

STANDARD OPERATING PROCEDURE

Leica Stellaris 5 DLS Microscope – Confocal SOP

INTRODUCTION

This document describes the operational setup and procedures for the Leica Stellaris 5 confocal and digital light sheet (DLS) microscope. This document offers users, particularly beginners, a brief step-by-step instruction on how to operate basic confocal imaging on the Stellaris.

Stellaris 5 DLS microscope combines laser scanning confocal and light sheet imaging technologies. The system is equipped with 405nm, 448nm diode lasers as well as a white light laser (WLL) covering a wide range of excitation from 465nm to 790nm. There are three HyD detectors for confocal fluorescence imaging all allowing free emission wavelength range setting. A conventional PMT detector is used for brightfield imaging.

This 2-in-1 system provides researchers the capacity to image different types of samples, including model organisms, organoids or cleared tissue, and the ability to take advantage of the full excitation spectrum.

SCOPE

This procedure applies to basic operation for confocal imaging mode including starting up the system, preparing the system, 2D image acquisition for fluorescence (both analog and photon counting detection) and bright field (BF), saving data and exporting, and shutting down the system. In addition, the document covers some advanced confocal applications including Z Stacking and tiling (Navigator).

For more detailed confocal operational instructions and other advanced applications such as time course, multi-area imaging, FRAP, and FRET, users are to refer to the LASX online help information which is accessible from the acquisition software installed on the Stellaris computer and seeking assistance from staff where needed. The DLS operational SOP is provided separately.

SAFETY

The operator must exercise caution during loading or removing of the slide to/from the stage, never put fingers into the sample loading area while the stage is moving or initialising.

In addition, safe operation of the instrument also requires the awareness of other risks e.g. biological hazard considerations, exposure to lasers and UV light, fatigue and gesture discomfort/injury etc. Refer to the latest Safe Work Procedure (SWP) published on the Westmead Imaging internet site, WIMR-SWP-WHS-GEN, "Using a fluorescence or laser scanning microscope"

Confocal acquisition of samples on the Stellaris 5 DLS microscope must only occur after the approval of an associated project in PPMS. Any projects to be run on the Stellaris involving

hazardous chemicals and/or biohazardous samples must have the appropriate PPMS project approval. Live biohazardous samples are to be imaged on the microscope in confocal mode when contained in a completely sealed vessel such as a capped culture flask or sealed culture plate. Proper cleaning is required at the end of the experiment.

OGTR requirements for safe work in a PC2 laboratory apply.

TRAINING / COMPETENCIES

All personnel require training prior to independent operation of the instrument. Training is conducted by staff with competency demonstration necessary before authorisation.

Competency is assessed via demonstration of independent instrument operation, in conjunction with verbal explanation of all aspects of operation and troubleshooting common faults. After training is carried out, a training competency quiz is to be completed and passed before instrument access is authorised by imaging staff. All instrument operation is to be conducted by trained operators.

EQUIPMENT & SUPPLIES

The Stellaris 5 confocal and DLS is located in J210, Level 2 WIMR.

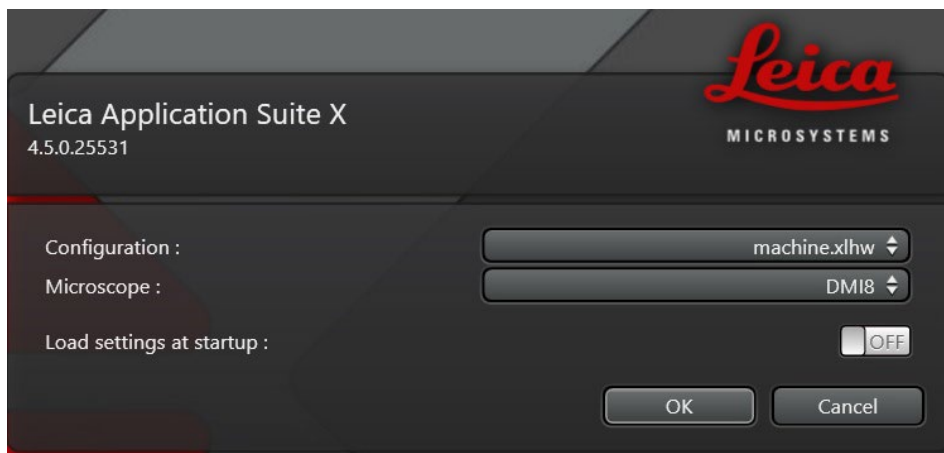
OUTLINE

1. System startup
2. Getting ready for your experiment
3. 2D confocal imaging (fluorescence)
4. Photon counting mode
5. 2D confocal imaging acquisition (transmitted light imaging: BF & DIC)
6. Saving and exporting data
7. System shutdown
8. Advanced application: Z stack imaging
9. Advanced application: Navigator

PROCEDURE

1. System startup

- Turn on the power of the following components in the specific order by following the numbers 1-4 marked next to the buttons/key of:
 1. Main power
 2. Power for Lasers
 3. Laser safety key (the indicator light will be lit afterwards)
 4. Microscope (on the wall behind the computer monitor)
- Make sure the knob on the top of the transmission light arm is switched to the “BF” position and not the DLS position.
- Log on to the computer and to PPMS.
- Open the acquisition software LASX using the following configurations for confocal imaging
 - Configuration: choose “machine” for confocal imaging
 - Microscope: DMI8
 - Load settings at startup: OFF



- Choose to initialise stage.
- Note:** stage initialisation is recommended as it is needed for Navigator application.

Warnings before initialisation:

- Make sure the current objective in position is lowered enough to avoid collision/cutting risk while initialising.
- Make sure there are no obstruction in the way of the stage initialisation movement.



