Leica SP8 Confocal User Manual

Microscopy and Imaging Facility

Shared Research Support Service

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Table of Content

Turn on the SP8 confocal system

1. Sign on the log sheet before starting the system.

2. Check if the four corners of the floating table floats freely.

3. Check if the stage is clean/oil free. If not, please clean it before you start.

4. Turn on the microscope in the order of 1→8 as labeled on the equipment. The mercury lamp needs a minimum 15 min on time before you can turn it off and vice versa. If you just turned it off, wait for at least 15 min before you turn it on.

5. Log in to your Pitt's account on the computer.

6. Click on the LAS X software icon on the desktop.

7. Click Ok when this window pops up.

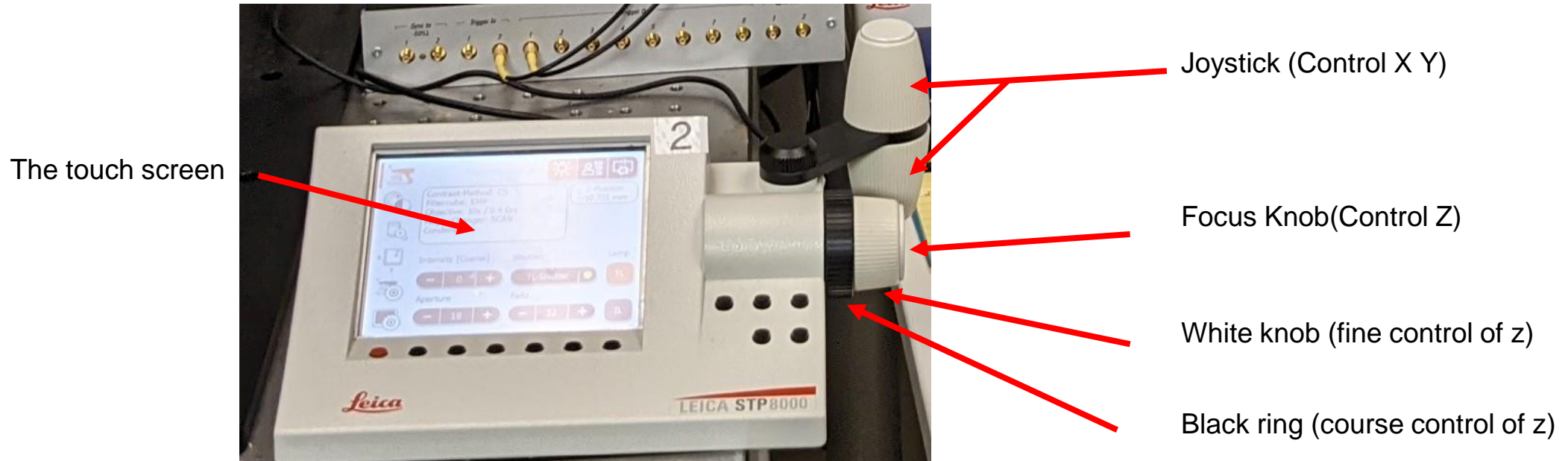8. If you need to use the Resonant Scanner, turn on Resonant at this step.

When do you need Resonant scan?

- Resonant scanning significantly increases image acquisition speed but compromises the resolution. It is often combined with averaging to increase resolution.
- Live cell imaging that requires ultra-fast acquisition speed.
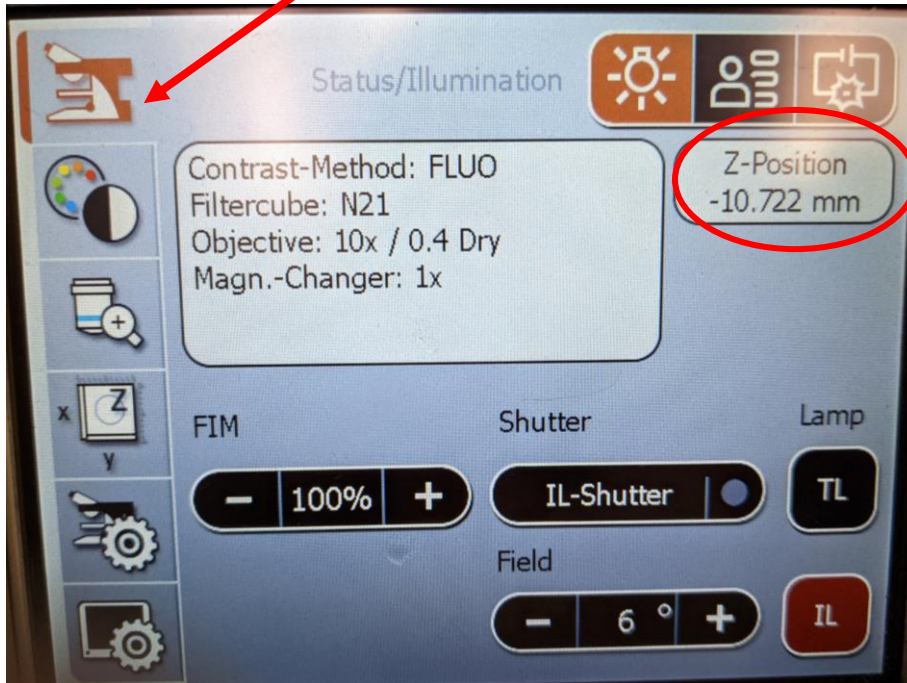- Huge data set that you need to reduce overall scanning time.
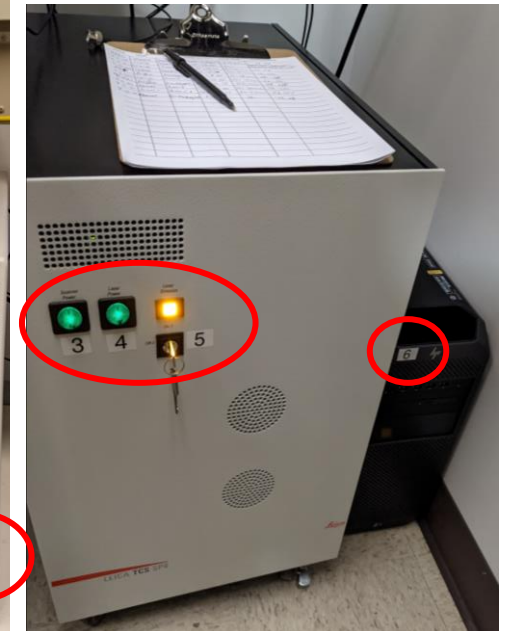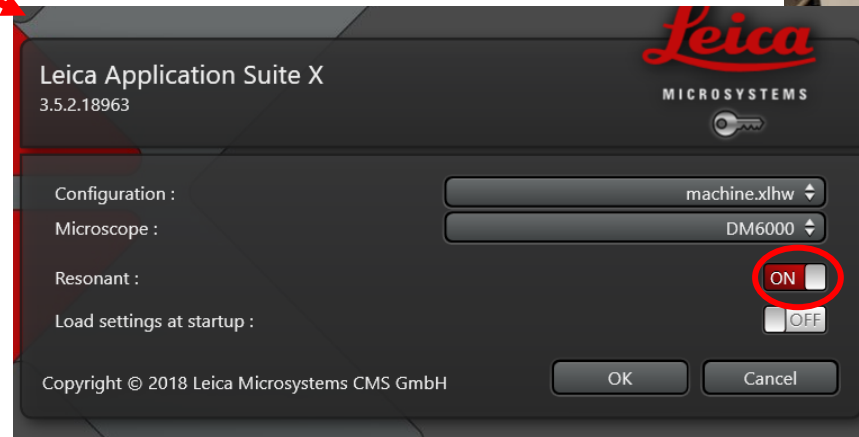
View samples through eyepieces

1. Place your sample coverslip-side up on the stage. (DO NOT press down on the stage, the stage has a delicate galvo system that controls precise z movement. It maybe destroyed if harsh pressure is applied.)

