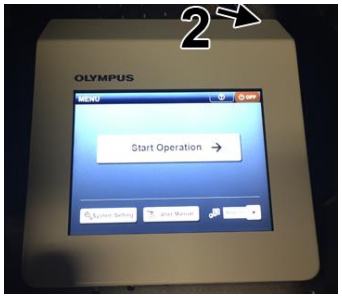
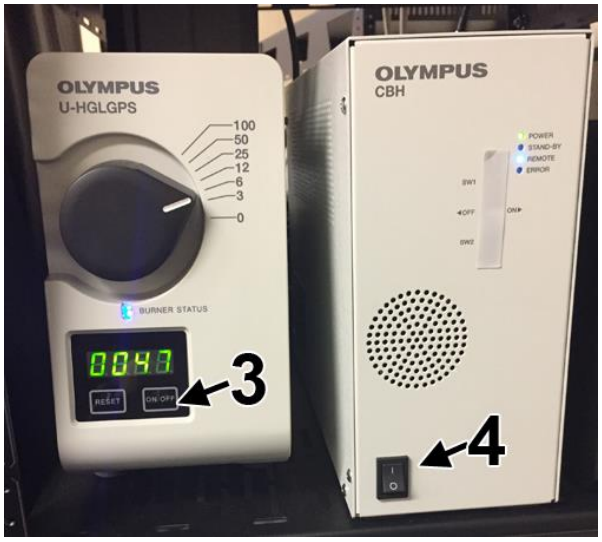
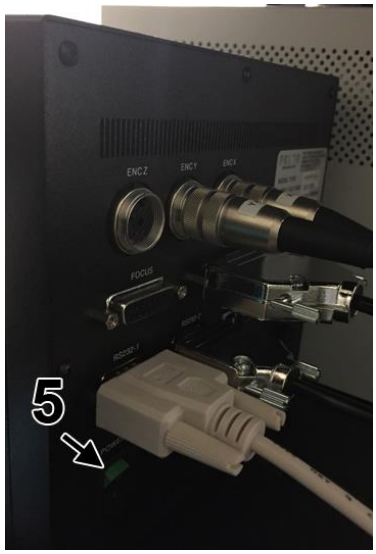
Olympus FV MPE-RS Multiphoton Laser Scanning Microscopy

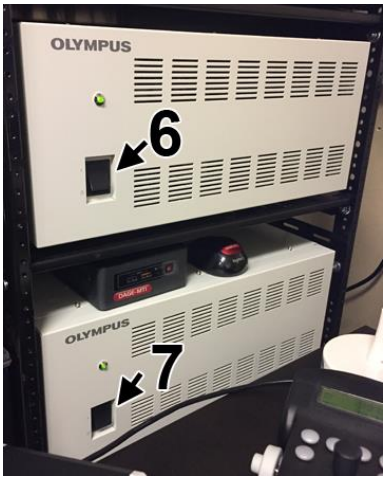
Turning on system.

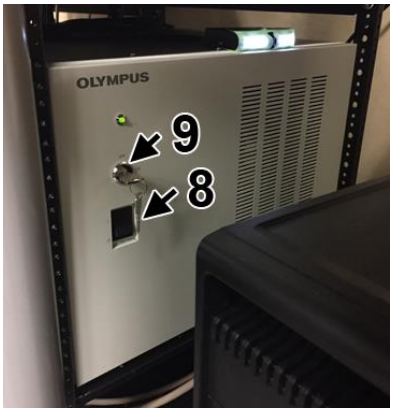
1. Turn on the computer first **(1).** Log into Windows desktop.
2. Turn on the Xenon arc lamp by pressing **ON/OFF button (3)** 2 seconds and releasing (U-HGLGPS).
3. Turn on the **control box CBH (4)**
4. Turn on the **XY stage power supply (5)** on the back side.
5. Turn on **FV30-PSU power supply unit (6)**.
6. Turn on the **Main scanner(7).**
7. Turn on the **SIM scanner** with **switch (8)** and **key (9).**
8. Turn on the remote **Microscope control panel (2)** (switch on the backside).
9. (Optional) if you are planning to do photomanipulation such as bleaching or activation, turn on the **Coherent visible laser sources (10, 11, 12).**

