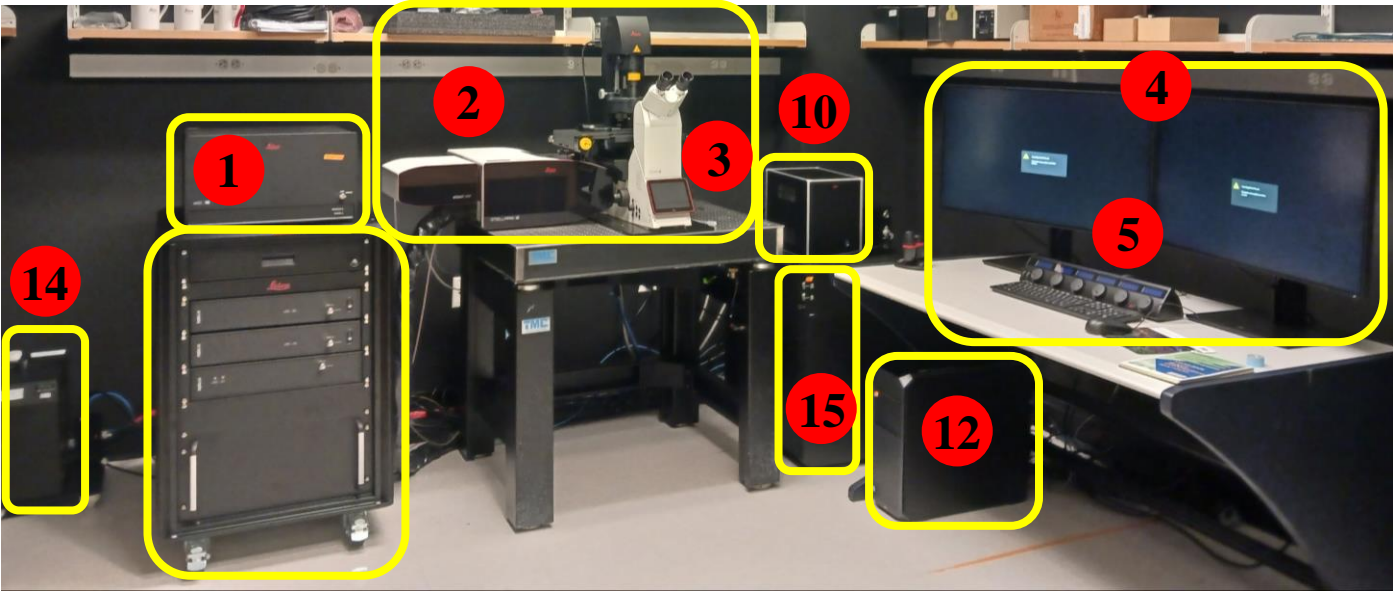
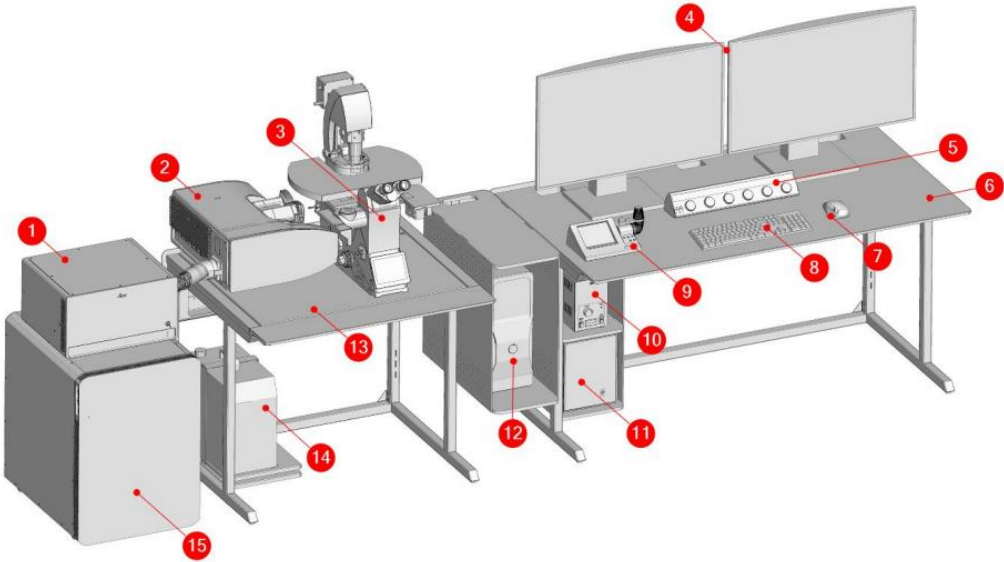


# Leica STELLARIS 8 Confocal/FLIM/tauSTED Operating Instructions

## System Overview (Confocal Application)



# Hardware information

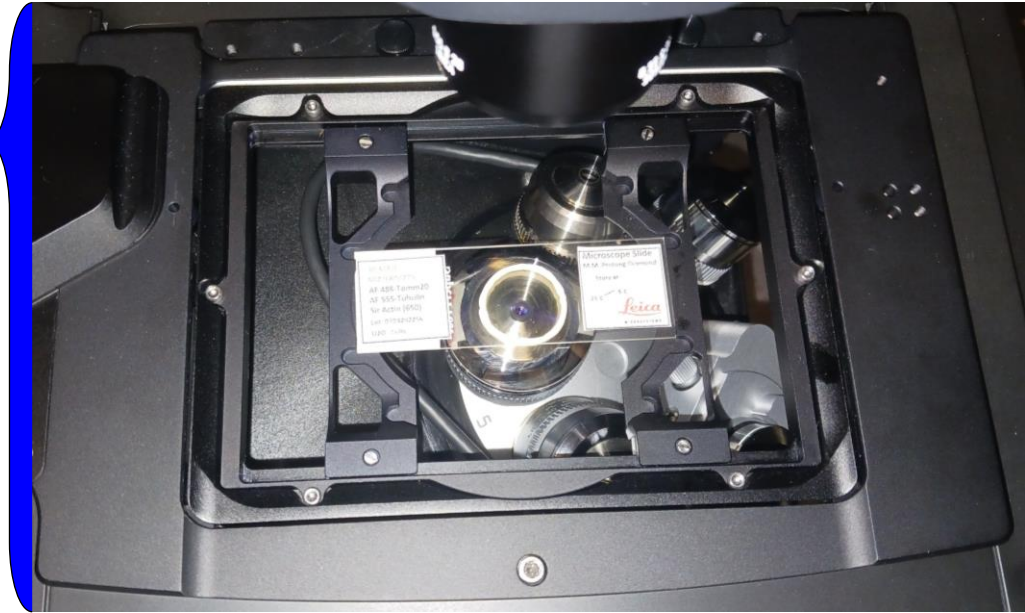


|   |                         |    |                            |
|---|-------------------------|----|----------------------------|
| 1 | White Light Laser (WLL) | 10 | External light source      |
| 2 | Scan Head               | 12 | Workstation                |
| 3 | Microscope              | 14 |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

# 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!**

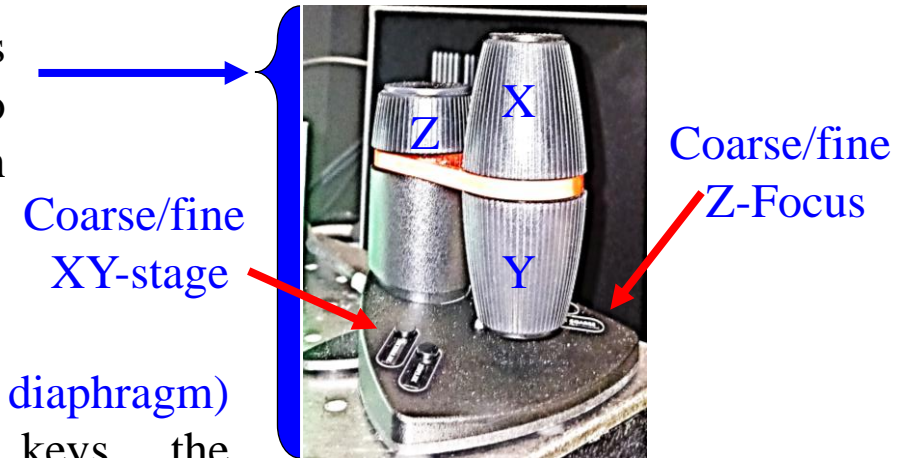


# Sample mounting and fluorescence observation through eyepieces

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.