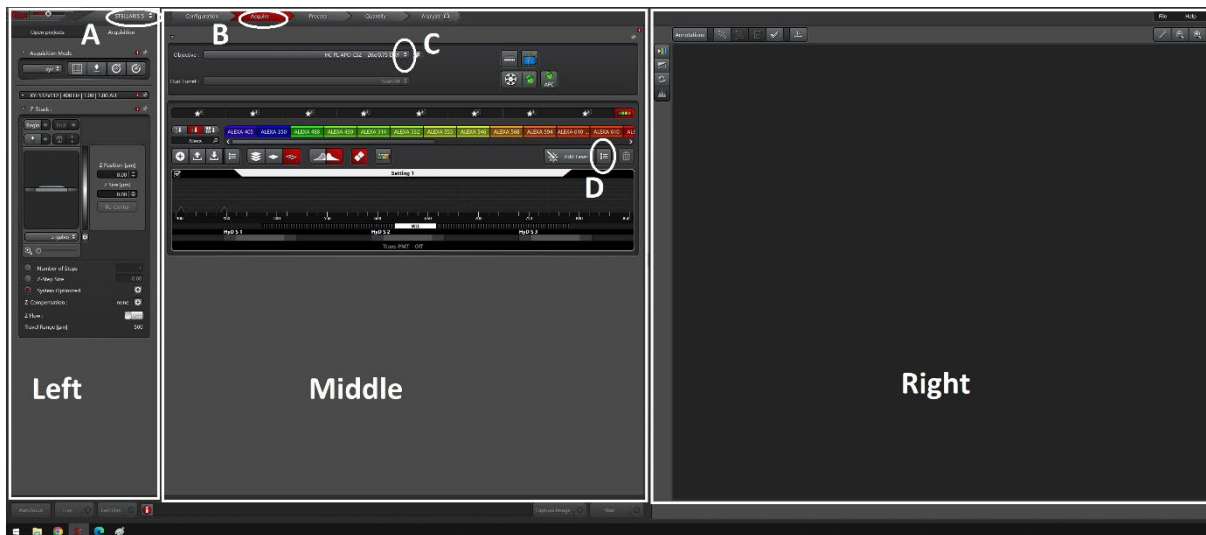
2. Getting ready for your experiment

- In LASX

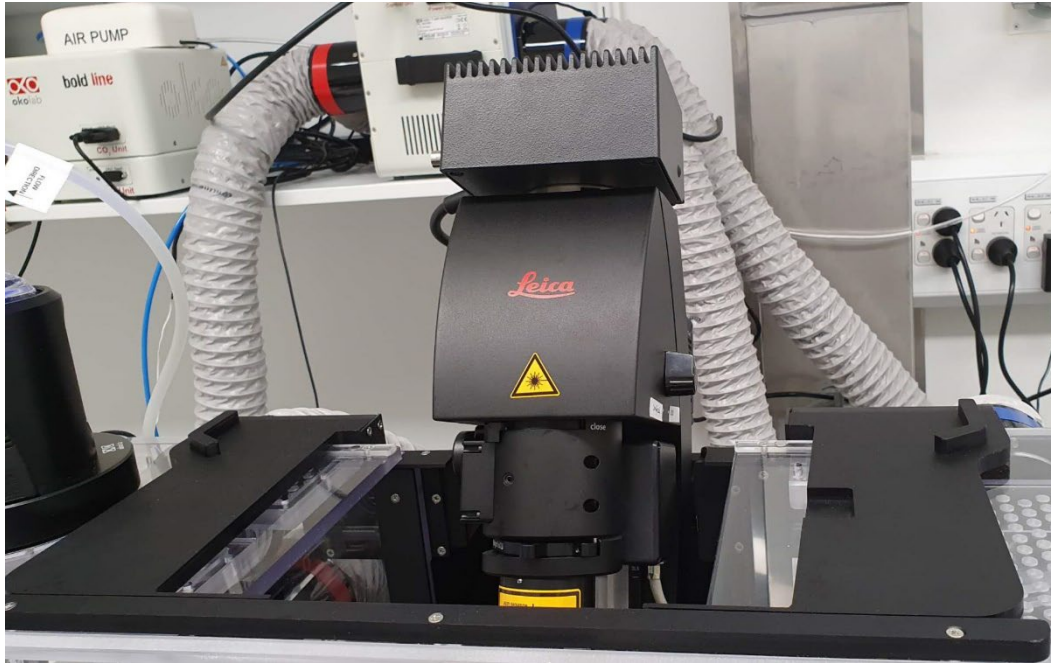
After opened, LASX looks like the below screenshot with left, middle, and right “panels”.

Follow the below steps to get LASX ready for imaging:

- Choose “Stellaris 5” for standard confocal imaging (A).
- Go to “Acquire” (B).
- Choose the objective needed for your experiment (C). Available objectives: 2.5x, 4x, 20x, 40x(W), 63x(O).
- Turn on the lasers that will be needed for your experiment (D). Available lasers:
405nm diode laser line
448nm diode laser line
485-790nm WWL (default laser power set at 85%)



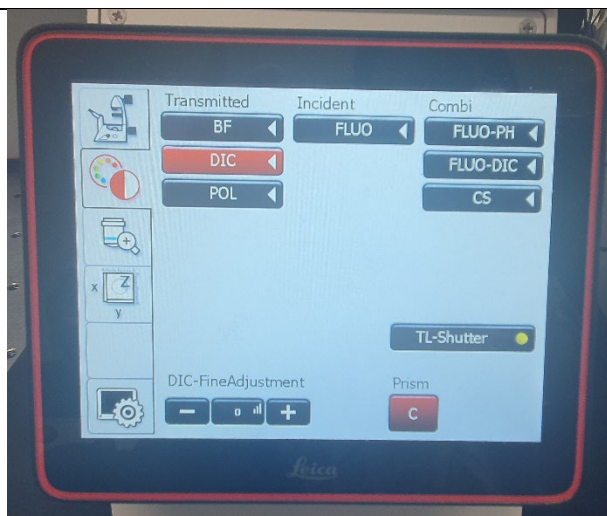
- On the microscope
- Open the incubator top window and gently tilt the transmission light arm down for better stage access.



- Clean your sample slide with the provided ethanol and cotton tips and check the objective is clean; use the right immersion medium for your chosen objective (dry/water/oil); place slide face-down.
- Open the front incubator windows and place the sample on the stage.
- Push the transmission light arm forward to sit straight.
- Set up the microscope on the touch screen (Steps A,B,C as below).

Step A: depending on your sample, choose either “BF” or “DIC” or “POL” or “FLUO” illumination to view your sample under the microscope.


Note: if BF/DIC/POL is chosen, the TL shutter will be automatically opened. If FLUO is chosen, the shutter needs to be manually turned on from the touch screen.



Step B: find your sample and bring it into focus using the device as shown in the right photo.

Note: it's recommended to push the transmission light arm forward to sit straight before focusing your sample. This is necessary for BF illumination.



You can set the current focus plane to zero if you like using the  button.



Step C: if desired, you can change to a different objective.



- For BF/DIC/POL, its recommended to optimise Köhler Illumination

Köhler illumination:

Note: It is recommended to run this procedure at least once at the beginning of your booking session. Detailed instruction on Köhler Illumination procedure can be found on Imaging webpage:

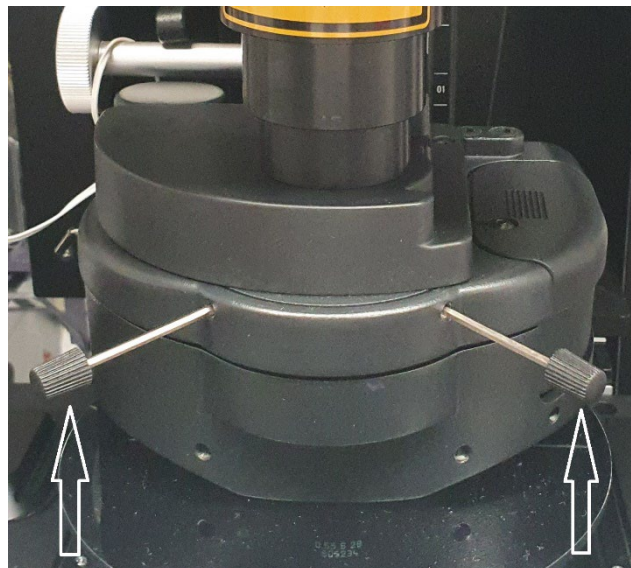
<https://sydneyuni.atlassian.net/wiki/spaces/WIF/pages/765397549/Tips+Tricks>.

- Close the field diaphragm (A) at the top of the microscope where the light is coming in until you can see the edges in the eyepiece.
- Focus the edges of the brighter polygon-shaped contour by adjusting the condenser height the knob (B).





- Centre the condenser by adjusting two centering pins until the polygon contour is located in middle of the field-of-view. The centering pins are located as shown below and are placed as shown.



- Open the field diaphragm until it is just outside the edge of the field of view.

Now, you are ready to image your sample.

3. 2D confocal imaging in analog mode (Fluorescence)

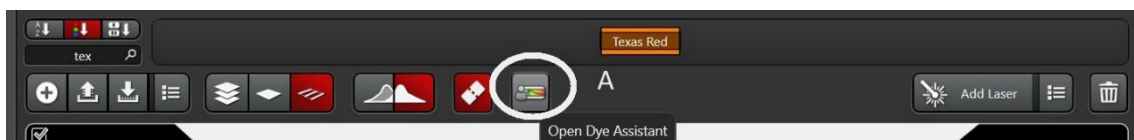
- Set up your fluorescence channels in LASX (Steps A-B)
 - If the dye you are going to image is not appearing in the Middle panel, you can search for it (A).
 - Now set up your first channel by dragging the specific dye (hold your left mouse button) to the “HYD1” bar and release the mouse (B).

