Quick Guide

ELYRA

Superresolution Systems

ELYRA PS.1

November 2013

ZEISS

We make it visible.

Contents

Introduction

This Quick Guide describes the basic operation of the ELYRA and LSM 7 ELYRA systems with the ZEN 2012 software.

The purpose of this document is to guide the user to get started with the system as quick as possible in order to obtain some first images from his samples.

This Quick Guide does NOT replace the detailed information available in the full user manual or in the manual of the respective microscope (Axio Observer).

Also, this Quick Guide is written for a user who is familiar with the basics of Laser Scanning Microscopy.

For your safety!
Observe the following instructions:

- The ELYRA and LSM 7 ELYRA microscopes, including their original accessories and compatible accessories from other manufacturers, may only be used for the purposes and microscopy techniques described in this manual (intended use).

- In the Operating Manual, read the chapter *Safety Instructions* carefully before starting operation.

- Follow the safety instructions described in the operating manual of the microscope and X-Cite 120 lamp.

Starting the System

Switching on the LSM 7 ELYRA system

- Switch on the main switch (Fig. 1/**1**) and the safety lock (Fig. 1/**2**).

- When set to **ON** the power remote switch labeled **System/PC** provides power to the computer. This allows use of the computer and ZEN software offline

After complete startup, the computer is ready for the components start.

- To completely switch on the system, now press the **Components** switch to **ON**. This starts the other components and the complete system is ready to be initialized by the ZEN software.
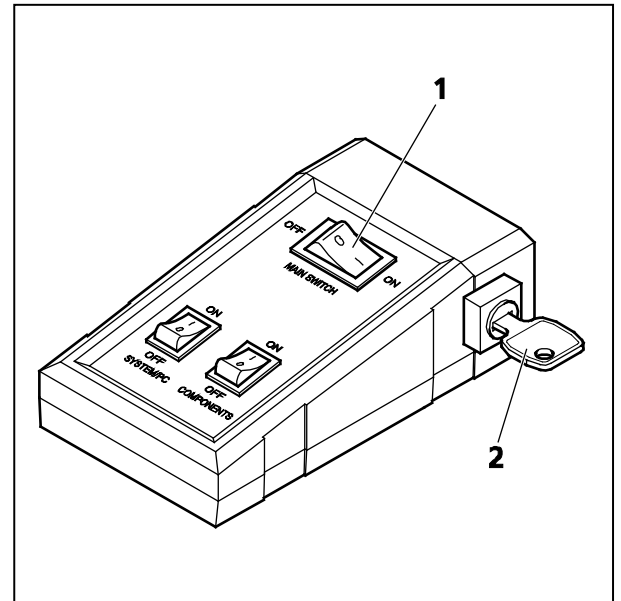
Switching on the ELYRA system

- If the system is provided with a power remote switch, proceed as described for the LSM 7 ELYRA system.

- If the system is not equipped with a remote power switch, switch on the three individual multipoint connectors.

Fig. 1   **Power remote switch**

Switching on the X-Cite 120 lamp

- Switch on the main switch of the X-Cite 120 lamp for reflected light illumination via the power supply as described in the respective operating manual.

Switching on the Ar-ML Laser

- If the Ar-ML laser is required, switch it on via the toggle switch (Fig. 2/**2**) on the power supply and turn the key (Fig. 2/**1**).
  The laser is automatically kept in standby mode for 5 minutes to warm up.

- Set the idle-run-switch (Fig. 2/**3**) to run. It takes about 50s until the laser has reached the set output power (green LED) provided the warm up time of 5 minutes is already completed.

- Adjust the required power level with the control knob (Fig. 2/**4**) (default position should be 11 o'clock).
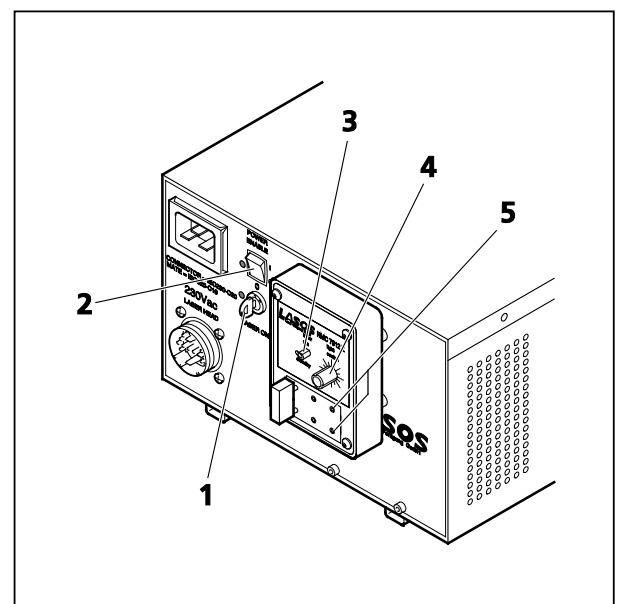

Fig. 2   **Power supply of Ar-ML laser**

Starting the ZEN software

- Double click the **ZEN 2012** icon on the WINDOWS desktop to start the Carl Zeiss LSM software.

The **ZEN** loading window (Fig. 3) followed by the Application window and the **Login "ZEN 2012"** window appear on the screen (Fig. 4).

Fig. 3   **ZEN loading window**

Fig. 4   **ZEN loading window - Boot Status display open**

In the small startup window, choose either to start the system (**Start System** hardware for acquiring new images) or in **Image Processing** mode to edit already existing images. Toggle the little ▶ symbol to view the Boot Status display and get the additional **Offline/Demo** button option:

- Choosing **Start System** initializes the whole microscope system and activates the entire software package for new image acquisition and analysis.

- The **Image Processing** mode ignores all hardware and activates only data handling and image processing functionality for already acquired images.

- The **Offline / Demo** mode reads the current hardware database but does not activate the system hardware for use. Instead, it simulates the system hardware for training purposes.

- Upon clicking the **Start System** button, the **Image Processing** button changes to a **Cancel** button. Click **Cancel** to interrupt/stop the **Startup** of the system.

After Startup, the ZEN Main Application Window (Fig. 5 and Fig. 6) opens. To benefit from all of ZEN's features, run the window in its full screen mode.

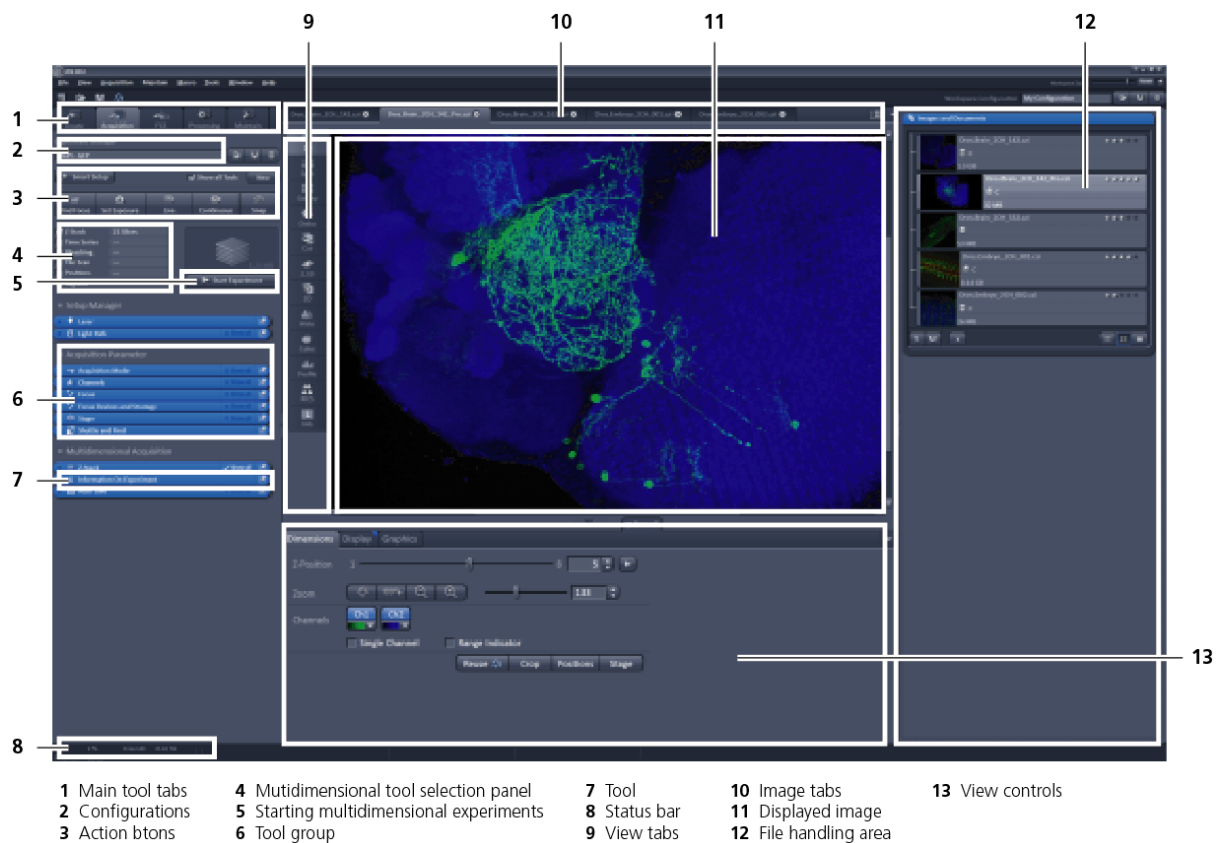Fig. 5   ZEN Main Application Window after Startup with empty image container

A Application bar
B Menu bar
C Main toolbar
D Left Tool Area
E Center Screen Area (hosts up to three Image containers)
F Right Tool Area
G Status Area

1 Main tool tabs	4 Mutidimensional tool selection panel	7 Tool	10 Image tabs	13 View controls
2 Configurations	5 Starting multidimensional experiments	8 Status bar	11 Displayed image	
3 Action btons	6 Tool group	9 View tabs	12 File handling area	

Fig. 6   ZEN Main Application Window after Startup with several images loaded

Introduction to ZEN – Efficient Navigation

The **ZEN 2012** interface is clearly structured and follows the typical workflow of the experiments performed with microscopy systems:

On the **Left Tool Area** (Fig. 5/**D**) the user finds the tools for sample observation, image acquisition, image processing and system maintenance, easily accessible via four **Main Tabs** (Fig. 6/**1**). All functions needed to control the microscope can be found on the **Ocular Tab**, to acquire images use the **Acquisition** tools (Fig. 6/**3** and **4**). Arranged from top to bottom they follow the logic of the experimental workflow. The area for viewing and interacting with images is centered in the middle of the **Main Application Window**: the **Center Screen Area**. Each displayed image can be displayed and/or analyzed with many view options available through view tabs which can be found on the left side of the image. According to the chosen view tab, the required view controls appear in View Control Tabs below each image. File management and data handling tools are found in the **Right Tool Area** (see Fig. 5 and Fig. 6).

Color and brightness of the interface have been carefully adjusted to the typical light conditions of the imaging laboratory, guaranteeing optimal display contrast and minimal stray light for high-sensitivity detection experiments. The **ZEN** software is optimized for a 30" TFT monitor but can also be used with dual-20" TFT setups.
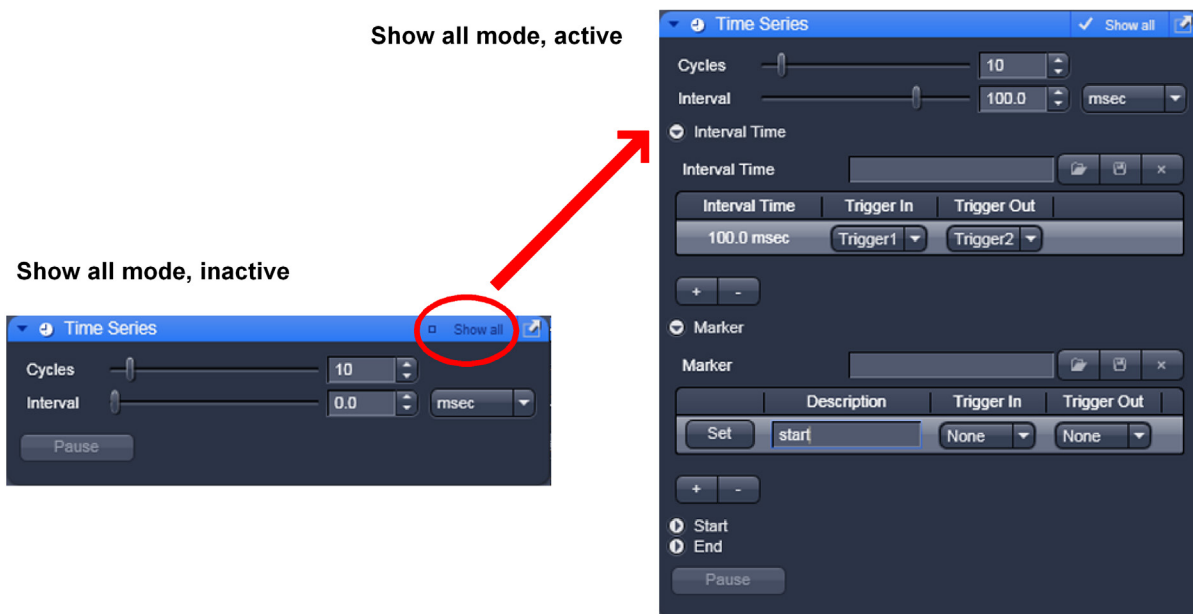

Fig. 7   Show all mode

A focus in the development of **ZEN 2012** was to fulfill the needs of both basic users and microscopy specialists. Both types of users will appreciate the set of intuitive tools designed to make the use of a confocal microscope from Carl Zeiss easy and fast:

The **Show all** concept ensures that tool panels are never more complex than needed. With **Show all** de-activated, the most commonly used tools are displayed. For each tool, the user can activate **Show all mode** to display and use additional functionality (Fig. 7).
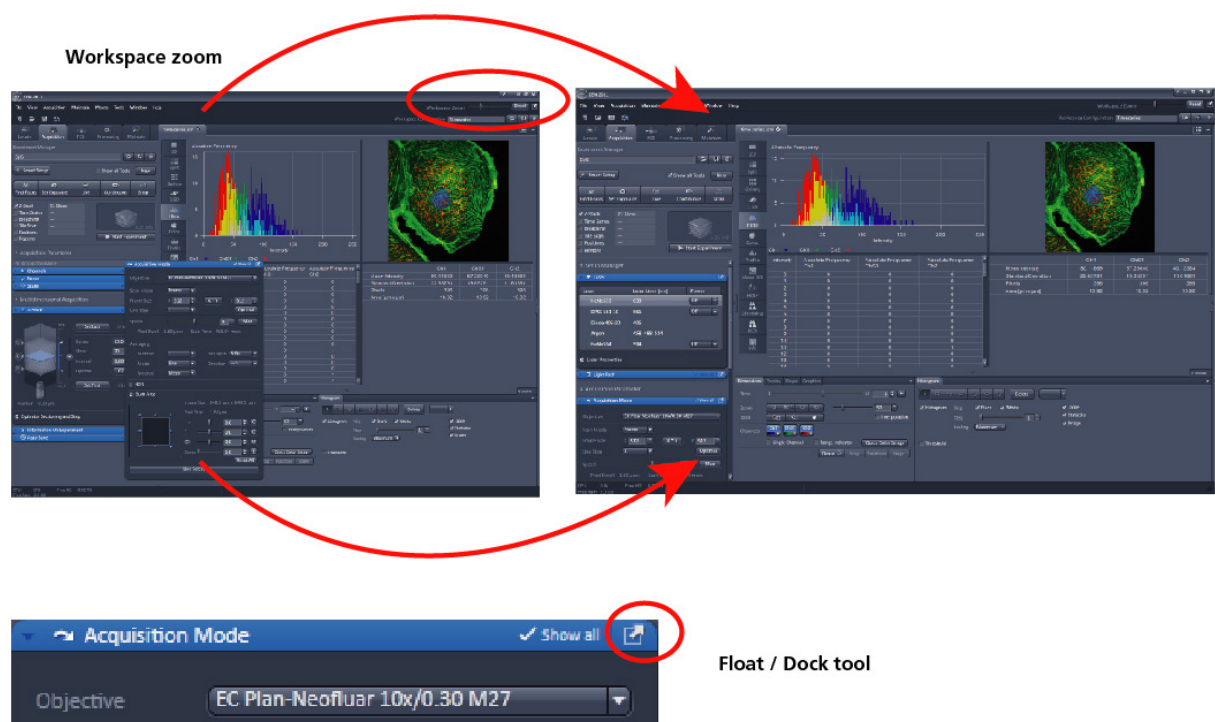
Fig. 8   ZEN Window Layout configuration

More features of **ZEN 2012** include:

– The user can add more columns for tools to the **Left Tool Area** or detach individual tools to position them anywhere on the monitor. To add a column, drag a tool group by the title bar (e.g., Acquisition Parameter) to the right and a new tool column automatically opens. Alternatively use the context menu "move toolgroup to next column". To detach a tool, click on the little icon on the very right end of the blue tool header bar (Fig. 8).

