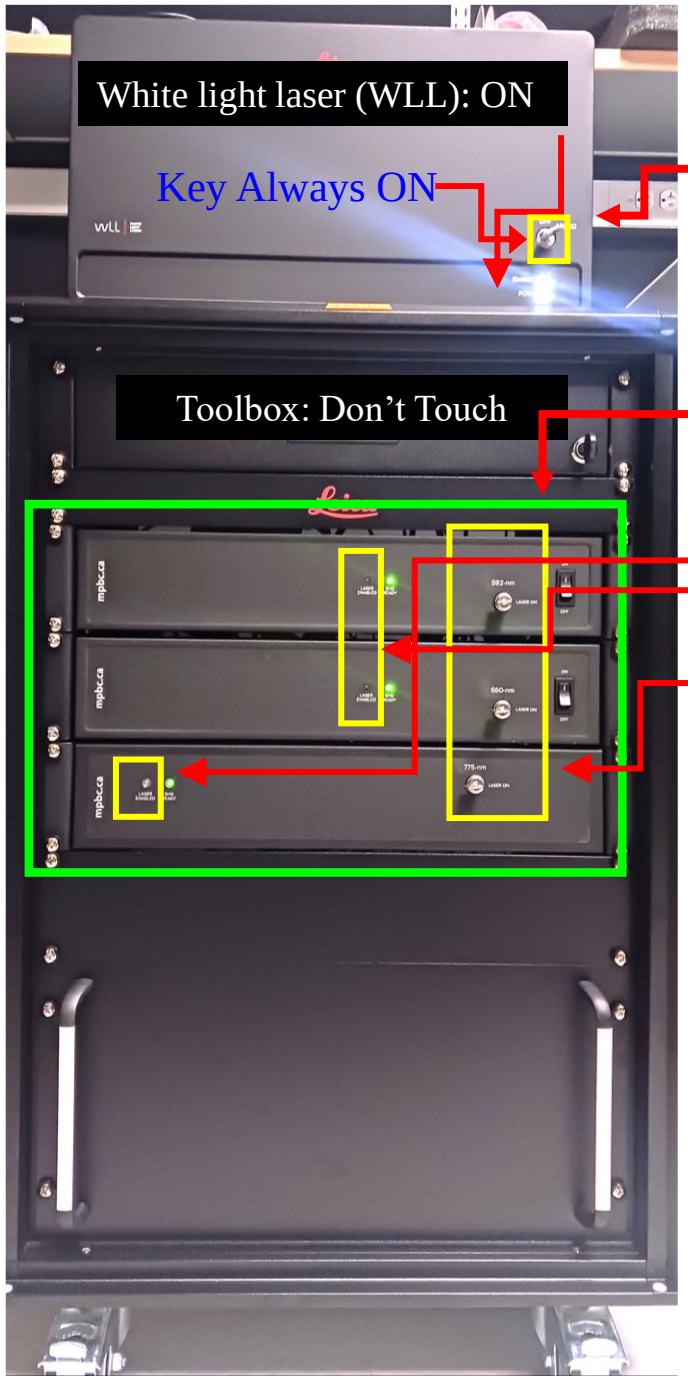
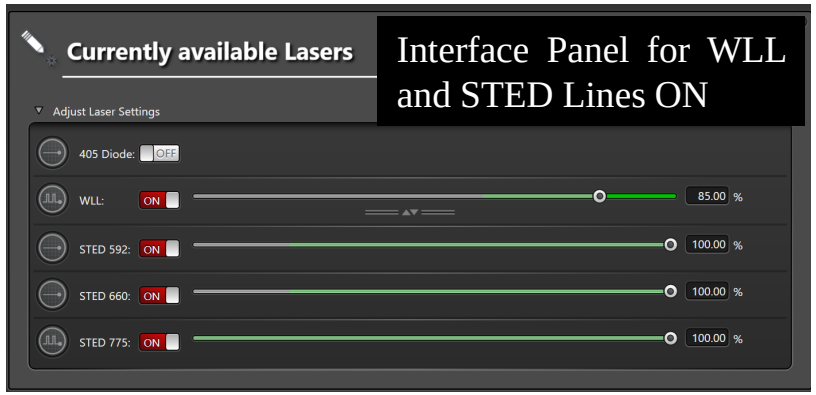
Leica STELLARIS 8 Confocal/FLIM/tauSTED Operating Instructions

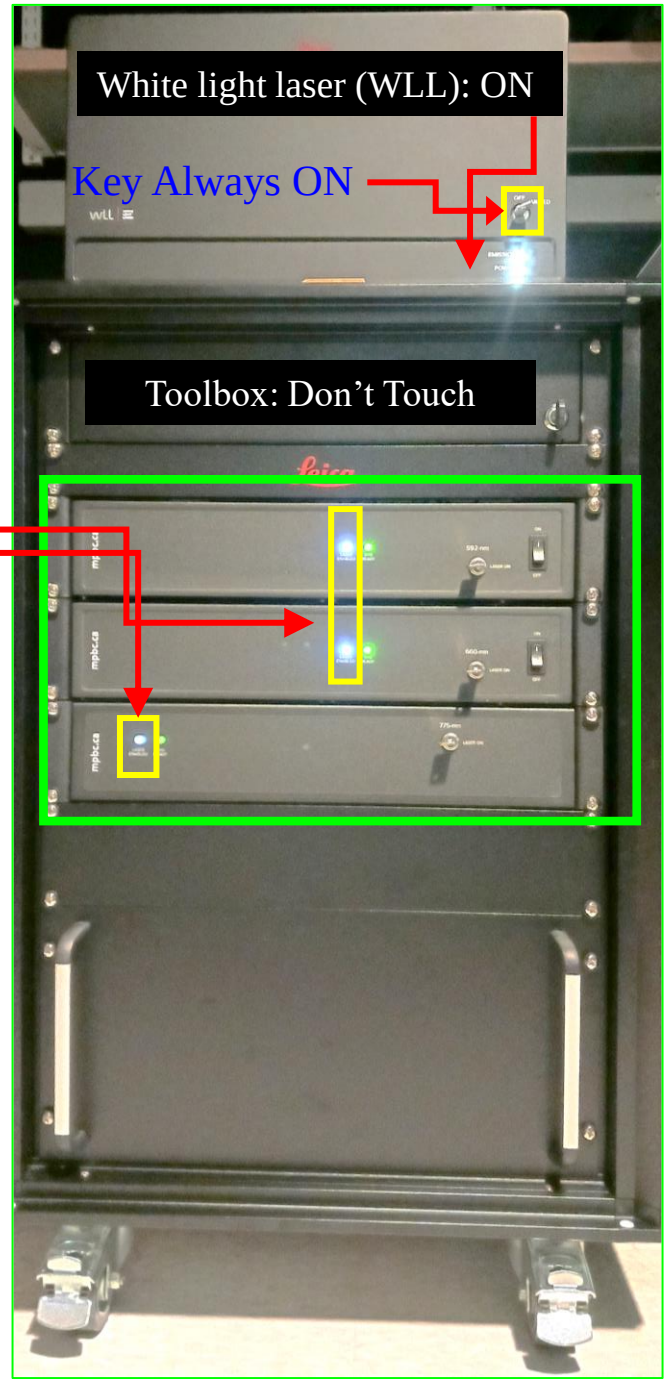
System Overview
(STED Application)

Hardware/Software Indications for STED Applications

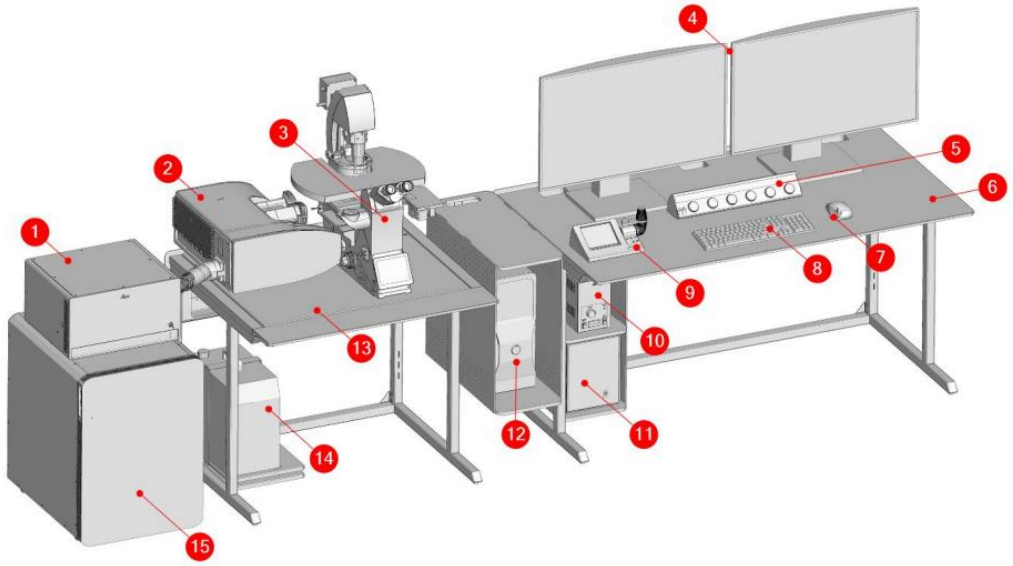
- White light laser (WLL) lines for conventional excitation: 440-790 nm.
- STED Depletion lines – There are three STED depletion laser lines: 592 nm, 660 nm, and 775 nm.

