BIOLOGICAL IMAGING DEVELOPMENT COLAB

UCSF University of California San Francisco

Pod A: Leica Stellaris 5

Laser Scanning Confocal Microscope

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Contents

1 Introduction

The Leica Stellaris 5 is an inverted laser scanning confocal with 4 laser lines (405 nm, 488nm, 561nm, and 638 nm). It features 3 next-generation Hybrid Detectors (HyDs).

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

Sign up for time using the **iLab** microscope scheduler.

The objectives available for use on the Stellaris 5 are:

Objective	Mag	Numerical Aperture	Immersion	Working Distance	ID Number
HC PL Fluotar	5x	0.15	Air	13.7 mm	506224
HC PL APO CS2	20x	0.75	Air	0.62 mm	506517
HC Fluotar VISIR	25x	0.95	Water	2.40 mm	506375
HC PL APO CS2	40x	1.30	Oil	0.24 mm	506358
HC PL APO	63x	1.40	Oil	0.14 mm	506350

2 Start-Up

1. Turn **On** the two power switches on the left side of the microscope control box. See Figure: 1.
   NOTE: The key should always be On and the microscope power box should always be On.

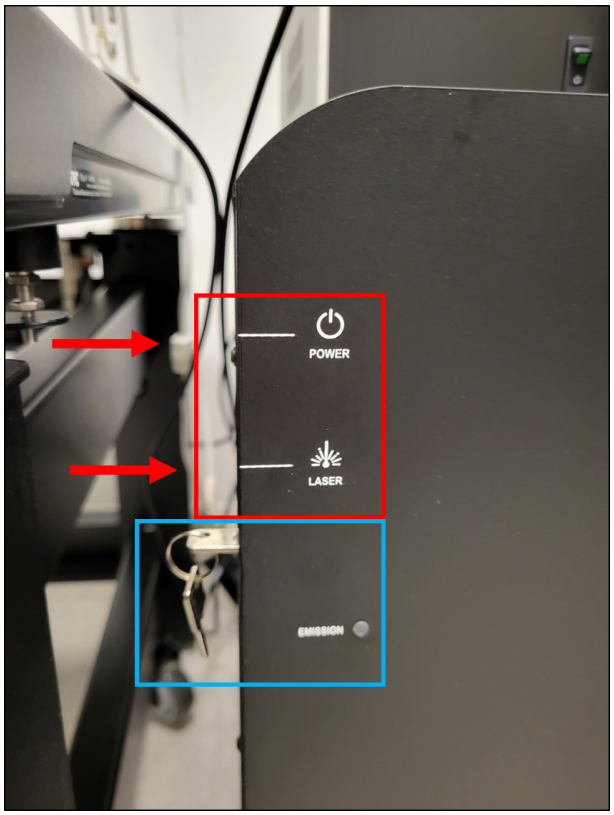

Figure 1: The power supply switches located to the left of the computer desk.

2. Turn **On** the computer tower.

3. Log into the **User** account.
4. Launch the **LAS X** software from the desktop.
5. Confirm that the **machine** configuration and the **DMI8** microscope are selected at the first prompt. See **Top** Figure: 2.
6. Ensure the stage area is clear of obstructions and **Initialize** the stage in the second prompt. See **Bottom** Figure: 2.

