

# Leica SP8 Resonant Confocal

Quick-Start Guide

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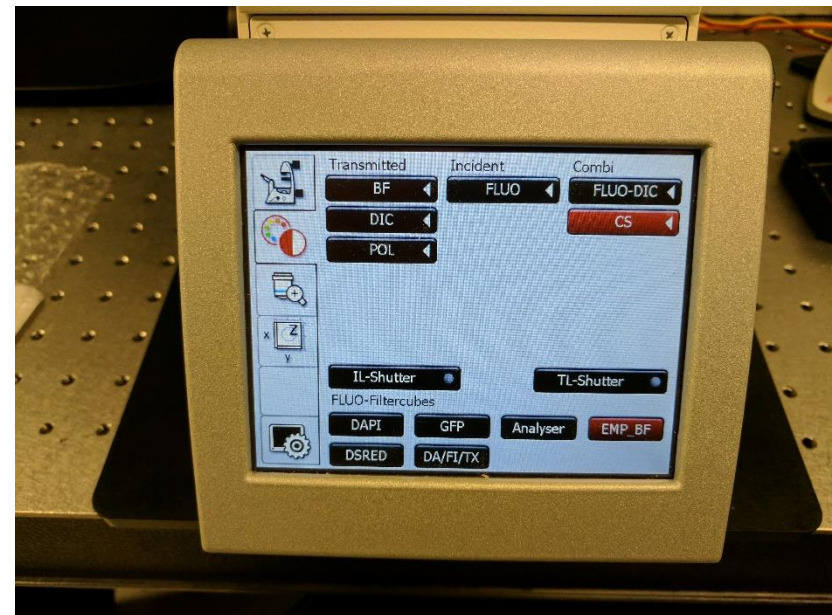
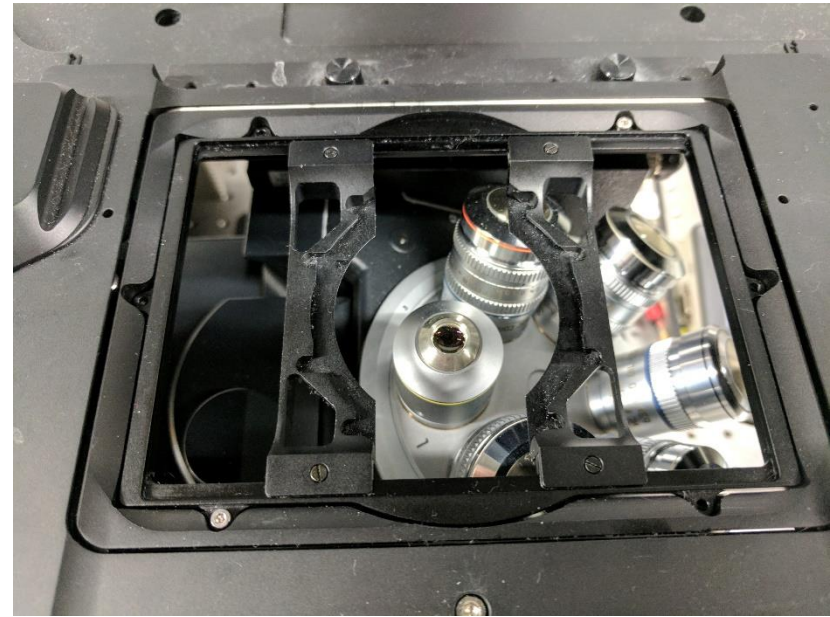


# Start-up: Turn on the Microscope / Computer

- Turn on 3 green switches from bottom to top
- Turn key to on position
- Turn on fluorescence excitation source for the eyepieces.
- Log-in using your ADS account name and password.
- For access to the network drive, select Run and then type:  
`\\microscopy-nas1.nri.ucsb.edu`
- Open LASX software
  - Choose resonant scanner to be on or off

