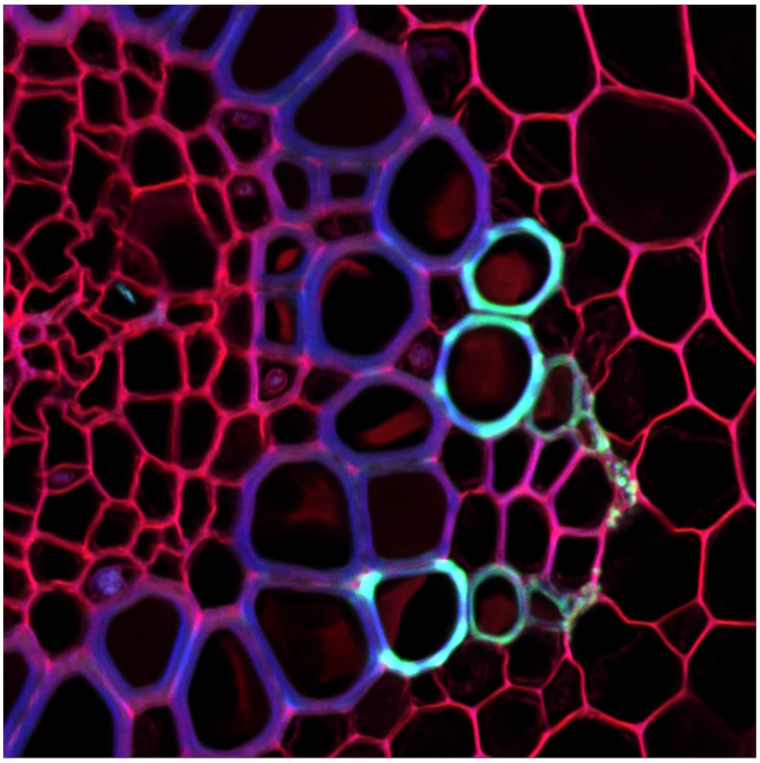
LEICA Stellaris 5

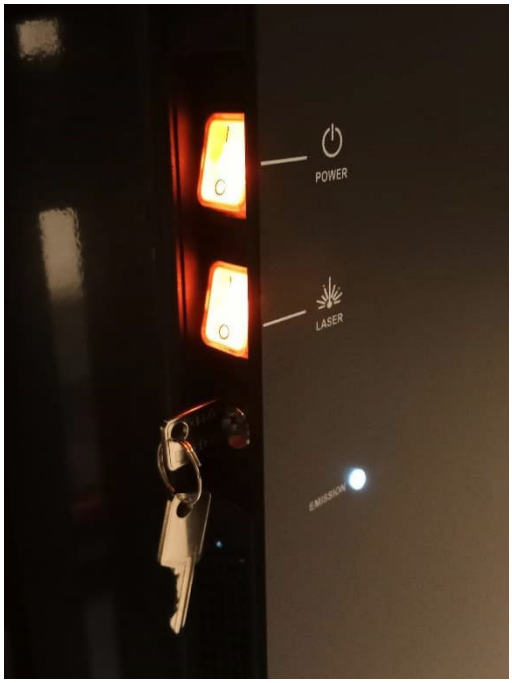
Confocal microscope

Manual/Quick guide

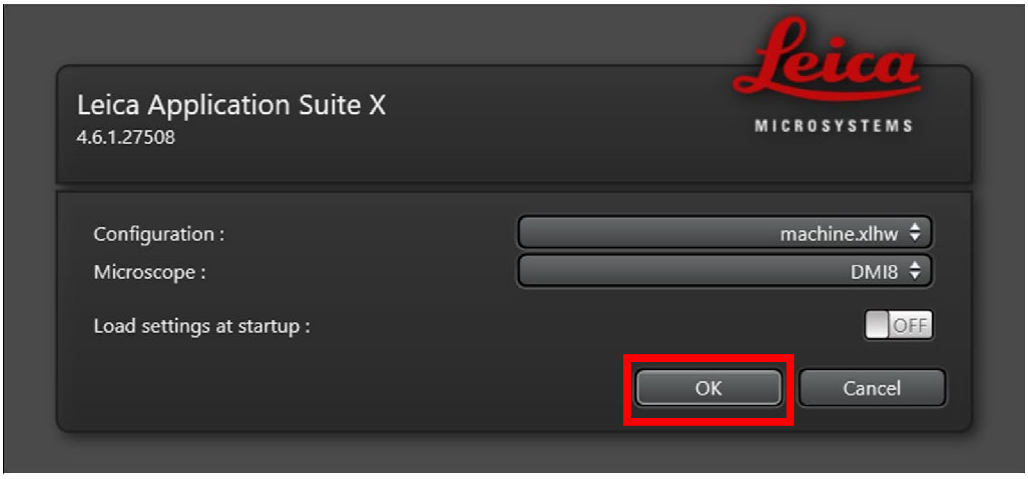


Starting the microscope

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

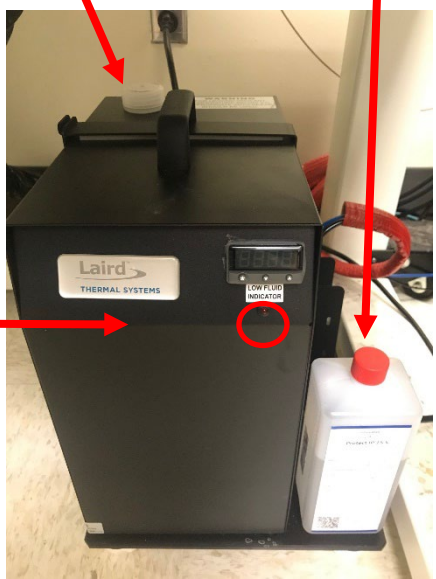


4. **Wait** approx. 3 minutes
5. **Start** the LAS X software and start "machine" configuration and "DM6" microscope:



