BIOLOGICAL IMAGING DEVELOPMENT COLAB

UCSF University of California San Francisco

Pod D: Leica Sp5

Laser Scanning Confocal Microscope with White-Light Laser

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July 2022

Contents

1 Introduction

The Leica Sp5 is an inverted laser-scanning confocal microscope with a 405 nm laser diode, a white-light laser (WLL), and four PMT (photo-multiplier tube) detectors. The system is enclosed with an incubation unit to regulate experimental temperature and air gas (5% CO2) for live cell imaging.

The objective turret houses five lenses including 10x Air, 20x Air, 40x Oil, 63x Water, 63x Oil, and 100x Oil immersion. The stage is motorized for large tiled stitching or marking and storing positions.

All new users need to be trained by a BIDC staff member before independent use.

Please sign up for time using the **iLab** microscope scheduler.

The available objectives are:

Mag	**Immersion**	**Numerical Aperture**	**Working Distance**	**ID Number**
10x	Air	0.40	2.20 mm	506285
20x	Air	0.70	0.76 mm	506513
40x	Oil	0.75	0.10 mm	506179
63x	Water	1.2	0.22 mm	506279
63x	Oil	1.4	0.10 mm	506188
100x	Oil	1.44	0.10 mm	506325

2 Hardware Start-Up

1. Turn on the three green power switches from left to right. See Figure: 1.
   (a) The first powers the **PC and the Microscope**.
   (b) The second powers the **Scanner**.
   (c) The third powers the **Laser**.
   (d) Once the three power switches are On, turn **On** the **Laser Emission** with the **Key**.

Figure 1: The power supply switches located at the right side of the computer desk.

2. Turn on the fluorescent lamp source See Figure: 2.

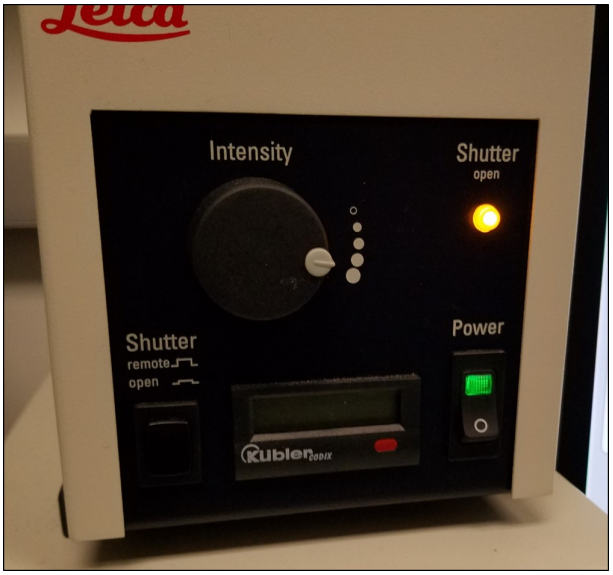

Figure 2: Fluorescent Lamp located on the computer desk.

3. **Note:** The Leica CTR6500 Control Box, located under the fluorescent lamp source, should always remain powered On, do NOT turn off.

3 Starting the Software

1. Check the stage and objectives for stray (not cleaned up) oil. Contact the BIDC if you find oil on non-oil objectives, or if you notice excessive oil. There should never be oil on the stage, or on non-oil objectives. Oil on non-oil objectives will permanently damage objectives. Improper use of immersion oil will not be tolerated.

2. Before starting the software, make sure the arm of the microscope (where the brightfield illumination source and transmitted detector are located) is down in its resting position over the stage. Additionally, no sample should be in the sample holder. See Figure:3

3. Start the **LAS AF** software

4. The software will pause, continue by clicking the **Ok** button.

5. A window will appear asking to initialize the stage. You may initialize the stage if you wish to do tiled images. If you select Yes, be sure that your sample is not on the stage, and that the objective turret is all the way down (Press and hold Z Down arrow button on the right-hand side of the microscope body). Damage to the objective or your sample may result if these precautions are not followed.

