BIOLOGICAL IMAGING DEVELOPMENT COLAB

UCSF University of California San Francisco

Pod B: Leica Sp8

Laser Scanning Confocal Microscope with White-Light Laser

Kyle Marchuk Austin Edwards Mohammad Naser Harrison Wismer

July 2022

Contents

1 Introduction

The Leica SP8 is an inverted laser scanning confocal system configured with an AOBS tunable detection pathway (2 PMTs and 2 HyD detectors able to detect from 380nm-800nm), and Argon laser, and a white light laser (470nm-670nm. The HyD detectors offer a "true black" background and are able to gate on fluorescence lifetime (allowing for the in-line reduction auto-fluorescence).

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

The objectives available for use on the SP8 are:

Objective	**Mag**	**Numerical Aperture**	**Immersion**	**Working Distance**	**ID Number**
HC PL APO CS	10x	0.40	Air	2.20 mm	506285
HC PL APO CS2	20x	0.75	Multi	0.66 mm	506343
Fluotar VISIR	25x	0.95	Water	2.40 mm	506375
HC PL FLUOTAR L	40x	0.60	Air	3.3-1.9 mm	506291
HC PL APO CS2	63x	1.40	Oil	0.14 mm	506350
HC PL APO	100x	1.44	Oil	0.10 mm	506325

The system also contains spectral scanning, three-dimensional (z) acquisition and time-lapse capabilities. A programmable stage allows automated return-to-site and multiple-field stitching.

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

2 Start-Up

1. Turn on the three green switches on the right side of table, turn emission key to on position. 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 the **Laser Interlock Key**.

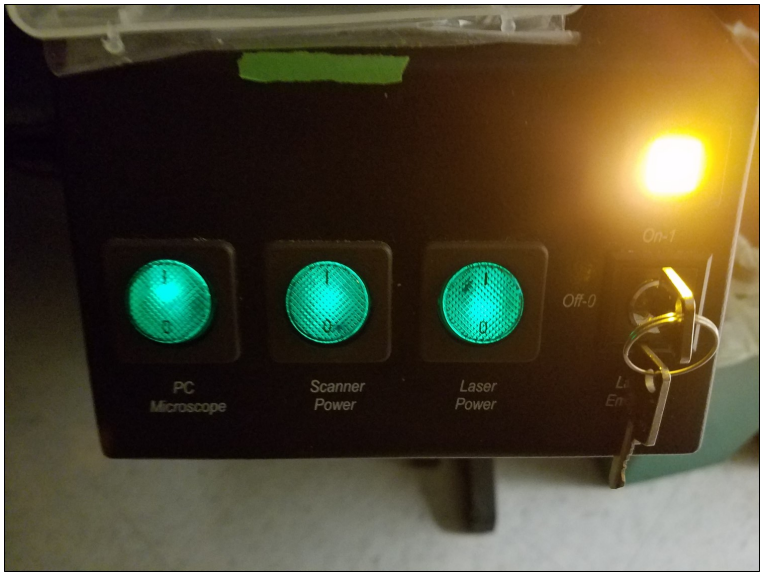

Figure 1: The power supply switches located at the right side of the computer desk.

2. Turn on lamp box (top box in the middle of the table) This lamp is an LED that can be switched on and off without cool down. See Figure: 2.

Figure 2: Fluorescent Lamp located on the computer desk.

3. Select **TCS-User** from the login screen.
4. Launch the **LAS X** software. See Figure: 3.

Figure 3

5. Select **OK** from the Configuration prompt. See Figure: 4.

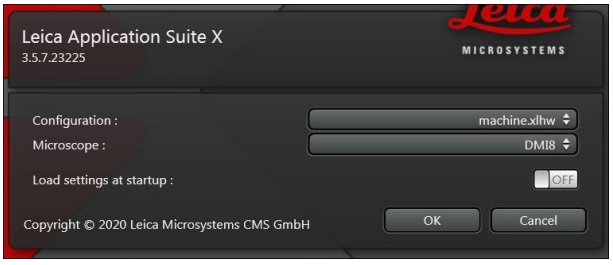

Figure 4

6. Ensure nothing is on the stage before accepting the initialization request.

7. Add the proper immersion liquid (water or oil) to the objective you will use to find the sample by eye.
8. Mount your sample to a stage insert and place it on the stage. Screwing the insert down is not necessary to have a stable image. **Please note:** you are adding your sample to the z-piezo so please be gentle.

