

Confocal LSM 700

Table of contents

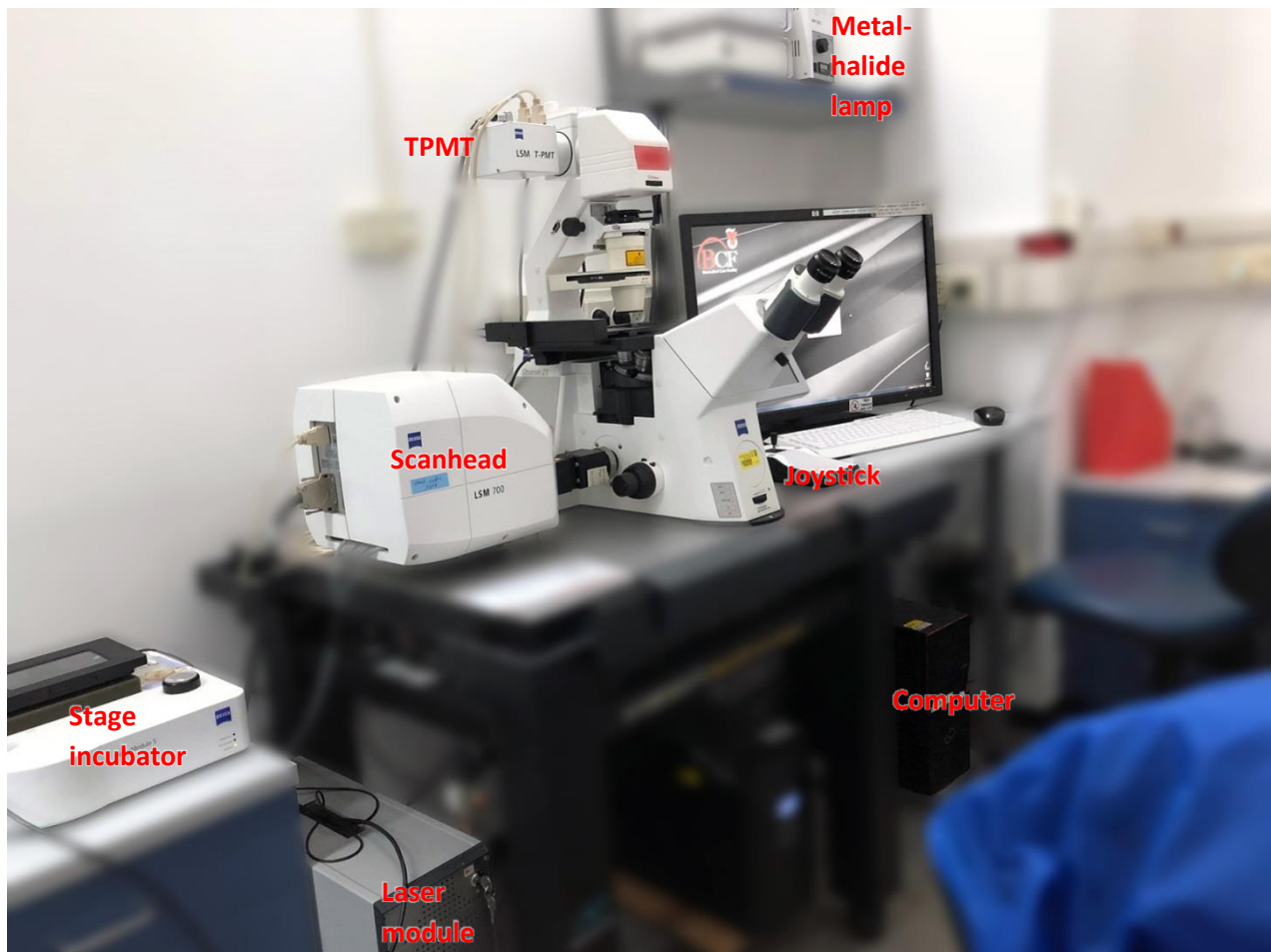
Introduction	2
Hardware	2
Objectives.....	3
Filter turret.....	3
Laser lines.....	3
Scanhead.....	4
Start up.....	5
Sample mounting and initial viewing.....	6
Software	6
Sample viewing	7
Differential Interference Contrast (DIC).....	8
Kohler illumination	9
Acquisition.....	10
LSM Channel mode confocal setting	10
Configuration:	10
Acquisition & parameter setting.....	12
Zoom	14
Single image acquisition	14
Multidimensional acquisition.....	15
Shutting down	19

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 system:



The LSM 700 is attached to an inverted motorized microscope, Axio Observer.

