CONFOCAL MICROSCOPE ZEISS LSM 980 AIRYSCAN 2 WITH 2-PHOTONS USER MANUAL

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START THE SYSTEM

1. Press the « Components » button on the ON

2. Press the button on the ON to activate the LED used to the ocular observation.

3. Switch on the PC and login into the session « USER ».

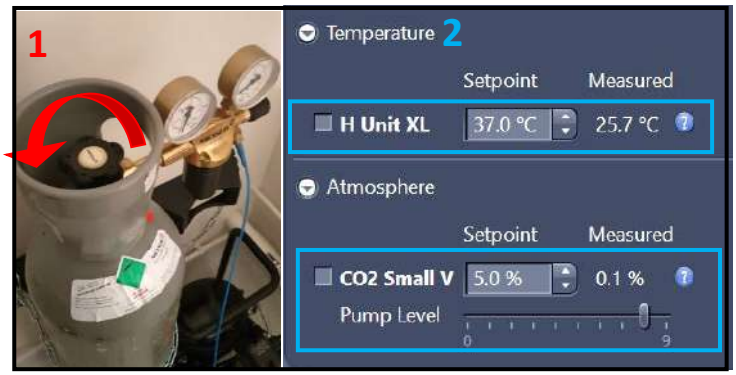
4. Start the software by clicking on the ZEN blue The icon on the window desktop.

5. Click on the ZEN SYSTEM button to access the acquisition menu.

START THE TEMPERATURE AND CO2

1. Open the bottle of CO2

2. On the right side of the ZEN software, activate the temperature and CO2



OCCULAR OBSERVATION

1. Select the LOCATE tab (first on the left).

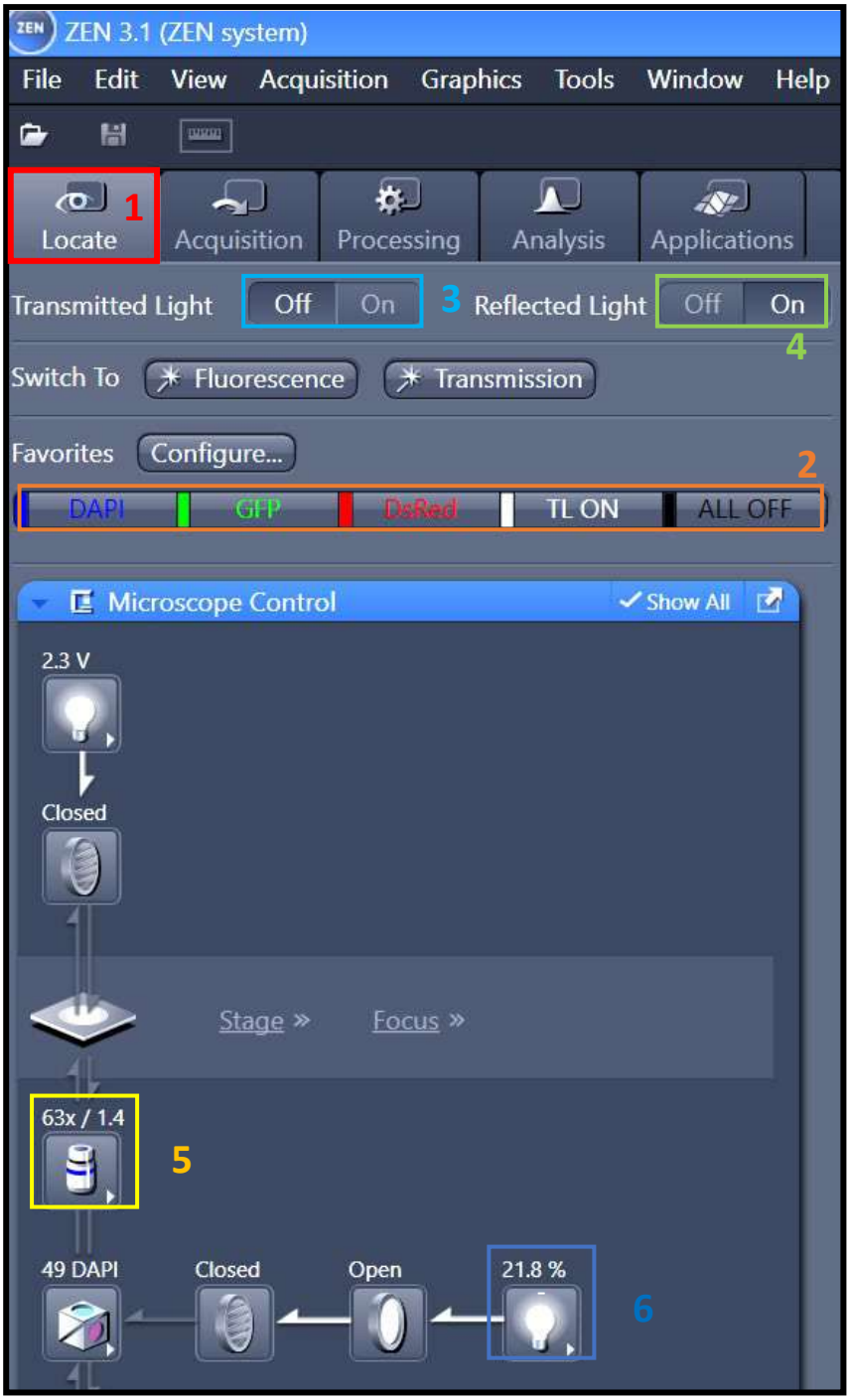
2. Select the desired illumination

3. Open/Close the transmission light

4. Open/close the reflected light

5. Adjust the fluorescent lamp intensity

6. Choose your objective
Depending on the type of illumination that you would like to.



Manual Command

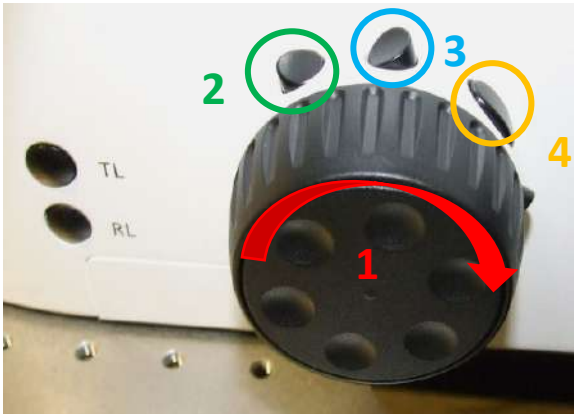
1. Wheel to adjust the focus

PS: The big one permits you to adjust fast, and the extremity one permits you to adjust with more precision.

2. put down the objective to install your sample

3. Put back the objective at the focus point

4. Open/Close the shutter of the transmission light

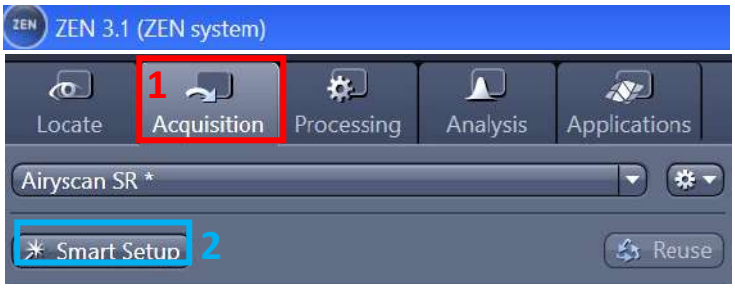


ACQUIRE ONE OR SEVERAL COLORS

I. Dye Assistant

1. Select the **ACQUISITION tab**

2. Open SMART SETUP



3. Then, in the DYE menu select the different fluorophores and their respective colors

Several methods are suggested:

-**FASTEST:** Allows simultaneous acquisition for all your channels; this is the fastest method, but cross-talk between consecutive channels can be important.

-**BEST SIGNAL:** Each channel is acquired separately. This method is the slowest one but it avoids (as far as it is possible) cross-talk between consecutive channels.

-**SMARTEST:** Compromise between speed and reduction of cross-talk. For example, both blue and red channels are acquired simultaneously, then the green one is acquired separately.