Fill neck

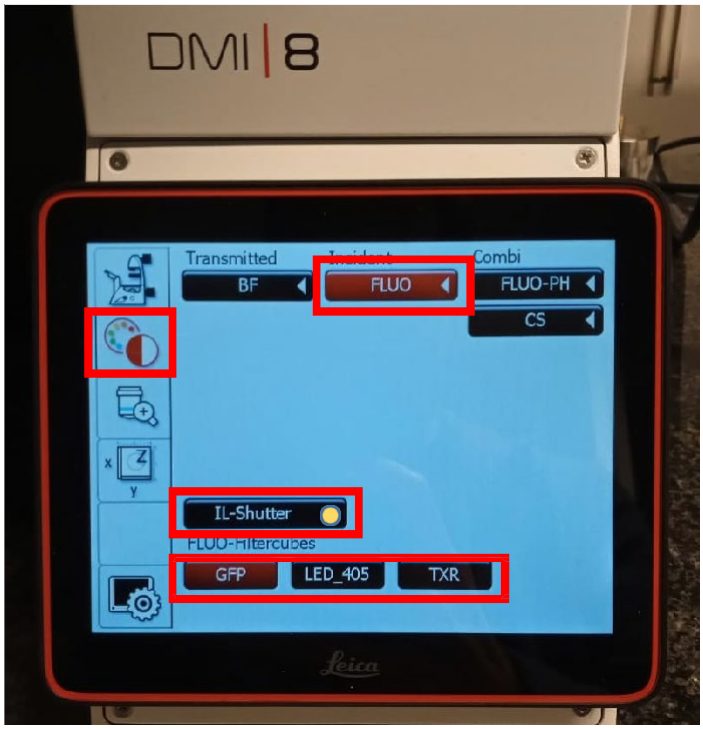
Coolant



Fill cooler to base of fill neck if low coolant light flashes

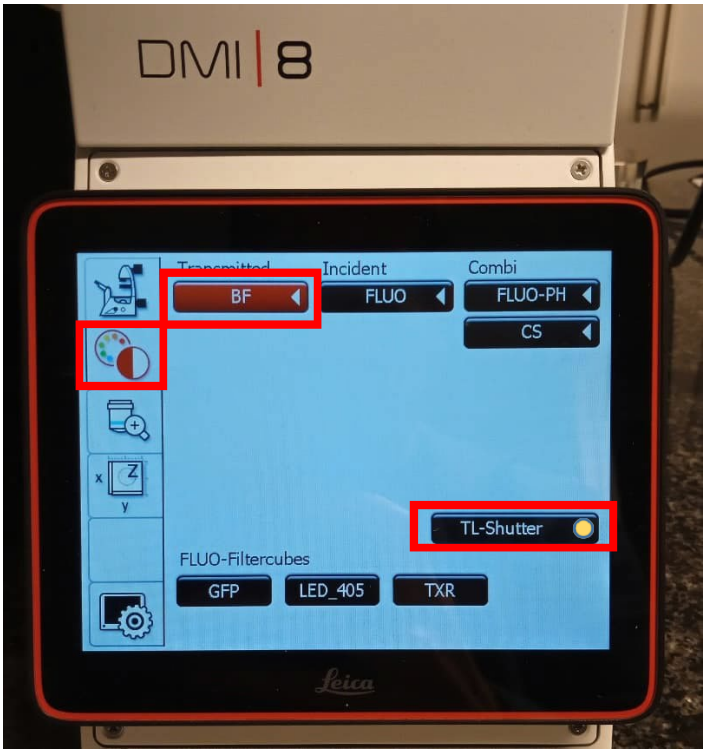
Visualizing the sample through the eyepiece using fluorescent light (IL)

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)

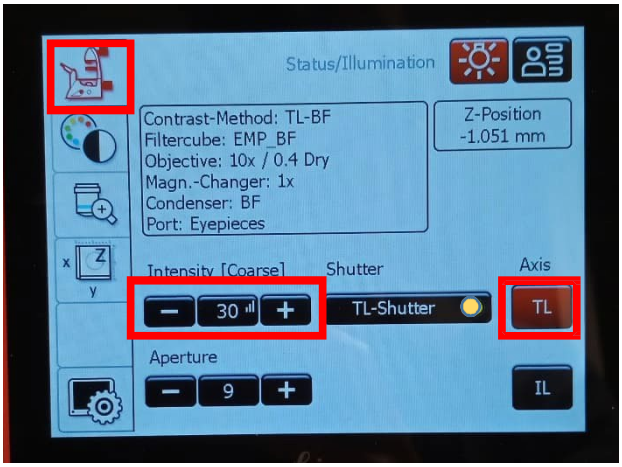
If you don't see any signal through the eyepiece, make sure that you are using the following settings on the TFT screen:

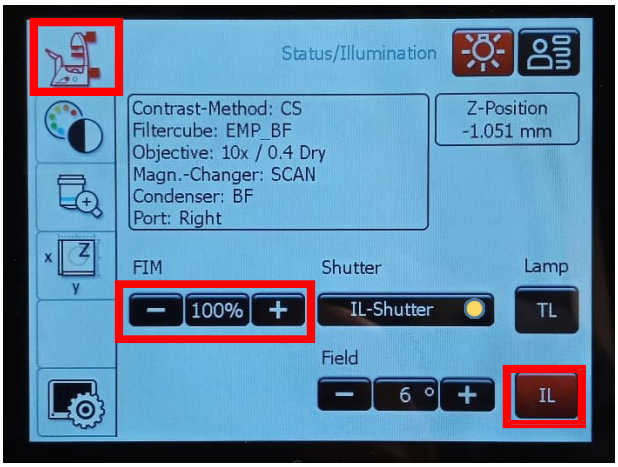


Follow these steps to have light through the eyepiece:

- 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:

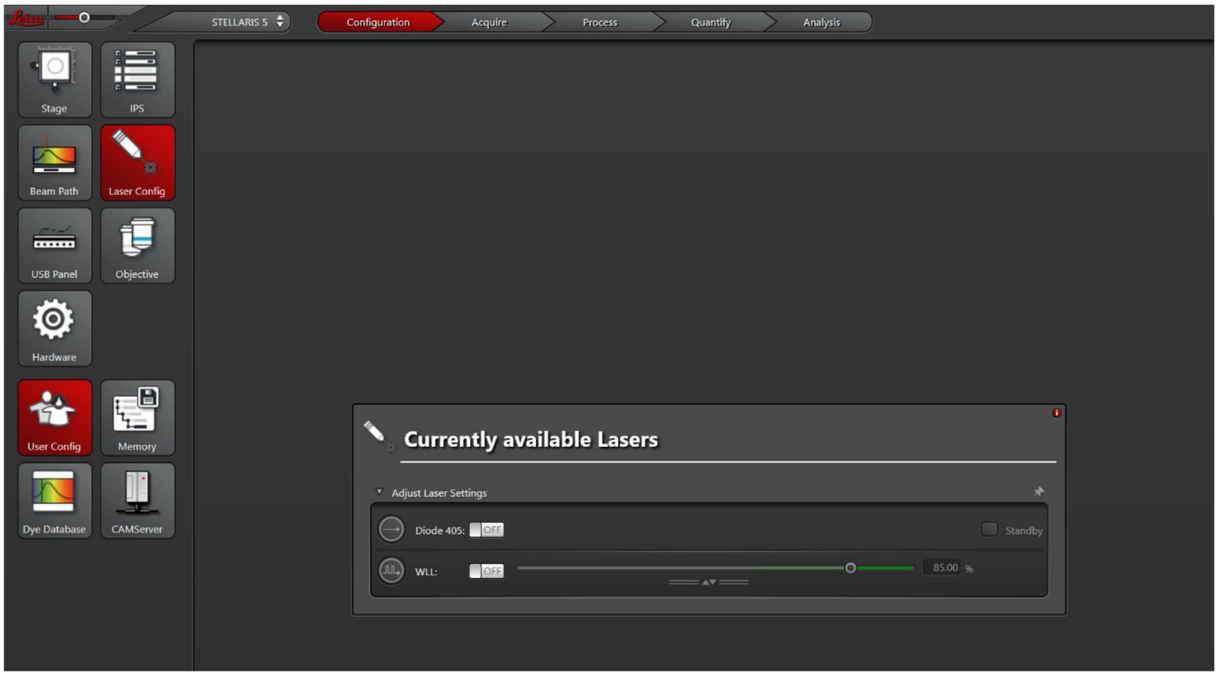




Using the LAS X software

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

Configuration tab

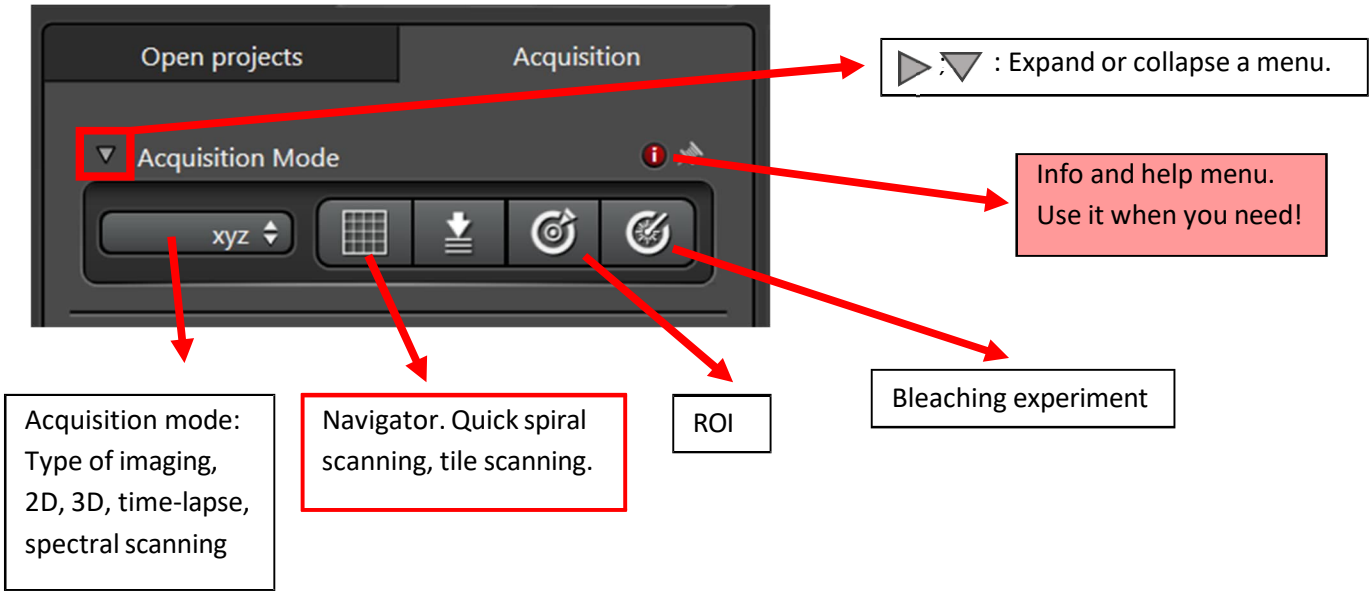


Under the configuration tab, several options can be changed and lasers can be turned ON and OFF.

- 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.



LAS X Navigator: The User Interface

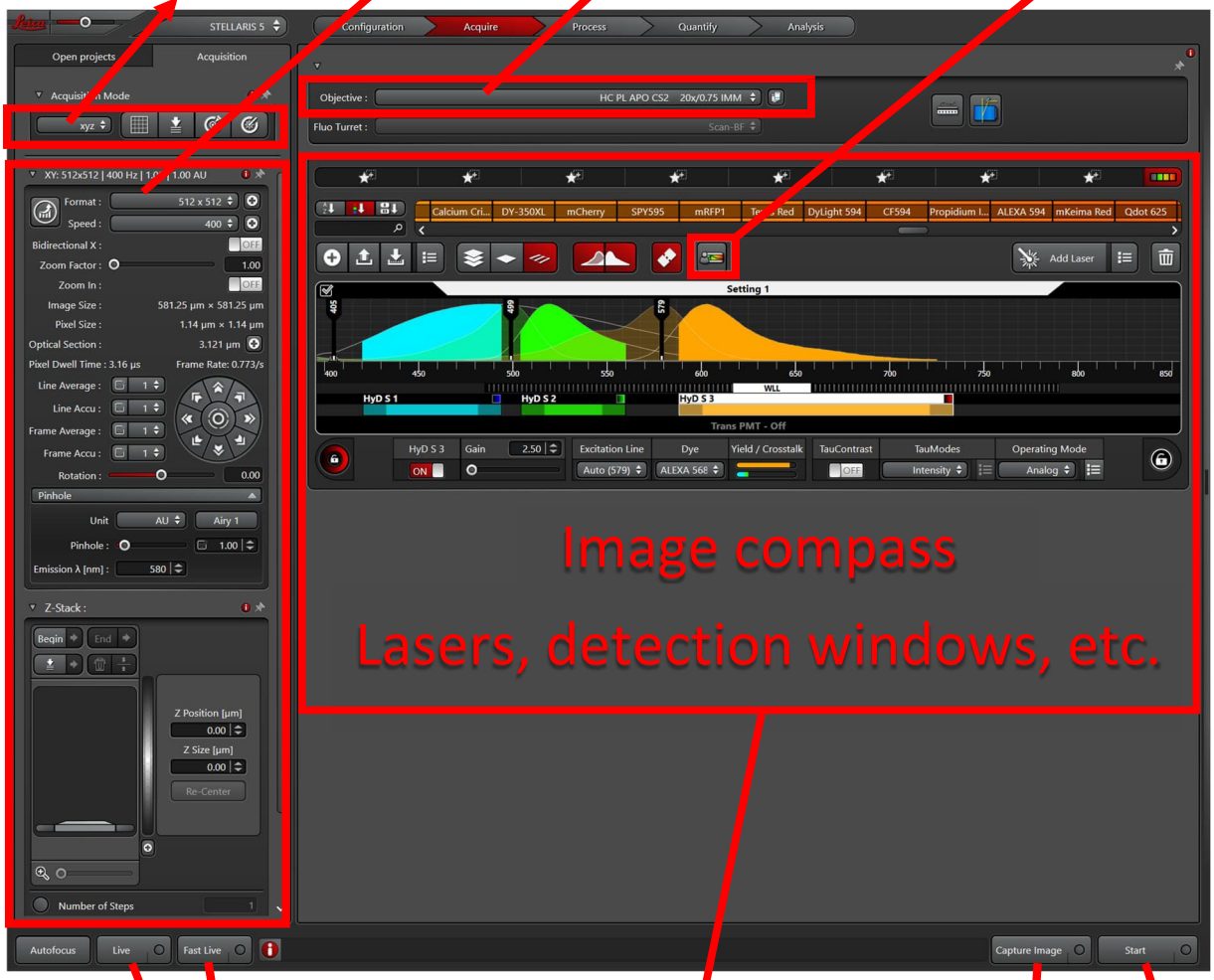
1. The viewer, functions using a mouse, functions using the context menu
2. Toolbar
3. Image Display
4. Mark & Find, scan region and focus map
5. Configuration of the Control Panel (Only for Confocal Systems)
6. Image Acquisition
7. Image List
8. Task List
9. LAS X Navigator Closing- transfers same region to Stellaris main window

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.