Objectives

Position	Objective lens	Magnification	NA	coverslip	Working distance	Immersion
1	EC Plan Neofluar	x10	0.3	0.17 mm	5.2 mm	Air
2	Plan Apochromat	x20	0.8	0.17 mm	0.55 mm	Air
3	LD LCI Plan Apochromat	x25	0.8	0-0.17 mm	0.57 mm	Oil Glycerin Water
4	LCI Plan Neofluar (optional)	x25	0.8	0-0.17 mm	0.21 mm	Oil Glycerin Silicone Oil Water
5	EC Plan Neofluar	x40	1.3	0.17 mm	0.21 mm	Oil
6	Plan Apochromat	x63	1.4	0.17 mm	0.19 mm	Oil

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,AI555
4	Fs49	G 365	FT 395	BP 445/50	DAPI,BFP
5	DIC				

Laser lines

In the electronic unit there are four solid state laser lines, entering the scanhead by an optical fiber; there is no need to turn on and off in the software.

Laser	Line
Solid state 5mW	633
Solid state 10mW	555
Solid state 10mW	488
Solid state 5mW	405

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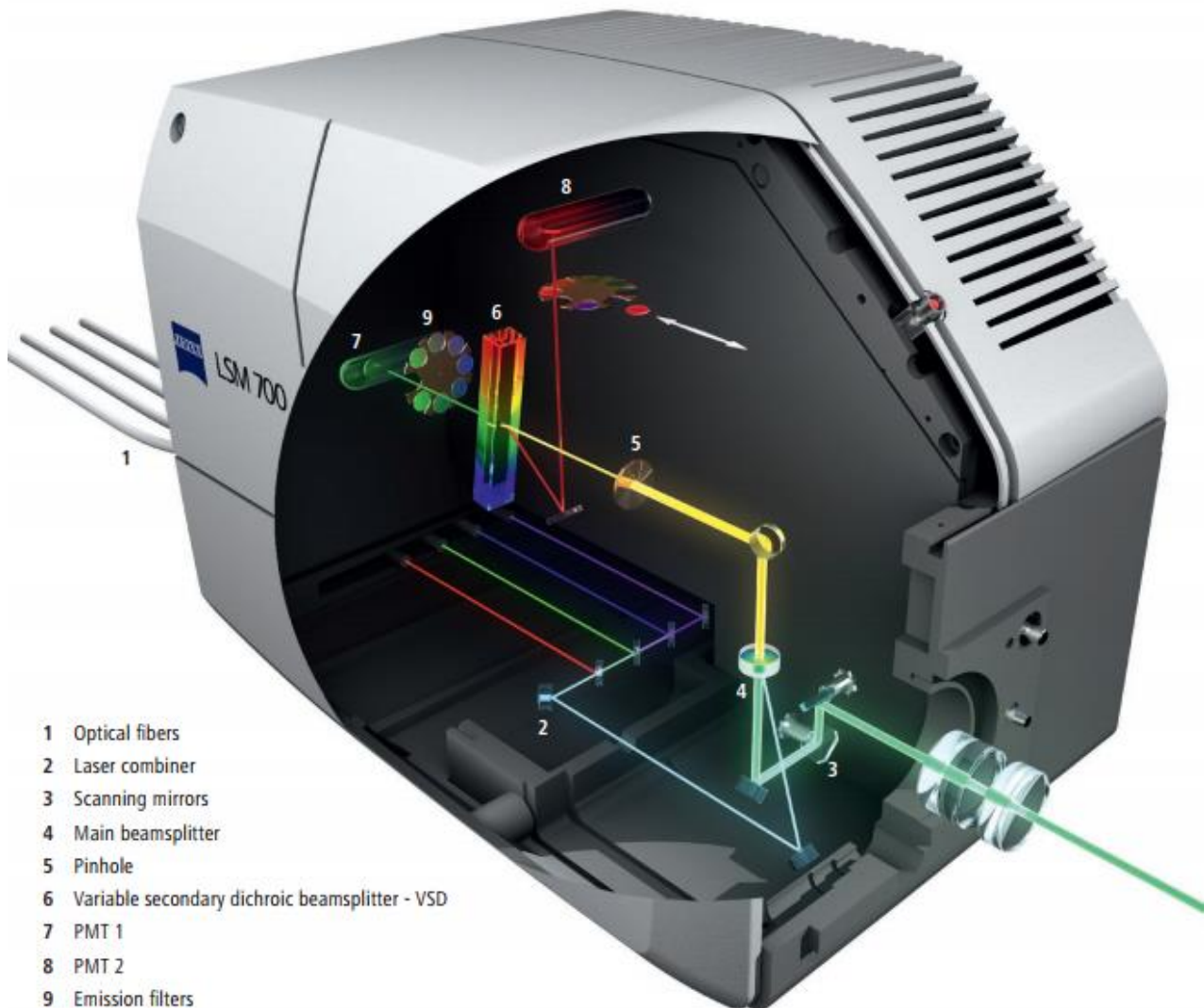
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Scanhead

The lasers come aligned from the manufacturer, all 4 lasers are attached to an optic cable and enter the confocal scan head (1)