## System Start-up



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

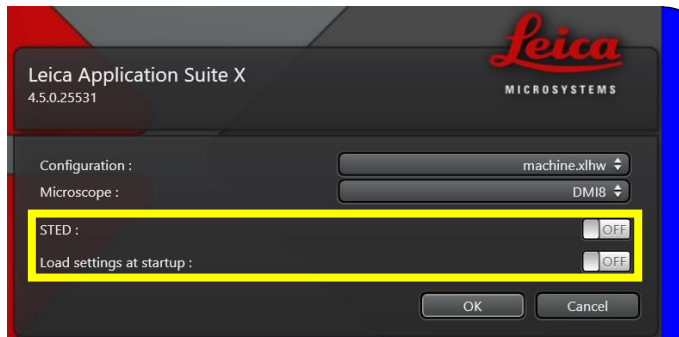
**\*Note:** Do not touch the key.

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



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

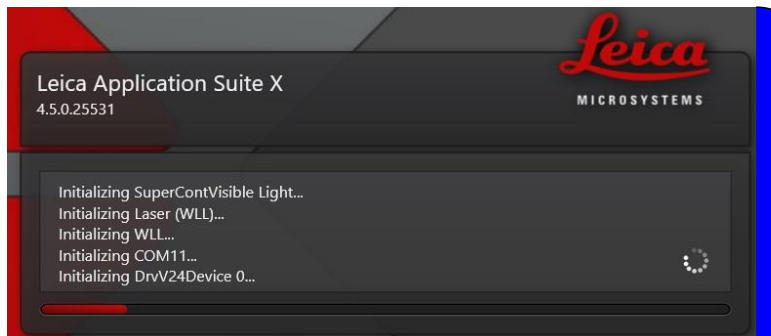
**\*Note:** The **LAS X** software is used to control all system functions and acts as the link to the individual hardware components. Image acquisition, image analysis and image processing are carried out using **LAS X**.



6. Select the **Machine** as configuration and **DM18** in the **Microscope** section as shown.

7. **Always OFF** for the confocal usage.

8. Now start **LAS X** by clicking the **OK** button.



9. Wait **Until the software initialization completes**.




10. Select **No**, if you are **NOT** using for **tiling, etc.** For Tiling you should initialize the stage.

11. Wait for the **LAS X Graphical User Interface** window.



# 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)

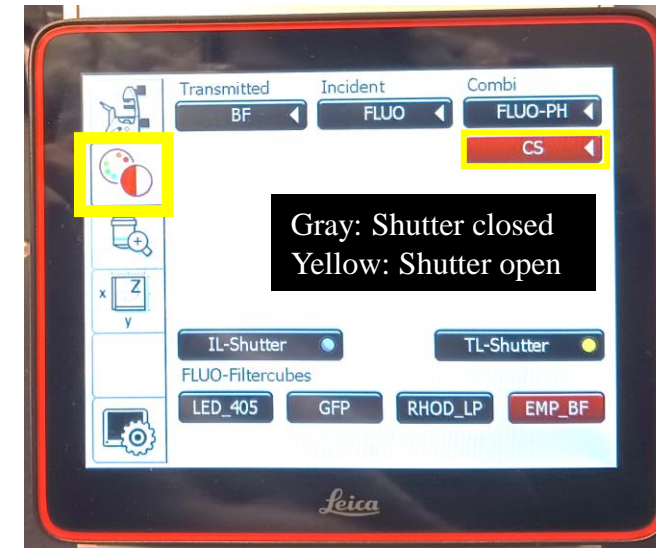
**Combination:** Fluorescence-Phase (FLUO-PH) and (CS)

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

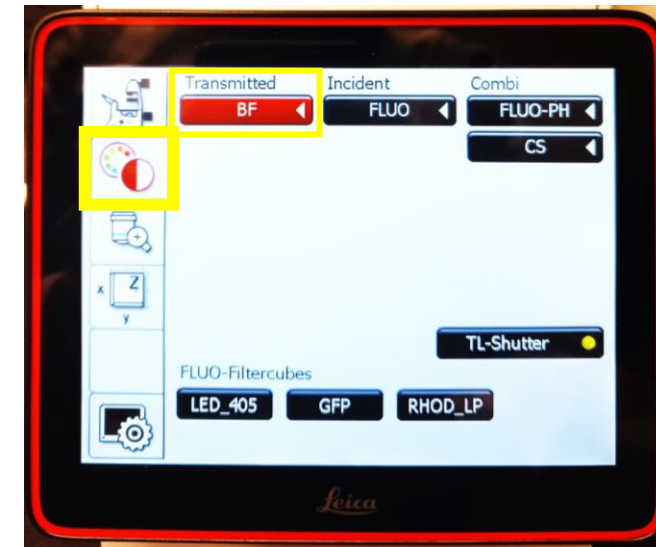
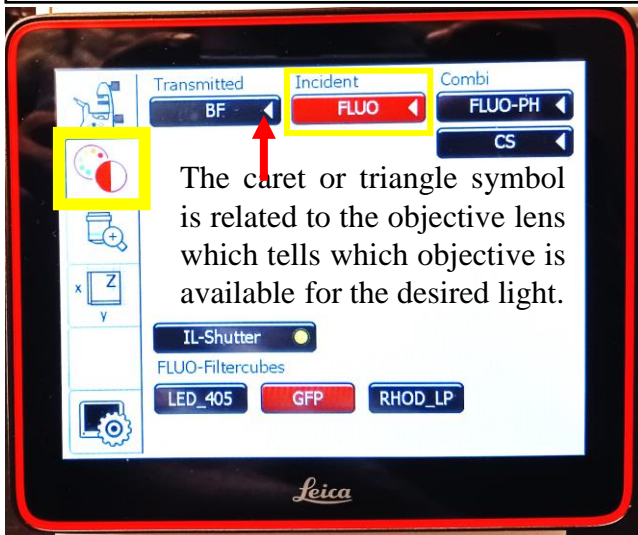
Click the FLUO for fluorescence incident light method

The available filter cubes are displayed. Select the desired cube using the corresponding button.

To see on the screen, use these settings.



To see through the ocular, use these settings.



# Using the microscope touchscreen

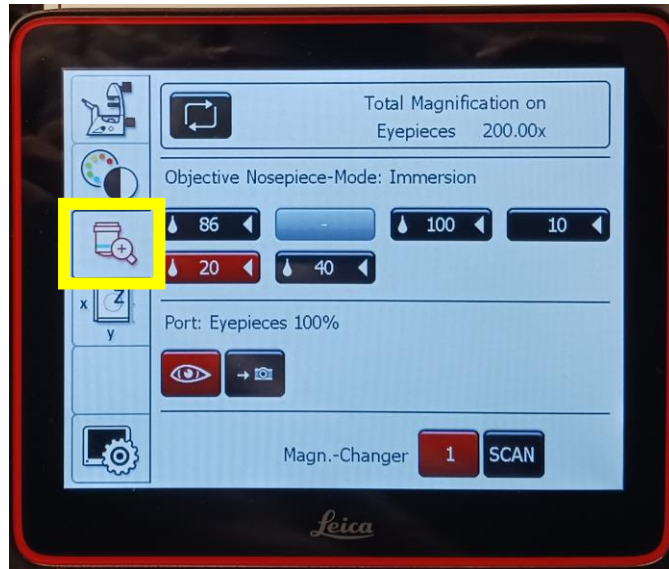
## Objective Tab

1. Shows the total magnification on the eyepieces.
2. Available objectives: Here 5.

Click the objective button from the touch screen and select the required objective.

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

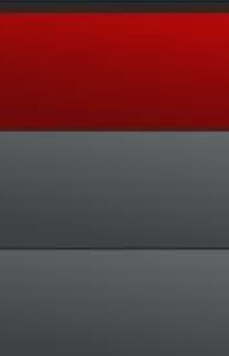
3. Next part of the panel shows whether the light is going to the eyepieces or to the camera.



The screenshot displays the Stellaris 8 software interface, which is divided into several panels. A workflow diagram is overlaid on the interface, showing the sequence of steps for a confocal imaging experiment:

- Panels**: Points to the left sidebar containing acquisition and Z-stack settings.
- Module Selection**: Points to the top toolbar where the imaging module is chosen. A yellow box highlights the **STELLARIS 8** dropdown menu, with a note: **\*Select the STELLARIS 8 for confocal imaging method.**
- Configuration**: Points to the central area showing the selected module's configuration, including a list of modules (ALEXA 405, ALEXA 350, ALEXA 488, etc.) and a graph of the acquisition path.
- Acquire**: Points to the **Acquire** button in the top toolbar.
- Process**: Points to the **Process** button in the top toolbar.
- Quantify**: Points to the **Quantify** button in the top toolbar.
- Analysis**: Points to the **Analysis** button in the top toolbar.

The interface also shows a list of modules on the right side, including **STELLARIS 8**, **Live Data Mode**, **FRAP**, **FRAP XT**, **FRET AB**, **FRET SE**, and **Lightning**.