Remarks: These modes are just a base to start a configuration. The software doesn't necessarily choose the best one. You have to check by yourself and then modify appropriately the settings.

II. Imaging Setup

1. In the IMAGING SETUP window, each TRACK corresponds to one sequence of one or several colors.

2. Add or remove sequences by clicking on +LSM Confocal/-

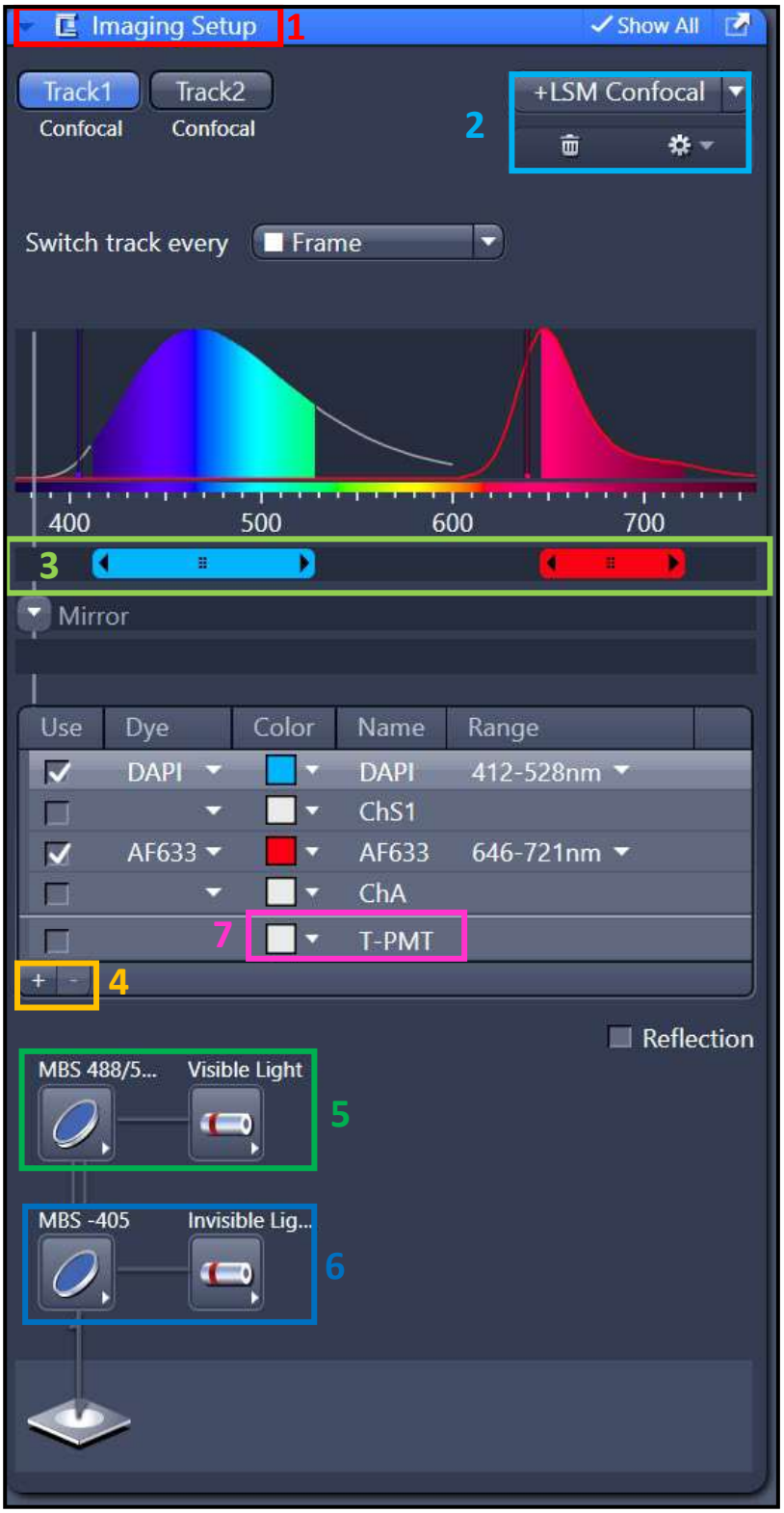
3. Adapt wavelength detection by adjusting the detection window for each channel.

4. Add or remove channels with +/- for each sequence.

5. Select, if possible, the same dichroic for each sequence to accelerate the change between sequences.
If no dichroic is required, select the « Plate » filter.

6. Activate the 405 dichroic for each sequence even if you don't need it.

7. Tick T-PMT to acquire transmission images in one of your sequences.



III. Acquision SetUp

1. In the "ACQUISITION MODE" window, choose the sampling of your image which has an impact on the resolution.

2. Zoom in If necessary, move your zoom zone to see your object of interest. If necessary, rotate your image

PS: Zoom can increase the photobleaching but can decrease the acquisition time

3. Choose the scan speed (the faster mode has the worst signal/noise ratio).

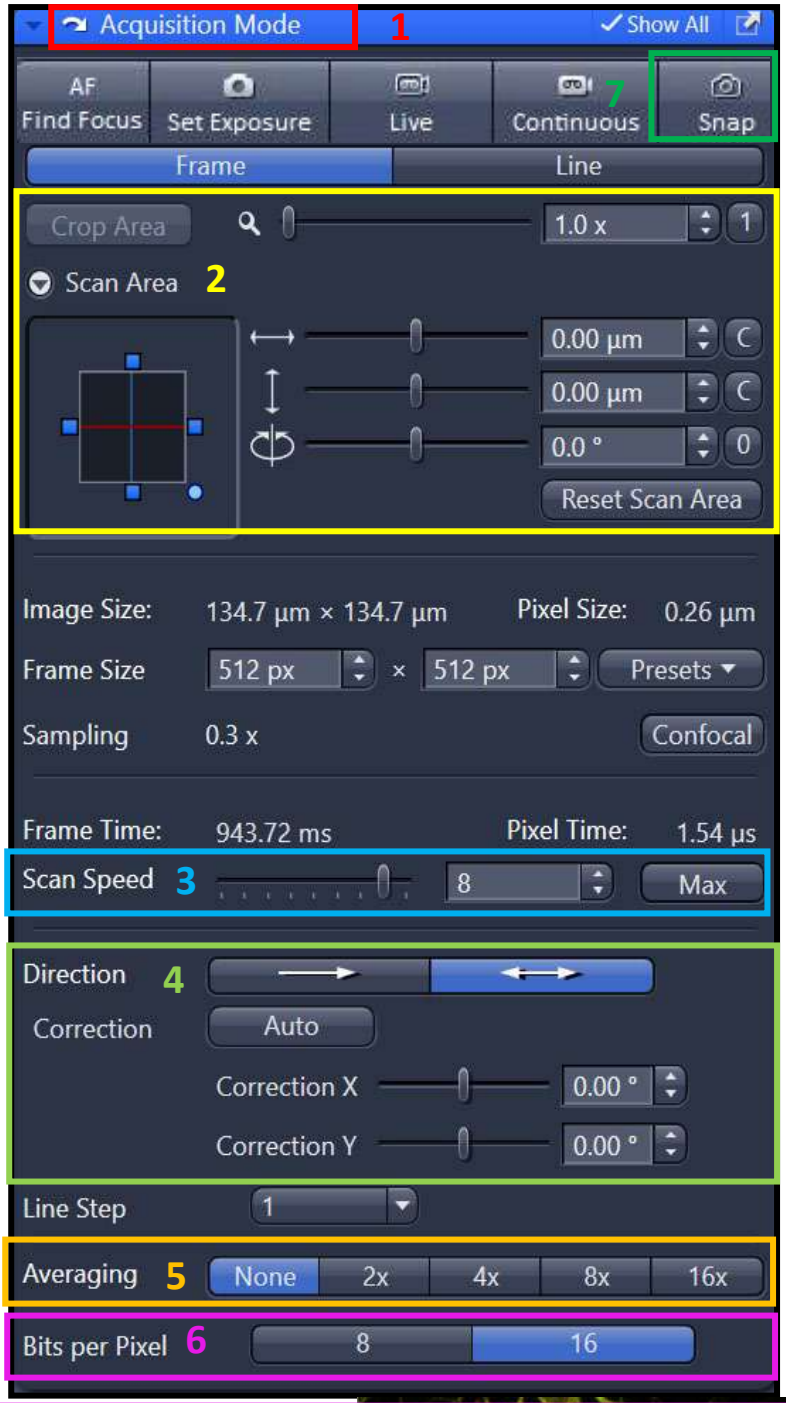
4. Bidirectional mode allows you to acquire 2 times faster.