3 Finding the Sample via the Eyepiece

1. Move the stage in the xy-directions until your sample is under the objective.
2. Carefully raise the objective to your sample until it is touching the immersion solution.
3. Choose the color wheel option on the **Front Panel** (second icon on the left). See Figure: 3.
4. Click **FLUO** tab (top middle).
5. Choose the filter you would like to use: DAPI, GFP, NXR. **Note:** use the brightest most ubiquitous fluorophore to find your sample.
6. Click the **IL-Shutter** button to open and close the shutter.

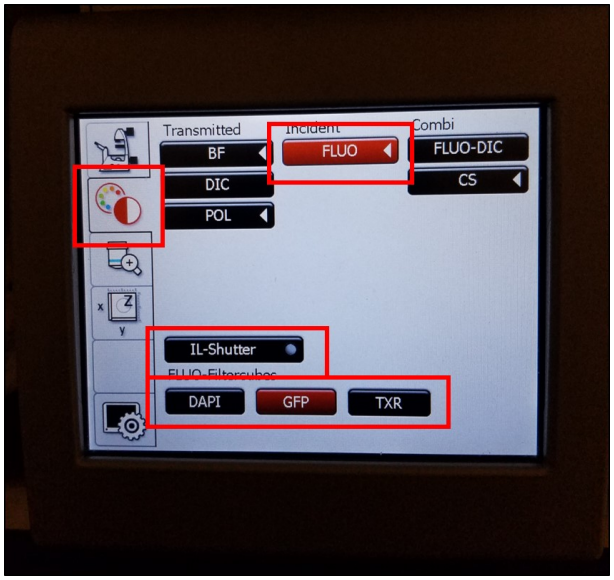

Figure 5: Located on the front of the microscope.

7. Using the fine adjust only, bring your sample into focus.
8. Once the sample can be seen through the eyes, close the shutter and go to the **LAS X** software for imaging setup.

4 Initial Imaging Setup

If there are fewer than 5 colors being imaged within the sample, the **Dye Assistant** can be used to quickly set up an imaging sequence. If the sample has more than 5 colors, the sequential scans will have to be set up manually.

4.1 Dye Assistant

1. Click on the **Dye Assistant** icon located in the middle of the page. See Figure: 6

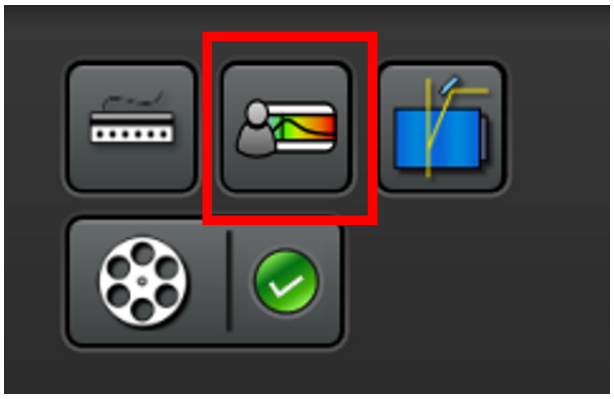

Figure 6

2. In the **Dye Assistant** window, click the **'...'** button in order to select the fluorescent markers that are present in the sample. See Figure: 7

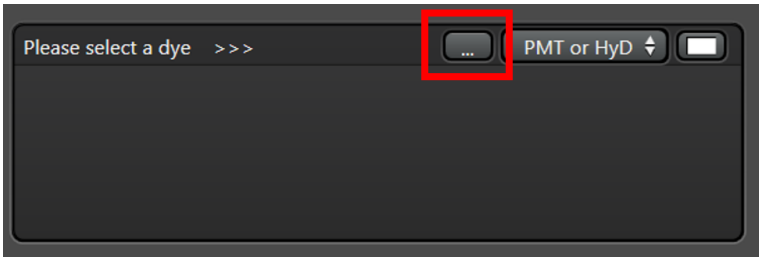

Figure 7

3. Choose which detector you want to pair up with the fluorescent marker in the **'PMT or HyD'** tab. **Note:** the HyD detectors are meant for detecting markers that are dimmer or are more sensitive to higher laser powers. If the marker is too bright and a HyD detector is used it can damage the detector. For this reason, the detector has a safety shut off and can only be turned on when the fluorophore intensity is low enough. This can be achieved by lowering the gain and laser power.

