

**April 2014**

**ZEN 2014 for Lightsheet**

**Lightsheet Z.1**  
Operating Manual

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

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# CHAPTER 1    **HARDWARE**

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## 1 Maintenance and Cleaning

The maintenance to be carried out by the customer is limited to cleaning the painted surfaces, replacing glass windows and rubber seals on the sample chamber, cleaning and disinfecting the system cavity and sample chamber, temperature sensors and detection optics unit, replenishment of coolants for the incubation device and liquid cooling of the detection module as necessary, as well as the replacement of CO<sub>2</sub> sterile filters for the incubation device.

a) To clean painted surfaces proceed as follows:

- Switch the device off completely and pull the mains plug.
- Make sure that no cleaning fluid is allowed to enter the system.
- Wipe the painted surfaces with a clean cloth moistened with water to which a small amount of cleaning agent has been added. Do not use a solvent. Dry off with a lint-free cloth.

b) To clean and disinfect the system cavity proceed as follows:

- Switch the device off completely and pull the mains plug.
- Make sure that no cleaning fluid is allowed to enter the system.
- Take care not to touch the front lens of the illumination and detection optics units. Remove these optics as necessary.
- Remove the sample chamber for cleaning.
- Cleaning the system cavity:
  - Wipe the inner surfaces of the system cavity with a clean cloth moistened with water to which a small amount of cleaning agent has been added. Do not use a solvent. Dry off with a lint-free cloth.
- Disinfecting the system cavity:

Carefully wipe the inner surfaces of the sample chamber with a lint-free cloth soaked in 70% ethanol or 70% isopropyl. Allow the solution to react for a few seconds. Repeat the procedure once or twice as necessary. Carefully wipe off the inner surfaces with a lint-free cloth soaked in distilled water.

c) To clean and disinfect the sample chamber proceed as follows:

- Remove the sample chamber and dismantle it into individual parts. Different procedures are required for cleaning and disinfecting the various parts of the sample chamber.
  - The cover glasses must be properly disposed of in the glass waste.
  - The steel parts of the chamber must be cleaned and disinfected as follows:
    - Rinse the parts with distilled water.
    - Lay the parts in an ultrasonic bath containing distilled water to which a few drops of detergent have been added.
    - Then rinse the steel parts thoroughly with distilled water until all detergent residue has been removed.
    - The parts may be laid in a disinfecting bath of 70 % ethanol (>1 hour) and then thoroughly rinsed with distilled water.
    - The steel parts of the sample chamber may also be autoclaved as necessary.
  - Sealing rings, hose connectors and blank plugs must be cleaned and disinfected as follows:
    - These parts may not be autoclaved.
    - First of all lay these parts in distilled water and then in a 70 % ethanol solution for max. one hour. Rinse them thoroughly with distilled water.

d) To clean and disinfect the temperature sensor proceed as follows:

- Remove the temperature sensor from the sample chamber and loosen the connections to the main system module Lightsheet Z.1.
- Once removed, thoroughly rinse the temperature sensor with distilled water and then suspend it for one hour in a 70% ethanol solution. Make sure that the contact plug remains dry. Complete the cleaning process by rinsing thoroughly with distilled water.

e) To clean and disinfect the detection optics unit proceed as follows:

- Make sure that no cleaning fluid is allowed to enter the system.
- To avoid scratches, do not wipe the front lens of the optics with dry lens paper and lens cloths.
- Remove these optics from the system cavity as necessary.
- Clean the front lens of optical elements according to general recommendations (see "The clean microscope" [www.zeiss.com/microscopy](http://www.zeiss.com/microscopy)) using an optical cleaning solution (Carl Zeiss optical cleaning solution: 85 % n - hexan, 15 % isopropanol. This solution is not sold by Carl Zeiss Microscopy GmbH) and a suitable lens cleaning paper or high purity cotton wool.
- In addition to the cleaning procedure described above, for special sterility requirements the front lens area of the detection modules can be cleaned several times with a lint-free lens cloth or lens paper soaked in 100% ethanol. Here again, observe the general notes on cleaning the optics ("The clean microscope").

## 1.1 Maintenance of the Liquid Cooling System

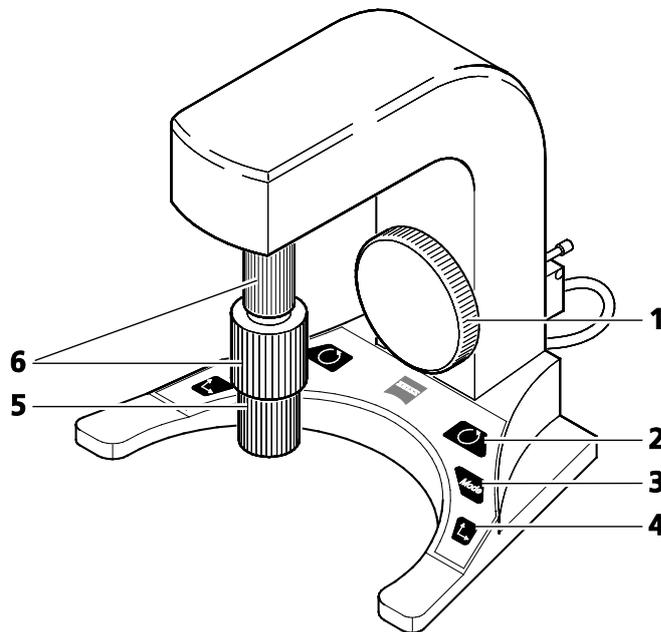
If detection modules in your Lightsheet Z.1 are connected to a liquid cooling system, consider that the pH value of the cooling liquid changes over time.

To prevent corrosion in the cooler of the detection module, the pH value of the cooling liquid must be checked at least once a year and the liquid replaced as necessary. Observe the directions of the cooling system manufacturer in the supplied operating manual.

If you have signed a service agreement with Carl Zeiss, our service staff will perform the check as part of the maintenance procedure.

The safety data sheet for the cooling liquid should also be observed.

## 2 ErgoDrive Operating Panel



- 1 Z-axis control
- 2 Rotation button (switches 6 to rotation drive)
- 3 Mode button (fine / coarse)
- 4 Axis button (switches 6 to y-axis drive)
- 5 X-axis control
- 6 Y-axis / Rotation control

**Fig. 1** ErgoDrive operating panel

With the ErgoDrive operating panel the movement of the sample holder and therefore the sample can be controlled. It is the manual equivalent of sliders in **the Specimen Navigator tool** in the ZEN software (Fig. 2), with which the movement of the specimen can be controlled as well.

The function to rotate around the center of the image, **Rotate around Center of image** (Fig. 2) is only available through the software interface. Even if chosen there, the rotation controlled by the ErgoDrive operating panel will always rotate around the axis of the motor drive.

The steering elements are two vertical rotary controls for the x, y and rotation movement. The large rotary wheel controls the z movement (Fig. 1).

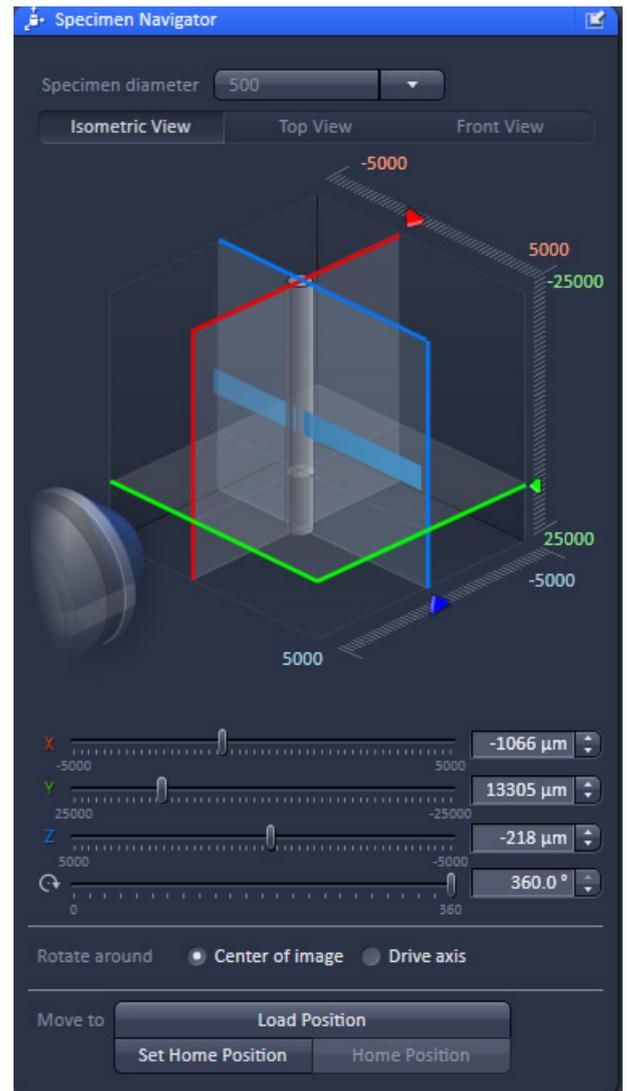


Fig. 2 ZEN software, Specimen Navigator tool window

The following control elements are part of the ErgoDrive operation panel:

**Buttons:**

The **Mode** button (Fig. 1/3) changes the speed of the drive from coarse to fine and back.

The **Rotation** button (Fig. 1/2) changes the upper rotary control to move the rotation drive (Fig. 1/6).

The **Axis** button (Fig. 1/4) changes the upper rotary control to move the y drive (Fig. 1/6).

**Rotation control:**

Upper rotary control (Fig. 1/6), after pressing the **Rotation** button (Fig. 1/2):

Clockwise: clockwise rotation of the sample; the angle is reduced in the software interface

Counter-clockwise: counter-clockwise rotation of the sample, the angle is increased in the software interface

**Y axis control:**

Upper rotary control (Fig. 1/6), after pressing **Axis** button (Fig. 1/4):

Clockwise: upward movement of the sample

Counter-clockwise: downward movement of the sample

**X axis control:**

Lower rotary control (Fig. 1/5):

Clockwise: right movement of the sample

Counter-clockwise: left movement of the sample

**Z axis control:**

Large rotary wheel (Fig. 1/1):

Clockwise: sample movement towards the detection optic

Counter-clockwise: sample movement away from the detection optic

### 3 User Interfaces



The ports of the detection modules, upper and lower system cavity doors and the upper system door are equipped with a safety interlock system to guarantee laser safety. These locking devices must not be manipulated. Other interfaces not described here are reserved for service and may only be used by authorized Carl Zeiss service personnel. The following devices may be mounted and dismantled by, or are accessible to, the user:

- Detection modules
- Sample chamber
- Sample Holder
- Incubation components
- Illumination optics
- Detection optics
- Reflector turret.

#### Customized Sample Chamber

The sample chamber delivered with the Lightsheet Z.1 can be used with a multitude of media (e.g. PBS, cell culture medium, artificial sea water) and is carefully designed to produce optimal results with the Lightsheet Z.1 system. However, if applications demand changes to the original sample chamber, Carl Zeiss Microscopy GmbH provides the CAD files and the corresponding technical drawing of our sample chamber for your convenience, in accordance with the following disclaimer:

*Carl Zeiss Microscopy GmbH (hereinafter “we”) hereby informs you that we will warrant the specified and agreed performance of the Lightsheet Z.1 system only if sample chambers are applied and used that either are delivered or explicitly approved by us.*

*The sample chamber design has been optimized to ensure the most established applications of Light Sheet Fluorescence Microscopy. Exceptional applications may require a slightly modified sample chamber design. In order to enable customized modifications of the existing sample chamber we also provide the corresponding CAD file and a technical drawing. We explicitly advise you that already minor deviations of the dimensions and tolerances specified in these documents will cause a significant loss of image quality and can potentially result in a liquid leakage. Therefore, you will not hold us or one of our affiliates liable for any damages caused by the employment of self-built or third-party-built sample chambers, the use of such self-built or third-party-built sample chambers will be solely on your own risk. Furthermore we want to inform you, that we will not render any assistance relating to the production and application of such self-built or third-party-built sample chambers.*

### 3.1 Installation and Deinstallation of the Detection Modules



A cooling liquid defined as a hazardous substance is used in the Lightsheet Z.1 to cool the detection modules (depending on configuration). The supplied safety data sheet with notes on hazards and safety measures must be observed when handling the cooling liquid.

The ports of the detection modules, reflection (Cam1) (Fig. 3/1) and transmission (Cam2) (Fig. 3/2), on the main system module Lightsheet Z.1 are equipped with a hardware interlock device. The contact rings (Fig. 3/9) are connected to the detection modules (Fig. 3/8) and adjusted in the factory. When handling the detection modules, ensure that neither tilting nor rotation forces are exerted on this connection to avoid misalignment. The detection module should be gripped as close as possible to the contact ring.

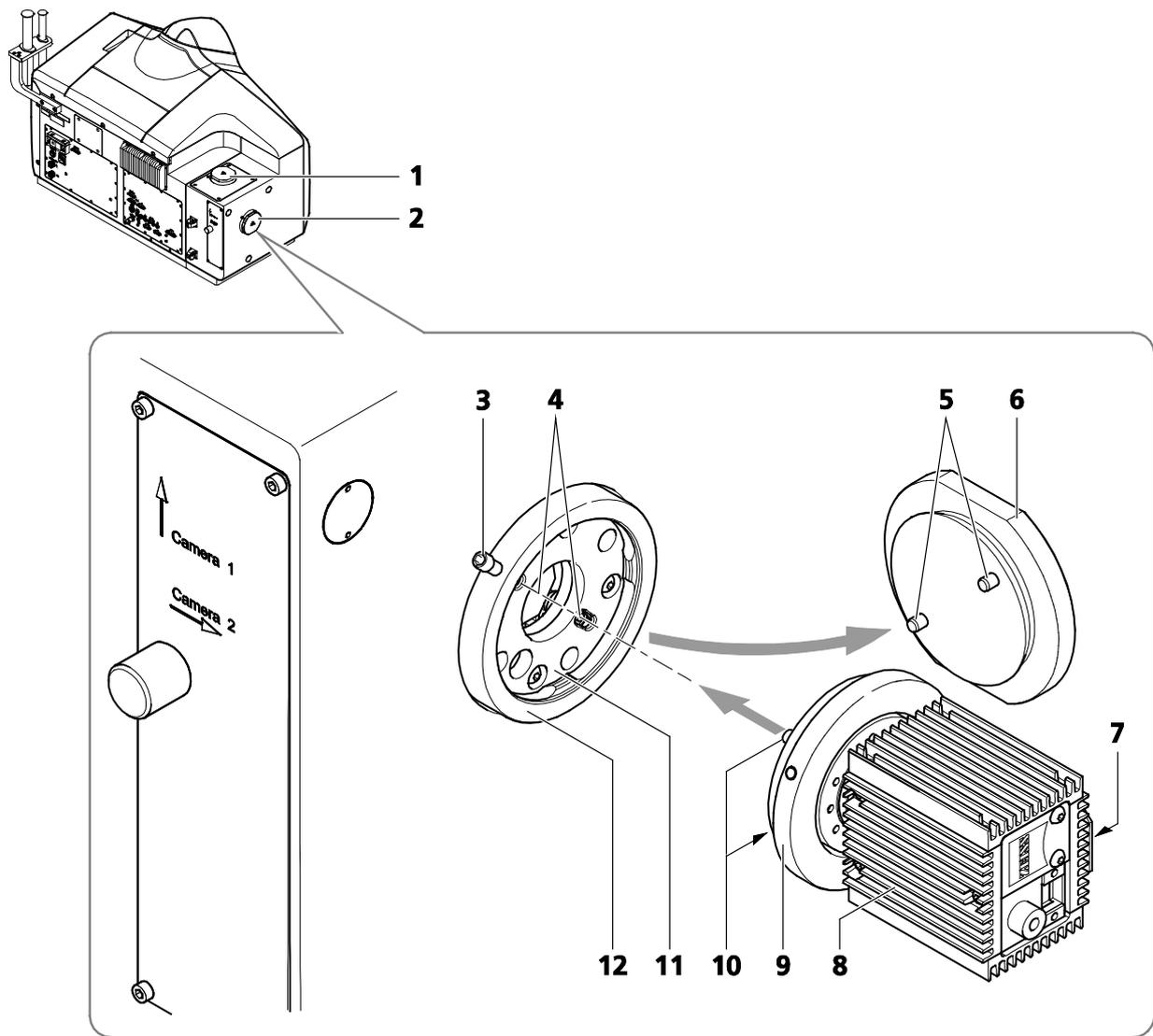
A rotation guard plate (Fig. 3/7) is mounted on the contact ring of each detection module. When mounting on both ports this must point toward the front side of the Lightsheet Z.1. It prevents rotation of the detection module and carries the laser protection notice.



Never remove the rotation guard plates of the detection module adapters. Do not modify or manipulate the detection module adapters.

The lock is functioning when the sensors (Fig. 3/4) of the sensor disk (Fig. 3/11) are pressed down by the pins of the contact ring (Fig. 3/10 or 5 for blind cap). If this is not the case, e.g. the space between the two devices is too large, the laser is blocked and the system cannot be used.

 If the system does not function after a detection module has been attached to or removed from a port with a safety interlock, check the connection of the contact ring on the respective detection module or blind cap (Fig. 3/9 or 6) to the sensor ring.



- 1 Detector port 1 (reflection, Cam1)
- 2 Detector port 2 (transmission, Cam2)
- 3 Securing screw
- 4 Sensors (2x) in sensor ring
- 5 Contact pins (2x) on blind cap
- 6 Blind cap
- 7 Rotation guard plate with laser protection notice
- 8 Detection module (varies)
- 9 Contact ring of the detection module
- 10 Contact pins (2x) on contact ring
- 11 Sensor disk
- 12 Sensor ring

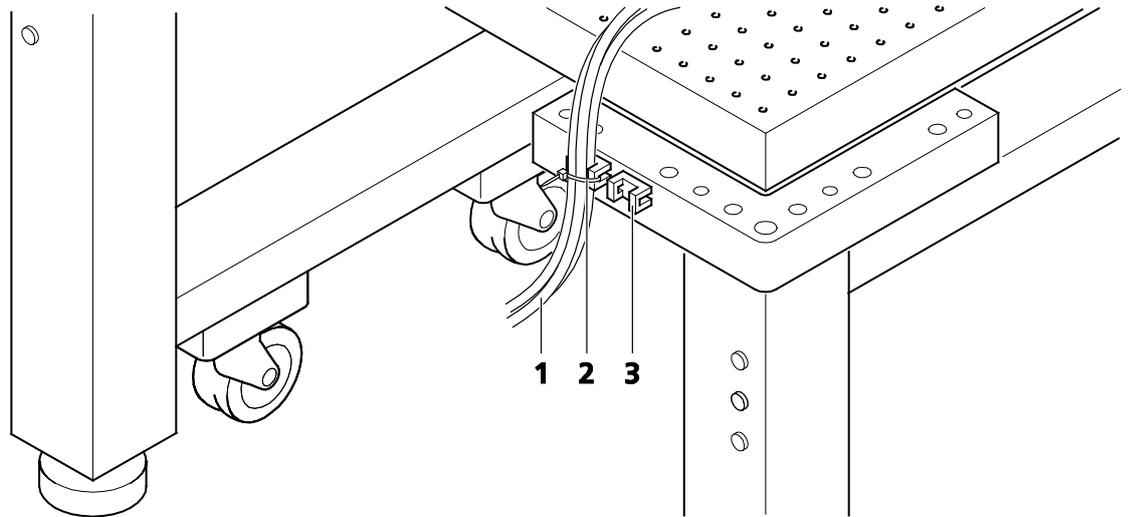
**Fig. 3 Mounting and dismounting the detection modules**

The exchange of the detection modules on your Lightsheet Z.1 can be divided into two parts. One deals with the actual mounting and dismounting of the detection modules and their connections to the system. The second part deals with the registration and alignment of the detection module using specific software tools.

-  The Lightsheet Z.1 must be **completely turned off** before changing detection modules.
-  No sample, capillary or similar object must be in the beam path while the alignment and calibration tasks are performed.

### 1) Dismounting and Mounting of Detection Modules

- Disconnect all cables from the detection module.
- The connection of the liquid cooling can stay in place, but the power plug for the liquid cooling unit must be pulled when the detection module is not in use.
- Now disconnect all cables from the Lightsheet Z.1 system and the PC for system control. Even if the detection modules are regularly switched no cable should remain on the system.
- Hold the detection module firmly in one hand.
- Loosen the securing screw with an Allen wrench (Fig. 3/3) on the sensor ring (Fig. 3/12) of the detector port (Fig. 3/1 and 2).
- Carefully pull away the detection module (Fig. 3/8) with contact ring (Fig. 3/9) using a slight tilting motion if necessary.
- Cover the detector port (Fig. 3/1 and 2) on the main system module Lightsheet Z.1 and on the detection module with the caps provided (Fig. 3/6).
- For mounting the new detection module, proceed in the reverse order. Tighten the securing screw (Fig. 3/3) on the sensor ring (Fig. 3/12) of the detector port (Fig. 3/1 and 2) without applying force.
- With the detection module "PCO.Edge," note that part of the optics projects beyond the contact ring. To protect it from damage and fingerprints, first push the optics into the port and then connect the contact ring to the sensor ring.
- Connect all necessary cables to the detection module, the Lightsheet Z.1 and the PC for system control (see sections **3.2.4 Cable Connections for the Detection Module "PCO.Edge"** and **3.2.5 Cable Connections for the Detection Module "Standard"**).
- Use the cable holder (Fig. 3/3) on the system table to connect the cables (Fig. 3/1) of the detection modules without tension.



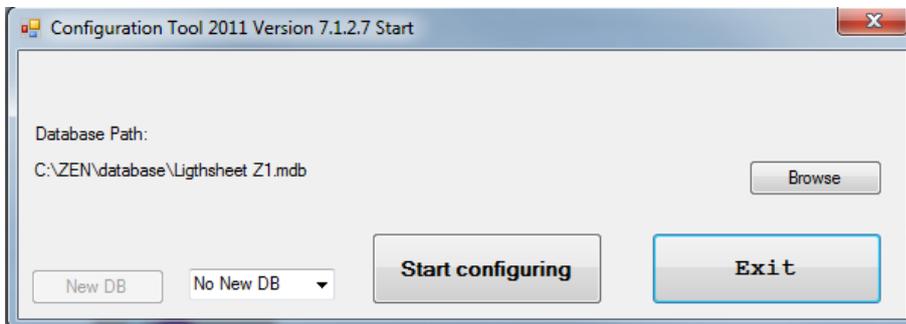
- 1 Cable (e. g. of the detection modules)
- 2 Cable tie
- 3 Cable holder on system table

**Fig. 4 Cable strain relief**

- Turn on the system (see **CHAPTER 4, SYSTEM OPERATION**).

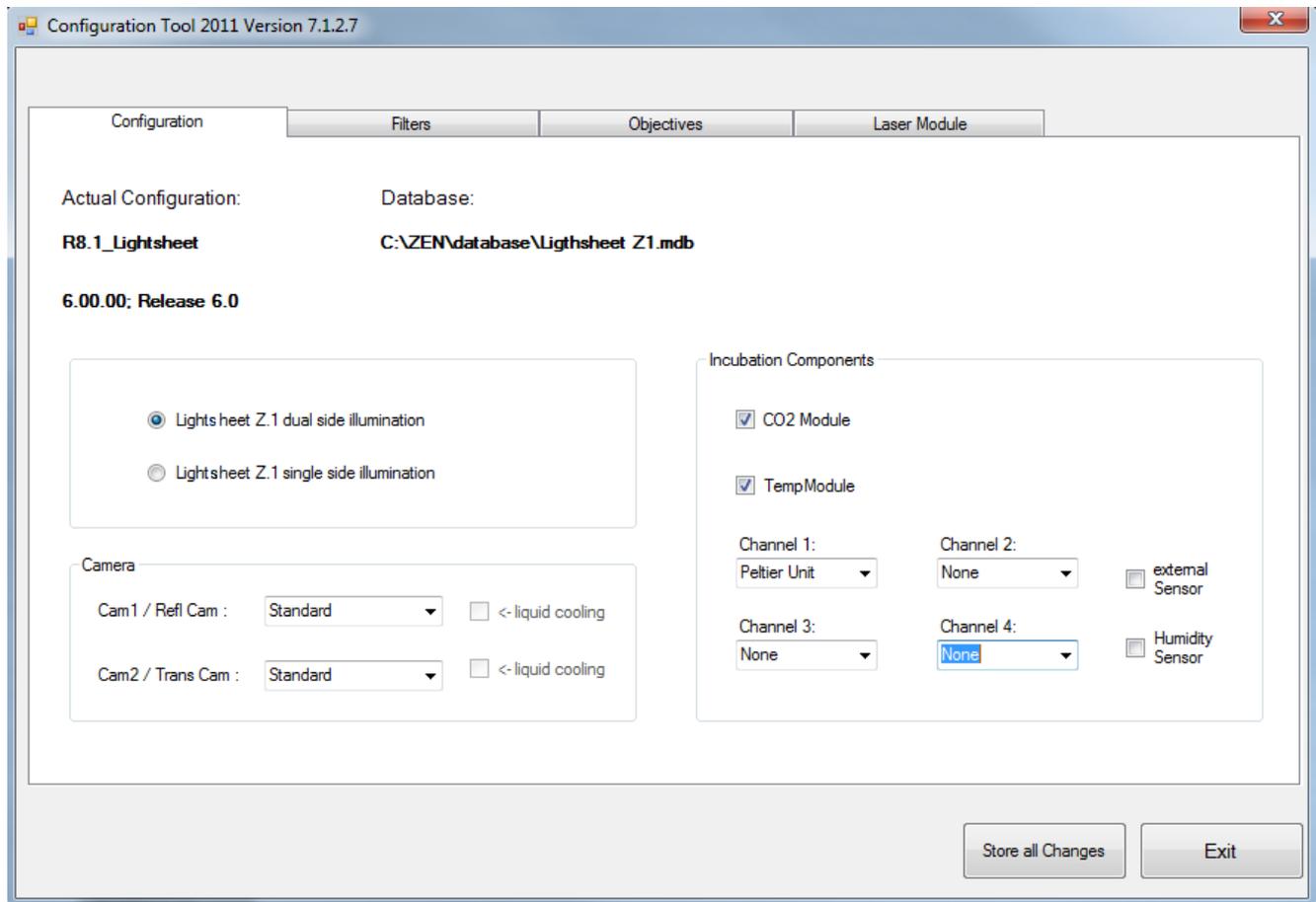
2) Registration and Alignment of the detection modules

- After the Lightsheet Z.1 and the PC for system control are turned on and the operation system has booted, start the software ZEN Configuration Tool . This can be found either as a shortcut on your desktop or in this directory: C:\ZEN\HWT as Configuration Tool 2012.exe.
- In the opening window press the Start configuring button (Fig. 5).



**Fig. 5 ZEN Configuration Tool Start-up window**

- On the **Configuration** tab (Fig. 6) you will find a **Camera** field with a drop – down menu for both detector ports (Cam1, Cam2). Select the detection module(s) you are planning to use. When you use the PCO.Edge, the checkbox liquid cooling will be marked automatically.



**Fig. 6 ZEN Configuration Tool, Configuration tab**

- Press the **Store all Changes** button and then exit the Configuration Tool. This will save the changes you have made into the database of your system.

 If you plan to exchange detection modules on a regular basis, it can be helpful to create two databases, one for each detection module type. To do so, copy the original database with a different name (you find it in the directory: C:\ZEN\database) and modify it with the Configuration Tool accordingly. Make sure to always keep the original database. Name both databases, to easily identify them. When ZEN is started open the **Boot Status** with the black arrow and then the **Hardware configuration database** the same way (Fig. 7). Here you can choose which database to use. The **Recent...** button shows all databases that have been used lately. The **Choose...** button opens a window explorer window which allows one to search for a database to use.

- Now start the ZEN (black edition) software and perform the following tasks found in the **Maintain** tab:
  - Detector recognition
  - Automatic Detector Alignment; and if necessary
  - Manual Detector Alignment.
- It might be necessary to adjust the focus of the calibration grating (Fig. 8) for the **Automatic** or **Manual Detector Alignment task**. Start one of these tasks to get a live image of the grating for evaluating and adjusting the focus if applicable (see section **3.2.2 Adjustment – Manual Detector Alignment**).



**Fig. 7** ZEN Start-up window

## 3.2 Adjustment – Detector Recognition

When the detection modules are exchanged or attached to the Lightsheet Z.1 system for the first time, the system must identify which detection module is present for which channel. Pressing the button **Detector recognition** will start an identification routine to do this job. A progress bar is visible during the process. When the **Cancel** button is pressed, the operation is aborted without saving any result. This routine must be conducted before the **Automatic** or **Manual Detector Alignment**. At the end of this process you be informed if the detector recognition had to perform any changes. If so, please restart the ZEN software before continuing.

### 3.2.1 Adjustment – Automatic Detector Alignment

This adjustment is necessary for one or two detection modules, when:

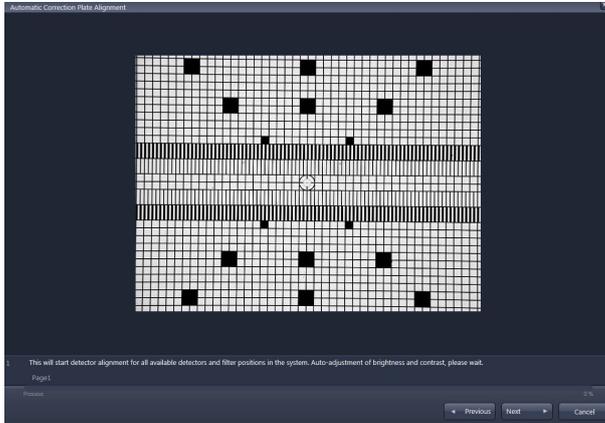
- detection modules have been exchanged
- reflector turrets have been removed and inserted (restart ZEN before the alignment task)
- recognition of a pixel shift between channels
- the zoom is not centered.

No sample should be present. The necessary correction plate and grating is present for each channel within the system and needs no additional activation.

The Automatic Detector Alignment will align the image center with the detection module center (zoom center). For two detection modules, it will additionally align both to have overlapping pixels.

 After detection module exchange, first press the **Detector recognition** button in the **Adjustment tool** window.

After pressing the **Automatic Detector Alignment** button, a wizard window (Fig. 8) will open that guides you through the process. Press the **Next** button after each step to continue or the **Previous** button to move one step back. If you press **Cancel** you will abort the process without saving any results.



**Fig. 8** Automatic Detector Alignment, first step

The first step (Fig. 8) automatically positions the necessary grating into the light path and adjusts brightness and contrast. A progress bar is present in the lower part of the window. You can follow the process in the displayed live image. You find the following text below the live image:

“This will start detector alignment for all available detectors and filter positions in the system. Auto-adjustment of brightness and contrast, please wait.”

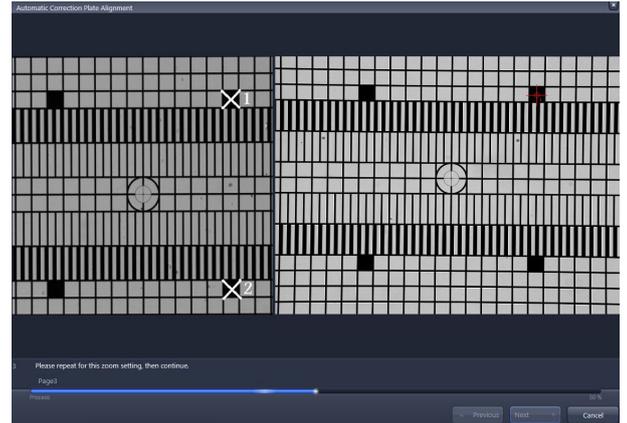
☞ If the live image of the grating shows the grid pattern out-of-focus, cancel the Automatic Camera Alignment wizard and first manually adjust the focus of the grating. The necessary steps are described in section **3.2.3 Adjust the Grating Focus for the Automatic or Manual Detector Alignment Tool.**

In Step 2 the movement of the correction plates is calibrated for both channels. There is no interaction needed. Press the **Next** button to continue after the task is done. The following text is displayed: *“Calibration of correction plate movement, please wait.”*

In Step 3 the zoom center is determined. A graphic of the grating is displayed at the left hand side of the window (Fig. 9). Two squares are labeled with white crosses in this image. On the right hand side, a live image of the grating is shown. Now mark the same squares as shown in the graphic in the live image by simply clicking on them with the left mouse button. This will be repeated for a second zoom step. The following text will be shown: *“Determining the detector center. Please click on the two squares in the right live image of the grid that are shown with the white cross within the left image display. Repeat for both zoom settings, then continue.”*

Step 4 is the final step in which the alignment for all filters and detection module channels is performed. A progress bar is present at the bottom of the window. When the procedure is finished the **Finish** button becomes available, press it to save all adjustments and leave the wizard. This text is displayed: *“Detector Alignment for all available filters in progress, please wait.”*

If you still encounter pixel shifts between the two channels after the Automatic Camera Alignment tool, you can fine tune settings using the Manual Detector Alignment tool, see section **3.2.3 Adjust the Grating Focus for the Automatic or Manual Detector Alignment Tool.**



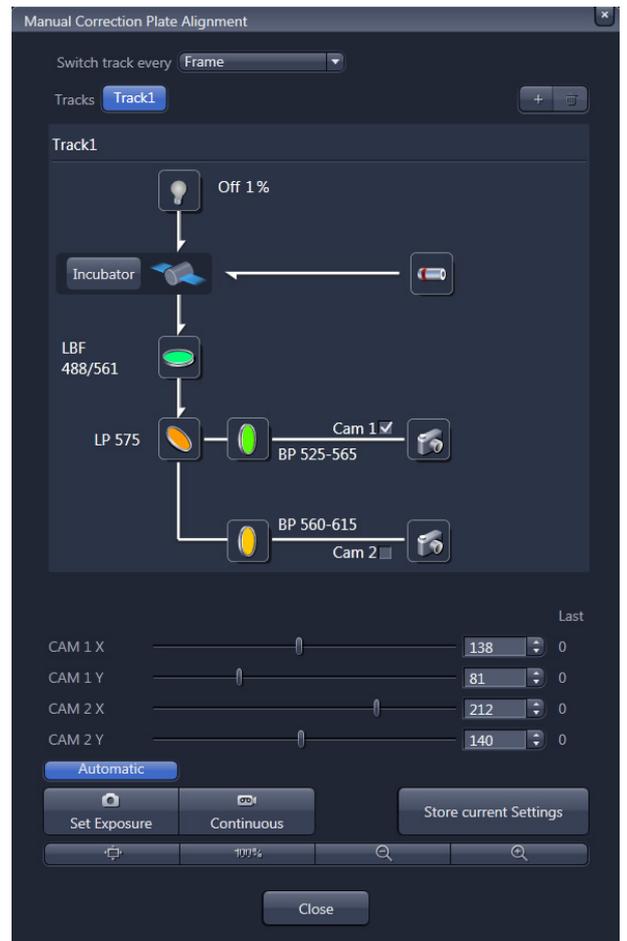
**Fig. 9 Automatic Detector Alignment, third step**

### 3.2.2 Adjustment – Manual Detector Alignment

This adjustment is necessary for one or two detection modules when you recognize a pixel shift between channels, or when the zoom is not centered.

 The **Automatic Detector Alignment** should always be performed first since it might already solve the problems. Furthermore it defines the settings for the combination Channel 1 (Cam1) and position 1 of the Emission Selection Filter, which should be used as the reference during the Manual Detector Alignment.

 The **Zoom** in the **Acquisition tool window** is fixed to 1 while performing the Manual Detector Alignment.



**Fig. 10 Manual Detector Alignment window**

No sample should be present when performing this task. When pressing the **Manual Detector Alignment** button, a window will open (Fig. 10). A grating is automatically inserted into the light path and a live image of it is shown in a new image container within the ZEN software. The **Set Exposure** button is activated automatically to adjust brightness. All components of the light path can be controlled in the **Manual Detector Alignment** window. The light source is the LED white light source to ensure the visibility of the grating independent of the filters in the light path. All other controls for adjusting imaging settings should be controlled in the tool windows of the main software.

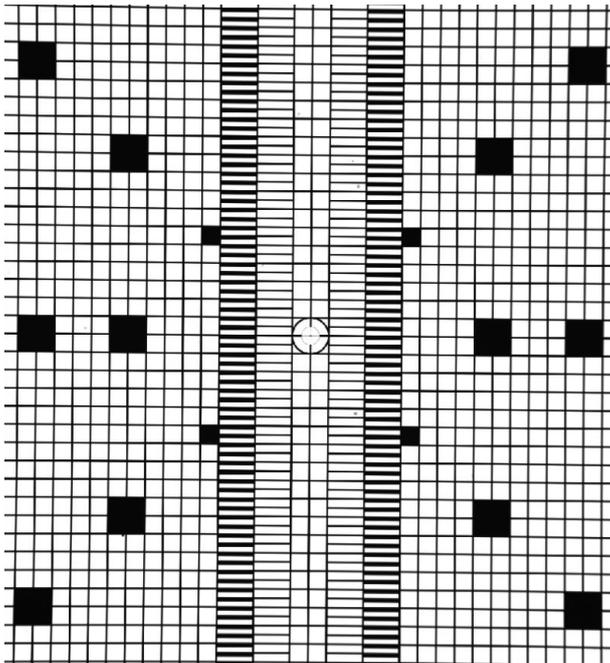


Fig. 11 Manual Detector Alignment,  
Grating at Zoom 1

The activated (blue) **Automatic** button loads the settings which have been previously defined with the Automatic Detector Alignment for each light path.

After pressing the **Continuous** button in the **Manual Detector Alignment** window or in the **Main tool tabs** a new image will open with a live image of the grating (Fig. 11)

☞ When the grating is out of focus, you need to refocus it for the relevant channel by performing the steps described in section **3.2.3 Adjust the Grating Focus for the Automatic or Manual Detector Alignment Tool**. To check the focus, zoom into the image using the **Zoom** function and look at the center cross within the square (Fig. 12). If the white portions within the inner most circle are recognizable, the grating is in focus.

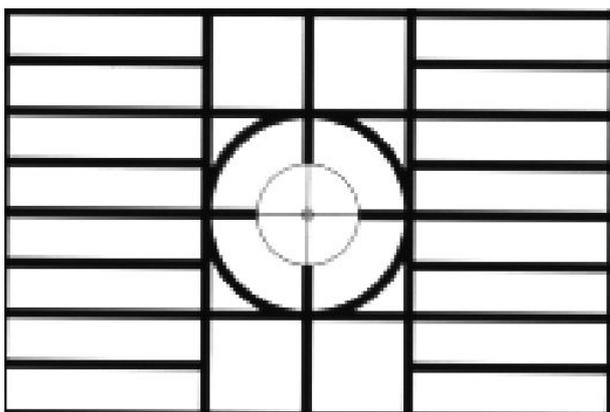


Fig. 12 Manual Detector Alignment,  
Grating, center part zoomed in

To zoom into the image you can use these buttons:



As a reference, the combination of Emission Selection filter position 1 and Cam 1 should be taken (see above). This combination should be the first live image. Here mark a structure close to the middle of the circled cross using the **Graphics View control**. This cross will be the reference for the following steps.

Now change the light path to the combination of Emission Selection filter and Channel (Cam 1 or Cam 2) that you wish to adjust. The live image will now show the grating with the current settings.

Use the sliders or the input box with arrows of **Cam 1 X** and **Cam 1 Y** (for Channel 1, Cam1) or **Cam 2 X** and **Cam 2 Y** (for Channel 2, Cam 2) to move the previously chosen structure of the grating to overlay with the cross. Finish this step by pressing the **Store Current Settings** button.

Repeat the procedure for all desired Emission Selection filters and channels, always using the cross marker as the reference.

It is helpful, especially after an **Automatic Detector Alignment** has been performed, to use the **Profile View** tab in order to evaluate if the image of Cam 1 and Cam 2 overlay. While the grating is continuously imaged, press the Profile View tab and draw a line or an arrow poly-line on the grating (Fig. 13). Use the sliders or the input box with arrows of **Cam 1 X** and **Cam 1 Y** (for Channel 1, Cam1) or **Cam 2 X** and **Cam 2 Y** (for Channel 2, Cam 2) to move the lines of the grating to overlay each other.



**Fig. 13** Manual Detector Alignment, Grating, Profile view tab

You can leave the Manual Detector Alignment by pressing the **Close** button at the bottom of the window.

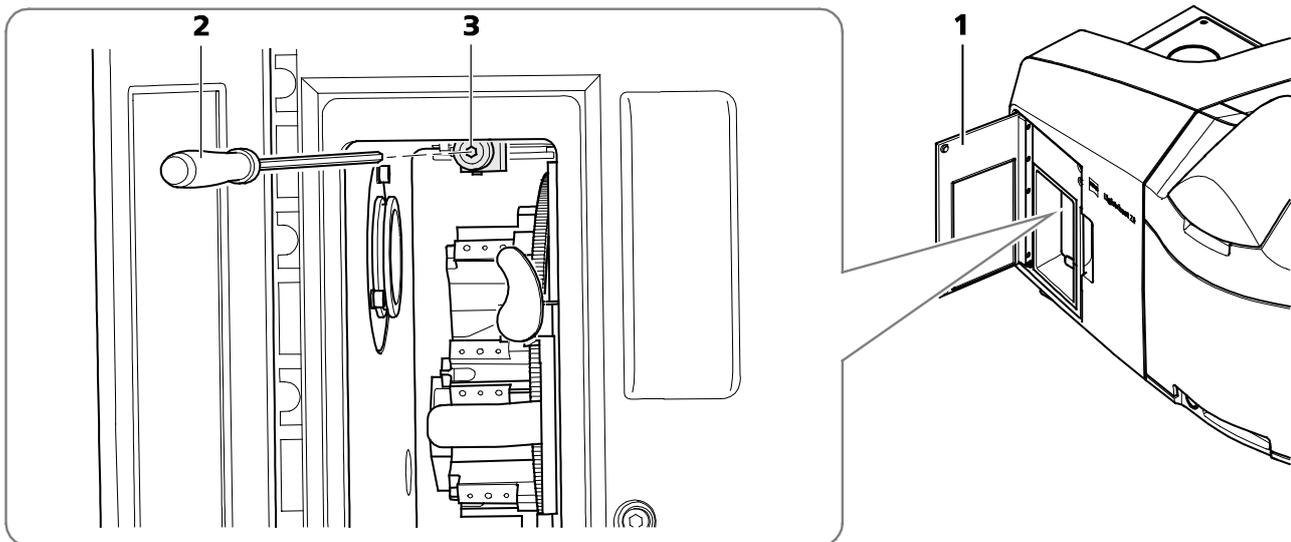
### 3.2.3 Adjust the Grating Focus for the Automatic or Manual Detector Alignment Tool

During the **Automatic Detector Alignment tool** wizard and the **Manual Detector Alignment tool** a grating is brought into the light path which can be imaged on each channel. When the grating is out-of-focus, you need to refocus it for the relevant channel. For checking the focus, it helps to zoom into the image using the **Zoom** function and look at the center cross within the square (Fig. 12) If the white portions within the inner most circle are recognizable, the grating is in focus.

To adjust the focus of the grating start the **Manual Detector Alignment** by pressing the button in the **Adjustment tool** window. The grating will be brought into the beam path and an image is generated and displayed in the image container of the main software.

### Set focus for Cam 1 (Channel 1)

- Use the **Light Path** of the **Manual Detector Alignment** to first activate channel 1 (Cam1) and press afterwards the **Continuous** button. The resulting live image shows the grating on channel 1 (Cam1).
- Use the **Zoom** tools of the Manual Detector Alignment to zoom into the center region of the image.
- With the live image running, open the **front system door** (Fig. 14/1) to the **reflector turret for emission filter selection**. This system door has no safety interlock, and opening it will not terminate image acquisition.
- Turn the screw which is above the turret (Fig. 14/3) with the long Allen wrench (Fig. 14/2).



- 1 Front system door
- 2 Allen wrench
- 3 Screw

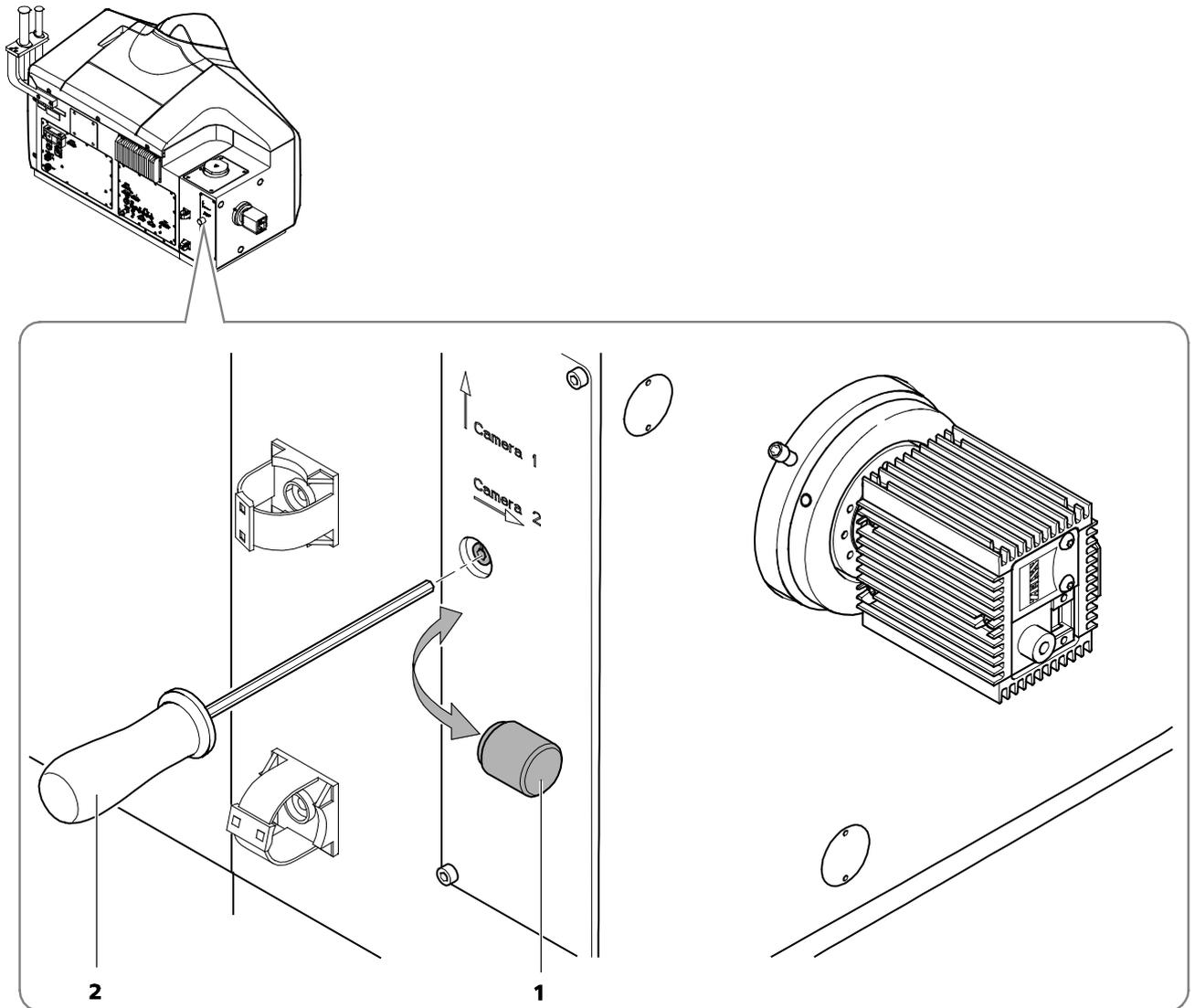
**Fig. 14** Position of the screw to adjust the focus of the grating for Cam 1 (channel 1)

- Monitor the live image of the grating while you turn the screw in any direction and find the position of the screw that results in the sharpest image.
- Stop the continuous scan and close the **front system door**.

### Set focus for Cam 2 (Channel 2)

- Use the **Light Path** of the **Manual Detector Alignment** to direct light towards channel 2 and activate channel 2 (Cam2).
- Press afterwards the **Continuous** button. The resulting live image shows the grating on channel 2 (Cam2).
- Use the **Zoom** tools for the Manual Detector Alignment to zoom into the center region of the image.
- While the imaging continues remove the black screw cap (Fig. 15/1) on the back of the main system module Lightsheet Z.1. This opening gives way to a screw that can be turned with an Allen wrench (Fig. 15/2).

- Monitor the live image of the grating while you turn the screw in any direction and find the position of the screw that results in the sharpest image.
- Stop the continuous scan and place the black screw cap back on the system.



- 1 Black screw cap
- 2 Allen wrench

**Fig. 15** Position of the screw to adjust the focus of the grating for Cam 2 (channel 2)

### 3.2.4 Cable Connections for the Detection Module "PCO.Edge"

The detection module "PCO.Edge" is equipped with a liquid cooling system. Please read the safety notes of the Lightsheet Z.1 system, as well as the separate safety data sheet for the liquid cooling system. The power supply unit (PSU) for the cooling is connected to the system's power strip at the rear of the laser module. The cooling hoses should be as short as possible. The connection creates a closed circuit, starting from the cooling unit leading to the detection module 1 (approx. 200 cm hose length) reflection port (Cam1). From the latter a hose (approx. 200 cm) leads either back to the cooling unit or, if a second detection module is used, first to the transmission port (Cam2) (approx. 100 cm) and then a third hose (approx. 200 cm) leads back to the cooling unit.

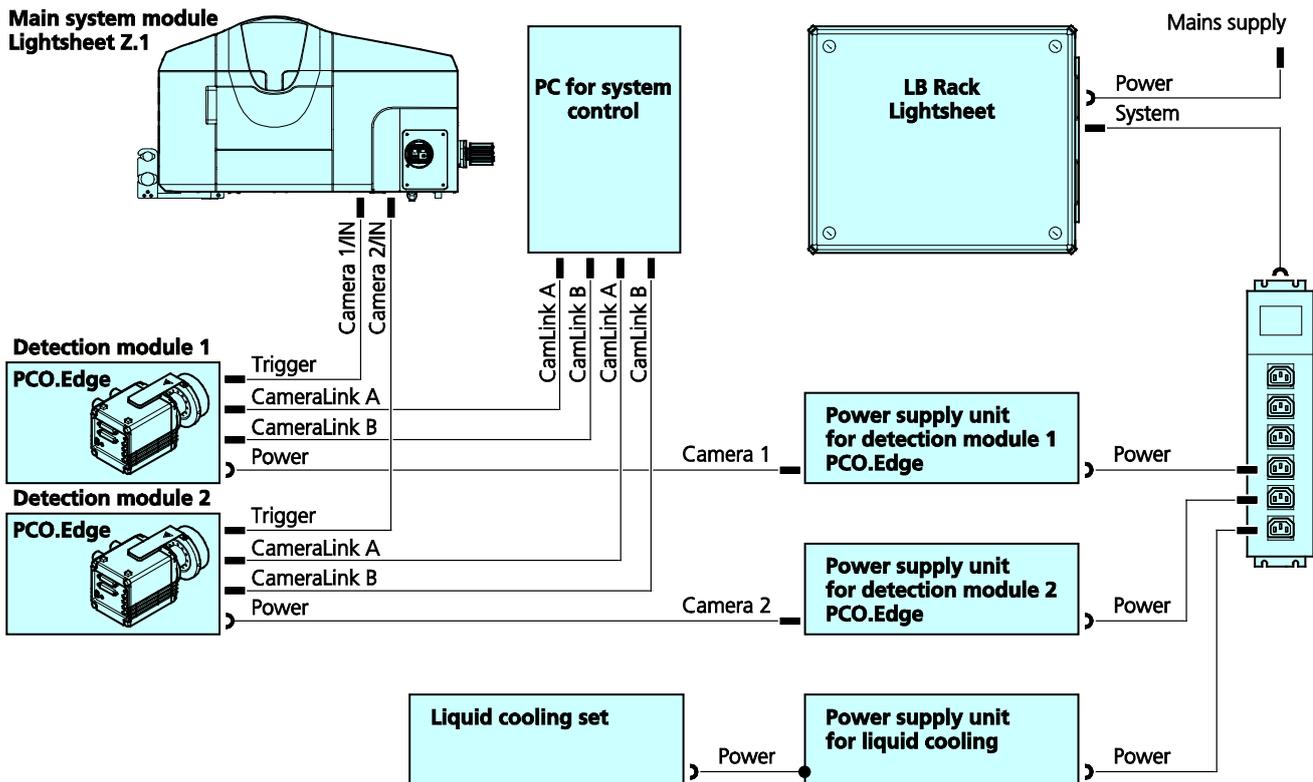


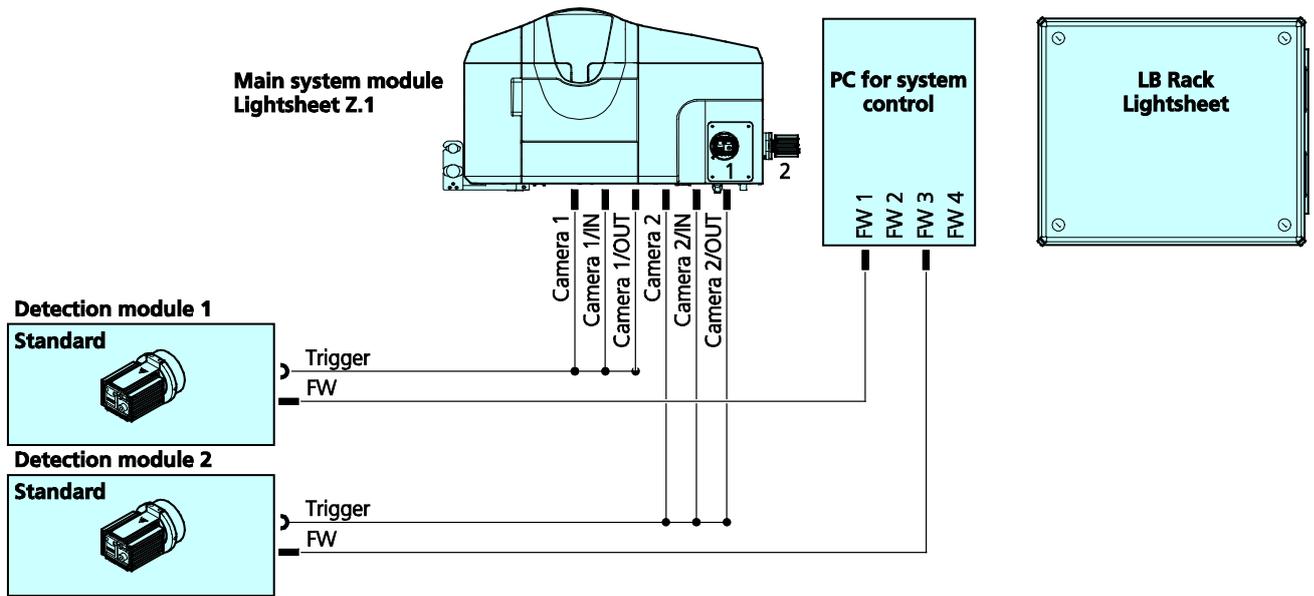
Fig. 16 Cable connections for the detection module "PCO.Edge"

The detection module cables are connected as follows:

- CameraLink cables A and B are each plugged into the PC for system control. The upper slots of the PC for system control are assigned to the detection module of the reflection port (Cam1), [CamLink A] right and [CamLink B] left. The lower slots are assigned to the transmission port (Cam2), [CamLink A] right and [CamLink B] left.
- The power cable is connected to the power strip of the system on the rear of the LB Rack Lightsheet.
- The trigger cable is always connected to the detection module with [1/IN] and on the rear side of the main system module Lightsheet Z.1 to [Camera 1/IN] (reflection port, Cam1) or [Camera 2/IN] (transmission port, Cam2).
- The detection module is switched on with the toggle switch on the module itself.

**3.2.5 Cable Connections for the Detection Module "Standard"**

- The following connections exist for this detection module:
- The firewire cable must be plugged into the PC for system control. There are four firewire sockets on the PC for system control; the detection module from the reflection port (Cam1) is connected to position [FW 1], from the transmission port (Cam2) to position [FW 3].
- The trigger cable is plugged into the respective detection module and the three ends on the rear side of the main system module Lightsheet Z.1 to [Camera 1] or [Camera 2], as well as [Camera 1/IN] and [Camera 1/OUT] (reflection port, Cam1) or [Camera 2/IN] and [Camera 2/OUT] (transmission port, Cam2).



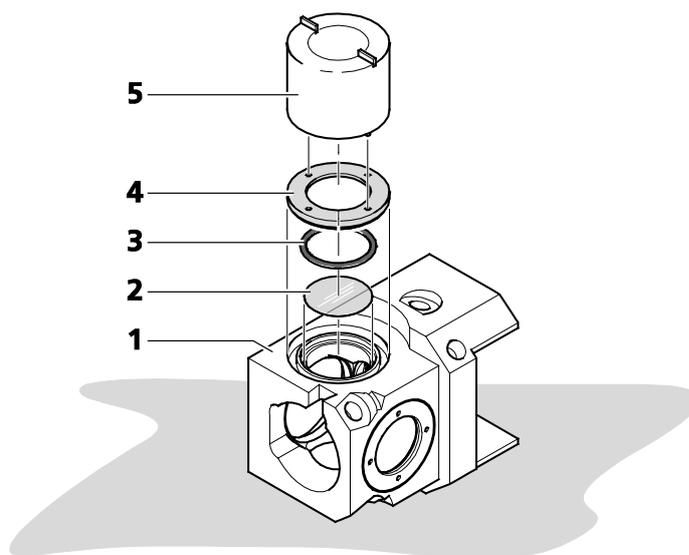
**Fig. 17 Cable connections for the detection module "Standard"**

### 3.3 Assembly of the Sample Chamber

- Note: It is crucial to assemble the sample chamber as described in this chapter. Wrong assembly might lead to a leaking chamber as well as to optical errors, affecting image quality.
- Note: the Sample Chamber and Lightsheet Z.1 system are designed for aqueous media, with a refractive index of 1.33. All other media will influence the image quality in an unpredictable manner.
- Note: the sample chamber should be filled only with aqueous media. Media based on organic solvents might evaporate within the system and can damage the optical surfaces as well as the motors of the system.
- Wear powder-free gloves to avoid fingerprints on the cover slips as well as to protect your hands from sharp edges.

#### 3.3.1 Assembly of the Sample Chamber Windows

- Assemble the three sample chamber body windows (for the **illumination optics on the sides** and the front chamber window for the **overview camera with LED white illumination**) using the following steps:



- 1 Sample chamber body
- 2 Cover slip
- 3 O-ring (18 mm O-ring, black)
- 4 Illumination adapter ring
- 5 Sample chamber window tool

**Fig. 18 Assembly of the three sample chamber windows for illumination optics and overview camera**

1. Take the sample chamber body (Fig. 18/1) and lay it onto the table so that one of the three smaller windows are facing you.

2. Using a forceps or gloved hands, remove one clean circular 18 mm cover slip; place cover slip into the empty sample chamber body window so it fits into the smallest groove (Fig. 18/2) – you will need a fine forceps.
3. Now place the O-ring (18 mm O-ring, black) into the corresponding groove (Fig. 18/3). Make sure not to disturb the positioning of the cover slip.
4. Take the silver illumination adapter ring (Fig. 18/4) and lay it into the window making sure the flat side with the four pinholes is facing you.
5. Using the sample chamber window tool (Fig. 18/5), position the circular pins into the pinholes and turn clockwise until finger-tight. Adapter should be flush with the sample chamber body.
6. Repeat step 1 - 6 for each of the three windows.

– Assemble the sample chamber window for the **detection optic**.

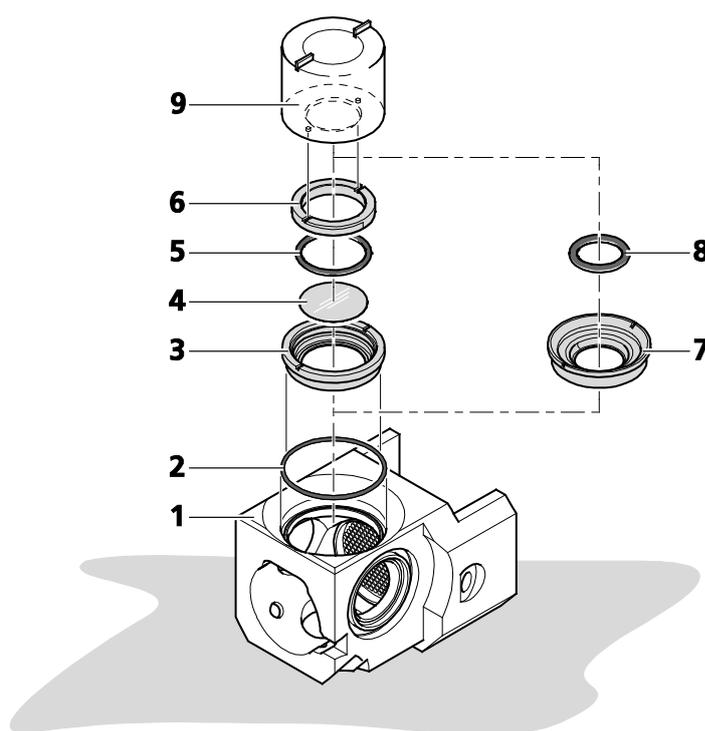
1. Turn the sample chamber body (Fig. 19/1) to the largest opening window for the detection optics.
2. Using a small spatula or the opposite end of a forceps position the blue O-ring tightly into the groove to ensure no leaking (Fig. 19/2).
3. Assemble the chamber window for the detection optic according to the following steps. Depending on the detection optics used, insert the correct detection optic adapter:

**4. For the detection optic 5x/0.16:**

- a) Place the large silver detection optic adapter 5x (Fig. 19/3) onto the table with the grooves facing you.
- b) Remove one clean circular 18 mm glass cover slip; place cover slip (Fig. 19/4) into the large silver detection optic adapter 5x (Fig. 19/3) making sure it is positioned into the smallest groove – adjust the coverslip position with the forceps if necessary.
- c) Now place the O-ring (18 mm O-ring, black) into the corresponding groove on top of the cover slip (Fig. 19/5).
- d) Take the silver retaining ring (Fig. 19/6) and place it inside the detection optics adapter 5x with the flat side with two notches facing you. Using the circular pin-side of the sample chamber window tool (Fig. 19/9), turn clockwise until finger-tight.
- e) Placing the assembled detection optic adapter 5x into the sample chamber body window. Using the squared pins of the sample chamber window tool, turn clockwise until finger-tight.

**5. For the detection optic 20x/1.0, 40x/1.0, 63x/1.0:**

- a) Use the corresponding detection optic adapter (see the detection optic adapter catalogue number and magnification engraving) (Fig. 19/7).
- b) Insert the detection optic adapter into the sample chamber body window for detection with the flat side facing you.
- c) Using the sample chamber window tool (squared pin-side) (Fig. 19/9) turn adapter clock-wise until finger-tight.
- d) Take a 15 mm black O-ring (Fig. 19/8) and position the O-ring into the groove inside the middle of the detection optic adapter using the blunt end of a forceps if necessary.



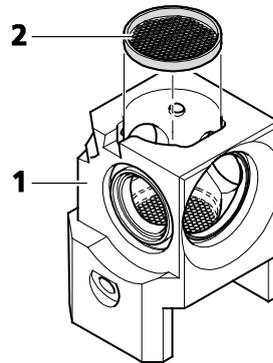
- 1 Sample chamber body
- 2 O-ring (blue)
- 3-6 **For the detection optic 5x/0.16**
  - 3 Detection optic adapter 5x/0.16
  - 4 Cover slip
  - 5 O-ring (18mm O-ring, black)
  - 6 Retaining ring
- 7-8 **For the detection optic 20x/1.0, 40x/1.0, 63x/1.0**
  - 7 Detection optic adapter (20x/1.0, 40x/1.0, 63x/1.0)
  - 8 O-ring (15mm O-ring, black)
- 9 Sample chamber window tool

**Fig. 19 Assembly of sample chamber window for the detection optic**

The following o – rings are used for the assemblage of the sample chamber:

O – Ring Description	Used for	O- Ring Reference number
O-Ring 26x1, Si840 VMQ 65° ± 5°, blue	Detection optics adapter window	5262039, C. Otto Gehrcken
O-Ring 17 x 1,5 NBR 50 Shore A, black	Cover slip, Sample Chamber window 5x detection optics	5262041, C. Otto Gehrcken
O-Ring 12x2 NBR 50 Shore A, black	20x – 63x detection optics	

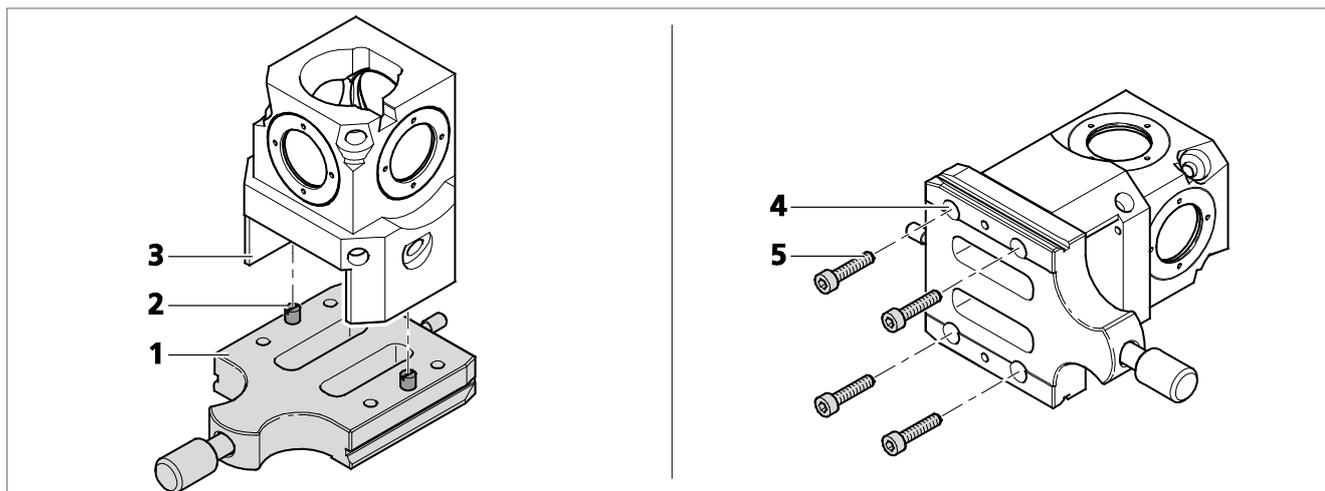
### 3.3.2 Assembly of the Sample Chamber Body and the Sample Chamber Dove Tail Slide



- 1 Mesh screen  
2 Sample chamber body

**Fig. 20** Placing the mesh screen

- Place the mesh screen (Fig. 20/2) into the *middle of the sample* chamber body (Fig. 20/1) to reduce turbulence stress on the specimen when the chamber is filled.
  - For cleaning, it can be carefully removed by a forceps or via a teflon-tip-free end of a capillary plunger.
  - If a **Heating component** is used, proceed first with chapter 3.7.1 (see PeCon manual for specific heating component) and screw it onto the bottom of sample chamber body.
- 
- The sample chamber body (Fig. 21/3) can now be attached to the sample chamber dove tail slide (Fig. 21/1).
  - Lay down the sample chamber dove tail slide with the two positioning pins facing upwards (Fig. 21/2).
  - Take the sample chamber body and position it on to the sample chamber dove tail slide with the detection optic adapter window facing to the back relative to the sample chamber dove tail slide grip.
  - Invert the sample chamber body with the sample chamber dove tail slide so that the four screw-holes (Fig. 21/4) that were on the bottom are now facing you.
  - Insert the four screws (diameter = M3, length = 14 mm) (Fig. 21/5) into the four screw-holes and tighten using an Allen wrench (2.5).

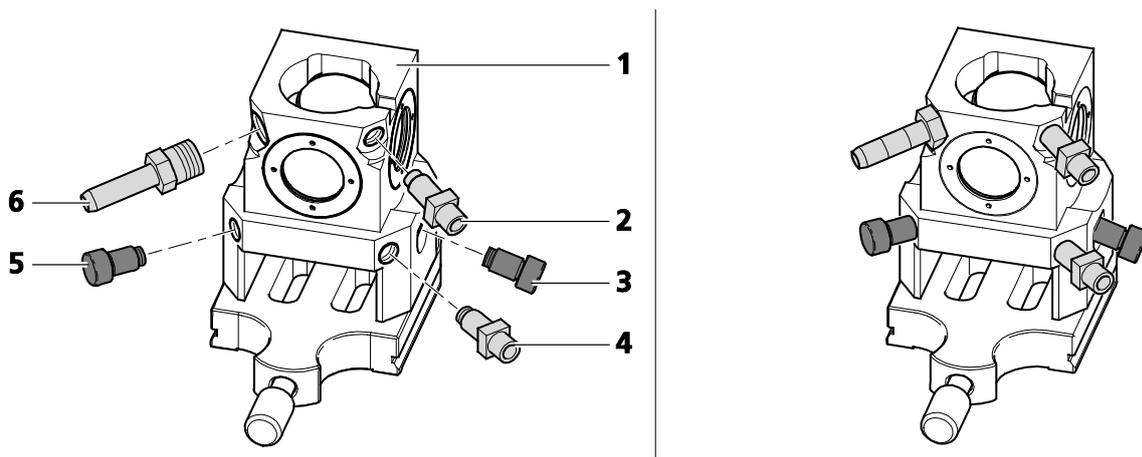


- 1 Sample chamber dove tail slide
- 2 Positioning pins
- 3 Sample chamber body
- 4 Screw-holes
- 5 Screws (diameter = M3, length = 14 mm)

**Fig. 21** Assembly of the sample chamber body and the sample chamber dove tail slide

### 3.3.3 Insertion of the Drain Connector, Luer-Lock Connectors and Blind Plugs

- Turn the assembled sample chamber body with dove tail slide so that the dove tail slide is on the table with the grip facing you (Fig. 22/1).
- Take the grey drain connector and screw this into the upper left hole of the sample chamber body until finger-tight (Fig. 22/6). This will guide excess liquid to the drain.
- Take a Luer-Lock-connector (white) and screw it into the lower right corner of the sample chamber body (Fig. 22/4). The Luer Lock connectors (white) are used to fill or perfuse the sample chamber with liquids and / or gasses.
- For CO<sub>2</sub> incubation, the Luer-Lock connector must be positioned at the upper right corner of the sample chamber (Fig. 22/2). For syringe based perfusion one can use any of the three lower screw holes. The lower right corner screw hole is recommended for convenience (Fig. 22/4).
- For perfusion pump use the right side bottom screw hole for the perfusion input and either of the front lower corner screw holes for the perfusion output.
- Close all remaining sample chamber screw hole openings by inserting the black blind plugs (Fig. 22/3; Fig. 22/5).

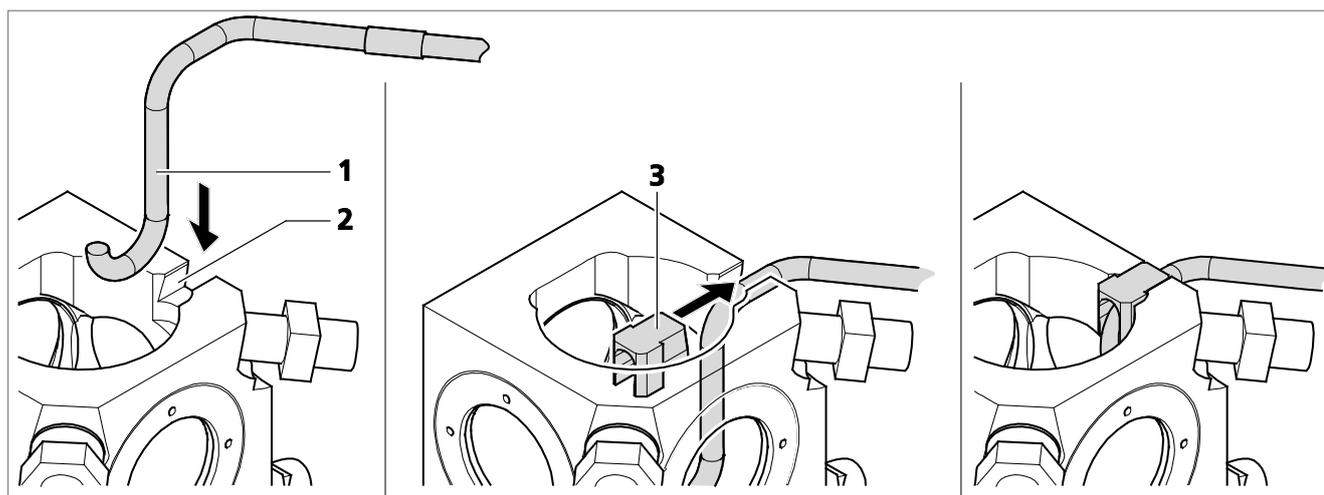


- 1 Sample chamber body with dove tail slide
- 2 Luer-Lock-connector (white); position recommend for CO<sub>2</sub> incubation
- 3 Blind plug (black)
- 4 Luer-Lock-connector (white); position recommend for syringe based perfusion
- 5 Blind plug (black)
- 6 Drain connector (grey)

**Fig. 22** Insertion of the Drain connector, Luer-Lock-connectors and Blind plugs

### 3.3.4 Insertion of Accessories for Incubation

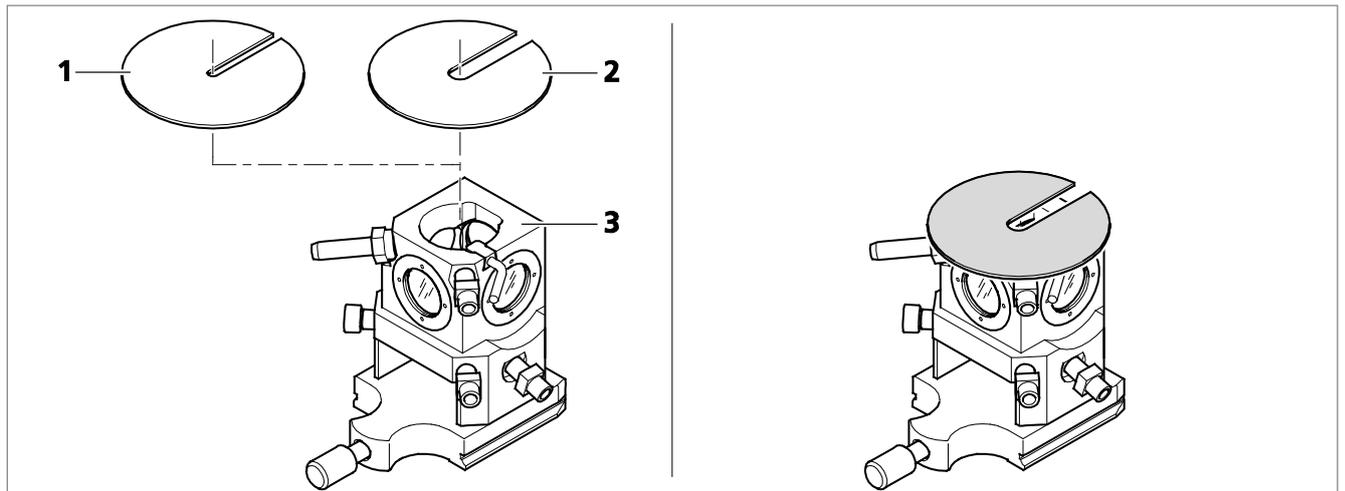
- A **temperature sensor** (Fig. 23/1) can be used in the sample chamber body.
  - Hold the temperature sensor so that it shows you the letter “J”. Slide the temperature sensor sleeve (Fig. 23/3) onto the top horizontal part of the J-shaped temperature sensor.
  - Dip the temperature sensor with sleeve into the sample chamber body and position the temperature sensor sleeve securely into the sample chamber body groove (Fig. 23/2).



- 1 Temperature sensor
- 2 Sample chamber body groove
- 3 Temperature sensor sleeve

**Fig. 23** Insertion of temperature sensor

- It is highly recommended to always place a **cover** on top of the sample chamber body (Fig. 24/3). This will minimize evaporation of medium or contamination of the sample chamber. If CO<sub>2</sub> module Lightsheet Z.1 is used (configuration dependent, chapter 2.7.2) the cover is recommended to maintain the CO<sub>2</sub> gas concentrations.
- Keep the opening as small as possible. Use the 3 mm opening cover for capillaries (Fig. 24/1) and 7 mm opening cover for syringes (Fig. 24/2). The cover only rests on top of the sample chamber body and is free to move.



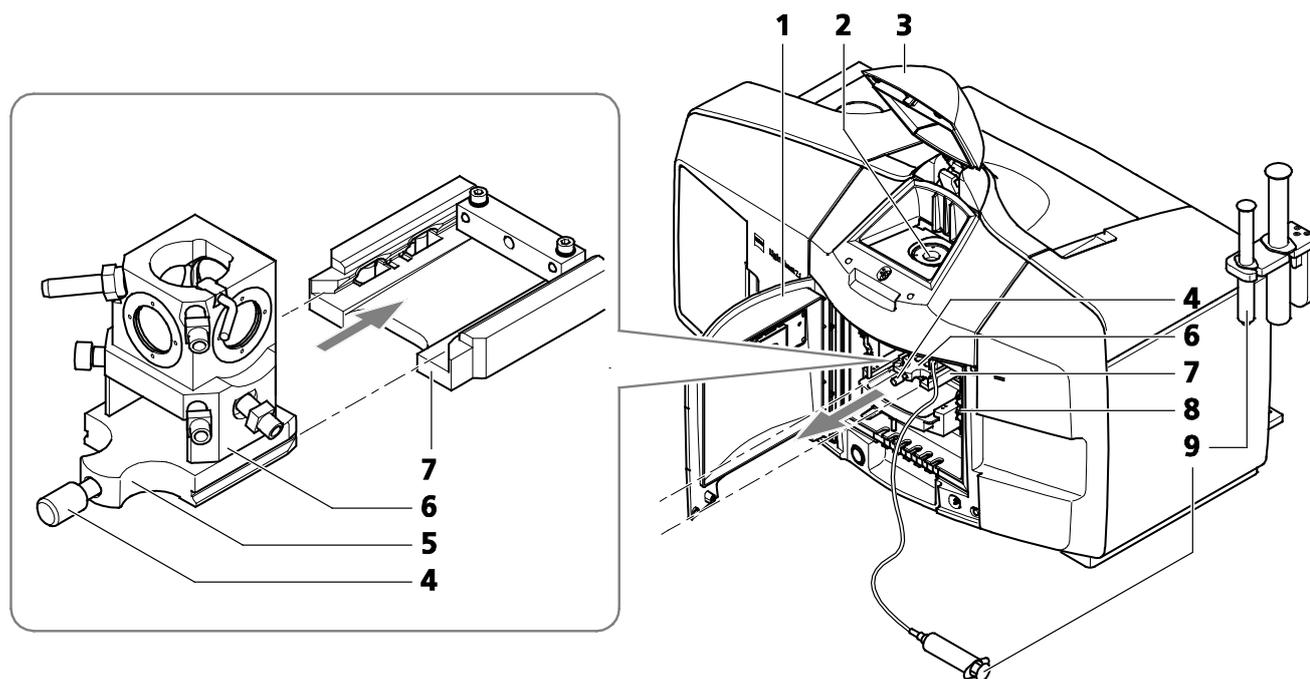
- 1 Cover for capillaries (3 mm opening)
- 2 Cover for syringes (7 mm opening)
- 3 Sample chamber body with dove tail slide

**Fig. 24** Placing cover on top of the sample chamber body

### 3.4 Removing and Inserting the Sample Chamber

The sample chamber can be removed from the system. For removal, proceed as follows:

- Open the front system cavity door (Fig. 25/1) of the main system module Lightsheet Z.1.
- Remove the liquid from the sample chamber (Fig. 25/6) using the hose with the corresponding syringe (Fig. 25/9).
- Ensure that no sample protrudes into the sample chamber. Remove the sample, if necessary, through the upper sample opening (Fig. 25/2) below the upper system cavity door (Fig. 25/3).
- If necessary, remove the connections for the incubation device (Fig. 25/8).
- Loosen the screw (Fig. 25/4) on the sample chamber and pull the sample chamber out by the grip (Fig. 25/5).
- Inserting the sample chamber:
  - Take hold of the sample chamber by the lower grip (Fig. 25/5) and push the sample chamber (Fig. 25/6) into the guide rails (Fig. 25/7).
  - Tighten the screw (Fig. 25/4) without exerting force. The sample chamber has been correctly inserted when its lower edge is flush with the guide rail.
- Plug in the cable to the incubation unit (Fig. 25/8) as necessary.



- |   |                          |   |                                  |
|---|--------------------------|---|----------------------------------|
| 1 | Front system cavity door | 6 | Sample chamber                   |
| 2 | Upper sample opening     | 7 | Guide rails/sample chamber mount |
| 3 | Upper system cavity door | 8 | Connections for incubation       |
| 4 | Securing screw           | 9 | Hose and syringe                 |
| 5 | Sample chamber grip      |   |                                  |

**Fig. 25** Removing and inserting the sample chamber

### 3.5 Assembly of the Sample Holder

Depending on the size of the sample there are two different types of sample holders available: sample holder for capillaries (inner diameter of capillary size 1 / ~0.68 mm, size 2 / ~1 mm, size 3/ ~1.5 mm, size 4 / ~2.15 mm) and syringes (1 ml). Always use the minimal cylinder diameter necessary for your specimen size to avoid excessive amounts of embedding medium.

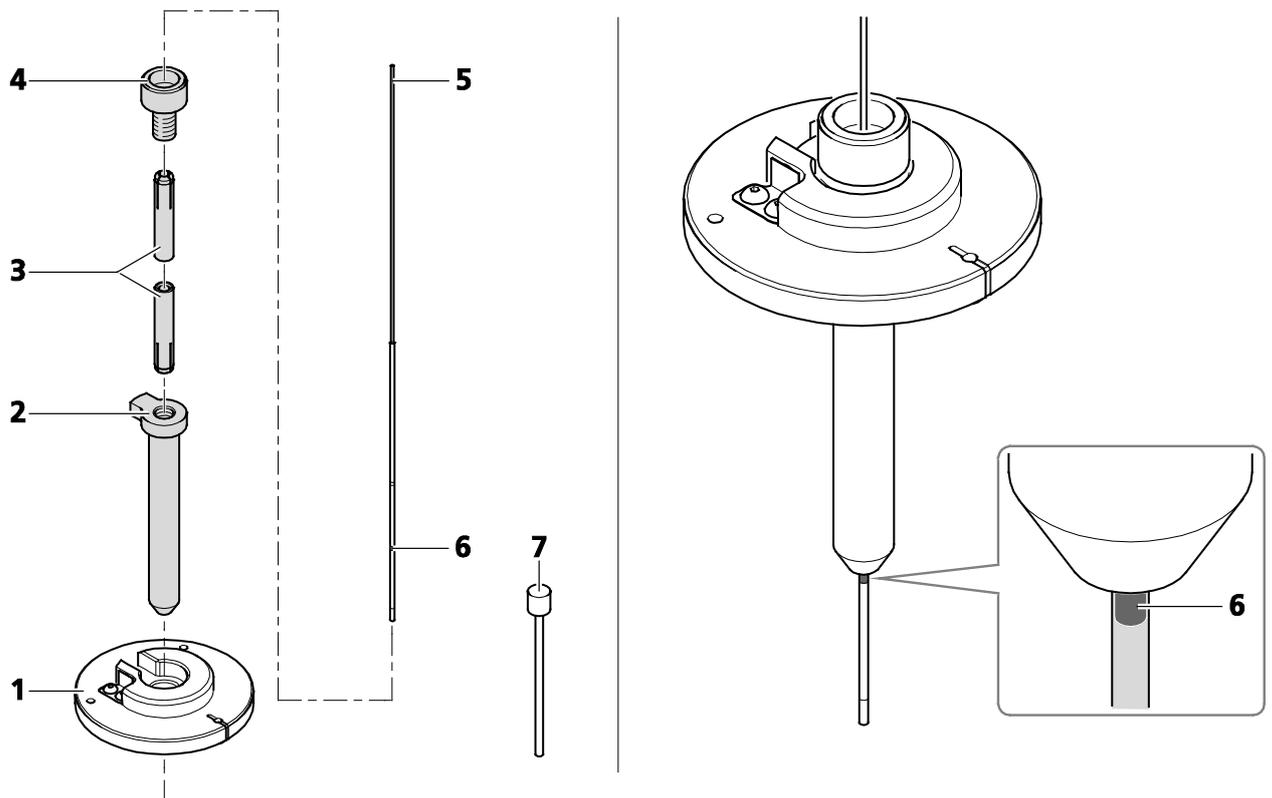
#### 3.5.1 Assembly of the Sample Holder for Capillary

1. Select the corresponding colored sleeves (Fig. 26/3) to match the color of the capillary of choice (Fig. 26/6).
2. The capillary should hold the sample and the appropriate plunger (Fig. 26/5), see as well the **CHAPTER 2 SAMPLE PREPARATION** in this manual. The plungers that fit into capillary size 2 - 4 have to be used with corresponding Teflon tips that are already assembled onto the plungers. Note that in addition 10x Teflon tips of each as well as matching Teflon tip tools are provided in the Chamber & Sample Holder Starter Kit Lightsheet Z.1 (**Tab. 1**). The Teflon tips can be assembled as a replacement onto the matching tip-less plungers.

Capillary size (inner diameter)	Capillary Color coding/ Reference number	Plunger Reference number	Teflon tip	Teflon tip tool Color coding
Size 1 (~0.68 mm)	Red/701902	#701996	-	-
Size 2 (~1mm)	Black/701904	#701997	Size 2	Clear
Size 3 (~1.5mm)	Green/701908	#701998	Size 3	Green
Size 4 (~2.15mm)	Blue/701910	#701999	Size 4	Blue

**Tab. 1 Capillary components in the Chamber & Sample Holder Starter Kit Lightsheet Z.1**

- The sleeves are tube-shaped with four slits at one end. Insert the sleeves into the capillary sample holder stem (Fig. 26/2) so that the two slit endings point outwards and the non-slit endings face toward the center.
- Take the clamp screw (Fig. 26/4) and position it onto the capillary sample stem holder turning clockwise three times (360° each turn).



- |   |                                    |   |                                    |
|---|------------------------------------|---|------------------------------------|
| 1 | Sample holder disc for capillaries | 5 | Plunger                            |
| 2 | Capillary sample holder stem       | 6 | Capillary with color-coded marking |
| 3 | Sleeves (color-coded)              | 7 | Ejection tool                      |
| 4 | Clamp screw                        |   |                                    |

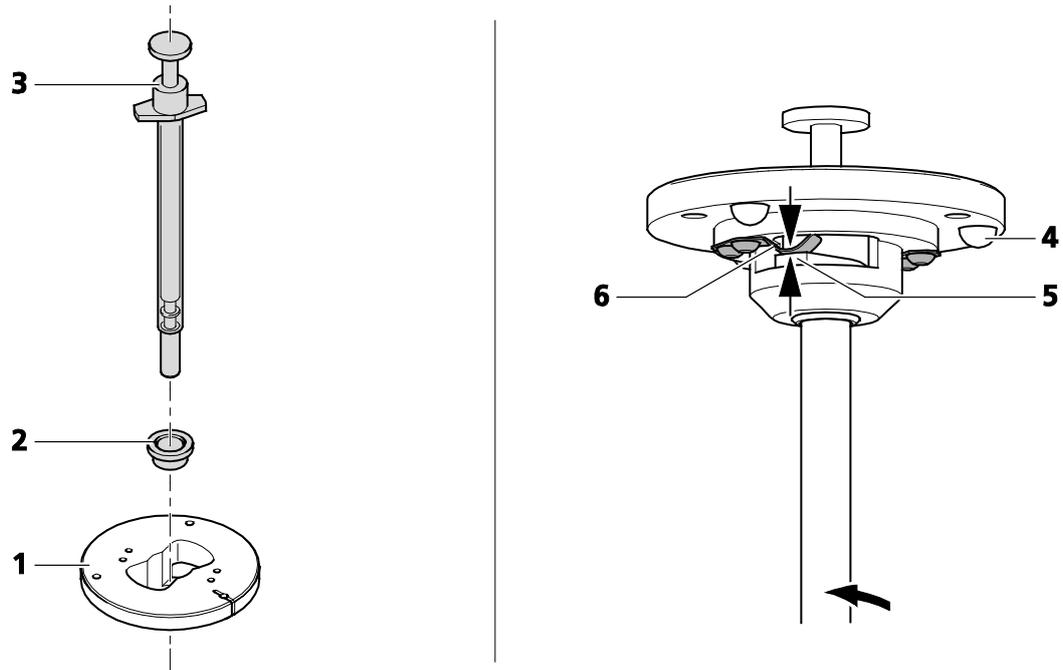
**Fig. 26 Assembly of the sample holder for capillary**

5. Take the capillary by the glass portion (avoiding the plunger) with the agarose embedded specimen inside and carefully place it into the center of the clamp screw sample holder top.
  6. Push the capillary within the holder downwards until the color-coded capillary marking becomes visible (Fig. 26/6 right hand side). This is the recommended position for later imaging. However, if there is more space needed in between the sample chamber and the sample holder positioning stage it can be useful to push the capillary downwards further. In these cases, carefully test if the capillary still can be placed tightly within the sample holder.
-  Newly bought sleeves can be slightly tight at their slit opening ends so that it can be difficult to push the capillary within the holder. In this case take the capillary with matching plunger and insert it carefully into the corresponding sleeves at their non-slit endings. Make sure that the slits point outwards. Position the capillary surrounded by the sleeves into the sample holder stem and place the clamp screw. You can proceed with step 6).
7. Tighten the clamp screw. Take the sample holder disc (Fig. 26/1) with the protruding side with positioning notch facing you. Carefully position the capillary stem holder with specimen into the sample disc holder until the ball bearing click-position is felt.
  8. Before taking the capillary out (chapter 2.6), retract the sample back inside the capillary glass via using plunger.
  9. For removal, loosen the clamp screw and carefully pull the capillary through the stem sleeve by using the glass end nearest the color-coded marking.
  10. Unscrew the clamp screw completely and remove the sleeves by using the ejection tool (Fig. 26/7) for the capillary sleeves removal.

The sleeves as well as the glass portion of the capillary can be cleaned with a lint-free cloth moistened with water.

### 3.5.2 Assembly of Sample Holder for Syringes

- Place the sample holder disc for syringes (Fig. 27/1) so the flat side is facing you.
- Take the adapter ring (Fig. 27/2) and place it into the center of the sample holder disc with the larger diameter of the ring facing you.
- Pick up the sample disc holder with adapter ring in one hand. Take the syringe carrying your specimen (Fig. 27/3) and insert it completely into the sample disc holder (avoiding touching the plunger).
- Touching the syringe body and turn clock-wise to position the syringe body into the two clamps (Fig. 27/6).

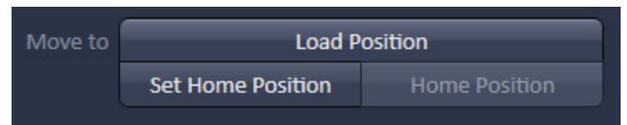


- |   |                                 |   |               |
|---|---------------------------------|---|---------------|
| 1 | Sample holder disc for syringes | 4 | Ball bearings |
| 2 | Adapter ring                    | 5 | Syringe body  |
| 3 | Syringe                         | 6 | Clamp         |

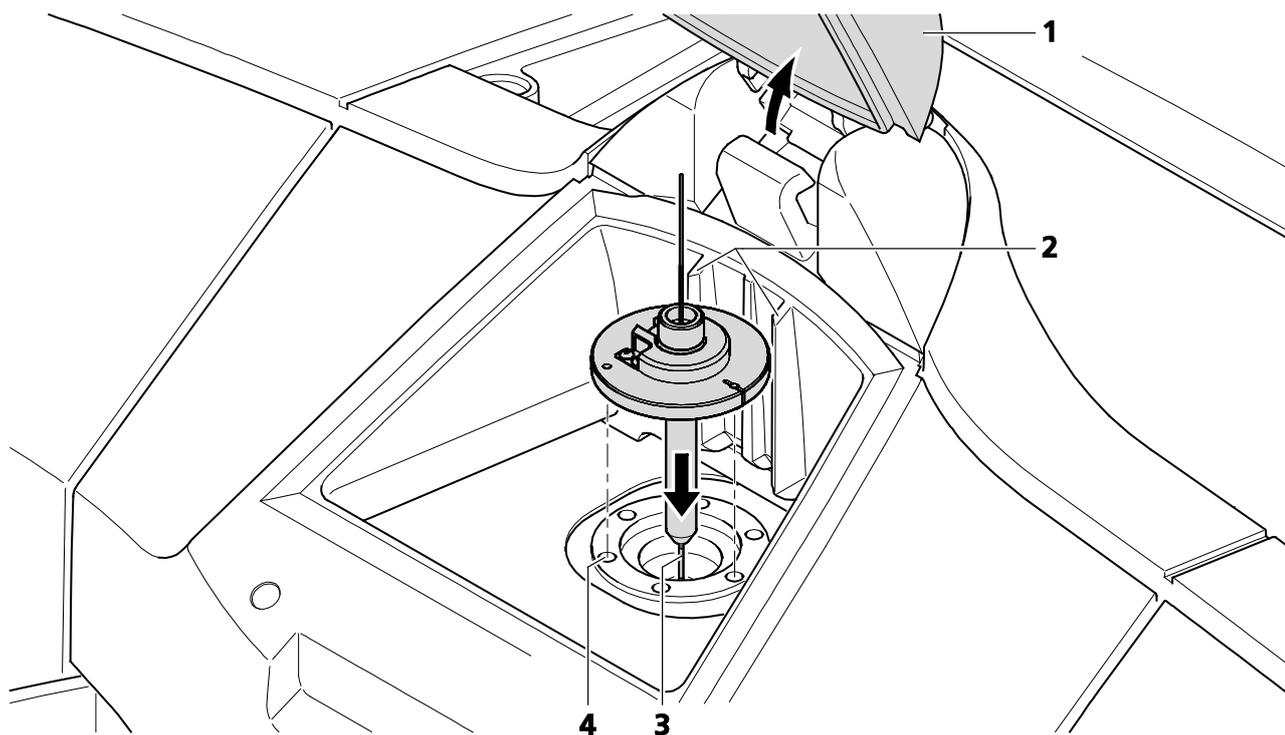
**Fig. 27** Assembly of sample holder for syringes

### 3.6 Inserting and Removing the Sample Holder

- To insert the sample holder move the stage of the Lightsheet Z.1 system to the **Load position** via the **Specimen Navigator** tool (Fig. 28) in the ZEN software (see CHAPTER 4 SYSTEM OPERATION).
- Open the upper system cavity door (Fig. 28/1).
- Take the sample holder for capillaries or syringes by the sample holder disc with the capillary or the syringe facing downwards (Fig. 29/3).
- Insert the sample holder gliding the sample holder disc along the guide rails (Fig. 29/2). It is placed correctly into a click position if the sample holder disc ball bearings lock into the three holes.
- Notice the white line marking at the outer edge of the disc. It can be used as a reference point for reorienting the sample holder disc once it was taken out.
- Close the upper system cavity door (Fig. 29/1) and position the capillary and the sample as described in **CHAPTER 4 SYSTEM OPERATION**.



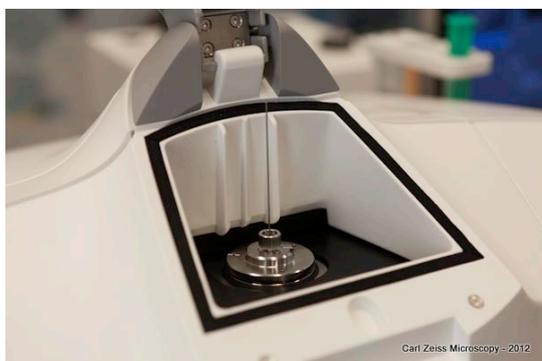
**Fig. 28** Load Position in the Specimen Navigator



- 1 Upper system cavity door
- 2 Guide rails
- 3 Capillary
- 4 Hole for disc ball bearings

**Fig. 29 Inserting the Sample Holder**

- Before taking the sample holder out, retract the sample back inside the capillary glass via using plunger.
- Move to the **Load position** via the **Specimen Navigator** tool (Fig. 28) in the ZEN software (see **CHAPTER 4 SYSTEM OPERATION**).
- Open the upper system cavity door (Fig. 29/1).
- Take the sample holder out by gliding the sample holder disc along the guide rails (Fig. 30).



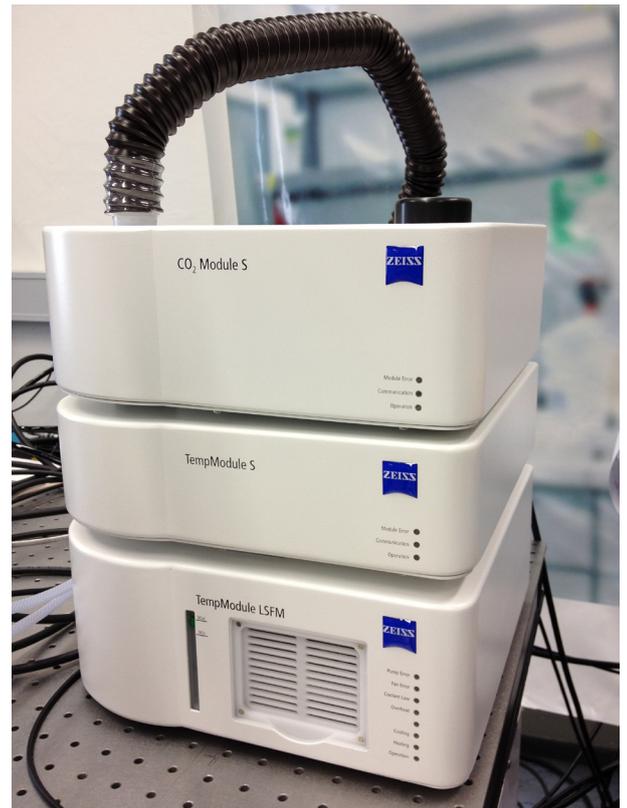
**Fig. 30 Removing the Sample Holder**

### 3.7 Installation of the Incubation Modules

The incubation of the Lightsheet Z.1 is configuration dependent. It is used to control and monitor temperature and CO<sub>2</sub> supply to the sample environment. The **TempModule S1** is required for all of the various configurations.

- The configuration dependent modules should be placed on a level surface next to the system and are stacked on top of each other (Fig. 31). For further information see the individual component manuals from PeCon.

The installation of incubation modules on the Lightsheet Z.1 can be divided into two parts. One deals with the actual assembly of hardware components of the incubation modules and their connections to the system. The second part deals with the registration of the incubation modules using specific software tools.



**Fig. 31** Incubation modules stacked on top of each other

#### 3.7.1 Heating Components

There are two different heating components available: **(1) Heating Block** Sample Chamber Lightsheet Z.1 enables to heat the sample chamber from ambient temperature to 42 °C. **(2) Peltierblock** Sample Chamber Lightsheet Z.1 permits heating and cooling of the sample chamber in the range from 10 °C to 42 °C.

**(1) Heatingblock** Sample Chamber Lightsheet Z.1 is controlled by the TempModule S1 providing electrical power supply and closed-loop temperature control. The system allows to heat the sample chamber from ambient temperature to 42 °C.

- 👉 Connect the Heating Block LSFM and the TempModule S via connecting cable. For proper electrical connection please follow the connection diagram in the individual component manuals from PeCon.

 Make sure that the temperature inside the sample chamber is in accordance with the desired temperature value required for the experiment. The ZEN software displays the temperature of the heating block itself and does not register the actual temperature in the sample chamber that can be lower (due thermal loss) or higher.

- To assemble the heating block to the sample chamber remove first of all the sample chamber dove tail slide from the assembled sample chamber (Fig. 1, section **3.3.2 Assembly of the Sample Chamber Body and the Sample Chamber Dove Tail Slide**).
- Take the heating block and screw it onto the bottom of sample chamber body following the instructions of the individual component manuals from PeCon.
- The sample chamber body can now be assembled together with the sample chamber dove tail slide (Fig. 1, section **3.3.2**).
- Insert the sample chamber as described in chapter 2.4 and plug in the 8 pin connector of the connection cable into the right side of the front system cavity / sample room following the individual component manuals from PeCon (Fig. 32/6) shows the connection exemplary for the Peltier Block S).

**(2) Peltier Block S** is part of the TempModule CZ-LSFM. The Peltier Block helps adjust the sample chamber temperature by heating or cooling the sample chamber within the range of 10 °C to 42 °C (up to 1.5°/min heating, up to 1.0°/min cooling). The temperature inside the sample chamber is monitored by a temperature sensor (Fig. 23/1; Fig. 32/3). For insertion of the temperature sensor in the sample chamber refer to section **3.3.4 Insertion of Accessories for Incubation**).

 The TempModule CZ-LSFM contains a water based coolant reservoir. For safety reasons (Risk of electrical shock as a result of leakage) the unit must be placed directly on a level table and is thus the base for all other incubation related modules (Fig. 31).

- Connect the TempModule S, the TempModule CZ-LSFM, the Lightsheet Z.1 and the Peltier Block according to the individual component manuals from PeCon.
- To assemble the Peltier Block to the sample chamber remove first of all the sample chamber dove tail slide from the assembled sample chamber (section **3.3.2**; Fig. 21).
- Take the Peltier Block and screw it onto the bottom of sample chamber body following the instructions of the individual component manuals from PeCon.
- The sample chamber body can now be assembled together with the sample chamber dove tail slide (Fig. 21) and insert it into the Lightsheet Z.1 as described (section **3.3.2**).
- Insert the accessories for incubation (temperature sensor, cover recommended) into the sample chamber (section **3.3.4**, Fig. 23, Fig. 24).
- Connect the Peltier Block (Fig. 32/6) to the Lightsheet Z.1 by the two provided silicone tubes (Fig. 32/7-8) and plug in the 8 pin connector of the connection cable into the right side of the front system cavity / sample room (Fig. 32). For proper electrical connections and tube connections follow the instructions of the PeCon manual.

Heating component	Temperature range	Incubation modules
Heating Block	Heating from ambient temperature to 42 °C	(1) TempModule S1 (2) Heating Block LSFM
Peltier Block	Heating and cooling in the range from 10 °C to 42 °C	(1) TempModule S1 (2) TempModule CZ-LSFM (including Peltier Block and Temperature sensor)

**Tabelle 1 Overview Heating components**

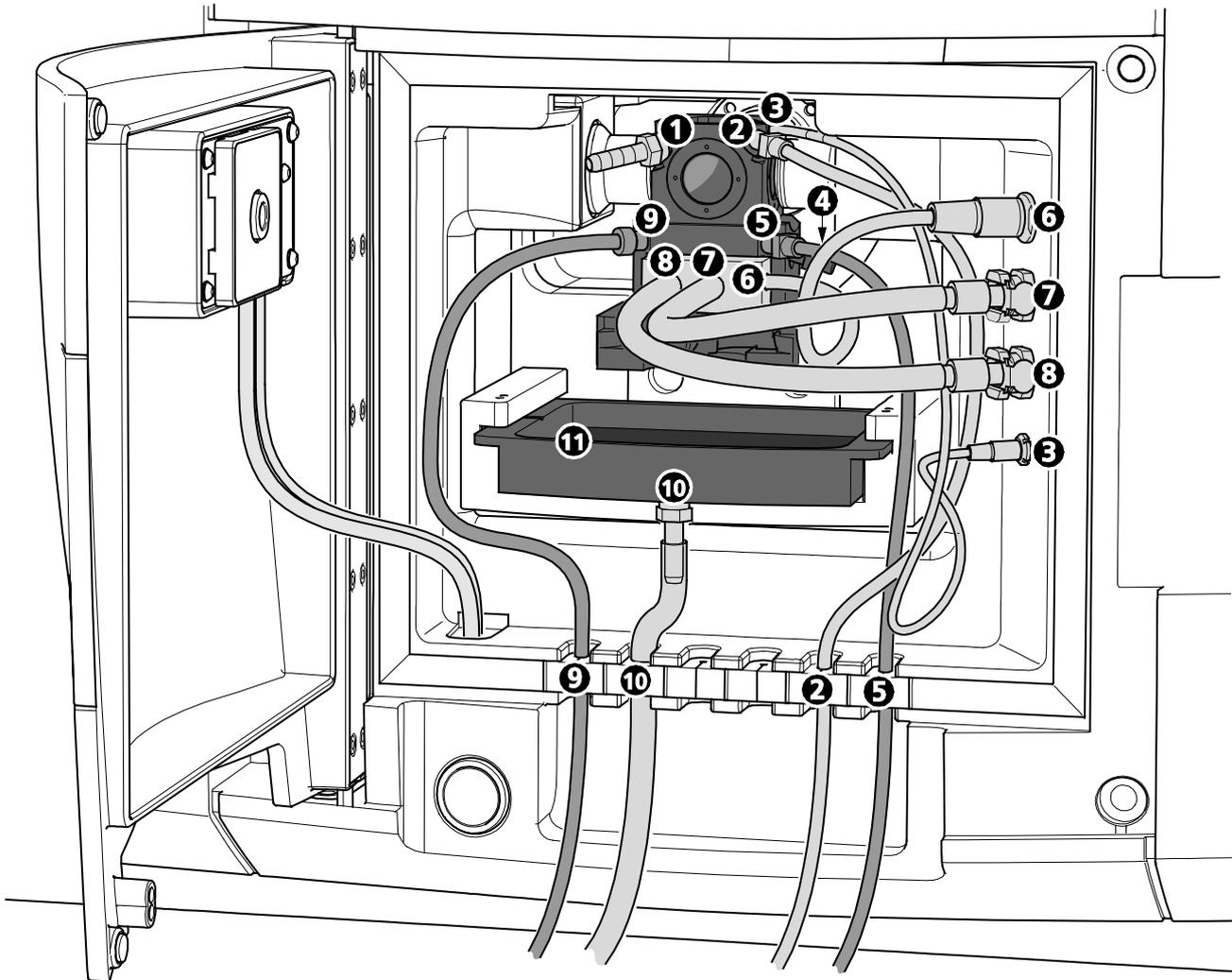
### 3.7.2 CO<sub>2</sub>-Module Lightsheet Z.1

The CO<sub>2</sub>-Module Lightsheet Z.1 controls and monitors adjustable CO<sub>2</sub> concentration (from 0 % to 10 %) in the sample chamber and requires CO<sub>2</sub> supply, sterile filter and Humidifier S1 to provide CO<sub>2</sub>-enriched air.

- At least 3h (or more) may be needed until a stable pH value is reached due to the small surface area in relation to sample chamber volume.
- For assembly of hardware components of the CO<sub>2</sub> Module and its connections to the system, as well as the calibration procedure needed follow the instructions from the PeCon manual.
- After CO<sub>2</sub> calibration, the Lightsheet Z.1 system must be completely turned off before work with the system can continue.
- Position the Luer-Lock connector for CO<sub>2</sub> incubation at the top right corner of the sample chamber. Place a **cover** on top of the sample chamber body (Fig. 24, chapter 3.3.4) to maintain the CO<sub>2</sub> gas concentrations.
- The sterility of the CO<sub>2</sub>-enriched air is given by the sterile filter. Take control of the sterility in your sample chamber by following the cleaning instruction of all parts of the CO<sub>2</sub>-module (see PeCon manual) as well as the components of the sample chamber (see **CHAPTER 1 HARDWARE**).

 Please clamp all incoming or outgoing tubing (Fig. 32/2, Fig. 32/5, Fig. 32/9, Fig. 32/10) into the provided clamping grooves. This will avoid possible disconnection problems after closing the front system cavity door.

 Note that for the sake of completeness Fig. 26 shows the connection diagram for the usage of a perfusion pump (Fig. 32/5, Fig. 32/9). Alternatively a syringe based-perfusion can be used (position Fig. 32/5). Consequently, Fig. 32/9 has to be replaced by a bling plug.



- 1 Drain connector
- 2 Luer-Lock connector for CO<sub>2</sub> incubation with tubing
- 3 Temperature sensor with 4-pin connection cable
- 4 Blind plug
- 5 Luer-Lock connector for perfusion pump-in with tubing (or alternatively for syringe based perfusion)
- 6 Peltier Block S with 8-pin connector
- 7 Cooling liquid (Silicon tube with male connector)
- 8 Cooling liquid (Silicon tube with male connector)
- 9 Luer-Lock connector for perfusion pump-out with tubing
- 10 Drain connector with tubing
- 11 Drain tray

**Fig. 32 Incubation connections for sample chamber body**

CO<sub>2</sub>-Module Lightsheet Z.1 can be extended with Heating Device Humidity S1 to improve thermal equilibrium in the sample chamber.

### 3.7.3 Heating Device Humidity S1

For a better humidification of incubation atmosphere the Humidifier of the corresponding **CO<sub>2</sub>-Module Lightsheet Z.1** can be inserted into the **Heating Device Humidity S1**. The Heating Device Humidity S1 enables to improve thermal equilibrium in the sample chamber and avoid condensation in the tubing.

### 3.7.4 Registration of the incubation modules

- After the Lightsheet Z.1 and the PC for system control are turned on and the operation system has booted start the software ZEN Configuration Tool. You find it either as a shortcut on your desktop or in this directory : C:\ZEN\HWT as Configuration Tool 2012.exe.
- On the opening window press the Start configuring button (Fig. 33):

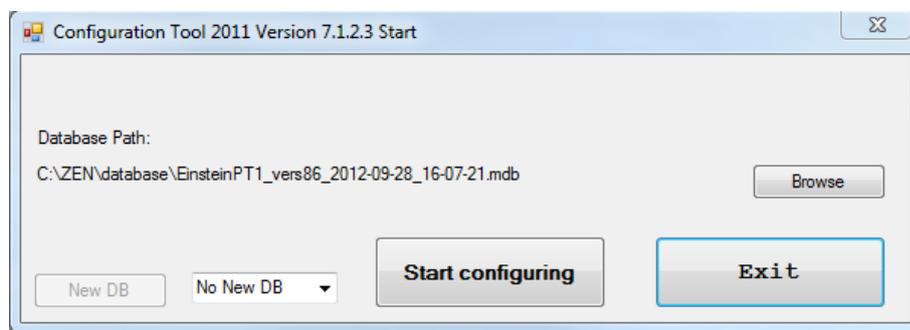
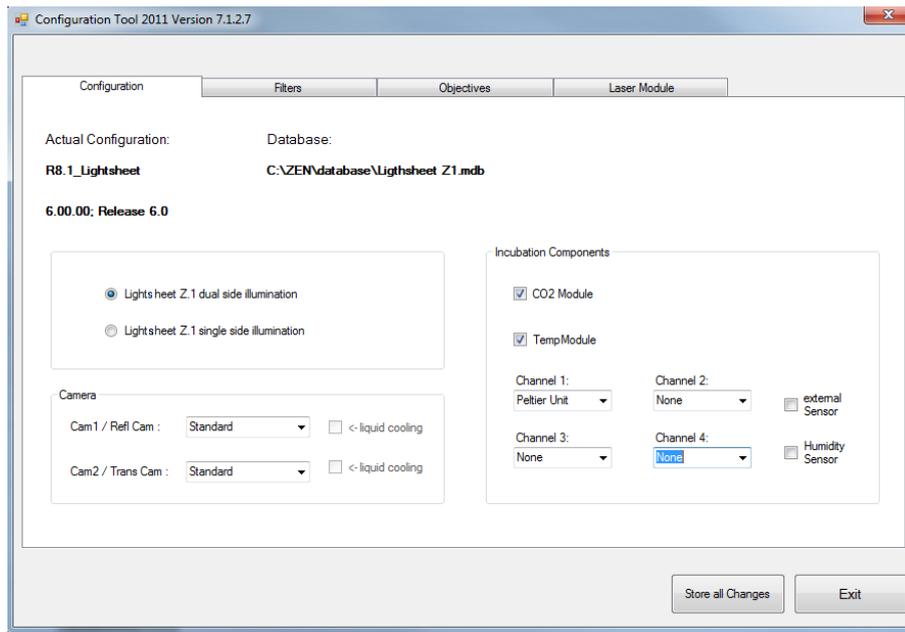


Fig. 33 ZEN Configuration Tool Start-up window

- On the **Configuration** tab (Fig. 34) you find an **Incubation Components** field with check boxes for **CO<sub>2</sub> Module** and **TempModule** that you can activate or deactivate depending on your incubation configuration. These modules will show up later as **Atmosphere (%)** panel and **Temperature (°C)** panel respectively in the ZEN software (see **CHAPTER 4 SYSTEM OPERATION**).



**Fig. 34 ZEN Configuration Tool Incubation Components field**

- A drop-down menu enables to assign heating components (Heatingblock, Peltier Block) as well as the Heating Device Humidity to four distinct channels.
- ☞ Make sure that the assignment of incubation components to the corresponding channels in the Configuration Tool is in accordance with the electrical connection of the incubation components into the TempModule S1 ("Channel 1-4", "Control Sensor").
- We recommend to assign Channel 1 to the heating component (Heatingblock, Peltier Block) and Channel 4 to the Heating Device Humidity if available.
- Do not check the check boxes for **external Sensor** as well as an **Humidity Sensor**. These components are not part of the Lightsheet Z.1 incubation modules.
- Leave the Configuration Tool with the **Store all Changes** button. This will write all changes into the database of your system.

### 3.8 Switch Incubation ON and OFF

- Before you turn **ON** the incubation, all incubation components you wish to use during the imaging session must be attached to the Lightsheet Z.1 system before starting ZEN software.
- Set the **"Incubation" switch** on the remote control to the ON position. Make sure that all incubation components are switched on.
- Once ZEN is running you can detach and reattach components.
- To turn **OFF** the incubation, uncheck the corresponding checkboxes in the **Incubator** tool window (see **CHAPTER 4 SYSTEM OPERATION**) or deactivate the incubation components by setting the **"Incubation" switch** to the OFF position.

 Be aware that all incubation components will continue working with the in **Incubator** tool window specified parameters after ZEN has been shut down or stopped working. This will keep your sample alive under precisely defined conditions, even if ZEN stops unexpected during an experiment.

### 3.9 Illumination and Detection Optics

In the Lightsheet Z.1, one or a pair of illumination optics plus one detection optic are always required.

We recommend the following combinations:

	Lightsheet Z.1 illumination optics 5× / 0.1	Lightsheet Z.1 illumination optics 10× / 0.2
Lightsheet Z.1 detection optics 5× / 0.16	yes	no
Lightsheet Z.1 detection optics 20× / 1.0	no	yes
Lightsheet Z.1 detection optics 40× / 1.0	no	yes
Lightsheet Z.1 detection optics 63× / 1.0	no	yes

The area that can be imaged with the combination of detection optics with the detection module on the Lightsheet Z.1 is summarized <sup>\*1)</sup> in the following tables:

	Zoom							
Detection Optic	0.36x		0.7x		1.0x		2.5x	
	x (μm)	y (y <sup>*2)</sup> μm)	x (μm)	y (μm)	x (μm)	y (μm)	x (μm)	y (μm)
5x / 0.16	4815	4815	2496	2496	1733	1733	693	693
20x / 1.0	1204	1204	624	624	433	433	173	173
40x / 1.0	602	602	312	312	217	217	87	87
63x / 1.0	382	382	198	198	138	138	55	55

**Tabelle 2** Detection module PCO.Edge

	Zoom							
Detection Optic	0.36x		0.7x		1.0x		2.5x	
	x (μm)	y <sup>*2)</sup> (μm)	x (μm)	y <sup>*2)</sup> (μm)	x (μm)	y (μm)	x (μm)	y (μm)
5x / 0.16	4157	5570	2138	2864	1497	2005	599	802
20x / 1.0	1039	1392	534	716	374	501	150	201
40x / 1.0	520	696	267	358	187	251	75	100
63x / 1.0	330	442	170	227	119	159	48	64

**Tabelle 3** Detection module Standard

\*1) Typical values for Lightsheet Z.1 systems

\*2) Be aware, a homogenous light sheet illumination with full intensity is limited to about 2800 μm in the y direction. Above and below this center, the illumination intensity will fade towards the edges in the y direction.

### 3.9.1 Removing and Inserting the Illumination Optics Unit

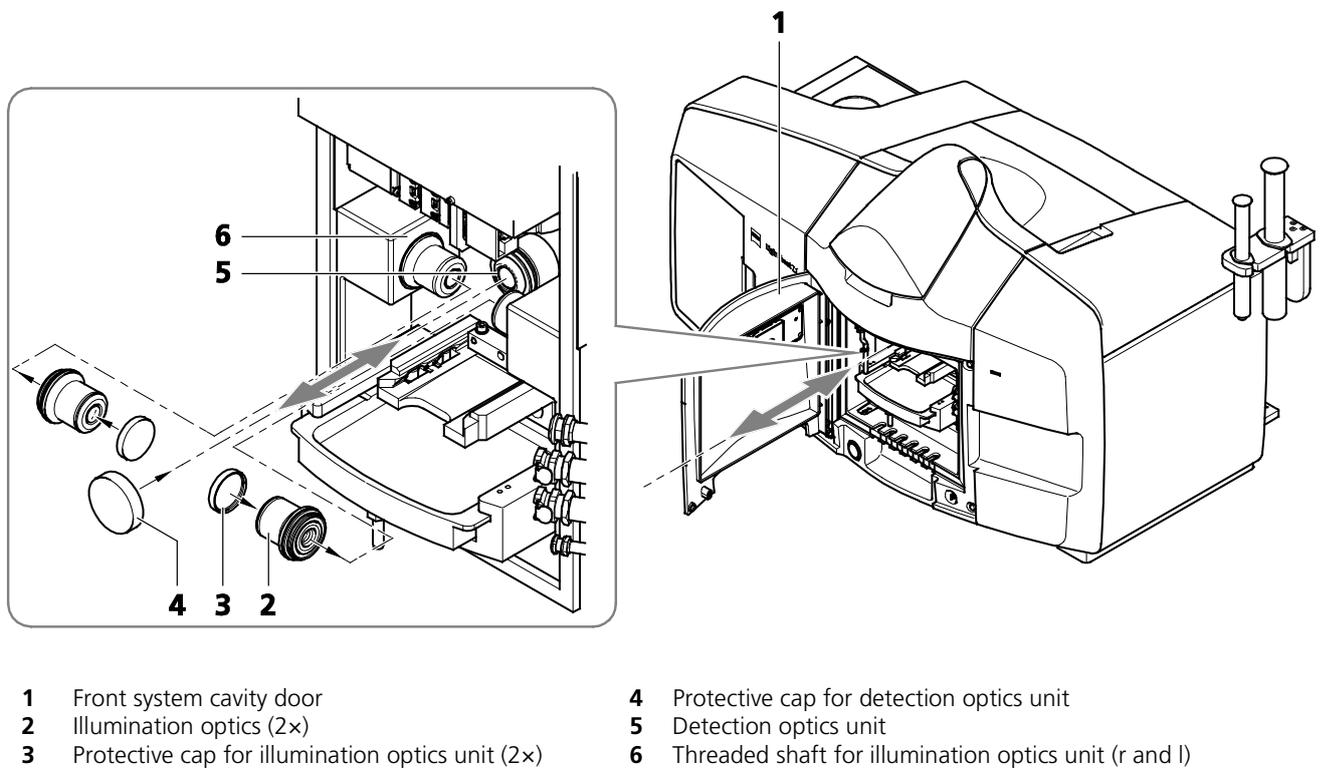
The illumination optics unit (Fig. 35/2) can easily be removed and inserted after the removal of the sample chamber (see section 3.4 Removing and Inserting the Sample Chamber).

To protect the front lenses, secure the appropriate protective caps (Fig. 35/3) over each of the illumination optics (Fig. 35/2) and protective cap (Fig. 35/4) over the detection optic (Fig. 35/5). The illumination optics can then be unscrewed by turning anticlockwise.

To insert the illumination optics, proceed in the reverse order by screwing it clockwise into the unit until finger-tight.

Finally, the protective caps can be removed and the sample chamber inserted.

After the illumination optic has been exchanged, the currently used optic must be registered in the ZEN software. The necessary software interface can be found in the Objectives tool window under the Maintain tab.



**Fig. 35 Removing and inserting the illumination optics**

### 3.9.2 Removing and Inserting the Detection Optics Unit

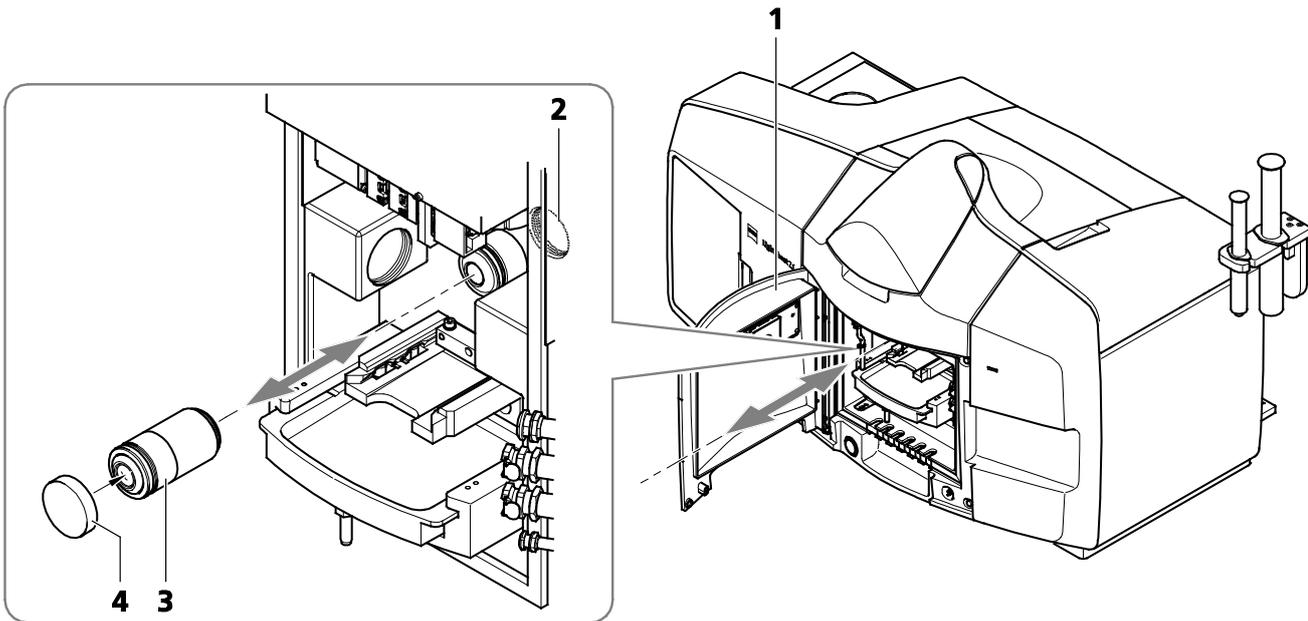
In order to remove the detection optics unit (Fig. 36/3) located in the rear zone of the system cavity, the sample chamber must first be removed (see section 3.4). To prevent damage to the illumination optics units, these must also be removed (see section 3.9.1). Secure the appropriate protective cap (Fig. 36/4) onto the detection optics unit.

Grab the detection optics unit as close as possible to the base and remove it by turning anticlockwise. Caution: the detection optics unit is heavy.

To insert the detection optics unit, it must be screwed in a clockwise direction without exerting force. Subsequently the protective cap should be removed before re-inserting the illumination optics unit and the sample chamber.

After the detection optic has been exchanged, the currently used optic must be registered in the ZEN software. The necessary software interface can be found in the Objectives tool window under the Maintain tab.

Each detection optic unit is labeled with an individual serial number. It ensures that the specific calibration file for this lens, necessary for optimal performance, is used when identified within the software. The calibration file is created by a Carl Zeiss service personnel representative during system installation, or when a detection optic is newly purchased. Ensure that the correct detection optic unit is chosen in the ZEN software interface, based on its name and the specific serial number of the detection optic in use.



- 1 Front system cavity door
- 2 Threaded shaft for detection optics unit
- 3 Detection optics unit
- 4 Protective cap

**Fig. 36** Removing and inserting the detection optics

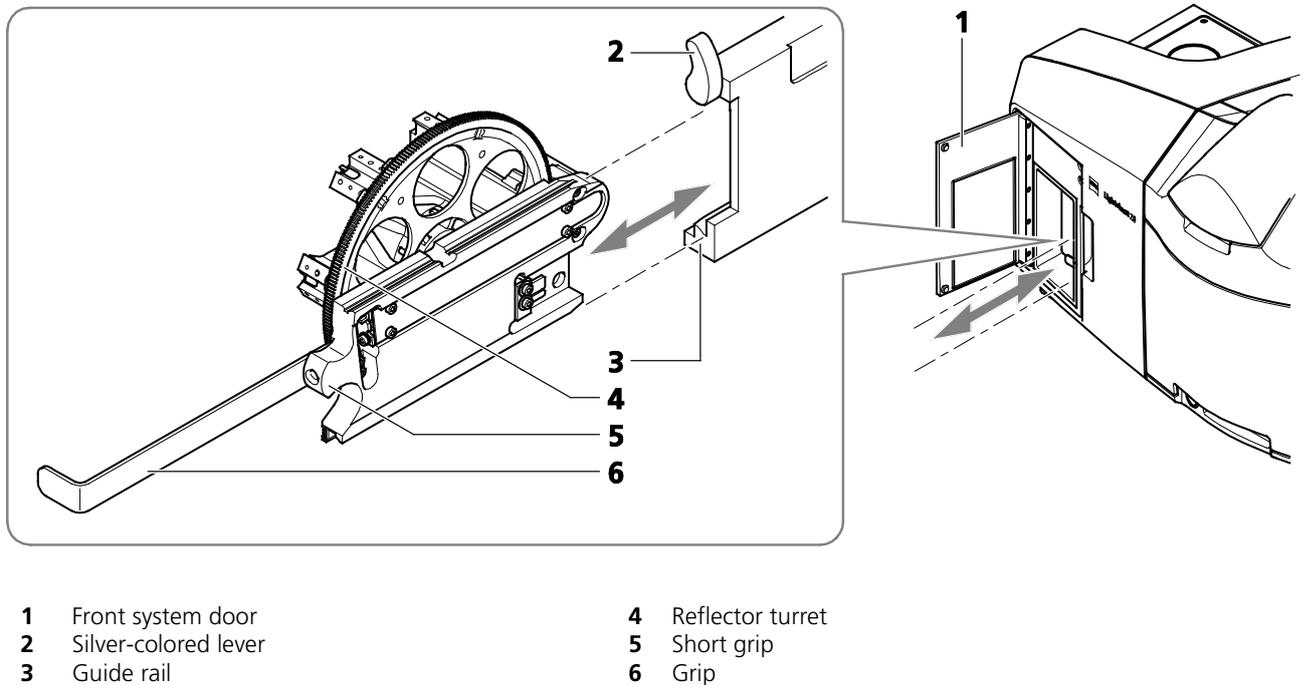
### 3.10 Removing and Inserting the Reflector Turret for Emission Selection

The reflector turret (Fig. 37/5) can be accessed by opening the left-hand-side front system door (Fig. 37/1).

 After the reflector turret for emission selection has been exchanged, the **Automatic Detector Alignment** should be performed. The ZEN software should be restarted if the reflector turret was removed, while ZEN was still running.

To remove, first of all turn the silver-colored lever (Fig. 37/2), then pull-out the reflector turret by using the grip (Fig. 37/6).

When inserting, it is recommended that the reflector turret first of all be held by the short grip (Fig. 37/5) and inserted into the guide rail (Fig. 37/3) and then pushed in as far as it will go using the long pull rod (Fig. 37/6). Return the locking lever to its original position. Finally, close the system door.



**Fig. 37** Removing and inserting the reflector turret for emission selection

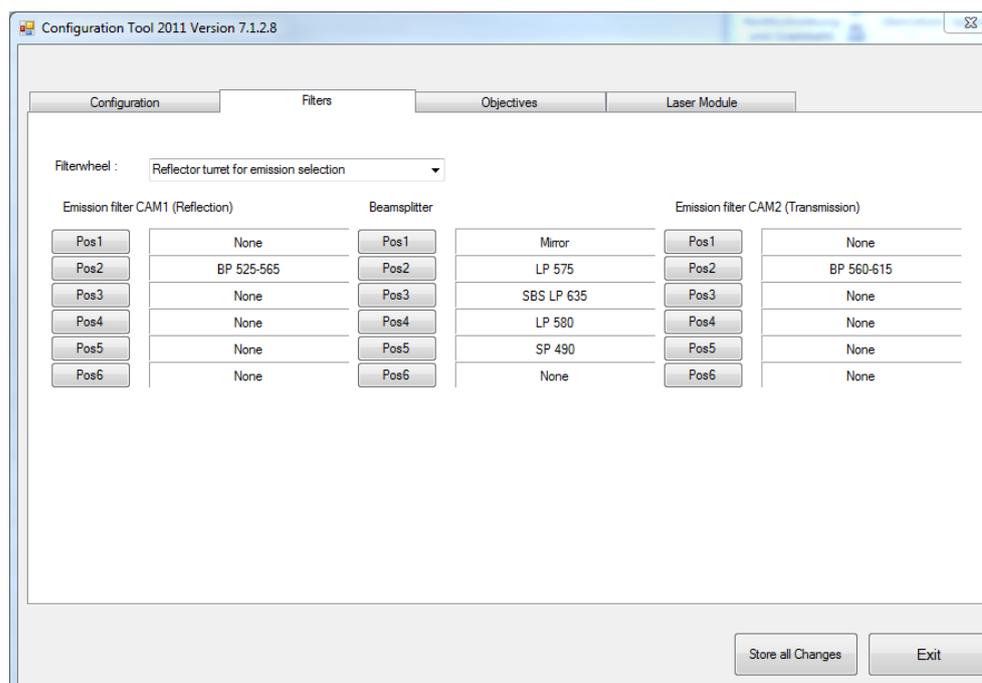
 The filters are not push and click filters and cannot be taken out as a whole. The filter cube and the beam splitter were installed and aligned in the factory. Do not try to take out single filter cubes or beam splitters, it will damage the filters and the alignment, rendering the system in the worst case unusable.

The emission filters can be exchanged on the filter turret (Fig. 37/4).

- Use the provided tool to loosen the retaining ring around the emission filter you wish to exchange and remove the ring.
- Take out the emission filter, best when wearing powder-free laboratory gloves to prevent fingerprints on the filter, by carefully tilting the filter wheel.
- Insert the new emission filter, the arrow mark on its edge must indicate the direction of the light beam. If no arrow marking is present, the reflective side of the filter must point towards the light source.
- Put the ring on top of the filter and tighten it with the same tool without applying force.

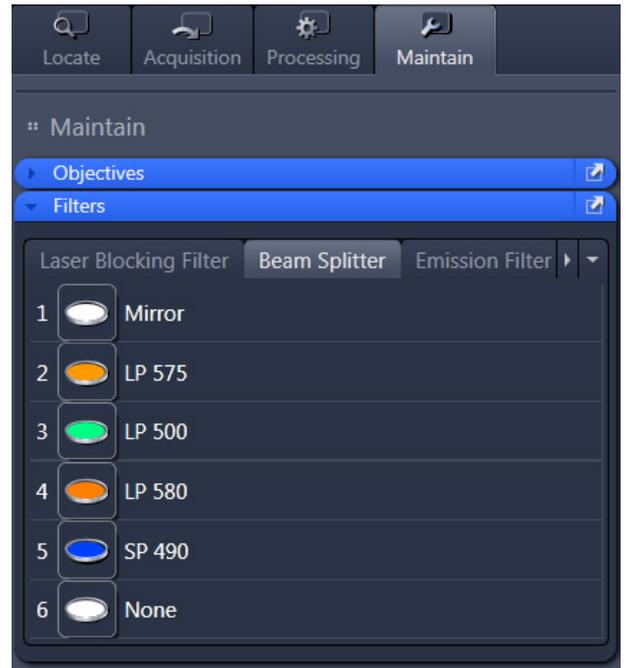
The emission filters facing towards the side of the turret are part of the channel 1 (Cam1, Emission Filter 1) light path; the filters facing the top are part of channel 2 (Cam2, Emission Filter 2).

After replacing the reflector turret for emission selection with new filters, the filter modules must be registered into the database using the Configuration Tool software (Fig. 38) or modified in the ZEN software under the Maintain tab, Filters tool window. The position number on the turret is on the left side of the filter. These numbers correspond with the numbering in the ZEN software.



**Fig. 38** Configuration Tool, Filters tab

 If you plan to exchange the reflector turret for emission selection on a regular basis, it can be helpful to create two databases, one for each reflector turret. To do so, copy the original database with a different name (you find it in the directory: C:\ZEN\database) and modify it with the Configuration Tool accordingly. Name both databases, to easily identify them. When ZEN is started open the **Boot Status** with the black arrow and then the **Hardware configuration database** the same way (Fig. 7). Here you can choose which database to use. The **Recent...** button shows all databases that have been used lately. The **Choose...** button opens a window explorer window which allows one to search for a database to use.



**Fig. 39** ZEN Software, Maintain tab, Filters tool window

### 3.11 Removing and Inserting the Reflector Turret for Laser Blocking Filter

To remove the reflector turret for laser blocking filter (Fig. 40/7), first open the upper rear system door (Fig. 40/4). Beneath this is an additional cover plate (Fig. 40/3) on which the knurled screws left and right (Fig. 40/1) can be loosened manually to lift off the cover by the black grip (Fig. 40/2).

Turn the silver-colored lever (Fig. 40/5) and pull the grip (Fig. 40/9 and 8) upwards to release the reflector turret.

To insert the reflector turret, slide it down using the guide rail (Fig. 40/6) as far as it will go and return the silver-colored lever to its original position. Place the cover over the opening and tighten the knurled screws carefully but without force. Close the upper rear system door.

 When the knurled screws are opened, the laser beam will be deactivated by the safety control. In the event that no laser beam appears after replacing the filter wheel, check the knurled screws.

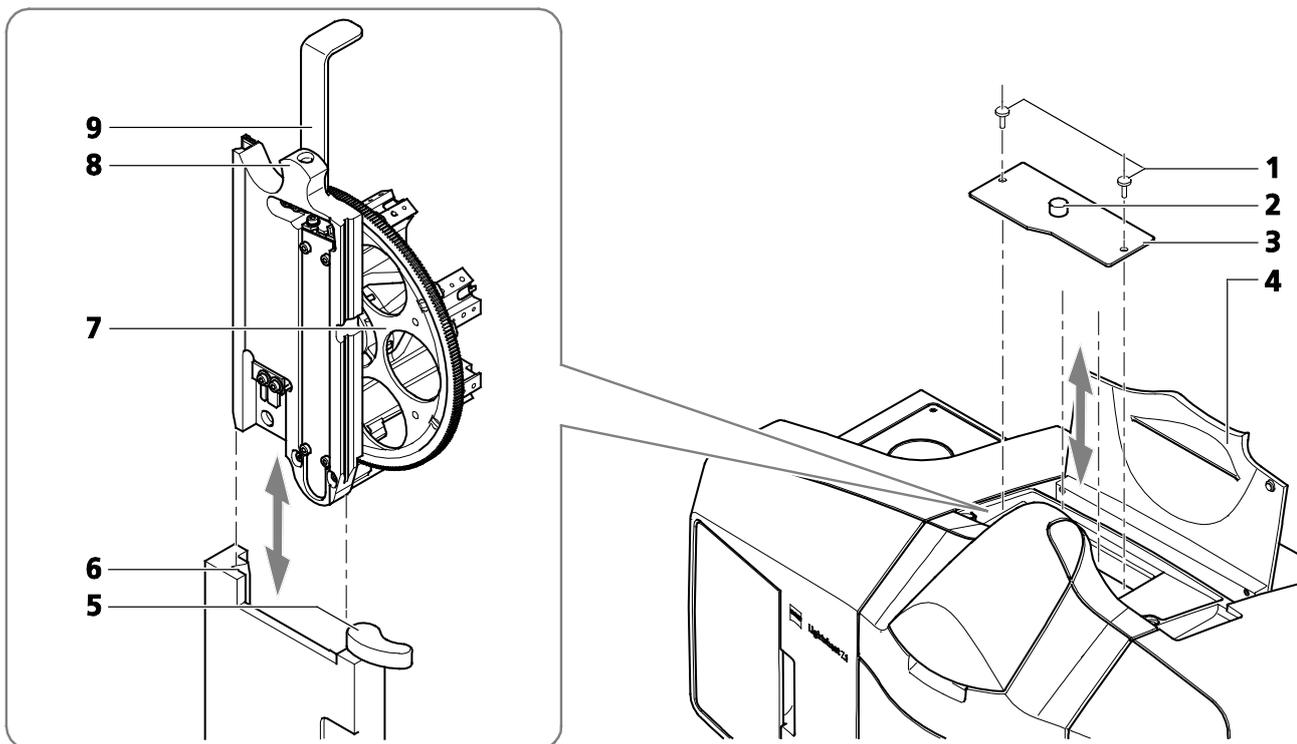
You can remove and replace filter modules by push and click.

How to uninstall a module:

- Disengage the filter module from the upper spring elements of the filter insert by tilting it forward, then lift it off the lower spring elements and remove the module.

How to install a module:

- Insert the filter module with the aid of its mounting elements on its left and right into the lower spring clips on the filter insert.
- Press the filter module against the upper spring clips until it engages firmly.



- 1 Knurled screws (2x)
- 2 Cover plate grip
- 3 Cover plate
- 4 Upper (rear) system door
- 5 Silver-colored lever

- 6 Guiding rail
- 7 Reflector turret
- 8 Short grip
- 9 Grip

**Fig. 40 Removing and inserting the reflector turret for laser blocking filter**

After replacing the reflector turret for laser blocking filter or individual filter cubes, it must be registered in the database with the Configuration Tool software or modified in the ZEN software under the Maintain tab within the Filters tool window (Fig. 42). The position number on the turret is on the left side of the filter. These numbers correspond with the numbering in the ZEN software.

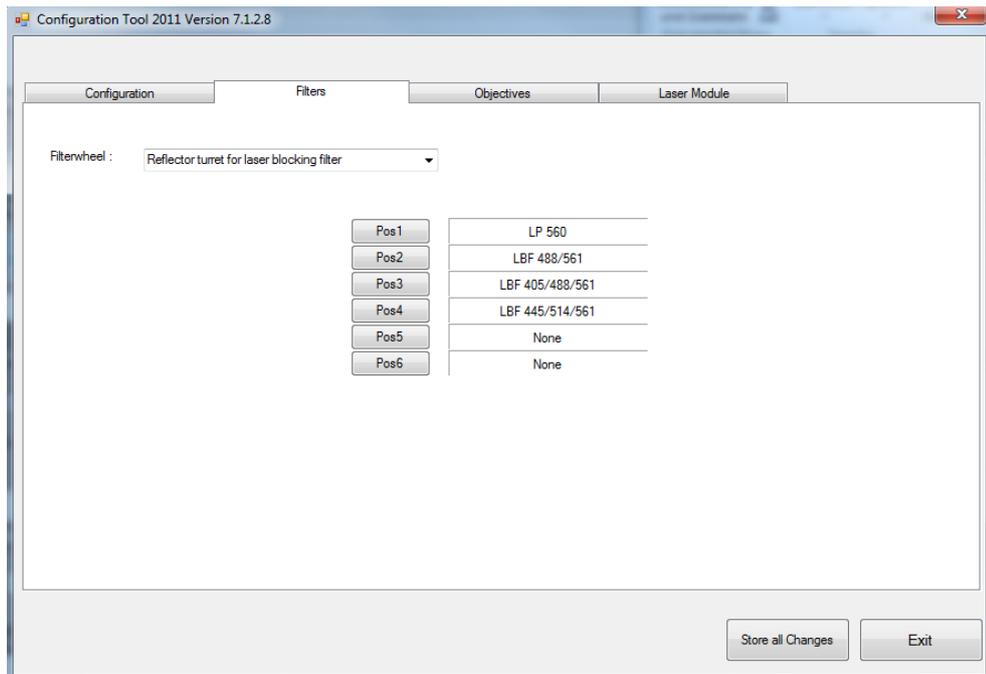


Fig. 41 Configuration Tool, Filters tab

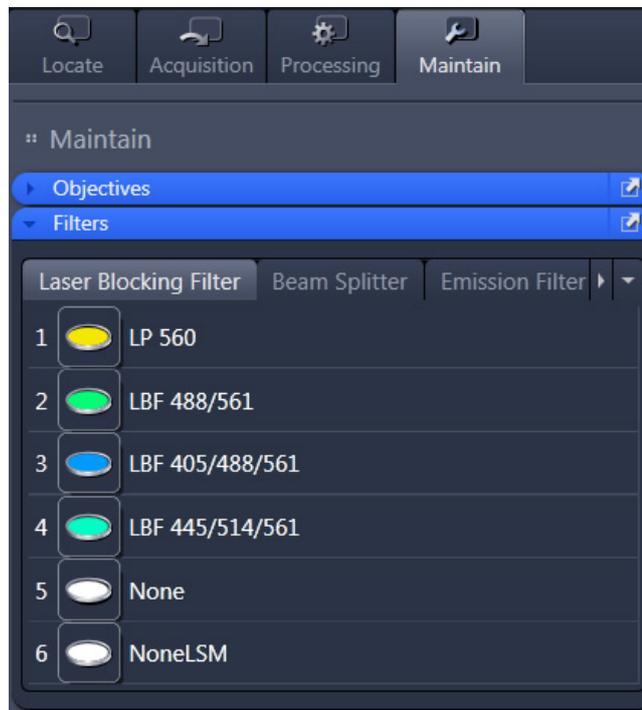


Fig. 42 ZEN software, Maintain tab, Filters tool window

### 3.12 Inter-Acquisition-Signal

The Inter-Acquisition-Signal (configuration dependent and not present with all Lightsheet Z.1 systems) is a level trigger with a high level of 3.3 V (nominal value of the high level:  $> 3.2 \text{ V} < 4.0 \text{ V}$ , and nominal value of the low level:  $0 \text{ V} \pm 0.4 \text{ V}$ ). The minimal working resistance is  $5 \text{ k}\Omega$ . The trigger can be gripped via a BNC connector, which can be found at the back side of the PC for system control. The BNC is connected to the PC via PIN 9 and 10 of the 44 pole SUB-HD port of an ISG adapter; Pin 18 is electrical grounding. The BNC connector comprises two outlets which transfer the same level trigger signal.