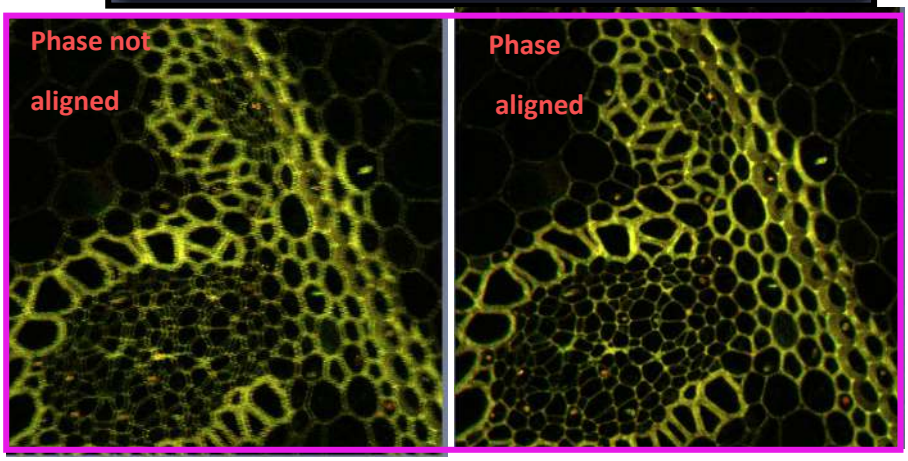
PS: the bidirectional mod could create phase issues that need correction X in phase settings

5. you can select the "AVERAGING" mode if the signal/noise ratio is not satisfying.
If you have a fixed sample the mode Frame is more appropriate. If you have a living sample the mode Line is preferable. (Method Mean is recommended)

6. Select 16 bits to increase your grey level and the quality of images.

7. Click on SNAP to acquire the image
PS: click on "Start Experiment" to begin your all acquisition (Tile; Z-stack etc).





IV. Channels

1. In the toolbox CHANNELS, adjust the pinhole at 1 AU (Airy unit) for each channel to obtain the best resolution/signal ratio.

2. Click on LIVE.

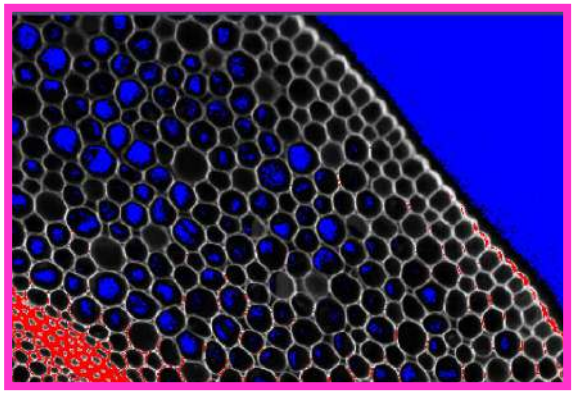
3. Adjust the laser power for each channel

4. You can increase the GAIN (MASTER) to decrease the laser power if it is too high. But the signal/noise ratio will be impacted. **Stay between 650-800**

5. You can adjust the DIGITAL OFFSET to improve the image by removing the background signal

6. Don't increase the DIGITAL GAIN except if the GAIN (MASTER) is high enough

7. To find adapted parameters for gain and laser power, it is recommended to use the « Range Indicator » display.
In this representation, saturated pixels are red and black pixels are blue.



ACQUIRE IN AIRYSCAN 2

I. Dye Assistant

1. Select the ACQUISITION tab

2. Open SMART SETUP

3. Then in DYE select the different fluorochromes and their respective colors.

4. Select « Airyscan »

5. **Several options are available:**

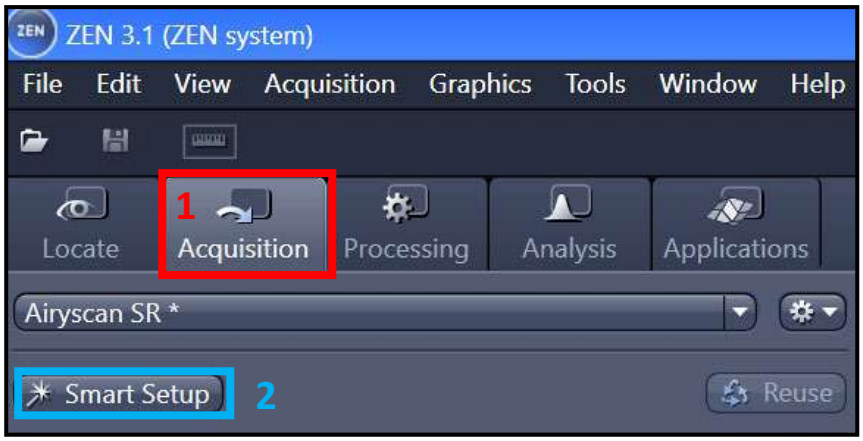
Resolution: allows to acquire the optimal resolution of the Airyscan 2.

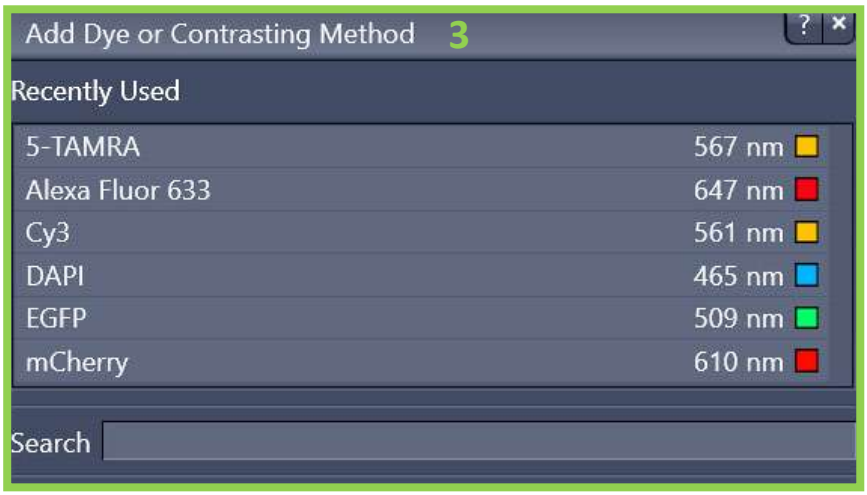
SNR/Sensitivity: Allows to acquire with the resolution of a confocal but with the sensitivity of the Airyscan

Speed: Allows to acquire using the fast modes (4Y or 8Y) of Airyscan 2.

Remarks: You can choose modes that make a compromise between the different options.

PS: The mode “Best or Smartest” is similar to the confocal mode.
These modes are just a base to start a configuration. The software doesn’t necessarily choose the best one. You have to check by yourself and then modify appropriately the settings.





