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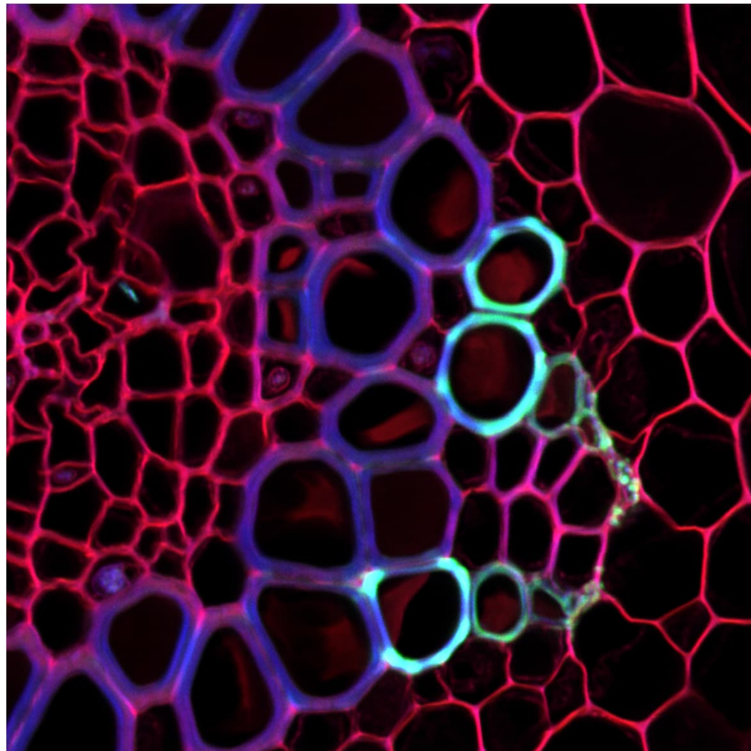
BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

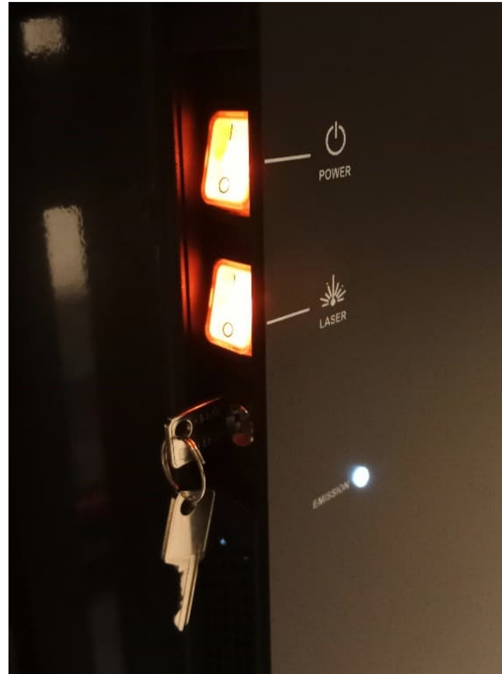
LEICA Stellaris 5
Confocal microscope
Manual/Quick guide



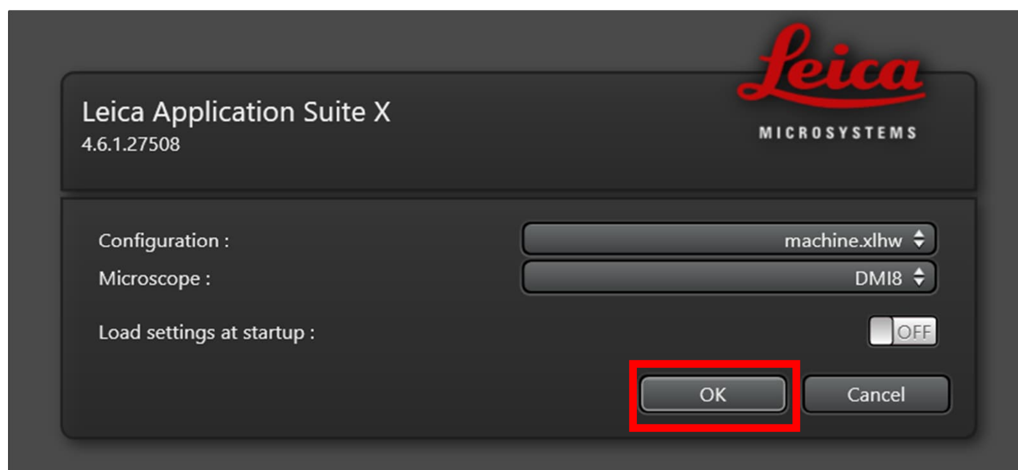
Matyas Molnar, Biovis 2024

Starting the microscope

1. Turn ON the computer, wait till Windows is booted
2. Log in with your UU AKKA account
3. Turn ON the scanner box switches below the table:
   i. Power button ON
   ii. Laser button ON
   iii. Turn the laser interlock key to vertical position (emission light ON)