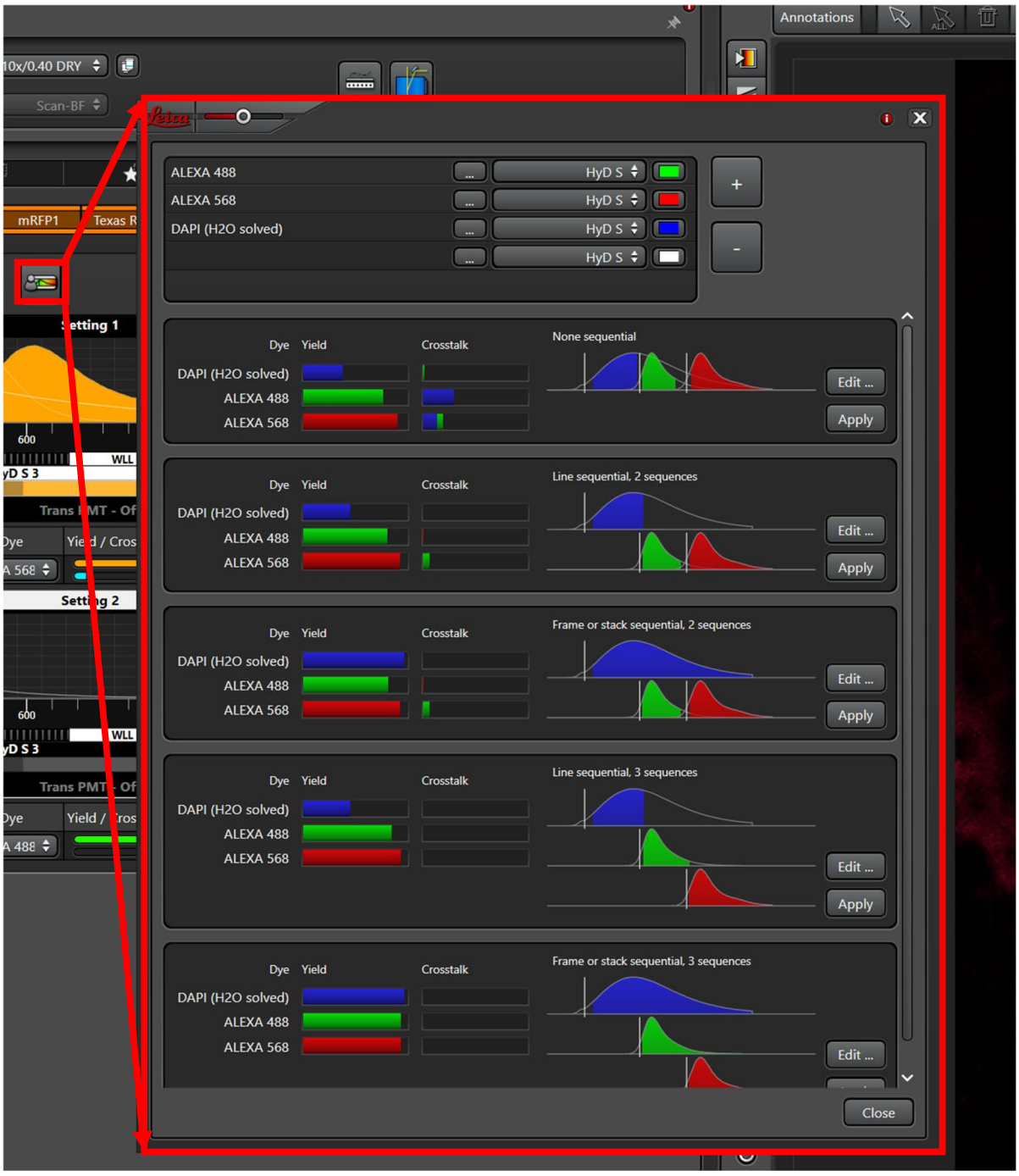
Start to acquire 3D; time-lapse;
or spectral scanning, etc.

Capture an image

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**

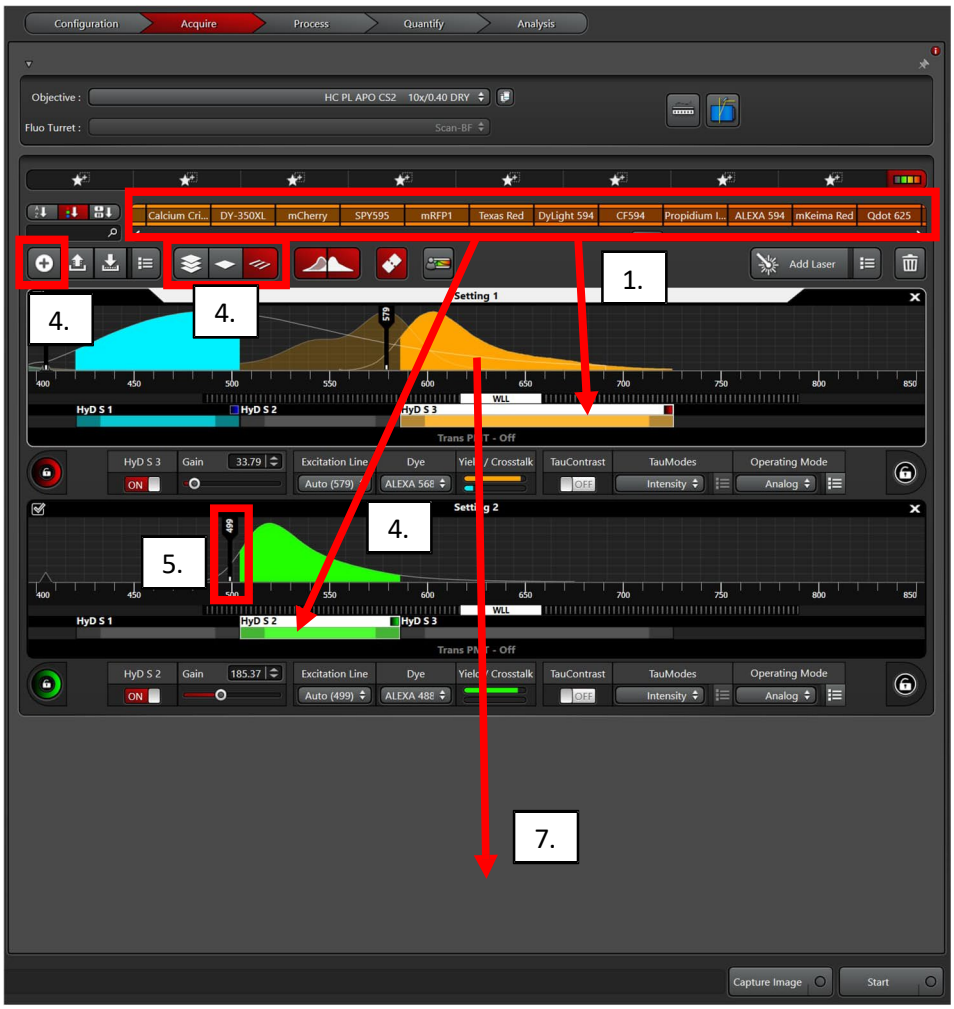


Manual mode, semi-manual mode (Image Compass)

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)