It is possible to choose the outlet to be used via the ZEN software (see as well **Time Series** in **CHAPTER 4 SYSTEM OPERATION**), so two different external individual installations can be setup, which can be chosen according to the experimental needs.

The level trigger marks the period between time points of a time series in which no image acquisition takes place.

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# CHAPTER 2 SAMPLE PREPARATION

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## 1 Introduction

*This section describes theoretical and practical aspects of sample preparation for Light Sheet-based Fluorescence Microscopy (LSFM). We present general rules for sample handling and mounting, as well as guidelines with respect to the best preparative technique to use, taking into account sample type, structure and properties. Step by step protocols and recommended materials for Lightsheet Z.1 samples are included. These protocols cover sample preparation ranging from micrometer-sized fluorescent beads to millimeter-sized insects, providing detailed information relating to preparation and observation techniques. Finally, this section identifies the main artifacts and problems that can result from the preparation techniques.*

A microscope generally performs best on suitable samples, and when the samples are optimally prepared for the imaging method and microscope type. In Light Sheet Fluorescence Microscopy (LSFM), the sample is commonly mounted in a liquid filled chamber and can be rotated easily. It is scanned through a sheet of light which illuminates the focal plane of a perpendicularly mounted objective lens. The resulting image of an optical section is observed through the objective and is usually detected on a camera-based detector. Since the geometry of the optical beam paths and the optics differ significantly from the conventional inverted and upright compound microscopes, the sample mounting protocols also differ significantly.

If the sample is perfectly transparent, like a block of 1 % agarose with beads inside, the light sheet can penetrate deeply and does not change its properties and shape along the illumination axis. Also, the fluorescent signal can reach the detector unperturbed by scattering effects in the specimen or hydrogel. However, if the sample is slightly opaque and diffracts or scatters the light sheet heavily (lipids, lipid vesicles or dense collagen fiber arrays that scatter light strongly) then the well-defined shape and thickness of the sectioning light sheet degrades along the illumination axis. In a second effect, the detected image from a well illuminated sample might still be degraded by such a poorly transparent sample. These effects can contribute independently to the final image quality of a LSFM and can be minimized or worked around by careful sample positioning in the microscope as well as by an optimized sample preparation protocol.

Ultimately, a fully opaque sample that can completely block the penetration of light and a light sheet (insect cuticular structures, bones...) will limit the imaging capabilities of Light Sheet Fluorescence Microscopes and the Lightsheet Z.1 to its surface.

Furthermore, the image quality in Fluorescence Microscopy in general – and in LSFM in particular - is not only determined by sample transparency that can be optimized by choosing a suitable model (transparent fish like Medaka), suitable growth conditions (no phenol red in the growth media to avoid autofluorescence) or, potentially, a clearing treatment (not suitable for the Lightsheet Z.1). It is also important to have a homogenous signal from a homogeneously labelled sample. Antibodies, for example, are rather large molecules that cannot penetrate deeply into tissue so it is difficult to image a complete juvenile fish after antibody as only the first 50 µm to 100 µm will be labelled, the interior showing reduced signal levels due to the limited diffusion of the antibody.

Samples must be carefully considered when using LSFMs such as the Lightsheet Z.1 as well as the label or dye used must be carefully chosen. In planning an experiment, it should be kept in mind that most labelling and imaging protocols have been developed for thin specimens and therefore many aspects are not adapted to imaging larger samples such as embryos, tissue slices or complete mosquitoes.

Many organisms have been imaged using Light Sheet Fluorescence Microscopy (Table 1) and you may want to read further specific papers to clarify sample preparation issues.

**Table 1**

Topic	Subtopic	Sample/Model Organism	Technique/ LSFM implementation	Reference
<b>Physics</b>	Technical set up of MISERB	Fluorescent beads	MISERB	Fahrback et al, 2010
	Structured illumination	Mouse cochlea	sTSLIM	Schroter et al, 2011
	Light Sheet Characteristics	Fluorescent beads	SPIM	Ritter et al, 2008
	Image formation	<i>Caenorhabditis elegans</i>	DSLIM	Olarte et al, 2012
	Image View fusion	live sea urchin embryo, live <i>Danio rerio</i> embryo	LSFM	Rubio-Guivernau et al, 2012
<b>Biochemistry</b>	Laser Microsurgery	In vitro microtubules	SPIM	Engelbrecht et al, 2007
	Microtubule dynamic instability	In vitro microtubules	SPIM	Keller et al, 2008
	mRNA nuclear export	<i>Chironomus tentans</i> Salivary Glands	SPIM	Siebrasse et al, 2012
	Heterochromatin dynamics	MCDK cells, <i>Drosophila melanogaster</i>	LSFM (FCS)	Capoulade, 2011
	Imaging of engineered gene expression	<i>Drosophila melanogaster</i>	SPIM	Ejsmont et al, 2009
<b>Microbiology</b>	Marine microbiology	Various bacteria, protozoa etc.	LSFM	Fuchs et al, 2002
<b>Cell biology</b>	Adaptive optics to improve imaging performance	Tumour spheroids	waoSPIM	Jorand et al, 2012
	Intracellular imaging	Mammalian cell organelles	Bessel beam plane illumination	Planchon et al, 2011
	Nuclear protein localisation	Cellular spheroids	SPIM	Zanacchi et al, 2011
	Imaging large living samples	MCDK cell cysts	SPIM	Verveer et al, 2007
<b>Plant Biology</b>	Live imaging of root growth	<i>Arabidopsis thaliana</i>	DSLIM	Maizel et al, 2011
	Consecutive imaging of vertically growing root	<i>Arabidopsis thaliana</i>	SPIM	Sena et al, 2011
<b>Developmental Biology</b>	Imaging of developing organs	<i>Danio rerio</i> heart valve	SPIM	Scherz et al, 2008
	Embryogenesis visualisation	<i>Drosophila embryo</i>	SPIM	Huisken et al, 2004
	Zebrafish development	<i>Danio rerio</i>	mSPIM	Kaufmann et al, 2012
	Cell identity lineaging and neurodevelopmental imaging	<i>Caenorhabditis elegans</i>	iSPIM	Wu et al, 2011
	Gene Expression: hour glass model verification	<i>Drosophila melanogaster</i>	SPIM	Kalinka et al, 2010
<b>Physiology</b>	Middle ear structure	Plastic Phantom Structure	(HR) OPFOS	Buytaert et al, 2007
	3D reconstruction of inner ear	<i>Cavia porcellus</i>	OPFOS; LSFM	Hofman et al, 2009
	Brain in vivo imaging	Microspheres	miniSPIM	Engelbrecht et al, 2010
	3D reconstruction for morphological analysis	Bast's valve	OPFOS	Hofman et al, 2007
	Scan of whole brain	Mouse brain	LSFM	Mertz and Kim, 2010
	Neural network imaging	Mouse brain	Ultramicroscope	Dotd et al, 2007
	Sectioning of thick tissues	Mouse cochlea/zebrafish inner ear, brain/ rat brain	TSLIM	Santi et al, 2009
	Imaging neuronal activity	Mouse vomeronasal cells	OCPI	Holekamp et al, 2008
	Imaging of immunolabelled receptors	Mouse	SPIM	Klohs et al, 2008
	Optical sectioning	<i>Meriones unguiculatus</i> cochlea, Hippocampus reidi head, <i>Xenopus laevis</i>	OPFOS	Buytaert et al, 2012

Topic	Subtopic	Sample/Model Organism	Technique/ LSFM implementation	Reference
<b>Large organism general biology</b>	Whole organism 3D reconstruction	<i>Ormia ochracea</i> ; <i>Emblemasoma auditrix</i>	LSP	Huber et al, 2001
	Whole organism 3D reconstruction	<i>Drosophila melanogaster</i>	Ultramicroscope	Jahrling et al, 2010
	Imaging copepod gut contents	<i>Calanus pacificus</i>	PLIF	Jaffe et al, 2009
	Whole organism 3D reconstruction	<i>Thermocyclops consimilis</i>	LSFM	Boistel et al. 2011

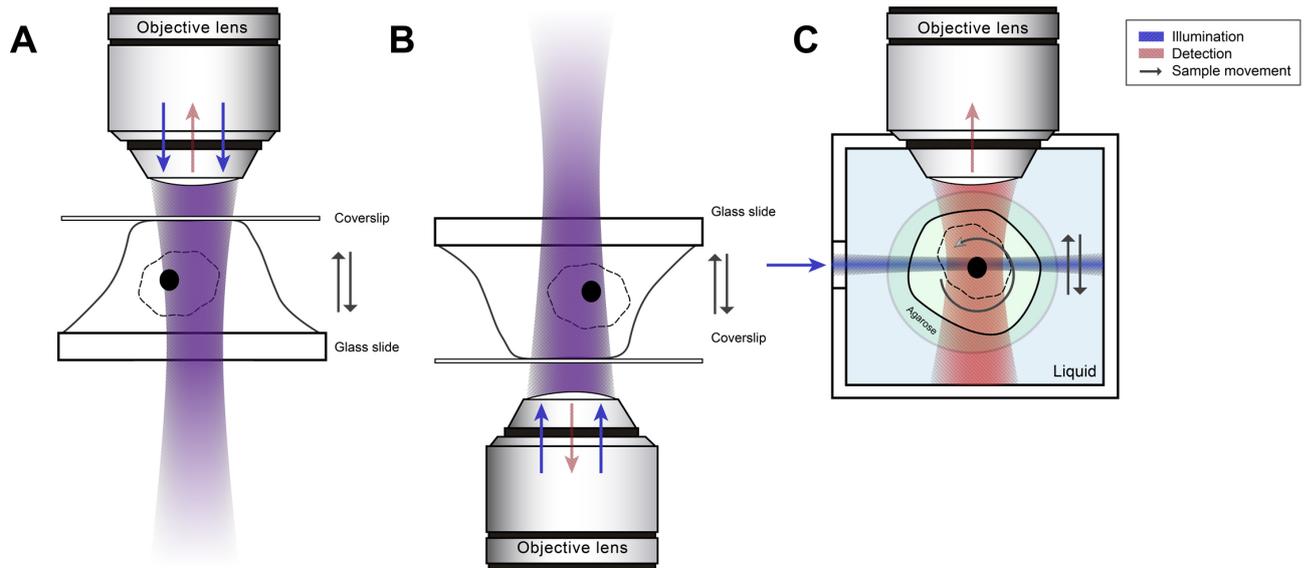
## 2 Sample Mounting for LSFM

-  For technical description of the sample chamber, incubation hardware and sample holder of the Lightsheet Z.1, you should refer to **CHAPTER 1 HARDWARE**.
-  The Lightsheet Z.1 system is optimized for gel embedded samples. The sample chamber must be filled with a watery solution (refractive index of 1.33) at all times, to ensure optimal image quality.
-  The Lightsheet Z.1 is not designed for the use with clearing reagents. The Lightsheet Z.1 is built for aqueous media. Also the sample chamber and the system cavity for the sample chamber and sample holder are not compatible with aggressive chemicals.

### 2.1 The perfect Sample for LSFM

Prior to observing a sample using a microscope, a preparation step is usually necessary. The sample properties and the microscope characteristics provide guidelines and also limitations to sample preparation and imaging. The classic method of mounting an object for microscopy requires the use of a slide and coverslip that in turn limits access to the sample from one side (Fig. 1/A and B). The sample is not touching the objective lens and the number of refractive barriers is at least two (coverslip, mounting medium) and can increase further if immersion oil is needed. The depth of the field of view is dependent on the type of objective lens and the sample properties, and will deteriorate with the thickness of the sample.

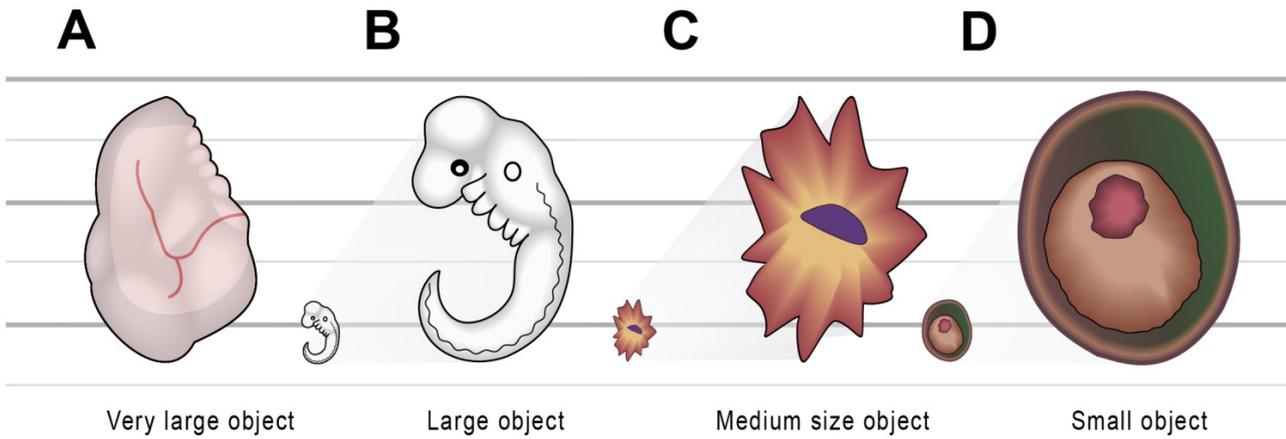
Light Sheet-based Fluorescence Microscopy (LSFM) utilizes illumination along an axis that is perpendicular to the detection axis (Fig. 1/C). Moreover, it usually allows sample rotation to record multiple image stacks that are acquired independently along different directions. To allow the high level of sample mobility required for such "Multiview" imaging, the sample is inserted in a sample holder from above. The sample holder is hanging in and linked to an x, y, z, and alpha positioning motor stage, allowing complete three dimensional translations and free rotation around an axis parallel to gravity. This configuration leaves the illumination and the detection paths completely open but requires the preparation of a sample that can be held from above or below in a medium-filled chamber. Such geometry goes hand in hand with the convenient use of water dipping or air objective lenses. As mentioned already in the introduction, another important aspect of sample preparation is the transparency of the specimen, especially when imaging large objects. Ideally, the light sheet penetrates as deeply as possible into the sample. Any obstacle or opaque medium will limit the light sheet penetration depth, generating shadows that will be read out as artifacts on the final image.



**Fig. 1 Relations between the sample and the objective in microscopy.** Samples are traditionally isolated from the objective by a glass coverslip (A and B) limiting access to one side only. (C). In LSM, the illumination is positioned at 90° compared to the detection axis and can be set up in a sideways geometry ("horizontal microscope").

In LSM, the sample is usually imaged in a water-based buffer. Generally, it can be kept dry and imaged in air but this has extensive limitations like diffraction due to the significant jump in refractive index from air to the sample material. This has several consequences for sample preparation. First, the refractive index of the mounting medium should be close to that of the sample buffer. The mounting medium should not scatter the illumination or the detection light. Second, the mounting medium should not dissolve in water. Third, its diffusive properties should be close to those of water/medium. Fourth, the medium should be non-toxic for live samples. Fifth, the medium should be flexible to allow the sample to develop. Finally, it should not change its mechanical properties during a period of observation (72 hrs and more).

The following part of this section will deal with sample as a general term but we have devised them in four main classes (Fig. 2) and you can check their size relationship (Fig. 2) and keep that in mind as different samples of different sizes will mean different sample preparation approaches and handling.



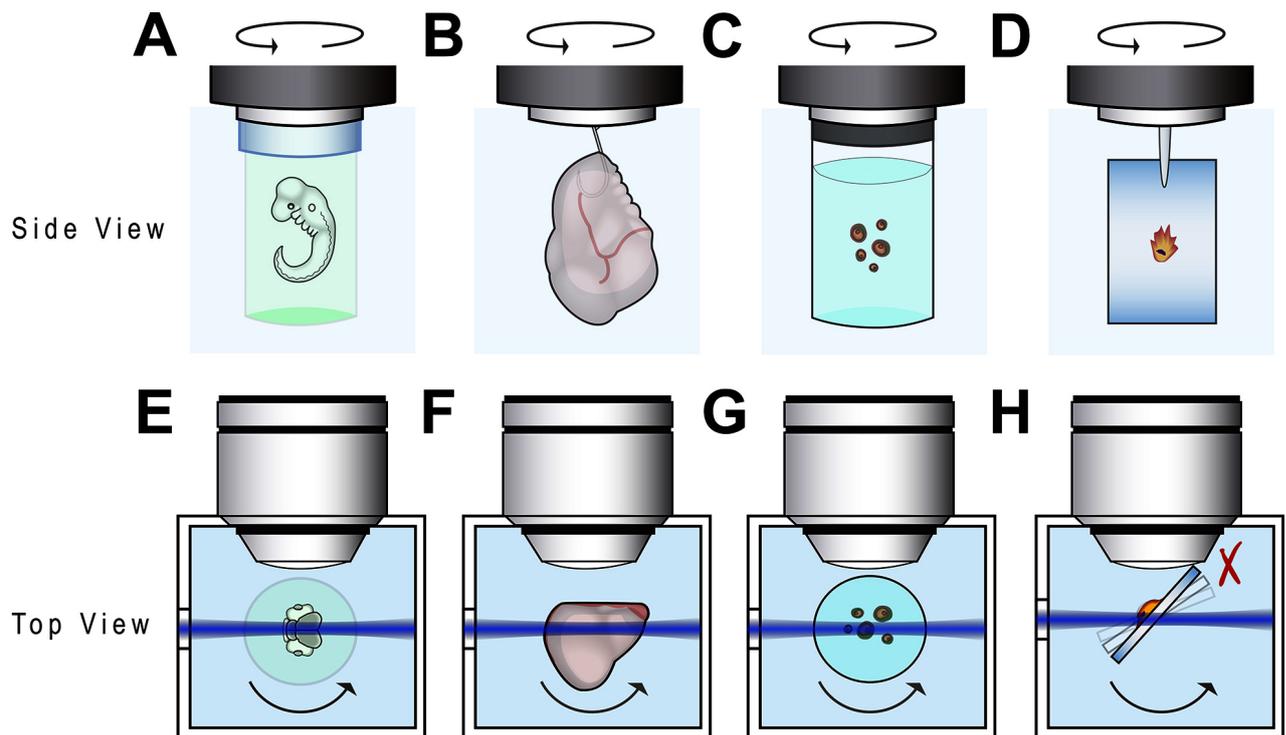
**Fig. 2 Different sample types.** (A) very large objects (cm), (B) large objects (mm), (C) medium size samples (100  $\mu\text{m}$ ) and small samples (10  $\mu\text{m}$ ) (D). Each is represented in relation to the following one to allow size comparison.

## 2.2 Holding the Sample

In LSFM, the detection axis is at  $90^\circ$  from the illumination axis. There are two main approaches to design such an optical configuration: horizontal or vertical, with respect to the detection axis. In both cases, the sample must be positioned at the intersection between the two axes in order to be observed. The Lightsheet Z.1 is a horizontal LSFM implementation and so the sample is presented from above, hanging along the gravitation axis to be scanned through the light sheet in order to acquire stacks of optical section images. Several possibilities exist to hold the sample in such an optical configuration.

In a vertical configuration, the simplest way is to place the sample on a slide or a cuvette filled with medium underneath the objective (Dodt et al., 2007), alternatively the sample can be embedded in a gel rod that can be rotated. In a horizontal configuration, like the Lightsheet Z.1, the sample can be either embedded in a stiff gel (Fig. 3/A) (Huisken et al., 2004) hooked and positioned in front of the objective (Fig. 3/B) (Engelbrecht et al., 2007), placed in a container (Fig. 3/C) (Engelbrecht et al., 2007, Kaufmann et al., 2012, Pampaloni et al., 2007) or placed on a slide and positioned at a  $45^\circ$  angle (Fig. 3/D). Alternatively, some investigators are using a system presenting the sample from underneath for better stability (Huber et al., 2001).

 The Lightsheet Z.1 is not designed to support mounting on a coverslip (Fig. 3/D).



**Fig. 3 Sample positioning in LSFM.**

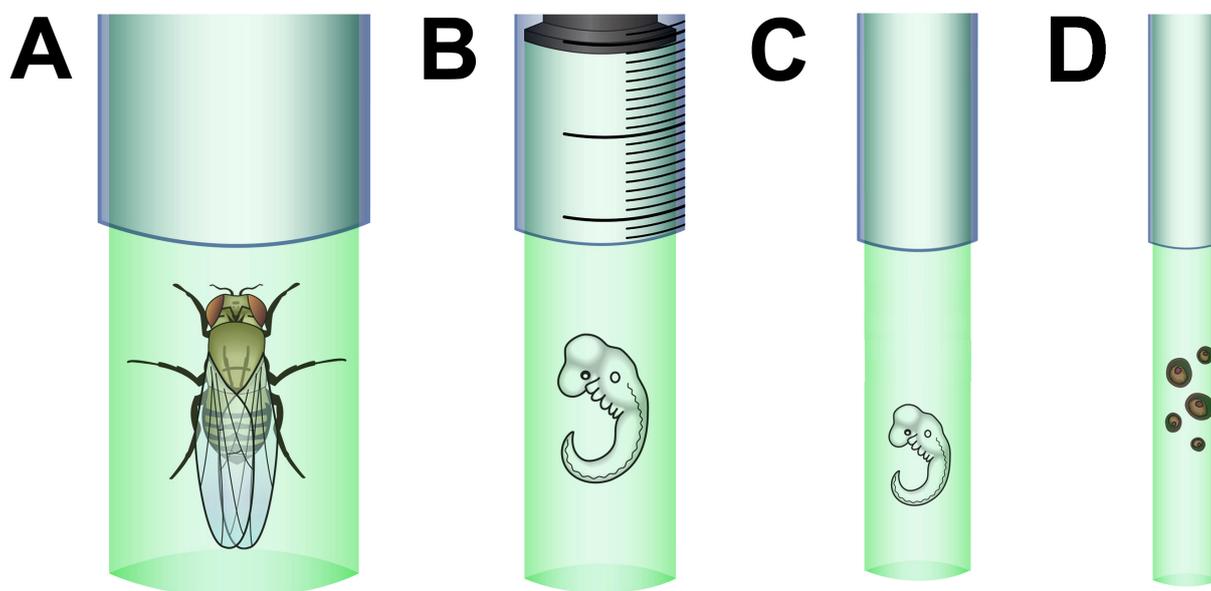
The sample can be held in front of the objective (A) embedded in gel, (B) by a clip, (C) in a container, or on a coverslip (D); Note: the Lightsheet Z.1 is not designed to support this way of mounting the sample). (E to H) show an eye bird view of the mounting (A to D).

Every mounting technique has some advantages and disadvantages. Here, we would like to mention one important parameter: the position of the sample relative to the objective lens. Gel embedding (Fig. 3/A and E) is usually safe but the capillary that holds the gel can potentially touch the detection objective. Such collisions are even more likely for hook (Fig. 3/B and F) and coverslip (Fig. 3/D and H) mountings. It is important to remember that in the Lightsheet Z.1 the sample can interact with the detection lens as well as the walls of the sample chamber and this could affect the imaging process.

One of the important advantages of the LSFM optics geometry is that it allows so-called Multiview imaging. In this case the sample must be mounted to support the required positioning. One approach used in Lightsheet Z.1 to support this experimental paradigm is to place the object in a gel rod that can be rotated (Fig. 3/A and E) in front of the objective. The hydrogel cylinder must be sufficiently stable to avoid movement during rotation. Typical preparation protocols use 0.8 % to 1.0 % agarose (see below in this section) to take this into account. The following sections will address the four main types of sample preparation that can be used: embedding, hanging, enclosing or flattening.

### 2.2.1 Embedded Samples

Embedding objects in plastic materials is a routine procedure widely used in the preparation of samples for electron microscopy. In the case of sample preparation for LSFM however, the immobilization of hydrated biological materials must not impair biological activity. It is necessary to keep the object we wish to observe in a perfect condition.



**Fig. 4 Embedded samples.**

Large samples, such as an adult *Drosophila melanogaster*, can be embedded in a large gel tube or a cut 1 ml syringe (A), intermediate size samples, such as a Medaka or Zebrafish embryo can be prepared by using either a cut 1 ml plastic syringe (see also Fig. 5) or a glass capillary (C and D) and small samples such as *Drosophila melanogaster* embryos or early stage cell clusters can be prepared using a smaller capillary (D).

In the case of LSFM, it is also necessary to contain the sample in such a way that it can be positioned and rotated in front of the objective. Furthermore, transparency of the mounting medium is essential to allow imaging. A basic technique of mounting objects for the LSFM is to shape them into a cylinder of gel (for example agarose, see also section **2.3.4 Gels and Polymers**) that can then be mounted on a dedicated holder. The Lightsheet Z.1 package provides four capillaries sizes adapted to the sample holder to embed objects of various sizes. The special sample holder of the Lightsheet Z.1 adapts to hold these capillaries for precise positioning (translation and rotation) of the cylinder-shaped object for observation through the detection optics. The used gel such as agarose behaves like mechanically stabilized water, supporting the object. It can be easily molded and the gel chosen should have an optical (refraction) index close to that of water. The object can be any size, as the gel can be molded accordingly (Fig. 4/A to D). The various gelling agents and polymers that can be used are discussed in greater detail in section **2.3 Materials and Equipment** (see below).

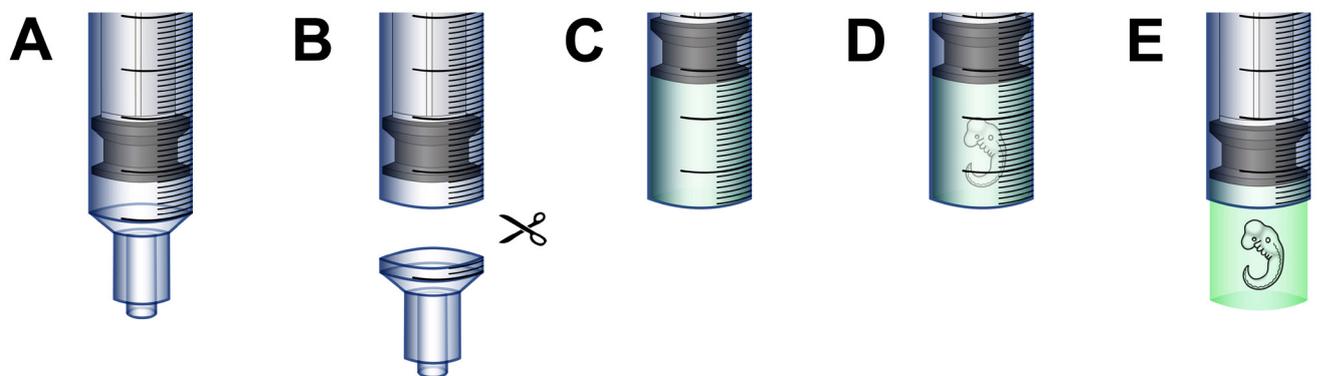
The preparation of embedded samples requires a container suitable for molding the gel. The simplest approach is to use any cylinder with a tight-fitting plunger to pump the molten gel into it and let it polymerize inside before pushing it out. The cylinder can be a syringe, a capillary or even a pipette.

In the case of Lightsheet Z.1, the Sample Starter Kit provides four types of color coded capillaries and a specific sample mounting device with color coded sleeves to fit each type of capillary perfectly to the sample holder. Moreover, a syringe sample holder is provided.

Below the preparation of a syringe for large sample and a basic protocol for capillary mounting are discussed.

An example of how to tailor a syringe for Lightsheet Z.1 sample preparation is illustrated in Fig. 5. The tip of the syringe is cut off to create an even cylinder, and the gel solution is pumped in using the plunger. The sample is then positioned precisely within the gel.

☞ After the gel has polymerized, the plunger is used to push out the specimen prior to imaging. Imaging is not done through the syringe or capillary, since this would impair the image quality due to the optical properties of the material.



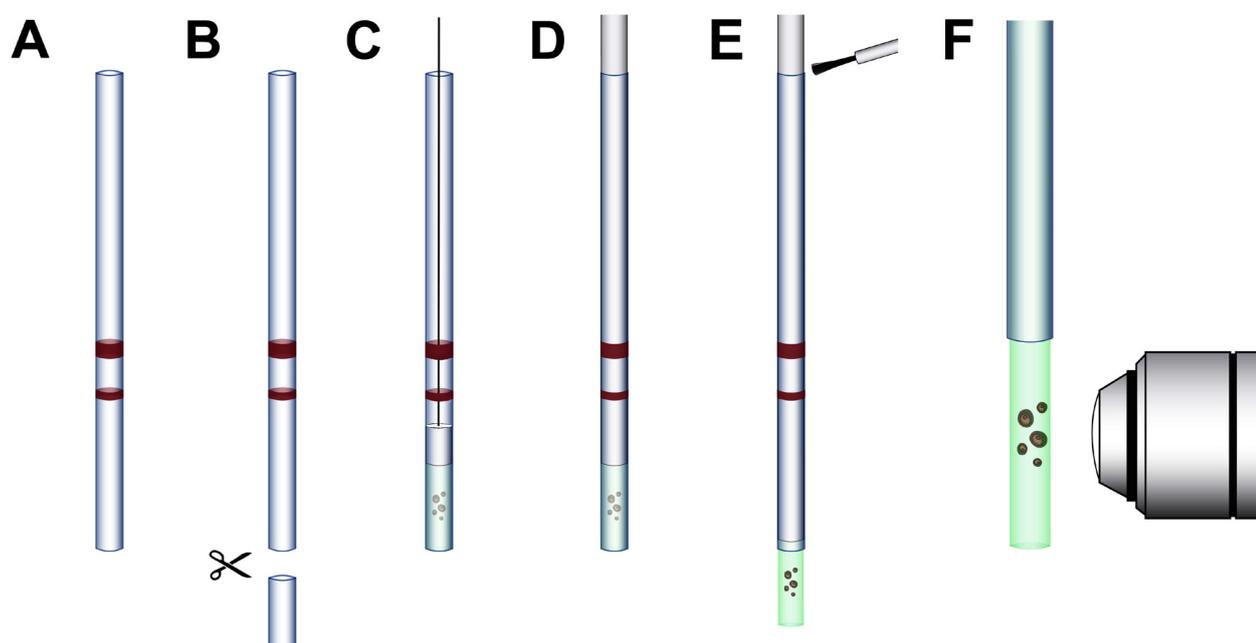
**Fig. 5** Preparing a sample embedding container with a syringe.

Many tubular objects can be used to make embedded samples. A simple technique is illustrated here. The tip of a syringe is cut away (A, B), agarose can then be easily pumped in using the plunger (C), and the sample can be positioned within the agarose tube (D). After polymerization the sample can be pushed out of the syringe for imaging (E).

For smaller samples, a capillary can be used as a sample embedding container. There are several commercial companies that provide glass capillaries with specific Teflon plunger. The Lightsheet Z.1 sample preparation kit comes with four sizes of capillaries and their specific plunger for this purpose.

☞ Make sure you use the right capillary for your sample. The sample size should not be more than  $2/3$  of the final agarose diameter and no less than  $1/3$ . You should also ensure that you use the right plunger for your particular capillary. Finally, the Teflon plunger should be handled carefully and checked regularly for integrity to avoid leaks that will lead to sliding of the gel rod.

The important points to consider are that the materials used do not interfere with the gel, the object to image or the sample preparation (chemical compatibility, melting point, transparency etc.), it must be easily prepared or easily purchased, it must be compatible to the LSFM sample holder as well as the x, y, z stage, it must fit to the sample chamber and should not cause damage to the objective lens once rotated or moved. It is also reasonable to consider reusable sample holders to limit waste. We have found that home-made sample embedding container using 1 ml syringes (BD Biosciences, Braun, Terumo or your local laboratory plastic ware supplier), 1 ml plastic pipettes (see your local laboratory plastic ware supplier such as Falcon or VWR), and glass capillaries (Brand, Sutter Instruments or check your local glassware specialist, see also section **4.3 Suggested Additional Sources of Information**) (Fig. 6) are particularly effective. The plungers usually come with the cylinders or can be made using metal rods, plastic or metal wires of an appropriate diameter.

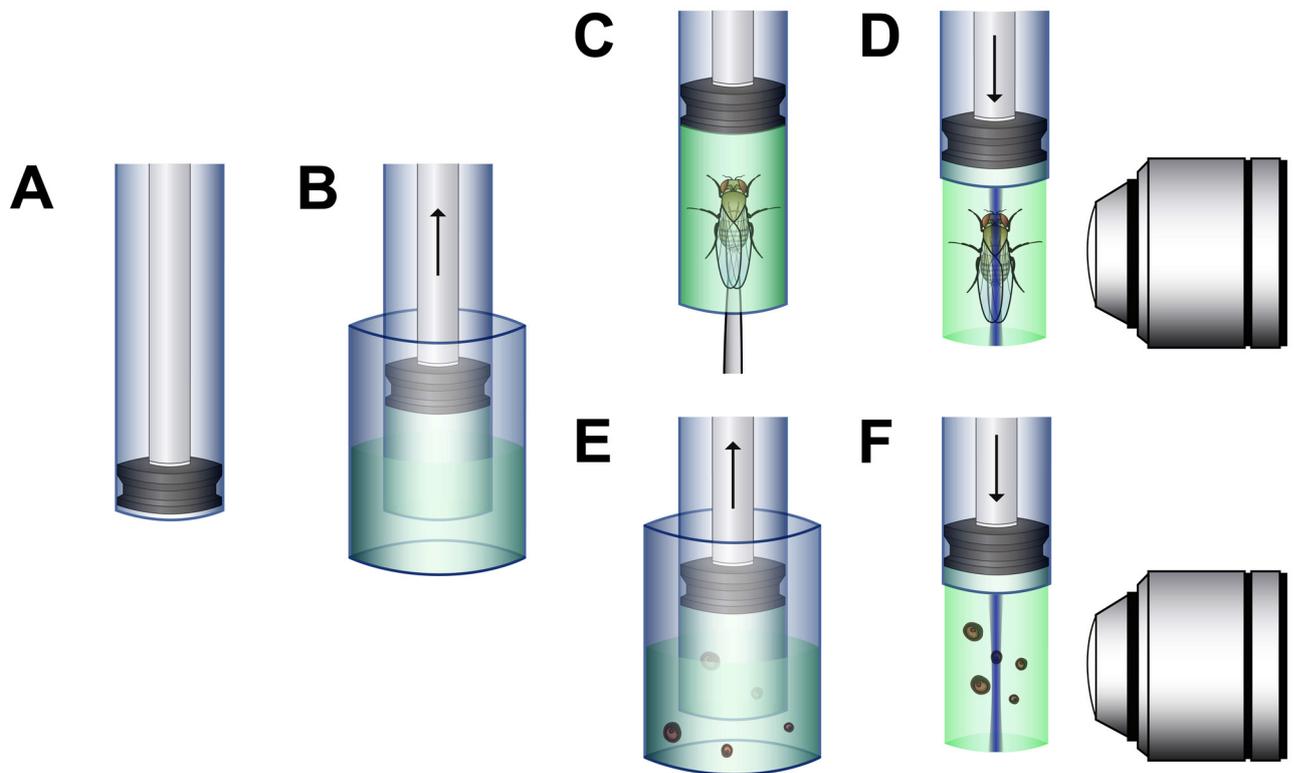


**Fig. 6** Preparing a sample embedding container with a capillary.

A glass capillary can be used. For the Lightsheet Z.1 they come from the manufacturer (Brand) in just the right length (A). Other capillaries can be cut to an appropriate length (B), the agarose with the object can be easily pumped in using the suitable plungers with Teflon tips (C). If no such plunger is available it can be made, for example, from a piece of electrical wire (D). To avoid leakage, such a plunger can be sealed with nail polish (E) once the sample is pushed out. The sample can then be imaged (F).

Once the sample embedding container is prepared, the sample preparation can begin. The first step consists of preparing the supporting agent at a suitable concentration and temperature. The gelling agent is usually a 0,7 to 1 % solution of low melting agarose in water or PBS, depending of the sample to be embedded (fixed, living, sensitivity to osmotic pressure etc.). If the sample needs to be maintained in a drop of solution or contains water or buffer it is advisable to use a higher concentration of agarose to obtain a final concentration of 1 % once the sample is embedded. The use of low melting-point agarose is recommended (Roth, n° 6351.1) as its melting temperature is only approx. 60 °C and it can be maintained liquid at just above 37 °C prior to embedding.

There are two principal methods of embedding an object. The first is to directly mix the object with the agarose then pump it into the sample embedding cylinder. This is a convenient way of embedding very small objects such as pollen grains (Swoger et al., 2007); yeast (Taxis et al., 2006) or cell clusters (Pampaloni et al., 2007) or even large objects like fish embryos. The action of pumping in the sample with the agarose results in a self-alignment of the specimen within the tube (Fig. 7/A, E and F).

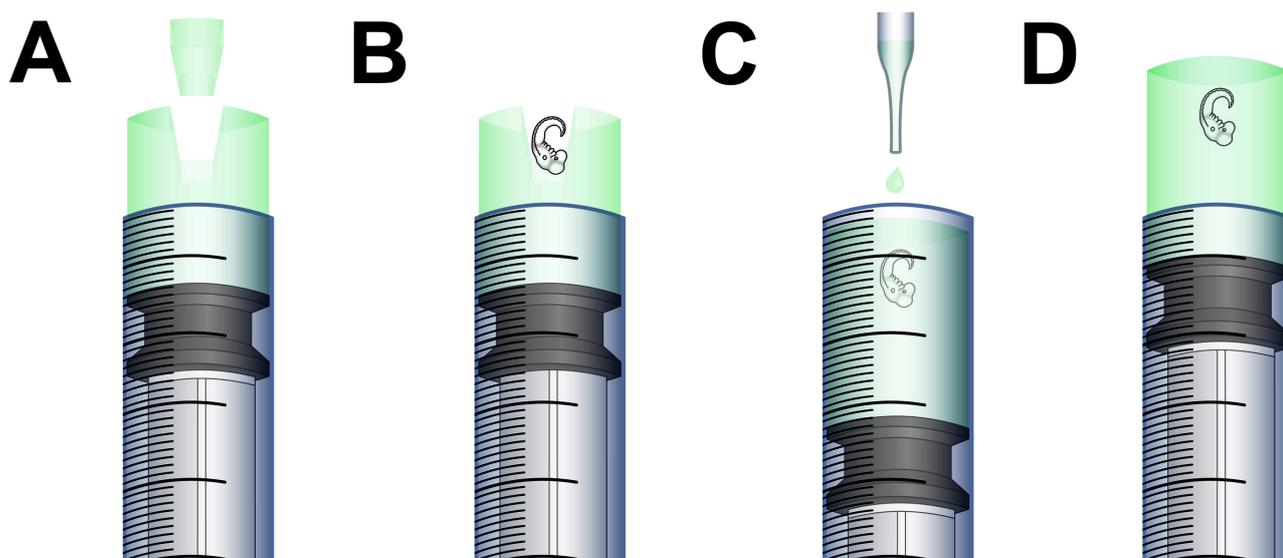


**Fig. 7 Basic principles of sample embedding.**

A cylinder with a suitable plunger is used as a mounting device (A). The 1 % low melting-point agarose is melted, then brought to 37 °C, then pumped into the cylinder. (B) The object is then introduced to the agarose with a needle or forceps. (C) Once solidified, the embedded sample can be pushed out and imaged (D). Alternatively, the object, devoid of water, or other solution, is added to a solution of 1 % low melting-point agarose at just above gelling temperature (typically 40 °C) and sucked into the cylinder (E) and then allowed to polymerize. The embedded sample can then be pushed out and imaged (F).

The second method is to fill the sample embedding container with the gelling agent, then to place the object within the gel using a needle or forceps (Fig. 7/A, B, C and D). This approach is more suitable for those samples that cannot be easily aligned using the first technique.

In some cases, it may still be challenging to align the specimen in the most suitable way for imaging. The orientation of the sample must then be optimized, so that interesting details are facing the surface of the agarose cylinder with as little material as possible in the optical path. One solution is to fill a syringe with agarose and allow it to cool until it solidifies. The agarose is then pushed out of the syringe (Fig. 8/A and B). A small V-shaped groove can be cut into the gel and the sample then positioned in the V-groove.



**Fig. 8** **Aligning an embedded sample.**

The sample can be aligned in a particular orientation to allow the details of interest to be close to the outer surface of the agarose. The solidified agarose is pushed out the syringe a few millimeters and a small v-groove is cut into the cylinder to take up the sample (A). The sample is placed into the v-groove (B). The sample on the agarose is pulled back into the syringe and more agarose is added (C). After the cylinder has completely solidified the sample is pushed out of the syringe allowing free sight on to the sample (D). The same approach can be used to carve a central tunnel in the middle of the agarose to align the sample along the agarose tube axis.

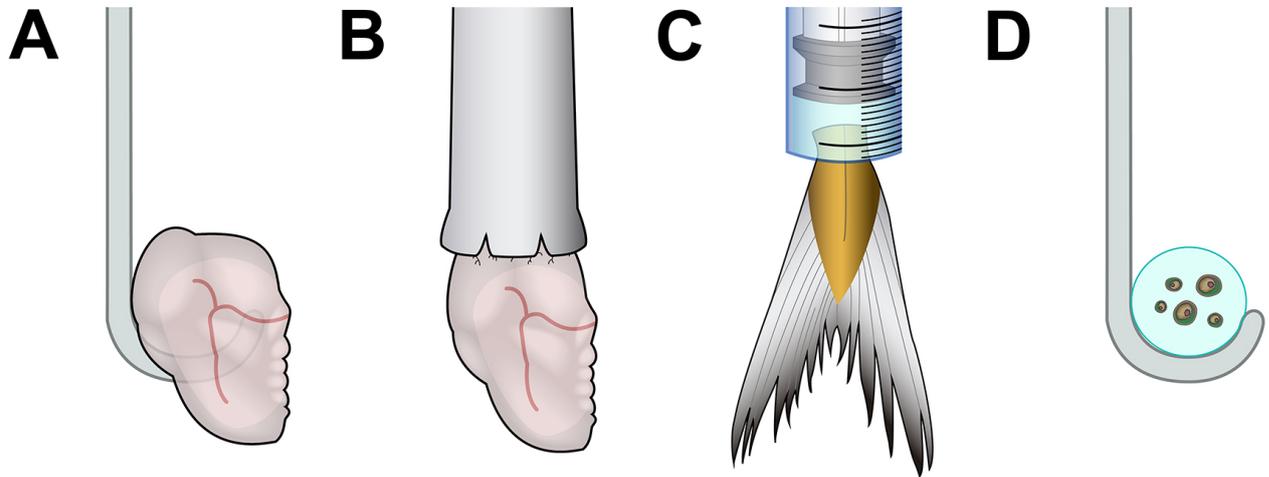
The gel can be cut into various shapes depending on the needs (cylinder, hole). Afterwards the gel with the specimen is pulled back into the syringe and is covered with more molten agarose. The agarose is allowed to cool and solidify, this time period can be shortened by cooling the whole sample. For example the housing of the sample can be rinsed with cold water, although care must be taken to ensure that the polymer does not come into contact with the water, otherwise the cooling agarose would become diluted and lose its stability necessary for holding the sample. After polymerization, the sample is ready for imaging.

### 2.2.2 Hanging Samples

- ☞ The Lightsheet Z.1 is optimized for gel embedding samples. The sample chamber must be filled with a watery solution (refractive index of 1.33) at all times, to ensure optimal image quality.
- ☞ This mounting technique can also be used, but will require some initial adaptations to the sample holder.

An intuitive way of imaging an object is to simply take it as it is and place it in front of an objective. In an LSM, this can be done by hanging the object in front of the objective where the axis of rotation and gravity are parallel. This can be achieved using a simple hook made of glass, stainless steel or plastic (Fig. 9/A). This mounting technique can be used for large samples such as organs (for example the brain) or complete organisms (insect, fish). One main drawback is the fact that the hook will partially damage the object and may also interfere with the field of view.

- ☞ The Lightsheet Z.1 has a maximum Field of View of approx. 2.5 mm (depending on zoom settings). This and the dimensions of the sample chamber might limit the size of the sample.



**Fig. 9** Different ways of hanging a sample.

Samples can be either hooked or deposited on a bent glass capillary (A, D), glued to a rod or capillary (B), or clamped on a syringe tip using the plunger and the syringe body as a holder (C).

Interestingly, such a hook can also be tailored to mount small objects embedded in agarose. The drop of agarose is more stable as it is closely held by the hook. This is very important when imaging at very high magnification (100x). The hanging method has been successfully used for imaging single *Saccharomyces cerevisiae* (Taxis et al., 2006) and holding fish fins during laser nanosurgery (Engelbrecht et al., 2007).

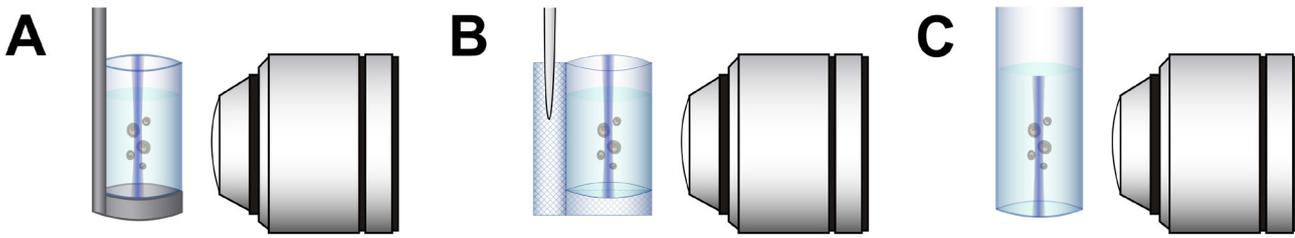
### 2.2.3 Enclosed Samples

- ☞ The Lightsheet Z.1 is optimized for gel embedding samples. The sample chamber must be filled with a watery solution (refractive index of 1.33) at all times, to ensure optimal image quality.

- ☞ This mounting technique can also be used, but will require some initial adaptations to the sample holder.

The last important technique of holding samples to be mentioned in this section is to create a container that can hold the object in front of the objective lens. This technique is particularly suitable for specimens that should not be embedded (for example due to temperature, physical constraints etc.) or that need to be constantly maintained in a specific buffer (for example in vitro assays, or living cells). The container must be suitable for LSFM imaging. It must be basically transparent and be suitable for the object but also for the imaging chamber and the sample holder. It can be hooked or clipped using specific holders.

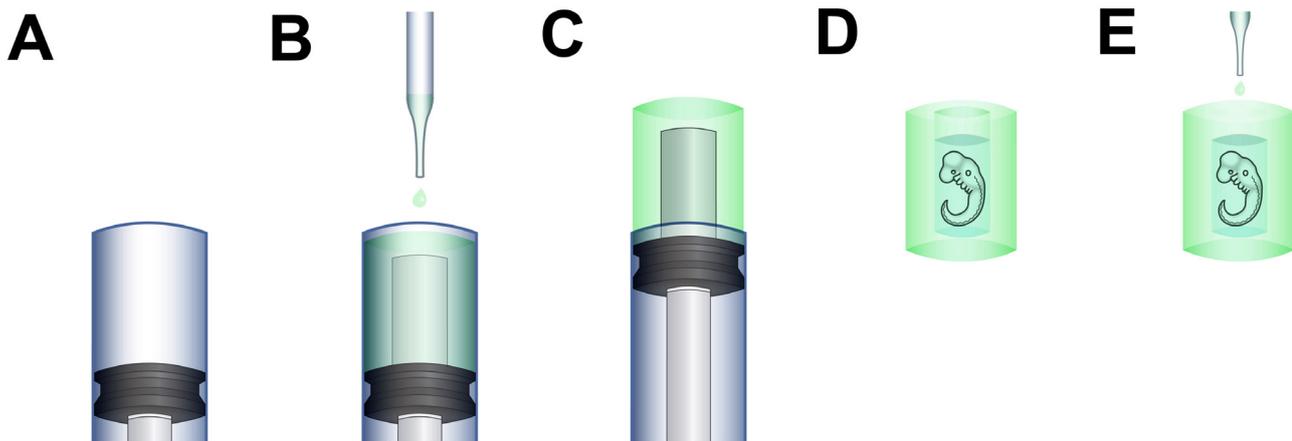
There are two main methods of generating such containers, using gelling agent to shape out a container (Fig. 10/A) or using polymers such as PTFE (Polytetrafluorethylen, Teflon) or FPE (Fluorinated Ethylene Propylene) to make it (Fig. 10/B and C).



**Fig. 10 Enclosed chambers for LSM.**

Incubation chambers can be made by molding an agarose beaker that can be mounted on a simple plastic holder and loaded with the sample prior to imaging (A). Another solution is to create a chamber with a specific polymer with a refractive index close to water (for example PFTE or FPE) using heat or glue to seal the chamber to the needed size and volume, and attach it to a suitable holder (syringe, capillary etc...) (B). A PDMS, FPE tube or glass chamber can also be considered (C).

The container can be easily molded using a gelling agent specifically chosen for its stiffness and transparency. The custom-made molding system is made from a syringe where the plunger has been modified to hold a cylinder of smaller diameter. This system allows tailoring of the size of the container wall and is easy to use for molding (Fig. 11/A). The plunger is pulled into the syringe body and filled with molten gelling agent. The plunger is further pulled to create the bottom part of the container (Fig. 11/B and C). Alternatively, the system can be used to generate a hollow tube that can be subsequently sealed (Fig. 11/D). The gelling agent is left to polymerize and then the tube is removed from the modified plunger. The containers can be used directly or kept in a water-based buffer for later use. The gelling agent must be transparent, and although the use of agarose is possible, the concentration will depend on the size of the container walls and the inside chamber. It is recommended to use a higher concentration of gelling agent to ensure the stability of the container. We have used a 1ml syringe as a molding system and a concentration of 1.5 % agarose for the container molding. The stability is good and the degradation of the optical path is minimal. Higher agarose concentrations may generate aberrations.

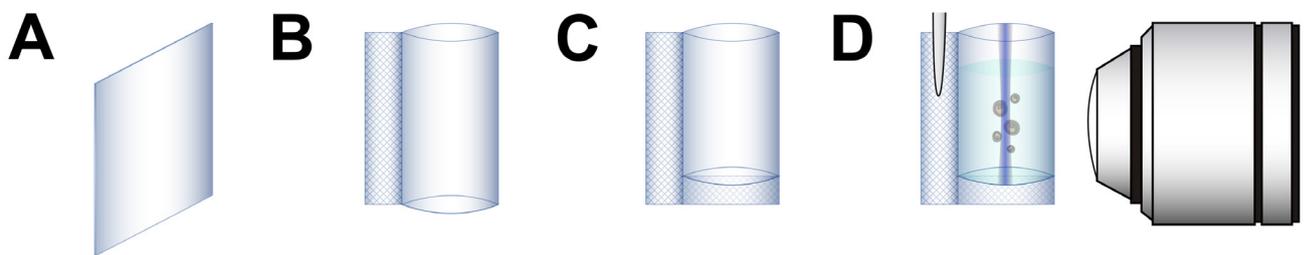


**Fig. 11 Making and mounting an agarose incubation chamber.**

A modified syringe plunger is made by inserting a smaller diameter cylinder on to the plunger (A, B). The tube is molded by simply pouring the molten agent into this device (C). Once removed, the open end of the container (D) can be closed with agarose (E).

Another possibility is to use a polymer to make the chamber. The polymer, similar to the gelling agent, must be transparent or at least have an optical index as close as possible to water or the buffer used during the experiment. The polymer is usually used as a sheet that can be formed as required. The other possibility is to approach commercial manufacturers to make polymer chambers at the specific sizes and lengths required.

Fusing polymer sheets can be done using a welding iron with controlled temperature or a welding device use for melting together plastic bags. As described in Fig. 12, the polymer foil is folded to an appropriate size. This can be made easier by using a guide or template, in this case a micropipette. The polymer is fused together. The tube generated is finally fused together on the other side to make a complete container. The polymer chamber can be easily mounted on the LSM by using a clip, a slotted metal capillary or glued to a micropipette. However, the last two options have the disadvantage of partially obscuring the field of view.



**Fig. 12 Making and mounting an incubation polymer foil chamber.**

A piece of polymer foil (A) is folded and either heat- or glue-sealed to generate a tube of a pre-defined size (B). Excess foil can be removed or used to glue the tube on to a specific holder (capillary, thread, metal rod...) (D). One side of the tube can be then glued or heat-sealed to close the chamber (C). The polymer used must be suitable for microscopy and easy to seal. The chamber can be glued to a support, held by forceps, or inserted into a slit rod.

This technique has been successfully used to image living cells (Engelbrecht et al., 2007) and cell clusters (Pampaloni et al., 2007).

#### 2.2.4 FEP Tubing

More recently, the availability of Fluorinated Ethylene Propylene (FEP) tube of different diameters has been successfully used for long term imaging of Zebrafish embryos (Kaufman et al., 2012). Here, we refer only to the paper by Anna Kaufmann, Michaela Mickoleit, Michael Weber and Jan Huisken in *Development* 139, 3242-3247 (2012) ("Multilayer mounting enables long-term imaging of zebrafish development in a light sheet microscope") and emphasize the fact that the mounting method described in this article is fully compatible with the Lightsheet Z.1.

## 2.3 Materials and Equipment

This section gives an overview on the materials and equipment for sample mounting and the sample chambers of the Lightsheet Z.1. The generalization of the concept is also mentioned.

### 2.3.1 Sample Chambers

In the Lightsheet Z.1, the sample is positioned within a chamber containing an aqueous solution. This chamber is tailored with O-rings to tightly fit the detection optics and avoid leakage. The upper part of the chamber is open to allow introduction of the sample. The bottom can be equipped with a Peltier Block or a Heatingblock (optional incubation). The remaining three sides are made in such a way that glass coverslips can be fixed allowing entrance of the light sheets from two sides and observation of the object by the user during the different steps of imaging using the appropriate software feature. The original chamber is made of medical steel, however, depending on the buffer used (salt, pH etc.), the experiment being performed (time lapse, live cell imaging etc.), there might be a need for more specific chambers. Carl Zeiss provides the technical drawing of the sample chamber for Lightsheet Z.1 so that users can develop their specific sample chamber<sup>1</sup>. You can refer to the sample chamber section of this manual for further information.

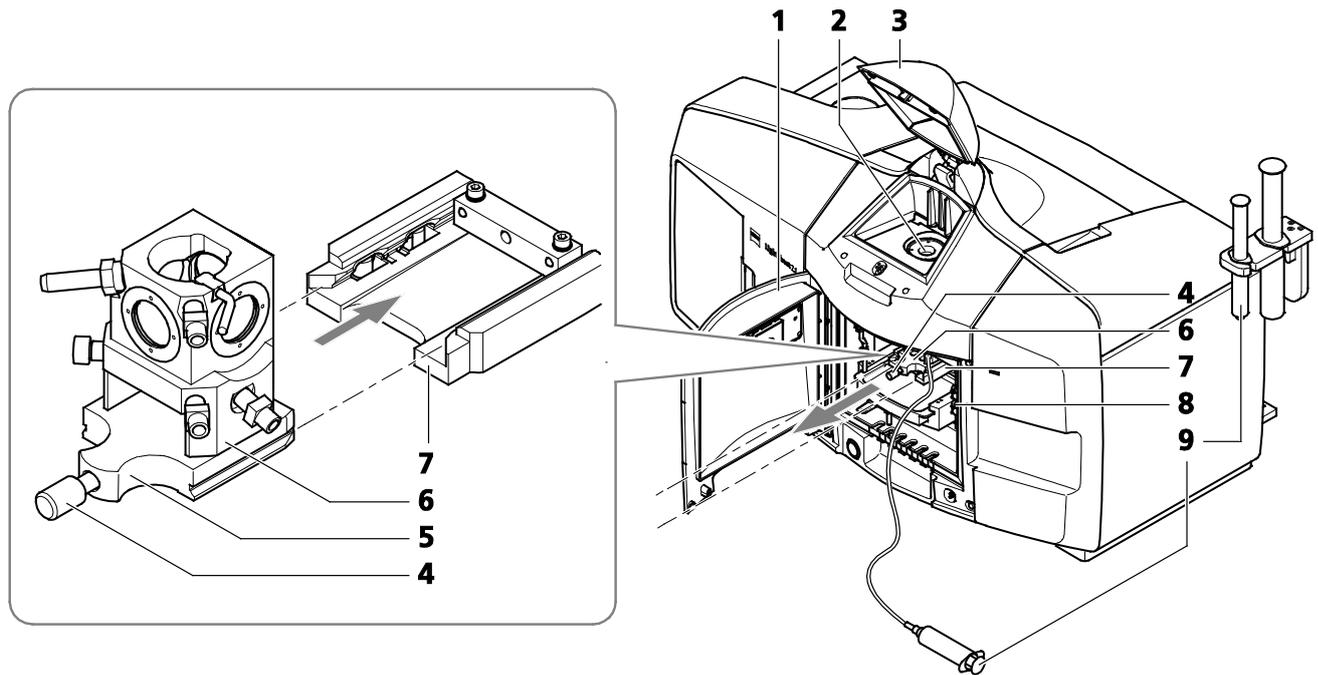
When designing a chamber for your particular application you must take into account the following points:

-  Transparency: user visual access, light sheet entry and exit routes, the distance of the coverslips for the light sheet – and the water filled space in between - are a crucial measure in the optics calculation of the Lightsheet Z.1 system. To ensure the functionality of the system these have to be maintained when a custom made chamber is designed.
  - Temperature control: heating devices, cooling devices
  - Volume: size of the sample, buffer used (cost), drug treatment (cost)...
  - Fitting: objective, illumination position, stage, heaters...
  - Material: buffer, heater, sterilization, UV protection...
  - Flow: flow entry and exit
  - Size
  - Cost

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<sup>1</sup> Carl Zeiss Microscopy GmbH (hereinafter "we") hereby informs you that we will warrant the specified and agreed performance of the Lightsheet Z.1 system only if sample chambers are applied and used that either are delivered or explicitly approved by us.

The sample chamber design has been optimized to ensure the most established applications of Light Sheet Fluorescence Microscopy. Exceptional applications may require a slightly modified sample chamber design. In order to enable customized modifications of the existing sample chamber we also provide the corresponding CAD file and a technical drawing. We explicitly advise you that already minor deviations of the dimensions and tolerances specified in these documents will cause a significant loss of image quality and can potentially result in a liquid leakage. Therefore, you will not hold us or one of our affiliates liable for any damages caused by the employment of self-built or third-party-built sample chambers, the use of such self-built or third-party-built sample chambers will be solely on your own risk. Furthermore we want to inform you, that we will not render any assistance relating to the production and application of such self-built or third-party-built sample chambers.



- |   |                          |   |                                  |
|---|--------------------------|---|----------------------------------|
| 1 | Front system cavity door | 6 | Sample chamber                   |
| 2 | Upper sample opening     | 7 | Guide rails/sample chamber mount |
| 3 | Upper system cavity door | 8 | Connections for incubation       |
| 4 | Securing screw           | 9 | Hose and syringe                 |
| 5 | Sample chamber grip      |   |                                  |

**Fig. 13 Removing and inserting the sample chamber.**

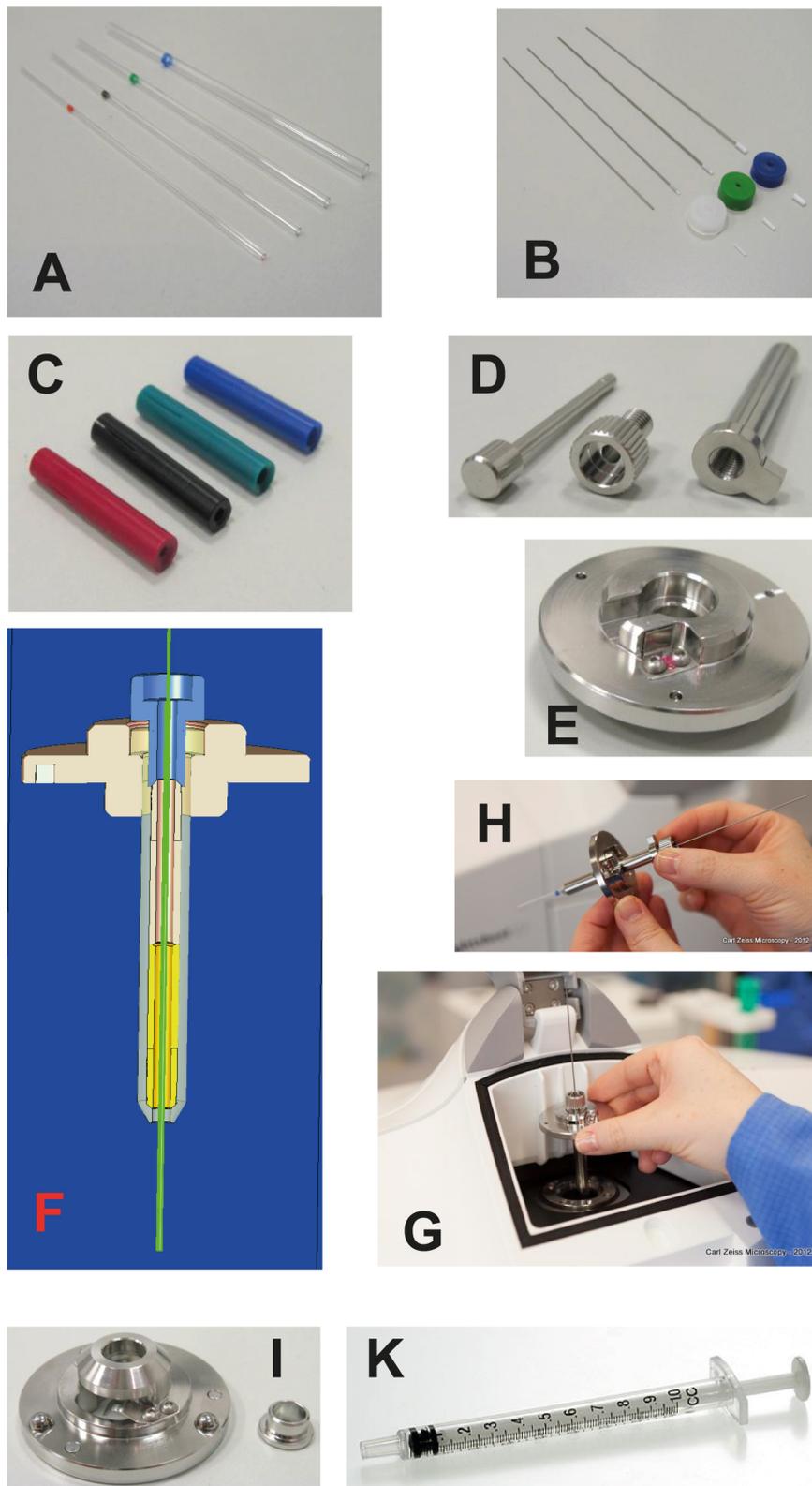
The chamber has five entry points allowing the positioning of the objective, the sample holder, the light sheet and the observation by the user. **Heated chamber.** The chamber can be equipped (optional) with a Peltier Block that can be tuned according to needs or a Heatingblock. For further details on the sample chamber handling, accessories, its cleaning and assembly please read the corresponding **CHAPTERS** of this manual.

### 2.3.2 Molding and Mounting Supports

As described previously, there are several options to prepare a sample and therefore several options to manipulate and mount it. Initially, readily available products found in cell biology laboratories: syringes, capillaries or pipettes were used to mount samples. These components are all commercially available, cheap and convenient for LSM sample preparation. However, they still need to be prepared for the specific needs. Plastic syringes exist in various sizes (0.2 ml, 0.3 ml, 0.5 ml, or 1 ml) and have tight plungers that easily allow pumping and movement of the agarose rod used to embed the sample.

They can also be used to hang the sample by effectively using the plunger and syringe body as forceps. The sample holder disc for syringes (Fig. 14/**I** and **K**) provided should be used in this case. Moreover, they can be purchased sterile for single use applications.

In the case of Lightsheet Z.1, the sample kit is provided with four types of color coded capillaries with matching plungers (Fig. 14/**A** and **B**) and color coded sleeves to fit perfectly each type of capillary to the sample holder (Fig. 14/**C**). The typical protocol of sample mounting is a two-step process of choosing carefully your sample mounting system based on your sample (size, agarose/sample ratio...) then to assemble it (e.g. plunger+tip+capillary) beforehand. Prepare it (e.g. sample + agarose) and insert it in the Lightsheet Z.1 upper sample opening (Fig. 14/**G** and **H**).



**Fig. 14** **Sample mounting accessories as part of the sample chamber and holder starter kit.**  
**A.** Capillaries (inner diameter of capillary size 1 / ~0.68 mm, size 2 / ~1 mm, size 3 / ~1.5 mm, size 4 / ~2.15 mm) **B.** Specific plungers and Teflon tips for each capillary. **C.** Specific color coded sleeves to adapt each capillary to the sample holder (F). **D.** Sample holder stem for capillaries, clamp screw, ejection tool. **E.** Sample holder disc for capillaries **F.** Sample Holder diagram showing the capillary, the stem and disc of the sample holder. **G** and **H.** Sample holder handling and insertion in the Lightsheet Z.1. **I.** Sample holder disc for syringes, adapter ring. **K.** syringe (1 ml).

A few points must be taken into account when choosing a particular mount:

- Compatibility. This is a crucial issue. The mount must be compatible with the object you want to image (chemistry, temperature etc.), but it must also be compatible with the stage holder.
- Stability (mechanical, optical, chemical).
- Tightness. In the case of embedded samples, once the gel has solidified, the cylinder of gelling agent is pushed through the capillary out of the distal end by a plunger fitting into the capillary. The system must be air tight to avoid air entry leading to a displacement of the gel rod. The plunger can be sealed with a drop of wax, acrylamide or nail polish, i.e. anything that prevents the plunger and hence the agarose containing the sample from moving.
- Cost.

### 2.3.3 Sample Holder

For technical description of the sample chamber, heating devices and sample holder, you can refer to **CHAPTER 1 HARDWARE**.

Once the specimen is prepared and properly labeled, it is ready to be imaged. While in conventional imaging there is a suitable platform on which to place the glass slide or the chamber, in LSFM the object must be held from above via the sample holder. Depending on the size of the sample there are two different types of sample holders available: sample holder for capillaries and syringes (Fig. 14/**C-F** and **I**). Always use the minimal cylinder diameter necessary for your specimen size to avoid excessive amounts of agarose.

The largest sample holder has been designed to accommodate a 1ml syringe that can be inserted from the top with a plunger that can be operated once the sample holder is mounted on the stage. Once inserted, the syringe is perfectly fitted to the sample holder as the two flaps used for injection fit the upper part of the holder. In this way the object support is well maintained, an essential issue for imaging and multiview imaging as the object is moved through the light sheet by the stage.

Capillaries have been extensively used to image small embedded objects, as hooks for very large objects, and as support for enclosed objects, so the capillary has become commonly used for LSFM sample embedding.



Capillaries are made of glass. They can break. They are also sliding when wet. Please handle them with care and dispose them properly.

### 2.3.4 Gels and Polymers

Gelling agents are commonly used for preparing semi-solid or solid tissue culture media. Gels provide support to tissues growing in static conditions. The gelling agent usually has several properties. In particular, it does not react with media constituents, is not digested by enzymes, and remains stable at all incubation temperatures. Gelling agents are very versatile and useful tools in LSM as they allow easier sample preparation. This section will present in more detail the properties, advantages and disadvantages of two well-described gelling agents and provides an additional list of gelling agents.

#### Agarose

Agarose is a complex carbohydrate polymer material, generally extracted from seaweed. It is used in chromatography and electrophoresis as a medium through which a substance can be analyzed by separating it into its components. The molecules are extremely water-soluble due to their large number of hydroxy groups, and solutions tend to be low-melting point aqueous gels. A wide range of different agaroses, of varying molecular weights and properties are commercially available. These include low melting types, (for example, Agarose Type VII, low melting temperature: gelling temperature below 30 °C, melting temperature above 65 °C) which can be used if the sample is sensitive to high temperatures. Interestingly, the refractive index of the low melting type is lower than that of normal agarose. However, to obtain the same strength, a higher concentration needs to be used. With a concentration of 1 % (w/w) the low melting point agarose has the same stability as a 0.5 % agarose (normal). The refractive index at this concentration is still lower than that of normal agarose, minimizing distortions when imaging. In our laboratory, we preferentially work with agarose as it is easy to handle, has good optical properties and is not expensive.

#### Gelrite

Gelrite gellan gum is a self-gelling hydrocolloid that forms rigid, brittle, transparent gels in the presence of soluble salts. Chemically, it is a polysaccharide comprised of uronic acid, rhamnose, and glucose. It is produced by the bacterial strain S-60 of *Pseudomonas elodea*. Gelrite is a trademark of Merck and Co, Inc (Rahway, NJ), Kelco Division, USA. One advantage of Gelrite is the lower scattering of light compared to an agarose gel with the same stability. It has a higher index of refraction but less scattering compared to agarose. Gelrite has a consistent batch-to-batch quality due to a stringent control of the fermentation process. Only half the amount of Gelrite is required for the same purpose. It hydrates rapidly and gel setting can be easily controlled. The stability of the gel depends on the concentration of divalent cations ( $Mg^{2+}$ ,  $Ca^{2+}$ ) therefore a gel made with Gelrite and pure water is unstable compared to a PBS- (buffer) based gel. Polymerisation is faster compared to agarose, which might be advantageous for some applications. The temperatures for gelling and remelting are similar to that of agarose.

Additional list of gelling agents

- Galactan
- Agar
- Gelatin
- Carrageenan
- Alginate
- Phytigel™
- Agargel™
- Transfergel™

### 2.3.5 Hydrogel Preparation

Every gelling agent is prepared following specific protocols that vary widely from supplier to supplier, and from laboratory to laboratory depending on the final application. We will not try to cover every single one of these but rather give a simple protocol that we have been using in our laboratory to prepare embedded samples in agarose as a gelling agent. The preparation is done as follows:

#### 1. Preparing a 1 % low melting agarose gel

Weigh 1 gram of low melting point ("low gelling") agarose ("Agarose Low Melt" (no 6351.1 from "<http://www.carlroth.com>") and place it in a flask. Add 100 ml of solvent (water, PBS) to the flask. Swirl to mix the solution.

Place the flask in the microwave. Heat above 95 °C until the solution is completely clear and no small floating particles are visible. Do not allow the agarose to boil over as this will affect the final agarose concentration. Swirl the flask frequently to mix the solution, prevent the agarose from burning, and prevent boiling retardation.

 Wear heat-protective gloves when handling the flask.  
The agarose can be also sterilized for sterile use (cell culture).  
It is also possible to remove dissolved air bubbles using a vacuum pump.

#### 2. Cooling the gel

Once molten the gel is left to cool to 37 °C (or just above gelling temp - read the material properties sheet) in a water bath or on a heating plate. It is very important, especially for sensitive samples, to ensure that the agarose is at 37 °C before use.

Note: Alternatively, you can aliquot your agarose solution into 1 ml or 2 ml Eppendorf tubes for later use. Label them and store them in a cool and dry place. In this case, each aliquot can be liquefied using a heating block (80 °C – 90 °C) then transferred to a heating block at 37 °C.

#### 3. Using the gel

At this stage follow the examples described in the section dealing with embedded sample preparation. Avoid bubble formation during handling and pipetting as they will impair the embedding process. Work quickly as the low melting point agarose will polymerize rapidly as it was kept at 37 °C close to the gelling temperature.

#### 4. Polymerization of the gel

Let the gel polymerize. Avoid contact with any water-based solution as it will dilute the gelling agent solution. The process of polymerization can be accelerated by cooling down the embedded sample (cold water, fridge...) – but keep in mind that this might affect viability of a living sample.

#### 5. Using the prepared sample

Once fully polymerized, the embedded specimen can be manipulated, but keep in mind that it is a gel and therefore fragile. Avoid any kind of friction, or shock etc. As it is a water-based object it must be kept wet at all times to avoid drying out and damaging the sample. Moreover, many types of gel may change their properties over time (e.g. swelling), and this can result in loosening of the gel in the support. It is therefore important to use your sample as soon as possible and monitor its quality over time if you plan to reuse it.

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## 2.4 Fixation and Fixatives

Many experimental samples will require fixation prior to imaging. The goal of fixation is to maintain cellular structure as close as possible to the native state. Proper fixation typically facilitates immunohistochemical analyses if desired, and is an important step prior to further processing. Specialized fixation procedures and processing may be required for certain tissues (e.g. bone de-calcification) or preserving specific target antigens.

The processing of most samples begins with fixation to preserve morphology. A fixation method must take into account two things: the preservation of cellular 3-D structure and maintenance of good access to antigenic sites. The goal is to preserve sufficient cellular organization to allow identification of the features of interest, but not to destroy the antigenicity of the target. Fixation is also frequently combined with permeabilization to allow the staining solutions used in later steps access to the cellular interior.

Commonly used histological methods of fixation and permeabilization often consist of treating the cells with solvents, such as methanol. While these methods are rapid-acting precipitating fixatives, they are also good permeabilizing agents, but have one significant negative consequence: cellular shrinkage. The degree of shrinkage may be almost insignificant for monolayers of cells, but will distort tissue samples dramatically. To take full advantage of the three-dimensional reconstruction capability of the LSM microscope, the use of a fixative that does not destroy *in vivo* structure and organization is imperative.

It is important to remember that different specimens may require different fixation methods. Testing and optimizing for each new sample type will ensure that the best balance between preservation and labeling is obtained. Fixing and permeabilizing your cells affects the cell morphology and the availability of the antigen you are trying to detect. You may get different results with different reagents, times and concentrations, hence the need for protocol optimization. The distortion of cell morphology is something to bear in mind when interpreting the images.

## 2.5 Stains and Staining

In LSM, like in any microscopy technique using fluorescence, the sample can be labeled using specific fluorescent dyes, fluorescent proteins or fluorescently coupled antibodies. Two basic techniques are generally used: direct labeling and indirect labeling. Both labeling methods are suitable for LSM microscopy. Direct labeling consists of using fluorescent proteins, fluorescently labeled primary antibody or a dye that cause the structure of interest to become fluorescent. Advantages of this method include speed and ease of application. A potential disadvantage is lack of sensitivity (low signal intensity). The indirect method involves binding a primary antibody to the epitope of interest, followed by a fluorescently labeled secondary antibody. The main advantage of using this technique is the great amplification of signal possible through an antibody cascade. The disadvantages include increased complexity, the method is more time consuming, and there are often problems with non-specific antibody reactions.

### 2.5.1 Choosing a Fluorescent Label

The choice of label depends upon the available equipment on your LSFM set-up (lasers, filters) and the availability of certain fluorescent protein variants, fluorochromes conjugated to required antibodies for use in multiple labeling schemes. In general, the laser lines available dictate which fluorophores or fluorescent proteins can be used. Recent advances in biochemistry have created new families of fluorophores with very favorable signal-to-noise and quantum efficiency (QE) properties. Similarly, many laboratories have developed a wide variety of fluorescent proteins that span the spectra from GFP<sup>2</sup> to Plum.

### 2.6 Antifading Agents

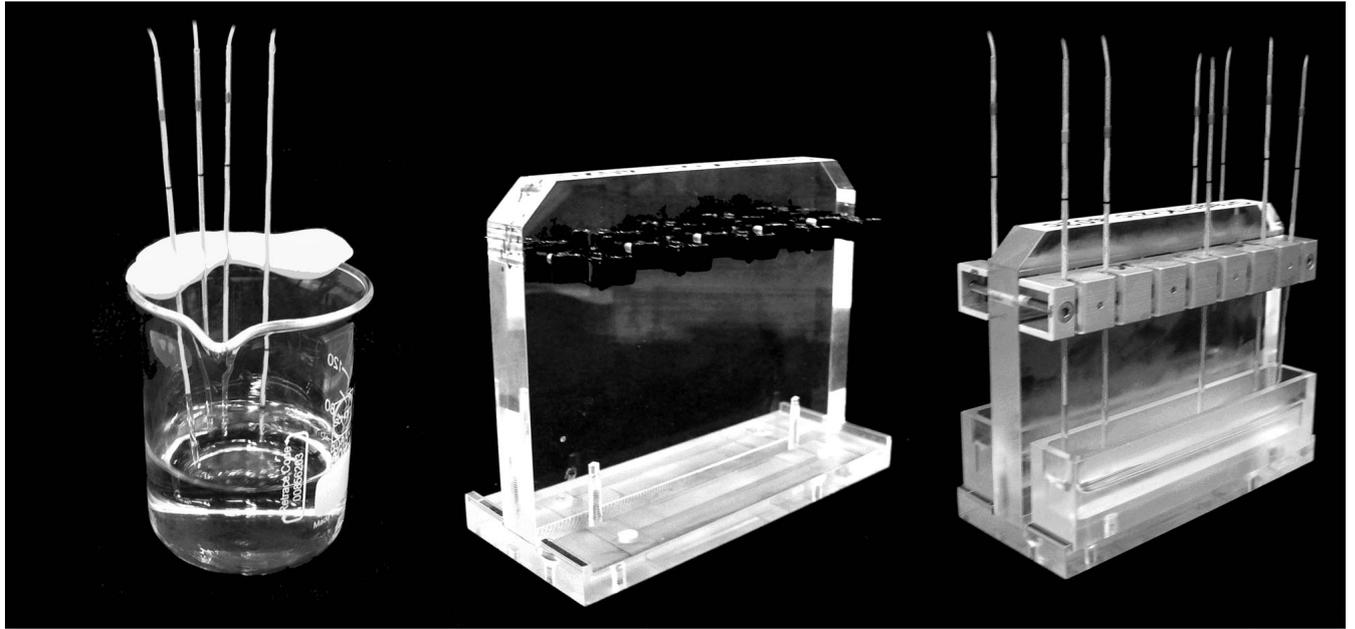
Fluorescently labeled cells and tissues exhibit a characteristic photobleaching curve in response to excitation by the light. Much of the photobleaching can be attributed to the generation of free radicals. The use of free radical scavengers has been shown to decrease the rate of photobleaching. Common scavengers include n-propyl gallate, p-phenylenediamine and DABCO (1,4-diazobicyclo-(2,2,2)-octane). Live systems have been reported to reduce photobleaching in the presence of vitamin C or Trolox. As the LSFM technology reduces greatly the phototoxicity and photobleaching effects during imaging, we never encounter samples that require the use of antifading agents so far. However, some applications may require the use of radical scavengers during long time imaging of GFP expressing samples as repeated exposure may lead to a regular increase of the free radical contents, which might affect its behavior over time.

### 2.7 Cleaning, Labelling and Storing Samples

One important point about samples is their handling. In the case of LSFM, all the samples are three dimensional objects that are mounted to be imaged in a chamber containing water based medium. They must then be maintained in a moist environment. Once prepared and prior to imaging, the samples can be held in a filled beaker or Falcon tube filled with the appropriate medium, e.g. water, PBS (Fig. 15). One simple solution we have developed in the laboratory is to use a beaker filled with the right buffer. The samples are maintained by using plasticine on the beaker border. An alternative is to cover the top of the beaker with an aluminum foil and accommodate the sample holders such as the 1 ml syringe by drilling a hole in the foil. This handling technique limits evaporation. More advanced holders can be designed and manufactured according to need. You will find a couple of examples that we have made in our laboratory<sup>2</sup> to handle various size of sample embedding containers. They include a water tank that keeps the samples moisture at all time. They are stable and can be easily move from the laboratory to the microscope as well as stored in the fridge.

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**Fig. 15** Supports for sample embedding containers.

Support are used to hold three dimensional objects that cannot be held flat easily. Moreover, embedded samples need to be kept in buffer to avoid gel shrinkage or sample damage. A simple system is to use a beaker filled with PBS and place plasticine on the upper border to support the sample embedding container (A). More elaborate supports can be made using clips of different sizes for holding syringes (B) or even capillaries (C).

As in LSM there is no need for oil or any specific chemical for imaging, the cleaning of samples is not necessary. However, you can rinse the sample within the capillary or syringe with water or your specific buffer after imaging if the chamber was containing particles, bacteria or other chemicals (dyes, drugs etcetera).

Labeling the samples can be an issue as it can be tricky to mark the name of every sample on the embedding container (capillary, syringe...). A simple marking technique is to use tape roll around the syringe plunger or the capillary. This must not affect the handling of the sample on the microscope. Another approach is to number the sample and to register the detail on a lab book. However, this can be a problem if you store many sets of samples in the same fridge day after day.

Sample preparation techniques usually allow long time storage (paraffin embedding, slides...). As long as a few basic rules are followed (keeping away from light, temperature...) they can be kept up to years. In the case of LSM, the samples are imaged in a water environment and must be always kept wet, even for long time storage. This can be a challenge. Usually, we keep fixed samples in the fridge using a sample embedding container support and we refill the buffer tank from time to time. However, we never kept samples for more than a month under such conditions. A longer storage possibility is to use a water tight container where the samples are kept with enough water not to dry out. One point to consider is the way the sample was prepared. Embedded samples may weaken with time as some gels may not maintain their strength over time at 4 °C. Hooked samples may as well be loosening and fall from their support. It may be better to unhook them and store them in a different type of container.

### 3 Specific Examples of Sample Preparation

In order to make this sample preparation section as useful as possible the following pages describe mounting techniques for specific samples, in particular describing the equipment needed, step by step protocols and illustrations based on our own laboratory<sup>3</sup> experiences.

 Fluorescent beads are used for later Landmark Registration processing of acquired multiview data, and should be included during embedding of samples of interest. Prepare agarose as described in section **3.1 Preparation of Fluorescent Beads** accordingly.

#### 3.1 Preparation of Fluorescent Beads

Samples with fluorescent beads are often used to characterize the imaging properties of a microscope such as the LSFM. Using a reproducible sample is an important tool to calibrate the instrument. This protocol describes how to handle fluorescent beads and to prepare optimal concentrations to image with an LSFM.

##### Equipment and reagents

- Fluorescent beads
- 1 % Low Melting Point (LMP) Agarose in deionised water
- Capillary (Size 4, Blue, #701910, BRAND GmbH)
- Sonicator
- Heating block- Vortex

##### Method

1. Vortex the bead solution to make a homogeneous dispersion.
2. Dilute a small volume of the bead dispersion in deionized or distilled water to a concentration 100x higher than the one desired for the specimen. Depending on the size of the beads and the magnification required it is first necessary to calculate the bead-agarose ratio (see below).
3. Sonicate the dilution for 5 minutes at maximum power.
4. Prepare a liquid agarose solution of a chosen concentration (0.5 % - 1 %) and cool it down to just above the gelling point (usually 38-40 °C).
5. Mix diluted fluorescent beads with the agarose in ratio 1:100 and vortex the mixture. Use a pipette or a capillary (by sucking in and out the liquid agarose several times) to mix the bead solution and the agarose thoroughly.
6. Insert an appropriate plunger and Teflon tip.
7. Push the plunger through the capillary, so the front end of the plunger is sticking out of the capillary by a bit before entering the liquid agarose and sucking the agarose in. This will avoid air bubble formation at the plunger.

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8. Suck in the agarose/beads by pulling the opposite end of the wire/plunger.
9. Let the gel polymerize (approx. 5 minutes) before imaging.
10. Make sure that only a very short part of the agarose block is pulled out of the glass capillary during image acquisition.
11. When multiple views are recorded, it is best to image from the centre of the agarose block.

 Beads should be fluorescent in the part of the spectrum you would like to analyze for 1 channel systems. With the Lightsheet Z.1, a two channel system, one can use one channel for the beads (e.g., red) and one channel for the specimen label (e.g., GFP.) Fluorescent beads covering whole visible spectrum are nowadays easily available from various different suppliers (e.g. Polysciences, Invitrogen, Estapor / Merck etc.). In our case, the density of the fluorescent beads is chosen to end up with several hundred beads in the imaged volume. For example, for a 40x magnification lens the volume of interest is around  $(200\ \mu\text{m})^3 = 8 \cdot 10^{-6}\ \text{ml}$ .  $(200\ \mu\text{m})^3 = 8 \cdot 10^{-6}\ \text{ml}$  If the fluorescent beads are shipped as a solution of  $5 \cdot 10^{13}$  particles/ml  $5 \cdot 10^{13}\ \text{particles/ml}$ , you have to dilute them 1:10<sup>6</sup> **1:10<sup>6</sup>** in agarose to have approximately 400 particles in the volume of interest. Having too few of them (less than 100) in the three-dimensional image will give you no or poor processing results, while too many of them (more than 1000) might considerably increase processing time without a significantly improving the final results.

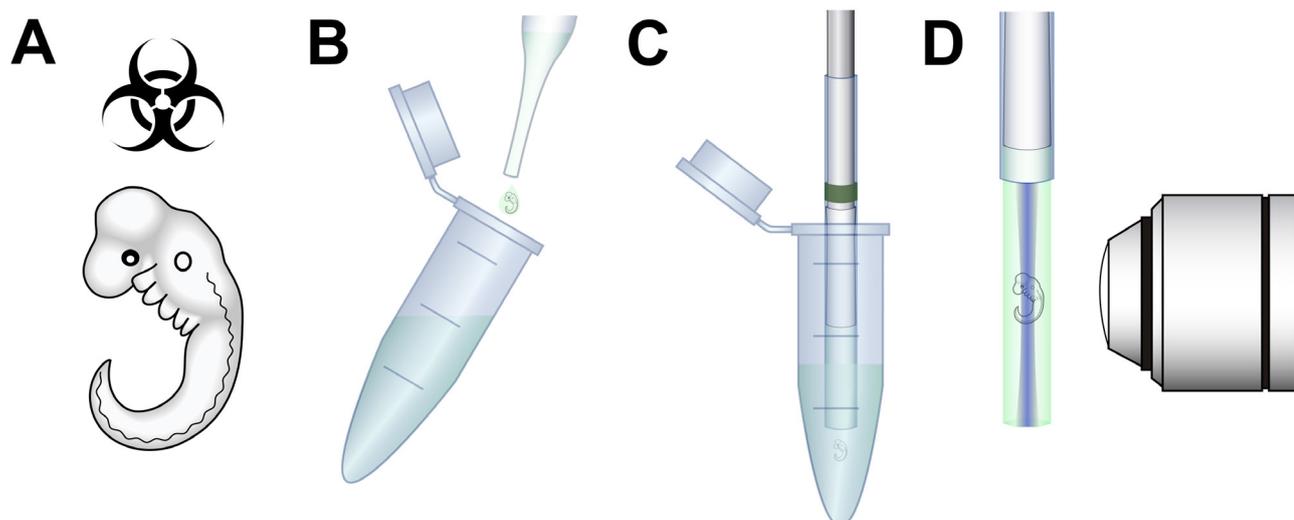
Moreover, a gel with sufficient stiffness but minimal impact on the image has to be used to immobilize the beads. 1 % low-melting agarose (Sigma, Type VII) is being routinely applied for lenses with numerical apertures up to 0.8 NA. For 1.0 NA objective lenses and above, a more diluted (e.g. 0.5 %) gel must be used to minimize gel-caused image aberration.

### 3.2 Preparation of a Medaka Fish Embryo (*Oryza latypes*)

Embryos have been extensively used for many decades to study developmental mechanisms as well as diseases. They can range from micrometers to centimeters depending on the species used (frog, fish, fly, worm, etc.). This protocol applies to most embryos. The important point is the temperature. The embryo must not be damaged by temperature shock during embedding. Moreover, the embryo should not be constraint by the stiffness of the gel. This may impair its normal development.

#### Equipment and reagents

- 1.5 % Low Melting Point (LMP) Agarose in E3 (Fish buffer)
- Mesab/Tricaine 0.4 % stock (3-Aminobenzoic Acid Ethyl Ester)
- Capillary (Size 4, Blue, #701910, BRAND GmbH)
- Electrical thread (1,6 mm) or plunger
- Heating block



**Fig. 16 Mounting an *Oryza latypes* embryo.** (A) The embryo is prepared (labelling, drug treatment, dissected...) (B) The embryo is deposited on the side of the Eppendorf tube and the excess of water is removed with a pipette. (C) The embryo is dropped into the agarose and pumped into the capillary. (D) The embryo can be imaged.

1. Select embryos for imaging, dechorionate. Melt 1.5 % LMP agarose, aliquot 0.5 ml into a 1.5 ml Eppendorf tube. Add 150  $\mu$ l of Mesab to ensure that the embryos do not move during imaging. Invert the tube to mix and allow agarose to cool to 40° C.
2. Add the embryo to the tube containing agarose using a Pasteur pipette, transferring as little buffer as possible.
  - ☞ Add the embryo to the Eppendorf tube as a drop on the tube wall. If necessary remove the extra buffer with a yellow tip before dropping the embryo into the agarose.
    - Or transfer the embryo to an empty Eppendorf tube, remove all medium and add the liquid agarose.
3. Let the embryo fall to bottom of the Eppendorf tube. Insert a capillary into the tube and suck the embryo into it by pulling out the thread or plunger like a syringe piston.
  - When sucking up the agarose, make sure that initially the plunger is sticking out of the capillary within the liquid agarose, to avoid air bubble formation. Furthermore leave some space between the plunger and the sample (see Fig. 16/D)
4. Allow the agarose to harden and place the capillary in a stand in water or PBS.
5. Mount on the capillary sample holder prior to imaging.

### 3.3 Preparation of a Fly Pupa (*Drosophila melanogaster*)

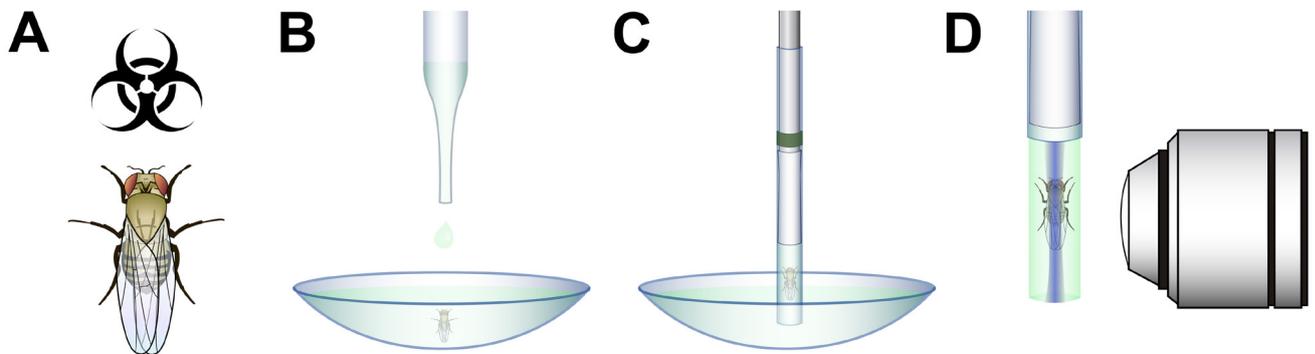
Some type of embryos are hydrophobic once dissected and cannot be mounted using the technique described above as they will float on the agarose and will be impossible to embed. The following protocol is suitable for this type of embryo such as fly embryos or pupas.

#### Equipment and reagents

- *Drosophila melanogaster* pupa or embryo
- 1 % Low Melting Point (LMP) Agarose in water or PBS
- Capillary (Size 4, Blue, #701910, BRAND GmbH)
- Heating block (90 °C and 40 °C)

#### Method

6. Choose a pupa *Drosophila melanogaster*. Melt 1 % LMP agarose, aliquot 0.5 ml into a 1.5 ml Eppendorf tube. Invert the tube to mix and allow agarose to cool to 40° C.
7. To allow sample preparation the pupa must be submerged in agarose by pouring it directly on top of it in a large drop of molten low melting point agarose.
8. The pupa can then be pumped into a capillary as previously described.
9. The insect can then be imaged.



**Fig. 17** Mounting a *Drosophila* pupa.

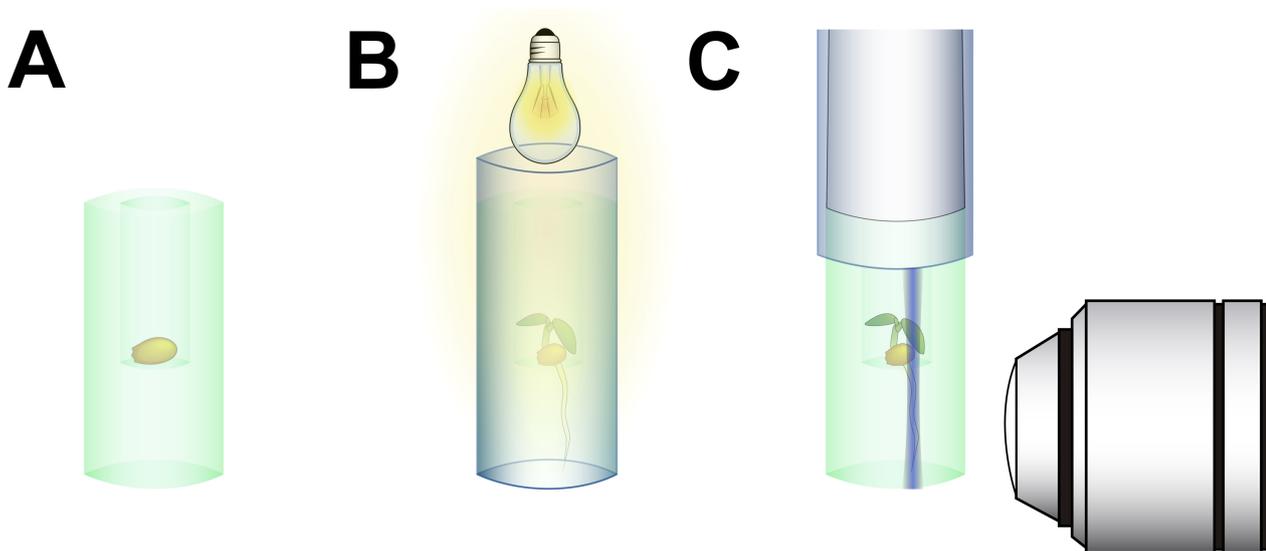
(A) The pupa is prepared (labeling, drug treatment, dissected...) (B) The pupa is deposited in a watch glass and covered by melted agarose to embed the hydrophobic pupa. (C) The pupa is then pumped into the capillary. (D) The pupa can be imaged.

### 3.4 Preparation of a Plant Root (*Arabidopsis thaliana*)

Plant research is an important field of investigation using plants as model systems. They are three-dimensional objects that are difficult to image fully and are usually dissected and sliced before being imaged and analyzed. This protocol has been used to mount complete young *Arabidopsis thaliana* plants for imaging root development directly on the microscope.

#### Equipment and reagents

- 1 % Low Melting Point (LMP) agarose in plant buffer
- 1 ml syringes
- *Arabidopsis thaliana* seeds
- Heating block



**Fig. 18** **Mounting an *Arabidopsis thaliana* root**

(A) An *Arabidopsis thaliana* seed is positioned at the bottom of an agarose cylinder (B) After a few days of development, the root can be seen in the agarose cylinder bottom. (C) The agarose cylinder is pushed out of the syringe for imaging.

#### Method:

1. Several agarose beakers are prepared as described in the enclosed sample section.
  2. Instead of pushing out the plunger to extract the agarose beaker, the plunger is pulled in to the end of the syringe where it can be released leaving the beaker inside the syringe.
- ☞ You need to make a long walled beaker to avoid inconvenient breakages and leakages that may be caused by the following steps.
3. A seed of *Arabidopsis thaliana* is put at the bottom of the beaker.
  4. The beakers are kept in the syringe in a humidified and well lit chamber to allow seed germination.

5. Once the root is visible within the bottom part of the beaker, a normal plunger is inserted in the top part of the syringe, where the open part of the beaker is present.
6. Push down the beaker into the syringe until the root can be seen outside of the syringe cylinder
7. The beaker is mounted in the sample chamber filled with distilled water or plant growth media at room temperature.

 As the plant depends on light to grow long-term imaging must take into account the illumination of the leaves between imaging sessions.

### 3.5 Imaging Cell Cysts in an Extracellular Matrix Gel

Live imaging of cells has been a major tool in cell biology. For this, cells must be maintained in optimal conditions during the complete time of the experiment. The incubation options for the Lightsheet Z.1 are described in another section of this manual (**CHAPTER 1 HARDWARE**). However, cells must be mounted in a way that allows them to hang in front of the objective from above. This protocol describes one way of imaging MDCK cells that naturally form cysts when grown in an extracellular matrix.

#### Equipment and reagents

- MDCK cells grown in an extracellular matrix (Matrigel, ExtraCell etc.)
- 1.5 % Low Melting Point (LMP) Agarose in PBS
- Modified plunger
- Sealing device
- Slitted capillary
- 1 ml syringe
- Capillary holder
- Heating block
- Polytetrafluorethylene foil or FPE tube

#### Method

1. An agarose beaker or a polymer foil chamber is prepared as previously described in the section **2.2.3 Enclosed Samples**.
2. MDCK cysts are grown in an extracellular matrix gel.
3. Cells can be stained at this stage with live markers (nuclear, mitochondrial, lysosomal etc.) before mounting.
4. Cells within the gel are transferred into the chosen chamber (agarose, polymer) and mounted in a 37 °C and CO<sub>2</sub> chamber on the LSM using a cut tip to limit shearing damage.

- ☞ If cells are grown in a different manner it is possible to mix them with a supporting gel prior to loading into a chamber. They can also be grown within the gel already present in an incubation chamber. This limits damage, shear and temperature changes during sample preparation and handling.
- 5. The agarose incubation chamber is mounted on a specific holder. The polymer chamber can be either clipped or glued to a supportive holder.
- ☞ Eukaryotic cells are highly sensitive to environmental change (temperature, pH, osmotic pressure etc.). The transfer steps must be rapid and carried out in a sterile manner (wherever possible) especially for long term time lapse experiments. It is important to be gentle and use cut tips and pre-warmed materials at all times, including the sample chamber.
- 6. Monitor the cell status during imaging to check viability and changes.

### 3.6 Immunostaining and Preparation of MDCK Cell Cysts

Immunofluorescence allows highlighting of specific proteins or structures using specific antibodies. This protocol is used to perform immunofluorescence on cysts which are three-dimensional cell structures that can be grown in extracellular matrix gel such as collagen.

#### Equipment and reagents

- 1.5 % Low Melting Point (LMP) agarose in water or PBS
- Capillary (Size 4, Blue, #701999, BRAND GmbH)
- Electrical thread (1.6 mm) or plunger
- 4 % paraformaldehyde solution
- Antibodies (primary and secondary)
- PBS
- Triton X-100
- Bovine Serum Albumin (BSA) or Foetal Calf Serum (FCS)
- Heating block

#### Method

1. MDCK cell cysts grown in extracellular matrix are collected and centrifuged at 500-1000g to pellet the cysts with the gel.
2. The supernatant is removed and replaced with 4 % paraformaldehyde and incubated for 15 minutes on a wheel or rocker to efficiently mix the gel pellet within the fixative.
3. The gel is pelleted and the supernatant is replaced by 0.1M glycine to quench the paraformaldehyde, and then incubated for 10 minutes.
4. The gel pellet is washed twice with PBS (500-1000 g, 5 minutes).

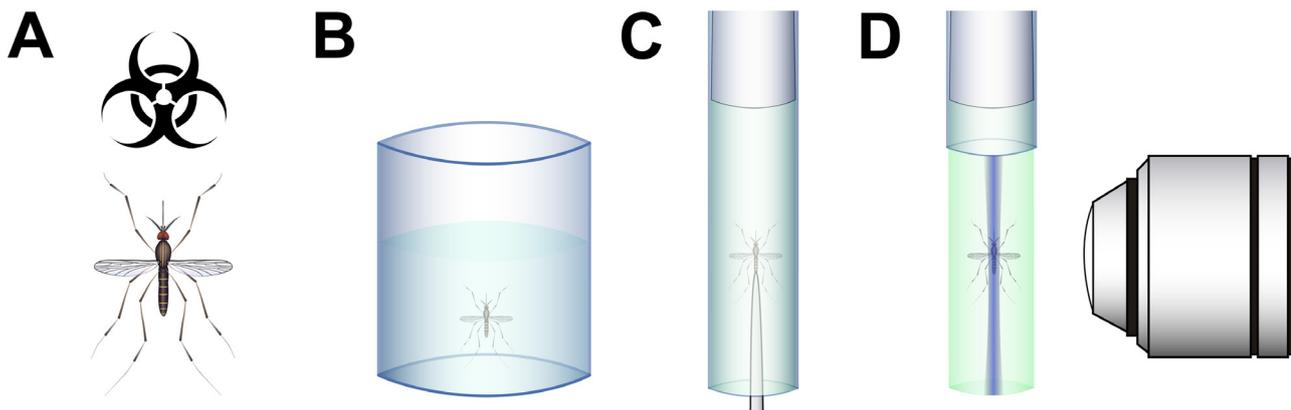
5. The pelleted cysts are permeabilized with PBS/1 % Triton X-100 for 10 minutes on a wheel or rocker to efficiently mix the gel pellet.
  6. The gel pellet is washed twice with PBS (500-1000 g, 5 minutes).
  7. The gel is incubated for 10 minutes in PBS/1 % FCS on a wheel or a rocker to block the extra epitopes and efficiently mix the gel pellet.
  8. The gel pellet is incubated with the primary antibodies at the concentration indicated by the supplier, using a wheel or rocker to efficiently mix the gel pellet.
  9. The gel pellet is washed twice with PBS (500-1000g, 5 minutes).
  10. The gel pellet is incubated with the secondary antibodies at the concentration indicated by the supplier, using a wheel or a rocker to efficiently mix the gel pellet.
  11. The gel pellet is washed twice with PBS (500-1000 rpm, 5 minutes).
  12. The cysts can be stained at this stage with Hoechst to label the nuclei.
  13. The gel is pelleted and as much of the supernatant as possible is removed.
  14. The gel pellet is mixed with low melting point agarose, mixed and pumped into a capillary.
-  The extracellular gel tends to clump once fixed and may stay as one piece when mounting. Care should be taken to quickly but efficiently resuspend the gel.
15. Allow the agarose to polymerize.
  16. Fill the chamber with PBS prior to introducing the sample for imaging.

### 3.7 Preparation of a Whole Mount of a Mosquito (*Anopheles gambiae*)

Adult insects are very large objects that can be up to 5 cm long. Moreover, they have an exoskeleton made of chitin which is hydrophobic and autofluorescent. This characteristic allows imaging without labeling by simply using the chitin autofluorescence to image the insect surface. This protocol applies to every type of adult insects as well as for various type of plankton.

#### Equipment and reagents:

- An adult *Anopheles gambiae*
- 1.5 % Low Melting Point (LMP) agarose in water or PBS
- 1 ml syringe (BD Biosciences)
- Ethanol (70 %)
- Glycerol (50 %)
- Sucrose
- Heating block



**Fig. 19** **Mounting a complete adult *Anopheles gambiae*.**  
(A) The insect is paralyzed. (B) The chitin surface is treated using 70 % ethanol. (C) The insect is positioned within the melted agarose. (D) The mounted insect is ready for imaging.

#### Method:

- Choose an adult *Anopheles gambiae* and immobilize it by cold treatment. Melt 1.5 % LMP agarose, aliquot 0.5 ml into a 1.5 ml Eppendorf tube. Invert the tube to mix and allow agarose to cool to 40° C.
- To avoid bubble formation on the insect surface that will affect imaging, the insect must be treated either with ethanol 70 % (animal death) or using 50 % glycerol or 1 M sucrose to cover the hydrophobic chitin surface prior to embedding (Fig. 19/B).
- The syringe is prepared as previously described and filled with molten low melting point agarose (40 °C).
- The insect can then be inserted into the agarose cylinder and aligned using a needle or forceps (Fig. 19/C).
- The insect can then be imaged (Fig. 19/D).

☞ This technique can be applied to any insect or similar type of organism possessing an exoskeleton. Depending on the animal part to be observed the insect can be aligned differently or dissected prior to embedding (head, wings, guts, salivary glands...).

---

## 4 Tips, Troubleshooting and Additional Information

### 4.1 Tips

#### Protocols

As a general rule, follow the protocol carefully and try it out well in advance as you may encounter difficulties and missing parts (chemicals...) that may hinder you to use the sample straight away. The protocols presented in this section are not the ultimate solution for every problem. They can be improved and adapted to your need. If you encounter a problem, check the scientific literature and the other protocols presented and see if you can find a tip that you may apply to solve your problem. Be creative.

#### Safety

Please refer to the safety instructions provided with the Lightsheet Z.1.

#### Setup Requirements and Maintenance

For optimal performance of the Lightsheet Z.1, please check the Setup Requirements information delivered with the system.

For information about maintenance and cleaning any part of the Lightsheet Z.1, including the optics, please refer to the according **CHAPTER** in this manual.

#### Refractive index mismatch

Light is refracted when it crosses the interface between two media of differing refractive indices (RI). Mismatching the refractive index of the objective immersion medium and mounting medium is one of the main causes of image degradation in microscopy. Refractive index mismatch results in stretching/compression of the z-axis. Also, spherical aberration is worsened by axial spreading of the point-spread function (PSF) resulting in reduced axial resolution. This phenomenon is exacerbated with depth and with a high numerical aperture objective, serious problems arise when imaging deeper than 10  $\mu\text{m}$  into an aqueous sample. The mounting medium and the immersion medium should be matched. It is not a major issue in LSFM but this has to be considered when filling the imaging chamber in regard to the sample preparation technique, especially for embedding as gelling agents are used.

### 4.2 Troubleshooting

#### Common problems

LSFM is a fluorescence microscopy technique so many troubleshooting guidelines from other microscopy techniques apply here as well. Do not hesitate to ask experts in the field, check the literature, as well as internet resources that may provide you with a more detailed solution to the problem you have encountered. Also check with your Lightsheet Z.1 specialist for FAQs and tips for troubleshooting.

**Sample image is unclear, blurred or has insufficient contrast.**

- This can be a simple optical problem: objectives or filters are dirty. Clean them accordingly. You can also check that all the components are well in place and aligned.
- Your light sheet might not be properly aligned. For Lightsheet Z.1, please realign the light sheet using the Light sheet auto-adjust function of the ZEN software.
- You could be imaging through an additional layer of material: glass, plastic... that belong to the mounting support and not the sample itself. Please, check the sample position or move it around to see if another angle solve the problem as a piece of glue, additional agarose or part of the mounting material is having a blurring effect.
- In the case of sample embedding it may occur that the gelling media is of low quality or badly polymerized. This leads to an uneven polymerization of the media that modify the optical path. Try again.
- The light sheet comes from the side and any obstacle modifies its quality. Check the illumination axis for any obstacle (capillary, objects...).
- Check if the medium level in the sample chamber has dropped below the imaging level. The specimen has to be fully immersed for good image quality.

**Sample image is partially obscured or unevenly illuminated.**

- This can be a simple optical problem: objectives or filters are dirty. Clean them accordingly. You can also check that all the components are well in place and aligned.
- Your light sheet might not be properly aligned. For Lightsheet Z.1, please realign the light sheet using the Light sheet auto-adjust function of the ZEN software.
- The light sheet comes from the side and any obstacle modifies its quality. Check the illumination axis for any obstacle (capillary, objects...).

**Sample signal is weak.**

- Human eyes have trouble quickly adjusting to the dark and it will be hard to discern a very dim fluorescent specimen immediately after darkening the room. You may want to check your sample using another microscope or a stereomicroscope equipped with a fluorescent lamp.
- It could be an optical problem, e.g. extra filter in the optical path, misaligned illumination or dying laser. You can also increase excitation energy (laser). However, the risk of bleaching and signal saturation will increase.
- In the case of immunofluorescence, you should increase antibody concentration or incubation time but this might in turn increase nonspecific background signal.
- In the case of GFP signal, the expression level might be too low or you have photobleached or damaged the GFP signal during sample preparation (fixation, ethanol treatment, excessive illumination during dissection...).

---

**High background signal within the sample.**

- In the case of immunofluorescence, you should decrease antibody concentration or incubation time but this may decrease the overall signal. You can also use blocking steps during the immunofluorescence (eg. BSA, FCS...). If preparing tissue section, you should increase the stringency of the washing steps.
- The imaging chamber may be dirty as well as the media inside. This contamination affects the quality of the light sheet and scattering occurs.
- You can apply deconvolution to your stacks afterwards.

**The sample is moving.**

- If you are imaging life samples, it may be simply due to life. The sample is moving so you may want to increase the anesthetic concentration or the agarose concentration to restrain any movement.
- The mounting is unstable. This occurs if the chosen material is not properly maintained (bad tweezers, leaking plunger, badly polymerized agarose...). For embedded sample preparation, you can improve the stability by limiting the amount of agarose emerging out of the syringe or the capillary, as the longer is the agarose tube the more unstable it will be. You can also tighten the plunger by sealing it with nail polish to avoid air leakage that will lead to gliding of the agarose tube.
- The system table might not be a float. Check that it is connected to the pneumatic supply and the air-dampening is active.
- The cables are not properly secured with the cable holders at the system table.
- Other instruments that produce vibrations, not completely dampened by the system table, are in close proximity (e.g. fridges, centrifuges, etc.).
- The stage is not properly fitted or damaged and prone to vibrations. This includes the sample holder and the imaging chamber support.

**Optical aberrations**

- As in any optical technique, the LSFM has advantages and disadvantages. Some optical aberrations are more general and can be dealt with easily. Do not hesitate to ask experts in the field, check the literature as well as Internet resources that may provide a more detailed solution to the problem you have encountered.
- A few optical aberrations are, however, typical for LSFM, as the optical axis are at a 90 degrees angles. Lines and stripes occur as any objects blocking the light sheet will reduce the light intensity leading to a discrepancy along the illuminated plane. This is often a problem with big samples, highly scattering samples or sample which have absorbing structures at the surface of the specimen volume. Rotating the sample to give a better path for the light sheet should be considered first. Dual Side illumination and/or Pivot scanning of the light sheet can often eliminate these effects (available in the Lightsheet Z.1) Second, the sample could be orientated differently during mounting or partially dissected to limit obstacles. The concentration of the objects can also be a problem especially with samples with optical properties (beads, tubes, glass capillaries...). For example, if you image large number of cell clusters, you may have a few of them in the light sheet path. By reducing the amount of objects, you will automatically reduce the lines and stripes.
- The use of image processing methods such as deconvolution may help to get rid of those aberrations.

### 4.3 Suggested Additional Sources of Information

#### Chemicals

- Agarose:
  - Molecular biology grade, for routine use, SIGMA, Ref. A-9539
  - Type VII, low gelling temperature, SIGMA, Ref. A-4018-50G
  - "Agarose Low Melt" (no 6351.1) from Carl Roth <http://www.carlroth.com> (from the US please contact Brunswig Chemie B.V., Amsterdam, NL (e-Mail: [brunswig@brunswig.nl](mailto:brunswig@brunswig.nl)))
- Gelrite Gellan Gum, SIGMA, Ref. G-1910
- Glycerine anhydrous, AppliChem, Ref. A3552,1000
- Nail Polish, any cosmetic shop near you
- PBS, local supplier
- Distilled water, local supplier
- Ethanol, local supplier
- Companies:
  - Sigma- Aldrich (<http://www.sigmaaldrich.com/>)
  - AppliChem (<http://www.applichem.de/>)
  - MP Blomedicals (<http://www.mpbio.com/>)
  - Merck KGa (<http://www.merck.de/>)

#### Materials

- Capillaries
  - 100 µl, color code Blue, Brand GmbH, Ref. 7087 45
  - 200 µl, color code Red, Brand GmbH, Ref. 7087 57
- Companies:
  - Brand GmbH (<http://www.brand.de/en/home/>)
  - SpectraGlass (<http://www.spectraglass.com/>)
  - Harvard Apparatus (<http://www.harvardapparatus.com/>)
- Syringes
  - 1 ml, BD Plastipak, BD Biosciences, Ref.300013
  - Braun Omnifix F Solo 1 ml Syringe (PZN 0569881, Ref. 61706)
  - Terumo 1 ml Syringe (Ref. BS-01T)

- Pipettes
  - 2 ml serological pipette, FALCON, BD Labware, Ref.35 7507
  - 1 ml serological pipette, FALCON, BD Labware, Ref.35 7521

### Equipments

For the following, please refer to your local lab supply companies

- Heating blocks
- Stereomicroscope
- Scalpels
- Tweezers
- Dissection Needles
- Watch glass
- Sonicator

## 4.4 References and Further Reading

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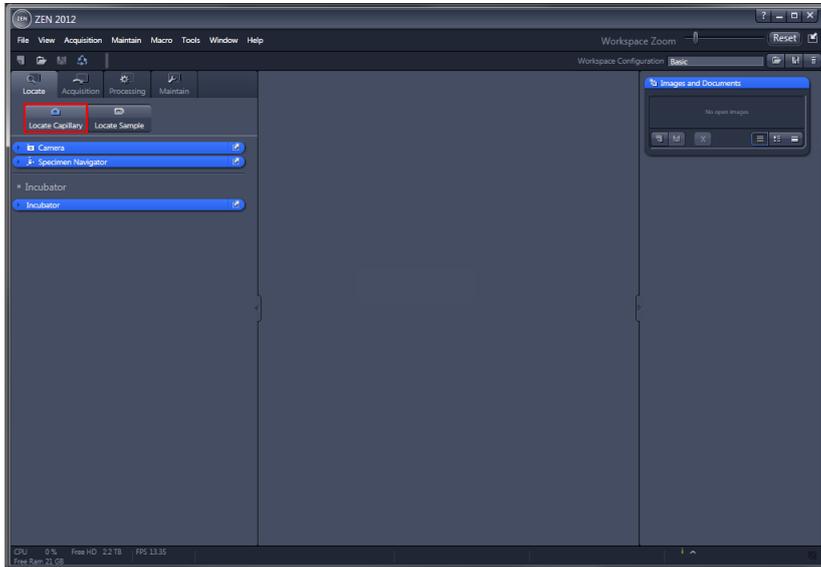


The content of this chapter is as well available as an e-learning tool, by which every chapter can be learned in an interactively step-by-step process. You will find the e-learning modules when starting the file:

***Introduction\_into\_Lightsheet\_Z.1-ZEN\_2014.exe***

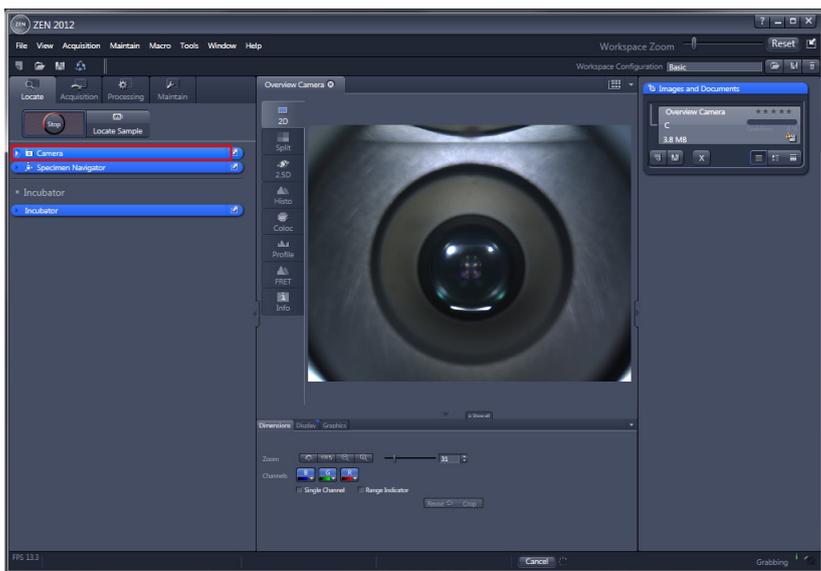
# 1 Locating Capillary and Sample

## 1.1 Locate Capillary



- To locate the capillary or similar device which is holding the sample, press the **Locate Capillary** button.  
The **white LED illumination** is turned ON and the **overview camera** acquires a live image from the inside of the sample chamber.

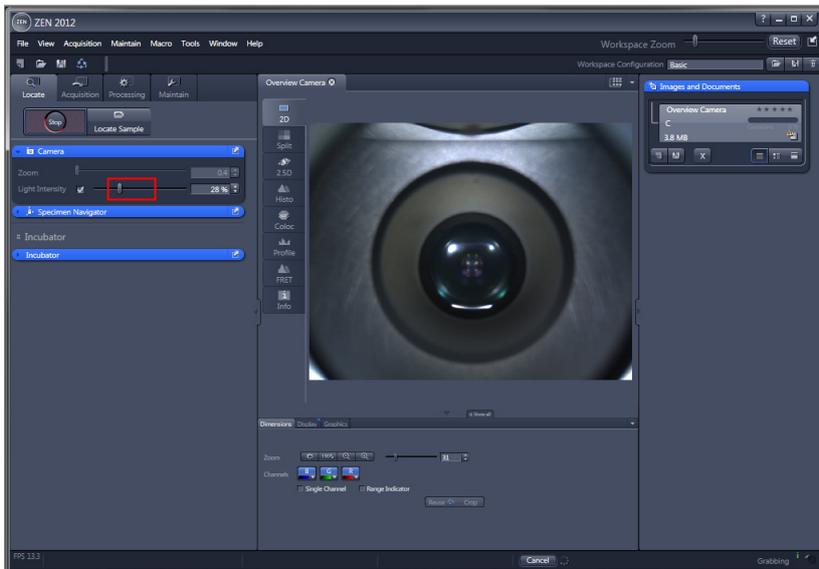
- Click Locate Capillary .



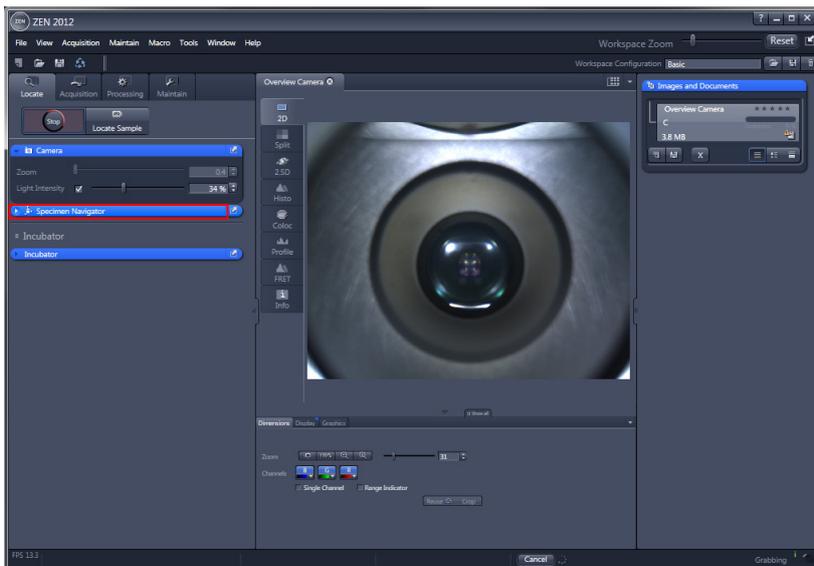
- The live image from the inside of the sample chamber is displayed in the **Center Screen Area**.

☞ Seeing the liquid level just at the very top of the displayed sample chamber image is a good way to monitor and be assured that there is no leakage within the sample chamber.

- Please open the **Camera Tool** window to access the light intensity adjustment for the displayed image.
- Click **Camera** 

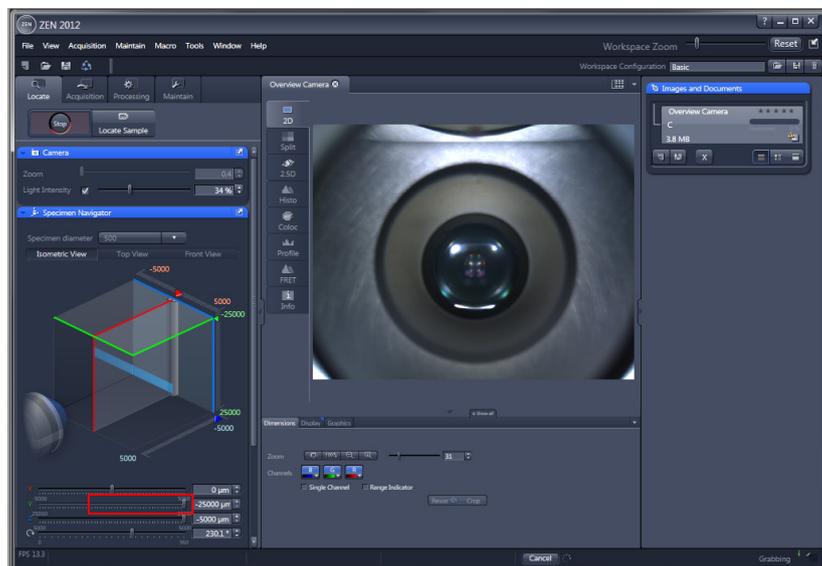


- Please adjust the light intensity by using the slider **Light Intensity** in the **Camera Tool** until details within the sample chamber become visible in the live image display.
- Click **Light Intensity** 

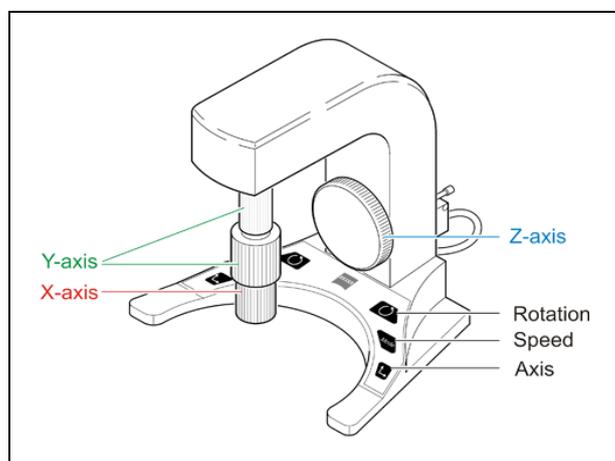


☞ The **Zoom** function is not available while using Locate Capillary.

- Use the **Specimen Navigator** to position the capillary from the **Load** position into the **Work** position.
- Click **Specimen Navigator**  Specimen Navigator



-  The **Specimen Navigator** is a graphical tool for accessing the relative positions of the **specimen** embedded in agarose (light-grey cylinder), the **illumination light-sheet** (light-blue), as well as the **X-axis** (red), **Y-axis** (green), **Z-axis** (blue) and the **Rotation** of the sample holder.



Instead of using the **Specimen Navigator**, you can also move and control the capillary containing the sample using the **ErgoDrive operating panel**.

#### Rotary controls:

- X-axis (red) control
- Y-axis (green) control (after pressing **Axis** button) Or **Rotary** control (after pressing **Rotation** button)
- Z-axis (blue) control

#### Buttons:

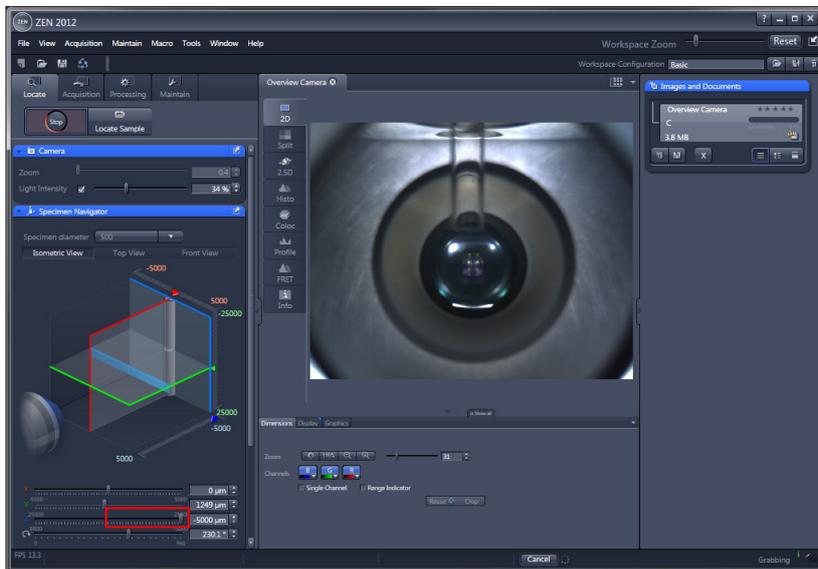
The **Rotations** button changes the upper rotary control to move the **rotation** drive.

The **Mode** button changes the **speed** by toggling the drive between coarse and fine.

The **Axis** button changes the upper rotary control to move the **Y drive**.

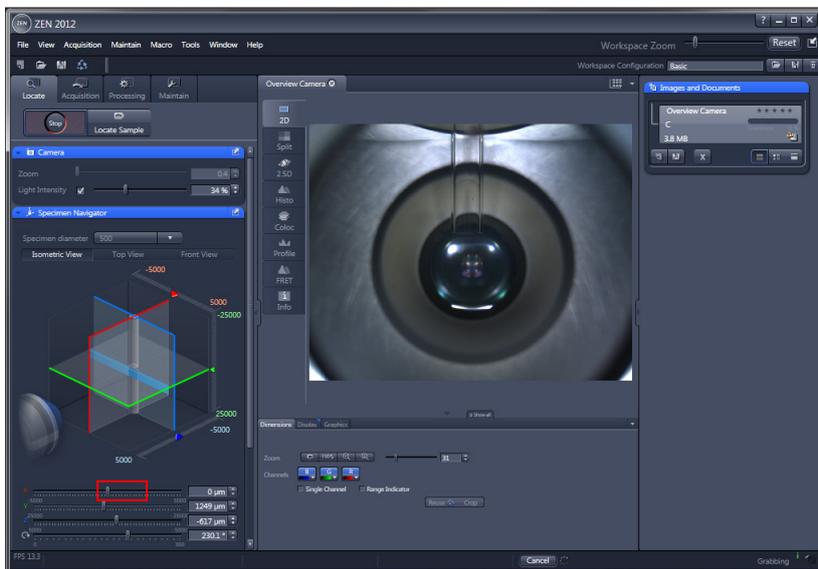
- First of all, move the **Y-axis** (green) until the capillary becomes apparent in the image display (from **Load** position to **Work** position).

- Click **Y-axis** 



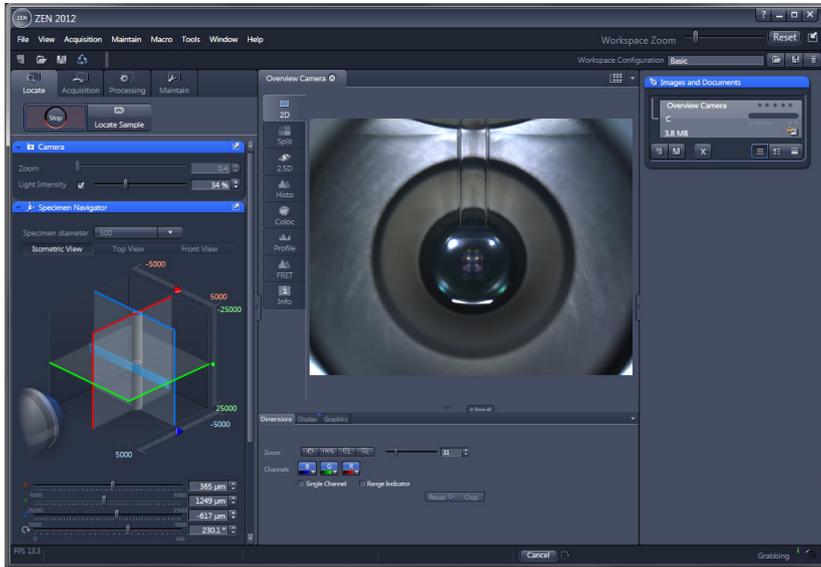
 The **capillary** becomes now apparent in the displayed image.

- Please bring the capillary in focus via the **Z-axis** slider.
- Click **Z-axis** 



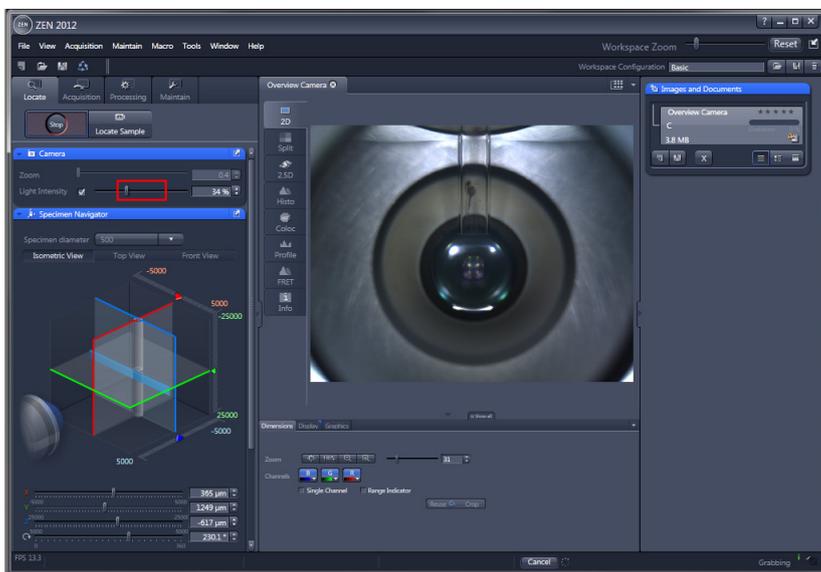
 Moving the light-grey cylinder, representing the embedding medium, into the **light sheet graphic** (light-blue) can be used as a **focus finding guide**.

- Please do the fine adjustment for the capillary in the **X** direction.
- Click **X-Axis** .

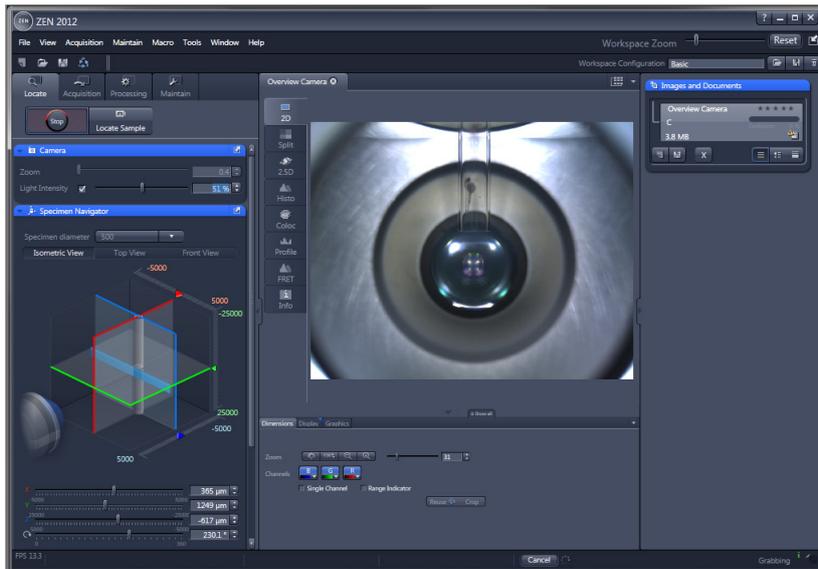


 The capillary is placed correctly when it is centered just above the detection optic lens.

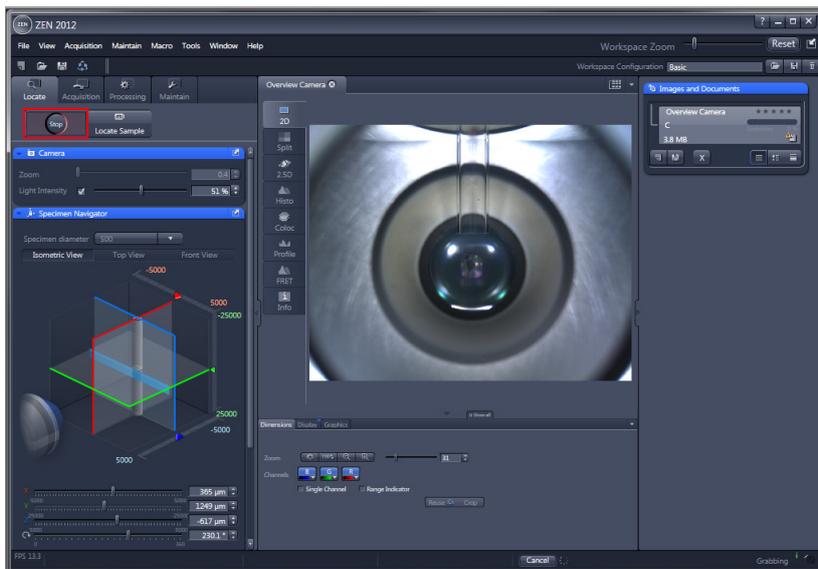
- Open the **upper system cavity door** and eject the **agarose cylinder**.



- Please adjust the light intensity by using the slider **Light Intensity** in the **Camera Tool** until the specimen becomes visible in the live image display.
- Click **Light Intensity** .



- Press the plunger of the capillary (or syringe) until the embedded **specimen** is roughly **centered in front of the detection optics lens**.

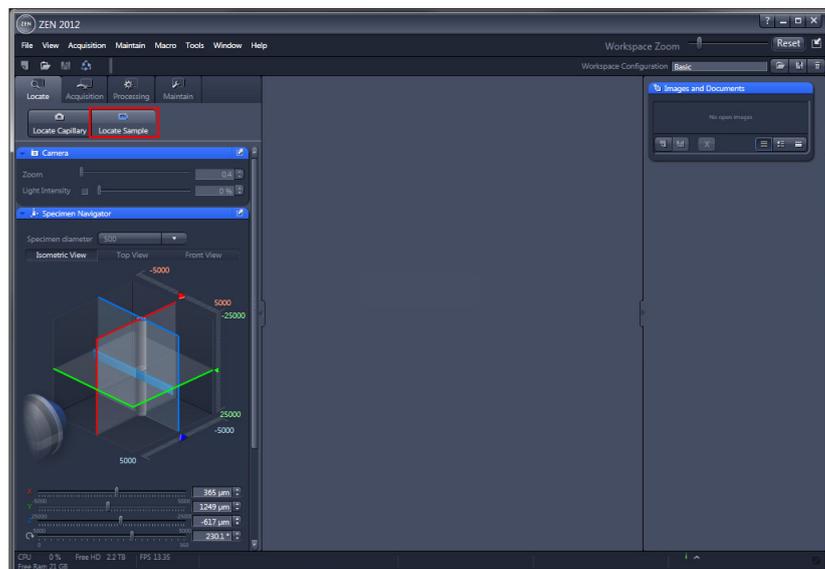


 The **Specimen** is now properly centered in front of the detection optic lens.

- Press the **Stop** button to end the image acquisition of the live image.

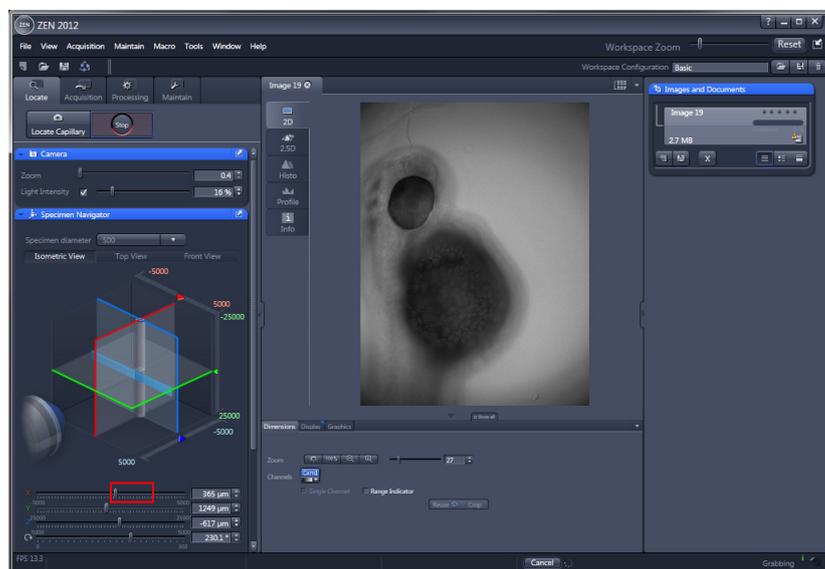
- Click **Stop** .

## 1.2 Locating Sample

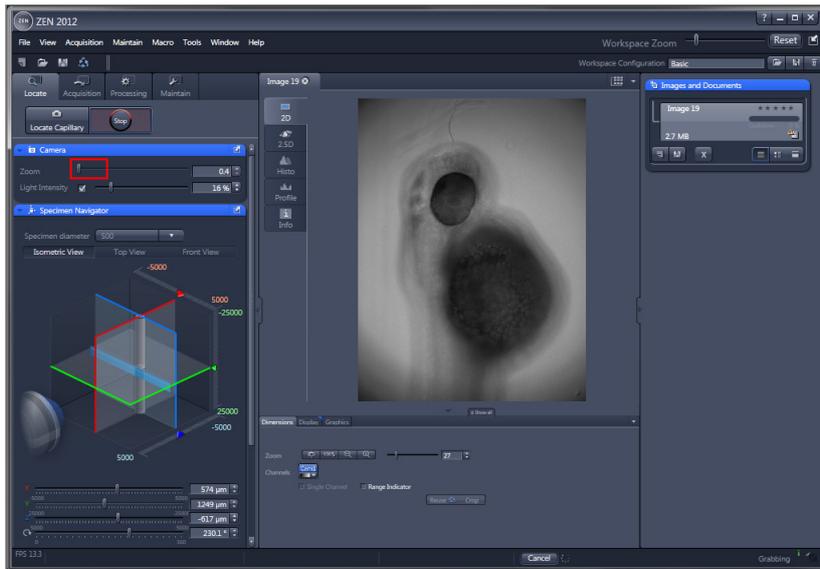


- To locate the sample within the sample chamber, the **Locate Sample** button activates an **infrared light source** and shows a live transmitted light image in the Center Screen Area. This image is taken using **Channel 1/ Cam1**.

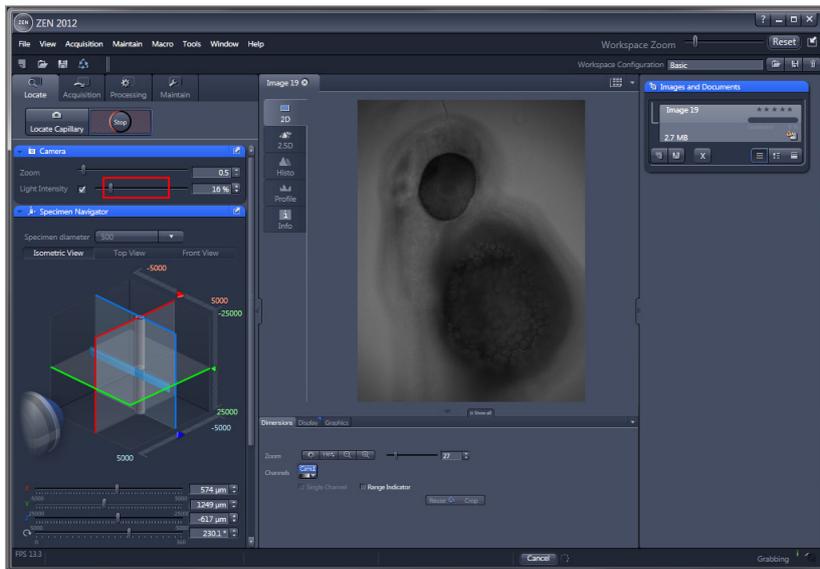
- Click **Locate Sample** .



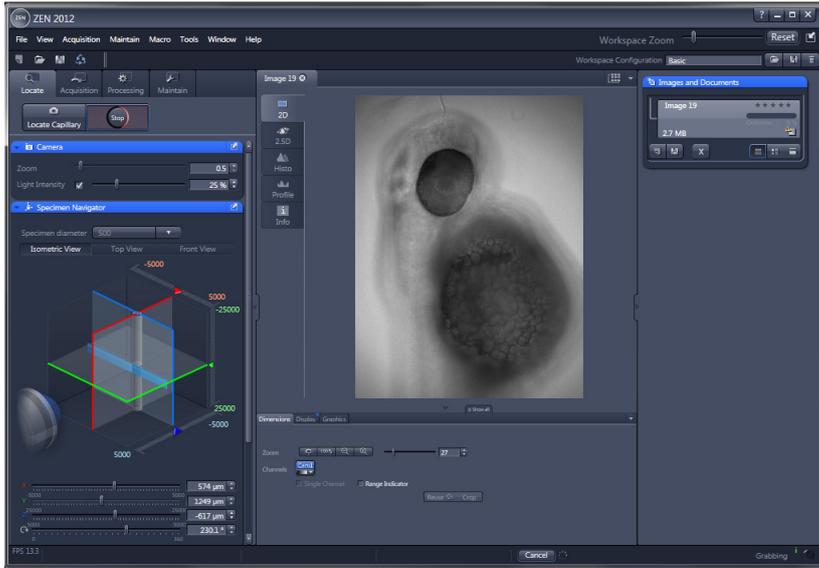
- Please fine adjust the **X**-position of the capillary and bring the specimen into the center of the image.
- Click **X-axis** .