– Another unique feature in Imaging Software is the scalable ZEN interface. This **Workspace Zoom** allows adjustment of the **ZEN 2012** window size and fonts to the situational needs or your personal preferences (Fig. 8).

– Setting up conventional confocal software for a specific experiment can take a long time and is often tedious to repeat. With **ZEN** these adjustments have to be done only once – and may be restored with just two clicks of the mouse. For each type of experiment one can now set-up and save the suitable **Workspace Layout**. These configurations can also be shared between users.

– For most controls, buttons and sliders, a **tool tip** is available. When the mouse pointer is kept over the button, a small pop-up window will display which function is covered by this tool/button.

These are just some of the most important features of the ZEN interface. For a more detailed description of the functionality for the **ZEN 2012** software, please refer to the User Manual that is provided with your system.

Setting up a new image document and saving your data

To create a new image document in an empty image container, click the **Snap** [Snap] or the **Set Exposure** [Set Exposure] button. For an empty image document press the **New** [New] or [⌐] buttons.

The new document is immediately presented in the **Images and Documents** panel of the **Right Tool Area**. Remember, an unsaved 2D image in the active image tab will be over-written by a new scan. Multi-dimensional scans or saved images will never be over-written and a new scan will then automatically create a new image document.

Acquired data is **not** automatically saved to disc. Make sure you save your data appropriately and back it up regularly. The ZEN software will ask you if you want to save your unsaved images when you try to close the application with unsaved images still open.

☞   There is no image database any more like in the earlier Zeiss LSM software versions.
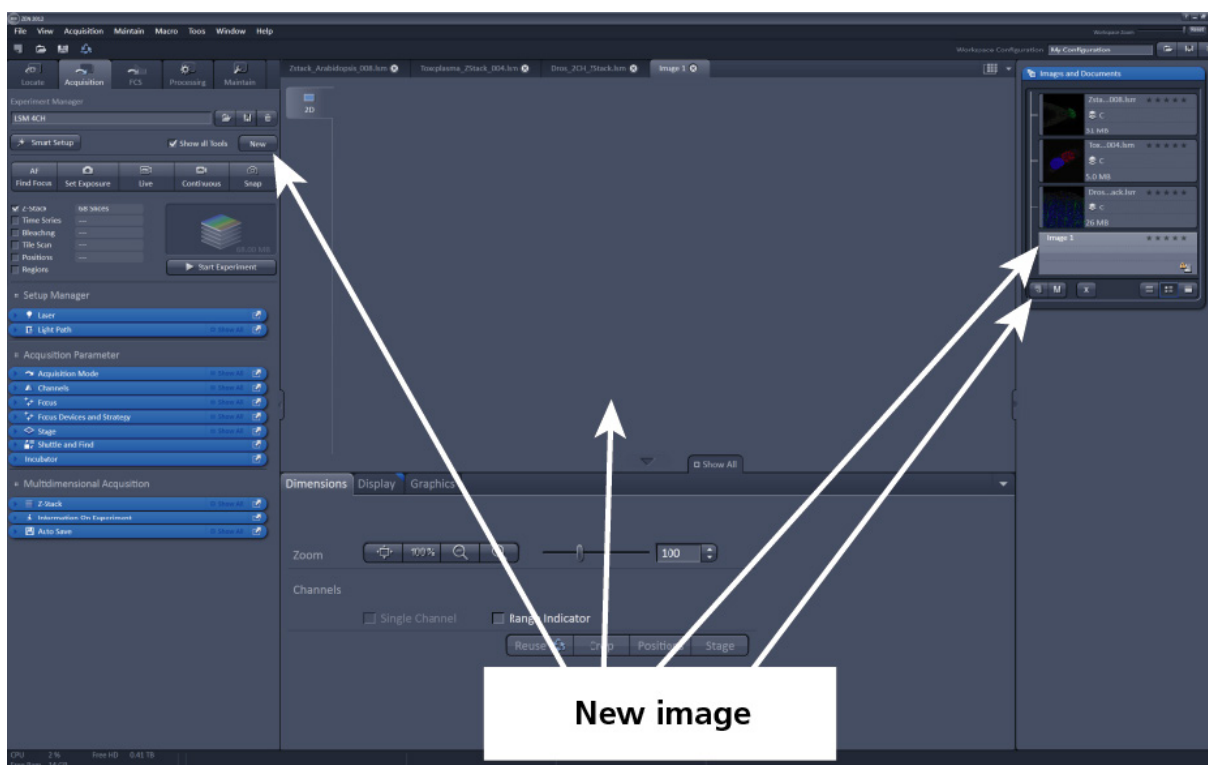
Fig. 9   New image document in the Open Images Areas

Advanced data browsing is available through the **ZEN File Browser** (Ctrl + F or from the **File** menu). The ZEN File Browser can be used like the WINDOWS program file browser. Images can be opened by double-click and image acquisition parameters are displayed with the thumbnails (Fig. 10). For more information on data browsing please refer to the detailed operating manual.
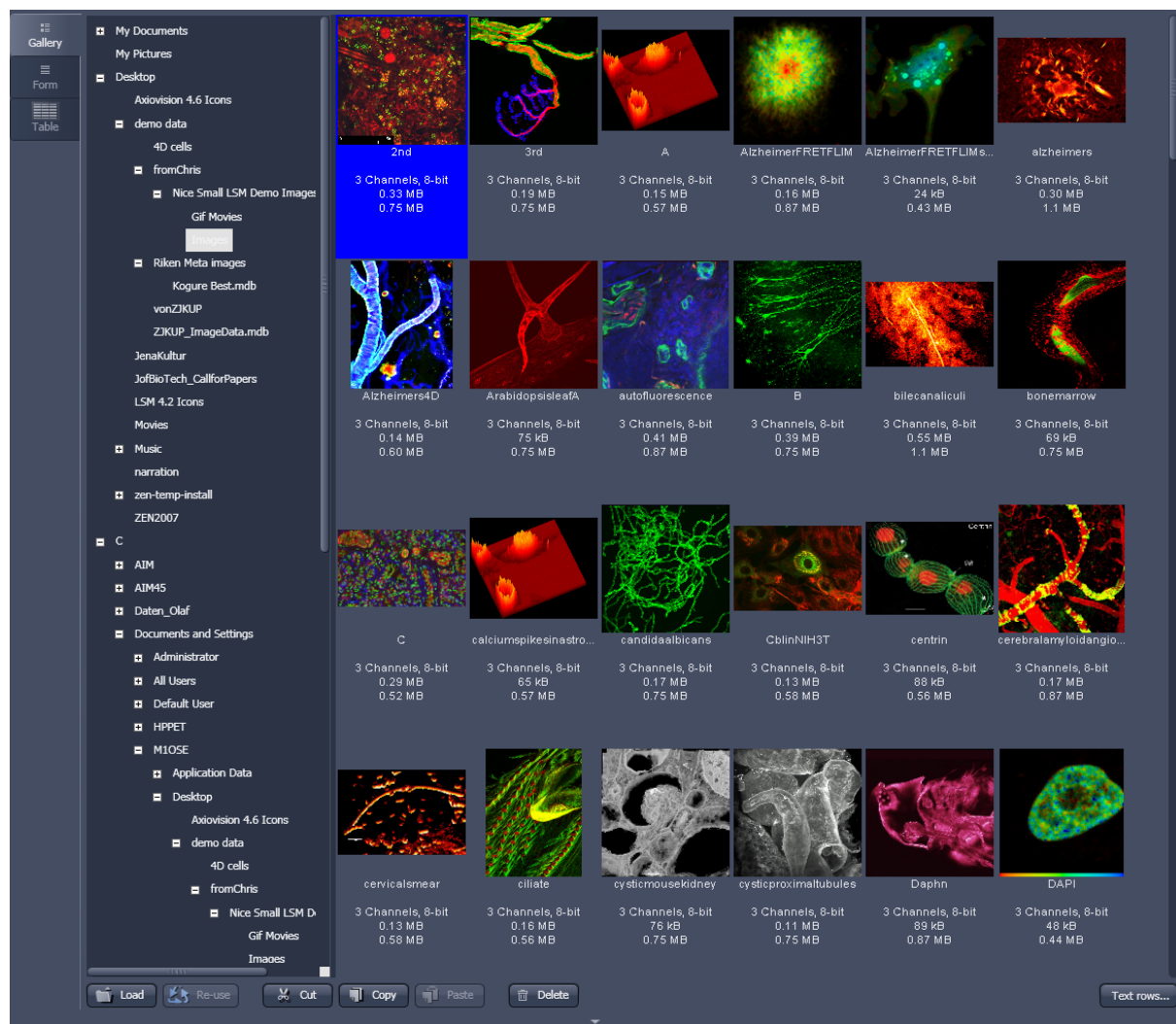

Fig. 10   File Browser

Turning on the lasers

ZEN 2012 operates all lasers automatically. Whenever they are used (manually or by the **Smart Setup** function) the lasers are turned on automatically. The **Laser Life Extender** function of the software shuts all lasers off if ZEN is not used for more than 15 minutes.

To manually switch lasers on or off:

- Click the **Show all tools** tickbox and open the **Laser** tool. All available lasers can be operated within this tool (Fig. 11).
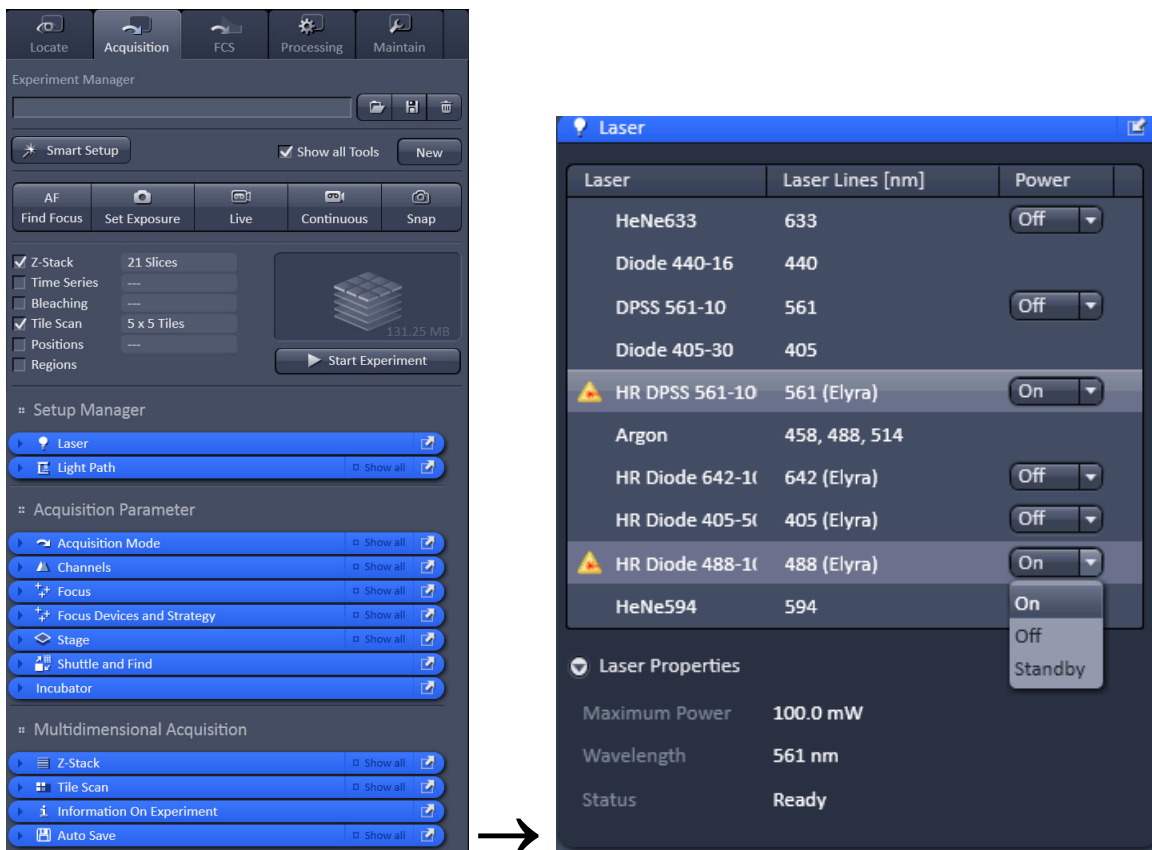
Fig. 11   Laser Control tool

Setting up the microscope

Changing between direct observation and laser scanning mode

The **Locate** and **Acquisition** buttons switch between the use of the microscope and the LSM 7 ELYRA:
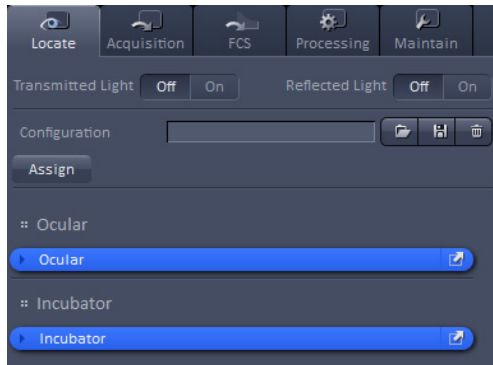
Locate selected

- Click on the **Locate** button to open the controls for the microscope beam path and for direct observation via the eyepieces of the binocular tube, lasers are blocked.