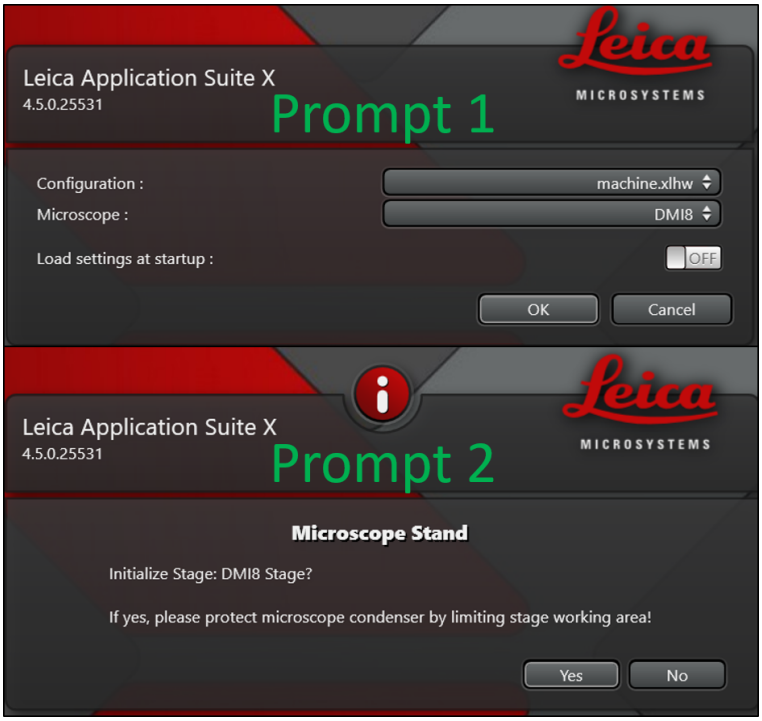

Figure 2: The two prompts to launch the software.

3 Focusing Your Sample

1. Select your objective in the software (See **Top** Figure: 3.) or on the front panel of the microscope (See **Bottom** Figure: 3.).

Figure 3: Options for selecting the objectives.

2. Add an immersion media to the objective if needed.
3. Place your sample into the appropriate sample holder.
4. Add the sample holder to the stage.
5. Use the **xy-stage controller** to align the sample above the objective.
6. On the front of the microscope navigate to the filter panel menu. See **Red** square Figure: 4.
7. Select **FLUO** for the **Incident** light. See **Blue** square Figure: 4.
8. Press the IL-Shutter to shine light on the sample. See **Green** square Figure: 4.
9. Slowly raise the objective using the **z-control** until your sample comes into focus.

Figure 4: Widefield illumination panel.

4 Initial Image Setup

4.1 Dye Assistant

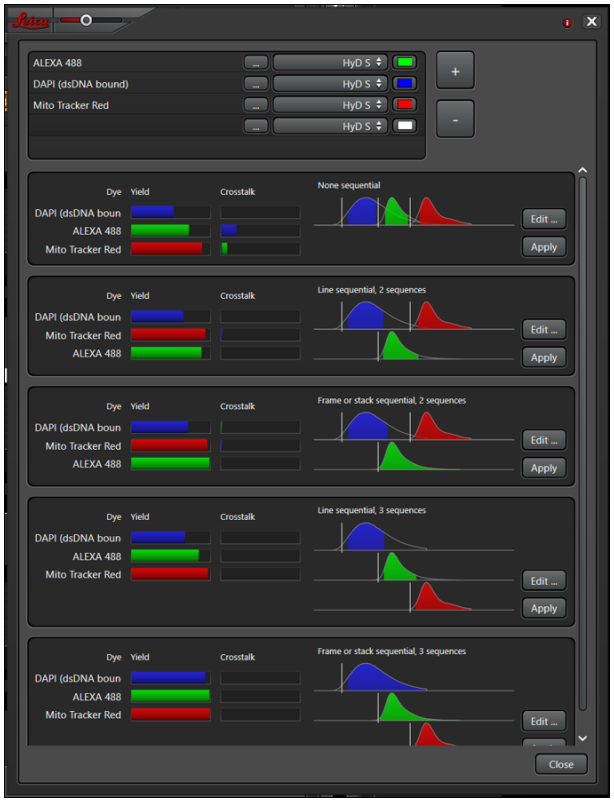

Figure 5: Dye Assistant Interface.

The **Dye Assistant** is a useful tool to quickly generate acquisition sequences for multiple fluorophores. The software can optimize for speed of acquisition or minimal fluorescence bleedthrough between channels.

1. Click on the **Dye Assistant Icon** near the top-middle of the main screen. See...
2. Press the **'...' Icon** and search for your fluorophore.
3. Continue until all fluorophores are added.
4. **Apply** one of the proposed sequences.

   None Sequential: All channels will be acquired simultaneously. This is the fastest option, but it is limited to 3 channels. This will likely have the most **Crosstalk** of the suggested settings.

   Line Sequential: Switches **Sequences** each line of the images scanned. To maximize speed, **Channels** that share a **Detector** need to have the same **Bandpass** width.

   Frame Sequential: Switches **Sequences** at the end of each image plane. No limitation on the width of the **Bandpass** window. Switching the hardware this way increases time.

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

5. Once a sequence in the **Dye Assistant** has been selected, the program will ask if you want to turn on the lasers needed for imaging. Select **Yes**.
6. Ensure the microscope arm is in place prior to going **Live**.