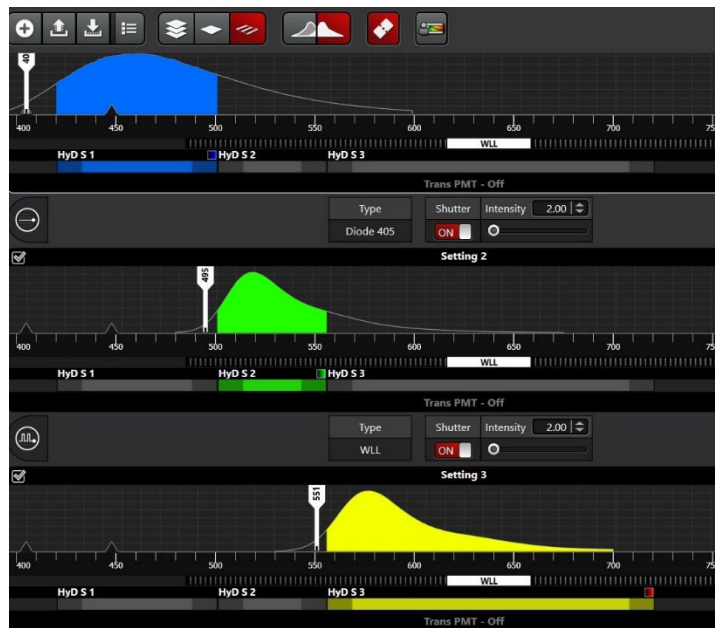


- Same way to set up the 2nd and 3rd channels.

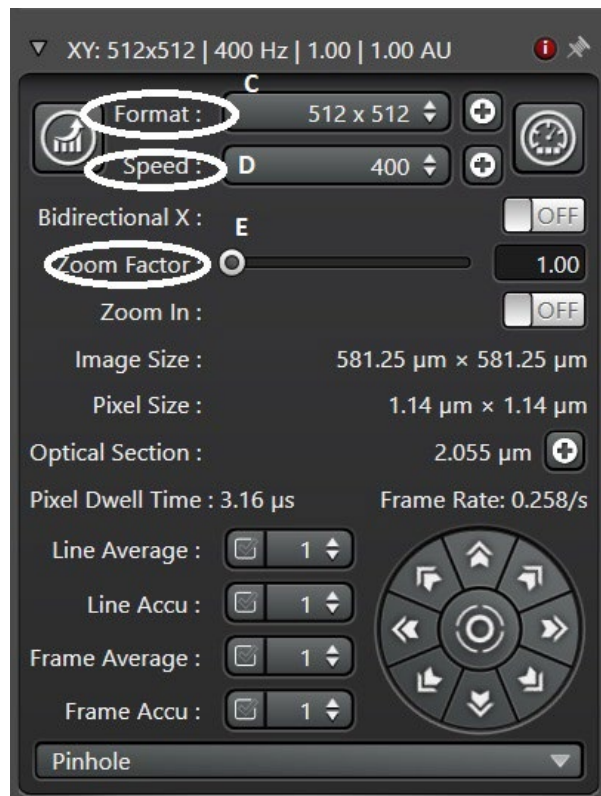
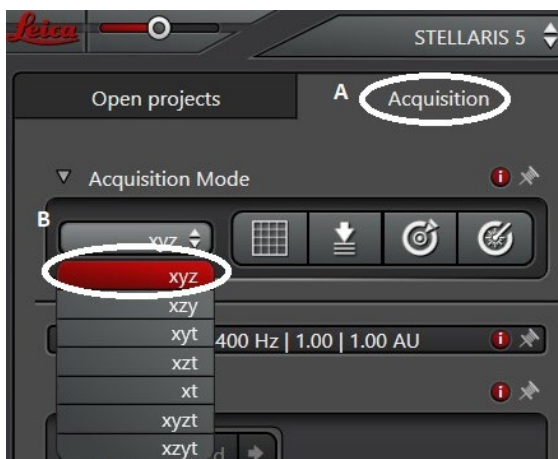


- Click “Dye assistant” (A) and pick up one of the Sequence options to minimize crosstalk between the channels, e.g. 3x line sequences.





- Preview in live window and to observe and adjust the image saturation/brightness
- In “Acquisition” tab (A), choose “XYZ” mode (B).
- Adjust imaging settings (C-E) to allow fast preview scan (there is no need high resolution images in live window at this point) i.e. low pixel format 512x512, no zoom in, no averaging nor accumulating etc.

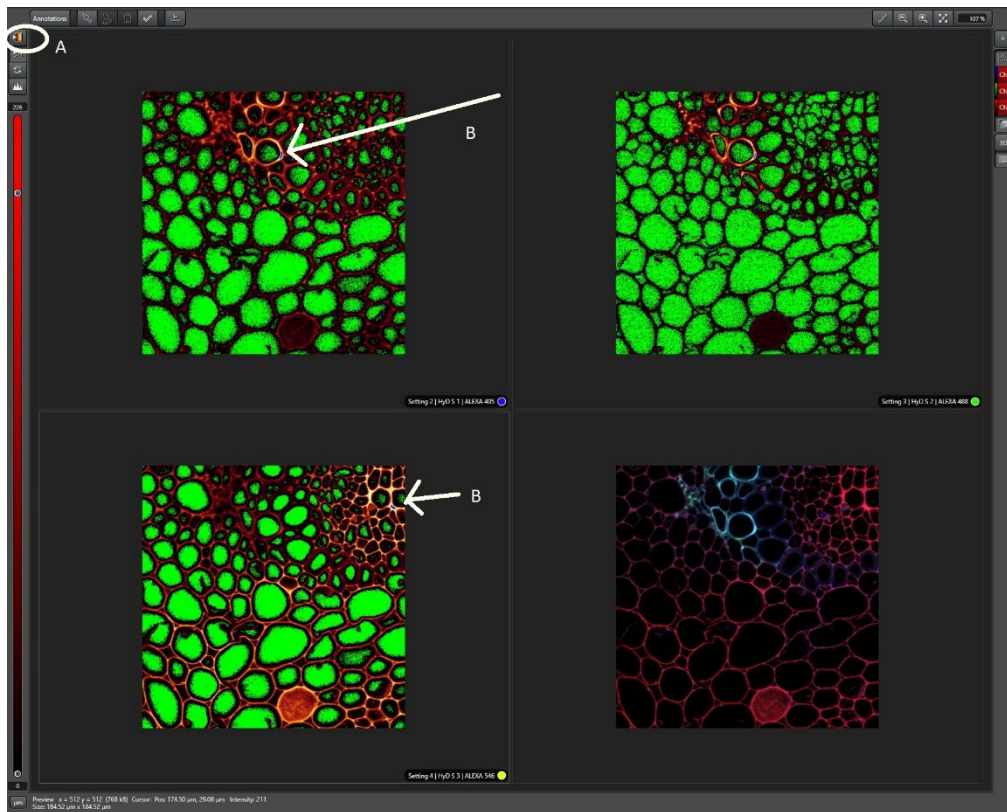
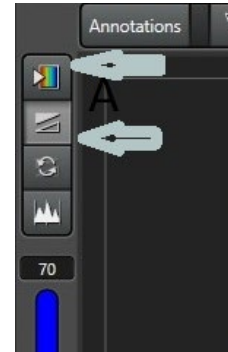


- Click “Live” to start preview scanning. Check how images look like in live window.
- Adjust laser “Intensity” and/or “Gain” (knobs on the USB panel) to get the right signal saturation in live window.