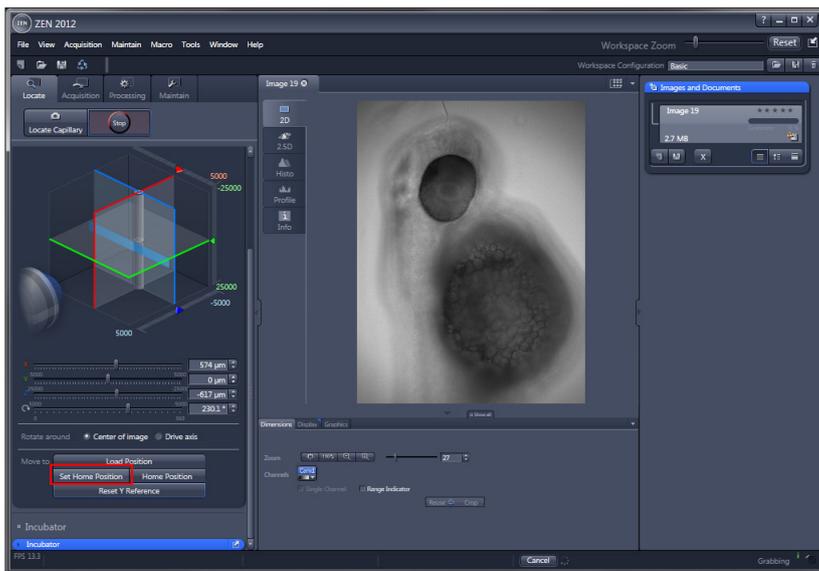
- Please use the **Zoom** slider to zoom into the image.
- Click **Zoom** .



- Please use the **Light Intensity** slider to increase the illumination on the sample.
- Click **Light Intensity** .

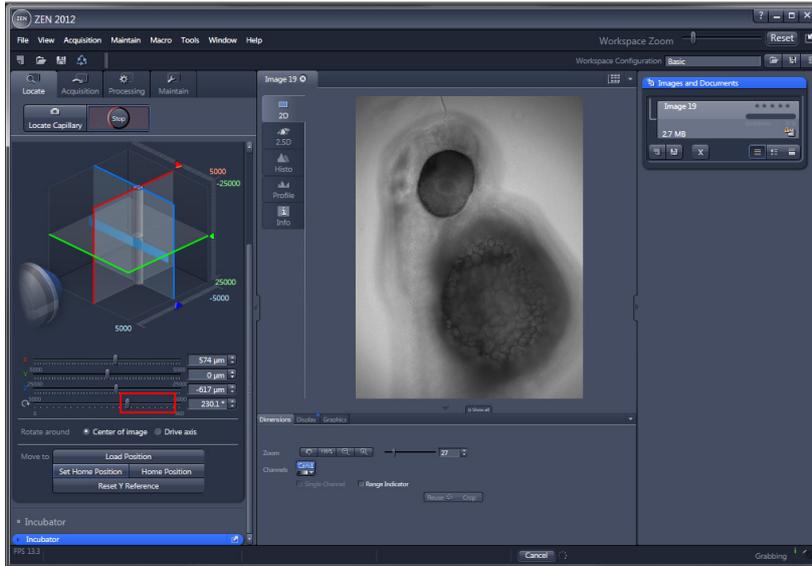


- Click on the **Arrow down button** to access the lower tool tabs.



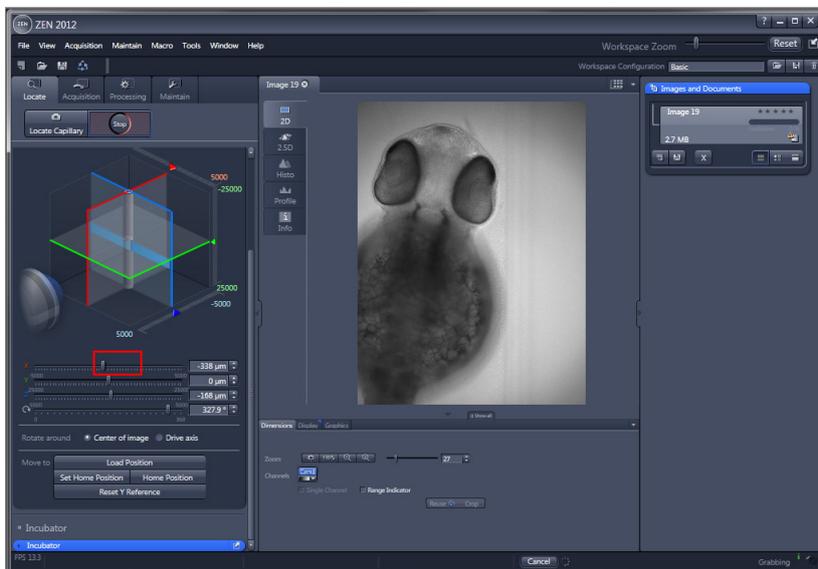
- You can define a position you might want to return to during the course of imaging by moving the sample to the desired point and pressing the **Set Home Position** button.
- Click **Set Home Position** Set Home Position.

 To return to this position, click the **Home Position** button.

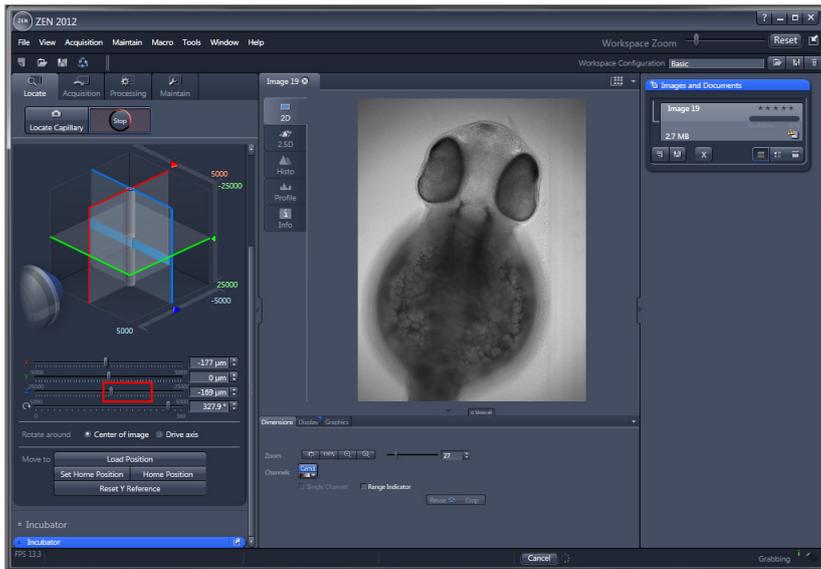


👉 Use **Rotate around Center of image** to automatically correct the **X** and **Y** positions for rotational movement and keep the sample within the chosen field of view. This can be helpful when the sample is not in the center of the embedding medium.

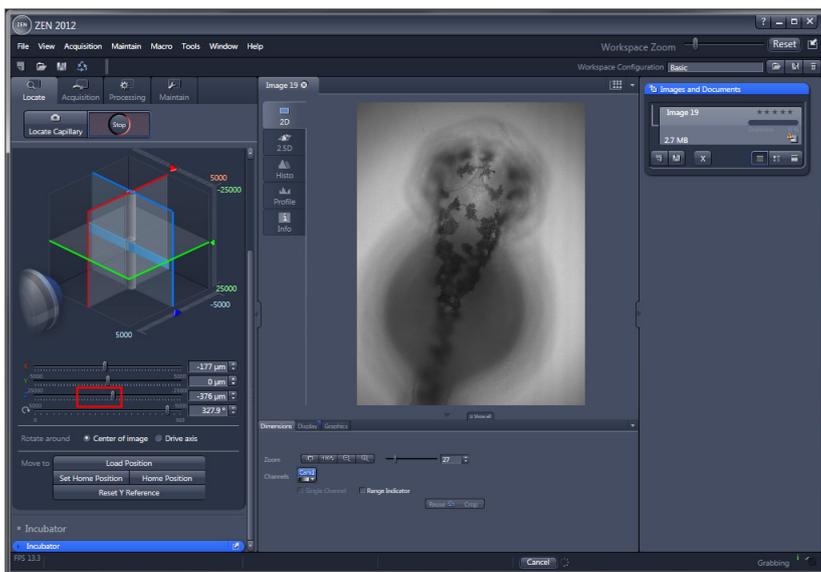
- Please rotate the capillary until the specimens back side is in front of the detection optic lens.
- Click **Rotation axis** .



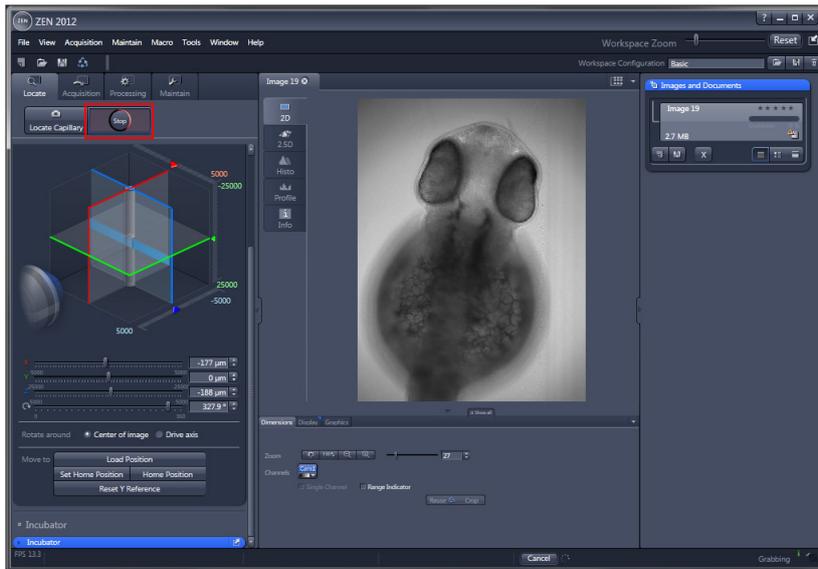
- Please fine adjust the **X**-position of the capillary and bring the specimen into the center of the image.
- Click **X-axis** .



- You can focus in **Z** through the specimen.
- Click **Z-axis** .

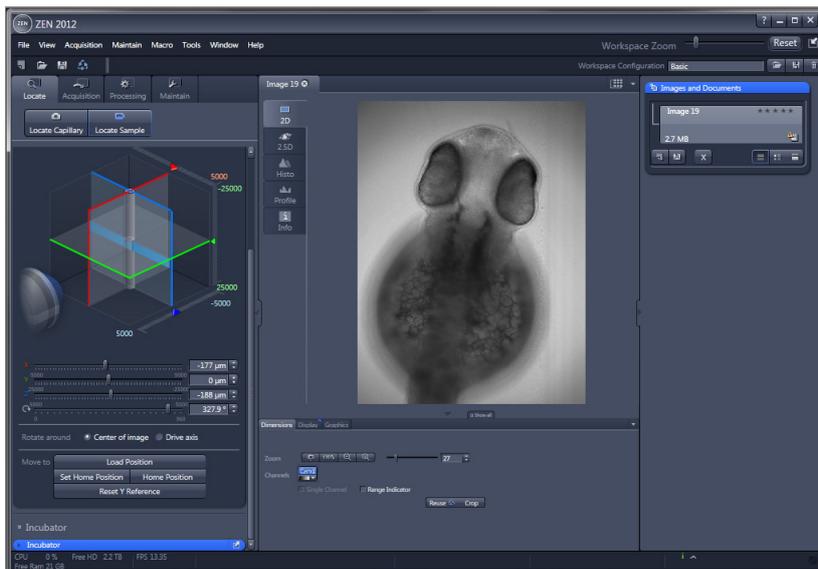


- Please re-focus in **Z** until you found the desired plane.
- Click **Z-axis** .



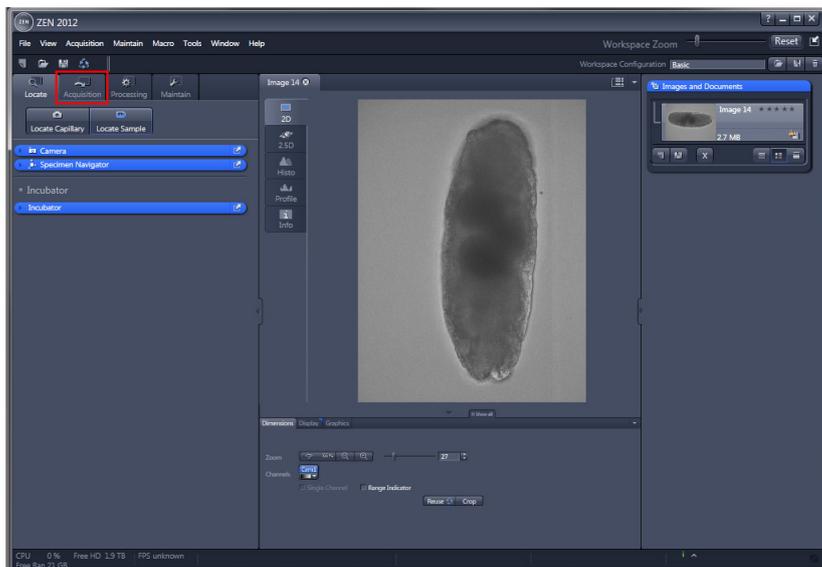
- Press the **Stop** button to end the live image acquisition and turn off the infrared light source.

- Click **Stop** 



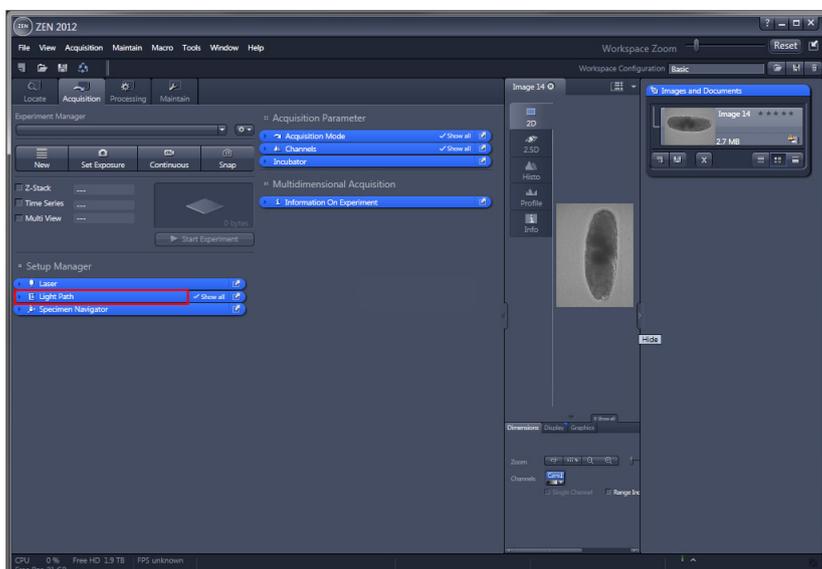
- ☞ **Your specimen is now properly orientated! You can now proceed with adjusting your image settings.**  
The **Zebrafish**, for illustrative purposes due to its larger size, was shown for positioning in this chapter. The following chapters demonstrate how to adjust image settings with **Drosophila melanogaster** as another possible organism.

## 2 Configuration of the Light Path

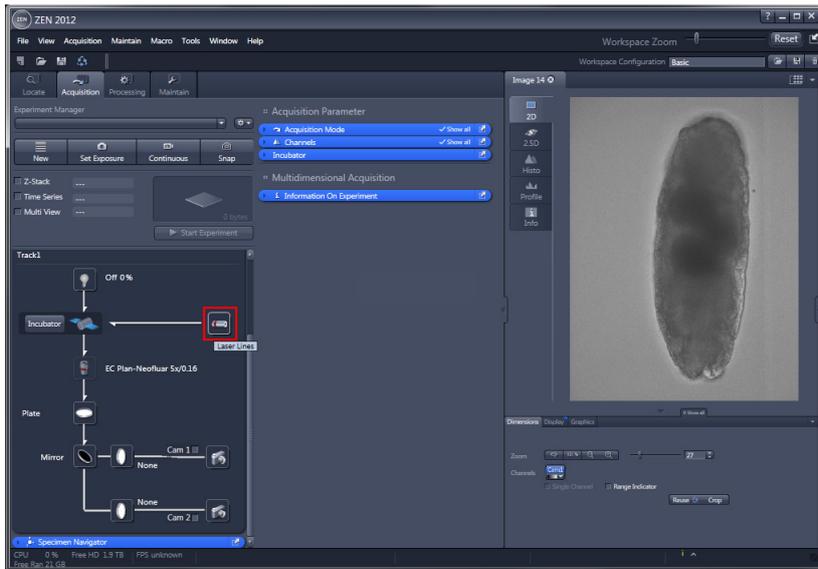


- Please click **Acquisition** to access all functions necessary for setting up and controlling light sheet fluorescent imaging and multidimensional data acquisition.

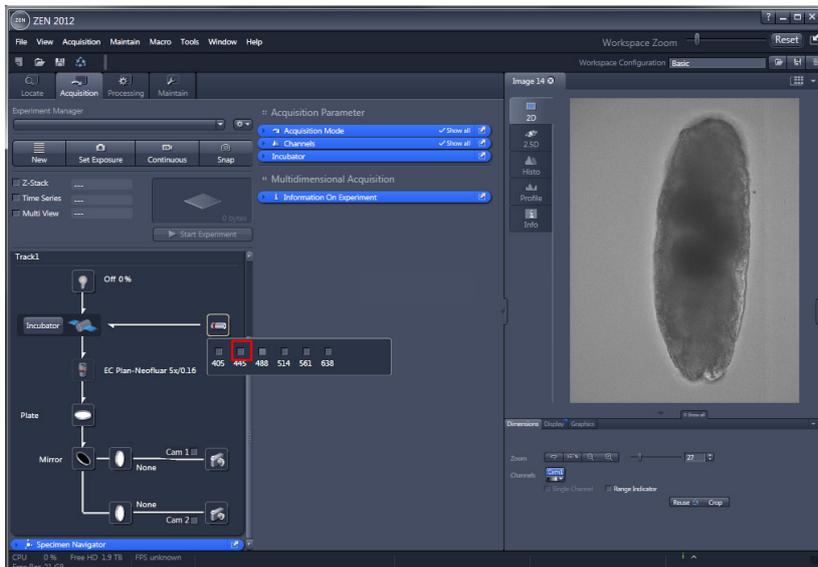
- Click **Acquisition** .



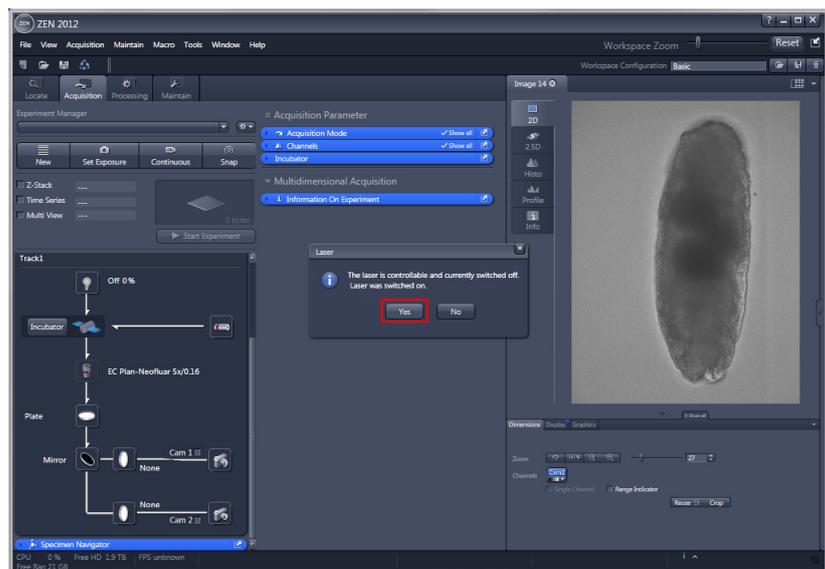
- The **Light Path** panel allows for new and/or existing beam path configurations to be created and/or edited, and saved.
- Click **Light Path** .

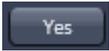


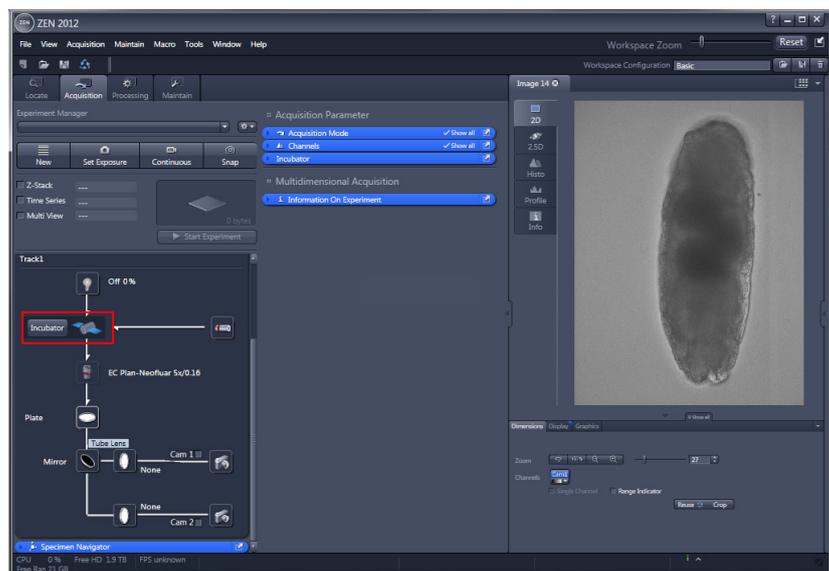
- Pressing the **Laser** icon displays the laser control submenu.
- Click **Laser Lines** .



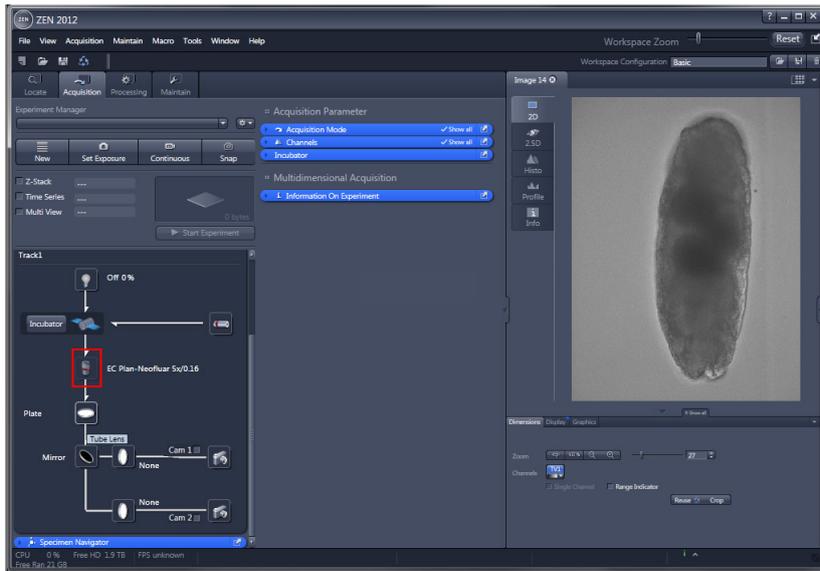
- Activate the **laser(s)** necessary for excitation by checking the corresponding boxes.
- Click **488** .



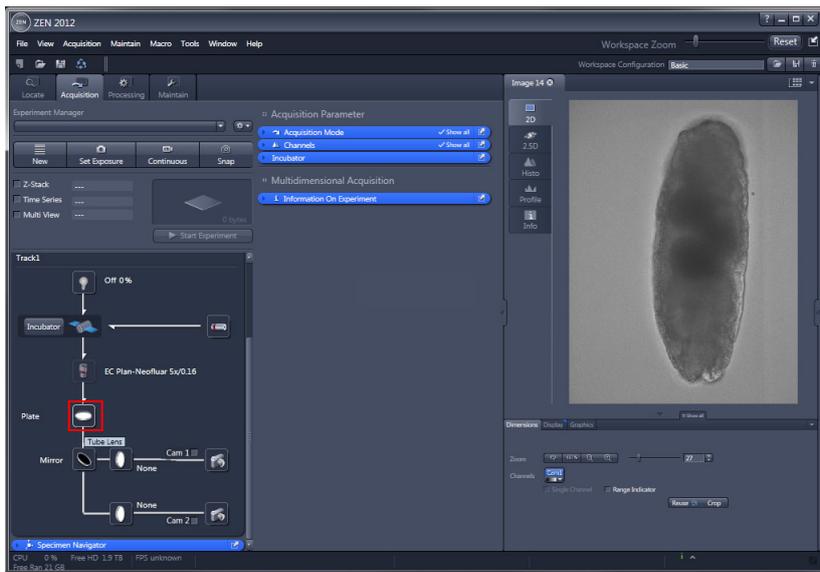
- A dialog box appears for switching ON the selected laser(s).
- Click **YES** .



 The **Incubator** button accesses all possible attached equipment (**Temperature** [°C] control and / or **Atmosphere** [%] control).

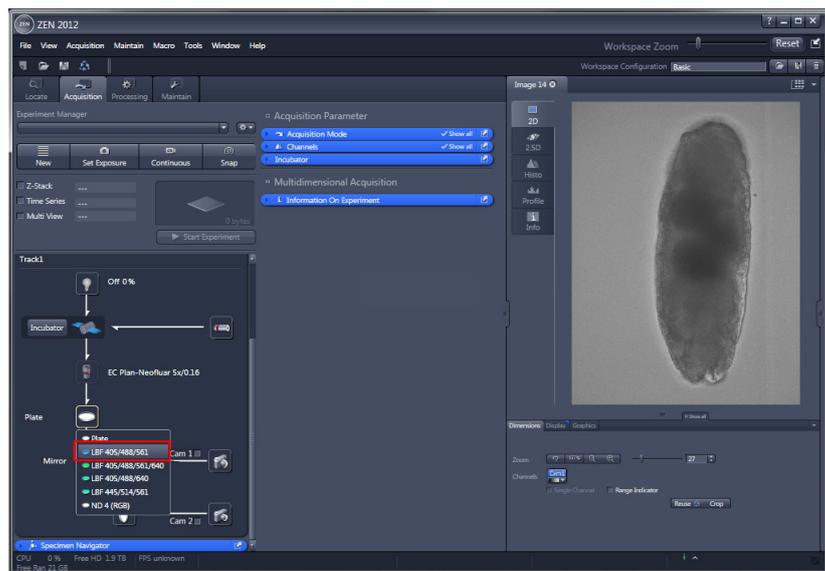


 **Detection Optics Display**

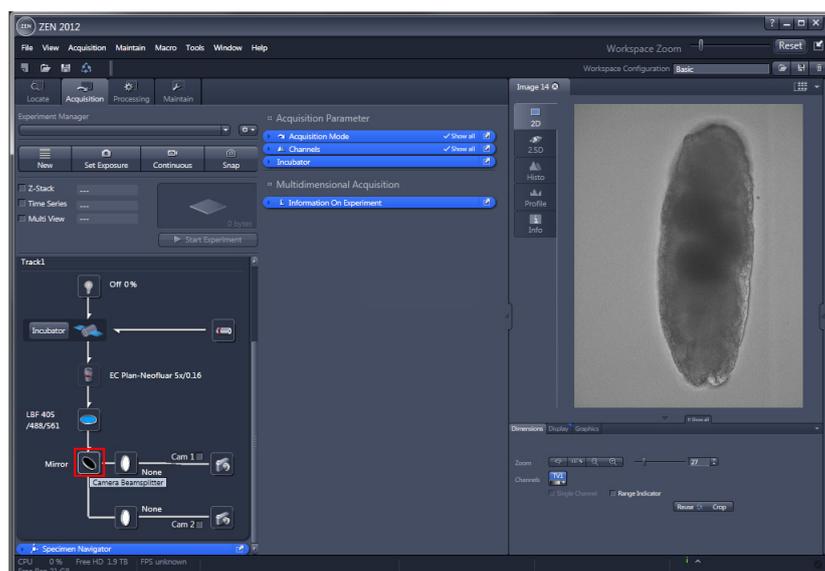


- Select a suitable **Laser Blocking Filter** for blocking the laser light.

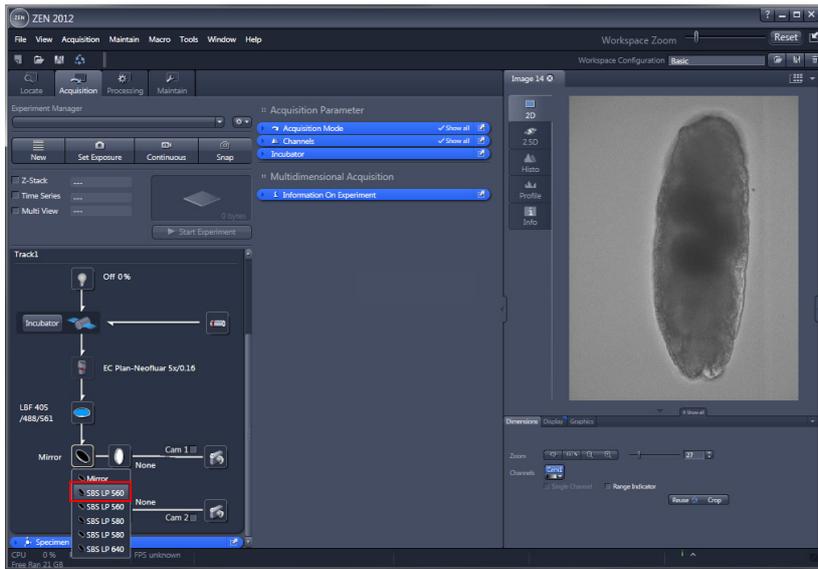
- Click **Laser Blocking** .



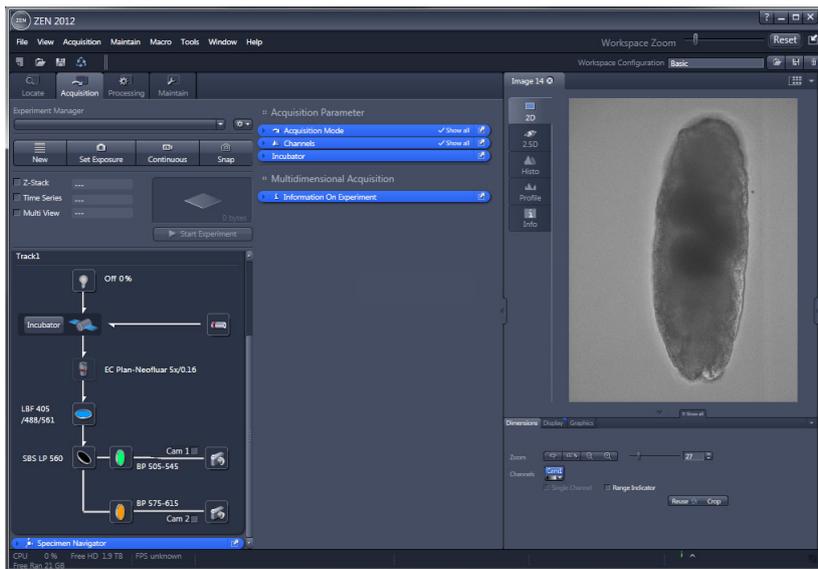
- According to the chosen laser lines, an appropriate **Laser Blocking Filter** can be selected.
- Click **LBF 405/488/561** 



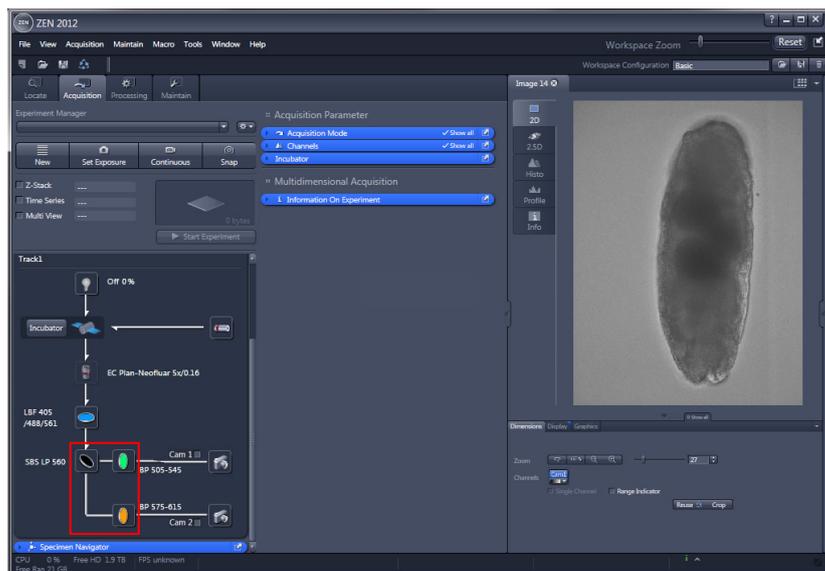
- The **Beam Splitter** button opens a drop-down menu for choosing the Beam Splitter to direct the emission light towards the desired detector module, **Cam1** or **Cam2**.
- Click **Beam splitter** 



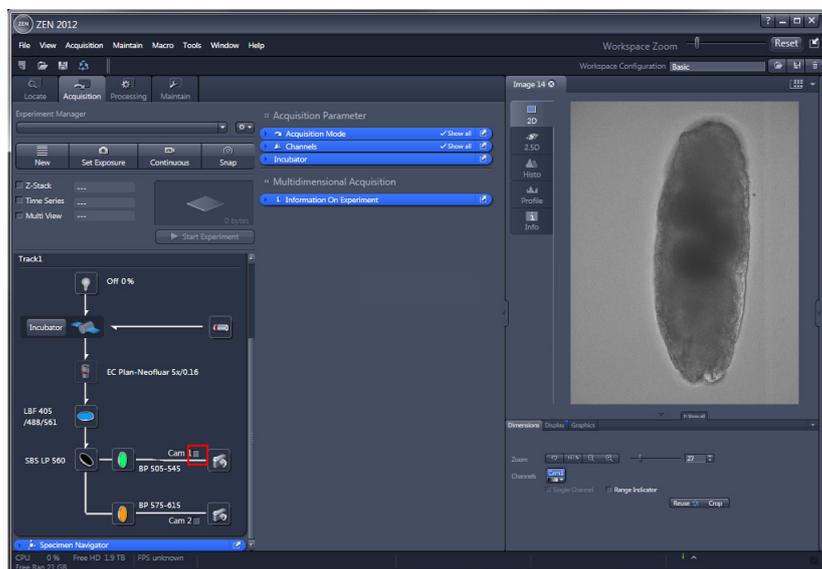
- Click **SBS LP 560** 



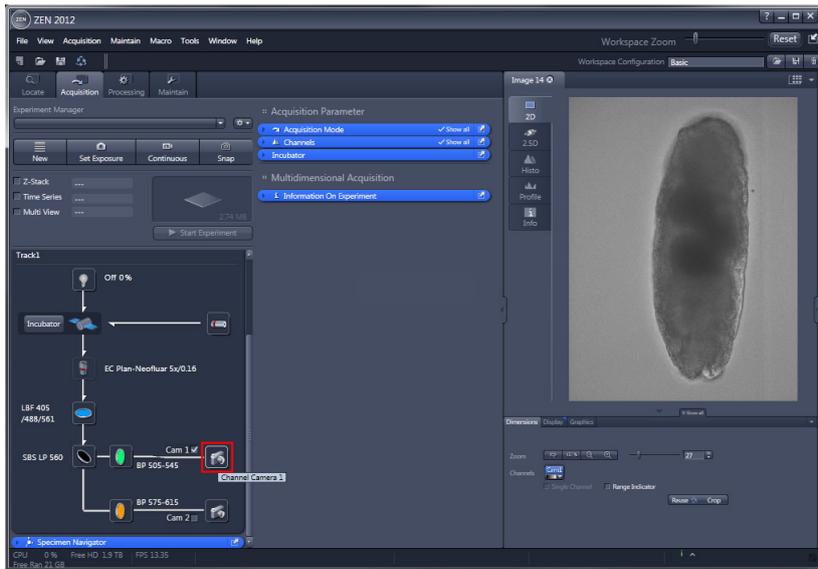
- The activated **Secondary Beam Splitter (SBS)** will move into position and its name is displayed in the light path.



- ✎ Since **Beam Splitter** and **Emission filters** form one unit, the corresponding **Emission Filter 1** (in front of Cam1) and **Emission Filter 2** (in front of Cam 2) will change as well in the light path.
- ✎ Even though the specimen is only stained with one dye, it is advisable to choose a Beam Splitter with an Emission Filter instead of only a Mirror for fluorescence detection.

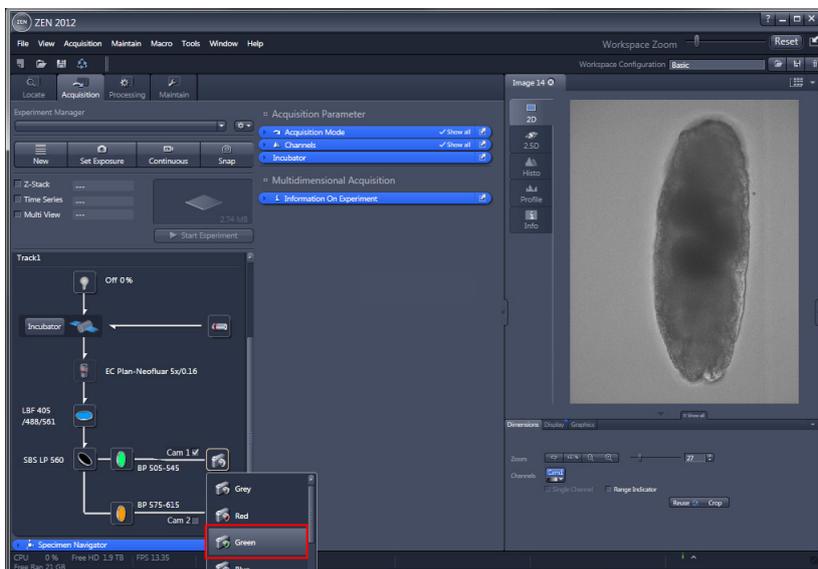


- Activate the imaging Channel by checking the box **Cam 1** next to the detection module icon. Make sure a suitable Emission Filter is selected for the specimen staining.
- Click **Cam1** .



- Assign a specific color to the channel image by clicking on the **detection module** icon "**Channel Camera 1**".  
A drop down menu opens for selecting a color or look-up-table (LUT).

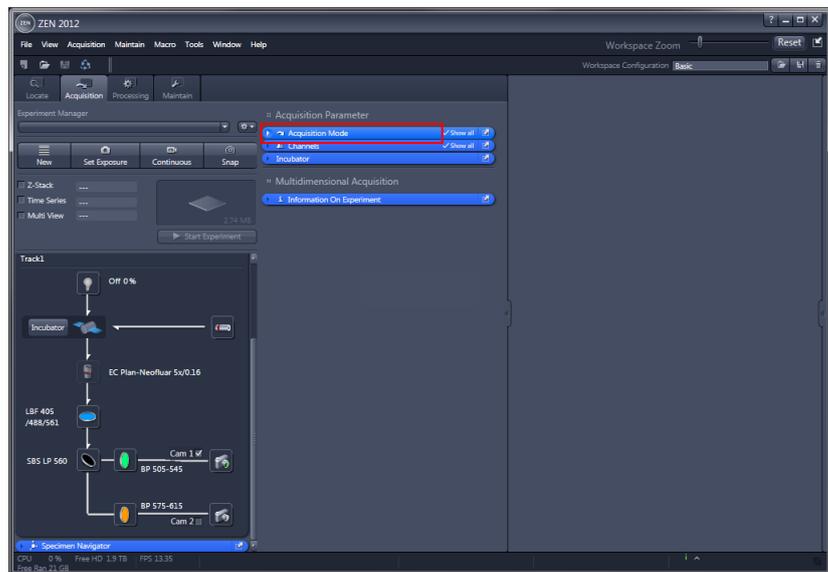
- Click Channel Camera 1 .



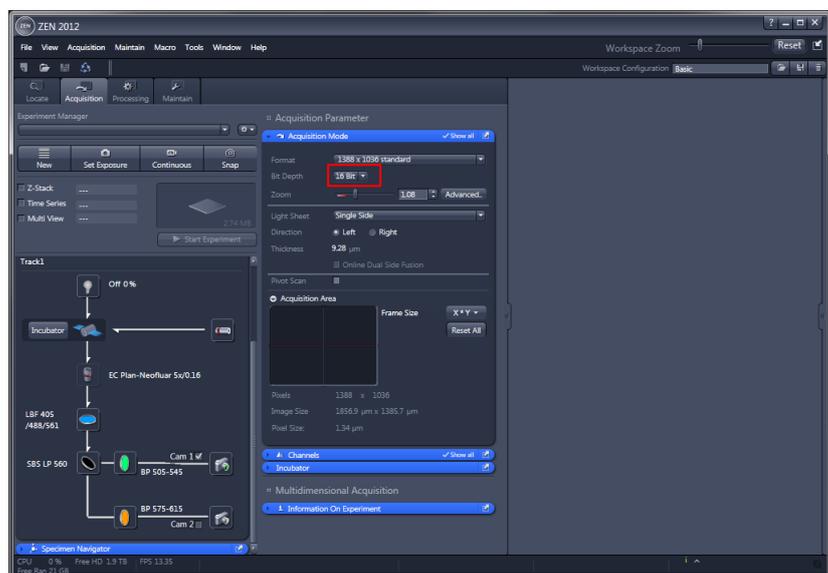
- Please select the green color for the dye shown in this presentation.

- Click **Green** .

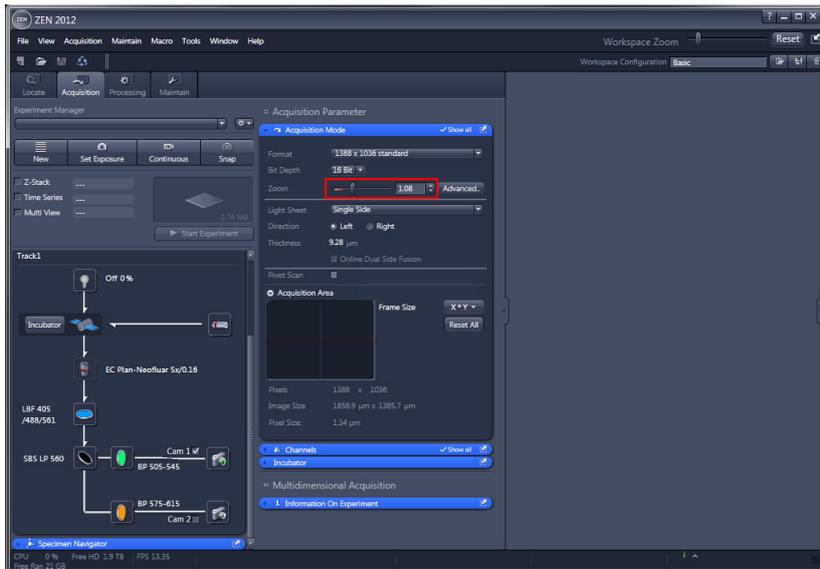
## 2.1 Adjust Acquisition Parameters



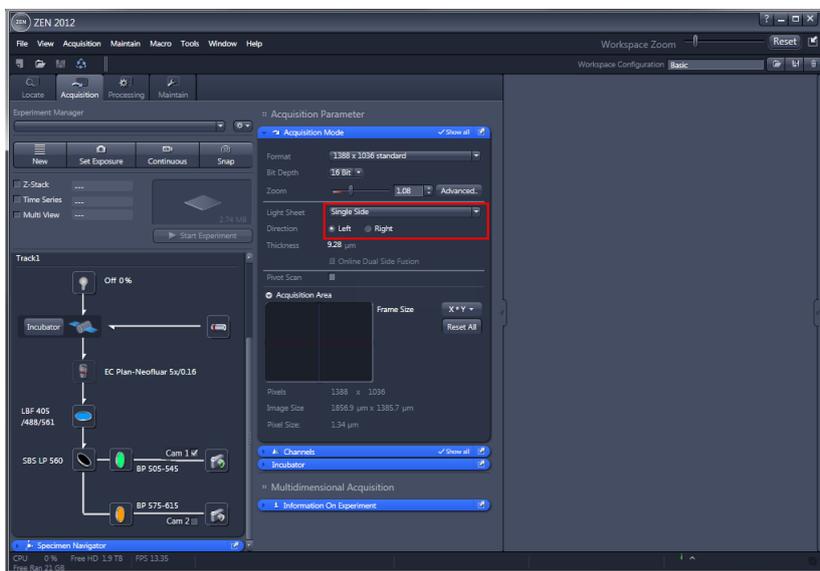
- In the **Acquisition Mode** tool tab, acquisition parameters can be set.
- Click **Acquisition Mode** 



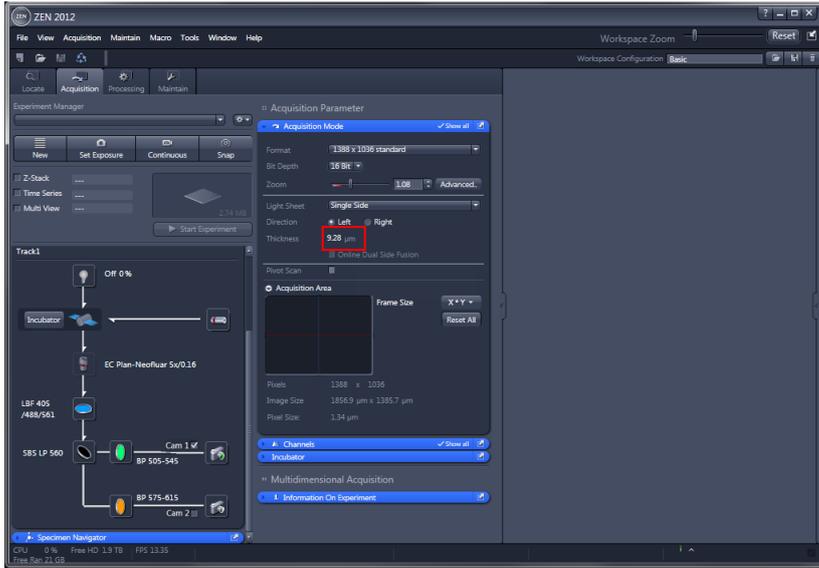
 Here the actual selected **Bit Depth** is displayed.



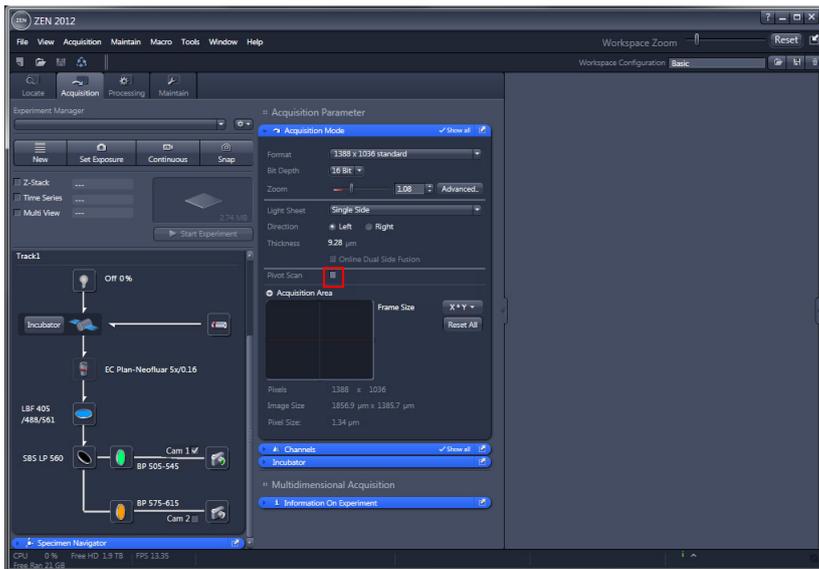
- ☞ You can change the imaged area using the **Zoom** function. This zoom utilizes hardware zoom optics within the system.
- ☞ Within the **Zoom range** from 0.36 to 0.7, the image quality towards the edges can decrease (**Zoom** numbers are displayed in **red**; range is indicated with a **red** line below the slider).
- ☞ Be aware that when you change the Zoom during an acquisition session, the light sheet alignment (Lightsheet **Auto-Adjust** in the **Channel tool** window) needs to be re-done.



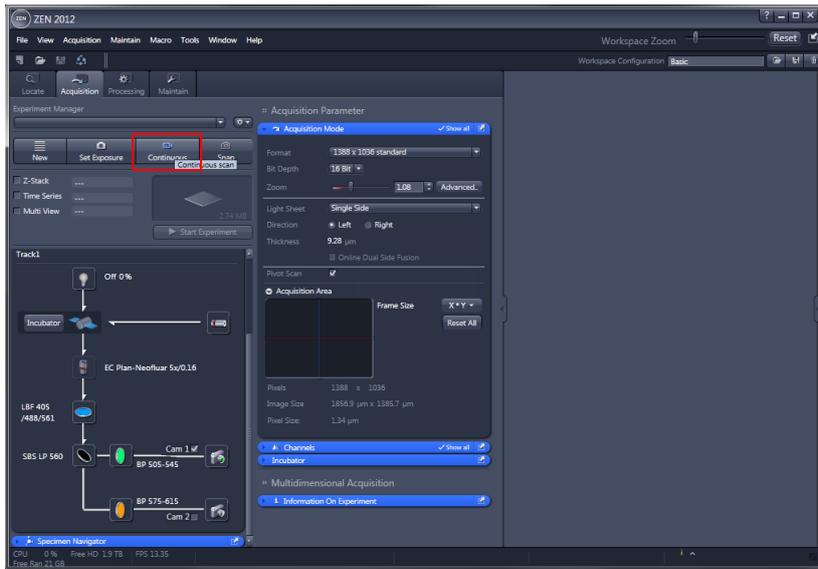
- ☞ The **Single Side** illumination is displayed by default from the **Left** direction irrespective of the configuration. This setting corresponds to the standard configuration where the light sheet only illuminates the sample from one side.  
If Single Side is chosen for Illumination, the direction, **Left** or **Right**, must be marked. When Dual Side illumination is chosen the sample is illuminated by the light sheet from the left and by the light sheet from the right sequentially, producing two images for each plane.



 The Light Sheet **Thickness** is displayed in µm.

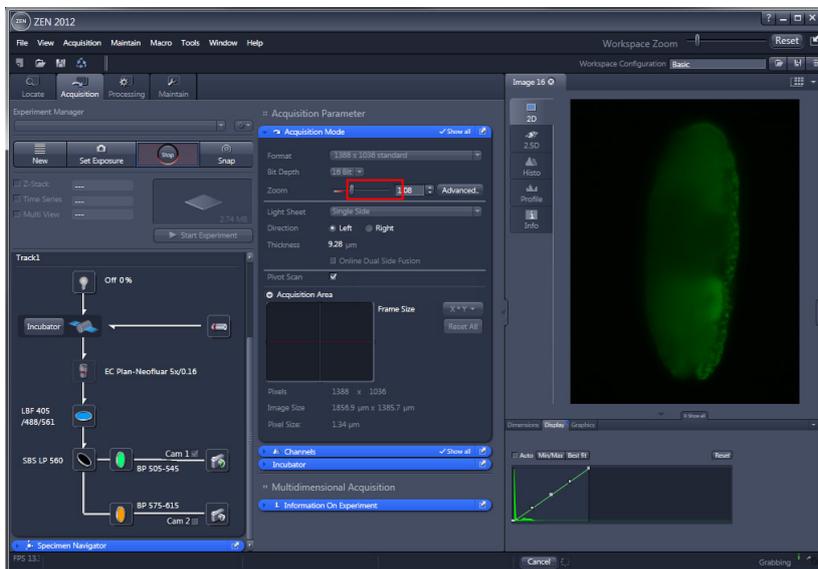


- The **Pivot Scan** is used to move the light sheet within the focal plane of the detection optics in a wavelike motion. This results in a reduction of shadows which might otherwise be cast by optically dense structures within the sample.
- Click **Pivot Scan** .



- **Continuous** produces images from all active channels and tracks. It thereby ignores the settings for multidimensional acquisition, but takes into account the imaging settings within the Acquisition Mode Tool and the Channels Tool.

- Click **Continuous** 

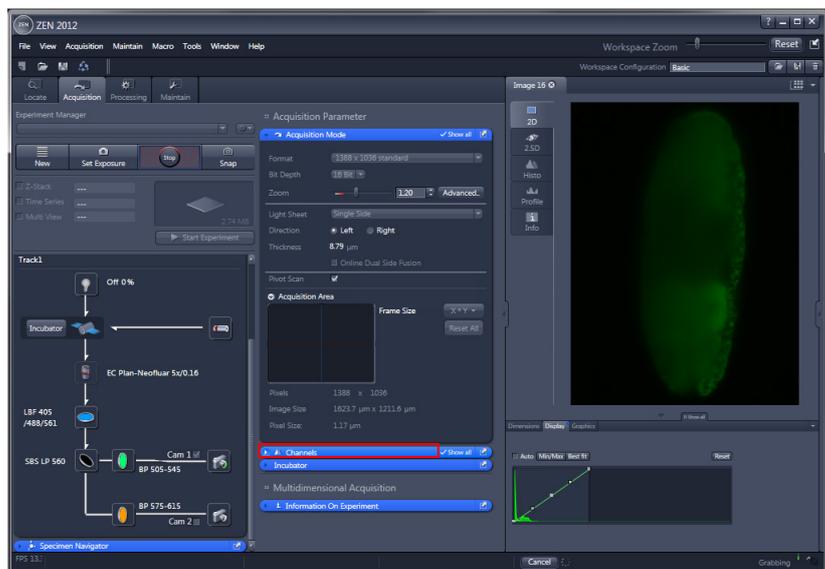


 After its activation, the **Continuous** button changes into the **Stop** button.

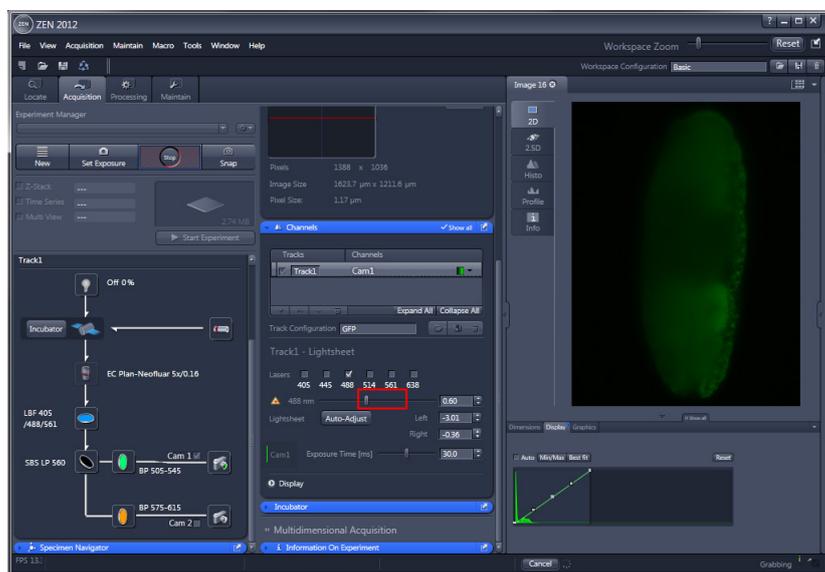
- Please adjust the **Zoom** to restrict the area you would like to image. It is recommended to use a Zoom > 0.7; this optimizes the image quality towards the edges.

- Click **Zoom** 

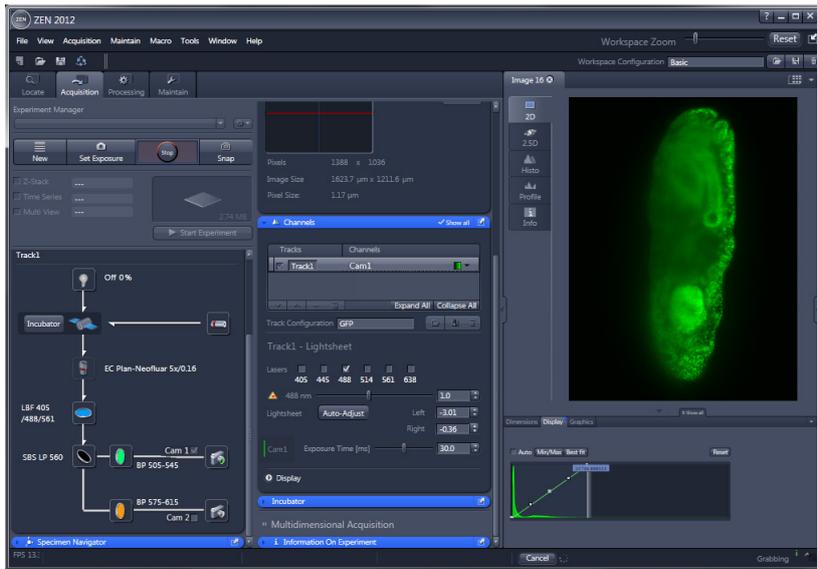
## 2.2 Define Channels and Tracks and adjust Detectors and Illumination Settings



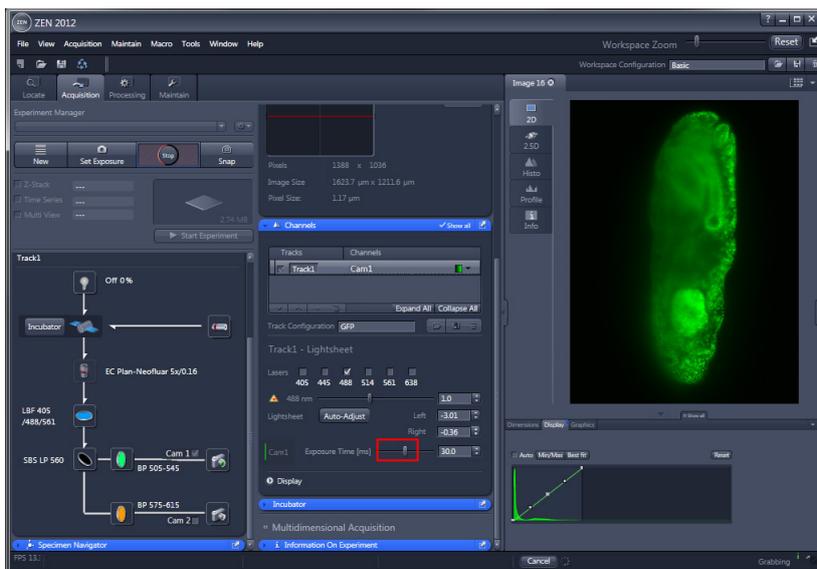
- The **Channels** tool provides definition of channels and tracks, adjustments for Detectors, Illumination settings, as well as Display options.
- Click **Channels**  **Channels**



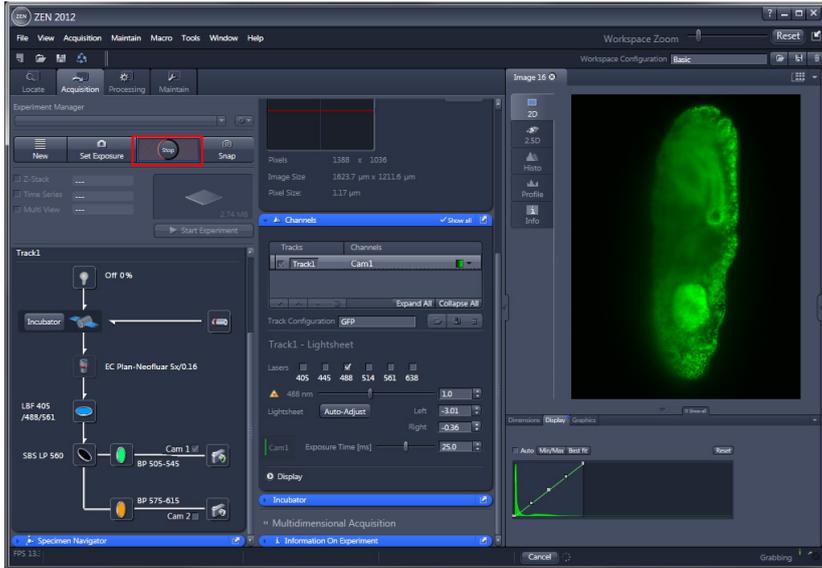
- For each chosen laser line, a slider with input box and arrows appears. Here you can set the laser power in percentage of total output.
- Click **Laser power** 



- Using the **Display characteristic curve** in the histogram, you can set the limit for the white value (right). This influences the contrast and brightness of the displayed image.



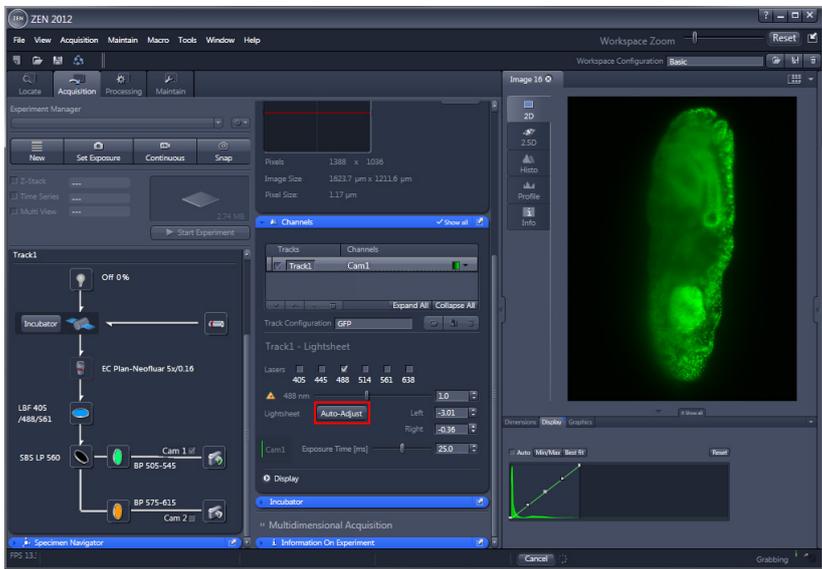
- The detection module controls are marked as **Cam1** or **Cam2** and show up with a vertical line in the selected Channel color.  
The **Exposure Time [ms]** is regulated with the slider, input box, and arrows.
- Click **Exposure Time** .



- To end **Continuous** acquisition, press the **Stop** button.

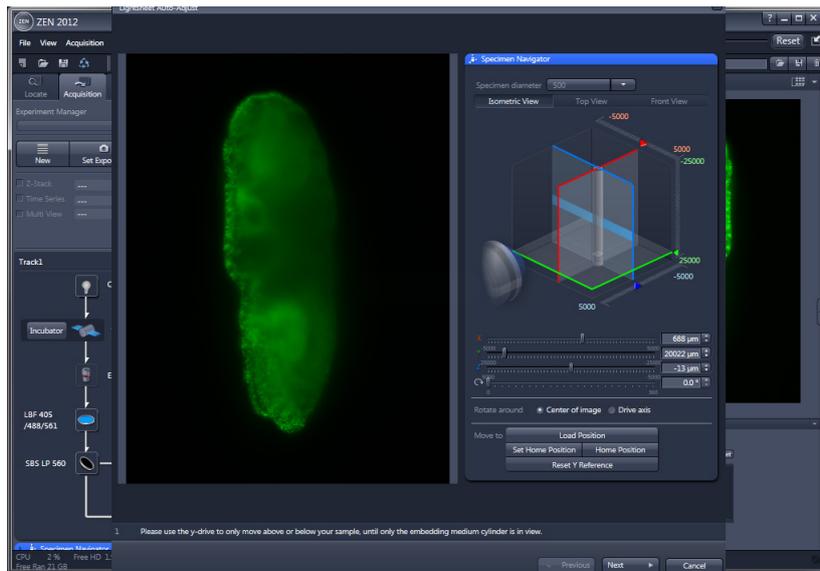
- Click **Stop** .

### 2.3 Lightsheet Auto-Adjust Wizard

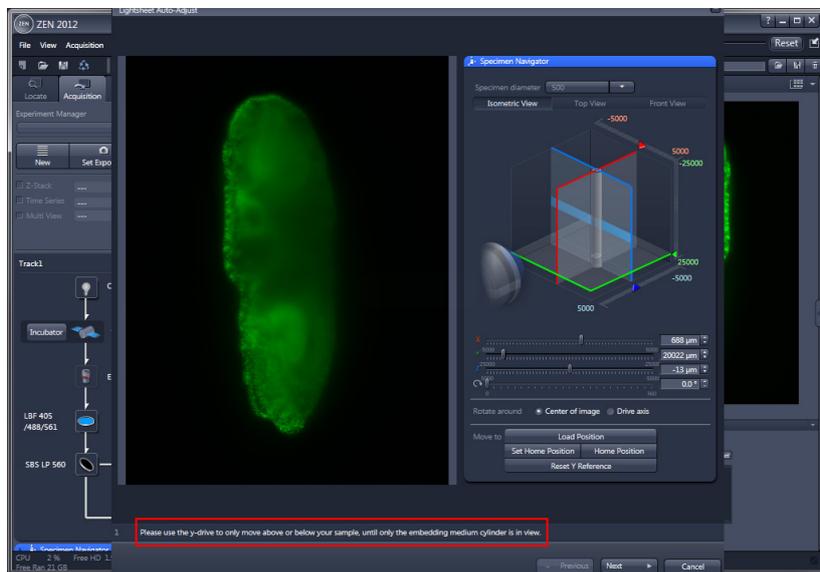


- Please adjust the light sheet right before you start the acquisition by using the Lightsheet **Auto-Adjust** wizard.

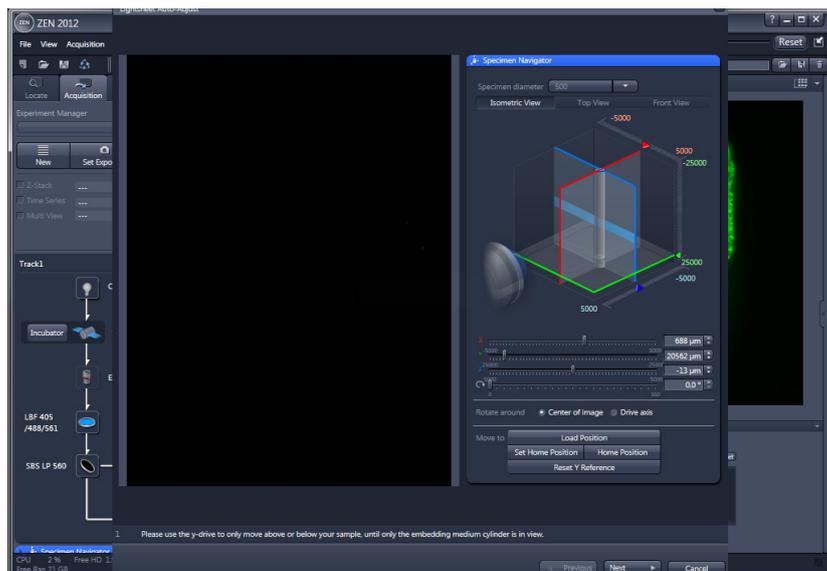
- Click **Auto-Adjust** .



- The initial window of the Lightsheet **Auto-Adjust** wizard shows a **live image** of your sample on the left hand side. The beam path and illumination settings are taken from the Channels Tool and Light Path Tool, so no additional corrections are necessary.
- The **Specimen Navigator** Tool is present on the right hand side.

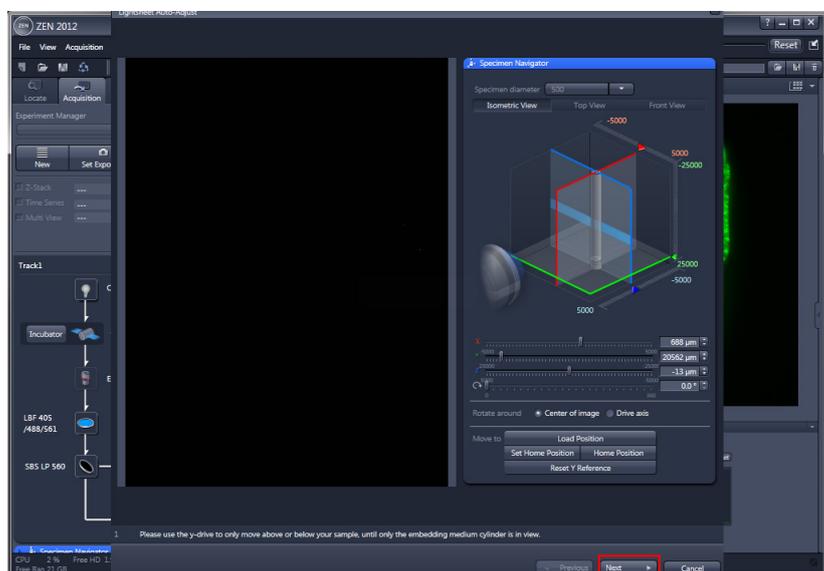


 A short description on what to do during this step is given at the bottom of the window.

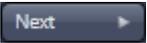


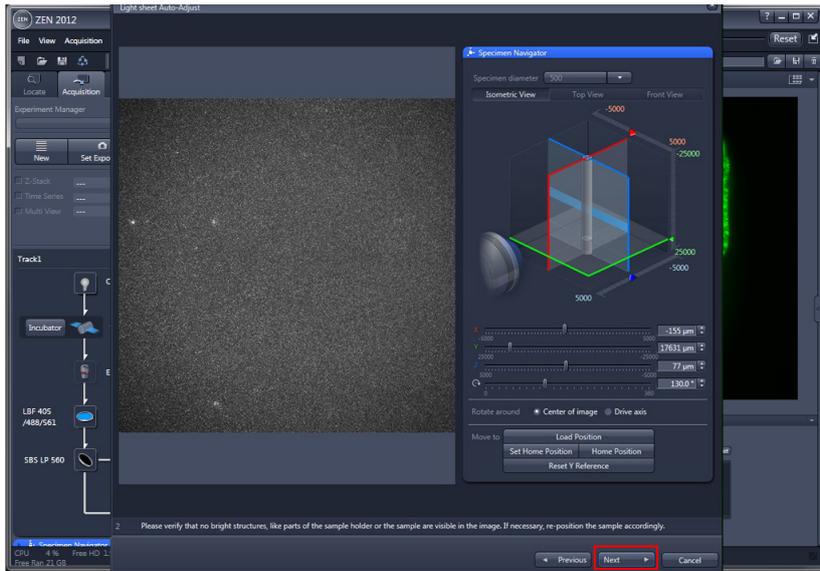
 **Only the Y-drive** should be used during this step since the conditions for the light sheet adjustment should be as close to the imaging conditions as possible.

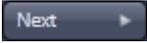
Since the embedding medium is an optical element that will influence the formation of the light sheet, for its adjustment, the position and thickness of the embedding medium should be the same as during imaging.

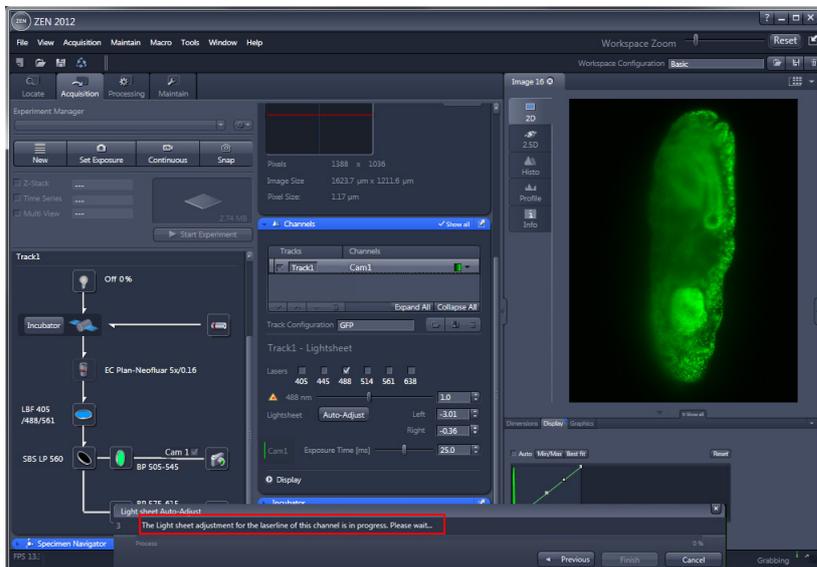


- When a position above or below your sample with only the embedding medium cylinder is in view, press the **Next** button.  
When you press **Cancel**, you will leave the wizard without any changes.

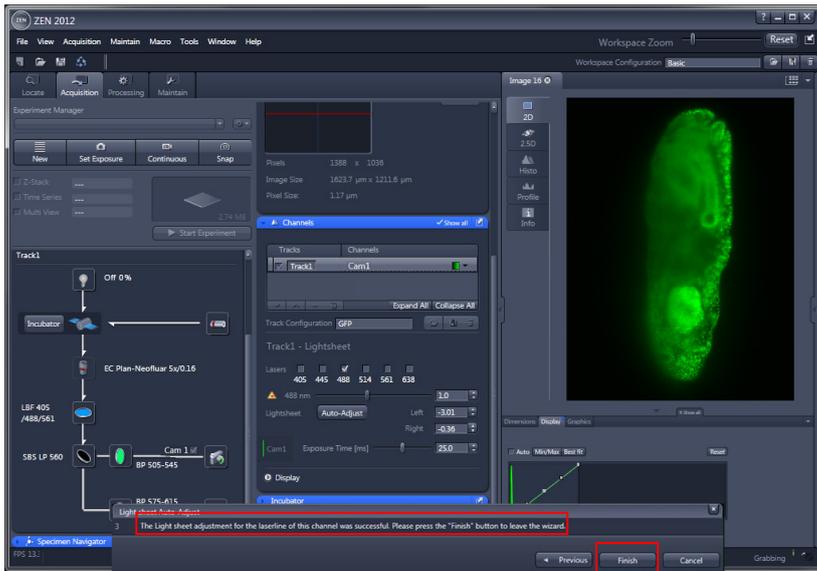
- Click **Next** 



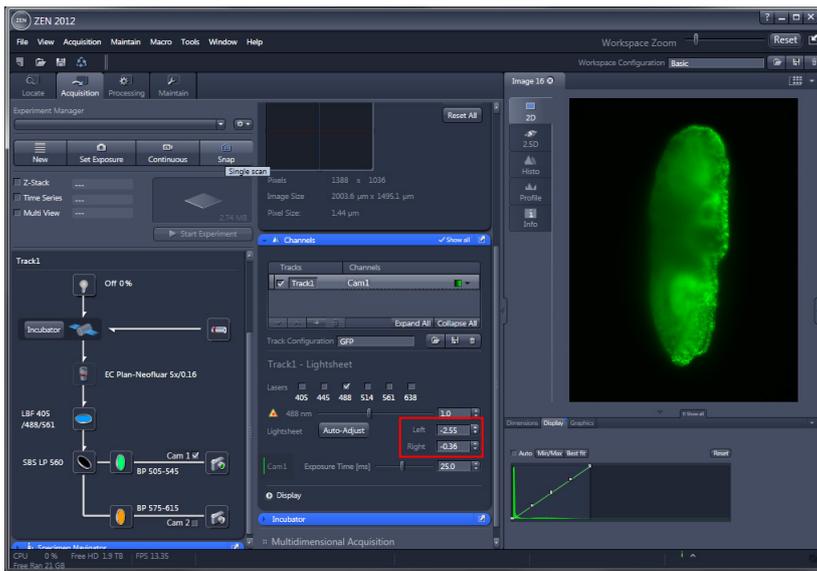
- The light path has been changed automatically, removing the laser blocking filter from the beam path, so stray light from the activated laser can be seen as a live image on the left hand side. Hence any bright or blocking structures, like the sample holder or the sample, are visible. If this is the case, the sample must be moved (optimally in the y direction), resulting in an evenly illuminated field of view.
- Click **Next** .



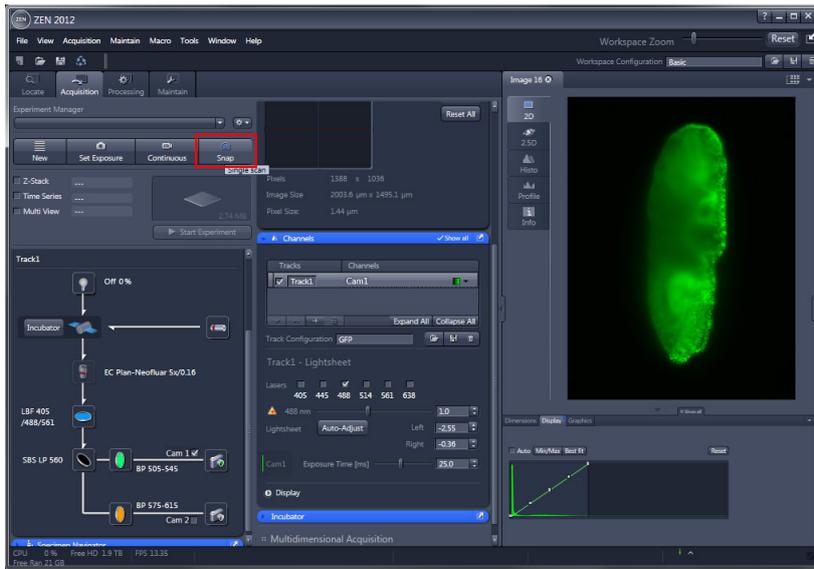
- You can follow the status of the adjustment on the progress bar.



- Click **Finish** 
- The original position of your specimen will be restored after the wizard is finished.

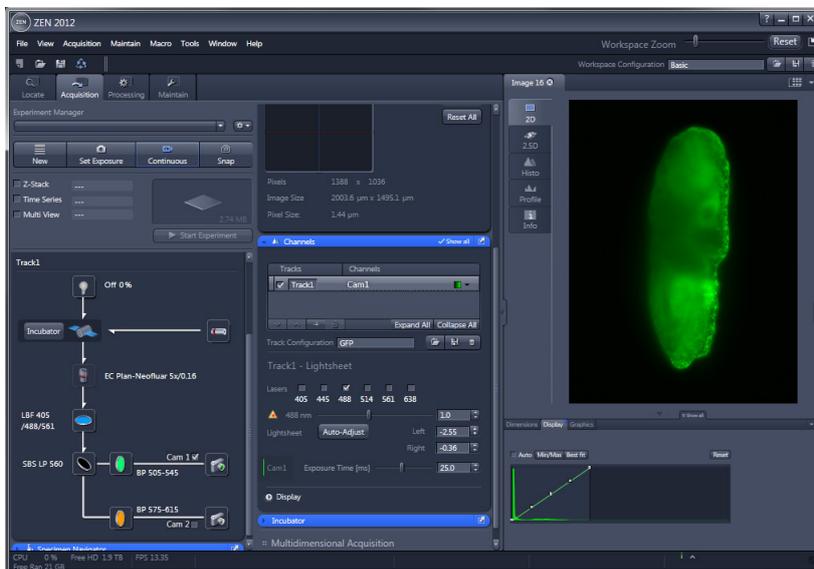


 The resulting settings from the light sheet alignment are automatically written into the input boxes **Left** and **Right** (or only one of them if Single side illumination is chosen in the Acquisition Tool window).



- **Snap** produces one single image using all active channels and tracks. It thereby ignores the parameters activated in the multidimensional acquisition area, but takes into account the imaging settings within the Acquisition Mode Tool and Channels Tool.

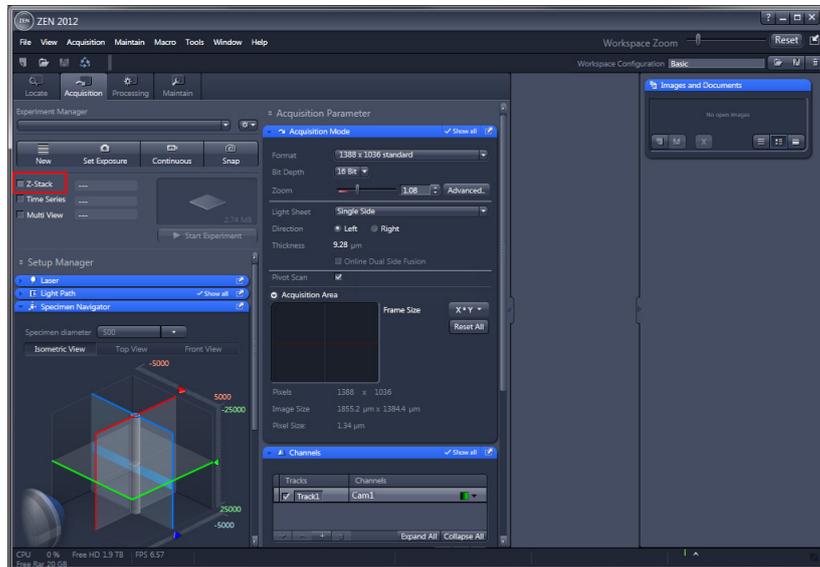
- Click **Snap** 

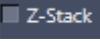


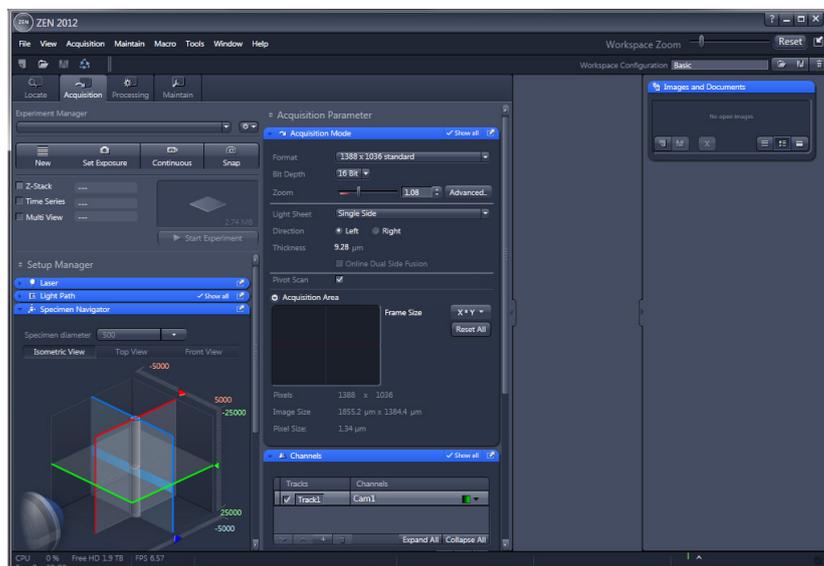
- 👉 **The basic image settings are now optimized!**  
**You can now proceed with a multidimensional image acquisition.**

## 3 Quick Steps to a Z-Stack

### 3.1 Defining the First Slice and the Last Slice

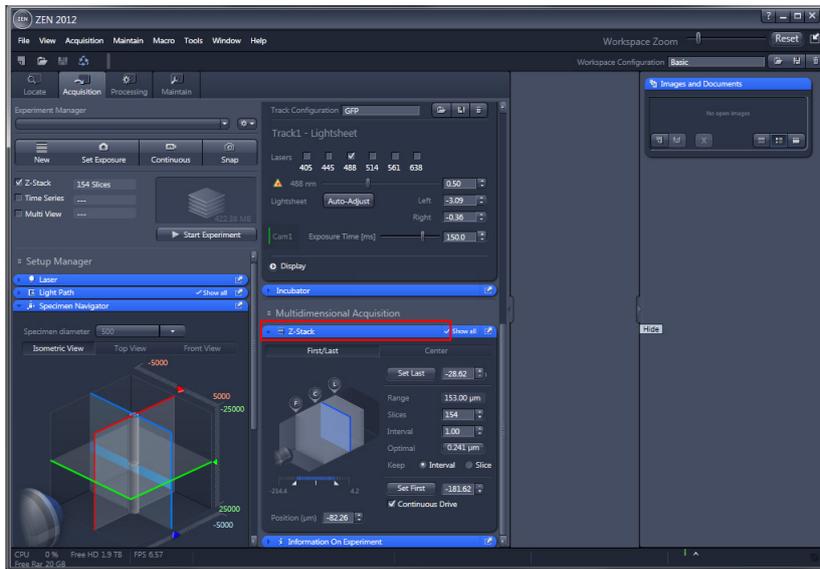


- Please activate the **Z-Stack** checkbox in the **Multidimensional acquisition** field to acquire an image series in z.
- Click **Z-Stack** .

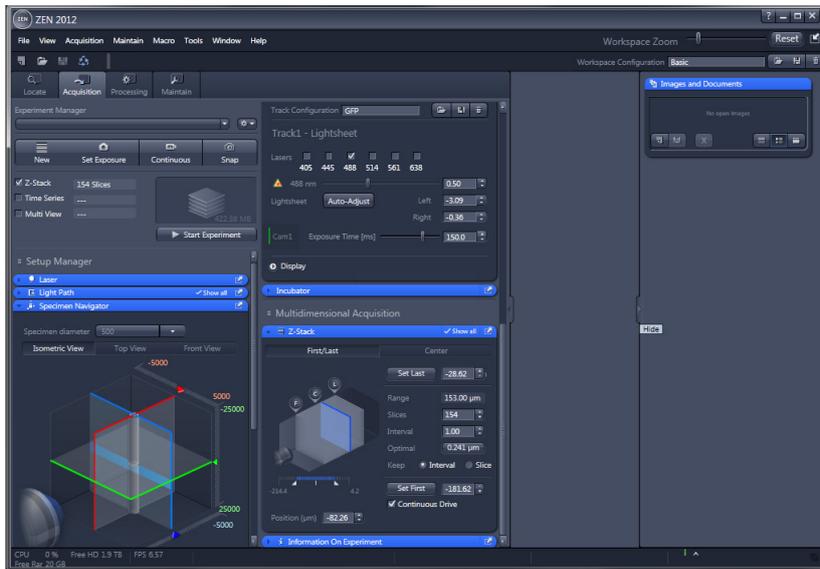


 The **Z-Stack** tool tab is only available when the **Z-Stack** checkbox is ticked.

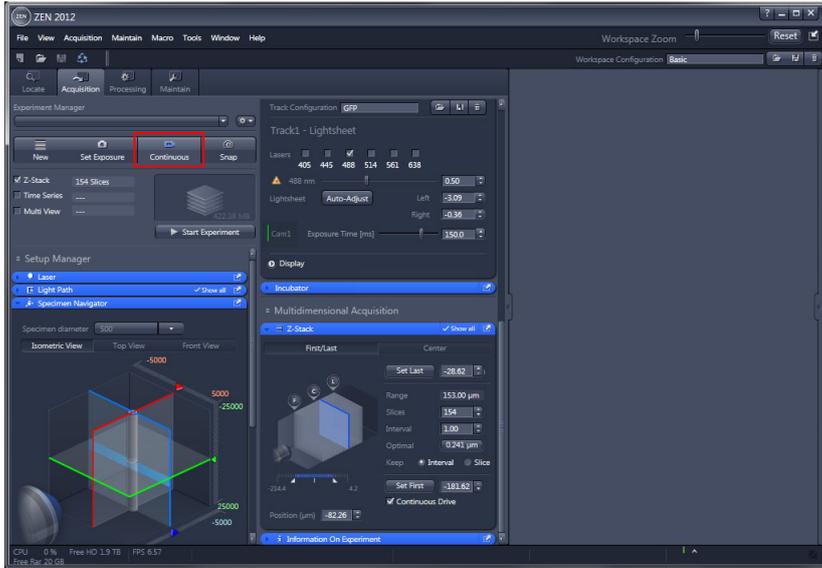
- Click on the **Arrow down button** to access the lower tool tabs.



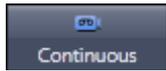
- If the **Z-Stack** tool bar is expanded, the **Z-Stack** control panel becomes available.



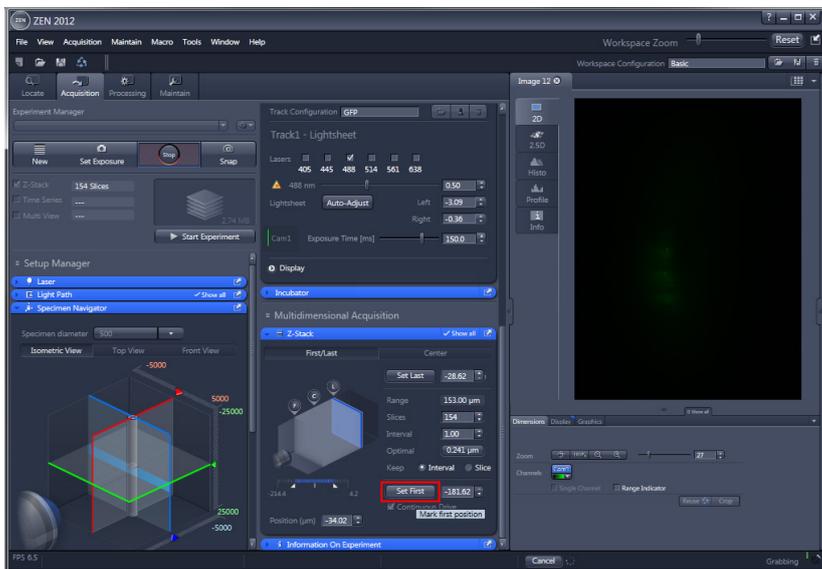
- Click on the **Hide** button to hide the Right Tool Area and expand the **Center Screen Area**.



- Click **Continuous** to start a live image acquisition using the currently active imaging parameters for defining the **First Slice** and the **Last Slice** within the **Z-Stack** control panel.



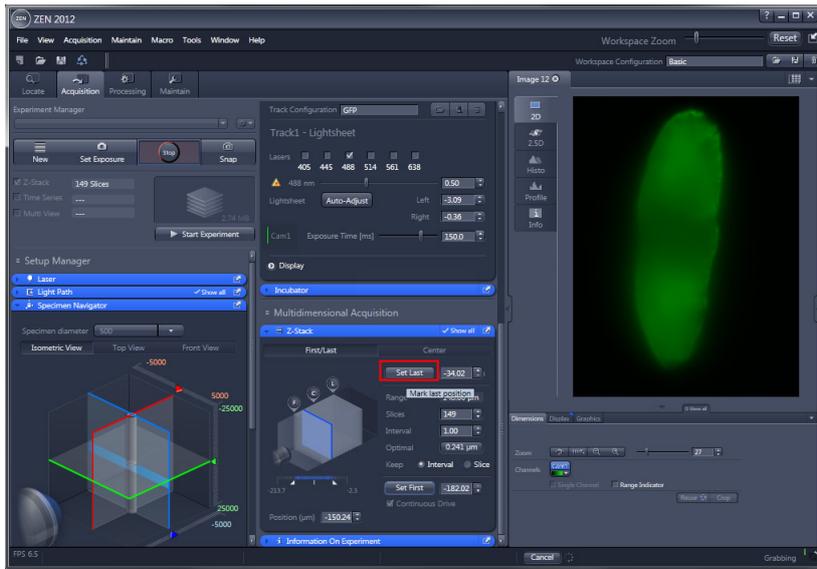
- Click **Continuous**



- When you reach the position within the specimen where you would like to start the stack, press **Set First** and this position will be taken as the first position in the stack.

- Click **Set First**

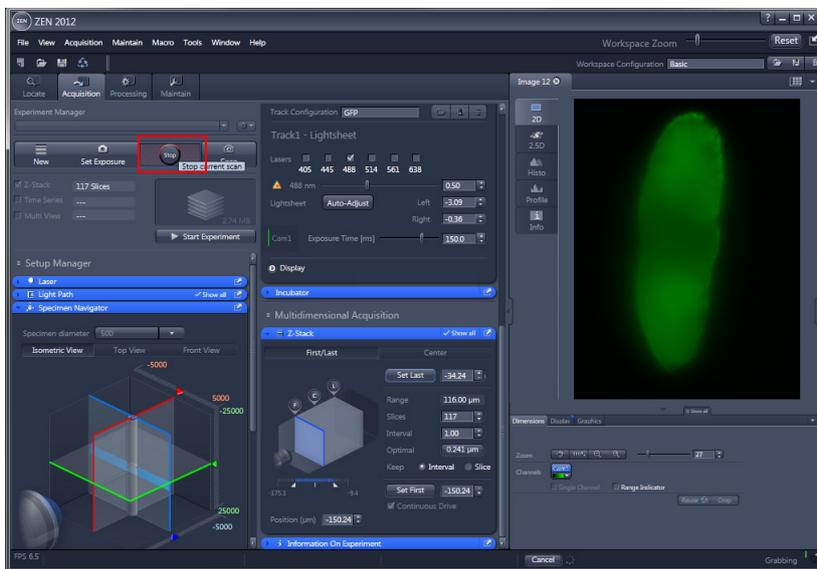




👉 Now move the focus in the other direction until the end position within the stack is reached, then press **Set Last**.

- Click **Set Last** 

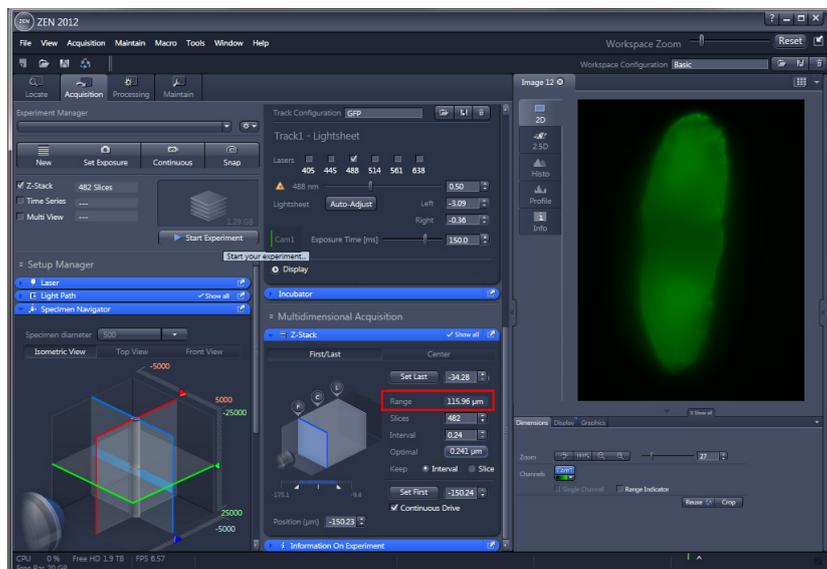
👉 Be aware that you might need a **wider range** for the z-stack which includes enough fiducials or other landmarks for the landmark alignment **processing** later.



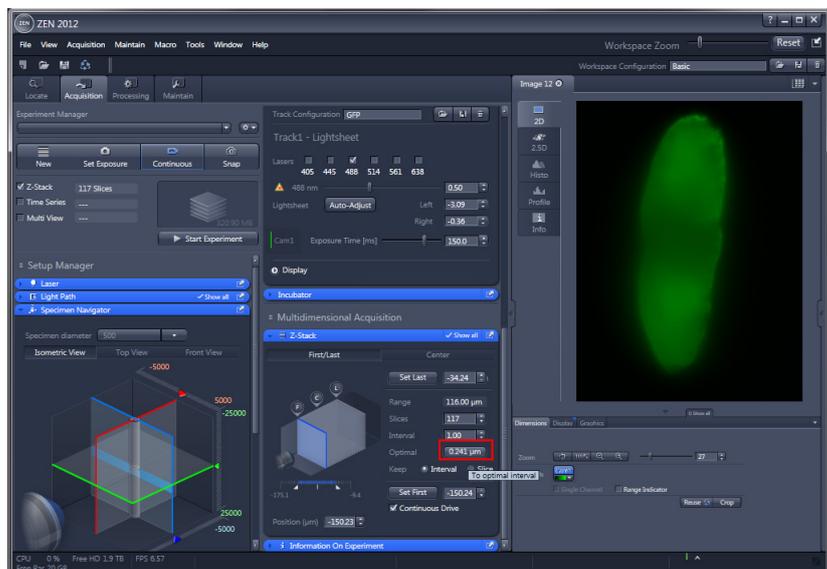
- To end the **Continuous** acquisition and save the specimen from unnecessary light exposure, press the **Stop** button.

- Click **Stop** 

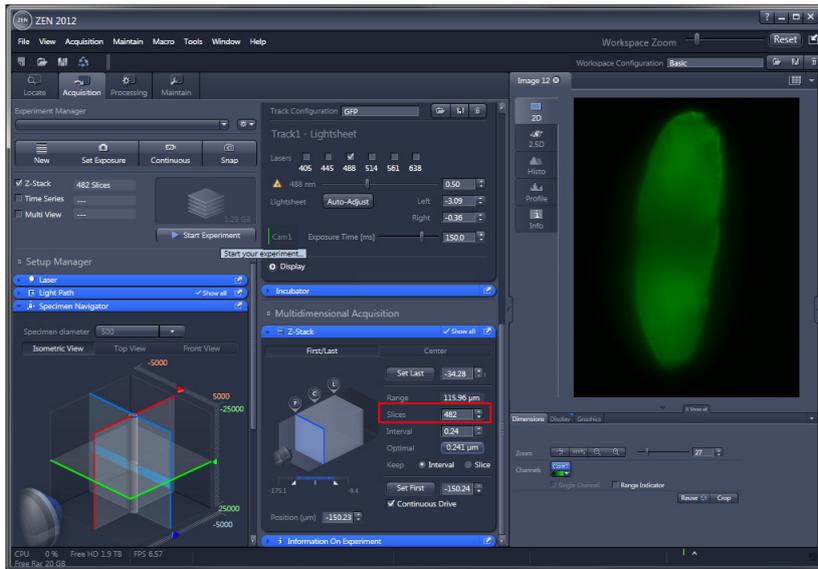
## 3.2 Adjustment of Z-Stack Settings



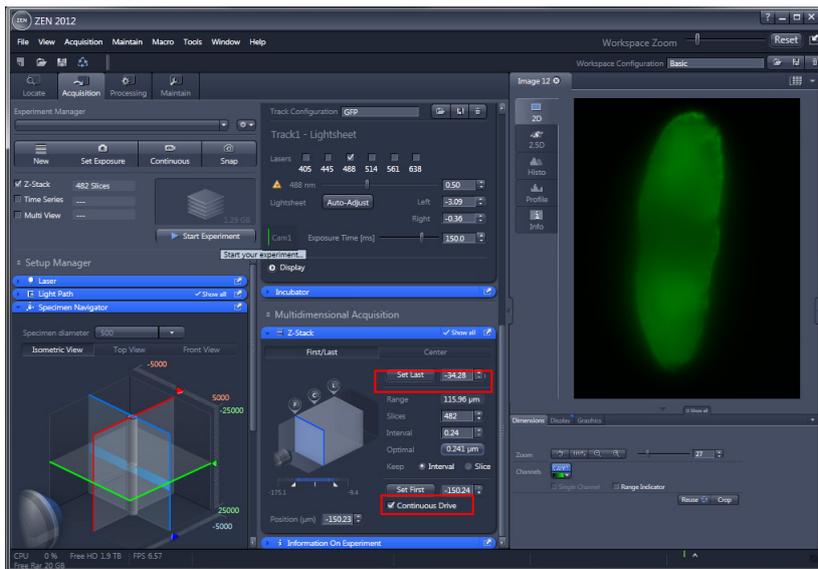
 The **Range** display box shows the total depth of the stack in micrometers.



- Press the **Optimal button** to set the number of slices to match the optimal Z-interval. This adjusts the number of **Slices** to keep within the **Range** set from marking Set First / Set Last.
- Pressing the **Optimal button** sets **Interval** to a value of half the optical slice thickness. Two adjacent slices therefore have always a 50 % overlap.
- Click **Optimal interval** **Optimal** 0.241 µm.



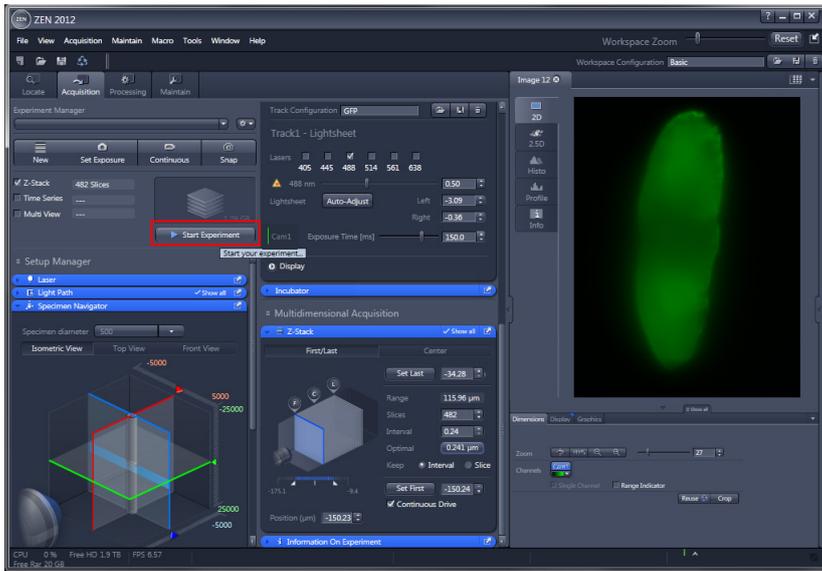
 The **Slices** input box displays the number of slices for the stack.

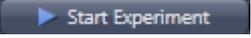


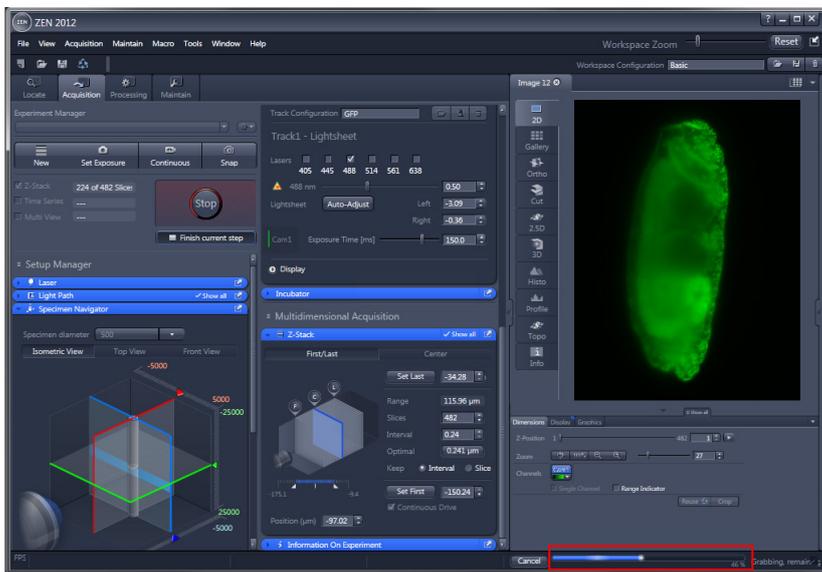
 Please use the **Continuous Drive** to speed up the Z-Stack acquisition significantly.

 When the **Continuous Drive** checkbox is ticked, the Z-Stack is performed by continuously moving the z-drive. The stack size is retained. The z drive will move the distance of the optical slice thickness during one exposure time that is set within the **Channels tool tab**. However, the overlap of two adjacent frames resulting from the interval distance is kept in this mode.

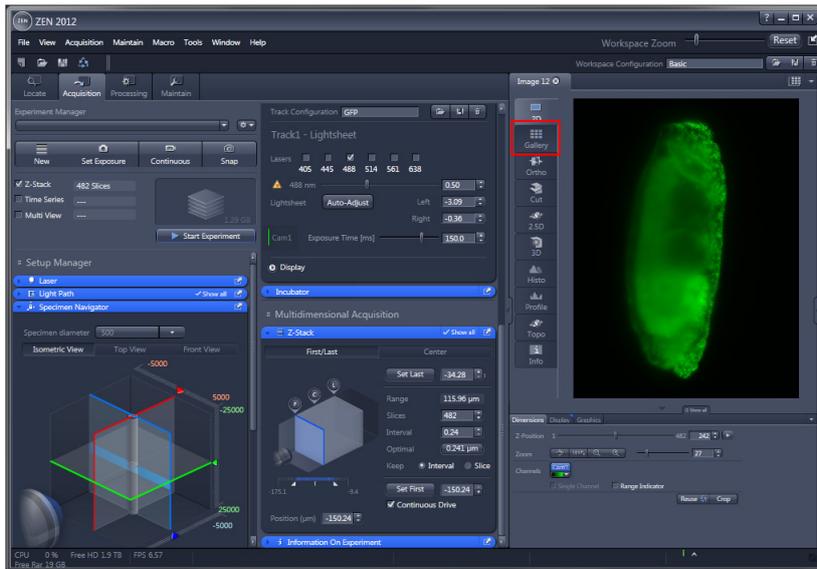
### 3.3 Start Z-Stack Experiment and evaluate Data



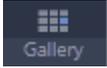
- Click **Start Experiment** to begin recording the **Z-Stack**.
- Click **Start Experiment** 

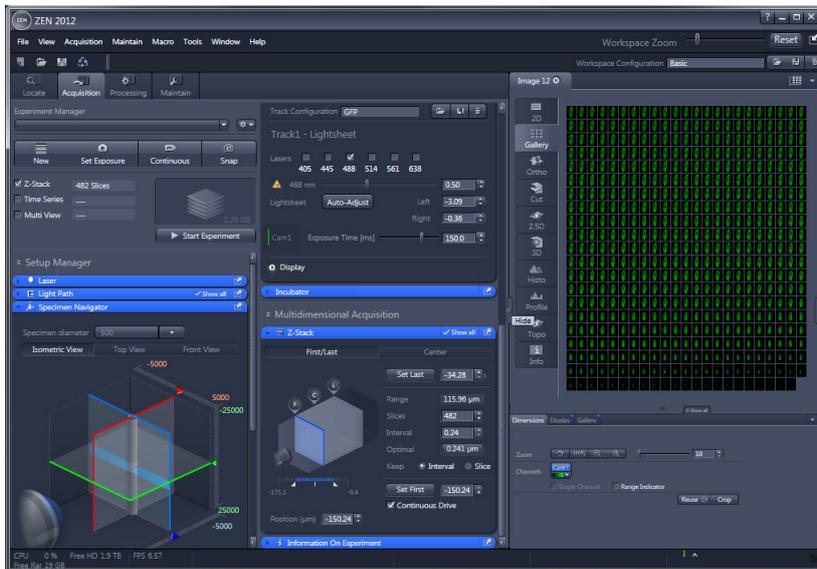


 You can follow the process by the displayed **progress bar**.

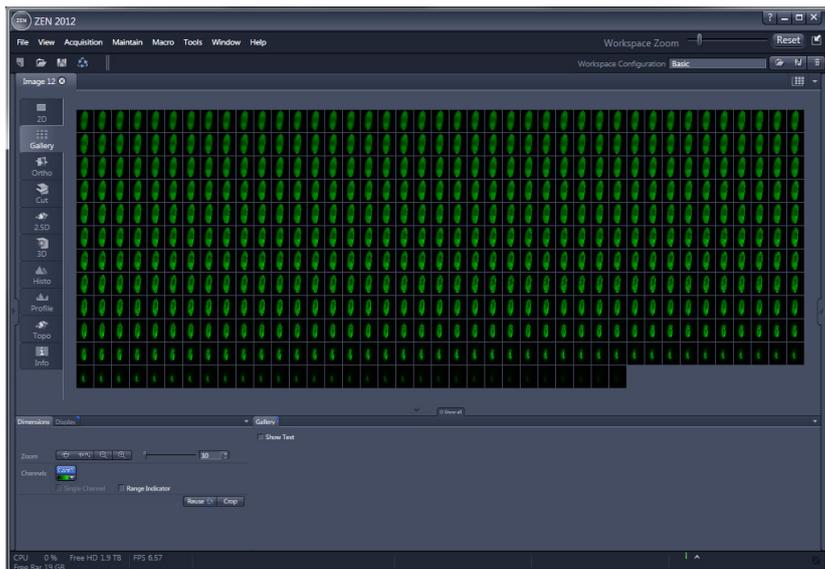


- The **Gallery view** displays images from the Z-Stack.

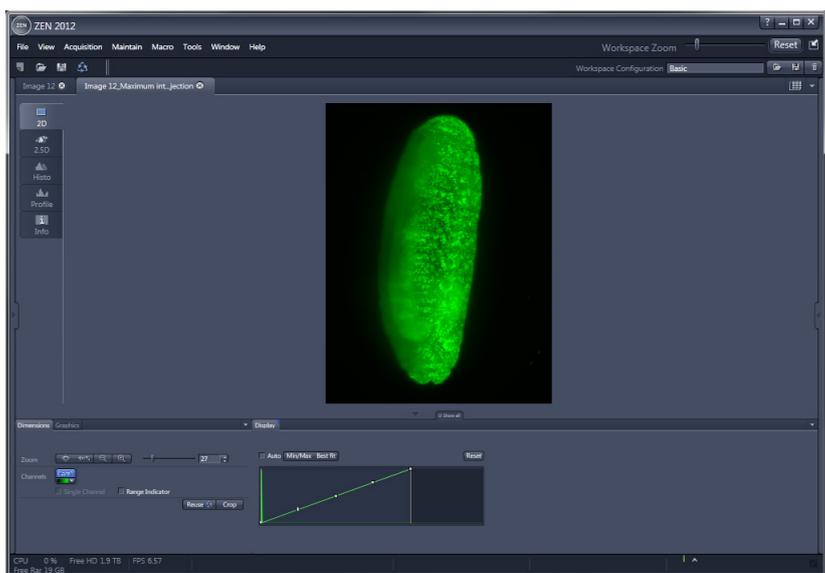
- Click **Gallery**  .



- Click on the **Hide** button to hide the Left Tool Area and expand the **Center Screen Area**.



 All the slices from the **Z-Stack** are represented in the **Gallery view** side-by-side in a tiled fashion.



- The **maximum intensity projection** shows an output image whose pixels contain the maximum value over all images in the stack at the particular pixel location.

 **You have successfully acquired a Z - Stack!**

You may now proceed with either:

**1. View tabs** in the Center Screen Area

- Ortho View
- Cut View
- 3D View (Image VisArt)

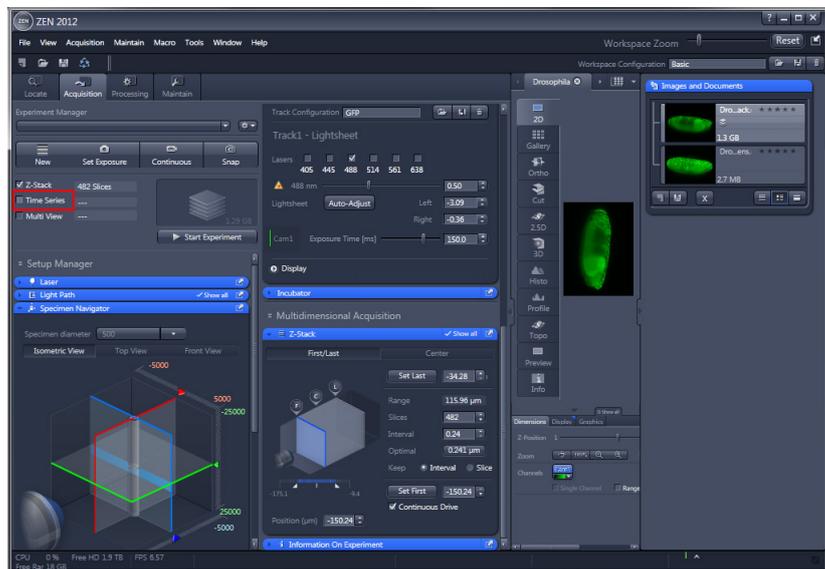
or

**2. Processing tab** in the Left Tool Area

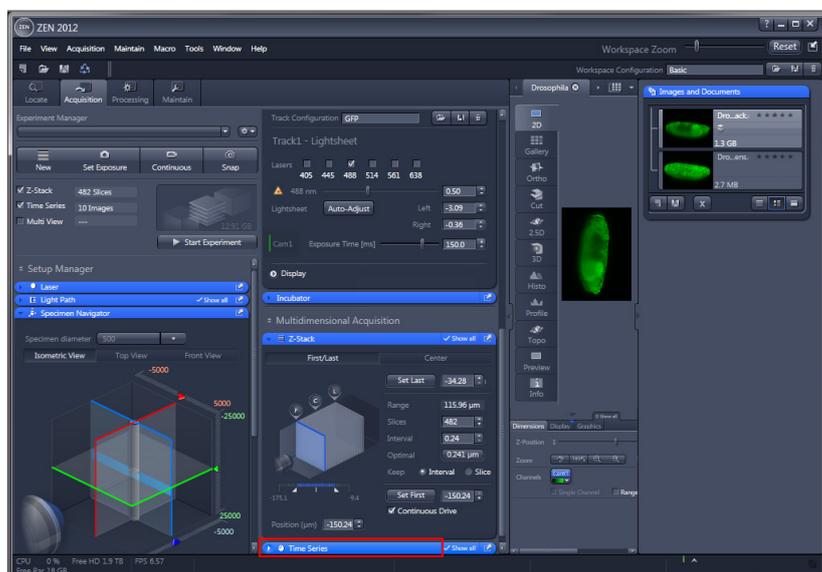
- Maximum Intensity Projection (as shown here)
- Lightsheet processing

## 4 Z-Stacks in Combination with Time Series

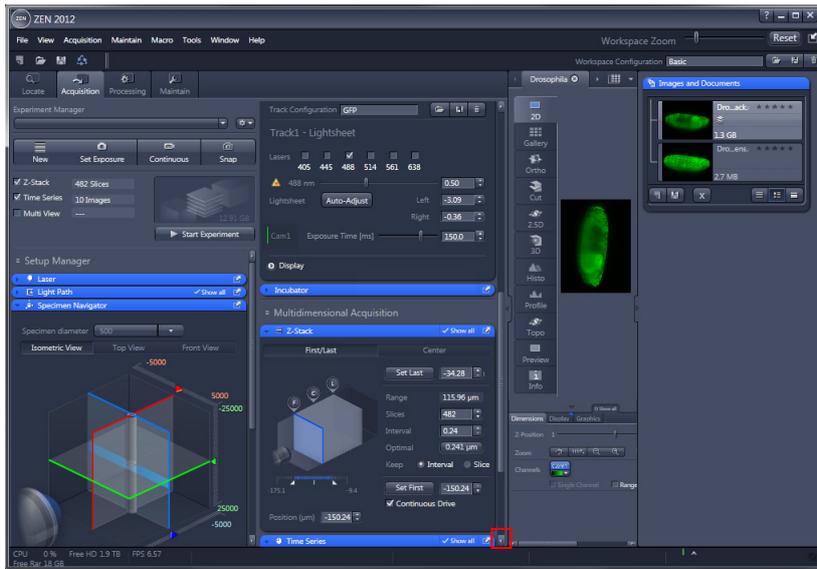
### 4.1 Defining the Time Series



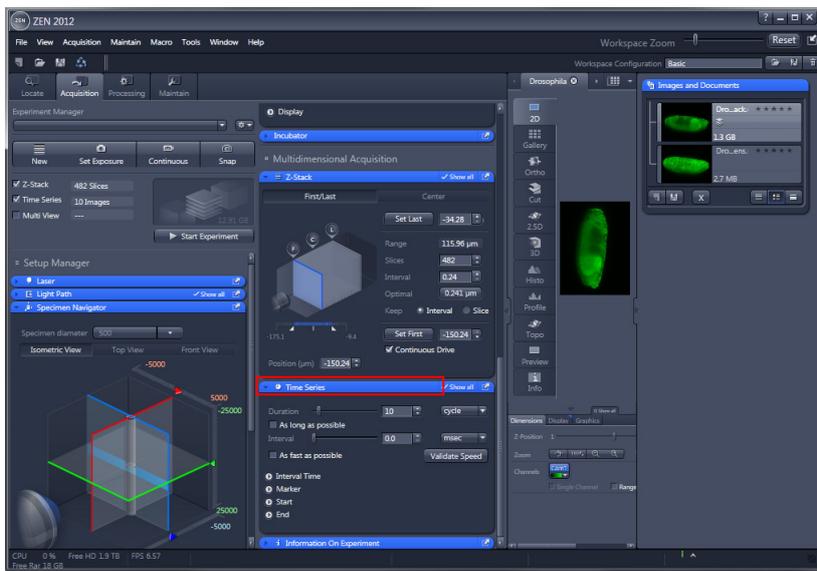
- Please activate in addition to the **Z-Stack**, the **Time Series** check box in the **Multidimensional acquisition** field to acquire an image series in Z in combination with a time series.
- Click **Time Series** .



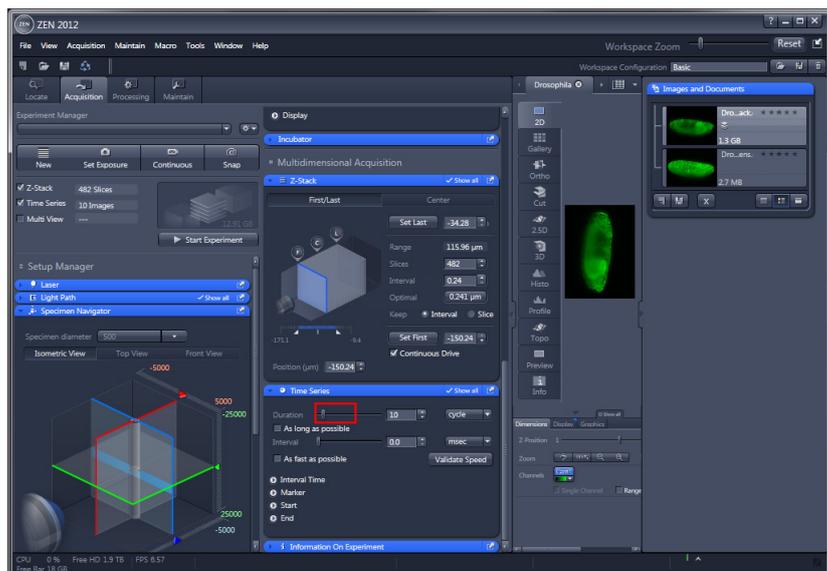
- In the **Time Series** tool, the parameters for the sequential image frame acquisition for a time series are defined.
- Click **Time Series** .



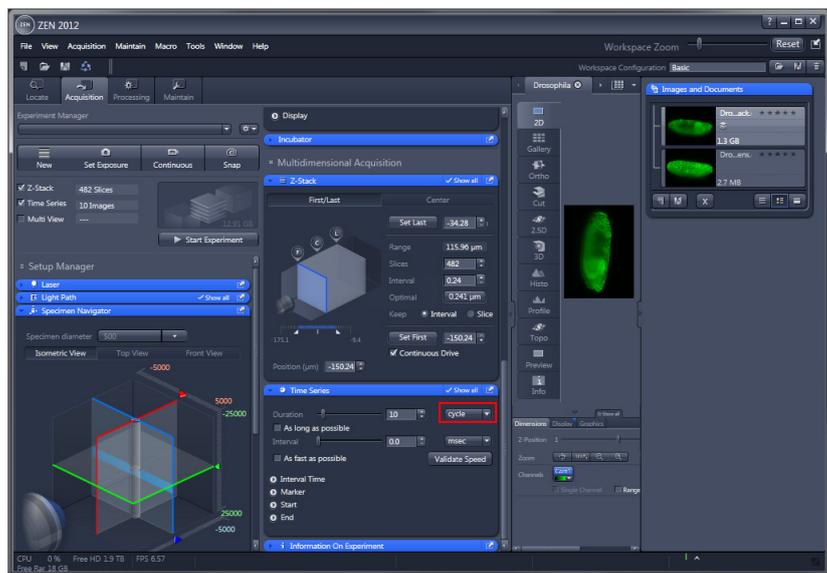
- Click on the **Arrow down button** to access the lower tool tabs.
- Click **Arrow down** .



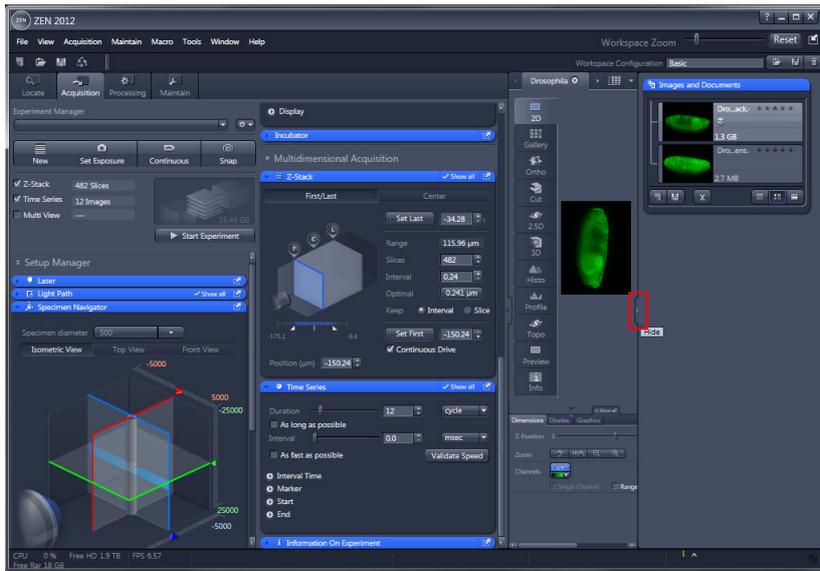
- If the **Time Series** tool bar is expanded, the **Time Series control panel** becomes available.



- To set up a time series acquisition, first define the **Duration** it should acquire the images by using the slider, or the text field, or the arrow buttons.
- Click **Duration** .

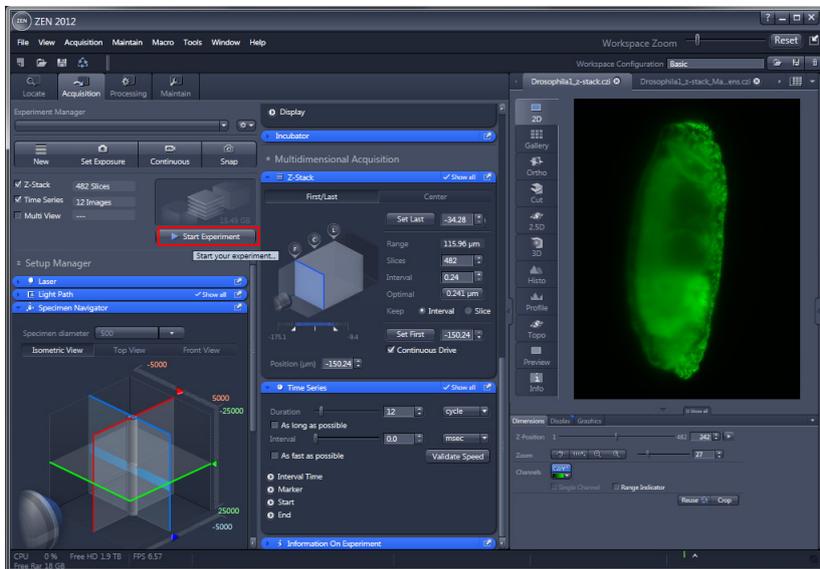


-  This selection box chooses the dimensions for setting the **Duration**; either cycle, msec (milliseconds), sec (seconds), min (minutes), h (hours), d(days).

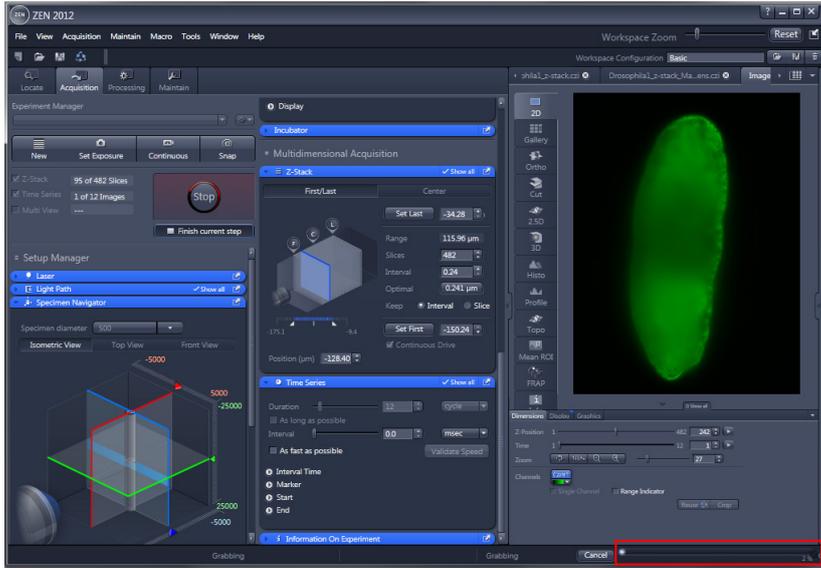


- Click on the **Hide** button to hide the Right Tool Area and expand the **Center Screen Area**.
- Click **Hide** .

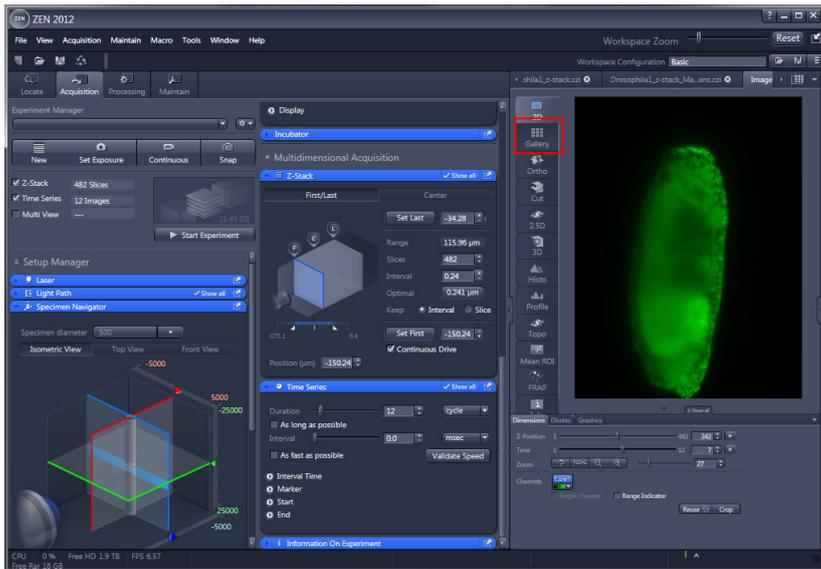
## 4.2 Start Z-Stacks Experiment in Combination with Time Series and evaluate Data



- Click **Start Experiment** .
- Click **Start Experiment** to begin recording the **Z-Stack** in combination with a **Time Series**.

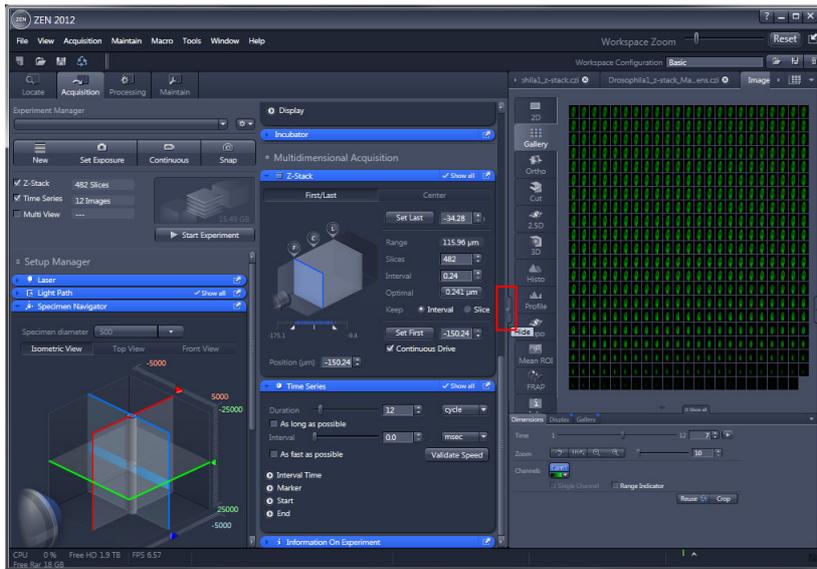


- You can follow the process by the displayed **progress bar**.

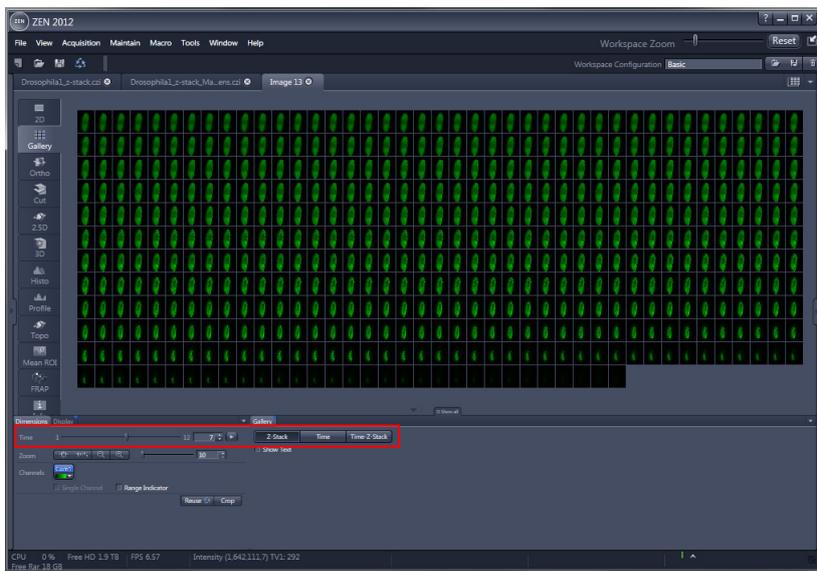


- The **Gallery view** displays images from the Z-Stack in combination with the Time Series.

- Click **Gallery** .



- Click on the **Hide** button to hide the Left Tool Area and expand the **Center Screen Area**.
- Click **Hide** .



-  The **Gallery** view displays images from the **Z-Stack** within the **Time Series**. The Gallery view in this example shows all slices from the Z-Stack acquired at Time point number 7.
-  The additional dimensions can be changed using the sliders and control blocks in the **Dimensions** View control block.

 **You successfully acquired a Z – Stack over Time!**

You may now proceed with either:

**1. View tabs** in the Center Screen Area

- Ortho View
- Cut View
- 3D View (Image VisArt)

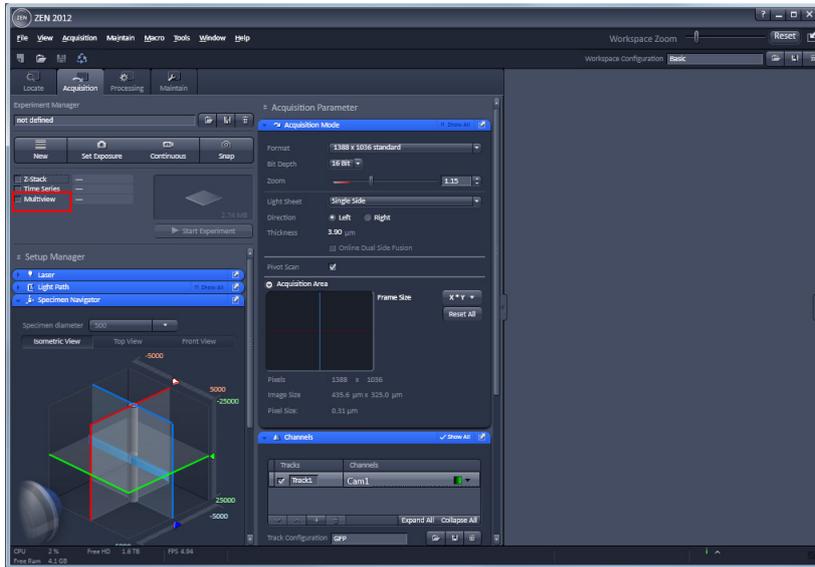
or

**2. Processing tab** in the Left Tool Area

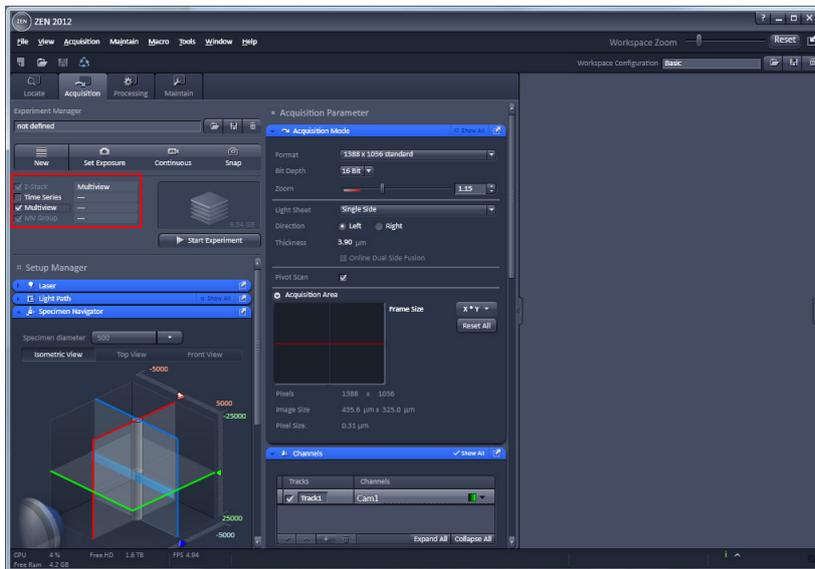
- Maximum Intensity Projection
- Lightsheet processing

## 5 See more from different Angles using Multiview Quick Setup

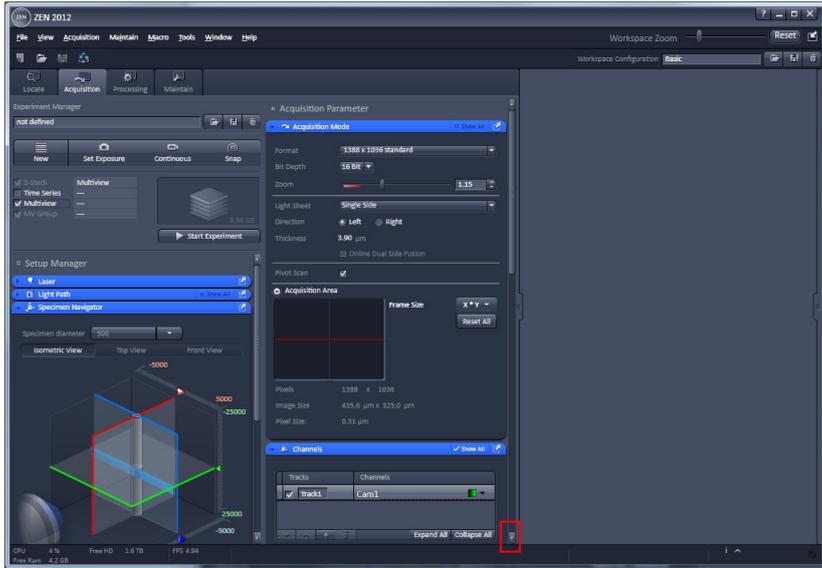
### 5.1 Multiview Quick Setup



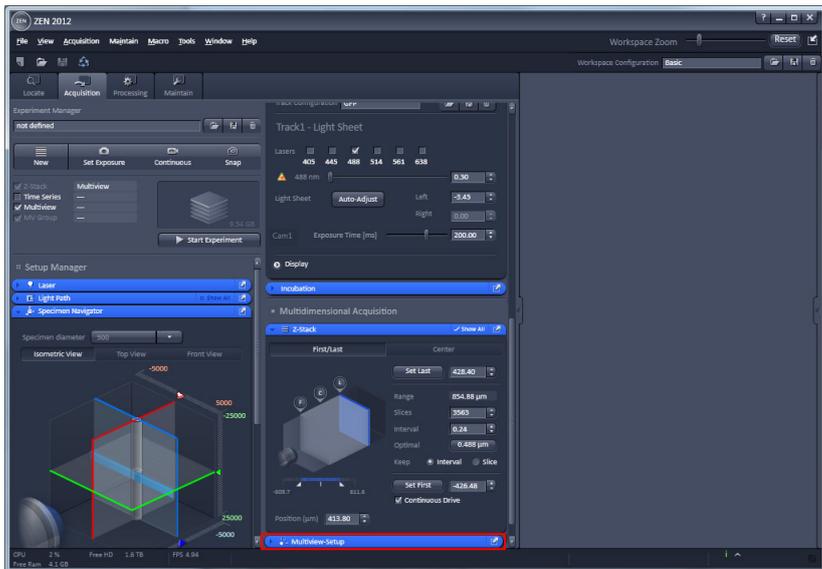
- Activate the **Multiview** checkbox in the **Multidimensional acquisition** field to acquire diversely sized Z-stacks using different Views.  
A **View** is determined by the **X**, **Y** and **Alpha** (rotation) motor position of the sample holder.
- Click **Multiview**  **Multiview**.



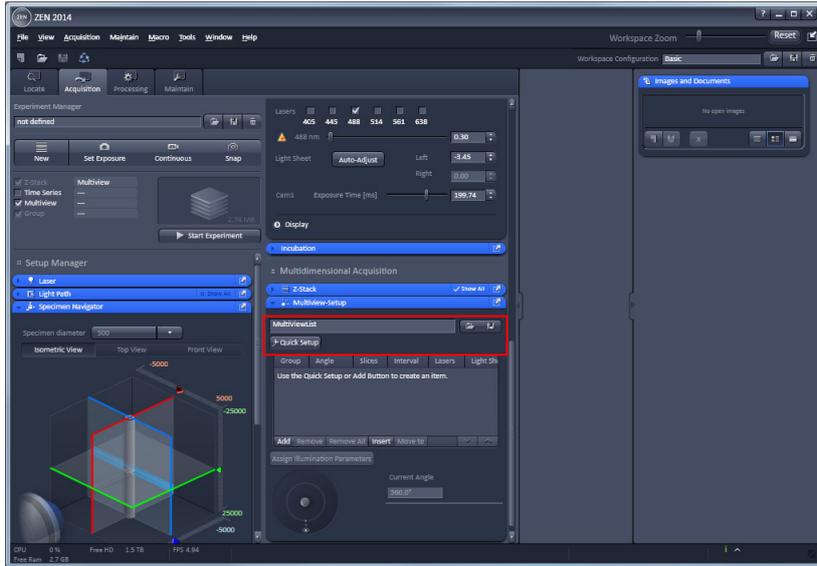
 Along with a **Multiview** experiment, a **Z-Stack** is automatically activated and greyed out.



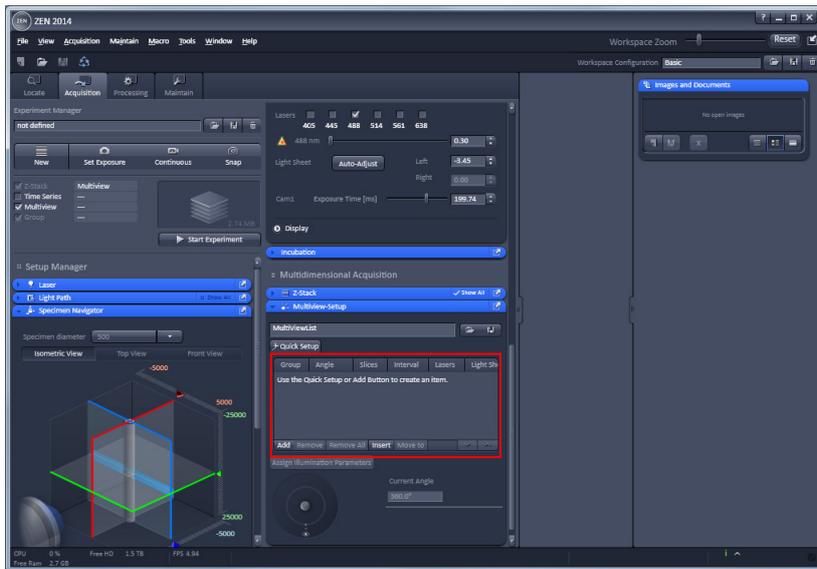
- Click on the **Arrow down button** to access the lower tool tabs.
- Click **Arrow down** 



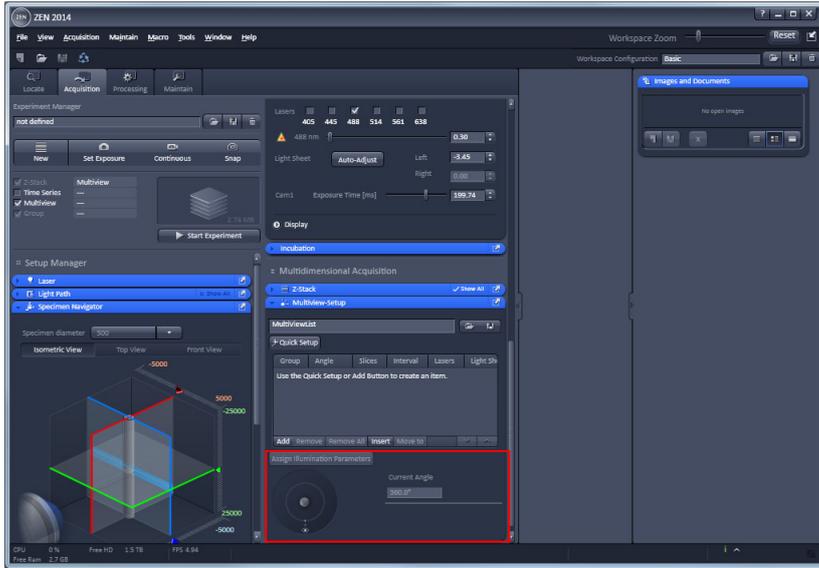
- When the **Multiview-Setup** tool tab is expanded, the **Multiview-Setup** control panel is available.
- Click **Multiview-Setup** 



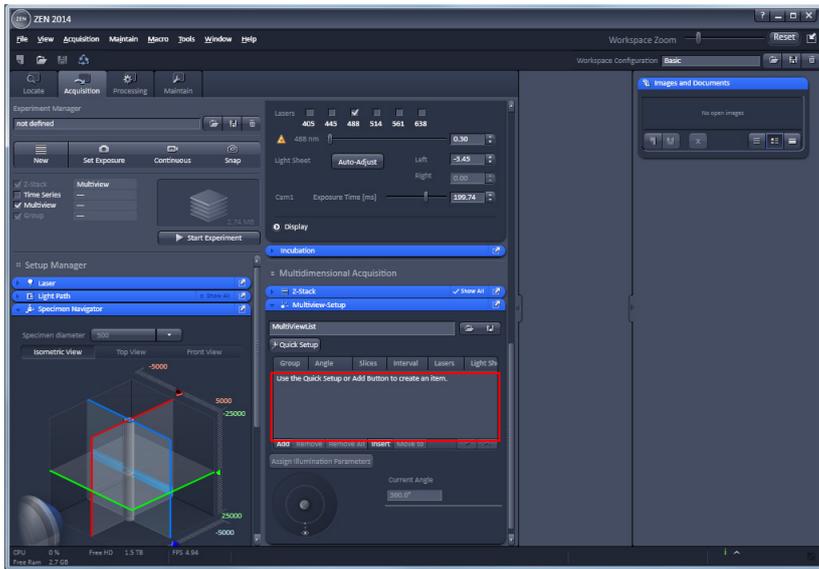
 The **Multiview-Setup** tool window is divided into three regions.  
**(1)** The upper region is the organization region and provides access to the **Quick Setup** wizard.