1. The laser lines are reflected by the main dichroic mirror (beamsplitter) (4)
2. The 2 Galvanometric mirrors scan the sample (3)
3. The return emission wavelength passes through the main dichroic mirror (4)
4. The emission passes through the pinhole aperture and the out of focus light is eliminated (5)
5. 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 which it will be reflected towards PMT2 (6)
6. 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)
7. The light is detected by one of the two PMT detectors - PMT 1 for shortpass emission wavelength, PMT 1 for longpass emission wavelength (7,8)



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Start up

Turn on the system in the following order:

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



- Turn on the microscope (On\Off button on the microscope left side) (3)



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



- Turn on the computer and log in (5):

User name: **multilabs**

Password: **123456**

- Turn on the metalhalide lamp (6)



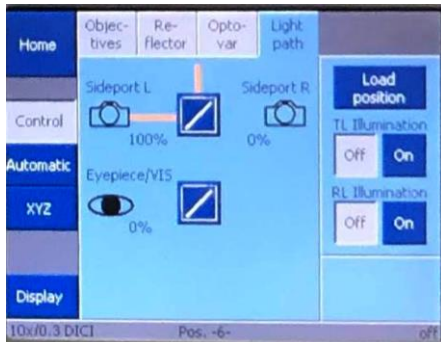
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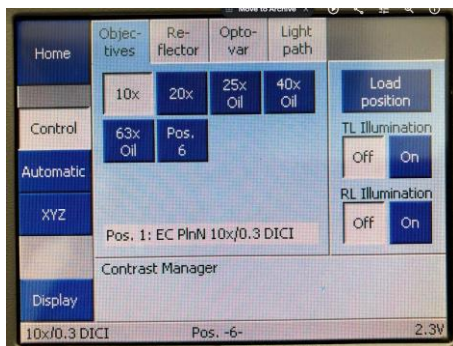
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Sample mounting and initial viewing

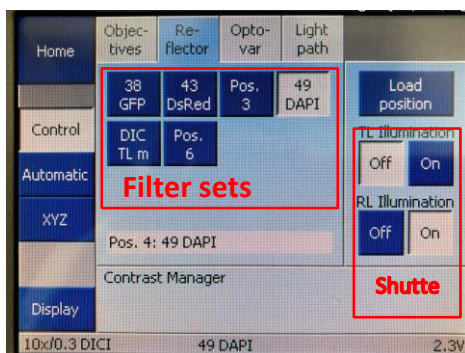
In the Touch Pad **Microscope** → **Control** → **Light path** → **Eyepiece/VIS**



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

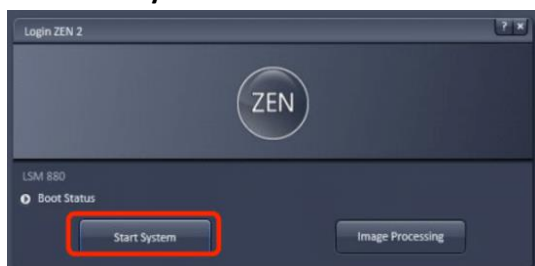


2. Choose the proper filter set and open the TL\RL shutter:



Software

- Log into **BookitLab** and activate your reservation to start **Black Zen 2.3**
- Click **Start System**



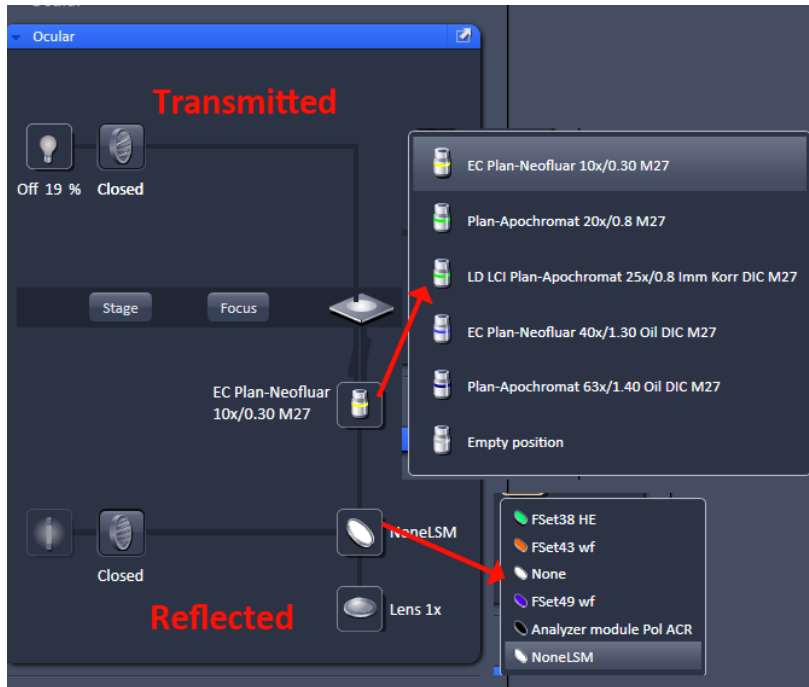
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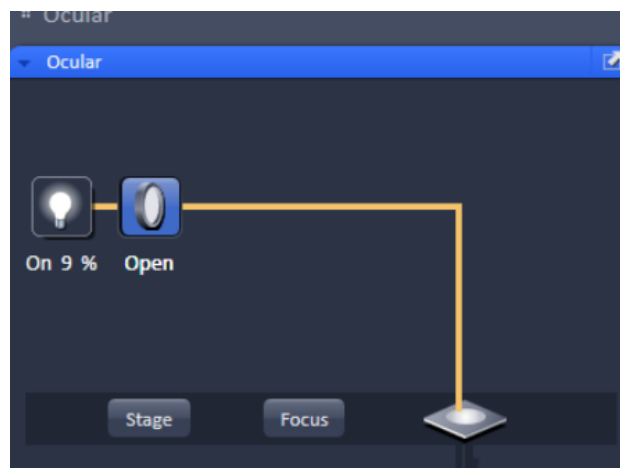
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Sample viewing

