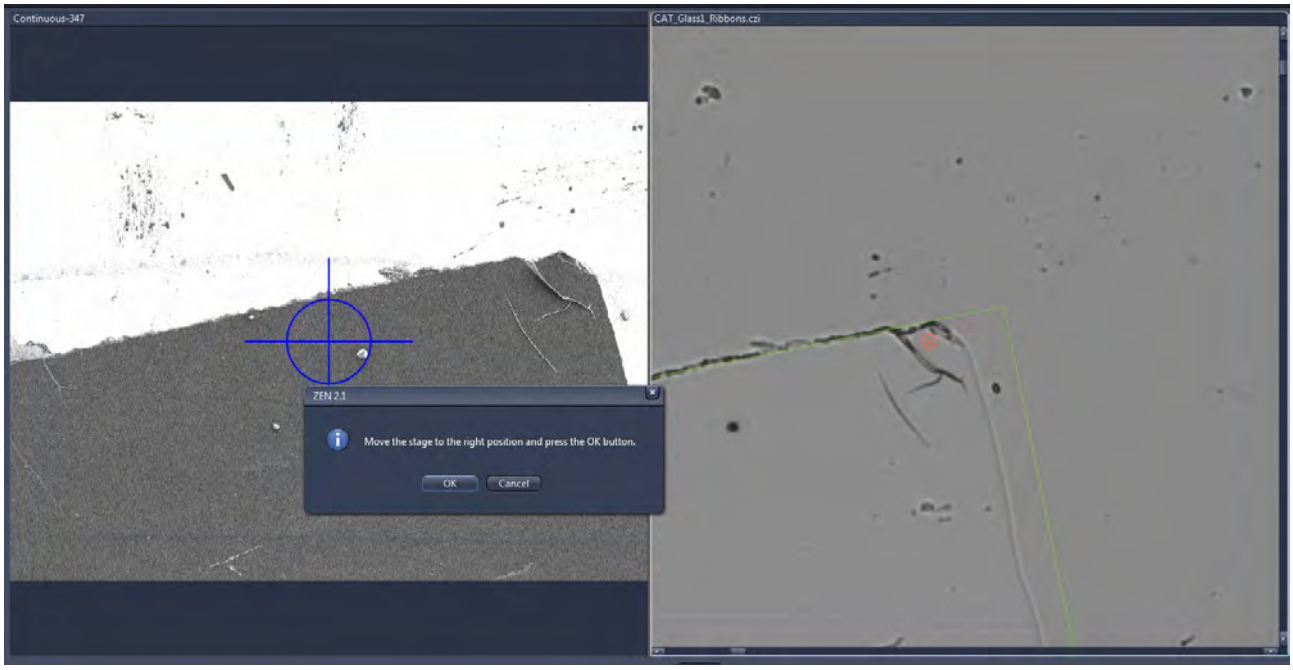
8 Move the correction points to prominent positions on the sample containing structures which are easy to recognize, e.g. the corner of the ribbons.



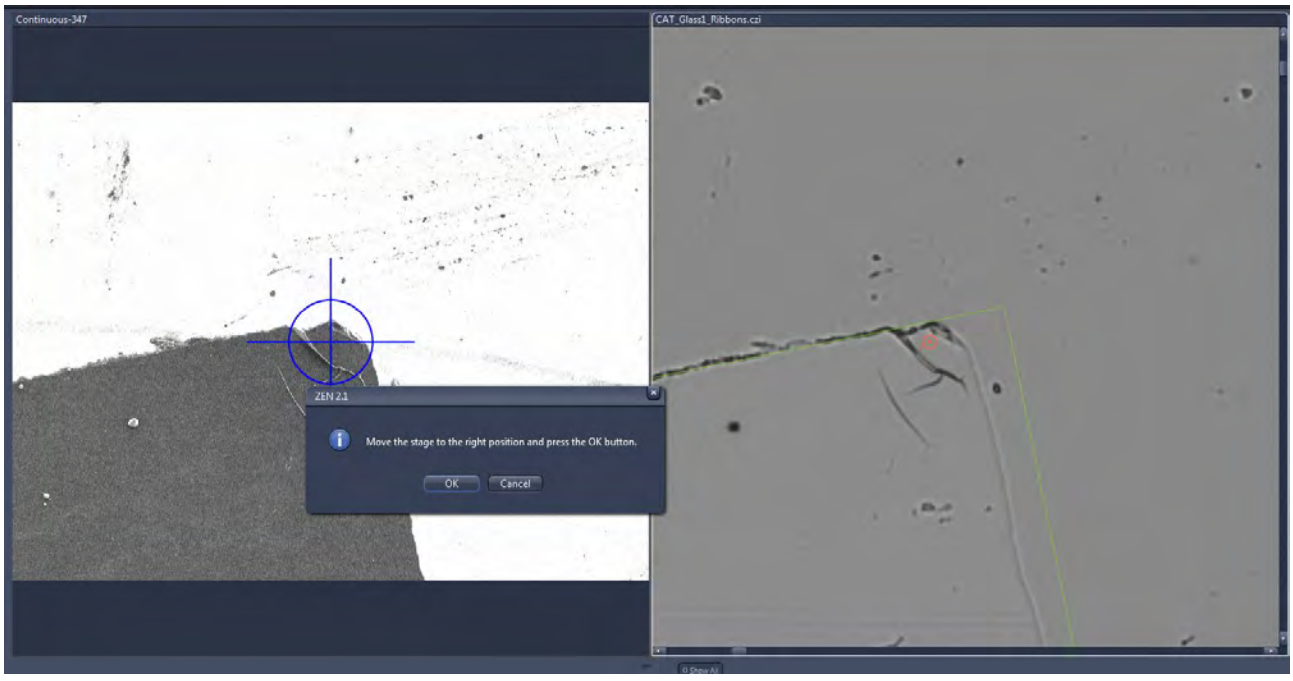
9 Click on **Set correction offset**. Note that for setting the offset correction you should use the same magnification which will be used for image acquisition later.

The stage moves to the first correction point.

In the left image you will see the SEM image position. In the right image you will see the correction point on the LM image. The positions do not match exactly.



10 Move the SEM stage so that its position will match the correction point in the LM image.



11 If the positions do match confirm the message by clicking **OK**.

The stage will move to the next correction point automatically.

Repeat the last 3 steps for the remaining correction points.

If you have finished the offset correction you should continue with verifying the support points again. As this is exactly the same procedure like described for the LM acquisition, read the chapters *Imaging the ribbons* [▶ 453] and *Imaging the ROIs* [▶ 461]. Note that you must use the control panel for the SEM to adjust the SEM parameter.

12 After you have verified the support points click on **Acquire**.

The ROIs will be imaged with the SEM now. Depending on your experiment settings this can take some time.

15.3.8 Aligning the Z-Stack Image

The wizard contains the following 6 steps:

- Image Import
- Pre-Processing
- Image Review
- Alignment
- Manual Correction
- Final Image Creation

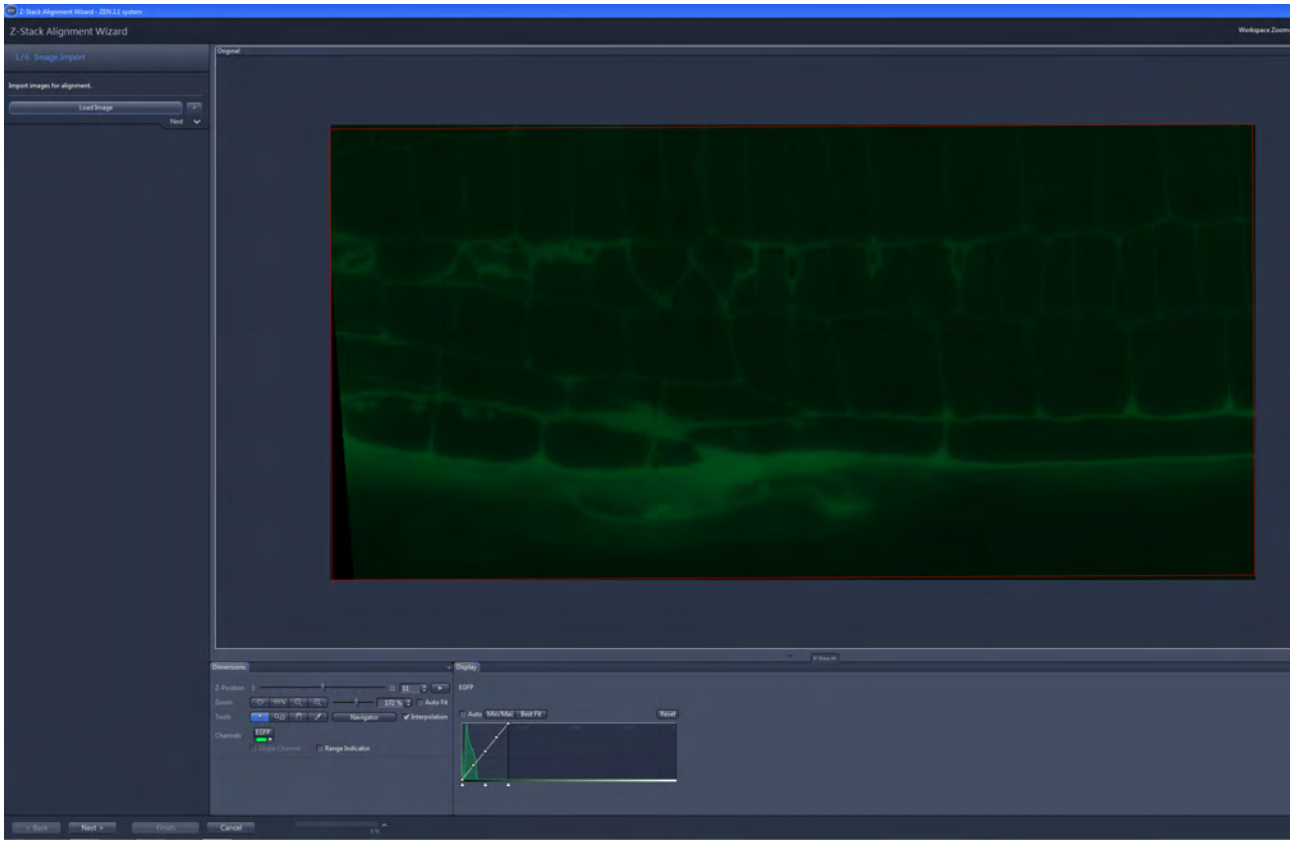
Procedure

1 In the **CAT** tool click on **Start Z-Stack Alignment Wizard**.

You will see the first step **Image Import** of the wizard.

2 Click on **Load Image** and select the acquired Z-Stack image from the file system. In our example we choose the LM image. The same process has to be performed for the SEM image afterwards.

You will see the Z-Stack image in the center screen area.



3 Click on **Next**.

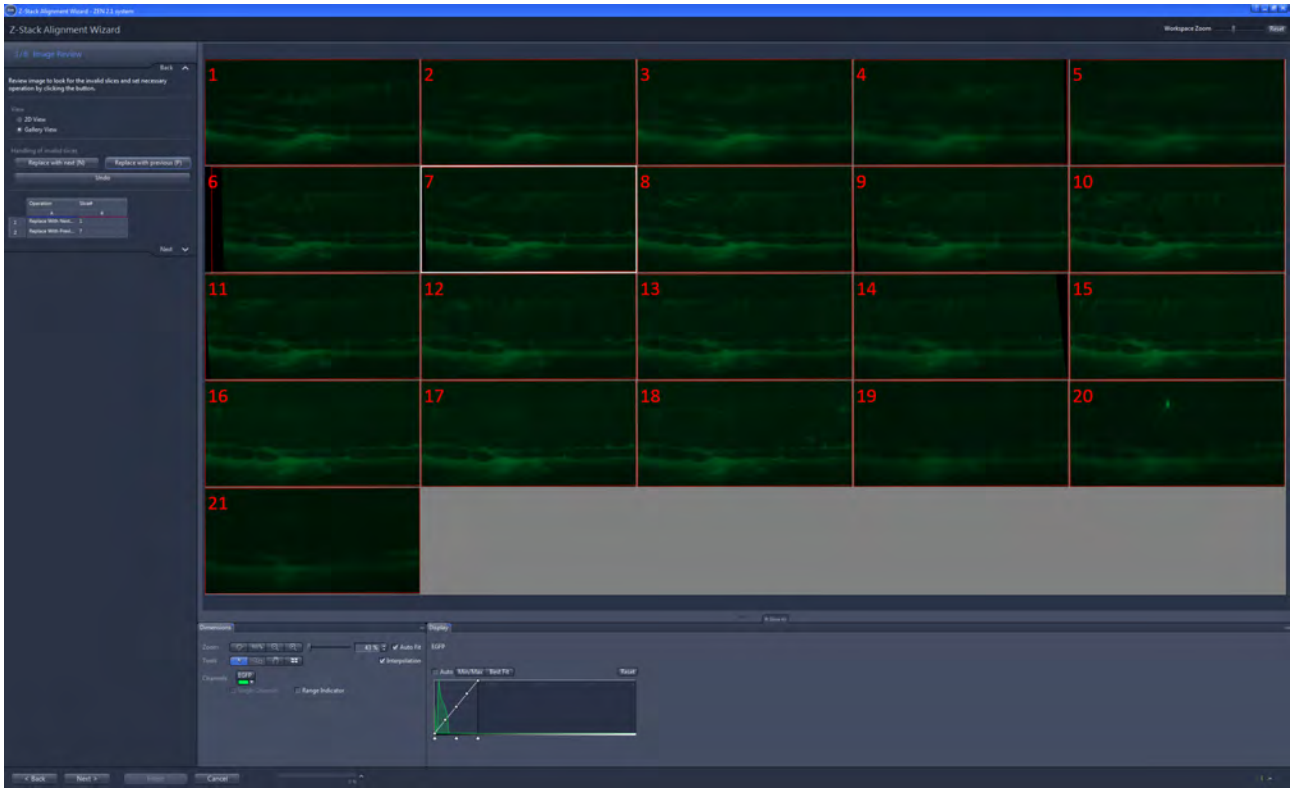
You will see step **2/6 Pre-Processing**.

4 If your image is a tile image click on **Apply Stitching** to correct the offset between the individual tiles.

5 If your image is a SEM image click on **Histogram Equalization** to adjust the image display.

6 Click on **Next**.

You will see step **3/6 Image Review**. Note that in this step no image acquisition is possible. You can just replace an image by the next or previous image of the Z-Stack.



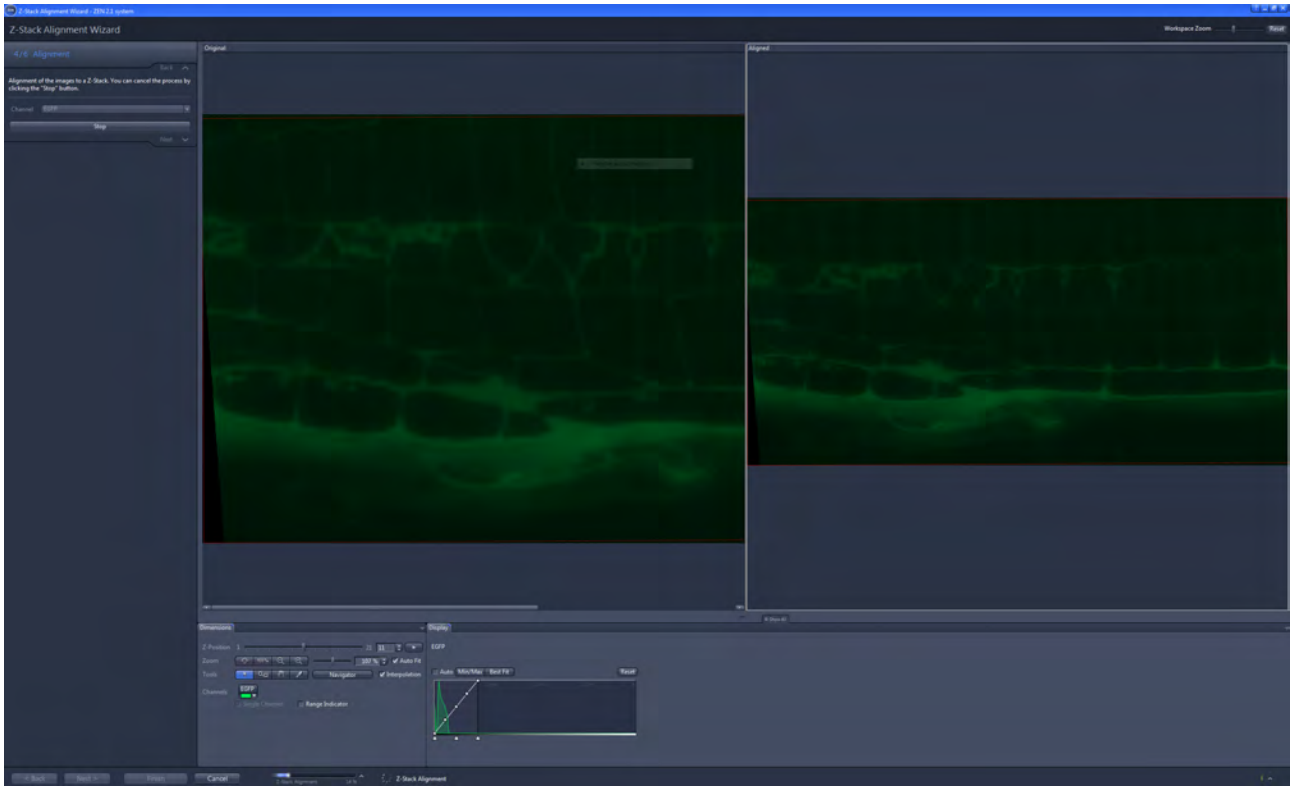
7 Select the image to be replaced by clicking on it with the mouse and click on **Replace with next** or **Replace with previous**. Alternatively you can press the *N* or the *P* key.

INFO

Note that the table of the replaced image will not be saved.

8 Click on **Next**.

You will see step **4/6 Alignment**.



9 If you have acquired a multi-channel image select the reference channel from the **Channel** list.

10 Click on **Start Alignment**.

The alignment of the Z-Stack image will be performed automatically. After the alignment you will see the original Z-Stack image in the left image and in the right image you will see the aligned Z-Stack image.

11 Click on **Next**.

You will see step **5/6 Manual Correction**.

If you browse through the Z-Stack by using the Z-Position slider and you still realize a shift between the single Z-Stack images, you can perform a manual correction of the single Z-Stack images. Therefore continue as follows:

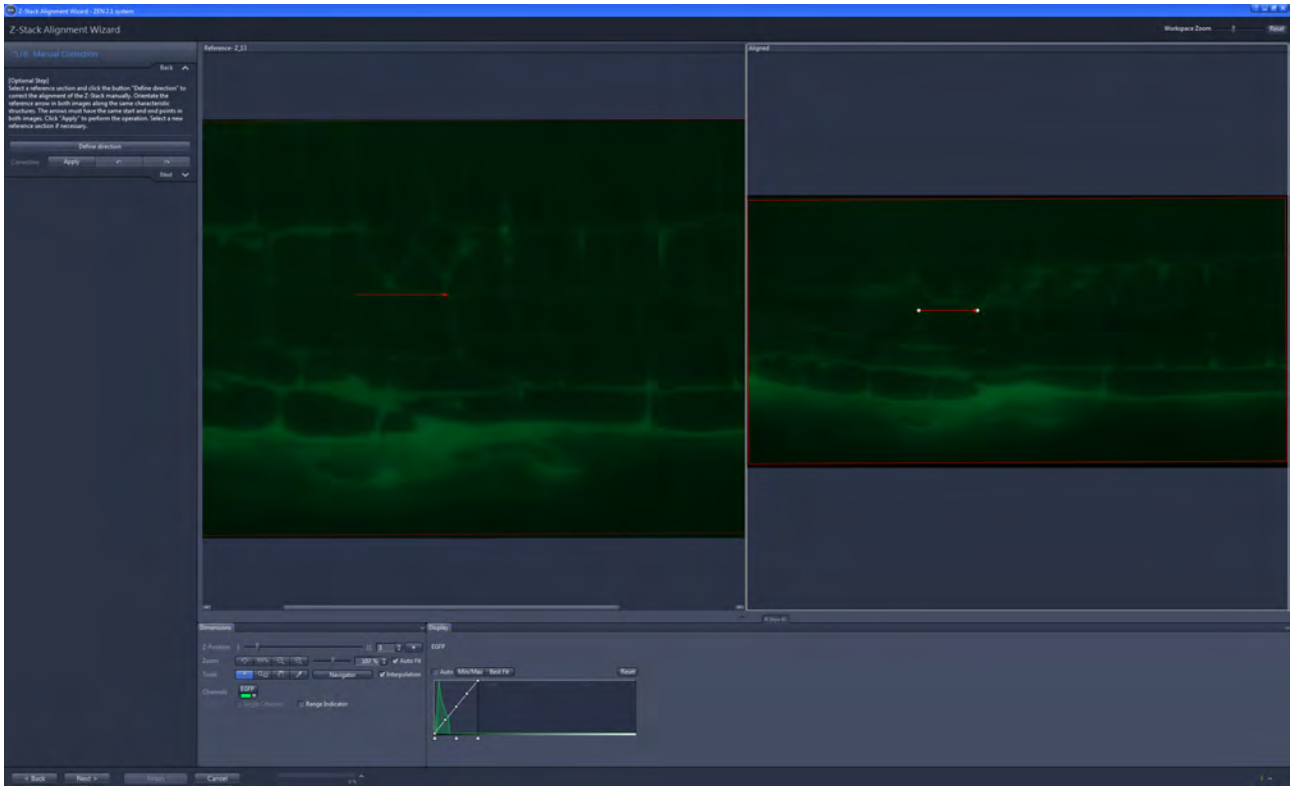
12 Click on **Define direction**.

A red arrow will appear in the right and in the left image.

13 Place the arrow in the left image at a prominent structure in the image which is easy to recognize through the full Z-Stack.

14 Select the right image and browse through the Z-Stack by using the Z-Slider on the **Dimensions** tab.

15 When you realize a shift in an image adjust the arrow in the right image so that it matches with the prominent structure marked with the arrow in the left image. Note that you have to check and adjust the arrow for each image of the Z-Stack which does not match the position.



16 Click on **Apply**.

The correction will be applied. If you want to correct the Z-Stack at another position, simply repeat the procedure with another prominent structure. Of course you can undo and redo actions by using the **Undo** / **Redo** buttons.

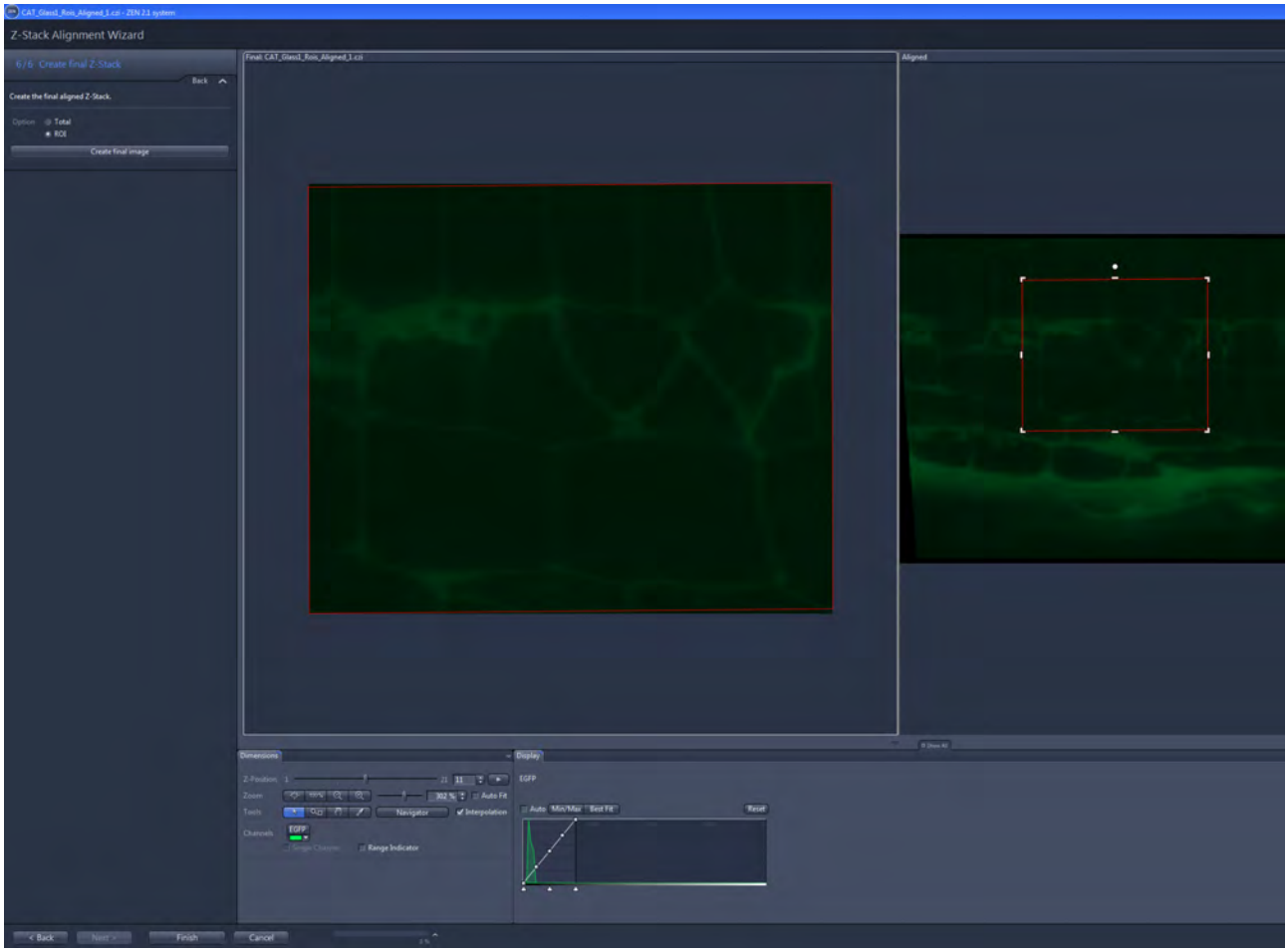
17 Click on **Next**.

You are in step **6/6 Create final Z-Stack**.

18 If you want to create the Z-Stack image from the complete image select **Total** and click on **Create final image**.

19 If you want to create the Z-Stack image from the marked ROIs, select **ROI**. Note that you can adjust the marked ROI in its size and position here. If you have marked more than one ROI you can switch between the ROIs by using the **Scene** slider on **Dimension** tab.

20 Click on **Create final image**.



You have successfully aligned and created the Z-Stack image. Of course you have to repeat the process for the SEM image that was acquired.

15.3.9 Correlating the LM and SEM images

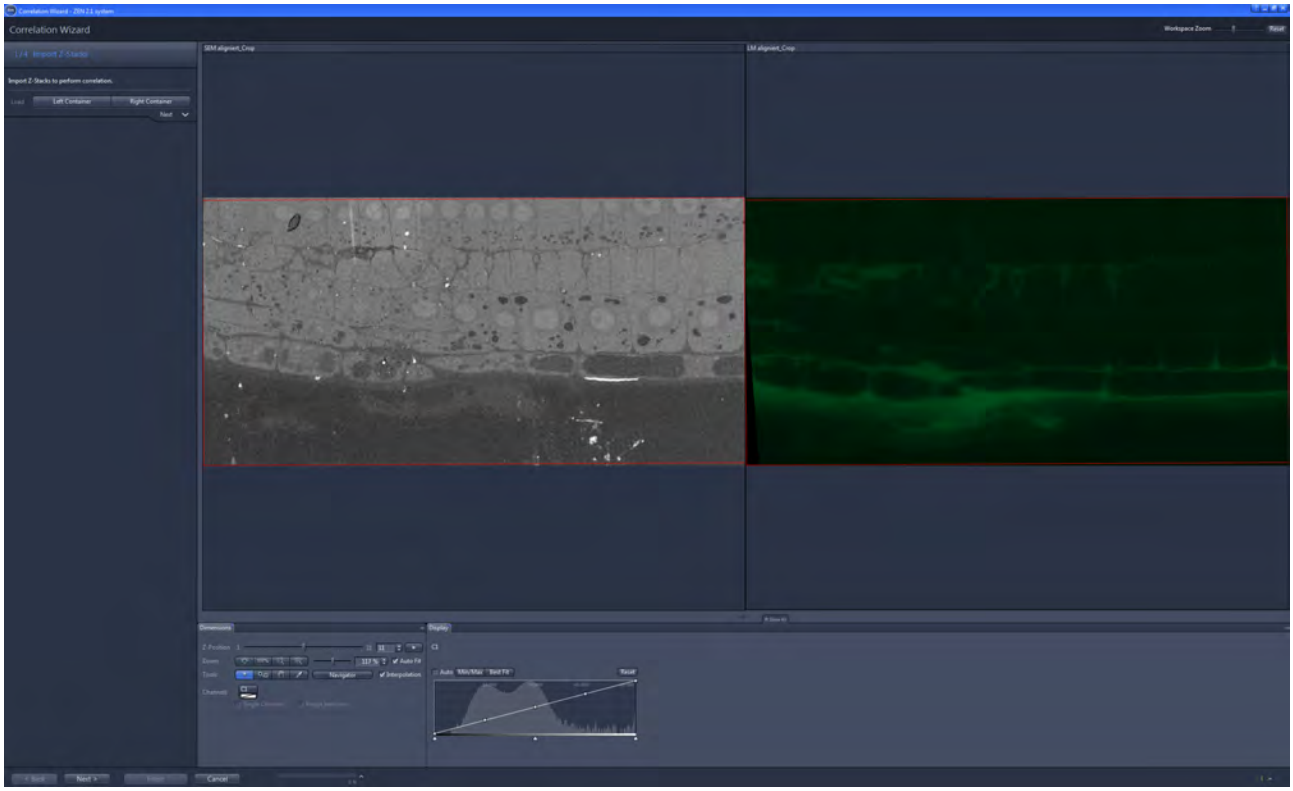
The wizard contains the following 4 steps:

- Import Z-Stacks
- Correlation
- Manual Correction
- Create Final Correlation Image

Procedure

1. In the **CAT** tool click on **Start Correlation Wizard**.

   You will see the first step **Import Z-Stacks**.
2. Click on **Left Container** to load the aligned Z-Stack image from the SEM.
3. Click on **Right Container** to load the aligned Z-Stack image from the LM.



4 Click on **Next**.

   You will see step **2/4 Correlation**.

5 Under **Transform** decide whether you want to transform the **Left Z-Stack** into the **Right Z-Stack** or vice versa.

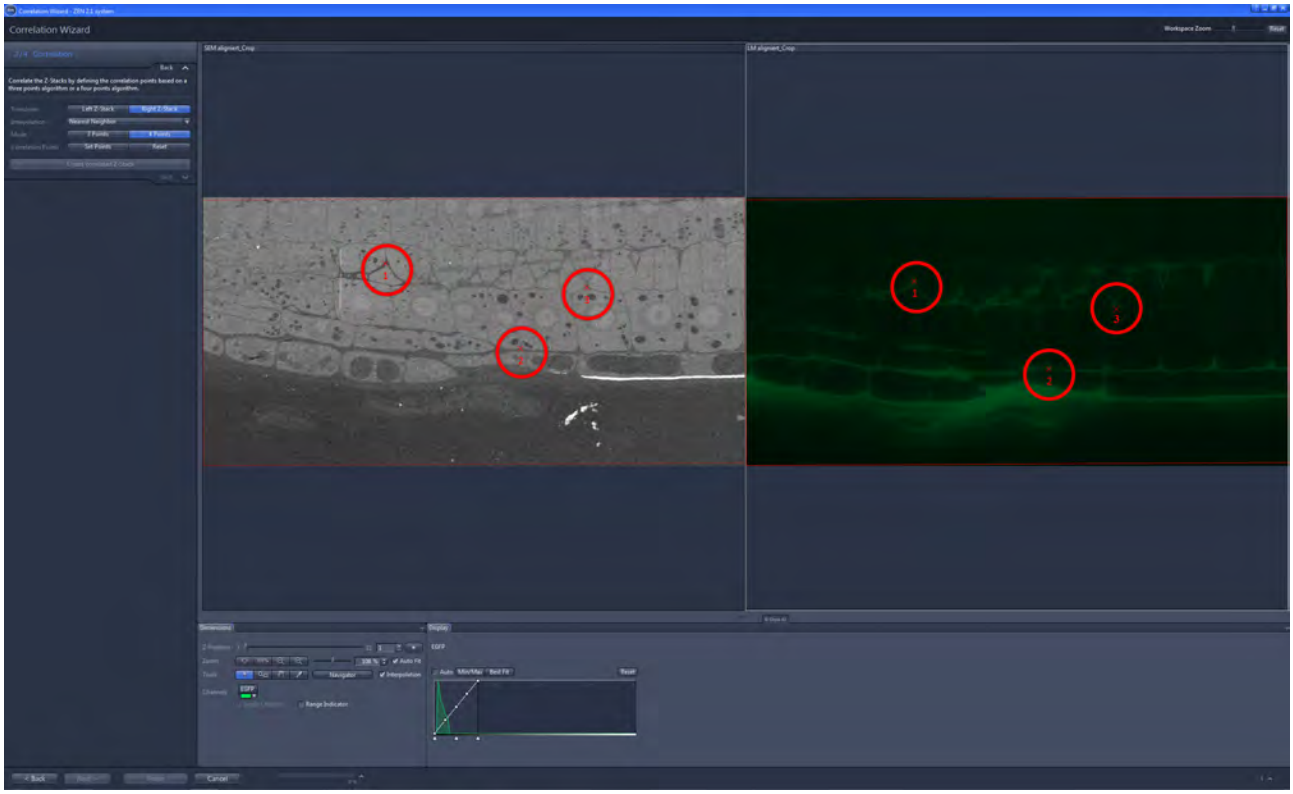
6 Under **Mode** select **4 Points**.

7 Click on **Set Points**.

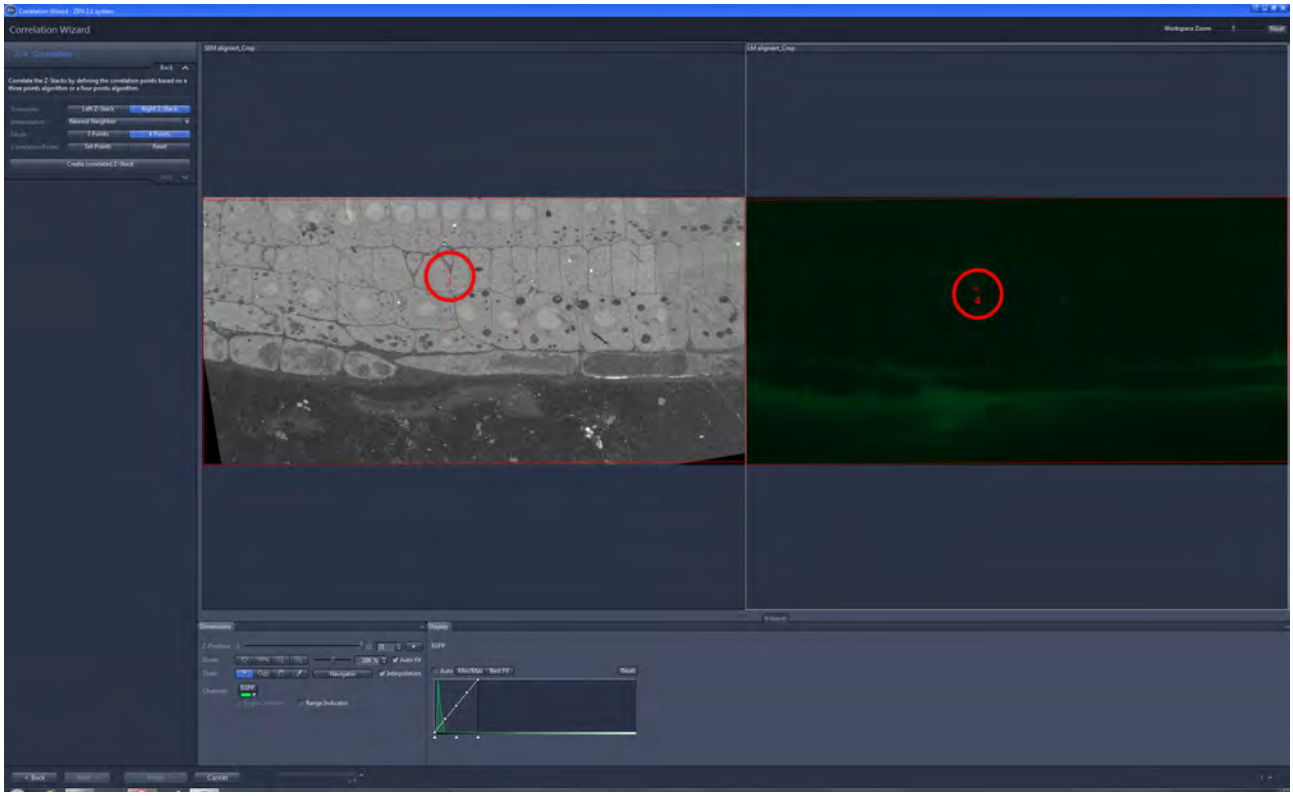
8 Set the first 3 correlation points in the first Z-Stack image of the left image.

9 Set the corresponding 3 correlation points in the first Z-Stack image of the right image.

   After the third point in the right image was set, the software automatically jumps to the last Z-Stack image.

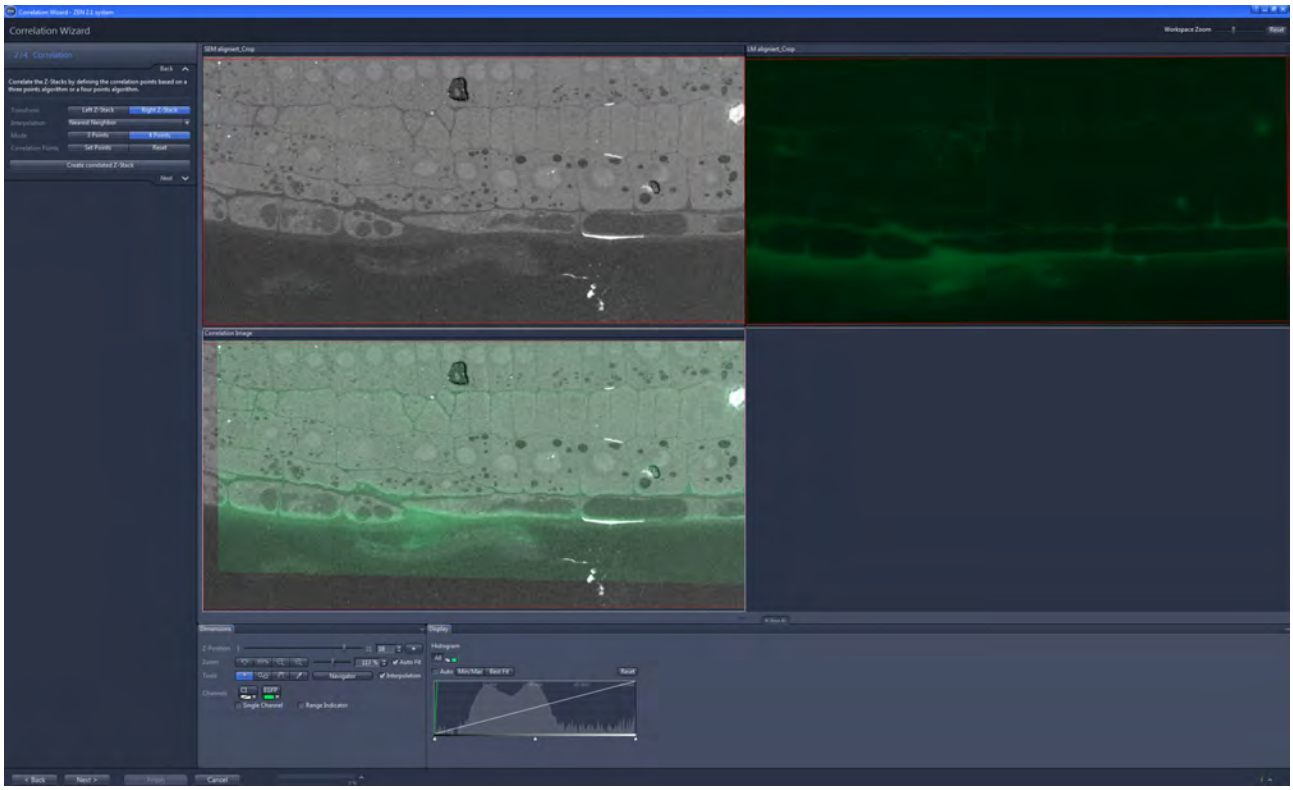


10 Set the fourth correlation point in the left image first and then set it in the right image.



11 Click on **Create correlated Z-Stack**.

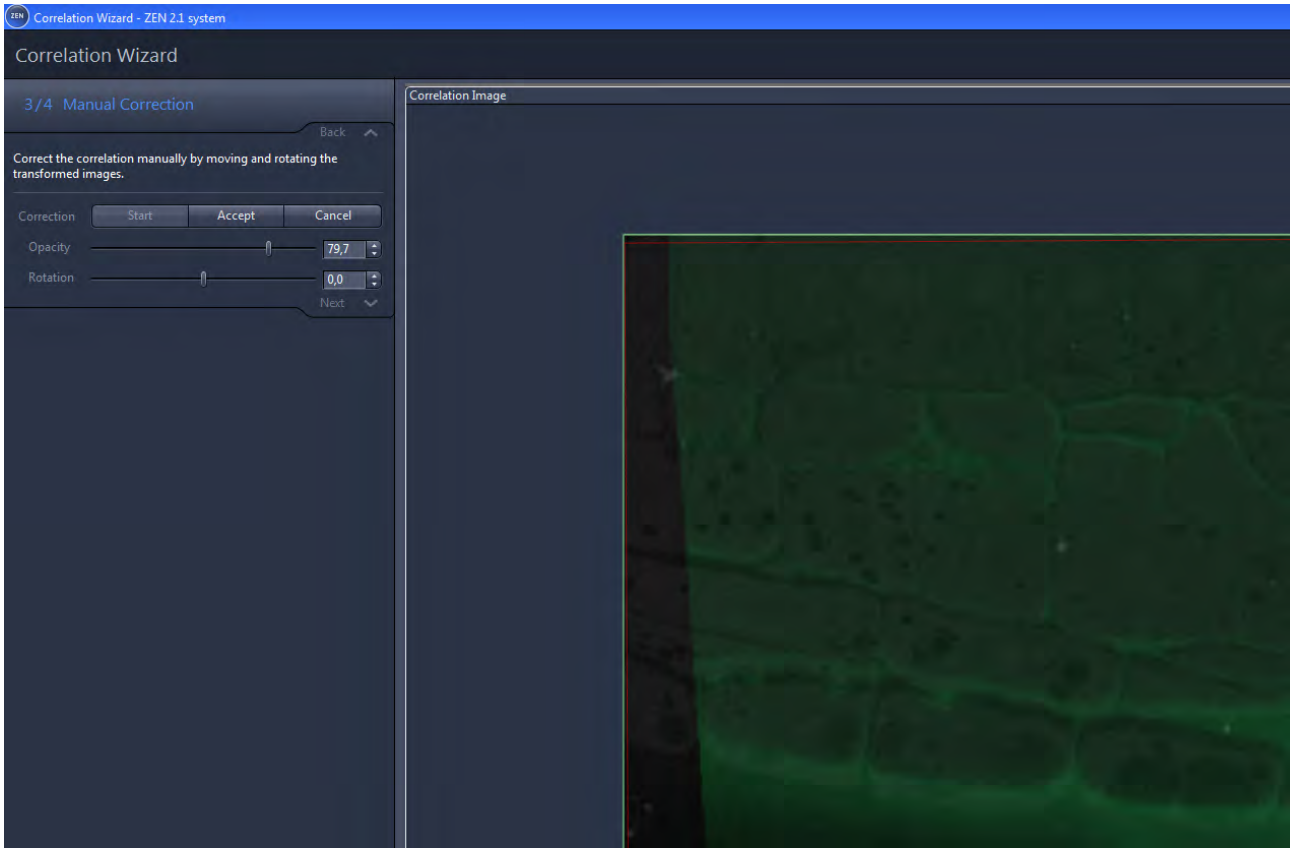
The correlated Z-Stack image will be generated and displayed in a separate image container.



12 Click on **Next**.

You will see step **3/4 Manual Correction**.

13 In this step you can manually correct the alignment of the images by moving and/or rotating the images according to each other. To rotate the image use the handle at the top of the image frame. To move the image simply left click on the image and hold the mouse button pressed while moving the image.



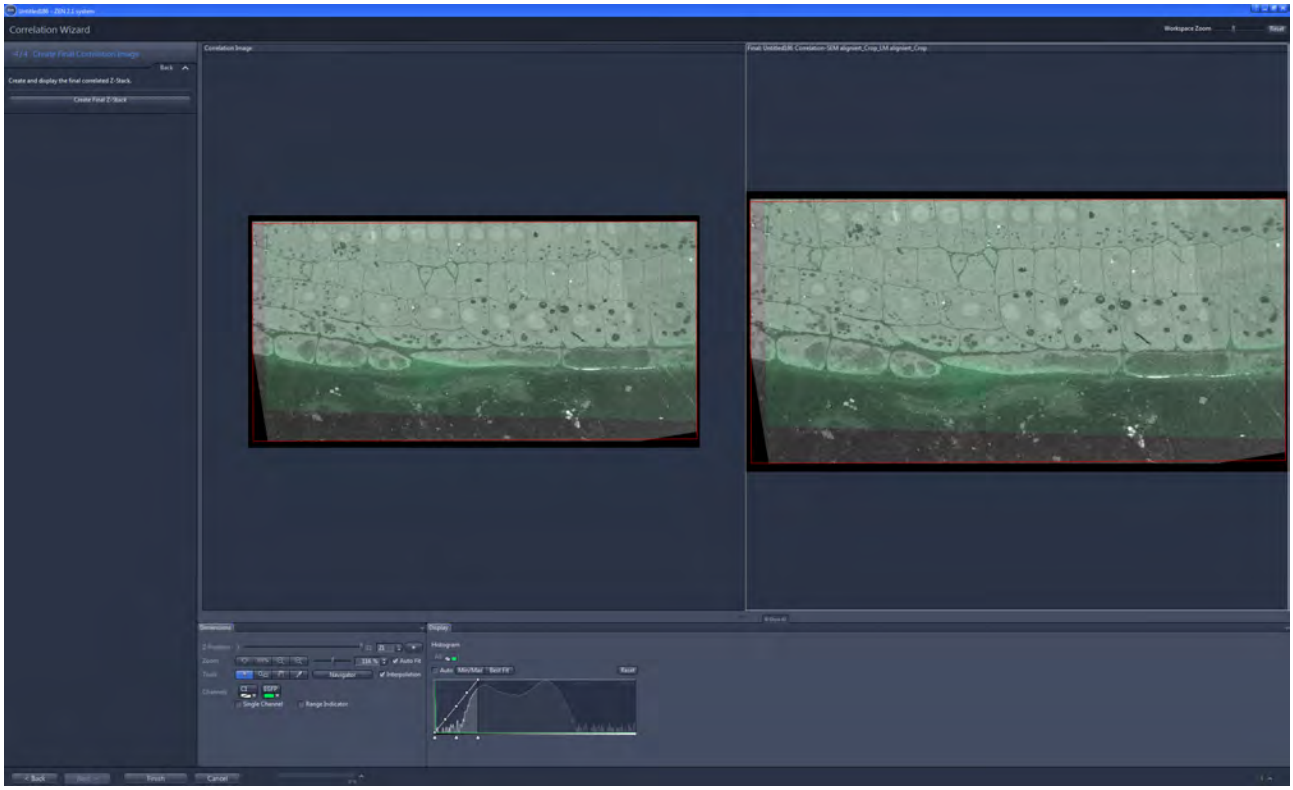
14 If you finished the alignment of an image click on **Accept**. You can browse through the correlated Z-Stack images by using the **Z-Position** slider in the **Dimension** tab.

15 Click on **Next**.

You will see step **4/4 Create Final Correlation Image**.

16 Click on **Create Final Z-Stack**.

The correlated Z-Stack image will be created.



17 Click on **Finish** to exit the wizard.

You have successfully created an correlated Z-Stack image.

15.4 Functions and Reference

15.4.1 Introduction

In this chapter you find the detailed descriptions of all parameters of the CAT module. This chapter is for beginners who want to get an overview of all software functions as well as for experts who search for information about a specific function.

15.4.2 CAT Tool

Using this tool you can calibrate and manage the sample holders and start the wizards which are used for acquiring images from serial sections, generating Z-stack images out of the single images and correlate two Z-Stack images from the light microscope (LM) and the scanning electron microscope (SEM).

Fig. 15.3: CAT Tool

Parameter		Description
Sample Definition (1)		
-	Select / Specify...	Opens the **Select Sample** dialog, see *Select Sample Dialog* [▶ 481]. There you can select a sample data sheet from the list or specify a new sample with user specific information. The specified sample information will be used for image processing (i.e. Z-Stack alignment) or for data management.
Sample Holder Calibration (2)		
-	Select...	Opens the **Select Template** dialog. There you select the preferred sample holder or define new holder templates, see Selecting the sample holder.
-	Calibrate... button	Opens the *Sample Holder Calibration Wizard* [▶ 483]. There you can calibrate the selected sample holder.
-	Apply to Image button	Only visible if the **Show All** mode is activated. **Use this button only, when you forgot to calibrate the holder before you acquire the image.**

Parameter	Description
	Applies a calibration to an acquired image. Do not remove the sample out of the correlative holder between image acquisition and calibration. Exception: correlative markers are on the sample holder.
Start Acquisition Wizard (3)	Starts the **Acquisition Wizard**, see *here* [▶ 486].
Start Z-Stack Alignment Wizard (4)	Starts the **Z-Stack Alignment Wizard**, see *here* [▶ 495].
Start Correlation Wizard (5)	Starts the **Correlation Wizard**, see *here* [▶ 497].

15.4.2.1 Select Sample Dialog

Here you can select or create a sample data sheet.

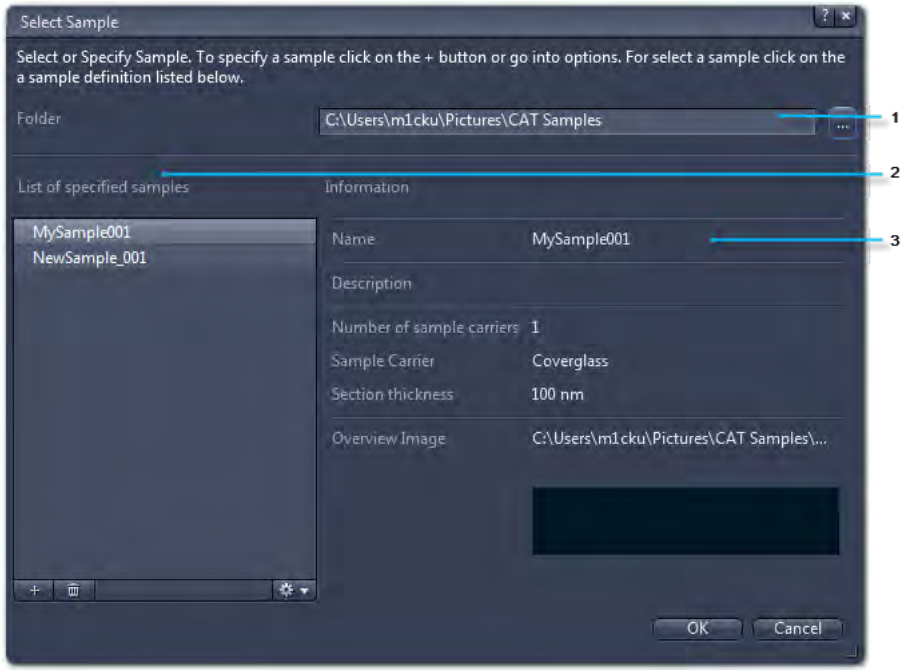
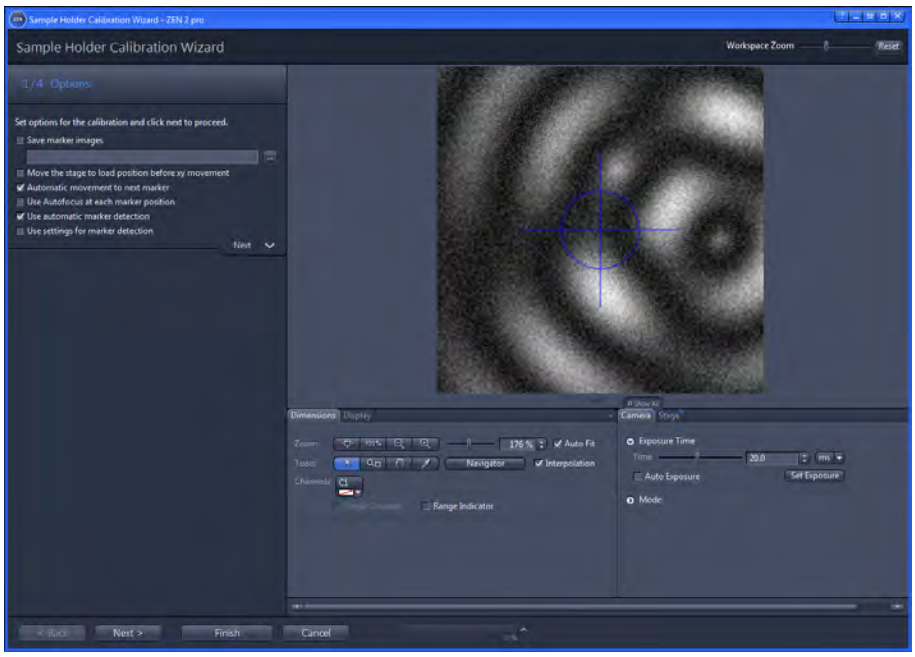


Fig. 15.4: Select Sample Dialog

No.	Parameter	Description
1	**Folder**	Shows the location, where the files are saved.

No.	Parameter	Description
		If you click on the  button you can change the storage location. The default path and folder is **C:\Users\user\Pictures\CAT Samples**. Within this folder, each sample is saved in a sub-folder. Images taken during image acquisition within the CAT Acquisition Wizard will be saved within the sub-folder, automatically. For a better clarity sub-folders with the name „[Date] [Time]“ will be generated when another CAT run is started within the CAT acquisition wizard. In case the file name will exceed a certain number of characters the name will be shortened using the sign „**°**“.
2	**List of specified samples**	Shows the samples which are already specified within the software. If you select a sample in the list and click **OK**, the sample will be used in your experiment. If you click on the  button, the **New Sample** Dialog opens. There you can create a new sample definition which will be added to the list, see *Creating a new sample* [▶ 443]. If you click on the **Options** button, you will see further options for managing samples like **Show/Edit** or **Refresh Sample List**.
3	**Information**	Shows the specified sample information, e.g. name, description, number of sample carriers, sample carrier, and section thickness.

15.4.3 Sample Holder Calibration Wizard



With the Sample Holder Calibration Wizard you calibrate the selected correlative sample holder. Make sure that you have selected the desired sample holder, see Selecting the sample holder.

15.4.3.1 Step 1: Options

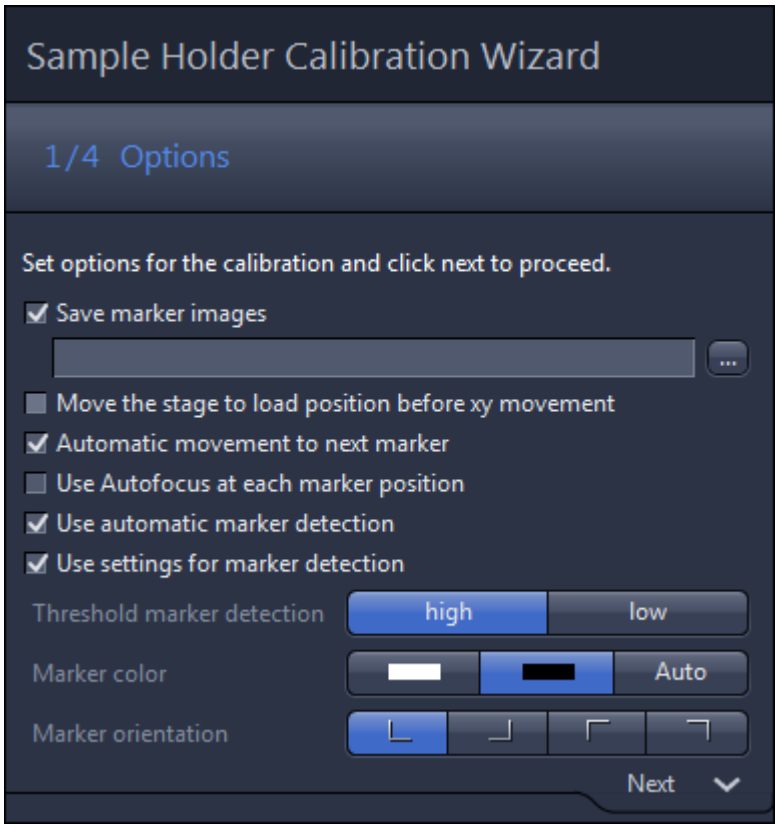


Fig. 15.5: Sample Holder Calibration Wizard Options

Option	Description
Save marker images	**Activated:** the marker images are saved during the calibration. The images can be used to check the calibration afterwards. Click on the **Select Folder (...)** button to select a storage folder.
Move the stage to load position before x/y movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Automatic movement to next marker	**Activated:** By clicking on the **Next** button within the wizard the stage moves automatically to the next calibration marker. **Deactivated:** You must use the joystick to navigate to the markers. This is necessary when using a correlative holder which has no holder data deposited.
Use Autofocus at each marker position	This option is active only if the **Automatic movement to next marker position** checkbox is activated.

Option	Description
	Activated: the focus is adjusted automatically after moving to the next marker position.
Use automatic marker detection	**Activated:** The software will detect the small calibration marker automatically.
Use settings for marker detection	This option is active only if the **Use automatic marker detection** checkbox is activated. **Activated:** shows settings for marker detection (see description below). Here you select the properties of the calibration markers.

Settings for marker detection

Only visible if the **Use settings for marker detection** checkbox is activated.

Option	Description
Threshold marker detection: high – low	A low threshold for marker detection is used when the dimensions of the correlative L markers cannot be recognized precisely, e.g. when the sample holder is slightly filthy.
Marker color	Here you select the color of the markers displayed in the live image. **White:** the marker is displayed white on a dark background. **Black:** the marker is displayed dark on light background. **Auto:** the marker color is set automatically.
Marker orientation	Here you need to set the orientation of the L-markers on your sample holder. Click on the corresponding button to select the orientation of the calibration marker which you can see in the live image

If you click on the **Next** button you will move to the next step of the wizard.

15.4.3.2 Step 2-4: Calibration

In steps 2-4 of the wizard you will be guided through the calibration procedure.

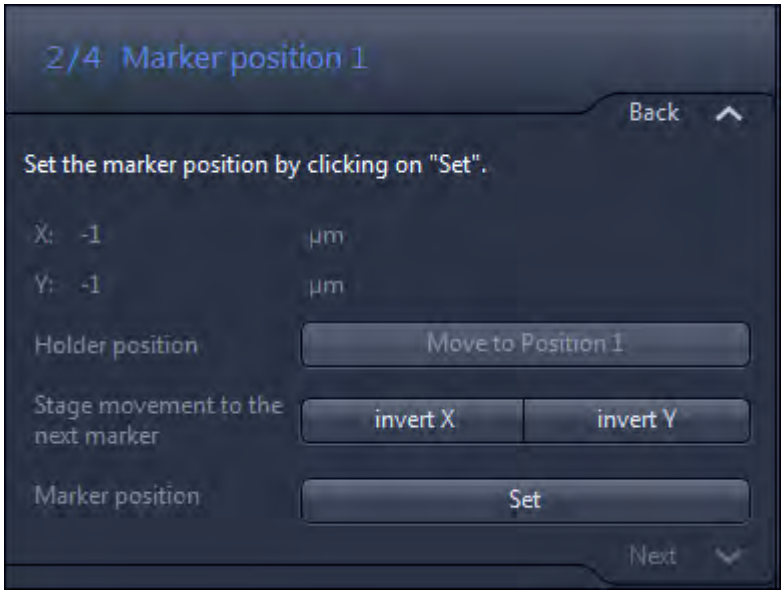


Fig. 15.6: Sample Holder Calibration Wizard

Option	Function
Holder position	**Move to Position 1** button Moves the stage to marker position 1. This is possible only if the first position was set before and x/y coordinates are given. **Current** button Only visible for marker position 2 and 3. Moves the stage to the current marker position. This is possible only if the current position was set before and x/y coordinates are given.
Stage movement to the next marker	Here you can change the movement of the stage in x or y direction. This is necessary if during calibration the stage moves in the wrong direction.
Marker position	By clicking on the **Set** button, the actual marker position will be confirmed.

Click **Finish** to leave the wizard.

15.4.4 Acquisition Wizard

This wizard is used to image the serial sections or user-defined region of interest within the sections.

The steps **Overview Imaging**, **Ribbon Imaging**, **ROI Imaging** and **Re-Shoot** are image acquisition steps. The step **Re-Shoot** gives you the opportunity to image parts of the ROI-series or tiles of a tile image, later.

The wizard consists of 7 steps which are described in the following chapters:

- *1/7 Overview Imaging* [▶ 487]
- *2/7 Ribbon Specification (optional)* [▶ 489]
- *3/7 Ribbon Imaging (optional)* [▶ 490]
- *4/7 Section Specification* [▶ 490]
- *5/7 ROI Definition* [▶ 493]
- *6/7 ROI Imaging* [▶ 494]
- *7/7 ReShoot (optional)* [▶ 494]

15.4.4.1 Overview Imaging

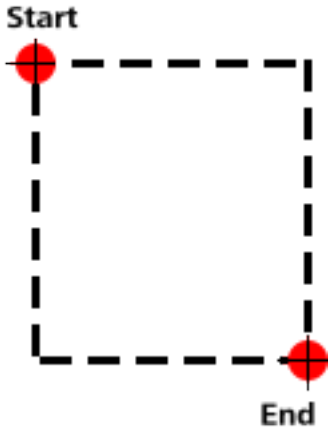
In this step you can acquire an overview image that allows navigation on the sample. You will see the positions of the serial sections on the sample carrier. In general, for the overview image an objective with low magnification is used. This makes the acquisition fast due to a large field of view and limited number of tiles.

Image acquisition with objectives with higher magnification is possible. But keep in mind, that the number of tiles will increase due a smaller field of view as well as the acquisition time.

i INFO

We recommend to use phase contrast images for the acquisition. The used algorithm for the automatic section specification, see step 4, is most reliable then.

Parameter	Description
Image Acquisition / **Load Image**	By selecting the corresponding button you can decide whether to acquire an overview image or load an image.
Image Selection	If you have selected **Load Image**, a saved image file from the file system can be chosen. Therefore simply click on the  button and navigate to your image file. The wizard will jump to the wizard step according to the information saved within the loaded image.
Experiment	If you have selected **Image Acquisition** you have to select an experiment from the **Experiment** list. Note that the experiment has to be set up and saved in advance, before you enter the wizard.

Parameter	Description
Objective	Here you can select the objective that you want to use for the acquisition of the overview image. As mentioned before, we recommend to use an objective with a low magnification (e.g. 2.5x or 5x).
Channels	Here you can select the channels that you want to use for the acquisition of the overview image. You can use more than one channel in one run, when your microscope is equipped with a motorized condenser.
Selected Light Source	Here you can select the light source that you want to use for the acquisition of the overview image. The light intensity can be adapted if a corresponding light source is selected.
Camera Settings	Here you can adapt the camera settings like changing the exposure time or activating / deactivating the shading correction. If a shading correction has been performed and activated in the selected experiment, the checkbox will also be activated automatically in the wizard.
Software Autofocus	Here you can activate the Software Autofocus functionality and apply it to the overview image. If activated, you can select the positions for focusing. During focusing no live image will be visible. Note that sensitive fluorescence labels might be bleached during the autofocus process.
Overview Image Definition	Here you can define the size of the overview image by defining a start and end position.  The software will calculate the area by means of the

Parameter	Description
	defined start and end position (= overview image). The number of tiles and the memory used will be displayed below the buttons.
- Set start position	Sets the current stage position as start position of the image area.
- Set end position	Sets the current stage position as end position of the image area.
- Move to start position	Moves the stage automatically to the defined start position. Note that the start position has to be defined before.
- Move to end position	Moves the stage automatically to the defined end position. Note that the end position has to be defined before.
No. of sample carrier	Only active, if you use more than one sample carrier for one correlative Z-Stack. Here you have to select the number of the used sample carrier. The total number of used sample carriers was defined in the CAT tool under **Select / Specify sample**.
Move the stage to load position before xy movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Acquire Overview Image	Starts image acquisition. The acquisition can be stopped in between. Then the button will change to **Restart**. Before you restart the image acquisition, you can modify the settings. The status of the image acquisition is shown in the status bar of the software. After the overview image is taken, the image can be stitched. If you click on the **Apply Stitching** button stitching will be carried out.
Next	Brings you to the next wizard step.

15.4.4.2 Ribbon Definition

This step is an optional step. It allows to image the ribbons with an objective with higher magnification, if necessary. E.g. when you would like to define the regions of interest by means of the sample structure that can only be identified using lenses with higher magnification.

Therefore you can mark the outlines of the ribbon on your sample. If you can`t see the sample structures, due to the overview image was acquired with a too low magnification, you can image your sample again using a higher magnification.

For marking the outlines in the image use the tools from the **Ribbon Definition** tab (e.g. Rectangle, Circle or Polygon).

15.4.4.3 Ribbon Imaging

For this step a split view will appear. On the left side you see the Live image. On the right side you see the overview image with the defined ribbons.

i INFO

To modify either the Live image or the image with the defined ribbons click on the corresponding container. The activated container will be marked with a white frame.

Again, like in step one for the overview image, you have to select the objective, channel, light source and adapt the exposure time. Additionally you have to generate a focus surface to ensure that your sample will be in focus during the image acquisition.

If you click on the **Create Ribbon Image** button, the ribbon image will be acquired.

15.4.4.4 Section Specification

To determine the positions of regions of interest (ROIs) within the sections, you have to define the sections. The section lines generate the reference system for the ROI positions. The sections are marked and outlined with a frame.

i INFO

Note that you must mark one section on each ribbon. Meaning when your sample has three ribbons, three sections have to be marked overall.

We recommend to use phase contrast images for the section specification. The algorithm used for the automatic section specification is most reliable when using phase contrast images. When bright field images are used, the algorithm might be sub-optimal. In that case you have the opportunity to add and to move the section contours manually.

On the **Section Definition** tab you will find tools and options for creating sections in the image.

Parameter	Description
Reference Section	
- Select	Activates the selection mode (default).
- Contour	If selecting this mode you can mark a contour line of a section.
- Keep	Keeps the selected tool active. You can then use the tool several times without interruption.
- Step Backward	Undo the last step
- Step Forward	Repeats the last step.
- Delete	Deletes a selected graphical element.
Section Index	Here you can determine the starting number of the ribbons. This is important when the ribbons of a sample are deposited on more than one sample carrier.
Selected Channel	Shows the selected channel which is used for the detection.
Detection Sensitivity	Here you can adjust the detection sensitivity from **Low** to **High** by using the slider. This will be done by modifying the contrast thresholds for the section detection algorithm. When setting a low sensitivity, sections will be recognized even if the contrast between the section and the substrate is low; disadvantage: sections will be recognized, even in areas where no serial sections are deposited. When setting a high sensitivity, the algorithm only recognizes sections, if there is a high contrast between section and substrate. If not all sections were recognized you have the possibility to copy section contours or to stamp section contours.
Section Detection	
- Apply	Starts the section detection on the sample. The software tries to detect each section within the ribbon.
Contrast Method	

Parameter		Description
-	Auto	Auto is used by default. The system recognizes the contrast method of the image automatically.
-	Ph.Contrast	Applies the phase contrast method. Even if you are using a brightfield image, phase contrast will be applied as contrast method.
-	Brigthfield	Applies the brightfield contrast method. Even if you are using a phase contrast image, brightfield will be applied as contrast method.
Use Internal Structure		Activate this checkbox only when sample structures are clearly visible within the sections. If activated, sample structures are used for section detection, additional to contrast differences between sections and substrate. In case that sections are not detected properly, you have the possibility either to stamp section contours or to copy section contours.
Post Definitions		
-	Stamp tool	If selecting this tool you can stamp in undetected sections after the section detection is finished. Therefore simply select the tool and move the mouse cursor in the area nearby the last detected section. The cursor will change to a stamp icon and you will be able to stamp in the missing section contours.
-	Accept Ref. Section	If you click on this button reference contours will transform into section contours.

When the section detection is finished you have different options for sorting the sections according to your needs, if necessary. Therefore right click on the detected sections. You will see a context menu with the following sorting options:

Parameter	Description
Sort	

Parameter	Description
	■ **Sort all sections in reverse order** Sorts all sections which have a section contour. The initial section with number 1 becomes the last section, the last section becomes the first section with the number 1 ■ **Sort selected Ribbon elements in reverse order** Sorts the selected sections on a ribbon. The initial section with number 1 becomes the last section, the last section becomes the first section with the number 1
Copy selected sections from here	Copies the selected sections.
Copy selected Ribbon sections from here	Copies all section contours on the selected ribbon.
Paste Section(s) to here	Pastes the section contours (selection of certain section contours or all sections of a ribbon) to the selected position.
Exchange numbering of selected Ribbon sections	Exchanges the numbering of selected sections on different ribbons.
Highlight Related Ribbon Sections	Highlights selected sections on a ribbon.
Merge two Ribbon Sections	Merges sections on two different ribbons to one.

15.4.4.5 ROI Specification

In this step you can screen your sample for interesting sample regions (ROIs) and mark this area by a graphical element. You can define several regions of interest within in one section.

On the **ROI Definition** tab you can draw either a rectangle, a circle or a freehand polygon/contour. Click on the **Apply** button to automatically identify the region of interest in all other sections. It is also possible to **Undo** / **Redo** an action using the corresponding buttons. To remove a graphical element select it and click on the **Delete** (bin icon) button.

INFO

With the arrow keys on your keyboard you can jump from one ROI to the next ROI along the series to check if the structure of interest is still within the defined region of interest.

15.4.4.6 ROI Imaging

With this step you can image the ROIs which are detected and marked in the previous step. The tile images will be generated from all defined region of interests automatically.

INFO

The size of the snapped tile images of a ROI series can change due to the number of tiles which are necessary to image the defined region of interest. The number of tiles can vary due to the bending of the ribbon.

15.4.4.7 Re-Shoot

This step is helpful, if some tiles or regions of interest are blurry. These tiles/regions can be replaced by repeating the acquisition of the selected tiles or tile images. The procedure is as follows:

- Select all blurry tiles, first.
- Adjust the focus for each tile position.
- Take new images.

Parameter	Description
Select Tiles	If this mode is active, you can select the tiles which you want to re-shoot. Use the **Z-Position** slider under **Dimension** tab or the arrows within the Image area to scroll through the acquired images. If you found a tile image that you want to re-shoot, simply click on it. Then the color of the image frame turns from red to green. Note that all tiles or blurry regions have to be defined, before the image acquisition can be repeated.
Acquire	If this mode is active you can acquire the selected tiles again after the focus was adjusted manually. If you click on this button, the stage will move to the first tile and the following buttons will appear:

Parameter		Description
-	Snap	Acquires a new image.
-	Replace	Replaces the old tile by the new tile.
-	Correct Brightness	In case the tile is brighter or darker, here you have the possibility to adapt the brightness of the tiles image

Click **Finish** to exit the wizard.

15.4.5 Z-Stack Alignment Wizard

This wizard is used to align the single images of a Z-Stack image. The wizard consists of 6 steps which are described in the following chapters:

- *1/6 Image Import* [▶ 495]
- *2/6 Pre-Processing* [▶ 495]
- *3/6 Image Review* [▶ 496]
- *4/6 Alignment* [▶ 496]
- *5/6 Manual Correction (optional step)* [▶ 497]
- *6/6 Final Image Creation* [▶ 497]

15.4.5.1 Image Import

In this step you can load your acquired Z-Stack images which you want to align. Therefore simply click on the **Load** button and select the image file from the file system.

15.4.5.2 Pre-Processing

In this step you can perform pre-processing functions on the loaded image, e.g. Stitching (only for tile images), Brightness and Contrast Correction (only for SEM images).

Parameter	Description
Apply Stitching button	Only visible if a tile image is loaded. If you click on this button, stitching is performed automatically on the image. The stitching can be canceled (Undo) or repeated (Redo) by using the arrow buttons.
Clip Limit	Reduces noise in the image. The higher the Clip Limit , the lower the noise. The clip limit can be adjusted between 0 and 10 %.

Parameter	Description
Region Size	Defines the region for histogram equalization. The smaller the area, the higher the contrast, but the noise will increase, too. The Region Size can be adjusted from 16 to 1024 px.
Histogram Equalization	If you click on this button, the SEM images are adapted to the selected values. The Histogram Equalization can be canceled (Undo) or repeated (Redo) by using the arrow buttons.

15.4.5.3 Image Review

This step is used for reviewing the single images of a Z-Stack. This is necessary because certain images might not be useful for 3D reconstruction due to problems during the image acquisition or sample preparation issues (wrinkles or ruptures within the section). These regions can be replaced either by the previous image or by the following image. To review the images, the images can be displayed as single 2D images in the **2D** view or as images series in the **Gallery** view.

Parameter	Description
2D View	If selected, you can review the single images of a Z-Stack image by using the 2D view. You can use the **Z-Position** slider to navigate through the single images. To replace an image select the image and click whether on the **Replace with next** or **Replace with previous** button. If you click on the **Undo** button the last action performed will be undone.
Gallery View	If selected, you can review the single images by using the **Gallery** view. The single images of a Z-Stack image are displayed as an image gallery. If you found an image that does not meet your expectations, simply select the image and replace it by the next or previous image.

15.4.5.4 Alignment

In this step you perform the image alignment. Therefore simply click on the **Start Alignment** button. To cancel the alignment click on the **Stop** button.

INFO

Before you start the alignment, select one channel as reference channel (e.g. DAPI, because it stains the nucleus and the nucleus is a proper structure for performing alignment).

During alignment a splitter view is visible. In the left container you can see the original images, in the right container you can see the aligned images.

15.4.5.5 Manual Correction

In this step (optional) you can navigate through the aligned images and check the result of the alignment.

In case the results are unsatisfactory, you have the possibility to correct the alignment of the images manually. Misalignment can occur, when no characteristic structures are visible within the images.

15.4.5.6 Final Image Creation

Last but not least, in this step you create the final image.

Parameter	Description
Total	If selected, the complete image will be used for the image creation.
ROI	If selected, only the ROI area will be used for the image creation.
Create final image button	Creates the final aligned Z-Stack image.

Click **Finish** to exit the wizard.

15.4.6 Correlation Wizard

This wizard is used to correlate a Z-Stack image from the Light Microscope (LM) with the Z-Stack image from the Scanning Electron Microscope (SEM). The wizard consists of 4 steps which are described in the following chapters:

- *1/4 Import Z-Stacks* [▶ 498]
- *2/4 Correlation* [▶ 498]
- *3/4 Manual Correction* [▶ 499]
- *4/4 Create Final Correlation Image* [▶ 499]

15.4.6.1 Import Z-Stacks

In this step you can import the aligned Z-Stack images from the LM and the SEM, e.g. the Z-Stack image from the LM in the left container and the Z-Stack image from the SEM in the right container.

If you click on the **Left Container** button, the image is opened in the left image container.

If you click on the **Right Container** button, the image is opened in the right image container.

15.4.6.2 Correlation

In this step you correlate the images.

Parameter	Description
Transform	Here you select which Z-Stack will be transformed. During transformation a pixel in the overlay image of the Z-Stack is calculated by using pixels of the two original images that shall be overlaid / merged.
Interpolation	Here you can select one of the following interpolation methods:
- Nearest Neighbor	The gray value of the resulting pixel in the overlay image is made of a pixel which is located next. This interpolation method is very fast.
- Linear	The resulting or calculated pixel in the overlay image is assigned to a gray value, which is the result of a linear combination of gray values derive from pixels located nearby (in the original image).
- Cubic	The calculated pixel in the overlay image is assigned to a gray value, which is calculated by means of a polynomial function using gray values of pixels in the original images; these pixels are located nearby the calculated pixel.
Mode	Here you can choose an algorithm mode:
- 3-Points	If selected, this mode enables you to set 6 correlation points after clicking on the **Set Points** button (3 points in each Z-Stack in each container)
- 4-Points	If selected, this mode enables you to set 8 correlation points after clicking on the **Set Points** button (4 points in each Z-Stack), 3 points in the first z-section, the last point in the last section
Correlation Points	If you click on the **Set Points** buttons you can set the correlation points.

Parameter	Description
	The number of correlation points is according to the selected algorithm. The cursor will change to a pipette symbol. Simply click in the image to set the points. Start with setting the first three points in the left container then set the corresponding correlation points in the right container. If a correlation point is set, a check mark icon will appear in front of the corresponding point. When you select the 4-Points-Algorithm the display will move automatically to the last image of the Z-Stack. Set the fourth correlation point in both containers. Make sure that the positions in both Z-Stacks are identical. After you have set all correlation points the cursor will be changed backwards from the pipette to the arrow. **Reset** deletes all correlation points in the image.
Create correlated Z-Stack	If you click on this button, the correlated Z-Stack will be generated and opened in a new image container.

15.4.6.3 Manual Correction

In this step you can correct the correlation manually by moving and rotating the transformed image.

Therefore simply click on the **Start** button. Then you can interactively move the image by dragging and dropping it with the mouse or rotate the image by clicking on the circle button attached on top of the green image frame or using the **Rotation** slider. You can also change the image opacity by adjusting the corresponding slider.

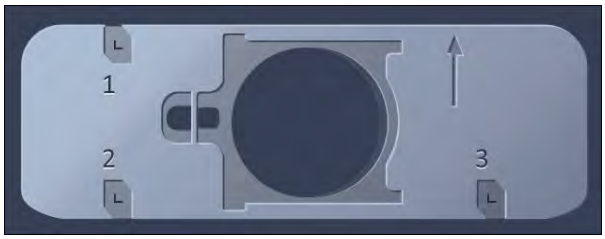
If you click on the **Accept** button the manual correction will be adopted to the correlated image.

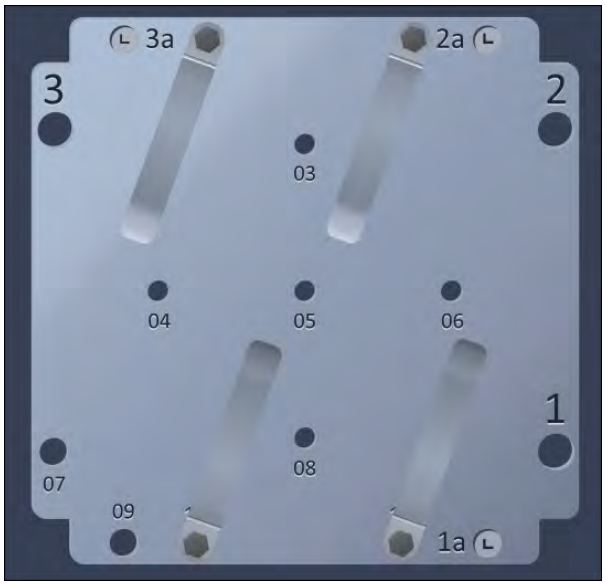
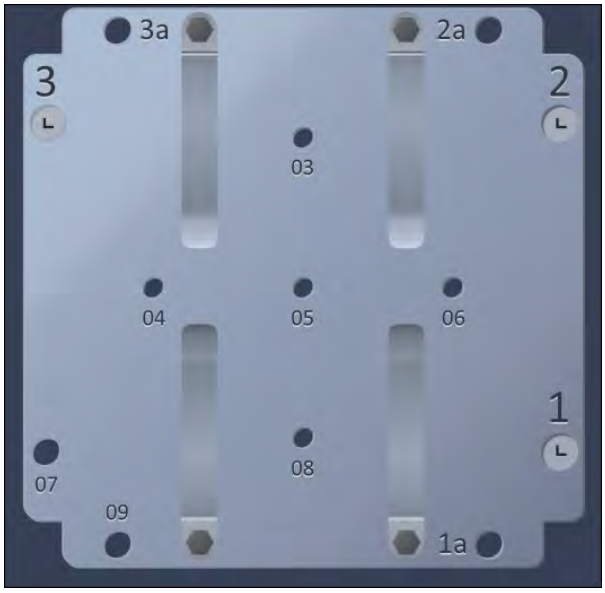
15.4.6.4 Create Final Correlation Image

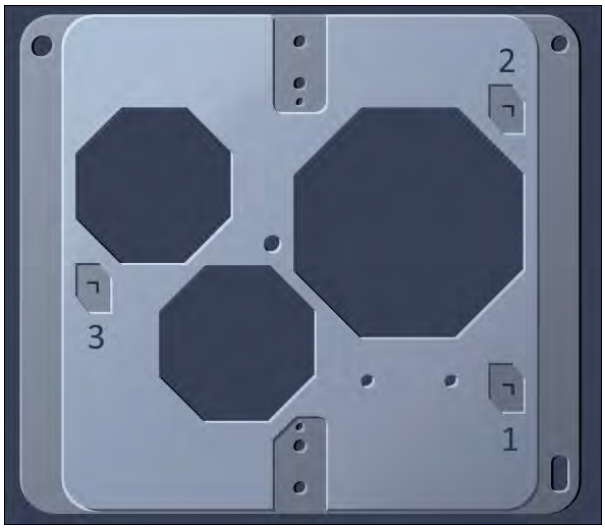
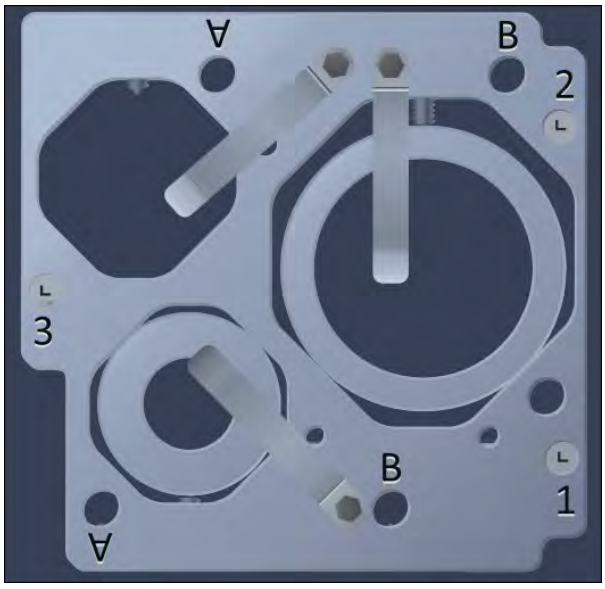
In this step create the final correlation image. Therefore simply click on the **Create final Z-Stack** button. Click **Finish** to exit the wizard.

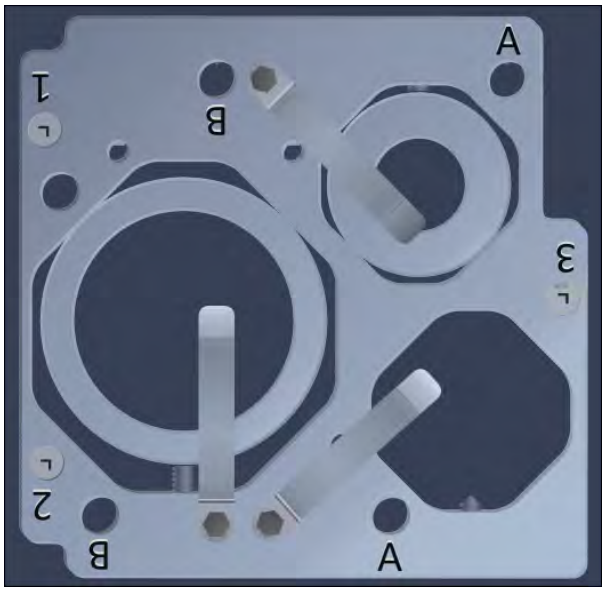
15.5 Appendix

15.5.1 Correlative Sample Holders

Name	Image
Life Science cover glass 22x22	
Life Science for TEM Grids	
Cover glass with fiducials 22 x 22	

Name	Image
MAT Flat Stubs A	
MAT Flat Stubs	

Name	Image
MAT Universal A	
MAT Universal B_A	

Name	Image
MAT Universal B_B	

16 Module Confocal Topography

16.1 Introduction

For our customers in material science, e.g. materials research labs, we have developed the module **Confocal Topography** for the ZEN software. The module includes the following functionality:

Topography Acquisition

With the **Topography Tool** you can acquire and inspect surfaces or surface structures of different sample types (e.g. wafer plates, solar cells). By the help of the **Topography Measurement Wizard** you can perform an confocal image acquisition of your sample. The advanced analysis of the topography image is performed by the help of the ConfoMap software which is included in the software package.

In the chapter **Functions & Reference** you find detailed functional descriptions of the *Topography Tool* [▶ 516] and the *Topography Measurement Wizard* [▶ 516].

In the chapter **Workflow Topography Acquisition** you find a detailed how-to guide for topography acquisition, see *Introduction* [▶ 504].

Layer Thickness Measurement

With the **Layer Thickness Measurement Tool** you can acquire z-stacks and perform layer thickness measurements of one or more transparent layers (e.g. coatings, alloys). Here as well, the **Layer Thickness Measurement Wizard** will guide you through the process. The measured values can be corrected by the refractive index of the coating.

In the chapter **Functions & Reference** you find detailed functional descriptions of the *Layer Thickness Measurement Tool* [▶ 517] and the *Layer Thickness Measurement wizard* [▶ 518].

In the chapter **Workflow Layer Thickness Measurement** you find a detailed how-to guide for layer thickness measurement, see *Introduction* [▶ 510].

16.2 Workflow Topography Acquistion

16.2.1 Introduction

In this chapter you find a how-to guide for the ZEN topography measurement workflow. The chapter is for users who search for an introduction to topography acquisition workflow. Starting from general preparations it will guide you through the acquisition until the data is transferred to the **ConfoMap** software for analysis.

Note that we will not explain the analysis in Confomap since this will be different which each sample you are interested in. Instead of that we recommend to read the Confomap tutorials and documentation, which describe the most important workflows in detail.

16.2.2 Acquiring Topography Images

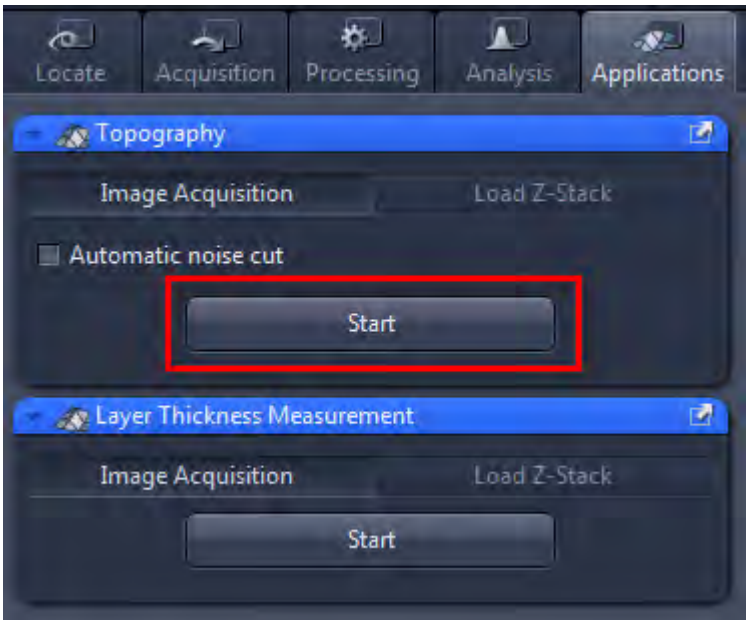
If you want to acquire a topography image in ZEN, you need to start the topography measurement wizard.

Prerequisites

- You are in the **Applications** tab and you see the **Topography** tool.

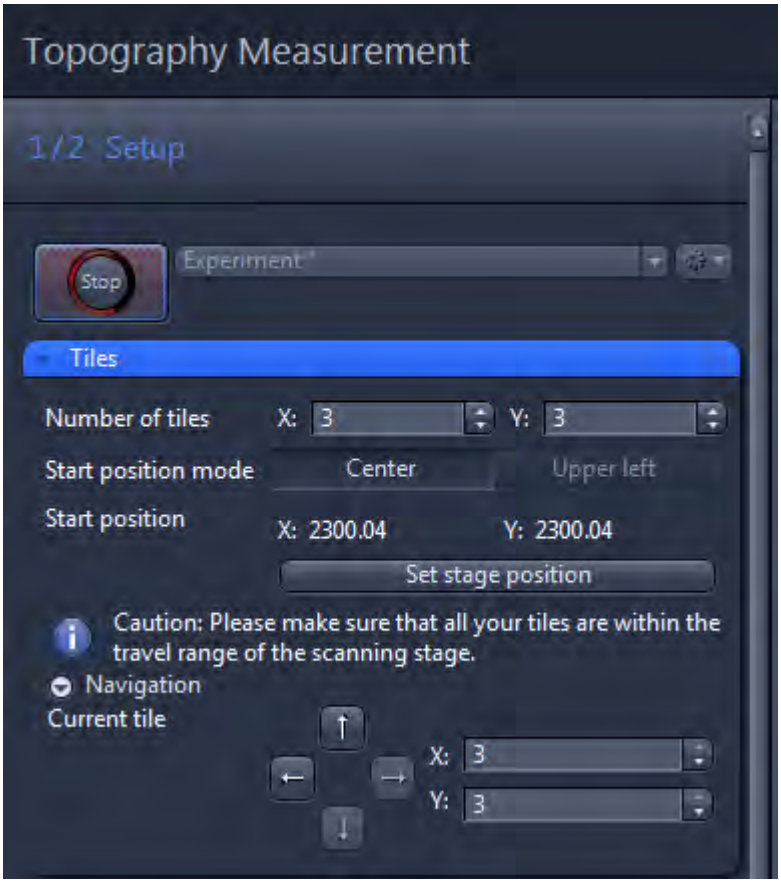
Procedure

1. Click on **Start**.



The wizard starts. In the left tool area you see the Step 1/2 **Setup**, in which you can adjust the acquisition parameters for the topography image. In the center screen area you see the live image from your sample in continuous mode.
As a default the system is set up to acquire only one single z-stack. In case your region of interest (ROI) is all within your field of view (FoV) continue with step 5 of this guide. If your ROI is bigger than your FoV, you can setup a tiles acqusition in the Tiles tool:

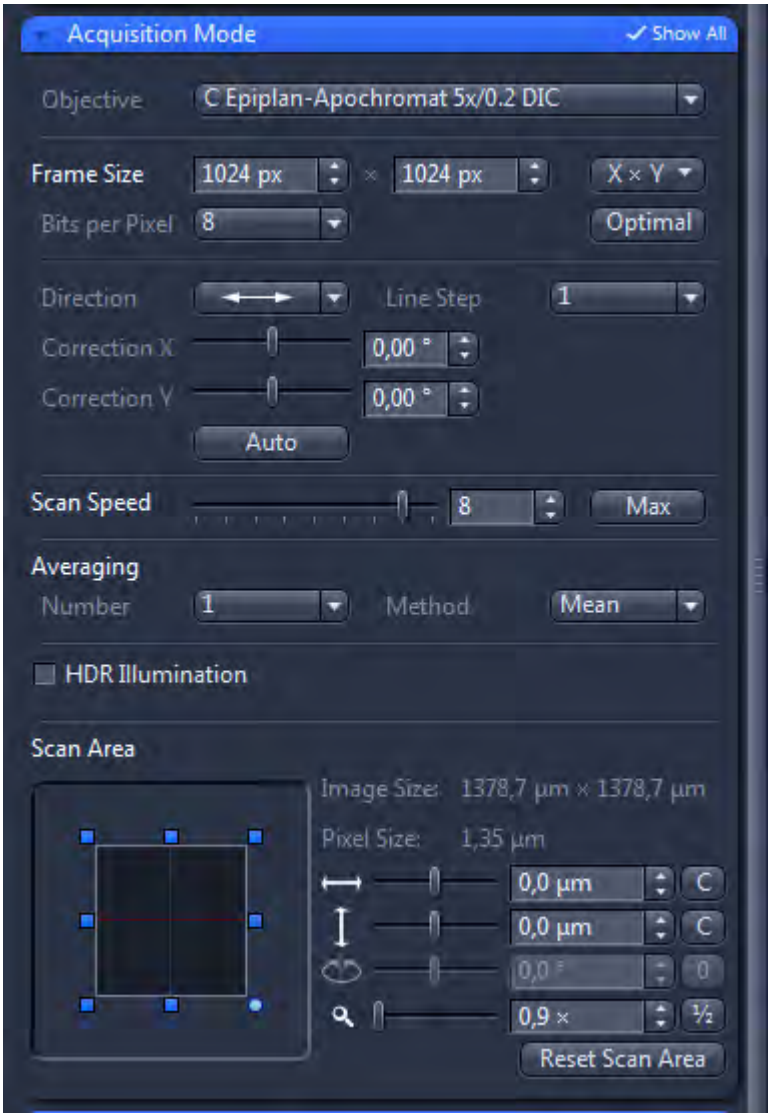
2 Enter the number of desired tiles in the **X:** and/or **Y:** direction input fields, e.g. 3 x 3. This will create a 3 x 3 tiles image with 9 single tiles.



3 Click on **Set stage position** to define the anchor point of your tile scan at the current stage position.

4 In the **Navigation** (must be expanded first) section click on the arrow buttons to navigate through the single tile images. This can help to check if the tiles cover the full ROI.

5 In the **Acquisition Mode** tool adjust the settings for **Objective**, **Frame Size** and **Bits per Pixel** according to your requirements.



INFO

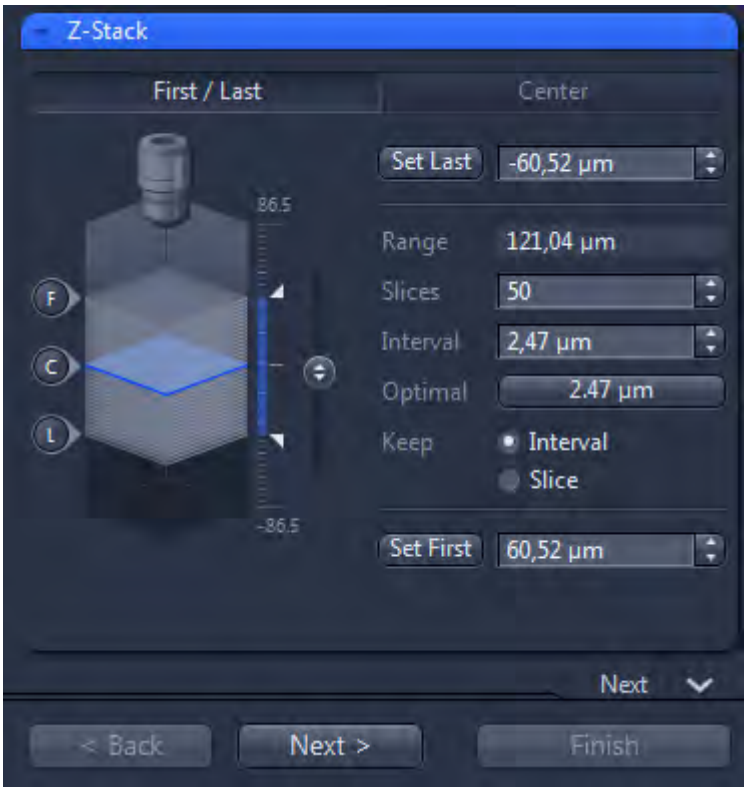
Note that you can not change the parameters **Frame size** and **Bits per Pixel** (Bit Depth) while **Continuous** mode (Live image) is active. To stop it click on the **Stop** button in the upper left corner.

6 Focus on the surface. The default pinhole setting is **Max**. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.

7 Adjust the pinhole to the required diameter. For best performance of the system we recommend to set the pinhole size to 1 Airy Unit (**1AU** button). Since the depth of field is reduced in confocal images you most probably need to slightly adjust the focus position.

8 Adjust the intensity of the laser. To do so focus on the brightest section of the z-stack. Then adjust the laser either via the **405 nm** slider or the **Master Gain** slider. Adjust the intensity so that you do not see any overexposed pixels. (shown in red).

9 Set up the **Z-Stack** by adjusting the z-range (upper and lower limit of your z-stack). We recommend to use **First / Last** mode for that.



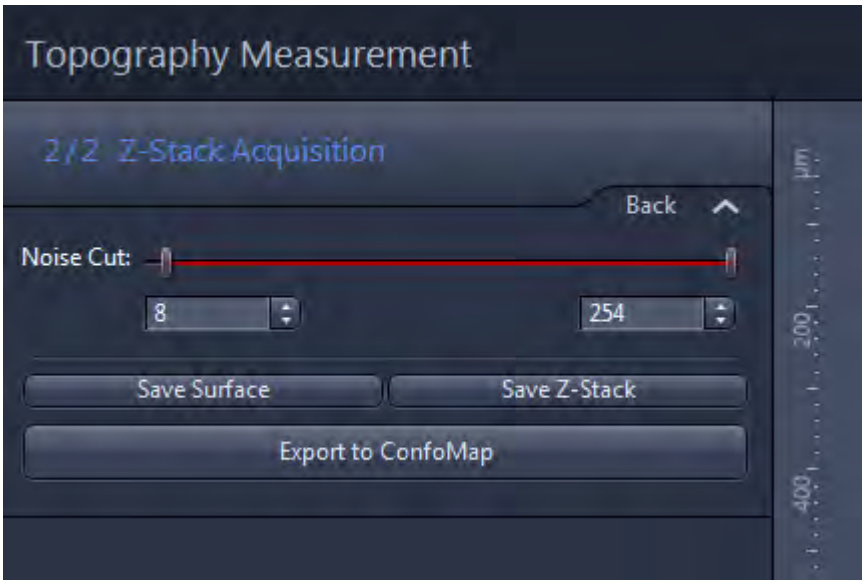
Make sure the whole surface, you want to measure, is within these limits. In case of a tile scan you can use the navigation arrows in the **Tiles** tool to jump to the different tiles to check.

10 Click on **Next**.

The wizard moves to **Step 2/2 Z-Stack Acquisition** and starts the image acquisition.

After image acquisition the topography image (height map) will be automatically generated. You will see the image in the center screen area after the acquisition and the processing is finished.

11 Adjust the thresholds by using the **Noise Cut** slider, if necessary. Note that pixels below the lower threshold are displayed in blue color and pixels above the upper threshold in red color.



INFO

In case you have activated **Automatic Noise Cut** in the **Topography** tool this step will be skipped and the topography image will be exported to **ConfoMap** automatically.

12 Finish your work in ZEN by choosing the following options:
- If you click on **Export to Confomap** the topography image will be directly opened in the **ConfoMap** analysis software.
- If you click on **Save Topography** you can save the topography image to the file system.
- If you click on **Save Z-Stack** you can save the raw data of the z-stack.

You have successfully acquired a topography image. As the image analysis is performed with ConfoMap software read the ConfoMap documentation for further information.

16.3 Workflow Layer Thickness Measurement

16.3.1 Introduction

In this chapter you find a how-to guide for the ZEN layer thickness measurement workflow. The chapter is for users who search for an introduction to layer thickness measurement workflow. Starting from general preparations it will guide you through the acquisition and layer thickness measurement.

16.3.2 Measuring Layer Thickness

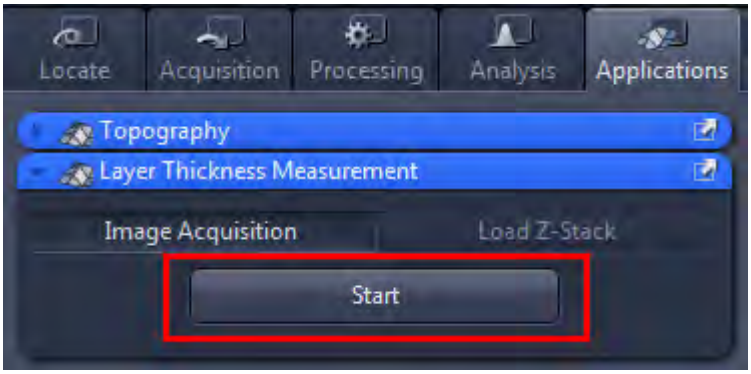
If you want to perform layer thickness measurement in ZEN, you need to start the topography measurement wizard.

Prerequisites

- You are in the **Applications** tab and you see the **Layer Thickness Measurement** tool.

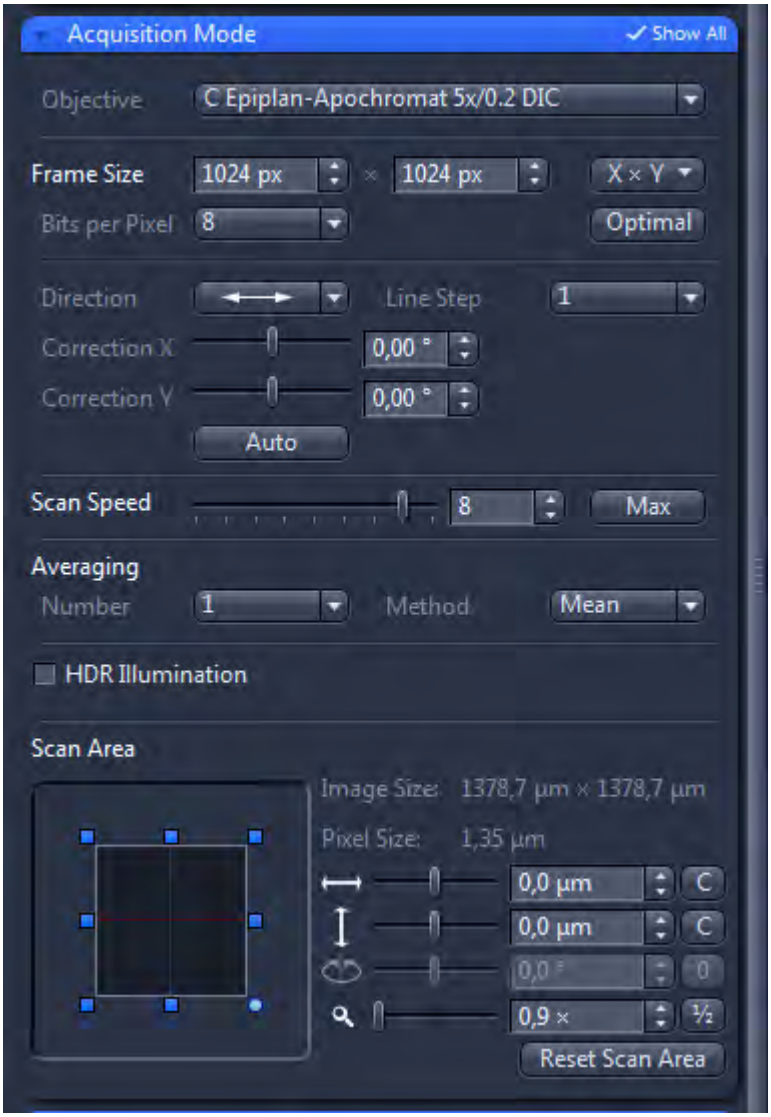
Procedure

1. Click on **Start**.



The wizard starts. In the left tool area you see the step **1/3 Setup**, in which you can adjust the acquisition parameters for the topography image. In the center screen area you see the live image from your sample in continuous mode.

2 In the **Acquisition Mode** tool adjust the settings for **Objective**, **Frame Size** and **Bits per Pixel** according to your requirements.



INFO

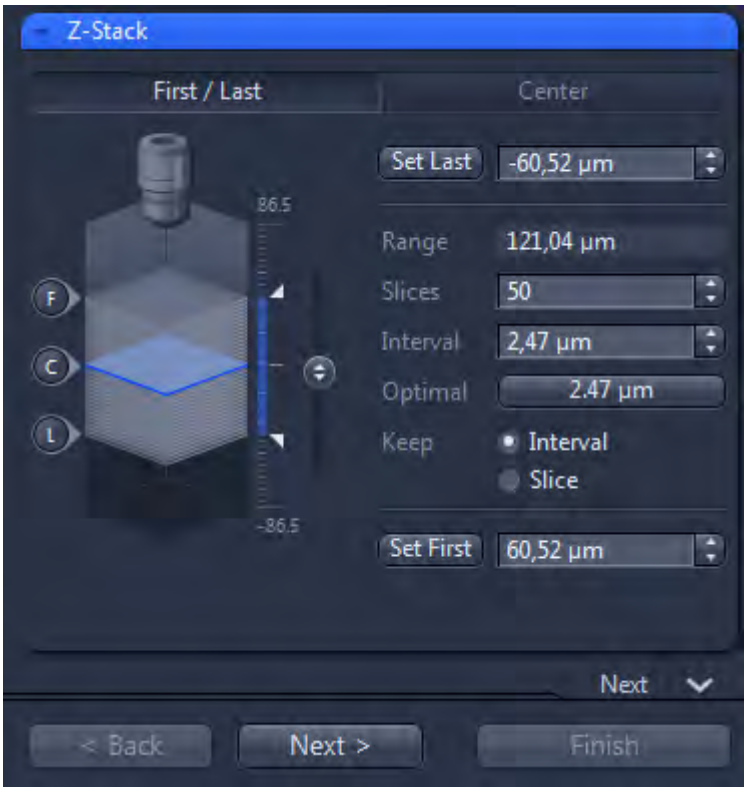
Note that you can not change the parameters **Frame size** and **Bits per Pixel** (Bit Depth) while **Continuous** mode (Live image) is active. To stop it click on the **Stop** button in the upper left corner.

3 Focus on the surface. The default pinhole setting is **Max**. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.

4 Adjust the pinhole to the required diameter. For best performance of the system we recommend to set the pinhole size to 1 Airy Unit (**1AU** button). Since the depth of field is reduced in confocal images you most probably need to slightly adjust the focus position.

5 Adjust the intensity of the laser. To do so focus on the brightest section of the z-stack. Then adjust the laser either via the **405 nm** slider or the **Master Gain** slider. Adjust the intensity so that you do not see any overexposed pixels. (shown in red).

6 Define the z-stack by adjusting the z-range (upper and lower limit of your z-stack). We recommend to use **First / Last** mode for that.



Make sure that all layers, you want to measure, are within these limits. In case of a tile scan you can use the navigation arrows in the **Tiles** tool to jump to the different tiles to check.

7 Click on **Next**.

The wizard moves to step **2/3 Sectioning** and starts the image acquisition.

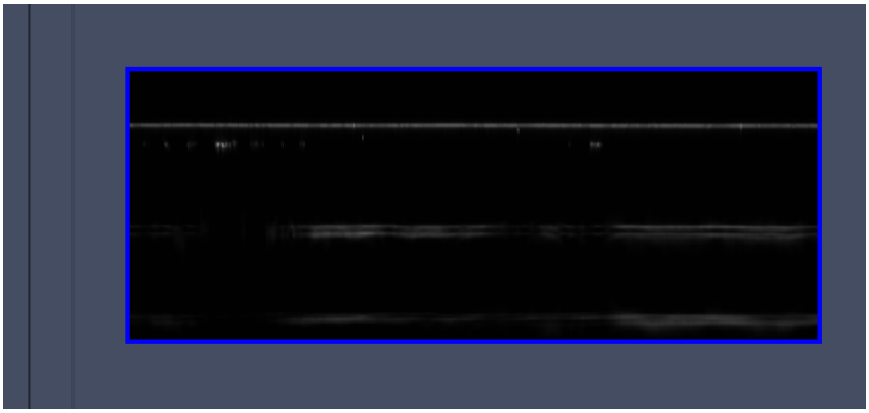
After image acquisition and processing you will see the image in the center screen area.

8 Click on **X-Z Layer** or **Y-Z Layer** to create the corresponding cross section which you want to analyze in detail.

9 Click on **Next**.

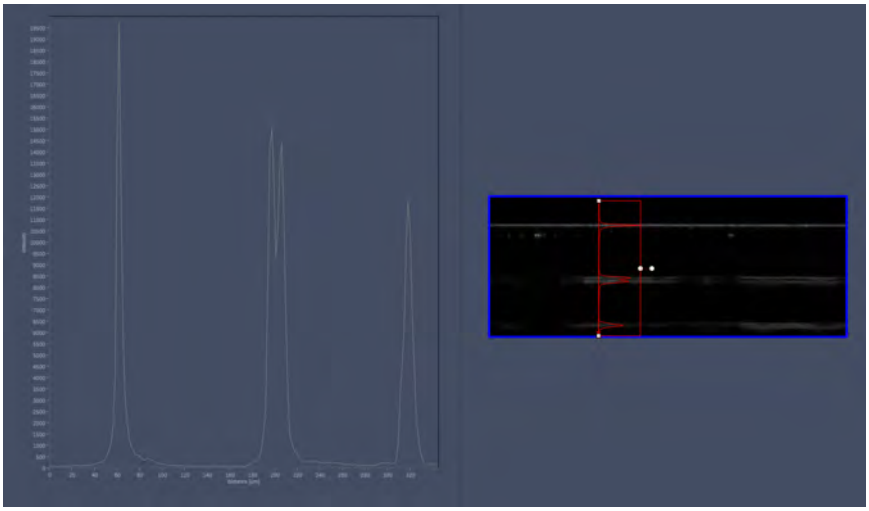
The wizard moves to step **3/3 Measurement**.

The selected cross section will appear on the right sight of the screen.



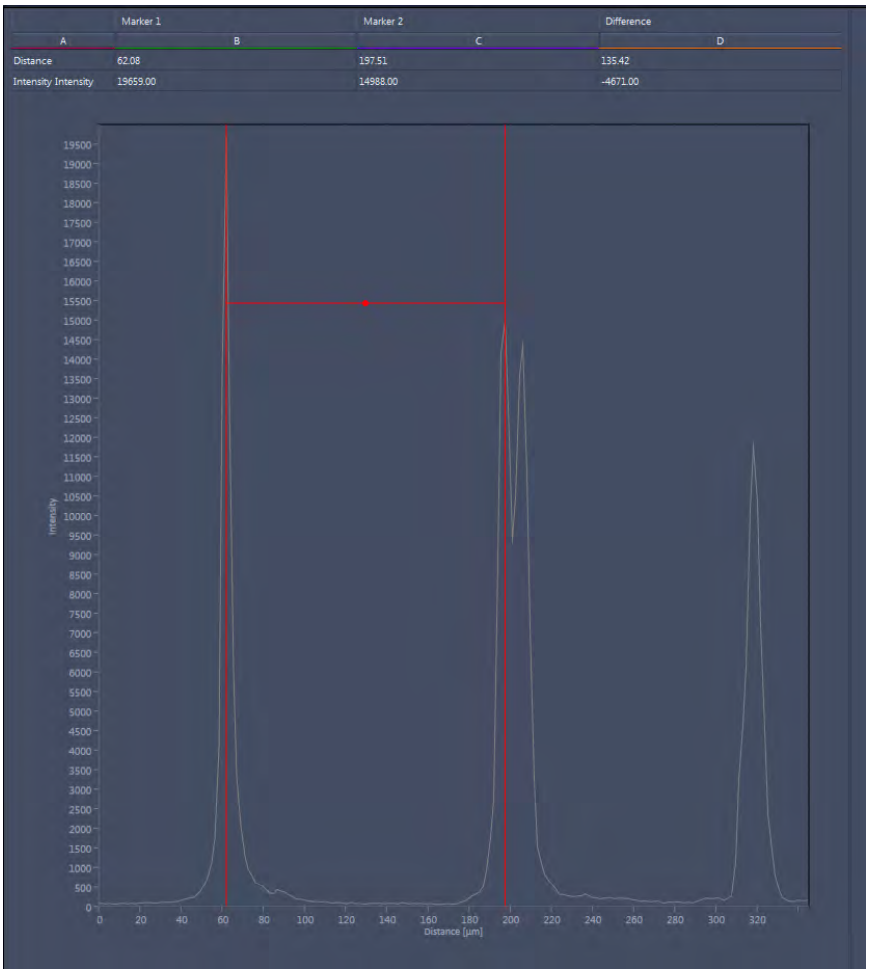
10 Draw in a profile perpendicular to your surface.

The intensity distribution of your profile will appear left to the cross section.



11 Position the calipers on the maximum intensity of the profile.

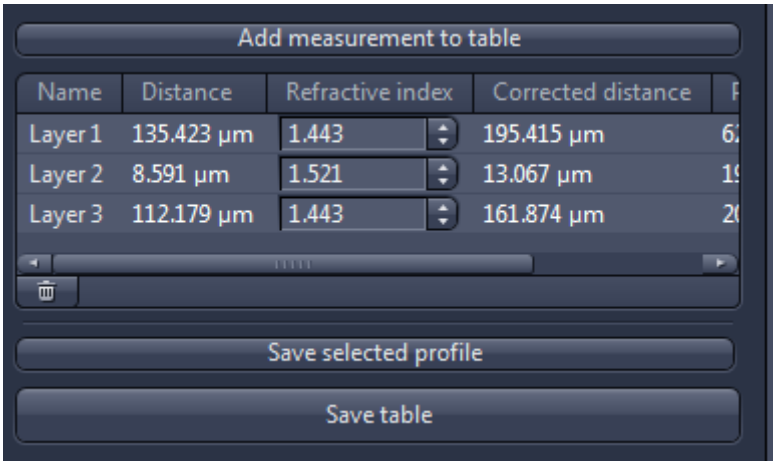
A table with the measurement data will appear on top of the profile.



INFO

The distance values within this table represent the layer thickness regarding to the light path. They are not corrected by the refractive index of the layer.

12 Click on **Add measurement to table** to transfer the data to the result table on the left. Within the table you are able to rename the layers and calculate the true layer thickness by adding the refractive index.



13 Finally you have three options how to save your results:
- If you click **Save table** the result table is saved on the file system.
- If you click **Save selected profile** the drawn in profile is exported for further investigation in third party software.
- If you click **Save Z-Stack** the raw data of the z-stack is saved on the file system.

You have successfully measured the layer thickness of your sample, saved the results table, the profiles and the raw data of the z-stack.

16.4 Functions and Reference

16.4.1 Applications Tab

On this tab you have the following tools available:

- **Topography** tool,
  for acquiring or loading an existing confocal z- stack and exporting it to ZEISS ConfoMap for further processing.
- **Layer Thickness Measurement** tool,
  for acquiring or loading an existing confocal z- stack and perform layer thickness measurements.

16.4.2 Topography Tool

Parameter	Description
Image Acquisition	If this option is selected you can acquire an image by the help of the **Topography Measurement** wizard. The wizard guides you through the image acquisition. Then you can export the image to **ConfoMap** for further processing. The wizard is started by the **Start** button at the bottom of the tool.
Load Z-Stack	If this option is selected, you can load an existing confocal z- stack from the file system. Therefore simply click on the folder icon and select the z-stack image from the file system. If you click the **Start** button, the **Topography** wizard will directly switch to the last wizard step to apply the noise cut and transfer the data to **ConfoMap**.
Automatic Noise Cut	If activated, the wizard will automatically perform the noise cut filtering with the preset parameters in the tool. If not activated, the noise cut filtering is an additional step in the **Topography Measurement** wizard.
- Thresholds	Here you can define thresholds for 8-bit and 16-bit images.
Start	If you click on this button, the *Topography Measurement Wizard* [▶ 516] is started.

16.4.3 Topography Measurement Wizard

When you have selected **Image Acquisition** in the **Topography** tool the **Topography Measurement** wizard consist of two steps:

Step 1/2: Setup

This step allows you to choose an experiment or setup imaging parameters for a new image acquisition, e.g. number of tiles, acquisition mode or z-stack creation, see *Step 1: Setup* [▶ 517].

Note that when you have selected **Load Z-Stack** in the **Topography** tool and you start the wizard, this step is not available.

Step 2/2: Z-Stack Acquisition

The second step is Z-Stack Acquisition. The acquisition will be performed automatically. Additionally you can apply noise cut filtering. Pixels which are effected by the thresholds will be displayed in red (upper threshold) or blue (lower threshold), see *Step 2: Z-Stack Acquisition* [▶ 517].

i INFO

The noise cut filtering will be skipped if the checkbox **Automatic Noise Cut** in the **Topography** tool is activated. The preset parameters will be immediately applied to the image and the wizard continues with the export to ConfoMap.

16.4.3.1 Step 1: Setup

After the wizard is started, you will see the live image (Continuous mode) from your sample in the Center Screen Area. You can setup the acquisition parameters in the left area of the screen. In the next chapters the individual steps are described.

Note that these are default ZEN blue tools customized for acquisition of topography images, see chapter Acquisition Setup Tools.

16.4.3.2 Step 2: Z-Stack Acquisition

Parameter	Description
Noise Cut	Here you can adjust the noise cut parameters manually. Therefore use the slider or enter the lower and upper values in the input fields.
Save Surface	Save the image of the surface to the file system.
Export to ConfoMap	Exports the image to the ConfoMap software. The ConfoMap software will be started automatically after you have clicked the button.

16.4.4 Layer Thickness Measurement Tool

Parameter	Description
Image Acquisition	If this option is selected you can acquire an image by the help of the **Layer Thickness Measurement** wizard. The wizard guides you through the image acquisition. Then you can export the image to **ConfoMap** for further processing. The wizard is started by the **Start** button at the bottom of the tool.

Parameter	Description
Load Z-Stack	If this option is selected, you can load an existing confocal z- stack from the file system. Therefore simply click on the folder icon and select the z-stack image from the file system.
Start	If you click on this button, the **Layer Thickness Measurement** wizard (see *chapter* > [▶ 518]) is started.

16.4.5 Layer Thickness Measurement Wizard

When you have selected **Image Acquisition** in the **Layer Thickness Measurement** tool the **Layer Thickness Measurement** wizard consist of three steps:

Step 1/3: Setup

In this step you choose an experiment or setup the imaging parameters for a new z-stack acquisition e.g., track setup, acquisition mode, z-stack settings, see *Step 1: Setup* [▶ 518].

Step 2/3: Sectioning

In this step (orthogonal sectioning) you define the cross section of the sample, you want to analyze. Here, in addition to the center view (**X/Y** axis; blue frame), you will also see the section views of the **X/Z** axes (top, green frame) and **Y/Z** axes (right, red frame), see *Step 2: Sectioning* [▶ 518].

Step 3/3: Measurement

In this step you draw one ore more intensity profiles in your cross section. In these profiles you can measure and create tables containing the measurement results. Finally you can save the profile, the z-stack or the result table, see *Step 3: Measurement* [▶ 519].

16.4.5.1 Step 1: Setup

After the wizard was started, you see the live image (Continuous mode) from your sample in the Center Screen Area. You can setup the acquisition parameters in the left area of the screen. In the next chapters the individual steps are described. Note that these are default ZEN blue tools customized for acquisition of layer thickness measurement images.

16.4.5.2 Step 2: Sectioning

Here you can adjust the sectioning parameters.

Parameter	Description
X-Z Layer (green)	If selected the X-Z section will be used for further investigation.
Y-Z Layer (red)	If selected the Y-Z section will be used for further investigation.
Cut Lines	Here you adjust the position of the desired cut line. Therefore use the slider or directly enter the position in the input field.
Mid button	If you click on this button, the corresponding cut line will be positioned in the middle (center) of the image.
Line Width	Here you adjust the line width of the corresponding cut line. Default value is 1 (in pixel).

16.4.5.3 Step 3: Measurement

Here you can adjust the measurement parameters.

Parameter	Description
Profile mode	Here you can select the profile mode for the measurement:
- **Arrow**	If selected, you can draw in an arrow in the profile. This will measure the profile along the drawn in line of the arrow.
- **Rectangle**	If selected, you can draw in a rectangle to the profile. This will measure the profile in the whole area of the rectangle.
Add measurement to table	If you click on this button, the measurement result will be added to the measurement table at the left side. In this table you can rename your profile and correct the result by the refractive index.
Save selected profile	If you click on this button, the selected profile will be saved as .czt file.
Save Z-Stack	If you click on this button, the Z-Stack image will be saved as .czi file.
Save table	If you click on this button, the table will be saved as .czt file.

16.4.6 Imaging Setup Tools

16.4.6.1 Tiles

Here you setup the tiles acquisition. The following parameters are available:

Parameter	Description
Number of Tiles	Here you can enter the desired number of tiles in X- and Y-direction.
Set stage position	If you click on this button, the current stage position is defined as starting position for the tiles acquisition.
Start position mode	Here you can define the alignment of the tile scan in respect of the defined position.
- Center	If selected, the start position is the center of the tile scan.
- Upper Left	If selected, the start position is the upper left corner of the tile scan.
Navigation	Click on the arrow button to show the section in full. Here you can check if each single image of your tile image is in focus before you start the acquisition. Navigate from on tile to another by using the arrow buttons or enter the X/Y value, to check the set-up of your z-stack.
Start position	Displays the X and Y value of the starting position for the tiles acquisition.

16.4.6.2 Channel

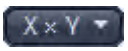
With this tool you control and adjust the laser. The following parameters are available:

Parameter	Description
405 nm	Here you can set the required attenuation (in %) of the laser using the slider, the arrows, or typing in the input field.
Pinhole	Adjusts the diameter of the pinhole. The diameter is specified in micrometer. The text below translates this diameter to Airy Units (AU) and section thickness. For confocal topography measurements a pinhole size of 1 AU is recommended.
- 1 AU	Sets the pinhole diameter to the recommended value of 1 AU (Airy Unit).
- Max (Default)	Opens the pinhole to its maximum diameter.

Parameter	Description
	This is also the default setting for the pinhole. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.
Master Gain	Here you can control the voltage of the PMTs. Higher voltage increases the gain of the PMT. The image becomes brighter and you may be able to reduce the laser power. At higher voltage, the noise level in the image increases. The optimum between gain and noise depends on your experimental requirements and on your sample. The maximum available voltage for multialkali PMTs is 900V.
Digital Offset	Here you can perform adjustments on the background of the image.
Digital Gain	Here you can digitally amplify the laser signal.

16.4.6.3 Acquisition Mode

Here you adjust scanning and acquisition parameters that you want to apply for the entire experiment.

Parameter	Description
Objective	Select the desired objective for acquisition here.
Frame Size	Adjust the frame size (in pixel) of the displayed image by entering the desired value in the two input fields. To change the frame size you must stop the live image acquisition.
- X x Y button 	By clicking on this button you can select from a list of default frame sizes (e.g. 128 x 128 or 512 x 512). In case of topography and layer thickness measurements we recommend to start with 1024 x 1024.
- **Optimal** button	By clicking on this button the frame size (image resolution) will be set to an optimal value corresponding to the optical magnification (objective) and the zoom factor . This provides an image where no information is lost and no empty data are generated as optimal sampling is achieved. The optimal value is calculated for the

Parameter	Description
	given objective and magnification settings matching a 2fold oversampling according to 2 fold Nyquist. Rectangular image dimensions are preserved.
- Bits per Pixel	In the dropdown list you can adjust the color bit depth to 8 Bit or 16 Bit (i.e. 256 or 65536 gray values). To change the bit depth you must stop the live image acquisition.
Direction	Following scanning directions can be selected:
- Unidirectional 	The laser scans in one direction only, then moves back to scan the next line.
- Bi-directional 	The laser also scans when moving backwards, i.e. the scan time is halved. In case a pixel shift between forward and backward movement (double image), resulting from bidirectional scanning, is visible, use the **Correction X** / **Correction Y** sliders to correct it. By clicking on the **Auto** button an automatic scan correction will be performed.
Scan Speed	Set the scan speed by adjusting the slider from 1 (slow) to 16 (very fast). Note that the available maximum scan speed depends on the selected **Frame Size** and zoom factor.
Averaging	
- Number	Select the number of images you want to average (1 - 16).
- Method	Select the method which will be used for averaging: ■ **Mean**: Uses the mean average of all images ■ **Sum**: Uses the sum of all images.
Scan Area	In this section, you can adjust the position of the scan area. The outer frame corresponds to the field of view of the microscope.

Parameter		Description
		The inner frame represents the scan area. All changes (Offset, Rotation, Zoom) made in this section will be immediately applied to the scan area. Following functions are available:
-	Offset	Adjust the offset by using the **Left** / **Right** or **Up** / **Down** sliders. You can also enter a specific value in the input field. If clicking on the **C** button behind the input filed the offset position will be reset to center position. If you left click on the inner frame and hold down the mouse button you can move the scan area freely. The positions in the input fields will be adopted according to your adjustments.
-	Rotation	Adjust the rotation degree by using the **Rotation** slider. You can also enter a specific value in the input field. If clicking on the **O** button behind the input field the rotation degree will be reset to default position (zero degree).
-	Zoom	Adjust the zoom level (from **0.5x** - **40x**) by using the **Zoom** slider. You can also enter a specific value in the input field. If you click on the **1/2** button behind the input field the zoom level will be reset to default (0.5x).
-	Reset Scan Area	Resets all adjustment to the system defaults.

16.4.6.4 Z-Stack

Here you setup the acquisition of the Z-Stack image.

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Z-stack images are always acquired from bottom to top automatically, irrespective of whether you have defined the top or bottom Z-plane of your stack as the first Z-plane. This acquisition sequence increases the accuracy of the Z-positioning.

For manually configuring Z-Stacks you have two modes available:

Parameter	Description
First / Last	If activated, you are able to configure the Z-stack via setting the first and the last positions of the Z-stack, see *Configuring a Z-Stack manually (First/Last Mode)* [▶ 52].
Center	If activated, you are able to configure the Z-stack via setting the center plane of the Z-stack, see *Configuring a Z-Stack manually (Center Mode)* [▶ 53].

Depending on which mode you have activated, you will see the following parameters for configuring the Z-stack:

Parameter	Description
Set Last/Set First	Only visible for **First/Last** mode. By clicking on the **Set Last** and on the **Set First** button you determine the current position as last or first position of the Z-stack.
Range	Displays the range of the configured Z-stack from the last to the first section plane.
Slices	Here you can enter the number of Z-slices that the Z-stack will have.
Interval	Here you can enter the desired distance between the Z-slices.
Optimal	The number on this button shows the distance calculated for the channels set and the current microscope according to the Nyquist criterion. If you click on the button, this value is automatically adopted into the **Interval** input field.
Keep	■ **Interval**: Keeps the set interval between the section planes constant if you change configuration parameters in the Z-Stack tool. ■ **Slice**: Keeps the set number of Z-slices constant.
Center	Only visible for **Center** mode. If clicking on this button the current position is set for the central Z-plane. You can also enter the value in the input field to the right of the button.
Offset	Here you can enter a value for an offset if desired.

17 Module Software Autofocus

17.1 Introduction

The **Software Autofocus** (Abbreviation: SWAF) module offers a configurable image based autofocus functionality that will search through a series of axially stepped images analyzing the “sharpness” of each. The z-value of the image returning the maximum sharpness is set as the new plane of observation.

The module requires the microscope to be fitted with a motorized Z-drive. It does not require a z-piezo actuator nor is the z-piezo used by the SWAF in the current implementation. On the **Acquisition** or **Locate** tab the settings for the SWAF can be adjusted in the **Software Autofocus** tool. These can also be called (and tested) by clicking the Find Focus (AF) button on the main button bar on Acquisition tab. SWAF settings are stored as part of an experiment on Acquisition tab.

The configuration allows the function of the SWAF run to be matched to the conditions under which the focus should be found. A basic description of the functions adjusted by the individual controls can be found in the chapter Functions & Reference. However, before going into the description of each parameter, we will try to address the following questions: How does the SWAF in the software attempt to locate the “focus”? And how do the parameters settings influence its behavior in this respect?

17.2 Terminology & Abbreviations

Perhaps the best place to start is with an explanation of the terms encountered when working with Focus Strategies in before looking at the individual strategies in detail. Many of these terms are also encountered in the **Tiles** and **Software Autofocus** module. The nomenclature takes some time familiarize with due to its subtleties. Here is a list of the more common terms:

Term / Abbreviation	Description
SWAF	Stands for Software Autofocus.
DF,**DF.1** or **DF.2**	Stands for Definite Focus, Definite Focus.1 or Definite Focus.2
Tile	One of the individual image fields that make up a tile region i.e. a 2x2 Tile region is made up of 4 tiles arranged as a grid. The tiles have a given overlap with their neighbors (default setting 10 %) allowing them to be stitched together as one image if necessary. Unless otherwise specified by a focus strategy, each tile has the

Term / Abbreviation	Description
	same z-value as the parent Tile region. After acquisition the individual tiles are displayed together as part of the tile region to which they belong, which in turn makes up one scene.
Tile Region	In a tile experiment a tile region refers to a ordered group of individual image fields (or tiles) that belong together and are arranged in the form of a grid (these arrangements can be based on quadrilaterals, circles, ellipses or freehand polygons) with a predefined overlap (default 10%) to facilitate stitching the images together. With the help of tile regions it is possible to acquire areas with dimensions that vastly exceed the size of an individual image field. Within an experiment a number of tile regions can be acquired at various localities/ wells/ containers on the sample. Each tile region is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive and are defined using the Tiles tool. After acquisition the individual tile regions are displayed as scenes to facilitate viewing.
Position	In a tile experiment positions refer to independent, individual image fields that placed at various locations on the sample. A position corresponds to (or is in some ways equivalent to) a tile region consisting of just one tile. Each position is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Individual positions or position arrays (grouped individual positions) are defined using the Tiles tool. After acquisition the individual positions are displayed as scenes.
Reference Channel	The channel selected as a reference z-value for focus strategies and events in particular a SWAF. The selected reference channel can be changed in the Reference channel expander or in the Channels tool. It is also possible to define a relative axial offset to the reference channel. This can be done for one or more other channels.
Focus Surface	Refers to the interpolated surface of z-values derived from support points (discrete z-values) defined by the user (or by functions such as SWAF or DF.2) prior to the experiment (or immediately before acquisition start). A focus surface can be “local” or “global”. The local form is confined to a single Tile region and attempts to describe the sample topography covered by the tile region such that all its image fields (tiles) will be in focus. The global surface form is technically identical, but is associated with a sample carrier, and defined in the sample carrier template

Term / Abbreviation	Description
	dialogue. Thus, tile regions or positions placed on this carrier will follow the slope or contour defined by a topography that covers part or most of the sample carrier. In both cases the surface is defined by interpolation from discrete z-values – so called "support points". Note that a positions z-value is used as its local surface, and as such does not require a support point. Global and local surfaces cannot be mixed in a single experiment (or block).
Support Point	To create a focus surface it is necessary to define one or more support points. Support points are user defined collections of z-values that correspond to the desired plane of observation at a given XY-coordinate. They can also be defined initially by a SWAF run or DF.2 recall focus function- initially, after the experiment is started, but before the first loop of images are acquired. The number of support points, defined by the user, can be distributed automatically by an algorithm, re arranged individually by hand or placed at the current stage position. The number of support points employed determines the degree of interpolation that can be used to generate the topography of the focus surface. Typically, the interpolation criteria (minimum number of support points required to generate a certain degree) should be over filled with support points, and a lower interpolation degree selected for more robust results. By default employs an interpolation degree of level 2 (which can generate a parabolic saddle surface with at least 9 support points). If too few support points are used the next lower level (a "tilted plane") will be use automatically. Higher interpolation degrees have to be manually selected, but for most use cases are typically not necessary.
Z-value	The current Z coordinate of the focus drive that is used to define a Tile region, position or support point when it is created by the user. Note that the individual tiles of a tile region all have the same initial z-value unless support points are used either in the context of a local or global focus surface, a software autofocus is used to determine them individually or a definite focus stabilization adjusts them. The z-value of a position defines its z-coordinate initially when a local focus surface is used. Positions spread on a global focus surface (carrier based) are adjusted accordingly as are the individual image fields of a tile region.

Term / Abbreviation	Description
Adapt Focus Surface/Z-values	The focus strategy “Use Focus Surface/Z Values Defined by Tiles Setup” allows the Focus surface or Z-values defined in the Tiles tool to be modified by the result of a SWAF or DF stabilization based on these initial values. These functions are not available when no SWAF module is present or no DF is configured. The function has several module / hardware dependent variations:
- As Additional Action	In focus strategies that use a focus surface or z-value defined by the Tile setup (tool) it is possible to optionally execute a so called “additional action” (a stabilization event) that adapts the focus surface/ z-values. This occurs after the reference z-value has been reached as defined in the tiles set-up for each discreet z-value (i.e. each tile/ position or the defining focus surface). Depending on the system configuration this can be a SWAF run or a DF stabilization. In the case of a SWAF run the initially defined reference z-value is used to center the search range defined in the SWAF settings. Thus, a SWAF run can be centered on the sample topology increasing the effectiveness and/ or speed at which a maximum is detected and subsequently used for image acquisition. In certain applications, such as Correlative array tomography (CAT) this function can be performed with DF instead. In this case a local focus surface is used to make sure that the DF stabilization stays within the catchment range of the device (only important for DF.1!). Complimentary to this is the number of support points needed to initially define the surface can be significantly reduced for a large elongated Tile region - which greatly reduces set-up time to image the extremely thin (typically 70 nm thick or less) “ribbon” of serial sections.
- Update with Single Offset	In combination with a Definite Focus or SWAF if a time series is used it is possible to make use of a focus surface or z-value defined by the Tiles setup and execute a so called “update” (a stabilization event) – this makes use of a SWAF run or a DF stabilization to update the Focus surface/ z-value defined initially by the Tiles set-up. In a time series the update action is performed once each time point (or every nth) at a single discrete “wait position” (default center of 1st Tile region / position). A change in Z (thermal or residual focal drift) at the wait position - if detected - is then applied to all the focus surfaces or Z-values defined in the Tiles Setup (adapting them all by the change in Z, applied as a common offset).

Term / Abbreviation	Description
	In some cases it is useful to be able to define a specific waiting position – for example when a special sample carrier is used were the DF reflex signal might be disturbed by its structure/optical properties at the first tile region/ position. Alternatively, if using a SWAF some kind of fiducial marker or such is available at this position that does not change (e.g. bleaching or movement) can be used.
- Update with Multiple Offset	For Definite Focus.2 only an additional function is available that allows the device to be initialized on each and every z-value prior to the experiment and hence stabilize and update these individually according to their location relative to the sample/ glass interface. This function can be used with or without a time series dimension. Thus, DF.2 enables true multi-location experiments in which the user defined z-values (including support points) are used by DF.2 to create a stabilization map that is monitored and updated throughout the experiment.
Initial Definition of Support Points/ Positions/Tile Regions	This function allows you to select how the initial z-values used in the experiment are defined. By default this is “By Tiles Setup” and the z-values specified there (in the Tiles tool) are used. However, it is possible to define or adjust these z-values directly before the experiment (after clicking 'Start experiment') either with a SWAF run or a with a DF.2 Recall Focus (Axio Observer). For the Celldiscoverer 7 this drop down offers the additional options 'Find Surface' or 'Find Surface + Additional Offset' to define the initial Z-values. In this case the Z-values are initially defined by the z-values resulting from this “pre-run” before the imaging loop starts. This can be particularly useful when working with multi well plates or chamber slides where the sample is located at a similar position relative to the carrier surface in each well or chamber. It also allows the imaging loop of the experiment itself to be speeded up and to be run in a triggered or compromised protocol (fast acquisition) thus reducing the time to complete the imaging loop of the experiment.
Stabilization Event Repetitions and Frequency	Defines the frequency and repetition of stabilization events within a given focus strategy. For the DF and SWAF focus strategies you can determine when and where in the experiment these event are executed in synchrony to the imaging loops – a loop here means time series, or positions for example, with the event synchronized to occur

Term / Abbreviation	Description
	immediately prior to the chosen loop. A general limitation of this implementation (to limit code complexity) is that these stabilization events can only be synchronized to iterate with a single imaging loop entity i.e. the selection is only possible in a mutually exclusive manner. These settings can be accessed only when 'Show all' is activated and expert mode is selected. Initially default settings are assigned and can be restored by clicking the 'Standard' button. In 'Expert' mode the settings are displayed and can be, if necessary, modified. Depending on the dimensions of the experiment or focus strategy different parameters can be modified to meet the experiment needs. For the Tile Region loop you can optionally select were the event occurs within the Tile region - either in the center or at the 1st Tile of the region (typically upper left hand corner). This is of use when using SWAF events as often the upper left hand corner of a Tile region might not contain sample, thus often the SWAF run will not return a suitable maxima (new z-value). Finally, focus strategies that include Definite Focus and are used with a time series dimension may also allow stabilization during the interval of the time series i.e. asynchronous to the imaging loops of the experiment. This might be necessary if the time interval is on the order of tens of minutes, or if a large thermal drift is expected (more significant for DF.1), or if the time series has no or a very short interval (i.e. fast as possible acquisition at a single position) allowing synchronized events to be disabled completely.
Focus Surface Outlier	Under **Tools** | **Options** | **Acquisition** | **Tiles** you find the option '**Enable Removing of Focus Surface Outlier**'. By two parameters you can define how so called “outlier” values are handled prior to calculation (interpolation) of a focus surface. This is particularly helpful when the z-values that will be used for this purpose contain one or more values that differ obviously from the others (for example if a SWAF run has returned a z-value that does not lie close the sample plane of interest). If not removed such values locally distort the focus surface potentially producing “blur” in the resulting images. By default a linear fit is used to detect such outliers in combination with a statistical threshold value (sigma). Values that do not meet these criteria (i.e. are significantly outside this) are classified as outliers and are not used to calculate the focus surface that will be subsequently generated for the experiment. In

Term / Abbreviation	Description
	extreme use cases it is possible to modify the sigma value or use a mean value instead of a linear fit for this purpose, but typically these default values never need to be changed.

17.3 When is focus the "right" focus?

In microscopy, the focus can be implied from image parameters, such as the contrast or intensity, that vary with the position of the objective's plane of observation in the sample and the level of detail at a given plane. However, an algorithm that tries to detect (and maximize) such values will only return an axial position that corresponds to a plane of interest if these coincide (which is typically the case with (thin) samples with a singular discrete plane of detail).

This becomes increasingly difficult with higher numerical aperture (NA) lenses, thicker samples, and less pronounced levels of detail (modulated as change in contrast or intensity in the resulting image). Hence, SWAF is not to be understood as a focus finder, but can be used as a method for reliably searching over a given axial range and locating such a plane in a sample. Thus, although not all samples and imaging conditions will be appropriate, SWAF is an approach that allows a useful detection of a focus plane as a start for further imaging activities.

17.4 Software Autofocus in ZEN

Basically, the SWAF searches, with a pre-set z step size, within a given range of z values for the image plane that returns the maximal "sharpness" value. The step size or sampling rate of the SWAF is determined by the objective NA and wavelength (more details are given below). In turn the (automatic) search range is also largely determined by the objective NA – obviously optics dictate that higher NA objectives have smaller search ranges and vice versa. For SWAF to be useful for the application in question the image plane that returns the maximal sharpness should ideally be equivalent to the plane of interest in the sample – i.e. thus sample characteristics determine whether SWAF is the appropriate method to reliably detect the desired plane of observation. The component algorithms and functions of the SWAF, their relationships and the basic SWAF workflow are visualized schematically in the image below:

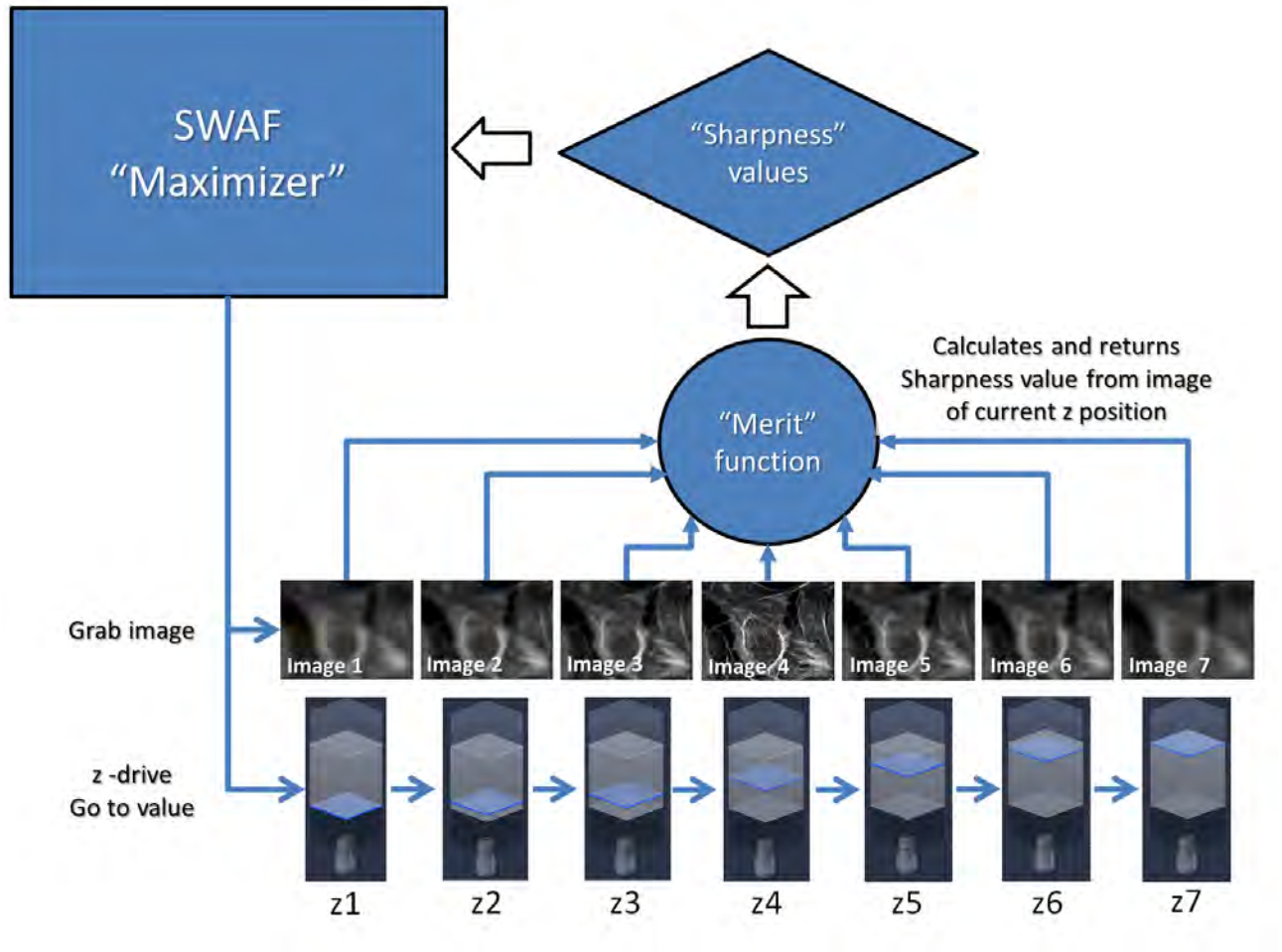


Fig. 17.1: SWAF Workflow

The SWAF run is driven and controlled by the Maximizer which in turn controls the z-drive and camera to acquire a sequence of images at predefined axial intervals. Each of these images is transferred in sequence to a merit function that calculates a sharpness value for the image. This value is returned to the maximizer were a table of sharpness values is maintained. The Maximizer searches for (using either unidirectional ("full") or bidirectional ("smart") axial travel) and determines when a maximal sharpness value is found. Next data fitting is performed locally around the returned maximum to refine its position. Subsequently, the SWAF sets this z-value as the resulting axial position of the z-drive. If no maximum is found the Maximizer detects an error condition and throws a corresponding exception (error message). In this case the z-drive returns to its position of origin, thus minimizing the likelihood of a sample/ objective collision incident.

17.5 Functions & Reference

17.5.1 Software Autofocus Tool

Parameter		Description
Mode		Here you can select the sharpness measurement mode.
-	Auto	This is the default setting. If selected, the software makes a choice based on the configuration of the microscope. Such that for Widefield approaches or transmitted light the sharpness measure is always **Contrast** based. On the other hand, for optical sectioning methods (e.g. Spinning Disk) the software will automatically select an **Intensity** based approach to determine the sharpness values. If the microscope configuration can`t be detected automatically you can manually select the sharpness measurement mode.
-	Contrast	If selected the contrast based mode will be used for sharpness measurement. This is the standard setting for **Camera** acquisition.
-	Intensity	If selected the intensity based mode will be used for sharpness measurement. This is the standard setting for **Confocal** acquisition.
-	Reflex	Only available for **LSM** systems. If activated, the reflex of the laser on the cover glass surface is detected. An offset is used to focus onto the sample. You need to add the offset once by clicking the **Find Offset** button while the sample is in focus. The method requires a refractive index mismatch between the immersion medium and the cover glass and is therefore not for oil immersion objectives. For **Reflex** mode, the **Search** parameter should ideally be set to **Smart**. After the offset was defined, you can manually reduce the search range in order to facilitate shorter focusing times. The acquisition settings (e.g. laser line, PMT gain, emission filters) are configured automatically.

Parameter	Description
Quality	This parameter determines the merit function that will be used to calculate the contrast value of the image when **Contrast** mode is used by the SWAF (Software Autofocus) run to measure sharpness.
- Default	If selected, a composite of weighted merit functions is used. Use this setting if the sample cover a greater part of the camera field of view.
- Low Signal	If selected, a single merit function to determine the value is used. Use this setting if the image is noisy or the sample covers a small area of the field of view. As might be the case if you work with a calibration slide or beads.
Search	There are two options Smart and Full. These define a different type of primary maximizer used to run the SWAF, which in turn determines a number of additional characteristics and parameters of the entire process. To learn more about this, read the *FAQ entry* [▶ 538].
- Smart	If selected, an alternative maximizer is used that can search in a bidirectional manner and will stop when a local maximum is found in the sharpness values (i.e. a significant decrease of sharpness in both z directions). Again if an error condition is detected the Smart maximizer will throw an exception. For specific information on the Software Autofocus using LSM Tracks in this context, also refer to chapter *Software Autofocus using LSM Tracks* [▶ 536].
- Full	If selected, the maximizer employed with this setting uses a unidirectional movement of the z-drive stepping through the entire relative or fixed search range defined in the SWAF tool (see Autofocus Search Range). The Full maximizer will return a global maximum for the autofocus run or throw an exception when an error condition is detected. For specific information on the Software Autofocus using LSM Tracks in this context, also refer to chapter *Software Autofocus using LSM Tracks* [▶ 536].
Sampling	Here you can select the step size of how the search range is sampled.

Parameter	Description
- Default	Uses the default step size (**dz = 1/sqrt(2) * 2 * n* lambda/NA**).
- Fine	Uses a small Z-distance (**0.5 * dz**) between the individual focus images that are used to calculate the best focus position. This doubles the number of z-slices for the given range.
- Medium	Uses a medium Z-distance (**2 * dz**) between the individual focus images that are used to calculate the best focus position. Halves the number of z-slices for given range.
- Coarse	Uses a large Z-distance (**4 * dz**) between the individual focus images that are used to calculate the best focus position. Reduces number of z-slices by a factor of four.
Autofocus Search Range	Here you can switch between two distinct approaches for the autofocus search range:
- **Relative Range**	This is the default mode. If selected, the software autofocus is calculated over a relative range. To learn more, read Relative Autofocus Search Range.
- Automatic Range	**Activated:** Calculates the range for the autofocus search automatically depending on the objective set.
- Range	Only active if the **Automatic Range** checkbox is deactivated. Here you can enter a range that you want to be used for the autofocus search.
- Step Size	Shows the distance between the individual focus images set under **Range**.
- **Fixed Range**	If selected, the software autofocus is calculated over a fixed range. To learn more, read Fixed Autofocus Search Range.
- Set Last	Defines the current Z-position as the end (last) point for the software autofocus. Alternatively, you can enter the desired value in the input field to the left of the button.