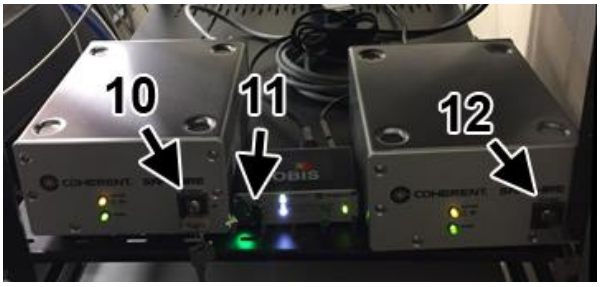






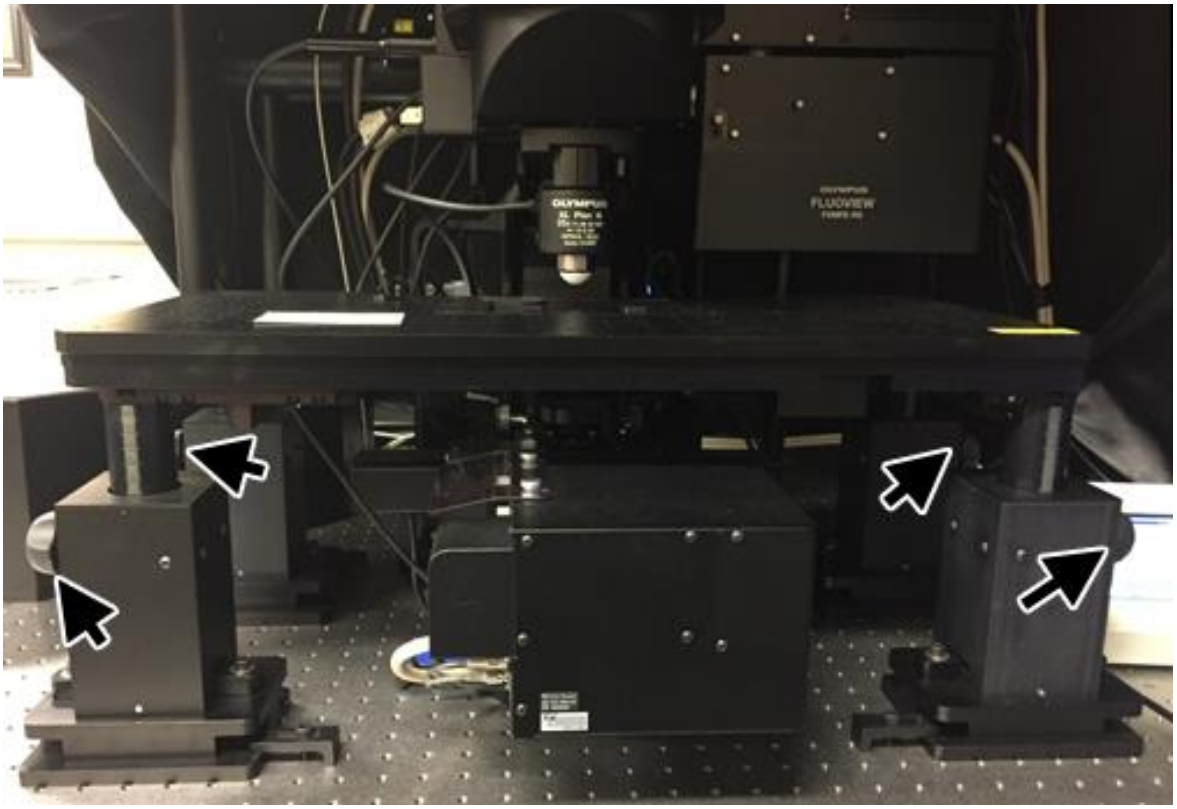




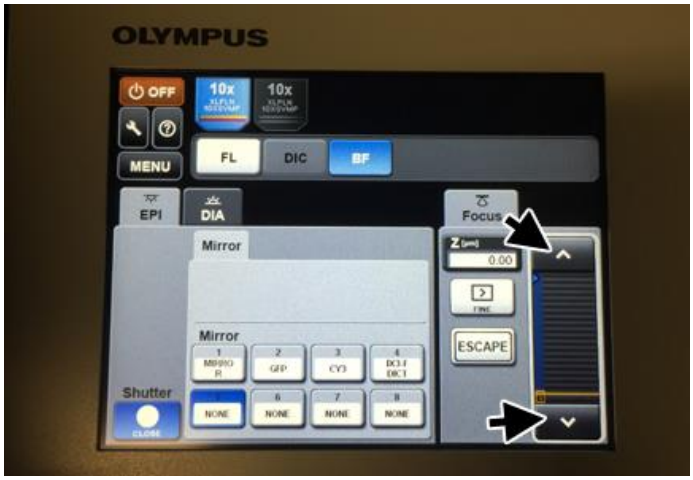


Microscope setting.

1. *Before you start imaging, check if the objective on the system is the one you want to use. If you need a different objective, contact Jake for installation.*



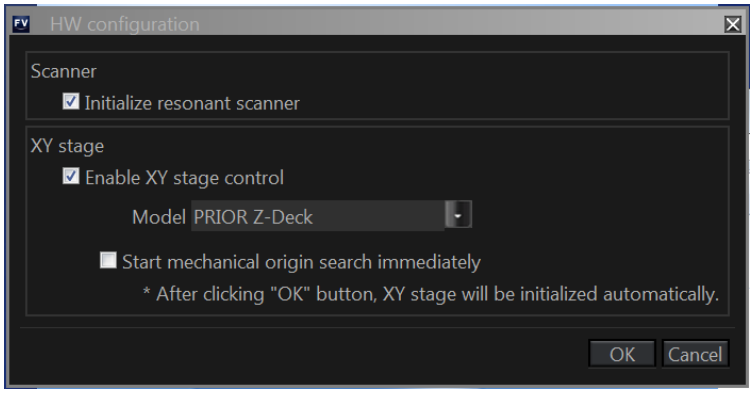
2. *Depending on the objective and your sample size, you may need to adjust the height of the stage in order to have enough space between them. Touch the **upper arrow head** on the microscope control panel to raise the objective.*

