BIOLOGICAL IMAGING DEVELOPMENT COLAB

UCSF University of California San Francisco

Gen 3

6-Channel 2-Photon Microscopy

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Contents

1 Introduction

The "Gen3" is the third generation of custom 2-photon laser scanning confocal microscopes in the BIDC. The instrument claims two IR laser excitation sources; 1) 18W Coherent Chameleon Vision II (effectively 780nm - 1080nm); 2) 8W MaiTai Ti-Saphire (effectively 780nm - 920nm).

An array of 6 PMTs allows for the simultaneous detection of violet, blue, green, yellow, red, and far red dyes at video rate.

The system uses an upright geometry, an automated Prior XY stage, and a piezo Z drive. Automation is controlled by µMagellan. Additionally, there is a perfusion system with an in-line and stage heater and an anesthesia setup making the microscope suitable for slide, slice, explant, and intravital imaging.

The objective is an Olympus 25x LWD 1.05 NA water immersion.

2 Safety

The Gen3 microscope is located in a **BSL2** rated room, which means that proper Personal Protective Equipment (PPE) must be worn at all times by anyone in the room. This specifically refers to **lab coats, gloves, and goggles** when there is a splash hazard. In addition to BSL2 regulations, **Laser Safety Goggles** must be worn at all times by all persons in the room when the lasers are on. Laser Safety googles are located in a case on a hook by the door, and a spare lab coat is on a hook by the curtain by the door.

All persons using the instrument and working in the room must complete the **Laser Safety Training** and send the PDF certificate to the BIDC (bidc@ucsf.edu).

3 PMT Power - IMPORTANT

The PMT power supply **MUST BE OFF WHEN THE ROOM LIGHTS ARE ON**. Only after the room lights have been turned off may you turn on the power to the PMT power supply box. See Figure 1. The box in Figure 1 is off, because the room lights are on.

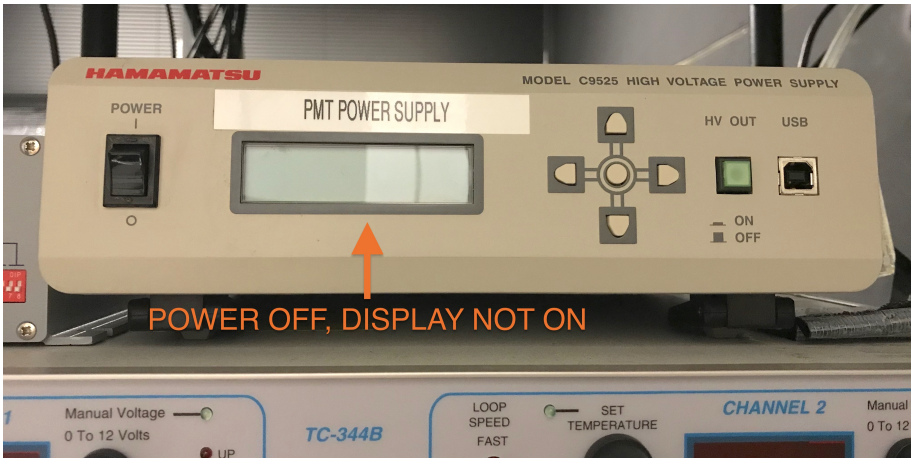

Figure 1: This PMT Power Supply Box is OFF, while the room lights are on.

4 Startup

1. Sign up for time at the BIDC MyCores page.
   (a) This can be done up to 2 weeks in advance.

2. When entering the room, **BEFORE** turning on the lights, check to see if the PMT power box is off.
   Note: *When the PMTs are on, the light on the control box will be orange.*

3. Turn on the 2 computers (if they are not already on).

   (a) Turn on the Acquisition Computer (located to the right on the shelf above the microscope).

   (b) Turn on the Laser Control Computer (located above the computer monitors).

4. Check the Sutter Mirror Control Box that the mirrors are in the "off" position. See Figure 2.

Figure 2: Sutter Mirror Control Box - mirrors in 'off' position.