Parameter	Description
- Range	Displays the area which is used for the autofocus search. Adopt the area via the Set Last/Set First buttons or the input fields.
- Step Size	Displays the selected **Sampling** distance between the individual focus shots.
- Set First	Defines the current Z-position as the start (first) point for the software autofocus. Alternatively, you can enter the desired value in the input field to the left of the button.
Autofocus ROI	Here you can define a Spot Meter or Focus ROI such that the pixels evaluated by the SWAF are limited to a user defined region of the image. This is particularly usefully if you use a fiducial marker such as a speck of dirt or other constant artefact that serves as a reference for the sample focus at a fixed position over time. The autofocus ROI is displayed in the live image of the sample enabling it to be positioned and resized as necessary. As an additional aid to help focusing you can also use the focus bar function of the live image that monitors the image contrast in the live image or Spot Meter ROI.
- Spot Meter / Focus ROI	**Activated:** Only uses the values from the Spot Meter / Focus-ROI to calculate the focus position. The Focus-ROI is displayed in the live image as an red dashed rectangle. You can adopt it by clicking on the frame and changing its size and position. Note that this option is not available for LSM acquisition.

17.5.2 Software Autofocus using LSM Tracks

Confocal Tracks are also suitable as reference **Channels** for the **Software Autofocus**. As LSM acquisition is by design slower compared to **Camera** acquisition, some optimizations are done in the background in order to speed up the focusing action.

The typical mesasure for the correct focus position in confocal images is the intensity. Hence the aim of the SWAF is here not to generate images of a certain quality, but only to evaluate relative image intensities along the z-stack. This allows us to use very coarse scanning parameters.

Generally, the SWAF for LSM uses a fixed **Frame Size** of 64*64 pixels in combination with the fastest possible scan speed at the currently configured zoom. To further speed up the acquisition, bidirectional scanning is used. Whatever **Laser power** you specify in the **Channels** tool window for this Track is used during the SWAF action. Of note, while you assing a reference Channel, the corresponding Track with all its channels will be active during the focusing.

Some behavior depends on the selected **Search Mode Full** or **Smart**.

In the **Full** search, the system will use the detector gain as configured in the **Channels** tool window.

In contrast, the **Smart** search aims to start close to the likely intensity maximum of the z-Stack. This focus position is approximated by a fast line z-stack in the center of the image frame. As the line scan generates less pixels and a higher noise level, a useful dynamic range needs to be ensured. To this end, the fast line z-stack is repeated several times with increasing PMT gain. After the line scan, regardsless if an intensity peak was found or not, a frame wise autofocus will follow.

In case no peak could be indentified, e.g. because of a sparsely distributed sample, the **Smart** search will start at the original z-position and not optimization of the starting position will take place. Essentially, the **Smart** search will outperform the **Full** search on high **Search Ranges** and a highly varying effective focus position.

If the focus fluctuations are preditably small, a narrow **Search Range** in combination with a **Full** search might be faster. As a final remark, Camera-based Autofocus can be time-saving, especially when the search range needs to be large and a Full Search is required. While Camera and LSM cannot be combined into one image document, the deactivated camera Track may be still be used as a reference Track.

17.6 FAQ

17.6.1 What should I do to adjust parameters of a SWAF run prior to using it in experiments?

In the first instance test the SWAF with the default settings and select an appropriate channel to be the reference channel – remember that you can uncheck the reference channel in the Channels tool i.e. so that it will not be imaged, but will still be used for a focus strategy or SWAF. Consider using a transmitted light channel if possible as this will not be subject to bleaching. Remember that the sample plane that returns a maxima from the SWAF run is not necessarily the same plane as that which you’re interested in imaging.

If this is the case, then you need to capture z-stacks with a suitable range or make use of the focus offset function that allows each channel to be offset by a relative value in z to the reference channel. Typically, once you have established that a maxima can be reliably found with the default SWAF parameters you might want

to consider optimising the SWAF run by modifying the parameters to reduce the time required to complete it or to reduce sample exposure (phototoxic stress levels).

17.6.2 The SWAF returns an error message. What does this mean and how can I correct this?

The most typical error encountered is that the SWAF run does not find (return) a clear maxima and hence fails (hits so called search boundary). This might be because the step size set is too course (i.e. the maxima is missed occasionally) or the search range does not contain a clear sharpness maxima e.g. due to lower contrast/ intensity (signal to noise) or a lack of signal.

This can happen, for example, at the 1st tile (upper left) of a tile region which is empty such as is often that case in the corners of such tile regions. In this case it is possible to modify the SWAF focus strategy in the Tile Region loop such that the SWAF will be executed at the centre of the tile region. In ZEN 2.3 and higher if a SWAF failure occurs at a given loop entity then a fall-back applies such that the initial or last known z value will be used.

17.6.3 SWAF returns a failure after reaching a search boundary – what`s wrong?

If you are able to optimize the SWAF and the reference channel is typically robust for this then the solution might require that you look at the overall stability of the focus due to environmental variables such as vibrational isolation, temperature flux, evaporation of fluid from sample vessels, or poor lighting conditions (extraneous light).

Generally, if these are optimized then better overall results can be expected, if this is not the case then the fluctuations they cause might be the source of such reliability problems when detecting the sharpness maxima. Other things to consider are optical disturbances such as loss or poor immersion of the objective (bubbles), or artefacts caused by sample characteristics (for example (cell) debris moving across the FOV during the SWAF run). Perhaps it is possible to use a fiducial marker if the sample allows this (see Auto Focus ROI above).

17.6.4 When should I use the Full or Smart setting?

This depends entirely on the sample in question and manner in which it is imaged (e.g. reflected or transmitted light). However, if we are to generalize then the answer might simply be given as follows. A **Full** search will return the global sharpness maxima from the entire search range defined in the SWAF tool. The search always runs in one direction i.e. the z-movement of the actuator is unidirectional always moving against gravity.

Smart on the other hand is intended for quickly* detecting a local sharpness maxima by allowing a bidirectional search pattern. Thus, the full setting is typically a more extensive search that takes longer to accomplish. However, the smart setting may be suitable and saves a great deal of time and reduces sample light exposure.

*Typically, it will be the case that **Smart** is faster, but under some circumstances it might be slower. This will likely be the case when the maximum is far away from the starting position.

17.6.5 Can I change camera parameters in a SWAF run e.g. exposure time or binning?

In the implementation of SWAF in ZEN blue version 2.1 and earlier it was not possible to change the acquisition parameters directly. The exposure time setting using by the SWAF is based on the exposure time setting of the reference channel. Thus, longer exposure times of the reference channel increase the exposure time employed by the SWAF up to a maximum of ca. 100 ms. Thus, weak signals in the reference channel may cause an increased likely hood that no clear maximum will be found. Binning settings of a camera used by the reference channel are not taken into account and SWAF always uses 1x1 binning – this may cause issues with cameras processing smaller pixels (e.g .new AxioCam models).

In ZEN Blue version 2.3 and higher the SWAF has be further adapted to address the described limitations. Thus, exposure times defined for the Reference channel are used even when these exceed 100 ms. Settings that apply to all the channels defined in the acquisition mode tool still apply to the SWAF i.e. an independent binning setting for the reference channel is not yet possible. Thus, please take this into account when setting up your SWAF parameters. In addition, the SWAF run has been streamlined and can make use of triggered acquisition when supported by the camera making the SWAF run ca. a factor of two faster than in previous versions.

18 Module Panorama (ZEN lite)

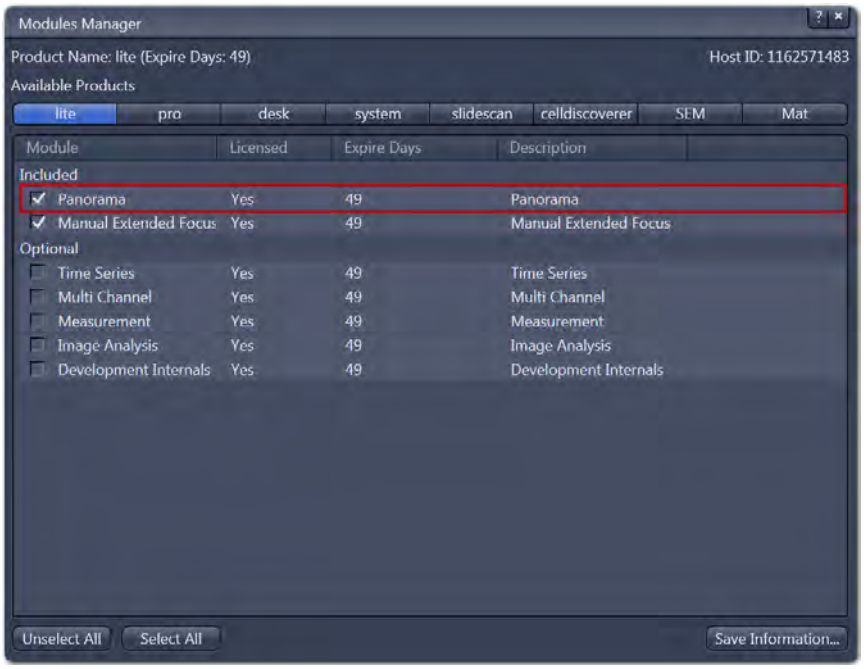
18.1 Introduction

By using the **Panorama** module for **ZEN lite** you can create overview images of large areas of your sample.

Prerequisites

For the interactive panorama acquisition following prerequisites are necessary:

- All available microscope components in the **MTB** (MicroToolBox) have to defined correctly.
- The **Panorama** module is activated in the menu **Tools** | **Modules Manager**.

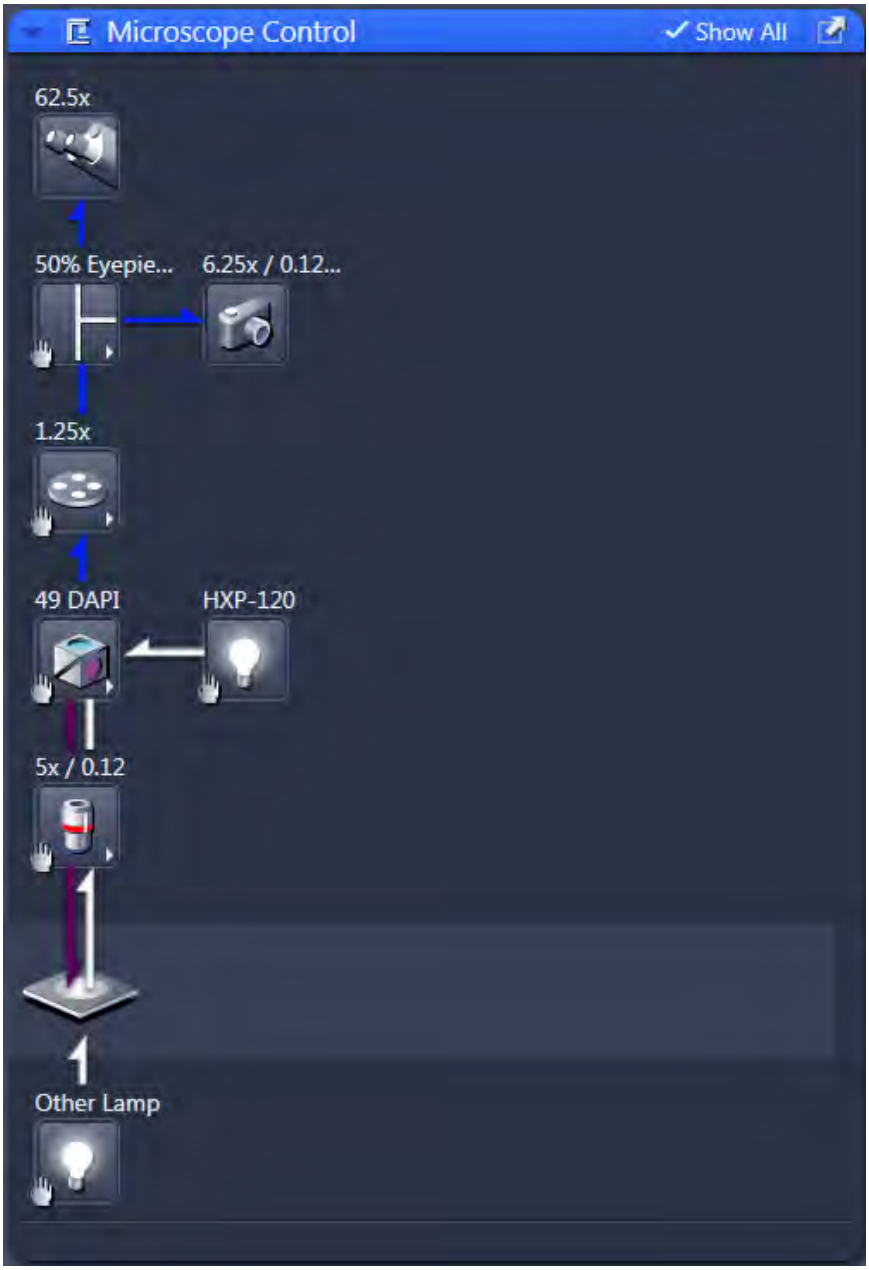


18.2 Prerequisites

Prerequisites

- You are on the **Locate** tab.

Procedure **1** In the **Lightpath** tool set the components that you want to use for the acquisition.



All these settings will be stored as metadata of the image.

Additionally the optical components will be used to automatically determine the pixel size for the scaling.

2 Click on the **Live** button.

Now you see the cameras live image of your sample in the **Center Screen Area**.

3 Click on the **Set Exposure** button.

The exposure time will be calculated automatically.

4 Alternatively you can set the camera parameters manually in the **Camera** tool.

5 Focus on your sample now.

You have completed the prerequisites for a panorama experiment.

18.3 Acquiring a Reference Image

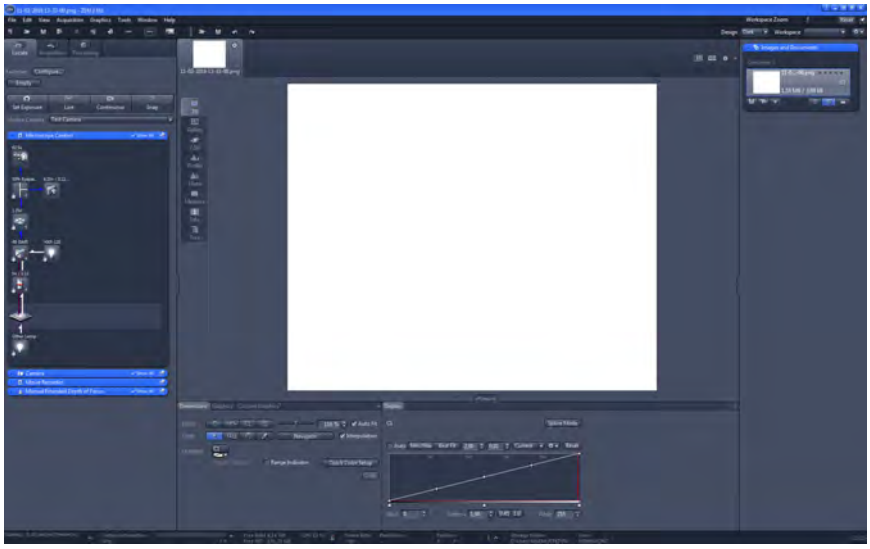
Before starting the experiment itself, we recommend to acquire a reference image for the shading correction first. This image will be used later for processing the panorama image.

Procedure

1 Move the sample to an empty field.

> **INFO**
>
> Ensure that the field of view is absolutely free of any structures (dirt); as this would cause errors in the images acquired later. If necessary, slightly de-focus the sample to suppress these structures.

2 Click on the **Snap** button to acquire this position as reference image.



3 Save the image and let it opened in the background.

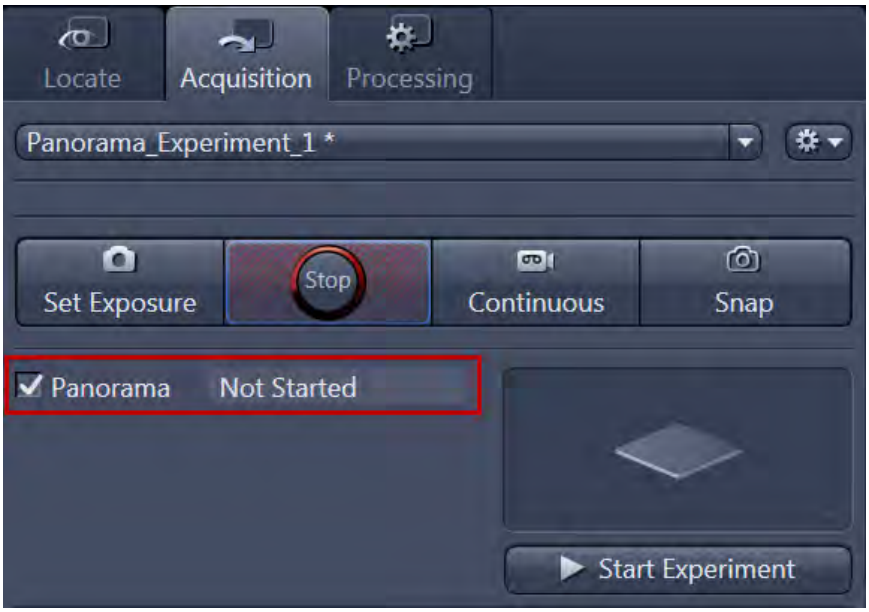
You have successfully acquired a reference image for the shading correction.

18.4 Settings for the Panorama Experiment

Prerequisites

- You are on the **Acquisition** tab.
- You have started the live mode via the **Live** button.

Procedure **1** Activate the **Panorama** checkbox.



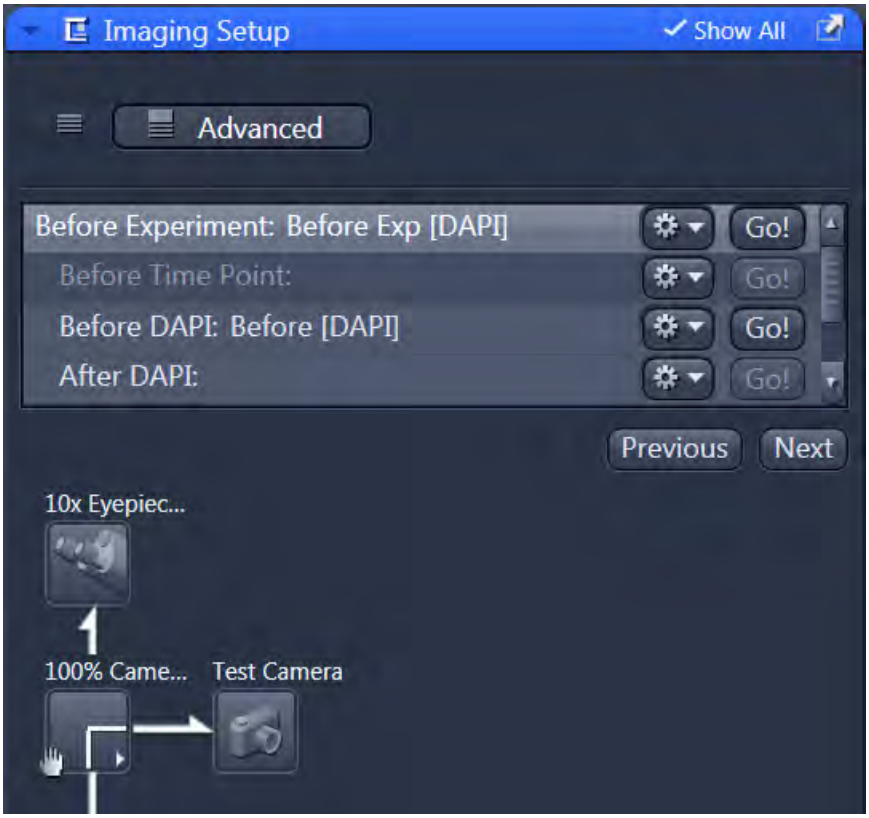
2 Move the sample to the desired start field of the panorama image to be acquired.

3 Check the defined exposure time again if necessary.

INFO

In the **Display** tab you can adjust the Display curve to avoid over-exposures in your images.

4 In the **Imaging Setup** tool, you can check the settings before/after the experiment.



INFO

The **Advanced Imaging Setup** is only displayed, if you have activated the **Enable Advanced Imaging Setup** checkbox under **Tools** | **Options** | **Acquisition** | **Acquisition Tab** | **Enable Imaging Setup**.

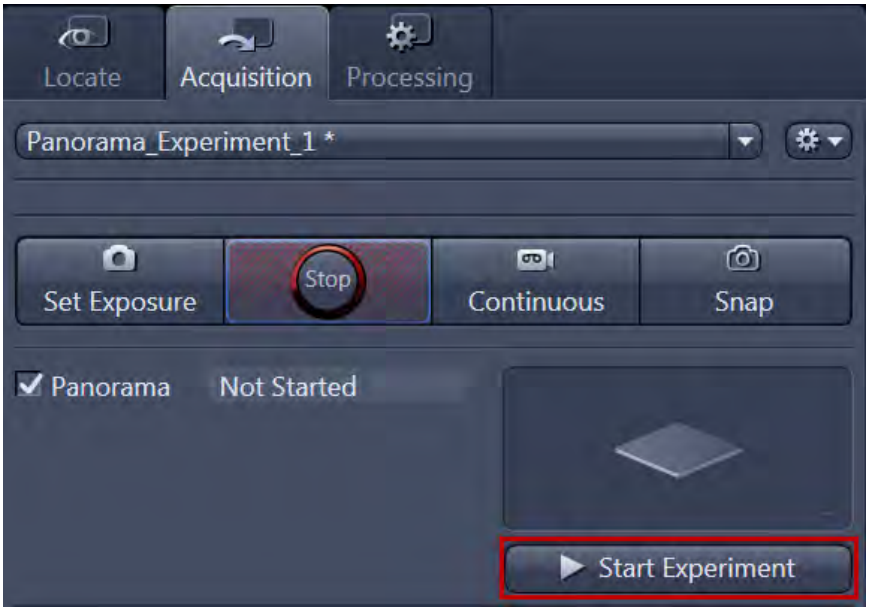
5 In the **Acquisition Mode** tool, click the **Get** button to transfer the active camera settings into the experiment.
As an alternative you can define your experiment settings here as well.

6 In the **Panorama** tool you can adjust several options for automatic or manual stitching, if desired.

7 Finally save your experiment with a suitable name in the **Experiment Manager**.

18.5 Acquiring the Panorama Image

Procedure **1** Click on the **Start Experiment** button to start the Panorama acquisition.



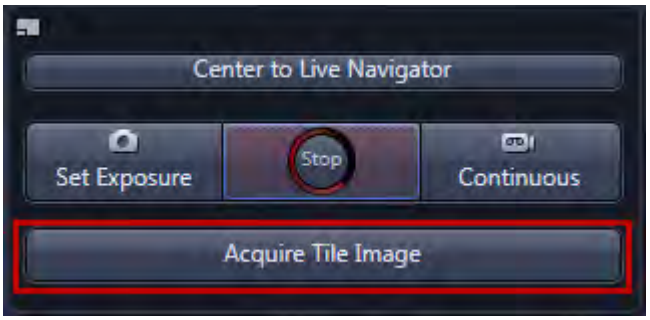
You will see the reduced display of the start image in the **Center Screen Area**.

INFO

You can adapt the size of image and surrounding area with the zoom keys *F7* and *F8* to your needs.

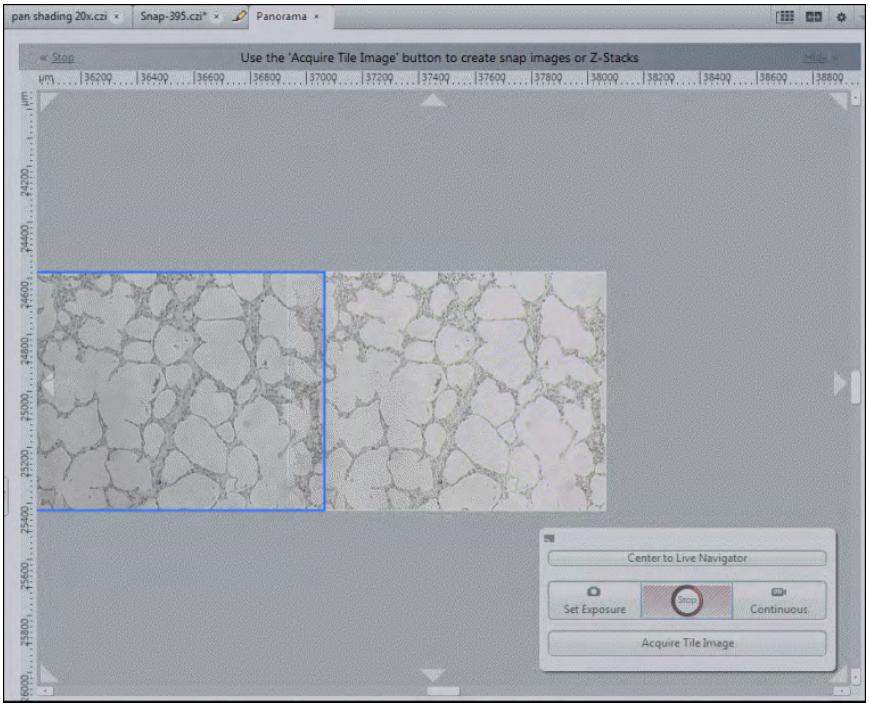
The displayed image is a live image. You still can change position and focus.

2 Click on the **Acquire Tile Image** button in the **Center Screen Area** to acquire the first tile image.



The image will be acquired and stored. The live image is still active as an overlay to the stored image.

3 Move the blue frame with the active live image in the desired direction aside the stored image by using the mouse.



INFO

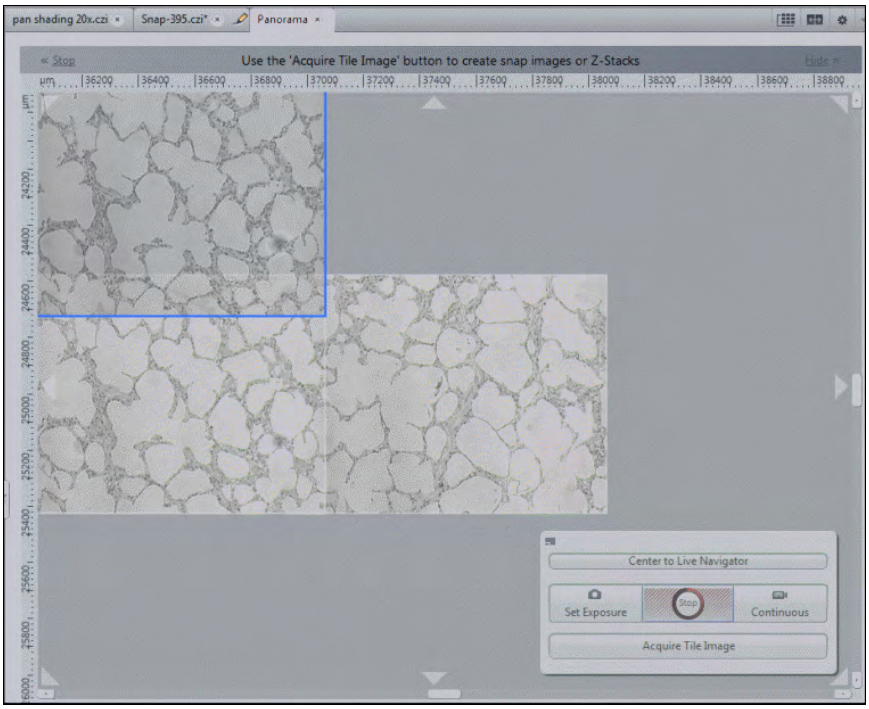
Keep a sufficient overlap area of the live with the stored image.

4 Now move the sample to the corresponding neighbor position using the microscope stage.

Try to position the structures in the overlap region as good as possible.

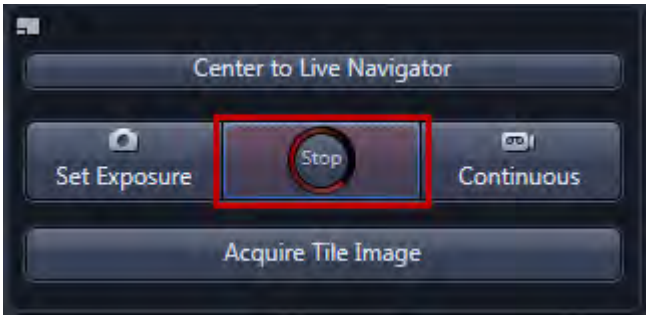
5 Click again on the **Acquire Tile Image** button to acquire this tile to the image.

6 Move the blue frame to the next position.

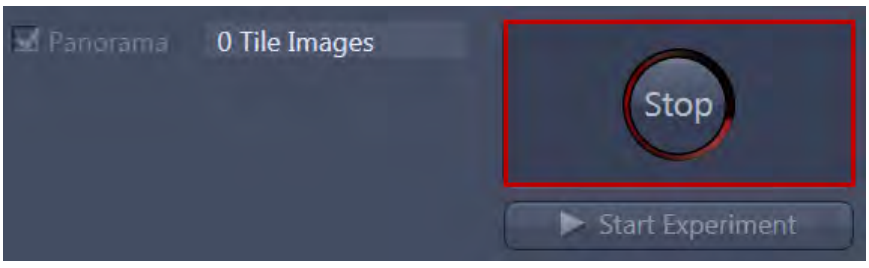


7 Continue these steps until you acquired the desired panorama image of your sample.

8 After acquiring the last tile image click on the **Stop** button to close the live mode.



9 Finally end the experiment via the **Stop** button on the **Acquisition tab**.



As a result you now see the recorded panorama image in a new image container.

10 Save the image as a raw image (*.**czi**).

INFO

In the case of errors during the following processing steps you therefore always have access to the original image data.

You have successfully acquired and stored a panorama image.

18.6 Processing the Panorama Image

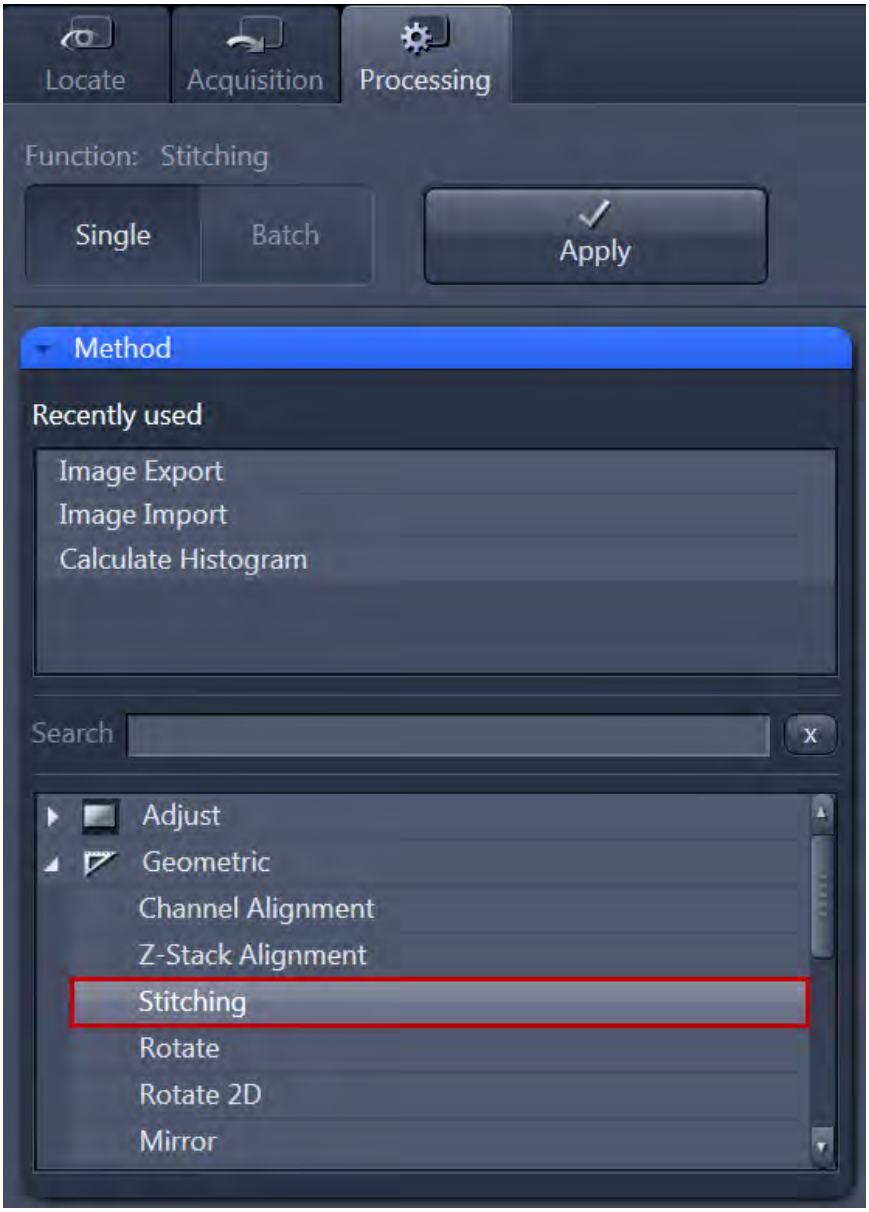
The next chapters will show you how to process panorama images with the **Stitching** processing function. Using this method you can correct an offset between the tile images. We will show you the different settings and make a comparison of the output images. So you can see which settings will give you the best result.

Prerequisites

Prerequisites

- You are on the **Processing** tab.

Procedure **1** Open the **Method** tool and select in the group **Geometric** the **Stitching** function.



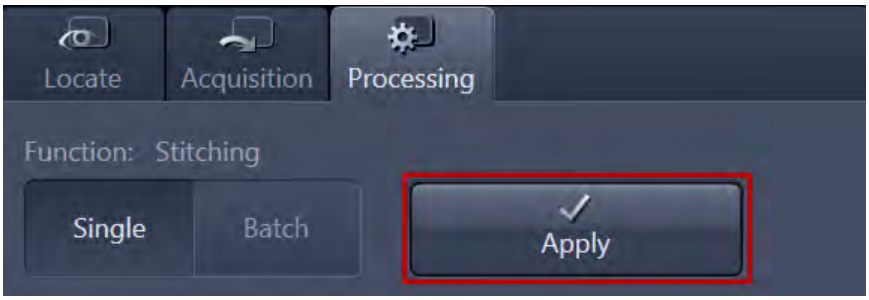
The following instructions will all base on this selection and show the different settings and results of this function.

18.6.1 Stitching (Defaults)

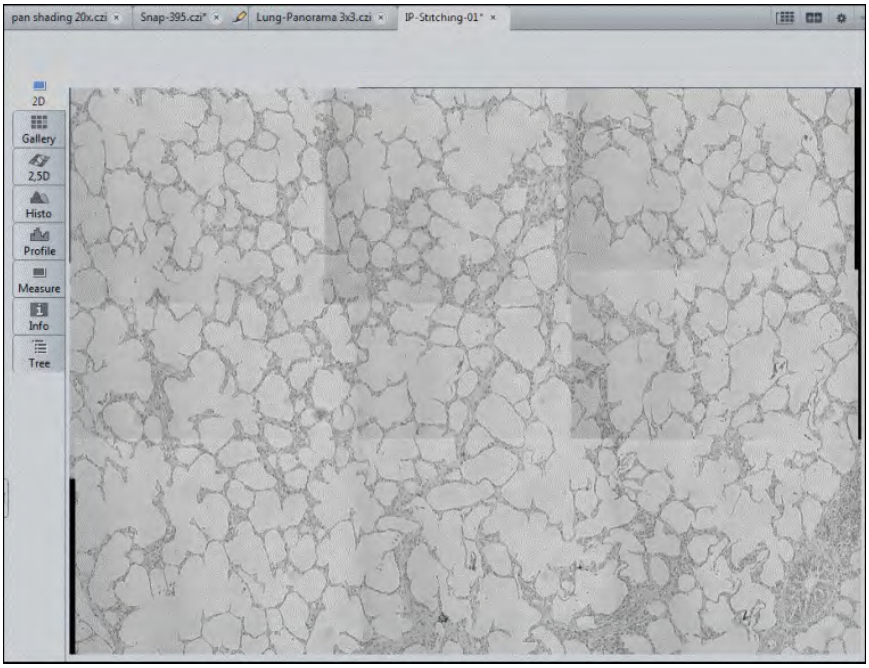
Procedure **1** In the **Method tool** open the **Geometric** group**.**

2 Select the **Stitching** function.

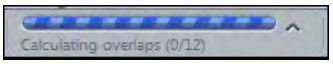
3 Click on the **Apply** button in the **Processing** tab to start the processing.



The stitching process will take a while depending on the image size.



In the **Status bar** you can see the work in progress.



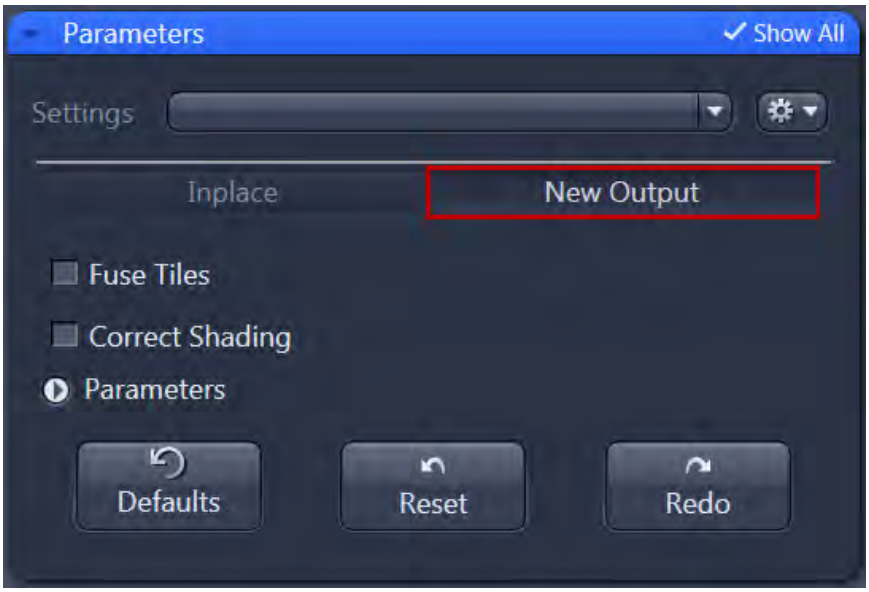
You have successfully used the **Stitching function**. As you still can see shadows and edges in the output image we will show you how to use the function receiving better results in the following chapters.

18.6.2 Stitching with Shading Correction

If your tile images contain a certain background shading you can correct this if you have acquired a reference image for the shading correction. This image has to be opened in the **Center Screen Area**.

Procedure 1 Select in the tile image for the stitching in the **Input** tool as first input.

2 In the **Parameters** tool select the **New Output** button.



This will keep the original image and create a new output image.

3 Activate the **Correct Shading** checkbox.

4 Select the **Reference** entry from the dropdown list.

This will let you select your reference image which is opened in Center Screen Area.´

INFO

If you do not have any image available for the shading correction, you alternatively can calculate a background image from the input image via the second selection **Automatic** under **Correct Shading**. This will only work, if your input image does not include large dark regions.

5 Now as a second input image select the reference image for the shading correction in the **Input** tool.

6 Click on the **Apply** button to start the processing.

As a result you will get a stitched panorama image without any shading influences. The next chapter will show you how to get rid of the edges which are still visible between the tiles.

18.6.3 Stitching with Fuse Tiles

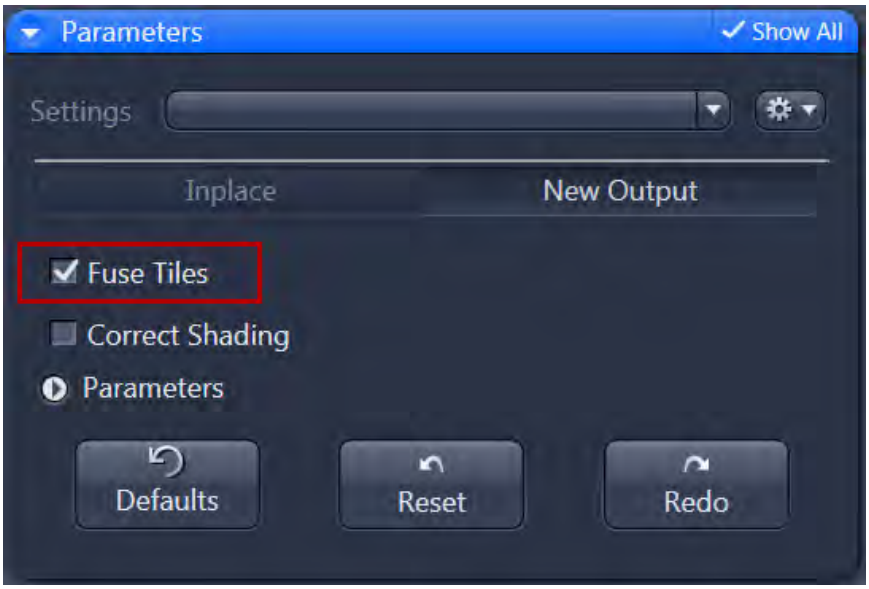
With very low shading content in the image you have an alternative method to homogenize the image transitions between the single tiles.

Procedure

1 Under **Image Parameters** in the **Input** tool select the tile image for the stitching.

2 In the **Parameters** tool, click on the **New Output** button.

3 Activate the **Fuse Tiles** checkbox.



4 Click on the **Apply** button to start the processing.

This function will smoothly average all edge regions of the tiles via their grey values in order to avoid sharp edges. With high background shading this function alone will not be sufficient.

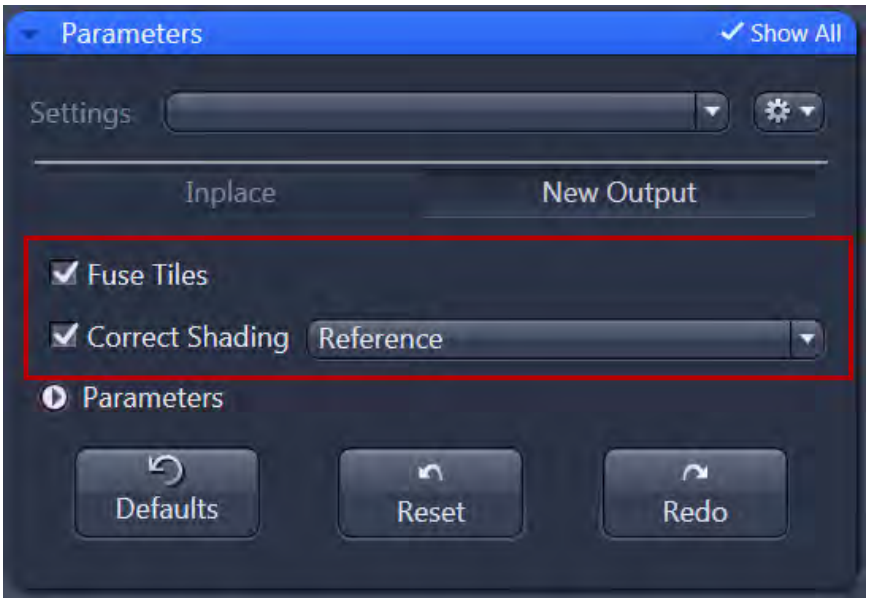
18.6.4 Stitching with Fuse Tiles and Shading Correction

For extreme cases you have the possibility to combine both transition corrections.

Procedure

1 In the **Parameters** tool click on the **New Output** button.

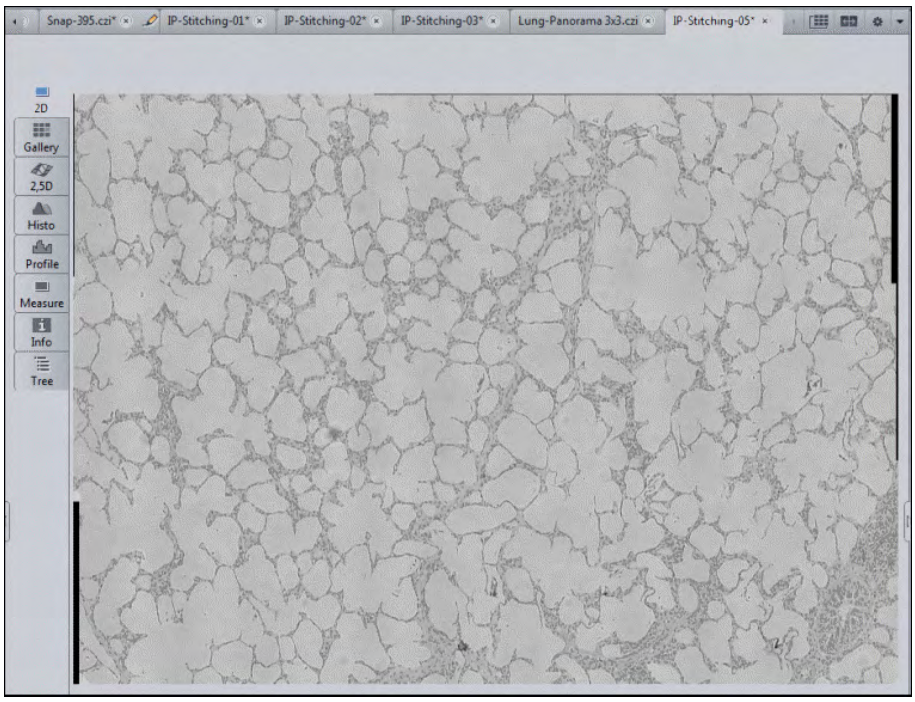
2 Activate the checkboxes **Correct Shading** and **Fuse Tiles.**



3 Under **Image Parameters** in the **Input** tool select the tile image for the stitching and the reference image for the shading correction.

4 Click on the **Apply** button.

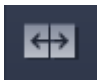
Both settings will be applied to the image. With this method you will receive a perfectly stitched image with no visible transition areas between the tiles any more.



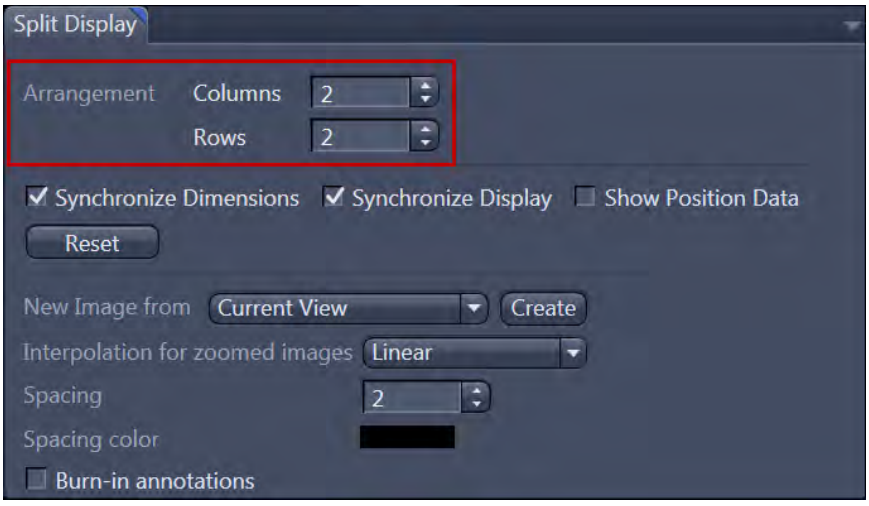
18.6.5 Image Comparison via Split Display

You can create a Multi Image to compare the different results of your processed images via the **Splitter-Mode.**

Procedure

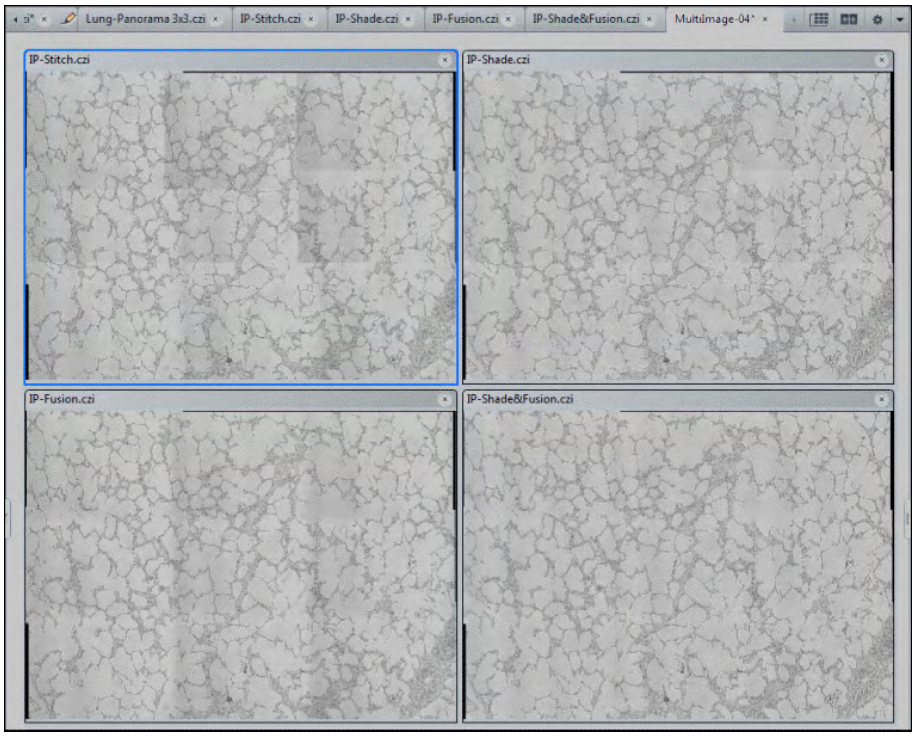
1 To compare different images, you can select the **Split Display** via the **Create New Multi Image**  button.

2 On the **Split Display** tab you can define how many images shall be displayed in X- and Y-direction aside each other and how they shall be synchronized, e.g. 2 **Columns** and 2 **Rows**.



3 Move each of the different panorama images via drag&drop from the **Right Tool Area** | **Images and Documents gallery** to an empty frame in **Center Screen Area**.

In our example we show the transition areas of the tile image as **raw image** (top left), as **fused tile image** (top right), as **shading corrected tile image** (bottom left) and finally the combination of **shading correction and fused tiles** (bottom right). In this last image no transitions between the tiles are visible any more.



18.7 Functions & Reference

18.7.1 Panorama Tool

Here you can adjust settings for the panorama acquisition.

After you have acquired the single tile images simply activate the **Perform stitching after the experiment** checkbox. If you want to stitch the images manually, click on the **Perform Stitching** button, after you have finished the experiment.

The panorama acquisition itself is performed interactively via in the **Panorama** view. If you click on '**Start Experiment**' the Panorama view will be opend and you can start to acquire the single tile images.

18.7.2 Panorama View

In this view you see the representation of the microscope stage. The **Live** image from the camera (blue frame) is automatically shown in the middle of the image area. Furthermore a tool window is displayed, that allows you to control the image acquisition, e.g. perform auto exposure or acquire an individual tile image.

18.7.2.1 Stage View

In the image area the full travel range of the microscope stage is displayed. You can control the stage view using the arrow icons at the edges of the image area. The view can be enlarged, reduced or moved using the general control elements.

Navigator frame

The current stage position is shown as a tile outlined in blue, the Navigator frame. In the Navigator frame you can see the camera's live image.

To move the frame, double-click on the position on the microscope stage to which you want to move it.

To acquire images, use the **Acquisition** buttons in the **Tools window.**

18.7.2.2 Tools window

The tool window for panorama view is normally visible in the lower right corner of the center screen area. It becomes active, if you move the cursor over it. You can use it to set acquisition parameters and acquire tile images for your panorama image.

Parameter	Description
Center to Live Navigator	Centers the stage view at the current position of the Navigator frame.
Action Buttons	With the three action buttons (**Live**, **Set Exposure**, **Continuous**) you're able to control acquisition parameters like you are used to do it on the **Acquisition** tab.
Acquire Tile Image	Acquires a tile image. This comprises all activated channels as well as Z-stacks. After the acquisition the tile image is placed in the corresponding location in the stage view.

19 Module ZEN Connect

19.1 Introduction

ZEN Connect allows working with images from multiple sources: zoom in from the full macroscopic view of your sample down to nanoscale details. The Correlative Workspace (CWS) is the efficient way to analyze and correlate images from multiple sources. It works with images from SEM, FIB-SEM, X-ray, light microscopes and any optical images, e.g., from your digital camera. Its sample-centric workspace lets you build a seamless multimodal, multiscale picture of your sample. Use it to guide further investigations and target additional acquisitions.

Understand your sample fully. **ZEN Connect** employs a novel graphical user interface concept that makes it easy to investigate all your samples. Design a workflow tailored precisely to the complexity of your experiment, no matter whether it's a simple one-step task or a compound experiment. A sophisticated workflow environment guides you all the way from the setup for automated acquisition to post processing and customized exports, and right on through to analysis.

ZEN Connect lets you manage, correct, align, and export images in correlation with images from other sources.

19.2 Licensing of ZEN Connect

For working with Connect projects or images, you might need a separate license file.

ZEN Connect Entry

This license includes the following:

- Connect including the display of images with their relations
- Manual alignment of captured images
- Auto-registration of images using stage coordinates
- Image acquisition into the project

ZEN Connect Advanced

This license includes all functionalities of the **ZEN Connect Entry** module. Additionally, the following functionalities are available:

- Algorithms for automatic overlay
- Import of Third-party images (BioFormats)
- Import of images to the Correlative WorkSpace

- Export of a Connect view as a single image

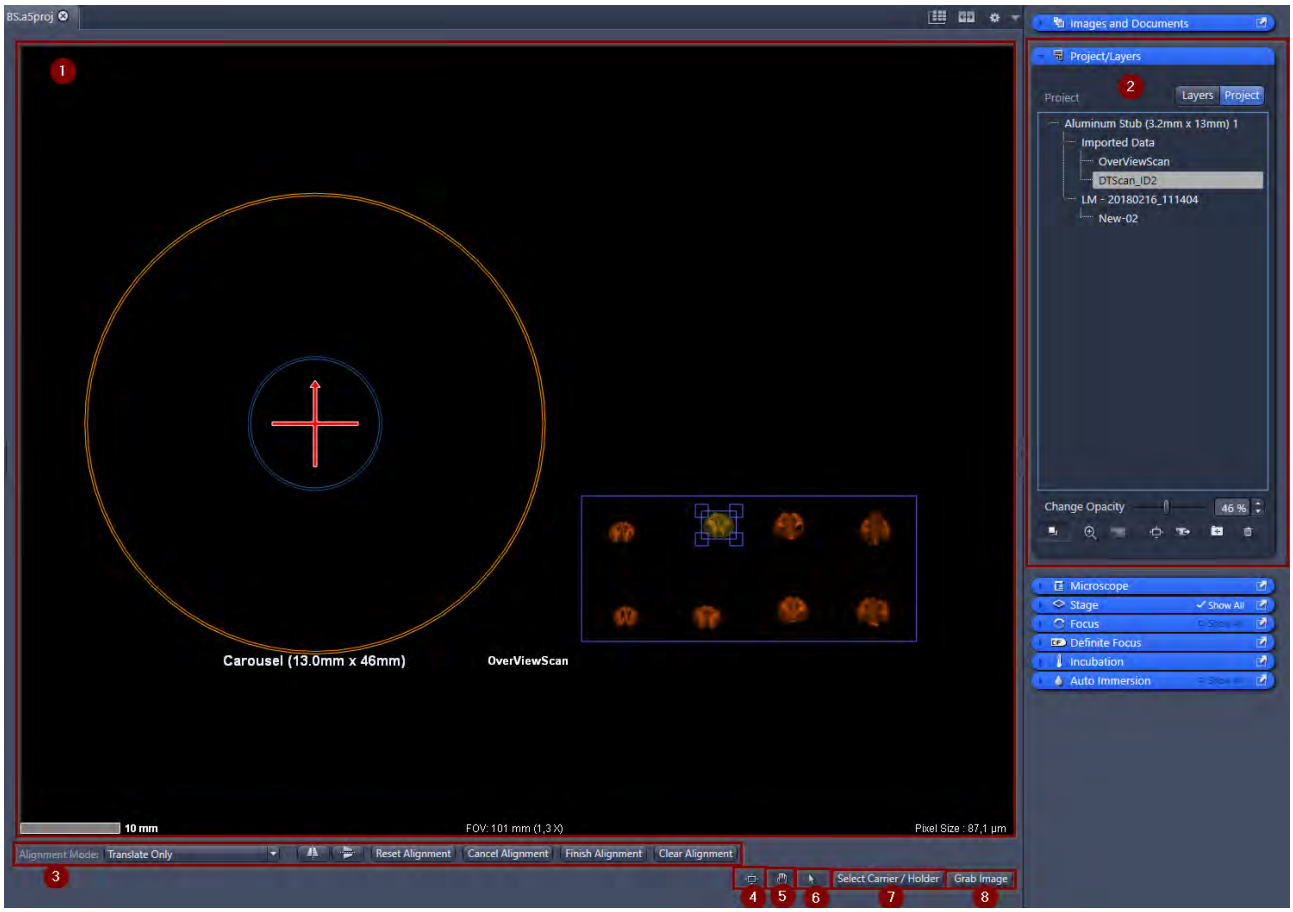
Overview

Product Name	ZEN Connect Entry	ZEN Connect Advanced	Third Party Import (BioFormats)
ZEN lite	**o**	**o**	**o**
ZEN pro	**x**	**o**	**o**
ZEN desk	**x**	**o**	**o**
ZEN system	**x**	**o**	**o**
ZEN slidescan	**n/a**	**n/a**	**n/a**
ZEN celldiscoverer	**n/a**	**n/a**	**n/a**
ZEN SEM	**x**	**o**	**o**

Note that **ZEN Connect Advanced** includes **Third Party Import**.

x=included; **o**=optional; **n/a**=not available

19.3 Introduction to the User Interface



Parameter		Description
1	**Image View**	Area where you interact with images. Here, for example, you can select images and align them.
2	**Project and Layers Tool**	Area where you manage your projects and images. For more information, see *Project and Layers Tool* [▶ 760].
3	**Alignment toolbar**	Functionalities for aligning images. For more information, see *Alignment Toolbar* [▶ 578].
4	 **Zoom To Extent**	Resets the view space of the Image View to be centered on the holder with a field of view (FOV) that includes all visible images in the project. For more information, see *Zooming to Extent* [▶ 575].
5	 **Pan & Zoom**	Activates the mouse for panning around and zooming in and out in the Image View.

Parameter		Description
		For more information, see *Panning & Zooming* [▶ 575].
6	 **Select & Navigate**	Selects a region in the Image View. For more information, see *Selecting region* [▶ 576].
7	**Select Carrier / Holder**	Opens a dialog to select a carrier or sample holder that matches. For more information, see *Selecting carrier / holder* [▶ 576].
8	**Grab Image**	Copies the currently displayed images to a new Image View to further work with the image. For more information, see *Grabbing an image* [▶ 577].

19.4 Workflow ZEN Connect

19.4.1 Opening the Correlative Workspace

Prerequisites

- You work with **ZEN (blue edition)**. You have licensed **ZEN Connect Advanced** or **ZEN Connect Entry** plus **Third Party Import**.

Procedure

1 Start the software. For more information, see *Starting the Software* [▶ 28].

The software opens and the Correlative Workspace (CWS) is available.

Note that before working with **ZEN Connect**, you need to create a Connect project. For more information, see *Creating a Connect project* [▶ 559].

19.4.2 Project and Image Management

19.4.2.1 Creating a Connect project

Within a Connect project, in a Correlative WorkSpace (CWS), you manage your data in a project structure tree combined with the viewer. Before acquiring or importing any images, you need to create the Connect project to display images correlatively. Only within a Connect project, you can use all Connect functionalities.

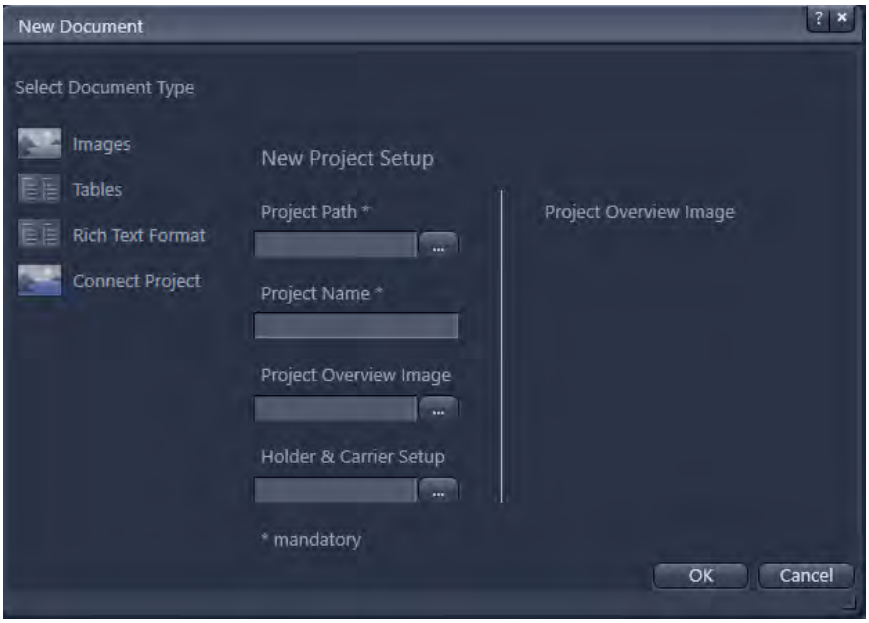
You can open only one Connect project at a time.

Prerequisites

- You work with **ZEN (blue edition)**. You have licensed **ZEN Advanced** or **ZEN Entry** plus **Third Party Import**.
- You have set up the sample.

Procedure

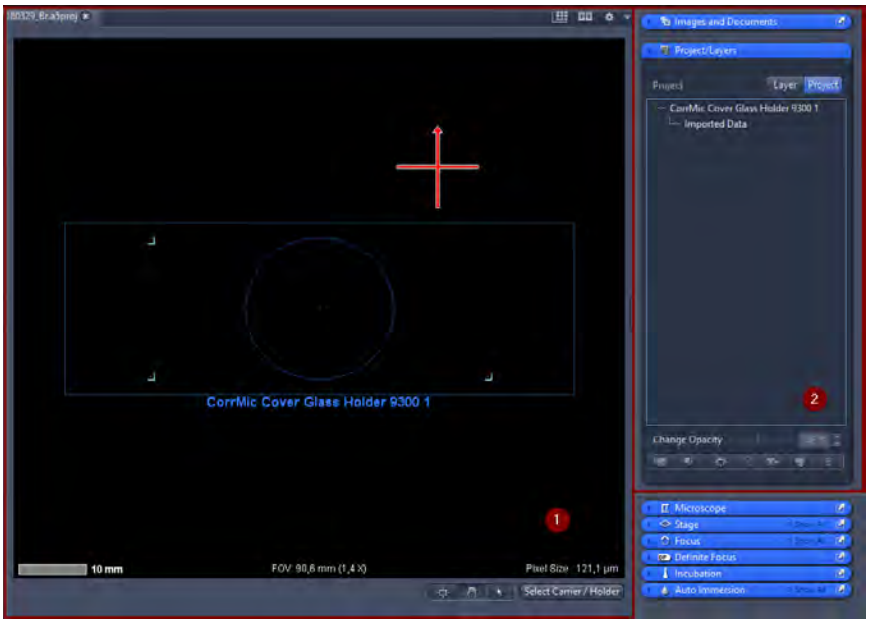
1 Select **File | New Document** and select **Connect Project**.



2 In the **New Project Setup** area, navigate to the folder, where you want to store the Connect project file. Select the relevant data to configure the Connect project . Click **OK**.

The Connect project is created with the project file name **<Projectname>.a5proj**. Note that all new images are saved in the subordinated folder <Projectname_data>. You can always check the path on the **Acquisition** tab in the **Auto Save** tool.

3 Click **Save**.



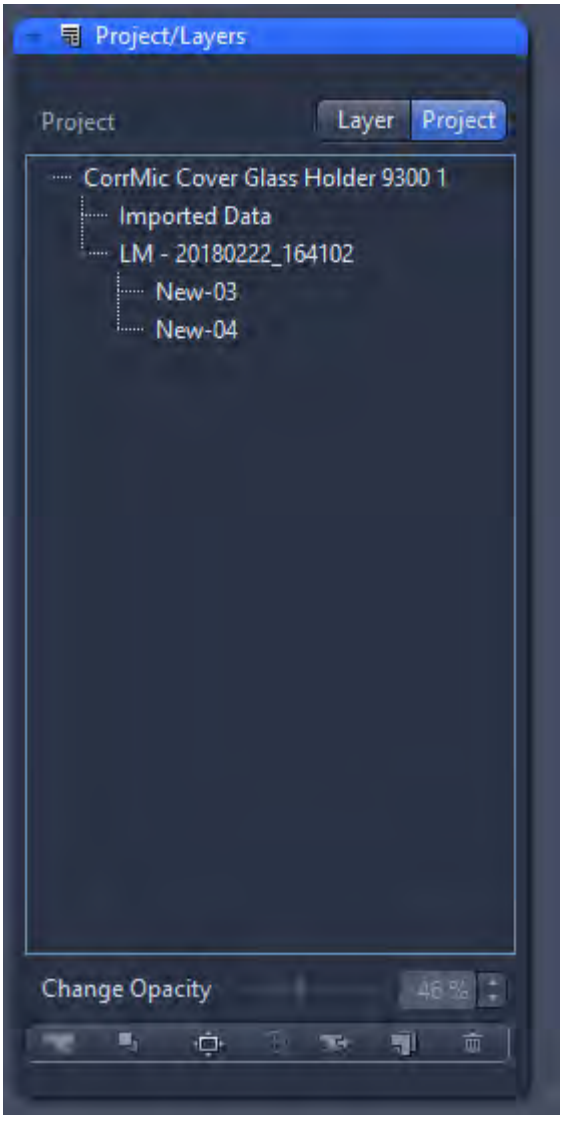
In the **Image View** (1), the sample holder and all acquired images are displayed.

In the **Project View** (2), the empty Connect project is displayed. Here, the structure of the Connect project will be displayed as soon as you acquire or import images. In the folder on your computer, the Connect project file **<CWS project name>.a5proj** is generated. A **<Connect project name>.a5lock** is generated to prevent more than one user to work on the project at the same time. It is generated any time you load a Connect project.

At the bottom of the **Image View**, a scale bar with size, the width of the field of view (FOV), and scaling is displayed.

You have created a Connect project.

4 Acquire an image.



In the **Project View**, a new session node is created, and each acquisition is displayed.

In the **Image View**, all images are displayed. They are signed with a colored frame.

5 When you close **ZEN (blue edition)**, you are prompted to save the project file.

For information on the **Project/Layers** Tool, see *Project and Layers Tool* [▶ 760].

19.4.2.2 Loading a Connect project

You can load any of your CWS projects to continue with your work. You can also load existing ZEISS Atlas 5 projects.

Prerequisites

- You have created a Connect project, or an ZEISS Atlas 5 project is available. ZEISS Atlas 5 projects belong to the ZEISS ATLAS 5 software. **ZEN Connect** supports these formats.
- The Connect project is saved to your computer. If your Connect project is saved to the data storage, load it from there. For more information, see *Opening or deleting a Connect project from the data storage* [▶ 566].

Procedure

1 Select **File | Open**, navigate to the Connect project and open it.

In the CWS **Project View**, the current state of the Connect project is displayed. In the **Image View**, the sample holders are marked, and previously acquired images are displayed. The current stage position is marked with a cross hair. If you want to acquire additional images to the project, align the new session with the existing data.

19.4.2.3 Adding an image to the Connect project

You can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, into your CWS project.

You can use an imported image as a backdrop to navigate the region. You can correlate imported images with sample holder marks, e.g., fiducials or other images through the alignment process. The imported image is displayed according to its position in the **Layers View** along with any other image in the project.

The metadata of .CZI-images are read natively and are also imported.

Prerequisites

- You have loaded a CWS project.

Procedure

1 Click **Project/Layers View** | **Project View** | right-click **Imported Data** | **Add image**. Alternatively, select **Project/Layers View** | **Project View** | **Import Data** icon.

You are prompted to select the image you want to add.

2 If an image is part of any of your sessions, you have the option to import the image into a certain session. Activate the **Import into sessions if possible** checkbox.

3 Click **Open**.

The image is imported to your Connect project.

In the **Project View**, it is displayed in the tree, subordinated to **Imported Data**.

If Shuttle & Find-calibration is available for the image and the image is at least in one session in the Connect project, the image is placed in the **Image View** according to the Shuttle & Find stage position. Take care that you select the correct sample holder when importing an S&F-calibrated image. The correct sample holder is the same sample holder that was used during acquisition.

If no Shuttle & Find data is available, the image is displayed centrally in the FOV of the **Image View**.

Alternatively, you have the following options to import data in your Connect project:

- *Importing Data* [▶ 565]
- *Importing Third-party images* [▶ 565]
- *Saving an image to the data storage* [▶ 568]

Adding datasets when adding images

Optionally, to organize your imported data, you can add datasets.

Prerequisites
- A Connect project is loaded.

Procedure
1. In the **Project View** tree, select **Imported Data** | right-click **Add dataset**.
2. Navigate to the image you want to add. Select it, and click **Open**.

The image is included in your Connect project, located below **New dataset**.

19.4.2.4 Removing images from the Connect project

Prerequisites
- You have loaded a Connect project.

Procedure
1. In the Connect **Project** view or in the Connect **Layer** view, select the image to remove.
2. Right-click the image, and select **Remove data**. Alternatively, in the **Project/ Layers View**, in the button bar, click the  **Remove Selected Data** icon.

The image is removed from the project. Removing data cannot be undone.

19.4.2.5 Opening images in the CWS

Prerequisites
- You have loaded a Connect project.

Procedure
1. In the **Project View** or in the **Layers View**, select the image you want to open, and right-click **Open image(s) in ZEN**.

The image is displayed on a separate tab.

19.4.2.6 Showing an image in the Explorer

You can locate an image on your computer.

Prerequisites
- You have loaded a Connect project.

Procedure 1 In the **Project View** or in the **Layers View**, right-click an image and select **Show in Explorer**.

The explorer opens and the folder with the selected image is displayed.

19.4.2.7 Moving or hiding images

In the **Layers View**, you can move images over and under other images, or hide them completely.

Prerequisites ■ You have loaded a Connect project with at least two images.

Procedure 1 To change the image order, in the **Layers View**, move the image by dragging it up or down.

In the **Image View**, the changed order is immediately visible.

2 To hide the image from the Connect project, in the **Layers View**, activate or deactivate the image by clicking the  **Eye** icon on the right of the image name. Alternatively, in the **Project View**, right-click **Show/Hide**.

The results of your changes are displayed immediately in the **Image View**.

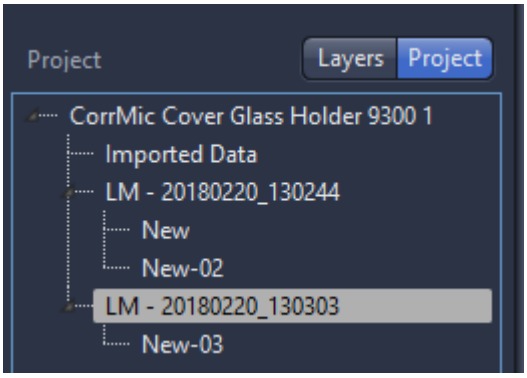
19.4.2.8 Starting a new session

To organize your work, you can start a new session within your Connect project any time.

Prerequisites ■ You have loaded a Connect project.

Procedure

1 In the Connect **Project/Layers** View, in the button bar, click the  **New Session** icon.



A new session is activated. As soon as you acquire a new image, a new session node is created, and the new image will be subordinated.

19.4.2.9 Closing a Connect project

You have the following options to close a Connect project:

Procedure 1 **File | Close**

2 In the **Image View**, click the cross next to the project name.

19.4.3 Import and Export

19.4.3.1 Importing Data

You can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, to your Connect project. For more information, see *Adding an image to the Connect project* [▶ 562].

Alternatively, you have the option to import BioFormats into your Connect project. For more information, see *Importing Third-party images* [▶ 565].

19.4.3.2 Importing Third-party images

ZEN uses BioFormats as an integrated library for reading and writing life sciences image file formats. It is capable of parsing both pixels and metadata for a large number of formats. It achieves this by converting proprietary microscopy data into an open standard called the OME data model. With BioFormats, you can read proprietary formats and convert them into an intermediate format, e.g., CZI or OME-TIFF. For example, it is possible to load simple images you can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, to your Connect project.

Prerequisites

- You have licensed the Third Party Import.

Procedure

1 Select **File | Export/Import | BioFormats Import**.

2 Navigate to the image with proprietary image format you want to import, select it, and click **Open**.

The image is converted and added.

Alternatively, you have the following options to import data in your Connect project:

- *Adding an image to the Connect project* [▶ 562].
- *Importing Data* [▶ 565]

For more information on BioFormats, see http://www.openmicroscopy.org/bio-formats/

19.4.3.3 Exporting single image data

You can export data of Connect projects as a single image for distribution to collaborators, or for the use in publications. The content can be a single image, tiles, a collection of images, or a view of the entire Connect project. You can drag or resize the region to control the area that you want to export. You can pan and zoom using the mouse in the **Image View** to get fine control of the export area.

Prerequisites

- You have loaded a Connect project.

- In the loaded Connect project, you have activated and deactivated the regarding areas of interest respectively.

Procedure

1. Click **Project/Layers View | Project View** or **Layers View**. From the context menu, select **Export**. Alternatively, in the button bar, click the **Export Selected Data** icon.

   A wizard opens.

2. Make your settings and click **Export Data**.
3. Navigate to the folder where you want to store the exported image. The default file name is the Connect project name. Click **Save**.

You have exported one image in a standard image format. The exported image is based on the export area you set up in the **Image View**.

19.4.4 Data Storage

You can store your data on your computer's file system. Additionally, you have the option to save your projects and images in a database. This makes the information more accessible, as you can search within the database and filter your results.

You have access to the database by opening **ZEN (blue edition)**.

19.4.4.1 Opening or deleting a Connect project from the data storage

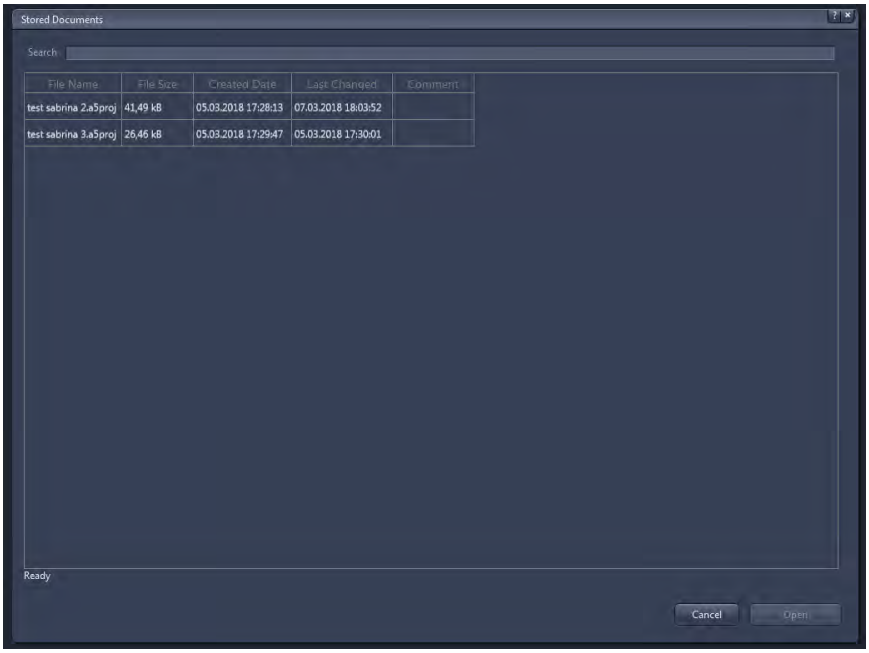
Prerequisites

- You have saved a Connect project to the data storage. For more information, see *Saving a Connect project to the data storage* [▶ 567].
- If your Connect project is saved to your computer, open it from there, and save it to the data storage. For more information, see *Loading a Connect project* [▶ 562].

Procedure

1. Select **File | Data Storage | Open Connect project**.

A list with stored documents is displayed.



2 Select the Connect project , and click **Open**.

In the Connect **Project** View, the current state of the Connect project is displayed. In the **Image View**, the sample holders are marked, and previously acquired images are displayed. Note that the project including its images is linked in the data storage. So please take care that these files are not moved or deleted as the links will be broken.

The current stage position is marked with a cross hair.

Deleting a Connect project from the data storage

Prerequisites
- You have opened the **Stored Documents** dialog.

Procedure

1 Right-click the project you want to delete, and click **Delete Document**.

You are prompted to confirm the deleting.

2 Click **Yes**.

The Connect project is deleted from the data storage.

19.4.4.2 Saving a Connect project to the data storage

You can save existing Connect projects that are currently saved on your computer to the data storage. If you save a Connect project with images, the image information is contained in the **Project View**. You can check it in the **Layers View**.

Optionally, you can create a new Connect project, and save it immediately to the data storage.

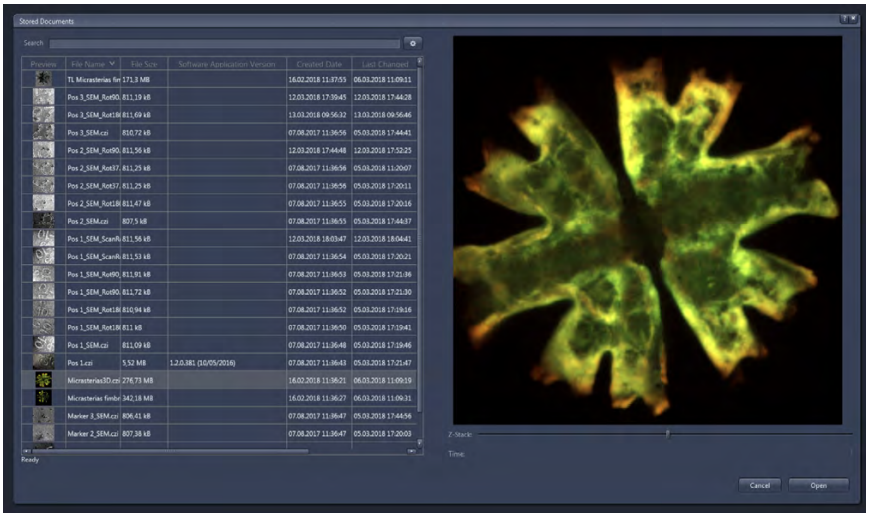
Prerequisites
- You have loaded a Connect project that is saved to your computer, or you are in the process of creating a new Connect project.

Procedure 1 Select **File | Data Storage | Save Connect Project**.

The Connect project is saved.

19.4.4.3 Opening or deleting an image from the data storage

Procedure 1 Select **File | Data Storage | Open Image**.



2 Double-click the image you want to open.

The image is loaded. A progress bar displays the progress. You can work with the images and save the update.

Deleting an image from the data storage

Prerequisites ■ You have opened the **Stored Documents** dialog.

Procedure 1 Right-click the image you want to delete, and click **Delete Document**.

You are prompted to confirm the deleting.

2 Click **Yes**.

The image is deleted from the data storage.

19.4.4.4 Saving an image to the data storage

You can save any image to the data storage. You can also open images from the data storage, update them, and save the updated image to the data storage.

Prerequisites ■ You have opened an image.

Procedure 1 Select **File | Data Storage | Save Image**.

The image is saved to the data storage.

19.4.4.5 Configuring the Stored Documents table

In the **Stored Documents** dialog, you select Connect projects or images to open or to delete. You can configure the columns of the table according to your needs.

Procedure

1. Select **File | Data Storage | Open Connect Project** or **File | Data Storage | Open Image**.

   The **Stored Documents** dialog opens.

2. Right-click into the header of the table, and activate the colums you want to see in the table.

19.4.4.6 Filtering Connect projects and images in the data storage

Prerequisites

- A Connect project is available in the data storage.

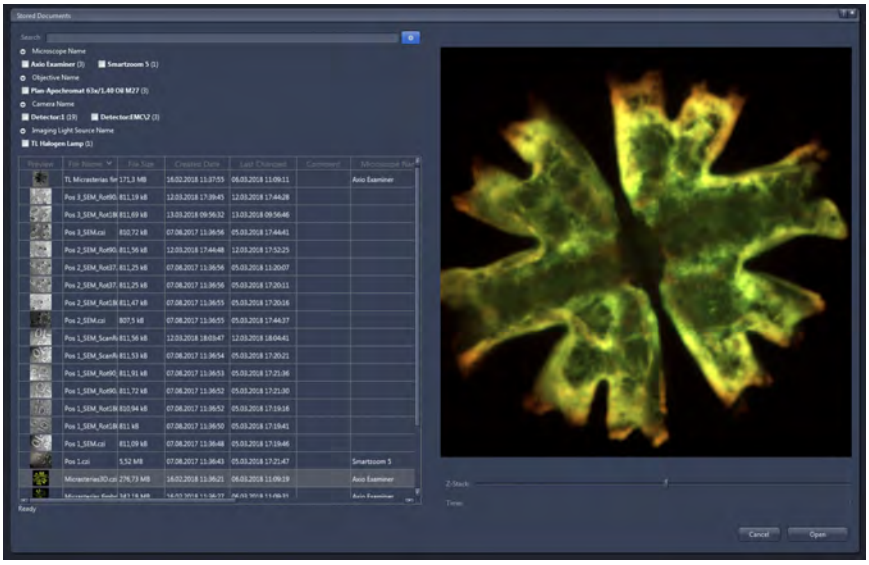
Procedure

1. Select **File | Data Storage | Open Connect Project** or **File | Data Storage | Open Image**.

   The **Stored Documents** dialog opens.

2. Right-click into the table header, and activate **Filter Data**.

   The search area with filter panel and metadata of the search results is displayed, e.g., **Smartzoom 5**. The number in brackets, here **(1)**, informs about the amount of search results.



3. To limit the search results, select a term of interest.

   The available values are limited accordingly.

4. Optionally, in the **Search** field, enter a term you are looking for, e.g., the file name.

The available values are limited accordingly.

19.4.5 Handling of images

19.4.5.1 Alignment

The module Shuttle & Find allows the correlation of two images. For more information on Shuttle & Find, see Module Shuttle & Find.

Additionally to that functionality, in a Connect project, you can manually align images in your workspace to correct their position or size with respect to the samples. Within a Connect project, you can calibrate your system using a sample holder with fiducial markers by moving between the markers and confirming their positions. To do so, you activate the alignment process and start aligning image data.

19.4.5.1.1 Activating the Alignment Process

The alignment process lets you align your current session with fiducial marks or previous images. You can align image data manually.

You should create a new session any time the alignment of the sample in the microscope has been disturbed.

Prerequisites

- A Connect project is loaded.

Procedure

1. In the **Layer View**, or in the **Project View**, select the image you want to align. Alternatively, you can select a region to select a couple of images.

   The image is marked with a square in each frame corner.

   As long as the alignment process is not activated, this is indicated with a little lock next to the cursor.

2. On the selected image, right-click **Align Data**. Alternatively, in the **Project/ Layers View**, in the **Layers View** or in the **Project View**, right-click and select **Align Data**.

You have activated the alignment process for one or more images. The **Alignment** toolbar is displayed below the **Image View**.

You can start aligning image data. If you start an alignment on a session node, the set alignment is used for all current and future images of the session. You can use this if you change your sample between different systems and want to align their coordinate systems to each other.

19.4.5.1.2 Aligning image data

In the alignment process, you have various options to align image data. Note that you can change the alignment mode during the alignment process. The alignment edits you have made are preserved, but you have to restart the pinning process if you have inserted any pins before changing the mode.

Note: The alignment process can be executed multiple times. Each time you run the alignment process, the end result of the last alignment is used as the starting point for the new alignment. If the initial image was far out of alignment at the start, it is easiest to do the alignment process once roughly, and then do the alignment

process a second time with more precision. The second alignment will use the first alignment as a starting point, and will allow you to establish a more precise alignment quickly.

Prerequisites

- You have loaded a Connect project and activated the alignment process.

Procedure

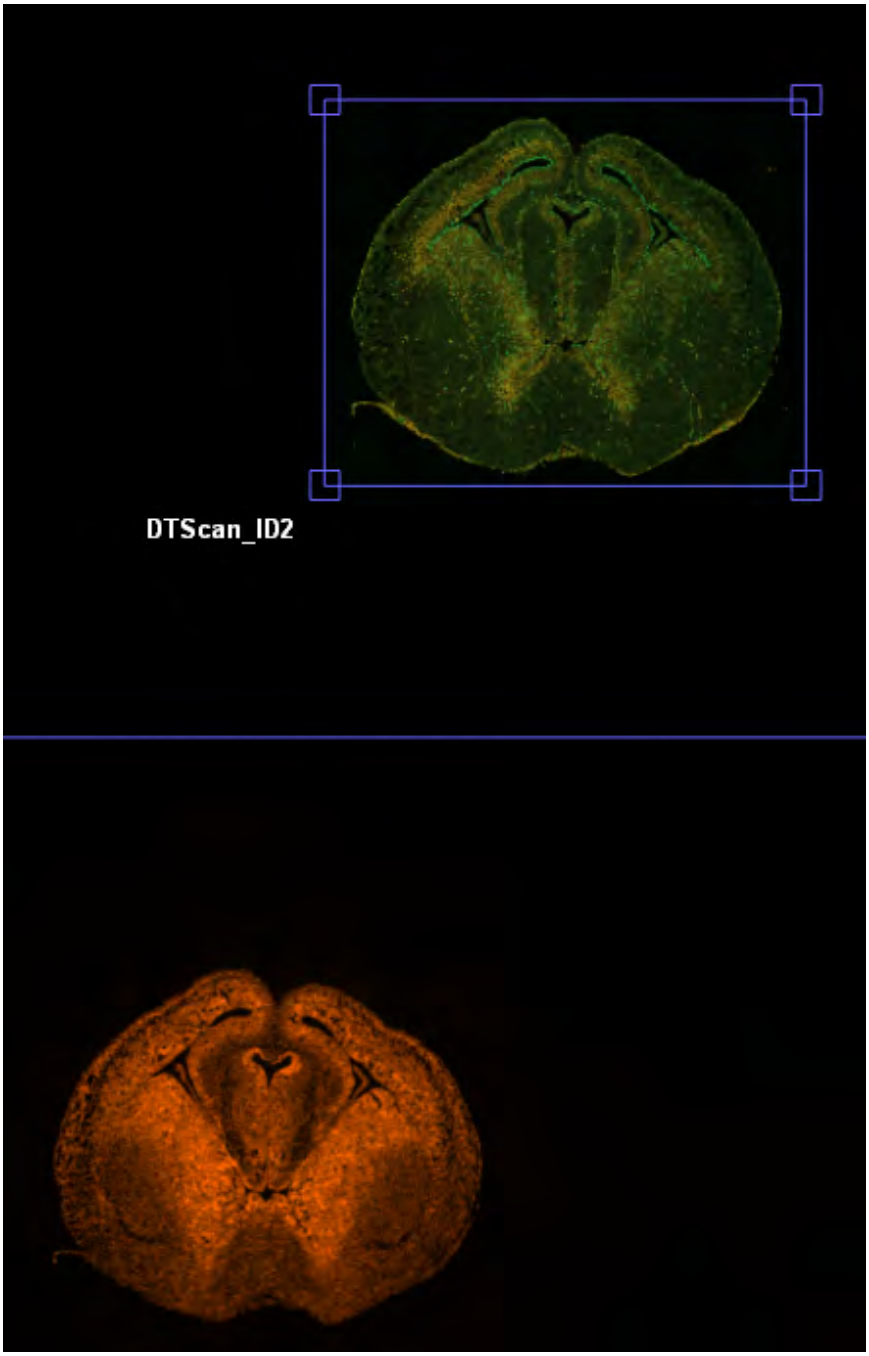
1. In the **Alignment** toolbar, select one of the following alignment modes, and select the region you want to align.

Translate Only

Procedure

1. Click and drag with the mouse to translate the image you are aligning with respect to everything else.

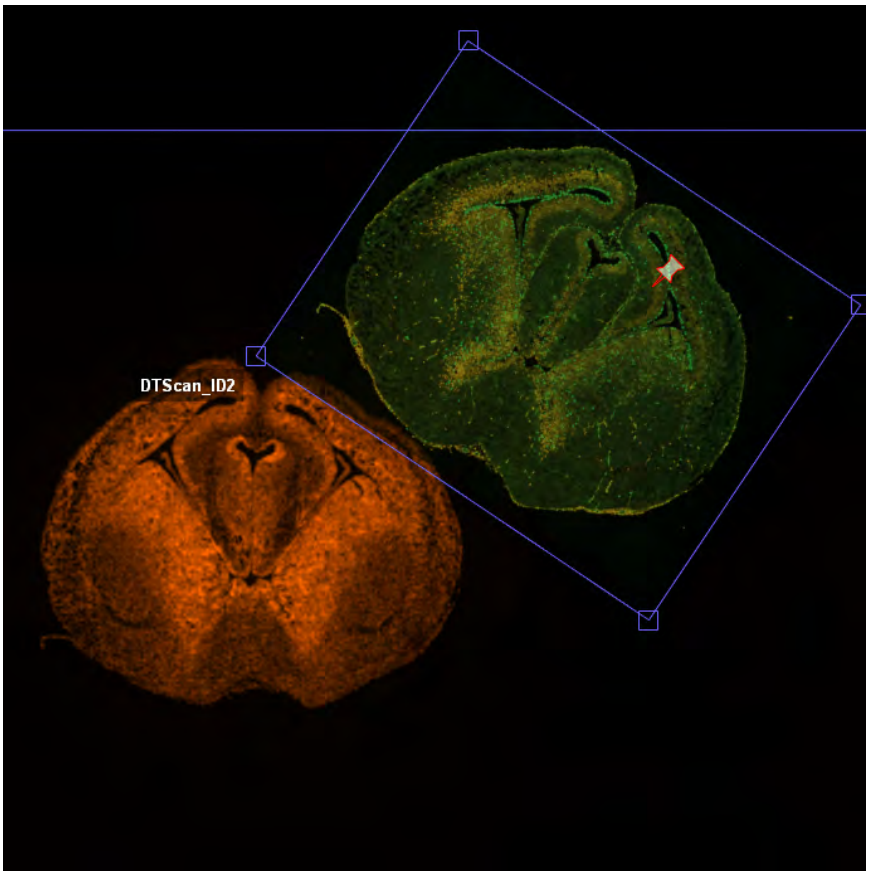
You can zoom in and out with the mouse wheel, or press and hold the *CTRL* key to pan while you are in the process of aligning the image.



Translate and Rotate Only

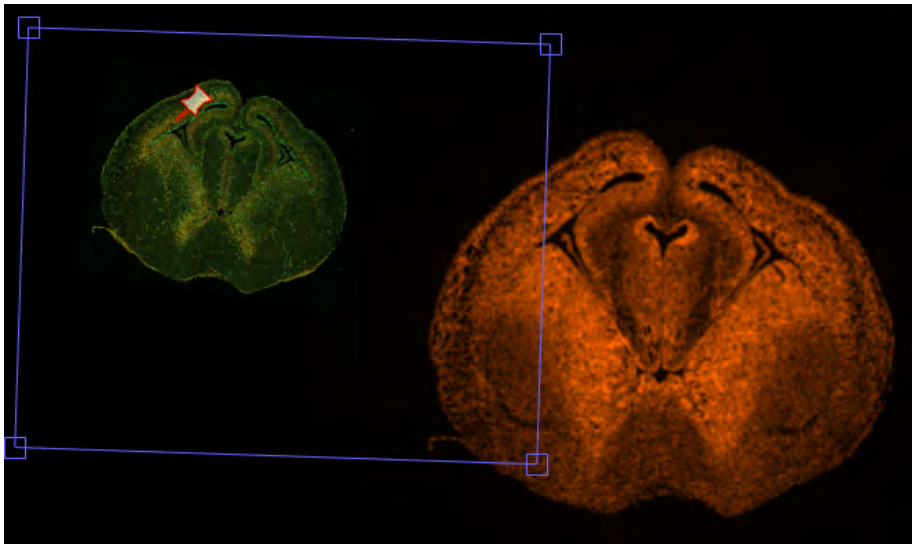
Procedure **1** Right-click at the location you have lined up to insert the first pin, a red and grey pin icon. The pin locks the image to the reference at this location. Press *DEL* key to remove the last pin you inserted.

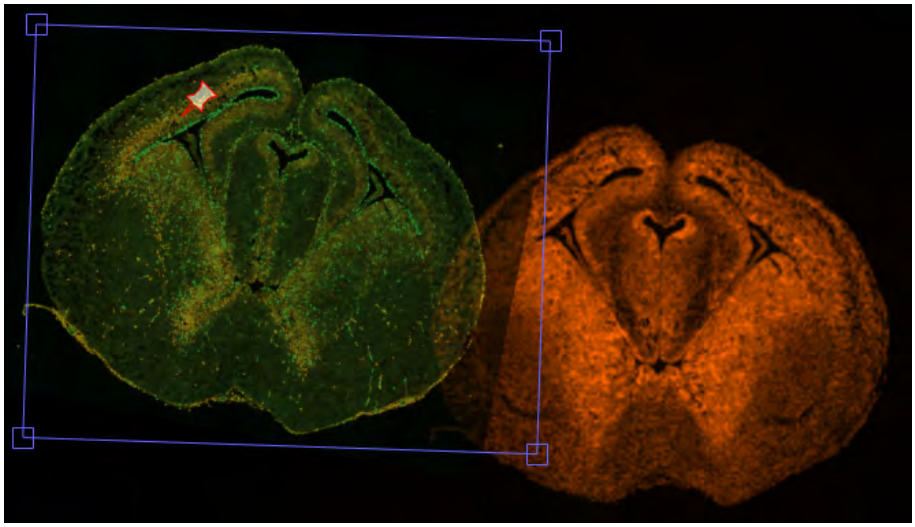
After you insert the first pin, your input will rotate the item around the first pin, when dragging it with the mouse.



Translate, Rotate and Scale Only

Procedure **1** If one of the images is smaller than the other, you can scale it. Right-click to insert a pin, and drag with the mouse to scale and rotate the image.





Translate, Rotate, Scale and Shear

Procedure

1 Right-click to insert a second pin, and drag with the mouse to shear the image.

After you insert the second pin, your input will also stretch and shear the item.

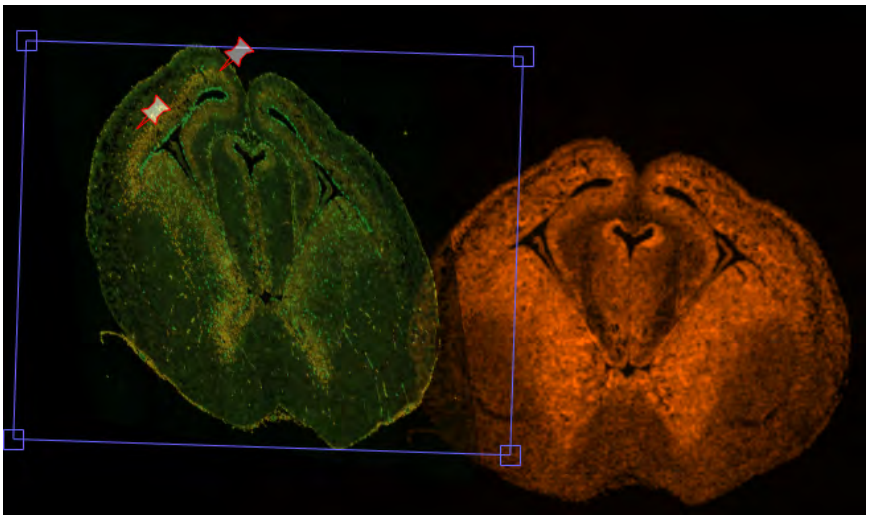


Image data from microscopes should not need to be stretched or sheared to perform alignment. If you need to provide much input after inserting the second pin, this might be an indication of other problems, such as equipment calibration issues.

Flipping the image horizontally and vertically

You can flip your image, to mirror it.

Procedure

1 To flip the image horizontally, click the  **Flip Horizontally** icon.

2 To flip the image vertically, click the  **Flip Vertically** icon.

Reset alignment

Procedure

1. Click the **Reset Alignment** button to reset the alignment you performed.

   The alignment is reverted as it was when you started aligning. The Alignment Mode ist still activated.

Cancel alignment

Procedure

1. Click the **Cancel Alignment** button to reset the alignment you performed.

   The current alignment is cancelled and reverted to the alignment in place before you started the Alignment Mode. The alignment mode is not activated any longer.

Finish alignment

Procedure

1. Click the **Finish Alignment** button to finish the alignment mode and to save the alignment information.

Clear alignment

Procedure

1. Click the **Clear Alignment** button.

   The session is restored to it's un-aligned state.

Saving alignment

Procedure

1. In the **Project View** or in the **Layers View**, select the image you have aligned, and right-click **Save Alignment**.

   The alignment data of an item is saved to an independent file. You can export the alignment information.

19.4.5.2 Zooming to Extent

Prerequisites

- You have loaded a Connect project.

Procedure

1. In the button bar below the **Image View**, click the **Zoom to Extent** icon.

   Alternatively, in the Connect **Project/Layers View**, click the  **Zoom to Extent** icon.

In the **Image View**, the sample holder is centered. All images in the Connect project are displayed.

19.4.5.3 Panning & Zooming

Procedure

1. In the button bar below the **Image View**, click the  **Pan & Zoom** icon.

   With your mouse, or alternatively, with the pressed *CTRL* key, you can pan and zoom in and out in the **Image View**.

19.4.5.4 Selecting region

You select a region to later apply the Alignment Mode to the image contained in this region.

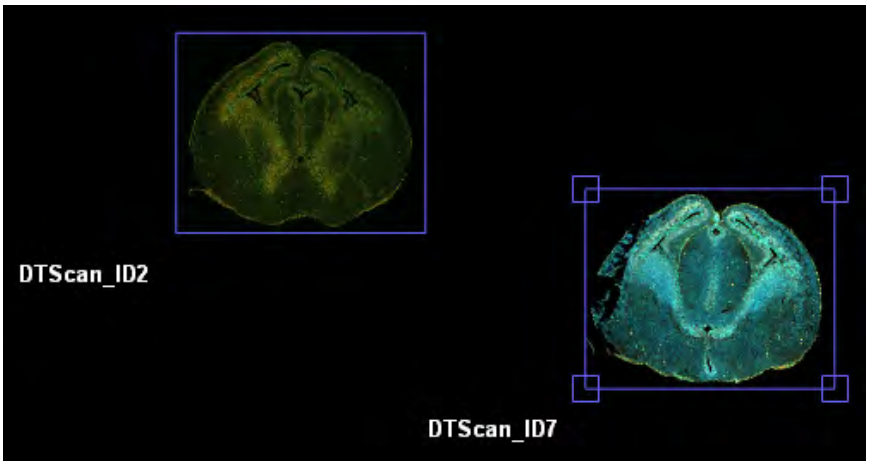
Prerequisites

- You have loaded a Connect project.

Procedure

1. In the button bar below the **Image View**, click the **Select Region** icon. Alternatively, press the *CTR* key, and click the desired image in the **Project View**.

   The image in the selected region is activated.



In the Connect **Project/Layers View**, both in the **Project View** and in the **Layers View**, the image within the selected region is highlighted.

19.4.5.5 Selecting carrier / holder

The sample is usually mounted on a carrier or directly on a sample holder. Select the appropriate sample holder for your configuration when you configure your project.

We offer specific sample holders and carriers with certain markers, e.g., "L"-markers or others. These CorrMic sample holders are necessary for a Shuttle & Find workflow.

Prerequisites

- You have loaded a Connect project.

Procedure

1. In the button bar below the **Image View**, click the **Select Carrier / Holder** button.

2 Select a template you want to add to your Connect project, and click **OK**.

The frame of the selected template is displayed in the **Image View** of your Connect project.

For information on correlative sample holders, see Correlative Sample Holders.

19.4.5.6 Grabbing an image

You can create an image from the loaded Connect project.

Prerequisites

- You have loaded a Connect project.

Procedure

1 In the button bar below the **Image View**, select the **Grab Image** button.

A new tab opens. In the **Image View**, the grabbed image is displayed.

2 Right-click and save the image to your computer.

You have saved the image. It is not part of your Connect project.

19.5 Functions and Reference

19.5.1 Select Template Dialog

In the **Select Template** Dialog, you select carriers and holders.

Parameter	Description
Tiles	Shows a list of all generic sample holders.
Correlative	Shows a list with all relevant correlative sample holders.

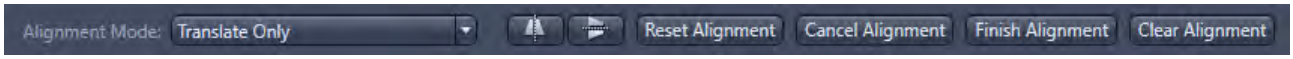
19.5.2 Alignment Toolbar

The alignment toolbar is visible, as soon as the alignment process is activated in the CWS project.

You can perform Three-Point Alignment:

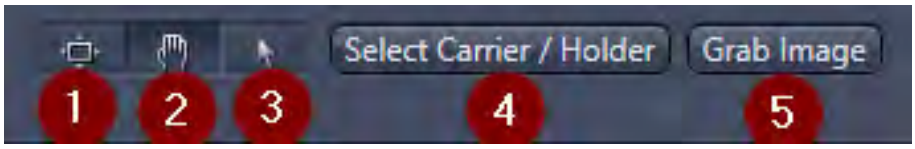
- Line-up an imported image with reference marks, such as the precision fiducials on a CorrMic Holder.
- Line-up features in an imported image with LM, EM and SEM images of the same features.
- Line-up a session of LM, EM and SEM imagery with previously acquired LM, EM and SEM imagery session.

With the three-point alignment process you can set the position, rotation, and scale of an image or tile. This is used to line the image or tile up with reference marks or other images. Once an image is lined up, it can be used as a reference (road map) to move the stage to control further image acquisition.



Parameter	Description
Alignment Mode	Sets which data properties you can change during the alignment.
- **Translate Only**	Moves the item you are aligning in X and Y only, without changing its size or orientation.
- **Translate and Rotate Only**	Moves the item in X and Y direction, and changes its orientation. It does not change the scale of the item you are aligning.
- **Translate, Rotate and Scale Only**	Moves, reorients and resizes the item you are aligning. It does not shear it.
- **Translate, Rotate, Scale and Shear**	Supports full three-point alignment.
 **Flip Horizontally**	Mirrors the image in the horizontal direction.
 **Flip Vertically**	Mirrors the image in the vertical direction.
Reset Alignment	Resets the alignment as it was when you started this alignment operation.
Cancel Alignment	Returns to the alignment as it was when you started, and exits from the alignment operation.
Finish Alignment	Exits from the alignment operation, keeping the alignment you have established.
Clear Alignment	Resets the alignment to where it was when the data was first acquired or imported.

19.5.3 Button bar below Image View



Parameter		Description
1	**Zoom To Extent**	Resets the view space of the Image View to be centered on the holder with a field of view (FOV) that includes all visible images in the project. For more information, see *Zooming to Extent* [▶ 575].
2	**Pan & Zoom**	Activates the mouse for panning around and zooming in and out in the Image View. For more information, see *Panning & Zooming* [▶ 575].
3	**Select Region**	Selects a region in the Image View. For more information, see *Selecting region* [▶ 576].
4	**Select Carrier / Holder**	Opens a dialog to select a carrier or sample holder that matches. For more information, see *Selecting carrier / holder* [▶ 576].
5	**Grab Image**	Grabs an image. For more information, see *Grabbing an image* [▶ 577].

19.5.4 Configure single image export wizard

With the **Configure single image export** wizard, you configure the parameters of the image you want to export.

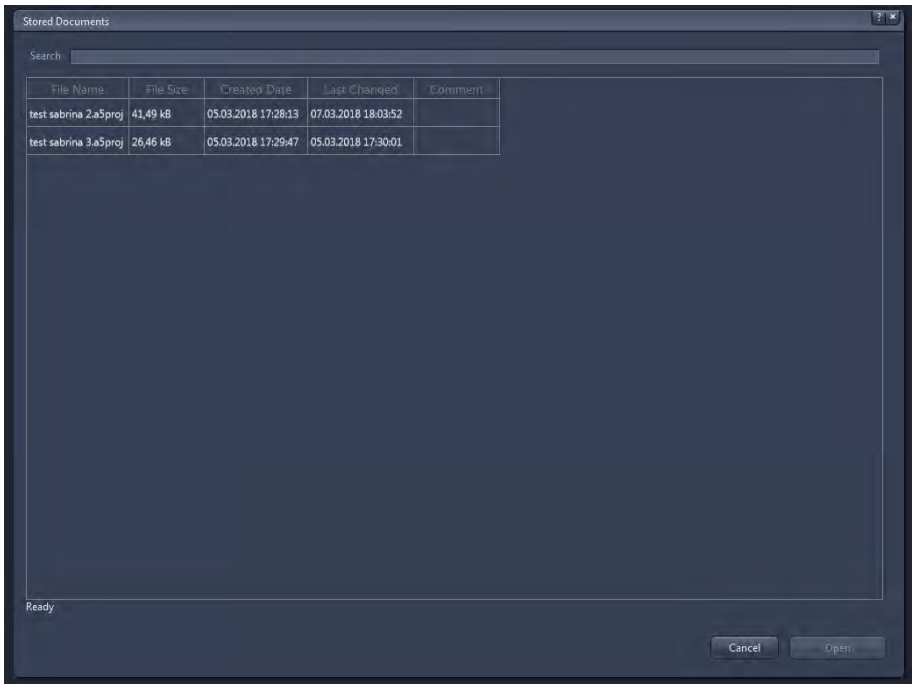
Parameter	Description
Color Style	Controls the color format of the export. Select **RGB** (color) or **Intensity** (black and white).
- **RGB**	Based on the RGB (Red-Green-Blue) color model. Be aware that color files may be up to three times as large as intensity files.
- **Intensity**	Images are saved in 8-bit format.

Parameter	Description
Export Format	Selects the format for the exported file. Only the file formats that support the number of pixels you are exporting will be shown. If your export is too large, formats like BMP, JPG and TIFF are not displayed. If you wish to export to one of these formats, you must pick a larger pixel size, or smaller export area for your export.
- **Raw image**	Raw binary dump of the pixel values. An XML file is also written detailing the image width and height in pixels, the pixel size in microns, the bits per sample and samples per pixel. Raw files are not limited in size.
- **CZI image**	A Carl Zeiss Image file. CZI files are not limited in size. The images are exported in a single channel CZI.
- **Tif image**	A standard TIFF file. TIFF files are limited in size.
- **Tif tiles**	With this option, the export is in TIFF format, but broken into 2Kx2K tiles saved as individual TIFFs. An XML file is written listing the file names of the tiles and their positions. This export option is unlimited in size, but designed for someone who is writing scripts to import the data into image processing applications or similar.
- **Bitmap image**	A standard Windows Bitmap. Bitmap files are limited in size.
- **Jpg image**	A standard JPEG file. JPEG files are limited in size.
Show Data Bar	Burns the currently configured data bar into the exported image. Activate the **Show Data Bar** checkbox
Rotation	Rotates the view to the desired orientation. Drag the slider, or in the text field, type in the value.
Pixel Size	Sets the pixel size of the export. Type the pixel size in the **Pixel Size** field. The smaller the pixel size, the more disk space your export will take. Click the **1:1** button to reset the pixel size to the native resolution.
Width (px)	Width value to directly alter the export pixel counts and export area (the pixel size is unchanged).
Height (px)	Height value to directly alter the export pixel counts and export area (the pixel size is unchanged).
Width (µm)	The full width of the export area in µm.
Height (µm)	The full height of the export area in µm.
Approx. Data Size	The actual file size after export may be less than the listed data size based on compression in some file formats.

Parameter	Description
Export Data	Starts the export with your settings.

For more information on exporting single images, see *Exporting single image data* [▶ 565].

19.5.5 Stored Connect projects in the data storage

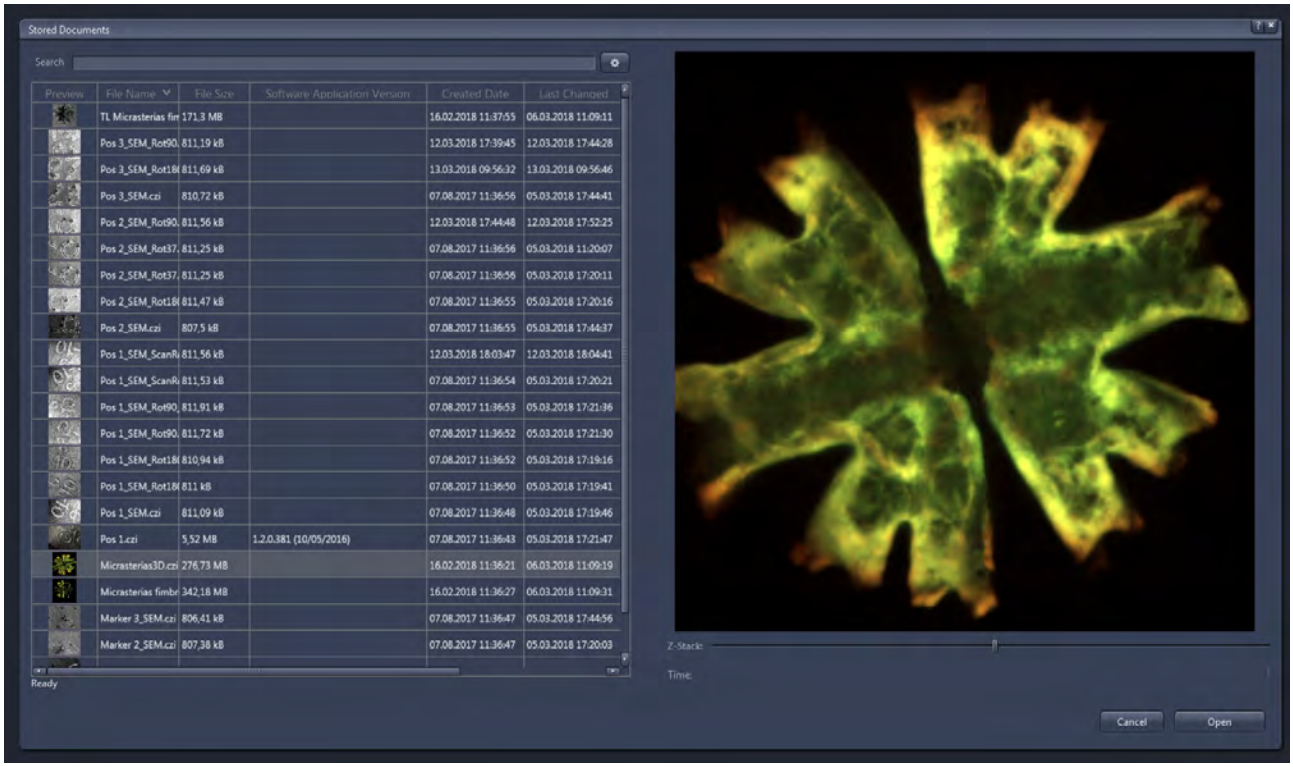


Parameter	Description
File name	Displays the Connect project file name. You can sort the file names alphabetically.
File Size	Displays the file size.
Created Date	Displays the creation timestamp.
Last Access	Displays at what date the image was last opened.
Comment	Displays a comment.
Cancel	Closes the dialog.
Open	Opens the Connect project. For more information, see *Opening or deleting a Connect project from the data storage* [▶ 566].

You can configure the table according to your needs. For more information, see *Configuring the Stored Documents table* [▶ 568].

You can filter data of stored documents. For more information, see *Filtering Connect projects and images in the data storage* [▶ 569]

19.5.6 Stored images in the data storage



Parameter	Description
Preview	Displays the preview of the image.
File Name	Displays the file name and the format of the image. You can sort the file names alphabetically.
File Size	Displays the file size.
Software application	Displays with which software application the image was acquired.
Software application version	Displays the version of the software application.
Created Data	Displays the creation day.
Last Change	Displays at what date the image was last changed.
Comment	Displays a comment.
Microscope Name	Displays the name of the microscope.

Parameter	Description
System Name	Displays the system name.
Objective Name	Displays the name of the objective.
Objective Magnification	Displays the objective magnification.
Reflector	Displays the reflector.
Channel Name	Displays the name of the channel.
Camera Name	Displays the name of the camera.
Scaling	Displays the scaling.
Imaging Light Source Name	Displays the name of the imaging light source.
Z-Slider	If the image contains a Z-stack, with the slider you can select a single Z-slice.
T-Slider	If a time series is contained in the image, with the T-slider you can select a certain point of time.

Double-click to open the image. For more information, see *Opening or deleting an image from the data storage* [▶ 568].

You can configure the table according to your needs. For more information, see *Configuring the Stored Documents table* [▶ 568].

You can filter data of stored documents. For more information, see *Filtering Connect projects and images in the data storage* [▶ 569]

20 Software Functions and Reference

20.1 Menus

20.1.1 File Menu

Menu item	Description	Short cut
New Document	Opens the New Documents selection dialog. You can create new images or new tables. For more information, see *New Document* [▶ 588].	*Ctrl + Shift +N*
New Image	Open an empty image container in the center screen area into which you can snap an image, for example.	*Ctrl+N*
Open...	Opens the **Open Document** dialog window. Here you can select the file you want to open.	*Ctrl+O*
Save	Saves the selected file.	*Ctrl+S*
Save As CZI	Saves the selected file under a new name. In case of an image only **.czi** file format can be used.	*Ctrl +Shift +S*
Save As with Options...	Saves the selected file under a new name. Advanced options can be selected: **File type**: czi, jpeg, jpg, png, tif, tiff, bmp, gif, wmp, wdp **Compression** (only for **czi** and **jpg/jpeg**): ■ **Original**: The image keeps the compression of the original image. ■ **Uncompressed**: The image is saved without compression. ■ **Compressed (JPEG XR)**: An uncompressed image will be compressed with the selected quality. A compressed image keeps the compression. ■ **Force Compression (JPEG XR)**: A compressed image will be decompressed and compressed with the selected quality.	

Menu item	Description	Short cut
	Zoom Level (only for pyramid images): Different zoom levels can be selected, depending on the image pyramid. **Set as default**: Sets the selected options as default saving options.	
Rename	Opens the **Rename** dialog window. Enter a new name for the file. Confirm the entry with **Yes**.	
Delete	Deletes the selected file.	
Export/Import	Opens the chosen parameter in the **Processing** tab | **Method** tool | **Export/ Import**. For more information, read chapter *Image Export* [▶ 215].	*Ctrl+6*
Send to ZEN Black	The **ZEN (blue edition)** installation includes a copy of **ZEN Black**. In some cases it might be necessary to transfer images from **ZEN (blue edition)** to **ZEN Black** to make use of special functions found only in **ZEN Black**.	
Close	Closes the selected file.	*Ctrl +F4*
Save All	Saves all open files.	
Open File Browser	Opens the file browser in the **Center Screen Area**.	*Ctrl+F*
Open Containing Folder	Opens the folder in which the selected file is located.	
Recent Files...	Opens the **Recent Files** dialog window. The **Recent Files** dialog displays the files you have used previously, separated according to file type.	*Ctrl+R*
Recently Opened Files	Opens a list containing the file paths of recently opened files.	
Print Preview	Opens the *Print Preview* [▶ 589] dialog for the selected file.	*Ctrl +F2*
Login	Opens the **Login** dialog window.	
Tiles	Opens a submenu with functions assocaited with the tiles tool. Here, parameters can be imported and exported.	

Menu item	Description	Short cut
	■ **Export Tiles Experiment** Opens a dialog to export the current Tiles experiment either to an XML or a CSV file. ■ **Import Tiles Experiment** Opens a dialog to import a Tiles experiment either from an XML or a CSV file. ■ **Import Stage Marks** Imports the existing positions from the **Stage** Tool (Marks) as single positions in the Tiles experiment. ■ **Import Preview Image** Imports an existing image document as a preview image into the **Navigation/Tiles** view. This function is also available with right mouse-click in the **Tiles - Advanced Setup** view. ■ **Save Preview Images** Saves existing preview/snap images into a separate image document. ■ **Extract Sample Carrier Template** Opens a dialog to extract the current sample carrier template from the Tiles experiment as a new template with the calibrated dimensions.	
Exit	Exits the software.	*Alt+F4*

20.1.1.1 New Document Dialog

Here you can create different types of new, empty documents (images, table, texts, Connect projects).

Select the desired document type and click on **OK**. The image, table or RTF file will be generated and opened in the current workspace.

Parameter	Description
Images	Creates a new, empty image file (*.czi).

Parameter		Description
Tables		Creates a new table (*.czt). The following elements are only visible if you have clicked on the **Tables** button:
-	Document name	Here you can enter the name of the new table-document.
-	Columns	Enter the number of columns that you want the new table to have in the input field.
-	Rows	Enter the number of rows that you want the new table to have in the input field.
-	Column Name	Here you can enter the title of the column.
-	Column type	Here you can select the desired data type of a column. The following types are available: ■ String ■ Integer ■ Real
-	Default Value	Here you can enter a default value that you want the column cell to contain.
Rich Text Format (RTF)		Creates a text file in the RTF format (*.rtf).
Connect Project		Creates a Connect project. For more information, see *Creating a Connect project* [▶ 559].

20.1.1.2 Print Preview Dialog

Parameter	Description
Printer	Here you can select the printer that you want to use.
Properties	Opens a dialog window containing the printer properties. Here you can configure advanced settings. This dialog window is dependent on the printer.
Format	Here you can select the page format e.g. A0 to A6, Letter or A4 Register.
Width	Displays the width of the page according to the chosen format.

Parameter	Description
Height	Displays the height of the page according to the chosen format.
All pages	**Activated:** Prints all pages of the report.
Selected Pages	**Activated:** Prints a certain number of pages of the report. In the input field to the right, enter the pages that you want to print. **Example:**The entry **1-3; 5** prints pages 1,2,3 and 5.
Pages per sheet	Here you can select how many pages you want to print on one sheet.
Number of copies	Here you can enter the number of copies that you want to print.
Collated	Only active if you have chosen to print several copies. **Activated:** Sorts the pages of each copy printed.
Auto fit	**Activated:** Adjusts the size of the report or image to the size of the page.
Scale pages	**Activated:** Adjusts the size of the report or image to the factor set in the input field to the right. Here you can set the desired enlargement/reduction factor for the report or image. A factor of 100% corresponds to the **Auto fit** option.
Print	Prints the report using the options set.
Down	Displays the next page.
Up	Displays the previous page.
Auto fit	Here you can select the zoom factor with which the page view is displayed in this dialog.

20.1.2 Edit Menu

Menu item	Description	Short cut
Undo	Undoes the last action.	*Ctrl+Z*
Redo	Redoes the last action.	*Ctrl+Y*
Cut	Cuts the selected graphic element out of the image.	*Ctrl+X* *Shift +Del*

Menu item	Description	Short cut
Copy	Copies the selected graphic element.	*Ctrl+C* *Ctrl +Ins*
Paste	Inserts the copied graphic element into the image.	*Ctrl+V* *Shift +Ins*
Delete	Deletes the selected element.	*Del*
Select All	Selects all graphic elements drawn into the image.	*Ctrl+A*
Display	Here you can manage image display settings. Functions include copy, paste, export or import of the display settings.	
ROI (Region of Interest)	Here you can draw a new rectangular selection region (ROI) into the image. It is subsequently possible to create a subset image from the selection.	*Ctrl+U*
Create Image from View	Creates an image from the current view.	

20.1.3 View Menu

Menu item	Description
Zoom	Here you can configure various zoom settings.
Player	Here you can navigate through a Z-stack or a time series image.
Text View	Displays the text name of a file in the **Document bar**.
Small Thumbnail View	Displays a small preview image and the name of a file in the **Document bar**.
Large Thumbnail View	Displays a large preview image and the name of a file in the **Document bar**.
1 Container	Displays one image container in the **Center Screen Area**.
2 Containers	Displays two image containers in the **Center Screen Area**.

Menu item	Description
3 Containers	Displays three image containers in the **Center Screen Area**.
Automatic Container Layout	Uses the predefined container layout.
Shared View Controls	General and specific view controls are shared for all containers and are active for the currently selected image container.
Separate View Controls	Each container has its own separate general and specific view controls that become active when the associated image container is selected.
Show All (Global)	Activates the **Show All** mode in every tool.
Show Macro Environment	Activates the *Macro Tool* [▶ 773] in the **Right Tool Area**. If you have licensed the **Macro Environment** module, the **Macro** menu appears in the **Menu bar**. The Macro Environment is deactivated by default.

20.1.4 Acquisition Menu

Menu item	Description	Short cut
Locate Snap	Acquires a single image with the active camera.	*F2*
Locate Live	Shows a live image from the active camera in the **Center Screen Area**. On the **Locate** tab the **Live** mode is activated.	*Shift+F2*
Acquisition Snap	Executes only if you have defined at least one channel on **Acquisition** tab in the **Channels** tool. Acquires and displays all the defined channels as an overlay.	
Acquisition Live	Executes only if you have defined a channel on **Acquisition** tab in the **Channels** tool. Uses the currently selected channel for a live image.	

Menu item	Description	Short cut
Set Exposure	Executes only if you have defined a channel on **Acquisition** tab in the **Channels** tool. Executes an exposure time calculation for the active camera.	
Set White Balance	Executes a white balance measurement.	*Alt+W*
Find Focus	Executes the Software Autofocus Running. On the **Acquisition** tab the **Find Focus** mode is activated.	
Start Experiment	Only active, if you have configured an experiment on **Acquisition** tab. Starts a defined experiment.	
Stop Experiment	Only active, if you have started an experiment on **Acquisition** tab. Stops a running experiment.	
Pause Experiment	Only active, if you have started an experiment on **Acquisition** tab. Pauses a running experiment.	
Continue Experiment	Only active, if you have paused an experiment on **Acquisition** tab. Continues the paused experiment.	
Dual Camera Calibration Wizard…	Only visible if a dual camera configuration is active. Starts **Dual Camera Calibration Wizard.**	
ApoTome Phase Calibration Wizard…	Only visible if a ApoTome configuration is active. Starts **ApoTome Phase Calibration Wizard.**	

Menu item	Description	Short cut
ApoTome Focus Calibration Wizard…	Only visible if a ApoTome configuration is active. Starts **ApoTome Focus Calibration Wizard.**	

20.1.5 Graphics Menu

Menu item	Description	Short cut
Select	Starts the selection mode.	*Alt+F1*
Draw Region of Interest	Allows you to draw in a region of interest (ROI).	*Crtl+U*
Text	Allows you to add a text field to the image.	
Scale bar	Allows you to add a scale bar to the image.	
Line	Allows you to add a line to the image.	
Arrow	Allows you to add an arrow to the image.	
Rectangle (aligned)	Allows you to add an aligned rectangle to the image.	*Alt+F2*
Circle (Diameter)	Allows you to add a circle to the image.	
Ellipse	Allows you to add a ellipse to the image.	
Contour (Spline)	Allows you to add a contour (spline) to the image.	
Profile	Adds an intensity profile along a drawn in arrow region.	
Rectangle Profile	Adds an intensity profile within the drawn in rectangle region.	
Scaled Profile	Adds an intensity profile with an scaling bar to the image.	
Grid	**Activated:** Adds a grid to the image.	
Frequent Annotations	Adds frequently used annotations to the image, e.g. Relative Time, Channel Name.	

Menu item	Description	Short cut
Distance	Adds distance annotations to the image, e.g. Length, Curve length.	
Multiple Distances	Allows you to add annotations for measuring multiple distances to the image.	
Region	Allows you to add region annotations to the image, e.g. Contour, Rectangle.	
Circle	Allows you to add circle annotations to the image.	
Angle	Allows you to add angle annotations to the image.	
Points	Allows you to add points of interest (POI) to the image such as events or markers.	
Format	Allows to open the dialog for formatting graphical elements. Also you will find options for resetting customized graphical elements.	
Burn-in Annotations...	Creates a new image with all annotations burned-in to the image.	
Show Bounding Box	Shows bounding boxes around graphics/annotations.	
Hide Bounding Box	Hides bounding boxes around graphics/ annotations.	
Bring to Front	Brings selected graphic/annotation to the front of the image.	
Send to Back	Sends selected graphic/annotation to the back of the image.	
Bring Forward	Brings selected graphic/annotation one layer forwards.	
Send Backwards	Sends selected graphic/annotation one layer backwards.	

20.1.6 Macro Menu

INFO

This menu is available only if you have licensed the **Macro Environment** module.

- Activate the Macro Environment controls in the **View** menu by klicking on **Show Macro Environment**.

Menu item	Description
Record a New macro	Starts recording a new macro.
Stop Recording	Stops recording the macro.
Macro-Editor…	Opens the *Macro Editor Dialog* [▶ 596].

20.1.6.1 Macro Editor Dialog

The macro editor represents the IDE (Integrated Developer Environment) to edit, execute, debug and manage macros. It is started via the menu **Macro** | **Macro Editor** or from the **Macro** tool in the **Right Tool Area**.

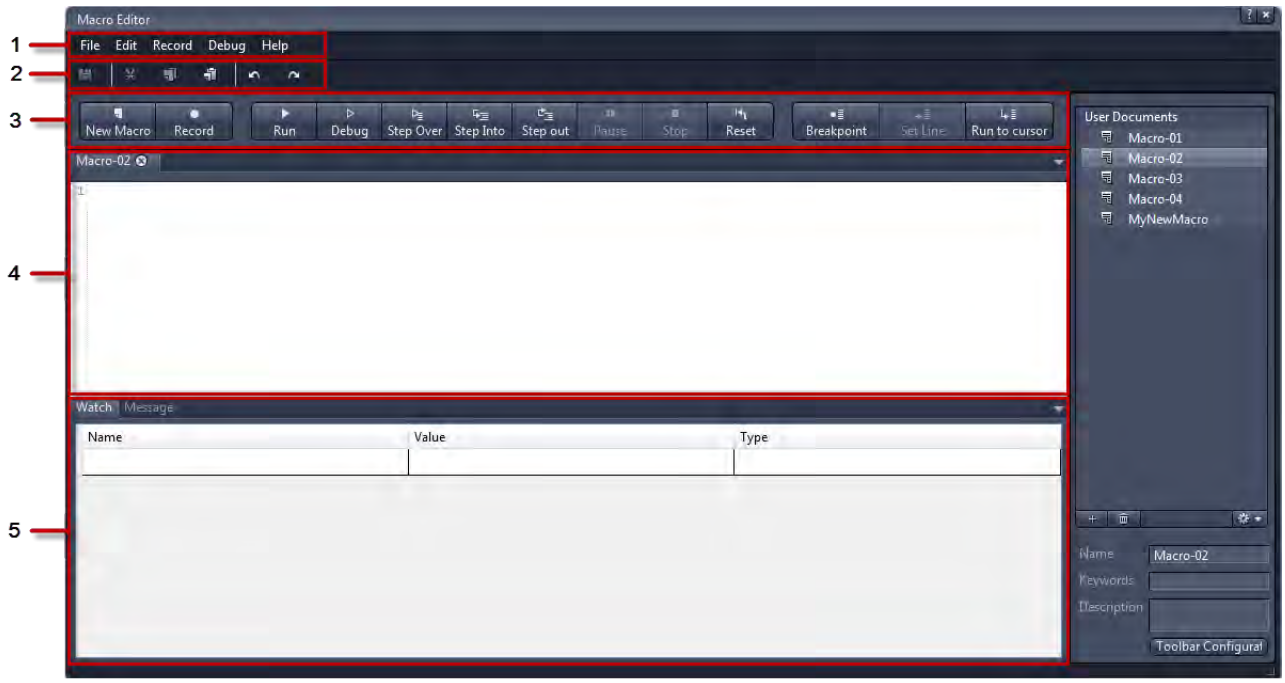


Fig. 20.1: Macro editor dialog

Parameter		Description
Menu bar (1)		For a detailed description of the menus, please read *Macro Editor Menus* [▶ 598].
Tool bar (2)		Via the icons you can quickly access the most important functions, like saving or editing macros.
Button bar (3)		Here you find the buttons for recording and controlling macros.
-	New Macro	Creates a new empty macro.
	Record	Starts macro recording.
	Run	Executes the active macro completely.
	Debug	Starts the debugger and executes the macro up to a breakpoint or error.
	Step Over	Starts the debugger stepwise, command by command, without stepping into function blocks.
	Step Into	Starts the debugger stepwise, command by command, and steps into function blocks.
	Step Out	Starts the debugger stepwise, command by command, and steps out of function blocks.
	Pause	Pauses macro recording.
	Stop	Stops the running macro at the active command.
	Reset	Resets all variables of the Python interpreter.
	Breakpoint	Sets/removes a breakpoint in the active line, to stop/ continue the macro in debug mode, in the active line.
	Set Line	Sets the pointer in the next active command line.
	Run to cursor	Sets the pointer to the current cursor position.
Code Window (4)		The central area of the Macro Editor shows the program code of the selected macro. Edit and write your macros in here. You can either use the **Record** button or type in the program code directly. Also a multi-document view is available, meaning that you can open several code windows at once.
Watch Window (5)		Observe variables of the macro program code here. Enter the variable directly in the column **Name**. You can also mark the variable in the macro and add it using **Add Watch** of the right mouse key context menu.

Parameter	Description
Message Window	Displays messages when using the print command in a macro.

20.1.6.1.1 Macro Editor Menus

File Menu

Menu item	Description	Short cut
New Macro	Opens the **New Macro** in the Macro programming area.	
Duplicate	Duplicates the selected macro to be saved under a new name.	
Save	Saves the selected macro.	*Ctrl+S*
Save As…	Saves the macro under a new name.	
Rename…	Opens the **Rename** dialog window. Enter a new name for the macro.	
Delete	Deletes the selected macro.	
Close	Closes the selected macro.	

Edit Menu

Menu item	Description	Short cut
Cut	Cuts the selected line out of the macro.	*Ctrl+X* *Shift +Del*
Copy	Copies the selected line in the macro.	*Ctrl+C* *Ctrl +Ins*
Paste	Inserts the copied line into the macro.	*Ctrl+V* *Shift +Ins*
Find	Finds the entered text.	*Ctrl+F*
Replace	Replaces the detected text with the new text..	*Ctrl+H*
Undo	Undoes the last action.	*Ctrl+Z*

Menu item	Description	Short cut
Redo	Redoes the last action.	*Ctrl+Y*

Record Menu

Menu item	Description
Record	Starts recording.
Stop Recording	Stops the recording of the active macro.

Debug Menu

Menu item	Description	Short cut
Start Debugging	Starts the debugger and executes the macro up to a breakpoint or error.	*F5*
Start Without Debugging	Executes the macro up to a breakpoint or error without debugging.	*Ctrl +F5*
Pause	Pauses debugging.	*Shift +F5*
***Continue (DEBUG)**	Continues debugging.	*Shift +F5*
Stop	Stops the running macro at the active command.	*Shift +F5*
Step Into	Starts the debugger stepwise, command by command, without stepping into function blocks.	*F11*
Step Over	Starts the debugger stepwise, command by command, and steps into function blocks.	*F10*
Step Out	Starts the debugger stepwise, command by command, and steps out of function blocks.	*Shift +F11*
Toggle Breakpoint	Sets/removes a breakpoint in the active line to stop/continue the macro in debug mode.	*F9*
Set Line To Execute	Sets the pointer in the next active command line.	*F8*
Reset	Resets all variables of the Python interpreter.	

Help Menu

Menu item	Description	Short cut
Contents...	Opens the **Online Help** dialog.	*Ctrl +F1*
Macro Object Model…	Opens the **Macro Object Model Online Help** dialog. This documentation includes descriptions of all objects available for the macro editor.	
Forum…	Opens the OAD forum in your web browser. Internet access required.	

20.1.7 Tools Menu

Menu item	Description
Axio Scan Calibration…	Only available for Axio Scan systems. Opens the **Axio Scan Calibration** wizard. By the wizard you can calibrate the following functions: ■ mapping of the preview camera to the scan camera, ■ parfocality of the objectives, ■ parcentricity of the objectives. ■ shading correction of the optical system (including fluorescence) and color calibration. It is not necessary to execute all steps, e.g. the shading correction for fluorescence and the color calibration can be skipped.
Diagnostics	Opens the **Diagnostics** dialog. There you receive detailed reports on your entire system state. *Ctrl+Shift+D*
Kitchen Timer…	Opens the **Kitchen Timer** dialog. There you can set a time period after which an alert is played.
Dosimeter…	Opens the **Dosimeter** dialog. There you can set multiple time points at which an alert is played.
Dye Editor…	Opens the *Dye Editor* [▶ 77] dialog.
Extension Manager…	Opens the **Extension Manager** dialog.

Menu item	Description
Modules Manager…	Opens the *Modules Manager* [▶ 601] dialog.
Users and Groups...	Opens the *Users and Group Management* [▶ 602] dialog.
Settings Editor...	Opens the **Settings Editor** dialog. Select from existing hardware settings or adopt the settings from the hardware being used into the software. You can also transfer settings from the software to the hardware that you are using.
System Maintenance and Calibration...	Opens the **System Maintenance and Calibration** dialog. Helps to keep your system in perfect working condition.
Calibration Manager…	Opens the **Calibration Manager** dialog.
Customize Application…	Opens the Customize Application dialog.
Scaling…	Opens the *Scaling* [▶ 604] dialog.
Sample Carrier/Holder Templates…	Opens the **Sample Carrier Templates** dialog.
Options…	Opens the *Options Dialog* [▶ 607].

20.1.7.1 Modules Manager Dialog

Here you can activate or deactivate the modules for which you currently own a license. Note that all the changes made here are implemented immediately.

Parameter	Description
Available Products	Here you can see the products available for your license. Click on the relevant button to select the product.
Included Modules	In this list you can activate/deactivate the modules that are included with your product. Klick on the checkbox to activate/deactivate a module.
Optional Modules	In this list you can activate/deactivate the modules that you have licensed as an option for your product. Klick on the checkbox to activate/deactivate a module.

Parameter	Description
Optional Hardware	In this list you see the hardware that you have configured.
Select All	Activates all available modules.
Unselect All	Deactivates all available modules.
Save Information...	Saves the current selection of modules within a **.txt** file.

20.1.7.2 User and Group Management Dialog

Here you can create new users and groups and manage their access rights. Activate the user and group management by activating the checkbox **Enable User Management**. For more details read also the chapter *Managing Users and Groups* [▶ 279].

20.1.7.3 Customize Application Dialog

Here you can customize the application layout, e.g. adopt the toolbar or shortcuts. To learn more about how to customize the application, read the chapter *Customizing Toolbar* [▶ 287].

Toolbar Tab

Here you can add menu items to the **Toolbar** as buttons for a quick access.

Parameter	Description
Available Toolbar items	In this list you see all menu items that you can add to the **Toolbar**.
Add 	Adds a selected item to the tool bar. It then appears in the **Selected Toolbar Items** list.
Selected Toolbar Items	In this list you see all the added menu items. Select the items here in order to sort them.
Delete 	Deletes a selected item from the **Selected Toolbar Items** list.
Up 	Moves a selected item one position up in the **Selected Toolbar Items** list.
Down 	Moves a selected item one position down in the **Selected Toolbar Items** list.

Parameter	Description
Separator	Inserts a vertical separator into the **Toolbar** after the currently selected item of the **Selected Toolbar Items** list.
Close	Closes the **Customize Application** dialog and saves the adjustments.

Shortcuts Tab

Parameter	Description
Available Commands	In this list you see all commands from the **Menubar** and edited Macros. Klick on the arrow on the left of the entry to show available commands.
Shortcut for the selected item:	If you have selected a command from the **Available Commands** list, the related shortcut is displayed here. If the field is empty, no shortcut yet exists for the selected command.
Remove	Deletes a shortcut of the selected command. Default shortcuts, e.g. *Strg+S* can not be removed.
Type a shortcut	Here you can type in a shortcut by clicking on the desired keys of your keyboard. If a shortcut is already used for another command, it is displayed in the **Shortcut is used by:** display field.
Add	Adds a shortcut to a chosen command.
Shortcut is used by:	If you typed in a shortcut which is already used, the related command is displayed here.
All commands with shortcuts:	In this list you see all the shortcuts and their related commands.
Close	Closes the **Customize Application** dialog and saves the adjustments.

Soft Keys Tab

Parameter	Description
Available Itemslist	In this list you see all items from the **Menubar**, Hardware Settings and edited Macros. Klick on the arrow on the left of the entry to show available items.
Soft Keys	The items from the **Available Items** list can be assigned to the buttons **Function0**-**Function9** via drag & drop.

Parameter	Description
Reset All	Resets all the adjustments.
Close	Closes the **Customize Application** dialog and saves the adjustments.

20.1.7.4 Scaling Dialog

Fig. 20.2: Scaling Dialog

Here you can specify how your images are scaled.

Option	Description
Active Scaling:	Shows the scaling that is set currently.
Units	Select the desired unit for the current scaling here.
Select Automatically	**Activated:** Calculates the scaling automatically from the microscope and camera configuration.

Available Scaling section

Option	Description
Scaling	Select scalings which are stored on your system e.g. Pixel, Theoretic. The scaling details will be displayed in the fields below the list. If a display field is empty, it will not be used in the calculation of the scaling.
Options 	By clicking on the you can perform the following actions :
- Activate Scaling	Activates the selected scaling. The scaling will be applied to all images that are acquired from this time point onward.
- Assign Scaling to Image	Assigns the selected scaling to the current image.
- Import	Opens the **Import Scaling** dialog window. Here you can select a scaling file (.czsc) that you want to import.
- Export	Opens the **Export Scaling** dialog window to export the selected scaling. Select the folder in which you want the exported scaling file to be saved and specify a file name (.czsc).
- Delete	Deletes the selected scaling.

Create new scaling section

Parameter	Description
Interactive Calibration...	Opens the **Open file for interactive scaling** dialog, if there is no image yet selected. Starts the *Scaling Wizard* [▶ 605] in the **Center Screen Area** for the currently selected image.

20.1.7.4.1 Scaling Wizard

Here you can create a new scaling. To do this, draw a reference line with a predefined length in the current image. An image of a calibration slide is best suited for this purpose.

Fig. 20.3: Scaling Wizard

Parameter	Description
Tool Bar	Here you can draw in two types of reference line. Therefor klick on one of the following buttons.
- Select 	If selected, the courser is in selection mode. You can move the dialog window or select a reference line to edit it.
- Draw Reference Line	This tool is selected by default. With that tool you draw in a line along a distance with a known length (e.g. using an object micrometer or calibration slide). Then you can adjust the length and units in the input field and dropdown list.
- Draw Parallel Reference Line	With that tool you draw in two parallel lines along a distance with a known length. The two parallel lines correct errors in the parallel axis resulting from the drawing of the lines. A third, corrected line is drawn in automatically from which the scaling is determined.
Automatic Line Detection	**Activated:** Automatically detects individual lines of the scale bar in the image close to the interactively defined distance. Using this method the centers of the lines are determined exactly, increasing the precision of the scaling.
Length input field	Here you must enter the length of the line you have drawn in.
Unit selection	Here you must select the corresponding unit of your drawn in line.
Scaling	Shows the calculated pixel scaling according to the drawn in line.

Parameter	Description
Name	Here you can enter the name for the scaling that will be created.
Save Scaling	Saves the scaling that has been created under the specified name. The scaling can be selected in the **Scaling** dialog under **Available Scalings**.

20.1.7.5 Options Dialog

Here you can configure the settings for general software options.

20.1.7.5.1 General Tab

Parameter	Description
Select Automatically	**Activated:** Automatically selects the user language of the operating system as the user language for the software.
Language Selection	Select the language from the dropdown list in which the software will be run next time it is started.

20.1.7.5.2 Startup/Shutdown Tab

Parameter	Description
Show Splash Screen	**Activated:** Displays the splash screen when the software starts.
Show Application Selection	**Activated:** Shows the application selection dialog when the software starts.
Reload Last Used Documents	**Activated:** Reloads all image documents, that were open when you last exited the system when the software starts.
Experiment	Selects the desired bahaviour on system start up with regard to the experiment management on the **Acquisiton** tab.
- **Load default experiment**	Loads an experiment with the set default values.
- **Load empty experiment**	Loads an empty experiment.

Parameter	Description
- **Load last used experiment**	Loads the last used experiment.
Request Stage/ Focus Calibration on Startup	**Activated:** Shows a message which asks you to perform stage/focus calibration.

20.1.7.5.3 Naming Tab

Here you can specify how images are (automatically) named and indexed. Changes will be stored after the session is ended.

Parameter	Description
Category	Select the category of the file you want to be named automatically, e.g. an Image, a Report or an Experiment.
Prefix	Here you can enter a prefix for the file name, e.g. IMG.
Digits	Select how many digits you want the counter to have.
Format	Here you can specify what information you want to include to the file name. From the **Format-IDs** list below you can add the desired attribute to the name. Therefore simply double click on the desired format in the list.
Initial Counter Value	Here you can enter the desired first value of the counter.
Suffix	Here you can enter a prefix for the file name.
Preview	Displays the preview of the naming format that will be allocated next for the selected category.
Save/Restore Counter Value	**Activated:** Saves the counter values for the individual categories. If the software is restarted, the values are restored.
Format IDs	Shows all the attributes which can be used for the **Format** field.

20.1.7.5.4 Saving Tab

Parameter	Description
Auto Save after Snap	**Activated:** Automatically saves images that are acquired on the **Locate** tab using the **Snap** button.
Don't Open a Document Window	Only active if the **Auto Save after Snap** checkbox is activated. **Activated:** Closes the automatically saved images immediately after acquisition.
File Type	Select the image format from the dropdown list that will be used when an image is saved automatically.
Auto Save Path	Here you can specify the folder into which the images are saved automatically.
Show "Discard All" Button in Dialog to Save Modified Documents	If activated, the **Discard All** button is displayed in the **Save Documents** dialog. **NOTICE** **If you click on this button all un-saved images will be deleted.**

20.1.7.5.5 Documents Tab

Default Settings for New Images section

Parameter	Description
Show Rulers	**Activated:** Displays rulers at the top and left-hand edge of the image – the units used are according to the scaling settings.
Auto Fit	**Activated:** Automatically adjusts the zoom factor of the image so that the entire image is visible and the view area is filled.
Use Interpolation for Image Display	**Activated:** Displays pixels in interpolated form.
Set Logarithmic Scale in Histogram	**Activated:** On the **Display tab** the frequency distribution (y-axis) of the histogram is plotted using a logarithmic scale.
Show Viewport Scalebar in 2D View	**Activated:** Shows a scale bar within a small window in 2D view.
Show Viewport Scalebar in Live Window	**Activated:** Shows a scale bar within a small window in the Live window.

Parameter	Description
Show Navigator in 2D View	**Activated:** Shows the Navigator window in the image area.
Use Pan Mode in 2D View for Tile Images	**Activated:** Pan mode will autoatically activated for tiled images opened in 2D view.

Display section

Parameter	Description
Enable Tree View	Additionally shows the **Tree** view in the Center Screen Area.
Show Time series/movie images without Bounding X/Y Area	**Activated:** Time series or movie images acquired when the stage coordinate is adjusted are shown without the bounding X/Y area (black boarder).

3D View section

Parameter	Description
Run Performance Assessment	Runs a test routine which evaluates the performance of the graphic card installed on the workstation. The result is an adjustment of the precision and accuracy parameters to allow fluid interaction with the rendered volume in 3D view.
Graphics Hardware Class	Selects the performance class of your graphics hardware. A higher performance class allows you to see your data in more detail but may lead to crasheson unsuitable hardware. The following classes are available: ■ **Very Low** ■ **Low** ■ **Normal** ■ **High** ■ **Very High**
Interactive Precision	Controls the interactive precision in %.

Parameter	Description
Interactive Accuracy	Controls the interactive accuracy in %.
Show Logo	**Activated**: Logo is displayed in the lower right corner of the 3D view.
Export Logo	**Activated**: Exports the logo in render series and snapshots.

20.1.7.5.6 Acquisition Tab

General section

Parameter	Description
Show a Request to Move Manual or Coded Hardware Components	**Activated:** Shows a dialog which asks you to move manual components. You have to confirm the dialog and move the component by hand.
Show a Confirmation Dialog for Channel/ Track Deletion	**Activated:** Shows a dialog which asks you to confirm to delete a channel or a track.
Hide Empty Objective Positions	**Activated:**Hides any empty position of the objective changer inside the user interface.
Lock Device Controls in Right Tool Area During Running Experiments	**Activated:**Prevents controls in the right tool area from be undocked during an experiment.
Show Stage Speed and Acceleration Options	**Activated:**Shows the options to adjust the stage travel speed and acceleration are shown in the stage tool in the right tool area.

Camera/Live section

Parameter	Description
Stop Live after Snap	**Activated:** Automatically closes the **Live** mode after an acquisition via the **Snap** button.
Enable Stage/Focus Control in Live/ Continuous View	**Activated:** Enables to navigate the stage and focus in **Live** and **Continuous** view.

Parameter	Description
	Configure the travel speed of the focus by adjusting the values in the corresponding fields from **Very Slow** = 0,005 to **Very Fast** = 50,0. Reset your adjustments by clicking on the **Default** button.
Automatically Add Scalebar Annotation at Snap	**Activated:** Automatically adds a scale bar to the image, if it was acquired via the **Snap** button.
Show Camera Expert Options	**Activated:** Shows advanced (expert) camera options on **Locate** tab within the **Camera** tool.
Use Centered Camera ROI only	**Activated:** Positions a camera ROI at the center of the camera chip regardless of its size. Centered Camera ROI = center of camera detector
Show Crop button	**Activated:** Displays the **Crop** button on **Locate** tab within the **Camera** tool.

Acquisition Tab section

Parameter	Description
Acquisition Tab without channel support	**Activated:** Enables the use and set-up of experiments without any channel support in the **Acquisition** tab.
Prevent Execution of After Channel Setting while Live Mode is Active	**Activated:** Prevents execution of after channel setting automatism while **Live** mode is active.
Automatically start Live Mode when Exposure Measurement was Started	**Activated:** Starts the**Live** mode when the **Set Exposure** button has been pressed such that the live image begins immediately after the **Set Exposure** measurement is complete. **Deactivated:** Takes a **Snap** subsequent to **Set Exposure**.
Switch to next Enabled Acquisition Block in Experiment Designer	**Activated:** Automatically switches to the next enabled acquisition block in the Experiment Designer during a running experiment.
Enabled Imaging Setup	Shows the **Imaging Setup** tool on **Acquisition** tab.

Parameter	Description
Enabled Advanced Imaging Setup	Only active, if the **Enable Imaging Setup** checkbox is activated. **Activated:** Shows the **Standard/Advanced** option on the **Acquisition** tab on top of the **Imaging Setup** tool. As the advanced options should be touched by experts only, this option is deactivated per default.

Z-Stack section

Parameter	Description
Adjust Auto-Z-Stack Focus Match on First Slice	Determines the degree of match between the image focus of the first image and that determined as the true focus (center plane of the resulting Z-stack).
Adjust Auto-Z-Stack Focus Match on Last Slice	Determines the degree of match between the image focus of the last image and that determined as the true focus (center plane of the resulting Z-stack).
Delay Time After Focus Move	Specifies a delay time after each focus movement during Z-Stack experiments in ms.