# Part 1: on the scope

- Lean condenser/bright-field arm back
- Choose objective from LASX software
  - Pay attention to immersion medium
- Place slide or dish in sample holder
- Use scope touchscreen to choose brightfield or fluorescence
  - Be sure to turn off fluorescence shutter when not observing through eyepiece
  - Small knob on left side will adjust fluorescence excitation intensity



# Part 2: Software interface

## Acquisition

## Excitation and Emission

## Image Viewing

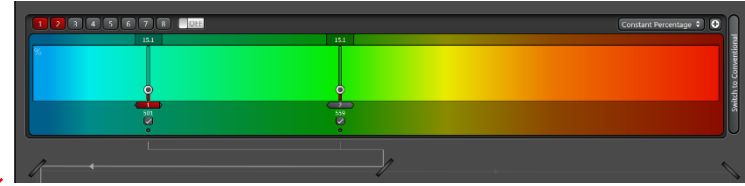
The screenshot displays the software interface for a microscope system, divided into three main sections:

- Acquisition:** This panel on the left contains settings for the acquisition mode. It includes fields for Format (512 x 512), Speed (400), Bidirectional X (checked), Zoom Factor (1.00), Zoom In (checked), Image Size (290.62 μm x 290.62 μm), Pixel Size (568.74 nm x 568.74 nm), Optical Section (1.27 μm), Pixel Dwell Time (1.2 μs), and Frame Rate (0.775%). It also features controls for Line Average, Line Accum, Frame Average, and Frame Accum, along with a Z-Stack section for Z-Position [μm], Z-Size [μm], and Z-Step [μm].
- Excitation and Emission:** This central panel shows the configuration for the microscope's optical path. It includes a diagram of the objective and fluo turret, a spectral graph showing the emission spectrum of the sample, and a table of excitation and emission lines. The table lists five lines (HyD 1-5) with their respective gain and offset values.
- Image Viewing:** This panel on the right is currently empty, showing a large black area where the acquired image would be displayed. It includes a toolbar with various icons for image manipulation and a status bar at the bottom.

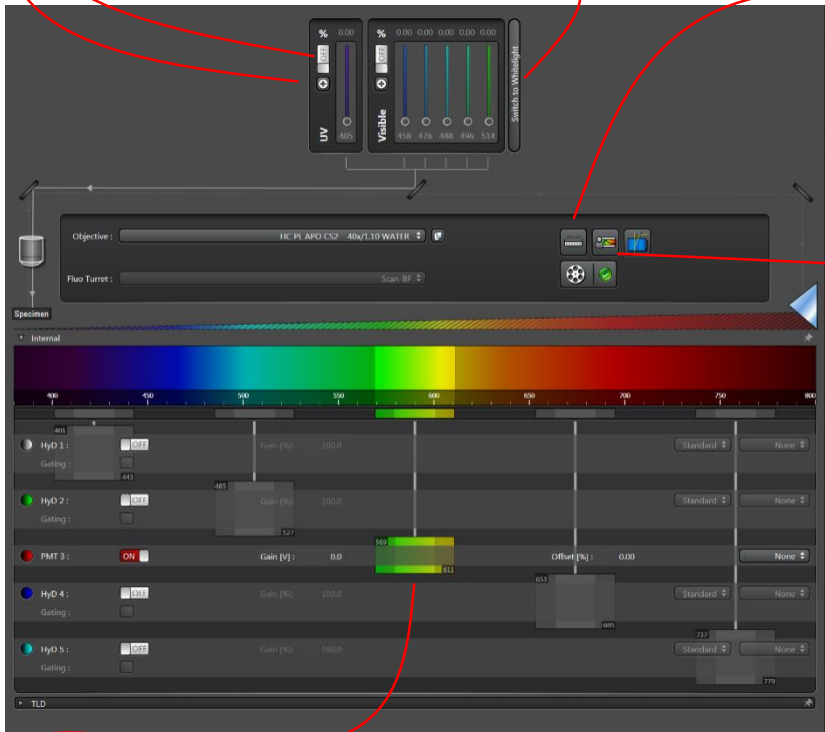
Line	Gain [%]	Offset [%]
HyD 1	100.0	0.00
HyD 2	100.0	0.00
PMT 3	0.0	0.00
HyD 4	100.0	0.00
HyD 5	100.0	0.00