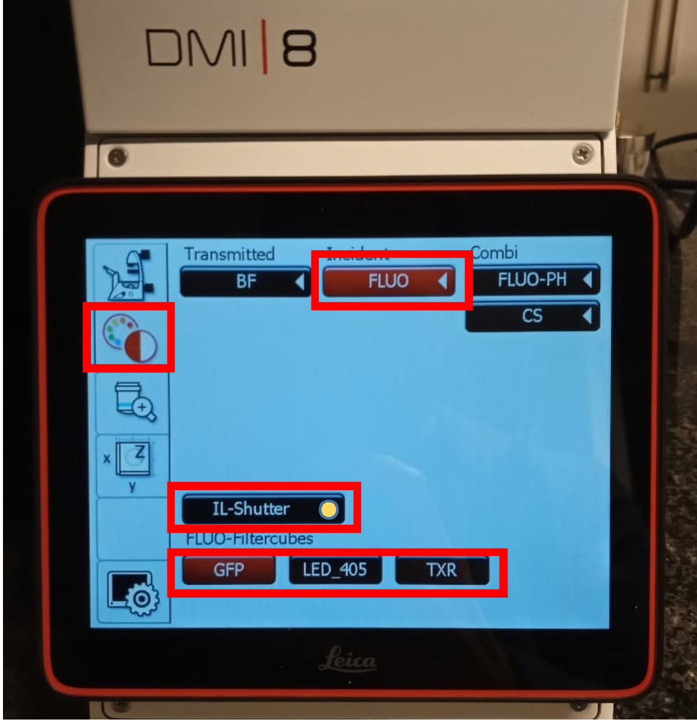


4. Wait approx. 3 minutes
5. Start the LAS X software and start “machine” configuration and “DMI8” microscope:

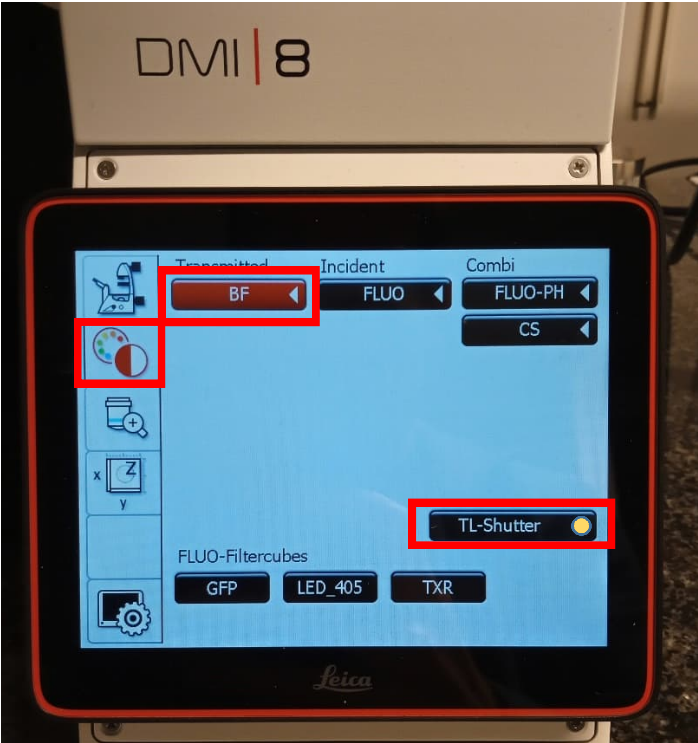


The microscope stand

Visualizing the sample through the eyepiece using fluorescent light (IL)
If you don't see any signal through the eyepiece, make sure that you are using the following settings on the TFT screen:

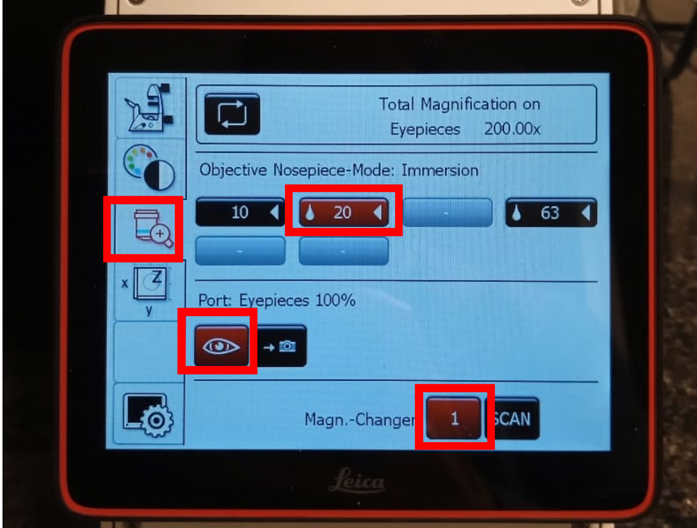


Visualizing the sample through the eyepiece using brightfield light (TL)
If you don't see any signal through the eyepiece, make sure that you are using the following settings on the TFT screen:

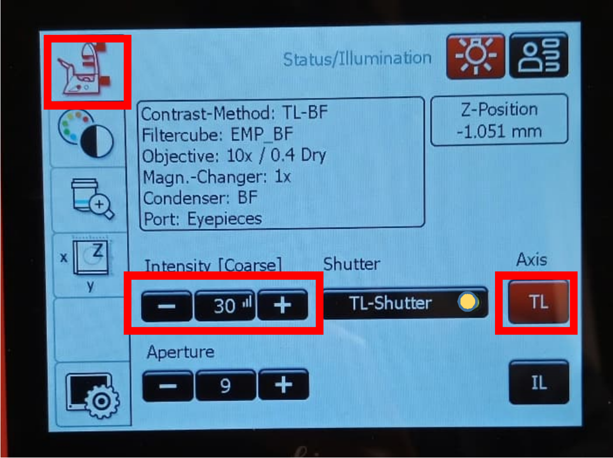


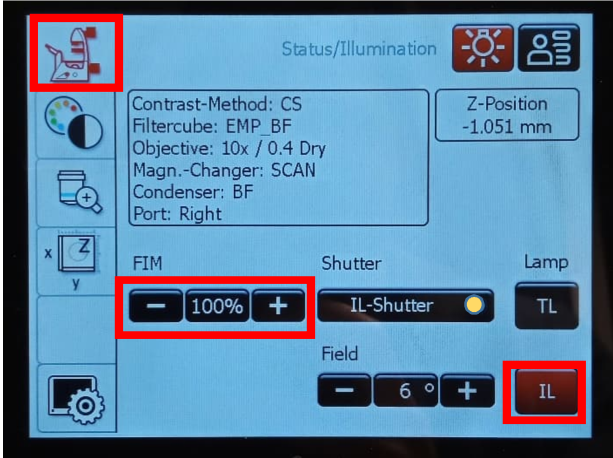
Follow these steps to have light through the eyepiece:

- Select the desired objective and use the correct immersion medium for it. On the TFT screen the objective buttons blink when there's a change in the immersion medium. Press them again after changed the immersion medium.
- Port: Eyepieces 100%
- Magn.-Changer: 1



To change the intensity for the TL or IL:

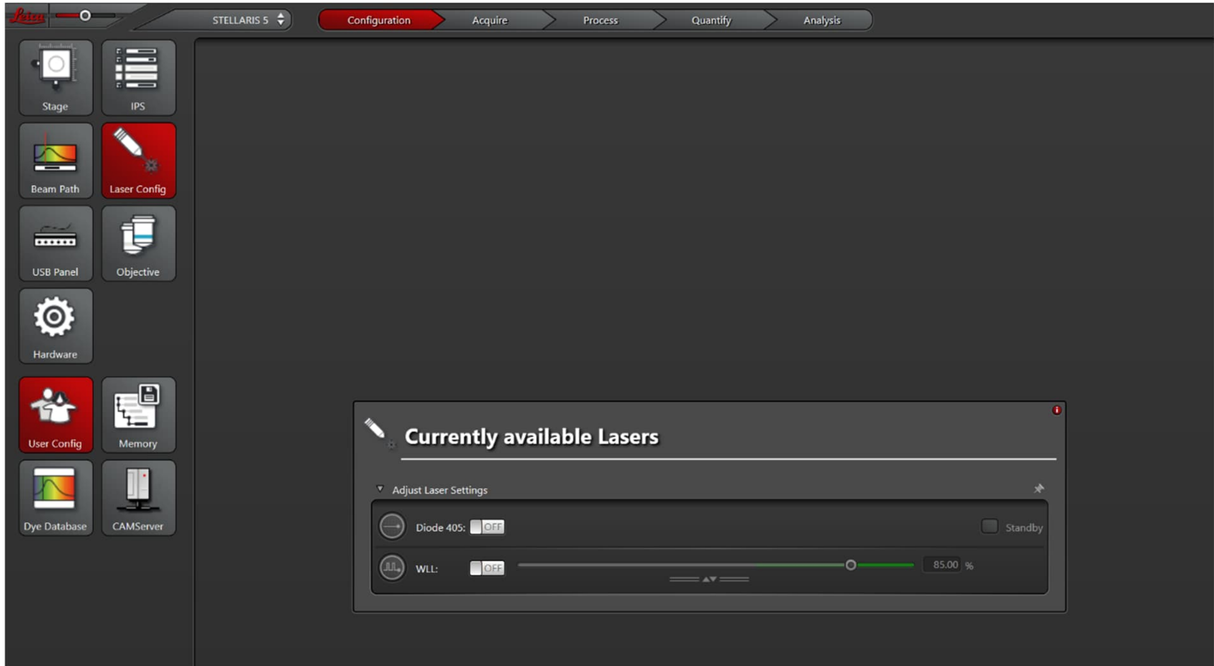




Using the LAS X software

In LAS X, hover the mouse on any button to get info about it.