4. After choosing the fluorescent markers and the detectors, the **Dye Assistant** will provide a list of imaging sequence options. The top option on the list will generally be the fastest sequence but will have the most amount of bleed through. Whereas, the last option on the list will be the slowest option but will have the least amount of bleed through. Users have the ability to edit their sequence choice (laser and detector settings) within the **Dye Assistant** or after the settings have been applied.

   None Sequential: All channels will be acquired simultaneously. Fastest option, but it limited to 5 channels. Will likely have the most **Crosstalk** of the suggested settings.

   Line Sequential: Switches **Sequences** each line of the image scanned. For there to be any speed in this setting, the collection **Bandpass** will be set at the same width.

   Frame Sequential: Switches Sequences at the end of each image plane. No limitation on the width of the **Bandpass**. Switching sequences does take time.

   Stack Sequential: Switches **Sequences** at the end of each **Stack**. Considerable faster than **Frame Sequential** and no limitation on **Bandpass**.

5. Once a sequence in the dye assist has been selected, the program will ask you if you want to turn on the lasers needed for imaging. Select **Yes**.

6. Ensure the microscope arm is down prior to going **Live**.

4.2 Manually Creating Sequences

1. Click on the **SEQ** button found in the Acquisition Mode tab. A sequential Scan window will appear.

2. Launch the laser power menu by pressing the laser **Menu** button. See **Red** box Figure: 8

3. Turn the **Power State** to **ON** for the lasers you will use.

4. Add laser lines by pressing the '+' button. See **Green** box Figure: 8

5. To add the 405 nm laser press the **405** button. See **Blue** box Figure: 8

Figure 8: Laser lines and power control panel.

6. In the Sequence menu press the '+' to add the next sequence to your acquisition. See Figure: 9

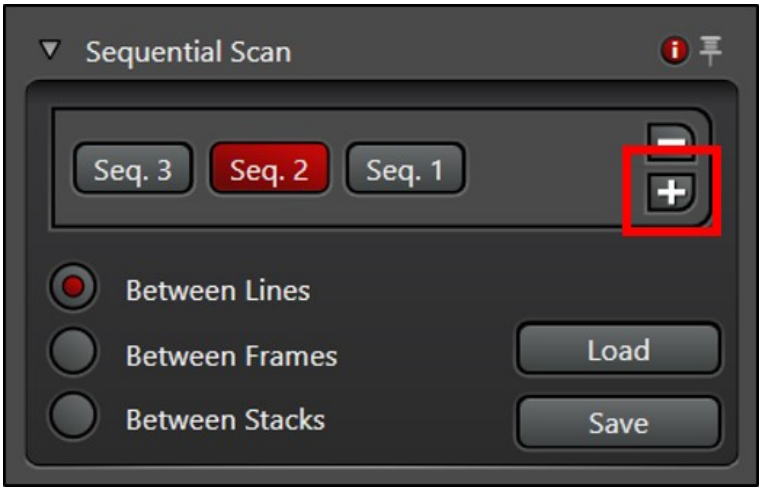

Figure 9: Add and remove sequences. Control when sequences are changed during acquisition.

7. Increase the laser power for the lasers you will use in **Seq 1** by moving the slider up.

8. Turn on the PMTs you will use for each laser line (**Blue** box) and adjust the bandpass window (**Red** box). See Figure: 10.
Note: the minimum setting for the PMT bandpass window should be set at least 5 nm above the excitation wavelength.

