Microscopy CoRE – Leica TCS SP8 (Icahn 13-71) and SP8 AOBS (Annenberg 18-250), Basic Operation

microscopy.core@mssm.edu; 212-241-0400
November 2024

The Leica TCS SP8 is an advanced confocal inverted imaging platform for fixed- and live-cell studies. This system features seven laser excitation lines; a motorized stage for multi-position imaging; an advanced detection system for spectral unmixing; and a full incubation setup for long-term research.

Prerequisites

1. **Before and after your session, you are responsible for cleaning <u>all</u> objectives.**

    a. Tilt the transmitted light arm (above the stage) gently backward to access the lenses.
    b. On the touchscreen (see System Overview), press to reach the Objective screen. Press on the lens to be cleaned (twice if switching between oil and dry lenses).
    c. **For Dry Lenses:**
        i. Tear off one sheet of lens paper and remove two cotton swabs from the package.
        ii. Soak the cotton tip of one swab with cleaning solution. Gently rub the cotton tip in a circular motion over the objective lens. Throw this swab out.
        iii. Place the lens paper, unfolded, over the objective. Using the second dry swab, use the cotton tip end to gently press the lens paper to the lens. Gently rub in a circular motion.
    d. **For Oil Immersion Lenses:**
        i. Tear off one sheet of lens paper.
        ii. Fold the lens paper twice, once in half widthwise and once in half lengthwise.
        iii. Wet one corner of the folded lens paper with cleaning solution.
        iv. Place the wet corner over the objective lens. Use your pointer finger to gently press the paper to the lens. Gently rub the lens in a circular motion.
        v. Move your pointer finger to a dry area of the same lens paper. Now gently press this dry area to the lens to dry it off. Move in a circular motion.

If the lens paper is shiny after cleaning, repeat the appropriate cleaning process until the paper is no longer shiny. Use fresh lens paper and cotton swabs each time – do not reuse supplies.

If you cannot get an objective clean, please talk with CoRE staff to have the lenses thoroughly cleaned.

2. **Before imaging, be certain that any mountants or sealants are <u>completely dry</u>.** This can take at least 24 hours from when the slide is prepared. Refer to the manufacturer's instructions.

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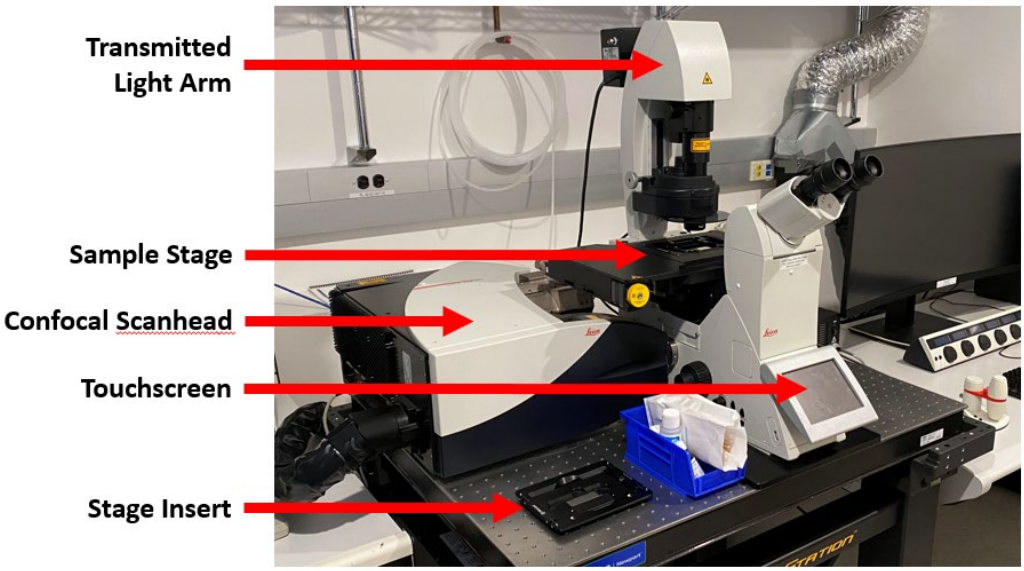
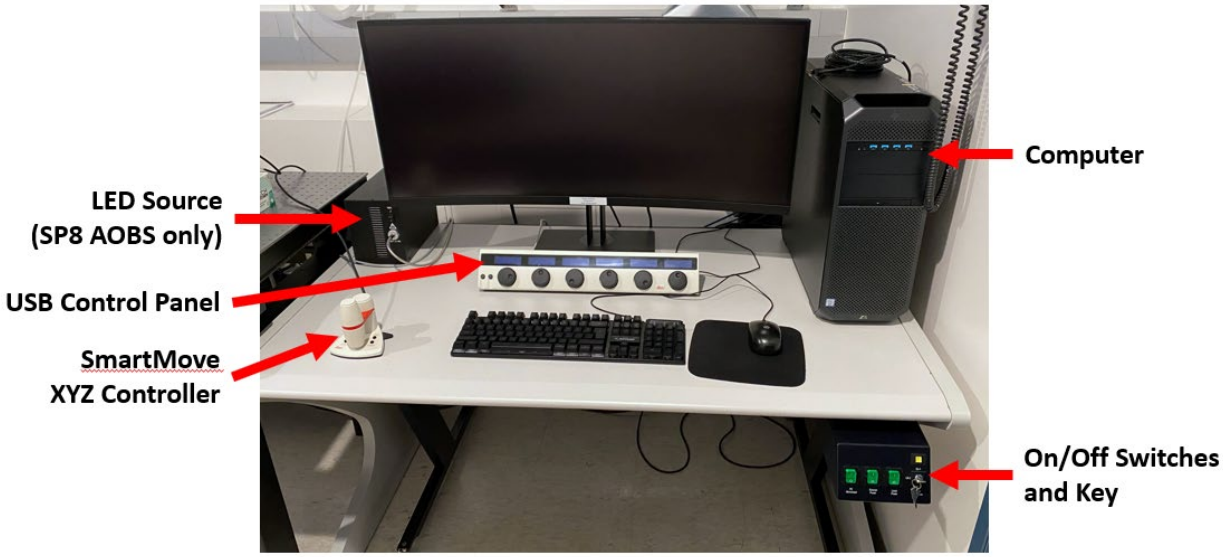
System Overview