Acquisition selected
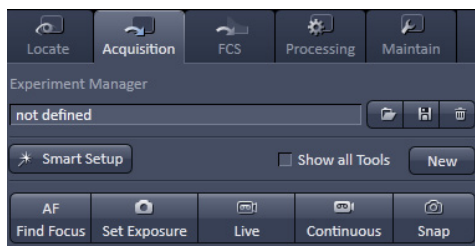
- Click on the **Acquisition** button to move back to the LSM system.
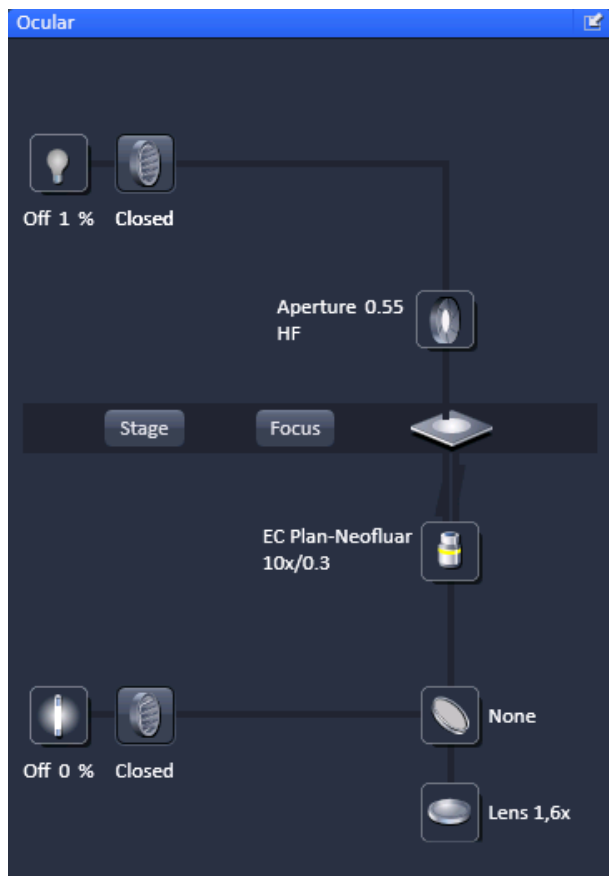
Fig. 12   **Microscope Control window of the Axio Observer.Z1**

Setting up the microscope and storing settings

Click on the **Locate** tab for direct observation; press the **Oculars Online** button for your actions to take effect immediately. Then open the **Ocular** tool tab to configure the components of your microscope like filters, shutters or objectives (Fig. 12).

Selecting an objective

- Open the graphical pop-up menu by clicking on the **Objective** symbol and select the objective lens for your experiment (Fig. 12).

- The chosen objective lens will automatically move into the beam path.

Focusing the microscope for transmitted light

- Open the graphical pop-up menu by clicking on the **Transmitted Light** icon (Fig. 13).

- Click on the **On** button. Set the intensity of the Halogen lamp using the slider.

- Clicking outside the pop-up control closes it.

- Place specimen on microscope stage. The cover slip must be facing the objective lens. Remember the immersion medium if the objective chosen requires it!

- Use the focusing drive of the microscope to focus the object plane.

- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control.

Setting the microscope for reflected light

- Click on the **Reflected Light** icon to open the X-Cite 120 Controls and turn it on.

- Click on the **Reflected Light** shutter to open the shutter of the X-Cite 120 lamp.

- Click on the **Reflector** button and select the desired filter set by clicking on it.

Storing the microscope settings

Microscope settings can be stored as configurations (Fig. 14) by typing a config name in the pull-down selector and pressing the save [💾] button. Fast restoration of a saved config is achieved by selecting the config from the pull-down list and pressing the [📂] load button. The current config can be deleted by pressing the delete [✕] button.

These configurations can be assigned to buttons that are easier to press.

☞ Depending on the microscope configuration, settings must be done manually if necessary.
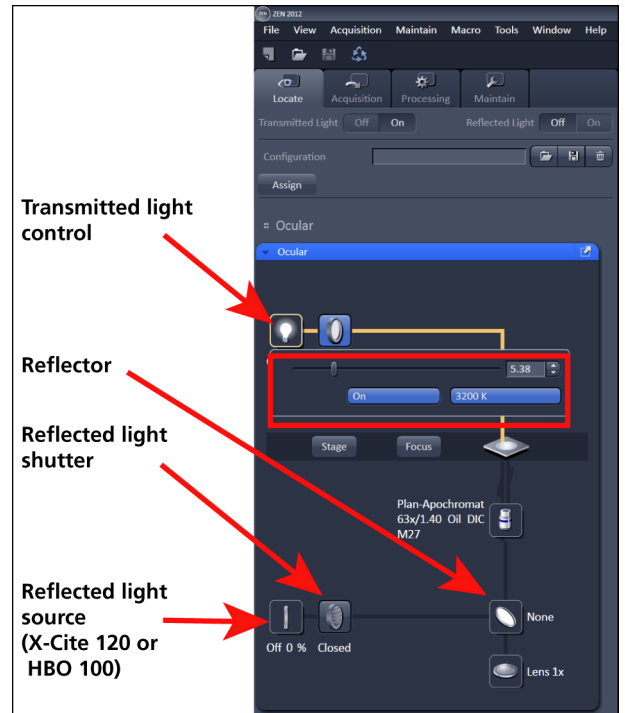
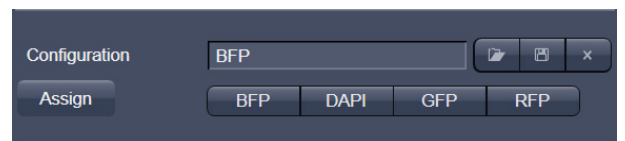**Fig. 13   Microscope Control window with Transmitted Light pop-up menu**

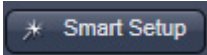**Fig. 14   Configuration panel**

Configuring the beam path and lasers for the LSM 780 / 710

- Click the **Acquisition** button.

Smart Setup

The tool **Smart Setup** is an intuitive, user-friendly interface which can be used for almost all standard applications. It configures all the system hardware for a chosen set of dyes.

- Click on the **Smart Setup** button to open the smart setup window. This window can be accessed any time from the software to change dye combinations.

- Click on the arrow in the dye list and simply choose the dye(s) you want to use in your experiment from the list dialogue. In this dialogue, the dyes can be also searched by typing the name in the search field.
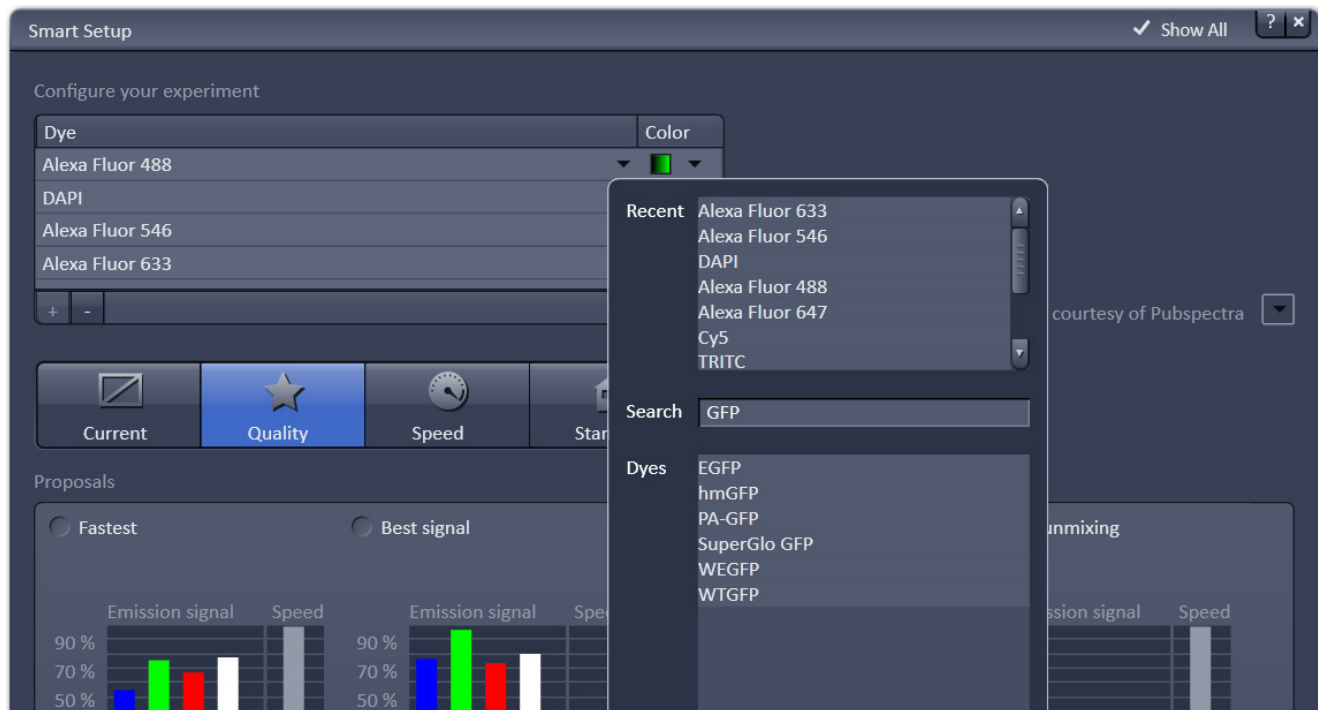
Fig. 15   **Smart setup tool**

Once finished with the input, **Smart Setup** suggests four alternative considerations (see below): One for **Fastest** imaging, one for the **Best signal**, **Smartest (Line)** between both speed and best signal and the optimal setup for later **linear unmixing** of the dyes.

The graphs display relative values for the expected emission signals and cross-talk. The resulting imaging scheme (single or multitrack) is shown below the graphs.
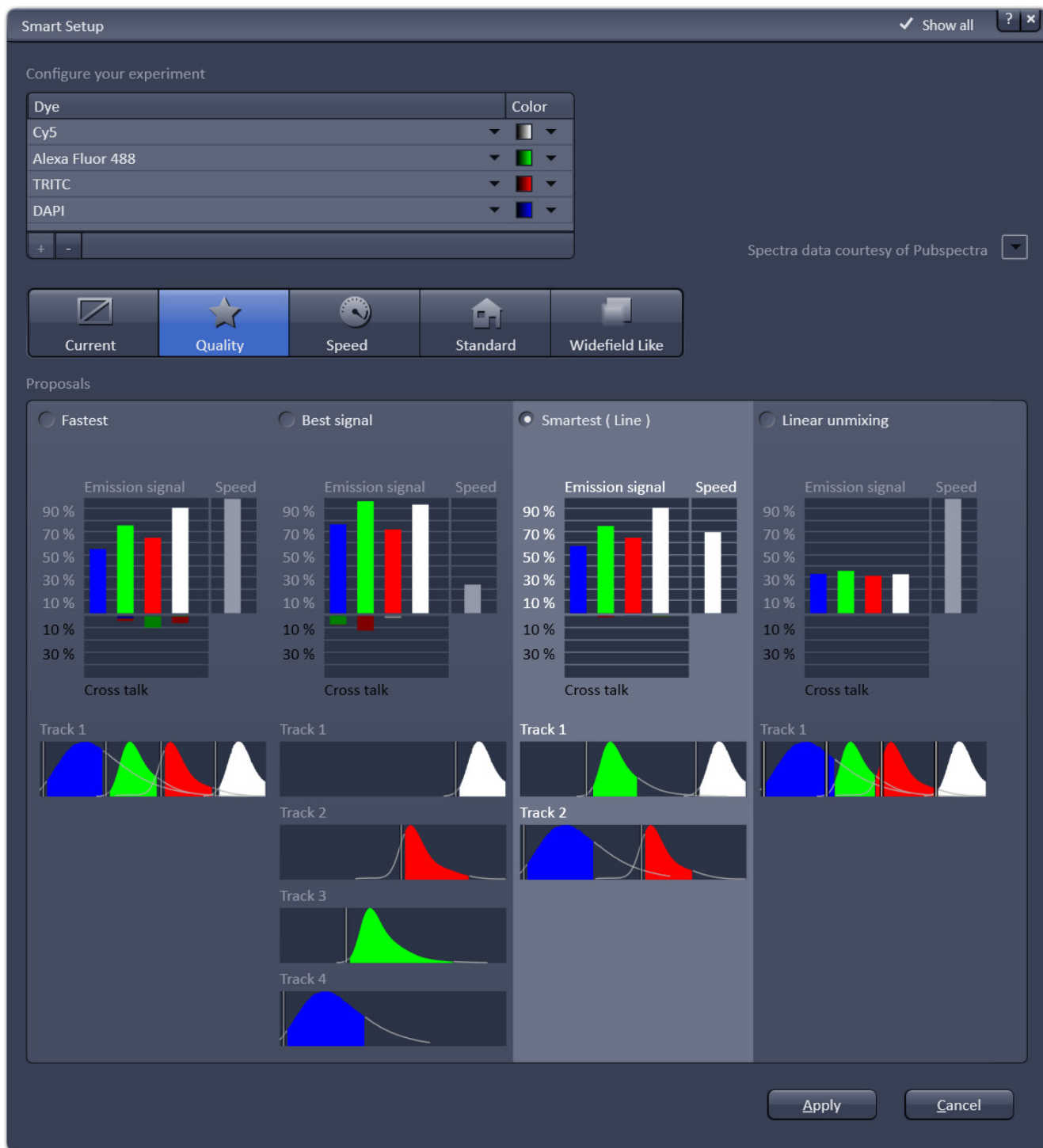
Fig. 16   Proposals panel of the Smart Setup tool

Motifs

The feature **Motifs** is part of the **Smart Setup** panel. Like in photography, common imaging conditions with typical requirements also exist in laser scanning microscopy.

The different **Motifs** are presets of scanning parameters (pixels size, pinhole diameter, scan speeds etc.) addressing such conditions (Fig. 17).

Upon selecting one of these options by mouse click the selected icon will be highlighted. If the motif **Current** is clicked, the current set of scanning parameters will be left untouched.

- Pressing the **Apply** button applies the selected proposal in Smart Setup as well as the motif.
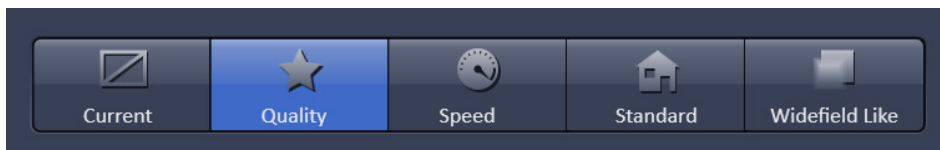
Fig. 17   **The Motifs are presets for typical imaging tasks. The selected motif is highlighted.**

If the option **Linear unmixing** is selected, the system is set in the lambda mode automatically.

Pressing the **Set Exposure** [Set Exposure] button will then optimize the settings of the Gain (Master) and offset for the given laser power and pinhole size. Further image optimization from this point can be done easily.

Setting up a configuration manually

Simultaneous scanning of single, double and triple labeling:

- − Advantage: faster image acquisition
- − Disadvantage: potential cross-talk between channels

Sequential scanning of double and triple labeling; line-by-line or frame-by-frame:

- − Advantage: Only one detector and one laser are switched on at any one time. This reduces cross-talk.
- − Disadvantage: slower image acquisition

- • Open the **Light Path** tool in the **Setup Manager** tool group and the **Channels** tool in the **Acquisition Parameter** tool group to access the hardware control window to set-up the beam path.