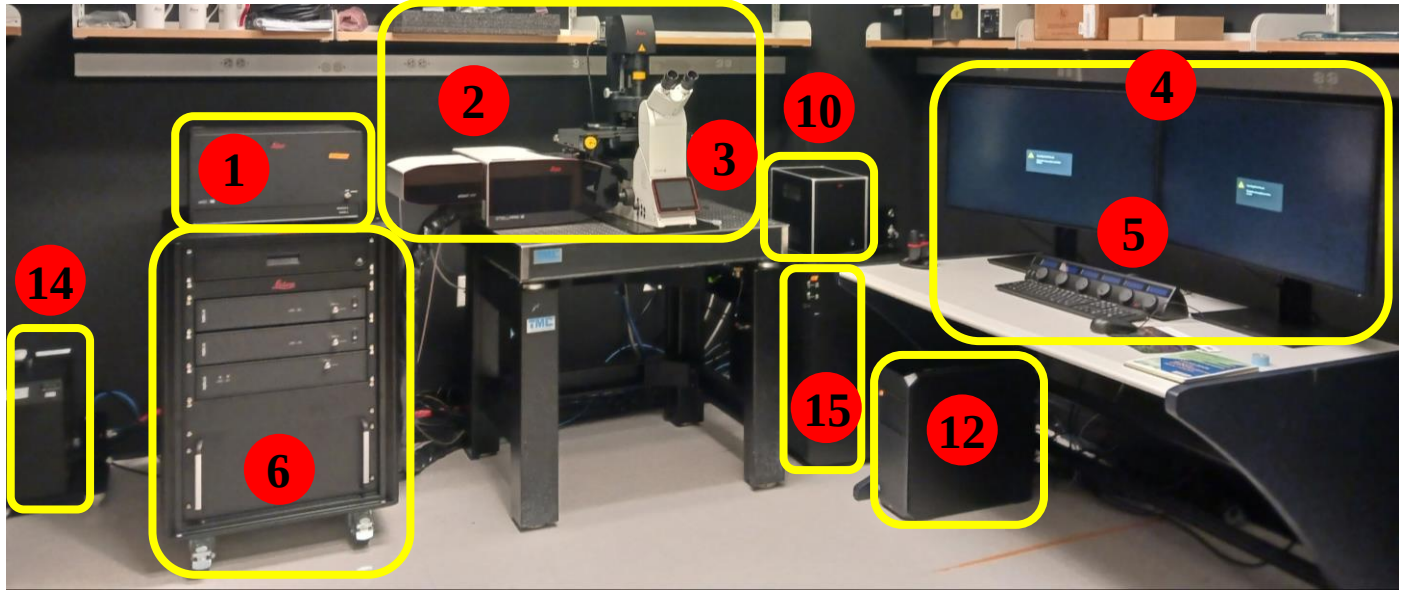






Hardware information





1	White Light Laser (WLL)	**6**	**STED Lines**
2	Scan Head	**10**	External light source
3	Microscope	**12**	Workstation
4	Monitor(s)	**14**	External cooling (chiller)
5	Control panel	**15**	Supply Unit

System Start-up

1. Turn on the power (wait for few seconds) and laser button as shown in the figure 1.

*Note: Do not touch any key.

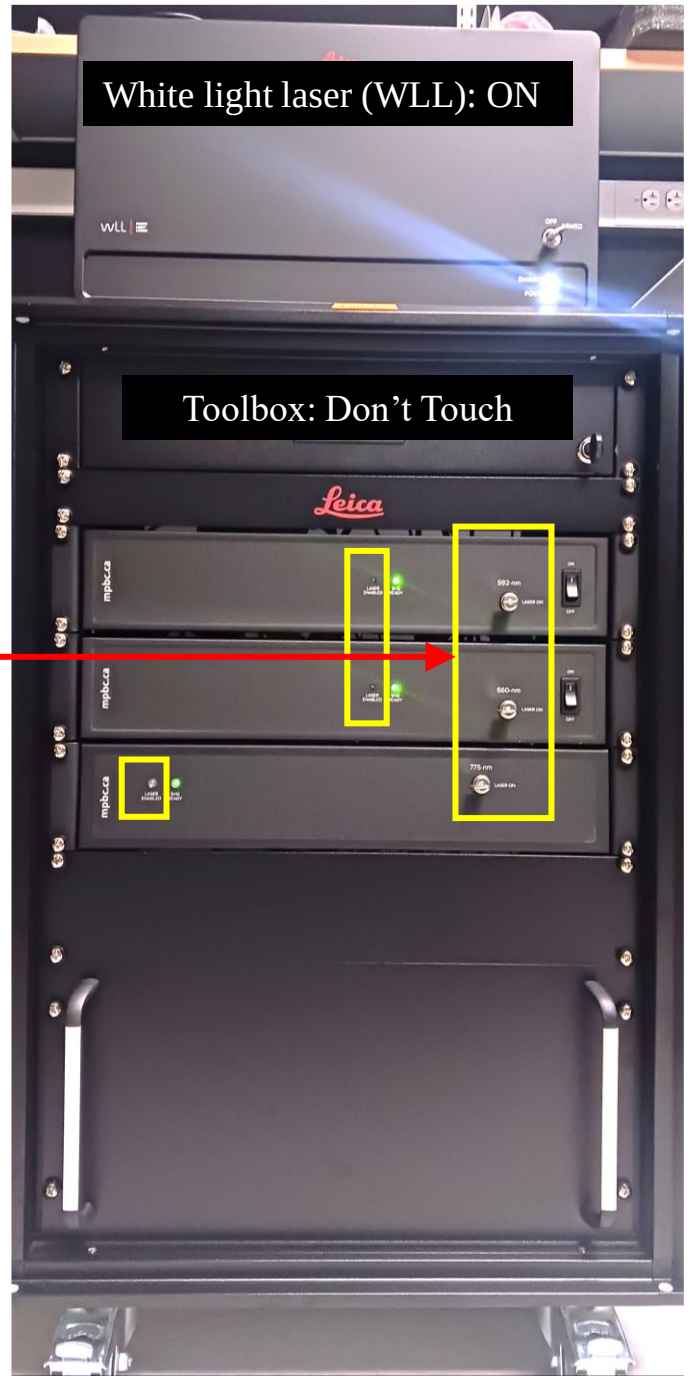
2. Turn ON the computer and wait.
3. Log into the computer with your own KCCI user account.
4. Record the starting time at the KCCI website

Keys Always ON but to before using the STED application follow the instruction 5.

5. Toggle the Keys (turn OFF and turn ON)

6. Start the system by double clicking the LAS X icon on the desktop.

***Note:** The LAS X software is used to control all system functions and acts as the link to the individual hardware components. Image acquisition, image analysis and image processing are carried out using LAS X.

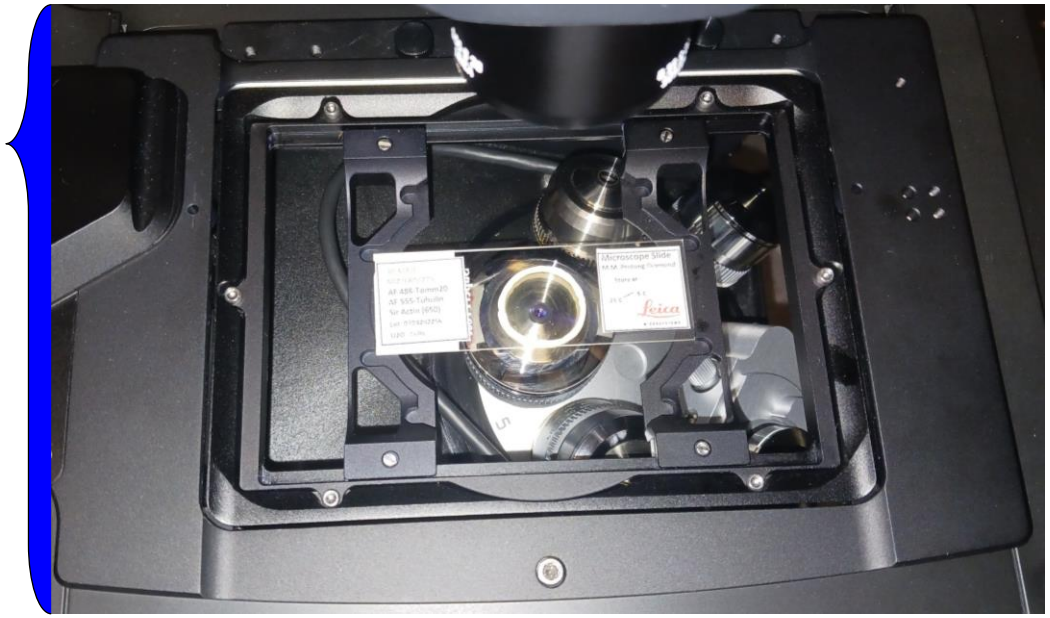


7. Select the Machine as configuration and DM18 in the Microscope section as shown.
8. **Toggle** the Turn OFF button to Turn ON from the panel for **STED** as wel as for the **Load Settings** at Start-up.
9. Now start LAS X by clicking the OK button.