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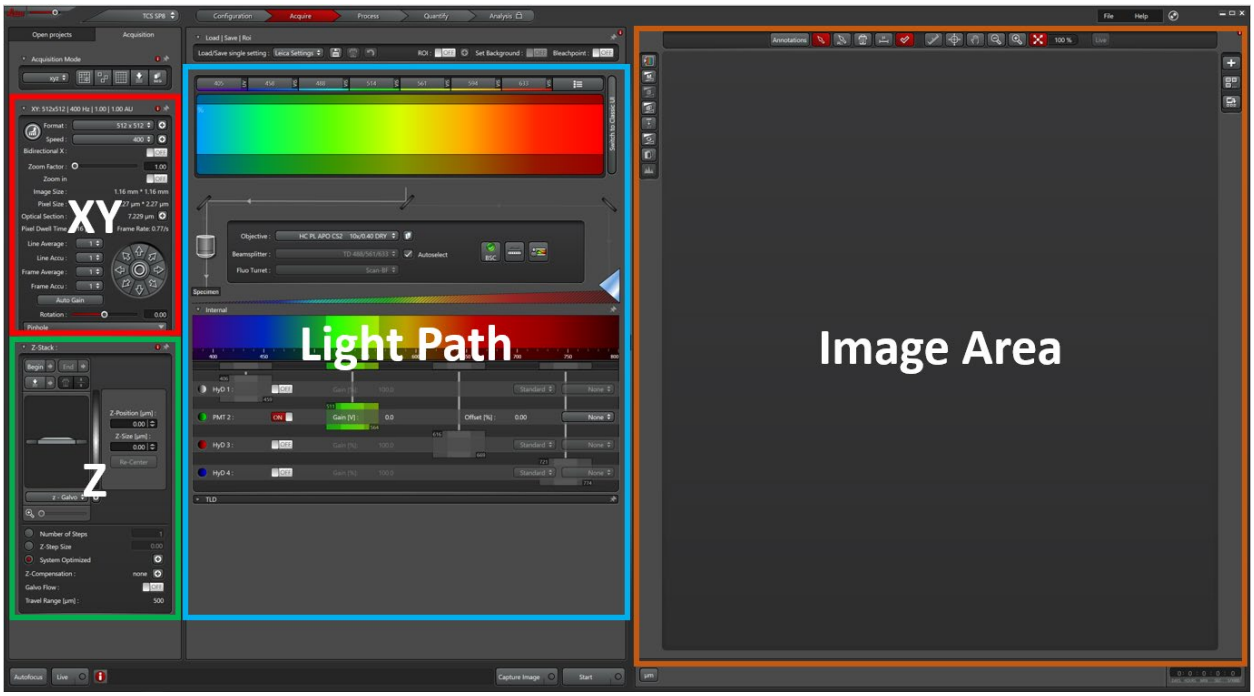

System Startup

1. At right, below the computer desk, flip on the three switches in sequence from left to right.
2. Next to the switches, turn the laser key clockwise to the ON position.
    a. Check that the fluorescence lamp, to the left of the computer monitor, comes on. If not, flip the Power switch to ON.
3. Log on to the computer using your credentials that you set up during the training.
4. SP8 AOBS only – turn OFF LED light source (left of monitor), then back ON.