The open **Light Path** is shown in Fig. 18.
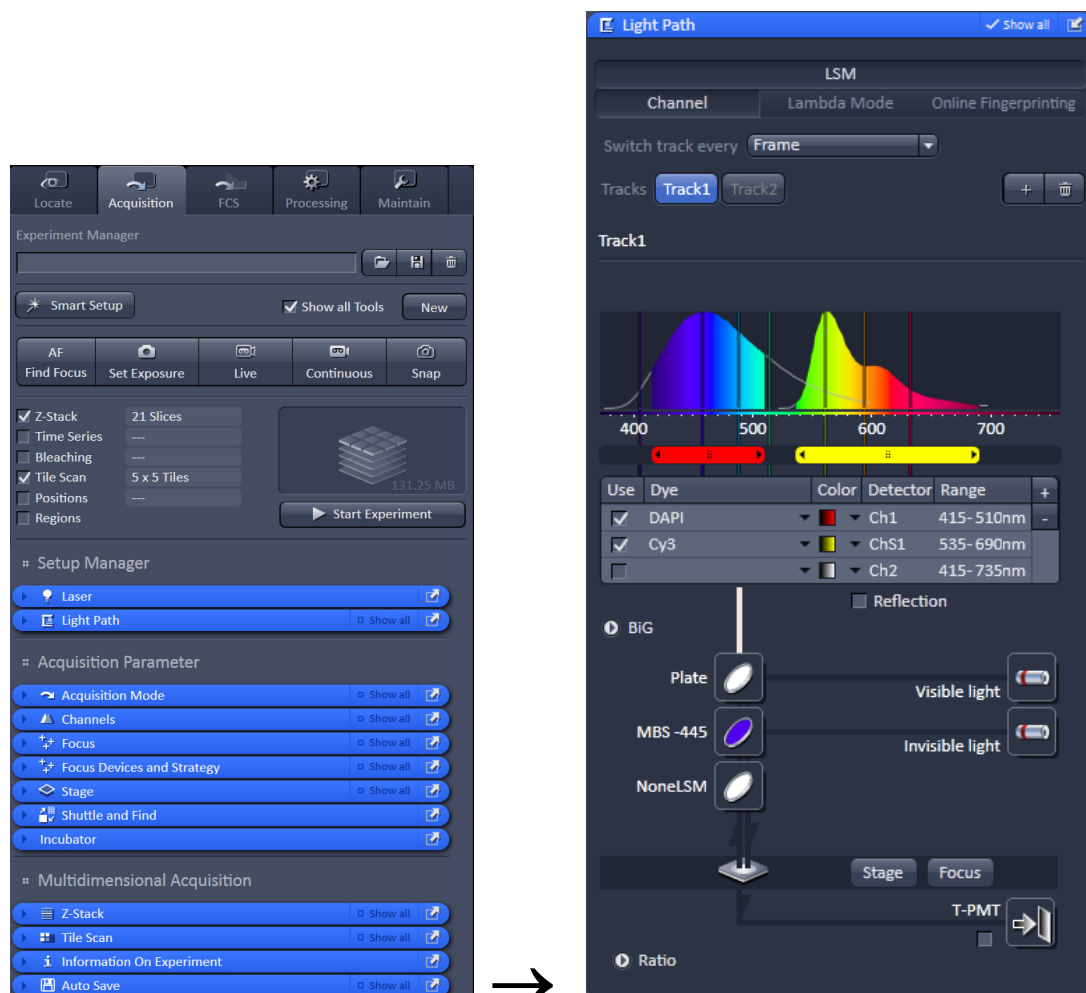
Fig. 18   **Light Path tool for a single track (LSM)**

Settings for track configuration in Channel Mode

- Select **Channel Mode** if necessary (Fig. 18).

The **Light Path** tool displays the selected track configuration which is used for the scan procedure.

- You can change the settings of this panel using the following function elements:

	Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). If necessary open the **Laser Control** tool (see above).
	Selection of the main dichroic beam splitter (MBS) for visible and invisible light from the relevant list box.
	Selection of an emission filter through selection from the relevant list box.
	Activation / deactivation (via check box) of the selected channel (Ch 1-4, monitor diode ChM, QUASAR detectors ChS1-8, transmission ChD) for the scanning procedure and assigning a color to the channel.
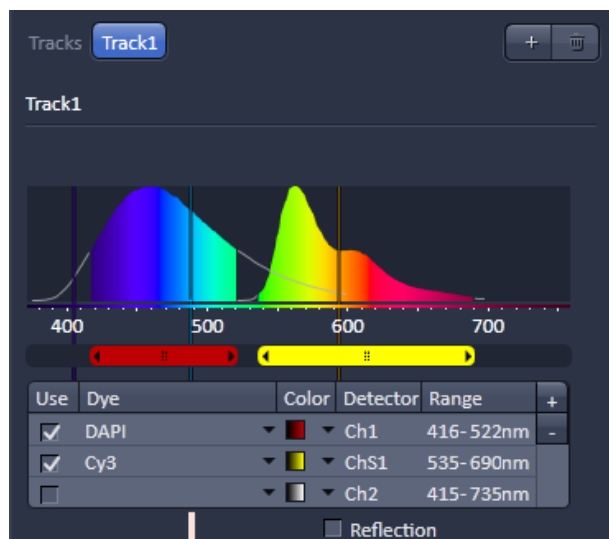
Fig. 19   Detection Bands & Laser Lines display

- Select the appropriate filters and activate the channels (Fig. 18).

- Click the **Laser** icon to select the laser lines and set the attenuation values (transmission in %) in the displayed window (Fig. 18).

- For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration (Fig. 19).

- The Detection Bands & Laser Lines are displayed in a spectral panel (Fig. 19) to visualize the activated laser lines for excitation (vertical lines) and activated detection channels (colored horizontal bars).

Fig. 20   Track configurations functions

- For storing a new track configuration open the **Channels** tool in the **Acquisition Parameter** tool group, click and enter a desired name in the first line of the list box (Fig. 20), then click **Ok** to store the configuration.

- For loading an existing configuration click then select it from the list box.

- For deleting an existing configuration click then select it from the list box and confirm the deletion with **Ok**.

Settings for multiple track configurations in Channel Mode

Multiple track set-ups for sequential scanning can be defined as one configuration (**Channel Mode Configuration**), to be stored under any name, reloaded or deleted.

The maximum of four tracks with up to eight channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently from the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), emission filters and dichroic beam splitters.

The following functions are available in the **Light Path** tool of the **Setup Manager** tool group (Fig. 18).

Switch track every selection box

Line   Tracks are switched during scanning line-by-line. The following settings can be changed between tracks: Laser line, laser intensity and channels.

Frame   Tracks are switched during scanning frame-by-frame. The following settings can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels incl. settings for gain and offset and the pinhole position and diameter.

Frame Fast   The scanning procedure can be made faster. Only the laser line intensity and the **Amplifier Offset** are switched, but no other hardware components. The tracks are all matched to the current track with regard to emission filter, dichroic beam splitter, setting of Detector Gain, pinhole position and diameter. When the **Line** button is selected, the same rules apply as for **Frame Fast**.

Tracks line
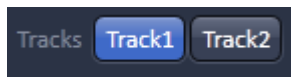
Track buttons: To change between the tracks settings click on the appropriate track button. The selected track is highlighted. The setting table for configuration will be shown behind.

Add Track button: An additional track is added to the configuration list in the **Light Path** tool. The maximum of four tracks can be used. One track each with basic configuration is added, i.e.: Ch 1 channel is activated, all laser lines are switched off, emission filters and dichroic beam splitters are set in accordance with the last configuration used.

Remove button: The track marked in the **List of Tracks** panel is deleted.

Scanning an image with the LSM 710 / 780

Setting the parameters for scanning

- Select the **Acquisition Mode** tool from the **Left Tool Area** (Fig. 21).

- Select the **Frame Size** as predefined number of pixels or enter your own values (e.g. 300 x 600) in the **Acquisition Mode** tool. Click on the **Optimal** button for calculation of appropriate number of pixels depending on objective N.A. and λ.
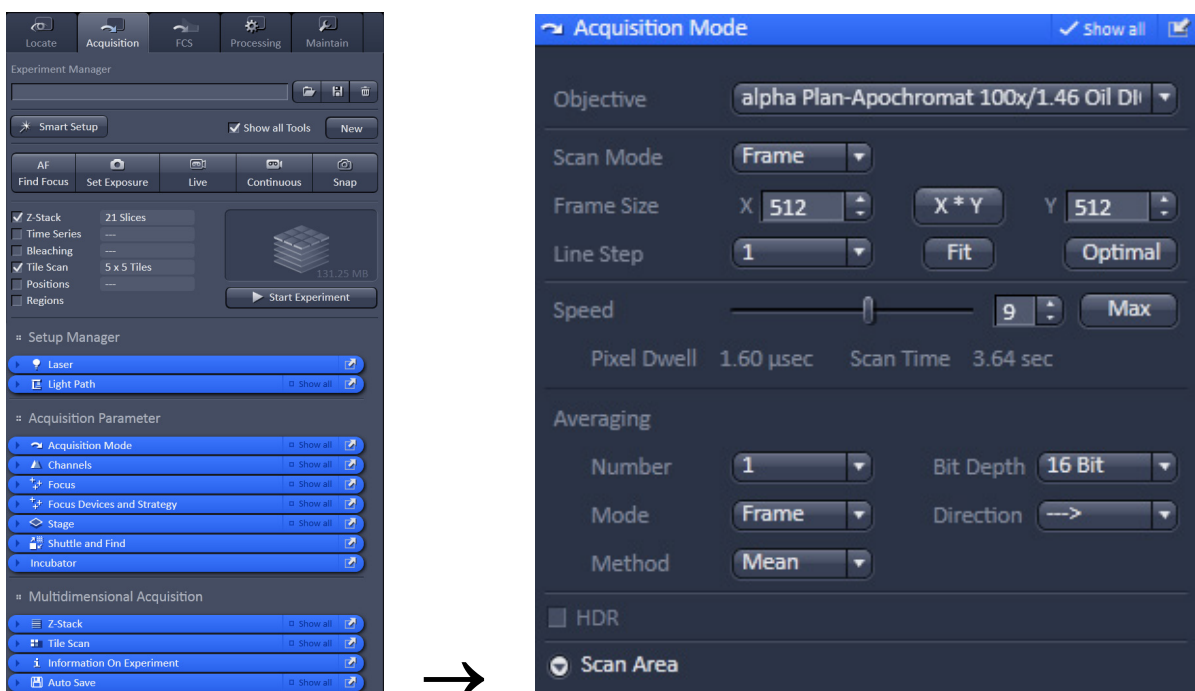
The number of pixels influences the image resolution!

Fig. 21   Acquisition Mode tool

Adjusting scan speed

- Use the **Scan Speed** slider in the **Acquisition Mode** tool (Fig. 21) to adjust the scan speed.

A higher speed with averaging results in the best signal-to-noise ratio. Scan speed 8 usually produces good results. Use speed 6 or 7 for superior images.

Choosing the dynamic range

- Select the dynamic range 8 or 12 Bit (per pixel) in the **Bit Depth** pull-down in the **Acquisition Mode** tool (Fig. 21).

8 Bit will give 256 gray levels; 12 Bit will give 4096 gray levels. Publication quality images should be acquired using 12 Bit data depth. 12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.

Setting scan averaging

Averaging improves the image by increasing the signal-to-noise ratio. Averaging scans can be carried out line-by-line or frame-by-frame. Frame averaging helps to reduce photo-bleaching, but does not give quite as smooth of an image.

- For averaging, select the **Line** or **Frame** mode in the **Acquisition Mode** tool.
- Select the number of lines or frames to average.

Adjusting pinhole size

- Select the **Channels** tool in the **Left Tool Area**.
- Set the **Pinhole** size to **1 AU** (Airy unit) for best compromise between depth discrimination and detection efficiency.

Pinhole adjustment changes the **Optical Slice thickness**. When collecting multi-channel images, adjust the pinholes so that each channel has the same **Optical Slice thickness**. This is important for colocalization studies.
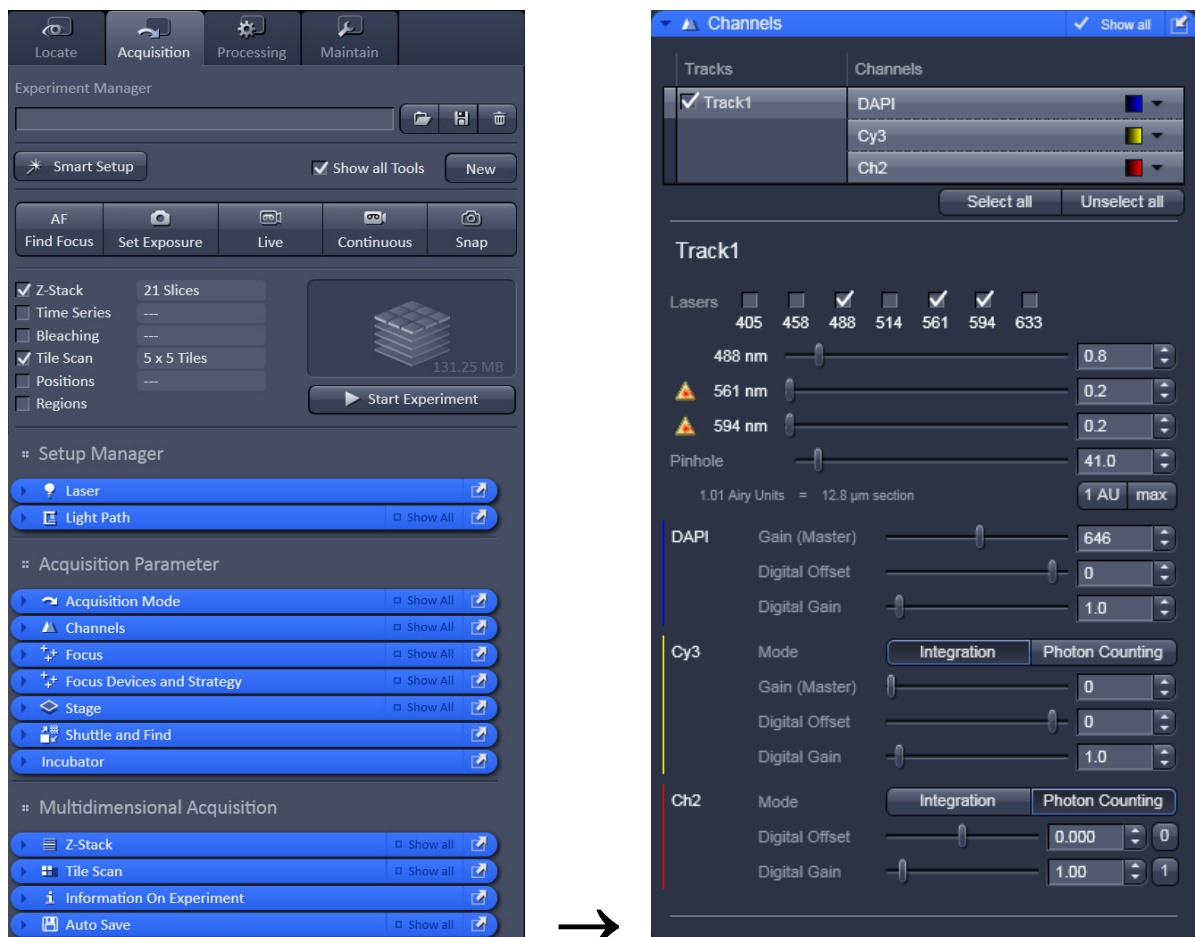
Fig. 22   Channels tool

Image acquisition
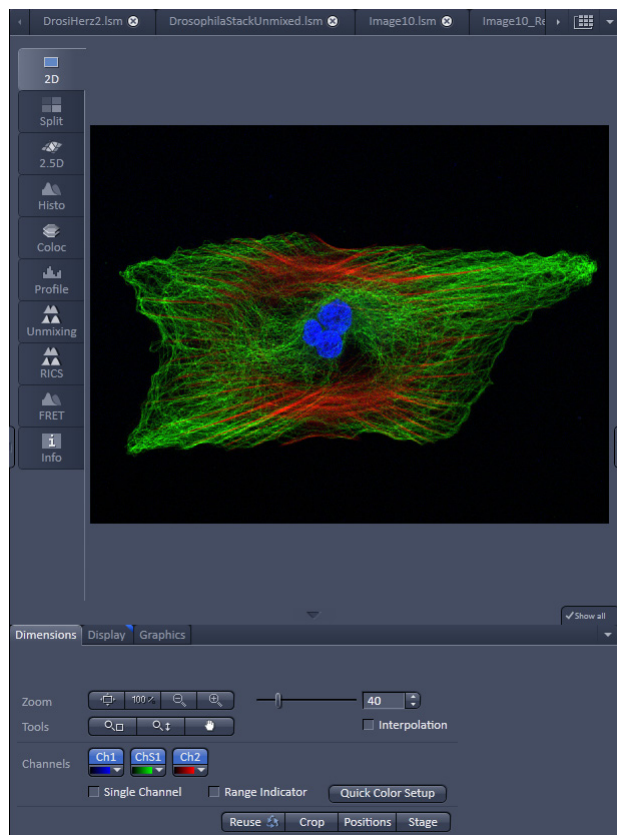
Fig. 23   **Image Display**

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Use one of the **Set Exposure**, **Live**, **Continuous** or **Snap** buttons to start the scanning procedure to acquire an image.