5. Turn on the Sutter Mirror Control Box using the black switch on the right hand side.
   Note: *It takes around 5 minutes to warm up.*

   (a) Use the toggle to choose between High Res and Low Res settings. Please do not touch pixel size knobs. Pixel size may change at time of calibration. Pixel size is recorded correctly with metadata as long as micromanager is set to match the mirror box setting. Frame rate should always be set to 30Hz.

      i. Down is LowRes (∼0.65 µm pixel) - The most common setting.

      ii. Up is HighRes (∼0.40 µm pixel) - Higher resolution resulting in a smaller field of view.

   (b) Turn on the mirrors with the bottom left switch, which will turn on the light above the 15V marker.

6. Turn on the MPC-200 Control Box located under the Mirror Control Box in order to use the secondary focus drive (the wedge)

7. Check the blue PI Piezo Z Drive Control Box located next to the PMT Power Supply (**do not** turn on PMT Power Supply at this time). The green LED light should be lit. If the red LED light is lit, ask BIDC personnel for assistance.

8. Turn on the Prior Stage control box, located on the shelf above the microscope.

9. Turn on the EOM controller box(es). (Located on the shelf towards the back of the room, above the lasers). You may turn on only the EOM control box(es) that is for the laser(s) you are using. See Figure 3.

10. Turn on the lasers.

    (a) MaiTai: **NEVER** turn off the power supply or chiller.

       i. Check the temperature controller below the optical table is around 21 degrees C.
       Note: *If the display says coolant is low, add ONLY distilled water to the chiller. It needs only a small volume (∼300 mL), and please wipe up any spills. If you are uncomfortable filling the chiller, please ask a BIDC member for assistance.*

Figure 3: EOM Power Supply boxes - turn on just what you need.

ii. To open the MaiTai Software on the Laser Control Computer, double click the Mai Tai software icon and select COM Port3 for the laser.

iii. Push and hold the "On" button to transition the laser from "Standby" to "Warming up". The small status line at the bottom of the window will change to indicate that the laser is starting. After a short period, the status line will indicate the laser is warmed up to 100% and display "Ready to Turn on" in the status line.

iv. Press and hold the "On" button for $\sim$ 3-5 seconds to fire the laser. The screen will then display "Emission" indicating the laser is active. The laser will now power up, which may take a few minutes. The laser is ready to use when the box under "Pulsing" is green (Figure 4).
Note: *The laser may start up faster if its wavelength is set above 850nm.*

v. To change the wavelength, drag the arrowhead on the control OR click the up/down arrows on the Set Wavelength control OR simply type in a new value in the "Set Wavelength" control box. The wavelength changes occur over several seconds and are gradually updated on this dialog.

vi. Press and hold the "Shutter" button to open the laser's internal shutter. The button should now be yellow (Figure 4).

(b) Chameleon:

i. Turn key on laser control box under the air table from "Standby" to "On".

ii. Start "Chameleon Vision" on Acquisition computer by double clicking the "Coherent GUI" software icon on the desktop.

iii. Wavelength Selection is on the upper portion of the GUI. Default wavelengths can be selected with preset buttons or input using the text box. (Can also be selected on the Chameleon Power Supply.)

iv. Shutter Control can be found in the System Control tab. (Can also be controlled on the Chameleon Power Supply.) See Figure 5.

Note: *Only when the lights are off and you are ready to image through the computer should you turn on the PMT array. Whenever you need to turn on the lights, the PMT array* **NEEDS TO BE OFF**.

