Confocal LSM 700

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Introduction

The Zeiss LSM 700 is equipped with 4 laser lines and can detect up to four color signals at frame rates approaching 5 frames per second at 512 x 512 pixels. Efficient separation of the fluorescence signals by selective laser excitation coupled to efficient splitting of the emission using the variable secondary dichroic (VSD) beamsplitter prevents crosstalk and enables spectral imaging as well as linear unmixing of highly overlapping fluorophores. Among the advanced features of the VSD beamsplitter is that all portions of the emission spectrum are utilized for determining each spectral data point.

Hardware

The microscope:

The LSM 700 is attached to an upright microscope (Axio Imager Z2).

Objectives

Position	Objective lens	Magnification	NA	coverslip	Working distance	Immersion	Parfocal length
1	W Plan Apochromat	X40	1.0	0	2.5 mm	Water dipping	45 mm
2	EC Plan Neofluar	x10	0.3	0.17 mm	5.2 mm	Air	45 mm
3	Plan Apochromat	x20	0.8	0.17 mm	0.55 mm	Air	45 mm
4	LD LCI Plan-Apochromat	x25	0.8	0-0.17	0.57 at cover glass 0.17	Water, Glycerine and Oil	45 mm
5	EC Plan-Neofluar	x40	1.3	0.17	0.21mm	Oil	45 mm
6	Plan Apochromat	x63	1.4	0.17	0.19 mm	Oil	45 mm

