

# Microscopy CoRE – Leica TCS SP8 STED 3X Basic Operation (Confocal)



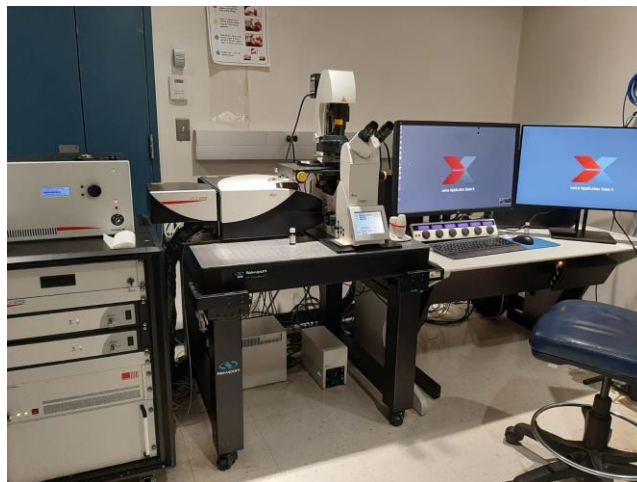
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June 2020

The Leica TCS SP8 STED 3X is an advanced confocal and super-resolution (STED) inverted imaging platform for fixed- and live-cell studies. This system features seven laser excitation lines, including a white light laser (WLL) that covers the whole spectrum from 470 to 670nm. The system also uses a motorized stage for multi-position imaging; an advanced detection system for spectral unmixing; and a full incubation setup for long-term research. Fluorescence lifetime imaging (FLIM) is also possible using the FALCON detection system.

## Prerequisites

- 1. Before and after your session, you are responsible for cleaning the objectives.**
  - a. Immersion lenses - Use lens paper and lens cleaner, located in the blue container, to wipe off any oil.
    - i. On the touchscreen (image on next page), press the lens control menu and switch to an immersion lens.
    - ii. Tear off one sheet of lens paper from the packet. Wet a small area of lens paper with the cleaner (pink liquid). **Do not use KimWipes** to clean any microscope optics.
    - iii. With your pointer finger, gently press the wet area to the front lens of the lens and wipe in a circular motion 1 or 2 times.
    - iv. Switch to a dry area of the same paper and gently wipe 1-2 times in a circular motion.
    - v. Repeat as necessary until the lens is clean. Use fresh lens paper and cleaner for each lens; use fresh paper as well if you must wipe off a lens more than once.
  - b. Dry/Air lens - Using dry lens paper, gently wipe (do not press into) the front lens of the objective using one circular swipe. Examine the paper for oil (shiny smears). If oil is noted, please talk with CoRE staff to have the lenses thoroughly cleaned. Do not attempt to clean dry/air lenses yourself.
- 2. Before imaging, be certain that any mountants or sealants are completely dry.** This typically takes at least 24 hours from the time the slide is prepared. Refer also to manufacturer's instructions.

## System Overview



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## System Startup

1. At right, below the computer desk, flip on the three switches in sequence from bottom to top.
2. Next to the switches, turn the laser key clockwise to the ON position.
3. Turn the fluorescence lamp, located under the microscope.
4. Log on to the computer using your credentials that you set up during the training.

## Basic Fluorescence Setup

1. On the microscope, gently tilt the transmitted light arm to the rear until it stops.
2. There are three sample holders available for this system. *The slide holder is typically installed. If you need the well plate holder or the incubation holder, please alert Core staff when booking the instrument. Do not push too hard on either sample holder due to their delicate nature.*
  - a. If you are using slides, place the slide coverglass face down in the center of the slide holder. Move the slide clips over the slide to secure it in place.
  - b. If you are using well plates, gently slide the well plate into the holder. There is a rubber plug along the top edge that will hold your plate securely in place.