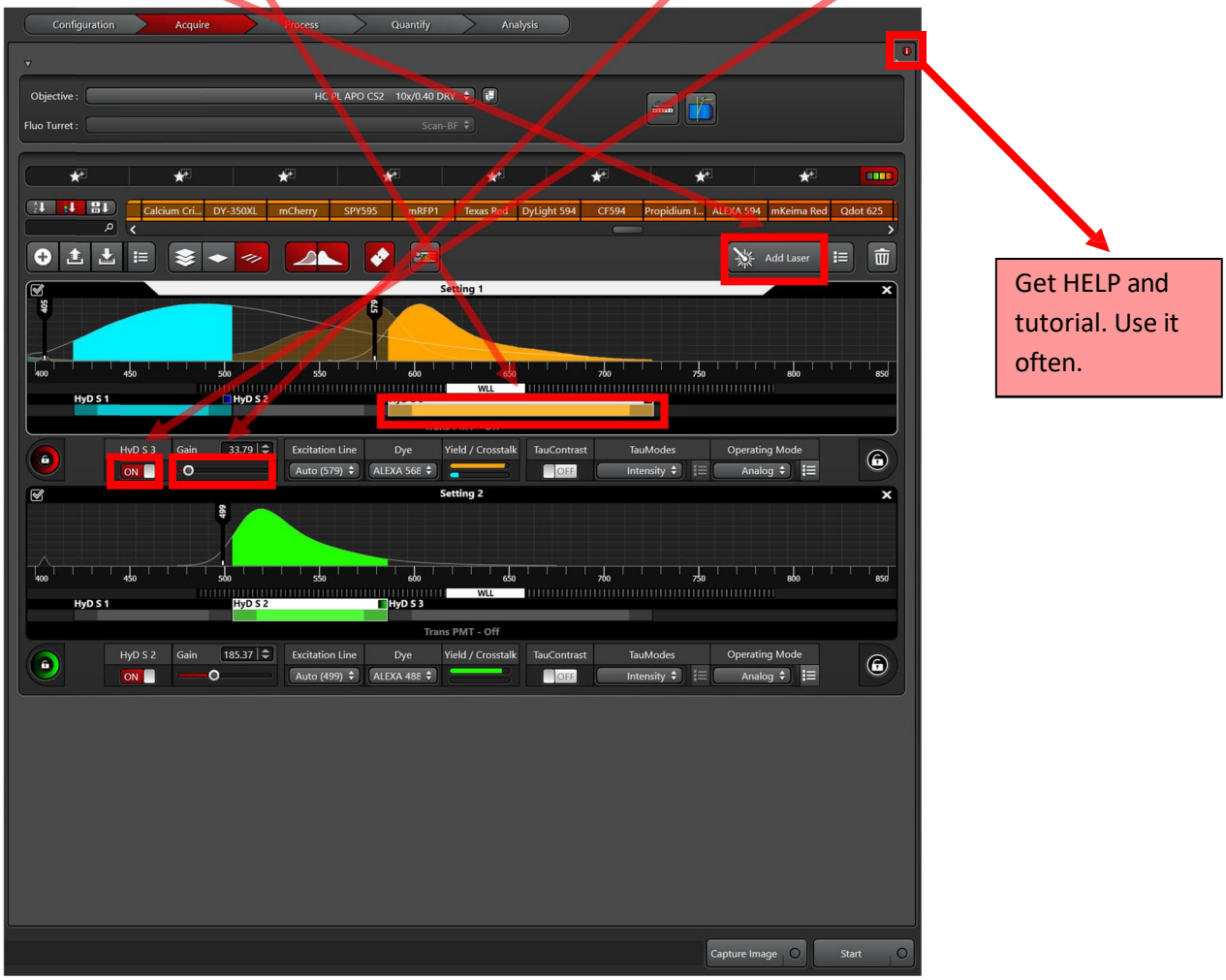
7. To delete a fluorophore from the settings, put your cursor on its emission curve and drag and drop it to an empty space. OBS ! If you want to only delete the laser line, click on it and push the thrash button (top right corner)
8. Navigation between Setting 1 and 2. You can:
   - Select a setting 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

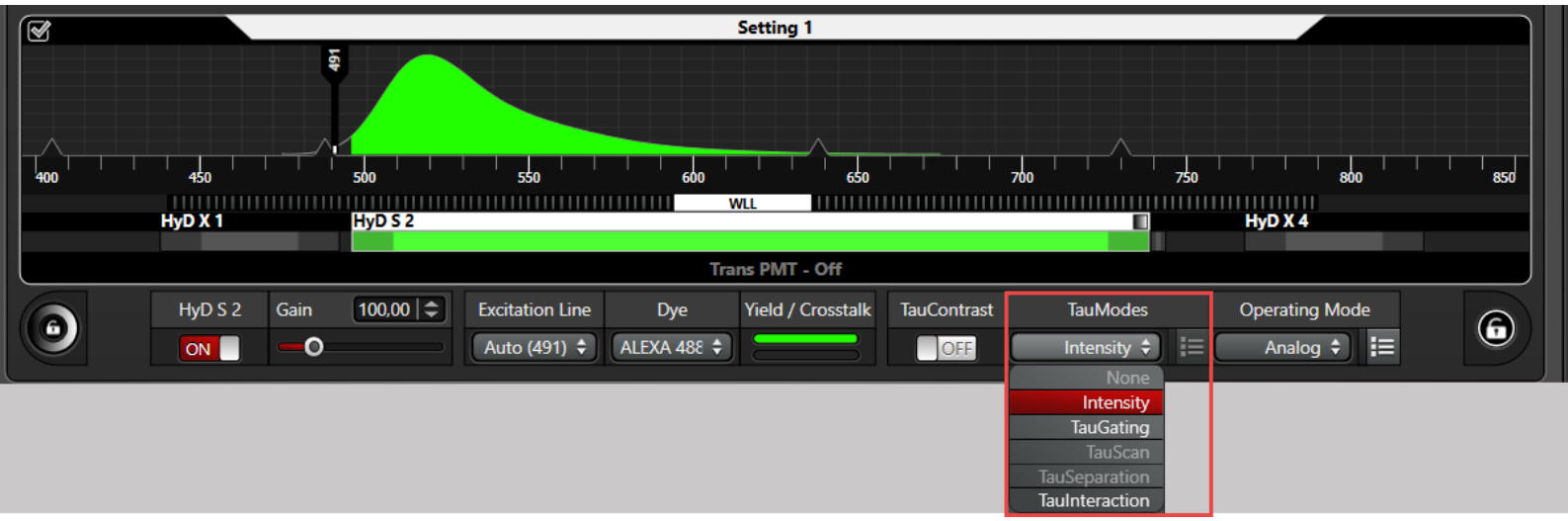
"Add Laser" (drag and drop this button to a desired laser wavelength) and turn ON a desired detector and set up the detection window, laser intensity and detector gain.



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.

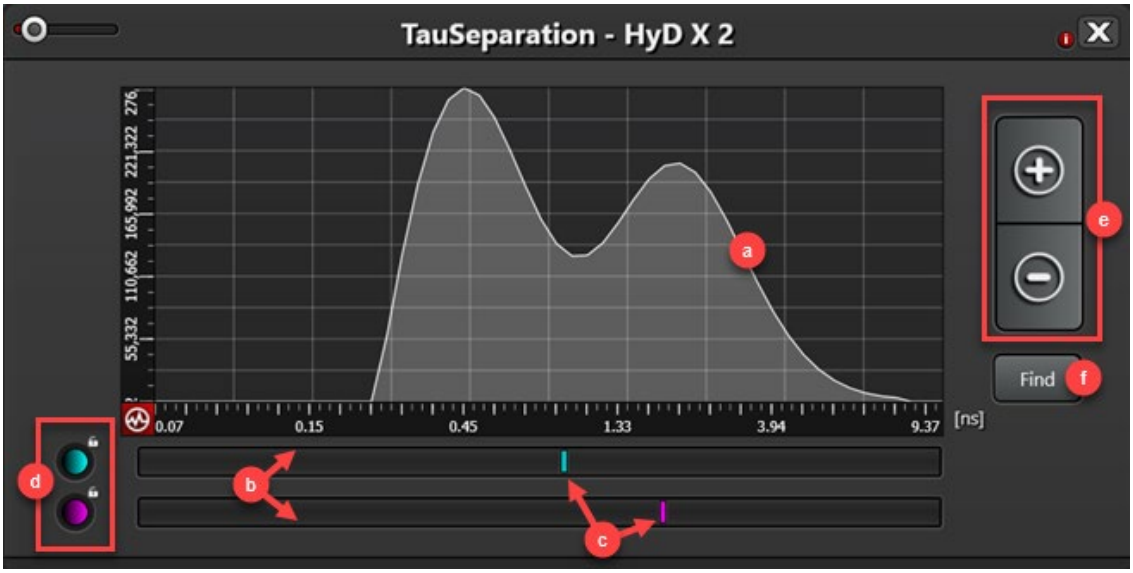
If lost, don't forget to use the built-in HELP function of the software by clicking on the closest "i" button. This is one of the best features of LAS X.