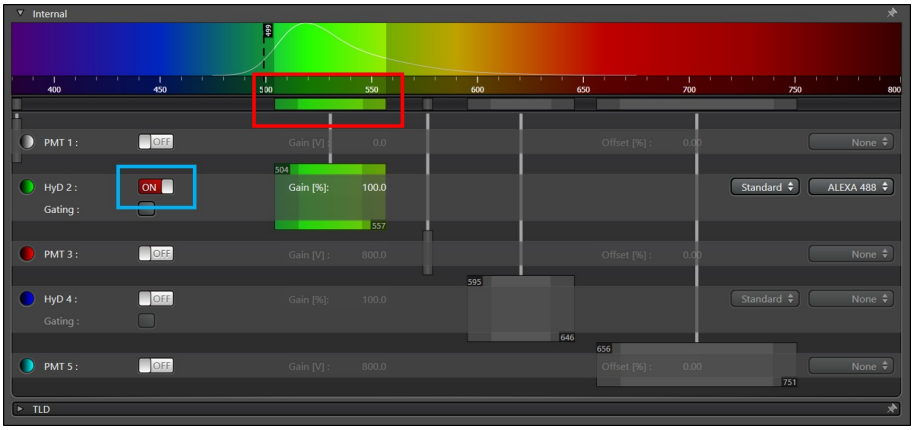

Figure 10: Turn on and off PMTs and adjust the AOBS bandwidth.

9. Repeat for as many sequences as needed.

4.3 Optimizing the Fluorescence Signals

1. Click **Live** in the lower left of the screen to start imaging the first of your sequences. The PMTs will be automatically set to 800V and the HyD detectors will be set to 100 percent.
2. Ensure the sample is in the imaging plane by adjusting the Z Position using the dial on the smart panel (not the microscope). You may need to adjust the **Look up Table (LUT) slider** to see signal.
3. Select the **Over/Under Saturation** display for the live images. See Figure: 11.

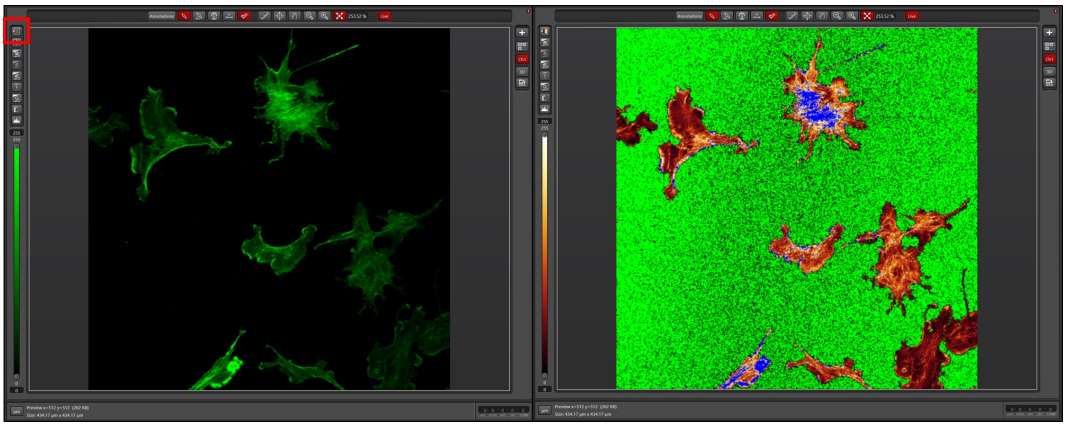

Figure 11: **LEFT:** Pseud-colored image. **RIGHT:** Over/under saturated pixels displayed.

4. Click on the displayed imaged to select that channel for gain adjustment.
5. Adjust the laser power and gain until you have a bright image just below saturation (blue pixels).
6. Click the **Capture Image** button to collect and save an image of that sequence.
7. Click the **Start** button to collect collect and save an image of all sequences.

5 Imaging Parameters

After setting up the laser and gain acquisition settings for the sequences, the user can set up the rest of sample acquisition parameters.

5.1 XY Tab

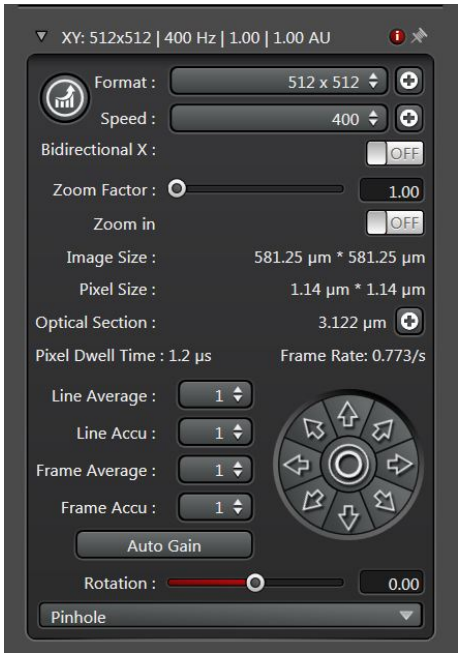

Figure 12: XY Tab

In general, used for setting up XY parameters pertaining to how images are captured.

