TAMU MIC Leica SP8 confocal/STED/FLIM user guide

Last updated Desc-1st-2021

You must read the MIC Facility Manual before training and satisfy the BSL-2 training requirements (outlined in a separate document from the instrumewnt web page). The MIC facility manual covers lab and laser safety, training policy, scheduling, and biosafety requirements.

USERS MUST RECEIVE TRAINING FROM MIC STAFF. Getting trained by another user is neither sufficient nor permissible for independent operation of the microscope. In other words, without the official training, you can be in the room and watch, but you are not allowed to touch the microscope.

Acknowledgment policies

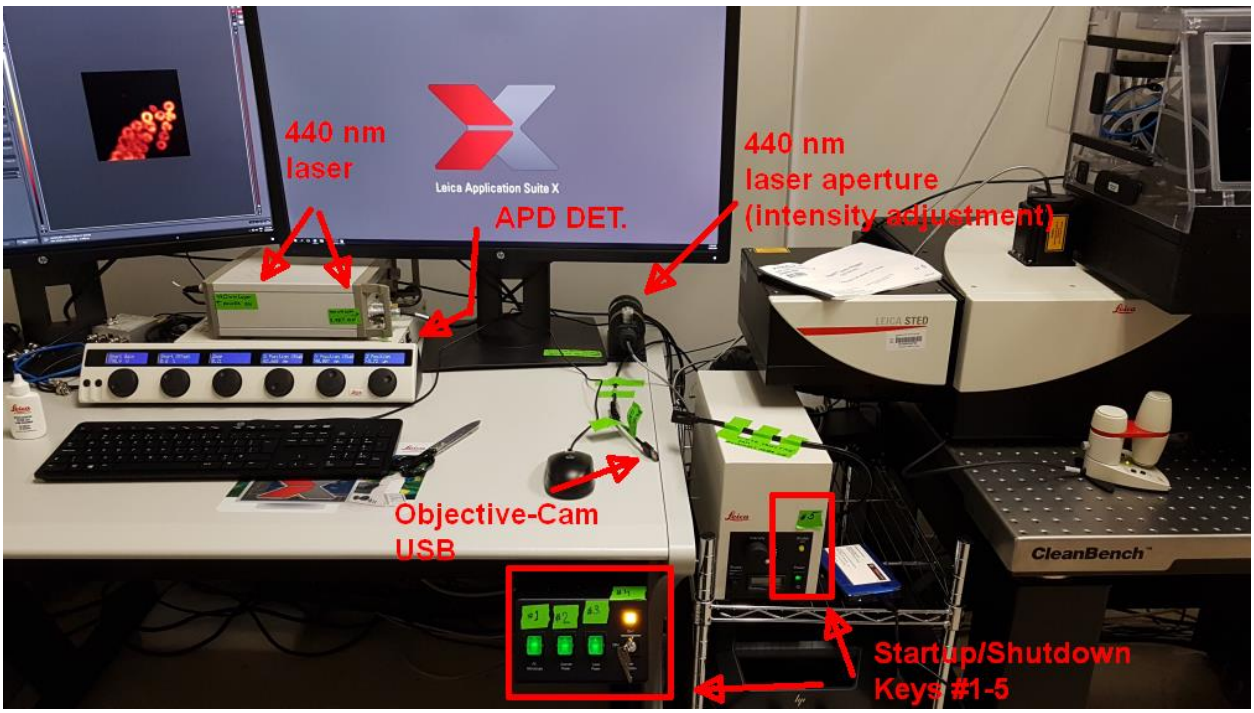
The use of the facility must be properly acknowledged in any publication (including web pages). You can use the following statement:

"The use of the Microscopy and Imaging Center facility at Texas A&M University is acknowledged. The Leica SP8 confocal microscope acquisition was supported by the Office of the Vice President for Research at Texas A&M University."

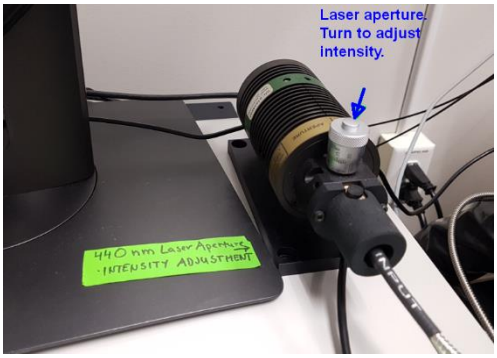
Users are also required to file a copy of any relevant publication containing the acknowledgment with the MCF administrative office.

- **If you need temperature control (37 °C), you must turn on the OkoLab heater 3-4 hours in advance for the temperature to be reached and stable. Make sure all doors on the enclosure are CLOSED.**
- **Microscope slides and other types of sample carriers:** Microscope objectives are designed to view the sample through a thin glass window, 0.17 mm thick (this is the #1.5 coverglass). ***If your samples are in a standard plastic Petri dish or a regular multi-well plate, you will not be able to get the best image quality with the 10x objective, and you will not be able to focus at all with the other objectives.*** Recommended types of samples: slides with a 0.17 mm thick coverglass, sealed with nail polish or other suitable sealer that has dried. Compatible sample carriers include: Coverglass-bottom Petri dishes 35mm and 60 mm size, chambered slides with optically clear, thin bottom (e.g. Ibidi microslides, Greiner CELLview cell culture Slides) LabTek II chambered coverglass, Eppendorf Cell Imaging Coverglass). Cell imaging 96-well plates with thin plastic or coverglass bottom.
- **Using 96-well plates: You must use plates where the bottom of these plates is NOT recessed**. Check with the MIC staff before using 96 well plates. If you use recessed plates, when the objective is in focus and you move to the well on the edge of the plate, the metal sample holder will hit the objective and damage it. You and your PI will be responsible for objective repair/replacement ($$$). Be extremely careful, and avoid using the edge wells.
- **The galvo z-stage is delicate; be gentle when loading and removing samples**
- **The focus controls on the microscope and joystick are for initial focus only. The z-galvo control on the USB panel must be used for 3D imaging, e.g. setting up z-stack positions. The travel range for the z-galvo is 500 µm. If you need to acquire a z-stack spanning more than 500 µm, switch the focus mode to z-wide in LASX software.**