2. The focus knob and stage control/ joystick are on the touch control pad.

3. Turning the focus knob towards you moves up the objectives. (Increases the distance between your sample and the objective.)
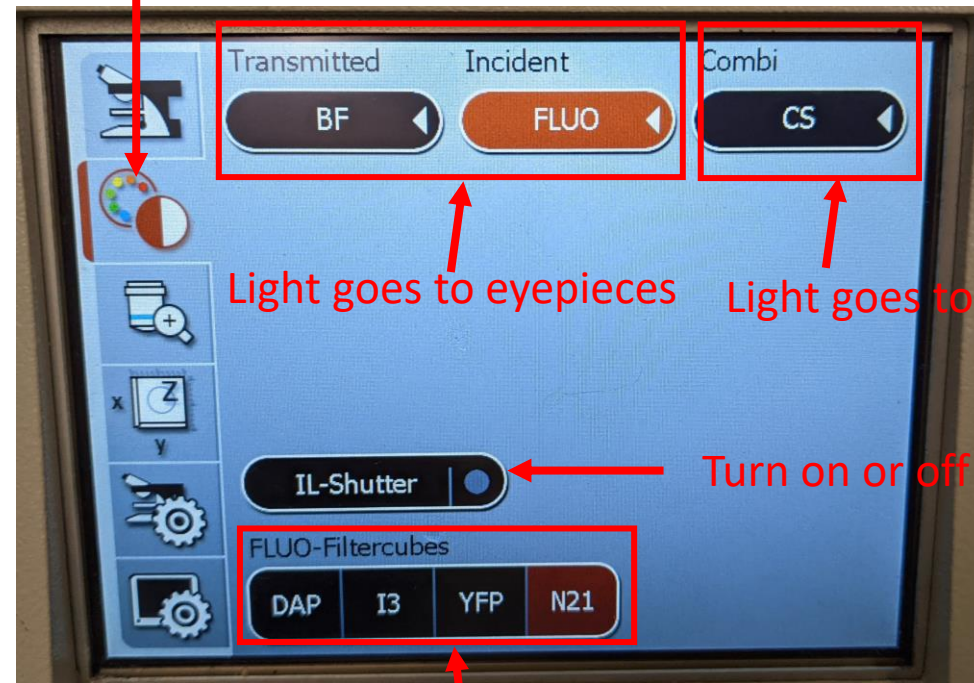
4. The joystick controls the stage position on XY.

The touch screen

Joystick (Control X Y)

Focus Knob(Control Z)

White knob (fine control of z)

Black ring (course control of z)

The touch screen

Microscope Section

Channel section

Light goes to eyepieces

Light goes to scanner

Turn on or off the light

Fluorescent channels for eyepieces

5. Click on either BF (Transmitted light) or FLUO (fluorescent light) to direct light to eyepieces.

6. Next, press on IL-Shutter (Fluorescent light) or TL-Shutter (Transmitted light) so the buttons turn yellow. Then you will see light turned on.

7. Change the focus and position of your sample through the focus knob and the joystick.

Objective Section

Stage Section

Objectives

Joystick fine & course adjustment
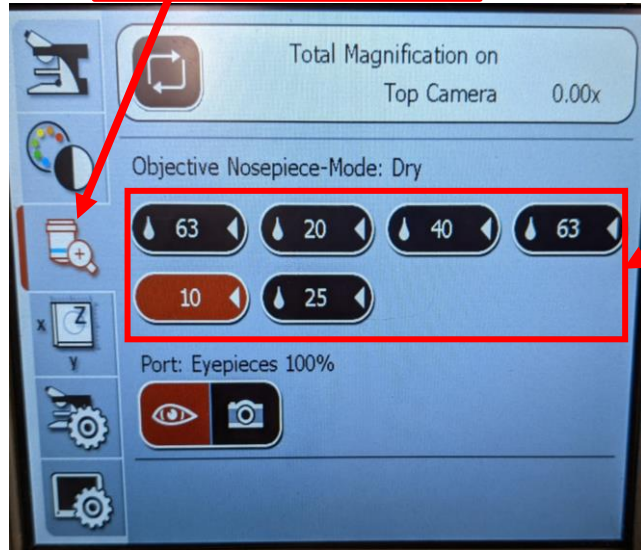
8. To change objectives, select the objective section, then press on the objective you want to use, Please make sure your sample does not have oil on it when using dry objectives. You can also change objectives through the software.

9. To change the course and fine movement of the joystick, select the Stage Section, then select XY, and switch between course and fine.

10. After observing through eyepieces, go back to the channel section, and turn off the IL/TL shutter. (This a good habit!!)

11. Now, you can start to view the image on the software.

LAS X software

Microscope Settings window on the left

Image window on the right

LAS X software

1. Click on the Configuration tab at the top of the software.
2. Then click on the Laser Config tab
3. Next, click on specific laser lines to turn them on.
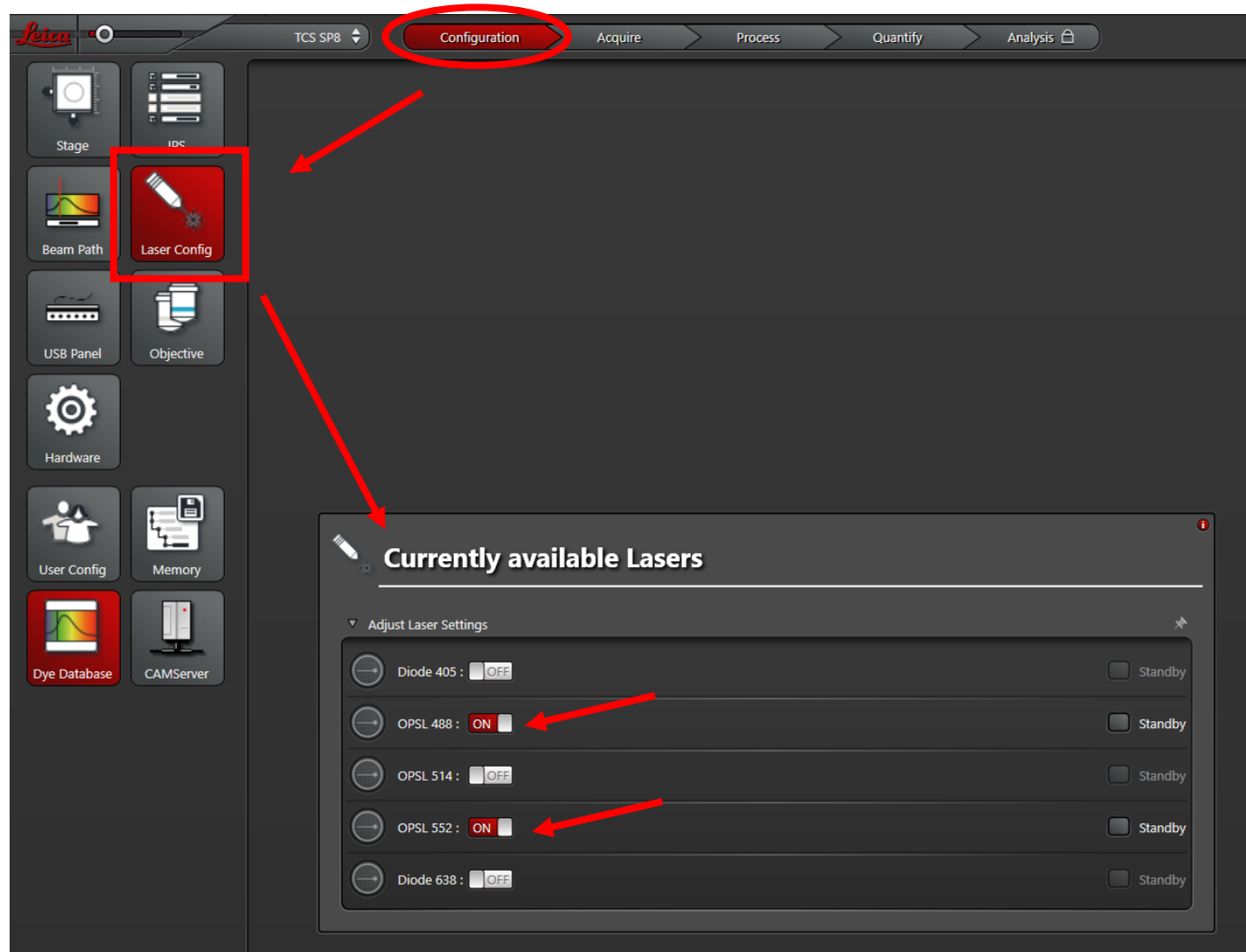
Create a new experiment setting