10. Wait Until the software initialization completes.
11. Wait for the LAS X Graphical User Interface window.

Sample mounting and fluorescence observation through eyepieces

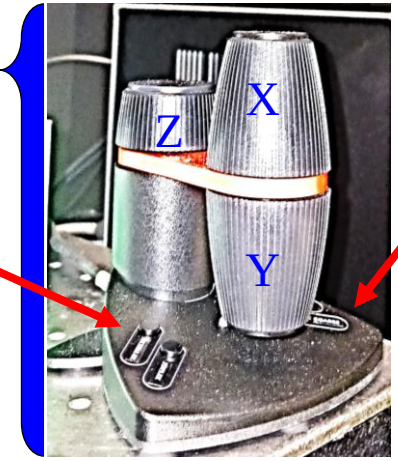
1. Tilt the illumination column.
2. Verify that the correct sample holder stage inset is correctly secured in the XY stage.



3. If using immersion lens, clean the lens and apply proper immersion.
4. Never clean dry lens!
5. If suspected dirty, or if immersion gets onto a dry lens, contact KCCI staff immediately!

Sample mounting and fluorescence observation through eyepieces

6. Secure your sample, thin coverslip Facing Down. The objectives cannot focus through a glass slide. If using immersion lens, quickly bring the immersion to contact with the sample using coarse/fast Z-focus (and XY-stage) adjustment on the "Smart Move" controller.



7. Using the Aperture (aperture diaphragm) or Field (field diaphragm) keys, the motorized diaphragms can be changed at any time. The display on the Touch Screen changes accordingly.

Using the microscope touchscreen

On touch screen click the highlighted icon  for basic microscope settings. Select the status/illumination menu using light source icon. Adjust the field diaphragms using + and – keys.
FIM: Fluorescence intensity manager.
IL: Incident light (Fluo: Fluorescence light)
TL: Transmitted light

Contrast Tab or Modality Tab: 

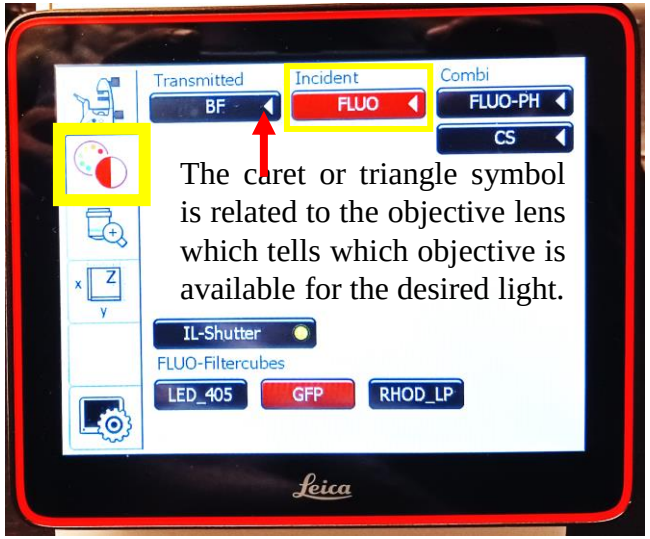
Transmitted light: Bright field (BF)
Incident light: Fluorescence (FLUO)
Combination: Fluorescence-Phase (FLUO-PH) and (CS)

The shutter can be operated using the buttons on the touch screen.

Click the FLUO for fluorescence incident light method
The available filter cubes are displayed. Select the desired cube using the corresponding button.

To see on the screen, use the highlighted settings.

To see through the ocular, use the highlighted settings.



Using the microscope touchscreen

Note: Use the microscope touch screen to select the objective lens not the software

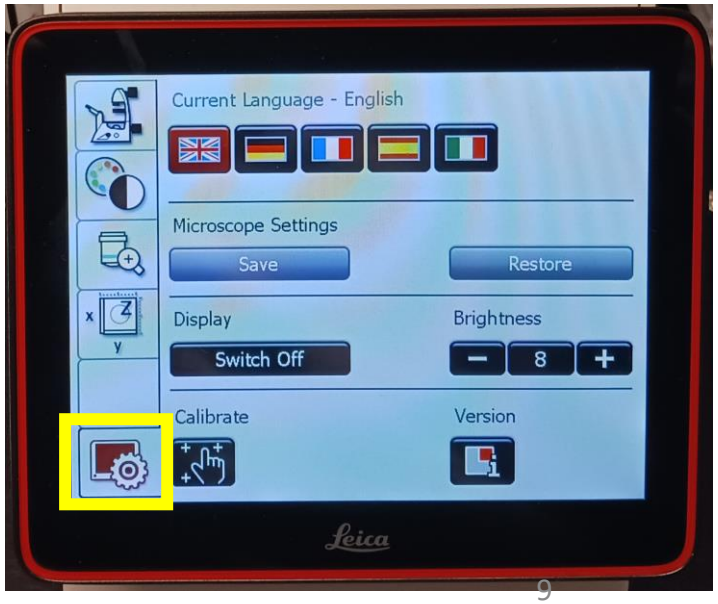
Objective Tab

1. Shows the total magnification on the eyepieces.
2. Available objectives: Here 5.
3. Click the objective button from the touch screen and select the required objective.
4. If the selection of the objective start blinking, press it one more time to confirm the selection.

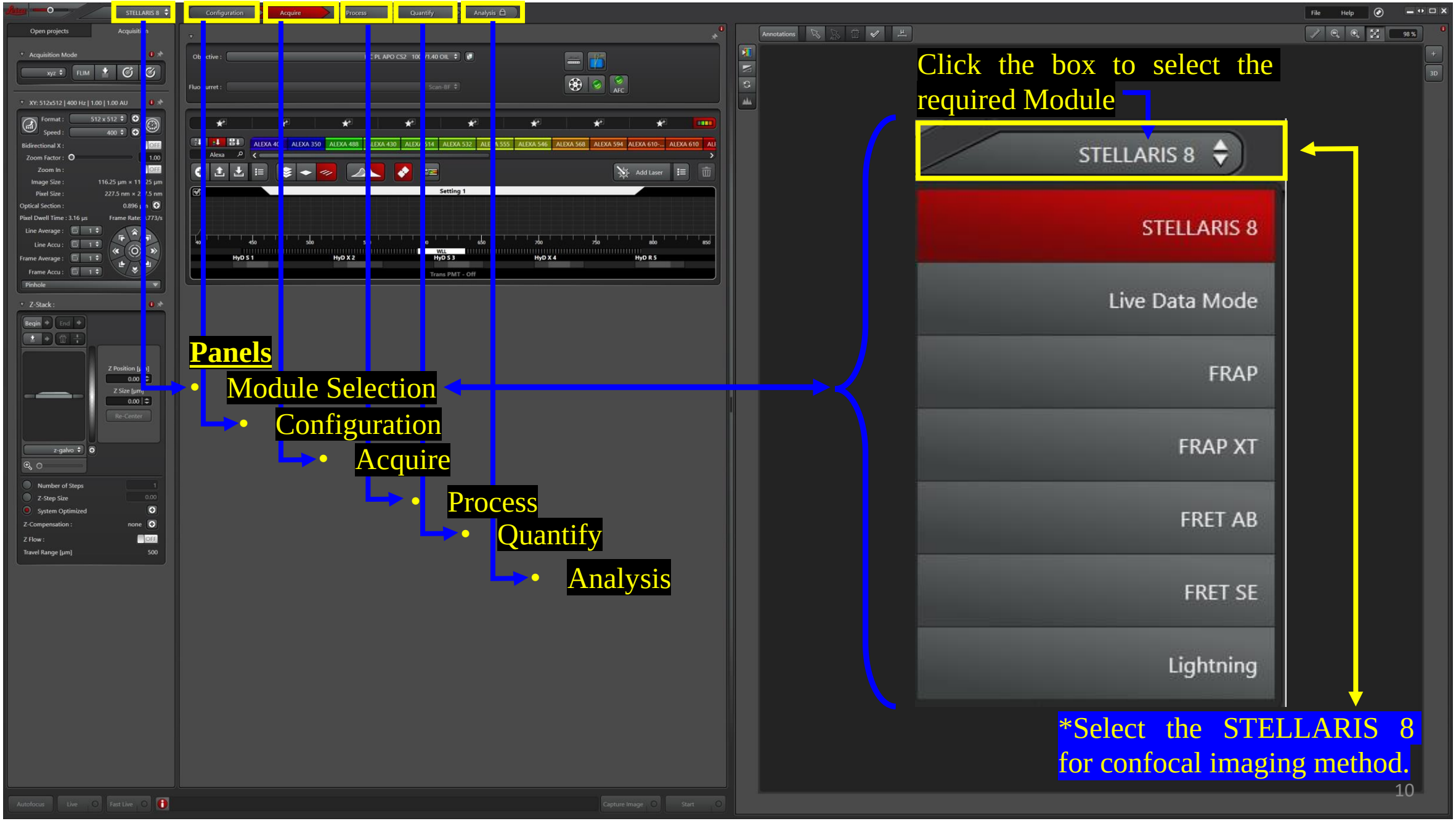
Note. For STED applications 100x/1.4 oil and 86x/1.2W for water are available.