Tiles & Positions section

Parameter	Description
Automatically Start Live Mode in the Advanced Setup View	**Activated:** Automatically starts the **Live** mode in the **Center Screen Area** if you klick in the **Acquisition** tab in the **Tiles** tool on the **Advanced Setup** button. Uncheck this option to prevent unnecessary specimen bleaching. The default is not activated.
Show Information Title in the Advanced Setup View	**Activated:** Displays a bar above the Advanced Setup view containing additional information, if you klick in the **Acquisition** tab in the **Tiles** tool on the **Advanced Setup** button.
Show Snap Animation	**Activated:** Shows the snap animated when snapping a new image in Advanced Setup.
Automatic Snap by Clicking the Live Navigator Buttons	**Activated:** Takes a snap in the **Tiles-Advanced Setup** tab every time the live navigator tool is moved with its navigation arrows.

Parameter	Description
Enable Stage Moving with Live Navigator Handle	In the Live navigator tool the current stage position including the live image is shown as a frame outlined in blue. To move the frame, double-click on the position to which you want to move it. The frame can also be used to control acquisition. **Activated:** Acquires an image if you click on one of the frame's blue arrow icons. The Live Navigator tool moves one frame width in the relevant direction. You can create tile images of your sample easily in this way.
Show Label on Sample Carrier Container	**Activated:** Shows a label on every container / well of a selected sample carrier.
Show Tool Tip on Sample Carrier Container	**Activated:** Shows a tool tip with the name of the container / well when the mouse is over it in the **Carrier** tab.
Show Stage and Focus Backlash Correction Setting in the Options	**Activated:**In the Tiles option, the setting to switch the backlash correction on or off is shown.
Delimiter for CSV Export/ Import	Specifies the delimiter for a CSV export or import. Select **Comma** (default), **Semicolon** or **Tab**.
Ask Whether Support Points/Positions Should be Overwritten	When the support points and/or positions are determined by a software autofocus run the existing points can be overwritten with the new **Z** values. **Activated:** Shows a message box asking if the points should be overwritten if there is a autofocus **Z** value.
Enable Removing of Focus Surface Outlier	**Activated:** Ignores support points that are significantly outside the interpolated focus surface. You have the following setting options available:
- Maximum Interpolation Degree for Outlier Detection	This value can be 0 or 1. If 1 then a linear fit is used to detect the outlier support points. This is the default. If 0 a simple average value is used to detect outliers.

Parameter	Description
- Threshold in Terms of the Standard Deviation (Sigma)	This parameter defines a threshold value to determine which of the support points are outliers from the fitting process. This is defined by the standard deviation (sigma value) set in the spin box. Support points not meeting this criteria are subsequently ignored when the focus surface is determined.
Activate Stitching During Acquisition for New Experiments	**Activated:** Activates stitching during acquisition as default for all new experiments. This value is overwritten by the corresponding option in the **Tiles** setup for a new experiment.
Use Local Focus Surface for Preview Scans	**Activated:** Uses local focus surface values (z-values of positions, tile regions and if defined interpolated focal surfaces defined by support points) for the acquisition of preview scan images. Note that on activation of the Tiles dimension the appropriate strategy **Use Focus Surface Defined by Tiles Setup** is pre-selected.
Binning Compensation of Exposure Time in Preview Scans	Defines the power to which the binning ratio is modified to automatically determine the exposure time value used for a preview scan were the binning setting between the experiment and preview scan differs. The default value is 2.0 i.e. quadratic. Thus, for example the exposure time would be reduced by a factor of four if the experiment binning is 1x1 and the preview scan binning is 2x2. The value can be varied between 1.0 and 2.0 in steps of 0.1.
Live Image in Sample Carrier Calibration Wizard (relevant only for systems with camera)	
- Use Imaging Device from Selected Channel with "Acquisition" Settings	**Activated:** Default setting for the live image that allows navigation and focus interaction during the carrier calibration wizard.
- Use Active Camera with "Locate" Settings	This option is only relevant for systems with a wide field (camera based) detector. **Activated:** Allows you to alternatively apply locate camera settings for use in the carrier

Parameter	Description
	calibration wizard (live image). By default the experiment settings for the currently selected channel/track will be used.

Panorama section

Parameter	Description
Automatically Start Live Mode in the Panorama View	**Activated:** Specifies that the live mode will start running automatically when you begin a panorama experiment.
Show Information Title in the Panorama View	**Activated:** Displays an additional information bar above the panorama view.
Show Acquisition Animation	**Activated:** Displays an animation when an image is acquired.
Automatically move Stage/Live after an Acquisition	**Activated:** Automatically moves the stage half a camera frame diagonally after acquisition of a snap image. Thus, the snap image can be inspected.
Enable Transparency Effect on Selected Tile Image	**Activated:** Displays the selected tile image with a transparency effect that enables you to see it in relation to the tiles underneath (lower layer = earlier acquisition) and those above (upper layer = more recent acquisition) at the same time. You can also adjust the degree overlap of the panorama grid. The default value is 20%, changes require a re-start to become affective. Note that this and the transprancy effect parameters are only relevant for manual stages.
Grid Overlap	Specifies the degree of overlap for the panorama grid in %. A software restart is requried.

Focus Strategy section

Parameter	Description
Show a Dialog to Prepare the Definite Focus Initialization	**Activated:** Reminds you to make appropriate adjustments to the focus prior for initialization at the start of experiments using Definite Focus.

Parameter	Description
Use Last Determined Z Position as Fallback for Focus Actions	**Activated:**Specifies whether a dialog to prepare the Definite Focus Initialization is shown when an experiment started.
Enable Definite Focus Stabilization on a Suitable Fallback Position	**Activated:**Specifies whether the last successfully determined z position is used if the primary focus action (Definite Focus or Autofocus) fails.
Enable Focus Strategy "Absolute Fixed Z Position"	**Activated:**Adds an addtional focus strategy to the **Focus Strategy tool**. This allows you to image positions at Z-values specified in the **Tiles** tool. Use this strategy if no **Tiles** module is available.
Show Definite Focus Setting "Resolution and Speed"	**Activated:**An additional section is displayed in the definite focus strategy. This allows selection of three definite focus modes for greater speed or accuracy of the stabilization.

Experiment Feedback section

Parameter	Description
Experiment Feedback	Sets the time until a deadlock of the synchronized script is assumed. Here you can adjust the time until ZEN assumes that a deadlock has occured after a synchronized script has been excuted. If this value is exceeded the function is aborted.

Dynamics section

Parameter	Description
Show Information Title in the Dynamics Setup View	**Activated:** Displays the information title at the top of the dynamics setup panel.

LSM section

Parameter	Description
Scanner Online Correction	**Activated:** Enables the scanner online correction. It ensures an optimal image quality at scan speeds > 13.

Parameter	Description
Airyscan Processing Baseline Shift	**Activated:** Adds an offset of 10.000 to the processed Airyscan images. This allows to display details in the processed Airyscan image that have negative intensity values and are therefore normally cut from the histogram.
Z-Piezo	**Activated:** Uses the Z-Piezo drive (if available) for the acquisition of Z-Stacks. Stacks with a range that is bigger than the specified working range of the piezo drive are automatically carried out using the objective focus drive.
Z-Piezo Range	Here you can select the range of the Z-Piezo drive. Note that the precision in the high range is lower compared to the default range.

20.1.7.5.7 User Tab

Here you can enter user and company information. These are written into the image metadata during acquisition. They are also used in reports.

Parameter	Description
User Information	Type in contact information of the software user here.
Company Information	Type in contact information of the company/institute/ facility here.
Logo	Upload a company logo to the company profile here. Therefore click on the  button. The logo is also used e.g. in reports.

20.1.7.5.8 Data Tables Tab

Data Table Import Options section

Parameter	Description
Start Import in Row No.	Defines the starting row of the data table into which the data will be imported.

Parameter	Description
Automatic CSV format detection	**Activated:** Automatically tries to detect the format of the data table when importing the table to the software.
Use column, decimal and list separator from windows regions settings	**Activated:** Uses the settings which are configured in the Windows regions settings when importing a table to the software.
Column Separators	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Configures the import options according to the format of your data table you want to import, e.g. specify the type of column or decimal separator.
Decimal Separator	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Select here, which decimal separator should be used.
Thousands Separator	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Select here, which thousands separator should be used.

Data Table section

Parameter	Description
Number of Decimal Places	Set the maximum number of decimal places for the numbers imported into the data table here.

20.1.7.5.9 Macro Editor Tab

Macro Configuration section

Parameter	Function
Show Inherited Members in Pop-up	**Activated:** Shows the inherited members of the ZEN class in a pop-up window.
Show line Numbers	

TCP Macro section

Parameter	Description
TCP Macro Section	**Activated:** Enables to enter the **TCP Port Number**.
Allow IPv4 Nat Traversal	Attention! For experts only. Do only activate this option if you know what you are doing.

Macro Recorder

Parameter	Description
Overwrite Interactive Recording Flag	**Activated:** Overwrites the parameter for interactive execution of a function during recording with the macro recorder.

20.1.7.5.10 Axeda Settings Tab

This option is visible only if you are using the **ZEN slidescan** application.

Here you can configure the settings for using Axeda remote service. This service is used for tracking statistical data of the system use and submitting the data to the service personnel. During installation of Axeda software, all of these settings can be entered as well. To change the settings you can use this dialog or open the Axeda software again. We recommend to change these settings only if you know what you are doing.

Parameter	Description
Use Axeda Agent	**Activated:** Axeda agent tracks system data. To switch of the service, simply deactivate the checkbox.

Parameter	Description
Use Proxy Server	**Activated:** Enter/edit data for Proxy Server settings like HTTP or SOCKS settings (see below).
HTTP or SOCKS	Only active, if the **Use Proxy Server** checkbox is activated. Here you can enter/edit HTTP or SOCKS settings.
Use Authentication	Only active, if the **Use Proxy Server** checkbox is activated. **Activated:** A user name and a password can be entered for authentication. You can enter/edit the user name and password in the corresponding input fields.
Use Proxy Auto Configuration	The **Proxy Server** settings will be deactivated, if the Auto Configuration is used. **Activated:** All settings will be configured automatically according to the information in the PAC file.

20.1.7.5.11 Correlative Workspace

Stage Size section

Parameter	Description
Stage Size in mm	Positions the images initially in a better way in the correlative workspace. For example, for 130x100mm stage an image at stage position 65x50mm will be placed in the center of the correlative workspace.

20.1.8 Window Menu

Menu item	Description	Shortcut
Full Screen	Sets the **Full Screen** mode to maximize the image view area size.	*F11*
Next Window	Displays the next open image in the **Center Screen Area** (direction of movement is to the right).	*F6*
Previous Window	Displays the previous open image in the **Center Screen Area** (direction of movement is to the left).	*Ctrl+F6*

Menu item	Description	Shortcut
Close	Closes the currently selected image.	*Ctrl+F4*
Close All	Closes all the open images.	*Ctrl+Alt+W*

20.1.9 Help Menu

Menu item	Description	Shortcut
Contents…	Opens the online help contents page.	*Ctrl+F1*
Index…	Opens the online help index page.	*Ctrl+F2*
Readme	Opens the file path to the Readme PDF-file.	
Create Service Report...	Opens the dialog to create the service report. For more details, read chapter *Creating a Service Report* [▶ 1087].	
About ZEN…	Shows notices of the producer.	

20.2 Main Tabs

20.2.1 Locate Tab

Depending on the system configuration and the licensed modules this tab can have a different appearance. In general you can use the locate tab for finding or "locating" interesting areas on your sample.

For mixed systems (e.g. LSM 800 including a microscope camera) the section **System Mode** is available additionally.

In the **Eyepiece** mode (for confocal systems) this tab contains only functions for controlling the light path and viewing the sample via the eyepiece, see *Microscope Control Tool* [▶ 641]. In the **Camera** mode this tab contains more control elements and tools, see Tools on Locate tab

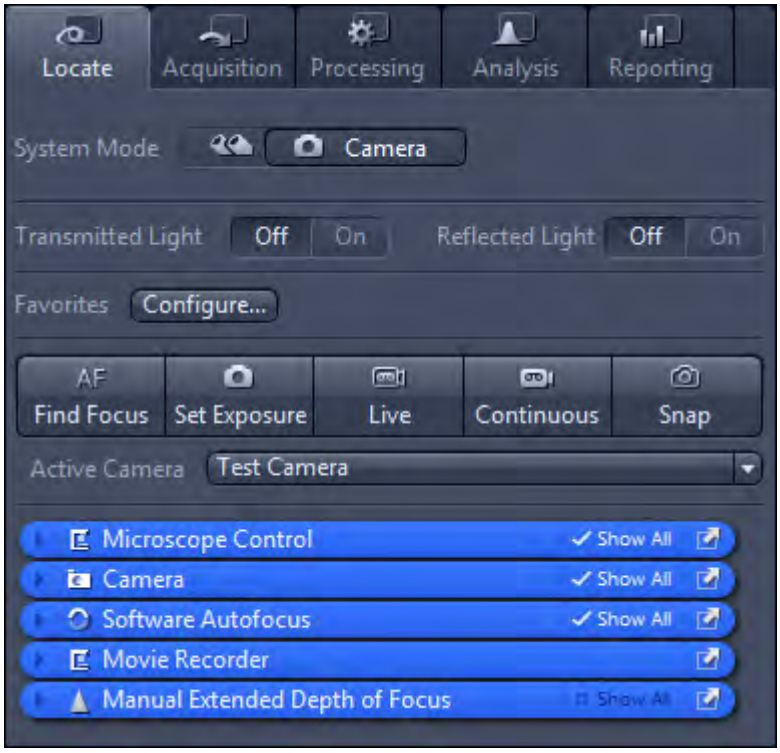


Fig. 20.4: Locate tab (Camera mode)

System Mode section

This section is only visible if a camera is installed with the system.

Here you can switch the system mode between **Eyepiece** and **Camera** mode. To switch between the 2 modes simply click on the corresponding button. In the table below you find a description of the two modes:

Mode	Description
Eyepiece mode	If you switch to Eyepiece mode the system adjusts the light path automatically to the eyepiece. The following list shows the changes in the ocular mode in detail: ■ All **Action** buttons are hidden ■ Only the **Microscope Control** tool is visible ■ Within the **Microscope Control** tool only the light path leading to the eyepiece is displayed. ■ All possible light paths to cameras are hidden.
Camera mode	■ All Widefield relevant components are displayed as usual. ■ The LSM Scan Head icon is not displayed in the light path.

Transmitted Light/Reflected Light section

Only visible if you have configured a motorized TL/RL shutter in MTB (MicroToolBox).

Parameter	Description
Off	Closes the shutter of the transmitted/reflected light source on a motorized microscope.
On	Opens the shutter of the transmitted/reflected light source on a motorized microscope.

Favorites section

Parameter	Description
Configure...	Here you can configure further buttons with your favorite hardware setting functions. Click on the button to open the Configuration dialog.

Action buttons section

With these buttons you control the microscope and the camera and acquire your images.

Fig. 20.5: Action buttons

Parameter	Function
Find Focus	Only visible if you have configured a motorized focus in the **MTB** (MicroToolBox). Starts an autofocus search using the current settings from the **Software Autofocus** tool.
Set Exposure	Starts an automatic exposure time measurement with the settings defined in the **Light Path** and **Camera** tool.
Live	Opens **Live View** and shows the live image from the active camera.

Parameter	Function
 **Continuous**	Starts a series of **Snaps** using the settings defined in the **Light Path** and **Camera** tool. In contrast to a live image, the exact same camera setting that has been set in the **Camera** tool is used. The result at the end of this mode is a single, acquired image that can be saved.
 **Snap** button	Acquires a single image.
 **Stop**	Only active if one of the acquisition buttons has been clicked. Stops the function of the relevant acquisition button.

Active Camera section

Parameter	Description
Link Cameras	Only active if you have connected two structurally identical cameras to your system. **Activated:** Acquires images using two cameras in parallel. This is often the case with 2-channel images for ratio measurements or FRET measurements.
Active Camera	Shows the active camera. If you have several cameras connected, you can select the detector to use here.

Tools section

Depending on which modules you have purchased you see different tools available in this section, see Tools on Locate tab.

20.2.1.1 Configure your hardware setting favorites Dialog

Here you configure up to 20 new buttons to get quick access to your preferred camera and hardware settings.

i INFO

To create and edit settings you need the settings editor. In **ZEN 2.3** and higher select **Tools | Settings Editor**.

Parameter		Description
Favorite Settings		If you have not yet defined any buttons, you will see an empty list here. To create a new button, click on the **Add**  button. Your Favorites are displayed as buttons on the **Locate** tab in the **Favorites** section. You can configure your favorite setting in the input fields:
-	Name	Here you can enter a name for the button.
-	Hardware Setting Ref.	Shows the selected hardware settings.
-	Camera Setting Ref.	Shows the selected camera settings.
-	Color	Here you can select a color for the related button. Click on the color dropdown list to choose a color.
-	Use Color also for Button Text coloring	**Activated:** Uses the selected color as the button text color.
Available hardware settings on disc		Here you see a list of all hardware settings that are saved on your hard drive. Select the hardware setting that you want to use with the configured button. To add a hardware setting, simply drag & drop them to the desired button configuration.
Available camera settings on disk		Here you see a list of all camera settings that are saved on your hard drive. Select the camera setting that you want to use with the configured button. To add a camera setting, simply drag & drop them to the desired button configuration.

20.2.2 Acquisition Tab

Here you configure and control your acquisition experiments.

i INFO

The content of the tab changes depending on the configuration of your imaging system and the options that you activate or deactivate.
Settings that you configure in the top part of the tab have an effect on settings in the bottom part of the tab. Settings that you configure in the **Acquisition Parameter** tool group, e.g. in the **Channels** tool also apply to the acquisition of all images that you configure in the **Multidimensional Acquisition** tool group.

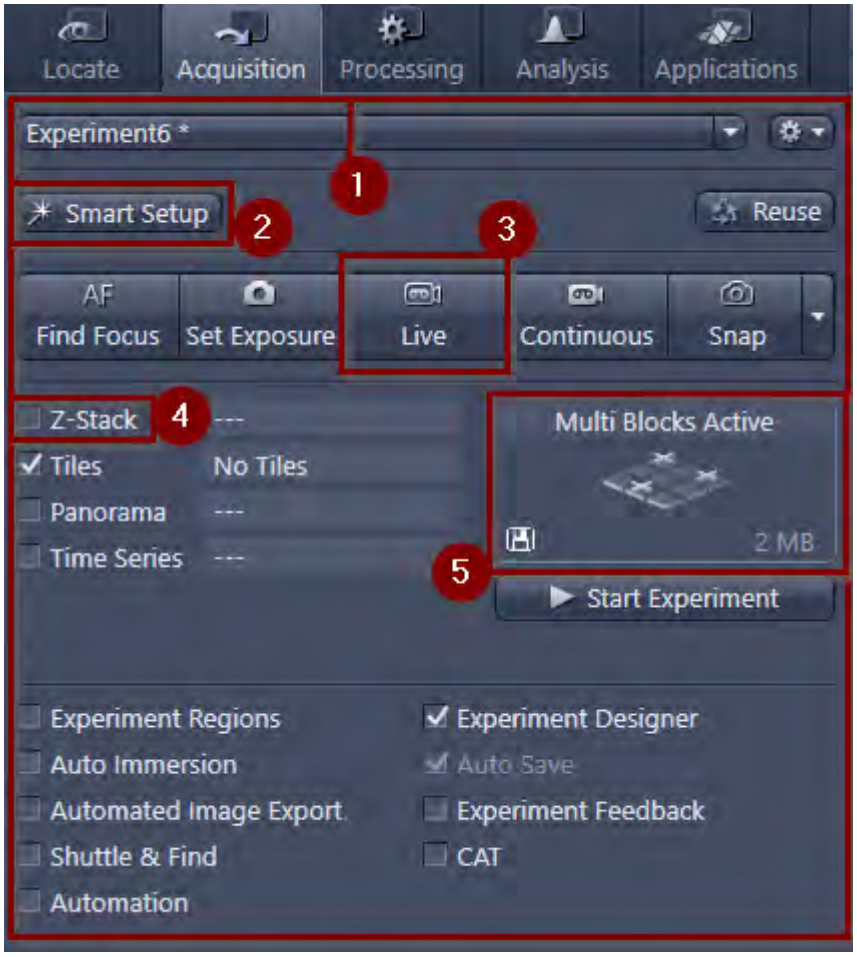


Fig. 20.6: Acquisition tab

No.	Parameter	Description
1	**Experiment Manager**	The area above the blue tools where you can load and save your experiments, control acquisition and decide which tools will appear in the certain tool groups.
	- Experiment Selection	From the dropdown list you can select saved experiments. If you make changes to an experiment, the name of the experiment is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") experiment, you will be asked whether you want to save the changes.
	- Options 	Opens the *Experiment Options* [▶ 629] shortcut menu.
2	**Smart Setup**	Opens the **Smart Setup** dialog, see Smart Setup

No.	Parameter	Description
3	**Action buttons**	With these buttons you control microscope and camera and acquire your images. The Acquisition buttons on the **Acquisition** tab differ from the Acquisition buttons on the **Locate** tab. The buttons on the Locate tab relate to an individual image. The buttons on the Acquisition tab relate to a multidimensional image with at least one channel.
	-  Find Focus	Only visible if you have configured a motorized focus (MicroToolBox). Starts an autofocus search using the settings from the **Focus Devices** tool. The autofocus search is performed for the selected reference channel in the **Channels** tool.
	-  Set Exposure	Starts an automatic exposure time measurement with the settings defined in the **Light Path** and **Camera** tool.
	-  Live	Starts the **Live Mode.** In the **Center Screen Area** you see the live image from the camera.
	-  Continuous	Starts a series of **Snaps** using the settings defined in the **Light Path** and **Camera** tool. In contrast to a live image, the exact same camera setting that has been set in the **Camera** tool is used.
	-  Snap	A so called "Snap" acquires a single image (snapshot). For widefield systems an additional tiles snap option is available. You can choose between 2x2, 3x3, 4x4 and 5x5 presets.
	-  Stop	Only active if one of the acquisition buttons has been clicked. Stops the function of the relevant acquisition button.
4	**Acquisition Dimensions**	Here you activate the desired dimensions (e.g. **Z-Stack** or **Tiles**) for your experiment. The corresponding fields to the right of each dimension show a preview of how extensive the experiment will be (e.g. **9 Tiles**).

No.	Parameter	Description
		Below the dimensions section you activate additional experiment features (e.g. **Auto Save**) or special modules (e.g. **Shuttle & Find**).
5	**Experiment Preview**	Here you can see a graphical representation of the configured experiment. The **Disc** icon indicates that you have enabled **Auto Save** function for the experiment.
	Start Experiment	Only active when an acquisition dimension was added to the experiment. Starts the experiment with the current configuration.

20.2.2.1 Experiment Options

In the **Options** shortcut menu you can create new experiments and rename, save, import, export or delete existing experiments.

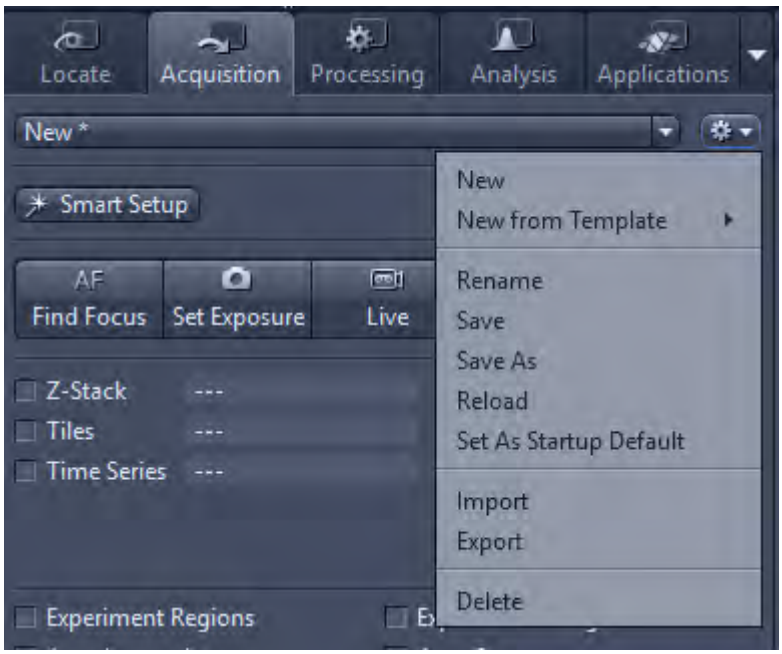


Fig. 20.7: Options shortcut menu

Menu item	Description
New	Creates a new, empty experiment. Enter a name for the experiment.

Menu item	Description
New from Template	Create a new experiment based on an existing experiment. The template experiment will not be modified. You can create your own template experiments by placing them in the folder **Carl Zeiss\ZEN\Templates\Experiment Setups**, which is located in the windows public documents folder and in the documents folder for each user.
Rename	Enables you to enter a new name for the experiment.
Save	Saves a modified experiment under the current name. An asterisk indicates the modified state.
Save As	Saves the current experiment under a new name. Enter a name for the experiment.
Reload	Reloads the selected experiment.
Set As Startup Default	Selecting this option will assign the currently loaded experiment as a default experiment, which is loaded every time the software is started. The startup default experiment is indicated by a special icon behind the experiment name.  You can decide to start the software with a particular default experiment, with the last used experiment or with an empty new experiment in **Tools** | **Options** | **Startup/ Shutdown** | **Experiment**.
Import	Imports an existing experiment. The experiment is shown in the **Experiment Selection** dropdown list.
Export	Exports the current experiment.
Delete	Deletes the current experiment.

20.2.2.2 Smart Setup

Smart Setup offers you support when configuring multichannel acquisition experiments. To start it click on the **Smart Setup** button on the **Acquisition** tab.

Select the fluorescent dyes and contrast techniques that you want to include in your experiment from a large dye database. Smart Setup takes the configuration of your microscope hardware and the properties of the selected dyes into account. Based on this information, it makes one or more suggestions for acquisition. You can adopt these into your experiment as required and make further changes to them there.

INFO

If **Smart Setup** is unable to make a proposal, it is not possible to use the selected dyes, contrast techniques, or current microscope hardware to make acquisitions. Select other dyes or another contrast technique or configure your acquisition experiment using the **Acquisition Mode** tool and the **Channels** tool.

For working with Smart Setup please note the following:

- Smart Setup tries to configure the motorized components of your system for the acquisition of multichannel images.
- Smart Setup does not change any parameters of other acquisition dimensions (e.g. Z-stack, Time series, or Multi-position acquisitions).
- For widefield tracks it does not influence any camera parameters (e.g., Exposure time or Resolution).
- For LSM tracks it adjusts parameters within the Imaging Setup, the Acquisition Mode and the Channels tool windows.

Depending on your system you will see two buttons on top of the dialog.

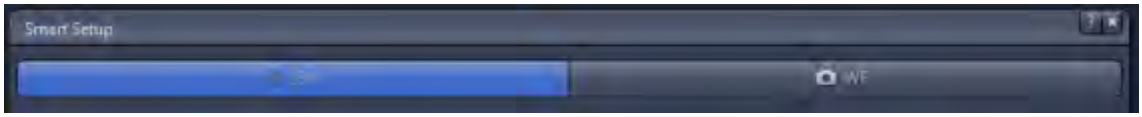


Fig. 20.8: Smart Setup Modes

If you select the **LSM** button you can use Smart Setup for configuring confocal experiments, see *Smart Setup (LSM)* [▶ 635].

If you select the **WF** button you can use Smart Setup for configuring widefield experiments, see *Smart Setup (WF)* [▶ 632].

20.2.2.2.1 Smart Setup (WF)

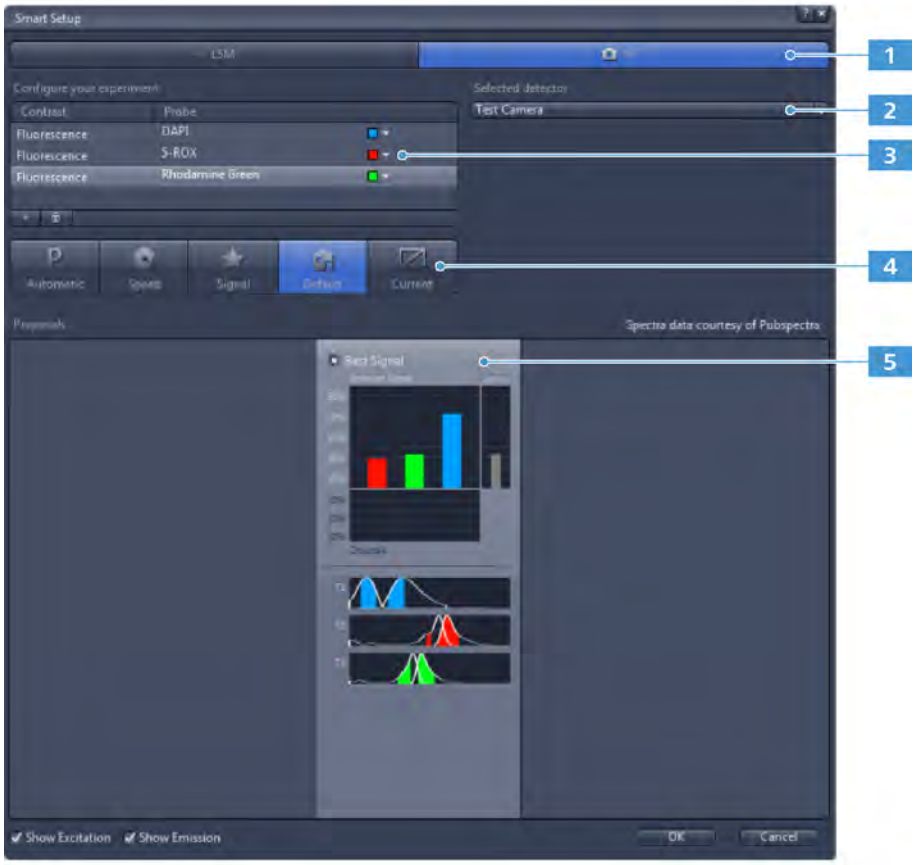


Fig. 20.9: Smart Setup (WF)

1 Imaging Mode Selection
2 Detector Selection
3 Experiment Configuration
4 Motif Buttons
5 Graphical Display of Proposals

Parameter	Description
Detector Selection (2)	Only visible if two or more cameras are configured for the system. Here you can select the desired camera for the experiment.
Configure your experiment (3)	Here you can add up to four reflected light fluorescence channels and one transmitted light contrast technique to your experiment. The added dyes or the contrast technique are shown in the list below.

Parameter		Description
		If you click on the **Add** button the *Add Dye or Contrast Technique* [▶ 776] dialog will be opened. There you can select the desired dye or contrast technique from the Dye Database.
Motif buttons (4)		Here you can optimize image acquisition regarding particular requirements like speed or quality. All parameters, e.g. camera resolution or dynamic range in the **Acquisition Mode** or the **Channels** tool, were set automatically. They will essentially influence the camera, detector, and lightning settings.
-	Automatic	■ The system will try to set the optimal resolution for the camera in the **Acquisition Mode** tool. The resolution will be calculated from camera parameters and numeric aperture; Microscanning will not be applied even if the camera supports this mode. ■ The dynamic range for all fluorescence channels will be set to 50% or 80% for all transmitted light channels.
-	Speed	■ If binning is supported, one binning category will be set for the camera under the optimal resolution in the **Acquisition Mode** tool. ■ Sets dynamic range of all fluorescence channels to 20% or 50% for transmitted light channels. ■ Sets power of all Colibri-LEDs to 100%. ■ Sets EMGain of the camera (if available) to half of Gain max. Sets read mode of camera to fastest. ■ Creates an acquisition configuration which removes all reducers or neutral filters. ■ Changes acquisition sequence for dimensions to fastest. Only effective with 2 or more dimensions.
-	Signal	■ Sets the optimal resolution for the camera in the **Acquisition Mode** tool. Microscanning will be applied if the camera supports this mode. ■ Sets the dynamic range for all fluorescence channels to 90% or 100% for all transmitted light channels. ■ Sets power of all Colibri-LEDs to 75%. ■ Sets EMGain of the camera (if available) to 10% of Gain max. Sets read mode of camera to slowest.

Parameter	Description
- Default	Sets all parameters in **Channels** and **Acquisition Mode** tool to the default values. All changes will be overwritten and reset.
- Current	No changes are made. Only the necessary hardware settings for acquisition are applied by **Smart Setup**. **NOTICE** **If you changed hardware settings in the Acquisition Mode tool manually and do not want to lose them, make sure you select the Current button.**
Proposals (5)	Here you can see the proposals made by **Smart Setup** displayed graphically. You can find a detailed description of the graphical display under *Graphical Display of Proposals* [▶ 637]. The proposals change the imaging settings in the **Imaging Setup** tool window accordingly. The number and type of proposals depend on the microscope hardware being used, the selected dyes, and the contrast technique:
- Best Signal	This proposal results in the best signal strength.
- Fastest	This proposal results in the fastest acquisition.
- Best Compromise	This proposal results in the best compromise between signal strength and fastest acquisition.
Show Excitation checkbox	Shows the excitation spectrum of the selected dyes in the graphical display.
Show Emission checkbox	Shows the emission spectrum of the selected dyes in the graphical display.
OK button	Adopts the proposal displayed as the current acquisition experiment. The suggestion overwrites existing experiments on the **Acquisition** tab.
Cancel button	Ends **Smart Setup**. The suggestions are not adopted into the experiment.

20.2.2.2.2 Smart Setup (LSM)

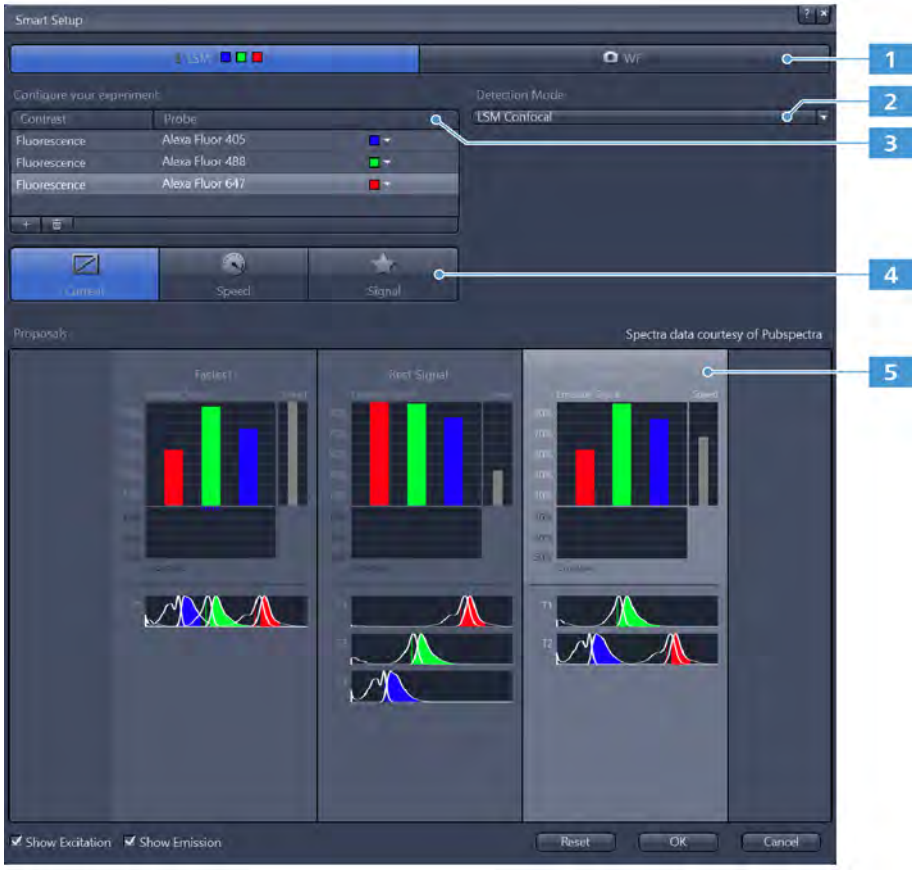


Fig. 20.10: Smart Setup (LSM)

1 Imaging Mode Selection
2 Detection Mode
3 Experiment Configuration
4 Motif Buttons
5 Proposals

Parameter	Description
Detection Mode (2)	If your sytstem is equipped with an Airyscan detector, you can use **Smart Setup** to generate proposals for Airyscan instead of confocal acquisition, see also*Airyscan Mode* [▶ 670].
Configure your experiment (3)	Here you can add up to 8 reflected light fluorescence channels and one transmitted light contrast technique to your experiment. The added dyes or the contrast technique are shown in the list.

Parameter		Description
		If you click on the **Add** button the *Add Dye or Contrasting Method Dialog* [▶ 776] dialog will be opened. There you can select the desired dye or contrast technique from the Dye Database.
Motif buttons (4)		Here you can optimize image acquisition regarding particular requirements like speed or quality. If clicking on a button different parameters in the **Acquisition Mode** or the **Channels** tool, were set automatically. The automatic settings will influence parameters like Frame Size, Speed, Direction, Bit Depth (in Acquisition Mode tool) and Pinhole Diameter, Gain, Laser Power (in Channels tool), depending on the selected button. Various proposals for further experiment settings are shown in the graphical display below the buttons.
-	Current	No changes are made. Only the necessary hardware settings for acquisition are applied by **Smart Setup**. **NOTICE** **If you changed hardware settings in the Acquisition Mode tool manually and do not want to lose them, make sure you select the Current button.**
-	Speed	■ Sets the frame size to 400x400 pixels ■ Sets the scanning speed to maximum value ■ Sets the scanning direction to bi-directional ■ Opens the pinhole to 2 Airy Units (AU)
-	Signal	■ Aims to provide high quality images with best signal to noise ratio. ■ Sets the frame size to a minimal value that fulfills the Nyquist criterion, but to a maximum of 2048x2048 pixel ■ Sets the scanning speed to 6 ■ Sets the scanning direction to uni-directional ■ Sets the Bit Depth to 16 bit
Proposals (4)		Here you can see the proposals made by **Smart Setup** displayed graphically. You can find a detailed description of the graphical display under *Graphical Display of Proposals* [▶ 637].

Parameter		Description
		The proposals change the imaging settings in the **Imaging Setup** tool window accordingly. The number and type of proposals depend on the microscope hardware being used, the selected dyes, and the contrast technique:
-	Fastest	This proposal results in the fastest acquisition.
-	Best Signal	This proposal results in the best signal strength and minimizes the level of cross talk.
-	Smartest (Line)	Combines the advantages of Fastest and Best Signal. It minimizes the number of tracks as well as cross talk.
Show Excitation checkbox		Shows the excitation spectrum of the selected dyes in the graphical display.
Show Emission checkbox		Shows the emission spectrum of the selected dyes in the graphical display.
OK button		Adopts the proposal displayed as the current acquisition experiment. The suggestion overwrites existing experiments on the **Acquisition** tab.
Cancel button		Ends **Smart Setup**. The suggestions are not adopted into the experiment.

20.2.2.2.3 Graphical Display of Proposals

i INFO

The bars in the graphs only show relative values. The actual strength of the emission signal and the crosstalk in the image can deviate substantially from this estimate, as Smart Setup has no knowledge of the strength with which the sample has been dyed with the individual dye components.

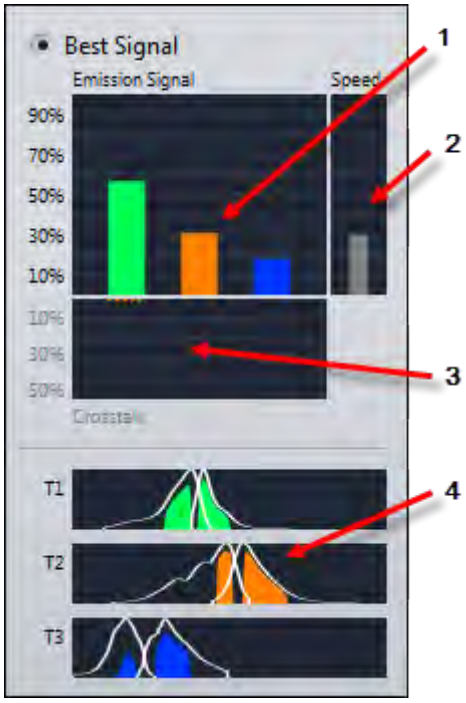


Fig. 20.11: Emission Signal, Speed, Crosstalk, and Tracks

No.	Name	Description
1	**Emission Signal**	A filled, colored bar in the **Emission Signal** display field shows the relative emission signal to be expected for the corresponding channel. The channel color corresponds to the color of the selected dye in the **Configure Experiment** section.
2	**Speed**	A gray bar in the **Speed** display field represents the approximate acquisition speed that can be expected. This is the time required for the movement of microscope hardware during multichannel acquisition. Camera exposure times or parameters for other acquisition dimensions are not taken into account here.
3	**Crosstalk**	A hatched bar in the **Crosstalk** display field shows the expected relative crosstalk originating from one or more dyes for other channels.
4	**Tracks** display	Only visible if the **Show Excitation** and / or **Show Emission** checkboxes are activated. The various tracks are labeled with **T1**, **T2** etc.. The white lines show the excitation and emission spectra of the dyes schematically. The spectra are filled in color in the places that will be acquired by

No.	Name	Description
		the acquisition configuration suggested by **Smart Setup**. Transmitted light channels are displayed as a white field.

20.2.2.3 Reuse

The Reuse functionality is only available if you have loaded an image in *.CZI image format. Then, the **Reuse** button will then appear on the **Acquisition** tab. Otherwise, the **Reuse** button is not active.

With this function you can apply the experiment setup of the acquired image to the current experiment. This will help you to easily reproduce the acquisition conditions for the next image. The function only works correctly if the system configuration at the time of acquisition is identical to the system configuration at the time when you execute the function.

Removing components (e.g. filter cubes, LEDs, cameras, etc.) can result in an experiment being created incorrectly. It is therefore essential that you check after executing the Reuse function whether the configuration of the experiment is in line with your expectations.

Using the Reuse function for a Z-stack promts a confirmation asking whether to place the Z- stack at the current focus position or the original focus position of the acqired image.

Note, that the original position may be way off the current position and starting an experiment right away can lead even lead to the destruction of your sample.

i INFO

Clicking on the **Reuse** button overwrites the current experiment without a prompt and marks it as having been modified. This can be seen from the appearance of an asterisk after the file name. If you want to keep the experiment in its previous form, you must save the modified experiment with a new file name under **Experiment Manager** | **Options** | **Save As**.

If you acquire images and save them in *.CZI image format, the following acquisition conditions are saved together with the image:

- Information on the type and status of your imaging system
- Time of acquisition
- Parameters set in the software.

20.2.3 Processing Tab

Here you apply processing functions to acquired or loaded images. For the detailed descriptions of the functions and the processing workflow please read more under *Image Processing Functions* [▶ 93].

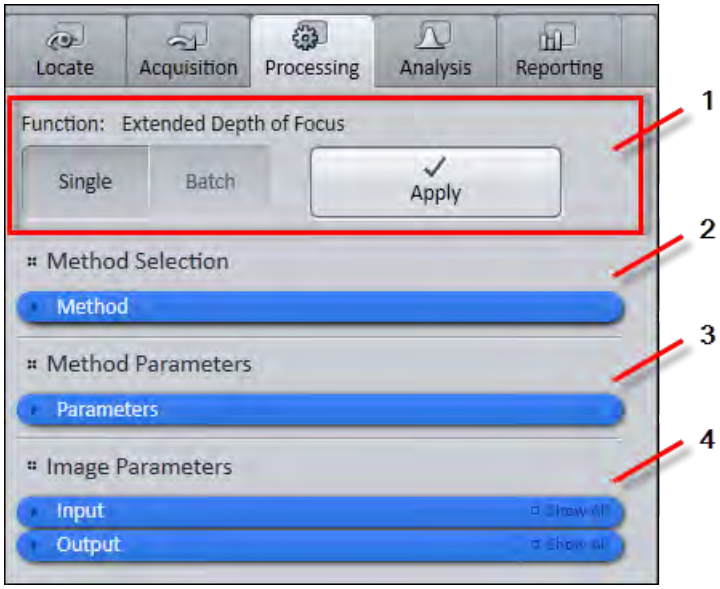


Fig. 20.12: Processing tab

No.	Parameter	Description
1	**Function**	Switch between Single and Batch mode here.
-	Single	In **Single Processing mode** you apply a selected processing method, with the relevant method and image parameters, to a single image.
-	Batch	In **Batch Processing mode** you apply a selected processing method, with the relevant method and image parameters, to the list of images. In this mode only a limited selection of processing functions is available.
-	Apply	Applies the selected method to the input image.
2	**Method**	Here you select the image processing functions. Click on the **Method** tool to show the list of IP functions.
3	**Method Parameters**	Here you configure the parameters of the selected image processing function. Click on the **Parameters** tool to show the parameters of the selected IP function.

No.	Parameter	Description
4	**Image Parameters**	Here you configure the image parameters of the input and output image. Click on the **Input** tool or **Output** tool to open input/ output image settings.

20.2.4 Analysis Tab

Here you find the tools available for image analysis, e.g. **Interactive Measurement** or **Image Analysis**.

20.2.5 Extensions Tab

This tab is visible only if you have activated an extension (e.g. ImageJ) under **Tools** | **Extension Manager**.

Our extensions concept allows to extend ZEN basic functionality by implementing third party extensions, e.g. ImageJ. The extensions concept is a part of **OAD (Open Application Development)** for ZEN, see Open Application Development (OAD).

Depending on which extension you have activated, you will see the extension´s functions and controls on the extensions tab. Please notice that we will not describe any functions of third party extensions here. Therefore use the third party documentation for each extension.

You can find more information on OAD and the supported extensions under **www.zeiss.com/zen-oad**.

20.3 Tools

20.3.1 Tools on Locate Tab

20.3.1.1 Microscope Control Tool

The configuration of your system according to your MicroToolBox (MTB) configuration setting is shown here. A valid microscope configuration has to be created first using the MTB2011 Configuration program. The light path follows the light starting with the light source to the specimen and from there to the camera or eyepiece. It displays control elments for all motorized and manually operated components. Here you can interactively adjust the microscope and its components.

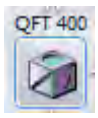
i INFO

If you are not using any motorized components, you will have to make the relevant adjustments manually.

Keep the following points in mind when working with this tool:

- To adjust a component, left-click on the relevant icon.
- Icons with an **arrow** symbol in the bottom right corner contain dialogs that allow you to configure additional settings. To open the dialogs, left-click on the corresponding icon.
- Icons with a **hand** symbol in the bottom left corner indicate components that have to be operated manually.

Icon	Parameter	Description
	**Eyepiece**	Displays the total magnification of the selected beam path with all activated intermediate magnifications above the **Eyepiece** icon. To direct the light path fully to the eyepiece, simply left-click on the icon.
 / 	**Shutter**	Here you can set the shutter to **Open** or **Closed**. The status is displayed in text form above the icon.
	**Reflector Turret**	Here you can select one of the configured filter cubes for reflected light techniques from the list.
	**Nosepiece / Objective**	Select the desired **Objective** from the list.
	**Stage**	Here you see the options for Stage Control and Focus Control. ■ **Stage >>:** Opens the *Stage Tool* [▶ 765] in the **Right Tool Area**. There you can move the microscope stage virtually with the help of a software joystick or by entering absolute coordinates. You can also calibrate the stage within that tool. ■ **Focus >>:** Opens the *Focus Tool* [▶ 768] in the **Right Tool Area**. There you can move the focus drive virtually with the help of a software joystick or by entering absolute coordinates. You can also calibrate the focus drive within that tool.

Icon	Parameter	Description
	**Aperture Diaphragm**	Adjust the diaphragm opening (0% to 100%) using the slider or spin box/input field.
	**Filter Wheel**	Here you can enter the first neutral density filter (e.g. 0.4%, 6%, 100%, 100%) that you require.
	**Condenser**	The condenser is only available in the **Transmitted Light** path. Select the contrast method from the list (e.g. brightfield, darkfield, phase contrast ring 1, 2, 3, DIC I, II, III).
	**Camera/ Eyepiece Switch**	Select whether you want to direct the light to the camera only (**100% Camera**), to the camera and the eyepiece (**30% Eyepiece/70% Camera**) or to the eyepiece only (**100% Eyepiece**) from the list .
	**Camera**	Shows the selected camera above the icon.
	**Microscope Manager**	Opens the *Microscope Manager* [▶ 644] dialog.
	**Reflected Light/ Transmitted Light Switch**	If your microscope has a halogen lamp for both reflected and transmitted light illumination, here you can select whether you want to control the halogen lamp for reflected light illumination or the halogen lamp for transmitted light illumination.
	6x Motorized Beam Splitter Wheel	This device is part of the Motorized Dual Filter Wheel. Select the desired Dichroic position from the dialog. Switching time is about 300 msec between neighboring positions.
	6x Motorized Emission Filter Wheel	This device is part of the Motorized Dual Filter Wheel. Select the desired Emission Filter position from the dialog. Switching time is between 60 and 240 msec between neighboring positions (depending on the speed configuration in the MTB2011 Configuration program).

Icon	Parameter	Description
	6x Motorized Excitation Filter Wheel	Select the desired Excitation Filter position from the dialog. Switching time is between 70 and 300 msec between neighboring positions (depending on the speed configuration in the MTB2011 Configuration program).
	12x Motorized vReflector Changer	If a Motorized Dual Filter Wheel and a Motorized Emission Filter Wheel is present, up to 12 virtual Reflektor positions can be configured in the MTB2011 Configuration program. Select the desired filter combination from the list of available positions. This is more convenient than adjusting excitation, dichroic and emission filters individually.

20.3.1.1.1 Microscope Manager

Parameter	Description
Contrast Manager	
Mode	Select the setting for the contrast mode from the **Mode** dropdown list.
- Off	The Contrast Manager is not used. All settings must be made manually or via a settings file.
- On Demand	The function of the Contrast Manager is activated via the touch screen on the microscope.
- Contrast Retaining	If core components (e.g. condenser, reflector, shutter) for a certain contrast technique are changed, dependent components are also changed accordingly.
Method	Select one of the available methods for the contrast mode here.
Light Manager	
Enabled	**Activated:** Activates the Light Manager. Activates the **Mode** dropdown list in the Light Manager.
Mode	Select a setting for adjusting the brightness of the light here.
- Objective	Adjusts the brightness of the light via the lamp voltage. The color temperature changes accordingly.

Parameter	Description
- Classic	Adjusts the brightness on the basis of the available filter wheels. The color temperature is retained. Only if the brightness adjustment cannot be achieved via the filter wheels does adjustment take place via the lamp voltage.
Dazzle Protection	**Activated:** Activates dazzle protection. Dazzle protection prevents light from passing through the eyepiece and dazzling the user, for example when reflector positions are changed. This is mainly achieved by closing the reflected or transmitted light shutter. If no shutters are installed, the lamp voltage is adjusted.
Parfocal Correction	**Activated:** Parfocal correction is activated.
Parcentral Correction	**Activated:** Parcentral correction is activated.

20.3.1.2 Camera Tool

Here you can configure all the settings for the selected camera.

Please note that the functions and settings in this tool depend on which camera you are using meaning not all cameras have all the functions described in here.

20.3.1.2.1 Exposure Time Section

The **Exposure Time** section enables you to control the exposure settings of your camera.

If you use automatic exposure, you can select an area on the camera sensor which is used to calculate the exposure time.

Parameter	Description
Time	Specifies the duration of the image acquisition. Select the unit of time (min, ms, s, µs) here.
Auto Exposure	**Activated:** The exposure time is calculated automatically every time an image is acquired. The exposure time in the corresponding input field fluctuates accordingly. **Deactivated:** The exposure time must be set manually.
Intensity	Enables you to compensate for underexposure or overexposure if you are not content with the auto exposure result.

Parameter	Description
	■ 5% - 100%: Darkens the image (compensates for overexposure) ■ 100% - 200%: Brightens the image (compensates for underexposure)
Set Exposure	Starts a one-off measurement of the exposure time, which is then used for all subsequent images. Deactivates the **Auto Exposure** checkbox. If you are not satisfied with the result, you can adjust the measured exposure time manually.
Spot Meter / Focus ROI	**Activated:** The exposure time and focus measurements use the intensity values within a specified area instead of the entire camera sensor area. This improves the results for the area to be acquired. If the red **Spot Meter / Focus ROI** frame is not visible in the live image, right-click in the live image and select **Spot Meter / Focus ROI** from the context menu.

Parameter	Description
Binning	Here you can set the binning. Binning combines the information of neighboring camera pixels into a single larger pixel. For example, if the binning is set to 2 × 2, four pixels are combined to one. Increasing the binning means weaker signals can be detected for a given exposure time. For CCD cameras, binning increases sensitivity by improving the signal-to-noise ratio, with resolution being decreased by the same factor. In the case of CMOS cameras, only the signal intensity is increased and the pixel count and resolution gets reduced correspondingly.
Binning-independent Brightness	Because **Binning** generally increases signal intensity, the brightness of the image normally also increases correspondingly. By activating this checkbox, the brightness level is automatically fixed (depending on the camera, either through exposure time adjustment or averaging), no matter the binning setting.

Parameter	Description
IP Quality	Here you can select the color interpolation quality (IP Quality) for the acquired image. Please notice that this function does not apply to Live mode. "**Fast"**: color interpolation for optimum speed (shorter computation) "**High**": color interpolation for optimum quality (less artifacts). This mode is only effective with binining factor 1.
Subsampling	Here you can reduce the amount of data acquired to acheive faster framerates. By subsampling 2x2, the effective pixel pitch is increased by sampling only every other pixel, thus reducing the overall data size of your image.
Resolution	Here you see the camera resolution, e.g. 1024 x 1024 px

20.3.1.2.2 Acquisition ROI Section

In this section you can define a **Region Of Interest (ROI)** on the camera sensor which will be used for acquisition. A smaller ROI can increase the acquisition speed.

The region of interest is indicated by a blue frame in the preview window and can be moved and resized freely. The preview window always shows the entire camera sensor area which can be acquired.

The **Pixel Size** shown below the preview window indicates the size in µm to which a pixel corresponds. This depends on the camera sensor properties and on the binning.

Parameter	Description
Maximize	Selects the entire available image sensor area as the region of interest.
Center	Positions the region of interest precisely at the center of the image.
Size	Sets the width and height of the region of interest in pixels.
Offset	Specifies the position of the top left corner of the **Acquisition ROI** (blue frame) with respect to the top left corner of the preview window.

Parameter	Description
Refresh Overview	Acquires and displays an image in the preview window with the current ROI settings. This has no effect on the image in the **Center Screen Area**.
Crop	Only visible if the checkbox **Show Crop Button** under **Tools** | **Options** | **Acquisition** | **Camera/Live** is activated. Allows you to specify a ROI (Region of Interest) in a snapped image. The "cropped" area is used as ROI for the next image acquisition. If no image is available in the **Center Screen Area** the button is deactivated.

20.3.1.2.3 White Balance Section

This section is only visible if you are using a color camera.
The section enables you to adjust the color balance to a neutral hue independent of the light source used.

Save suitable white balance settings using the **Settings** section to ensure color reproducibility of images acquired in the future.

Parameter	Description
Auto	Compensates for the color temperature of the light source automatically to yield a neutral hue. The entire camera sensor area is measured. If there are no pure white areas on the sample and **Auto** does not yield the desired results, measure and compensate for the color temperature of the light source as follows: ■ Transmitted light: Move the sample such that a clear and transparent region is illuminated or remove the sample from the microscope. Click the **Auto** button to perform the auto white balance. ■ Reflected light: Use a neutral surface (e.g. a piece of white paper) as a sample. Click the **Auto** button to perform the auto white balance. You can now acquire white balanced images of your sample with the above settings.
Pick...	Enables you to select a reference pixel for white balance from the live image. The selected pixel should be neutral white.

Parameter	Description
3200 K	Applies a predefined color balance setting to compensate for the color temperature of a halogen light source at approximately 3200 K.
5500 K	Applies a predefined color balance setting to compensate for the color temperature of an LED light source at approximately 5500 K.
Show Channels	Enables you to set the color balance of each color channel (red/cyan, green/magenta and blue/yellow) individually to make the image appear neutral.
Color Temperature	Changes the overall color temperature of the image from cool (blue cast) to warm (red cast). The color channels (red/cyan, green/magenta and blue/yellow) are adjusted automatically. The **Color Temperature** setting can work against the settings applied using **Show Channels**. Use **Color Temperature** for fine tuning in combination with **Pick...** if **Pick...** does not give perfect results.
Saturation	Changes the colorfulness of the image.
Reset	Resets any color changes and sets the white balance value to 6500 K.

20.3.1.2.4 Gain Section

Using the gain adjustment amplifies the signal intensity and brightness of the camera image while at the same time reducing the available dynamic range.

20.3.1.2.5 Post Processing Section

Here you can apply basic image processing functions while acquiring the image. This can be helpful if certain image processing steps are necessary for any acquired image and saves image processing work later in a job.

Depending on the camera model, different settings are available.

Parameter	Description
Black Reference	Influences the live image and each image acquired. For the black reference to work, you first need to acquire a reference image. Define a corresponding reference image using the **Define** button. **Activated:** Applies the measured black reference to the image.

Parameter		Description
		Deactivated: The measured black reference is not used. The reference image is retained. If longer exposure times are used (from exposure times of approx. >5s, depending on the camera), individual bright pixels may become visible with CCD or CMOS sensors. With the help of the black reference these effects are measured and corrected in accordance with the exposure time employed. It is recommended that you repeat this measurement at certain intervals. This correction is recommended in particular for applications that involve long exposure times, i.e. for which very little light is used (live cell imaging, fluorescence images). The availability of a black reference for the selected camera can be checked on the menu **Extras** | **Calibration Management**.
-	Define	Automatically defines the black reference. The measurement lasts for several seconds. The **Black Reference** checkbox is then activated automatically.

INFO

For the measurement the camera must see a completely dark image. The light path to the camera must therefore be blocked. Set the eyepiece switch to 100% eyepiece and close the reflected light/transmitted light shutter. To define the black reference, click on the **Define** button.

Parameter	Description
Shading Correction	Shading correction is used to correct optical effects, such as minor differences in illumination or static contaminants in the beam path, with the help of a reference image. The reference image must be acquired without a sample. You can select between two modes **Global** and **Specific** from the dropdown list (see description below). After you have selected the mode simply click on the **Define** button. **Activated:** Applies the defined shading correction to the image. The applied correction mode is multiplicative.

Parameter		Description
		Deactivated: The measured shading correction is not used. The reference image is retained.
-	Define	Automatically calculates the shading correction.
-	Global	Performs an objective specific shading correction. This is the default method for shading correction. The following components will be considered: ■ Magnification: Objective and Optovar ■ Camera bit depth and RGB/BW mode ■ Camera type and port position Fluorescent filters or other fluorescence specific components are not considered. In principle, shading correction is objective specific. A separate reference image has to be created for each objective. Once calibration has been completed, the correction image associated with the objective being used is loaded automatically if shading correction is active. If no correction image is available for an objective, the **Shading Correction** checkbox is automatically deactivated when the objective in question is swung in. Objective recognition on a motorized or encoded microscope is required for these automatic actions.
-	Specific	Performs channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. If the fluorescence channel is changed, a previously created reference image is also loaded. The availability of created reference images can be checked on the menu **Tools** | **Calibration Manager**. Incorrect reference images can also be deleted there. The following components will be considered: ■ Contrasting method and condenser ■ Fluorescence reflector and beam splitter ■ Spinning disc fluorescence filter

INFO

An empty image without structures at a medium illumination intensity is required for the shading correction measurement. To create this image, locate an empty position on the slide outside the sample and acquire an image for shading correction there. There must be no visible structures on the slide, as these will be incorporated into the correction image and could then lead to a visible artifact at other positions. It may be necessary to clean the slide and defocus the microscope slightly. You should bear in mind that Köhler illumination needs to be set correctly. No part of the image must be overexposed.

Parameter	Description
Enable Noise Filter	**Activated:** Filters the noise in the acquired image according to the adjusted threshold. Affects acquired images only. The live image does not change.
- Threshold	The higher the value, the greater the tolerance for noise. The lower the value, the stronger the noise reduction. The noise filter reduces the extent to which individual pixels deviate from the average value of their nearest neighbors. The **Threshold** corresponds to a tolerance value. If the deviation of the middle pixel value from the average value of the pixels immediately surrounding it exceeds the tolerance value (i.e. it is interpreted as noise), it is replaced by the average value. This technique reduces the noise of individual pixels that are produced, in particular with EMCCD cameras and CMOS cameras. The selected technique prevents any changes being made to object edges, as in most cases these are larger than individual pixels. This filter is also suitable for removing individual "hot pixels" from an image without having to acquire a reference image in advance.
Enable Unsharp Mask	Enhances contrasts at fine structures and edges. Thus, the resulting image appears clearer and enriched in detail.
- Strength	Controls the amount of contrast enhancement applied to fine structures and edges. The higher the strength, the darker or lighter the resulting edges, compared to the original image.
- Radius	Determines the size of detail to be enhanced. A small radius enhances smaller details.

	Parameter	Description
		The radius also affects the appearance of enhanced edges. A large radius leads to a visible halo along enhanced edges. The larger the radius, the broader the halo.
-	Color Mode	Determines the calculation method, which affects the appearance of the output image. ■ **RGB**: – The **Unsharp Mask** filter calculates the sharpness for each color channel individually. – The color saturation and the color of structures may be changed and color noise may occur. ■ **Luminance**: – The **Unsharp Mask** filter calculates the sharpness based on the luminance signal computed from the RGB channels. – This mode avoids possible color noise or shift in color saturation, which could be induced by certain image textures.
-	Auto Contrast	**Activated:** Enables you to adjust the **Contrast Tolerance** (0-20). **Auto Contrast** only works in RGB color mode.
-	Contrast Tolerance	Increasing the contrast during unsharp masking is achieved by broadening the distribution of intensities. This corresponds to a spread of the image histogram. Controls how much the intensity distribution is spread and thus how strong the contrast is increased. ■ **Contrast Tolerance = 0** : No spread of intensities, no increase of contrast ■ **Contrast Tolerance = 20**: Maximum spread of intensities, maximum increase of contrast
-	Clip To Valid Bits	**Activated:** Composes the processed image of the same colors as the original image (i.e. the value range of the output image is adjusted to the color range of the input image). **Deactivated:** Colors not present in the original image may appear in the processed image.

20.3.1.2.6 Settings Section

In this section you can save all the settings you have made in the camera tool to a settings file (*.**czcs**). This is very helpful because you can restore/load your saved settings very quickly when starting the software again.

Parameter	Description
Default	Resets all camera settings in the **Camera** tool to the factory default settings. These settings can also be selected from the dropdown list of available camera settings to the right of the **Default** button. To do this, select the **Original Settings** entry.
Options 	Opens the *Options* [▶ 654] menu.
Reload	Undoes the changes you have made to a loaded setting and restores the original status of the loaded setting.

20.3.1.2.6.1 Camera Tool Options

Parameter	Description
New	Creates a new camera setting. Enter a name for the camera setting in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Rename	Renames the current camera setting. Enter another name for the camera setting in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Save	Saves the current camera setting.
Save As	Saves the current camera setting under a new name. Enter a new name in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Import	Imports an existing camera setting.
Export	Exports the selected camera setting.
Delete	Deletes the selected camera setting.

20.3.1.2.7 Mode Section

In this section you can adjust how the software retrieves the camera sensor data.

Parameter	Description
Color Mode	This parameter is available for color cameras only.
– RGB	Transmits the image data of a color camera unchanged. This corresponds to the standard operating mode of a color camera.
– B/W	Treats the image data of the color channels as grayscale. The data of related color channels are averaged. The saturation of the camera appears reduced as a result. This process does not change the spectral properties of a color camera. The image information of the color sensor still undergoes color interpolation. An infrared filter also restricts the spectral sensitivity of the color camera compared to the spectral sensitivity of a genuine black and white camera.
Live Speed	Here you can select the live image update speed (slow, medium, fast). Enables you to focus or to find regions of interest on a sample quickly. A high live image update speed reduces the exposure time of the live image, even at longer exposure times used for image acquisition. To achieve a similar impression of image brightness, however, the image data supplied must be adjusted digitally, which may generate a certain amount of noise or reduce the resolution of the live image.
IP Quality	Only available for **Axiocam 503** color and **Axiocam 506** color. Here you can select the color interpolation quality (IP Quality) for the recorded image. Please notice that this function does not apply to **Live** mode. If you reset the settings via the **Default** button the **IP Quality** is set to high.
– Fast	Represents the image optimized and requires a shorter computation time.
– High	Represents the image without artifacts and with a higher image quality. This mode is only effective with binining factor 1.
NIR Mode	This function is only available for cameras which offer this feature (e.g. **AxioCam MRm**). **Activated:** Uses the camera in the near infrared range (NIR=Near-Infrared).

Parameter	Description
	Sensitivity for signals in the near infrared range (approx. 700nm to 1000nm) is increased. However, the tolerance for overexposure is reduced and, if very bright structures are present, overexposure artifacts (blooming) can result. We recommend that you use this mode mainly for very weak signals.

20.3.1.2.8 Trigger Control Section

Only visible if the selected camera has a trigger input/output.

Trigger Out section

Using the trigger output you set how the camera sends a trigger signal to an external component (e.g. shutter).

INFO

Activate both checkboxes if you want the trigger signal to be generated both during the live image and during acquisition.

Parameter	Description
Enable for Snap	**Activated:** Generates the trigger signal during the acquisition of an image.
Enable for Live	**Activated:** Generates the trigger signal during the live image.
Control Signal	
- Active High	The **Control Signal** jumps from 0 Volts to 5 Volts when the camera's exposure begins. Following exposure it returns to 0 Volts.
- Active Low	The **Control Signal** jumps from 5 Volts to 0 Volts when the camera's exposure begins. Following exposure it returns to 5 Volts.
Shutter Open Delay	Here you can enter the delay before acquisition.

Trigger In section

The trigger input allows you to trigger acquisition by the camera using an external trigger signal.

INFO

Due to its inertia, a mechanical shutter needs a certain amount of time to change from the closed to the open position after the control signal has been generated. To ensure that this transitional state is not recorded during the exposure of the sensor, the start of actual acquisition can be delayed by an adjustable period of time.

Parameter	Description
Enable for Snap	**Activated:** Only acquires the image after the trigger signal has been received.
Control Signal	
- Active High	The **Control Signal** jumps from 0 Volts to 5 Volts when the camera's exposure begins. Following exposure it returns to 0 Volts.
- Active Low	The **Control Signal** jumps from 5 Volts to 0 Volts when the camera's exposure begins. Following exposure it returns to 5 Volts.

20.3.1.2.9 Model Specific Section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button  .

In this section you see additional, model-specific camera settings depending on which camera you use on your system.

Reset button

Resets all entries to the original values.

20.3.1.2.9.1 Axiocam 506

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Acquire section

Readout Speed (MHz)

Readout speed can be varied between 39 MHz and 13 MHz if the camera is operated on the USB3.0 bus. In case the camera is connected to the slower USB2.0 Bus only the 13 MHz mode is available. At the slower 13 MHz the signal quality is slightly improved due to a reduced noise of the signal transmission.

Cooling

Status information, if camera cooling is active. The Axiocam 506 can be operated without active cooling. Cooling is deactivated if the USB2.0 connector of the camera is not supported to the PC or to a USB compatible power supply.

Readout Port

The Axiocam 506 uses a high performance CCD sensor with four readout ports. It can be adjusted to quadport, dualport, singleport and Auto mode. Maximum speed is reached by using all four ports and short exposure times. When exposure time gets larger than the readout time the benefit for using multiple ports is getting insignificant. By switching the readout mode to single port, the most homogenous signal quality can be reached as all data is sent through one single processing chain. In Auto mode, the number of used readout ports is selected automatically depending on the exposure time.

Readout time (ms)

The valid camera readout time is given in this status window which is defined by the number of used ports or by defining a sensor sub region window (ROI).

Temperature

The valid CCD sensor temperature is shown here. It is adjusted to 18 C°. It can not be changed. If a black reference is used it should be used at the same sensor temperature when it was created.
If free air circulation for the camera housing is blocked, it may happen that the sensor temperature is increased and the dark current of the sensor may be higher than normal. If the camera is operated without cooling (USB2.0 port of camera not

connected) the sensor temperature is increased and dark current will be higher than normal. This should be considered when using the camera at longer exposure times.

Expert section

Abort on missed frames

In case of high speed time lapse acquisition the camera is sending an enormous amount of data to the PC. If the PC is not fast enough in handling the image data it may happen that dropped frames occur. By this parameter the behavior of the acquisition routine can be defined: it is the default mode to abort a acquisition sequence in case of a dropped frame. If unchecking this the event of a dropped frame is ignored and the acquisition is continued without further notification.

Main LED

The LED in the camera lid shows the general operation mode of the camera by showing different colors. In special low light applications it may be desirable to minimize stray light in the microscopy workspace. Therefore the intensitiy of the status LED can be dimmed or switched off completely. The following status informations are available:

Red: after plugging the main USB3.0 cable of the camera to a PC, camera firmware is not yet loaded by driver. As soon as the camera gets loaded with firmware, the color changes one of the following colors:

Yellow: connected to USB2.0 bus, only 13 Mhz clock speed available, no cooling (right USB port connected to USB2.0, left USB port not connected),

Green: connected to USB 2.0 bus, only 13MHz available, cooling active (right USB port connected to USB 2.0 bus, left USB port connected to computer USB port, or separate USB power supply),

Pink: connected to USB3.0 bus, full 39 MHz clock speed available, no cooling (right USB-port connected to USB3.0, left USB-port not connected)

Blue: connected to USB3.0 bus, full 39 MHz clock speed available, cooling active (right USB-port connected to USB3.0, left USB port connected to computer USB port, or separate USB power supply)

Red: wile exposure of sensro is active, in Live mode or acquisition of image series the LED is blinking, therefore.

Trigger LED

A second LED is at the camera back, above the Micro-d connector for the trigger cable. This LED indicated the status of the trigger port. It is only active, in case the trigger port is used.

In special low light applications it may be desirable to minimize stray light in the microscopy workspace. Therefore the intensitiy of the status LED can be dimmed or switched off completely. The following status informations are available:

Green: asynchronous triggering is possible (no jitter in line timing),

Yellow: synchronous triggering is possible (some jitter in line timing),

Red: no trigger accepted,

Blue: external trigger signal active,

Tile Adjustment

Camera expert section shows camera parameters which need deeper understanding of functionality.

The Axiocam 506 camera is using a special CCD sensor with four readout ports in order to read out the image data from the CCD area as fast as possible. It is assured by proper alignment of the electronics that all associated signal paths are absolutely equal and no quadrants get visible as this is very undesirable for good image quality. In order to offer the best quality possible, a special algorithm is activated by default as an additional safety measure to suppress remaining residuals. This correction can be deactivated in order so minimize image processing on the camera raw data.

Acquire (Expert) section

Camera **Expert** section shows camera parameters which need deeper understanding of functionality.

8 bits compression

In case other devices are using bandwidth on the connected USB 3.0 data bus, it is possible to reduce the amount of image data sent by the Axiocam 506 by activating data compression from 14 bit to 8 bit. By converting the data through a square root loaded lookup table the 14 bit values are converted to 8 bits per pixel. This is handled transparently as the data is decompressed automatically by the camera driver in the PC while receiving it. As compression has some slight impact on data quality it is deactivated as default.

Frame time (ms)

In case of fast time lapse acquisition the camera is sending huge amount of image data to the PC within a short time. The data needs to be handled by the PC without delay. There is a certain risk of dropped frames, if the camera is sending data faster, than the PC can handle it, especially at very short exposure times. By setting a frame time larger than zero, an acquisition delay can be defined for continuous acquisitions. Max. value is 5000 ms. Default value is zero.

Readout mode

This is a status information and shows the currently used number of used sensor ports. Values are: quad port, dual port, single port.

Adjust Live Frame Rate

The camera live image can send a lot of image data which need to be processed on the fly. In case of less powerful computers this can overload the PC and cause a slow reaction of the ZEN user interface. By activating this function the slider below

gets accessible and a maximum limit for the live frame rate can be adjusted. Abundant image data is then discarded accordingly and not processed to be displayed.

Live Frame Rate Max

Adjustment of maximum accessible frame rate for live display, only. Only accessible if "Adjust Live Frame Rate" checkbox is activated.

Live Frame Rate

This is a display field only. It shows the measured live image speed.

20.3.1.3 Movie Recorder Tool

Here you can acquire image sequences in the form of videos using the camera's fastest burst mode.

> **INFO**
>
> To play the acquired Movie, use the **Player** tab in the **Center Screen Area** (Only visible in **Show All** mode).

Parameter	Description
Start Movie	Starts the acquisition. The button changes into the **Pause** button. The animated **Stop** button appears in the window above the button.
Pause Movie	Pauses the acquisition. The button changes into the **Continue** button.
Continue Movie	Continues acquisition if it has been paused. The button changes into the **Pause** button.
Stop	Stops acquisition. Save the acquired movies either ■ in the internal **CZI** format via **File** menu | **Save as...** ■ as a series of individual images via **File** menu | **Export/ Import** | **Export** ■ or as an **AVI** file via **File** | **Export/Import** | **Movie Export**.

20.3.1.4 Manual Extended Depth of Focus Tool

Parameter	Description
Z-Stack	If activated, an EDF image is acquired out of a Z-Stack image.
Quality	Here you can select the quality level that you want the function to work with.
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Mode	
- Timer	Acquires an EDF (Extended Depth of Focus) image automatically after the interval you have set.
- F12 Key	Acquires an EDF image when you press F12 key.
Interval	Only active, if you have clicked on the **Timer** button. Set interval (in sec.) here, after which the automatic acquisition begins.
Start	Starts the acquisition of an image series. Press the **Pause** button to pause acquisition. Press the **Continue** button, to continue acquisition. Press the **Stop** button to stop acquisition. The image with extended focus will be calculated from all single images.

20.3.2 Tools on Acquisition Tab

20.3.2.1 Imaging Setup Tool

Only visible, if the **Enable Imaging Setup** checkbox in the **Tools** menu | **Options** | **Acquisition** | **Acquisition Tab** is activated.

Here you can view and adjust the hardware parameters used for confocal (LSM) or camera (WF) experiments. All hardware parameters set to detect one or more specific signals simultaneously are defined as a track.

INFO

Note that the **Microscope Control** tool on the **Locate** tab has a similar appearance and similar control elements but its function differs from the **Imaging Setup** tool described here.

If there is no track configured, you need to add a first track to the experiment by clicking on the drop down button. To see a list of the availalbe acquisition modes, click on the arrow on the right side of the button.

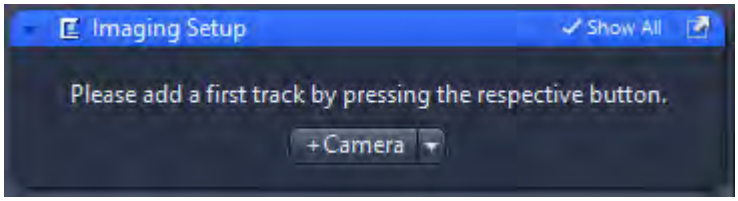


Fig. 20.13: Imaging Setup without tracks

If there are tracks available you will see buttons with the respective name (**Track1**, **Track2**, etc.) on the top of the tool. The active track is always highlighted in blue color and the acquisition type of the track is indicated below the button. You can switch between the channels by clicking on the corresponding track button. Using the **Delete** button you can delete tracks or access further options for editing via the **Options** button (Add, Delete, Duplicate, Rename).

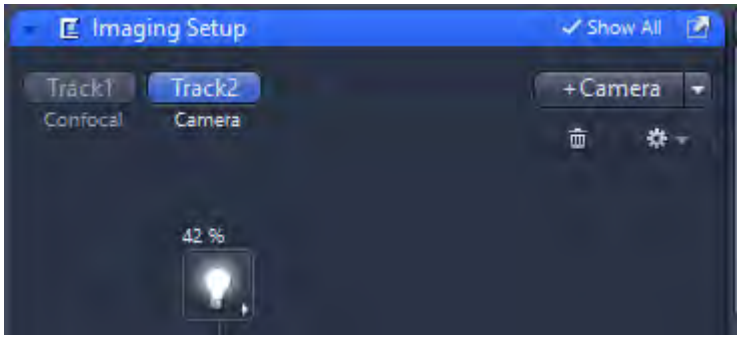


Fig. 20.14: Imaging Setup with tracks

Depending on what kind of track you have selected the **Imaging Setup** tool has a different appearance. For a detailed description of the specific parameters read the chapters *Imaging Setup (WF)* [▶ 664] and/or Imaging Setup (LSM).

Tracks of different kind are not always compatible with each other. It is e.g. not possible to combine camera and LSM acquisition into one single image. The Imaging Setup and Channels tool will automatically disable incompatible tracks upon creation of new tracks. To re-enable incompatible tracks, you need to deactivate first the active tracks. Once all incompatible tracks are disabled, the remaining tracks can be activated again.

20.3.2.1.1 Imaging Setup (WF)

By selecting a WF track you can see the graphical display of the acquisition light path with various icons. The arrangement of the icons represents the typical set-up of the microscope components configured on your system. For a description of the most common icons, read Reflected/Transmitted Light Path.

The associated hardware settings are shown above the icons and can be changed here. To change the relevant hardware settings, left-click on the icons. In the shortcut menus you will see numerous selection and setting options for adjusting your settings.

INFO

Any change you make is automatically adopted and written to the corresponding hardware setting of the experiment. If you want to undo these changes, do not save the experiment. Instead, reload the experiment in the **Experiment Manager**.

If you change the hardware settings in this section, please bear the following points in mind:

- If the checkbox **Include in this setting** is activated the component is activated. Activated components are included into the hardware settings of the experiment and subsequently applied in the experiment. Activated components are highlighted in blue color.
- Components with a deactivated checkbox are not adopted into the hardware settings of the experiment and are not subsequently applied in the experiment. These components are displayed with a grayed-out icon.
- Components with a filled-in checkbox and a triangle underneath are only partially adopted into the hardware settings of the experiment and subsequently applied in the experiment. To show the sub-components, click on the triangle under the checkbox. To adopt the sub-components into the hardware settings of the experiment and subsequently apply them in the experiment, activate the relevant checkboxes for the sub-components.

20.3.2.1.2 Confocal Acquisition

In this mode the current hardware settings within the scan head are displayed. This mode uses for each channel one discrete detector. Please note the following:

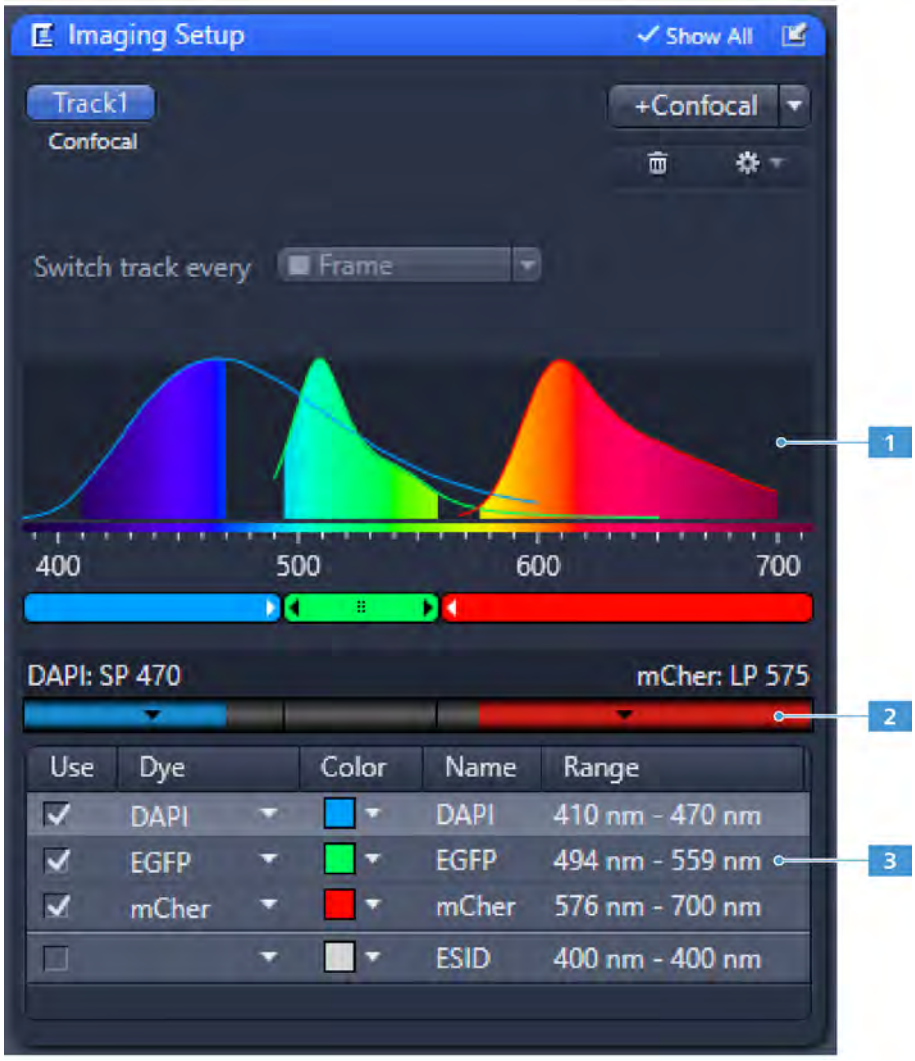


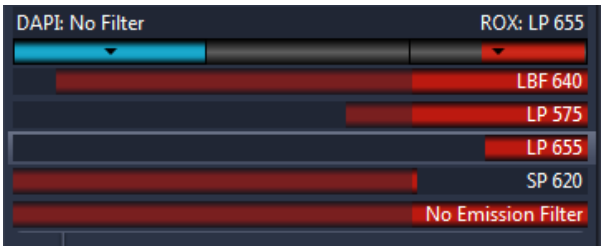
Fig. 20.15: Imaging Setup

- On a two channel system, both detectors are either multialkali PMTs or GaAsP PMT detectors.
- On a three channel system, Channel 1 and 3 are either multialkali PMT or GaAsP PMT detectors. Channel 2 can be a multialkali or GaAsP PMT or an Airyscan.

Parameter	Description
Switch track every	Here you can adjust how the available tracks are switched:
- Line	Switches the track every line. This setting is used for fast acquisitions. It is especially useful for applications where a high temporal correlation of the different fluorescence signals is required.

Parameter	Description
- Frame	Switches the track every frame. It also allows to change emission filters, VSD positions and detector gain between the tracks.
- Frame Fast	Switches the track every frame (fast). It is not possible to move hardware and to set individual PMT gain for the different tracks.
- Z-Stack	A full Z-Stack is acquired per track. Using this setting the acquisition is faster because the hardware is only moved once per stack. This setting is ideal for fixed specimen.

No.	Parameter	Description
1	**Spectral Display**	Shows the spectral range of the activated channels in a spectral display. All channels cover a spectral range from 400 to 700 nm. Below the spectral display, a slider is displayed for each activated channel. The slider has the same color that was chosen for the channel. Channel 1 to Channel 3 are always aligned from left to right. These sliders represent the emission range that is covered with the respective channel. When gripping them with the left mouse button, you can change the range and position to the imaging needs. If you point the mouse on a slider, the cursor changes to a pen icon. If you click on the slider now a sub-menu is opened. Here you can type in the split positions between channels. The flexible direction of light is done with two Variable Secondary Dichroic Beam Splitters (VSD), positioned between the three Channels (one VSD in a two Channel system, respectively).
	- Two channel system	For a two channel system the sliders are fixed at the left and right side respectively and the middle sides can be moved but cannot be separated. So no gap in between the channels is possible.
	- Three channel system	For a three channel system, the behaviors is similar. The range of the middle channel (Channel 2) is freely selectable between 450 and 650 nm. When you need to image the Range below 450 or above 650 nm with

No.	Parameter	Description
		Channel 2, you may not use it in the same track Channel 1 or Channel 3 respectively. To be able to select this range, you have to disable these detectors first.. If adjacent channels are activated, there is never a gap in between them but their spectral range join at their borders.
2	**Emission Filters**	Shows if filters are used for the relevant dye. If you click on the filter bar with the small arrow button you can select an emission filter for the selected dye. Note that there is no emission filter configurable for Channel 2 on a three channel system. The Channel 2 is using a fixed build-in Laser Blocking Filter for 640 nm. 
3	**Detectors List**	Shows all configured detectors in a list. There you can activate the detectors for the experiment and adjust the channels display.
	- Use	If activated, the corresponding detector will be used for the experiment.
	- Dye	If you click on the small arrow button you can select a dye from the dye database. The emission spectrum of the selected dye is displayed in the channel display if the **Use** checkbox is activated.
	- Color	If you click on the rectangle, you can select a pseudo color for the dye. There are different color schemes available (**Color**, **LUT**, **Custom** or **None**).
	- Name	Shows the name of the selected detector channel. If you add a new dye, its name will be adopted to this field. You can change the name of the dye in the channels list of the**Channels** tool.

No.	Parameter	Description
	- Range	Shows the emission range for the selected detector. This range is affected by the VSD position and the emission filter. You can adjust the range by using the corresponding slider under the channel display and by using the emission filters.

20.3.2.1.3 Lambda Mode

The Lambda mode is used for imaging heavily overlapping emission signals. If you have activated this mode, the display changes to show the currently active laser line(s) and the detection range for spectral imaging.

Fig. 20.16: Imaging Setup Lambda Mode

In this mode the system is recording the overall emission from the sample by moving the VSD (Variable Secondary Dichroic) sequentially to different positions over the selected spectral range. During this movement in steps the lower part and the higher part of the spectrum is detected by PMT 1 and PMT 3, respectively. In a

two channel system PMT 1 and PMT 2 are used. These accumulated signals represent a spectral signature of the dye(s) which can be used for spectral linear unmixing.

With this imaging mode, it is possible to acquire an intensity image which displays the intensity of the fluorochrome(s) with down to 10 nm steps in a spectral range from 450 nm to 650 nm. According to the detection range and the chosen number of steps, a certain number of such images is acquired sequentially, which is called a Lambda stack.

In between the spectral range the number of steps the VSD performs to record the spectral information can be set by typing in a number into the box next to **Channels**. The number displays the actual resulting channels, but the VSD will take one step more and the system will perform one scan more as well.

The image data provide the intensity information within the selected detection range for each pixel. Therefore, the data allow deducing an emission spectrum for each pixel corresponding to the emission spectrum of a specific dye.

This calculation is done by linear unmixing using the acquired Lambda stack and allow to clearly separate even heavily overlapping emission signals.

The images acquired in Lambda mode are displayed in the Lambda View, see *Lambda View* [▶ 832].

Parameter	Description
Channels	Here you can set the number of resulting channels (max. 20). If a new channel is added, you can see it in the graphical display above. Per default the step width will be equally distributed.
Start / End	Sets the starting /ending position of the spectral range. You can also use the slider below the graphical display to adjust the positions.
Channel Width	Displays the current step width (in nm) of a channel.
Flexible Split Positions	If activated, non linear step widths can be adjusted. To adjust the position select a marker in the graphical display. The current value is displayed in the **Current Split** input field. Now you can move the marker by holding the left mouse button down. You can also enter a new value in the Current Split input field.

Please refer to chapter *Confocal Acquisition* [▶ 664] for a detailed explanation of the Spectrum and the handling of emission filters.

NOTICE **Using emission filters in Lambda stacks can lead to false results and is considered as an expert setting.**

20.3.2.1.4 Airyscan Mode

This mode is only available if the system is equipped with an Airyscan module.

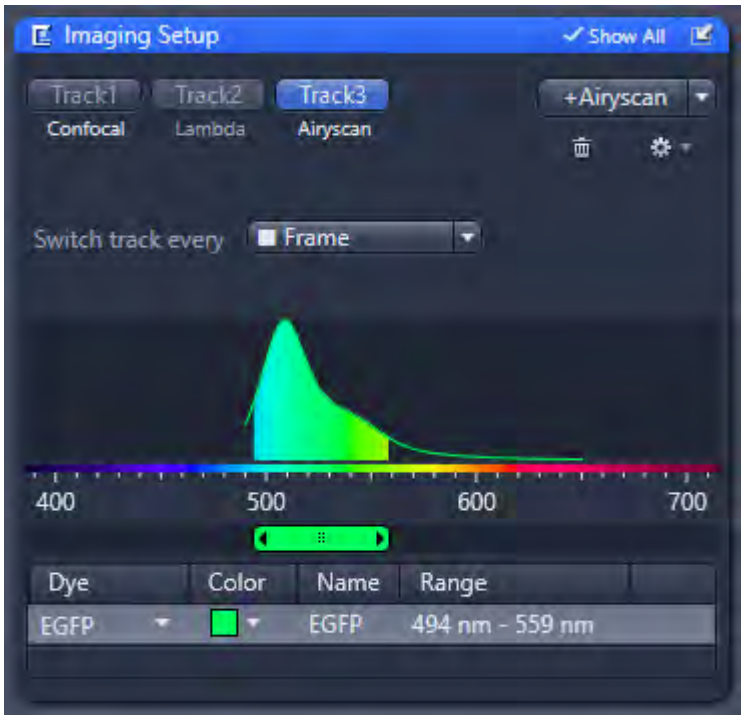


Fig. 20.17: Imaging Setup Airyscan Mode

- In the graphical display of the spectral range only one channel (Channel 2) remains for editing.
- The detection width and position can be chosen within the range of 400 nm - 700 nm.
- For multi-color Airyscan images, multi-track acquisition must be used.
- Line-wise multi-track acquisition using Airyscan is possible when all active tracks are Airyscan tracks.

20.3.2.1.5 Advanced Imaging Setup

Only visible, if the **Enable Advanced Imaging Setup** checkbox in the **Tools** menu | **Options** | **Acquisition** | **Acquisition Tab** | **Enable Imaging Setup** is activated.

There is a switch on top of the tool. By clicking on the button you can switch from the **Standard** to the **Advanced** Imaging Setup.

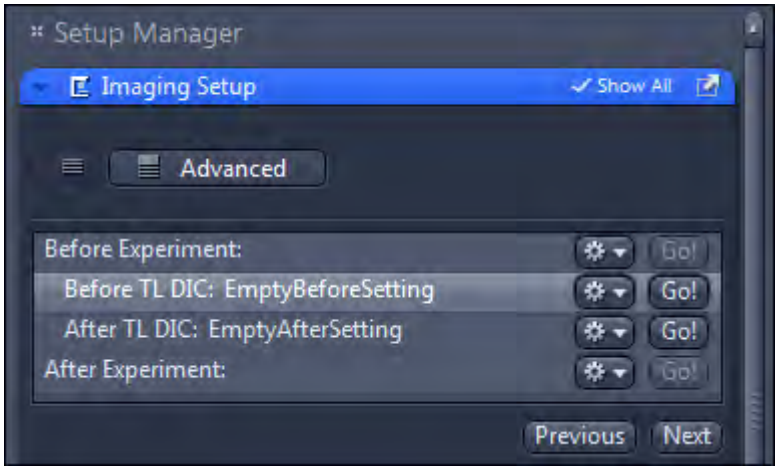


Fig. 20.18: Advanced Imaging Setup

The Advanced Imaging Setup offers additional options for controlling an experiment. The **Before/After Experiment** settings can be found right under the Standard/Advanced button.

Additionally the **Experiment Settings Pool** can be found at the bottom of the tool. We recommend that you talk to ZEISS Service staff or Imaging Specialists before you try to change settings here, as they should be changed only if you know what you are doing.

Before/After Experiment settings

Parameter	Description
Before/After Experiment	Shows the name of the hardware setting that will be applied immediately before or after the experiment.
Options 	Opens the **Options** menu for the specific hardware setting.
Go!	Applies the selected hardware settings.
Previous/Next	The buttons allow you to navigate through the various hardware settings.

Experiment Settings Pool

Parameter	Description
Clear all unused hardware settings from experiment	By clicking on the **Clear** button you can delete all unused hardware settings from your experiment.

Parameter	Description
All available hardware settings in experiment	Here you can see all available hardware settings.

20.3.2.2 Acquisition Mode Tool

In the **Acquisition Mode** tool you can set the various acquisition parameters that you want to apply for the entire experiment.

INFO

If you have created an experiment using the **Experiment Designer** tool, the settings in the **Acquisition Mode** tool only apply to the relevant experiment block and may differ in the next block.

In terms of content and appearance, the Acquisition mode tool is largely dependent on which imaging mode was chosen in the **Imaging Setup tool**, either LSM tracks or widefield channels.

If you have configured LSM tracks, including e.g. Airyscan or Lambda Tracks, please read the chapter *Parameters for LSM Mode* [▶ 672].

If you have configured widefield channels, please read the chapter *Parameters for Widefield Mode* [▶ 677].

20.3.2.2.1 Parameters for LSM Mode

Here you adjust scanning and acquisition parameters that you want to apply for the entire experiment.

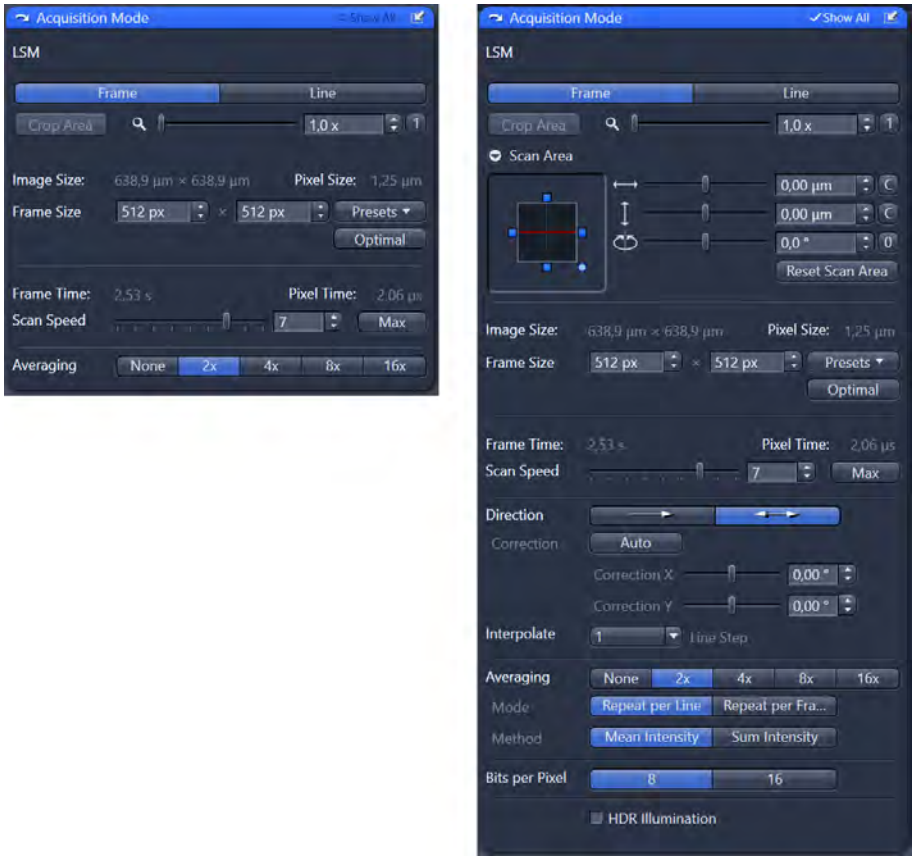
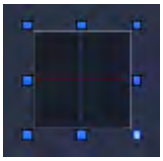
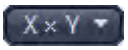


Fig. 20.19: Acquisition Mode tool - Show All options deactivated or activated, respetively

Parameter		Description
Scan Mode		
-	Line	Activates the line scan mode. If this mode is selected you will see the representation of the scanning line in the **Scan Area** section at the bottom of the tool.  Not available for **Airyscan** acquisition, as at least 32 lines are required to perform Airyscan processing.
-	Frame	Activates the frame scan mode. If this mode is selected you will see the representation of the scanning frame in the **Scan Area** section at the bottom of the tool. 

Parameter	Description
Frame Size	Adjust the frame size (in pixel) of the displayed image by entering the desired value in the two input fields.
- X x Y button 	By clicking on this button you can select from a list of default frame sizes (e.g. 128 x 128 or 512 x 512). We recommend to start with 512 x 512 px.
- Optimal button	By clicking on this button the frame size (image resolution) will be set to an optimal value corresponding to the optical magnification (objective), the zoom factor and the emission range detected excitation wavelengths included in the experiment. This provides an image where no spatial information is lost and no empty data is generated as optimal sampling is achieved. The optimal value is calculated for the given objective and magnification settings matching a 2fold oversampling according to 2 fold Nyquist. Rectangular image dimensions are preserved. When you press this button once, it will change its color to blue. This indicates that the optimal sampling will be maintained as you continue to change acquisition parameters like zoom, Laser lines or add more tracks. This active state of the button will be lost once you manually edit the frame size or click the button again.
- Bits per Pixel	Use the buttons to adjust the color bit depth to 8 Bit or 16 Bit (i.e. 256 or 65536 gray values).
Direction	Following scanning directions can be selected:
- Unidirectional 	The laser scans in one direction only, then moves back with beam blanked and scans the next line.
- Bi-directional 	The laser also scans when moving backwards, i.e. the scan time is reduced by about a factor of two. Please note that the pixel shift between forward and backward movement (double image) resulting from bi-directional scanning must be corrected. To do that use the **Correction X** / **Correction Y** sliders. By clicking on the **Auto** button an automatic scan correction will be performed. For optimal results this correction should be repeated every time scan parameters like rotation, frame size, zoom or speed are changed.

Parameter	Description
Interpolate	Only available if the **Frame** Scan Mode is selected. Select the desired line step size (from 1-10). According to your selection only every n-th line is scanned. The lines in between are interpolated. This fast scan mode is called Step Scan. This feature is not available for Airyscan acquisition.
Scan Speed	Set the scan speed by adjusting the slider from 1 (slow) to 16 (very fast). The corresponding values for **Pixel Dwell** and **Scan Time** will be displayed below the slider. Please note that the available maximum scan speed depends on the selected **Frame Size** and zoom factor. Maximum Speed is available with a zoom factor of 6.5x. By clicking on the **Max** button the maximal possible scan speed will be set automatically. When you click this button once, it will change its color to blue. This indicates that the system will always use the highest possible scan speed as you continue to change acquisition parameters like zoom or frame size. Click the button again to deactivate this permanently active state.
Averaging	
- Number	Select the number of images you want to average (2 - 16).
- Method	Select the method which will be used for averaging: ■ **Mean**: Uses the mean average of all images ■ **Sum**: Uses the sum of all images.
- Mode	Select the mode for averaging :

Parameter	Description
	■ **Frame**: The calculation of the average is based on individual full frames. Each frame is scanned quickly, but there is a higher delay between the individual data sets taken for the averaging. ■ **Line**: The calculation of the average is based upon individual lines, which are acquired sequentially before moving on. By this, the data used for the averaging are is closely connected, but the acquisition of the full frame takes respectively longer.
HDR Illumination	This parameter is only available if you have licensed the **HDR Blue Confocal Basic** module. If activated, a HDR effect will be applied to the image. This effect will boost weak structures without saturating bright areas in the image and enable an optimal representation of the morphology of weak and bright objects within the same image. To achieve this, the image will be scanned three times with increasing the excitation intensity. Areas in the image, that displayed overexposure will be excluded in the following scans in order to avoid photobleaching. It is recommended to use 16bit for the acquisition of HDR datasets. *HDR was developed based on ideas and a concept of O. Ronneberger and R. Nitschke (Albert-Ludwigs-University Freiburg, Department of Computer Science and Life Imaging Center at ZBSA).*
Scan Area	In this expandable section, you can adjust the position of the scan area. The outer frame corresponds to the field of view of the microscope. The inner frame represents the scan area. All changes of **Offset** and **Rotation** made in this section will be immediately applied to the scan area. Following functions are available:

Parameter	Description
- Offset	Adjust the offset by using the **Left** / **Right** or **Up** / **Down** sliders. You can also enter a specific value in the input field. If clicking on the **C** button behind the input filed the offset position will be reset to center position. If you left click on the inner frame and hold down the mouse button you can move the scan area freely. The positions in the input fields will be adopted according to your adjustments.
- Rotation	Adjust the rotation degree by using the **Rotation** slider. You can also enter a specific value in the input field. If clicking on the **O** button behind the input field the rotation degree will be reset to default position (zero degree).
- Reset Scan Area	Resets all adjustment of **Rotation**, **Offset** and **Zoom** to the system defaults.
Zoom	Adjust the zoom level (from **0.5x** - **40x**) by using the **Zoom** slider. You can also enter a specific value in the input field. If clicking on the **1** button behind the input field the zoom level will be reset to default (1,0x) for confocal acquisition and 1.3x if an Airyscan track has been configured.

20.3.2.2.2 Parameters for Widefield Mode

20.3.2.2.2.1 Camera Section

In this section you can adopt camera settings from the active camera to your experiment and adjust basic camera settings.

Parameter	Description
Get Settings from Active Camera	
- Get	Applies the settings from the active camera to your experiment.
- Default	Resets the camera settings to factory default.

Parameter	Description
Binning	Here you can set the binning. Binning combines the information of neighboring camera pixels into a single larger pixel. For example, if the binning is set to 2 × 2, four pixels are combined to one. Increasing the binning means weaker signals can be detected for a given exposure time. For CCD cameras, binning increases sensitivity by improving the signal-to-noise ratio, with resolution being decreased by the same factor. In the case of CMOS cameras, only the signal intensity is increased and the pixel count and resolution gets reduced correspondingly.
Binning-independent Brightness	Because **Binning** generally increases signal intensity, the brightness of the image normally also increases correspondingly. By activating this checkbox, the brightness level is automatically fixed (depending on the camera, either through exposure time adjustment or averaging), no matter the binning setting.
IP Quality	Here you can select the color interpolation quality (IP Quality) for the acquired image. Please notice that this function does not apply to Live mode. "**Fast"**: color interpolation for optimum speed (shorter computation) "**High**": color interpolation for optimum quality (less artifacts). This mode is only effective with binining factor 1.
Subsampling	Here you can reduce the amount of data acquired to acheive faster framerates. By subsampling 2x2, the effective pixel pitch is increased by sampling only every other pixel, thus reducing the overall data size of your image.
Resolution	Here you see the camera resolution, e.g. 1024 x 1024 px

20.3.2.2.2.2 Acquisition ROI Section

In this section you can define a **Region Of Interest (ROI)** on the camera sensor which will be used for acquisition. A smaller ROI can increase the acquisition speed.

The region of interest is indicated by a blue frame in the preview window and can be moved and resized freely. The preview window always shows the entire camera sensor area which can be acquired.

The **Pixel Size** shown below the preview window indicates the size in µm to which a pixel corresponds. This depends on the camera sensor properties and on the binning.

Parameter	Description
Maximize	Selects the entire available image sensor area as the region of interest.
Center	Positions the region of interest precisely at the center of the image.
Size	Sets the width and height of the region of interest in pixels.
Offset	Specifies the position of the top left corner of the **Acquisition ROI** (blue frame) with respect to the top left corner of the preview window.
Refresh Overview	Acquires and displays an image in the preview window with the current ROI settings. This has no effect on the image in the **Center Screen Area**.
Crop	Only visible if the checkbox **Show Crop Button** under **Tools** | **Options** | **Acquisition** | **Camera/Live** is activated. Allows you to specify a ROI (Region of Interest) in a snapped image. The "cropped" area is used as ROI for the next image acquisition. If no image is available in the **Center Screen Area** the button is deactivated.

20.3.2.2.2.3 Fast Acquisition Section

In this section you can set 3 different modes for acquisition.

Parameter	Description
Interactive	Using this mode you can intervene manually at certain points during acquisition. The acquisition is comparatively slow.
Compromise	This mode is activated automatically if only individual hardware components, but not the whole system, are compatible with the Triggered mode for acquiring an experiment.

Parameter	Description
Triggered	Fast acquisition via the hardware.
- Validate	To establish whether the system is able to perform an experiment in **Triggered** mode, click on the **Validate** button. The validation result is displayed in the info box below the button bar.

20.3.2.2.2.4 Post Processing Section

In this section on the **Acquisition** Tab in the **Acquisition Mode** tool you can apply basic image processing functions while acquiring the image. This can be helpful if certain image processing steps are necessary for any acquired image and saves image processing work later in a job. Depending on the camera model, different parameters can be available.

Parameter	Description
Black Reference	Influences the live image and each image acquired. For the black reference to work, you first need to acquire a reference image. Define a corresponding reference image using the **Define** button. **Activated:** Applies the measured black reference to the image. **Deactivated:** The measured black reference is not used. The reference image is retained. If longer exposure times are used (from exposure times of approx. >5s, depending on the camera), individual bright pixels may become visible with CCD or CMOS sensors. With the help of the black reference these effects are measured and corrected in accordance with the exposure time employed. It is recommended that you repeat this measurement at certain intervals. This correction is recommended in particular for applications that involve long exposure times, i.e. for which very little light is used (live cell imaging, fluorescence images). The availability of a black reference for the selected camera can be checked on the menu **Extras** | **Calibration Management**.

Parameter	Description
- Define	Automatically defines the black reference. The measurement lasts for several seconds. The **Black Reference** checkbox is then activated automatically.

INFO

For the measurement the camera must see a completely dark image. The light path to the camera must therefore be blocked. Set the eyepiece switch to 100% eyepiece and close the reflected light/transmitted light shutter. To define the black reference, click on the **Define** button.

Parameter	Description
Enable Noise Filter	**Activated:** Filters the noise in the acquired image according to the adjusted threshold. Affects acquired images only. The live image does not change.
- Threshold	The higher the value, the greater the tolerance for noise. The lower the value, the stronger the noise reduction. The noise filter reduces the extent to which individual pixels deviate from the average value of their nearest neighbors. The **Threshold** corresponds to a tolerance value. If the deviation of the middle pixel value from the average value of the pixels immediately surrounding it exceeds the tolerance value (i.e. it is interpreted as noise), it is replaced by the average value. This technique reduces the noise of individual pixels that are produced, in particular with EMCCD cameras and CMOS cameras. The selected technique prevents any changes being made to object edges, as in most cases these are larger than individual pixels. This filter is also suitable for removing individual "hot pixels" from an image without having to acquire a reference image in advance.
Enable Unsharp Mask	Enhances contrasts at fine structures and edges. Thus, the resulting image appears clearer and enriched in detail.
- Strength	Controls the amount of contrast enhancement applied to fine structures and edges. The higher the strength, the darker or lighter the resulting edges, compared to the original image.
- Radius	Determines the size of detail to be enhanced. A small radius enhances smaller details.

	Parameter	Description
		The radius also affects the appearance of enhanced edges. A large radius leads to a visible halo along enhanced edges. The larger the radius, the broader the halo.
-	Color Mode	Determines the calculation method, which affects the appearance of the output image. ■ **RGB**: – The **Unsharp Mask** filter calculates the sharpness for each color channel individually. – The color saturation and the color of structures may be changed and color noise may occur. ■ **Luminance**: – The **Unsharp Mask** filter calculates the sharpness based on the luminance signal computed from the RGB channels. – This mode avoids possible color noise or shift in color saturation, which could be induced by certain image textures.
-	Auto Contrast	**Activated:** Enables you to adjust the **Contrast Tolerance** (0-20). **Auto Contrast** only works in RGB color mode.
-	Contrast Tolerance	Increasing the contrast during unsharp masking is achieved by broadening the distribution of intensities. This corresponds to a spread of the image histogram. Controls how much the intensity distribution is spread and thus how strong the contrast is increased. ■ **Contrast Tolerance = 0** : No spread of intensities, no increase of contrast ■ **Contrast Tolerance = 20**: Maximum spread of intensities, maximum increase of contrast
-	Clip To Valid Bits	**Activated:** Composes the processed image of the same colors as the original image (i.e. the value range of the output image is adjusted to the color range of the input image). **Deactivated:** Colors not present in the original image may appear in the processed image.

20.3.2.2.2.5 Mode Section

In this section you can adjust how the software retrieves the camera sensor data.

Parameter	Description
Color Mode	This parameter is available for color cameras only.
– RGB	Transmits the image data of a color camera unchanged. This corresponds to the standard operating mode of a color camera.
– B/W	Treats the image data of the color channels as grayscale. The data of related color channels are averaged. The saturation of the camera appears reduced as a result. This process does not change the spectral properties of a color camera. The image information of the color sensor still undergoes color interpolation. An infrared filter also restricts the spectral sensitivity of the color camera compared to the spectral sensitivity of a genuine black and white camera.
Live Speed	Here you can select the live image update speed (slow, medium, fast). Enables you to focus or to find regions of interest on a sample quickly. A high live image update speed reduces the exposure time of the live image, even at longer exposure times used for image acquisition. To achieve a similar impression of image brightness, however, the image data supplied must be adjusted digitally, which may generate a certain amount of noise or reduce the resolution of the live image.
IP Quality	Only available for **Axiocam 503** color and **Axiocam 506** color. Here you can select the color interpolation quality (IP Quality) for the recorded image. Please notice that this function does not apply to **Live** mode. If you reset the settings via the **Default** button the **IP Quality** is set to high.
– Fast	Represents the image optimized and requires a shorter computation time.
– High	Represents the image without artifacts and with a higher image quality. This mode is only effective with binining factor 1.

Parameter	Description
NIR Mode	This function is only available for cameras which offer this feature (e.g. **AxioCam MRm**). **Activated:** Uses the camera in the near infrared range (NIR=Near-Infrared). Sensitivity for signals in the near infrared range (approx. 700nm to 1000nm) is increased. However, the tolerance for overexposure is reduced and, if very bright structures are present, overexposure artifacts (blooming) can result. We recommend that you use this mode mainly for very weak signals.

20.3.2.2.2.6 Model Specific Section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button .

In this section you see additional, model-specific camera settings depending on which camera you use on your system.

Reset button

Resets all entries to the original values.

20.3.2.2.2.6.1 Axiocam 105

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 180**

Acquire section

Gain Boost checkbox
If activated, the image signal is amplified so that the image becomes brighter. The gain factor is 1.7x. This factor is in addition to the standard Gain control.

20.3.2.2.2.6.2 Axiocam 506

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Acquire section

Readout Speed (MHz)

Readout speed can be varied between 39 MHz and 13 MHz if the camera is operated on the USB3.0 bus. In case the camera is connected to the slower USB2.0 Bus only the 13 MHz mode is available. At the slower 13 MHz the signal quality is slightly improved due to a reduced noise of the signal transmission.

Cooling

Status information, if camera cooling is active. The Axiocam 506 can be operated without active cooling. Cooling is deactivated if the USB2.0 connector of the camera is not supported to the PC or to a USB compatible power supply.

Readout Port

The Axiocam 506 uses a high performance CCD sensor with four readout ports. It can be adjusted to quadport, dualport, singleport and Auto mode. Maximum speed is reached by using all four ports and short exposure times. When exposure time

gets larger than the readout time the benefit for using multiple ports is getting insignificant. By switching the readout mode to single port, the most homogenous signal quality can be reached as all data is sent through one single processing chain. In Auto mode, the number of used readout ports is selected automatically depending on the exposure time.

Readout time (ms)

The valid camera readout time is given in this status window which is defined by the number of used ports or by defining a sensor sub region window (ROI).

Temperature

The valid CCD sensor temperature is shown here. It is adjusted to 18 C°. It can not be changed. If a black reference is used it should be used at the same sensor temperature when it was created.
If free air circulation for the camera housing is blocked, it may happen that the sensor temperature is increased and the dark current of the sensor may be higher than normal. If the camera is operated without cooling (USB2.0 port of camera not connected) the sensor temperature is increased and dark current will be higher than normal. This should be considered when using the camera at longer exposure times.

Expert section

Abort on missed frames

In case of high speed time lapse acquisition the camera is sending an enormous amount of data to the PC. If the PC is not fast enough in handling the image data it may happen that dropped frames occur. By this parameter the behavior of the acquisition routine can be defined: it is the default mode to abort a acquisition sequence in case of a dropped frame. If unchecking this the event of a dropped frame is ignored and the acquisition is continued without further notification.

Main LED

The LED in the camera lid shows the general operation mode of the camera by showing different colors. In special low light applications it may be desirable to minimize stray light in the microscopy workspace. Therefore the intensitiy of the status LED can be dimmed or switched off completely. The following status informations are available:

Red: after plugging the main USB3.0 cable of the camera to a PC, camera firmware is not yet loaded by driver. As soon as the camera gets loaded with firmware, the color changes one of the following colors:

Yellow: connected to USB2.0 bus, only 13 Mhz clock speed available, no cooling (right USB port connected to USB2.0, left USB port not connected),

Green: connected to USB 2.0 bus, only 13MHz available, cooling active (right USB port connected to USB 2.0 bus, left USB port connected to computer USB port, or separate USB power supply),

Pink: connected to USB3.0 bus, full 39 MHz clock speed available, no cooling (right USB-port connected to USB3.0, left USB-port not connected)

Blue: connected to USB3.0 bus, full 39 MHz clock speed available, cooling active (right USB-port connected to USB3.0, left USB port connected to computer USB port, or separate USB power supply)

Red: wile exposure of sensro is active, in Live mode or acquisition of image series the LED is blinking, therefore.

Trigger LED

A second LED is at the camera back, above the Micro-d connector for the trigger cable. This LED indicated the status of the trigger port. It is only active, in case the trigger port is used.

In special low light applications it may be desirable to minimize stray light in the microscopy workspace. Therefore the intensitiy of the status LED can be dimmed or switched off completely. The following status informations are available:

Green: asynchronous triggering is possible (no jitter in line timing),

Yellow: synchronous triggering is possible (some jitter in line timing),

Red: no trigger accepted,

Blue: external trigger signal active,

Tile Adjustment

Camera expert section shows camera parameters which need deeper understanding of functionality.

The Axiocam 506 camera is using a special CCD sensor with four readout ports in order to read out the image data from the CCD area as fast as possible. It is assured by proper alignment of the electronics that all associated signal paths are absolutely equal and no quadrants get visible as this is very undesirable for good image quality. In order to offer the best quality possible, a special algorithm is activated by default as an additional safety measure to suppress remaining residuals. This correction can be deactivated in order so minimize image processing on the camera raw data.

Acquire (Expert) section

Camera **Expert** section shows camera parameters which need deeper understanding of functionality.

8 bits compression

In case other devices are using bandwidth on the connected USB 3.0 data bus, it is possible to reduce the amount of image data sent by the Axiocam 506 by activating data compression from 14 bit to 8 bit. By converting the data through a square root loaded lookup table the 14 bit values are converted to 8 bits per pixel.

This is handled transparently as the data is decompressed automatically by the camera driver in the PC while receiving it. As compression has some slight impact on data quality it is deactivated as default.

Frame time (ms)

In case of fast time lapse acquisition the camera is sending huge amount of image data to the PC within a short time. The data needs to be handled by the PC without delay. There is a certain risk of dropped frames, if the camera is sending data faster, than the PC can handle it, especially at very short exposure times. By setting a frame time larger than zero, an acquisition delay can be defined for continuous acquisitions. Max. value is 5000 ms. Default value is zero.

Readout mode

This is a status information and shows the currently used number of used sensor ports. Values are: quad port, dual port, single port.

Adjust Live Frame Rate

The camera live image can send a lot of image data which need to be processed on the fly. In case of less powerful computers this can overload the PC and cause a slow reaction of the ZEN user interface. By activating this function the slider below gets accessible and a maximum limit for the live frame rate can be adjusted. Abundant image data is then discarded accordingly and not processed to be displayed.

Live Frame Rate Max

Adjustment of maximum accessible frame rate for live display, only. Only accessible if "Adjust Live Frame Rate" checkbox is activated.

Live Frame Rate

This is a display field only. It shows the measured live image speed.

20.3.2.2.2.6.3 AxioCam ERc5s

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Sharpness

Using this function you can increase the impression of sharpness in an image.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 180**

20.3.2.2.2.6.4 AxioCam ICc5

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

20.3.2.2.2.6.5 AxioCam HRc

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**

- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Readout Speed (MHz)

The **High Speed** mode activates the faster 24 MHz mode, for which the digitization accuracy is set to 12 bits per pixel. This mode offers advantages if sufficient light is available and situations need to be acquired quickly: fast time series or tile images.

In **High Accuracy** mode the readout speed is 12 Mhz and the digitization accuracy 14 bits per pixel. This mode offers advantages if very little light is available and you want the camera to acquire very weak signals just above the camera's noise level.

20.3.2.2.2.6.6 AxioCam MRm

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

20.3.2.3 ApoTome Mode Tool

Parameter	Description
Enable ApoTome	**Activated:** Uses the ApoTome for acquisition and experiments.
Phase Images	Select here the number of phase images per optical sectioning. 5 phase images are the default value.
Live Mode	Here you set the display of the **Live Mode**. Default value is **Grid Visible**.

20.3.2.4 Channels Tool

INFO

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**.

In the **Channels** tool you can configure channels for Confocal or Widefield acquisition. The tool offers you the option of entering the hardware settings for acquisition manually or performing the configuration automatically.

If there is no channel available, you will be asked to add a **LSM** or a **WF** channel to the experiment. Therefore click on the corresponding button.

Fig. 20.20: Channels Tool (without channels)

The following tool appearance is only visible if you have added one or more channels:

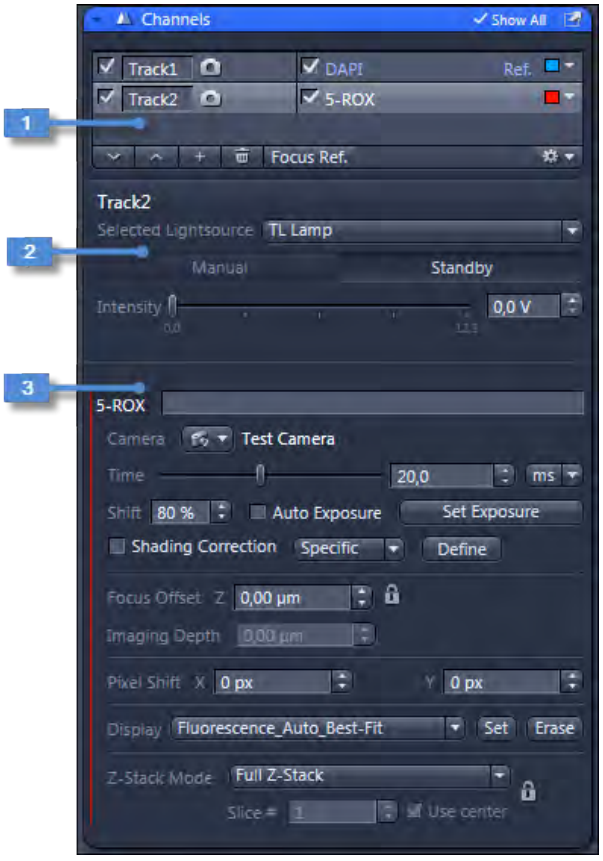


Fig. 20.21: Channels tool

1 *Channels list* [▶ 692]
2 *Light Source section (WF)* [▶ 695]
3 *Channel Settings (WF)* [▶ 695]

20.3.2.4.1 Channels list

Please bear the following points in mind for the channels list:

- The selected channel is highlighted by a light gray bar.
- The reference channel for the auto focus is highlighted by a blue font color and marked with **Ref.**.
- The preview color (LUT) for the channel is shown on the right side of the list. To change the preview color for the channel, click on the colored rectangle with the arrow icon and select an alternative color from the shortcut menu. The preview color is also shown in the sections for channel-specific hardware settings as a thin line on the left side of the list.

INFO

If you select a dye or contrast technique in the **Add Dye or Contrasting Method** dialog, a suggestion for the hardware settings for the acquisition of this channel is made automatically. If no suggestion can be made, a channel without hardware settings is added. You will then see a corresponding indication in the status area of the program interface.

List control elements

Parameter	Description
 **Down**	Navigates one row down.
 **Up**	Navigates one row up.
 **Add**	Opens the *Add Dye or Contrasting Method Dialog* [▶ 776].
 **Delete**	Deletes the selected channel.
Focus Ref.	Sets the selected channel as reference channel for focus actions or stitching during acquisition.
 **Options**	Opens the *Options* [▶ 694] shortcut menu.

Laser Range section

Parameter	Description
High Intensity Laser Range	**Activated:** Uses a high intensity laser range , where you can adjust the lasers between 0.2 and 100 % of their power. This is especially relevant for bleaching experiments. The setting affects all tracks. While switching, the system is trying to keep the intensities at a similar level. If the currently selected intensity is outside of the overlapping range (0.2 % to 3.5-5 %) the closest possible value is used.

Parameter	Description
	The LSM 800 is working by default in a laser power range between 0.01 % and 3.5-5 % of the available laser intensity. The available maximum in the default range is depending on the laser wave length.

20.3.2.4.1.1 Channels Tool Options Menu

Parameter	Description
Add New...	If **WF** channels are added this function opens the *Add Dye or Contrasting Method Dialog* [▶ 776] to add more WF tracks. If **LSM** channels were added this function will add a new LSM track.
Duplicate	Creates a new track with the same settings and dye as the currently selected track.
Rename	Assigns a new name to the channel of the currently selected track. To change the name of the track, you can directly click into the respective field of the channels list.
Reset Color	Resets the color of the selected track(s) to default.
Select All	Selects all tracks of the list.
Delete	Deletes the selected track.
Delete All	Deletes all existing tracks.
Set as Reference Channel	Defines the selected channel as the reference channel for focusing actions. Note that, you can also set Channels of inactive Tracks as Reference Channels. The Autofocus will in this case be performed on this channel, but the Channel or Track will not be part of the resulting image document. Using this approach, e.g. Camera tracks may be used for fast focusing within a Confocal experiment, while the acquisition of Camera and Confocal into one image is not possible.
Compare...	Opens the **Compare channels** dialog, where the active channels are displayed horizontal so you can easily compare and adjust key parameters of the active tracks.

20.3.2.4.2 Channel-specific settings (WF)

The settings always relate to the channel you have selected in the **Channels** list.

To show the settings for all channels, click on the button | **Select All** in the **Channels** list.

20.3.2.4.2.1 Light Source section (WF)

Fig. 20.22: Light Source Section

In this section you can select the available light sources from the **Selected Lightsource** dropdown list and adjust the corresponding settings. You can adjust the parameters of the light sources without having to save these in the hardware settings. You can therefore adjust the intensity of the laser lines or LEDs, for example, immediately before starting an acquisition.

If your system is equipped with a TIRF slider, the TIRF angle and type of illumination can also be set here.

If you select the **Use Setting** entry, the settings for the light sources disappear. The light source parameters from the hardware settings are used instead for the acquisition of the channel.

20.3.2.4.2.2 Channel Settings (WF)

Parameter	Description
Dye name	In the input field after the selected dye you can enter an additional name.
Camera	Select the desired camera for the channel from the dropdown list.
Time	Adjust the exposure time for the camera using the slider or spin box/input field. Select the unit of time from the dropdown list at the right of the spin box/input field.
Shift	Only visible if the **Show All** mode is activated. Here you can enter the range of the camera's dynamic range that is utilized.

Parameter	Description
Auto Exposure	**Activated:** Automatically determines the camera's exposure time for the selected channel. The value set manually is ignored.
Set Exposure	Starts an exposure time measurement for the channel. After the measurement the value is adopted as the exposure time setting.
Shading Correction	**Activated:** Uses the calculated shading correction for this channel. To learn more about shading correction read the chapter, *Post Processing Section* [▶ 649].
- Define	Automatically calculates the shading correction.
- Specific	Performs channel-specific shading correction.
- Global	Performs an objective specific shading correction. This is the default method for shading correction.
EM Gain	Only visible for EMCCD camera models. Sets the EM gain value.
Focus Offset	Only visible if the **Show All** mode is activated. Here you can enter the focus offset from the channel to the Z-position of the reference channel or to the current position. The **Lock** icon shows that this setting will be synchronized between all channels of this track.
Pixel Shift	Only visible if the **Show All** mode is activated. Here you can define the pixel shift in **X** and **Y**. The defined pixel shifts are applied to images collected with Snap, contentious or in an experiment.
Display	Only visible if the **Show All** mode is activated. Here you can select an existing predefined display setting to be automatically applied to this channel after acquisition. Please note, that this does not apply for Live acquisition. There always the last used setting will be applied.

Parameter	Description
Z-Stack Mode	Only visible if the **Z-Stack** checkbox is activated in the **Experiment Manager** and while **Show All** mode is active.
- Full Z-Stack	Acquires the Z-stack as defined in the **Z-Stack** tool.

Parameter		Description
-	Single slice only	Acquires a single slice of the Z-stack only. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
-	Single slice, rest black	Acquires an image of a single slice of the Z-stack only. All other Z-slices of the stack are filled with black images. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
-	Fill with single slice	Acquires an image of a single slice of the Z-stack only and fills all other Z-slices with this slice. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.

20.3.2.4.3 Channel-specific settings (LSM)

The settings always relate to the channel you have selected in the **Channels** list.

To show the settings for all channels, click on the  button | **Select All** in the **Channels** list.

20.3.2.4.3.1 Lasers section

Fig. 20.23: Lasers Section

Parameter	Description
Laser	Here you can activate the desired laser line for the selected track. Activate the required lasers by activating the corresponding checkbox. The laser lines along with sliders will appear. Set the required attenuation (%) using the sliders, the arrows, or typing in the input field.

Parameter		Description
Pinhole		Adjusts the diameter of the pinhole. The diameter is specified in micrometer. The text below translates this diameter to Airy Units and section thickness for the configured wavelength. Note that this control is not available for Airyscan tracks. Here the physical pinhole is automatically opened and set to the optimal diameter.
-	1 AU	Sets the pinhole diameter to a value that is corresponding to 1 Airy unit for the configured detection wavelength. When you click this button once, it will change its color to blue. This indicates that the system will always use a pinhole diameter corresponding to 1 AU as you continue to change acquisition parameters like excitation or emission wavelengths. Click the button again to deactivate this permanently active state.
-	Max	Opens the pinhole to its maximum diameter. This can be useful to find the focal plane.

20.3.2.4.3.2 Channel Settings (LSM)

Parameter	Description
Master Gain	Here you can control the voltage of the PMTs. Higher voltage increases the gain of the PMT. The image becomes brighter and you may be able to reduce the laser power. At higher voltage, the noise level in the image increases. The optimum between gain and noise depends on your experimental requirements and on your sample. The maximum available voltage is depending on the type of the detector and is 1200V for multialkali PMTs, 900V for GaAsP PMTs and 1000V for the Airyscan. GaAsP PMTs and the Airyscan have a minimum voltage of 500V.
Display	Here you can select an existing predefined display setting to be automatically applied to this channel after acquisition. Please note, that this does not apply for Live acquisition. There always the last used setting will be applied.

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Digital Offset	This control element is not available for Airyscan tracks. Here you can perform adjustments on the background of the image.
Digital Gain	Here you can digitally amplify the signal.

Parameter		Description
Z-Stack Mode		Only visible if the **Z-Stack** checkbox is activated in the **Experiment Manager** and while **Show All** mode is active.
-	Full Z-Stack	Acquires the Z-stack as defined in the **Z-Stack** tool.
-	Single slice only	Acquires a single slice of the Z-stack only. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
-	Single slice, rest black	Acquires an image of a single slice of the Z-stack only. All other Z-slices of the stack are filled with black images. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
-	Fill with single slice	Acquires an image of a single slice of the Z-stack only and fills all other Z-slices with this slice. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.

20.3.2.5 Focus Strategy Tool

Here you can select the focus strategy that you want to apply. The strategies that are available depend on the dimensions selected (Z-stack, time series, tiles), the hardware devices present (e.g. Definite Focus) and software licenses (e.g. **Software Autofocus** module).

In general focus strategies determine and/or update a Reference Z-Position, which in most cases is used directly for acquisition.

i INFO

The software automatically selects the most appropriate focus strategy e.g. **Use Focus Surface/ Z Values Defined by Tiles Setup** when you activate the tiles dimension and no focus strategy i.e. other than **None** has been selected by you previously.

Exceptions

- When Z-stacks are acquired the center of the Z-stack determines the Reference Z-Position.
- Defined offsets for channels and Z-stacks shift acquisition in relation to the Reference Z-Position.
- If two focusing methods are combined, the Reference Z-Position of the first method is used as the starting point for the subsequent method.

Depending on your system, experiment configuration and licensed modules, the following strategies are available:

Strategy	Description
None	No focus strategy has been selected. This is the default setting for all experiments that do not include a tiles dimension. In this case the software automatically selects the **Use Focus surface/ Z Values Defined by Tiles Setup** strategy. The current Z-position at the time the experiment is started is set as the Reference Z-Position and remains unchanged during the experiment. **Exception:** By default, Z-stacks are acquired at the fixed Reference Z-Position that has been defined as the center in the Z-Stack tool. You can change this setting in the **Z-Stack Acquisition** section of the **Focus Strategy** tool.
Software Autofocus	Only available if you have licensed the **Software Autofocus** module. Note that any focus surface / z-values defined in the tiles setup are ignored if this strategy is selected. If you want to use the values setup in the **Tiles** tool select the strategy **Use Focus Surface/ Z Values defined by Tile setup strategy** The focus position is determined via the sharpness calculation or intensity calculation of a series of images (Z-stack) and set as the Reference Z-Position. The settings are configured in the **Software Autofocus** tool.
Definite Focus	Only available if your microscope system has attached a **Definite Focus** device.

Strategy	Description
	Note that any focus surface / z-values defined in the tiles setup are ignored if this strategy is selected. If you want to use the values setup in the **Tiles** tool select the strategy **Use Focus Surface/ Z Values defined by Tile setup strategy** Definite Focus attempts to maintain a certain distance to the cover glass of the sample in order to compensate for mechanical and thermal movements. The Definite Focus is initialized at the start of the experiment by setting the current distance as the reference distance. You will be requested to define this value at the start of the experiment. When the focus is stabilized during the experiment, the current distance is adjusted to the reference distance. This is achieved by moving the focus drive accordingly. The new Z-position resulting from this is used as the Reference Z-Position for acquisition. Two modes are available:
Stabilization Event Repetitions and Frequency	Only available for **Definite Focus** in combination with **Tiles** or **Time Series** experiments.
- Standard	This mode uses default settings for stabilization which we are recommend to use if you are not familiar with the Definite Focus device.
- Expert	This mode allows advanced settings for using Definite Focus stabilization. Under **Synchronized with Image Acquisition** you can select how Definite Focus is used: ■ **Time Series** If activated, this setting repeats Definite Focus at certain predefined points within a image acquisition loop (e.g. every 2nd time point). ■ **Tile Regions / Positions** If activated, this setting repeats Definite Focus at certain **Region / Positions** (e.g. every 2nd Position). You can also select to run the stabilization for each tile region optionally at either the center or the first (acquired) tile of the region.

Strategy	Description
	■ **Tiles** If activated, this setting repeats Definite Focus at a certain **Tile** (e.g. every 2nd Tile) Under **During Time series interval** you can enable Periodic Stabilization. **Periodic Stabilization** is available for experiments that include **Time Series** only. If activated, a stabilization with a defined **Period** (e.g. every 10 s) will be performed. This mode is useful if long intervals are needed between image acquisition loops. This mode can be combined with the stabilization events before discrete imaging loops.
Combine Software Autofocus and Definite Focus	Only available for **Tiles** or **Time Series** experiments. This strategy allows you to combine the functions of Definite Focus and Software Autofocus. This can be done in two ways, see descriptions below. Note that in both cases it is possible to modify the time point and frequency at which these events occur. The repetitions and frequency of these events is performed with predefined standard settings. If you wish to adjust these for a particular experiment select the **Expert** mode (only visible in **Show all**).
- Software Autofocus as Reference for Definite Focus	Software Autofocus moves the focus drive to the focus position that has been calculated. Taking this as the starting point, a new reference distance is defined for the next distance stabilization performed by Definite Focus. This can reduce the likely-hood of a stabilization failure when the sample is long and elongated and the carrier possibly tilted.
- Definite Focus as Start for Software Autofocus.	The last valid Reference Z-Position defined by Definite Focus is the starting position for the Software Autofocus search. This allows you to optimize the search range and step size of Software Autofocus.
Use Focus Surface/Z Values Defined by Tile Setup	Only available if you have licensed the **Tiles** module. This strategy is selected automatically when the **Tiles** dimension is activated and no previous strategy was selected i.e. **None**.

Strategy		Description
		In the **Tiles** module (optional module) a focus surface can be defined in two ways: Local (for tile regions and/ or positions) Global (based upon a carrier e.g. petri dish, slide, plate)
-	Local (per Region/ Position)	**For Tile Regions:** A local focus surface can be defined for a Tile region in two ways: ■ 1) For large tile regions or samples in which the plane of interest ("focus") is described by a slope or parabolic you can define one or more "Support points" for each tile region. These can be arrange in a regular or irregular manner. Each support point is assigned a discrete z-value ("focus"). From these the software is able to interpolate a local focus surface with a chosen degree of complexity in an attempt to describe the contour of the plane of interest for the Tile region. ■ 2) When no support points are used the z value assigned to the Tile region defines the "focus" for all the tiles it contains. For small tile regions on a suitable sample this might be sufficient. **For Positions:** For positions the local focus surface is defined by the discrete Z-value assigned to it. A position cannot have support points.
-	Global (Carrier based)	A global focus surface is defined based on a selected carrier template. To create a global focus surface you need to add support points by creating or editing a carrier template from the appropriate section of the Tiles tool. Thus, a group of support points are used to help describe the tilt or curvature of the carrier (again by a process of interpolation). Tile regions or position "placed" upon this global focus surface are mapped onto it accordingly.
Adapt Focus Surface/ Z-values		If activated, you can adapt the focus surface / z values by the following options (if available):
-	**Definite Focus** / **Software Autofocus**	Here you select if you want to adapt the settings wether by using Definite Focus or Software Autofocus (SWAF). Depending on this selection and the available dimensions of your experiment the following additional functions can be selected from the second dropdown list:

Strategy	Description
- SWAF / **As additional action**	The **Reference Z-Position** calculated from the Local Focus Surface is used as the starting point for an additional software autofocus search which updates the Reference Z-Position. This allows you to reduce the search range and step size of Software Autofocus (faster).
- SWAF / **Update with single offset**	This option is available for **Time Series** experiments only. This option allows a focus surface (local or global) to be updated by the activity of a software autofocus run. The focus surface is regularly adjusted by means of a software autofocus search which is performed exclusively at a single defined waiting position. A resulting correction of the **Reference Z-Position** is adopted for all focus areas. This strategy is only relevant if **Tiles** and **Time Series** experiments are combined. Again the repetition and frequency of the software autofocus stabilization can be modulated to meet the experiment needs.
Definite Focus / **As additional action**	The Reference Z-Position calculated from the Local Focus Surface is used as the starting point for a Definite Focus stabilization, which updates the Reference Z-Position (adjusts this to a single stabilization offset, defined at the start of the experiment such that the distance between the coverslip and objective will be the same). This method only makes sense in the context of a very thin sample, where the sample lies at a constant close distance to the interface. For Definite Focus.1 this also reduces the likely-hood of a stabilization failure.
Definite Focus / **Update with single offset**	This option is available for **Time Series** experiments only. This option allows a focus surface (local or global) to be updated by the activity of a Definite Focus stabilization. The focus surface is regularly adjusted by means of a definite focus stabilization, which is performed exclusively at a single defined waiting position. A resulting correction of the Reference Z-Position is adopted for all focus areas. This strategy is only relevant if **Tiles** and **Time Series** experiments are combined. Again the repetition and frequency of the definite focus stabilization can be modulated to meet the experiment needs.
Definite Focus / **Update**	This option is available for Definite Focus.2 only.

Strategy	Description
with multiple offsets	This approach allows a focus surface (local or global) to be updated by the activity of a Definite focus stabilization with multiple unique offsets. These unique stabilization offsets are determine immediately before the start of the experiment so that the z-values of all the support points/ positions/ tile regions are adapted individually respective to their initial z-value. This strategy can be applied to tiles experiment with or without a time series dimension. The repetition and frequency can only be adjusted for the time series loop.
Initial Definition of Z-values for Support Points/ Positions/Tile Regions	By default this is given by the **Tile Setup** (user defined values from the **Tiles** tool) from the Support Points / Positions / Tile regions list. It is optionally possible to use the **Software Autofocus** function or the Definite Focus.2 **Recall Focus** function to initially define these value prior to the start of the image acquisition. The resulting z-values can overwrite the existing listed values in the support points/ positions list.

20.3.2.6 Experiment Regions Tool

This tool allows to define Regions of Interest (ROIs) which are used for image acquisition, sample manipulation (bleaching) and image analysis.

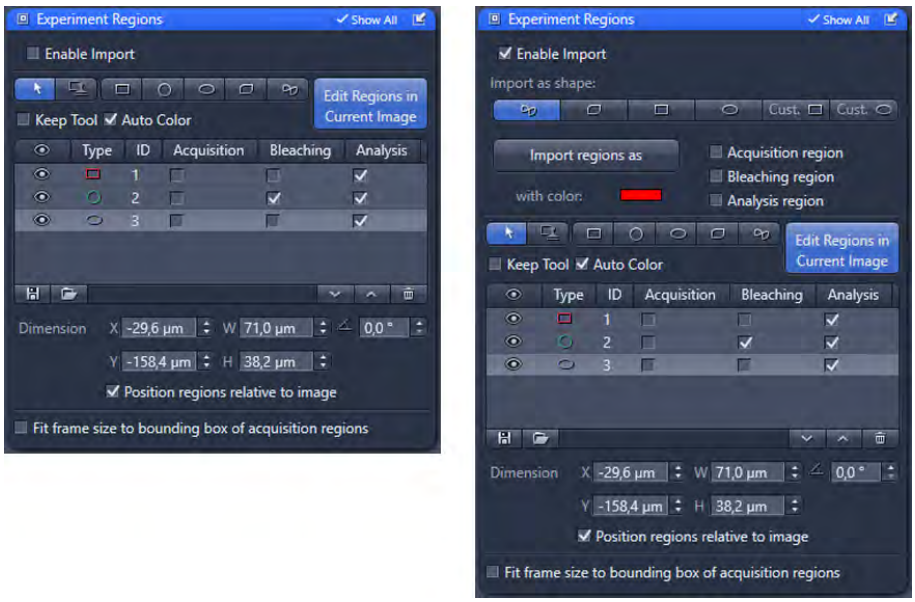


Fig. 20.24: Experiment Regions Tool (Show All) - Enable Import deactivated and activated

Parameter	Description
Enable Import	Activate this option to show additional controls for the import of Regions which were created by an Image Analysis workflow upfront. This option is only available in **Show all** mode.
Import as shape: Toolbar	Select here, which type of Shape the imported. The shape of the created Experiment Region can either be a polygon, a simplified polygon or a rectangle or ellipse with the approximate size of the bounding box from the analysis region. Alternatively you can define a custom rectangle or ellipse with specific size and an offset relative to the center of the bounding box of the analysis result.
Import regions as	When you press this button, the currently selected regions from an Image Analysis result will be imported into the list of Experiment Regions. The properties, e.g. if the regions will be used for bleaching and analysis, are defined by the neigboring checkboxes.
Color	Select here the color which will be used to indicate the imported regions in the image.
Toolbar	Use the tools from the toolbar to draw ROIs into the image.
Edit Regions in Current Image	To add a new Experiment Region to your experiment, you need to click the **Edit Regions in Current Image** button first. The button will turn to blue to indicate that Experiment Regions are displayed in the image and can be added or manipulated. When the button is not enabled, all standard graphical elements are shown in the image, but not Experiment Regions. Standard graphical elements are used for image analysis and annotation, but will not have an impact on the next image acquisition. Experiment regions which are used e.g. for bleaching, are automatically converted into standard graphical elements while the experiment is performed. It is not possible to edit Experiment Regions when the image is displayed in certain viewers. Switch back to the 2D view, in case the button is disabled.
Keep Tool	**Activated**: Keeps the selected graphic tool for multiple actions.
Auto Color	**Activated:** Assigns different colors for each drawn ROI.

Parameter	Description
	Only available in Show all mode
ROIs List	This list shows all drawn in ROIs . Their **Type** (form and color), **ID** (number) are listed as identifiers. For each ROI certain properties can be chosen by activating the according checkbox:
- Acquisition	**Activated:** Acquires images only within the ROI(s). Use this parameter in combination with **Fit frame size to bounding box of acquisition regions**.
- Bleaching	**Activated:** Performs a bleaching experiment will be within the selected ROI(s). The bleaching parameters are specified in the **Timed Bleaching Tool**, sse chapter *Timed Bleaching Tool* [▶ 726].
- Analysis	**Activated:** Uses data for Mean of ROI analysis in the **MeanROI** view from the corresponding ROI(s) .
Save	Saves the selected ROI(s) to the file system.
Load	Loads ROI(s) from the file system.
Delete	Deletes the selected ROI(s) from the ROIs list.
The following parameters are only visible in the **Show All** mode.	
Dimensions	
- X / Y	Shows the X / Y position of a selected ROI. Enter new values in the input fields.
- W / H	Shows the Width (W) and Height (H) of the selected ROI. Enter new values in the input fields.
- Angle	Shows the rotation angle of the selected ROI. Enter a new value in the input field.
- Position regions relative to image	The experiment region is placed in relation to the current field of view. Use this option if the regions should maintain their position within the image in case the stage is moved. When this option is deactivated, the regions are defined by their stage coordinates. In this case, regions can be placed on certain structures of your sample and the regions will stay there when the stage is moved. Note, that the system will not attempt to automatically move to these positions e.g. during bleaching experiments, if they are not accessible in the current scan area.

Parameter	Description
Fit frame size to bounding box of acquisition regions	**Activated:** Fits all ROIs that are marked as Acquisition ROIs in the table to the total frame that the scanner will cover. This can decrease imaging time, since the scanner has not to move over the complete frame of the original image, in which the ROIs were drawn. While the frame size is reduced, the scan speed will maintain its value as configured in the Acquisition Mode tool. In order to achieve maximum acquisition speed, you should use the **Acquisition Mode Tool** to manually reduce the frame size and adjust the speed correspondingly.

20.3.2.7 Experiment Designer Tool

INFO

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**.

In the **Experiment Designer** you can create experiments for multidimensional acquisition. Experiments can consist of any number of components. A component is referred to as an experiment block. Each experiment block has a distinct number, which is shown above the block. To create a new experiment block click on the **Create Acquisition** button and select the desired type of block (Acquisition, Delay, Wait, Execute blocks) then click on the button again. The block will appear in the **Timeline** of the experiment which you see below the blocks.

Please note the following when working with experiment blocks:

- Each acquisition block can be seen as its own independent single experiment with its own individual settings.
- Each experiment block can have its own dimensions (e.g. channel settings like exposure time, active camera, camera parameters; Z; T).
- Focus strategies are block specific as well.
- You can change the order of experiment blocks via drag & drop in the experiment timeline.

Special actions that influence the course of an experiment are performed by means of a special block. In the **Show All** mode you can define Loops and Repetitions and specify the number of image files.

Parameter	Description
Import	Opens the *Import experiment blocks from other experiments Dialog* [▶ 710]. Here you can choose blocks to import from existing experiments.
Export	Opens the **Export Experiment Blocks** dialog. You can choose here, which experiment blocks you want to export to to the file system.
Create Acquisition Block	Adds a new, empty acquisition block to the experiment timeline.
Create Delay Block	Adds a Delay block to the experiment timeline. A delay block pauses the experiment for a predefined period. After that period the experiment continues automatically. You can set the length of the pause in the **Properties** section with the **Delay** slider or input field by clicking on a Delay block. The delay will be shown within the block. If the **Synchronize with preceding blocks** checkbox is activated, the duration of the delay block is reduced by the measured execution time of the preceding blocks.
Create Wait Block	Adds a Wait block to the experiment timeline. A wait block holds the experiment in idle status as long as clicking on the **Continue Experiment** button in the message box. This can be used for adding a solution or changing the buffer of the specimen. The message box will be shown when a wait block is reached. You can enter a message text into the input field in the **Properties** section by clicking on a **Wait!** block.
Create Execute Block	Adds an Execute block to the experiment timeline. A execute block executes a selected hardware setting. You can change the blocks properties by clicking on the corresponding block. If the **Sequential** checkbox is activated the experiment will continue while the hardware setting is executed. If it is deactivated the experiment will wait until the hardware setting was executed. By clicking on the  *Options* [▶ 710] button the options for configuring and selecting the hardware settings will appear.

Parameter	Description
	If a hardware setting was selected, the **Go!** button is active. If clicking on this button the selected hardware setting will be applied immediately.
Duplicate	Duplicates the selected block and inserts the newly created block after the last block.
Delete	Deletes the selected block from the timeline.

20.3.2.7.1 Import experiment blocks from other experiments Dialog

Here you can import experiment blocks from existing experiments.

Parameter	Description
Choose Experiment	Here you select the experiment you want to import experiment blocks from. The experiment must have been saved on your computer before.
Select desired blocks	Here you can select the experiment blocks you want to import. Simply click on an experiment block to select it. The selected block is marked in blue. If you want to import all blocks of an experiment don't select a block but continue by clicking on the **Import** button directly. This will import all experiment blocks at once.
Import	Imports the experiment or the selected experiment blocks to the experiment timeline.
Cancel	Cancels the import.

20.3.2.7.2 Create Execute Block Options

Parameter	Description
Edit Setting/Light Path	Opens the light path dialog in which you can change the relevant hardware setting.
Set to None	Removes the existing hardware setting.
Get Current Hardware	Adopts the current device status.
Experiment Settings Pool	The shortcut menu shows a list of the existing hardware settings.
Harddrive Folder	The shortcut menu shows a list of the saved hardware settings.

Parameter	Description
From File...	Opens the Import Hardware Settings dialog window. Select a ZIS hardware settings file (*.czhws).
Export to User Folder	Exports the current hardware setting.

20.3.2.7.3 Loops and Repetitions section

Only visible if the **Show All** mode is activated.

Here you can specify which experiment blocks should be repeated during the experiment. You can define as many repetitions as you like for each experiment. An experiment block may only appear once within the repetitions defined.

INFO

If you define several repetitions, the following conditions must be met:

- Repetitions must form a complete unit.

- One repetition may not be placed within another.

If these conditions are not met, the repetition cannot be performed. In this case a yellow warning symbol appears under the **Active** field.

Parameter	Description
Loops	Enter the number of loops that you want to be performed.
Start	Enter the number of the starting block.
End	Enter the number of the end block.
Active	If the checkbox is activated, this repetition is performed in the experiment.
 **Add**	Adds a new repetition to the experiment.
 **Delete**	Deletes the selected repetition.

20.3.2.8 Z-Stack Tool

INFO

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**.

In the **Z-Stack** tool you can configure acquisitions that comprise several Z-planes of your sample. You can set all the parameters manually using two different modes (see *Manual Configuration* [▶ 713]) or have configuration performed automatically (see *Z-Stack Automatic Configuration* [▶ 713]).

Z-Stack Graphical Display

The graphical display in the left area of the tool represents the configured Z-stack. In the case of inverse microscopes the objective appears in stylized form at the bottom of the Z-stack. In the case of upright systems it appears at the top.

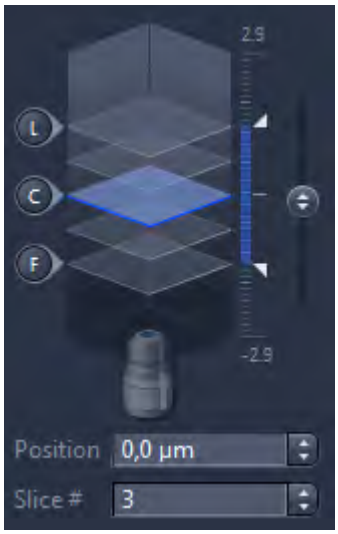


Fig. 20.25: Graphical Display of the Z-Stack

The blue plane indicates the current section plane. The round **L**, **C** and **F** buttons refer to the corresponding planes (**L** = Last, **C** = Center, **F** = First). To change the current Z-position, click on the relevant buttons. The blue plane then jumps to the desired position.

The values at the top and bottom of the measurement scale on the right-hand side of the graphic indicate the distance to the center of the Z-stack.