# Part 2: Software interface – Excitation Emission

- Click + button to access laser power menu
- Toggle from off to on for each laser
- Visible argon laser is mostly for bleaching
- Click “Switch to Whitelight” to see WLL interface



- Activate laser lines by clicking on 1,2,...,8
- Move line to desired wavelength
- Top number is laser % (5 is a good place to start)
- Click checkbox to turn on



You can set which properties the control panel controls, and how coarse or fine the control is



- Dye assistant will set excitation and emission for chosen fluorophores
- Use the crosstalk column to decide if sequential scan is necessary



- For each detector you can set the starting and ending position of the emission window
- The only limitation is that the channel windows have to be arranged in order from left to right, 1 is leftmost, then 2 to the right of 1, etc.



# Part 2: Software interface – Scan Settings, Z-stack, timelapse

## Scan Settings

- **Format**: number of pixels, drop down menu gives choices, “+” button gives exact control.
- **Speed**: For resonant it is fixed at 8000. For galvo there are many options, speeds greater than 600 will limit field-of-view.
- **Bidirectional**: doubles scan speed, phase may need adjustment if image appears blurry.
- **Pixel size**: spatial size of pixels. To achieve best resolution zoom or increase pixel count to Nyquist limit. Button to the left of **Format** will set pixel count to Nyquist.
- **Line Average**: If low signal or noisy image increase line average to improve signal/noise. You will almost always need to use this with the resonant scanner.

## Z-stack

- Set “**Begin**” and “**End**” to desired positions
- **System optimized**: Nyquist z-spacing
- **Z-Compensation**: adjust excitation or detection gain to compensate for brightness changes with focal depth changes.

## Time

- **Minimize**: sets minimum time interval limited by image acquisition time
- Set length of timelapse in number of stacks, duration, or acquire until stopped