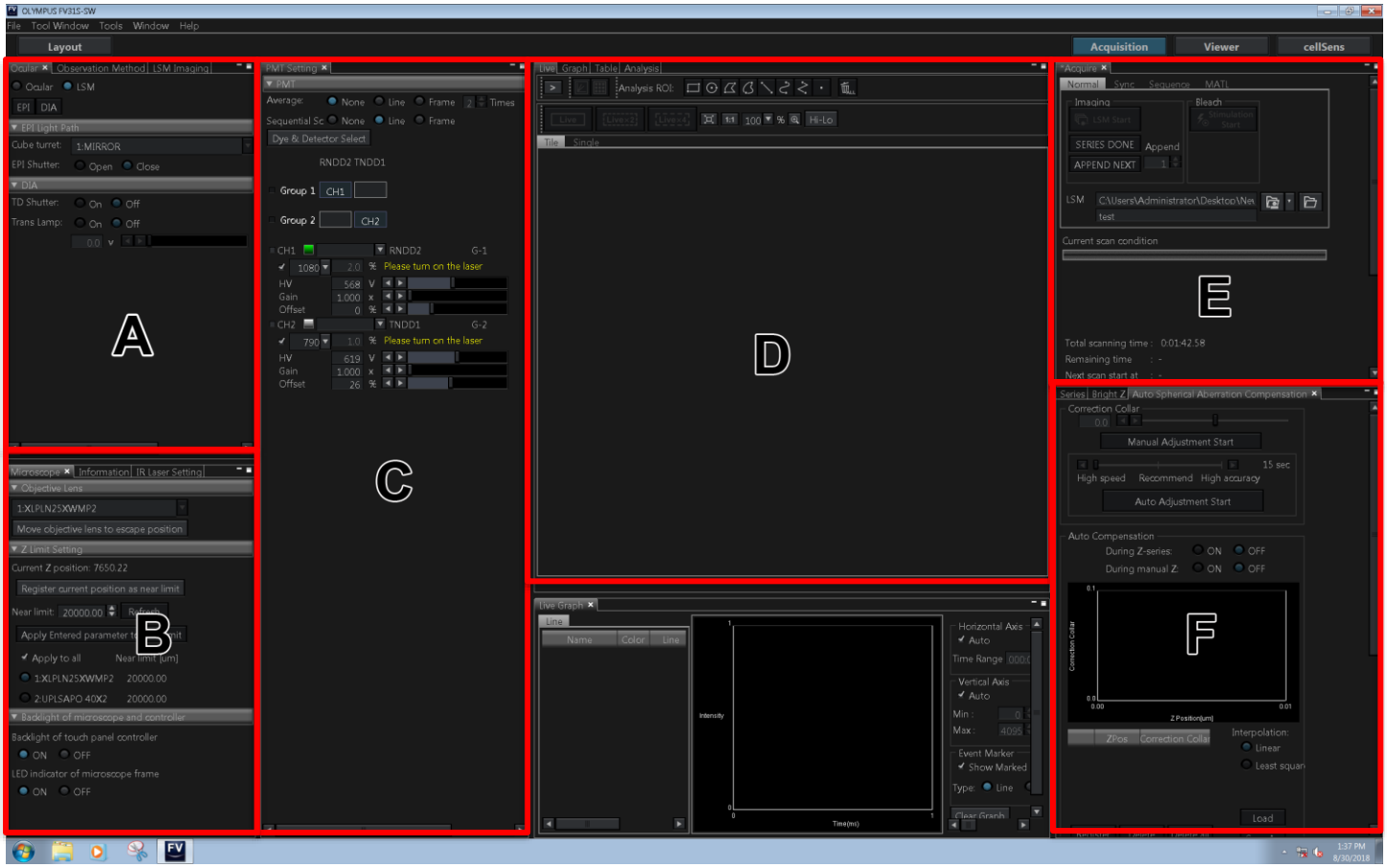


3. *If you can place your sample without touch the objective, then the current setting will be good to go.*
4. *Use the **joystick to move the stage**.*
5. *If you can't fit your sample in or the objective is too far away from the sample, loosen the **four knobs on the stage** (arrows) and gently push down or pull up the entire stage. **Make sure the stage is on level with T-level.***
6. *Tighten the **stage knobs**.*

Confocal Scanning Setting

Double click on the **FV30S-SW**  icon on the desktop. It will ask if you want to initialize xy stage and resonance scanner. Click **OK**.
The initial user interface of the program will show up.



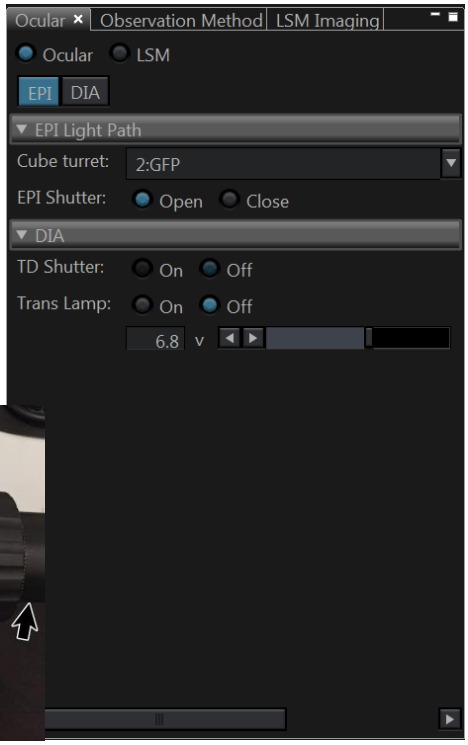


You will use the area **A~F** most during typical image acquisition.

- **(A)** Control the viewing mode (eye or scanning), select filter cubes for viewing.
- **(B)** Control the setting of microscope objectives, z movement, and IR laser line and alignment.
- **(C)** Set up the scanning mode, such as sequential, scan averaging, and laser power and detector sensitivities.
- **(D)** Control live image scanning, ROI setting, and other tools.
- **(E)** Set up the image acquisition.
- **(F)** Set up z and time series, Bright Z, and spherical aberration compensation.

Basic Image Acquisition

Place your sample on the stage and place immersion medium if needed.



Click **Ocular** tab for direct observation through microscope eyepieces.

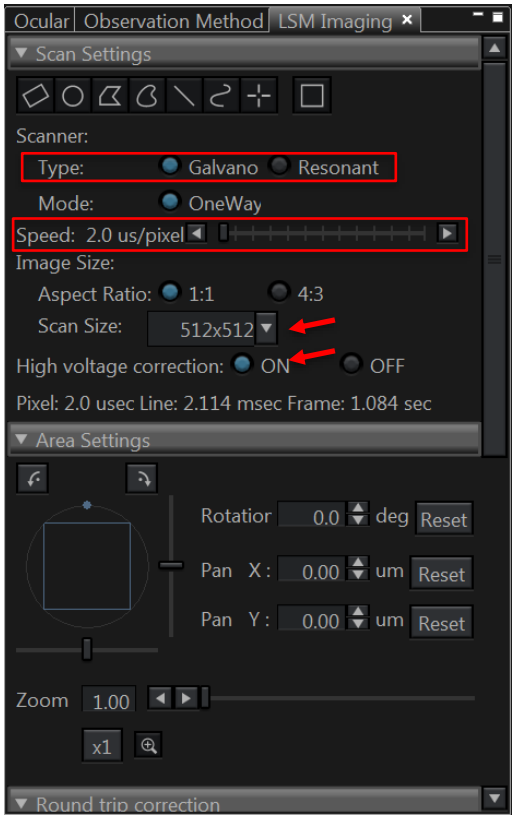
Click **Epi** button for fluorescence observation. Select a filter from the drop down list of **Cube Turret** .

Click Open on EPI Shutter to illuminate your sample with fluorescence light.

Focus onto the sample by moving objective fast up or down with **arrowheads**, and fine control with **focus handle.**

Once you find a region to image, click **LSM** on the **Ocular** tab to change the lightpath to the laser scanning mode.

Before acquiring images, there are a few steps in configuration to set up how to acquire your images, such as types of IR lasers, number of detectors, scanning type (Galvano vs Resonant) etc.



Scan Settings

Select either Galvan or Resonant scanner (Galvano scanner scans slowly to generate better quality images, whereas Resonant scanner scans extremely fast but sacrifice image quality. At 512x512 resolution, the fastest of Galvano scanner is 2.0 us/pixel, whereas the resonant scanner scans at a fixed rate of 0.067us/pixel).

With Galvano scanner, you can choose the scan speed by sliding the bar. Galvano with OneWay scanner is recommended for routine high-resolution image acquisition.

