

## Leica SP8 HyVolution Confocal Microscope: **LIVE CELL** IMAGING OPERATION

Light Microscopy Imaging Core, Robbins building, 6<sup>th</sup> floor, Rm. E-632  
Case Western Reserve University

### SYSTEM SET UP

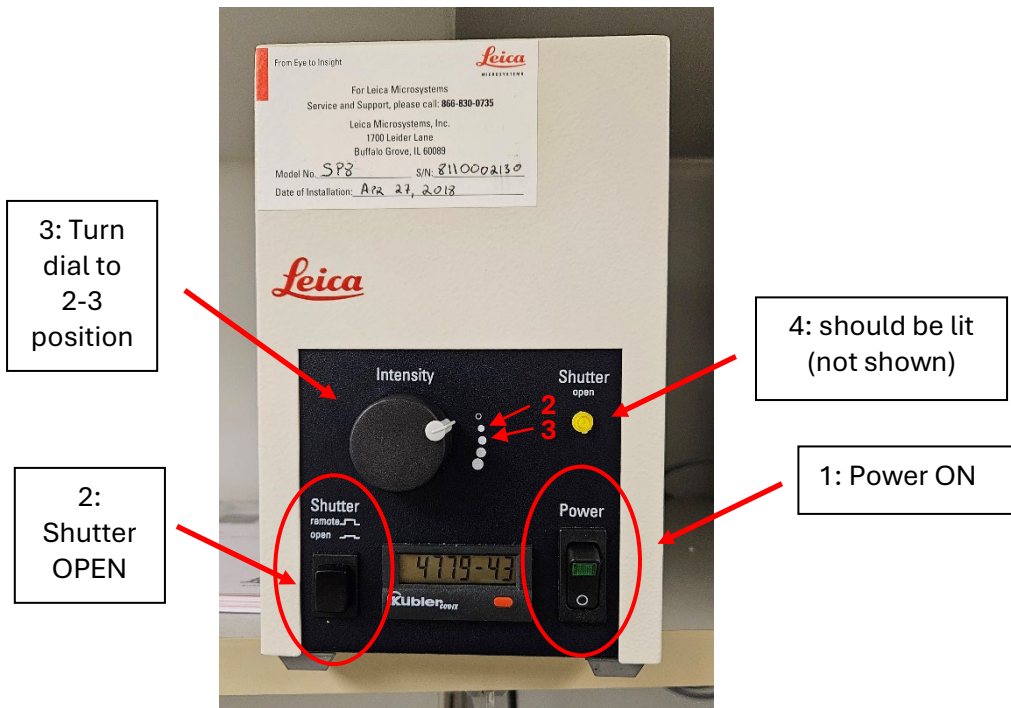
Follow the **EXACT ORDER** listed:

1. **UNCOVER** the microscope
2. **TURN ON 3 green switches** under the computer table (from **LEFT TO RIGHT**)
3. **TURN the LASER KEY** to **ON** position

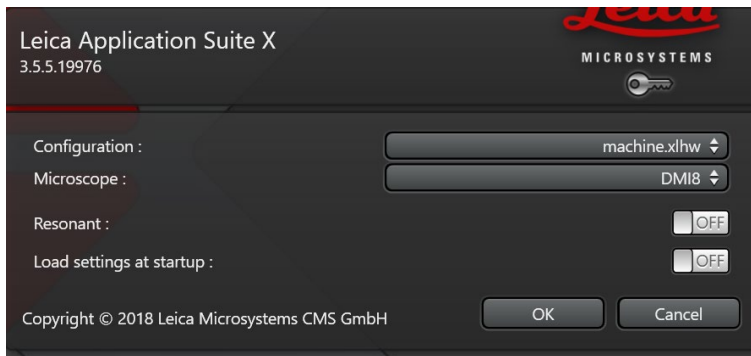


4: Turn key to **ON** position

4. **TURN ON Mercury lamp** → press shutter button to open if not already open (light should be lit) → turn the intensity dial to ~2-3 position

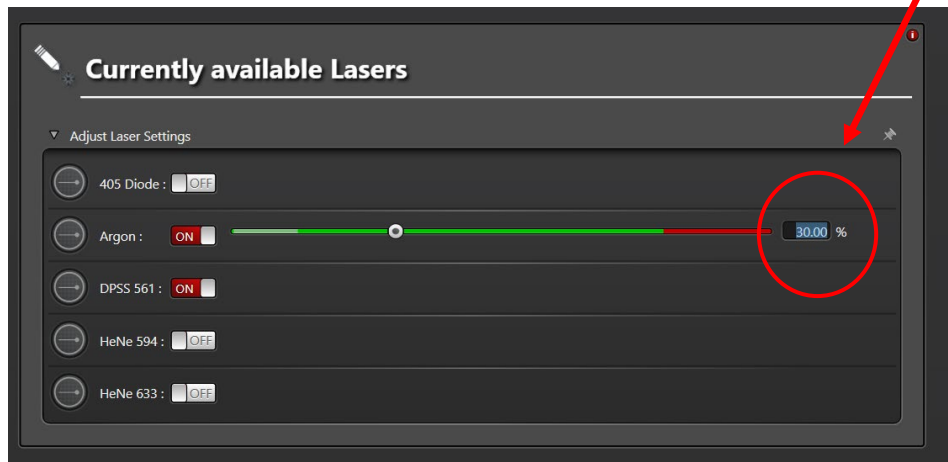


5. Open up **LAS X** program
6. In the **START-UP SCREEN**:
  - a. Check the following setting → click OK



7. **CONFIGURATION** tab → **LASER CONFIG** icon

- a. **TURN ON ALL** the lasers you need for your experiment
  - i. 405 Diode → use this for DAPI
  - ii. Argon: 458, 476, 488, 496 and 514nm
    1. *Note: The default intensity for Argon laser is 0%. Start with 30% power and adjust according to signal intensity of your sample. Can go down to 10%.*
  - iii. DPSS 561nm
  - iv. HeNe 594nm
  - v. HeNe 633nm