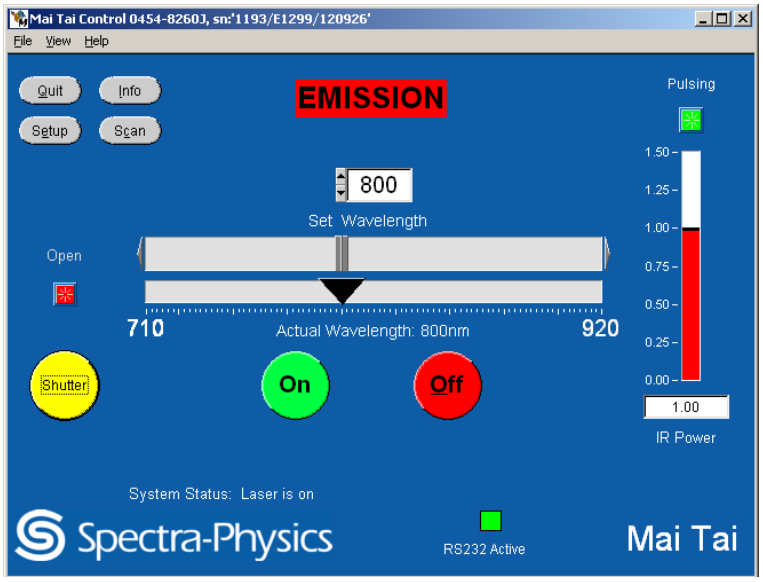

Figure 4: Mai Tai Laser GUI when laser is pulsing and shutter is open.

5 Data Acquisition

There are two effective ways to collect data on the Gen3; through the Micro-Manager interface or the Micro-Manager plugin extensions known as µMagellan. Both have their pros and cons and the choice ultimately comes down to the user. However, µMagellan must be open to run both Micro-Manager and µMagellan. µMagellan is where settings for PMT voltage, EOM settings, and Z position will be determined. If you are using Multi-Dimensional Acquisition, you must still open µMagellan first.

5.1 Micro-Manager

1. Open the latest version of MicroManager v1.4 and the latest configuration file.
   Note: *If you are unsure which configuration file to open, it's typically the last one opened without a specific person's name attached.*

2. Along the left side of the screen there are 5 buttons (Figure 6).

   (a) **Snap:** Takes a single image using the live settings (must save with bottom floppy-disk button).

   (b) **Live:** Continuously takes and displays images (no saving).

   (c) **Album:** Adds images to a series each time it is pressed (must save with bottom floppy-disk button).

   (d) **Multi-D Acq:** Multi-Dimensional Acquisition opens up a new window that controls your acquisition settings (more below).

   (e) **Refresh:** Refreshes the current state of the hardware.

3. Camera and Configuration Settings (Figure 6):

   (a) **Exposure:** The PMT exposure is always set to 33 ms. This window selects the number of frames to average.

   (b) **Binning:** Not applicable on this instrument.

   (c) **Shutter:** Automatically set on this instrument.

   (d) Be sure your **Image Construction** is on the appropriate setting – use Snap/Live for Snap, Live, and Multi-Dimensional Acquisition. Use Magellan for imaging with µMagellan. Using the wrong setting may cause the software to crash.

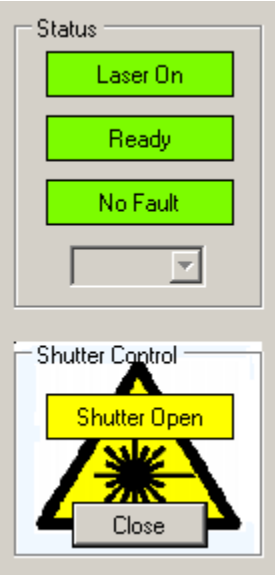

Figure 5: Chameleon settings found in the Coherent GUI.

(e) Be sure your **Resolution** is on the appropriate resolution setting. It should match the setting you chose on the Sutter Mirror Control Box (See Figure 2). Mismatching resolutions will result in a heavily aliased image that cannot be corrected without matching resolutions.

4. Histogram or L.U.T.

   (a) The histogram or Look Up Table is displayed at the bottom of the Micro-Manager window when an image is present.

   (b) This microscope creates an 8-bit image, meaning there are 256 different grey levels.

   (c) You can set the level of which is displayed as the brightest (white) or dimmest (black) with the sliders (see arrows in Figure 7). Alternatively you can click Auto-Stretch and the computer will set this for you.

   (d) The L.U.T. does not affect what data is recorded; only how it is displayed. All channels are pseudo-colored.

5.1.1 Setting Up Detector gain and Excitation Intensity

To get the microscope set up, open the µMagellan plugin (Plugins > µMagellan). There will be four tabs at the top of the window – Device Status/Control, Setup Multiple Acquisitions, Grids, and Surfaces. See Figure 8.

1. Under the leftmost tab, **Device Control/Status**, set your PMT voltages for each channel. It is recommended to start at a voltage of 0.9, and then adjust each channel from there.

