

Leica TCS SP5 Confocal Microscope (341-H)

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START UP

1. Turn on the portable AC
2. Switch on the PC/Microscope button on the control panel. If the microscope box is off, switch it on.
3. After Windows boots up, log in to your account.
4. Flip on the Scanner switch. Wait about 60 seconds for it to establish full connection to the computer.
This is crucial; otherwise the software will not load
5. Open the LAS AF program. When prompted, click OK.
6. After the software begins to load (you'll see text scrolling in the LAS AF start up window), flip on the laser power switch on the control panel
7. After about 60 seconds, flip the laser ignition key on the control panel.



MOUNTING A SAMPLE

1. Tip the microscope condenser head back and place your sample **COVERSLIP DOWN** onto the stage.
This is an inverted microscope; you will not be able to focus if you have glass thicker than a coverslip between the objective lens and your sample
2. Tip the microscope condenser head back over the stage.
3. Use the X and Y knobs on the joystick to center your sample above the objective lens
4. Press the TL/IL (transmitted/illuminated light; on left side of microscope) button and look through the microscope eyepieces. Adjust the Z position of the objective lens using the joystick until your sample comes into focus.
5. If you cannot see your sample because it is completely colorless, you may need to use the Hg fluorescent lamp in order to focus on your sample. Flip the Hg lamp power supply button. It will take about 60 seconds for the lamp to warm up and ignite (indicated by green light on power supply). Press either the N2.1 or I3 buttons on the front panel of the microscope to insert a fluorescent filter (FLUO mode will be indicated on the microscope LCD screen). Press the shutter button on the front microscope panel to illuminate your sample with light from the Hg lamp. Try to focus on your sample once more. Close the shutter when finished.

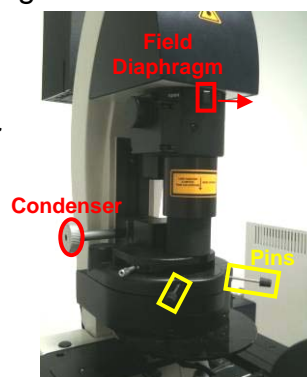


If you turn on the Hg lamp, it needs to be left on 30 minutes before you turn it off in order to not cause damage to the light bulb. The lifetime of the bulb is only 200 hours, so pay careful attention to the lifetime counter on the power supply box. When it approaches 200 hours, please contact me so I can replace the bulb before it becomes hazardous.

ADJUSTING THE KOHLER ILLUMINATION

Aligning the lenses within the microscope properly ensures optimal illumination of your sample so that you have the best possible data. This procedure must be done every time you change objective lenses.

1. After focusing on your sample, completely close the field diaphragm (slider at the top of the microscope). You will see a small illuminated circle in the center of the field of view surrounded by the shadow of the field diaphragm.
2. Adjust the condenser height (silver knob on left side of the microscope) until the interface between the shadow and the illuminated circle comes into focus. When it is out of focus, you will see either a green or red halo at the interface. When it is in focus, the red/green halo will disappear and the interface will be crisp and sharp.
3. Center the condenser in the XY plane using the silver pins above it. It helps to judge whether the condenser is centered by opening and closing the field diaphragm after you adjust its XY position. If it is centered, the shadow will disappear at the same rate (shadow appears to have the same thickness) throughout all of the field of view as you open the field diaphragm.
4. After the condenser is centered, open the field diaphragm just enough so that the shadow completely disappears. This ensures that you are only illuminating the area in your sample that can be seen in the field of view.



SETTING UP AN EXPERIMENT

1. After LAS AF is loaded, go to the Configuration tab and click on the laser icon. Check the boxes next to the lasers you wish to turn on (the Ar laser has 458, 476, 488 and 514 nm). For the Ar laser, drag the power slider to about 30-40%.
2. In the Acquire tab, choose a saved or pre-set laser beam setting in the Leica Settings window. This will activate the specified laser lines, load the appropriate dichroic mirror, and set the PMT detectors to the appropriate detection ranges for the specified lasers/dyes.
3. Alternatively, you can create your own beam setting by
 - 1) Sliding the power slider to a reasonable power level for each of your desired lasers,
 - 2) Choosing a dichroic mirror that matches the lasers you turned on, and

- 3) Manually activating and moving the PMT detectors to appropriate detection ranges for your sample.

****IMPORTANT: never set a PMT to detect underneath a laser line. This will damage the detector.****

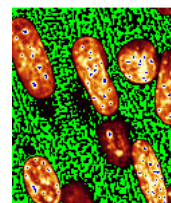
4. In the left panel of the Acquire screen, choose the appropriate data acquisition mode (usually XYZ), desired image size, and desired scanning speed.