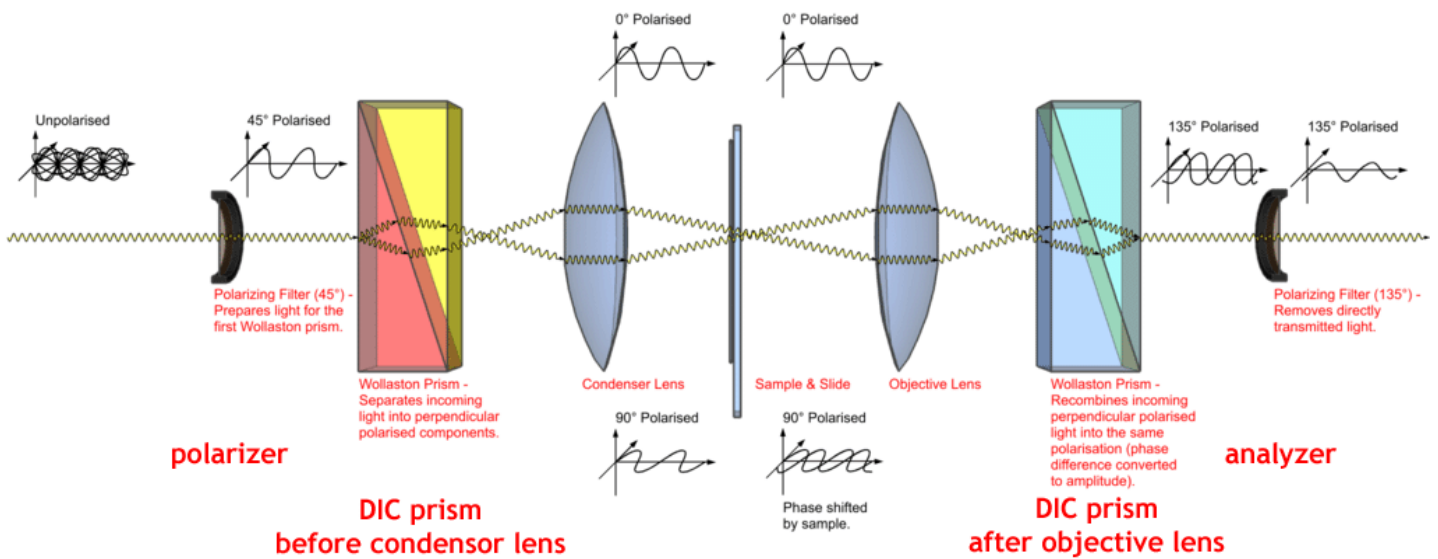
Go to the **Locate** tab that sets the light path to the eyepiece, choose the preferred objective and filter set and open the appropriate illumination shutter reflected or transmitted



- If DIC is desired, set the Transmitted light:
Click the transmitted light shutter icon, click ON and set desired brightness (or via the microscope base at the front).
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).



Differential Interference Contrast (DIC)



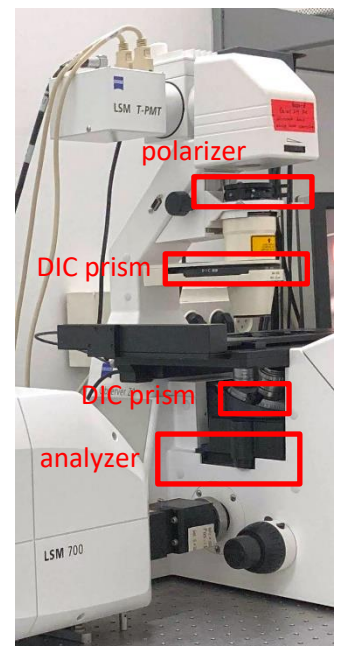
To establish DIC contrast, four optical components need to be aligned, two before the sample and two after the sample:

- Polarizer
- DIC prism at the condenser according to the objective's NA
- DIC prism mounted at the base of the DIC able objective
- Analyzer at the reflector turret

Sample vessel/coverslip should be made of glass or special low-birefringence plastic.