5. Use only Type F immersion liquid for the objective if required.

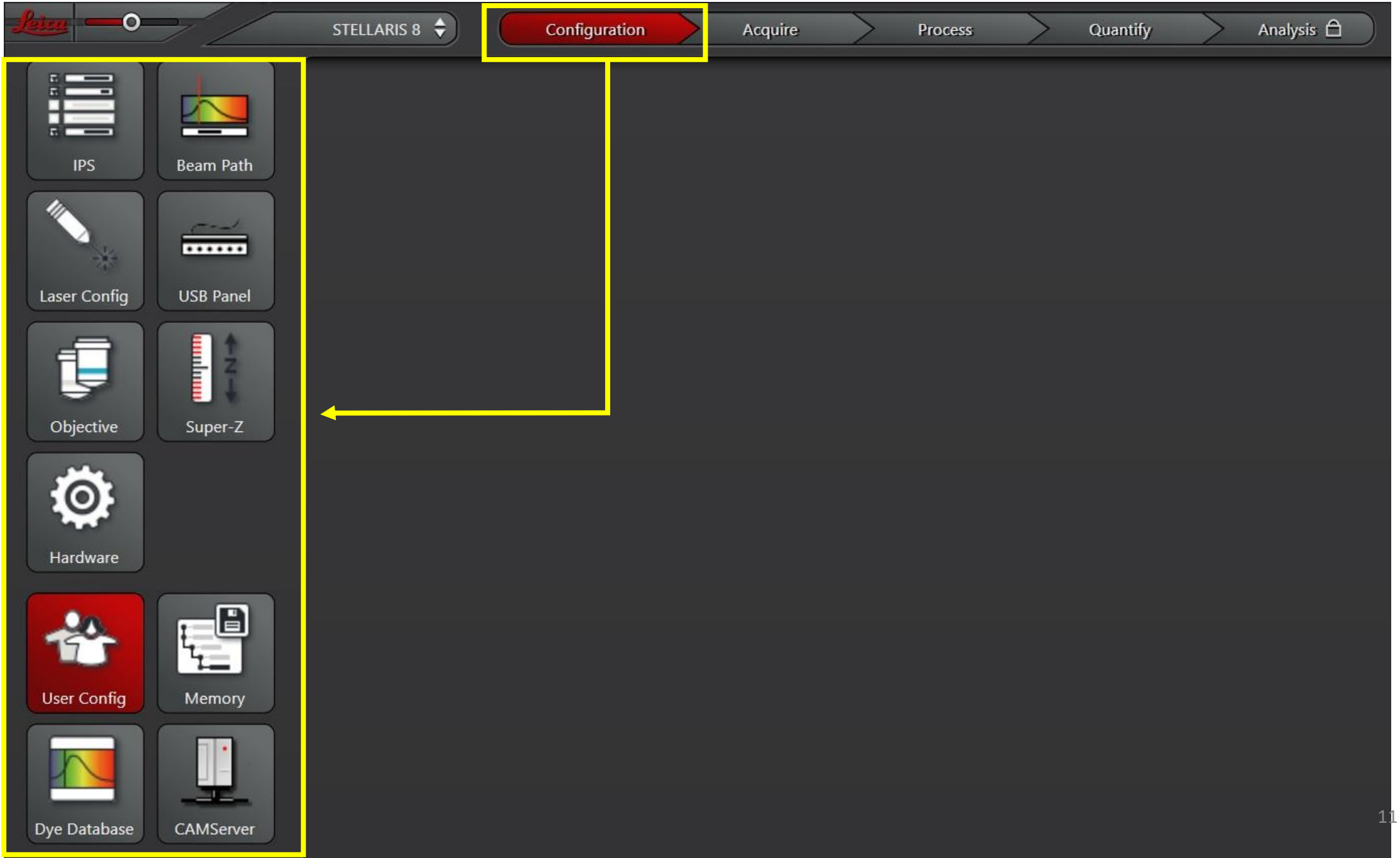
6. In the objective Tab: Port: Eyepieces 100 % indicates the whether the light is going to the eyepieces or to the camera.



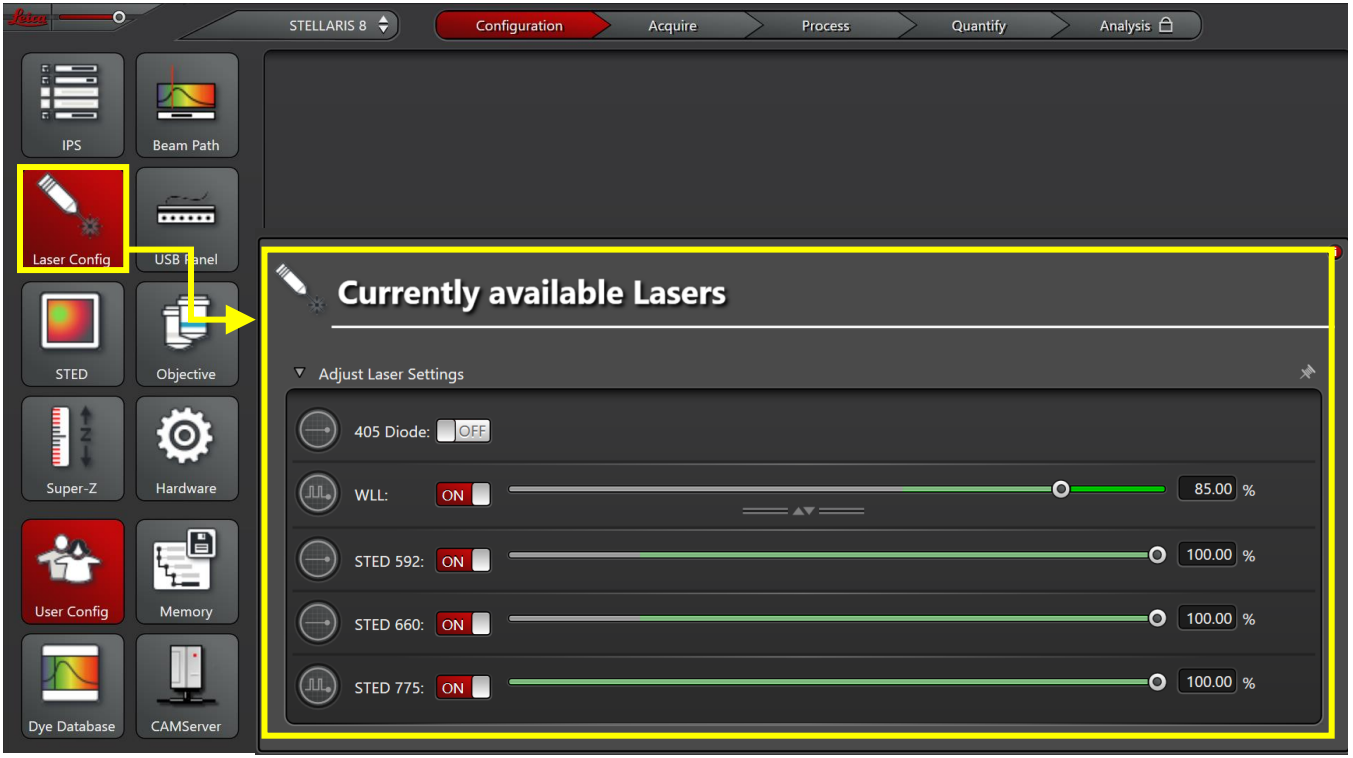
Structure of the LAS X Graphical User Interface



Click the configuration button to activate the configuration



STEP:1
Turning ON the LASER



- Turn ON the white light laser (WLL) from the software.
- The STED configuration has three STED depletion laser lines: 592 nm, 660 nm, and 775 nm.