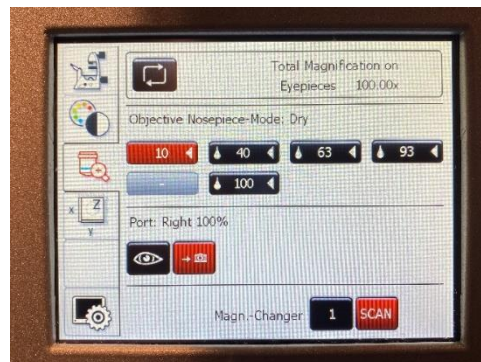
3. Gently tilt the transmitted light arm back into place over the stage.  
Center your specimen using the XY controls over the objective lens.

Stage movement knob: Y-Axis  
Stage movement knob: X-Axis  
Fine stage movement button  
Coarse stage movement button



4. *On the touchscreen* – Along the left edge, tap the **Lens Control** tab (objective lens with a “+” symbol).

Tap the 10x objective (twice if it does not move right away).



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5. *On the touchscreen* – Along the left edge, tap the **Filter Control** (colorwheel/black-white wheel). Along the top edge, tap **FLUO** under “Incident.” Alternatively, focus your sample using brightfield illumination by tapping **BF** (“Transmitted”).

Along the bottom edge, select one of the three available widefield filters – DAPI (blue), FITC (green), or RHOD (red).



6. *On the touchscreen* – Tap **IL Shutter**. Fluorescent light should now appear on the stage.
7. Use both hands to set the correct interpupillary distance: when you look through the eyepieces, you should see one image circle instead of two.

*If you do not see light through the eyepieces, briefly return to the Lens Control page and tap the “eye” icon under **Port** (see screenshot in Step 4).*

8. Use the XYZ controller to center and focus on your sample.

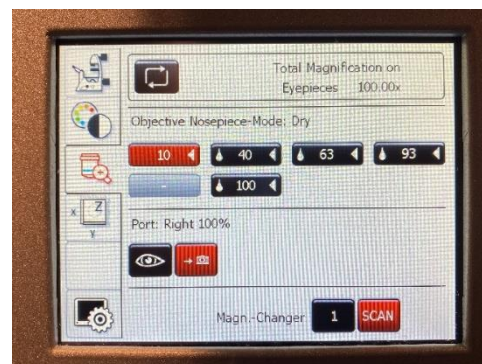


9. *On the touchscreen* – Tap either of the two remaining widefield filters to ensure there is signal and specificity for all fluorophores used in your sample.

*If your sample does not appear to be exhibiting fluorescent signal where you would expect, or as brightly as you may expect, this may be a good time to return to the lab to review your sample preparation methods to see if anything can be adjusted for a better result.*

10. When initial observation is complete, tap **IL Shutter** (Filter Control) again to turn off the fluorescence lamp.

11. To switch to an oil immersion lens – Tap on the **Lens Control** tab. Tap on the required magnification TWICE. *The oil immersion lens will swing into place. Carefully tilt the transmitted light arm back and remove your sample as per Steps 1 and 2. Apply one drop of immersion oil using the paddle to the front lens of the objective. Replace your sample and refocus.*



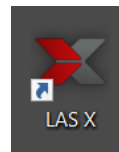
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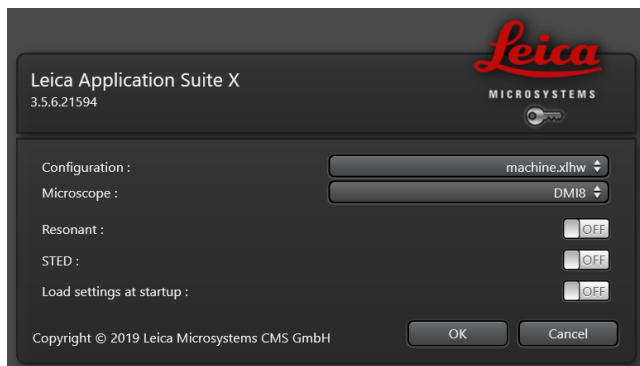
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## Software Startup

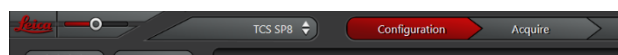
1. On the desktop, launch **LAS X**.