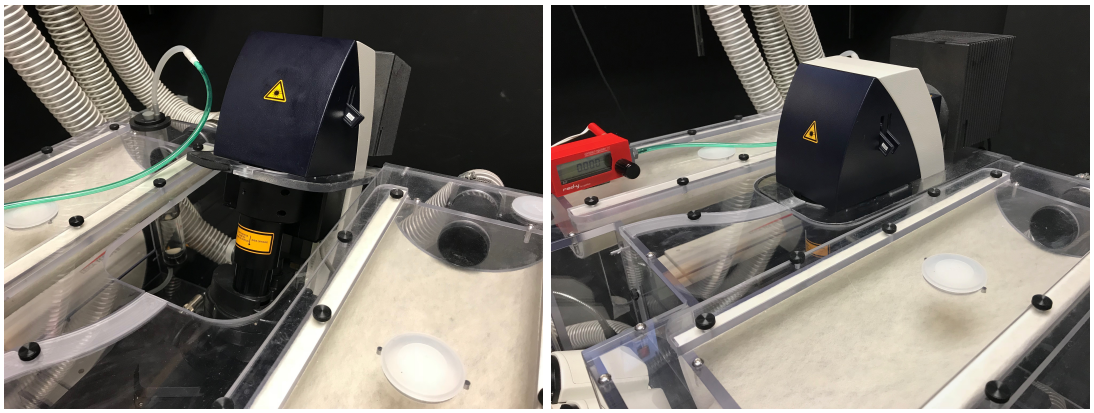

Figure 3: The image to the left shows the arm of the microscope lifted. The image on the right shows the arm of the microscope down in its resting position.

4 Finding the Sample by Eye

1. Choose the objective you want to image with using the side panel or the **Objective** text in the **Acquire** tab.
2. Add water or oil if using that immersion objective.
3. Place your sample in the appropriate sample holder and set on the stage.
4. To focus on fluorescence, use the panel on the lower front of the microscope. See Figure: 4. Select the desired **filter** from the list below.

   I3 = GFP

   N2.1 = RFP

   A = DAPI

   CFP/YFP = CFP/YFP
5. Press the **shutter** button on the same panel to open the shutter.

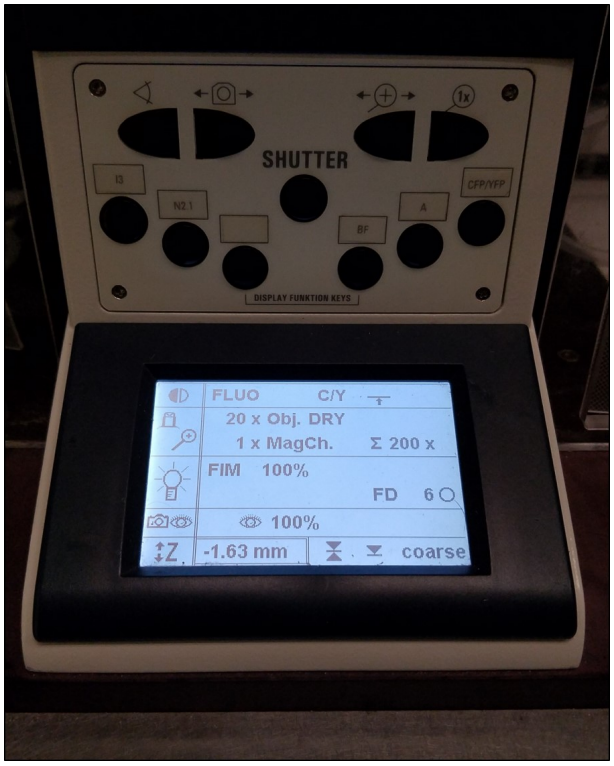

Figure 4: Panel at the front of the microscope for controlling filters for the eyes.

Note: if you notice that the light coming out of the objective appears dim and you are having a hard time finding your sample through the eyes, press the shutter button on the fluorescent light source to disengage the shutter and then once again to re-engage it. See Figure: 2. You should now notice the light coming from the objective to be much brighter.

6. To focus on brightield see Figure: 5:
   (a) Press the **TL/IL** button on the lower left-side of the microscope body to turn on the light source
   (b) Adjust the brightness using the **INT** button.

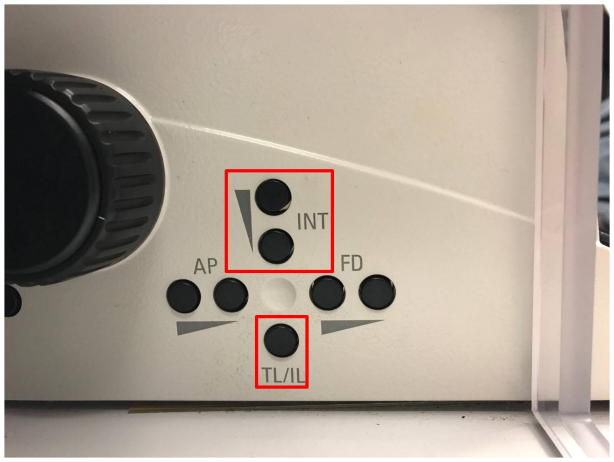

Figure 5: Brightfield controls located on left side of microscope.