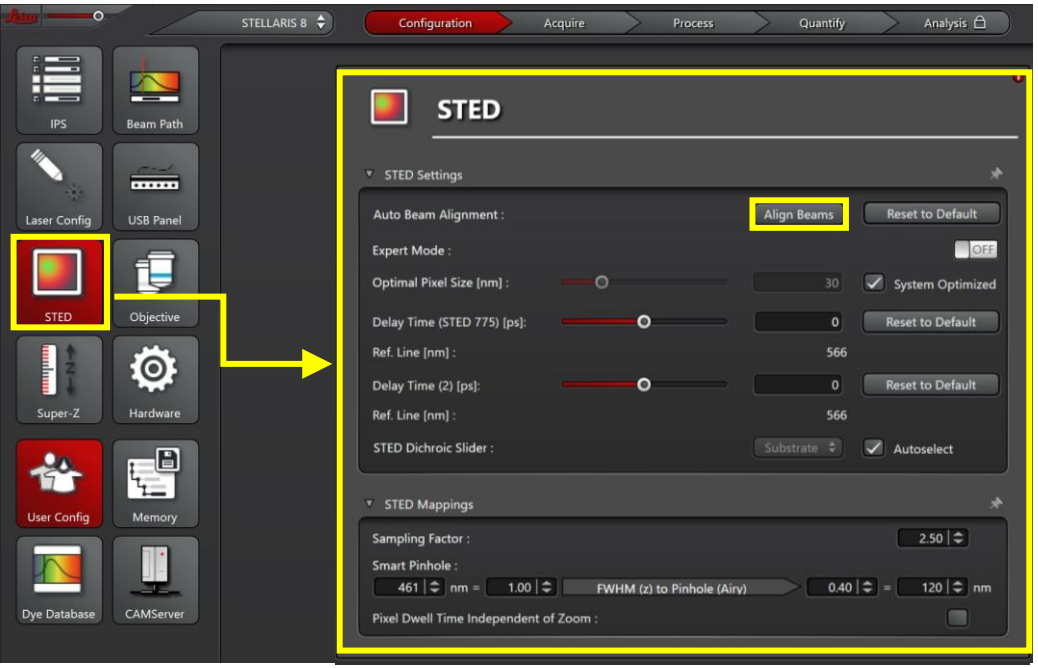
- Toggle the OFF button to turn ON to switch ON the WLL and Required STED Lines from the Laser Configuration sub-panel under the Configuration panel.

- Note: Keep the STED lines power (100 %) as indicated the Left panel.

- Wait for at least 30 minutes to warm up the LASERs.

STEP 2.
Beam Alignment

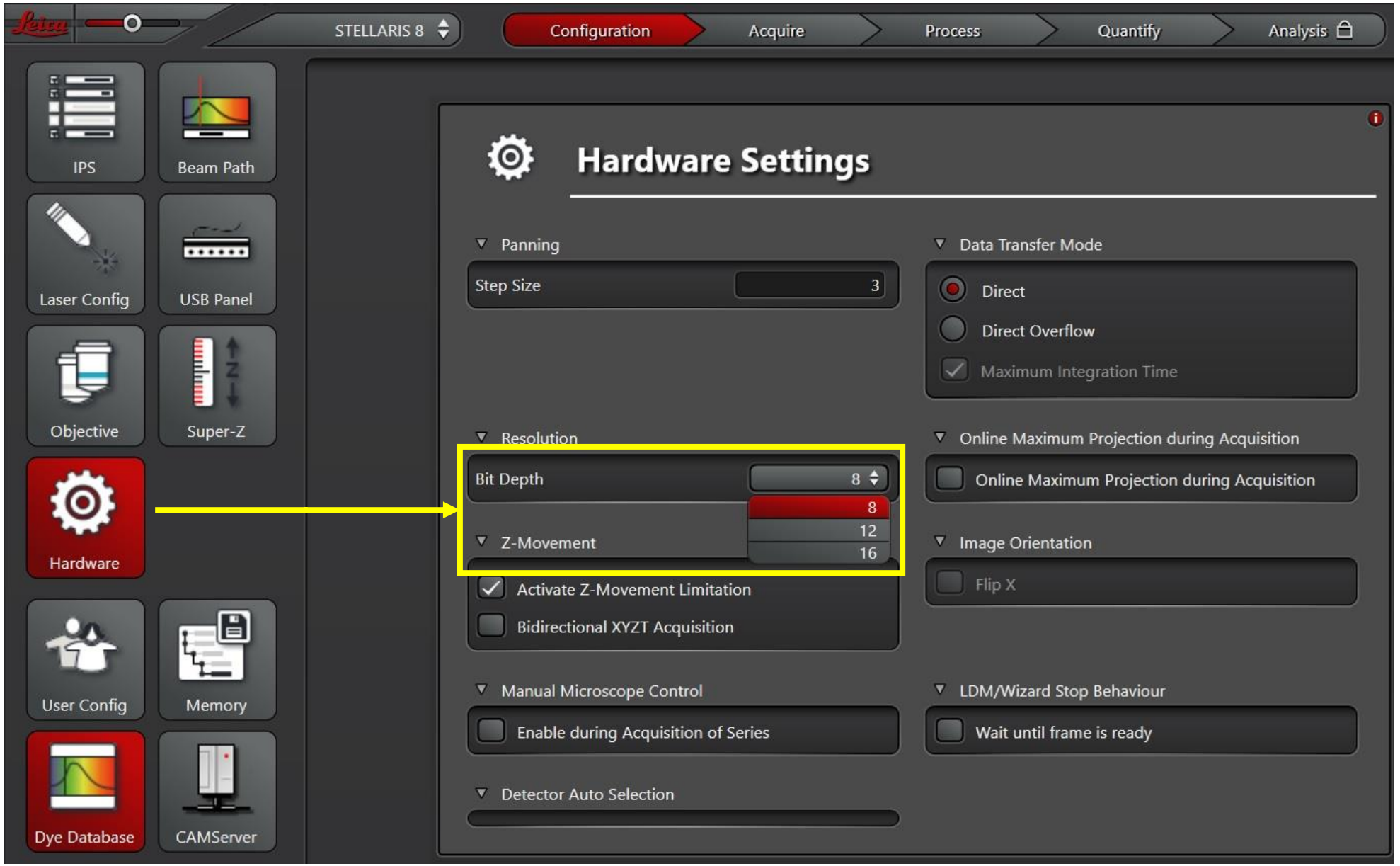
*Note: Beam alignment is the essential condition for the STED applications.



Wait for 3-4 min

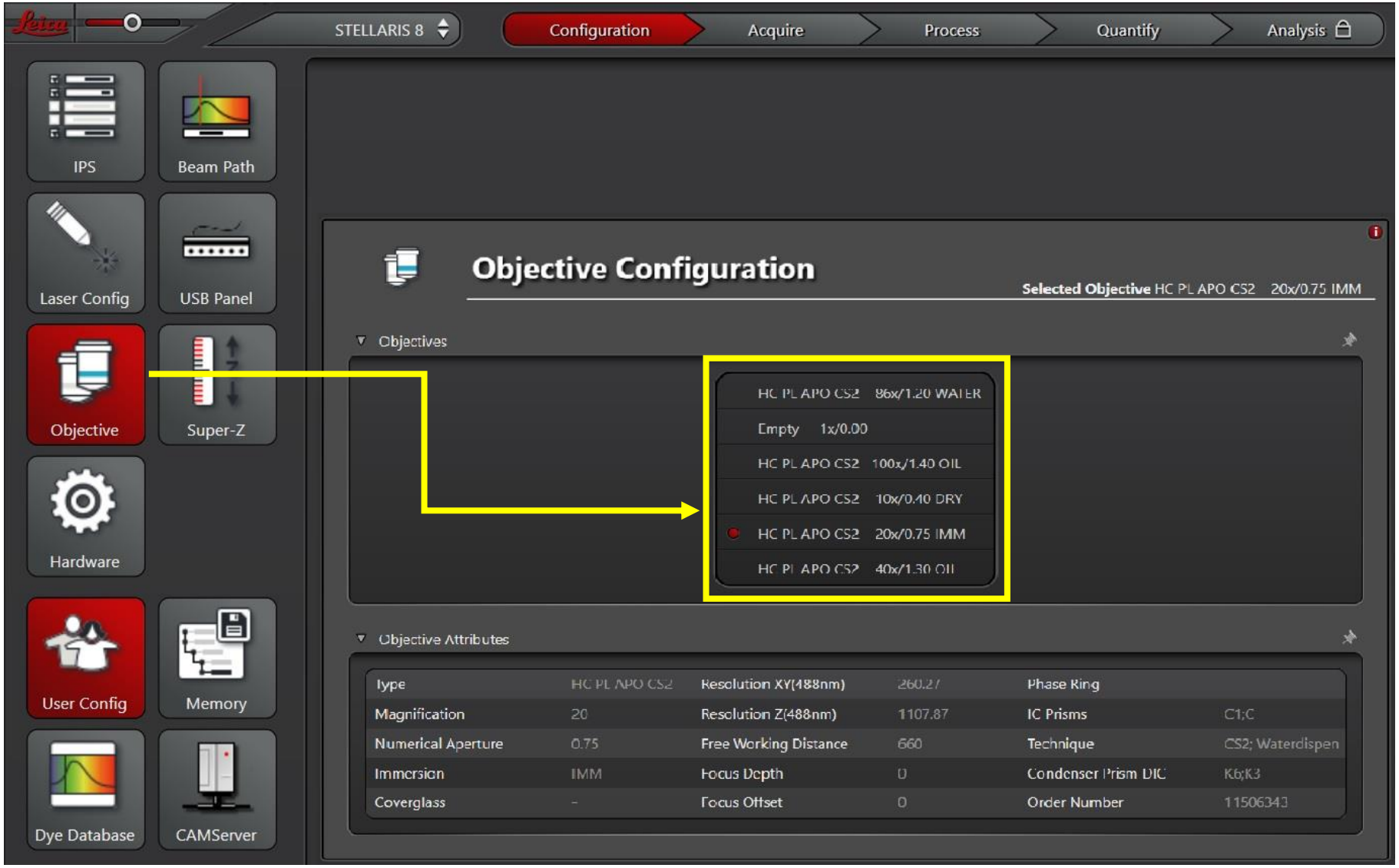
- Click the STED option under the configuration panel.
- Click on Align Beams button and wait for 3-4 minutes to complete the process.
- In case Alignment Fails. Repeat the NOTE 1 and NOTE 2.
- After successfully completing the beam alignment close the dialog box and go to the Acquire panel.