The **Position** display field below the graphic indicates the Z-position at which the section plane is located. Here you can navigate precisely to the relevant Z-positions.

The **Slice #** display field below the graphic indicates the number of the current slice.

20.3.2.8.1 Z-Stack Automatic Configuration

INFO

Before you perform automatic configuration, the current focus position must be at the center of the sample. The camera's current field of view must always be at a position on the sample that shows a signal in the selected channel.

If you click on the **Z-Stack Auto Configuration** button of the tool the automatic configuration is performed.

Note that Z-stack automatic configuration only works with microscopes and systems that do not use an optical sectioning technique. If you use an **LSM**, **ApoTome**, **VivaTome**, **Spinning Disc (CSU)** or another technique for generating optical sections, the Z-stack must be configured manually.

The following parameters are set automatically:

- Z-position of the central plane
- Distance between the individual planes
- Number of section planes

Parameter	Description
Start Auto Configuration	Automatically configures the Z-stack using the current sample.

20.3.2.8.2 Manual Configuration

INFO

Z-Stack images are always acquired from bottom to top automatically, irrespective of whether you have defined the top or bottom Z-plane of your stack as the first Z-plane. This acquisition sequence increases the accuracy of the Z-positioning.

For manually configuring Z-Stacks you have two modes available. Please note that these modes are only available if **Show All** is activated.

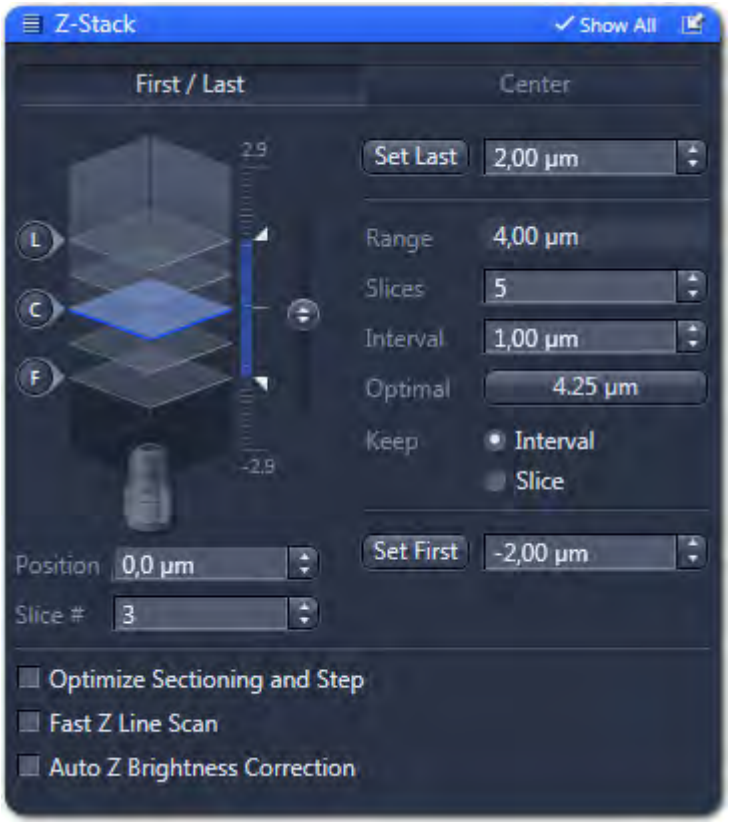


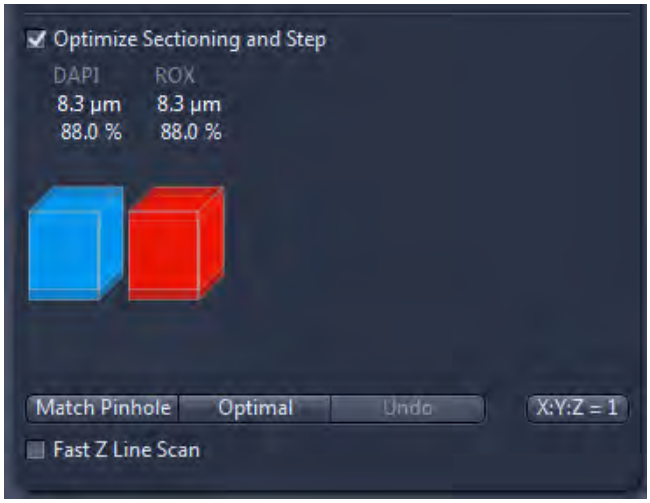
Fig. 20.26: Z-Stack Tool (Show All activated)

Parameter	Description
First / Last Mode	If activated, you are able to configure the Z-Stack via setting the first and the last positions of the Z-Stack, see *Configuring a Z-Stack manually (First/Last Mode)* [▶ 52].
Center Mode	If activated, you are able to configure the Z-Stack via setting the center plane of the Z-Stack, see *Configuring a Z-Stack manually (Center Mode)* [▶ 53].

Depending on which mode you have activated, you will see the following parameters for configuring the Z-Stack:

Parameter	Description
Set Last/Set First	Only visible for **First/Last** mode. By clicking on the **Set Last** and on the **Set First** button you determine the current position as last or first position of the Z-Stack.
Range	Displays the range of the configured Z-Stack from the last to the first section plane.

Parameter	Description
Slices	Here you can enter the number of Z-slices that the Z-Stack will have.
Interval	Here you can enter the desired distance between the Z-slices.
Optimal	The number on this button shows the distance calculated for the channels set and the current microscope according to the Nyquist criterion. If you click on the button, this value is automatically adopted into the **Interval** input field. When you click this button once, it will change its color to blue. This indicates that the system will always use the optimal interval as you continue to change acquisition parameters. Click the button again or manually edit the interval to deactivate this permanently active state.
Keep	■ **Interval**: Keeps the set interval between the section planes constant if you change configuration parameters in the Z-Stack tool. ■ **Slice**: Keeps the set number of Z-slices constant.
Center	Only visible for **Center** mode. If clicking on this button the current position is set for the central Z-plane. You can also enter the value in the input field to the right of the button.
Offset	Here you can enter a value for an offset if desired.
Optimize Sectioning and Step	This function is for LSM only. Here you can set the optimal Interval for more than one channel or track.

Parameter		Description
		Each channel is represented by a graphical display of two Z-slices. The Channel name, the Z-slice thickness and the current overlap of two Z-slices in percent are given above the graphic. 
-	Match Pinhole	Changes the pinhole of the tracks to match the smallest optimal interval of all channels or tracks. If manually a interval was set for all tracks, this value will be used to match the pinhole diameter.
-	Optimal	Sets the interval for all tracks to the optimal value, this might result in different intervals for the tracks. The optimal value is based on the current values for the pinhole diameter of the tracks and therefore the currently valid Z-resolution
-	Undo	Resets all parameters to the default values.
-	X:Y:Z = 1	Matches the settings in Z to the settings in X and Y. This produces a cubical voxel. This can be useful for later import into third party software for rendering.
Fast Z Line Scan		Only available if the **Line** Scan Mode is chosen in the **Acquisition Mode** tool. If activated, the focus of the microscope will move continuously during a line scan to acquire the Z-Stack, hence the Z-Stack is acquired with enhanced speed but with no distinct Z-steps. A Z-Stack of a line scan will be displayed in the **Ortho** View.

The settings of **Auto Z Brightness Correction** are part of the image acquisition and are reused with other settings of an image. They are also part of an experiment setting. However, the function is not activated for reuse or when loading an experiment as the settings apply to the absolute Z-position in µm used when the (previous) image was aquired. If the new stack is acquired in a different position using the previously defined settings (extrapolate) can lead to a an extreme overexposure of the sample. Enable test will always be deactived when an image stack is reused or an experiment is loaded.

Worflow:

In order to reuse the Auto Z Brightness parameters for subsequent Z-Stacks make sure to manually set the center (or first or last) position of the first Z-Stack to zero (Focus TW, Z-position: Set Zero) before defining the Auto Z parameters. Set the center (or first or last) position of all following Z-Stacks also to zero to be able to reuse the Auto Z Brightness parameters accordingly. When saving and loading the parameters, the same logic applies. When switching between linear and spline interpolation during continous scan, the current acquisition parameters are not updated until the Z-position is changed.

Parameter		Description
Auto Z Brightness Correction		This function is for LSM only. If activated, certain acquisition parameters (laser power, Master gain, digital offset and digital gain) are automatically adjusted according to the predefined settings while the Z-Stack is being acquired. The settings are typically adjusted while focussing throught the sample when the system is in continuous scan mode. Activate the option **Enable Test** to allow an update of the settings during focussing either with the focus control of the microscope or using ZEN. ZEN does not show the update of the values during the acquisition of the experiment. Note that resetting the focus position in the **Focus** tool does not affect or shift the position values in the list.
-	Add	Adds the current Z-position to the list and stores the currently configured settings. If the position is already in the list, the values are updated. The positions don't need to be added in a certain correct order.
-	Move to	Changes the focus position to the selection in the list. This can also be done by a double click on the list item. If **Enable Test** is activated, the parameters are immediately applied during a continuous scan.
-	Remove	Removes the currently selected position from the list.

Parameter		Description
-	Remove All	Removes all positions from the list.
-	Load../Save..	Loads or saves the stored position parameters to/from a *.ABC file.
-	Spline Interpolation	If activated, a spline interpolation instead of a linear interpolation is used. Note that spline interpolation with only few positions and relatively large distances in between the intermediate images can show an overshoot of the applied acquisition settings.
-	Extrapolate	If activated, the interpolation between the Positions in the list can be extrapolated to the actual first and last slice of a Z-Stack, if those are not part of the range of the Positions in the list.
-	Enable Test	If activated, the parameters in the **Channels** tool are updated while changing the current Z-position and applying the parameters during a continuous scan.

20.3.2.9 Time Series Tool

INFO

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**.

Here you can configure acquisitions that allow you to acquire an image series consisting of a number of time points. Here you can enter, for example, the acquisition interval, the length of the experiment and other specifications to control the experiment.

Parameter	Description
Duration	Here you can define the duration of your experiment. You can either specify the number of time points (in cycles) or the duration (in milliseconds, seconds, minutes, hours or days).
As Long as Possible	**Activated**: The acquisition of the time series continues "as long as possible" depending on the free disk space of the hard disk. That means the following: ■ For hard disks with **more than 20 GB free disk space** the acquisition always runs until **2 GB disk space** is left.

Parameter	Description
	Example: An experiment using a hard disk with 250 GB of free disk space will run until 2 GB are left. The calculated/required disk space for the experiment is 248 GB. ■ For hard disks with **less than 20 GB free disk space**, at least **10 % disk space** will be always left free. **Example:** An experiment using a hard disk with 15 GB of free disk space will run until 1,5 GB are left. The calculated/required disk space for the experiment is 13,5 GB.
Interval	Here you can define the interval from individual image to individual image in an image series. You can specify the interval to set the gap between individual time points (in milliseconds, minutes, hours or days).
Use Camera Streaming if Possible	Only visible in **Camera/Widefield** Mode. **Activated**: The software tries to use the free running/ streaming mode of the active camera.
Use Burst Mode if Possible	Only visible in **Camera/Widefield** Mode. **Activated**: The system buffers acquisition data in the main memory. For more info, see Burst Mode.
Measure Speed	Not available for **LSM** acquisition. Checks whether the experiment can be performed using the interval which is set. If the interval is too small, the shortest possible value is defined automatically for the interval.

TIP

The shortest possible interval is calculated by performing a blind experiment. The camera exposure time, number of steps of a Z-stack and the number of acquisition channels are taken into consideration in the calculation. Depending on the number of Z-stacks and channels and whether long exposure times have been set, it may take some time to calculate the shortest time interval.

20.3.2.9.1 Experiment Conditions section

The following functions are only visible if the **Show All** mode is activated:

Here you can define the **Start**, **Stop** and **Pause** conditions for your experiment. Select the parameters for the corresponding condition from the dropdown list:

Parameter	Description
Manual	The experiment is started immediately when clicking on the **Start Experiment** button in the **Experiment Manager**.
At Time of Day	The experiment is started, stopped or paused at the entered time. Enter the desired time in the spin box/input field to the right of the dropdown list.
After Delay	The experiment is only started, stopped or paused once the length of time entered has passed.
On Trigger	The experiment is started, stopped or paused once a TTL signal has been received.

INFO

If you define times as start, stop and pause conditions, these apply once for the entire experiment. This also applies to experiments that use the **Experiment Designer**.

20.3.2.9.2 Switches section

The following functions are only visible if the **Show All** mode is activated:

Here you can add and configure **switches** that can be used to execute certain actions during your experiment. To add a new switch, simply click on the **Add**  button.

Left-click on a new or existing switch to open the dialog in which you can configure the button parameters:

Parameter	Description
Name	Here you can enter a name for the button.
Description	Here you can enter a description for the button.
Color	**Activated:** Shows a colored line at the left edge of the switch.
Color Selection	Opens the **Color Selection** dialog. Here you can select a color for the line at the left edge of the switch.
Action	Here you can select one of the following actions. This action will be executed when you click on the button: ■ **None** ■ **Set Interval** ■ **As Fast as Possible**

Parameter	Description
	■ **Trigger** ■ **Hardware Setting** ■ **Jump to previous block** ■ **Jump to next block** ■ **Jump to block #**
Delete Switch	Deletes the selected switch.

20.3.2.10 Experiment Information Tool

Here you can find an overview of your experiment parameters, e.g. the memory requirement of the experiment or its duration.

Parameter	Description
Required Disk Space	Indicates the calculated memory space that the experiment will take up on your hard drive. All the activated blocks of an experiment created using the **Experiment Designer** are taken into account.
Duration (Theoretical)	The system adds together all the exposure times arising during acquisition in the experiment and indicates this value. In the case of time series the intervals set are also taken into account. The actual acquisition duration will always turn out longer, however, as switching times for components (diaphragms, reflectors) and positioning times (Z-plane, stage position) also come into play.
Maximum Acquisition Rate	If the **Time Series** acquisition dimension is activated in the **Experiment Manager**, you can measure the maximum possible frame rate of the system in the **Time Series** tool. In that case the frame rate is shown here. Otherwise "not available" is displayed. After any change is made to the experiment the frame rate must be determined again in the **Time Series** tool.
Elapsed Time (Last Experiment)	If you have already run the current experiment before on the system, the duration actually required for it is displayed here. This information disappears again if you change the experiment.
Next Time Point in	Shows duration to next time point.

Parameter	Description
Tile Size	Shows the X/Y dimensions of your experiment. In the case of a single position this value is identical to the size of the camera field.

20.3.2.11 Experiment Feedback Tool

INFO

This tool is only visible if you have licensed and activated the module **Advanced Processing** in the **Modules Manager** and additionally activated the **Experiment Feedback** checkbox in the **Experiment Manager** on the **Acquisition** tab.

Parameter	Description
Edit Feedback Script...	Opens the *Script Editor* [▶ 723] dialog. There you can create scripts for an Experiment Feedback.
Select script runtime conditions	
- Free Run	Upon the experiment start the acquisition and the feedback script are started but run from here in an completely unsynchronized manner. The online image analysis or the script run itself will not slow down the actual image acquisition.
- Synchronized	This mode will lead to strictly determined order of events depending on the chosen level of synchronization. The online image analysis and/ or the feedback script will be started after current acquisition is finished. In contrast to the **Free Run** mode, a synchronized run can slow the whole acquisition down. The big advantage of the mode is, that the synchronized run ensures a predictable workflow.
Define Script Slot	Here you define the experiment feedback sequence by arranging the slots (represented by blue buttons). The blue slots run one after another the non blue slots are run separately. ■ The **Acquisition** slot represents the actual image acquisition. ■ The **Analysis** slot represents the online image analysis.

Parameter	Description
	■ The **Script Run*** slot represents the execution of the experiment feedback loop script. Note that the loop script will be only executed when triggered by a used observable inside the loop script. ■ The **HD Writing** slot represents the slot for writing the image data to your hard drive.
Allow additional loop script runs	If activated, additional loop script runs for hardware and environment observables are allowed. This only applies when acquisition is idle.

20.3.2.11.1 Script Editor for Experiment Feedback Dialog

Input Windows

The three windows on the left allow you to input scripts based on the programming language Python:

Window	Description
Pre Loop - Single Execution on Experiment Start	Script elements in this window are run through and executed once at the beginning of the experiment. This area can be used to define global variables, for example.
Loop Script - Repetitive Execution During Experiment Runtime	Script elements in this window are run through and executed continuously during the experiment. This area contains the instructions for the dynamic control of the experiment.
Post Loop Script - Single Execution on Experiment Stop	Script elements that are in the lower input window are run through and executed once at the end of the experiment. This area can be used to issue notifications or audible alerts at the end of the experiment, for example.
Accept	Adopts the script within the experiment
Cancel	Leaves the dialog without adopting the script.

Specification area - Commands tab

On the right is the specification area for observables, actions and editor tools:

Parameter	Description
Available Observables	Observables are conditions or parameters that can be determined and observed during the course of the experiment. Select observables, actions and tools by clicking on the black triangle at the right-hand edge and dragging the desired action from the list to the desired input area. You can adopt sample scripts by double-clicking. By doing this, however, you will overwrite the content currently available in the script window entirely (after receiving a prompt).The following observables are available:
- Analysis	Analysis observables contain measurement parameters from image analyses that have been previously defined (eg in Analysis Wizard). Only those variables that are specified through the selection of the measurement program (in the list) appear.
- Experiment	Experiment observables relate to the experiment currently running and display the current time point or the current Z-stack plane, for example.
- Hardware	Hardware observables include, for example, the status of connected devices, such as trigger devices, or of incubation.
- Environment	Environment observables describe the system environment, such as the time or available memory space on the computer.
Available Actions	Actions are possible actions and reactions that can be performed during the experiment. These can vary greatly and include, for example, performing hardware actions on the imaging system, changing camera parameters, generating notifications or audible alerts, calling up other programs or canceling the experiment.
- Experiment Actions	This section contains commands that can be used to modify a running experiment on-the-fly. It also includes modifying hardware parameters which are typically part of an acquisition experiment, like exposure times or light source intensities.
- Hardware Actions	Hardware Actions are modifications of a running experiment concerning the incubation, XYZ positions and the Digital IO ports.

Parameter	Description
- Extra Actions	This section contains commands that can be used to log any kind of data into a text file or to start an external application outside ZEN at any time during a running experiment.
Editor tools	The Editor tools include sample scripts and an option for checking the script syntax.
- Examples and Templates	Contains a small collection of python snippets to illustrate some basic ideas of the feedback script itself.
Validate script	Using the **Validate Script** button you can check your script for errors. If clicking on the button there will appear an info text below the button if your script is ok or not.
Information	
- IO Card Port Labels	Contains the exact naming of the available IO ports for the current system.

Specification area - Debugging tab

Parameter	Description
General	
- Show Warning Popup	Allows to open warning popups.
- Show Output Messages	Opens the output messages field. Here the messages defined below are displayed during skript run for debugging purposes.
Output Messages	
- Show	Displays the corresponding message in white Highlight: Displays the corresponding messages with different colors.
- Highlight	Displays the corresponding messages with different colors.
User Defined Messages	
- Write to Output	Writes user defined messages (ZenService. Xtra.System. WriteDebugOutput (string message)).
Auto Messages	

Parameter	Description
- Observable Change	Writes a notification each time an observable changes.
- Action Called	Writes a notification each time an action is called.
- Action Executed	Writes a notification each time an action is executed.
- Action Warning	Writes action warning messages.
- Exception	
- Script Run	Writes which part of the sript (preloop, loop script, post loop script) is run.
Additional Information	
- Show Message Type	Allows to additionally display the message type.
- Show Time Stamp	Allows to additionally display the time stamp for each message.
- Follow Debug Message	Follows the output if activated.
Clear output	Clears the output messages field.

20.3.2.12 Timed Bleaching Tool

This tool permits setting the bleach parameters for a bleaching experiment in combination with a time series. Bleaching or photomanipulation is done in between acquisition.

Fig. 20.27: Timed Bleaching Tool (Show All Deactivated)

Parameter	Description
Bleaching Method	
- LSM	If no other devices are attached, this is the only list entry and cant be changed. If other devices are attached you can select them from the list.
Start after # of images	**Activated:** Enables you to set the number of frames that are imaged before the bleaching process.
Repeat after # of images	**Activated:** Enables you to set the number of images that will separate a repetitive bleaching procedure.
- Iterations	Sets the number of images that will be repeated. This indicates the total amount of scans which are performed for bleaching the selected region during each bleaching process.
Excitation of Bleach	
- Use different settings for different regions	**Activated:** For each previously drawn ROI a tab is present, in which laser line and laser power can be chosen.
All Regions	Only visible if the checkbox above is not activated. Note that for high laser power the **High Intensity Laser Range** must be set in the **Imaging Setup** or **Channels** tool.

Parameter	Description
	Activates the according checkbox for a laser line. Use the slider to adjust the power for bleaching/photo-manipulation.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Bleaching Settings	Here you can manage bleaching settings, e.g. save, load or delete settings on the hard disk.
Stop on intensity below	**Activated:** Enables you to bleach the sample to an intensity value calculated as % of the initial intensity within the region to bleach, during an experiment with repetitive bleaching. The intensity value is determined with every taken image.
Set Different scan speed	**Activated:** Enables you to set the scan speed for bleaching can differently than the imaging scan speed. A lower speed results in a longer pixel dwell time, which increases the efficiency of bleaching.
Set different Z-Position	**Activated:** Enables you to perform the bleaching at a different z-level than currently set. Enter the difference in µm into the input field.
Zoom Bleach (fast, less accurate)	**Activated:** Accelerates the bleaching process. In this case the scanner movement will be restricted to the bleaching region zooming in onto this region. This may result in a less accurate positioning of the region as the definition of the region has been made in a different zoom in the image. The bleach process will be faster than without this option. The gain in speed is dependent on the speed that is used for bleaching. If a high speed has already been chosen for bleaching the gain in speed will be minimal.
Protect detectors during bleach	**Activated:** the PMT gain is set to 0 V during the bleaching step. Note that, changing the PMT gain will significantly slow down the acquisition. Typically, small bleach ROIs will not be causing a critically high signal for the detectors. However, even when this option is not activated, the detectors will shut down automatically in case of significant over exposure.

20.3.2.13 Interactive Bleaching Tool

This tool allows to bleach interactively during a **Continuous** scan or during a **Time Series** experiment while image acquisition is performed. The bleach region is determined by pointing the mouse onto the position in the image.

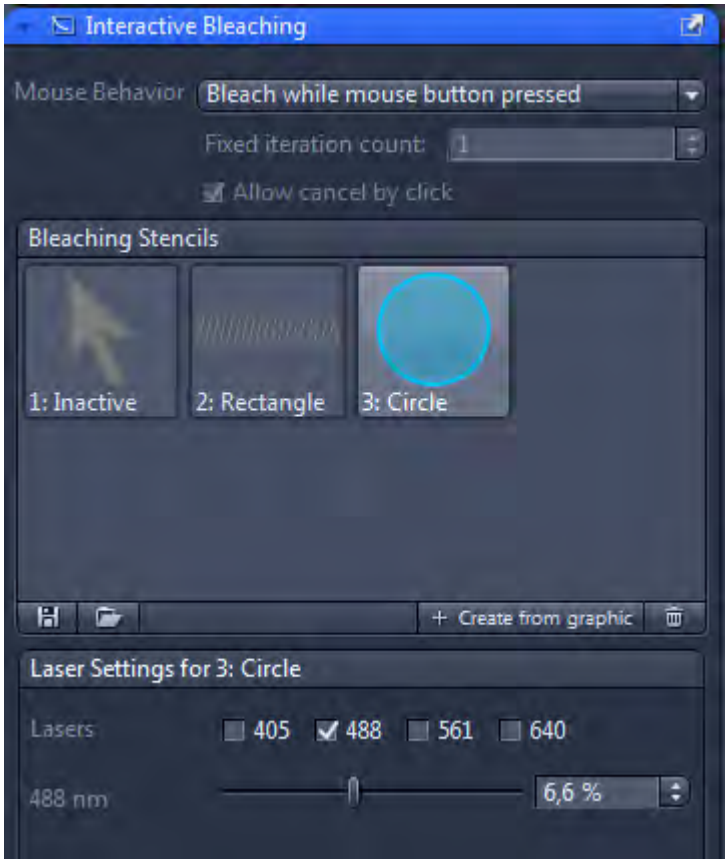


Fig. 20.28: Interactive Bleaching Tool

Parameter		Description
Mouse Behavior		Change the display of the selected clipping plane using the dropdown list to the right of the **Activate** checkbox. The following settings are available:
-	Bleach while mouse button pressed	The bleaching process is continued while the mouse button is pressed.
-	Bleach fixed number of iterations	The bleaching process is continued for a fixed number of times after the mouse is pressed. The number of iterations can be entered below.
-	Fixed iteration count	Determines the number of iterations.

Parameter	Description
- Allow cancel by click	If activated, the bleaching process can be stopped before the fixed number iterations is accomplished. Simply click on the left mouse button again to stop the bleaching process.
Bleaching Stencils list	The available bleaching stencils (equivalent to ROIs for bleaching) are listed here. A stencil is displayed in its graphical form, the color displays the laser line that is assigned for bleaching to this stencil. You can add a stencil by clicking the **+ Create from graphic** button. This will import an activated graphical element from the **Graphics** tool tab. A stencil can be saved, loaded or deleted. A stencil is active, when clicked on and highlighted. To use an activated stencil for bleaching, move the mouse cursor onto the image and click on the left mouse button.
Laser Settings for section	Here you can set the laser line and laser power for the activated stencil.

20.3.2.14 Dynamics Tool

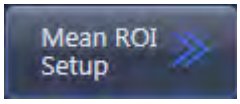
INFO

This tool is only visible if you have licensed and/or activated the module **Physiology (Dynamics)** in the **Modules Manager** and additionally activated it on the **Acquisition** tab in the **Experiment Manager**.

The checkbox to activate the tool is only visible if **Time Series** acquisition is active and **Z-Stack** or **Tiles** are not activated.

Using the tool you can open the **Mean ROI Setup** where you can configure dynamics functions for a **Time Series** experiment. Deactivate the **Dynamics** checkbox to deactivate dynamics functions.

If you click on the **Mean ROI Setup** button the setup view will be visible in the **Center Screen Area**. There you will see a snapshot containing all channels of the currently active image.



20.3.2.14.1 Mean ROI Setup

The **Mean ROI Setup** is in essence a modified version of the **MeanROI** view with several minor additions. It allows experiment pre-settings to be made on Snapshots of the cells/ specimen on which the measurements will be made. These settings include:

- image and chart layouts;
- chart axis settings;
- generation, placement and management of measurement and Background ROIs
- measurement settings

A ratio preview image is also displayed that allows an assessment of the current ratio settings on the Online Ratio tab. To update the images in the Mean ROI Setup a new Snap can be made at any time.

20.3.2.15 Auto Save Tool

i INFO

The Auto Save tool is not visible, if you have activated the **Panorama** checkbox in the Experiment Manager.

When you execute an experiment or click on the **Snap** button, the Auto Save tool is disabled until the experiment is cancelled or finished. The tool also stays disabled when the experiment is paused. This behavior should prevent operation errors during experiments.

If the **Auto Save** checkbox in the **Experiment Manager** is activated, all images which are acquired from the Acquisition tab, are automatically stored as ***.CZI** format during acquisition.

Generally, all images are automatically written to the hard disk during acquisition. This is to prevent data loss in case of technical problems. The folder path for these files is displayed in the status bar under **Storage Folder**. The location can easily be opened by double-clicking on this field. The path can be changed in the **Tools** menu | **Options** | **Saving**. Even though these files are stored physically on the disk, they are indicated with an asterisk and you will be prompted to either rename and store them in a different place, or to delete them. They are maintained only if the software crashes. In case you want to store the files directly in a location of your choice, activate the **Auto Save** checkbox. In this case the files are not written to the temporary folder anymore.

The automatically saved images are contained in the subfolder **temp** within the currently chosen image storage path (default path is: C:\Users\<username>\Pictures \temp). When saving such temporarily saved images via the **File** menu | **Save** you

will be asked to specify a document name and a storage location. If you close such an image document without saving it, it will be permanently deleted from the Temp folder in order to prevent the accumulation of unnecessary images.

Parameter	Description
Folder	Shows the directory for the images. The text box is read-only. No values can be entered or pasted by the user. This ensures that the text box contains always a valid directory To change the directory click on the  button to select a new folder for the auto saved images.
Automatic Sub-Folder	**Activated:** Creates automatically a top level sub folder in the given directory. The sub folder name is based on the actual date, e. g. 2014-07-04.
Name	Here you can specify the image name. An index number is automatically appended to the image name.
Close CZI after acquisition	**Activated:** Closes the CZI image in the center screen area when the experiment is finished.
File Name Preview	Shows the currently chosen storage path as well as a preview of file name being used next.

20.3.2.16 Automated Image Export Tool

INFO

It is not possible to automatically export images created by the **Snap** function. If you require to use the Auto Export feature for individual images, you must create a Time Series experiment with a single cycle.

If the **Automated Export** checkbox in the **Experiment Manager** is activated before an experiment is executed, the generated images will be stored in the defined directory with the given parameters and options, provided by the options under the checkbox. This option was developed for automatically exporting images with a user defined format (TIFF or JPEG).

INFO

When you execute an experiment, the tool is disabled until the experiment is cancelled or finished. The tool also stays disabled when the experiment is paused. This behavior should prevent operation errors during experiments.

For technical reasons images acquired from the Acquisition tab are always auto-saved temporarily as CZI files. If the application requires images to be stored in external common file formats, it is necessary to run the export function. The Automatic Image Export facilitates this in a convenient und automatic way giving the choice of single page TIFF or JPEG file formats. It is also possible to automatically close and discard the auto-saved CZI file to streamline the acquisition workflow.

Parameter	Description
Folder	Shows the directory for the images. The text box is read-only. No values can be entered or pasted by the user. This ensures that the text box contains always a valid directory To change the directory click on the  button. Select a new folder for the auto saved images in the dialog.
Automatic Sub-Folder	**Activated:** Creates automatically a top level sub folder in the given directory. The sub folder name is based on the actual date, e. g. 2014-07-04.
Prefix	Here you can define a prefix for the image file name and a name for the sub folder. If the text box is empty an image gets a localized default prefix (“Untitled”) and a folder gets a localized default name (“New folder”). If an image or folder with a name still exists, the new image or folder gets the same name with an increasing index in accordance to the standard Windows Explorer behavior, e. g. New Folder (1), New Folder (2).
Format	Here you select the format for the export images. Two formats are supported: ■ **TIFF**: For the TIFF format (lossless, bigger file size) you can additionally select the **Compression** method. – None – LZW – ZIP ■ **JPEG**: For the JPEG format (lossy, smaller file size) you can set the **Quality** level by adjusting the slider between **Low** (lower quality, smaller images) to **High** (higher quality, bigger file size).

Parameter	Description
Original data	For **TIFF** format only: **Activated:** Generates an additional raw data TIFF image. Its bit depth depends on the original camera image (Gray 8/16 bit or RGB 24/48 bit)
Gray Scale Linear	For **JPEG** format only: **Activated:** Generates an additional raw data JPEG image. The bit depth depends on the original camera image. In case of 8 bits, the JPEG image has 8 bit gray scale or 24 RGB. In case of 16 bits, the JPEG image is reduced to the "Valid Bits" of the camera. If the camera image is a 16 bit gray scale image, the resulting JPEG image is a 8 bit gray scale image. In case of a 48 bit RGB image the JPEG image has 8 bit RGB. Both additional image types are marked with an **ORG** suffix in their file names.
Apply display curve and channel color	**Activated:** Applies the display curve and channel color to the JPEG or TIFF image
Use channel names	**Activated:** The name of the resulting image contains the name of the defined channel.
Add XML Metadata	**Activated:** Saves an additional xml file with image meta data. Its name has the following nomenclature: Prefix_Metadata(image format).xml Test_Metadata(tif).xml If more than one xml file with the same name exists the file gets an index, e.g. Test-02_Metadata(tif).xml.
Close CZI image after acquisition	**Activated:** Closes the CZI image in the center screen area when the experiment is finished. **NOTICE** **If Auto Save is not activated, this will lead to the loss of the original .czi file for the experiment.**
Dimension/Sub-directory	If you check one of the Channels, Time Series, Z-Stack or Scenes checkboxes, an additional sub-directory will be created if the corresponding dimension exists in the experiment block. The sub-directory will be created in the same image dimension order as the CZI image created, e.g. T-C-Z. The top level folder within the "Dimension" folders is always the B ("Block") folder [new].

Parameter	Description
	Each sub-directory gets a letter that represents its image dimension (T for time series, C for channel, etc.) and an index, if more than one dimension of the same type exists (T=0, T=1).

20.3.2.17 Automation Tool

With this tool you can select OAD macros which will be executed before or/and after an experiment. The default folder for macros is **User/Documents/Carl Zeiss/ZEN/Documents/Macros**. Any other folder is selectable.

Parameter	Description
Run OAD macro before experiment execution	If activated, the selected macro is executed before the experiment starts. If you click on the **...** button you can select a macro from the file system.
Run OAD macro after experiment execution	If activated, the selected macro is executed after the experiment. If you click on the **...** button you can select a macro from the file system.

20.3.3 Tools on Analysis Tab

20.3.3.1 Interactive Measurement Tool

Feature Set section

Parameter	Description
Feature Set	Here you select and load previously saved feature definitions/feature sets. If you have made changes to a feature definition, the name of the feature selection is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") feature selection, you will be asked whether you want to save the changes.
 **Options**	Opens the Options shortcut menu.
Define	Opens the *Feature Selection Dialog* [▶ 737].

Feature Subset section

Only visible if the **Show All** mode is activated.

Parameter	Description
Feature Subset	Here you can select and load previously saved definitions of subsets. If you have made changes to a subset definition, the name of the feature subset is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") feature subset, you will be asked whether you want to save the changes.
 **Options**	Opens the Options shortcut menu.
Define	Opens the *Feature Subset Definition Dialog* [▶ 738].

Measurement Sequence section

Only visible if the **Show All** mode is activated.

Parameter	Description
Measurement Sequence	Here you can select and load previously saved measurement procedures. If you have made changes to a measurement procedure, the name of the measurement procedure is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") measurement procedure, you will be asked whether you want to save the changes.
 **Options**	Opens the Options shortcut menu.
Define	Opens the *Interactive Measurement Sequence Definition Dialog* [▶ 739].
Run	Starts the selected Measurement Sequence in the Interactive Measurement Sequence Execution Dialog.

Parameter	Description
Create Measurement Table	Creates a measurement data table. This contains the measurement data from the **Measure** view of the current image.

20.3.3.1.1 Feature Selection Dialog

Here you can specify which features are measured with the available graphic elements. This selection is adopted into the current feature definition. The feature definition is then marked ("asterisked") as having been changed.

Available Elements section

In this section you can specify for each available graphic element which features you want to be measured. The graphic elements are ordered by type. The following types are available for selection:

Element Type	Description
Regions (2D)	Here you will find all the graphic elements that define a closed region.
Single Distances	Here you will find all the graphic elements with which you can measure a single distance.
Multiple Distances	Here you will find all the graphic elements with which you can measure several distances at once.
Angle	Here you will find the graphic elements with which you can measure an angle.
Point	Here you will find the graphic elements with which you can perform measurements at a pixel.
Events	Here you will find the graphic elements with which you can count various events in an image.

Selected Features section

The features that you have selected for each individual graphic element are listed in this section.

Activate the **Display** checkbox for each feature to display the value of the measured feature in the graphics plane of the image.

Parameter	Description
 **Delete**	Deletes the selected feature.
 **Upwards**	Moves the selected feature one position higher.
Downwards	Moves the selected feature one position lower.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter		Description
Search Features		Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
-	All	All features are listed.
-	Geometric Features	All geometric features are listed.
-	Intensity Features	All features that analyze intensity values are listed.
-	Image Features	All features that contain meta information about the measured image are listed.
-	Position Features	All features that describe the position are listed.
-	Position Features Unscaled	All features that describe unscaled positions are listed.

Parameter	Description
+ **Add**	Click on the button to select a feature for the measurement.

20.3.3.1.2 Feature Subset Definition Dialog

Here you can specify which features are available in the *Feature Selection Dialog* [▶ 737]. These features are adopted into the current subset definition. The subset definition is then marked ("asterisked") as having been changed.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter		Description
Search Features		Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
-	All	All features are listed.
-	Geometric Features	All geometric features are listed.
-	Intensity Features	All features that analyze intensity values are listed.
-	Image Features	All features that contain meta information about the measured image are listed.
-	Position Features	All features that describe the position are listed.
-	Position Features Unscaled	All features that describe unscaled positions are listed.

Parameter	Description
Feature	There is a checkbox in front of the name of each of the listed features. Activate the checkbox in front of the features that you want to be offered in the **Feature Selection** dialog.
Shortcut menu	Right-click in the Features section to open the shortcut menu.
- Select All Features	Activates all checkboxes.
- Deselect All Features	Deactivates all checkboxes.

20.3.3.1.3 Interactive Measurement Sequence Definition Dialog

Here you can define an interactive measurement procedure. You can specify the order in which you want the individual graphic elements to be drawn in and which measurement parameters you want to have calculated for them. The definition is adopted into the measurement procedure currently selected. The measurement procedure is then marked ("asterisked") as having been changed.

Available Elements section

In this section you can specify for each available graphic element which features you want to be measured. The graphic elements are ordered by type. The following types are available for selection:

Element Type	Description
Regions (2D)	Here you will find all the graphic elements that define a closed region.
Single Distances	Here you will find all the graphic elements with which you can measure a single distance.
Multiple Distances	Here you will find all the graphic elements with which you can measure several distances at once.
Angle	Here you will find the graphic elements with which you can measure an angle.
Point	Here you will find the graphic elements with which you can perform measurements at a pixel.
Events	Here you will find the graphic elements with which you can count various events in an image.

Double-click on a available element to select it and adopt it into the **Selected Elements Sequence** list.

Selected Elements Sequence section

This list displays the selected graphic elements in the order in which they will be drawn in during the measurement, from top to bottom. To display the value of the measured feature in the image's graphics plane, activate the corresponding checkbox of the graphic elements.

Parameter	Description
 **Delete**	Deletes the selected feature.
 **Upwards**	Moves the selected feature one position higher.
 **Downwards**	Moves the selected feature one position lower.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter		Description
Search Features		Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
-	All	All features are listed.
-	Geometric Features	All geometric features are listed.
-	Intensity Features	All features that analyze intensity values are listed.
-	Image Features	All features that contain meta information about the measured image are listed.
-	Position Features	All features that describe the position are listed.
-	Position Features Unscaled	All features that describe unscaled positions are listed.

Parameter	Description
+ **Add**	Click on the button to select a feature for the measurement.

20.3.3.2 Image Analysis Tool

Parameter	Description
Setting	Select and load previously saved analysis programs here.
Options	Opens the options menu.

Parameter	Description
Setup Image Analysis	Opens the *Image Analysis Wizard* [▶ 742] to define a new analysis program or to change an existing program.
Analyze Interactively	Runs the selected analysis program with all the interactive steps.
Analyze	Runs the selected analysis program without interruption.

INFO

Steps that you have not marked as interactive in the **Image Analysis Wizard** are run with the values set in the analysis program. The program does **not** stop to allow you to change these interactively.

20.3.3.3 Image Analysis Wizard

20.3.3.3.1 Classes

In this step you can define the classes into which the measured objects in the image are divided.

Parameter	Description
Classes	The defined classes are listed here. If you create a new measurement program, a predefined set of classes is created automatically. Each class consists of two entries. The first entry concerns all the objects belonging to the class. The second entry represents an individual object.
Add Class	Adds a new individual class to the list on the Base-level.
Add Subclass	Adds a new subclass to the selected class.
Remove Class	Deletes the selected class from the list.
Name	Here you can enter a name for the selected class in the **Classes** list.
Channel	If you create a measurement program for a multichannel image, in this selection field you need to select the channel for the selected class in the **Classes** list. This channel is used for image segmentation.
Color	Here you can select a color to mark the objects of a class.

Parameter	Description
Next	Moves on to the next step of the wizard.

Parameter	Description
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.2 Frame

In this step you can define one or more measurement frames. Only the area within the measurement frames will be analyzed. Furthermore you can define how the analysis treats objects that are cut by the border of the image or the frame.

Parameter	Description
Interactive	**Activated:** The measurement frame definition can be changed interactively while the measurement program is running in **Analyze Interactively** Mode.
Tool bar	
-  Select Frame	Use this to select measurement frames that you have already created. To select a measurement frame, click inside it. To select several measurement frames, hold down the *Ctrl* key and click inside the desired measurement frames. Once you have selected a measurement frame, you can change its size. Delete frames with the *Del* key.
-  Draw Rectangle	Enables you to create a rectangle as a measurement frame in the current image.
-  Draw Circle	Enables you to create a circle as a measurement frame in the current image.
-  Draw Contour (Polygon)	Enables you to create a contour as a measurement frame in the current image.
-  Remove All Frames	Removes all drawn-in measurement frames in the current image.
Maximize circle	Only active if you have defined precisely one circle. **Activated:** Maximizes the drawn-in circle to the full image size. In the case of rectangular images the circle is adjusted to the shorter side.

Parameter	Description
Center circle	Only active if you have defined precisely one circle. **Activated:** Centers the drawn-in circle to the full images size.
Mode	Here you can select how you want the measurement frame to be applied. The following modes are available:
- Inside Only	Measures only those objects, that are lying completely within the measurement frame. Objects that are touching the frame or are intersected by the frame are not analyzed.
- Cut at Frame	Measures all objects that are lying within the measurement frame. Objects that are intersected by the measurement frame are measured precisely up to the measurement frame.
The following fields are only active if you have selected a drawn-in graphic element:	
Left	Here you can enter the start point for the frame on the X axis in pixels.
Top	Here you can enter the start point for the frame on the Y axis in pixels.
Width	Here you can enter the width of the measurement frame in pixels.
Height	Here you can enter the height of the measurement frame in pixels.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.3 Automatic Segmentation

In this step you can choose the segmentation method to be applied and set parameters for the segmentation of the objects that you want to measure.

Parameter	Description
Execute	**Activated:** Sets the defined threshold values when the measurement program is run.
Interactive	**Activated:** The defined threshold values can be changed while the measurement program is running in **Analyze Interactively** mode.
Classes	Here you can select the class for which you want to define the segmentation.
Ring Element Class	This additional parameter is only available if you have selected the **Zone of Influence** (ZOI) method.
- Ring Distance	Distance from surface of the primary object. Negative values mean that the ring starts at the defined distance within the primary object.
- Ring Thickness	Defines the thickness of the ring.
ZOI Class	This additional parameter is only available if you have selected the **Zone of Influence** (ZOI) method.
- ZOI Width	Allows you to set the distance of border of the ZOI from the border of the ring, or the main object, respectively.
Select	Opens the selection of segmenters. For more information, see *Segmentation Method Selection* [▶ 750].

The following parameter are visible depending on the selected segmentation method.

Smoothing section

Parameter	Description
Smoothing	Here you can select how you want to smooth the image before the threshold values are set. The following methods are available:
- None	The image is not smoothed.
- Low Pass	Applies the Low Pass Method.
- Gauss	Applies the Gauss Method.
- Median	Applies the Median Method.
Size/Sigma	Only visible, if you have selected **Low Pass** or **Median**. Enter the size of the filter matrix in the X and Y direction using the slider or input field.

Parameter	Description
Sigma	Only visible, if you have selected **Gauss**. Enter the sigma value using the slider or input field.

Subtract BG section

Parameter	Description
Subtract BG	Here you can select what kind of background subtraction is performed. Only visible if **Segmentation with Background Subtraction** is selected.
- None	No background subtraction is performed.
- Rolling ball	The rolling ball background subtraction is performed.

Sharpen section

Parameter	Description
Sharpen	Here you can select how you want to improve the sharpness of the image before the threshold values are set. The following methods are available:
- None	The sharpness of the image is not changed.
- Delineate	Applies the Delineate Method.
- Unsharp Masking	Applies the Unsharp Masking Method.
Threshold	Only visible, if you have selected **Delineate**. Enter the threshold value for edge detection using the slider or input field. The threshold value should correspond roughly to the gray value difference between objects and the background.
Size	Only visible, if you have selected **Delineate**. Enter the size of the edge detection filter using the slider or spin box/input field. The value should correspond to the size of the transition area between objects and the background.
Strength	Only visible, if you have selected **Unsharp Masking**. Enter the strength of the Unsharp Masking using the slider or input field. The higher the value selected, the greater the extent to which small structures are enhanced.

Threshold section

Here you can define the threshold values for the selected class in the **Classes** list.

Parameter	Description
Reset	Resets all threshold value settings.
Undo	Undoes the last change made to the threshold values.
Redo	Restores the last undone change to the threshold values.
Color Model	Only visible if the image is a color image. *Color Model* [▶ 751]
- RGB	In RGB Mode you can define the threshold values for the red, green and blue color channels.
- HLS	In HLS Mode you can define the threshold values for hue, saturation and lightness.
Histogram	In the histogram you can change the lower and upper threshold value for the activated value. Drag the lower or upper adjustment handle or shift the entire highlighted area between the lower and upper threshold value.
Threshold Definition	
- Click	Click in the image on the regions that you want to define as objects.
- Automatic	The threshold values are determined automatically from the histogram. During setup only the part of the image displayed in the viewport is taken for the calculation of the threshold.
Pick Behavior	Only visible, if you have selected **Click** for **Threshold Definition**.
- **+**	Enables you to expand the currently segmented regions by the gray values/colors of the objects subsequently clicked on.
- **-**	Enables you to reduce the currently segmented regions by the gray values/colors of the objects subsequently clicked on.
Tolerance	Only visible, if you have selected **Click** for **Threshold Definition**. Enter the tolerance range by which the gray/color value read out when you click is expanded to define the threshold value, using the slider or input field.

Parameter		Description
Neighborhood		Only visible, if you have selected **Click** for **Threshold Definition**. Enter a neighborhood range around the pixel clicked on, using the slider or input field. The threshold value is calculated from the average of the gray/color values in this neighborhood range.
Method		Only visible, if you have selected **Automatic** for **Threshold Definition**. Select the method from the dropdown list that you want to use for the automatic calculation of the threshold values. The following methods are available:
-	Otsu	The threshold value is calculated according to the Otsu method.
-	Maximum Peak	
-	Iso Data	The threshold value lies in the middle between two maximums in the histogram.
-	Triangle Threshold	The threshold value is calculated from the sum of the average and three times the sigma value of the histogram distribution.
-	Three Sigma Threshold	

INFO

After the automatic calculation of the threshold values you can further modify the threshold values found interactively by selecting **Click** for threshold value definition.

Minimum Area section

Parameter	Description
Minimum Area	Enter the minimum area in pixels that an object must have in order to be segmented, using the slider or input field.

Fill Holes section

Parameter	Description
Fill Holes	**Activated:** Fills holes in segmented objects.

Binary section

Parameter	Description
Binary	Performs morphological operations on the segmented (binary) image.
- None	No operation is performed.
- Open	Opening performs first erosion and then dilation. The effect is smoothing and removing of isolated pixels.
- Close	Performs first dilation and then erosion. The effect is smoothing of the objects and filling of small holes.
- Dilate	Enlarges the boundaries of segmented regions. Areas grow in size and holes within the regions become smaller.
- Erode	Erodes boundaries of the segmented regions. The areas shrink in size and holes within the areas become larger.
Count	Enter how often the selected binary operation is performed, using the slider or input the field.

Separate section

Parameter	Description
Separate	Here you can select whether you want to process the image further after segmentation. Objects that are touching one another can be separated using different methods.
- None	Objects are not separated.
- Morphology	This method separates objects by first reducing and then enlarging them, making sure that once objects have been separated they do not merge together again.
- Watersheds	Using this method you can separate objects that are roughly the same shape. This method may however result in the splitting of elongated objects.

Parameter	Description
Count	Enter how often the method is applied successively to the result at the location of the separation, using the slider or input field.

Suppress Invalid section

Parameter	Description
Suppress Invalid	**Activated:** Discards invalid pixels at the border of the image.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.3.1 Segmentation Method Selection

Parameter	Description
Segmentation Source	Here you can select the segmentation source. Depending on the selected segmentation source, the functionalities in the Image Analysis Wizard change accordingly. *Automatic Segmentation* [▶ 744].
- From Image Channel	Uses the channel of the multi-channel image defined in step Classes for segmentation of the selected region class.
- From Measurement Frame (s)	Takes the regions from the measurement frame. You can modify the region in the interactive segmentation step.
- Remaining to Frame	Takes the regions from the measurement frame when the regions of the other classes are subtracted from the measurment frame.
- Take from parent regions	Takes the regions that fulfill a certain condition, defined in step "Region Filter", from the parent regions.

Parameter	Description
Image Segmenter	Here you can choose which segmentation method will be applied.
- Segment by global thresholding	A global threshold is applied to the image channel for image segmentation.
- Segmentation with Background subtraction	A background subtraction is performed prior to a threshold-based image segmentation.
- Segment binary images	Segments binary images.
- Variance-based thresholding	Image segmentation via variance-based thresholding. Detects objects where there is a strong change in pixel intensities independent of the absolute intensity.
- Dynamic thresholding	Dynamic thresholding or adaptive thresholding calculates a local threshold for small regions of the image. This is expecially helpful for inhomogenious illumination or background structures.

20.3.3.3.3.2 Color Model

RGB

Here you can set the RGB channel threshold values.

Parameter	Description
 **Red**	Activates the red channel in the Expander **Histogram**.
 **Green**	Activates the green channel in the Expander **Histogram**.
 **Blue**	Activates the blue channel in the Expander **Histogram**.
Low	Here you can enter the lower threshold value for the corresponding channel.
High	Here you can enter the upper threshold value for the corresponding channel.
Invert	Swaps the **Lower** and **Upper** values for the corresponding channel.

Parameter	Description
Full Range	Sets the **Low** value to 0 and the **High** value to the maximum possible gray value for the corresponding channel.

HLS

Here you can set the hue, lightness and saturation threshold values.

Parameter	Description
H **Hue**	Activates the hue in the Expander **Histogram**.
L **Lightness**	Activates the lightness in the Expander **Histogram**.
S **Saturation**	Activates the saturation in the Expander **Histogram**.
Low	Here you can enter the lower threshold value for hue, lightness and saturation.
High	Here you can enter the upper threshold value for hue, lightness and saturation.
Invert	Swaps the corresponding **Lower** and **Upper** values.
Full Range	Sets the corresponding **Lower** value to 0 and the **Upper** value to the maximum possible value.

20.3.3.3.4 Region filter

In this step you can define the conditions under which you want an object to be measured.

Parameter	Description
Execute	**Activated:** Uses the measurement conditions when the measurement program is run.
Interactive	**Activated:** The measurement conditions can be changed while the measurement program is running in **Analyze Interactively** mode.
Classes	Here you can select the class for which you want to define the conditions.

Parameter	Description
Edit	Opens the *Region Filter Editor* [▶ 753].
Copy to All	Copies the defined region filters to all classes.
Conditions	If you have defined one or more blocks with conditions in the **Region Condition Editor**, you can select the block for which you want to set the condition. Select the relevant block and set the maximum/minimum values either by clicking on the objects in the image you want to include in the measurement, or by entering the maximum/minimum values separately.
Undo	Undoes the last change made to the condition.
Redo	Restores the last undone change to the condition.
Reset	Resets all settings for the conditions.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.4.1 Region Filter Editor

Here you can add features to a block with measurement conditions.

Parameter	Description
Selected Features for Condition	In this list, the features that you have selected for the condition are displayed block by block. All features in a block are "And"-linked for the condition, i.e. an object is only measured if the values of each individual feature fall within the defined range.
Add Block	Adds an "**Or**" block. If several "Or" blocks have been defined, an object is measured if it meets the condition in at least one block.
Clear Block	Deletes all features in an "**Or**" block.
Remove Block	Deletes the selected "**Or**" block.
Remove All	Deletes all "**Or**" blocks.

Parameter		Description
Search Features		Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
-	All	All features are listed.
-	Geometric Features	All geometric features are listed.
-	Intensity Features	All features that analyze intensity values are listed.
-	Image Features	All features that contain meta information about the measured image are listed.
-	Position Features	All features that describe the position are listed.
-	Position Features Unscaled	All features that describe unscaled positions are listed.

Parameter	Description
+ **Add**	Click on the button to select a feature for the measurement.

20.3.3.3.5 Interactive Segmentation

In this step you can post-process the segmented objects interactively. You can modify the results of the automated segmentation when you use **Run Interactive** to analyze your image data.

Parameter	Description
Interactive	**Activated:** The segmented objects can be post-processed interactively while the measurement program is running.
Classes	Here you can select the class whose objects you want to process.

Edit Region section

With the tools below you can modify the objects of the currently selected class.

Parameter	Description
Draw	Draw new objects of the selected class.
Erase	Using this button you can erase parts of an object. Holding down the left mouse button, outline the parts of the object that you want to erase. Right-click to erase these parts of the object.
Cut	Use this button to separate connected objects. Holding down the left mouse button, draw in the separation line between the objects. Right-click to cut the objects.
Merge	Use this button to connect objects. Holding down the left mouse button, outline the parts of the object that you want to merge. Right-click to merge the objects.
Fill	Fills a hole. To fill a hole, left-click on the hole.
Remove	Removes a drawn in object by clicking on it.
 **Remove All**	Deletes all drawn in objects.
Tool bar	
-  Rectangle	Enables you to add a rectangular object or cut a rectangular region from an object.
-  Circle	Enables you to add a circular object or cut a circular region from an object.
-  Contour	Enables you to add an object or cut a region from an object.
-  Contour (Spline)	Enables you to add an object or cut a region from an object.
-  Active Contour	Enables you to add an object or cut a region from an object.
-  Polyline Region	Enables you to add a line-object.
-  Point	Enables you to add a point object.

Region Growing section

Parameter	Description
Mode	
- **+**	Click on areas in the image you want to add to the selected object class.
- **-**	Click on areas in the image you want to remove from the selected object class.
Intensity	Here you can enter a tolerance value for the intensity using the slider or input field. The tolerance value specifies how much the intensity of a pixel may deviate from the average intensity of the object in order to still "grow" to become part of the object.
Color	Only active if your input image is a color image. Here you can enter a tolerance value for the color using the slider or input field. The tolerance value specifies how much the color value of a pixel may deviate from the average color value of the object in order to still "grow" to become part of the object.
Fill	Fills holes that are created during region growing.

Parameter	Description
Undo	Undoes the last action.
Redo	Restores the last undone action.

Post Processing section

Parameter	Description
Region Filter	Reapplies the region filter you defined in the previous step to the post-processed image.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.6 Features

In this step you can select the measurement features that you want to measure.

Parameter	Description
Classes	Here you can select the class for which you want to define measurement features. For each class there are two entries for which you can define features. The first entry concerns all the objects belonging to the class. The second entry represents an individual object.
Region Features	
- Edit	Opens the **Feature Selection** View.
- Copy to All	Copies the defined features to all other classes.
Display	If you activate **Display** in the **Feature Selection** window for a feature, the result of the measurement is displayed next to the corresponding object in the analyzed image.
Annotations	
- Edit	Opens the **Feature Selection** window.
- Copy to All	Copies the annotations to all other classes.
Annotations	Only visible if a "child" class is active. The list shows all annotations that are drawn in for the current class.
Annotation Options	
- Color	Allows you to select the color for the region annotations.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard program.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.3.3.7 Results Preview

In this step you will see the preview of the measurement result. The results table contain only the measurements performed in the current view port. The measured image is displayed in the *Analysis View* [▶ 821].

Parameter	Description
Classes	Here you can select the class for which you want to see the measured features. For each class there are two entries: The "parent" class, which shows the features for all objects together, and the "child" class, which shows the features for each individual object.
Highlight Box	
- Color	Allows you to set the color of the highlight box surrounding the selected object in the image.
- Line Width	Allows you to set the line width of the highlight box around the selected object in the image.

Parameter	Description
Back	Moves back to the previous step of the wizard.
Finish	Saves the analysis program created and ends the wizard.
Cancel	Cancels the analysis program and closes the wizard.

For more information, see *Introduction* [▶ 238] to Image Analysis.

20.3.4 Tools in Right Tool Area

20.3.4.1 Images and Documents Tool

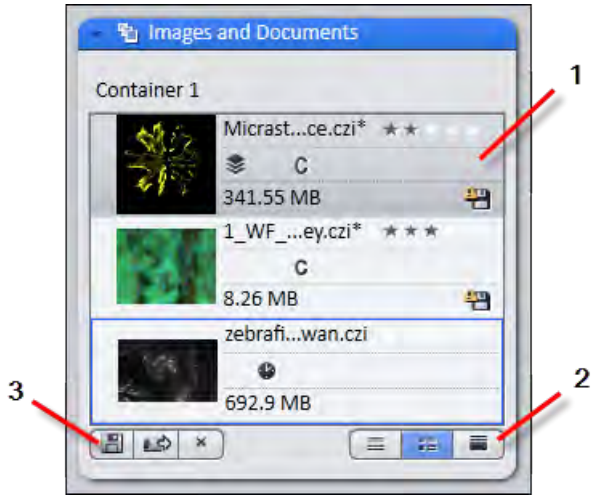


Fig. 20.29: Images and Documents Gallery

1 List of opened images and documents

Here you find a list of all images and documents which are currently opened in the **Center Screen Area**. The disk symbol with a small warning sign means, that you changed and/or have not saved the chosen image or document.

2 Button bar (right)

No.	Parameter	Description
1	**Text view**	Shows text of file only.
2	**Thumbnail view**	Shows a small preview image (thumbnail) of file.
3	**Image view**	Shows a preview image of file.

3 Button bar (left)

No.	Parameter	Description
1	**Save**	Saves he chosen file. **Save as** dialog will open if you haven't saved the file yet.
2	**Quick Export**	Automatically exports the active image with the default settings of **Single File Export** method to**.../user/pictures** (Windows default folder for images). Images of time series or z-stacks will be automatically exported with the default settings of **Movie Export** method to**.../user/movies** (Windows default folder for movies).
3	**Close**	Closes the active image or document.

20.3.4.2 Project and Layers Tool

The Project and Layers Tool provides a layers view and a tree view of image data that you have acquired for the Connect project. Every image that you have acquired for the Connect project is listed. As you acquire or import more image data, the new image data will be listed in the views.

The **Project/Layers** Tool displays the following:

- Images that have been acquired for the Connect project.
- Images that have been imported for the Connect project.
- Position of the image in the project or the layers.

Connect Project tree view

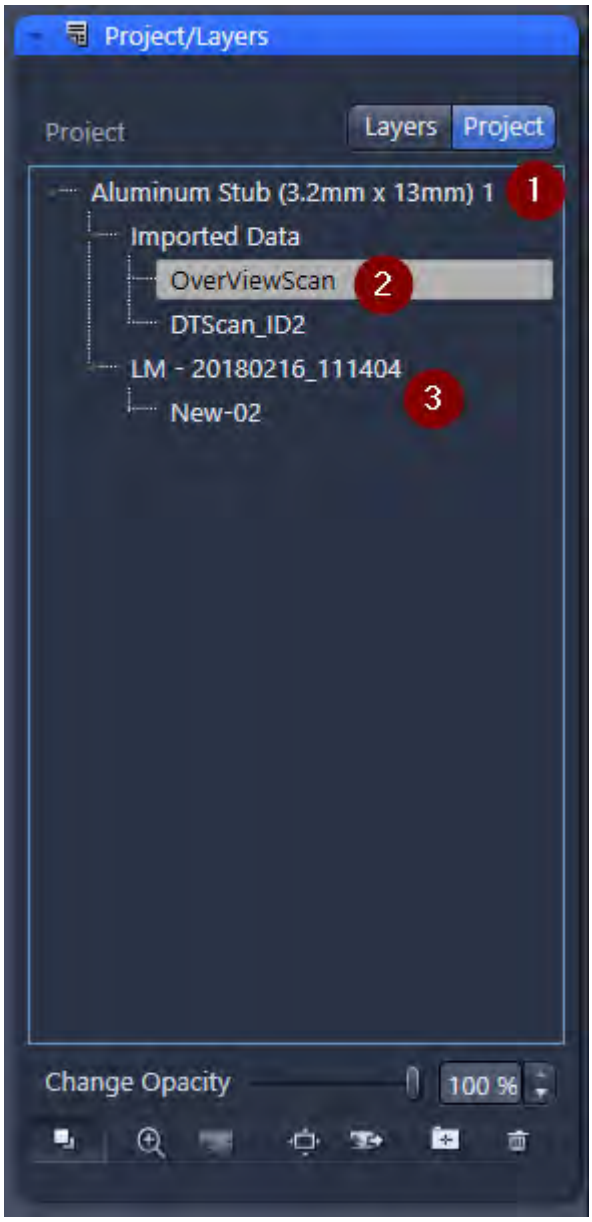


Fig. 20.30: Tree view: Connect project (Example)

Parameter		Description
1	Here: **Aluminium Stub (3.2mm x 13mm) 1**	Sample holder
2	**Imported Data**	Subordinated, the data added to the Connect project is displayed. These images are not acquired within the Connect project.

Parameter		Description
3	Here:	On top, the session node is displayed. It contains the following information. Subordinated, the acquired images are listed.
	LM	Type of microscope, e.g., here:**LM** (light microscope). **EM** (electronic microscope) or **LSM** (laser scanning microscope)
	20180216	Date <yyyymmdd>
	111404	Time <hhmmss>
	New-02	Image taken in this session.

Connect Project Layer View

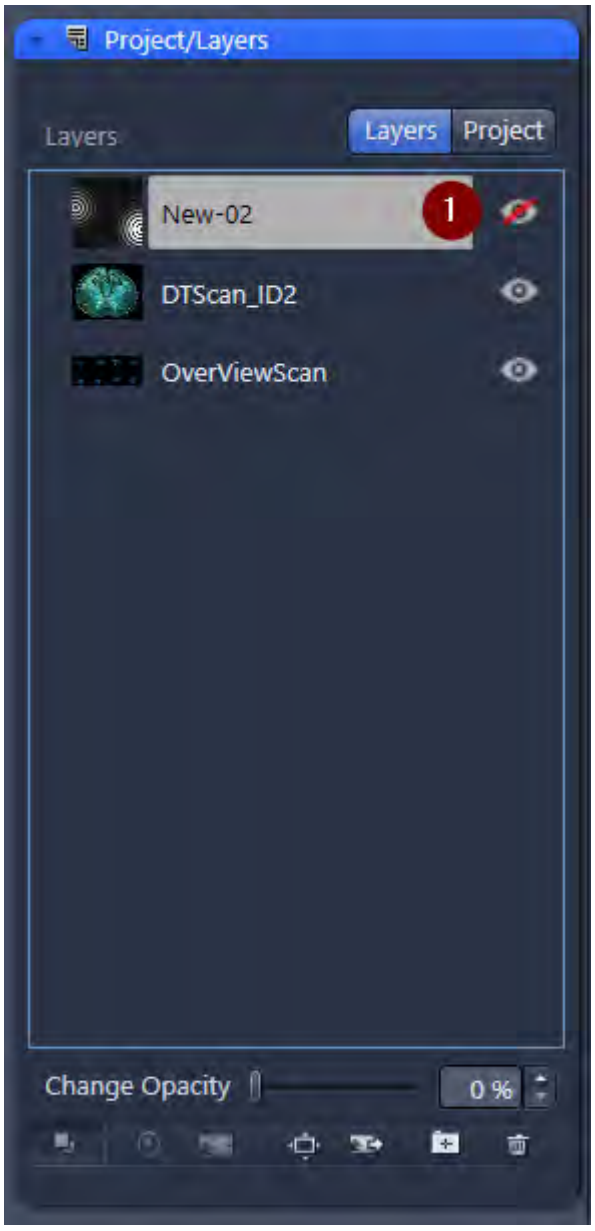


Fig. 20.31: Layers view: Connect project (Example)

Images in your Connect project are displayed in the **Image View** according to its position in the **Layers View**. With drag & drop, you can move images over and under other images. You can also hide them completely (1).

For more information, see *Moving or hiding images* [▶ 564].

Adjustment of Opacity

You can change the opacity of layers. A layer's overall opacity determines to what degree it obscures or reveals the layer beneath it. A layer with 1% opacity appears nearly transparent, whereas one with 100% opacity appears completely opaque.

Button bar

Parameter		Description
1	**Align Selected Data**	Activates the alignment process for the selected data. For more information, see *Activating the Alignment Process* [▶ 570].
2	**Zoom to 100%**	Zooms to the selected image in the **Image View** and displays it at 100% scale.
3	**Add Image**	Imports one or more images to the Connect project. For more information, see *Importing Data* [▶ 565].
4	**Zoom To Extent**	Zooms the **Image View** to show all of the data in your project For more information, see *Zooming to Extent* [▶ 575].
5	**Export Selected Data**	Exports the selected data in one single image. For more information, see *Exporting single image data* [▶ 565].
6	**New Session**	Starts a new session. The images acquired next will be subordinated below a new session node. For more Information, see *Starting a new session* [▶ 564].
7	**Remove Selected Data**	Removes the selected image form the Connect project, but does not delete it from the computer. For more information, see *Removing images from the Connect project* [▶ 563].

20.3.4.3 Microscope Tool

Parameter	Description
Objective List	Here you can easily switch between the objectives and pre-magnification. The color bar on the objective buttons indicates the color for the respective stage limit indicator inside the **Navigation** tab. If you select **autocorr** objectives (motorized correction collar) you can additionally adjust the relevant settings like **Correction Mode, Bottom Thickness** or **Imaging Depth**.

20.3.4.4 Stage Tool

CAUTION

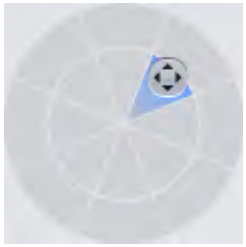
Risk of Crushing Fingers

The drive of a microscope stage with a motorized horizontal stage axis (stage drive) is strong enough to crush fingers or objects between the stage and nearby objects (e.g. a wall).

- Remove your fingers or any objects from the danger area before moving the stage drive.
- Release the joystick immediately to stop the movement.

This tool enables you to navigate the sample in a microscope equipped with a motorized stage. You can use the **Navigation Circle** (software joystick) to move the stage or enter the coordinates directly.

Parameter	Description
Navigation Circle	Enables you to move the stage freely in the X and Y direction and in both diagonal directions. To move the stage, drag the **Navigation Circle** icon in the desired direction. If released, the icon snaps back to the **Navigation Circle** center and the stage stops. The **Navigation Circle** allows four speeds:

Parameter	Description
	■ Normal modes: – Inner segments: Slow – Outer segments: Medium  ■ High-speed modes: – Inner segments: Fast – Outer segments: Very Fast  To enter the high-speed mode, right-click the **Navigation Circle** icon. The **Navigation Circle** turns red. To return to normal speed, right-click the **Navigation Circle** icon again.
Stop	Stops any stage movement immediately. Use this button if you entered **X-Position** and/or **Y-Position** and wish to interrupt the stage movement immediately (e.g. to prevent a collision).
X-Position, Y-Position	Specifies the target coordinates for the stage movement. The stage starts moving immediately after the coordinates have been entered and confirmed; either by pressing the **Return** key or by clicking anywhere outside the current input field.

TIP

You can also control the **Navigation Circle** and thus the motorized stage with the keyboard. To activate keyboard control left-click anywhere inside the segmented **Navigation Circle**. To change between the two speed modes, right-click the central**Navigation Circle** icon.

- To move the stage at the lower speed, use the arrow keys (diagonal movements are also possible).
- To move the stage at the higher speed, use **Shift** + arrow keys.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Speed	Sets the moving speed of the stage in percent (100%=maximum possible speed). Note that the speed setting does not change the speed graduation of the SW joystick.
Acceleration	Sets the acceleration of the stage in percent (100% = maximum acceleration value).
X/Y-Position	
- Set Zero	Sets the current position as the new zero point for the x/y coordinates.
- Calibrate	⚠ **CAUTION** **Risk of Crushing Fingers.** Performs an automatic stage calibration. For this the stage moves to the limit switches to determine the zero points in the x and y direction and then returns to its starting position, which is now defined with its absolute coordinates.

Marks section

This section shows a list where you can define **X** / **Y** positions (optional z value), so called marks. It is also possible to import a list of positions from the list into an experiment including the '**Tiles**' tool.

20.3.4.5 Focus Tool

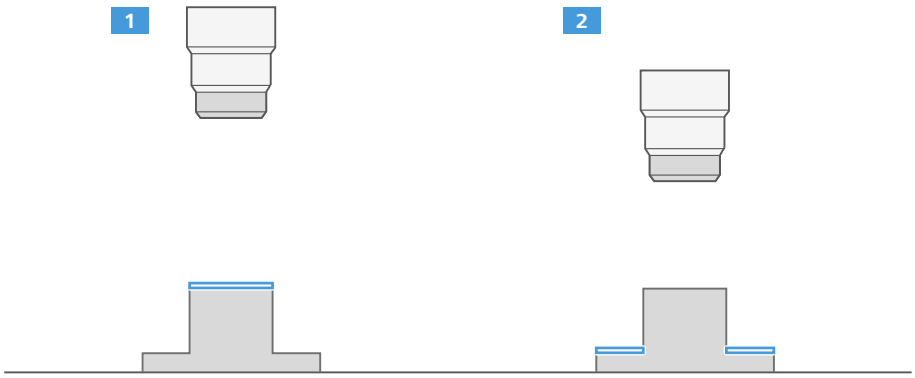
CAUTION

Risk of Crushing Fingers

The drive of a microscope stage with a motorized vertical axis (focus drive) is strong enough to crush fingers or objects between the stage and the microscope stand.

- Remove your fingers or any objects from the danger area before moving the focus drive.
- Release the joystick immediately to stop the movement.

This tool changes the vertical distance (i.e. Z direction) between stage and objective. This enables you to focus the sample, or, for a sample with an uneven surface, to focus the area of interest.



1 Higher objective position, higher sample features in focus
2 Lower objective position, lower sample features in focus

Parameter	Description
Current	Displays the stage position in µm Initially, when you use the **Focus** tool for the first time after switching on the microscope, the exact position of the stage is not known. Therefore, the position indicated by **Current** is initially set to zero. If you enter a value, the stage moves by the entered amount relative to the current position. If you want to move the focus to an absolute position, you must first click **Home** to move the focus to one of the end positions. The value of **Current** is set to this known position. You can then enter an absolute position.

Parameter	Description
Position control	Enables you to set the stage position. You can either use the **Navigation Bar** to move the stage up or down or you can enter the target position in the **Current** input field.
– Navigation Bar	Enables you to move the stage freely in Z direction To move the stage, drag the **Navigation Bar** button in the desired direction. If released, the **Navigation Bar** button snaps back to the center and the stage stops. The **Navigation Bar** allows four speeds.
	 Normal modes: ◆ Inner segments: Slow ◆ Outer segments: Medium
	 High-speed modes: ◆ Inner segments: Fast ◆ Outer segments: Very Fast
	To enter the high-speed mode, right-click the **Navigation Bar** button. The **Navigation Bar** turns red. To return to normal speed, right-click the **Navigation Bar** again.
– Current	Defines the target position of the stage in µm. The stage starts moving immediately after the coordinates have been entered and confirmed by pressing the *Enter* key or by clicking anywhere outside the **Current** input field.
– Stop	Stops any stage movement immediately.
Backlash Correction	Activated: Enhances the positional accuracy by performing an extra movement. When activated the focusing takes slightly longer
Handwheel on	■ Activated: Turning the handwheel also adjusts the focus ■ Deactivated: The handwheel is deactivated: turning it does not affect the focus
Step Size	Defines the difference in µm by which the stage moves at each step. Indirectly this defines the speed of the stage movement.

Parameter	Description
	The **Step Size** also determines the accuracy of the focus position.
Home	Moves the focus to one of the end positions. The value of **Current** is set to this known position. This ensures that the position shown as **Current** corresponds to the actual stage position.
Work	Moves the stage back to the position it was in before using the **Load** button (i.e. the work position) If you have moved the stage (e.g. using the **Navigation Bar**) after moving it into the load position, the work position is lost and the **Work** button will not work.
Load	Increases the distance between objective and stage by 8,000 µm This aids you in exchanging the sample. After exchanging the sample, you can move the stage back into its work position by using the **Work** button. Make sure not to move the stage (e.g. using the **Navigation Bar**) after moving it into the load position. Otherwise, the previous position is lost and the **Work** button will not work.
Measure	This function allows to measure distance in Z direction.
Z-Position	Specifies which position of the motorized z drive is used as the origin (zero value)
– Set Zero	Sets the current focus position as the origin (zero value)
– Calibrate	Performs an automatic calibration

20.3.4.6 Definite Focus Tool

Parameter	Description
Status	Displays the actual status of the device, e.g. Standby or Monitoring.
Find Surface	Tries to find the surface of the cover glass and adjusts the focus position accordingly. If the signal is not strong enough, the actual focus position remains.
Store Focus	Sets (and saves) the current focus position as the stabilizing position.

Parameter	Description
	Note that if you change an objective, the saved position will be deleted.
Recall Focus	Restores the saved focus position.
Lock Focus	If activated, Definite Focus holds the current focus distance by starting a continuous focus stabilization.

20.3.4.7 Incubation Tool

In the Incubation Tool you can define and control parameters for temperature, atmosphere and the Y-Module. The available parameters depend on which components you have configured on your system.

INFO

The symbols behind measured values indicate if the measured and the set values are
- the same = green check mark,
- different = red or blue triangle with exclamation point, or
- not activated = blue circle with question mark.

Parameter	Description
Temperature	Here you can control up to 4 independent heating channels that are linked to certain devices (e.g. incubator XL, heating insert P, objective heater etc.). The devices are assigned to different channels in the Micro Tool Box (MTB).
- Channel (1-4)	**Activated:** The channel will be used for the experiment. Under **Setpoint** you can set the temperature of the channel in °C. Under **Measured** you see the currently measured value.
- Sensor	Shows the current temperature inside the incubation chamber.
Atmosphere	Here you can define the O2 and CO2 concentration, as well as the temperature for an Air Heater module. Note that the meaning of the symbols behind the measurement values is the same like described above.
- O2 Channel	**Activated:** The O2 channel will be used for the experiment. Under **Setpoint** you can set the O2 concentration of the chamber in percent (1-100%).

Parameter		Description
		Under **Measured** you see the currently measured value.
-	CO2 Channel	**Activated:** The CO2 channel will be used for the experiment. Under **Setpoint** you can set the CO2 concentration of the chamber in percent (1-100%). Under **Measured** you see the currently measured value. ■ **Fan Speed**: Sets the rotation speed of the fan.
-	Air Heater	**Activated:** The air heater will be used for the experiment. Under **Setpoint** you can set the temperature of the air heater in °C. Under **Measured** you see the currently measured value. ■ **Fan Speed**: Sets the rotation speed of the fan.
Y-Module		The Y-Module panel allows setting the temperature for two independent modules (thermostats).
-	Selected	Here you can select which module you want to control (**Module 1** or **Module 2**).
-	Circulator 1-2	**Activated:** The channel will be used for the experiment. Under **Setpoint** you can set the temperature of the channel in °C. Under **Measured** you see the currently measured value. For each module two circulator channels can be activated.

20.3.4.8 Macro Tool

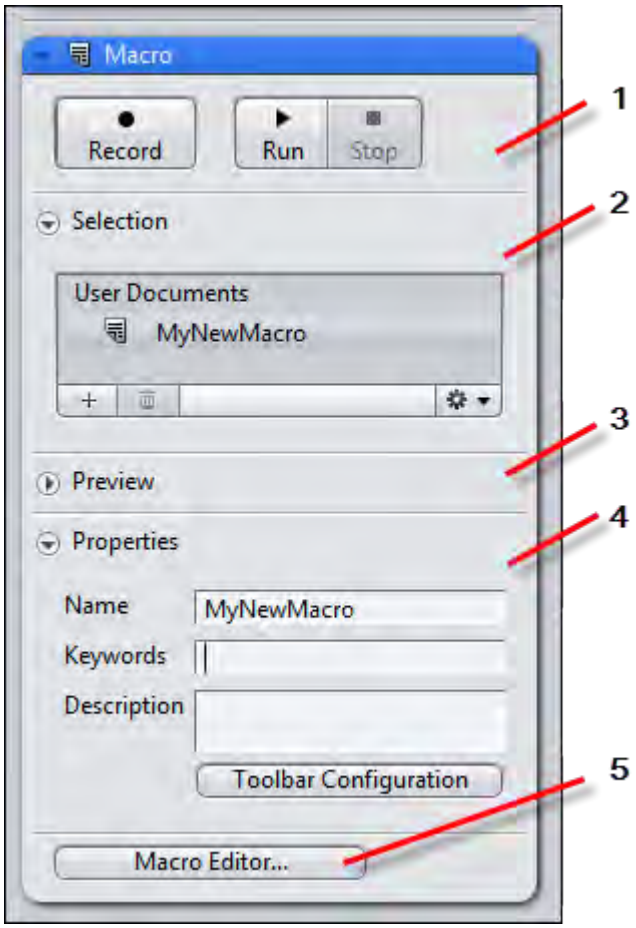


Fig. 20.32: Macro tool

No.	Parameter	Description
1	**Button bar**	
	- Record	Records a new macro. The **Record** button will change to a **Stop** button.
	- Stop	Stops the macro recording.
	- Run	Executes the selected macro.
	- Stop	Stops the running macro at the active command.
2	**Selection**	Here you find a list of all existing macros. Via the  **Options** shortcut list you can create, duplicate, rename and safe new macro files or delete existing macros.
3	**Preview**	Here you see a preview to the macro program code of the selected macro. Editing the macro here is not possible.

No.	Parameter	Description
4	**Properties**	Here you see information of the selected macro. You can edit the fields **keywords** and **description** in here.
	- Toolbar Configuration	When you click on the button you will enter the **Customize Toolbar** dialog. There you can add macro buttons or functions to the toolbar for a quick access. How you can configure the toolbar is described under *Customizing Toolbar* [▶ 287].
5	**Macro Editor…**	Opens the *Macro Editor Dialog* [▶ 596].

20.4 Dialogs

20.4.1 Stage/Focus not calibrated Dialog

If you see this dialog, after you have started the software and the hardware was initialized, you should consider to calibrate the stage and focus drive immediately.

The calibration is necessary, if

- a motorized stage and/or focus drive are used, and
- the stage and focus drive are not calibrated.

To start the calibration procedure, simply click on the **Calibrate Now** button.

⚠ CAUTION

Risk of Crushing Fingers !

Before starting the calibration procedure, ensure that people stand clear of the instrument and that the full travel range is not obstructed by any objects.

If you skip the calibration, you can calibrate the stage and focus drive afterwards within the **Stage Control** and **Focus Control** dialogs accessible via the **Lightpath** tool, see Stage Control and Focus Control. Make sure that the **Show All** mode is activated, to see the **Calibrate** button within the dialogs.

Note that for fully automated system like Axio Scan the axes are calibrated automatically. The calibration is not necessary in that case.

20.4.2 ApoTome Dialog

Parameter	Description
Recommended Grid	In this section you can set the grid with which you want the ApoTome to be operated.
Automatic Grid Control	Activated by default. **Activated**: The appropriate grid for the selected objective is selected automatically (in the case of ApoTome.2). **Deactivated**: It is possible to select another grid from the dropdown list that is now active, e.g. to create a thicker optical section thickness. A list of recommended objectives and suitable wavelengths can be found here.
Calibration Status	Here you can see whether your ApoTome has been calibrated successfully or whether calibration needs to be performed.
Theoretical Thickness	The theoretical section thickness for the selected filter set and the objective used is displayed here.

20.4.3 ApoTome Settings Dialog

Parameter	Description
Camera	Here you can select the camera you wish to use to acquire your **ApoTome** images. As soon as you have selected a camera, **ApoTome** images are generated automatically during acquisition (**Snap**). The selected camera also applies to the **Acquisition** tab.
Live Mode	Here you can choose between the **No Combination**, **Optical Section** and **Conventional Fluorescence** modes for the live image.
Acquisition Mode	Here you can choose between the **No Combination**, **Optical Section** and **Conventional Fluorescence** modes for acquired images.
Phase Images	Here you can choose between no fewer than 3 and no more than 15 phases. Each phase corresponds to a grid position. By default, 5 phases are acquired.
Filter	Here you can set a filter which can be used to filter out residual streaks from the image. You have a choice between no filtering (**Off**) and three strength levels.

Parameter	Description
Image Normalization	**Activated**: The gray values are extended to the maximum available dynamic range following the calculation, see Normalization .

20.4.4 Add Dye or Contrasting Method Dialog

Here you add dyes and contrast techniques to your experiment. The dyes in the database contain important information that is saved in the image document (e.g. spectral characteristics). This information can be used later during image processing (e.g. deconvolution).

INFO

You can add additional dyes to the database via the **Dye Editor** under **Tools | Dye Editor...**.

Fig. 20.33: Add dye dialog

No.	Parameter	Description
1	**Recently used**	Shows the six recently used dyes or contrasting methods in a list. This ensures that you have quick access to the dyes or contrasting methods that you frequently use.
2	**Search**	Here you can enter the name or initial letters of the dye or contrasting method that you want to search for. The search results are displayed immediately in the **Dye Database** list or the **Contrasting Methods** list.

No.	Parameter	Description
		If no search filter is active, the lists of dyes or contrast techniques are arranged in alphabetical order. If you cannot find a certain dye, try using a related dye name or a general name.
3	**Dye Database**	Choose fluorescent dyes here. Double click on the dye or click on the **Add** button to add it to the experiment. The left column shows the name of the dye. The right column contains its color and main emission wavelength. The "**Custom**" entry adds a channel to your experiment without any additional information. This means that the resulting image cannot be used for certain processing operations.
4	**Contrasting Methods**	Only available for **WF** tracks. Here you can choose a contrasting method. Double click on the contrasting method or click on the **Add** button to add it to the experiment.
	Add	Adds the selected dye to the experiment. You can add several dyes/contrasting methods in a row.
	Close	Closes the dialog.

20.5 Image views

The software offers a lot of different image views. The **general image views** are visible for each image. The **specific image views** are available only if the image has the appropriate characteristics (eg multiple channels, Z-stack, etc.) or you have licensed a specific module (e.g. for 3D View). Each image view has general and specific controls which you can use to work with the view.

20.5.1 General image views

These image views are available with any image. Depending on the type of image in question, the **general control elements** may have additional or more limited functions.

20.5.1.1 2D View

This view is the default view for images in **ZEN (blue edition)**. For this view the *General View Controls* [▶ 844] are available to you. To open the view's *context menu* [▶ 779], right-click in the *Center Screen Area* [▶ 31].

20.5.1.1.1 2D View Context Menu

Menu item	Description	Short cut
Full Screen	Switches to full screen mode. To exit full-screen mode, press *F11* or *ESC*.	*F11*
Zoom Group	Here you have access to the main zoom functions (Dimensions tab | *Zoom section* [▶ 846]).	
Rulers	Shows rulers at the top and left edge of the image.	
Show Floating Scale Bar	Shows a scale bar, which you can position freely in your image.	*Alt + S*
Navigator	Shows the **2D view** Navigator window.	
Spot Measurement / Focus ROI	This function is only active in the live image or during **Continuous** mode. Shows a region in which the exposure time is measured and the software autofocus is focused.	
Graphics	This function is activated by default. Shows graphic elements that have been drawn into the image, e.g. annotations or scale bars.	
Show Bleach ROI	This function is only visible with FRAP images. Shows graphic elements that were used during acquisition for bleaching (FRAP).	
Grid	Adds a grid to the image.	
Copy Display Settings	Copies the display settings from an image (Display tab).	
Paste Display Settings	Inserts copied display settings into an image (Display tab).	
Paste Display Settings and Channel Colors		

Menu item	Description	Short cut
**ROI (Region of Interest)	Draw Region of Interest**	Draw a certain region that particularly interests you into the image. You can draw several regions into an image.	*Ctrl+ U*
**ROI (Region of Interest)	Create Subset Images From ROI**	Creates new image documents from the selection regions you have drawn in. All dimensions of the image are taken into account here.	*Ctrl+ Shift + C*
Create Image from View	Creates an image from the current view.	
Paste	Inserts a graphic element into the current image from the clipboard.	*Ctrl + V, Shift + Ins*

20.5.1.2 Gallery View

In this view you see an overview of your multidimensional images. The individual images of the images concerned are presented in a gallery. It is possible to show any combination of dimensions, e.g. channels against time. When you view images for the first time in the **Gallery** view, they are displayed as follows:

Image type	Display
Multichannel image	All the channels present in an image are shown, including the mixed color image.
Time lapse image	All the time points present in an image are shown.
Z-stack image	All Z-planes are shown.
Multichannel & time lapse image	All the time points present in an image are shown. All channels are shown as a mixed color image.
Multichannel & Z-stack image	All Z-planes are shown. All channels are shown as a mixed color image.
Time lapse & Z-stack image	All Z-planes are shown.

Image type	Display
Time lapse, Z-stack & multichannel image	All Z-planes are shown. All channels are shown as a mixed color image.

20.5.1.2.1 Gallery Tools Tab

Here you can specify which dimension you want to be displayed on which axis of the Gallery view. To do this, click on the corresponding dimension´s button.

Displayed Dimensions section

Each of the buttons is only visible if the current image contains the corresponding dimension.

Button	Function
Channels	Shows the channels present as individual images.
Z-Stack	Shows the Z-Planes present as individual images.
Time Series	Shows the time points present as individual images.
Chann.& Z (Channels and Z-Stack)	Shows the channels present in relation to the Z-stack images present.
Chann.& Time (Channels and Time Series)	Shows the channels present in relation to the time lapse images present.
Z&Time (Z-Stack and Time Series)	Shows the Z-Stack images present in relation to the time lapse images present.

Options section

Checkbox	Function
Show Dimension Labels	Inserts annotations into each individual image that provide information on the time point or Z-plane.
Invert X/Y axis	This checkbox is only available if the **Show All** mode is deactivated. It is active only if two dimensions are shown in relation to each other (Chann.&Z, Chann.&Time, Z&Time). If activated, this function inverts the X and Y axis of the view.
Show Graphics	Shows graphics / annotations within the images (in case if graphics / annotations are drawn in).

Checkbox	Function
Show Merged	Only visible for multichannel images. Only active if the channels present are shown. Shows the pseudo colored (mixed) images of all channels in addition to the individual images.

Advanced Functions

The following functions are only visible if the **Show All** mode is activated:

X Axis / Y Axis settings

From the first 2 dropdown list you can select which dimension (depending on which dimensions are available in the active image, e.g. channels, z-stack, etc.) will be shown on the X or Y axis (X axis = horizontal direction, Y axis = vertical direction).

In the second dropdown lists you can select wether you want to display all images of each dimension or if you want to display a certain range of images on the X or Y axis. Therefore you find the following options :

Option	Description
All	Displays all images of the active image in the Gallery view.
Subset by Step	If selected, you can enter a step size in the **Step** input field. If 2 steps are entered, only every second image will be shown. In the **Max.** input field you can enter the desired number of images which will be shown. The step size will be calculated automatically.
Subset by Range	If selected, you can adjust a range of images (e.g. from image 4-10) which is displayed in the view. Use the slider or the input fields to enter the desired range.

Image Creation

Here you can directly create images out of the Gallery view. Select the type of image that you want to create from the **Create image from** dropdown list. If you click on the **Create** button the image will be generated and opened in a new image document. The resulting image contains all the information of the input image; the pixel data are not changed. Following options are available:

Option	Function
Gallery View	Creates an image of the current Gallery view. If this option is selected, the option **Gallery Image from** is available. Here you can additionally select a dimension

Option	Function
	that is not currently displayed (e.g. Single Image will export each single image additionally). The resulting image is always a 24 bit RGB color image. The pixel data of the original image are changed. If the **Burn in graphics** checkbox is activated, all graphics or annotations will be burned into the output images.
Selection Subset	Creates an image from the images that have been selected in the current view. To select an image simply click on the image in the Gallery view. Hold **Crtl**-Key while clicking to select more images at once.
Range Subset	Creates an image from the defined selection range. If this entry is selected, sliders for the selected dimensions appear (**Start**, **End** and **Interval**). Use the sliders to set the selection range you want.

20.5.1.2.2 Gallery Appearance Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Dimension Labels	Here you can define the font and the style, color, position and size of the text for the dimension details that are shown.
Layout	Here you can set the background color of the **Gallery view** and the distance between the individual images (from 1-10 pixels).

20.5.1.3 2.5D View

In the **2.5D view** intensity values in a two-dimensional image are converted into a height map. Here the highest intensity values are represented by the greatest extension in the Z-direction. Overall this results in a so-called 2.5D or pseudo-3D image.

i INFO

Type and source of risk

If you are viewing a multichannel image, you can have the intensity values of the individual channels displayed. To do this, activate or deactivate the desired channels on the **Dimensions** tab.

20.5.1.3.1 2.5D View Tool bars

The tool bars are arranged to the left of and underneath the image area. You can use the tools to control the display of the 2.5D volumes in the image area.

Left Tool Bar

Icon	Parameter	Description
	**Top thumb wheel**	Enlarges or reduces the image area.
	**Rotate**	Use this to rotate the 2.5D volume in any way you wish within the space. This is the default mode when you switch to **2.5D view** for the first time.
	**Zoom**	Use this to increase the zoom factor of the image area.
	**Bottom thumb wheel**	Rotates the 2.5D volume around the horizontal (X) axis.

Bottom Tool Bar

Icon	Parameter	Description
	Left thumb wheel	Use this to rotate the 2.5D volume around the vertical (Y) axis.
	**Bounding Box**	Use this to show or hide a bounding box around the 2.5D volume.

Icon	Parameter	Description
	**Show X/Y Axis**	Use this to show or hide the X/Y axis.
	**Show Z Axis**	Use this to show or hide the Z axis.
	- **Start View**	Use this to switch back to the start view. A top view of the 2.5D volume is displayed. Lateral movements and the zoom factor are adjusted so that the 2.5D volume can be seen at the center of the image area.
	**Right thumb wheel**	Use this to compress the 2.5D volume on the (Z) axis perpendicular to the screen plane.

20.5.1.3.2 2.5D Display Tab

On the **2.5D Display** tab you have 4 **Render mode** options for displaying your 2.5D image.

Parameter	Description
Render mode	
- Profiles	Displays the relief divided into a number of profiles with an equal distance. Set the number of profiles using the **Grid distance** slider.
- Grid	Displays the relief overlaid with a grid. This view supports gray levels only. Make the grid more closely or more coarsely meshed using the **Grid distance** slider.
- Filled	Displays the height relief by upwardly discrete, layered pillars.
- Surface	Displays the relief as a continuous, flowing landscape. Make the surface coarser or finer using the **Grid distance** slider.
Invert Z axis	Use this function for images that contain many large, bright regions. **Activated:** Displays the lowest intensity values by means of the greatest extension in the Z direction.
Use palette	**Activated:** Overlays the relief with the pseudo colors that have been set on the **Dimensions** tab.

Parameter	Description
Show Faces at Side	Only available in the **Surface** render mode. **Activated:** Closes the sides of the relief.
Show plane	**Activated:** Shows two blue, transparent planes in the 2.5D volume. Set the position of the planes using the **X/Y** sliders.
Extract image	To save an individual image in the current view, click on the **Save As** button.

20.5.1.3.3 Series Tab

On the **Series** tab in the **2.5D** view you can create a series of images in the 2.5D view. These series can be played back later as a video clip, for example.

Parameter	Description
Render Series	Here you can select the desired series mode:
- Turn Around X	Here you can define the start/stop angle and the rotation direction around the X axis.
- Turn Around Y	Only visible in the **3D** view. Here you can define the start/stop angle and the rotation direction around the Y axis.
- Turn Around Z	Here you can define the start/stop angle and the rotation direction around the Z axis.
- Start/Stop	Here you can define the angle and zoom settings for the start and end position of your series. The intermediate positions are interpolated evenly.
- Position List	Here you can define any number of positions. The positions can each have completely different rotation, zoom and illumination settings.
- Over Time	Only visible in the **2.5D** view. Here you can define the start time point and end time point for a series. All other settings (rotation, zoom, etc.) remain unchanged.
Stored	
 **Options**	Opens the Options shortcut menu.
Apply	Creates a series image with the current settings.

Parameter	Description
Preview	To obtain a preview of the series, click on  **Play**. To end the preview, click on  **Stop**.
No. of Frames	Here you can enter or select the number of individual images in the series.

20.5.1.3.4 2.5D Display Options Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Angle X	Enter the rotation angle in the X direction with a precision of 1 degree using the slider or input field.
Angle Y	Enter the rotation angle in the Y direction with a precision of 1 degree using the slider or input field.
Z Scaling	Enter the Z scaling using the slider or input field.
Ambient	Reduces or increases the intensity of the ambient lighting in the 2.5D view.
Reflection	Reduces or increases the proportion of the ambient light reflected on the relief.
Shine	Reduces or increases the effect of the ambient light shining on the relief.
Light height	Reduces or increases the intensity of the lighting in the 2.5D view. A small distance means a circular light source at the center, while a large distance illuminates the scene evenly.
Reset lights	Resets all settings to the default values.

20.5.1.4 Profile View

In the **Profile** view you can create intensity profiles of certain regions in your image. In the right image area you can see your image. In the left image area you can see the **Profile window**. The raw data for each channel are displayed in the **Profile table** below the **Profile window**. The measured values of measurements in the profile are shown in the **measurement data table** below the original image.

INFO

To create an intensity profile of a certain region, select a tool on the **Profile Definition** tab. Use this to highlight a region in your image. An intensity profile of the region is generated automatically and displayed in the **Profile window**. To enlarge the view in the Profile window, drag out a rectangular frame using the left mouse button in the Profile window. The selected region is displayed in enlarged form. Right-click to return to the original view.

20.5.1.4.1 Profile Definition Tab

Tool bar

Using the tools you can add certain measurement lines to your image. The intensity profile of each line is shown in the Profile window.

Parameter	Description
Select	Changes the mouse pointer to Selection mode. You can use this to select graphic elements in the image.
Clone	Use this to copy the last selected element and insert it at another position in the image.
Arrow	Use this to insert a measurement line in the original image. The measurement is shown in the **Profile window** in the direction of the arrow.
Polygon	Use this to insert a polygonal measurement line in the original image.
Freehand	Use this to insert a measurement line with a shape of your choice.
Rectangle	Use this to insert a rectangular measurement region.
Keep Tool	**Activated:** Keeps the last selected tool active.
Auto Color	Only visible if the **Show All** mode is activated. **Activated:** Highlights each drawn-in measurement line with a random color.
Stroke Thickness	Here you can enter the line width of the measurement line.

Parameter	Description
Show profile in graphics	**Activated:** Also displays the profile of a measurement line in the original image.

Profile Measurements section

Parameter	Description
Normal	Switches the Profile window back to the view display.
Measurement	Use this to perform a point measurement in the profile. To adopt the value into the measurement data table, click on the **Insert Values** button.
Caliper X	Use this to perform a measurement of a region in the X direction in the profile. . To adopt the value into the measurement data table, click on the **Insert Values** button.
Caliper Y	Use this to perform a measurement of a region in the Y direction in the relevant profile. To adopt the value into the measurement data table, click on the **Insert Values** button.
Reset Table	Empties the measurement data table below the original image.
Add to table	Adds the current measurement in the Profile window to the measurement data table below the original image.

Grid Distance section

Parameter	Description
Grid Distance	Enter the grid distance of the measurement line using the slider or input field.

20.5.1.4.2 Profile View Tab

Here you can configure the display for the Profile view.

Show section

Parameter	Description
Profile Table	**Activated:** Shows the profile table.
Int. Measurement	
Values	
Image	Only visible if the **Show All** mode is activated.

Parameter	Description
	Activated: Shows the original image in the image area.

Channel section

Here you can activate or deactivate the profiles for each channel.

X/Y Axis section

Only visible if the **Show All** mode is activated.

Here you can determine the limits for the **X axis** and **Y axis**.

Parameter	Description
Auto	Sets the limits for the axes automatically.
Norm	Normalizes the profile display to the maximum values of the distribution.
Fixed	Enter the min/max values for the profile display in the **Min**/**Max** input fields.
Log X Scale	
Log Y Scale	

Data Table section

Click on the **Create** button to create a data table from all the measured values displayed. To save the table, click on the **Save As** button.

New Image from section

Here you can create a new image document. Select the type of image from the dropdown list. To save the image, click on the **Save As** button.

20.5.1.5 Histo View

The **Histo** (Histogram) view shows you the gray value histogram of your image. In the right image area you can see your current image and in the left image area you can see the **Histogram window**. At the side you will also find four **data tables**:

- In the first table from the left you will find all the **raw data** for each channel.
- In the second table from the left you will find all the **limits** for each channel of the image next to the image name.
- In the third table from the left you will find the **statistical values** for the gray value distribution, e.g. average, standard deviation, minimum and maximum value.

- The fourth table shows the values of measurements in the histogram. The results (Integral) show the percentaged fractions of the occurences.

20.5.1.5.1 Histo Definition Tab

Tools

With these tools you can add specific ranges to your image. The histogram window displays the gray value histogram for each area.

Parameter	Description
 **Select**	Changes the mouse pointer to Selection mode. You can use this to select graphic elements in the image.
Clone	Use this to copy the last selected element and insert it at another position in the image.
 **Draw Rectangle**	Use this to insert a rectangular measurement region.
 **Draw Circle**	Use this to insert a circular measurement region.
 **Draw Spline Contour**	Use this to insert a measurement region with a shape of your choice. The line is closed automatically.
 **Draw Polygon**	Use this to insert a polygonal measurement region in the original image.
Keep Tool	**Activated:** Keeps the last selected tool active.
Auto color	Only visible if the **Show All** mode is activated. **Activated:** Highlights each drawn-in measurement region with a random color.
Normal	Switches the Profile window back to the view display.
CaliperX	Use this to perform a measurement of a region in X direction in the histogram display. To adopt the value into the measurement data table, click on the **Add to Table** button.
Add to Table	Only active if a measurement (using CaliperX mode) was drawn into the histogram. Adds the current measurement into a measurement data table below the original image.

Parameter	Description
Reset Table	Deletes the measurement data table below the original image.

Histo Table section

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Histo Table	Select the type of gray value distribution from the dropdown list. The following types are available:
- Frequency	If selected, the histogram is displayed according to the relative frequency of the gray values in percent.
- SumUp	
- SumDown	
Relative Frequency	

Parameter	Description
Bin count	Enter the Bin count using the slider.
Bin size	Only visible if the **Show All** mode is activated. Enter the Bin size using the slider.
Logarithmic binning	Only visible if the **Show All** mode is activated. **Activated:** Switches from the linear to a logarithmic class width. The class size is calculated automatically.
Lower Threshold	Enter the lower threshold value for the gray value distribution using the slider or spin box/input field. All regions in the image with gray values below the lower threshold value are overlaid in blue and all those with gray values above the upper threshold value are overlaid in red.
Skip Black	**Activated:** Automatically subtracts the lowest value of the gray distribution. If activated, the settings for the lower threshold value are deactivated.
Upper Threshold	Enter the upper threshold value for the gray value distribution using the slider or spin box/input field. All regions in the image with gray values below the lower threshold value are overlaid in blue and all those with gray values above the upper threshold value are overlaid in red.

Parameter	Description
Skip White	**Activated:** Automatically subtracts the highest value of the gray distribution. The settings for the upper threshold value are deactivated.
Show thresholds	Only visible if the **Show All** mode is activated. **Activated:** Shows the threshold values as colored overlays in the original image.

20.5.1.5.2 Histo View Tab

Here you can configure the display for the Histo view.

Show section

Parameter	Description
Statistic table	**Activated:** Shows the table containing the statistical values in the image area.
Int. Measurement	**Activated:** Shows the measurement data table below the original image.
Frequency Table	Only visible if the **Show All** mode is activated. **Activated:** Shows the table containing the raw data for each channel.
Image	Only visible if the **Show All** mode is activated. **Activated:** Shows the original image in the image area.

Channel section

Here you can activate or deactivate the histograms for each channel.

X/Y Axis section

Only visible if the **Show All** mode is activated.

Here you can determine the limits for the **X axis** and **Y axis**.

Parameter	Description
Auto	Sets the limits for the axes automatically.
Norm	Normalizes the histogram display to the maximum values of the distribution.

Parameter	Description
Fixed	Enter the min/max values for the histogram display in the **Min**/**Max** input fields.
Logarithmic	

Channel Transparency section

Parameter	Description
Channel Transparency	

Data Table section

Click on the **Create** button to create a data table from all the measured values displayed. To save the table, click on the **Save As** button.

New Image From section

Here you can create a new image document. Select the type of image from the dropdown list. To save the image, click on the **Save As** button.

20.5.1.6 Measure View

In this view measured values from images are displayed in a table. The table is only visible if there are annotations/measured values in the image. To highlight the row of the table containing the measured values of a graphic element, click on a graphic element in the image. To highlight a graphic element in the image, click on the measured value in the row of the table.

20.5.1.6.1 Measurement Tab

Here you can specify how to draw the graphic elements for measurements into an image and how the measurement data are displayed. You can also add user-specific features to individual graphic elements.

Graphic Elements Section

Only visible if the current image is a multidimensional image.

Here you can decide, whether to draw a graphic element "globally" into all channels, Z-positions, time points, etc., or whether to draw in separate elements for the view currently displayed.

Parameter	Description
Channel	**Activated:** Activates the Single Channel mode. Only draws graphic elements into the channel currently displayed.
Time	**Activated:** Only draws graphic elements into the time point currently displayed.
Z-Position	**Activated:** Only draws graphic elements into the Z-position currently displayed.
Copy in All Following	**Activated:** Draws a new graphic element into the view currently displayed and into all subsequent time points or Z-positions.

New Feature section

Here you can add a defined feature to the selected graphic element.

Parameter	Description
Name	Here you can enter a name for the feature.
Value	Here you can enter the desired value for the current graphic element.
Unit	Here you can enter the desired unit for the feature.
Add	Adds the feature. The measurement data table is expanded to include this feature.
Remove	Removes the selected feature.

Data Display section

Here you can specify how you want the measured values for the drawn-in graphic elements to be displayed.

Parameter	Description
Format	
- Table	Displays the measured values in a row of a table. As you can specify the features individually for each graphic element, the number of columns containing measured values may differ from graphic element to graphic element (i.e. from row to row).
- List	Displays each measured value in a separate row. The measurement data table then has the following defined columns:

Parameter	Description
	■ **Name:** Name of the graphic element (e.g. line). ■ **Feature:** Name of the feature (e.g. distance). ■ **Value:** Value of the feature. ■ **Unit:** Unit of the feature (e.g. µm).
Current View	Only displays the measured values of the current view.
All Views	Displays all measured values contained in the image.
Create Document	Creates a measurement data table from the measured values displayed. The table is saved as a separate document.

20.5.1.7 Info View

The **Info View** allows you to display extensive information about your image. Using the  buttons in each of the sections you can show additional fields in the sections or hide fields that are currently showing. To show or hide individual sections, click on the  button to the left of the headings for each of the sections.

i INFO

The **Info View** only shows the fields that actually contain data. Using the  buttons in each of the sections you can show additional fields. To do this, activate the corresponding checkboxes in the shortcut menu.

20.5.1.7.1 General section

Parameter	Description
Title	Here you can enter a title for your image.
Description	Here you can enter a description for your image.
Comment	Here you can enter a comment.
Keywords	Here you can enter keywords for your image.
Rating	Here you can enter a rating for your image. To enter a rating, click on the star icons.

20.5.1.7.2 File section

Parameter	Description
Name	Displays the file name of the image without file extension.
File Type	Displays the file type of the image.
File Path	Displays the location where the image is saved in your file system.
File Size	Displays the file size of the image.
Created	Displays when the image was created.
Last Modified	Displays when the image was last changed.
User	Displays the name of the user. You can enter the user name in the **Extras** menu	**Options**	*User* [▶ 618].

20.5.1.7.3 Image Dimensions section

Parameter	Description
Time Series	Displays how many time points the image contains. The value in brackets shows the full duration of acquisition.
Z-Stack	Displays how many Z-planes the image contains. The value in brackets shows the full size of the Z-stack.
Channels	Displays how many channels the image contains.
Tiles	Displays how many individual images (tiles) the image is composed of.
Scaling (per Pixel)	Displays the scaling per pixel. **Edit** button: Opens the *Edit Scaling dialog* [▶ 798].
Image Size (Pixels)	Displays the image size in pixels. The first number indicates the horizontal dimension and the second the vertical dimension.
Image Size (Scaled)	Displays the scaled image size. The first number indicates the horizontal dimension and the second the vertical dimension.
Bit Depth	Displays the bit depth of the active image, e.g. 24 Bit. The bit depth depends on the camera settings when acquiring the image.

Parameter	Description
Stage Position	Displays the stage position. Within the image this is the center point. In the case of tile images this is the center point of the first tile.
Scanning Mode	Displays the scanning mode. This can either be the image field, an image line or a pixel.
Scanner Zoom	Displays the zoom factor. The value 1 corresponds to the standardized image field of all confocal systems.
Rotation	Displays the rotation of the image field around the optical axis.
Crop Offset	Displays the shift of the scanned region from the center of the image.
Pixel Time	Displays for how long the emission signal is collected per pixel. This is the so-called integration time.
Line Time	Displays how long the system needs to scan an image line.
Frame Time	Displays how long the system needs to scan the image field displayed in X and Y in full.
Averaging	Displays the number of individual measurements per image or line. The average of the individual measurements produces the pixel intensity values for the image.

20.5.1.7.3.1 Edit Scaling Dialog

The **Edit Scaling** dialog is divided up into table form. The columns contain the **Scaling Factor** and **Scaling Unit** and the rows the dimensions.

Parameter	Description
Scale Factor column	Enter the desired scaling factor in the input fields.
Scale Unit column	Select the desired scaling unit from the dropdown list. The metric units **Meter**, **Centimeter**, **Millimeter**, **Micrometer**, **Nanometer** and **Picometers** are available as options, as well as the imperial units **Inch** and **Mil**.
Row X	Shows the scaling in the horizontal direction.
Row Y	Shows the scaling in the vertical direction.
Row Z	Shows the scaling in the 3rd dimension. This is usually the focus direction.

INFO

Row **Z** for the third dimension is only displayed if the image has a third dimension.

20.5.1.7.4 Acquisition Information section

Parameter	Description
Acquisition Start	Displays the date and time when the acquisition of the image took place.
Microscope	Displays which microscope was used to acquire the image.
Objective	Displays which objective was used to acquire the image.
Optovar	Displays which Optovar was used to acquire the image.
Reflector	Displays which reflector cube was used to acquire the image.
Beam Splitter	Displays which beam splitter was used to acquire the channel.
Emission Wavelength	Displays the main emission wavelength of the channel or dye used.
Excitation Wavelength	Displays the main excitation wavelength of the channel or dye used.
Contrast Method	Displays the contrast technique. In transmitted light this is the condenser setting, while in reflected light it corresponds to the selected reflector cube.
Ligth Source Intensity	Displays the lamp intensity with which the image was acquired.
Pinhole	Displays the diameter of the pinhole.
Laser Power Percent	Displays the percentage of laser power used for acquisition.
Laser Blanking	Blanking of the laser during scanner movement without acquisition.
Laser Atten. Bleach.	Displays the laser power used for bleaching.
Channel Name	Displays the name of the channel.

Parameter	Description
Channel Description	Here you can enter a description of the channel. Describe the exact use of the channel or what can be seen in this channel.
Dye Name	Displays the name of the dye.
Channel Color	Displays the pseudo color allocated to the channel.
Camera	Displays which camera was used to acquire the image.
Camera Adapter	Displays which camera adapter was used to acquire the image.
EM Gain	Displays the factor by which the camera signal was increased.
Exposure Time	Displays the exposure time with which the image was acquired.
Depth of Focus	Displays the depth of focus. This is calculated according to the following formula: Depth of field = (2 * n * λ) / $(NA)^2$ = (2 * refractive index * emission wavelength) / $(\text{numerical aperture})^2$
Section Thickness	Displays the thickness of the optical section.
Binning Mode	Displays whether binning was applied during acquisition and how much.
Detector	Displays which detector was used for acquisition.
Detector Gain	Displays the gain setting of the detector for acquisition.
Detector Digital Gain	Displays the digital gain of the detector during acquisition.
Detector Offset	Displays the offset settings of the detector during acquisition.

i INFO

In the case of multichannel images the channel-dependent information is saved in a table. Here the sorting of the individual information fields may differ.

20.5.1.8 Tree View

The **Tree View** is visible only if you have activated the **Enable Tree view** checkbox under **Tools** | **Options** | **Documents**. The checkbox is deactivated by default.

The tree view shows a detailed list containing all meta data of the selected image.

20.5.2 Specific image views

These image views are only visible if the image has corresponding features. The **3D view**, for example, is only visible for Z-stack images.

20.5.2.1 Split View

Only visible for multichannel images and not during the acquisition of LSM images.

In this view you see all channels of a multichannel image. The channels are displayed side by side, in the channel colors that have been assigned to them. You also see the mixed image view in which all the channels are overlaid.

i INFO

By double-clicking on an acquired multi-channel image, you can switch quickly to the **2D** view.

Double-clicking on the image in the **2D** view switches you back to the **Split** view. If you double-click on one of the displayed channels, only this channel will be shown in **2D** View.

20.5.2.2 Ortho View

Only visible for Z-stack images.

In this view (orthogonal section) you can analyze your Z-stack images. Here, in addition to the top view (X/Y axis), you will also see the section views of the X/Z axes (top) and Y/Z axes (right).

20.5.2.2.1 Ortho Display Tab

Parameter	Description
Cut Lines	Enter the positions (pixel values) for the section lines using the **X/Y/Z** sliders or input fields. Alternatively you can also adjust the positions directly in the image area. To adjust the positions, move the mouse over a section line in the image. Hold down the left mouse button and move the mouse.
- Mid	Positions the relevant slider at the center of the view.
Line Width	Only visible if the **Show All** mode is activated. Enter the thickness of the section lines in pixels using the sliders or input fields. This results in a maximum intensity projection being displayed over the selected pixel width.
Cut Line Opacity	Only visible if the **Show All** mode is activated.

Parameter	Description
	Here you can enter the degree of opacity of the section lines from 0% (invisible) to 100% (completely opaque).
Maximum Intensity Projection (MIP)	**Activated:** Displays a maximum intensity projection (MIP) across all planes for all 3 views. The section lines are hidden and the control elements that are not relevant in this view are deactivated.
Measure 3D Distance	Only visible if the **Show All** mode is activated. **Activated:** Activates the 3D distance measurement. The **Set Start** and **Set End** buttons and the **Distance** display field are visible. To set a starting point for the measurement, navigate the cutlines to the desired starting point and click on the **Set Start** button. The **Set End** button will become active. To set an end point for the measurement, navigate the cutlines to the desired end point and click on the **Set End** button. The pixel coordinates of the measurement points are displayed next to the buttons. The measured distance is displayed in the **Distance** display field.
New Image	Here you can create a new image document. Select the desired view from the dropdown list (only in **Show All** mode). To save the image, click on the **Create** button.

INFO

The resulting image contains the image data in the same dynamics (bit depth) as the original image and consists of the same number of channels (in the case of multichannel images) or time points (in the case of time lapse images) as the original image, but only contains the Z-plane currently displayed.

20.5.2.3 Cut View

Only visible for Z-Stack images.

This view allows looking from a different x/y/z-position/pitch/yaw to the slices of your Z-Stack image.

20.5.2.3.1 Cut Display Tab

Parameter	Description
X/Y/Z	Moves view in X/Y/Z-direction.
Pitch	Adjusts view pitch.

Parameter	Description
Yaw	Adjusts view yaw.
Navigator	Shows the **Navigator**-Window in the **Center Screen Area**. Adjust settings directly in graphical illustration.
Reset	Resets each setting to default.
Reset All	Resets all settings to default.

20.5.2.4 3D View

This view is only available if:

- you have licensed and activated the **3D Visualization** module.
- you have loaded or acquired a Z-Stack image.

The 3D view displays Z-Stack images three-dimensionally as a 3D volume. Using the toolbars on the left, right and bottom of the image area (**1**) you can directly control and move the 3D volume. With the View Specific Tabs (**2**) you find a lot of parameters to adjust the appearance and further settings of the 3D volume.

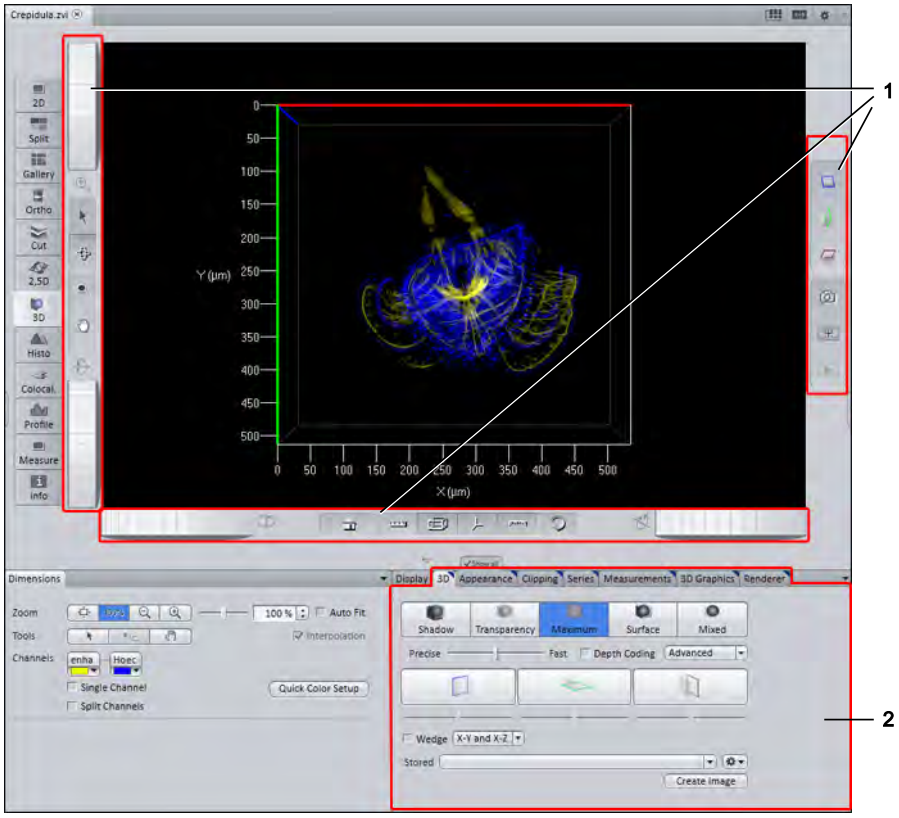


Fig. 20.34: 3D View

1 Tool bars	see *Tool bars* [▶ 804]

2 View Specific Tabs	see *3D Tab* [▶ 808], *Appearance Tab* [▶ 810], *Clipping Tab* [▶ 813], *Series Tab* [▶ 815], *Measurements Tab* [▶ 818], *3D Graphics Tab* [▶ 820]

20.5.2.4.1 Tool bars

The tool bars are arranged to the left and right of the image area and underneath it. You can use the tools to control and adjust the display of the 3D volumes in the image area.

20.5.2.4.1.1 Tool Bar (Left)

Parameter	Description
 **Top thumb wheel**	Zooms in or out of the 3d image.
 **Arrow**	Use this to select end points of measurement tools that have been drawn into the 3d image (**Measure** tab). You can then edit the position of the end points.
 **Rotate**	Use this to rotate the 3d image in any way you wish within the space. This is the default mode when you switch to **3D view** for the first time.
 **Zoom**	Use this to increase or reduce the zoom factor of the image area.

Parameter	Description
 **Move**	Use this to move the 3d image laterally.
 **Fly**	Clicking on this button enables the flight mode. This mode allows to fly virtually through the 3d image. Use the keys from the list below to control your flight.
 **Bottom thumb wheel**	Rotates the 3d image around the horizontal (X) axis.

Flight Mode Key Layout / Controls

Key	Function
W	Forward
S	Backward
A	Left
D	Right
Space	Up
C	Down
E	Rotate (clockwise)
Q	Rotate (counter-clockwise)
X	Precision Mode, enables slower movement

20.5.2.4.1.2 Tool Bar (Right)

Parameter	Description
 **Toggle X/Y clipping plane (blue)**	Hides the X/Y clipping plane.
 **Toggle X/Z clipping plane (green)**	Hides the X/Z clipping plane.
 **Toggle Y/Z clipping plane (red)**	Hides the Y/Z clipping plane.
 **Snap**	Creates a 2D image of the current view. The image is a 24 bit color image. All annotations are burnt in automatically.
 **Add**	Adds the current view to a position list as a new position. With the help of position lists you can have your view calculated as a series of individual images. This series can then be exported as a movie, for example.
 **Play**	Only active if a position list containing at least two saved positions exists. Plays back a preview of the series that is calculated. To stop the preview, click on the button again.

20.5.2.4.1.3 Tool Bar (Bottom)

Control element	Description
Left thumb wheel 	Rotates the 3D volume around the vertical (Y) axis.

Control element	Description
Home view button 	Switches back to the start view from any view. A top view of the 3D volume is displayed. Lateral movements and the zoom factor are adjusted so that the 3D volume can be seen at the center of the image area.
Show measurements button 	Shows or hides drawn-in measurements. If measurements are drawn-in, a table of the measurements appears at the right side of the image area.
Show bounding box button 	Shows or hides a bounding box around the 3D volume.
Show coordinate axes in color button 	Shows or hides the coordinate axes. ■ X axis = red ■ Y axis = blue ■ Z axis = green
Show scaling button 	Shows or hides the scaling on each axis.
Spin Mode button 	Enables the spin mode. This allows to set the 3D volume in continuous motion. You will find a short description on how to use the spin mode below.
Right thumb wheel 	Rotates the 3D volume around the (Z) axis perpendicular to the screen plane.

20.5.2.4.1.4 Animating the 3D Volume

Prerequisites
- The **Rotation** mode in the left tool bar is selected.

Procedure

1 Click on the **Spin mode** icon.

2 Move the mouse inside the image area.

3 Hold down the left mouse button and move the mouse slightly to the left or right.

4 Release the left mouse button again.

The 3D volume rotates continuously in the direction in which you moved the mouse. If you move the mouse quickly, the 3D volume rotates quickly. If you move the mouse slowly, the 3D volume rotates slowly.

To stop the animation, left-click again in the image area.

20.5.2.4.2 3D Tab

Here you can specify which projection/rendering mode you want to use to display the 3D volume. There are 5 view modes available. To activate the desired view mode, click on the corresponding button. An activated button (respectively the mode) appears in blue color.

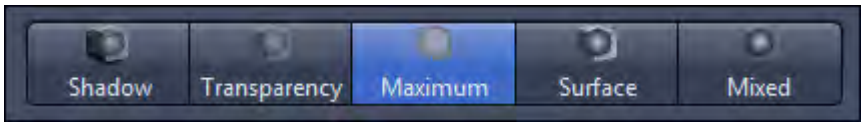


Fig. 20.35: Mode Buttons

Parameter	Description
Shadow	Activates Shadow projection mode.
Transparency	Activates Transparency rendering mode.
Maximum	Activates Maximum intensity projection mode.
Surface	Activates Surface reconstruction mode
Mixed	Activates combination of transparency rendering and surface reconstruction mode

Parameter	Description
Precise/Fast	Adjust the level of detail of the 3d volume here.

Parameter	Description
	■ If you select the **Precise** setting, all the information present in the image is used to achieve the best possible display. The calculation time can increase accordingly. ■ If you select the **Fast** setting, the image data are significantly reduced before the calculation. The calculation is fast, but only a very coarse 3D display of the volume is achieved.
Depth coding	Only active if **Transparency**, **Maximum** or **Mixed** mode is activated. **Activated:** Replaces the channel colors of the volume with a rainbow color table. This is also shown as a palette with an indication of the depth (in scaled units).
Toggle Clipping Planes	By activating or deactivating the buttons you can show or hide the corresponding clipping planes in the 3D volume. If you right-click on an activated button, a shortcut menu opens. Here you can select whether you want the back (**Clip Back**), front (**Clip Front**) or both sides of the 3D volume to be clipped. You can also specify the **Style** of the clipping plane . Under each button is a slider. You can use this to move the relevant clipping plane within the volume.
Wedge	**Activated:** Activates two texture planes. Only the sector between the planes is cut out. You can select which planes you want to be used for the wedge function from the dropdown list. The selection is also visible in the relevant buttons.
Stored	Here you can select saved 3D settings.
 **Options**	Opens the options menu, see list below.
Create Image	Creates a new image from the current view. This image is a 24 bit RGB color image. All graphic elements, such as annotations, are burnt in.

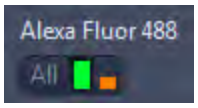
Options

Parameter	Description
New	Creates a new settings file that is given a name automatically and has the file extension *.cz3dr. The settings file can be found in the user path under \My Documents\Carl Zeiss\ZEN\Documents\3D render settings
Delete	Deletes the selected settings file from the hard drive.
Rename	Renames the selected settings file. Enter a new name in the input field and confirm with **OK**.
Save As	Saves the selected settings file under a different name.
Import	Imports a ***.cz3dr** file and applies it to the current image.
Export	Exports a ***.cz3dr** file to a different location.

20.5.2.4.3 Appearance Tab

Here you can define the appearance of the 3D volume. On the tabs available on this tab, select the setting that you want to change (e.g. Transparency). Depending on which mode you have activated on **3D** tab, different tabs and parameters are available.

20.5.2.4.3.1 Transparency Tab

Parameter	Description
 **Channel selection**	Here you can select the channel of a multichannel image for which you want to set the transparency.
Threshold	Sets the lower threshold value in percent of the gray levels displayed. With this setting you specify the gray value range for the relevant channel that you want to be included in the rendered image.
Only available in **Shadow** and **Transparency** projection mode:	
Ramp	Sets the extent of the transition from completely transparent to completely opaque (0-100 percent).
Maximum	Sets the level of opacity (0-100 percent).

Parameter	Description
Histogram	Displays the settings that you enter using the sliders schematically. The X axis represents the gray level values and the Y axis the opacity. You can also change the position of the curve using the mouse.
Reset	Resets all parameters to the original values.

20.5.2.4.3.2 Surface Tab

Only visible if **Surface** or **Mixed** view mode is activated on the **3D** tab.

Parameter	Description
 **Channel selection**	Here you can select the channel of a multichannel image for which you want to adjust the surface settings.
Threshold	Sets the lower threshold value in percent of the gray levels displayed. With this setting you specify the gray value range for the relevant channel that you want to be included in the rendered image.
Ambient Light	Sets the ambient light on a scale from 0 to 100%.
Spectacular Light	Sets the spectacular light from 0 to 100%. This value influences the differences between bright and dark structures.
Shininess	Sets the surface shininess.
Reset	Resets all parameters to the original values.

20.5.2.4.3.3 Channels Tab

Only visible if **Mixed** view mode is activated on the **3D** tab.

Here you can specify how **Transparency** and **Surface** settings are mixed. In the case of multichannel images you can also configure these settings differently for each channel.

Activate the corresponding checkboxes for Transparency and Surface in the list.

20.5.2.4.3.4 Background Tab

Parameter	Description
Background Color	Sets the background color for the 3D view. To do this, click on the color field and select the desired color.

20.5.2.4.3.5 Light Tab

Parameter	Description
Brightness	Sets the brightness of the light source (from 0 - 100 %).
Azimuth	Here you can enter the angle of the light source above the virtual horizon.
Elongation	Here you can enter the light source's horizontal angle of incidence.
Light source	As an alternative to the slider or input field, you can set the **Azimuth** and **Elongation** together by using the mouse to move the point within the light source display.
Reset	Resets all parameters to the original values.

20.5.2.4.3.6 Projection Tab

Parameter	Description
View angle	Sets the projection angle at which you want to view the scene freely between 0° and 80°. The effect of this on the perspective display is as if you are viewing the 3D image through a telephoto or wide-angle lens.
Scale Z	Here you can set the scaling of the volume in the Z direction (value range 10% - 600%).
Stereo anaglyph	**Activated**: Displays the 3D volume as anaglyphs. You can choose between a ■ **Red/Green** display, or a ■ **Red/Cyan** display.
Camera separation	Sets the distance between the two virtual cameras (0-20%).
Parallax shift	Sets the degree of movement that is necessary to bring the two camera images back into line (-100 to +100%).

Parameter	Description
Reset	Resets all parameters to the original values.

20.5.2.4.4 Clipping Tab

Only visible if the **Show All** mode is activated.

Here you can edit clipping planes. To select a clipping plane, click on the corresponding button. The editing functions which you can use to modify the selected clipping plane become visible when you activate the specific clipping plane.

INFO

On the **Clipping** tab you can edit the clipping planes. On the **3D** tab you can activate or deactivate the relevant clipping planes in the 3D volume.

Parameter	Description
Show All Clipping Planes	**Activated:** Automatically inserts all 3 clipping planes into the 3D volume. Additionally the editing functions for each clipping plane were activated automatically.
 **X/Y**	Activates the editing functions for the X/Y clipping plane.
 **X/Z**	Activates the editing functions for the X/Z clipping plane.
 **Y/Z**	Activates the editing functions for the Y/Z clipping plane.
Activate	**Activated:** Activates the selected clipping plane in the 3D volume. The corresponding settings become visible. You will find a detailed description of the settings in the list below. **Note:** Each plane is positioned at the center of the 3D volume and aligned orthogonally (in the X/Y, X/Z, Y/Z direction).
Reset All	Resets all parameters to the original values.

The following parameters are only visible if the **Activate** checkbox is activated and a clipping plane has been selected.

Parameter	Description
Clipping Plane Style	Change the display of the selected clipping plane using the dropdown list to the right of the **Activate** checkbox. The following settings are available:
- Invisible	The plane is invisible.
- Colored	The plane is displayed in color. The frame color is used with 50% transparency here.
- Binary	The data above the threshold value that are touched by the clipping plane are displayed in binary form as a white area. Black pixels are non-transparent.
- Transparent	The data that are touched by the clipping plane are displayed as they are in **Transparent** view mode, but in 2 dimensions. The ramp for the transparency is linear here. Black pixels are transparent.
- Textured opaque	The display appears as it does with the **Textured** setting. Black pixels do not let any light through, however, meaning that the render data behind them are not displayed.
Outline	**Activated:** Displays the frame of the selected clipping plane. Enter the frame color via the color field.
Clip Front	**Activated:** Clips the front of the 3D volume.
Clip Back	**Activated:** Clips the back of the 3D volume.
Clip Transparency	Only active if **Mixed** view mode is activated. **Activated:** In addition to the surface data, also clips the transparency data.
Clip Surface Channels	Only visible if **Surface** or **Mixed** view mode is activated. Here you can enter which channel you want to be clipped using the channel buttons.
Position	Here you can enter the position of the selected clipping plane.
<X (X Angle)	Here you can enter the X angle for the selected clipping plane.
<Y (Y Angle)	Here you can enter the Y angle for the selected clipping plane.

Parameter	Description
Reset Orientation	Resets the selected clipping plane to the original position.

20.5.2.4.5 Series Tab

Here you can create render series of individual views, which you can later view and export as a movie. The tab contains different control elements depending on the Render Series type. The following parameters are the same for all render series types: **Render Series** section, **Stored** section, **Apply** button and **Fixed Resolution** checkbox.

Parameter	Description
Render Series	Here you can select the desired series mode. Depending on the chosen render series type, different parameters are displayed.
- Turn Around X	Define the start/stop angle and the rotation direction around the X axis.
- Turn Around Y	Only visible in the **3D** view. Define the start/stop angle and the rotation direction around the Y axis.
- Turn Around Z	Define the start/stop angle and the rotation direction around the Z axis.
- Start/Stop	Define the angle and zoom settings for the start and end position of your series. The intermediate positions are interpolated evenly.
- Position list	Define any number of positions. The positions can each have completely different rotation, zoom and illumination settings.
- Over Time	Only visible in the **2.5D** view. Define the start time point and end time point for a series. All other settings (rotation, zoom, etc.) remain unchanged.
Apply	If clicking on this button the series will be calculated. A new image document will be opened in the **Center Screen Area**. You can view the series by clicking on the **Play** button in the **Dimensions** tab.
Stored	Only visible if **Show All** is activated. Here you manage your series settings. Via the dropdown list you can select a saved settings file.

Parameter	Description
 **Options**	Clicking on the button opens a shortcut menu with the following options:
- New	Creates a new settings file (***.czsht**). This file can be found in the user's local document path (e.g. \My Documents\Carl Zeiss\ZEN\Documents, in a corresponding subfolder).
- Delete	Deletes the selected settings file.
- Rename	Opens a dialog to rename the selected settings file.
- Save As	Saves a copy of the currently selected settings file under a different name.
- Save	Saves changes to a currently selected settings file.
- Import	Imports a settings files from the hard disk.
- Export	Exports a settings files to the hard disk.
Preview	Shows a preview of the series to be created. Use the  **Play** /  **Stop** button to start or stop the preview.
Frames	Sets the number of individual frames that the series consist of after the calculation. The more individual images that you specify here, the more fluidly the scene transitions will be displayed later. Select predefined values from the dropdown list (e.g. **20** or **100** frames).
Fixed Resolution	Only visible if **Show All** is activated. As a rule, the image series is calculated using the current screen resolution. If you want to set a different format for the series, activate the checkbox. In the input fields that are now visible you can enter the width and height in pixels with which you want the series to be created.

The following parameters are only available if you have selected **Turn Around X / Y / Z** under **Render Series**:

i INFO

The X rotation, Y rotation and Z render series types all have the same control elements and differ only in the axis around which the rotation is calculated.

The preview function is not available for these types of series.

Parameter		Description
360° Panorama		Select 360° panorama, if you want to generate a complete rotation series.
Partial Panorama		If you select partial panorama, you can specify the starting angle and stopping angle that you want to use for the series. To do this, enter the desired values in the input fields or adjust it in the graphical representation of the rotation circle at the right of the input fields.
-	Start Angle	Determines the starting angle.
-	Stop Angle	Determines the stop angle.
-	Direction	Determines the direction of rotation.
-	 Angle Definition	When you are configuring a partial panorama, the desired angles can also be determined easily using the circular control element: Grab the white start/stop points with the mouse and position these accordingly on the circle. The number of individual images is also displayed here.

The following parameters are only available if you have selected **Start/Stop** under **Render Series**:

Parameter	Description
Start Position	You can position the volume in the image area as required using the mouse. The geometric parameters are displayed in the input fields. You can also determine the **Camera Position** and the **Look At** parameters for X, Y or Z and the angle directly using the input fields or the slider. All changes are displayed immediately in the image area.
Stop Position	You can position the volume in the image area as required using the mouse. The geometric parameters are displayed in the input fields. You can also determine the **Camera Position** and the **Look At** parameters for X, Y or Z and the angle directly using the input fields or the slider. All changes are displayed immediately in the image area.

The following parameters are only available if you have selected **Position list** under **Render Series**:

Parameter	Description
Add	Adds the current position to the position list.
Insert	Inserts a new position between two existing positions.
Position list	Each position is displayed in the list with its X, Y, Z angle and zoom level.  Using the control elements at the bottom of the list you can change the order of the positions (**Arrow** buttons), cut positions (**Scissors** icon) or copy and paste them again at another position (**Copy / Paste** icons). If you want to delete all positions, click on the **Clear List** button.
Further Parameters	You can determine which of the following parameters you want to be taken into consideration when the series is calculated. To do this, activate the corresponding checkbox:
- Light	Includes illumination parameters.
- Transparency	Includes transparency settings (not active in **Surface** mode).
- Background	Includes color and distance of the background.
- Time	Includes time series parameters (only active for time series images).
- Camera	Includes camera settings, e.g. viewing angle (from the 3D / virtual camera).
- Planes	Includes planes settings (not active in **Shadow** mode).
- Surface	Includes surface settings (only active in **Surface** and **Mixed** mode).

20.5.2.4.6 Measurements Tab

Only visible if the **Show All** mode is activated.

Here you can perform interactive measurements in the 3D volume. Note that measurements are not possible in **Shadow** projection mode. The measurements can be drawn in directly in the 3D volume using different tools. The measurement results are displayed in a list at the right of the image area.

Parameter		Description
Tool bar		 Using the tools you can perform interactive measurements in the 3D volume. The following tools are available:
-	Select	Changes the mouse pointer to Selection mode. Use this to select measurements in the 3D volume in order to change them.
-	Line	Use this to measure the length of a line in µm. Click once on the starting point and hold down the mouse button. Then drag the mouse to the end point and release the mouse button again. The measurement is complete. The result of the measurement is displayed in the list to the right of the image area.
-	Angle	Use this to measure the angle between two connected legs. First define the starting point. Then use the mouse to drag the first leg to the desired first end point. Define the second leg by clicking on the second end point. The angle measurement ends with a display of the angle measured (in degrees). The result of the measurement is displayed in the list to the right of the image area.
-	Polygon Curve	Use this to measure along a line with any number of segments. Click from corner point to corner point. Complete the measurement by right-clicking. The result of the measurement is displayed in the list to the right of the image area.
-	Color selection	Here you can select a color for the tool you want to draw in. Simply click on the colored rectangle and choose a color from the list.
-	Keep Tool	**Activated:** Keeps the selected tool active.
-	Auto Color	**Activated:** Automatically changes the color of the drawn-in tool.

Parameter	Description
Show Measurements	**Activated:** Shows the measurements in the 3D volume or in the list of measured values at the right of the image area.
- On top	**Activated**: All drawn-in measurement tools appear in the foreground, even if these are in fact obscured by image structures.
Display Values	
- on the objects	**Activated:** Displays the measured values in the 3D volume.
- as list	**Activated:** Displays the measured values in the measurement data table.
Delete Selected	Only active if a measurement tool has been selected in the 3D volume. Deletes selected measurement tools from the 3D volume.
Delete All	Deletes all measurement tools from the 3D volume.

20.5.2.4.7 3D Graphics Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Tool bar	 Using the tools you can perform interactive measurements in the 3D volume. The following tools are available:
- Select	Changes the mouse pointer to Selection mode. Use this to select measurements in the 3D volume in order to change them.
- Line	Use this to measure the length of a line in µm. Click once on the starting point and hold down the mouse button. Then drag the mouse to the end point and release the mouse button again. The measurement is complete. The result of the measurement is displayed in the list to the right of the image area.
- Angle	Use this to measure the angle between two connected legs. First define the starting point. Then use the mouse to drag the first leg to the desired first end point. Define the second leg by clicking on the second end point. The angle

Parameter		Description
		measurement ends with a display of the angle measured (in degrees). The result of the measurement is displayed in the list to the right of the image area.
-	Polygon Curve	Use this to measure along a line with any number of segments. Click from corner point to corner point. Complete the measurement by right-clicking. The result of the measurement is displayed in the list to the right of the image area.
-	Color selection	Here you can select a color for the tool you want to draw in. Simply click on the colored rectangle and choose a color from the list.
-	Keep Tool	**Activated:** Keeps the selected tool active.
-	Auto Color	**Activated:** Automatically changes the color of the drawn-in tool.

Parameter		Description
3D Measurements		All measurement contained in the 3D volume are displayed here. The list contains the following columns:
-	Eye icon	Here you can select whether or not a measurement tool is displayed in the image. If you click in the title field of the column, the setting is made simultaneously for all entries.
-	Lock icon	Not activated for the 3d view.
-	Type	Displays the type of a tool. If you click on the icon, you can change the color of the tool.
-	ID	Displays the unique identification number of the measurement tool.
-	A	No function
-	M	**Activated:** Displays the measurement data in the image. If you click in the title field of the column, the setting is made simultaneously for all entries.
-	Name	Displays the name of the tool. To change the name, double-click on the entry. Then enter a new name. Confirm the entry with the Enter key.

20.5.2.5 Analysis View

Only visible if an interactive measurement has been performed.

The **Analysis** view displays the image from the interactive measurement and the table containing the measurement results.

INFO

To highlight the row of the table containing the measured values of an object, click on a measured object in the image.

To highlight the corresponding measured object in the image, click on a row in the table.

20.5.2.5.1 Analysis Tab

On the **Analysis** tab you can define how the measured objects are displayed in an image.

Parameter	Description
Show Objects	**Activated:** Displays the measured objects in the graphics plane.
Fill	**Activated:** Displays the objects in filled form. **Deactivated:** Displays only the contours of the objects.
Opacity	Here you can set the opacity with which the measured objects are displayed in the graphics plane.
Delete Measurement Data	Deletes all objects and measurement data from the image.
Show All Classes	**Activated:** Displays the objects of all classes. **Deactivated:** Displays the objects of the selected class.
Create Measurement Data Tables	Creates two measurement data tables. One measurement data table contains the field features for all classes and the other the object features for all classes.
Classes	In the Classes section you can select the class whose measurement features you want to be displayed in the measurement data table. For each class there are two entries: the first entry concerns all the objects belonging to the class (field features) and the second represents an individual object (object features).

20.5.2.6 Mean ROI View

In the **Mean ROI** view you can draw ROIs and measure their intensity profile after acquiring time series experiments. The intensity profiles are displayed as charts and can be exported to data tables.

INFO

The **Physiology** module activates addition features to those of Mean ROI for the offline analysis of dynamics (physiology) experiments, e.g. the **Time Line View Panel**.

INFO

In this view the Image area is always to the left, charting area always to the right. Depending on which Region layout you have selected in **Layout** tab the Mean ROI view can have a different appearance.

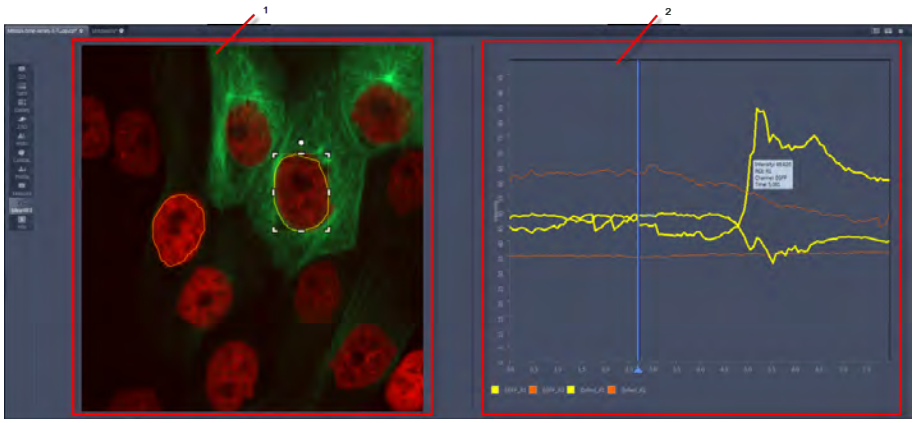


Fig. 20.36: Mean ROI View

1 Image area

Here you see the time series image.

2 Charting Area

Playhead (blue line)

Indicates the current frame of the time series visible in the image panel(s). The position of the playhead is synchronised with the displayed image frame number and vice versa. The playhead can be dragged. Hover over the playhead with the mouse to reveal a horizontal double headed arrow. Press and hold the left mouse and drag the playhead in the desired direction. The current time point of the visible frame is displayed to right in the middle of the playhead line in the same time units as the chart x-axis.

Data intensity plot of correspondingly colored ROI (yellow graph line)
Note that this ROI is selected and therefore the plot line thickness is increased as a highlight.

Data intensity plot of correspondingly colored ROI (orange graph line).

Note that the this ROI is un-selected and therefore the plot line thickness is normal.

INFO

Tool tip info box

Hover with the mouse (crosshair) over the plot. A tool tip appears with details of the intensity value at this position, ROI ID #, Channel, and time point (in currently set time unit of x-axis). Note these values (intensity and time) are interpolated.

20.5.2.6.1 Time Line View panel

Only visible if you have licensed the **Physiology** module.

This chart supports that same functions as detailed for charts in **Mean ROI** e.g. zoom, data selection and tool tips.

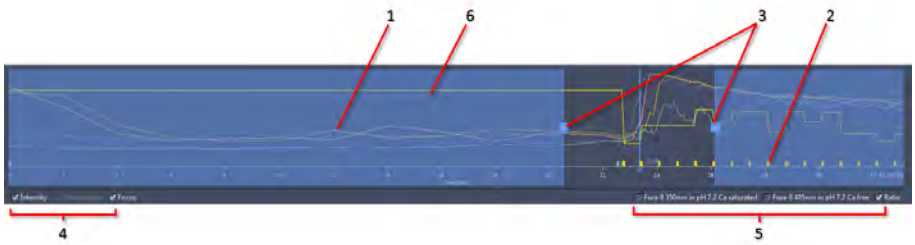


Fig. 20.37: Time Line View panel

No.	Parameter
1	Intensity data traces from measurement ROIs
2	Switch markers with corresponding color code.
3	Experiment pause marker
4	Zoom tool control The center line (blue line) displays the playhead. The transparent area (blue) is the user definable zoom range. The zoom range translates into the display range of the x-axis of the other charts displayed above the time line view. To edit the zoom range hover over the bounding lines at either end. A double headed arrow will appear. Click and hold the left mouse to drag and resize the area width.

No.	Parameter
5	Chart data/intensity parameter selection check boxes Depending on the available metadata different check boxes are available. Only two parameters can be displayed at a given moment. The combination can be chosen as required. When intensity parameters are displayed, it is possible to further select which of the available channels and or ratio. Channel intensity and ratio intensity can be displayed in a mutually exclusive manner.

20.5.2.6.2 Mean ROI Tab

Parameter		Description
Measurements		Change the display of the selected clipping plane using the dropdown list to the right of the **Activate** checkbox. The following settings are available:
Recalculate		Starts a recalculation of the selected measurement. For example when loading a data set that contains ROIs from a previous session.
Background Correction		A background correction can be performed on a channel by channel basis. Selection of a background correction method modifies the ratio set-up formula accordingly. Following modes are available:
-	None	No background correction is performed.
-	Constant	Allows a user defined numeric value to be entered for each channel in the appropriate spin box.
-	ROI	Allows to select the background ROI defined in the **Mean ROI Setup**. Note that for dual wavelength protocols the same ROI is used in each case, but its channel specific values are applied for the correction.

20.5.2.6.3 Layout Tab

Parameter	Description
MeanROI View Layouts	Here you can adjust how the image, chart and table will be displayed.

Parameter	Description
- Image and Chart	Select one of three different layouts of how an image together with a chart will be displayed. If you click on one of the buttons the layout will be changed.
- Image and Chart with Table	Select one of three different layouts of how an image and a chart together with a table will be displayed. If you click on one of the buttons the layout will be changed.
Show Markers/ Switches	**Activated:** The temporal position of any switches and markers are always displayed on the charts both during acquisition or post-acquisition.
Show Time Line View	Only visible if you have licensed the **Physiology** module. **Activated:** The Time Line View panel is displayed below the other image chart panels of the **Center Screen Area**. The Time Line View panel is designed to provide an overview of the experiment whilst allowing the user to define the detail displayed in the other chart panels by means of an integrated zoom tool. The Time Line View can be hidden by unselecting the check box as required both during or after an experiment.
Show View Captions	**Activated:** Displays the channel name clearly with the image of each channel in the multichannel view layout.

20.5.2.6.4 Charts Tab

Parameter	Description
Chart Settings (X- / Y-Axis)	Note that a function is active when the button is highlighted in blue. The settings for **X-** and **Y-Axis** (only if Show All is activated) are the same, see description below:
- Auto	The scaling of the respective axis is automatic, allowing for an optimal, and appropriate adjusting display of the all values.
- Norm	The scaling of the axis is normalized to fit the maximum value in the data set.
- Fixed	The upper and lower limit of the axis can be defined using the min and max spin boxes.
X-Units	
- Auto	The units are selected automatically.

Parameter	Description
- Fixed	You can select the desired unit for the x-axis from the dropdown list.
Show Tick Marks	Shows tick marks in the chart. If activated, you can set the **Form** and **Size** of the tick marks.
Show Legend	Shows the chart legend.
Show Axis Captions	Shows captions of the axis.

20.5.2.6.5 Export Tab

Parameter	Description
Data Table	
- As New Document	Opens the measurement data table in a new document tab. The table displays all measurement values and area for all ROIs in each channel. If event markers are present these are also listed here at the appropriate time points. This function provides access to the ZEN charting functions.
- Save as *.csv	Opens the **Save As** dialog and allows the measurement data to be exported as a comma separated value (***.csv**) file. The following values are exported for each ROI and channel: Intensity, area and if present event markers.
Ratio image	
- As New Document	Opens the ratio image in a separate new document as a *.czi file (current Z only).
- Save as	Opens the **Save As** dialogue to save the ratio image directly to a ***.czi** file.

20.5.2.6.6 Ratio Tab

This view option is almost identical to the **Online Ratio** tab in the **MeanROI Setup**, see *Online Ratio Tab* [▶ 828]. In fact when an experiment is finished the exact same values used for the display of the online ratio are transferred to the offline ratio tool of the **MeanROI** view.
For offline ratio assessment the settings can be changed and applied to recalculate the ratio image and measurements. The following descriptions will describe the differences on the Ratio tab:

Reference image (Ft_0) Set-up

This difference only applies if the Single wavelength method is selected:

Parameter	Description
Range	The numbers in the spin boxes refer to the frame number of the experiment currently been viewed in MeanROI view. The desired frame numbers can be entered by the spin buttons or directly by typing a number into the box.

20.5.2.6.7 Online Ratio Tab

Only visible in **MeanROI Setup**.

Parameter	Description
Method	Select the ratiometric method you want to use. **Single** or **Dual wavelength** dyes are supported. The ratio set-up will change in accordance with your selection.
Calculation	
- Single Wavelength Method	Select the channel in the dropdown menu. The Ft_0 value is the averaged fluorescence from the specified number of image frames. The number of frames to average is defined in the spin box of the reference image set-up (see 10). The spin box at the far left is a multiplication factor
- Dual Wavelength Method	Select the channels in the dropdown list required to calculate the ratio values/image e.g. for Fura-2, a dual excitation dye, the numerator is the 340 nm image the denominator the 380 nm image. For dual emission dyes the function is identical. The spin box at the far right is a multiplication factor.
Color	Select the color (LUT) used to display the ratio image during the online measurement. Per default the Rainbow LUT is used as it allows intensity changes to be followed easily.
Background Correction	A background correction can be performed on a channel by channel basis. Selection of a background correction method modifies the ratio set-up formula accordingly. Following modes are available:
- None	No background correction is performed.
- Constant	Allows a user defined numeric value to be entered for each channel in the appropriate spin box.
- ROI	Allows to select the background ROI defined in the **Mean ROI Setup**.

Parameter		Description
		Note that for dual wavelength protocols the same ROI is used in each case, but its channel specific values are applied for the correction. For single wavelength ratio, no ROI background correction is available.
Threshold		
-	Enable	**Activated:** Allows the threshold values to be set for the ratio calculation.
-	Channel / Threshold	A threshold value can be applied in the form of a constant integer value for each channel individually. Thresholds help to reduce noise anomalies that are cause by pixel to pixel variations in areas between cells or near cell boarders during the ratio calculation. Enter the desired threshold value for each channel into the spin boxes provided.

20.5.2.7 Unmix View

This view is only visible for multi-channel or lambda stack images. It is used for:

- display the spectra corresponding to defined ROIs (mean ROI intensity over Lambda),
- show the intensity values in table form, copy the table to clipboard or save the table as a text file and
- generate linear unmixed multi-channel images.

In this view you will see 2 areas as default. The intensity-over-lambda diagram **(1)** to the left and the image display **(1)** to the right. The specific view options **(3)** below the image area are described in the following topics.