1. If you want to use existing settings, you can load it from your previously saved settings or images acquired with that setting (See instructions on page 15). Otherwise, you need to create a new experiment setting.
2. Create a new experiment setting under Acquire→Acquisition.
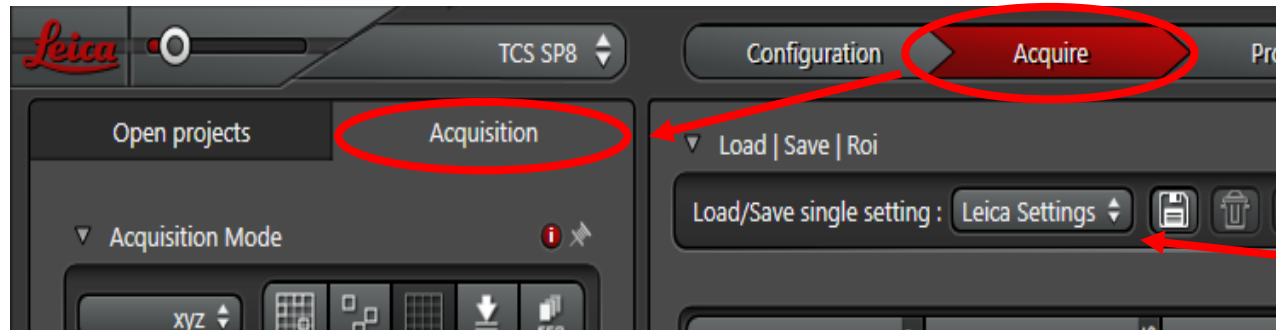

3. Make sure you create a Sequential Scan setting when you have more than one fluorescent channels.
4. Click on the seq tab to turn on sequential scan setups.
5. Acquisition Mode is set to xyz by default.
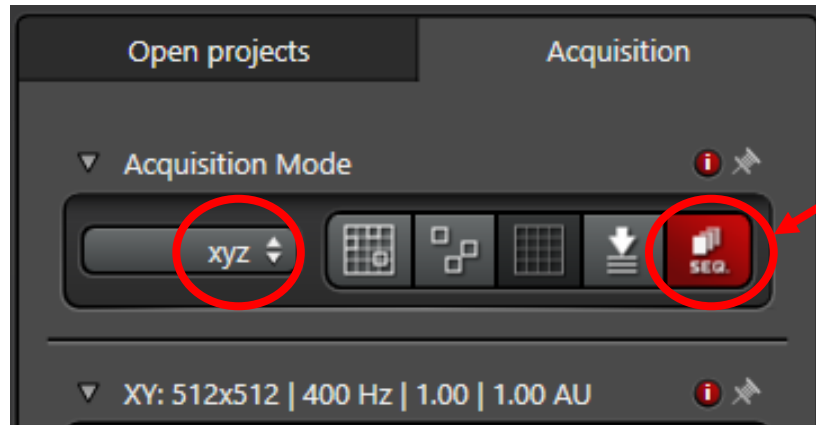
If you only have one fluorescent channel, you can select it from the Load/Save single settings dropdown menu. In this case you do not need to set up a sequential scan, but sequential scan is suggested for most experiments.
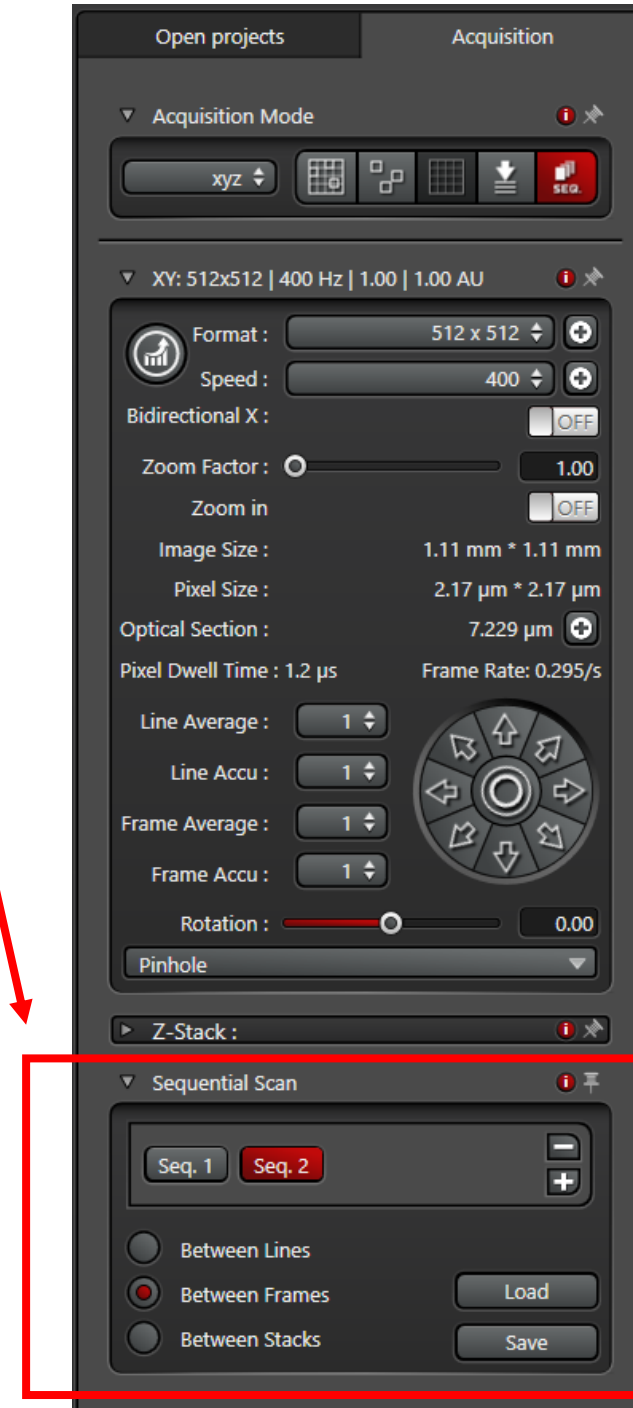
6. After clicking on the Seq tab, you will see a Sequential Scan section at the bottom. Your sequential scan settings will show up in this section.