Overview, layout of the system:



The microscope is equipped with an external **445 nm** laser. Turn on only if needed (e.g., for CFP imaging). Laser intensity is not controlled by the software. It is adjusted by opening or closing the aperture on the optical fiber (right back corner of the table)



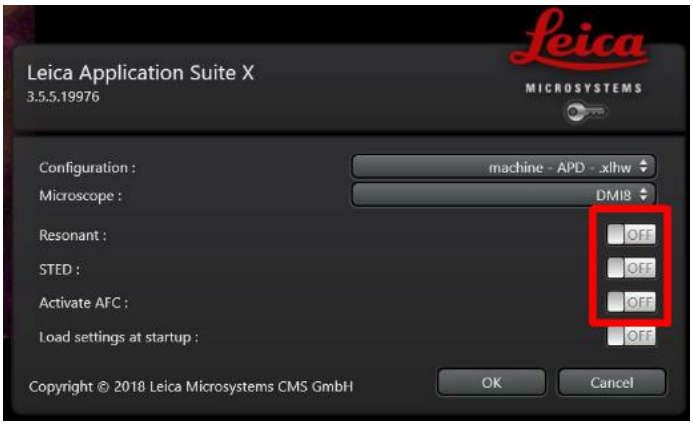
For NIR and IR signals, the system is equipped with Avalanche Photodiode (APD) detectors. These external APD detectors are turned on only if needed and will require a dedicated emission filter cube specific to your dye of choice. You must be trained by MIC staff to use these detectors.

For Live Cell imaging, the Okolab enclosure should be turned on several hours before the imaging session. Press and hold the on/off button on the OkoLab controller (on top of the laser tower, not shown in the picture. OkoLab stage-top gas chamber will need to be installed if you need CO2 and humidity control. The system uses 5% CO2/air pre-mixed gas.

1. Startup

1) Check the microscope room to see whether the microscope looks operational and not occupied by another user. If you previously started the OkoLab enclosure heater, check that the desired temperature has been reached and is stable (wear gloves when touching any surface).
2) **START YOUR ILAB SESSION IN KIOSK (computer by the main office)**
3) Avoid using your phone while in the room, otherwise you will need to sanitize the phone before exiting.
4) Put on gloves and a lab coat.
5) Perform surface sanitization with 70% ethanol
6) Fill out the paper log
7) Switch on the three green buttons in a sequence as indicated, also turn the laser key on (#4). Do not rush.
8) Switch on the LED fluorescence illuminator (label #5)
9) If you need the 445 nm laser (e.g., for CFP imaging), turn it on now (power switch in the back and a key in the front)
10) Log into the computer as Leica SP8 User, launch the **LAS X acquisition software.**

11) In the startup dialog, select the options if you want to use them: Resonant Scanner, STED, AFC. Do not change other settings.
12) Watch for a message asking if you want to initialize the microscope (stage). Stage initialization is needed for Tiling (Navigator) feature. **If you select "yes", make sure there is nothing on the stage that will hit the condenser or objectives! This option will time out after 1 minute, so pay attention. If not initialized, you will not be able to control the stage from the software and will not be able to move the stage using the buttons on the USB console.**



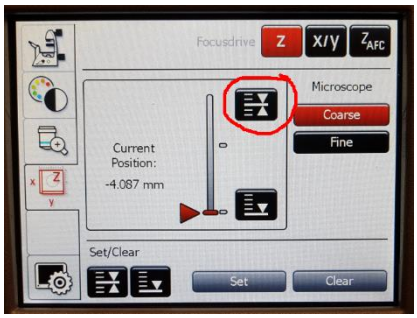
13) If you plan to use the 20x multi-immersion objective or the 63x glycerol immersion objective, now it is a good time to check whether the correction collar on the objective is set to the desired value. See Objectives with manually-adjusted correction collars.

Shutdown:

1. Remove the sample from the microscope stage. Place the samples in a secondary container that you brought them in and surface sterilize the container with 70% ethanol.
2. Clean the immersion objectives if they were used. See the section Cleaning Objectives for proper procedures.
3. Switch to a 10x objective.
4. Close all doors on the enclosure. If you were using temperature control and the next user did not indicate that they need the heat on, turn the OkoLab controller off (hold the on/off buton for several seconds).
5. If you were using multi-well plates, switch the sample holder back to the regular adjustable holder. Stow the multi-well plate holder in the corresponding drawer.
6. Save your Project files to the data disk D, exit LasX software.
7. Copy the project files (the .lif and .lifex) from your folder on DATA disk D: to the remote PC harddrive. DO NOT connect any portable storage drives to the imaging computer. Old data files will be purged periodically from the D: drive by the system administrator.
8. Shut down the computer (from the Windows system). Only after the computer is off, you may turn off switches in a reverse order (#5, #4, #3, #2, #1).
9. Sanitize the work surface, any surfaces you might have touched, light switches and the door handle, and also microscope with paper towels soaked in 70% ethanol. NEVER use paper towels on the optics. Sanitize the eyepieces using LENS PAPER soaked with 70% ethanol
10. Write the end time to the log book.
11. Remove lab coat and gloves, wash hands, turn the lights off, take your items and go to the kiosk to finish your iLab session.