CHANGING OBJECTIVE LENSES

1. In the LAS AF program, in the Acquisition window, click on the name of the objective lens installed on the beam path diagram to open up a menu of available objectives
2. Choose desired objective
3. The instrument will automatically lower the first objective, rotate the new one into position, then raise it to the last z position
4. Adjust the focus on the microscope
5. Re-adjust the Kohler illumination for this objective

ADJUSTING THE GAIN/OFFSET OF IMAGES

1. Press the Live button in the bottom left corner to view a live image
2. In the right monitor, you will see an image for each PMT that is activated.
3. Press the overlay icon on the right side of the image screen to activate the overlay image channel which combines the images from all PMTs into one.
4. Double click on each image to enlarge it to full screen.
5. Press the QLUT button (looks like a square rainbow) on the middle left side of the image monitor to put the image into the gain/offset adjustment mode by clicking the QLUT button once (turns orange). This mode is called over-glow/under-glow. All pixels that are at the maximum intensity readable by the detector will be blue. All pixels that show no signal on the detector will be green.
6. Adjust the Smart Gain knob until you see only a few blue pixels (may need to increase or decrease gain; if you have maxed out the gain at 1250 and still see no blue pixels, increase the laser power for that image). Having only a few blue pixels in the over-glow/under-glow image ensures that you have the best gradient of contrast between the brightest and dimmest areas of your image.
7. Next, adjust the Smart Offset knob until about 50% of the black pixels turn green (usually turn the knob to a negative value).
8. Press the QLUT button twice more to return the image to its normal color
9. Repeat gain/offset adjustment for each image



****You should do this before capturing any image on your sample to ensure you are collecting the truest, most believable fluorescent images****

CAPTURING AN IMAGE

1. Move the stage to an area of interest on your sample
2. Adjust the zoom factor if you wish (either by the slider in the Acquisition tab or using the Zoom knob)
3. Adjust the gain and offset for each image

4. Press the Capture button at the bottom of the left monitor.
5. An image will appear in the queue in the Experiment tab in the Acquire window
6. To save the image, right click on it and choose Export
7. Export the image as a TIFF
8. You can choose to add a scale bar, timestamp, dimension data, etc. to the image (normally just scale bar) that will appear in the exported image

ACQUIRING A Z-STACK

1. Ensure the microscope is not in the live imaging mode; if not, press Stop (bottom left of left monitor)
2. In the Z-stack window (bottom left side of the Acquire window's Acquisition tab), select Z-wide (we do not have the Z-galvo stage, so this setting will not allow you to collect a Z-stack)
3. Press the Live button
4. Using either the Z knob on the joystick or the Z knob under the monitors, move to the brightest Z position in the area you want to image
5. Adjust the gain/offset at this Z-position
6. Next, move the Z position to either the upper or lower limit you wish for your stack
7. In the Z-stack window, click the black Begin arrow on the left so that it turns orange; then click the Set Plane button.
8. Move the Z position to the other extreme limit you wish for your stack
9. In the Z-stack window, click the black End arrow on the right so that it turns orange; then click the Set Plane button
10. In the Z-stack window you should see a crude rendering of the volume of the section you will be imaging. Ensure that the Begin and End Z positions are different.
11. The system has optimized the number of slices and their thickness according to the objective lens you've chosen in order to have the best possible Z resolution in the stack. You can change the step size or number of steps by first pressing Stop, and then clicking the circle next to "Nr. of steps" in the Z-stack window. Make any adjustments you desire.
Using a smaller step size than the optimized step size will not improve resolution, but instead will make your 3D image blurry because the images will be overlapped in the Z direction.
12. Press the Start button to collect the Z stack

MAKING A 3D PROJECTION OF A Z-STACK

1. Once the Z stack acquisition is complete, a new Series will appear in the queue in the Experiment tab in the Acquisition and Process windows. Select your Z stack.
2. Click on the Process tab at the top of the left monitor to open the Process window.
3. In the Tools tab of the Process window, choose Visualization → 3D Projection
4. In the 3D projection window, a crude rendering of your Z stack will appear. Click and drag your mouse in this window to change the viewing angle. Releasing the mouse button will create a fully rendered image of your 3D projection

5. You can change the rendering mode to maximum, average, transparent or color-coded
6. If you want to save this projection, press the Apply button (bottom right of left monitor) and a Projection of Series image will appear in the queue in the Experiment tab.
7. If you want to make a movie of the rotation of your 3D projection, click the "Create Movie" box.
8. Set the start and end angles by either inputting the numbers or clicking/dragging the crudely rendered projection to the desired angle and pressing Set Start/Set End.
9. Choose the number of frames, slice thickness, and rendering mode.
10. Press Apply to create movie. A movie file will be added to your queue in the experiment tab. These can be exported as an AVI file.

SHUT DOWN

1. Load the FITC-TRITC-Cy5 beam setting (loads 488/543/633 triple dichroic mirror) ***This is imperative so that the dichroic mirrors do not get jammed***
2. Load the 10x objective
3. In the Configuration tab, click on the laser icon. Drag the Ar laser slider to 0% and uncheck the boxes for all lasers.
4. Turn off the laser key
5. Ensure you've exported all images. Do not save the experiment as a .lif file unless absolutely necessary (time constraints) because these are HUGE files and slow down the computer's speed.
6. Close LAS AF
7. Flip off Scanner button on control panel
8. Download your data onto a USB drive or external hard drive
9. Shutdown the computer
10. Once computer screen goes dim, turn off PC/Microscope button
11. Remove sample from stage
12. Wait 20 minutes for laser to cool down before turning off the laser switch on the control panel and the portable AC

Lasers

Type	Wavelength (nm)	Max power at source (mW)	Max power at focal plane (mW)	Beam type
Ar	458, 476, 488, 514	< 200	< 30	Continuous wave
HeNe	543	< 1.5	< 0.5	Continuous wave
HeNe	633	< 15	< 4	Continuous wave

Objective Lenses

Magnification	Numerical Aperture	Dry/Oil Immersion
63x	0.7	D
63x	1.4	O
40x	0.85	D
10x	0.3	D