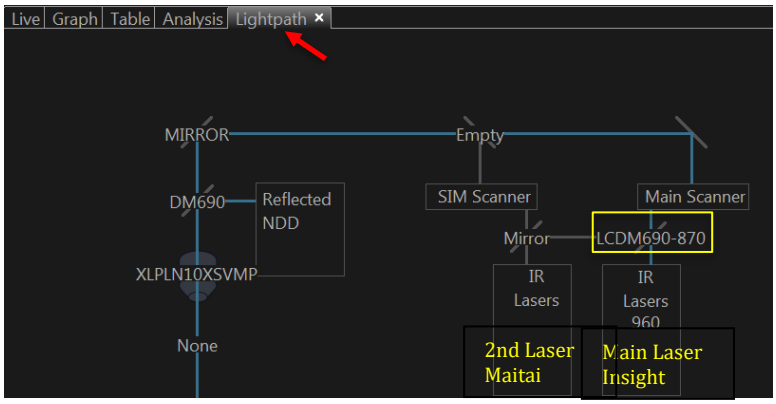
Scan (image) size is 512x512 as default, and you can change it from the drop down list.

Select High voltage correction ON.

Area Settings

Here, you can **rotate** sample display without actual rotation of the sample. You can **zoom** in to get better resolution (keep it under 5x, beyond it may not increase resolution.)

Laser beam path setting

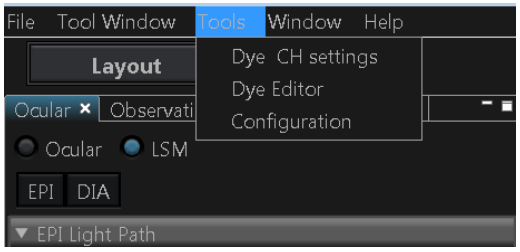


To check the current setting of dichroic mirrors and emission filters, use menu command, ***Tool Window/Lightpath***. The **Lightpath** tab will be added to **D** area. Click the tab and check laser coupling mirror (yellow box) if the correct one is selected.
If not, select one from the Coupling mirror of IR laser tab.

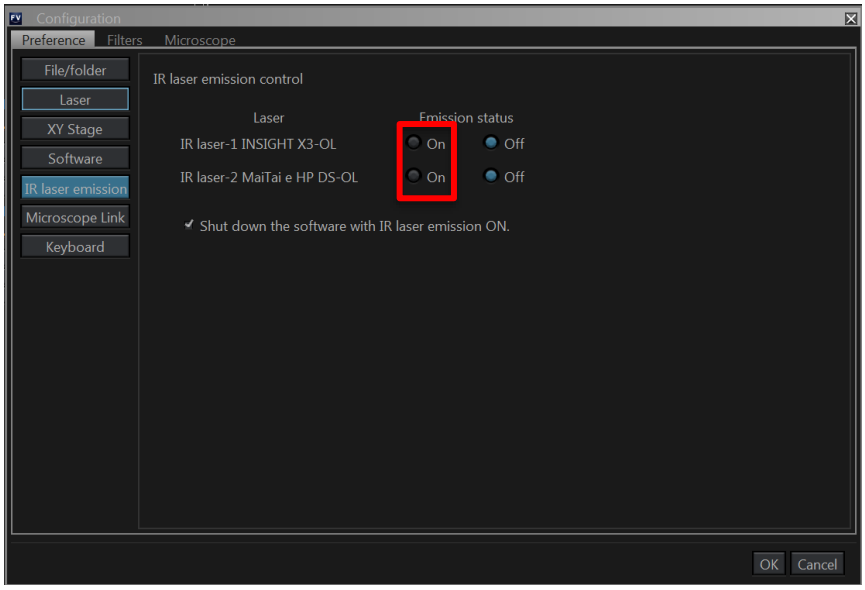
- **Glass** for use of Main laser only.
- **LCDM690-870** for two lasers with Main (>920 nm) and 2nd (<870 nm)
- **LCDM1030-1300** for two lasers with Main (<940nm) and 2nd (>1030nm)
- **LCDM690-1050** for two lasers with Main (>1120nm) and 2nd (<1050nm)

PMT Setting

On the PMT Setting panel, there will be a message “Please turn on the laser”, which means the IR laser emission is not activated.



Open **Configuration** window from **Tool/Configuration** menu.



Select **IR laser emission** tab and click **On** for IR laser that you will use.
Click **OK.**

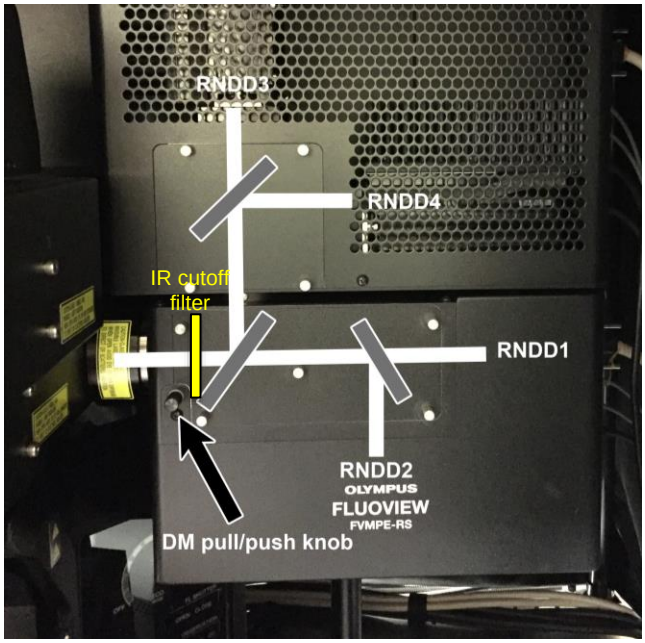
A few moments later, the message will change into “the Laser is ON” and you can change the power level.

Now decide which detector (CH1~CH4) will be used for your experiment.
This system has 4 detectors for fluorescence; 2 conventional PMTs (RNDD1 and RNDD2) and 2 high sensitive GaAsP detectors (RNDD3 and RNDD4).

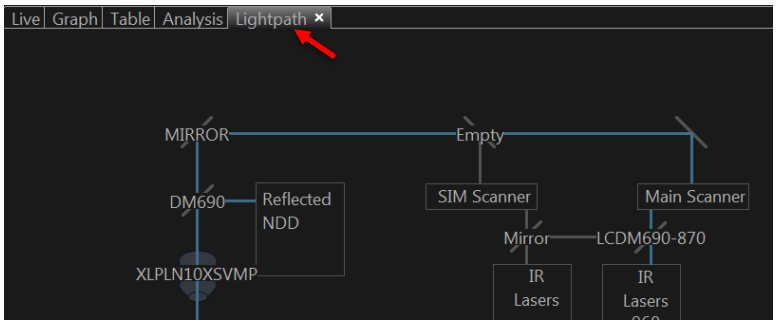
You can use only **2 PMTs** or **all 4 detectors** by DM knob on the microscope (arrow).

With the knob is pushed **in**, you will use **RNDD1** and **RNDD2**.