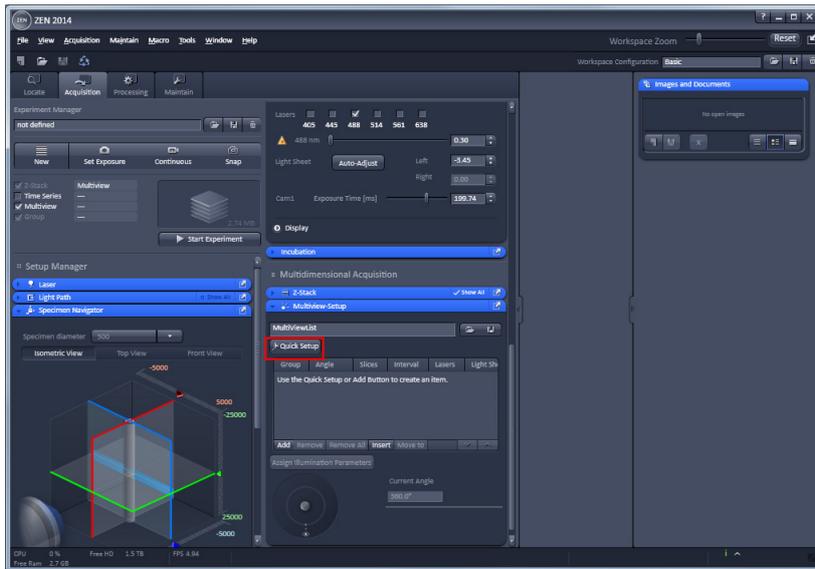
 **(2)** In the middle you find a list of created Views (if already available) and their control buttons.

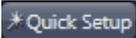


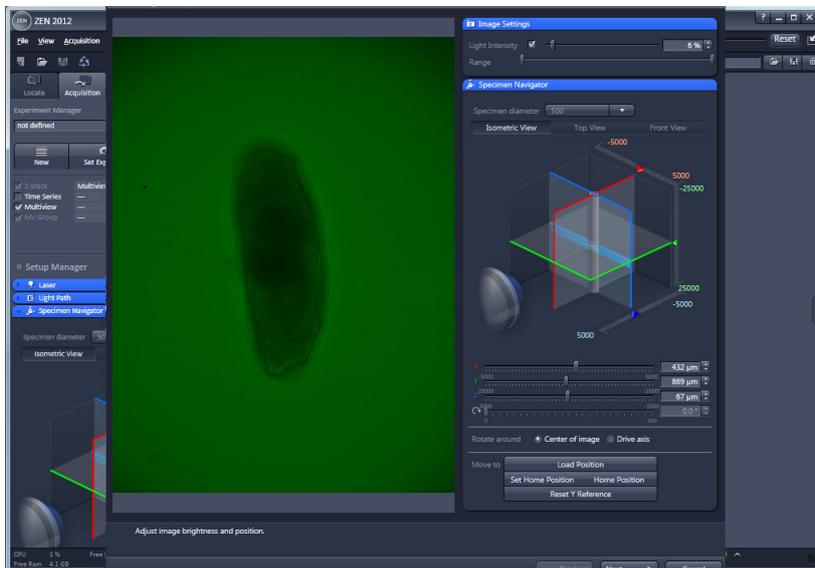
**(3)** In the lower part is an illustration showing the angle from which the detection optic (eye symbol) is facing the sample (middle cylinder). The zero angle is indicated by the dot at the bottom of the outer circle.



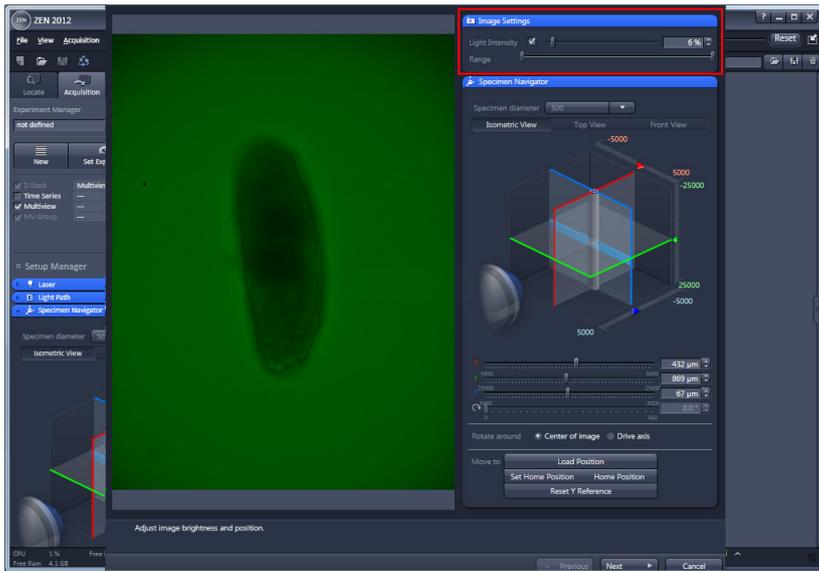
**!** When Views are already defined when starting Quick Setup, you can choose to overwrite them or add a new Group of Views to the list.



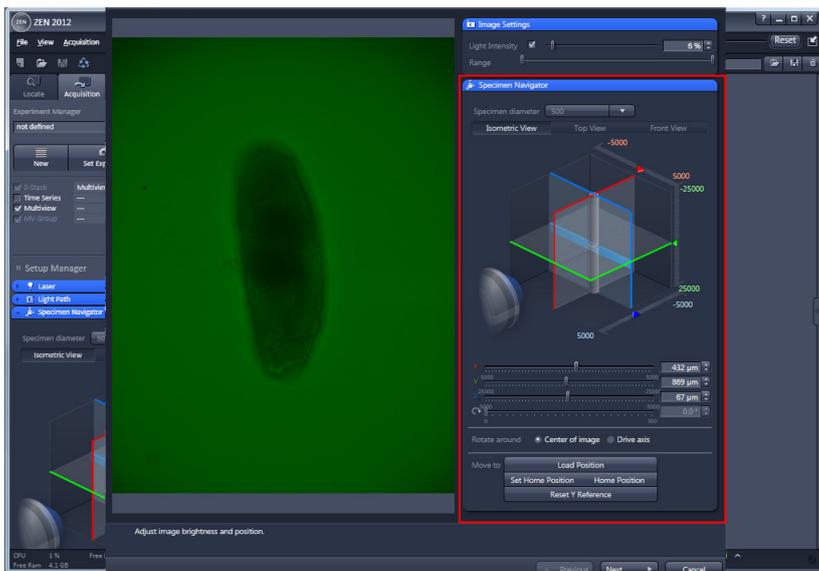
- The **Quick Setup** button opens a new window with a software interface that guides you through the process of setting up a multiview experiment. This includes the **positioning of the sample**, defining the necessary **Z-stacks** (Range, Slices, and Interval), and the **number of views**.
- Click **Quick Setup** 



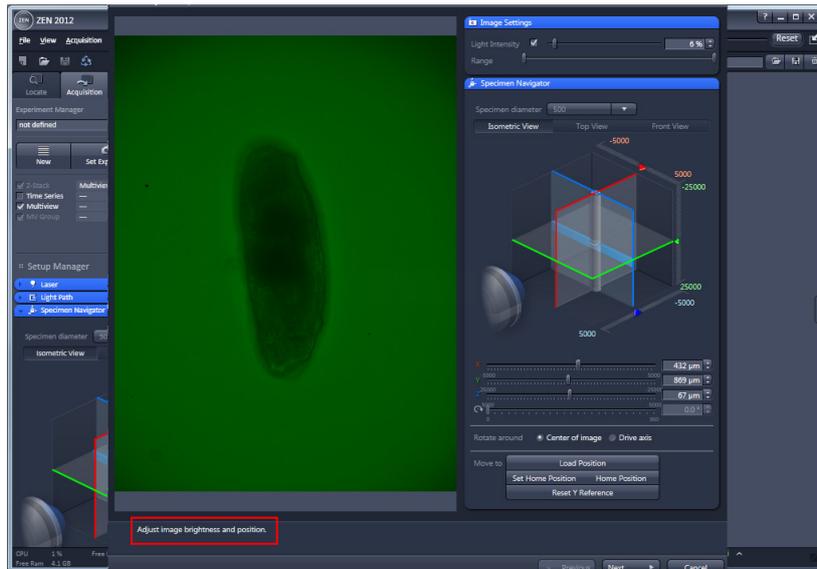
- ☞ The initial window for the Lightsheet **Quick Setup** wizard shows a live transmitted infrared light image of your sample on the left-hand side.



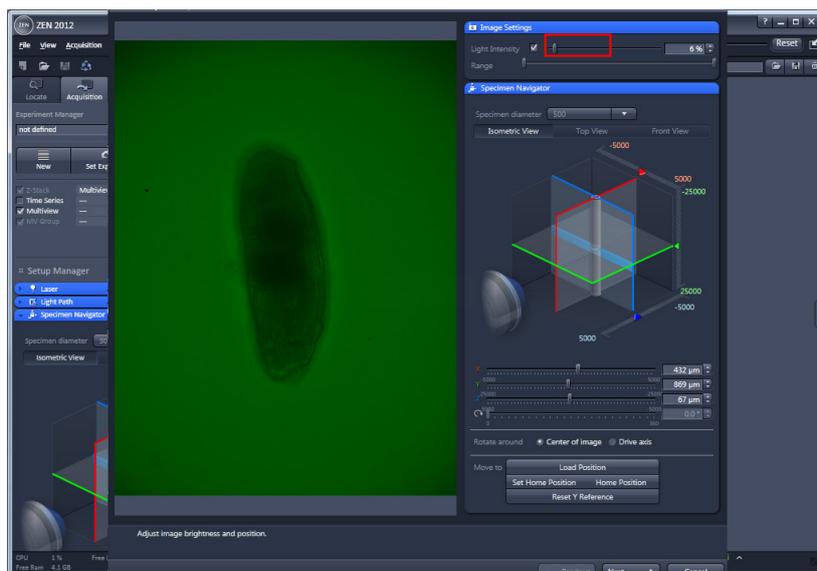
👉 On the right-hand-side is the **Image settings** tool tab. Here you have easy access to the **Light Intensity** slider for adjusting the **infrared illumination**.



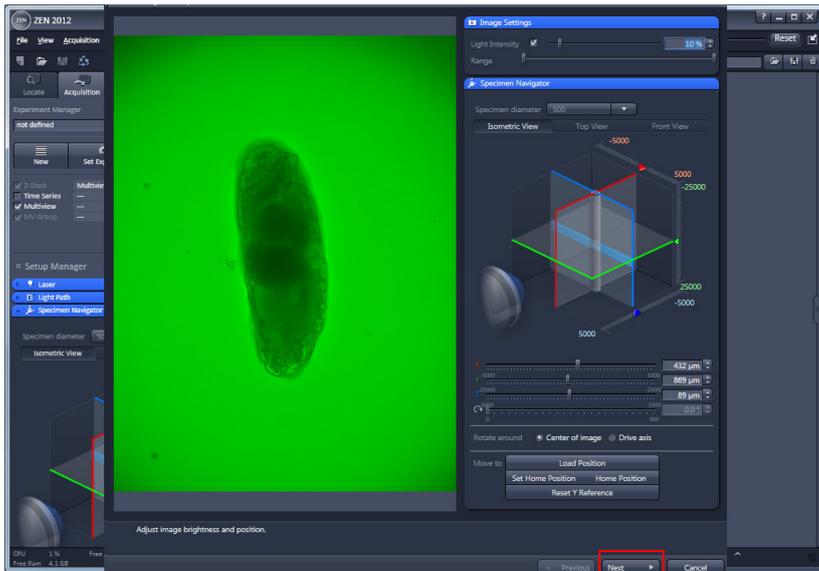
👉 The **Specimen Navigator** provides access to the focus and specimen position.



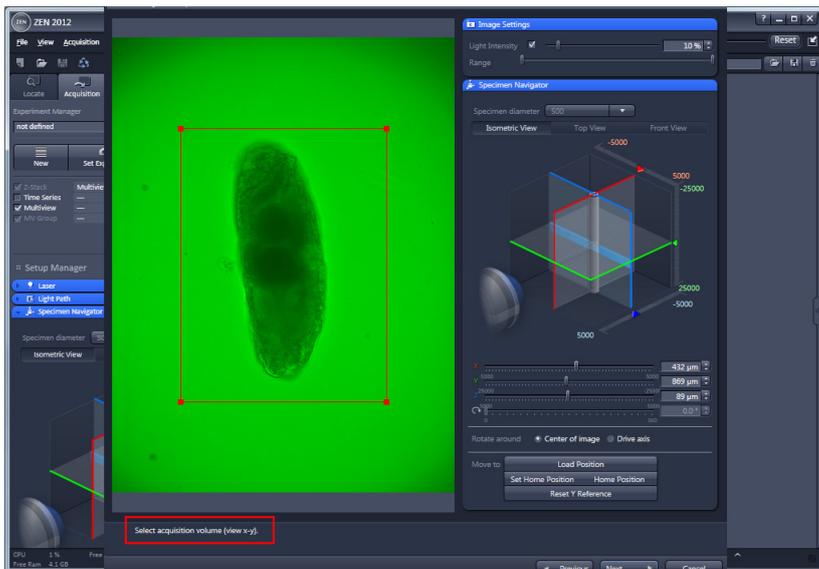
 A short description of what to do during this step is given at the bottom of the window.



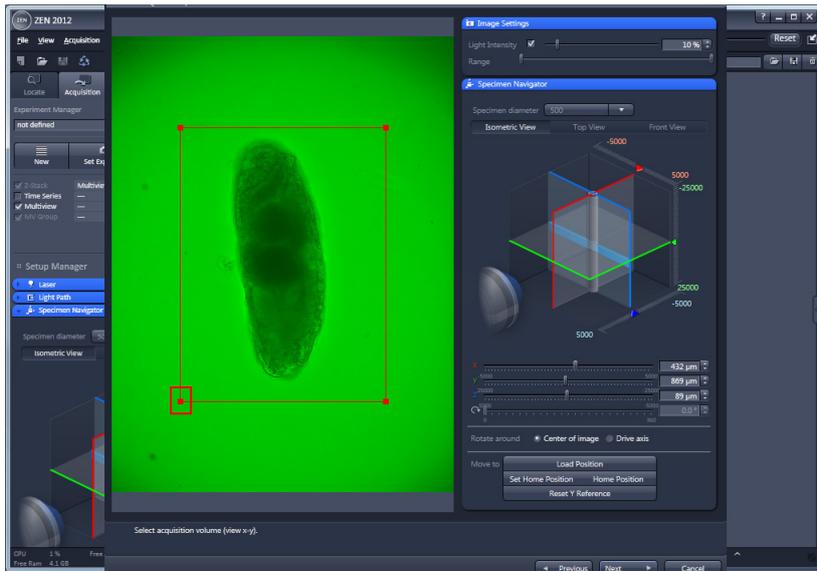
- Move the **Light Intensity** slider to adjust the **infrared illumination**.
- Click **Light Intensity** .



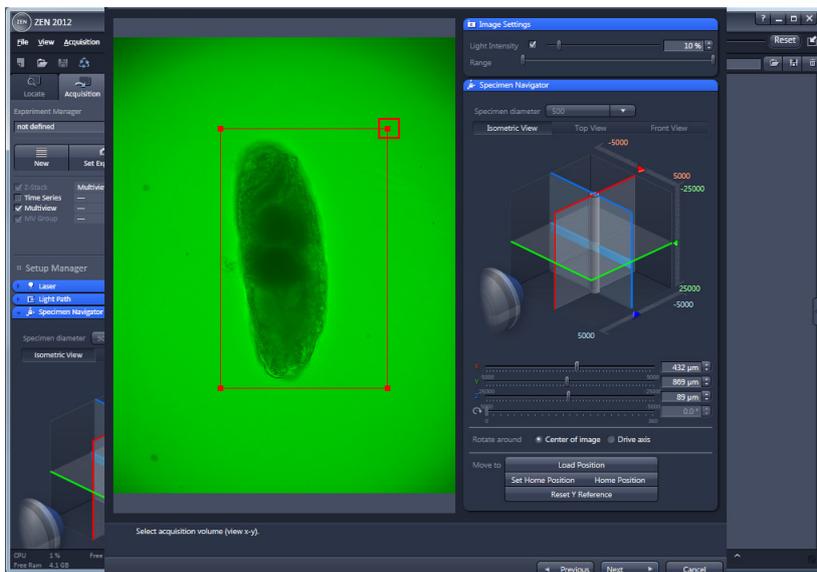
- If you have finished the image brightness and position adjustment, please press **Next**.
- Click **Next** .



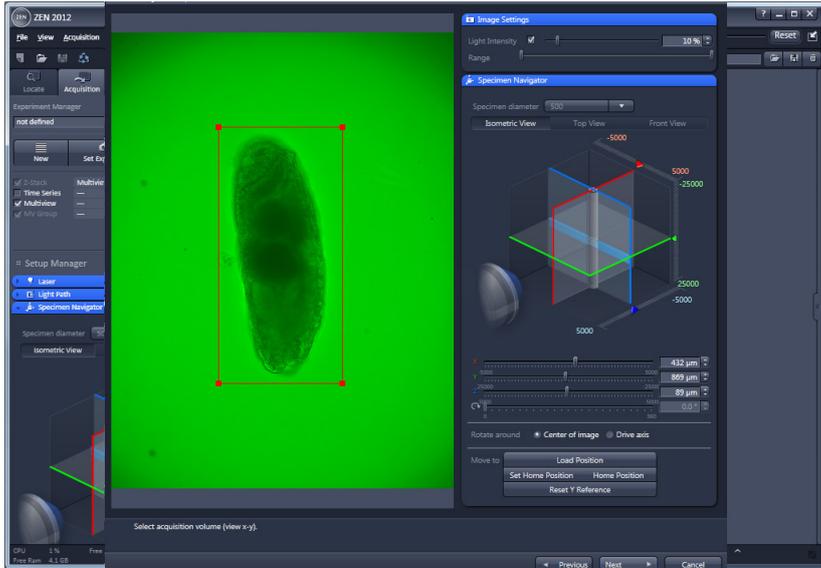
- To select the **acquisition volume** (view x-y), a **red rectangle** is displayed in the live image window. The **red rectangle** must be adjusted to completely surround the sample.



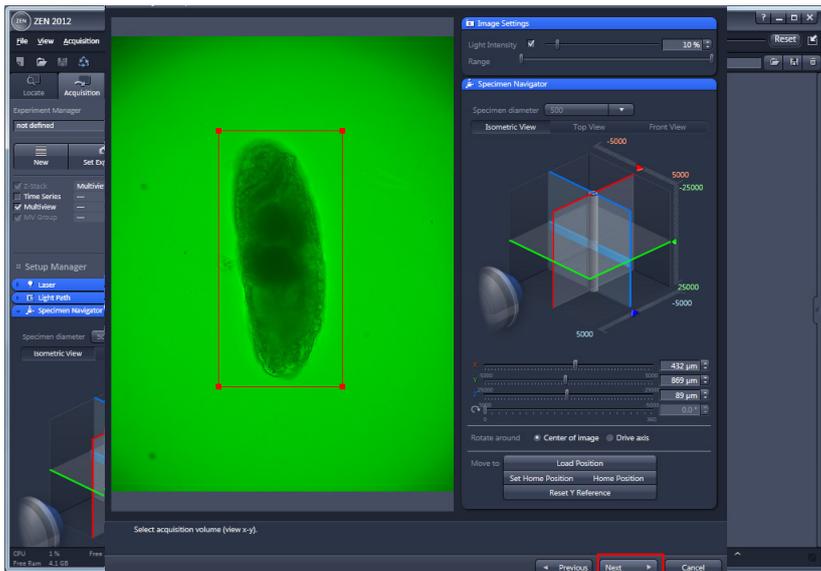
- To change its size, grab it with the left mouse button on one of the corners and adjust the length of the adjacent sides.
- Grab **Corner** .



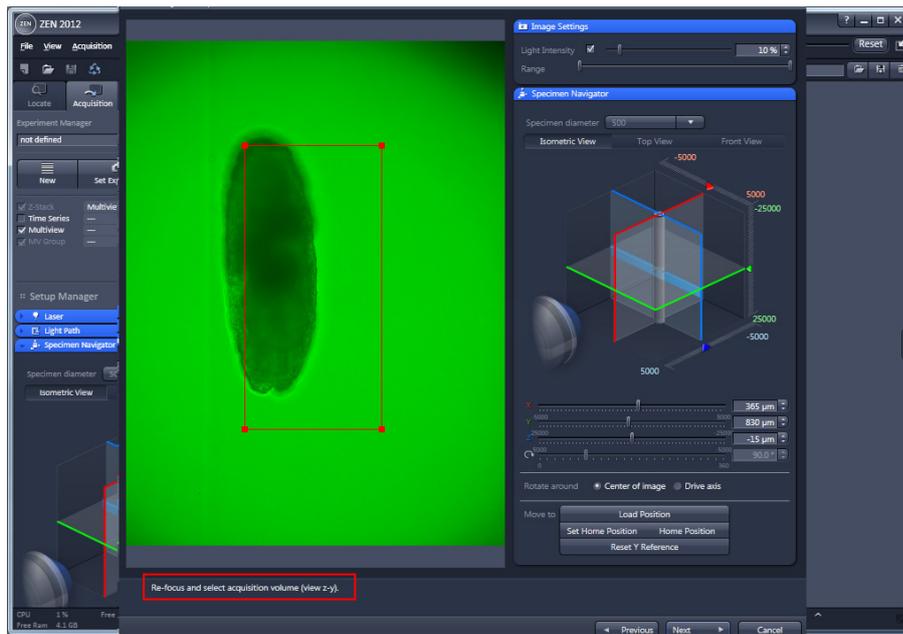
- To change its size, grab it with the left mouse button on one of the corners and adjust the length of the adjacent sides.
- Grab **Corner** .



Check, by moving the Z-drive, to see if the red rectangle encircles the whole sample along its Z-axis.



- After pressing the **Next** button, the sample is turned by 90° before proceeding to the next step.
- Click **Next** 



- (1) Re-adjust the sample focus by using the Specimen Navigator tool tab if necessary.  
It might be necessary to reposition the specimen using the X- and Y-drives as well.

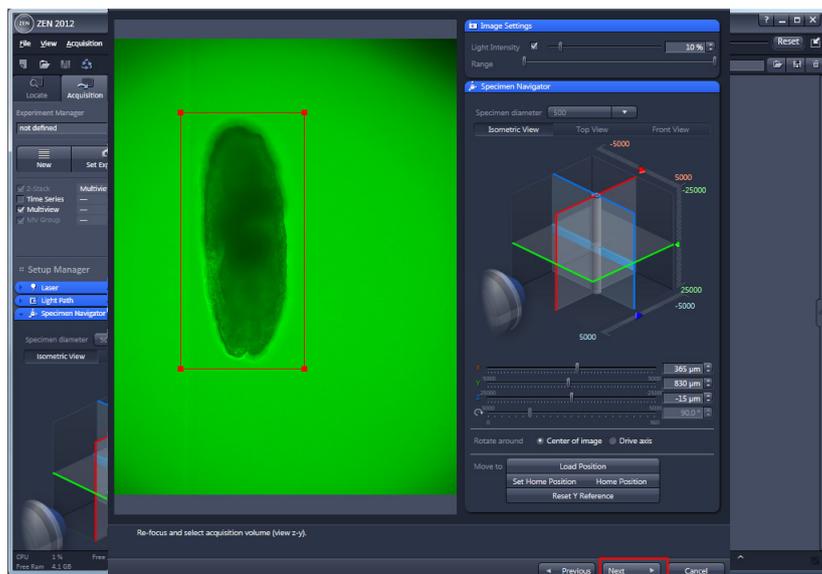


**Do not change the angle!**

- To move the whole rectangle, grab it by holding the left mouse button on one of the sides and drag it to the wanted position.

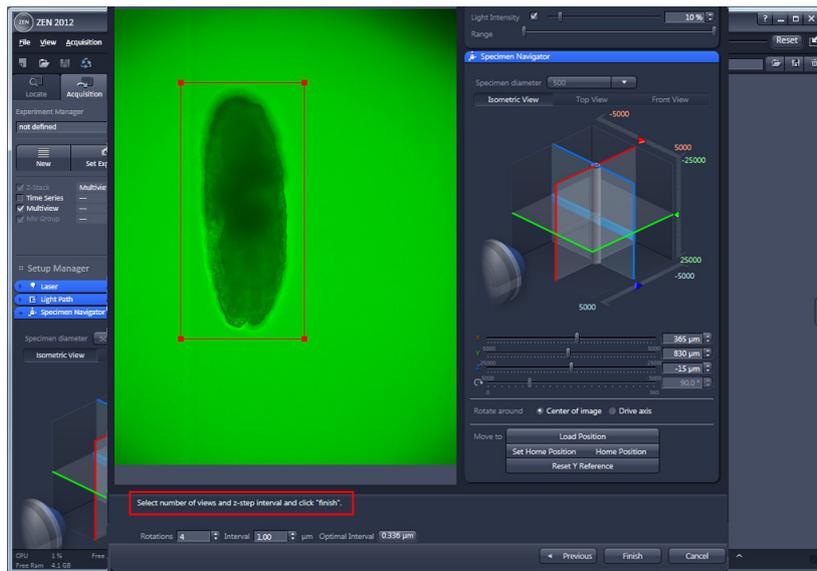
Alternatively, you can reposition the sample into the middle of the rectangle by using the **Specimen Navigator**.

- Move the rectangle by grabbing the side .

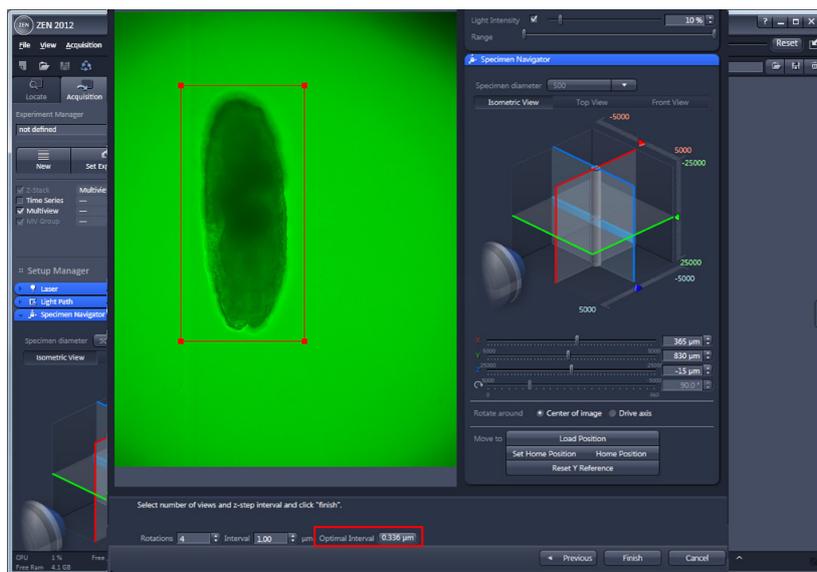


Check, by moving the Z-drive, to see if the red rectangle encircles the whole sample along its Z-axis and continue with **Next**.

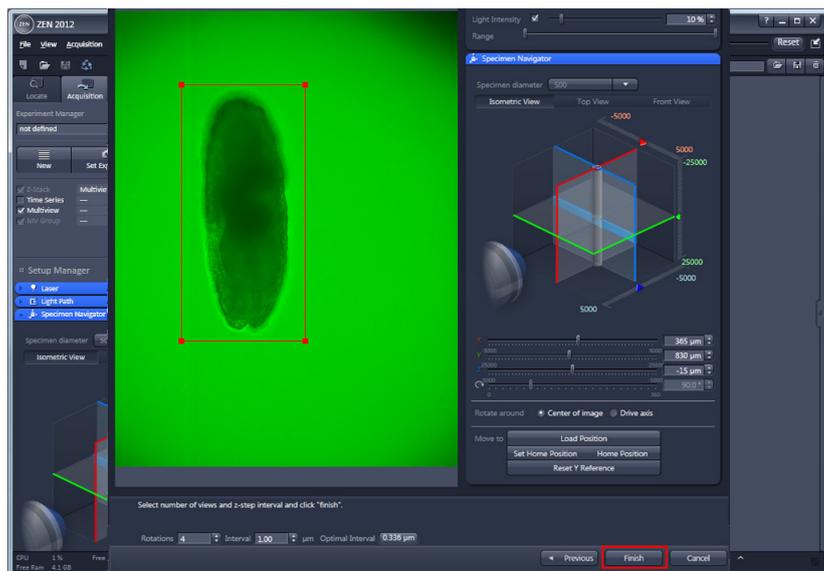
- If you have finished Re-focussing and selecting the acquisition volume (view z-y), please press **Next**.
- Click **Next** .

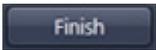


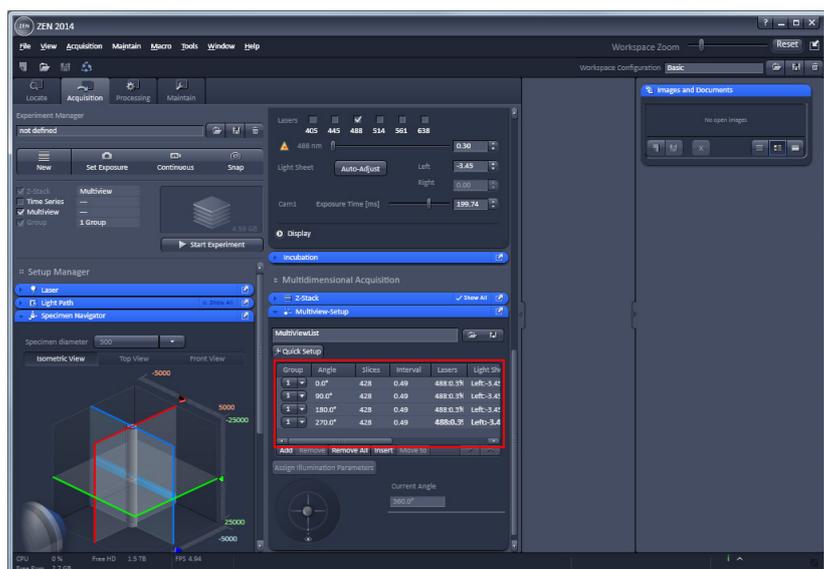
 This interface defines the number of views by entering the number of **Rotations** within the input box or by using the arrows.



- Pressing the **Optimal button** sets **Interval** to a value of half the optical slice thickness.
- Click **Optimal Interval** **Optimal Interval** 0.336 µm.

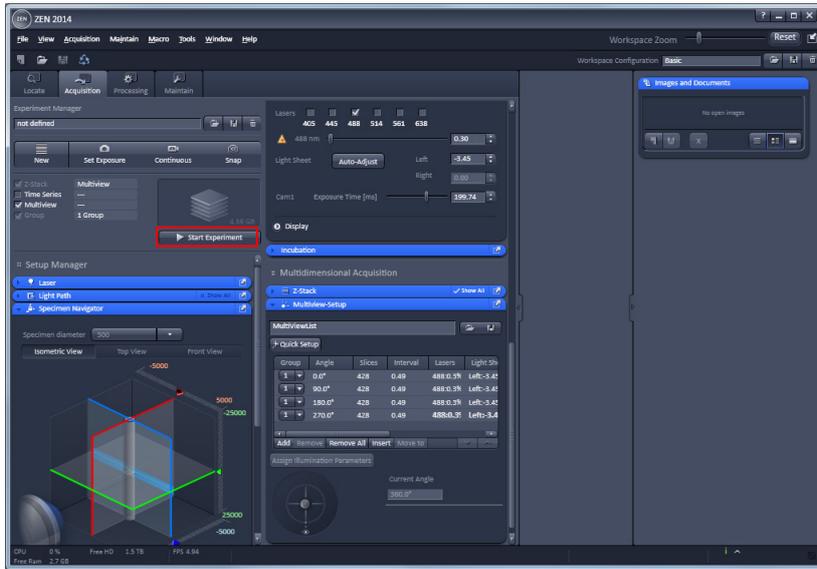


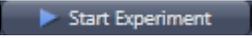
- Please press the **Finish** button to complete the **Quick Setup** and close the window.
- Click **Finish** 

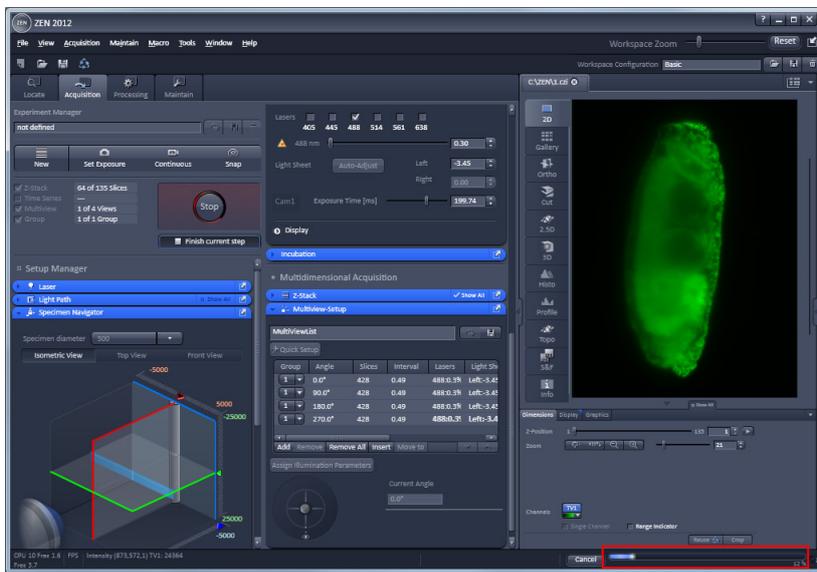


- ☞ The resulting Views are now displayed in the **Multiview Setup** tool tab View list.
- ☞ Be aware that the **Zoom** setting will change when the volume, defined in step 2 and 3 of the Multiview Quick Setup, requires a larger field of view then with the **Zoom** set in the **Acquisition** tool window. The Multiview Quick Setup uses the higher value of either the largest possible diagonal through the volume or the length of the y-axis as a reference to determine the needed field of view, hence the Zoom.
- ☞ When the **Zoom** setting is changed during the Multiview Quick Setup, the alignment of the light sheet must be checked and adapted. Following the rule, the light sheet adjustment is the last step before image acquisition. After adapting the light sheet offset, press the **Assign Illumination Parameters** button.

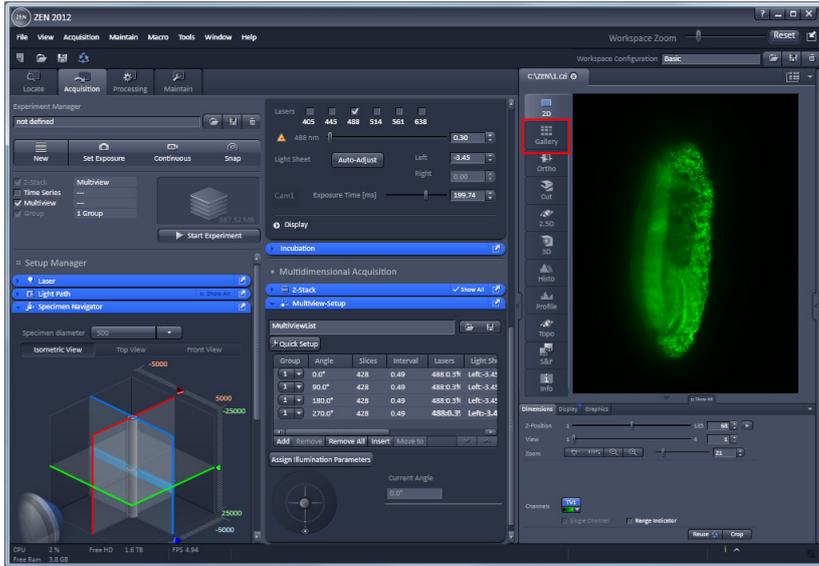
## 5.2 Start Multiview Experiment and evaluate Data



- Click **Start Experiment** to start acquiring the **Multiview** experiment.
- Click **Start Experiment** 

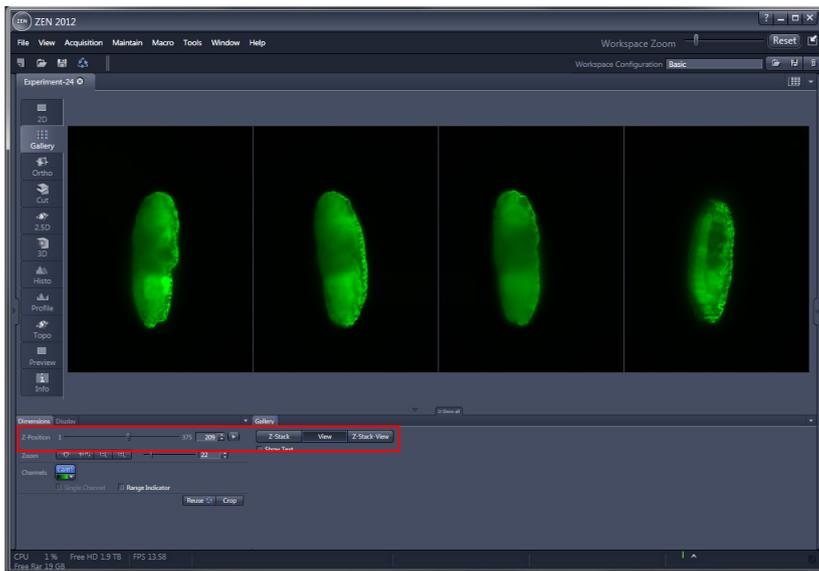


 You can follow the process by the displayed **progress bar**.



- The **Gallery** view displays all **Views** and corresponding slices from the **Z-Stacks**.

- Click **Gallery** 



- ☞ All the **Views** and corresponding slices from the **Z-Stacks** can be represented in the Gallery view side-by-side.  
The **Gallery view** in this example shows all **Views** from the Experiment at **Z-Position** 209.

- ☞ The additional dimensions can be selected with the sliders and control blocks in the **Dimensions** View control block.

**You successfully acquired a Multiview experiment!**

You may now proceed with either:

**1. View tabs** in the Center Screen Area

- Ortho View
- Cut View
- 3D View (Image VisArt)

or

**2. Processing tab** in the Left Tool Area

- Maximum Intensity Projection
- Lightsheet processing

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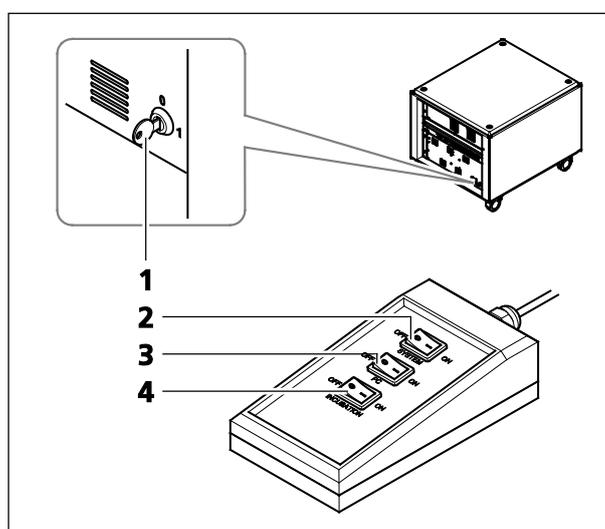
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# 1 Startup and Shutdown of the System

## 1.1 Startup of the System

The Lightsheet Z.1 system is equipped with a Laser key-operated switch on LB Rack Lightsheet and a remote control to switch on the system, the PC for system control, and the incubation. "**System**", "**PC**" and "**Incubation**" switches are accessible via the power remote switch (Fig. 1). The storage and analysis PC has to be turned on separately.

As an option, the sample can be temperature controlled and supplied with CO<sub>2</sub>. To activate the incubation components, set the "**Incubation**" switch to the **ON** position.



- 1 Laser key-operated switch on LB Rack Lightsheet
- 2 System ON / OFF
- 3 PC ON / OFF
- 4 Incubation ON / OFF

**Fig. 1** Power remote switch

### 1.1.1 Switching on the Laser Module

- The laser key switch (Fig. 1/1) at the front of the LB Rack Lightsheet laser module must be set to the **1 (ON)** position to be able to switch the lasers on.

### 1.1.2 The Power Remote Switch

- To switch on the system, set the "**System**" switch (Fig. 1/2) to the **ON** position.
- When set to **ON** the power remote switch labeled "**PC**" (Fig. 1/3) provides power to the computer and ZEN software offline. The storage and analysis PC has to be turned on separately and can be run independently of the Lightsheet Z.1 system.
- To activate also the incubation components, set the "**Incubation**" switch to the **ON** position (Fig. 1/4).

The control LED on the system lights up as soon as the system is ready for operation.

- ☞ The storage and data analysis PC should be completely booted before the PC for system control is turned on. This way the drive and directory of the storage and data analysis PC, on which data is streamed during image acquisition, will be automatically mapped when the PC for system control is turned on.
- ☞ It is not recommended to shut down and restart the storage and analysis PC. If possible, it should run continuously.

- 
- After booting the PC for system control, type in the user name and password to log into the computer.
  - After entries, confirm by clicking the **OK** button or **Enter**.

The WINDOWS operating system desktop appears on the screen, showing a number of icons.



**ZEN** icon

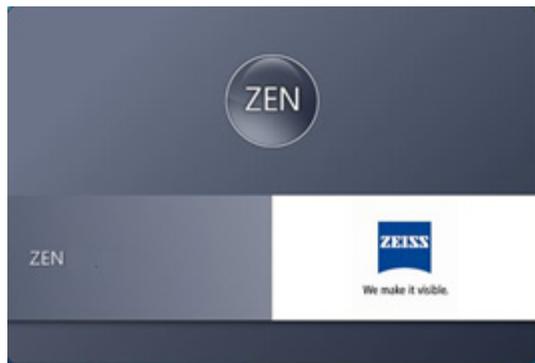
Starts the ZEN software for operating the Lightsheet Z.1 system.



**Configuration Tool** icon

The **Configuration Tool** permits alterations to the database of the Lightsheet Z.1 that is used by the ZEN software program. This function should preferably be performed by trained personnel.

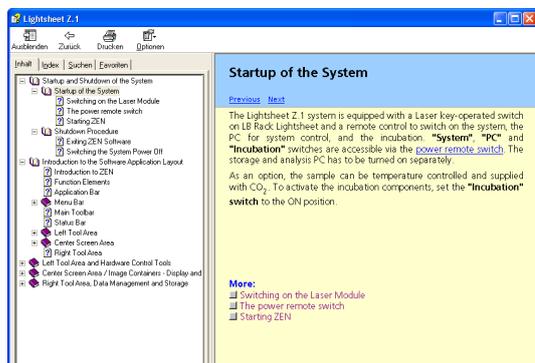
Now the complete system is ready to be initialized with the ZEN Software.



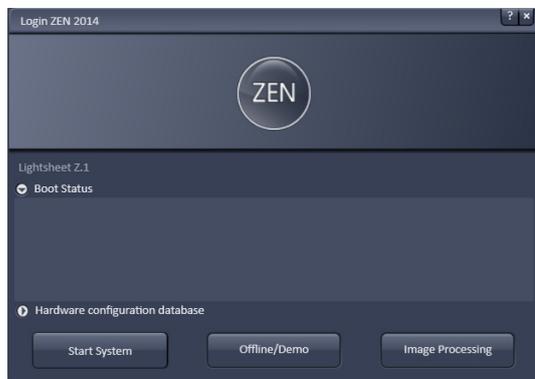
**Fig. 2 Starting the ZEN software – The Loading "ZEN" window**



**Fig. 3 Starting the ZEN software – The Login "ZEN" dialog**



**Fig. 4 The Help menu dialog**



**Fig. 5 Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded**

### 1.1.3 Starting ZEN



#### ZEN icon

Start ZEN software for operating the Lightsheet Z.1 system.

- Your Windows operating system is ready for use.
- ZEN is installed successfully.
- To start the software, double click the black **ZEN** icon on your desktop!

During loading, the **ZEN Access** window will appear (Fig. 2). It displays the ZEN symbol in the upper part, the designation of the current ZEN software version (lower left) and the Zeiss logo (lower right).

Once the program is loaded, the **Login ZEN** dialog will appear (Fig. 3) in front of the ZEN interface. The number after ZEN designates the current version.

- By pressing the **? (question mark)** symbol, the context sensitive **Help menu** will appear (Fig. 4). Close the menu with the Windows commands if you do not want to search the help database by pressing the **x (cross)** symbol.
- Pressing the **x (cross)** symbol, the **Login ZEN** dialog will be closed and the ZEN software will not be started.

The **Login ZEN** dialog allows you to boot the Software in different modes. If the **Boot Status** is closed, the options are **Start System** and **Image Processing**.

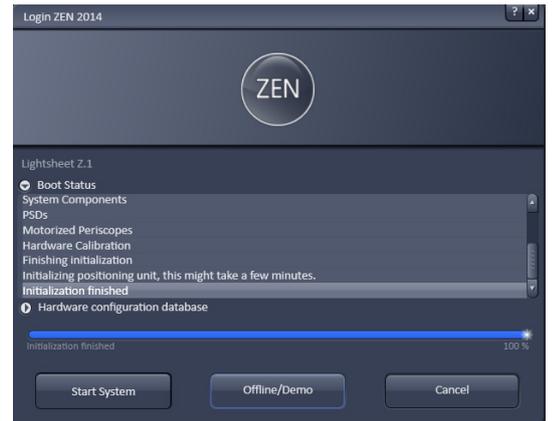
A third option, **Offline/Demo**, becomes available (Fig. 5), once the **Boot Status** field is expanded by pressing the **expansion** symbol (triangle).

You can collapse the field by pressing the expansion symbol again. Select:

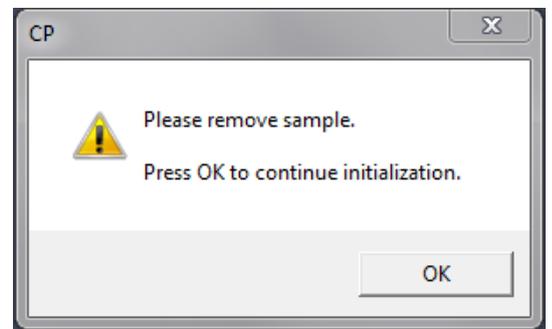
- **Start System** for system control, data / image acquisition / recording and analysis. The whole microscope system will be initialized. The progress of the boot process will be displayed by the blue time tab. During booting, the status of the system will be displayed in the **Boot Status** window and the single components will be listed which have been or are currently being initialized during the boot process (Fig. 6).

 When ZEN software is started for the first time, the positioning units need to be initialized. All doors of the system must be closed and no sample can be present in the system. The boot process will be interrupted and a message (Fig. 7) will state to remove the sample; do so if necessary and press **OK** to continue with the boot process.

- **Offline/Demo** for data analysis and simulation. **Offline/Demo** reads the active hardware database and simulates the respective hardware. This button is only available after opening the **Boot Status** display by clicking the little arrow left of the text "Boot Status". This option should be chosen when working at an offline station and if you want to test functionalities of the system. The progress of the boot process will be displayed by the blue time bar. The single components will be listed, which are initialized during the boot process. Note that the software will not control the hardware in this mode.
- **Image Processing** for data analysis only. This mode ignores the hardware and activates only data handling and image processing functionality to analyze stored images. Note that only those software tools will be displayed that are needed for processing and analysis of the data. No hardware control tools will be shown.
- **Cancel** to abort the booting process and to leave the dialog. Note that the **Cancel** button will appear in the place of the **Image processing** button only if **Start System** or **Offline/Demo** was selected (Fig. 6).
- You can leave the software by choosing the **File** menu from the main tool area and selecting **Exit**.
- You can select a database before booting, if you wish to change the current one. To change the database, open the **Hardware configuration database** expansion field (Fig. 8). The expansion field displays the currently loaded database and its location on the hard disc.



**Fig. 6** Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded and during the process of booting



**Fig. 7** Message to remove sample, prior to initialization of the positioning unit



**Fig. 8** Hardware configuration database - The Login "ZEN" dialog allows you to boot the Software in different modes. If the Boot Status is closed, the options are Start System and Image Processing.

- If **Recent...** is pressed the last couple of databases that were in use will be displayed (Fig. 9). Click on the one you want to select. By pressing the **Choose...** button, Windows Explorer will open. Search for the file folder where the database is stored and open it. Select it for use. Select a database suited for your purpose from the Explorer window.
- You can also change the database after having booted the system. To do this, choose from the **File** menu of the main tool area **Login...** and the **Login Zen** dialog will appear. Select the database by pressing **Choose...** or **Recent...** and the system restarts.

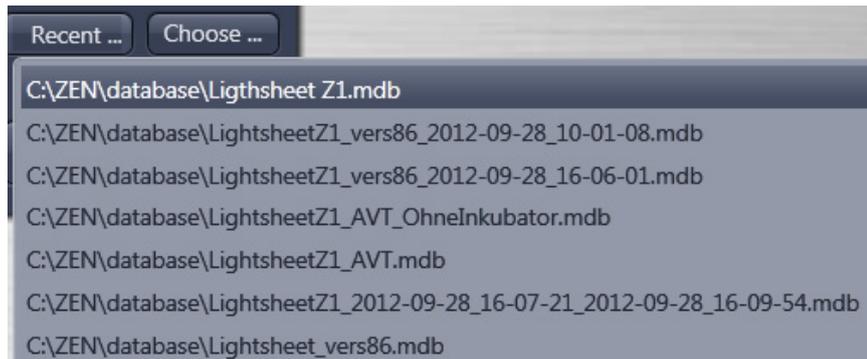


Fig. 9 Recent databases

## 1.2 Shutdown Procedure



Do not switch the computer off until the ZEN software is closed and the Windows 7 operating system has completely shut down. The program and/or data files may otherwise be lost.



In the **Maintain** tab / **System Options** dialog window, activate **Laser off** on **Exit** in the **Shutdown** tab (see section **3.4.3 Maintain – System Options**). The lasers will then automatically be switched off when you exit the ZEN software.

### 1.2.1 Exiting ZEN Software

The Software can be shut down from **File** menu of the Main toolbar by selecting **Exit** (Fig. 10) or by pressing the **Window Close** button (symbolized by a cross) in the ZEN window.

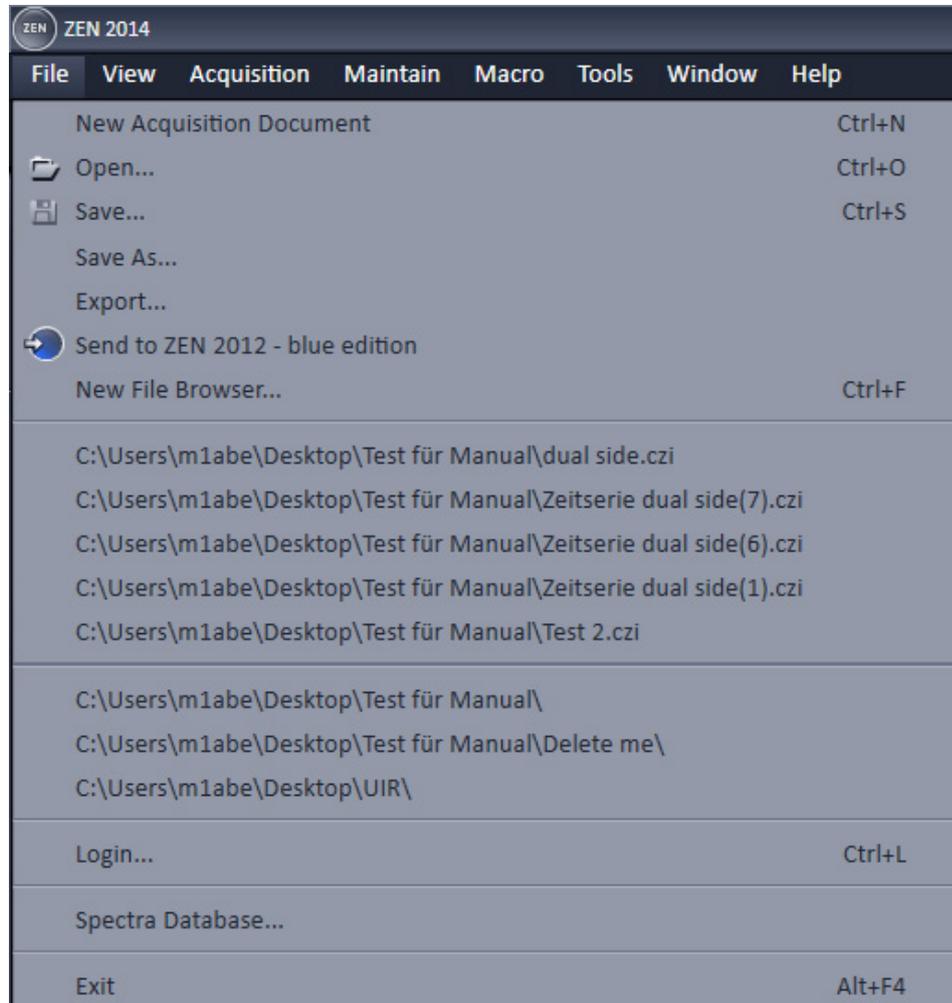
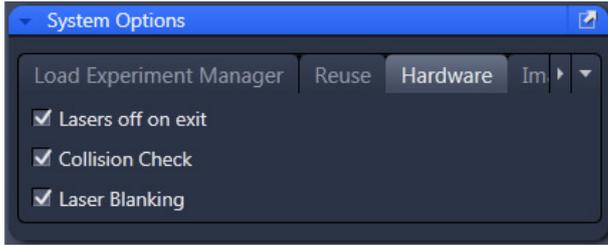


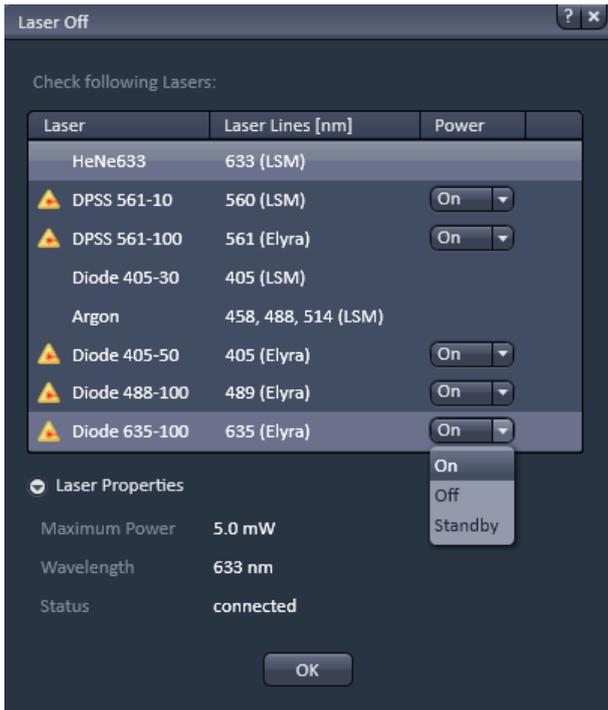
Fig. 10 Exit in the File menu



**Fig. 11** Laser off on Exit option in System Options window

In the **Maintain** tab under the **System Options** dialog window, the box for activating the **Lasers off on Exit** is located under the **Hardware** tab (Fig. 11). When checked, the lasers will be automatically switched off when exiting the ZEN program.

If the box is not checked, the **Lasers Off** dialog box will appear allowing one to check on/off the listed lasers (Fig. 12).



**Fig. 12** Laser off dialog box

By expanding the Laser properties field, one can view the current status of the selected laser. Press **Ok** after having made the desired changes for exiting the ZEN software.

### 1.2.2 Switching the System Power Off

ZEN software is exited successfully.

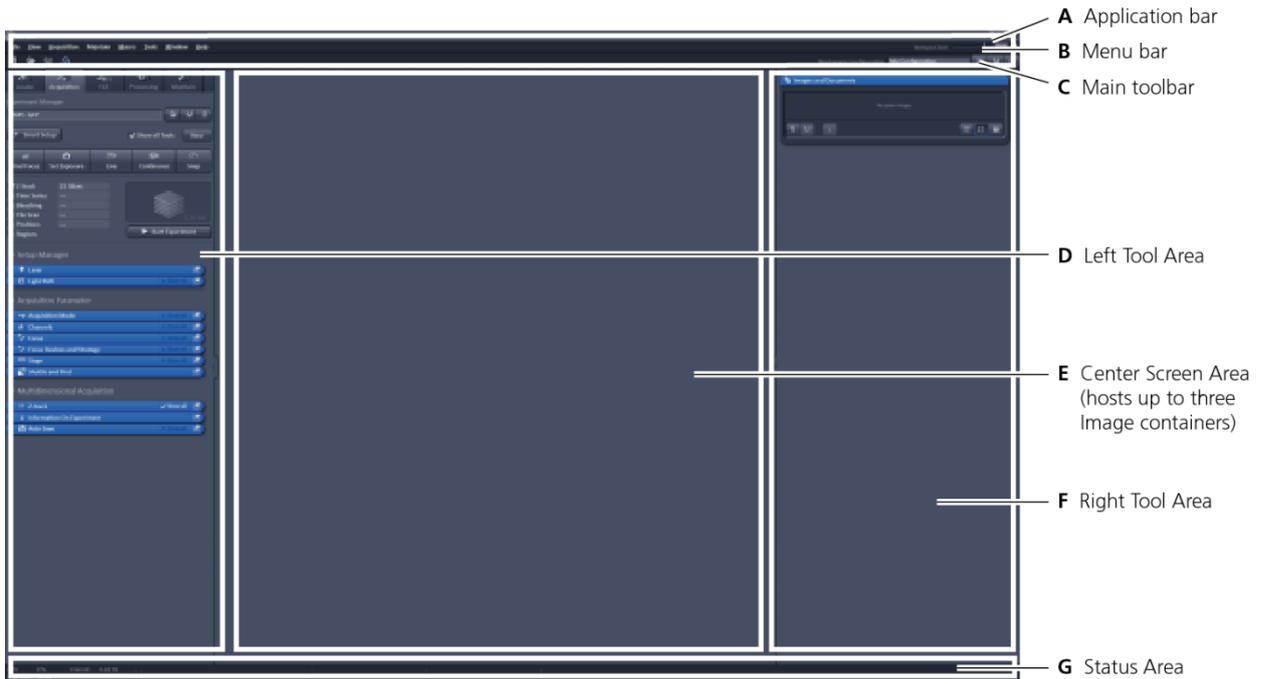
After having exited the ZEN software, you may turn off the whole system with the following **Shutdown** procedure.

- Shut down WINDOWS.
  - About 20 seconds after WINDOWS is shut down, the computer turns off.
- On the remote power switch, turn off the "**System**", "**PC**", and "**Incubation**" switches.
- Set the laser key switch (Fig. 1/1) on the front of the LB Rack Lightsheet laser module to the **0 (OFF)** position.
- Shut down the storage and analysis PC.
 

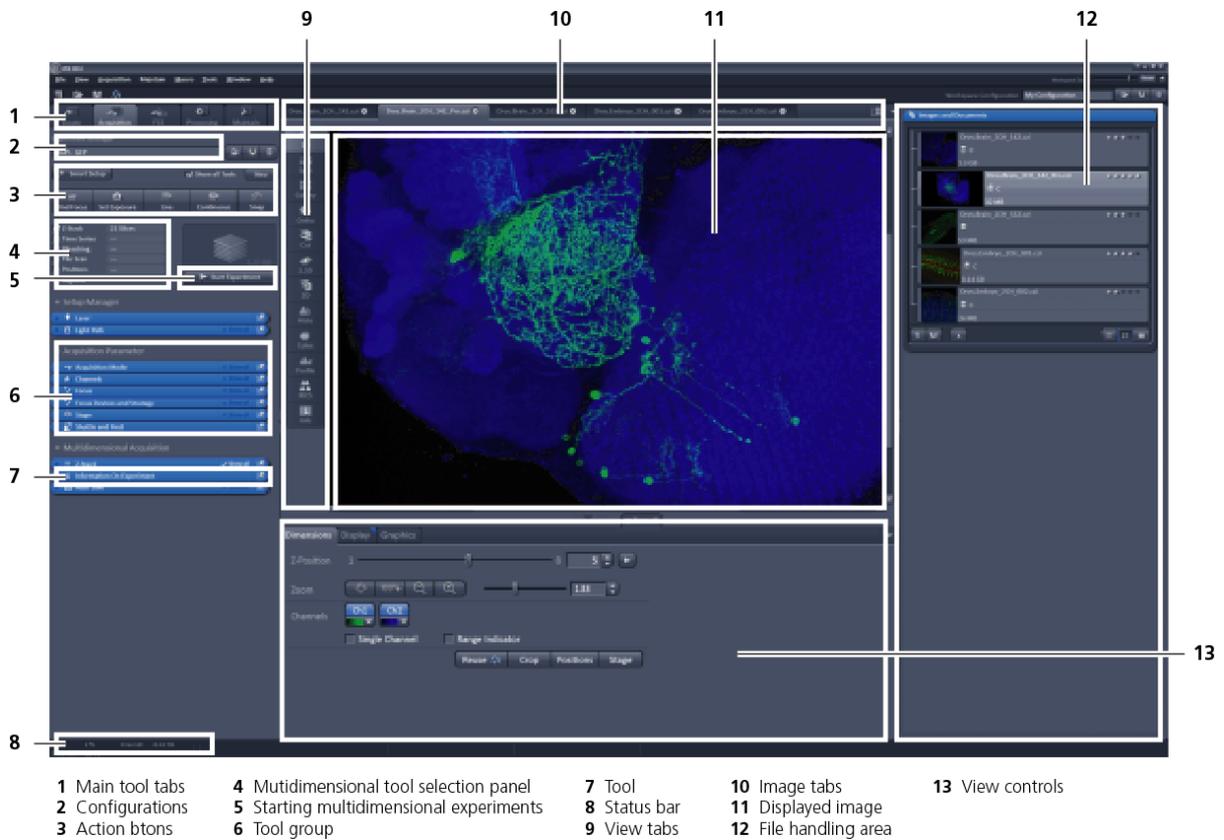
It is not recommended to shut down and restart the storage and analysis PC. If possible, it should run continuously.

## 2 Introduction to the Software Application Layout

### 2.1 Overview on the Screen Layout



**Fig. 13** ZEN Main Application window after Startup with empty image container



**Fig. 14** ZEN Main Application window after Startup with several images loaded

## 2.2 Introduction to ZEN

**ZEN - Efficient Navigation** - is the software from Carl Zeiss which sets new standards in application-friendly usage for microscopy systems.

The ZEN Interface is clearly structured and follows the typical workflow of the experiments performed with Light Sheet Fluorescence Microscope Systems (LSFM):

On the **Left Tool Area** (Fig. 13/D) the user finds the tools for image acquisition, image processing, and system maintenance, easily accessible via the **Main** tabs (Fig. 14/1). All functions needed to position the sample and control the incubation components can be found on the **Locate** tab. To acquire images, use the **Acquisition** tools (Fig. 14/3 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 the View control blocks below each image. File management and data handling tools are found in the **Right Tool Area**.

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.

A focus in the development of **ZEN** 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 Light Sheet Fluorescence Microscope from Carl Zeiss easy and fast.

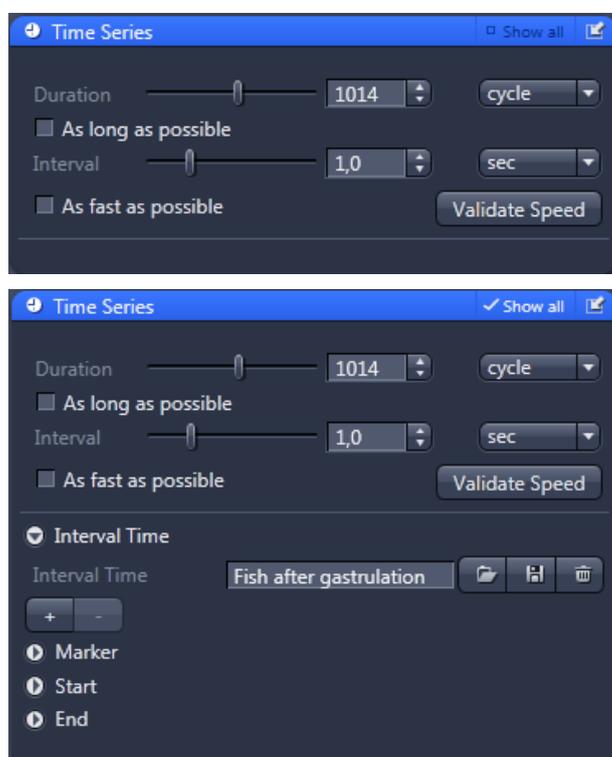


Fig. 15 Show all mode

The **Show all** concept ensures that tool panels are never more complex than needed. If **Show all** is deactivated the tools show only the most relevant functions, covering approximately 80 % of the user's application. For each tool, the user can activate **Show all** to display and use additional functionality (Fig. 15).

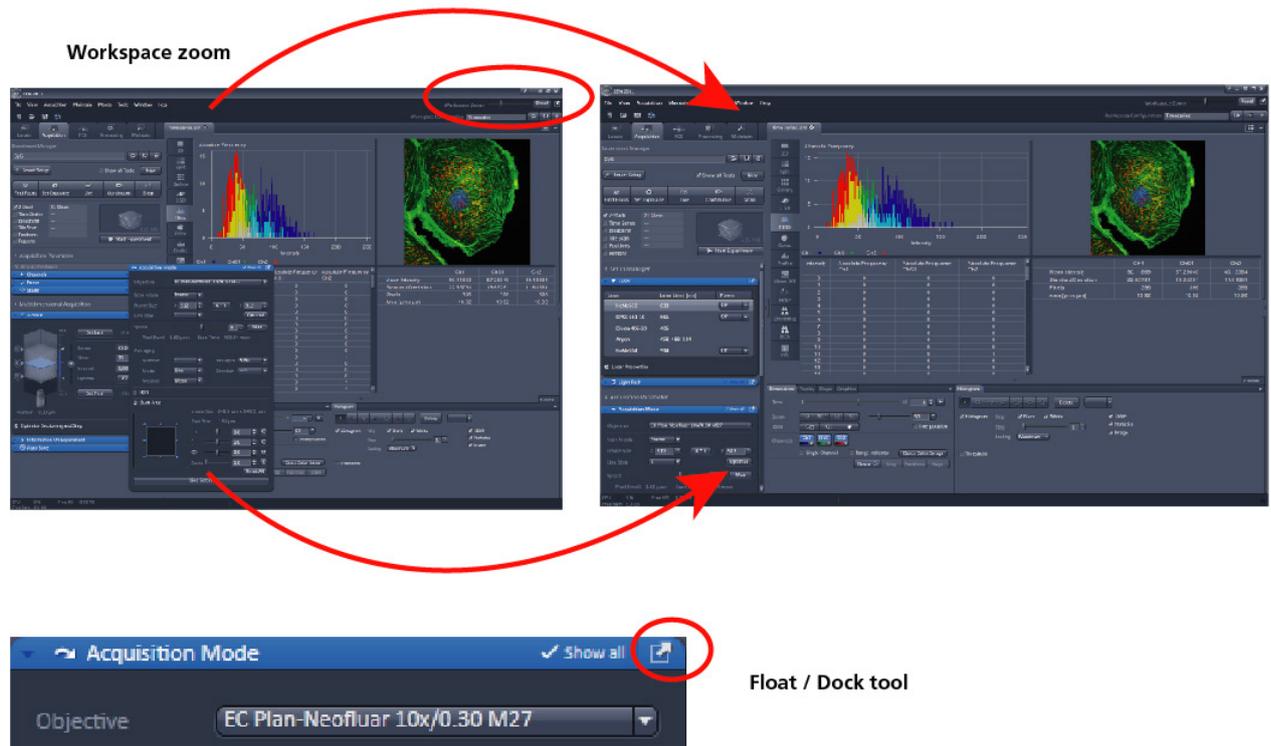
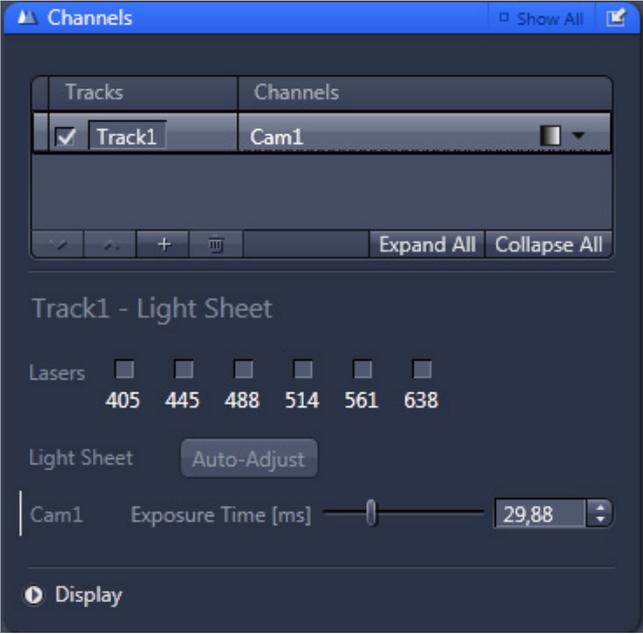
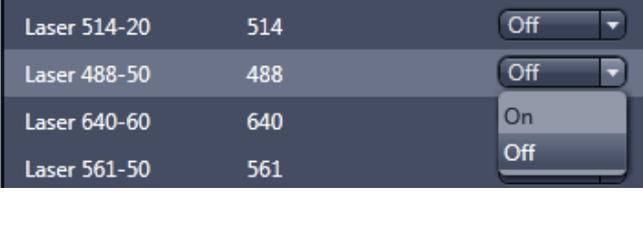
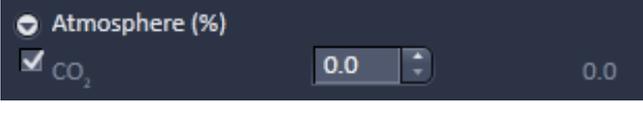


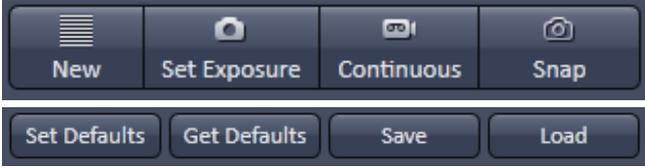
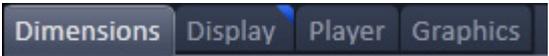
Fig. 16 ZEN window Layout configuration

More features of ZEN include:

- The user can add more columns 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.
- To detach a tool, click on the little icon on the very right end of the blue tool header bar or drag it by this blue header bar to where you want it (Fig. 16). To bring the detached tool back to its original position in the tool group you can drag the tool by its blue header to its original position, which is shown by an identical grey header; or click on the dock symbol at the right hand side of the blue or grey header. A fourth option is to double click on the grey header at the original position in the tool group.
- Another unique feature is the scalable ZEN interface. This **Workspace Zoom** allows adjustment of the **ZEN** window size and fonts to the situational needs or your personal preferences (Fig. 16).
- Setting up conventional microscopy 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.

### 2.3 Function Elements

Function element	Description / Explanation
	<p>Tool group</p>
	<p>Tool (closed) or tool window (opened)</p>
	<p>Panel (e.g.: <b>Zoom</b> panel) Field with a subset of tools of a tool window</p>
	<p>List box or selection box</p> <ul style="list-style-type: none"> <li>- Selection of one of the displayed options via a mouse click. The selected option is highlighted.</li> <li>- Open the box by clicking on the arrow button.</li> </ul>
	<p>Input box with arrows ("spin box")</p> <ul style="list-style-type: none"> <li>- Input of text or numeric values via the keyboard or using the arrows.</li> </ul>

	<p>Slider with input ("spin") box and arrows</p> <ul style="list-style-type: none"> <li>– Setting of numbers in the relevant input box by moving the slider or clicking on the arrow buttons or clicking on the slider and moving via the arrow keys of the keyboard. Press the Shift or Ctrl key while clicking on the arrow button to change the numeric values in coarse or fine steps.</li> </ul>
	<p>Button</p> <ul style="list-style-type: none"> <li>– Selection / performance of a function via mouse click.</li> </ul>
	<p>Tab</p> <ul style="list-style-type: none"> <li>– Selection of functions of a tab via mouse click (e.g.: Dimensions or Display tab).</li> </ul>
	<p>Load – Save – Delete</p> <ul style="list-style-type: none"> <li>– Buttons to load, save or delete a configuration, overlay set, track setting etc.</li> </ul>
	<p>New – Save – Close</p> <ul style="list-style-type: none"> <li>– Buttons to open a new acquisition document, save an image document, or close it.</li> </ul>

 When stacks or time series are exported, each frame is stored as an individual image.

## 2.4 Application Bar

The application bar includes the following control elements in the ZEN software application window:

-  starts the online help
-  minimizes the application window
-  closes the window

## 2.5 Menu Bar

The menu bar contains the menu items and the controls for the **Workspace Zoom**, including the **Reset** button for the **Workspace Zoom** and the button to re-dock all free floating tools to the **Left Tool Area**.



Fig. 17 Menu bar, left side

The menu functions are shown in the following menu overview (see next page).

### Workspace Zoom

- Moving the slider to left or right, changes the display size of the Left/Right Tool Area and the Center Screen Area.
- Clicking the **Reset** button, sets the standard zoom factor for all areas.



Fig. 18 Menu bar, right side

- Clicking on  moves all undocked tools back to the Left Tool Area (see also section **2.8.1 Tool Groups and Tools**).

**Menu overview**

<b>File</b>	<b>View</b>	<b>Acquisition</b>	<b>Maintain</b>	<b>Macro</b>	<b>Tools</b>	<b>Window</b>
<b>New Acquisition Document</b> Opens a new and empty image document.	<b>Text View</b> Displays the image tabs with text only.	<b>New Acquisition Document</b> Opens a new and empty image document.	<b>Hardware Administrator</b> For service only.	<b>Macro</b> Opens the Macro Control window for creating, loading or handling macros	<b>Paste Image</b>	<b>Close</b> Closes the active window.
<b>Open</b> Opens an existing document in the active image container.	<b>Small Thumbnail View</b> Displays the image tabs with small thumbnails.	<b>Set Exposure</b> Starts the Find procedure. This balances Gain settings.				<b>Close All</b> Closes all windows, a separate save dialog will be displayed if images have not been saved.
<b>Save</b> Saves the active document.	<b>Large Thumbnail View</b> Displays the image tabs with large thumbnails.	<b>Countinuous</b> Scans an image continuously with the currently active scanning parameters.				
<b>Save As</b> Saves the selected image with entering new formats.	<b>1 Container</b> Sets ZEN to work only with one image container.	<b>Snap</b> Scans one single image.				<b>Help</b> <b>Help</b> Opens the ZEN help.
<b>Export</b> Exports the currently active document using various image formats.	<b>2 Containers</b> Sets ZEN to work with two image containers.	<b>Starts Experiment</b> Starts a multidimensional experiment (Z, T, ...).				<b>About</b> Opens a panel with information about the ZEN release.
<b>Open Containing Folder</b> Opens the folder, where the image file is saved.	<b>3 Containers</b> Sets ZEN to work with three image containers.	<b>Stop</b> Stops the scanning immediately.				
<b>Send to ZEN 2012 - Blue edition</b> Sends the currently active document to ZEN blue edition.	<b>Automatically layout container</b> The size of the containers is automatically adjusted depending on the screen space available.					
<b>New File browser</b> Opens a document containing a ZEN File Browser.	<b>Shared view Controls</b> Shares the view controls below the images between containers.					
<b>Login</b> Opens the Login ZEN dialog to change the software operation mode.	<b>Separate view Controls</b> Each container is displayed with a separate view control panel.					
<b>Spectra Database</b> Opens the Spectra Database.	<b>"Show all" (global)</b> Sets all windows, tools, and control panels to "Show all", all controls are always visible in all tools.					
<b>Exit</b> Closes the ZEN software.						



For detailed information about the image acquisition functions see section **3.2 Acquisition Tab**.

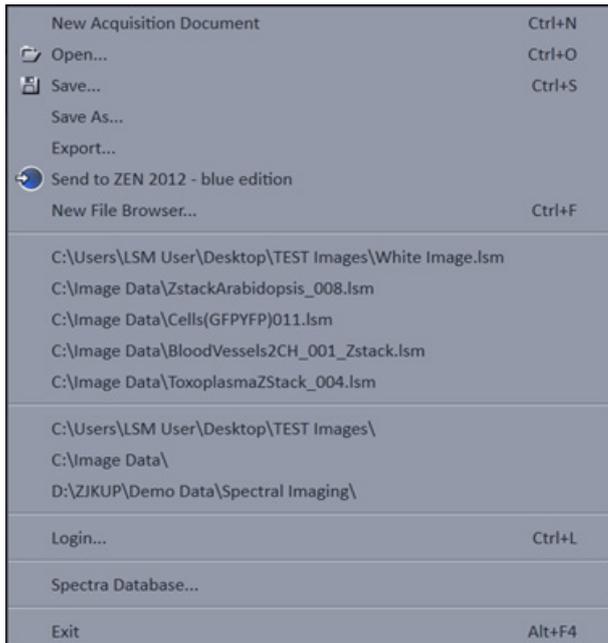


Fig. 19 File menu

### 2.5.1 File

- In the menu bar, click on **File**.
  - This displays the **File** menu shown in Fig. 19.
  - The items in this list reflect the standard windows files menus with the following exceptions:

**New File Browser** will open a ZEN File Browser in the currently active container (see section **5.3 ZEN File Browser**).

In the group below **New File Browser**, it lists the five last accessed files. Selecting one of the list entries will open the respective file.

**Login** displays the Login screen and permits one to change the operation mode of ZEN, e.g. from Online (with hardware started) to Offline (Image Processing or Demo mode), without closing ZEN.

Spectra database starts the dialog used to handle previously recorded reference spectra. This function is for LSM systems only.

☞ See also section **5 Right Tool Area, Data Management and Storage** for image file handling.

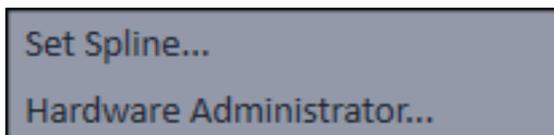


Fig. 20 Maintain menu

### 2.5.2 Maintain

The **Maintain** menu from the menu bar contains additional modules to check and guarantee the interference-free operation of all the software and hardware components of laser scanning systems (LSM).

- In the **Menu** bar, click on **Maintain**.
  - This opens the maintain list (Fig. 20).

The maintain list is bipartite. The upper two sections list user accessible tools for LSM (Set Spline) but not for Lightsheet Z.1 systems, the lower section lists tools reserved for service personnel which are password protected.

The **Hardware Administrator** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

**2.5.3 Help – About**

- The **About** window can be accessed via the **Help** item in the menu bar (Fig. 21).
- Clicking **About** opens the **About** window (Fig. 22).

The panel hosts important information about the software version number, the license number and the available software modules.

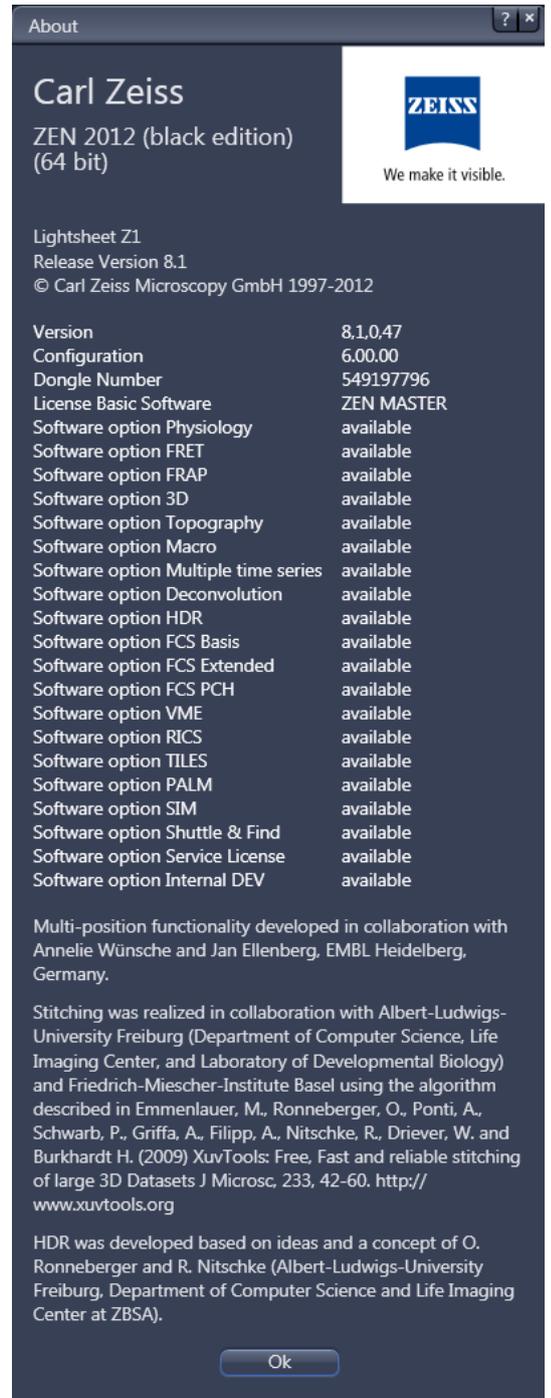
The software version number follows the following rules:  
Main Version number, Service Pack number, Hotfix number, Build

For example 7.1.3.352 reads

Main Version: 7, Service Pack: 1, Hotfix: 3, Built: 352



**Fig. 21 Help menu**



**Fig. 22 About window**

## 2.6 Main Toolbar

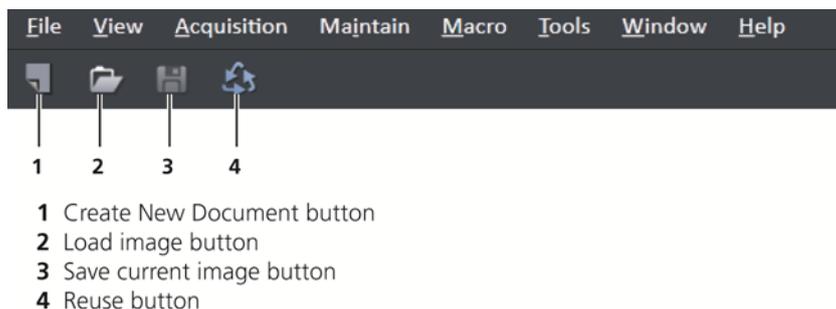


Fig. 23 Main toolbar, left side

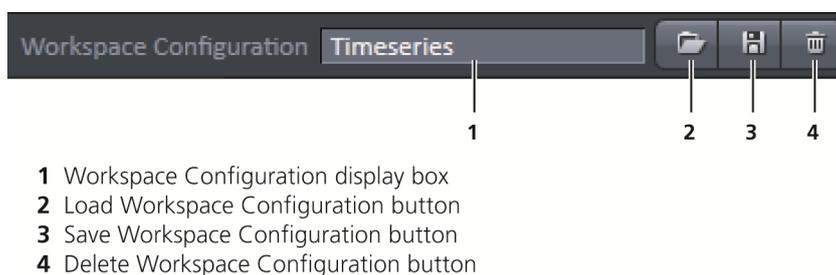
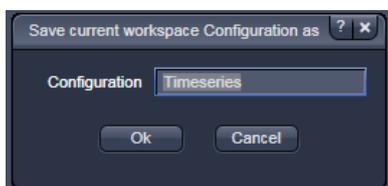


Fig. 24 Main toolbar, right side

### Workspace Configuration

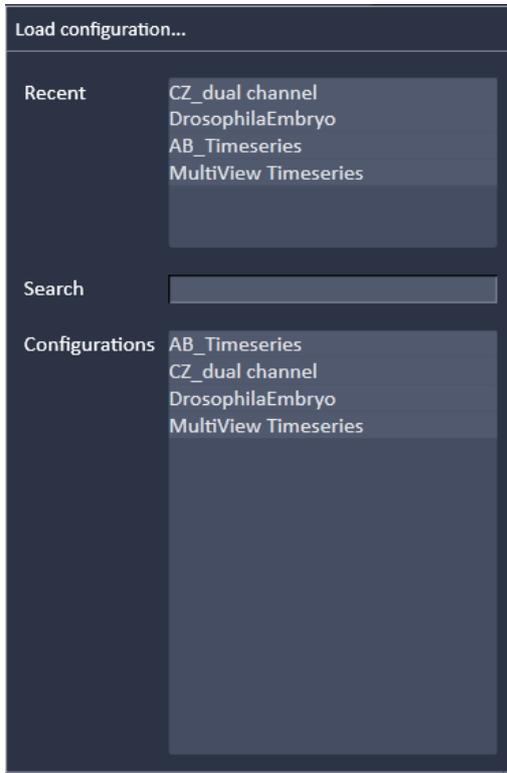
This function allows loading, saving or deleting a workspace configuration. These workspace configurations are saved settings that allow one to restore a pre-defined layout of the whole ZEN application window, including status, size and position of tools and windows, workspace zoom, number of tool columns.

- To save a workspace configuration, click on the  button. The following dialog will appear:



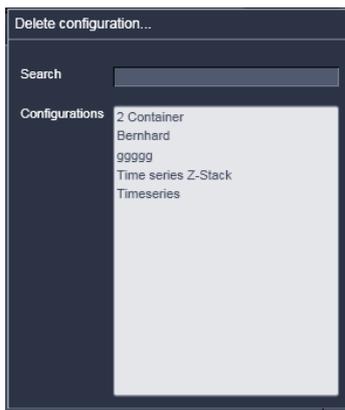
The name of the workspace configuration can be entered. Clicking **OK** saves the configuration.

- To load a workspace configuration, click on the  button. The following list box will appear:



Selecting one of the list entries will load the respective configuration.

- To delete a workspace configuration, click on the  button. The configuration to be deleted can be selected from a list.



## 2.7 Status Bar



Fig. 25 Status bar

### System information

- Frames per second (FPS)
- Position and pixel intensity

### System information

Shows information of the currently system tasks (state). The possible states are:

- CPU usage
- free hard disc capacity
- free RAM capacity

### Position and pixel intensity

Shows the intensity values of the existing channels for the current X, Y, and Z position of the mouse cursor in the image.

### Frames per second (FPS)

Shows the current frame rate in frames per second (FPS).

### Progress bar

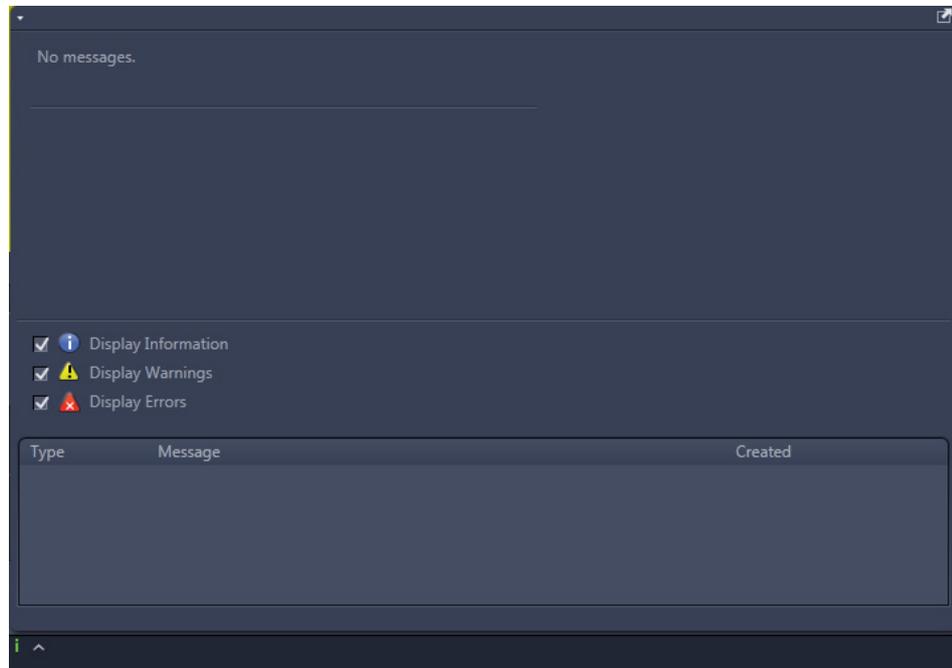
Shows the process of the running processes. Either for rendering or processing actions or during acquisition of experiments. Additional information is given on the action in progress and an estimation of the remaining time.



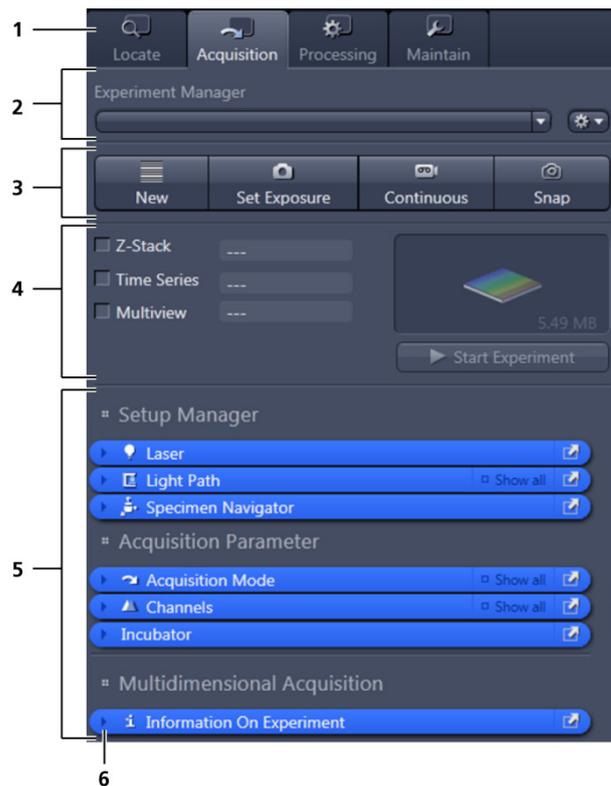
Fig. 26 Progress bar

## System messages

Next to the progress bar you find a green upside down exclamation mark and a white arrowhead . When you click on the arrowhead a window opens in which the history of system messages is shown (Fig. 27).



**Fig. 27** System messages



- 1 Main tool tabs
- 2 Experiment Manager
- 3 Action buttons
- 4 Selection panel for multidimensional acquisition
- 5 Tool groups
- 6 Expand slider with arrow button pointing left to collapse (pointing right to expand)

**Fig. 28** Left tool area

## 2.8 Left Tool Area

### Main Tool tabs

Switches between the **Locate**, **Acquisition**, **Processing**, and **Maintain** main tools to operate the included tool

### Action buttons

Action buttons are only available for the **Acquisition Main** tool to control the image acquisition process.

### Tool groups

Groups special tools, e.g. **Setup Manager** or **Acquisition Parameter**.

### Setup Manager

These tools (Laser and Light Path) allow one to choose settings for illumination and detection.

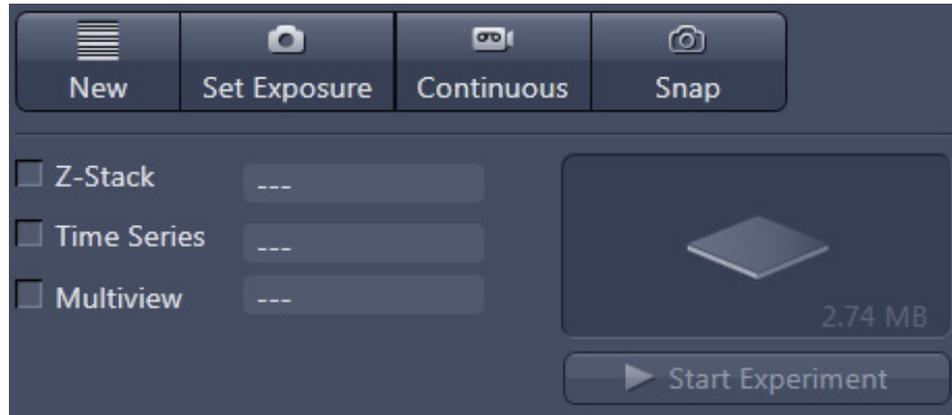
### Acquisition Parameter

Tools continuously needed for all acquisition procedures.

### Multidimensional Acquisition

Tools for all multidimensional imaging

The tools in this tool group will only appear if selected in the selection panel below the action buttons. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after pressing the **Start Experiment** button. The experiment type is represented graphically above the Start Experiment.

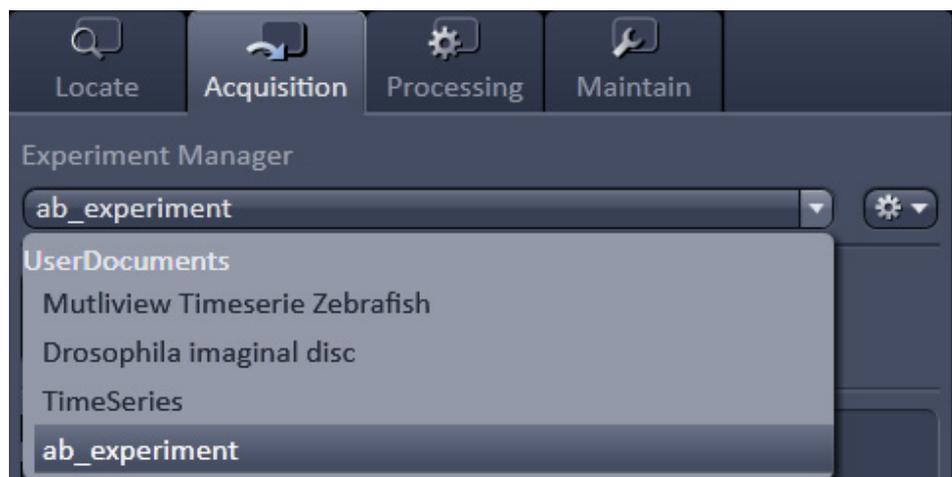


### Tools / tools window

Used for setting up the system and software functions.

### Experiment Manager

This function allows selecting an Experiment Manager imaging configuration. The configuration can be chosen from a drop-down list. Clicking on a configuration loads the selection.





To add, edit or delete configurations, click on the  button next to the configuration drop-down list.



### 2.8.1 Tool Groups and Tools

#### Multiple Columns Layout of tool groups

This function allows moving one or several **tool groups** into a second or third column in the **Left Tool Area**. This is done by dragging and dropping the **tool groups** header. The second column opens automatically if a group is moved and closes if all groups are removed from it.

#### Undock function of tool windows

This function allows undocking and moving one or several tool windows from its home position in the Left Tool Area to any position on the Monitor. This can be done by clicking the  button. The tools can also be undocked by simply dragging the header bar of the respective tool to the desired position. To place a tool back into its home position, click the  button again, or drag the tool back to the **Left Tool Area**, or click on the grey place holder at the home position of the tool. The last option is particularly useful if the screen gets too crowded and one can't find the tools anymore. To float all tools back with one click, use the  button at the top right corner of the menu bar.

To position the tool window freely on the desktop, use the drag and drop function.

#### Opening a tool window

Clicking on the tool header opens or closes the tool pull-down window. The state is indicated by a changing icon (, ) at the left hand side of the tool window header.

If a tool is undocked from the **Left Tool Area** (float function) it will be opened automatically and cannot be closed, the icons (, ) will not be displayed in this case.

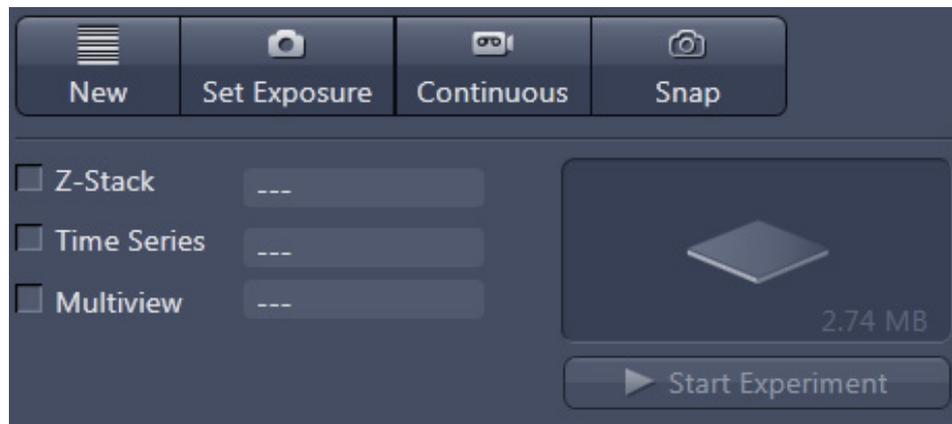
### Show all functions within the tool window

Clicking on the  Show all button shows or hides additional functions within the tool.

<input type="checkbox"/> Show all	Only the basic functions are shown; this functionality is adequate for most applications.
<input checked="" type="checkbox"/> Show all	All functions are activated in the tool window. The full functionality of the tool is available.

### Activation of tools with respect to a multidimensional image acquisition using the tool window

The tools for multidimensional image acquisition can be activated and selected by ticking the respective tick boxes in the selection panel:



Clicking on the  check box next to the appropriate tool selects, activates, and displays the tool for multidimensional image acquisition. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after pressing the **Start Experiment** button. The experiment type and the final file size is represented graphically above the button.

<b>New Acquisition Document</b>	
<b>Set Exposure</b>	<b>Ctrl+E</b>
<b>Continuous</b>	<b>Ctrl+T</b>
<b>Snap</b>	<b>Ctrl+Space</b>
<b>Start Experiment</b>	
<b>Stop</b>	

Fig. 29 Context menu for multidimensional acquisition area

## 2.8.2 Context Menu for the Left Tool Area

There are two different context menus in the Left Tool Area with different functionalities available.

The first one (Fig. 29) is opened by clicking with the right mouse button next to the action buttons or in the area of the Experiment Manager.

**New Acquisition Document** opens an empty acquisition document in the active image container.

**Set Exposure** starts a procedure to find balanced Exposure Time settings for all activated channels.

**Continuous** starts the uninterrupted imaging procedure with the currently active imaging parameters.

**Snap** takes one image.

**Start Experiment** starts a multidimensional experiment (Z-Stack, Time Series, Multiview, ...).

**Stop** stops imaging immediately.

<b>Auto Close Mode</b>
<b>Move group to next column</b>
<b>Move group to prev. column</b>

Fig. 30 Context menu for the tool groups area

The second context menu (Fig. 30) appears with a right mouse button click in the area of the tool groups.

**Auto Close Mode** means that the "oldest" open tool window will be closed automatically if opening a new tool window leads to a full tool column.

A de-activated **Auto Close Mode** will lead to a scrollbar on the right hand side of the Left Tool Area, once a tool column is filled with open tools.

**Move tool group to next column** shifts an entire tool group to the adjacent column.

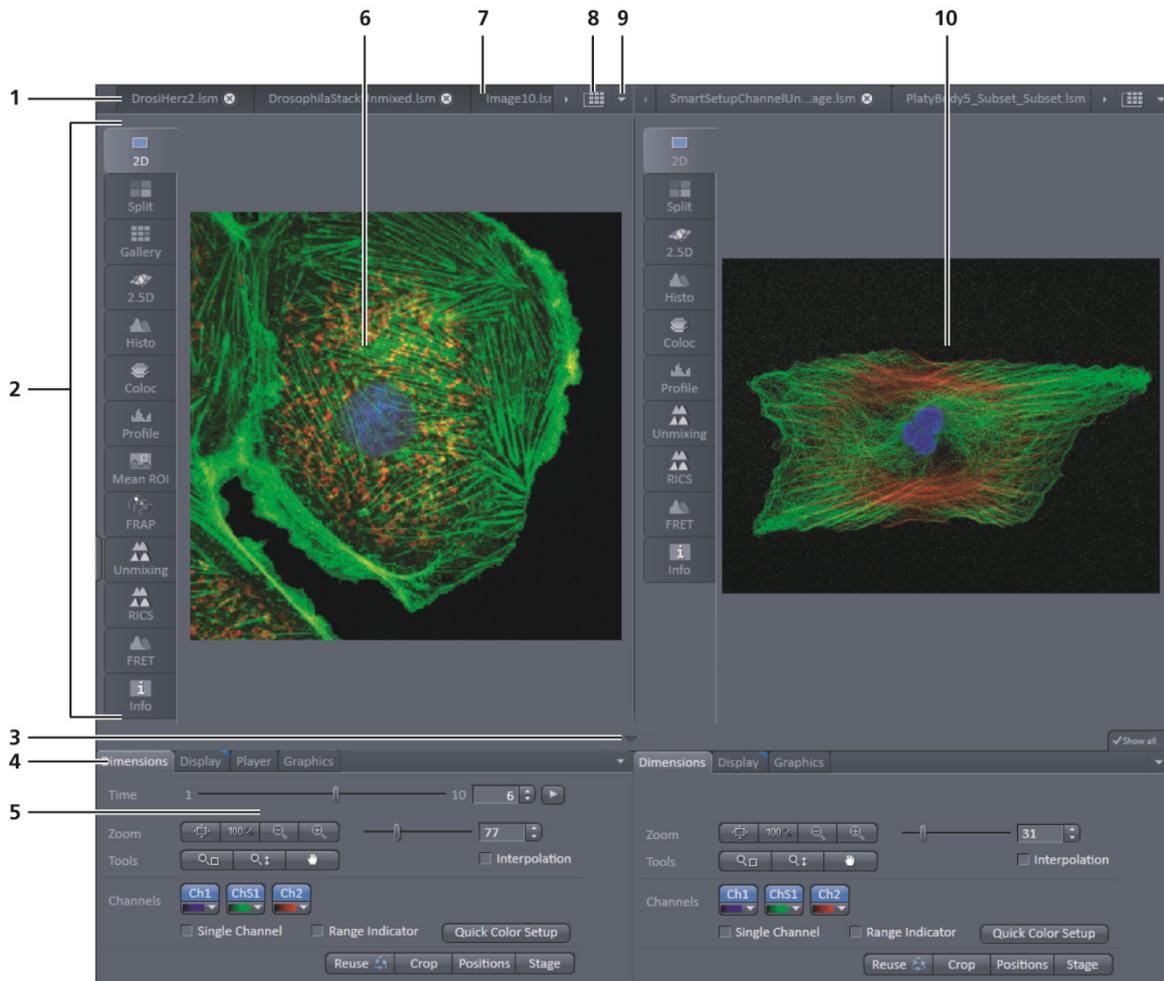
## 2.9 Center Screen Area

The Center Screen Area is used for displaying acquired images or to show images in the available image views. Use the context menu for the Center Screen Area by clicking the background of a container, the view of this area can be varied individually.

The Center Screen Area can be split into 1, 2, or 3 container views. So, it is possible to show several images in parallel.

Images can be moved from one container to another using the drag-and-drop function.

### 2.9.1 Overview for the Center Screen Area



- 1 Image tabs with scroll arrow buttons left and right of container 1
- 2 Image view tabs of container 1
- 3 Arrow down or arrow up button to hide or show the image control area
- 4 Image view controls
- 5 Image control area
- 6 Image container 1 (with opened image window)
- 7 Background area of container 1
- 8 Expose mode button (overview)
- 9 Arrow down button for fast image selection
- 10 Image container 2

**Fig. 31 Center screen area**



Fig. 32 Image tabs

### Image tabs

For each opened image, one image tab is shown in the header of the actual container.

Three modes can be selected using the context menu of the **Center Screen Area** (see also section **2.9.2 Context Menu for the Center Screen Area**).

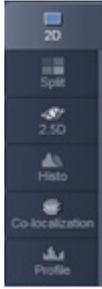


Fig. 33 Image view tabs

### Image view tabs

Used to select one of the various image views (see also sections **4.2 2D View** to **4.13 Information View**).

### Arrow down or arrow up button to hide or show the image control area

Clicking on the arrow down button hides the image control area and increases the image view field of all containers in the vertical direction for a larger image display.

Clicking the arrow up button shows the image area in all containers once again.

### Image view controls

Activates one or more of the various image display and analysis functions (see also section **4.2 2D View**).

### Image control area

Shows the tools for the selected image display or analysis functions.

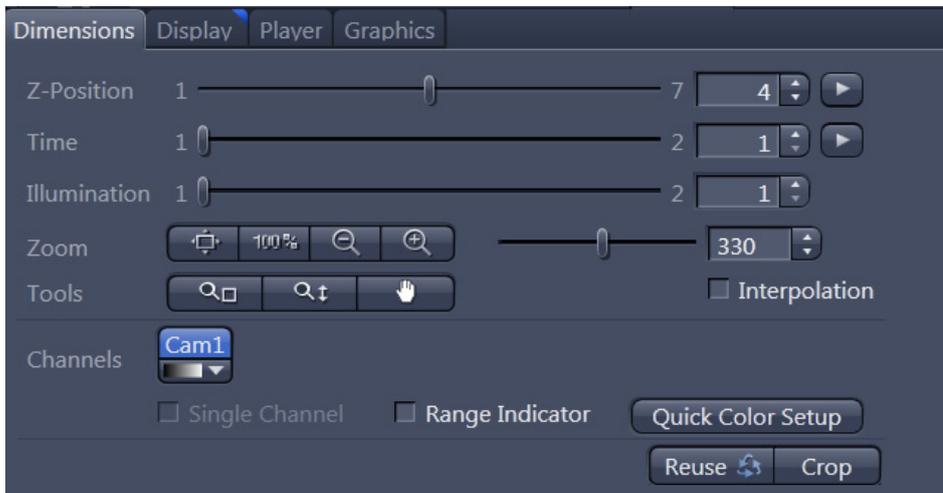


Fig. 34 Image control area

**Expose Mode**



Changes the single image display to the Expose display, showing thumbnails of all open images. Clicking on one of the image headers changes back to the single image display.

The main purpose of this function is to quickly find an image that might be only one of many open images which are all represented by image tabs on the top. Especially if the image tabs are configured to display only the image name.

**Arrow down button**



Clicking on this button shows a list with all opened images for fast image selection.



Using the drag and drop function, one or several images can be moved into another opened container. If closing a container, the included images will be moved automatically to another open container.

**2.9.2 Context Menu for the Center Screen Area**

Clicking the right mouse button on the background of the container opens the context menu for the **Center Screen Area** (Fig. 35).

The following functions are available:

**Display of the image tabs in the container header**

- Text View
- Small Thumbnail View
- Large Thumbnail View

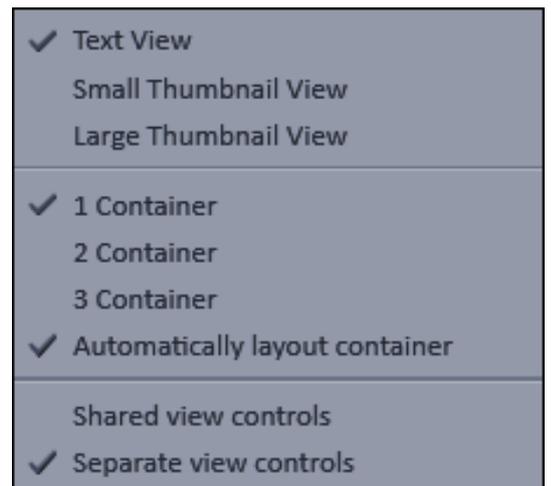
**Number of opened containers**

- Up to three containers can be opened in the Center Screen Area.

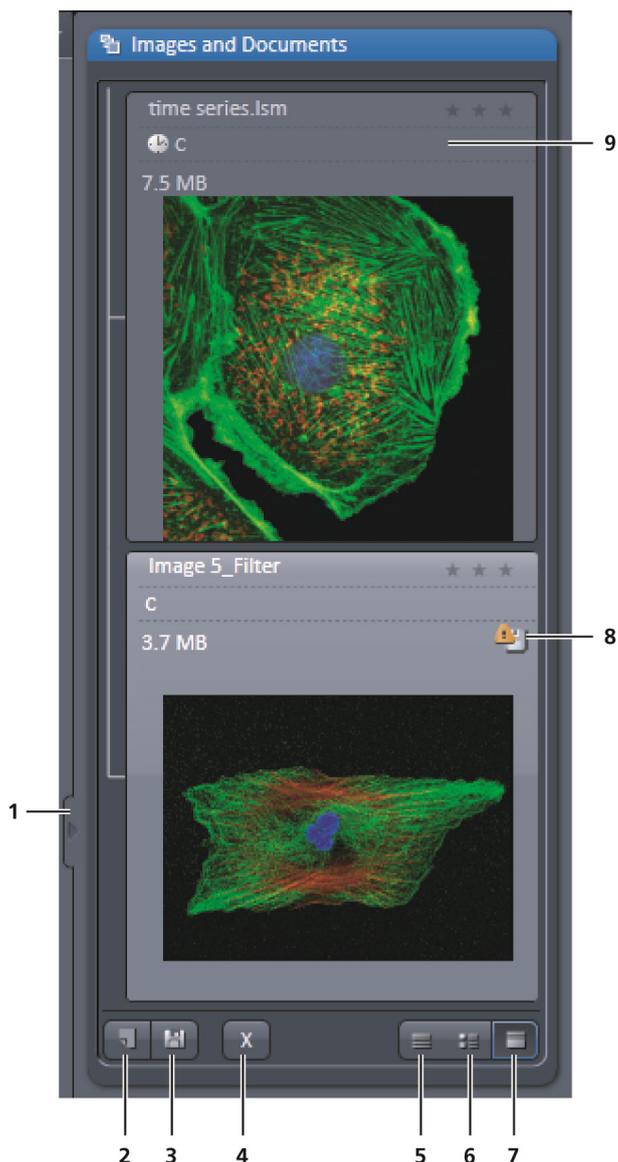
**Shared or separate view controls**

The display of the Center Screen Area can be changed between shared view controls mode and separate view controls mode.

**Separate view controls** shows a set of view control tabs for each container. In shared view controls mode, only one set of view control tabs is displayed, spanning the whole bottom area under all 2 or 3 containers. They always apply to the active image/container.



**Fig. 35 Context menu**



- 1 Expand slider
- 2 New image document button
- 3 Save button
- 4 Close button
- 5 Textual view button
- 6 Thumbnail view button
- 7 Big view button
- 8 Save status button
- 9 Image information field

**Fig. 36 Right Tool Area**

## 2.10 Right Tool Area

The Right Tool Area is used for displaying and handling the opened images, e.g. save or close. The view of this area can be varied individually, e.g. expand or hide the area, change between the view modes.

### Expand slider

Dragging this slider to the left side expands the Right Tool Area by decreasing the center tool area.

Dragging the slider to the right side decreases the Right Tool Area up to the default size.

### Save button

Saves the selected imaged or changed image. The **Save as** dialog appears.

### New image document button

Opens an empty image document in the active container.

### Close button

Closes the selected image. The **Close image** dialog appears to close the image with or without saving or to cancel the closing.

### Textual View button

Shows images only with the textual characters.



### Thumbnail View button

Shows images with the textual characters and an image thumbnail preview.



**Big View button**

Shows images with the textual characters and a big image preview.

**Save Status icon**

This icon appears in the image that is not saved yet.

**Image information**

Displays the name, type, and file size of the image.

### 3 Left Tool Area and Hardware Control Tools

The Left Tool Area contains all tools for system operation, image acquisition, image processing, and maintenance.

The major functions are organized into the four main tool tabs (Fig. 37):

#### Locate

Inspection of sample chamber to either locate the capillary or the sample.

#### Acquisition

Acquiring images with the Lightsheet Z.1.

#### Processing

Processing images and multidimensional (multiview) images taken with the Lightsheet Z.1.

#### Maintain

Setting imaging, system, and firmware parameters.

By pressing the arrow left symbol on the **Expand tab** at the right-hand-side border, the **left tool area** will collapse to the left side. The symbol changes to an arrow right symbol. By pressing the latter, the **left tool area** will expand again.

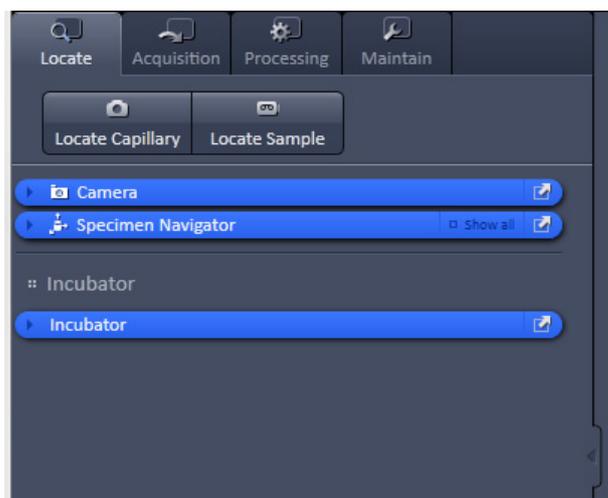


Fig. 37 Locate tab

#### 3.1 Locate Tab

**Locate** contains all functions of the system to locate the capillary or similar device held by the sample holder and to also locate the sample within its embedding medium.

### 3.1.1 Locate Capillary

To locate the capillary or similar device which contains the sample, press the **Locate Capillary** button. The white LED illumination is turned on and a live image is acquired showing the inside of the sample chamber. This image is displayed in the Center Screen Area. To adjust the light intensity, the **Light Intensity slider** in the **Camera Tool** window can be used. The zoom function is not available during this process and remains deactivated. The upper system cavity door can be open during this live image.

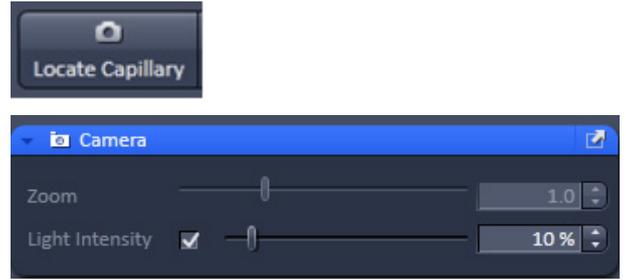


Fig. 38 Locate Capillary interface

### 3.1.2 Locate Sample

To locate the sample within the sample chamber, the **Locate Sample** button activates the infrared light source and a live transmitted light image is shown in the Center Screen Area. This image is taken with channel 1/ Cam1. **Zoom** and **Light Intensity** can be adjusted by using the sliders in the **Camera Tool** window. The upper system cavity door can be opened during this live image.



Fig. 39 Locate Sample interface

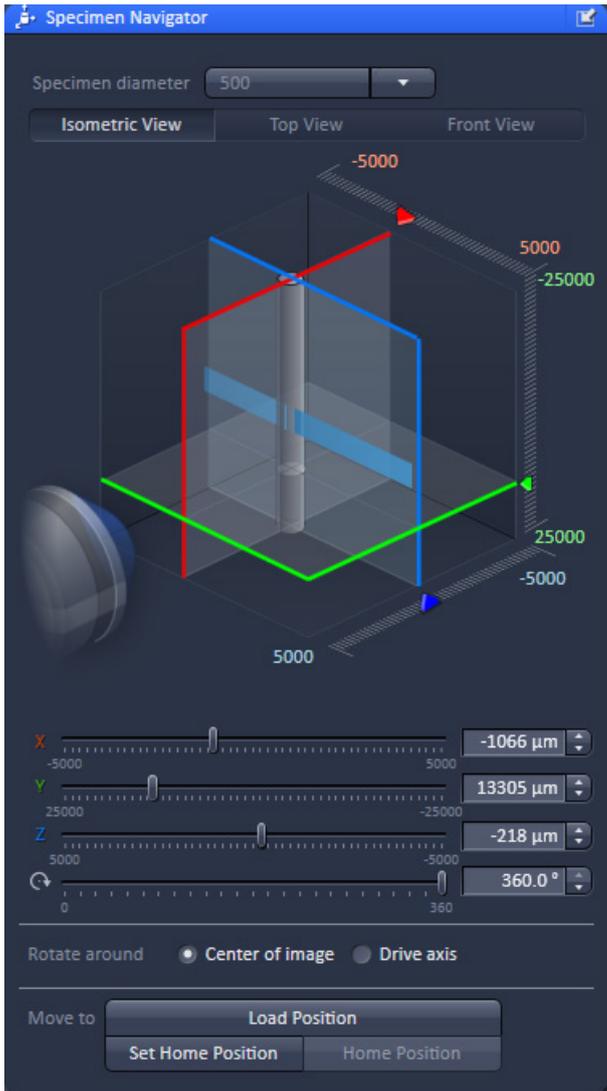


Fig. 40 Specimen Navigator tool window

### 3.1.3 Specimen Navigator Tool

To position the sample holder for imaging while using the **Locate Capillary** or **Locate Sample** function, the **Specimen Navigator** tool window is available in the Locate tool tab (Fig. 40).

Here the relative positions of the sample (light-grey cylinder), the illumination light-sheet (light-blue), as well as the x-axis (red), y-axis (green), and z-axis (blue) are graphically displayed. You can choose between an **Isometric View**, a **Top View** or a **Front View** for orientation.

In all of these Views, you can zoom into the graphical display by clicking once into the graphic with the left mouse button and afterwards pressing the **Ctrl** key on your keyboard (Fig. 41). For increased zooming, press the Ctrl key with the Shift key. The zoom will last as long as the key or combination of keys are pressed. To remain in the zoom mode, press the Alt key at the right side of the keyboard also. The zoom will remain until the Ctrl key is pressed again.

To move the sample holder in the x-, y-, or z-direction, you can either place the mouse over the according colored line in the graphic, hold it with the left mouse button and drag it to the required position; or you can use the sliders and spin boxes for each axis below the graphic. To rotate the sample holder, use the slider or spin box.

There are two options for rotating the sample. With **Rotate around Center of image** selected, the x and y positions are additionally corrected for rotational movement to keep the sample within the chosen field of view when rotating. This can be helpful when the sample is not in the center of the embedding medium. If the option **Rotate around Drive axis** is chosen, the sample holder is rotated around its main axis without any correctional movements for the other axis.

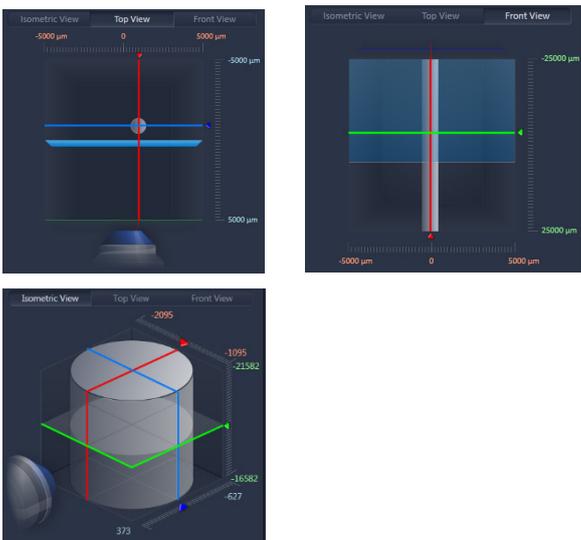


Fig. 41 Zoom into the Isometric, Top and Front View

To define the size of the sample embedding cylinder, you can adjust its diameter. Use the drop-down list at the top of the **Specimen Navigator** tool window next to **Specimen diameter**. You can pick default values or manually insert a diameter. Recently used specified diameters are listed next to **Recent** (Fig. 42). The diameter specified is directly influencing the **Collision Check** (see **3.4.3.3 System Options – Hardware**) and should match the actually used sample.

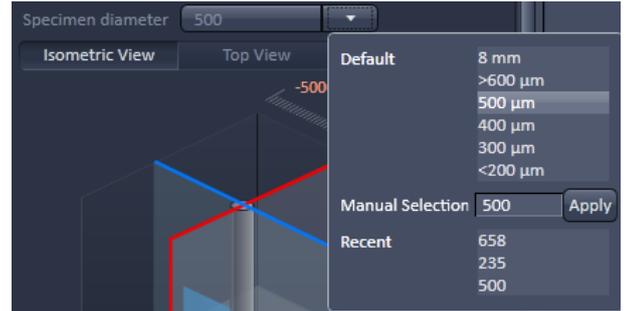


Fig. 42 Specimen diameter interface

For an exchange of a sample holder click the **Load Position** button. The same holder will be driven to the upper most position. Now an easy access of the sample holder through the upper system cavity door is possible.

You can define a position which you might want to return to during the course of imaging by pressing the **Set Home Position** button after putting the sample to the desired spot. To return to this position you click the **Home Position** button.

This will not affect the coordinates in the **Multiview-Setup** tool window (see section **3.2.12 Multidimensional Acquisition – Multiview-Setup**). Here the y axis parameters will retain their original naming and need no changing.

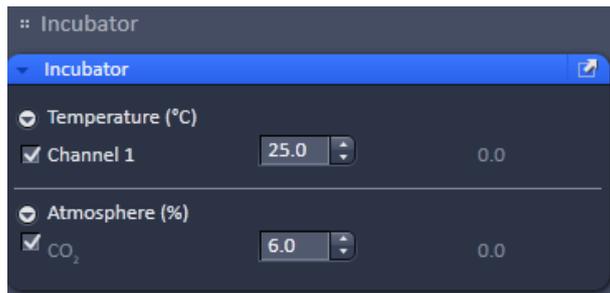
### 3.1.4 Incubator Tool

To have access to incubator functions for defining the atmosphere and temperature, expand the **Incubator** panel by pressing the triangle in the **Incubator tool** tab.

- ☞ All incubation components you wish to use during the imaging session must be connected to the Lightsheet Z.1 system and turned on before starting the ZEN software. Once ZEN is running you can detach and reattach components.
- ☞ Be aware that all incubation components will continue working with the **Incubator** tool window specified parameters after ZEN has been shut down or has stopped working. This will keep your sample alive, even if ZEN stops unexpectedly during an experiment. To turn off the incubation, uncheck the corresponding checkboxes in the **Incubator** tool window or turn off the Incubation on the remote control.

You will have access dependent on the attached equipment to

- Temperature (°C) control
- Atmosphere (%) control

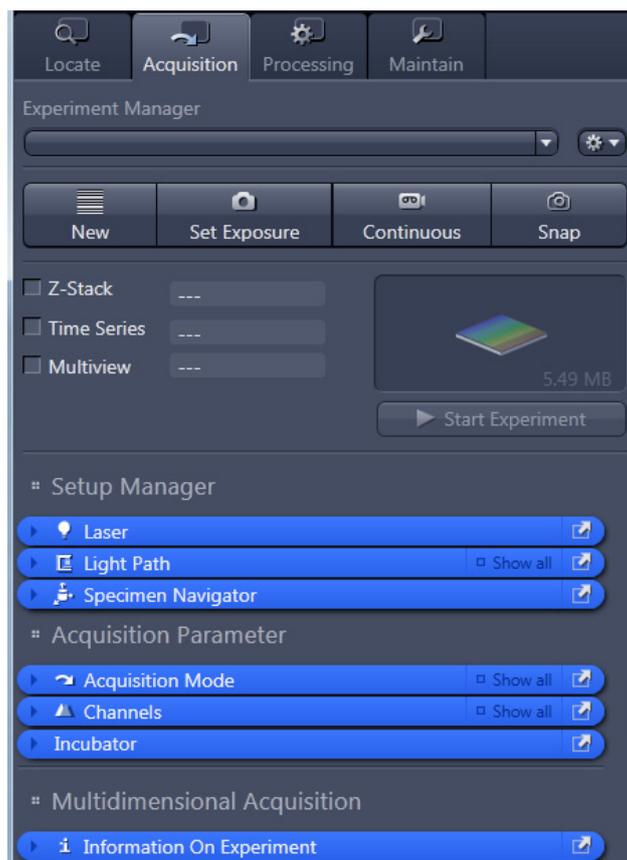


**Fig. 43** Incubator panel – Expanded fields for atmosphere and temperature control

To have access to the respective controls, expand the fields by pressing the triangles (Fig. 43).

The **Temperature (°C)** panel allows you to control up to 2 independent heating channels that are linked to certain devices (Peltierblock Sample Chamber Lightsheet Z.1, Heatingblock Sample Chamber Lightsheet Z.1) that are assigned to different channels. Enter the desired nominal temperature via the input boxes by typing in the numbers or by using the arrows to set the parameters. Check the corresponding box to activate the desired **Channel**. The grey numbers on the right will display the actual temperature.

The **Atmosphere (%)** panel allows defining CO<sub>2</sub> concentration via the input boxes. Either type in the numbers or use the arrows to set the parameters. Actual values will be displayed on the right.



**Fig. 44** Acquisition panel – appearance

### 3.2 Acquisition Tab

If **Acquisition** is selected, the **Acquisition** panel becomes available (Fig. 44).

**Acquisition** contains all functions of the system that are needed to set up and control light sheet fluorescent imaging and multidimensional data acquisition.

Depending on the hardware configuration determines which of the described functions are available.

For excitation, all imaging modes use lasers. The **Acquisition** panel is divided into the

- **Experiment Manager** field for loading predefined configurations, action buttons and definition of the multidimensionality of the experiment and
- **Tool Group** field with the **tool tabs** for setting the imaging parameters.

### 3.2.1 Experiment Manager

You can load a predefined experiment from the **Experiment Manager** field by pressing the arrow to access the list box (Fig. 45).

Select the appropriate one by clicking on the experiment name which is highlighted when moused-over.

To save, delete, or edit experiments, open the selection box next to the experiment list box (Fig. 46).

To define a new experiment, set all parameters according to the acquisition needs in the Tool Group fields. Press **New** in the menu list and type in a name for your experiment in the editing line. which will open at the place of the experiment list box (Fig.49). Afterwards, press the disc icon to save the new configuration or press the cross icon to abort the process.

The process is similar when renaming an experiment by pressing **Rename** or using the **Save As** option. After typing the wanted name the changes can be either saved or the process can be aborted.

To save changes done in the tool tabs to a previously defined experiment, press the **Save** option.

Experiments can be imported or exported as .czlsmexp files. When using Import or Export, windows explorer will open. Either the location of the file to be imported can be chosen or a location where the exported file should be saved.

To delete an experiment, press the **Delete** button. This opens the **Delete configuration...** panel (Fig. 47).



Fig. 45 Experiment Manager field with experiment drop down list

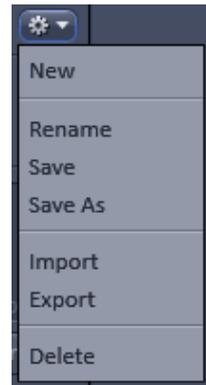


Fig. 46 Menu to save, delete or edit experiments

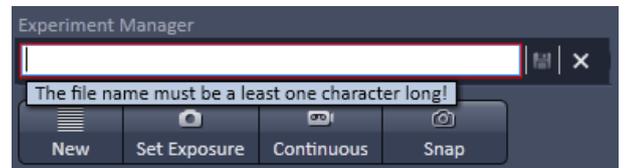


Fig. 47 Delete configuration panel...

### 3.2.2 Action Buttons

**Action** buttons provide control for how the image is acquired (Fig. 48).

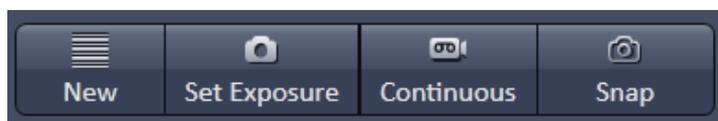


Fig. 48 Action buttons

They have the following functions, regardless of the imaging mode:

The **New** button opens a new **Image window** in the **Image Container** into which the new image will be displayed. The next acquired image is displayed in this image frame, when it is the selected one. This function can be used to avoid that an old unsaved image (acquired by **Set Exposure**, **Continuous** or **Snap**) will be overwritten by a new image.

**Set Exposure** optimizes the exposure time settings of the detection units for all active channels and tracks dependent on the given laser power. **Set Exposure** can be disrupted by pressing the **Stop** button, which the **Set Exposure** button changes to upon activation (Fig. 49).

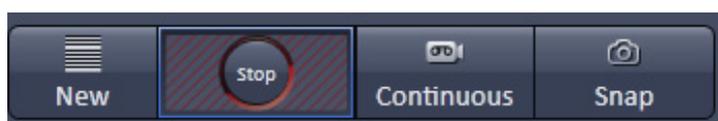


Fig. 49 Action buttons – Set exposure button active

**Continuous** produce images from all active channels and tracks, thereby ignoring the settings for multidimensional acquisition, but taking into account the selected imaging settings. The last frame taken will stay in the image window. To disrupt **Continuous** acquisition, the **Stop** button has to be pressed which is what the **Continuous** button changes into after its activation (Fig. 50). The stop of the continuous image acquisition can take a couple of seconds, due to the fact that hardware components need a certain reaction time and it is a normal behavior of the system. During this time the **Stop** button will be displayed.



Fig. 50 Action buttons – Continuous button active

**Snap** produces one single image using all active channels and tracks thereby ignoring the parameters activated in the multidimensional acquisition tool group, but taking into account the imaging settings. **Snap** can be disrupted by pressing the **Stop** button which is what the **Snap** button changes into upon activation (Fig. 51).

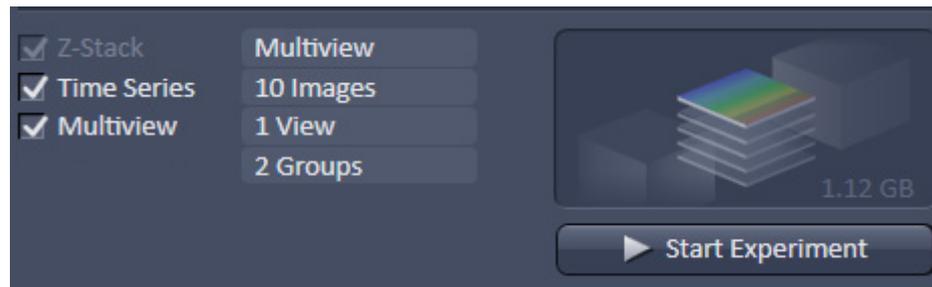


Fig. 51 Action buttons – Snap button active

-  All actions buttons (with the exception of **New**) turn into **Stop** buttons once pressed. They can be used to stop the current process or experiment.

### 3.2.3 Multidimensional Acquisition Settings

In the **Multidimensional acquisition** field (Fig. 52), the multidimensionality can be defined by checking the corresponding boxes.



**Fig. 52** Multidimensional input field – Z-Stack, Time Series (with 10 time-points) and Multiview (with 1 views and 2 groups) active

The following dimensions are available:

- **Z-Stack** – Acquisition of an image series in z
- **Time series** – Acquisition of an image series in time
- **Multiview** – Acquisition of diversely sized Z-Stacks at different Views. A View is determined by the x, y and alpha (rotation) motor position of the sample holder.

The settings for multidimensional acquisition are accomplished within the tool group **Multidimensional Acquisition** for all functions. If available, the settings are displayed beside the function, e.g. how many images are taken for the time series (Fig. 52). Corresponding tool bars within the tool groups only become available if the box of a multidimensional function is checked; otherwise the tool bar is not displayed (Fig. 53). The **graphical display** field above the **Start Experiment** button shows a representation of the experiment type with its dimensions shown. It also lists the estimated size of the final multidimensional image.



Fig. 53 Multidimensional Acquisition Tool group – all possible tool tabs displayed

Note that the Multiview function needs the Z-Stack function for a useful experiment and will activate the Z-Stack function as well. The latter is activated and greyed out.

Pressing the **Start Experiment** button starts the image acquisition, taking into account all activated functions. The progress of the experiment in regards to which image the system currently acquires will be shown updated to the right of the functions if available (Fig. 54).

When **Streaming** is activated in the **Streaming tool** window under Maintain (see section **3.4.4 Maintain – Streaming**), pressing Start Experiment will open a Windows explorer window. Here the file name and location can be defined as to where the experiment will be saved during the acquisition.

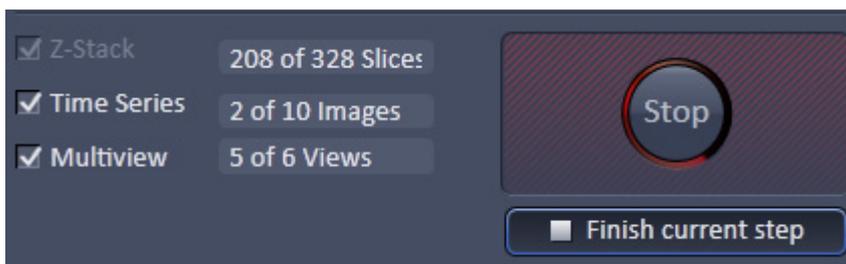


Fig. 54 Multidimensional input field – display of the current status 208 from 328 slices taken from the 2nd of 10 images of the time series and the 5th view of 6

Upon pressing the **Start Experiment** button, it changes into a **Finish current step** button, whereas the **graphical display** field changes into a **Stop** button. Pressing the **Stop** button immediately finishes the acquisition. Pressing the **Finish current step** button will interrupt the multidimensional acquisition after the currently performed step; e. g. when performing a Z-Stack over time it will finish the current Z-Stack and then stop the experiment.

### 3.2.4 Setup Manager – Laser

Open the **Laser** tool (Fig. 55) by clicking the **Laser** tab in the **Setup Manager** tool group. Close the tool box by clicking again.

Lasers are listed with the **Laser names**, the **Laser lines (nm)** and the **Power** states. The **Laser names** are a combination of laser line (nm) and the power in mW.

All lasers of the Lightsheet Z.1 are solid state lasers and are controlled by the software. To turn them on, select the power state from the power state switch box. The **Power** state switch box shows the state of the laser: **On** or **Off**. To switch a laser on, select **On** from the respective pull down menu.

For each laser, the properties are displayed if the **Laser Properties** window is opened. After turning the Laser on, the Status will change from connected, to Warming up, to Ready.

Upon exiting the Software, the **Laser Off** panel will be displayed - if the **lasers off on exit** checkbox in the **Hardware** tab of **Systems Options** for the **Maintain** tab is not ticked -, showing the current power state of the lasers (Fig. 55). The user has the possibility to manually switch off still active lasers before the software closes.

 After switching on the lasers in the laser control window and their status shows Ready, the system can be used for imaging. However, for quantitative imaging it is recommended to let the system warm up for at least 3 hours.



Fig. 55 Laser tool window



Fig. 56 Light Path configuration panel

### 3.2.5 Setup Manager – Light Path

The **Light Path** panel allows new / existing light path configurations to be created / edited and saved.

- Click on the **Light Path** tab to open the **Light Path** configuration panel (Fig. 56). Click on any other tab to leave the panel.

The **Light Path** panel is divided into the

- Track definition field
- Beam path configuration field

### 3.2.5.1 Track Definition

In this tool one can create a configuration with up to 4 tracks (Fig. 57). The **sequential** imaging of **tracks** permits several tracks to be defined as one configuration for the imaging procedure, can be stored under any name, can be reloaded or deleted configurations for a track are thereby defined in the **Beam path configuration** field for the selected track. To create a new track configuration, highlight the track by pressing the according **Track** button. Go to the **Beam path configuration** to set up the beam path as well as the **Channel** tool to set up exposure time and laser power. You can store the track configuration in the **Experiment Manager** field.

Tracks can be added or deleted by pressing the **+** or **trash can** button, respectively. The **trash can** button is inactive if only one track is defined. Up to 3 tracks can be added for a total of 4 tracks. The **+** button becomes inactive after the 4<sup>th</sup> track has been added. An added track will have the same filter settings as the first track, which can be subsequently changed in the **Beam path configuration** field. The **Track** button of the selected **Track (1-4)** will be highlighted in blue and the **Track display** area below designates the track that is displayed in the **Beam path configuration** field. All alterations will be specific for that track

With the **Switch track every** drop down menu (only available if **Show all** is active), you can select the way tracks are switched. The different tracks can be switched every **Frame**, **Fast Frame** and every **Z-Stack** (Fig. 58).

**Frame** will image every frame with all available tracks before moving to the next slice of a defined Z-Stack. Up to 4 tracks can be imaged. The Continuous Drive in the Z-Stack tool window is deactivated.

In **Fast Frame** mode (up to 2 tracks can be defined), switching of hardware components that require mechanical movement like filter changes is prevented and hence the setting for the first track remains in place, even if the other tracks have different configurations. The latter parameters are therefore overwritten by those from the first track. Only those settings that do not require mechanical movement are accepted like activation/deactivation of laser lines, and laser power settings. You will be prompted in an **Info** window to allow parameters to be overwritten when using **Frame Fast** switching modes (Fig. 59). Press **Yes** to accept parameters to be overwritten. Press **No** to return to **Frame** switching mode, where all settings will be kept for each track. Note, that if mechanical switching is required, switching time will be accordingly slow.

When the **Z-Stack** option is activated for the experiment the **switch track every Z-Stack** becomes available. When chosen, a Z-Stack is imaged completely with the first track, afterwards the same Z-Stack is acquired with the configuration of the next track and so on. This will reduce acquisition time noticeably. Up to 4 tracks can be configured.

 The total set of parameters for one or multiple tracks can be stored as an acquisition configuration using the save option in the **Experiment** field of the **Experiment Manager**. Using multiple tracks is the preferred method to clearly separate weakly overlapping emission signals.



Fig. 57 Track definition field



Fig. 58 Switch track every drop down menu

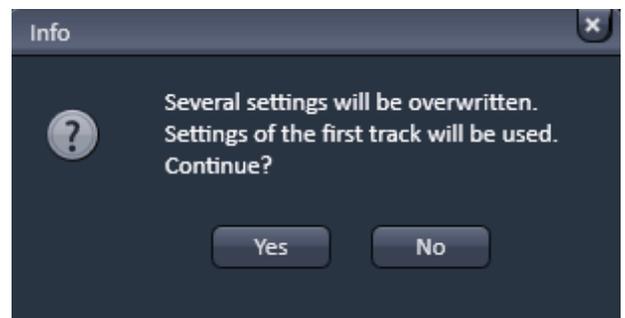


Fig. 59 Info window to confirm

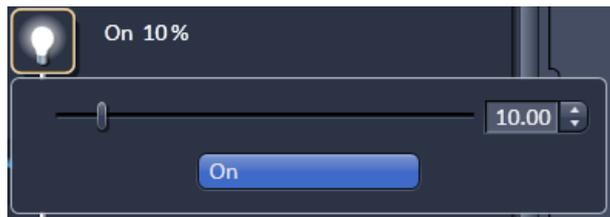


Fig. 60 Transmitted Light Lamp panel

### 3.2.5.2 Transmitted Light Lamp Control

When the **Transmitted Light Lamp** icon for transmitted light from the infrared lamp is pressed, the control panel for the light settings is displayed (Fig. 60).

If you press the **On** button, the lamp is turned on and the **On** button is highlighted in blue. The light intensity of the **Transmitted Light** lamp can be varied via the input box or slider. Press the **On** button again to turn off the light.



Fig. 61 Laser panel

### 3.2.5.3 Laser Control

Press the **Laser** icon to display the laser control expansion submenu (Fig. 61).

Activate the lasers you want to use by checking the corresponding boxes.

The used laser line with sliders will appear below.

Set the requested laser attenuation (%) using either the sliders, the arrows, or typing into the display field.

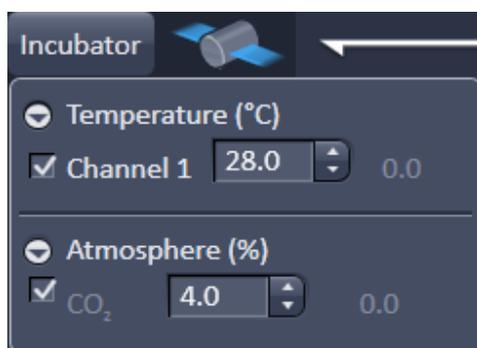


Fig. 62 Incubator panel

### 3.2.5.4 Incubator

You have access to incubator functions by pressing the **Incubator** button.