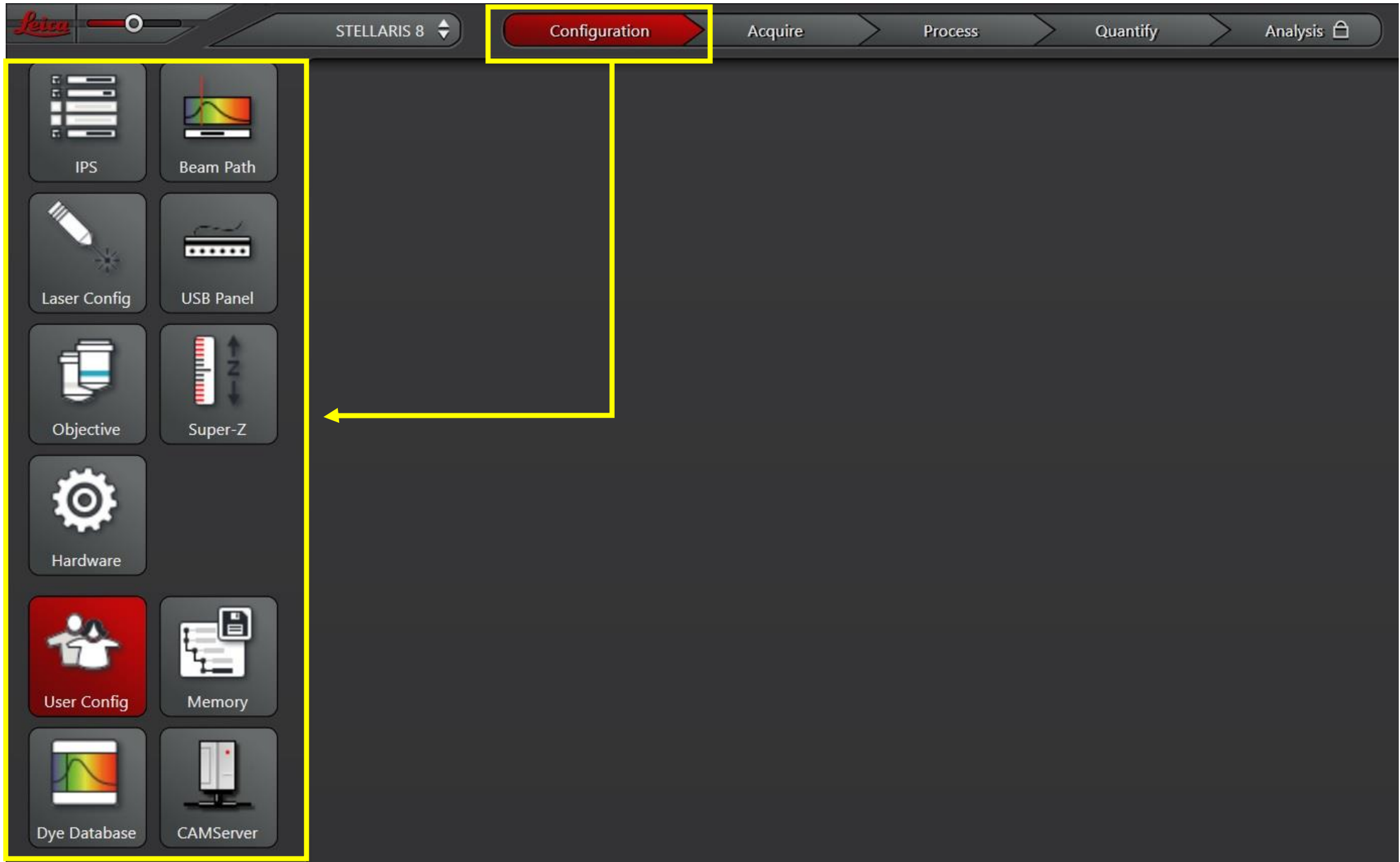
The screenshot shows the Stellaris 8 control panel. At the top, a yellow box highlights the 'STELLARIS 8' label and a small blue arrow points to a diamond-shaped button. Below this, a red bar also displays 'STELLARIS 8'. The main menu consists of several grey buttons: 'Live Data Mode', 'FRAP', 'FRAP XT', 'FRET AB', 'FRET SE', and 'Lightning'.

| Mode           |
|----------------|
| STELLARIS 8    |
| Live Data Mode |
| FRAP           |
| FRAP XT        |
| FRET AB        |
| FRET SE        |
| Lightning      |

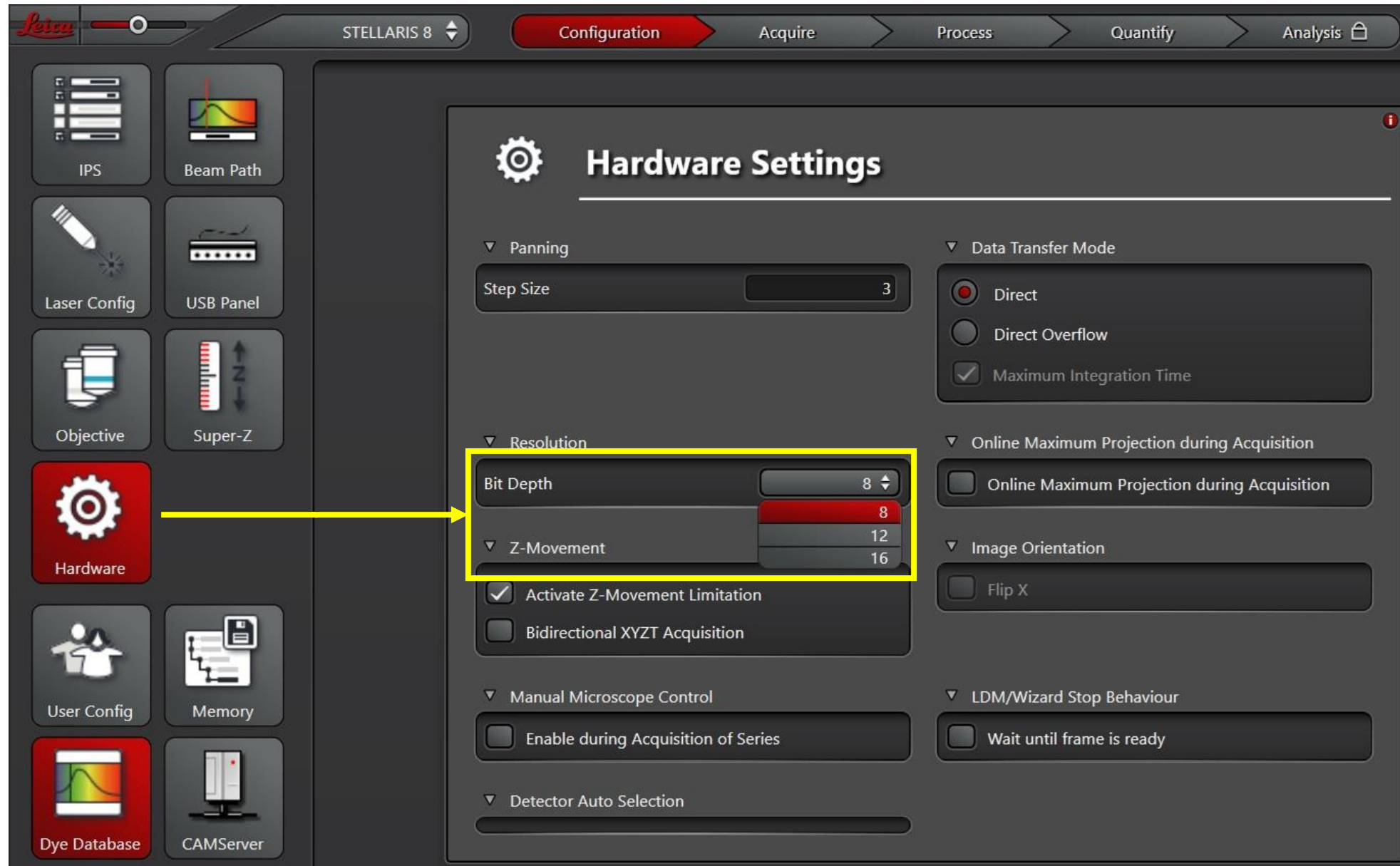
\*Select the STELLARIS 8 for confocal imaging method.