II. Imaging Setup

1. Each "T**RACK**" represents a sequence of one or more colors.

2. Add or delete sequences by pressing +SR/-

3. Check/modify the filters selected for the acquisition of each Track.

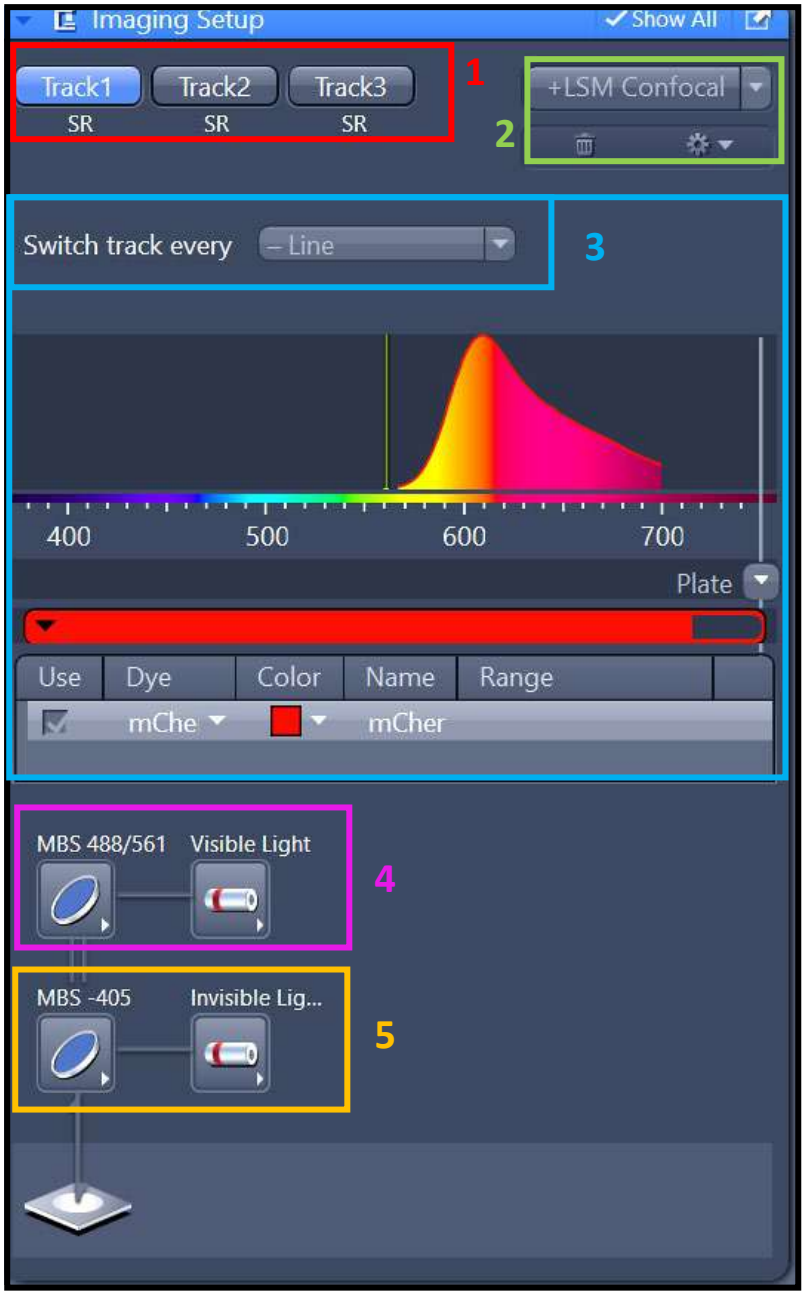
PS: the filter selection corresponds to a combination of 2 filters.

The spectrum acquires corresponding at the zone colored.

- If you use Line or Frame fast mode you have to choose the same filter combination for all the tracks.
- However, if you select the Frame mode you can set up a combination different for each filter.

4. Select if possible, the same dichroic for each sequence, to speed up the transition from one sequence to the other.
If you do not need to use a dichroic for an acquisition, you must choose the filter « plate ».

5. Activate the 405 dichroic for each sequence even if you do not use it.



II. ACQUISITION MODE

1. The Frame size defines your image resolution, but a high resolution involves a longer acquisition time.

PS: some Airy Scan mode has some definite parameters

- Select **"Confocal"** to keep the optimal resolution of a confocal.
- Select "SR" to maintain the optimal resolution of an airscan.

2. Modify the scan speed, a high speed decreased the signal/noise ratio (if you are “in vivo” select the Max)

3. Two-way mode allows you to go twice as fast.

PS: as in confocal mode the bidirectional mode could create phase issues. To resolve it you can correct it as explained previously (page 6, Number 4)

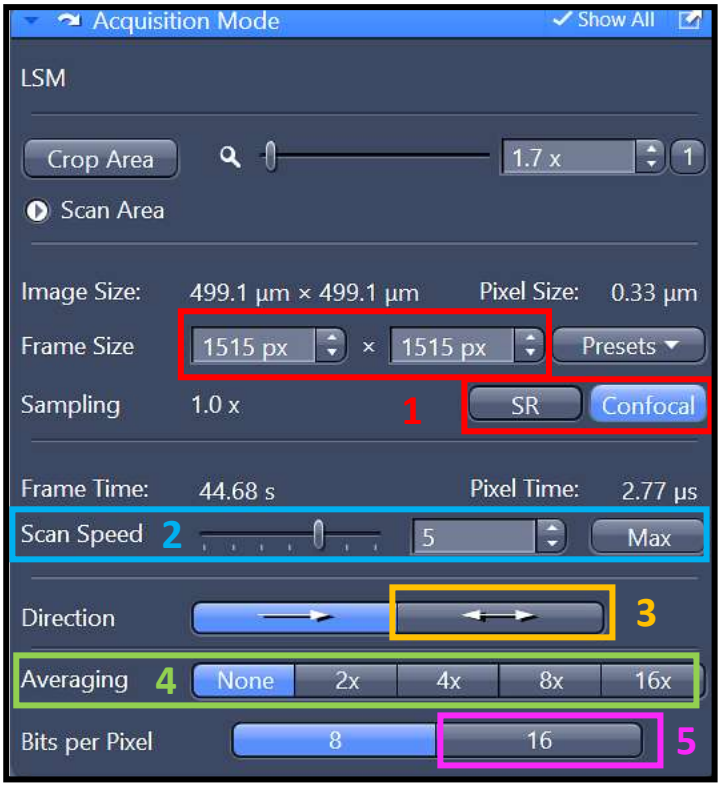
4. AVERAGING is a way to reduce noise ratio is not satisfactory. If the sample is fixed the Image mode is more appropriate. If the sample is alive the Line mode is preferable.

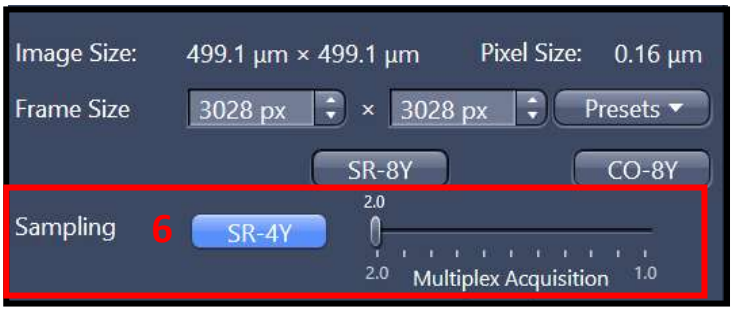
5. Select the 16 bits mode to obtain a better image quality.

6. in Fast SR, select the speed mode in the "Multiplex Acquisition" mode **SR-4Y**: SR reso with simultaneous 4-point scanning

7. Save your configuration.

PS: You can reuse the settings of an acquired image later by selecting the image and clicking on REUSE.





III. Set Up the Airy scan Sensor

1. To calibrate the **AiryScan 2** sensors, start by selecting the red sensor icon.

2. On the new tab, select « Adjust in live and continuous scans ».

3. Select "**Live**" or "continuous scans". Wait until "quality and status" says "OK".

PS: Be sure that your signal intensity is high enough (you can increase the laser power or the gain).

4. Stop the acquisition, and **uncheck the « Adjust in live and continuous scans » box.**

IV. Channel Set Up

The channel setup is similar to the confocal mode explained on page 7

1. Adjust the laser power for each channel
The power of the laser is different in the confocal mode

2. In the toolbox CHANNELS, adjust the pinhole at 1 AU (Airy unit) for each channel to obtain the best resolution/signal ratio.