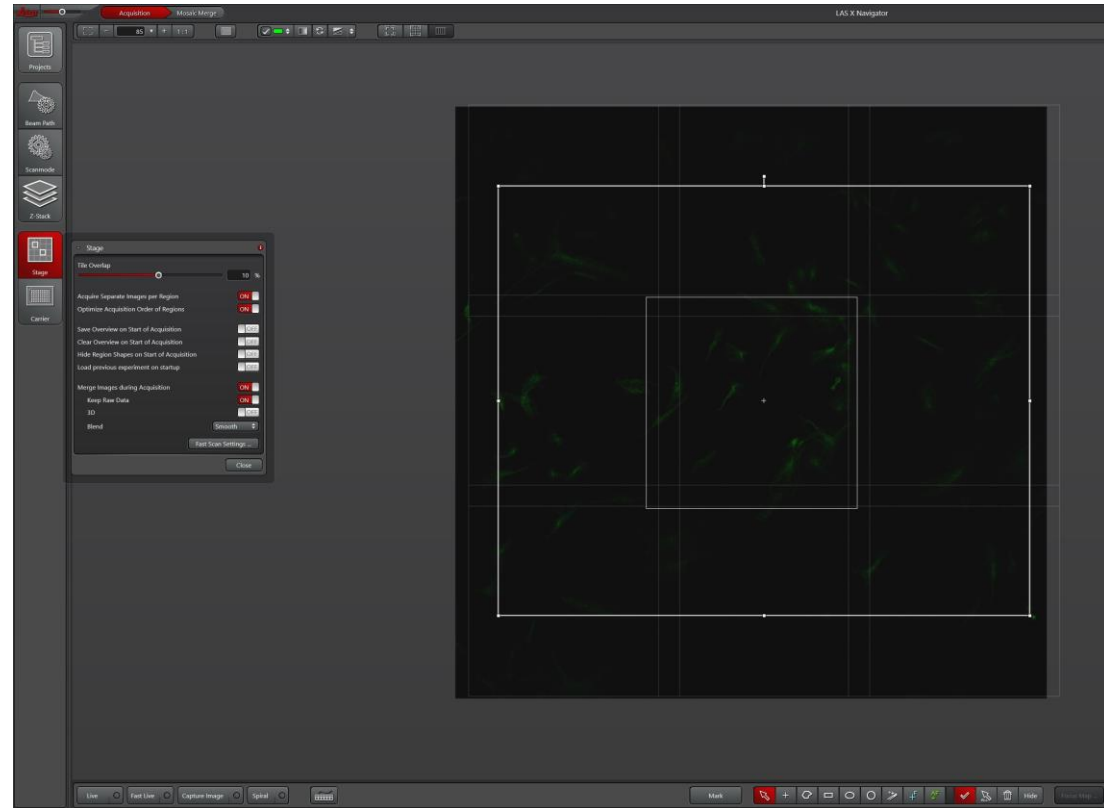






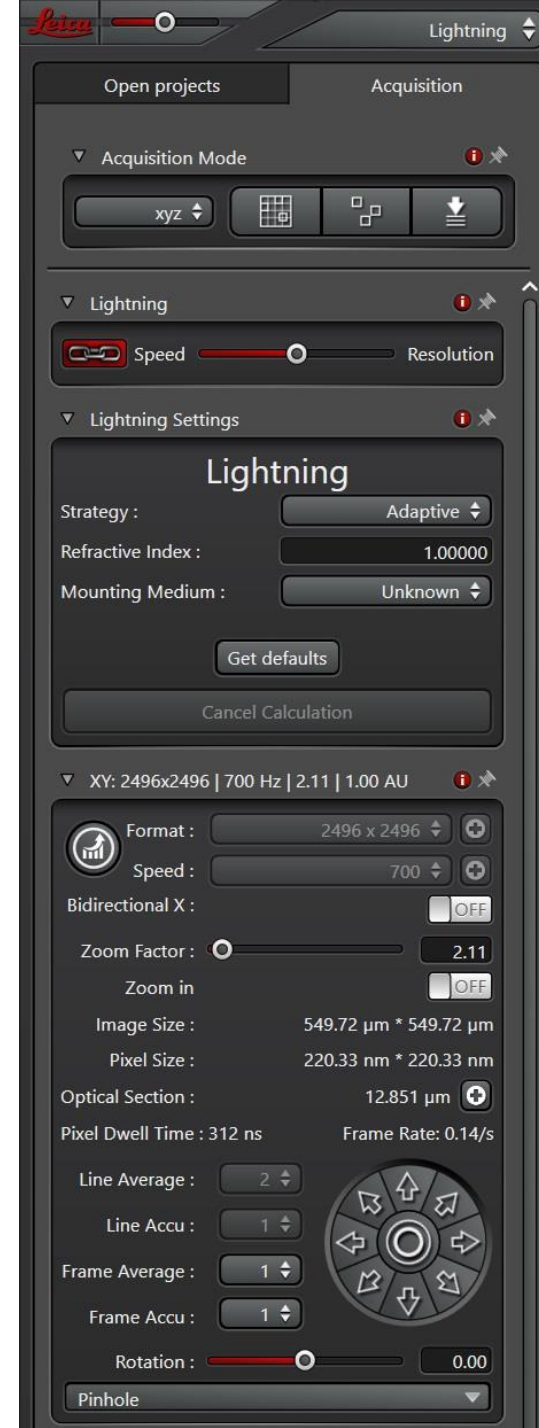
# Part 2: Navigator

- Click on “Navigator” button to access stage overview interface
- Original interface elements are located as tabs on the left side
- Live button will start live-scan like normal
- Spiral will start a live scan where the stage position spirals around the starting position filling out the stage overview
- The mouse wheel lets you zoom in and out
- Click and drag to move overview around
- Double-click at any position to move stage to that position and acquire one scan
- Region-of-Interest (ROI) tools will let you select large areas and acquire a tilescan for images larger than one field-of-view (FOV)
- ROI tools will also let you select many individual FOVs for “mark and find” timelapses
- Be sure to select automatic stitching option
- If you’ve set a z-stack then the tilescan will do a z-stack at each FOV
- For large tilescans you will likely need to set focus points to keep all FOVs at the same focal plane