Operating the SP8

Focusing:

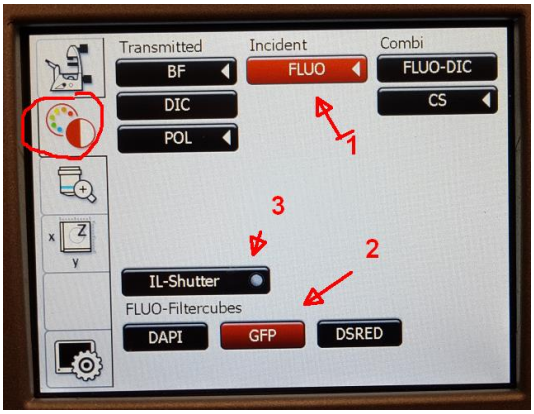


- At Start, the objective turret is in low position. With a 10x objective, move close to focus (on a typical sample), by selecting the XYZ button on the LCD panel and holding the "focus" button. The objective turret will move up and stop at "0 mm". If you have an unusual sample that protrudes down towards the objectives, be very careful not to crash into the objective.
- Use the focus knob on the XYZ joystick, or the focus knob on the microscope itself for coarse focusing. This moves the objective turret up and down. This is not normally used by the LASX software for z-stacks, so you cannot use these controls to set up a Z-stack, unless you changed the focus drive from z-galvo to z-wide in z-stack settings.
- Use the z-position knob on the USB control panel for fine focus and to set up a z-stack

Finding the samples, using the microscope for brightfield and fluorescence imaging:

Confocal scanning must be stopped if you want to look through the eyepieces. Most functions are controlled thorough the front panel. You can turn on the Brightfield (BF) or Fluorescence (FLUO) mode, open and close the illumination shutter, move the objective turret to a pre-set focus (zero) position, remember and recall XY coordinates on the motorized stage, change the intensity of illumination,

1. **Start with a 10x dry objective. Bring the objective close to focus using the Z control on the microscope front panel.**
2. **Set up Köhler illumination to get a good brightfield image (see next section).**
3. **Switch to Fluorescence mode (FLUO), select a suitable filter set (DAPI, GFP, RFP) and open the IL shutter to view the sample**
4. **Once you found the area of interest, close the illumination shutter and set up the confocal imaging parameters.**

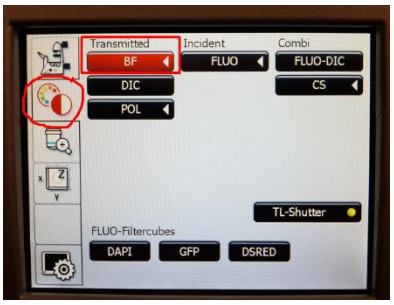


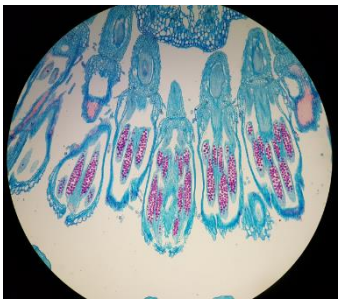
Setting up Köhler illumination

Kohler illumination produces an even field of illumination in TRANSMITTED LIGHT and does not show dust and dirt that accumulated on optical surfaces in the illumination path.

Adjust the illumination using the 10x dry objective. When you switch objectives, you do not need to repeat the process, except to check that the condenser aperture is set properly to get a good balance between contrast and resolution.

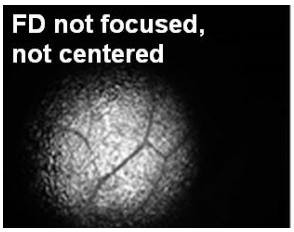
1. Place specimen on the stage, turn on the Brightfield mode. (BF button on the microscope front panel) and focus on the specimen.

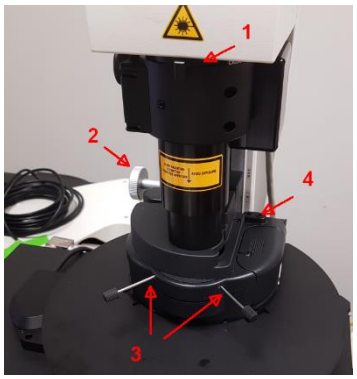




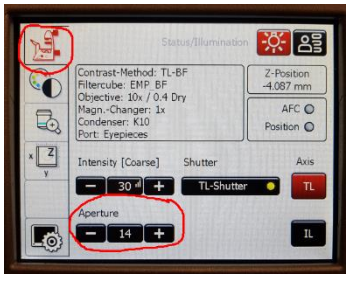
2. Close the Field Diaphragm (1) – FD is on the condenser arm, hidden by the OkoLab enclosure. You have to open the sliding covers on top of the enclosure and tilt the arm back to see the FD control.

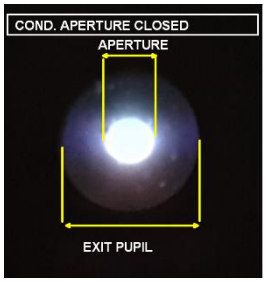
3. Focus the FD by turning **the condenser** knob (2) up or down. If necessary, center the FD using two removable wrenches (3) that are stowed in two slots on the back of the condenser (4). Open FD just enough to to illuminate the entire field of view. Stow the wrenches when finished.

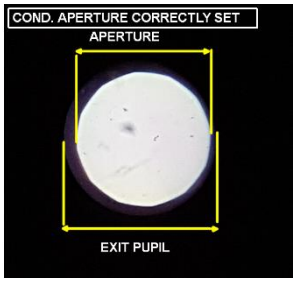




4. Adjust Condenser Diaphragm (aka Aperture) using the front panel of the microscope. Decrease the aperture size to minimum, then start increasing it. View the aperture by removing an eyepiece. When the Aperture size almost reaches the size of the objective exit pupil, stop. Closing the Condenser Aperture provides more contrast but less resolution.