7. Click on the setup icon, and the setup window will pop up.
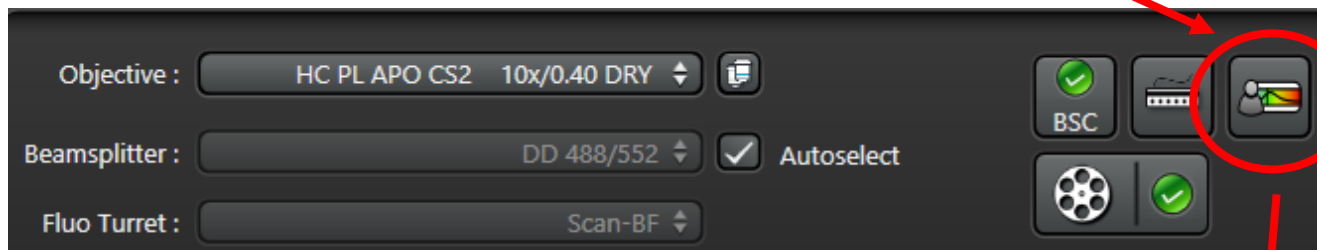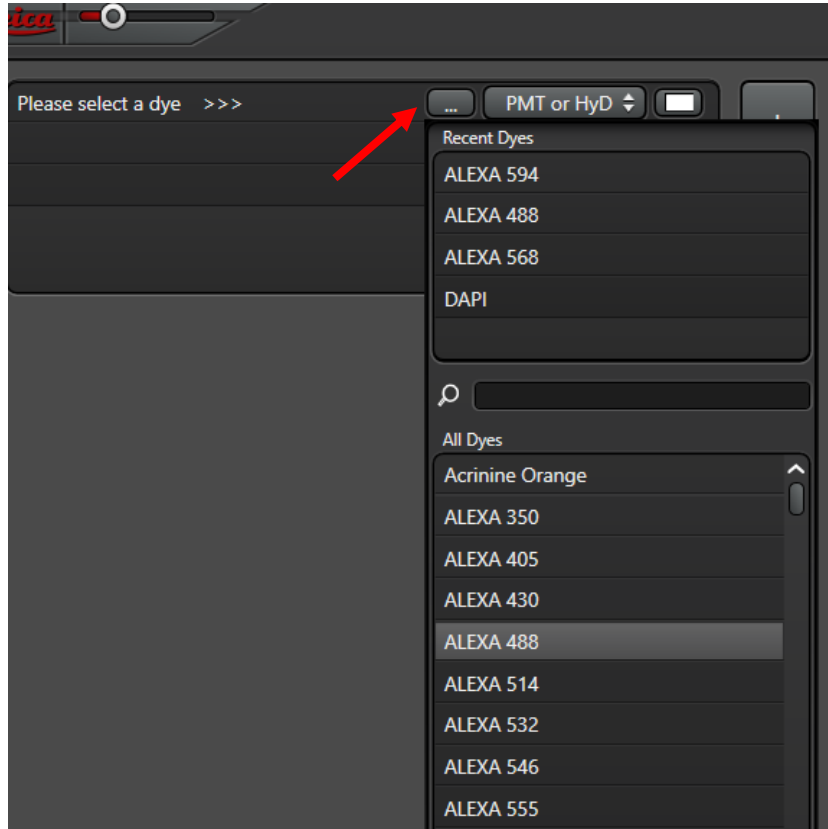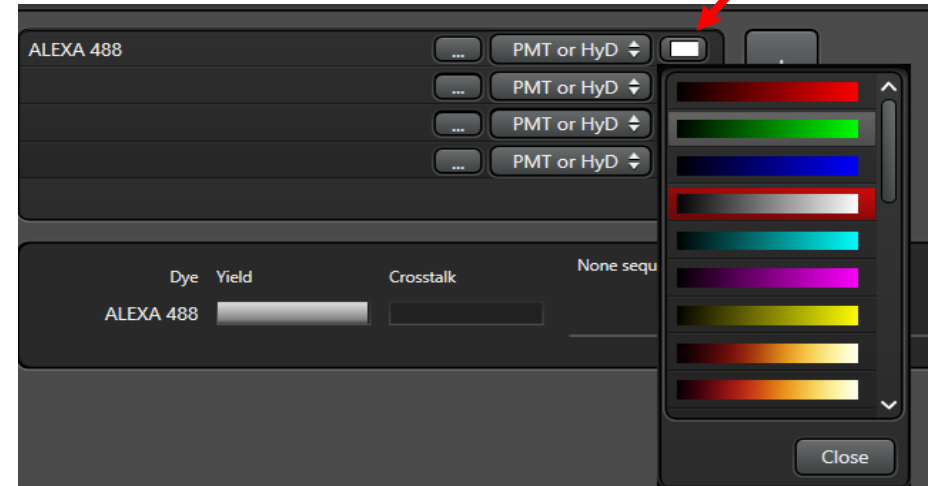

A new window pops up
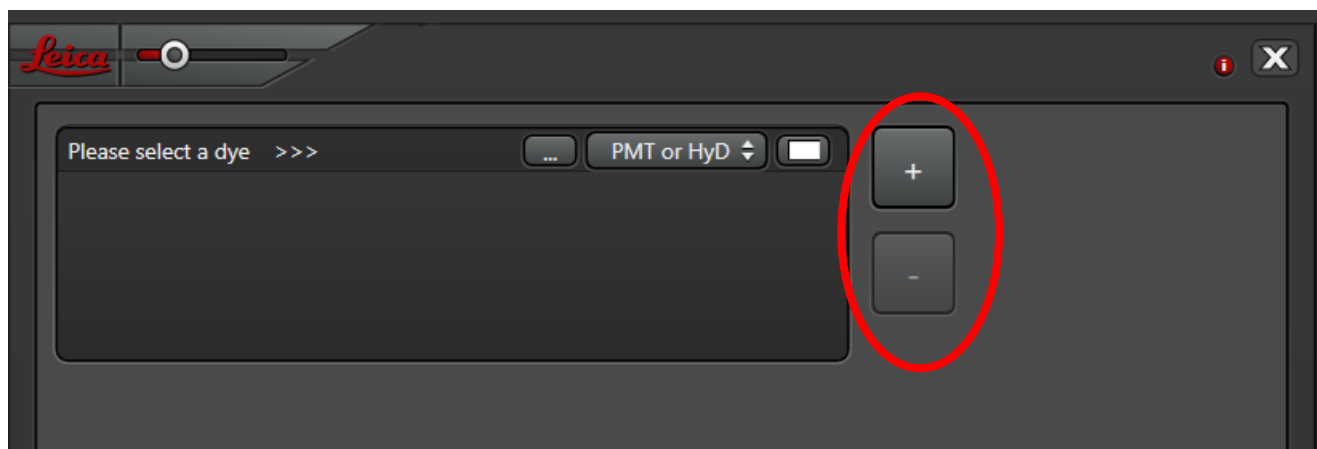
8. Click on "+" or "-" to add or delete channels.

9. Select a channel: Click on [icon] icon to select the channels.

10. Choose a color: Click on the [icon] icon to select a color for that channel.

11. Choose a detector (PMT or HyD)

Select HyD for visible lasers (488, 514, 552)
Select PMT for invisible lasers (405, 638)

12. After setting up a multi channel experiment, you can adjust the parameters.

Name of the scan

Simultaneous scan--**DO NOT** use this

Partial Sequential Scan.

Sequential Scan (Recommended).

Click on Apply to select the scan you prefer.
Click Edit to adjust the emission light band width.