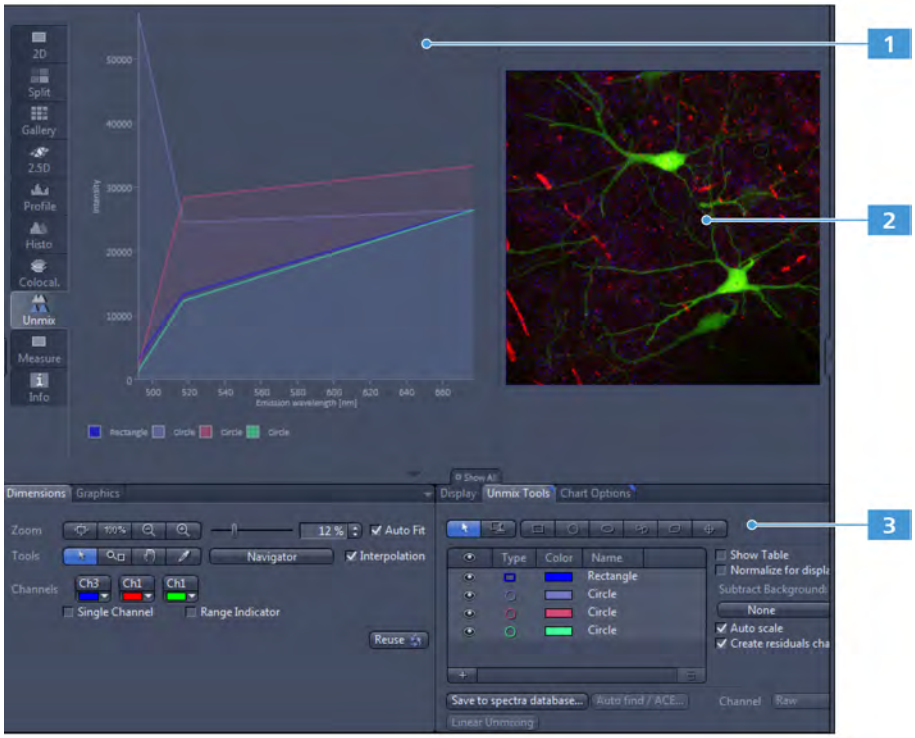


Fig. 20.38: Unmix View

20.5.2.7.1 Unmix Tools Tab

Here you can select various tools and use these to draw graphic elements into your images, similar to the tool bar on the **Graphics** tab. You can also obtain an overview of the graphic elements that you are using in your image.

The following list will describe the specific parameters for this tab:

Parameter	Description
Toolbar	Using the tools you can draw in certain regions of interest which are then displayed in the intensity-over-lambda diagram and will be used for linear unmixing.
List of Spectral Data	The list gives you an overview of the spectral data in the image, which will be used for linear unmixing. The names indicates the origin, e.g. if manually or automatically picked by **ACE** (see below) or loaded from the spectra database. To load a spectrum from the spectral database, press the Add **+** button for a new row. Click into the Name column and select the according name for the needed spectrum.
Save to spectra database	If you click on this button you can save the selected entry to the spectra database.

Parameter	Description
Auto find / ACE...	ACE stands for Automatic Component Extraction. If you click on this button the software automatically searches for regions with distinct spectral signatures and tries to find the defined number of spectra.
Linear Unmixing	Performs the linear unmixing processing of the image with the selected spectra. Note: The channels of the Lambda stack which are de-selected in the **Dimensions** tab are not included in the calculation.
Show Table	**Activated**: Displays a table of intensity values over Lambda below the default image area.
Normalize for display	**Activated:** Normalizes the graphs of the spectra to 1.
Subtract background	Here you can select the list entry of a marked spectrum that should be subtracted before linear unmixing.
Auto scale	**Activated:** Automatically balances the intensity of unmixed channels to equal levels.
Create residuals channel	**Activated:** Generates an additional channel in which the intensity value represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra for each pixel. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed.
Channel	
- Raw	The raw data acquired during a lambda stack is used as channels and for spectral display.
- Spectral	The intensity data of the lambda stack is calculated into channels for each detector (Channel 1 and Channel 2).

20.5.2.7.2 Chart Options Tab

This tab provides several parameters to change the appearance and contents of the spectral graph. For the beginning we recommend to use the default settings here.

20.5.2.8 Lambda View

In this view you can display images that are acquired in Lambda mode, see *Lambda Mode* [▶ 668]. the resulting images are called Lambda stacks. For that type of image the **2D** View is not available.

Instead the Lambda View displays a Lambda Stack in a wavelength-coded color view as default. A color palette, mimicking the emission wavelength of the channel, is automatically assigned to the individual lambda images which are then displayed in a merge-type display.

On **Display** tab, the channel-specific settings of brightness, contrast and gamma can be handled as described for channels in the **2D** View.

In order to use other views (e.g. Split or Gallery view) or to view lambda stack data sets in ZEN black, convert the data set using the **Convert to Lambda** image processing function.

The general view options on **Dimension** tab are adapted to the Lambda View with the following changes:

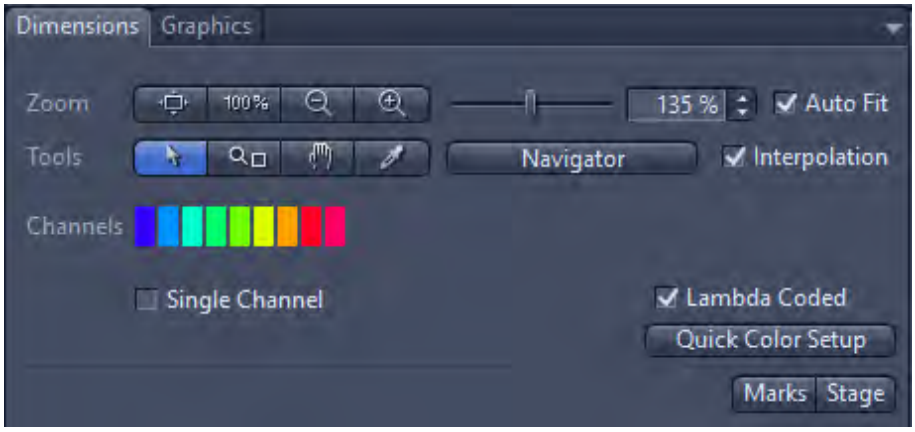


Fig. 20.39: Dimensions tab changes for Lambda view

Parameter	Description
Channels	Displays the single channels of a Lambda stack image as a colored button. You can handle the channels like in the 2D View. E.g. if you click on a channel button you can show or hide the channel in the image area.
Lambda Coded	**Activated:** All channels are displayed as a merged image. Each channel is assigned to a channel color that represents the recorded emission wavelength in the lambda stack. **Deactivated:** Only one channel of the Lambda Stack is displayed without pseudo coloring. Additionally the **Single Channel** checkbox is activated and cannot be changed. To

Parameter	Description
	display a different channel of the Lambda Stack, click on the according channel. This will display the chosen channel and deactivate the previously displayed channel.

20.5.2.9 Colocalization View

Only visible for multichannel fluorescence images.

In the **Colocal.** (Colocalization) view, you can analyze the extent of colocalization quantitatively in two fluorescence channels. The view consists of two main areas: the **X/Y scatter plot** on the left and the actual image (2 channels are displayed) in the right image area. Using the **Coloc. Tools** specific view control, you can also display the **Colocalization table** in the lower image area. To do this activate the **Table** checkbox in the **Extract** section.

INFO

The channels that you are comparing with one another are displayed in the image area in the form of a color overlay. The channel color of the image is used here. If the images have more than 2 channels, you can add additional channels on the **Dimensions** tab. This temporary selection is deactivated, however, when you select the channels to be compared on the **Coloc. Tools** tab.

20.5.2.9.1 X/Y Scatter Plot

The pixel intensities of two channels are plotted against one another in the diagram and each pixel pair with the same X/Y image coordinates is displayed as a point. The frequency with which pixels of a certain brightness occur is visualized by means of a color palette that is displayed at the bottom of the diagram. The relative value range lies between 0-255.

The vertical and horizontal axes show the gray value range that applies for the relevant channel.

The diagram is overlaid with two lines that subdivide it into 4 quadrants, numbered from 1-4. Using the mouse you can position the lines freely and therefore adjust the threshold values to the data.

The quadrants have the following meanings:

- 1: Non-colocalizing pixels from channel 1
- 2: Non-colocalizing pixels from channel 2
- 3: Colocalizing pixels
- 4: Background

20.5.2.9.2 Colocalization table

Only visible if the **Table** checkbox is activated on the **Coloc. Tools** tab.

For each quadrant of the scatter plot there is a correspondingly labeled row in the table. The **Global** row contains the values for the entire image. The table contains columns for the following measured values:

20.5.2.9.2.1 Region

Once a region has been selected it has a number assigned to it. This number appears in the image and in the table.

20.5.2.9.2.2 Quadrant

Indicates the measured values for the four quadrants of the scatter plot.

20.5.2.9.2.3 Pixel Number

Shows the total number of pixels of each quadrant. The sum of all pixels in this column for all 4 quadrants corresponds to the product of the height x width of the original image.

20.5.2.9.2.4 Area (µm²)

Area = number of pixels x scaling factor for X/Y
If there is no scaling for the original image, the following applies: 1 pixel = 1 µm.

20.5.2.9.2.5 Relative Area (%)

Relative area = area of quadrant/total area

20.5.2.9.2.6 Pearson's Correlation Coefficient

Provides information on the intensity distribution within the colocalization region. Value range: -1 to 1.

1: All pixels are on a straight line in the scatter plot from bottom left to top right (if, for example, you have used the same channel twice for the colocalization, you will find the value 1 in this column).

0: The pixels in the scatter plot are distributed in a cloud without a preferred direction.

-1: The pixels do not overlap. The scatter plot stretches from top left to bottom right. This situation can be described as negative colocalization and means "exclusion".

The calculation formula is as follows:

$$\frac{\sum((GreyCh1_i - MeanCh1) \times (GreyCh2_i - MeanCh2))}{\sqrt{\sum(GreyCh1_i - MeanCh1)^2 \times \sum(GreyCh2_i - MeanCh2)^2}}$$

GV: Gray Value; AV: Average Gray Value; C: Channel

20.5.2.9.2.7 Manders' Correlation Coefficient

Insensitive to differences in the signal intensity between the two channels and bleaching.

Value range: 0 to 1

The calculation formula is as follows:

$$\frac{\sum GreyCh1_i \times GreyCh2_i}{\sqrt{\sum GreyCh1_i^{\,2} \times \sum GreyCh2_i^{\,2}}}$$

Fig. 20.40: C: Channel

20.5.2.9.2.8 Coloc. Coefficient 1

This coefficient indicates the relative number of colocalized pixels in channel 1 in relation to the total number of pixels above the threshold value:

$$\frac{\sum PixelsCh_{1,colocalized}}{\sum PixelsCh_{1,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Number of pixels in quadrant 3

Denominator = Number of pixels in quadrant 3 + number of pixels in quadrant 1

20.5.2.9.2.9 Coloc. Coefficient 2

This coefficient indicates the relative number of colocalized pixels in channel 2 in relation to the total number of pixels above the threshold value:

$$\frac{\sum PixelsCh_{2,colocalized}}{\sum PixelsCh_{2,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Number of pixels in quadrant 3

Denominator = Number of pixels in quadrant 3 + number of pixels in quadrant 2

20.5.2.9.2.10 CC (weighted) 1

Weighted correlation coefficient channel 1. Calculated like the simple colocalization coefficient, but using the sum of the gray value intensity rather than the number of pixels.

$$\frac{\sum SumGreyCh_{1,colocalized}}{\sum SumGreyCh_{1,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Sum of intensity of all pixels in quadrant 3

Denominator = Sum of intensity of all pixels above the threshold value

20.5.2.9.2.11 CC (weighted) 2

Weighted correlation coefficient channel 2. Calculated like the simple colocalization coefficient, but using the sum of the gray value intensity rather than the number of pixels.

$$\frac{\sum SumGreyCh_{2,colocalized}}{\sum SumGreyCh_{2,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Sum of intensity of all pixels in quadrant 3

Denominator = Sum of intensity of all pixels above the threshold value

20.5.2.9.2.12 Average Intensity 1

The sum of all gray values from channel 1, divided by the total number of pixels in this channel:

$$\frac{\sum GreyCh1_{i}}{AreaCh_{1}}$$

20.5.2.9.2.13 Average Intensity 2

The sum of all gray values from channel 2, divided by the total number of pixels in this channel:

$$\frac{\sum GreyCh2_i}{AreaCh_2}$$

20.5.2.9.2.14 Standard Deviation 1

Displays the standard deviation of the gray values in channel 1:

$$\sqrt{\frac{\sum (GreyCh1_i - MeanIntensityCh_1)^2}{AreaCh_1 - 1}}$$

20.5.2.9.2.15 Standard Deviation 2

Displays the standard deviation of the gray values in channel 2:

$$\sqrt{\frac{\sum (GreyCh2_i - MeanIntensityCh_2)^2}{AreaCh_2 - 1}}$$

20.5.2.9.2.16 Z Index

Displays the Z index for Z-stack images.

20.5.2.9.2.17 T Index

Displays the time index for time lapse images.

20.5.2.9.2.18 Relative Time

Displays the time of acquisition for all dimensions of a multidimensional image, beginning at 0h:00min:00sec:00msec.

20.5.2.9.2.19 Relative Focus

Displays the relative focus position at which an image has been acquired.

20.5.2.9.3 Coloc. Tools Tab

Here you will find all the control elements that you need to perform a colocalization analysis.

20.5.2.9.3.1 Tool Bar section

Only visible if the **Show All** mode is activated.

Use the tools to draw regions into the image in which you want the analysis to be performed. A description of the tools can be found on the *Graphics* [▶ 853] tab.

Once a region has been drawn in, it is automatically treated as an active region. The scatter plot shows the pixel value frequencies for this region.

The **Colocalization table** displays the data for the entire image and for the selected region. To select several regions, hold down the **Ctrl** key and click on the desired regions.

Apart from drawing regions into the image, you can also draw them into the **X/Y scatter plot**. If you have used the function in the *Regions section* [▶ 840], only those pixels that are framed by a region in the scatter plot are taken into consideration. This means that you can correlate interesting point clouds quickly with the corresponding pixels in the image.

If you have drawn regions into the scatter plot, the ROI (region of interest) button will also appear in the tool bar. As long as this button is activated (highlighted in blue), you can select, move and change the regions in the scatter plot using the **Selection** tool. If you want to change the quadrant lines again, you will need to deselect the ROI button beforehand.

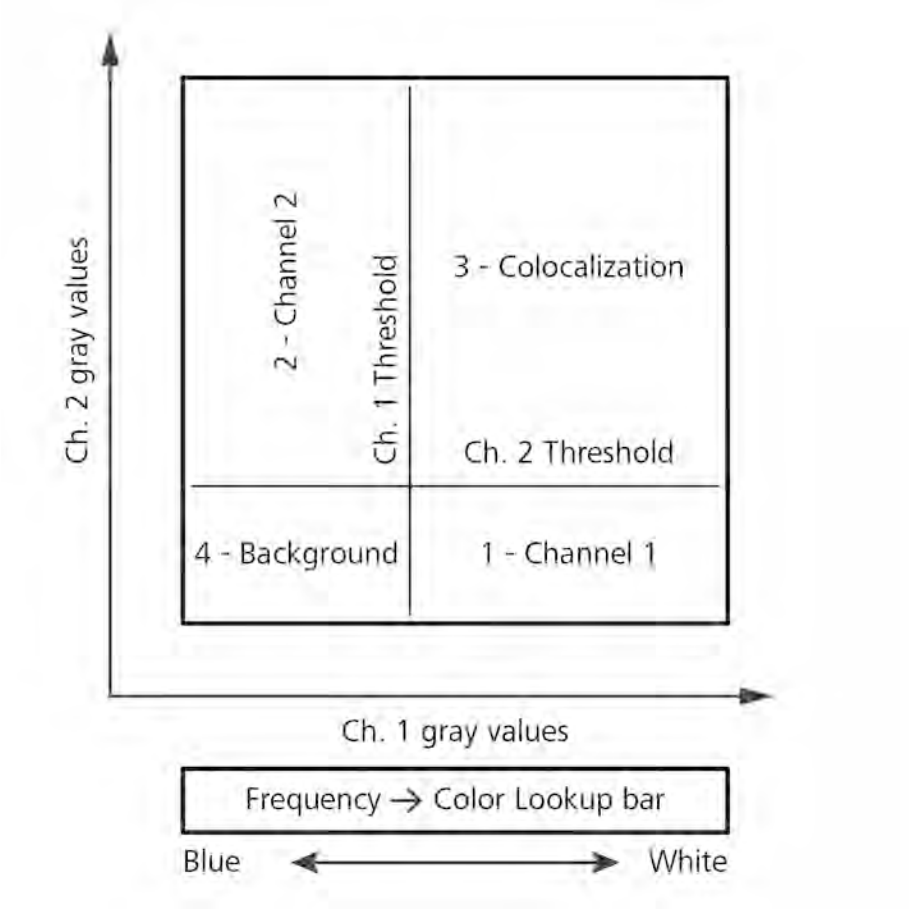
20.5.2.9.3.2 Channels and scatter plot control section

Channels dropdown lists

Here you can select which channels of a multichannel image are compared with one another. Select a channel for both the horizontal and vertical diagram axis from the **Channels** dropdown list. The first and second channel are always selected by default. As soon as you have made a selection, all other channels are automatically removed from the image display. You can, however, add other channels temporarily on the **Dimensions** tab.

Threshold sliders

Using the two Threshold sliders and the two spin boxes/input fields, you can set the threshold value (in gray levels) for both channels.



Range dropdown lists

Only visible if the **Show All** mode is activated.

Here you can define the gray value range that the scatter plot axes will display. **Auto** is selected here by default, which means that the range is automatically set to the brightest pixel in the image. You can, however, enter a fixed gray value range between 256 (8 bits) and 65535 (16 bits). If the image is a time lapse or Z-stack image, **Auto** has been selected, you can select the dimension for which you want the gray value range to be automatically determined from another dropdown list. In this way you can easily determine a valid diagram setting for an entire time series, for example, without having to analyze each individual time point.

Costes button

Calculates the optimal threshold value according to Costes et al.

20.5.2.9.3.3 Regions section

Channels buttons

Here you can mask pixels in the image according to which one of the four quadrants they belong to. The numbers on the buttons correspond to the numbering of the quadrants in the **X/Y scatter plot**. The color selection window is accessed by clicking on the **color field**. Using the **Opacity** slider you can determine the degree of transparency of the masking.

Cut Mask button

Only active if a quadrant has been masked.

Creates a new image exclusively containing the masked pixels.

20.5.2.9.3.4 Extract section

Scatter Plot button

Creates a new image document from the X/Y scatter plot. In the case of time series or Z-stacks the dimensions are also created automatically.

The following functions are only visible if the **Show All** mode is activated:

Table button

Creates a new table document. The document contains all measurement data from the colocalization analysis. All dimensions, such as T and Z, are also taken into account. This table can be saved as a *.csv document for further processing in other programs.

Table checkbox

Activated: Displays the colocalization table in the image area.

20.5.2.10 FRAP View

The FRAP view (FRAP = Fluorescence Recovery after Photobleaching) is only visible for a time series data set which includes a minimum of one bleach event. It permits interactive analysis of bleaching experiments, including:

- Fit Formula
  Fitting of FRAP data to a mono exponential or double exponential model for intensity
- Fit Range
  Selection of data points for the fitting
- Photofading Factor
  Determining the fading factor from a reference region (Ref.) from the present experiment or a control experiment and reusing it for subsequent experiments

- Defining Background and Reference Regions for the analysis (fitting) of FRAP data

The Image Display in the FRAP View shows 4 panels:

- the intensity-over-time diagram with the fitted curve per channel **(1)**
- the image display with the ROI graphics **(2)**
- the table with the fit parameters for each channel and analysis ROI Group (one or more ROIs can be grouped for analysis, up to three groups can be analyzed) **(3)**
- an optionally viewed table of the average intensity values of each ROI Group (corrected for Background and Reference) for each time point and channel

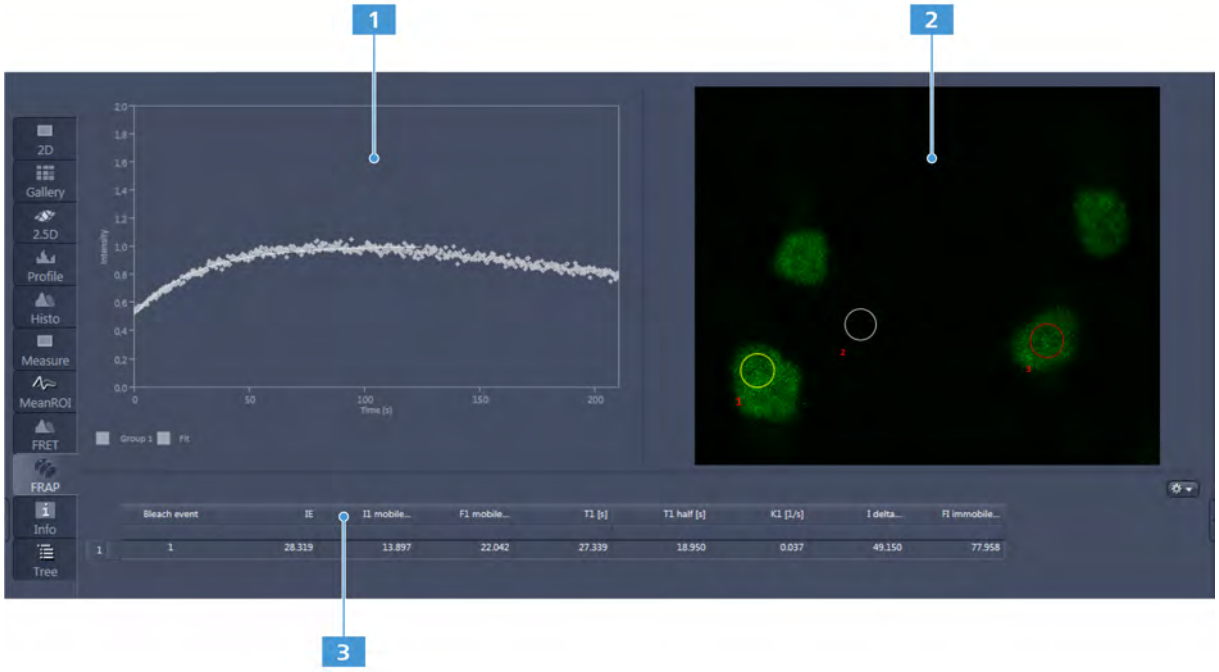


Fig. 20.41: Image display of a time series with bleach event in the FRAP View Tab

20.5.2.10.1 FRAP View Options

The additional view-specific FRAP View Options are available in the **FRAP** View Options Tab.

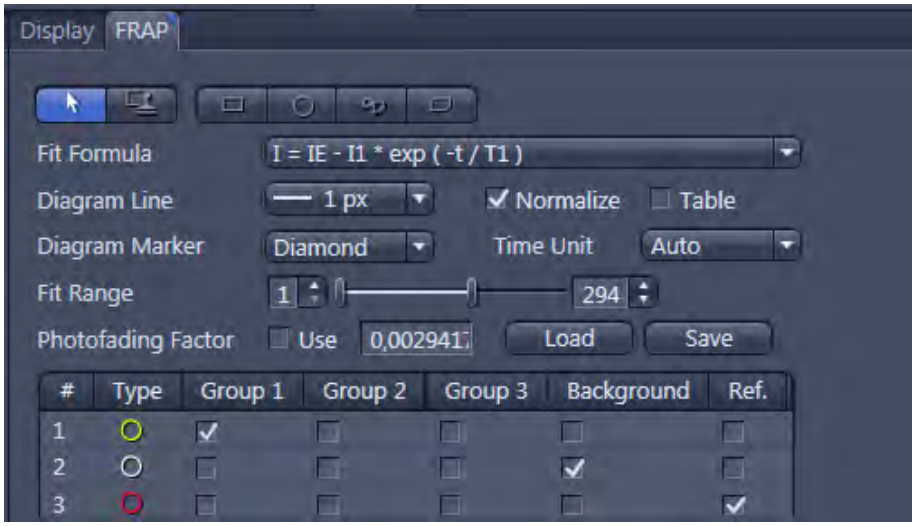


Fig. 20.42: FRAP View Options Tab

Any changes performed with this view options tab are effective immediately.

The settings of the Dimensions, Display, Player and Graphics View Options tabs apply for FRAP View, too.

Available tools in the **FRAP** View Options Tab are:

Parameter	Description
Regions of Interest (ROIs)	The **FRAP** View Options Tab includes drawing tools that work identically to the Graphics View Options Tab. ROIs from both control blocks can be combined.
Fit Formula	Drop down menu to select the mathematical model (mono or double exponential model) for data fitting
Diagram Line	Drop down menu to format the line thickness of the fit curve(S) in the diagram
Diagram Marker	Drop down menu to format the time point markers of the fit curve(s) in the fit diagram
Normalize	Checkbox to choose between absolute or normalized fit data. This updates the table of the intensity values and the fit diagram
Table	Checkbox to activate/deactivate the display of the intensity value (for each channel) per time point corrected for background and reference region if applied
Time Unit	Drop down menu to format the time units of the fit diagram, the fit data table and the intensity table
Fit Range	Edit boxes and slider to define the data range for the fit algorithm

Parameter	Description
Photofading Factor	Checkbox to Use or not use the photofading factor for the data fitting algorithm; Edit box to type in a photofading factor; Load and Save function buttons to save and retrieve a factor (.xml file). The photofading factor is either calculated from the current reference region(s) or it is loaded from a previous experiment. The edit box shows the currently (applied) value. The photofading factor = reference(t)/reference(t=0) and then fitted by: intensity(t) = exp(-kappa * t). The fitted value of kappa is displayed in the edit box.
Background Region	Check box in the list of ROIs: Mark the region of interest which represents the mean background intensity to be used for data correction. The mean intensity value of the background region is subtracted from the data prior to fitting.
Reference Region	Check box in the list of ROIs: Define and select a ROI which represents the fluorescence intensity of a reference area that has not been bleached and has not been affected by the leach event. The mean intensity within that region is used to correct the data at each time point for any bleaching artifact that occurred during the imaging process. For this the data are divided by [reference(t)/ reference(t=0)]
Combine Regions Group 1, 2, 3	Check boxes in the list of ROIs which allow to choose more than one ROI for analysis and to group them according to the experimental set up. Mean Intensity values for the regions combined are used as data input.

20.5.2.10.2 Fit Model

The Fit Model applied provides the following data/parameter:

- The final signal intensity in the analyzed ROIs following recovery **IE** (of the fitted curve)
- The amplitude of the fitted curve (which equals the mobile fraction) **I1** mobile fraction
- The proportion of the mobile fraction: **F1 mobile fraction (%)**
- The fitted parameter **T1(s)**
- The half time of recovery **T1 half (s)**

- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1 (1/s)**
- The part of the immobile fraction of the protein **I delta immobile fraction**
- The proportion of the immobile fraction: **F1 immobile fraction (%)**

A double exponential displays the mean of the fitted values for the two different mobile fractions as the fit curve. The following (additional) parameters are provided

- The amplitude of the two curves, displayed as one (which corresponds to each part of the mobile fractions) **I1** and **I2**.
- The fitted parameters **T1 (s)** and **T2 (s)** for each mobile fraction.
- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1 (1/S)** and **K2 (1/s)** for each mobile fraction.
- The half time of recovery for each fraction **T1 half (*s*)** and **T2 half (*s*)**

The table displays the result of the data fit. The result can be copied to the clipboard (right mouse click) and directly pasted into excel or saved as text.

The calculation of the parameters is based on the bleached ROI(s) unless other ROIs or selected or the ROIs are moved.

The analysis is then part of the image and the type of analysis is displayed again when opening the FRAP view for the image.

20.5.3 General View Options

The general view options are visible in any view. Some of the view options are only visible when you open a particular file type. E.G. see the **ApoTome** view option only if you have opened a **ApoTome** image.

20.5.3.1 Dimensions Tab

Here you configure the settings for how the image will be displayed on the screen. You can select the size of the display and call up information about the content of the image. In the case of multidimensional images you can select here which dimension is displayed. The dimension sliders (e.g. time, channels) help you to navigate through the single images of an experiment.

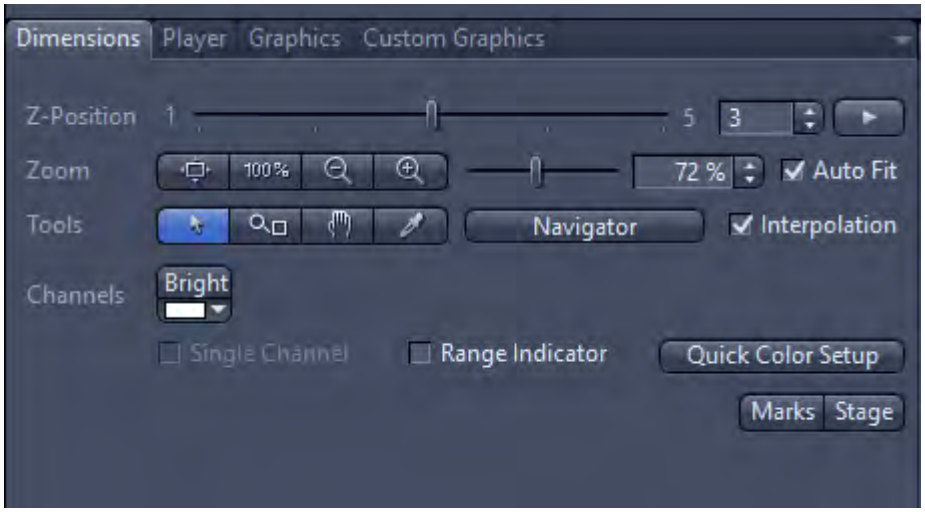


Fig. 20.43: Dimensions Tab (Show All)

20.5.3.1.1 Slider section

Depending on how many dimensions your image contains, several sliders can be available in this section. Using the sliders you can adjust the position that you want for each dimension available in the image. You will see the current position number in the input field to the right of the relevant slider. You can also enter the position number directly here.

Fig. 20.44: Slider Section (example)

The  **Play** buttons to the right of the input fields enable you to play back the dimension automatically. This takes place at a rate of 5 images per second by default. You can change the speed on the *Player Tab* [▶ 852].

i INFO

For images with more than 3 dimensions a scrollbar is displayed which you can use to access the other sliders.

Depending on the available dimensions, the following sliders can be visible:

Parameter	Description
Follow Acquisition	By activating the **Follow Acquisition** checkbox during acquisition of multi dimensional experiments (time series, tiles and z-stacks) the actual image is displayed (default value).

Parameter	Description
	By moving the **Time** slider in the **Dimensions** tab, this feature is disabled and a button for performing a manual update of the slider range is shown.
Z-Position	Only visible in the case of Z-stack images. Here you can adjust the desired Z-position.
X-Position	Only visible for images acquired in Line scan mode. Here you can adjust the desired X-position.
Time	Only visible in the case of time series images. Here you can adjust the desired time point.
Phase	Only visible when using the Airyscan for imaging. Here you can adjust which of the 32 channels (phase) from the Airycan detector is displayed. Note, that only the Raw data channel ChA of unprocessed Airyscan images has the Phase dimension. This channel is automatically not activated during and after the acquisition. The phase dimension of the so-called Sum channel ChA# does not have a Phase dimension.
Scene	Only visible if the image contains different scenes. Here you can adjust the desired scene. If you deactivate the **Scene** checkbox, all scenes are displayed in an overview.
Block	Only visible if you have used the **Experiment Designer** and created several experiment blocks. Here you can adjust the desired experiment block.
Total Time	Only visible if you have used the **Experiment Designer**. Here you can adjust the duration across all blocks.

20.5.3.1.2 Zoom section

This section contains tools that you can use to adjust the size of the image region displayed.

Fig. 20.45: Zoom section

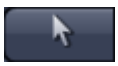
INFO

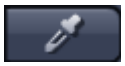
If you hold down the *Ctrl*-key, you can zoom in and out of the image using the mouse wheel.

Parameter	Description	Shortcut
 **Fit to View**	Automatically sets a zoom factor at which the entire image can be displayed visibly on the screen.	*Ctrl + 0*
 **Normal View (100%)**	Shows the image without increasing or decreasing the zoom factor. One pixel of the image corresponds to one pixel on your screen.	*Ctrl + 1*
 **Decrease Zoom**	Decreases the zoom factor.	*F8, Ctrl + F8*
 **Increase Zoom**	Increases the zoom factor.	*F7, Ctrl + F7*
 **Zoom factor**	Here you can set the display size steplessly. The desired zoom factor can be entered in the input field in percent.	
 **Auto-Fit**	**Activated:** Adjusts the display size automatically and continuously to the size of the window.	

20.5.3.1.3 Tools section

Fig. 20.46: Tools section

Parameter	Description
 **Selection**	Activates the selection mode.

Parameter	Description
 **Zoom Rectangle**	Activates the zoom mode. Hold down the left mouse button and drag out a selection rectangle. When you release the left mouse button, the region within the rectangle is displayed in enlarged form. If you have a mouse with a mouse wheel, you can also use this to move enlarged/reduce image regions.
 **Move**	Activates the move mode. Left-click inside an enlarged image to move the zoomed region. If you have a mouse with a mouse wheel, position the mouse pointer inside the image region and hold down the mouse wheel. The mouse pointer will then appear automatically as a hand icon. You can now move the image region.
 **Inner Zoom**	Only visible in **Gallery** view. This function allows you to set a new zoom level for all images simultaneously using the mouse wheel. The size of the Gallery view does not change here. This allows you to limit the view to one interesting image region. Use the **Move** tool to move the view.
 **Show Pixel Values**	Activates the show values mode. If you move the mouse pointer into the image region, a vertical arrow and a display field will appear. The pixel values of the position to which the arrow is pointing are displayed in the display field. In the first line of the display field the X/Y coordinates are shown. The second line shows the X/Y coordinates in scaled units. In the other lines the gray values for each channel are shown.
Navigator	Opens the in the image area. There you will see an overview of your image and you can navigate to different positions using a rectangular window.
Interpolation	**Activated:** The pixel elements of the image are shown in an interpolated display. This makes it possible to avoid the pixelated display of small or greatly enlarged images. **Deactivated:** The pixel elements of the image are displayed as they are. This function is activated by default. You can deactivate this function via **Tools** menu | **Options** | *Documents Tab* [▶ 609].

20.5.3.1.4 Channels section

This section contains all channels that you are using in your image. You can switch the display of channels in images on or off and change the channel colors (pseudo color assignment).

Fig. 20.47: Channels section

A button is displayed for each channel. Each button has two functions:

1 The channel name is displayed in the top section. To switch a channel off/on again, left-click on this section of the button.

2 The bottom section of the button shows the channel color. The display changes depending on the status of the button:

- When switched off, you will see a colored line below the button.
- When switched on, you will see a color field with a dropdown list below the button. Clicking on the dropdown list opens the color selection, see *Color Selection Dialog* [▶ 851].

INFO

For images with 8 or more channels, the **Channel** buttons are reduced in size. In this case it is no longer possible to change the color channel by channel.

Parameter	Description
Single-Channel	**Activated:** Only a single channel is displayed.
Show Channel Colors	Only visible in **Split** view. **Activated:** Displays the individual channels of multichannel images with the assignment of pseudo colors.
Range Indicator	**Activated:** Changes the display to single channel mode. The channel is displayed in monochrome. At the same time you will see pixels that are saturated (displayed in red) and pixels that have no signal (values = 0; displayed in blue). Note that with camera systems it is normally not possible to achieve pixel values of 0. The blue indicator is therefore normally not displayed.

Parameter		Description
		This function helps you to set your acquisition settings, camera exposure or detector gain, so that saturation of the detector is avoided. The range indicator function is not available for the sum channel of the Airyscan ChA#.
Quick Color Setup		Opens a dialog that allows you to select a color quickly for all channels of a multichannel image. The following options can be set:
-	None	All channels are displayed without a pseudo color.
-	Grayscale	All channels are displayed in monochrome (this applies in particular to multichannel images that have been acquired using color cameras).
-	BGR	Channel 1: blue, channel 2: green, channel 3: red, no color assigned to any other channels.
-	GRB	Channel 1: green, channel 2: red, channel 3: blue, no color assigned to any other channels.
-	RGB	Channel 1: red, channel 2: green, channel 3: blue, no color assigned to any other channels.
-	Via LUT	Colors for all channels are selected using a reference look-up table. The LUT is divided up into as many sections as there are channels, with the channel color being used at the separation point. You can select the reference LUT using the **Reference LUT...** button.
-	Custom	The colors defined by the user are restored.
-	Dye	The color of the dye used during the experiment is restored

On the bottom of the tab further controls are available:

Parameter	Description
Marks	**Activated:** Stage coordinates (Marks) can be defined by clicking into the image. The coordinates are written into the Marks table in the **Stage** tool . The Marks button stays available during image acquisition
Stage	**Activated:** You can move the stage with the mouse during a continuous acquisition by clicking and dragging the displayed red cross in the image container.

20.5.3.1.4.1 Color Selection Dialog

Here you can select a pseudo color for the selected channel. In the lower area of the dialog you will see four buttons that offer various methods of color selection. The selected button is highlighted in blue. To change the method, simply click on the appropriate button.

Fig. 20.48: Color Selection dialog

Parameter	Description
Weight	Sets the weighting of one channel to another channel. This is only possible with multi-channel recordings.
Color	Here you can choose the desired color from a default color chart. The selected color is displayed on the color button.
LUT	LUT = Look-Up Table Here you can choose the desired color from a more complex color look-up table.
Cust...	Cust = Custom

Parameter	Description
	Here you can define an own color and assign it to a color field.
None	Assigns no color to the channel. Images of monochrome cameras are black/white display. images of color cameras are displayed in real colors.

20.5.3.2 Player Tab

Only visible if the **Show All** mode is activated.

Using the functions on this tab you can play back multidimensional images. The functions largely correspond to the functions for playing back films.

Player Options

The following control elements are available:

Parameter	Description
 **Play**	Plays back the image series forwards from first to last image. The dimensions are played back one after the other in the sequence specified.
 **Stop**	Stops the play-back of the image series.
 **Play**	Plays back the image series backwards from last to first image. The dimensions are played back one after the other in the sequence specified.
 **Play alternately**	Plays back the image series forwards and backwards alternately.
 **Jump to first**	Jumps to the start of the image series.
 **Jump to last**	Jumps to the end of the image series.

Parameter	Description
Speed (FPS)	Here you can adjust the speed at which an image series is played back.

Parameter	Description
	The speed is displayed in frames per second (FPS) in the input field. You can also enter the desired speed directly in the input field. The maximum play-back speed is 25 FPS.
Follow Acquisition	**Activated:** Always displays the last acquired image during an ongoing acquisition procedure, as well as the slider for the corresponding dimension.
Dimensions	Depending on the available dimensionsin the active image a slider is displayed here for each dimension. Possible sliders: ■ **Z-Stack** ■ **Time** ■ **Scene** ■ **Block** The sliders have each two adjustment handles, which you can use to define the start and end point of the playback. If there are several dimensions, you can determine, by activating the corresponding checkbox, if you want the dimension to be taken into account during the play-back. Each slider offers as many steps as there are individual positions in the specified dimension. A third adjustment handle indicates the current position and cannot be controlled directly.

20.5.3.3 Graphics Tab

Here you can select various tools and use these to draw graphic elements into your images. In the list you see the graphic elements that you have drawn in to the image.

Global Graphics

In general there are two classes of graphic elements: global and custom. Each global graphic element has a set of properties such as style or type of measurement values displayed, which can be changed system wide for each element. Each global element can only have one formatting style which is used every time, this element is being used. All graphic elements can be accessed through the **Graphics** menu, a selection of the most important tools is also available in the **Graphics** view options tab for quick access (see image below). The content of the Graphics tab cannot be modified however.

Custom Graphics

Custom graphic elements are available in the **Custom Graphics** tab, see *here* [▶ 860]. Here it is possible to configure a collection of graphical elements according to personal preference. It is also possible to create multiple copies of the same tool type with different formatting styles and measurement values which is not possible for global graphic elements.

INFO

Graphic elements are characterized by their formatting style, can be annotated with a free text and can contain measurement values such as geometric or gray value measurements. Add free text by double clicking any graphic element and typing in the desired text.

A selection of global graphic elements to work with are available to you here. For more tools, open the *Graphics* [▶ 594] menu.

Parameter	Description
 **Select**	Use this to select the graphic elements in the image area. If you are currently in another mode, you can switch back to the Selection mode using this button.
 **Clone**	Use this to create an identical copy of the last graphic element drawn in by simply clicking anywhere into the image area. To exit this mode, either switch back to the Selection mode or press the *ESC* key.
 **Draw Region of Interest**	Use this to draw in a Region of Interest (ROI) into the image. ROI's are only used to create subsets of images, not for annotation of images. To create a subset, hold the *Ctrl* key pressed and move the mouse cursor within the ROI. Then press the left mouse key and wile keeping it pressed down drag the mouse outside the ROI. A new subset image document only containing the pixels within the ROI but with all other dimensions is being created.
 **Draw Text**	Use this to insert a text field into the image. With the field drawn in start typing to add text.
 **Insert Scale Bar**	Automatically inserts a scale bar into the bottom right corner of the image. The size is set automatically to approximately 5% of the width of the image size. The length can be modified by selecting the scale bar in the image and changing the length.

Parameter	Description
 **Draw Arrow**	Use this to draw in an arrow.
 **Draw Rectangle**	Use this to draw in a rectangle that is always parallel to the edges of the image. This element by default also shows the mean gray level of the image region.
 **Draw Circle**	Use this to draw in a circle. This element by default also shows the mean gray level of the image region.
 **Draw Spline Contour**	Use this to draw in a freely selectable contour. You can either define the corner points by a series of clicks or you can trace a contour by keeping the left mouse key pressed. Close this contour by right-clicking. Corners are always rounded with this tool. This element by default also shows the mean gray level of the image region.
Format	Only active, if you have selected a graphical element in the image or the **Annotations/Measurements** list. Opens the *Format Graphic Elements dialog* [▶ 859]. There you can format the selected graphic element according to your preference. Alternatively you can double-click on the list entry or right click on the graphical element in the image and select the **Format** entry.
 **Relative Time**	Adds a text box to the top left of the image with information on the relative acquisition time per channel. Relative means, that the time value is set to 0 at the time point, where the element is drawn in. This makes it easier to analyze time series images.
 **Acquisition Time**	Adds a text box to the top left of the image with information on the absolute acquisition time per channel.
 **Relative Focus**	Adds a text box with information about the relative focus position. Relative means, that the focus value is set to 0 at the z-plane the element is drawn into. This makes it easier to interpret focus changes when playing through the image dimensions.
 **Focus Position**	Adds a text box with information about the absolute focus position as recorded from the focus drive of the microscope.

Parameter	Description
 **Exposure Time**	Adds a text box with information about the exposure time used by the camera given in the format „00.000“ [ss.msec].
 **Multi Channel Name**	Adds a text box with the names of all the active channels.
 **Carrier Container Name**	Adds a text box with information on the carrier container name. This is only useful for images acquired from multiple scenes using the Tiles module. Examples are multiwell plates. With this annotation an image can be related to the well it came from.
Keep Tool	**Activated:** Keeps the selected tool active. This allows you to draw in a number of the same elements one after the other.
Auto Color	This parameter is only visible if **Show All** is activated. **Activated:** Uses a new color for each element drawn in.
Snap to Pixel	This parameter is only visible if **Show All** is activated. **Activated:** Draws in the graphic elements in a way that connects them to the image pixels. If image-pixel precise measurements shall be done, this option must be used. If this option is not active, the graphic elements are drawn into the graphics layer independently of the actual image pixels.

Layers

Only visible if the **Show All** mode is activated.

Here you can specify which graphic element layers are active and visible in the image. To open the shortcut menu, click on the Layers dropdown menu.

Parameter	Description
Active Layer	Here you can specify which graphics layer is active in the image. The other layers are visible but blocked. To activate a layer click on the menu entry.
- automatic	Sets the active layer automatically. This is the default setting.
- Selection	Sets the Selection layer as the active layer. This layer contains graphic elements such as ROI selection, Grid, etc.

Parameter	Description
- Annotations/ Measurements	Sets the Annotation/Measurement layer as the active layer. This layer contains most of the graphic elements which can be drawn in such as all annotation elements or interactive measurement tools.
- Acquisition	Acquisition elements are elements which have been used in experiments to specify acquisition ROI's or photomanipulation ROI's such as used for FRAP experiments.
Layers	Here you can specify which layer is visible in the image. The other layers are not visible.
- Selection	Displays the Selection layer in the image.
- Annotations/ Measurements	Displays the Annotation/Measurement layer in the image.

Table

Here you can see almost all the graphic elements that exist in your image. You can also control the behavior of the graphic elements here, e.g. lock or hide them. You can format each graphic element as you wish.

i INFO

In the list you will only see the graphic elements relating to the active graphics plane. To change the active graphics plane, click on the **Layers** button. This button is only visible in **Show All** mode. Select the layer that you want to display under **Active Layer**.

The columns of the list contain the following entries:

Parameter	Description
 **Visibility**	Shows or hides a graphic element.
 **Fix Position**	Locks a graphic element to prevent changes being made.
Type	Displays the icon for the tool type. To format a graphic element, double-click on the icon. The Format Graphic Elements dialog then opens.
ID	The parameter is only visible if **Show All** is activated.

Parameter	Description
	Displays the **ID** for the graphic element. To do this, activate the checkbox at the corresponding list entry. Note that each graphic element has its unique ID.
A	The parameter is only visible if **Show All** is activated. Displays **Annotations** for a graphic element. To do this, activate the checkbox at the corresponding list entry. „Annotations" refers to all user defined text as well as text from frequent annotations.
M	The parameter is only visible if **Show All** is activated. Displays **Measurement** data for a graphic element. To do this, activate the checkbox at the corresponding list entry.
Name	Displays the name of the graphic element. To change the name, double-click in the Name field. Then enter the text of your choice. This can be used to label elements in your image.
 **Save**	Saves the selected graphic element for reusing it with other images.
 **Load**	Loads an existing graphic element into the current image.
 **Delete**	Deletes the selected graphic element.

Dimension

The coordinates and dimensions of the selected graphic element are displayed in the corresponding input fields (standard unit = image pixels):

Parameter	Description
Scaled µm	**Activated:** The dimensions are shown in scaled unit.
X	Displays the X coordinate of the center point of a graphic element. Edit the X coordinate here.
Y	Displays the Y coordinate of the center point of a graphic element. Edit the Y coordinate here.
W	Displays the width of graphic elements. Change the width here.

Parameter	Description
H	Displays the height of graphic elements. Change the height here.
Angle	Displays the angle of rotation of graphic elements. Here the measured angle is displayed for the graphic element Angle. Change the angle here.

20.5.3.3.1 Format Graphical Elements Dialog

This dialog can be called up via the menu **Graphics** |**Format** or via the **Graphics** view option tab.

Parameter	Description
Zoom with image	If activated, the size of the graphic element (e.g. line width, given in nr. of pixels) is related to the pixel size in the image. Therefore, when zooming into the image, the line width increases in the same way as the image pixels. If deactivated, the size relates to the monitor pixel size. That means when zooming into the image, the line width e.g. does not change.
Line	Here you change the line color, width and the line style (none, solid, dashed and dotted).
Text	Here you select the text font, color, size and style by selecting the appropriate options. Also select the desired horizontal and vertical alignment from the dropdown list. Reading direction is only active for measurement annotations of the **Line** element. **One direction** aligns the text annotation to the edge of the image. **Two directions** aligns it parallel to the line itself.
Fill	Here you can adjust, if the background of the annotation should be filled or not. Several filling options are available.
Opacity	Here you can adjust the degree of opacity of the graphic element in percent. 100% makes the graphical element completely opaque (covering the underlying image pixels), while 0% makes it completely transparent.
Annotation	
Set as new global default	If you click on this button the formatting style of the graphic elements currently selected in the image is set to the new default.

Parameter	Description
Reset	If you click on this button the formatting style of the currently selected graphic element is reset to factory default.

20.5.3.4 Custom Graphics Tab

Only visible if the **Show All** mode is activated.

Here you can create your own preferred collection of graphic elements. You can add multiple instances of the same element but assign different formats or measurement values to them. Custom graphics should not be confused with the standard graphic elements the formatting style of which can only be changed globally (meaning one default style per element is used system wide). When opened for the first time, no elements exist. Click **Customize** to add your own graphic elements.

Parameter	Description
 **Select**	Use this to select the graphic elements in the image area. If you are currently in another mode, you can switch back to the Selection mode using this button.
 **Clone**	Use this to create an identical copy of the last graphic element drawn in by simply clicking anywhere into the image area. To exit this mode, either switch back to the Selection mode or press the *ESC* key.
Customize	Opens the **Customize Tools** dialog. In this dialog you can add up to 35 graphic elements which are organized in up to 5 tool bars. You can make changes to their formatting style and also define which measurement values they should show.
Keep Tool	**Activated:** Keeps the selected tool active. This allows you to draw in a number of the same elements one after the other.
Auto Color	This parameter is only visible if **Show All** is activated. **Activated:** Uses a new color for each element drawn in.

20.5.3.4.1 Customize Tools Dialog

This dialog is called up via the **Custom Graphics** view option tab.

Parameter	Description
User Toolbar	This list shows all custom graphic elements added to the **Custom Graphics** tab. Select a tool and double click on it's icon to open the **Format New Custom Graphic Tool** dialog, see *here* [▶ 859]. You can rearrange the order of elements by using the **Up** or **Down** arrows at the bottom edge of the list. To delete a selected element use the **Delete** icon at the bottom edge of the list.
Search	Type in text to search for specific graphical elements.
Tools	This list contains all graphical elements available. If you select an element and double click on it, it will be added as a new entry to the top row of the **User Toolbar** list. Alternatively the Button with the Plus symbol at the bottom edge of the list can be used.
Frequent Annotations	This list contains all frequent annotations available. Frequent annotations are aligned rectangle elements preconfigured to show image metadata such as acquisition time or focus position used during acquisition. If you select an element and double click on it, it will be added as a new entry to the top row of the **User Toolbar** list. When finished, click the **Close** button. The newly added elements are now shown in the **Custom Graphics** tab.

20.5.3.5 Display Tab

Here you can adjust the image display. This function is particularly important if you want to display images with a very high dynamic range on the screen.

The histogram shows the brightness distribution of the pixels that are present from all channels simultaneously. The Y axis represents the relative frequency and the X axis indicates the brightness. A curve showing the corresponding distribution, the so-called display characteristic curve, is displayed for each channel.

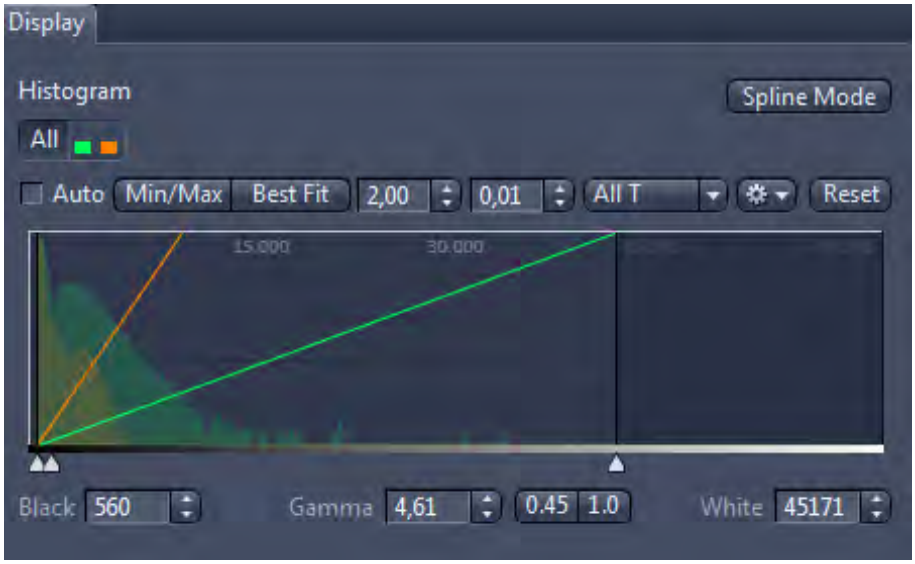


Fig. 20.49: Display tab (Show All)

If you want to adjust the histogram for an individual channel, activate this via the relevant color field in the **Channel Selection**. Alternatively you can also click on the corresponding distribution curve directly in the histogram.

Display characteristic curve

Each channel has a display characteristic curve. Using the display characteristic curve you can set the limit for the black value (left) and the limit for the white value (right). This allows you to influence the contrast in the image. Move the mouse pointer over the corresponding adjustment handles at the bottom edge of the display histogram or to the small rectangles on the display characteristic curve. Hold down the left mouse button and move the adjustment handles or rectangles as required.

The curvature of the display characteristic curve influences the gamma value. To change the curvature, move the mouse pointer to the second or fourth small rectangle on the display characteristic curve. Hold down the left mouse button and move the rectangles up or down. The setting is used immediately for the display. Using the middle rectangle you can move the whole display curve. This changes the brightness of the image.

Parameter	Description
 **Channel Selection**	Only visible for Multichannel Images. Here you can select for which channel you want to adjust the display on the screen. To select all channels, click on the **All** button. To select a certain channel, click on the corresponding channel field. Hovering the mouse pointer over a color field displays the relevant channel name.

Parameter	Description
	If the image consists of more than 29 channels, a scrollbar will be displayed which you can use to switch to the desired channel.
Spline Mode	Only visible if the **Show All** mode is activated. Clicking on this button allows you to add up to 8 points to the display characteristic curve. You can then bend the curve around these points. To do this, click on the desired section of the display curve and move it as required. Clicking on the display curve again adds another point. You can delete points by moving them along the display curve until they lie on top of another point. In this way, even in difficult situations you can adjust the display curve so that all important image regions can be displayed well.
Auto	Adjusts the image display automatically. This is particularly useful in the case of a live image, in **Continuous** mode or if you play back a time series image that contains changing brightnesses. The automatic adjustment is not available during the acquisition of LSM experiments.
Min/Max	Adjusts the display characteristic curve so that the darkest pixel is black and the brightest pixel is white in the display.
Best Fit	Adjusts the display characteristic curve so that 0.1% of the darkest pixels contained in the image are black and 0.1% of the brightest pixels are white in the display.
Input fields	Only visible if the **Show All** mode is activated. By the two input fields to the right of the **BestFit** button you can adjust the black/white values from 0.1% to values from 0 to 90% according to your requirements.
Dimension Selection	Only visible if the **Show All** mode is activated. If your images contain time series, Z-stacks or both, here you can select the aspect of an image for which the display settings should be applied. Note that with all settings other than **Current** there may be several seconds of calculation time until the setting is applied, depending on the number of time points/Z-planes. The following options are available:

Parameter	Description
- Current	Adjusts the display for the current image and keeps this setting for all other time points or Z-planes.
- All T	Collects the intensity values from all time points and adjusts the display according to the brightest and darkest pixels within the entire time series.
- All Z	Collects the intensity values from all Z-planes and adjusts the display according to the brightest and darkest pixels within the entire Z-stack.
- All T+Z	Collects the intensity values from all Z-planes and time points and adjusts the display according to the brightest and darkest pixels within the entire Z+T series.
 **Options**	Only visible if the **Show All** mode is activated. Here you can copy display settings to the clipboard, insert them into other images from there or save and reload settings. This allows you to apply identical display settings to several images in order to produce comparable display conditions.
Reset	Resets all parameters to the default values.
Only visible if the **Show All** mode is activated.	
Black	Displays the gray value currently set up to which all pixels are shown as black. You can also enter a certain value here.
Gamma	Displays the gamma value currently set. You can also enter a certain value here.
0.45	Sets a gamma value of 0.45. This is the recommended setting for most color images.
1.0	Sets a linear display characteristic curve with a gamma value of 1.0.
White	Displays the gray value currently set from which all pixels are shown as white. You can also enter a certain value here.

20.5.3.6 PSF tab

In most image views you will see the **PSF Display** tab as soon as a PSF image has been loaded. PSF images differ from the data types of normal images. They are saved, for example, in the high-precision floating point format. A series of important values that allow conclusions to be drawn about the microscope system and sample conditions can also be read from PSF images.

Export PSF button

Generates a new PSF document in 16 bit gray level format, which can be processed as required to allow it to be used in other programs.

Display Mode settings

Choose between three display options from the dropdown list:

- **Intensity PSF**: The PSF is displayed in the position space, gray values are displayed in floating point format.
- **Intensity OTF**: The optical transfer function (OTF) displays the 3D PSF in the frequency space following a 3D Fourier transformation. Gray values are displayed in floating point format.
- **Intensity Slice OTF**: Displays the 2D Fourier transformation of each individual Z-plane.

Axial cut view checkbox

Activated: Displays the PSF in axial section view.

Deactivated: A slider for Z appears on the Dimensions tab. This allows you to move through the various Z-planes.

List of PSF values

A series of important values relating to the PSF are displayed here in a table:

- **Storage type:** Format in which the PSF is saved
- **Source:** Shows whether the PSF has been generated by measuring a bead stack (External) or from the theoretical calculation (Internal).
- **Used Dimensions:** shows whether the PSF is 3D or 2D.
- **Instrument:** shows the type of microscope used.
- **Illumination:** shows the illumination conditions that applied.
- **NA Objective:** Numerical aperture of the objective
- **Lateral Magnification:** shows the objective magnification.
- **Working Distance:** shows the working distance of the objective.
- **Illumination Wavelength:** shows the wavelength of the excitation light; in the case of multichannel PSFs the values for all channels are shown here.
- **Detection Wavelength:** shows the wavelength of the detected emission light**;** in the case of multichannel PSFs the values for all channels are shown here.
- **Transverse Resolution (Rayleigh)**: shows the actual lateral resolution achieved according to Rayleigh; in the case of multichannel PSFs the values for all channels are shown here.

- **Axial Resolution:** shows the actual axial resolution achieved, determined according to Full Width Half Maximum (FWHM); in the case of multichannel PSFs the values for all channels are shown here.

i INFO

Please note that the resolution values for measured PSFs show the performance of the entire system, consisting of all optical and electronic components. The sample, with its optical properties and possible aberrations, therefore has a significant impact on the resolution. This means that these values are not suitable for making statements about the quality of the objective.

20.5.3.7 ApoTome tab

Here you will find various settings for displaying the resulting image following ApoTome acquisition.

Display mode

Here you can select which combination mode is used for the image view. This impacts on how the image is displayed.

If you have selected the Raw Data combination mode, the **Phases** slider will also appear on the **Dimensions** tab. This enables you to select the various phases of the raw images.

Create Image button

Creates a new image document. The available settings are taken into consideration here.

The following functions are only visible if the **Show All** mode is activated:

Normalization checkbox

Activated: The resulting images always fill the entire 16 bit dynamic range of the image histogram, see normalization .

Apply Correction checkbox

Activated: Applies streak correction to the resulting image. Here an attempt is made to remove streak artifacts which may be caused by bleaching of the sample during acquisition or by slight deviations in the grid phase position. Select one of the following corrections from the **Correction** dropdown list:

- Local Bleaching : Corrects the bleaching for each pixel individually (default setting). This is usually the best method.
- Global Bleaching : Corrects bleaching by means of global bleaching correction, which applies equally to the entire image.
- Phase Errors : Corrects phase errors in the image without additional bleaching correction.

Phase Correction checkbox

Only visible if you have selected one of the two bleaching corrections as the correction method.

Activated: Performs a correction of any phase deviations present in addition to the selected bleaching correction.

Fourier filter

The Fourier filter attempts to remove residual streaks. You can choose between **Off**, **Weak**, **Medium** and **Strong**.

Grid

Here you can see the grid frequency used for the image in lines/mm.

Deconvolution checkbox

Activated: Access to the key parameters for **ApoTome** deconvolution is activated.

Adjust Strength checkbox

Activated: Enter the degree of restoration using the slider.

To achieve strong image restoration, move the slider towards **Strong**.

To achieve less image restoration, move the slider towards **Weak**.

If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear.

Deactivated: The restoration strength for optimum image quality is determined automatically.

The restoration strength is inversely proportional to the strength of so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

Aberration Correction checkbox

Activated: The entries for aberration correction are taken into account when the image is created.

- **Index of Embedding:**
  Here you can enter the refractive index of the embedding medium used.
- **Distance to Cover Slip:**
  Here you can enter the distance of the acquired structure from the side of the cover slip facing the embedding medium.

Apply Deconvolution

Applies the deconvolution to the ApoTome image. The result is displayed directly without a new document being created. If you wish to create a separate document, click on the **Create Image** button.

20.6 View Modes

20.6.1 Full Screen mode

In this mode the image will be displayed in the full monitor size.

To start the full screen mode, position the cursor on the image area and open the context menu via right mouse click. Click on **Full Screen**. You can also press **F11** or click on menu **Window** | **Full screen** as an alternative.

Toolbar

In the toolbar at the bottom you find several buttons for general and image specific functions, like zoom function (**Zoom** button) or image informations (**Info** button). When you open a multidimensional image, you find buttons for specific functions, etc. **Z-Stack**, **Channels**. To open the functions, click on the button.

Previous button

Displays the previous document in full screen mode. You can page step by step backwards through all open documents.

Next button

Displays the next document in full screen mode. You can page step by step forwards through all open documents.

Exit Fullscr. button

Closes the full screen mode.

20.6.2 Exposé mode

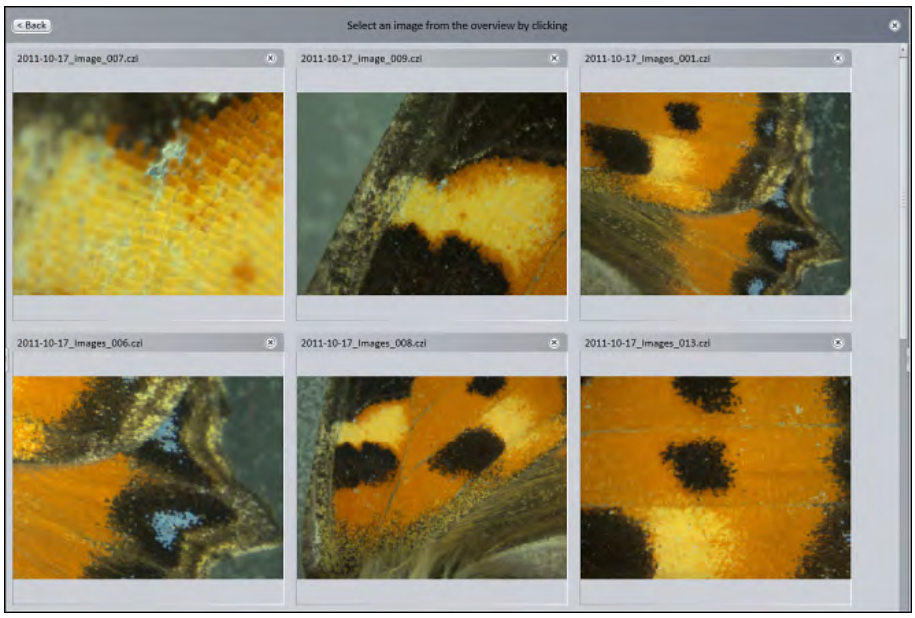


Fig. 20.50: Expose mode

To open the **Exposé** mode click on the expose mode icon in the *document bar* [▶ 33].

In this mode all open documents will be displayed in an overview.

When you click on an image in the overview the **Exposé** mode will be closed and the image will be displayed in standard view.

Back button

Jumps back to the standard image view.

Close button

Closes the den **Exposé** mode.

20.6.3 Splitter mode

In this mode you can generate a multi image of one or several images in order to compare them. Drag an image of the **Images and Documents** gallery in **Right Tool Area** and drop it in a splitter position. The standard setting for the splitter are 2 columns and 1 row. You can modify these setting in the **Split-View**.

Proceed similarly with further images to be displayed in the multi image. The same image can be dropped several times in the splitter view, i.e. to compare different image scenes.

The multi image can be saved as **CZSPL** (Zeiss Multi Image Files) image type in the menu **File | Save As**. The stored multi image is no image document, but rather a reference of the images displayed in the splitter mode.

Use the **Split Display tab** for further adjustments (i.e. arrangement) of the splitter mode. Here you can create a single image of the multi image to be saved as **CZI** image type.

20.6.3.1 Split Display Tab

Arrangement section

Here you can set how much columns and rows the splitter image should have. Therefore simply enter the desired number in the **Columns** / **Rows** input fields.

Dimensions / Display settings

Parameter	Description
Synchronize Dimensions	**Activated:** The settings of the **Dimensions** tab (i.e. Zoom) will be applied synchronously to all images in splitter mode.
Synchronize Display	**Activated:** The settings of the **Display** tab (i.e. Gamma) will be applied synchronously to all images in splitter mode.
Show Position Data	**Activated:** The cursor changes to an arrow symbol and a cross marker in the image. The **X/Ycoordinates** with scaling unit and **gray value** of the current cursor position are displayed below the image. Furthermore additional information is displayed for multidimensional images: i.e. the **gray value for each channel** of a multichannel image, the **time of each time point** of a time lapse image, the **focus position of each Z position** of a Z-Stack.
Reset	By clicking on this button you can reset all adjustments applied to the images in splitter view.

Image Generation

Parameter	Description
New Image From	Here you can select the type of image to be generated. The available options are depending on the dimensions of the displayed image.
- Current View	Creates a 2-dimensional image of all opened images visible in the splitter mode.
- Time Series	Creates a multi-dimensional image containing each time point of a time series image.

Parameter	Description
- Z-Stack	Creates a multi-dimensional image containing each Z-plane of a Z-stack image.
- Rotation	Creates a multi-dimensional image containing each rotation plane of a rotation series image.
Create	Generates an single image of the multi image displayed in splitter mode with the corresponding settings. The image can be saved in the ***.CZI** format in **File** | **Save as** menu.
The following functions are only visible if the **Show All** mode is activated:	
Interpolation for zoomed images	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Spacing	Here you can adapt the size of the distance between the single images to values 0 up to 20. The background (distance) will be displayed in black color and appears, if the splitter image is generated by the **Create** button.
Spacing color	Here you can change the background color of the splitter image.
Burn-in annotations	**Activated**: All existing annotations and graphical elements within the images will be burned into the resulting splitter image.

20.7 File Browser

Here you see an overview of all image or data files stored on your computer. In the left column you see a file structure which is associated with the common image or data containing folders in your file system (Images and documents). In the right area you see the preview to the selected folder.

i INFO

The ZEN folders contain automatically the **Auto Save** folder. Here you see all auto-saved images from ZEN. Set Auto Save path in **Tools** | **Options** | **Saving**.

Gallery View

Here you see all files of a folder as small preview images (thumbnails). Use **Tool** tab to adjust preview images size, sorting, etc..

Info View

Here you see a detailed list with all data the selected image contains. Find a detailed description of all possible data under *Info View* [▶ 796].

Table View

Here you see all files of a folder well-arranged in a table. This view is perfect for folders which contain many files.

20.7.1 Tools Tab

Parameter	Description
Icon size	Set size of thumbnail images here.
Text Rows	Select entries which you want to have displayed as additional text row under the thumbnail image.
Record	Switch from file to file in the selected folder by using the slider.
Sorting	Arrange your files to certain properties (i.e. file name, type, etc.)
Folders	Manage selected file folders here (i.e. new folder, rename folder, etc.).
Selection	Manage selected files here (i.e. copy file, or delete file).

21 Software Extensions

21.1 ImageJ Extension

21.1.1 Introduction

Our ZEN software includes the no-charge **ImageJ** extension, which offers following possibilities:

- The extension allows an easy exchange of images, from simple two-dimensional images, to more complex, multidimensional entities, like Z-stacks, time series and so on. The exchange can go both ways, from ZEN to ImageJ, as well as from ImageJ to ZEN.
- The user can execute ImageJ functions on ImageJ, without having to leave the ZEN environment.
- Lastly, the ImageJ extension allows the user to combine the two benefits, introduced above: the users can send a ZEN image to ImageJ, have it processed there, and then return the resulting image back to ZEN in one single step.

Note that different versions and variants of ImageJ and Fiji exist. This document is based on the ImageJ/Fiji version 1.46. See notes for specifics of other versions and variants. For the sake of simplicity, Fiji is implied also, wherever ImageJ is mentioned in the following text.

21.1.2 Preparations

i INFO

Note that the extension for ImageJ is not available in **ZEN lite**.

Procedure

1. Install **ImageJ** on your computer. Make sure that you use the latest version (check for online updates after installation).
2. Download **loci_tools.jar** and drop it into in the **ImageJ**/**plugins** folder.
3. Note the name of the folder with your preferred alternative. While you can switch freely among them all, it makes sense to stick to one and the same environment, once you have started to add your own programs and macros.
4. The ImageJ/Fiji folder you will eventually decide on, can either belong to you alone or be shared among other users of the system. It is up to you decide, what you prefer: if you are the only user, nobody will meddle with its contents (images, macros etc), but then, you will need to copy and distribute the contents, if they are of interest to others as well.

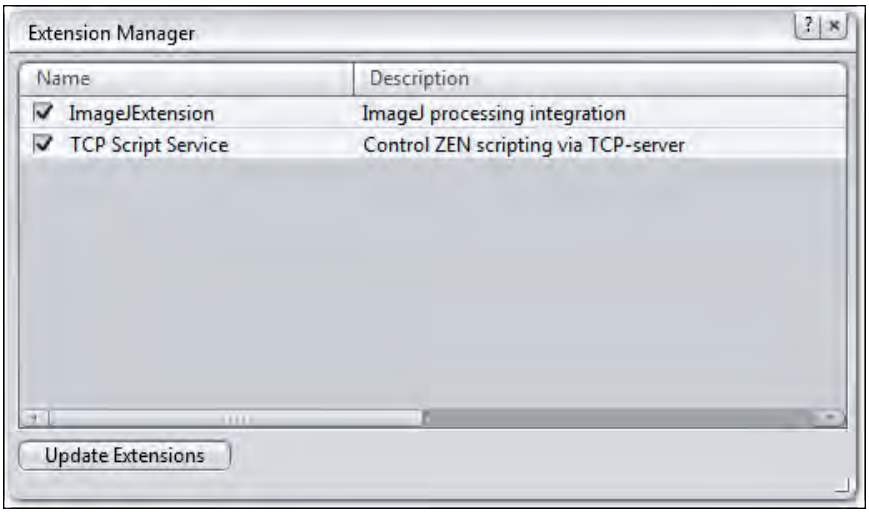
You have successfully fulfilled all prerequisites. You can now continue with setting up ImageJ within ZEN software.

21.1.3 Activate ImageJ Extension

The extension is automatically included in the ZEN installation. To set it up, start the software and then proceed as follows:

Procedure

1 For activating the extension go to **Tools** | **Extension Manager…** and activate the checkbox **ImageJExtension.**

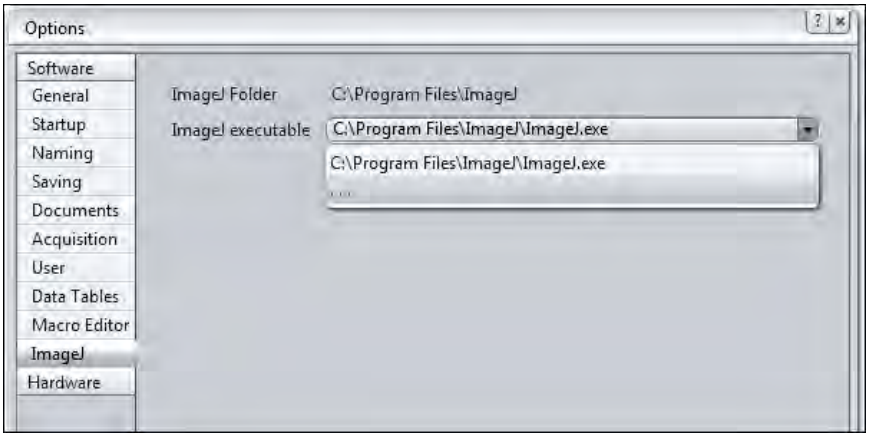


2 Click on the **Apply** button.

The extension looks through the usual places, where an existing installation could be found, and makes a sensible suggestion. To select the environment that you would prefer, proceed as follows:

3 In the **Tools** menu | **Options** | **ImageJ**, select **ImageJ.exe** in the **ImageJ executable** dropdown list.

4 If you prefer another executable or your preferred executable is not in the list click on the last entry **...** to search for it.



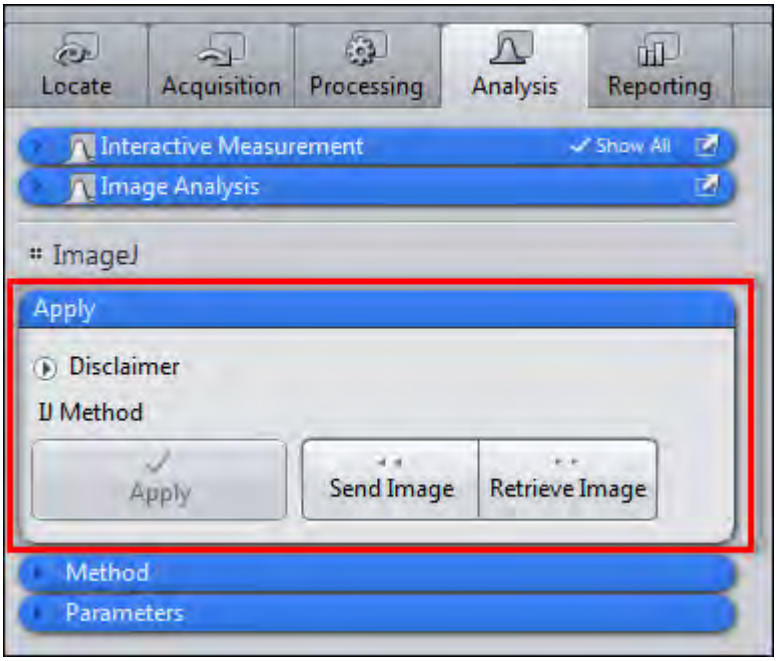
You have successfully set up ImageJ extension within the software. Now you can start working with the extension.

21.1.4 Send and Retrieve Images

Procedure

1. In the **Left Tool Area** click on the **Analysis** tab.

   The tool group **ImageJ** will appear containing the **Apply** tool.



Procedure

1. To send an image from **ZEN** to **ImageJ** select the image in ZEN.
2. Click on the **Send Image** button.

   The image will be sent to ImageJ and opened within a new image frame. You can now edit the image within the ImageJ application.
3. To retrieve an image from **ImageJ** to **ZEN** select the image in ImageJ.
4. Click on the **Retrieve Image** button.

   The image will be opened in ZEN.

You have successfully exchanged images between ZEN and ImageJ.

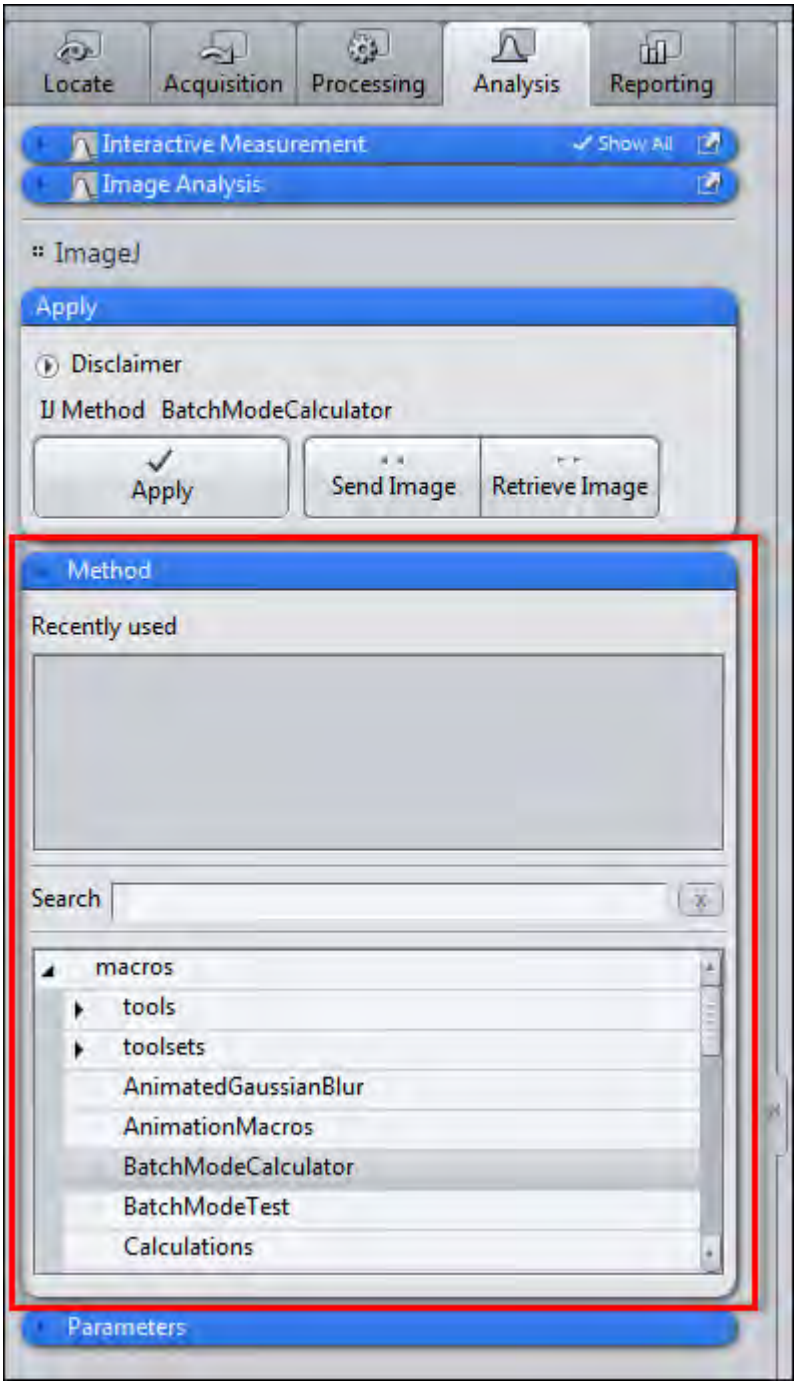
21.1.5 Use ImageJ Methods

The extension offers the possibility of sending images to ImageJ to get processed, to retrieve the result of the operation or both. The following instruction will show the basic steps which are necessary to apply ImageJ methods on any images.

Prerequisites

- You are on the **Analysis** tab in the **ImageJ** group.

Procedure **1** In the **Methods** tool select the command or method to be executed, e.g. an ImageJ macro.



2 In the **Parameters** tool you specify if the method selected will need an input image and/ or provide a resulting image.

3 In the **Apply** tool click the **Apply** button to execute the command.

You have successfully applied an method to an image.

21.1.6 Image Type Send/Retrieve Conventions

ZEN to ImageJ

Image Type	Received as…	Comments
.tif, .jpg, .bmp, .png, .gif	Original	
.ome.tif	Original	
2D image B/W .czi	32-bit (RGB)	Convert the image in **ImageJ** to the required pixel type using Image > Type command
2D image 24/48 bit color .czi	32-bit (RGB)	
2D image 36/42 bit color .czi	-	Convert the CZI image to 24/48 bits before sending it or using it in a method
Multi-channel x Z-Stack x T-series	MD image	If necessary reassign the dimensions using Image > Hyperstacks for instance. Channel colors may be different from those set in ZEN
Tiled images	-	Only the first tile gets loaded.
12bit B/W images	Error in ImageJ	Workaround: convert the pixel type of the image to 16 bits in ZEN

ImageJ to ZEN

Image Type	Received as…	Comments
.tif, .jpg, .bmp, .png, .gif	Original	
.ome.tif	Original	
2-D images, B/W and RGB	B/W, RGB TIF	
Multi-channel x Z-Stack x T-series	MD image	Hint: select RGB in Quick Color Setup to get the same colors for channels as in ImageJ
Tiled images	-	Only the first tile gets loaded.

21.2 Intellesis Extension

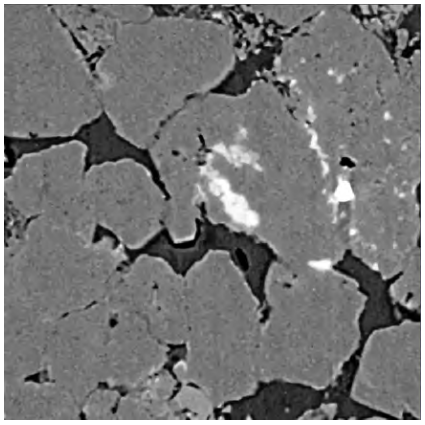
21.2.1 Introduction

The **Intellesis Trainable Segmentation** module for **ZEN (blue edition)** enables you to use machine-learning algorithms for segmenting images using pixel-classification. It uses different feature extractors to classify pixels inside an image based on the training data and the labeling provided by the user. There are a variety of use cases because the functionality itself is "data-agnostic" meaning it can be used basically with every kind of image data.

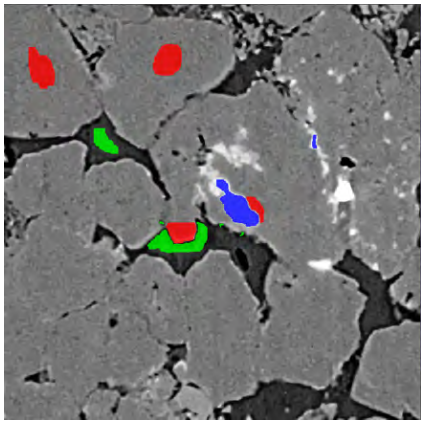
The extension has the following main functionality:

- Any user can intuitively perform image segmentation without advanced training by simply labeling what shall be segmented
- Import of any image format readable by **ZEN (blue edition)** software, incl. **CZI**, **OME-TIFF**, **TIFF**, **JPG**, **PNG** and **TXM** (special import required)
- Creation of predefined image analysis settings (*.czias) using machine-learning based segmentation that can be used inside the ZEN 2D measurement framework
- Integration of the Trainable Segmentation processing function within the OAD environment

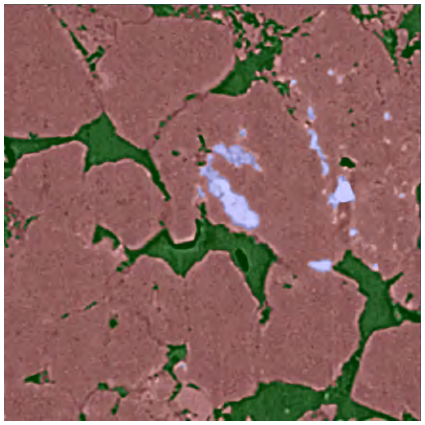
Application Example: XRM (X-Ray Microscopy) image from sand stone showing the main steps when working with the **Intellesis Trainable Segmentation** module.



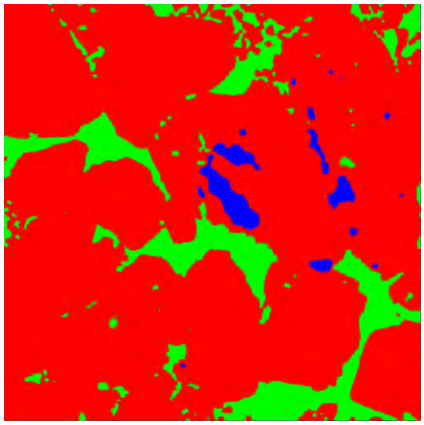
1 Original Image



2 Labeled Image

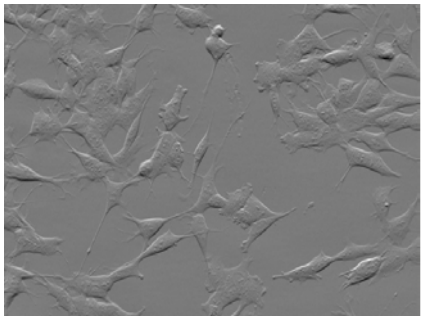


3 Overlay of Original Image and Segmentation Result

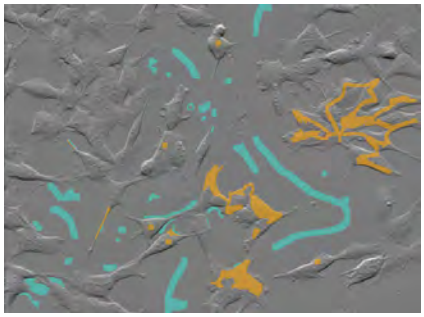


4 Segmented Image

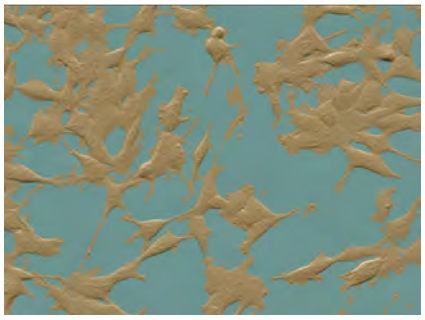
Application Example: Cells image with Phase Gradient Contrast on the Celldiscoverer 7 and segmented using **Intellesis** Trainable Segmentation.



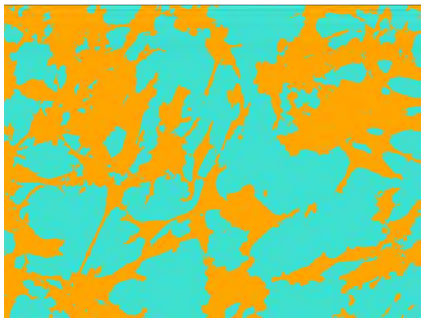
1 Original Image



2 Labeled Image



3 Overlay of Original Image and Segmentation Result



4 Segmented Image

21.2.2 FAQ /Terminology

Term / Question	Description
Machine Learning	The **Intellesis Trainable Segmentation** module uses machine learning to automatically identify objects within an image according to a predefined set of rules (the model). This enables any microscopy user to perform image segmentation even on complex data sets without programming experience or advanced knowledge on how to set up an image segmentation.
What is a "Model" ?	A model is a collection of rules according to which the software attributes the pixels to a class. Such a class is mutually exclusive for a given pixel, i.e. a pixel can only belong to one class. The model is the result of (repeated) labeling and training a subset of the data. After the model is trained (the labels provided by the user were used to "train" the classifier), it can be applied to the full data set in image processing or it can be used to create an image analysis setting (*.**czias**) to be used with the ZEN image analysis module. In image processing the trained model can be applied to an image/or data set and perform segmentation automatically. As result you will get two images, the segmented image on the one hand and a probability map on the other hand.
What is a "Class" ?	A class is a group of objects (consisting of individual pixels) with similar features. According to the selected model the pixels of the image will be attributed as belonging to a certain class, e.g. cell nuclei, inclusions in metals, etc.. Every model has by default already two classes built-in, because at least two classes are needed (e.g. cells and background or steel and inclusions). Of course, more classes can be defined if necessary.
What is "Labeling" ?	Instead of using a series of complex image processing steps in order to extract the features of the image, you just need to label some objects in the image that belong to the same class. Based on this manual labeling the software will attribute the pixels of the image as belonging to a certain class. In order to refine the result, you can re-label wrongly attributed pixels and this way assign them to another class.

Term / Question	Description
What is "Training" ?	During the training process (within the training user interface) you can repeatedly label structures as belonging to one class, run the training, check if the result matches your expectation and if necessary refine the labeling in order to improve the result. The result is a trained model (a set of rules) which produce the desired result when applied to the training data. With the labeled pixels and their classes a classifier will be trained. The classifier will then try to automatically assign single pixels to classes.
Training UI (User Interface)	The user interface for training is the starting point of the automatic image segmentation process. Here you import images, label and train the model on a subset of your data which you can later use for automatic image segmentation. Within this interface you can load the training data, define the classes of objects found in your data and train the classifier to assign the objects to the correct classes.
What is "Segmenting" or "Segmentation"?	In general segmentation is the combination of pixels of the same class within an image. Before you can perform segmentation the segmentation model has to be trained. Within the Training UI you train the software by labeling specific objects or structures that belong to different classes. A pseudo-segmentation is performed each time you train the model so that you see if the feature extractor works for your image. One output of the **Intellesis Trainable Segmentation** processing function is the fully segmented image or data set using the trained model. The second output is the probability map.
Probability Map	The probability map is one of two resulting images when you apply a trained model to an image by using the processing function **Intellesis Trainable Segmentation**. The (resulting) grayscale image encodes the reliability of the segmentation. Areas which can be addressed to a certain class with a high probability will appear bright, whereas areas which have a lower probability to belong to a certain class will appear dark. The probability is represented by a percentage value, where 0 means "Not confident at all" (dark) and 100 "Very confident" (bright).

Term / Question	Description
What is a "Feature"?	A feature is a specific property of an image, that will be calculated by using a predefined set of filters and processing functions. This process results a so-called "Feature Vector" for every pixel. This is the information that will be used for training the model.
What is a "Feature Extractor"?	A feature extractor is a predefined set of processing functions that is used to create the feature vector for every pixel. As feature extractor a specific layer of a pre-trained neuronal network can be used as well.
Prediction	When the model that was trained on example data is applied to a new unlabeled data set the result is called a prediction.
Multi-Channel Images	The **Intellesis Trainable Segmentation** module supports multi-channel data sets. It is important to understand that in case of a multi-channel image still every pixel can only belong to one class, i. e. the classes are mutually exclusive. The additional information of having more than one intensity value per pixel (e.g. one for every channel) is also used for classification. **Example**: If you have overlapping regions A and B in the image that you want to classify then consider labeling three independent classes: ■ Class 1: A ■ Class 2: B ■ Class 3: A overlapping with B If you want to segment an individual channel from a multi-channel image, use the **Create Subset** IP function first to extract the desired channel.

21.2.3 Operating Concept

The operating concept can be generally split in three parts:

Training

The **Training User Interface** which is accessed via the **Intellesis Trainable Segmentation** tool on **Analysis** tab. Within the training user interface you can label the images to be used as input for training a specific model, see *User Interface - Training* [▶ 885].

Processing

The **Image Processing** (IP) function **Intellesis Trainable Segmentation**, which can be used to segment images resulting in binary masks. Those masks can be used in subsequent ZEN workflows, such as 2D or 3D analytics or they can be exported for further use in external 3rd party software packages. You will find more details under *Using a Trained Model for Image Processing* [▶ 901].

Analyzing

The automatic creation of **Image Analysis** (IA) settings (*.**czias**), which allows to use a trained model for automated segmentation and measurement of image data within the ZEN **Image Analysis Wizard**. To familiarize with the basic steps take a look at our step-by-step guide in the chapter *Using a Trained Model for Image Analysis* [▶ 904].

21.2.3.1 Intellesis Fact Sheet

- Machine-Learning Tool for Pixel Classification powered by Python (Py3, Dask, Scikit-Learn and Tensorflow)
- Client / Server Architecture with REST-API
- Engineered Default Feature Set (CPU) with 33 Features
- Neural Network (vgg19) Layers for Feature Extraction (GPU)

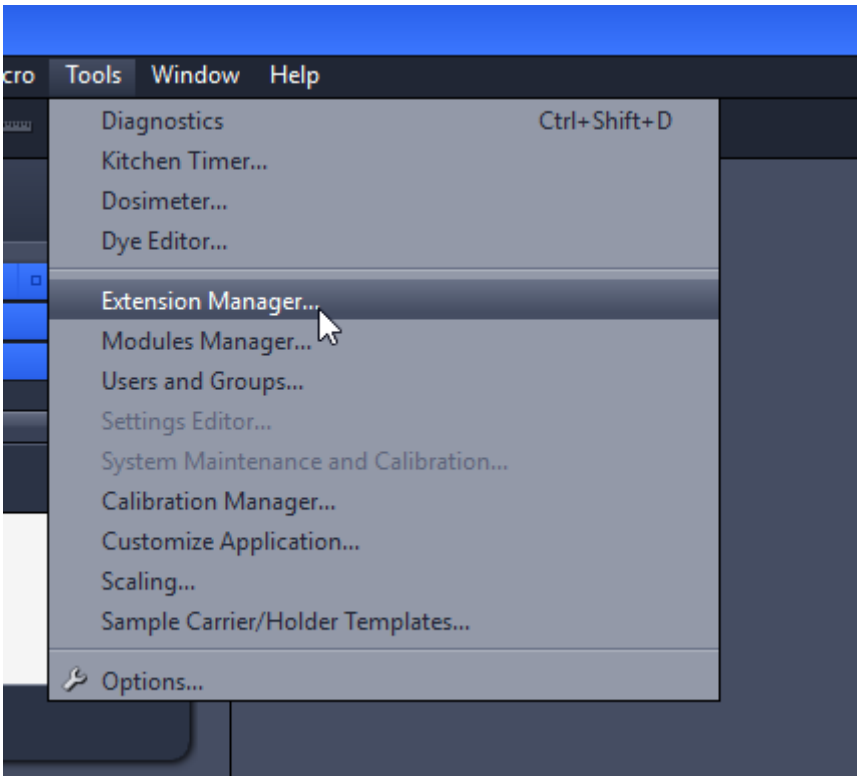
  64, 128 (red. 50) or 256 (red. 70) Features for 1st, 2nd or 3rd layer
- Classifier: Random Forrest
- Simple User Interface for Labelling and Training and Integration into ZEN Measurement Framework
- **Supports multi-dimensional Datasets**

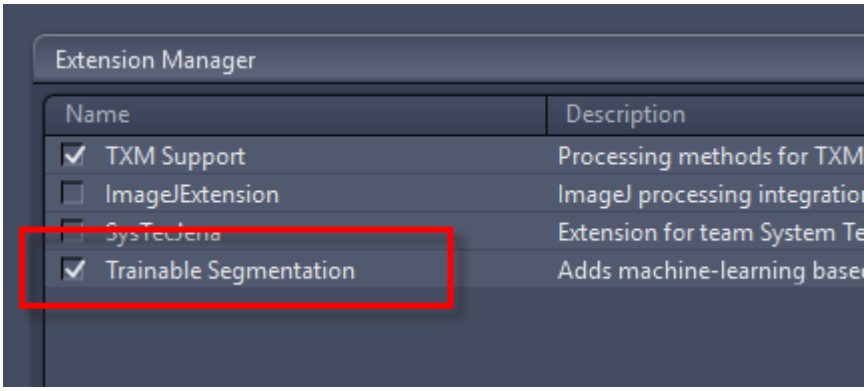
21.2.4 Activating the Intellesis Extension

Prerequisites

- You have installed and licensed the **Intellesis** extension.
- You have started the **ZEN (blue edition)** software.

Procedure **1** Activate the extension under **Tools** | **Extension Manager**.



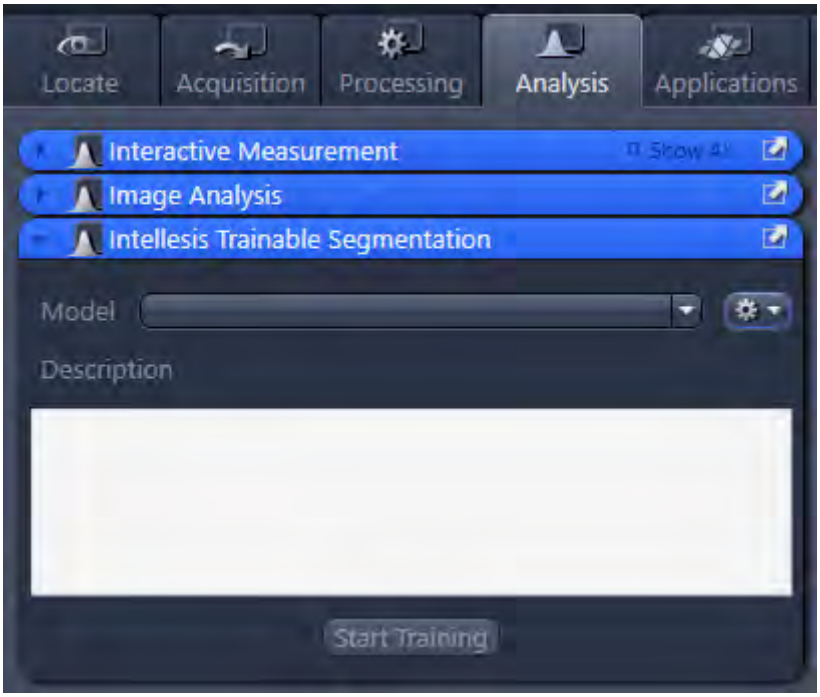


INFO

Note that if you want to import **TXM** images, the **TMX Support** extension must also be activated.

2 Go to the **Analysis** tab and click on the **Trainable Segmentation** tool.

The tool will be extended and you see the opened tool window.



You can now start creating a new model for image segmentation.

21.2.5 User Interface - Training

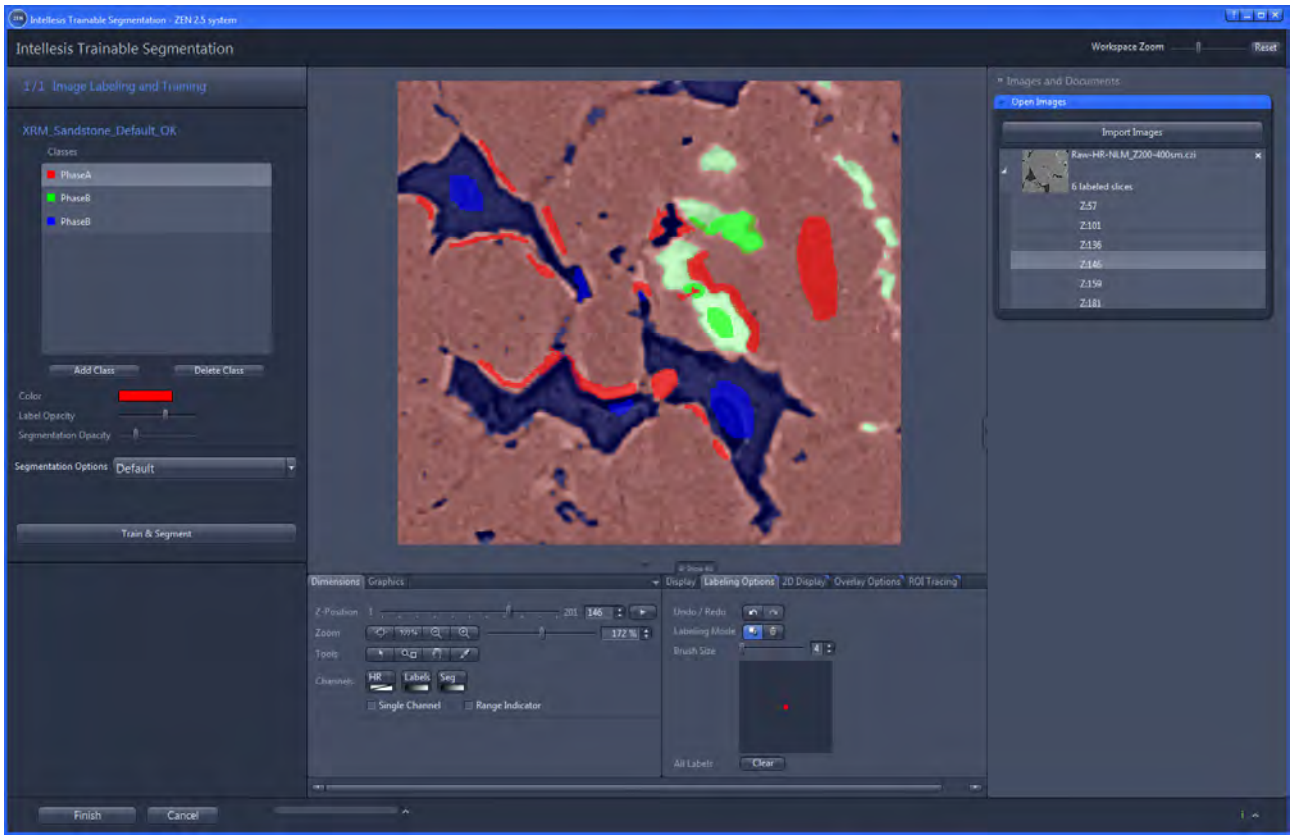
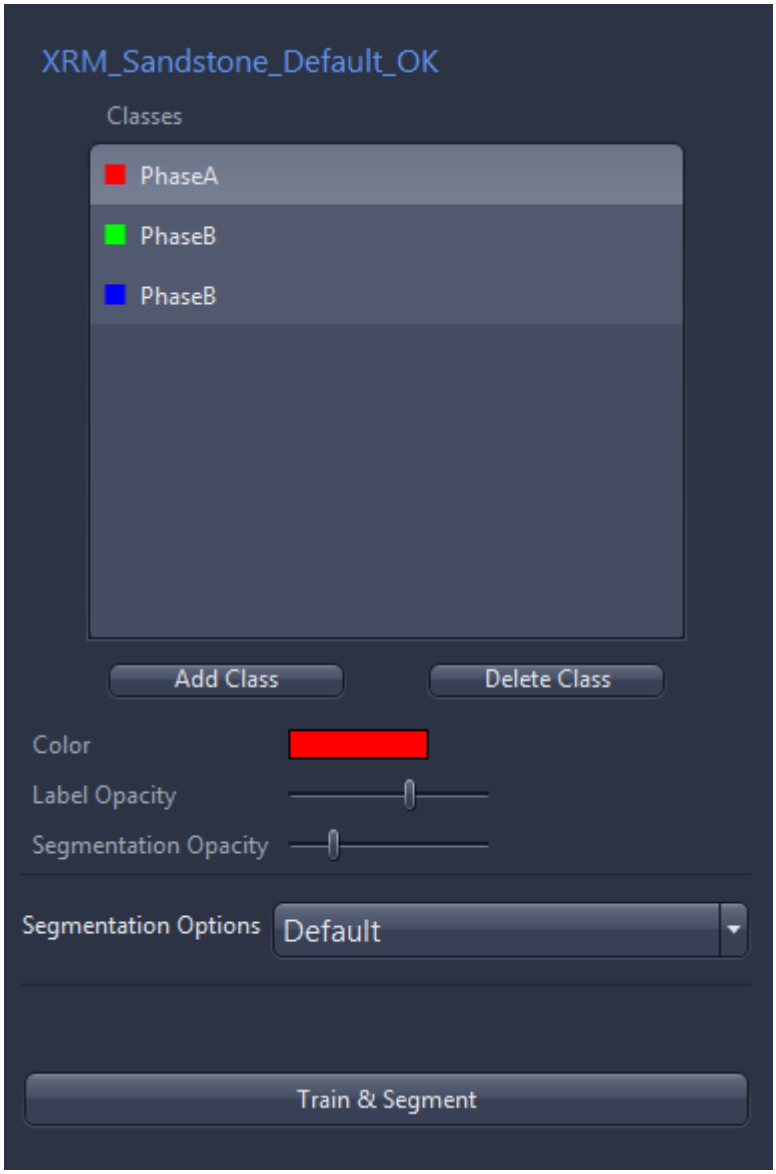


Fig. 21.1: User Interface for Training

The Training user interface is accessed via the **Analysis** tab. Open the **Intellesis Trainable Segmentation** tool, select or create a new a model and click on **Start Image Training**.



On the left side under **Image Labeling and Training** you find elements for managing the classes. You can add and delete classes and select them for labeling an image.

You can change the label opacity and the segmentation opacity by adjusting the corresponding slider. Opacity determines to what degree it obscures or reveals labels or segmentations. Opacity of 1% appears nearly transparent, whereas 100% opacity appears completely opaque.

You will also find the **Train & Segment** button which starts the automatic training algorithm and then performs the pseudo-segmentation of classes.

INFO

When you use images with large X/Y dimensions, e.g. large tile images, the segmentation will be only performed on a subset of the whole image in order to avoid long waiting periods. The current image subset maximum size in X/Y is 5000 pixels and is centered on the current view port. Nevertheless all labels inside the complete image will be used for training, but the segmentation preview (pseudo-segmentation) will be only applied to that subset.

In the center you see the image area. Load the desired images you wish to segment and start the labeling of classes there. Under the image you find the **Labeling Options**, where you can adjust certain parameters like **Labeling Mode** or **Brush Size**.

On the right side you find the **Image Gallery** where you can import and select the images you want to use for training and segmenting.

21.2.5.1 Labeling Options

Parameter	Description
Undo / Redo	When you click on the arrows you can undo/redo the last actions you have performed.
Labeling Mode	Here you can select between labeling and erase mode.
Brush Size	Here you can set the brush size of the labeling / erasing tool. Note that the brush size can be changed alternatively by holding the *Strg* key and using the mouse wheel (when the cursor is inside the image area.)
All Labels	When you click on **Clear**, all labels in the active image will be deleted.

21.2.5.2 Segmentation Options

Parameter	Description
Default	A predefined, default feature set is used to create the feature vectors.
Deep Features 1 **Deep Features 2 All** **Deep Features 2 Reduced**	The complete (All) or reduced feature set from either the 1st, 2nd or 3rd layer of a pre-trained network is used to create the feature vectors.

Parameter	Description
Deep Features 3 All	
Deep Features 3 Reduced	

For the selection of the parameters note the following:

- There is no "right" selection. We recommend to always try different parameters for the same image to see which one works best.

21.2.5.3 Image Gallery

In the right tool area under **Images & Documents** you find the area for handling the images to be used for training. Here you can load and select the images you want to use for training. When you click on a loaded image, the image will be visible in the **Center Screen Area**.

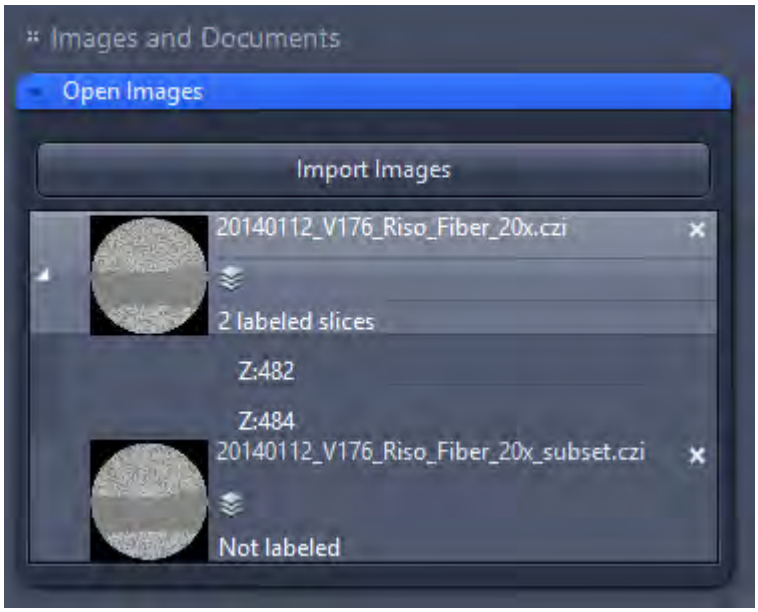


Fig. 21.2: Image Gallery with additional info

In the list of images you have certain possibilities to gain advanced information about the image. If you load a new image only the preview image, file name and type of image are displayed.

When you have started to label an image of a larger data set, a small arrow appears on the left side of the preview image. If you click on the arrow, a list of the images that contain labels will be displayed, containing dimension and image number (e.g. for a Z-stack, Z:400 indicates that the slice number 400 contains labels).

If you click on this information the corresponding image will be automatically displayed in the center screen area. This is very helpful when you are working with large data sets such as z-stacks, scenes or time-series and you want to quickly load the image which you have already labeled.

21.2.6 Workflow Overview

ZEN Intellesis Trainable Segmentation offers three main workflows. The general workflows and the basic steps involved are shown inside the diagram.

- Labelling and Training – results in a **Trained Model**.
- Segmentation – Using a **Trained Model** to segment images and results in Binary Masks.
- Use Trained Model inside Image Analysis Setting – Using a **Trained Model** to classify pixel for subsequent segmentation and measurements of objects.

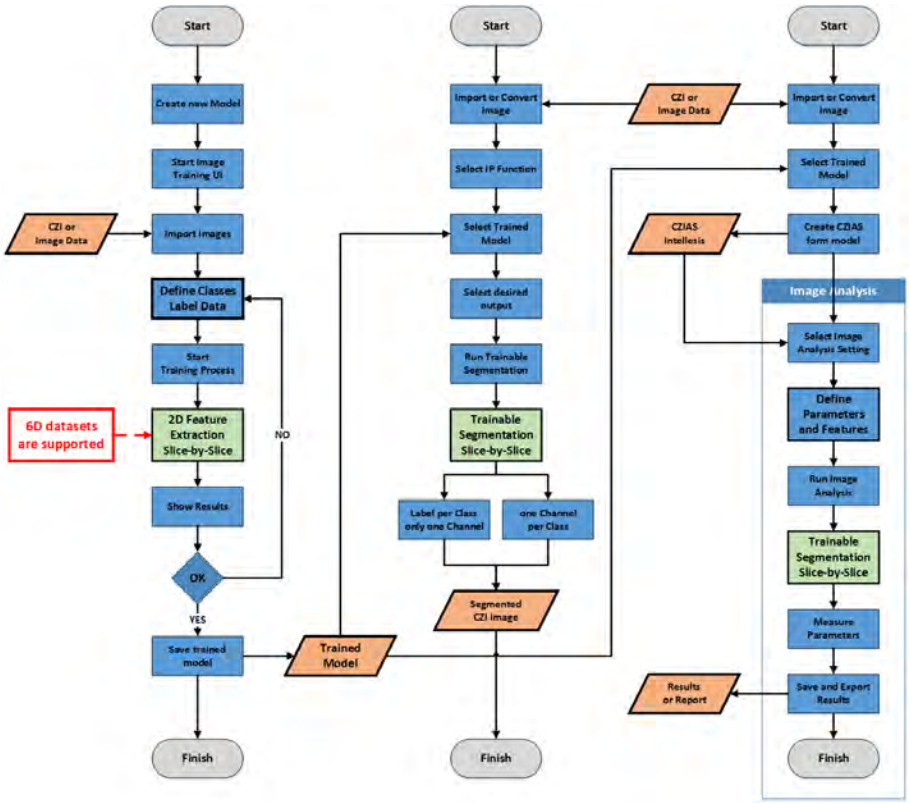


Fig. 21.3: Process description of the Intellesis workflow

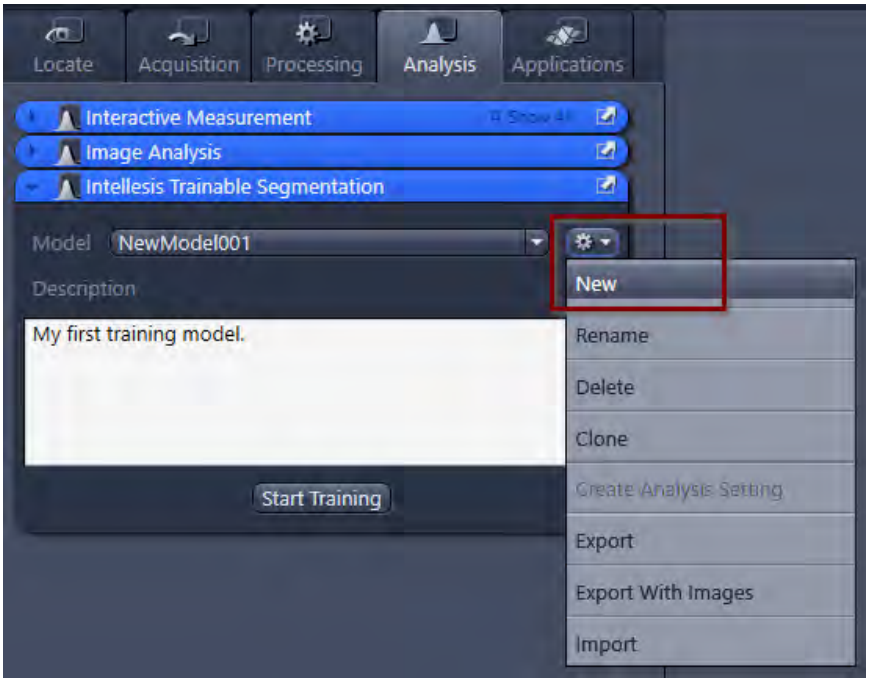
21.2.7 Creating a New Model

Prerequisites

- You have completed the general preparations.

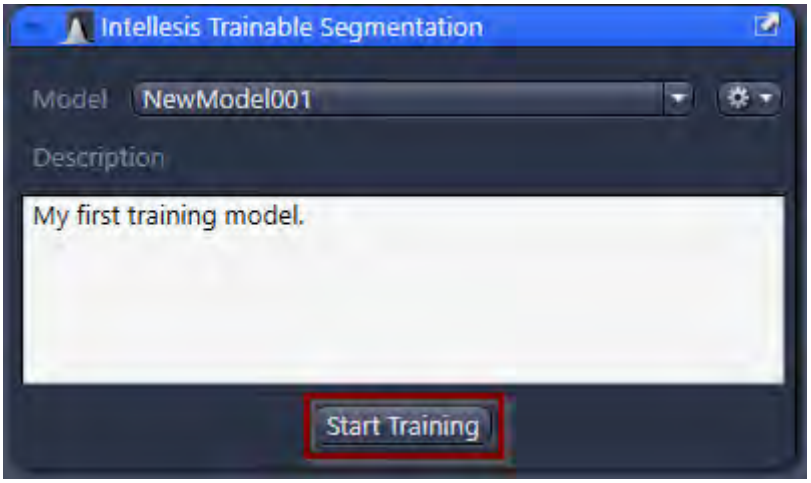
Procedure

1 On the **Analysis** tab in the **Intellesis Trainable Segmentation** tool, select **Options** | **New**.



2 Enter a name and a description for the new model.

3 Click on **Start Image Training**.



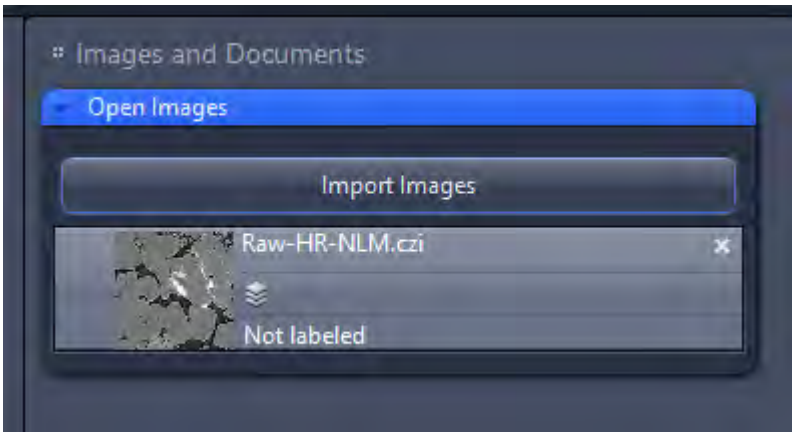
The Training UI opens.

4 In the **Right Tool Area** under **Open Images** click on **Import Images**.

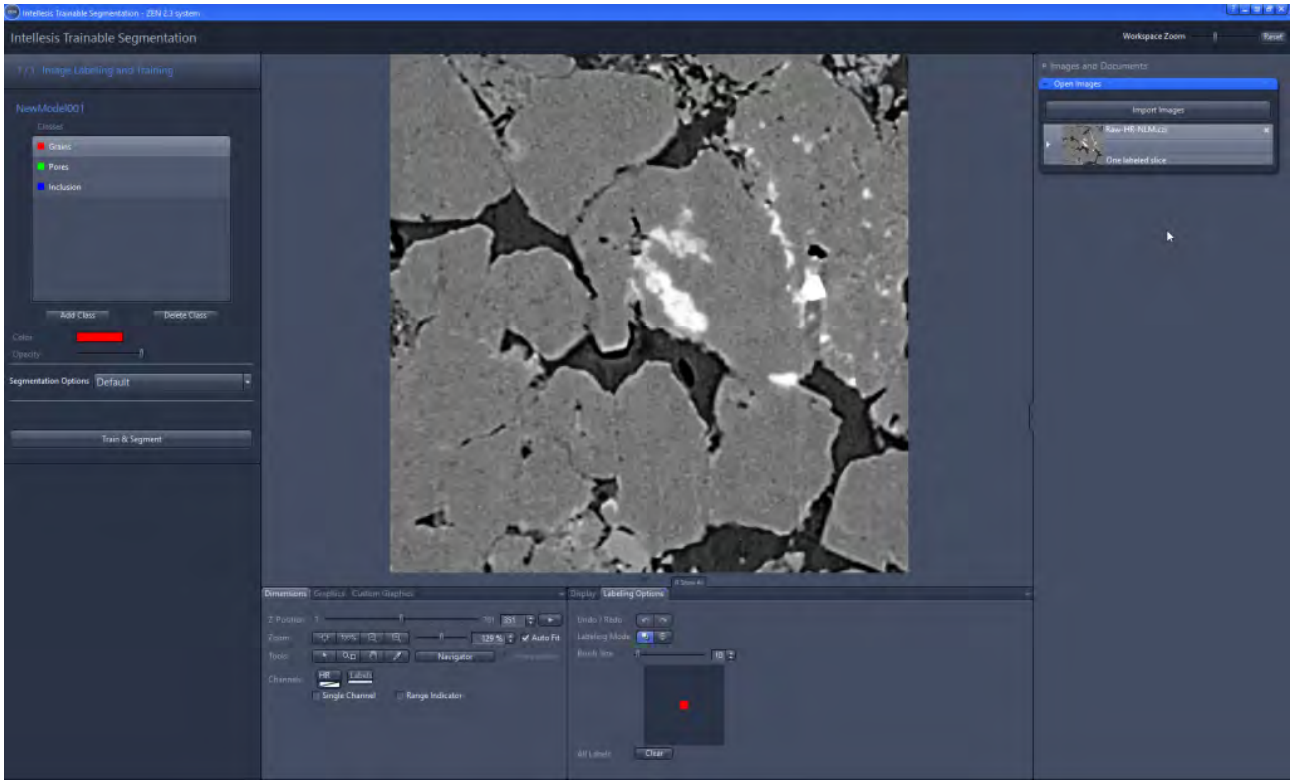
5 Select the image for training from the file system and click on **Open**.

The image will be visible in the list. Note that all imported images will be included to your training model.

6 Select the image from the list.



The image will be displayed in the **Center Screen Area**.

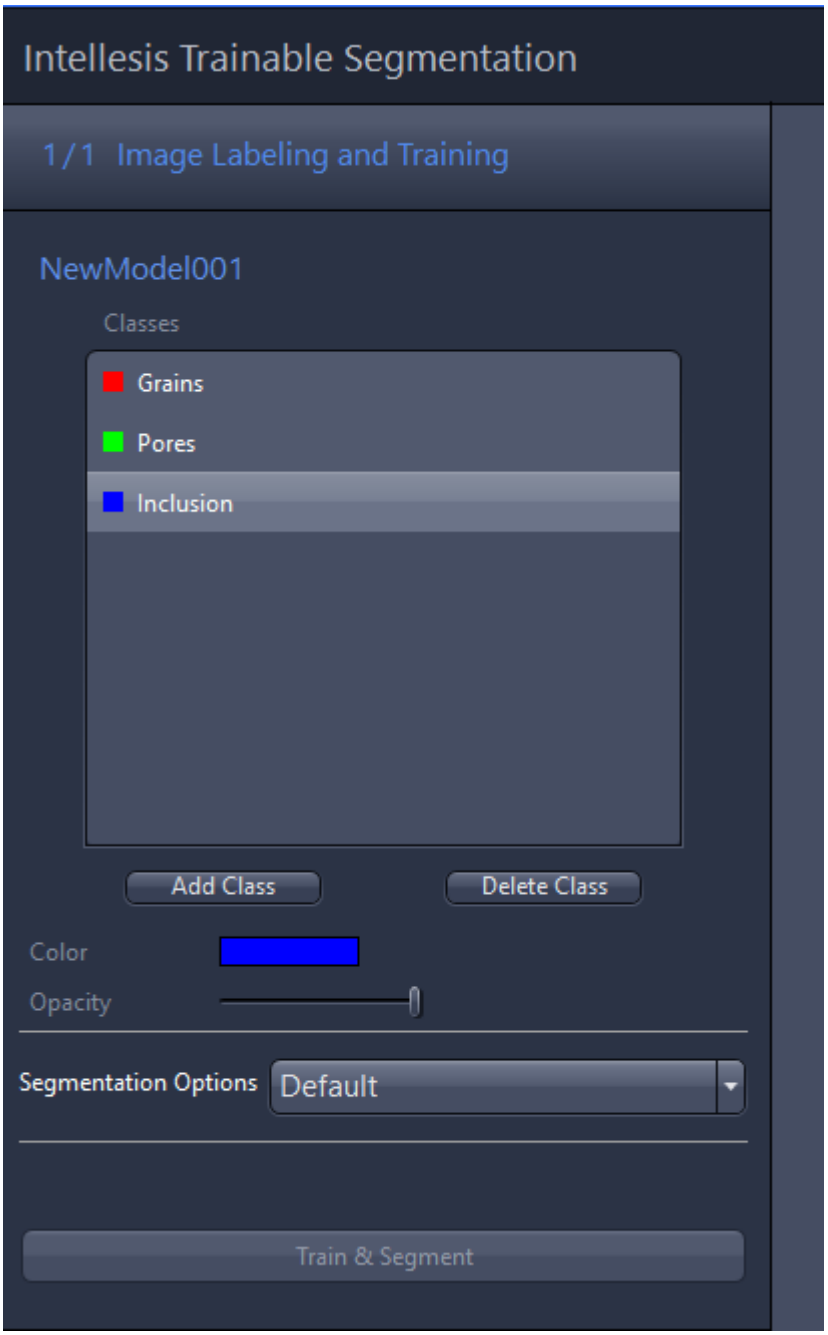


INFO

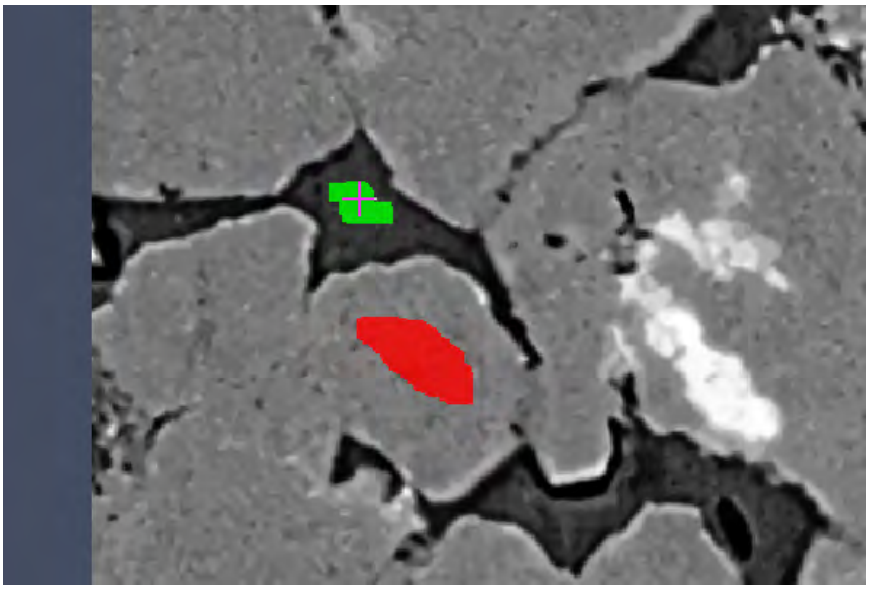
Note that on a later stage you can add more images to refine the training via **Import Images**.

7 Switch to the **Left Tool Area** and continue with the definition of the classes. Depending on your image and what you want to segment you can define a certain amount of classes. When you start with a new model you will see the two predefined classes "Object" and "Background". If you click on **Add Class** a

new class is added. You can rename these classes by a double-click and entering a new name.



8 Now move the courser inside the image and start labeling the areas which you want to assign to the selected class. To label within the image simply hold down the left mouse key and move the mouse.



INFO

For a good training result always note the following:

- The more accurate you perform the labeling the better the result will be. You can start with a coarse labeling (as indicated in the image above) and then check the result for problematic areas where you should refine the labeling (as shown in Fig. 6.1)
- Accurate labeling is generally preferred over "just labeling everything" roughly.
- Take care to also label some areas which contain edges of objects and transitions between two classes.
- Really use an iterative approach: check the segmentation / training results before labeling huge amounts of pixels.

9 After labeling a few areas with different classes click on **Train & Segment**.

The software will now start the training. The system will try to automatically recognize other areas of the same classes. Depending on the image, the pixel classification can take a while. When finished the image will have the additional channel **Seg**(menation) containing the segmentation preview.

10 If you are not satisfied with the result you can label more details of the corresponding classes. Therefore you can zoom into the image or change the brush size of the courser. The more accurate you label the different classes within the image, the better the recognition will be. When you finished the labeling you have to click on **Train & Segment** again. You can repeat that process until you are satisfied with the segmentation result.

Note that at this point as a result you will only see a pseudo segmented image and only the area visible in the main window will be segmented (max. area 5000x5000 px). The full segmentation of an image/data set is performed on the **Processing** tab by using the trained model within the **Trainable Segmentation** processing function.

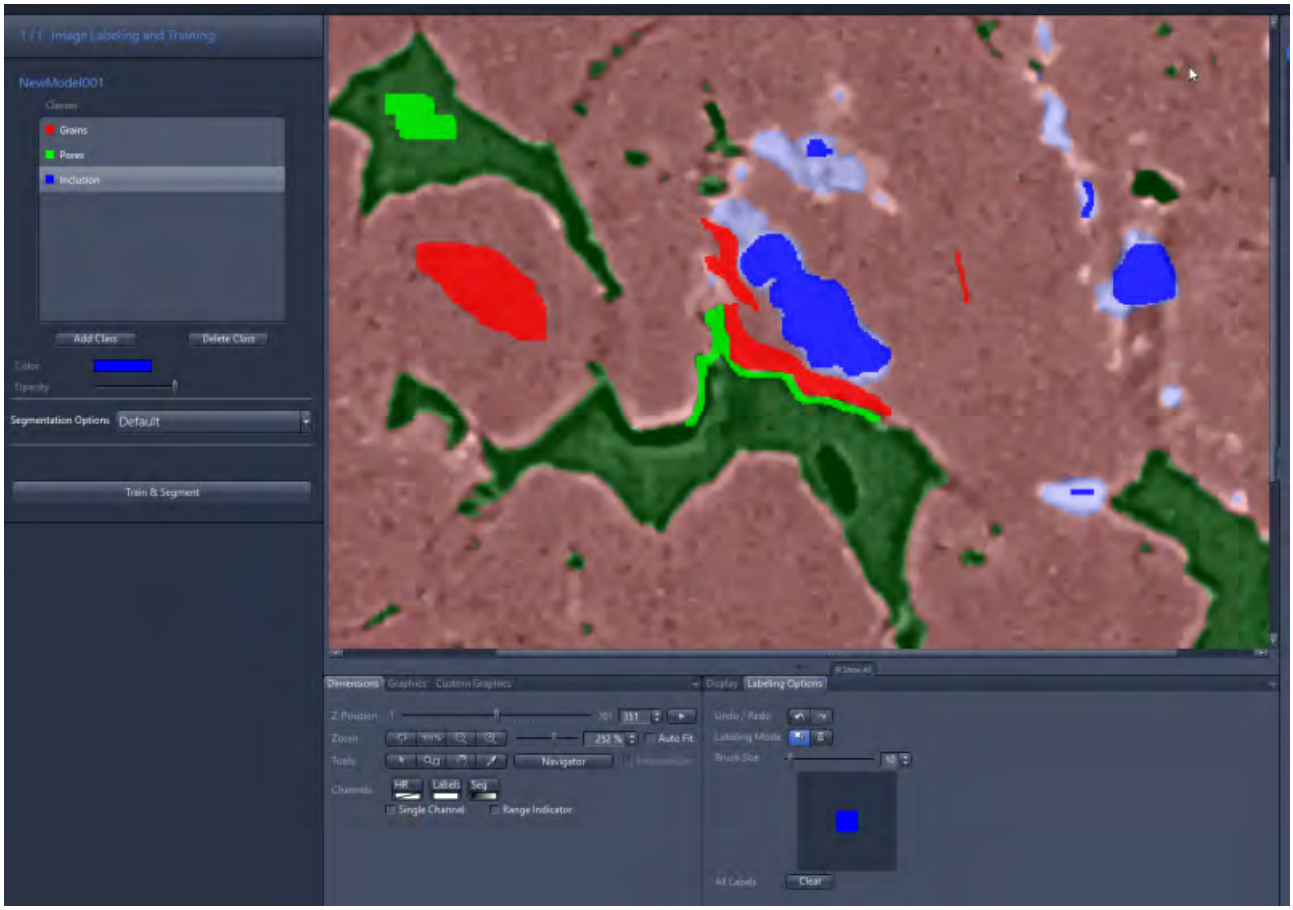
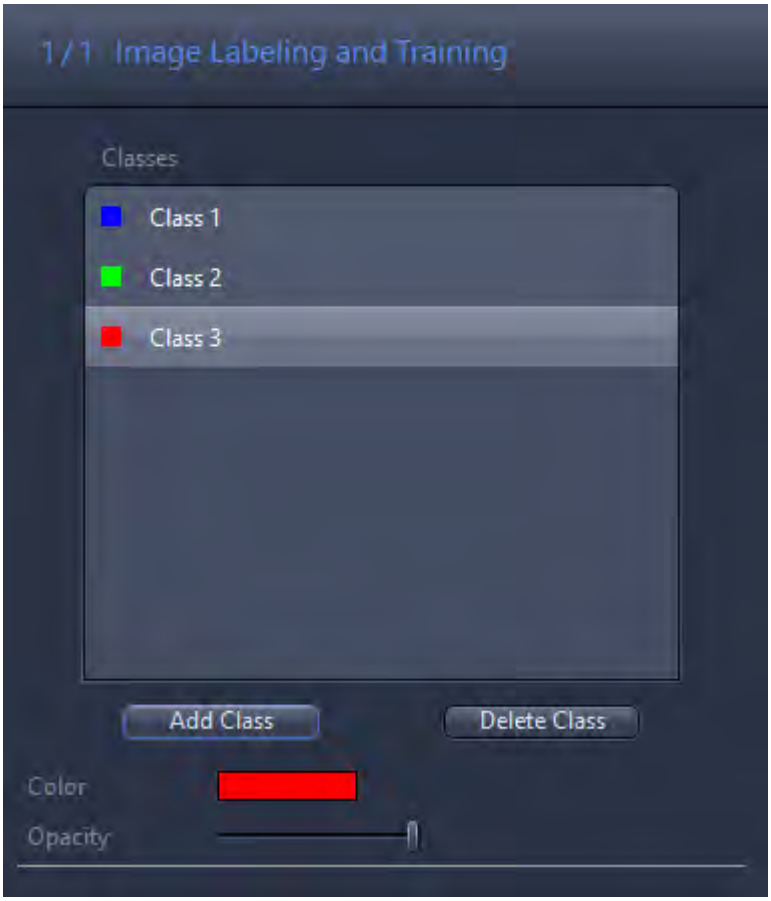


Fig. 21.4: Trained model with refined labeling and pseudo segmentation results

21.2.8 Editing Classes

Procedure **1** To add a new class to the **Classes** list, click on **Add Class**.

The classes have a random color by default.



2 To change the color of a class, select the class and click on the colored rectangle next to **Color**.

You see the **Color Selection** dialog.

3 Select a new color from the list.

4 To change the opacity of the labels within the image, adjust the **Opacity** slider.

5 To rename a class double, click on the class entry and enter a new name. Press *Enter* or click on the **Save** icon to save the new name.

6 To delete the selected class, click on **Delete Class**.

21.2.9 Importing Labels from Binary Mask

With this class specific function you can import binary images from an external source as labels for the current selected class. This is helpful when the "ground truth" for a specific image is available or when you want to use an image obtained by a different modality.

INFO

Be aware that this function overwrites existing labels for this class and that this functionality can possible create a huge number of labels that might lead to memory issues depending on the system configuration and the selected feature extractor.

Prerequisites

- The label image to be imported has exactly the same dimension in XY as the currently selected training image.
- You have opened the **Intellesis Trainable Segmentation** Wizard. For more information, see *Creating a New Model* [▶ 889].

Procedure

1. Right-click a class and select **Import Labels from Binary Mask**.

   The Explorer opens.

2. Navigate to the label image you want to import, and click **Open**.

The imported image is displayed in the **Image** view. The displayed labels have the color of the selected class and fit exactly with the class of the loaded image.

21.2.10 Converting segmentations to labels

With this function you can convert the result of a segmentation directly to labels and thereby increase the number of labels for the next training step.

Prerequisites

- You have opened the **Intellesis Trainable Segmentation** Wizard. For more information, see *Creating a New Model* [▶ 889].
- You have performed a segmentation.

Procedure

1. Right-click a class and select **Segmentation to Labels**.

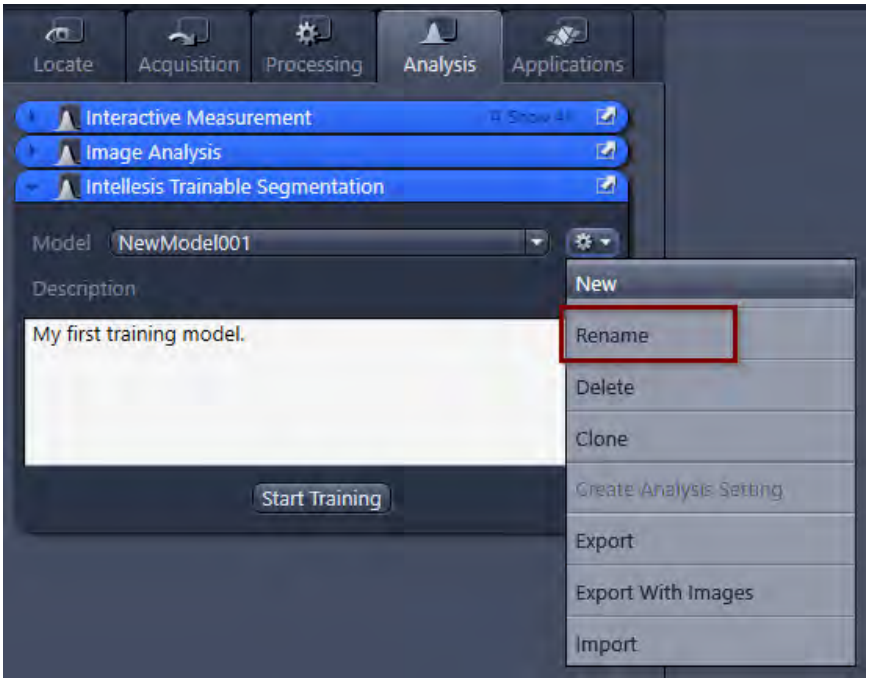
The segmentations are converted to labels.

21.2.11 Renaming a Model

Prerequisites

- You have selected a training model.

Procedure **1** On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, select **Options** | **Rename**.



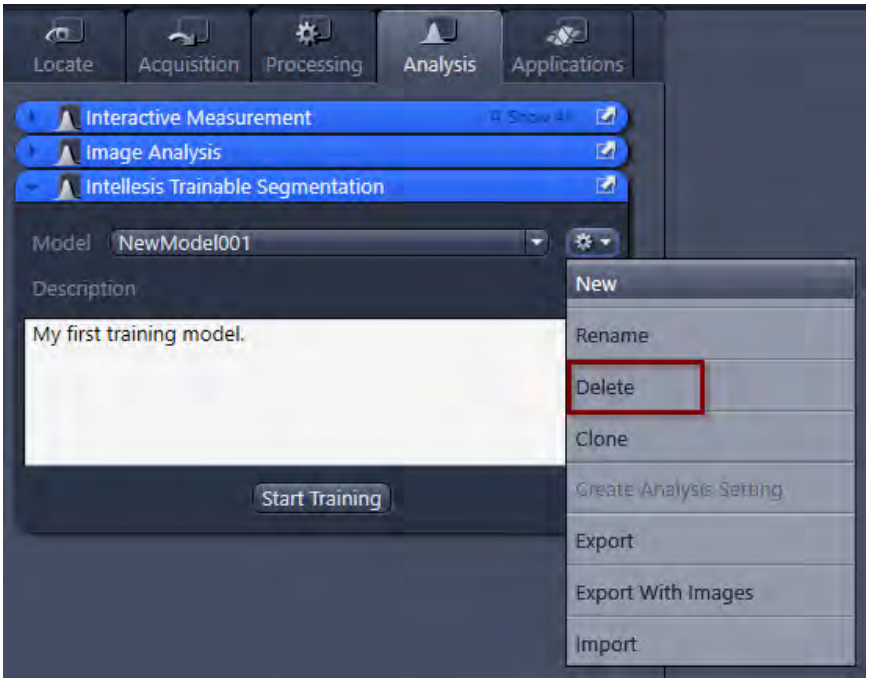
The **Model** field is editable.

2 Enter a new name for the model and save it.

21.2.12 Deleting a Model

Prerequisites ■ You have selected a training model.

Procedure **1** On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, select **Options** | **Delete**.



You have deleted the model. The model you worked with before is selected.

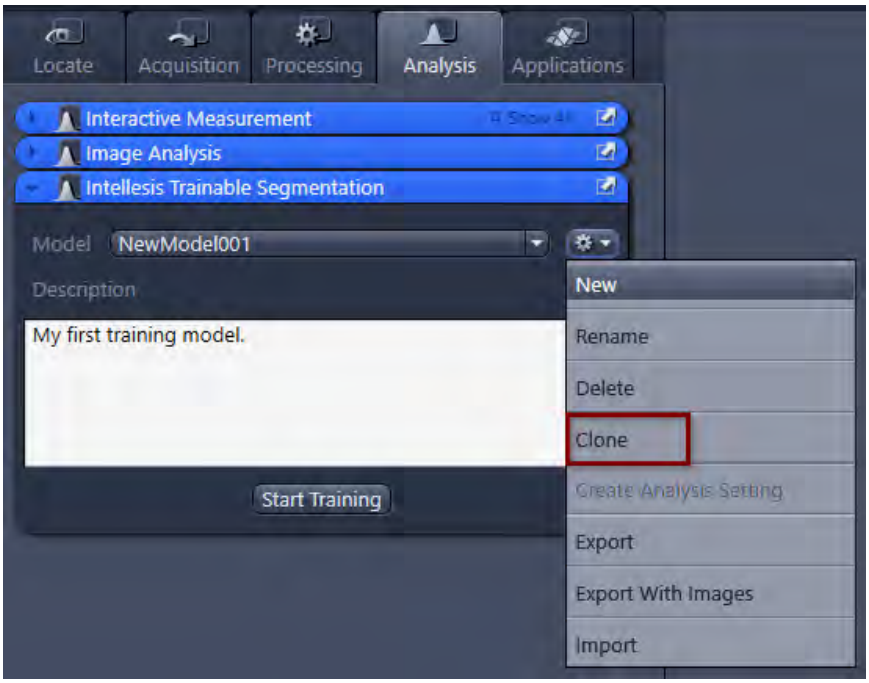
21.2.13 Cloning a Model

Prerequisites

- You have selected a training model.

Procedure

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, select **Options** | **Clone**.



   The name of the currently loaded model disappers and the **Model** field is editable.

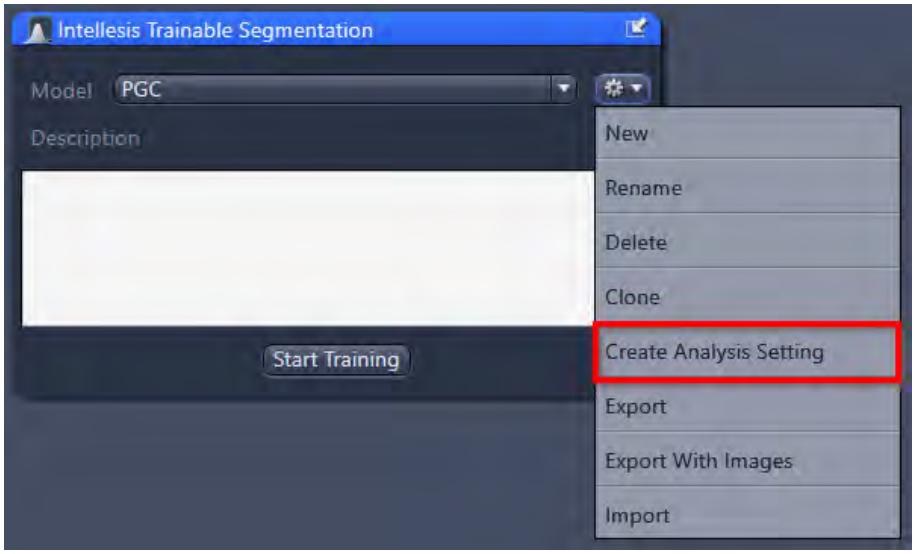
2. Enter a new name for the model and save it.

You have cloned an existing model.

21.2.14 Creating Analysis Setting

Procedure

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, select **Options** | **Create Analysis Setting**.



For more information, see *Using a Trained Model for Image Analysis* [▶ 904].

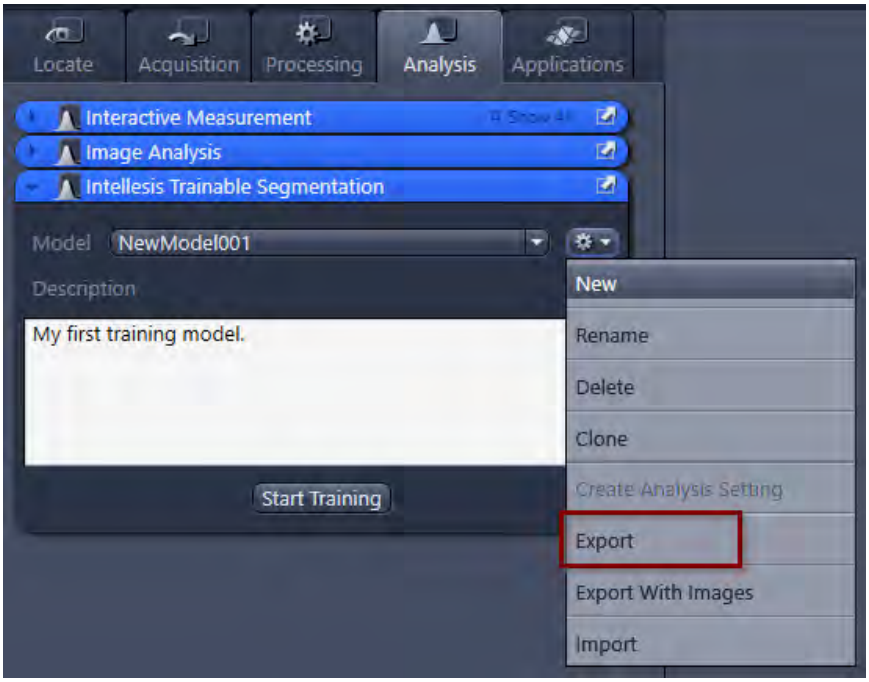
21.2.15 Exporting a Model

Prerequisites

- You have created a model for advanced image segmentation.

Procedure

1. Select the model in the **Trainable Segmentation** tool and click on **Options** | **Export Model**.
   If you want to export the full model containing all images select "**Export Model with Images**".



2. Select the file location and click **Save**.

The model will be exported as ***.zip** file which contains the trained segmenting routine as well as the images which were used for training. In case of the option where the images are nor included, only the model files itself will be exported. Such a model is meant to be used for segmentation purposes or to create an Image Analysis Setting, but not for the Training Process anymore.

21.2.16 Exporting with Images

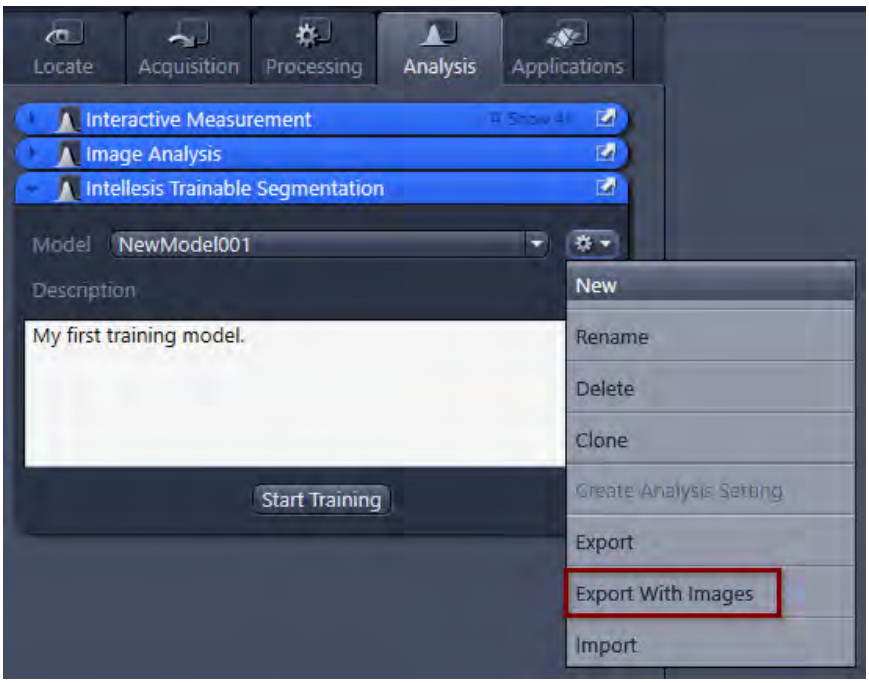
You can export a training model as a zip file, so that you can continue to use them in other programs.

Prerequisites

- You have selected a trained model with images.

Procedure

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, select **Options** | **Export With Images**.



   The Explorer opens.

2. Navigate to the folder where you want to store the training model, and press the **Save** button.

You have exported your model with all images.

21.2.17 Importing Models

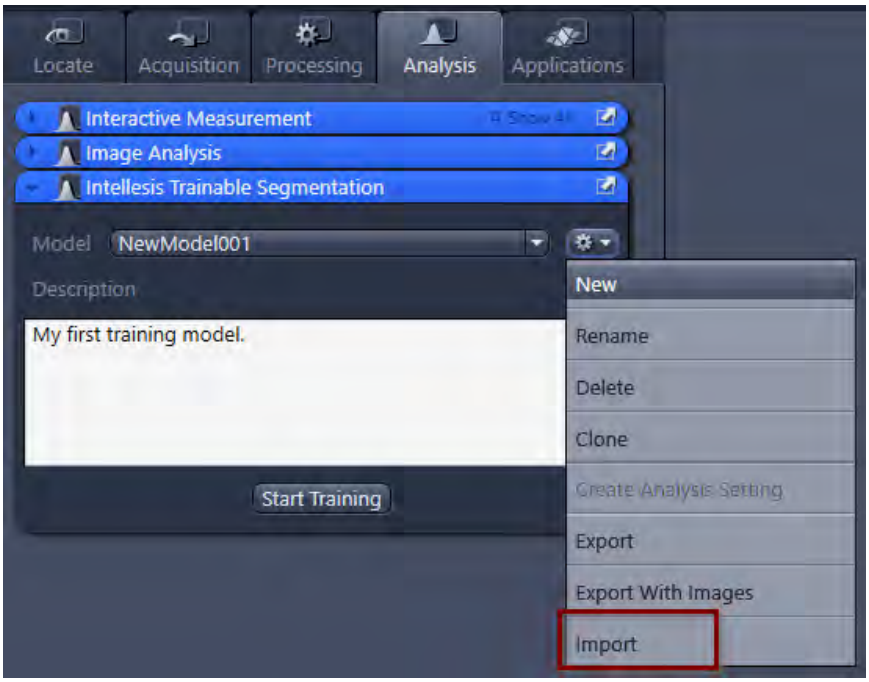
Prerequisites

- You have completed the general preparations.
- You have a trained model available which you want to import (model *.zip file).

Procedure

1. In the **Trainable Segmentation** tool open the settings menu by clicking on the **Options** icon  .

2 Click on **Import** and select the model *.zip file from the file system.



3 Click on **Open**

The model will now be available in the dropdown list.

4 Select the model and click on **Start Image Training** to work with the model, e.g. if you want to train more details.
If you want to use the model for image processing switch to the **Processing** tab. In the **Trainable Segmentation** processing function you can select the imported model and apply it to the desired images/data sets.