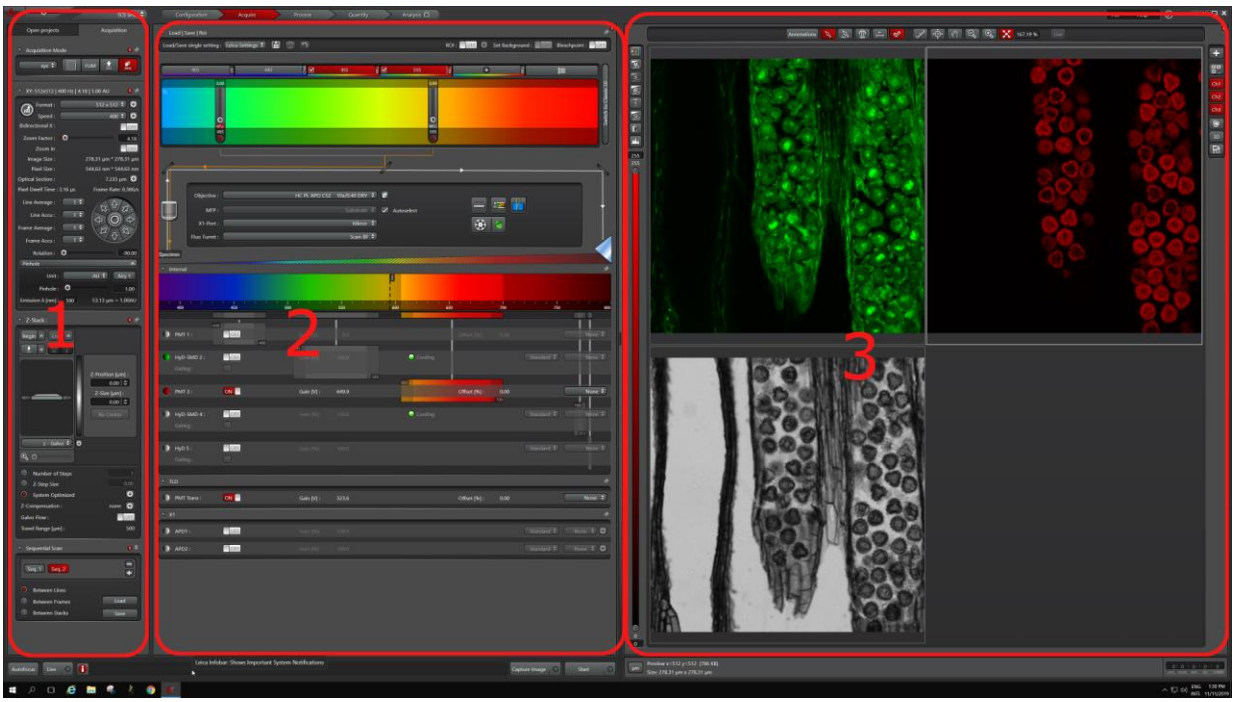


The LASX user interface for Image Acquisition has three sections:

1: Acquisition (Scanning) parameters and file/Project settings

2: Light Path, Detector settings

3: Image display



Getting started with confocal image acquisition:

First check the following

1. **LASERS**

- Go to Configuration, Lasers, and turn on the White Light Laser (WLL) to 70%.
- If needed, turn on the 405 nm laser.
- If needed, turn on the external 440nm pulsed laser: You have to turn on the power supply itself, on top of the laser stack (switch in the back) and turn the key to ON position. Do not play with the laser power adjustments, this affects the pulse characteristics (important for FLIM). Output intensity is adjusted using the mechanical shutter knob that is hiding behind the left computer monitor (ask if you do not know what and where it is).

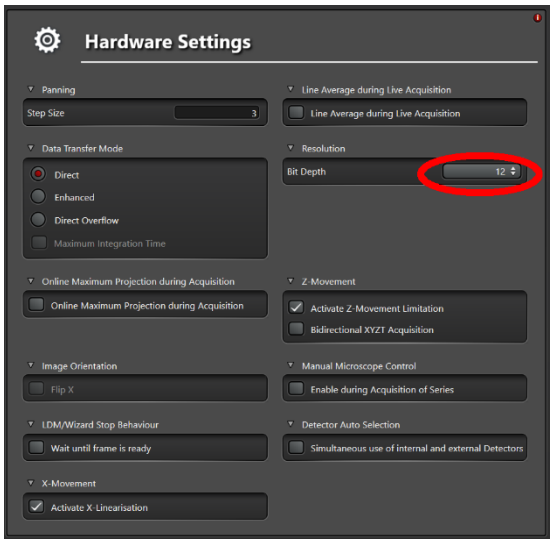
2. **IPS, Instrument Parameter Setting: Configuration – IPS**

- IPS determines what parameters that were recorded with image files or with dye combination presets will be applied to the hardware. ***Some of these settings may be dangerous to apply blindly and can cause damage to the microscope, so be very careful***. Do NOT select parameters like the objective, stage position, Z-position.
- Do select settings like Scan size, line and frame averaging, …
- Special note about Resolution (=bit depth) - see next section for details

- Once you selected the desired IPS parameters, you can save these choices to a file (somewhere in your folder on the data disk, so that next time you can load the IPS presets.