Click the configuration button to activate the configuration



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



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

Leica STELLARIS 8 Configuration Acquire Process Quantify Analysis

IPS Beam Path Laser Config USB Panel Objective Super-Z Hardware User Config Memory Dye Database CAMServer

### Objective Configuration

Selected Objective HC PL APO CS2 20x/0.75 IMM

Objectives

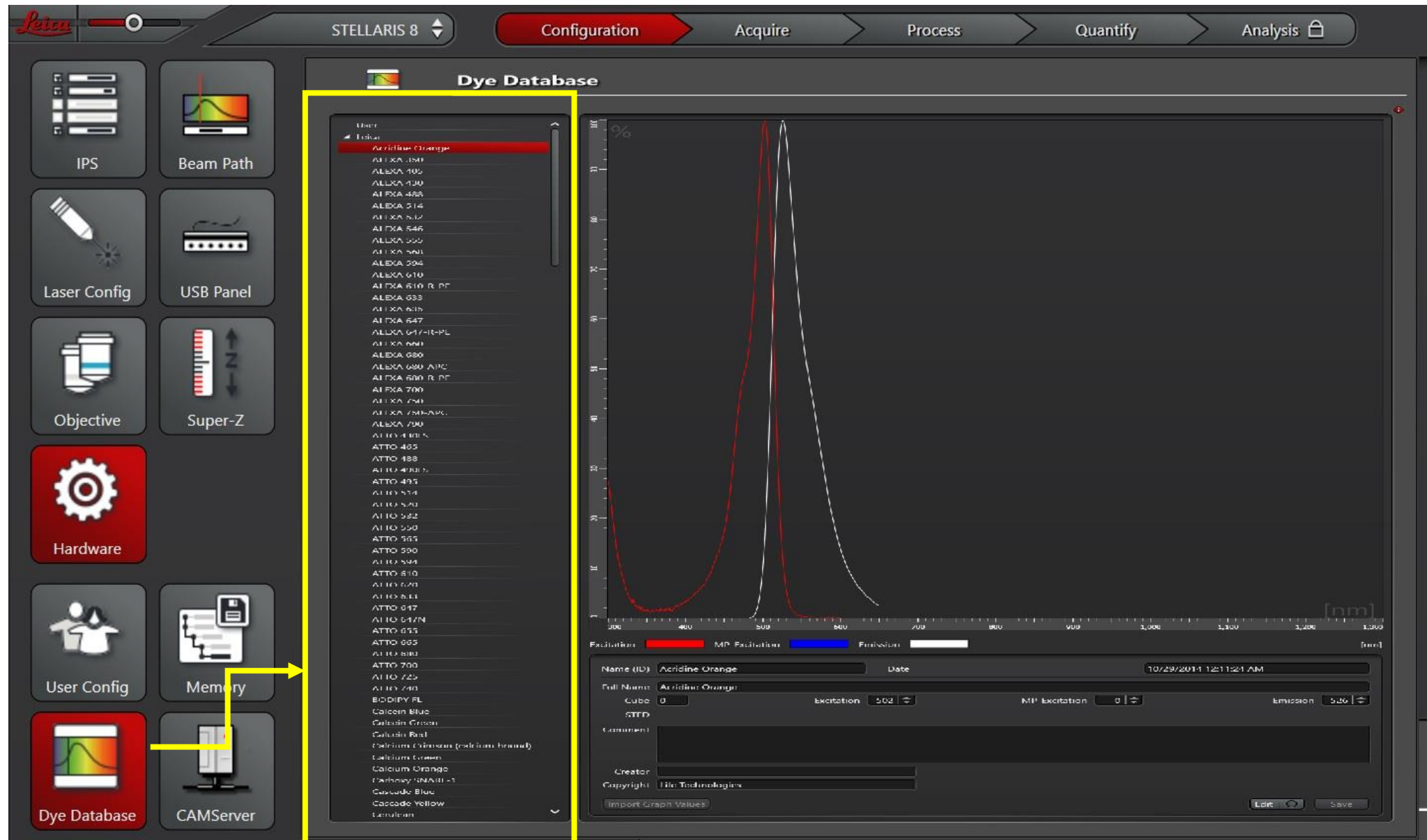
- HC PL APO CS2 86x/1.20 WATER
- Empty 1x/0.00
- HC PL APO CS2 100x/1.40 OIL
- HC PL APO CS2 10x/0.40 DRY
- HC PL APO CS2 20x/0.75 IMM**
- HC PL APO CS2 40x/1.30 OIL

Objective Attributes

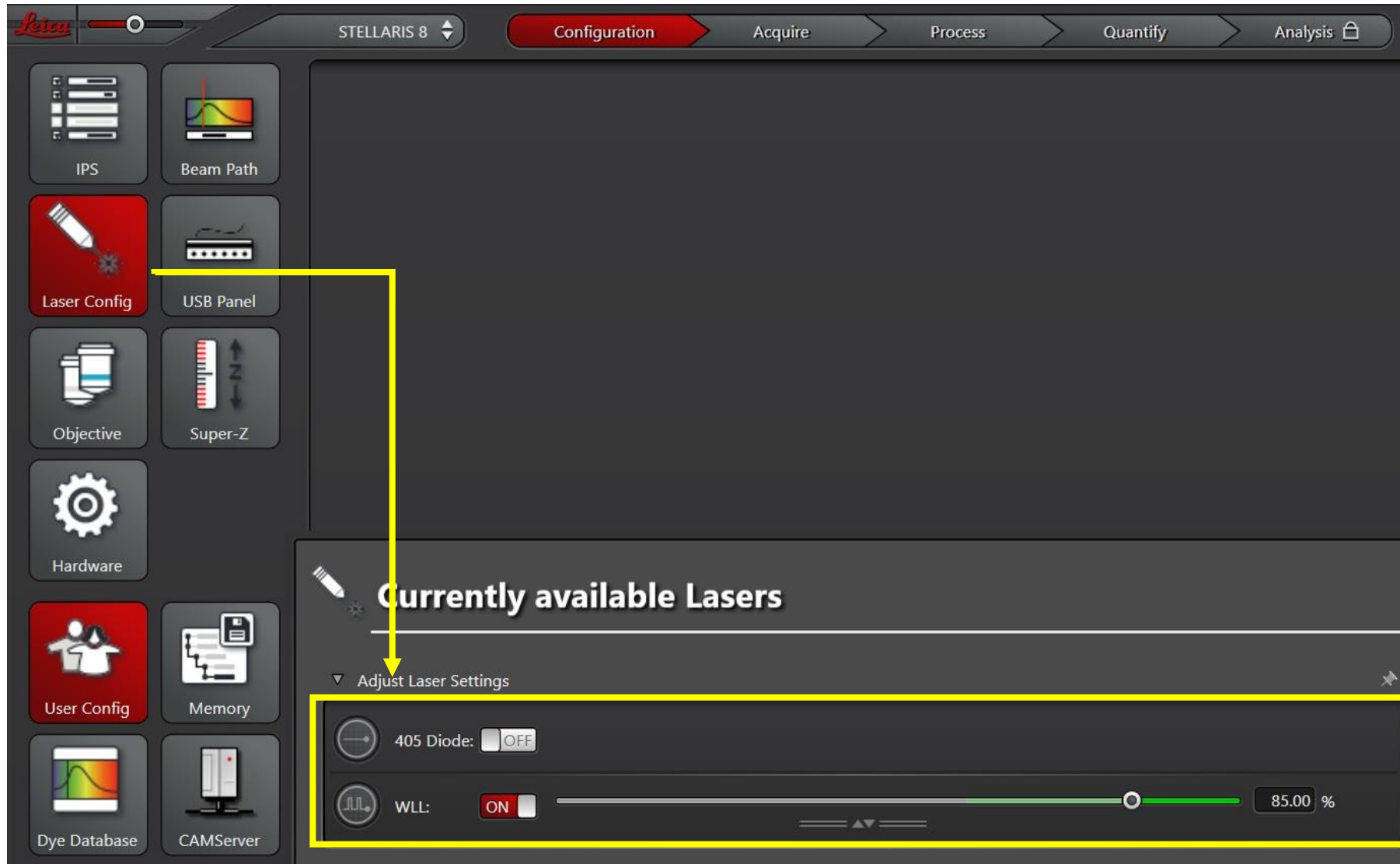
|                    |               |                       |         |                     |                  |
|--------------------|---------------|-----------------------|---------|---------------------|------------------|
| Type               | HC PL APO CS2 | Resolution XY(488nm)  | 260.27  | Phase Ring          |                  |
| Magnification      | 20            | Resolution Z(488nm)   | 1107.87 | IC Prisms           | C1;C             |
| Numerical Aperture | 0.75          | Free Working Distance | 660     | Technique           | CS2; Waterdispen |
| Immersion          | IMM           | Focus Depth           | 0       | Condenser Prism DIC | K6;K3            |
| Coverglass         | -             | Focus Offset          | 0       | Order Number        | 11506343         |



To select the required fluorophore, click the **Dye Database**.



Click the **Laser configuration icon** and turn on the **white light laser (WLL)** from the dialogue box appeared on the screen.



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

The screenshot shows the Stellaris 8 software interface. The 'Acquire' panel is highlighted with a yellow border. Within this panel, two horizontal panels are highlighted with yellow boxes: the top one for selecting fluorophores (ALEXA 405, ALEXA 350, ALEXA 488, ALEXA 430, ALEXA 514, ALEXA 532, ALEXA 555, ALEXA 546, ALEXA 568, ALEXA 594, ALEXA 610, ALI) and the bottom one for selecting excitation wavelengths (HyD S 1, HyD X 2, HyD S 3, HyD X 4, HyD R 5). A yellow arrow points from the 'Acquire' panel to the 'Open projects' and 'Acquisition' buttons on the left sidebar. Another yellow arrow points from the 'Acquire' panel to the '2 vertical sub-panels' section of the text.