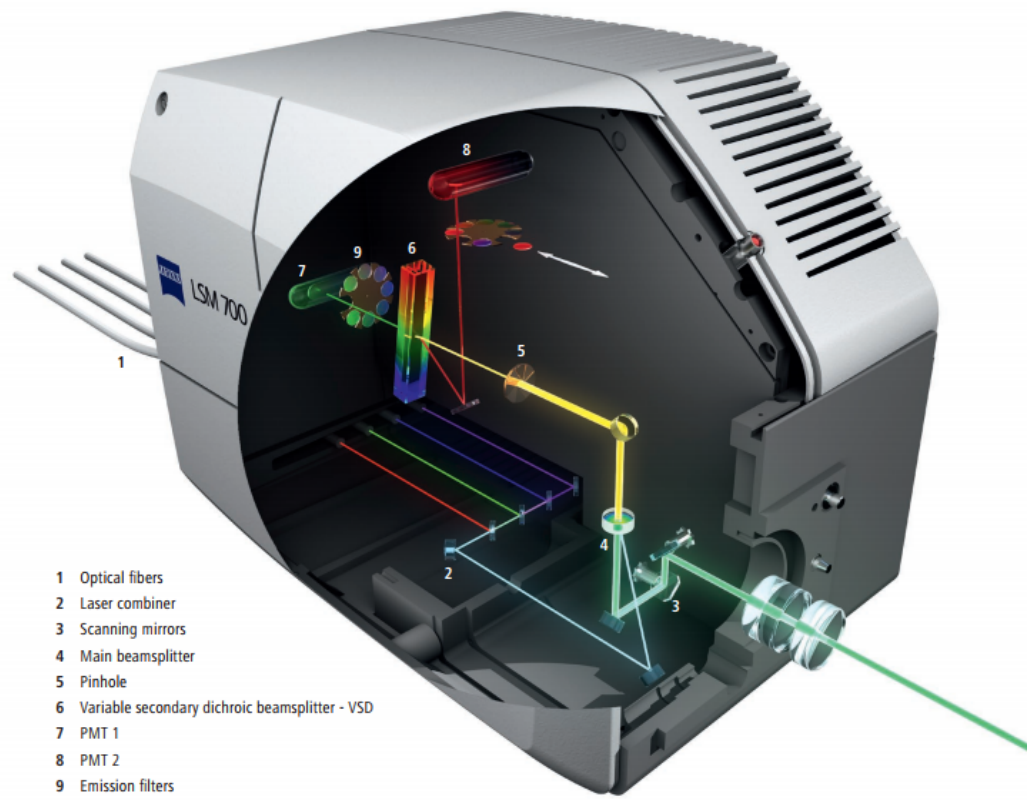
Filter turret

position	Filter set	Excitation filter	Beam splitter	Emission filter	Fluorophore family
1	FS38	BP 470/40	FT 495	BP 525/50	Cy2,GFP
2	FS43	BP 545/25	FT 570	BP 605/70	Cy3,Al555
4	Fs49	G 365	FT 395	BP 445/50	DAPI,BFP
5	DIC				

Laser lines

Laser	Line
Solid state 5mW	639
Solid state 10mW	555
Solid state 10mW	488
Solid state 5mW	405

Light Path



1. The lasers come aligned from the manufacturer, all 4 lasers are attached to an optic cable and enter the confocal scan head. (**1**)
2. The laser lines are reflected by the main dichroic mirror (beamsplitter). (**4**).
3. The 2 Galvanometric mirrors scanthe sample. (**3**).
4. The return emission wavelength passes through the main dichroic mirror (**4**).
5. The emission passes through the pinhole aperture and the out of focus light is eliminated.(**5**)
6. The variable secondary dichroic beamsplitter (VSD) is coated with a variable coating that between 420nm - 630nm, according to the position of light incidence determines the cutoff below which wavelength the light will pass to PMT1 and above this wavelength it will be reflected towards PMT2.(**6**)
7. Before the detection an emission filter can be inserted from the filter wheel before the PMT, only longpass (LP)/shortpass (SP) filters are available. (**9**)
8. The light is detected in one of the two PMT detectors- PMT 1 for SP emission wavelength, PMT 1 for LP emission wavelength.(**7,8**)

Start up

Turn on the system in the following order:

- In the power strips turn on switches 1 → 2.

- Turn on the stage controller (3)

- Rotate (clockwise) the silver key in the lasers electronic unit(**4**)

- Turn on the computer and log in

  User name: **multilabs**

  Password: **123456**

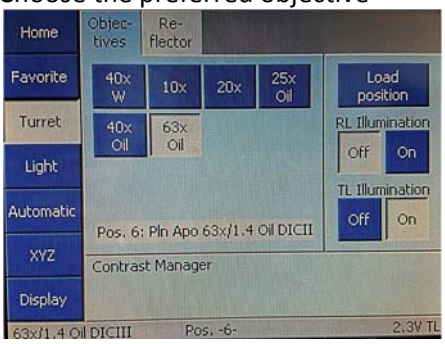
- Turn on the metalhalide lamp (**6**)

Sample mounting and viewing

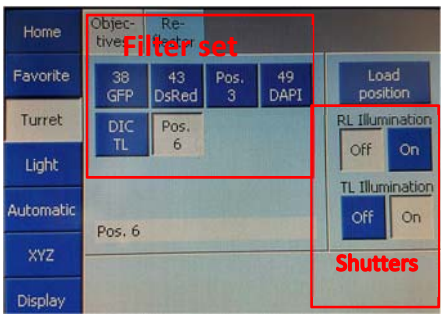
Insert the slide or dish to the stage holder.

Push in the Light switch between the ocular and the confocal.