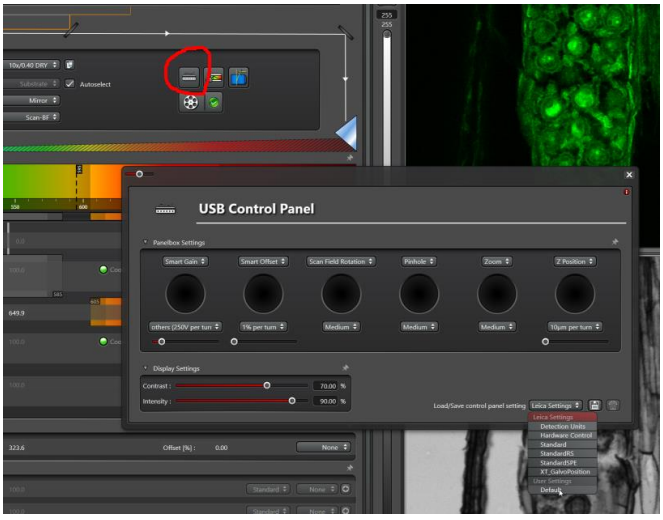
3. Choosing 8-bit versus 12-bit dynamic range ("Resolution" in LASX)

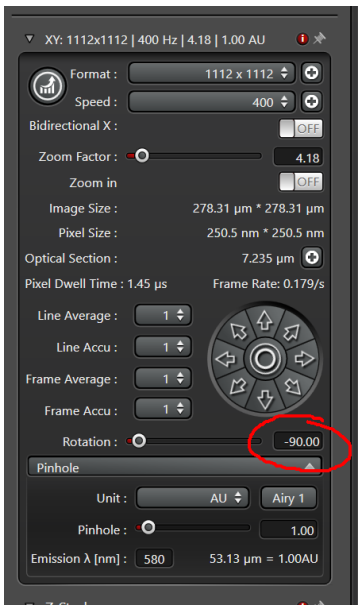
- By default, LASX is set to do 8-bit image acquisition (pixel intensities or photon counts between 0 and 255). For images with high contrast, or when you do line or frame accumulation, and for images that you will be processing and adjusting after acquisition, this is not ideal. **SWITCH TO 12-BIT "RESOLUTION" AT THE START OF YOUR SESSION.**
- Configuration – Hardware – Resolution, choose 12-bit
- CAUTION: if you selected "Resolution" as one of IPS parameters, and load settings from an image file that was acquired with 8-bit resolution, the bit resolution setting will revert to 8-bits. The Leica dye combination presets are all defined with 8-bit resolution as well. Your own dye combination presets are saved with whatever bit depth was selected at the time.



4. Configuring the USB Control Panel

- You can customize the control panel and save the panel settings so that you can load them next time, or you can use the "User-Default" configuration. It has the stage XY movements assigned to two of the buttons.





- Since the scanner head is rotated 90 degrees, the image is rotated compared what you see in the eyepieces and the "X" movement seen in the eyepieces would show as "Y" movement on the screen. Therefore, in Scan settings, set the Rotation to -90 degrees. Note: rotation is not available in STED mode.

5. **Choosing fluorochromes, selecting detectors.** Standard PMTs have reasonable sensitivity at the ends of the visible spectrum. HyD detectors are much more sensitive in the middle of the spectrum, are cooled for lower noise, and allow FLIM imaging and detection gating. The external APD detectors are preferred for NIR and infrared signals, but are damaged when exposed to strong light, and show higher noise. Additional training is required before you can use the APDs.

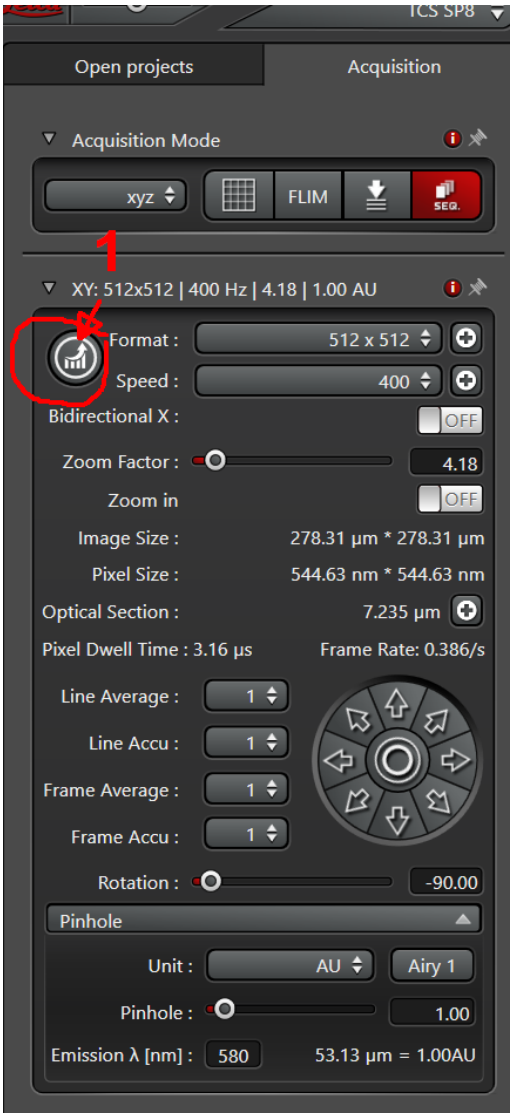
a. ***Choose an existing acquisition setting***, using the "Load Setting " pull-down list in above the laser spectral panel. Note that this may reset the bit depth setting. You can save your own setting using this function.

b. ***Load parameters from an image file***: in the "Open project" tab, open a project (.lif file) and right click on an image file. Choose the "Apply image settings" option. Note: this will load only parameters that you chose in the Configuration-IPS (see previous sections)

c. ***Use the Dye Database*** to select fluorochromes. You can choose the detector type (HyD or PMT) to use, and with multiple dyes choose sequential imaging modes (to minimize spectral crosstalk).