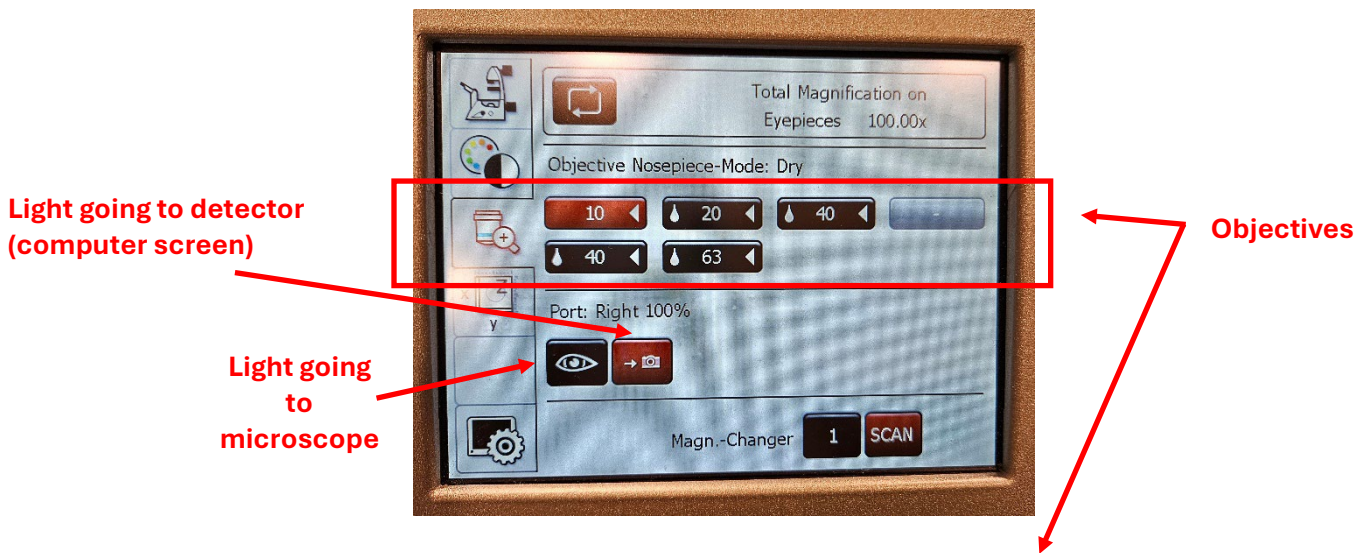
## SAMPLE SET UP ON MICROSCOPE

### Cleaning up objectives

8. Gently tilt back the transmitted light arm
9. Check if there is any oil residue on the objective you want to use
  - a. Wipe the front of the lens lightly with **lens paper** (**not** Kimwipes) moving in one direction
    - i. You can gently press down on the lens when wiping (should feel some spring tension)
  - b. Wipe the sides of the lens (can wipe in rotating motion)
  - c. **When changing objectives during experiment:**  
**ALWAYS** clean oil residue **BEFORE** changing to a different objective



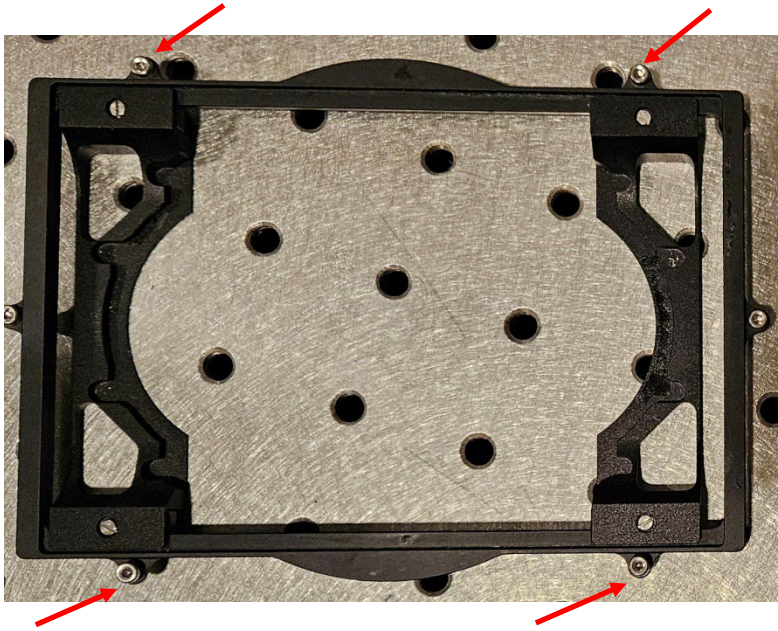
10. **Change to desired objective lens** either on microscope touchscreen or through LAS X software
  - a. 10x / 0.40 (dry)
  - b. 20x / 0.75 (multi-immersion)
  - c. 40x / 1.10 (water)
  - d. 40x / 1.30 (oil immersion)
  - e. 63x / 1.40 (oil immersion)





**Setting up a Humid chamber for live cells**

11. Unscrew 4 corners of slide stage with an Allen wrench



12. **Keep the screws in place** (still on the slide stage) → slowly and carefully remove the slide stage from microscope

a. Do **NOT** lose screws



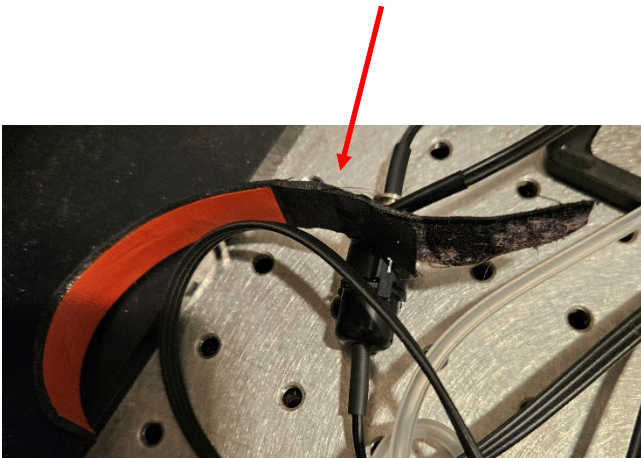
**Slide stage**

13. Power **ON** the **Incubator** and turn **ON** the **Lens Heater**
  - a. This incubator will keep the humid chamber warm and objective lens that will be touching the sample warm to keep cells happy and alive