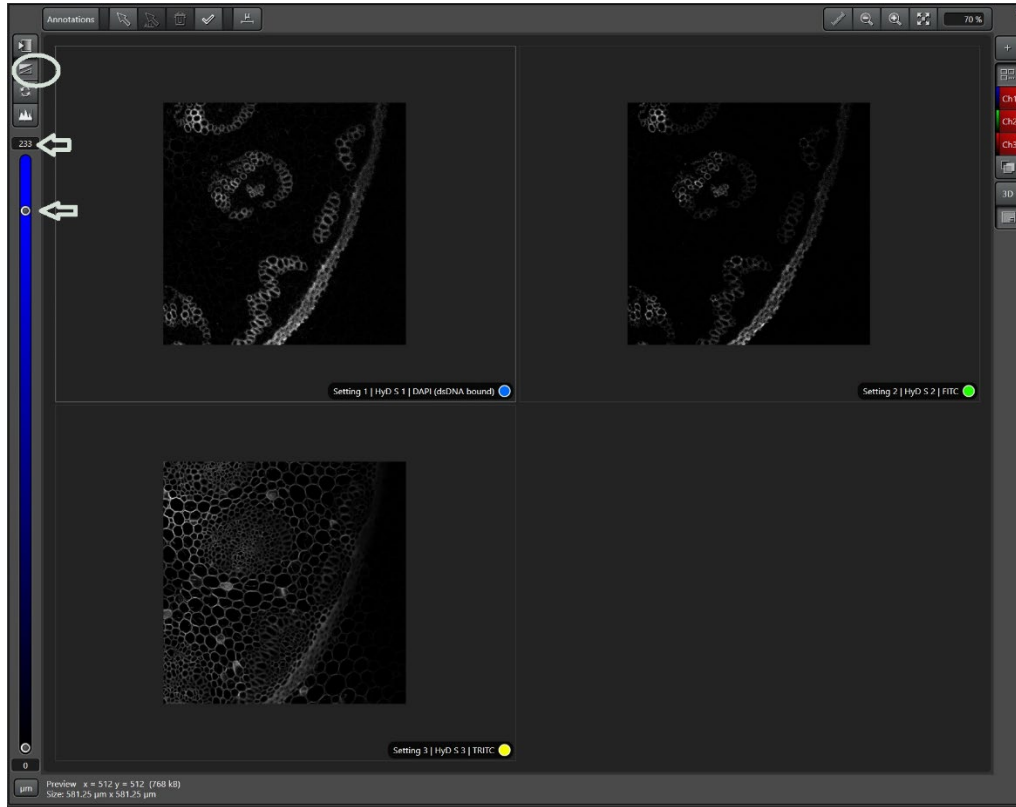
Note: there are two tools you can use to check whether your images are under or oversaturated.

Tool 1: Over-saturation (top arrow).
Tool 2: AutoScale (bottom arrow)

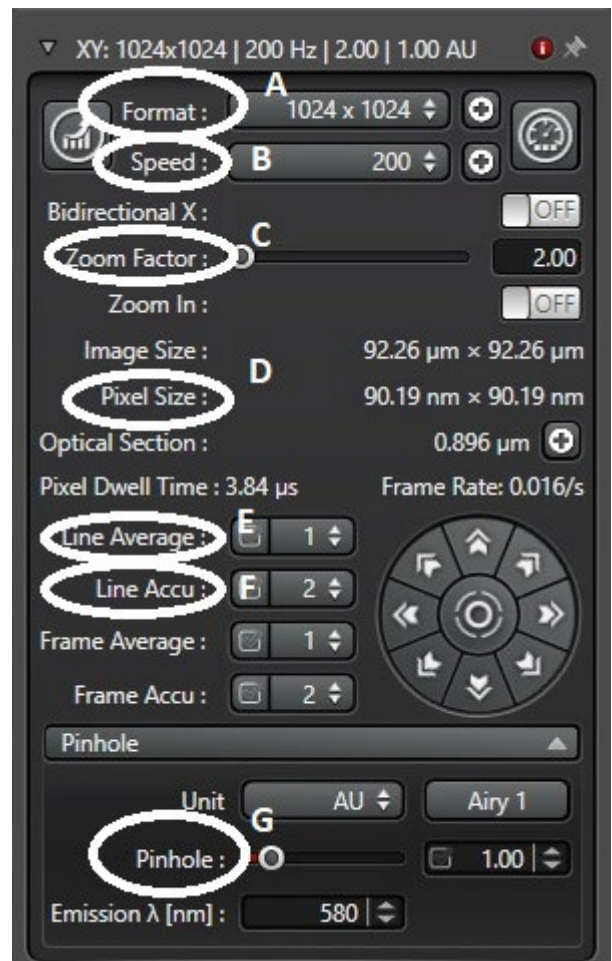
If you use Tool 1 (Oversaturation, A), the signals should be bright enough, but no oversaturation can be seen (blue pixels indicate oversaturation—Bs).



If you use Tool 2 (AutoScale), the highest pixel reading should be less than 255 (greater pixel number, brighter signal).



- Final scan
 - Adjust imaging settings to ensure outcome images will be of high resolution by choosing high pixel format (A) i.e. 1024x1024 and/or applying zoom in (C).
 - You can also choose to set up averaging (E) to smooth up images, or accumulating (F) to enhance faint signals, etc.
 - Choose your final scanning speed (B). Slower speed, smoother images.
 - Set up your pinhole (G) i.e. 1x unit.
 - Click “Start” to acquire the final image.

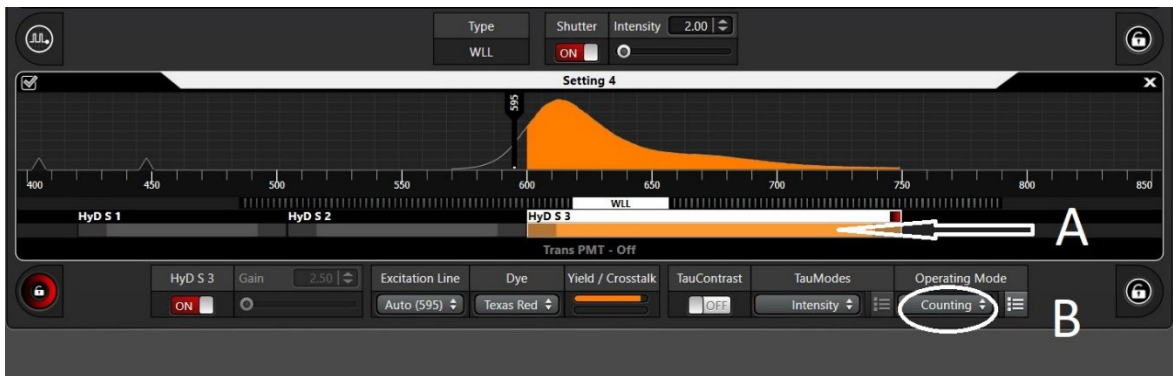


4. Photon counting mode

The Stellaris 5 system allows you to operate confocal imaging by binning and counting emitting photons resulting in reliable data for quantitative analysis and improved sensitivity with less background due to the ability to do on the fly lifetime analysis (Tau modes). It is recommended to test both conventional analog and photon counting modes so you can decide which to use of the modes.

Here we use one channel Alexa 594 as an example to demonstrate the acquisition procedure with photon counting mode.

- Click the detector bar 'HyD S3' (A) to highlight the channel.
- Select 'Counting' in the drop-down list (B).



- Adjust laser intensity to check the signal saturation as usual.

Note: in photon counting mode, laser intensity is the only parameter to adjust signal saturation. Offset is deactivated at default.

5. 2D confocal imaging acquisition (transmitted light imaging: BF & DIC)

Note: Transmitted light imaging is detected with a conventional PMT (not a HyD detector). You can choose to do either brightfield (BF) or differential interference contrast (DIC) imaging.

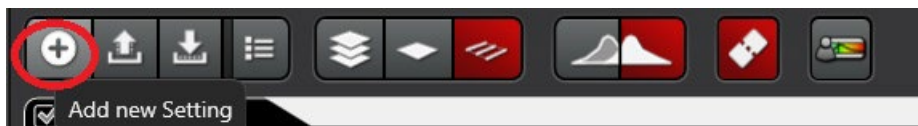
BF imaging

There are two options for BF imaging:

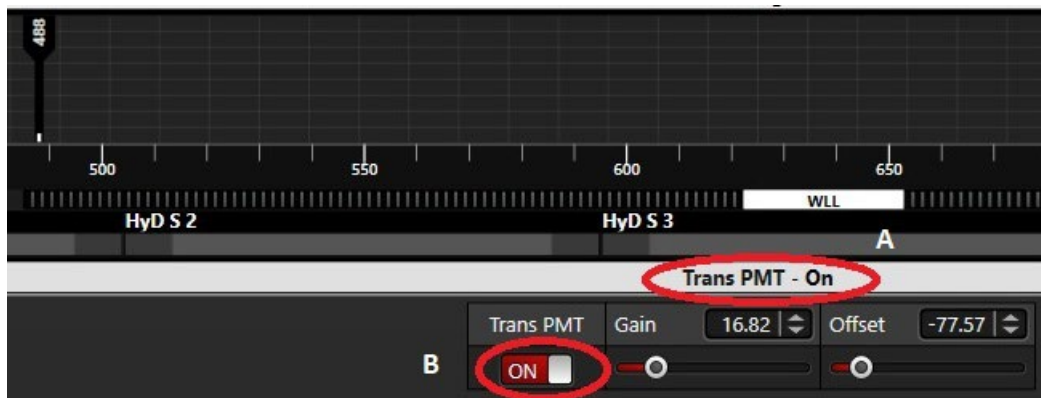
Option A: adding a new BF channel in addition to the existing fluorescent channels.