7. Once your sample is in focus set up your acquisition in the software.

5 Turning on the Lasers

1. Go into **Configuration** tab, select **Laser icon**.
2. Turn on desired lasers by clicking checkboxes. The WLL should automatically turn on at 70%. See Figure: 6

Figure 6: Laser settings under the configuration tab.

3. Click **Settings** icon, and adjust bit depth resolution if necessary (default is 8 bit, other options are 12 and 16 bit) If you are quantifying your image intensities, it is recommended to use 16 bit. See Figure: 7

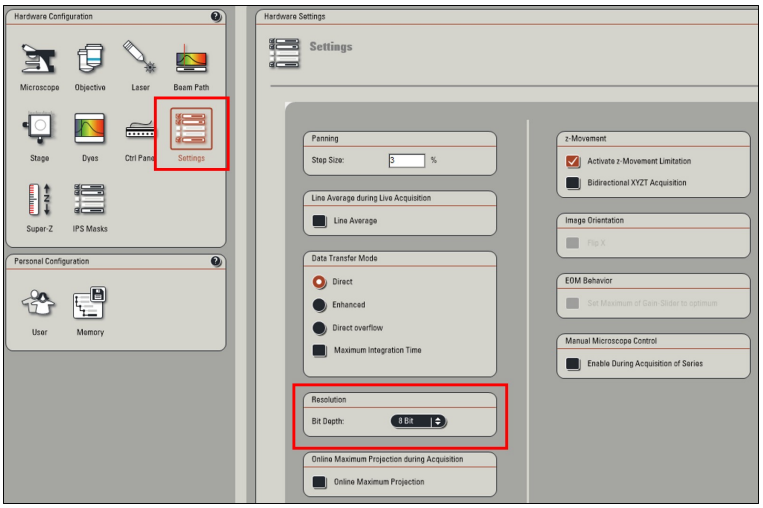

Figure 7: Bit depth settings.

4. It is **not** recommended to change any other settings.

6 Acquiring an Image

This microscope is configured with a WLL and AOBS tunable emission filter. These features enable a high degree of control in excitation and emission parameters. Additionally, a user can control whether fluorophores are imaged simultaneously, sequentially, or in combination.

6.1 Simultaneous Image

This microscope can image up to 4 fluorophores (plus Scan BF) simultaneously.

1. Add the laser lines you wish to use.
   (a) The 405 nm laser line.
      i. Click on the **UV** button to open the shutter. See Figure: 8
      ii. Increase the laser power using the slider. **Note:** 10% is a good starting point.

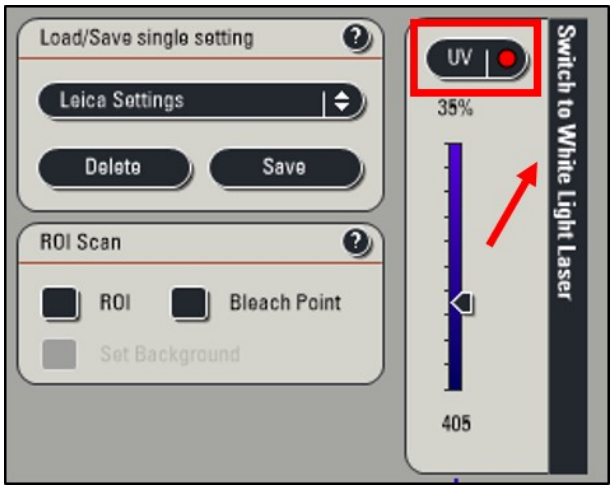

Figure 8: Control of the 405 nm laser.

2. Add lines from the WLL.
   (a) Click on the **Switch to White Light Laser** panel. See Figure: 8
   (b) Click on the **WLL Shutter** button to open shutter. See Figure: 9
   (c) Add or Remove laser lines by clicking the numbered squares at the top.
   (d) Check the box to engage the laser line.
   (e) Slide or double-click and type the correct wavelength for each line.
   (f) Increase the laser power for each line to a starting percentage. The laser lines should now appear on the spectrum below.

Figure 9: Control of the WLL laser.

3. For each laser line engage a PMT and set the bandpass with the sliders. See Figure: 10
   Note: The minimum parameter for the PMT should be set at least 5 nm above the excitation wavelength.
   Note: A reference spectrum can be loaded to help with choosing the channel bandpass.