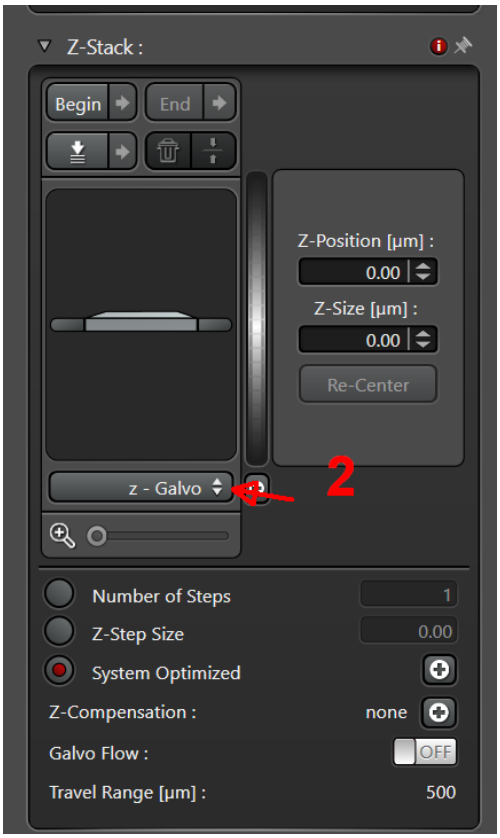
d. You can ***set up and modify the detector and laser settings manually***.

The Acquisition Tab – Imaging Parameters



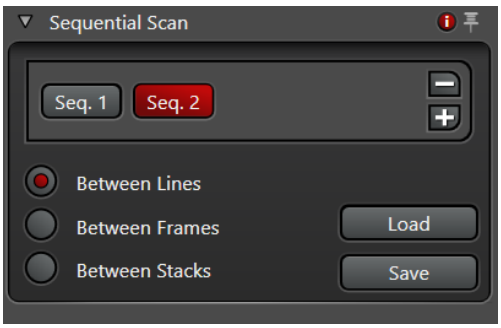
- **Format:** how many pixels are scanned. Start with 512 x 512, to tweak detector settings in live view. The more pixels, the longer it takes to get an image.
- **Speed:** (line frequency): Usually 400 or 600 is good for final acquisition. Go faster for initial focusing and adjusting parameters in live view
- **Bidirectional:** for higher speed imaging/moving objects. If you need a really high speed, you will want to start the microscope in a resonant scanning mode.
- **Zoom factor:** the more you zoom in, the smaller area of the sample is scanned. The combination of Format (number of pixels) and Zoom determines the pixel size, which is a critical parameter for recording images at high resolution.
- **Pixel Size:** To record images at full resolution, the pixel size must be at or slightly below one half of the optical resolution of the optics (this is called the Nyquist criterion). To see the optical resolution information, click on the objective icon in the Light Path area of the software. You can use the Auto-setting button next to the Forma control (labeled as "1" in the screenshot) to set the scan format (number of pixels) for full resolution. Quite often you end up with very large scan format (=very slow imaging). Zoom in as much as you can to still see the area you need tyo image, and click the Auto-setting button again. Also note that you do not always have to scan at maximum resolution.
- **Line, Frame Average:** when scanning fast and with high detector gain, reduces the nopise in the image.
- **Line, Frame Accumulation:** scans the line or frame several times and sums (adds) the photon counts or pixel intensities. Used for very weak signals. Note that if you left the scanning bit depth at 8-bits, it is easu tyo end up with saturated pixels (= not good).
- **Rotation:** Set it to -90 degrees so that the movement of the stage works the same way when looking through the eyepieces and when looking at the screen.
- **Pinhole:** determines the Z-resolution and signal brightness. Setting to 1 AU (Airy Unit) gives reasonable resolution and signal. Better resolution is achieved by closing the pinhole to 0.6 AU, but the signal is then much lower. For very weak signals, you can open the pinhole; The Z-resolution is then much worse.

The Acquisition Tab – Z stack



- in Live view, focus the microscope **USING THE Z-POSITION KNOB ON THE USB PANEL** to the starting position of the z-stack and click the "Begin" button.
- Focus the microscope to the end position of the stack and click the "End" button.
- You should now see how many slices will be in the stack ("Number of Steps"), and also what is the z-step size. Use the "System Optimized setting for automatic z-step size (based on the objective, signal wavelength and pinhole size).
- If number of slices remains at 1, confirm that you use the right focusing controls – The Z-galvo,stage insert (indicated by "2" in the screenshot) is controlled only through the knob on the USB panel. FZocusing by the jopystick or by the focus wheel on the microscope is not detected by the software.
- If you need to acquire stacks that cover more depth than the z-galvo range (500 um), switch from z-galvo to "z-wide" setting. This will control the objective turret movement (slower, but bigger range). Now you will be able to use the joystyick and the focus know on the microscope to set the z-stack, but not the z-position knob on the USB panel.

The Acquisition Tab - Sequential Scan



- When using multiple fluorochromes, it is recommended to use sequential scanning to eliminate spectral overlap between dyes, unless you can demonstrate that spectral overlap is not a problem with a given sample.
- If you use the Dye Database, it will show the predicted amount of spectral overlap and allow you to choose which dyes will be imaged simultaneously and which sequentially.
- Since you will be scanning the same sample several times, imaging will take longer.
- In most cases, sequential switching between lines and between frames will show similar results. If you want to capture transmitted light images in the DIC (differential Interference Contrast) mode, you will have to use the "between Stacks" mode and put the Transmitted Detector (TD) DIC acquisition in a separate sequence.
- **Important note:** You can adjust detector gain for any detector, regardless of what Sequence you are in (Seq1 or Seq2 in the screenshot) **BUT CHANGE LASER POWER ONLY FOR LASER THAT IS SUPPOSED TO BE ON IN A GIVEN SEQUENCE**. Otherwise you would be turning on a laser that should not be on in the given sequence, and you no longer are doing sequential imaging.

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Using deconvolution (Leica LIGHTNING) for increased resolution

Both regular wide-field fluorescence and confocal fluorescence images can benefit from computational image restoration (3D deconvolution). Deconvolution aims to reassign the out-of-focus signal back to the source and thus improve resolution and contrast in the axial direction (z) as well as laterally (XY). When done properly, resolution improvement of about 1.4 x can be achieved.

Deconvolution is NOT a method that will take images from badly prepared and incorrectly imaged sample and magically give you beautiful images. Garbage in, garbage out.