- Via the touch pad:
    1. Choose the preferred objective



    2. Choose the proper filter set and open the TL\RL shutter:

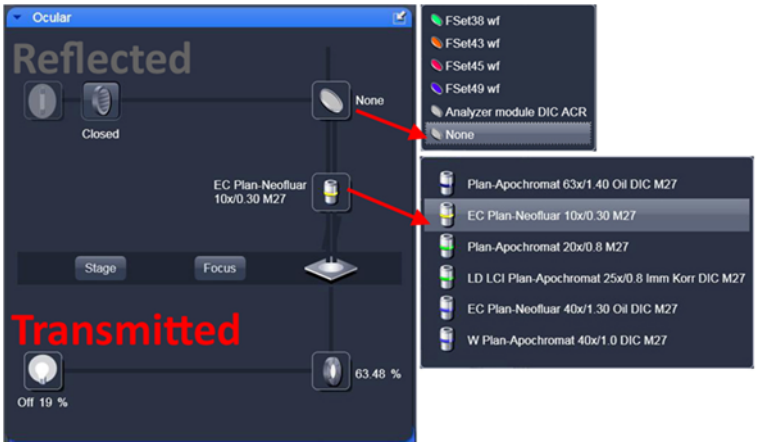


Software

- Log into **BookitLab** and activate your reservation to start **Black Zen 2.3**
- To acquire images click **Start System**

Sample viewing

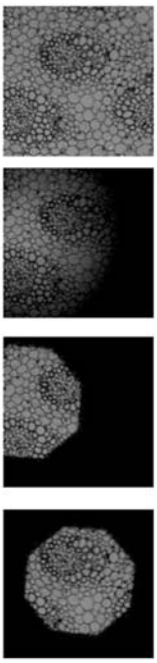
In the **Locate** tab choose the preferred objective filter set and open the illumination shutter RL\TL

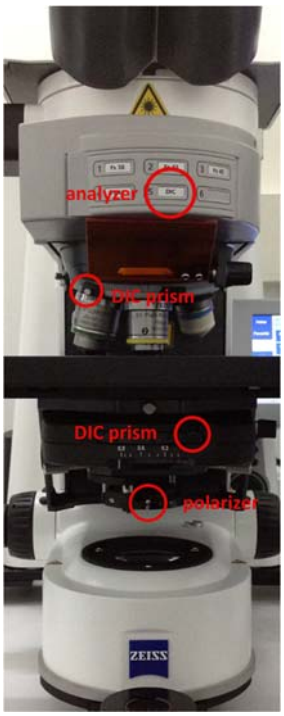


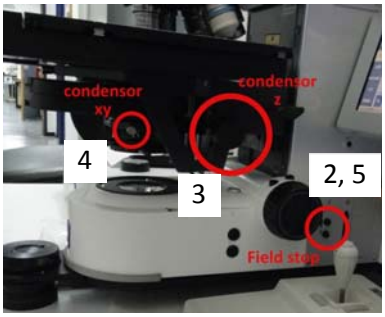
- Set the Transmitted light (In case the TL is needed)
  Click the transmitted light lamp icon, click ON and set desired brightness (or via the microscope).
  Set the filter turret position to DIC and the condenser below the stage to I, II or III according to the objective lens (see objective lens info on touchpad).

  If you are acquiring transmitted channel set up Kohler illumination:

1. Bring the sample into focus.
2. Close the field stop using the motorized aperture button (F) located at the right of the microscope until you can see at least one edge
3. Adjust the condenser height until the edges of the diaphragm image are crisp.
4. Center the diaphragm image using the two centering screws.
5. Open the field diaphragm, just until the image fills the field of view.

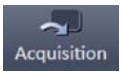



analyzer
DIC prism
DIC prism
polarizer
ZEISS


condensor
xy
condensor
z
4
3
2, 5
Field stop

Acquisition

LSM Channel mode confocal setting

- In the software go to the Acquisition main tool tab 

- Pull out the Light switch between the ocular and the confocal.
- 

Configuration:

It is highly recommended to seek BCF staff's advice for new fluorophores (exact name) or new combinations.