This opens the expansion submenu for access to the possible attached equipment

- Temperature (°C) control
- Atmosphere (%) control

The **Temperature (°C)** panel allows you to control up to 2 independent heating channels that are linked to certain devices (Peltierblock Sample Chamber Lightsheet Z.1, Heatingblock Sample Chamber Lightsheet Z.1) that are assigned to different channels.

Enter the desired nominal temperature via the input boxes by typing in the numbers or by using the arrows to set the parameters. Check the corresponding box to activate the desired **Channel**. The grey numbers on the right will display the actual temperature.

The **Atmosphere (%)** panel allows setting the CO<sub>2</sub> concentration via the input boxes. Either type in the numbers or use the arrows to set the parameters. Actual values will be displayed on the right.

### 3.2.5.5 Detection Optics Display

The Detection Optic previously selected in the Maintain tab (see section 3.4.1.1 **Detection Optics**) is displayed as the **Detection Optics** icon (Fig. 63).

The full name of the detection optic is written next to the graphical display.



Fig. 63 Detection optics display

### 3.2.5.6 Laser Blocking Filter Selection

According to the chosen laser lines, the appropriate filter from the Reflector Turret for Laser Blocking Filter can be chosen from a list when pressing the **Laser Blocking Filter** button (Fig. 64).

There are six positions available in the turret. To choose one, click on it. The turret will move to the correct position and the filter and its name will be shown in the light path.



Fig. 64 Laser Blocking Filter selection

### 3.2.5.7 Emission Selection Filter Selection

The **Emission Selection Filter** button opens a drop-down menu in which you can choose the Beam Splitter to direct the emission light towards the desired detection module, **Cam1** or **Cam2**. The activated filter will move into position and its name will be displayed in the light path. Since Beam Splitter and Emission filters form one unit, the corresponding Emission Filter 1 (in front of Cam1) and Emission Filter 2 (in front of Cam 2) will change as well in the light path.

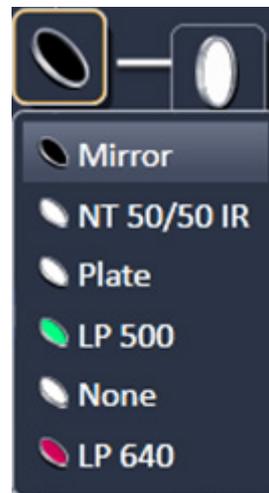


Fig. 65 Emission Selection Filter control panel



Fig. 66 Detection module icon

### 3.2.5.8 Channel Selection

#### Activation/Deactivation of Channels

- To activate a channel for imaging, check the check box next to the detection module icon, either **Cam 1** or **Cam 2**. To deactivate the channel uncheck this box (Fig. 66).
- To assign a specific color to the image of a channel, click onto the detection module icon and select a color or look-up-table (LUT) from the drop down menu (Fig. 67).



Fig. 67 Look up table to choose Channel display color

### 3.2.6 Specimen Navigator Tool

To position the sample holder for imaging the **Specimen Navigator** tool window is available (Fig. 68).

Here the relative positions of the sample (light-grey cylinder), the illumination light-sheet (light-blue), as well as the x-axis (red), y-axis (green), and z-axis (blue) are graphically displayed. You can chose between an **Isometric View**, a **Top View**, or a **Front View** for orientation.

In all of these Views, you can zoom into the graphical display by once clicking into the graphic with the left mouse button and afterwards pressing the **Ctrl** key on your keyboard (Fig. 69). For additional zooming, press the Ctrl with the Shift key. The zoom will last as long as the key or combination of keys are pressed. To remain in the zoom mode, press the right Alt key. The zoom will remain until the Ctrl key is pressed again.

To move the sample holder in x-, y-, or z- direction you can either place the mouse over the according colored line in the graphic, hold it with the left mouse button and drag it into the required position; or you can use the sliders and spin boxes for each axis below the graphic. To rotate the sample holder, use the slider or spin box.

There are two options for rotating the sample. With **Rotate around Center of image** selected, the x and y positions are additionally corrected for rotational movement to keep the sample within the chosen field of view when rotating. This can be helpful when the sample is not in the center of the embedding medium. If the option **Rotate around Drive axis** is chosen, the sample holder is rotated around its main axis without any correctional movements for the other axis.

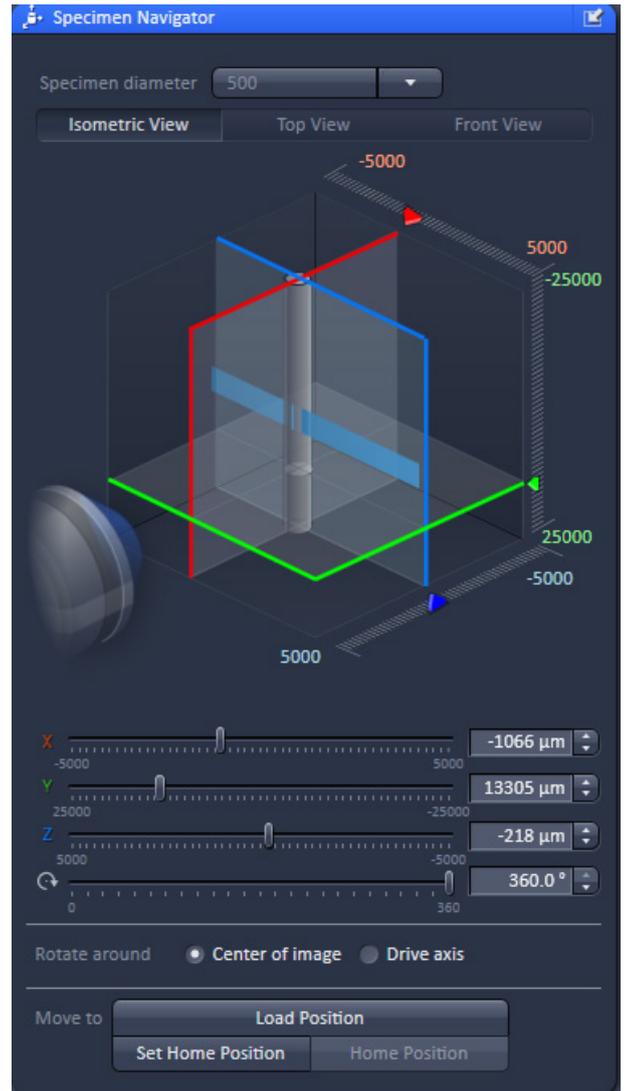


Fig. 68 Specimen Navigator tool window

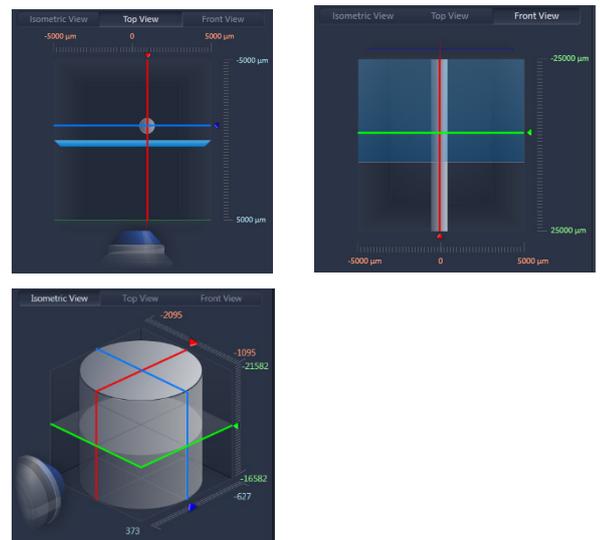


Fig. 69 Zoom into the Isometric, Top and Front View

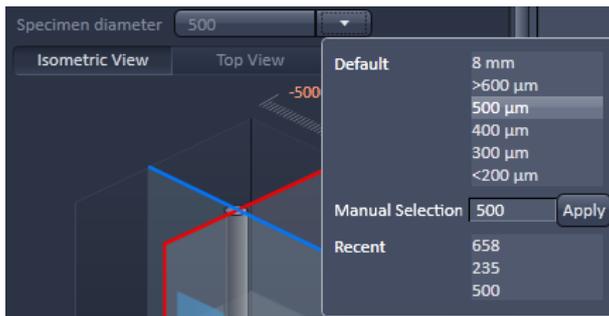


Fig. 70 Specimen diameter interface

To define the size of the sample embedding cylinder, you can adjust its diameter (Fig. 70). Use the drop-down list at the top of the **Specimen Navigator** tool window next to **Specimen diameter**. You can pick default values or manually insert a diameter. Recently used specified diameters are listed next to **Recent**. The diameter specified is directly influencing the **Collision Check** (see **3.4.3.3 System Options – Hardware**) and should match the actually used sample.

For an exchange of a sample holder click the **Load Position** button. The same holder will be driven to the upper most position. Now an easy access of the sample holder through the upper system cavity door is possible.

You can define a position which you might want to return to during the course of imaging by pressing the **Set Home Position** button after putting the sample to the desired spot. To return to this position you click the **Home Position** button.

This will not affect the coordinates in the **Multiview-Setup** tool window (see section **3.2.12 Multidimensional Acquisition – Multiview-Setup**). Here the y axis parameters will retain their original naming and need no changing

### 3.2.7 Acquisition Parameter – Acquisition Mode

In the **Acquisition Mode** tool tab, parameters for the acquisition can be set.

Controls include

- Format selection
- Bit depth selection
- Zoom selection and Light Sheet Thickness selection
- Auto Exposure settings
- Light Sheet Illumination Settings
- Pivot Scan Selection
- Acquisition area settings

### Format Selection

The total number of pixels of the currently used detection module is displayed in the **Format** drop down menu and is labeled as standard. Binning of the camera pixels can be set as well using the **Format** drop down menu which is labeled with the binning factor and the resulting number of pixels.

### Bit depth Selection

Select **8 Bit**, or **16 Bit** Data Depth, for 256, or 65536 gray values, using the corresponding drop down list.

### Zoom selection and Light Sheet Thickness selection

You can regulate the area to be imaged with the **Zoom** function, adjust the value with the slider, type in numeric values into the adjacent box, or use the arrows. This zoom is realized with hardware zoom optics within the system. When using the **Zoom**, the number of pixels stays unchanged. But, the size of the total area imaged (**Image Size**) and the area represented by a pixel (**Pixel Size**) will change accordingly. You can look at the image and pixel dimensions in  $\mu\text{m}$  in the Acquisition Area.

-  The **Zoom** numbers are displayed in red when set to values from 0.36 to 0.7; the same range is indicated with a red line below the slider. Within these zoom settings, the image quality towards the edges can decrease.
-  Be aware, when you change the Zoom during an acquisition session, the light sheet alignment (Light sheet **Auto-Adjust** in the **Channel tool** window) needs to be done again.
-  When the Zoom slider is used to change the **Image Size**, a zoom of the illumination beam path is also activated to ensure the ideal light sheet thickness ratio of 1:2 for the center to border ratio.

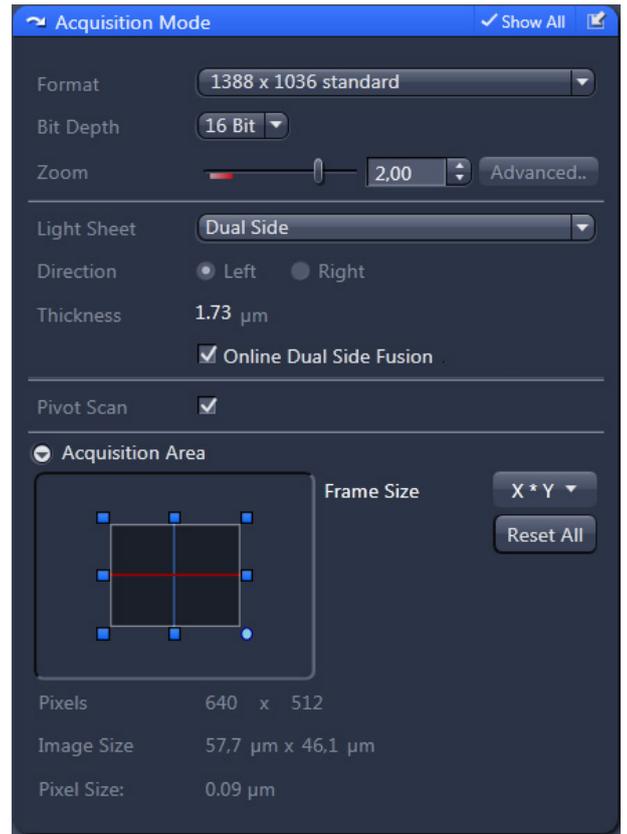


Fig. 71 Acquisition Parameter - Acquisition Mode



Fig. 72 Advanced... light sheet thickness menu

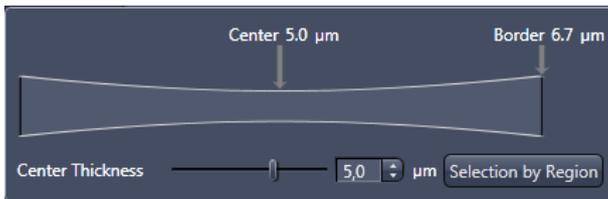


Fig. 73 Increased thickness at the center



Fig. 74 Decreased thickness at the center

Information about the thickness of the light sheet with the selected zoom is available when pressing the **Advanced..** button. A graphical display (Fig. 72) informs you about the thickness in the center and the border of the created light sheet in  $\mu\text{m}$  within the field of view. To manually change the thickness of the **Center Thickness** use either the slider, enter a value into the box, or use the arrows.

Be aware, this will change the ratio of the light sheet thickness center to border of the image frame. If you decrease the **Center Thickness**, the ratio center to border will be  $1: >2$ ; so the light sheet will be thinner in the center but wider at the border of the image field (Fig. 74). If you increase the **Center Thickness**, the ratio will change to  $<1:2$  accordingly, so the light sheet will have a more homogeneous thickness in the image field (Fig. 73).

 To return to the ideal light sheet thickness ratio, use the **Zoom** slider in the **Acquisition Mode** tool window.

Be aware that the Zoom and the Center Thickness settings are influencing the dimensions of the light sheet overall. The ratio or the thickness in the center of the light sheet and at its border should be ideally 1:2 for the used frame.

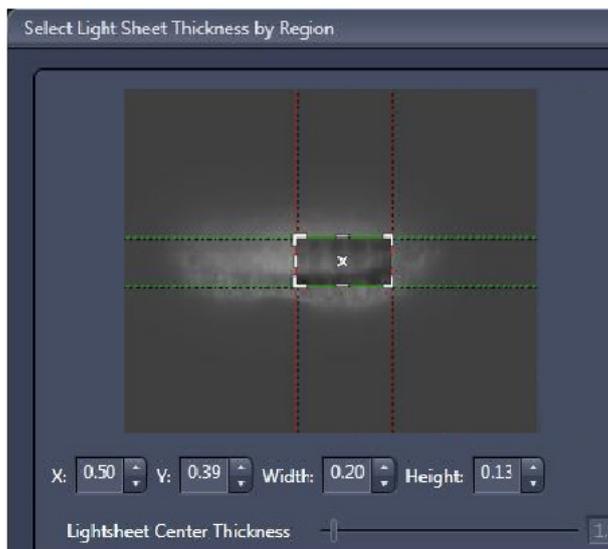


Fig. 75 Select Light Sheet Thickness by Region

The **Select by Region** button opens an additional window displaying a live image of the sample.

A square region is defined within the frame with red (y-axis) and green (x-axis) markings. The region can be enlarged along these axis by dragging the according side with the mouse. But the square cannot be moved to an offset within the image.

Within the chosen sub-frame, the optimal light sheet thickness ratio of the center to the border of 1:2 is created. The thickness of the light sheet at the center of the chosen square is displayed next to the **Lightsheet Center Thickness** slider within the input box.

## Light Sheet Illumination Settings

The first choice to make regarding the **Light Sheet illumination** is given in the drop down menu and determines if the light sheet should only illuminate the sample from one side (**Single Side**) or if the light sheet is sequentially generated from both sides (**Dual Side**) (configuration dependent). The third mode is **Dual Side when Experiment**. Here a single side illumination is kept during the setup of the experiment, hence when using the **Continuous** or **Snap** action buttons, which facilitates the setup of the imaging parameters. The dual side illumination is only activated when the **Start Experiment** button is pressed in the Multidimensional tool selection panel.

If **Single Side** is chosen, the direction, **Left** or **Right**, must be marked. This part of the panel is inactivated when the **Dual Side** illumination is selected.

The **Thickness** of the Light Sheet at its center is displayed in  $\mu\text{m}$ .

If **Dual Side** illumination is selected for each image (z-slice, time point) two frames are acquired, each with one illumination direction. The corresponding frames can be processed during image acquisition and combined into one frame when activating the **Online Dual Side Fusion** checkbox. The raw data will not be kept when **Online Dual Side Fusion** is selected, but only the resulting fused images. Or the raw data can later be fused using the **Lightsheet Processing** tools in the **Processing** tab.

## Pivot Scan Settings

The **Pivot Scan** (configuration dependent) is used to move the light sheet within the focal plane of the detection optics in a wavelike motion. This results in a reduction of shadows which might otherwise be cast by optically dense structures within the sample. It can be used when activating the checkbox next to **Pivot Scan**.

## Acquisition area settings

Information about the used number of **Pixels** within the frame is given as well as the dimensions of the **Image Size** and the **Pixel Size** are displayed in  $\mu\text{m}$ . The dimensions will change when using the Zoom function described above. **Frame Size** and therefore the number of **Pixels** to be used can be adjusted by opening the drop down sub menu. Here you can choose either from a list of **Rectangular** or **Square** predefined crop settings. The last five used pixel numbers from either list will appear in the **Recent** section of the sub menu. For individual adjustment you can type in the desired pixel amount for x and z next to **Manual Selection**.

When the **Reset All** button is pressed, the full frame with all available pixels will be imaged. This button has no influence on the zoom settings.

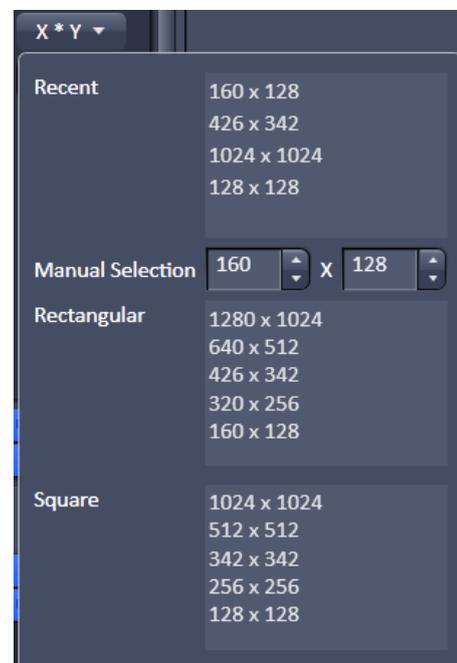
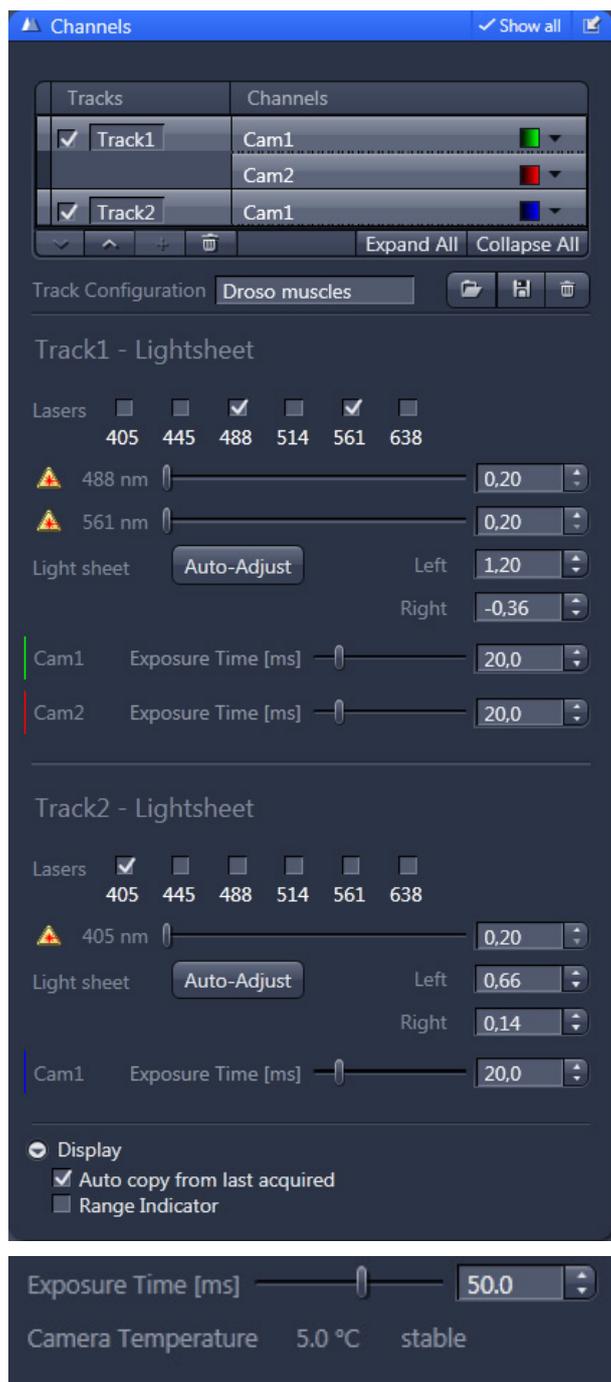


Fig. 76 Frame Size



**Fig. 77** Channel tool – Appearance with two tracks

### 3.2.8 Acquisition Parameter – Channel

In the **Channels** tools, the following options are available:

- Defining channels and tracks
- Defining Detector and Illumination settings
- Defining Display options

Expand the **Channels** tool tab to have access to these options.

#### 3.2.8.1 Defining Channels and Tracks

The **Channel** tool displays every track in the **Track Selection display** area that is currently open in the **Light Path** tool.

The **Track** names and the used **Channels** (Cam1 or Cam2) for that track are indicated. Each track that is selected by mouse click is highlighted and is displayed in the **Track Configuration display** area, which allows for individual settings for each track (Fig. 77).

A track will only be active when the **Continuous**, **Snap**, or **Start Experiment button** is pressed if the track check box is ticked.

Changes in the selection of the tracks in the **Channel** tool will automatically update the selection in the **Light Path** tool and vice versa.

E.g. if Track 1 and Track 2 are selected in the **Channel** tool, they will be selected in the **Light Path** tool as well.

You can either display one track or all of the tracks. There is no possibility to display two or three out of all four tracks.

If the triangle of the **Look Up Table (LUT)** drop down menu is pressed, a color can be assigned to a channel by highlighting it (Fig. 78).

The **Range Indicator** is used to easily visualize black and white pixels. This display mode shows white regions in red and black regions in blue. Some red and some blue pixels should be visible to ensure the signal is spread over the dynamic range of the detector and no image information is lost.

The scroll bar allows access to all predefined colors.

By pressing the **upwards pointing open arrow**, the highlighted track will move one position up in the displayed track array (Fig. 79).

Likewise, by pressing the **downwards pointing open arrow**, it will move one position down. The arrows are only active, if an upward or downward movement is possible.

You can add up to 4 tracks by pressing the **+** button. You can delete tracks by highlighting them and pressing the **trash can** button.

You can load a predefined track configuration as a profile in the position of the highlighted track from the **Track Configuration** field (only available in the **Show all** mode) by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 80).



Fig. 78 Track LUT drop down menu

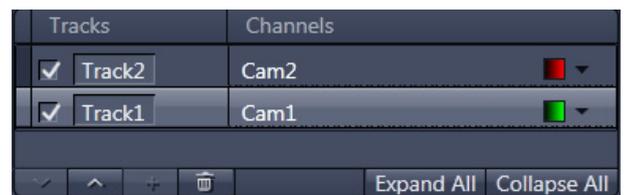


Fig. 79 Track selection display – Track 2 moved to the top, no further upward movement is possible

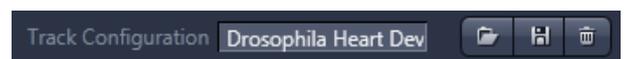


Fig. 80 Track configuration

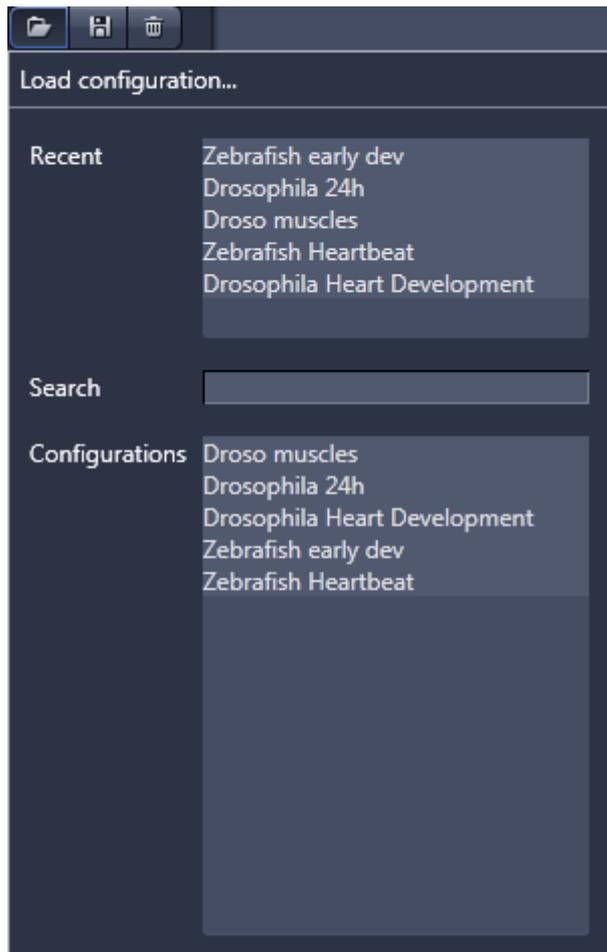


Fig. 81 Load configuration... panel

All saved track configurations will be listed in the **Load configuration...** panel (Fig. 81).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used track configurations or from the **Configurations** section, where all saved track configurations are displayed.

To search for a track configuration, type in its name or part of its name into the **Search** field and only those track configurations will be listed that contain the typed in string of letters.

To delete a track configuration press the **Delete** button (symbolized by the **trash can** icon). This opens the **Delete configuration...** window (Fig. 82).

Select the configuration from the **Configurations** list by highlighting it or search for it by typing its name into the **Search** field.

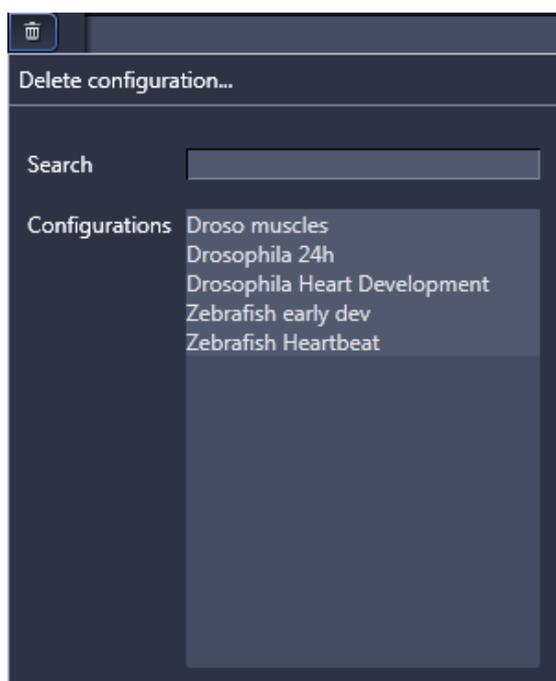


Fig. 82 Delete configuration... panel

The **Info** window pops up (Fig. 83).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a configuration press the **Save** button (symbolized by the **disc** icon).

This opens the **Save current Track configuration as** panel (Fig. 84).

Type in an appropriate name into the **Configuration** field and press **Ok** to save the configuration into the data base or press **Cancel** to abort the process.

The name of the track can only be changed when saving the track (Fig. 85).

If a highlighted track is saved with a certain name or a predefined track with a name is loaded into a highlighted track, the new name will appear in the **Tracks** designation box automatically.

Track designation in the **Light Path** tool will automatically be updated (Fig. 86).

The total set of parameters for one or multiple tracks can be stored as an acquisition configuration using the save option in the **Experiment Manager** field of the **Acquisition** panel.

Using multiple tracks is the preferred method to clearly separate weakly overlapping emission signals.



Fig. 83 Delete information window

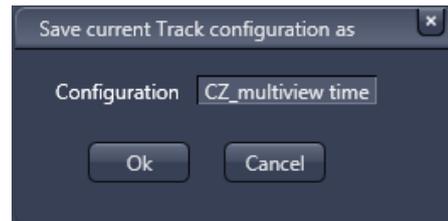


Fig. 84 Save current Track configuration as window

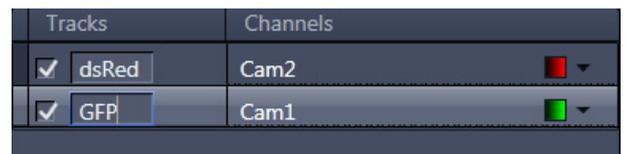


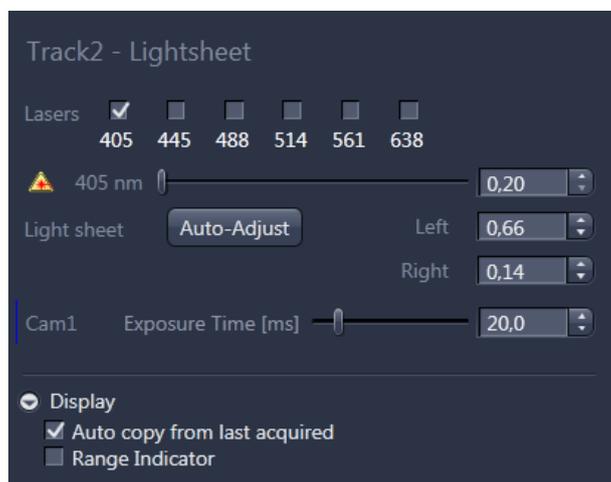
Fig. 85 Track selection display – Tracks renamed



Fig. 86 Light Path tool Tracks display– Tracks renamed updated from the Channel tool

### 3.2.8.2 Defining Detector and Illumination Settings

To define the detector and illumination settings, you can either highlight the track or channel in the **Track selection display** by clicking on the track or channel bar next to the track name or the colored square of the channel. This will display the detector and illumination settings for the marked track or channel. To open the detector and illumination settings for all tracks and channels, press the **Expand All** button; to close the display for all, press the **Collapse All** button.



**Fig. 87** Detector and Illumination settings display

Within the **Detector and Illumination settings** display (Fig. 87) the options for Illumination are given in the upper half and the options for the detection module are in the lower half.

You can activate a laser line by marking the according **Lasers** checkbox. For each chosen laser line, a slider with input box and arrows appear. Here you can set the laser power in percentage of total output.

With one or more activated laser lines, the tools to adjust the light sheet, the Light Sheet **Auto-Adjust** button, and the input boxes **Left** and **Right**, become visible. If **Single Side Illumination** is chosen in the **Acquisition Mode** tool window the respective input box is accessible while the other one is inactive. This way only the chosen illumination side can be changed.

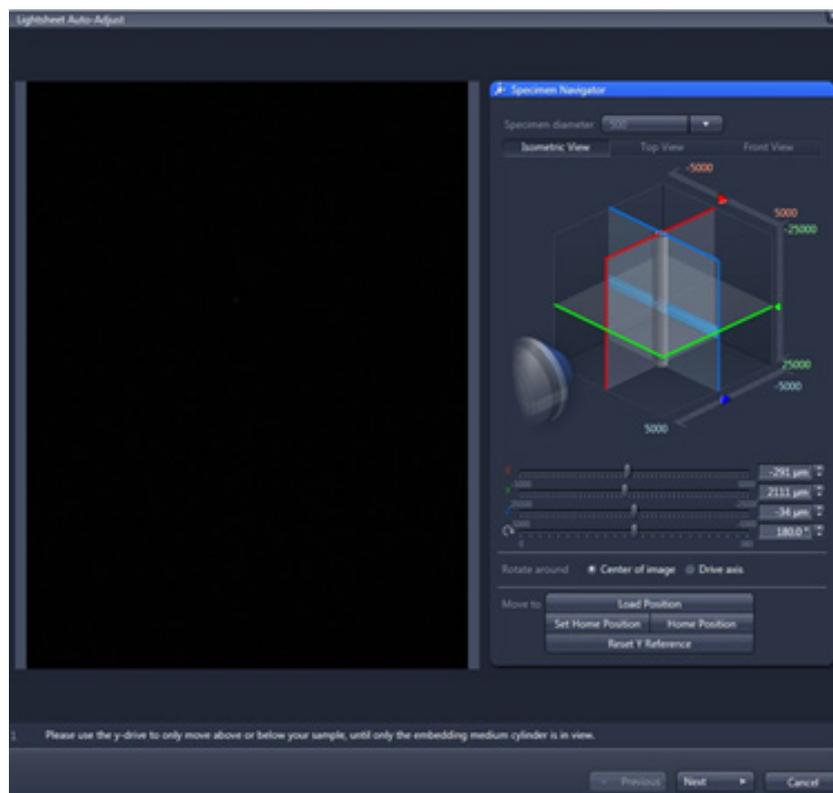
The numbers of the input boxes correspond to the Light sheet adjustment settings for the left and for the right illumination side. They can be changed manually. For proper adjustment, the Auto-Adjust wizard can be used. For each Track this alignment must be done separately.

The light sheet adjustment is needed to guarantee a slim light sheet with the liquid in the sample chamber and the embedding medium of the sample present in the beam path. The adjustment is always done for the laser with the lowest wavelength, when multiple lasers are used in the same track.

This adjustment should be performed right before the image acquisition or experiment is started. The sample should be in position and all other settings in the Light Path, Acquisition Mode, Channel Tool, etc. set. After the Light sheet Auto-Adjust procedure, the original position of the sample is restored and imaging can start without further modification. If the image quality is not as expected, it might be necessary to perform this correction again during an imaging session. The **Auto-Adjust** button leads you through a wizard-based step-by-step process.

 Be aware that Light sheet **Auto-Adjust** is done right before the acquisition starts. If you change the Zoom settings (e.g. during Multiview Quick Setup), you need to repeat the Light sheet **Auto-Adjust** process.

The initial window (Fig. 88) of the Light sheet **Auto-Adjust** wizard shows a live image of your sample on the left hand side. The initial beam path and illumination settings are taken from the Channels Tool and Light Path Tool, so no additional corrections are necessary. The **Specimen Navigator** Tool is present on the right hand side. Here, use the y-drive only to find a position below or above your specimen where only the embedding medium is visible. You can control this step by observing the live image. Only the y-drive should be used during this step, since the conditions for the light sheet adjustment should be as close to the imaging conditions as possible. Since the embedding medium is an optical element that will influence the formation of the light sheet, for its adjustment the position and thickness of the embedding medium should be the same as during imaging. That is easiest to achieve when only the y-position is changed. The original position of your specimen will be restored after the wizard is finished.

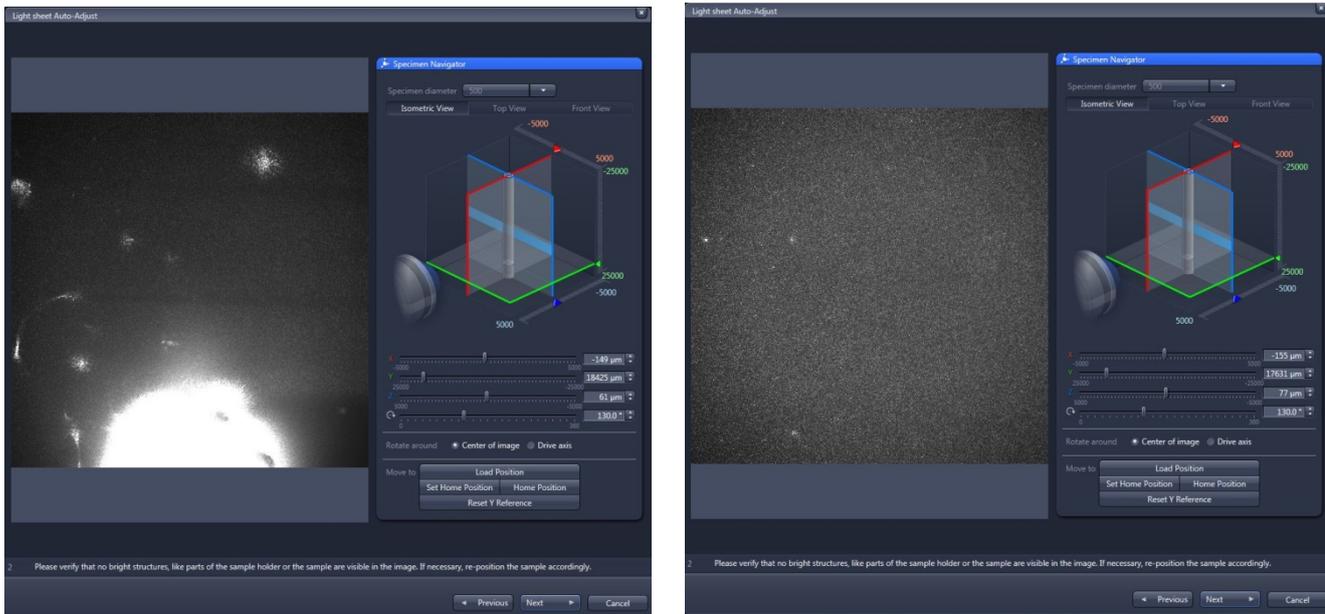


**Fig. 88** Light sheet Auto-Adjust first window

A short description on what to do during this step is given at the bottom of the window (Please use the y-drive to only move above or below your sample, until only the embedding medium cylinder is in view.). When done press the **Next** button. When you press **Cancel** you will leave the wizard without any changes.

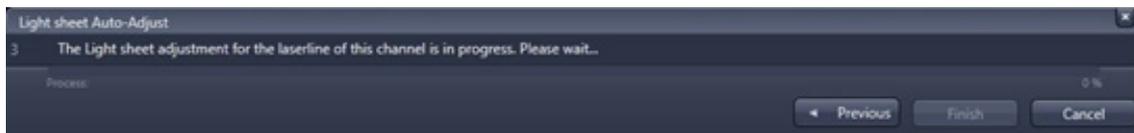
The second window of the **Auto-Adjust** wizard displays a live image of the sample and it describes which action is necessary during this step (Please verify that no bright structures, like parts of the sample holder or the sample are visible in the image. If necessary, re-position the sample accordingly.).

The light path has been changed automatically, removing the laser blocking filter from the beam path, so stray light from the activated laser can be seen. Hence any bright or blocking structures, like the sample holder or the sample, are visible. If this is the case (Fig. 89), the sample must be moved (optimally in the y direction), resulting in an evenly illuminated field of view. To proceed press the **Next** button.

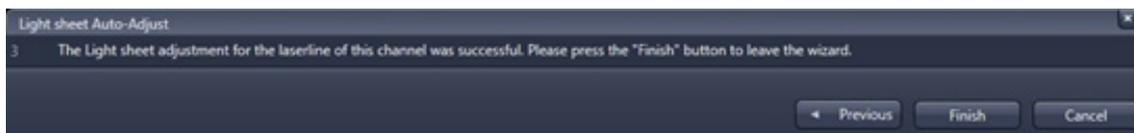


**Fig. 89** Light sheet Auto-Adjust second window, bright elements in the beam path (left), even illumination (right)

The window of the Auto-Adjust wizard is now minimized and a smaller control panel remains (Fig. 90). A progress bar shows the progress of the light sheet alignment. There is no interaction needed. A short text describes the process (The Light sheet alignment for the laserline of this channel is in progress. Please wait ...).



**Fig. 90** Light sheet Auto-Adjust procedure in progress



**Fig. 91** Light sheet Auto-Adjust procedure finished

When the light sheet adjustment is finished it is indicated with the displayed information (The Light sheet adjustment for the Laserline of this channel was successful. Please press the **Finish** button to leave the wizard.) and the **Finish** button (Fig. 91) can be pressed. The resulting settings are automatically written into the input boxes **Left** and **Right** (or one of them, if Single Side Illumination is chosen in the Acquisition Tool window).

The detection module controls are marked as Cam1 or Cam2 and are with a vertical line in the chosen channel color.

The **Exposure Time [ms]** is regulated with the slider, input box, and arrows (Fig. 87). The exposure times need to be synchronized with the scanning of the light sheet to ensure an even illumination of the whole image field. The software will automatically prevent sub-optimal exposure times and might change the chosen exposure time to the nearest suitable value.

For very short exposure times, below 10 ms, the image might display artifacts in some cases; these critical exposure times are displayed in yellow.

When a detection module with liquid cooling is used, the working temperature is displayed below the Exposure Time.

### 3.2.8.3 Defining Display Options

By pressing the black arrow on white background next to **Display** at the bottom part of the **Channel** Tool window, you access two checkboxes which influence the display of your images.

When **Auto copy from last acquired** is checked, the settings of the **Display View Option** control block (see section 4.2.2 **Display**) from the last taken image will be used directly for each newly acquired image.

With **Range indicator** activated, all images will be displayed with this look up table (LUT) regardless of the chosen color in the Track configuration display of the **Channel** Tool or in the **Light Path** Tool window (see section 3.2.5 **Setup Manager – Light Path**).

### 3.2.9 Acquisition Parameter – Incubator

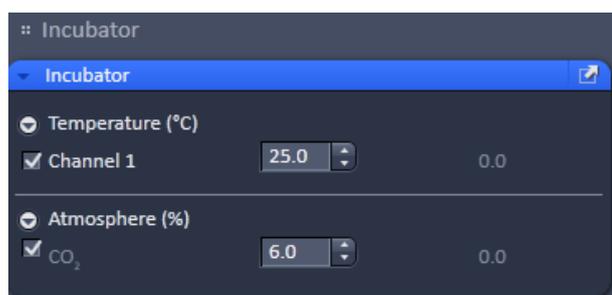
To have access to incubator functions for defining the atmosphere and temperature, expand the **Incubator** panel by pressing the triangle in the **Incubator tool** tab.

 All incubation components you wish to use during the imaging session must be attached to the Lightsheet Z.1 system before starting ZEN software. Once ZEN is running, you can detach and reattach components.

 Be aware that all incubation components will continue working with the **Incubator** tool window specified parameters after ZEN has been shut down or stopped working. This will keep your sample alive, even if ZEN stops unexpectedly during an experiment. To turn off the incubation, uncheck the corresponding checkboxes in the **Incubator** tool window or turn off the Incubation via the remote control.

You will have access depending upon the attached equipment to

- Temperature (°C) control
- Atmosphere (%) control



**Fig. 92** Incubator panel – Expanded fields for atmosphere and temperature control

To have access to the respective controls, expand the fields by pressing the triangles (Fig. 92).

The **Temperature (°C)** panel allows you to control up to 2 independent heating channels that are linked to certain devices (Peltierblock Sample Chamber Lightsheet Z.1, Heatingblock Sample Chamber Lightsheet Z.1) that are assigned to different channels. Enter the desired nominal temperature via the input boxes by typing in the numbers or by using the arrows to set the parameters. Check the corresponding box to activate the desired **Channel**. The grey numbers on the right will display the actual temperature.

The **Atmosphere (%)** panel defines the CO<sub>2</sub> concentration via the input boxes. Either type in the numbers or use the arrows to set the parameters. Actual values will be displayed on the right.

### 3.2.10 Multidimensional Acquisition – Z-Stack

The **Z-Stack** tool tab is only available if the **Z-Stack** checkbox is ticked in the **Multidimensional acquisition** field. If the tool bar is expanded, the **Z-Stack** control panel becomes available (Fig. 93). When **Start Experiment** is pressed, the system will perform a z-stack.

 Be aware that you might need a wider range for the z-stack to include enough fiducials or other landmarks for landmark alignment processing later.

With the z-stack function, a series of XY-images in different focus positions can be acquired resulting in a z-stack. In this way, a 3 dimensional data set from the specimen is obtained.

The way in which the z-stack is acquired can be selected by pressing either the **First/Last** or **Center** mode buttons. The modes differ in the way the first and last images of the stack are selected. The **Center** mode is only available in the **Show all** mode.

#### 3.2.10.1 Performing a Z-Stack Using First/Last Mode

- Press the **First/Last mode** button (Fig. 93) to open the **First/Last definition** panel.
- Press the **Continuous** action button and focus onto the specimen.
- While imaging, change the focus using the focus drive of the Ergo Drive control panel or the z-drive control within the **Specimen Navigator Tool tab**.
- When you reach the position within the specimen where you would like to start the stack, press **Set First** and this position will be taken as the first position in the stack.
- Move the focus in the other direction until the end position within the stack is reached, then press **Set Last**. These focus positions are then indicated next to **Set First** and **Set Last** buttons. The **Range** display box shows the total depth of the stack in micrometers.

The **Slices** input box displays the number of slices for the stack. This number can be edited by typing or using the arrow keys. Changing the value for **Slices** automatically adjusts the **Interval** in a way that the total **Range** for the stack is maintained.

The **Interval** input box displays the z-distance (interval) between slices in  $\mu\text{m}$ . This number can be edited by typing or using the arrow keys. Changing the value for the **Interval** automatically adjusts the **Number of Slices** while keeping the total **Range** for the stack within the possibilities of fitting the calculated number of slices within the Range. The total Range may therefore vary with higher and lower numbers around the **initial value of the Range**.

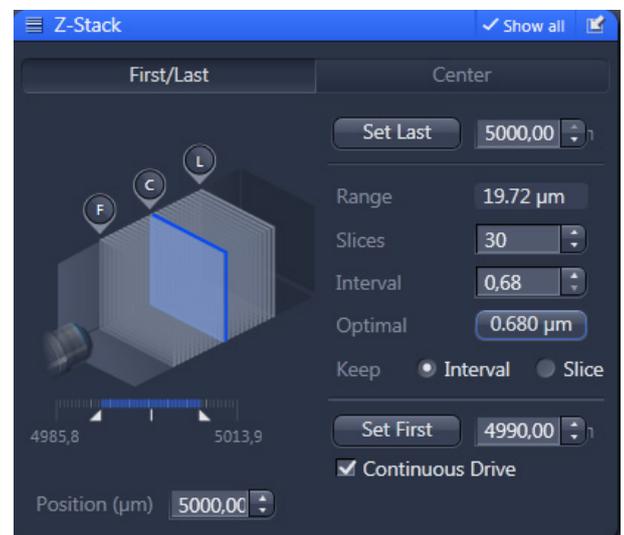


Fig. 93 Z-Stack Navigation appearance, performing a Z-Stack using First/Last Mode

**Pressing the Optimal button** sets **Interval** to a value of half the optical slice thickness. Two adjacent slices have therefore always a 50% overlap. The value in **Slices** is adjusted accordingly to keep the **Range** within the limits defined by **Set First** and **Set Last**. The optimal interval is displayed within the button in  $\mu\text{m}$ .

Within the **Keep** display field (only available in **Show all** mode) you have the option to switch between **Keep Interval** and **Keep Slice**. If **Keep Interval** is checked, the number of slices is adjusted to a new **Range** defined by pressing **Set First** or **Set Last**. Likewise, when **Keep Slice** is checked, the value for the **Interval** between the slices is adjusted to a new Range defined by pressing **Set First** or **Set Last**.

The **Position ( $\mu\text{m}$ )** input box indicates the current position of the focus. The number can be edited by using the arrow keys. The focus drive will automatically move to the new position.

When the **Continuous Drive** checkbox is ticked the z-stack is performed using a continuous movement of the z-drive. The stack size is retained. The z drive will move the distance of the optical slice thickness during one exposure time that is set in the **Channels tool tab**. However, the overlap of two adjacent frames resulting from the interval distance is kept in this mode.

When the **Continuous Drive** is deactivated, each image is taken after the z-drive has come to a stop. After the frame is imaged, the interval distance is travelled before stopping to take the next image frame of the z-stack.

Using the Continuous Drive will therefore speed up the acquisition of a z-stack immensely.

The **Z-Stack Navigation** field displays a graphical representation of the z-stack. The **white frames** represent the number and localization of the slices. The **blue frame** indicates the current focus position, also during image acquisition. By dragging the **blue frame** with the cursor, the current focus position changes accordingly. The **ruler** immediate below the graphical representation indicates the dimensions (range) of the z-stack.

The **F**, **C** and **L** buttons indicate the positions of the first slice, center slice, and last slice of the stack, respectively. If you press one of the buttons, an image will be acquired at the respective focus position

It is not relevant which of the chosen slices is assigned first and last position. The system will always move the sample away from the detection optics when acquiring the stack.

### 3.2.10.2 Performing a Z-Stack Using Center Mode

Press the **Center** mode button to open the **Center definition** panel (Fig. 94). This mode allows setting the Range of the z-stack in relation to the current **Focus Position** which is indicated as **Offset**.

It is useful when several z-stacks of the same range should be acquired at different positions within the specimen, as the whole range of the stack can be set to a different position at once using the **Offset** function.

The values for number of **Slices** and **Interval** are taken over from any settings done in **First/Last** mode. The **Offset** defines the **Center** position of the stack in relation to the **Focus Position**.

The **Slices** input box displays the number of slices of the stack. This number can be edited by typing or using the arrow keys. Changing the value for **Slices** leaves the **Interval** untouched, but the total **Range** is adjusted.

The **Interval** input box displays the z-distance (interval) between slices in  $\mu\text{m}$ . This number can be edited by typing or using the arrow keys. Changing the value for the **Interval** automatically adjusts the total **Range**. The **Slices** number will remain unchanged.

**Pressing the Optimal button** sets **Interval** to a value of half the optical slice thickness. Two adjacent slices have therefore always a 50% overlap. The optimal interval is displayed in the button in  $\mu\text{m}$ .

Within the **Keep** display field (only available in **Show all** mode) you have the option to switch between **Keep Interval** and **Keep Slice**. If **Keep Interval** is checked, the number of slices is adjusted to a new **Range** defined pressing **Set First** or **Set Last**. Likewise, when **Keep Slice** is checked, the value for the **Interval** between the slices is adjusted to a new Range defined by pressing **Set First** or **Set Last**.

The **Position ( $\mu\text{m}$ )** input box indicates the current position of the focus. The number can be edited by using the arrow keys. The focus drive will automatically move to the new position.

Pressing the **Center** button will define the current focus position (as defined in the **Position ( $\mu\text{m}$ )** input box) as the center position of the stack. After pressing **Center**, the whole range will move by the difference between of the previous center position and the new one. The number in the **Center** input box will then equal the number in the **Position ( $\mu\text{m}$ )** input box.

The **Offset input** box allows one to define an offset between the actual focus position as displayed in the **Position ( $\mu\text{m}$ )** input box and the current center position as displayed in the **Center** input box. One can edit the number by typing or using the arrow keys. When defining a specific **Offset**, the **Center** position will be recalculated as the actual position plus the offset. Accordingly the range of the z-stack is shifted to a different position in Z. Pressing the **C** button of the **Z Navigation** representation moves the actual **Focus Position** to the **Center** position and acquires an image at that position. A z-stack is then acquired at the new position when starting the experiment. Pressing **Center** moves the **Center** position back to the actual **Focus Position** setting the **Offset** again to zero.



Fig. 94 Z-Stack Navigation appearance, performing a Z-Stack using Center Mode

Use the **F**, **C** and **L** buttons to get images from the first slice, center slice and last slice of the stack. The **Focus Position** updates accordingly. The **Offset** value then might be set to half of the Interval depending on the interval value and the number of slices, as with a lower number of slices or a large interval the real center position of the stack might be just between two slices.

When the **Continuous Drive** checkbox is ticked the z-stack is performed using a continuous movement of the z-drive. The stack size is retained. The z drive will move the distance of the optical slice thickness during one exposure time that is set in the **Channels tool tab**. However, the overlap of two adjacent frames resulting from the interval distance is kept in this mode.

When the **Continuous Drive** is deactivated, each image is taken after the z-drive has come to a stop. After the frame is imaged interval distance is travelled before stopping to take the next image frame of the z-stack.

Using the Continuous Drive will therefore speed up acquisition of a z-stack immensely.

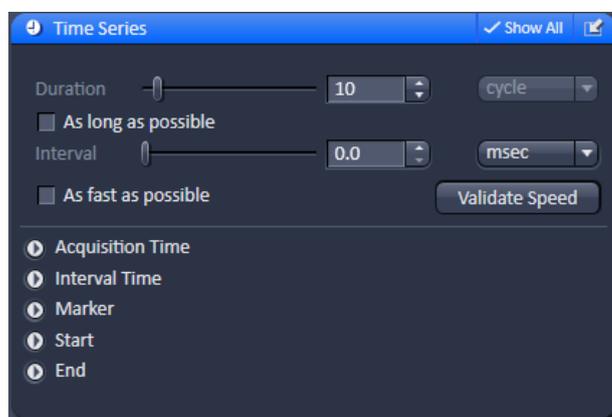


Fig. 95 Time Series tool

### 3.2.11 Multidimensional Acquisition – Time Series

When the **Time Series** tool tab is expanded, the **Time Series** control panel becomes available (Fig. 95). In the **Time Series** tool the parameters for the sequential acquisition of image frames as time series are defined.

When time series are acquired with the Lightsheet Z.1 a master file is created, with the following appearance:

Name.czi (e.g. FishEmbryogenesis.czi)

which is as well the first time point of the series. For each additional time point, a separate file is formed in the same folder with the nomenclature:

Name(time point-1).czi

(e.g. FishEmbryogenesis(221).czi would be the image taken at time point 222).

To set up a time series acquisition, first you define the **Duration** it should acquire images by using the slider or type into the text field, or use the arrow buttons. Next to these is a selection box in which you can choose the dimensions corresponding to the set **Duration**; either cycle, msec (milliseconds), sec (seconds), min (minutes), h (hours), d (days).

A maximum value of 100,000 can be set. The amount of data that can be handled by the system is limited. If the maximum amount is exceeded, a warning will appear. In this case, it is necessary to either change the destination for data storage and/or reduce the amount of cycles. The limit also depends on the imaging setup and the acquisition mode.

When the **As long as possible** checkbox is activated the system will continue taking images until the available storage is filled.

**Interval** defines a potential time interval, set in milliseconds (ms), seconds (sec) or minutes (min) by the **Interval** drop down menu, to separate the beginning of two cycles. A time shorter than the imaging time for a frame is not effective. The interval time can be changed by using the **Interval** slider or **Interval** input box, also while imaging.

The **As fast as possible** checkbox sets the **Interval** slider to 0,0.

To get information on how long the image acquisition of one time point will take, you can press the **Validate Speed** button. The system will simulate all actions of the time point specified in the experiment (tracks, exposure times, z-stack, multiview).

Hence the process will take approximately as long as it will take for one time point during the experiment itself, up to several minutes. You can follow the process by the displayed progress bar (Fig. 96) and **Cancel** if it takes too long.

After the **Validate Speed** process is finished, information is given on how long the whole experiment will last (Fig. 97).

If a number of cycles is chosen for the experiment, the text on the left reads: **The Experiment runs for ...h**; when a duration is chosen it shows **The Experiment runs for ... cycles**.

On the right hand side the duration for one time point is shown.

There are different advanced tools (only available in Show all mode) including

- Inter-Acquisition-Signal (Configuration dependant)
- Interval Time
- Marker
- Start
- End

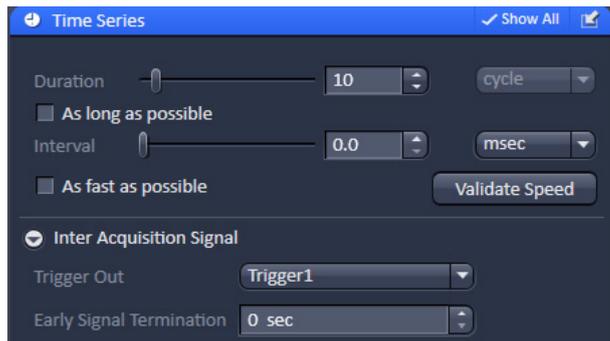
For the Lightsheet Z.1 system, only the Inter-Acquisition-Signal (Trigger) is supported; other Trigger In or Trigger Out signals are not available. All settings within the advanced tools: Interval Time, Marker, Start and End must be activated and changed manually.



**Fig. 96** Time Series tool – Validate Speed progress bar



**Fig. 97** Time Series tool – showing the experiment time



**Fig. 98** Time Series tool – Inter-Acquisition-Signal



**Fig. 99** Time Series tool – Inter-Acquisition Signal, Trigger Out drop down menu



**Fig. 100** Level Trigger – Trigger Out End Delay

### 3.2.11.1 Inter-Acquisition-Signal

The **Inter-Acquisition-Signal** function is configuration dependent. Only with the hardware present and the according information within the systems database does the **Inter-Acquisition-Signal** interface appear in the graphical interface of the software.

The **Inter-Acquisition-Signal** is a trigger signal with its level elevated while there is no image acquisition during the interval of a time series. An external device can be controlled to be either inactive (or active) during this acquisition pause.

The **Inter-Acquisition-Signal** is available for timer series of z-stacks and multiview experiments.

Expand the Interval field by clicking the triangle next to **Inter Acquisition Signal** (Fig. 98). To choose which Trigger out lines should be used for the experiment, open the drop down menu and select either **Trigger 1** or **Trigger 2** (Fig. 99). It is not possible to use both Trigger out lines in one experiment.

If the **Early Signal Termination** (Fig. 100) is set to 0, the level of the trigger is elevated during the complete acquisition pause of the interval time. The trigger level is lowered with the end of the interval and the start of image acquisition. To lower the trigger level earlier within the acquisition pause, use the **Early Signal Termination** selection box to define the time period (in seconds) the level is lowered before the start of a new image acquisition. The external device is therefore inactivated (or activated) before the interval of the time series is completed.

### 3.2.11.2 Interval Time

Expand the Interval field by clicking the triangle (Fig. 101). An **Interval Time** is a potential time interval between the end of one cycle and the beginning of the next cycle. The time and time unit for the interval time is set using the slider or input box of **Interval Time**.

A total of six different interval times can be defined by adding additional lines by clicking on the **+** button.

Clicking the **-** button deletes the currently highlighted interval time.

The interval time can be changed while imaging manually by highlighting the interval time in the list of the **Interval** field. A change of interval time will be indicated by a blue marker in the time series (see also section **3.2.11.3 Marker**).

You can load a predefined timer configuration from the **Interval Time** field by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 102).

All saved timer will be listed in the **Load configuration...** panel (Fig. 103). Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used timer configurations or from the **Configurations** section where all saved timer configurations are displayed. To search for a timer configuration, type in its name or part of its name into the **Search** field and only those timer configurations will be listed that contain the typed in string of letters.

To delete a timer configuration, press the **Delete** button (symbolized by the **trash can** icon). This opens the **Delete configuration...** window (Fig. 104).

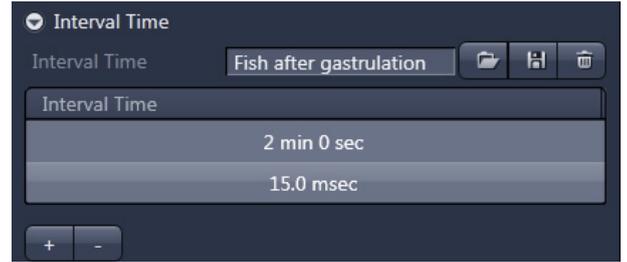


Fig. 101 Time Series tool – Interval Time

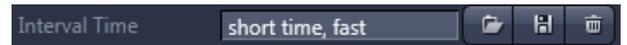


Fig. 102 Interval Time

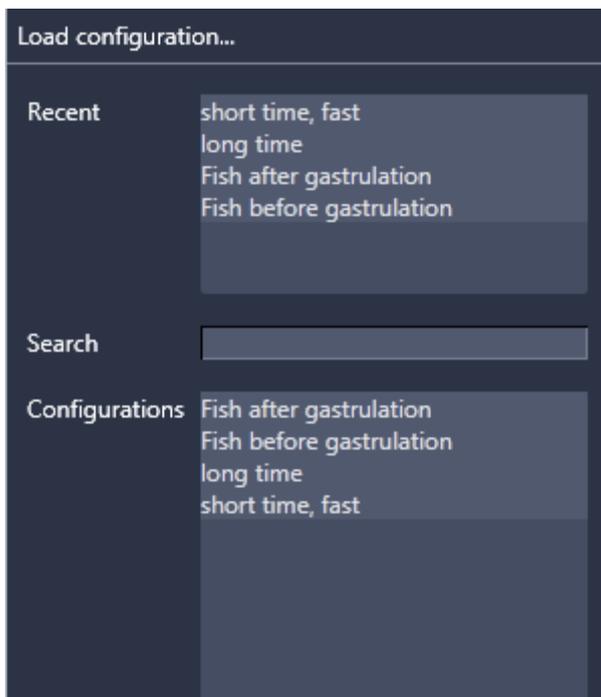


Fig. 103 Load configuration... panel

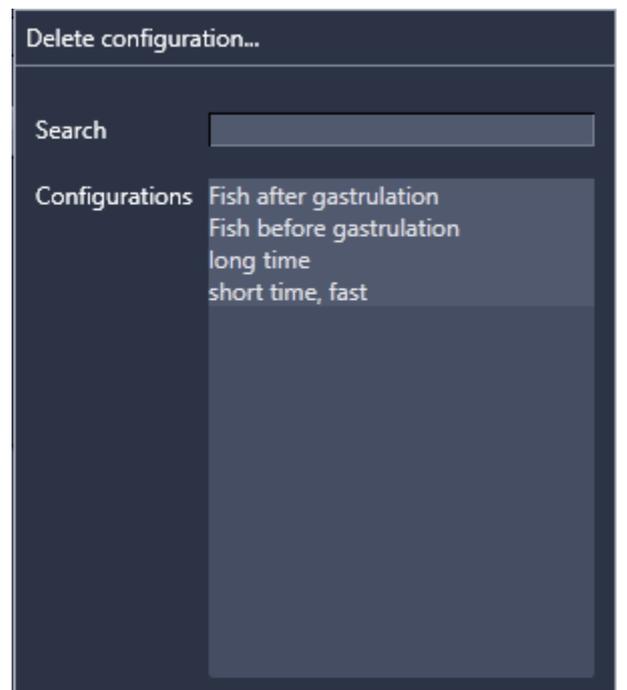


Fig. 104 Delete configuration... panel



Fig. 105 Delete information window

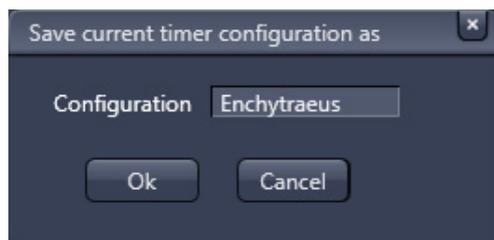


Fig. 106 Save current timer configuration as window



Fig. 107 Time Series tool – Marker

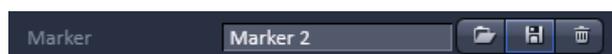


Fig. 108 Marker

Select the timer configuration from the **Configurations** list by highlighting it or search for it by typing its name into the **Search** field. The **Info** window pops up (Fig. 105).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a configuration press the **Save** button (symbolized by the **disc** icon). This opens the **Save current timer configuration as** panel (Fig. 106).

Type in an appropriate name into the **Configuration** field and press **Ok** to save the configuration into the data base or press **Cancel** to abort the process.

### 3.2.11.3 Marker

Expand the **Marker** field by clicking the triangle (Fig. 107).

The **Marker** functionality provides the possibility to indicate the start or end of an external process in the data of the image series.

You can edit a description of that marker in the **Description** box of the Marker which will be visible in the image data using the Mean ROI functionality or the image gallery display mode.

By clicking **Set** a marker is set manually at any time during image acquisition.

A maximum of seven different markers can be defined using the **+** button. The **-** button deletes the currently highlighted marker.

You can load a predefined marker configuration from the **Marker** field by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 108).

All saved marker configurations will be listed in the **Load configuration...** panel (Fig. 109).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used marker configurations or from the **Configurations** section where all saved marker configurations are displayed.

To search for a marker configuration, type in its name or part of its name into the **Search** field and only those marker configurations will be listed that contain the typed in string of letters.

To delete a marker configuration press the **Delete** button (symbolized by the **trash can** icon). This opens the **Delete configuration...** window (Fig. 110).

Select the marker configuration from the **Configurations** list by highlighting it or search for it by typing its name into the **Search** field.

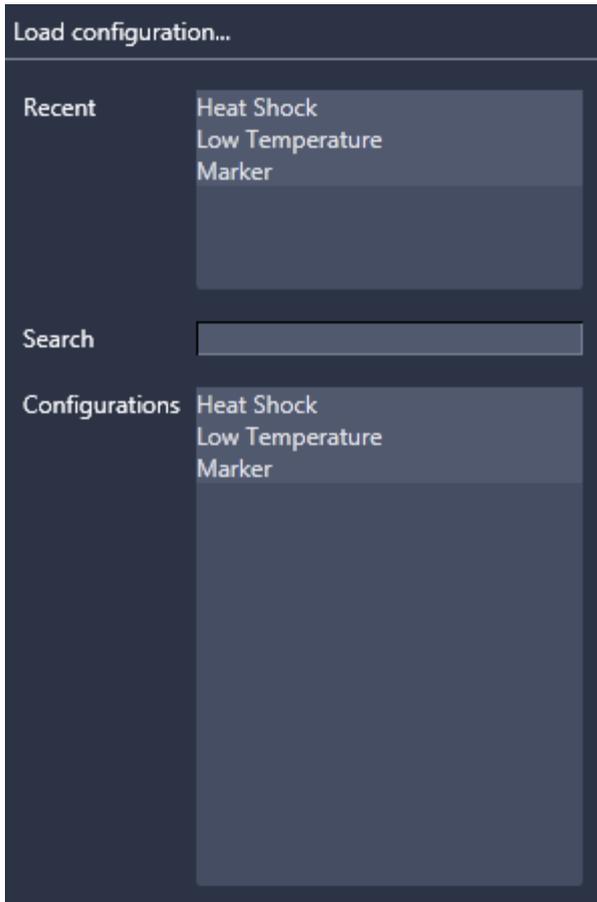


Fig. 109 Load configuration... panel

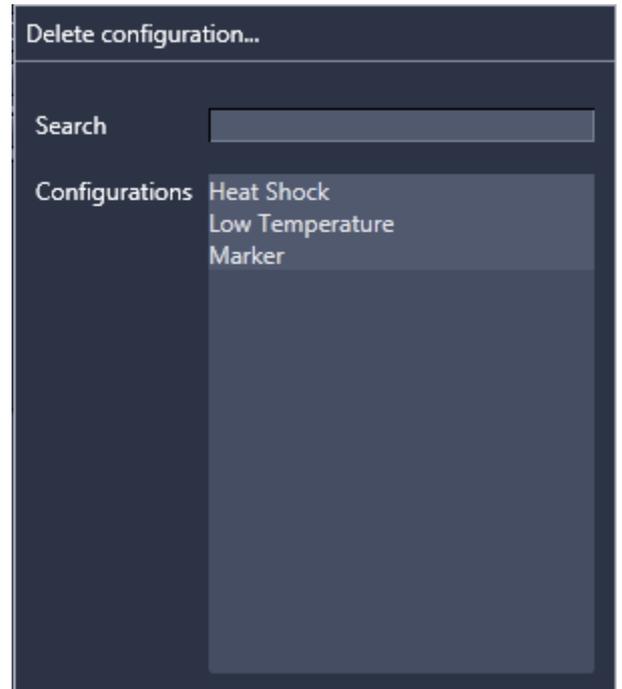


Fig. 110 Delete configuration... panel

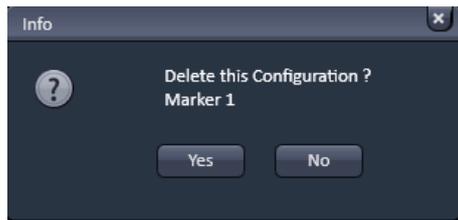


Fig. 111 Delete information window



Fig. 112 Save current marker configuration as window

The **Info** window pops up (Fig. 111).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a marker configuration press the **Save** button (symbolized by the **disc** icon).

This opens the **Save current marker configuration as** panel (Fig. 112).

Type in an appropriate name into the **Configuration** field and press **Ok** to save the configuration into the data base or press **Cancel** to abort the process.

When the image series is stored, all the markers, including the time indication and the comments, are stored along with the image contents.

The markers visible in the image series have different colors with the following meaning:

- **red**: manually set marker with time indication and comments
- **blue**: automatically set marker with change of interval

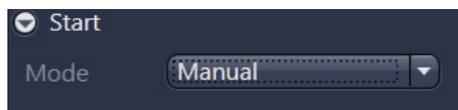


Fig. 113 Start field

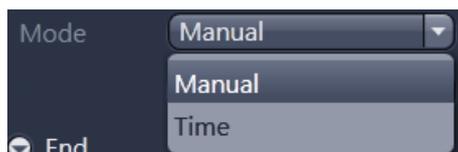


Fig. 114 Start Mode drop down menu



Fig. 115 Start Mode drop down menu – Time selected

#### 3.2.11.4 Start

Expand the **Start** field by clicking the triangle (Fig. 113).

In the **Mode** drop down menu (Fig. 114) you can select the way the acquisition is triggered. You have the choice between **Manual** and **Time**.

If **Manual** is selected, the acquisition of a time series is started using the **Start Experiment** action button.

If **Time** is selected the **hh (hour)**, **min (minute)** and **sec (second)** input boxes become available (Fig. 115), in which the hours, minutes and seconds can be edited. After pressing **Start Experiment** button, the system will display the message **Waiting for Start Time** and starts acquisition after the defined computer time has been reached.

### 3.2.11.5 End

Expand the **End** field by clicking the triangle (Fig. 116).

In the **Mode** drop down menu (Fig. 117), you can select the way the acquisition is triggered. You have the choice between Manual and Time.

If **Manual** is selected the acquisition of a time series ends using the **Stop button** or after the defined number of cycles.

If **Time** is selected the **hh** (hour), **min** (minute) and **sec** (second) input boxes become available (Fig. 118), in which the hours, minutes and seconds can be edited. The system interrupts the acquisition after the defined computer time has been reached.



Fig. 116 End field

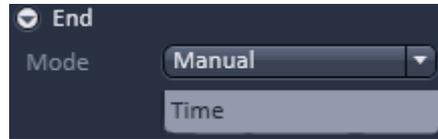


Fig. 117 End Mode drop down menu



Fig. 118 End Mode drop down menu – Time selected

-  A Time Series is finished when the end Time is reached. If the entered number of Cycles has been processed, the Time Series is finished before the end Time. If the number of cycles has not yet been processed until the end Time, the Time Series is also finished. The end time for the Time Series can be changed online.
-  If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interruption of the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.

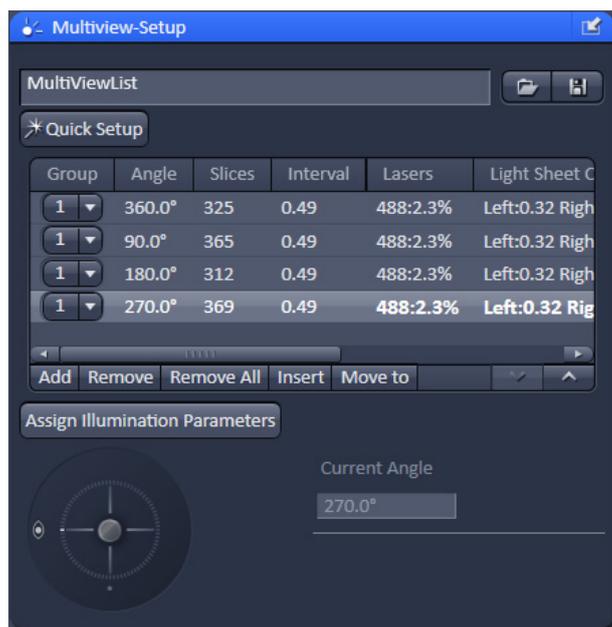


Fig. 119 Multiview-Setup panel

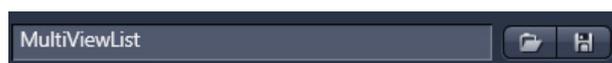


Fig. 120 Multiview Setup, MultiViewList organisation panel

### 3.2.12 Multidimensional Acquisition – Multiview-Setup

The **Multiview-Setup** tool tab is only available, if the **Multiview** checkbox is ticked in the **Multidimensional acquisition** field. The **Z-Stack** checkbox will be automatically activated along with the **Multidimensional acquisition** field and greyed out since every Multiview setup requires a z-stack. Multiview experiments can be combined with a time series by activating the **Time Series** checkbox within the **Multidimensional acquisition** field. When the **Multiview-Setup** tool tab is expanded the **Multiview-Setup** control panel becomes available.

With the **Multiview tool** it is possible to define z-stack acquisition from multiple viewing angles; acquiring information covering up to 360° degrees around the y-axis of the sample. A series of such views, with adjacent viewing angles and adequate overlap can be used in **Lightsheet Processing** (see also section **3.3.18 Processing – Lightsheet Processing**) to create a fused 3D dataset of the sample.

If more than one sample or position should be imaged from multiple angles during one experiment, it is possible to assign views to individual **Groups** (Fig. 119), the group index is shown in the first column of the Multiview list. Each group of views is saved as one individual masterfile with associated files (default setting for separation: time points) hence each group can be used individually for **Lightsheet Processing**.

The file name for multiview experiments are labelled with the **Group index** as following: Filename\_Gx.czi with "x" being the group index.

The **Multiview-Setup** tool tab is divided into three regions (Fig. 119). The upper region is the organization region and the access to the **Quick Setup** wizard. In the middle you find a list of created Views and their control buttons.

In the lower part of the tool tab is an illustration of the angle from which the detection optics (eye symbol) is facing the sample (middle cylinder). The zero angle is indicated by the dot at the bottom of the outer circle. To easily get an overview of the assigned angles, this graphic shows a stable sample and translates the relative movement of the detection optics, whilst in the system the sample is moved and the detection optics remains in place.

Next to this graphical display is the **Current Angle** shown.

You can load a predefined MultiviewList (Fig. 120) by pressing the **Load** button (symbolized by the **folder** icon). This opens a Windows explorer window in which you can open the folder and select the MultiviewList file (.mvl) to open. Its name will appear in the input box.

To save a list of views, press the save button (symbolized by the disc). This opens a Windows explorer window. Here you can choose a folder and name for the Multiview List file (.mvl).

A **View** that shall be used for image acquisition is defined by its group, angle, x- and y-position; additionally for each view a z-stack with its interval and number of slices must be defined. The information and settings of a **view** are shown in the multiview list table:

**Group:** group of views that are acquired at one y-position and can be registered and fused with Lightsheet Processing. Each group is saved as an individual master file with associated files (default setting for separation: time points).

**Angle:** angle position of the specimen in regards to the detection optics.

**Range:** first and last z-position of the defined z-stack for that view.

**Slices:** Number of z-slices of the z-stack defined for that view.

**Interval:** Interval used in the z-stack defined for that view. One group of views that will be used for Lightsheet Processing must share the same interval.

**Position:** displays the motor coordinates defining x, y, and z to position the sample (x,y,z). The values for x and y will change when the sample is moved in x, y, or rotated (only if **Center of Image** is activated) with the controls in the **Specimen Navigator** tool tab, or for x and y with the Ergo Drive control panel. Values for z can only be influenced by the z-stack defined within the **Z-Stack tool window** for that view.

Furthermore specific settings for a **group** is as well displayed in the multiview list table:

**Laser:** shows which laser power is set for the group of views (either in the **Light Path** tool window or in the **Channels** tool window). It is not possible to select different lasers for different groups; only the laser power of activated lasers can be defined group specific.

**Light Sheet Offset:** for each group of views the light sheet offset can be adjusted. This is done in the **Channels** tool window, using either the manual settings or the **Auto-Adjust** wizard.

Views and groups can be created either manually or using the **Quick Setup** wizard.

When adding views manually to the multiview list, the settings specific for a view must be defined before the view is added.

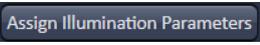
-  Settings specific for **groups** (e.g. laser power) will be taken from the last added view of one group. This last entry is displayed in bold lettering for identification.
-  **Laser power** and **Lights Sheet Offset** can be changed for the whole group by selecting one view of the group; changing the settings accordingly in the **Channels** tool window and pressing the **Assign Illumination Parameters** button (Fig. 119, ).
-  All imaging settings, that are not listed in the multiview list table are valid for the complete experiment and are not specifically bound to a view or group. Hence when settings (e.g. light path settings, activated laser lines, zoom, illumination side, pivot scan, continuous drive of the z-stack) are altered, these changes will be applied to the complete list of views.



Fig. 121 Generating a new group



Fig. 122 Choosing group index

**A) Creating a multiview list manually**

To add a view, place the sample using the **Specimen Navigator** tool tab or the Ergo Drive control panel hardware, define the position and z-stack within the **Z-Stack tool window** and then press the **Add** button. The view is always added at the very end of the list.

The first view of the list will automatically be sorted into Group 1. Any additional view added will be joined to the group index of the last entry in the list.

After a view is added to the list, it can be defined as a new **Group** by opening the drop down menu in the table (Fig. 121) and choosing **new**. Once a new group index is created it will be listed in the drop down menu (Fig. 122).

By pressing **Remove**, the highlighted view will be deleted. Pressing **Remove All** will delete, after a short information about this, all views in the list.

You can insert a view within the list by pressing the **Insert** button; the new view will be placed above the highlighted view entry. The newly generated view will automatically get the group index of the highlighted view entry. The group index can be changed by opening the drop down menu within Multiview table and selecting the desired group index or creating a new one (Fig. 121 and Fig. 122).

Pressing the **Move to button** will bring the specimen holder back to the highlighted view.

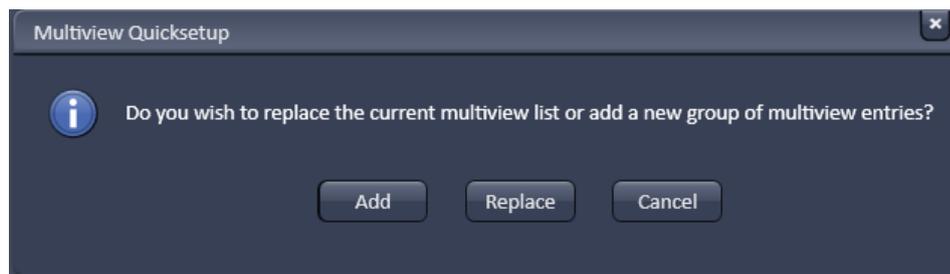
You can move a view within the list by marking it and pressing the **arrow buttons up or down** until the view has reached the wanted position within the view list.

 Be aware to change the group index accordingly if necessary.

## **B) Creating a multiview list using the Quick Setup wizard**

The **Quick Setup** button opens a new window with a software interface that guides you through the process of setting up a multiview experiment; including the positioning of the sample, defining the necessary z-stacks (Range, Slices and Interval), the zoom, and the number of views.

If the Quick Setup is used with views are already present in the multiview list, the new entries will be either added to the list as a new group with the corresponding group index, or they can replace the previous entries (Fig. 123). Be aware that the **Quick Setup** is a tool to facilitate the creation of a multiview experiment but the resulting z-stacks might have a wider range than absolutely necessary to image the specimen. If data size is an issue in your experiment, or time considerations, it can be more efficient to manually setup the multiview experiment with tightly fitting z-stacks.



**Fig. 123** Multiview Quick Setup information window on replacing of adding views

When the **Quick Setup** tool window opens, the optical zoom will be changed to half the value (if possible), so that the field of view of the specimen within the **Quick Setup** tool window is enlarged.

The Multiview **Quick Setup** tool window is divided into four sections. On the right hand side are the **Image settings** tool tab and the **Specimen Navigator** tool tab (see section 3.1.3 **Specimen Navigator Tool**).

The **Image settings** tool grants easy access to the **Light Intensity** slider for adjusting the brightness of the infrared illumination.

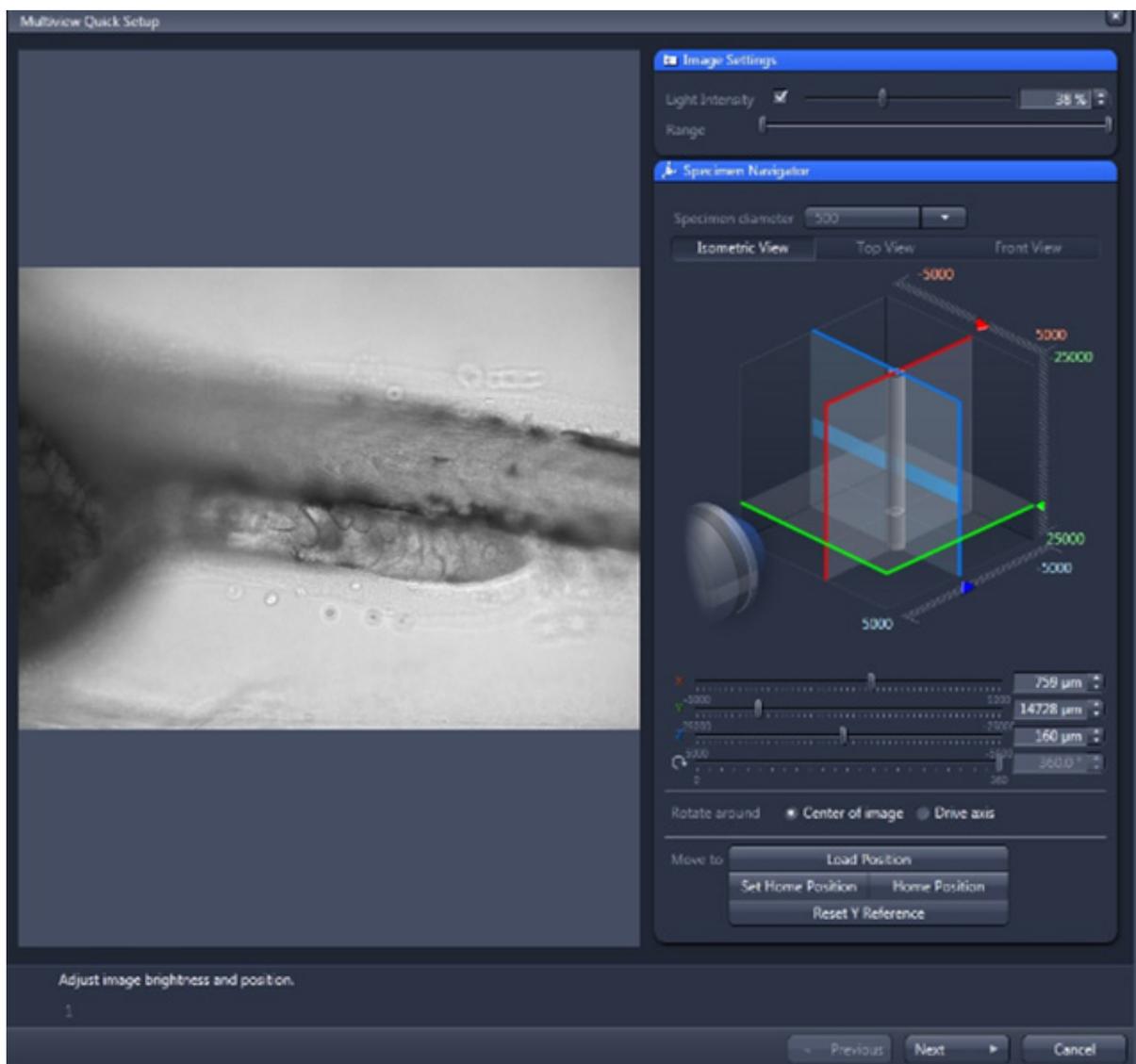
With the **Range** slider you can control which part of the dynamic range should be used by moving the restriction sliders on both sides. The **Range** slider represents simplified low grey values on the left side and high grey values on the right side. On the left hand side, the resulting live image is displayed.

The middle section gives information on the current step in the process (numbered 1 to 4) and describes which task needs to be done by the user. The lower section holds the buttons **Previous** to go one step back, **Next** to proceed to the next step and **Cancel** to end the process without result.

The **Previous** button is inactive in the first step and the **Next** button is replaced by the **Finish** button in the last step.

**Step 1: Adjust image brightness and position**

Position your sample with the **Specimen Navigator** tool tab (Fig. 124). The middle of the sample should be in focus. Use within the **Image Settings** tool tab the **Light Intensity** slider or input box with arrows and the **Range** sliders to adjust the brightness of the live image on the left hand side.



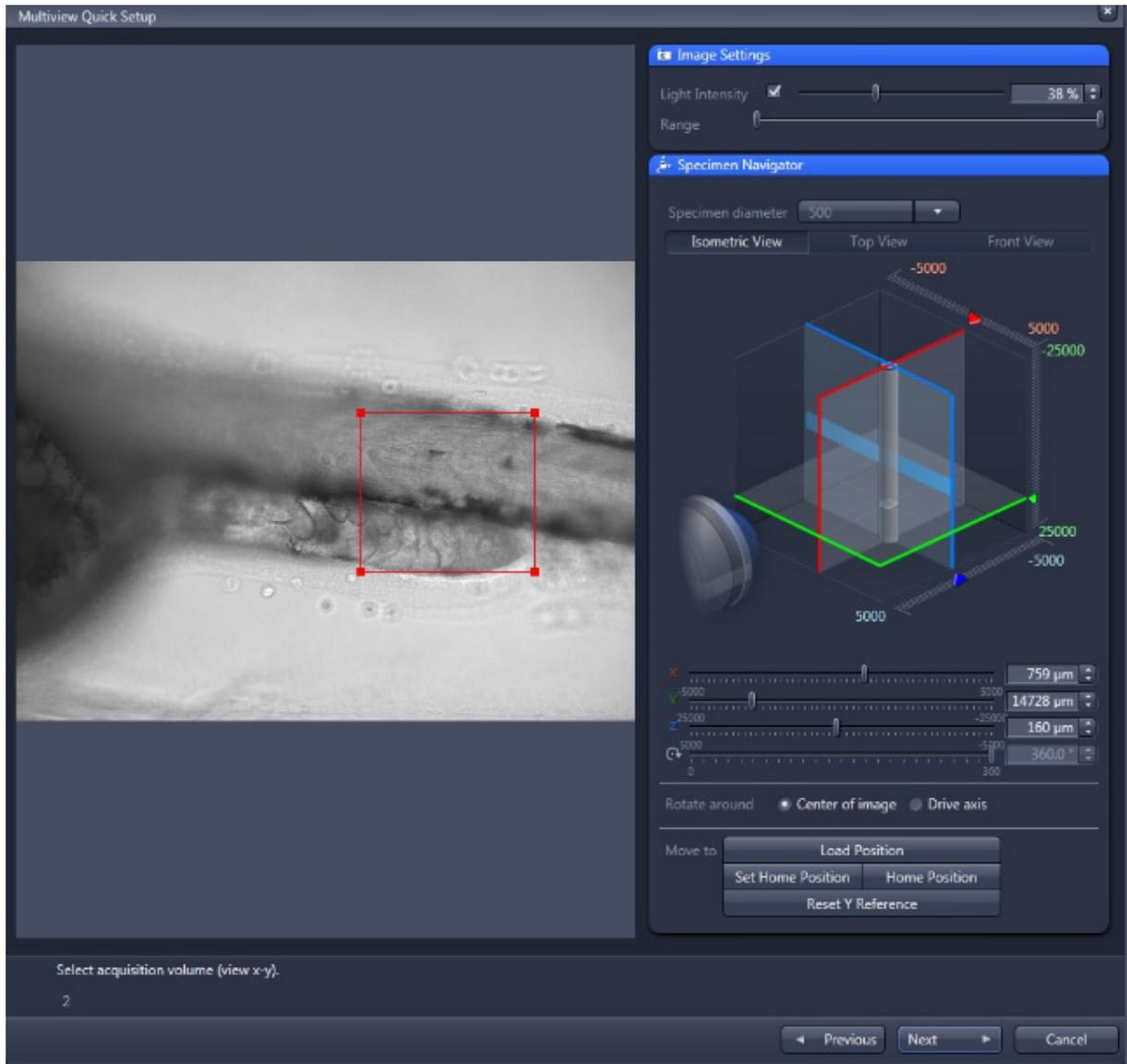
**Fig. 124** Multiview-Quick Setup, Step 1

**Step 2: Select the acquisition volume (view x-y)**

A red rectangle is displayed in the live image window (Fig. 125). The red rectangle must be adjusted to surround the sample completely. To move the whole rectangle, grab it by holding the left mouse button on one of the sides and dragging it to the wanted position. To change its size, grab it with the left mouse button on one of the corners and adjust the length of the adjacent sides.

 Check by moving the z-drive if the red rectangle encircles the whole area of interest along its z-axis.

After pressing the **Next** button, the sample will be turned by 90° before proceeding to the next step.



**Fig. 125** Multiview Quick Setup, Step 2

**Step 3: Re-focus and select the acquisition volume (view z-y)**

Adjust the focus for the sample with the **Specimen Navigator** tool tab or the Ergo Drive control panel (Fig. 126). It might be necessary to reposition the specimen with the x- and y-drive as well. Do not change the angle at this step. As in Step 2, the red rectangle must surround the region of interest, again adjust position and size of the rectangle.

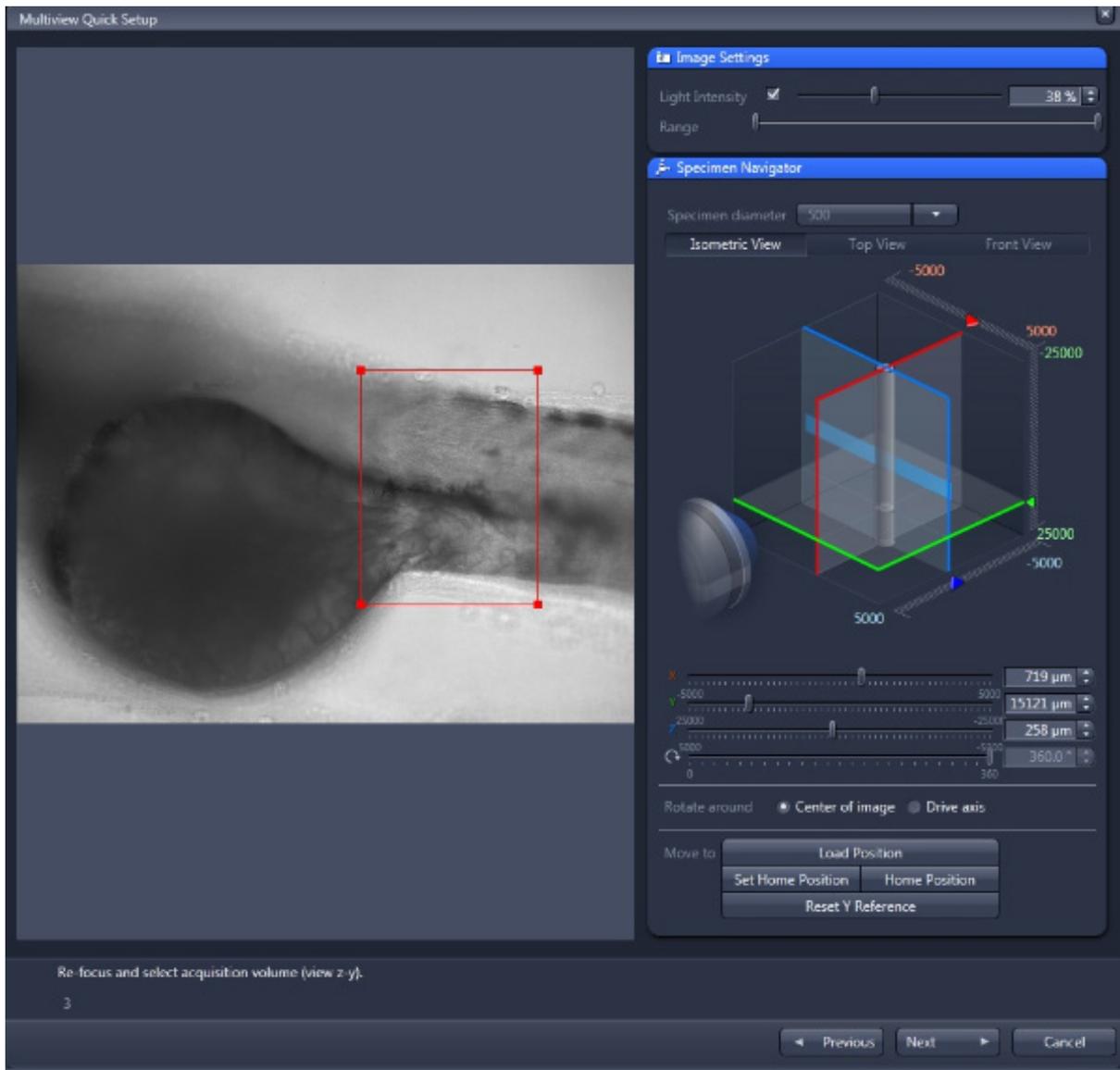


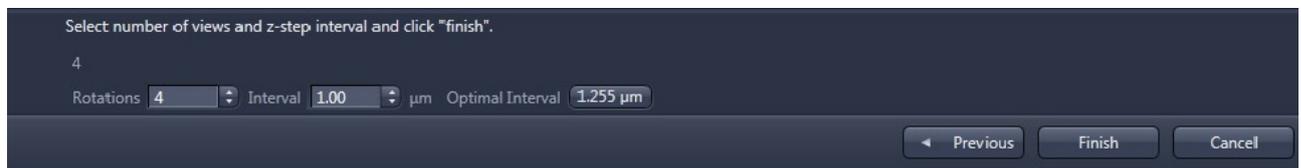
Fig. 126 Multiview Quick Setup, Step 3

**Step 4: Select number of views and z-step interval and click "finish"**

In this interface you define the number of views by entering the number of **Rotations** within the input box or using its arrows (Fig. 127). This will lead to the following angles:

- Rotations: 2 → 0° angle and 90° angle
- Rotations: 3 → 0° angle, 90° angle and 180° angle
- Rotations: > 3 → a full 360° rotation is evenly divided by the number of rotations.

You can set the **Interval** for the z-stacks either by typing a value into the box or using its arrow buttons, or use the **Optimal Interval** button (see as well Z-Stack tool tab section **3.2.10 Multidimensional Acquisition – Z-Stack**). Afterwards, press the **Finish** button to complete the **Quick Setup**; the window will close and the resulting views are displayed in the **Multiview Setup** tool tab multiview list.



**Fig. 127** Multiview Quick Setup, Step 4

-  Be aware that the **Zoom** setting will change when the volume, defined in step 2 and 3 of the Multiview Quick Setup, requires a larger field of view than with the **Zoom** set in the **Acquisition** tool window. The Multiview Quick Setup uses the higher value of either the largest possible diagonal through the volume or the length of the y-axis as a reference to determine the needed field of view, hence the Zoom. When the **Quick Setup** is used several times to create several groups, the smallest zoom value for all groups will be used for the experiment.
-  When the **Zoom** setting is changed during the Multiview Quick Setup, the alignment of the light sheet must be checked and adapted. Following the rule, the light sheet adjustment is the last step before image acquisition. To assign different light sheet offset settings to the individual groups, select one view of the group by clicking onto it and highlight. Then use the spin box within the **Channel** tool window to adjust the light sheet alignment. Please see as well section **3.2.8 Acquisition Parameter – Channel** for light sheet adjustment and the **Light Sheet Auto-Adjust** routine. Afterwards press the **Assign Illumination Parameters** button (Fig. 119) to use these settings for the whole group. Repeat the process for every generated group.

To **start the experiment** after all views and groups are created, press the **Start Experiment** button in the **Multidimensional acquisition** field. Before acquisition starts, the groups are sorted automatically, to prevent a mixture of group entries during acquisition.

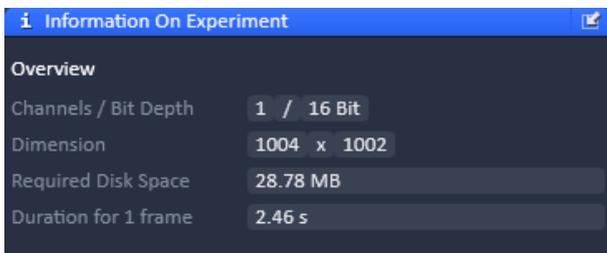
If the angles of one group are not already sorted, an information window is providing the option to sort the angles of each group to ensure that they are acquired in an ascending sequence (Fig. 128). It is crucial to acquire adjoining angles, to use the multiview experiment for registration and fusion of views with **Lightsheet Processing**.



**Fig. 128** Multiview information window to confirm or decline sorting of angles

If different groups were created, all entries of one group are saved in an individual file. In combination with a **Time Series**, each group is saved as a master file and its associated files, one for each time point. One group, hence one of the master files, can be chosen for **Online Multiview Processing**.

 Note, when you create multiple z-stacks with the Multiview-setup tool, they might have different numbers of slices. In the **Dimension View** tab, the maximum number of slices present in the dataset will be displayed, even if a view with fewer slices is chosen.



**Fig. 129** Information on Experiment tool tab

### 3.2.13 Multidimensional Acquisition – Information on Experiment

If the **Information on Experiment** tool tab is expanded, the Information on experiment panel becomes available (Fig. 129). This tool displays the information on the current experiment in terms of

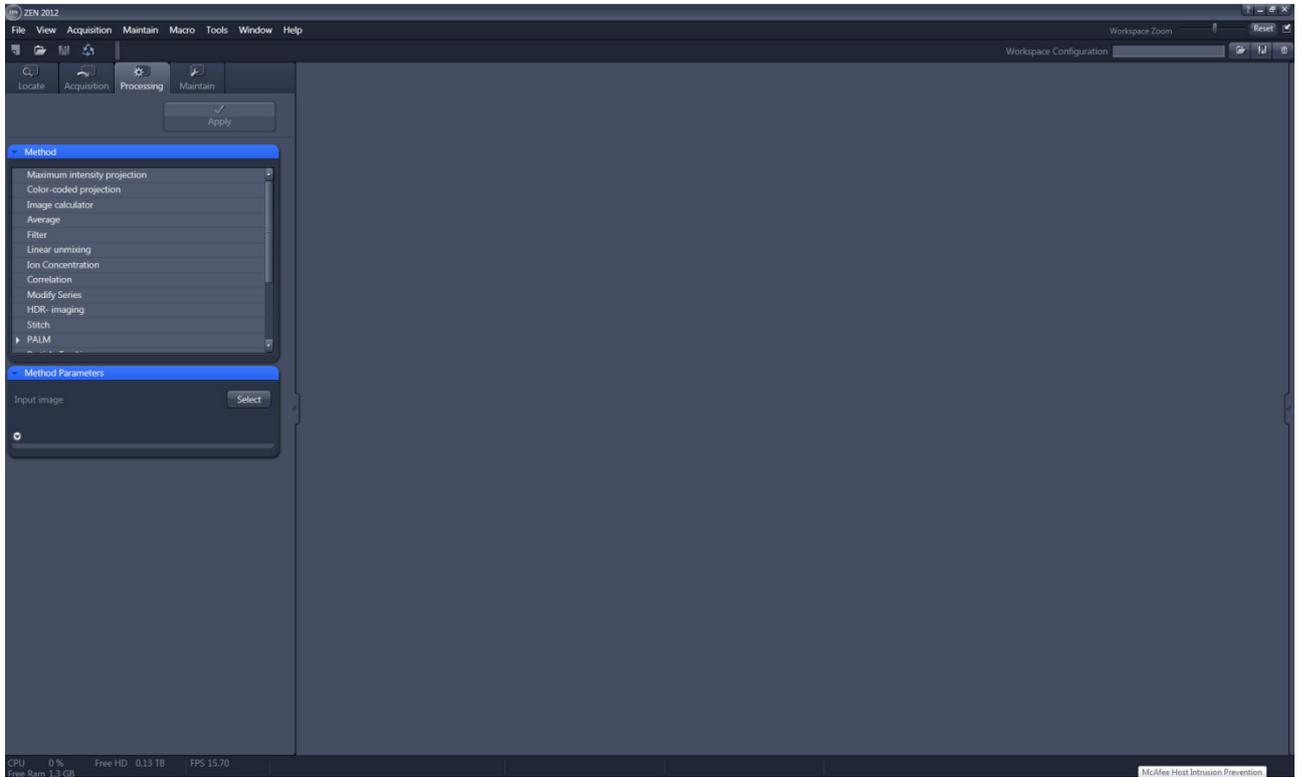
- Channels / Bit Depth
- Dimension
- Required Disk Space
- Duration for 1 frame

Any changes in the settings will be automatically updated in regard to the displayed parameters.

### 3.3 Processing Tab

#### 3.3.1 General Structure of the Processing Tab

Click on the **Processing** tab to have access to the processing functions. The list of available Processing tool will be displayed (Fig. 130). The scrollbar must be used to have access to all functions.



**Fig. 130 Processing tool**

The **Processing** tool provides a number of methods for image processing and analysis using mathematical operators and algorithms, which include:

– Maximum Intensity projection	– Stitch
– Color-coded projection	– PALM
– Image calculator	– Particle Tracking
– Average	– Experimental PSF
– Filter	– Structured Illumination
– Linear Unmixing	– Channel Alignment
– Ion Concentration	– Lightsheet Processing
– Correlation	– Copy
– Modify Series	– ICS
– HDR-imaging	– Adjust

-  All processing tools can be used with stored and reloaded images or with newly acquired images (that need not be stored before processing).
-  Please note that for PALM images, although stored in a .czi format, only the PALM and Channel Alignment Processing tools are available, which can recognize tables. If you want to apply other Processing tools, the PALM image based on the table has to be converted to a true image. Such a function is available as a sub-function in the PALM Processing tool. Calculated SIM images, on the other hand, have the same format as normal wide field images.
-  Note that all .lsm and .czi images can be loaded into a function, but in order for that function to make sense, the .lsm or .czi image format must have the right dimensions.

The **Processing** tool tab (Fig. 131) is structured into different fields.

The top field or header (just beneath the **Main tool tab** area) contains the **Apply** button. The button is only active when an image is selected. If the **Apply** button is pressed, the selected or active processing tool operations will be applied to that image.

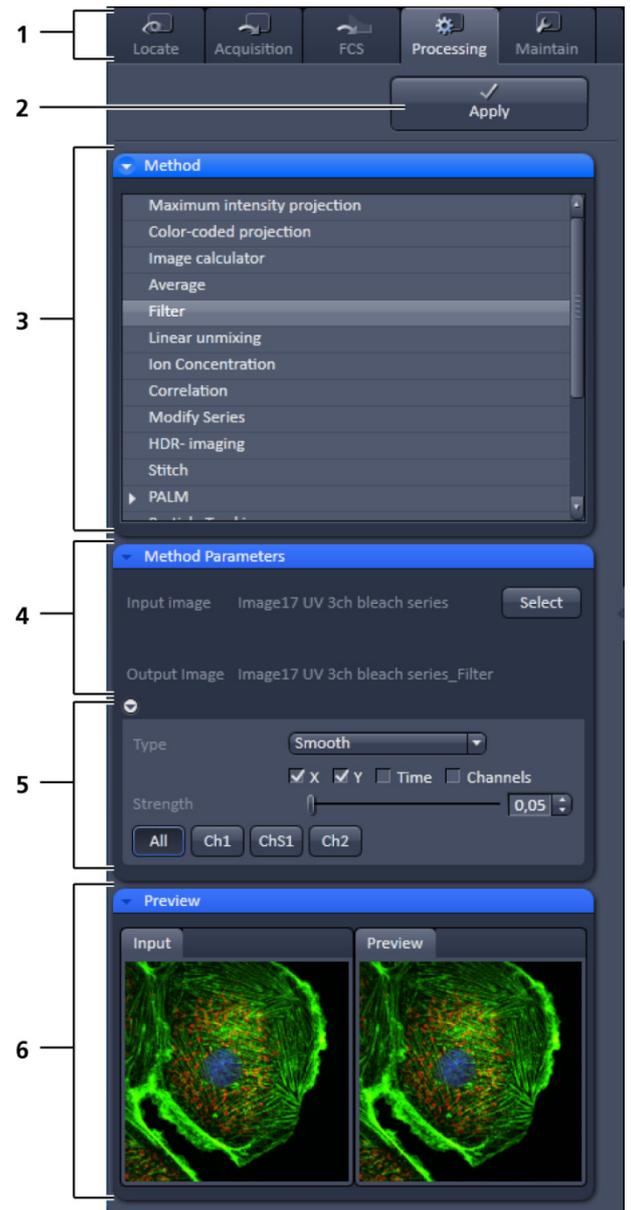
The next field contains the list of **Processing** tools. Select the desired tool by clicking on it. The active tool will be highlighted.

 Next is the image selection field. Highlight the image in the image container that you want to select. Load the **Input image** by pressing the **Select** button. **Select** will load the active image in the container. Alternatively, first press **Select** and then click on the image in the image container. With some image processing tools, two images can be loaded. Then select the first image to be loaded in the image container and press the **Select** button for **Input image**. Then select the second image to be loaded in the image container and press the **Select** button for **Input image 2**.

 Note that once an image is loaded by pressing **Select**, it cannot be unloaded but only be replaced by another one. This holds also true if the Processing tool is changed; the previous loaded image will stay selected. If another image is to be loaded, just highlight that image in the image container and press **Select** again. The newly selected image will replace the old one.

 If two input images are selected (**Input image** and **Input image 2**), they have to match each other in their dimensions. If they do not match, a warning is given. Acknowledge the warning to continue with the processing. The message will also be displayed if you load a new image pair that does not match in dimensions to the old pair. Since you can only load one image at a time, this will lead to a mismatch between the old **Input Image 2** and the new **Input Image**. Acknowledge the warning to continue loading the second new image.

 It is the responsibility of the user to check if an operation is suited for an image.



- 1 Main tool tabs
- 2 Apply button
- 3 Method panel with the available processing methods
- 4 Method Parameters panel with the input image and output image selection field
- 5 Method Parameters panel with with the method-specific settings
- 6 Preview panel with input and preview thumbnails

**Fig. 131 Processing tool structure**

Once an image is selected by pressing the **Select** button, the **Output Image** name field with the name of the processed image will appear. The name can be later changed when the processed image is saved.

The next field is the method specific **Settings** panel that can be expanded / collapsed by pressing the triangle repeatedly. The triangle only appears when a processing tool is highlighted. It displays all the operational options for a specific processing function, if applicable, for the loaded image. Note that some **Processing** functions do not contain additional options and the field remains empty. Only those functions will be displayed that match the dimensions of the image parameters and that can be applied to the loaded image.

Once an **Input image** is selected, the **Preview** panel becomes available at the bottom field. It shows the **Input image** (or **Input Image** and **Input Image 2**, which can be selected by pressing the respective thumbnail) on the left and the **Preview** image on the right. In addition, a new View tab – the **Preview** tab – is opened for the image in the **Center Screen Area** (Fig. 132).

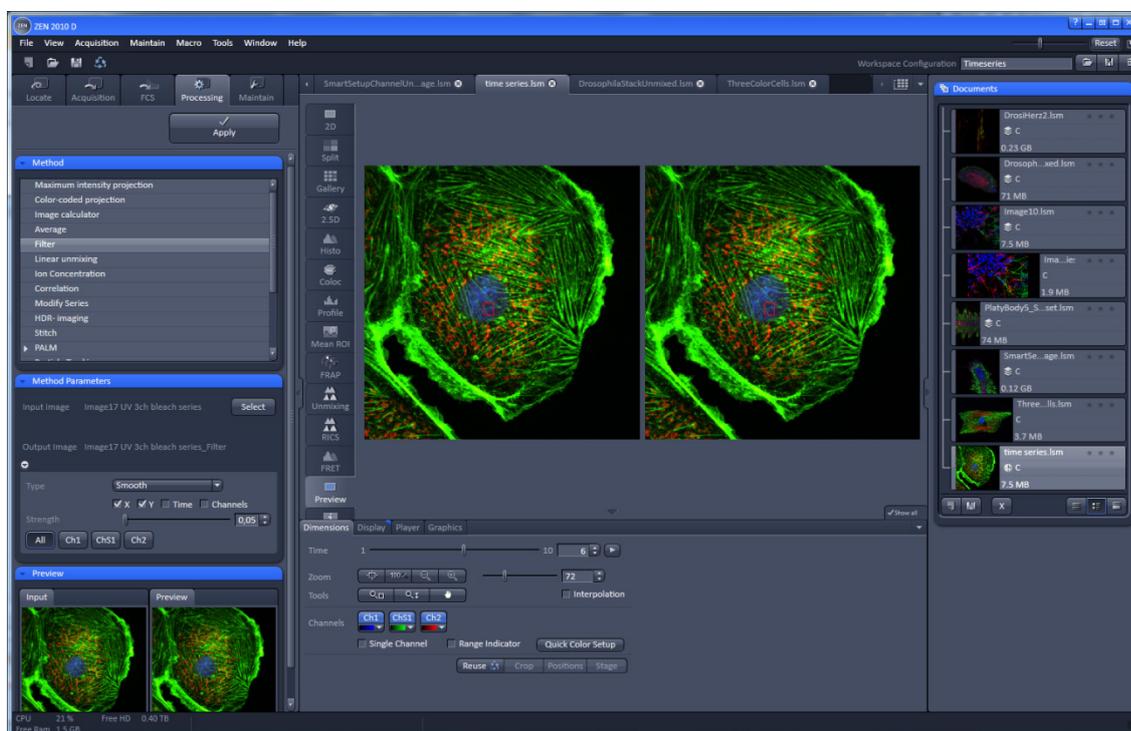


Fig. 132 Preview view tab in the Left Tool Area

The **Preview** tab shows the preview in more detail. The **Preview** thumbnail is always displayed in the **Processing** main tool tab, even when the view in the **Image Display** is changed. If in doubt which Image is selected as processing input, always refer to the displayed thumbnails. Both Previews – in the thumbnail as well as in the automatically generated Preview View tab in the Center Screen Area – are immediately updated.

### 3.3.2 Processing – Maximum Intensity Projection

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1
Tool accepts Z-Stacks and time series (incl. combinations with several other dimensions). See full description of tool for details.				

The **Maximum Intensity Projection** function generates a maximum intensity projection image along the z-, time- or channel dimension of a multidimensional image data set (Fig. 133).

- To open the **Maximum Intensity Projection** Processing tool, select it from the list in the **Processing** main tab.
- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels of the function.
- Select the **Input Image** with the **Select** button and choose the dimension along which you want to generate a maximum intensity projection from the **Coordinate** pull down menu (z, time, channel) in the Settings panel.
- Clicking the **Apply** button creates the maximum intensity projection as a new image document using the name shown in the Output image display for the Select field.

The maximum intensity projection creates an output image whose pixels contain the maximum value over all images in the stack at the particular pixel location.

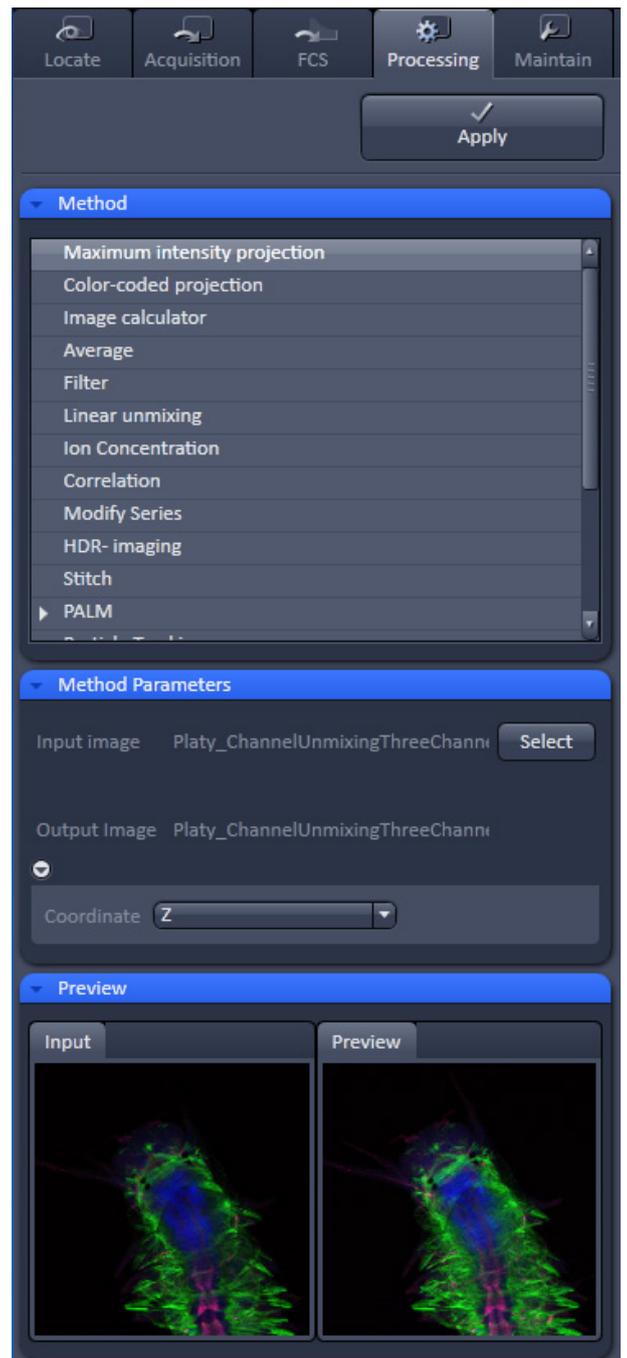


Fig. 133 Processing – Maximum Intensity Projection

### 3.3.3 Processing – Color-coded Projection

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1

Tool accepts Z-stacks and time series (incl. combinations with several other dimensions). See full description of tool for details.

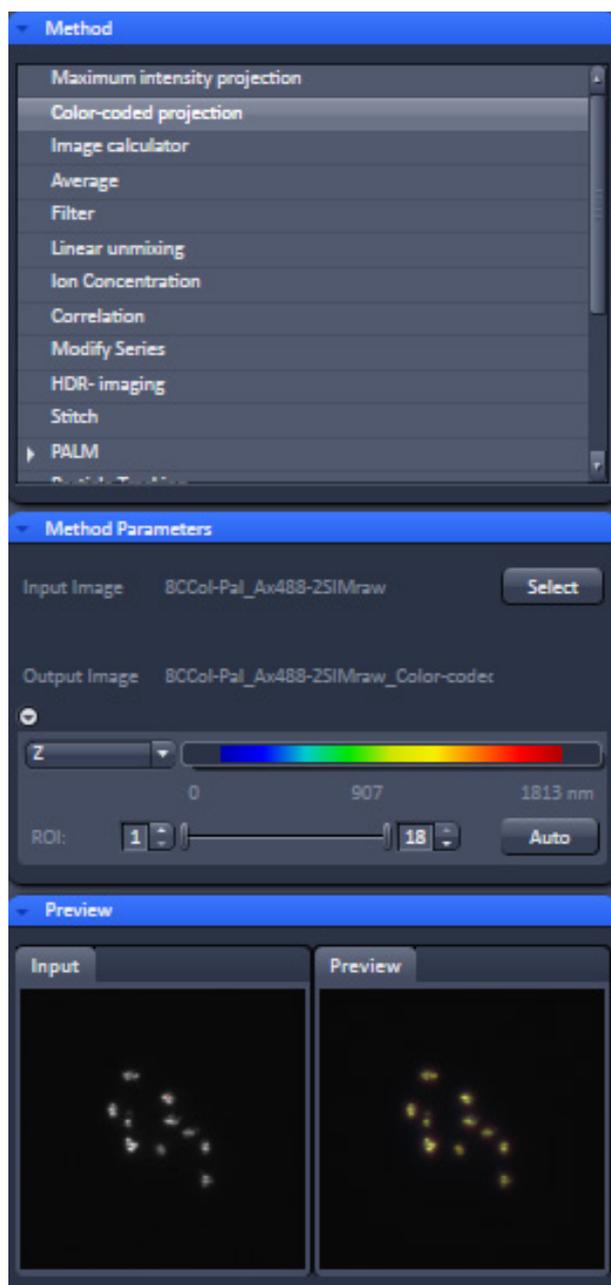


Fig. 134 Processing – Color-coded projection

The **Color-coded projection** function generates a maximum intensity projection image along the z-, or time- dimension of a multidimensional data set (Fig. 134). Instead of using the colors assigned to the channels, it displays the position in z or in time with a color gradient.

- To open the **Color-coded projection** Processing tool, select it from the list in the **Processing** main tab.
- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels of the function.
- Select the color gradient by choosing an appropriate look up table (LUT) from the **LUT** drop down menu.
- You can select from the **dimension** drop down menu any dimension of the image. The color gradient will apply for the selected dimension. These include **Z** (for a z-stack) or **T** (for a time series).
- Below the color gradient the corresponding depth or time is indicated in grey numbers and letters.
- Define the range (**ROI**) of the Z-Stack or time series by using the sliders or the input boxes with arrows.
- Clicking the **Apply** button creates the color-coded projection as a new image document using the name shown in the Output image display for the Select field.

### 3.3.4 Processing – Image Calculator

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1
Tool accepts all types of image data recorded with the listed instruments. See full description of tool for details.				

The **Image calculator** tool provides a calculator-style interface to apply arithmetic operators to the selected image.

- To open the **Image calculator** tool, click on the **Image calculator** in the list of the **Method** panel in the **Processing** main tool tab.
- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 135) for the function.

The two **Select** buttons in the **Selection** panel select the active image in the Image Display as **Input Image** and **Input Image 2**. The Output Image is automatically fed into a new image document. A specific input channel from a multi-channel image or series can be selected using the pull down menus next to the Select buttons.

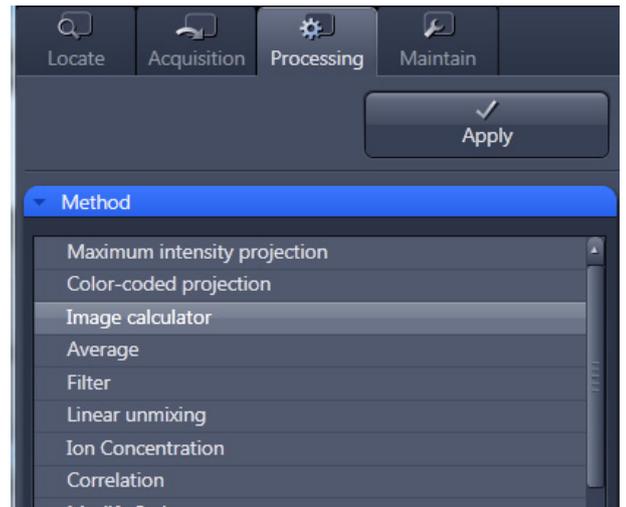


Fig. 135 Processing – Image Calculator

The number in the **First image** display box, which can be edited, reduces the **Input 2 Image** series to the selected first frames of a time series. This can be used to normalize a time series to the values of its first image(s).

After loading 2 input images (source images S1 and S2) specifying the input channels, the calculator keyboard in the **Settings** panel allows the definition of an operator or a combination of operators which is represented by a formula in the formula display box. The operators will be inserted into the formula at the cursor position. The formula can also be typed in directly. By pressing **Input 1** or **Input 2** source image 1 or 2 will be loaded, respectively at the position of the cursor.



Fig. 136 Operators window

For an overview of the available more advanced operators, please refer to the formula panel that is shown in a pop-up window after clicking the **Operators...** button (Fig. 136).

By highlighting the selected operator, a description of the function of this operator is displayed in the lower part of the operators list (Fig. 136).

All image calculations in this tool work pixel by pixel and start with the upper left pixel regardless of the image size for the two images or image series.

 When choosing images of different data depth, the check box next to **Intensity range 0...1** should be marked. This normalizes the image intensity for all images to values between 0 and 1.

All actions performed in the Image Calculator have immediate effect on the previews. After pressing the Apply button on the top of the Processing **main tab**, a new image document with the resulting image is created.

A good example application for the Image Calculator is offsetting/normalizing two images or an image series with a reference image to enhance contrast for small changes in intensity:

- Select a time series as Input 1 and the first frames of these series (or another offset image) as Input 2.
- Then type/edit an operator as shown in Fig. 137 that enhances the relative changes in the image with respect to the relatively bright signal level, for example:  

$$S1 - S2/2 = \text{source 1} - \frac{1}{2} * \text{source 2}.$$

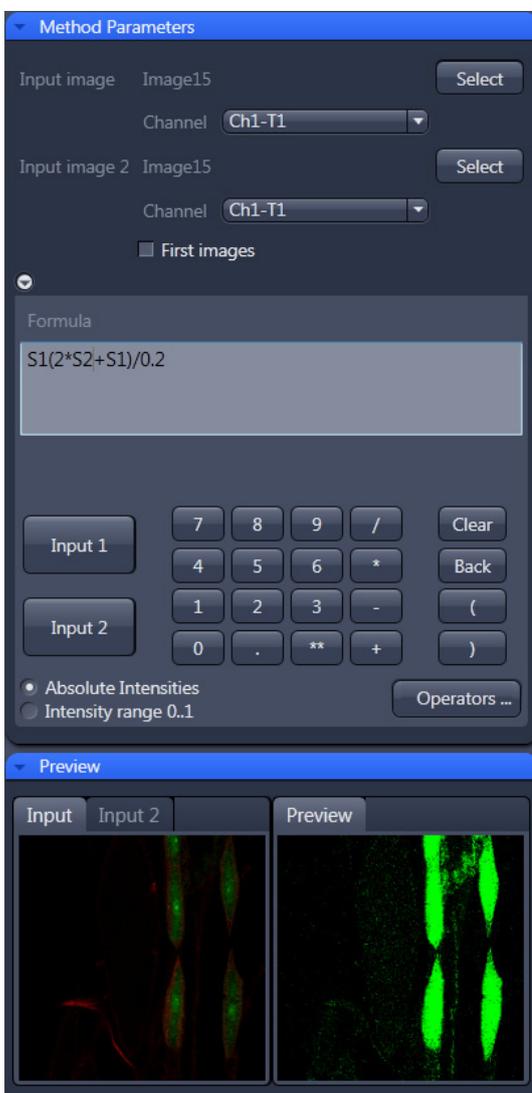


Fig. 137 Image calculation window

### 3.3.5 Processing – Average

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1

Tool accepts all types of image data recorded with the listed instruments. See full description of tool for details.

The **Average** processing function will calculate an image that can be averaged in pixel and time.

- Activate **Average** in the **Processing** tool.

The **Average** menu will appear. The **Average** processing function will calculate an average image.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 138) for the function.
- To select an image, click on the image in the container and press the **Select** button. This will be your **Input** image.
- Select how many pixels you want to bin in x-direction, y-direction, and time points by setting the respective values in the **X Average Pixels**, **Y Average Pixels**, **Z Average Pixels** and **Time Average Pixels** input boxes, which can be opened by clicking on the arrows.

 Note that **Z-Average Pixels** and **Time Average Pixels** are only available for Z-Stacks and time series. You can either enter a value, or use the sliders, or arrows of the input boxes.

 If a moving average for **Time** should be performed, the respective **Moving Average** box must be checked. In this case, consecutive averages comprised of the specified number of frames in **Average Pixels** will be computed.

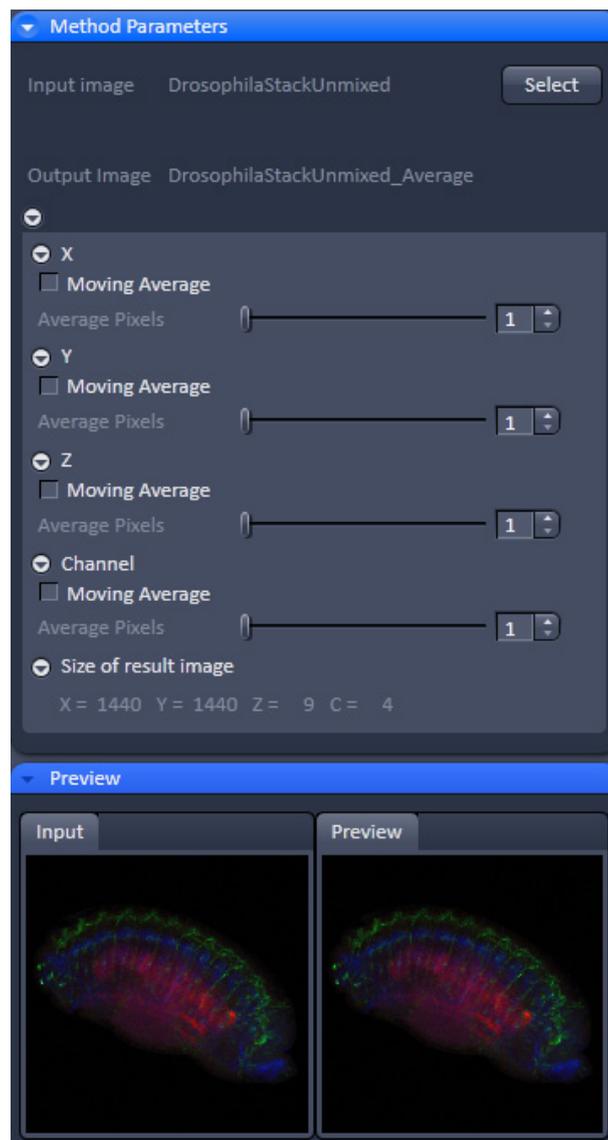


Fig. 138 Processing – Average

An average output image will be calculated according to the settings and the result presented in the **Preview** window.

The size of the resulting image in **X**, **Y**, **Z**, and **T** can be viewed in the **Size of result image** display. If the **Apply** button is pressed, the averaged image will be opened as a new image in the container.

### 3.3.6 Processing – Filter

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1

Tool accepts all types of image data recorded with the listed instruments. It is generally advisable to perform certain processing steps (e.g. linear unmixing or SIM processing) before applying filters. See full description of tool for details.



Fig. 139 Processing – Filter

The filter function permits the processing of images with a Median, Smooth, Sharpen, or Bandpass filter.

- To open the **Filter** tool, select it from the list under the **Processing main tab** (Fig. 139).
- Select the Input Image with the **Select** button and choose the filter of your choice from the Type pull down (**Median, Smooth, Sharpen, Band,** and **Gradient**).
- Select the channels you want to process with the **All, Ch-T1** or **Ch-T2** buttons.

Once a filter has been selected from the type drop down menu, filter specific settings are available.

#### Median Filter

With the median filter, the gray value of each center pixel is replaced with the median value of the neighboring pixels. The viewed neighbor pixels are defined by a square of a certain size called the kernel. The modified pixel now is the center pixel of this pixel matrix.

The median value is defined as the middle value (not average) of all the gray values sorted in ascending order within a matrix.

Image noise will be reduced by the application of the median filter. The cut-off of regions will slightly blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

The effect of the filter increases with the matrix size. This matrix / kernel size can be set for each dimension with the three sliders **Kernel size X**, **Kernel size Y**, and **Kernel size Z** in the **Filter Type** panel or by setting the size in the spin-box on the right hand side of each slider (Fig. 140). A larger kernel, however, also increases the processing time. Other settings of this filter cannot be modified.



Fig. 140 Median filter

### Smooth Filter

The smooth filter is a low pass filter with Gaussian characteristics. The gray value of each center pixel is replaced with the weighted average value of the surrounding neighbor pixels. The "neighborhood" is limited by the cut-off in pixels, set by the **Strength** slider. It has an analogous effect as the kernel size (Fig. 141).



Fig. 141 Smooth filter

If a dimension should be processed by this tool the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked. The modified pixel now is the center pixel of the filter matrix.

Image noise will be reduced by the application of the low pass filter. Sharp edges of regions will blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

### Sharpen Filter

With the sharpen filter, the original image is filtered with a low pass filter first. The result of this filtering is then subtracted from the original image. This will improve image sharpness.

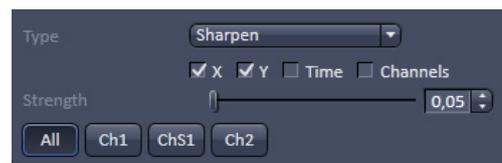
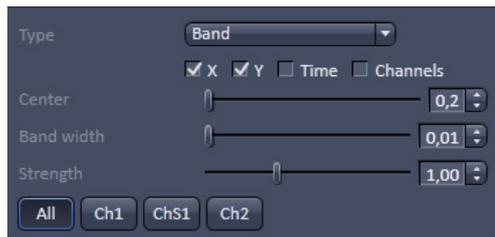


Fig. 142 Sharpen filter

The "neighborhood" is limited by the cut-off in pixels, set by the **Strength** slider. It has an analogous effect as the kernel size (Fig. 142). If a dimension should be processed by this tool, the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked.

**Fig. 143** Band filter

### Band Filter

With the band filter (Fig. 143), the original image is filtered with a "band blocker" filter. It filters out a certain frequency band with the position set by **Center**, width set by **Band Width** and a steepness of the edges set by **Strength**. This is particularly useful to remove periodic perturbances in the image.

If a dimension should be processed by this tool, the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked.

**Fig. 144** Gradient Filter

### Gradient Filter

The Gradient Filter visualizes the direction and rate of brightness changes from one pixel to the other. It illustrates if brightness changes smoothly or abruptly in the image (Fig. 144).

The gradient filter can be used for all dimensions present in one dataset. You can select it if you wish to perform it for all channels with the **All** button or individual channels with the according (e.g. Cam1 or Ch1) buttons.

The dimensions can be chosen by checking the according check-boxes.

### 3.3.7 Processing – Linear Unmixing

Licensing		Instrument		
✓ standard	optional	✓ LSM	ELYRA	Lightsheet Z.1
<p>Tool accepts lambda stacks and multi-channel image data (single images and multi-dimensional image data). <i>Linear Unmixing</i> was developed for processing LSM data. Successful processing of multi-channel images obtained with other systems is potentially possible as well (provided this input meets the requirements for unmixing). See full description of tool for details.</p>				

The Linear Unmixing processing tool permits extracting the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in multi-channel images and so-called "Lambda stacks" (only available in LSM imaging mode).

In brief, 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. 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 (available only in **LSM imaging** mode) from the multi-labeled sample is acquired.

Finally, the individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be fed into the least-square-fit based algorithm to produce a 10-channel multi-fluorescence stack without any partial overlap between the channels.

If no mono-labeled samples are 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** tab).
  - Automatically by software analyses of what the individual spectral signatures are. This processing function is called "Multi-channel Unmixing" or "Automatic Component Extraction" (ACE). Note: in some cases, spectrally acquired images are not appropriate for ACE or "Multi-channel Unmixing" and linear unmixing can lead to wrong results.
- To open the **Linear unmixing** tool, click on **Linear unmixing** in the **Processing** tool list under the **Processing** tab (Fig. 145).

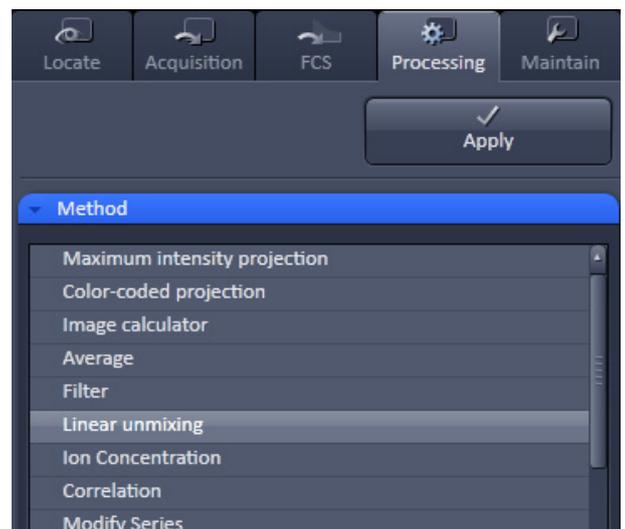


Fig. 145 Processing – Linear unmixing

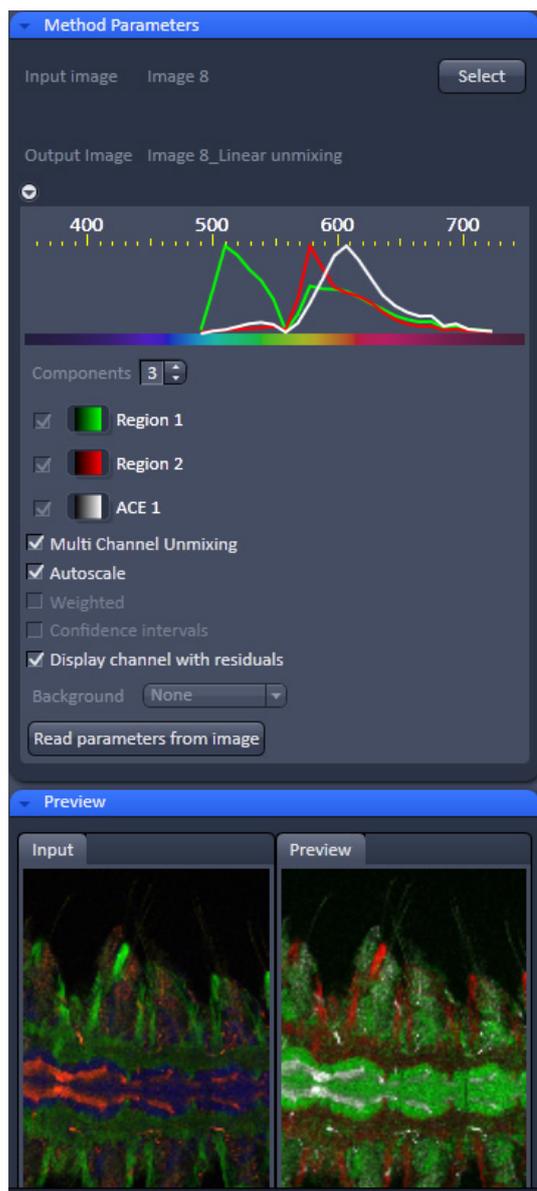


Fig. 146 Linear unmixing panel

- Pressing the **Select** button loads the active image from the **Image Display** and displays it as Input Image. Only Lambda stacks (only available in **LSM imaging** modes) or multi-channel images can be loaded.
- In the **Linear unmixing** panel (Fig. 146), the number of spectrally distinguishable fluorescent components within the imaged sample can be selected from the **Components** selection box. The number of extractable components cannot be higher than the number of acquired channels. The ZEN software is limited to a maximum of 10 components.

- Select the sample-specific fluorescence dye reference spectra from the Spectra Database (Fig. 147).
- Assign appropriate colors to the fluorescence channels by clicking on the color flag and choose from the available lookup tables (LUTs)
- If no predefined reference spectra exist:
  - Prepare reference samples and create spectra via the **Save to Spectra DB** button in the Unmixing View (see section **3.3.7 Processing – Linear Unmixing**).
  - or
  - Use the "Multi-channel Unmixing" functionality described below. (Note: in some cases, spectrally acquired images are not appropriate for "Multi-channel Unmixing" and linear unmixing can lead to wrong results – see discussion below.)
- After definition of the required reference spectra click **Apply**.

A new image with the resulting (unmixed) channels will be created (Fig. 148 and Fig. 149).

The following additional settings and functions are available for linear unmixing:

Auto scale balances the intensity of the unmixed channels to equal levels.

Display Channel with 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 dataset. This shows that either the acquired data is not representing the sample (e.g., when information is lost by pixel saturation) or wrong/insufficient reference spectra have been chosen. In this case, new image acquisition with no overexposed/ saturated pixels is strongly recommended. In the case of wrong reference spectra, choosing/acquisition of different/new reference spectra can be necessary to improve the results.

Background: Select a background spectrum from the list of selected components. This spectrum is then subtracted from the images prior to unmixing. For example: three components selected and one of them marked as background will subtract the selected background from the acquired images and generate a 2-channel unmixed image. For the generation of background spectra refer to the Unmixing View. Even small background contributions to the image should be indicated for the unmixing algorithm to ensure best unmixing results.

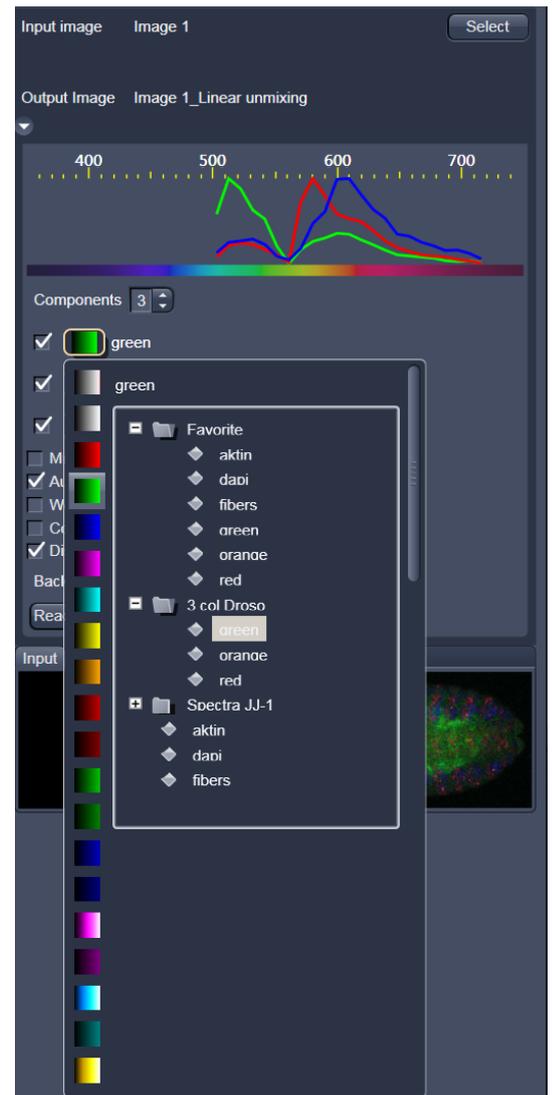
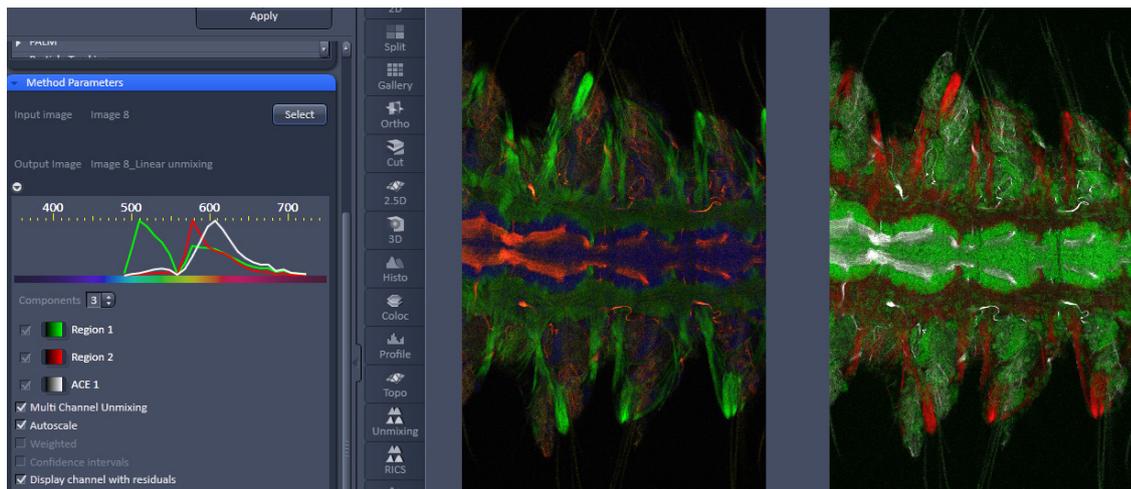


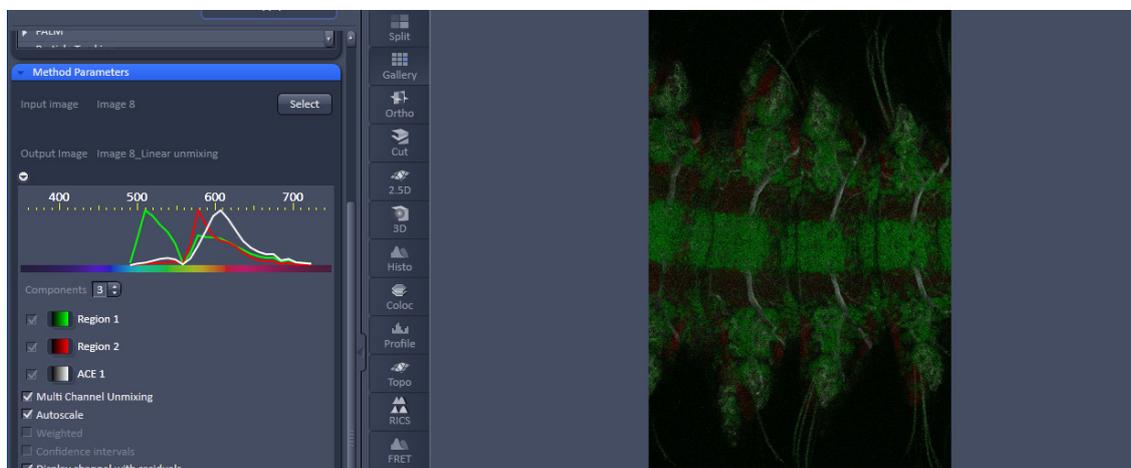
Fig. 147 Spectra and LUT assignment

**Weighted unmixing:**

When this option is checked, spectral channels with high noise contribute less to the unmixing result. This option includes a statistical analysis of the signal-related (Poisson-) noise and weighs the respective contribution for the fitting with the combination of reference spectra to the experimental data. Note: this option involves a more sophisticated unmixing algorithm and therefore takes longer than the basic unmixing analysis. Weighted unmixing generates improved unmixing results when acquisition channels are not so well balanced but still have a good signal-to-noise ratio.