Note: It is best to select Line or Frame sequential scan. Partial sequential scan may still induce cross talk, even if theoretically the two wavelengths do not overlap.
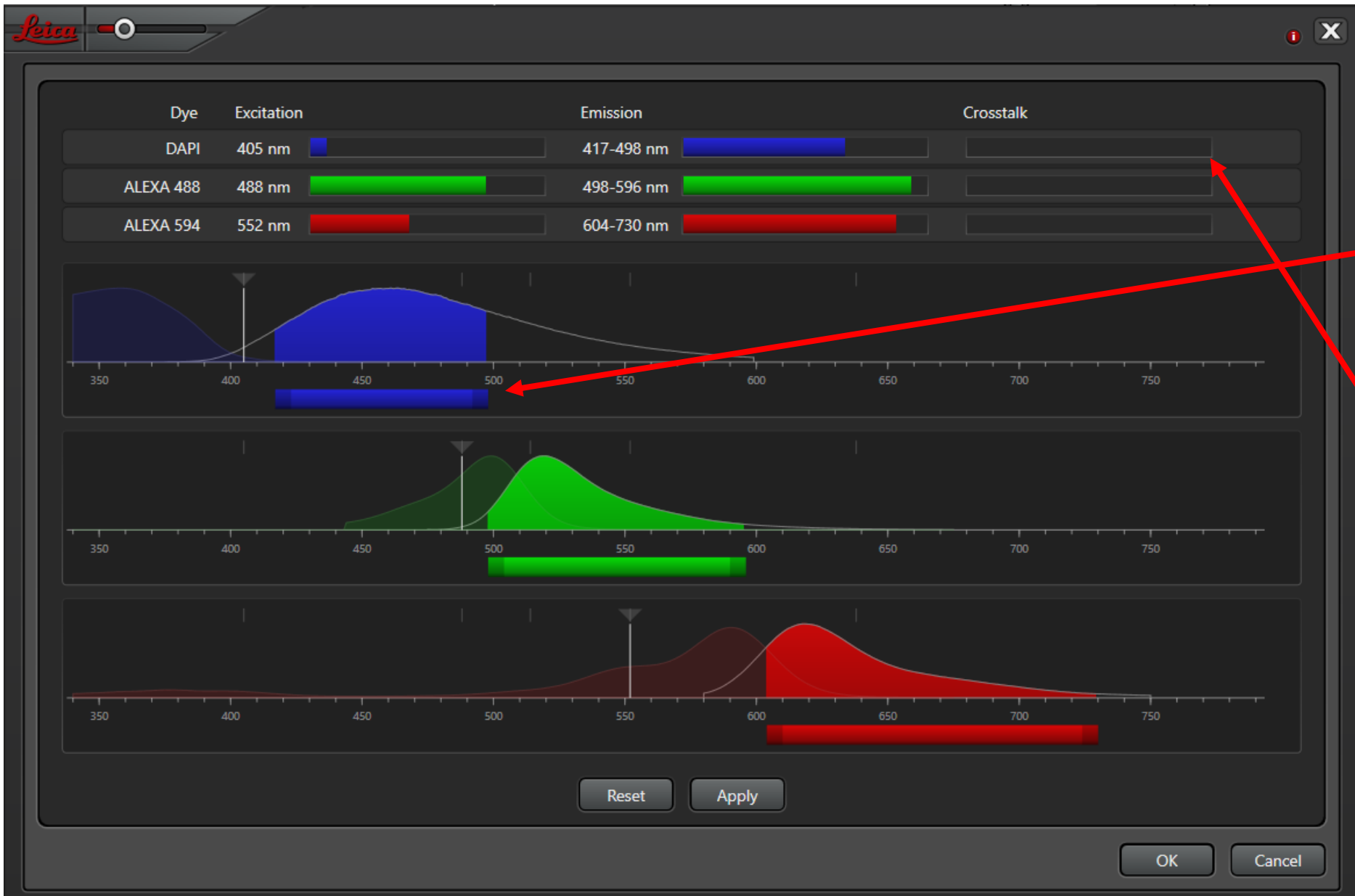
You can move the bar around or change the length of the bar to adjust emission light band width.

It is best to adjust the width to a point that there is no crosstalk.

For DAPI, it is always good to start from 417 nm.

When adjustment is done, click Apply, then click OK.

13. Now, in the sequential scan section, you will find the channels that you set up.

14. Clicking on each Seq., you will find the corresponding laser line and detector. Important: One laser and one detector for one Seq.

Laser

Adjust laser power through the bar

Detector

Seq 1

Seq 2

Load pre-existing experiment settings

1. Once you've set up an experiment setting, you can load it from an image that you acquired with this setting.

2. To load from an image, click on Open projects, then click on the open folder icon to load your image.

3. Click on an image, then click on the Apply icon to apply the settings of this image. Gain and offset values, averaging and accumulation, as well as laser power values are all applied in this way.

4. Another way of loading pre-existing experiment settings is to first save the setting as .seq format through Save, then load this .seq file through Load. This .seq file is not an image file, it does not contain the gain, offset, averaging, accumulation and laser power values.
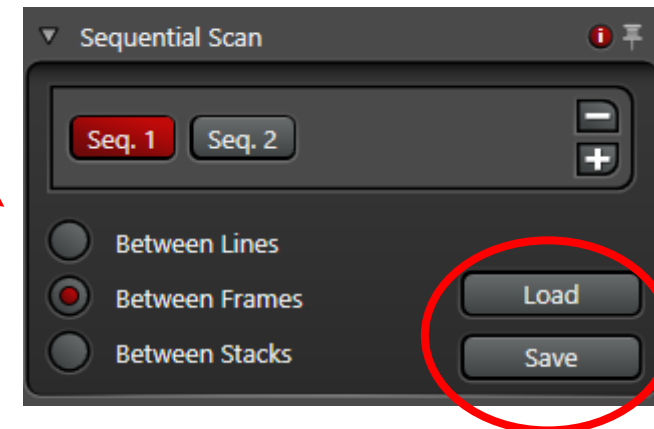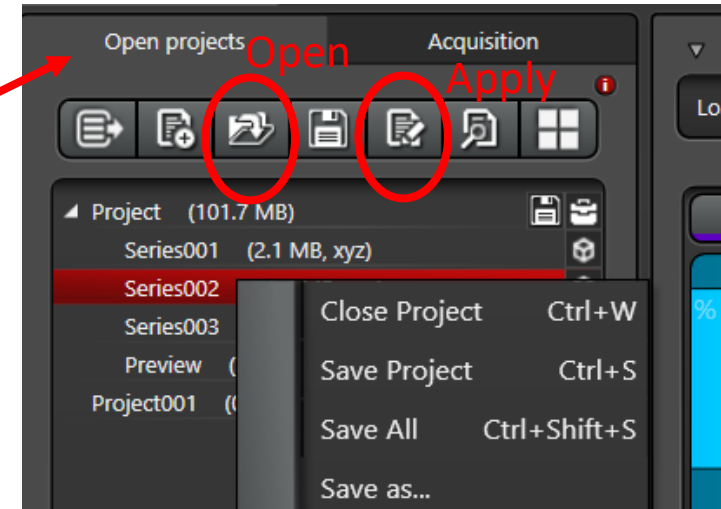
Image Acquisition

1. By default, the image for acquisition mode is set to xyz. This allows you to capture 2D and 3D images. Usually you do not need to change it. You can change it to other format such as xyt, xt, if you do time lapse imaging.

2. The image format is set to 512x512, scanning speed is 400HZ. This is good for fast live view. But before you start to capture the image, make sure you change the format to at least 1024x1024.

3. Select one Seq, then click on Live at the bottom left of the software.

4. To zoom in, change the zoom factor.

5. You will not see an image if the Gain value is 0. Gain and offset adjustment is on the dial controller. Increase the Gain to about 500 on PMT to start with, or until you start to see an image on the screen. The image won't be in best focus even if you focused it while looking through the eyepieces. Make sure to refocus.

Gain   Offset   Z focus
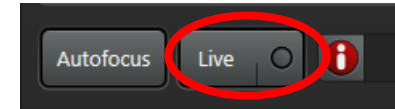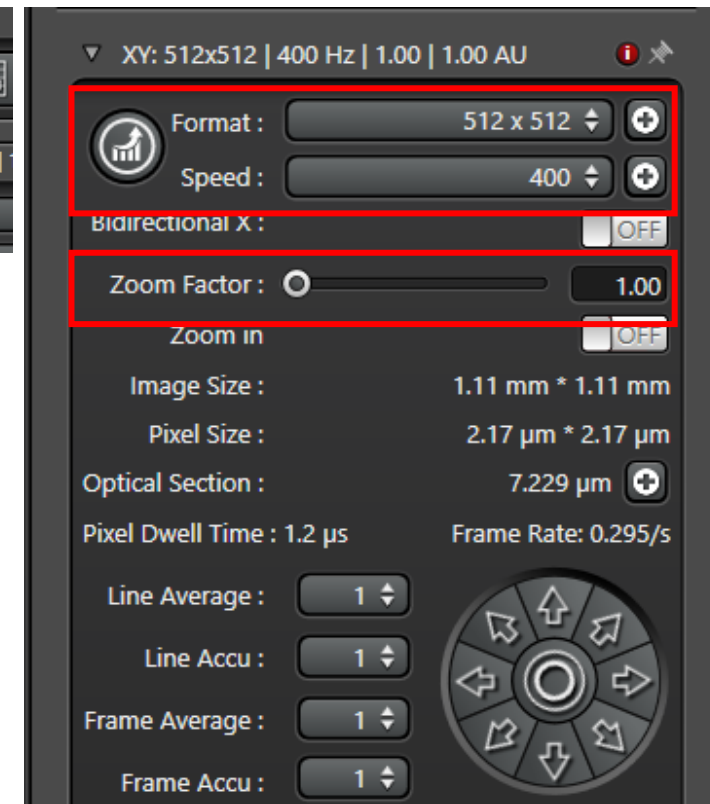
Dial controller

6. There are three ways of viewing the live image. Colored image, black and white, and saturation indication mode.
7. When adjusting signal brightness, switch the view mode to saturation indicating mode by clicking on this icon on the top left of the image.