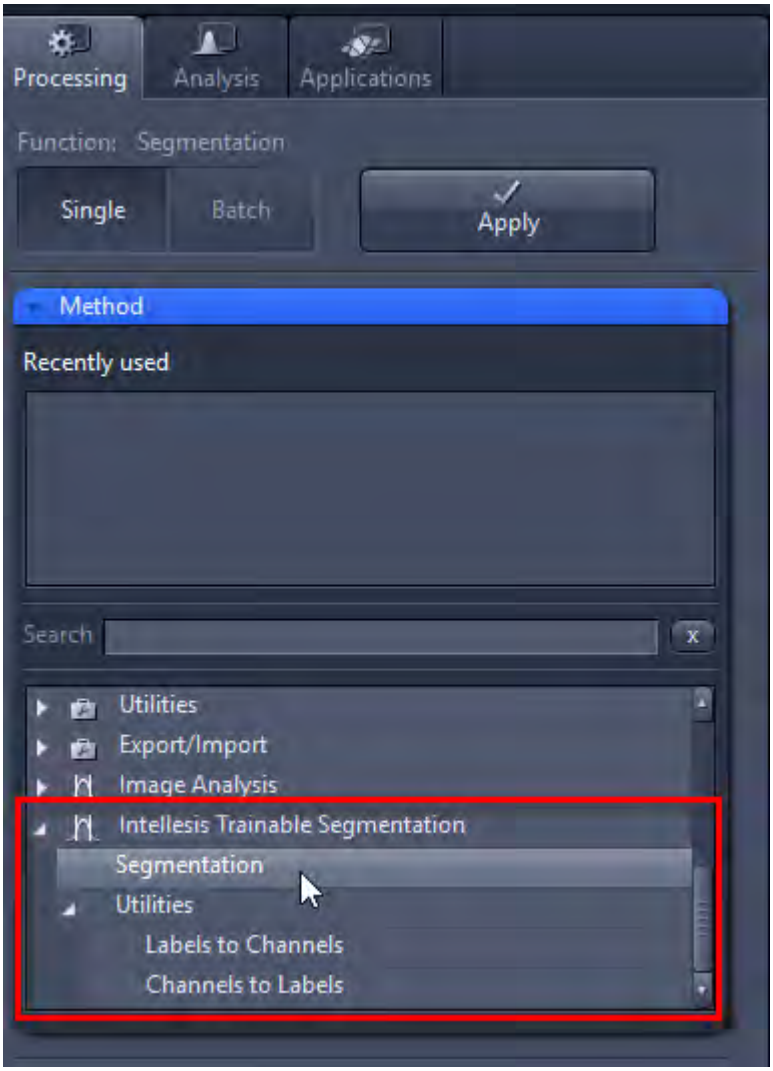
21.2.18 Using a Trained Model for Image Processing

Prerequisites

- You have a trained model available for automatic image segmentation.
- You have opened the image which you want to segment under **Images & Documents**.

Procedure

1 On the **Processing** tab under **Method** open the group **Intellesis Trainable Segmentation** and select the **Segmentation** entry.



2 Open the **Method Parameters** and select the trained model from the **Model** list. Note that the model must be trained on images with similar features otherwise the segmentation will not work properly.

3 Select the desired **Output Format**.
If you select **Multi-Channel**, the result will be a multi-channel image, where every class that was defined in the trained model will be in their own channel. This output format can be easily viewed inside the ZEN **3D view** and can be combined with the original image data easily.
If you select **Labels**, you will get an image with one channel, where the pixels belonging to the different classes will be labeled with different colors and will be represented by distinct pixel values.
Note: Currently such an label image cannot be displayed inside the 3D view directly without any further processing steps.

4 Under **Input Parameters** select the image which you want to segment. Note that it must be already opened in the ZEN software, otherwise it will not be available in the list.

5 Click on **Apply**.

The automatic image segmentation using the trained model is performed.

After a short while you will get two resulting images, depending on the output format:
- the multi-channel or labels image and
- the probability map.

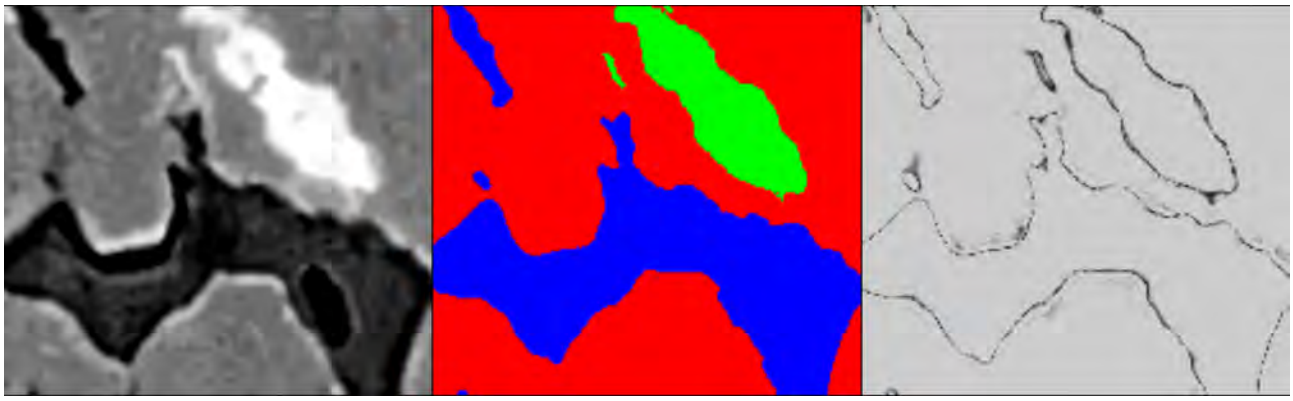


Fig. 21.5: The image shows (from left to right): original image, segmented image, probability map

21.2.19 Intellesis Trainable Segmentation (IP function)

Using the **Segmentation** image processing function you can apply a trained segmentation model to an image/data set.

Parameter		Description
Model		Select the trained model here.
Output Format		When applying the **Segmentation** processing function to an image you will always get two output images. The processed image and the probability map. The following output formats for the processed image are available.
-	Multi-Channel	If selected, the output image will be a multi-channel image. Each class which is defined within the model will result in a separate channel.
-	Labels	If selected, the output image will be a single-channel image.

Utilities

Here you can convert an output image generated with the **Segmentation** IP function according to your needs:

Parameter	Description
Labels to Channels	Converts the resulting image with the output format "**Labels**" to a multi-channel image.
Channels to Labels	Converts the resulting image with the output format "**Multi-Channel**" to an image containing a single channel image with labels. Under **Parameters** you can additionally adjust the Unlabeled Pixel Value and the Output Pixel Type (8 Bit B/W or 16 Bit B/W).

21.2.20 Using a Trained Model for Image Analysis

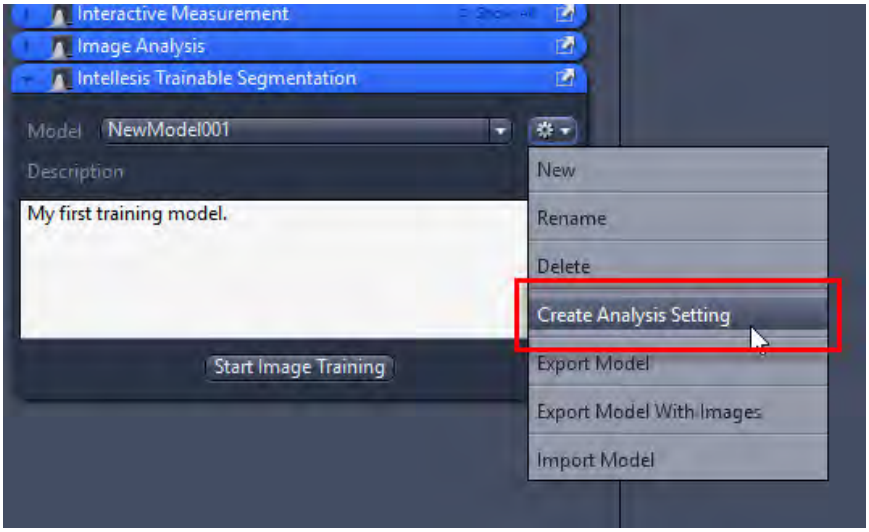
Once having trained a model for segmentation you can use it also in the **Image Analysis** wizard of **ZEN (blue edition)** for further analysis. In order to use the trained model, you must first create a new image analysis (IA) setting (*.**CZIAS** format) first.

Prerequisites

- You are in the **Trainable Segmentation** tool.

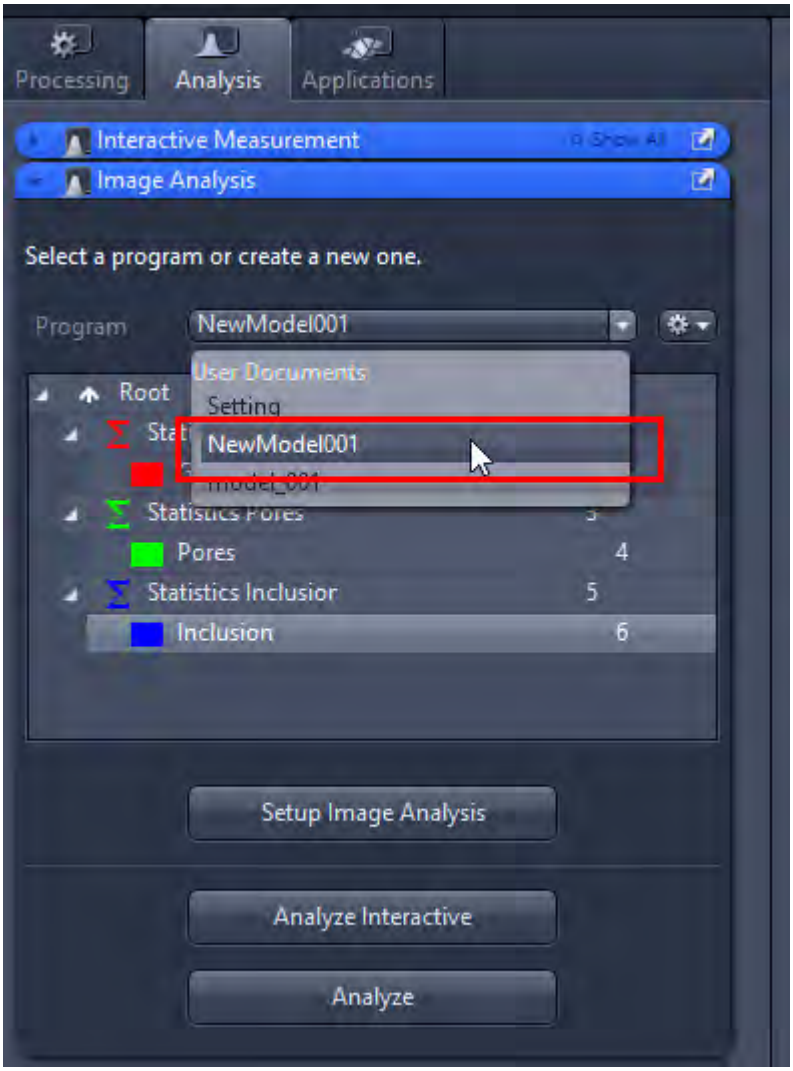
Procedure

1. Select the trained model from which you want to create an analysis setting.
2. Click on the **Options** icon and select **Create Analysis Setting**.



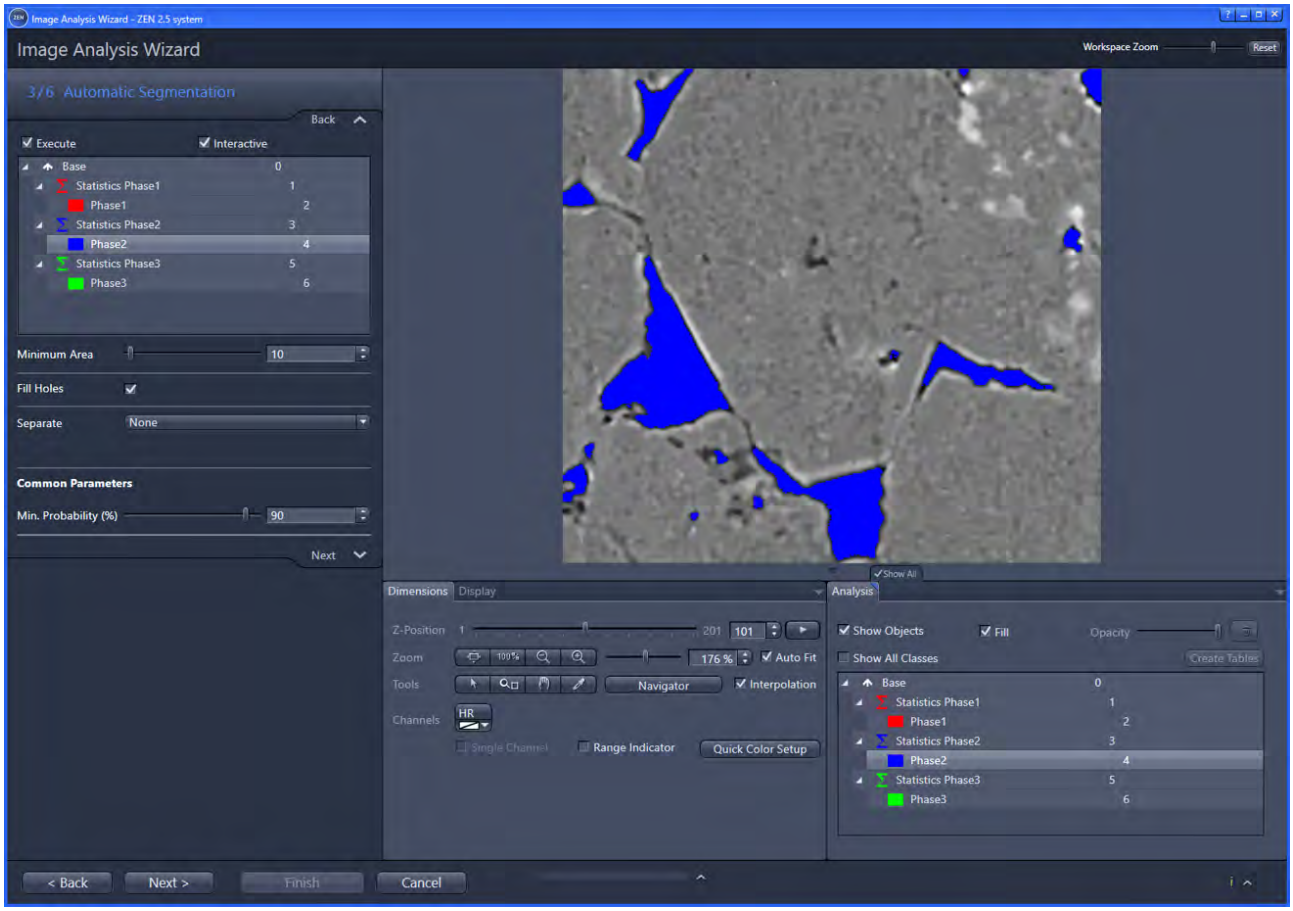
   The dialog for saving the setting opens. The setting will be saved as *.czias file in the ZEN default folder for image analysis settings (usually under **User/Documents/Carl Zeiss/ZEN/Documents/Image Analysis Settings**).
3. Click on **Save**.

4 Now change to the **Image Analysis** tool and select the setting from the dropdown-list. Note that the setting will be only available in the dropdown-list when you have used the default folder for saving. Otherwise the setting must be loaded from the file system (specific location) via the **Import** option.



The model will be loaded with its predefined classes.

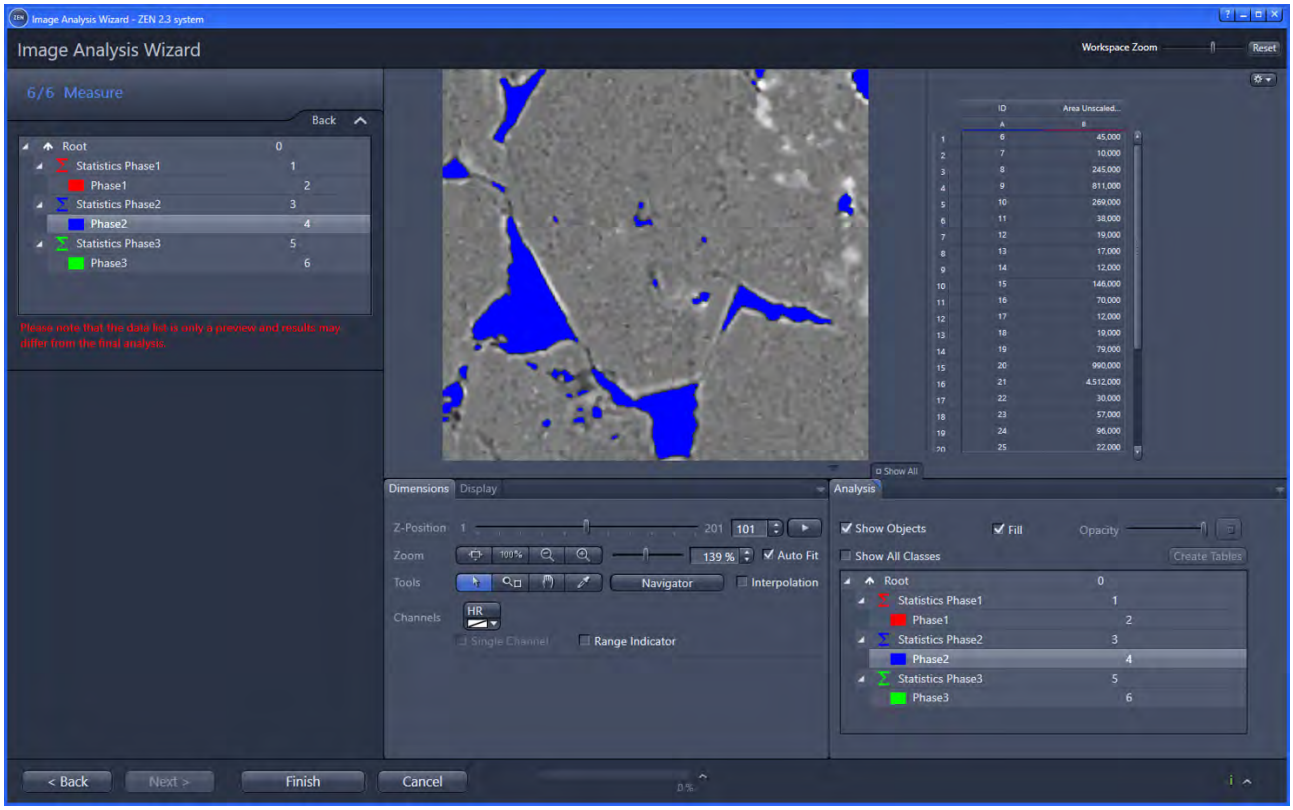
5 You can now continue with setting up an image analysis. For more information about the **Image Analysis Wizard**, see *Image Analysis Wizard* [▶ 742].



Sandstone Dataset segmented using **Intellesis** inside the **Image Analysis Wizard** showing the actual segmentation step. Instead of conventional thresholds, the classifier will be used to identify pixels.

It is possible to allow only for pixel above a certain classification probability (valid for all classes) using the Min. Probability (&) parameters.

The binary functions Fill Holes and Separate will be only applied on the resulting binary masks from the classification and are therefore independent from the actual classification process.



Sandstone Dataset segmented using **Intellesis** inside the **Image Analysis Wizard** showing the measurement results for one particular class (shown in Blue).

You can also use the IP function inside the **ZEN Blue Batch** Tool similar to all the other functions to segment several images using different models at once in one run.

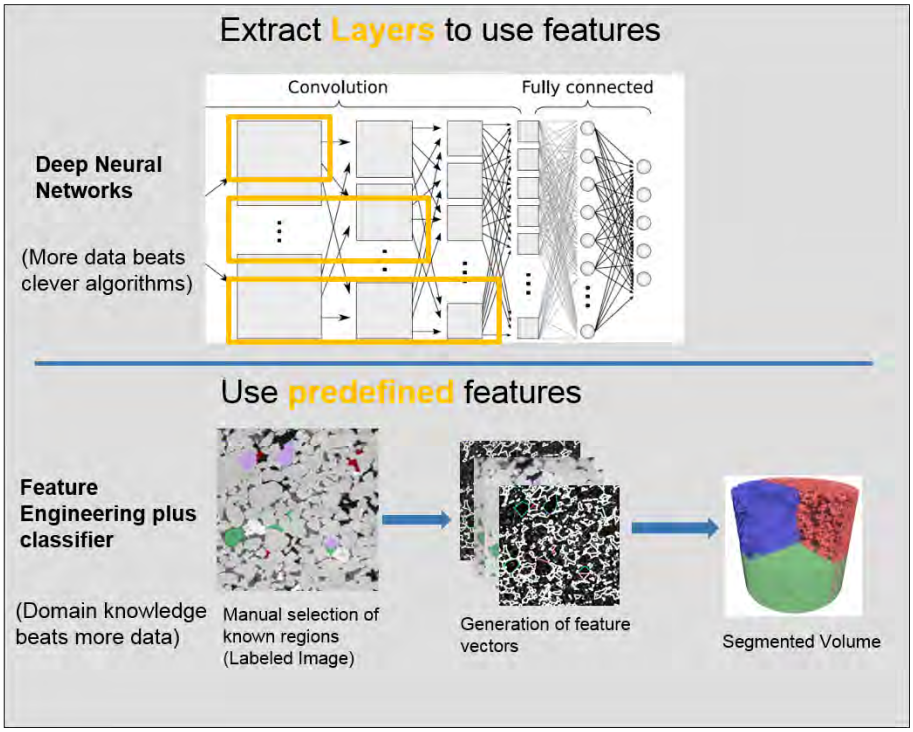
21.2.21 Using Intellesis within OAD

The **Intellesis Trainable Segmentation** module allows to use the **Trainable Segmentation** processing function within the ZEN **Open Application Development (OAD)** environment.

Method / Command	Description
ZenTrainableSegmentation.**Segmentation** (Input, Model, Output Format)	Addresses the trainable segmentation function.
■ Input	ZenImage - Defines the input image to be segmented.
■ Model	ModelName - Defines the name of the model.

Method / Command	Description
■ Output Format: SegmentationFormat.**MultiChannel** SegmentationFormat.**Labels**	SegmentationFormat - Optional argument; Defines the desired output format, e.g. Multi-Channel or Labels

21.2.22 Feature Extractors



21.2.22.1 Intellesis Default Features

- For calculating the features various filters with various filter sizes and parameters are applied to the region around this pixel (2D Kernels).
- Results are concatenated yielding the final feature vector describing the pixel.
- Considering the filter sizes yields in a dimensionality of 33 for the Default Features.
- Used Filters:
  - Gaussian filter (20 different sigma) = 20 feature dimensions
  - Sobel filter (1 sigma) = 1 feature dimension
  - Gabor filter (1 theta, 2 different sigma, 2 different frequencies) = 4 feature dimensions
  - Mean filter (5 different sizes) = 5 feature dimensions

- Hessian filter (1 sigma) = 3 feature dimensions (one for derivative in direction xx, one for derivative in direction xy and one for derivative in direction yy)

21.2.22.2 Intellesis Deep Features

- Entire image as input for pre-trained network
- Take the output from an intermediate layer of that network as feature vector, e.g. output from layer 3 was processed by preceding layers 1 and 2
  - DF Layer 1: Feature dimension = 64
  - DF Layer 2 All: Feature dimension = 128
  - DF Layer 2 Reduced: Feature dimension = 50, reduced by random transformation
  - DF Layer 3 All: Feature dimension = 256
  - DF Layer 3 Reduced: Feature dimension = 70, reduced by random transformation

21.2.23 Remarks and Additional Information

- Segmentation Performance in general depends among other factors on the system performance, the **available and free RAM and GPU memory**.
- **Whenever using ZEN Intellesis Trainable Segmentation it is strongly recommend not to use other memory- or GPU-intensive applications at the same time**.
- Deep Feature Extraction will use the GPU (NVIDIA only) if present on the system. It is recommended to use an GPU with at least 4GB of RAM.
- The installation of the GPU version of **Intellesis** can be selected during the installation process of ZEN itself.
- When installing the GPU libraries it is required to use the latest drivers.
- In case of using an approved ZEISS workstation the latest drivers can be found on the installation DVD.
- When using Deep Feature Extractor on a GPU system, Tensorflow will occupy as much memory of the GPU as possible. When the segmentation is finished this GPU memory released automatically (with the current version).
- Therefore, when starting another GPU-intensive application, for example GPU-DCV, the GPU memory cannot be used by this new process and a CPU fallback will be used or performance issues may occur.
- In such a case, restart ZEN to free all possible GPU memory and then start using GPU-DCV (or similar applications).

22 Applications & Components

22.1 ApoTome.2

22.1.1 Introduction

In the following chapters you will learn how to calibrate the **ApoTome** for a two-channel experiment and acquire a two-channel image. This image will be used as a basis for demonstrating the processing options. After this, a Z-Stack image will be acquired and processed with the help of ApoTome deconvolution.

Phase calibration, if it has not yet been performed, is carried out from the **Locate** tab, while the other steps are all performed from the **Acquisition** tab.

Grid Focus Calibration is an important step. It is best to perform this using the sample that you will want to acquire later, to guarantee identical optical conditions. If your sample is prone to significant bleaching, you can also use the calibration slide provided.

Background information on the ApoTome can be found here:

- *Principle of imaging using fringe projection* [▶ 910]
- *Optimum acquisition conditions* [▶ 914]
- List of recommended objectives

22.1.2 Principle of imaging using fringe projection

The optics of a microscope are optimized for analyzing very thin samples. For a cover-slip-corrected objective, all optical calculations are performed for very thin objects that lie directly beneath the cover slip. All cover-slip-corrected objectives from ZEISS are optimized for this particular usage, and exhibit an optimum point spread function (PSF) for the wavelengths for which the corresponding objective has been specified.

In biological applications, however, the vast majority of specimens used do not satisfy these optimum requirements. Sometimes thicker biological tissue slices are used, e.g. to analyze cells in the tissue using specific fluorescent markers.

In such cases, during microscopic analysis, and particularly during documentation, the set focal plane is hidden by parts of the image that originate from above and below the actual focal plane. As a result the image appears "faded", the contrast is reduced, and the background becomes bright. In extreme cases important structures and image details may be completely hidden.

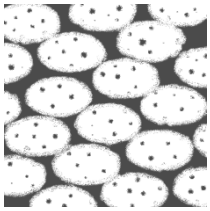


Fig. 22.1: Input image (schematically)

The above representation of a microscopic image of cell nuclei in tissue shows this effect. A number of methods can be used to prevent or reverse this effect, such as confocal laser scanning microscopy or 3D deconvolution.

Principle of fringe projection

With the **ApoTome 2** the principle of fringe projection has been employed. This technology involves inserting a grid structure with grid lines of a defined width into the plane of the luminous field diaphragm of the reflected light beam path. As the plane of the luminous field diaphragm is conjugated to the focal plane, when you look into the eyepiece you can therefore see the grid, overlaid with the actual sample.

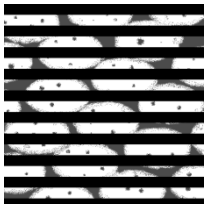
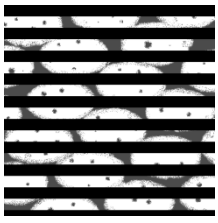


Fig. 22.2: Grid image

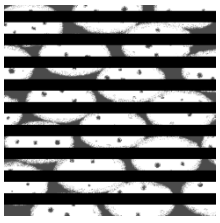
The image of the grid is shown schematically in the figure above. In reality the grid lines are much thinner.

Moving the grid structure

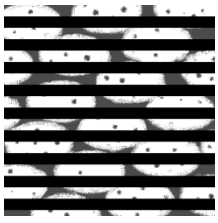
A scanning mechanism in the ApoTome 2 slider is used to move the grid structure in at least three defined steps within the specimen plane. The grid is moved very quickly (in less than 20 ms). A digital image is acquired at each grid position. The next figure shows the movement of the grid schematically:



Position 1



Position 2



Position 3

Optical section

The three raw images are combined online on the PC and displayed as an optical section. This combined resulting image is an optical section through the sample with the following properties:

- The grid structure has been removed from the raw images.
- The parts of the image that are out of focus are no longer visible.
- The sharpness and contrast of the image have been increased.
- The image's resolution in the axial direction has been increased.

Fig. 22.3: Output image (schematically)

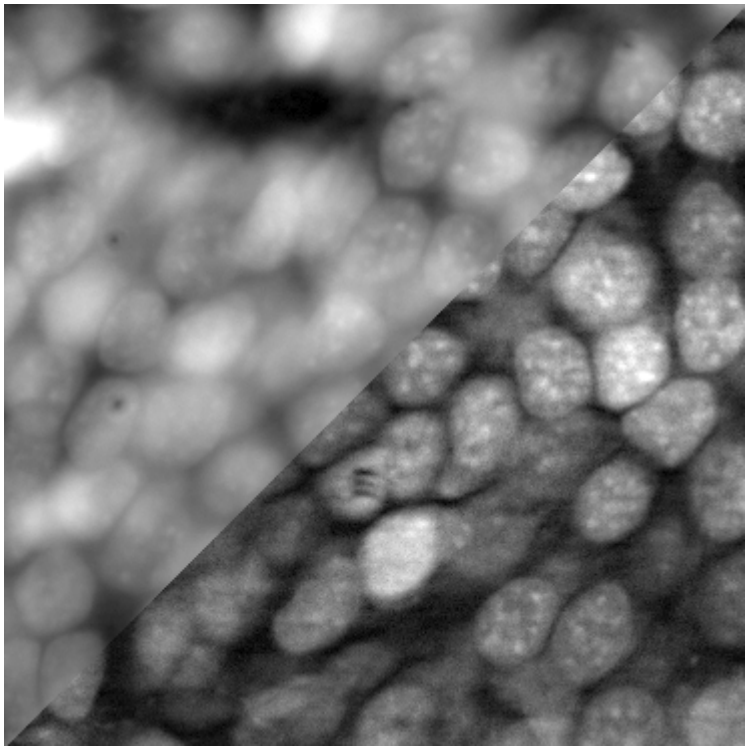


Fig. 22.4: Sample image: cell nuclei

The figure above shows an application image of cell nuclei (tadpole brain section) in black and white. Above left: conventional fluorescence Below right: optical section

Why is the resulting image an optical section?

Schematic image of the grid

One possible way to explain this is to use the image of the grid in the sample:

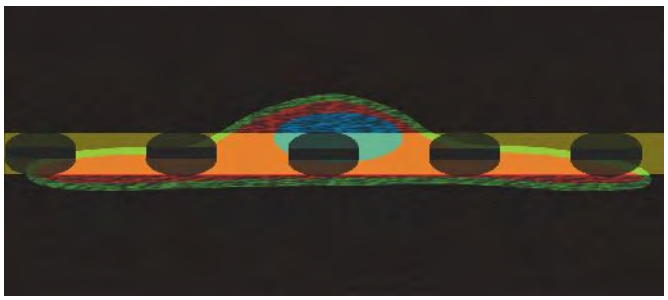


Fig. 22.5: Grid image (schematically)

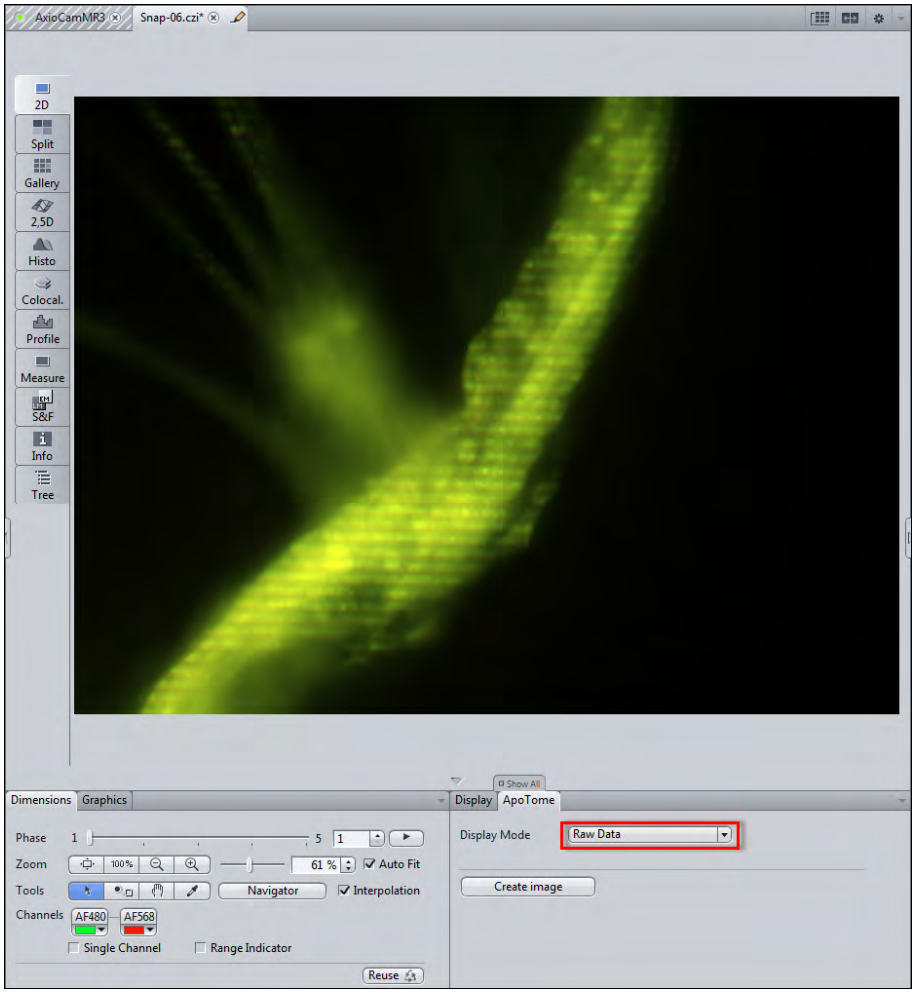
The image of the grid provides the necessary information on the distance of the various specimen structures from the set focal plane (see figure above). Some specimen structures are in focus, while others lie above or below the focal plane, and enter the set focal plane.

The technique of grid projection makes use of the fact that the image of the grid above and below the actual focal plane is blurred, and enters the blurred areas of the specimen. When the grid line is moved, significant brightness differences (= contrast) appear in the focal plane.

Outside the focal plane only minor differences are produced, as the sample and the image of the grid are practically "blurred" together. The brightness differences are detected by the algorithm used to combine the raw images, and are used to remove the parts of the image that are out of focus.

The Raw Data mode

To view the raw data directly switch to the **Raw Data** display mode on the **ApoTome** tab.



You will now see the **Phase** slider on the **Dimensions** tab. Here you can locate the individual grid positions. This view can be useful when looking for errors, e.g. to find out where residual streaks in the processed resulting image originate from.

22.1.3 Optimum acquisition conditions

The following requirements should be met in order to produce optimum ApoTome images:

- **Exposure time of the camera**: this should be set so that approx. 80% of the camera's dynamic range is used. The smaller the dynamic range of the images of the lines, the more noise the combined resulting images will contain.
- **Correct calibration**: Good results can only be achieved if calibration has been performed correctly. Ideally you should use your own sample for calibration. If this does not lead to good results, use the calibration slide provided.
- **Sufficient grid contrast in the sample**: Good section image results can only be achieved if the grid lines in the live image can be clearly identified in all object areas. Under certain circumstances samples with very homogeneous staining throughout may not be suitable for ApoTome images.
- **Avoid vibrations** during acquisition, as any movement of the grid position during acquisition can lead to streak artifacts.
- **Number of phases**: Although 3 grid positions (also called phases) can completely cover the object structures that are in focus and are therefore sufficient for creating optical sections, the results are significantly better when 5 or more phases are acquired. For this reason 5 phases are acquired as standard.
- **Selection of the correct grid frequency**: Under normal circumstances, the automatic grid selection yields the best results. In the case of difficult samples, e.g. if the staining is weak, selecting a different grid manually can lead to better results.
- **Avoid electronic interference**: The ApoTome's scanner unit is equipped with highly precise control. Avoid electrical interference, e.g. leaving cell phones close to the ApoTome, to prevent incorrect positioning of the grid.

22.1.4 List of recommended objectives

The following list gives an overview about the recommended objectives and the compatibility with several microscopes. The links give detailed information about the features of each listed objective.

Overview

- EC Plan-Neofluar (Axio Observer / Axio Zoom.V16) *EC Plan-Neofluar* [▶ 916]
- LCI Plan-Neofluar (Axio Observer / Axio Zoom.V16) *LCI Plan-Neofluar* [▶ 916]
- Plan-Apochromat (Axio Observer / Axio Zoom.V16) *Plan-Apochromat* [▶ 917]
- LD LCIPlan-Apochromat (Axio Observer) *LD LCIPlan-Apochromat* [▶ 917]
- C-Apochromat (Axio Observer) *CApochromat* [▶ 917]
- LD C-Apochromat (Axio Observer) *LD CApochromat* [▶ 918]
- A Plan-Apochromat (Axio Observer) *a Plan-Apochromat* [▶ 918]
- A Plan-Fluar (Axio Observer) *a Plan-Fluar* [▶ 918]

Microscopes

- Objectives for Axio Observer
- Objectives for Axio Zoom.V16

22.1.4.1 EC Plan-Neofluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.3	Air	2.9 / 31.9	1.7 / 18.2	0.9 / 9.9	OK	OK
20x	0.5	Air	2.4 / 9.2	1.4 / 5.3	0.7 / 2.9	OK	OK
40x	0.75	Air	1.6 / 2.8	0.9 / 1.6	0.5 / 0.9	OK	OK
40x	1.3	Oil	2.5 / 2.2	1.4 / 1.2	0.8 / 0.7	OK	OK
63x	0.95	Air	1.0 / 1.1	0.6 / 0.7	0.4 / 0.4	OK	No
63x	1.25	Oil	1.6 / 1.5	0.9 / 0.9	0.5 / 0.5	OK	OK
100x	1.3	Oil	1.0 / 0.9	0.6 / 0.5	0.4 / 0.3	OK	OK

22.1.4.2 LCI Plan-Neofluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
25x	0.8	Oil, water or glycerin	2.9 / 6.6	1.7 / 3.7	0.9 / 2.0	OK	OK
63x	1.3	Water or glycerin	1.5 / 1.3	0.9 / 0.7	0.5 / 0.4	OK	OK

22.1.4.3 Plan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.45	Air	4.2 / 20.4	2.4 / 11.5	1.3 / 6.2	OK	OK
20x	0.8	Air	3.2 / 4.9	1.8 / 2.8	1.0 / 1.5	OK	OK
40x	0.95	Air	1.6 / 1.7	0.9 / 1.0	0.5 / 0.5	OK	OK
40x	1.3	Oil	2.5 / 2.2	1.4 / 1.2	0.8 / 0.7	OK	OK
40x	1.4	Oil	2.4 / 1.8	1.4 / 1.0	0.7 / 0.6	OK	OK
63x	1.4	Oil	1.6 / 1.2	0.9 / 0.7	0.5 / 0.4	OK	OK
100x	1.4	Oil	1.0 / 0.8	0.6 / 0.5	0.4 / 0.3	OK	OK

22.1.4.4 LD LCIPlan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
25x	0.8	Oil, water or glycerin	2.9 / 6.5	1.7 / 3.8	0.9 / 2.0	OK	OK

22.1.4.5 CApochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.45	Water	4.2 / 20.2	2.4 / 11.7	1.3 / 6.1	OK	OK

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
40x	1.2	Water	2.1 / 1.9	1.3 / 1.1	0.7 / 0.6	OK	OK
63x	1.2	Water	1.4 / 1.3	0.8 / 0.7	0.5 / 0.4	OK	OK

22.1.4.6 LD CApochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
40x	1.1	Water	2.1 / 2.3	1.3 / 1.4	0.7 / 0.7	OK	OK

22.1.4.7 a Plan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
63x	1.46	Oil	1.5 / 1.0	0.9 / 0..6	0.5 / 0.3	OK	OK
100x	1.46	Oil	1.0 / 0.7	0.6 / 0.4	0.3 / 0.2	OK	No

22.1.4.8 a Plan-Fluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
100x	1.45	Oil	1.0 / 0.7	0.6 / 0.4	0.3 / 0.2	No	No

22.1.5 Preparation: Phase calibration

Before you can use the **ApoTome** for your experiments, the optimum angle of deflection of the scanner unit must be set on the **ApoTome 2**. This fine adjustment of the scanner unit only has to be performed once after the system has been set up. The mirror slide and special reflected light reflector cube, both of which are supplied with the ApoTome 2, are used for this purpose.

Calibration only needs to be performed for one grid and one objective. It is advisable to calibrate the grid for the low magnification range (grid marked with an "L" for "Low magnification") using a 20x objective.

The positioning of the camera is also optimized in the dialog for phase calibration. To achieve optimum performance, the camera horizontal should be aligned parallel to the ApoTome 2 grid lines with as much precision as possible.

The calibration process is supported by a wizard. Start the function by selecting the ApoTome Phase Wizard function from the Acquisition menu.

The wizard guides you through the calibration process in 5 steps. Follow the instructions in the text field of the wizard.

i INFO

For phase calibration you will need the mirror slide provided and the calibration filter (424930-9902-000). The filter is designed for use with white light sources such as the **HXP120C** and cannot be used with the LED light source **Colibri**. If your ApoTome system was ordered and supplied exclusively with Colibri, a suitable calibration filter (424930-9000-000) has already been provided. If Colibri has been retrofitted and the calibration filter is not available, it is not possible to perform phase calibration. In this case please contact your ZEISS sales representative.

22.1.6 Step 1: Define channels using Smart Setup

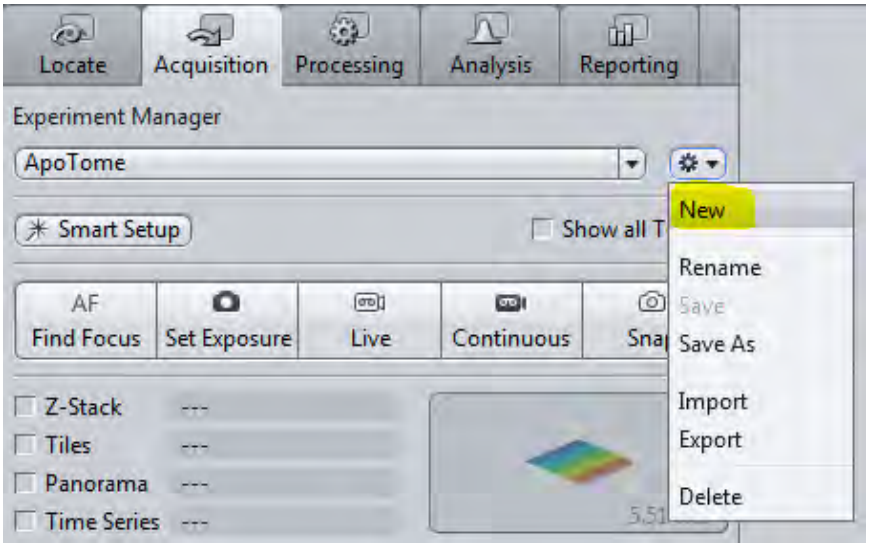
Aim

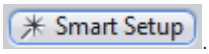
In this step we will set up a two-channel experiment. To do this, we will use the Smart Setup function. The ApoTome is in the first click-stop position, i.e. in the empty position without a grid.

Procedure

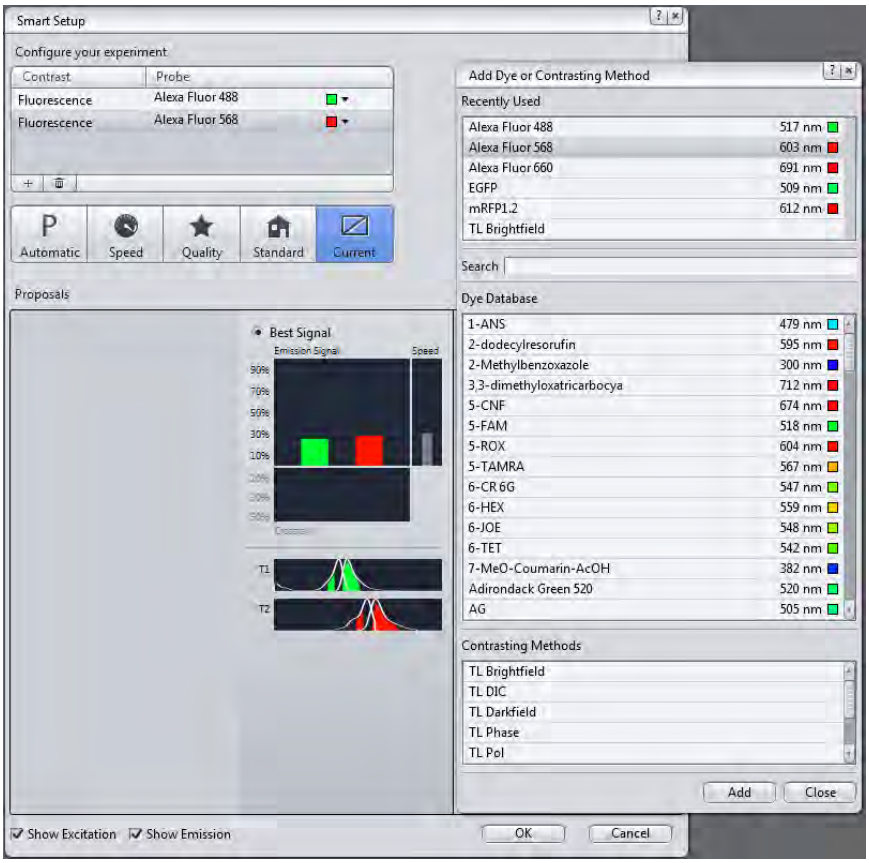
1. Place your sample onto the microscope stage, localize it with the help of the functions on the **Locate** tab and bring it into focus.

2 Now go to the **Acquisition** tab and create a new experiment in the **Experiment Manager**:



3 Open the **Smart Setup**  .

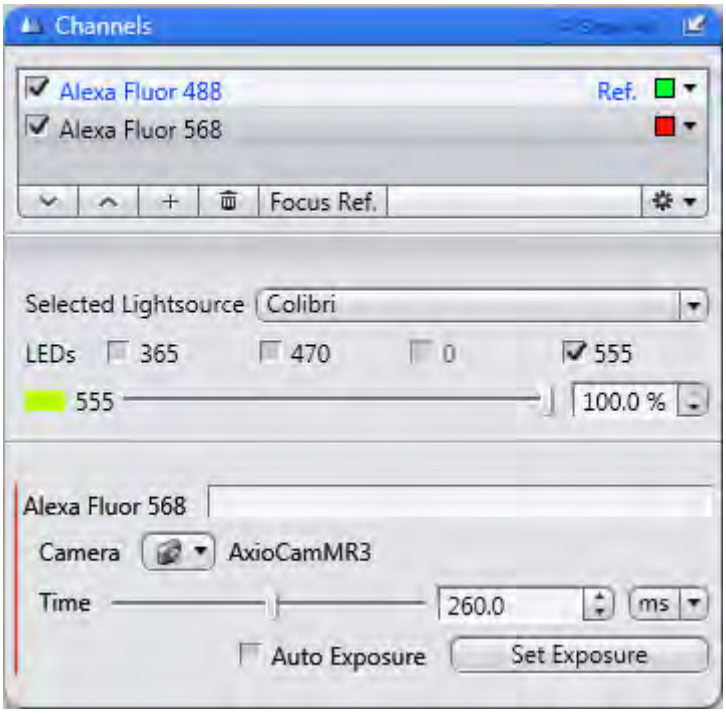
4 Select the appropriate dyes (in our case Alexa 488 and Alexa 568):



5 Close the dialog by clicking on **OK**.

You will now see two channels in the **Channels** tool.

6



You have successfully set up the channels. In the live mode you can now check the focus and exposure time for the two channels.

22.1.7 Step 2: Grid focus calibration

Aim

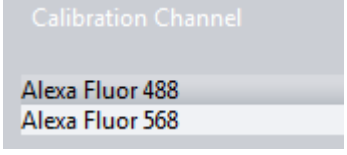
In this step you will calibrate the focus position of the ApoTome grid for the selected channels. This step is essential, as without a valid calibration it is not possible to perform an ApoTome experiment. Provided that no changes are made to the device settings that are important for calibration (objective, filter and illumination source, camera) the calibration remains valid for future experiments. We nevertheless recommend that you repeat the calibration from time to time, especially if the sample type you are analyzing changes. Calibration takes place in a wizard, which guides you through 3 simple steps.

Procedure

1 Move the ApoTome to the second click-stop position so the grid is positioned in the beam path.

2 From the **Acquisition** menu item open the **ApoTome Focus Calibration Wizard ...** entry.

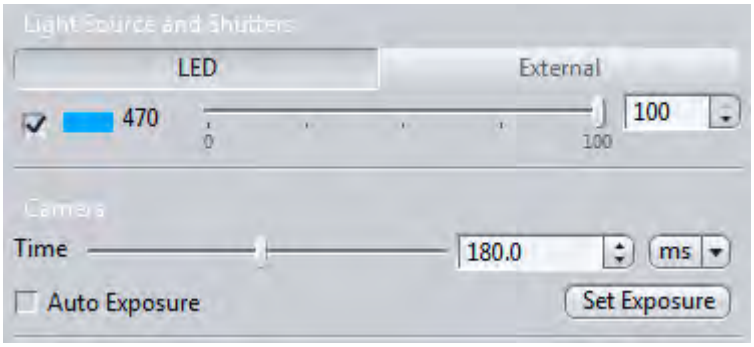
3 In the first step select the channel for which you want to perform calibration.



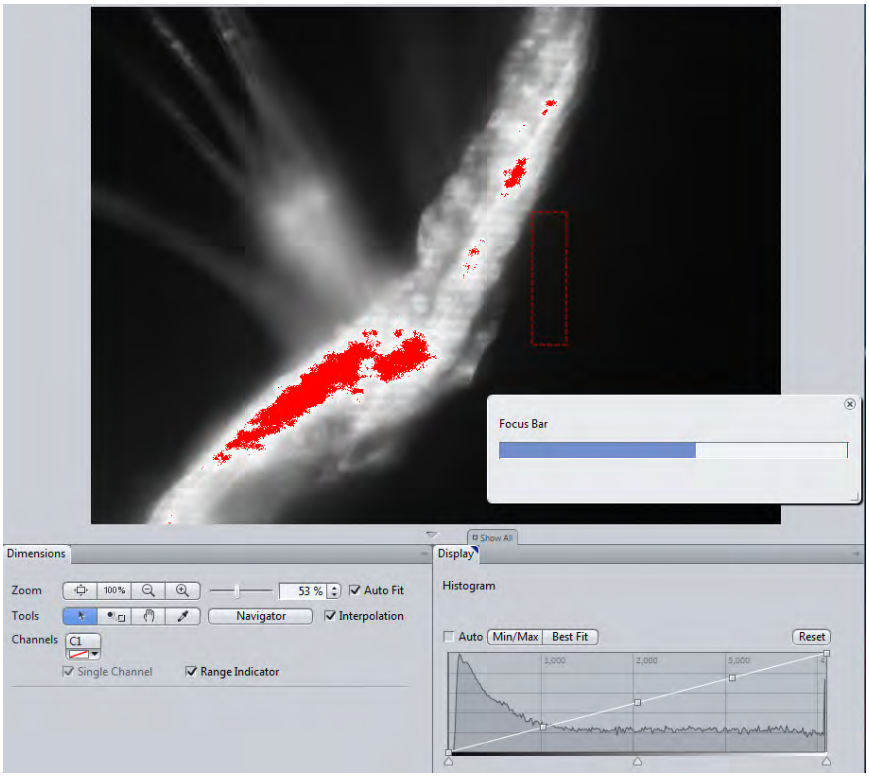
4 Click on **Next** to proceed.

You will now see the **Live Image**. Bring the sample into focus. The ApoTome grid has been moved to an end position to make it easier for you to focus on the sample. If you can nevertheless see the grid lines, something that can never be avoided entirely if you are using a 100x objective for example, click on one of the two buttons **Grid To Start** or **Grid To End** and use the position at which the fewest grid lines can be seen.

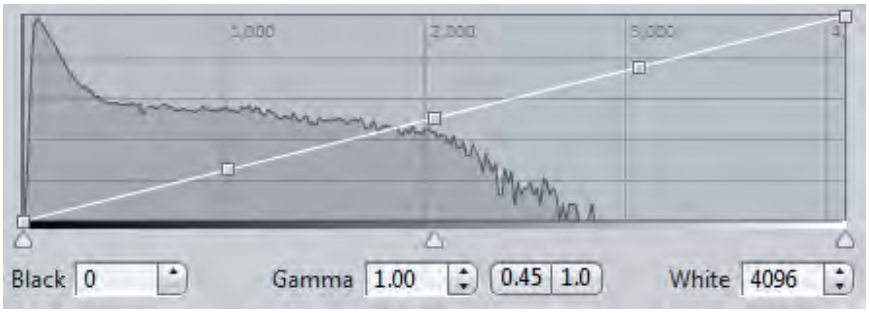
5 On the left you have the option of adjusting the exposure time and, if necessary, the illumination intensity (with corresponding light sources only).



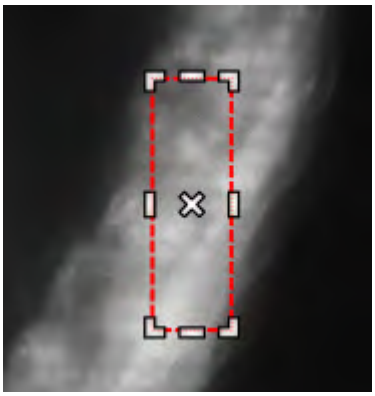
6 To adjust the exposure time correctly, select the saturation display on the **Dimensions** tab. Regions overlaid in red indicate that pixels are saturated.



7 Reduce the exposure time accordingly. The ideal situation is where approx. 70% of the histogram is filled for the brightest region of the sample.



8 Position the rectangle in the live image and adjust its size in such a way that it covers fairly homogeneous fluorescent structures and does not lie over the background. The grid focus is only determined within this rectangle.

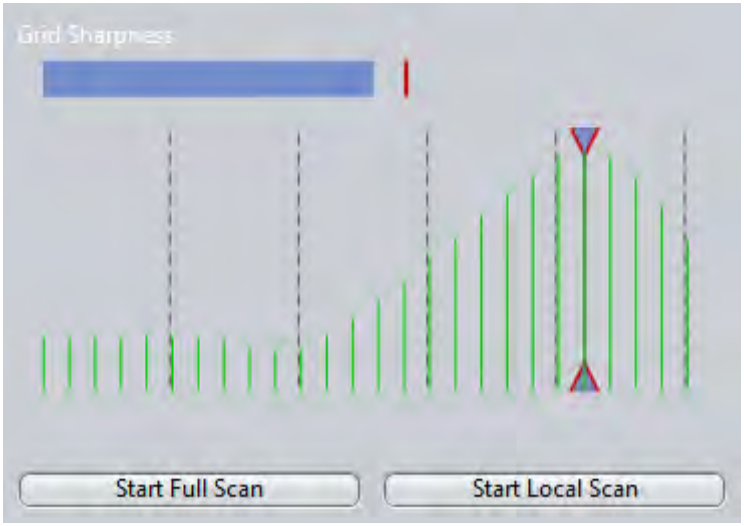


9 Click on **Next** to proceed to the final step.

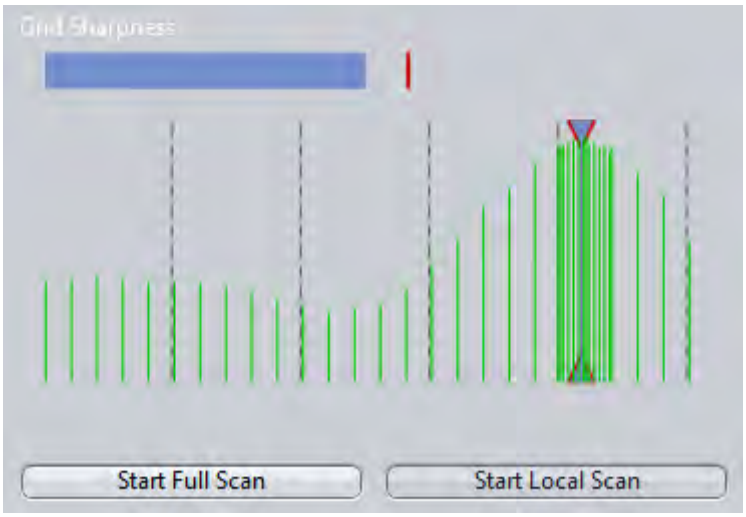
10 Now click on the **Start Full Scan** button to start the grid focus search.

The grid contrast is displayed in the histogram.

11



More precise grid focusing can be achieved if you click on the **Start Local Scan** button.



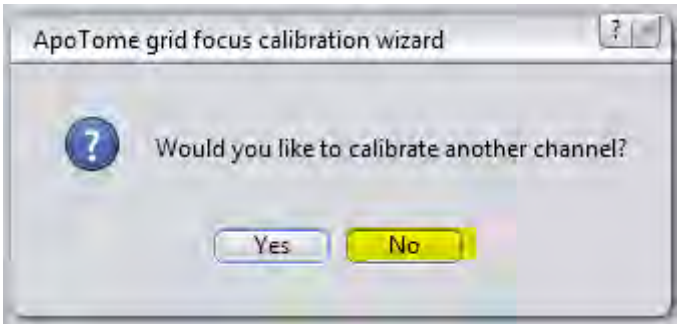
However, this is only recommended for samples that are not particularly prone to bleaching. For samples prone to significant bleaching, the results of the **Local Scan** would measure considerably lower intensities and distort the result.

12 Click on the **Finish** button.

13 To perform calibration for another channel, answer **Yes** to the question "Do you want to calibrate another channel?" in the dialog that is now displayed.

The wizard then begins again from Step 1.

14 As soon as you have calibrated all channels, exit the wizard by clicking on **No**.



You have successfully performed grid focus calibration. Now continue with the next step.

22.1.8 Step 3: Perform ApoTome experiment

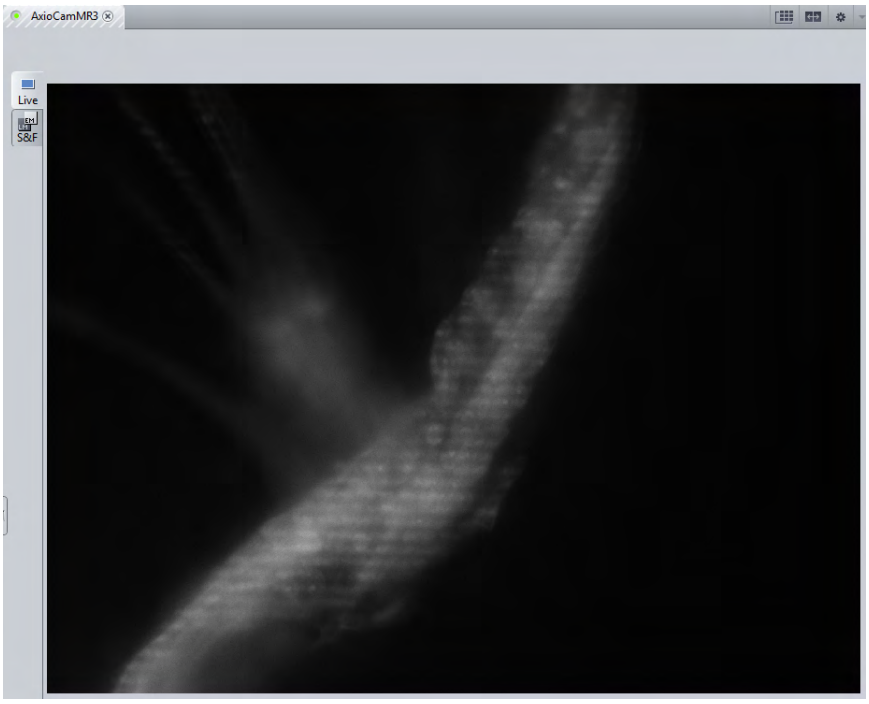
Aim

In this step you will perform acquisition for a two-channel experiment. You will use the same channels that were set up in Step 1. The objective must also be the same one used to calibrate the grid focus.

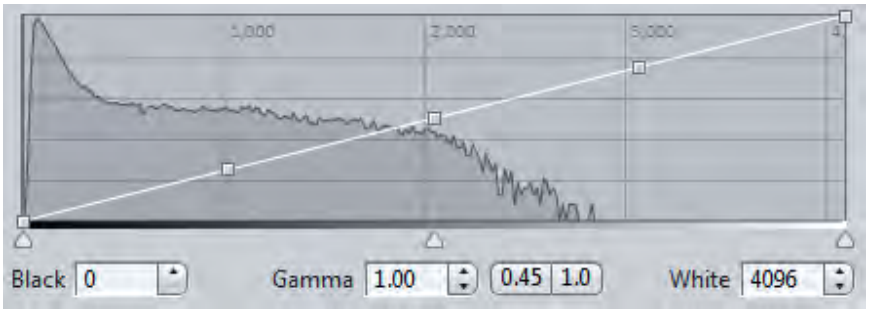
Procedure

1 Insert the desired sample into the slide holder.

2 Open the **Live** Image and focus on the specimen.

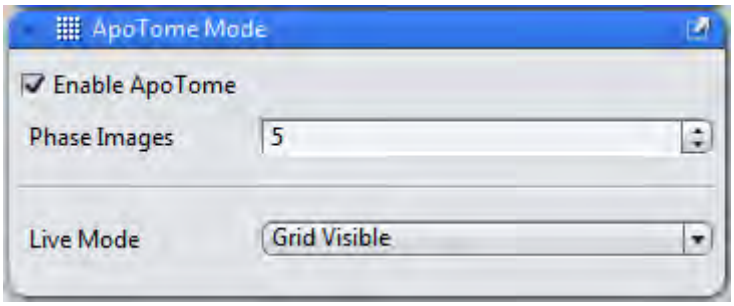


3 Adjust the exposure time for the two channels in such a way that approx. 70% of the **Histogram** is used.



4 Close the Live Image to protect the sample.

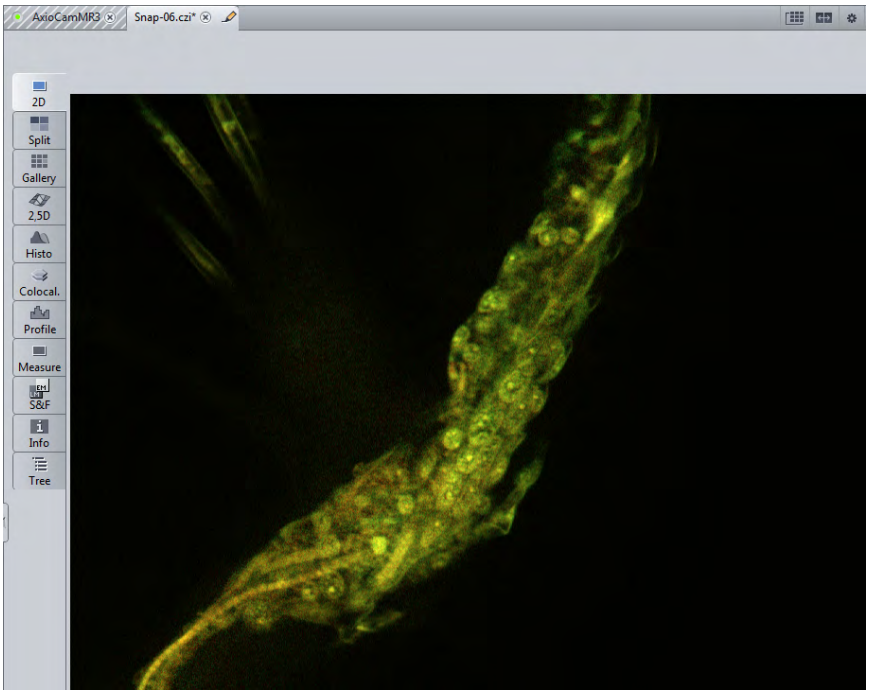
5 Open the **ApoTome Mode** tool on the **Acquisition** tab and enable the **ApoTome**.



6 Start acquisition by clicking on the **Snap** button.

The result is a two-channel image of your specimen.

7



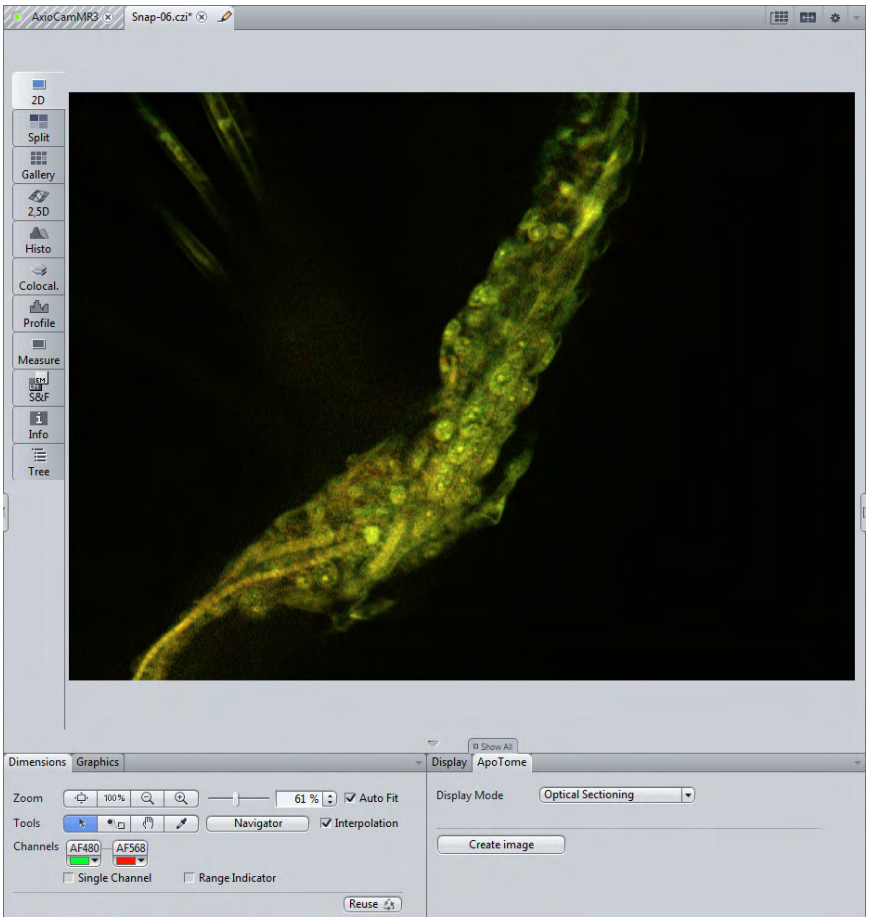
You have successfully performed an ApoTome experiment.

22.1.9 Step 4: Process the resulting image

Aim

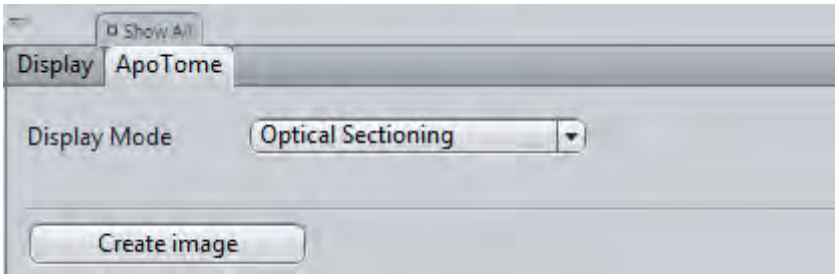
ApoTome images that are acquired from the **Acquisition** tab always take the form of raw data. In this step, with the help of the image you acquired in Step 3, we will look at the various display options available for ApoTome raw images. We will also create a processed resulting image, which you can process further as required.

Prerequisites ■ For this step you need to be in the *2D view* [▶ 778].



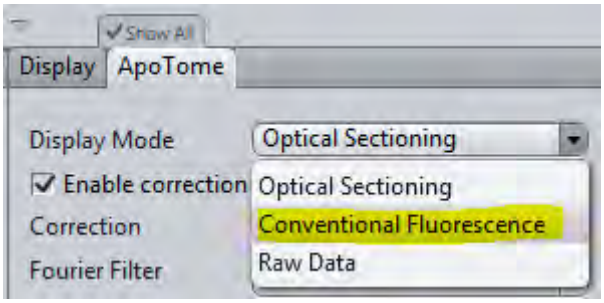
Procedure **1** In the Center Screen Area go to the **ApoTome** tab. This view option is only displayed for ApoTome raw images.

If the Show all mode is deactivated you will see two view options: the **Display Mode** settings & the **Create Image** button.

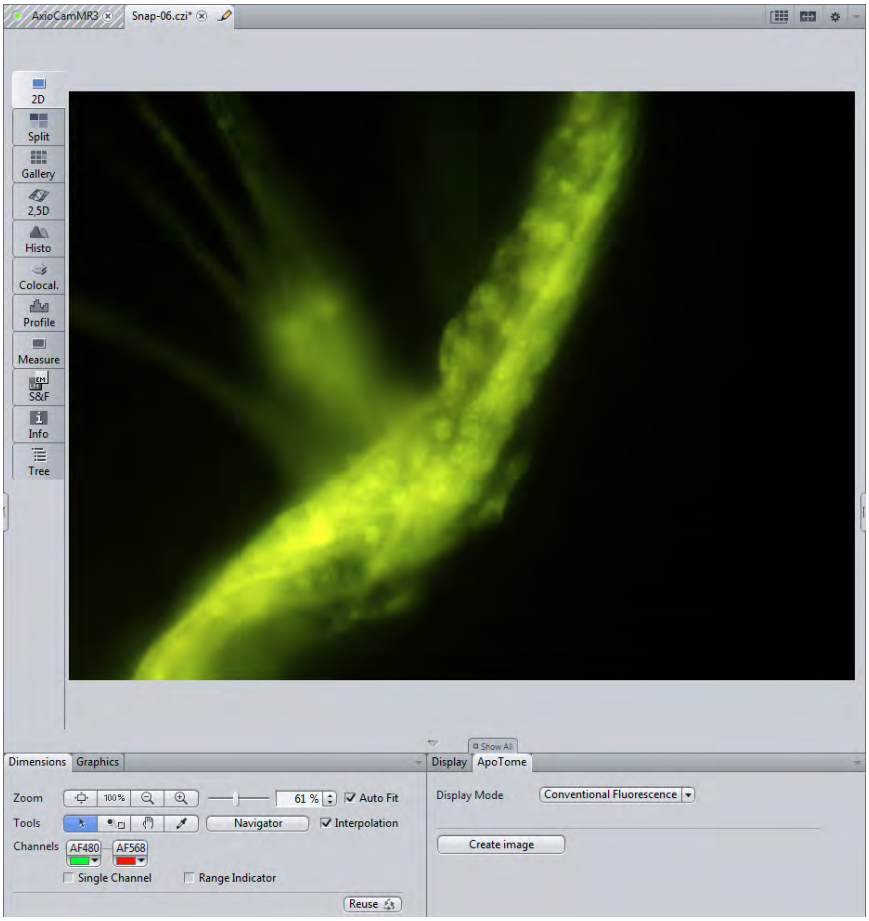


The default selection for Display Mode is the **Optical Section** view.

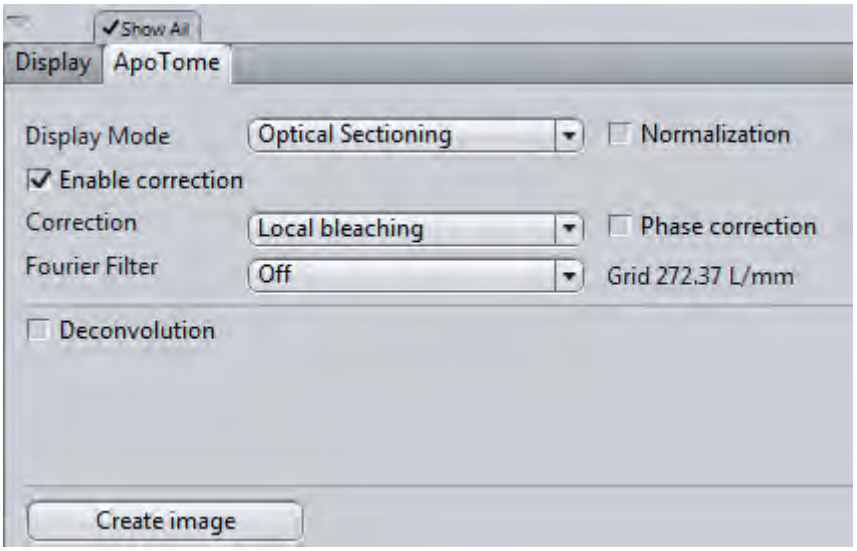
2 Select the **Conventional Fluorescence** option from the **Display Mode** dropdown list.



The image is now no longer displayed as an optical section, but as a conventional fluorescence image ("widefield"):



3 Activate the **Show All** mode to see additional settings for the calculation of the optical section image.



INFO

The main cause of streak artifacts is the bleaching of the fluorescence signal during acquisition of the grid images (= phase images). Depending on the degree of bleaching, fine streak artifacts can appear in the resulting image when the grid images are combined. These can be easily corrected, however.

4 To see the difference between a corrected and uncorrected image, deactivate the **Enable Correction** option. Detailed information on the individual options can be found in the online help.

5 If you have not yet done so, enable the correction using the **Local Bleaching** option.

6 Create a new, processed resulting image by clicking on the **Create Image** button.

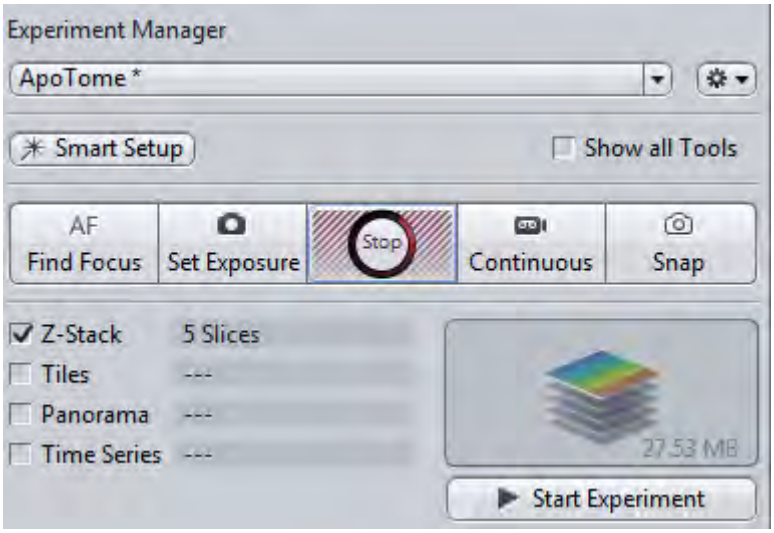
You have successfully processed the ApoTome image and created a resulting image for further processing.

22.1.10 Step 5: Perform Z-stack acquisition

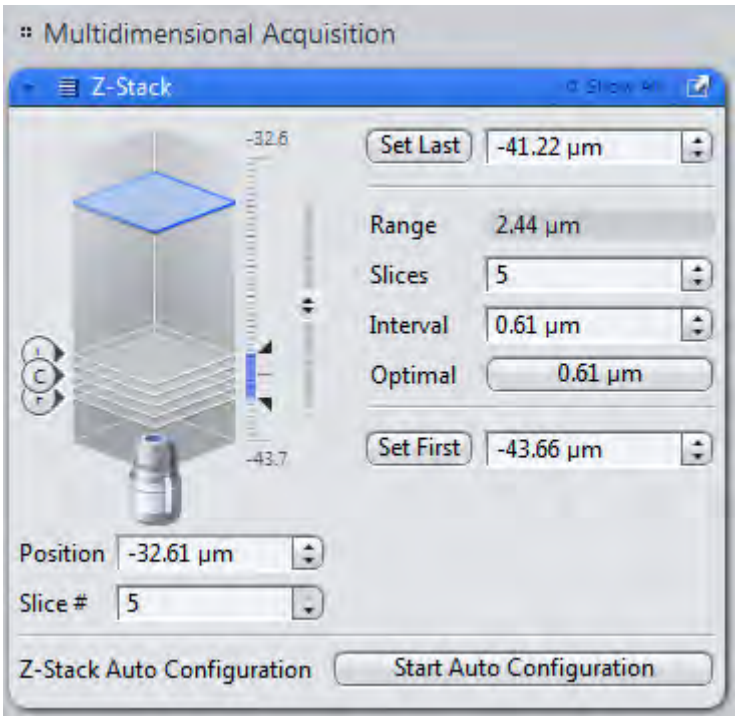
Aim

In this step you will acquire a Z-stack image with the same channel settings as in Step 3.

Procedure **1** On the **Acquisition** tab activate the **Z-Stack** acquisition dimension in the Experiment Manager.

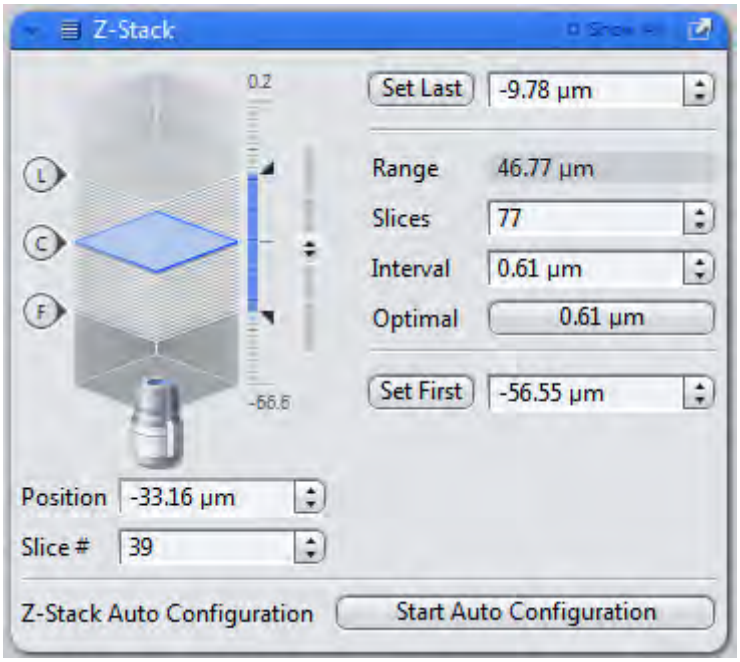


2 Open the Z-Stack tool.



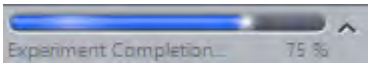
3 Start the **Live Mode** and define the dimensions of the Z-stack for your specimen. To capture the entire object three-dimensionally, you should set the upper and lower limit in such a way that object structures can no longer be

seen in focus. Set the interval between the individual Z-planes using the **Optimal** button:



4 Start acquisition by clicking on the **Start Experiment** button.

You can follow the progress of the experiment in the progress bar on the status bar:



You have successfully acquired a Z-stack image. Save the resulting image under a meaningful name via the **File** menu | **Save** (**Ctrl+S**).

22.1.11 Step 6: Perform ApoTome deconvolution

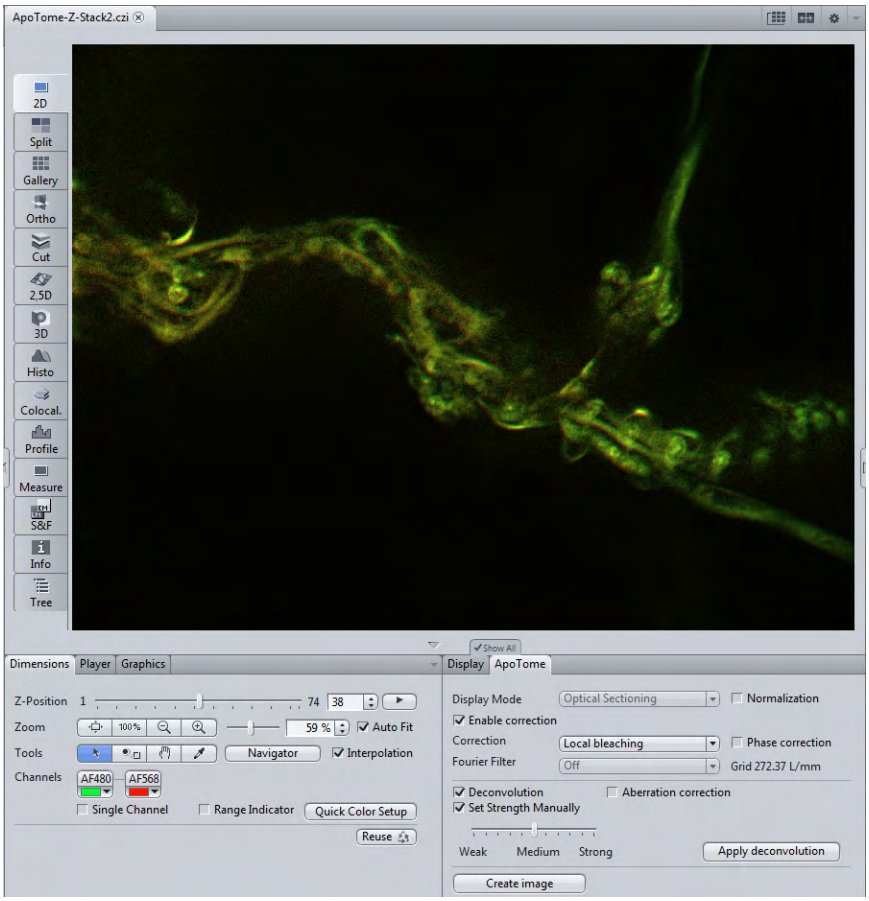
Aim

In this step ApoTome deconvolution will be performed for the Z-stack acquired in Step 5. This enables you to significantly enhance the image, beyond what is possible using the normal **ApoTome** processing functions.

Prerequisites

- The Z-stack image must be in the foreground and in the 2D view. Go to the **ApoTome** tab (view option). Make sure that the **Show All** mode has been activated.

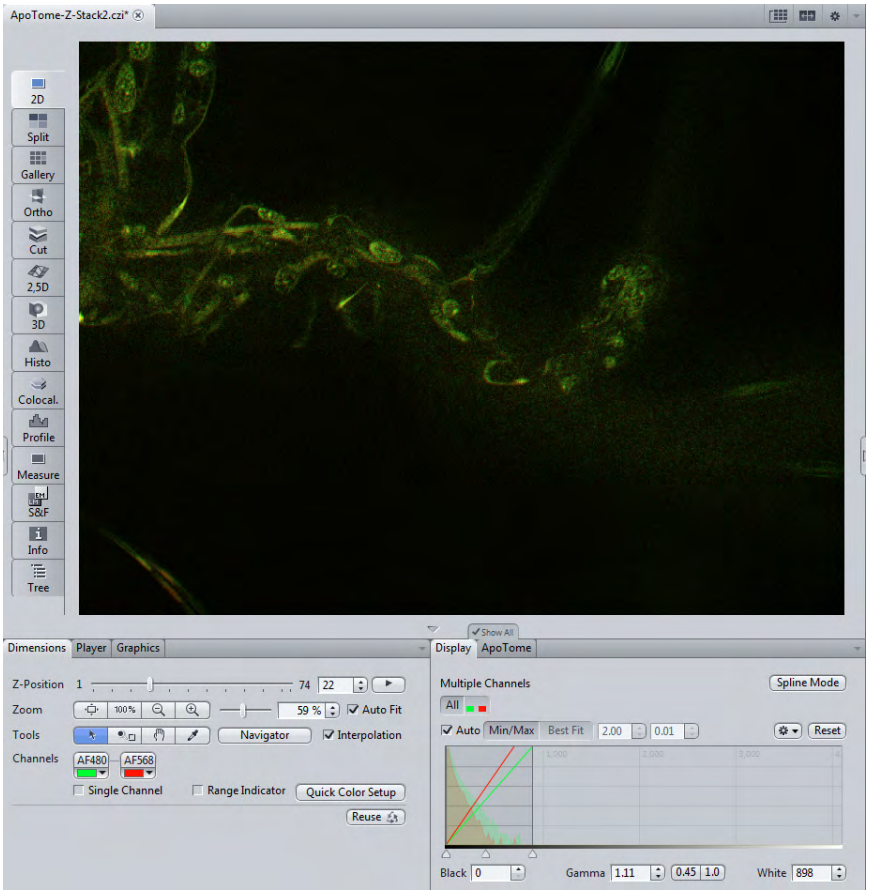
Procedure **1** Activate the **Deconvolution** checkbox.



Make sure that the **Set Strength Manually** option is also activated. The **Strength** slider is set to **Medium** by default. Retain this setting for the time being.

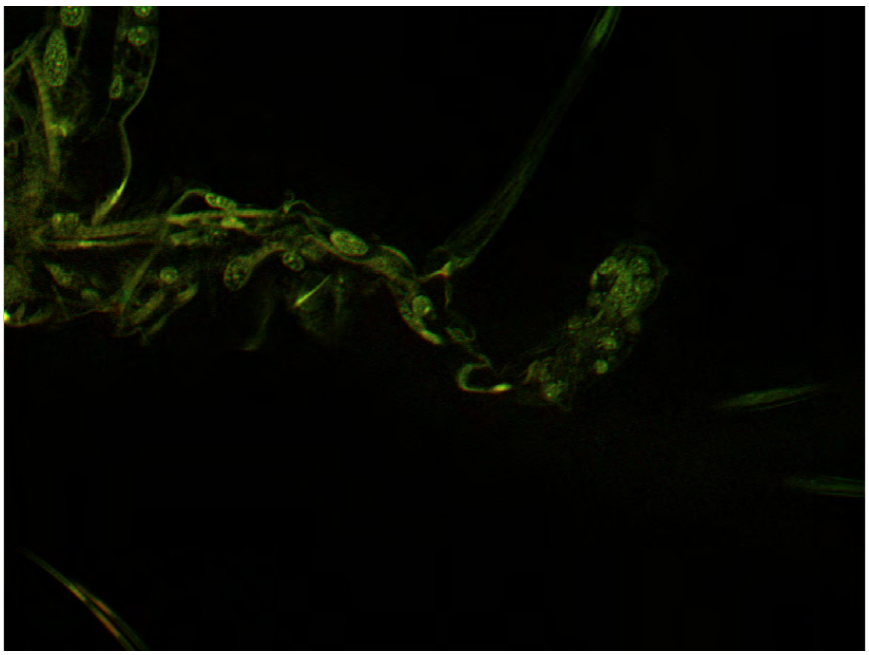
2 Click on the **Apply deconvolution** button.

Depending on the image size and the specifications of the computer, the processing can take anything between a few seconds and a few minutes. Make sure that you also adjust the brightness and contrast using the settings on the **Display** tab (tip: try out the **Min/Max** button).

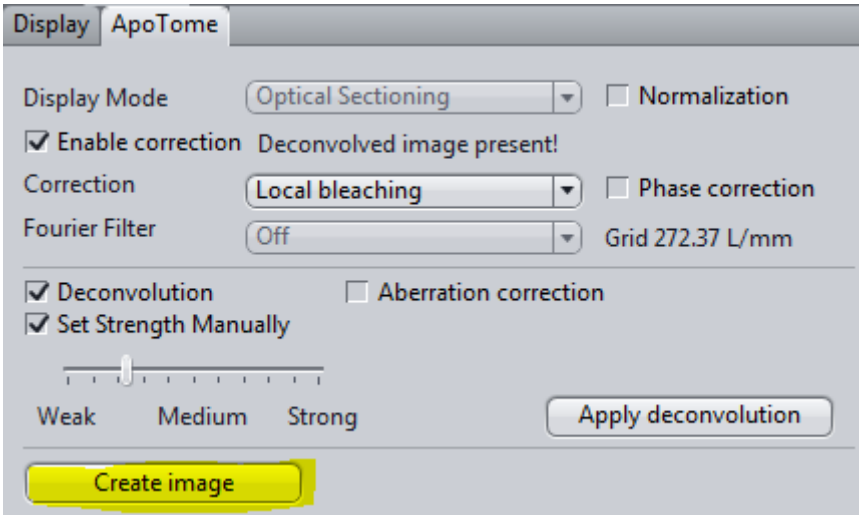


3 Examine the result by navigating through the Z-stack using the **Z-Position** slider on the **Dimensions** tab.

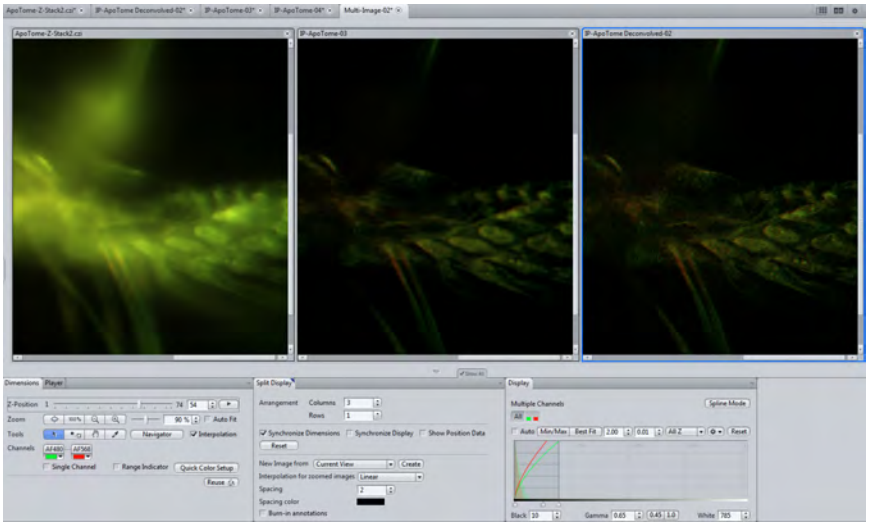
4 Change the **Strength** setting on the **ApoTome** tab until there is an obvious improvement and there is no disruptive background noise.



5 To obtain the result as a separate image document, click on the **Create image** button.



6 Using the *Splitter mode* [▶ 869] you can now compare the resulting image with the widefield and the ApoTome processed version.



You have successfully performed ApoTome deconvolution, created a resulting image and compared different resulting images.

22.2 Celldiscoverer 7

22.2.1 Introduction

Celldiscoverer 7 - Your Automated Platform for Live Cell Imaging

The **Celldiscoverer 7** is an automated inverted imaging platform for research applications. The system calibrates itself, detects and focuses your samples and has adaptive optics to provide the optimal optical settings in an automated fashion. Thanks to its next generation opto-mechanical concept, a brand new line of dedicated objectives and a multitude of innovative built-in automation features **Celldiscoverer 7** delivers benchmark data quality and easy operation. It allows to work with a huge variety of sample carriers like wellplates, petri dishes and slides.

The system can automatically detect and calibrate the sample carrier category, measure the bottom material thickness and adapt the optics automatically to deliver maximum image quality. A built-in incubation chamber, auto immersion allow for long-term lice cell imaging experiments. The new hardware based focus system can not only hold the focus over time but find the sample carrier surface.

The system is controlled by the ZEN Blue software package which offers all tools and functions you need to operate the **Celldiscoverer 7** efficiently and automate your imaging workflow. The software contains some additional functions allowing the user to navigate in a safe, easy and efficient way without unnecessary bleaching.

- **Tiles & Positions** - in combination with focus strategies this allows for easy and flexible image acquisition especially for multi-position and wellplate experiments.
- **Advanced Processing & Analysis** - Use the built-in image analysis functions, create pipelines to run online image analysis and modify experiments based on those results on the fly.
- **Macro Environment** - Powerful Python scripts allows to automate all kind of workflows, export data and connect to 3rd party application required for the workflow.
- **Automation GUI** - Automate routine experiments and using scan profiles that can be started with just one button.

22.2.2 Sample Tab (Interactive mode)

The **Sample** tab is the central point of operating the **Celldiscoverer 7** system. On top of the tab you can activate two different modes, the **Interactive** or the **Automation** mode. Usually the Interactive mode is used for the default workflow when no plate loader is present or required. The Automation mode is especially suited for running routine experiments in an automated fashion. To learn more about the Automation mode, read *Sample Tab (Automation mode)* [▶ 940].

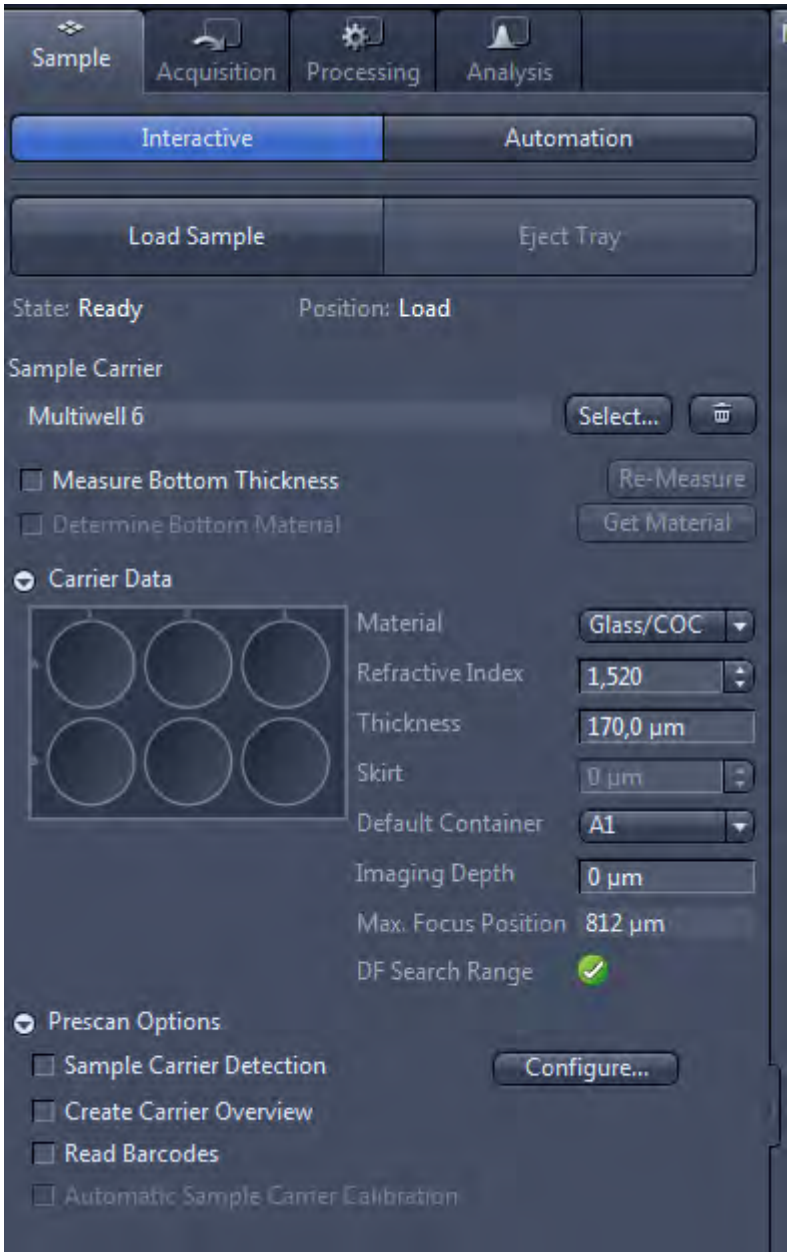


Fig. 22.6: Sample tab - Interactive mode

If you have selected **Interactive** mode, the following controls and functions are available:

Parameter	Description
Load Sample	Loads the inserted sample and performs various actions depending on the selected options under **Sample Carrier** and under **Prescan Options**. The detection of the bottom surface to measure the skirt of the sample carrier is always executed for every individual insert separately.

Parameter	Description
Eject Tray	Ejects the loaded tray with the sample carrier.
State	Shows the current system status.
Position	Shows the current position of the sample carrier. When the tray is ejected the position is Load, e.g. the system is ready to be loaded with a sample carrier. When the carrier is in scan position (above the objectives) the stated position depends on the current carrier type. As an example it could state **WellPlate** or **Petri Dish 6x, A1**.
Sample Carrier	
- Select...	Here you can select the sample carrier template you want to work with from list. Inside the selection dialog it is also possible to define and specify your own custom sample carriers, for example your favorite brand for a 384-well plate carrier. Be aware of the fact, that one must use the provides tray insert at any time in order to avoid collisions .Click on the **Delete** button to remove the selected sample carrier template. It is not possible to leave the selection empty, since the Celldiscoverer relies on having a valid sample carrier template all the time.
- Measure Bottom Thickness	**Activated**: The bottom thickness of the sample carrier is measured using special optics. Make sure the used sample carrier has the correct bottom material selected. When glass or COC is selected as a material the thickness measurement will have much smaller search range. In case of Polysterol as the selected material the search range will be large in any case. Note that the measurement may not be correct for embedded samples.
- Re-Measure	Performs the bottom thickness measurement again at the current XY position.
- Determine Bottom Material	**Activated**: Attempts to determine the use bottom material on demand. The method can differentiate Glass/Cycle Olefin Copolymer (COC) / "glass-like" materials and Polysterol (PS).
- Get Material	Performs the material determination again at the current XY position.

Parameter		Description
Carrier Data		After the sample carrier detection during the prescan (see **Prescan Options** below), a graphical sketch the sample carrier data will be displayed here. Additionally detailed information about the carrier are shown. If the sample carrier detection option is not activated, you can enter the sample carrier data here manually e.g. **Material**, **Refractive Index**, **Thickness** etc..
-	Material	Here you can select the correct bottom material, e.g. **Glass/COC** or **Polystyrene** (PS).
-	Refractive Index	Here you can modify the pre-selected values if required.
-	Thickness	Shows the measured bottom material thickness. You can adjust the value if required.
-	Skirt	Per default this parameter can not be edited. You can switch it on under **Options** | **Celldiscoverer** | **General** | **Allow manual adaptation of skirt**. This value is measured automatically for every sample as soon as the tray is loaded. It defines the distance from the surface of the tray to the bottom surface of the actual carrier, e.g. the well plate.
-	Default Container	Specifies the container which is approached initially, e.g. to detect cover glass thickness and measure the skirt height.
-	Imaging Depth	Specifies the desired penetration depth for autocor objectives.
-	Max. Focus Position	Shows the current upper z-limit of the focus drive.
-	DF Search Range	This field shows whether the Definite Focus (DF) search range is restricted by the z-limit. If a warning is displayed you can do the following: ■ Increase the z-limit (Under **Options** | **Celldiscoverer**) ■ Reduce the imaging depth ■ Check the refractive index
Prescan Options		
-	Sample Carrier Detection	**Activated**: The system automatically detects which type sample carrier category is used. The result is displayed in the **Carrier Data** section. The **Configure** button allows to assign a special carrier template to carrier category. For

Parameter		Description
		example the Pre-Scan recognizes a 96 well plate (category). This "recognition event" can be assigned to sample carrier template "MyFavorite96Plate".
-	Create Carrier Overview	**Activated**: An overview image of the sample carrier is acquired with the Pre-Scan camera. The overview image is displayed in the image document area after the Pre-Scan.
-	Read Barcodes	**Activated**: The system automatically detects barcodes on the sample carrier. Currently the system can read codes places on the short sidewalls of well plates or on top of the carrier, e.g. on a slide.
-	Automatic Sample Carrier Calibration	**Activated**: An automatic sample carrier calibration is performed during pre-scan. In case of reflecting or non-transparent covers (for well plates) the option Use LED Illumination for Carrier Calibration can be activated as an option.

22.2.3 Sample Tab (Automation mode)

With the Automation mode it is possible to create scan profiles theat combine all the prescan options with an actual ZEN experiment. The Automation mode can be used with or without a plate loader attached to the **Celldiscoverer 7** system. In case of not having a plate loader some of the UI elements will not be visible.

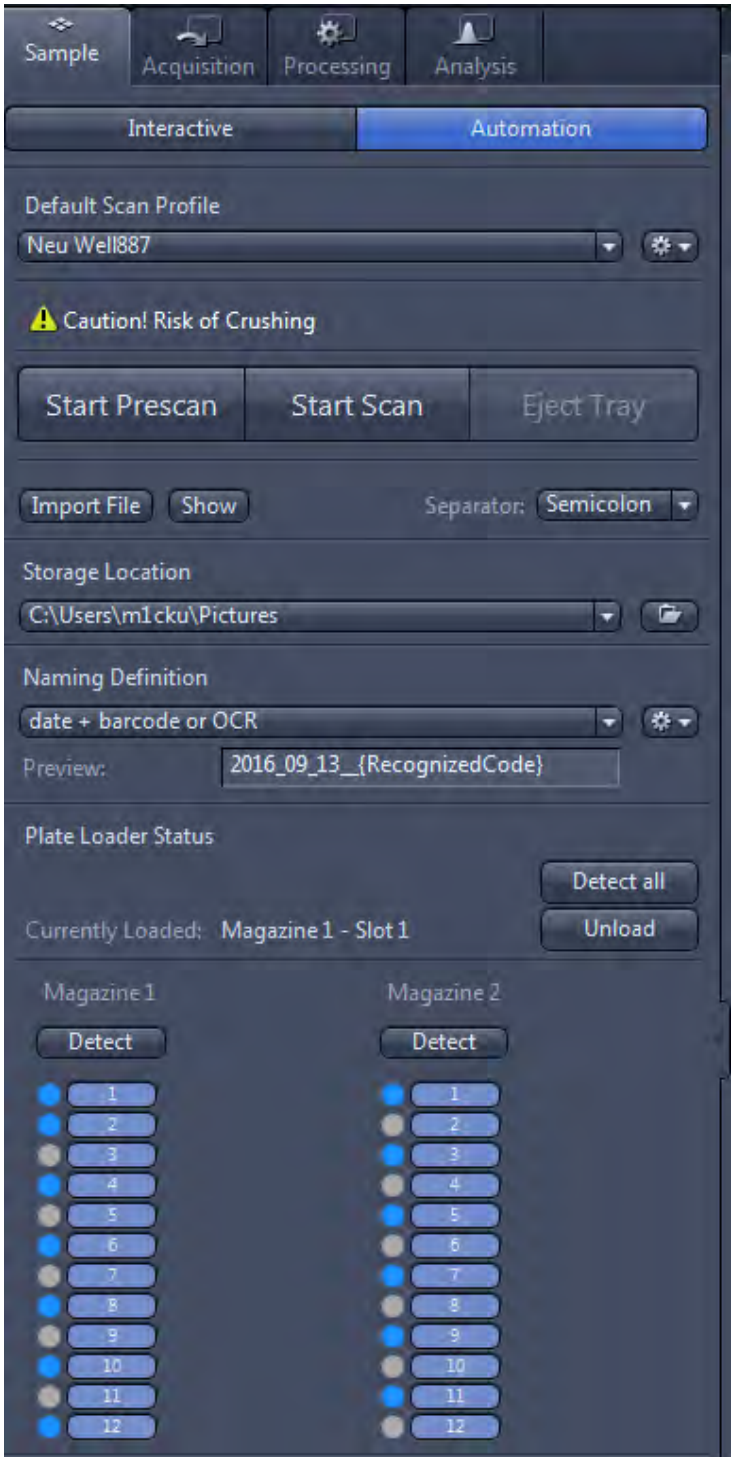


Fig. 22.7: Sample tab - Automation mode

If you have selected **Automation** mode, the following controls and functions are available:

Parameter	Description
Default Scan Profile	Here you can set a default scan profile. This profile will be used if new sample holders are inserted.

Parameter	Description
	If no profiles exists, you first have to create a new profile via the **Options** menu  , see below:
- **Options** 	■ **New Scan Profile…** Creates a new scan profile. ■ **Open Profile Configuration** Opens the *Profile Configuration* [▶ 944] dialog. There you can edit an existing scan profile. ■ **Rename** Renames the selected profile. ■ **Save as** Saves the user profile under another file name. ■ **Delete** Deletes the selected profile.
Start Prescan	Starts a prescan of all selected slots or the inserted sample (if no plate loader is used). To select a slot for a prescan, go to **Magazine** view and activate the corresponding checkbox in the **Process** column.
Start Scan	Starts the scan of all selected slots or the inserted sample.
Eject Tray	Ejects the tray which contains the sample holder.
Import File	If you click on this button you can import a file (*.csv format) which contains configuration data for the system. Typically the following data can be imported: ■ **Magazine**, **Slot**, **Barcode**, **Scan Profile**, **ImageFileName**, **SubPath** Under **Separator** you can select the separator used in the *.csv file (e.g. Semicolon, Tab) If you click on '**Show**' a preview of the imported data will be displayed.
Storage Location	Here you can specify the storage location (file path) for all created images. All image sub paths, which might be read from the configuration file will be inserted below the defined file path.

Parameter	Description
Naming Definition	Here you can define naming definitions for the acquired images. You can select several definitions for the file names from the dropdown list. The name automatically contains the detected barcode content if the barcode detection is active for the active profile. Click on the **Options** button  to define, edit or delete a definition.
Plate Loader Status	The display of this section depends on your system configuration. If you do not use a plate loader only the sample carrier which you have configured is displayed in the **Magazine** view. If you work with a plate loader, its magazine with the different slots is displayed. Common plate loaders can have up to 4 magazines. One magazine can contain up to 12 slots. In the slots you put your sample carriers containing the sample. The functions mentioned below only apply for the plate loader.
- Currently Loaded:	Shows the location (in the hotel of the loading robot) of the sample holder which is loaded currently.
- Detect All	Checks all magazines for slots which contain sample carriers automatically.
- Unload	Unloads the currently selected sample carrier from the Celldiscoverer back to its slot in the magazine.
- Reset	This button is only shown in case of an error with the plate loader (e.g. Plate loader is blocked). The respective message is shown in red. In this case all controls will be disabled and the plate loader will stop working. **NOTICE** **In case of an physical obstruction, e.g. by a misplaced sample carrier, you first have to remove the sample carrier before you click on 'Reset'.** By clicking on **Reset** you can try to restore the system status of the plate loader. The system then tries to solve the problem using some internal error checking routines and additional actions.

Parameter	Description
Magazine 1 - ...	Here you see the graphical display of the magazine(s) and its lots. The magazines are numbered from 1 - 4 (depending on the available magazines). Under a magazine each slot is displayed as blue button. The slots are numbered as well (e.g. from 1- 12). The icon in front of a button shows the loading status. Following status are possible: ■ gray = empty slot ■ blue = occupied slot ■ blue blinking = carrier is currently on the stage ■ yellow = problem detected ■ red = Error occurred during processing ■ green blinking = Slot has status 'prescanned' ■ green = Scanning the slot is finished. If you double-click on a slot button, the slot will be loaded or unload from the tray (depending of the loading status). If the tray is loaded with a carrier from a certain slot and you load a carrier from a different slot, the currently loaded slot will be unloaded first.

22.2.3.1 Profile Configuration

Parameter	Description
Selected Profile	Shows the name of the selected profile. Under **Profile Description** you can enter a short description, if desired.
Carrier Data	Contains information about the sample carrier
- **Automatic Carrier Detection**	In case of the option Automatic Carrier Detection the scan profile can contain data for different carrier types. They are visible inside the Sample Carrier list view.
- **Fix Carrier Assignment**	Only one specific carrier type is assigned to the scan profile. This is useful when working with the same carrier type all the time.
Options	
- **Create Carrier Overview**	Created and save an sample carrier overview image during the prescan which is store inside the actual image data file as an attachment.

Parameter	Description
- **Detect Occupied Positions**	In case of a sample carrier with multiple inserts, the systems automatically checks for empty positions.
- **Read Barcodes**	Read the barcodes from the respective position on the sample carrier itself (position depends on the carrier).
- **Use configuration file**	Three different options available: ■ **No configuration file**: no configuration will be used. ■ **Imported Data** : use a configuration that must be imported before. ■ **Control Barcode**: Defines rules how a barcode will be interpreted.
Sample Carriers	This list view allows managing the sample carrier types supported by the current scan profile. The **+** button allows to add more carrier types. The **Delete** button deletes the carrier type including all assigned scan experiments.
Carrier Configuration	This area shows the respective carrier configurations and allows to modify them. For normal wellplates there will be only one data set visible, but for carriers with multiple inserts it is possible to define different configuration for every available insert
- Use same sample carrier configuration for all positions	When activated the same configuration will be used for all sample carriers during the processing.
Assigned Scan Experiments	Here you can assign the actual ZEN experiments. For carriers with multiple inserts it is possible to assign an individual experiment to every insert, e.g. it is possible to run a different experiment for every petri dish when using the 6x petri dish.
- Use same scan experiment for all positions	Allows assigning the same experiment to every individual position of the carrier, e.g. use the same experiment for all petri dishes of a 6x petri dish holder.

22.2.4 Magazine View

In general the **Magazine** view is used to get an overview of the status of the sample carrier(s) / scan items in the tray of the **Celldiscoverer 7** system or in the magazine(s) of the plate loader.

The display of this view depends strongly on the system configuration. If you use the **Celldiscoverer 7** system without a plate loader the configured sample carrier is displayed.

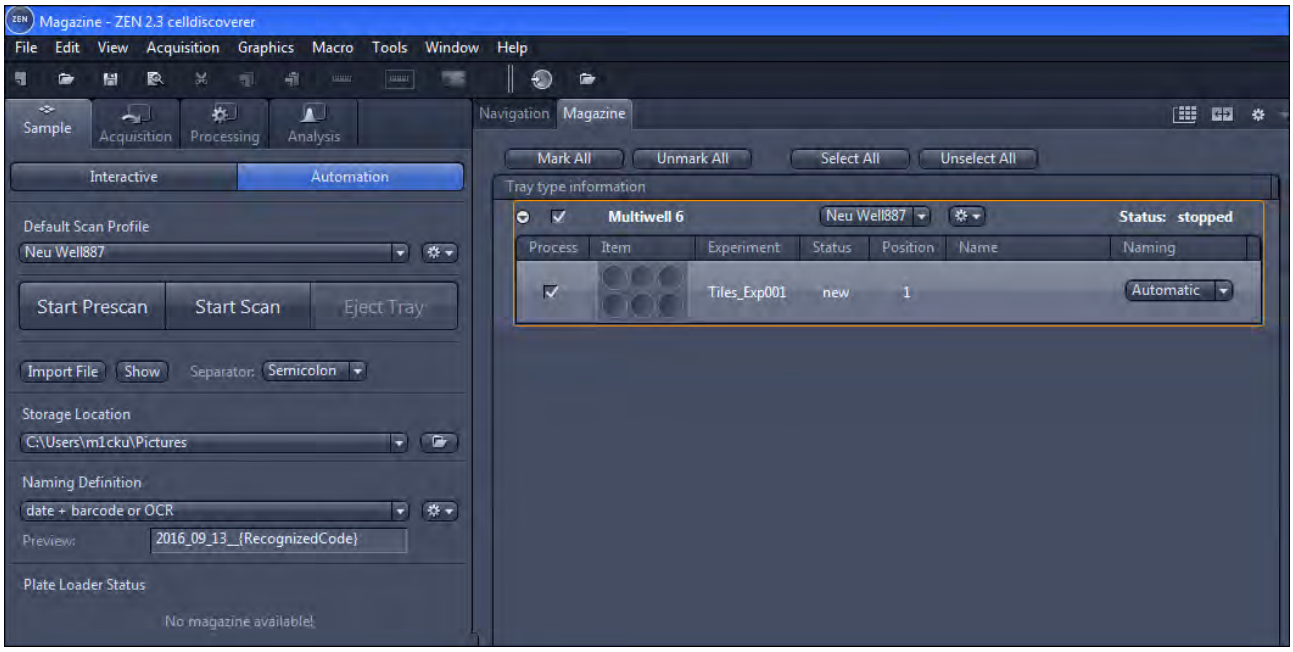


Fig. 22.8: Magazine View without Plate Loader

If you work with a plate loader you will see a list of the available magazines and slots. Note that only slots which are occupied are displayed. Empty slots are not displayed in the list. We recommend to perform a check for occupied slots by clicking on **Detect all** in **Sample** tab under **Plate Loader Status**.

The currently loaded slot / scan item is marked by an orange rectangle.

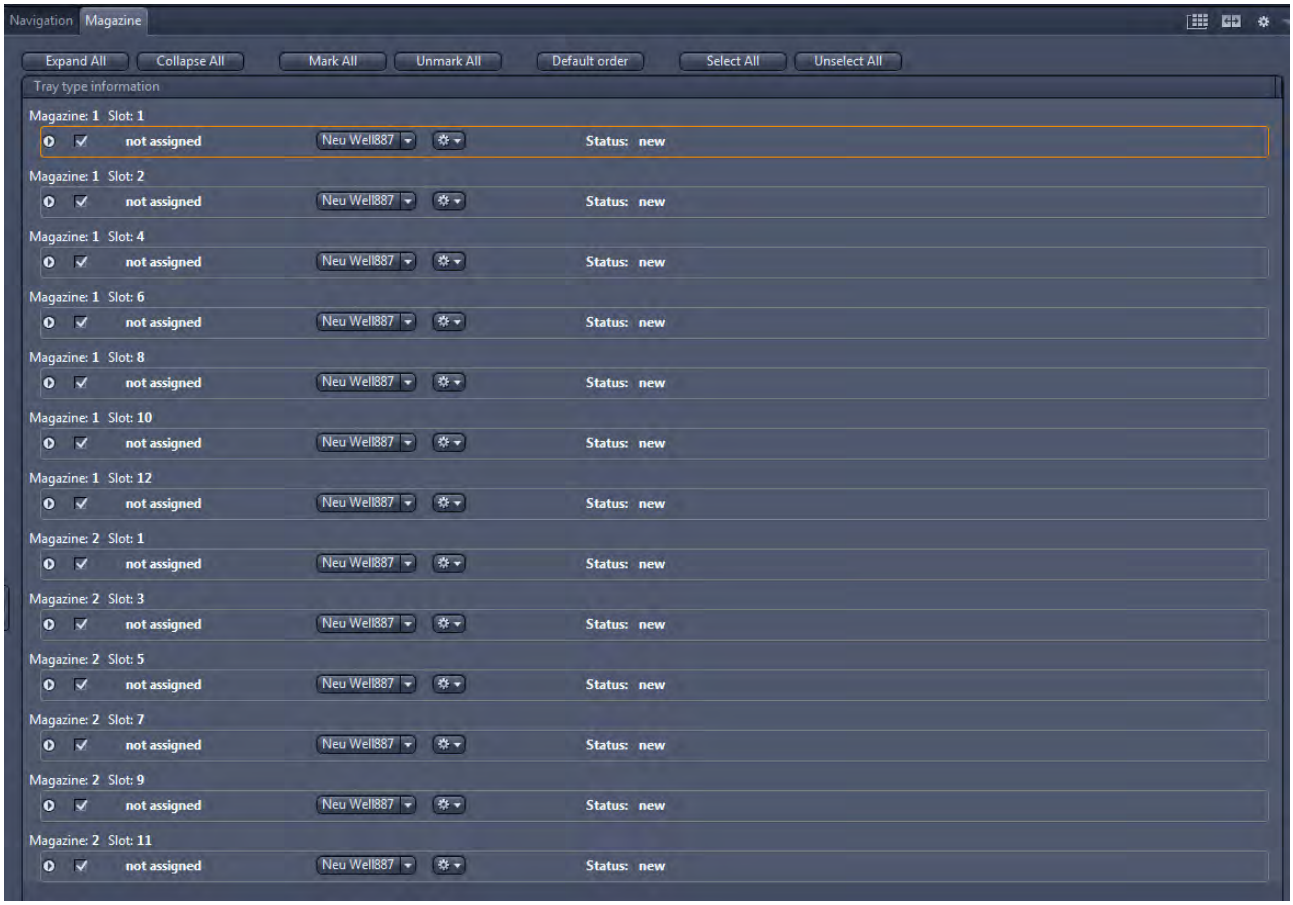


Fig. 22.9: Magazine View with Plate Loader

The following controls are available for Magazine view:

Parameter	Description
Expand All	Expands all scan items so that all of them are visible including there configurations. (This button is only visible when a plate loader is present.)
Collapse All	Collapses all scan items so that only the short information overview and the processing status is visible. (This button is only visible when a plate loader is present.)
Mark All	Activates the processing markers for all scan items. When clicking '**Start Prescan**' or '**Start Scan**' those items will be processed, if their status allows for those actions (new, prescanned, stopped).
Unmark All	Deactivates all processing markers.
Default Order	Restores the default order of slots. Their order can be changes via Drag & Drop. Newly detected carriers will be added at the end.

Parameter	Description
Select All	Selects all items and allows the execution of a context menu entry for all shown elements at once.
Unselect All	Unselects all items.
Tray type information	
- Show Information	Shows the results of the processing, if existing.
- Show Profile	Open a XML viewer to inspect the complete scan profile configuration.

22.2.4.1 Magazine View Options

The options below are opened via right click on the corresponding slot (e.g. Magazine:1 Slot:1).

Parameter	Description
Assign Scan Profile	Open a list with all available scan profiles. A click on the desired profile assigns it to the currently selected items.
Mark all scan items of highlighted trays for processing	Activates the processing checkbox for all selected slots and their scan items.
Unmark all scan items of highlighted trays for processing	Deactivates the processing checkbox for all selected slots and their scan items.
Show Overview Image	Opens the sample carrier overview image.
Expand all highlighted holders	Opens the sample carrier overview image.
Collapse all highlighted holders	Opens all selected sample carriers.
Reset carrier status to 'New'	Closes all selected sample carriers.

The options below are opened via right click on an individual sample carrier inside the list (scan item).

Parameter	Description
Move to Scan Position	Moves the stage to the respective scan position. Depends from the sample carrier and a possible insert and therefor from the scan item type.
Mark all highlighted scan items for processing	Opens the resulting scan image as an individual document inside the normal document area.
Unmark all highlighted scan items for processing	Resets all precessing checkboxes for all selected scan items.
Reset Scan status to New	Resets the status of the scan items to "new".

22.2.5 Navigation View

In the **Navigation** view you see the graphical display of the loaded sample carrier. In general the view is used for navigation purposes. Depending on the carrier, on **Selection** tab you can navigate to the single containers of the carrier (e.g. of a petri dish) simply by double clicking on the desired container. The stage will move automatically to the center of the selected container. In case of carriers with multiple inserts (e.g. 6x petri dish insert) the selection tab allows you the switch between the different inserts.

Fig. 22.10: Navigation view

The **Celldiscoverer 7** system is equipped with an automatic collision prevention system. Based on the geometry of the objective front, the working distance of the objective, the actual carrier or insert geometry and the skirt height the safe XYZ traveling ranges will be calculated and adapted automatically. In **Navigation** view the currently possible XY travelling ranges are always displayed. The current active limit for the selected objective is displayed by a thick, dashed line using the respective color. The limits for the other objectives are also shown using a thin line using the respective color.

The blue cross hair shows the current stage position and the blue rectangle (only visible at higher zoom levels) shows the field of view of the objective.

22.2.6 Celldiscoverer Tool

The **Celldiscoverer** tool is located in the **Right Tool Area**. Note that the tool is not visible in **Automation** mode.

Fig. 22.11: Celldiscoverer tool

The tool is used for controlling the systems hardware components, like objectives, beam splitter, filter wheels, light path etc..

Parameter	Description
Active Camera	Here you can select the active camera or detector. The respective port switcher moves automatically.
Objective List	Here you can easily switch between the objectives and pre-magnification. The color bar on the objective buttons indicates the color for the respective stage limit indicator inside the **Navigation** tab.

Parameter	Description
	If you select AutoCorr objectives (motorized correction collar) you can additionally adjust the relevant settings like **Correction Mode, Bottom Thickness** or **Imaging Depth**.
Beam Splitter List	Here you can select the desired beam splitter from the list. If you change the beam splitter the corresponding emission filter from the list below is changed as well.
Filter List	Here you can select the desired emission filter from the list. A change here will not affect the selected beam splitter.
Pipette Position	If you click on the ON button the system moves in the pipette position, where it is possible to add reagents to the sample. The tip of the pipette will be located at the center of the optical axis indicated by the blue crosshair inside the **Navigation** tab. Make sure the height adjustment for the pipette tool is correctly adjusted to the current carrier geometry.
Microscope Control	Opens the **Microscope Control** dialog. There you can see and adjust the full light path of the system. We recommend to only adjust settings in the light path, if you know what you are doing.

22.2.7 Dispensing Tool

This tool is used to set up dispensing events for certain container(s) on a sample carrier. To perform dispensing, the system moves the sample carrier to the dispensing position where you can easily add dispensing fluid to the selected container (s).

Parameter	Description
Sample Carrier	Shows the selected sample carrier which will be used for dispensing.
Options	
- Repeat every	Here you can adjust the interval between dispensing events during an experiment. E.g. if you adjust the value to 5 min, the software will pause the running experiment after 5 minutes and allows you to add a substance to your sample.
- Dispensing events	Here you can adjust the total number of dispensing events for an experiment.

Parameter	Description
- Select containers	If you click on the **Select** button, the **Container selection** dialog opens. There you can select the containers in which you want to add the fluid.

22.2.8 Celldiscoverer Options

The Celldiscoverer **Options** menu can be found under **Tools** | **Options** | **Celldiscoverer**.

Parameter	Description
General	
- Use Automated Camera Frame Correction	When using the 0.5X after-magnification the chip window on the camera is adjusted automatically in order to remove the dark areas inside the corners due to the limited FOV for (only!) this after-magnification.
- Enable Automation Mode (restart required)	If activated and you work with a plate loader, on **Sample** tab the **Automation** mode is automatically activated.
- Unload last used sample carrier after scan job is finished	If activated, after the experiment the currently loaded sample carrier will be unloaded from the tray.
- Allow manual skirt adaption	
- Don't show a 'Warning' at experiment execution, if the validation result can be ignored	

Parameter	Description
Cover Glass Thickness Detection Options	
- Use Default Thickness Detection (recommend ed)	If deactivated (**not recommended**), you can adjust the cover glass thickness detection options manually for trouble shooting.
- Use Aberration Correction	Activated: Attempts to improve the result of the thickness measurement by considering aberration effects. Per default this setting is deactivated.
Microscope Manager	
- Parfocal Correction	Activated: Focal Shifts are corrected automatically when changing the magnification based on the calibration file. We recommend to leave this checkbox always activated.
- Parcentral Correction	Activated: Lateral XY Shifts are corrected automatically when changing the magnification based on the calibration file. We recommend to leave this checkbox always activated.
Prescan Options	
- Show Images Used for Recognition	Activated: Displays the images used for recognition inside the Document area after the Pre-Scan. This option should be only activated for trouble-shooting the Pre-Scan sample carrier recognition. Normally this option can be deactivated.
- Show live container during sample carrier calibration	Activated: Displays the live images used for the automatic sample carrier calibration inside the Document area after the Pre-Scan. This option should be only activated for trouble-shooting the Pre-Scan sample carriers recognition. Normally this option can be deactivated.
Z-Limit above Surface	Allows adjusting the default Z-Limits for different objectives to increase the available XY traveling range. The icons inside the DF (Definite Focus) Search Range column indicate if the available Z-range is sufficient for performing a **Find Surface** operation.

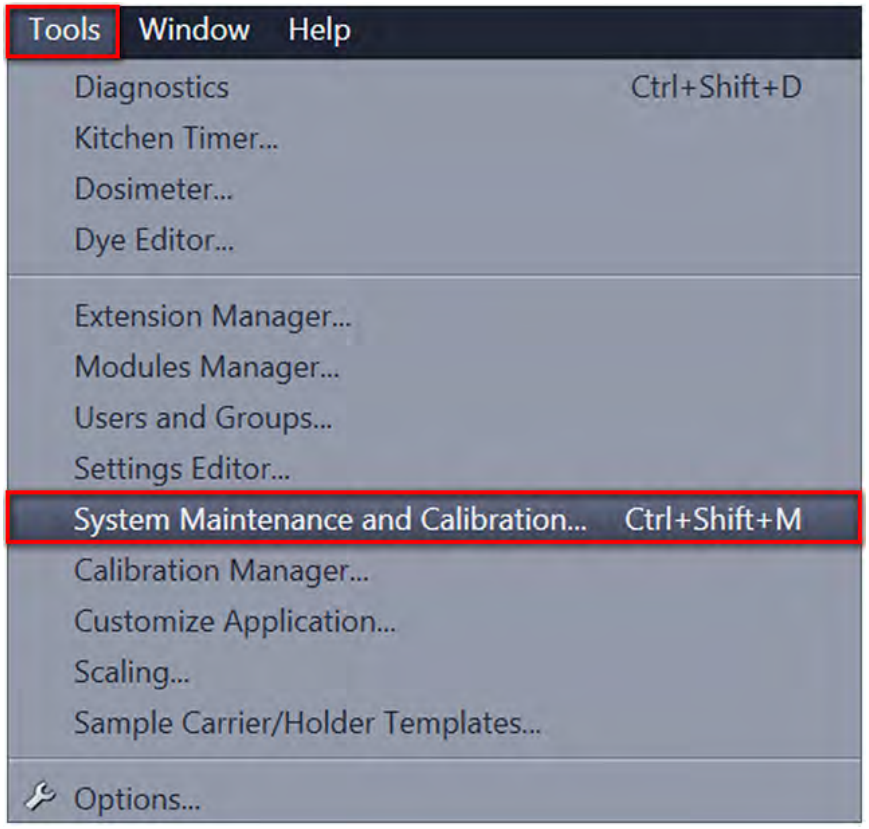
Parameter	Description
Auto Immersion Refill Options	
- Remove Water	If you want to use Auto Immersion for an experiment, here you adjust the duration for the removal of the applied water. The set time defines the duration for which the suction pump is switched on (default: 3000 ms). For use of Auto Immersion, in **Left Tool Area** | **Experiment Manager** activate **Auto Immersion**.
- Apply Water	If you want to use Auto Immersion for an experiment, here you adjust the duration for the application of water (default: 2000 ms).
- Refill every...	Regardless of an experiment, here you adjust the interval time of the global periodic timer for renewing the immersion (default: 45 minutes). This prevents the system from drying out if it is switched on and in a rest period for a long time, e.g. over night.
- Reset	Resets the parameters to the default values.
Phase Gradient Contrast Settings	
- Automatic Half Pupil Angle Adaptation	A phase gradient contrast image is calculated from two single images acquired with two different angles of the half pupil. If activated, the optimal choice of the angles is calculated depending on the current X/Y Position insides the well.

22.2.9 Performing a Celldiscoverer calibration

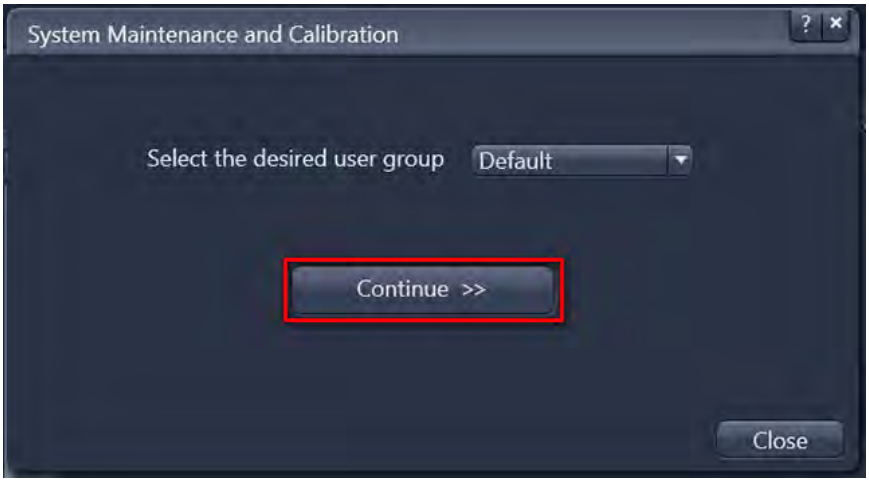
The Celldiscoverer calibration wizard is used for hardware calibration of the system. The individual components can be selected for re-calibration. Calibration data are stored for comparison.

If you want to perform a calibration using the wizard, the following steps are necessary.

Procedure **1** In the **menu bar** | **Tools** select **System Maintenance and Calibration...**

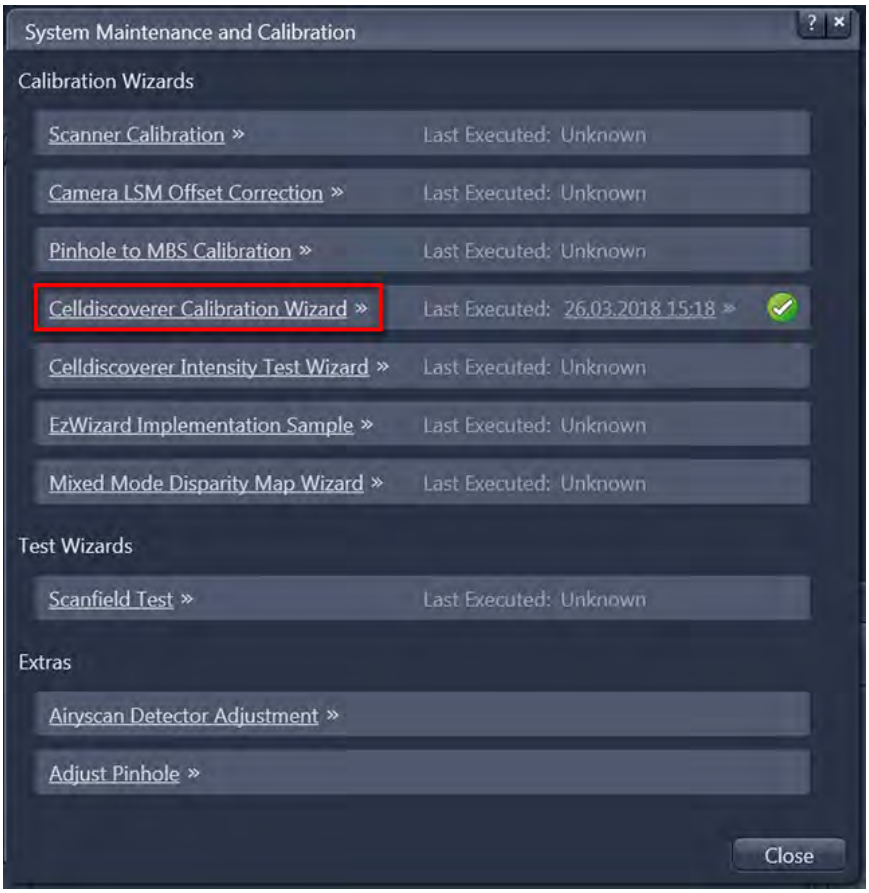


The **System Maintenance and Calibration** dialog opens.



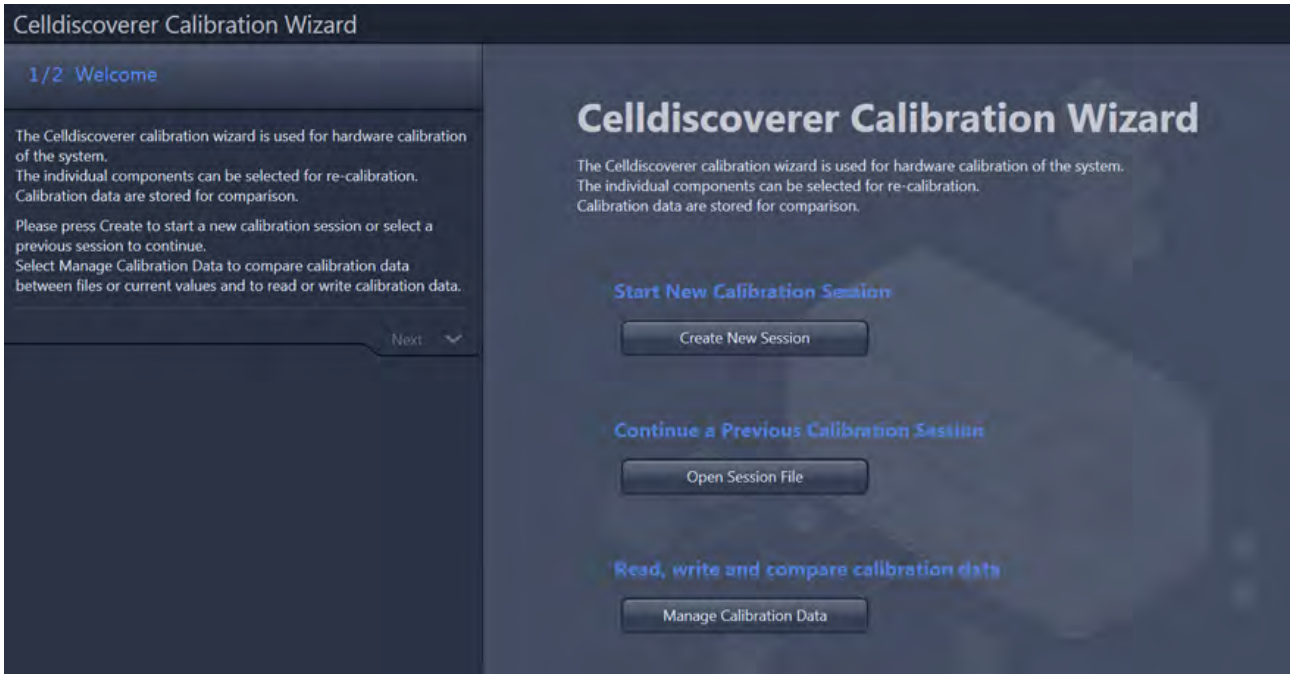
2 Click on **Continue >>**.

In the **System Maintenance and Calibration** dialog, the available Calibration procedures will be shown.



3 Click on **Celldiscoverer Calibration Wizard >>**.

The Celldiscoverer Calibration Wizard (1/2 Welcome) opens.



Three option are available to continue:
- start a new calibration session by clicking on **Create New Session**
- continue with a previous calibration session by clicking on **Open Session File**
- read, write and compare calibration data by clicking on **Manage Calibration Data**

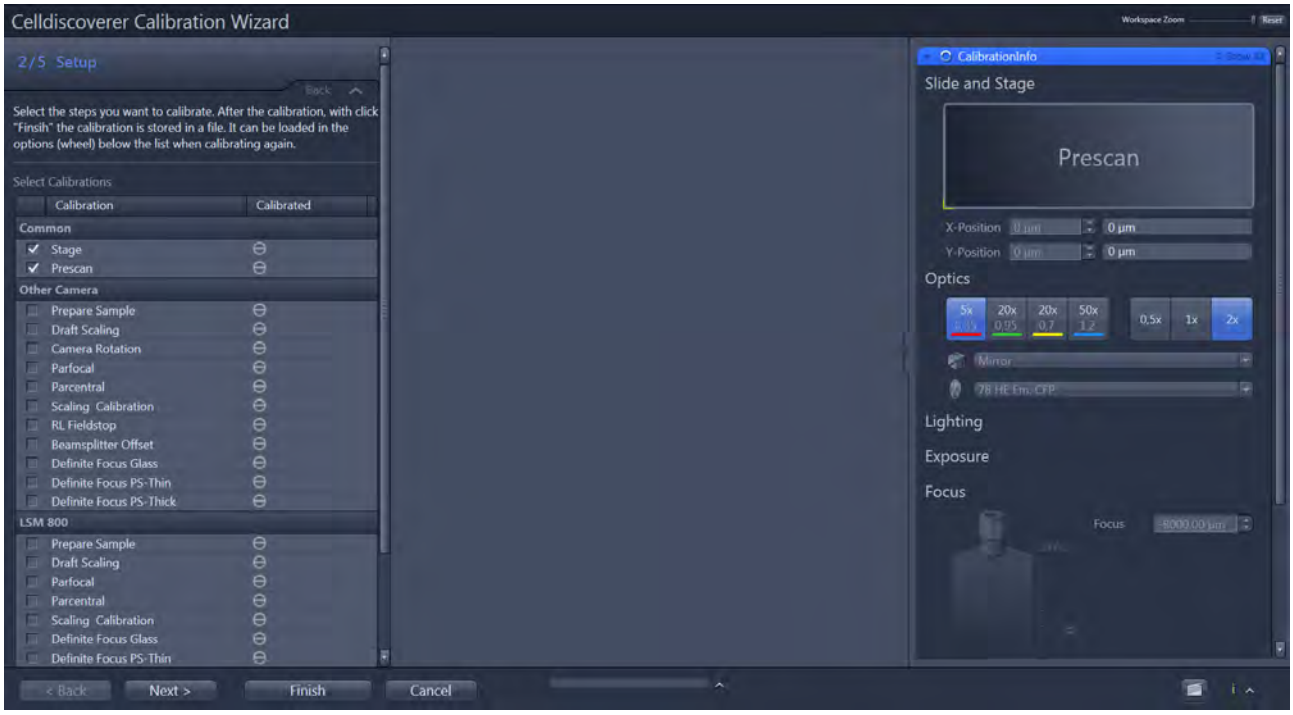
4 To perform a new Celldiscoverer calibration, click on **Create New Session**.

Additionally, an edit field with a proposed session name and the **Exclude Immersions Objectives** checkbox are hidden.

5 If required, edit the name of the calibration session and activate the **Exclude Immersions Objectives** checkbox.

6 To start the calibration procedure, click on **Create**.

The next screen of the wizard will be shown.



7 Select the steps for the calibration procedure by activating the appropriate checkboxes.

8 Click on **Next >** to run the calibration.

9 After the calibration is done, click **Finish**.

The calibration results will be stored in a file and the **System Maintenance and Calibration** dialog appears again. The **Celldiscoverer Calibration Wizard >>** item is marked by a green checkmark.

10 Close the **System Maintenance and Calibration** dialog with **Close**.

If the window closes, you have successfully performed the Celldiscoverer calibration.

22.2.10 Auto Immersion for Celldiscoverer

22.2.10.1 Introduction

The **Auto Immersion** functionality in **ZEN (blue edition)** is used for adding immersion fluid (water) to water immersion objectives and automatically renewing the immersion fluid during experiments.

Working with Auto Immersion in ZEN is easy. Firstly, create the immersion or renew it, when the immersion fluid starts to dry out. This is performed using the **Auto Immersion** tool in the **Right Tool Area**. The tool can be compared with the Auto Immersion controls available on the TFT display of the Axio Observer.

You can also configure Auto Immersion functionality for experiments. This can be done in the **Auto Immersion** tool in the **Left Tool Area** on **Acquisition** tab. E.g. for time series experiments you can set a time interval after which the created immersion will be renewed automatically during the experiment.

In the following chapters we describe step-by step how you prepare working with Auto Immersion objectives and how you can use Auto Immersion for experiments.

22.2.10.2 Preparing Auto Immersion

When using a water immersion objective, the immersion process can be controlled manually.

The following steps have to be performed:

Procedure

1. In **Right Tool Area**, open the **Auto Immersion** tool.

2. For controlling the immersion manually, activate the **Enable Immersion** checkbox.

   The **Create Immersion** and **Remove Immersion** buttons will be available.

3. Select the water immersion objective.

4. Click on **Create Immersion**.

   This initiates the creation of one immersion procedure.

5. Click on **Remove Immersion**.

   This initiates the removing of the immersion fluid.

The immersion creation or removing procedures are independent from the configured settings in the **Auto Immersion Refill Options**, see chapter *Celldiscoverer Options* [▶ 952].

22.2.10.3 Using Auto Immersion for an experiment

If you want to use Auto Immersion during an experiment, the following steps are necessary.

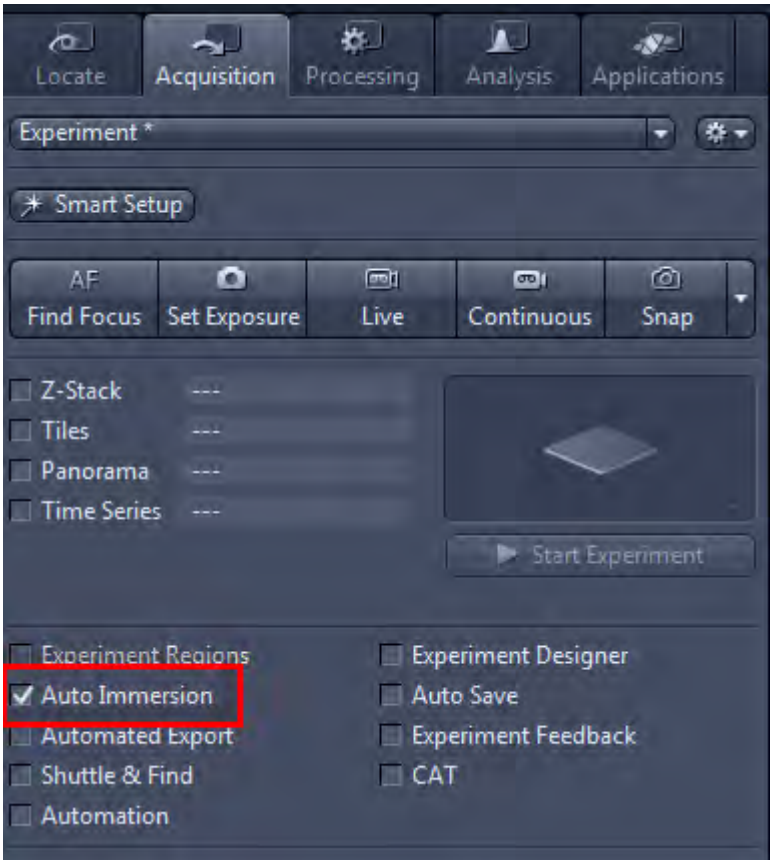
Prerequisites

- You have set up an experiment (e.g. a time series experiment).
- You have read the chapter *Preparing Auto Immersion* [▶ 959]. Basically you have to perform the steps described in this chapter before each experiment. The immersion amount which will be automatically applied during an experiment is the same like if you click on the **Renew** button.
- You have set the Remove Water and Apply Water parameters for Auto Immersion Refill during an experiment, read chapter *Celldiscoverer Options* [▶ 952].

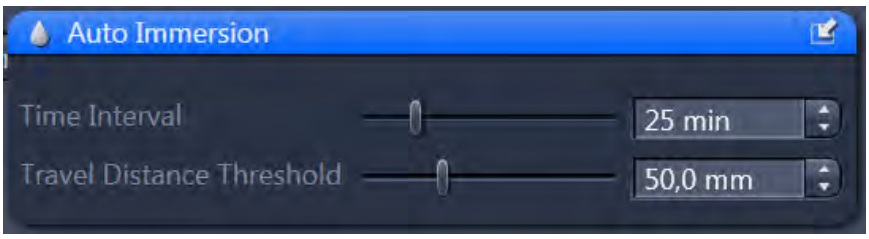
Procedure

1. Switch to the water immersion objective you want to use in the experiment.
2. In **Right Tool Area** | **Auto Immersion** tool click on **Create Immersion**

   The immersion will be created.
3. Select **Acquisition** tab.
4. In **Left Tool Area** | **Experiment Manager** activate **Auto Immersion**.



The **Auto Immersion** tool is displayed.



5 Configure the parameters for **Time Interval** and/or **Travel Distance** for the experiment. That event which will take place first will activate the renewing of the immersion during the experiment. For time series experiments we can recommend time intervals for up to 20 minutes, if necessary.

You have successfully configured auto immersion for an experiment.

22.2.10.4 Auto Immersion Tool

The Auto Immersion tool in the **Right Tool Area** in general is used to check the immersion status and to operate the immersion functions.

Parameter	Description
Tank Level	Shows the current filling level of the immersion fluid tank.
Enable Immersion	If activated, the controls for immersion appear.
Create Immersion	If you click on this button, the system will automatically add the immersion fluid to the selected immersion objective. If the fluid was added once, the button is grayed out.
Remove Immersion	If you click on this button, the system will automatically remove the immersion fluid from the selected immersion objective.

22.2.10.5 Auto Immersion Tool

The Auto Immersion tool in the **Left Tool Area** | **Acquisition** tab is used for experiments only. You can configure the time interval and travel distance for automatic immersion events in here.

Parameter	Description
Time Interval	Here you can adjust the time interval for auto immersion. E.g if you set the value to 30 min, the immersion is automatically renewed every 30 minutes during a running experiment.
Travel Distance	Here you can adjust the travel distance for auto immersion. E.g. if you set the value to 80 mm, the immersion is automatically renewed after the stage has moved a distance of 80 mm.

22.3 Slidescan

22.3.1 Introduction

Fig. 22.12: Axio Scan.Z1 system

ZEN slidescan is a software package that offers all tools and functions you need to operate the Axio Scan.Z1. E.g. it includes the possibility of creating z-stack and extended depth of focus images. With the software you can automatically generate high-resolution images (virtual slides) from your specimens. The system is able to perform these scans in brightfield and also multi-channel fluorescence mode
The Axio Scan.Z1 is mainly driven by software means. For details regarding the operation of the hardware and other hardware related procedures (like exchange of filter sets, etc.) refer to the latest hardware manual of the Axio Scan.Z1. As the Axio Scan.Z1 includes a built-in magazine with a 12 or 100 slide capacity, the software deals with scanning the specimens, operating the magazine, and handling the slides (including the status of the slides). This is visualized via an intuitive graphical user interface.
This software guide describes the specific operation of the ZEN slidescan software and not the general ZEN functionality; for this, please consult the general ZEN help. The information included in this document is also available via the online help (F1). Here you can conveniently access the information in context within the tabs or the wizards.

22.3.2 Working with ZEN slidescan

22.3.2.1 Setting up brightfield, fluorescence or polarization profiles

The following steps will show you how to set up brightfield, fluorescence or polarization profiles. In our example we choose a brightfield profile in the Smart Profile Selection. For fluorescence or polarization profiles choose the corresponding profiles from the profile pool.

22.3.2.1.1 Step 1: Smart Profile Selection

Procedure

1 Click on the **Smart Profile Selection…** button on the **Scan** tab (the button is located in the top half of the tab).

You will now be guided through a decision tree to find the suitable profile for your application. The steps are illustrated with pictograms to simplify the visualization process. Once this is complete, the software will present you with the most appropriate profile for your application/specimen. You can change the name and description of the profile if necessary.

i INFO

The profiles used in the **Smart Profile Selection** are fixed and part of a profile pool which cannot be changed by the user.

2 Click on the **Finish** button.

The profile will be copied to the profile folder. You can see the profile as an additional item in your profile list.

The profile can now be used as it is. It is recommended to execute the **Scan Profile Wizard** to adapt this profile to your specific needs. This can be done directly after the Smart Profile Selection by activating the corresponding checkbox.

22.3.2.1.2 Step 2: Scan Profile Wizard

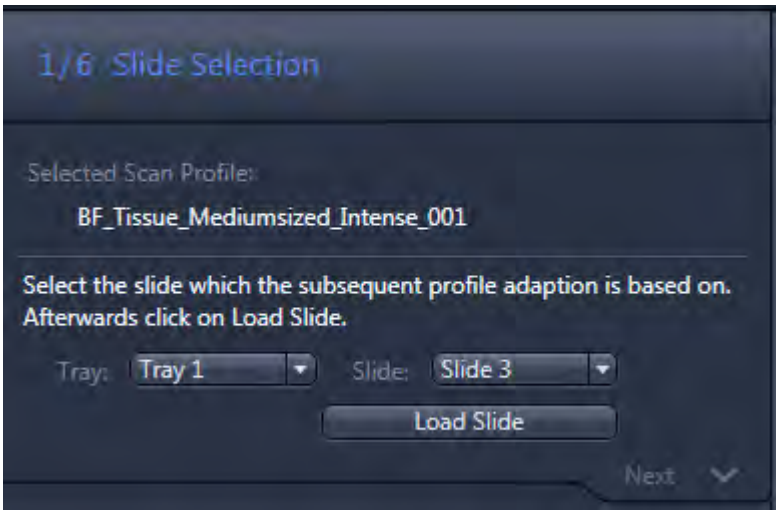
Prerequisites

- Open the door of your **Axio Scan.Z1** e.g., via the **Open/Close** button. Put a tray with a typical glass slide (the specimen should represent a larger group of similar specimens) inside the magazine and close the door. Wait until the main status indicator on the device is continuously green.