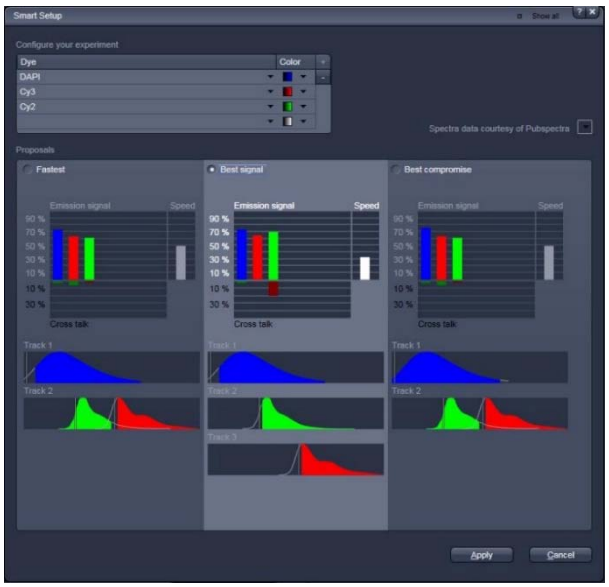
Choose one:

- Load a saved configuration from the **Experiment Manager**

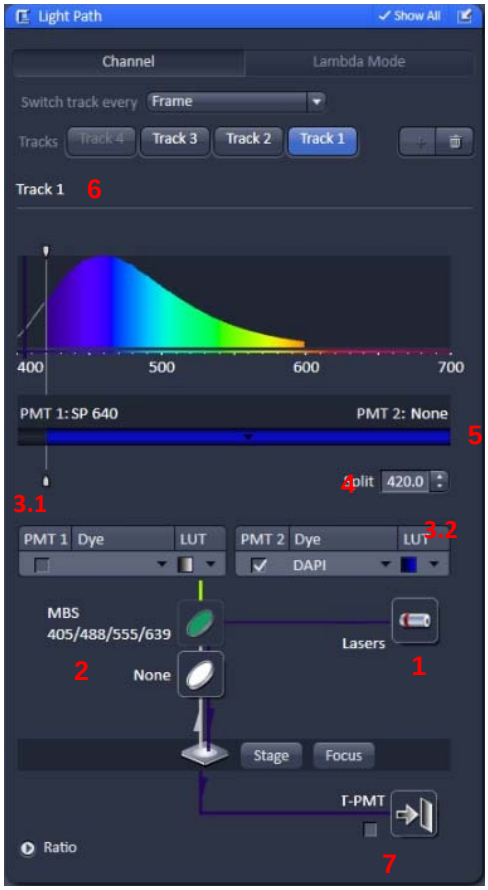
- Reuse settings of an image: open an image and below the image click **Reuse** 

- New setup: click 

, choose the desired dyes from the list, choose the scanning method and click **Apply**



- Select **show all tool** Show all Tools and open the **Imaging set up** tab
  Note: make sure that the chosen configuration is correct and suits the fluorophores; make changes if needed it is highly recommended to check all dyes spectrums in a spectra viewer
  ThermoFisher
  Chroma



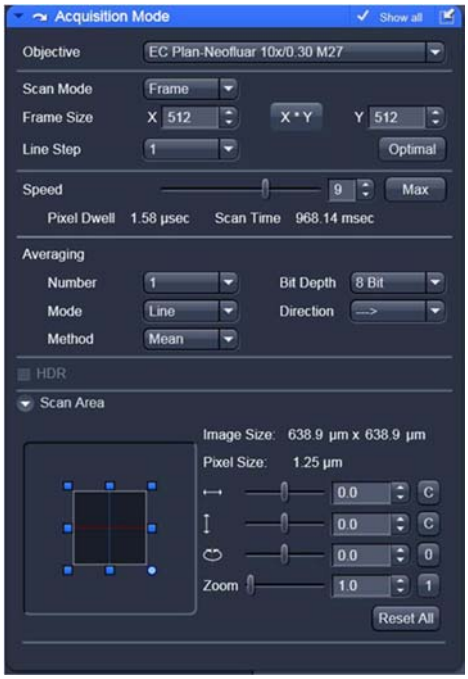
1. Laser lines
2. Main dichroic mirror
3. PMTs: PMT1 and PMT2
4. Variable secondary dichroic beamsplitter (VSD).
5. Emission Filter selection
6. Emission spectrum with Graphic representation of the laser line, the position of the VSD and emission filter.
7. Transmitted light PMT- a transmitted light image (non confocal) can be added to the confocal image (it recommended to add to the longest wavelength track).