8. Blue signal indicates oversaturation, and green signal indicates absolutely no signals. <u>Make sure you have no blue in your region of interest</u>, and some green in the background, but not full of green.

9. There are three ways to adjust the signal intensity:
- Gain and Offset on the dial controller
  (Gain adjust signal intensity. Offset adjusts background)
- Laser power
- Line/Frame accumulation.

10. Line or Frame Average decreases noise, and increases image quality, but do not change resolution.
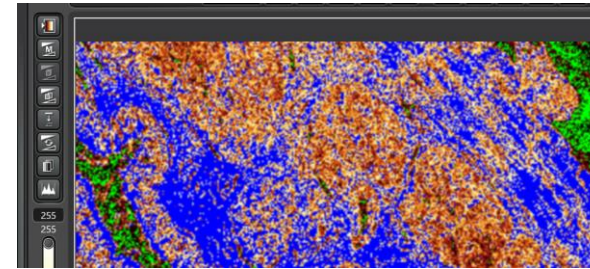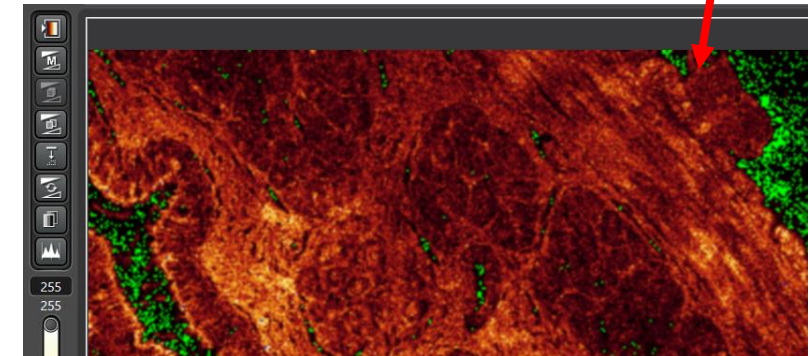
This is the recommended outcome, in which your most interested region is in yellow-orange color.
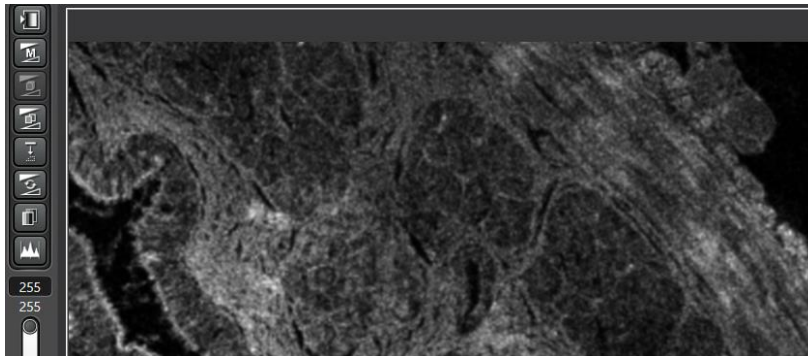
Black and White mode
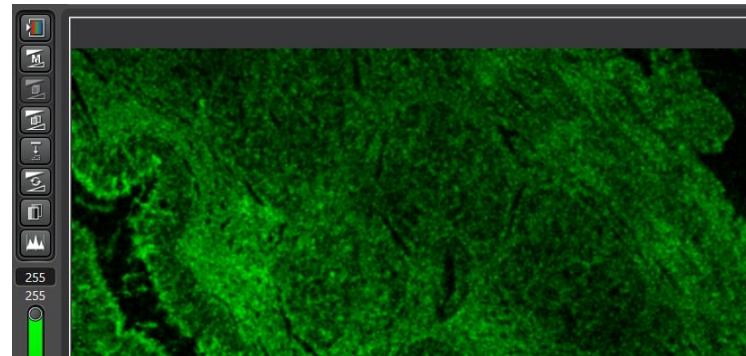
Color mode

Saturation indication mode

10. Do adjustment for each channel (or each .Seq). If you selected line sequential scan, you cannot set up different averaging and accumulation numbers for different channels. Frame sequential scan allows different setups in averaging and accumulation between channels.

11. Then switch imaging format to at least 1024x1024, scanning speed to 100HZ. (DO NOT FORGET THIS STEP!)

12. Click on Start at the bottom of the software to capture images. If you clicked on Capture Image, it only captures the live view image.

10. To view a multichannel image, you can select channels through the icons on the right side of the image.

Channels

Merge channels
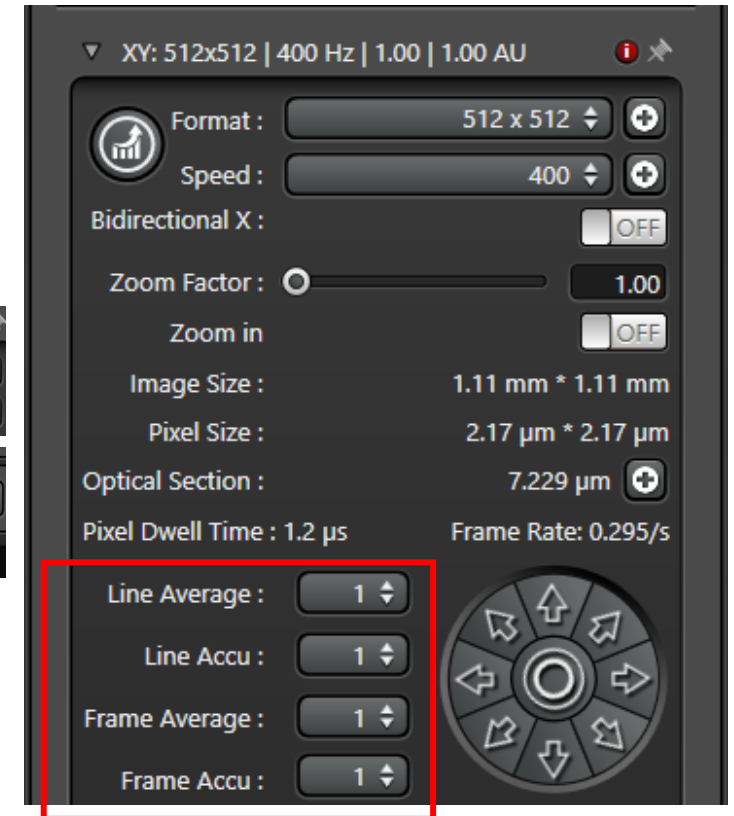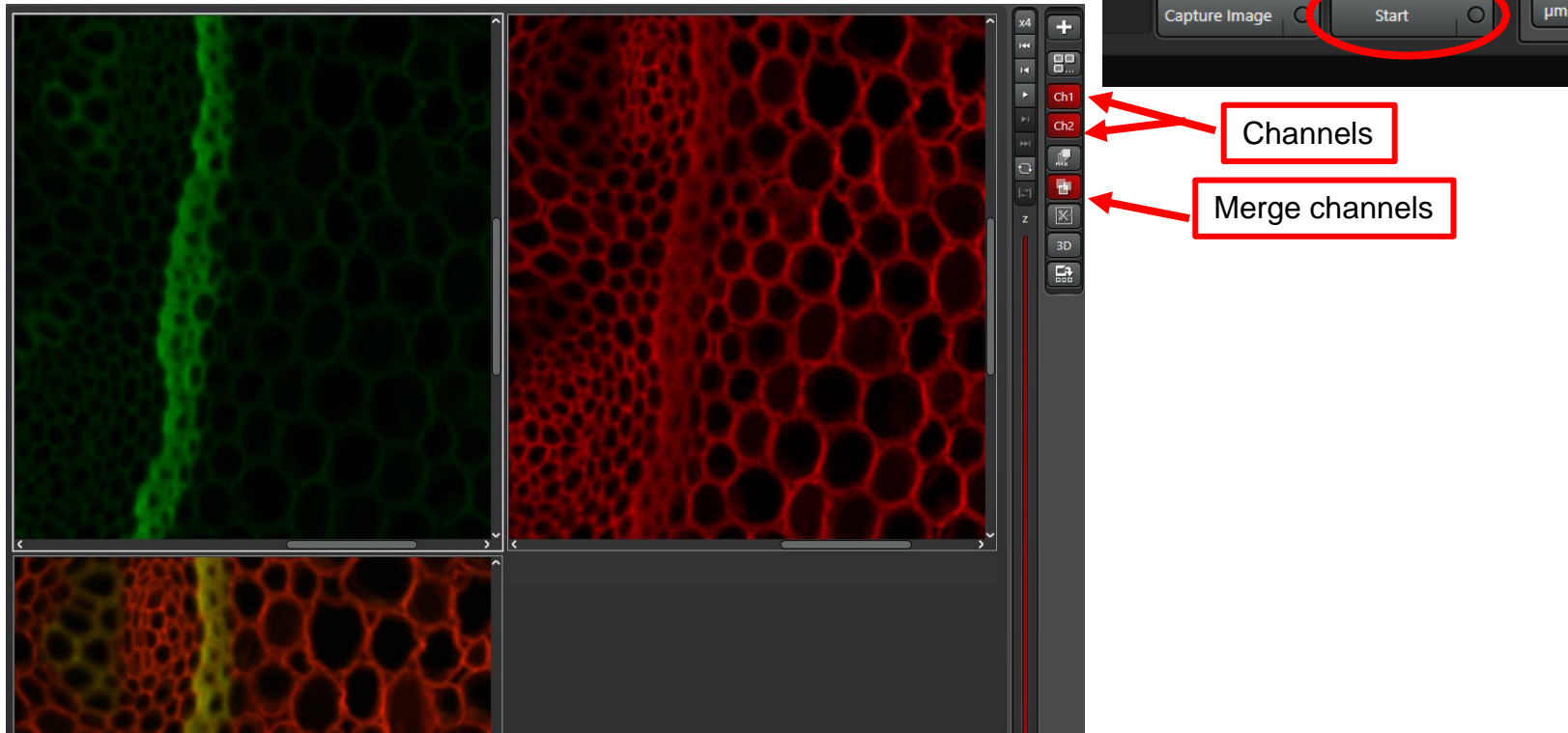
Z-stack images