2. When prompted, set the options as follows and click OK.



3. Switch to **Configuration** along the top edge.



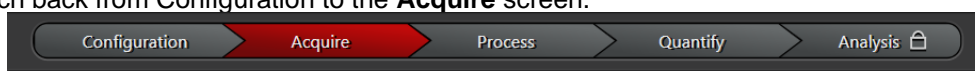
4. Click on **Laser Config** along the left edge. Set the following percentages for activated laser lines (see next page). *Only activate lasers that will be needed for that experiment – leave others turned off.*



Laser Line	Excitation Wavelengths	Recommended Percentage	Compatible Fluorophores ( <i>limited list; many more options available</i> )
405nm	405nm	N/A	DAPI, Hoechst
Argon	458, 476, 488, 496, 514nm	20%	CFP, AF488, EGFP, FITC, YFP
WLL	470 to 670nm	70%	Any fluorophore from green to far red

## Imaging Setup (Software)

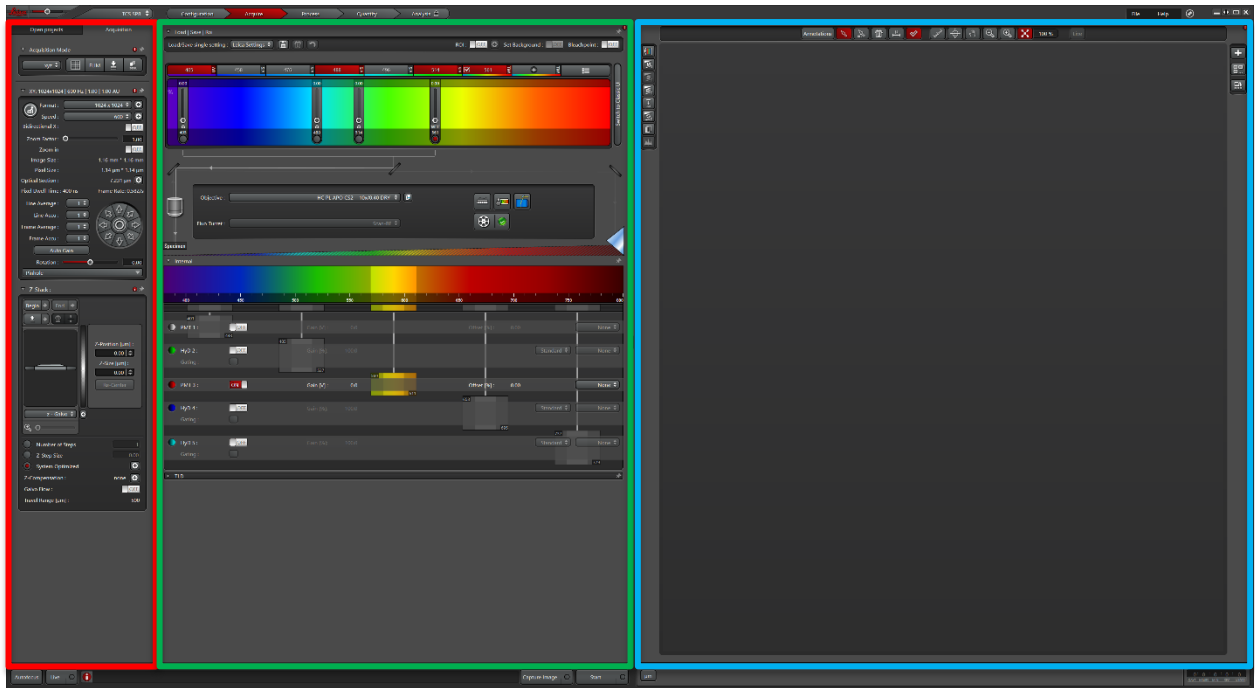
1. Switch back from Configuration to the **Acquire** screen.