Acquisition & parameter setting

Pull out the lightpath switch between the ocular and the confocal

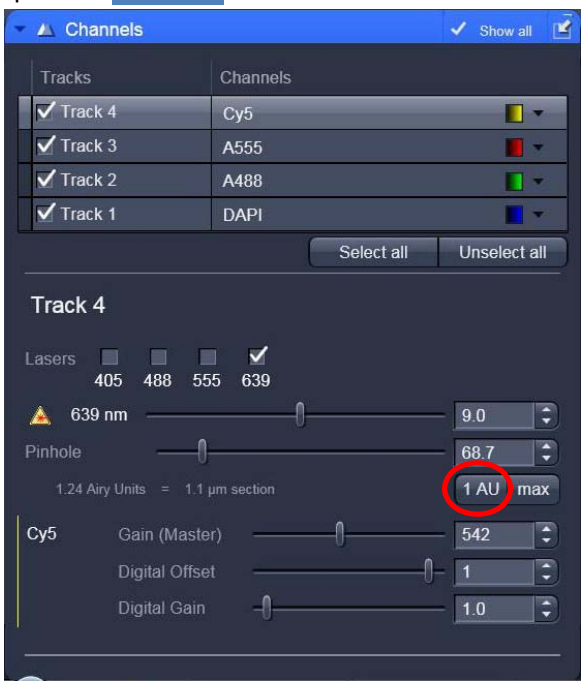
Parameter setting

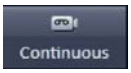
- Open the **Acquisition mode** tab



- **Scan mode**: frame (in most cases);
  line scan mode can be useful for physiology experiments.
- **Frame size**: 512X512
- **Speed**: 8
- **Averaging**:
  - Method: mean (in most cases):
    in low signal with low noise Sum method may be useful
  - Number: 1
  - Mode: frame for fixative sample, for live cells and in case of unstable dye line mode will be more suitable
  - Bit Depth: defined the dynamic range of the image, choose 8bit\12 bit according to the biological question.
  - Direction: ----> (more precise)
- **Scan area:** slight nudge, zoom and rotation

- Open the **Channels** tab



- Check and select only the shortest wavelength track and click the **1AU** button
- Start scan in continuous mode 

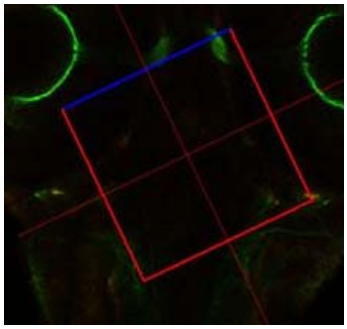
- Check the Range indicator 

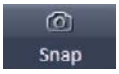
(below the image container in the Dimensions tab).
- Set your image parameters to achieve image without saturation (saturation=red pixels in the image) and the correct black level (black= blue pixels in the image)
    - Set the PMT gain to 550-600.
    - Set the Laser % - it is recommended to use the lowest % as possible and to increase gain (master).
    - In case of low signal with high laser % and high gain you can add digital gain (up to 2).
    - Set the offset level until you reach the PMT threshold (blue pixel will appear in the image while scanning in range indicator mode).
- Stop the scan 

- Select another track; set its pinhole to the same **µm section** as the first track and set its parameters and so on
- Stop the scan 

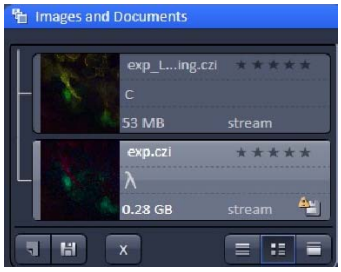
Single image acquisition

- In the **Acquisition Mode** tab select the preferred zoom (alternatively you can easily position the cropping frame to zoom in via the **crop tool** Crop below the image in the Dimensions tab)