1. Acquire panel contains

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

and

2 vertical sub-panels

- Open projects
- Acquisition

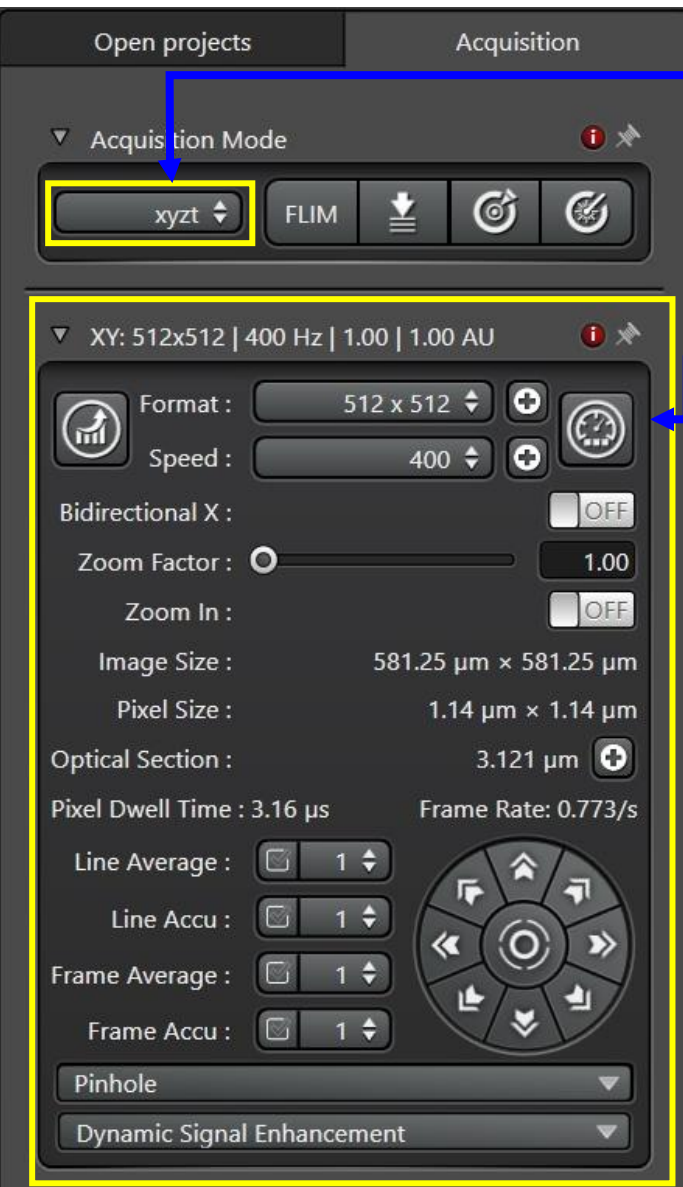



Configuration

Acquire

Process

Quantify

Analysis 

Open projects

Acquisition

Acquisition Mode

xyz

FLIM

XY: 512x512 | 400 Hz | 1.00 | 1.00 AU

Format: 512 x 512

Speed: 400

Bidirectional X: OFF

Zoom Factor: 1.00

Zoom In: OFF

Image Size: 581.25  $\mu\text{m}$  x 581.25  $\mu\text{m}$

Pixel Size: 1.14  $\mu\text{m}$  x 1.14  $\mu\text{m}$

Optical Section: 3.121  $\mu\text{m}$

Pixel Dwell Time: 3.16  $\mu\text{s}$  Frame Rate: 0.773/s

Line Average: 1

Line Accu: 1

Frame Average: 1

Frame Accu: 1

Pinhole

Dynamic Signal Enhancement

- Choose the acquisition mode from the dropdown list.
- Use the XY panel to set the image format, zoom, line averaging, pinhole.

## Z-stack imaging



Z-Stack:

Begin End

z-galvo

Z Position [ $\mu\text{m}$ ]: 0.00

Z Size [ $\mu\text{m}$ ]: 0.00

Re-Center

Number of Steps: 1

Z-Step Size: 0.00

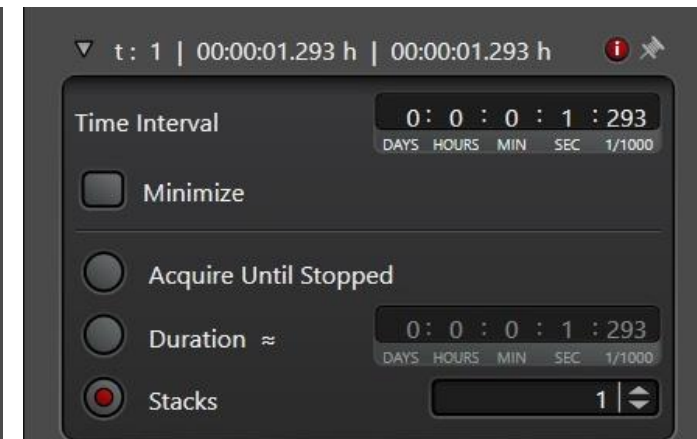
System Optimized: ON

Z-Compensation: none

Z Flow: OFF

Travel Range [ $\mu\text{m}$ ]: 500

## Time-lapse



t: 1 | 00:00:01.293 h | 00:00:01.293 h

Time Interval: 0:0:0:1:293

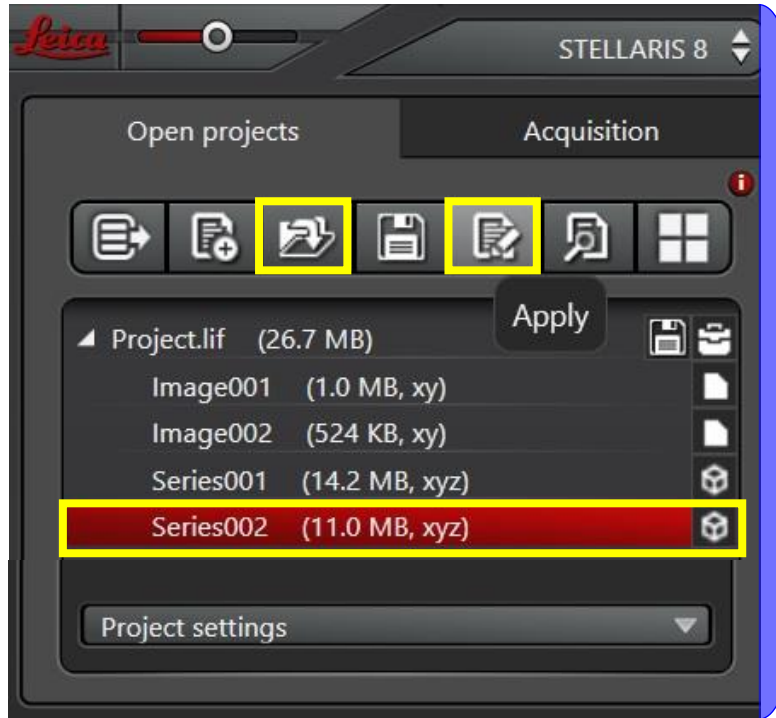
Minimize

Acquire Until Stopped

Duration: 0:0:0:1:293

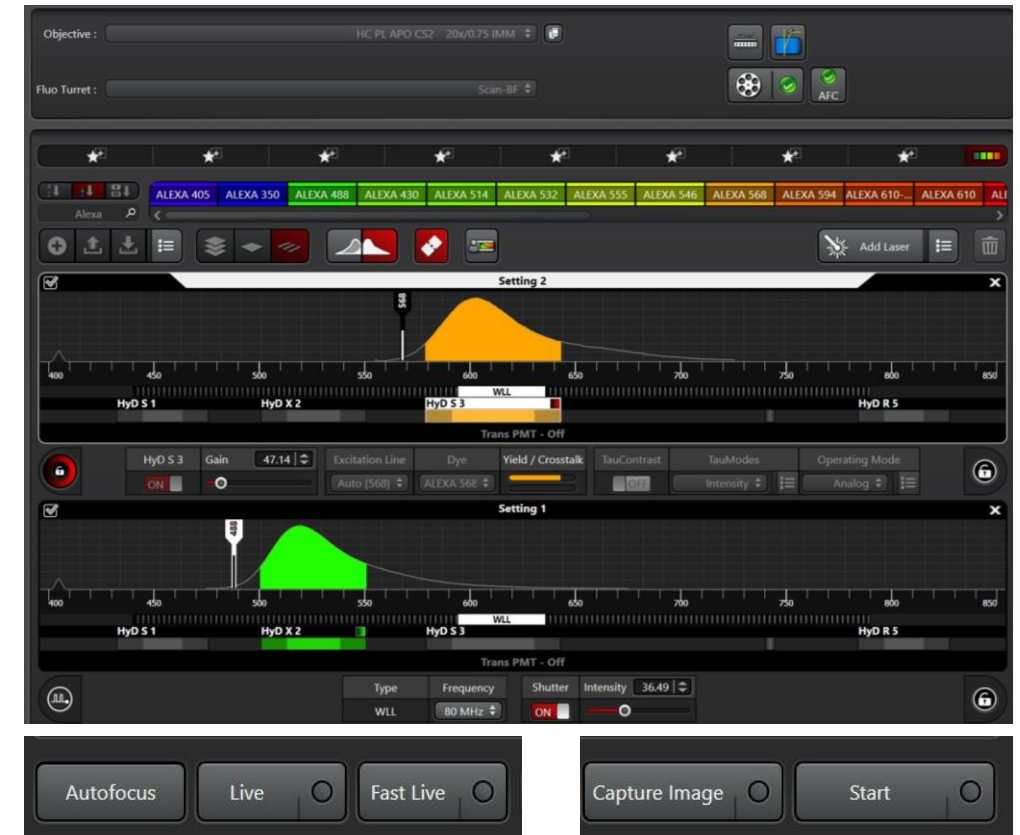
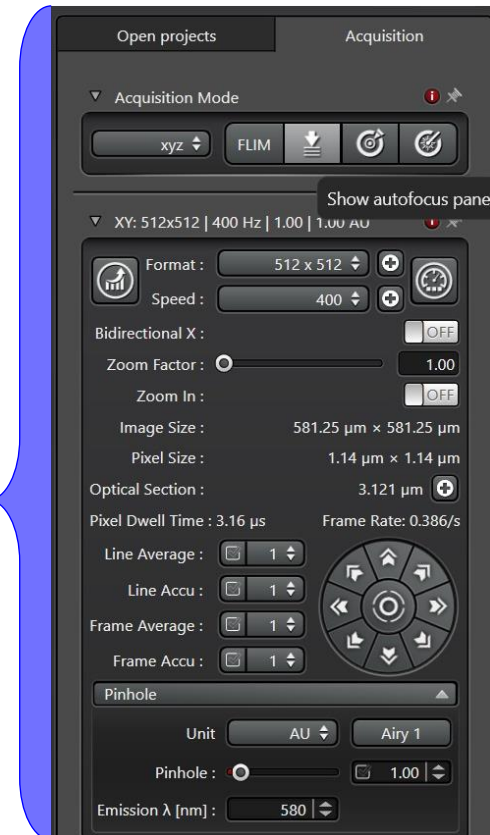
Stacks: 1

## Loading your imaging method

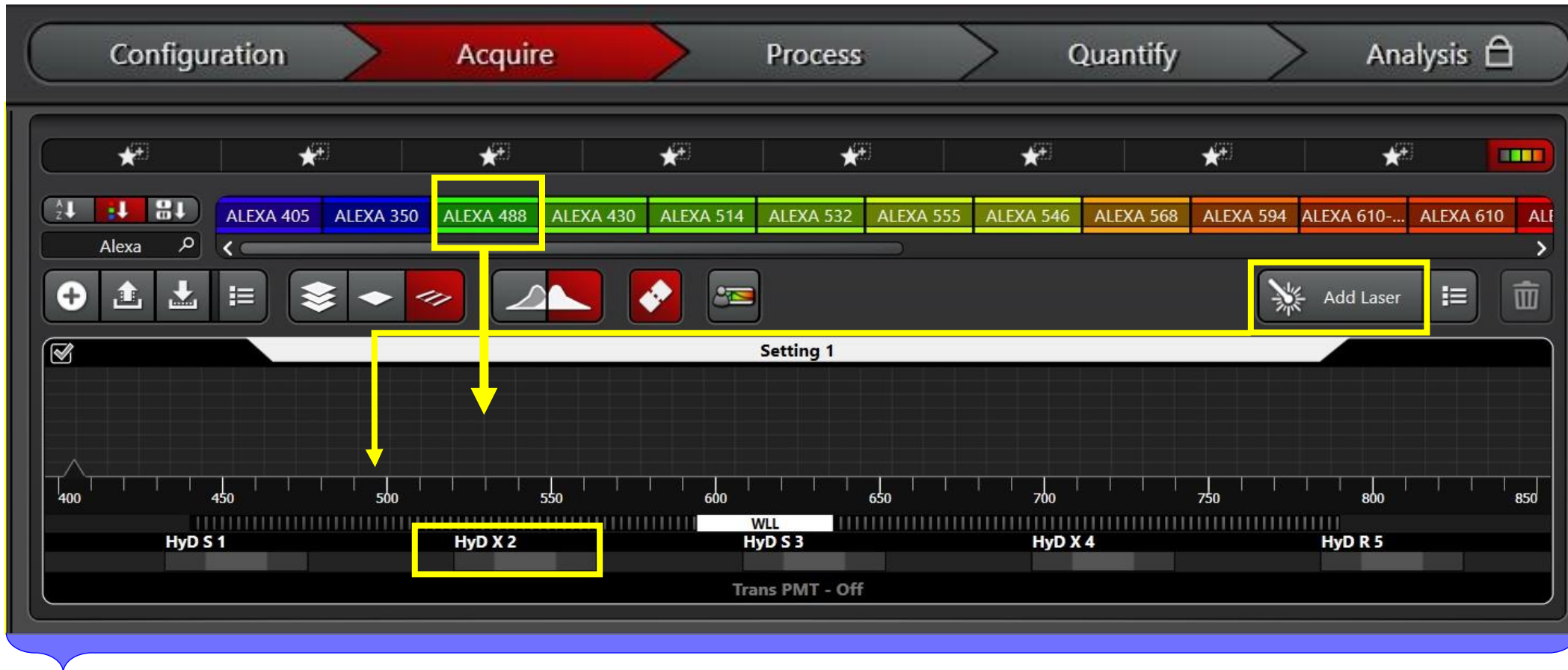


- **NOTE:** The method must be set up by the KCCI staff and you must be trained by the KCCI staff to use the method.
- The easiest way of loading a method is to open a previously saved image ('lif') under the **Acquire 'Open Projects'** and then click the **'Apply'** button to load the imaging method.

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



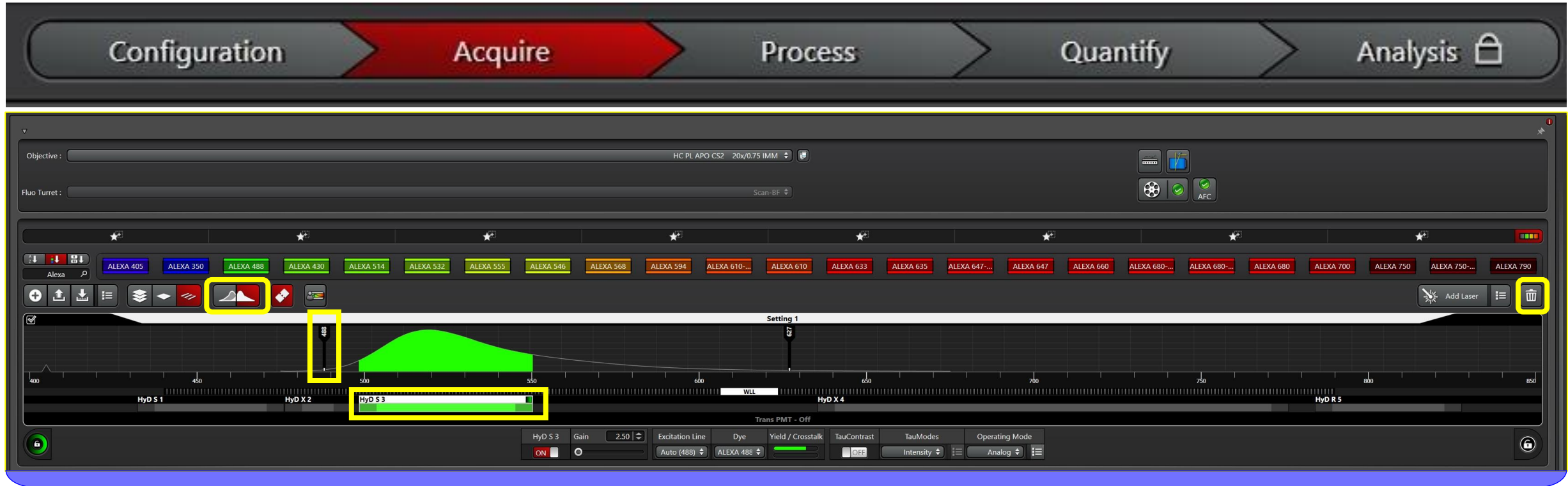
## Acquire panel: Selection of **the detectors**, **Dye**, and **Excitation wavelength**.



- To select the fluorophore from the available top ribbon, hold the required dye button and drag it to Setting 1 (bottom panel).
- The particular hybrid detector will appear at the bottom of the selected fluorophore.