Option B: activating BF imaging on to one of the existing fluorescent channels.

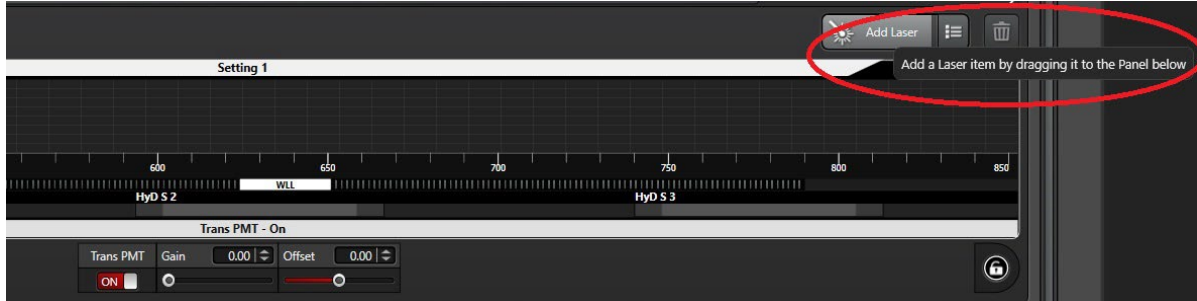
- Option A: adding a BF channel in addition to exiting fluorescent channels.
 - Click on “+” to add a new channel.



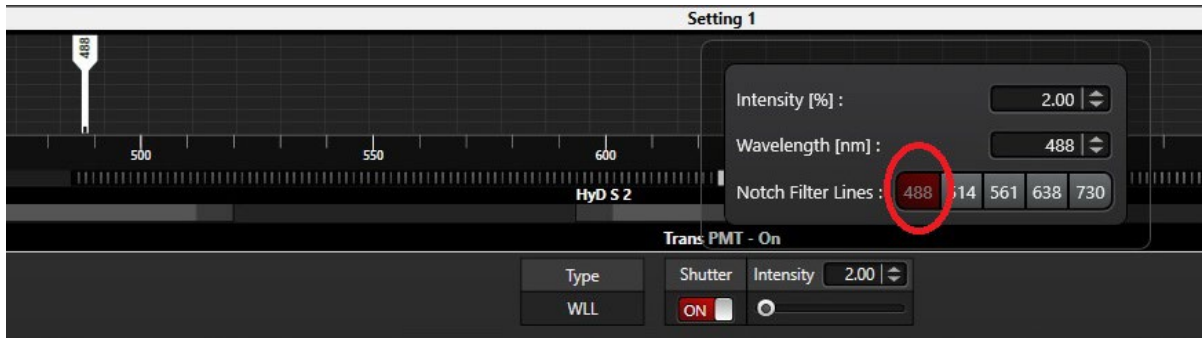
- Turn on the PMT detector (Steps A-B).



- Add a laser line for your BF channel i.e 488nm by dragging the “Add Laser” to the below panel. For now, do not worry about which laser line you ‘ll end up with once you release the mouse. The laser wavelength can be specified in the next step.



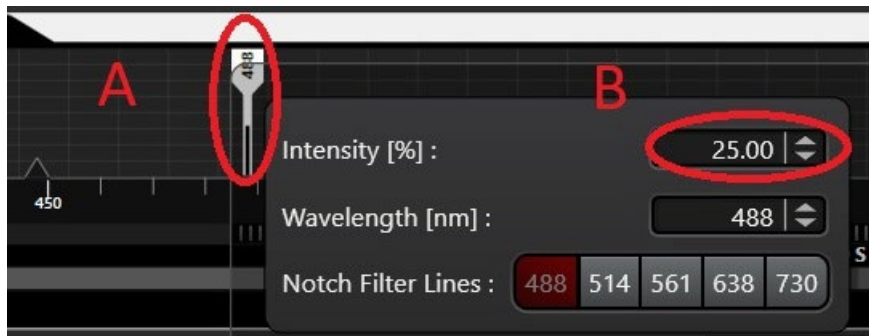
- Double click the excitation bar to open a window where you can select a specific laser line i.e. 488 nm in this case.



- Click “Live” to view how the BF image looks like on the screen. You may need to change the display image colour to grey.



- While continuously scanning, double click on the laser bar to adjust image brightness. Laser intensity adjustment can also be done using the Intensity knob on the USB panel as usual.



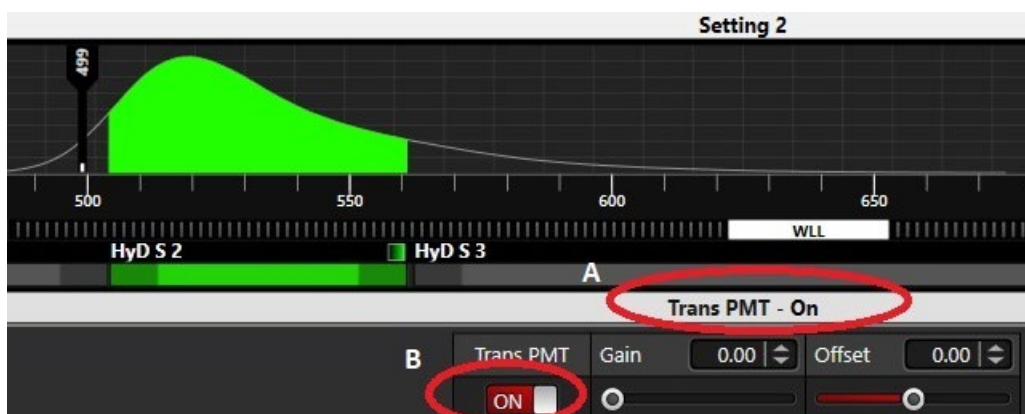
- You can further adjust PMT Gain (A) and/or Offset (B) to adjust image brightness and/or background.



- Option B: adding a BF channel in addition to exiting fluorescent channels.

Here we use one channel Alexa 488 as an example.