Tau modes



Tau contrast

Tau Seperation



You can use the **TauSeparation** method to separate species present in the specimen based on their fluorescence lifetimes and acquire the resulting images in separate channels. If a species has multiple lifetime components, you can also detect them separately.

a. Lifetime diversity curve: Graphical display of the frequency of lifetime components (t measured in ns) in the entire image. Each peak indicates a lifetime component present in the image.

b. Each line represents a channel. Each species is assigned a separate channel

c. The line represents a lifetime component for the respective channel. Move this line below a peak to assign a lifetime component to a channel and, consequently, to a species.

d. Here, you can adjust the color for the channel display. Double-clicking the color field opens a dialog for selection of a LUT. To prevent changes to the LUT assignment, you can lock it. To do so, click the open lock icon. The lock icon is closed and the LUT assignment is protected from further changes. Refer also to Beam Path Settings: Setting the Detectors.

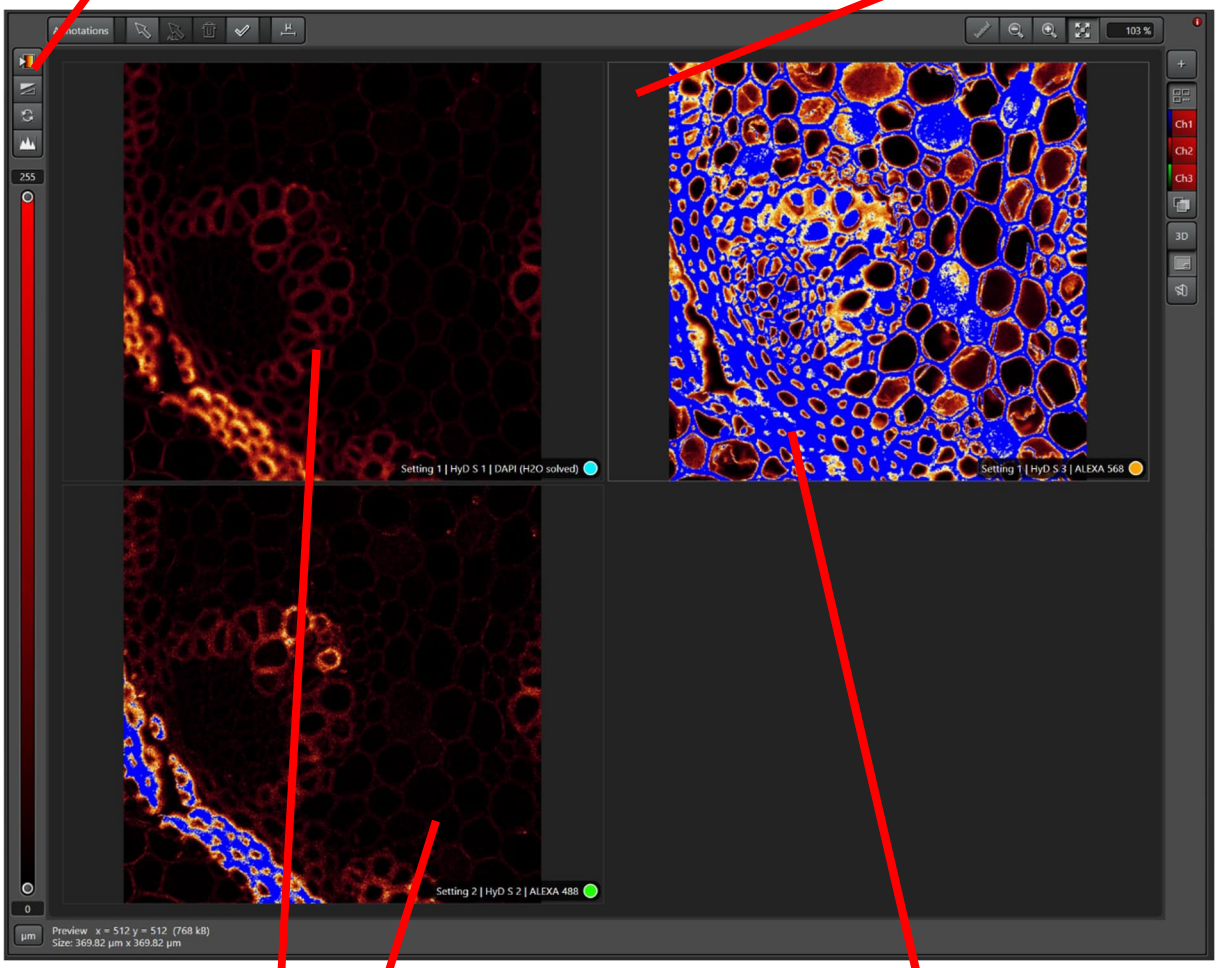
e. Using the + and - buttons, you can add further channels or species and remove them.

f. By clicking Find, the analysis is carried out for all configured channels, and optimum settings for the lifetimes are determined.

Note: The TauSeparation method is not applicable in case of reflections. If the detection range is too close to the excitation line, you might see a plateau in the lifetime distribution diagram. In this case, move the detection range further to the right to increase the distance to the excitation line.

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. There is no Offset for underexposed pixels in LAS X!



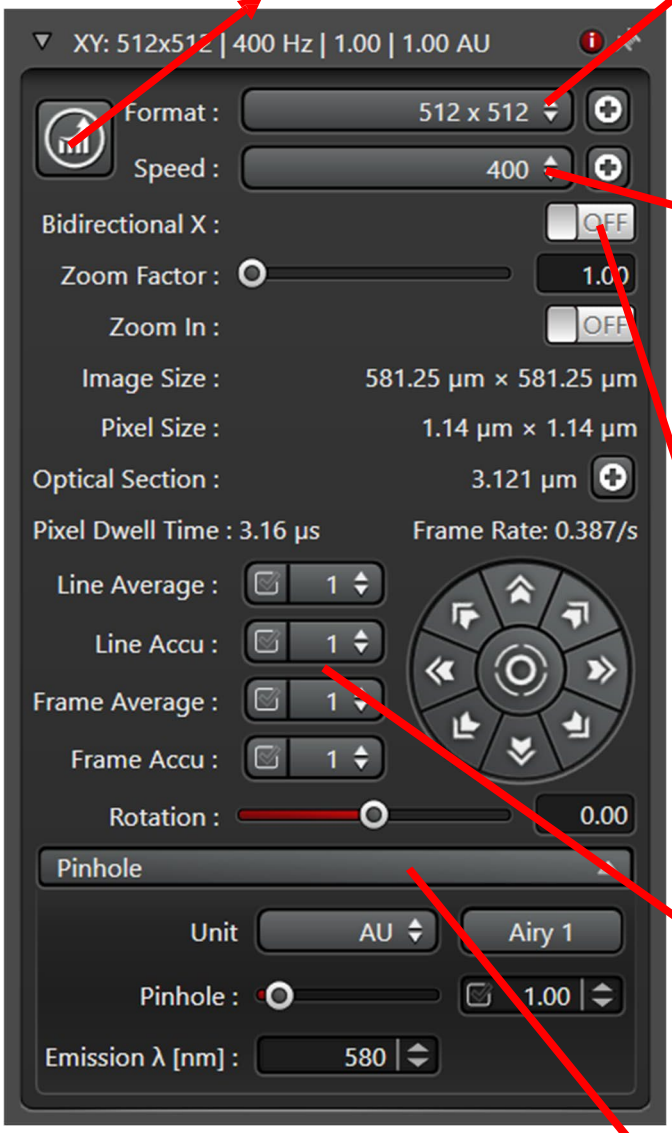
The overexposed pixels are out of the detector range, their intensity information is lost. We must avoid seeing overexposed (blue) areas. By changing (decreasing) the detector gain (voltage) or the laser intensity, we can move the overexposed areas into the range of the detector. For multichannel simultaneous 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.