Condenser setting according to objective NA:

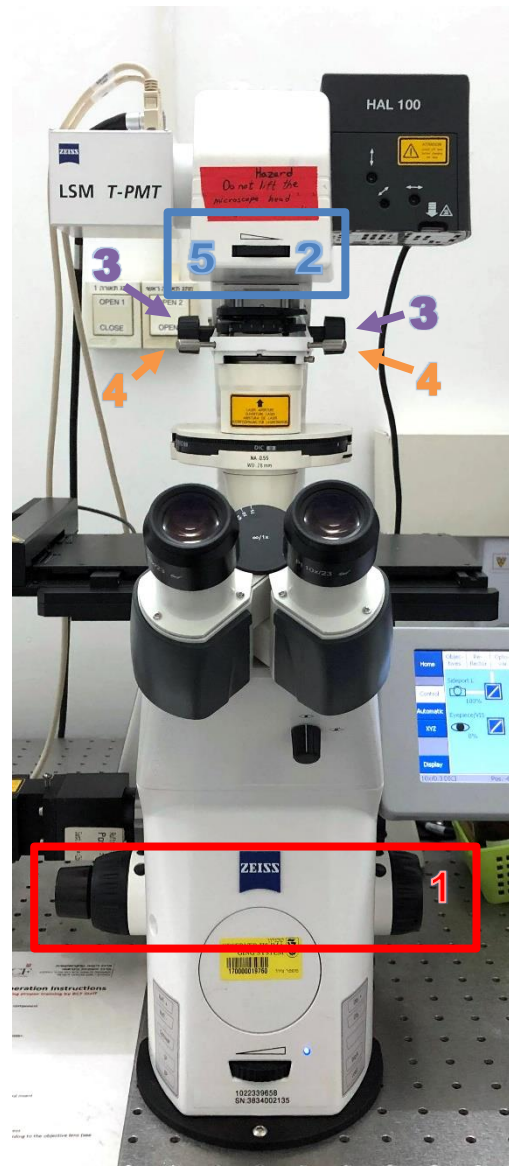
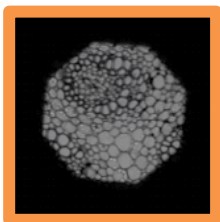
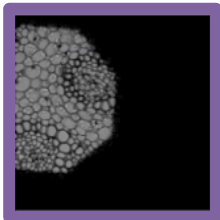
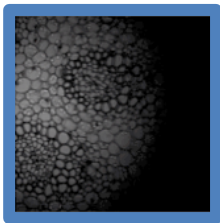
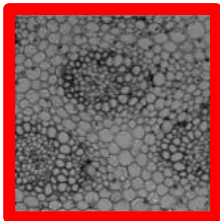
Magnification	NA	Condenser DIC prism
X20/X25	0.8	DIC II
X40/X63	1.2/1.3	DIC III



Kohler illumination

If you acquire transmitted light you will have to set up Kohler illumination:

1. Bring the sample into focus.
2. Close the field diaphragm 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 both centering screws.
5. Open the field diaphragm, just until the image fills the field of view.



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Acquisition

LSM Channel mode confocal setting


- In the software go to the Acquisition main tool tab




Configuration:

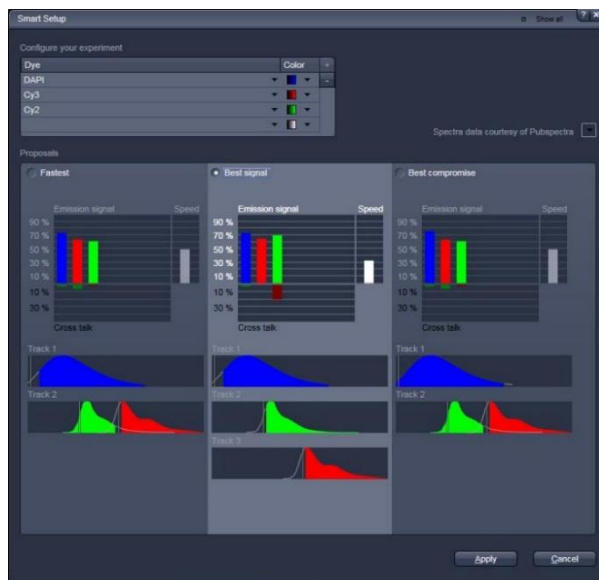
It is highly recommended to seek advice from BCF staff for new fluorophores (please provide exact name) or new combinations.