- Scanned images are shown in separate windows.

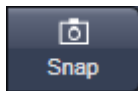- Click on the **Stop** button to stop the current scan procedure if necessary.
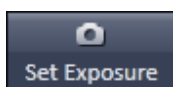
Select **Set Exposure** for automatic pre-adjustment of detector gain and offset.
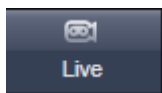
Select **Live** for continuous fast scanning – useful for finding and changing the focus.
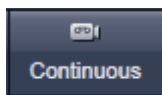
Select **Continuous** for continuous scanning with the selected scan speed.

Select **Snap** for recording a single image.

Select **Stop** for stopping the current scan procedure.

Image optimization
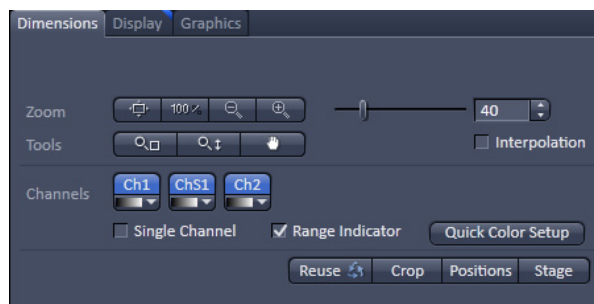
Fig. 24   **View Dimensions Control Block**

Choosing Range Indicator

- In the **View – Dimensions** View Option Control Block, activate the **Range indicator** check box (Fig. 24).

☞ Clicking on the right hand side of the button leads to a list of colors.

The scanned image appears in a false-color presentation (Fig. 25).

If the image is too bright, it appears red on the screen. Red = saturation (maximum).

If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).

Adjusting the laser intensity

- Set the **Pinhole** to **1** Airy Unit (Fig. 26).

- Set the **Gain (Master)** high.

- When the image is saturated, reduce AOTF transmission in the **Laser** control section of the **Channels t**ool (Fig. 26) using the slider to reduce the intensity of the laser light to the specimen.

Adjusting gain and offset

- Increase the **Digital Offset** until all blue pixels disappear, and then make it slightly positive (Fig. 26).

- Reduce the **Gain (Master)** until the red pixels only just disappear.
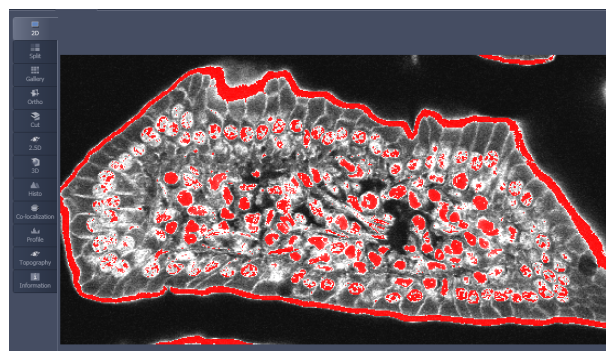
Fig. 25　　　**Image Display**
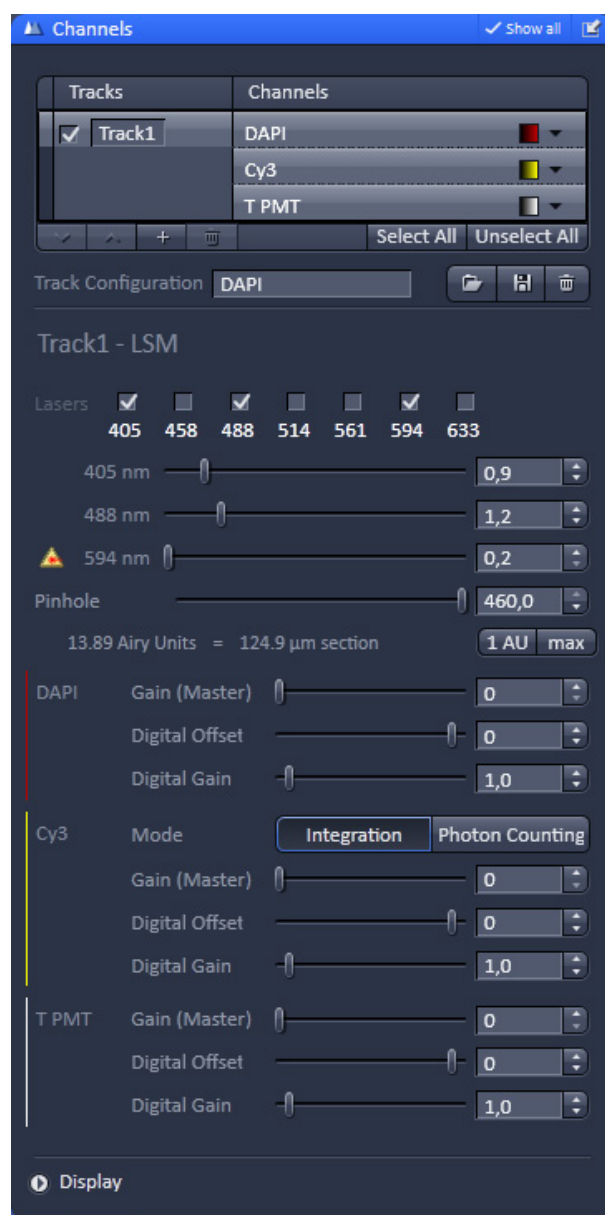
Fig. 26　　　**Channels tool**

Scanning a Z-Stack
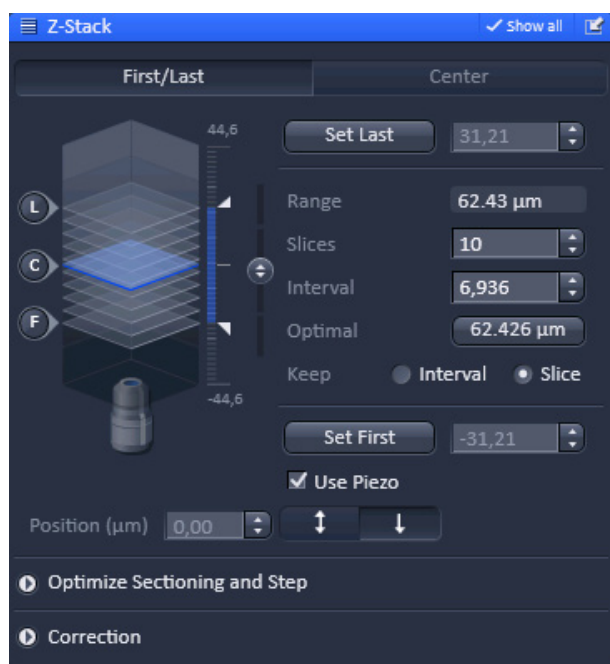
Fig. 27   Z-Stack tool

- Select **Z-Stack** ☑ Z-Stack in the main tools area.

- Open the **Z-Stack** tool in the **Left Tool Area**.

- Select **Mode First/Last** on the top of the **Z-Stack** tool.

- Click on the Continuous button in the **Action Button** area.

A continuous XY-scan of the set focus position will be performed.

- Use the focus drive of the microscope to focus on the upper position of the specimen area where the Z-Stack is to start.

- Click on the **Set First** button to set the upper position of the Z-Stack.

- Then focus on the lower specimen area where the recording of the Z-Stack is to end.

- Click on the **Set Last** button to set this lower position.

- Click on the Optimal   62.426 µm button to set number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter.

- Click on the ▶ Start Experiment **Start Experiment** button to start the recording of the Z-Stack.

☞   When a multi-dimensional acquisition tool is not selected, the respective tool and its set parameters are not included in the multidimensional image acquisition. If no multidimensional tool is activated, the ▶ Start Experiment **Start Experiment** button is grayed out and only single images can be scanned.

Configuring the beam path and lasers for SIM

Setting up configurations

Sequential imaging of double, triple and quadruple labeling frame-by-frame:

- Advantage: Only one detector is needed and one laser is switched on at any one time. This reduces cross-talk.

- Disadvantage: slower image acquisition

- Open the **Light Path** tool in the **Setup Manager** tool group to access the hardware control window to set-up the beam path. Select **SIM**.

- The open **Light Path** is shown in Fig. 28.

- The **Light Path** tool displays the selected track configuration which is used for imaging. Each track can be configured individually by activating the track.

- You can change the settings of this panel using the following function elements:

|	
	Activation / deactivation of the excitation wavelengths (check box). Click the **Laser** icon to select the laser lines and set the attenuation values (transmission in %) in the displayed window.
	Selection of FOV size is done automatically.
	Selection of grid is done automatically, but can be overruled.
	Selection of the filter cube with main beam splitter and emission filter. Please refer to the spectrum of the used dyes for the best filter setting.
	Selection of objective.
	Selection of camera is done automatically
	Selection of emission tube lens is done automatically, but can be overruled
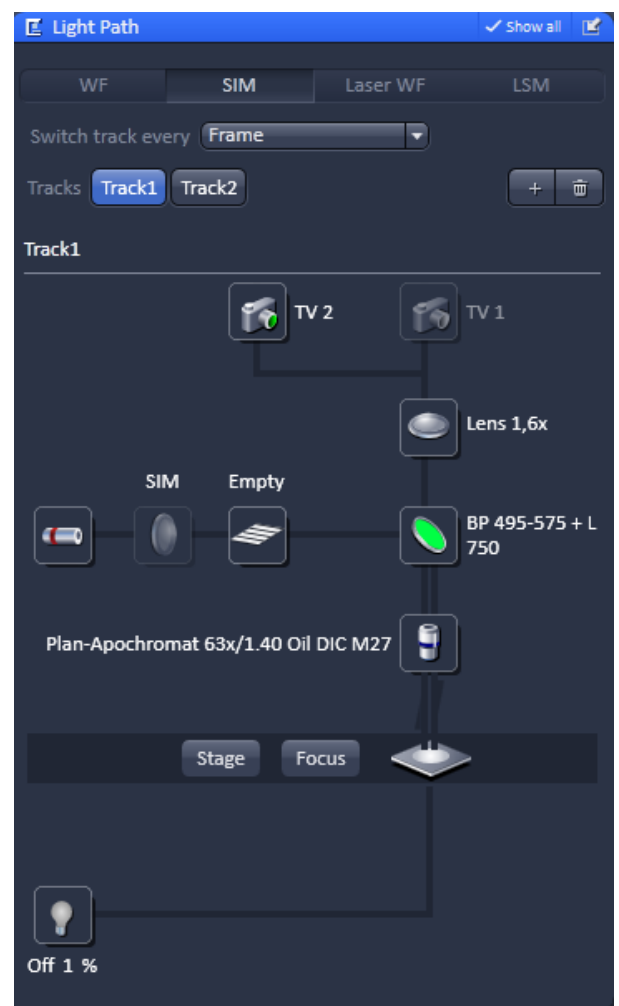
Fig. 28   **Light Path tool for SIM**
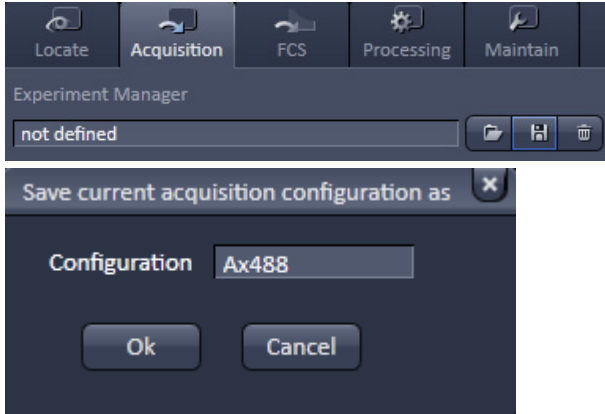
Fig. 29 Saving Configurations via the experiment Manager

- **Multiple track** set-ups for sequential scanning can be defined as one configuration, to be stored under any name, reloaded or deleted.

- For storing a new configuration click [icon] of the **Experiment Manager** of the Acquisition **tab** and enter a desired name in the first line of the list box (Fig. 29), then click **Ok** to store the configuration.

- For loading an existing track configuration click [icon] then select it from the list box.

- For deleting an existing track configuration click [icon] then select it from the list box and confirm the deletion with **Ok**.

- **Switch track every** selection box

Frame	Tracks are switched during imaging frame-by-frame. The following settings can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels incl. settings for gain.
Frame Fast	The image acquisition can be made faster. Only the laser line intensity is switched, but no other hardware components. The tracks are all matched to the current track with regard to filter cube and setting of Detector Gain.

Setting up tracks

- Select the **Channels tab** from the Acquisition parameter tool group (Fig. 30).

- The maximum of four tracks can be defined simultaneously and then imaged one after the other. Each track is a separate unit and can be configured independently from the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), and filter cubes.

The following functions are available

- Activate/Deactivate Lasers as necessary and set the laser power via AOTF using the **Laser** slider.

- Set the FOV. For SIM iamging the setting SIM should be chosen.

- Set the Exposure time in ms. A good start value is 200 ms.