- Scan one frame to check position.
- Check all tracks in the **Channel** tab, In the **Acquisition Mode** tab click Optimal to set the frame size.
- Set scanning speed to 6
- Set averaging number to 2-4 (according to the SNR)
- Press Snap 

in the start buttons
- Save the image: press  in the **Images and Documents** tab (right hand side of the window) and save in the directory:
D:\Users data\PI name\MM-YYYY\User name\YYYY-MM-DD



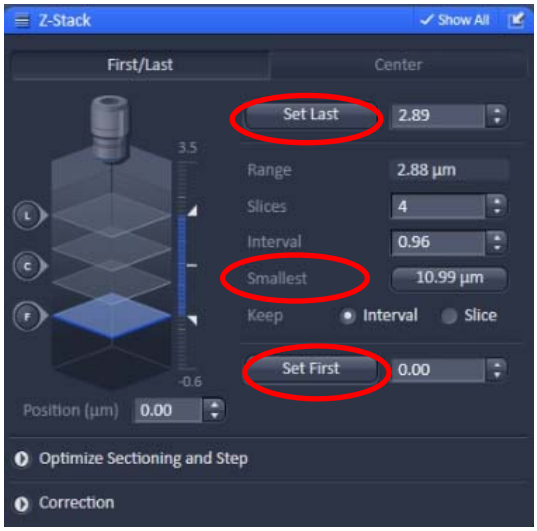
Multidimensional acquisition

Z stack

- In the Multidimensional Acquisition options below the start buttons check **Z stack**; a new tab (**Z-stack**) will appear under the **Multidimensional Acquisition Section**.
- Set the interval: press **Smallest** for the system recommendation (Nyquist criterion)
- With one channel checked and In the **Acquisition mode** tab:

Frame size	512X512
Scanning speed	8
Averaging number	1

- Start scan in continuous mode
- Define the signal boundaries by moving the focus wheels and pressing **Set First** in one boundary and **Set Last** in the second boundary.



- With all channels checked, in the **Acquisition mode** tab:

  Frame size Press optimal
  Scanning speed 7
  Averaging number 2-4

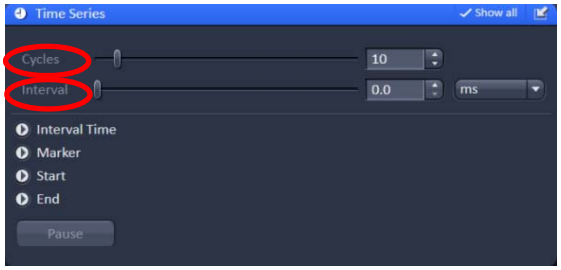
- Press ▶ Start Experiment
- Save the Image

  Note: there are more advanced Z stack options in this tab such as brightness correction over z.

Time series

- In the Multidimensional Acquisition options check **Time series**; a new tab (**Time Series**) will appear under the **Multidimensional Acquisition Section**.
- Define number of cycles and the interval

  Note: in case of live cell imaging avoid high laser intensity



- In the **Acquisition Mode** tab:

  Frame size Press optimal

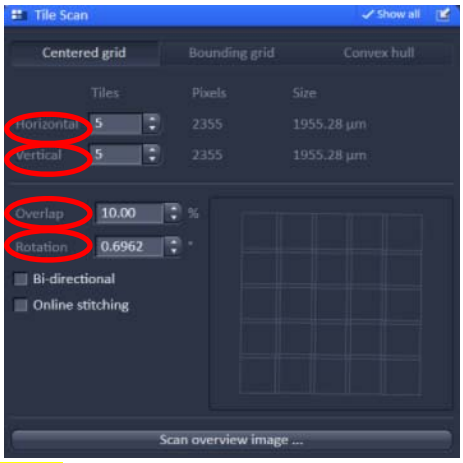
  In case of big frame size with long frame scan time it recommended to increase the zoom and set a new frame size with less pixels by pressing optimal again.