**Fig. 148** Image Display window before unmixing in Preview



**Fig. 149** Image Display window after unmixing

**Display channels with statistical confidence:**

This option displays an additional channel per unmixed component which shows the relative statistical error in each unmixed component channel. This statistical uncertainty of the pixel intensity in the unmixed channel is calculated based on the (Poisson-) noise from the acquired (input-) channels, the bandwidth and -position and the quality of the reference spectra. The display can be interpreted best when an appropriate false color LUT is chosen. The error is displayed as percentage of unmixed channel pixel intensity. For example: A "confidence value", i.e., pixel intensity of 50 represents a 50% statistical uncertainty of the corresponding intensity in the unmixed channel. The higher the statistical error for the acquired pixel intensity (the shot noise), the higher is the intensity value of this pixel.

**Multichannel Unmixing:**

When this option is chosen, the unmixing algorithm is applied to a multi-channel image (up to 10 channels) without the use of reference spectra.

The calculation of residuals and the subtraction of background based on a background spectrum are not available. For heavily overlapping signals this method will yield poor results.

-  Avoid saturation of fluorescence signal in the data set to be unmixed. Saturation will generate a high signal in the residual channel. To get the best unmixing results, define an extra background channel, if possible.
  
-  Reference Spectra used for Linear unmixing of the Lambda stacks processed in the Unmixing View are stored with the resulting image and can be re-loaded using the **Processing → Linear unmixing** tool with the Read parameters from image button. This facilitates processing of a series of images or Lambda stacks all with the same parameters / reference spectra.

### 3.3.8 Processing – Ion Concentration

Licensing	Instrument
standard <input type="checkbox"/> optional <input checked="" type="checkbox"/>	<input checked="" type="checkbox"/> LSM    ELYRA    Lightsheet Z.1

Tool accepts single-channel or multi-channel, multi-dimensional data (typically time series data) of samples labeled with fluorescent ion indicators. *Ion Concentration* was developed for analyzing LSM data. Successful analysis of image data obtained with other systems is potentially possible as well (provided the input meets the requirements for intensity quantification). See full description of tool for details.

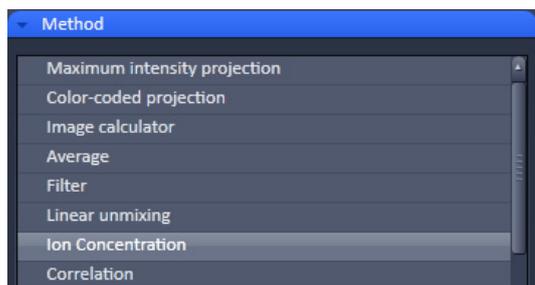


Fig. 150 Processing – Ion concentration

This Processing function permits the calibration of ion concentrations in physiological experiments.

To open the **Ion Concentration** tools click **Ion concentration** (Fig. 150).

The two  buttons select the active image in the **Image Display** as **Input Image** and **Input Image 2** (for background subtraction). The name of the automatically generated Output Image is shown under the name of the Input Images.

In the **Preview** panel at the bottom of the Ion concentration tool window, thumbnails of the input images as well as a preview of the output image are displayed (Fig. 151).

After opening the **Method Parameters** panel full window by clicking the little arrow, the full tool window opens and the following functions are available (Fig. 152).

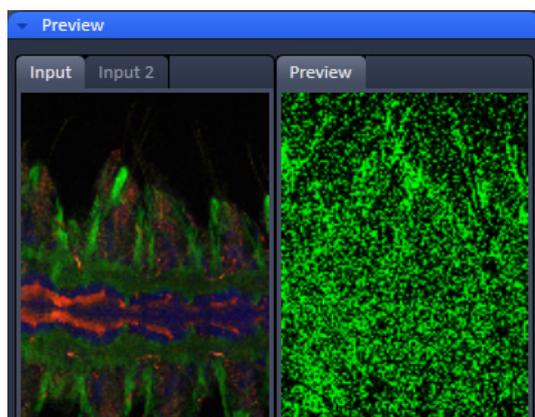


Fig. 151 Ion concentration panel



Opens the display for the resulting calibration curve.



To load & save calibration curves from / to files.

buttons



To select Input Image 2 as background image.

tick box



Spin boxes to set the Min and Max in the Output Image

Image scaling

Pull down menus for



To set three different calibration options, according to the dyes (single wavelength, vs ratiometric), the calibration method and if the calibration is carried out in vitro/situ. Ch 1 and 2 select the channels from Input Image 1.

From these pull downs, the following combinations of parameters can be chosen:

Dye: single wavelength vs. ratiometric

Available methods for single wavelength: Titration or equation (both, **in vitro** and **in situ calibration**).

Available methods for **ratiometric** dyes: **Ratio**, **titration**, **equation**, and **unmixing**.



Fig. 152 Fully opened Ion Concentration tool window - Ratiometric dye in equation mode

### 3.3.8.1 Single Wavelength Dyes – Offline Calibration

- Subtract background/ autofluorescence image from raw images to obtain better raw data to start with (Fig. 153).
- Perform equation or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).

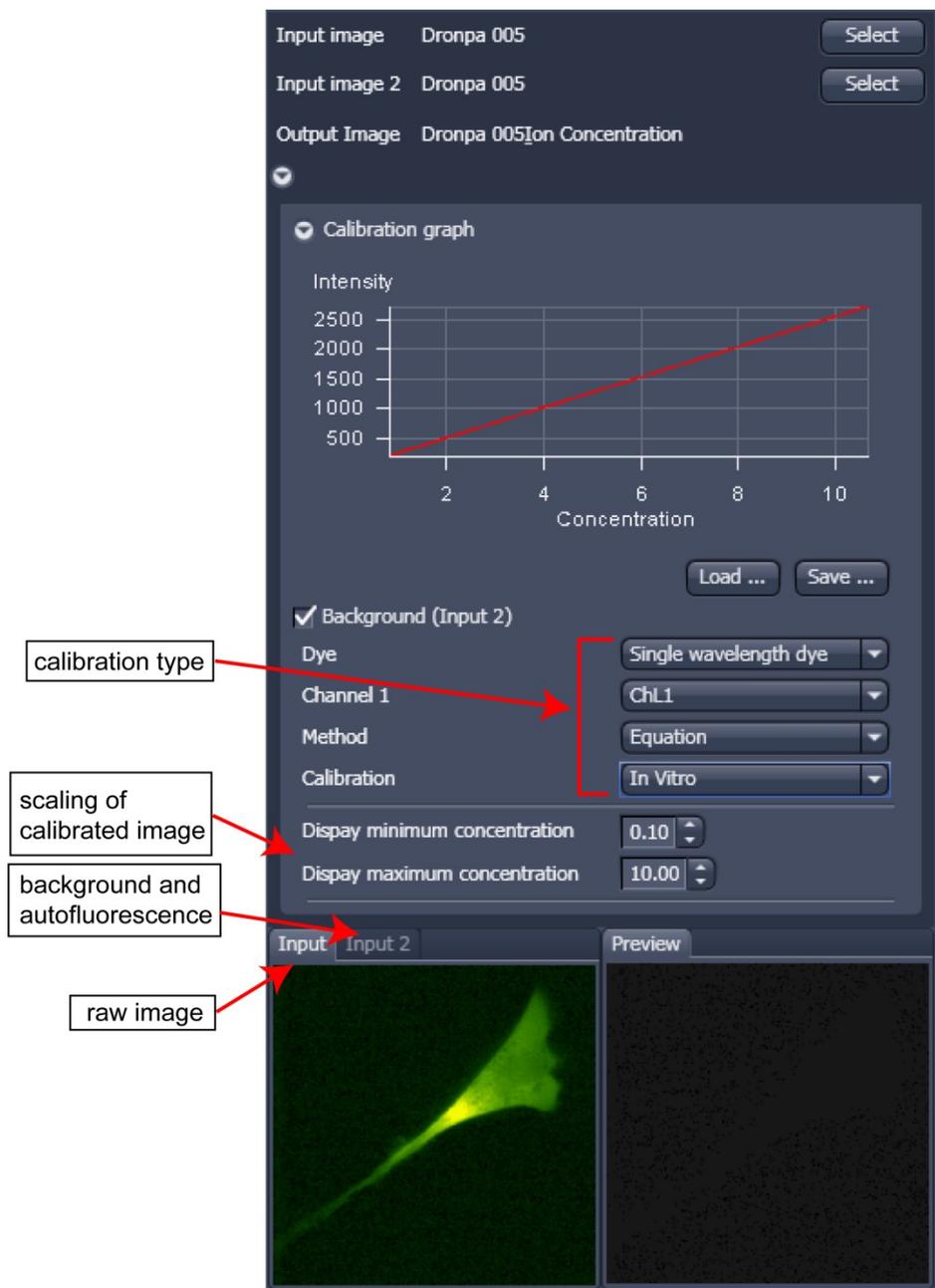


Fig. 153 Ion concentration panel

### 3.3.8.2 Ratiometric Dyes

- Fura-2, Indo-, SNARF, Cameleon, Ratiometric Pericam, Phluorin.
- Display fluorescence ratio  $R$  over time
- Display fluorescence ratio  $R$  corrected for background/autofluorescence over time
- Calculate absolute ion concentrations (pixel by pixel) via titration calibration (known ion concentrations applied to the cells – in situ – or in solutions – in vitro or equation calibration where possible [Fura-2, Indo-, SNARF])
- Calculation of  $R$  eliminates artifacts and uncertainties caused by
  - inhomogeneous dye distribution
  - photobleaching
  - may be applied with moving cells.

#### Ratiometric Dyes - Online Ratio (Fig. 154)

$$R(t_1) = F_1(t_1) / F_2(t_1), R(t_2) = F_1(t_2) / F_2(t_2) \dots$$

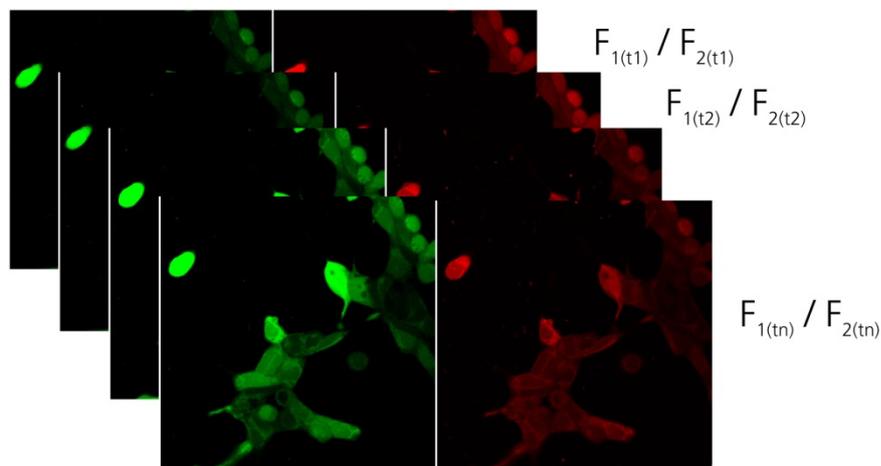


Fig. 154 Ratiometric Dyes – online ratio

**Ratiometric Dyes - Calibration** (Fig. 155)

- Subtract background/autofluorescence images from raw images to obtain

$$R_{korr} [=F1-F1Background)/(F2-F2Background)]$$

when calibration reference is not obtained with the experimental sample (in situ).

- Calculate ratio R.
- Perform equation- or titration calibration (compare R with a calibration curve → titration calibration or put R values in calibration formula).

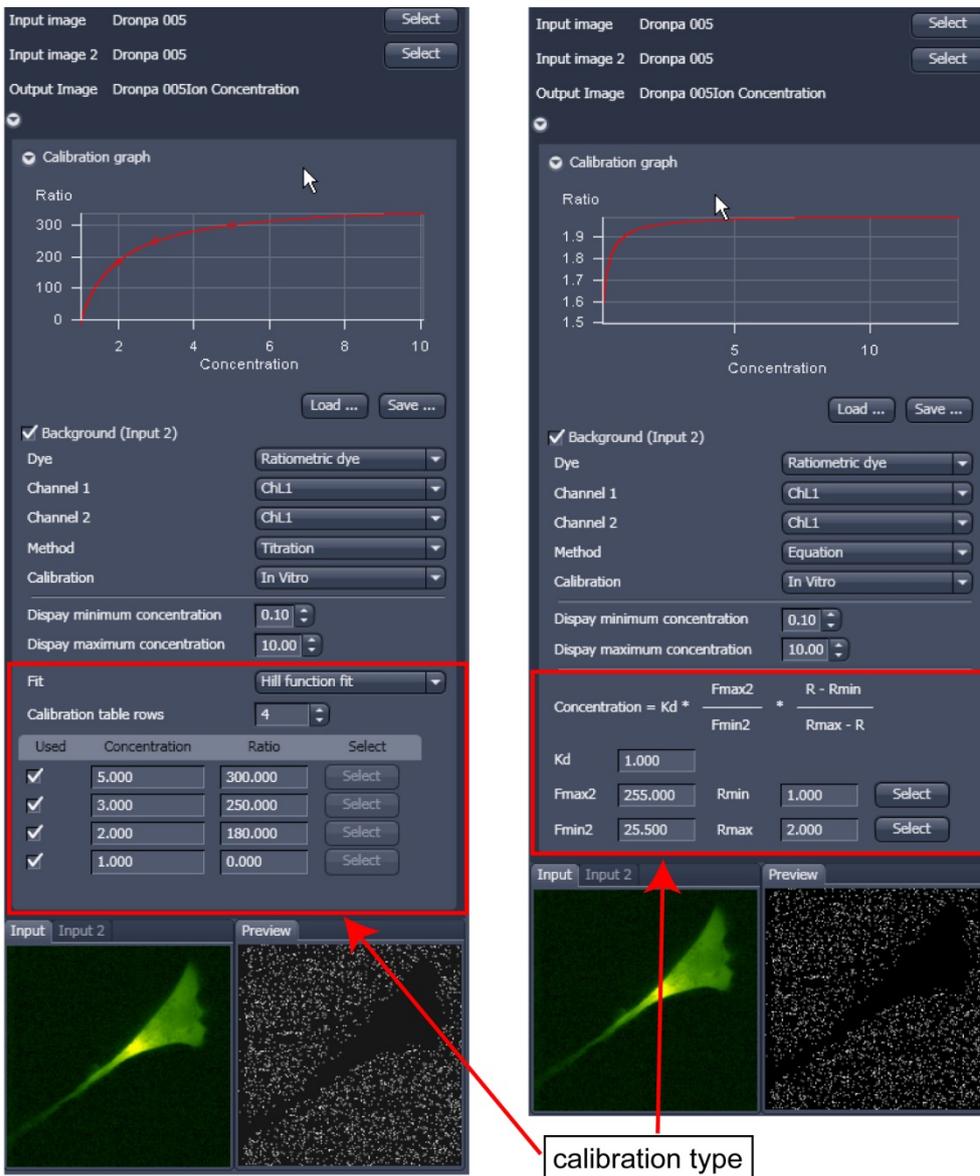


Fig. 155 Ion concentration panel

**Ratiometric Dyes - Equation Calibration (Grynkiewicz)** (Fig. 156)

Fura-2, Indo-1, ..

$K_D$  (dissociation constant) taken from literature

$R_{min}$ : derived from ion-free state of the dye (e.g. 0  $Ca^{2+}$ )

$R_{max}$ : derived from ion-bound state of the dye (e.g. saturated with  $Ca^{2+}$ )

$F_{min2}$  and  $F_{max2}$  are the minimum and maximum fluorescence intensities at wavelength 2

$R_{min}$ ,  $R_{max}$ ,  $F_{min2}$  and  $F_{max2}$  may be determined in the cells under investigation (in situ) or in solutions (in vitro)

Calibration parameters may be saved and reloaded (\*.cal)

Dye: Ratiometric dye

Channel 1: ChL1

Channel 2: ChL1

Method: Equation

Calibration: In Vitro

Display minimum concentration: 0.10

Display maximum concentration: 10.00

$$\text{Concentration} = K_d * \frac{F_{max2}}{F_{min2}} * \frac{R - R_{min}}{R_{max} - R}$$

Kd: 1.000

Fmax2: 255.000

Fmin2: 25.500

Rmin: 1.000

Rmax: 2.000

**Fig. 156** Ion concentration - Ratiometric dye, Equation Calibration

**Options for Calibration Image Selection (Equation- or Titration Calibration)**

- Click into image window.
- Select source channel(s).
- Optional background subtraction
- Optional calculation of parameters from overlay region(s)

### 3.3.9 Processing – Correlation

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1

Tool accepts potentially all types of image files. See full description of tool for details.

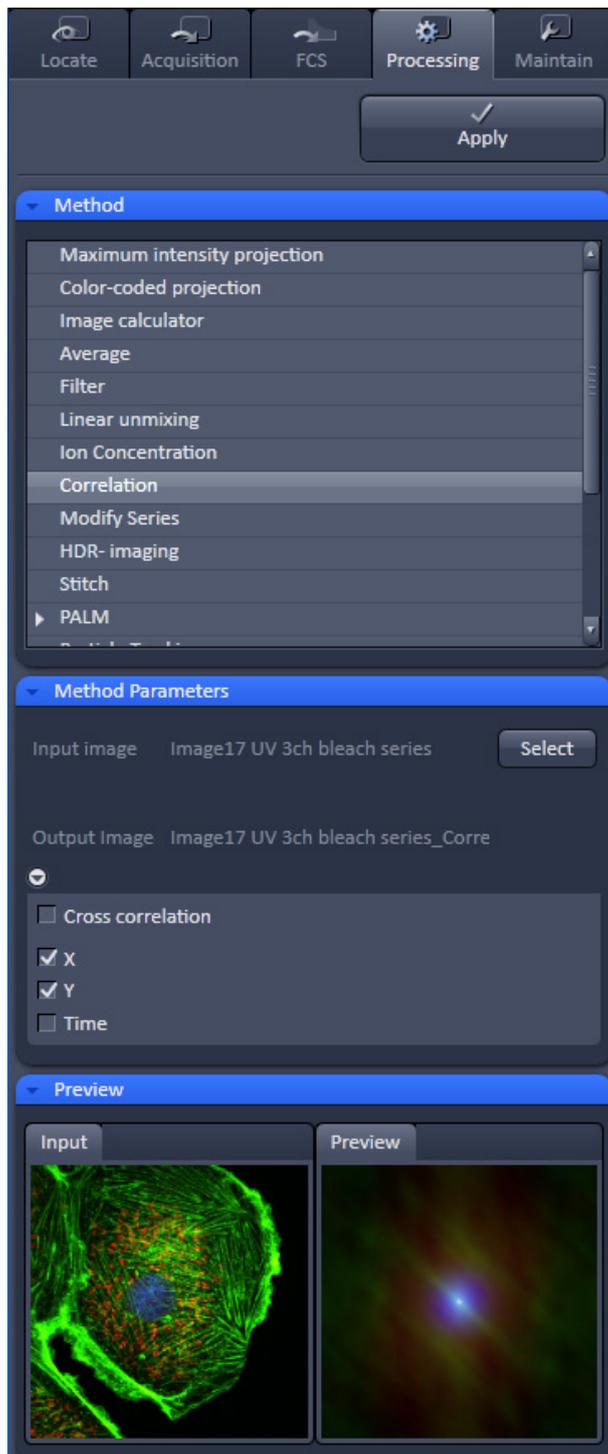


Fig. 157 Processing – Correlation

- Activate **Correlation** in the **Processing** tool. The **Correlation** menu will appear (Fig. 157).

The **Correlation** processing function will display the spatial (only available in the **LSM imaging** mode) or temporal correlation of an image or image stack.

- To select an image, highlight the image in the image container and press the **Select** button. This will be your **Input** image.

As an output image the correlation image is computed and the result presented in the **Preview** window.

- You can select which kind of correlation you want to perform by activating the **X**, **Y**, **Z**, and **Time** check boxes to perform spatial correlations in x, y, and z-direction or a temporal correlation.

 Note **Z** and **Time** check boxes are only available for Z-Stacks or time series stacks.

- If the **Apply** button is pressed, the correlation image will be opened as a new image in the container.

### 3.3.10 Processing – Modify Series

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1

Tool accepts single image files (for concatenation) and multi-dimensional image series (typically time series and Z-stacks). See full description of tool for details.

This function allows modifying dimensions of images or image series (Fig. 158).

Depending on the input image (for example **Z-Stack** or **Time Series**) the available modifications vary, which are available in the Modify mode drop down menu (Fig. 159 and Fig. 160).

It allows modifying the acquisition date, rotating or mirroring the image(s), converting or reversing dimensions, renaming channels or making time series from Z-Stacks, tiles, or images from various positions. Furthermore it allows to concatenate time series or z-stacks when two input files are selected.

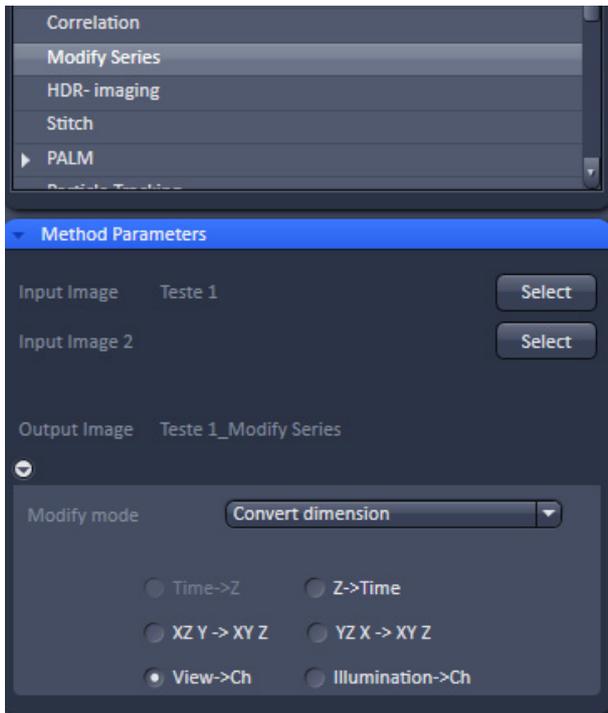


Fig. 158 Processing – Modify Series

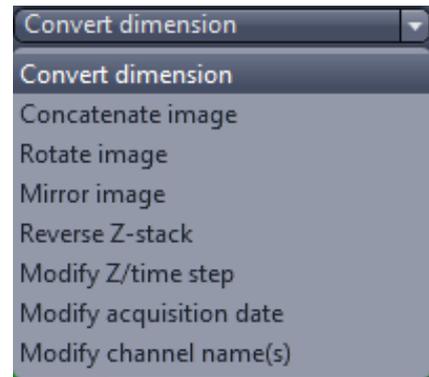


Fig. 159 Modify mode drop down menu – Z-Stack options

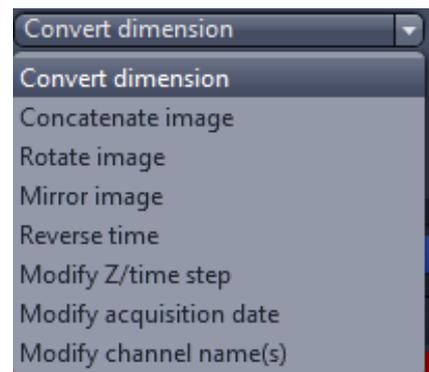


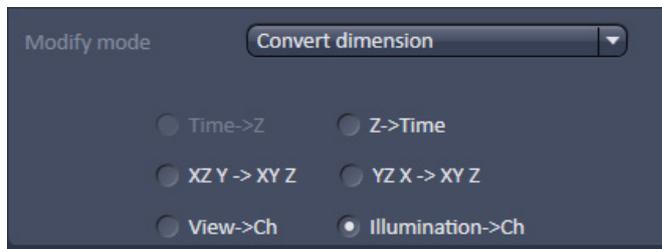
Fig. 160 Modify mode drop down menu – Time series options

Data acquired with the Lightsheet Z.1 system might have additional dimensions, the Illumination as well as the view resulting from a multiview experiment. If the data includes the dimension the option convert **View into Channel** and/ or **Illumination into Channel** becomes available.

The function **Illumination into Channels** enables to display the single illumination sides in individual colors for inspection and analysis (e.g. correct overlap). The resulting channels are named with the following nomenclature: Cam1-I1 = Camera 1-Illumination 1 (left); Cam1-I2 = Camera 1-Illumination 2 (right) and will look as follows:



To evaluate the views of a multiview experiment, the **View into Channel** conversion is a useful tool. As a result, different colors can be assigned to each view. The channels will have the following nomenclature: Cam 1- V1 = Camera 1-View1, Cam 1-V2 = Camera 1-View2 and will look as follows:



**Fig. 161** Convert dimensions – View into Channel and Illumination into Channel option

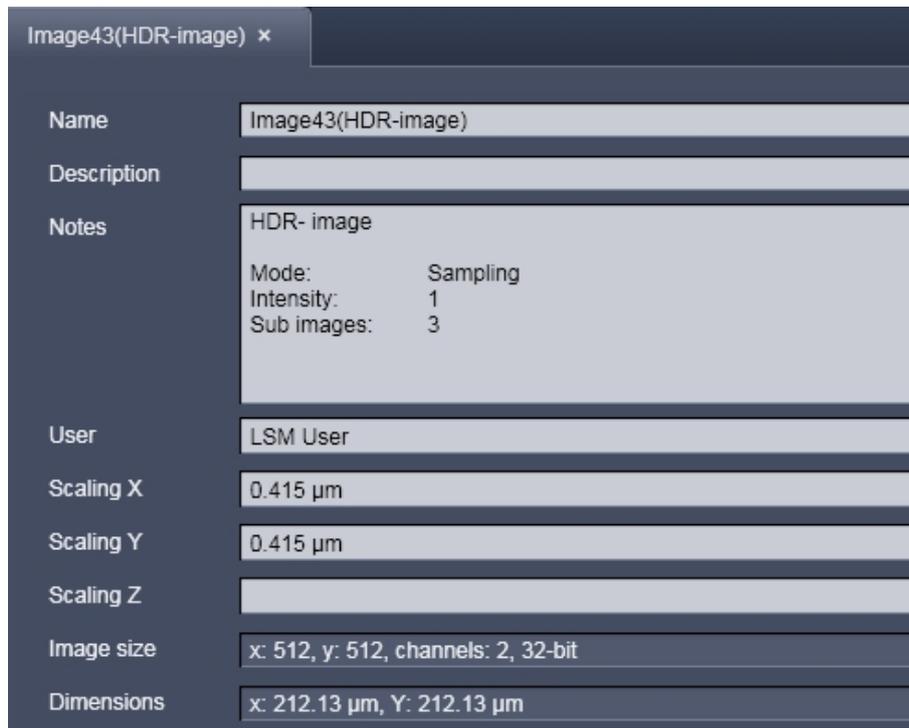
**3.3.11 Processing – HDR-imaging**

Licensing	Instrument
standard <input checked="" type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM    ELYRA    Lightsheet Z.1

Tool accepts image data recorded with the optional feature "HDR" of the acquisition tool *Acquisition Mode*. In addition, input acquired either without using the feature "HDR" or on instruments other than LSM systems is potentially suitable for this processing tool as well (provided the input meets the requirements for HDR-imaging). See full description of tool for details.

Please see the description of the HDR (High Dynamic Range) function (part of the Acquisition Mode tool) for details on HDR image recording with LSM systems.

HDR creates an image with a depth of 32-bit and therefore covers a far broader dynamic range than conventional images (8 to 16 bit).



**Fig. 162 Image info of a HDR image**

As a result of the HDR process, the grey scale is independent from the initial bit depth, but covers a range from 0-1.

Also, the grey stepping is much finer, with much better visibility of weak structures (Fig. 163).

The HDR method employed is also shown in the image info (Fig. 162).

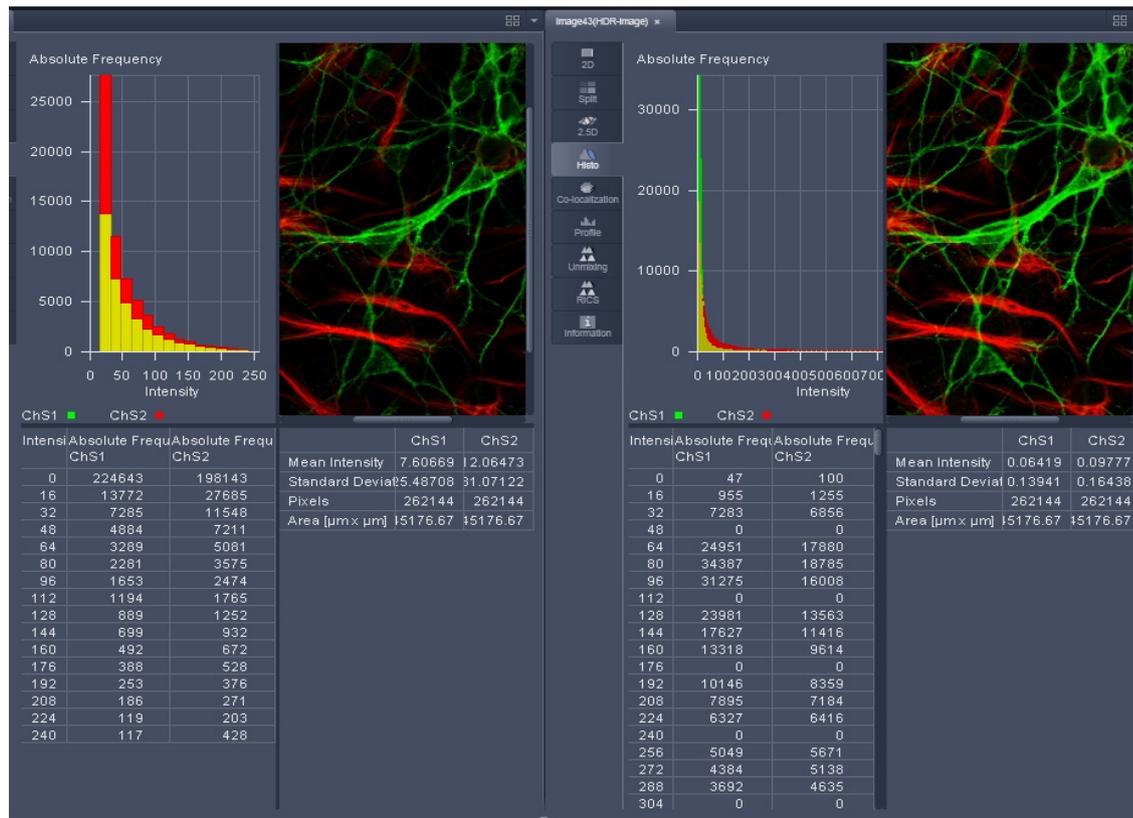


Fig. 163 HDR image display (left side without, right with HDR)

The **Sampling** mode is meant to improve the image display as it equalizes the differences between the very high and the very low intensities in the image. However, it is not suitable for quantitative analysis as the level steps become non-linear.

The image re-calculation works already with a single image (**Frame** = 1) and is done in the following way taking **Intensity** 3.

Image x 1; cut off of grey levels above the dynamic range; Result divided by 1

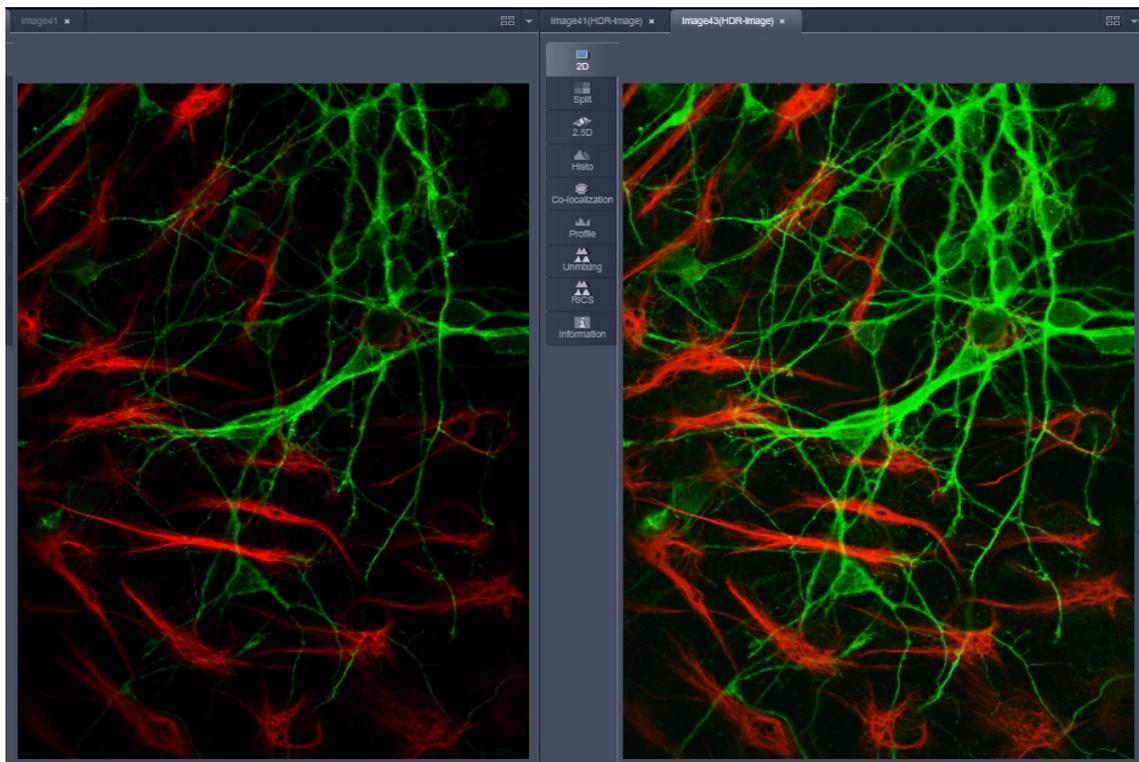
Image x 2; cut off of grey levels above the dynamic range; Result divided by 2

Image x 3; cut off of grey levels above the dynamic range; Result divided by 3

The three resulting images are summed up and again divided by 3. This procedure is adapted accordingly if a higher **Intensity** number is set. If two images are **acquired** (**Frame** = 2) and Intensity is still at 3, the second image is rated lower and is multiplied and divided in three steps with the value 4,5 and 6; The then 6 resulting images are summed up and divided by 6.

The image acquisition parameters are not changed for the acquisition of the two (or more) **Frames**.

After acquisition, the grey dynamics of the image are automatically linearized (see description above) to match the range of the output display. This step visibly intensifies weak signals (Fig. 164).



**Fig. 164** HDR image display using Sampling mode (left side without, right with HDR)

Unlike the **Sampling** method, the **Illumination** method of HDR still provides a linear grey stepping which allows quantitative measurements. For the acquisition step with increased laser power, those regions of the image are excluded from exposures when no reasonable data can be obtained with any higher laser power.

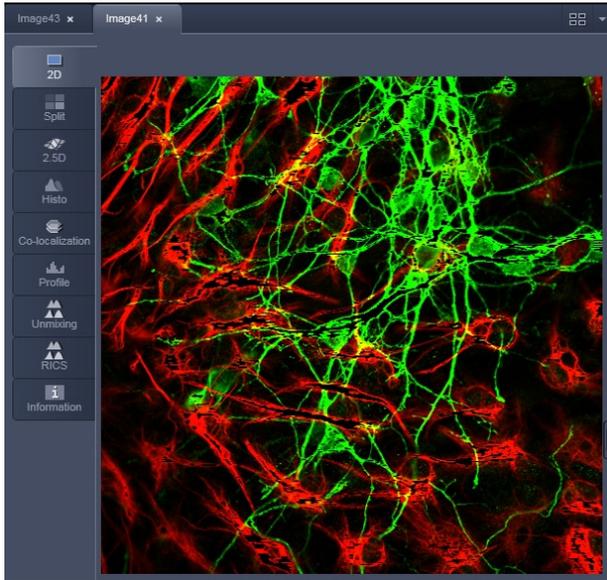


Fig. 165 HDR image display after using Illumination mode

During data recording with the **Illumination** method, masks are applied to protect the sample from laser irradiation. The respective ROIs are displayed in an additional image (Fig. 165).

The calculation of the resulting image follows the same procedures as for the sampling mode. However, the regions, which are excluded from a second and/or third illumination step are divided by 2 instead of 3.

After acquisition, the grey dynamics of the resulting image are not automatically compressed to match the range of the output display.

Use the **Gamma** and **Contrast** sliders of the image display (Fig. 166) to adjust the image.

For manual adjustment, a result can be achieved which fits the individual requirements, without affecting the actual raw data stored with the image.

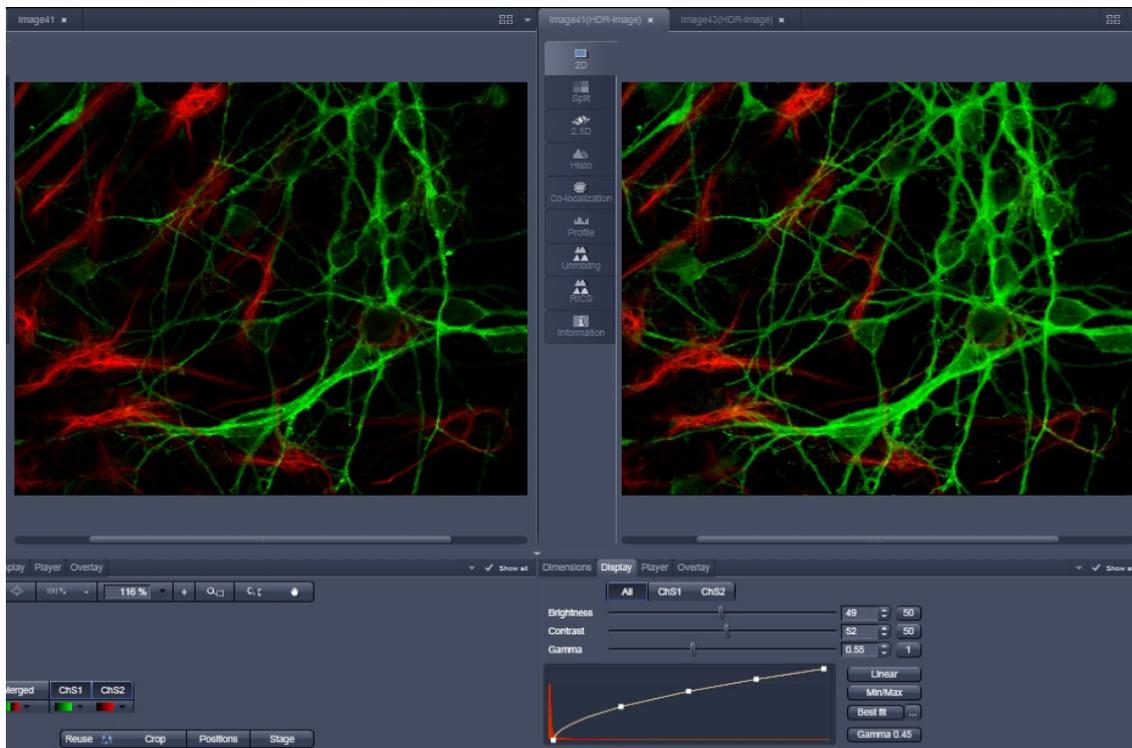


Fig. 166 Image adjustment of the HDR image (left side without, right with HDR)

### 3.3.12 Processing – Stitch

Licensing	Instrument
standard ✓ optional	✓ LSM   ✓ ELYRA   Lightsheet Z.1

Tool accepts image data recorded data recorded with the acquisition tool *Tile Scan* (incl. combinations with other multi-dimensional acquisition tools). Images recorded in SIM mode on ELYRA (SIM data) must be processed with the tool *Structured Illumination* before stitching. For optimal results, image data should have some degree of lateral overlap. See full description of tool for details.

With this function tiled images acquired with Versions of ZEN 2012 and upwards and the tiling function can be stitched together in 2D and 3D.

Select a tiled image (or stack) with the **Select** button (Fig. 167).

The **Correlation Threshold** is defining the accuracy with which the algorithm detects similarities in the adjacent image planes.

If a 3D tile scan image has been acquired (Tile Scan combined with Z-Stack) then additional functions for the stitching procedure are available.

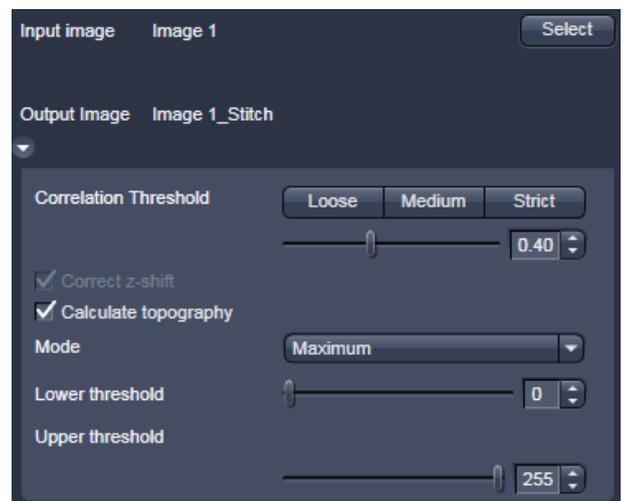


Fig. 167 Processing – Stitch

**Ignore Z Correction** will in some cases reveal better results for the 3D stitch. Depending on the image data the last step of the stitch algorithm, which makes a correction of the Z-Stacks in Z looking at the overall image, can lead to unwanted shifts of the stacks in Z. This can be avoided when this option is checked.

**Calculate Topography** provides a topographic image of the 3D stitched image data. This calculation can be done in several modes.

#### First / Last maximum

- Select **First maximum** to calculate the topography surface by using the first maximum coming from the top. This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multi-layers with subsurface layers of higher signal intensity.
- Select **Last maximum** to calculate the topography surface by using the last maximum coming from the top.

**First / Last threshold**

- Select **First threshold** to calculate the topography surface by using the first slice coming from the top, where the intensity reaches the value defined by the lower intensity threshold.
- Set the **Threshold** to define the lower and upper intensity thresholds used for calculation of the topography surface. Use of this function is recommended to find the real surface in the case of images with pronounced noise. All image pixels with intensity less or higher than the thresholds set are ignored for the surface calculation. **Correct bleaching** applies a correction factor to the double-exposed pixels. Crossfading is the only method available here and does an interpolation of the brightness values over a number of pixels in X or Y direction. The **Correction Width** in pixels can be adjusted.

### 3.3.13 Processing – PALM

Licensing	Instrument
standard ✓ optional	LSM ✓ ELYRA Lightsheet Z.1

Tool exclusively accepts PALM data. See full description of tool set for details.

The **Processing PALM** tool (Fig. 168) is applied to data acquired in the **PALM imaging** mode. It subdivides in 4 subcategories.

- **PALM**: Peak finding and localization
- **Remove outliers**: Filter to select for molecules surrounded by a threshold number of other molecules
- **Import PALM molecules**: Import function for molecule tables
- **Convert to Image**: Converts the table (vector map) of a PALM image to a bona fide image.
- **Localization Precision**: Attaches to an Experimental PSF a localization precision look up table based on simulations.

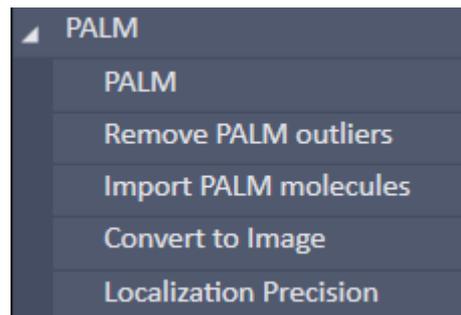


Fig. 168 Processing – PALM

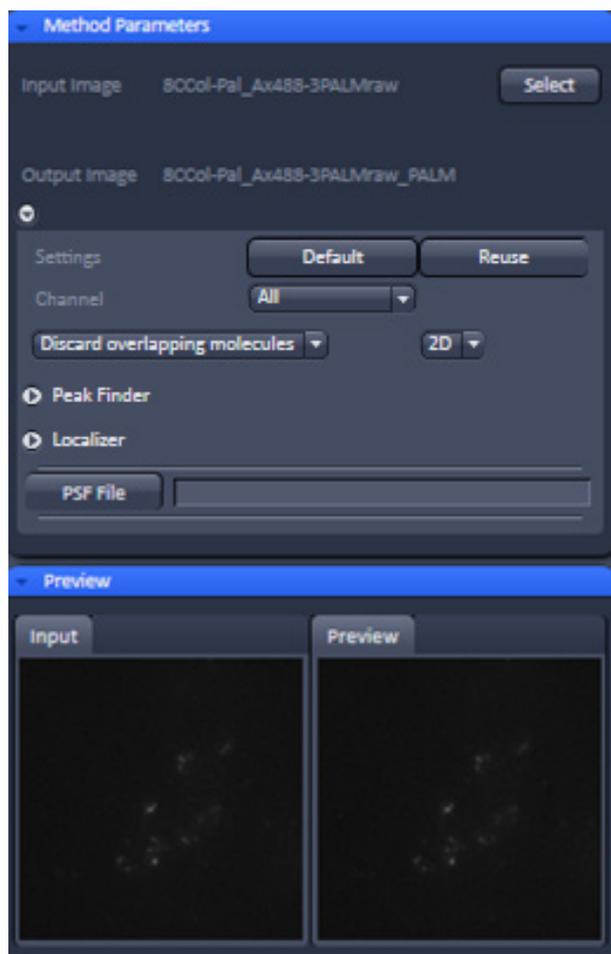


Fig. 169 Processing – PALM – PALM



Fig. 170 Channel drop down menu of a dual channel data set

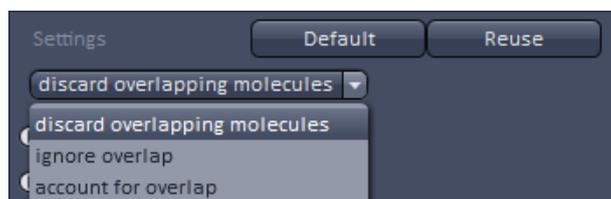


Fig. 171 Settings drop down menu

### 3.3.13.1 PALM – PALM

The **PALM** function allows setting filters for peak finding and choosing a fit model for the localization calculations.

Select the **PALM** tab to select the **PALM** Processing tool (Fig. 168). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the **Peak Finder** and **Localizer** (Fig. 169).

 You have to select the subdirectory **PALM** tab in order to have the PALM functionalities available. The directory **PALM** tab will not give access to the PALM tools.

Pressing the **Settings Default** button will reset all filter and parameter settings to the default values.

Pressing the **Settings Reuse** button will recall the parameter settings of the actively selected image.

The **Channel** drop down menu allows you in a multi-channel experiment to apply different settings to different channels, which are all listed (Fig. 170). If you select **All**, all channels will be fitted the same way. If you select a channel, and change settings, these setting will apply only to this channel. Note, even if only one channel is selected, every channel will be fitted according to the last settings.

The **Settings** drop down menu (Fig. 171) will allow you to define how peak events will be fitted. You have three options.

- **Discard overlapping molecules** (single emitter): only single emitters will be taken into account and fitted, whereas all multi-emitter events will be discarded.
- **Ignore overlap** (ignore emitter): all single- and multi-emitter events will be taken into account and fitted as single emitter events.

- **Account for overlap** (multi-emitter): multi-emitter events will be fitted taking into account the overlap. If selected, the **Maximum cluster size** slider and input box become available (Fig. 172). Here you can select the maximum number of molecules in the cluster that are used for computation. So a number of 10 means, for example, that clusters with 10 or less peaks will be taken, and those exceeding 10 will be discarded. We recommend to limit cluster size to 10 as otherwise computation can become time consuming.



Fig. 172 Maximum cluster size field

White circles indicate that the particular emitters are used (if channel color is grey; otherwise the channel color is used), red circles that the emitters are discarded for the fitting process. For account of overlap the color stays white or in the channel color regardless if a cluster may be discarded due to the **Maximum cluster size** setting.

The **Dimension** drop down menu will display the dimensionality (2D or 3D) of the selected PALM image, which is automatically recognized if a .czi format is loaded (Fig. 173). It is possible to overrule the automatically chosen value.



Fig. 173 Dimension drop down menu

In the **Channel** drop down menu you can select the channel for which the Processing should be conducted for a multi-channel image.

Expand the **Peak Finder** field to have access to the peak selection filters (Fig. 174). Two filters are available:

- **Peak mask size**
- **Peak intensity to noise**

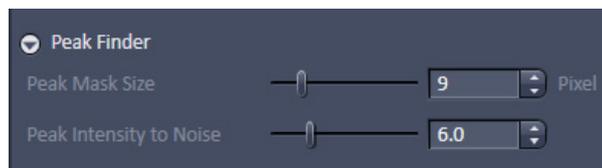


Fig. 174 Peak Finder Expansion field

Use the sliders or input spin boxes to set the corresponding values.

 We recommend to start with the default values for the peak mask size (9 for 2D; 19 for 3D data) and then optimize on behalf of the results. Note that in 3D-PALM using PRLIM (phase ramp localization imaging microscopy) with a double phase ramp, the PSF is split into two lobes (Fig. 175) that have a larger expansion than the classical PSF.

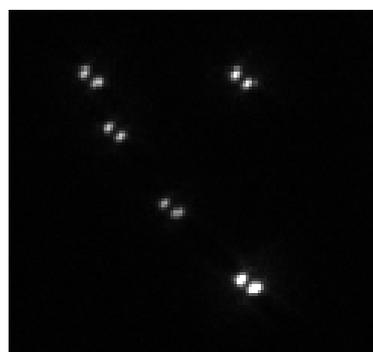


Fig. 175 Double lobed PSF created by a double phase-ramp in the pupil plane

The **Peak mask size** filter defines the radius in **pixel** units for a detected peak intensity that will be used to cut out the peak and put the peak into a new coordinate system. The size will also be used when fitting a 2D Gauss function to the intensity distribution of the peak.

If the image was selected by pressing the **Select** button, the **Preview** window of the Image in the image container will show all identified peaks circled (Fig. 176). The radius of the circles will correspond to the set value of the **Peak mask size** (Fig. 177).

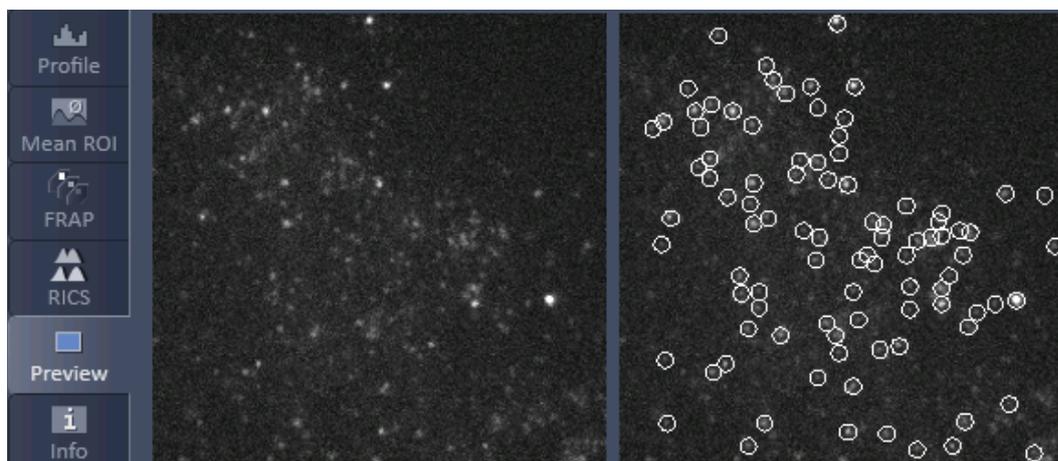


Fig. 176 Preview window of the image container for PALM processing

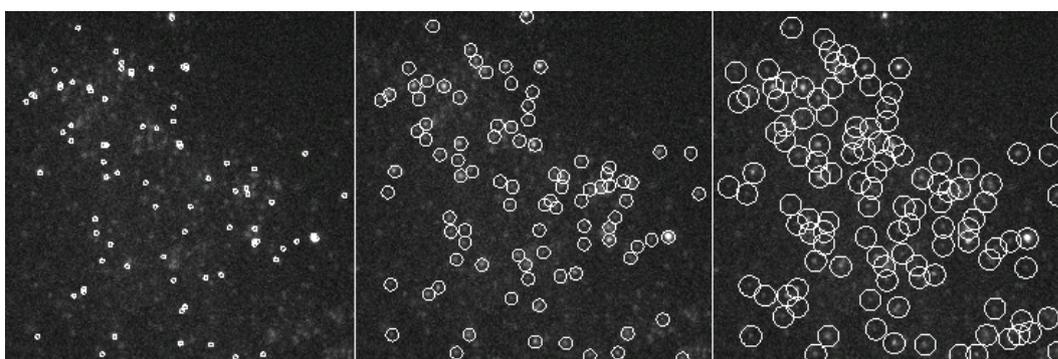


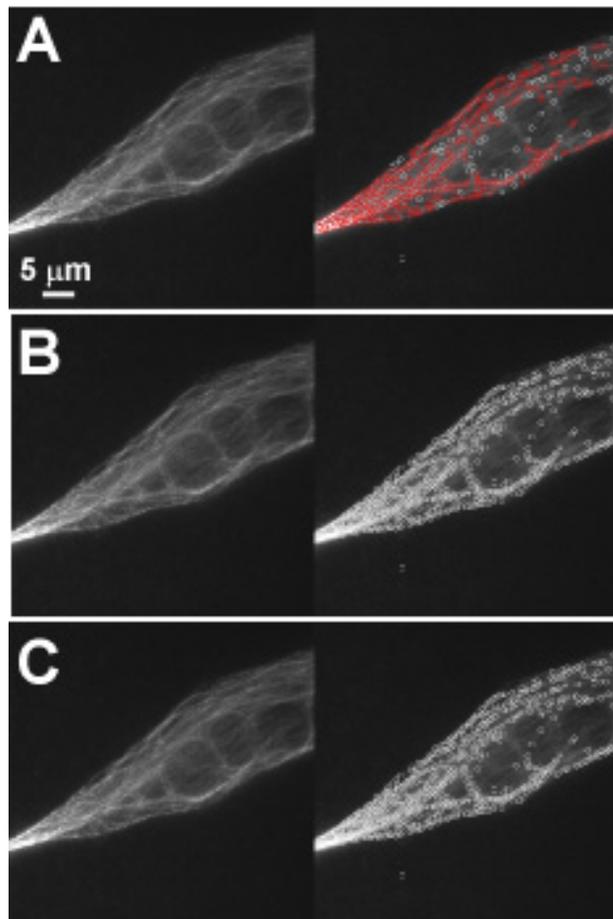
Fig. 177 Preview window of the image container for PALM processing with the Peak mask size set to 3 (left), 9 (middle) and 15 (right) pixels

☞ The bigger the radius the more prone are closely juxtaposed peaks to overlap. The smaller the circles the more likely it is that one peak with a broad intensity distribution will be taken as two or multiple peaks. The preview can be taken to decide what the best radius will be. 9 and 19 pixels will work in > 95 % of the cases for 2D and 3D PALM experiments, respectively.

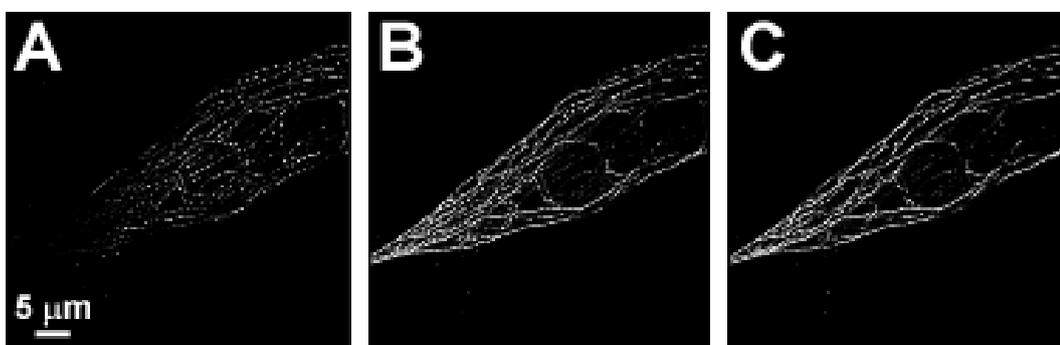
☞ If a circle around a peak lying at the borders of the image does not fit completely within the image, the peak is discarded.

If the **discard overlapping molecules** was selected from the **Settings** drop down menu, all identified peaks with overlapping circles will be indicated in red and discarded (Fig. 178). If **ignore overlap** or **account for overlap** was selected, overlapping peaks will not be indicated and all peaks will be circled in white.

- If **discard overlapping molecules** is selected, all overlapping peaks, indicated by red circles will be discarded. This will result in the highest accuracy, which is defined as the deviation of the fitted to the real peak position and localization precision (the standard deviation from the fit) (Fig. 179, **A**). This method is therefore the method of choice for single emitters and little overlap.
- If **ignore overlap** is selected, all single and overlapping peaks will be taken into account. However, fit accuracy will be decreased when multi-emitter events are fitted as the distribution of one emitter will influence the distribution of an overlapping one without this fact taken into account. Hence accuracy might suffer for multi-emitter events whereas localization precision will be sustained. (Fig. 179, **B**). This method is most suitable for slightly overlapping molecules.
- If **account for overlap** is selected all single and multi-emitter events will be taken into account. For multi-emitters the overlap of neighboring molecules is taken into account so losses in accuracy are minimized. There will be a slight decrease in the localization precision (Fig. 179, **C**). This method should be used, when molecules heavily overlap.



**Fig. 178** Preview window of the image container for PALM processing with the discard molecules (A), ignore overlap (B) and account for overlap (C) selected

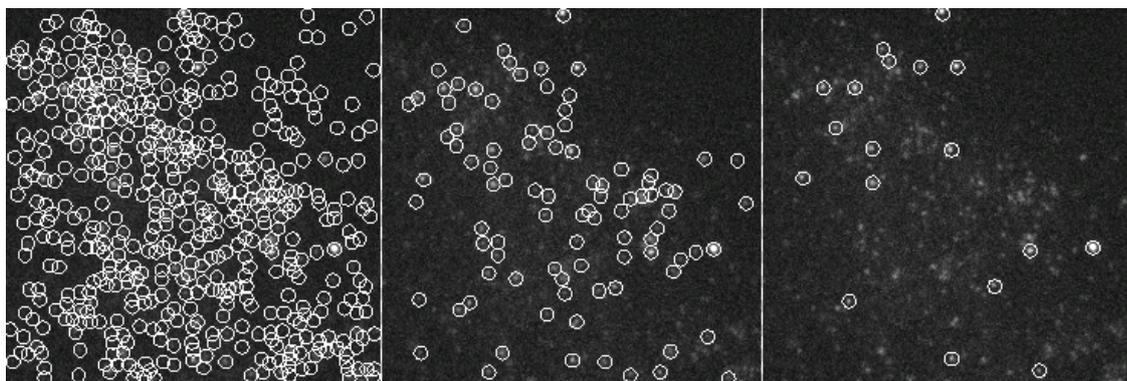


**Fig. 179** Analyzed data set of Fig. 178 using discard overlapping molecules (A), ignore overlap (B) and account for overlap (C).

The **Peak intensity to noise** filter defines the threshold of the signal strength of an identified peak over background, at which the peak is regarded to be genuine. The filter works as follows:

- The highest intensity peak with the intensity  $I$  is identified in the frame
- The mean intensity  $M$  and the standard deviation  $SD$  is calculated from the frame
- The Peak intensity to noise (PI) is set.
- If  $I - M > SD \times PI$  than the peak is regarded to be genuine
- The peak is cut out of the frame and the next highest intensity peak is selected.
- The procedure is repeated until  $I - M < SD \times PI$ . Than the peak finding process will end for that frame.

All identified peaks above the threshold will be displayed per frame in the Preview window (Fig. 180).



**Fig. 180** Preview window of the image container for PALM processing with the Peak intensity to noise set to 3 (left), 6 (middle) and 10 (right)



The higher the **Peak intensity to noise** factor, the more stringent is the peak selection. Too low numbers will result in noise being falsely identified as peaks; too high numbers will miss some genuine peaks. The preview can be taken to decide what the best value will be for the particular image series. The default value of 6 will work in > 95 % of the cases.

Expand the **Localizer** field to have access to the fit model used to localize peaks (Fig. 181). Two fit models are available that can be selected by the **Fit Model** drop down menu (Fig. 182)

- **x,y 2D Gauss Fit**
- **Mask fit**
- **File PSF**

If **3D** is selected from the **Dimension** drop down menu, only **File PSF** is available. You should load an Experimental PSF with linked look up table for localization precision in order to obtain localization precisions in z in addition to x and y.

If **x,y 2D Gauss Fit** is selected, the intensity distributions of the identified peaks are fitted to a full 2D Gaussian function. The fit in each direction takes into account the influence of the other direction (x,y 2D Gauss).

If **Mask Fit** is selected, a Gaussian mask with a defined PSF width is used to match as best as possible the intensity distribution of a peak in x and y (Fig. 183). The PSF width can be set via the PSF Width slider or input spin box. Default value is the theoretical calculated PSF based on the objective lens and laser line used.

 Note that with the **Mask Fit** only one predefined PSF width will be used. Computation of the Fit will be faster for the **Mask Fit**, but less precise.

If **File PSF** is selected, you can select a PSF that will be used (Fig. 184). To this end press the **PSF file** button. This will open Windows Explorer, where you can select an appropriate file, the name of which will be displayed in the **PSF file** display box.

 The fit model in 2D defines the PSF width, which will be used to fit to the experimental results. It does not correspond to the size of the peak that is defined by the Peak Mask Size and that in the end will define the overlapping degree of neighboring peaks. In 3D the PSF with the attached look up table is a simulation where the PSF was modulated.

 The settings of the **Localizer** will not influence the peak finding process and hence the preview image.

 In the Processing **PALM** function, peak finding and localization are always linked. If you want to just view localized peaks (molecules), you might select in the **PAL-Render** tab of the PALM image in the **Display** drop down menu **Centroids**.



Fig. 181 Localizer Expansion field with x,y 2D Gauss Fit selected

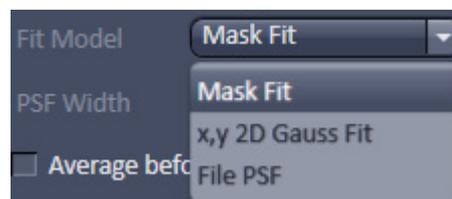


Fig. 182 Fit Model drop down menu



Fig. 183 Localizer Expansion field with Mask Fit selected

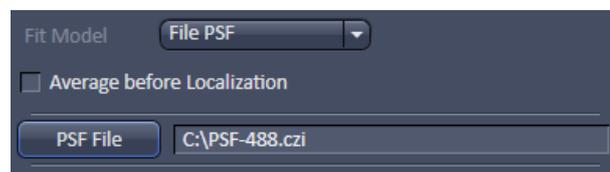


Fig. 184 File PSF menu



**Fig. 185** Localizer Expansion field with Average before localization selected

If the **Average before Localization** check box remains inactive, every peak (event) will be localized independently and is regarded as being derived from one molecule. Later on, peaks can be grouped belonging to one molecule in the image container in the **PAL-Grouping** tab of the image (**localization before grouping**). If the check box is ticked, however, the system will group peaks (events) based on the filter settings that become available (Fig. 185) before localizing the grouped peaks (**grouping before localization**).

When **localization before grouping** is performed, the single peaks are first localized and then the localizations averaged. When **grouping before localization** is performed, then the peaks are first averaged and then localized.



**Fig. 186** Drift correction panel

There will be two filter settings when one performs grouping before localization:

- Filters for **Drift Correction** based on fiducial markers (like gold particles).
- Filters for **Grouping** the peaks (events).

Expand the **Drift Correction** field to have access to the **Drift Correction** tool (Fig. 186). If you want to perform a drift correction the **Drift Correction** check box must be ticked.

Note that for lateral drift correction the system needs to identify at least one fiducial marker based on the **Drift Correction** filter settings. Fiducial markers could be gold particles deposited on the glass surface of the dish. If the system cannot find a fiducial, no drift correction will be performed and in the **PAL-Drift** tab of the image no fiducials will be listed in the **Fiducial** table.

You can set two parameters to be associated with a fiducial by using the sliders or the spin input boxes:

- **Min on time [%]**. This defines the minimal number of frames out of the total frames acquired in percentage the molecule has to be on in order to be regarded as a fiducial. E. g. 75 % means that the molecule has to be on at least  $\frac{3}{4}$  of all the frames.
- **Capture radius [pixel]**. This defines the radius around a fiducial in which no other fiducial should be localized in order to be taken as a fiducial. E. g. if 2 pixels was set and within two pixels another fiducial is present, than both fiducials will be discarded.

Note that the settings of the **Drift Correction** will not influence the **Preview** image.

Note that using **Drift Correction** in the Processing tool is irreversible. Therefore, in the created image all drift related functions in the **PAL-Drift** tab will be inaccessible and greyed out.

Expand the **Grouping** field to have access to the **Grouping** tool (Fig. 187).

You can set two filters to be taken into consideration when peaks (events) are grouped:

- **Off Gap.** If peaks within the defined **Capture Radius** will be interrupted by more than the set value of consecutive frames, they will not be regarded belonging to the same molecules.
- **Capture radius [pixel].** If peaks in consecutive frames are not localized within the specified radius they are not regarded as belonging to the same molecule.

 Note that the settings of the **Grouping** will not influence the **Preview** image.

 Note that using **Grouping** in the processing tool is irreversible. Therefore, in the created **PAL-Grouping** tab all functions will be greyed out and not accessible.

If all parameters and filters are set press the **Apply** button. This will create the PALM image in the image container and the **PALM** view tab becomes available that allows further processing and analysis of the image. The container will display PALM image (**HR** channel) as well as the sum wide field image (**SWF** channel – not available for 3D-PALM) (Fig. 188).

 Note that in contrast to the TIRF image, which is the raw image of the point spread functions (PSFs), the sum wide-field image is the background corrected image of the PSFs and the PALM image is a vector map of centroids.

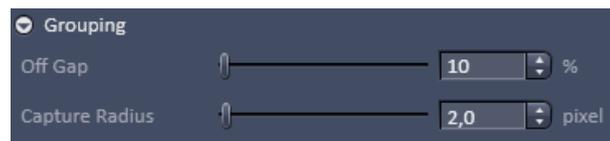


Fig. 187 Grouping panel

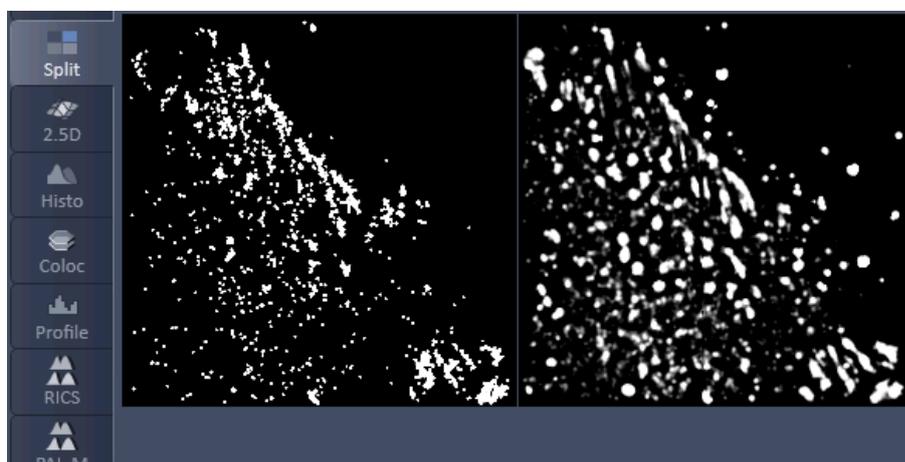


Fig. 188 PALM image (left) and sum widefield image (right) after PALM Processing

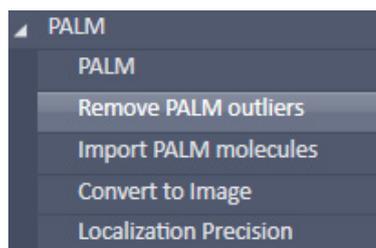


Fig. 189 Processing – Remove PALM outliers tool selected

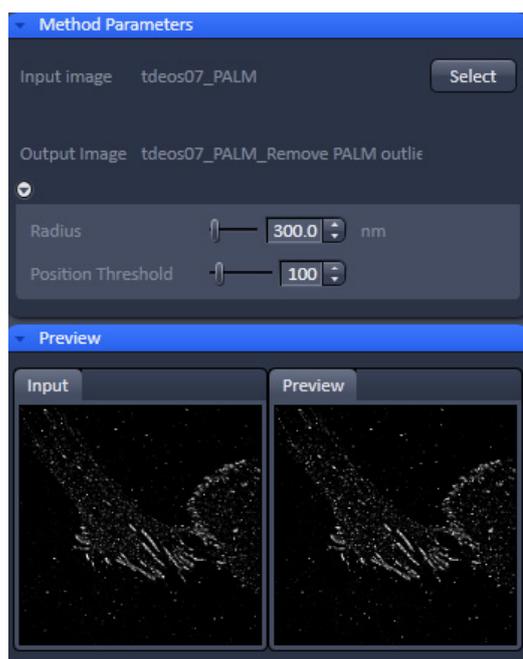


Fig. 190 Processing – PALM Remove PALM outliers



Fig. 191 Processing – PALM Remove Outliers message box

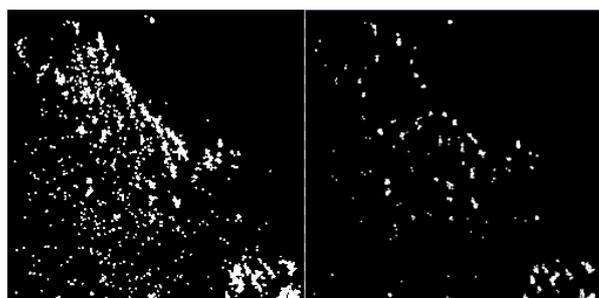


Fig. 192 PALM images before (left) and after (left) removal of outliers; Radius set to 3000 and Position Threshold to 100. Only those peaks / molecules in denser structures remain.

### 3.3.13.2 PALM – Remove PALM Outliers

The **Remove PALM outliers** function allows removing all molecules that within a defined radius will have less than the specified numbers of neighboring molecules.

Select the **Remove PALM outliers** tab to select the **Remove PALM outliers** processing tool (Fig. 189). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the filter functions (Fig. 190).

You can set two parameters by using the sliders or the spin input boxes:

**Radius [nm].** The radius defines the distance from a localized position of a peak (molecule) in which all peaks falling within that distance are counted.

**Position threshold.** If the number of counted neighboring peaks (molecules) falls short of the set value, the respective peak (molecule) will be removed from the list and the PALM image.

This tool will emphasize densely labeled structures and can help remove floating molecules and ghost signals.

This tool works only for PALM images.

After setting the thresholds, press **Apply** to create the new image. A message will be displayed telling how many molecules have been removed from the input PALM image (Fig. 191).

Press **Ok** to have access to the newly computed image (Fig. 192).

Please note that for 3D-PALM removing outliers applies only in 2D for a maximum intensity projection of the Z-Stack. Removing outliers does not apply for the z direction.

### 3.3.13.3 PALM – Import PALM Molecules

The **Import PALM molecules** function allows importing PALM molecule tables saved as text files (extension .txt) and reconstruct a PALM image from the table.

Select the **Import PALM molecules** tab to select the **Import PALM molecules** processing tool (Fig. 193).

Press **Select** (Fig. 194) to open **Windows Explorer** and select the molecule table you want to import. Press **Open** to load the table and then press **Apply** to create the PALM and sum wide field images (Fig. 195).

 There are no tools available if the **Methods Parameters** tool area is expanded.

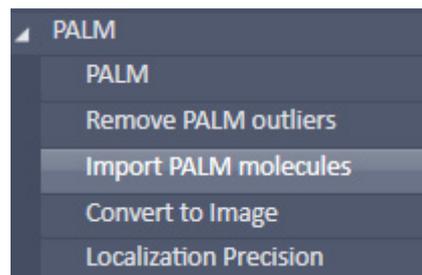


Fig. 193 Processing – Import PALM molecules tool selected

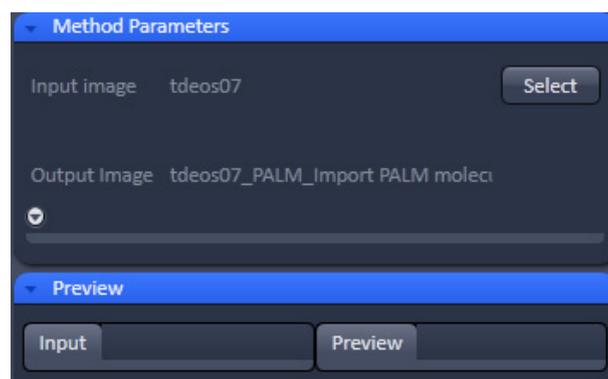


Fig. 194 Processing – Import PALM molecules

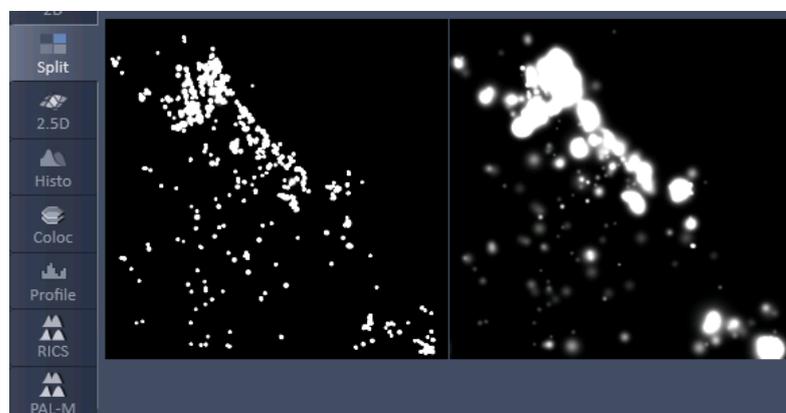


Fig. 195 PALM image (left) and sum wide field image (right) in the Split mode after importing a molecule table

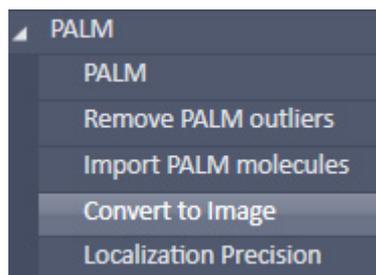


Fig. 196 Processing – Convert to Image tool selected

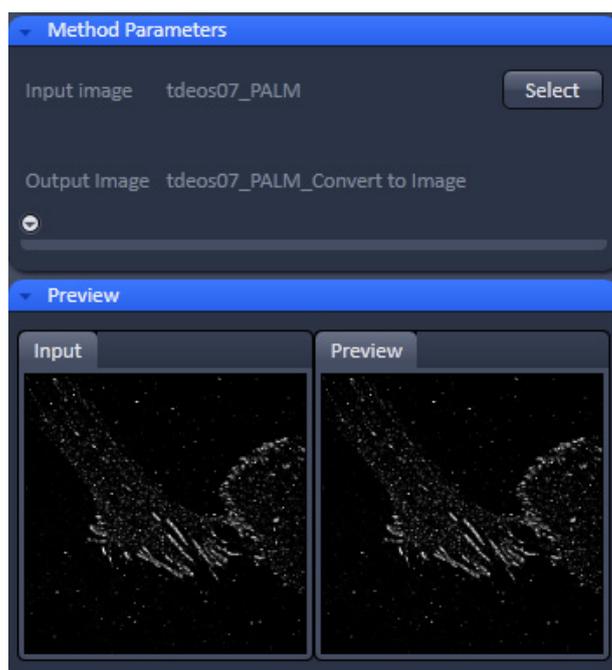


Fig. 197 Processing – Convert to image

### 3.3.13.4 PALM – Convert to Image

The **Convert to Image** function allows converting the vector map of centroids to be transformed to a bona fide image.

Select the **Convert to Image** tab to select the **Convert to Image** processing tool (Fig. 196).

Highlight the PALM image in the image container you want to convert and press **Select** (Fig. 197). Press **Apply** to convert the HR channel to a true image.

The new file has the extension "name\_PALM-ConverttoImage" as a default.

It contains the **HR** channel and **SWF** channel as grey value pixel images for 2D PALM. For 3D PALM it will contain the z-stack HR image file; no SWF image is available for 3D PALM.

### 3.3.13.5 PALM – Localization Precision

Select **Localization Precision** to attach a Localization Precision look up table (LUT) to an Experimental PSF (Fig. 198).

Highlight the Image with the Experimental PSF (should be derived from the PSFs obtained by a 4 µm stack of 100 nm beads) and press **Select** (Fig. 199).

Press apply to obtain a file of the Experimental PSF with LUT attached. The file bears the extension "LocalizationPrecision".

 Please note that the localization precision will be estimated using a Monte-Carlo Simulation of the experimental PSF including noise. The generated lookup table will then contain the localization precision depending on the three parameters photon number, background variance and Z position.

 The Experimental PSF with attache LUT should be used when fitting 3D PALM data. If only an Experimental PSF without LUT is used, a warning message pops up (Fig. 200). Press **No** to cancel processing or **Yes** if you want to continue. In this case, only a rough estimate of the localizatzion precision will be given.



Fig. 198 Processing – PALM Localization Precision tool selected

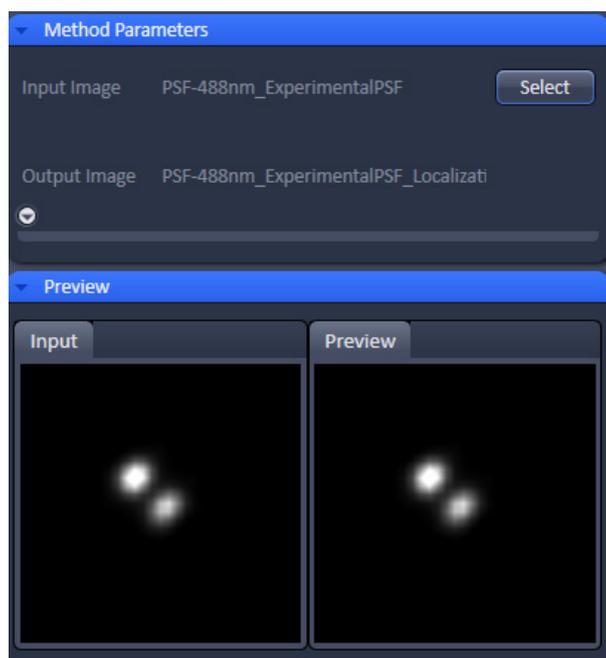


Fig. 199 Processing – PALM - Localization Precision with double-lobed Experimental PSF loaded

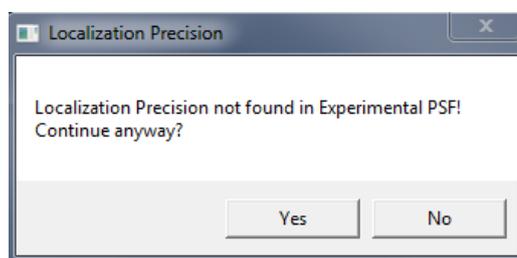


Fig. 200 Localization precision warning

### 3.3.14 Processing – Particle Tracking

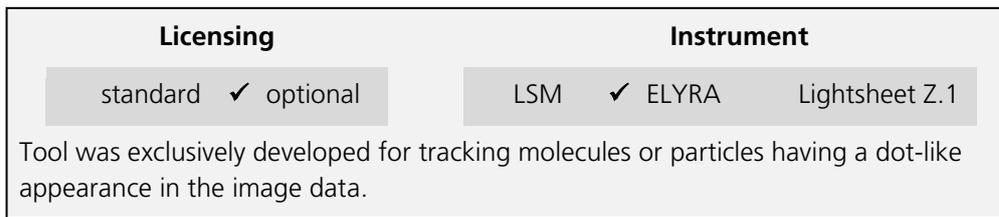


Fig. 201 Processing – Particle Tracking tool selected

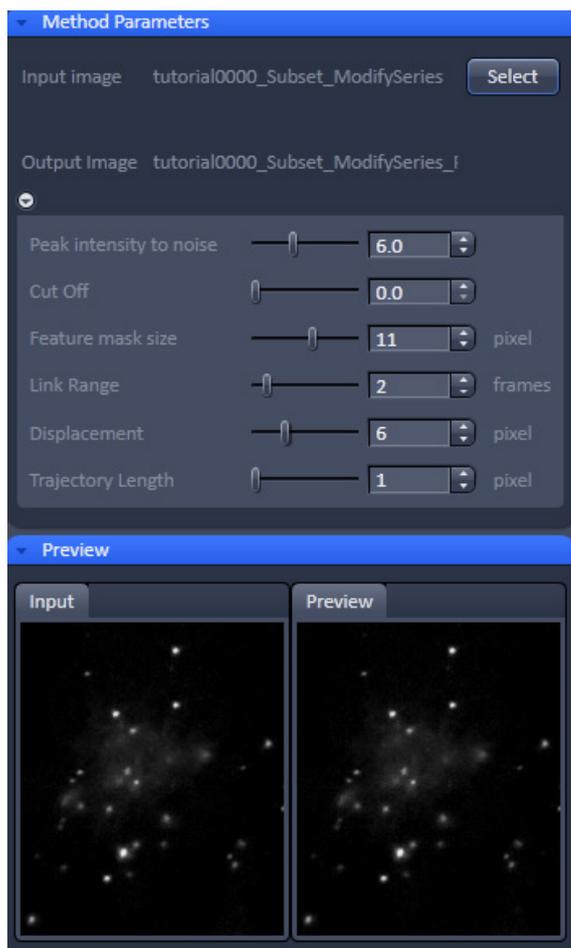


Fig. 202 Processing – Particle Tracking tool field

The Processing **Particle Tracking** tool allows the identification of single molecules and follows their movement. Trajectories will be displayed.

In the image you have than the possibility in the **Particle Tracking** tab to calculate the mean square displacement and diffusion coefficients of the single molecules.

Highlight the **Particle Tracking** field to have access to the **Particle Tracking** tool (Fig. 201).

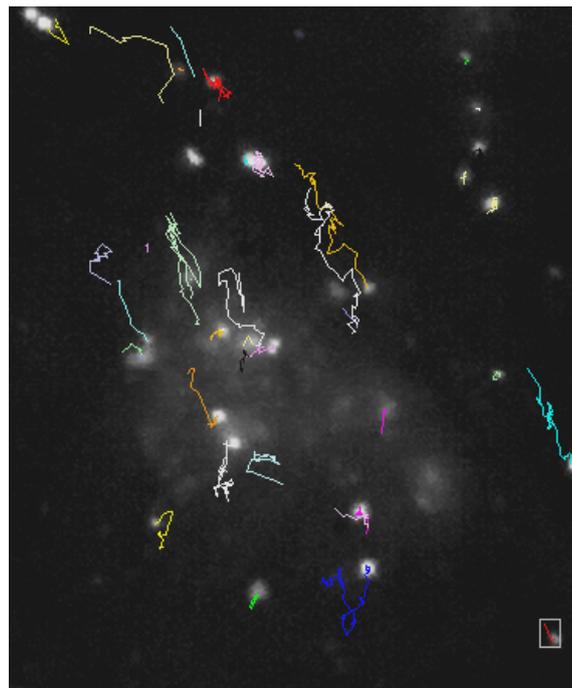
Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the **Particle Tracking** tools (Fig. 202).

You can set different parameters to define the molecules and the calculation of the trajectories:

- **Peak intensity to noise.** Sets the signal over noise threshold according to  $I-M > SS \times PI$  ( $I$  = intensity of particle;  $M$  = mean intensity of image frame;  $SD$  = standard deviation of mage frame;  $PI$  = peak intensity to noise).
- **Cut off.** Sets a filter to suppress molecules that deviate from the ideal Gauss shape, for example it there is a background structure.
- **Feature mask size [pixel].** Determines the mask used to cut out the particle.
- **Link range [frames].** Sets the lower limit in frames over which the particle has to be detected.
- **Displacement [pixel].** Sets the upper limit of distance the particle is lowed to have from one frame to the next.
- **Trajectory Length [pixel].** Sets the lower limit in the trajectory length a particle must have over the observed time period in order to be considered a particle.

Once the settings are complete press **Apply** to obtain the trajectories of the single molecules (Fig. 203).

 Please note, that Particle Tracking only works for 2D data sets.



**Fig. 203** Image with trajectories of single molecules. The selected trajectory is boxed

### 3.3.15 Processing – Experimental PSF

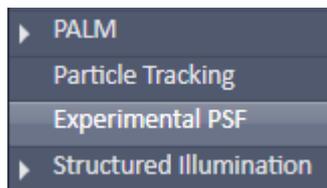
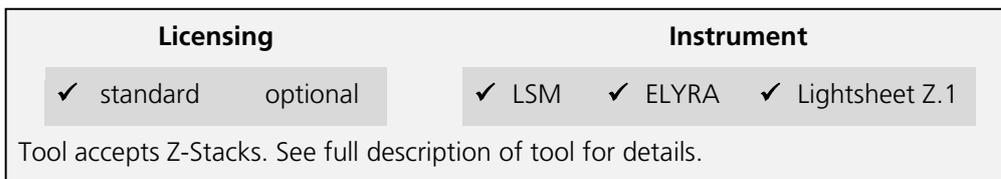


Fig. 204 Processing – Experimental PSF tool

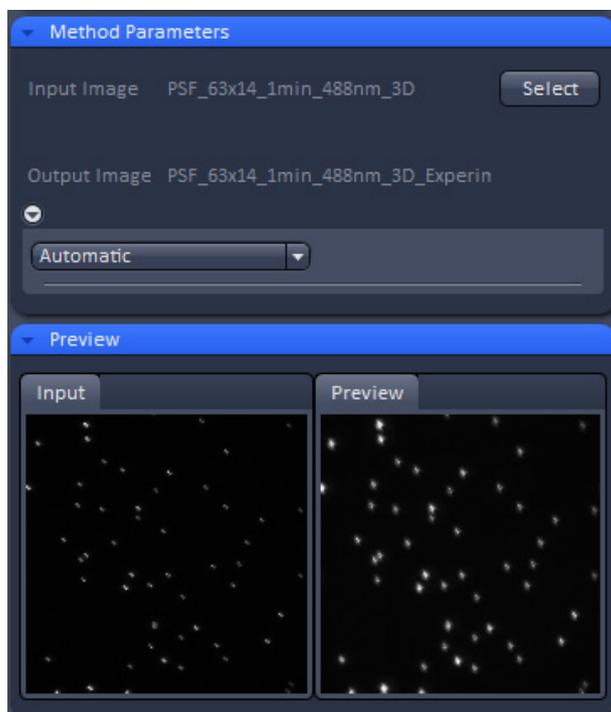


Fig. 205 Experimental PSF – automatic mode

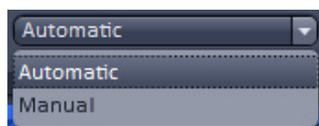


Fig. 206 Mode drop down menu

This function allows determining the point-spread function (PSF) of sub-resolution beads and using this experimentally determined PSF as input for Structured Illumination processing.

Select the **Experimental PSF** tab to select the **Experimental PSF** Processing tool (Fig. 204). Activate the image (a 3D stack of sub resolution beads) you want to process in the image container and press the **Select** button for **Input Image** to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 205).

 You can load a 3D stack of a wide field image or a non-processed raw data SIM image. LSM image formats are not accepted to determine the **Experimental PSF**.

You have the possibility to automatically compute the Experimental PSF by choosing **Automatic** from **Mode** drop down menu (Fig. 206).

If you want to process manually and set quality parameters, select **Manual** (Fig. 207).

Pressing the **Default** button will load Standard values. Data will only be reused, if the result image is selected. Than pressing the **Reuse** button will load the values of the selected image. The options **Symmetrical** and **Damp Edge** are not included in the Reuse function.

When the **Symmetrical** check box is ticked, a radial symmetrization of the PSF will be performed. That means that a perfectly round PSF is calculated, even out of an oval PSF.

When the **Damp edge** check box is ticked, the edge of the PSF will be smoothed (with a smooth transition to zero at the edge).

The **PSF sampling** pull down menu allows you to interpolate the result to different degrees. Since the resulting PSF is an average of many PSFs corresponding to the localized beads and every bead is localized with the sub-pixel accuracy, one can obtain after averaging a PSF with higher resolution.

You can average the PSF by **1** (pixel size of the resulting PSF is the same or **1** times as in the original image), **3** (pixel size of the resulting PSF is **3** times smaller as in the original image), **5** (pixel size of the resulting PSF is **5** times smaller as in the original image) or **7** (pixel size of the resulting PSF is **7** times smaller as in the original image) times (Fig. 208).

The **3D PALM** box will be automatically checked, if the image data was acquired with the 3D-PALM slider inserted. In this case the double lobe structure of each bead will be aligned to the right lobe.

In the **Background** drop down menu you can decide the way background is determined (Fig. 209).

You have three options:

- **Local:** Background is computed from the periphery area of the mask defined in **Peak Mask Size**.
- **Histogramm:** Background is calculated from the histogram as at 10% cut off value.
- **ROI:** Background is computed from ROIs in the image (**drawn** with the Graphics tool). Note, this option is only available if ROIs have been defined in the image.

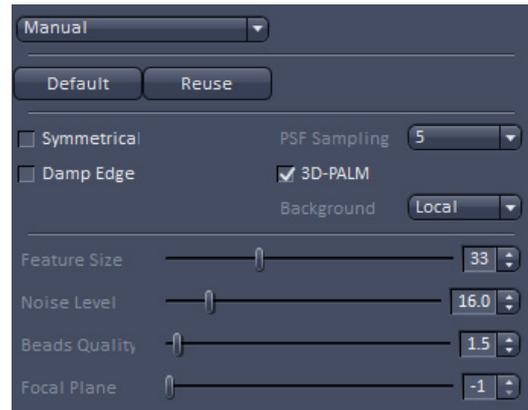


Fig. 207 Experimental PSF – manual mode

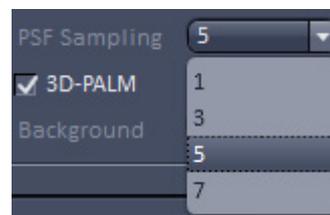


Fig. 208 PSF sampling drop down menu

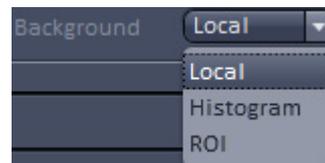
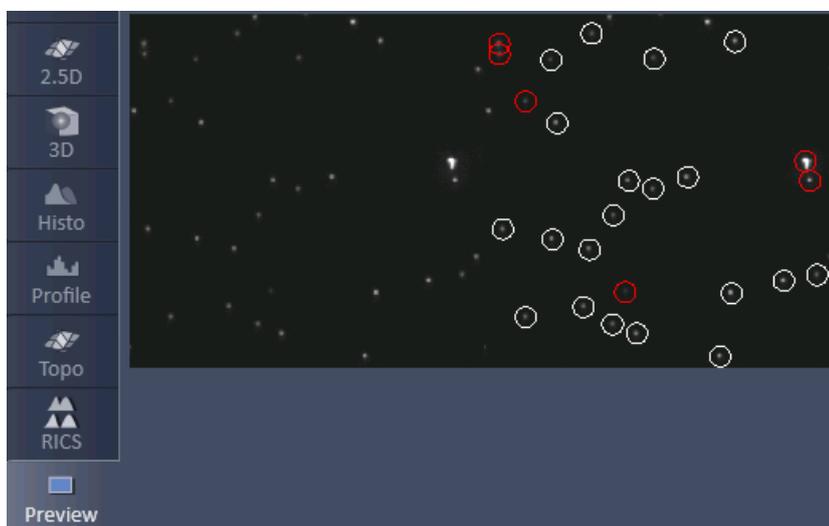


Fig. 209 Background drop down menu

There are different filters that can be set by corresponding sliders or spin input boxes to select sub-resolution beads.

**Feature size** sets the mask size that is used to cut out the bead and to create the **Experimental PSF**. The extensions are displayed in the **Preview** tab of the image container as circles (Fig. 210).

White circles indicate that the particular PSFs are used (if channel color is grey; otherwise the channel color is used), red circles that the PSF are discarded for the calculation of the Experimental PSF.



**Fig. 210** Selection of beads in Preview tab

**Noise Level** sets the threshold of the intensity of the sub-resolution beads over background according to  $I - M > SD \times NL$  ( $I$  = intensity of bead;  $M$  = mean intensity of image frame;  $SD$  = standard deviation of image frame;  $NL$  = noise level). Lowest number means that everything (even noise) is taken into account; highest number means only the brightest beads will be used.

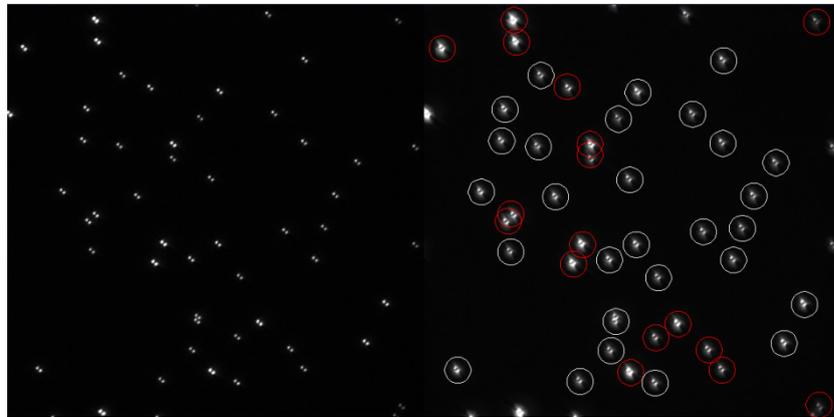
**Beads quality** evaluates two characteristics, the intensity (first moment) and the size (second moment). These two moments are calculated for every localized bead. Then the beads are sorted according to these characteristics, resulting in two sorted lists of beads. The beads that are closer to the medians in both orderings are assumed to have higher quality. The **Beads quality** defines how far the beads characteristics (intensity and size) may be away from the median value. The smaller the threshold, the closer beads should be to the median. Beads quality equals to 0 is likely to result in a single bead right in the middle. In this way also the bead shape (aspect ratio) is assessed. Lower input number means that only perfectly shaped round beads will be taken into account. Highest input numbers will take into account all beads, even ellipsoid shaped ones.

**Focal Plane** determines which plane is the focal plane. The default is **-1** (automatic). Here the system determines the focal plane automatically. The user can also select a frame, which is presumably the focal plane, by input the number of the frame within the stack.

All sub-resolution beads that will be taken into account will be circled by a white line. Those that do overlap with their circles or are considered unsuitable (beads beyond the quality thresholds) are outlined in red and will not be taken into account (Fig. 210).

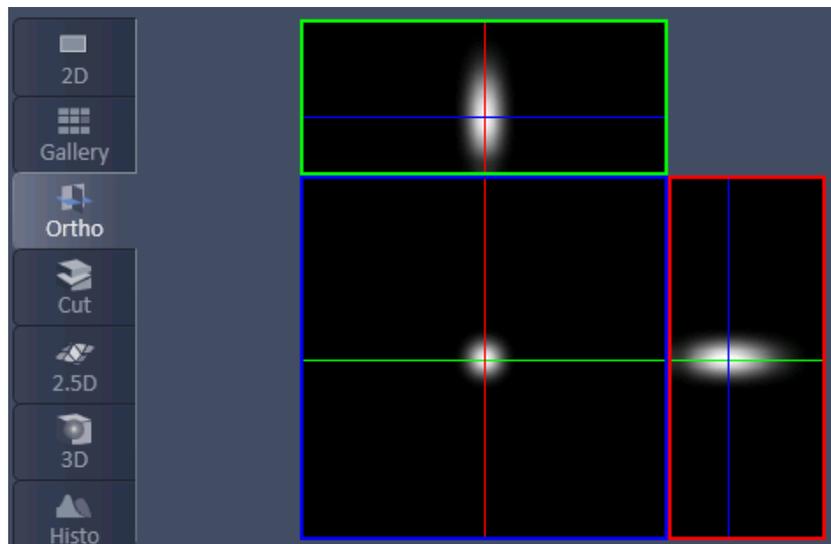
 Please note that you should use a 4  $\mu$ m stack of 100 nm beads, if you want to use the Experimental PSF for 3D PALM.

Once settings have been completed the **Preview** window displays beads that are not taken into account as red circles and those that are used in white circles (if channel color is grey) or in their channel color (if channel color is other than grey) (Fig. 211). Press apply to compute the Experimental PSF. The created file will have the extension "... .ExperimentalPSF. ...".



**Fig. 211** Preview window for bead selection using 3D PALM

☞ Best results for an Experimental PSF for 3D data will be obtained, if beads are seeded sparsely, for example 10 - 20 beads evenly distributed in a 512 x 512 image.



**Fig. 212** Experimental PSF in Ortho view

If **Apply** is pressed an average PSF from all the beads taken into account will be computed (Fig. 212). For each channel a separate PSF will be displayed. The **Experimental PSF** can be saved (format .czi) and used for the **Structured Illumination - Structured Illumination** Processing tool in place of the **Theoretical PSF**. The size and pixel size of the **Experimental PSF** are recalculated and the PSF is interpolated to match the processed image.

### 3.3.16 Processing – Structured Illumination

Licensing	Instrument
standard ✓ optional	LSM ✓ ELYRA Lightsheet Z.1
Tool accepts data recorded in SIM mode on ELYRA. In data sets containing other types of image data as well ("mixed mode" files), this tool selectively processes the SIM data. "Experimental PSF" (requires Z-stacks of point-like objects) and "Fourier Transform" accepts input other than SIM data as well. See full description of tool set for details.	

The **Processing Structured Illumination** tool works exclusively for data acquired in the **SIM imaging** mode. It subdivides in 4 subcategories (Fig. 213):

- **Structured Illumination:** calculates the high resolution image from the set of structured images
- **TIRF filtered:** Will apply the TIRF sectioning to the SIM image if the TIRF was added as the last frame.
- **Modulation contrast:** Determines the modulation contrast from an image with beads to serve as a diagnostic tool
- **Fourier transform:** Calculates the spatial frequencies and displays them in an image. No phase information will be available.

### 3.3.16.1 Structured Illumination – Structured Illumination

The **Structured Illumination** function allows automatic or manual processing of a set of structured images obtained by phase shift and rotation of the grid.

Select the **Structured Illumination** tab to select the **Structured Illumination** Processing tool (Fig. 213). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 214).

 You have to use the subdirectory **Structured Illumination** tab when selecting the image to have access to the **Structured Illumination** tools. You will have no tools available is the directory **Structured Illumination** tab is selected.

If a region of interest (**ROI**) is defined in the image, then this **ROI** will be exclusively taken for image processing and the rest of the image will not be processed.

You can instead of processing only one selected image also load more images to be processed sequentially. To this end press the **Run Batch** button (Fig. 214). This will open **Windows** Explorer from which you can select all images you want to process. All selected files will be displayed in the **Batch display** window, which will appear when the files are opened by Windows explorer. The **Batch display** window also shows the current state of batch processing.



Fig. 213 Processing – Structured illumination tool selected

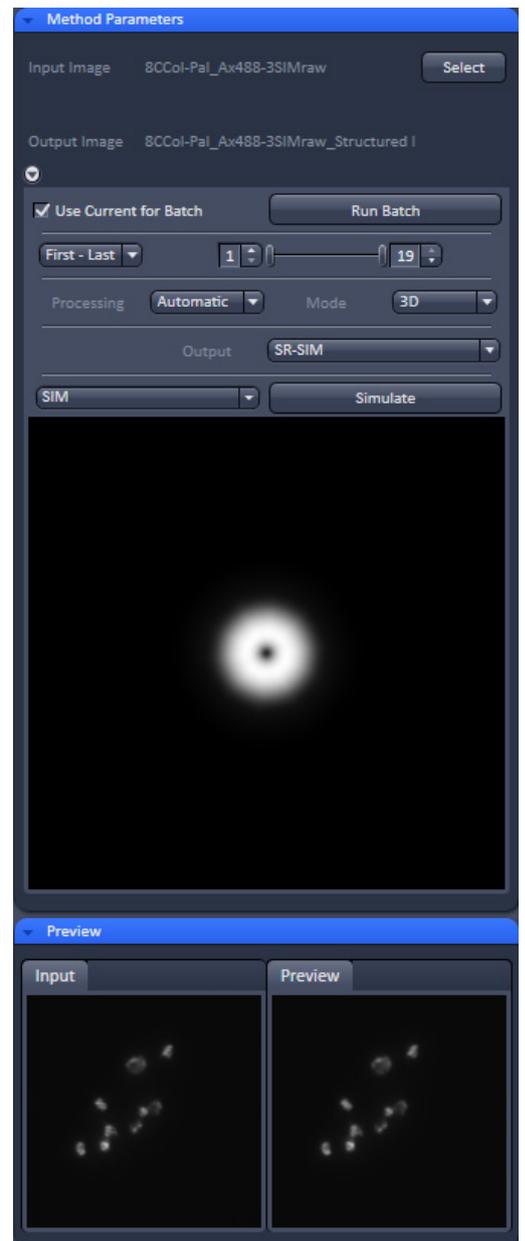


Fig. 214 Structured Illumination – Structured Illumination

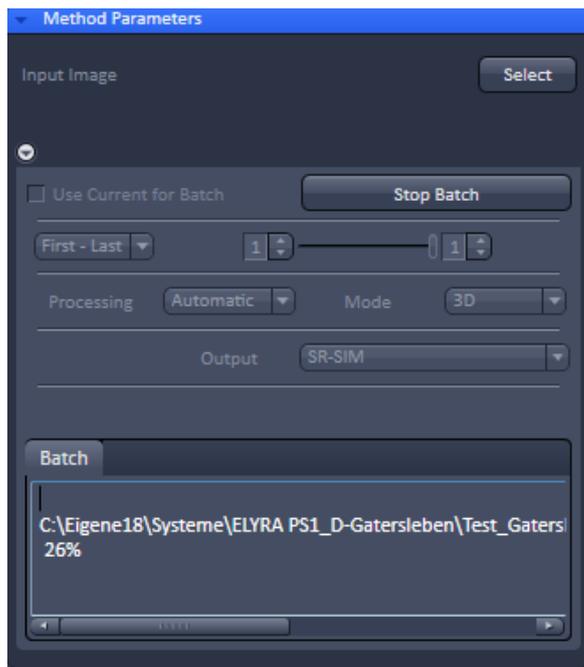


Fig. 215 Structured Illumination – Batch processing



Fig. 216 Stack processing mode drop down box



Fig. 217 Setting of the first and last frame of a stack to be processed



Fig. 218 Selection of one frame from a stack be processed



Fig. 219 Mode drop down box

If **3D** is selected, the information of the neighboring frames in regard to structuring information and out of focus contribution will be used in addition to the information of the frame itself. This will result in better x, y and z resolution and better contrast by removing the out of focus light to a greater extent at the cost of more computational power requirement.

The **Run Batch** button will turn into a **Stop Batch** button (Fig. 215) once pressed. If the button is pressed, batch processing will be stopped, no matter if **Apply** was pressed or not and the button turns back to **Run Batch**.

☞ In batch processing all images can only be processed with one parameter setting. If images, however, have been processed already once and are linked to processing parameters by pressing the **Attach** button, those settings will be taken and the general setting overruled. If you want to overrule attached settings and fit all Batch files with the set and displayed parameters, you have to check the **Use current for Batch** box.

If a Z-Stack was acquired you have the option to tell the system how the frames of the stack should be processed from the **Stack processing mode** drop down box (Fig. 216).

You have the option between **First-Last** and **Single**.

When **First-Last** is selected the double-slider or the left and right spin input boxes can be used to define the first and last frame of the stack that should be processed (Fig. 217).

When **Single** is selected, the slider or spin input box can be used to define one frame in the stack that should be processed (Fig. 218).

With the **Mode** drop down menu you have the option to select, how the single frames of a stack are processed (Fig. 219). You have the option between **2D**, **3D** and **3D large**.

☞ Note that **3D** and **3D large** are only available, when the image to be processed contains a stack of >1 and **First-Last** was selected as the **Stack processing mode**. Otherwise, the Mode drop down menu shows only **2D** and is greyed.

When **2D** is selected, every frame is reconstructed with the information of that frame only and the structuring in the thirds dimension is not used.

If the stack is large, available memory might be limited. In this case a **Warning** is issued, recommending the usage of the **3D large** mode (Fig. 220). In this case, the stack is subdivided into individual smaller stacks, which are individually processed. The result images of the individual stacks are then automatically combined into one output image.



Fig. 220 Warning for memory shortage

If one selects a region of interest (**ROI**) in the image, than that **ROI** will be used for image processing. This also can help in reducing memory problems when processing huge stacks.



Fig. 221 Processing drop down box

In the **Processing** drop down menu one can select between **Automatic** and **Manual** processing (Fig. 221). If **Automatic** is selected, pre-set filter and parameter values will be used.

If **Manual** is selected these filters and parameters become accessible to be changed and the **Manual selection** field becomes accessible (Fig. 222).

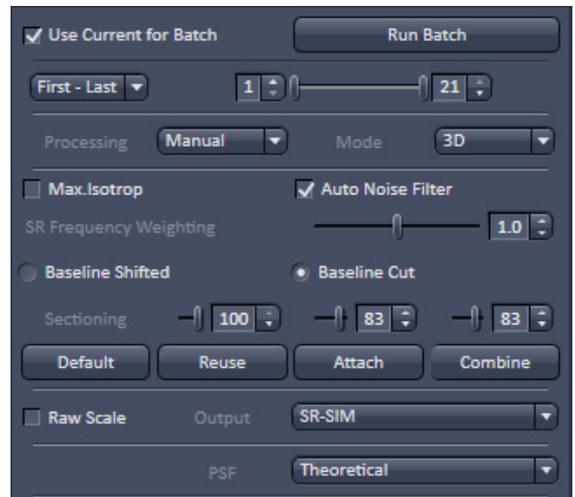


Fig. 222 The Manual selection field

 The **Automatic** mode is recommended for images with high modulation contrast. In case the modulation contrast is low one might benefit from changing the pre-set values.

In the **Channel** drop down menu (Fig. 223) **All** or single channels can be selected from a multi-channel image that should be processed. The **Channel** drop down menu is not displayed if the loaded image has only one channel.

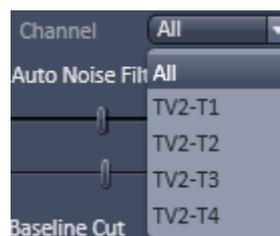


Fig. 223 Channel drop down box

Due to the limited number of rotations more frequencies and hence higher resolution information can be obtained exactly at the rotation angles. The directions between the rotational angles contain lesser frequencies. All available frequency information will be used if the **Max.Isotrop** checkbox is not ticked. This gives rise to the flower like appearance of the Fourier transform, which extends wider at the rotational angles.

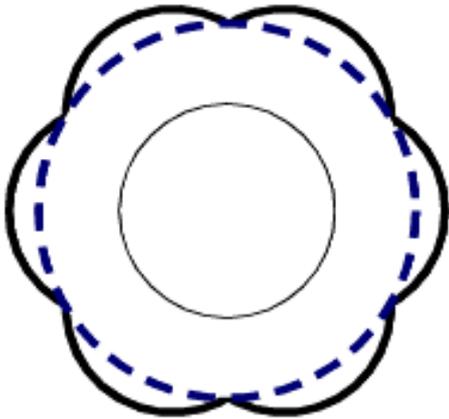


Fig. 224 Fourier transform with Max.Isotrop disabled (strong solid line) and enabled (blue stippled line). The inner circle (light solid line) is the Fourier transform of the non-structured image

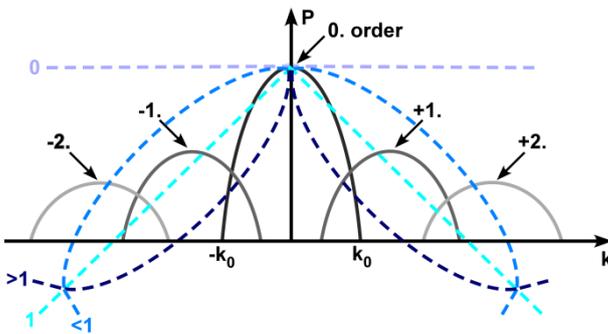


Fig. 225 Sketch of the influence of SR Frequency weighting filter (blue stippled lines) on the spatial frequencies

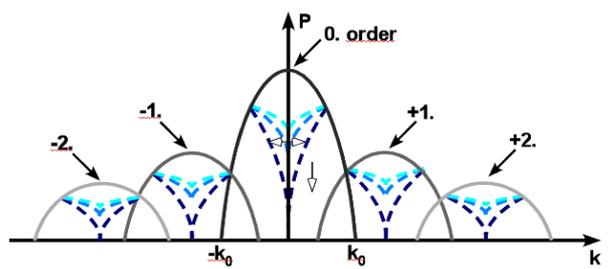


Fig. 226 Influence of sectioning filter (blue stippled lines) on the spatial frequencies. The open arrow indicates increasing strength, the double arrow indicates the variance, which is pre-defined

If the **Max.Isotrop** checkbox is ticked, the software will compute the high resolution image with the same resolution in every direction that is it does not use the highest frequencies in the rotational directions and the Fourier transform will be circle like (Fig. 224).

If the **Auto Noise Filter** checkbox is not ticked, the **Noise Filter** slider is available, that can be used to set the noise filter for the so called Wiener de-convolution performed. The negative number indicates the power to 10 for the filter. The smaller the values (towards -11), higher frequencies are allowed leading to noisier and crispier images. If the **Auto Noise Filter** is ticked, the **Noise Filter** slider is not displayed.

The **SR Frequency weighting** filter (also known as HD goal filter) will affect more prominently the higher frequencies. You can set the value by using the slider or the spin input box. The filter is a kind of cone that multiplies the Fourier transform of the resulting image. Weighting changes the slopes of the cone from the convex (towards 0) to concave (towards 2) (Fig. 225). 0 is no filtering, 1 is a straight line, <1 is convex, >1 concave.

You can toggle between **Baseline Shifted** and **Baseline Cut**. In **Baseline Shifted** negative values that can arise as an artifact by the **Noise** or Wiener filter will be kept and an offset is added to the whole image, so that the minimal (negative) intensity becomes zero intensity (baseline shifted). In **Baseline Cut** negative values will be clipped by setting them to zero. No offset is applied.

The **Sectioning** (or Strength) filter removes the 0-frequencies for the 0<sup>th</sup> order with a pre-defined variance. It also removes the 0-frequencies from the non-shifted 1<sup>st</sup> (+1, -1) and 2<sup>nd</sup> (+2, -2) orders. Therefore bumps and deeps might be occasionally detected in the Fourier transformed image at the positions defined to the rotation angle and grating frequency. The higher the value, the more stringent the filtering will be and more of the lower frequencies will be removed (Fig. 226). Three filter sliders and spin input boxes for the **0** order, the **1<sup>st</sup>** orders and the **2<sup>nd</sup>** orders are available (from left to right) for input.

All parameters will influence the Optical Transfer Function (OTF) used for reconstruction. The shape of the OTF in x, y is displayed in the **OTF display** window below the **Manual selection** field.

The **Preview** tab shows the **Input** image and a **Preview** of the output image. By pressing **Default** the current parameter values will be stored as default values. By pressing **Reuse** the last stored default values will be loaded.

Pressing **Attach** allows parameter values to be attached to an image. In normal case if any of the SIM parameters will be changed, they will be applied accordingly for the processing, but may not be saved into the image. Then if the image is saved, the values of the parameters applied for the image will be lost. If you attach the corresponding tag to the image by pressing the **Attach** button parameter values used to process the image will be attached to the image, even if it is not explicitly saved again. They will then be available for further processing, in particular for Batch processing.

In the **Output** drop down menu you can set, what kind of channels you want to display (Fig. 227). You have the option to display

- **SR-SIM + DCV + Widefield**
- **SR-SIM + DCV**
- **SR-SIM + Wide Field**
- **SR-SIM.**

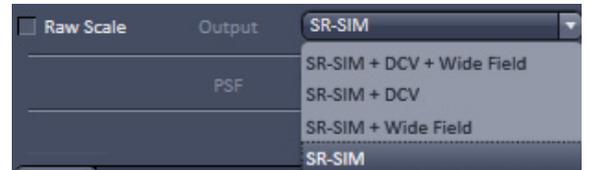


Fig. 227 Output drop down menu

Highlight the option you want to display. Channel designations are as follows: **SR** for SR-SIM, **WF** for Wide Field and **D** for Deconvolution (DCV).

If the **Raw Scale** box is unchecked, output images will be normalized to the maximum dynamic range. Please note that in this case quantitative information is lost, however, images are displayed with best brightness and contrast. If you want to keep the original scaling for quantitative studies, you have to check the **Raw scale** box. In this case the original dynamic range is kept.

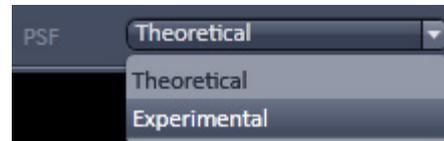


Fig. 228 PSF drop down menu

You can select between theoretically computed or experimentally determined PSF by choosing **Theoretical** or **Experimental** in the **PSF** drop down menu (Fig. 228). In case **Experimental** is selected, the **PSF File** button becomes available (Fig. 229). Pressing the button will open Windows Explorer, which allows you to select any pre-saved Experimental PSF. The file name of the loaded file is displayed in the **PSF File** display window.

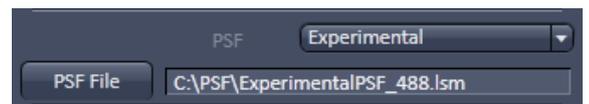


Fig. 229 PSF selection menu

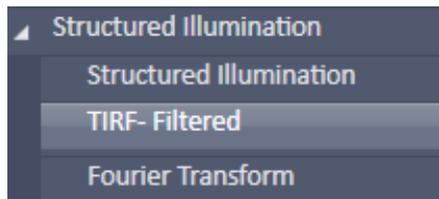
 Note, if the number of the Experimental PSF channels is less than the number of the WF or SIM channels, the theoretical PSF will be used for the missing channels and a warning is generated. The program also checks the match of the excitation wavelengths between the PSF and WF / SIM channels. In case of mismatching, it generates a warning, but the experimental PSF will still be used.



Fig. 230 SIM image (left) and wide field image (right) in the Split mode after SIM processing

By pressing **Apply** the high resolution image will be computed with the current parameter values (Fig. 230).

If a value is changed you have the possibility to press **Combine**. Then a new calculation is only done for the changed parameter and combined with the previous calculations on the unchanged parameters, which will save dramatically computational time. If **Apply** is pressed, calculations will be done on all parameters regardless of being changed or unchanged.



**Fig. 231** Structured illumination – TIRF-Filtered tool selected



**Fig. 232** Structured Illumination – TIRF-Filtered tool

### 3.3.16.2 Structured Illumination – TIRF-Filtered

This function works for SIM images (stack) in combination with a TIRF image. The lowest frame (first frame) of the stack, preferentially one taken close to the coverslip, will be taken and combined with the TIRF. The SIM image contributes the x,y enhancement, the TIRF image the z-resolution of the TIRF.

Select the **TIRF-Filtered** tab to select the **TIRF-Filtered** Processing tool (Fig. 231). Activate the SIM image you want to process in the image container and press the **Select** button for **Input image** to load the SIM image. Activate the TIRF image you want to use as the reference in the image container and press the **Select** button for **Input image 2**. Then expand the **Methods Parameters** tool window to have access to setting parameters (Fig. 232).

You can toggle between Input image 1 and 2 in the **Preview** tab window, which also displays a preview of the output image.

The **Image input** description field lists all the channels in the **SIM** column, the used lasers of a channel in the **Laser** column and the employed camera coded by a number in the **Camera** column.

The **Image input 2** description field lists all the channels in the **TIRF** column and the used laser of a channel in the **Laser** column.

It is important to load the SIM image as **Input image** and the TIRF image as **Input image 2** as the software expects it that way. If in **Input image 2** an image stack is selected, the system gives an error message as it expects a single plane (Fig. 233).



**Fig. 233** Error message for falsely loaded image dimensions in Input image 2

The **Link** drop down menu available for each channel displays all channels of the SIM **Input Image** (Fig. 234).

You can now link the TIRF channel to one of the SIM channels by highlighting the latter. Then the respective TIRF channel will be used to compute its z-resolution into the linked SIM channel.

When **Apply** is pressed, the new SIM image with the improved z-resolution in the lowest frame will be computed.

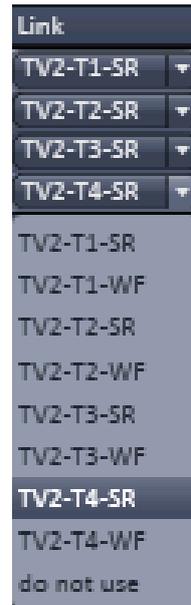


Fig. 234 Link drop down menu

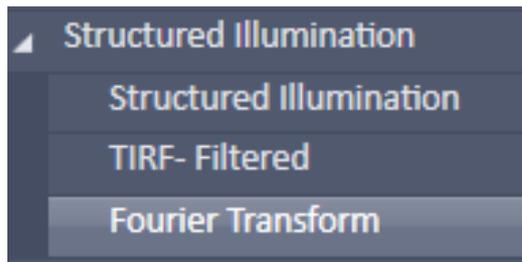


Fig. 235 Structured illumination – Fourier Transform tool selected

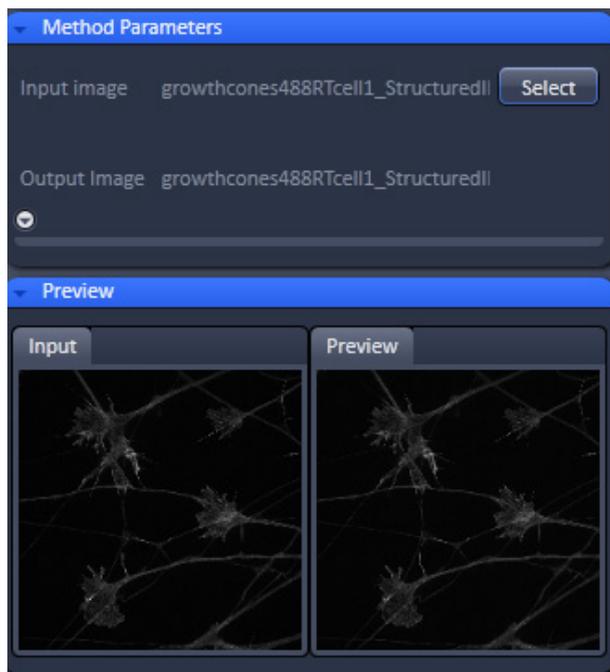


Fig. 236 Structured Illumination – Fourier Transform tool

### 3.3.16.3 Structured Illumination – Fourier Transform

The **Fourier Transform** tool computes and displays the Fourier transform of the image (frequency space).

 Note that the Fourier image will contain the information on the spatial frequencies, but does not contain phase information.

Select the **Fourier Transform** tab to select the **Fourier Transform** Processing tool (Fig. 235). Activate the image you want to analyze in the image container and press the **Select** button for **Input image** to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 236).

If the **Log FT** check box is inactive, the result is the absolute value of the Fourier transform [ $\sqrt{\text{Real} \times \text{Real} + \text{Imag} \times \text{Imag}}$ ] with Real and Imag being the real and imaginary part of the Fourier transform]. If **Log FT** is ticked, the result is the log-transform of the shifted absolute values [ $\log(1 + \text{Real} \times \text{Real} + \text{Imag} \times \text{Imag})$ ]. Log-transformation squeezes the dynamic range of the Fourier-transformed image, so that weaker intensities may become better visible.

Press **Apply** to compute the Fourier transforms of all image channels (Fig. 237).

 The Fourier images can be used to assess the quality of the SIM calculations and retrieve the resolution enhancement.

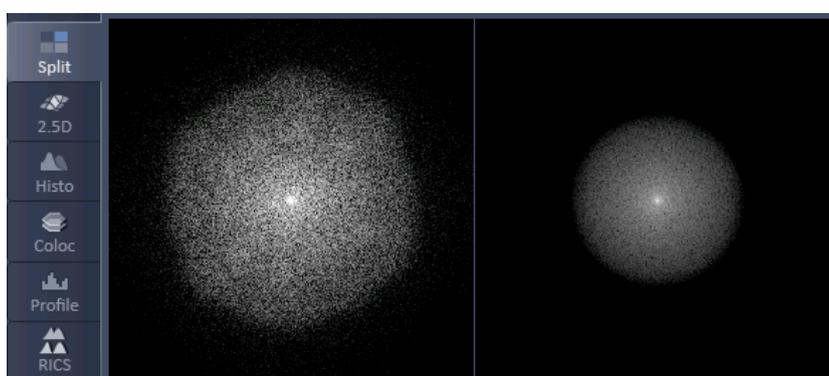


Fig. 237 Fourier transform images of SR (left) and WF (right) image in the Split view

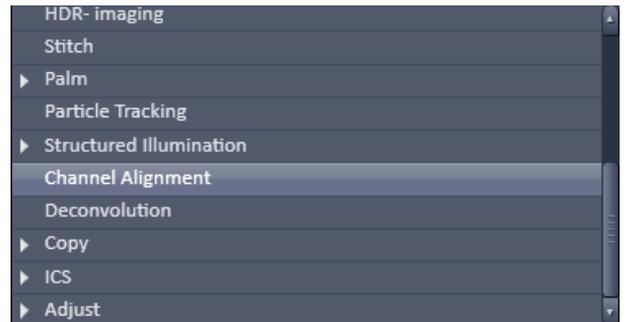
### 3.3.17 Processing – Channel Alignment

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1

Tool accepts multi-color image data (including multi-dimensional data sets). See full description of tool set for details.

The Processing **Channel Alignment** tool allows for correction of lateral drift and chromatic aberration between different channels of an image or between images.

Highlight the **Channel Alignment** field to have access to the alignment tools (Fig. 238).

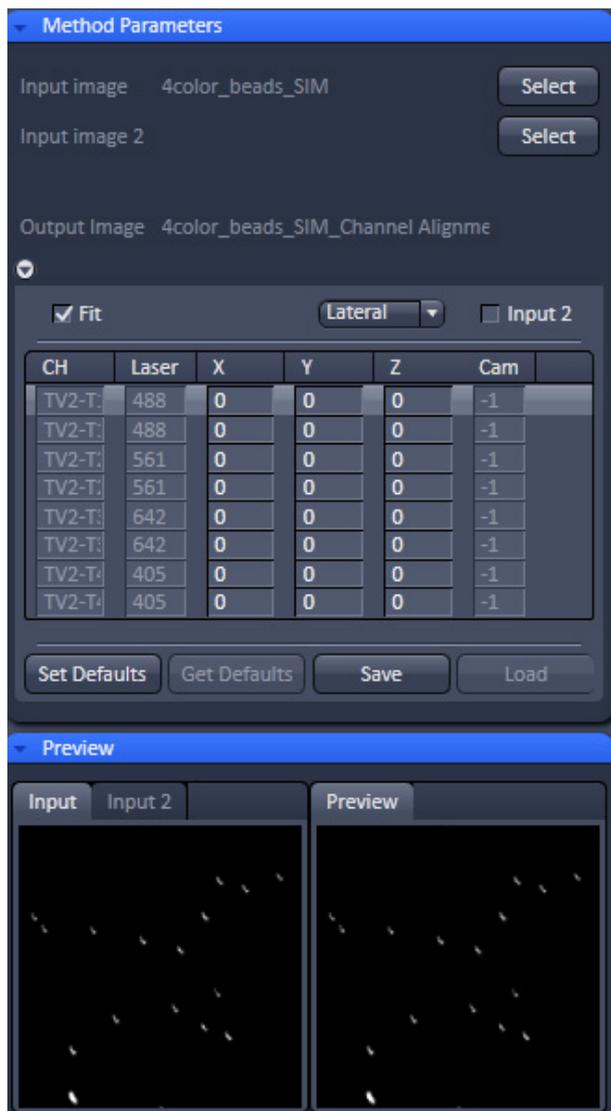


**Fig. 238** Processing – Channel Alignment tool selected

Activate the first image you want to process in the image container and press the **Select** button for **Input image** to load the image.

If you want to load a second input, highlight it in the image container and press the **Select** button for **Input Image 2**.

You can toggle between the two input images in the **Preview** tab display field. Then expand the **Method Parameters** tool window to have access to the **Channel Alignment** tools (Fig. 239).



**Fig. 239** Processing Channel Alignment tool field with one input image (with SIM and paired WF channels) selected

If the **Fit** check box is ticked and the **Input 2** checkbox inactive, then the software will align all channels of **Input image** having the same format to the first listed channel of the same format.

Care should be taken about what channels will be aligned and in which order they appear. For SIM images the super resolution (**SR**) and the wide field (**WF**) channels are listed alternatively (pair wise). Since all channels are of the same format each channel (**SR** and **WF**) would be aligned to the first **SR** channel, since **SR** channels are normally listed before their corresponding **WF** channel. If this should be avoided, subsets of images containing only **SR** or **WF** channels have to be created in the Processing Tool **Copy Subset** and independently aligned. The situation is different for PALM images. The high resolution (**HR**) and sum wide field (**SWF**) channels are also listed alternatively, with the **HR** channels preceding their corresponding **SWF** channels, however, the format of the **HR** channels is different to the one of the **SWF** channels. In this case the software aligns all **HR** channels to the first listed **HR** channel, which will be the first displayed channel, and all **SWF** channels independently to the first listed **SWF** channel, which is second in the list.

With the **Fit** check box ticked, the **coefficient table** lists as the first column the Channel name **Ch** and as the last two columns the used **Laser** line and camera channel **Cam**. In between are listed the corresponding correction coefficients. You have the choice from the **Alignment mode** drop down menu between **Lateral**, **Affine** and **Parabolic** alignment.

**Lateral** (Fig. 240) is used to correct for linear lateral and axial drifts, that is in all directions. Linear alignments are performed in the x, y and z direction, when the **Applied** button is pressed. The respective offsets (coefficients) are displayed in the coefficient table in the **X, Y** and **Z** columns, respectively (Fig. 241).

For **SIM, WF** and **SWF** images, the software will use significant structures for performing the alignment. You can set a ROI and then the structure within the ROI is used. For **HR (PALM)** images at least one fiducial is expected. Without a fiducial, no alignment will be performed in this case.

**Affine** is used to correct for linear lateral (**X, Y**) and axial (**Z**) drifts, rotational offsets in the image plane in x and y (**RX, RY**) as well as a 1<sup>st</sup> order stretching in the image plane in x and y (**SX, SY**). The respective offsets (coefficients) are displayed in the **coefficient table** in the corresponding columns (Fig. 242). The scrollbar can be used to have access to all columns. For **SIM, WF** and **SWF** images, the software will use significant structures for performing the alignment. You can set a ROI and then the structure within the ROI is used. For **HR (PALM)** images at least three fiducials are expected. Without fiducials, no alignment will be performed in this case.

**Parabolic** is used to correct for linear lateral (**X, Y**) and axial (**Z**) drifts as well as chromatic aberrations, where a 2<sup>nd</sup> order stretching in the image plane in x (**X1, X2**) and y (**Y1, Y2**) is performed. The respective offsets (coefficients) are displayed in the **coefficient table** in the corresponding columns (Fig. 243). The scrollbar can be used to have access to all columns. **Parabolic** only works with **HR (PALM)** images and is displayed only in the **Alignment mode** drop down menu, when a **HR (PALM)** image is selected as the **Input image**.

 The calculated coefficients in the **coefficient table** can be edited by hand. The correction will be exactly performed when pressing **Apply** according to the coefficients in the **coefficient table**.

 If the **Save** button is pressed, the **coefficient table** (with the coefficients) will be saved in a .bin file. These .bin file can be reloaded by pressing the **Load** button via the **Windows Explorer**. The **Load** button becomes only active when the **Fit** check box is not active.



Fig. 240 Alignment mode drop down menu for selected PALM image

CH	Laser	X	Y	Z	Cam
TV2-T1-SR	488	0	0	0	-1
TV2-T1-WF	488	-0.023	-0.009	-0.099	-1
TV2-T2-SR	561	-0.423	0.450	0.005	-1
TV2-T2-WF	561	-0.576	0.305	-0.087	-1
TV2-T3-SR	642	-1.234	-4.614	-0.080	-1
TV2-T3-WF	642	-1.859	-4.767	-0.087	-1
TV2-T4-SR	405	4.489	4.177	-4.432	-1
TV2-T4-WF	405	4.459	4.305	-4.246	-1

Fig. 241 Correction table in the Lateral alignment mode

CH	Laser	X	Y	RX	RY	SX	SY	Z	Cam
TV2-T1-SR	488	0	0	0	0	0	0	0	-1
TV2-T1-WF	488	0.059	0.095	-0.000	0.000	0.000	-0.000	-0.098	-1
TV2-T2-SR	561	-0.234	1.130	4.082	-0.000	-0.001	-0.002	0.002	-1
TV2-T2-WF	561	-0.196	0.980	-0.000	0.000	-0.000	-0.002	-0.087	-1
TV2-T3-SR	642	-0.574	-3.535	-0.000	-0.000	-0.002	-0.003	-0.080	-1
TV2-T3-WF	642	-1.297	-3.311	-0.000	0.000	-0.002	-0.005	-0.089	-1
TV2-T4-SR	405	4.489	4.177	0	0	0	0	-4.432	-1
TV2-T4-WF	405	4.459	4.305	0	0	0	0	-4.246	-1

Fig. 242 Coefficient table in the Affine alignment mode

CH	Laser	X	Y	X1	Y1	X2	Y2	Z	Cam
HR 1	0	0	0	0	0	0	0	0	-1
SWF 1	0	0	0	0	0	0	0	0	-1
HR 2	0	193.0	175.7	-1.506	-3.874	1.477	1.040	0	-1
SWF 2	0	0	0	0	0	0	0	0	-1

Fig. 243 Coefficient table in the Parabolic alignment mode

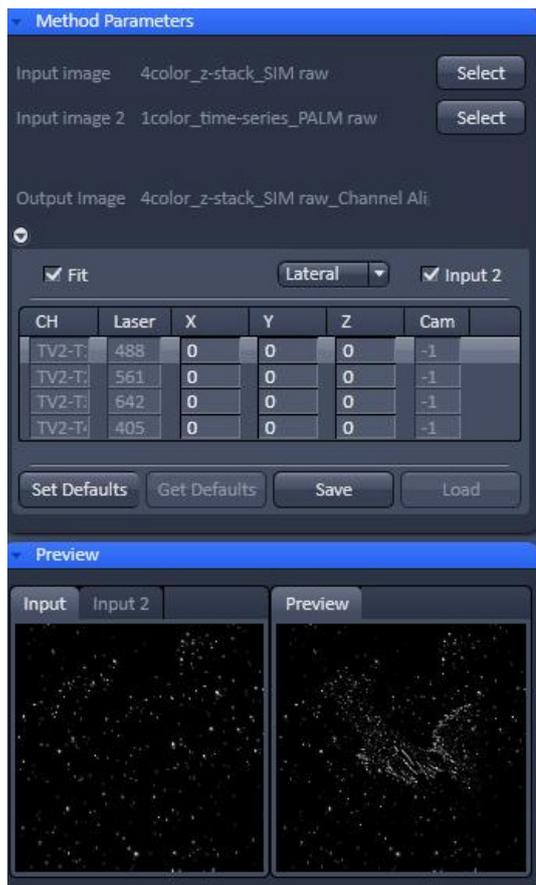


Fig. 244 Two images mismatched in their dimensions (Z-Stack SIM raw and time-series PALM) images loaded

When the **Save Default** button is pressed, the present coefficients will be stored and can be recalled by pressing the **Restore default** button, which becomes active once the **Save default** button was pressed once.

If the **Input 2** check box remains inactive, the image selected for **Input image 2** is not considered for alignment. If the **Input 2** check box is ticked, then the channels of **Input image 2** are aligned to the corresponding channels of the **Input image** that is to those channels at corresponding positions in the list.

It is the responsibility of the user to align only images of the same dimensions and channels of the same format. If images will have different dimensions, for example a raw SIM Z-Stack and a raw PALM time the system will give no warning message telling the user that the images do not match (Fig. 244). It will load both images and aligns the channels according to their position as long as there are matching channels.

If no **Input image** was selected and only an **Input image 2**, no alignment will be performed.

Loaded images cannot be unloaded but replaced by other images in selecting the respective image in the image container and then pressing the **Select** button for **Input image** or **Input image 2**.

If the **Fit check** box is not ticked a second table, the **assignment table** will be displayed below the **coefficient table** (Fig. 245). In this case the selected image will not be fitted directly, but a loaded alignment will be performed on the image. Columns in the **coefficient** and **assignment** tables can be expanded by dragging the borders of the columns.

The **coefficient table** in this case has to be loaded from a .bin file by pressing the **Load** button or is still available from a previous alignment or obtained by pressing the **Restore Default** button, if values have been previously restored by pressing the **Save default** button. In any case, the listed coefficients (no matter what **Alignment mode** is associated) will be applied to the selected image. If the **Input 2** check box is inactive, this will be the image selected for **Input image**. If the **Input 2** check box will be ticked, it will be the image selected for **Input image 2**.

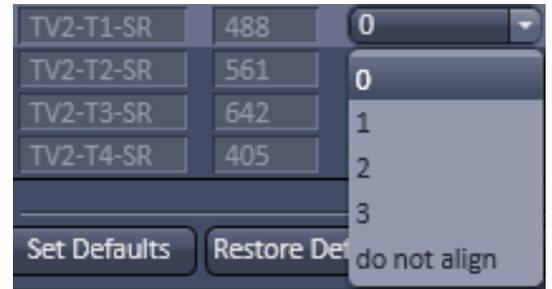


Fig. 245 Coefficient table and assignment table

In either case, the other loaded image does not play a role. In the **coefficient table** the channel (**Ch**) assignment column is replaced by an identity (**Id**) column that numbers the channels in their ascending order starting with 0.

The assignment table represents the selected image, either **Input image** (**Input 2** check box inactive) or **Input image 2** (**Input 2** check box ticked).

Note that the dimensionality must not be the same as in the **coefficient** table. The **assignment table** lists the channels (**Ch**) and the laser lines used (**Laser**). With the identity (**ID**) drop down menu (Fig. 246), an ID from the **coefficient table** can be assigned individually to a channel. That channel will then be corrected exactly with the coefficients of that **ID**.



**Fig. 246** ID drop down menu

If no alignment should be performed **do not align** can be chosen.

 The **channel alignment** tool is generically programmed to have high flexibility. Care should therefore be exerted in the control which channels are how aligned as there are no warnings, if unrelated channels are aligned to each other.

If you draw Regions of Interests (ROIs) in the image, they will be taken for alignment. For parabolic fit, you should have at least 3 well spaced regions. If you have fiducial markers, you can use them by setting the ROIs around them. Please be aware that in the z-direction the algorithm scans the whole z-stack and takes the brightest area in the z-direction. If your beads are not the brightest structure, then the alignment will fail.

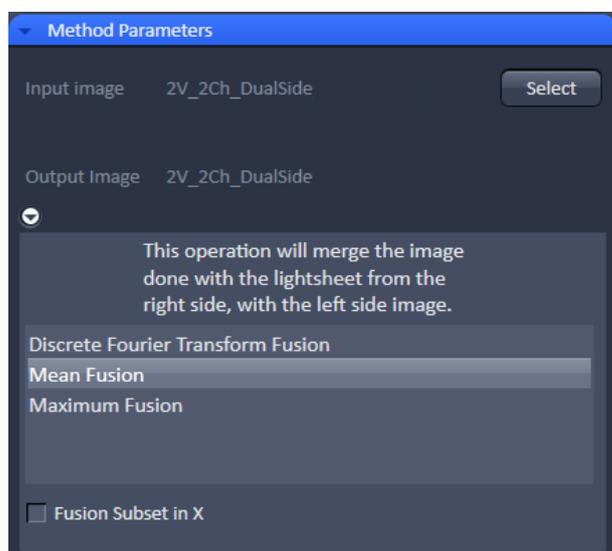
### 3.3.18 Processing – Lightsheet Processing



The **Lightsheet processing** module allows one to process images that were acquired with the Lightsheet Z.1 using **Dual Side** illumination and/or with the **Multiview tool** tab.

Files need to be saved prior to any Lightsheet Processing task. If this is not the case the software will ask to save the data before proceeding.

 All images and registration files resulting from Lightsheet Processing can be found in a Result folder that is created in the folder of the original file, that was used for processing.



**Fig. 247** Processing – Lightsheet Processing. Dual Side Fusion

#### 3.3.18.1 Dual Side Fusion

This processing task can be used for the following datasets acquired with **Dual Side** illumination:

- Image (Snap function)
- Time Series
- Z-Stacks
- Combinations of Time Series and Z-Stacks
- Multiview experiments as well in combination with Time Series

When a dataset has been acquired using **Dual Side** illumination, the dataset will have the illumination dimension saved in its metadata and the **Dimension slider** for Illumination is visible in the **Image Control area**. Both illumination sides are handled as single frames within the image. **Dual Side Fusion** provides three main options for merging both images from the different illumination sides into a single frame.

Choose the dataset you wish to perform the task by mark either option (Fig. 247).

#### Discrete Fourier Transform Fusion:

The fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003).

Fine structures and details are more visible after the fusion which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

**Mean Fusion:**

The average intensity for each pixel is calculated using the values from both illumination sides.

**Maximum Fusion:** The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X** can be activated (Fig. 248). With these controls it is possible to choose which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** within the image container (Fig. 249). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X** the currently chosen View is displayed in the **Current View** box.

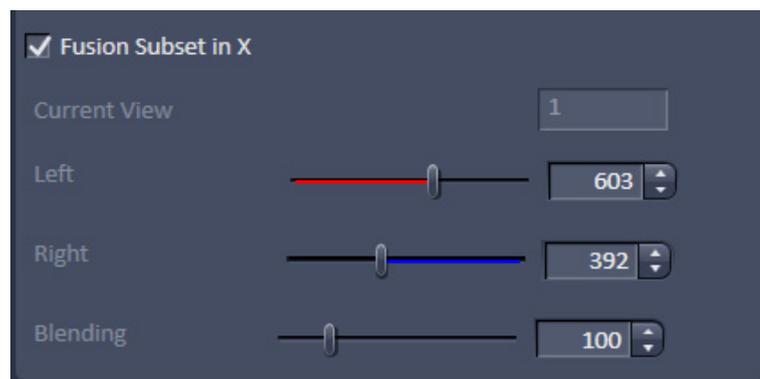
With the **Left** slider or spin box the area of the left illumination side is defined. This is marked at the slider itself with a red line, the spin box shows the pixel value of the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 249).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.



**Fig. 248** Dual Side Fusion – Fusion Subset in X



**Fig. 249** Dual Side Fusion – Fusion Subset in X, Preview and Image control area

After pressing **Apply**, a new .czi file will be generated. The original file name will be extended with "\_Dual Side Fusion". For this resulting dataset, the illumination dimension is no longer available.

### 3.3.18.2 Multiview Processing

#### 3.3.18.2.1 Deconvolution

For the multiview datasets acquired with the Lightsheet Z.1, **Deconvolution** is an available option (Fig. 250).

The following datasets are available for this processing option:

- a) Multiview experiments before registration and fusion (single or dual side illumination)

In this case, deconvolution is performed for each frame from all Z-Stacks and all Views. The process uses a point spread function (PSF) valid for single view images.

- b) Multiview experiments after registration and fusion.

The deconvolution is performed on the fused dataset, hence only one Z-Stack needs to be processed. It uses a different point spread function (PSF) than what is used for the deconvolution before registration and fusion of a multiview experiment dataset.

The software automatically recognizes if the image has been registered and fused or not and adjusts the interfaces accordingly.

Two tabs are available for detailed configuring (**Deconvolutions** tab and **PSF settings** tab):

On the **Deconvolution** tab, select the desired algorithm and define the precise settings for it.

Use the drop-down list next to Algorithm to choose from the available deconvolution algorithms:

**Nearest Neighbour:** The Nearest neighbor method uses the simplest and fastest algorithm (Castleman, K.R., Digital image Processing, Prentice-Hall, 1079). Its function is based on subtraction of the out-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 Z-Stack. It allows you to enhance contrast quickly, even if image stacks have not been put together optimally.

**Regularized Inverse:** 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 or the PSF, which can be performed very quickly. In the real space, this corresponds to a 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. Z- stacks must have been acquired at the correct (Nyquist or smaller) distance. The additional acquisition of z-planes above and below the structure of interest is recommended.