Format: The size of the image in pixels. Suggested size is **1024x1024** for most applications.

Speed: Image acquisition speed. Suggested speed is **600 Hz**. Speed faster than 600 Hz will require increasing the Zoom of the image.

Bidirectional X: Data is collected on both movement directions of the X galvo. Some acquisition parameters are tied to this setting.

Zoom Factor: Digital Zoom of the image. Increasing the magnification of the objective will give a better image and optical section capability.

Zoom In: Press this button to draw an ROI on the image. The microscope will zoom on the ROI.

Image Size: Describes the size of the image in **microns**. This is dependent on the **Objective**, **Format**, and **Zoom Factor**.

Pixel Size: Describes the size of the pixel in **microns**.

Optical Section: Thickness of the image plane.

Pixel Dwell Time: Length of data collection for each pixel.

Frame Rate: Time required to collect a single sequence at a given image plane.

Line Average: Number of times each line of the image will be acquired and averaged to produce the image. Used to reduce noise.

Line Accu: Number of times each line is accumulated (summed) to produce and image. Can be used on very dim samples.

Frame Average: Number of times each frame of the image will be acquired and averaged to produce the image. Used to reduce noise.

Frame Accu: Number of times each frame is accumulated (summed) to produce and image. Can be used on very dim samples.

Auto Gain: The system will increase the gain to optimize the image with the current laser power.

Rotation: Rotation of the image.

Pinhole: Used to adjust the pinhole size. Increasing the pinhole produces a brighter signal at the expense of your optical section capability.

6 Imaging Modules

6.1 Z-Stack

Used for creating the volumes of a 3D stacked image.

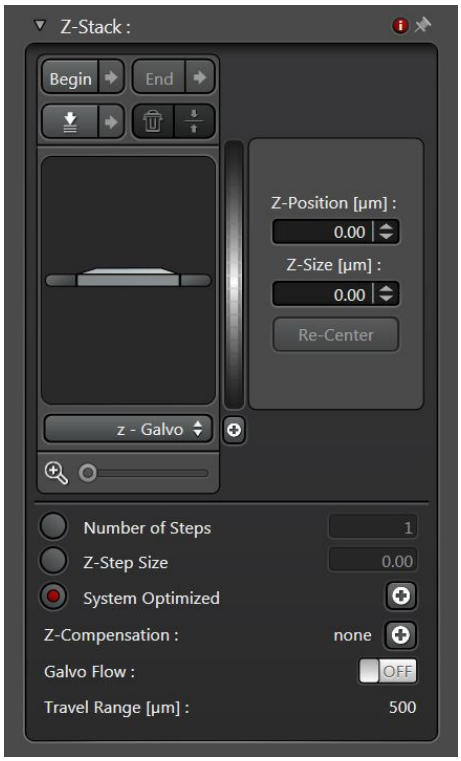

Figure 13: Z-Stack Tab

Begin: Using the **Z Position** knob, move the image plane to the 'Top' of your sample and press this button to assign this position.

End: Using the **Z Position** knob, move the image plane to the 'Bottom' of your sample and press this button to assign this position.

Trash Can Icon: Deletes your position assignments.

Stack Direction (Z): Scanning direction for the volume acquisition. Typically want to move the stage in the **Up** direction to scan from the Top of your sample to the Bottom.

z-Galvo: Uses the z-piezo actuator for movement. Fast, but limited to 500 microns of travel range.

z-Wide: Uses the z-stepper motor for movement. Slower, but has 13 mm of travel range.

Number of Steps: Within the given volume, determines z-step size by number of image planes.

Z-Step Size: Within the given volume, determines the number of steps by step size.

System Optimized: Determined by the objective; the smallest step size before over-sampling the volume.

Z-Compensation: Is used to associate laser power and gain with a z-position. Typically used to compensate for scattering in a thick sample.