- Set the EMCCD gain. For SIM imaging it is recommended to stay below 10.
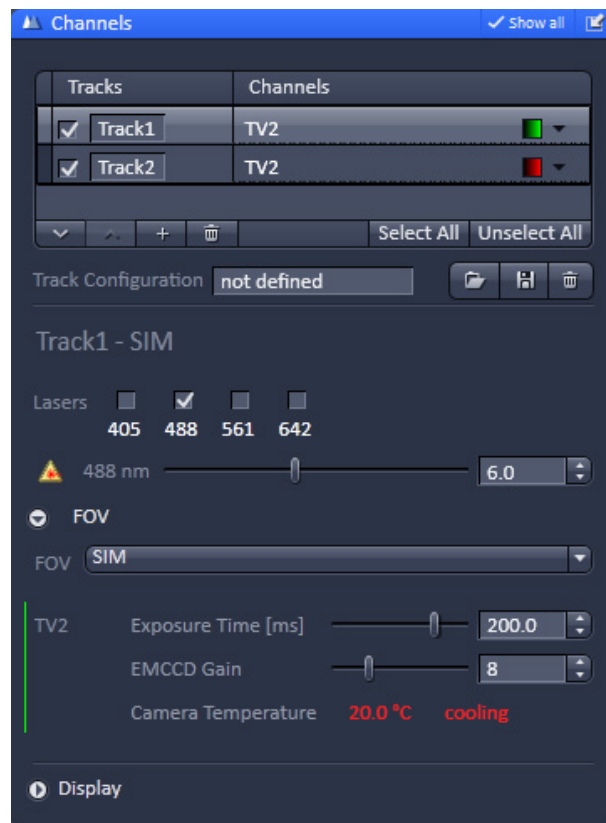
Fig. 30   **Channels tool for SIM with two tracks**

Add Track button	An additional track is added to the configuration list by pressing the button. A maximum of four tracks can be used. Beam configuration of a track is defined in the **Light Path** tool.
Remove button	The track marked in the **List of Tracks** panel is deleted.
	A click on this arrow button will move the selected track (highlighted in light grey) one position upwards in the list box.
	A click on this arrow button will move the selected track (highlighted in light grey) one position downwards in the list box.

- For storing a new track configuration click ![save icon] of the **Track Configuration** and enter a desired name in the first line of the list box (Fig. 31), then click **Ok** to store the configuration.

- For loading an existing track configuration click ![open icon] then select it from the list box.

- For deleting an existing track configuration click ![delete icon] then select it from the list box and confirm the deletion with **Ok**.
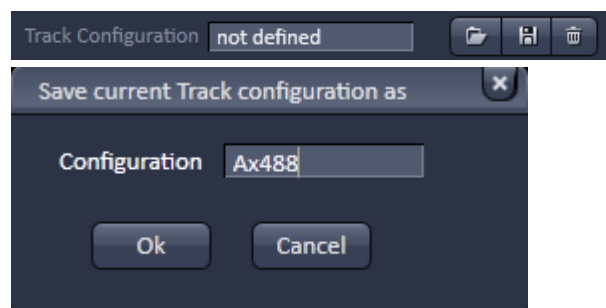

Fig. 31   **Saving Configurations via the experiment Manager**
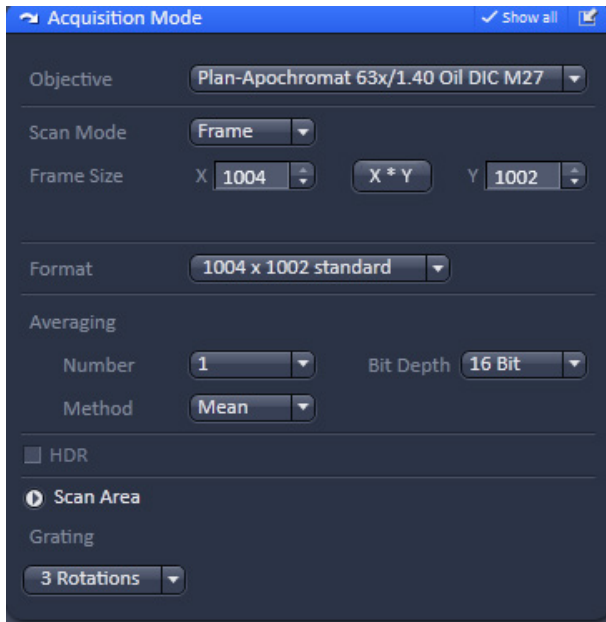
Fig. 32   Acquisition mode tab

Setting up parameters

The following parameters can be set in the **Acquisition Mode** tool (Fig. 32):

- **Objective**: For best performance use Plan-Apochromat 63x / 1.4 oil objective

- **Scan mode**: is by default Frame and is fixed

- **Scan Size**: is by default 1004 x 1002 and is fixed

- **Format**: is by default 1004 x 1002 standard and should not be altered.

- **Average**: Number of 1 and Method Mean are the defaults. Averaging can help to increase signal-to-noise.

- **Bit Depth**: is by default 16 Bit. It is recommended not to alter the value.

- **Scan Area**: is by default the full chip and fixed. The scan area can only be altered by Cropping and Panning.

- **Grating**: is by default **3 Rotations**. **5 Rotations** is recommended for better isotropic resolution enhancement. **3 Rotations** will have the benefit of faster image acquisition.

Taking a SIM image

Image acquisition

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Use one of the **AF Find Focus**, **Set Exposure**, **Live**, **Continuous** or **Snap** buttons to start the procedure to acquire an image (Fig. 33).

- New images are shown in separate windows.

- Click on the **Stop** button to stop the current scan procedure if necessary.

- The set up should be such that the grid can be seen during image acquisition (Fig. 34). It might help to change the phase and rotation by moving the corresponding sliders in **Dimensions** view control tab of the original image (Fig. 35). If the grid cannot be seen, resolution enhancement will be limited. In case no grid is seen, it might help to switch to the grid with the next higher spacing.
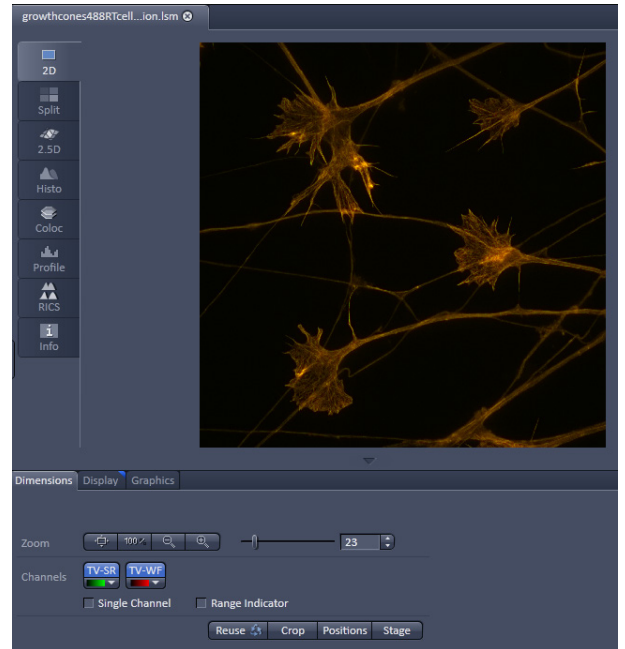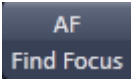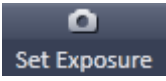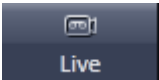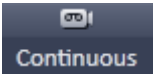
Fig. 33   **Image Display**

Select **AF Find Focus** to screen for the focus position based on contrast

Select **Set Exposure** for automatic pre-adjustment of detector gain and offset.

Select **Live** for continuous fast acquisition – useful for finding and changing the focus.

Fig. 34   **Image with grid pattern**

Select **Continuous** for continuous scanning with the selected scan speed.

Select **Snap** for recording a single image.
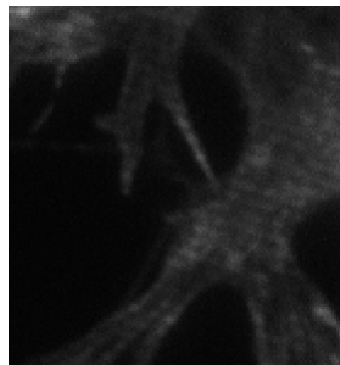

Select **Stop** for stopping the current scan procedure.

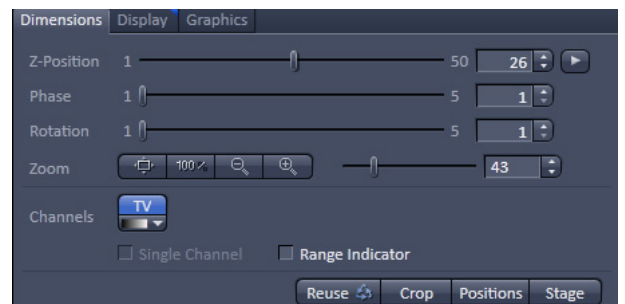**Fig. 35**   **Dimensions view control tab for a raw SIM image**

Image optimization

Fig. 36 Dimensions view control tab for a SIM image
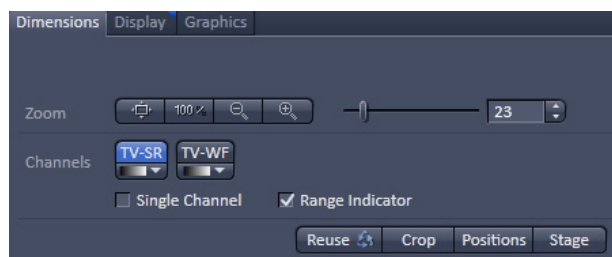

Fig. 37 Image with Range Indicator

Fig. 38 Display view control tab

Choosing Range Indicator

- In the **Dimensions** view control tab check the Range Indicator box (Fig. 36).

- The scanned image appears in a false-color presentation (Fig. 37). If the image is too bright, it appears red on the screen. Red = saturation (maximum). If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).
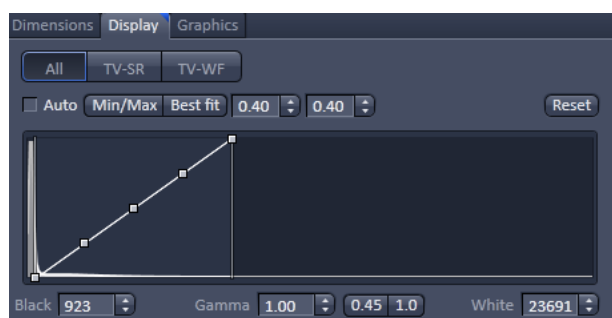
Adjusting the laser intensity

- When the image is saturated with the slider in the **Display** view control tab at **Reset** (linear), reduce AOTF transmission in the **Laser** control section of the **Channels** tool (Fig. 38).

Adjusting gain

- Reduce the **EMCCD Gain** until the red pixels just disappear.

Adjusting display settings

- If the slider in the Display view control tab is offset from the Reset (linear) position, red as well as blue pixels can also occur. Use the slider to set contrast and brightness to values that avoid red and blue pixels. (Fig. 38).

Acquire a Z-Stack

- Select **Z-Stack** [☑ Z-Stack] in the main tools area.

- Open the **Z-Stack** tool in the **Left Tool Area**.

First/Last Mode (Fig. 39)

- Select **Mode First/Last** on the top of the **Z-Stack** tool.

- Click on the [Continuous] button in the **Action Button** area. A continuous XY-scan of the set focus position will be performed.

- Use the focus drive of the microscope to focus on the upper position of the specimen area where the Z-Stack is to start.

- Click on the **Set First** button to set the upper position of the Z-Stack.

- Then focus on the lower specimen area where the recording of the Z-Stack is to end.
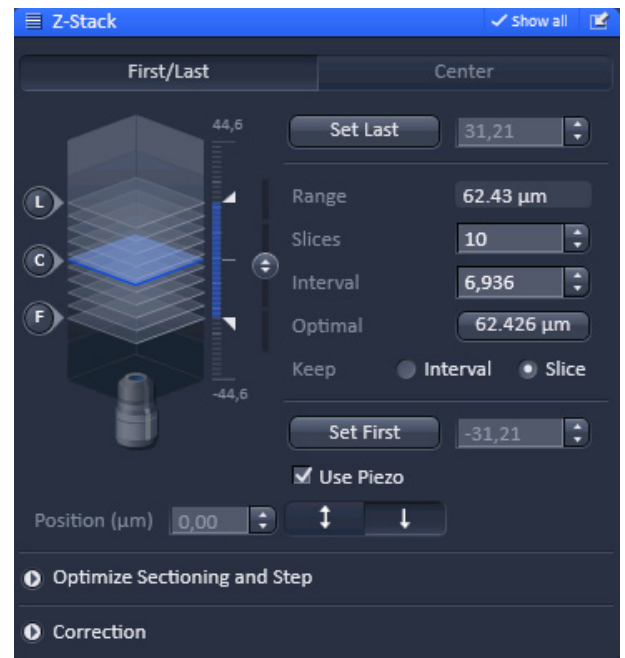
- Click on the **Set Last** button to set this lower position.

Fig. 39   Z-Stack tool - First/Last active

Center Mode (Fig. 40)

- Select **Mode Center** on the top of the **Z-Stack** tool.

- Click on the [Continuous] button in the **Action Button** area. A continuous XY-scan of the set focus position will be performed.

- Use the focus drive of the microscope to focus on the center position of the specimen.

- Click on Center to center the Z-Piezo stage insert to the present focus position of the microscopes focus drive.
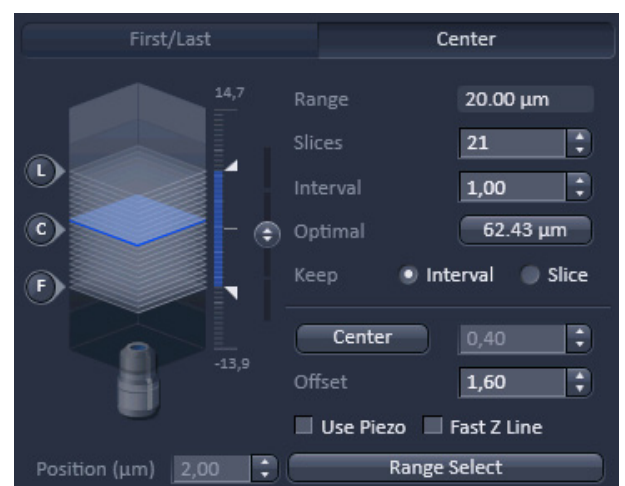

Fig. 40   Z-Stack tool - Center active

Trigger the z-Scan

- Click on the [Smallest   0.43 µm] button to set number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter.

- Click on the [▶ Start Experiment] **Start Experiment** button to start the recording of the Z-Stack. The recording will be performed with the Z-Piezo stage insert.

☞ When a multi-dimensional acquisition tool is not selected, the respective tool and its set parameters are not included in the multidimensional image acquisition. If no multidimensional tool is activated, the [▶ Start Experiment] **Start Experiment** button is grayed out and only single images can be scanned.

Processing of SIM images

Loading the image

- Click on the **Processing** tab (Fig. 41).

- Open the **Method** tab.

- Scroll to **Structured Illumination** (SIM) and open the tool.

- Select **Structured Illumination**.

- In the image container click on the Image you want to process.

