**Leica Confocal SP5 Operation Protocol**

Important information to know before working on the Leica SP5 Confocal Microscope:

* Sign up for this instrument on Booked Scheduler.
* Schedule a training session with FMIC staff if you have not yet been trained.
  + You must complete 5 supervised hours before enjoying independent access.
* Make selections on the microscope touchpad using your fingernail (without a glove!).
* The fluorescent lamp must be on for at least 30 minutes during a session or off for 30 minutes before being turned on again.
* If somebody has the instrument booked within an hour after you, leave the fluorescent lamp on.
* The last step of shutting down the instrument involves leaving the Laser Power on for 10 minutes as the instrument cools down.

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1. Turn on the fluorescent lamp rocker switch.
2. Turn on PC Microscope.

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* 1. Wait *10 minutes* as the instrument warms up.
  2. Select the TCS-User account, password: True!Confocal55

1. Turn on Scanner Power – wait 15 seconds.
2. Turn on Laser Power – wait 15 seconds.
3. Turn the key clockwise into the vertical position.
4. Select FLUO on the touchpad Illumination Control Menu (see next page).
5. Ensure that the microscope stage is set to its lowest position and that the lower stage height limit is set (see next page).



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**Objective**

**Lens**

**Paper**

**Stage**

**Immersion Oil**

***Convallaria***

**Slide**

**ddH20**

**Touchpad**

Microscope



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Stage Control Menu

Illumination Control Menu

1. Launch LAS AF.
2. Select **Ok** on Leica Application Suite.
3. Select ***No*** when asked to initialize the DM6000 Stage.

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**Laser Lines**

**Argon**

**Laser**

**Acquisition**

**Settings**

**Detectors**

Main LAS AF Page

1. Select the Configuration tab.
2. Select the Laser icon.
3. Activate your lasers of interest (reference the table of common fluorescent labels below).
   1. If the Argon laser is selected, drag the laser power to 50%.

**11**

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| --- | --- |
| **Laser** | **Common Labels** |
| 405 Diode | DAPI, Hoescht |
| 458 (Argon) | CFP |
| 476 (Argon) | May work for GFP |
| 488 (Argon) | Alexa 488, FITC, GFP |
| 496 (Argon) | May work for YFP, and a little for GFP |
| 514 (Argon) | YFP |
| HeNe 543 | Alexa 543, Alexa 568, Cy3, Rhodamine, TRITC |
| HeNe 594 | Alexa 594, Texas Red |
| HeNe 633 | Alexa 633, Cy5 |

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1. Select the Acquire tab.
2. Select Visible.
3. Increase the power of your laser line to 3%.
4. A picture containing indoor

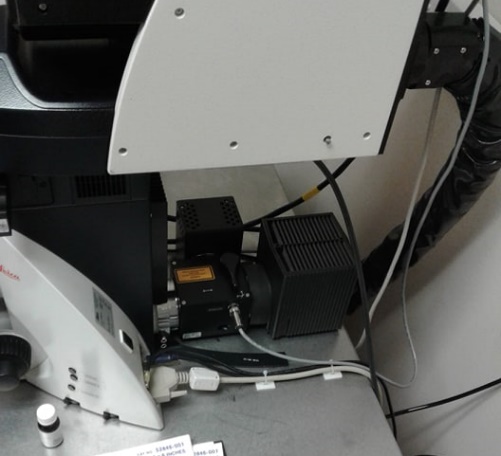
   Description automatically generatedGently collapse the detection bands of unused detectors and place them to the side. Double-click the detection band of your detector to bring up a Spectral Range box.

**19**

1. Enter an appropriate Min value that is at least 10 nm to the right of a laser line and a Max value that captures most of your spectral range. Select **Ok**.
2. Secure your slide onto the stage using the stage clips.
3. Select BF from the Illumination control menu. Ensure that the TL button is selected on the touchpad and that the TL shutter switch is turned towards the front of the microscope.
4. ***Cautiously*** raise the stage until your sample comes into focus with the eyepieces under the 2.5x objective. Use the Z-Axis focus knob on the Microscope Controller under Fine mode for more precise movement. Do not allow the stage (parallel orange lines) to be raised so high that it is at risk of crashing into the objective. Frequently check to make sure that the objective is not touching the specimen.
5. When your sample is in focus, select Focus Step Set/Clear
6. Select Set. You have now set both Z-axis height limits for the touchpad.
7. Select the 10x objective and add a few drops of ddH20 to your slide. Select 10x again. Carefully tweak the focus and set this adjusted upper height limit on the Stage Control Menu.