4.2 Manually Creating Sequences

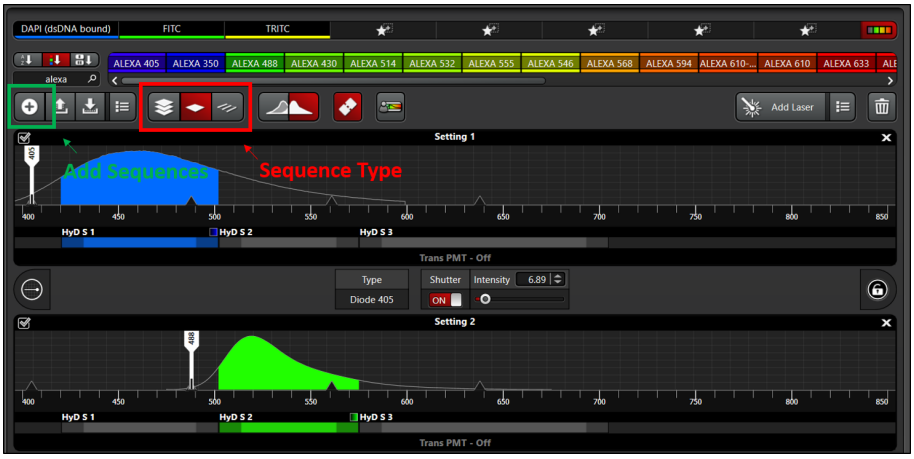

Figure 6: Sequence Interface.

1. Press the **'+' Icon** to add the number of **Sequences** you would like.
2. Search for a fluorophore in the **Search Bar**.
3. Drag the fluorophore **Icon** down to the **Sequence** you want to place it in.
4. Continue adding all your fluorophores to the **Sequences**.
5. Choose whether you want **Line Sequential**, **Frame Sequential**, or **Stack Sequential** by choosing the appropriate **Icon** (see above).
6. When you are happy with your **Sequence** you can Save it to your directory by pressing the **Down Arrow Icon**.

4.3 Optimizing the Fluorescence Signals

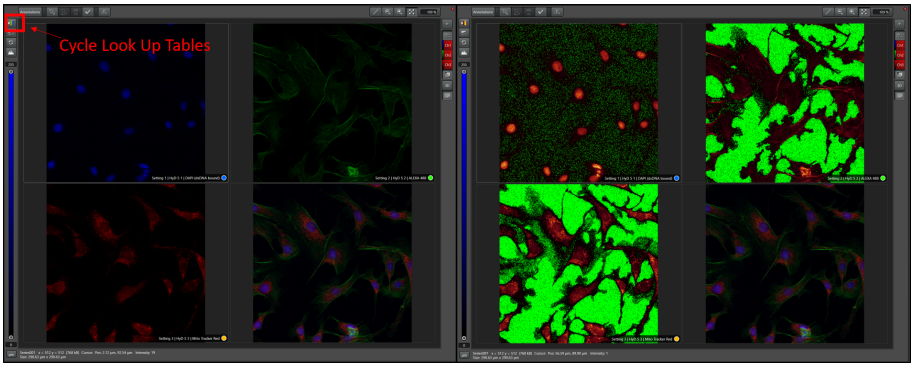

Figure 7: Tools to quickly optimize your image intensity.

1. Click **Live** or **Fast Live** in the lower-left of the screen to start imaging the selected **Sequence**.
2. Ensure the sample is in the imaging plane by adjusting the **Z-position** using the dial on the smart panel. 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.

4. Click on a displayed image 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 on the **Capture Image** button to collect and save an image of that **Sequence**.
7. Click on the **Start** button to 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