With the knob is pulled **out**, you will use all 4 detectors, if **FV30-SDM570** dichroic mirror is installed (see below)

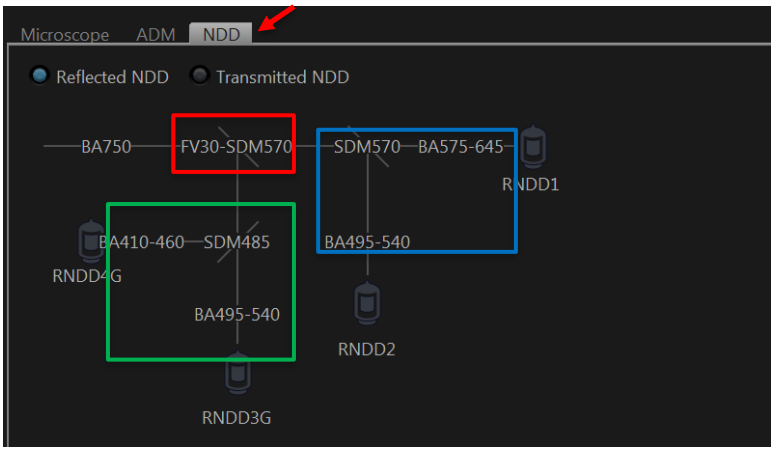


To check the current setting of dichroic mirrors and emission filters, use menu command, ***Tool Window/Lightpath***.
The **Lightpath** tab will be added to **D** area.



Select **Lightpath** tab and **NDD** tab (red arrows).
Click **Reflected NDD**.

If the red box (SDM) shows **FV30-SDM570**, the current setting allows two or four detector configuration.



SDM	
FV30-SDM570	>570 nm to RNDD1/2 and <570 nm to RNDD3/4
FV30-SDM-M	Mirror all emission to RNDD 3/4
Empty	All emission to RNDD1/2

It also shows the current fluorescence NDD filter cubes for the detectors (green and blue boxes)

Filter cube	Colors	Emission filter 1	DM	Emission filter 2
FV30-FVG	Violet & Green	BA410-460	DM485	BA495-540
FV30-FCY	Cyan & Yellow	BA460-500	DM505	BA520-560
FV30-FGR	Green & Red	BA495-540	DM570	BA575-645
FV30-FOCY5	Orange & Far red	BA575-645	DM650	BA660-750
FV30-F475R	475 nm & Red	SHG465-485	DM570	BA600-670

The filter cubes need to be changed depending on user's experiment. Please contact the core staff for filter changes.

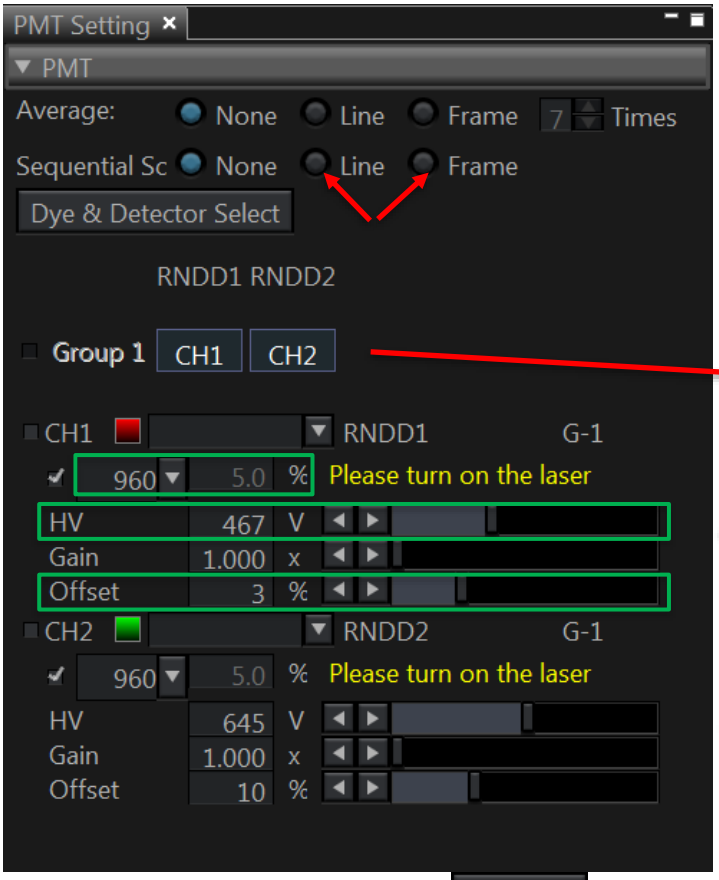
PMT Setting: Setting Image Acquisition condition.

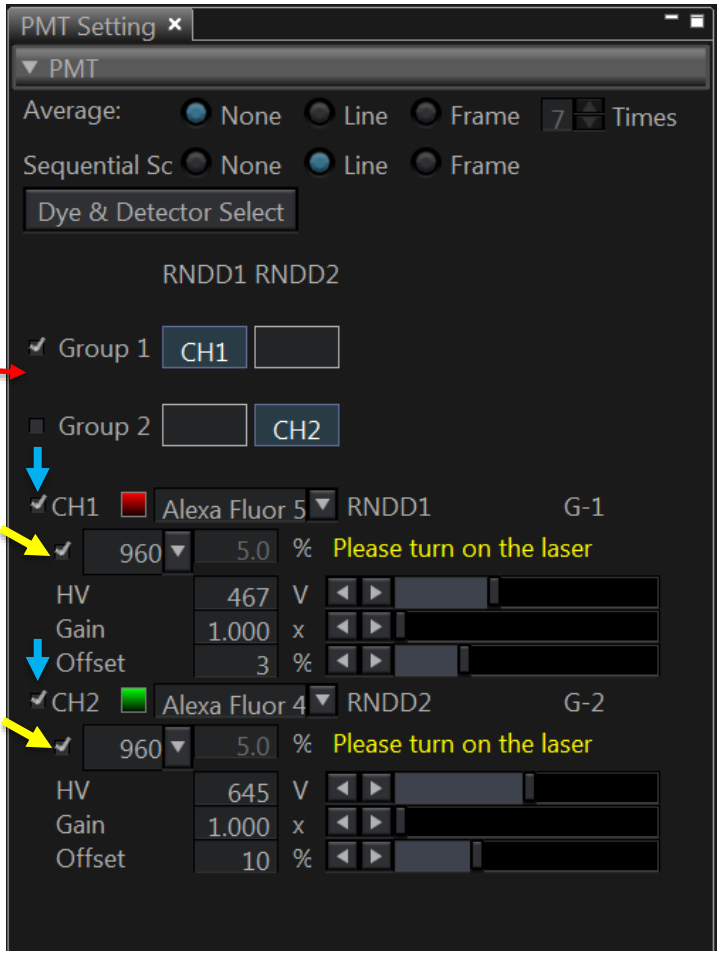
For better quality image, it is often useful to use **Average** in either **Line or Frame** mode. Put a number to the **Times**, and the system will scan the same line or frame as many as you specify and display the image with average intensity on the screen.