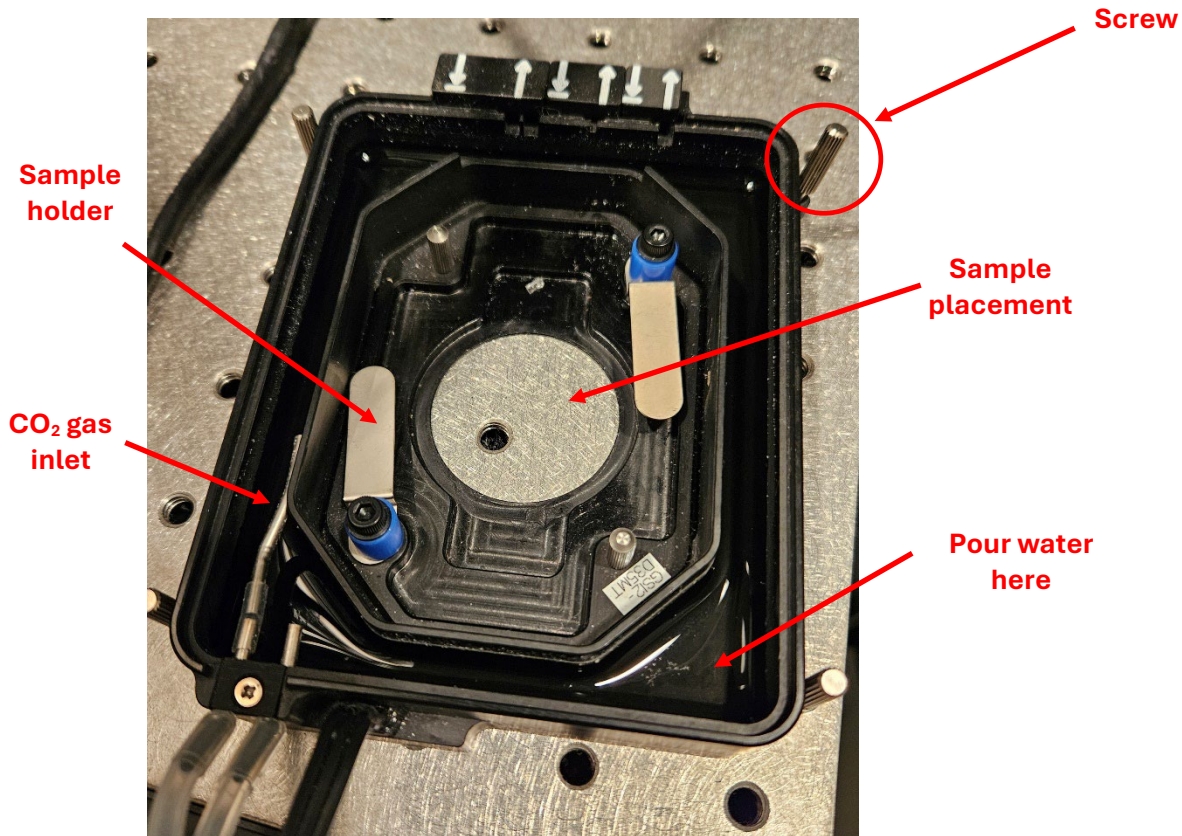
**Do NOT touch (should be OFF)**

14. Wrap the **Lens Warmer** velcro **around the objective lens** to keep warm

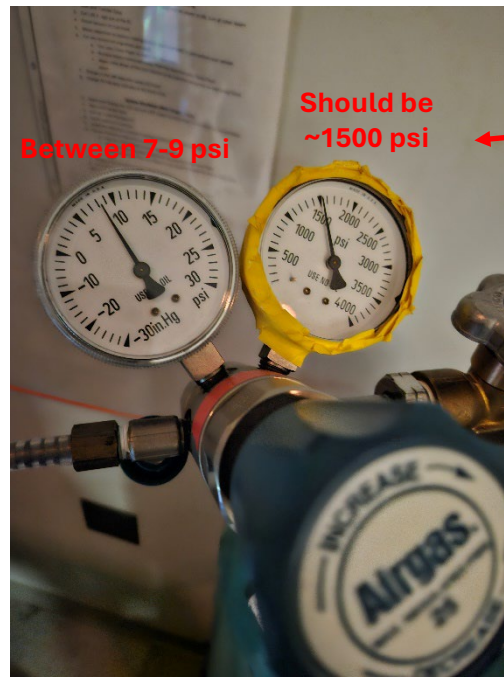




15. If using an **oil objective**, **put some oil** (a few drops of oil) **directly** on the **objective lens**
  - a. Just enough to cover the front (hole) of the lens
  - b. Do not apply too much oil that it drips to the sides
16. Pipette **~5-10ml** or pour some **DI water** into a corner of the humid chamber
17. Place the **humid chamber** onto microscope where slide stage was
18. **Tighten** 4 screws by hand – should be tightened enough but not overtight



19. Open valves in order from #1 to #3 of CO<sub>2</sub> gas tank



Should be  
Between 7-9 psi

Should be  
~1500 psi

Should read  
these  
pressure

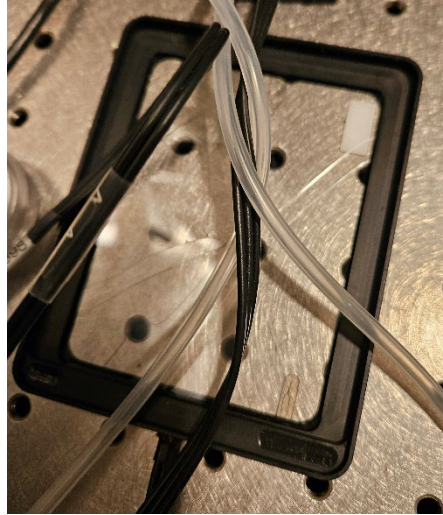
20. Check the CO<sub>2</sub> level and adjust the level of CO<sub>2</sub> by using the dial on incubator