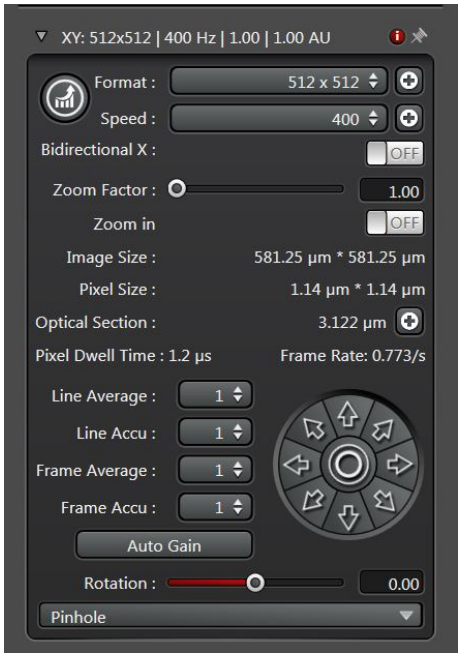

Figure 8: XY Tab

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

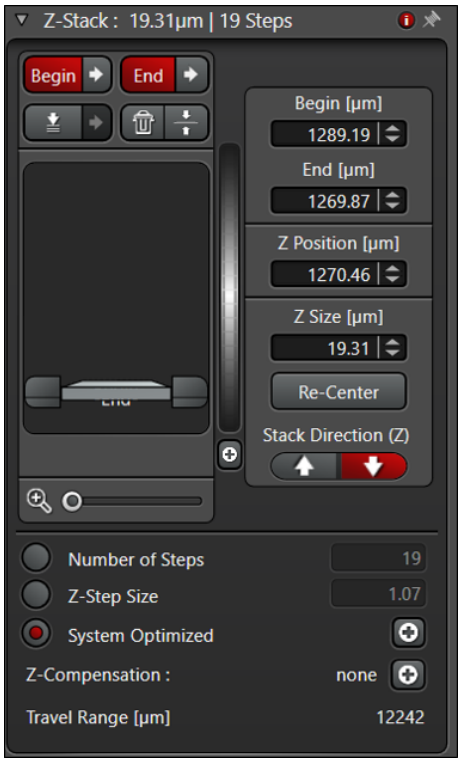

Figure 9: Available settings for the volume acquistion.

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

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

End: Using the **Z-Position** knob, move the image plan 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, you want to move the stage toward the objective to scan **into** your sample.

Number of Steps: Volume divided by **Z-Step Size**.

Z-Step Size: Volume divided by **Number of Steps**.

System Optimized: Determined by the objective; the smallest **Z-Step Size** before over-sampling the volume.

Z-Compensation: Is used to associate laser power and gain with z-position. Typically, it is 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 HyD gain.
2. Navigate to an imaging plan within your volume and adjust the laser intensity and HyD gain to your satisfaction.
3. Move to other planes 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.

Travel Range: Full distance the z-stage can travel.

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.

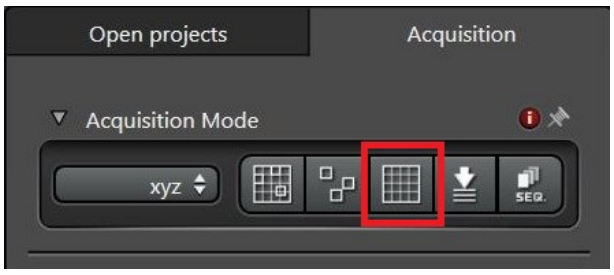

Figure 10: Navigator Icon

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.

Beam Path Tab On the Stellaris, individual settings can be temporarily turned on and off by checking and unchecking the box in the top left corner of each setting in the **Beam Path** tab. Having less settings active when spiraling will greatly increase the tile it takes to create a tiled preview.

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 11: 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.

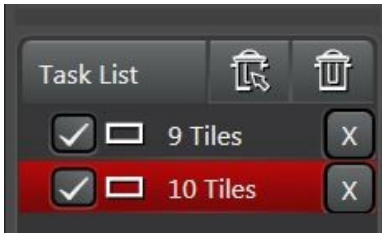

Figure 12: List of all created ROI shapes to be imaged.

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 11.
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.