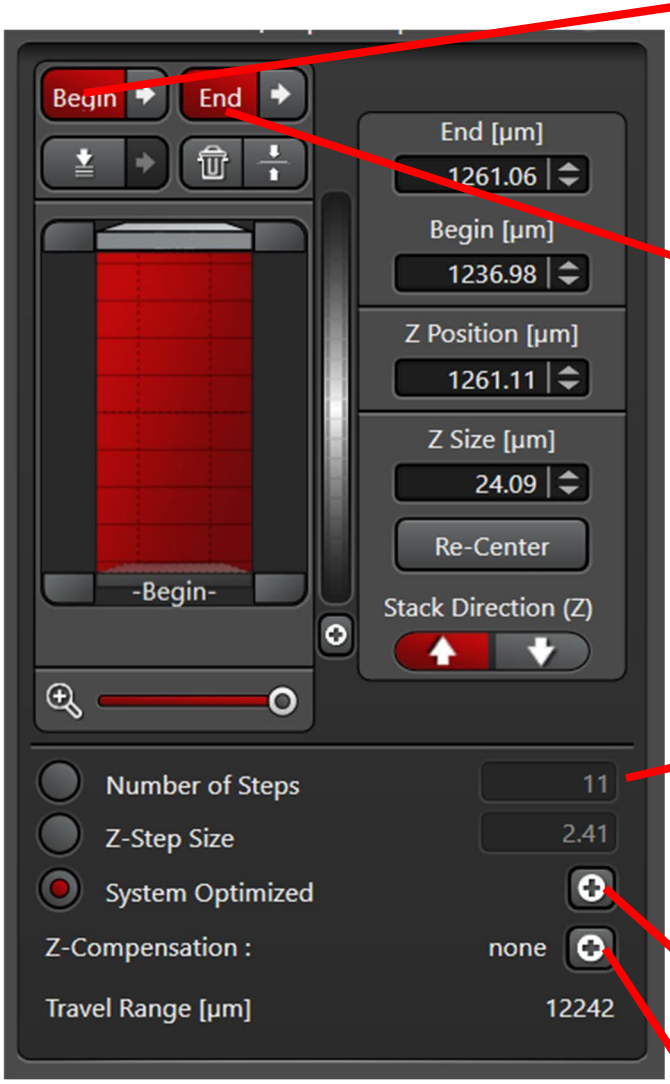
Scanning speed (Hz): go up with the speed to decrease, go down to increase the quality. No golden rule, it is sample dependent, if unsure try the sample with different speeds, and use the best for the real acquisition. Again COMPROMISE between time and quality, decide what is more important.

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

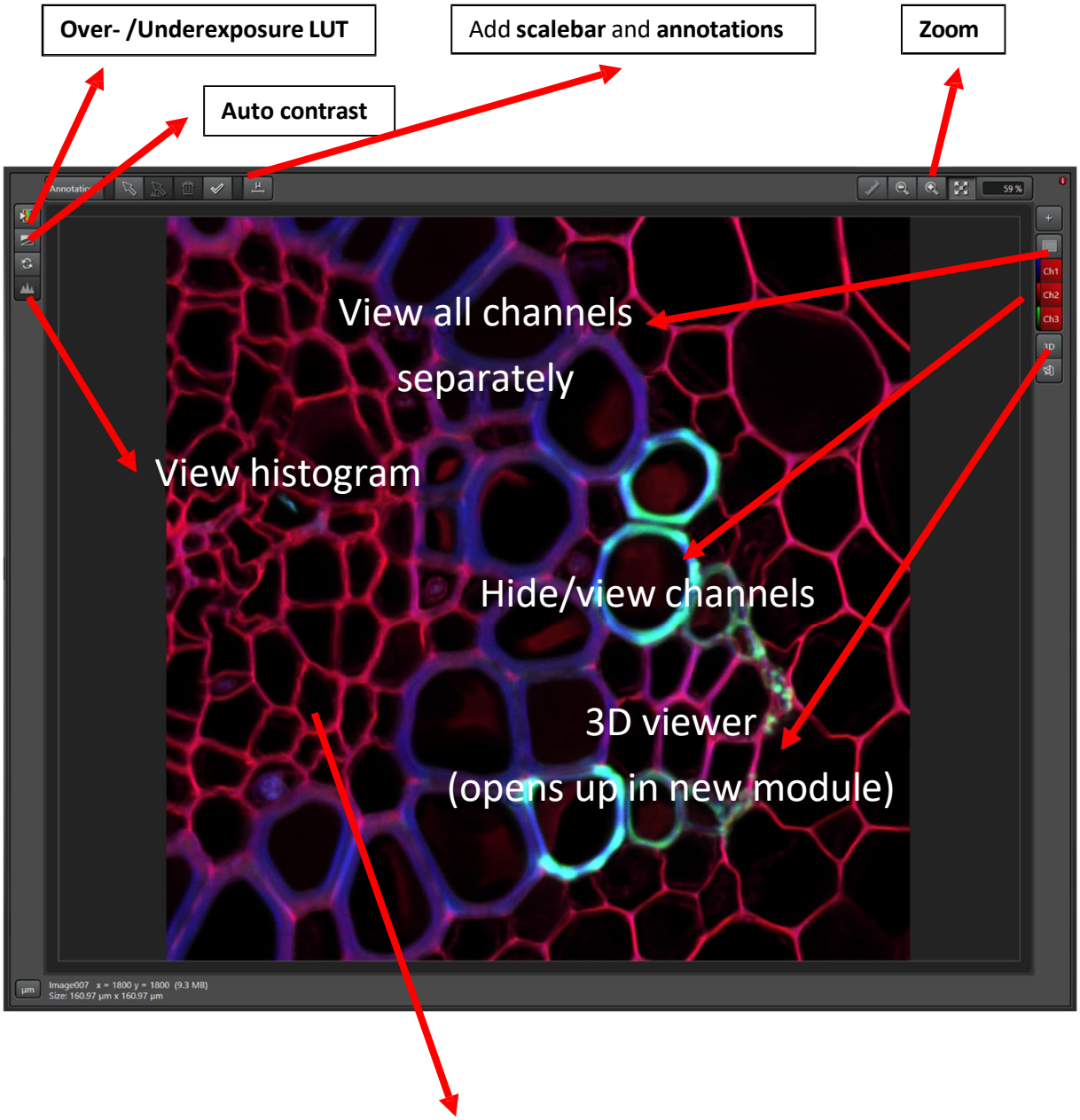
5. Setup for linear laser or detector gain compensation when signal is getting lost in deep samples.