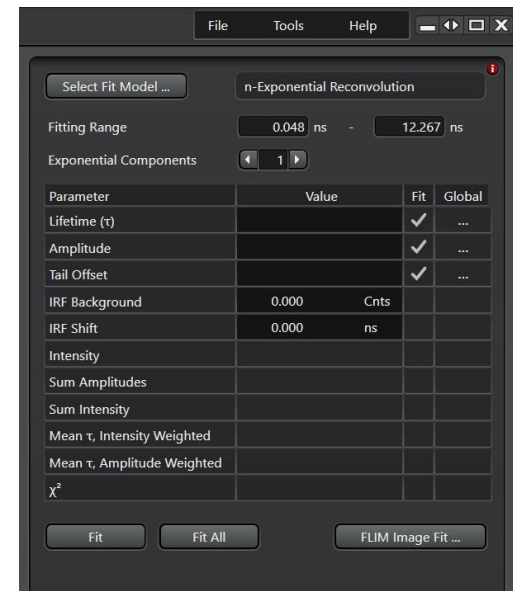
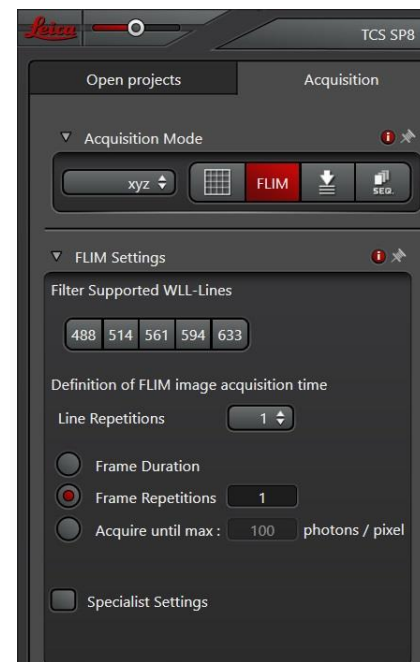
# Part 2: Deconvolution

- Click on SP8 button and choose Lightning from the drop down menu
- Left panel now has options to help you choose ideal setting for deconvolution
- Choose refractive index of sample (water, glycerol, antifade type, etc.)
- The slider lets you choose between speed and resolution
  - If you want to manually adjust setting then unselect the chain icon
- Note that at high resolution settings the pixel count is very high and the confocal aperture is set to 0.5 AUs
  - Acquisition will take much longer
  - Check that image brightness is still okay with 0.5 AU aperture
- Deconvolution is very useful to improve low signal images due to fast timelapses. If you need faster timelapses but the image quality is very low then you should test out the deconvolution. You might be surprised how much it can improve a low signal-to-noise image.
- Always compare to original unprocessed images and watch out for deconvolution artifacts



# Part 2: FLIM basics

- If you haven't used FLIM before then a training with Ben is recommended
- Click on "FLIM" button, FLIM interface opens up on second monitor
  - Be sure that live scan is NOT active when you open the FLIM interface
- FLIM only works with White Light Laser (WLL)
- If possible, use one of the notch filter line wavelengths for excitation
- Use HyD 2 first. It is cooled and is more sensitive therefore best for FLIM
  - Only HyD detectors can be used for FLIM
- Click Live, then adjust excitation intensity so that the photon counts per pulse histogram peaks at less than 1. (lower left plot on second monitor)
- Right-click on fast FLIM image and choose data cursor.
  - Observe number of counts in pixels of fast FLIM image
  - set frame summing number so that counts are at least 500-1000
  - acquire images
- Analysis
  - Choose number of components, then fit
  - Check lifetime of components for reasonableness
  - Then choose Image Fit → Precise Fit
  - Components will separated into separate channels
- Save FLIM data
- FLIM data can be exported as picoquant filetype for analysis in the free FLIMfit software