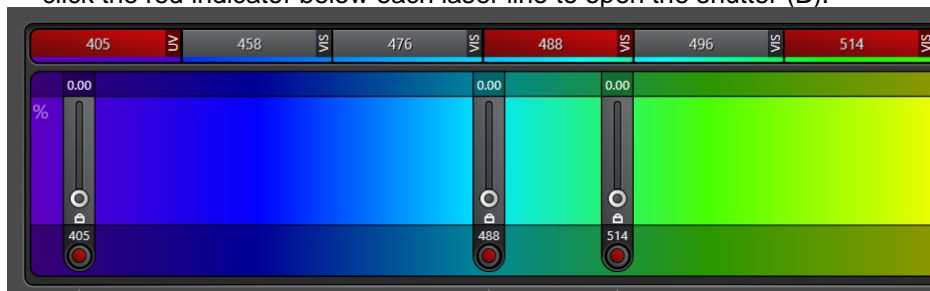
PANEL A	PANEL B	PANEL C
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2. In **Panel A: Scan Parameters**, set **Format** to 1024 x 1024 and **Speed** to 600 Hz
3. In **Panel B: Light Path**, activate the lasers needed to excite the fluorophores in the sample (A).
  - a. For the 405nm (“UV”) or Argon (“VIS”) lasers, click once on the laser to activate (A). Also click the red indicator below each laser line to open the shutter (B).

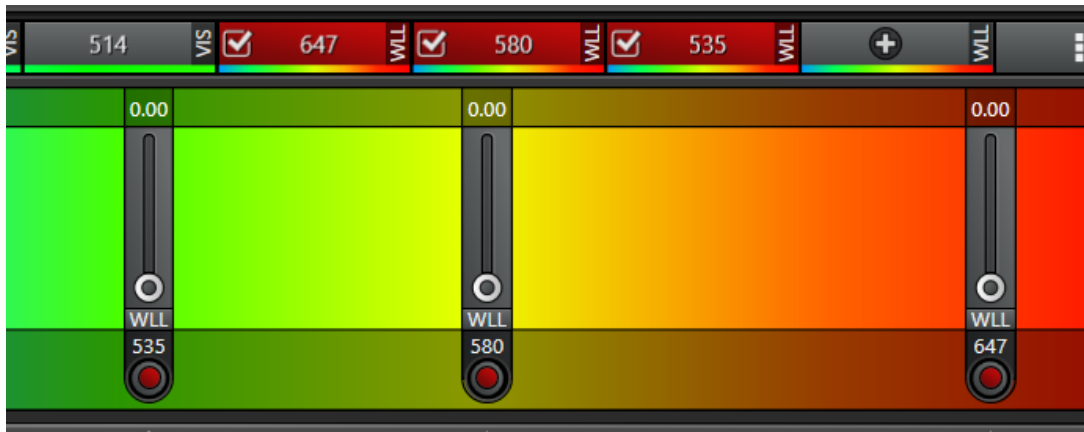


- i. For the WLL, click “+” to add up to eight additional laser lines (practical advice: do not exceed 2-3 lines). WLL lines must be further activated by adding a checkmark next to the laser line. Lastly, activate the red indicator under the laser line and double-click on the number to set the precise excitation wavelength.