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



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**.

Carrying Out a ΛΛ Scan

For an excitation/emission wavelength scan (**ΛΛ-Scan**), an excitation wavelength scan (**Λ-Scan**) is combined with an emission wavelength scan (**λ-Scan**). During this process, in each detection step of the λ scan, a complete Λ scan is carried out. This means the detection range remains unchanged for the individual detection steps, while the excitation wavelength runs through the entire spectrum every time.

The acquisition of an excitation/emission spectral image series can be used to determine both the excitation and emission spectrum of a fluorochrome at the same time. This way, a profile of a fluorochrome can be created under various conditions or in various specimens, environments, organisms, etc.

These instructions guide you through the procedural steps for acquiring an excitation/emission spectral image series.

The ΛΛ scan is recorded in the **Acquire** step.

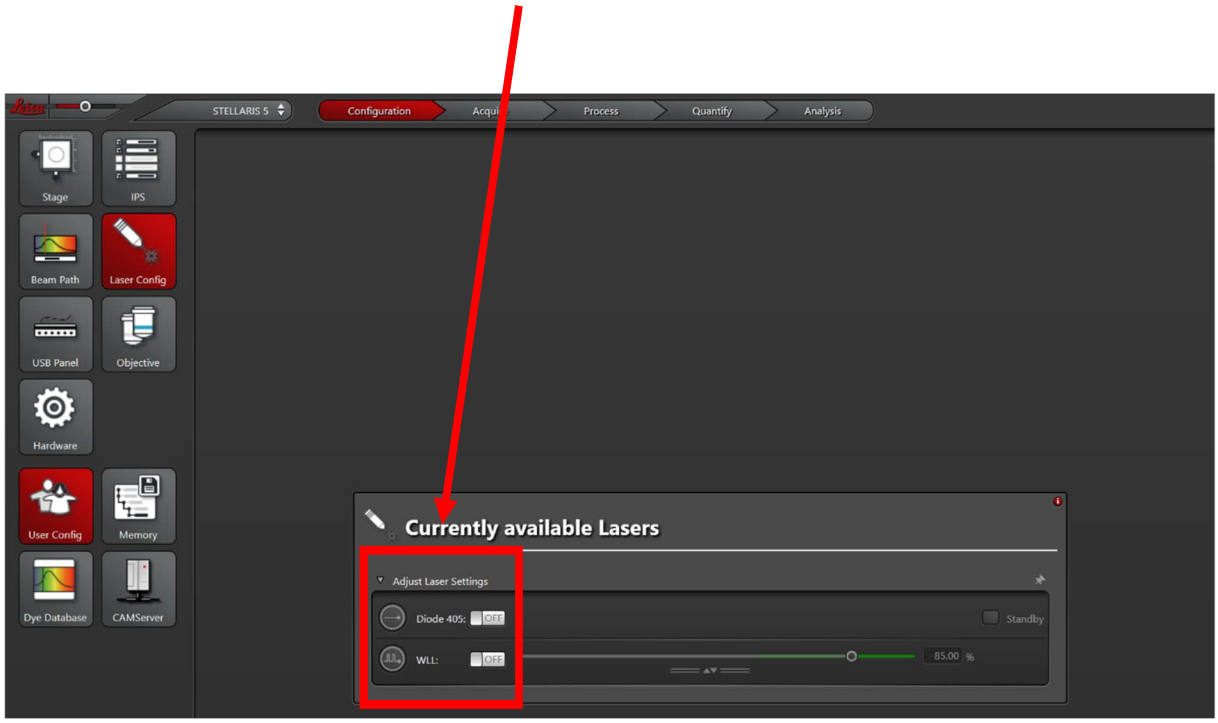
Requirement: The specimen is in focus.

Note
A ΛΛ scan is carried out with a white light laser. Make sure that no notch filter is in the detection range.

- **1. Selecting Acquisition Mode xyΛλ**
- **2. Adjusting Wavelength and Detection Range**
- **3. Additional Settings**
- **4. Showing the Graphic Display of the Scan and Detection Range**
- **5. Adjusting the Intensity Control for the White Light Laser**
- **6. Carrying Out a ΛΛ Scan**
- **Possible cause of reflections in the fluorescence image**

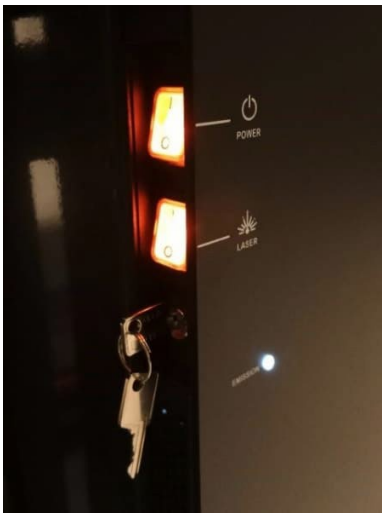
Shutting down the microscope

1. **Clean** after yourself and **cover** the microscope stand with its dust cover.
2. 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**).

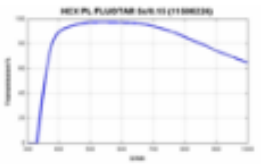
4. **Close** down the LAS X software, **wait** till fully OFF
5. **Shut down or log off** the computer, **wait** till fully OFF. There is a billing script that runs on login and logoff. If there is an error let someone know so time can be accurately recorded.
6. **Turn OFF** the scanner box switches below the table:
   i. Laser button OFF
   ii. 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. Clean any oil immersion objectives with lens paper and ethanol. REPLACE COVER OVER MICROSCOPE!!!

HC PL FLUOTAR 5x/0,15 #506224

5x	0.15	13.7
Mag	NA	WD (mm)



TL-BF | TL-DIC | TL-P | TL-DF | TL-IMC | IL-BF | IL-DIC | IL-P | FLUO

Techniques

IL, LightSheetIllumination, TL

HC PL APO 10x/0,40 CS2 #506424

10x	0.4	2.56
Mag	NA	WD (mm)

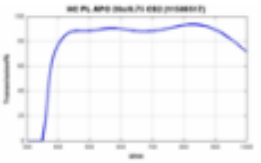
TL-BF | TL-DIC | TL-DF | FLUO

Techniques

Confocal, CS2, TL

HC PL APO 20x/0,75 CS2 #506517

20x	0.75	0.62
Mag	NA	WD (mm)



TL-BF | TL-DIC | TL-DF | TL-IMC | FLUO

Techniques

Confocal, CS2, TL

HC PL APO 40x/1.30 OIL CS2 #506428

40x	1.3	0.17
Mag	NA	WD (mm)

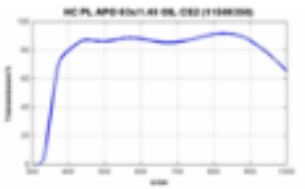
TL-BF | TL-DIC | TL-IMC | FLUO

Techniques

Confocal, CS2, TL

HC PL APO 63x/1,40 OIL CS2 #506350

63x	1.4	0.14
Mag	NA	WD (mm)



TL-BF | TL-DIC | TL-IMC | FLUO

Techniques

Confocal, CS2, TL