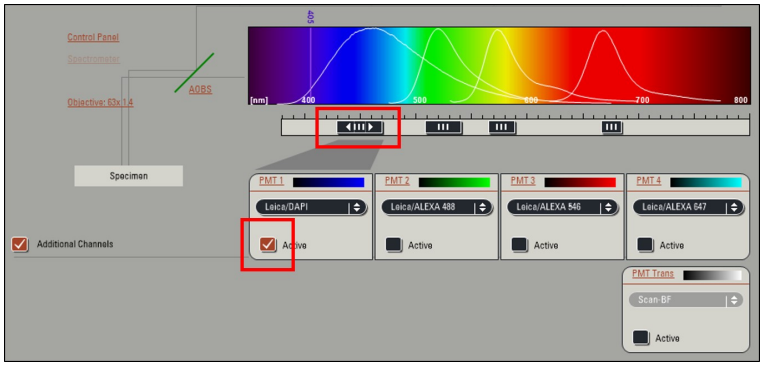

Figure 10: Engage PMTs and set the bandpass.

4. Click **Live** to view your sample on the computer and adjust the **Smart Gain** between 700-1250V , although the lowest gain possible is recommended to reduce noise. See Figure: 11

5. After setting the gain, adjust the **Z Position** (focus) using the dial on the smart panel, Not on the microscope. See Figure: 11

Figure 11: The image above shows the smart board where the Smart Gain and Z- Position knobs are located.

6. Select Quick LUT (look-up table) on the upper left-hand side to cycle through display options. See Figure: 12

7. Using the over/under saturation **LUT** adjust the laser power and PMT gain for each channel until you have a bright signal but no pixels are blue. **Note:** Under saturated pixels appear green and can indicate the PMT has no gain set.

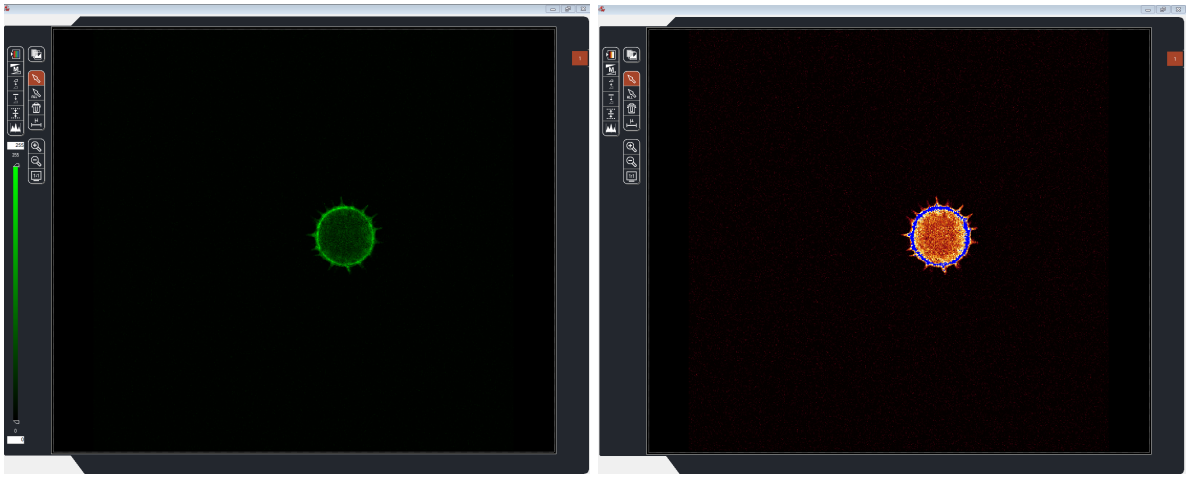

Figure 12: Left: Pseudo-color look up LUT. Right: Over/under saturation LUT with over saturated pixels in blue.

8. Press **Capture** or **Start** to take a single image.

Note: Under Beam Path Settings, and the Load/Save single setting window, you may use the drop-down menu to find preset settings for common fluorophores, such as DAPI, GFP, or TRITC. See Figure: 13

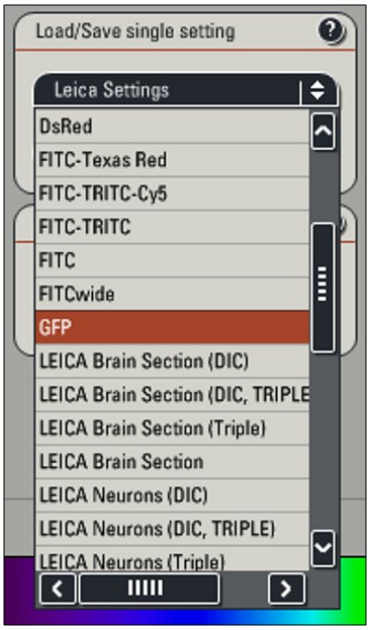

Figure 13: Selecting the GFP preset setting from the Load/Save Single Setting window.