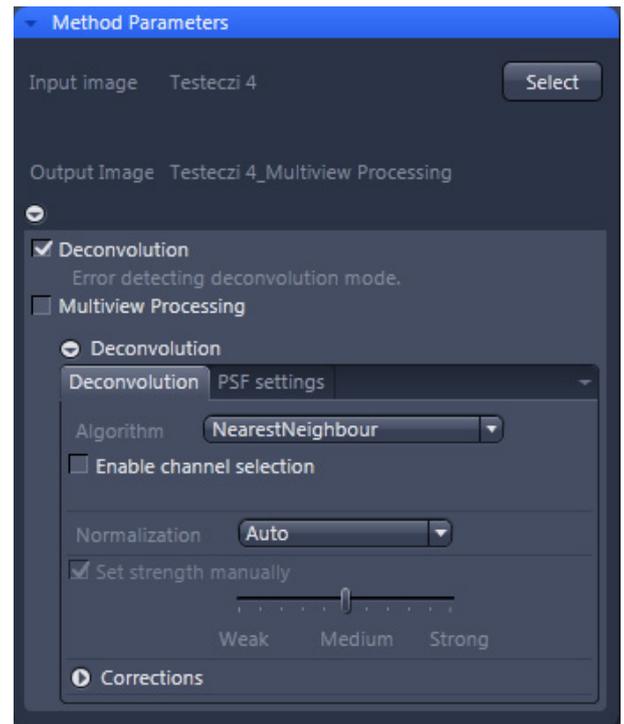
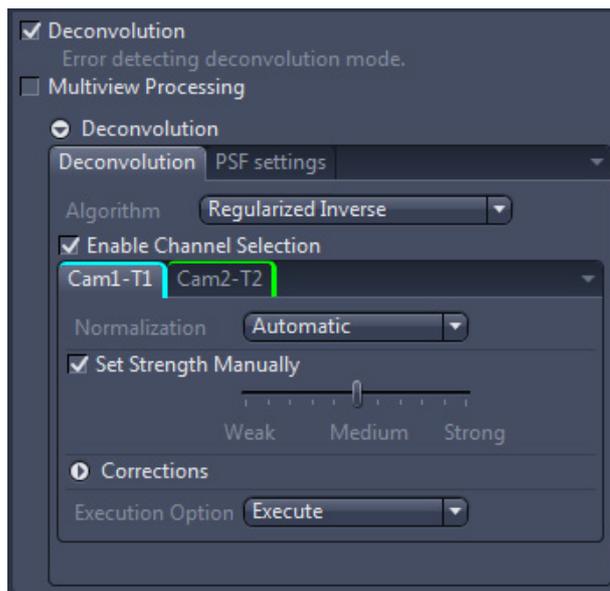


Fig. 250 Processing – Lightsheet Processing. Multiview Processing, Deconvolution

**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 (&), 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 create quantitative brightness conditions in the image. If under-sampled images are present, artifact formation may also result.

**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, Vo. 204, Pt2, 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.



**Fig. 251** Processing – Lightsheet Processing. Multiview Processing, Deconvolution, Enable channel selection

If the checkbox **Enable channel selection** is activated, the settings are applied on a channel-specific basis. This allows you to set parameters in a targeted way for each channel. You will see a separate colored tab for each of the channels (Fig. 251).

In the **Normalization** drop-down list, you can specify how the data from the resulting image are handled:

**Clip:** For non-registered and non-fused multiview images, this should be used as the default setting. It 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).

**Automatic:** Normalizes the output image automatically. Not to be used for non-registered and non-fused multiview images.

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

**Adjust Strength checkbox:**

If you have selected the Nearest Neighbor algorithm or Fast Iterative, the set strength manually checkbox is always activated.

With the other Algorithm options, you can activate or deactivate this option using the check box next to **Set strength manually**. When activated, you can enter the degree of restoration using the slider.

To achieve strong contrast enhancement, move the slider towards Strong.

To achieve less contrast enhancement, move the slider towards Weak.

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

If **Set strength manually** is deactivated, it determines the restoration strength for optimum image quality automatically. The restoration strength is inversely proportional to the strength of the so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

To expand the options for **Corrections**, use the black arrow-head button. Here you can find three additional corrections which can be activated or deactivated using the adjacent check boxes.

**Lamp Flicker checkbox:** 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 in brightness.

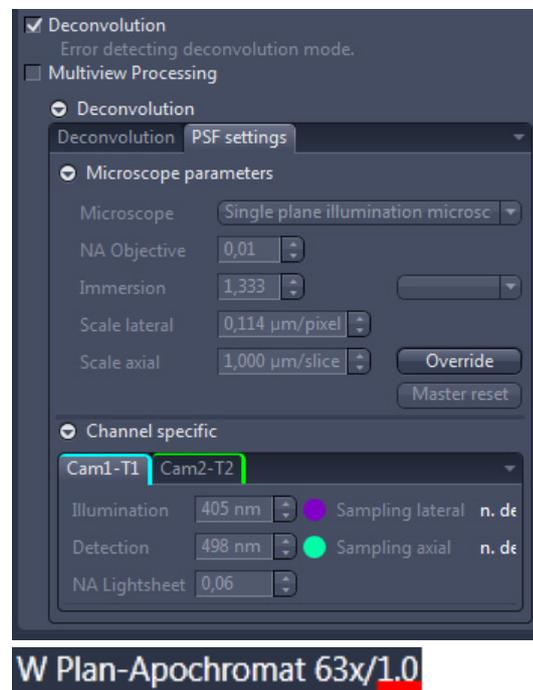
**Decay:** Corrects changes in brightness (e.g. bleaching) of the sample during acquisition of the Z-Stack. This function should only be activated for widefield images. Use it if your sample undergoes strong bleaching during acquisition.

**Background Correction checkbox:** Analyzes the background component in the image and removes it before the DCV calculation. This can prevent background noise being intensified during DCV.

On the **PSF settings** tab (Fig. 252), you can see and change all key parameters for generating a theoretically calculated Point Spread Function ("PSF").

 If an image is used that is not registered and not fused, a point spread function (PSF) is used that is specific for single view images (single view PSF) for the Lightsheet system. If an image is loaded that has already been registered and fused, a specific PSF is used for this dataset. This multiview PSF is calculated using the information on how many views and which rotations were used to acquire this particular dataset, taken from the image metadata.

Ordinarily, images that have been acquired using ZEN (stored as \*.czi) automatically contain all system parameters and image information, 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.



**Fig. 252** Processing – Lightsheet Processing. Multiview Processing, Deconvolution, PSF settings

To edit any of the input boxes, press the **Override** button. To get back to the settings provided by the software, press the **Master Reset** button.

In the **Microscope Parameters** section are the most important system parameters for PSF generation that are not channel-specific displayed.

You can select the system used for data acquisition in the **Microscope dropdown** list. There are three options: Single plane illumination microscope, conventional microscope (also known as a widefield microscope), and confocal microscope for which the additional pinhole diameter parameter applies.

#### **Dimension:**

You can view and change the numerical aperture for the detection, which results as a combination of the detection optic and the used zoom setting, in the **NA Obj.** input field. So, it is not the numerical aperture of the detection optic alone which is indicated on the lens itself or displayed in the software.

The **Immersion input** field 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.

The **Scale lateral** input field displays the geometric scaling in the X/Y direction.

The **Scale axial** input field displays the geometric scaling in the Z direction.

When scaling in this window is changed it will override the settings chosen in the **Multiview Processing** window (Fig. 253).

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

The **Illumination** input box holds the excitation wavelength for the channel [in nm]. The maximum is assumed. The color field corresponds to the wavelength (as far as possible).

The **Detection** input box represents the maximum of the emission wavelength for this channel dye. The color field corresponds to the wavelength (as far as possible).

The **NA Lightsh.** Input box displays the numerical aperture (NA), which is resulting from a combination of the illumination optics and the zoom settings used during image acquisition.

### 3.3.18.2.2 Registration Options

The **Registration Options** check-box next to **Multiview Processing** opens the interface to define the settings for the Registration Parameters, the **Input settings**, and the **Fusion options** for the data (Fig. 253). Each interface is accessed by pressing the  button next to it.

The following datasets can be processed with this task:

- Multiview datasets, single side illumination
- Multiview datasets, dual side illumination, Dual Side without Fusion
- Multiview datasets, dual side illumination, Dual Side with Fusion
- Multiview datasets, with Deconvolution
- Multiview datasets, without Deconvolution

The first step is to select the **Registration Options** using the drop-down menu. The choice will influence the **Parameters** interface.

The **Registration** will generate the transformation matrices so that all views are matched to overlay. As a result, a dataset is provided in which all Z-Stacks of all views should already largely overlay. The reference at which registrations starts is always View 1 of the dataset. This image can be used as a control. If one or more views do not have the same alignment, registration was not successful and the parameters must be adjusted.

When **Apply** is pressed and the registration process has finished, the transformation matrices are saved as an .xml file (Filename.czi.xml). Only the transformation matrix is saved if **Apply** is pressed and **None** is selected under **Fusion** (see below). This .xml file can be used for the **Use Registration From File** option.

The **Registration** must always be done before the **Fusion** of the data.

**Registration Options** are:

- Landmark Alignment
- Intensity Based Alignment
- Use Registration From File
- Interactive Registration

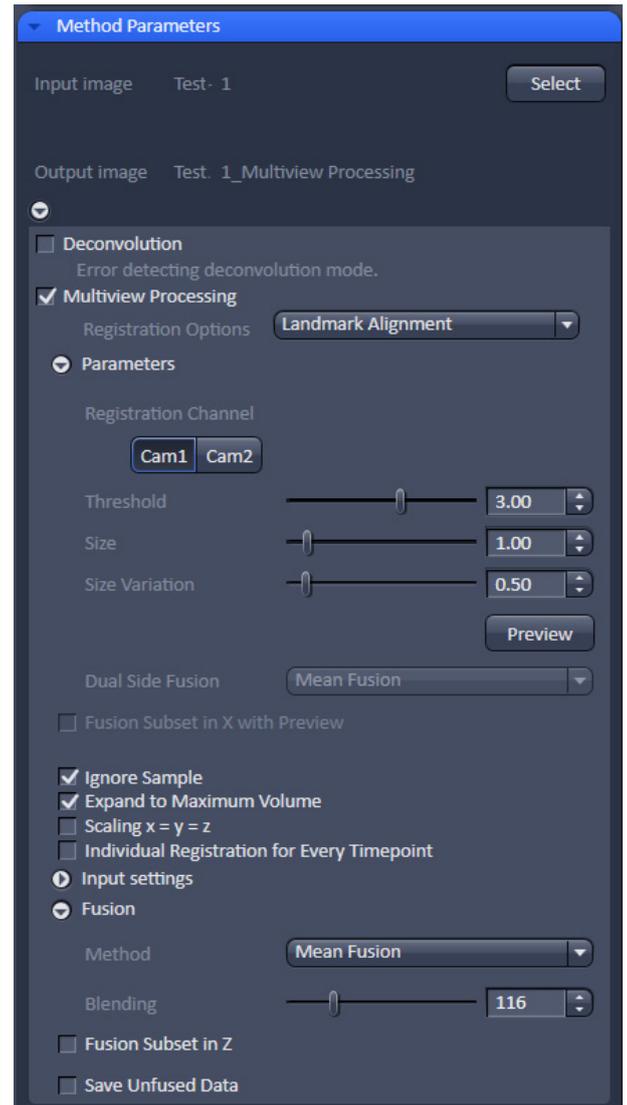
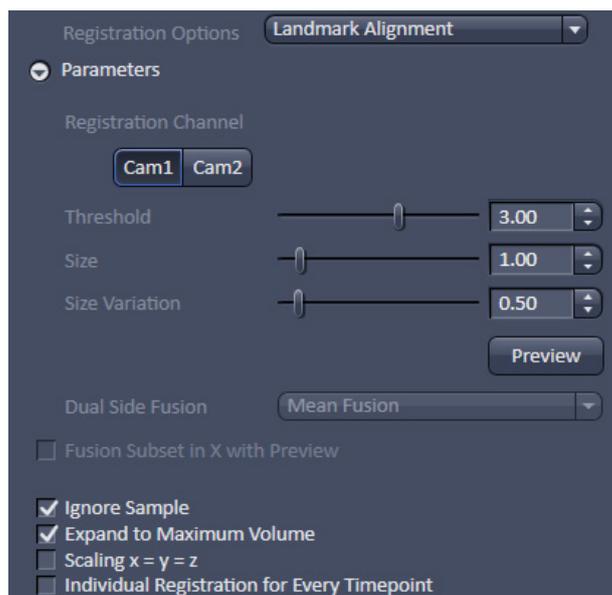


Fig. 253 Processing – Lightsheet Processing. Multiview Processing



**Fig. 254 Processing – Lightsheet Processing. Multiview Processing, Landmark Alignment Parameters**

### 3.3.18.2.2.1 Landmark Alignment

For the **Landmark Alignment** (Fig. 254), the sample must have been embedded with appropriate fluorescent beads (fiducials) which can be recognized by the software. The beads can be present in the same channel as the image information. Another option is to image the beads alone in a second channel.

Select the Channel in which the beads are present by clicking the according **Registration Channel** button. One can select more than one channel for the registration process. For each chosen channel and individual registration will be performed; it is therefore advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be chosen for later comparison of the results.

To define which structures within the images are the beads to be used for the Landmark Alignment, use the sliders and input boxes with arrows of

**Threshold:** brightness of beads,

**Size:** size of the beads, and

**Size Variation:** how much do the beads differ in size.

All values with these parameters are arbitrary units.

To check if the settings recognize the beads of the image, press the **Preview** button. This will circle all elements recognized as beads in the chosen and displayed image frame. Use the sliders in the **Dimensions View** control to change to a different view, z-slice, and time point and press again the **Preview** button. Control by using several different frames to see if the beads are recognized correctly before proceeding. Adjust the settings for **Threshold**, **Size**, and **Size Variation** until this is accomplished.

If your data was acquired with dual side illumination which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion**, **DFT Fusion**, **Maximum Fusion**).

**Mean Fusion:** The average intensity for each pixel is calculated using the values of both illumination sides.

**DFT Fusion** (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after the fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

**Maximum Fusion:** The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 255). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 256). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 256).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.

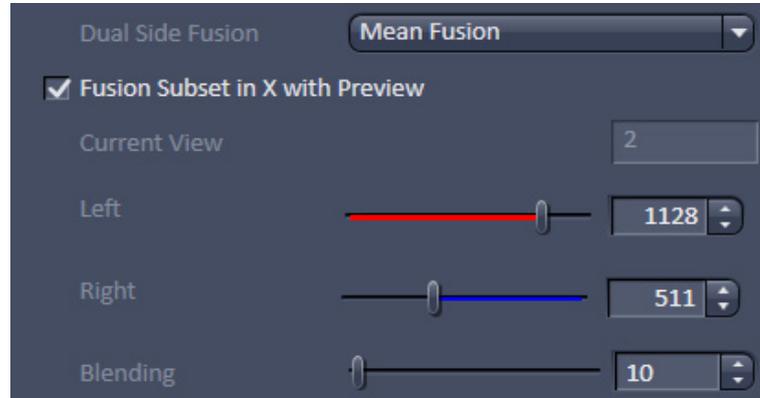


Fig. 255 Dual Side Fusion – Fusion Subset in X



**Fig. 256** Dual Side Fusion – Fusion Subset in X, Preview and Image control area

**Ignore Sample:** This option activates an additional function to find structures within the sample that were incorrectly labeled as beads (fiducials). These structures are not taken into account during the Landmark Alignment process; but they are not removed from the **Preview**.

**Expand To Maximum Volume:** This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

**Scaling  $x=y=z$ :** The dimensions are downsized to the smallest to have the same size. If the scaling in **Deconvolution, PSF settings** (Fig. 252) is changed, the latter will override the scaling settings here.

**Individual Registration For Every Time point:** If the multiview experiment has been performed over time, this option becomes available.

- If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the Individual Registration For Every Time point option becomes necessary.
- If **not activated**, one transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.

### 3.3.18.2.2 Intensity Based Alignment

The **Intensity Based Alignment** option does not need any fluorescent beads (fiducials) present in the sample since registration is done based on structural information within the image content. It is therefore advisable to select the **Registration Channel** which contains clearly defined fluorescent structures within the image. One can choose more than one channel for registration. For each chosen channel and individual registration will be performed; it is therefore advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be chosen for later comparison of the results.

Registration can be done as a two-step process with a preceding **Pre-Registration** and afterwards the **Registration** (Fig. 257).

The **Pre-Registration** performs the process in a 2D maximum intensity projection of the z-stacks; while the **Registration** algorithm uses the complete 3D information of the z-stacks.

 It is advisable to perform first a **Pre-Registration** to move similar structures already into close proximity, which might facilitate the **Registration**.

You can select the following **Pre-Registration Methods** from the drop down menu:

**None:** no Pre-Registration is performed. Registration is then only done with the settings chosen in the Registration Method menu.

**Hardware Rotation:** The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata.

**Trans Hardware Rotation:** The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata. Additionally the x, y, and z axis are moved to match the Views.

**Correlation Hardware Rotation:** The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata. Additionally the x, y, and z axis are moved to match the Views. Cross-correlation is used to determine the translation in x, y, and z.

To select the **Registration Method**, use the drop-down menu.

**Use Pre Register Only:** No Registration process is performed; only the result of the Pre-Registration is used.

**Translation:** The registration will correct along the x, y, and z axis only.

**Trans Rotation:** The registration will correct along the x, y, and z axis and also the rotation along x and y.

If your data was acquired with dual side illumination which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion, DFT Fusion, Maximum Fusion**).

**Mean Fusion:** The average intensity for each pixel is calculated using the values from both illumination sides.

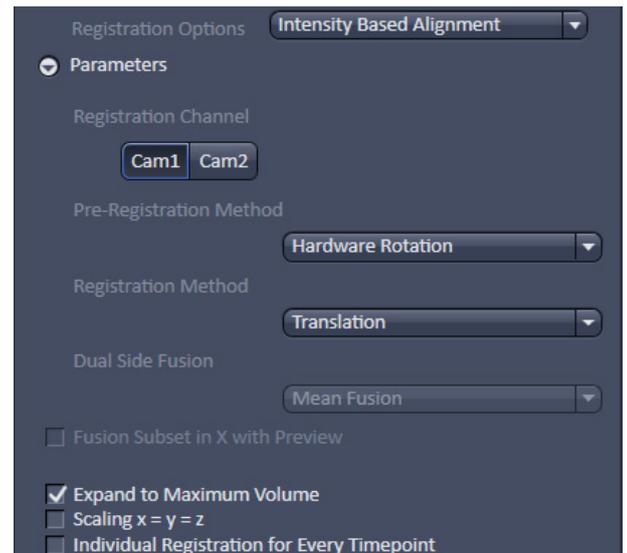


Fig. 257 Processing – Lightsheet Processing. Multiview Processing, Intensity Based Alignment, Parameters

**DFT Fusion** (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

**Maximum Fusion:** The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 258). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 259). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 259).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.

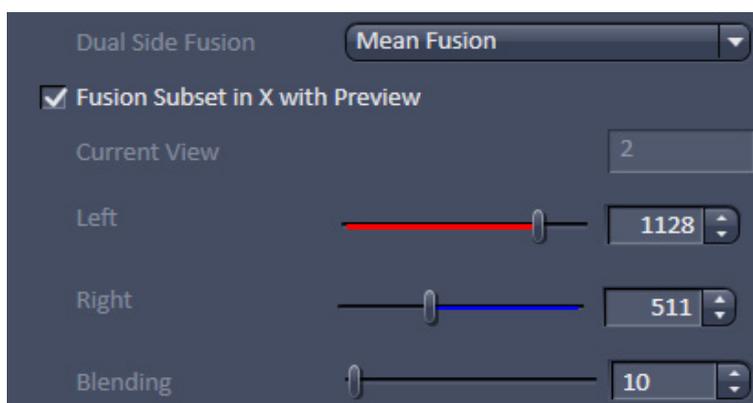


Fig. 258 Dual Side Fusion – Fusion Subset in X



**Fig. 259 Dual Side Fusion – Fusion Subset in X, Preview and Image control area**

**Expand To Maximum Volume:** This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

**Scaling x=y=z:** The dimensions are downsized to the smallest to have the same size.

**Individual Registration For Every Time point:** If the multiview experiment has been performed over time, this option becomes available.

- If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the Individual Registration For Every Time point option becomes necessary.
- If **not activated**, one transformation matrix is generated for all the following time points based upon the first time point. As a result, processing for registration has only to be done once which reduces processing time.

### 3.3.18.2.2.3 Use Registration from File

When the Registration for the dataset has been determined already and the transformation matrix has been saved as an .xml file (Filename.czi.xml), this option can be chosen. Click on the button next to **Registration File** to open a Windows Explorer window in which you can find and select the file.

With the drop down menus **Dual Side Fusion** processing can be chosen (see as well **Landmark Alignment or Intensity Based Alignment** section).

**Expand To Maximum Volume:** This checkbox is activated as the default. In a multiview experiment, the Z-Stacks are often of different sizes. When **Expand To Maximum Volume** is activated, it will use the maximum slice number (from one View) present in the dataset for registration. The additional z-slices needed in other views will be filled with black frames. This will ensure that no data is lost from views with larger z-dimensions. If this checkbox is not activated, the number of z-slices from the first View is taken as the default and larger Z-Stacks will be cut; there is a risk of data loss.

**Scaling x=y=z:** The dimensions are downsized to the smallest to have the same size.

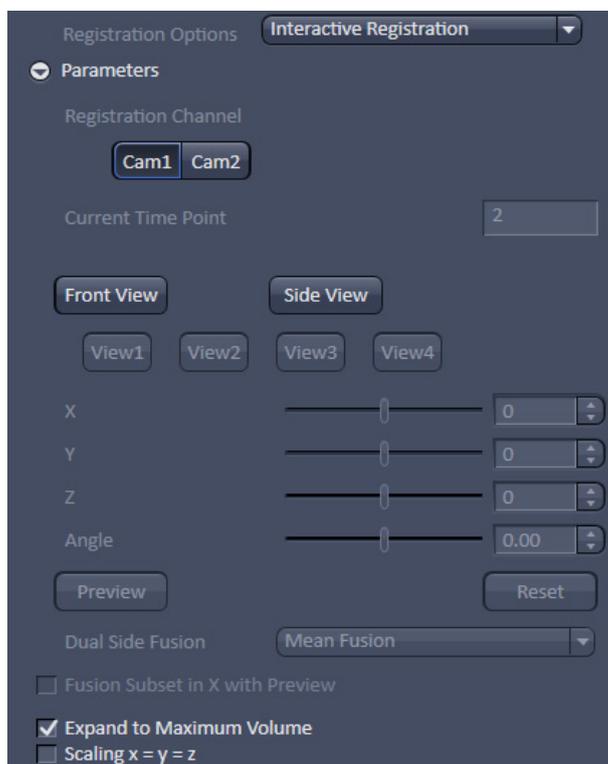


Fig. 260 Registration Options: Interactive Registration

### 3.3.18.2.2.4 Interactive Registration

**Interactive Registration** allows to register the recorded views of an experiment manually, when the other alignment options fail for that dataset (Fig. 260). The views of one channel at one time point of the experiment are the basis for the procedure. The z-stacks of the individual views are rotated based on the rotation information of the files meta data and transformed into channels resulting in one maximum intensity projection image. Within this newly generated image the views, now behaving as channels, can be moved in x, y, z and rotated to overlay each other. When all views are layed on top of each other, the **Apply** button will use the adjusted parameters to register, and when required fuse, the views for all time points of the experiment. An .xml file is created which can be used for the “**Use registration from file**” option. This .xml file can be found along with the processed data in the Result folder.

The first step is to chose the **Registration Channel** that should be used to manually overlay all views of the experiment by pressing the according button, e.g. **Cam1**. Only one channel can be used for this procedure, all other channels will not be displayed. If the experiment consists of a time series, one time point is selected with the **Time** slider and spin box of the **image control area**. This chosen time point is indicated in the **Current Time Point** box (Fig. 260).

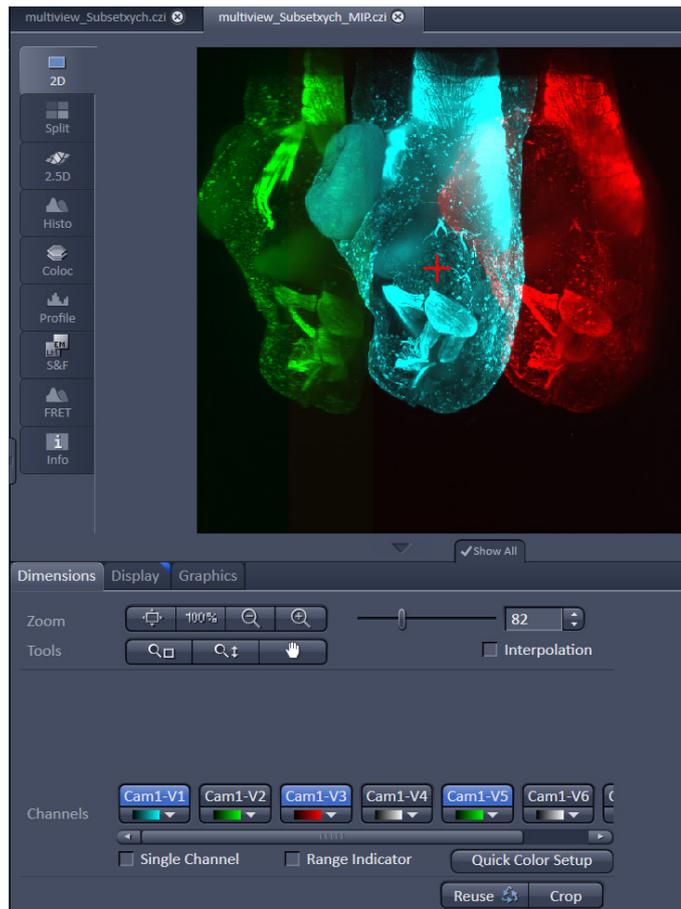
To generate the maximum intensity projection image either the **Front View** or the **Side View** button can be pressed. In the resulting maximum intensity projection image, each view is handled as a channel (e.g. Cam1-V1, Cam1-V2) of the image, a color can be assigned to each channel and it can be turned on or off in the **image control area** (Fig. 261).

 Be aware, the calculation of the maximum intensity projection of the **Front View** or **Side View** will take some processing time, depending on the size of the used dataset.



**Fig. 261** Interactive Registration – Views into Channel conversion of the maximum intensity projection image

It is of no consequence with which of the maximum intensity projections the process is started. In case of using the **Front View** button, the maximum intensity projection is calculated along the z-axis and the x,y axis are displayed in the resulting image (Fig. 262). The **Side View** button results in a maximum intensity projection along the x-axis, displaying the z- (horizontal) and y- (vertical) axis. The maximum intensity projection image is opened in a new **image window**, named Filename\_MIP.czi. The following steps are done on the basis of the maximum intensity projection, hence in this **image window**.



**Fig. 262** Interactive Registration – Maximum intensity projection (Front View) image with red cross overlay for view rotation (views Cam1-V1, Cam1-V3 and Cam1-V5 are visible in the image)

**View 1** is the master view and cannot be moved during the process, the button **View 1** is therefore inactive and grayed out. Any of the other views can be activated by pressing the according button (e.g. **View2**, Fig. 260). The activated view is automatically displayed in the image (the associated channel is turned on). To move the view relatively to View 1 the sliders and spin boxes can be used; **X** for the x-axis, **Y** for the y-axis, **Z** for the z-axis and **Angle** for a rotation. The axis that is not present in the maximum intensity projection is grayed out and inactive (**Z** for the **Front View** and **X** for the **Side View**).

Additionally to the rotation **Angle**, the position of the rotation point can be defined. This is done by positioning the **red Cross overlay** within the maximum intensity projection image window to the desired position, the view should be rotated around (Fig. 262).

The **Reset** button moves all views back to the position they were at, as the maximum intensity projection was generated.

Each view should be activated once with its corresponding button and positioned, resulting in a matching overlay to **View 1**. Afterwards the maximum intensity projection is switched to either **Front View** or **Side View**; displayed in the same image window.

Again all views must be moved using the according sliders **Y**, **Z** and **Angle** to produce a perfect overlay to View 1.

Switching from **Front View** to **Side View** should be repeated at least twice, to adjust the positioning of the views.

To control if the manual alignment is satisfactory, the **Preview** button produces a new image of the channel and the time point, which is registered with the provided settings. The **Preview** image will contain fused views, when **Mean Fusion** has been selected at the **Fusion Method** drop down list.

If the result is satisfactory, one can proceed with the remaining settings for **Multiview Processing**.

If the data was acquired with dual side illumination, which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion**, **DFT Fusion**, **Maximum Fusion**).

**Mean Fusion:** The average intensity for each pixel is calculated using the values of both illumination sides.

**DFT Fusion** (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after the fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

**Maximum Fusion:** The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 263). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 264). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 264).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each View individually.

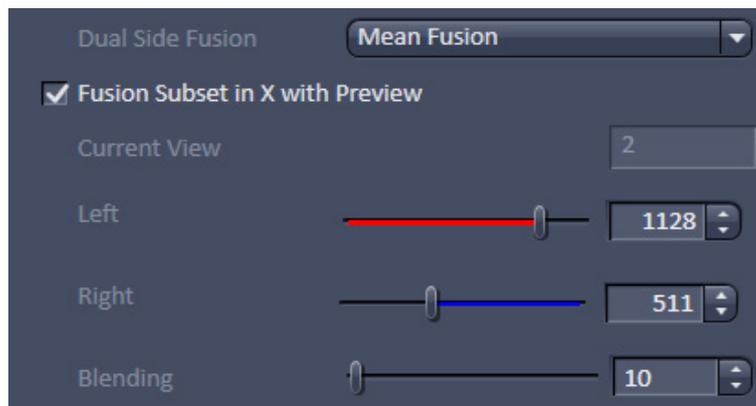


Fig. 263 Dual Side Fusion – Fusion Subset in X



Fig. 264 Dual Side Fusion – Fusion Subset in X, Preview and Image control area

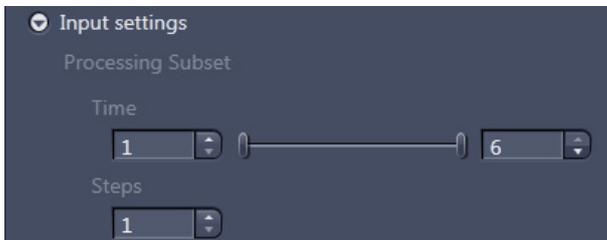
**Ignore Sample:** This option activates an additional function to find structures within the sample that were incorrectly labeled as beads (fiducials). These structures are not taken into account during the Landmark Alignment process; but they are not removed from the **Preview**.

**Expand To Maximum Volume:** This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

**Scaling x=y=z:** The dimensions are downsized to the smallest to have the same size. If the scaling in **Deconvolution, PSF settings** (Fig. 252) is changed, the latter will override the scaling settings here.

**Individual Registration For Every Time point:** If the multiview experiment has been performed over time, this option becomes available.

- If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the **Individual Registration For Every Time point** option becomes necessary.
- If **not activated**, one transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.



**Fig. 265** Processing – Lightsheet Processing. Multiview Processing, Input Settings

### 3.3.18.2.3 Input Settings

In this sub-menu, a subset of a present time series can be chosen for the Registration and Fusion processing (Fig. 265).

Use the two sliders or input boxes with arrows on each side to truncate the **Time** points. If not every time point in this period should be taken, the **Steps** can be defined with the input-box with arrows.

If a subset of a time series is defined this way, the resulting images will be numbered according to their new sequence, which might not be the same as in the input file.

### 3.3.18.2.4 Fusion

After Registration of the Views, the **Fusion** process can be performed (Fig. 266). This will lead to one z-stack or z-stack over time.

You can choose the following methods using the drop-down menu:

**None:** no Fusion is performed after Registration and Dual Side Fusion.

**Mean Fusion:** The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.

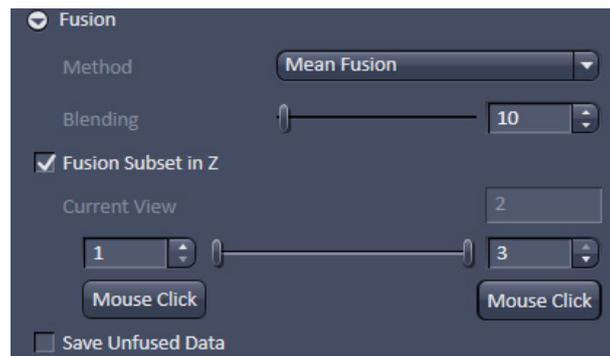


Fig. 266 Processing – Lightsheet Processing. Multiview Processing, Fusion

**Mean Fusion + Multiview DCV:** This option is only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The **Deconvolution** interface becomes available when chosen (see above).

When all z-stacks (hence all views) are fused into one z-stack, the intensity values at the z-stack borders can lead to visible edges. To prevent this, the borders of all z-stacks can be faded during the Fusion process. With the **Blending** slider and input box with arrows, the amount of blending (smoothing) can be controlled. The value for **Blending** should be as low as possible, with typical values from 20 to 80; but this needs to be tested for every dataset.

It is possible to define which portion of the z-stacks of the contributing views of the experiment shall be used for the fusion process. To do so the checkbox **Fusion Subset in Z** must be activated (Fig. 237). The view can be chosen with the according dimension slider of the **image control area**, and is displayed as **Current View**. For each of the views the z range can be chosen with the spin boxes or the sliders from each side. Alternatively a click on the **Mouse Click** button and afterwards onto the image (e.g. in the **Gallery View**) can be used to select the images to define the z-range.

Whiles the whole z-stacks are still used for registration (e.g. fiducials for Landmark based registration remain accessible), only the image information of the selected z-range will be used for **Fusion**.

Portions of the z-stacks, highly scattered and/or blurry content, can be discarded from the resulting fused image, to overall enhance the image quality. It must be considered for later deconvolution, that the calculated point spread function (PSF) of a multiview experiment, is based on the assumption that all views equally contribute to all areas of the image. This is not the case when using **Subset Z** and might result in artefacts of the deconvolution result.

If you want to keep the registered image as an interim result, even if the registration and fusion are done in one routine, activate the **Save Unfused Data** checkbox.

### 3.3.18.3 Online Multiview Processing

Processing of a multiview experiment dataset can be done online during data acquisition while the data is streamed onto a storage and analysis PC. ZEN software must be started on the storage and analysis PC and the master file (\*.czi) of the experiment can be opened.

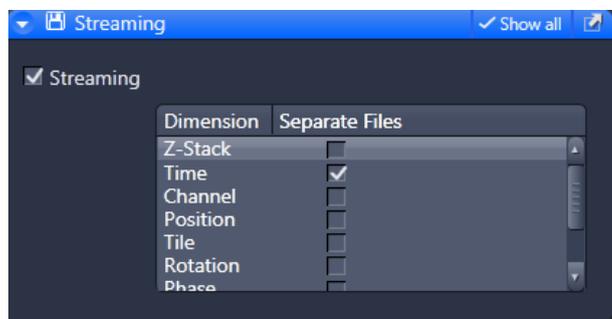


Fig. 267 Streaming tool window

It is possible to save dimensions of the experiment as individual files. To do so, the dimensions can be chosen with the according check box in the **Streaming** tool window (Fig. 267) of the **Maintain** tab (see as well section 3.4 **Maintain Tab**).

 It is important that only the **Time** checkbox is activated for **Online Multiview Processing**, which is the default setting. If other dimensions are separated (e.g. channels) they are not included in the processing procedure and will be missing in the resulting file.

When time series are acquired with the Lightsheet Z.1, the first time point is a master file with the following appearance: Name.czi (e.g. FishEmbryogenesis.czi). For each time point, a separate file is additionally formed in the same folder with the nomenclature: Name(time point-1).czi (e.g. FishEmbryogenesis(221).czi). This master file must be selected in the **Method Parameters** window. There, the settings for processing can be chosen as described in the section 3.3.18.2 **Multiview Processing**.

When pressing the **Apply** button, processing will start for the already acquired images of the time series.

The registration option: **Interactive registration** is not available for **Online Multiview Processing** and is missing from the according drop down menu. To use this registration during the acquisition of the time series, please proceed as following. Open one time point of the experiment and choose the **Multiview Processing** option and press the **Select** button. Follow the description in section 3.3.18.2 **Multiview Processing** to perform an interactive registration. When pressing the **Apply** button, an .xml file is created in the **Result** folder, which holds all registration information. The **Result** folder is created within the folder of the original dataset.

Proceed by opening the master file (first time point) of the ongoing experiment and choose **Online Multiview Processing**. Press the **Select** button and choose **Registration from file** from the drop down menu. Follow the instructions in section 3.3.18.2 **Multiview Processing**.

### 3.3.19 Processing – Copy

Licensing		Instrument		
<input checked="" type="checkbox"/> standard	<input type="checkbox"/> optional	<input checked="" type="checkbox"/> LSM	<input checked="" type="checkbox"/> ELYRA	<input checked="" type="checkbox"/> Lightsheet Z.1

Tool accepts all types of image data as input. See full description of tool set for details.

The Copy function provides means to copy or duplicate images.

**Copy Channel** (Fig. 268) produces a copy of the selected image.

The first **Select** button in the selection panel selects the active image in the **Image Display** as Input Image. The **Channel** selection pull down menu specifies which channel to copy.

The second **Select** button is used to choose a destination image if the copied channel is to be added to an existing image document. In the second **Channel** selection pull down menu the destination channel is specified – or a new image document can be chosen as destination.

Pressing **Apply** starts the copy process.

 For Z-Stacks or Time Series, the entire series for the selected channel is copied.

#### Duplication

**Duplication** creates a new image document which is a duplicate of the selected input image.

#### Delete Image

Single images or series of images can be deleted from the selected image series (input image) acquired as time series or Z-Stacks or combinations of those.

The **Select** button selects the active image in the **Image Display** as the **Input Image** to be processed.

In the tool-specific settings panel, the parameters for the interpolation procedure are set.

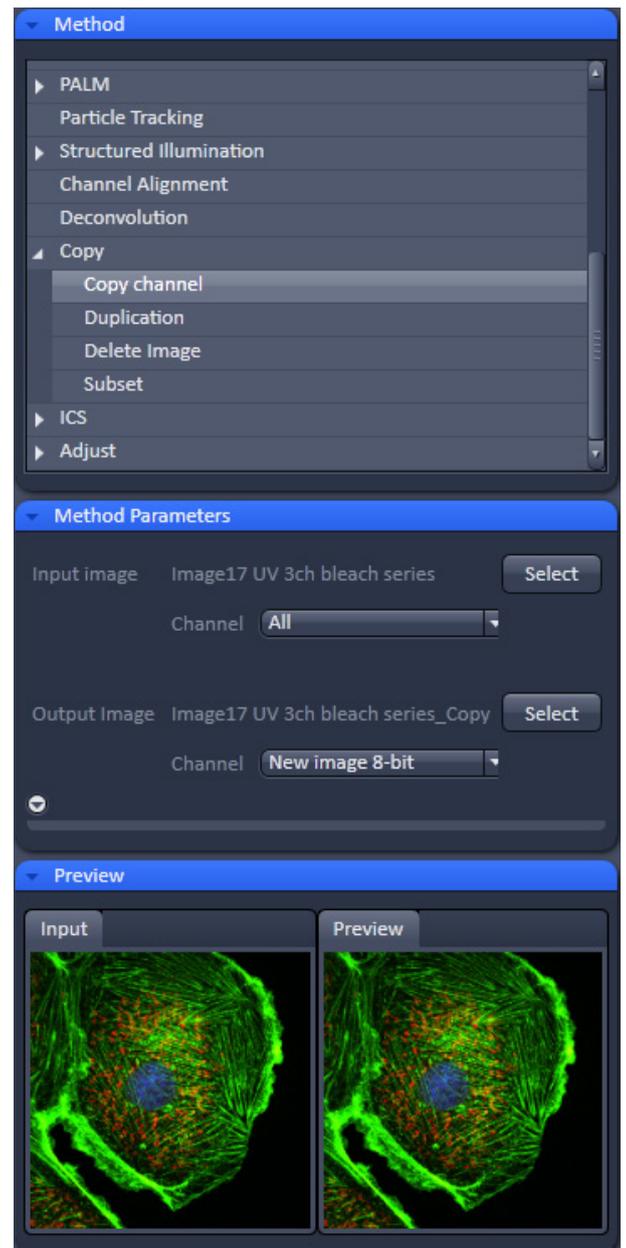


Fig. 268 Processing – Copy

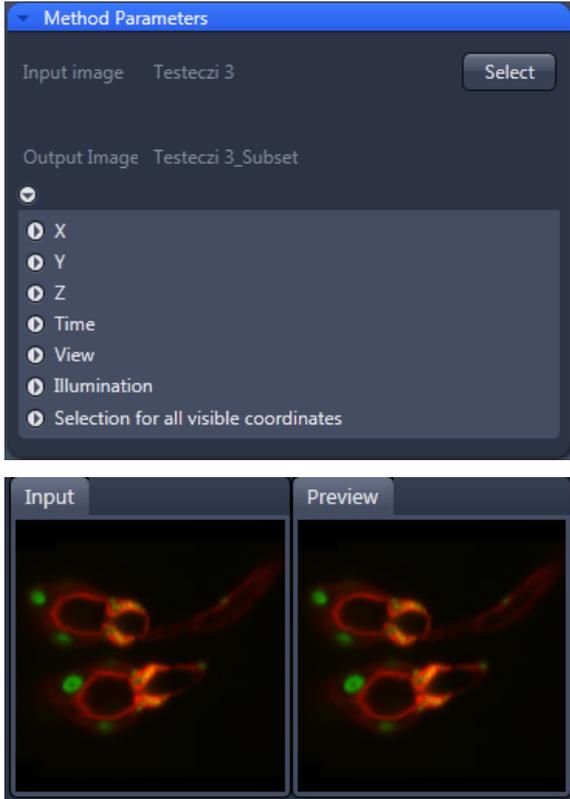


Fig. 269 Subset tool with loaded Multiview (Lightsheet Z.1) image



Fig. 270 Subset panel for x-coordinate

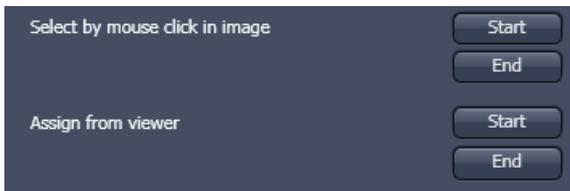


Fig. 271 Subset panel for "visible"

Opening for example the x-subset panel (Fig. 269) brings up three sliders to set the **Start** and **End** coordinates of the new data set.

Pressing the **Mouse click** button selects the respective coordinate by clicking on the original image in the **Preview View** in the image display area (Center Screen Area). The **Step** coordinate determines how many data points in the respective dimension are dropped out. For example: setting **Step** to 3 takes 1 and drops 2 pixels along the respective dimension, reducing the data to 1/3. The scaling is calculated to remain correct in the resulting image.

### Subset

The **Subset** function is a set of tools to truncate a multidimensional data set in all available coordinates to the desired size (Fig. 270).

These subset panels are available for all coordinates of the selected image (x, y, z, t, view, illumination). The panel **Selection for all visible coordinates** is shown in Fig. 271. It always applies only to those coordinates open within the subset-panel (above).

The **Select by mouse click in image** option allows one to click in the Image Display and the respective coordinates are set (if displayed in the image: x, y in the 2D view, z, view and illumination in the gallery view, etc.).

The **assign from viewer** selects the coordinate from the slider in the **Dimensions View Options** control block.

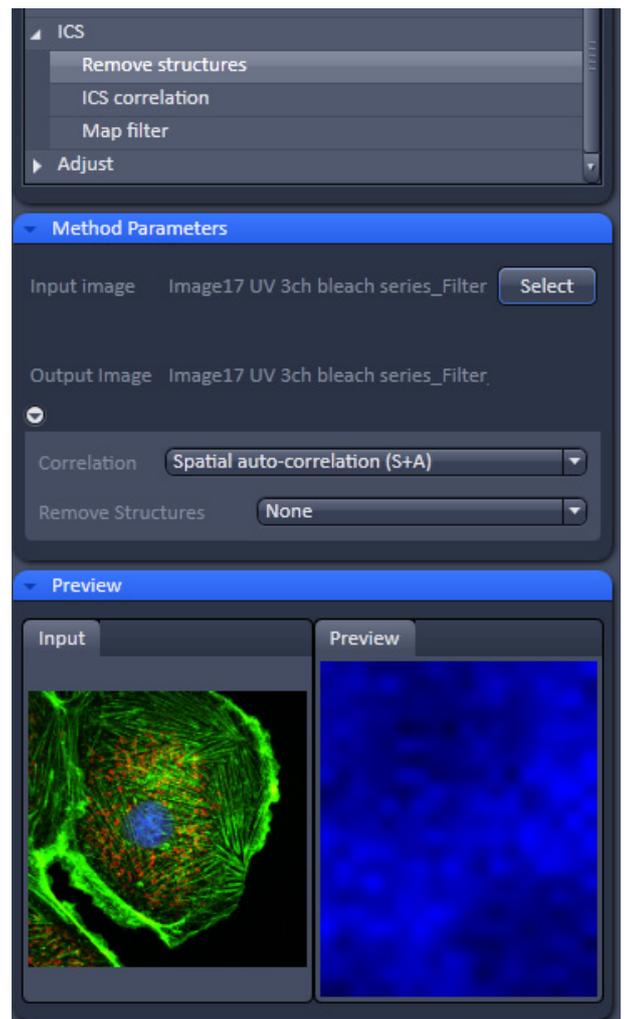
**3.3.20 Processing – ICS**

Licensing		Instrument		
standard	✓ optional	✓ LSM	ELYRA	Lightsheet Z.1

Tool accepts exclusively input acquired with LSM 710 and LSM 780. See full description of the tool set and section on *RICS (Raster Scanning Image Correlation Spectroscopy)* for more details on requirements to be met.

- Activate **ICS** in the Processing tool by clicking on the arrow.

The **ICS** menu will appear with three sub-processing functions if expanded: **Remove structures**, **ICS correlation** and **Map filter** (Fig. 272).



**Fig. 272 Processing – ICS Remove structures**



**Fig. 273** Remove structure drop down menu

### 3.3.20.1 Remove Structure

- Activate **Remove structures** in the **ICS** tool. The **Remove structures** menu will appear.
- To select an image, highlight the image in the image container and press the **Select** button. This will be your **Input** image.

As an output image an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- If the **Apply** button is pressed, the averaged subtracted image stack will be opened as a new image in the container.

You have four options that can be selected via the **Remove Structures** drop down menu (Fig. 273):

**None, Slowly moving structure, Immobile structures (+Stack) and Immobile structures (+Plane).**

If **None** is selected, no average will be subtracted and the original image is kept. If **Slowly moving structure** is activated the **Average frames** input box becomes available. Type in the required number (minimum is 2) or use the slider or arrows for input. The number defines the number of frames that will be averaged and subtracted in a gliding way from the original stack in the following way:

- From a stack of  $n$  frames take successive  $m$  moving average frames with an overlap of  $m-1$  and average these. The outcome is a new stack with  $n-m+1$  gliding or moving average frames. Calculate from each of the average frames the overall average of all pixels, which is a scalar.
- Subtract the  $i$ -th of the gliding average frame from the  $(i-1)+m/2$  original frame and add the  $i$ -th scalar. In this way, the first frame to be used from the original stack is  $m/2$  and the last  $m/2+(n-m+1)$ . This creates a new stack of  $n-m-1$  frames that is the output image.

If **Immobile structures (+Stack)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. An average value of all pixels from all frames is computed, which is a scalar, and added to each pixel of the single frames of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

If **Immobile structures (+Plane)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. Average values of all pixels from each of the original frames is computed, which is a scalar, and added to each pixel of each corresponding single frame of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

### 3.3.20.2 ICS Correlation

- Activate **ICS correlation** in the **ICS** tool. The **ICS correlation** menu will appear (Fig. 274).
- Highlight the image in the image container and press the **Select** button. This will be your **Input** image.
- This function is only available in the LSM imaging mode

A correlation image will be computed based on the settings and displayed in the **Preview** window. As an output image an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- The correlation to be computed can be selected from the **Correlation** drop down menu (Fig. 275). For one channel recordings, only **Spatial auto-correlation (S+A)** is available, for a two channel recording in addition **Spatial cross-correlation (S+C)**.

The way of average subtraction can be selected from the **Remove structures** drop down menu (Fig. 276).

- You can select between **None** (no average subtraction), **Slowly moving structures** (subtraction of moving average, the frame size of which can be selected in the **Average frames** input box), **Immobile structures (+Stack)** and **Immobile structures (+Plane)** (subtraction of total average and adding average scalar of all pixels from one frame to each corresponding frame).

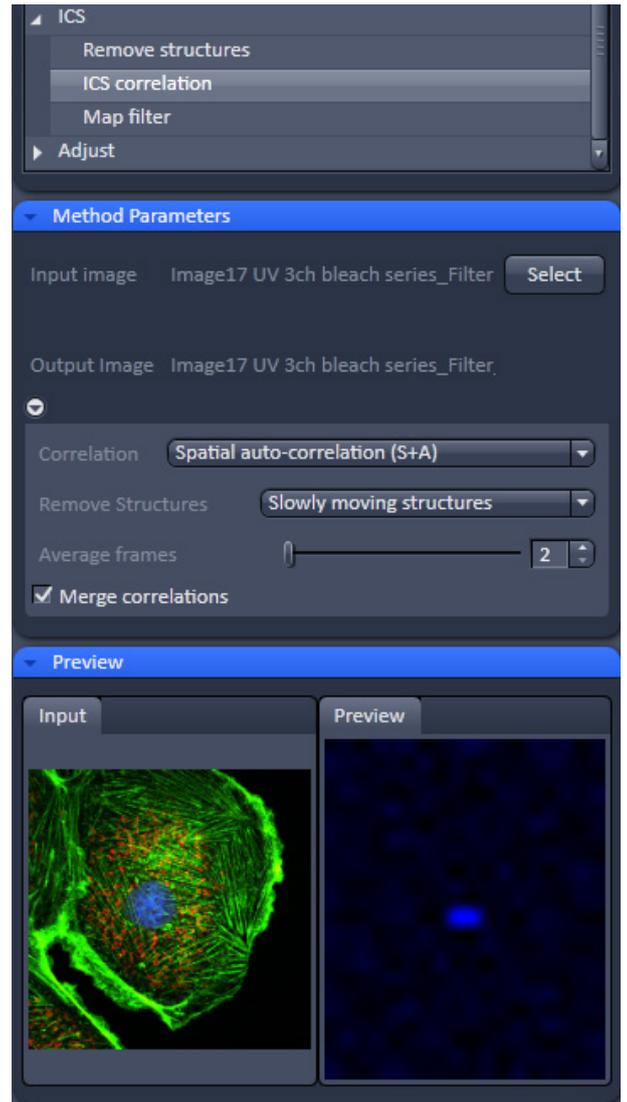


Fig. 274 Processing – ICS correlation

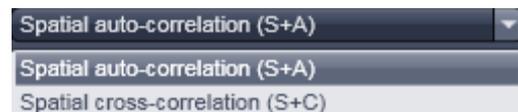


Fig. 275 Correlation drop down menu

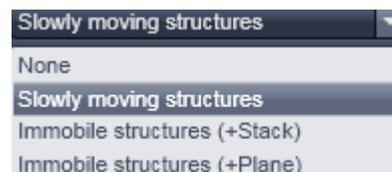


Fig. 276 Remove structures drop down menu

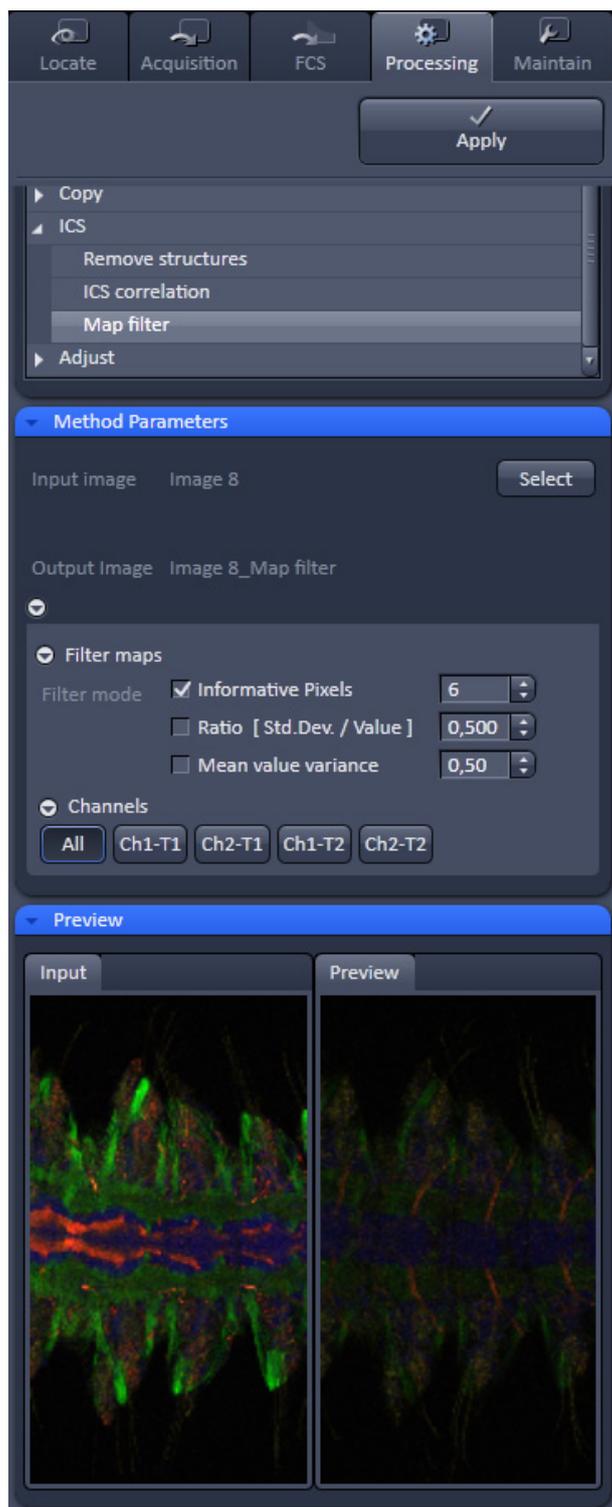


Fig. 277 Processing – ICS Map filter

### 3.3.20.3 Map Filter

- Activate **Map filter** in the **ICS** tool. The **Map filter** menu will appear (Fig. 277).
- To select an image highlight the image in the Image container and press the **Select** button. This will be your **Input** image map.

As an output image map an image map with the selected filters applied is computed and the result filtered image maps will be opened as a new image maps in the container.

The way of filters to be applied can be selected in the **Filter maps** menu, which can be opened by the arrow, by checking the **Ratio [Std.Dev./value]** and the **Mean value variance** check boxes.

 Note that both filters can be applied together by checking both boxes.

- Thresholds for the filters can be entered in the corresponding input boxes by typing or by using the arrows. Pixels with values exceeding the threshold will be displayed in black and will be discarded for the scaling of the map.
  - You can define if all maps should be filtered by pressing **All** button in the available filter display area.
  - If you want to apply filtering only to a specific map (**Number of particles**, its standard deviation **Std.Dev.**; **Diffusion coeff. [ $\mu\text{m} \times \mu\text{m} / \text{s}$ ]**, its standard deviation **Std.Dev.** and  **$X^2$** ) the **Filter all maps** box must not be ticked and the corresponding map button pressed, which is indicated by highlighting in blue.
-  Note that filtering is never applied to the  **$X^2$**  map, even if it was selected.

### 3.3.21 Processing – Adjust

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1*

Tool accepts all types of image data as input. See full description of tool set for details and potential restrictions.  
\* Except Auto Adjust Shading Correction

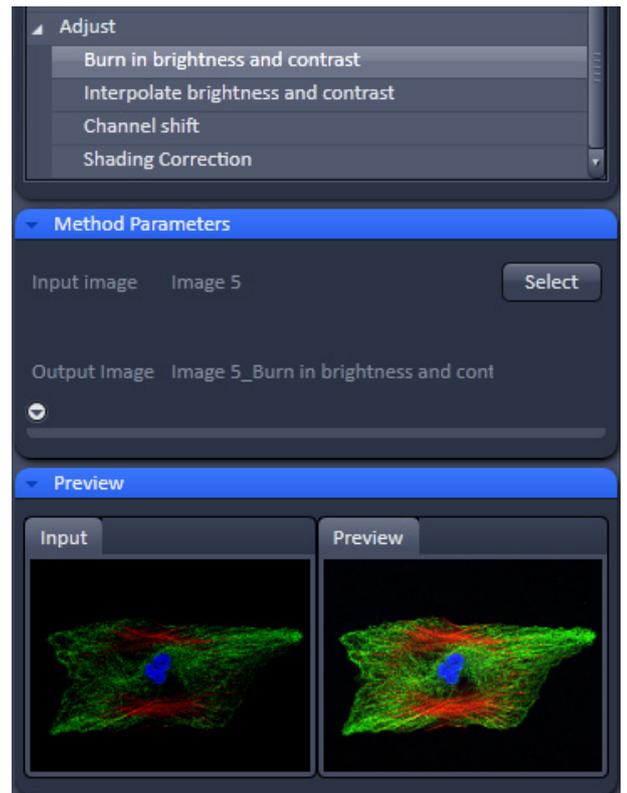
This tool allows brightness and contrast adjustments to your image as well as shifting two images against each other (Fig. 278).

#### 3.3.21.1 Burn in Brightness and Contrast

The **Burn in brightness and contrast** function creates a new image document in which the current brightness and contrast settings from the Display View Options control block (see section **4 Center Screen Area / Image Containers - Display and Image Analysis**) are permanently written into the image file (Fig. 278).

The **Select** button selects the active image in the **Image Display** as the Input Image to be processed.

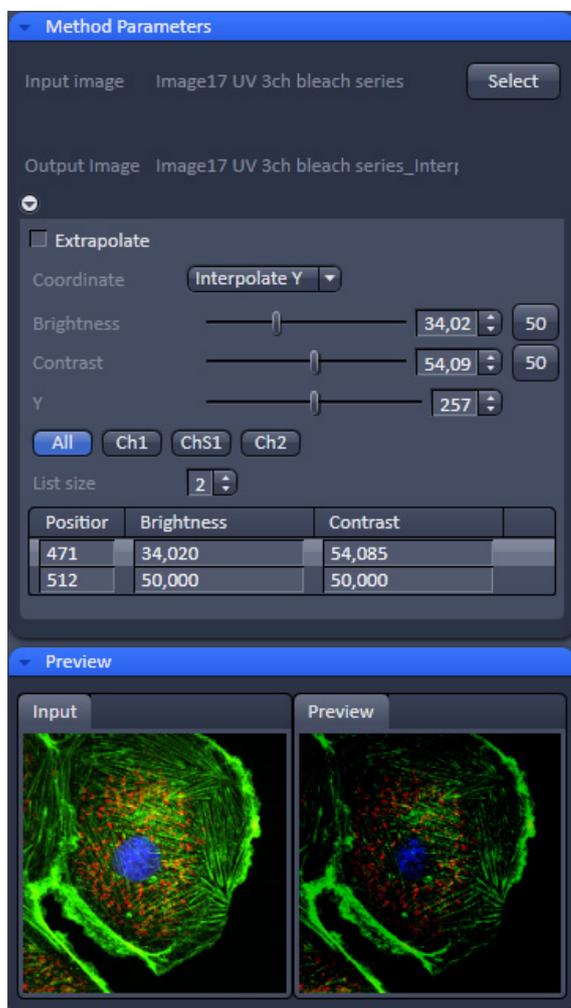
- After adjusting the brightness and contrast in the Display View Options control block, click Apply to create the new image document with the burnt in contrast and brightness settings.



**Fig. 278 Processing – Adjust with Burn in brightness and contrast**



**Fig. 279** Processing – Adjust with Interpolate brightness and contrast



**Fig. 280** Processing – Adjust with Interpolate brightness and contrast tool with loaded Z-Stack

### 3.3.21.2 Interpolate Brightness and Contrast

This function permits a continuous contrast and brightness adjustment in a Z-Stack or Z-Stacks over time by interpolation between the starting and end values of the respective image series. This permits a post-acquisition compensation of signal loss in, for example, thick tissue imaging where excitation and detection efficiency decrease significantly. Interpolation can be defined for the entire image or only for individual channels. In addition, a set of coordinates for which intensities are kept fixed during the interpolation calculation can be defined (Fig. 279).

The **Select** button selects the active image in the **Image Display** as the Input Image to be processed.

In the **Interpolation** panel (Fig. 280), the parameters for the interpolation procedure are set.

- In the **Coordinate** pull down, select the dimension for the interpolation. Depending on the image data type, x, y, z, and time are the possible options.
  - Use the **List size** spin box to set the number of fixed data points. The table in the **Interpolation** panel will be extended to the selected number of rows.
  - Highlight a row of your choice by clicking on it. Then use the **Brightness**, **Contrast** and Z (or t) slider to set the values for this fixed data point. The numbers in the table will be updated according to the slider position. Then highlight the next row, set the parameters and keep setting the fixed data points for all rows.
  - Use the available **All**, **Ch1-T1** and **Ch2-T2** channel buttons to select the channel for interpolation or click on the **All** button if the entire image is to be interpolated.
  - Having set the parameters, click on the **Apply** button. Interpolation will be performed in a new **Image Display** window.
  - The newly created image (series) can be stored using the **Save As** function.
-  If you de-activate the Extrapolate check box, only the slices lying between the first and last fixed data point (table rows) will be taken into account for interpolation. Otherwise, brightness and contrast will also be changed for the other slices.

### 3.3.21.3 Channel Shift

**Channel shift** is used to produce a congruent image with relation to the pixels of the various channels (Fig. 281).

This pixel correction function is particularly important in UV applications.

 This is a manipulation of your data that has to be considered very carefully when co-localization analysis is part of your experiment.

The **Select** button selects the active image in the **Image Display** as the Input Image.

The image has to be a multi-channel image.

- Select the channels you want to shift by ticking the Ch1-T1 or Ch2-T2 check box:
- Use the X and Y sliders or spin boxes to define the shift in pixels to select the pixel shift in the horizontal and vertical direction. Clicking the Zero next to the spin-boxes resets the shift to the original position.

A preview is automatically generated at the bottom of the **Channel Shift** tool in the **Processing** tab of the Left Tool Area. At the same time, a large preview is shown in the **Preview** view tab in the Image display in the Center Screen Area.

- Click on the **Apply** button to generate a new image with the set pixel shift.

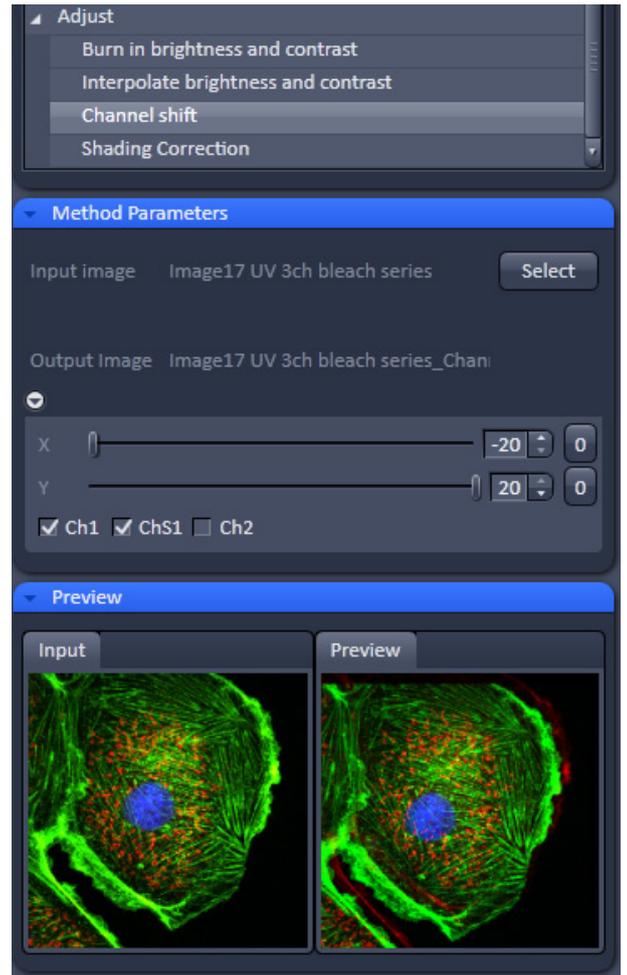
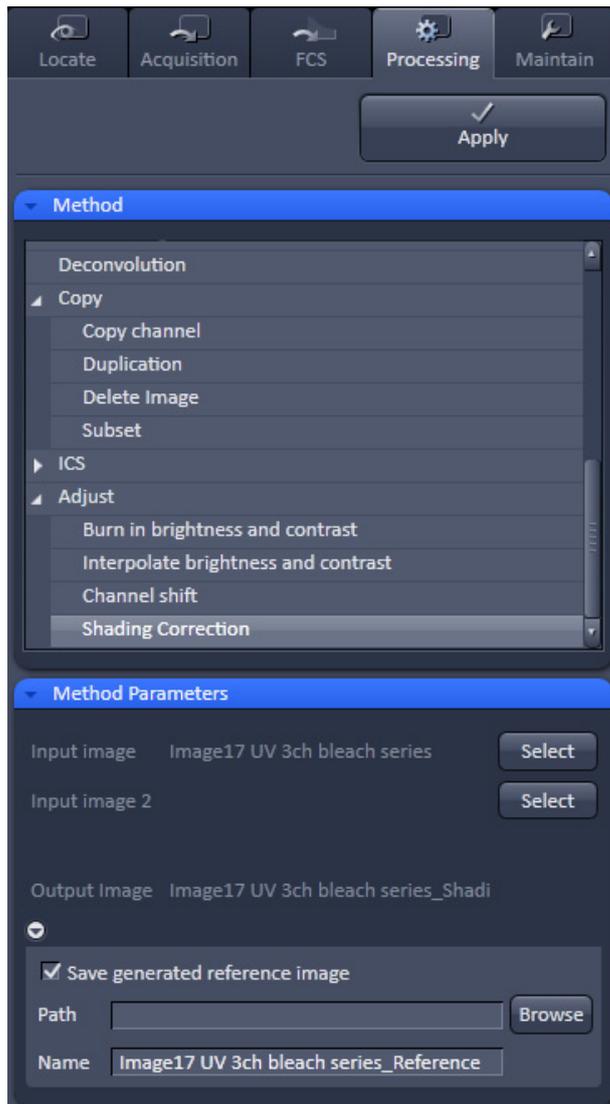


Fig. 281 Processing – Adjust with Channel shift



**Fig. 282** Processing – Adjust with Shading correction

### 3.3.21.4 Shading Correction

Shading Correction is a tool to manipulate images which are unevenly illuminated over the field of view. This works with single images, time series, Z-Stacks, or tiled images. Click on **Shading correction** to open the interface for using that function (Fig. 282).

**Select** the image (or image series, tiled image, or Z-Stack) for which the shading correction should be applied by clicking **Select** next to **Input image**. When clicking **Apply** the system calculates a reference image from the input image(s) which is used to correct the input image(s). The corrected image is displayed in the center screen area.

In addition, the calculated **reference image** can be stored and used for further shading corrections of other images. Therefore the check box in the lower part of the tool box must be checked. Specify the folder where the image should be stored in the text box next to **Path**. **Browse** opens the windows folders from which you can select. A suggestion for the name of the image is made based on the original name of the image. Change this if needed.

This reference image can then be loaded and selected as a reference image for the shading correction of subsequent images, which are then processed much quicker.

The reference image must be taken from an image that was acquired with identical settings for objective, zoom, image format, and orientation.

### 3.4 Maintain Tab

Press the **Maintain** tab to have access to the **Maintain** tool group area (Fig. 283). The maintain tool group allows adjustment of hardware settings.

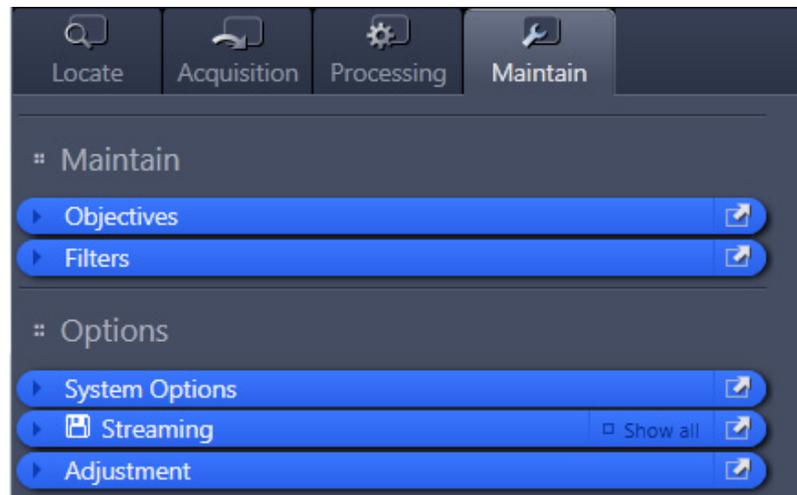


Fig. 283 Maintain tool group area

The Maintain area is divided into the **Maintain** and **Options** field.

The tool tabs in the **Maintain** field allow the calibration and adjustment of hardware settings. The following options are available:

- Define **Objectives**
- Define **Filters**

The tool tabs in the **Options** field allow customized hardware and software settings. The following options are available:

- **System Options**: Options for Lightsheet Z.1
- **Streaming**: Defining the directory and the dimensions for data saving during acquisition.
- **Adjustment**: Tools to adjust the Lightsheet Z.1 and the detection modules.

#### 3.4.1 Maintain – Objectives

Expand the **Objectives** tab to have access to the objective definition and selection.

With this function one can assign a detection optic or illumination optics, e.g. when an detection optic or both illumination optics were exchanged within the Lightsheet Z.1 system without having to leave the ZEN software.

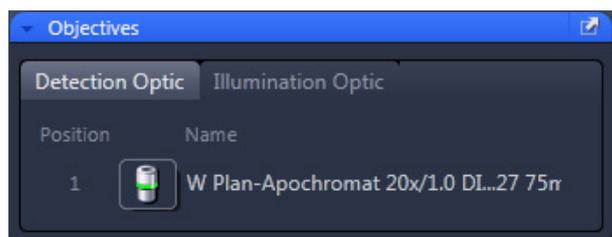


Fig. 284 Detection Optic tab

### 3.4.1.1 Detection Optics

Press the **Detection Optic** tab to have access to the Detection Optics definition window (Fig. 284).

Press the Objective icon to have access to the **Change Objective** expansion window for the respective objective with the following functions

- Add Objective
- Show/Edit Objective
- Remove Objective
- Select Objective.

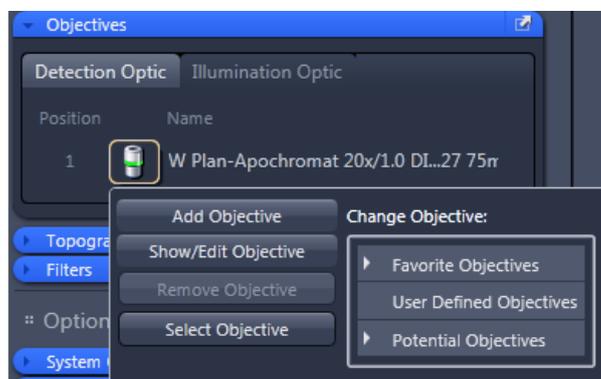


Fig. 285 Objective selection menu

### 3.4.1.2 Select Detection Optic

Select the **Detection Optic** tab from the **Objective** tool, which will show the **Name** of the detection objective. After screwing the detection or illumination optic into your system you continue as follows:

Click on the **Objective icon** button. This will open the Objective selection menu (Fig. 285).

In the Change Objective area open one of the available directories of **Favorite Objectives** (showing all the objectives that have been previously selected), **User Defined Objectives** (showing all the objectives that have been previously defined by a user) or Potential Objectives (showing all objectives listed in the used ZEN database).

Expanding the field opens the respective objective directory list. An expansion triangle is only available when objectives are available in the respective directory. The scroll bars allow having access to all entries within the list.

- Select the correct objective by highlighting it. Press the **Select Objective** button. Note that the button becomes only active if an objective was selected. The Name of the objective will appear in the corresponding position.

Each detection optic is labeled with an individual serial number. It ensures that the specific calibration file for this lens is used when identified within the software. Make sure that the correct detection optic is chosen in the ZEN software interface, based on the name and the serial number of the detection optic in use.

- Click anywhere outside the expansion window to close it.

### 3.4.1.3 Add Objective

This function allows adding objectives to the **User Defined Objective** database.

- Click on the Add Objective button. The **Create new Objective** expansion window will appear (Fig. 286). The scrollbar allows access to the whole field.
- Enter the parameters of the new objective in the appropriate display boxes or drop down menus. The text of those boxes that are inactive for editing, are greyed.
- Press the **Apply Add** button to store the newly defined objective in the **User Defined Objective** directory.

If the **Non Zeiss** check box is ticked, objectives from other manufacturers can also be included in the database.

If the name of the objective already exists, a warning is displayed telling the user to rename (Fig. 287).

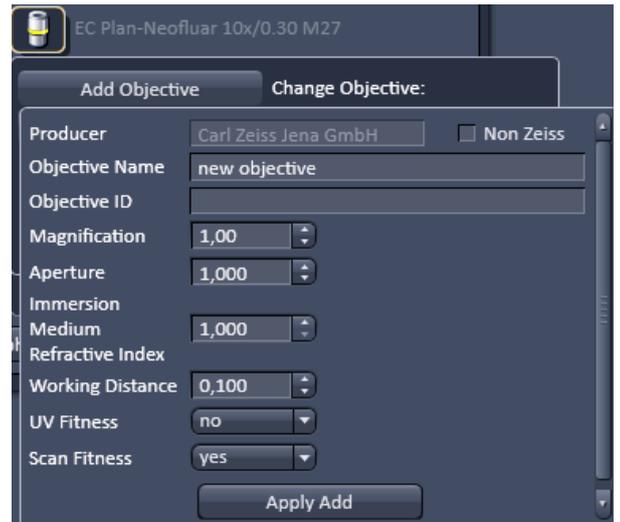


Fig. 286 Adding a new Objective – Editing field

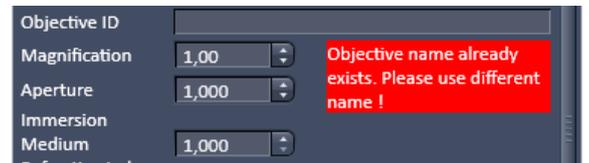


Fig. 287 Objective Name warning message

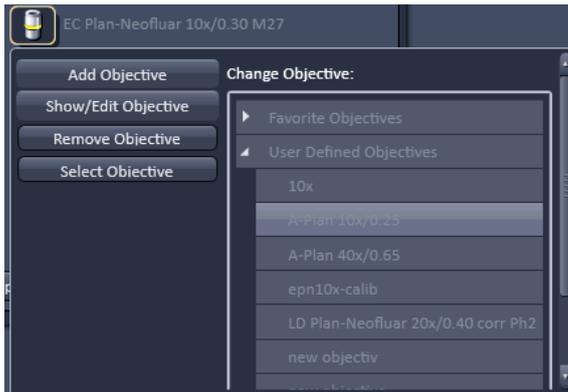


Fig. 288 Remove objective

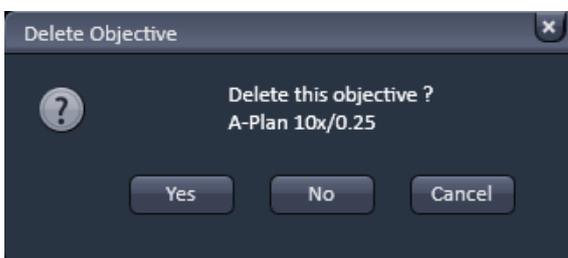


Fig. 289 Delete objective window



Fig. 290 Show/Edit objective

### 3.4.1.4 Remove Objective

This function allows removing objectives from the User **Defined Objective** database (Fig. 288).

Expand the **User Defined Objective** directory and highlight the objective you want to delete.

Press the **Remove Objective** button in order to delete the selected objective. Note that the Remove Objective button only becomes available if an objective from the user Defined Objectives directory was chosen. You will be prompted to confirm the deletion of the objective by the **Delete Objective** pop up window (Fig. 289). Press **Yes** to delete the objective, **No** to keep it in the database, or **Cancel** to abort the process.

### 3.4.1.5 Show/Edit Objective

This function allows viewing the parameters of an objective from all objective directories. Editing the parameters is only possible for the **User Defined Objective** database (Fig. 290).

Expand the **Favorite Objective**, **User Defined Objective**, or **Potential Objective** directory and highlight the objective you want to view. This will open the View tab.

In case of an objective listed in **User Defined Objective** directory, parameters can be edited within the display boxes (those parameters not to be edited will be greyed out) or the drop down menus. Press the **Apply Edit** button (only available for user defined Objectives) to make the changes take effect and store the new annotations.

### 3.4.1.6 Illumination Optics

Press the **Illumination Optics** tab to have access to the Illumination Optics definition window.

Press the Objective icon to have access to the **Change Objective** expansion window for the respective objective with the following functions:

- Add Objective
- Show/Edit Objective
- Remove Objective
- Select Objective.

Please see details in section **3.4.1.1 Detection Optics**.

### 3.4.2 Maintain – Filters

Expand the **Filters** tool to define the filters present in the Reflector Turret for Laser Blocking Filters as well as in the Reflector Turret for Emission Selection for your Lightsheet Z.1 system as will be shown in the ZEN software.

Laser blocking filters are easily exchangeable push and click filter cubes. The beam splitters cannot be exchanged, but the associated emission filters can be exchanged.

On the top is a row of tabs for every filter position. To reach the right most tab, press the white arrow on the right side of the row. If you press the white arrow on the right side that points downwards, a list of all possible tabs are displayed. Here you can chose which filters to edit.

In each tab you find a list of positions that relate to the positions in the corresponding reflector turret.

The **Laser Blocking Filter** tab represents the Reflector Turret for Laser Blocking Filters (Fig. 291). These are push & click filters which can be easily exchanged.

The **Beam Splitter**, **Emission Filter 1**, and **Emission Filter 2** tabs (Fig. 292) describe the filters of the Turret for Emission Selection. Each row of these tabs represents one position in the turret, and all three together represent one filter combination. Only the **Emission Filter 1** (in front of Cam1) and the **Emission Filter 2** (in front of Cam2) can be exchanged by the user (see CHAPTER 1 HARDWARE).

To update the filter information in the software, press on the **filter symbol** you would like to edit. This will open a box listing the **Recent** used filters in the upper part (Fig. 293). In the lower part are listed all available **Filters** which can be searched by entering the full or part of the name into the **Search** field. Simply clicking on the Filter within the list will enter its name into the software interface.

The following abbreviations are used:

- LBF: Laser blocking filter
- SBS: Secondary beam splitter
- BP: Band pass filter
- LP: Long pass filter
- SP: Short pass filter

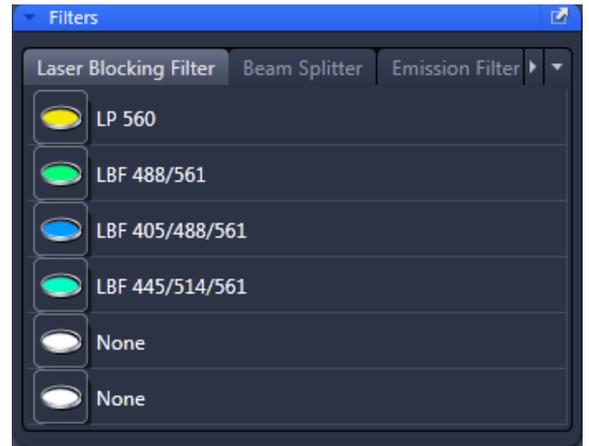


Fig. 291 Laser Blocking Filter Tab



Fig. 292 Beam Splitter Tab

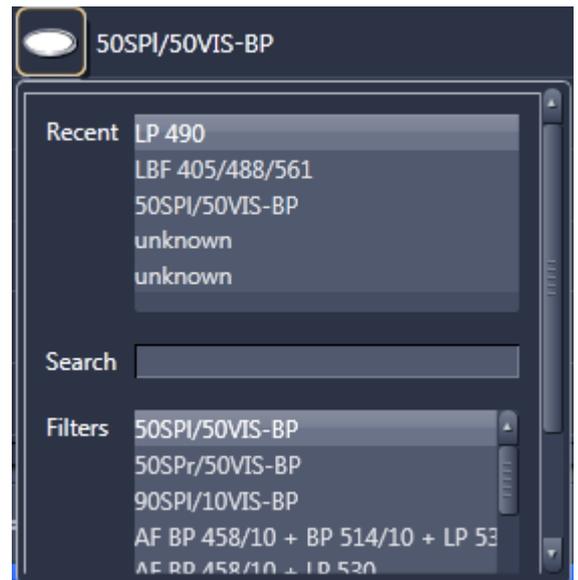


Fig. 293 Recent and Filters list box

### 3.4.3 Maintain – System Options

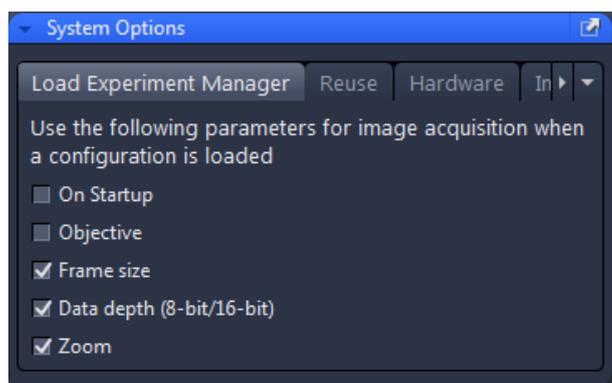
The **System Options** tool allows for setting hardware parameters. Expand the tool to have access to the **System Options** settings window.

The left and right pointing triangles give access to all functions by sliding the tabs to the left and right.

The following available user-accessible program **Settings** for the ZEN software are:

- **Load configuration**
- **Reuse**
- **Hardware**
- **Image display**

Click on the respective tab or select the function from the Settings drop down menu to open the desired panel.



**Fig. 294** System Options – Load Experiment Manager

#### 3.4.3.1 System Options – Load Experiment Manager

Press the **Load Experiment Manager** tab to open the panel (Fig. 294).

**Load Experiment Manager** lists parameters that, when their respective check box is ticked, will be taken into consideration when an experiment configuration based on a saved image is loaded. The following parameters are available:

- On Startup (the last open configuration is loaded when ZEN is started)
- Objective
- Frame size
- Data depth (8-bit/16-bit)
- Zoom

### 3.4.3.2 System Options – Reuse

Press the **Reuse** tab to open the **Reuse** panel (Fig. 295).

The **Reuse** function can be found in the **Dimensions View** control and the **File Browser** window.

Using an acquired image you can load its hardware settings and start imaging.

The following parameters are loaded when the **Reuse** button is pressed:

- Continuous drive (a function in the Z-Stack tool)
- LED illumination settings
- Tracks/Channels and their light path
- Laser lines and their respective power in %
- Streaming

**Reuse** lists parameters that can be altered for use in the Reuse function. When their respective check box is ticked, they will be taken into consideration when the Reuse button for a loaded image is pressed. The following parameters are available:

- Incubation
- Binning
- Frame size
- Multiview Acquisition (settings not including the Z-Stacks)
- Light Sheet Illumination Mode (single or dual side illumination)
- Light Sheet Direction (left or right side)
- Pivot scan activation
- Bit depth

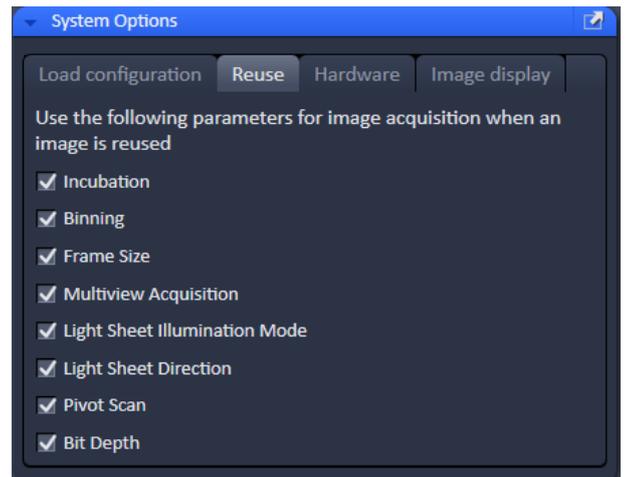


Fig. 295 System Options – Reuse panel

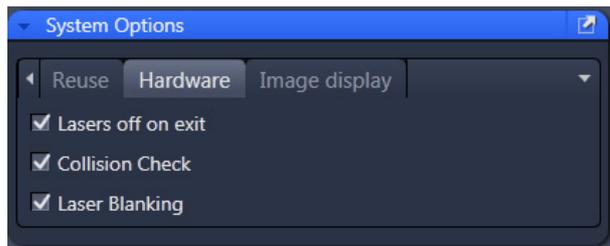


Fig. 296 System Options – Hardware panel

### 3.4.3.3 System Options – Hardware

Press the **Hardware** tab to open the **Hardware** panel (Fig. 296).

**Hardware** lists parameters that, when their respective check box is ticked, will set several hardware defaults.

By activation of the **Lasers off on exit** check box, the lasers are automatically switched off when the ZEN software is closed. The lasers are allowed to cool for five minutes before they are switched off.

The option **Collision Check** is activated by default with every start of the software. When active the software limits the movement of the sample holder to prevent the direct contact of either a capillary, syringe or the embedding medium with the front lens of the detection optic. This safety measure might prevent you from properly focusing on your sample. If so, you should uncheck this box and try to focus on your sample again.

**Laser Blanking** is a service task for your service engineer and is secured via a password. Please do not attempt to use it.

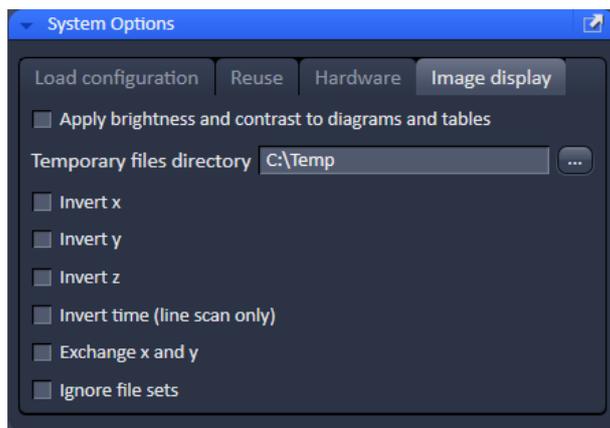


Fig. 297 System Options – Image display

### 3.4.3.4 System Options – Image Display

Press the **Image display** tab to open the **Image display** panel (Fig. 297).

If the **Apply brightness and contrast to diagrams and tables** is ticked, any changes in brightness and contrast of the image will also affect the image data shown in diagrams or tables. If not ticked, any changes in the **Image Display** will not affect the original image data. The default setting is off.

The **Temporary files directory** can be chosen manually by pressing the ... button via Windows explorer. For large datasets, it is advisable to use a drive **other** than the C-Drive of your computer.

Ticking the **Invert x** check box will invert the x-coordinate in the display. The original data set is not changed.

Ticking the **Invert y** check box will invert the y-coordinate in the display. The original data set is not changed.

Ticking the **Invert z** check box will invert the z-coordinate in the display. The original data set is not changed.

Ticking **Invert time (line scan only)** displays the line scan result with an inverted time axis. This function is usable for line scanning systems (LSM).

Ticking the **Exchange x and y** check box will exchange x for y coordinates and vice versa. The original data set is not changed.

When time series are acquired with the Lightsheet Z.1, the default setting will generate individual files for each time point (see as well section **3.4.4 Maintain – Streaming**). The first time point is the master file, which will open all associated time points together. If the first time point shall be opened individually, the checkbox **Ignore files sets** must be activated. The same logic holds true, when other dimensions are saved separately (e.g. channels, views).

### 3.4.4 Maintain – Streaming

Open the **Streaming** tool window (Fig. 298) to activate this option by checking the box next to **Streaming**. This will activate the function that data is saved and automatically written to the hard drive during the acquisition process, so when image acquisition is disrupted, the data up to this point will be available.

- ☞ This is advisable and should be activated by default since large datasets can be acquired with the Lightsheet Z.1 system.
- ☞ If you have a storage and analysis PC you wish to stream onto, make sure that the directory is mapped. When you turn on the PC for system control after the storage and analysis PC is completely booted, the directory is mapped automatically.

Activated Streaming will enhance the speed by which .czi datasets are opened.

Before an Experiment is started, a Windows explorer window will open. Here the destination of the streaming and a file name can be chosen. The image series will then be saved with this file name extended by the exact date and time of the image acquisition.

The data can be separated into separate files, defined by chosen dimensions. More than one dimension can be selected. As a default for later **Lightsheet Processing**, Time is activated and should remain this way. It will generate the file structure for later Lightsheet Processing, Multiview Processing and Online Multiview Processing.

In the **Dimension selection** field, you can define how the image series is saved. For each dimension, you can define by ticking the respective **Separate Files** checkbox to save this dimension into separate files. The file names of the single files will have extensions of a letter indicating the dimension (t for time series, z for Z-Stack, etc.) preceding a number that indicates the frame number from the series. If none is checked, the whole image series will be saved into one file.

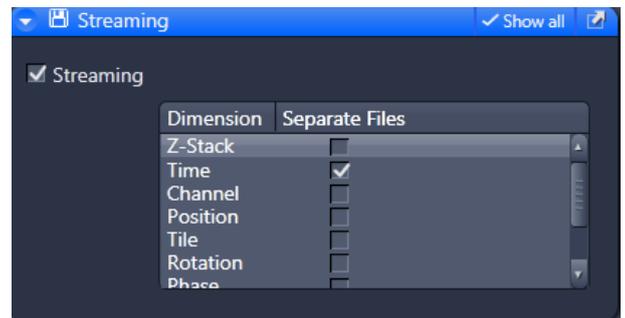


Fig. 298 Streaming tool window

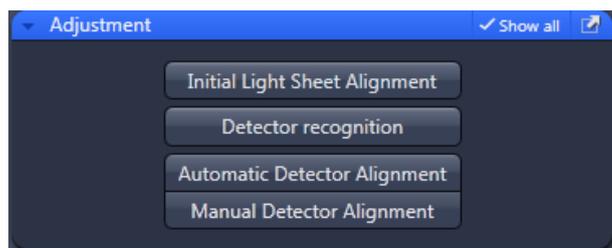


Fig. 299 Adjustment tool window

### 3.4.5 Maintain – Adjustment

In the **Adjustment** tool window, there are three buttons that start tools for the detection module recognition and alignment (Fig. 299).

**Detect Camera Order**, **Automatic Detector Alignment**, and **Manual Detector Alignment** are inherently part of the detection module exchange procedure.

☞ When you wish to **exchange detection modules**, please follow the complete descriptions in **CHAPTER 1 HARDWARE**.

Using the show all mode for this tool window, a fourth button appears: **Initial Light Sheet Alignment**. This is a task for authorized service personnel of Carl Zeiss Microscopy GmbH only and is therefore password protected.

☞ Do **not** use the **Initial Light Sheet Alignment** service task. You run the risk of altering elementary settings for your Lightsheet Z.1 system that have been optimally set upon delivery and installation. Please contact your local Carl Zeiss representative regarding this point only if you have designed and built your own sample chamber.

#### 3.4.5.1 Adjustment – Detector Recognition

When the detection modules are exchanged or attached to the Lightsheet Z.1 system for the first time, the system must identify which detection module is present for which channel.

Pressing the button **Detector recognition** will start an identification routine to do this job. A progress bar is visible during the process.

When the **Cancel** button is pressed, the operation is aborted without result.

This routine must be conducted before the **Automatic** or **Manual Detector Alignment**.

At the end of this process, you will be informed if the detector recognition had to perform any changes. If so, please restart ZEN software before continuing.

### 3.4.5.2 Adjustment – Automatic Detector Alignment

This adjustment is necessary for one or two detection modules, when:

- detection modules have been exchanged
- reflector turrets have been removed and inserted (please restart ZEN before the alignment task)
- when you recognize a pixel shift between channels
- when the zoom is not centered.

No sample should be present. The necessary correction plate and a grating is present for each channel within the system and needs no additional activation.

The Automatic Camera Alignment will align the image center with the detection module center (zoom center). For two detection modules, it will additionally align both to have overlaying pixels.

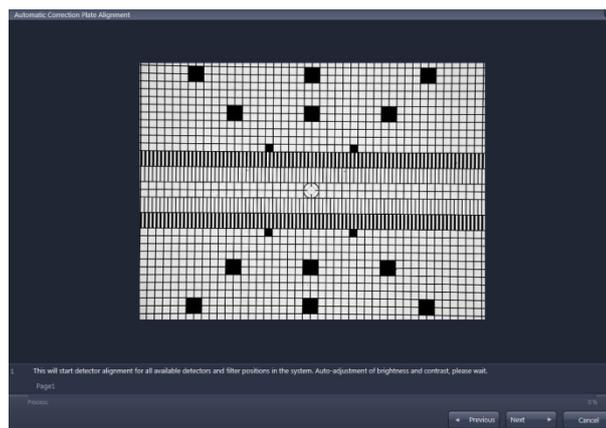
 After the detection module exchange, first press the **Detector recognition** button in the **Adjustment tool** window.

After pressing the **Automatic Detector Alignment** button, a window with a wizard will open that guides you through the process. Press the **Next** button after each step to continue or the **Previous** button to move one step back. If you press **Cancel** you will abort the process without result.

The first step (Fig. 300) automatically positions the necessary grating into the light path and adjusts brightness and contrast. A progress bar is present in the lower part of the window. You can follow the process in the displayed live image. You will find the following text below the live image:

"This will start detector alignment for all available detectors and filter positions in the system. Auto-adjustment of brightness and contrast, please wait."

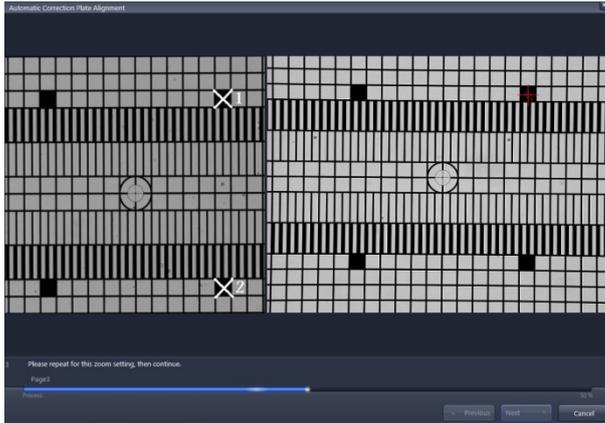
 If the live image of the grating shows the grid pattern out of focus, cancel the Automatic Camera Alignment wizard and then manually adjust the focus of the grating. The necessary steps are described in **CHAPTER 1 HARDWARE**.



**Fig. 300 Automatic Detector Alignment, first step**

In step 2 the movement of the correction plate is calibrated for both channels. There is no interaction needed. Press the **Next** button to continue after the task is done. The following text is displayed:

"Calibration of correction plate movement, please wait."



**Fig. 301** Automatic Detector Alignment, third step

In step 3 the zoom center is determined. A graphic of the grating is displayed on the left hand side of the window (Fig. 301). Two squares are labeled with white crosses in this image. On the right hand side, a live image of the grating is shown. Now mark the same squares as shown in the graphic in the live image by simply clicking on them with the left mouse button. This will be repeated for a second zoom step. The following text will be shown: "Determining the detector center. Please click on the two squares in the right live image of the grid that are marked with the white cross within the left image display. Repeat for both zoom settings, then continue."

Step 4 is the final step in which the alignment for all filters and detection module channels is performed. A progress bar is present at the bottom of the window. When the procedure is finished, the **Finish** button becomes available. Press it to save all adjustments and leave the wizard. This text is displayed: "Detector Alignment for all available filters in progress, please wait."

If you still encounter pixel shifts between two channels after the Automatic Camera Alignment tool, you can fine tune the settings using the Manual Detector Alignment tool, see section **3.4.5.3 Adjustment – Manual Detector Alignment**.

### 3.4.5.3 Adjustment – Manual Detector Alignment

This adjustment is necessary for one or two detection modules when you recognize a pixel shift between channels or when the zoom is not centered.

 The **Automatic Detector Alignment** should always be performed first since it might already solve the problems. Furthermore, it defines the settings for the combination Channel 1 (Cam1) and position 1 of the Emission Selection Filter (see section **3.2.5.7 Emission Selection Filter Selection**), which should be used as a reference during the Manual Detector Alignment.

 The **Zoom** in the **Acquisition tool window** is set to 1 while performing the Manual Detector Alignment.

No sample should be present when performing this task. When pressing the **Manual Detector Alignment** button, a window will open (Fig. 302). A grating is automatically inserted into the light path and a live image of it is shown in a new image container within the ZEN software. The **Set Exposure** button is activated automatically to adjust brightness. All components of the light path can be controlled in the **Manual Detector Alignment** window. The light source is the LED white light source to ensure the visibility of the grating independent of the filters in the light path. All other controls for adjusting imaging settings should be controlled in the tool windows of the main software.

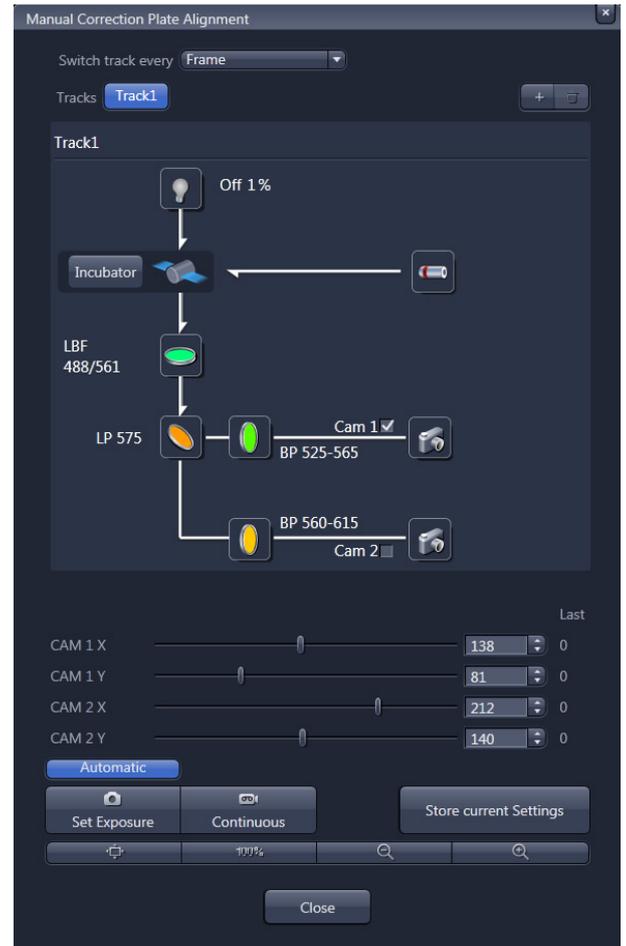


Fig. 302 Manual Detector Alignment window

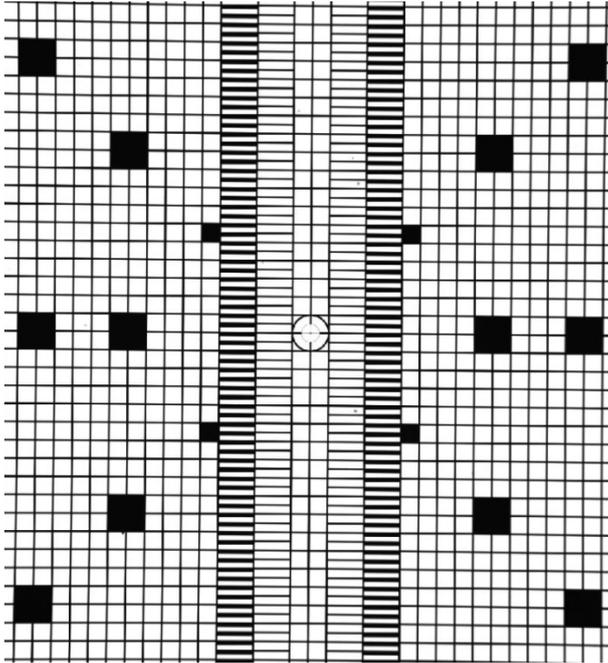


Fig. 303 Manual Detector Alignment, Grating at Zoom 1

The activated (blue) **Automatic** button loads the settings which have been previously defined with the Automatic Detector Alignment for each light path.

After pressing the **Continuous** button in the **Manual Detector Alignment** window or in the **Main tool tabs**, a new image will open with a live image of the grating (Fig. 303).

☞ When the grating is out-of-focus, refocus it for the relevant channel by performing the steps described in **CHAPTER 1 HARDWARE**. To check the focus, zoom into the image using the **Zoom** function and look at the center cross within the square (Fig. 304). If the white portions within the inner square are recognizable, the grating is in focus.

To zoom into the image, use these buttons:



As a reference, the combination of Emission Selection filter position 1 and Cam 1 should be taken (see above), so this combination should give the first live image. Here mark a structure close to the middle with a cross using the **Graphics View control**. This cross will now be the reference for the following steps.

Now change the light path to the combination of Emission Selection filter and Channel (Cam 1 or Cam 2) that you wish to adjust. The live image will now show the grating with the current settings. Use the sliders or the input box with arrows of **Cam 1 X** and **Cam 1 Y** (for Channel 1, Cam1) or **Cam 2 X** and **Cam 2 Y** (for Channel 2, Cam 2) to move the previously chosen structure of the grating to overlay with the cross. Finish this step by pressing the **Store Current Settings** button.

Repeat the procedure for all desired Emission Selection filters and channels, always using the drawn cross marker as the reference.

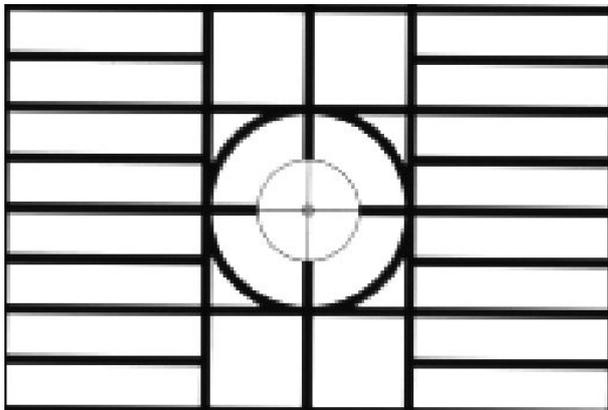


Fig. 304 Manual Detector Alignment, Grating, center part zoomed in

It is helpful, especially after an Automatic Detector Alignment has been performed, to use the Profile View tab in order to evaluate if the image of Cam 1 and Cam 2 overlay. While the grating is continuously imaged, press the Profile View tab and draw a line or an arrow poly-line on the grating (Fig. 305). Use the sliders or the input box with arrows of **Cam 1 X** and **Cam 1 Y** (for Channel 1, Cam1) or **Cam 2 X** and **Cam 2 Y** (for Channel 2, Cam 2) to move the lines of the grating to overlay each other.

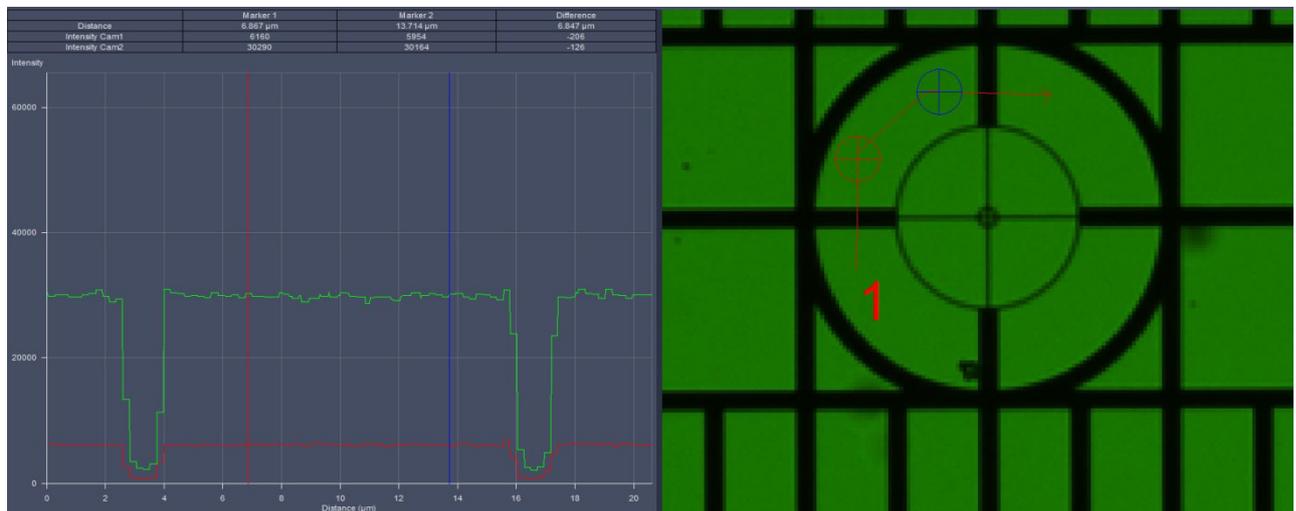


Fig. 305 Manual Detector Alignment, Grating, Profile view tab

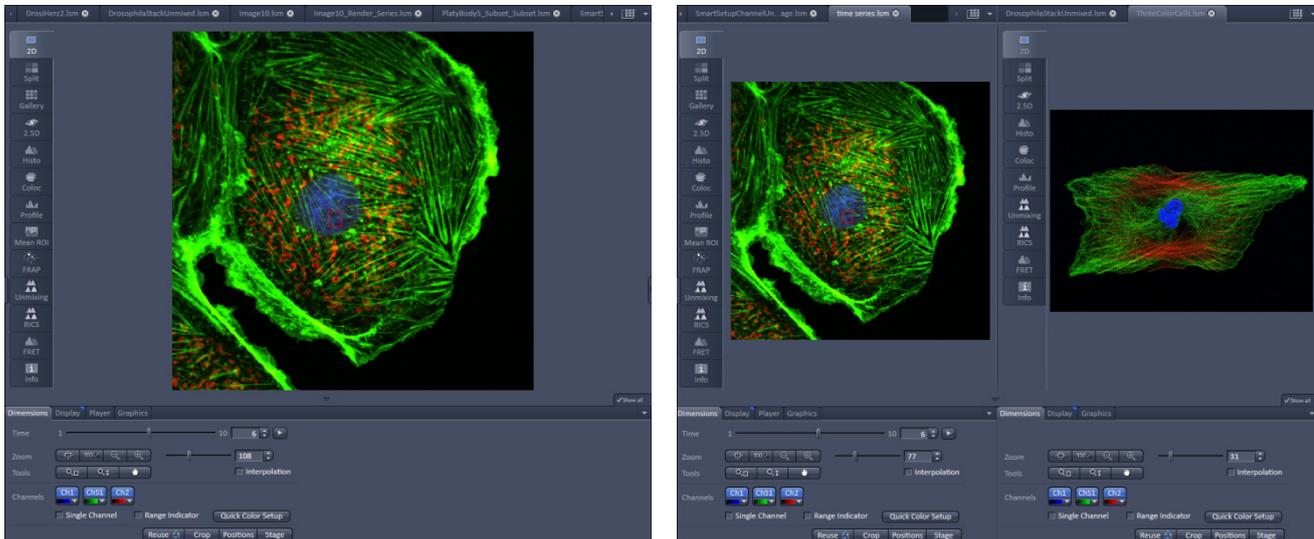
Leave the Manual Detector Alignment by pressing the **Close** button at the bottom of the window.

## 4 Center Screen Area / Image Containers - Display and Image Analysis

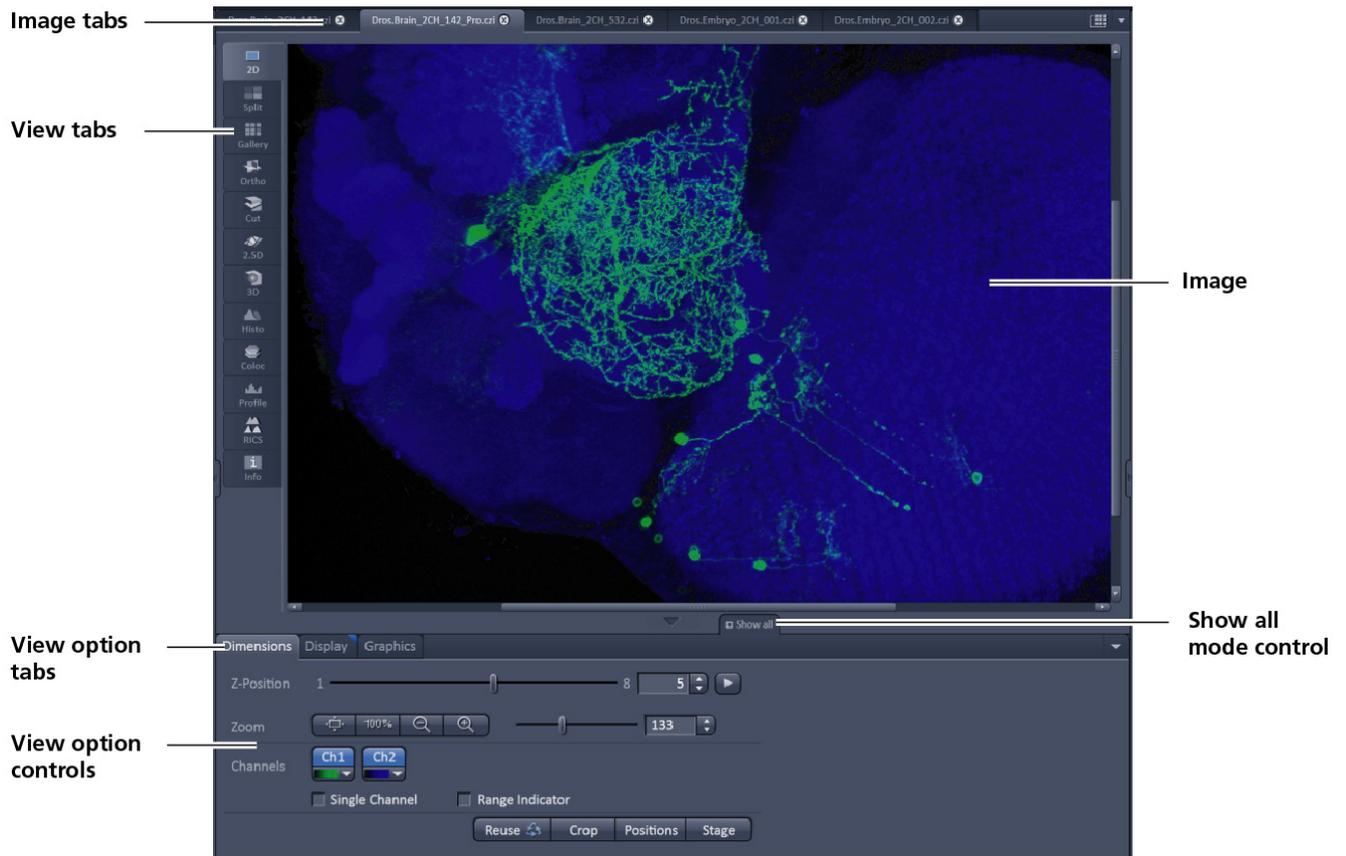
### 4.1 Structure and Functional Concept of the Center Screen Area and the Image Display Container

#### 4.1.1 General Structure

In this section, the Center Screen Area of the **ZEN Main Application** window is described. The **Center Screen** area can be set up to hold 1, 2 or 3 **Image Containers**. Fig. 306 shows the layout with one or two Image Containers.



**Fig. 306** The Center Screen Area of the ZEN Main Application window – Left: 1 Image Container, Right: 2 Image Containers



**Fig. 307** Image Display window – image tabs show options

### View tabs:

The View tabs make all viewing options and image analysis functions directly available from the main view. Switching from one View tab to another changes the view type for the currently active image, keeping the image in the foreground. This avoids several display windows for different analysis tools and keeps all information always right at hand.

- ☞ After switching between several open images, upon returning to a previously activated Image tab, the image document "remembers" which view type was activated before and displays the same parameters upon return to this image.

**Image tabs:**

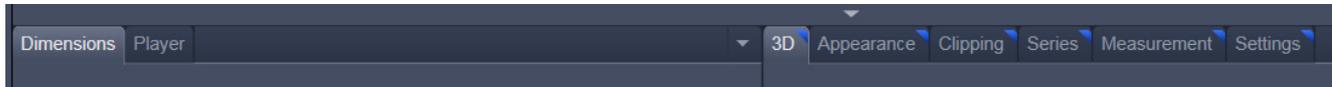
The Image tabs show every open window so they are easy to find and access even when the **Open Images** panel in the **Right Tool Area** is hidden/minimized. This way of organizing open images avoids the problem of many open image display windows lying on top of each other. The **Image Container** concept allows large numbers of images open at the same time and having them all easily accessible while keeping the workspace organized and tidy. **Image** tabs can show the image file name only (Fig. 308/a), the name and a small image thumbnail (Fig. 308/b) or the file name and a larger image thumbnail (Fig. 308/c). This can be configured in the Container Context menu (see Fig. 314).



Fig. 308 Image Display window; –image tabs show options

**View Option control tabs:**

These tabs allow individual activation / deactivation of the available **View Option** control blocks by clicking on the tabs. Available but hidden View Option Control tabs are grey. Active tabs are displayed in front of the others:



The **View Option** control tabs:

The **View Option** control tabs are placed in the area under the image display. Each block hosts functionally related tools for image analysis, display modification, and data manipulation. There are two groups of **View Option** control tabs: A general one which contains the blocks **Dimensions**, **Display**, **Player**, and **Graphics**. The second group consists of **View** tab-specific tool tabs hosting tools that are functionally linked to only one view type. The View tab specific control tabs are marked with a blue triangle on their upper right corner. **Histogram** The control tabs of the general group and their respective tools are described in the section for **2D View** (the first and default **View** tab).

**Tab mechanics:**

All tabs of the two groups can be dragged out of their groups and placed freely in the **View Options Area**. Wherever it is possible to drop a grabbed tab – the underlying area is displayed in blue (Fig. 309):

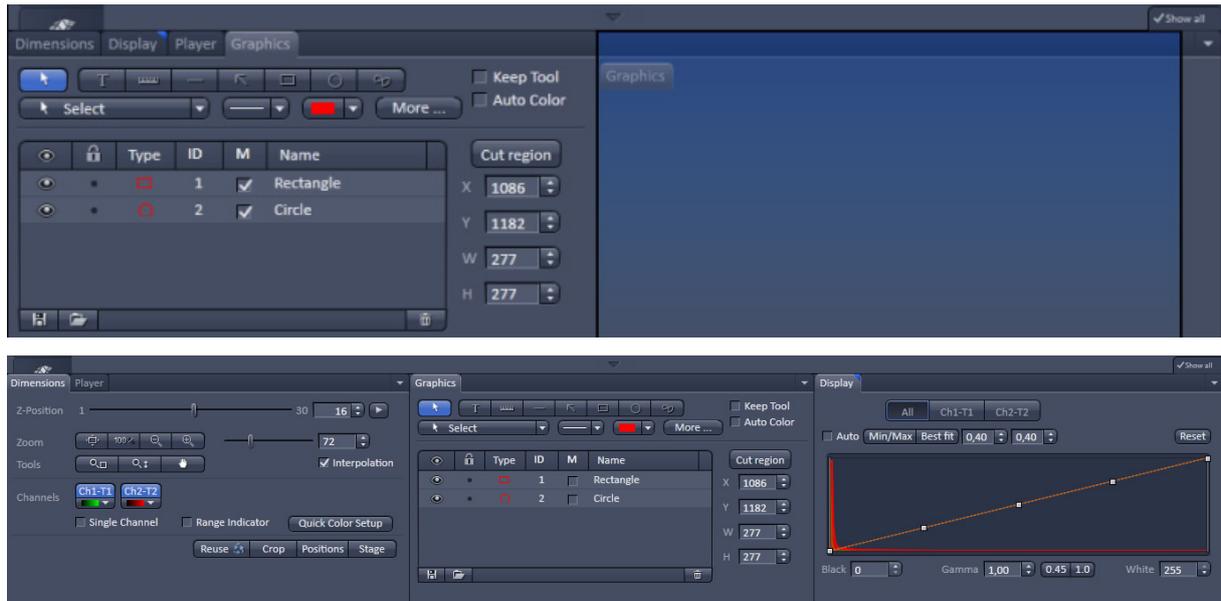
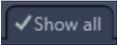


Fig. 309 General View Options control blocks

In order to reset the tab layout – use the entry **Reset Tab Layout** in the context menu which opens with a right mouse click within the View option controls, or click on the triangle  at the right hand side to access the View Options Area menu (Fig. 310).

### Show all mode for the View Options Area:

Activating the **Show all** mode  from the View Options Area has two effects:

- In every view options control tab, all the available tools are shown; de-activating the **Show all** mode hides less often used tools.
- All available view options control tabs are shown; de-activating the **Show all** mode hides less often used tabs.

Any changes done with these tools have immediate effect on the image display but not on the saved data. To permanently store your results, save the data as described in section **Right Tool Area, Save** button.

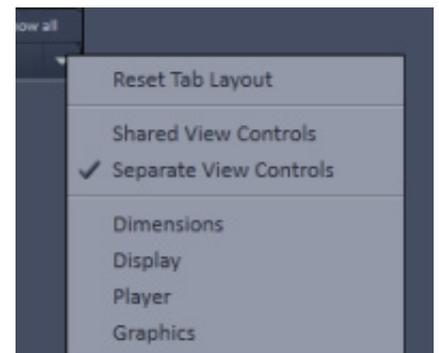
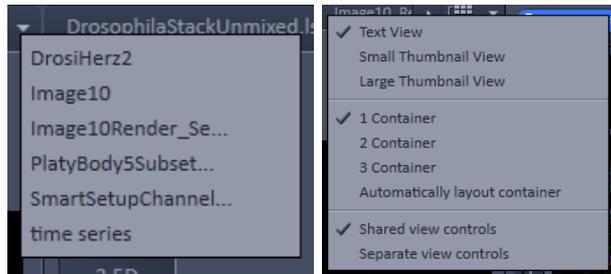


Fig. 310 View Options Area menu



**Fig. 311** Control area of the top of the image container



**Fig. 312** Open Image pull down menu (left) and context menu of the Center Screen Area (right)

### Image Handling and Container Layout Configuration:

The control area (Fig. 311) on the top right corner of the Image Display is for scrolling (click on the left or right triangle button) and listing the Image tabs which cannot be displayed if too many images are opened (left click on the down triangle).

This is just an alternative way of handling the open images (Fig. 312, left).

The main tool for this purpose is the **Open Images** panel (see section **5 Right Tool Area, Data Management and Storage**).

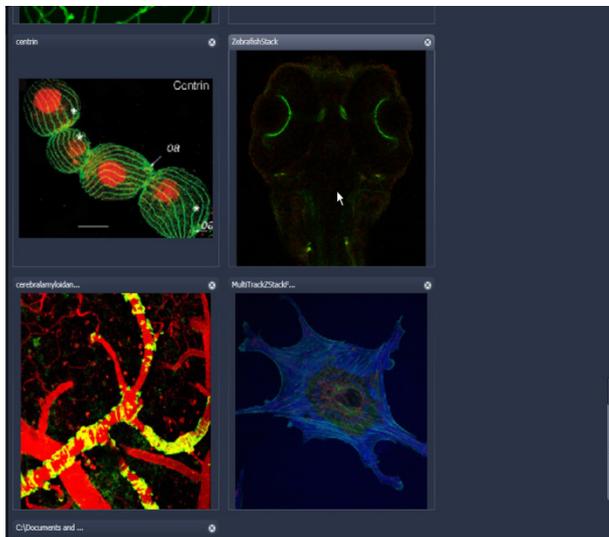
A right click on the image tab row opens the context menu (Fig. 312, right) for the layout of the Center Screen Area.

### The Image Display:

The Image Display contains and displays the image data or –depending on the active view type – a combination of image data, overlays, graphs, and tables. The content is automatically maximized to the available image or display size.

### Expose Mode:

Clicking the **Expose Mode** button  in the top right corner of the Image Display opens a field of thumbnails in the image display with one large thumbnail for each open image. Whenever you lost track of your open images, open the Expose Mode and click once on the image you are looking for and it will be immediately brought to the foreground of the Image Display (Fig. 313).



**Fig. 313** Expose Mode Display

#### 4.1.2 Container Configuration with the Container Context Menu

Clicking on the background of the control area on top of the image container (Fig. 314) with the right-mouse button opens a context menu for:

- setting the view for the Image tabs (see above),
- choosing to split the Center Screen Area into 1, 2, or 3 containers and setting the automatic container layout
- choosing between separate or shared view controls between the containers.

The same menu is available within the menu bar in the **View** part of the menu.

Splitting the Center Screen Area into several containers has the advantage that side-by-side comparison of image data becomes very easy.

The disadvantage is that the individual container necessarily becomes smaller. Up to 3 containers can be chosen. The "Automatically layout container" option is switched on by default and fixes the container width in layouts with multiple containers. In two-monitor setups it also fixes the right edge of the first container to the monitor edge.

If **Separate view Controls** are chosen, each container has its own set of View Options control blocks. If a **Shared view Controls** is chosen, the set of View Options control tabs spans the whole area under the containers. The tools and functions always apply to the currently active container / image.

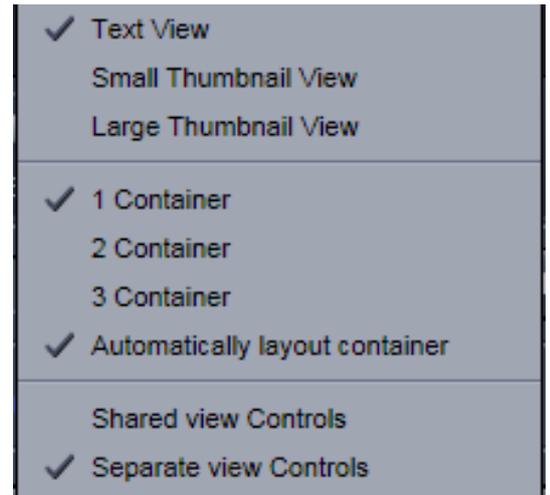


Fig. 314 Container Context menu

## 4.2 2D View

This function allows to

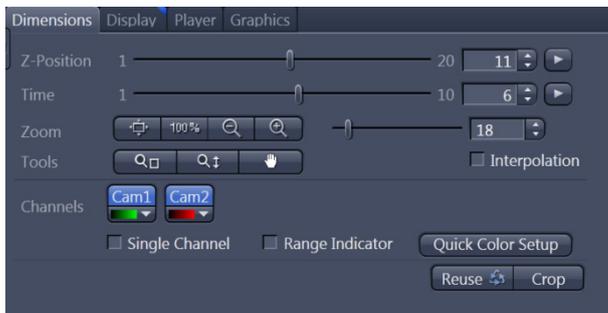
- display a single image in frame mode,
- display multiple channel images in superimposed mode.

The following **View Option** control blocks apply to this View Type:

**Dimensions, Display, Player, and Graphics.**

In the 2D view, only the general **View Option** control blocks are displayed. These view option blocks are also available in those other view options in which the functions are applicable.

The general view option control blocks are only described in this section.



**Fig. 315 View Options control block - Dimensions**

### 4.2.1 Dimensions

The **Dimensions View Options** control block holds the tools to modify the image display with respect to the (multiple) dimensions of the image data set.

The sections (slices) in the dimensions can be scrolled with sliders (**Z-Position**, **Time**, and **Illumination** in Fig. 315) and also directly addressed with setting numbers in the spin-boxes next to the sliders.

When more than three dimensions are present in the image, a scroll bar at the right side of the sliders appears. Scroll to the slider you wish to use.

 Note, when you create multiple Z-Stacks with the Multiview-setup tool, they might have different numbers of slices. In the Dimension View control, the maximum number of slices present in the dataset will be displayed, even if a view with less slices is chosen.

The small  button on the right hand side of the spin-boxes (**Z-Position** and **Time**) starts and stops an animation in the respective dimension. This basic animation cannot be customized. For all other player-functionality, refer to the **Player View Block**.

**Interpolation check box:** activates / deactivates the interpolation of neighboring pixels within the image. This is only a display option and will not affect the original data.

## (1) Dimensions – Zoom

In the **Dimensions View Options** control block, there are the **Zoom** and **Tools** function lines related to changing the zoom factor of a displayed image.

Any changes on these elements affect the displayed image immediately. The zoom function can be performed online.

The functions of the **Zoom** and **Tools** lines are:



**Zoom Normal:** The image is fitted automatically into the **Image Display** window which is always the default displayed size.



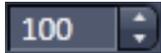
**Zoom 100 %:** displays one pixel of the image also as one pixel on the screen.



**Zoom -** reduces the image.



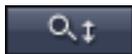
**Zoom +** enlarges the image.



**Spin-box and slider:** type in your magnification value in percent directly. The zoom factor can be set alternately by moving the slider. The spin-box displays the current zoom factor in %. 100% corresponds to the original size.



**Zoom selection via mouse:** draw the area to zoom-in directly on the image.



**Zoom-Mouse:** allows you to enlarge or reduce the zoom factor of an image using the left mouse button. When the mouse is moved forward while holding the left mouse button within the image, it will zoom into the image. When the movement of the mouse is reversed, it will zoom out of the image. When you release the mouse, the zoom will stay at its last position. To return to Normal Zoom click the right mouse button into the image.



**Navigation hand:** allows you to scroll the position of a zoomed image inside of the image window.

## (2) Dimensions – Channels

The **Channels** functions are designed to switch on/off channels or the display of the merged image as well as to assign color look-up tables (LUTs) to the individual channels:



Clicking on the **Ch1-T1**, **ChS1-T2**, **Ch2-T3** buttons switches the respective channels on/off in the display.

Clicking on the  buttons or white arrow opens the channel color lock-up-table to select the color, LUT or no color for this channel (see Fig. 316).



Fig. 316 Image Display - Dimensions - Channel color lock-up-table

The **Single Channel** check box activates / deactivates single mode operation, and only one channel will be displayed.

The **Range Indicator** check box activates / deactivates the **Range Indicator** function.

Clicking on the **Quick Color Setup** button opens a selection menu. Here you can change the image view between **BGR**, **GRB**, **RGB**, **Grey** and **Range** indicator mode quickly.



**Fig. 317** Image Display - Dimensions

Any changes on these functions affect the displayed image immediately.

If the display area of the channels is not enough to display all channels, a slider becomes available below the listed channels allowing you to scroll through all channels (Fig. 317).

### (3) Dimensions – Reuse

Clicking the  button transfers acquisition parameters from the stored image data to the **System Hardware Settings / Control tools** and applies those parameters directly to the system (for details see section **3.4.3.2 System Options – Reuse**).

In the **Maintain** tab (section **Maintain – System Options – Reuse** tab), some of the parameters used for **Reuse** can be selected or deactivated.

The acquisition parameters of an image are displayed in the Information View (section **4.13 Information View**).

#### (4) Dimensions – Crop

This function allows one to interactively define the size and orientation of a rectangular Frame Size for the image displayed in the **Image Display** window.

The defined area is synchronized with the **Frame Size** parameters in the Acquisition Area panel of the Acquisition Mode tool (section **3.2.7 Acquisition Parameter – Acquisition Mode**).

Click on  will display the **Crop Rectangle** in the **Image Display** (Fig. 318). Any changes done with the **Crop Rectangle** are updating the imaging parameters immediately. On the next execution of an image acquisition (**Set Exposure, Continuous, Snap**) the new Image Size will be applied. To reset the crop function and use default values, click the **Reset** button in the Acquisition tool window. When the  button is de-selected, the imaging parameters are reset as well.

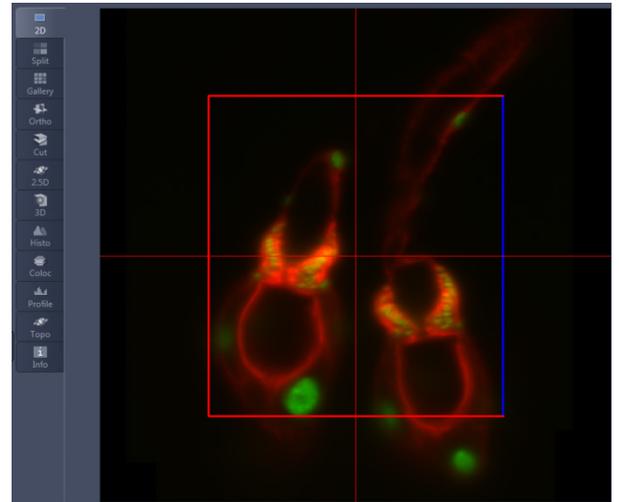


Fig. 318 Crop rectangle in image container

The **Crop Rectangle** is controlled via the following functional elements:

- Offset**  To move the rectangle within the image, creating an offset. The stage will be moved in x and y.
- Zoom**  Click on a corner of the crop rectangle, keeping the left mouse button pressed, set the required size. Release the mouse button. The zoom is equivalent to the Zoom slider in the Acquisition Mode tool window until its maximal value has been reached.
-  Be aware, if the Zoom is changed due to the Crop function, the light sheet alignment (Light sheet **Auto-Adjust** in the **Channel** tool window) needs to be redone.
- Only when the maximum Zoom is reached does the reduction of the Crop Rectangle lead to a reduction of pixels.
- Rotation**  The Crop Rectangle cannot be rotated in a Lightsheet Z.1 systems.
- Side ratio**  Click on any of the intersection points between the crossline and crop rectangle, keep the left mouse button pressed and change the side ratio as required. Release the mouse button. This function is only available when the maximum Zoom is reached within the Zoom range(0.36 – 2.5), the Crop region remains

## 4.2.2 Display

In the **Display** View Option control block, the intensity minimum can be set for the Black value and the intensity maximum for the White value of the image using the **Min/Max** button.

Clicking on the **Best fit** button sets the intensity minimum for Black to the percentile value and the intensity maximum for White to the percentile value.

Activating the  **Auto** check box sets the intensity minimum and maximum values automatically.

With the **0,40** spin-boxes, you can set the lower and upper percentile values.

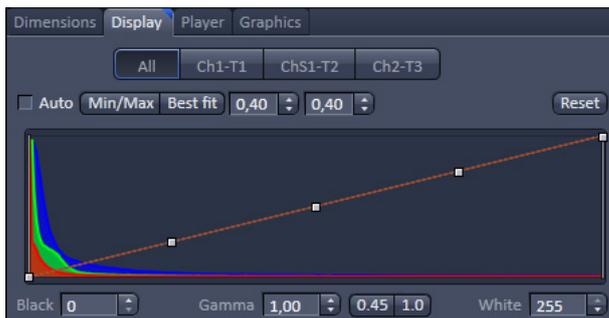


Fig. 319 View Options control block - Display

The **Black**, the **Gamma**, and the **White** values of the displayed image can be adjusted (Fig. 319) individually.

With the **All**, **Ch1-T1**, **ChS1-T2**, **Ch2-T3** buttons, the effect of the settings can be restricted to an individual channel or be applied to all channels. By default, the settings apply to all channels simultaneously.

The parameters can be changed using the spin-boxes – or directly by typing the numbers into the number-field. With the **Reset** button, the original settings are easily re-set.

With a right mouse button, click into the area which displays the histogram, a menu opens (Fig. 320) to change the displayed bit depth as well as the scaling of the histogram.

The settings in this View control block apply only to the Image Display and are NOT changed in the original data set. To write the changed settings to the image data set, use the **Burn in Brightness and Contrast** function in the **Processing** tab (section 3.3 Processing Tab).



Fig. 320 View Options control block – Display, settings

### 4.2.3 Player

This function allows one to

- animate frames of a Z-Stack or a time series
- specify animation parameters such as range and animation speed

When the image displayed in the **Image Display** window is neither a Z-Stack nor a time series, this View Option control block is empty.



Fig. 321 View Options control block - Player

The **Player View Option** control block is controlled by the following elements:

	<b>The current position</b> slider is displayed in the View Option control block <b>Dimensions</b> and can also be moved manually or by entering the slice number into the input box. The slider can be accessed only when the player animation is off.
	<b>Start</b> and <b>End</b> slider: The setting of the <b>Start</b> and <b>End</b> sliders limits the number of slices to be used for the animation. Slices before <b>Start</b> and after <b>End</b> are not taken into the animation. These sliders can be changed during automatic animation.
	Starts the forward motion of the automatic animation. After the last slice has been passed, restart is made at the first slice.
	Starts backward motion of the automatic animation. After the first slice has been passed, restart is made at the last slice.
	Starts the combined forward / backward motion of the automatic animation, i.e. when the last slice has been reached, the backward motion is activated and the forward motion is activated again on reaching the first slice.
	Stops the automatic animation.
	Moves to the first slice.
	Moves to the last slice.
	<b>Speed</b> can be set through a slider or spin-box (selection box).
	<b>Multidimensional</b> animations can be selected by ticking the individual dimensions tick boxes.



Fig. 322 View Options control block - Graphics

#### 4.2.4 Graphics

This function allows one to

- select from a set of drawing functions such as rectangles and arrows,
- add a scale bar to the image, as well as text annotations,
- use a set of interactive measurement functions for length, angle, area, and size,
- add a text box that displays the coordinate of a hidden dimension (e.g., the z-position in a Z-Stack or the timestamp in a time series).

#### Functional Description

The graphics function uses a plane separate from the image plane (the graphics plane) and therefore does not change the content of the image(s).

The **Graphics** view option control block is available in all View Types except **2.5D** and **Preview**.

Any changes done with this function are effective immediately.

The overlay graphics can be stored in the images or separately in a file from where they can be re-loaded.

#### Available functions in the Graphics View Control Block

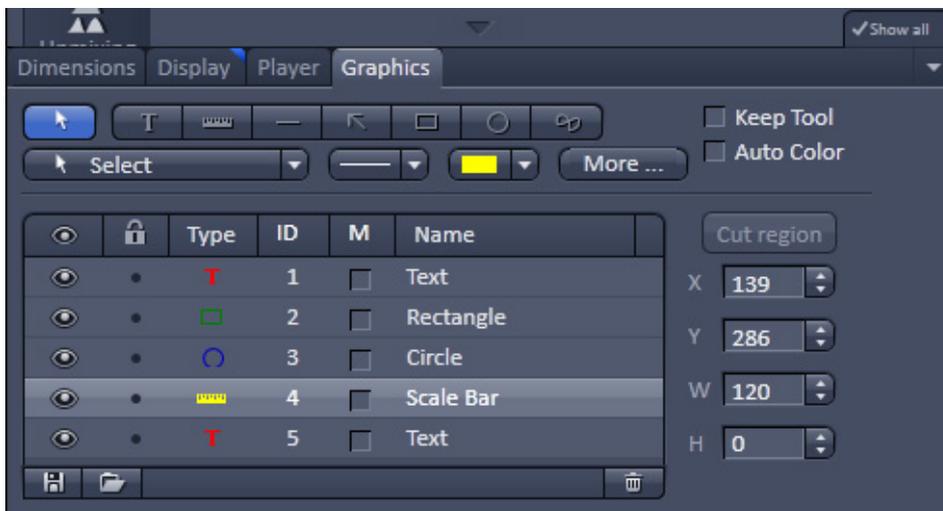


Fig. 323 Graphics view control block

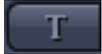
The following list describes the most common functions in the **Graphics** view control block. More can be found under the  pull down menu.



**Select tool:** Activation of the mouse cursor for selection, resizing, or movement of an graphic element in the Image Display.

Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.

Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.



**Text (Annotation) tool:** Creation of a text box in the Image Display.

After clicking on T, the Text window will be displayed and text can be entered via the keyboard. To alter the font size and style, click on the **More...** button (see below) to open a menu. Here the drop down list next to Text size enables you to select the font size. Likewise you can change Font name, Font style, and Font character set. With the S and L buttons you can easily reduce or enlarge the font size. The entered text will be displayed in a ROI within the image. The Text window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.



**Scale bar:** Creation of a horizontal or vertical scale bar with default increments in the Image Display. Click and hold the mouse button for the starting point, drag for the horizontal or vertical scale, release the mouse button to end the procedure.



**Line tool:** Creation of a straight line in the Image Display.

Click and hold down the mouse button, draw a line in any required direction, release the mouse button to end the procedure.



**Line with arrow button:** Creation of a line with arrow in the Image Display window.

Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.



**Rectangle tool:** Creation of a rectangle in the Image Display.

Click and hold down the mouse button, draw a rectangle in any required direction, release the mouse button to end the procedure.



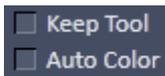
**Circle tool:** Creation of a circle in the Image Display.

Click and hold down the mouse button, draw a circle in any required direction, release the mouse button to end the procedure.



**Closed Bezier tool:** Creation of a closed bezier figure in the Image Display.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure and closes the figure.



**Keep Tool check box:** keeps the selected tool for creation of further objects, if activated.

**Auto Color check box:** sets the color of the object automatically, if activated.



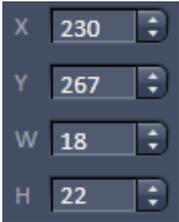
**Line thickness pull down:** sets the line thickness of the selected element.



**Color selection pull down:** In this pull down, different colors can be assigned to the graphic elements with a click of the mouse. A selected color is automatically assigned to the currently selected graphic element and to all elements created afterwards.



**Cut Region tool:** The region of a Z-Stack or 4D-image surrounded by a graphic element is extracted and displayed separately in a new Image Display. This function is only active if the active graphic element defines a closed contour or volume. If multiple regions are present in the image, only the active (by mouse click) region will be taken into account. If none of multiple regions is activated, all will be extracted and presented in the smallest fitting rectangle.



**Coordinate and dimension display:** shows the XY coordinates of the object center and the width and height of the graphic element.

#### Function elements in the graphics list box:



Activate/deactivate the display of the graphic element in the image.



Locks / unlocks the graphic element in the image.



Shows the types of the graphic elements as an icon.



Shows the number of the graphic elements.



Activate/deactivates the display parameters of the graphic elements, e.g. area, etc.



Shows the types of graphic elements written.

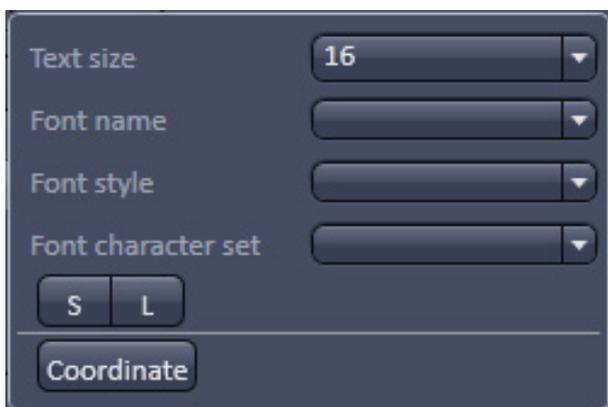


Fig. 324 View Options Graphics - More



**The More** options (Fig. 324) allow the selection of the following text parameters for the appropriate list box:

- Text size
- Font name
- Font style
- Font character set

Clicking on the  or  buttons toggles between small or large font size.

To insert a **Coordinate Label** into the image display, click the **Coordinate** button in the **More ...** dialog. The coordinate settings dialog (Fig. 325) offers the available settings and upon clicking OK, the selected coordinate is displayed as a text field in the image display. This text field is always immediately updated when the slice/section/time point is changed in the display by changing settings in the **Display View Option** control block or, for example, starting the **Player** animation. This feature is particularly useful to display time or z-position in an exported animation movie for presentations (see Fig. 323).

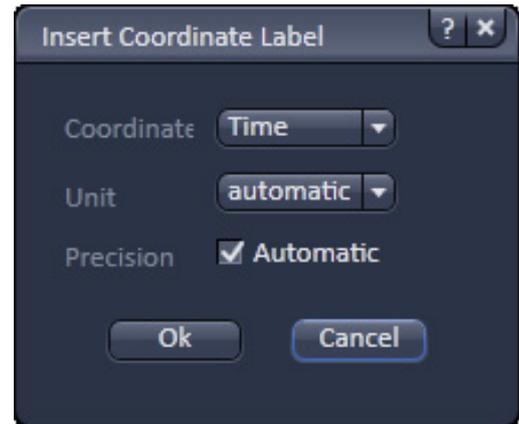


Fig. 325 View Options Graphics - Coordinate

To load / save graphics from / to a file, use the **Load**  / **Save**  buttons in this View Options control block. To delete a graphic element from the image, select this element and click on the **Delete**  button.

Naming and location in the file system can be set individually.

#### 4.2.5 Preview View

The Preview View Type is an enlarged copy of the thumbnail Previews described in the section **3.3 Processing Tab**.

### 4.3 Split View

This function allows one to

- display the individual channels of a multi-channel image as well as the superimposed image.

The settings of the **Dimensions**, **Display**, **Player**, and **Graphics** view options control blocks apply with the following additional features:

- The **Dimensions** View Options control block shows the **Merged** tick box to activate / deactivate the display of the channel overlay and a **Zoom All** button is added synchronize zooming for the Dimensions view control block.
- Graphic overlay elements are always displayed in all channel displays.