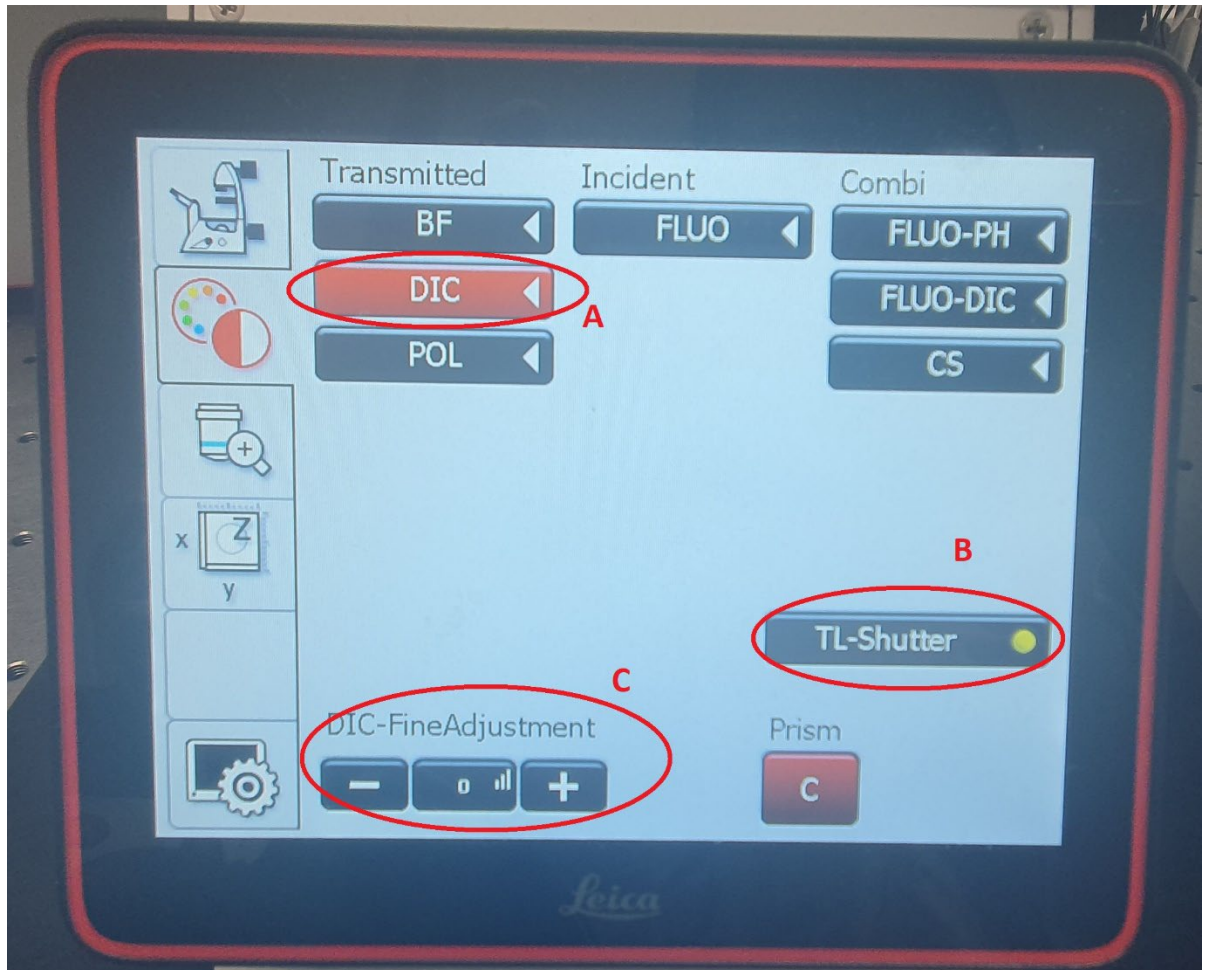
- Turn on the PMT detector (Steps A-B)



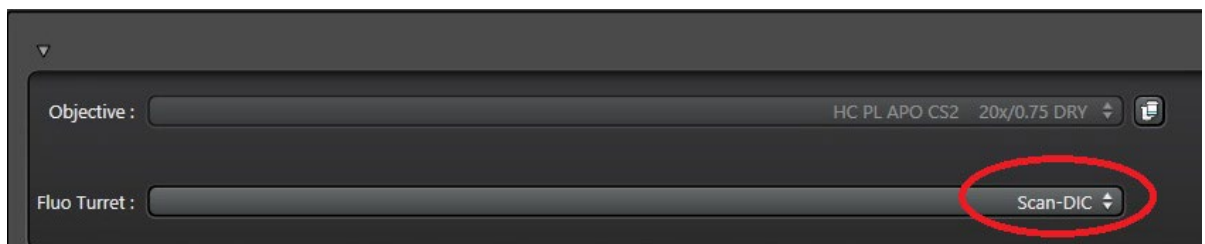
- The rest of image acquisition procedure is same as Option A.

DIC imaging

- On the touch screen of the microscope:
 - Select “DIC” (A).
 - Make sure the shutter is on (B).
 - You can adjust the contrast level here (C).



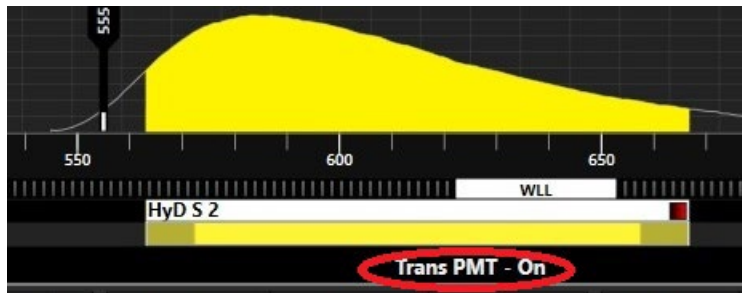
- In LASX
 - Choose “Scan-DIC” in the Fluo Turret list.



- You can adjust contrast level here in the software with the ‘Bias’ knob.



- Turn on the PMT as you do for BF imaging.



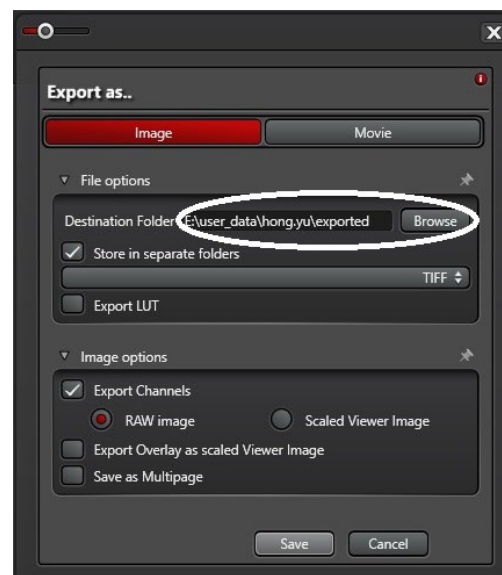
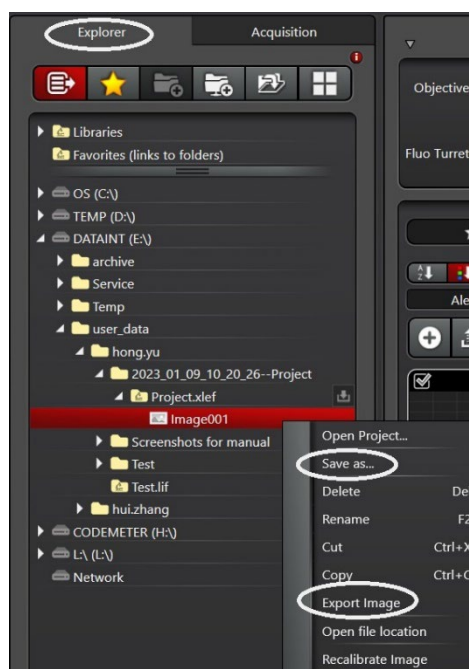
- Then proceed with previewing (click “Live”) and final imaging (Click “start”) as usual.

6. Saving and exporting data

- Go to ‘Explorer’. You can choose to save the whole project or individual images. Just highlight it and right click. It’s recommended to ‘Save as’ .lif file first as your raw data and then ‘Export image’ to other general formats i.e. .tiff if needed.

Note:

- When exporting images, you need to make sure the image destination is correct. Otherwise, your images may be saved to another user’s folder.
- Refer to ‘DATA & RECORD MANAGEMENT’ section for data management policy.



7. System shutdown

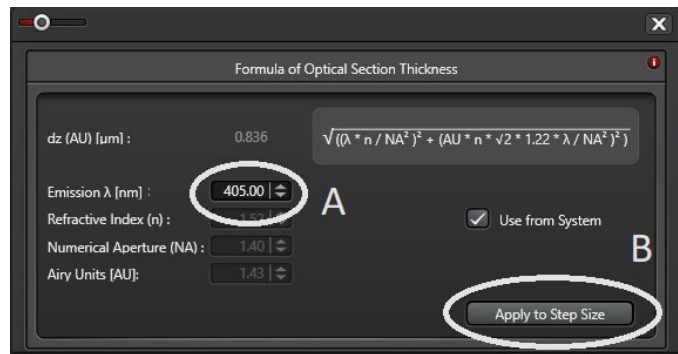
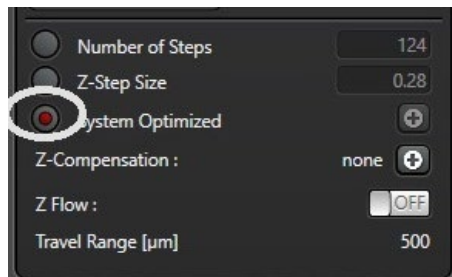
- Lower the objective to the lowest point from the touch screen of the microscope.
- Take off your slide and clean the objective.
- Clean the objective with provided single use lens cleaning wipes ONLY if water or oil was used.
- Bring up the condenser height to a safe position.
- Exit the LASX software and log off PPMS.
- Turn off the components in opposite order as start-up: Steps 4-3-2-1.