Procedure

1. On the **Scan** tab in the **Default Scan Profile** section, select the profile you want to modify. We recommend using a profile from the **Smart Profile Selection**.
2. Click on the **Scan Profile Wizard…** button.

   The Scan Profile Wizard opens. You will see the description of the profile (e.g., what kind of preview detection is used). This is important for preparing the slide correctly (e.g., if the marker is needed, the slide has to have a marker on it).
3. The first step is the **Slide Selection**. Select the **Tray** and **Slide** position and click on the **Load Slide** button.

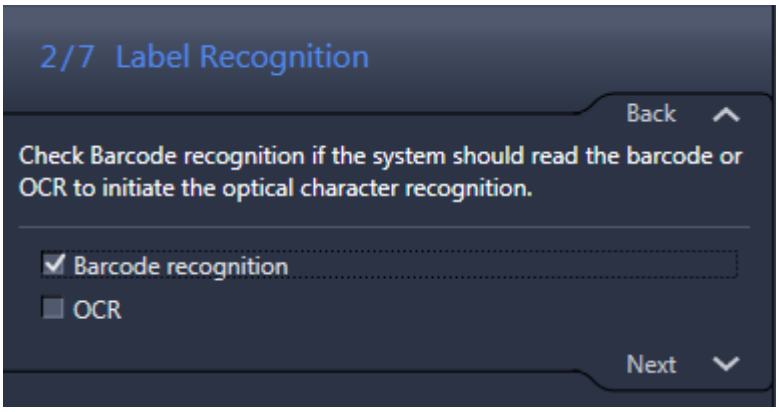


   The system will now move the tray to the operating position. If the tray is not already on the stage, the software will load the tray from the magazine.

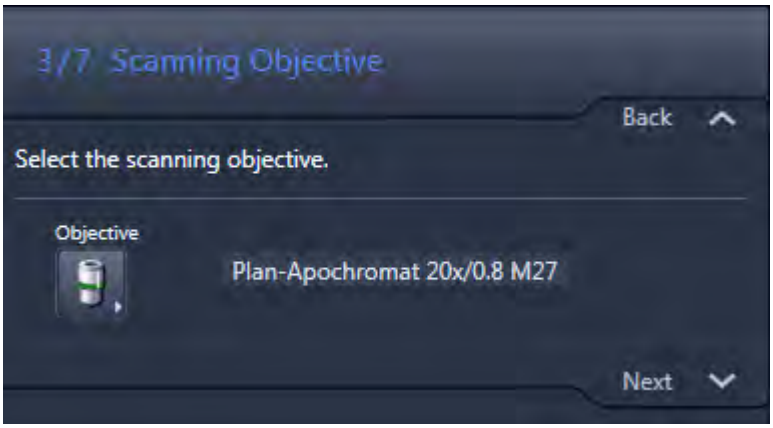
> **INFO**
>
> If you see a view to set up a grid in the next step and the profile contains the information that a grid should be used, please refer to the Grid definition.

4. The next step is the **Label Recognition**. Here you can select if the system uses **Barcode recognition** or **OCR** (Optical Character Recognition). If barcode recognition is selected, the system will check for all possible barcode types by

default. For detailed settings (e.g., if only a specific barcode should be read) use the **Advanced Scan Profile Wizard**.



5 In the next step, you have to select the **Scanning Objective**.



6 The next step is to adapt the image brightness in the **Exposure settings**. This can be influenced by setting the **Flash intensity** and the **Flash duration**.

To adapt these settings (this is particularly necessary if the scanning objective has been changed) you should move the stage containing the specimen into

the light path (e.g., by clicking inside the preview image in the **Navigation** tab or using the virtual joystick in the **Stage** tab).

7 You now need to focus within the specimen, e.g., by clicking on the **Smart Focus** button. You can also use the manual focus adjustment options.

i INFO

In most cases, the **Smart Focus** (which is the combination of the Coarse Focus and the Fine Focus) should work. If the **Smart Focus** does not work, check the detailed focus settings in the **Advanced Profile Wizard**.

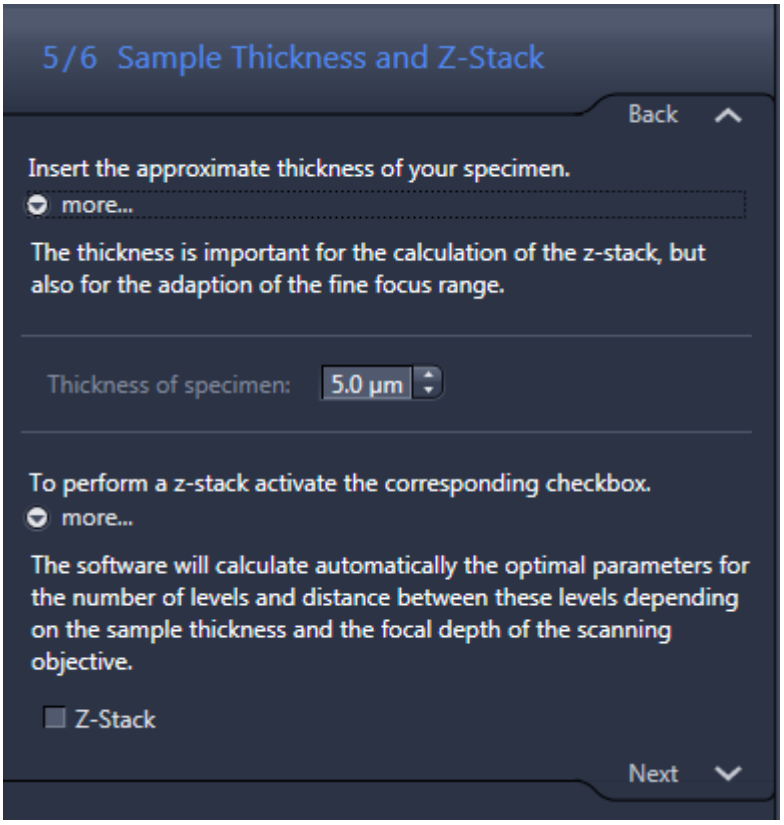
8 Once the specimen is focused, you should visit a region that does not include part of the specimen inside the field of view. You can now adapt the settings until you achieve a suitable appearance, meaning you have no overexposure and no significant underexposure. Please refer to the Display curve to check the intensity distribution.

i INFO

Please keep in mind that adjustments to the flash intensity are fine, while those to the flash duration are coarse. The flash duration should not exceed 10 µs.

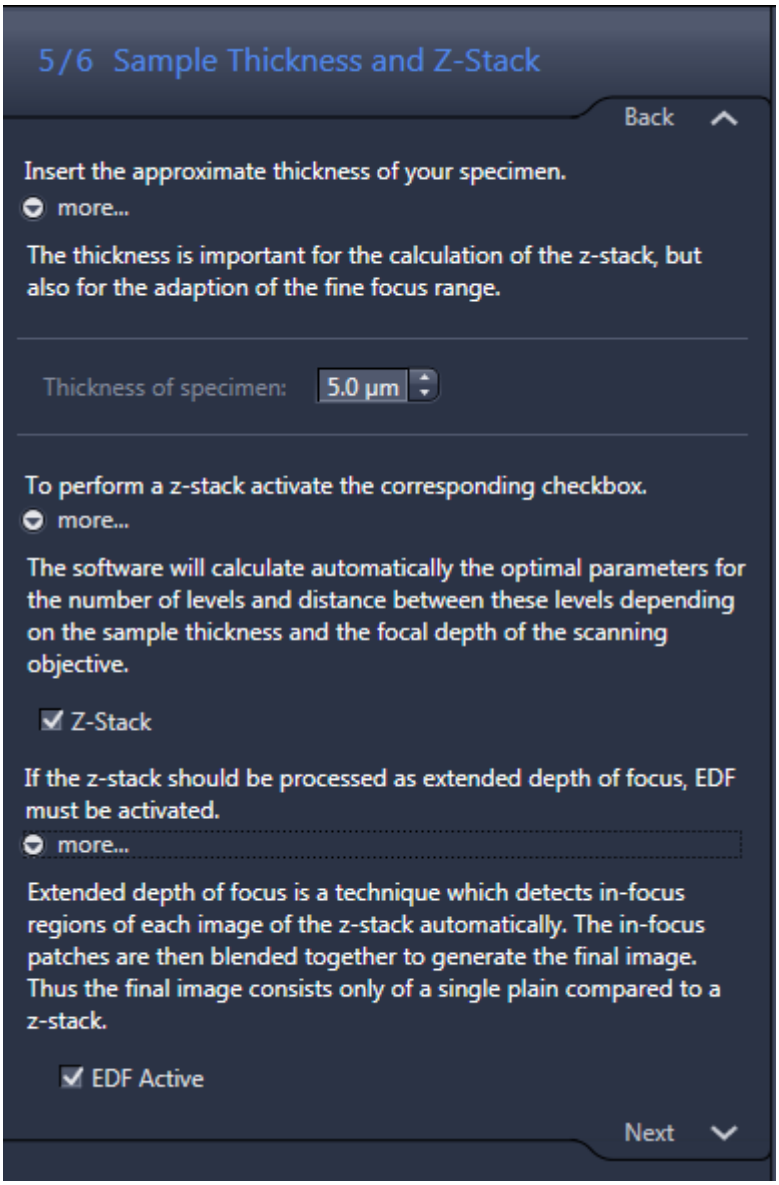
9 The next step is to select the **Sample Thickness** and **Z-Stack**. To use a z-stack, activate the **Z-Stack** checkbox. The thickness is used to calculate not only the z-stack parameter, but also the fine focus range. This means that even if no z-stack is activated, this value has to be double-checked and adjusted.

10 If the user was already working on a profile from this pool in the smart profile selector, the z-stack can be activated if this is necessary based on the application. Nevertheless, the parameters also have to be double-checked. In this case, the parameter is the thickness of the specimen.



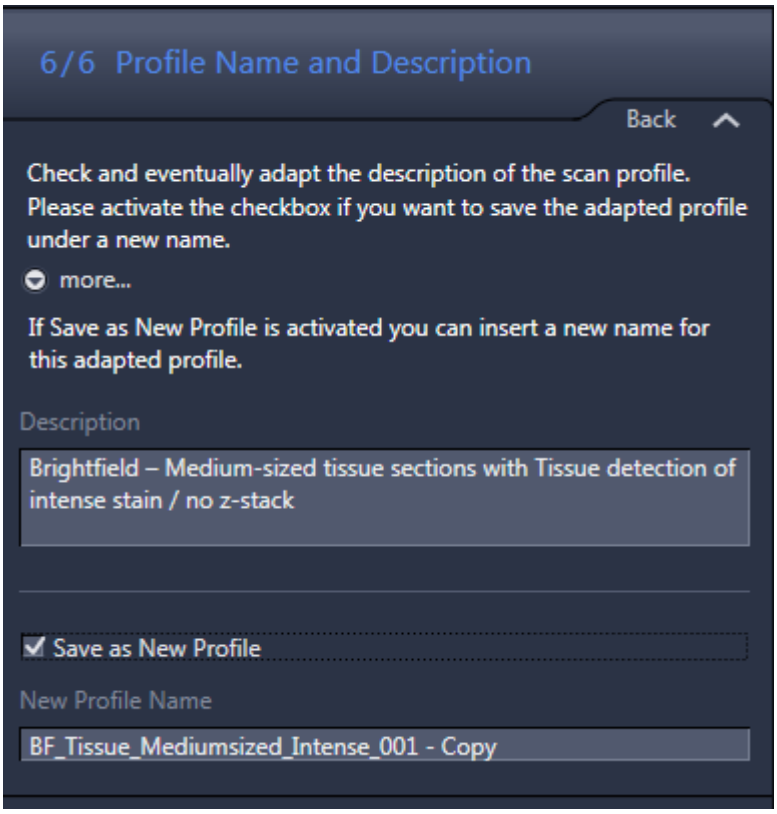
Based on this thickness, the software will calculate the number of focus levels and the distance of these levels based on the objective used.
Finally, you can decide if the z-stack should be conserved or if an **EDF**

(Extended Depth of Focus) should be applied. If so, the **EDF** checkbox must be checked.



11 The final step is **Profile Name and Description**. Here you can save the scan profile under a new name if the **Save as New Profile** function is activated. You can also change the description or provide a new one. It is recommended to

change at least the description of the profile so that it is possible to track the basic setup of the profile with ease.



12 Click on the **Finish** button to save the changes and close the wizard.

You have successfully set up a brightfield scan profile.

22.3.2.2 Importing names, profiles and scenes

> **INFO**
>
> Before you start the import you have to insert the trays with the slides first in the magazine as only with this information the system can make logical double-checks.

The import is done via a comma-separated values file (csv). For each slide one row has to be used. Each line for each defined slide should have following format:

- Tray number (absolute),
- slide number (absolute),
- image name,
- profile name,
- scene1, scene2, scene3, etc.

Tray and slide number must be inserted the other parameters are optional. It is possible to skip certain entries and these entries will not be changed by the import. The naming of the scenes starts at the upper left object and continues to the right side. After all objects are assigned with a specified scene name the software will start at left at the second line of objects and continues to the right side, etc.

Example for the scene name order assignment:

1	2	3	4
5	6	7	8
9	10	11	12

Example A

1,1,Mouse1,Profile001,brain1,brain2,brain3

For the first slide of the first tray the system will define Mouse1 as image name and will apply the Profile001. If the preview detects several scenes the system will name the first scene as brain1, the second scene as brain2, etc.

Example B

1,1,Mouse1

1,2,Mouse2

1,3,Mouse3

Here the system will only apply the image names to the specified tray and slide position.

Example C

1,1,,Profile001

1,2,,Profile002

1,3,,Profile003

Here the system will only apply the profile names to the specified tray and slide position.

Notes

- If in the import file a profile is specified which is not in the list of the available profiles, the software will generate a warning and the profile will not be applied.
- If in the import file a position is defined which is not occupied by a slide the software will generate a warning and will skip the import for this slide position.

- If you defined more scenes than the system detects during the preview the system will not generate a warning, the system will stop the naming assignment after the last detected scene.
- If you defined less scenes than the system detects during the preview the system will not generate a warning, the system will name all the scene where names are available and will stop the naming but all scenes will be scanned.
- By default the comma (,) is used as separator. It is possible to select another separator (tabulator, semicolon, space) via Tools -> Options -> Acquisition -> Scan

22.3.2.3 Using the Label Scan Settings with 4"x3" slides

If you inserted 4”x3” slides the Label Scan Settings section will have some additional functions. This is based on the fact that the preview image for the label will be created by two images from the preview camera.

INFO

In this mode for the 4”x3” slides the **Recognition** and **Label Orientation** expander is only available in the **Snap** mode, thus the two stitched images are seen.

If you open the step the system will activate automatically the **Live** mode for the upper image.

With buttons **Upper Side** and **Lower Side** you can toggle between a live view from the two views.

Here you can judge the exposure time, white balance, light intensity and adapt these settings which are listed in the expanders below the graphical user elements. This handling is the same as for the other type of slides (1”x3” and 2”x3”).

Once the settings are done (normally the standard settings should work and an adjustment is not necessary) you can switch now to the Snap mode by clicking on the **Snap** button.

Now the system will create two images and stitch these two images together. This image will be presented and the user can adapt the captured area via the red frame. The content of the red frame will be captured during the preview / scan of the slide and will be stored together with the image.

INFO

The other settings like **Recognition** are the same as for the other slide types.

22.3.2.4 Using the Preview Scan Settings with 4"x3" slides

If you insert 4”x3” slides the section will have some additional functions, this is based on the fact that the preview image for the specimen area will be created by two images from the preview camera.

If you open the step the system will activate automatically the LIVE mode for the upper image

With buttons **Upper Side** and **Lower Side** you can toggle between a live view from the two views.

Here you can judge the exposure time, white balance, light intensity and adapt these settings which are listed in the expanders below the graphical user elements. This handling is the same as for the other type of slides (1”x3” and 2”x3”).

Once the settings are done (normally the standard settings should work and an adjustment is not necessary) you can switch no to the Snap mode by clicking on the button **Snap**.

Now the system will create two images and stitch these two images together. This image will be presented and the user can adapt the captured area via the red frame. The content of the red frame will be captured during the scan of the slide and will be stored together with the image.

i INFO

The other settings, like **Recognition**, are the same as for the other slide types.

22.3.2.5 Implementing your own tissue detection algorithm

You have the possibility to integrate your own tissue detection algorithm with in the standard workflow. This realized via an extension written in C# the extensions has to be provided as a dynamic linked library (DLL). The wrapper has to be in C#, the code underneath for the tissue detection can be of a programming language of your choice.

The basic workflow will be that the system created a CZI and provide this image to your extension. Based on this image your algorithm should perform the tissue detection and as a feedback for the standard workflow you will provide the image with annotations. These annotations will mark the regions to be scanned.

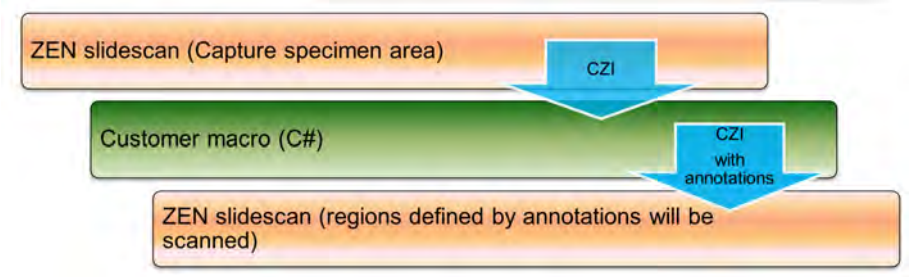


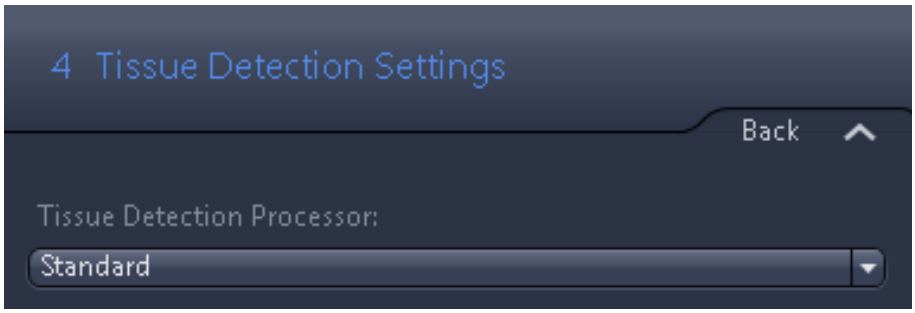
Fig. 22.13: Basic workflow

Procedure

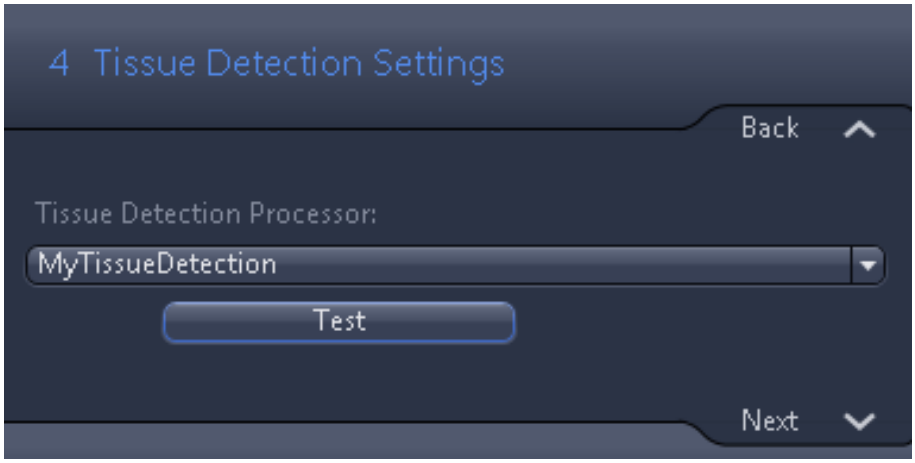
1. Close ZEN, if its still running.
2. If necessary create a directory with the name **Extensions** under **C:\Program Files\Carl Zeiss\ZEN 2\ZEN 2 (blue edition)**
3. Copy your DLLs in this directory

> **INFO**
>
> Steps 1 to 3 has to be executed only the first time.

4. Open ZEN
5. Now you will see a new entry under **Tissue Detection Processor** with in the tissue detection section of the wizard as pull-down menu (this pull-down menu is only seen if the software finds DLLs in the directory).



6. Standard is the default tissue detection built-in with the ZEN slidescan, thus you will see still the tools from the specific tissue detection set-up
7. Now you have to select your tissue detection algorithm via the pull-down menu.



8 If you select your extension all the other possibilities to adjust the tissue detection settings will disappear.

9 With the button **Test** you can execute a test with your algorithm to check the performance of your algorithm and if the implementation works.

INFO

For further information regarding our file format please visit **www.zeiss.com/czi** and register to receive the necessary information.

22.3.2.6 Using barcode to define name, profile and subfolder

If the you want to use the information from a barcode to define the name of the image, the profile used or the sub-path, you have to activate the function **Name and Profile from barcode**. It is located in the **Naming Definition** tool in the Left Tool Area.

A barcode can be used to define the output subdirectory, the name and the profile to be used. To do this, the positions of the characters in the string (=barcode) are defined in the mask fields. It is possible to define areas using the character –. Furthermore, characters located at different places of the string may be combined by means of a comma.

Example A

Character string: AXIO_SCAN_123

Mask: 1-4 AXIO

Mask: 6-9 SCAN

Mask: 1-4,11-13 AXIO123

Example B

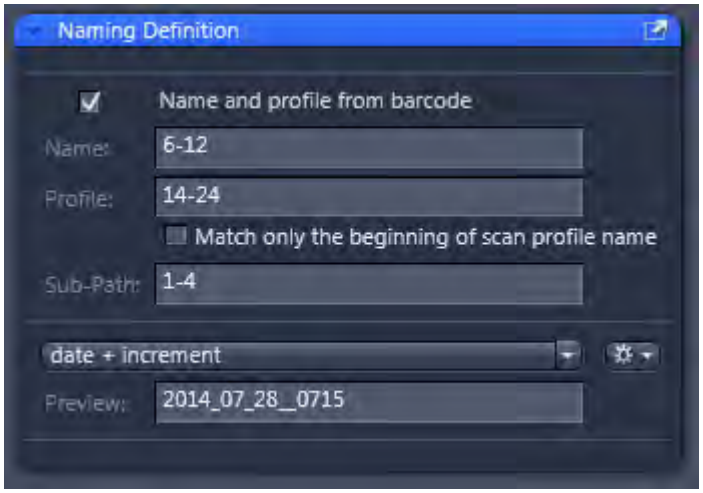
Barcode: Path_DoeJohn_003_FAST_HE

Path is the path for saving

DoeJohn is the name of the image

003_FAST_HE is the profile name

The barcode control for the example above would have to look like this:



It is not necessary to utilize all three possibilities, you can also use only one or two of these possibilities. If you do not want to use a certain setting please leave this setting simply empty.

If under **Name** a range is defined the software will insert under **Naming** (in the **Magazine** view) the text **Name from barcode**, as the system does not know until now the barcode once the barcode of this slide is read the system insert the correct name. The same applies for the profile, in this case the text is **Profile from barcode**.

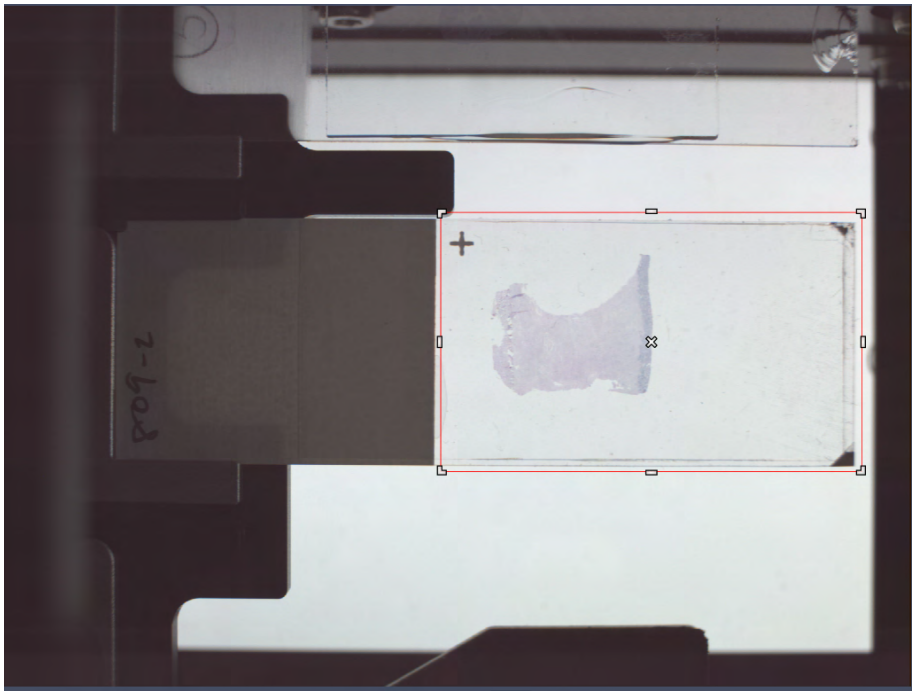
If the profile is derived from the barcode the system will take the label capture information (this includes the kind of barcode, etc.) from the profile selected under **Default Scan Profile** on the Scan tab.

In administrating and creating profiles, you will prefer descriptive names to recognize them easier (e.g.: FF04_CF08_HE_23). However, coding of such profiles in the barcode is very memory-intensive and also prone to errors. For this case, the option **Match only the beginning of scan profile name** may be enabled. Then, the system will use only the first characters to identify a profile. The name may begin, for example, with an unequivocal number used as identification. The profile could be named, for example, 005_FF04_CF08_HE_23. You would then enter 1-3 into the profile mask and enable the option *Match only the beginning of the profile name*. Now, only the number 005 would be used to identify the profile. Make sure that only one profile name beginning with the characters 005 exists.

22.3.2.7 Setting up the ROI for the preview image

An important adjustment to be made is the definition of the ROI (Region Of Interest) for the preview image of the specimen area. The system will take this region of interest and capture it. The tissue detection (if selected) will also be

applied to this region of interest. This includes the saved image along with the scanned image and the image of the label area. The region of interest can be adjusted in the **Live** image via the red rectangle:



The red rectangle can be moved by dragging it. The size can be changed by moving the cursor to the edge or the borders, clicking and holding the left mouse button, and moving the edges or borders of the rectangle until the appropriate size is achieved.

i INFO

For best results the red frame should cover the complete slide and some air around. Especially for the detection of an empty area on the slide, for the automated shading correction on glass, it is important that the red frame is not set too small (e.g. around the tissue only). Otherwise the sample could be considered as empty region. This would result in a sup-optimal shading correction. If you want to limit the range for the tissue detection use the red frame in the **Tissue Detection Settings** section in the sub-sequent step. Avoid that the red frame covers the label area of the slide.

i INFO

You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the specimen area is displayed in the Magazine view!

22.3.2.8 Creating a Shading Reference from Tile Image

The function **Shading Reference From Tile Image** is used to generate shading reference images. The function will create for every channel of the processed image a separate shading correction image. This image can be stored directly to the Calibration manager. Note that this processing function cannot process time lapse-images and images with z-stacks.

> **INFO**
>
> As we need around 300+ tiles this function is limited mainly to a calibration with an objective with a magnification of 20 and higher. Other objectives (especially 2.5x and 5x) need still the manual calibration via the **Locate** tab.

Prerequisites

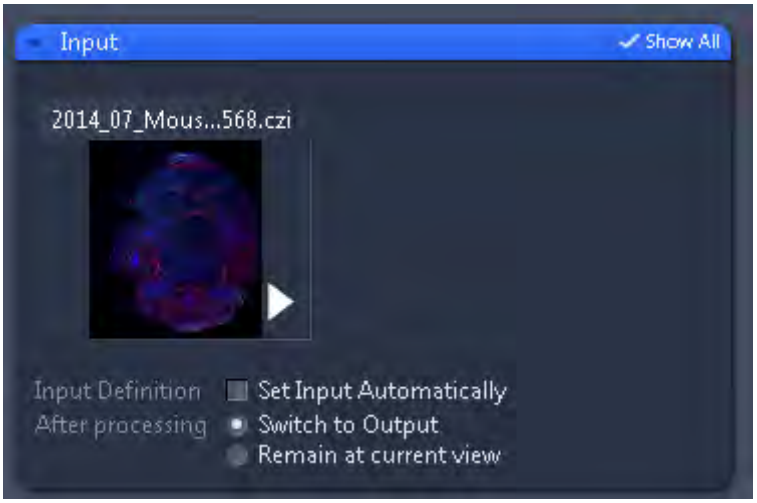
- You have created an image with all the channels for which you want to create a shading reference image for. For a good reference image it is necessary to have a number of over 300 tiles per image / scene.
- Avoid any over- or underexposure in the resulting image. If the signal is only very dim it is possible to use the functions **Multiply Factor** or **Autoadjust Intensity** thus the resulting reference image get brighter. Depending on the camera type you need a certain degree of coverage of the dynamic range.
- For the **Orcaflash** camera it should be at least 30% of the total dynamic range. Otherwise the resulting reference image will not be accepted as a valid reference image. For color cameras used for brightfield the minimum value would be 40%.
- Be aware that the acquisition must be done **without** activated Shading correction.
- The use of fluorescence slides for the creation of reference images for fluorescence has sometimes disadvantages (e.g. sparse distribution of the signal, bleaching). Thus it has to be considered to use at least for standard fluorescence channels (like DAPI, GFP, Cy3) a standard H&E sample and utilize the autofluorescence of this sample.
- As it is advised to scan without overlap, besides the other above mentioned settings, the Smart Profile Wizard provides a special profile for this purpose. Open the Smart Profile Wizard - Select Reference Slides and subsequent FL Slide for shading correction. Use this profile as a start profile for this kind of calibrations.
- As it is advised to scan without overlap, besides the other above mentioned settings, the Smart Profile Wizard provides a special profile for this purpose. Open the **Smart Scan Profile Selection** Wizard, select **Reference Slides** and subsequent **FL Slide** for shading correction. Use this profile as a start profile for this kind of calibrations.

Procedure

1. Open the image with ZEN software.

2 In the **Processing** tab under **Method** open the function **Shading Reference From Tile Image**.

3 In the **Input** tool open the created image.



4 Set the desired parameters. For a detailed description of the parameters , see chapter Shading Reference From Tile image.

5 Independent from the imaging modalities you can see a slider / spin box if the image contains more than one scene.

6 In the **Output** tool define the name of the created files (this has no influence on the naming if the files are stored directly to the calibration manager). The functionality is the same as for the other image processing functions offering the naming possibility under **Naming…**.

7 Click on **Apply** button to start the processing function.

INFO

If you have a multi channel image and you define for at least one channel the attribute Skip Channel you will still see under **Output** all channels from the selected image.

22.3.2.9 Shading Reference from Tile Image

With this method you can create shading reference images for multi-channel tile images.

Parameters (Brightfield Image)

For a **Brightfield** image you can select following parameters:

Note that the parameter **Selected Scene** and the checkbox **All Scenes** are only available if you process a multi-scene image.

Parameters	Description
Save directly as Shading Reference	**Activated**: The software creates the shading reference image and stores it directly to the Calibration Manager (Shading Reference). The software provides no possibility to check the image before they will be saved, thus it is recommended to deactivate the checkbox and execute this function. The system will create the reference images and present it to you. If the images have a good quality, you can activate the checkbox and run the function again.

Parameters	Description
	Note that, if you activate this option it is important that you check afterwards the system messages (the "i" in the lower part of the screen). If e.g. the resulting shading reference image is too dim the system will not use it as valid shading reference image, this will be shown under the system messages. Also if the import of the reference database were successful this will be shown!
Channel-specific	**Activated**: The software performs channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. The following components will be considered: Contrasting method and condenser, fluorescence filter, magnification: Objective and Optovar; camera bit depth and RGB/BW mode, camera type and port position. **Deactivated**: The system creates an **All Channel** calibration and perform an objective specific shading correction. The following components will be considered: magnification (Objective and Optovar); Camera bit depth and RGB/BW mode and camera type and port position
Multiply Factor	Here you can apply a multiply factor, thus the software will multiply the pixel intensity for each pixel of the shading reference image by this value. If you use an own sample it is mostly the case that the images are very dim and the intensity does not reach the value needed to be used within the shading reference calibration manager, thus it will be rejected.
Auto Adjust Intensity	If activated, this option calculates automatically the multiply factor based on the gray values of the image and the needed gray values for using it in the shading reference calibration manager. If activated, the setting for **Multiply Factor** has no influence anymore on the image generation.
Apply Gaussian Filter	If activated, a Gaussian filter is applied after the averaging of the field of views from a tiled image is done. This enables to smooth the shading reference image. The **Sigma** factor defines the strength of the smoothing.

Parameters	Description
	Use this filter and the Sigma factor very carefully as it could remove also features which are real shading structures. This feature could be recommend if the number of tiles in the scanned image is low and cannot be increased for certain reasons

Parameters (Multichannel Image)

For a multichannel image (e.g. fluorescence image) all parameters (description see above) can be adjusted channel specific.

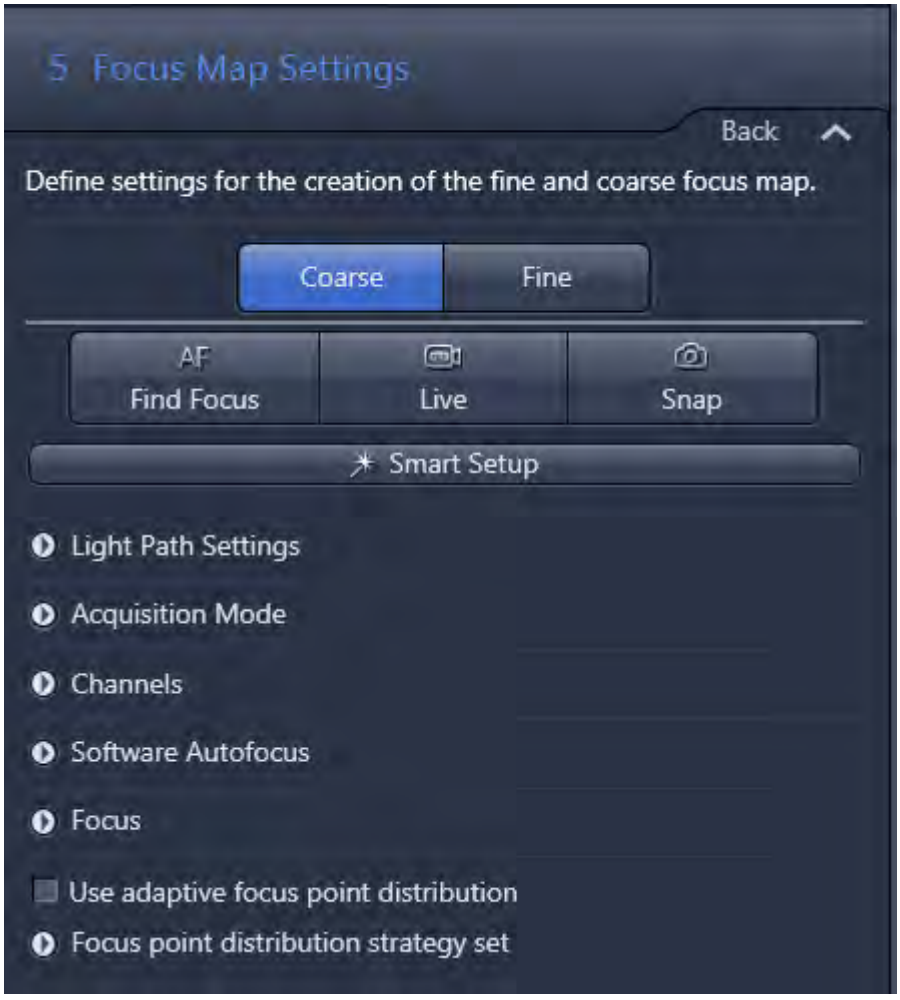
Note that the parameter **Selected Scene** and the checkbox **All Scenes** are only available if you process a multi-scene image.

Parameters	Description
Adjust per Channel	If activated, you can adjust the settings for every channel separately. To use a channel select **Process Channel** or select **Skip Channel** if you don`t want a channel to be processed. The settings **Save directly as Shading Reference** and **Channel-specific** are applied separately for each channel. If you want to use the same settings for all channels deactivate the checkbox.

22.3.2.10 Focus Map Settings (FL)

22.3.2.10.1 Adjusting Coarse Focus Map Settings (FL)

This chapter explains how to adjust settings for the **Coarse** focus map settings in the **Advanced Scan Profile Wizard** for fluorescence profiles.



Prerequisites

- In Step **1 Global Data** under **AF (Autofocus) Contrast Type Coarse** you have selected **Channel**.

Procedure

1 Start **Smart Setup** and define the channel you want to use for focusing. For more information, see *here* [▶ 630]. A good channel for coarse focus is defined by a short exposure time (to minimize bleaching and reduce the focus time) and it is very important that the fluorophore is evenly distributed over the specimen so that the software will always find a signal to focus on. After the configuration with **Smart Setup**, the software will automatically calculate the **Channel configuration**.

2 By default, the system will insert the objective which is in the first position of the objective revolver as the coarse focus objective. Under **Light Path Settings** you can change the objective, if necessary. Therefore activate **Before Experiment: Before Exp.** You can now see the light path configuration. Select another recommended objective (5x or 10x) and click on **Go!**. The system will put the selected objective in the light path.

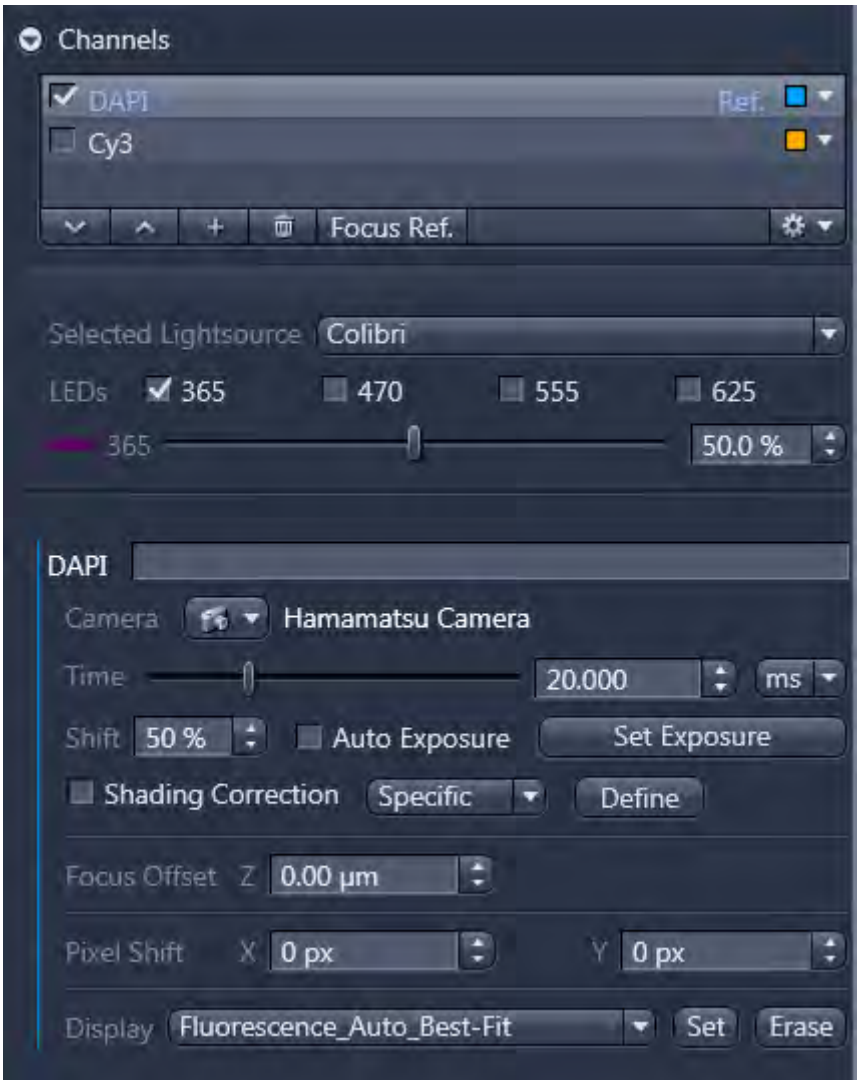
3 Under **Acquisition Mode** change the binning from **1x1** to **2x2** or higher as the image quality is not important for the focus. This will decrease the exposure time and thereby minimize the bleaching and the time for focus afterwards.

4 Now navigate to the specimen with the **Stage** tool. You can also click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position.

5 Under **Channels** in the list, you can see all defined channels. If you highlight a certain channel, the hardware will change the light path.

6 To select a channel as Focus Reference highlight the channel and click on **Focus Ref.**. Make sure that this channel is selected via the checkbox and the others are de-selected. You can still select the light source if you have two

(e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source. Lower light source intensities normally result in less bleaching even if the exposure time is longer.



7 The next step is to focus on the specimens and adjust both the exposure time (**Time**) and light source intensities. To see a live image from the camera, you have to activate **Live**, which will display a live image, or else click on **Snap** to take a single snapshot of all selected channels. It is important to take extra care in Live mode as this can result in bleaching of the sample. It is therefore recommended to use this feature with caution and, if possible, to use a representative region outside the scanning region.

8 You can use the **Find Focus** (**AF**) button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you should use the manual focus control (under **Focus**). You

may also need to adjust the exposure time. (under **Channels** /**Time**) and/or the intensity of the light source(s).

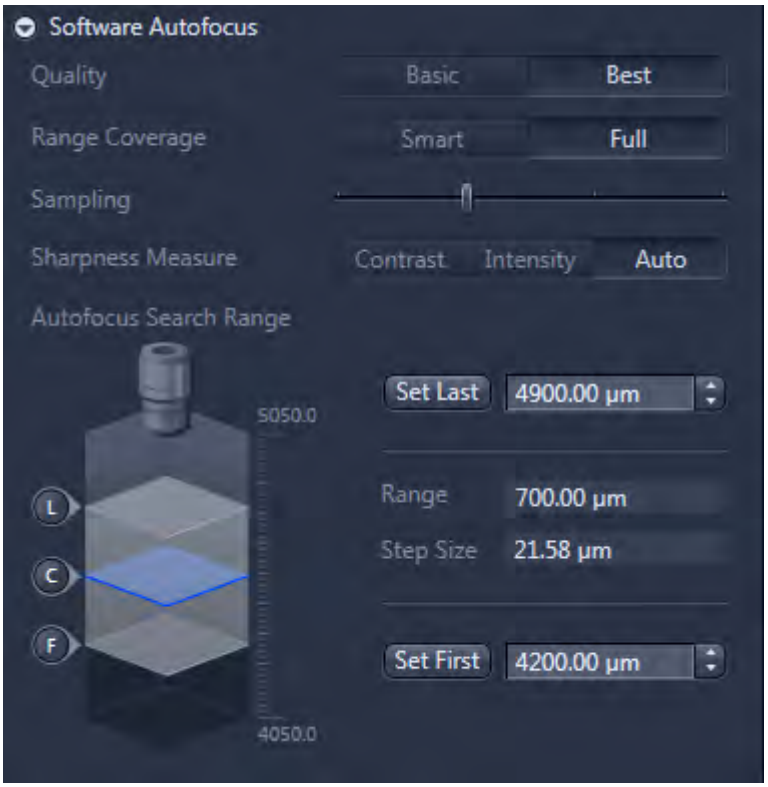
9 It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.

10 Once the focus has been established, you can then adjust the exposure time (under **Channels** / **Time**)or automatically by clicking on **Set Exposure**. The exposure time will be determined so that the range of the camera will be covered by the value stated under **Shift**.

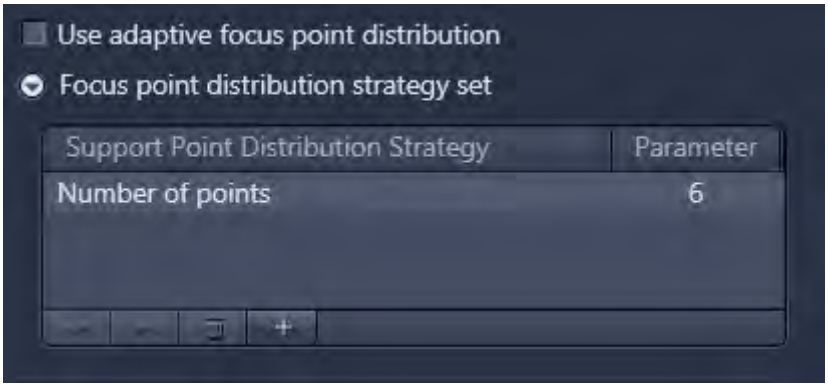
11 The next step is to set up the **Software Autofocus**. The setting under **Quality** defines the type of autofocus algorithm in use. The **Range Coverage** defines whether the complete z-stack is performed (**Full**) or if it is only captured until a local maximum is detected first (**Smart**). **Full** is the more robust principle but the **Smart** is the faster one. It is an option if the system detects two maxima with **Full**. The **Sampling** will define the step size of the z-stack. The currently selected value can be seen under **Step size** (the available range and steps depend on the objective and other optical parameters). A step size of approx. 20 µm is recommended for 2.5x and 5x, and approx. 15 µm for 10x. The **Sharpness Measure** defines whether the systems are using the average **Contrast** for determining the autofocus or **Intensity**, or if the software decides which of the two are used (**Auto**).

12 Look up the current z-position (under Position, in our case 4550 µm). This z-position should be in the range specified under **Set First** and **Set Last**. In our case, it fits very nicely in this range. If it does not fit within the range, you have to adjust the range. You can increase the number of slices (under **Slices**). This

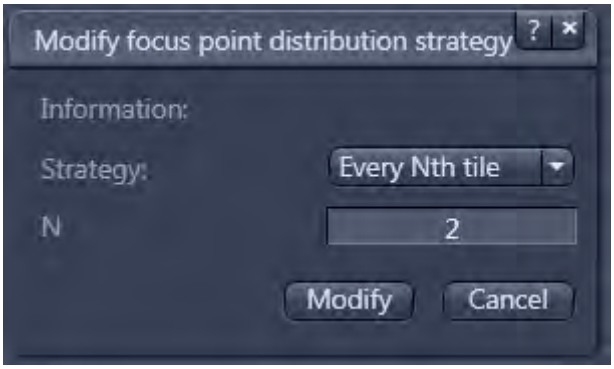
will increase the range, thus the focus will take longer. Otherwise, you can shift the range by changing **Set First** and **Set Last**.



13 The next step is to adjust the **Focus point distribution strategy set**. The default setting (**Number of points** = **6**) is the best value for a wide variety of specimen (in the table view you can see only one parameter if you want to see also the other parameters (if available; e.g. for Onion skin) double-click on the entry). An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended. A description of all strategies available can be found in the chapter *Focus point strategy set* [▶ 1042].



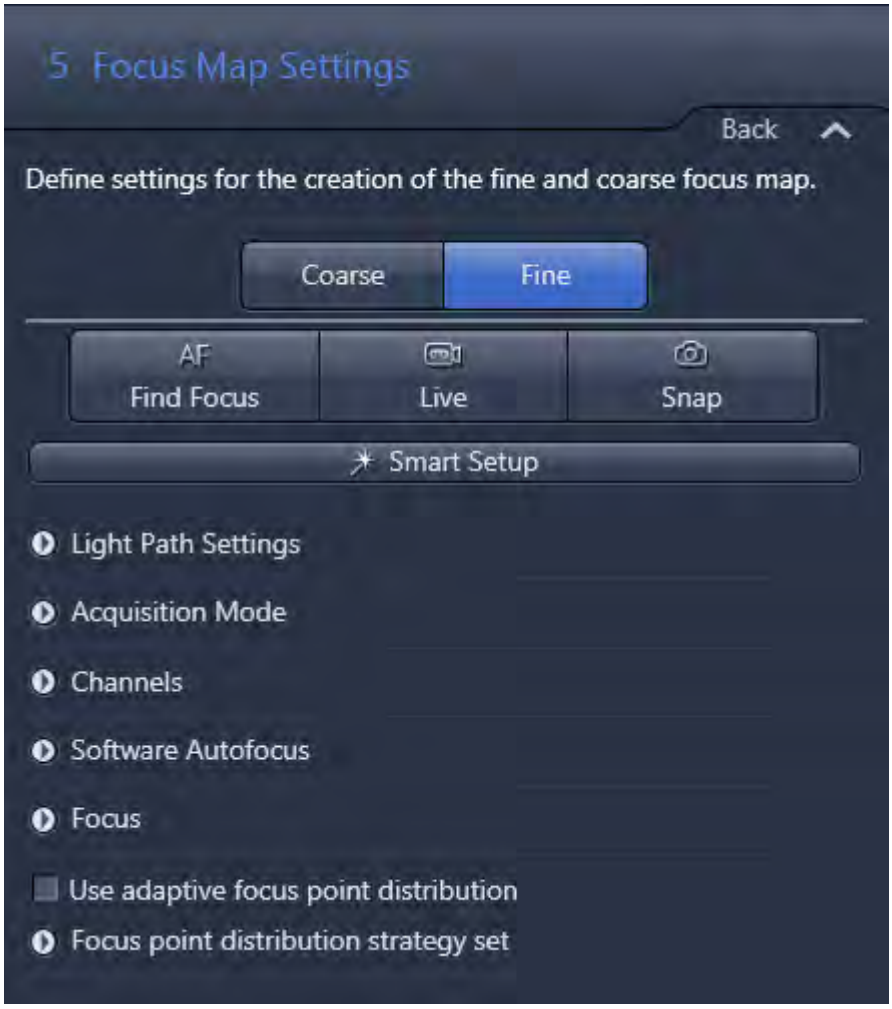
14 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another strategy via the dropdown menu and you can adjust the parameters for this setting. If you click on **Modify** the changes will be applied.



You have adjusted the settings for the coarse focus map. You can now continue with adjusting the settings for the fine focus map in the same wizard step.

22.3.2.10.2 Adjusting Fine Focus Map Settings (FL)

This chapter explains how to adjust settings for the **Fine** focus map settings in the **Advanced Scan Profile Wizard** for fluorescence profiles. Note that you should adjust the Coarse Focus Map Settings first.



Prerequisites

- In Step **1 Global Data** under **AF (Autofocus) Contrast Type Fine** you have selected **Channel**.

Procedure

1. Start **Smart Setup** and define the channel you want to use for focusing. For more information on **Smart Setup**, see *here* [▶ 630]. For **Fine Focus**, use the same channel as you used for the **Coarse Focus**. After the configuration with **Smart Setup**, the software will automatically calculate the **Channel configuration**.
2. By default, the system will insert the **20x** as the fine focus objective. Under **Light Path Settings** you can change the objective, if necessary. Therefore activate **Before Experiment: Before Exp.**. You can now see the light path configuration. Select another objective and click on **Go!**. The system will put the selected objective in the light path. Note that the objective for the **Fine Focus** should be the same as the one you want to use for scanning.

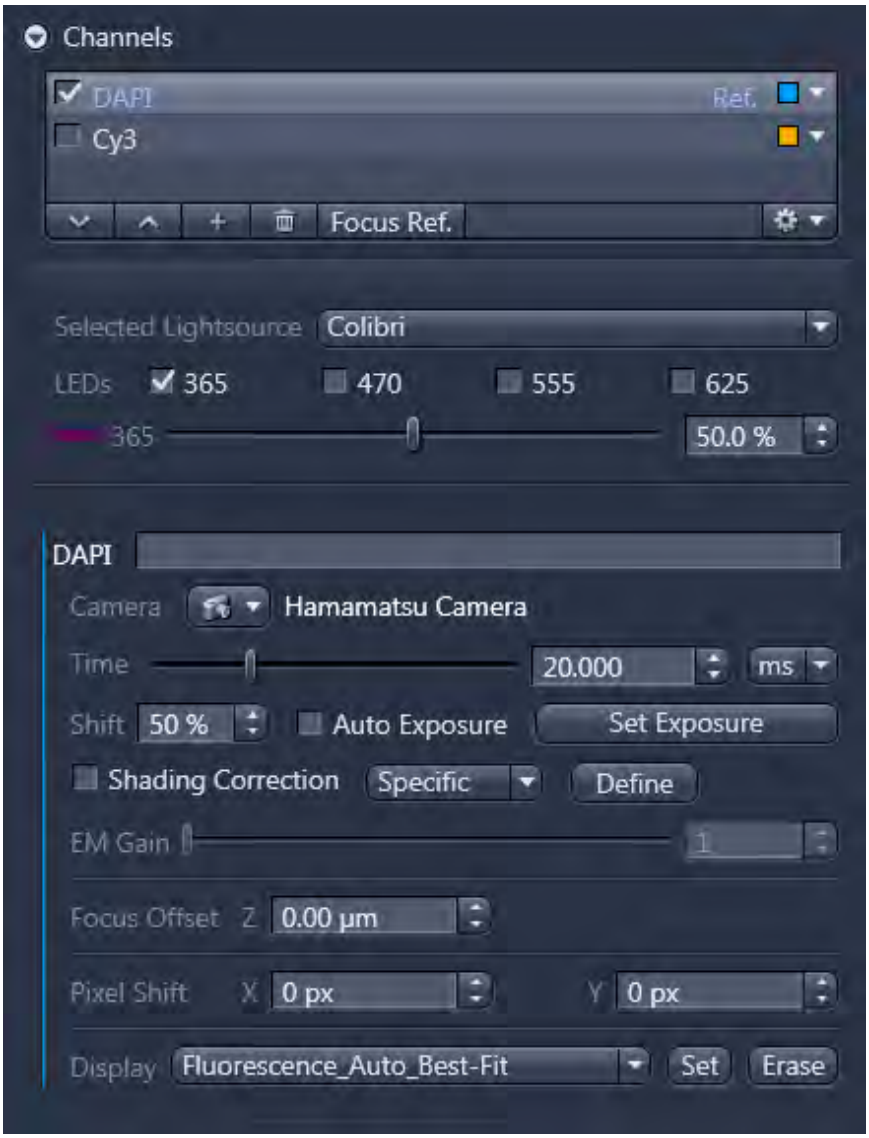
3 Under **Acquisition Mode** change the binning from **1x1** to **2x2** or higher as the image quality is not important for the focus. This will decrease the exposure time and thereby minimize the bleaching and the time for focus afterwards.

4 Now navigate to the specimen with the **Stage** tool. You can also click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position.

5 Under **Channels** you can see all defined channels. If you highlight a certain channel, the hardware will change the light path.

6 To select a channel as Focus Reference highlight the channel and click on the button **Focus Ref.**. Make sure that this channel is selected via the checkbox and the others are de-selected. You can still select the light source if you have two (e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source.

Lower light source intensities normally result in less bleaching even if the exposure time is longer



7 The next step is to focus on the specimens and adjust both the exposure time (under **Channels / Time**) and light source intensities. To see a live image from the camera, you have to activate **Live**, which will display a live image, or else click on **Snap** to take a single snapshot. It is important to take extra care in Live mode as this can result in bleaching of the sample. It is therefore recommended to use this feature with caution and, if possible, to use a representative region outside the scanning region.

8 You can use the **Find Focus** (**AF)** button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you should use the manual focus control (**Focus** section). You

may also need to adjust the exposure time. (under **Channels / Time**) and/or the intensity of the light source(s).

9 It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.

10 Once the focus has been established, you can then adjust the exposure time manually (under **Channels / Time**) or automatically by clicking on **Set Exposure** button. The exposure time will be determined so that the range of the camera will be covered by the value stated under **Shift**.

11 The next step is to set up the **Software Autofocus**. The setting under **Quality** defines the type of autofocus algorithm in use. **Range Coverage** defines whether the complete z-stack is performed (**Full**) or if it is only captured until a local maximum is detected first (**Smart**). **Full** is the more robust principle but the **Smart** is the faster one. The **Sampling** will define the step size of the z-stack. The currently selected value can be seen under **Step size** (the available range and steps depend on the objective and other optical parameters). A step size of approx. **2 µm** is recommended for **20x**. **Sharpness Measure** defines whether the systems are using the average **Contrast** for determining the autofocus or **Intensity**, or if the software decides which of the two are used (**Auto**). **Range** defines the search range of the autofocus based on the coarse focus found in the first step. If you use a 2.5x for the coarse focus, a search range of up to 250 µm for the fine focus is needed. If you use a 5x or 10x, the

search range can go down to around 80 µm. This also depends on the focus settings and the evenness of the sample.

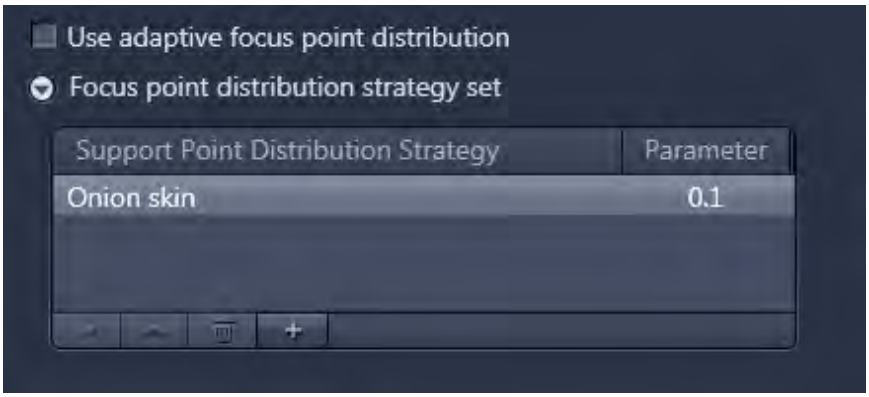
NOTICE

Risk of damaging the objective

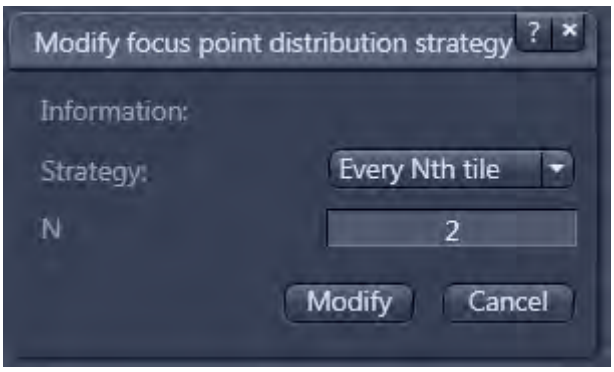
Based on the working distance of the objectives the maximum focus range should be **100 µm** for a **Plan-Apo 40x** and for every other objective a focus range of below **600 µm** is acceptable. This considers a typical cover slip thickness (170 µm) and a thickness of a specimen of around 10 µm. If you use only a fine focus range of 60 µm, the maximum thickness of the specimen can be 50 µm. A larger working range could lead a damage of the objective and in extreme cases to a damage of the slide.

12 The next step is to adjust the *Focus point strategy set* [▶ 1042]. The default setting (**Onion skin** with a density of 0.1 and a maximum number of 24 focus points; in the table view you can see only the parameter for the Density if you want to see also the other parameters (if available) double-click on the entry) is

the best value for a wide variety of slides with large specimens. An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.



13 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.



14 To double-check, click on **Find focus**. If all settings are correct, the system should focus on the sample without providing any warnings/errors. If you still experience problems, please double-check the settings above.

i INFO

The described procedure is applicable for specimens where the objects have an equal size. As the focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive focus point distribution, refer to the chapter Adaptive focus point distribution for more detailed information on this topic.

22.3.2.11 Adjusting Scan Settings for FL Profiles

Procedure

1 The first step is to adjust the **Light Path Settings**. This can be done manually or with the help of **Smart Setup**, see *here* [▶ 630].
We recommend to use **Smart Setup** as it not only includes double-checks, but is also very easy to use. Once you open **Smart Setup**, you add your dyes by double-clicking the dyes from within the extensive list available. The system then suggests one or more potential configurations. The number of

suggestions provided by the system depends on your hardware configuration. If you select a setup (e.g., **Best Signal**) the system will automatically adapt the Light Path Settings. **Smart Setup** allows you to define up to four channels. If you want to define more than 4 channels, you have to access the Light Path Settings and add additional channels manually.

By default, the system will insert the **20x** as a scanning objective inside the Light Path Settings. You can change the objective by opening the expander for Light Path Settings and activating the Before Experiment: Before Exp. You can now see the light path configuration. The objective should be checked and the 20x should be selected as this is the default setup. If you want to use another objective, select this and click on the **Go!** button. The system will then put the selected objective in the light path.

NOTICE

Risk of damaging the device

Use only the **Colibri.2** or the **HXP 120** in one scan, thus in the Scan Settings setup. The switch within the Colibri to switch between the LEDs and the HXP is not laid out for a large number of cycles.

INFO

You can select more channels than you actually need for the experiment to make the profile more universal, and you can select and deselect single channels at a later stage.

INFO

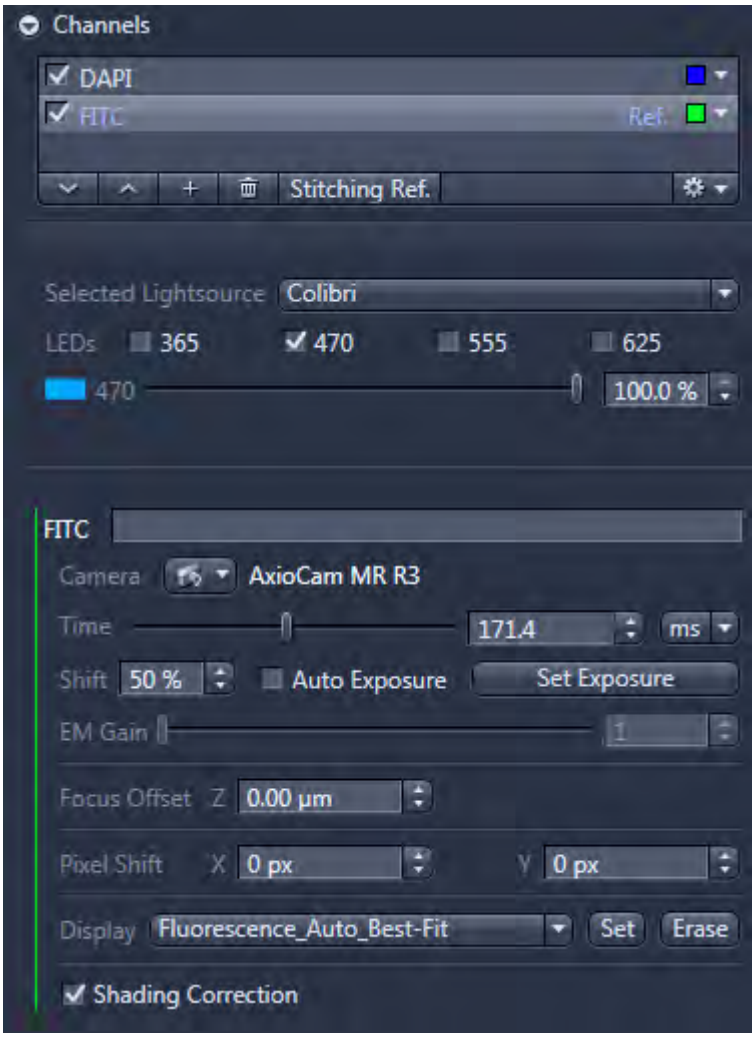
If you change the objective, ensure that you also use the same objective for your **Fine focus**.

2 Under **Acquisition Mode** you find advanced settings, but these are normally set to the optimal levels and do not require any manual intervention. E.g. you have the possibility to decrease settings such as the exposure time to minimize bleaching or even decrease the scan time. This can be achieved by increasing the **Gain** of the camera (if available for the specific camera) or increasing the **Binning**. However, it must be noted that this will decrease the resolution and/or increase the noise level of the image.

3 Navigate to the specimen with the **Stage** tool. You can also click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position.

4 Under **Channels** in the list, you can see all defined channels. You can activate (or deactivate) each channel by activating/deactivating he checkbox. If you highlight a certain channel, the hardware will change the optical light path. Here you must also define the channel which will be used for Stitching. This channel is marked with **Ref.**. To change the stitching channel, highlight a channel and click on **Stitching Ref.**. Make sure that the selected stitching channel is activated. An appropriate stitching channel is one in which the dye covers the specimen evenly (thus the system has enough structure for stitching). Large structures with fine details (such as cytoskeletons) are preferable if they are evenly distributed across the specimen. Under the

channels list you can still select the light source, if you have two (e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source.



5 The next step is to focus on the specimens and adjust the exposure time (under **Time**) and light source intensities. To see a live image from the camera, you have to activate **Live** view, which will display a live image, or else click on **Snap** to take a single snapshot of all selected channels. It is important to take extra care in Live mode as this can result in bleaching of the sample. We recommend to use this feature with caution and to use a representative region outside the scanning region.

6 You can use the **Find Focus** (**AF**) button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you can use the manual focus control (under **Focus**). You may also need to adjust the exposure time. (**Time**) and/or the intensity of the light source(s).

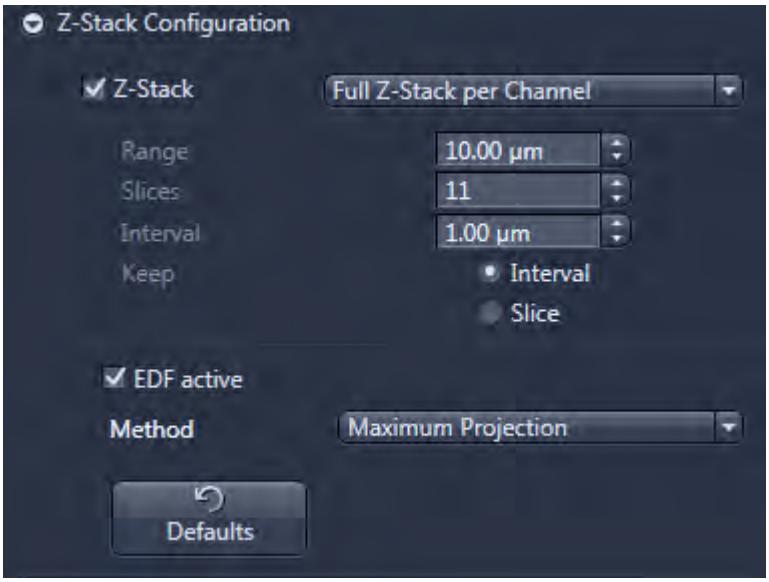
It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.

7 Once the focus has been established, you can then adjust the exposure time again (**Time**). This can be done manually by moving the **Time** slider or automatically by clicking the **Set Exposure** button. The exposure time will be determined so that the range of the camera will be covered by the value stated under "Shift".

8 Make sure to check several regions of the specimen (using Navigator or Stage control) to establish whether certain regions have a much higher fluorophore concentration. Repeat this step to determine the exposure time for all other checked channels.

9 The section **Z-Stack Configuration** is important if you want to automatically acquire a z-stack with or without extended depth of focus (EDF). This can be considered for uneven specimens or very thick samples. Note that – depending on the objective, condenser, and camera – a 20x Plan-Apo has a depth of focus of around 1 µm and the 40x Plan-Apo has a depth of focus of around 0.5 µm. The unevenness can be the result of a sub-optimal preparation or if the user aims to scan thick or cytological specimens.
The best way to evaluate the optimal settings is to go to the representative region of the specimen, autofocus from a blurry image to an image where the first structures come into focus, and note the z-value. Go through the specimens until the last regions of the specimens in the field of view are switching from being focused to blurry, then note the z-value once again. The difference between the z-values is the height of the z-stack. It is recommended to repeat this procedure for different regions of the specimen. The largest z-range will be used. Empirical research has shown that it is recommended to add 20% to the z-range as the autofocus is not always exactly in the middle of the specimen. It is now necessary to determine the step size based on the z-range. Theoretically, the step size is between the depth of focus and half of it, thus 0.5 to 1 µm for a 20x Plan-Apo objective. However, this would result in a larger number of z-steps and a low scanning speed. If you do not want to

preserve the z-stack and work with smaller image sizes, you can activate the extended depth of focus (**EDF active**). If active, the software will use the captured images in different focus planes for each image field and combine the regions of strongest contrast within given z-stack images into an image that contains the maximum contrast portions of all images recorded for this image field. This method guarantees maximum depth of sharpness. It is strongly recommended to use the standard parameters as these have been selected for their optimal fit. For a detailed description of the single parameters, please refer to the help for standard ZEN functionality of EDF. A scan with EDF is slightly faster than the same scan with a Z-stack. It is important to note that an extended depth of focus can also be applied at a later stage; however, if the extended depth of focus is applied in the profile then the z-stack cannot be restored afterwards.



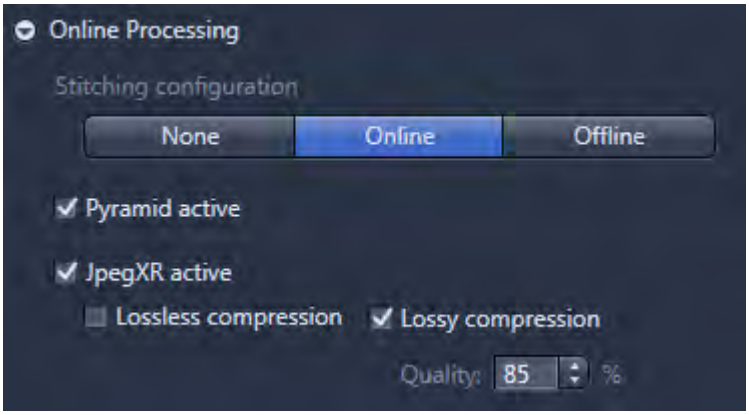
NOTICE

Risk of damaging the objective

Based on the working distance of the objectives the maximum range should be **50 µm** for a **Plan-Apo 40x** and for every other objective a range of below **300 µm** is acceptable. This considers a typical cover slip thickness (170 µm). A larger working range could lead a damage of the objective and in extreme cases to a damage of the slide.

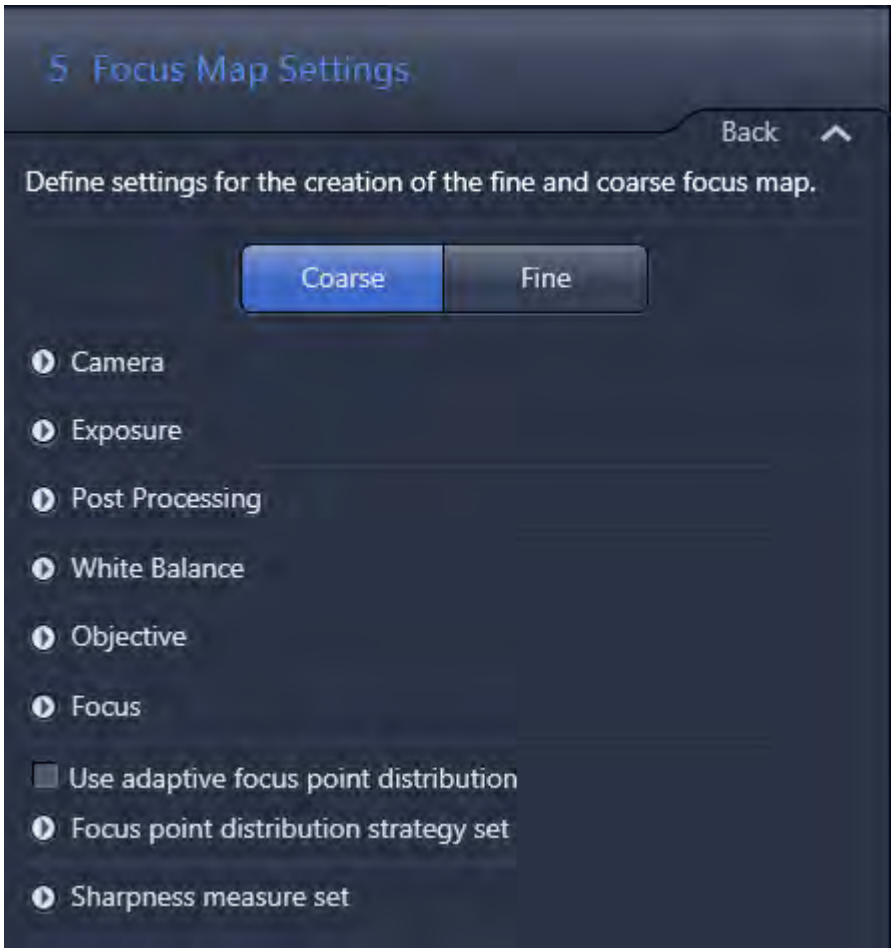
10 The final step to adjust is the **Online Processing** section. Here you can define whether the stitching is carried out **Online**, i.e., during the acquisition, **Offline**, or if no stitching (**None**) will be applied at all. The standard setting is online stitching as this provides the best performance in terms of the processing time of the slide.
The **Offline** mode will initially perform the scan, with the stitching applied at a later stage. This procedure is slower than the Online stitching. The difference is relative to the size of the specimen, i.e., if a specimen is very large, the offline

mode is very slow compared to the online mode. For smaller specimens, there is barely any difference in processing time. It is only possible to use no stitching (**None**) if the stitching is to be applied afterwards using ZEN measures or a separate program after an export. As stitching for fluorescence is critical, offline mode should at least be considered for small samples. If the offline mode is selected, the user has the possibility to activate **Fuse**. With **Fuse**, the system creates new tiling and thus the shading improves.
The option **Pyramid active** should always be activated, as this will speed up the viewing of the image afterwards. However, a pyramid can also be created afterwards. If a non-pyramid image of a certain size is opened, the software will always ask if the pyramid should be created.
These final settings allow you to adjust the compression which will be applied as part of the online processing. If **JpegXR active** is deactivated, the image will be saved uncompressed. If the **JpegXR active** is activated via the checkbox, you can save them lossless (**Lossless compression**), thus the image will only be compressed to the extent that no information is lost. If **Lossy compression** is activated, the compression always involves a loss of information. If this checkbox is activated, you can determine the degree of **Quality**.



22.3.2.12 Focus Map Settings (BF)

22.3.2.12.1 Adjusting Coarse Focus Map Settings (BF)



Procedure

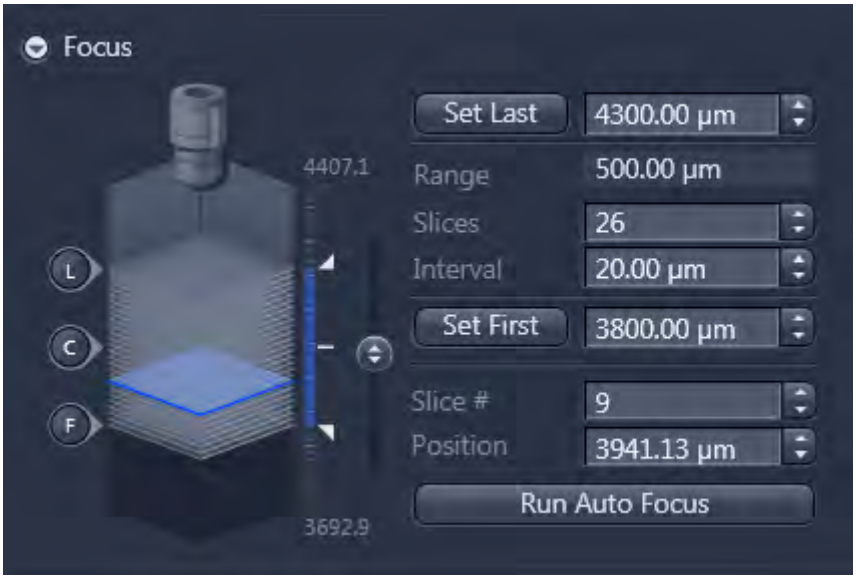
1. In the **Camera** section select the active camera. **NOTICE** **Select the Hitachi color camera here only. Do not select a monochrome camera or the AxioCam IC as this will result in a not working profile!**
2. Under **Objective** select the appropriate objective. In this case, a **5x** objective is recommended. If this is not available, use the 10x or 2.5x.
3. Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the

virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.

4 In the **Focus** section click on **Run Auto Focus**. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack.

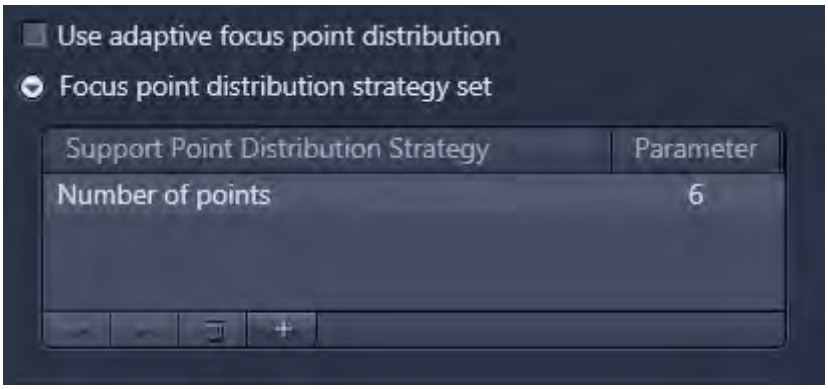
5 Once the specimen is in focus, double-check that the image is not too bright or too dark and that the white balance is correct.

6 Look up the current z-position (under **Position**, in our case 4200 µm). This z-position should be in the range specified under **Set First** and **Set Last**. In our case it fits very nicely in this range. If it does not fit within the range, you have to adjust the range. You can increase the number of slices (under **Slices**). This will increase the range, thus the focus will take longer. Otherwise, you can shift the range by changing **Set First** and **Set Last**. The following settings are recommended for the **Interval**: 2.5x: 20 µm; 5x: 20 µm; 10x: 15 µm.

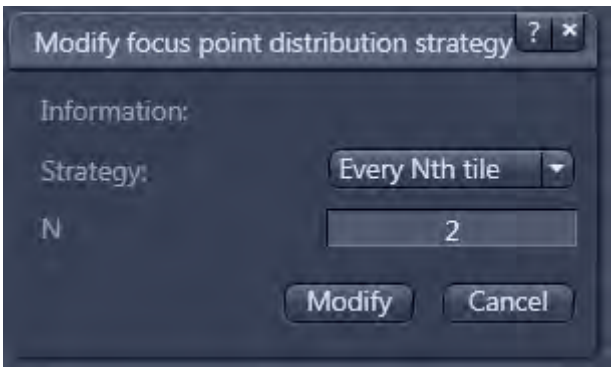


7 The next step is to adjust the *Focus point strategy set* [▶ 1042]. The default setting (**Number of points** = **6**) is the best value for a wide variety of specimen (in the table view you can see only one parameter if you want to see also the other parameters (if available; e.g. for Onion skin) double-click on the entry).

An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.



8 To change the strategy double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.



9 The last step is to adjust the *Sharpness measure set* [▶ 1059]. In our case, **Best** or **Basic** are the best choices for the coarse focus in brightfield.

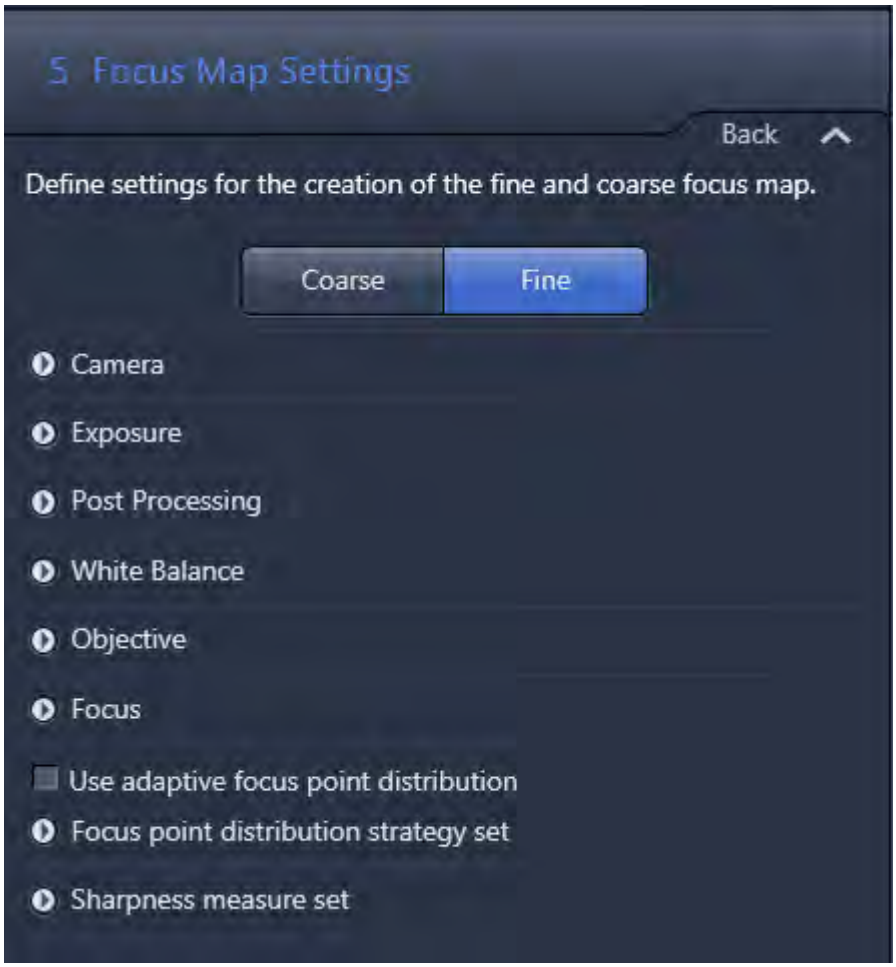
10 To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings. You can interrupt the focus run by clicking on **Stop Auto Focus**.

11 By default, the next step would be to adjust the **Fine Focus**.

i INFO

The described procedure is applicable for specimens where the objects have an equal size. As the focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive focus point distribution, refer to the chapter Adaptive focus point distribution for more detailed information on this topic.

22.3.2.12.2 Adjusting Fine Focus Map Settings (BF)



Prerequisites

- You have adjusted the **Coarse Focus** settings in advance, see *Adjusting Coarse Focus Map Settings (BF)* [▶ 1005]

Procedure

1. In the **Camera** section select the active camera. **NOTICE** **Select the Hitachi color camera here only. Do not select a monochrome camera or the AxioCam IC as this will result in a not working profile!**
2. Select the appropriate objective in the **Objective** section. This is the same objective that you would use for scanning the specimen.
3. Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the

virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.

4 In the **Focus** section click on **Run Auto Focus**. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack. If the Coarse focus step was executed previously and the software does not find a focus, this suggests that the focus parameters for the fine focus are not correct.

5 Once the specimen is in focus, double-check that the image is not too bright or too dark and that the white balance is correct.

6 You now have to adjust the **Range**. You could change the **Interval**, but it is not normally necessary to make any significant changes. The following settings are recommended for the **Interval**: **2.5x**: 20 µm; **5x**: 8 µm; **10x**: 4 µm; **20x**: 2 µm; **40x**: 1.5 µm), thus it is advised to change the number of **Slices**. The final optimal range depends on several parameters, such as the number of focus points, specimen evenness, and the objective used for the **Coarse Focus**. If 5x or 10x is used as the objective for Coarse focus, then the search range for the Fine focus can be around 70 µm. If you use 2.5x as the Coarse focus objective, the range should be at least around 150 µm.

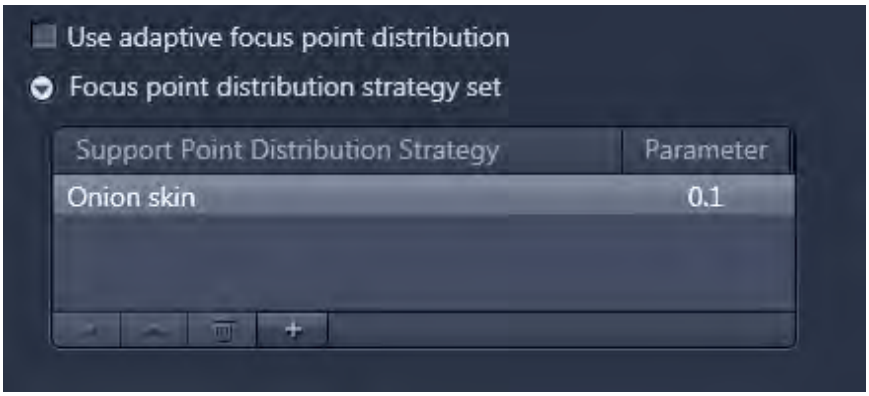
NOTICE

Risk of damaging the objective

Based on the working distance of the objectives the maximum focus range should be for a Plan-Apo 40x 100 µm and for every other objective a focus range of below 500 µm is acceptable. This considers a typical cover slip thickness (170 µm) and a thickness of a specimen of around 10 µm. If you use only a fine focus range of 60 µm, the maximum thickness of the specimen can be 50 µm. A larger working range could lead a damage of the objective and in extreme cases to a damaged of the slide.

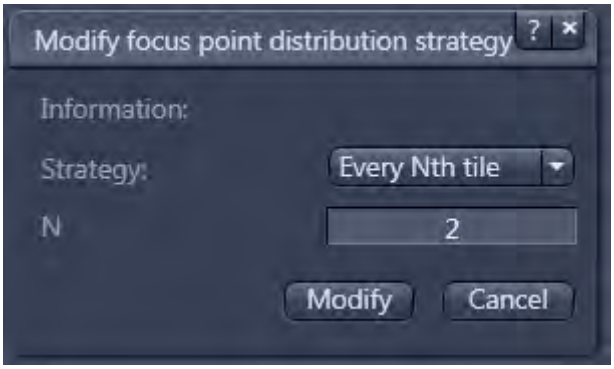
7 With the parameter **Offset**, you can apply a fixed offset value (in µm) to the focus map. This offset value will be added to all z-values of the focus map.

8 The next step is to adjust the *Focus point strategy set* [▶ 1055]. The default setting (**Onion skin** with a density of 0.1 and a maximum number of 24 focus points; in the table view you can see only the parameter for the Density if you want to see also the other parameters (if available) double-click on the entry) is the best value for a wide variety of slides with large specimens. An exemption could be Tissue Micro Arrays, for which the strategyy **Center of gravity** is recommended.



9 To change the strategy, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a

click on **Modify** the changes will be applied.



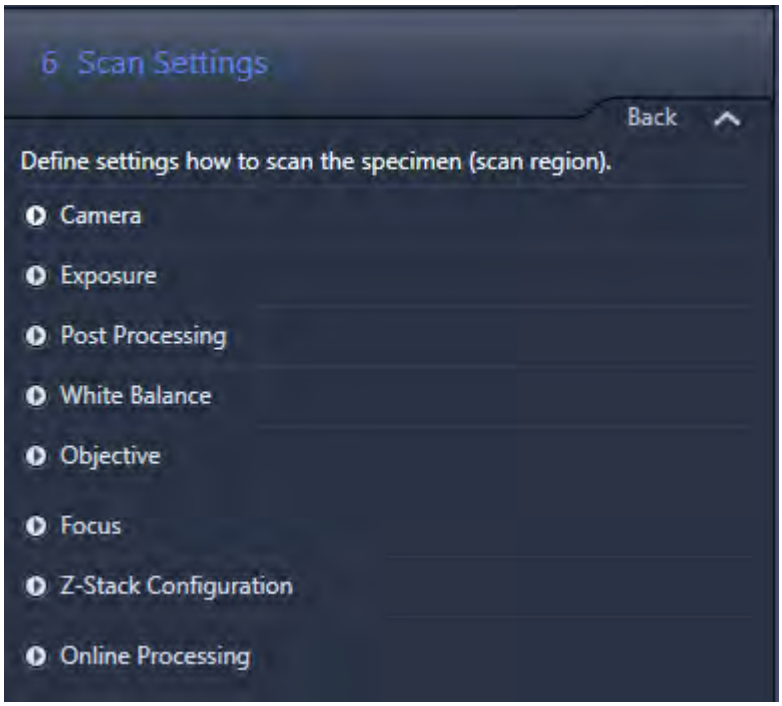
10 The last step is to adjust the *Sharpness measure set* [▶ 1059]. In our case, **Best**, **Basic**, or **HG 2^8** are the best choices for the fine focus in brightfield. The latter focuses more on the nuclei.

11 To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings. You can interrupt the focus run by clicking on **Stop Auto Focus**.

INFO

The described procedure is applicable for specimens where the objects have an equal size. As the focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive focus point distribution, refer to the chapter Adaptive focus point distribution for more detailed information on this topic.

22.3.2.13 Adjusting Scan Settings for BF Profiles

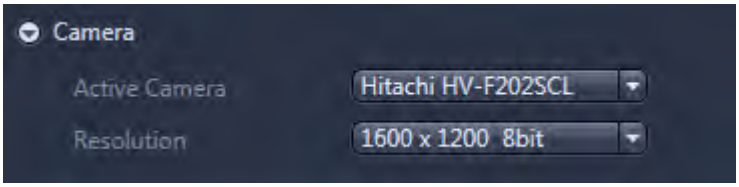


NOTICE

Under **Camera** do not switch to a monochrome camera or the AxioCamIC, as this will result in a not working profile.

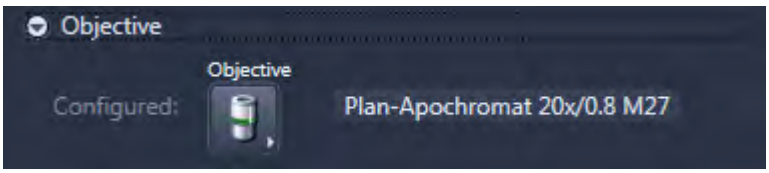
Prerequisites

- In the case of using the **Hitachi-HV-F202** camera, you can select whether you want to capture the image in 8-bit, 10-bit or 12-bit. Note that a bit depth above 8-bits will result in images with a much larger image size.



Procedure

1. Under **Objective** check whether the correct objective is activated. This should be the same as the objective used for the fine focus or at least an objective with a lower magnification.



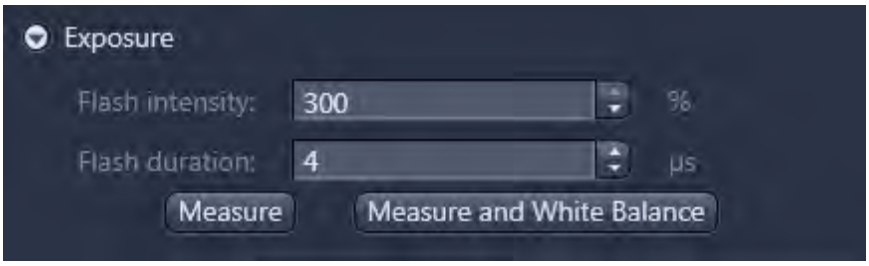
2. Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the

virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.

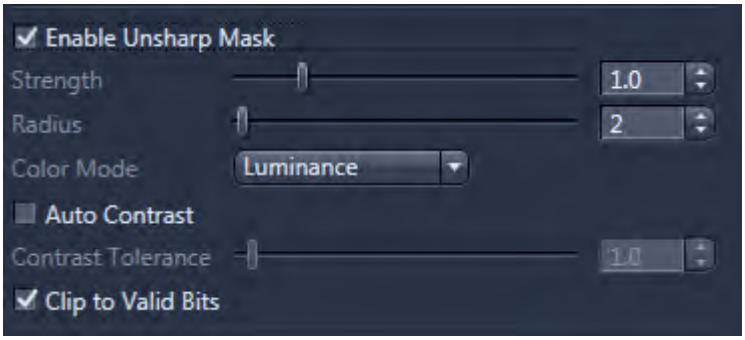
3 To be able to judge the quality, you first have to focus (under **Focus**). This section is only for focusing and has no influence on the focusing in the subsequent scan as the settings have already been made in the previous step. Use the **Run Auto Focus** button to focus automatically. If the autofocus is running you can interrupt the focus run by clicking on **Stop Auto Focus**.

4 To adjust the **White Balance** and the **Exposure** time, navigate to a region outside the specimen. Use the **Measure and White Balance** button in the **Exposure** expander to adjust the exposure and white balance automatically (the software will only determine the exposure automatically using **Measure**). If the intensity is inadequate, you can adjust the exposure under the **Exposure** expander. It is not possible to adjust the exposure time for the camera as the exposure time is fixed. You can only change the lamp settings. As the system is equipped with a flashlight LED, you can adjust the **Flash duration** and the **Flash intensity**. The flash duration is the duration for each flash, and the flash intensity is the intensity of the flash. The flash intensity can be increased up to 300%. With the flash intensity, you have a measure to make very fine adjustments to the resulting intensity. Changes to the flash duration constitute a coarse adjustment. In the case of manual adjustment, it is therefore advisable

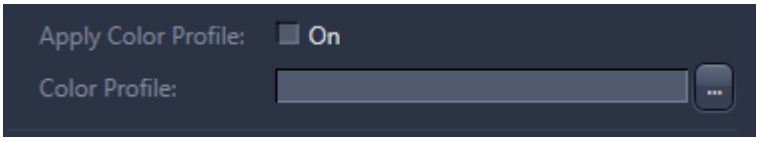
to start with a flash intensity of around 250% and adjust the flash duration to achieve a decent signal before increasing the flash intensity as appropriate.



5 For the description of the settings for **White Balance** and **Post Processing**, please refer to the general ZEN description of these features as these are the same. In the **Post Processing** section, you can enable **Unsharp Mask**. The unsharp mask will increase the contrast of the resulting images, but has to be applied carefully to prevent the images from looking artificial. This can be a particularly useful tool for a 40x magnification, as the 40x magnification provides oversampling and an unsharp mask could enhance the structures. If you apply sharpening, the scan speed will decrease slightly. Shading should always be activated.



6 In the **Post Processing** section, it is also possible to activate color correction if you activate **Apply Color Profile**. When you activate the **On** checkbox, you can select a color profile. The **srgb** color profile will be applied by default. If the software does not find a profile, it is very likely that the color profile was deleted or no color calibration was executed. In this case, you have to repeat the color calibration with the help of the **Axio Scan Calibration Wizard**.



7 In the **Z-Stack Configuration** isection you can automatically acquire a Z-Stack with or without extended depth of focus (EDF). This can be considered for uneven or thick specimens. Note that – depending on the objective, condenser, and camera – a 20x Plan-Apo has a depth of focus of around 1 µm and the 40x Plan-Apo has a depth of focus of around 0.5 µm. The unevenness can be the result of a sub-optimal preparation or if the user aims to scan thick or cytological specimens. The best way to evaluate the optimal settings is to go to the representative region of the specimen, focus from a blurry image to an image where the first structures come into focus, and note the z-value. Go

through the specimens until the last regions of the specimens in the field of view are switching from being focused to blurry, then note the z-value once again. The difference between the z-values is the height of the z-stack. It is recommended to repeat this procedure for different regions of the specimen. The largest z-range will be used.

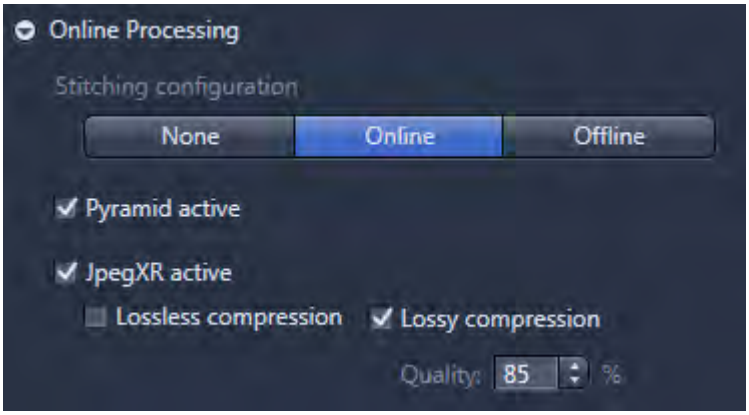
Empirical research has shown that it is recommended to add 20% to the z-range as the autofocus is not always exactly in the middle of the specimen. It is now necessary to determine the step size based on the z-range. Theoretically, the step size is between the depth of focus and half of it, thus 0.5 to 1 µm for a 20x Plan-Apo objective. However, this would result in a larger number of z-steps and a low scanning speed. If you do not want to preserve the z-stack and work with smaller image sizes, you can activate the extended depth of focus (**EDF active**). If active, the software will use the captured images in different focus planes for each image field and combine the regions of strongest contrast within given z-stack images into an image that contains the maximum contrast portions of all images recorded for this image field. This method guarantees maximum depth of sharpness. It is strongly recommended to use the standard parameters as these have been selected for their optimal fit. For a detailed description of the single parameters, please refer to the help for standard ZEN functionality of EDF. A scan with EDF is slightly faster than the same scan with a Z-stack. It is important to note that an extended depth of focus can also be applied at a later stage; however, if the extended depth of focus is applied in the profile then the z-stack cannot be restored afterwards.

NOTICE

Potential risk to damage the objective

Based on the working distance of the objectives the maximum Range should be for a Plan-Apo 40x 50 µm and for every other objective a Range of below 300 µm is acceptable. This considers a typical cover slip thickness (170 µm). A larger working Range could lead a damage of the objective and in extreme cases to a damage of the slide.

8 The final step to check is the **Online Processing** section. Here you can define whether the stitching is carried out **Online**, i.e., during the acquisition, **Offline**, or if no stitching (**None**) will be applied at all. The standard setting is online stitching as this provides the best performance in terms of the processing time of the slide. The Offline mode will initially perform the scan, with the stitching applied at a later stage. This procedure is slower than the Online stitching. The difference is relative to the size of the specimen, i.e., if a specimen is very large, the offline mode is very slow compared to the online mode. For smaller specimens, there is barely any difference in processing time.
No stitching (**None**) should only be applied if the stitching is to be applied afterwards using ZEN measures or by a separate program after an export.
Pyramid active should always be activated, as this will speed up the viewing of the image afterwards. However, a pyramid can also be created afterwards. If a non-pyramid image is opened by ZEN, the software will always ask if the pyramid should be created.
These final settings allow you to adjust the compression which will be applied as part of the online processing. If **JpegXR active** is deactivated, the image will be saved uncompressed. If the **JpegXR active** is activated via the checkbox, you can save them lossless (**Lossless compression**), thus the image will only be compressed to the extent that no information is lost. If **Lossy compression** is activated, the compression always involves a loss of information. If this checkbox is activated, you can determine the degree of **Quality**.



9 In the lower part of the window below the area of the live image, you have the tab **Display**. In this tab you can adjust the display curve. The adjusted display will be saved along with the generated image, thus if you open the image this

display curve will be applied. This has no influence on the image data itself, but it is e.g. a possibility to suppress the grayish background.

INFO

The checkbox for the shading correction in the **Post Processing** section should always be activated. The applied shading correction workflow depends on the magnification of the objective, e.g., if the magnification <=10x, the software will capture the shading correction image outside the slide, i.e., through the air. The advantage of this is that the shading correction image is not influenced by dirt or other objects.
If the magnification is >=20x, the system will capture the shading correction image through the glass. To do so, the software will determine an empty region with approx. 100 fields of view. The software will scan this region and determine an average image as the shading correction image. If the system cannot find an empty region (for example, if the slide is completely covered with the specimen; e.g., blood smears), the system will use the most recently captured shading correction image and will display a warning.
In the **Post-Processing** section you can create a shading correction image manually, but this will be overwritten by the automated.

22.3.3 Slidescan Wizards

22.3.3.1 Introduction

The ZEN slidescan software includes the following 6 wizards:

- *Smart Scan Profile Selection (offline)* [▶ 1018]
- *Scan Profile Wizard (online)* [▶ 1020]
- *Advanced Scan Profile Wizard (online)* [▶ 1021]
- *Advanced Scan Profile Wizard (offline)* [▶ 1071]
- *Axio Scan Calibration Wizard (online)* [▶ 1074]

- *Tissue Detection Wizard (offline)* [▶ 1071]

Each wizard has a certain functionality and/or provides a certain level of access to the functionality of the Axio Scan.Z1. A wizard always contains several steps. These steps guide you through all necessary settings, e.g., to generate a scan profile or calibrate the scanner. The **Tissue Detection Wizard** contains only one step.

Two principal wizards are implemented – the online type and the offline type. The offline type works without having a physical slide mounted on the stage, meaning that no live image is produced and there is no access to the functionality which is normally associated with a live image. The online type requires a slide to be placed on the stage (or the wizard places the slide on the stage) thereby also generating a live image.

i INFO

For the **Advanced Profile Wizard (online/offline)**, it is not necessary to go through the wizard step by step; it is possible to jump directly to a certain step of the wizard by double-clicking the desired step.

If certain features/settings are missing, this may be because the **Show all** mode is not active. If so, activate the **Show all** checkbox. On the other hand, several settings are only available for very specific applications. If these settings cause confusion, switch off the Show all checkbox.

You can generate new profiles from scratch using the default settings (via the **Scan** tab/**Default profile** section/**New**), but in most cases it is recommended to modify existing profiles. This makes it easier and faster to adapt a profile for a new application without checking each step of the wizard.

If you do not have a adequate profile as a source to modify, it is recommended to use the **Smart Profile Selection** first to select a base profile for a certain application and modify settings afterwards using the **Scan Profile Wizard**.

22.3.3.2 Smart Scan Profile Selection (offline)

In this wizard you can select pre-defined scan profiles. You can select the **Contrast Method** (Step 1), **Sample Type** (Step 2, ff.) and enter a **Profile Name and Description** (last step).

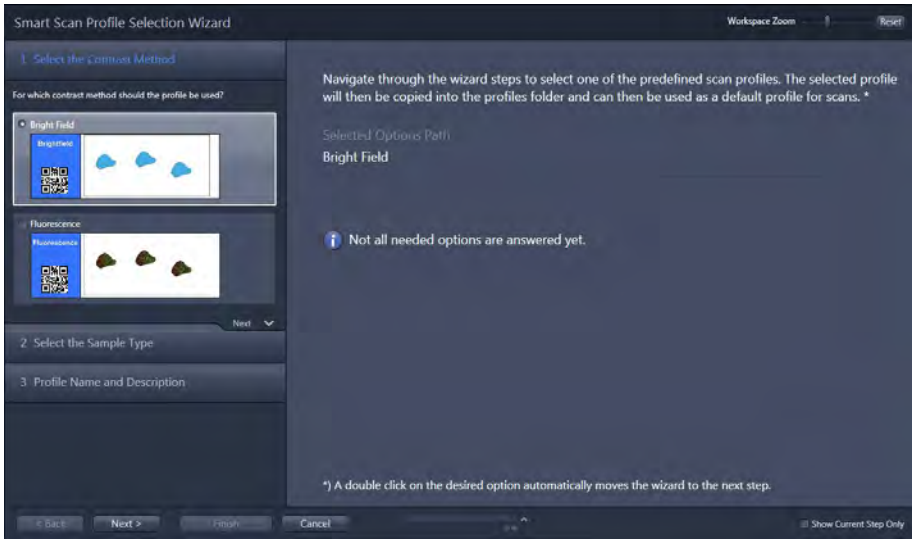


Fig. 22.14: Smart Scan Profile Selection Wizard

The system has pre-configured profiles (known as pool profiles) for a large number of applications including **Polarization** and profiles for inference objects. These profiles are stored in a safe location and cannot be changed by the user.

INFO

Note that the default scanning objective for all pool profiles is the 20x objective, with the exemption of two profiles under Reference slides which are respectively named.

In the wizard you will be guided through several steps to find the best profile as a preset for further adaptations. Each choice is followed by a selection of individual options paths. You can choose the desired option by double-clicking on it or by selecting the option and clicking on the **Next** button at the bottom.

When you finish the wizard, the selected profile from the profile pool will be copied to the user accessible folder. From here, you can select it as a scanning profile. In the last step, you can change the name and activate the option that the **Scan Profile Wizard** will be started right after the **Smart Scan Profile Selection**.

You can then modify the profile using the **Scan Profile Wizard** or the **Advanced Scan Profile Wizard**.

Reference Slides

The wizard contains a section called **Reference slides**. This section contains seven predefined profiles. Five of them are used for quality control.

In the factory two specimens (if the system has the **Polarization** option 3 specimens) are scanned:

1. **BF 20x** -> Specimen BF 03 (rat kidney; H&E) from the Sample-set brightfield (**474032-9010-000**) at 20x (if a 20x objective is available with the system)
2. **BF 40x** -> Specimen BF 03 (rat kidney; H&E) from the Sample-set brightfield (**474032-9010-000**) at 40x (if a 40x objective is available with the system)

3 **FL 20x** -> FluoCells® Prepared Slide #3 (mouse kidney; F-24630) at 20x (if a 20x objective and fluorescence is available with the system)

4 **FL 40x** -> FluoCells® Prepared Slide #3 (mouse kidney; F-24630) at 40x (if a 40x objective and fluorescence is available with the system)

5 **Polarization 20x** -> Specimen POL 01 (rat knee; Sirius red) from the Sample-set polarization (**474032-9020-000**) at 20x (if Polarization is available with the system)

Thus you can scan directly without any modification with these profiles the mentioned samples. The remaining two profiles are for special purpose regarding the shading correction for fluorescence (for more information refer to chapter *4.8* [▶ 980] and *4.9* [▶ 982]).

22.3.3.3 Scan Profile Wizard (online)

In this wizard you can edit settings for existing scan profiles.

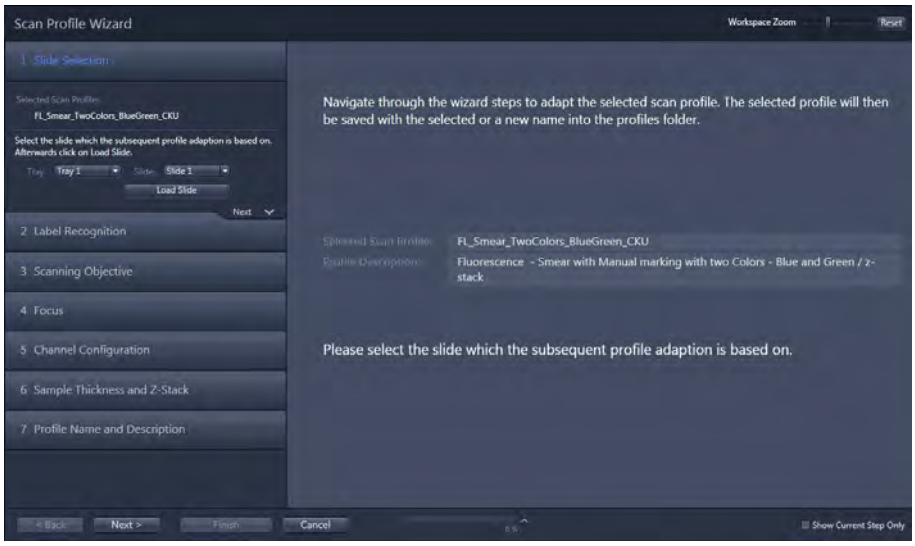


Fig. 22.15: Scan Profile Wizard

The following settings can be edited:

Settings for Brightfield Profiles

- Grid definition if grid is part of the profile
- Barcode reading
- OCR (optical character recognition) functionality
- Selecting the scanning objective
- Adjusting flash intensity and flash duration
- Z-stack functionality (with the definition of the number of slices and interval automatically via the specimen thickness)
- EDF (extended depth of focus) functionality

Settings for Fluorescence Profiles

- Grid definition if grid is part of the profile
- Barcode reading
- OCR (optical character recognition) functionality
- Selecting the scanning objective
- Selecting/deselecting channels
- Defining stitching channel
- Adjusting exposure times
- Adjusting light source intensities
- Z-stack functionality (with the definition of the number of slices and interval automatically via the specimen thickness)
- EDF (extended depth of focus) functionality

22.3.3.4 Advanced Scan Profile Wizard (online)

This wizard is an online wizard, thus the system will take the tray containing the slide from the magazine if the tray which contains the slide is not already on the stage. The system will always show you a live image from the corresponding cameras (preview camera / scan camera) while also moving the slide according the input from the operator.

This mode is important if you want to adjust settings such as exposure times and regions of interest including labels and preview areas. This means that you will always work on a physical glass slide. On the other hand, this can be time-consuming because the physical glass slide has to be moved. If you want to change settings that are not directly associated with a live image, you can use the **Advanced Profile Wizard (Offline)**.

As the settings for this wizard are different for fluorescence and brightfield profile types we have separated the descriptions in two chapters.

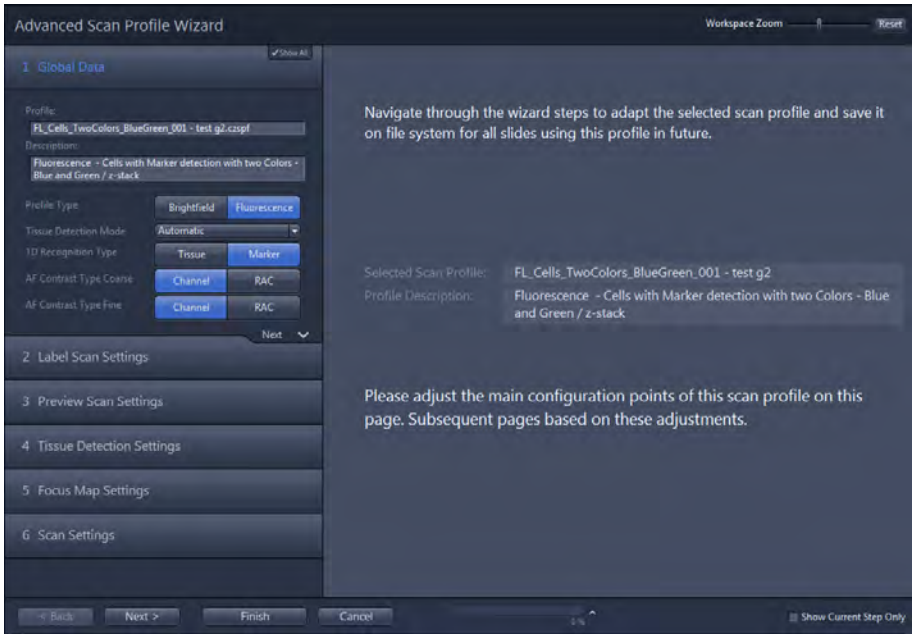


Fig. 22.16: Advanced Scan Profile Wizard

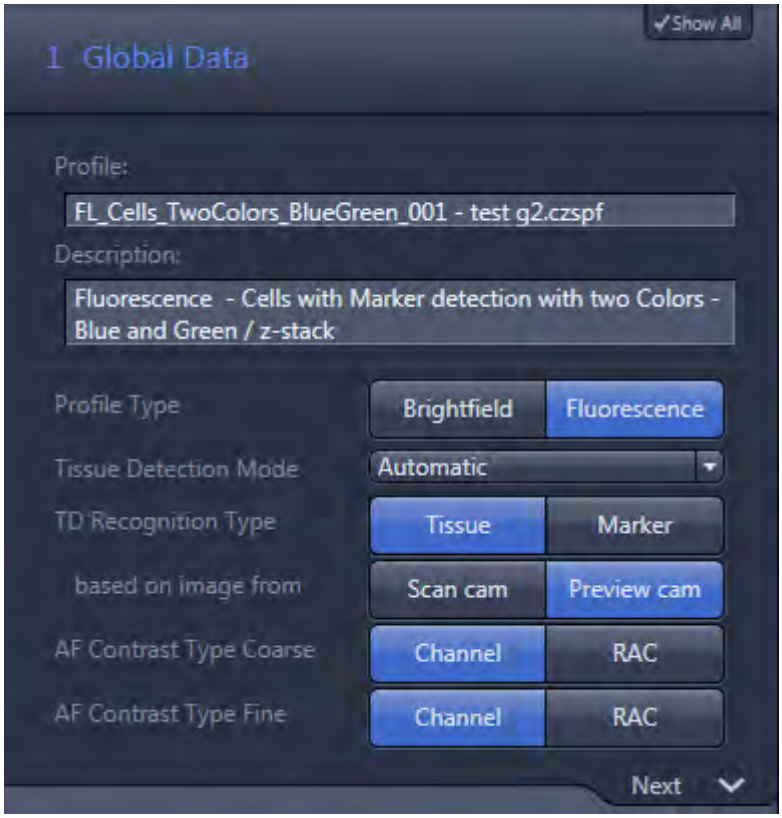
22.3.3.4.1 Settings for Fluorescence Profiles

22.3.3.4.1.1 Global Data

INFO

To access all of the functions and properties described in this guide, you have to activate the **Show All** mode by activating the checkbox in the upper right-hand corner of the left tool area.

Here you can define global settings for your scan profile. Depending on these global settings, both the wizard and the workflow will be adapted automatically.



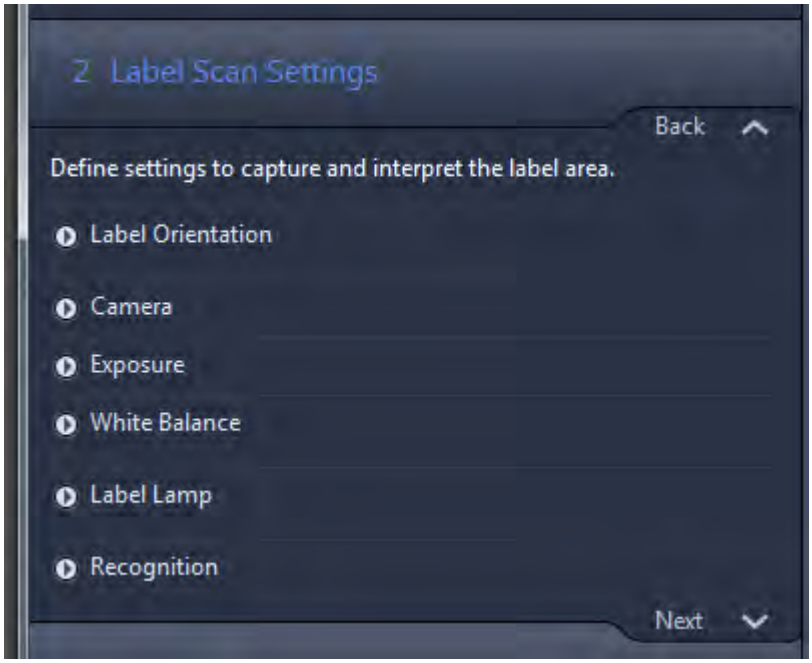
Parameter	Description
Profile	Displays the name of the selected scan profile. This name cannot be changed here.
Description	Shows the description of the scan profile. As the information content of the profile name is always limited, you can insert additional information here to highlight the most important profile settings (e.g., magnification/z-stack settings/focus map settings).
Profile Type	Here you can select between **Brightfield** and **Fluorescence** profile types. If you choose brightfield, AF Contrast Type settings will not be shown.
Tissue Detection Mode	This is where you select the kind of tissue detection you want to apply. The following three modes are available:
- Automatic	In this mode, the system detects the tissue automatically. This is the most common selection (together with **Marker**) as it works independently of the kind of specimen and specimen preparation.

Parameter		Description
-	Interactive	In this mode, the system will pause the scan process if a batch is started and waits for your input to define a region of interest manually. As the system pauses until this interaction is complete, this function has to be used very carefully.
-	Manually	In this mode, you can define a region of interest to be scanned manually or a grid in a subsequent step of the wizard. This is an option if you only want to select a subset of the tissue on the slide, e.g., if the tissue is very faint, the system cannot detect the specimen reliably, or if the specimen has a regular pattern on the slide. If you use this function, the defined region/regions will always be exactly the same for all slides assigned with this profile, thus it can be only applied if you have a fixed scan region (e.g., PAP smears).
TD Recognition Type		Not available if you set **Tissue Detection Mode** to **Manual**. Here you select whether the tissue detection is carried out via thresholds (**Tissue**) or if you want to use a **Marker** to encircle the specimen manually on the front of the physical slide. In this case, the threshold will be used to detect the marker. Everything inside this marker will be scanned.
-	Marker	If selected, the system will always generate a preview using the separate preview camera.
-	Tissue	If selected, you can select under **based on image from** whether the input image is created by: ■ **Preview cam** Creates the image using the preview camera. This mode results in a lower resolution, no special contrast method is used. We recommend this mode if the specimen is clearly visible on the slide (e.g., if the specimen is very thick). ■ **Scan cam** Creates the image with the scan camera and a selected objective with the Ring Aperture Contrast (RAC). If the specimen is faint (i.e., hard to see with the naked eye) we recommend to use this mode.

Parameter	Description
AF Contrast Type Coarse & Fine	Only available for **Fluorescence** profile types. Here you can define what contrast type is used to perform the Coarse and Fine focus.
- Channel	If selected, the system will use a fluorescence signal to perform the focus. This is the best choice if the specimen provides a fluorescent counterstain (e.g., DAPI); you must ensure that the fluorescent stain is evenly distributed over the sample so that the system detects enough signals at the focus point to preform a reliable autofocus.
- RAC	If selected, the system will use the Ring aperture contrast (RAC) to perform the focus. This principle is recommended if no fluorescent counterstain is available and/or if the fluorophores are very sensitive to bleaching. It is also important for the kind of specimen to be visualized using this brightfield contrast method. This means that cells spreads cannot be visualized with this contrast method.

22.3.3.4.1.2 Label Scan Settings

Here you can select the capture parameters for the label area.



The label area ROI (Region Of Interest) is defined by the red rectangle shown in the live image. It is possible to adjust the frame in size and position freely to fit with the label on your slide. The label area is captured with reflected light and a separate camera (called **AxioCam IC**).

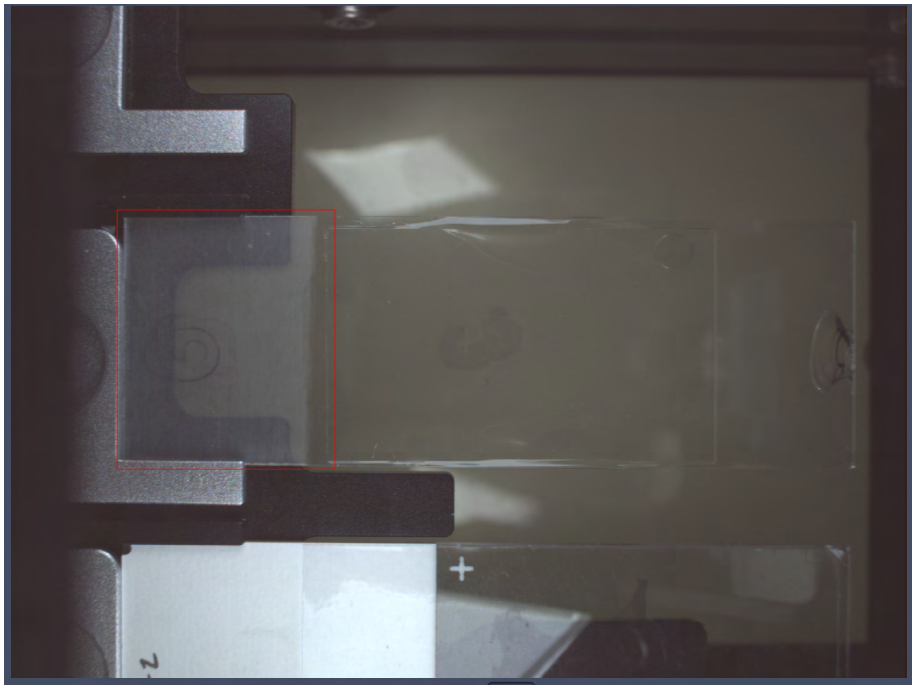


Fig. 22.17: Label area of a slide (red rectangle)

i INFO

It is not usually necessary to adapt the camera settings and/or light source as these settings are factory defaults and should represent the optimal settings. For this reason, these settings (**Camera**, **Exposure**, **White Balance** and **Label Lamp**) are not described in detail within this section.

Parameter	Description
Label Orientation	Here you select how the label is aligned on a slide. You can rotate the label image in 90° steps clockwise (CW) or keep the original orientation (**Original**). If you select **Use Barcode Orientation**, a barcode sticker has to be attached on the slide. The software will then determine the orientation of the barcode and apply this to the orientation of the label area of the current slide. To use this function, a barcode label has to be attached and you also have to activate the option **Barcode Recognition active** in the **Recognition** section.

Parameter	Description
	You can also change the label orientation later on in the software if you browse through the slides. If all of the slides have a rotated label, however, it is recommended to change the label orientation here.
Recognition	Here you can check if the barcode or characters can be recognized. If you click on **Test**, the system will show the region of interest with the orientation given and apply the barcode or OCR recognition. The result will be presented in the **Recognized** field. 
Barcode	If the **Barcode Recognition active** checkbox is activated, the system recognizes the barcode and saves the barcode information as metadata within the image. If you want to use the barcode information as part of the image name, you have to apply automated naming and use the keyword "**RecognizedCode (%N)**" to make the barcode part of the image name. By default, the software will check for all implemented barcode types and all orientations. It is not necessary to define a specific barcode. If the label contains two or more barcodes with a different barcode type, we recommend that you define the specific barcode type you are looking for. To activate a specific barcode, open the expander for **Barcode types** and activate only the barcode you want to be read.

Parameter	Description
OCR (Optical Character Recognition)	Note that the OCR works with **English** text only. It works best with clearly printed numbers and/or letters. We recommend to use OCR_A or OCR_B as the font for the OCR. We do not recommend using underscores (_) or special characters within the text. You can select different options to optimize the recognition quality, but the standard parameter should work. The printed text should be very precise in terms of its orientation. There should be minimal text tilt after the image orientation.

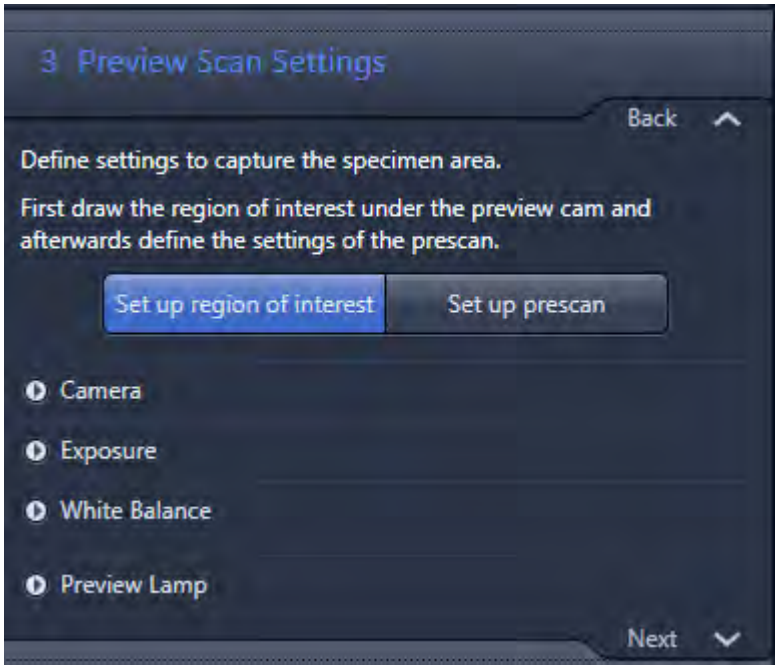
INFO

You have the possibility to adjust the display curve (see **Display** tab on the bottom of the window). The display settings will stored within in the profile. This has also an impact on the display of the label in the **Magazine** view.

22.3.3.4.1.3 Preview Scan Settings

Depending on the settings under **Global Data**, the appearance and functions on the user interface can be different. In the following 2 sections, we describe the two most common settings for a preview scan with an objective (prescan) or the preview camera (preview).

22.3.3.4.1.3.1 Preview Scan with Objective (Prescan)



Prerequisites

- In Step 1 **Global Data** under **Tissue Detection Mode**, you must have activated either the **Automatic** or **Interactive** setting.
- Under **Tissue Recognition Type**, you must have activated the **Tissue** setting and the **Scan cam** setting for **based on image from**.

With these settings, the system will perform a preview scan with the scanning objective (known as Prescan) and will also determine the **Coarse Focus**. The following parameters are available:

Parameter	Description
Set up region of interest	If you have selected this button, you can use the **Center Screen Area** to set up the area for the prescan by changing the position and the size of the red frame which defines the prescan region. As a prescan is much more time-consuming than a preview, it is recommended to select a rather small area.
Set up prescan	If you have selected this button, you can set up how the system will scan the region of interest using a prescan. You have the choice of RAC or fluorescence (channel). The settings depend on the setting for **Coarse Focus** under **Global Data**. If the coarse focus is set to **RAC**, the prescan will be carried out with RAC; if it is set to **Channel**, the prescan will be carried out with fluorescence. This principle is based on the connection between prescan and coarse focus. For an in-focus prescan, the system needs an autofocus. As this autofocus is already carried out using the prescan, it is not necessary to carry out any subsequent steps for the coarse focus. For this reason, the coarse focus will be disabled under **Focus Map Settings** if a prescan is activated. This is where you set up the acquisition parameters for the prescan and the settings for the z-stack (for the autofocus). As the process for creating the acquisition parameters is similar to that for setting up the Focus settings, please refer to this section for more details.
Camera settings (Camera, Exposure, White Balance)	This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. The camera settings are described in the appropriate chapters in the ZEN Online Help. It is not possible to change the camera for the preview as this is fixed.

Parameter	Description
Preview Lamp	Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light). Once again, we recommend that you do not change the value.

22.3.3.4.1.3.2 Preview Scan with Preview Camera (Preview)

Prerequisites

- In Step 1 **Global Data** under **Tissue Detection Mode** you must have selected the **Automatic** or **Interactive** option.
- Under **Tissue Recognition Type**, you must have activated the **Tissue** button, as well as the **Preview cam** button under **based on image from**.

Or:

- In Step 1 **Global Data** under **Tissue Detection Mode**, you have selected the **Manually** option.
- Under **Tissue Recognition Type**, you must have activated the **Marker** button.

In all cases, the system will perform a preview taken with the separate preview camera, thus the system generates the preview image (called preview) with one snapshot.

Parameter	Description
Camera, Exposure, White Balance	This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. As the controls are similar to the standard ZEN controls for cameras, please refer to the

Parameter	Description
	appropriate chapters in the Online Help. It is not possible to change the camera for the preview (under **Camera**), as this is fixed.
Preview Lamp	Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light). Once again, we recommend that you do not change the value.

22.3.3.4.1.4 Tissue Detection Settings

The appearance of the Tissue Detection (TD) Settings depends on the settings which you have made in the first step of the wizard under **Global Data**. In the following sections four possible cases are described.

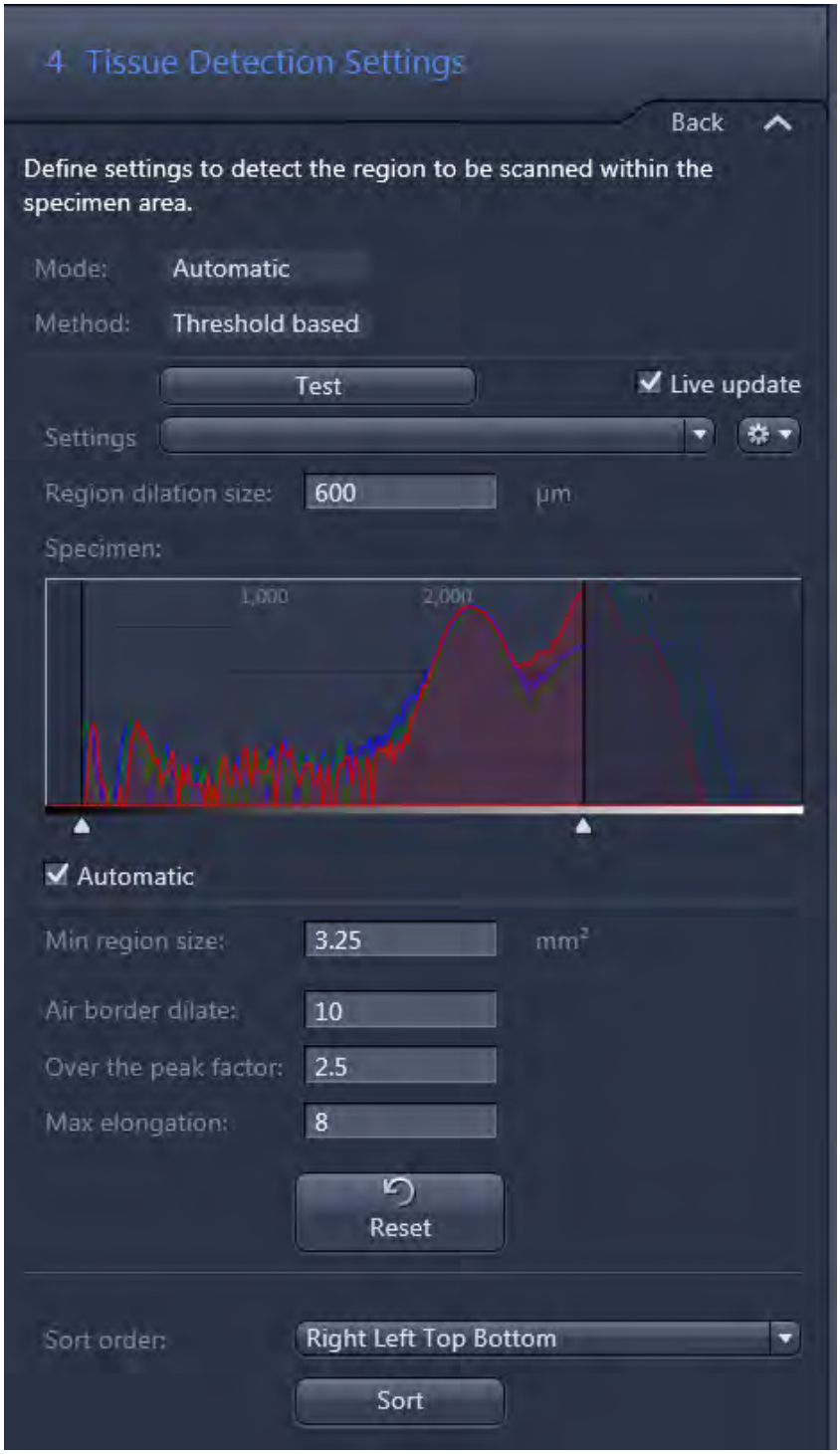
INFO

You have the possibility to implement your own tissue detection algorithm and make this algorithm part of the workflow, see *Implementing your own tissue detection algorithm* [▶ 975].

22.3.3.4.1.4.1 Case A: Automatic Mode / Threshold based

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Automatic**, as well as
- under **TD Recognition Type** you have selected **Tissue**, the TD settings will appear as follows:



Region of Interest for Tissue Detection

i INFO

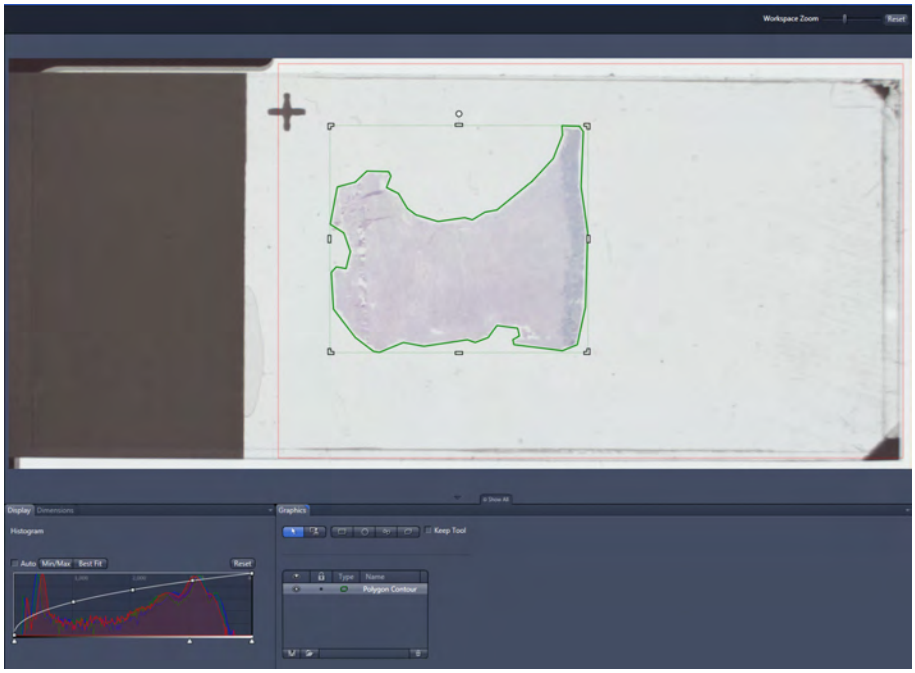
If you draw your own regions, make sure that these contain tissue. Empty space could lead to an incorrect focus map as the software will use the entire drawn region to create the focus point distribution.

The red rectangle in the live image which you can see in the **Center Screen Area** represents the ROI for the Tissue Detection. Only the regions that are inside the ROI will be presented as regions which can be scanned. Other regions outside the ROI and/or touching them will be ignored. You can change the size of the ROI by adapting the size of the red rectangle.

INFO

If two or more objects are very close to each other and thus the two or more objects are seen as one objects it is perhaps advisable to split these objects with the **Split** function which is available in the context menu seen after a right-mouse click.

If you click on **Test**, the software will execute the inserted parameters and show the results in the middle part of the window. The detected objects (every object is a single scene) will be presented with a green border.



All objects are listed in the **Graphics** tab. You can delete objects and even add new ones. However, the result will be only valid for this slide.

To achieve a continuous update, you can activate **Live update** via the checkbox. Keep in mind that a refresh can take some seconds after changing a parameter. The main parameters of this step are described below:

Parameter	Description
Mode & **Method**	Here you can see the mode and method for tissue detection which you have selected under **Global Data**.

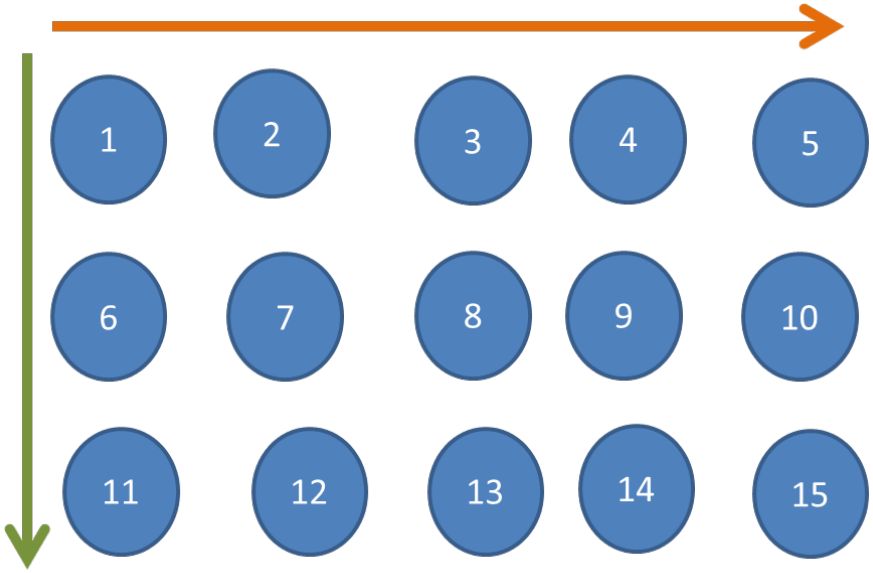
Parameter	Description
Region dilation size	Here you can enter a value for the spacing of the border around the detected tissue.
Specimen	Here you define the upper and lower borders of the specimen via the thresholds. By clicking on one of these borders and pressing the right mouse button you can move the borders up and down. If you have activated the **Live update**, you will see the result toward the left of center with a certain delay. The upper border (right side) is the border for the lighter stains and the lower border (left side) is the border to influence the darker stains. Thus moving the right side further to the right marks the lighter stains. Moving the left side further to the left marks the darker stains.
Min region size	Here you can enter the minimum region size to be scanned. The system will not detect regions if they are smaller than this value.
Air border dilate	The air border is the border around the physical glass slide, i.e., the area outside the glass slide and inside the red frame. This value determines the dilation of this air border. Everything inside this dilated air border will not be recognized as tissue/a marker. This is a measure for ensuring that slide edges and coverslip edges are not detected as needing to be scanned.
Over the peak factor	This parameter is only visible if the **Automatic** checkbox is activated. It defines the distances between the glass peak of the slide and the upper threshold value for the specimen detection. Thus if **Automatic** is activated, the upper value will be determined automatically.
Max elongation	This setting is important to remove coverslip edges. Coverslip edges are normally structures with a large number for the ratio of the longer side and the shorter side. This means that every structure that exceeds this parameter for the ratio between the longest side and the shortest side will be excluded.
Reset	Reverts the values to their default settings.

Once you have determined the appropriate settings, you can save them by selecting **New** via the **Options** icon under **Settings**. The settings can then be used in another profile simply by selecting the respective entry from the dropdown list. It is not essential to save these settings as they will be saved within the profile. It is only necessary if the settings are to be used in another profile.

Sorting Order Section In the **Sort order** section you can select the sorting schema (numbering) for the detected objects (regions / scenes). By clicking an **Sort** the selected schema will be applied. You can select from 8 different schemes. The first entry (e.g. Left Right) provides the first sorting schema and the second entry (e.g. Top Bottom) the second sorting schema.

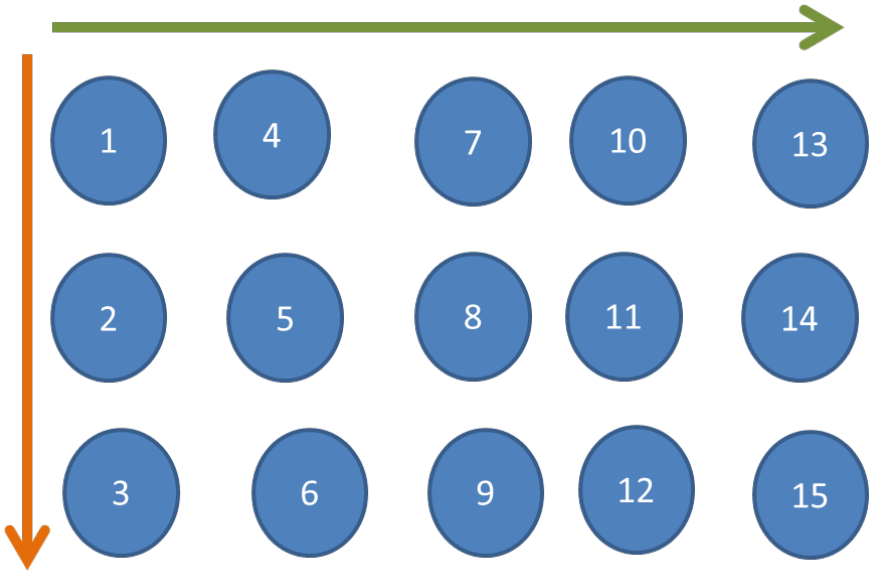
Example 1: Left Right / Top Bottom

The first sorting schema is from Left to Right (red arrow) and the second sorting schema is from Top to Bottom (green arrow):



Example 2: Top to bottom / Left to right

The first sorting schema is from Top to bottom (red arrow) and the second sorting schema is from Left to right (green arrow):



22.3.3.4.1.4.2 Case B: Automatic Mode / Marker recognition

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Automatic**, as well as
- under **TD Recognition Type** you have selected **Marker**, the TD settings will appear as follows:

The settings are limited to the definition of the marker threshold (comparable to the tissue threshold described above). The only further adjustable value is the **Min region size**, whose functionality is the same as described in Case A.

Note that the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

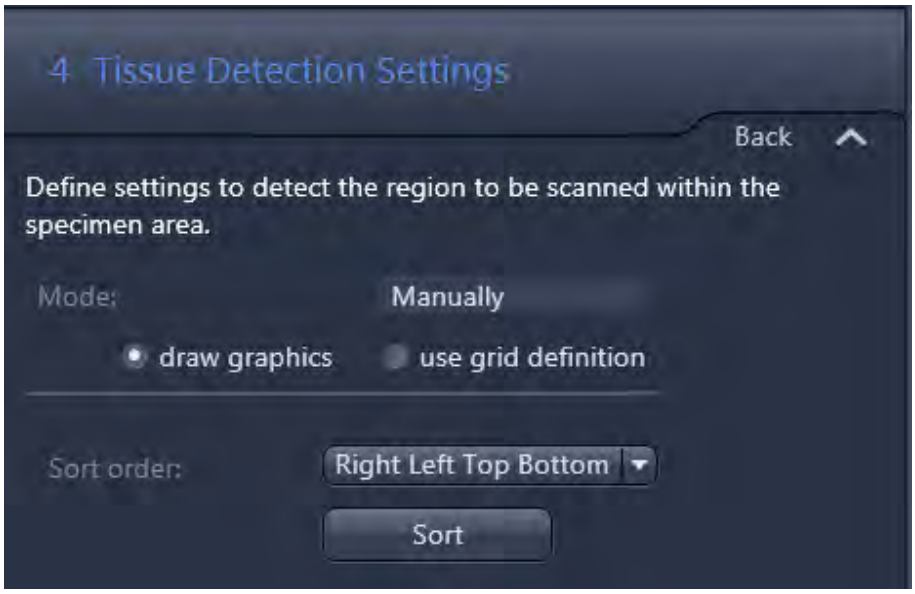
i INFO

If you use the marker on the physical glass slide, make sure that the regions marked contain specimens. Empty space could lead to an incorrect focus map as the software will use the entire marked region to create the focus point distribution.

22.3.3.4.1.4.3 Case C: Manual Mode / Draw Graphics

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Manually**, the TD settings will appear as follows:



Drawing in TD ROIs manually

If you have selected **draw graphics** it is possible to draw regions manually within the region of interest if you activate draw graphics. To draw regions, you can use the tools on the **Graphics** tab in the general view options of the **Center Screen Area**. You can use it in the same way you would generally annotate images within ZEN. The drawn regions will be saved and the same areas will be used for the entire time the profile is applied, hence this option can only be used if the regions are always defined precisely on the slide (e.g., PAP smears).

Note that the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

i INFO

If you want to mark a sample that is very faint, you can use the **Display** curve to change the display settings (particularly by adjusting the gamma curve) to make it possible to see even unstained sample of a reasonable thickness.

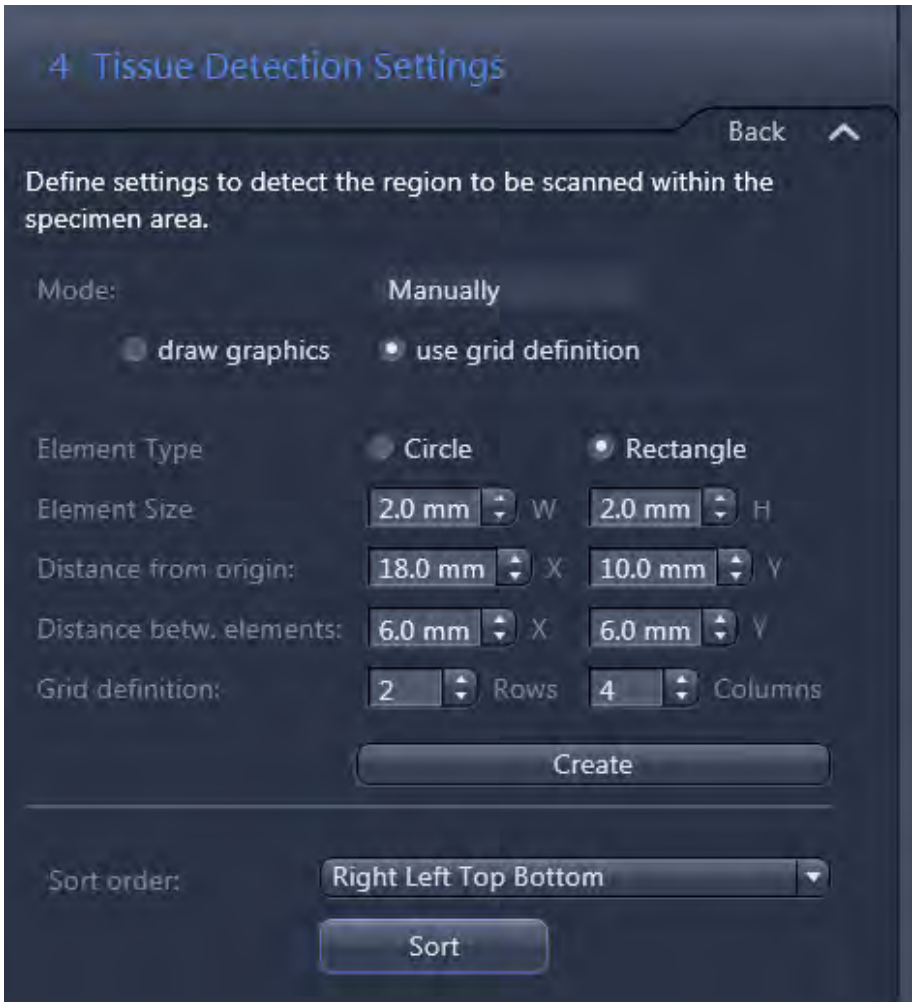
i INFO

You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile. This has also an impact on the display of the label in the Magazine view. This has also an impact on how the sample area is displayed in the Magazine view!

22.3.3.4.1.4.4 Case D: Manual Mode / Grid definition

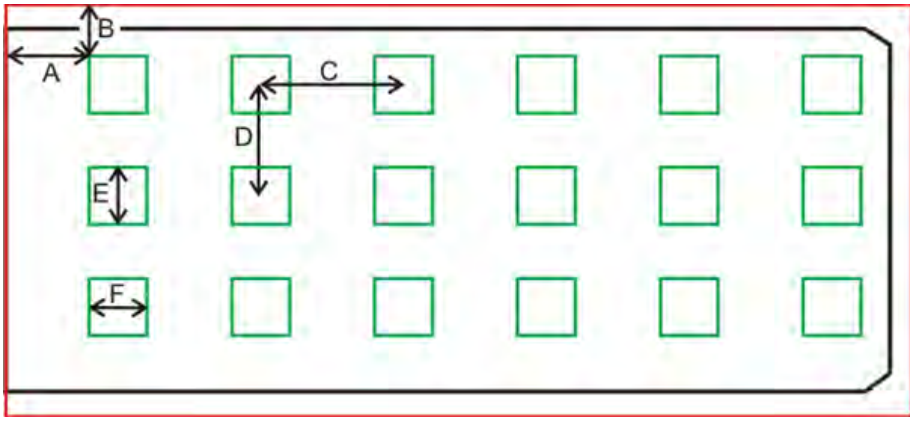
Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Manually**, the TD settings will appear as follows:



If you have selected **use grid definition** you can define a grid with rectangles or circles as **Element Types** in a regular pattern. The reference point for this grid is the upper right-hand corner of the specimen area (red rectangle). Based on this, you can define on offset from the upper left-hand corner in x (A) and y (B) in mm and also the pitch of the grid in x (C) and y (D).