  Scanning speed: 7-8
  Averaging number 1-2

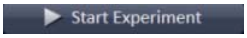
- Press ▶ Start Experiment
- Save the Image

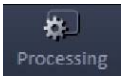
Tile

- In the Multidimensional Acquisition options check **Tile scan**; a new tab (Tile Scan) will appear under the **Multidimensional Acquisition Section**.
- Define the number of tiles
- Define the overlap % - recommended is 8%-15%
- Calculate slide rotation: In the main menu choose **Macro → Tile scan rotation**
  In the popup window press **Calibrate** and wait until the process is finished (the rotation angle will be exported to the tile scan tab) then press **close.**



Note: Online stitching is not a recommended option, the process can be configured in the processing main tool tab.

- In the Acquisition mode tab:
  Frame size: Press optimal
  Zoom: It is recommended to set the zoom above 1.2
  Scanning speed: 7
  Averaging number: 2
- Press 

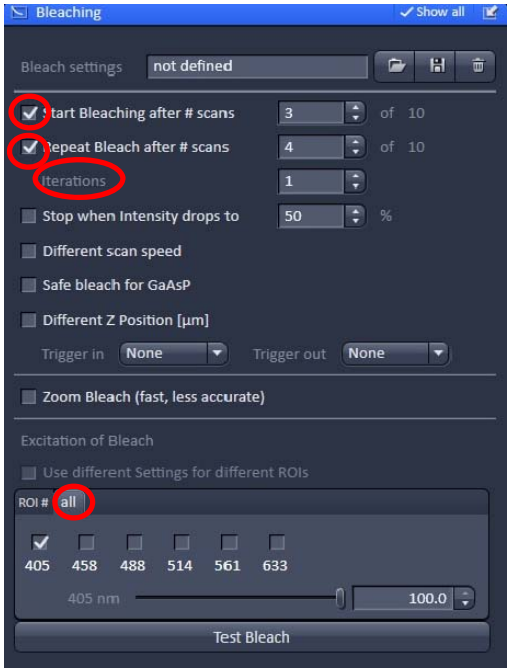
- Save the Image
- **Stitching**: In the Processing main tool tab 

→ Stitch → select your input image → press Apply.
  In case of uneven illumination the stitching process can be done with more options in the **ZEN blue** software.

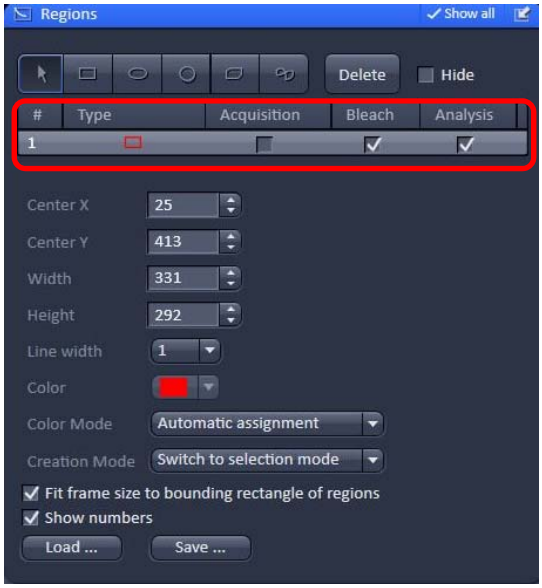
Regions and Bleaching

- In the Multidimensional Acquisition options check **Bleaching**; the Bleaching, Time series and Regions tabs will appear in the **Multidimensional Acquisition Section**.
- In the Bleaching tab define:
  Start bleaching after number of scans

Repeat bleach after number of scans
The amount of iterations
The laser and its intensity
More setting can be defined in the bleaching dialog.