Before you attempt deconvolution, confirm that your specimen and your imaging settings are as good as possible for regular imaging:

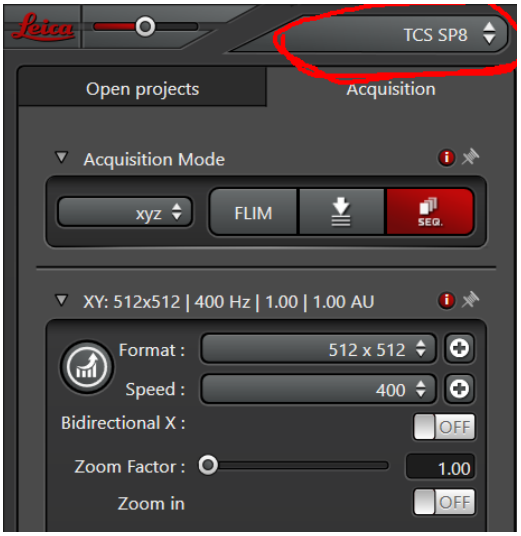
1. You are already using the objective best suited for the sample, and of the highest available numerical aperture.
2. The specimen is refractive index-matched to the immersion fluid of the objective – aqueous samples are best imaged with water immersion objectives; fixed samples mounted in glycerol-based non-hardening media are imaged with glycerol-immersion objectives; Samples mounted in high refractive index media, like 2,2-thiodiethanol, or the hardening media, are suited for oil immersion objectives
3. If using the multi-immersion 20x objective, the correction collar on the objective is properly set to the medium being used (oil, glycerol, or water with a coverglass)
4. The imaging chamber has the correct thickness (0.17 mm glass for most objectives), and that if the objective has a coverglass correction, , the collar is set to the correct setting for the temperature used, 23 C or 37 C (on the 63x/1.3 GLYC IMM) or to get maximum brightness of signal (40x/1.1 MOT CORR water immersion objective) .

Please note that in order to achieve higher resolution, the confocal pinhole has been closed below 1 AU, the z-step in 3D stacks is smaller and the scan format or zoom will need to change to achieve proper Nyquist sampling (smaller pixel size than for a regular confocal image).

That means that: 1. You will be collecting fewer photons (images will be dimmer, and have larger photon shot noise), and 2) Scanning will take longer because of increased scan format.

You can compensate for dimmer signal by:

- frame or line accumulation
- using the HyD detectors
- lowering the scan speeds
- Increasing laser power (usually not a good idea, causes photodamage/photobleaching),

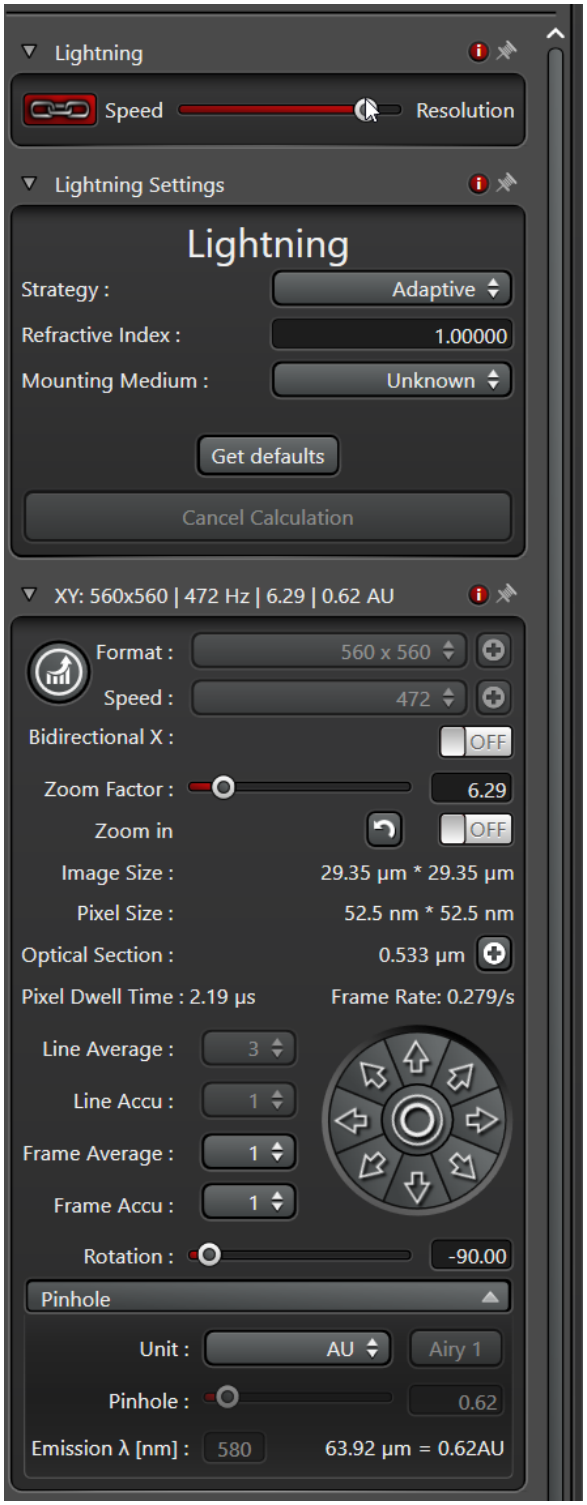


To use Lightning Deconvolution, change the imaging mode (circled in the screenshot) from TCS SP8 to “LIGHTNING”. This will bring out a scan parameter panel that has some extra options

- Choose the balance between speed and resolution. When maximizing resolution, the scan size (number of pixels) will increase, pinhole size, z-step and signal intensity will decrease, and the system may use averaging (multiple scans) to reduce noise.