Basic Fluorescence Setup

1. *SP8 Icahn only* – On the microscope, slide open the two top covers surrounding the transmitted light arm. If the live-cell imaging cover is in place, also slide open the front two doors.
2. Gently tilt the Transmitted Light Arm to the rear.
3. There are two styles of sample holders available for these systems.
    a. **SP8 AOBS, "click-in holder"** – Rotate the holder in your hands until the red dot is face up and in the bottom-left corner (similar to the stage dot). Place the holder onto the stage a little to the right of center, then slide it to the left until it drops fully into place.

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Icahn School of Medicine at Mount Sinai

b. **SP8 Icahn, piezo insert** – The stage holders are NOT user-exchangeable. You must arrange with the CoRE ahead of time for the holder you need. Typically, the holder for slides and 35mm dishes is installed. For well plates, send an email to the CoRE BEFORE your session to arrange a swap. ***Do not push on the sample holder due to its delicate nature.***

4. Insert your sample carrier:
   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.

5. Gently tilt the transmitted light arm forward into place. Close all doors to the incubation chamber.

6. *On the touchscreen* – Along the left edge, tap the **Lens Control** tab (objective lens with "+").

7. *On the touchscreen* – Tap the 10x objective (twice if it does not move right away), and under Port, tap the picture of the eye.

8. *On the touchscreen* – Along the left edge, tap the **Filter Control** (colorwheel/black-white wheel).

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

10. *On the touchscreen* – Tap **IL Shutter**. *Fluorescent light should now appear on the stage.*

11. Use both hands to set the correct interpupillary distance for the eyepieces: when you look through them, you should see one image circle instead of two.

12. Use the SmartMove XYZ controller to center and focus on your sample. Use Coarse (fast) and Fine (slow) speed buttons on the SmartMove to reach the destination.

13. *On the touchscreen* – Tap the two remaining widefield filters to ensure there is signal and specificity for all fluorophores used in your sample, if applicable. *NOTE: Far reds ~633nm are not visible with the installed filter sets.*

NOTE: 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.

REMEMBER: If you are setting up the imaging parameters for the first time, you must screen your samples and set the parameters based on your brightest samples!

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14. When initial observation is complete, tap **IL Shutter** again to turn off the fluorescence lamp.

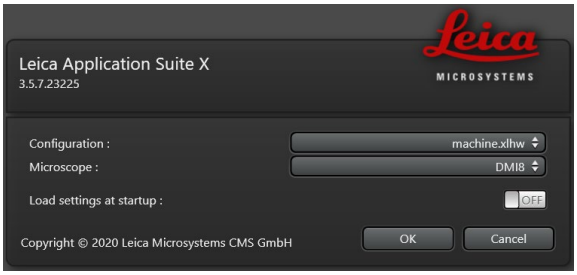
15. To switch to an oil immersion lens – Tap on the **Lens Control** tab. Tap on the required magnification; once the lens "blinks," tap it once more.
    The oil immersion lens will swing into place. Carefully tilt the transmitted light arm back and remove your sample. Apply one drop of immersion oil using the paddle on the lid of the

immersion bottle to the front lens of the objective. Replace your sample, then repeat Steps 9-13 to reconfirm focus and location.

Software Startup

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

2. When prompted, set the options as follows and click OK: **get screenshot of launch window**



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

4. Click on **Laser Config**. Set the following percentages for activated laser lines. *Only activate lasers that will be needed for that experiment – leaves others turned off.*

Laser Line	Excitation Wavelengths	Recommended Percentage	Compatible Fluorophores (limited list; many more options available)
405nm		N/A	DAPI, Hoescht
Argon*	458, 488, 496**, 514	30%	CFP, AF488, EGFP, FITC, YFP
561nm		N/A	AF555, AF568, DsRed
594nm		N/A	AF594, mCherry
633nm		N/A	AF633, AF647, Cy5
*Argon laser takes five minutes to warm up AND cool down. **496nm laser available exclusively on SP8 AOBS.			