2. Set applicable EOM settings for each laser. These settings will be dependent on your sample and the wavelength used.

3. Set your starting Z range to approximately half of the Z piezo's working distance (1000 µm), so about 500 µm.

4. Once your PMT gains and EOM settings have been chosen, it's time to check the channel offsets.

   (a) Place a pollen slide on the microscope.

   (b) Making sure your image construction (see Figure 6) is on "Snap and Live," click "Live" button in the Micro-Manager window. Use the hex key to change stage position and focus your sample. Be mindful of the Live window freezing.

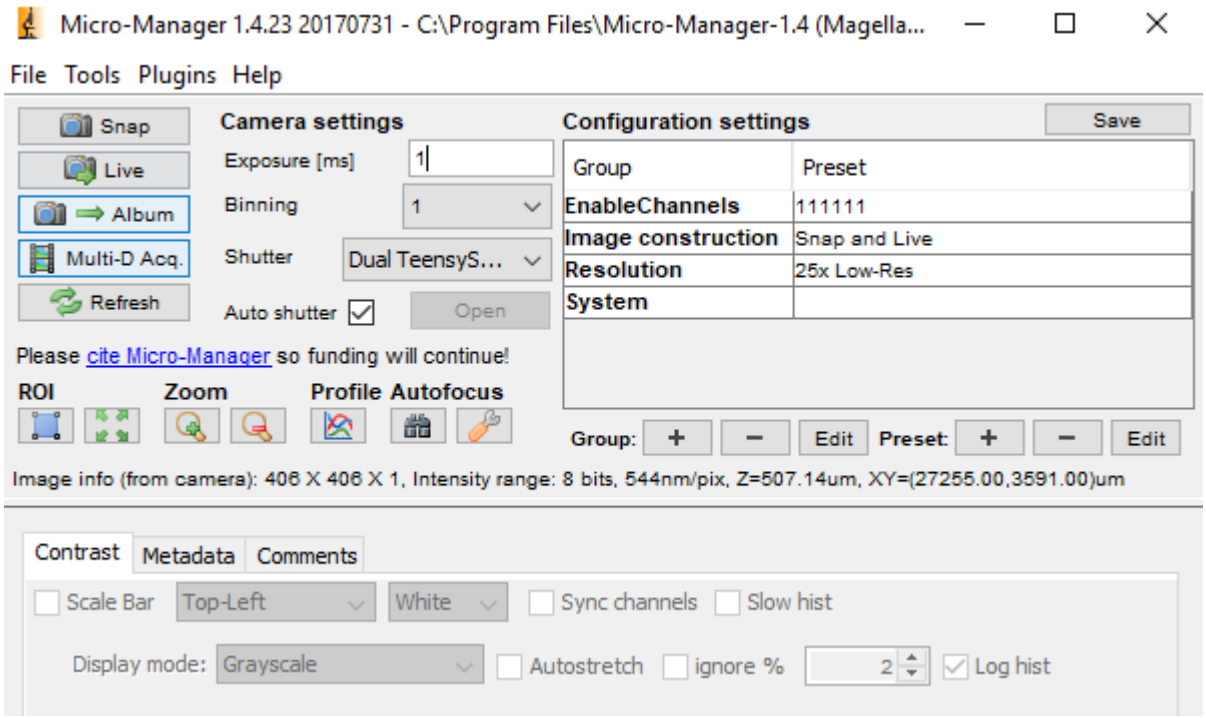

Figure 6: The top portion of the Micro-Manager window.

(c) Once your sample is in focus, go to the Micro-Magellan window and find the tab labelled "2Photon Settings" under Acquisition Settings. While Live mode is running, use the channel offsets to correct for image "fraying." See Figure 9 for examples.

5.1.2 Multi-D Acquisition

This window allows you to set up data collection parameters. Check the box to choose which elements you wish to use. All of these elements can be done in μMagellan with a bit more ease. See Section 5.2.