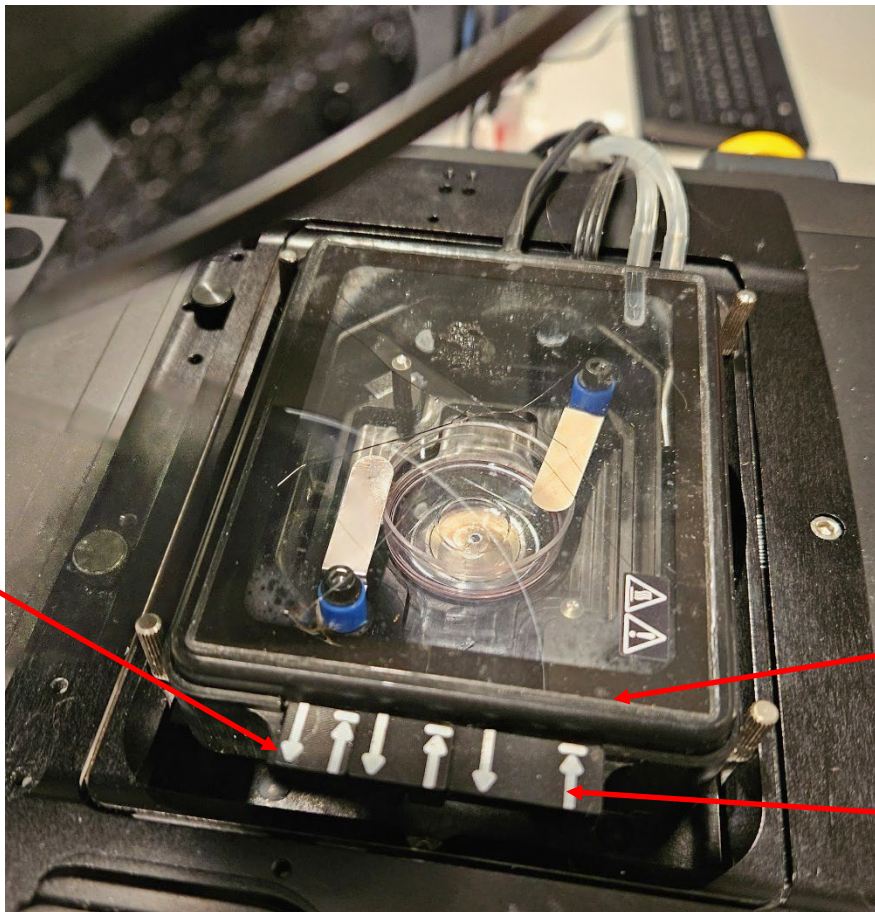
This black floater  
should be ~15  
(between 10 and 20)



21. **Place your sample** (cells contained in a glass bottom dish) onto the middle circle area inside the humid chamber. **Secure it in place** with two metal sample holders.
22. Place (lay) the **chamber cover** on top of the humid chamber



**LIVE CELLS LOADED AND READY TO BE IMAGED**



Humid chamber cover on

Note: as time passes, there will be condensation since it is kept at 37°C during imaging.

Sample secured in place by sample holder arms

Sample dish

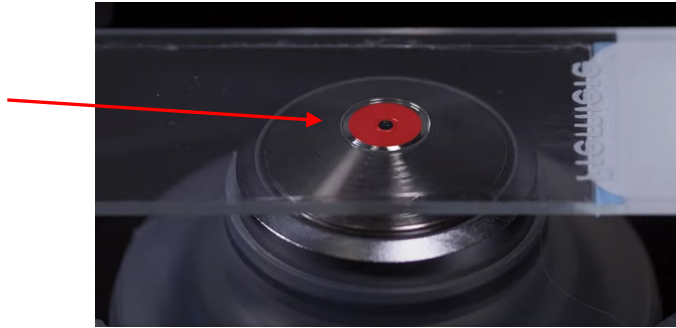


23. Use **joystick dials** to position the humid chamber close to the objective lens
- X dial: moves humid chamber left and right
  - Y dial: moves humid chamber towards you or away from you
  - Z dial: moves objective lens up and down
    - Turn left (i.e. like loosening cap) → lens moves down
    - Turn right (i.e. like tightening cap) → lens moves up
  - Keep the transmitted light arm tilted back so you can see the humid chamber and lens movement
  - Press **XY FAST** button (**coarse**) a few times then move slide stage in X and Y directions to line up the sample with the lens
  - Press **Z COARSE** button a few times then bring up the lens in Z direction
  - When slide is close to the lens, press **XY PRECISE** button and **Z FINE** button **several times** for fine movement to position the sample slide very close to the lens

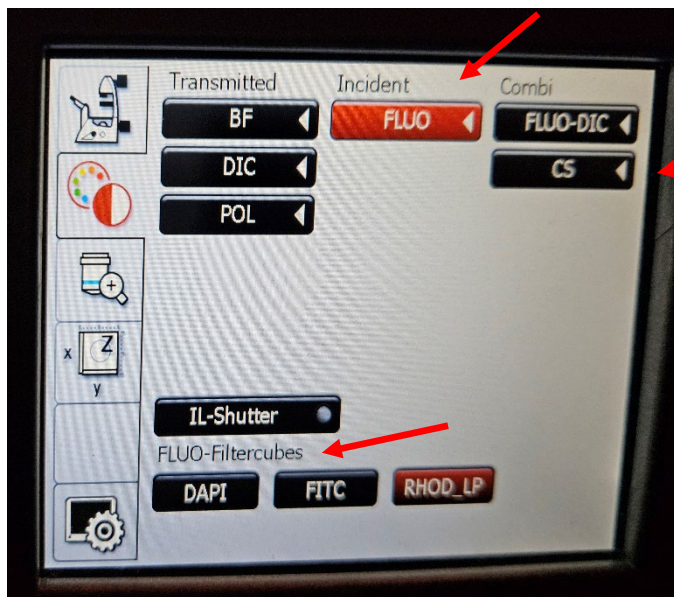


24. If using **oil objectives**, lens has to make contact with the bottom of the glass dish
- Use **coarse** movement to bring up the lens **first**
  - Once objective lens makes contact with the bottom of the dish, **oil droplet will expand**
  - The minute the lens and dish make contact, change to **Precise** and **Fine** movement on joystick
  - Keep moving lens up slowly to a point where **oil droplet stops expanding** (this is the maximum closeness between the lens and the dish) → at this time, oil droplet should cover the front part of the lens (indicated in red circle below), but not the area beyond this point

Oil droplet (red area) will expand as slide and objective lens make contact



25. Set the microscope with one fluorescent setting (i.e. **Fluo** → **DAPI**, etc.) on touchscreen → turn Z dial (still on precise and fine movements) counterclockwise to lower the lens → keep lowering lens until you find sample through eyepieces on microscope
26. Once you have found your sample, select **CS** (Computer Screen) on microscope touchscreen → this will turn off the shutter so it will shut off the fluorescent light to protect the sample from photobleaching → go to software