Configuration tab



Under the configuration tab, several options can be changed and lasers can be turned ON and OFF.

- In the Laser config menu: laser can be turned ON or OFF. We have two lasers, a fixed 405nm and a tunable White light laser (WLL) 485-790nm.
- In the Hardware menu: dynamic range can be set to 16-bit (the default is 8-bit)
- In the USB Panel, the sensitivity of the knobs can be changed

Acquire tab, confocal imaging

This is the most used tab, here the imaging setup can be configured and imaging can be started.

Open projects
Acquisition
Acquisition Mode
xyz

▷; ▽ : Expand or collapse a menu.

Info and help menu. Use it when you need!

Acquisition mode: Type of imaging, 2D, 3D, time-lapse, spectral scanning

Navigator. Quick spiral scanning, tile scanning.

ROI

Bleaching experiment

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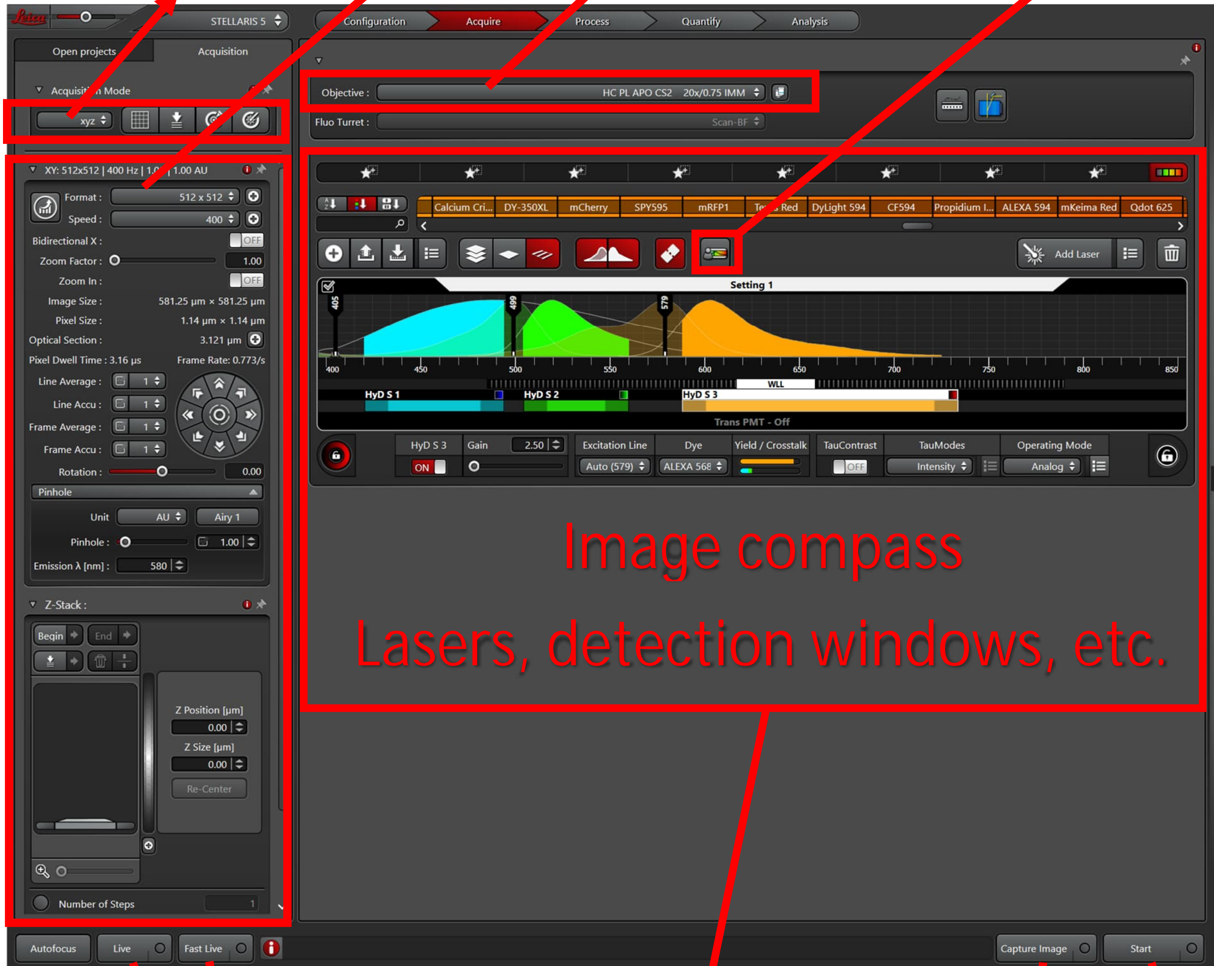
Acquisition mode:
Type of imaging, 2D, 3D, time-lapse, spectral scanning, simultaneous or sequential imaging

Acquisition menus

Objectives

Auto mode. If you are unsure what you are doing, start setting up your imaging here.