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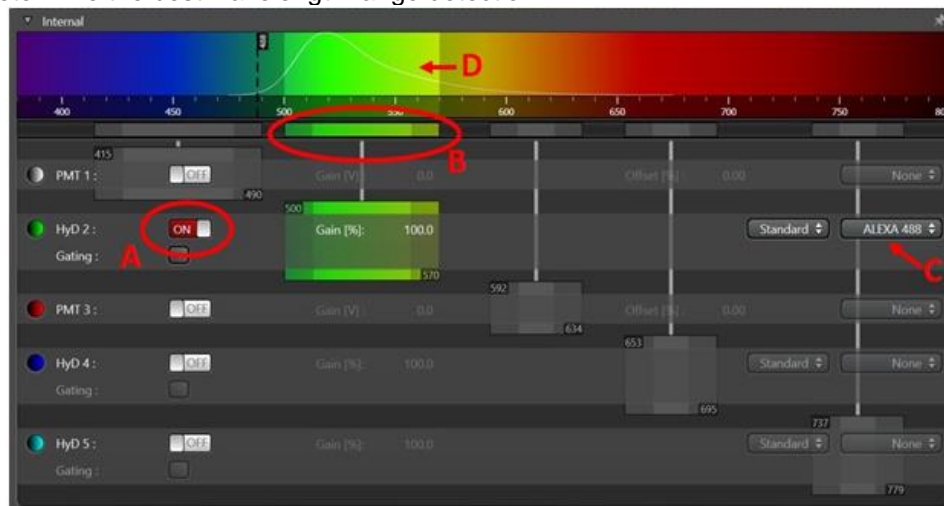
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- b. *WLL-specific:* To add more WLL lines click “+”. Double-click on the wavelength to change it or slide the laser bar to the correct wavelength (B). Set the intensity to 1% to start and make sure the circle below the wavelength (D) is red (shutter ON).



- c. Activate the detector/s needed (A). Adjust the slide bar to collect the proper wavelength range for each of the fluorophores (B). The drop down menu on the right side for each detector shows a list of the most common fluorophores (C). Choose the fluorophore of interest to plot the emission spectra in the light path scheme (D). This will help to determine the best wavelength range detection.



## \*\*When to use a PMT or HyD:

- HyDs are lower noise detectors and therefore recommended for weaker signals. Also, when combined with the WLL it allows to “gate” the signal detection: the timing for photon detection can be adjusted to discard autofluorescence and light reflection.

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- PMT are more suitable for stronger signals (good signal to noise ratio)

## \*\*Choosing the acquisition mode:

- **None sequential:** fastest mode for acquisition. All fluorophores are detected at the same time using different detectors. Since all the lasers needed to excite the fluorophores are “on” at the same time, this can lead to signal crosstalk or bleed-through (detection of two different fluorophores in the same channel) --> set all lasers and detectors together.
- **Line sequential:** to avoid crosstalk, the excitation-detection of the different fluorophores can be alternated. In this case, the excitation-detection of the different fluorophores switched between lines until the whole frame is scanned. This mode is optimal for scanning fast events since the switch between sequences is very fast. In this mode, the hardware settings (detector position, averaging, accumulation...) need to be the same for all sequences need to be the same to allow the fast switching --> click SEQ in the Acquisition mode. Set the laser/s and detector/s for each sequence. Choose “Between Lines” in the Sequential Scan
- **Frame sequential:** similar to line sequential but the switch between sequences is done after acquiring the whole frame. For this mode, hardware settings can be adjusted individually for each channel giving more flexibility for the detection of each fluorophore --> click SEQ in the Acquisition mode. Set the laser/s and detector/s for each sequence. Choose “Between Frames” in the Sequential Scan



When imaging on sequential mode: All lasers should be always on (red button) but lowered to 0% during the sequences that are not needed.

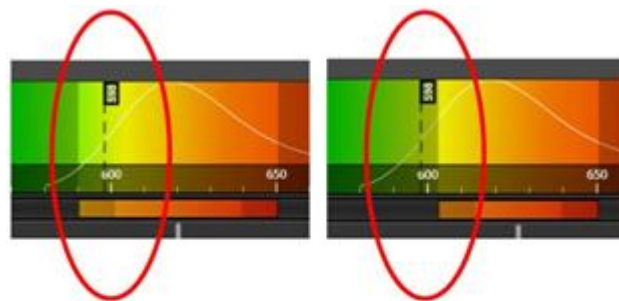
In the example below, lasers 405 and 594 are set at 0% in the sequence for A488 detection

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**CAUTION: MAKE SURE THAT THE EXCITATION LIGHT IS NOT BEING DETECTED BY THE PHOTODETECTOR, SPECIALLY THE HyD. IT CAN DAMAGE THE SYSTEM**



**DO NOT** extend the detector over the excitation laser

**Always** keep the excitation laser outside the detection range

## Image Acquisition

1. In the bottom left of the screen, click “Live” to start your preview scan.
2. Click the Quick LUT button  to toggle through the three available pseudocolor options: (1) current channel’s color, (2) Glow Scale (Over/Underexposure), and (3) grayscale.