8. Advanced application: Z stack imaging (Fluorescence)

- Check signal saturation in all channels as usual.
- Click 'Live' to start preview scan. In the meanwhile, look for the top of your sample by adjusting the Z Position knob (Z Galvo, not Z wide) of the USB panel. Click 'Begin' to set the top position.
- Turn the Z Position knob (Z Galvo) to opposite direction to look for the bottom of your sample. Click 'End' to set the bottom position.



- After the top and bottom are set, you can set the step size (determining the resolution in Z axis). The simplest way is to use the 'System Optimized' tool.
 - Activate 'System Optimized', then a window will pop up.
 - Type in the shortest laser wavelength (Step A) to be used for Z stack and click 'Apply to Step Size' to confirm (Step B).



- Similar to 2D imaging, before acquiring a final scan, check the settings to ensure good resolution for XY.
- Click 'Start' to run Z stacking imaging.
- Once Z stack acquisition is completed, click "3D" on the right edge of the live window to view the stack images in 3D viewer.



9. Advanced application: Navigator (Tiling)

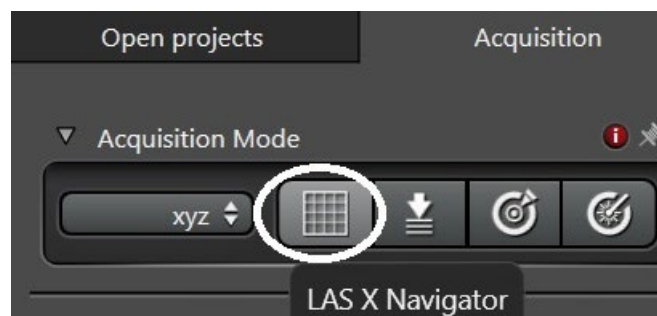
There are two approaches to set up tiling experiment in Navigator.

Method 1: Set up channel saturation in a standard XYZ mode as usual and then open Navigator for tiling setup.

Method 2: Open Navigator first and then set up channel saturation in Navigator.

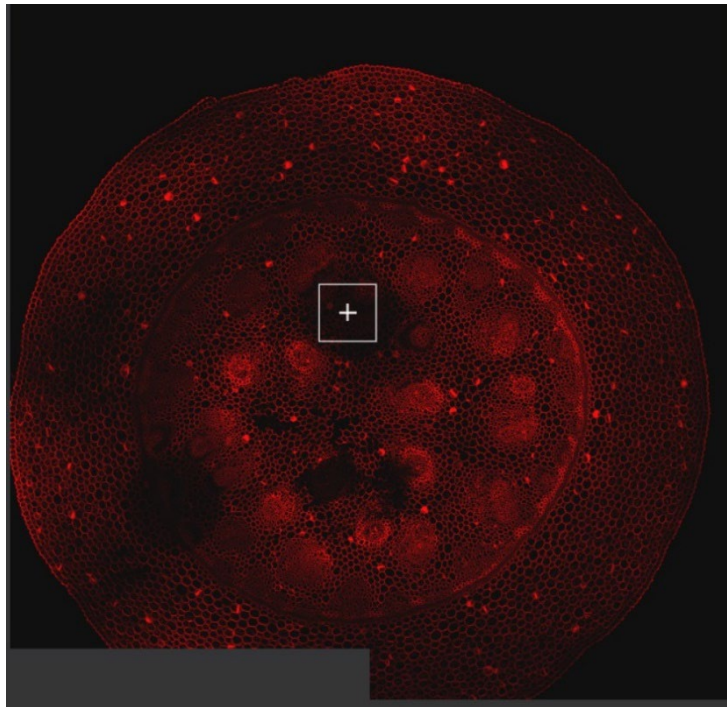
The below procedure uses Method 1 as an example to demonstrate the Navigator application. This is a simple approach to set up multi-position imaging or Tiling. For full details of other options to set up Tiling in Navigator i.e. using the 'Carrier' tool, please refer to LASX online help.

- Click the 'LASX Navigator' icon under 'Acquisition' tab to activate the Navigator.

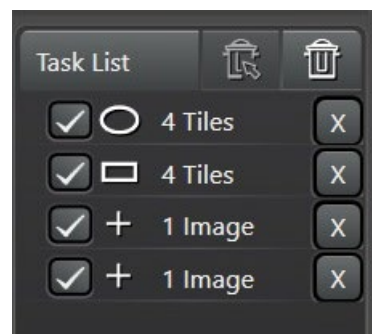
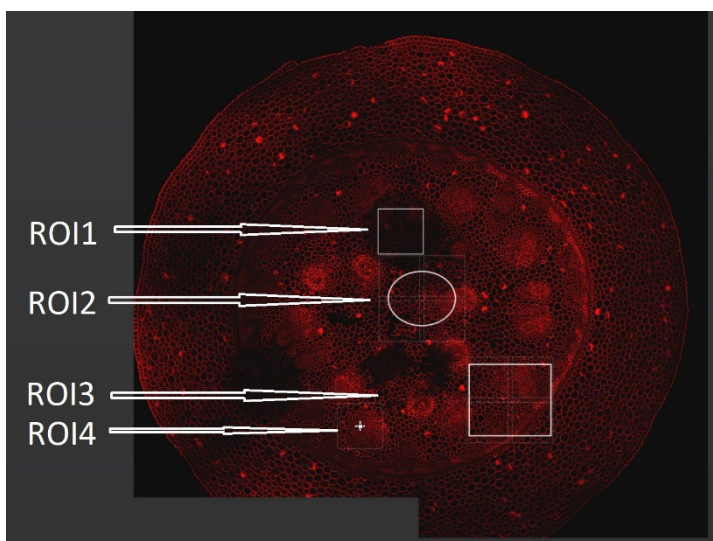


- Click 'Spiral' to start scanning an overview image across the sample. This could take a while. You can stop it at any point if the overview area in live window looks big enough for you to find interesting positions or tiling area(s).

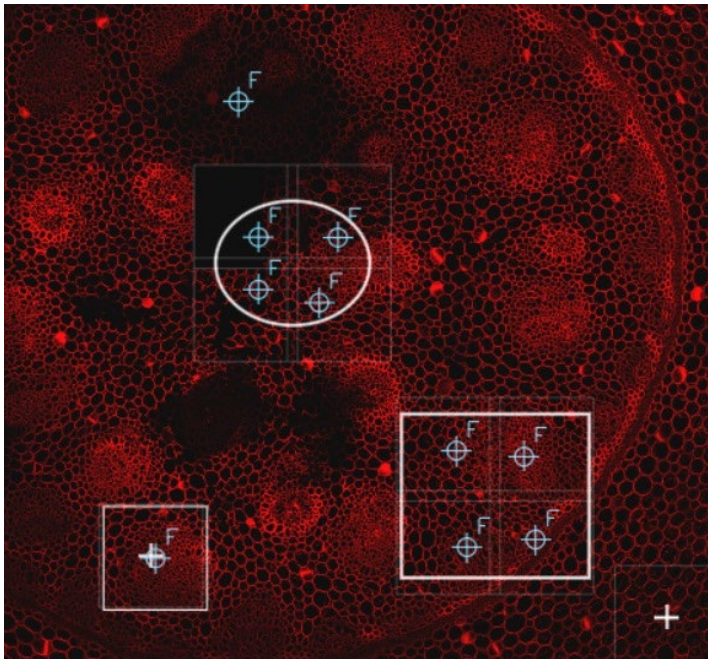




- Click '+' to start adding/drawing ROIs. In this case, there are 4 ROIs added including 3x areas (ROIs 1-3) and a single position ROI4. The ROI 1 area is too small to be split into multiple tiles so this ROI1 is going to be ended up with a single image. ROIs 2 & 3 will be 4 tiles. The selected 4 ROIs can be found in the right 'Task list' section.