1. **Time Points:** Select the number of images to take and the interval between them.
2. **Multiple Positions (XY):** This will open up the positions dialog box for you to add multiple areas to revisit or create/change your grid size. **Note:** *It is important to clear all positions that were set prior to the latest sample alignment. The computer does not know the size and position of your sample, and it is easy to accidentally run the objective into something solid and break it.*
3. **Z-stacks (slices):** Determines how you scan through your sample. You can set the starting and ending position along with the step size. Relative Z will change the positions as you adjust focus in live mode. To minimize radiation on your sample, do not check the "Keep shutter open" box.
4. **Channels:** Since we use MultiChannel Mode, Channels is not longer used.
5. **Acquisition Order:** This tells the program the order in which to acquire images.
6. **Save Images:** Select the directory in which to save your files. The BIDC is not responsible for archiving data. Please remove it from the D: drive as soon as possible.

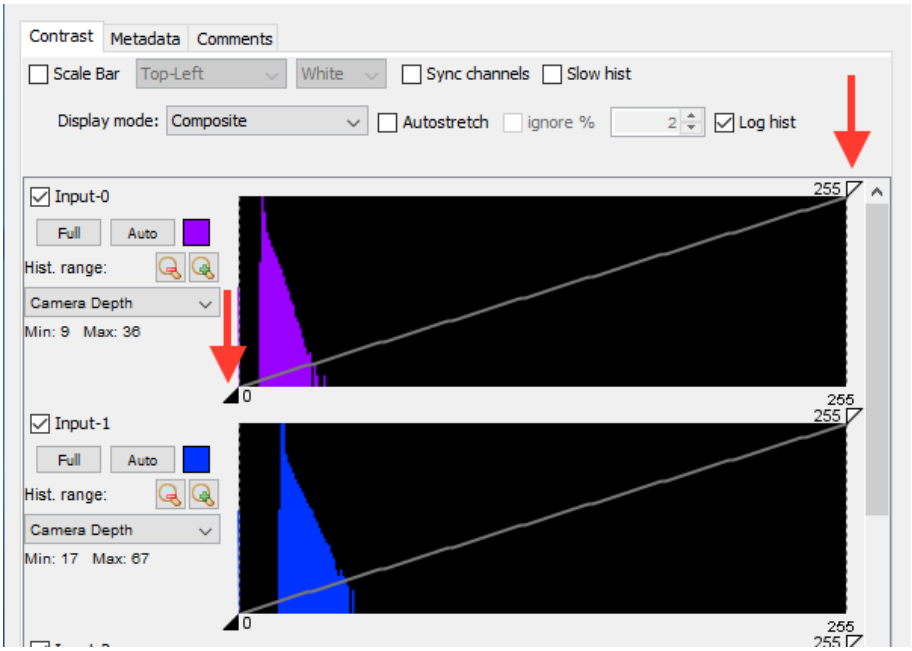

Figure 7: The arrows indicate the sliders that you can left-click and drag to change the display of your channel. This does not affect the data being collected.

5.2 µMagellan

The Micro-Manager plugin µMagellan was written by the former BIDC employee Henry Pinkard. It has many built-in features designed to speed up sample exploration, region of interest defining, and sample acquisition time while limiting sample irradiance. Data is recorded in a special folder that includes image stacks of the regions of interest in TIFF format along with a .XML file that contains the metadata. To stitch the files together for data analysis in either ImageJ/FIJI or Imaris, the folder needs to run through a separate plugin known as "Imaricµmpiler". Imaricµmpiler is also available for download via the BIDC website. Please note that Imaricµmpiler runs best with no other programs running on the computer.

Below is a condensed user's guide for µMagellan. A more in depth article can be found at:

`https://www.micro-manager.org/wiki/MicroMagellan`

µMagellan must be open in order for Gen3 to run.

5.2.1 Explore Mode

Explore mode allows the user to make a "map" of their sample, allowing them to quickly find regions of interest, define z-stack thickness, and create grids for image acquisition. See Figure 10.

1. Initial Setup:
   (a) Assign the saving directory to your user folder, making a folder for the date to keep organization.
   (b) Assign a saving name appended with _explore (µMagellan will save your exploration areas).
   (c) Enter a z-step (this is independent from data acquisition z-step).
   (d) Change "Channel Group" to "Enable Channels."
   (e) Enter a "Tile overlap" percentage (6-10% is a typical amount).
   (f) Press "Explore!" (a new window will open).
2. Your cursor is indicated by a blue box moving along a grid (Figure 11).