3. You can increase the GAIN (MASTER) to decrease the laser power if it is too high. But the signal/noise ratio will be impacted. **Stay between 650-800**

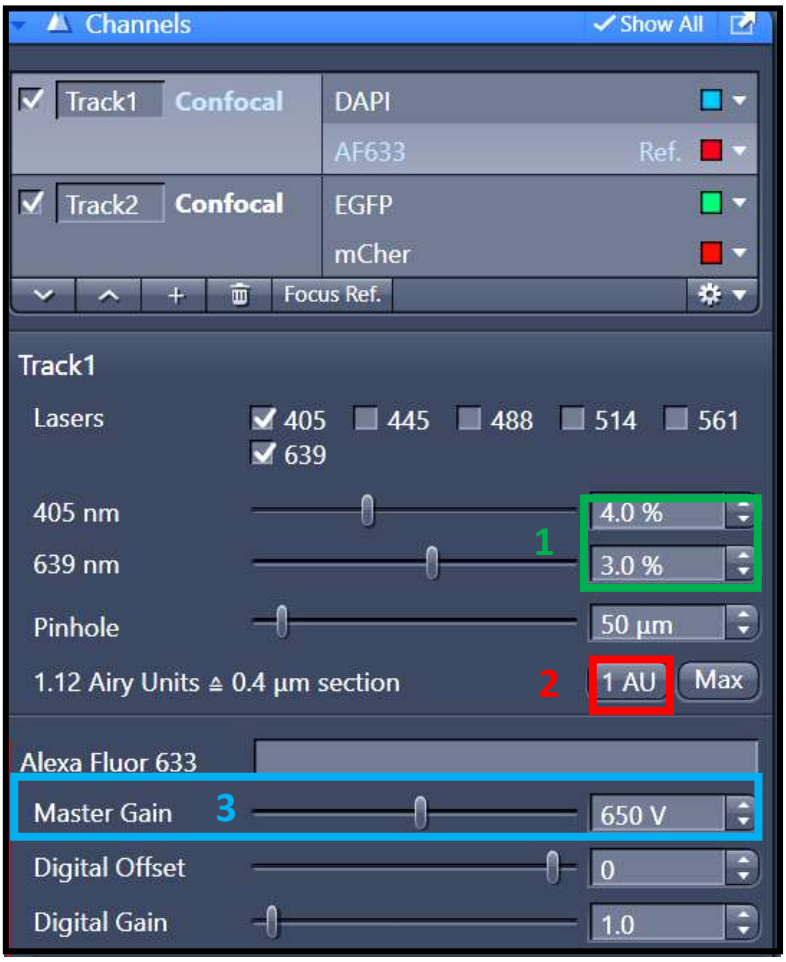


IMAGE PROCESSING.

1. Select the **PROCESSING** tab

2. In « *Method* » select « ***Airyscan Processing*** »

3. In « *Image Parameters* » select the image to be processed

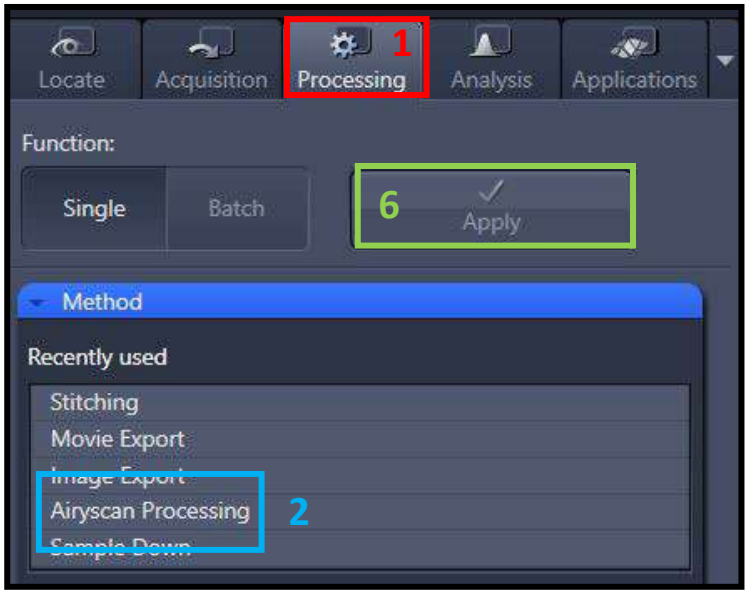
4. In « *Parameters* » tick « *Auto filter* »

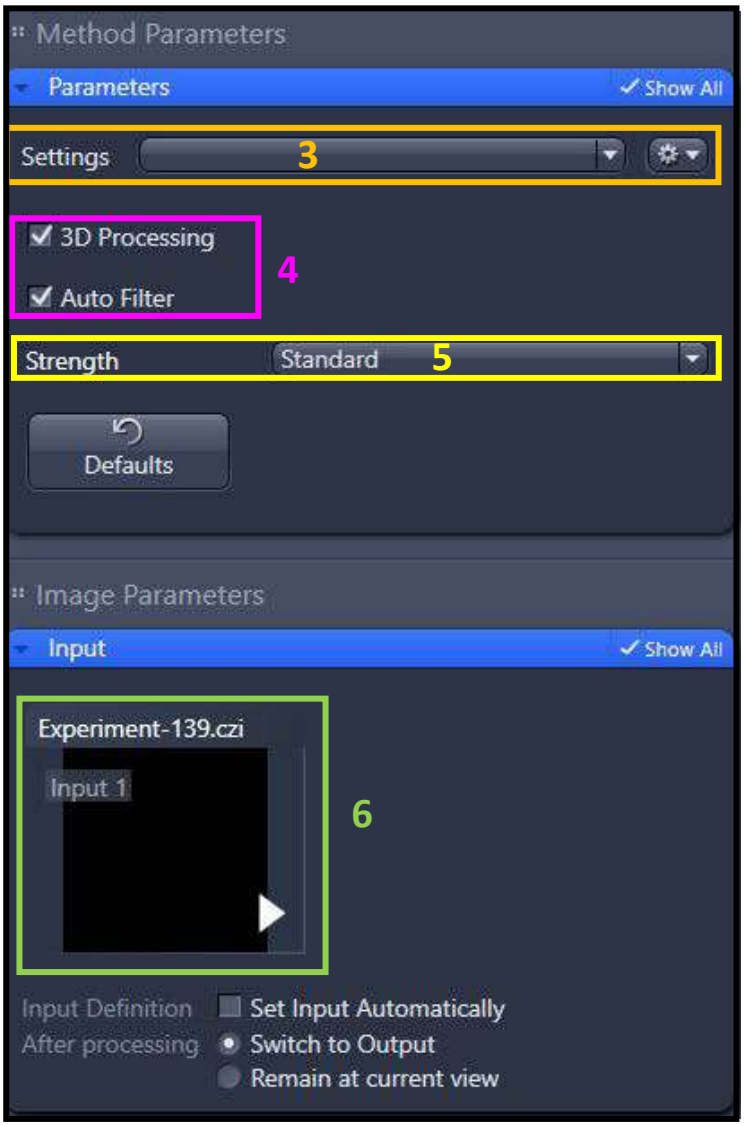
When the acquisition is a Z-stack, the option is replaced by the option « *3D Processing* ». Thick this option when the Z interval is optimal.

5. Select the filtering mode from the 3 proposed:

- Low
- Standard
- Strong

6. In the **« Input »** Window choose the file that you want to process Click on « *Apply* »





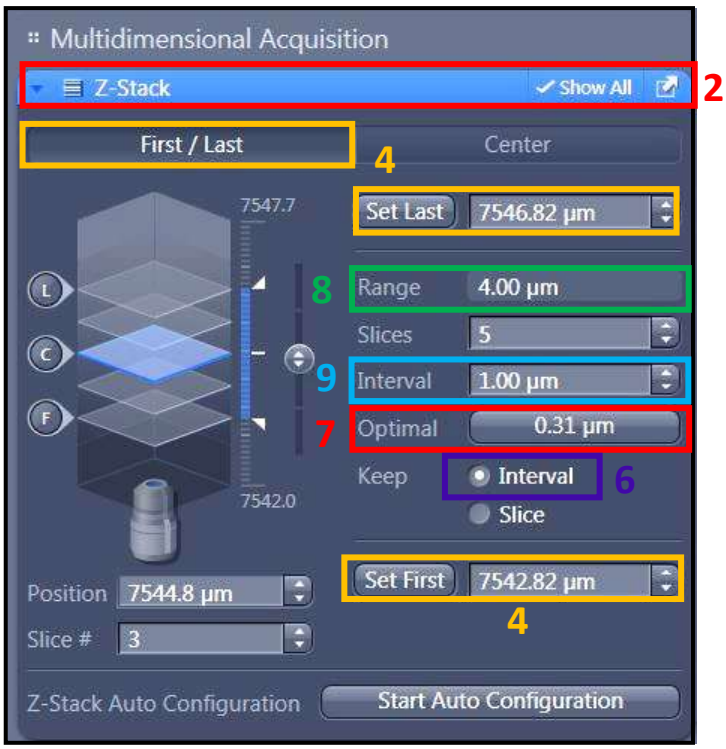
ACQUIRE Z-STACK SERIES

1. Check the « *Z-Stack* » option.

2. Check « *Show all Tools* ».

3. For multicolor acquisitions, make the entire z-stack of one channel, then switch to the other one « Full Z-Stack per Channel » or all channels per plane « All Channels per Slices ».