Click the Hardware button to select the Panning, Resolution etc.



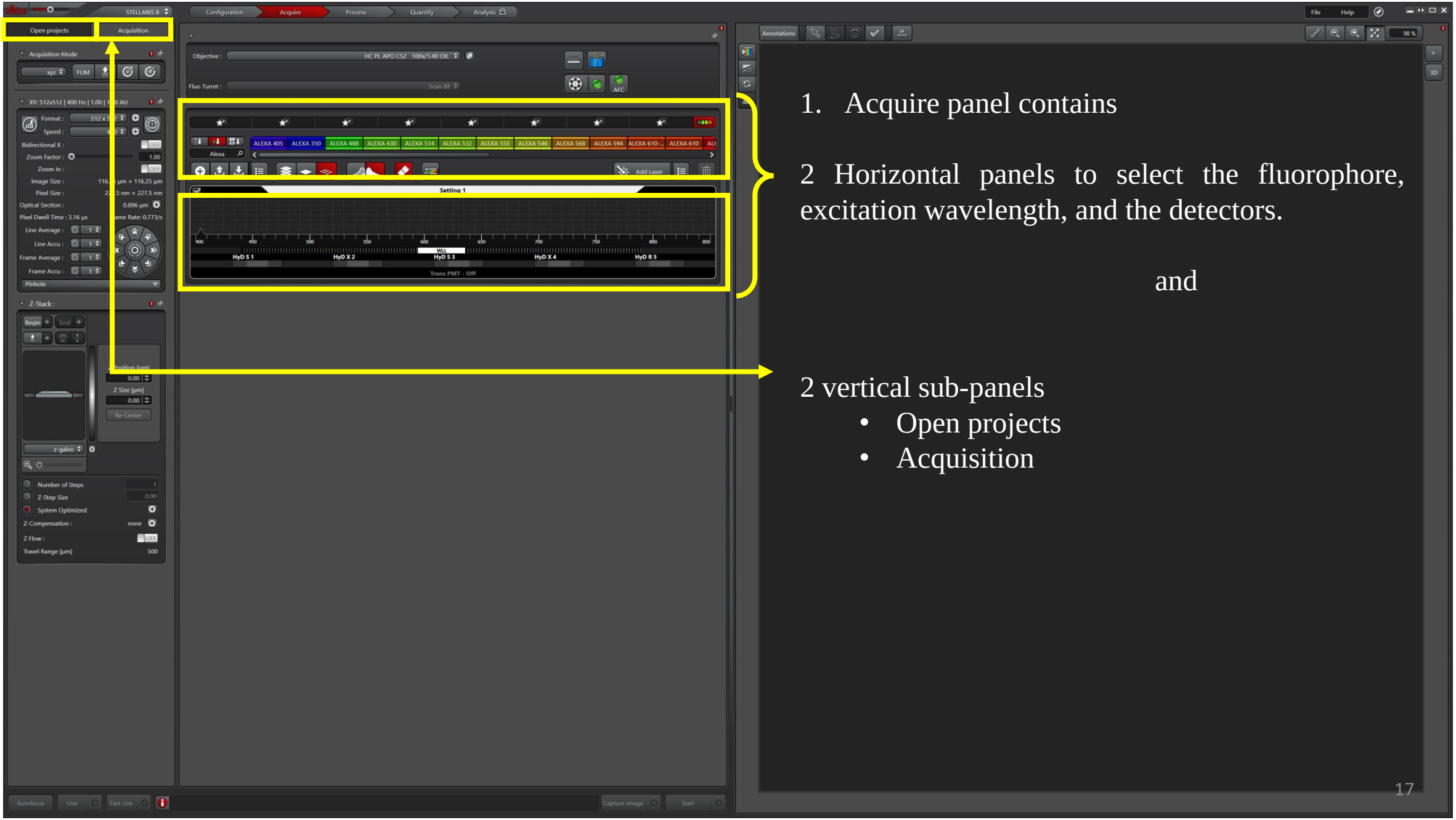
Click the Objective Icon to select the desired objective the configuration panel.

***Note: Use the touch screen to select the desired objective lens**



To see the overview of the fluorophores, click the Dye Database.

Click the Acquire panel to image the specimen. The Acquire panel is shown in the figure.



1. Acquire panel contains

2 Horizontal panels to select the fluorophore, excitation wavelength, and the detectors.

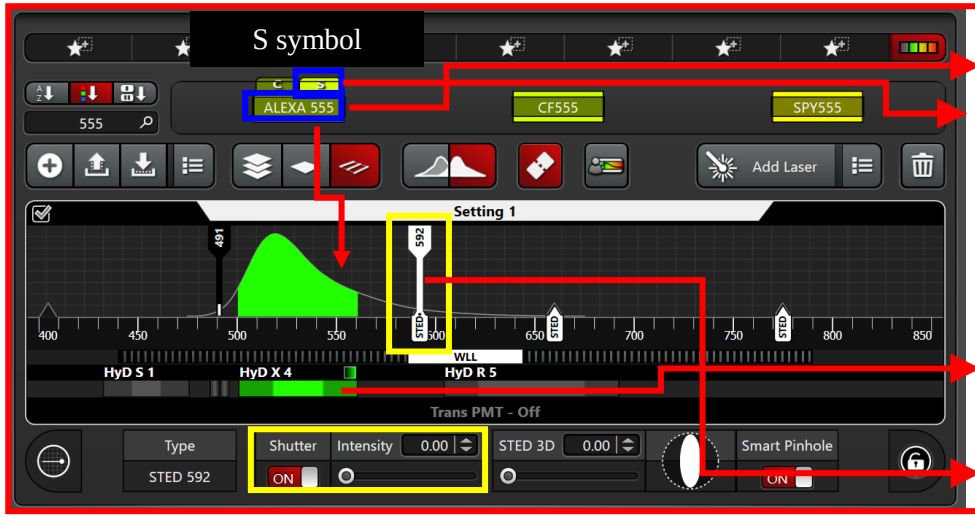
and

2 vertical sub-panels

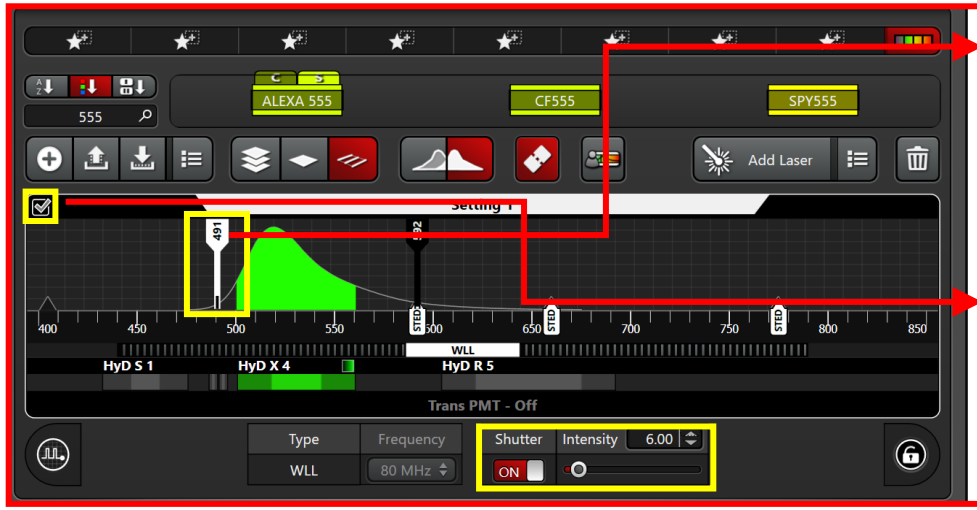
- Open projects
- Acquisition

STEP 3.
Selection of dye, excitation light (WLL), and STED Line

* The instructions below are for the single-color STED applications



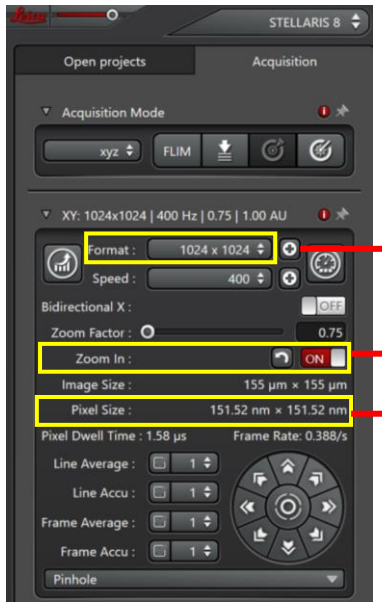
- **Dye selection**: Search e.g. 488 (ALEXA 488) on the search bar.
- Click the **S symbol** available just above the **fluorophore icon** and drag the fluorophore into the setting 1 panel and place it over the appropriate **detector**.
- If necessary, add the corresponding **STED line** by dragging the **Add Laser** button (e.g. as shown in case of ALEXA 488 i.e. STED 592) and **set the intensity 0** as shown in the left panel.
- Click the **ribbon (here green)** under the spectrum to see the **detector parameters** (e.g. Gain).
- Click the **STED line** to see the **depletion laser parameters** (e.g. intensity).