5. Click on **Hardware**. Switch the **Resolution** to the required bit depth.

6. Click on **IPS** – Select **Load**. Go to **Desktop** > select "default ips.xml," click **Open**.

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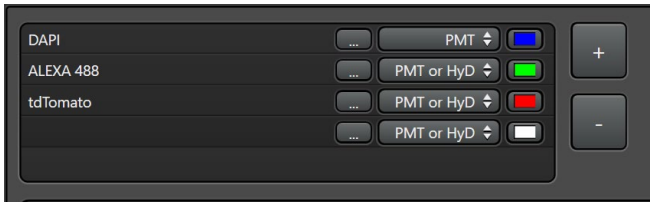
7. Switch back from Configuration to **Acquire**.

8. Open **Dye Assistant**.

9. Click the ellipsis ("...") to add your first fluorophore. Decide if you would like to specifically send this signal to a PMT or a HyD detector.



PMT's are ideal for brighter signals; HyD's are for weaker signals, or signals where the Signal-to-Noise Ratio (SNR) is low.

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10. Repeat Step 9 for additional fluorophores.

11. Choose the imaging sequence appropriate for your sample. Click **Apply**.

None sequential – fastest imaging; greatest risk of crosstalk; rarely recommended
Line sequential – moderate speed; better crosstalk reduction, limited hardware control; recommended for well-spectrally separated fluorescence panel
Frame sequential – slowest imaging; best for weak signal capture; independent hardware control per sequence

Channels divided across fewer sequences will speed up imaging as well (example: 2x 2-channel sequences instead of 4x 1-channel sequences).

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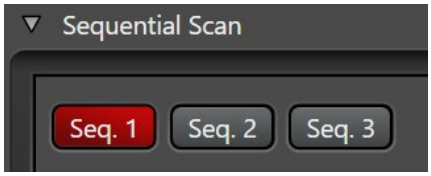
12. Under **Sequential Scan**, select the first Sequence.



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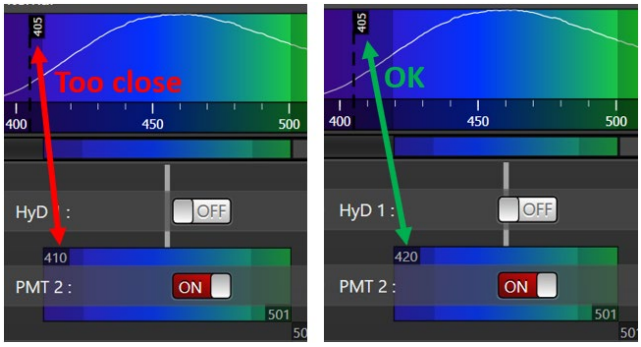
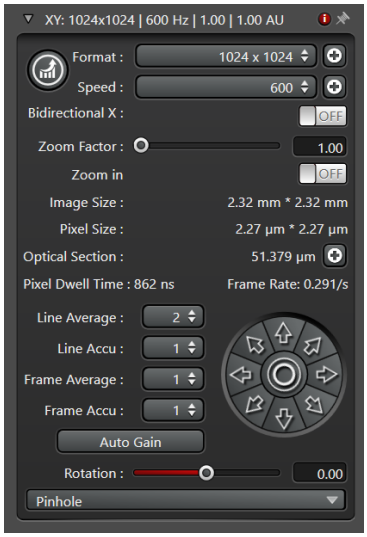
13. Double-check the light path of the Sequence: make sure the *left* side of the emission window (per Sequence) is *at least 10 nanometers from the laser excitation wavelength*. Adjust the window as necessary.



14. Under **XY**, set the **Format** to 1024x1024 and the **Speed** to 600. A **Line Average** of 2 is recommended.

Ideal settings are highly-sample dependent.



15. Click **Live**.

16. Switch to **Under-/Overexposure** Mode (Glow Mode).

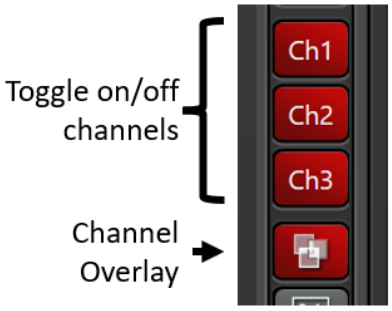
17. Adjust laser power and Detector Gain until overexposure is removed (a few blue pixels is OK). *Focus through the layers of the sample to ensure you are looking at the brightest plane of the brightest sample.*

Focus control is only from the far-right knob on the USB Control Panel – do not use SmartMove or microscope focus knobs for imaging.