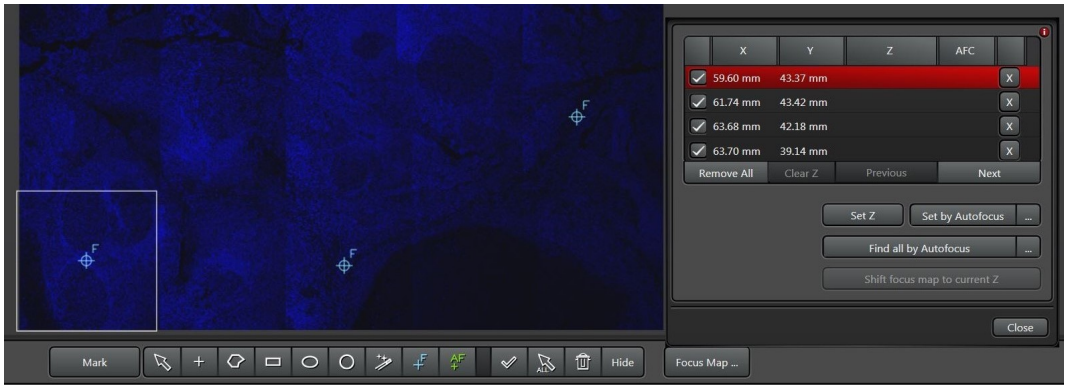

Figure 13: Focus Map Tab

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 to your hard drive.
4. Please remove old files from the Stellaris computer once they have been saved on your hard drive or Box.

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-stack, tiles, timelapse, etc. This will make it easier to 'unpack' the images later.

8.2 USB Control Panel

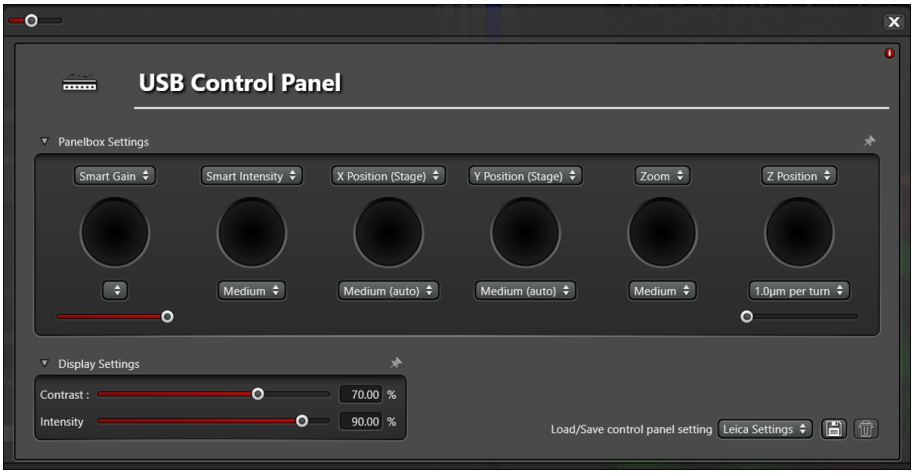

Figure 14: Can be customized for your workflow.

The **USB Control Panel** can be customized to include controls you may want to readily access. 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.
2. Select a dropdown and change it to **x Position (Stage)**.
3. Select another dropdown and change it to **y Position (Stage)**.

To change the speed at which the z-stage moves:

1. Located below the **Z-Position** dropdown (far right):
2. Select the speed you wish the stage to move per full turn of the wheel on the USB bar.

8.3 Fast Live Settings

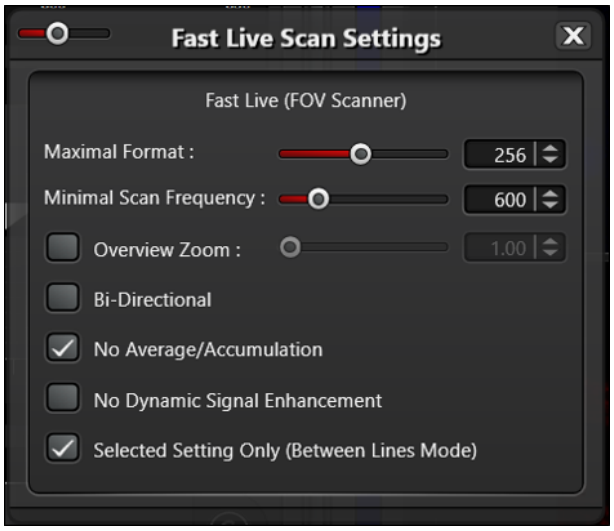

Figure 15: Interface for the Fast Live settings.

Pressing the **Fast Live** 'gear' icon allows you to change the XY imaging settings while in **Fast Live**.

The BIDC recommends 256x256 at 600 Hz with **Bi-Directional** scanning **On**.

9 Shut Down

1. After saving, close the **LAS X** software.
2. Shut down the computer using **Shut Down** option in the **Start Menu**.
3. Remove samples and clean objectives as needed.
4. Press the two **Power Switches** to turn off the entire system.
5. Replace dust cover and clean the area.

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.