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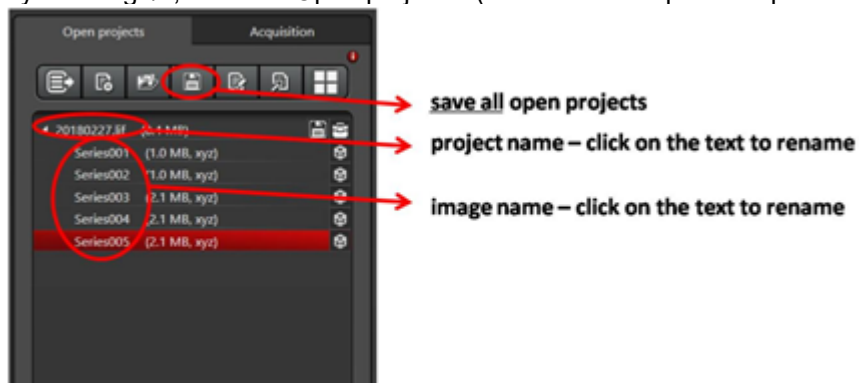
3. Select the Glow Scale, the image color will be red/orange/yellow and may have patches of blue or green. Blue denotes saturation while green indicated absolute black.
4. Set your gain:



- a. Using the Smart Gain knob (A), adjust the gain until you see saturation (blue pixels)
  - b. Use the z-position knob (C) to find your brightest focal plane in your specimen of interest
  - c. Readjust the gain to minimize the amount of blue pixels until you have a few left in your image
5. If using a PMT, set your offset: using the Smart Offset knob (B), adjust the offset until you see green pixels (absolute black) then readjust the offset to minimize the amount of green pixels until you have a few left in your image
  6. Click “Stop” in the bottom left (“Live” button) to stop your live scan
  7. If different channels are being acquired. Click on the next sequence and repeat steps 1-6 for each channel.



8. If signal is still low, adjustments on “Line accumulation” and laser power might be needed.
9. Adjust format/speed again if needed. Additionally, line/frame average can be increased to reduce background.
10. Click “Start” (bottom) to capture your image (all the channels). Click “Capture image” to only acquire the active sequence.
11. To save your image/s, click on “Open projects” (tab next to “Acquisition” parameters):



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12. The software stores all the images in a database file (.lif). please save your folder within the User Data folder in the Y:\ drive (HIVE server).

## Export images

ImageJ/Fiji can read lif files. Just drag the lif file on the ImageJ bar and the Bioformats plugin will read the images. You can choose to open all the files or just select from the list of images.

In LAS X, select the image or images you want to export on the “Open projects” tab and click the right button on the mouse. From all the list of options, choose “Export” – “as Tiff”. Select the options you want to export: choose directory, overlay channels, micron scale...

## Apply previous imaging settings

To reuse the same settings from a previous image, open the lif file that contains the image. Click the right button on the mouse and select “Apply image settings”. This reads the metadata of the file and copies the information on the acquisition window.

## System Shut down

1. Save all your images/projects.
2. Switch to Configuration along the top edge.
3. Click on Lasers along the left edge and turn off all the lasers.
4. Close the software. It will take a few minutes to shut down.
5. Turn off the computer or sign out. If another user is coming after you, do not continue with the next steps.
6. Turn the fluorescence lamp, located under the microscope.
7. If using the Argon laser, wait until the fan (cooling system) stops.
8. At right, below the computer desk, flip off the three switches in sequence from bottom to top.
9. Next to the switches, turn the laser key clockwise to the OFF position.