1. A z-stack image must be set up under live mode. All settings are in the Z-Stack section.

2. After clicking Live, change the z position, either through the dial controller or the focus knob, to the lowest position you need. Then click on Begin. Once a position is defined as Begin, the button will turn red.

3. Similarly, change the z-position to the highest z position that you need, then click on End. Now both Begin and End has been defined.

4. Next, you can either use System optimized number of Z steps, or type in a number that you desire. It is suggested that you do not set Z-Step Size smaller than system optimized number. Use half of the system optimized number of steps is a common practice. (If Deconvolution will be done in image analysis, do not use step size bigger than recommended.

Real time Z position
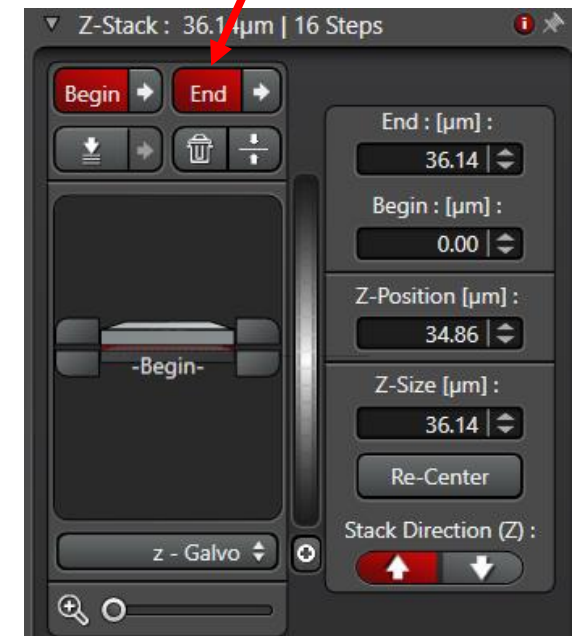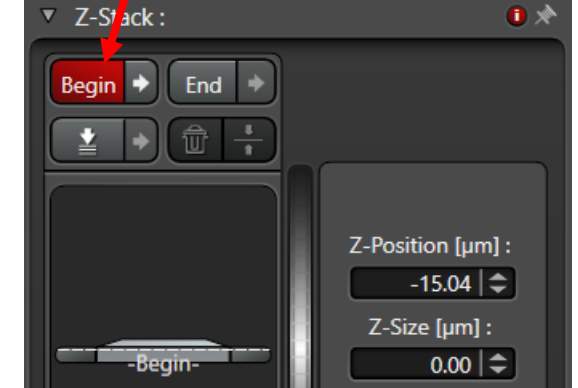
Tile scan/Large image scan

1. A tile scan image must be set up under live mode.

2. First, click on the Tile Scan icon and the Stage section will appear.

3. You can zoom in on the stage map for a bigger view the square representing the current field of view.

4. Click on live and move the stage to a different position, you will see the square in the map move at the same time.

5. Move the field to a desired position, then click on [icon] to memorize this position. Then move the field again to a different position and click on [icon] again to memorize the second position. Now you will see that the area between the two positions are all covered up.