- Open the **Methods Parameters** tab.
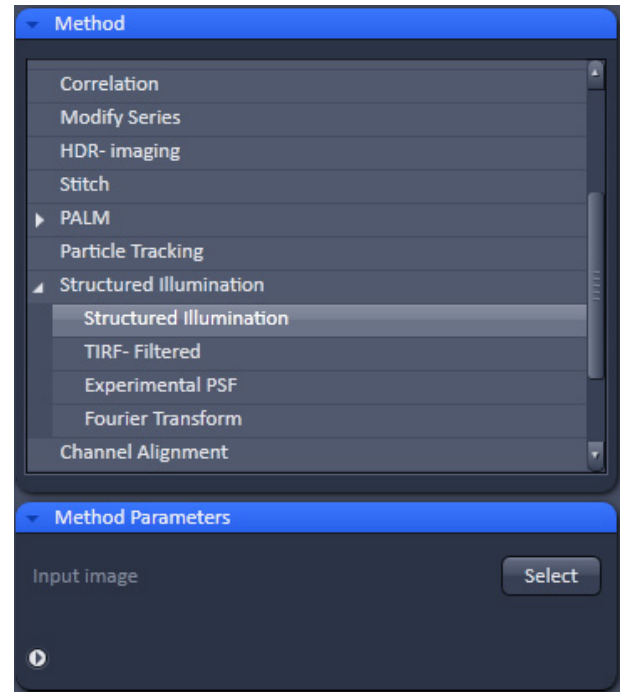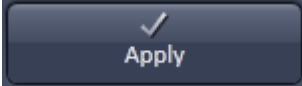
- Press **Select** to load the image.

Fig. 41   Processing tab, Method tab

Processing the image

Fig. 42   **Methods Parameters tab**

- Expand the parameter definition field in the **Methods Parameters** tab (Fig. 42).

- Select **First-Last** and define the slice of the stack you want to process. If you want to process only one slice select **Single**. It is recommended to process a stack, since this will also enhance x,y resolution.

- Select for **Processing - Automatic**. If you want to refine processing and want to set parameters manually, select **Manual**.

- As **Mode** select 3D if you process a stack. Select 2D if you do not want to get resolution enhancement in z.

- As **Outpu**t select **SR-SIM + Wide Field**. If you also want to display a deconvolved wide field image (DCV) select **SR-SIM +DCV+Wide Field**.

- Press [Apply] to compute the SIM image.

Configuring the beam path and lasers for PALM

Setting up a configuration

- Open the **Light Path** tool in the **Setup Manager** tool group to access the hardware control window to set-up the beam path.

- Select **Laser WF**.

- The open **Light Path** is shown in Fig. 43.

The **Light Path** tool displays the selected track configuration which is used for imaging. Each track can be configured individually by activating the track.

- You can change the settings of this panel using the following function elements:

Activation / deactivation of the excitation wavelengths (check box). Click the **Laser** icon to select the laser lines and set the attenuation values (transmission in %) in the displayed window.

Selection of FOV size is done automatically, but can be overruled.

Collimator adjustment. Set to obtain evenly illuminated field.

TIRF Angle adjustment. Select **EPI** for epi fluorescence, **TIRF** for total internal reflection. In TIRF mode adjust angle so that epi fluorescence light is minimized.

Selection of the filter cube with main beam splitter and emission filter. Please refer to the spectrum of the used dyes for the best filter setting.

Fig. 43   Light Path tool for PALM

Moving the 3D PALM slider between load and work positions.

Selection of objective.

Selection of camera is done automatically, but can be overruled by pressing the TV#:Andor iXon xxx buttons. The Andor iXon 897 is recommended for PALM applications. For systems without LSM an AxioCam camera for IR transmitted light is optionally available. Activate the camera by checking the box if required.

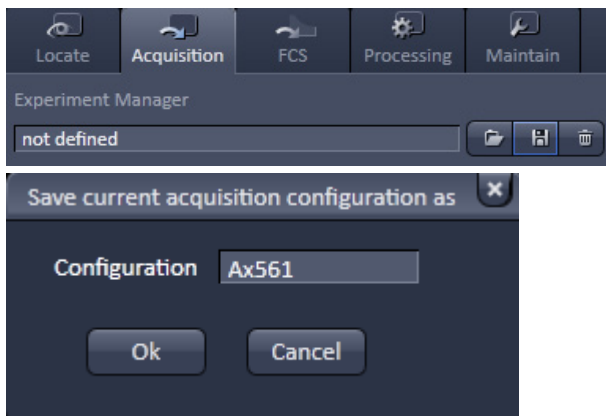Selection of emission tube lens is done automatically, but can be overruled


Fig. 44   Saving Configurations via the experiment Manager

- For storing a new configuration click of the **Experiment Manager** of the **Acquisition** tab and enter a desired name in the first line of the list box (Fig. 44), then click **Ok** to store the configuration.

- For loading an existing track configuration click then select it from the list box.

- For deleting an existing track configuration click then select it from the list box and confirm the deletion with **Ok**.

Setting up a track

- Select the **Channels tab** from the Acquisition parameter tool group (Fig. 45).

- It is recommended for PALM to just define one track. The track can be configured with regard to channels, Acousto-Optical Tunable Filters (AOTF), and filter cubes.

The following functions are available:

- Activate/Deactivate Lasers as necessary (activation and imaging laser) and set the laser power via AOTF using the **Laser** slider.

- Set the **FOV**. For PALM imaging the setting TIRF should be chosen.

- Set the **TIRF** Angle (°) by activating TIRF and adjust the slider. Press **Save TIRF angle** to save the setting. For finding cells it can be beneficial to use epi illumination. To this end press the **EPI** button to screen and go back to TIRF or HILO for 2D or 3D PALM imaging, respectively. TIRF and HILO will result in a better signal-to-noise ratio and better localization precisions.

- Press **3D PALM** button to move 3D PALM slider from load position (button appears unpressed) to work position (button appears pressed) and vice versa.

- Adjust the **Collimator Camera ELYRA** to have a homogeneously illuminated filed. Store the settings by pressing the Save collimator position(s) button.

- Set the **Exposure time** in ms. A good start value is 100 ms.

- Set the **EMCCD Gain**. For PALM imaging it is recommended to stay above 100.

- If beneficial set the **Laser trigger** of the activation laser to **transfer** and the imaging laser to **integration**.
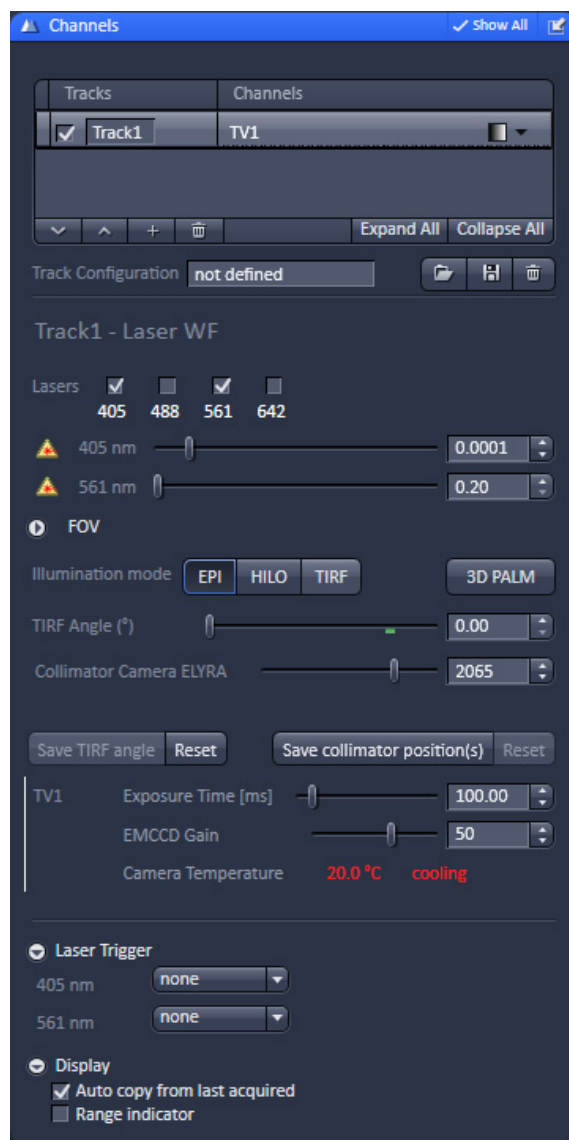

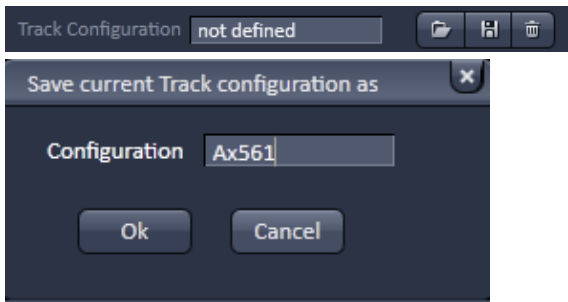**Fig. 45**   Channels tool for PALM with a single track

Fig. 46 Saving Configurations via the experiment Manager

- For storing a new track configuration click [icon] of the **Track Configuration** and enter a desired name in the first line of the list box (Fig. 46), then click **Ok** to store the configuration.

- For loading an existing track configuration click [icon] then select it from the list box.

- For deleting an existing track configuration click [icon] then select it from the list box and confirm the deletion with **Ok**.
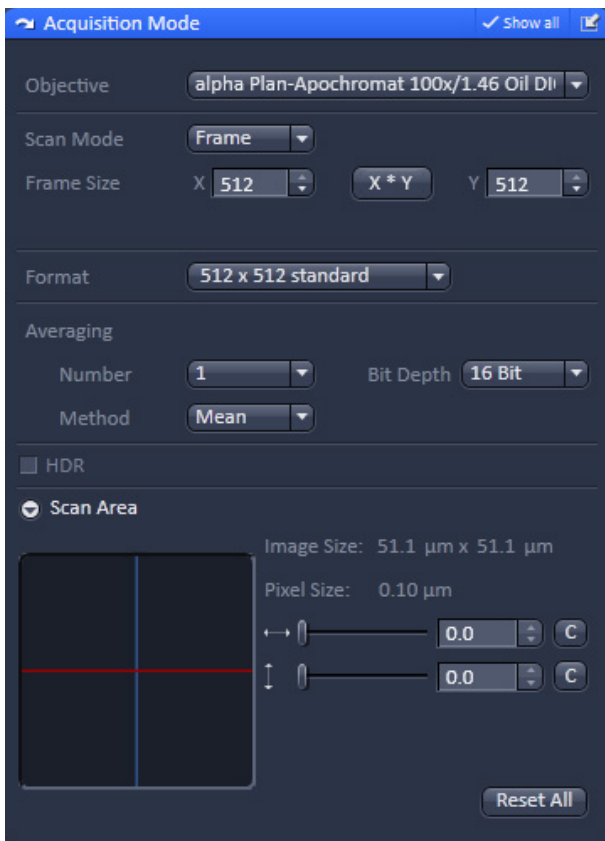
Setting up parameters

Fig. 47 Acquisition mode tab

The following parameters can be set in the **Acquisition Mode** tool (Fig. 47):

Objective: For best performance use alpha Plan-Apochromat 63x / 1.46 oil objective

Scan mode: is by default **Frame** and is fixed

Scan Size: is by default **512 x 512** and is fixed

Format: is by default **512 x 512 standard** and should not be altered.

Averaging: **Number** of **1** and **Method Mean** are the defaults. Averaging should not be used for PALM.

Bit Depth: is by default 16 Bit. It is recommended not to alter the value.

Scan Area: is by default the full chip and fixed. The scan area can only be altered by cropping and panning.

Taking a PALM image

Image acquisition

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Use one of the **AF Find Focus**, **Set Exposure**, **Live**, **Continuous** or **Snap** buttons to start the procedure to acquire an image (Fig. 48).

- New images are shown in separate windows.

- Click on the **Stop** button to stop the current scan procedure if necessary.

- The set up should be such that, once you found your cells, you have a regime where you bring molecules to blink. This is done by balancing the laser powers of the activating (low power) and imaging (high powers) lasers (Fig. 49).
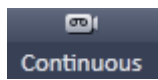
Select **AF Find Focus** to screen for the focus position based on contrast.
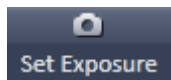
Select **Set Exposure** for automatic pre-adjustment of detector gain and offset.

Select **Live** for continuous fast acquisition – useful for finding and changing the focus.

Select **Continuous** for continuous scanning with the selected scan speed.

Select **Snap** for recording a single image.

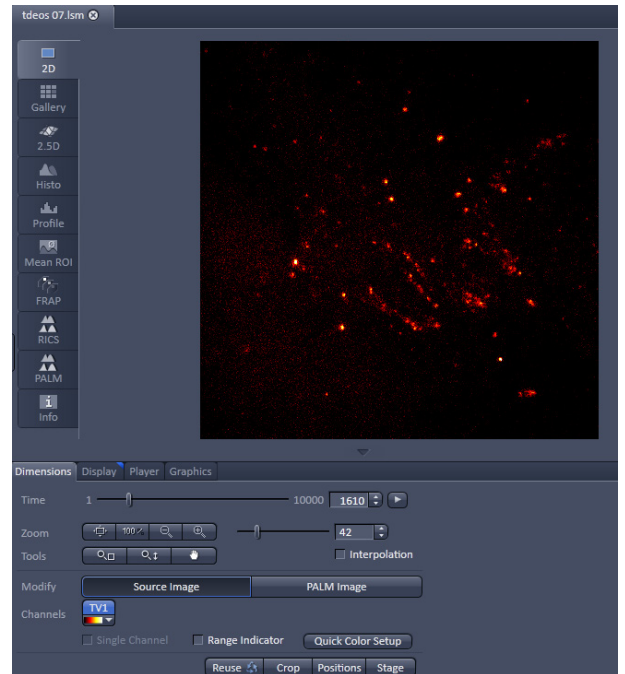Select **Stop** for stopping the current scan procedure.

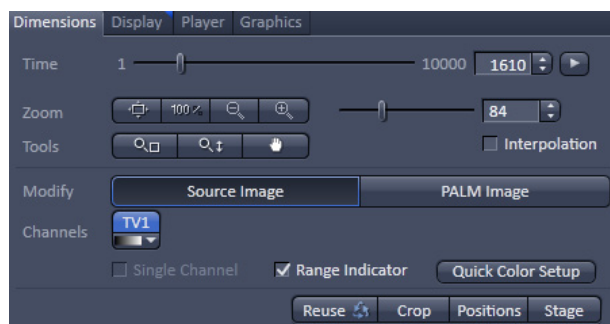**Fig. 48**   **Image Display**

Image optimization

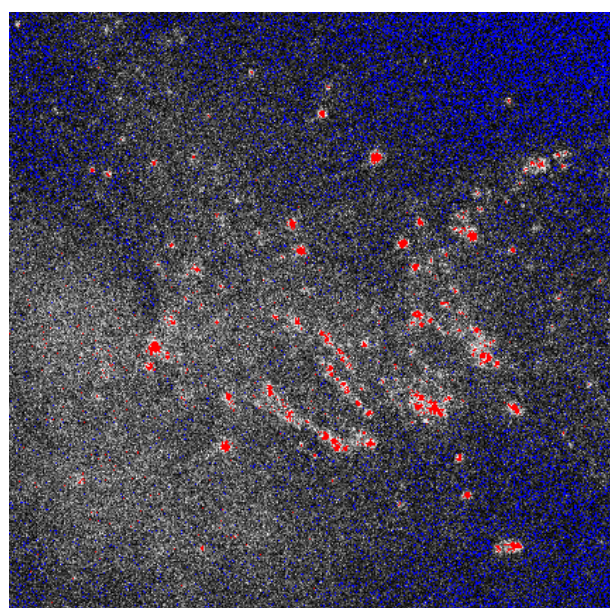**Fig. 49**   **Dimensions view control tab for a PALM image**

Fig. 50   **Image with Range Indicator**

Fig. 51   **Display view control tab**

Choosing Range Indicator

- In the **Dimensions** view control tab check the Range Indicator box (Fig. 49).