To start working, choose one (in order of preference):

- Reuse settings of an image: open an image and below the image click **Reuse** 
- Load a saved configuration from the Experiment Manager



- New setup: click  **Smart Setup**, choose the desired dyes from the list, choose the scanning method and click **Apply**

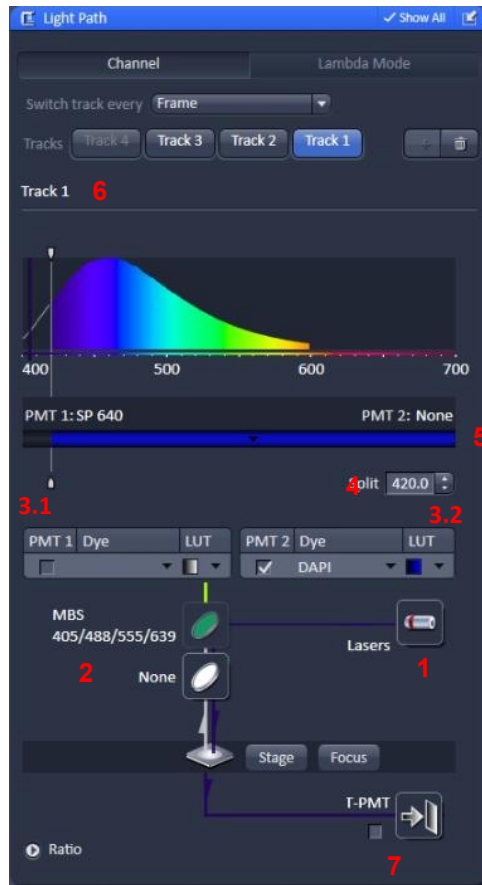


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- Select **show all tool** Show all Tools and open the **Imaging set up** tab
Note: make sure that the chosen configuration is correct and suits the fluorophores; make changes if needed; it is highly recommended to check all dyes spectrums in a spectra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



1. Laser lines
2. Main dichroic mirror
3. PMTs: PMT1 (shortpass) and PMT2 (longpass)
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 is recommended to add to the longest wavelength track).

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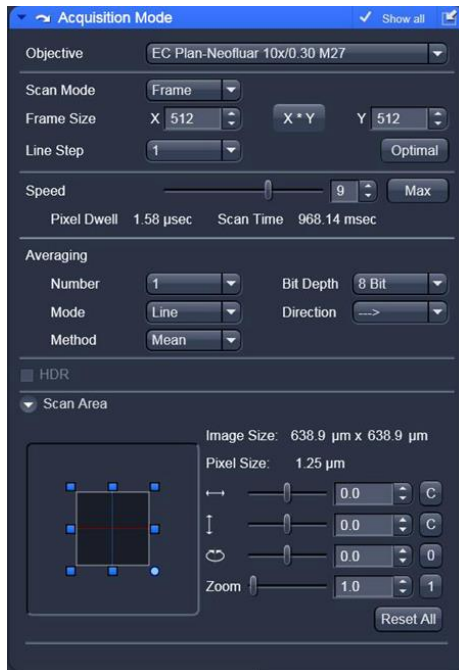
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Acquisition & parameter setting

Parameter setting

- Open the **Acquisition mode** tab



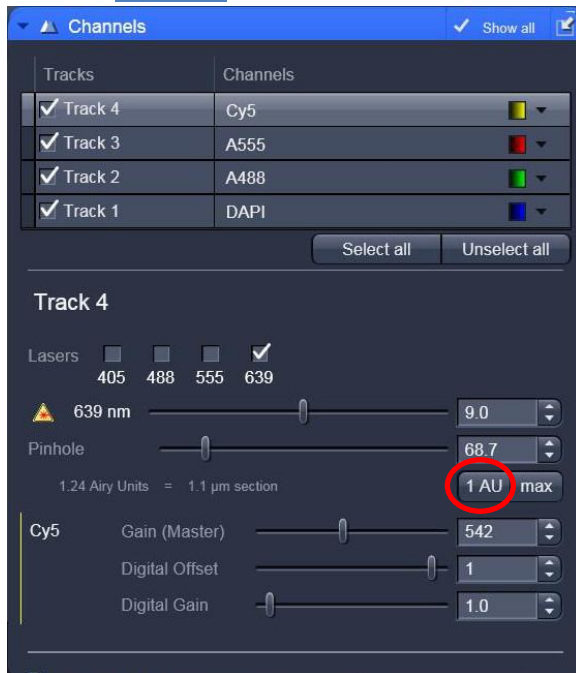
- **Scan mode:** frame (in most cases);
line scan mode can be useful for physiology experiments.
- **Frame size:** 512x512
- **Speed:** 8
- **Averaging:**
 - Method: mean (in most cases);
in low signal with low noise Sum method may be useful
 - Number: 1
 - Mode: frame for fixed sample, for live cells and in case of unstable dye line mode will be more suitable
 - Bit Depth: defines the dynamic range of the image, it is preferable to choose 12 bit, 4096 shades of grey.
 - Direction: ----> (more precise)
- **Scan area:** slight nudge, zoom and rotation

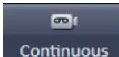
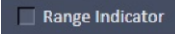