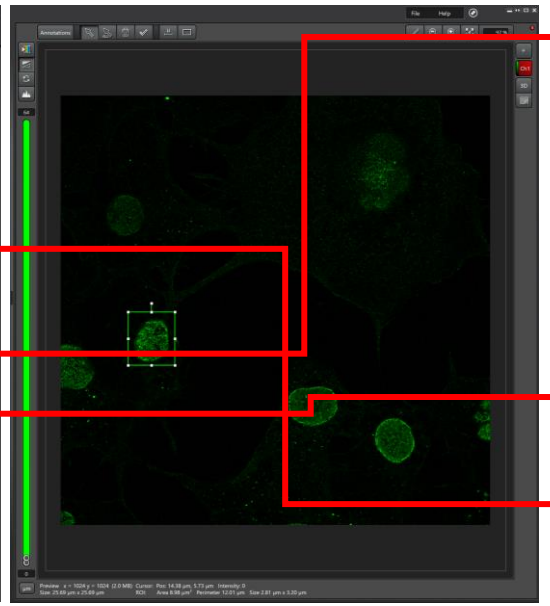


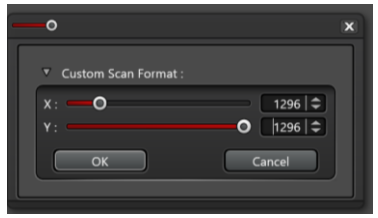
- Click the **conventional excitation WLL line** and tune the intensity **(to 6 %)** as shown in the left panel.
- Click the Live button to locate the specimen.
- Click the Capture button to record the specimen image.

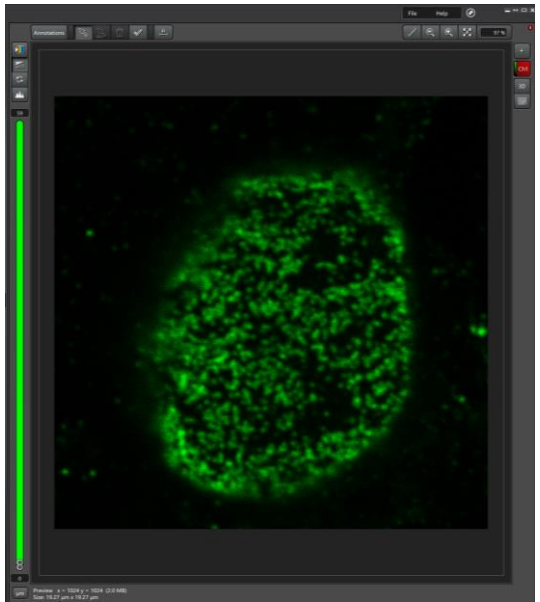
- Uncheck the extreme left corner of the setting panel to deactivate the particular channel.

STEP 4.
Selecting location for STED applications









- Toggle the **zoom button (ON)** and select the location by drawing the **rectangle** around it.
- Click Live or capture.

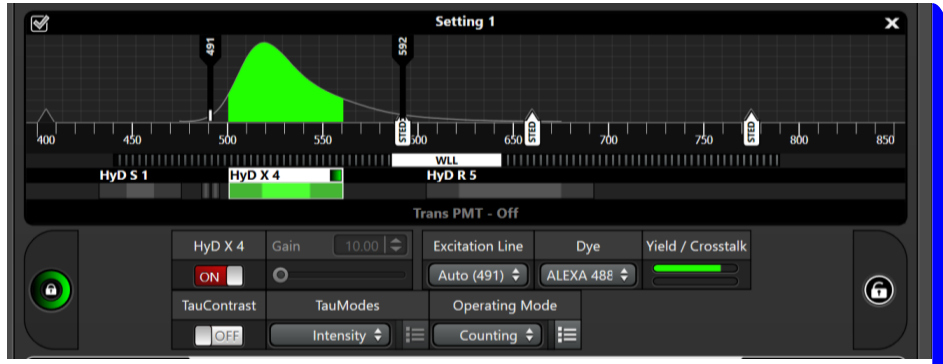
Image Size :	155 µm × 155 µm
Pixel Size :	151.52 nm × 151.52 nm

- **Pixel Size** in the **XY sub-panel** under **Acquisition** is important parameter for the Effective Resolution.
- To get the reasonable pixel size use the appropriate Format.
- *Note: To get the resolution of 60 nm, the pixel size should be approximately 3 times smaller (i.e. 20) than the resolution (i.e. 60).

- Manual option: Click the **(+) symbol** available in the **Format option** for customized values.
- Fill the appropriate entries in the Dialog Box.

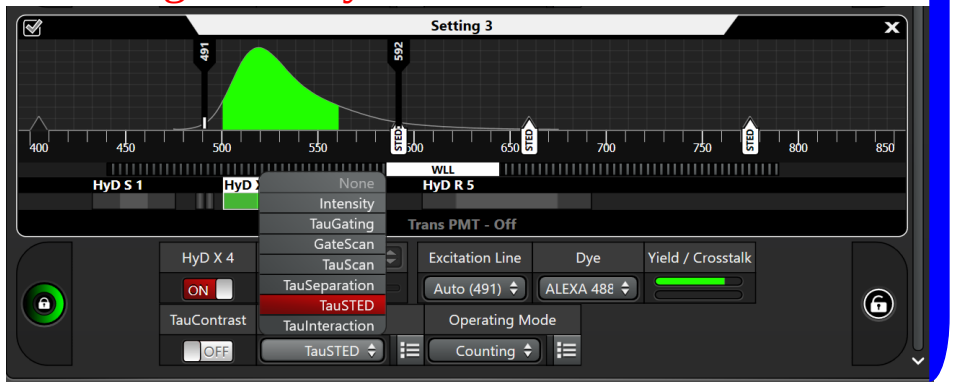
STEP 5. Selecting Modes for the STED: Intensity, TauGating, and TauSTED

Selecting Intensity Mode



Selecting Intensity TauGating

Selecting Intensity TauSTED



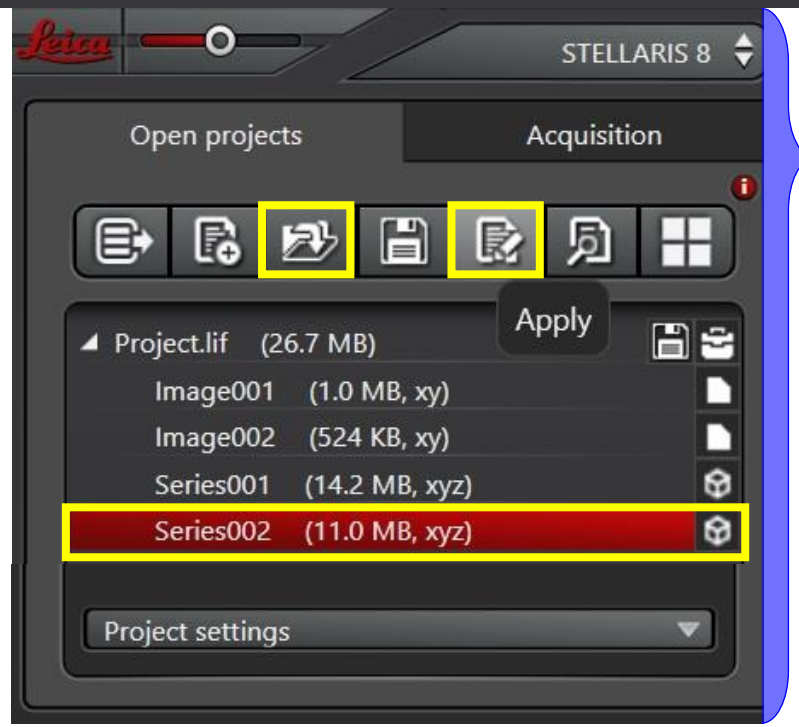
- Create a **2 more additional duplicate panels** as shown in the left panel.
- Click the detector bar and change the **TauModes** in the corresponding panels as **Intensity, TauGating and TauSTED**.
- Note: Don't try to find the FOV using STED beam. Reduce the STED beam intensity to 0.