18. Switch to the next Sequence. Repeat Steps 14-17 for remaining Sequences. *When frame-switching, you must click Stop on Live first to switch Sequences. Switch, then restart Live.*

19. Click **Start** to capture an image.

20. To the right of the images, activate the **Channel Overlay**.



21. To capture more fields, click **Live** and use the SmartMove controller or the microscope's widefield mode (covered above, in Basic Fluorescence Setup) to locate a new region.

22. Under **Open Projects**, arrange images in any manner that you like. Create new Projects to form new image groups.

Recall Previous Image Settings

1. Connect to the HIVE or a preferred cloud storage website. No USB drives are to be connected to computers, as per CoRE policy.

2. Under Open Projects, click Open.

3. Load the relevant LIF file.

4. Right-click on an image that used the desired settings.

5. Select **Apply image settings**.

NOTE: To correctly apply settings, be sure you have loaded the "default ips.xml" configuration file first!

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Software Setup – Z-stack

1. Select any **Sequence** to begin.
   It is recommended to use the Sequence imaging the fluorophore that occupies the largest structure in the cell, such as a membrane marker. This ensures all focal planes of the cell are imaged.

2. Click **Live**.

3. Focus to one side of the sample using the Z Position knob of the USB Control Panel. *Do not use the SmartMove controller or microscope focus knobs for this operation!!*

4. Under **Z-Stack**, click **Begin**.

Do not move too far out of focus or you will unnecessarily image focal planes with no data – wasted time and disk space. It will also affect the quality of certain image processing.

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5. Focus through the specimen to the other side. Click **End**. *The same advice as above applies here.*

6. Use **System Optimized** for the step size when generating three-dimensional datasets.

Exceptions exist: For non-3D datasets, the Number of Steps can be cut in 1/2 to 1/3 of the System Optimized number of steps. No Extended Depth of Field will be possible, only Projections (MIP, etc.)

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7. Click **Start**.

Tiling/Navigator

Please see our separate guide on this module for more information.

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System Shutdown

1. Under **Open Projects**, save all data in the LIF format on the HIVE.
2. Switch to **Configuration** > **Laser Config**. Turn off all lasers.
3. Close LAS X.
4. Clean all objective lenses and inspect for damage.
5. On the computer:
    a. If another user is imaging after you (check iLabs), log off the computer. Leave all switches and the key ON.
    b. If there are no users after you, shut down the computer fully. This will log you off.
6. For full shutdown (no other user after you): Wait for computer to turn off.

IMPORTANT NOTE: If you had turned on the Argon laser, wait for the fan to shut off (you will hear it). This takes about five minutes from when you deactivate the laser in LAS X. Do not turn off any switches or the key before this fan shuts off.

7. When computer and Argon are shut off fully: turn off the key, then turn off the three switches in reverse order (right to left).

Instrument	**Leica TCS SP8 AOBS**
SN	**508797**
SW Version	**LAS X 3.5.7.23225**

Objectives	**Magnification/NA**	**Immersion**	**Coverglass**	**Manufacturer PN**	**Working Distance**
	1) HC PlanApo 10x/0.4 "A"	air/dry	0.17mm ("#1.5") coverglass	506407	2740um
	2) HC PlanApo 20x/0.75 "C"	air/dry	0.17mm ("#1.5") coverglass	506517	620um
	3) HC PlanApo 40x/1.3 "D"	oil immersion	0.17mm ("#1.5") coverglass	506358	240um
	4) HC PlanApo 63x/1.4 "E"	oil immersion	0.17mm ("#1.5") coverglass	506350	140um
	5) HC PlanApo 100x/1.4 "D"	oil immersion	0.17mm ("#1.5") coverglass	506372	130um
	6) (none)				

A/B/C/D/E - DIC objective prism "class," based on BFP location

Excitation Lasers	405nm
	458nm
	488nm
	496nm
	514nm
	561nm
	594nm
	633nm

		All detectors
Detectors	2x HyD (ultra-sensitive detectors for weak signals)	*Frequency Range: 10-1800Hz*
	2x PMT	*Detection Range: 400-800nm*
	1x T-PMT (for transmitted light)	
Largest Field Size	1.55mm X 1.55mm (10x objective, 0.75 zoom)	
Dichroics	458/514/561	*Dichroics allow for simultaneous acquisitions of multiple florophores.*
	488/561/633	
	458/514	
	488/594	
	RT 15/85	
Techniques Available	Simultaneous confocal	
	Sequential confocal	
	Lightning - 40FPS @ 512x512; 120nm XY resolution	
	Z-stack (13.0mm full; 500um fast Z)	
	Tiling (Navigator)	
Compatible with:	standard slides (76x26mm)	
	skirtless well plates	