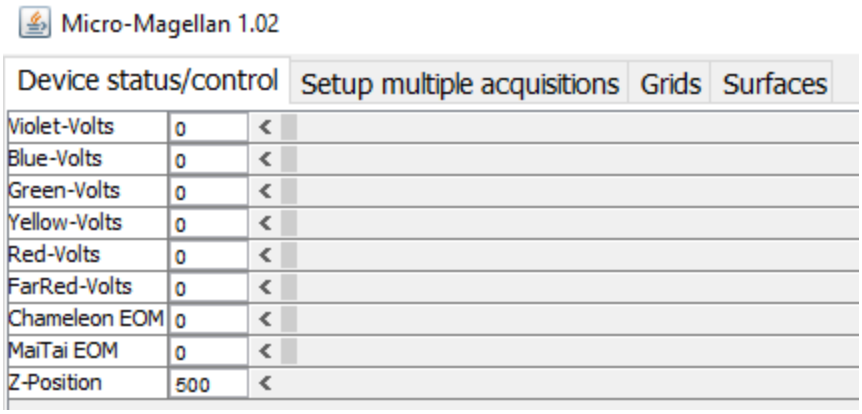

Figure 8: The top portion of the Micro-Magellan window, where you will set PMT settings, EOM settings, and Z Position before imaging.

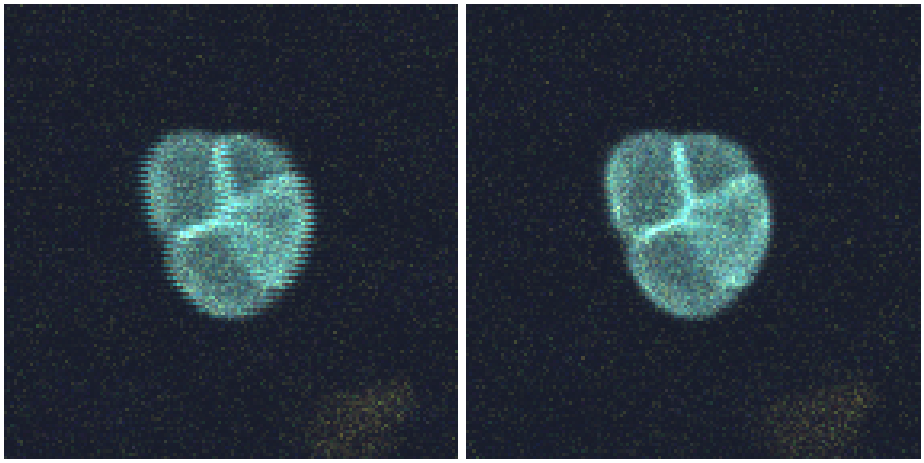

Figure 9: Image with incorrect (left) vs correct (right) offset settings.

3. Click anywhere in the black space (a magenta box will appear).
4. Click on the magenta box to make it green and confirm image area (you should now see an imaged ROI in the colors you selected).
5. Use the look up tables (LUTs) on the right to adjust contrast for each color.
6. Use the scroll wheel to zoom in and out in the explore window. Click and hold the right cursor to move around your sample.
7. You can left click and drag to select multiple fields of view to be imaged.
8. Adjust the z-limits by either pressing the arrow keys (which will step by the pre-defined step size) or dragging one of the z-limit scroll bars. Imaging a region will now include multiple image planes. See Figure 12.
   (a) The white bars represent the volume that you will image next – white bars on top of each other represent a single z-plane being imaged.
   (b) The light green area within the Z limit area represents other acquired z-planes.
   (c) The dark green bars represent the z-plane that you are currently viewing within the Explore window. Slide the Z bar (below the C bar) to change the z-plane viewed.
9. Define regions to be imaged:

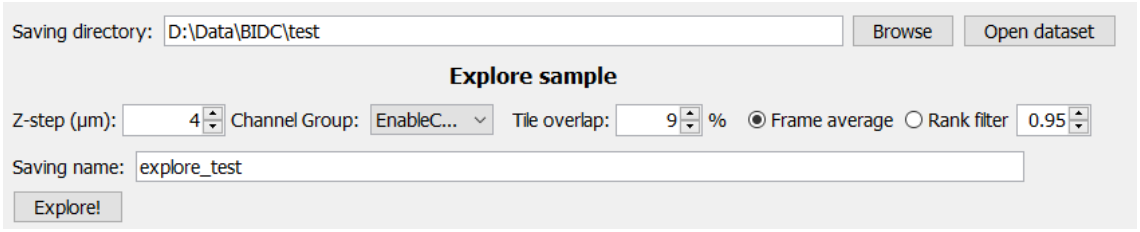

Figure 10: Explore Settings

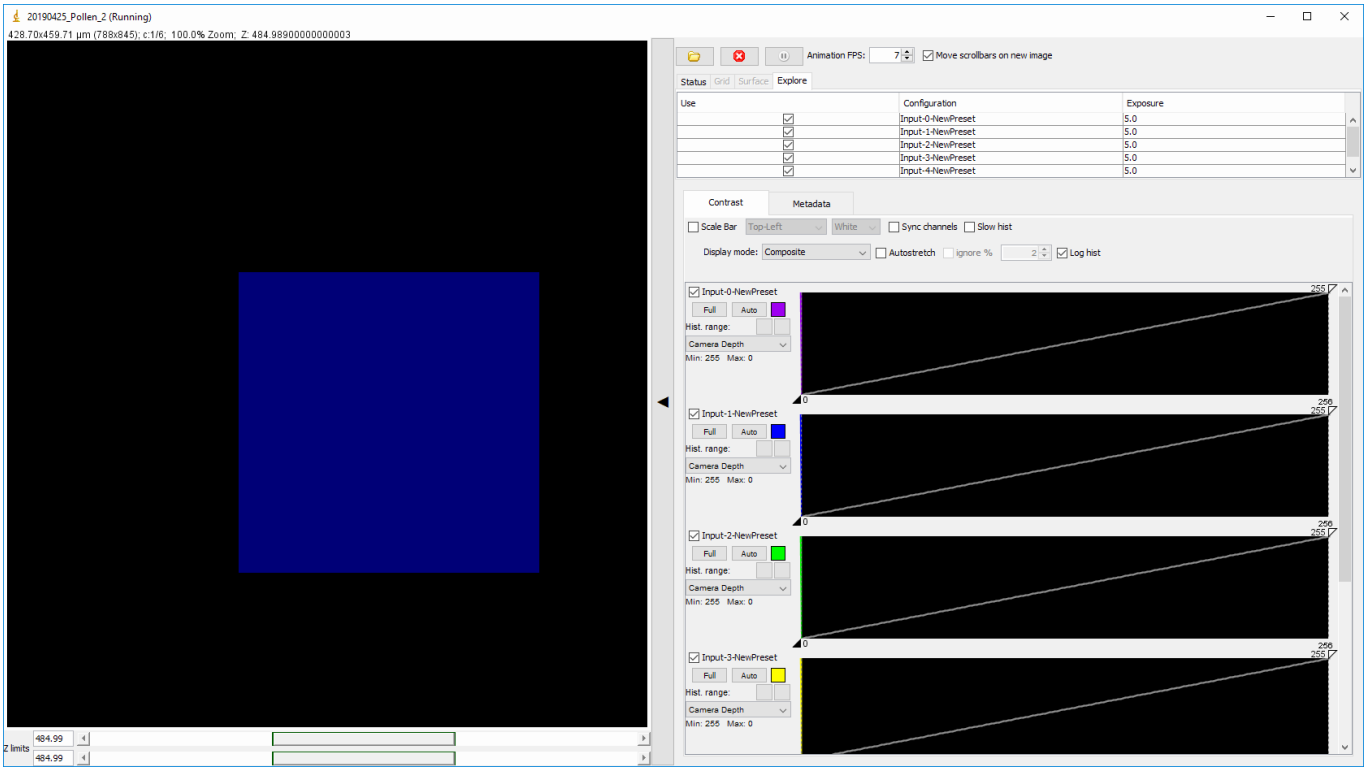

Figure 11: Explore Window

(a) Click on the "Grid" tab in the upper right.