## SOFTWARE SET UP

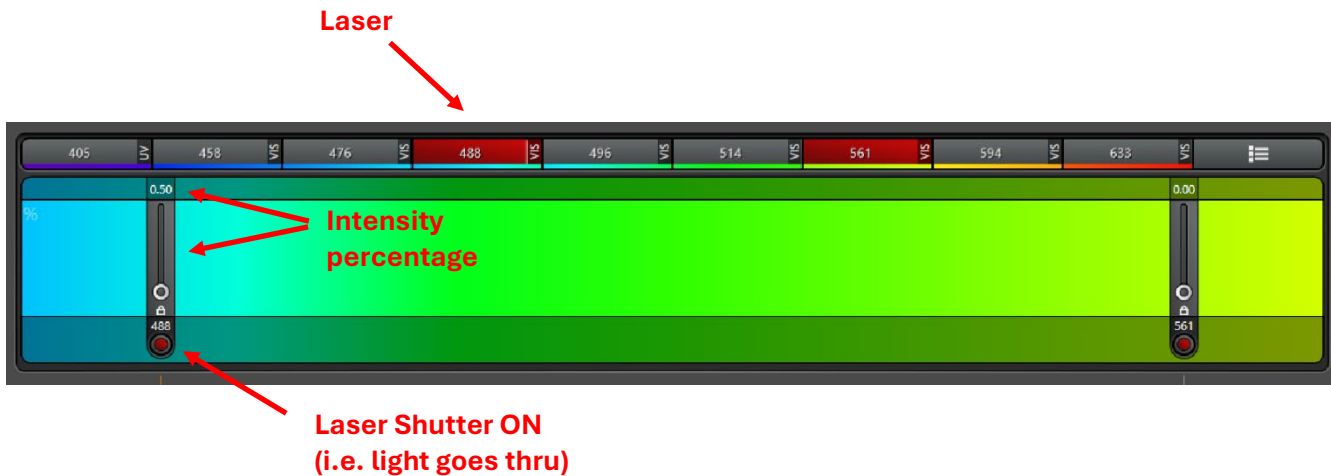
27. Set up **laser / detection setting** in **Acquire** tab / **Acquisition** section in software

- a. Things to keep in mind:
  - i. Set up a setting for one dye first THEN add sequences (**SEQ**) for additional dyes
    1. **One dye per sequence per detector**
  - ii. Make sure there is **no or very minimal crosstalk** between channels
  - iii. Assign a **different detector** for each sequence (i.e. dye)
    1. Do not use the same detector for all dyes → will wear out detector
  - iv. Assign a **different color** to each detector
    1. This is only to visualize your images on screen (not necessarily the same as fluorescent signal color of your sample)

### Setting up the 1<sup>st</sup> Sequence

28. In the **laser section at the top**:

- a. Select a laser at the top of the screen (**wavelength**) → laser bar will appear
- b. Change laser intensity to **2-3%** to start (double click on % to type in number → **press Enter**)
  - i. Less than 10% of any laser power is considered ok (i.e. not too strong)
- c. **Open** the shutter on laser bar
- d. A **dotted line (= excitation wavelength)** in the visible spectrum in the detector area will appear



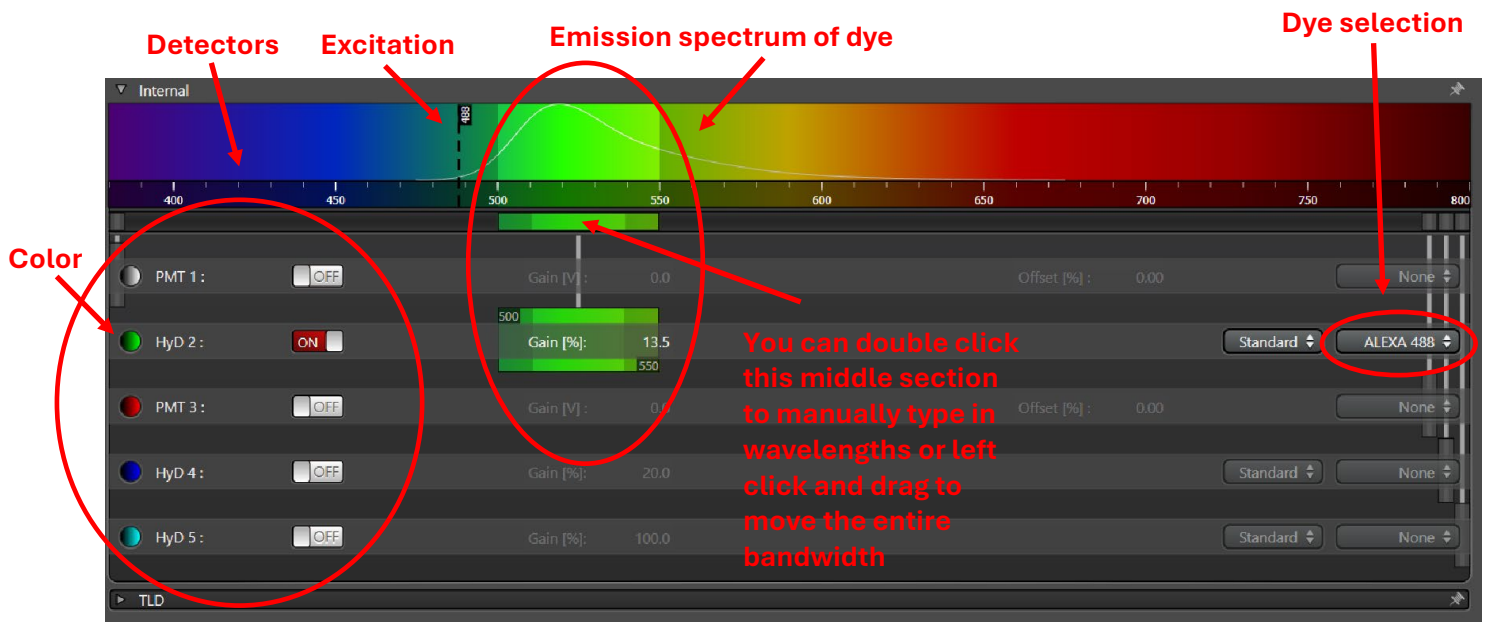


29. In the **detector section at the bottom**:

- a. Turn **ON** whichever detector you want to use
  - i. Use **PMT** for **bright dye** like **DAPI**
    1. Gain is 0% so you have to increase it manually to see the image on computer screen when live imaging. You can change offset (i.e. background).
    2. If your sample has high background, try using PMT detector and lower background manually (changing offset)
  - ii. Use **HyD** for **most fluorophores**
    1. Gain is already set at 100% so you can see the image when live imaging. You can adjust gain manually. You cannot adjust offset.
    2. High resolution and low background
- b. **Select the dye** on the right hand side (drop down menu), i.e. DAPI, Alexa Fluor 488, etc.
  - i. The emission spectrum of that dye will appear