Live mode. Same as "Capture Image" but on continuous mode (speed and resolution settings are utilized here).

Fast live mode to check the sample and find locations with quick scanning (speed and resolution settings are NOT utilized here).

Detection configuration. Three HyD detectors can be turned ON-OFF, and their parameters such as detection range, gain and operating mode can be modified.

Capture an image

Start to acquire 3D; time-lapse; or spectral scanning, etc.

Auto mode (Dye Assistant)

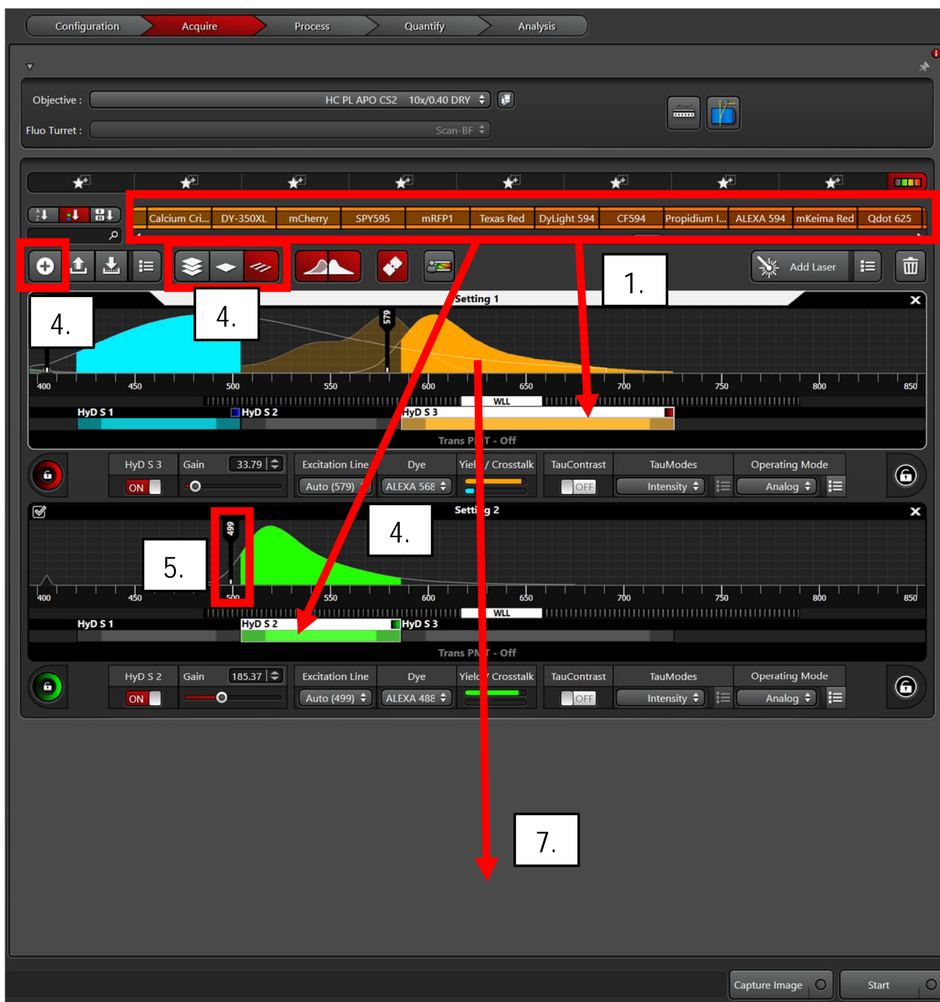
Auto mode is a quick and easy way to create the settings for fluorophores and channels. Unexperienced users can start with the auto mode. Please note that auto mode might not work perfectly in special cases or has to be fine-tuned manually. Users shouldn't rely on auto modes, the safest way is to set up the imaging as manually as possible.

- Select your fluorophores
- Select between simultaneous or sequential imaging, line or frame

Manual mode, semi-manual mode (Image Compass)

Setting up the channels with the image compass is a love and hate thing. It was made user friendly and super-overcomplicated at the same time where you need to alternate between clicking buttons and dragging-dropping items. Watch out for high numbers of buttons, check-in marks, padlocks, menus and small signs. They all have a feature.
Sometimes you need to delete settings by dragging and dropping it to and empty space, sometimes to click on it and then click on the delete button.