(b) Press "New Grid".

(c) Define the number of rows and column in the grid coresponding to fields of view.

(d) Left click and drag the grid on the screen to the desired region of interest.

(e) Repeat for as many areas you would like imaged.

5.2.2 Acquisition

There are many advanced features in the acquisition setting. Please follow the link above for more information.

1. Navigate back to the main µMagellan window.
2. Remove any unwanted grids from the auto-populated list.
3. Press the "Saving" tab to direct data to the appropriate folders.
4. Press the "Time" tab to set up a time lapse.

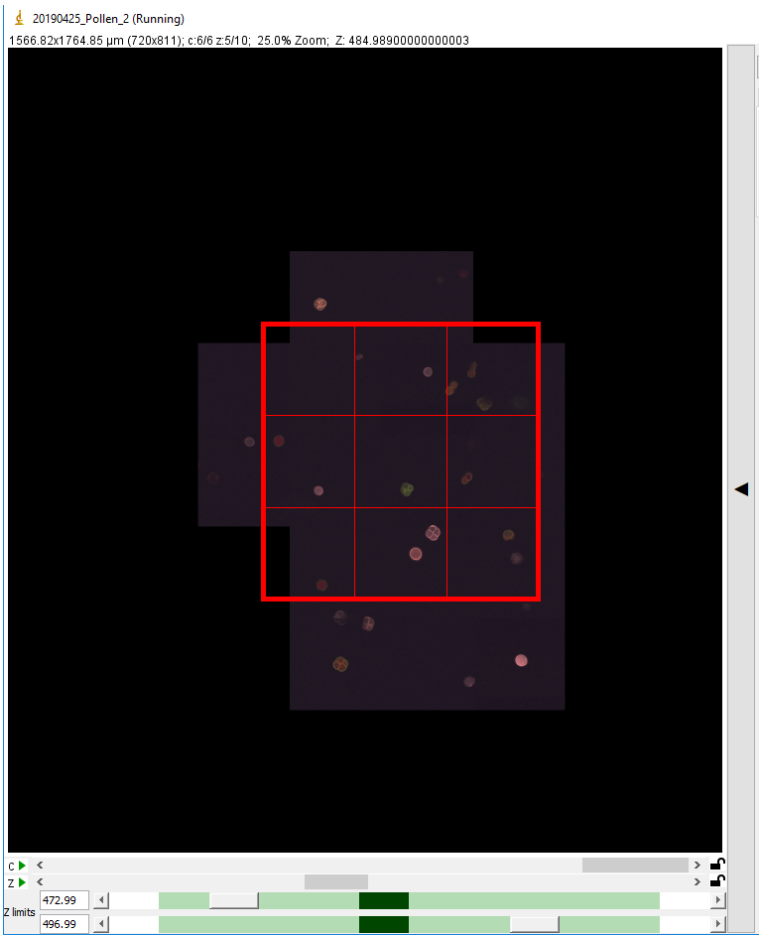

Figure 12: Explore Grids Image

5. Press the "Space" tab, select "Simple Z Stack" and input the appropriate z-limits for each grid.
6. To run a single grid, press the "Run Acquisition" button at the bottom of the window, otherwise press the "Run all" button under the "Setup multiple acquisitions".

More options for acquisition include:

1. Surfaces (3D grids)
2. Individual time lapse for each grid
3. Space (To reduce collection of "blank" data)
4. Covaried Setting (Changing hardware parameters based on imaging position)

Please read the online guide or talk to a BIDC employee for more information.

6 Shutdown

Shut down is fairly simple, and can be done in really any order. It is advised to close the software and transfer data to a hard drive while completing the other steps and cleaning up.

1. Close Software

2. Transfer data (may take time)
3. Lasers
4. EOM
5. Prior Stage
6. PI Piezo Z Drive
7. Mirrors
8. Sutter MPC-200 control box
9. Clean stage, table, hood area, countertop.
10. Report any broken or messy items, or problematic issues, to the BIDC as soon as possible.

7 Troubleshooting

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.