**c. Move the bandwidth of emission spectrum at least 10 nm downstream (towards the red zone) AWAY from the dotted line**

- i. If not, this will destroy detector since laser will be shooting the light directly at the detector
  - ii. Ex) DAPI: excitation is set at 405 nm, so move the emission bandwidth to the right to 415 nm and set the end of emission wavelength **50 nm** from the start of the emission wavelength →  $415 + 50 = 465$  nm. So, the detector for DAPI will collect emission data in 415 – 465 nm range.
  - iii. Ex) Alexa 488: excitation is 488, so emission should be set to ~500-550.
- d. Assign a **color** to the detector → this is only to visualize your images on screen (not necessarily the same as fluorescent signal color of your sample)



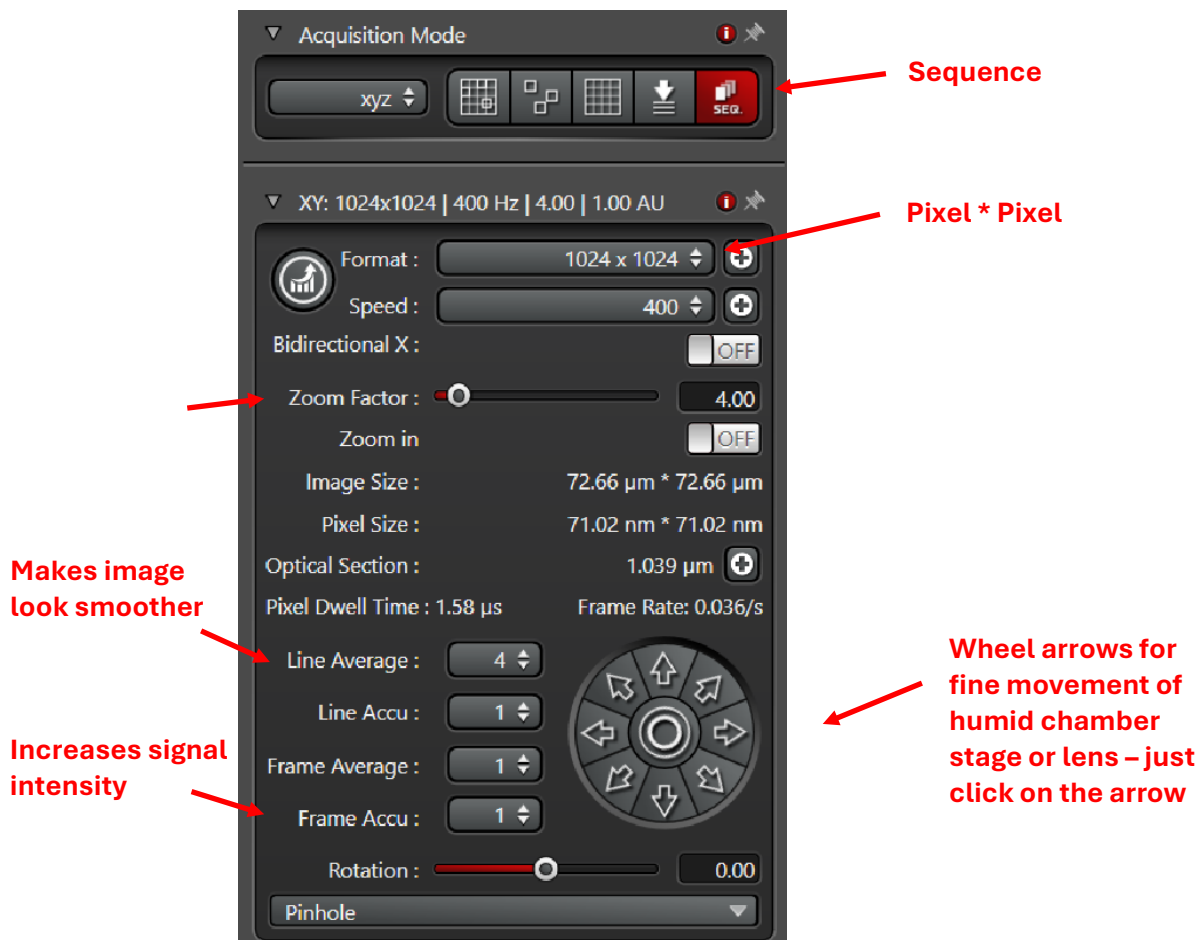
30. Click **LIVE** to see real time image and adjust settings

- a. Check saturation level → click on **LUT** icon on Image panel on the right side of screen (1<sup>st</sup> icon on the top left)
  - i. Blue: saturation
  - ii. Green: background
  - iii. Get image in dynamic range (i.e. from low to high signals)

**b. WAYS TO ADJUST SIGNAL INTENSITY**

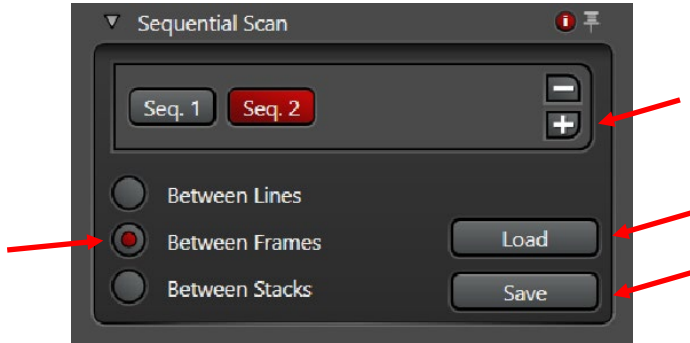
- i. **Gain / Offset on dial controller**
  - 1. **Gain: adjusts signal intensity**
  - 2. **Offset: adjusts background**
- ii. **Laser power**
- iii. **Line / Frame accumulation**
- iv. **Argon laser power if being used**

- c. You can zoom in by changing **Zoom Factor**
- d. Increase resolution to **1024x1024** instead of default 512x512. Keep the speed the same (**400 Hz**).
- e. If image is blurry, **re-focus** by moving **Z position dial**
- f. You can move image on screen by clicking on **wheel arrows** in the software (very fine movement)



**Setting up additional Sequences for different dyes**

31. When you see a good quality image (i.e. all the settings are optimized) in the first channel, this is Seq. 1 by default → click on **SEQ.** icon at the top under **Acquisition Mode** panel first then Sequential Scan panel appears at the bottom of screen → click on **+** icon to create Seq. 2. This Seq. 2 is a duplicate of Seq. 1 → it has all the same settings that you just set up in Seq. 1.