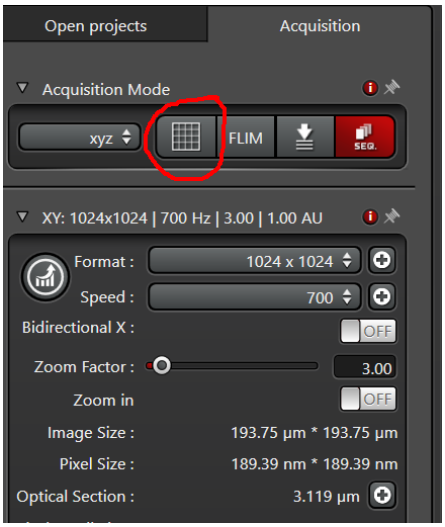
- **Important:** Select what mounting medium was used for your sample, or enter the refractive index manually

- In the screenshot on this page, note the Pixel Size, Line Average settings, Pinhole Size.



Navigator: Tiled Scans, image stitching

The motorized stage allows imaging adjacent fields of view (or z-stacks) and stitch them together for extended area imaging.



Training will be provided as needed. Please refer to the LASX help file if you need more information on the navigator options.

STED: Superresolution imaging

Additional instruction is provided to users who passed the basic training

Objectives with manually-adjusted correction collars

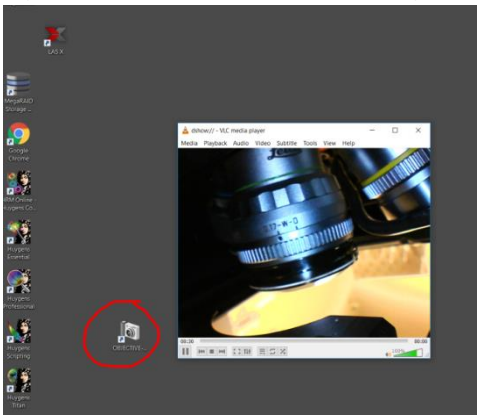
The 20x/0.7 IMM objective can be used with either oil, glycerol, or water immersion. The objective has a correction collar that must be set correctly to the immersion medium being used. Since you do not know what the user before you did, you must check the setting on the objective before you use it.

Similarly, the 63x/1.3 glycerol immersion objective has a correction collar for coverglass thickness and temperature. You must know what is the coverglass thickness in your slides/imaging chambers. Most often it is 0.17 mm if you used coverglass #1.5, or Labtek II chamered coverglass, or it may be something else – find out what it is. This objective has two different scales: one for room temperature (23 °C) and for 37 °C.

YOU WILL NOT GET GOOD IMAGES IF YOU USE THE WRONG SETTING ON THE OBJECTIVE.

Because the objective markings on the correction collar are hard to see, use the miniature camera that has been installed on the system:

1) Remove the specimen, if it is present
2) Switch to the immersion objective (20x IMM or 63x GLYC IMM)
3) Minimize the LASX software so that you see the desktop
4) Plug in the objective camera into the USB connector (in front of the APD detector box).
5) Start the Objective-Cam application (icon on the desktop)
6) Use the thumbwheel on the camera cable to adjust lighting to see the objective markings. It may be necessary to unscrew the objective half-turn to see the marks.
7) Set the correction collar to the desired setting
8) Screw the objective back, taking care not to change the settings on the collar.
9) Exit the Objective-Cam application
10) Unplug the camera from the USB cable.



Cleaning Objectives

Clean immersion objectives after use. The dry objective should not be cleaned this way since it has a concave front lens.

Use dry lens paper to blot off larger amounts of oil or other immersion medium:

• Take a sheet of lens paper, fold it in three into a long rectangle; do not run your fingers over the middle of the paper that will touch the lens

• Blot the lens by holding either end of the lens paper, holding it on the objective lens with very light pressure. Lift the paper and move to a fresh area of the tissue several more times.

• Repeat as needed with a fresh lens paper to remove excess of immersion oil.

Use wet lens paper to remove traces of oil, other dirt:

• Fold as above, put a drop or two of lens cleaner on the lens paper (or use the lens cleaner spray bottle to squirt some cleaner on the paper).

• Wipe the lens gently by holding either end of the wet lens tissue dragging it gently straight across the objective lens three times. Use a fresh area of the tissue each time.

NEVER wipe the lens in a circular pattern; NEVER apply any pressure directly to the lens.

Note: If you find something on the lens that you can't remove with this procedure, please contact MIC staff as soon as possible, and discontinue use of the objective until the problem is solved. Also write a comment in the log book.

Choosing slides, chambered coverglass and other imaging vessels.

This section is a work in progress

1. Most objectives are designed to be used with coverglass 0.17 mm thick. This corresponds the coverglass labeled as #1.5 (thickness range 0.16 to 0.19 mm)

2. ***If your samples are in a standard plastic Petri dish or a regular multi-well plate, you will not be able to get the best image quality with the 10x objective, and you will not be able to focus at all with the other objectives.***

3. Using the wrong thickness coverglass will degrade resolution.

4. Recommended types of samples:

    a. Slides with a 0.17 mm thick coverglass, sealed with nail polish or other suitable sealer that has dried.

    b. Coverglass-bottom Petri dishes 35mm and 60 mm size.

    c. Home-made coverglass-bottom Plexiglass® imaging chambers

    d. Chambered slides with optically clear, thin bottom (e.g. Ibidi microslides, Greiner CELLview cell culture Slides)

    e. LabTek II chambered coverglass, Eppendorf Cell Imaging Coverglass).

5. **Cell imaging 96-well plates with thin plastic or coverglass bottom.**

    You must use plates where the bottom of these plates is NOT recessed. Check with the MIC staff before using 96 well plates. If you use recessed plates, when the objective is in focus and you move to the well on the edge of the plate, the metal sample holder will hit the objective and damage it. You and your PI will be responsible for objective repair/replacement ($$$). Be extremely careful, and avoid using the edge wells.