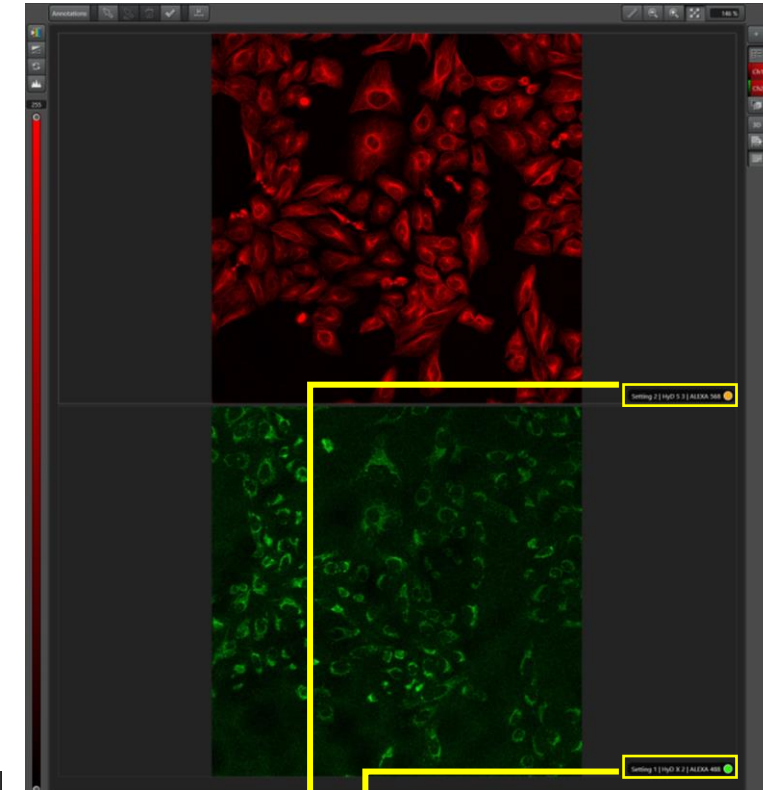
# Acquire panel: Selection of **the detectors**, **Dye**, and **Excitation wavelength**.



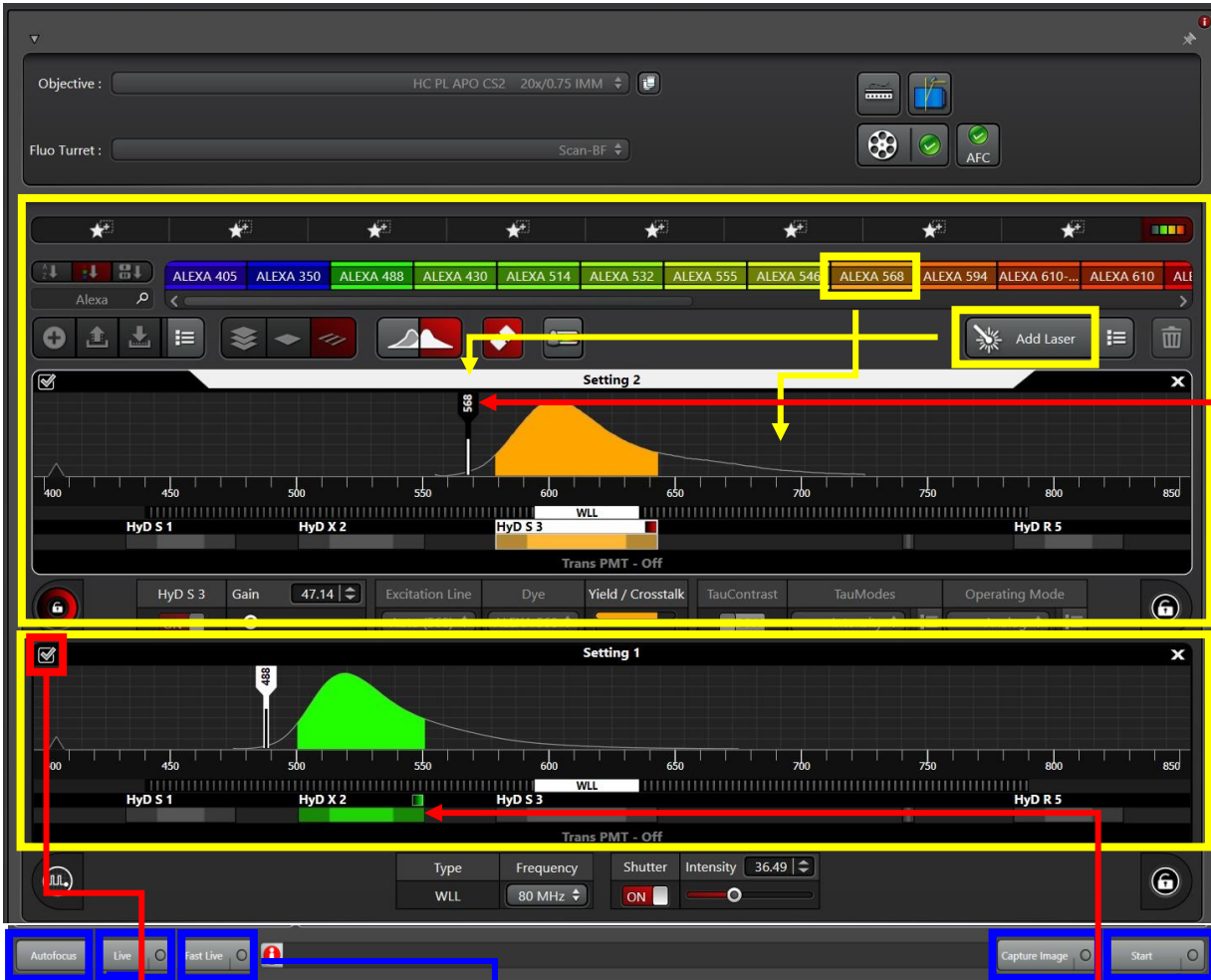
- To remove the unwanted fluorophore active the detector ribbon (below the fluorophore) from the setting 1 panel and click the delete button.
- To remove the unwanted excitation wavelength, click the excitation line from the setting 1 panel and click the delete button.

# Multi-fluorophores: Sequential Imaging Method

To image the **additional fluorophore**, create a **duplicate panel** by **right click** on the setting 1 panel. Delete the already occupied parameter via **Reset** (Right click on the panel and choose Reset). Select **the fluorophore** by dragging it to the duplicate panel and **suitable excitation wavelength** from the 'Add Laser' option.



2 different image panels indicates the selection of 2 different fluorophores (**ALEXA 568** and **ALEXA 488**) and the 2 different PMTs (**HyD S** and **HyD X**).



**Autofocus** **Live** **Fast Live**

**Capture** **Start**

Uncheck here to deactivate the particular channel.

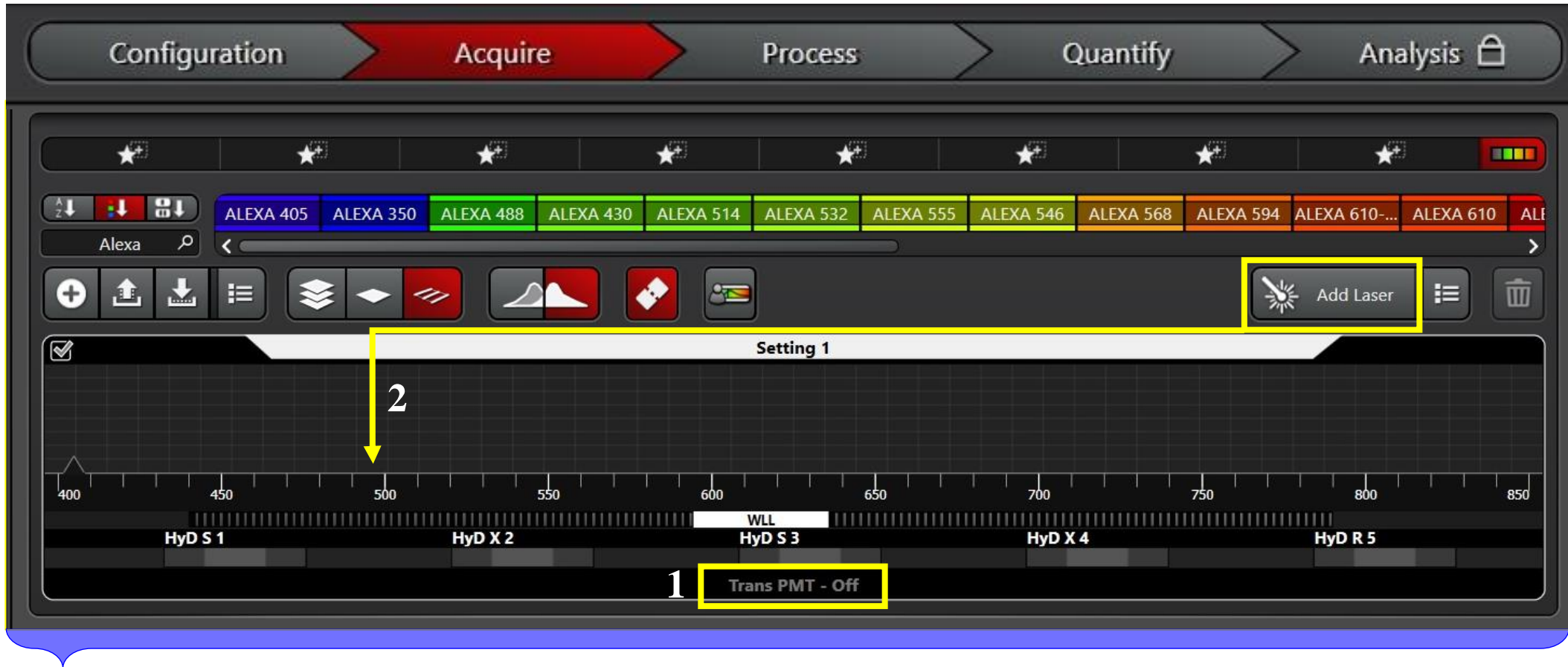
Click the **Live** to locate the specimen and **Capture Image** button (available at the boron of the Acquire panel) to record the desired selected field of view.