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- Open the **Channels** tab



- 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 (master) to 550-600 (preferably).
 - Set the Laser % - it is recommended to use the lowest % as possible and to increase gain (master or digital gain up to 1.5).
 - 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 (a few blue pixels 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 

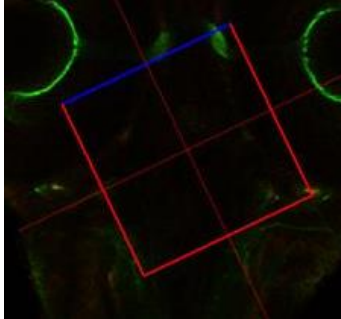
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Zoom


- In the **Acquisition Mode** tab select the preferred zoom (alternatively, after stopping continuous acquisition, press **Crop** below the image in the **Dimensions** tab and position the cropping frame by moving it in the image



- Scan one frame to check position.

Single image acquisition

- 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 signal to noise ratio)
- Press **Snap** in the start buttons
- Immediately save the image in czi format:

In the **Images and Documents** tab (right hand side of the window) press  and save in the directory:

D:\Users data\PI name\User name\YYYY-MM-DD



Make sure you are saving in the local folder and not on the server!

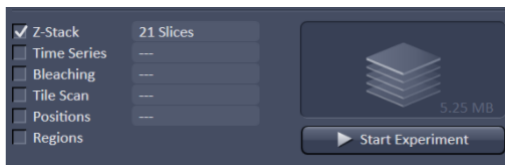
Data will be periodically erased from the local computer. It is the user's responsibility to back data up first to the server and from there to the preferred mode of data storage (more than one copy). The server has finite volume for each PI and is not intended for long time storage.

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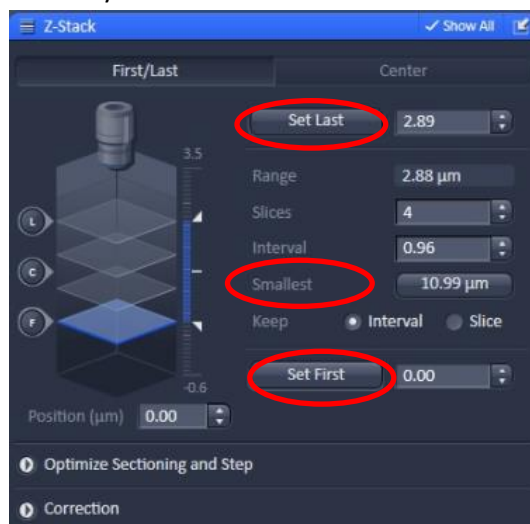
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Multidimensional acquisition



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
- In **Z-stack** tab, press First/Last
- 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 (usually)
- Press **Start Experiment**
- Immediately save the Image in czi format
Note: there are more advanced Z stack options in this tab such as brightness correction over z in case of thick stacks.

Time series

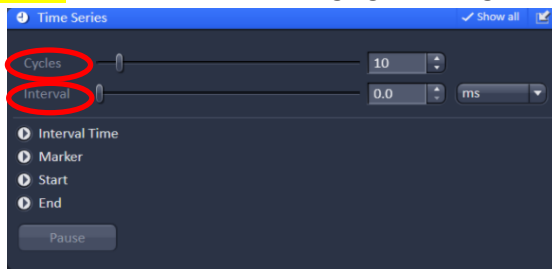
- In the Multidimensional Acquisition options below the start buttons check **Time series**; a new tab (**Time Series**) will appear under the **Multidimensional Acquisition Section**.

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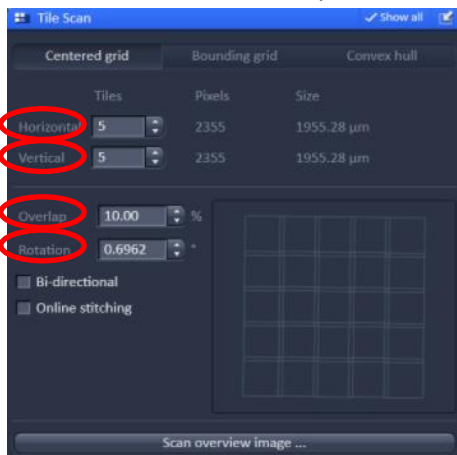
- Define number of cycles and interval
- Note:** in case of live cell imaging avoid high laser intensity



- In the **Acquisition Mode** tab:
 - Frame size Press optimal
 - In case of big frame size with a long frame scan time it is 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**.
- It is recommended to set zoom to 1.2 or more to avoid shading
- Define the number of tiles
- Recommended overlap is 8%-15%



- Preview number of tiles by quickly scanning (**Start Experiment**) in one channel, 256x256 pixel frame, speed 8, averaging 1.
- 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); press **close**.