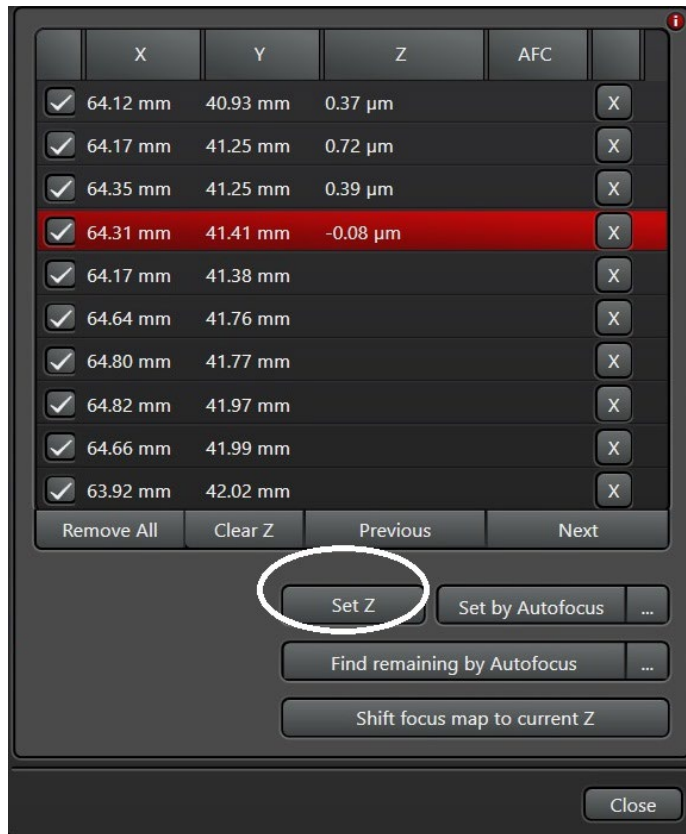
- Click the blue 'F +' icon to manually add focus points in the selected ROIs (the green 'F+' icon is to automatically add focus point).



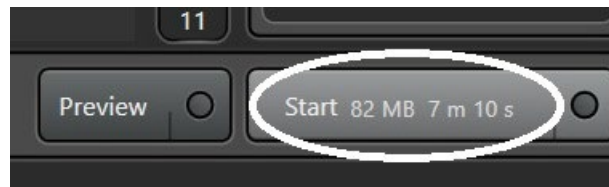
- Click 'Focus map' to open up the focus point list/map. This is where you can go through all the selected focus points and fine tune focus for individual points.



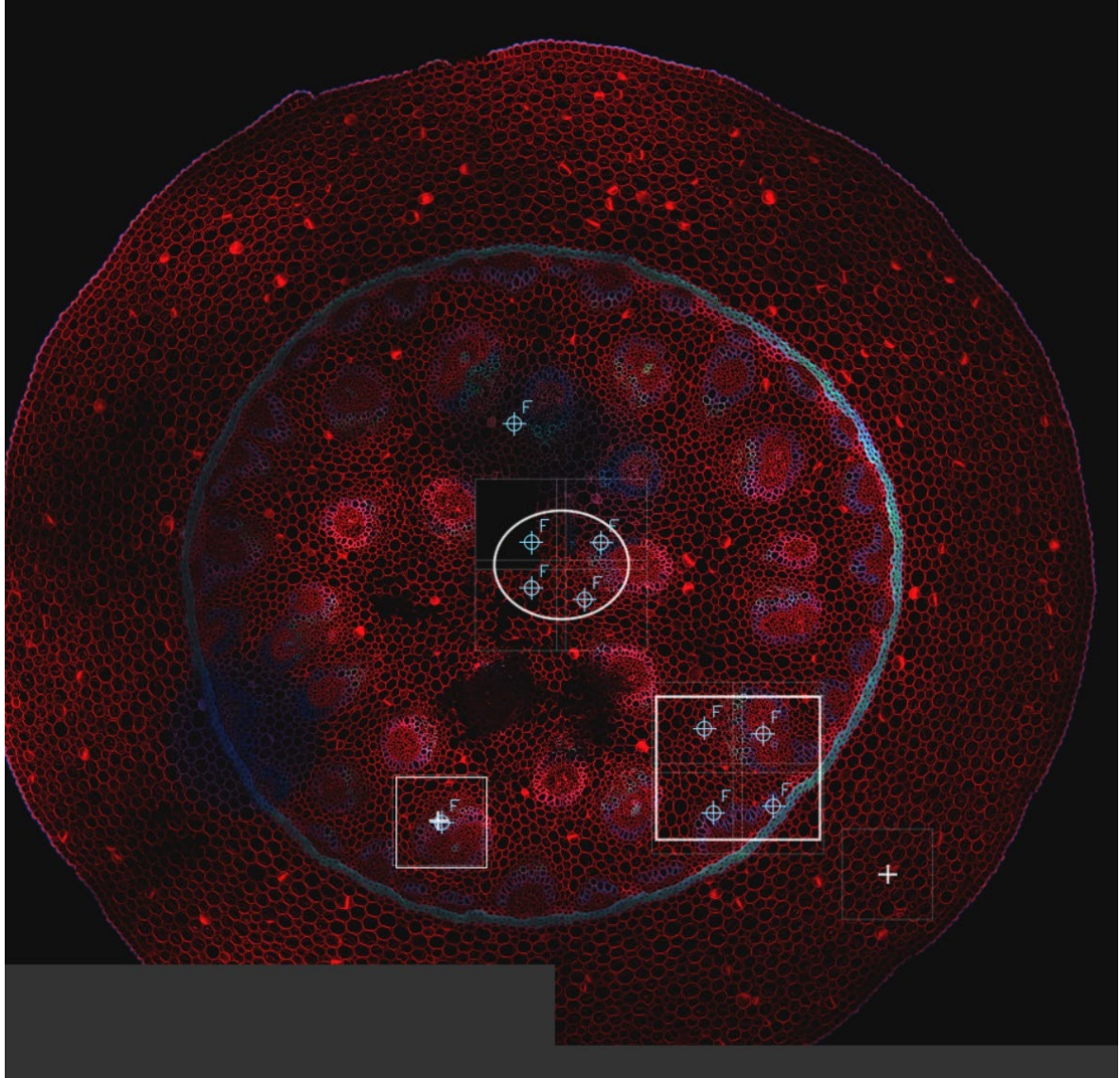
- In the Focus map, double click each focus point to visit its position. Fine tune its focus by adjusting Z Position knob of the USB panel (Z Galvo) then click 'Set Z' to update its focus plane (it takes a little while to set).



- Click 'Start' to acquire tiling data. It shows you how much memory it will take for this experiment and how long to complete acquisition.



- Post-acquisition.
 - View your data. Once acquisition is completed, your data can be found under “Images” tab and the stitched image will be displayed in the monitor (with preview on or off).



- Save your data. You can save your data in Navigator or back in the standard XYZ image workflow interface.

DATA & RECORDS MANAGEMENT

All data must be saved onto the local computer where it is kept for 7-14 days before automatic deletion.


Save data to E:\users data\“your_folder”. Your data is synchronized to the WIMR Imaging Onedrive account and transferred to the Scientific Platforms server. Scientific Platforms (\\Scientific_Platforms\Cell Imaging\Stellaris\“your_folder” where it will be held for 14 days before deleting without warning. You need to therefore move your data promptly. Staff can share to non-WIMR researchers a onedrive link to access your folder remotely if required.

REFERENCES

For more information about confocal imaging and other advanced applications i.e. lightening, Tausense, please refer to LASX Online Help.

This SOP is also published on the Westmead Imaging Website.

DOCUMENT CONTROL

Document Reference:	WIMR-SOP-SP-CI-34.01
Status:	Approved
Endorsed by:	Hong Yu
Approval Authority:	Suat Dervish
Signature of Approval Authority:	
Approval Date:	4/1/2024
Issue Date:	4/1/2024
Review Date:	4/1/2024
Author:	Hong Yu
Policy Owner:	Scientific Platforms
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