Two acquisition mode exists: you can define the top and the bottom of your stack, or define only the center of your stack.

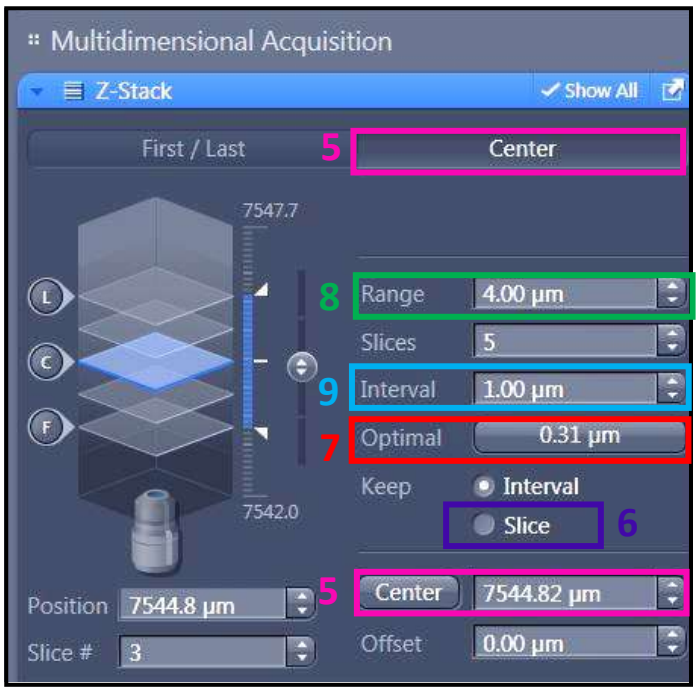


4. First /Last Mode

- Check the tab « ***First/Last*** »
- Make a « *Live* ».
- « *Set First* » defines one extremity of your stack and « *Set Last* » the other one

5. Define Center Mode

- Check the tab « ***Center*** ».
- Make a « ***Live*** ».
- Define the center of your stack with « ***Center*** ».



6. « *Interval* » has to be checked to ensure a fixed and chosen interval value in µm.

7. To choose the optimal resolution, you can use the « *Optimal* » option.

8. « *Range* » indicates the thickness of your stack.

9. The number of planes can be changed in « *Slices* ».

10. Start the acquisition by using « *Start Experiment* ».

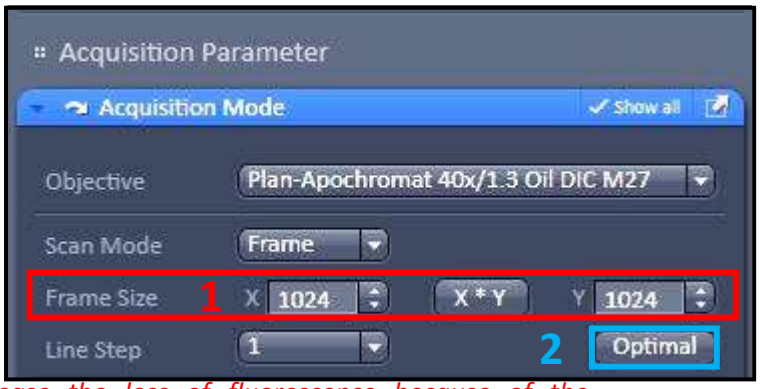
SPATIAL SAMPLING

To obtain the optimal resolution for your image, the image voxel size must be equal to half of the resolution of the objective that you use (Nyquist criteria). On a laser scanning microscope, the lateral resolution (in XY) is better than the axial resolution (in Z).

I. Choose the pixel size by modifying

1. Sampling image means several pixels composing your image.

2. To choose automatically the best resolution depending on the objective and the zoom, click on OPTIMAL.



PS: Having pixel size smaller than the resolution is useless (over-sampling).

However, you can under-sample the image to increase the speed of the acquisition and decrease the loss of fluorescence because of the photobleaching effect.

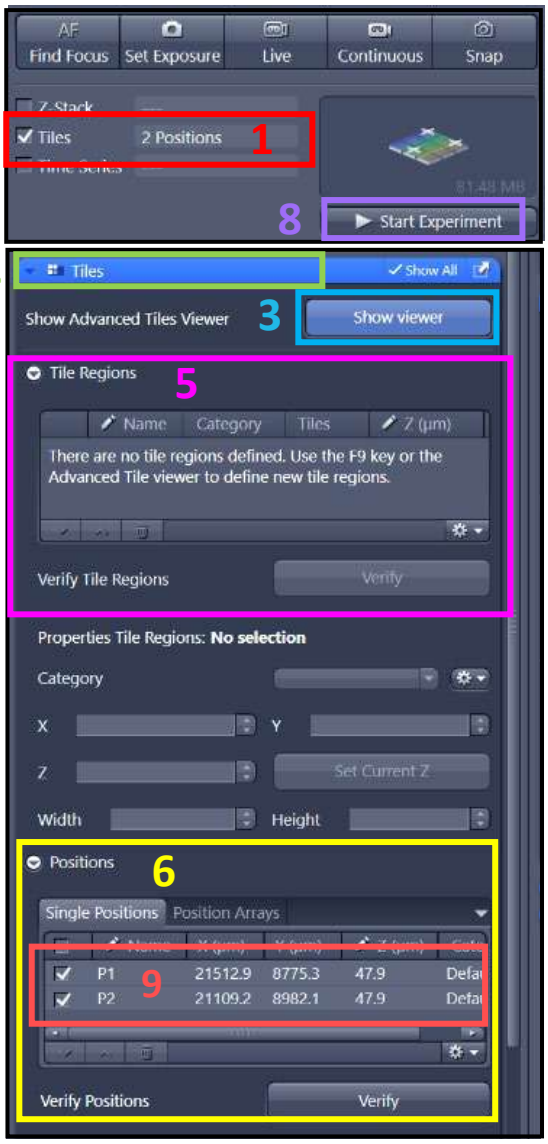
TEMPORAL SERIE

1. Select the **Time Series** box in the Acquisition Tab.

2. Choose the number of **cycles** and the **Interval** between each time point.

3. Click on START EXPERIMENT to start the acquisition

Tile scan/Multi-Position acquisition



1. Check « *Tiles* ».

2. Open the menu « *Tiles* ».

3. Click on « *Show Viewer* ». Make a « *Live* ».

4. The navigation space is displayed in the center of the software interface. Double-click where you want to acquire an image or use the joystick to move the stage

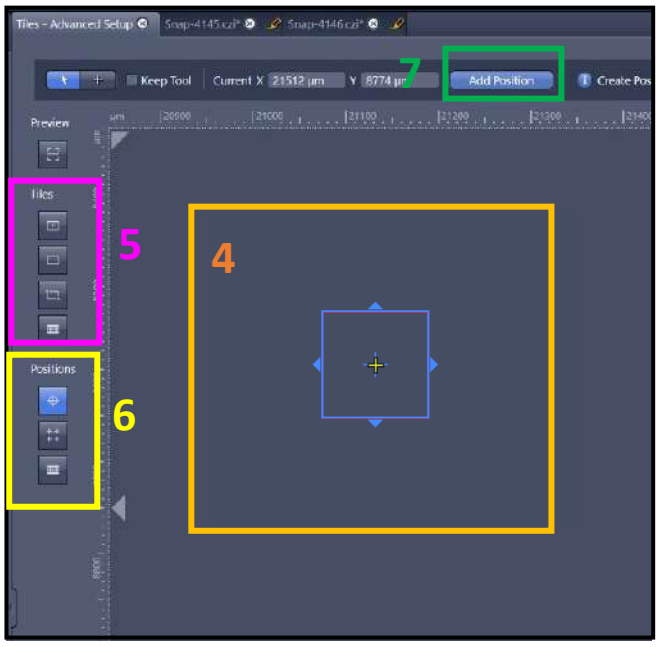
5. In the menu « *Tile Regions* », click on « *Tiles* » and double-click on your position. Your current position will correspond to the center of the tile.