- The scanned image appears in a false-color presentation (Fig. 50). If the image is too bright, it appears red on the screen. Red = saturation (maximum). If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).

Adjusting the laser intensity

- When the image is saturated with the slider in the **Display** view control tab at **Reset** (linear), reduce AOTF transmission in the **Laser** control section of the **Channels** tool (Fig. 51).

Adjusting gain

- Reduce the **EMCCD Gain** until the red pixels just disappear.
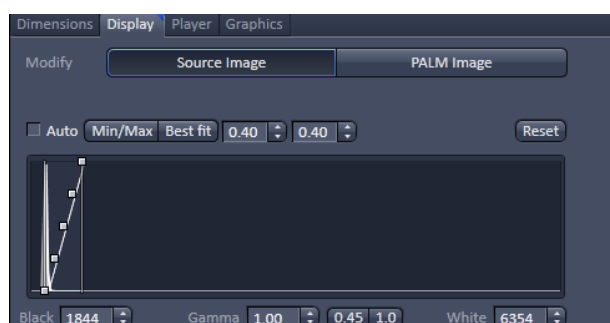
Adjusting display settings

- If the slider in the Display view control tab is offset from the Reset (linear) position, red as well as blue pixels can also occur. Use the slider to set contrast and brightness to values that avoid red and blue pixels. (Fig. 51).

Acquire a time series

For PALM imaging a time series has to be set up.

- Select **Time Series** ☑ Time Series in the main tools area.

- Open the **Time Series** tool in the **Left Tool Area** (Fig. 52).

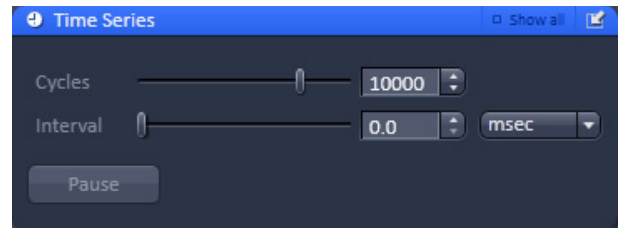- Set the required **Cycles**. For PALM you will need 5000 - 15000 images.

Fig. 52   Time series

Online Processing

Data can be processed during acquisition already online (Fig. 53).

- Open the **Online Processing Options** tab

- Check the Online Processing PALM box

- Check the Fit 2D Gauss 2D box to allow for full 2D Gauss fitting.

- Set the Peak mask size [pixel] to 9, which is the default value.

- Set the Peak intensity to noise to 6, which is the default value.

If a fiducial is present, it can be used for a holding focus.

- Check the Use holding focus box

- Check the Use region(s) box and set Regions to 1.

- Press Continuous and mark the fiducial with the displayed ROI.

Fig. 53   Online Processing Options tab

- Press **Find Focus** that will appear. The system will determine the z-focus of the fiducial.

- Press the STOP button.

- Press the Start Experiment button. Data will be processed online and the developing PALM image is displayed side by side to the time series.
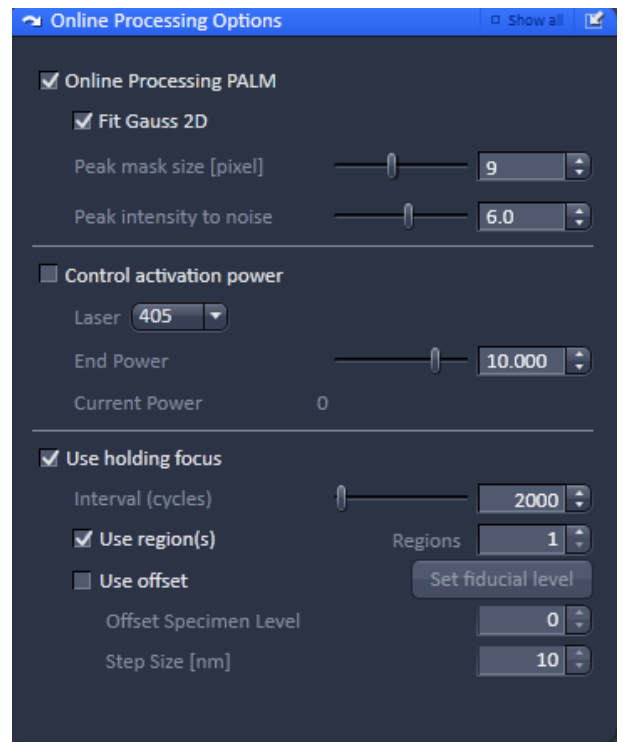
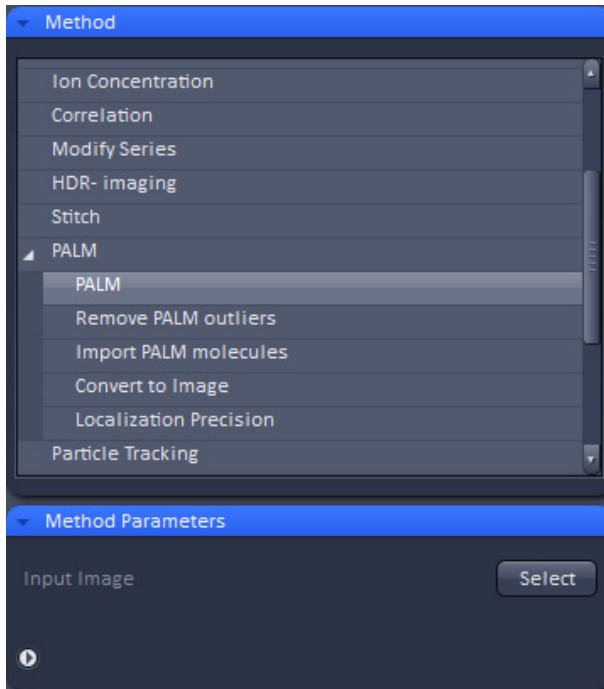# Processing of PALM images

Loading the image

Fig. 54   **Processing tab**

- Click on the **Processing** tab (Fig. 54).

- Open the **Method** tab.

- Scroll to **PALM** and expand the tool.

- Select **PALM**.

- In the image container click on the Image you want to process. You can use a time series or an online processed time series.

- Open the **Methods Parameters** tab.

- Press **Select** to load the image.

Processing the image

- Expand the Parameter definition field in the **Methods Parameter** tab and select the image. The respective settings for a 2D PALM (Fig. 55) or a 3D PALM data set (Fig. 56) will be available.

- In the **Peak Finder** set the **Peak mask size** to **9** (default for 2D) or **19** (default for 3D) and the **Peak intensity to noise** to **6** (default). You can view in the preview window which peaks the system will identify (Fig. 57).

- For 2D PALM data set select in the **Localizer** field **x,y 2D Gauss Fit** (default). For 3D PALM data set load an appropriate Experimental PSF with attached Localization Precision LUT in the **PSF File** selection field.
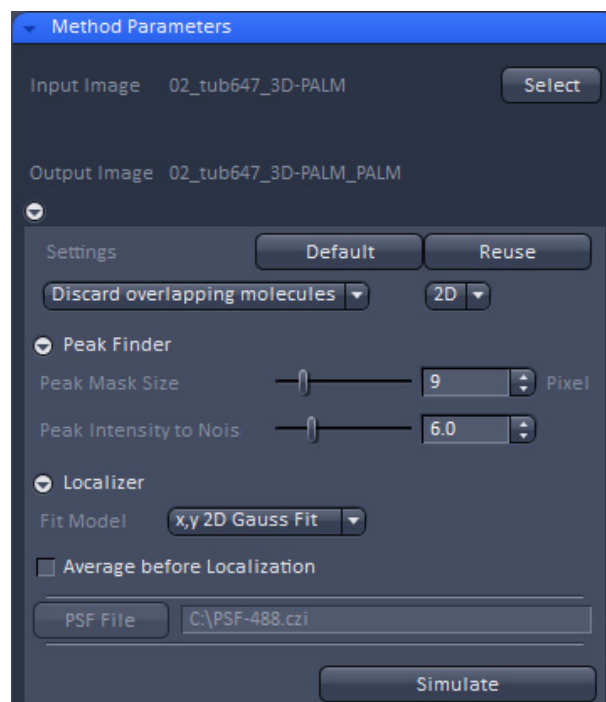

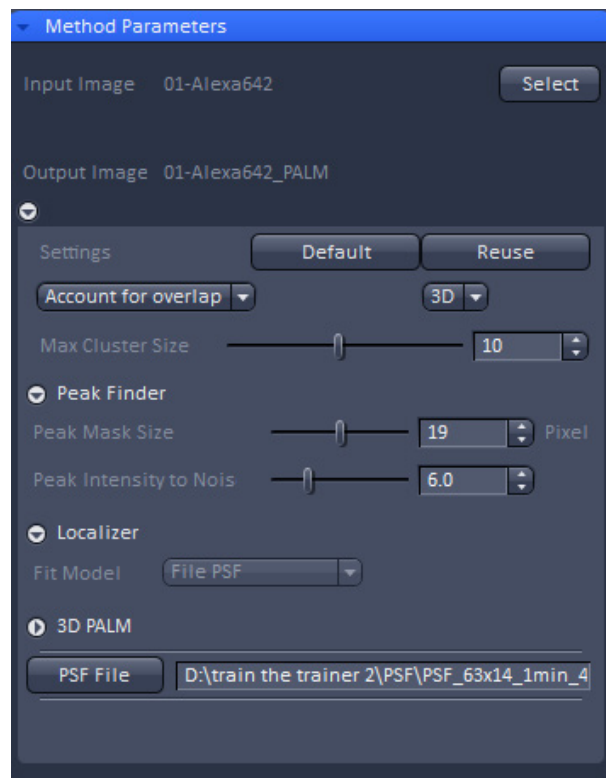**Fig. 55**   Methods Parameter tab for 2D PALM data set

Fig. 56   Methods Parameter tab for 3D PALM data set

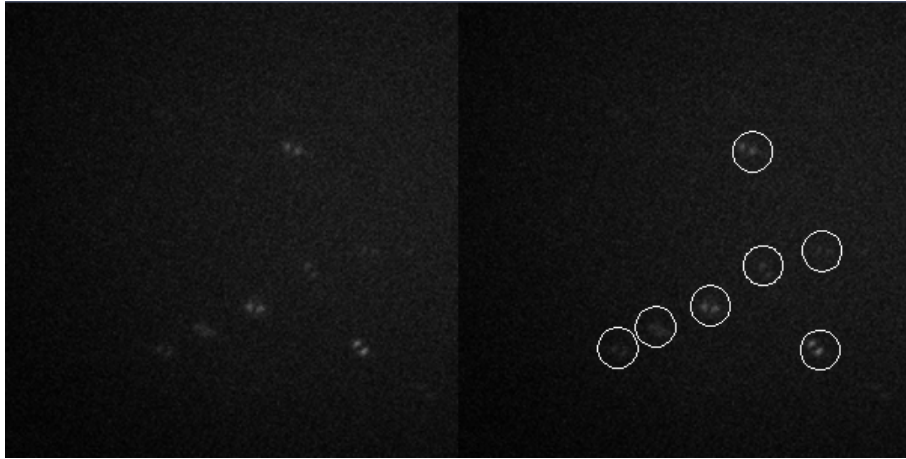- Press ![Apply] to compute the PALM image.

Fig. 57 **PALM Processing Preview window. Circles indicate identified peaks (displayed for a 3D PALM data set)**

Storing and exporting image data

- To save your acquired or processed images, click on the **Save** or **Save As** button in **File** menu, or click the ▣ button in the **Main Toolbar** (Fig. 58/**1**), or click on the 💾 button at the bottom of the **Images and Documents** area (Fig. 58/**2**).
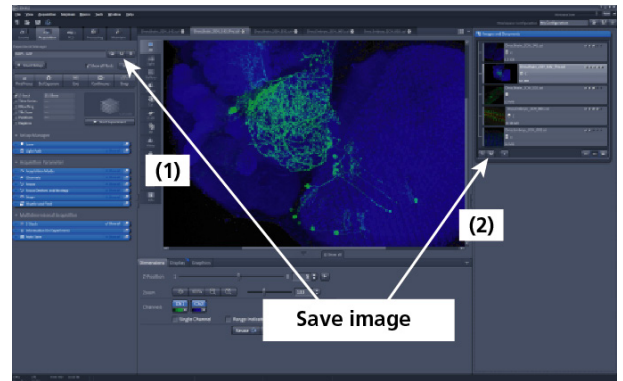

Fig. 58　　**Save image buttons in ZEN**

- The WINDOWS **Save As** window appears.

- Enter a file name and choose the appropriate image format. Note: the LSM 5 format is the native Carl Zeiss LSM image data format and contains all available extra information and hardware settings of your experiment.

- Click on the **Save** button.

If you close an image which has not been saved, a pop-up window will ask you if you want to save it. Choosing **yes** will lead you to the WINDOWS **Save As** window.
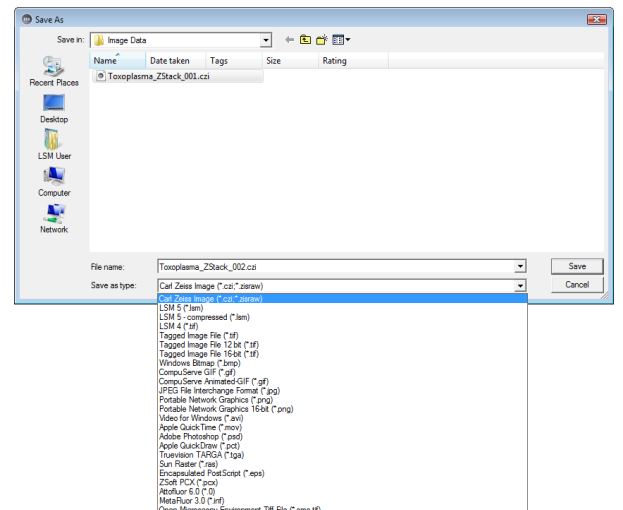

Fig. 59　　**Save as window**

To export image display data, a single optical section in raw data format or the contents of the image display window including analysis and overlays, choose **Export** from the **File** menu. In the **Export** window you can select from a number of options and proceed to the WINDOWS **Save As** window to save the exported data to disk.
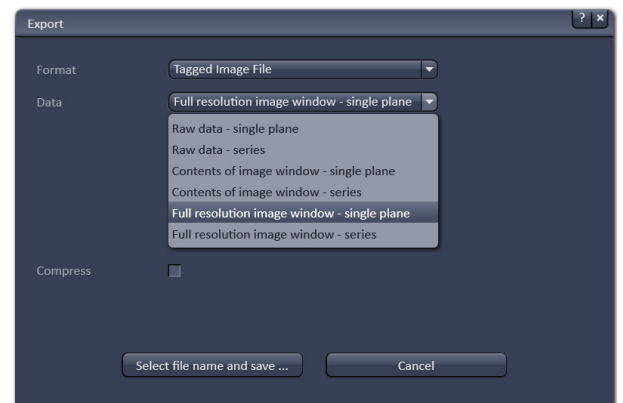

Fig. 60　　**Export window**