1. Check which options you'd like to associate with z-position. **Excitation Gain** is laser intensity. **Emission Gain** is the PMT gain.
2. Navigate to an imaging plane within your volume and adjust the laser intensity and PMT gain to your satisfaction.
3. Move to other plane and repeat the process.
4. Delete any positions you do not need, or may have loaded when you started.
5. Select your next **Sequence** and repeat the process.

Galvo Flow: When **On**, the z-stage does not stop at each individual step. Instead it continuously moved from the **Begin** to **End** point. Useful when needing super fast acquisitions.

Travel Range: Travel range of the selected z stage.

6.2 Navigator

The **Navigator Module** is a large-area exploration tool that is capable of setting up multiple ROIs of different sizes for tiled-image acquisition. The navigator can be opened by clicking the grid icon in the top left corner of the main LAS X window.

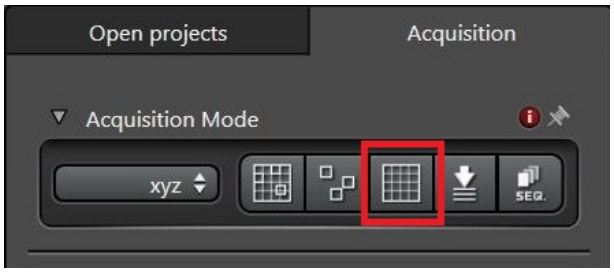

Figure 14: Navigator Icon

Once open, you should see a white box on a gray background. This box represents the current field of view of the stage. When opening the navigator for the first time, this box will be located at last field of view seen in the main software window.

Moving the Stage: Double-click on the viewing area and the stage will move and take a preview tile using the currently selected sequence.

Live: Press the **Live** button to continuously view the current stage position depicted by the white box.

Fast Live: Press the **Fast Live** button to continuously view that stage position in a lower resolution (faster than **Live**).

Spiral Press the **Spiral** button to engage the stage in an expanding pattern for exploration.

ROIs Using the ROI shape tools, draw **ROIs** on the stage area for exploration or experiment acquisition.

Figure 15: ROI & Focus Map tools located at the bottom-center of the screen

Preview Press **Preview** and the microscope will do a fast acquisition of the drawn ROIs.

Start Press **Start** to run the experiment from the **Navigator**. Any ROIs created for tiling will be listed in the tab in the bottom right of the screen. Shapes can be either deleted or temporarily turned off using this tab.

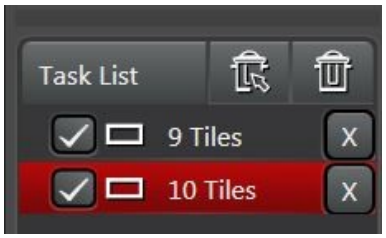

Figure 16: List of all created ROI shapes to be imaged.

Focus Map For uneven tissues, individual focus points can be manually placed and each associated with a unique Z position in order to correct for the uneveness of the sample.

1. Select either the blue focus point or green auto-focus point from the options next to the ROI tools. See Figure 15.
2. The **Focus Map** option will now be illuminated. Select **Focus Map**, and click on the first focus point listed.
3. The software will zoom in on a live view of the field of view surrounding the selected focus point. Adjust the Z position until the sample is in the desired focal plane.
4. Once satisfied, select **Set Z** to associate the focus point with the current Z position.
5. Hit **Next**. Then repeat steps 3-5 for each focus point placed.

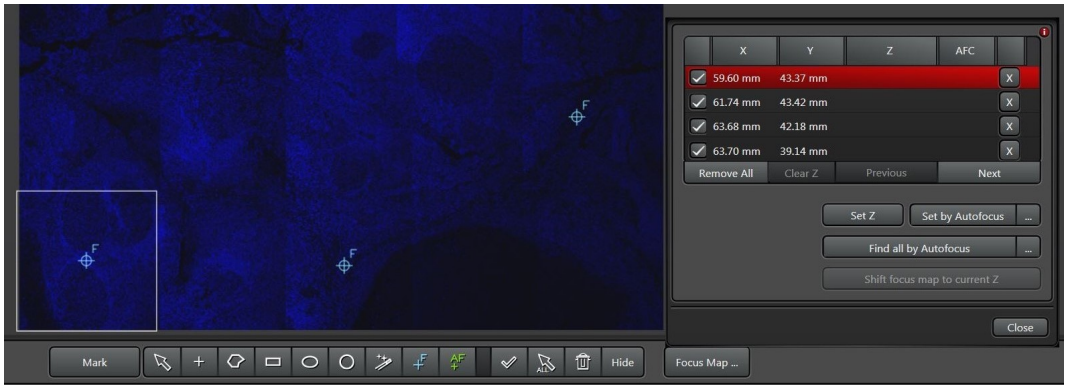

Figure 17: Focus Map Tab

6.3 Time Series

Used for setting up a **Time Series**, **Time Lapse**, or Movie for 'live' samples.