## Part 3: Heat and CO<sub>2</sub> incubation stage

- Turn on switch on Okolab touch screen.
  - Turn on switch on Lauda water bath.
  - Open CO<sub>2</sub> tank valve (if needed).
  - Set valves correctly for incubation stage and incubator.
  - Set desired temperature and CO<sub>2</sub> percentage.
    - Turn off airflow if CO<sub>2</sub> is not needed .
  - Remove z-galvo stage, set standing (do not lay flat).
    - Two thumbscrews and one hex screw (red screwdriver).
  - Move objective to lowest possible position.
  - Slide in incubation stage so that spring contacts are used.
  - Make sure water flow tubes are not pinched.
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- Remember z-galvo stage is no longer attached so for your z-stack be sure to switch from z-galvo to z-wide.

# Part 4: Other options

- HyD 2 is a special cooled detector. It can be used in any circumstance, but should be your first choice for low signal conditions.
- Immersol water-index fluid is available for long timelapses with the 40x water objective.
- Live Data Mode is available for automating more complicated tasks involving several different imaging jobs.
- Lightning is the deconvolution software, and can be used to slightly improve resolution and significantly improve signal-to-noise ratio.
- Navigator is the interface for tiling and mark-and-find acquisitions.
- There are sample holders for standard well plates at room temperature and in the incubation chamber.

# Shut-Down Procedure

- Check the online schedule
  - Shut-down if nobody is scheduled for the rest of the day.
  - Leave the system on if somebody is using the system today but do the following.
    - Log-off the computer
    - Remove sample
    - Wipe immersion oil off of objectives with lens paper
    - Return to the 10x objective
- Adjust your online reservation end-time if you finished early or late
- Shut off the computer
- Turn off key, then each switch from top to bottom
- Turn off eyepiece excitation light. (Do not turn off box underneath light box)
- Put dust cover over microscope



# Specifications

- 5 objectives installed
  - 10x/0.3 air (Working Distance = 11 mm)
  - 20x/0.75 Multi-Immersion (WD = 660  $\mu\text{m}$ )
  - 40x/1.1 water motorized correction collar (WD = 650  $\mu\text{m}$ )
  - 63x/1.3 glycerol (WD = 300  $\mu\text{m}$ )
  - 63x/1.4 oil (WD = 140  $\mu\text{m}$ )
  - Available on request: 5x/0.15 air and 40x/1.3 oil
- 3 laser sources
  - UV: 405 nm
  - Argon: 458, 476, 488, 496, 514
  - White Light Laser: 470-670 nm
- 3 HyD and 2 PMT detectors
  - HyD 2 is cooled for better low signal detection

# Common Issues and Solutions

## Is software bootup stuck at Klondike scanner step?

➡ Flip middle green switch off then on and wait about a minute.

## Is the touchscreen interface on the microscope off?

➡ Turn on hardware control box behind the left side of the monitor.

## Are you using the incubation stage?

➡ Be sure the hoses aren't kinked and the stage feels appropriately warm or cold.  
Make sure the stage is properly seated and doesn't wobble.  
Change z-stack setting from z-galvo to z-wide.

## Are your images poor quality?

➡ Increase the pixel count up to that recommended by the button left of format.  
Increase laser excitation intensity (may require decrease in detector gain).  
Increase line averaging to improve signal-to-noise ratio.

## High background or laser reflections? (Particularly a problem on the 10x)


➡ Make sure there is at least 10 nm between excitation wavelength and the start of detection range.  
Move excitation wavelength to one of the notch filter wavelengths  
Turn on gating for the HyD detector

## Is the frame rate too slow?

➡ Restart software and turn on resonant scanner option (some line averaging will probably be needed)  
Reduce Image height  
Reduce pixel count

# Common Issues and Solutions (Page 2)

**Do you need a brightfield-like image?**

 In the software interface click on TLD, turn on detector, hit live, then turn up the gain until you see the brightfield image.