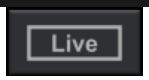
It is often necessary to acquire different fluorescence signal sequentially to minimize bleed through between different color channels.

Click **Line or Frame** button on the **Sequential Scan** (red arrows). Then the RNDD1 and RNDD2 will be grouped as Group 1 and Group2.

Check the CH1 and CH2 boxes (blue arrows) to activate the detectors and Laser sources (yellow arrows) if they are not checked.

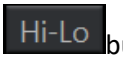




Now you are ready to scan. Click **Live** 

button on **Live** Tab. It will change into **Stop** 

button to click to stop scanning.

To set dynamic intensity range for imaging, click **Hi-Lo** 

button while scanning. It will change the image in gray scale with some red and blue pixels. The red pixels represent the pixels with maximum intensity (saturate d) and the blue pixels ones with zero intensity. The proper image will have a few red pixels and blue background.
This condition can be achieved by adjusting **laser power** (in %) and **the HV** and **Offset** of each detector (green boxes)
The higher laser power and HV are, the brighter the signal intensity.
Increasing Offset will suppress (reduce) background.
Try to minimize the laser power and maximize the detector's HV without generating electronic noise (keep HV <800).

Stop Live Scanning by click 

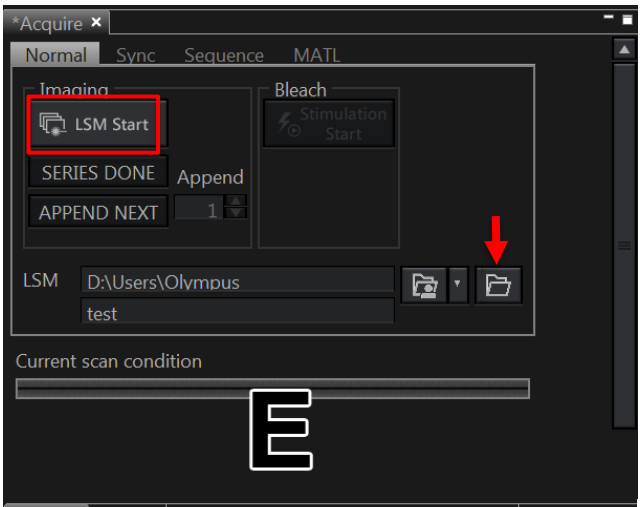
button.
Go to **Acquire** tab in area E and select **Normal** tab.

Click **File folder** icon (arrow) and under the Data (D:)/Olympus, select a folder (create one for you if you have not created one) to save images.

The acquired images are saved automatically to the folder you specified.

Click **LSM Start** 

to start image acquisition.

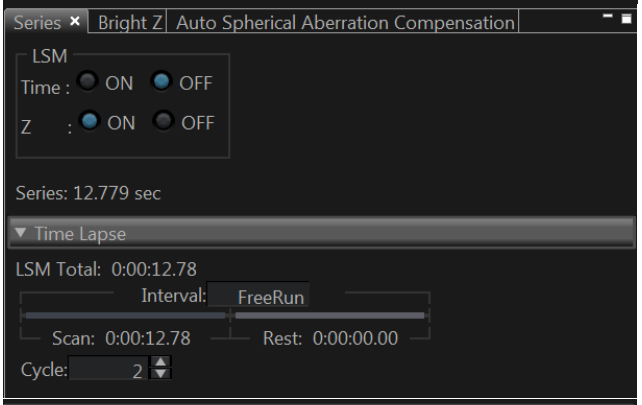


Acquiring Z-series

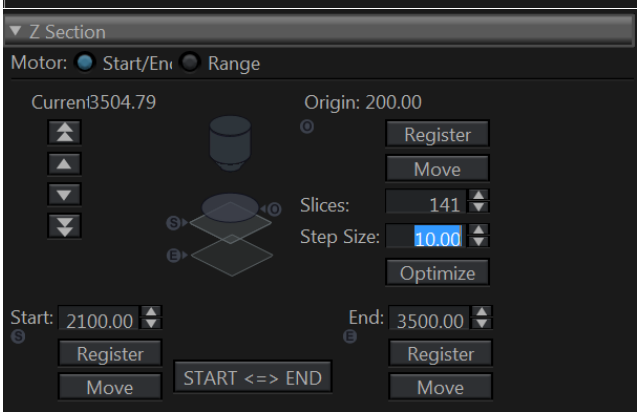
Select **Z: ON** at the **Series** tab.

There are two ways to define the upper and lower limits of a z-series; (1) Start/End or (2) Range.

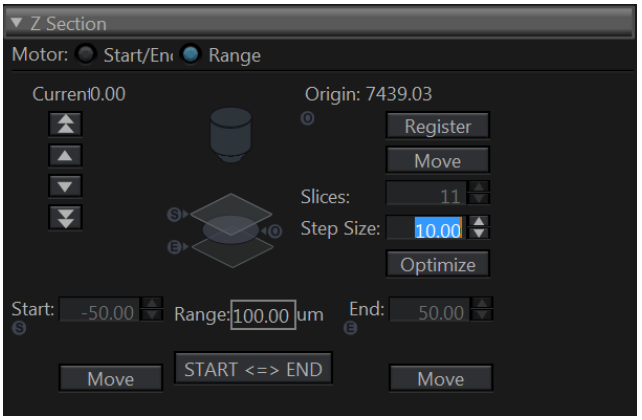
(1) Click **Start/End** button in **Z Section** Window.
Move the focus one side (either lower or upper part) of your sample.
Click **Register** this focus for **Start** (or **End**).
Move the focus the other side of the sample.
Click **Register** this one for **End** (or **Start**).
Choose the **Step Size**, the number of **Slices** will be changed according to the total thickness of the z dimension.