1. Under **Acquisition Mode** select **'xyzt'** from the drop down menu.

6.4 Tiling

The **Tiling Module** will create a 2D grid for acquisition based upon saved stage positions.

1. Locate an initial corner position (upper right, lower left, etc) for your grid either through the eyepiece or while **Live**.
2. Press the **Add Position** button to save that position.
3. Move the stage to another edge of the ROI and add that position.
4. A grid will appear in the **Stage** window. **Note:** move the Zoom slider if you do not see a grid appear.
5. To increase the grid size, add more points outside the current grid.
6. To decrease the grid size, add points within the current grid, and delete the unecessary point by selecting that point in the drop down menu and pressing the **Trash Can Icon**.
7. Turn **Merge Images**, **Auto Stitching**, and **Linear Blending** to **ON** to automatically create a stitched imaged at the end of acquisition.
8. To **Use Focus Map** a focus map will need to be made in the Navigator (See Below).

6.5 Mark and Find

The **Mark and Find Module** is a way to quickly save points and take a single-viewing-area image or volume.

1. Identify a position of interest while moving the stage in **Live** mode.
2. Add the position of interesting by pressing the **Add Position** button.
3. To remove an unwanted position select the position from the **Positions** drop down menu and press the **Trash Can Icon**.
4. Select **Same stack for all** if you want to use the same imaging plane or **Z-Stack** setup for each position.
5. To define separate volumes for each position:
   (a) Select your first position from the dropdown.
   (b) Define your volume in the **Z-Stack** panel.
   (c) Press **Redefine Stack**
   (d) Select your next position and repeat the process.

7 Spectral Unmixing Modules

The Leica SP8 equipped with AOBS detectors and a white light laser is an ideal microscope for imaging beyond 4 colors. Even with optimized settings, however, there is likely to be some bleedthrough between channels either from excitation or emission overlap. The software has multiple options for mathematically unmixing this overlap.

8 Other Imaging Tips

8.1 Project Naming and Saving

It is important to adopt a project naming and saving convention to avoid future headaches when trying to analyze the data.

1. Save your **Project** file with a name that identifies the experiment. We recommend this to be either a slide identifier, or an experiment identifier with a date.
2. **Rename** the images within the **Project** file with descriptions of the image type. We recommend making note of whether the images are z-stacks, tiles, timelapse, etc. This will make it easier to 'unpack' the images later.

8.2 USB Control Panel

The USB Control Panel (Figure ...) can be customized to include controls you may need. For example; the xy stage position can be controlled similarly to the z-piezo. To add additional features:

1. Press the USB Panel shortcut button (Figure...).
2. Select **Scan Field Rotation** and change it to **x Position (Stage)**.
3. Select **Pinhole** and change it to **y Position (Stage)**

8.3 Line Averaging While 'Live'

As mentioned previously, the **Live** button will show an image at your selected resolution without line average, frame averaging, etc that you may have selected. To change the **Live** option to reflect these acquisition parameters:

1. Select **Configuration** new to the **Acquisition** tab on the top of the screen.
2. Press the **Hardware** icon.
3. Check the **Line Average during Live Acquisition'** box.

9 Saving and Shut Down

1. Save images to your folder on the **Data** drive and remove data when finished.
2. Close **LAS X** software.
3. Shut down computer through the **Start** menu.
4. **Clean** objectives that were used.
5. Turn off **lamp box** (top box in the middle of the table).
6. Turn **key** to off then turn off **three green switches** on the right side of table.
7. Ensure the area is **clean and tidy** for the next user.
8. Inform the **BIDC** if any materials needs to be replaced or replenished.

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/emergency assistance and no one is available in the office, or it is after business hours, please call the **BIDC Hotline** at 415-745-2432.