Semi-manual mode:



1. Drag and drop your fluorophore to a detector
2. The excitation laser and detection window for the detector is selected automatically
3. Continue doing this with all your fluorophores starting from the blue range (HyD S 1 detector) till the red-infrared range (HyD S 3 detector)
4. To image sequentially, add a new “Setting” with the “+” sign and drag and drop a fluorophore to a detector in the new setting. Select “Stack, frame or line” mode.
5. Change the laser intensity by clicking on the laser line and changing the “intensity” (knob can be used)
6. Change the detector gain by clicking on the detector and changing the “gain” (knob can be used)

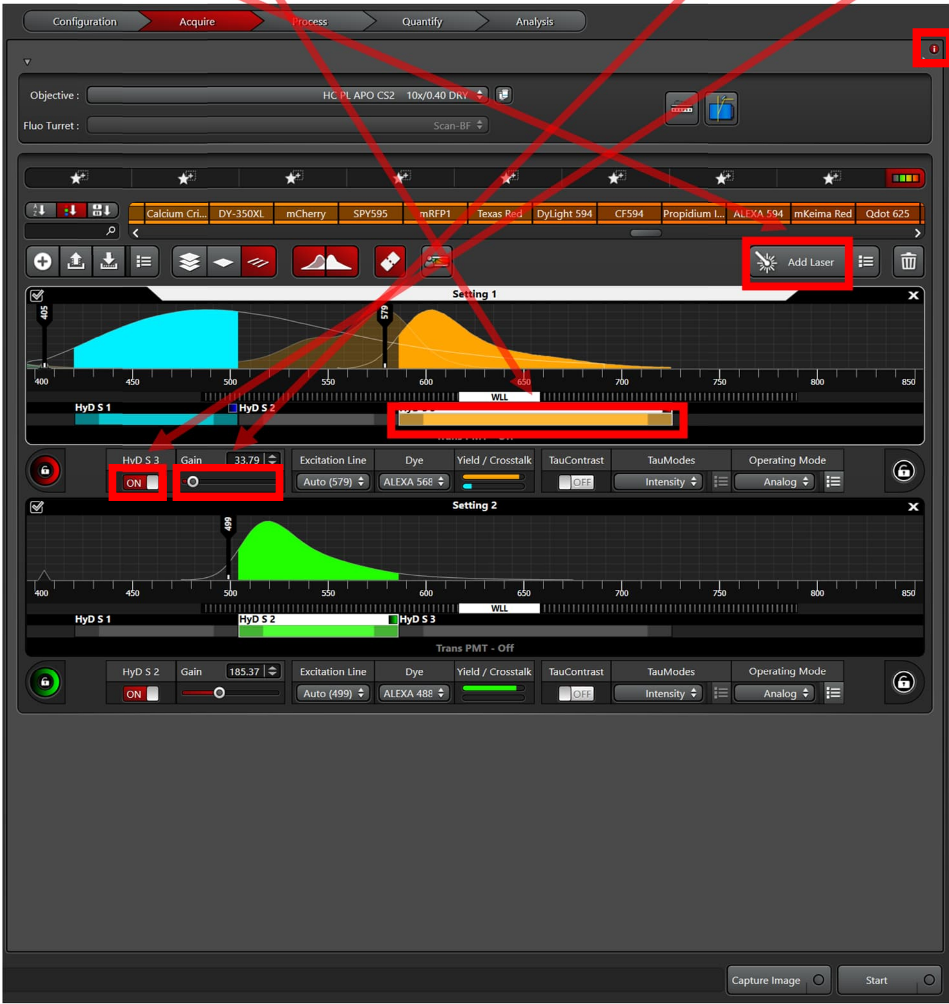
7. To delete a fluorophore from the settings, put your cursor on its emission curve and drag and drop it to an empty space. OBS ! If you want to only delete the laser line, click on it and push the thrash button (top right corner)
8. Navigation between Setting 1 and 2. You can:
   - Select a setting by clicking on it
   - Uncheck (deactivate) a setting with the check-in box on the top left corner
   - Remove/delete a setting by the “X” button on the top right corner.

Manual mode:

Start with the semi-manual mode (by dragging and dropping a fluorophore to a detector) and modify the automatically generated channel (detection range, tuning the WLL onto a different laser wavelength, etc.).

OR

"Add Laser" (drag and drop this button to a desired laser wavelength) and turn ON a desired detector and set up the detection window, laser intensity and detector gain.



Get HELP and tutorial. Use it often.

It's encouraged to try out what all the different buttons and features do. Hover the mouse over a button to see the info about it.

If lost, don't forget to use the built-in HELP function of the software by clicking on the closest "i" button. This is one of the best features of LAS X.

Adjusting the correct intensity with the Detection configuration menu or the knobs

The best way to adjust the correct intensity of a channel is to use the Over-/Underexposure tool on the left side of the image window (first button). If Over-/Underexposure tool is activated we see the overexposed pixels in blue.

This channel is selected (clicked), we change the detector gain for this channel

Over-/Underexposure tool

These channels are not selected (not clicked), we change the detector gain for the other channel

Overexposure (blue color); the histogram is clipped on the right side. To avoid clipping and to remove the blue, lower the "gain" or "laser power"

The overexposed pixels are out of the detector range, their intensity information is lost. We must avoid seeing overexposed (blue) areas. By changing (decreasing) the detector gain (voltage) or the laser intensity, we can move the overexposed areas into the range of the detector. For multichannel simultaneous imaging, be sure to click on the image of the channel you want to change, the other channel's detector is not changing meanwhile. Which one should we change, the laser or the

detector? There are no rules here, both have advantages and disadvantages. If we use high laser power with low gain we see a good quality image with low noise, but we can bleach the sample, so as always, COMPROMISE between quality and time/bleaching! Don't use a default laser or gain settings, always change them freely to get the best image without ruining the sample.