6. Tile Position: you can add the position one by one to be more specific in your choice.
For this, you have to Click on « + », of the position window to add the position selected on the navigation space

7. Click on « + or Add position/Tile», to validate the choice of your Region of Interest.

8. When all your positions and tile are selected you can click on « *Start Experiment* » to start the acquisition.

Focus correction



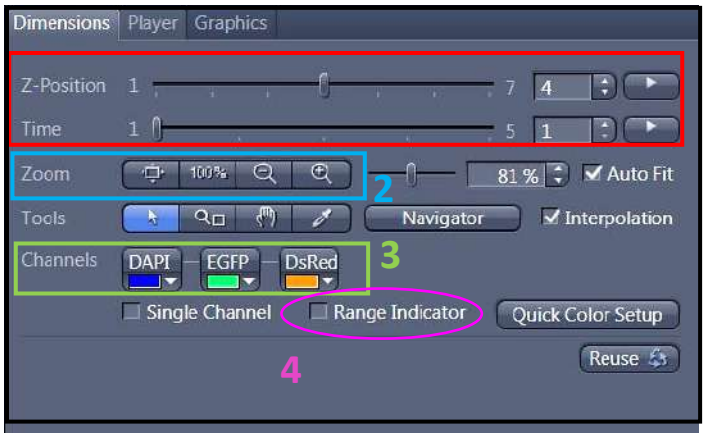
9. Make a right click on the position in the position window (tile window) and select “set current X/Y/Z or Current Z

PS: when you define the Z-stack it is important that you define with the Center mode as described previously to permit a z-stack adapted at the different Z set up of your different position.

For specific operations, you can ask the engineers of the platform or ask for assistance hour on the microscope

GRAPHIC TOOLS

In the « *Dimensions* » Tab



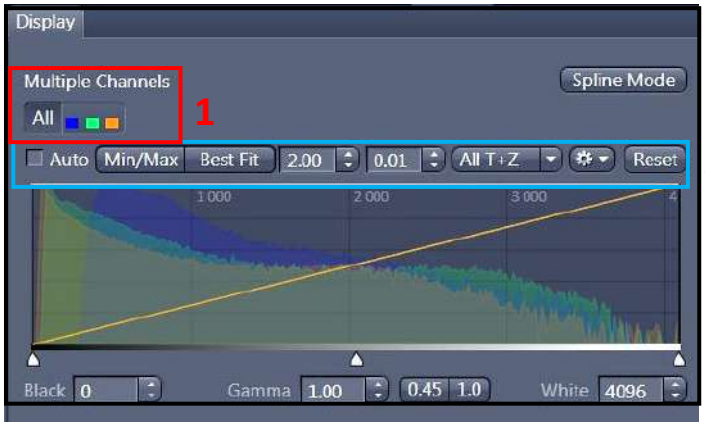
1. Move in the Z axe or in the Time series by the displacement of the adapted.

2. Image size adjustment. With 100% 1 pixel in image acquisition corresponding to 1 pixel of the image obtained

3. Show / Hide the channel on the screen.

4. Saturation observation (Red is the saturated pixel).

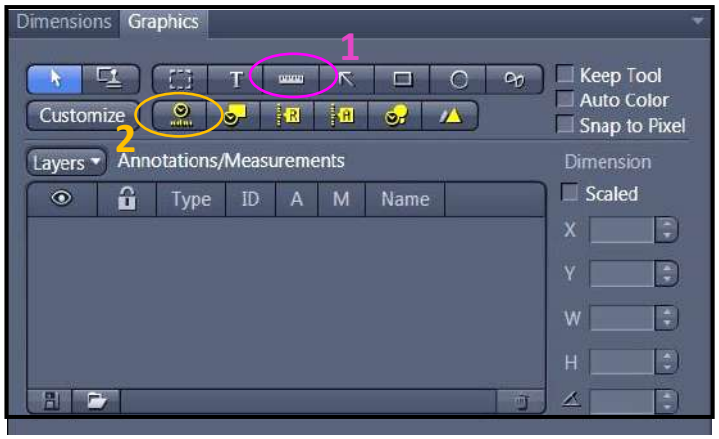
In the tab « *Display* »



1. Choose the channel or all to modify the image quality.

2. Contrast adjustment. Check on Reset to reinitialize it.

In Tab « *Graphics* »



1. Show the scale bar.

2. Show the time.

SIGNAL QUANTIFICATION

1. To quantify a fluorescence signal, you have to acquire them with the same acquisition parameters (same laser power, same gain, and Offset for PMTs).

2. To don't change the parameter you have to adjust your acquisition's settings with the sample whose fluorescence signal is the brightest. To limit the Over exposition effect.

IMAGE AND PARAMETER IMAGING

Settings Saving

1. select « *Save As* » in the « Parameter » logo. Name your settings and register its

2. to charge your parameter, open the menu « *Experiment Manager* » and choose your configuration.

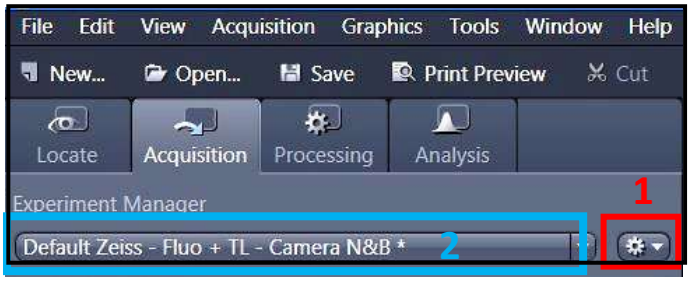


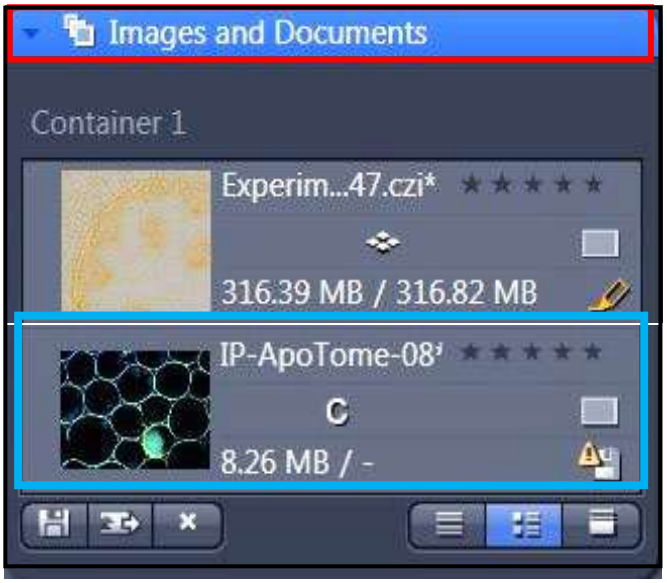
Image Saving

1. All images are visible on the right side called « *Images and Documents* ».

2. to save double-click on the images.

3. Click on the diskette logo, name your images, and save them in the folder « *Users* ».

Ps: create a folder with the date and your name.