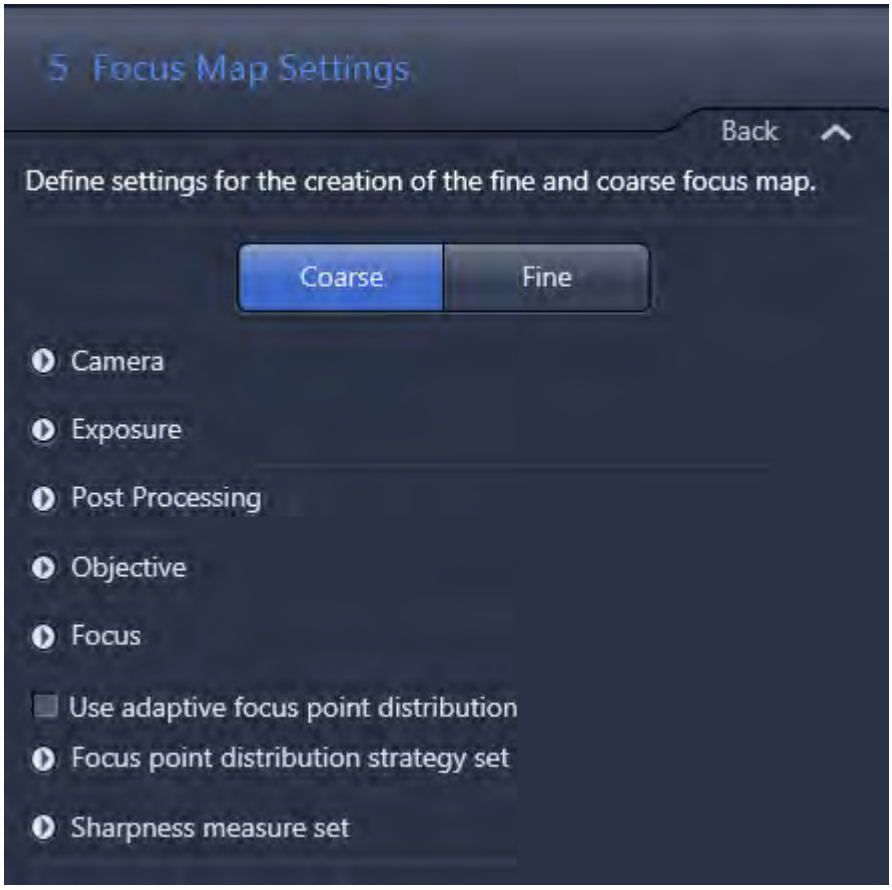
Based on the selected geometrical shape (circle or rectangle), you can define a radius or the dimensions in x (F) and y (E). The number of elements in x (in this example, 6) and y (in this example, 3) defines the number of elements.



When you click on the **Create** button, the grid will be generated. You will see the grid as an overlay in the image. It is also possible to make manual adjustments such as those used for standard graphics in ZEN.

Note that the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

22.3.3.4.1.5 Focus Map Settings



Appropriate focus map settings are important for ensuring good focus quality throughout the specimens. The focus map generation is done in two steps:

- With a low magnification objective to generate the **Coarse** focus map. For this coarse focus map, only some focus points are necessary. We recommend the use of the 5x Fluar for the Coarse focus as the offset between the 5x and the scanning objective (mostly 20x) is minimal.
- With a high magnification objective (normally the scanning objective) to generate the **Fine** focus map. In this case, more focus points are needed and this is normally defined via a density. The Fine focus map is based on the coarse focus map.

The focus is not a traditional autofocus that needs to hit the sharpest z-value precisely. The system will acquire a z-stack (defined in the next step) and calculate a curve through the focus value (e.g., contrast value). This curve is used to determine the peak value. This makes it possible to cover a large range while still maintaining fast focusing.

If the profile is configured in a way that involves a prescan (scan with an objective) being carried out, the **Coarse** focus is disabled as this is already carried out while prescanning the region of interest.

As the settings under **Camera**, **Exposure**, **Post Processing**, **Objective** and **Focus** are described in the ZEN Online Help in the corresponding topics, we only describe the slidescan specific settings in the following sections.

We also recommend to read the following topics where you can find detailed how to guides for the focus relevant topics:

- *Adjusting Coarse Focus Map Settings (FL)* [▶ 986]
- *Adjusting Fine Focus Map Settings (FL)* [▶ 991]

22.3.3.4.1.5.1 Sharpness measure set

Item	Description
Basic	The autofocus is contrast-based, hence the algorithm will focus on the highest contrast along the z-stack. It is a simple but fast autofocus.
Best	The autofocus is also contrast-based in this case, thus the algorithm will focus on the highest contrast along the z-stack. Compared to Basic, the focus is slightly slower but more accurate for certain samples. Please be aware that Best does not mean that this algorithm is always superior to Basic, as this depends on the kind of tissue/specimen.
FFT	This autofocus setting applies to the Fourier transform algorithm to determine the best focus value. This algorithm is slower than the other algorithms and should be used only in the case of the

Item	Description
	Ring aperture contrast. It is not ideal for standard brightfield contrast or fluorescence as this algorithm looks for two contrast peaks and the local minimum between them.
HG 2^8	This is an advanced algorithm compared to Basic and Best. This algorithm focuses less on the larger stained structures and more on the smaller local structures, thus this algorithm will focus more on nuclei, for example, than on unstained structures (e.g., immunohistochemistry stains). Based on the current status of evaluation, this means that HG2^8 is a good autofocus method if a user wants to focus on nuclei. **Drawback: The weight of dirt also increases, hence it should only be used for the fine focus, not the coarse focus.**
Contrast Power	This algorithm is comparable to the HG 2^8.

22.3.3.4.1.5.2 Focus point strategy set

i INFO

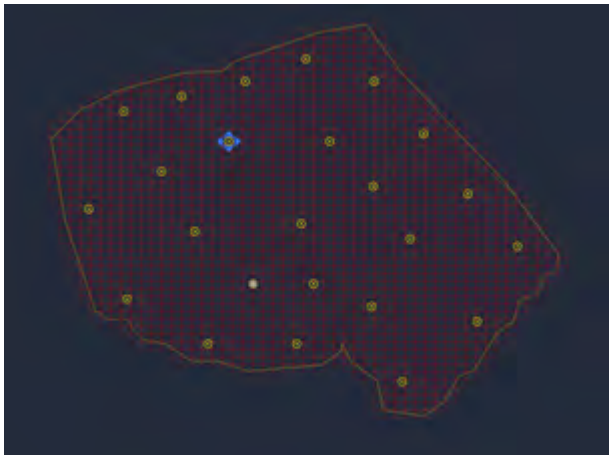
The focus point strategy set will be applied for each scene, i.e., as a separate detected object in each case. If you select six fixed points for the Coarse focus, the software will apply 6 focus points for each scene.

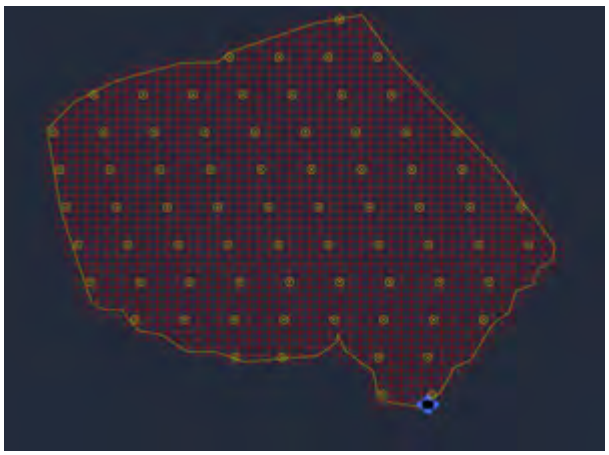
Certain **Focus point strategies sets** have an additional checkbox called **Prefer border**. If this checkbox is activated the system will put preferable more focus points to the border of the detected specimen. This is advisable if the border of the specimen has compared to the other parts of the object significant other focus values.

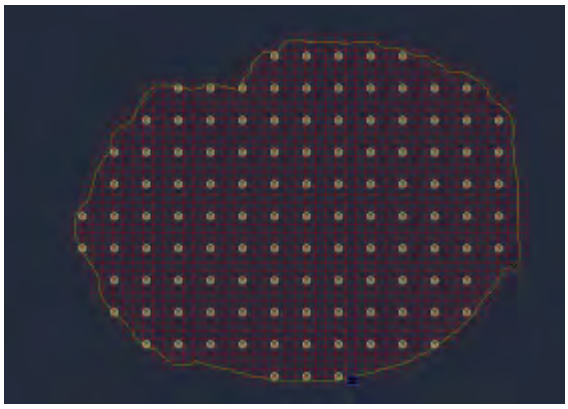
If the setting density is part of the **Focus point strategy set** this value gives the percentage of generated focus points in relation to the total number of tiles of the object (e.g. if the object has 2000 tiles and the setting is set to 0.1 (= 10%) the system would generate 200 focus points). As especially for larger objects too many focus point will be generated (you have to keep in mind that not always a larger number of focus points will automatically result in higher overall focus quality) you can define also a maximum number of focus points (**max. number**) which is advisable.

If you use the automatic tissue detection and you define a border dilation the system will subtract this dilatation before the focus points are distributed, thus no focus point will be put in the dilation zone. This applies only for the automatic tissue detection.

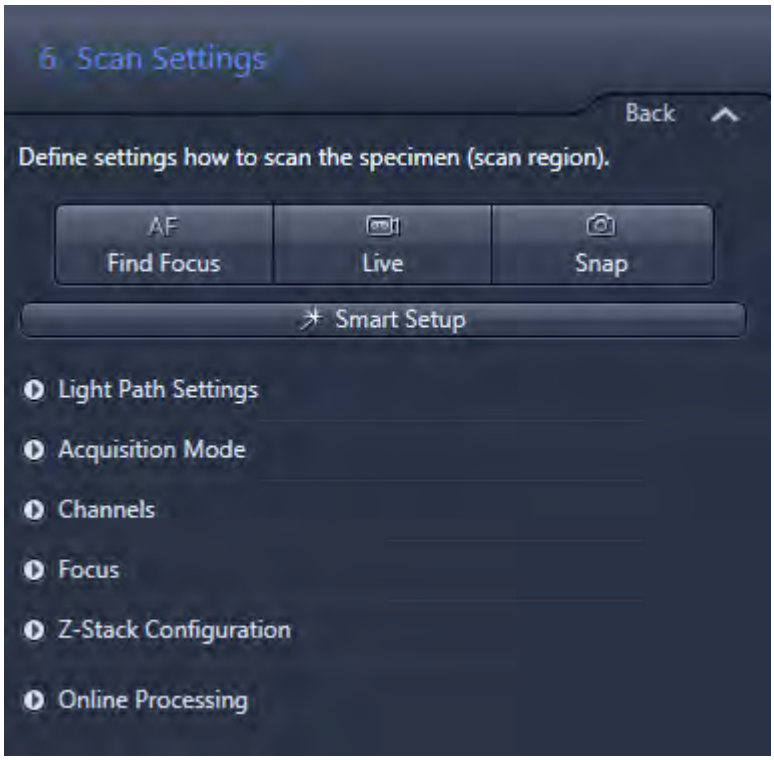
To ensure that the focus points are within the tissue the system will move the focus points a certain number of field of views away from the border. This value can be adjusted under **Tools** | **Options …** | Tab **Acquisition** | Expander **Axio Scan** | **Minimum Margin of focus point distribution in tile(s)**. The default setting is 1. This gives with a wide variety of specimens a very good result. It has to be kept in mind that this value for the software version ZEN 2012 slidescan and ZEN 2012 slidescan SP2 was 2 (and a fixed not shown parameter).

Item	Description
Onion skin	This is a density focus schema. The resulting focus points are displayed as layers of onion skin, providing an even distribution and also ensuring that the border in particular has enough focus points. This is the standard setting for the fine focus map. The parameter is a density with a standard setting of 0.1. This setting means that 10% of the total number of viewing fields will be used as focus points to calculate the focus surface. For large specimens this number can get very large, but a larger number of focus points does not always automatically mean a better quality calculation of the focus surface. For this reason, the user can also define a maximum number of focus points. This number is normally in the range of 24 to 36 points. This focus point strategy set is recommended for mid-sized to large objects. Example (Density: 0.1; max number 24): 
Every Nth tile	The system will put a grid over the specimen with a distance between two focus points of the specified value (N) for the number of camera viewing fields in the x and y direction. This is a clearly defined way to create the focus point, even it creates more focus points and will take longer to create the focus map. The focus point strategy set is recommended for mid-sized and smaller objects.

Item	Description
	Example (every 5th tile): 
Every tile	The system will focus on every tile. This is particularly time-consuming for scans with a large magnification and/or large samples; however, it yields the best results for very uneven specimens.
Number of focus points	Here the user defines a fixed number of focus points per object (scene). The default setting for the Coarse focus is six focus points per scene. Example (n = 24): 
Density	The density is similar to that of an onion skin; the difference is that the pattern is not distributed like the layers of an onion. The distribution is more evenly spread over the specimen/scene. This setting can also be used to define a maximum number of focus points. The creation of this focus map is more random – as shown in the example – and thus the onion skin is recommended over the density.

Item	Description
	Example (density: 0.1; max number 24): 
Grid cell size	This setting defines a fixed grid over the specimen to establish the focus points for the focus surface. The value is in µm. This setting is independent from the current frame/sensor size of the camera used. If you want to use a setting that is based on the chip size of the camera, please use “Every Nth tile”. Example (1000 µm 1000 µm): 
Center of Gravity	The system will place one focus point within each scene/ object. The position of this focus point is the center of gravity, thus the whole object has only one focus point. This is a useful setting for small objects (e.g., Tissue micro arrays). Be aware that for certain structures (such as a half moon), the center of gravity may lie outside the tissue, hence the system has nothing to focus on. In such cases, select “Number of focus points” with 1 as the parameter. The center of gravity is a valid option, e.g., for Tissue Micro Arrays to place only one focus point in each core.

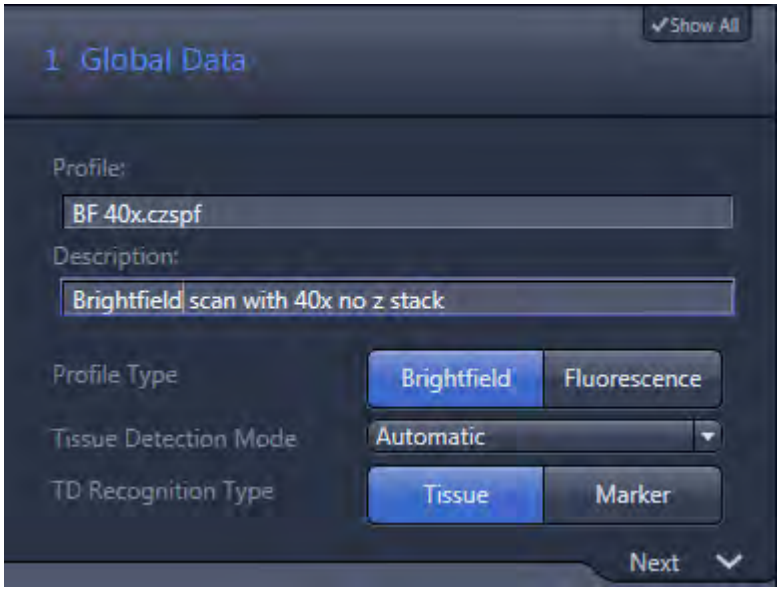
22.3.3.4.1.6 Scan Settings



In this wizard step you can adjust the scan settings. You can read how to adjust the settings in the chapter *Adjusting Scan Settings for FL Profiles* [▶ 997].

22.3.3.4.2 Settings for Brightfield Profiles

22.3.3.4.2.1 Global Data



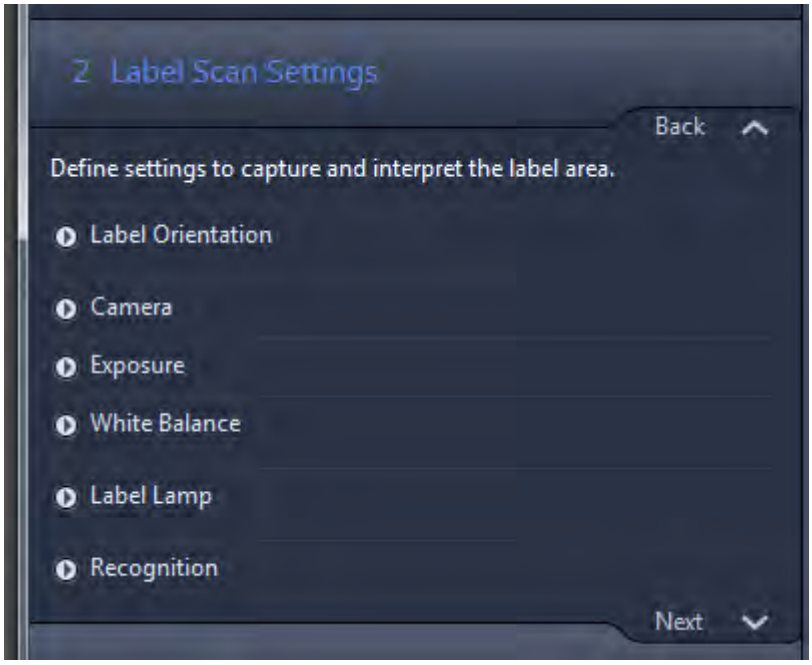
From here, you can define global settings. Depending on these global settings, both the wizard and the workflow will be adapted automatically.

Parameter	Description
Profile	Displays the name of the selected scan profile. This name cannot be changed here.
Description	Shows the description of the scan profile. As the information content of the profile name is always limited, you can insert additional information here to highlight the most important profile settings (e.g., magnification/z-stack settings/focus map settings).
Profile Type	Here you can select between **Brightfield** and **Fluorescence** profile types. If you choose brightfield, AF Contrast Type settings will not be shown.
Tissue Detection Mode	This is where you select the kind of tissue detection you want to apply. The following three modes are available:
- Automatic	In this mode, the system detects the tissue automatically. This is the most common selection (together with **Tissue**) as it works independently of the kind of specimen and specimen preparation.
- Interactive	In this mode, the system will pause the scan process if a batch is started and waits for your input to define a region of interest manually. As the system pauses until this interaction is complete, this function has to be used very carefully.
- Manually	In this mode, you can define a region of interest to be scanned manually or a grid in a subsequent step of the wizard. This is an option if you only want to select a subset of the tissue on the slide, e.g., if the tissue is very faint, the system cannot detect the specimen reliably, or if the specimen has a regular pattern on the slide. If you use this function, the defined region/regions will always be exactly the same for all slides assigned with this profile, thus it can be only applied if you have a fixed scan region (e.g., PAP smears).
TD Recognition Type	Not available if you set **Tissue Detection Mode** to **Manual**. In both cases the system will generate a preview using the separate preview camera.
- Marker	A **Marker** is used to encircle the specimen manually on the front of the physical slide. In this case, the threshold will be used to detect the marker. Everything inside this marker will be scanned.

Parameter		Description
-	Tissue	The tissue detection is carried out via thresholds.

22.3.3.4.2.2 Label Scan Settings

Here you can select the capture parameters for the label area.



The label area ROI (Region Of Interest) is defined by the red rectangle shown in the live image. It is possible to adjust the frame in size and position freely to fit with the label on your slide. The label area is captured with reflected light and a separate camera (called **AxioCam IC**).

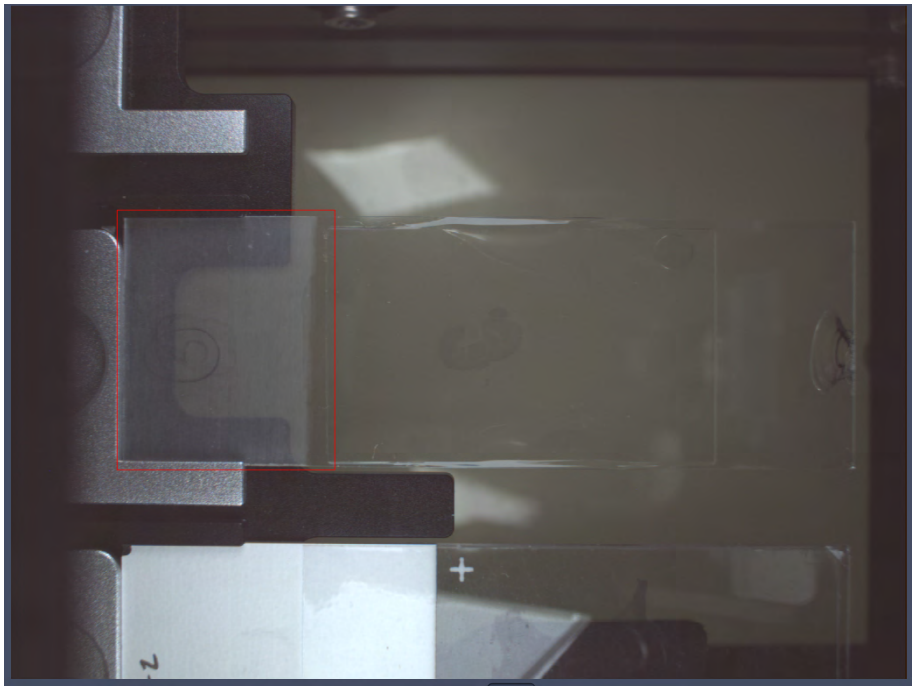


Fig. 22.18: Label area of a slide (red rectangle)

i INFO

It is not usually necessary to adapt the camera settings and/or light source as these settings are factory defaults and should represent the optimal settings. For this reason, these settings (**Camera**, **Exposure**, **White Balance** and **Label Lamp**) are not described in detail within this section.

Parameter	Description
Label Orientation	Here you select how the label is aligned on a slide. You can rotate the label image in 90° steps clockwise (CW) or keep the original orientation (**Original**). If you select **Use Barcode Orientation**, a barcode sticker has to be attached on the slide. The software will then determine the orientation of the barcode and apply this to the orientation of the label area of the current slide. To use this function, a barcode label has to be attached and you also have to activate the option **Barcode Recognition active** in the **Recognition** section. You can also change the label orientation later on in the software if you browse through the slides. If all of the slides have a rotated label, however, it is recommended to change the label orientation here.
Recognition	Here you can check if the barcode or characters can be recognized.

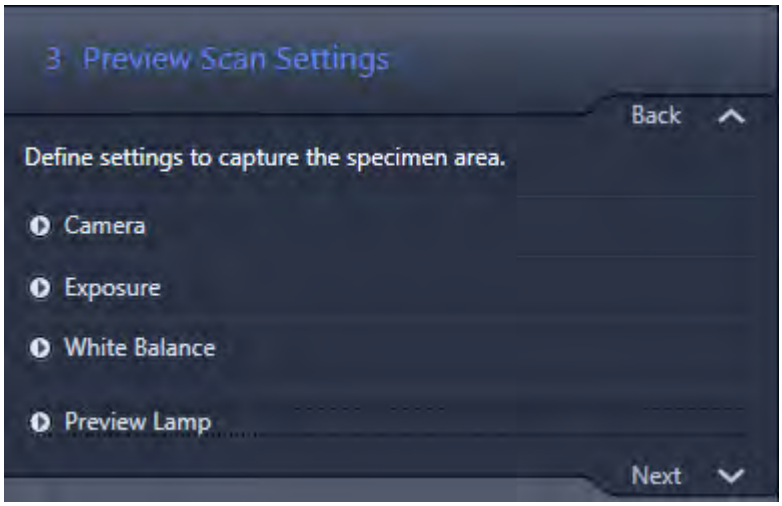
Parameter	Description
	If you click on **Test**, the system will show the region of interest with the orientation given and apply the barcode or OCR recognition. The result will be presented in the **Recognized** field. 
Barcode	If the **Barcode Recognition active** checkbox is activated, the system recognizes the barcode and saves the barcode information as metadata within the image. If you want to use the barcode information as part of the image name, you have to apply automated naming and use the keyword "**RecognizedCode (%N)**" to make the barcode part of the image name. By default, the software will check for all implemented barcode types and all orientations. It is not necessary to define a specific barcode. If the label contains two or more barcodes with a different barcode type, we recommend that you define the specific barcode type you are looking for. To activate a specific barcode, open the expander for **Barcode types** and activate only the barcode you want to be read.
OCR (Optical Character Recognition)	Note that the OCR works with **English** text only. It works best with clearly printed numbers and/or letters. We recommend to use OCR_A or OCR_B as the font for the OCR. We do not recommend using underscores (_) or special characters within the text. You can select different options to optimize the recognition quality, but the standard

Parameter	Description
	parameter should work. The printed text should be very precise in terms of its orientation. There should be minimal text tilt after the image orientation.

i INFO

You have the possibility to adjust the display curve (see **Display** tab on the bottom of the window). The display settings will stored within in the profile. This has also an impact on the display of the label in the **Magazine** view.

22.3.3.4.2.3 Preview Scan Settings



The preview for brightfield scans will always be taken with the separate preview camera, thus the system generates the preview image with one snapshot.

Parameter	Description
Camera, Exposure, White Balance	This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. As the controls are similar to the standard ZEN controls for cameras, please refer to the appropriate chapters, see **ZEN (blue edition)** Online Help / Camera tool. It is not possible to change the camera for the preview (under **Camera**), as this is fixed. See also the following information regarding the best setting for an exposure time.
Preview lamp	Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light). But this value should not be changed and should be 100%.

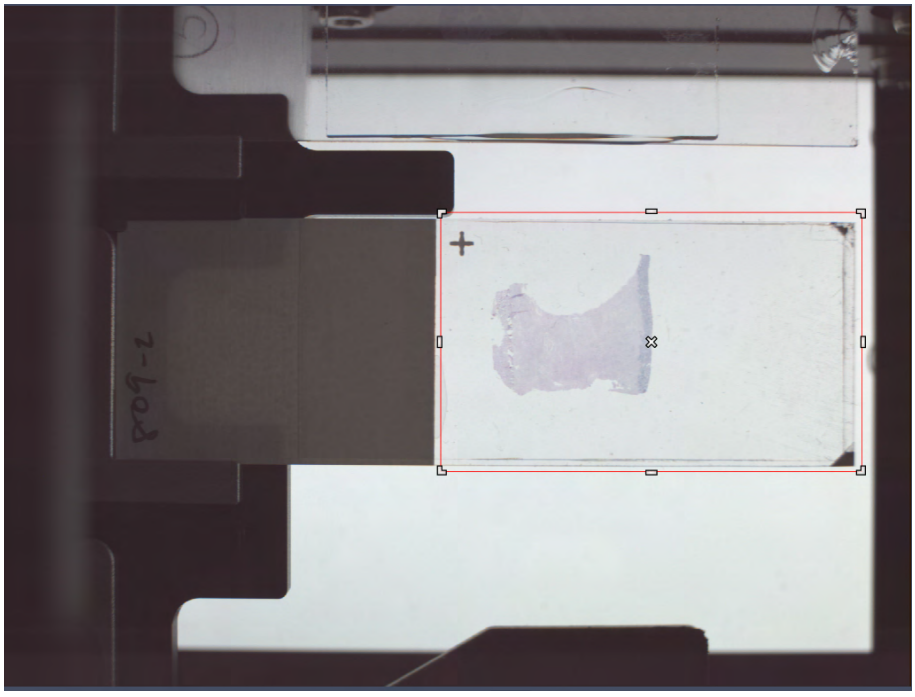
INFO

Avoid any kind of over exposure of the image (especially outside the slide). An overexposed image can result in a sub-optimal shading correction. The same applies if the image is too dim, also this will result in a sub-optimal shading correction. To avoid these issues activate the checkbox to view the overexposure (lower part of the window in the tab **Dimensions** the checkbox **Range Indicator**). Adjust the exposure time of the camera (expander **Exposure** and the setting **Time**) to a value until you see the first red pixels showing the overexposure, now decrease the exposure time in 1 ms steps until the red pixels disappear. Once the red pixels disappear decrease the exposure time additionally by 1 ms to be sure that for all cases no overexposure will be seen.

Notes on setting up the region of interest for the preview image

An important adjustment to be made is the definition of the region of interest for the preview image of the specimen area. The system will take this region of interest, capture it, and apply tissue detection (if selected) to it. This includes the saved image along with the scanned image and the image of the label area.

The region of interest is adjusted on the middle part of the **Center Screen Area** via the red rectangle:



The red rectangle can be adjusted by editing its frame.

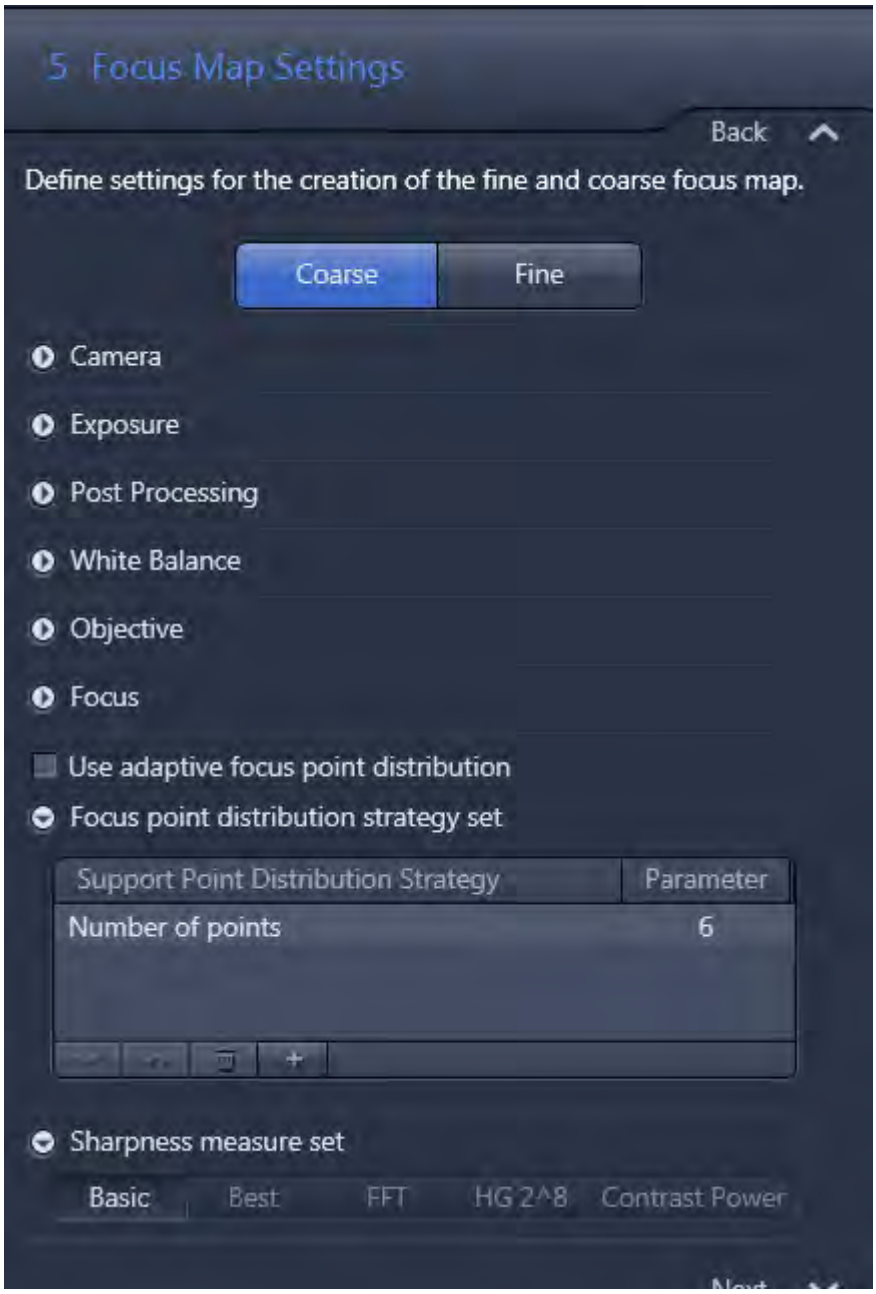
INFO

For best results the frame should cover the complete slide and some air around. Especially for the detection of an empty area on the slide, for the automated shading correction on glass, it is important that the red frame is not set too small (e.g. around the tissue only). Otherwise the sample could be considered as empty region. This would result in a sup-optimal shading correction. If you want to limit the range for the tissue detection use the red frame in the **Tissue Detection Settings** section in the sub-sequent step. Avoid that the red frame covers the label area of the slide.

INFO

You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the specimen area is displayed in the Magazine view!

22.3.3.4.2.4 Focus Map Settings



Appropriate focus map settings are important for ensuring good focus quality throughout the specimens. The focus map generation is done in two steps:

- With a low magnification objective to generate the **Coarse** focus map. For this coarse focus map, only some focus points are necessary. We recommend the use of the 5x Fluor for the **Coarse** focus as the offset between the 5x and the scanning objective (mostly 20x) is marginal.
- With a high magnification objective (normally the scanning objective) to generate the **Fine** focus map. In this case, more focus points are needed and this is normally defined via a density. The Fine focus map is based on the coarse focus map.

The focus is not a traditional autofocus that needs to hit the z-value with the sharpest image precisely. The system will acquire a z-stack (defined in the next step) and calculate a curve through the focus value (e.g., contrast value). This curve is used to determine the peak value. This makes it possible to cover a large range while still maintaining fast focusing.

As we recommend leaving most of the settings untouched, here we will focus on the two most important settings, which are the **Focus Point Strategy** and **Sharpness Measure sets**. You will find a detailed description of these two settings in the following chapter.

We also recommend to read the following topics where you can find detailed instructions on how to adjust the coarse and fine focus settings:

- *Adjusting Coarse Focus Map Settings (BF)* [▶ 1005]
- *Adjusting Fine Focus Map Settings (BF)* [▶ 1008]

22.3.3.4.2.4.1 Focus point strategy set

INFO

The focus point strategy set will be applied for each scene, i.e., as a separate detected object in each case. If you select six fixed points for the Coarse focus, the software will apply 6 focus points for each scene.

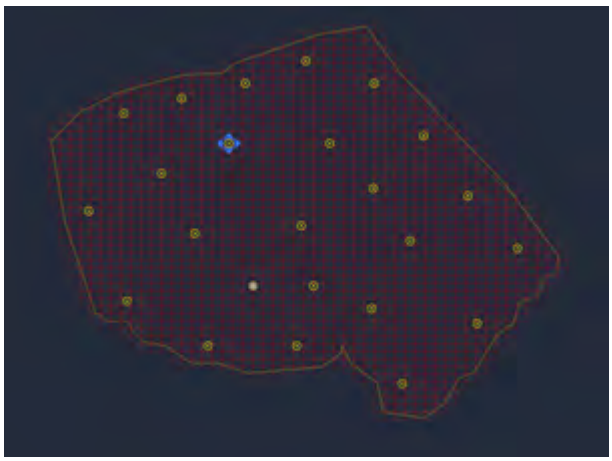
Certain **Focus point strategies sets** have an additional checkbox called **Prefer border**. If this checkbox is activated the system will put preferable more focus points to the border of the detected specimen. This is advisable if the border of the specimen has compared to the other parts of the object significant other focus values.

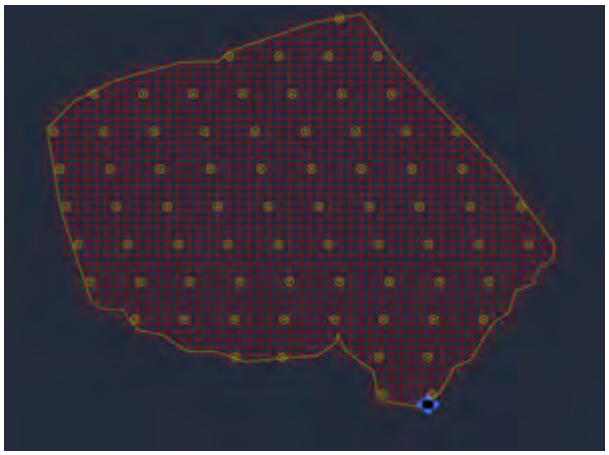
If the setting density is part of the **Focus point strategy set** this value gives the percentage of generated focus points in relation to the total number of tiles of the object (e.g. if the object has 2000 tiles and the setting is set to 0.1 (= 10%) the system would generate 200 focus points). As especially for larger objects too many focus point will be generated (you have to keep in mind that not always a larger number of focus points will automatically result in higher overall focus quality) you can define also a maximum number of focus points (**max. number**) which is advisable.

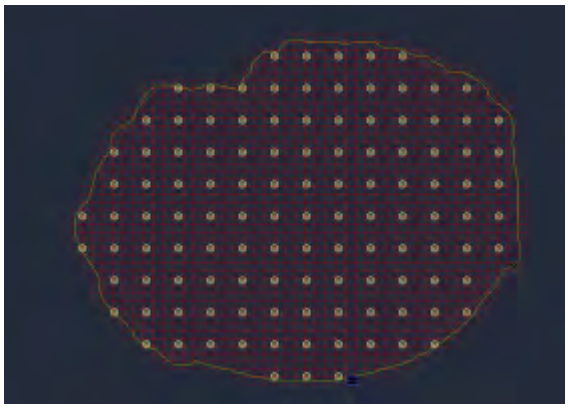
If you use the automatic tissue detection and you define a border dilation the system will subtract this dilatation before the focus points are distributed, thus no focus point will be put in the dilation zone. This applies only for the automatic tissue detection.

To ensure that the focus points are within the tissue the system will move the focus points a certain number of field of views away from the border. This value can be adjusted under **Tools** -> **Options …** -> Tab **Acquisition** -> Expander **Axio Scan**: **Minimum Margin of focus point distribution in tile(s)**. The default setting is 1.

This gives with a wide variety of specimens a very good result. It has to be kept in mind that this value for the software version ZEN 2012 slidescan and ZEN 2012 slidescan SP2 was 2 (and a fixed not shown parameter).

Item	Description
Onion skin	This is a density focus schema. The resulting focus points are displayed as layers of onion skin, providing an even distribution and also ensuring that the border in particular has enough focus points. This is the standard setting for the fine focus map. The parameter is a density with a standard setting of 0.1. This setting means that 10% of the total number of viewing fields will be used as focus points to calculate the focus surface. For large specimens this number can get very large, but a larger number of focus points does not always automatically mean a better quality calculation of the focus surface. For this reason, the user can also define a maximum number of focus points. This number is normally in the range of 24 to 36 points. This focus point strategy set is recommended for mid-sized to large objects. Example (Density: 0.1; max number 24): 
Every Nth tile	The system will put a grid over the specimen with a distance between two focus points of the specified value (N) for the number of camera viewing fields in the x and y direction. This is a clearly defined way to create the focus point, even it creates more focus points and will take longer to create the focus map. The focus point strategy set is recommended for mid-sized and smaller objects. Example (every 5th tile):

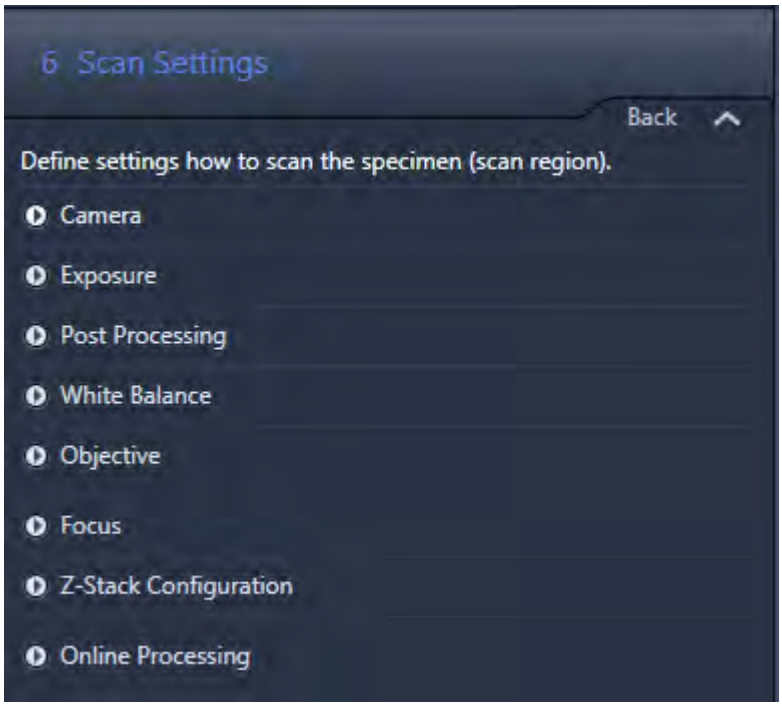
Item	Description
	
Every tile	The system will focus on every tile. This is particularly time-consuming for scans with a large magnification and/or large samples; however, it yields the best results for very uneven specimens.
Number of focus points	Here the user defines a fixed number of focus points per object (scene). The default setting for the Coarse focus is six focus points per scene. Example (n = 24): 
Density	The density is similar to that of an onion skin; the difference is that the pattern is not distributed like the layers of an onion. The distribution is more evenly spread over the specimen/scene. This setting can also be used to define a maximum number of focus points. The creation of this focus map is more random – as shown in the example – and thus the onion skin is recommended over the density. Example (density: 0.1; max number 24):

Item	Description
	
Grid cell size	This setting defines a fixed grid over the specimen to establish the focus points for the focus surface. The value is in µm. This setting is independent from the current frame/sensor size of the camera used. If you want to use a setting that is based on the chip size of the camera, please use “Every Nth tile”. As seen on the sample, this schema is not as strict as Every Nth tile, thus Every Nth tile is recommended over the Grid cell size. Example (1000 µm 1000 µm): 
Center of Gravity	The system will place one focus point within each scene/ object. The position of this focus point is the center of gravity, thus the whole object has only one focus point. This is a useful setting for small objects (e.g., Tissue micro arrays). Be aware that for certain structures (such as a half moon), the center of gravity may lie outside the tissue, hence the system has nothing to focus on. In such cases, select “Number of focus points” with 1 as the parameter. The center of gravity is a valid option, e.g., for Tissue Micro Arrays to place only one focus point in each core.

22.3.3.4.2.4.2 Sharpness measure set

Item	Description
Basic	The autofocus is contrast-based, hence the algorithm will focus on the highest contrast along the z-stack. It is a simple but fast autofocus.
Best	The autofocus is also contrast-based in this case, thus the algorithm will focus on the highest contrast along the z-stack. Compared to Basic, the focus is slightly slower but more accurate for certain samples. Please be aware that Best does not mean that this algorithm is always superior to Basic, as this depends on the kind of tissue/specimen.
FFT	This autofocus setting applies to the Fourier transform algorithm to determine the best focus value. This algorithm is slower than the other algorithms and should be used only in the case of the Ring aperture contrast. It is not ideal for standard brightfield contrast or fluorescence as this algorithm looks for two contrast peaks and the local minimum between them.
HG 2^8	This is an advanced algorithm compared to Basic and Best. This algorithm focuses less on the larger stained structures and more on the smaller local structures, thus this algorithm will focus more on nuclei, for example, than on unstained structures (e.g., immunohistochemistry stains). Based on the current status of evaluation, this means that HG2^8 is a good autofocus method if a user wants to focus on nuclei. **Drawback: The weight of dirt also increases, hence it should only be used for the fine focus, not the coarse focus.**
Contrast Power	This algorithm is comparable to the HG 2^8.

22.3.3.4.2.5 Scan Settings



In this wizard step you can adjust the scan settings. These settings are important as they determine the image quality that is subsequently experienced by the user in terms of exposure time, white balance, etc. The settings under **Post Processing** and **White Balance** are similar to the standard ZEN tools. You can read how to adjust the settings in the chapter *Adjusting Scan Settings for BF Profiles* [▶ 1012].

22.3.3.4.2.5.1 Comparison compression regime vs. file size

Compression regime	File size
No compression	9124 MB/cm^2
Lossless (JPEG XR)	4997 MB/cm^2
90% JPEG XR quality	916 MB/cm^2
80% JPEG XR quality	392 MB/cm^2
60% JPEG XR quality	263 MB/cm^2
40% JPEG XR quality	198 MB/cm^2
30% JPEG XR quality	157 MB/cm^2

22.3.3.4.2.6 Tissue Detection Settings

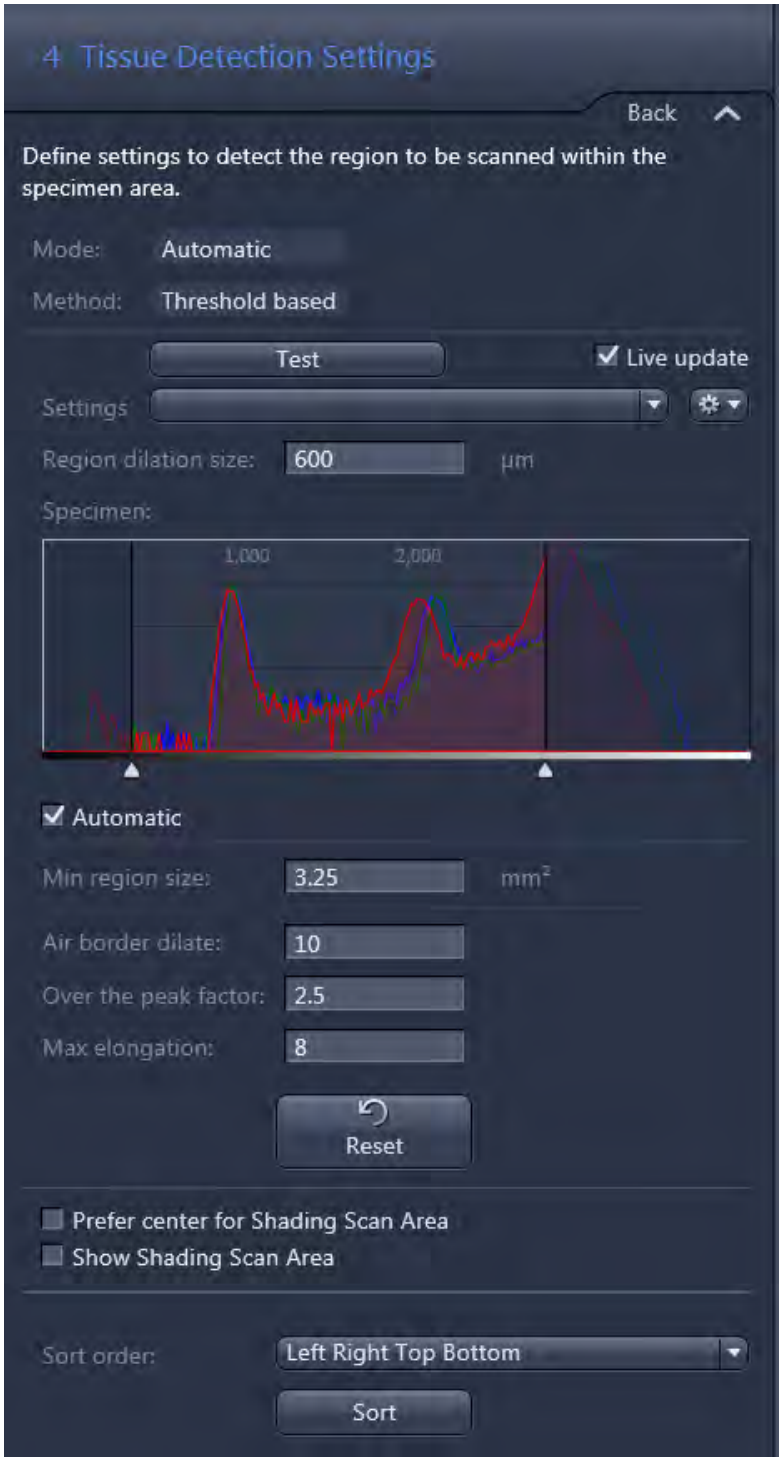
The appearance of the Tissue Detection (TD) Settings depends on the settings which you have made in the first step of the wizard under **Global Data**. In the following sections four possible cases are described.

> **INFO**
>
> You have the possibility to implement your own tissue detection algorithm and make this algorithm part of the workflow, see *Implementing your own tissue detection algorithm* [▶ 975].

22.3.3.4.2.6.1 Case A: Automatic Mode / Threshold based

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Automatic**, as well as
- under **TD Recognition Type** you have selected **Tissue**, the TD settings will appear as follows:



Region of Interest for Tissue Detection

INFO

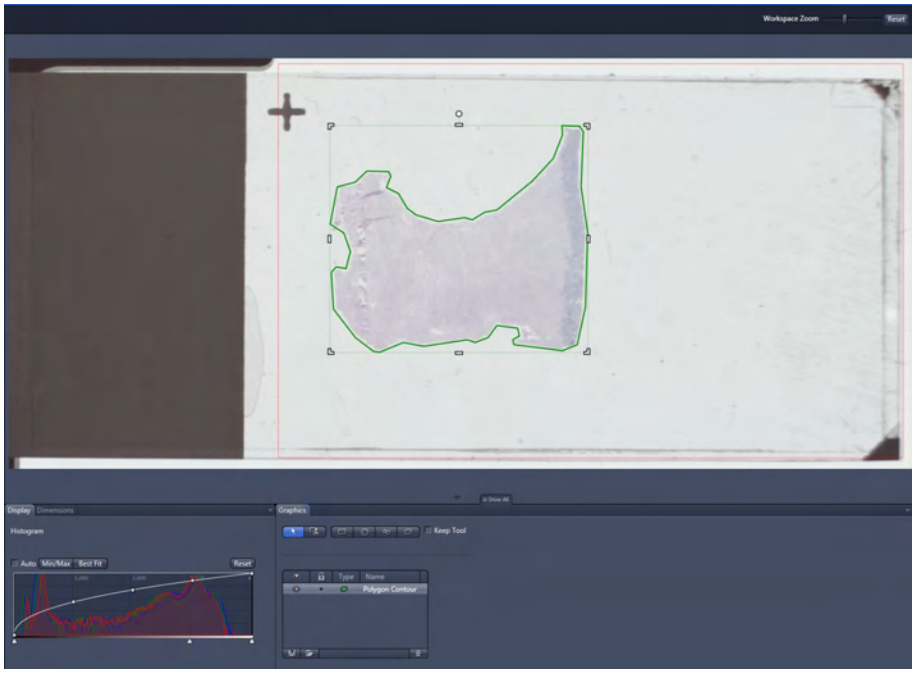
If you draw your own regions, make sure that these contain tissue. Empty space could lead to an incorrect focus map as the software will use the entire drawn region to create the focus point distribution.

The red rectangle in the live image which you can see in the **Center Screen Area** represents the ROI for the Tissue Detection. Only the regions that are inside the ROI will be presented as regions which can be scanned. Other regions outside the ROI and/or touching them will be ignored. You can change the size of the ROI by adapting the size of the red rectangle.

INFO

If two or more objects are very close to each other and thus the two or more objects are seen as one objects it is perhaps advisable to split these objects with the **Split** function which is available in the context menu seen after a right-mouse click.

If you click on **Test**, the software will execute the inserted parameters and show the results in the middle part of the window. The detected objects (every object is a single scene) will be presented with a green border.



All objects are listed in the **Graphics** tab. You can delete objects and even add new ones. However, the result will be only valid for this slide.

To achieve a continuous update, you can activate **Live update** via the checkbox. Keep in mind that a refresh can take some seconds after changing a parameter. The main parameters of this step are described below:

Parameter	Description
Mode & **Method**	Here you can see the mode and method for tissue detection which you have selected under **Global Data**.

Parameter	Description
Region dilation size	Here you can enter a value for the spacing of the border around the detected tissue.
Specimen	Here you define the upper and lower borders of the specimen via the thresholds. By clicking on one of these borders and pressing the right mouse button you can move the borders up and down. If you have activated the **Live update**, you will see the result toward the left of center with a certain delay. The upper border (right side) is the border for the lighter stains and the lower border (left side) is the border to influence the darker stains. Thus moving the right side further to the right marks the lighter stains. Moving the left side further to the left marks the darker stains.
Min region size	Here you can enter the minimum region size to be scanned. The system will not detect regions if they are smaller than this value.
Air border dilate	The air border is the border around the physical glass slide, i.e., the area outside the glass slide and inside the red frame. This value determines the dilation of this air border. Everything inside this dilated air border will not be recognized as tissue/a marker. This is a measure for ensuring that slide edges and coverslip edges are not detected as needing to be scanned.
Over the peak factor	This parameter is only visible if the **Automatic** checkbox is activated. It defines the distances between the glass peak of the slide and the upper threshold value for the specimen detection. Thus if **Automatic** is activated, the upper value will be determined automatically.
Max elongation	This setting is important to remove coverslip edges. Coverslip edges are normally structures with a large number for the ratio of the longer side and the shorter side. This means that every structure that exceeds this parameter for the ratio between the longest side and the shortest side will be excluded.
Reset	Reverts the values to their default settings.
Prefer Center for Shading Scan Area	If activated, the software will force the region which will be used for the shading correction in the middle of the slide for magnifications of the scan of >= 20x to avoid to be influenced by e.g. the coverslip edges. By default this option is deactivated.

Parameter	Description
	If you see in the scanned image tiling artefacts we recommend to activate this option and recheck the shading quality. If this option is activated it can be that the region is splitted in smaller regions (as the software is limited regarding the search area because the outer regions are out of limits), this has as consequence that the shading correction scanning is slower, but compared to the total scanning the reduction of several seconds has no major influence on the scanning time.
Show Shading Scan Area	If activated, the software will show the area used to generate the shading reference image for this slide. For certain scan magnifications the region will be shown even it will be not used.

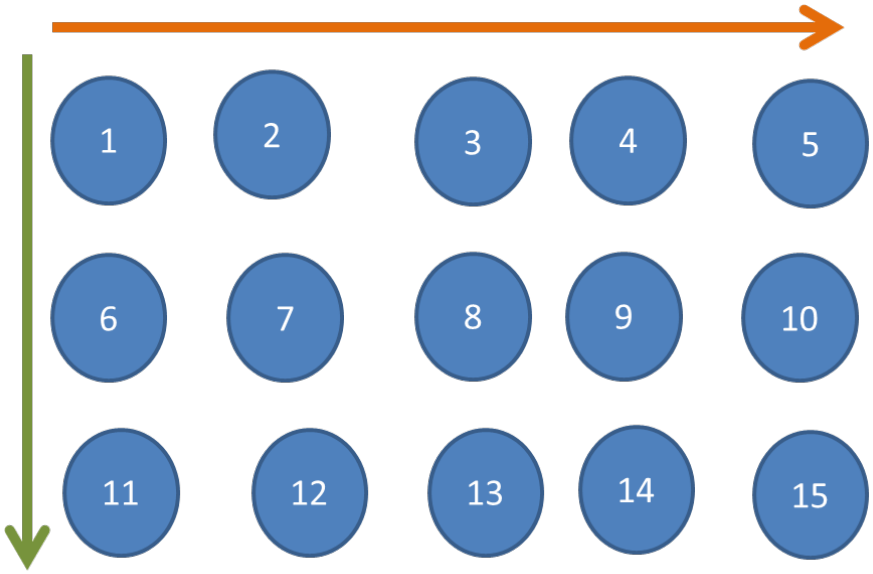
Once you have determined the appropriate settings, you can save them by selecting **New** via the **Options** icon under **Settings**. The settings can then be used in another profile simply by selecting the respective entry from the dropdown list. It is not essential to save these settings as they will be saved within the profile. It is only necessary if the settings are to be used in another profile.

Sort Order Section

In the **Sort Order** section you can select the sorting schema (numbering) for the detected objects (regions / scenes). By clicking an **Sort** the selected schema will be applied. You can select from 8 different schemes. The first entry (e.g. Left Right) provides the first sorting schema and the second entry (e.g. Top Bottom) the second sorting schema.

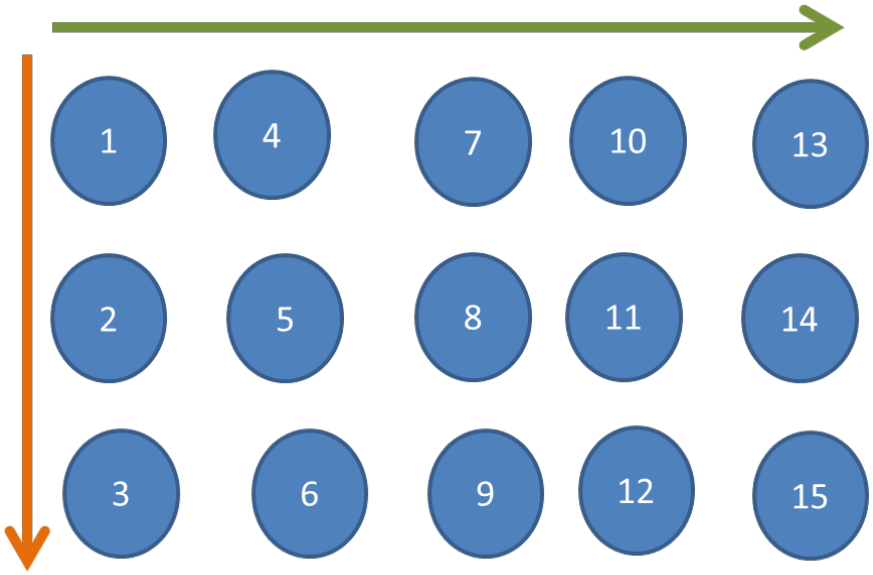
Example 1: Left Right / Top Bottom

The first sorting schema is from Left to Right (red arrow) and the second sorting schema is from Top to Bottom (green arrow):



Example 2: Top to bottom / Left to right

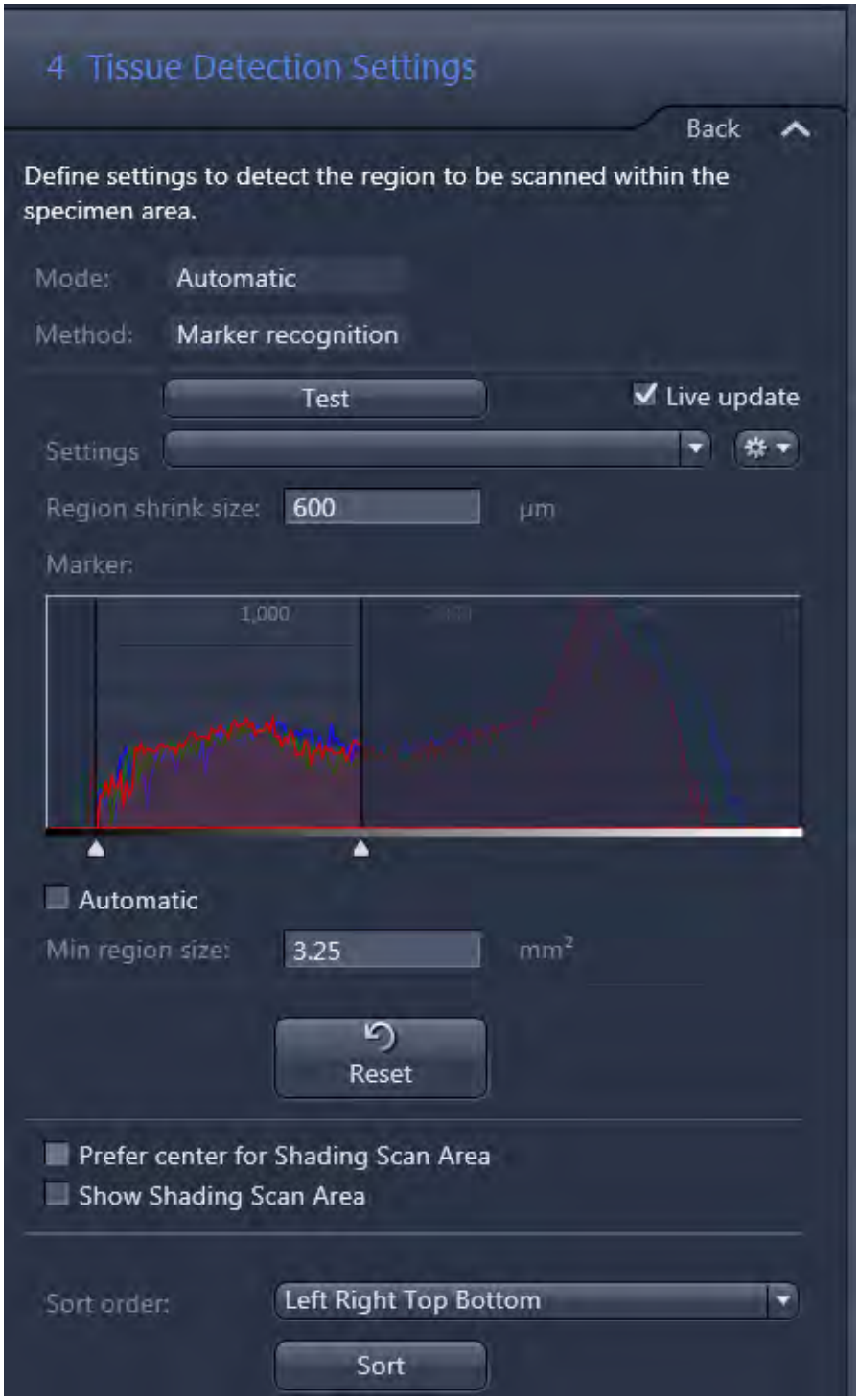
The first sorting schema is from Top to bottom (red arrow) and the second sorting schema is from Left to right (green arrow):



22.3.3.4.2.6.2 Case B: Automatic Mode / Marker recognition

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Automatic**, as well as
- under **TD Recognition Type** you have selected **Marker**, the TD settings will appear as follows:



The settings are limited to the definition of the marker threshold (comparable to the tissue threshold described above). The only further adjustable value is the **Min region size**, whose functionality is the same as described in Case A.

Note that also the **Shading** options, the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

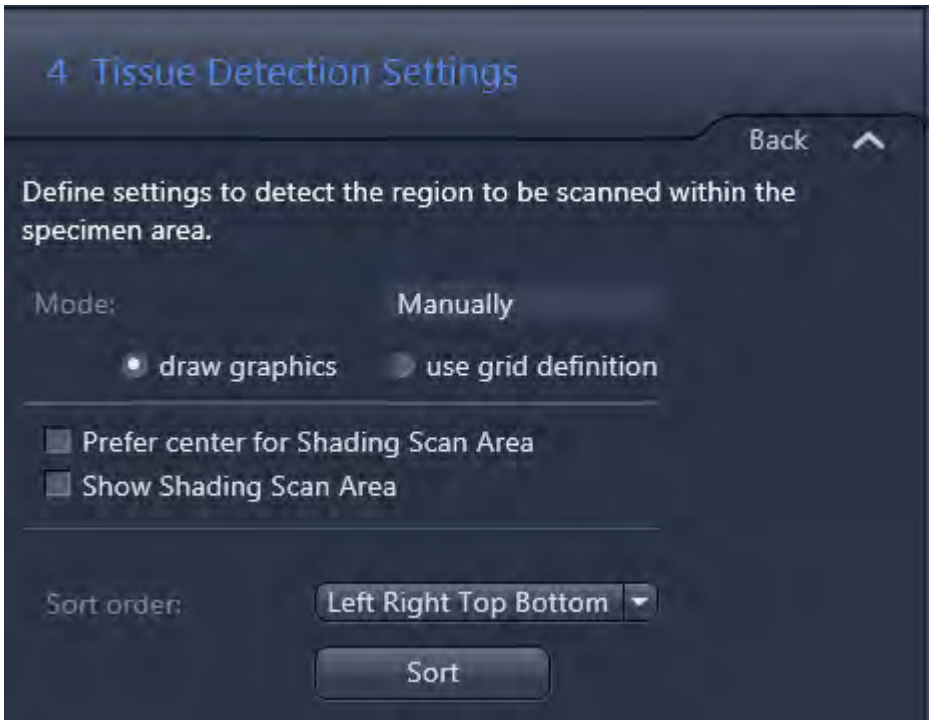
i INFO

If you use the marker on the physical glass slide, make sure that the regions marked contain specimens. Empty space could lead to an incorrect focus map as the software will use the entire marked region to create the focus point distribution.

22.3.3.4.2.6.3 Case C: Manual Mode / Draw Graphics

Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Manually**, the TD settings will appear as follows:



Drawing in TD ROIs manually

If you have selected **draw graphics** it is possible to draw regions manually within the region of interest if you activate draw graphics. To draw regions, you can use the tools on the **Graphics** tab in the general view options of the **Center Screen Area**. You can use it in the same way you would generally annotate images within ZEN. The drawn regions will be saved and the same areas will be used for the entire time the profile is applied, hence this option can only be used if the regions are always defined precisely on the slide (e.g., PAP smears).

Note that also the **Shading** options, the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

INFO

If you want to mark a sample that is very faint, you can use the **Display** curve to change the display settings (particularly by adjusting the gamma curve) to make it possible to see even unstained sample of a reasonable thickness.

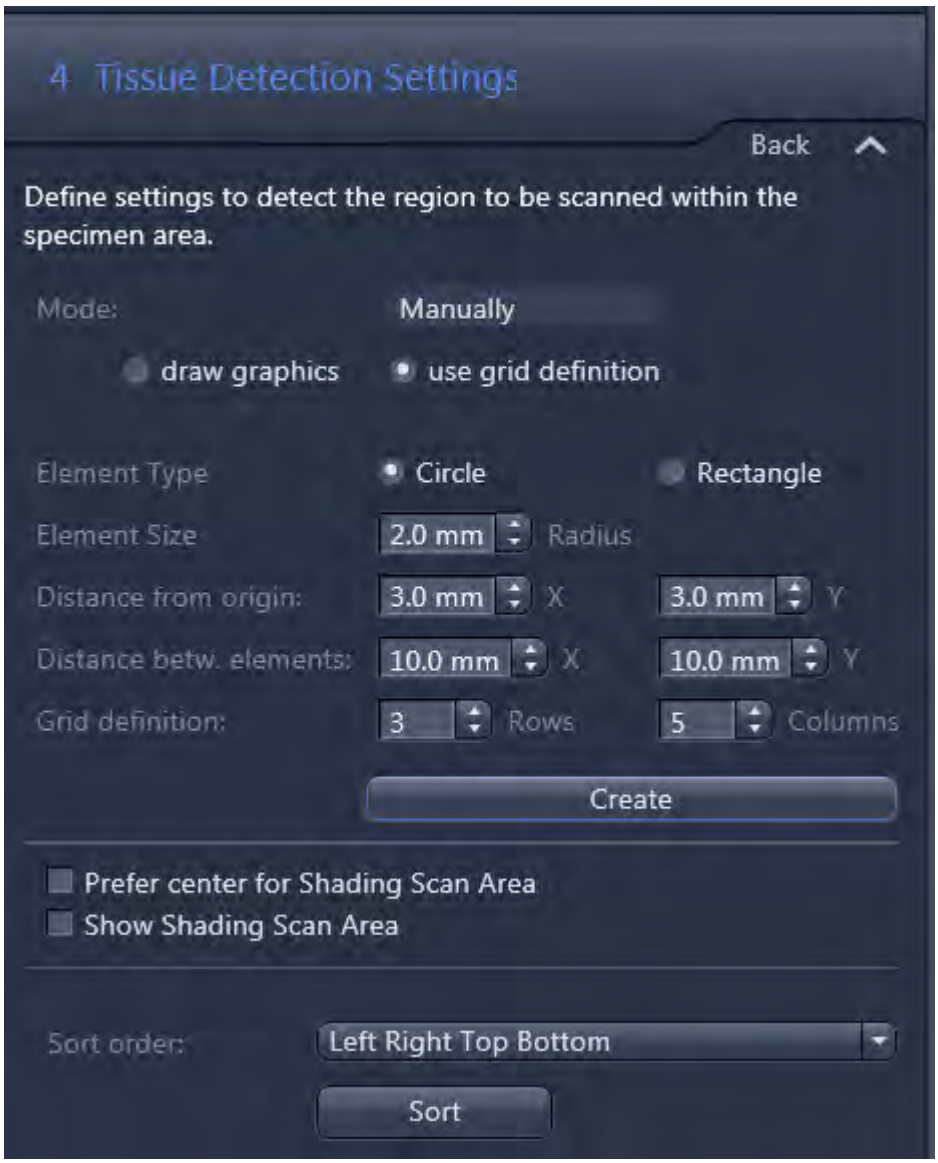
INFO

You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile. This has also an impact on the display of the label in the Magazine view. This has also an impact on how the sample area is displayed in the Magazine view!

22.3.3.4.2.6.4 Case D: Manual Mode / Grid definition

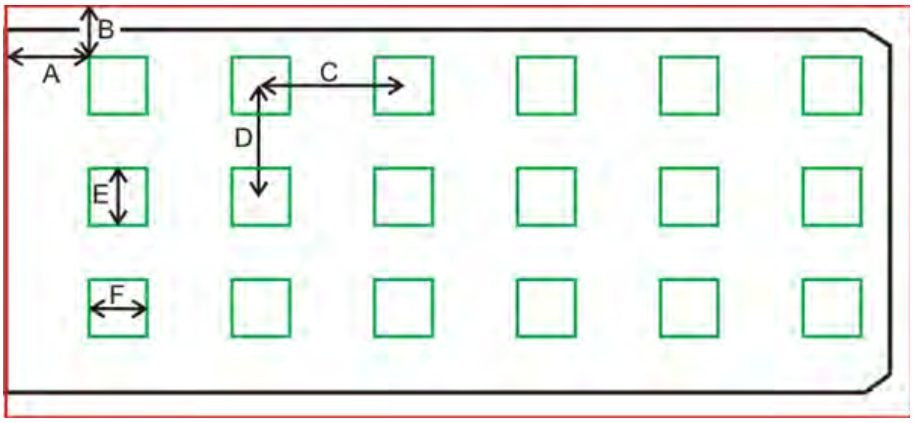
Prerequisites

- If in Step 1 **Global Data** under **Tissue Detection Mode** you have selected **Manually**, the TD settings will appear as follows:



If you have selected **use grid definition** you can define a grid with rectangles or circles as **Element Types** in a regular pattern. The reference point for this grid is the upper right-hand corner of the specimen area (red rectangle). Based on this, you can define on offset from the upper left-hand corner in x (A) and y (B) in mm and also the pitch of the grid in x (C) and y (D).

Based on the selected geometrical shape (circle or rectangle), you can define a radius or the dimensions in x (F) and y (E). The number of elements in x (in this example, 6) and y (in this example, 3) defines the number of elements.



When you click on the **Create** button, the grid will be generated. You will see the grid as an overlay in the image. It is also possible to make manual adjustments such as those used for standard graphics in ZEN.

Note that also the **Shading** options, the creation of the **Sort Order** and the **Sort** button have the same functionality as described in Case A.

22.3.3.5 Advanced Scan Profile Wizard (offline)

This wizard is an offline wizard, which means that the system will not move the tray (slide) and you will not see any live images from the cameras. But you can still change the parameters. This is a mode that is appropriate if you want to change parameters that are not directly associated with a live image; e.g., Z-stack and EDF (extended depth of focus) settings.

It is not necessary to move the physical glass slides to adjust these settings. As a result, the functionality is limited compared to the **Advanced Profile Wizard (online)** but does not contain more settings than this wizard. Please refer to the Advanced Profile Wizard (online) for help with certain functions.

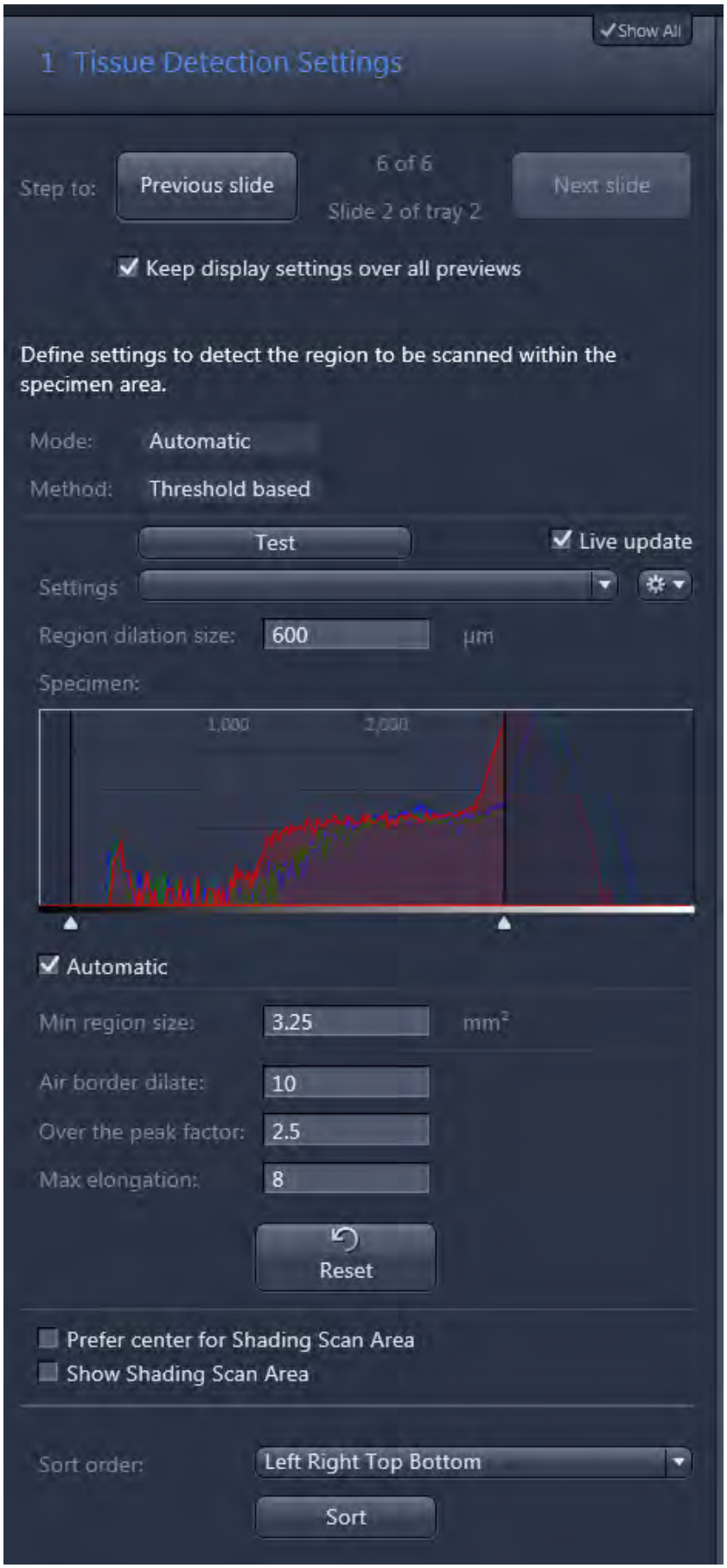
22.3.3.6 Tissue Detection Wizard (offline)

i INFO

This wizard is only active if a preview scan was generated previously. Once the system has started the preview generation, you can start the Tissue detection wizard immediately and work in parallel.

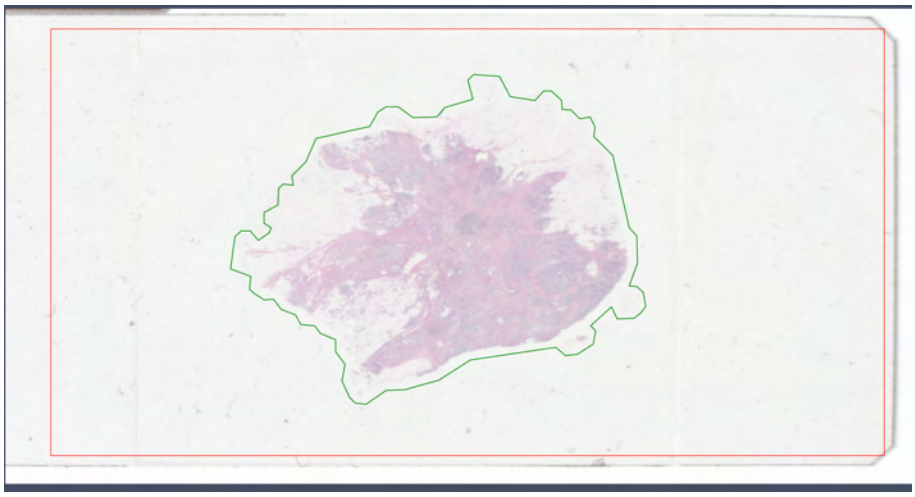
The wizard is accessed via the Scan Profiles options menu.

Here you can change the region that the system will scan afterwards in a single step. It is not possible to change any settings other than tissue detection; however, the option is available to navigate directly to the next slide (**Next slide**) or previous slide (**Previous slide**). This makes the switch between the slides fast and convenient.

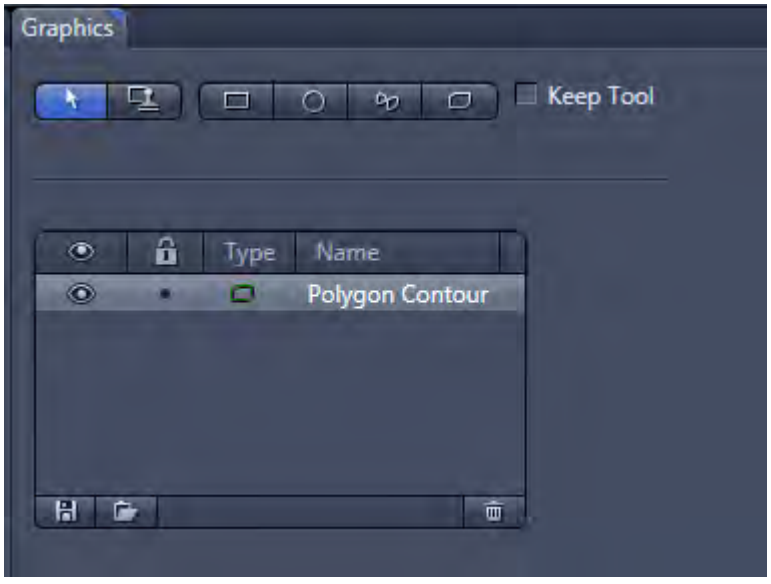

Show All
1 Tissue Detection Settings
Step to:
Previous slide
6 of 6
Slide 2 of tray 2
Next slide
Keep display settings over all previews
Define settings to detect the region to be scanned within the specimen area.
Mode:
Automatic
Method:
Threshold based
Test
Live update
Settings
Region dilation size:
600
µm
Specimen:
1,000
2,000
Automatic
Min region size:
3.25
mm²
Air border dilate:
10
Over the peak factor:
2.5
Max elongation:
8
Reset
Prefer center for Shading Scan Area
Show Shading Scan Area
Sort order:
Left Right Top Bottom
Sort

This option does not exist for the other wizards. It is not necessary to move the physical glass slides to adjust these settings.

Generally speaking, you will not change the settings in the wizard on the left-hand side. Instead, you will normally work directly in the image of the specimen area to mark the region(s) of interest or delete regions of interest that have been wrongly detected. To delete a region of interest, simply select the region and hit "**Delete**" on the keyboard. You can also use the multi-select function.
The hotkey CTRL+Z allows you to undo the most recent actions.



The red rectangle is not a graphic; it is the frame to define the region where the automated tissue detection is looking for specimens. It is possible to draw graphics manually outside this frame. In the **Graphics** tab in the lower part of the window, you can view and manage the graphics (regions of interest) that you have drawn.



As the regions of interest are standard graphics within ZEN, you have access to the advanced editing options depending on the graphics type, such as Split, Merge, Edit points, etc.

The tissue detection settings are basically the same as described in the **Advanced Scan Profile** wizard in the Tissue Detection Settings chapter.

22.3.3.7 Axio Scan Calibration Wizard (online)

> **INFO**
>
> The first position of the tray must hold the Geometric calibration slide (474029-9030-000), the second position the Color calibration slide (474029-9041-000).
>
> The tray with the calibration slides must be placed in the first position within the magazine.

The following settings can be calibrated or checked:

- Parfocality of the objectives
- Parcentricity of the objectives
- Adjustment between preview camera and scan camera
- Scaling of the objectives
- Determination of the camera rotation (this is a double-check); if the camera rotation is not in the expected range (<= 0.5°), please contact ZEISS service
- Color calibration (you need the Color calibration slide (474029-9041-000) for this calibration) – this step can be skipped

The wizard guides you through the process step-by-step. The steps do not normally require user interaction (only the color calibration step needs an user interaction, loading the calibration, thus this calibration should be available). It is only necessary to start the action for each step. Once the action is complete, this will be indicated by a green light. You can then continue with the next step. The last step is to save the data to the system.

22.3.4 Functions and Reference

22.3.4.1 Scan Tab

Here you will find the most important functions needed to operate the **Axio Scan.Z1** system. The tab is closely interlinked with the Magazine view, which can be found in the **Center Screen Area**. On top of the tab, you will find the **Default Scan Profile (1)** section where you can select predefined scanning profiles or create new profiles. Additionally, the **Smart Profile Selection (2)** and the **Scan Profile Wizard (3)** will help you with finding a suitable profile or changing and creating a new one. You can start your preview scans or scans with the prominent **Scan buttons (4)**.

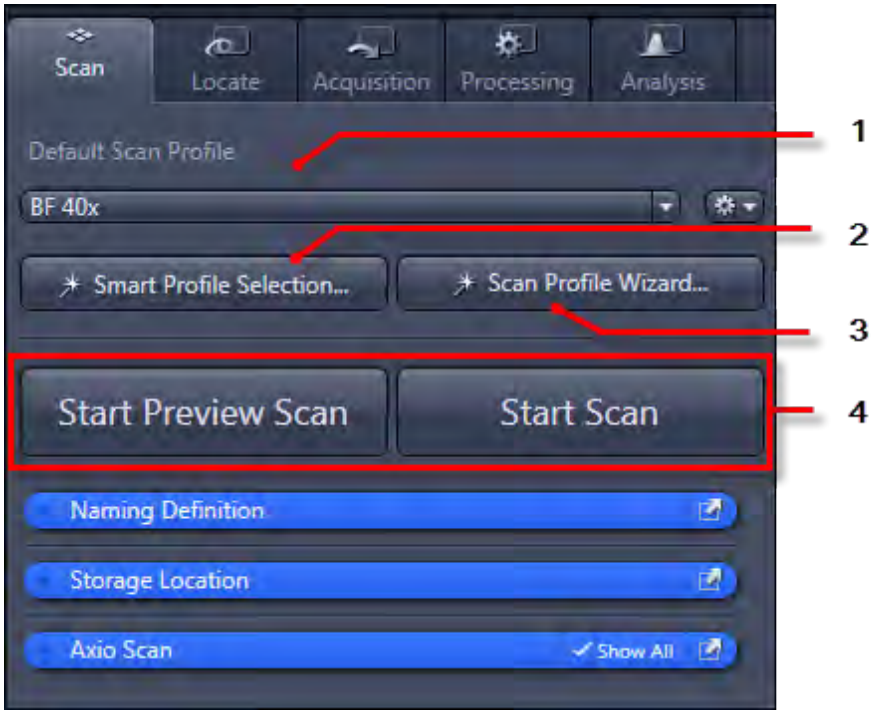


Fig. 22.19: Scan tab

Parameter	Description
Default Scan Profile section (1)	From the dropdown list, you can select the default scan profile which will be used if new mounting frames are inserted. The system assigns the selected profile to all newly inserted slides. Click on the **Options** button to open the *Default Scan Profile Options* [▶ 1076] context menu.
Smart Profile Selection…(2)	Clicking on this button opens the Smart Scan Profile Selection wizard. This wizard helps you to open predefined profiles. This is the fastest and most intuitive way to assign and generate scanning profiles.
Scan Profile Wizard…(3)	Clicking on this button opens the Scan Profile wizard. This wizard helps you to change/modify existing scanning profiles very easily. You can change the profile for each mounting frame afterwards in the **Magazine** view. If the default profile is changed, this profile will also be assigned to all unprocessed slides.
Scan buttons (4)	
- Start Preview Scan	Starts a preview scan of all selected slides.

Parameter		Description
		To select a slide, go to **Magazine** view and activate the slide's checkbox in the **Process** column. This checkbox will be activated by default if mounting frames are inserted. The settings to generate the preview will be used as defined by the assigned profile. For brightfield specimens, the preview consists of a snapshot of the label area (reflected light) and of the specimen area (transmitted light). The result will be shown in the **Preview** column of a slide. To enlarge the view, double-click on the image. The software opens the image in a separate tab. To adapt the regions to be scanned, please use the **Advanced Scan Profile Wizard** or the **Tissue Detection Wizard**.
-	Start Scan	Starts a scan of all selected slides. The scan consists of the preview generation (label area and specimen area), tissue detection, focus map, and high resolution scan of the specimen. The settings will be used as defined by the assigned profile. To open an image, double-click on it and it will open in a separate tab in the center of the screen.

22.3.4.1.1 Default Scan Profile Options

Menu entry	Description
New Scan Profile…	Creates a new profile. If a new profile was created, it will be automatically assigned to all slides in **Magazine** view.
Smart Profile Selection…	Opens a wizard to select a predefined profile. The user will be guided through a series of questions about the specimen, resulting in the most appropriate profile for the digitization process. It is recommended to process this profile afterwards with the Scan Profile Wizard.
Advanced Scan Profile Wizard…	Opens a wizard to adapt all possible profile settings and allows the user to utilize the full flexibility of the system. This wizard is recommended for experienced users.
Scan Profile Wizard…	Opens a wizard to adapt basic settings of the profile; e.g., barcode/OCR/scanning objective/z-stack.

Menu entry	Description
Rename	Renames the selected profile.
Save as	Saves the user profile under another file name.
Delete	Deletes the selected profile.

22.3.4.1.2 Naming Definition Tool

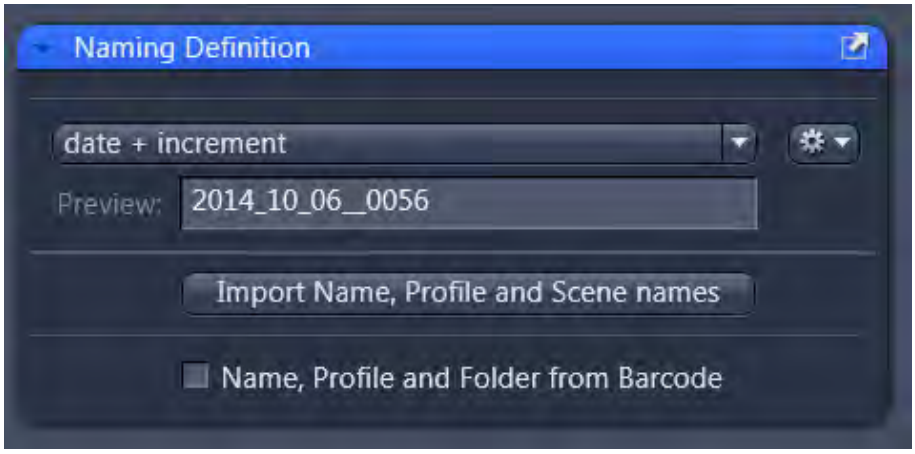


Fig. 22.20: Naming Definition tool

Here you can define naming definitions for the acquired files/images. You can select several definitions for the file names from the dropdown list. The name automatically contains the detected barcode content if the barcode detection is active for the active profile. Click on the **Options** button to define new, editor delete existing naming definitions.

INFO

The **Preview** field shows a preview of the configured file name.

Parameter		Description
Naming Definition options		
-	**New**	Here you can create a new naming definition. The system will ask for the name of the definition and you can then set up the naming definition in the naming dialog.
-	**Edit**	Here you can edit the current naming definition.
-	**Rename**	Here you can rename the current naming definition.

Parameter	Description
Save As	Here you can save the current naming definition under another name.
Delete	Here you can delete the currently selected naming definition.
Import Names, Profiles and Scenes names	Clicking on this button allows you to import the image names and used profiles. You also have the option to apply scene names. When starting an import, it is necessary to insert all your slides beforehand. The software will only apply the imported parameters for slides that have been inserted with the status "**new**". You can read more details on the import under Importing names, profiles, and scenes.
Name, Profile and Folder from Barcode	Activating this checkbox allows you to use barcode information to name image, assign profiles and sub directories. You can read more details on the import under *Using barcode to define name, profile and subfolder* [▶ 977].

22.3.4.1.3 Storage Location tool

Fig. 22.21: Storage location tool

Here you can specify the storage location of the created images. A location can be a local path on the computer in use as well as a network share to store the data on a remote server. It is recommended to store the images locally, as depending on the network properties, the connection may not be sufficiently reliable to store the data. This may result in the scan workflow stopping if the network is interrupted, or slowing down if the network connection is performing at a lower rate. If a network share is selected, a transmission rate of 100 MB/s is recommended. If they are available, you can select paths that have already been used from the dropdown list. Clicking on the **Create New Folder** button in the **Browse Folder** dialog creates a new storage folder.

22.3.4.1.4 Axio Scan tool

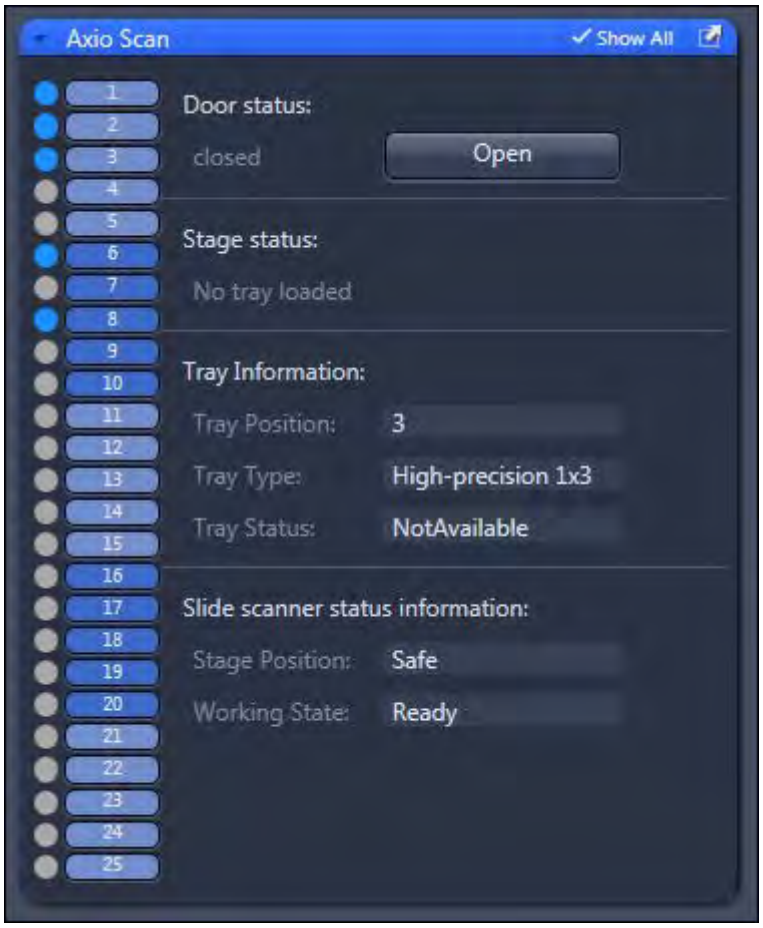


Fig. 22.22: Axio Scan tool

Parameter	Description
Door status	Here you can see whether the door is open or closed. With the **Close**/**Open** button nearby, you can open or close the door depending on the current status. This function is equivalent to the **Open**/ **Close** button on the device itself.
Stage status	Here you see if a tray is loaded or not. You can load a tray by double-clicking on the tray buttons on the left-hand side of the tool. If a tray is loaded, the **Unload tray** button will be shown . Clicking on this button will unload the tray located on the stage into the magazine.
Tray Information	Here you can see information on the tray and the mounting frame. Hover over the tray buttons on the left to see the information in the display fields. The signal colors in front of the buttons indicate the current status of the trays. This is the same kind of signal that can be found on the device itself. Refer to the operation manual for a

Parameter		Description
		detailed description. Double-clicking on the tray button inserts the mounting frame. If another frame is mounted, it will be removed automatically.
-	Tray Position	Shows the position of the tray/mounting frame inside the magazine.
-	Tray Type	Specifies the inserted mounting frame type (e.g. High-precision 1x3 or High-precision 2x3).
-	Tray Status	Shows the status of the frame (e.g. processed, preview done).
Slide Scanner status information		This sections gives you information on the **Stage Position** (e.g. **safe**) or **Working State** (e.g. **ready**) of the device. It is the same information as shown by the main indicator on the device itself (e.g. ready, processing, warning).

22.3.4.2 Magazine View

This view gives you an overview of the **Axio Scan.Z1** magazine. You can assign profiles and naming conventions to slides, show the status, and move trays up and down (using drag and drop) to change the scanning order. The system will start processing slides from top to bottom for all slides that have been activated (via the checkmark in the **Process** column). The view is closely interlinked with the **Scan** tab in **Left Tool Area**.

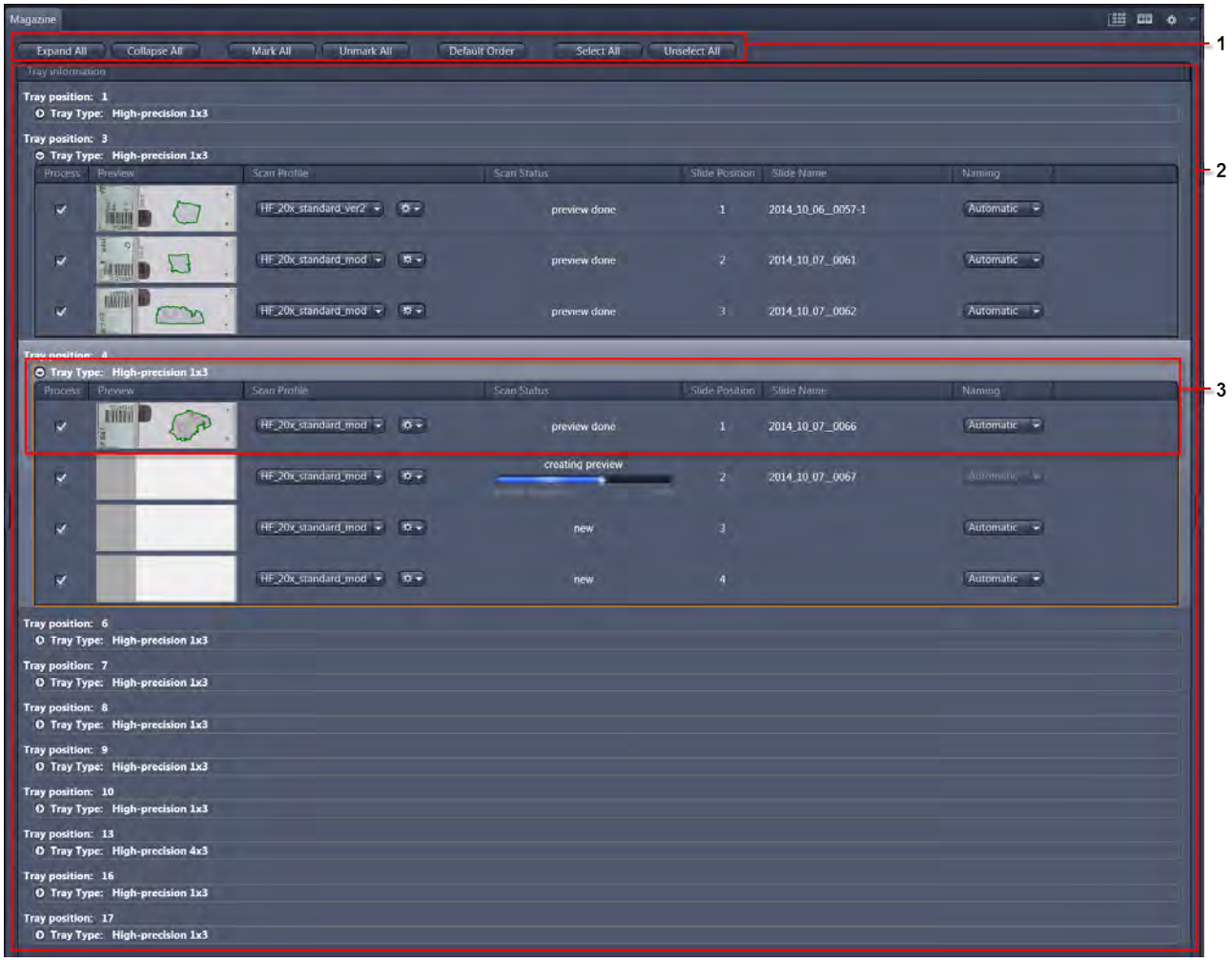


Fig. 22.23: Magazine view

Notes

- If the door is closed (using hardware or software), the system detects all trays inside the magazine and establishes both the tray type and the positions in the tray that are occupied with a physical slide.
- The system will also check all slides by default (see **Process** column) and assign the **Scan Profile** (defined in the **Scan** tab) to every slide if new slides are inserted. It is still possible to change the profile and other settings afterwards (if the slides are not processed or currently scanned), even if the batch process has already started.
- Only the inserted slides will be shown. Empty places in the tray will not be shown. The loaded tray will be visualized with an orange frame around the mounting frame (in our example this would be Tray 4).

Main buttons (1)

These buttons allow you to adjust the display and settings of the magazine view with ease:

Name	Description
Expand All	Expands all trays so that you can see the slides contained within them.
Collapse All	Collapses all trays so that you can only see the tray positions.
Mark All	Activates all slides for processing.
Unmark All	Deactivates all slides for processing.
Default Order	Resets the default positions of trays and slides. Only if you have changed the order of trays or slides (e.g., by drag and drop).
Select All	Highlights all slides within the magazine
Unselect All	Deactivate the highlighting within the magazine

Tray information section (2)

Here you can see an overview of all trays and the inserted slides. Every tray position has the same structure:

The headline for every tray is the physical **Tray Position** (e.g. **Tray position: 1**). Below the tray position information you will see the **Tray Type** which is inserted (e.g. **High-precision 1x3** or **High-precision 2x3**). Right-clicking on a tray will open the Magazine View context menu with several options for magazine view or the individual trays. If you click on the little expander button in front of the tray type, you can expand or collapse the view to see a tray's **Slides** . All trays are expanded by default.

Slide layout (3)

Each row within a tray represents a slide. Right-clicking on a slide will open the Slide context menu with several options for the selected slide. The slide columns are always the same:

Column	Description
Process	Indicates whether the slide will be scanned (checkbox activated) or not (checkbox deactivated).
Preview	Shows the preview image of the slide if a preview was executed. It shows the label area and the specimen area.
Scan Profile	Assigns a specific scan profile to each slide. If a profile was adapted, the system indicates this with a gearwheel in front of the profile name.

Column	Description
	If you click on the **Options** button  behind the scan profile, the Scan Profile Options context menu will be opened.
Scan Status	Gives information about the status of the slide scanning process. This can be: ■ **new**: no preview was executed and no scan ■ **preview done**: preview was already executed and will be displayed (in the **Preview** column) ■ **finished**: the scan was successfully executed If an error/warning comes up, this will be shown here. If you click on the error/warning message, it will be displayed.
Slide Position	Represents the physical position of this slide inside the tray.
Slide Name	Represents the name of the image which will be generated. You can insert a name or the system can create a name according to an automated naming rule. The selection of the procedure to be executed depends on the **Naming** column.
Naming	Here you can select whether the system creates the name automatically using a naming rule or specify a name manually. It is also possible to change the name of the image once the preview has been generated by simply clicking on the name and then editing it. Depending on the settings under **Naming Definition** you can also import names via a CSV file or you can use the barcode information to define a name as substring of the barcode.

i INFO

To enlarge the label image or the preview image of the specimen area simply click on the small preview image. You will see an enlarged view of this image for a better recognition of the content. If the scanning for a slide is finished you do not see the overview image of the specimen you will see a low magnification representation of the resulting image. If you want to close the image click on the image again, move the mouse pointer of the image or click another slide to enlarge another preview / scanned image .

Fig. 22.24: Preview image enlarged

22.3.4.2.1 Magazin View Context Menu

Menu Item	Description
Load tray	The system will move the selected tray from the magazine to the stage and any tray on the stage that has already been loaded will be removed automatically. A loaded tray is marked with an orange rectangle.
Assign scan profile	Opens a dropdown menu where the user can assign an existing profile to all highlighted slides
Mark all slides in highlighted trays for processing	Selects all slides in the highlighted trays for processing (it is possible to select multiple trays).
Unmark all slides in highlighted trays for processing	Deselects all slides in the highlighted trays for processing (it is possible to select multiple trays).
Expand all highlighted trays	Expands all highlighted trays by clicking on the mouse once. To highlight a tray, hold the Ctrl key and click on the trays you want to highlight.
Collapse all highlighted trays	Collapses all highlighted trays by clicking on the mouse once.

22.3.4.2.2 Slide Context Menu

Menu Item	Description
Move to Prescan position	Moves the selected slide to the prescan position.
Move to Scan position	Moves the selected slide to the scan position.
Assign scan profile	Here you can select a scan profile and assign it to the selected slide. If you have selected several slides, the selected profile will be assigned to all of them.
Open image(s)	Only active if a preview was generated or the scan was finished. The software will open the preview or scanned image of the slide. If you have selected several slides, all images from these slides will be opened.
Mark all highlighted slides for processing	Marks all selected (highlighted) slides for processing.
Unmark all highlighted slides for processing	Unmarks all selected (highlighted) slides for processing.
Reset Scan status to new	Resets the scan status of the selected slide to “new”.
Reset scan status to previewed	Resets the scan status of the selected slide to “previewed”.

22.3.4.2.3 Scan Profile Options

Menu entry	Description
Open advanced wizard	Opens the **Advanced Scan Profile Wizard** (Online Mode). In this mode, the system will take the tray containing the slide from the magazine (only if the tray which contains the slide is not already on the stage). You can see a live image from the different cameras (preview camera/scan camera) and navigate on the slide. This mode is useful if you want to adjust exposure times, for example, and set the windows directly in real time. Thus you will always work on a physical

Menu entry	Description
	glass slide. On the other hand, this can be time-consuming because the physical glass slide has to be moved.
Open advanced wizard in offline mode	Opens **the Advanced Scan Profile Wizard** (Offline mode). In this mode, the system will not move the tray (slide) and the cameras will not display a live image. This mode is useful if the user wants to change parameters that are not directly associated with a live image, e.g., Z-stack and EDF (extended depth of focus) or focus settings. It is not necessary to move the physical glass slides to adjust these settings.
Open tissue detection wizard	Only active if a preview was generated. Opens the **Tissue Detection Wizard**. With the help of this wizard, you can change the region that the system will scan afterwards. Within the wizard, you can switch directly to the next or previous slides. This makes it fast and convenient to switch between the slides. It is not necessary to move the physical glass slides to adjust these settings. This wizard can be launched even if the preview generation for other slides is still running.
Save adapted scan profile	Only active if the scan profile was adapted. Here you can save an adapted scan profile (an adapted scan profile is visualized via a gear wheel in front of the profile name). Once you have activated this function, a **Save As…** dialog appears which allows you to select and save an appropriate name for the adapted profile.
Properties	Here you can check the properties of the profile. An XML viewer appears, which contains all of the details for the scan profile. This information is only of interest to more experienced users.

23 Service / Maintenance

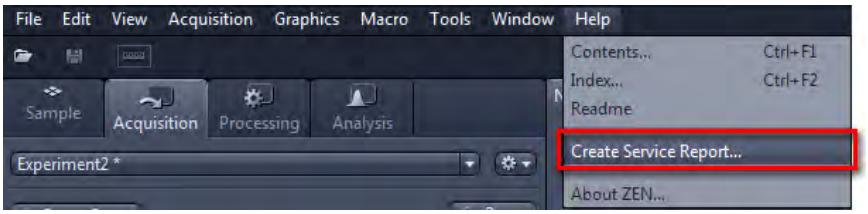
23.1 Creating a Service Report

The service report contains log files from MTB and ZEN.

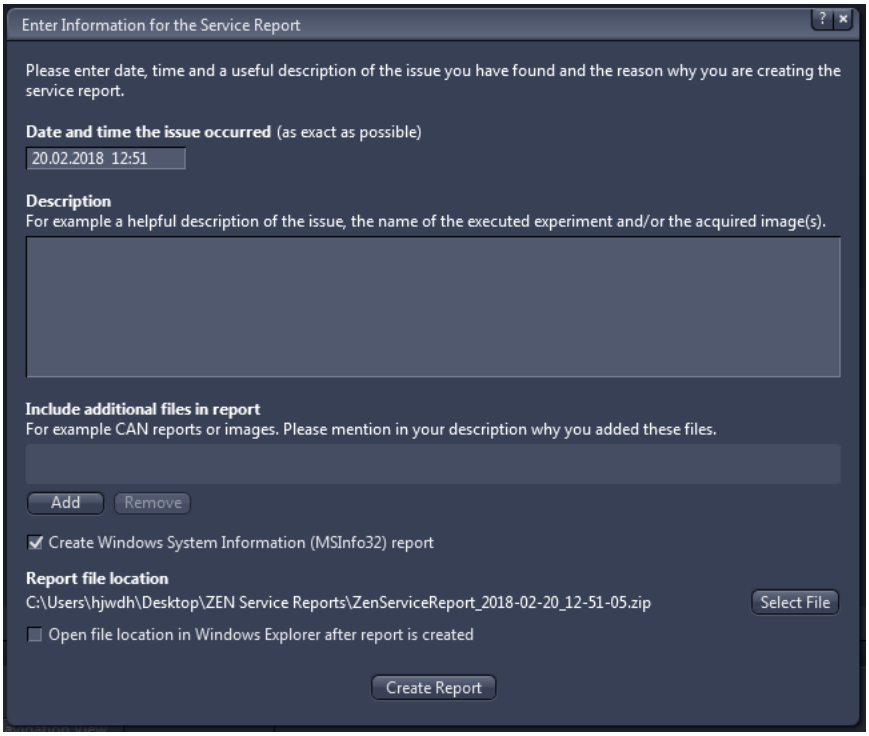
If you want to create a Service Report, the following steps are necessary.

Procedure

1 In the **menu bar** | **Help** select **Create Service Report...**



The **Enter Information for the Service Report** dialog opens.



2 Enter date, time and an useful description of the issue occured and the reason for creating this service report.

3 To include additional files to the report, click **Add**.

The Windows Explorer opens.

4 Open the folder with the file to be added.

5 Select the file.

6 Click on **Open**.

The Windows Explorer closes. Location and file name of the added file are shown in the display field.

7 To remove a file from the display field again, select it and click **Remove**.

8 If required, activate **Create Windows System Information (MSInfo32) report**.

9 To change the default location for the report file, click **Select File**.

The Windows Explorer opens.

10 Select the location for storage of the report file.

11 If required, edit the name of the report file.

12 Click **Save**.

The Windows Explorer closes.

13 If required, activate **Open file location in Windows Explorer after report ist created**.

14 Click **Create report**.

The **System information** window appears, showing the storage progress.

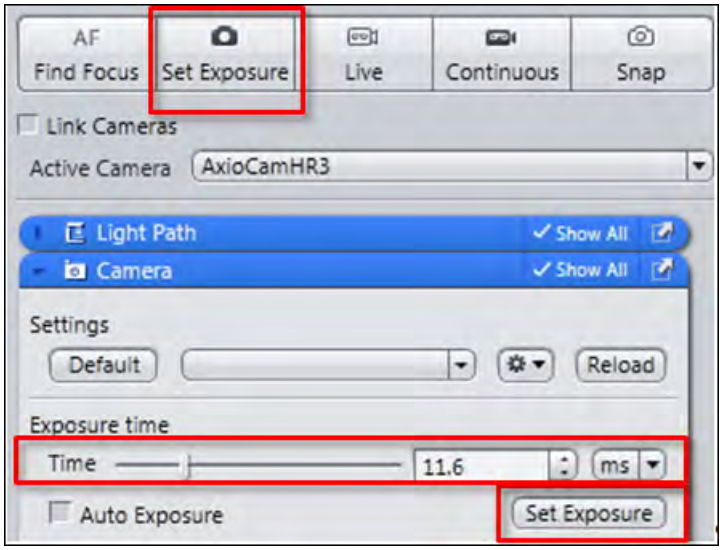
If the window closes, you have successfully created the Service Report.

24 FAQ

24.1 What can I do If my image is too dark?

Try to increase the exposure time by performing the following settings:

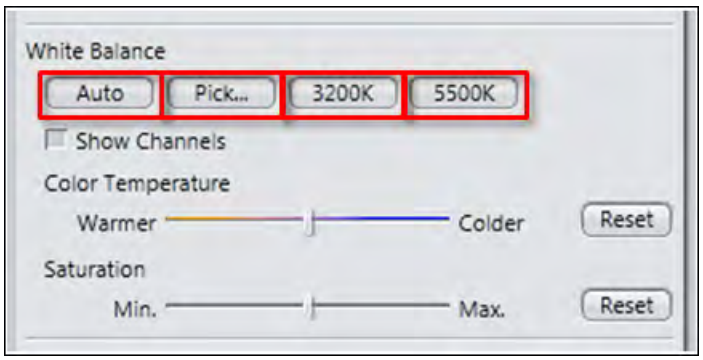
- in the **Locate** tab click on the **Set Exposure** button. This will calculated the correct exposure time automatically.
- in the **Camera** tool manually adjust the **Time** slider until you achieve the desired result.



- in the **General View Options** | **Display tab** adjust the display curve, see chapter *Adjusting Live Image Settings* [▶ 42].

24.2 How can I balance my images color?

To perform an automatic or manual white balance you must use a color camera. In the **Left Tool Area** | **Locate tab** | **Camera tool** the **White Balance** section appears. There you can perform a white balance with one of these methods:



Auto Method:

Procedure
1. Move the sample out of the **Live** window's field of view, so that you only see the background (essentially the light source).
2. Click on the **Auto** button.
3. The white balance will be calculated automatically. Afterwards move your specimen back into the field of view.

Interactive/Pick… Method:

Procedure
1. Click on the **Pick…** button.
2. Click on a white area of the **Live** window which should be represented as white.

This area will be used as a reference for the white balance.

3200K Method:

Use this method if working with a halogen bulb.

Procedure
1. If available set the light source to 3200K by pressing the **3200K** button located on the body of the microscope.
2. Click on the **3200K** button.

This method is also largely depending on the quality/age of your bulb. If the color rendition is not as desired, try the **Auto** or the **Interactive/Pick**… methods above.

5500K Method:

Use this method if working with a LED.

Procedure
1. Click on the **5500K** button**.**
2. If the color rendition is not as desired, try the **Auto** or the **Interactive/Pick**… methods above.

If none of the above methods produce a satisfactory result, one can additionally manually adjust the **Color Temperature** slider.

24.3 How can it be that my image has dust or a shadow, although my specimen is clean?

If the dust is not on your specimen, then the best method is to clean the optical elements that lay in the imaging pathway of your microscope. However if that poses a problem, alternatively, you can perform a Shading Correction as shown below. This solution has some limitations, especially if the dust is very dark or thick.

Procedure
1. Move your sample out of the Field of View until you see nothing but the light source/dust.

2 Click in the **Camera** Tool in the **Post Processing** section on the **Channel Specific** button.

3 Move your sample back into the Field of View.

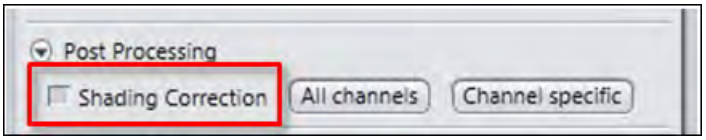
24.4 Why my image seems to look that something have burned in? (i.e. a shadow of a previous specimen?)

Check that The Shading Correction of the previous experiment is **not** already adjusted.

Procedure

1 In the **Camera** Tool open the **Post Processing** section

2 Deactivate the **Shading Correction** checkbox.



24.5 How can I fix a color gradient cast?

To fix a color gradient cast, you can try the following:

Procedure

1 Move your sample out of the Field of View until you see nothing but the light source.

2 Click in the **Camera** Tool in the **Post Processing** section on the **Channel Specific** button.

3 Move your sample back into the Field of View.

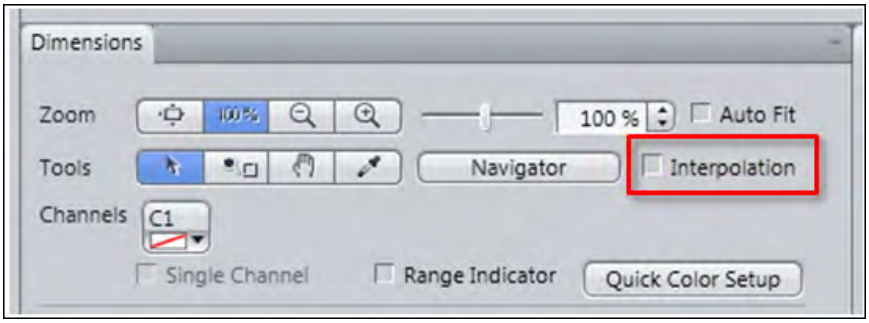
4 Perform a **White Balance,** see chapter *How can I balance my images color?* [▶ 1089]

24.6 What can I do if my live image is of a low quality and looks pixelated?

Procedure

1. In the **Camera** Tool | **Mode** section in the **Live Speed** dropdown list select the entry **Slow**.

2. Right-click on the live image and select the **Fit to View** entry.
3. You can also optionally, in the **Dimensions** tab, activate the **Interpolation** checkbox.



24.7 What can I do if my live image is slow?

Solution A

The live speed of your image is possibly set too slow. Increase the Live Speed.

Procedure

1. In the **Camera** tool in the **Mode** section, select a faster speed from the **Live Speed** dropdown menu. There are at most three choices, depending on the camera: **Slow**, **Medium**, **Fast**.

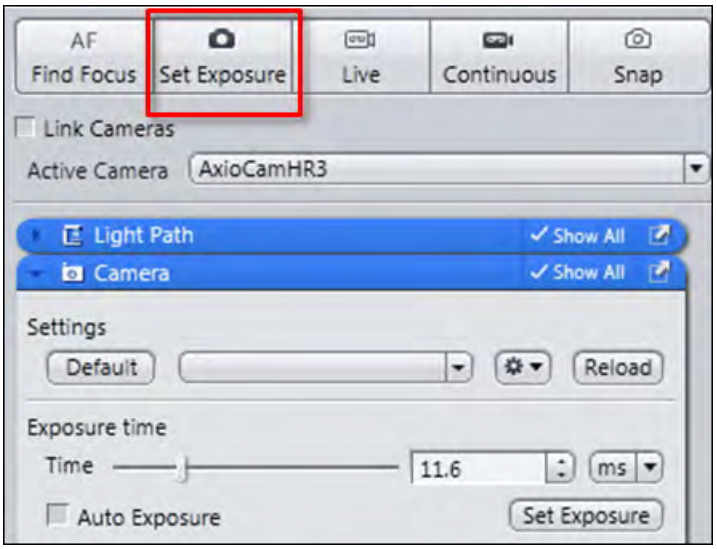
Solution B

The exposure time is possibly set to high, respectively improper. Optimize your settings in the **Camera** tool, in the **Exposure time** section

Procedure

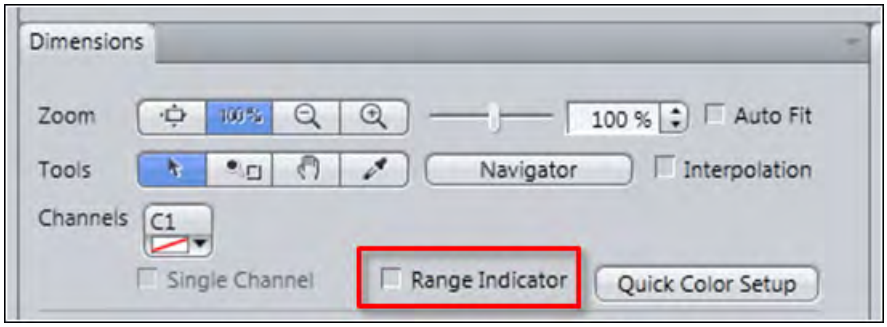
1. On the **Locate** tab click on the **Set Exposure** button.
2. Alternatively in the **Camera** tool click on the **Set Exposure** button.

3 Manually adjust the **Time** slider until you achieve the desired result.



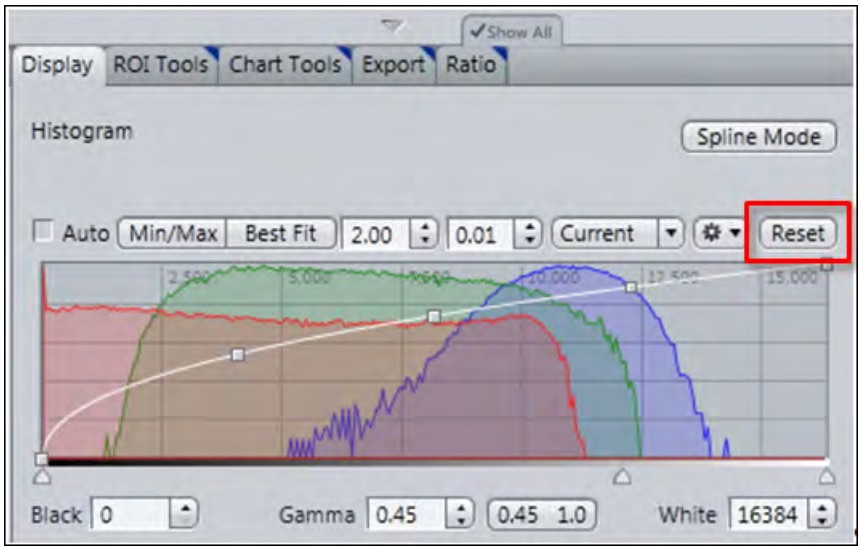
24.8 What can I do if my live image is mostly red/blue?

Check whether the checkbox **Range Indicator** is activated. If this is the case, the display switches to the **Single Channel** mode. The channel will be displayed monochrome. Simultaneously you see areas where the camera sensor is saturated, shown in red. Areas in which the pixel values = 0, are shown in blue. If this is not needed anymore deactivate the checkbox.



24.9 What can I do if my live image is still black or white after setting the exposure?

Check to see that your display curve is not set all the way to the left/right. Try to reset the display curve by clicking in the **Display** tab on the **Reset** button to achieve the default setting.

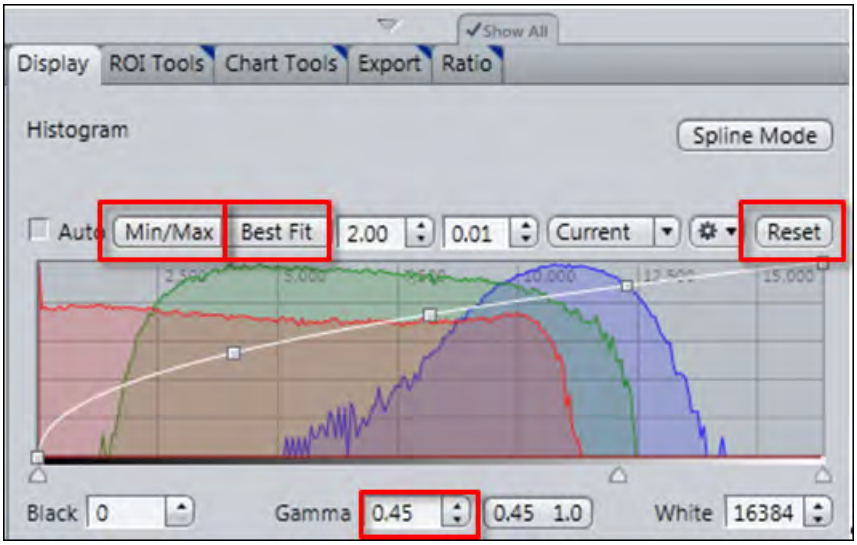


24.10 Why my live image shows extreme colors in comparison to what I see in the eyepieces?

The reason could be, that your **display curve** is not adjusted.

Procedure

1. Click on the **Reset** button.
2. Click on the **0.45** button or set Gamma 0.45.
3. You can additionally click on **Min/Max** or **Best Fit** button.

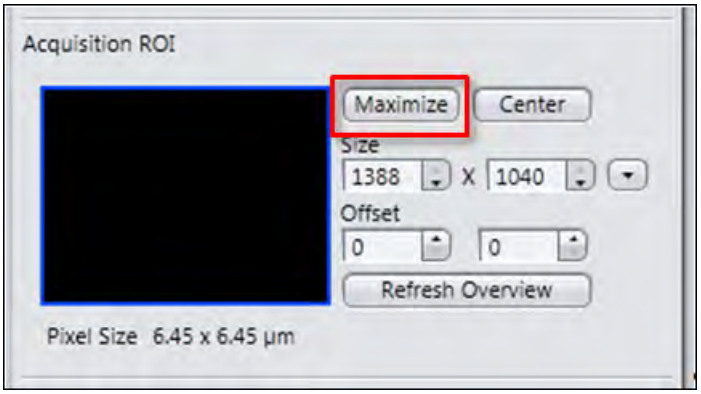


24.11 Why is my image resolution lower than the given camera specification?

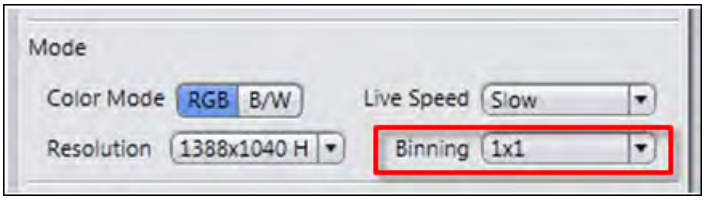
Because you chose a wrong or improper setting.

Procedure

1 In the **Camera** tool in the **AcquisitionROI** section click the **Maximize** button.



2 In the **Mode** section check that **Binning** is set to **1x1.**



24.12 What can I do if I do not see a focused live image?

Refocus the specimen on the microscope. You may activate the **Focus Bar** as an additional aid.

Procedure

1 Open the context menu via right-click in the **Live image.**

2 Select the entry **Focus Bar**.

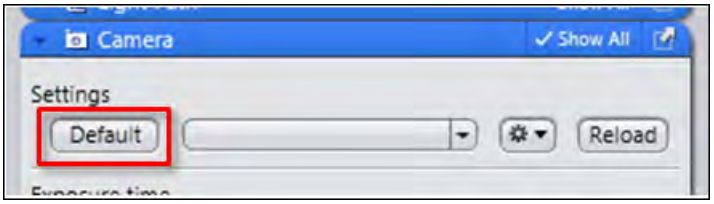
The **Focus Bar** will be shown within the **Live image** now.

24.13 Why is my image color not the same that I see through the eye pieces?

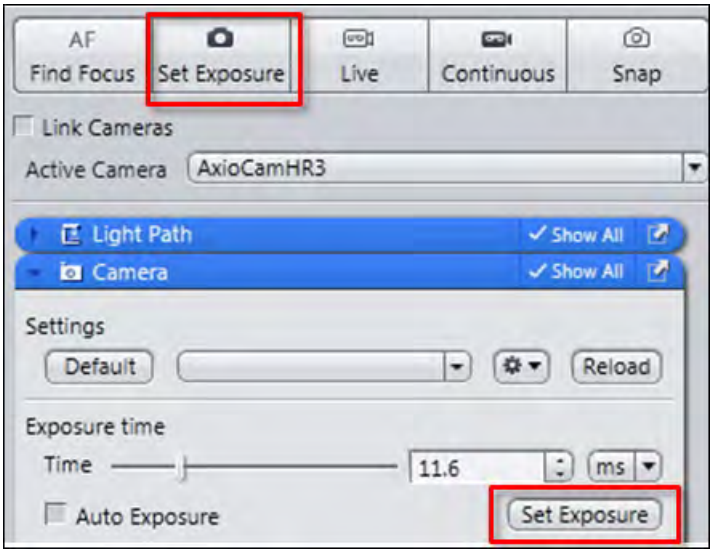
This is largely dependent on the color of your light source. The following instruction assumes that your light source is set to white.

Procedure

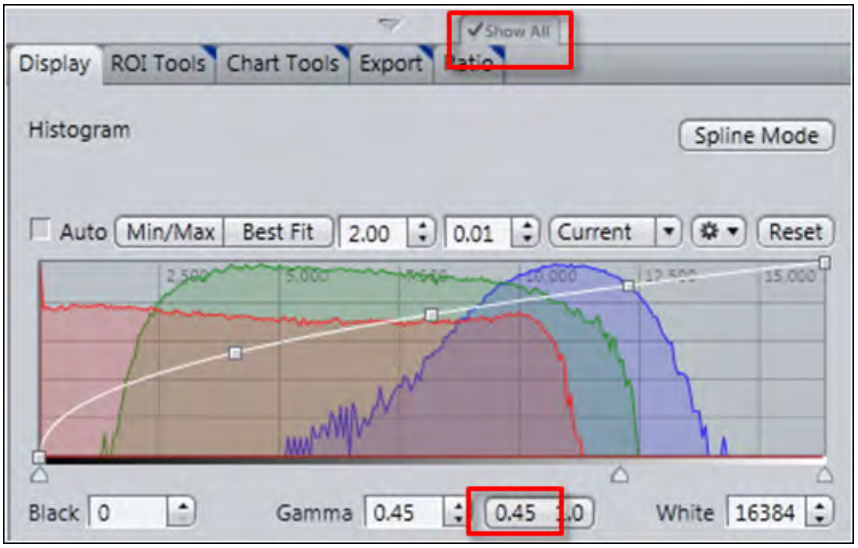
1 Click in the **Camera** Tool in the **Settings** section on the **Default** button, to set the camera back to factory default.



2 Click on the **Set Exposure** button.



3 In the **Display** tab, click the **0.45** button. If you do not see this button, activate the Show All mode.



A

Airyscan Principle

A classic confocal microscope illuminates one spot on your sample to detect the emitted fluorescence signal. Out-of-focus emission light is rejected at a pinhole, the size of which determines how much of the airy pattern reaches the detector. You can increase the resolution by making the pinhole smaller, but signal-to-noise drops significantly since less valuable emission light is passing through. With Airyscan ZEISS introduces a new concept. Instead of throwing light away at the pinhole, a 32 channel area detector collects all light of an Airy pattern simultaneously. Each detector element functions as a single, very small pinhole. Knowing the beampath and the spatial distribution of each Airy pattern enables a very light efficient imaging: you can now use all of the photons that your objective collected.

ApoTome - Global bleaching correction

For each raw image the total (sum) intensity is measured and a global decay curve determined. This decay factor is used to correct the brightness of all pixels in the raw image. This method is suitable for samples with only one fluorescent dye present.

ApoTome - Local bleaching correction

If more than one fluorescent dye is present in the sample, a single decay curve cannot be used to correct bleaching. This is the case for most biological samples especially when considering the contribution of autofluorescent substances to the total signal detected. The solution is to use a local bleaching correction determining a decay factor for each individual pixel position in each raw image which effectivel removes artifacts also for complex dye combinations.

ApoTome Bleaching correction

The main reason for residual stripe artifacts in ApoTome images is the fact, that acquiring at least 3 grid images for one resulting processed image leads to bleaching of the fluorescent dyes. This bleaching leads to brightness differences in the raw images and to artifacts when processing the data uncorrected. The principle for correcting bleaching in widefield data is the fact, that no matter, how far away from the focal plane the detector is placed, the sum intensity emitted from the sample remains the same. This is also true for the grid images. This fact is being used for the ApoTome. Two methods exist, both of which are patented.

B

Binning

Binning is understood to mean the combination of neighboring image elements (pixels) on the image sensor itself, e.g., the CCD sensor in a digital camera. Source: Wikipedia

Bleaching Correction

The characteristics of a widefield fluorescence microscope are based on the assumption that all Z-planes have the same total brightness, irrespective of the focus position. Use is made of bleaching correction by applying a correction factor to each Z-plane. However, this assumption does not apply to techniques that result in the generation of optical sections, such as confocal images.

Burst Mode

Burst Mode is some kind of optimization to enable the recording of the fastest framerates, which can be achieved by the used camera hardware. This mode requires some compromises for the sake of speed: it supports only single channel time lapse acquisition, an update of the image display is suppressed while recording and the maximum time lapse duration is depending from the size of available main

memory (minus some space to breathe for the operating system). If a multi channel image needs to be acquired, Burst Mode will be disabled and maximum frame rate can be slower then specified in the camera hardware performance documentation.

C

Clipping planes

The purpose of clipping planes is to cut open the calculated 3D image so that elements on the inside can be visualized. Clipping planes can cut the volume in such a way that either the front, back or both sides of the volume data are no longer visible. In addition, the clipping plane itself can be given various textures. This is a very important modeling option for analyzing 3D data.

Colocalization

Acquiring fluorescence images in several channels makes it possible to visualize the relationship between biological structures. A combined display of two channels in color overlay mode makes it easier to assert whether the components are "colocalized", i.e. whether they are located at the same position. Conventionally, two fluorescence channels are displayed in the form of a color-coded overlay. The most common form is the red/green overlay. Regions in which both fluorescent dyes are present at the same place are displayed in yellow. It is not possible, however, to make quantitative statements concerning the extent of colocalization on the basis of this display. At best, a qualitative statement is possible with regard to whether or not two dyes are colocalizing. The Colocalization module is able to fill this gap and presents the user with a tool that enables colocalization to be determined quantitatively. Principle: It is always the colocalization of two channels that is analyzed. Colocalization results from the pixel-by-pixel comparison of intensities for each channel.

Constrained Iterative

The best image quality is achieved using the iterative maximum likelihood algorithm (see Schaefer et al.: "Generalized approach for accelerated maximum likelihood based image restoration applied to three-dimensional fluorescence microscopy", J. of Microscopy, Vol. 204, Pt 2, November 2001, pp. 99ff.). This algorithm is able to calculate light from various focal planes back to its place of origin. Consequently, with this method it is possible to derive the 3D structure from fluorescence images with the correct brightness distribution and to visualize optical sections. It is also possible for missing information to be partially restored from neighboring voxels. The spatial resolution can be increased without artifacts up to a theoretical limit (one voxel). It is essential for Z-stacks to have been acquired in accordance with Nyquist. Acquiring sufficient planes above and below the structure of interest is also imperative for achieving good results. As this is a complex mathematical method, the calculation can take longer, depending on the image size and the PC being used.

Costes

Costes et al. (Biophysical Journal, 2004, vol. 86, pp 3993-4003) have published a statistical method with the help of which an attempt is made to determine an optimal colocalization threshold automatically. This takes place by initially maximizing the threshold for both channels and then gradually reducing it. With each step Pearson's Correlation Coefficient is determined for all pixels below the set value. These steps are repeated until the Pearson value is minimized (ideally a value of 0 for perfectly colocalizing channels). See the publication for further details. This method has been implemented in Colocalization. Clicking on Auto initiates the

above iterative process, which, depending on the sample, can take several seconds. The threshold now set corresponds to the confidence criterion calculated. This method works very well with large, diffusely stained structures such as nucleoplasm or diffuse cytoplasmic structures. Under certain circumstances it does not function so well for small structures (e.g. nuclear speckles or vesicular structures), particularly in the case of widefield images, where the signal to background ratio is not as good as it is with methods that involve the generation of optical sections (e.g. LSM, TIRF or ApoTome). The Regions button becomes active as soon as a region is inserted into the scatter plot. It remains active as long as regions are selected or moved there. Activating and deactivating the button makes it possible to switch between threshold selection using the mouse and the selection/moving of selected regions in the scatter plot image. If regions are defined in the scatter plot, the corresponding data appear in the table in addition to the overall image.

D

Deconvolution

Deconvolution is a method that is used to improve fluorescence images in particular. Image information acquired using a microscope system can never fully reproduce the structures of the actual object. This is because unavoidable distortions occur during acquisition due to the optics and electronics. In addition, particularly in the case of fluorescence microscopes that do not offer any methods for generating optical sections, light from areas of the object outside the objective's focal plane is also always acquired. This covers the structures that the user actually wants to see to a varying degree and therefore leads to a reduction in the contrast and consequently in the visible resolution. These optoelectronic effects can be described mathematically in the form of the point spread function (PSF). If the PSF is known, it is possible to correct the negative effects to a large extent using deconvolution. This produces a completely sharp image of the object that is richer in contrast. Deconvolution is usually performed on Z-stacks, i.e. it is used as a 3D method. However, it can also be used to a limited extent to improve 2D images. A good review of deconvolution can be found in Wallace et al., 2001: A Workingperson's guide to deconvolution in light microscopy; Biotechniques 31: 1076-1097.

Display characteristic curve

The display characteristic curve allows you to define the range of the gray value histogram of an image that you want to display on the screen. The limit on the left defines the gray value up to which all pixels are displayed as pure black (black value), while the limit on the right defines the gray value from which all pixels are displayed as pure white (white value). The curvature of the curve defines the so-called gamma value.

Drag&Drop

Literally translates to "drag and drop". Does the moving of objects (eg, files, icons, etc.) on the screen as from one folder to another. Clicking the object with the left mouse button, holding down these, the object moves with the mouse to the desired location.

Dynamic range

The dynamic range describes the number of brightness gradations that a camera or another detector is able to distinguish. Modern, scientific digital CCD cameras, for example, have a dynamic range of up to 2^16 gray levels. In this case we talk of 16 bit cameras.

E

Experiment Feedback

Experiment feedback allows the definition of specific rules and actions to be performed during an experiment. This allows changing the course of the course of an experiment depending on the current system status or the nature of the acquired data on runtime. Moreover, it is possible to integrate certain tasks like data logging or starting an external application, directly into the imaging experiment. Typically, but not exclusively, such an experiment connects the image pickup with an automatic image analysis.

F

Fast Iterative

The "Fast Iterative" method is an iterative restoration method that uses only one iteration per convolution step (see Meinel, E. S.: Origins of linear and nonlinear recursive restoration algorithms. J.Opt.Soc.Am, 3 (6), 1986, 787-799). No regularization is used in this case. Due to the fast processing and convergence after just a few iterations, this method is suitable in particular for the processing of larger time lapse images. The results of the method can quickly lead to good results and remove most of the out-of-focus light. They do not, however, create quantitative brightness conditions in the image. If undersampled images are present, artifact formation may also result.

Field Feature

A field feature is calculated for all segmented objects of a class. The geometric or intensity parameters of all objects of the class, e.g. the area or the average intensity, are added together. In addition, all objects can be counted, for example, or the area of the objects in relation to the total image area can be calculated as a percentage.

Fluorescent beads

Fluorescent beads are often used to measure the point spread function (PSF). The diameter of these beads is usually significantly below the resolution limit of the objective used. Based on the known shape and size, various optical parameters of the microscope system can be determined with the help of such objects. To measure the PSF when using an objective with a numerical aperture of 1.4, beads with a diameter of 50-170 nm should be used.

Fourier Filter

The processed result ApoTome image can sometimes still show fine residual line artifacts. This is due to sample, staining, dynamic range during acquisition or exposure time used. These artifacts can be removed using the Fourier Filter option. This function makes use of the fact, that stripe artifacts appear as a group of dots in frequency space. The filter masks those dots and can in this way remove stripe artifacts from the image. It only works however, if the camera had been aligned parallel to the grid lines during phase calibration. Also, it is quite a crude method and should not be needed if all other preconditions for using the ApoTome have been met.

G

Gamma value

The gamma value makes it possible to correct the display of images on computer screens which do not allow the linear display of gray value curves. By changing the gamma value you can emphasize certain intensity ranges within your image when it

is displayed on the screen. A value <1 emphasizes the ranges of medium pixel intensity (medium gray values), while a value >1 emphasizes the dark and bright pixel intensities and therefore increases the contrast. The recommended settings are 0.8 for fluorescence images, 1.2 for phase contrast or DIC images and 0.45 for true color images. Please bear in mind that a "correct" gamma value setting depends on numerous parameters, such as screen settings, ambient brightness, etc., and a universal setting cannot therefore be given.

Gaussian Distribution The emission of fluorescent light in fact follows a Poisson distribution. If, however, detector noise predominates during imaging, or the image data are only just above the camera noise and therefore very dark, a normal distribution according to Gauss tends to apply to such images.

Generalized Cross Validation (GCV) Regularization, which lessens the influence of noise during restoration, is normally controlled by a parameter that in most cases is determined heuristically via trial and error. The "generalized cross validation" (GCV) method makes it possible to estimate this parameter even under the complex conditions of Poisson maximum likelihood minimization.

H

Halo effect A halo is a (usually unwanted) effect in digital image processing ("halo" around the image object).

I

Image Analysis Wizard Using the Image Analysis Wizard you can create automatic measurement routines very easily. The wizard delivers precise results without any need to spend time on programming. This allows you to complete even complex measurement tasks in just a few minutes.

Image display A maximum range of 256 gray levels (black and white image) or 16 million colors can normally be displayed on a screen. Modern digital cameras capture a much larger range: black and white cameras up to 65536 values and color cameras theoretically up to (65536)3 colors (281 billion). The display of these gray values/ colors therefore needs to be adjusted for the monitor by the user. For this adjustment an upper and a lower gray/color value are defined. All gray/color values between these limits are displayed on the monitor within the 256 gray values/16 million colors that can be represented.

Image Normalization The processing algorithm for ApoTome raw images acts in a subtractive manner effectively reducing the gray value range of the output image. Since all calculations are done internally in the high precision 32 bit floating point image format the normalize option can help to generate better output data. The floating point numbers are back-converted into 16 bit integer numbers using the full 16 bit dynamic range normalized to the brightest pixel. This option makes it impossible however to make quantitative comparisons between images.

K

Kymograph A Kymograph is a twodimensional representation of a moving object over time. The movement of the object is traced using a line or curve of a given thickness and an intensity plot is then generated over time. The kymograph image displays the intensities along the line in X direction and the time points are plotted in the Y direction. With this method one can visualize and analyze speed and acceleration of moving objects with a simple 2D representation.

L

Lamp Flicker This phenomenon mainly occurs if fluorescent arc lamps are operated for a long period of time. Under certain circumstances alternating darker and brighter layers can then appear in the Z direction in Z-stacks. This effect may prevent 3D deconvolution from being usefully applied, for example.

M

Maximum mode In the case of a maximum intensity projection, only the pixels with the highest intensity are displayed along the observation axis. This view is well suited to the two-dimensional display of three-dimensional images, e.g. in publications, one reason being that a maximum transparency effect is only visible in this mode.

Microscanning Microscanning is a technological process for the production of high-resolution images using a CCD or CMOS sensor. For a sequence of images, the sensor is moved in two dimensions by micro-mechanics in very small intervals between acquisitions.The distances are smaller than a pixel dimension and allow the inclusion of detailed information that would otherwise not be seen by the sensor.

Mixed mode In Mixed mode, a volume can be displayed in both Surface mode and Transparency mode. In the case of multichannel images, for example, structures inside a cell, such as FISH signals or nucleoli, can be displayed in Surface mode and the cytoplasm around these structures can be displayed transparently in another channel. This means that even highly complex spatial relationships can be shown convincingly.

Motif buttons With the Motif buttons you can optimize image acquisition regarding particular requirements like speed or quality. All parameters e.g. camera resolution or dynamic range in Acquisition Mode or Channels tool were set automatically. They will influence basically camera, detector and lightning settings.

MTB The software MicroToolBox (MTB) is used to generate and manage microscope configurations. Information about microscope components (e.g. nosepieces, reflector turrets, shutters etc.) and, if necessary, additional external units (e.g. motorized xy stages, external light sources etc.) is stored in these configurations. Furthermore, the software can also be used to enter information about microscope components, such as objectives, fluorescence filter cubes etc., in a simple way and to save this information in the microscope (depending on the type of microscope in question). In this case, the information is saved directly in the microscope, allowing it to be displayed on the microscope's TFT screen, for example. Various configurations can be created, of which only one is activated at any time. The

active configuration is used by imaging software such as ZEN to provide graphic control dialogs for the configured microscope units (e.g. lightpath or microscope components control).

N

Nearest Neighbor The Nearest Neighbor method uses the simplest and fastest algorithm (Castleman, K.R., Digital Image Processing, Prentice-Hall, 1979). Its function is based on subtraction of the out-of-focus information in each plane of a stack, taking the neighboring sections above and below the corrected Z-plane into account. This method is applied sequentially to each plane of the entire 3D stack. It allows you to enhance contrast quickly, even if image stacks have not been put together optimally.

Nyquist Criterion The Nyquist criterion states that a signal must be detected with at least double precision in order to reliably acquire all the frequencies in the signal. In the case of images acquired with coarser resolution, undesired effects such as aliasing may otherwise result. For the deconvolution of microscope images, this means, in practical terms, that images should be acquired with a pixel resolution that is at least double the optical resolution, both in the lateral and axial direction.

O

Object Feature An object feature is calculated for an individual segmented object. It describes a geometric or intensity property of the object, e.g. its area or its average intensity.

P

Phase Correction The ApoTome grid is moved in precise steps during acquisition in order to cover the sample fully for one section. The used steps are stored in the image metadata and used for all subsequent processing steps. In most cases this will work very well. However, there can be cases e.g. caused by vibrations, when the actual grid position deviates from the reported position causing artifacts during processing. Phase correction analyses the actual grid positions in the raw images and uses the determined values instead for processing. This option will add a bit of processing time however.

Point Spread Function (PSF) All optoelectronic effects that influence the creation of a microscope image can be described mathematically in the form of the point spread function (PSF). If the PSF is known, deconvolution can be used to largely remove the negative effects from microscope images. There are three possible ways to determine the PSF: theoretically through knowledge of the key optical parameters, experimentally through measurement using fluorescent beads of a known diameter, or blindly using a method that works with less prior knowledge. In ZEN the theoretical model according to Lanni and Gibson has been implemented, which also models asymmetries like those that can arise due to spherical aberrations (see S. F. Gibson, F. Lanni, "Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy", J. Opt. Soc. Am. A, vol. 8, no. 10, pp. 1601-1613, October 1991).

Poisson Distribution The emission of photons by fluorochromes follows a statistical distribution, known as a Poisson distribution. This is the preferred model taken as the basis for the deconvolution calculation. It applies if the predominant proportion of image noise is caused by shot noise ("salt and pepper noise"). This assumption applies to images that have been acquired using good, low-noise detectors, the dynamic range of which has been utilized to a certain extent.

Position In a tile experiment positions refer to independent individual image fields (tiles) that are localized at various places on the sample. A position corresponds to a tile region consisting of just one tile. Each position is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Individual positions or position arrays (grouped individual positions) are defined using the Tiles tool. After acquisition the individual positions are displayed as scenes.

Pseudo color assignment In fluorescence microscopy, pseudo color assignment describes the assignment of any artificially selected color to the channel of a multichannel fluorescence image. As it is mostly monochrome cameras (which produce black and white images rather than "true colors") that are used in this area of application, we talk of pseudo coloring.

R

Raw Data Mode The ApoTome combines the advantages of widefield imaging systems with the advantages of optical sectioning. Images acquired from the Acquisition tab always contain all images acquired from the grid. These grid images are also called phase- or raw-images. This principle offers several advantages: 1) all informations acquired are kept and not discarded; 2) the acquisition itself is not slowed down by processing overhead; 3) you get access to various correction methods giving you flexibility in treating your sample in the right way after acquisition; 4) Phase (=grid-position) errors occuring during acquisition such as caused by vibrations of the microscope can be likely corrected using the phase correction option without having to redo the acquisition; 5) you can achieve a marked improvement in resolution and contrast by using the specially adapted ApoTome deconvolution option bundled with all systems; 6) the raw mode facilitates easy analysis of images which show errors or artifacts in the sectioned image which would otherwise remain obscure.

Reference Z-Position By default the current Z-position at the time the experiment is started is set as the Reference Z-Position for acquisition. Z-stack experiments, for which the center of the defined Z-stack is set by default as the fixed Reference Z-Position, form an exception to this. Offsets for channels and Z-stacks shift acquisition in relation to the Reference Z-Position. If a focus strategy is used, this determines and updates the Reference Z-Position during the experiment.

Regularization Working with real microscope images that are affected by noise leads to considerable difficulties with the practical application of deconvolution methods, which is why regularization (e.g. according to Tikhonov-Miller-Phillips) is essential. Regularization is a method that lessens the influence of noise by means of various penalty terms. Stronger regularization leads to weaker restoration and weaker regularization to stronger restoration, although in this case noise is also intensified.

Regularized Inverse Filter The inverse filter is a genuine 3D method and generally achieves better results than the Nearest Neighbor algorithm. It essentially involves dividing the Fourier transformation ("FT") of the volume by the FT of the PSF, which can be performed very quickly. In the real space this corresponds to deconvolution. In addition, a statistical method ("General Cross Validation – GCV") is applied, which determines the noise component of the image and automatically sets the restoration strength to the optimum level in line with this. This process is also known as regularization. The method is very well suited to the processing of several image stacks in order to preselect images for the application of the iterative "high-end" method. Z-stacks must, however, have been acquired at the correct (Nyquist) distance. The additional acquisition of Z-planes above and below the structure of interest is recommended.

Render Series To display a 3D volume on the screen, each image must be recalculated. This takes time and, in the case of large images, cannot be done interactively. You can, however, have a series of individual images calculated which represent the animation that you want. Such an image series can be displayed considerably faster and more fluidly than is possible interactively on the screen as, in this case, the views no longer have to be rerendered. Furthermore, an image series like this lends itself extremely well to being exported as a film.

S

Shading Correction The Brightness of microscopic images often declines to the edges. This is caused for example, by misaligned, or inhomogeneous lighting, inconstant light conditions or dirty optics. ZEN is able to correct this interference with the so called Shading Correction. First you need a white image. This functions as a reference for the background of your image, which shall be corrected.

Shadow mode In Shadow mode the structures in the image are illuminated by means of a virtual light source. The image stack is viewed from above, as if through the microscope's eyepiece, and a shadow is projected onto a virtual base (in the image background). This gives the data a reference in relation to the space, which makes visualization easier. The impression of a three-dimensional structure is created from the combination of light being reflected and opacity (degree of impenetrability to light) and the casting of a shadow.

Smart Setup Smart Setup is the intelligent and convenient control center for your fluorescence images. Simply select a fluorochrome from the more than 500 dyes stored and ZEN will automatically provide the optimal filter combinations and acquisition settings for your experiment.

Spherical Aberration Every objective requires the use of a defined immersion medium to deliver the best optical resolution. In microscopy practice, particularly in the area of biosciences, it is not always possible, however, to embed the sample in a medium with the correct refractive index. When light enters the embedding medium with the wrong refractive index this results in "spherical aberration". The PSF becomes more asymmetrical the further away from the cover slip it is measured. In practical terms this becomes noticeable in the form of an increasing loss of brightness as the distance from the cover slip increases. It is possible to compensate for spherical aberration either by using objectives with correction rings or objectives that have

been calculated for certain embedding media (e.g. aqueous solutions). Within certain limits, however, spherical aberration can also be compensated for during deconvolution, by taking the parameters responsible for this effect into consideration when calculating the theoretical PSF. For further details see S. F. Gibson, F. Lanni, "Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy", J. Opt. Soc. Am. A, vol. 8, no. 10, pp. 1601-1613, October 1991.

Surface mode The two modes previously described display the data with soft transitions or with a transparent character, depending on the setting. In Surface mode, the program calculates solid surfaces ("isosurfaces") from the gray values, which emphasizes particularly flat structures (e.g. cell walls of plant cells). This display can be used if you want to draw attention to certain structures, while other, internal structures are hidden.

T

Threshold Which threshold is the correct one is a question that is frequently asked. Unfortunately it is not possible to give a definitive answer to this question, particularly because this often depends on the problem and the properties of the sample. Generally speaking it can only be said that the best approach is to determine the thresholds using appropriate control samples, e.g. samples without colocalization as a negative control and samples with biologically relevant colocalization as a positive control. Thresholds determined in this way can, under certain circumstances, be transferred to the sample of interest.

Tile region In a tile experiment a tile region refers to a group of individual image fields (tiles) that belong together and are arranged in the form of a grid. With the help of tile regions it is possible to acquire areas with dimensions that exceed the size of an individual image field. Within an experiment a number of tile regions can be acquired at various positions on the sample. Each tile region is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Tile regions are defined using the Tiles tool. After acquisition the individual tile regions are displayed as scenes.

Transparency mode In Transparency mode a three-dimensional image is calculated with a transparency effect. At least two 2D texture stacks are calculated for this from different views, which are used depending on the position angle. In contrast to Shadow mode, in this case the scene is illuminated from behind by diffuse, white light. Using the setting options, in this mode you can "mix" several channels with one another and also make information visible inside a structure. This view is therefore particularly well suited to visualizing the spatial relationship between structures within the image.

W

Widefield Classical microscopes frequently are called "widefield" microscopes in order to distinguish them from microscope systems with optical sectioning capability such as laser scanning microscopes. In contrast to such systems widefield microscopes do not possess the ability to discriminate betwee image information in the axial (=Z)

direction leading to blurred images and therefore are only poor 3D imaging systems per se. There are methods to add this missing axial sectioning ability to widefield microscoped such as 3D deconvolution or structured illumination (ApoTome, Elyra-S)

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A

B

C

D

E

F

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H

I

L

M

N

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R

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V

W

Z

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