Note that if you change the pinhole or detection range, the signal is collected in a different Z/spectral range (intensity is changed), therefore new intensity adjustment is needed.

Acquisition menu

Optimize resolution (pixel size)

Set resolution (pixel size) manually. If you are unsure, use the optimize button. For thick 3D imaging use small, like 512x512 to save time/avoid bleaching.

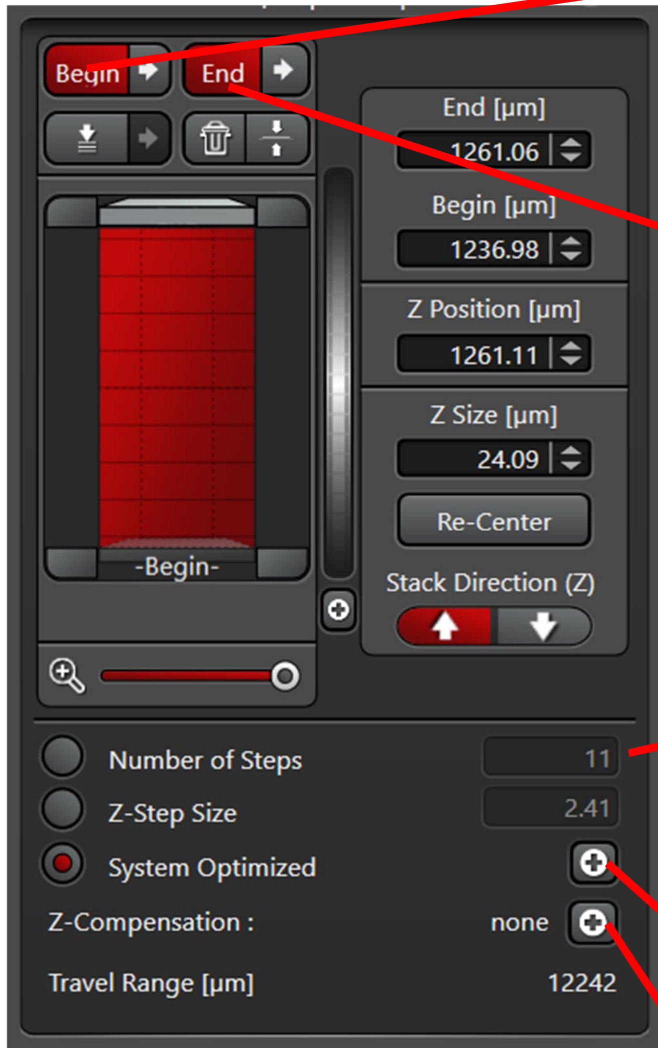
Scanning speed (Hz): go up with the speed to decrease, go down to increase the quality. No golden rule, it is sample dependent, if unsure try the sample with different speeds, and use the best for the real acquisition. Again COMPROMISE between time and quality, decide what is more important.

Bidirecional scanning: Before starting a scan always test if X phase is correct. If unsure, don't use it, it can ruin you imaging.

Averaging: noise (random pixels) are removed with averaging process. Select the number of image to be used for averaging (again time vs. quality!) and choose between line and frame averaging. Use the check-in box to apply the settings to all sequences.

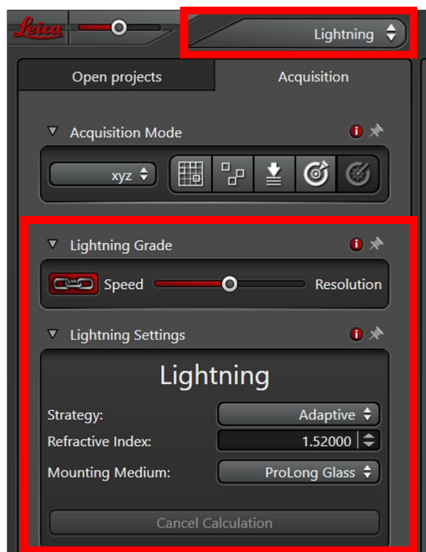
Pinhole: changes the thickness of the optical section. Click 1 AU button to get confocal imaging. If the signal is weak, open the pinhole more to collect photons from a thicker optical plane.

Z-stacking (3D imaging)



1. Go to Live or Fast Live mode, use the focus knob to find the end of the sample and push Begin to select the starting position of the sample where the first 2D stack should be made.
2. Focus to the other end of the sample and hit End to select the position where the last stack should be made.
3. Select the number of 2D stacks. Two ways of doing it, one can select Z-step Size and type in the interval between two stacks or select the Number of steps and type in the desired slice number. If unsure, go for the System Optimized button to not lose any information between two stacks.
4. Advanced optimization setup
5. Setup for linear laser or detector gain compensation when signal is getting lost in deep samples.