6. You can keep moving around and memorize positions until the entire tissue is covered up.

7. Then click Start to acquire the selected field of view.

Stage map

Current field of view

1st position

2nd position
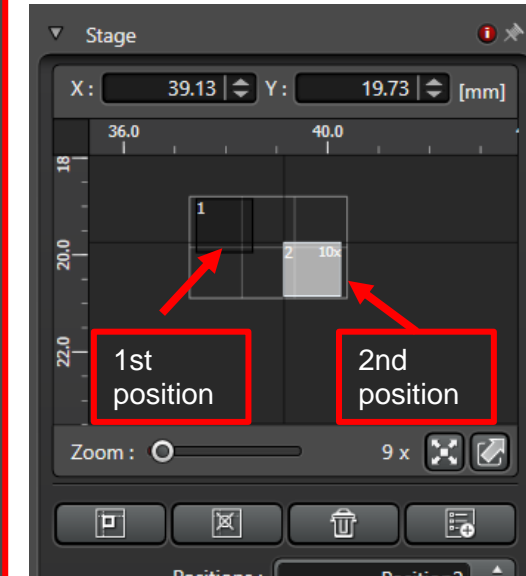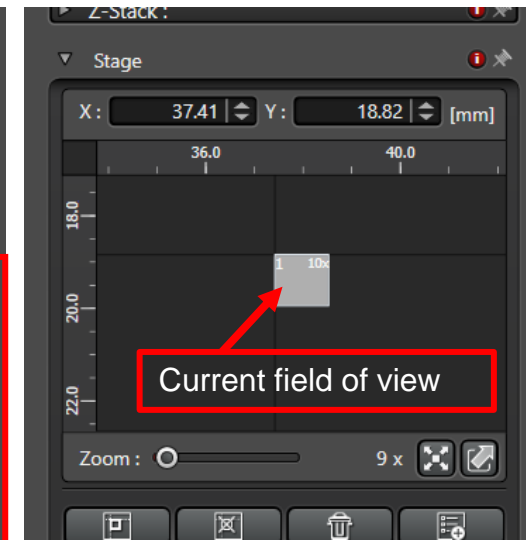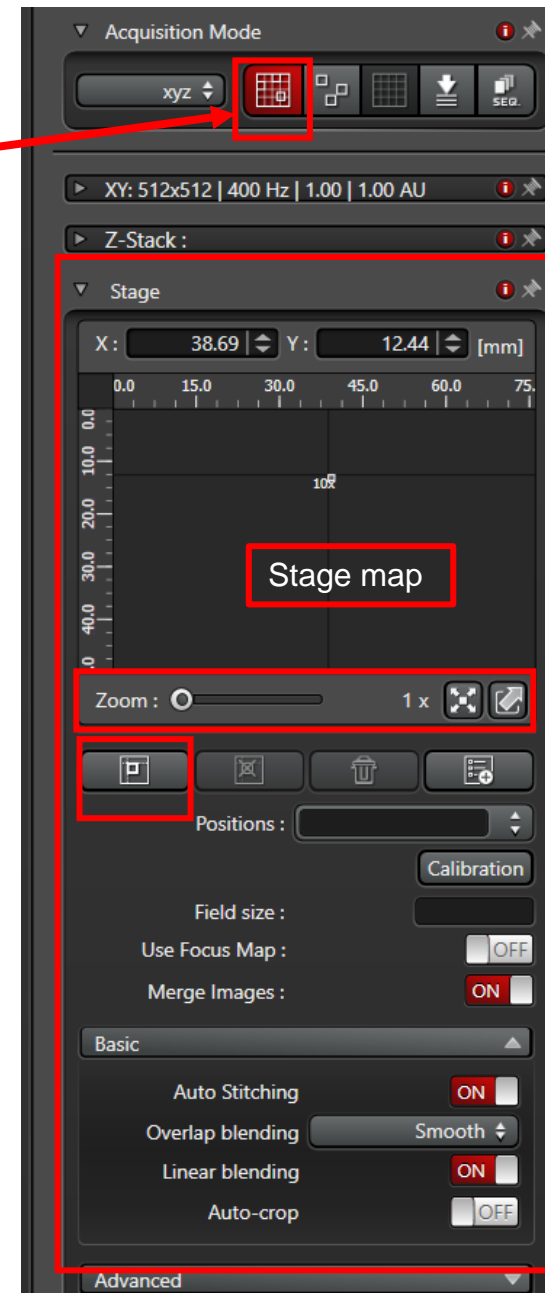
Save and export images

1. After an image is captured, you can find it in the Open Projects section. The default name of an image is Series XXX.

2. You can click on the Project or each individual image and then click Save All.

3. **Please save all your date in the E drive (Data Drive)** Do not save anything on the desktop or C drive.

4. Your original image format is .lif.

5. To export to tiff files, click on the project or the image, then click Export.

6. In the export window, setting 1 creates a merged color image. Setting 2 creates colored individual images. Do both one after the other if you need both merged and individual files.

7. It is recommended to transfer your data through your personal One Drive which you can access through my.pitt.edu.
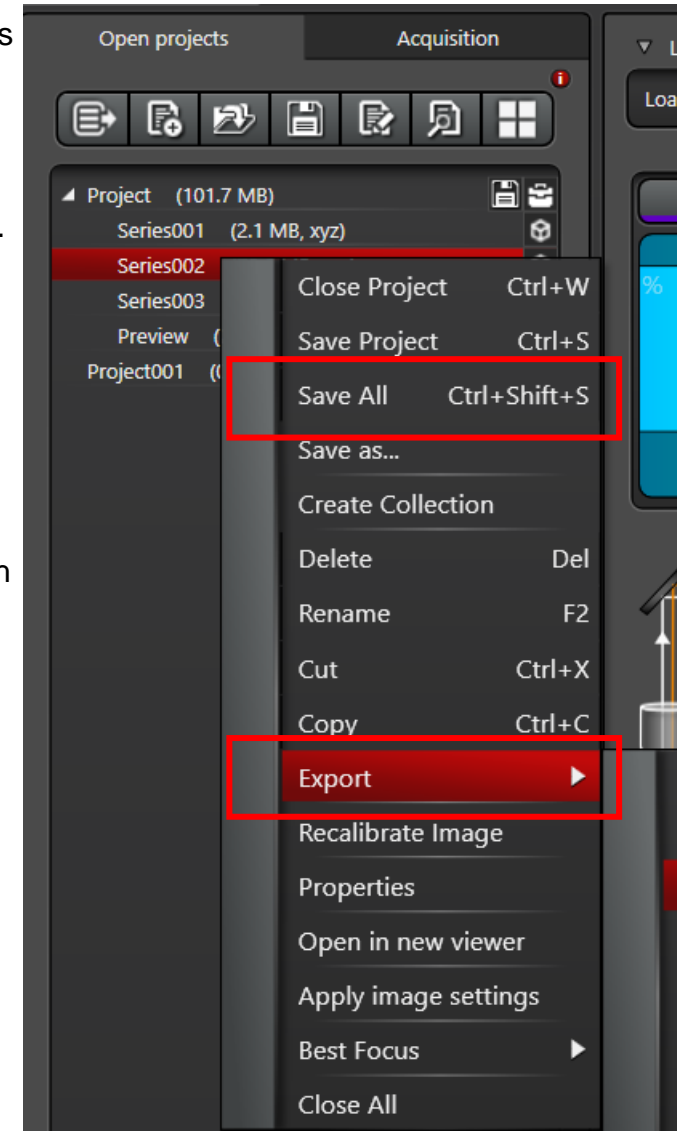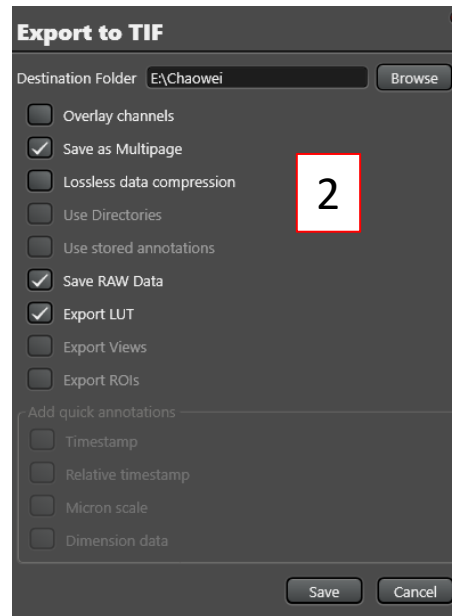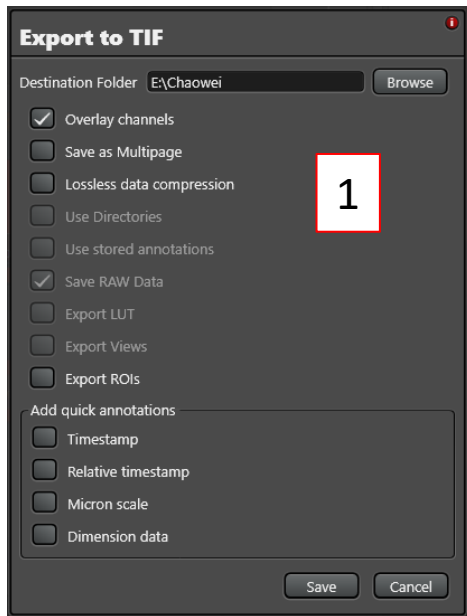
Shut down the SP8 confocal system

1. Clean the objectives if you used oil objectives. Please also check whether the dry objectives accidentally caught oil if you used both oil and dry objectives. Use lens paper to clean objectives.

2. After cleaning, change the objective to 10x and raise the objectives to the top.

3. Clean your sample slide if you added oil on it. Use kimwipes to clean slides.

4. Clean the stage if oil is caught on stage. (Do not apply harsh force onto the stage)

5. Turn off the lasers from the software configuration.

6. Turn off the computer.

7. Turn off the Microscope in the order of 8→1 as labeled on the equipment.

8. Clean up the desk and do not leave any samples behind.

9. Log out on the log sheet.