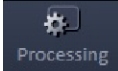


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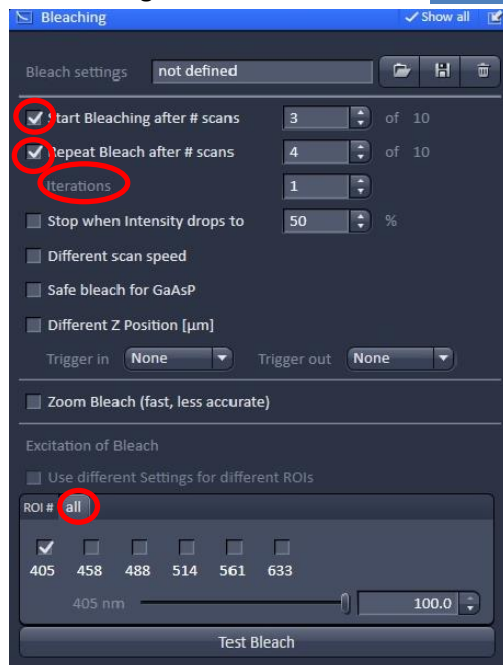
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Note: Do not check “online stitching”, the process can be configured in the **processing main tool** tab.

- In the **Acquisition mode** tab:
 - Frame size: Press optimal
 - Scanning speed: 7
 - Averaging number: 2
- Press **Start Experiment**
- 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 set:
 - Number of scans after which bleaching starts
 - Number of scans after which bleaching repeats
 - Number of iterations
 - The laser and its intensity
 - More settings can be defined in the **bleaching** tab.



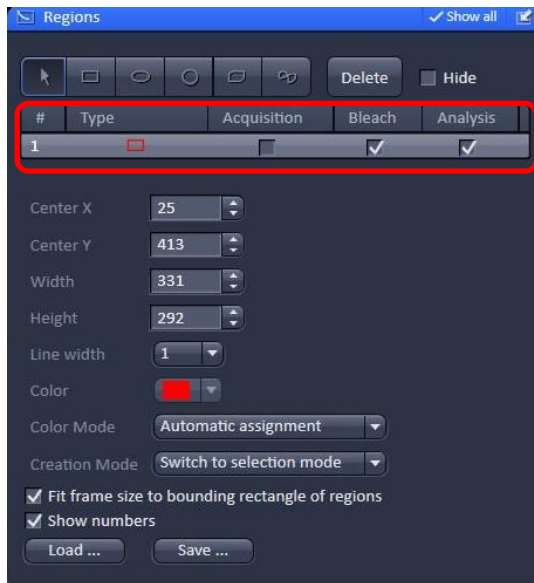
- Define one or more ROIs:
 - After a single scan in the **Regions** tab choose one of the ROI tools and draw on the acquired image; the dimensions can also be set manually in the Width/Height textboxes.

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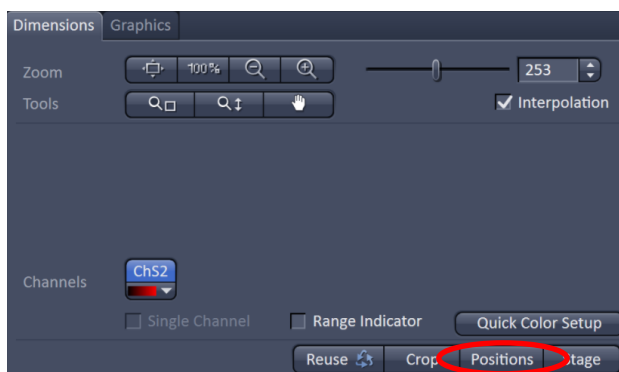
Define which process will be performed in the ROI – Acquisition/Bleach/Analysis.



- Define Time series:
- In the **Acquisition mode** tab:
 - Frame size Press optimal
 - Scanning speed 7 or faster
 - Averaging number 1-2
- Press **Start Experiment**
- Save the image
- The intensity data from the **ROI** can be viewed in the **ROI mean** tab in the image window
Note: Regions 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 smaller field of view.



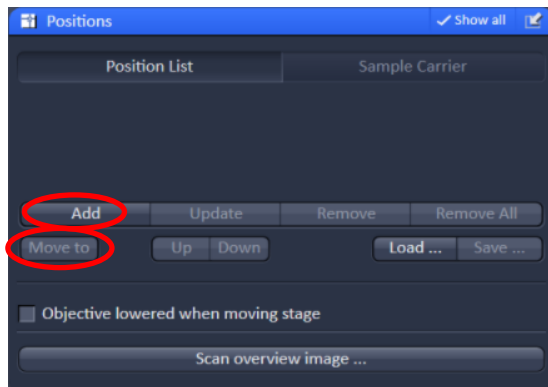
- Add a position by pressing **Positions** below the image container and clicking on the image container that was just acquired at the current stage position

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- 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.

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 your data is saved. The data will be automatically saved to the server during the night if the computer is on. You can manually copy them using the icons on the desktop.



PLEASE DO NOT USE any USB storage device drive on this computer.

- Clean any oil immersion objectives you used with clean 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 microscope(3)
- Turn off switches (2) → (1) In the switch box
- Cover the microscope

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