32. **STOP** live imaging → go to **Seq. 2** and **turn off** everything: turn **OFF** the detector → decrease the laser intensity to **0%** → **CLOSE** shutter → **DESELECT** that laser

a. Sequential scans should be **“Between Frames”**, not Lines

33. **Set up Seq. 2** by repeating above steps (as in setting up Seq. 1) → **repeat for all other sequences**

a. If you have DAPI → set up Seq 1 with DAPI first then set up all additional Seq’s. Then arrange Seq’s (left click and drag around the Seq. buttons) so that you start with longer wavelength Seq. (less energy) first to minimize photobleaching → set DAPI as last Seq.

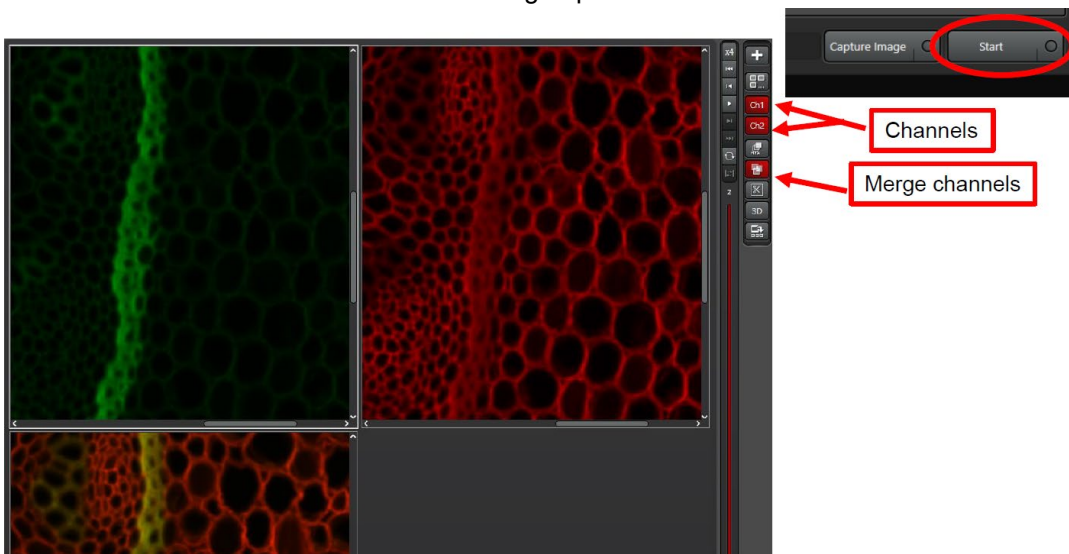
34. Once all **sequential channels** have been defined, you can collect images:

a. **Save** Seq. settings

b. A single channel image: select one Seq. → click **“Capture Image”**

c. Multichannel (sequential) scan: click **“Start”**

i. Leica will go through each channel in sequence and take images → can be viewed by individual channel or as in merged picture





**Z-stack images**

35. Set the boundaries:
  - a. Go to **Live** image
  - b. Go to **LUT** (saturation level icon)
  - c. Move down on Z until you get only background (totally green)
  - d. Select **Begin**
  - e. Move up on Z until you get only background (totally green)
  - f. Select **End**
  
36. Set **Z step size** to 1 um or type in how many “**number of steps**” you want (this will automatically calculate the z-step size)
  - a. Number of images should not be too many (i.e. should be ~10-15 images)
  
37. When you Start, Leica will take images in all channels with all Z-stack information

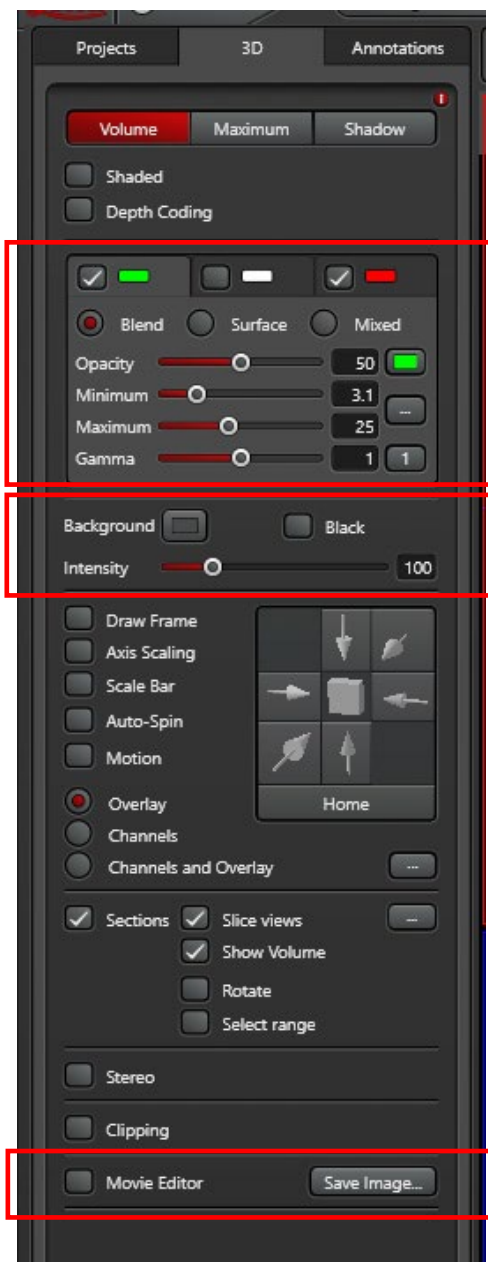


38. You can click on **Maximum Projection** icon on the Image Panel on the right
  - a. This function combines all Z-stacks and display in a 2D image → right click on image → take a “**snap shot**” → saves this Z-stack merged image