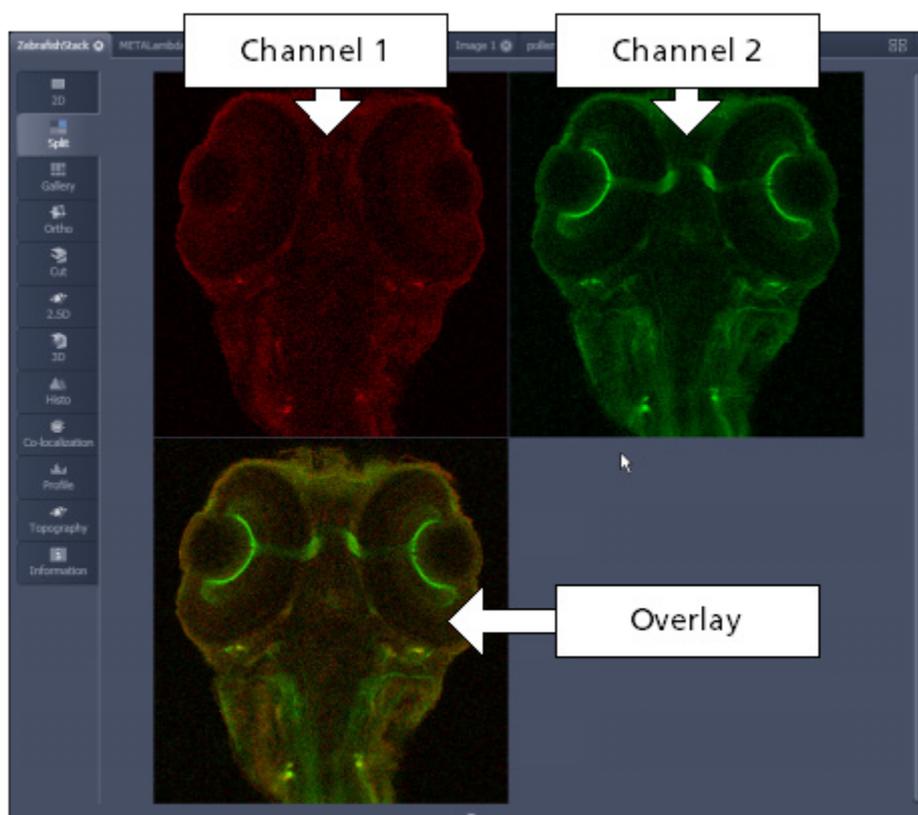


Fig. 326 Image Display, Split view type

- ☞ This function is useful to optimize the individual channels in a multi-channel image acquisition using the **Range Indicator** palette.

## 4.4 Gallery View

This function allows to:

- display images (Z-Stack, Time Series, View, Illumination, and the combinations Z-Stack-View, Time-View, Z-Stack-Illumination, Time-Illumination, and View-Illumination) side by side in a tiled fashion. The additional dimensions are controlled with the sliders and control blocks in the **Dimensions** View control block.
- add data relevant to the displayed images (Z-Stack slice distance, time of acquisition, angle, or illumination side),
- extract a subset of images from the original stack and store the result as a new image (controls for this function are in the **Processing** tab function **Copy** → **Subset** (see section **3.3.19 Processing – Copy**).

☞ Switching to the Gallery View while imaging can be demanding for the system since all dimensions that are part of an experiment need to be loaded. We therefore do not recommend doing so during image acquisition.

The settings of the **Dimensions**, **Display**, **Player**, and **Graphics** View Options control blocks apply.

The additional view-specific **Gallery** View Option control block includes a tick box to switch on and off the display of the slice coordinates in the Gallery display. If this text is activated, transparent mode and color can be selected in this control block too (see Fig. 327).

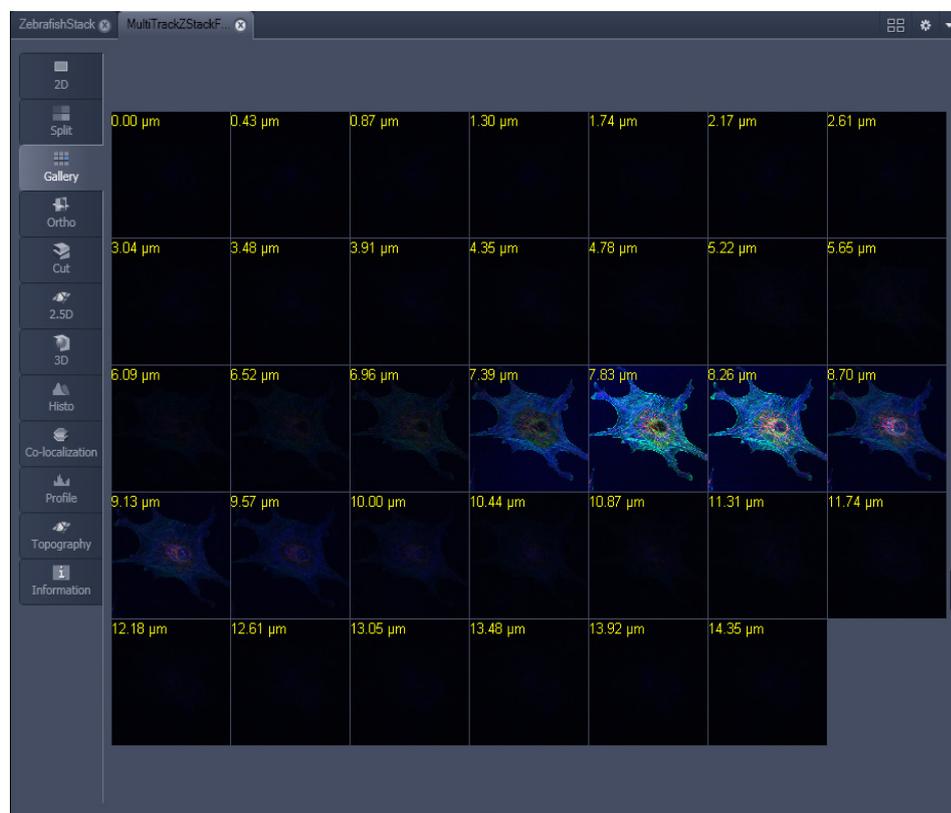


Fig. 327 Image Display window - Gallery display

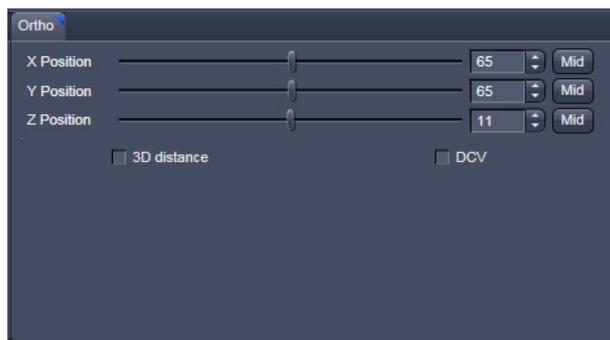


Fig. 328 View Options control block - Ortho

## 4.5 Ortho View

This function allows one to

- display a Z-Stack of images in an orthogonal view
- measure distances in three dimensions

The settings of the **Dimensions**, **Display**, **Player**, and **Graphics** view options control blocks apply.

In addition to the 4 general View Option control blocks, the view-specific **Ortho View Option** control block is available (Fig. 328).

In the **Ortho** View, section sliders appear in the **Ortho View Option** control block together with orthogonal projections in the image (Fig. 329).

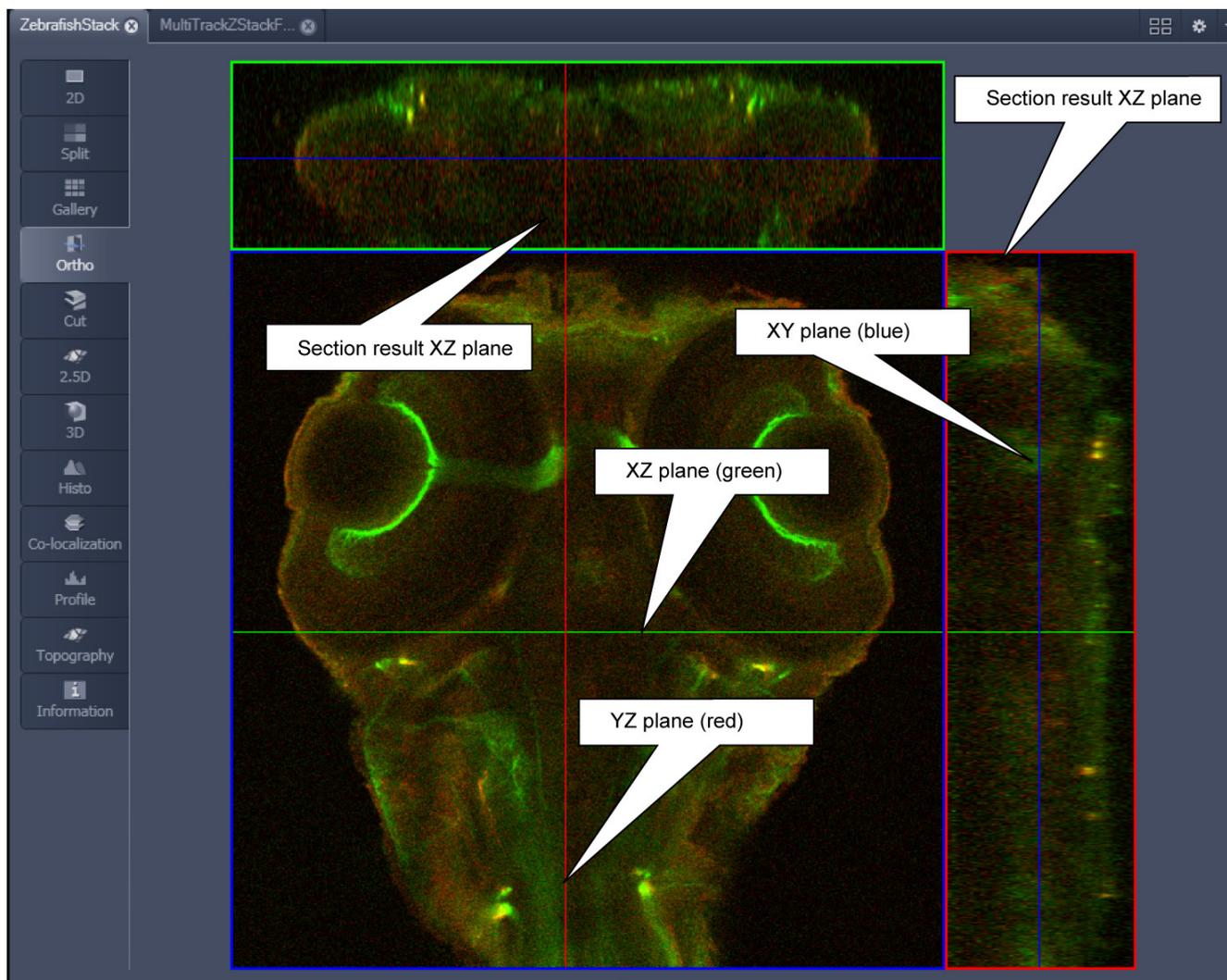


Fig. 329 Image Display - Ortho View

### 4.5.1 Ortho – Select Function

- By changing the parameters X, Y, and Z in the **Ortho** View Option control block, the section plane can be positioned to any XYZ coordinate of the Z-Stack.

The position of section planes can be changed in various ways:

- By moving the sliders on the **Ortho** View Option control block.
  - X and Y settings may range from 1 up to the maximum number of pixels present (in the example shown: 512).
  - Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.
- By directly entering the relevant number value into the X-, Y-, or Z-input box and pressing the **Enter** key.
- If you move the cursor into the **Image Display** window, it changes into a crossline symbol  $\oplus$ . By positioning this symbol with the mouse you can move the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.
- If you move the crossline symbol  $\oplus$  onto the intersection of the red and green section planes, it changes into the:  $\leftrightarrow$  symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.
- If you move the crossline symbol  $\oplus$  onto the green section plane, it changes into the  $\updownarrow$  symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.
- You can reposition the (red) YZ plane in the same way using the  $\leftarrow\rightarrow$  symbol.

The result of an orthogonal section is visible at the image margin.

- Section of the XZ plane (green line) through the stack: above the XY image.
- Section of the YZ plane (red line) through the stack: right of the XY image.
- Section of the XY plane (blue, slice plane of the stack): center image.

 If you zoom in one plane, the other planes will be zoomed with the same factor.

### 4.5.2 Ortho – Distance Function

- Activating the  3D distance tick box permits length measurements in 3D space.
- Click on the **Mark** button to set the first XYZ-point for the measurement of the spatial distance.
- Set the second XYZ-point for measurement by moving the X-, Y-, Z-sliders or by moving the green, red and blue lines in the image.
  - The projections of the spatial distance are shown in the image planes by yellow lines (Fig. 330). The actual spatial distance is calculated and shown in  $\mu\text{m}$  next to the **Mark** button.

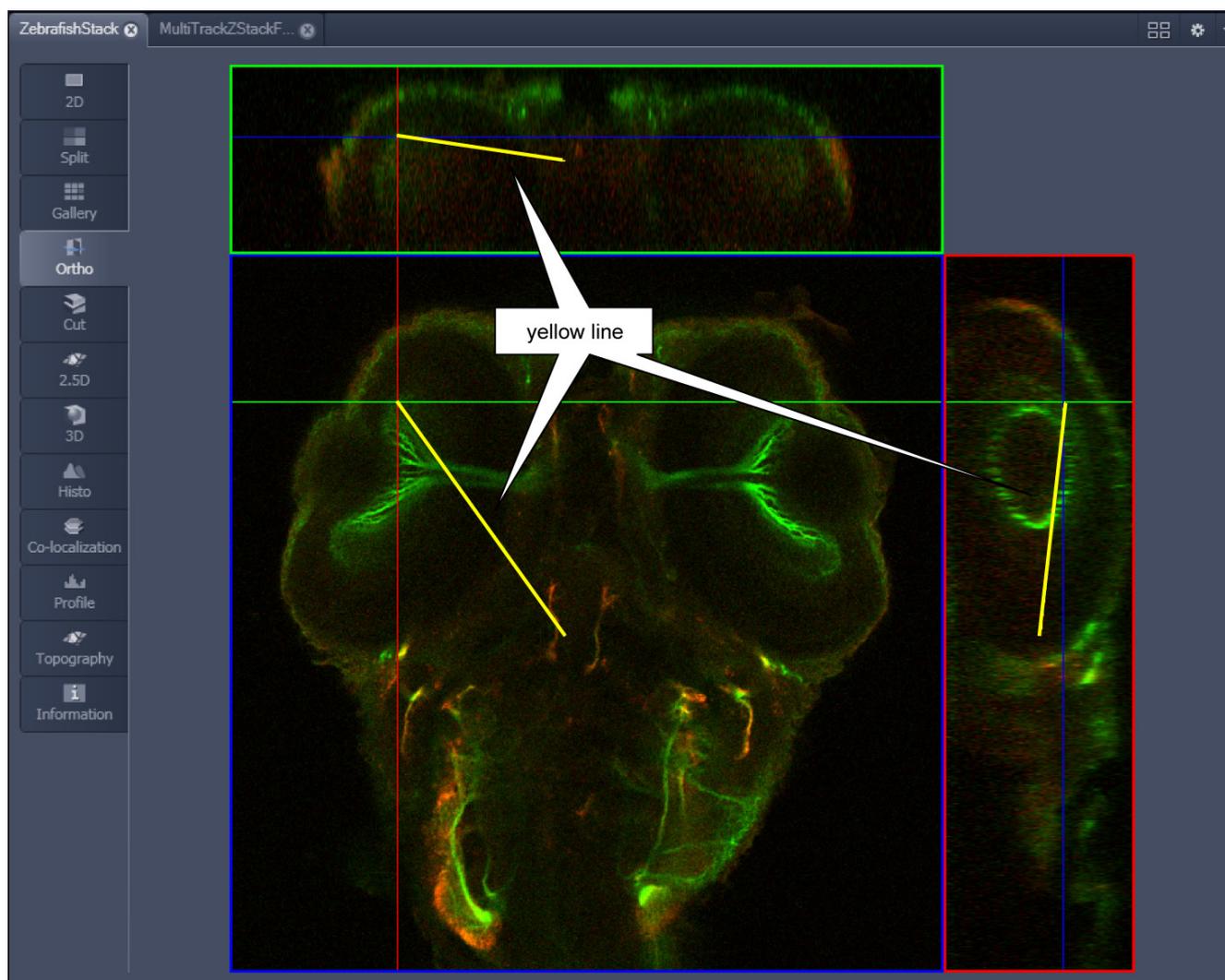


Fig. 330 Image Display – Ortho View - 3D Distance Measurement display

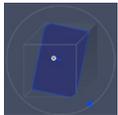
## 4.6 Cut View

This function allows to display a user defined section plane (= cut plane) of a Z-Stack. It automatically improves the image of the section plane by trilinear interpolation.

The settings of the **Dimensions**, **Display**, **Player**, and **Graphics** View Options control blocks apply.

Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

- By varying the parameters **X**, **Y**, **Z**, **Pitch**, and **Yaw**, you can position a section plane of any orientation within the stack volume.
- Clicking the **Mid** **Mid** **Mid** **0** **0** buttons restores the original position.



In order to place the Cut View Plane interactively use the interactive graphic in the cut view control tab.



Fig. 331 Cut View – control block

## 4.7 2.5 D View

This function allows one to

- display the two-dimensional intensity distribution of an image in a pseudo 3D mode,
- show the intensity values in profile, grid, or filled mode,
- show separate distribution for each channel in a multi-channel image.

 The **2.5 D** button can also be used online during imaging.

The settings of the **Dimensions** View Options control blocks apply. The settings of **Display, Player,** and **Graphics** View Options control blocks do not apply.

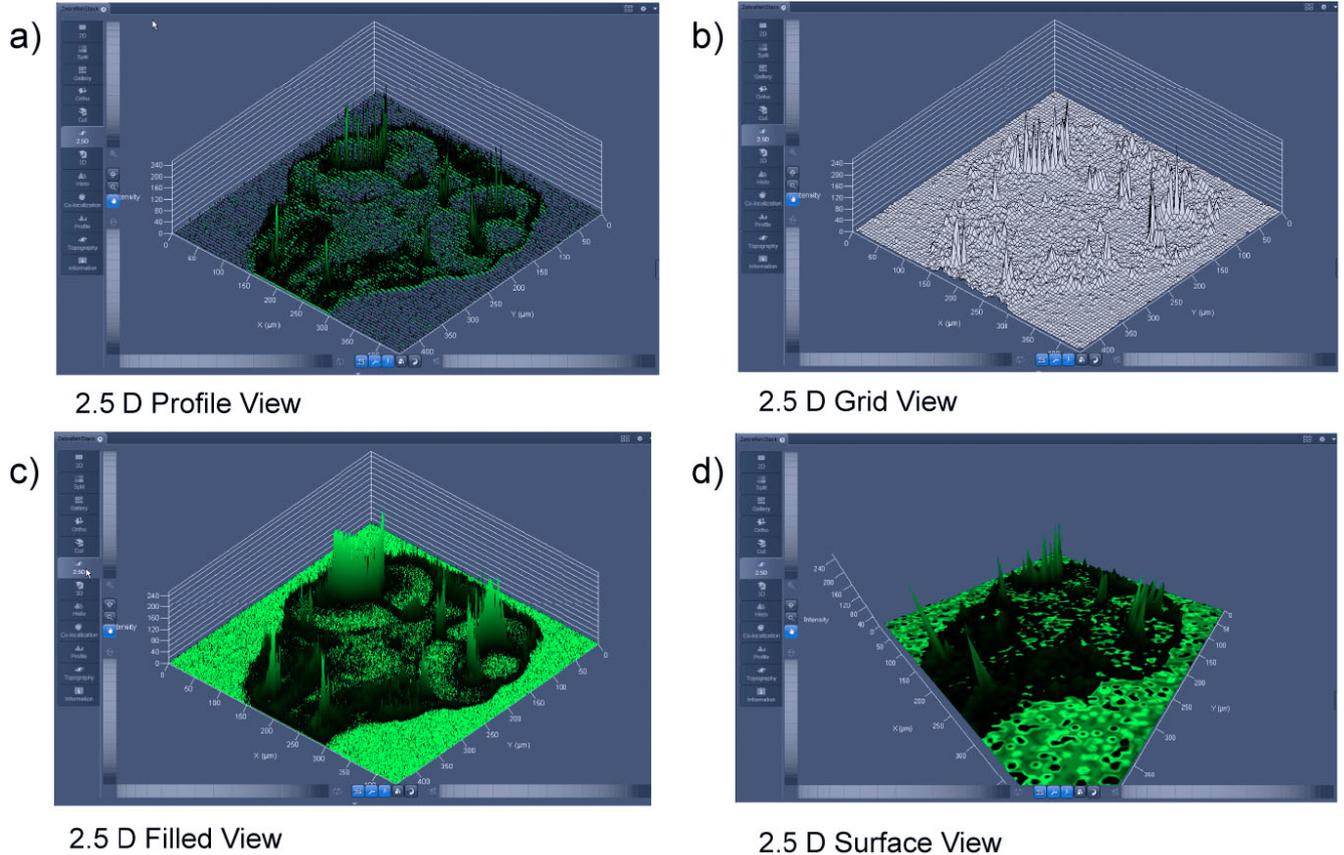
Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

The image display in the **2.5 D View Type** is the same as in the **3D (VisArt)** View Type. For a description of the controls in the display window, see section **4.8.8 3D Rendering Settings in VisArt**.

In brief, the viewing plane of the **Image Display** window can be rotated, tilted either directly with the mouse or by the scroll bars on the right-hand side and the bottom of the **Image Display** window.



Fig. 332 2.5 D View Option control tabs



**Fig. 333** 2.5 D Image Display

The 2.5 D view control tab contains the following functional elements:

- |                       |   |
|-----------------------|---|
| <b>Profile</b> button | Profile display (vertical polygon display). Setting of the <b>Profile Distance</b> between 1 and 20 using the slider.       |
| <b>Grid</b> button    | Grid display (horizontal grid display). Setting of the <b>Grid Distance</b> between 1 and 20 using the slider.              |
| <b>Filled</b> button  | Color diagram (filled 3D diagram). Selection between the <b>Mono</b> , <b>Rainbow</b> , and <b>Six Step</b> color palettes. |

## 4.8 3D View (3D VisArt)

With 3D VisArt, the Z-Stack of images generated by using the Lightsheet Z.1 system can be displayed in three dimensions. Display and rendering of Z-Stacks with up to eight color channels are supported and the data can be a time lapse series or a multiview experiment after registration and fusion.

The parameters of the **Channels** (including the selected LUT) set in the Dimensions View Options are applied. All other parameters from the **Dimensions, Display, Player, and Graphics View Option** control blocks are not applied.

A click on **3D** will display the **3D View Controls**. Any changes done within these controls are effective immediately. A status bar within the Status Area indicates the advancement of the rendering process.



☞ Depending on the selected mode and hardware configuration, it can take several seconds to minutes until the 3D reconstruction is refreshed on the monitor after any alterations.

☞ The **3D** View should not be used online during imaging.

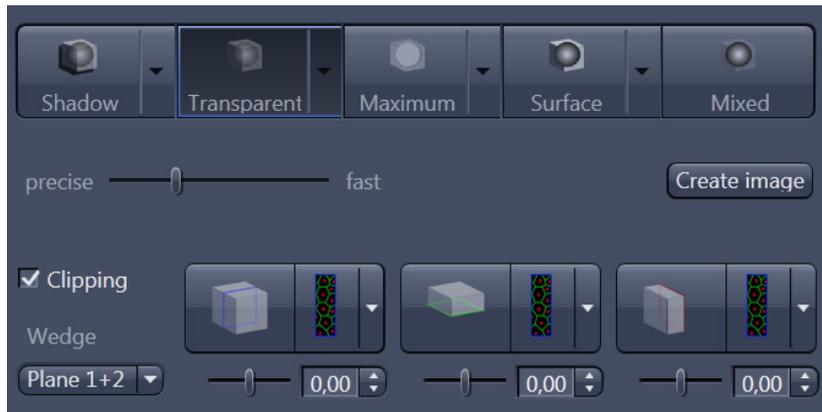


**Fig. 334** Image Display window - 3D display

The 3D view Controls contain the following functional elements:



They are displayed when the 3D tab is pressed in the order shown above.

**3D:**

On the **3D** View control panel, the different render modes are accessible:

**Shadow**, **Transparent**, **Maximum**, **Surface**, and **Mixed**. The rendering quality can be adjusted by using the **precise** ————— **fast** slider. The **Create image** button **Create image** opens a new image window and produces a 2D image of the currently used render mode. It uses the screen resolution unless it is otherwise defined in the **Settings** tab (see Settings).

**Clipping**  **Clipping** activates the Clipping Plane functionality. 3 different Clipping Planes are displayed and can be operated individually via 3 Buttons:



The Cut button toggles between different clipping behaviors for the respective planes: 

On the Clipping Planes, different surfaces can be chosen from a pull down list: 

The Position of the clipping planes can be adjusted by the **Position Slider**.

Further functionality is available in the **Clipping Plane View control tab** → see there.

A wedge between 2 Planes can be clipped using the pull down  underneath the activation check box for this clipping functionality.

**Appearance:**

On this view control panel, the settings for the respective render mode are available.

**Clipping:**

3D VisArt has the option to cut off certain parts of the 3D information by using so called "clipping planes". All the necessary tools to operate the clipping planes are found in this control panel.

**Series:**

3D Data can be visualized online by simply grabbing and turning the data stack with the mouse. In addition to this interactive way of interacting with the data, 3D VisArt is able to perform render series where the dataset is turned around one of the axes, along a freely defined position list, or a freely definable start and end point. The resulting series can be saved as movie files for presentations. A preview of the render series is available in the position list mode.

**Measurements:**

Measurements of distances, angles, and polylines can be measured using the Measure panel in 3D VisArt.

**Settings:**

In the Settings panel, global settings for the 3D VisArt Module can be found.



Fig. 335 3D panel - Shadow button

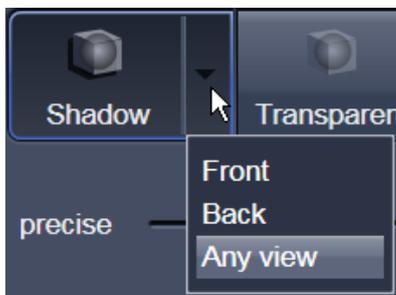


Fig. 336 Shadow pull down menu

### 4.8.1 Shadow Projection

In the Shadow render mode (Fig. 335), the structures in the image are illuminated by a virtual light source.

The volume is viewed as if looking through the eyepieces of the microscope and the light source projects a shadow onto a background plane.

This gives the data a reference in space, making visualization much easier. The rendering effect itself consists of a combination of light reflection and opacity (degree of transparency).

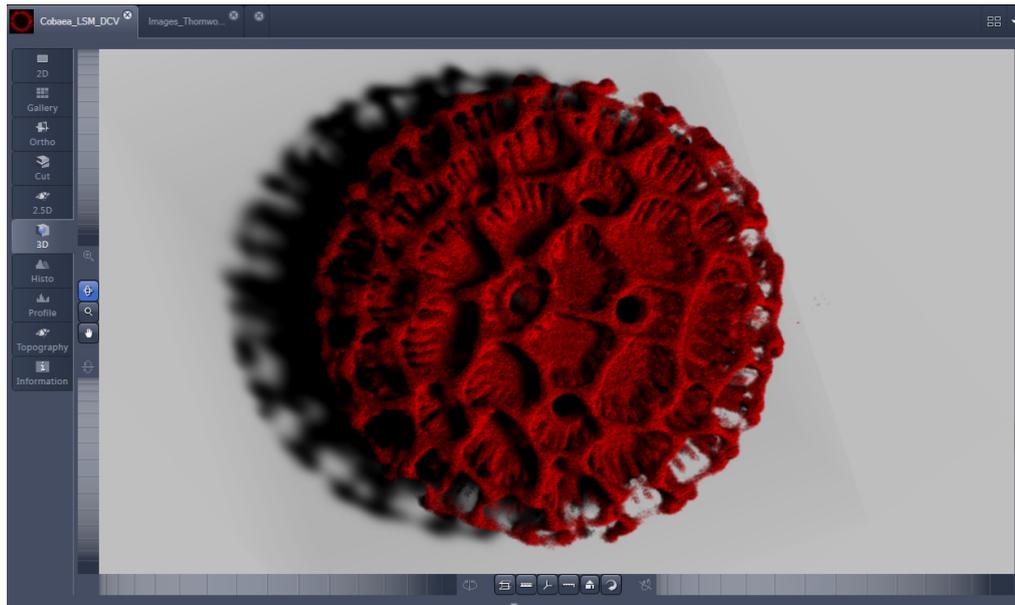
The display in this mode is calculated by the main processor (CPU) of the computer (Voxel mode). The different modes can be accessed by clicking on the pull down symbol at the right hand side of the **Shadow** button (Fig. 336).

- **Front** button: Shadow rendering front view
- **Back** button: Shadow rendering back view
- **Any View** button: Shadow rendering with user defined view

With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from the back left.

Detailed settings for shadow projection can be accessed via the **Appearance** tab.

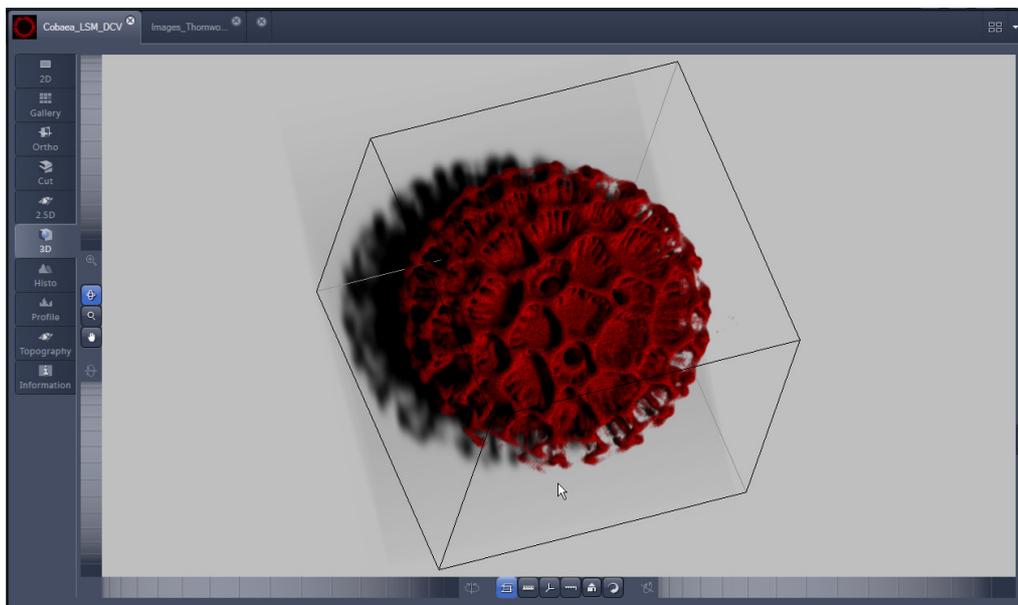


**Fig. 337** Image Display window, 3D display, Shadow projection, Front view

The zoom wheel to the left of the **Image Display** window allows continuous zooming of the 3D reconstructed image.

A click on the Any View button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.



**Fig. 338** Image Display window, 3D display, Shadow projection, Any View

The following additional buttons are available in the **Any View** shadow projection mode:



- After activation of the **Frame** button  (below the image), a bounding box is drawn around the 3D reconstructed image.
- A click on the **Coordinate System** button  displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red, the Y axis in blue, and the Z axis in green.
- A click on the **Scale** button  display an X-, Y-, and Z-scale in the **Image Display** window.
- A click on the **Home** button  resets the display parameters to the default values.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

- A click on the **Animation** button  activates the animation mode. The object can be pushed by dragging it in the **Image Display** window and it rotates continuously. Any new push with pressed left mouse button changes the rotation direction and speed of the animation.

## 4.8.2 Transparency Render Mode

Principle:

In the **Transparent** mode, a three-dimensional image with a transparent effect is calculated. At least two two-dimensional texture stacks (computed from two different angles) are used depending on the volume orientation. In contrast to the Shadow mode, the scene here is illuminated by diffuse white light from the rear. By changing the available parameters, one can mix different channels and reveal relationships between information in those channels. This view is particularly useful for visualizing the three-dimensional relationships between structures within the volume. This mode can be displayed both in CPU based Voxel mode (**Basic**) and also (with the help of a suitable graphics card) with accelerated calculations in so-called OpenGL mode (**Advanced**).

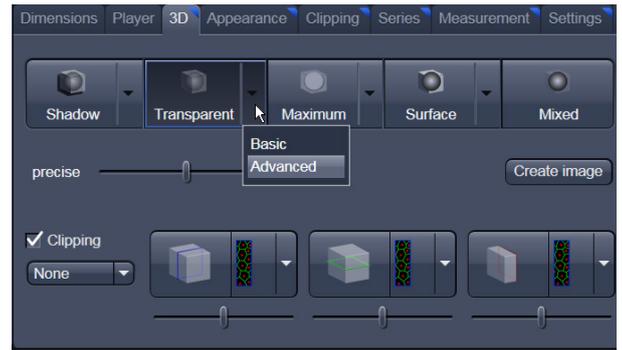


Fig. 339 3D View Control Panel, Transparent

**Basic** Transparency rendering (voxel based) (CPU)

**Advanced** Transparency rendering (voxel based) with textures (GPU)

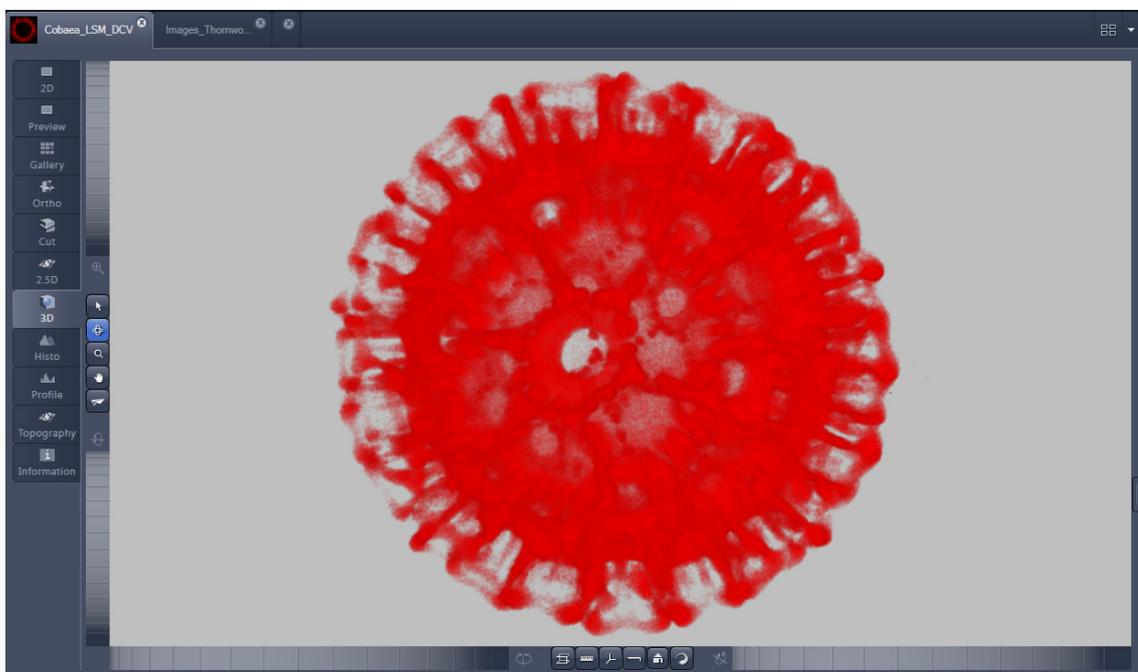
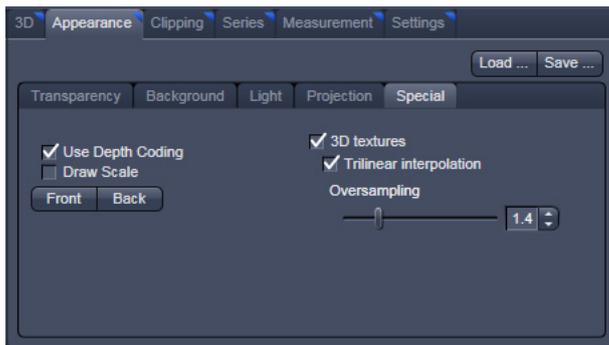


Fig. 340 Image Display window, 3D display, Transparency projection, Advanced



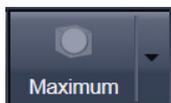
**Fig. 341** 3D toolbar – Transparent – Depth Coding

By means of the **Depth Coding** function (Fig. 341), the Z-information contained in a sequence can be colored with the colors of the rainbow, in which case "blue" stands for higher values and "red" stands for lower values.

The **3D Textures** option can be used for faster and better rendering of transparency renderings.

 Note that this function is only available in Transparency and Maximum Intensity Render Modes.

### 4.8.3 Maximum Mode

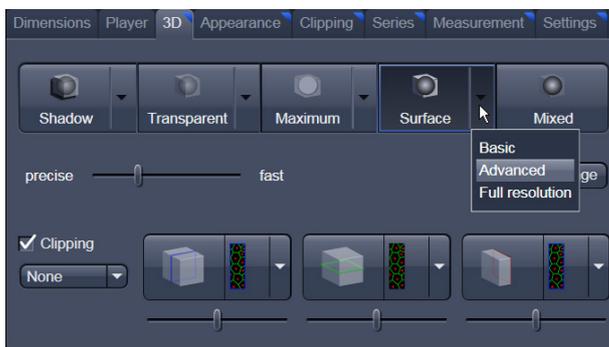


Clicking on the **Maximum** button switches to **Maximum projection** mode, which calculates a three-dimensional view of the data by only showing pixels of the highest intensity along the projection axis. This view is particularly useful for displaying three-dimensional images in two dimensions, e.g. in publications, and guarantees a maximum possible degree of transparency. The display in this mode can be calculated by the main processor (CPU) of the computer (Maximum basic) or the graphics card (Maximum advanced) in OpenGL mode.

 Note that this is a rendering. If desired 1:1 Maximum Intensity Projections can also be done in the **Processing** tab in **Maximum Intensity Projections** (see section 3.3.2 **Processing – Maximum Intensity Projection**). With these Images from the **Processing – Maximum Intensity Projection**, exact Intensity Measurements can be performed.

**Maximum Basic**                      Maximum Intensity CPU based

**Maximum Advanced**            Maximum Intensity GPU based



**Fig. 342** 3D toolbar – Surface

### 4.8.4 Surface Render Mode

In the **Surface** mode (Fig. 342), grey values in the image are computed as solid surfaces (also known as Iso surfaces) instead of soft transparent shades.

This display mode is useful to focus the attention on certain structures (e.g. plant cell walls, nucleoli, intracellular vesicles) while potentially hiding additional structures deeper within.

In this mode you also can select between Voxel (CPU) [basic] and OpenGL (graphics card) [advanced] display.

The [full resolution] projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

<b>Basic</b> button	Surface rendering (voxel based)
<b>Advanced</b> button	Surface rendering (triangle based)
<b>Full Resolution</b> button	High accuracy surface rendering (triangle based)

#### 4.8.5 Mixed Render Mode

In **Mixed** mode, both transparency and surface mode can be mixed in one volume display.

Using this mode one can render small structures within cells such as vesicles or speckles (e.g. FISH signals) as surfaces in one channel of a multichannel image while rendering the surrounding cytoplasm from another channel in transparency mode.

This way one can also visualize more complex relationships convincingly.

The render mode (**Transparent**, **Surface**, **Surf.+Transp.**) can be chosen per channel.

 The channels can also be switched off individually by de-activating the channels in the **Dimensions** view control panel.

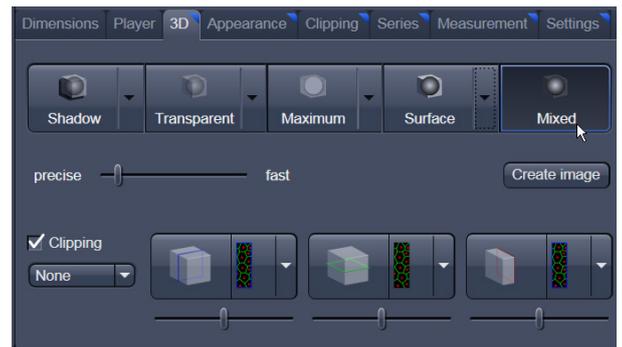


Fig. 343 3D toolbar – Mixed

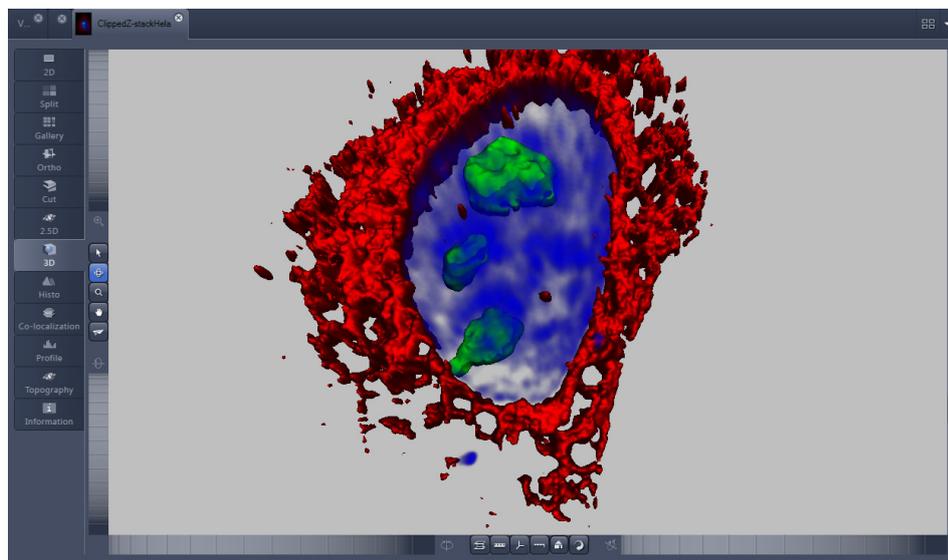


Fig. 344 Image Display window, 3D display, Mixed projection

### 4.8.6 Clipping Planes

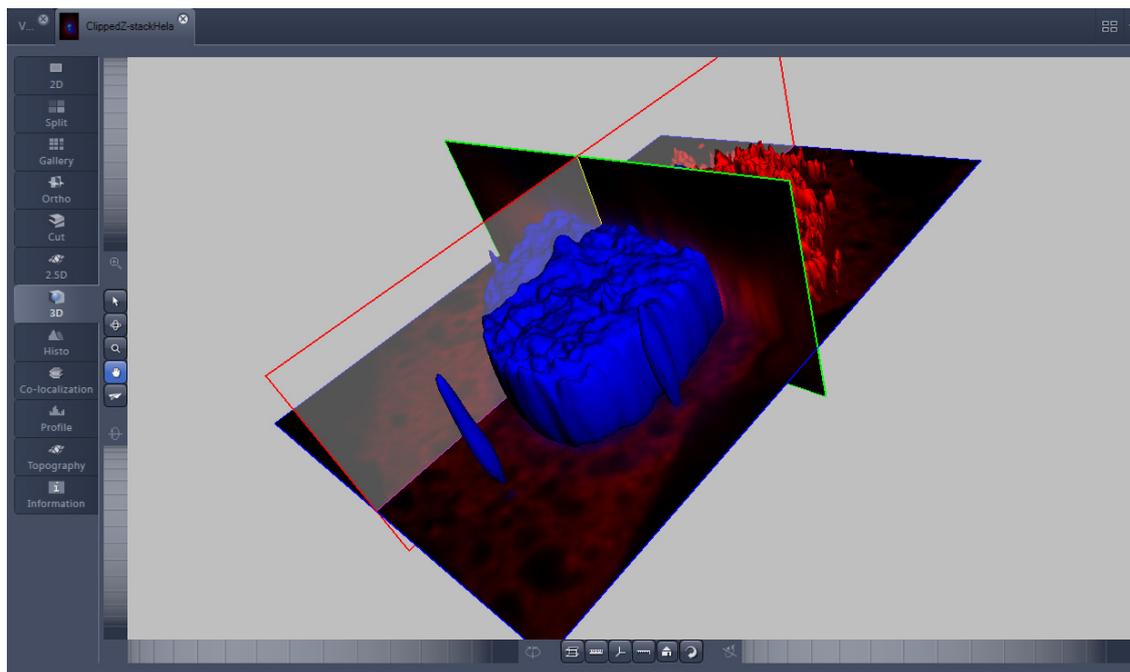
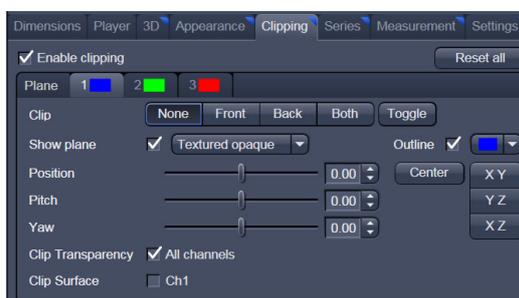
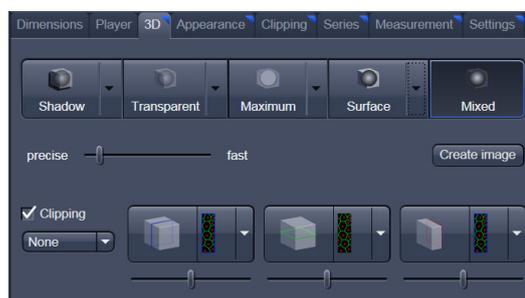


Fig. 345 Image Display window, 3D display, Clipping planes

3D VisArt can display up to three so called **Clipping planes**. These planes can be used to cut the volume open in order to visualize structures that are within a given volume. Clipping planes make most sense when used in the **Surface** and **Mixed** mode but can also be used with limited functionality by the rendering modes **Maximum** and **Transparency** as well as in the Split mode (see below)

The clipping planes can be accessed via the **3D** tab or the **Clipping** tab. On the 3D tab, only the most commonly used functionality is available. The full functionality can be accessed on the **Clipping** tab.

 Note that the **Clipping** tab is only available with the **Show all** mode activated.

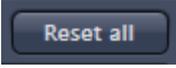


In **Surface** mode, clipping planes can be used in a channel specific manner. This means that in a multichannel image, one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique is also applicable in **Mixed** mode.

A further possibility is to use clipping planes in order to display only the grey values of the volume which touch the clipping plane.

All these functions can be used for creating a render series in order to show them and to make movies for presentations.

There are three clipping planes which are labeled for ease of use with colored outlines. These outlines do NOT represent the channels. The standard orientations of the planes are **blue**: XY plane, **green**: YZ plane, and **red**: XZ plane. This is linked to the default orientation of the volume axis.

Activating the **Clipping** panel displays the clipping planes and displays those with default settings if the image was not opened in the 3D module before.  is setting back the appearance of the planes to the following settings:

- Each plane is placed into the center of the volume and oriented in an orthogonal fashion.
- The planes have an opaque surface and colored outlines.
- The clipping functionality of the planes is not activated in this state.

### Controlling the clipping functionality of the 3 planes

The clipping planes are operated by three buttons on the **3D** tab which are color coded in the same way the respective planes are color coded. The buttons are located below the **Render Mode Buttons** and can be activated by ticking the **clipping** tick box.

 Note that full functionality of the clipping planes can be found in the **Clipping View Control** tab!

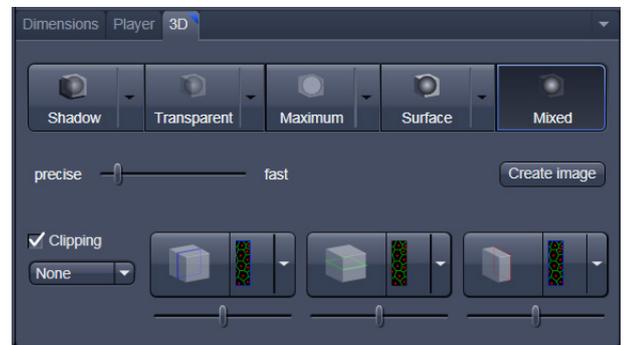


Fig. 346 Toggle buttons

By toggling these



buttons, the clipping behavior of the planes can be set in the following manner:

- **None**: hides the plane completely 
- **Front**: clips away the data on the front side of the plane 
- **Back**: clips away the data on the back side of the plane 
- **Both**: clips away the data on both sides of the plane 
- **Show Plane** only displays the plane – no clipping 

These adjustments can be made individually for each plane. The clipping planes can be chosen by selecting the respective button.

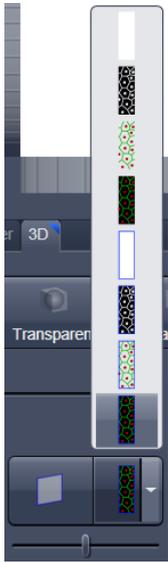


Fig. 347 Clipping planes

### Appearance of the clipping planes

By default, the clipping planes have an opaque surface and a colored outline. This can be changed by using the dropdown in the **3D** View Control tab. In the **Clipping** View Control tab, this function can be found under a pull down.

 This function is applicable to all 3 clipping planes separately.

**Colored:**  
white - opaque surface of the plane

**Binary:**  
displays the binary data on the clipping planes

**Textured:**  
Data which are touched by the plane are displayed on the clipping plane as in the render mode **Transparent** but as a two-dimensional projection. This does include the settings (threshold, ramp etc) applied to the **Transparent** render mode. Black pixels are transparent.

**Textured fine:**  
Same as above but with 4x times the resolution

### Transparent:

Data which are touched by the plane are displayed on the clipping plane as in the render mode **Transparent** but as a two-dimensional projection. This does not include the settings applied to the **Transparent** render mode. Black pixels are transparent.

### Textured opaque:

as with **Transparent** (including the settings) but in this case black pixels are non-transparent, rendered volume data in the background does not shine through.

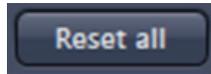
### Interactive positioning of the clipping planes

The clipping planes can be moved interactively with the mouse by activating the  button. If hovering over a plane outline, the plane turns into a different color, the cursor turns into a crosshair indicating that the plane can now be moved. Clicking and dragging then moves the plane. The clipping behavior (e.g. "front") is maintained during the movement.

 These movements can also be "recorded" using the render series function.

### Clipping Planes Settings

Activate or deactivate the clipping functionality globally by the check box **Enable clipping**.



displays the three clipping planes in the default mode (opaque surface, colored outlines, clipping planes active, no clipping).

With the **Plane** tabs, the planes can be addressed individually and their settings can be adjusted independent from each other.

**Outline** displays or hides the colored outlines. The actual color can be chosen from a color chooser for each plane individually.

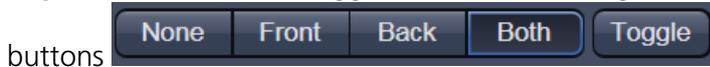
**Show Plane** hides or displays the plane.

A plane can cut away information while being "invisible".

For distance or angle measurements on a clipping plane, the plane has to be displayed and a color has to be chosen for the clipping plane.

The appearance of the plane can be selected from a drop-down menu (see Fig. 347).

**Clip** names a row of toggle buttons switching between different clipping modes as named on the



buttons

Using the **Pitch**, **Yaw**, and **Position** sliders, each plane can be freely orientated in three-dimensional space.

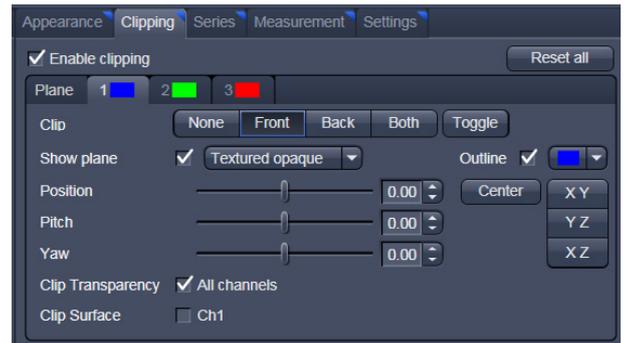


Fig. 348 Clipping planes settings



Fig. 349 Sliders Pitch, Yaw, and Position

### 4.8.7 Flying Mode

The **Flying** mode is an interactive way to explore three-dimensional data. By pressing the mouse wheel you can fly into and through a dataset. Moving the mouse left / right and up / down controls the flying direction. Pressing the mouse wheel and the right mouse button at the same time allows for flying backwards.

☞ Once the **Flying** mode is activated, the rotation of the data switches modes: Whereas normally the data turns around an imaginative point in the center of the dataset, in the fly mode, the data turns (if moved interactively with the mouse once one stops flying) around an imaginative point "behind"

the operator. Pressing the  button switches back into the normal mode.

If a wheel-mouse is not available, pressing the  button turns the left mouse button into the **Flying** button.

☞ The **Flying** mode is only available in graphics card accelerated render modes.

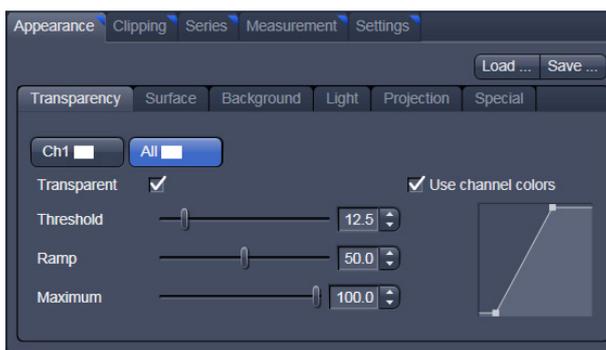


Fig. 350 3D Rendering window (e.g. Surface Advanced rendering mode)

### 4.8.8 3D Rendering Settings in VisArt

Depending on the render mode selected, the following adjustments can be applied to the datasets:

All settings can be saved and re-applied to other datasets by using the  buttons.

- **Threshold** specifies a lower threshold as a percentage of the gray values displayed. This decides which data enter the rendered image. If one fluorescence channel contains a homogeneous fluorescence signal with high signal strength, one would set the threshold higher in order to "protect" signals from weaker channels from being quenched.
- **Ambient** describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 100).
- **Specular** sets a directional brightness (values on a scale between 0 and 100). Influences the differences between dark and bright structures, which are strongest at a value of 0.
- **Shininess** changes the surface gloss (on a scale between 0 and 100).
- **Ramp** controls the degree of the transition from completely transparent to completely opaque.
- **Maximum** influences the degree of opacity.

The settings are combined into a histogram . The x axis corresponds to the gray values and the y axis to the degree of opacity. You can also use the mouse to interact with the histogram curve.

- **Roughness** (for Shadow mode only): You can also influence the roughness used to display the surface of the image structures. This changes the plasticity of the display (relative scale between 0 and 1). This setting quickly can produce artifacts if pixels reach saturation. The degree of roughness should be reduced accordingly.
- **Brightness** can be controlled via input field or slider bar.
- **Distance** determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").
- **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range: 0° to 90°).
- **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range: -180° to +180°).

- **Channels:** The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel, click on the corresponding button (labeled with the channel color and number).

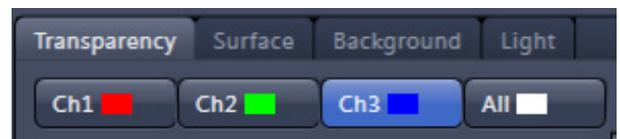


Fig. 351 Channels

- **Distance** (for Shadow mode only) sets the distance between the 3D object and the virtual background on a scale from 0.5 to 3.0.
- **Color:** The color of the background can be freely chosen from a table which appears when clicking the color chooser button .

- **View angle / Scale Z:** In hardware accelerated modes, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

The Z-Scale of the dataset can be altered interactively by using the slider. The slider ranges from a factor of 0.1 to a factor of 5.

- **Background:** The background color can be altered by using the pull down menu. For some 3D Objects, it can be beneficial to display a **Structured background** for better orientation within the dataset.



Fig. 352 Viewing angle

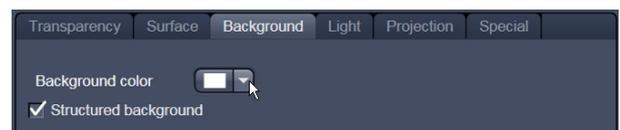


Fig. 353 Background



Fig. 354 Series tab



Fig. 355 Render Series window (e.g. Turn around X mode)



Fig. 356 Render Series modes

**(1) Turn around X and Turn around Y mode**

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for number of **Total frames**, **Difference angle** and **First angle** can be selected accordingly.

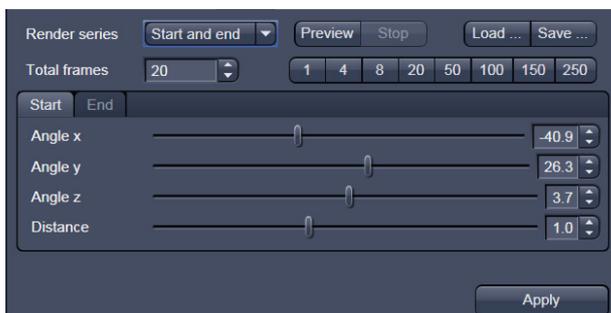


Fig. 357 Start panel in the Render View control block

**4.8.9 Series**

The **Series** tab (Fig. 354) displays the **Render Series** control panel. This panel allows settings for the axis to be used for rotation of the 3D reconstructed images. This feature is applicable to all render modes available in 3D VisArt.

There are four different modes for producing render series (see Fig. 356).

Depending on the activated mode, directly set the parameters for animation in the **Render Series** window and the position of the 3D image in the **Image Display** window (zoom, rotation axes, rendering parameters). A maximum of 1000 **Total frames** can be used for all Series options.

Click on **Apply** to start the animation

The animation is performed in a separate **Image Display** window, which permits the animation to be saved afterwards.

**(2) Start and End mode**

In the **Start and End** mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y, and Z and the distance (zoom) can be determined using the sliders.

The value for **Total frames** can be varied. The frames are distributed equally between the start and end points (Fig. 357).

### (3) Position List mode

In the **Position List** mode, the image is reconstructed between any required number of interim positions to be determined individually. Just rotate the reconstructed image with the mouse or do any other adjustment and add this as a "position" in the position list. The module will create a movie using those positions as a guideline. It will interpolate all the parameters ticked in the list.

The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

Every required interim position is included in the list of the **Render Series** window with a click on the **Add** button.

**Remove** permits the contents of the list to be deleted. **Insert** inserts a position above the highlighted list entry.

The value for **Total frames** can be varied. A value of 20 produces a render series with 20 frames in total. Interpolate lists variables that are interpolated during the Render Series.

Pressing the  button creates a short and low resolution (no interpolation!) preview of the rendering using the positions currently in the list. This serves as a help to quickly review the rendering without actually starting the process (which might be time consuming in cases of long position lists).

Click on the **Apply** button calculates a spline along all the defined positions from the list and starts an animation along this spline track in space.

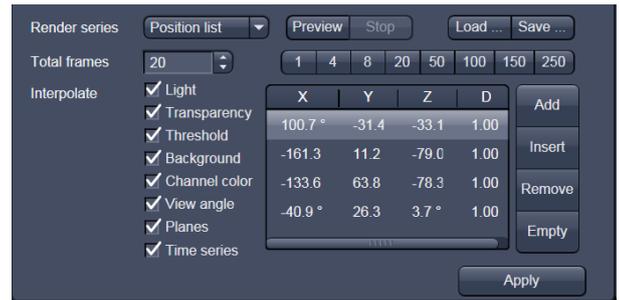


Fig. 358 Render Series window - Position List mode

### 4.8.10 Interactive Measurements

3D VisArt features three tools for interactive measurements in Euclidian space. Measurements can be performed in all graphic-card accelerated modes (**Transparency**, **Surface**, **Maximum**, and **Mixed**). The measured tools are shown as shapes in the rendered volume and can be changed here interactively. Measurement values can be exported as a table to the clipboard for downstream processing in other programs.

First, a suitable tool has to be chosen. In order for measurements to work, the mouse cursor must "recognize" a structure. In the **Surface** mode this is a uniquely identifiable surface structure, for the transparent modes the tools look for the highest local gray level maximum.

As a sign, that the mouse cursor has "found" a structure, the cursor shape changes from  into .

The found measurement point is drawn as a blue circle .



Fig. 359 3D Measurement View control block

#### Interactive 3D-Measurement

The measurement dialog contains a toolbar with the available measurement tools.

The **Line** tool measures the length along a line in µm. First, click on a starting point and move the mouse to the desired end point while keeping the left mouse key depressed. The measurement is concluded upon releasing the left mouse key.

The **Angle** measurement tool defines an angle between two connected line segments. First define the starting point by clicking, and then draw the first line segment until you click on the first endpoint. Now draw the second line segment and conclude the measurement by clicking onto the second end point. This measures the angle in degree.

The **Curve** tool measures the total length (µm) along a variable multi-segmented polygon line. Please click from point to point and finish the measurement by clicking with the right mouse key.

Measurement values are compiled in a list. In order to delete a measurement, select it in the list and press the **Remove** button. One can also delete all measurements by clicking on **Remove all**.

The measurements can be copied into the clipboard with the **Copy** button.

Measurements are only transiently drawn into the volume and are not stored automatically with the image.

## 4.8.11 Settings

### Flying mode

Ticking the tick box **Flying** mode activates the mode, unticking deactivates it.

To show or hide an overview image, tick the **Show overlay image** box.

The fly-speed can be adjusted in a range from 1 to 100.

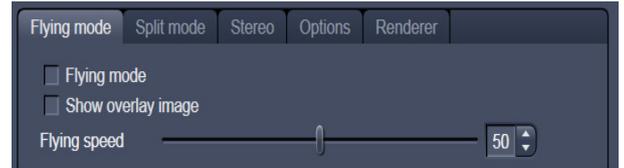


Fig. 360 3D Renderer Settings – Flying mode

### Split Mode

Ticking this tick box activates a channel wise 3D split view of the data. This is available in surface render mode only. An overview image with all channels combined can be shown optionally by ticking **Draw combined image**.

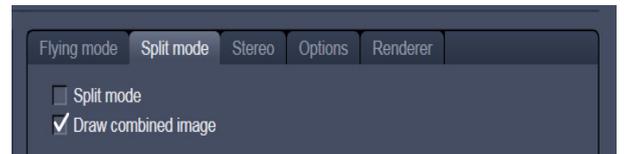


Fig. 361 3D Renderer Settings – Split mode

### Stereo

Two Stereo views can be chosen from this sub-menu:

**Anaglyph** is a view in which the data can be examined in 3D using red/green glasses. The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

The stereoscopic effect can only be seen with the aid of red/green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.

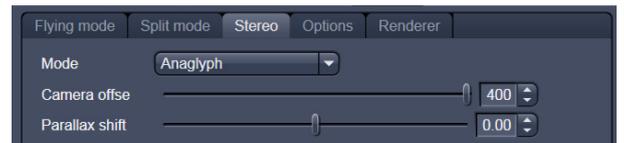


Fig. 362 3D Renderer Settings – Stereo

**Camera offset:** Defines the distance of both cameras from each other (units in meters).

**Parallax shift:** Determines the degree of shift which is necessary to correlate both camera views. The slider should be adjusted to maximize the stereo effect. Please note, that this setting will be different for each observer.

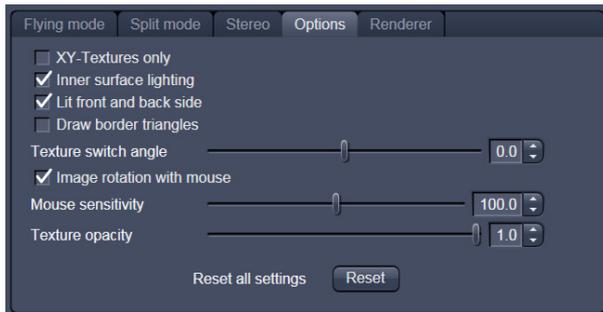


Fig. 363 3D Renderer Settings – Options

#### 4.8.12 Options

**XY Textures only:** Instead of working with three texture stacks per dataset in transparent render mode, the system will only use one texture stack. This will result in faster renderings. Looking at the dataset from a side although will make the individual z-planes visible.

**Inner surface lighting:** If checked, illuminates the inside of a surface rendered volume. If unchecked, the inside is dark. If one views the inside of a volume by flying or zooming into it, the inside illumination is helpful. This option is only available for the **Surface** mode.

**Lit front and back side:** With activated **Inner surface lighting**, this check box colors the inside lighting with a bluish hue. This makes distinguishing inside from outside easier. This option is only available for the **Surface** mode.

**Draw border triangles:** When displaying the data touching the six sides ("borders") of the Z-Stack in 3D, the triangles used to do this are all located in one plane and orientation. This can cause artifacts, therefore the display of those border triangles can be switched off.

**Texture switch angle:** This parameter influences the volume angle, upon which the next set of 2D-Textures will be loaded into the graphics card (value range 1 to 100 degree). Depending on the object orientation, the number of z-slices and the chosen render settings, this loading process can be visible and may be disturbing especially when rendering a series for movie export. Changing this angle can postpone or even avoid this switch during a series rendering. This option is only available for **Transparency** and **Maximum** modes.

**Image Rotation with mouse:** Activates a method to rotate the dataset interactively with the mouse. In addition to rotating it freely, when clicking and dragging at the sides of the image window the dragging rotates the data around one axis only. Just like the wheels on the 3D window frame.

**Mouse Sensitivity:** Influences the mouse sensitivity (value range: 1 to 200). Small values make the mouse actions precise but slow, large values less precise but faster.

**Texture Opacity:** With this slider the  $\alpha$ -values of the dataset can be adjusted. The rendering is getting more or less transparent using this setting.

**Reset:** Sets all render settings back to the factory standard settings.



Fig. 364 3D OpenGL Renderer settings

#### Renderer Options:

**Renderer:** OpenGL Software is rendering the 3D data with the CPU; OpenGL Hardware uses the Graphics card to do this. Especially when having trouble with non Zeiss-certified graphics cards, it is sometimes useful to switch to a pure CPU based rendering.

**Palette textures:** This feature is an optimization feature not supported by modern graphics cards any more. It used to optimize display of single channel images.

**Intensity Alpha textures:** This feature optimizes the usage of the graphic ram with single channel images.

**Display Lists** activates a batch processing of the operations the graphics card is doing. It results in faster rendering.

**Hardware accelerated series rendering:** Un-ticking this feature uses the Computer Processor for render series. In case of trouble with non-certified graphics cards, render series can be performed with the CPU without risk. The use of memory of the graphics card can be adjusted in a dialog box.

**Window independent series image size:** Using this feature creates render series (see section **4.8.9 Series**) with the output size indicated in the two input boxes. The maximal resolution is 4096 x 4096 pixels. This feature is available in all Render Modes (CPU and GPU accelerated). This applies also to the "**Create Image**" button on the **3D View Options** tab.

**Allocate Graphics Card RAM:** The amount of Graphics card RAM can be freely defined in the last Item on the **3D | Settings** view control panel. Default sets this value to ~ 80 % of the available RAM of the currently used graphics card.

### 4.8.13 3D View – Basic

Without an active license for 3D VisArt, ZEN offers the following 3D functionality:

The available basic 3D options are described in section **4.8 3D View (3D VisArt)**. The following features are available:

- Shadow view - Front view,
- Transparency render mode - Basic, rotate around x or y, render series,
- Maximum brightness projection – Basic Mode only, only rotate around x or y, or start to end movies,
- Surface - Basic Render Mode, rotate around x or y movies,
- Stereo Anaglyph Mode.

## 4.9 Histogram View

The **Histogram** function (**Histo View**) allows to

- display a histogram (distribution of pixel intensities) of an image or Region of Interest,
- show the histogram values in table form,
- copy table to clipboard or save as text file,
- measure the area and mean gray value and standard distribution in an area.

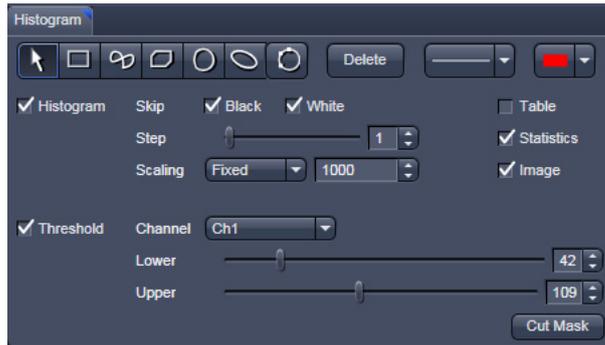


Fig. 365 View Option control block - Histo View

The settings of the **Dimensions**, **Display**, **Player**, and **Graphics** view options control blocks apply. The additional view-specific **Histo View Option** control block is shown in Fig. 365.

Any changes done with this View Option control block are effective immediately.

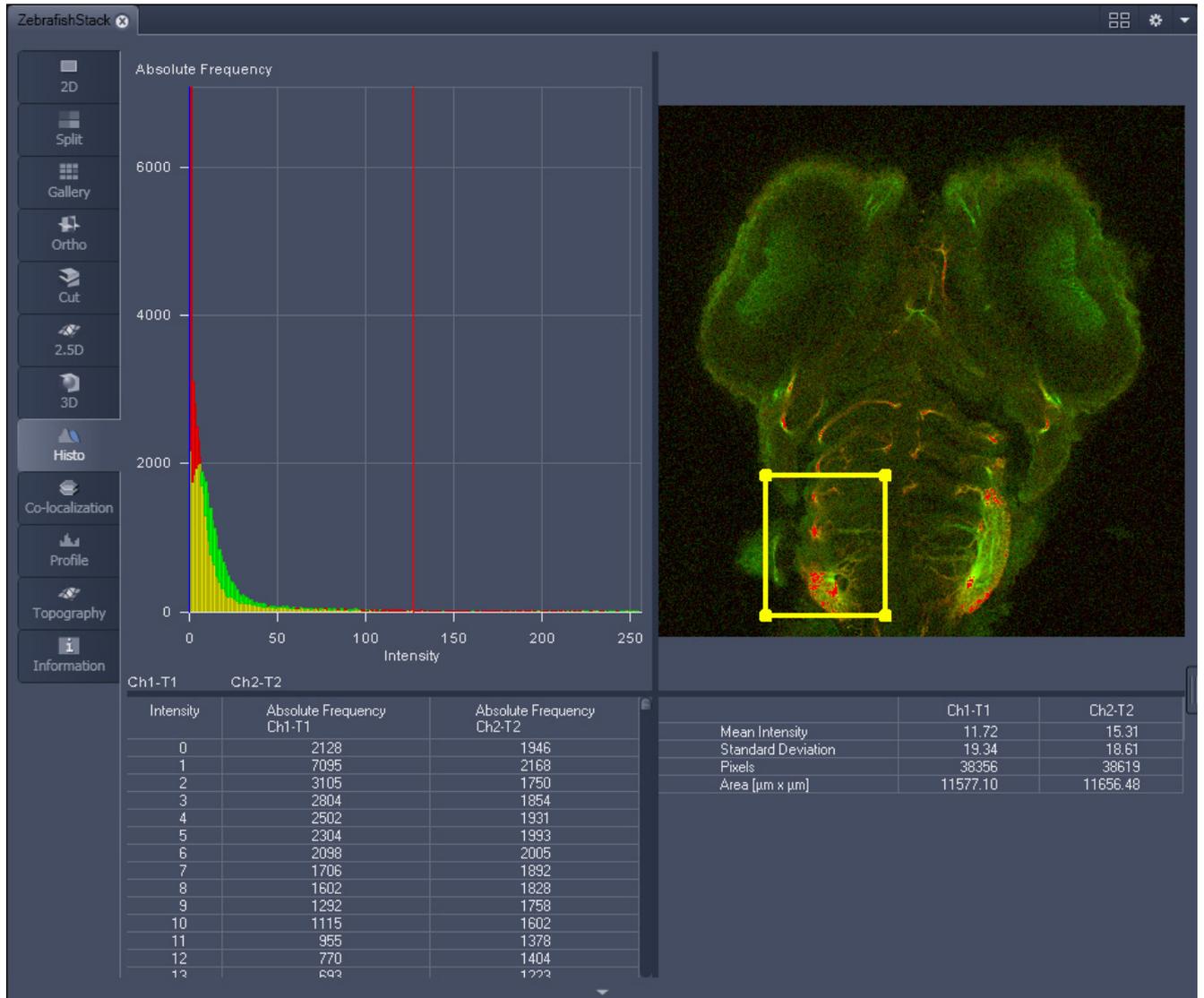


Fig. 366 Image Display, Histogram view

 The **Histo** button can also be used online during image acquisition.

**Histogram functions:**

**Skip Black** and **Skip White** tick boxes: Ignore black pixels (gray value 0) and ignore white pixels (gray value 255 or 4096) in the histogram.



**Step** slider and spin box: Sets the number of intensity steps ("trays") of the histogram display. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps (for 8 bit images). Reduction is made by averaging.



**Scaling** pull down menu: Sets the histogram graph scaling of the Absolute Frequency to either dynamic (adjusting to the maximum value) or fixed



**Show Table** tick box: A table of the intensity values is shown in the **Image Display** window.



**Show Statistics** tick box: Displays statistical parameters (Mean Intensity, Standard Deviation, Number of Pixels, and Size of Area) in an additional table. Area measurements of very small areas (<10 pixels) give only approximate values.



**Show Image** tick box: Shows the image in the **Image Display** window with the histogram graph.



The **Cut Mask** tool creates a new image document which sets every pixel outside ROIs to Zero. Within the ROIs, only the pixels with values between lower and upper threshold are transferred to the new image.

 Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

**Histogram functions and Overlays:**

The functionality of the **Graphics View Option** control block fully applies to the **Histo View** Type. If a ROI is marked/selected in the Histo View Image display, only this ROI will be taken into account for the histogram display, the table, and the statistics. If there are several ROIs, but none is marked/selected, the histogram display, the table, and the statistics analyze all the pixels included in all ROIs.

### Histogram Threshold:

When the **Threshold** tick box is selected, the controls to set a lower and upper threshold for the histogram calculation are displayed (Fig. 367). The threshold can be set for each channel (channel selection pull down menu).

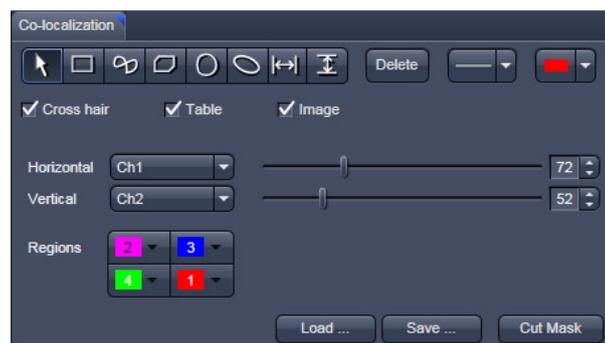


**Fig. 367** Histo View Option control block – Threshold

## 4.10 Colocalization View

The **Colocalization** function permits interactive analysis of two channels of an image by computing a scatter diagram (co-localization).

The settings of the **Dimensions, Display, Player,** and **Graphics** view options control blocks apply. The additional view-specific **Colocalization View Option** control block is shown in Fig. 368.



**Fig. 368** Colocalization View Option control block

Any changes done with this View Option control block are effective immediately.

The Image Display in the Colocalization View shows 3 panels: the scatter diagram, the data table, and the pseudo-colored image display (see Fig. 369 and Fig. 370).

 Colocalization is defined by the presence of two or more different molecules at the same location in a specimen. However, in the context of digital imaging, the term colocalization refers to colors emitted by fluorescent molecules detected by the same pixel in the image. It is important to be aware of the fact that colocalization cannot be analyzed for fluorophores with similar emission spectra. Accurate colocalization analysis is only possible if the fluorescence emission spectra are well separated between fluorophores and the correct filter sets are used for data acquisition. If spectral bleed-through artifacts are present because of spectral overlap between the fluorophore emission spectra, or due to the use of incorrect filter sets, colocalization measurements will be meaningless. To avoid this, the fluorophores must be carefully chosen and matched to the excitation laser lines to obtain the maximum excitation efficiency while still maintaining a useful degree of separation between emission wavelengths. The choice of fluorophores is crucial for colocalization analysis.

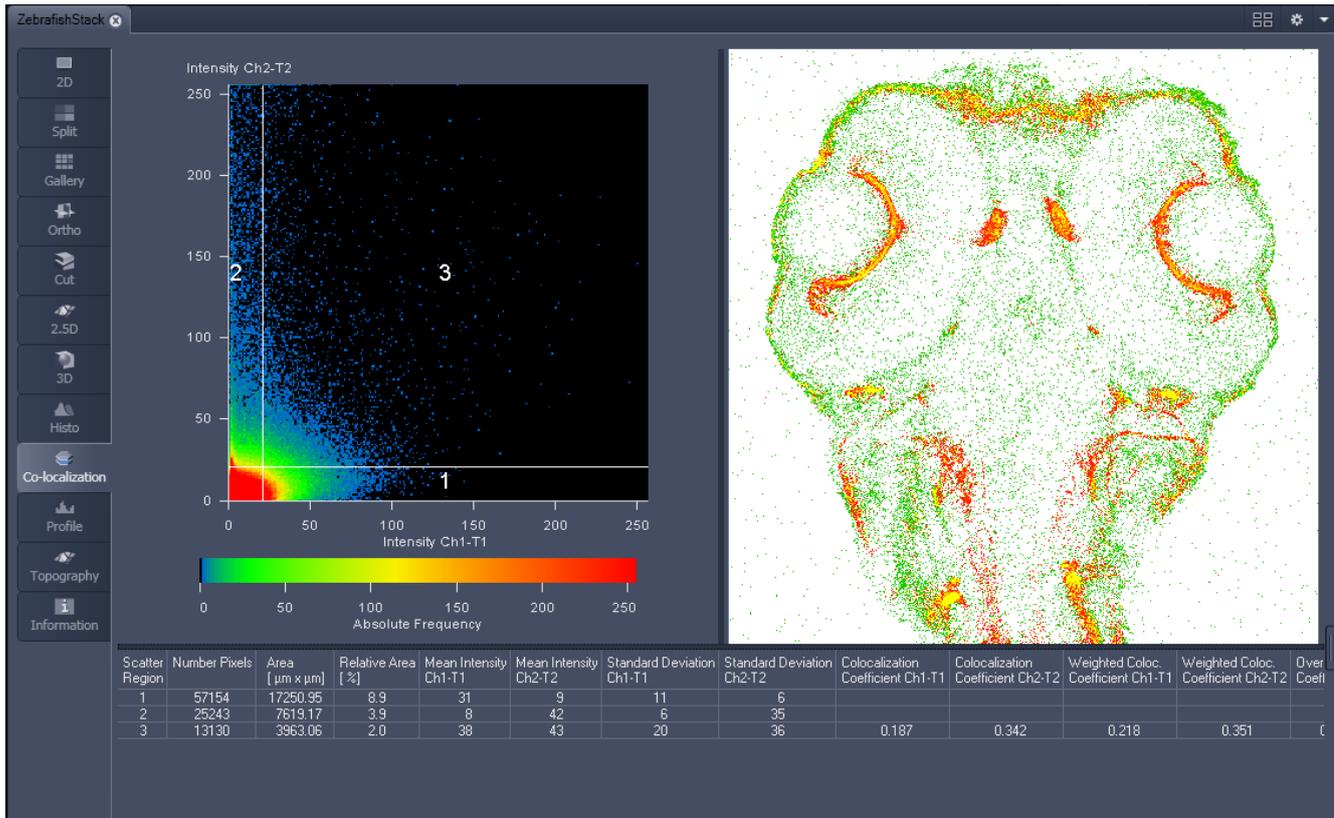


Fig. 369 Image Display, Colocalization view

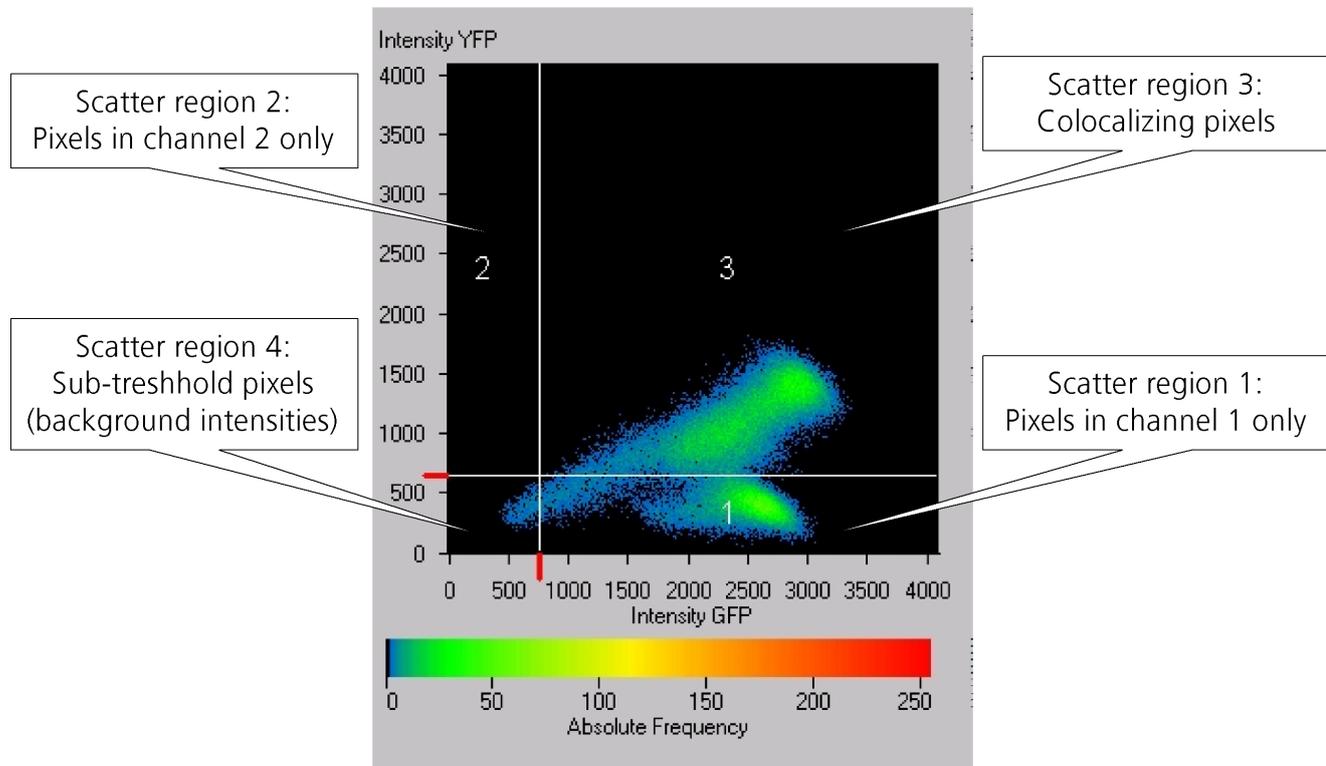


Fig. 370 Scatter diagram and threshold with crosshair

### 4.10.1 How a Scatter Diagram is Generated

All pixels having the same positions in both channels are considered a pair. Of every pair of pixels (P1, P2) from the two source channels, the intensity level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

 Differences between the channels cause irregular spots in the scatter diagram.

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) with the same intensity can occur. Differences between the images cause an irregular distribution in the scatter diagram.

 Scatter diagram, image display, and data table are interactively linked and immediately updated when settings are changed.

#### Available Controls:

##### Drawing tools



With the drawing tools, the colocalization analysis can be restricted to a region of the image. Tools in the Colocalization View Option control block and in the Graphics View Option control block work the same and can be combined.



Cross hair, Table, and Image selection tick boxes: when selected, the respective element is displayed in the Image Display area. If off, the element is hidden.



With the pull down menu, select the Channels you wish to perform colocalization analysis with. The sliders or input boxes control the x – and y-axis of the crosshair or the red markings in the scatter diagram.



Set the color LUTs for each quadrant in the image display. When the button is pressed on the colored field, the pixel of this quadrant are marked within the image in the according color.



The Load and Save buttons can be used to save colocalization settings (like color settings for the quadrants and crosshair-positions) to a \*.col file and re-load it when analyzing a different data set. This allows using the same parameter for the colocalization analysis of different images.



The Cut Mask tool creates a new image document which sets every pixel outside Quadrant 3 (not colocalized) to Zero. ROIs don't play a role for this Cut Mask function (as opposed to the Cut Mask in Histo View).

 Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

#### 4.10.2 Quantitative Colocalization Parameters Shown in the Data Table

- **Number of pixels** in image ROI or scatter region
- **Area / relative area** of image ROI or scatter region
- **Mean intensities / Standard Deviation** within image ROI or scatter region
- **Colocalization coefficients**
- **Weighted colocalization coefficients**
- **Overlap coefficient** after Manders
- **Correlation coefficients** (R and R<sup>2</sup>)

#### 4.10.3 Colocalization Coefficients

$$c_1 = \frac{\text{pixels}_{Ch1,coloc}}{\text{pixels}_{Ch1,total}} \quad c_2 = \frac{\text{pixels}_{Ch2,coloc}}{\text{pixels}_{Ch2,total}}$$

- Relative number of colocalizing pixels in channel 1 or 2, respectively, as compared to the total number of pixels above threshold.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- All pixels above background count irrespective of their intensity.

Weighted colocalization coefficients

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_{i,total}} \quad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_{i,total}}$$

- Sum of intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- Bright pixels contribute more than faint pixels

Correlation coefficient, Pearson's correlation coefficient

$$R_p = \frac{\sum_i (Ch1_i - Ch1_{aver}) * (Ch2_i - Ch2_{aver})}{\sqrt{\sum_i (Ch1_i - Ch1_{aver})^2 * \sum_i (Ch2_i - Ch2_{aver})^2}}$$

- Provides information on the intensity distribution within the colocalizing region
- Value range -1 to +1
  - 1,+1: all pixels are found on a straight line in the scatter diagram
  - 0: pixels in the scattergram distribute in a cloud with no preferential direction

#### 4.10.4 Overlap Coefficient after Manders

(Manders, Verbeek and Aten, J. Microscopy 169:375-382, 1993)

$$r = \frac{\sum_i Ch1_i * Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 * \sum_i (Ch2_i)^2}}$$

- Another parameter used to quantify colocalization in image pairs
- Insensitive to differences in signal intensities between the two channels, photo-bleaching, or amplifier settings

Value range 0 – 1 (0: no colocalization, 1:all pixels colocalize)

#### 4.11 Profile View

This function allows to

- display the intensity distribution of an image along a straight or curved line,
- show the intensity values in table form and copy table to clipboard or save as text file,
- show separate profiles for each channel in a multi channel image.

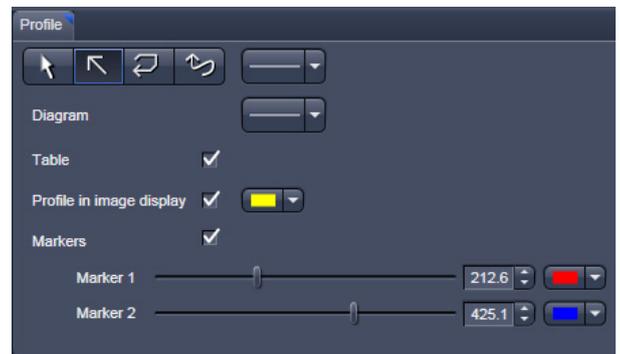


Fig. 371 View Option control block - Profile View

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply. The additional view-specific **Profile View Option** control block is shown in Fig. 371.

Any changes done with this View Option control block are effective immediately.

The content of the overlay plane is temporarily hidden while the toolbar is displayed.

 The **Profile** View can also be used online during imaging.

- Draw a Profile line in the image display to define a profile. The intensity curves are shown in a separate graph (see Fig. 372).
- In the **Profile** View Option control block you can select the width and type of the profile line. The color setting is taken from the **Graphics** View Option control block.
- You can place two markers on the profile line to measure differences in intensities and distances in the XY plane.
- Tick the **Profile in Image Display** block to see an intensity graph superimposed on the image (see Fig. 373).

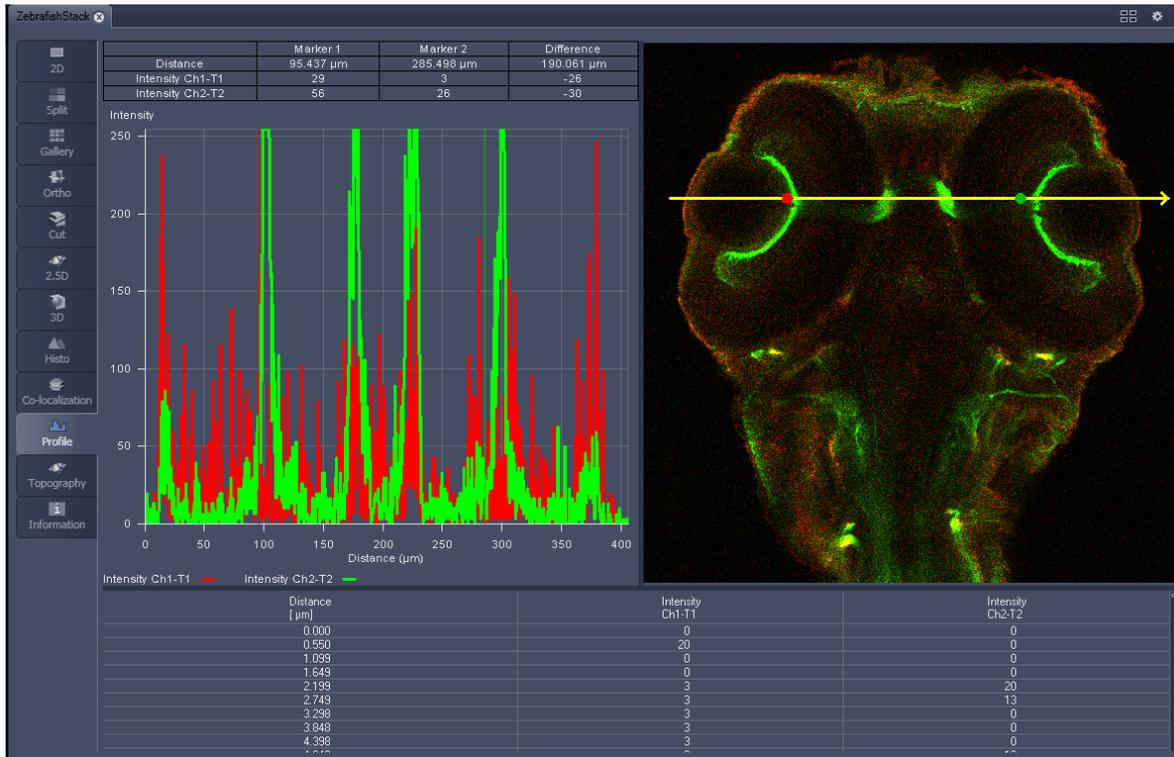


Fig. 372 Image Display - Profile View, Line Profile with markers

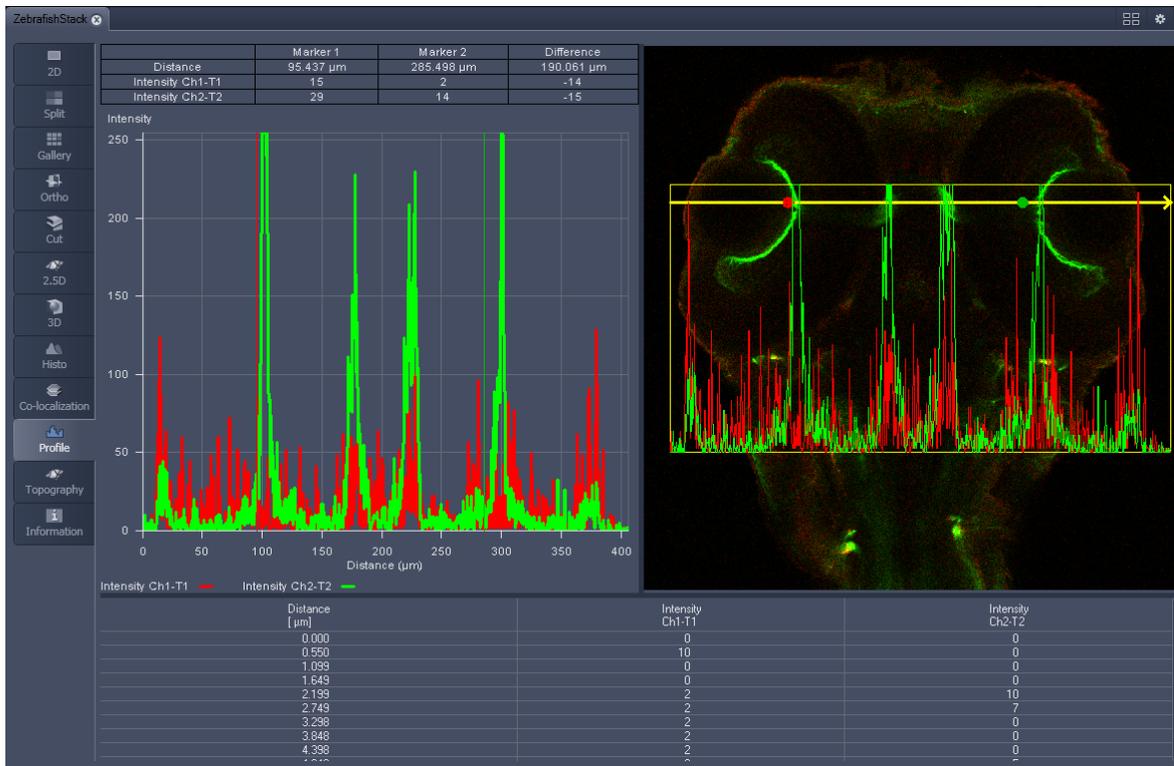


Fig. 373 Image Display - Profile View, Profile displayed in Image

## Control block tools

The **Profile** toolbar contains the following tools:



**Select** button: Activates the mouse button for selection, resizing or movement of the profile line in the **Image Display** window.

Resize: Click on handle and hold down the mouse button, move the handle, release mouse button.

Movement: Click on line and hold down the mouse button, move the entire line, release mouse button.



**Line with arrow** button: (open arrow): Activates the straight profile drawing mode.

Click into the image and hold the mouse button, drag a line in any direction and release the mouse button to end the procedure.



**Open polyline arrow** button: Activates the open polyline drawing mode.

The first click into the image sets the starting point, each additional click adds a further line, right mouse click ends the procedure.



**Line** button: This button allows you to determine the line thickness of the profile line. It has no influence on the way the intensity profile is generated.



**Show Table** button: The profile diagram is displayed as a table at the bottom of the **Image Display** window.

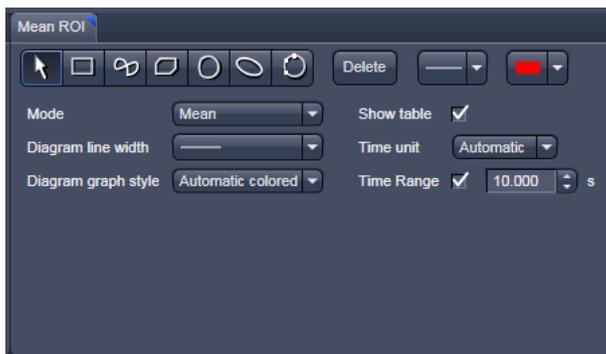


Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

## 4.12 Mean of ROI: Additional View Type for Time Series

The **Mean of ROI** View Type allows one to

- display the intensity - time diagram (mean intensity in user defined ROIs over time),
- use frame - time series and frame - Z-Stack - time series as input,
- show the intensity values in table form and copy table to clipboard or save as text file.



**Fig. 374** Mean of ROI View Option control block

The **Mean of ROI** function permits interactive analysis and monitoring intensity over time.

The settings of the **Dimensions, Display, Player,** and **Graphics** View Options control blocks apply. The additional view-specific **Mean of ROI View Option** control block is shown in Fig. 374.

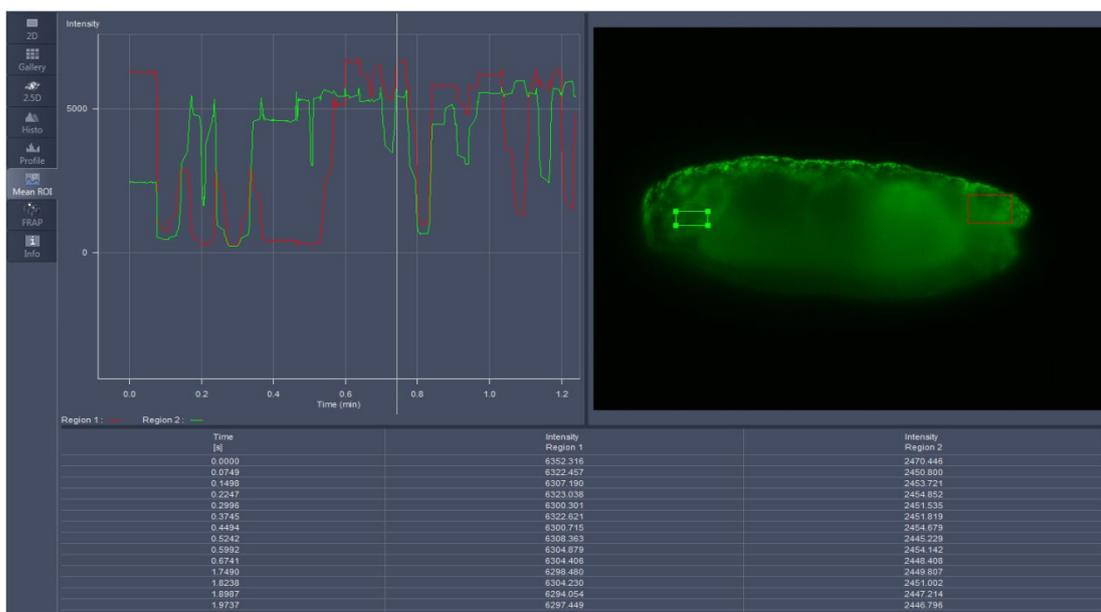
Any changes done with this View Option control block are effective immediately.

The Image Display in the Mean of ROI View shows 3 panels: the intensity-over-time diagram, the data table with the intensity values for each ROI over time and the image display with ROIs in overlay (see Fig. 375 and Fig. 376).

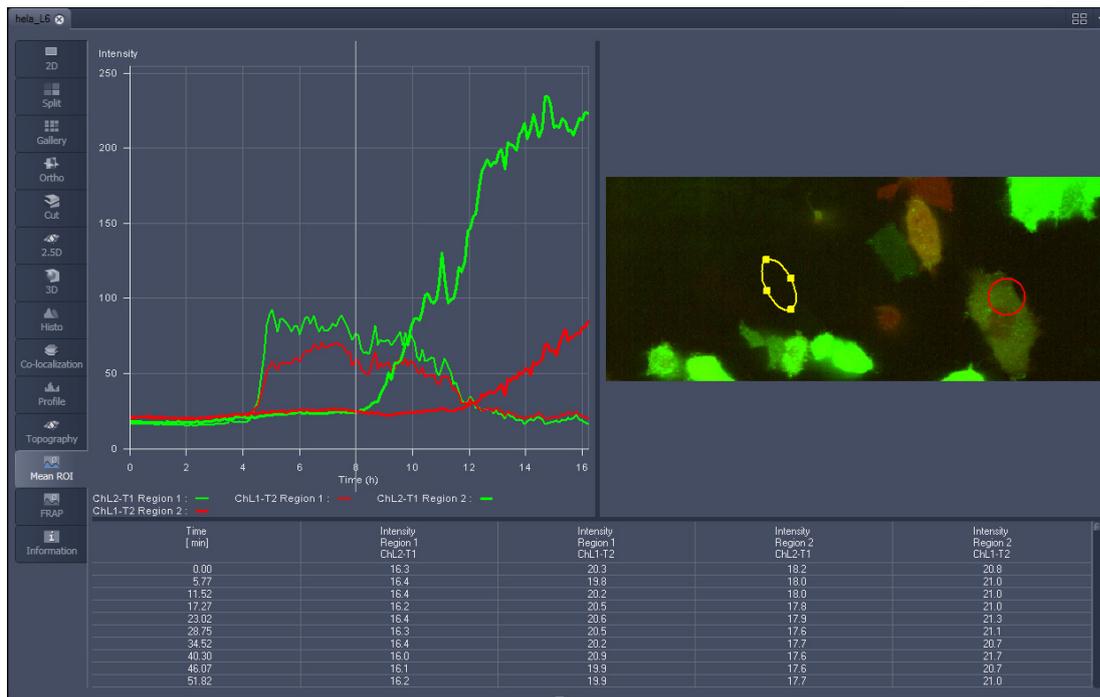
To access the **Mean of ROI** View Type, load or acquire a time series data set and click on the **Mean ROI** View tab.

The mean intensity of one or several regions of interest is displayed over time. Time flags of markers or of trigger events are marked with little symbols on the time-axis of the graph (see Fig. 375).

The **Mean of ROI** analysis always refers to the currently displayed z-section of a Z-Stack (see **Dimension** View Option control block for which section it is).



**Fig. 375** Image Display window, Mean ROI display for time series in single plane



**Fig. 376** Image Display window, Mean ROI display for time series of Z-Stack

The **Mean of ROI** view options control block contains the following function elements:

#	Type	X	Y	Width	Height	Lock
1		147	65	51	37	<input type="checkbox"/>
2		239	176	40	40	<input type="checkbox"/>
3		376	121	84	113	<input type="checkbox"/>

**ROIs** can be managed in the **Graphics View** Option control block.



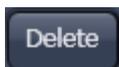
**Arrow** button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.



**Bezier** button: Activates the Bezier figure drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.



**Circle** button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.



**Delete** button: All the ROIs in the image are deleted.



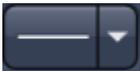
**Rectangle and 3D Rectangle** button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.



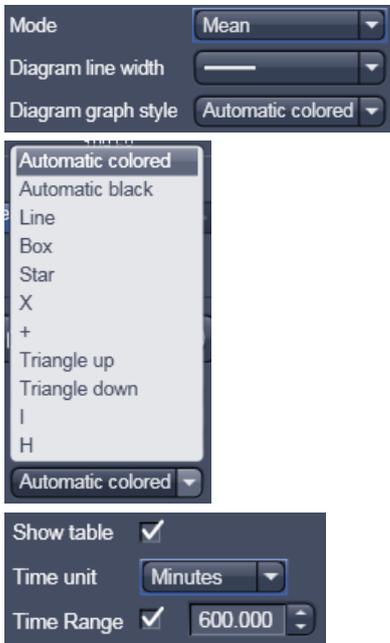
**Ellipse** and **3D Ellipse** button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.



**Polyline** and **3D Polyline** button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.



**Line** button: This button allows you to determine the line thickness of the ROI outline.



#### Buttons for diagram display options:

**Mode** pull down: choose between area and mean mode

**Area:** Display of the area of the ROI in the intensity time diagram, depending on the set threshold values. Area measurements of very small areas (< 10 pixels) give only approximate values.

**Mean:** Displays the mean values of the relevant ROI in the intensity time diagram.

**Diagram line width** pull down: sets the line width of the curves in the diagram.

**Diagram graph style** pull down: choose from a set of graph styles

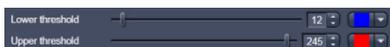
**Show Table** tick box: The table of intensity values is displayed on the bottom of the Image Display window.

**Time unit** pull down: choose between seconds, minutes, hours, or Automatic

**Time Range** check box and spin box: reduce the displayed time range to the selected value



**In Area Mode: Ch1 / Ch3 / Ch4** pull down: Selection of the channel to be used.



**In Area Mode: Threshold low and high** slider: The intensity values below /above threshold are not displayed for the **Area** function.

### 4.13 Information View

The **Information View** is a view type that shows a summary information sheet of all relevant image acquisition parameters. Parts of it can be edited and are labeled with an (E).

The Information View displays:

Name	File Name of the data set	E
Description	A brief description of the data set	E
Notes	A Notes field in which the user can note down essential information on the experiment	E
User	The user name of the person who acquired the image	N
Scaling x	The pixel scaling in X	E
Scaling y	The pixel scaling in Y	E
Scaling z	The pixel scaling in Z	E
Dimensions	Dimensions of the dataset (x, y, z, views, illumination side, time points, channels, bit depth)	N
Image Size	The image size x, y, z in $\mu\text{m}$	N
Data Volume	File size	N
Microscope	System name (Serial Number)	N
Detection Module	Type of detection module used	N
Detection Optics	Name of detection optic used	N
Emission Selection	The emission filters and beam splitters of the Emission Selection turret	N
Laser blocking filter	The laser blocking filter used	N
Lasers	The used lasers and their respective power in %.	N
Exposure Time	Detection module exposure time	N
Light Sheet Thickness	Calculated light sheet thickness as displayed in the GUI	N
Z-Stack	Information if continuous drive was used	N
Zoom	Detection zoom settings	N
Track	Number of tracks	N
Illumination Mode	Single or Dual Side Illumination used	N
Light Sheet Pivot	Information if Pivot was activated	N

## 5 Right Tool Area, Data Management and Storage

### 5.1 General

#### Document Type

Images are stored as files (\*.czi format) in folders just like in any other Windows application.

All information (metadata) will be saved with the .czi file. When exporting .czi files as .lsm files this metadata cannot be completely exported. When exporting .czi files as .lsm files, each Z-Stack will be saved as an individual .lsm file. The .czi file must be saved, closed and opened again to save it as .lsm file.

We will gladly send you further Information about the .czi file format. Please use the following link to fill out the necessary form and receive the pdf document:

<http://www.zeiss.com/C12579A30038958A/LicenceAgreement.1?OpenForm&lang=eng>

When time series are acquired with the Lightsheet Z.1 the first time point is created as a master file with the following appearance: Name.czi (e.g. FishEmbryogenesis.czi). For each time point, a separate file is additionally formed in the same folder with the nomenclature: Name(time point-1).czi (e.g. FishEmbryogenesis(221).czi).

In ZEN there are three ways to access your data:

- The File menu
- The ZEN File Browser
- Open Images are accessible in the Right Tool Area

#### ZEN File Browser

The File Browser allows access to image data of variable image formats and permits displaying them in three views:

- Gallery View,
- Table View and
- Form View.

Images can be loaded, copied, and deleted within the ZEN File Browser.

The **Reuse** (see section **4.2.1 Dimensions**) function is available in the ZEN Image Browser: Hardware parameters can be loaded without opening the files by clicking the **Reuse** button.

#### Images and Documents

In the Right Tool Area, the **Images and Documents** section displays the images which are loaded in the system memory. Double clicking brings the image to the front. Files can be saved and deleted/closed on this section.

#### Export

With the **Export** dialog in the **File** menu, the acquired data can be directly exported to various file types.

 In the **Save As** dialog in the **File** menu, these formats can also be chosen as file type.

## 5.2 Comments on Data Management

Using the Lightsheet Z.1 system which is designed to acquire fast Z-Stacks from multiple views over a period of time will easily produce large datasets. To give a rough idea, here are some typical examples:

Parameters	Experiment 1	Experiment 2
Z-Stack:	300 slices	300 slices
Views:	4	4
Time:	1	300 (overnight experiment)
Channel	1	1
Illumination	dual-side	dual-side
Frame	1600 x 1600 pixel	1600 x 1600 pixel
Size	12 GB	3.6 TB

When using two channels, the data will double.

You may want to consider limiting one or the other of the mentioned parameters to reduce data before starting the experiment. For example:

Cropping the image frame to the necessary pixels without comprising the image information. Keep in mind that processing of Multiview data might require sufficient landmarks imaged with the specimen

 Binning the pixels, if possible, if high resolution is not necessary.

Keeping Z-Stacks small, but taking into consideration enough fiducials in the different views in case a landmark alignment processing is planned.

To achieve the specified performance of the system, the **Streaming** option in the **Streaming tool** window of the **Maintain tab** has to be active.

Please ensure the following for the drive you will be streaming your data onto:

- If you have no storage and analysis PC, use the D drive from your PC for system control. Using the C drive will lead to an unstable system.
- If you have a storage and analysis PC:
  - Ensure that you are using the optical fiber cable to connect the PC for system control to the storage and analysis PC.
  - Ensure that the drive of the storage and analysis PC is mapped to the PC for system control. This will happen automatically when the storage and analysis PC is booted before the PC for system control is turned on.
  - While streaming data (experiments) onto the storage and analysis PC, do not try to remove or load any other data from or to the storage and analysis PC. It will slow down the data stream from the PC for system control and will influence the acquisition speed of the experiment.

- Do not use the network of your institute or laboratory to stream data to the storage and analysis PC or to your servers. Most likely, data transfer will be too slow and will jeopardize your experiment and will lead to an unstable system. If the PC for system control and the storage and analysis PC are both connected to an internal network, ensure that the preferred streaming path is via the optical fiber cable.
- Do not use any USB devices (external hard drives) to stream onto; data transfer will be too slow.
-  **Do not exchange single Raid elements** of your storage and analysis PC. The system is designed to function as one unit. To remove already saved data from your storage and analysis PC (not during data acquisition), you can use USB devices (external hard drives) with USB 2.0 and 3.0 connections; internal network connections, and the optical fiber cable.
-  To configure individual data repositories or to connect to storage services of the institute, solely for data storage, the type of PCI board and the information on the cable can be obtained from Carl Zeiss Microscopy GmbH. However, ensure that all additional storage devices are disconnected from the PC of system control or the storage and analysis PC during image acquisition to ensure the full acquisition performance of the system.

### 5.3 ZEN File Browser

The ZEN File Browser is a versatile tool to access your image data.

- In the menu bar, click on **File** and select the **New File Browser ...** item for opening the **ZEN File Browser**.

It displays the hard drives and connected network drives available on the computer. Clicking on a folder opens it and lists the image files therein.

The ZEN File Browser can be operated in three views:

- Gallery View,
- Table View and
- Form View.

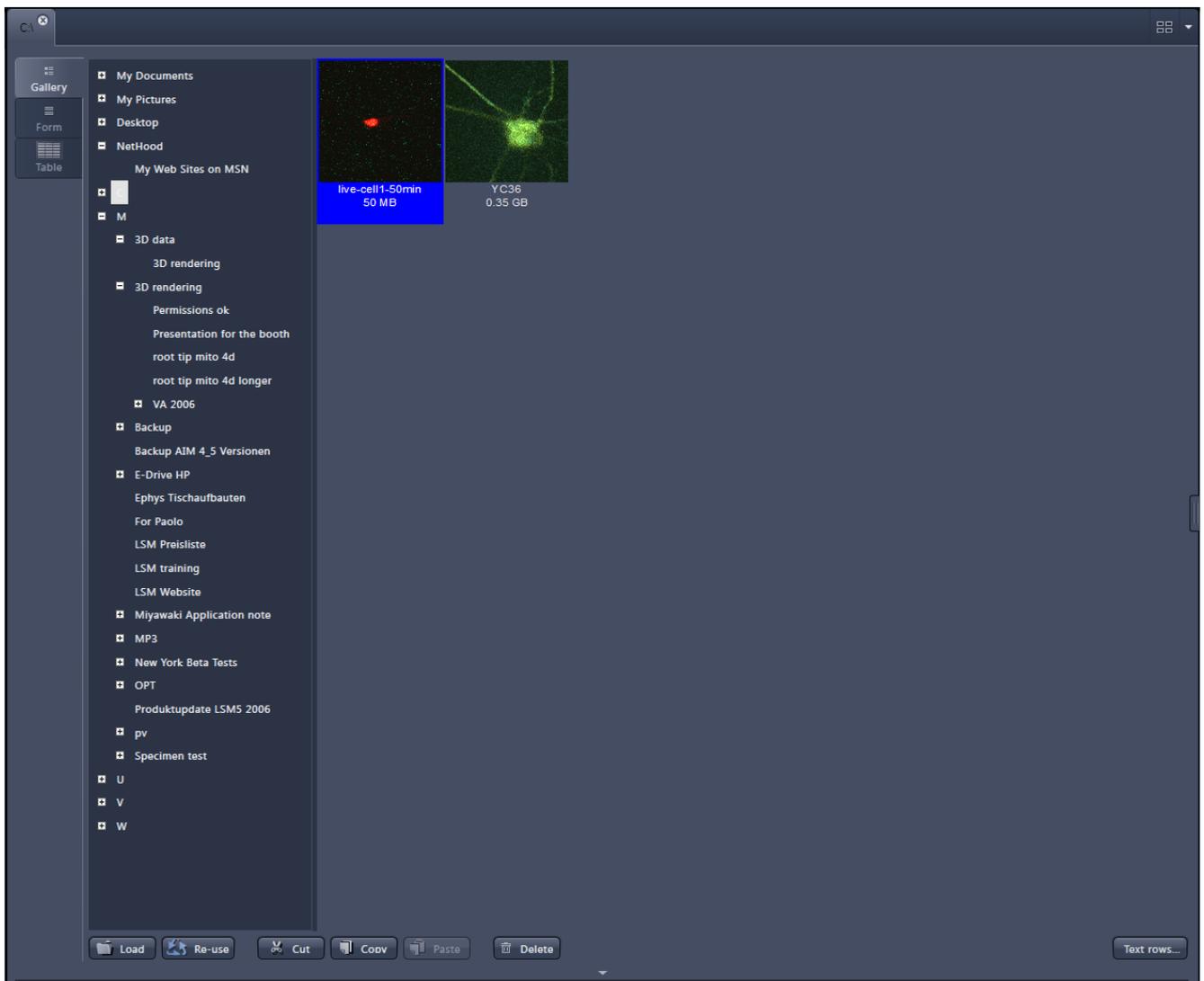


Fig. 377 ZEN File Browser

With the use of the button bars underneath, the files can be loaded, cut, copied, pasted, and deleted.

Pressing the **Reuse** button loads the hardware settings of the .czi image highlighted (blue) in the browser window. You do not have to open the image to apply the Reuse function.

- ☞ Sometimes an information message may appear (Fig. 378) in the right hand corner to inform one about missing hardware if applicable. The message box will disappear after ~ 10 seconds.

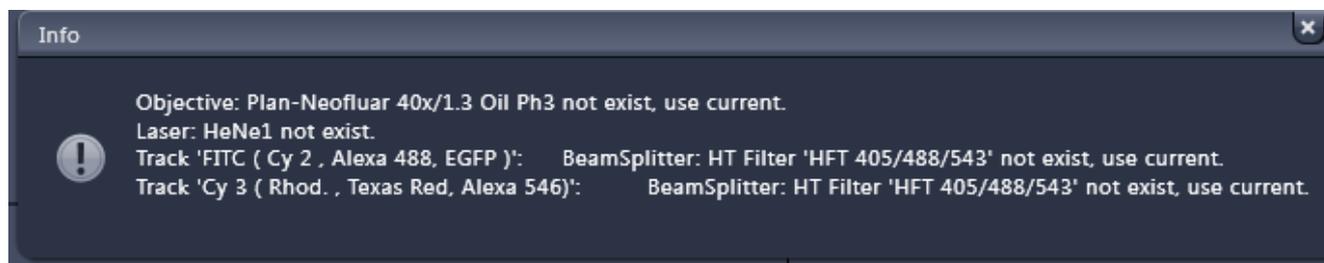


Fig. 378 Error message

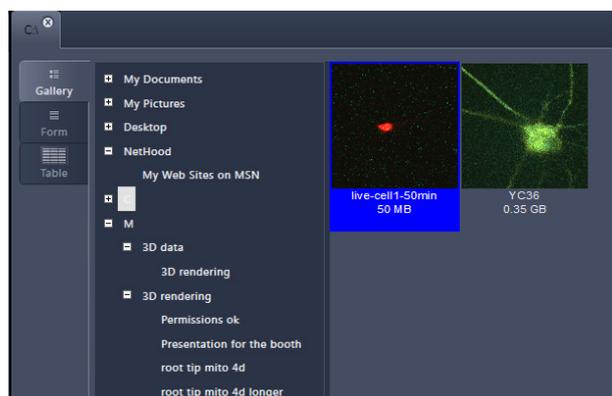


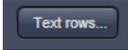
Fig. 379 Gallery view

### 5.3.1 Gallery View of the ZEN File Browser

In the **Gallery View** of the browser, the images are displayed as thumbnails. One click selects a single image, press Shift and click to select multiple images. If the CTRL key is pressed, all images are selected.

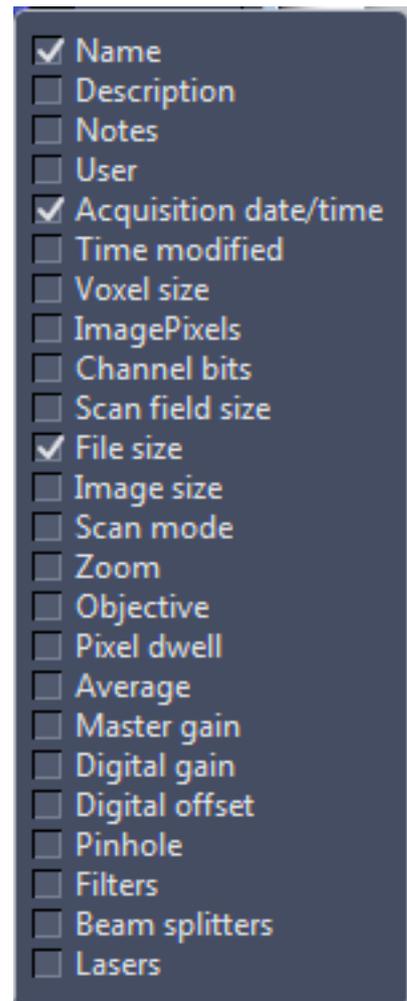
**Opening Images:**

- To open an image, double click on it.
- For opening multiple images at a time, select the desired images and press Load.

The information stored in the .dzi file format can be displayed underneath the thumbnail. By pressing the  button, a choice of information is available (see Fig. 380).

The example below (Fig. 381) shows the use of the display of:

- file name,
- acquisition date,
- file size.



**Fig. 380** Image information selection

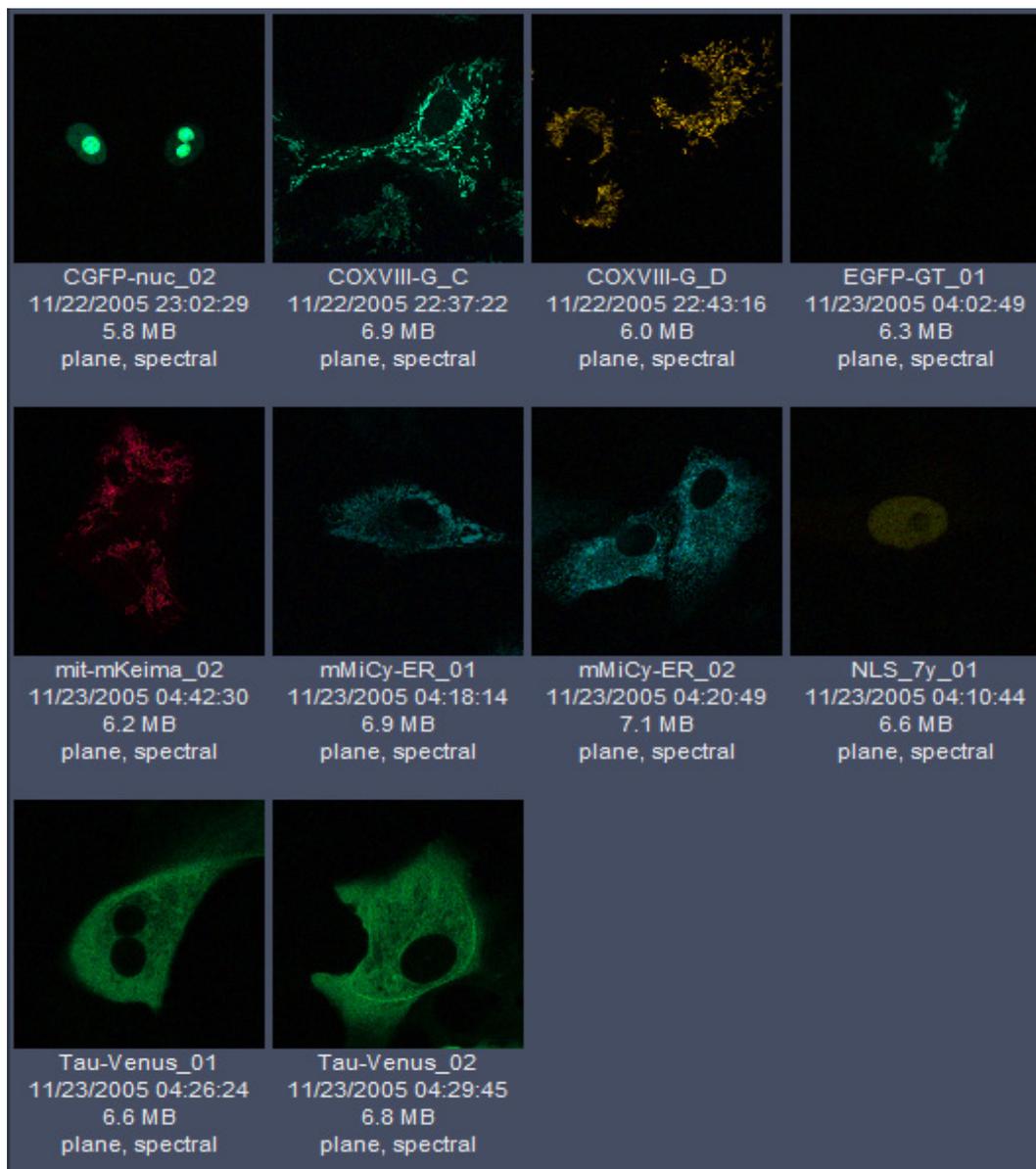


Fig. 381 Images with information

### 5.3.2 Form View of the ZEN File Browser

All images in a given folder can be accessed by using the slider below the Info-Panel or by using the stepper on the right side of the slider. The total number of images in a folder is indicated on the right



side of the slider. In the **Form View** of the browser, all information available (or entered) in the Info-tab of the image is displayed.

Note that the upper four fields (Name, Descriptions, Notes, and User) are currently not saved. To edit these fields, please load the image and edit the form in the info-view of the image. The information in those fields are – however – accessible by copy and paste.

The buttons for copying and loading images underneath are available in the Form view as well.

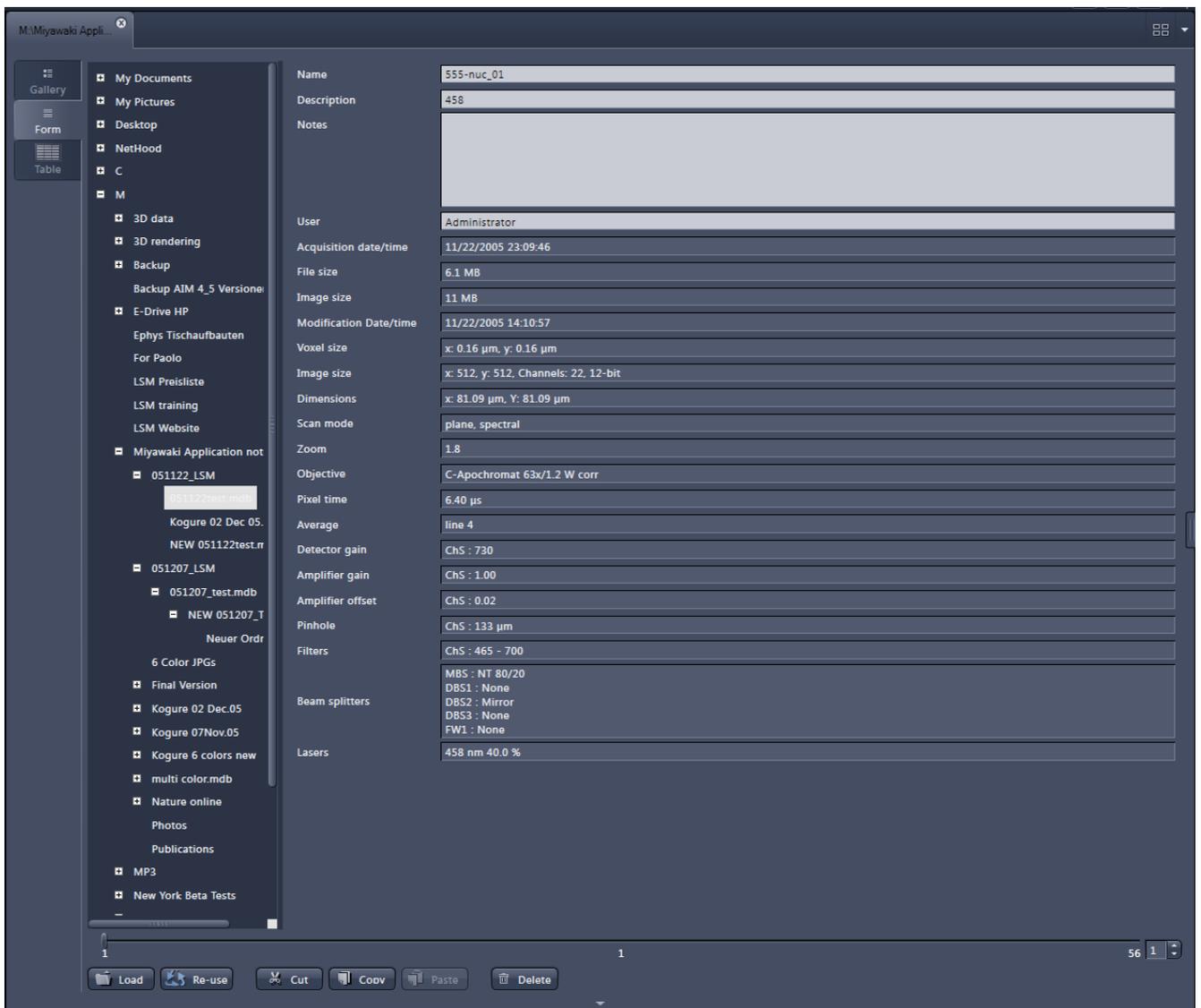


Fig. 382 Form view

### 5.3.3 Table View of the ZEN File Browser

The **Table View** is especially useful if a lot of files are present in one folder.

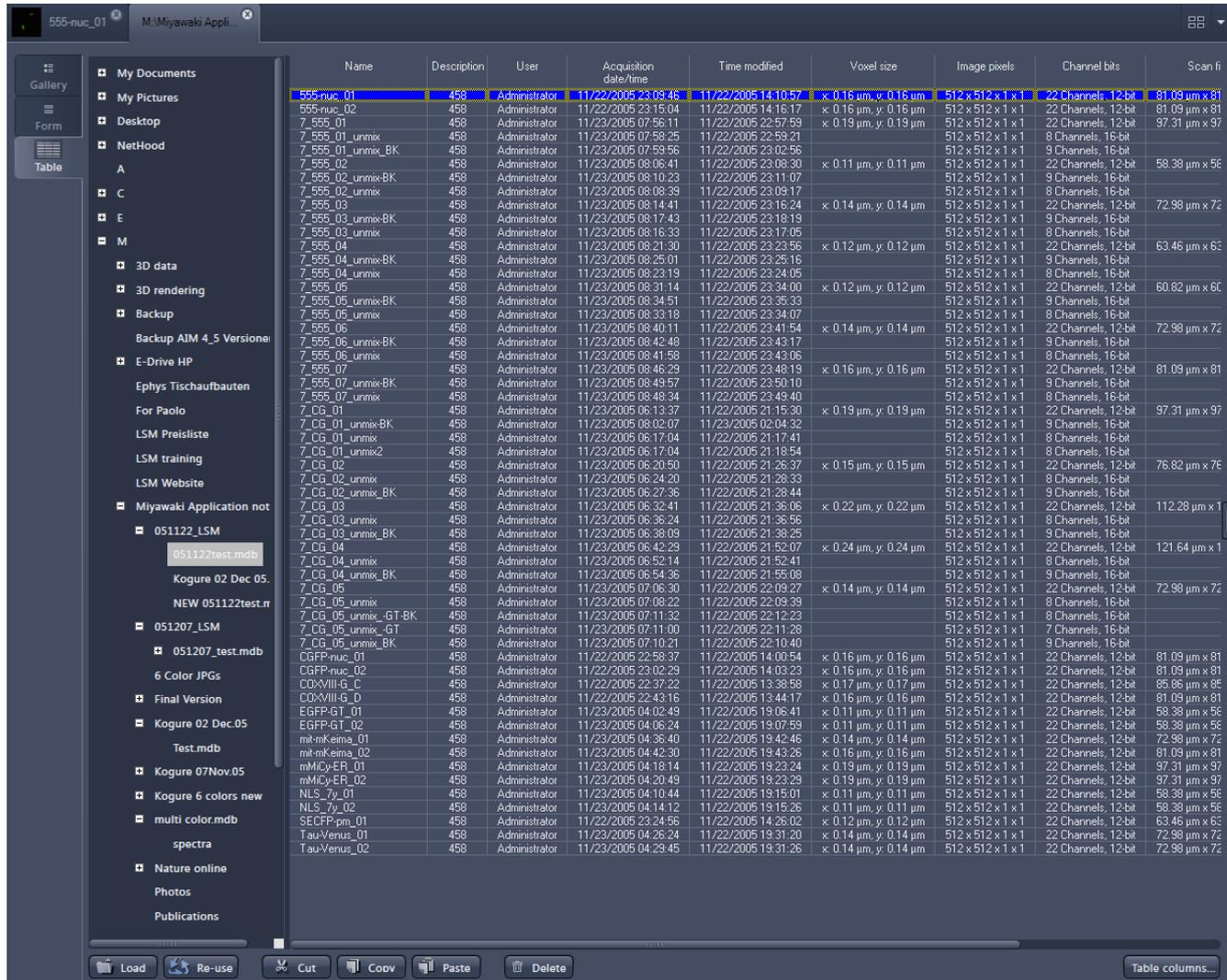


Fig. 383 Table view

The columns displayed in the table are selectable from the Table columns button. The options shown in Fig. 384 are available.

The width of the columns is editable and the order of the columns can be freely shifted by dragging the header of the columns.



Fig. 384 Table view options

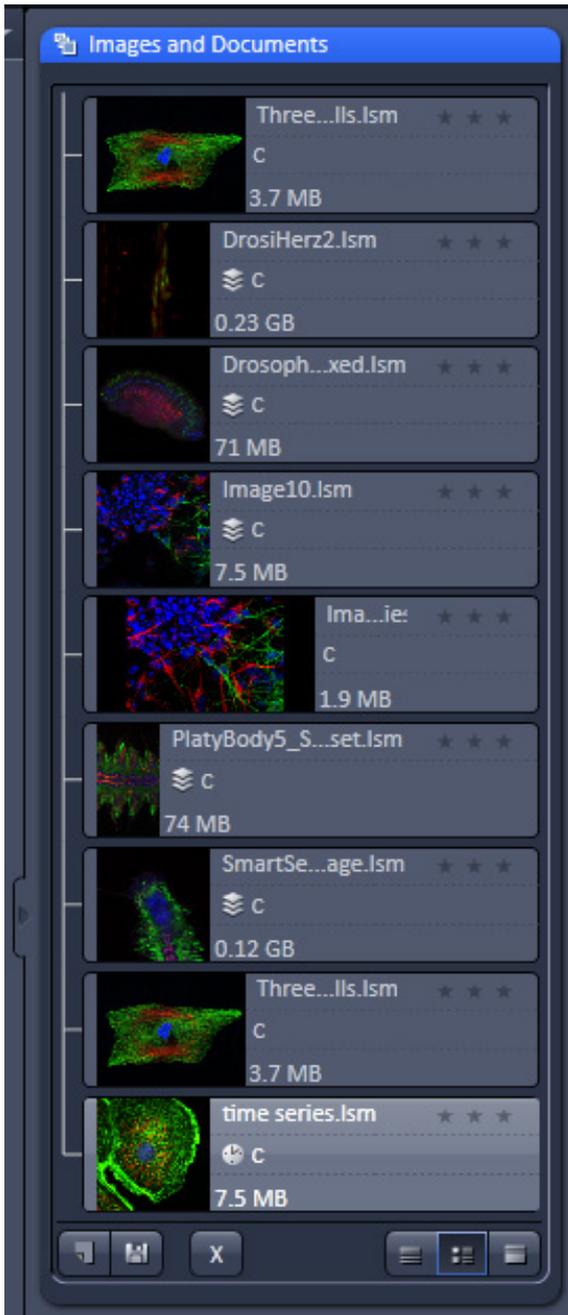


Fig. 385 Images and Documents panel

## 5.4 Images and Documents Panel

The **Images and Documents** panel (Fig. 385) is located in the Right Tool Area of ZEN. It displays all images and data which are open in ZEN. Its purpose is multi-fold:

- All opened images can be brought to the front by double clicking on them which makes the navigation through lots of images easy and fast.
- Basic file handling functions are implemented for fast operation with the acquired images. The images can be saved and closed using the following buttons: 

- A new acquisition document can be opened using the  button.

- The  button saves the selected image.

- The  button closes the selected image.

- On already saved images the  button is closing the image, not deleting it. Multiple images can be selected by holding down the CTRL key on the keyboard and clicking on the list entries.

- The whole section can be hidden by clicking on the black triangle at the left side of the panel



Once hidden, a click on the right boarder of ZEN lets the **Images and Documents** panel slide back into the application.

- All files can be displayed in three ways by using the  buttons underneath the section.

-  Textual View

-  Thumbnail View

-  Big View

The default setting is **Thumbnails view** as indicated in Fig. 386.

The context menu of the **Images and Documents** section allows the following functions:

- Selecting one of the view options **Textual View**, **Thumbnail View**, or **Big View**
- Selecting all opened image files by clicking on **Select All**
- Opening a **New Acquisition Document**
- Saving or closing the selected image files by clicking on **Save** or **Close**
- Clicking **Delete** to delete the selected image file
- Clicking **Open Containing Folder** opens the folder where the selected image file is saved.
- Clicking **Send to ZEN 2012 – blue edition** sends the currently active document to ZEN blue edition.

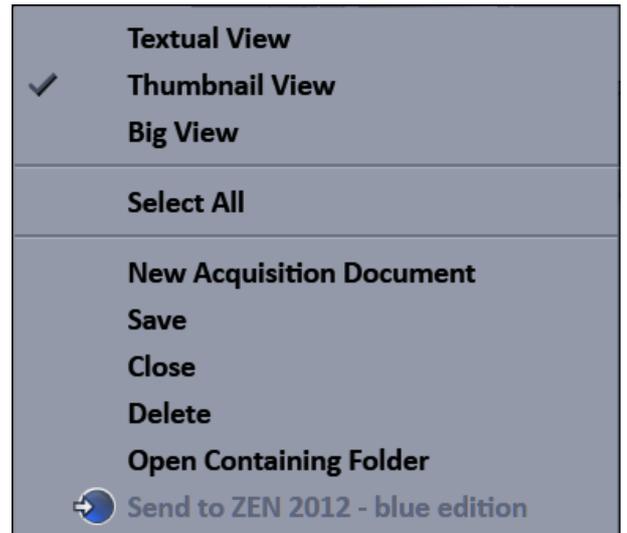


Fig. 386 Context menu of the Images and Documents section

Regardless of which display type is chosen, the individual images contain the following information:

- File name
- Data type indicated by icons:  like e.g. Stack, Time, Bleach, Lambda, or C for multichannel images. These icons are the same as underneath the start button when starting multidimensional experiments (section 3.2.2 Action Buttons).

The file size is indicated in MB, GB or TB.

- Example of a 2 Ch. Z-Stack displayed with a small thumbnail in the **Images and Documents** section (Fig. 387).



Fig. 387 2 Ch. Z-Stack image

- In case the image is not saved or has been processed, a warning symbol is displayed on the right side (Fig. 388).

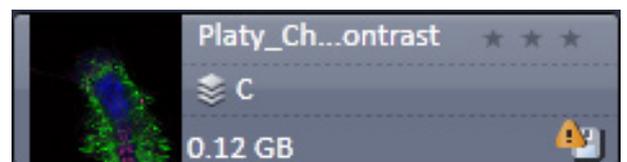


Fig. 388 Image with warning symbol

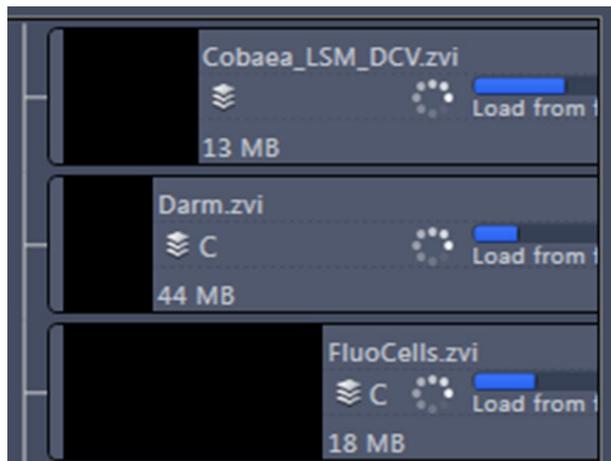


Fig. 389 Image progress bar

- A progress bar (Fig. 389) is shown for each image facilitating the overview if e.g. many processing functions are running at the same time.



Fig. 390 Enlarging the image view

- In case long filenames are used, the whole section can be dragged to the left and thereby enlarged (Fig. 390).

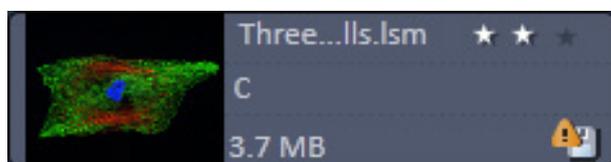


Fig. 391 Image rating star function

- The stars can be used to rate the pictures. They are activated by clicking on them.

## 5.5 Opening of Files via the "Open" Command in the File Menu

- Click on the **File** menu of the menu bar and select Open **or click on the  icon of the main toolbar.**
  - The standard windows open dialog appears.
- Select the desired file and click Open.

## 5.6 Save

The **Save** function allows one to store an image together with the acquisition parameters (and processing information) and is stored as an \*.czi file.

Proceed as follows to save an acquired or an edited / processed image:

- Click on the **Save** or **Save As** button in the **File** menu of the menu bar or **click on the  icon of the main toolbar.**
  - The **Save Image and Parameter As** window appears on the screen.

### Save:

Stores a newly created or changed image. Newly created images must be given a name

### Save As:

Stores a previously stored and called up image under a different name. If images are called up and stored again, the original data and time display will be retained.

- Clicking on either of these Menu Items opens the **Save As** window to create and open an image database.
- Click on the **OK** button in the **Save Image** window.

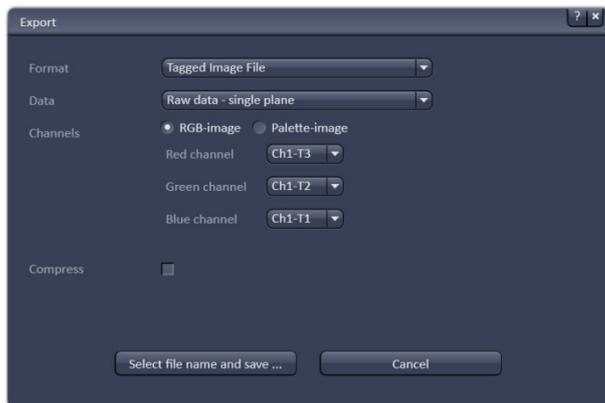


Fig. 392 Export window

## 5.7 Export of Images

The **Export** function allows the export of acquired images and images loaded

- Select the image to be exported.
- Click on the **Export** menu Item in the **File** menu of the menu bar
  - This opens the **Export** window (Fig. 392).

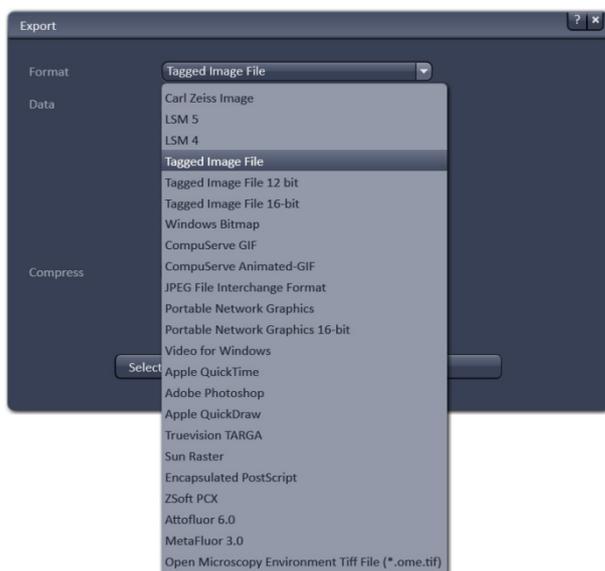


Fig. 393 Format selection

- Under **Format**, select the format to which the image is to be exported as (Fig. 393).



Fig. 394 Data type selection

- Select the data type for how the image is to be exported under **Data** (Fig. 394).
- Choose a compression level. For some file formats lossless compression or various other compression levels are available. The degree of losses for the image quality is listed according to the type of compression.
- Click on the **Select file name and save ...** button. The standard Windows File saving dialog appears. Click **Save** to complete the export process.

☞ When stacks or time series are exported, each frame is stored as an individual image.

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# Lightsheet Z.1 - Overview