Microscope Controller



**21**

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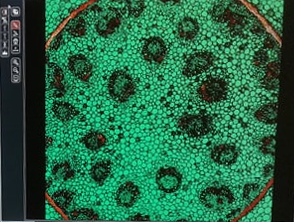
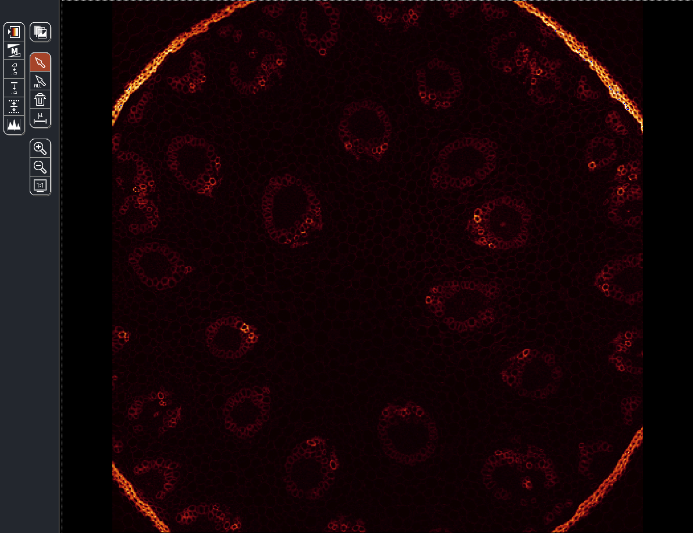
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1. Select Live to begin scanning your sample.
2. Increase Smart Gain to 600 V (500-700 V is optimal).
3. Increase the laser power until the sample is visible on the viewing window.
4. Switch to the Lookup table option (LUT) termed Glow, where you will see red and orange pixels. Adjust the laser power until just a few blue, overexposed pixels are present.
5. A close up of a computer

   Description automatically generatedTurn the Detector Offset knob to a slightly negative value to reduce background noise.

**29**

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**Smart**

**Gain**

**Detector**

**Offset**

**Rotate**

**Pinhole**

**Size**

**Zoom**

**Z-focus**

**28**

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1. A close up of a computer

   Description automatically generated(Optional) Select the default LUT then stop Live scanning. Turn on the PMT Trans detector. After selecting Live, click on the new window and increase Smart Gain until the brightfield image is sufficiently illuminated. Add an overlay/combined image.

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1. Before capturing an image, improve acquisition settings (e.g. by increasing Image Format to 2048 x 2048 pixels, reducing Scanning Speed to 100 Hz, and increasing Line Average to 3).
2. A screenshot of a cell phone

   Description automatically generatedSelect Start to begin image acquisition.
3. A data file with a generic name is added to the active experiment. Both objects should be renamed with meaningful descriptions then saved. Experiments, often renamed with a date, are stored under a personal directory that you add to the desktop file, Users. Data files should be renamed, saved, and exported as a useful file type.

**33**

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**35**

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1. Export your file by right-clicking it, selecting export, and then selecting the desired file type (.tif).

**36**

1. Select the folder that you created on Datadrive (D:) for the destination folder.
2. Ensure that Micron scale is selected. Select OK. If you captured a brightfield image to produce an overlay, repeat step 34. This time select Overlay channels. Select OK.
3. You may transfer data files onto your flashdrive via the system unit on the floor. FMIC staff routinely remove files left on computers longer than 3 months in order to maintain computer performance.
4. To begin shutting down the program, save all files under the Experiments tab or delete them. Ensure that Live is not selected. Deselect detectors, reduce laser lines to 0, deselect Visible or UV, and turn off lasers under the Configuration tab.
5. Bring the stage down to the lowest height using the touchpad. Remove your sample, switch the set objective to 2.5x, and clean objectives with lens paper.
6. A picture containing indoor

   Description automatically generatedExit out of the LAS AF software.
7. Start > Shutdown

**42**

1. Turn key counterclockwise to Off-0. Wait 15 seconds.

**44**

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1. Turn off Scanner Power. Wait 15 seconds.
2. Turn off PC Microscope. Wait 15 seconds.
3. Turn off the fluorescent lamp rocker switch.
4. Wait *10 minutes* for the system to cool down before turning off Laser Power. In the meantime, ensure that the work environment is clean to be courteous to the next user.

**46**