STOP THE SYSTEM

1. Press « Components » button on ON

2. Press the button on the ON to activate the LED used to the ocular observation.

3. Switch on the PC and login into the session « USER ».

4. Start the software by clicking on the ZEN blueThe icon on the window desktop.

5. Click on the ZEN SYSTEM button to access the acquisition menu.

Module FLIM

I Start the System

1. Start the FRET / FLIM Computer

2. Start the software by clicking on the icon « Symphotime ».

3. On the LSM980 computer, open « ZenbluePicoQuantapp ».

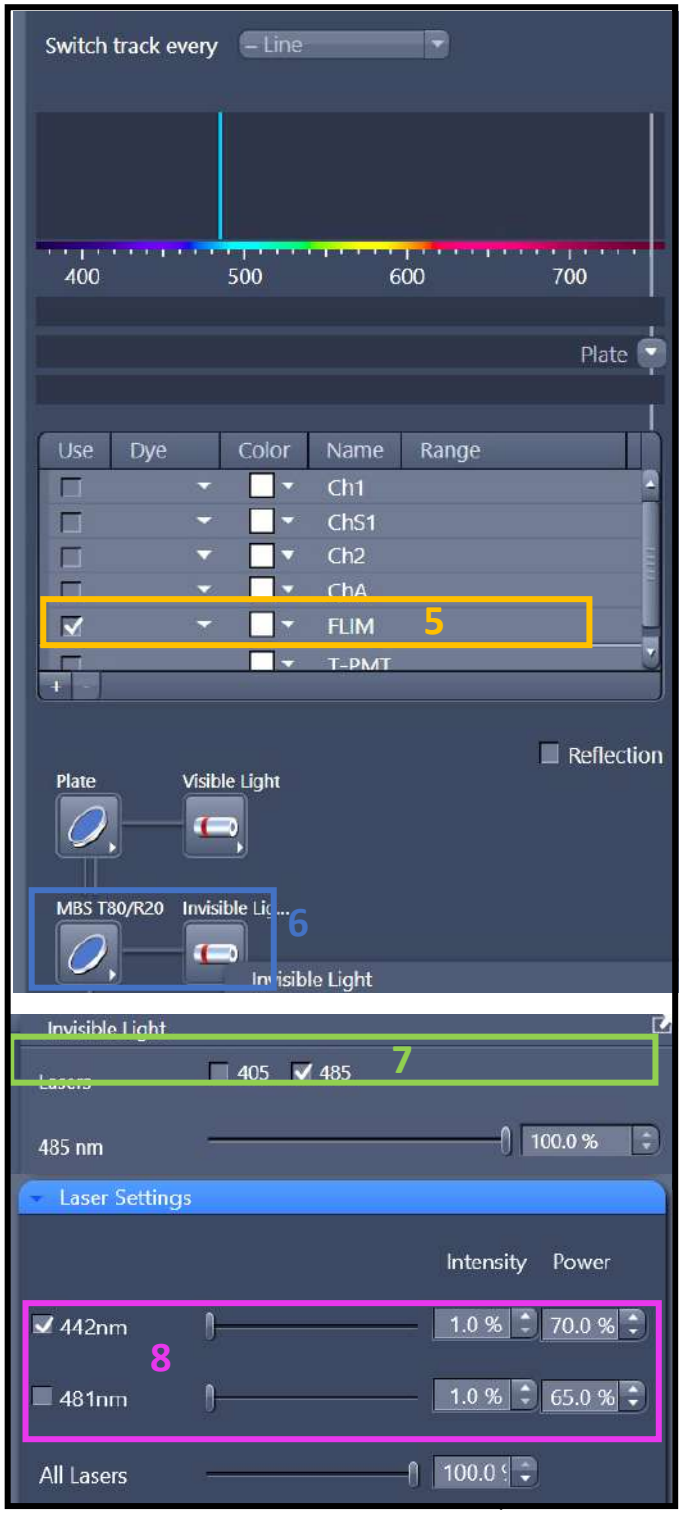
4. Search the sample by the confocal mode to make your focus.

5. Select the « FLIM » detector in « Imaging Setup ».

6 Select the dichroic filter in the "Invisible light" section.

7. Select the « 485 » laser, it's the PicoQuant laser. Set to 100%.

8. Select one of the two lasers in the "Laser settings" tab of the "FLIM" window and choose the laser power.



II Application Settings

1. the frame size has to be **512** or **1024** not upper.

2. In the « Acquisition» tab, select « unidirectional ».

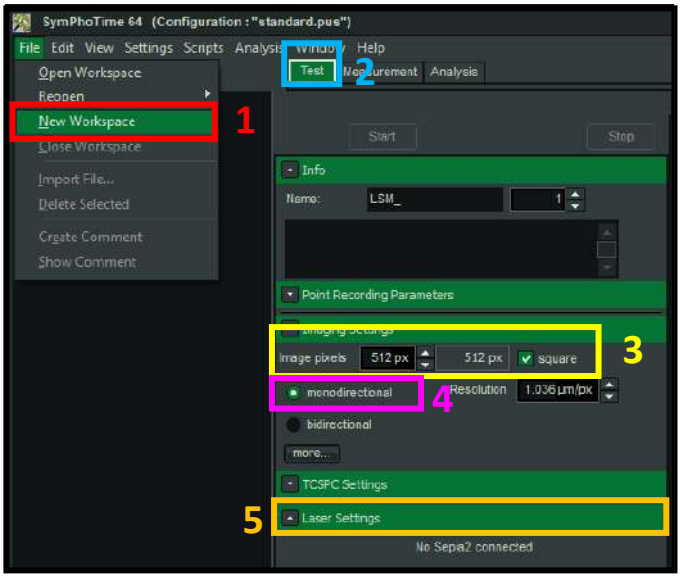
3. Select « Stop Manually » in « Measurement Settings ».

4. In the « PicoQuant » tab, set the « Repetition Rate » to 40MHz in « Laser Settings ».

This is the excitation frequency, it depends on the wavelength of the fluorescent labeling.

You need the excitation frequency allows seeing the fluorescence decay.

In the « Symphotime » software during « Measurement »



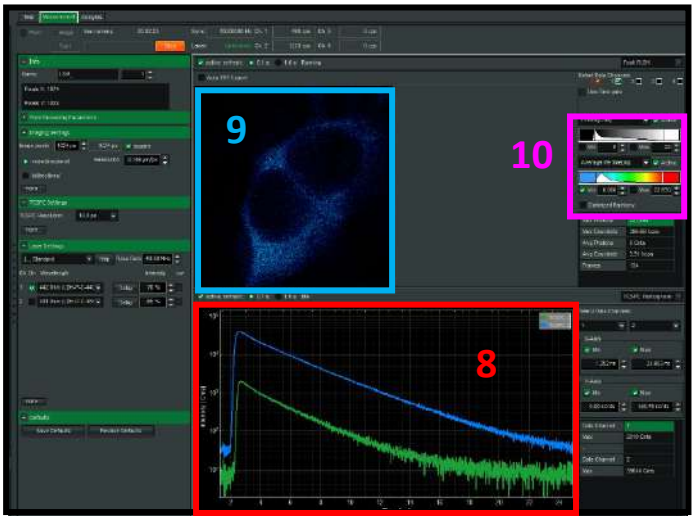
1. On the FLIM computer, in the "Symphotime" software, select «File" and create a new workspace.

2. Select the « Test » tab.

3. In « Imaging Settings », define the same pixel number as the frame size (see previously)

4. Choose the « Monodirectional » mode.

5. Select the same laser as the one chosen in Zen software.



6. In Zen software and start « test » in « Zeiss PicoQuant Application ». A set of parameters will have to be modified in order to obtain an optimal FLIM acquisition. This depends on the tagging, the medium, and the conditions.

7. in Zen, launch "Measurement" in the "Zeiss PicoQuant Application" tab

8. Fluorescence decay curve of detectors 1 and 2

9. Fluorescence lifetime Topography of detector 1

10. Fluorescence lifetime Histogram for each detector. And photons amount histogram.

11. Fluorescence lifetime Topography of detector 2

12. The maximum number of photons detected in the acquisition of a pixel. It will be the benchmark for stopping the experiment**. Count 1000 minimum to have a good signal for the analysis.**

13. In the Zen software, click on 'Stop' in the 'PicoQuant' tab to stop the acquisition.

Analysis of the FLIM acquisition

1. Go to « Analysis ».

2. In the « Imaging » tab, select « FLIM » mode and click on « Start ».

3. Select the acquisition folder.

In the new window, you will find a set of parameters and information about your FLIM Acquisition:

4. Fluorescence lifetime Histogram.

5. Fluorescence lifetime Topography.

6. Fluorescence decay curve (fluorescence intensity by time).

All of this information has been matched against the data set recovered during the acquisition. For further analysis please refer to the PicoQuant manual, and articles or contact the platform engineers directly.