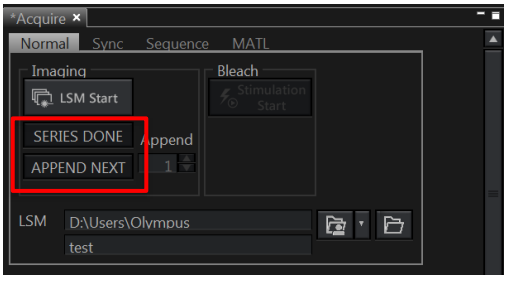


(2) For using **Range**, click **Range** button in **Z Section** Window.
Move the focus to the middle of the your sample in z dimension.
Type the number for the micrometer range (for example 100 um, which means it will capture 50 um range up from the current position and 50 um range down from the current position) and step size.



Once the z series range is set up, click **LSM Start** button.
Once the image acquisition is done, the SERIES DONE and **APPEND NEXT** button will flash. Click SERIES DONE to finish or append more sections if necessary by adding number and clicking **APPEND NEXT**.

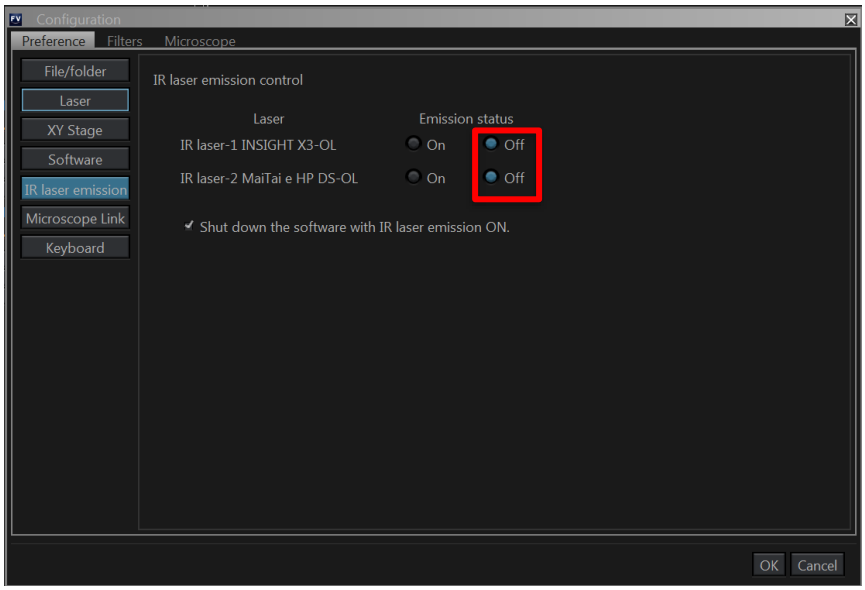


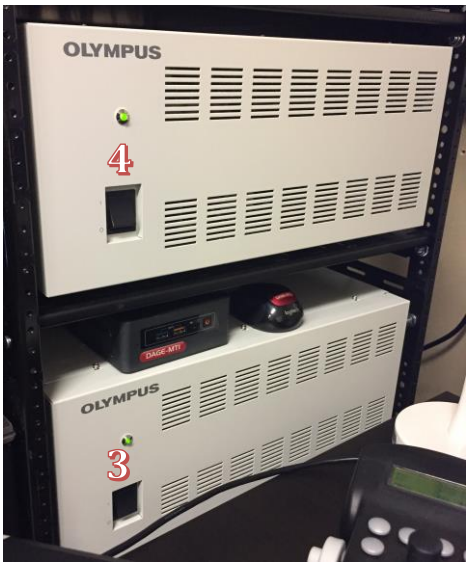


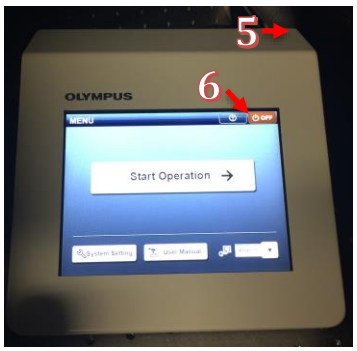
System Turn off

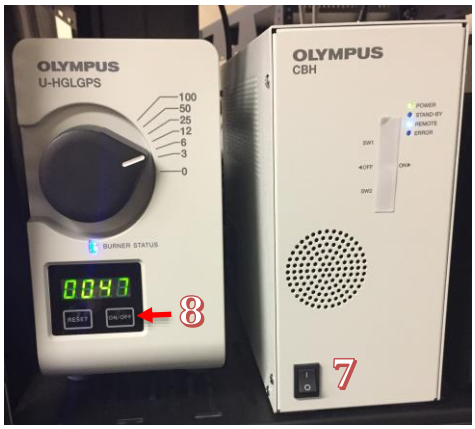
If you will be the last person to use the scope for the day, please turn off the system.

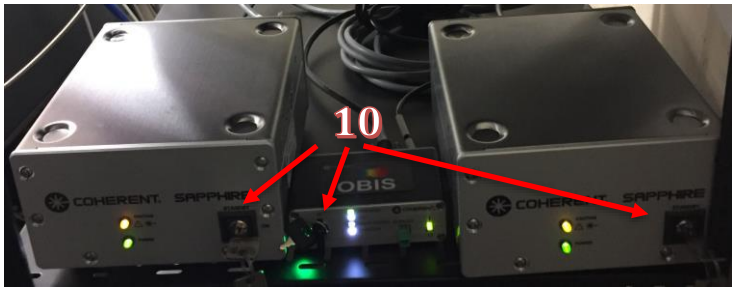
1. Before exit the FV30S-SW imaging program, go to menu command **Tool/Configuration.** Click **IR laser emission** in the **Preference** tab**.** Click **Off** on both IR lasers and click **OK**.
2. Exit the **FV30S-SW** program.
3. Shut down the window program.
4. Turn the key off (1) and then switch(2) of the SIM scanner.
5. Turn off the main scanners (3 and 4).
6. Touch OFF button (5) on the touch panel and exit. Power off with the switch on the back (6).
7. Turn off the CBH unit (7).
8. Turn off the fluorescence arc lamp by pressing the ON/OFF button (8) for 2~3 seconds and releasing it.
9. Turn off the xy stage controller power supply (9)
10. If you used the bleaching visible lasers, turn off them (10)