Click the ribbon under the spectrum to see the detector parameters (e.g. Gain).

Click the Laser excitation line to see the excitation laser paratmers.

If No signal at the detector window tune the laser and detector parameters (excitation intensity or Gain)

# Bright Field



- Turn ON the Trans PMT.  
\*Note: Do not select the dye in the case of Bright Field.
- Drag the Add Laser button to the setting 1 panel to select the excitation light. Make sure the white light laser is ON.
- Click Live to view the specimen and click Capture to record the image.



# Take a Z-Stack

*\*Note: Z-stack is different from Z-galvo*

1. Choose the 'xyz' imaging mode.

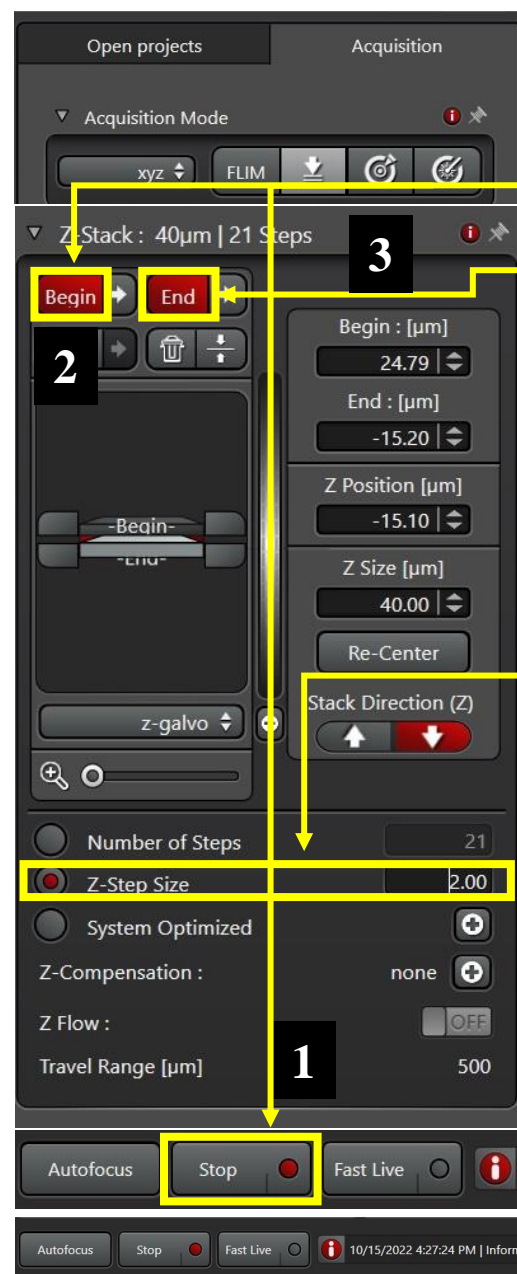
## 2. Open the Z-stack panel

- Step 1. Press the 'Live' Button (1) and go to the **top** position of your stack using Z-focus. Click the 'Begin' button (2), which will then turn red, meaning that the 'Begin' position is locked in the top position of your stack.
- Step 2. Press the 'Live' Button (1) again and go to the **bottom** position of your stack using Z-focus. Click the 'End' button (3), which will then turn red, meaning that the 'End' position is locked in the bottom position of your stack.
- **Now, your stack is defined.**

- Define the Z-step size (4), e.g.  $0.5\ \mu\text{m}$  for taking the image sections every half micron.
- Click the Number of Steps option and press the Enter Key. You will be able to see the number of sections to be acquired in your stack.

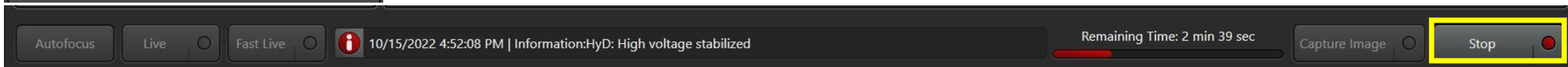
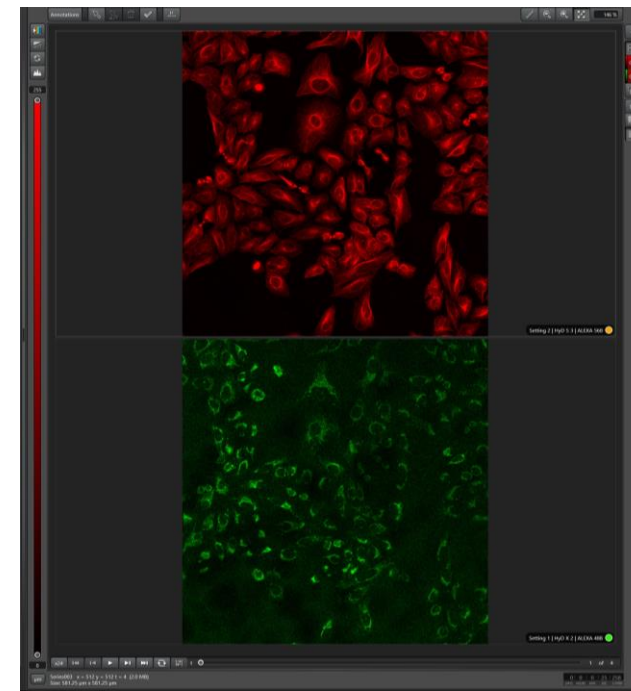
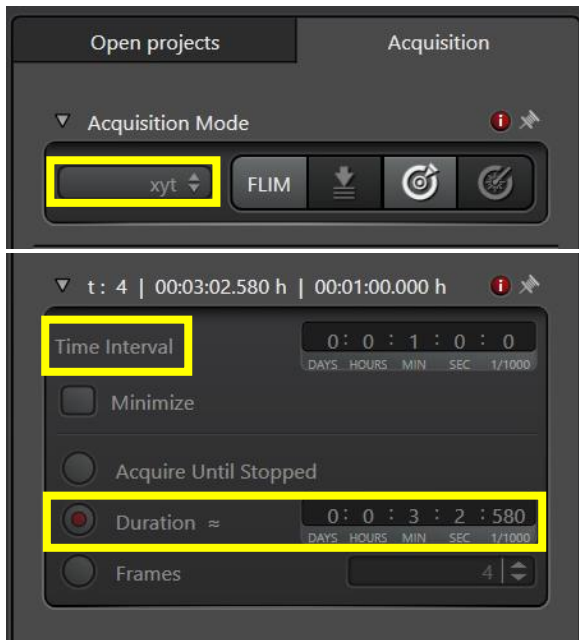
3. To change the stack direction click the desired button under the 'Stack Direction' (5) option.

4. Click the start button at the bottom of the Acquire panel to image the specimen.



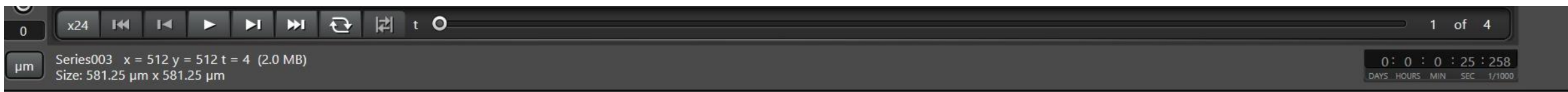
# Take a Time-lapse

- 2-D Time-lapse: Choose the 'xyt' imaging mode.
- Open the 'time-lapse' panel,
- Define the 'Time Interval', e. g. 1 minute for taking images every 1 minute.
- Define the 'Duration', e. g. 3 minutes for taking time-lapse images for 3 minutes.
- Click the 'Start button' (at the bottom of the Acquire panel) to start the acquisition



Video play button

Remaining Time



Number of Frames

# 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 laser 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.