6.2 Sequential Image

To image more than 4 channels (plus Scan BF) and/or to reduce channel bleedthrough, a user can choose to image with multiple sequences.

1. Press the **Seq** button in the **Acquisition Mode** tab in the upper left of the screen. See Figure:14

Figure 14: This tab defines the overall acquisition parameters for the experiment.

2. This will open the **Sequential Scan** tab. See Figure: 15

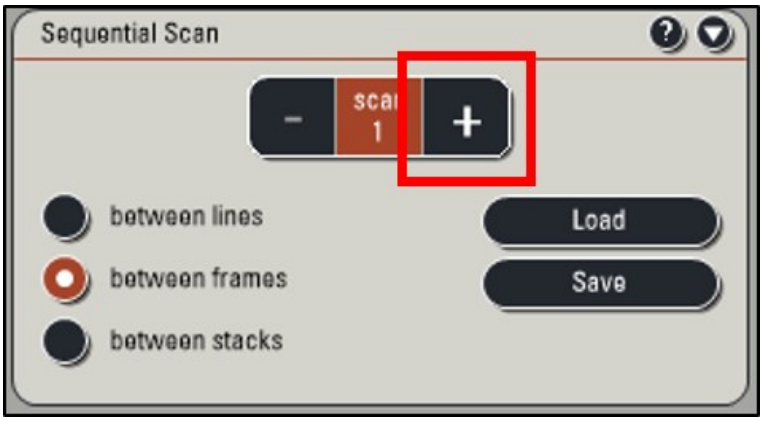

Figure 15: Add sequences, save/load sequence files, control when the sequences change.

3. Add all the laser lines you will use, but do not add any power.
4. Press the '+' button in the **Sequential Scan** tab to add as many sequences as you want.
5. For each sequence increase the laser power for the laser lines you want to use within that sequence and engage the PMT and bandpass you want to use. **Note:** It is best practice to assign sequence from Red to Blue (DAPI last). This method means the highest energy photons interact with the sample last.
6. Select either **between lines**, **between frames**, or **between stacks**. This is when the sequences are changed during the experiment.
7. Once your settings are satisfactory, you may save this personalized sequence for future use using the Save/load buttons within the **Sequential Scan** tab.

6.3 XY Panel

Settings as they pertain to the image in XY.

1. Choose your pixel resolution. The default settings will be 512 x 512 but can be changed to a higher resolution such as 1024 x 1024 for imaging.
2. Select your scan speed. The default scan speed setting is 400Hz. Decreasing the speed will decrease the noise. Increasing the speed will increase the noise, and may result in a smaller field of view of the scan head cannot scan the area in the time requested.
3. Set your line averaging or integration. This can be done on a by sequence basis.

6.4 Z-Stack Panel

Volume settings. **Note:** To Image a Z stack, **Acquisition Mode** must be in xyz. See Figure: 14

1. In Z-Stack panel, set the beginning and end of your z-stack using the Z Position control knob on the smart panel, not the microscope, while in Live mode.
2. Once the limits are set, turn off Live mode to set the number of steps within the selected volume, or the z-step size to determine the number of steps.
3. There is also a **System optimized** option to use **Nyquist** sampling methods.

7 Saving Acquired Images

1. Click the **Experiments** tab to view all images taken.
2. Delete or Rename images as needed.
3. Click **Save All** to save images as a .lif file in your hard drive.
4. Please remove old files from the Sp5 computer once they have been saved to your hard drive.

8 Shut Down

1. After saving, close the LAS AF software.
2. Shut down the computer using Shut Down option in the Start Menu.
3. Remove samples and clean objectives as needed.
4. Shut off Leica shutter box.
5. Turn emission key for laser to off.
6. Turn off the 3 power switches.
7. Confirm the space is clean and ready for the next user.

Contact the BIDC

The BIDC office is located in Medical Sciences Building Room S1109.
The BIDC office phone number is 415-476-4550.
If you need immediate assistance and no one is available in the office, or it is after business hours, please call the **BIDC Hotline** at 415-745-2432.