39. **Unclick Maximum Projection** and **select one good overlay Z-stack layer**

40. Click on **3D** icon to see **3D rendering**

- a. Another program opens up
- b. Change **background** to black
- c. Change **intensity** to 200
- d. Can change **signal intensity** for **each channel** by adjusting **minimum** and **maximum** scroll bars
- e. Can rotate, move around, make movies (Movie Editor) – add frames (400)
- f. **Saving a movie**: MPEG-4 format, 60 frames / second
  - i. **Save in YOUR folder** → **D: drive** → **Users** → **Your folder**



**Exporting data**

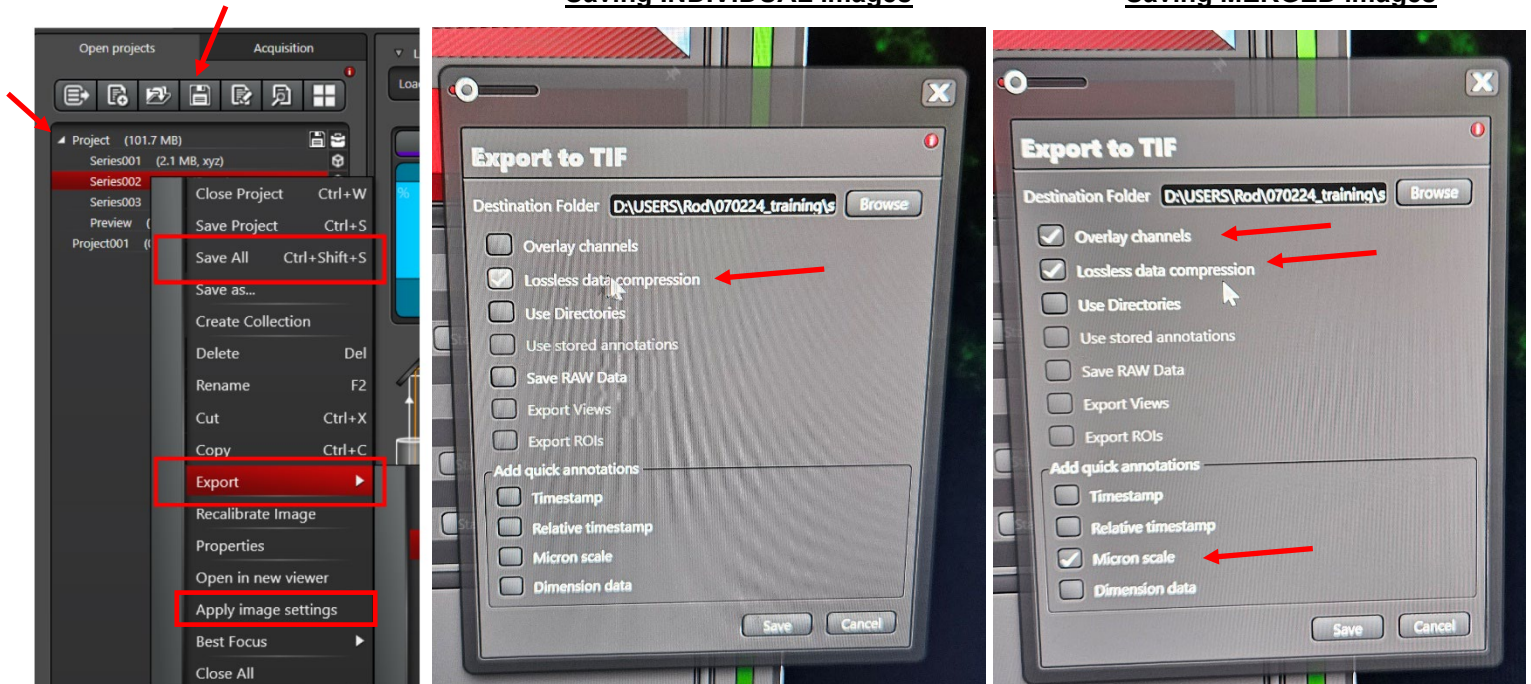
41. Bring your USB drive
42. Go to **Open projects** tab
43. **Save project** (click on **floppy disk icon** at the top, in the middle)
  - a. **Save in YOUR folder** → D: drive → Users → Your folder
  - b. **Rename the Project** when saving
  - c. Raw image format is **.LIF**
44. **Right click on Project** → **Save All**
45. **Right click on Project** → **Export TIFF files**
  - a. To save **Individual** images, select:
    - i. **Lossless data compression**
    - ii. Micron scale if you want
    - iii. Do **NOT** select Save Raw Data → black and white images
    - iv. Do **NOT** select Use Directories → will not save images
  - b. To save **Overlay** channels (merged), select:
    - i. **Overlay channels**
    - ii. **Lossless data compression**
    - iii. **Micron scale**

\* **Note:** This is where you can **load entire experiment setting**

46. Once you have set up an exp. setting, you can load it from an image that you acquired with this setting → open the image first then click on the image → click **Apply image settings**
  - a. Contains gain, offset, averaging, accumulation, and laser power values

**Saving INDIVIDUAL images**

**Saving MERGED images**





**\*\*\*\*SHUT DOWN**

47. **CONFIGURATION** tab → turn **OFF** all lasers
48. Turn **LASER KEY** under computer to **OFF** position → do NOT turn off the 3 green buttons yet
49. Turn **OFF** Mercury lamp
  
50. **LOWER** the objective lens
51. Get your **SAMPLE DISH** out
52. Power **OFF** the **INCUBATOR** (also **LENS HEATER**)
53. Turn **OFF CO<sub>2</sub>** gas by **CLOSING valves** in **REVERSE order** (close valve **#3, #2, then #1**)
54. **ASSEMBLE back** the **SLIDE STAGE** onto the microscope
  
55. **CLEAN** the **OBJECTIVE LENS**
  - a. Only use lens paper, not Kimwipes (harsh, may scratch the lens)
  - b. Wipe clean all the oil residue (wipe in one direction)
  - c. Use lens cleaner → one drop on the objective → wipe off with lens paper
  
56. Turn the **OBJECTIVE to 10x, DRY** (use the touchscreen or software)
57. **CLOSE LAS X**
58. Transfer data onto **USB**
59. **EJECT** your USB
  
60. **SHUT DOWN COMPUTER COMPLETELY** (wait until all computer and monitor lights are turned off)
61. End session in **iLab**
  
62. **When fan shuts off automatically**, turn **OFF** the 3 remaining **green buttons from right to left** (do this 5 min. after you turn off the laser key)
63. Put the microscope **COVER** back on
64. Fill out the **log book**