Deconvolution (Lightning)



With the Lightning module, on-the-fly deconvolution can be used to increase resolution and signal to noise ratio.

Use the slider to decide a balance between speed and resolution.
Strategy: Adaptive is always better, use this (numbers are based on and calculated for parts of the image with different signal to noise ratio).
Select the used mounting medium or select “custom” and type in manually the correct refractive index.

The image window

Over- /Underexposure LUT

Add scalebar and annotations

Zoom

Auto contrast

View all channels separately

View histogram

Hide/view channels

3D viewer (opens up in new module)

Image007 x = 1800 y = 1800 (9.3 MB)
Size: 160.97 µm x 160.97 µm

Right click on the image to Snapshot a view (for MIP or orthogonal view for example)

Saving, exporting

In LAS X projects can be created and saved. In a project, many images, datasets, snapshots can be saved and later re-used. Saved data will end up in .lif files that can be opened in LAS X, ImageJ, and Imaris.

Always save your image in RAW format (.lif) as it contains all the settings and information. If image file is needed, right click on a dataset and select Export.

New project

Open project

Save all

Show/Hide gallery

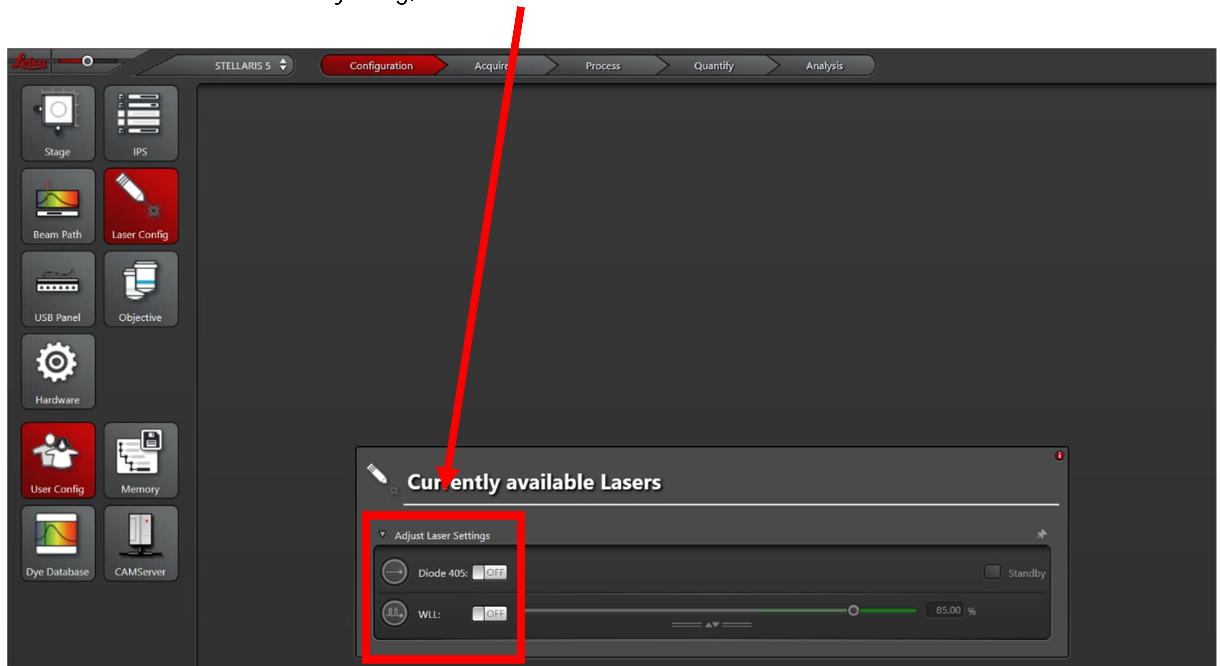
Save project

Right click on an Image and select Properties to see all the imaging settings for the dataset. To re-use the settings for a new scanning, hit Apply settings in the pop-up window.

Right click on an Image and select Export if image files or video files are needed of the dataset

Shutting down the microscope

1. Clean after yourself, put in the smallest objective and cover the microscope stand with its dust cover.
2. Use the logbook and type in your imaging session's details.
3. In LAS X software save everything, and turn OFF all lasers



To turn OFF the lasers, use ONLY the ON-OFF sliders in the Laser Overview window (in the Acquire tab) or in the Laser Config window (in the Configuration tab).

4. Close down the LAS X software, wait till fully OFF
5. Shut down the computer, wait till fully OFF
6. Turn OFF the scanner box switches below the table:
   i. Turn the laser interlock key to horizontal position (emission light OFF)
   ii. Laser button OFF
   iii. Power button OFF

6. Wait 10-15 sec so the instrument turns itself off. Don't turn off anything else that is not mentioned here.