Selecting STED Lines Intensities

- Tune the **STED line intensities** from **preset 0 value to 30 %** in each panel.
- **Increase the Line Accumulation to 4.**

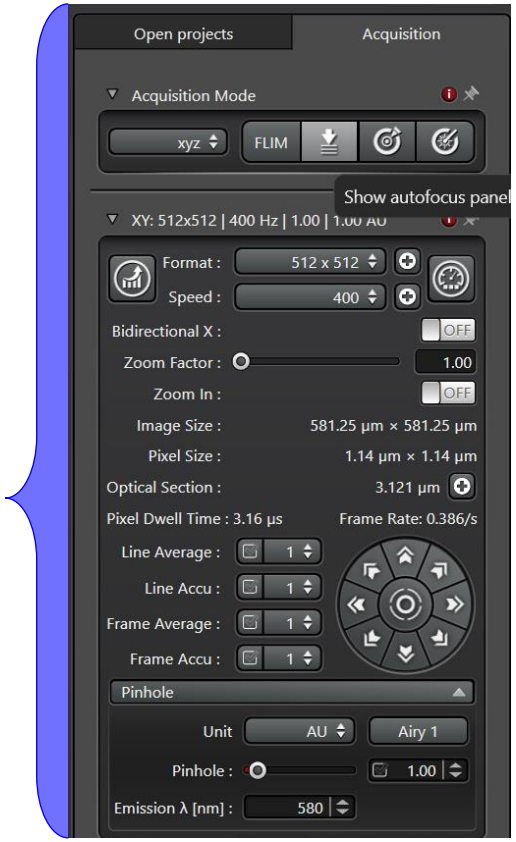
- Click the Capture button.

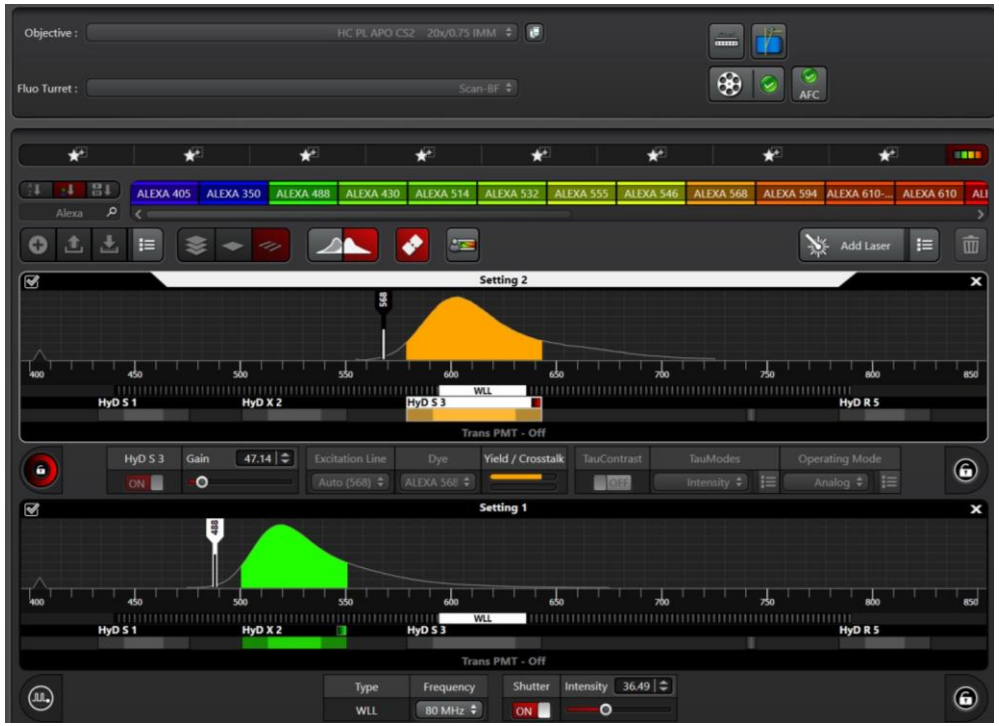
Loading your imaging method



- The easiest way of loading a method is to open a previously saved image (‘lif’) under the Acquire ‘Open Projects” and then click the ‘Apply’ button to load the imaging method.

- Click the Acquisition panel under Acquire and click the Live to check the specimen or Capture Image button to record the image.





Shutdown the system

1. Save the data and transfer the data to KCCI/data server
2. Stop the record time at the KCCI website.
3. Turn OFF the white light laser and STED lines from the software.
4. Close the software and shutdown the system.
5. Wait.
6. First turn OFF the Laser button and then Power button.

*Note: Remember, do not touch the key.

Troubleshooting Microscope for STED Application (NOTE: 1)

STED lines Turning ON

First warning: Laser could not be activated, please refer to the user's Manual of MP8-Section 'Radiation Emission Indicators'

Troubleshooting: Toggle the ignition keys (Hardware panel available at the bottom of the) from Tun ON to Turn OFF and Toggle again from Turn OFF to Turn ON.

Go to the STED button from the configuration. Turn ON the white light laser (WLL). Turn ON the STED 592 line by toggling it from OFF to ON from the Laser Overview panel. Turn ON the STED 660 line. If the second dialog box appears as shown in the figure 2, indicating the warning

STED 660 laser need to be turned automatically to ensure guaranteed laser lifetime. The procedure will take approximately 45 minutes.

STED mode will not be accessible during that time. Confocal functionality can be used as usual.

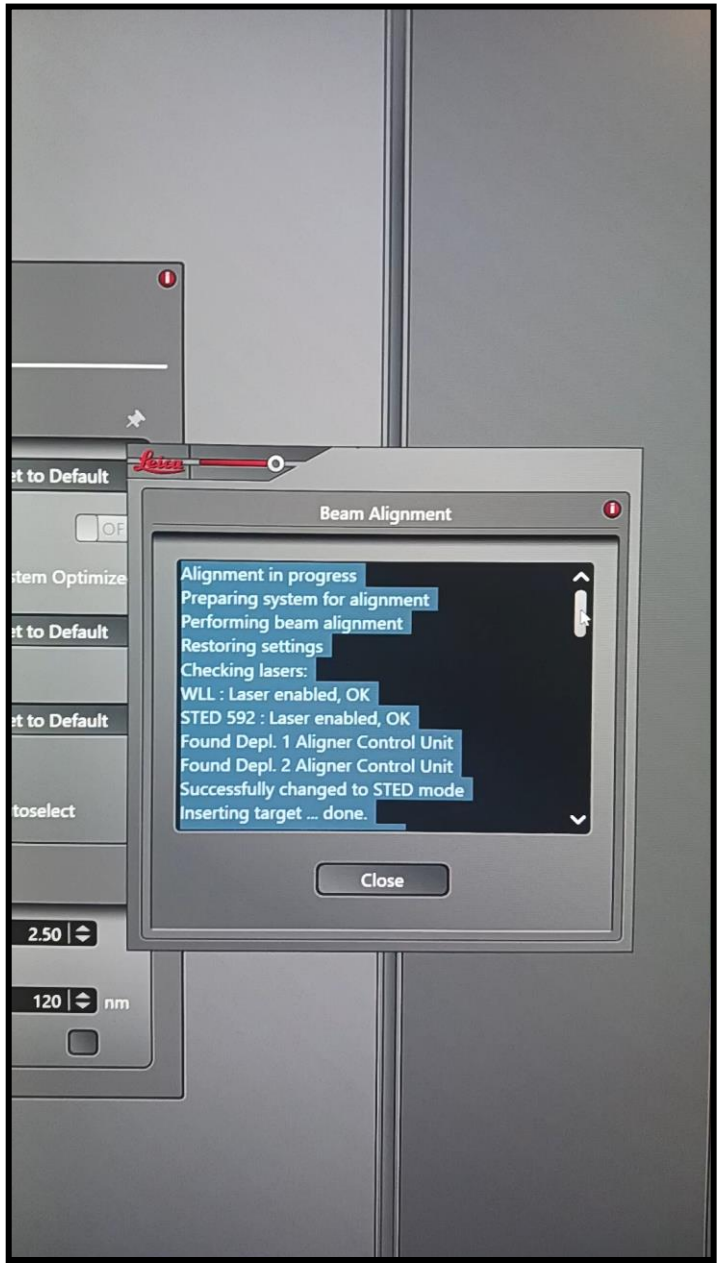
Start SHG tuning now?

Troubleshooting: Click Yes button available on the appeared dialog box and wait till the procedure ends.

Turn OFF the WLL and STED lines from the Laser Overview panel and exit the software.

Troubleshooting Microscope for STED Application (NOTE: 2)

Beam Alignment



Beam alignment troubleshooting (Beam alignment of the WLL and the STED lines): The system does the beam alignment with respect to the shortest wavelength available for the STED applications (i.e. 592 nm).

If the beam alignment fails after even after the following the STEP 1 and STEP 2, Tun OFF the WLL and STED lines.

- Exit the software.
- Restart the computer.
- Toggle the Emission keys from Tun ON to Turn OFF and Toggle again from Turn OFF to Turn ON.
- Open the LAS X software.
- Go to the STED button from the configuration panel. Turn ON the white light laser (WLL).
- Turn ON all the STED lines and required STED lines.
- Do the beam alignment again.

Note: For the beam alignment there is not need to Turn ON the 660 and 775 STED lines.