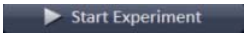


- Define one or more ROIs:
  After a single scan in the **Regions** tab choose one of the ROI tools and draw on the acquired image; the dimensions can also be set manually in the Width/Height textboxes.
  Define which process will be performed in the ROI – Acquisition/Bleach/Analysis.



- Define Time series:
- In the Acquisition mode tab:

Frame size	Press optimal
Scanning speed	7 or more
Averaging number	1-2

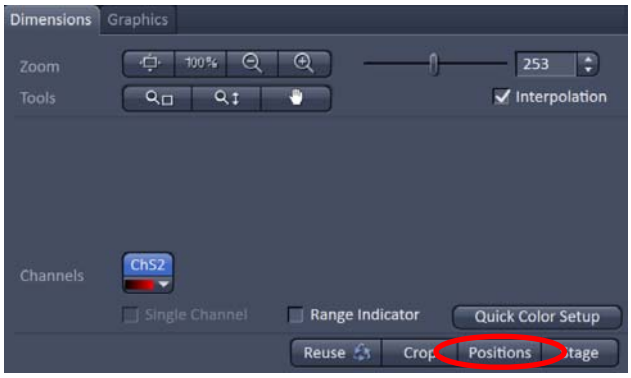
- Press 

- Save the image
- The intensity data from the **ROI** can be viewed in the **ROI mean** tab in the image window

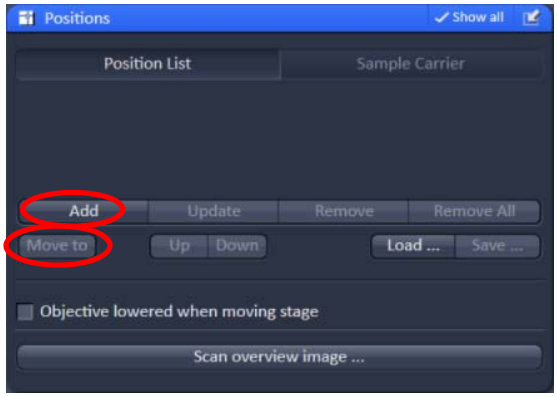
Note: The region can be defined in other types of experiments with or without time lapse and can also be used as a tool of zooming in by checking Fit Frame size to bounding rectangle of regions.

Positions

- A positions list can be added to a multidimensional experiment in which all of the settings will be acquired (channel, acquisition mode, Z stack, tile, time series).
- The **Positions** tab can be a useful tool in managing a large tile and for quick slide scanning (via the ocular) with low magnification before moving to high magnification with small field of view.



- Add a position by pressing **Positions** below the image container and clicking on the image container that was just acquired at the current stage position
- Check **Positions** at the Multidimensional Acquisition options.
- At the Positions tab move to a position in the list by selecting and pressing **Move to**
- A position can also be added by pressing **Add** in the Positions tab



Note: If you use the positions list only to locate saved positions uncheck Positions from the Multidimensional Acquisition options before pressing Start Experiment, otherwise your settings will be acquired at every position in the list.

Note you can use multiple options in the multidimensional scanning for example Z stack & tile Time series, bleaching, ROI & Z stack and positions.

Shutting down

- Make sure all the data is saved. The data will be automatically saved to the server during the night. **PLEASE DO NOT USE ANY USB FLASH DRIVE on this computer.**
- Clean any oil immersion objectives you used with lens paper and petroleum ether, twice.
- Close the ZEN software
- Log off from your account in the BookItlab window
- Check if another user has a reservation; if yes verify his/her arrival. If you are the last user for the day or the next reservation is in two hours or later, continue with system shutdown.
- Turn off the metalhalide lamp (**6**)
- Turn off the computer (**5**) **only on weekends**
- Turn off the laser electronic unit (**4**)
- Turn off the stage controller (**3**)
- Turn off switches 2 → 1 In the switch box
- Cover the microscope