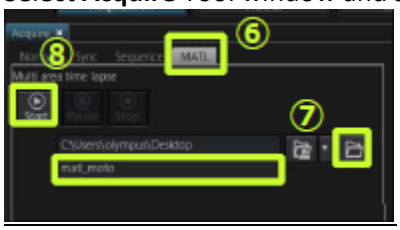




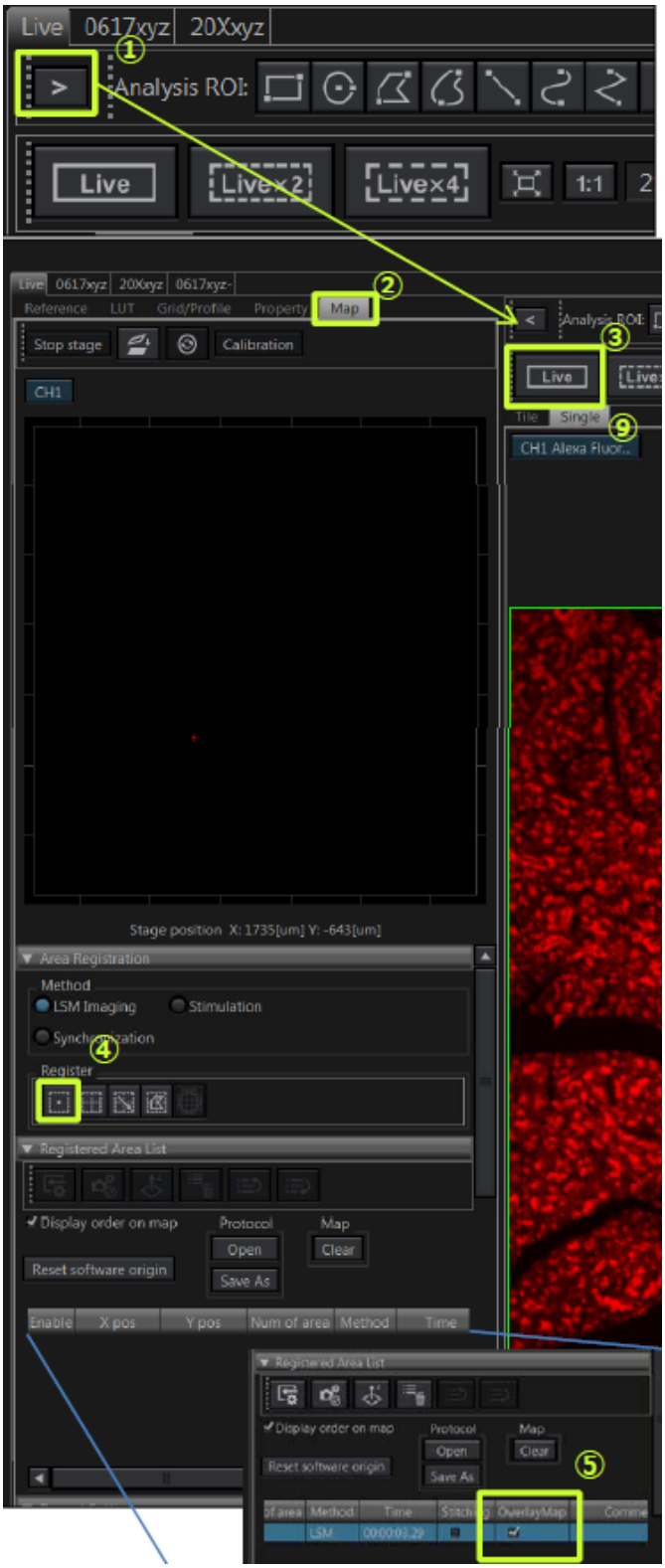


Multi Area Imaging with Map Image

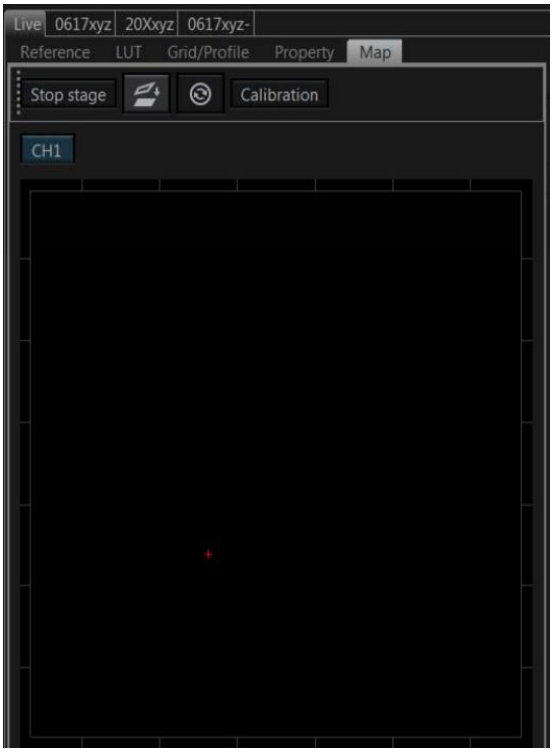
- Click > button in **Live** tab to expand the sub pane.
- Select **Map** tab.
- Focus onto a ROI and adjust the acquisition parameters. Click button to register the position and the acquisition parameters.
- Repeat for additional ROIs.
- Scroll to the right in the registered ROIs list and check “OverlayMap”.
- Select **Acquire** Tool window and select **MATL** tab.



- Press the file folder icon and select (or make) a folder to save the images.
- Press Start button to captures the images of the registered ROIs.
- After image acquisition is finished, the images will appear on the **Map** area.



Acquiring the stitched image with MAP Imaging



- Click **Map** icon or > button and select **Map** tab to define the multiple ROIs or tilting area.
- Move the stage to a region of your sample and press “register” button to register the position. (Note: all image acquisition conditions such as xy position, focus, laser intensity, PMT setting etc are registered when you press register button.
  So, register after you finish adjusting all conditions.
- Move to the other side of your sample and register.
- Use the or or button to create tiles for imaging areas.
- If you change the acquisition parameters after registration, you must update by pressing update Button.
- Select MATL tab in the Acquire Tool Window and Press Start button to acquire images.
- If your sample is not flat, use **Focus Map**. Click **Focus Map** button, it will define 3 tiles. You can move the tile according to the slope of your sample. Go to the one defined focus point, adjust the top or bottom of your sample as reference point for z-series and click button. Repeat for the other two points. The program will calculate the offset along the focus point.

Annotating the images

- To display annotation of main feature (e.g. time stamp for time lapse image series), click the **Active Screen Overlay button** (arrow 1).
- If you want to display specific annotation (e.g., time slice number), click the **Create a Text Annotation for Analysis button** (arrow 2) and then click on the image.
- A popup window will show up with a list of annotation. Select the annotation from the dropdown list and click **Add** and **OK**.
- You can change the **format of the annotation** by selecting it and **right-click** for context menu.