Leica SP8 HyVolution Confocal Microscope Operation

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

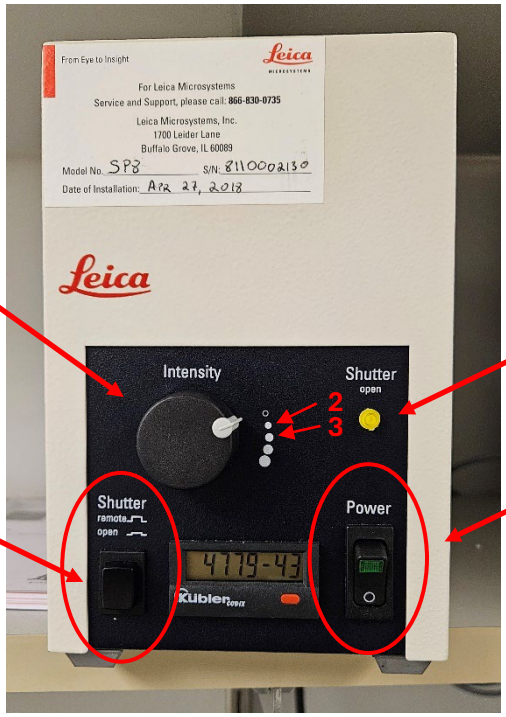
SYSTEM SET UP

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



3: Turn dial to 2-3 position

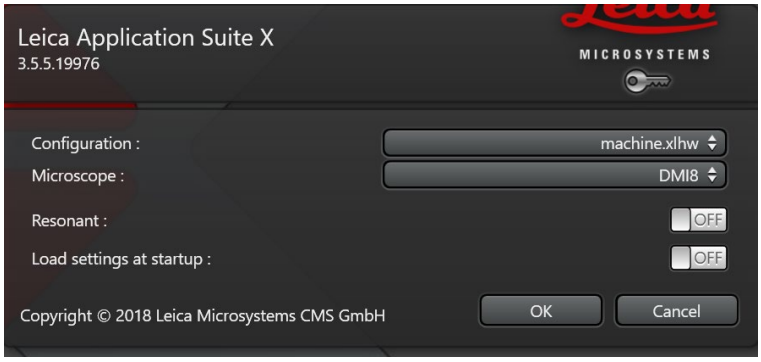
4: should be lit (not shown)

2: Shutter OPEN

1: Power ON

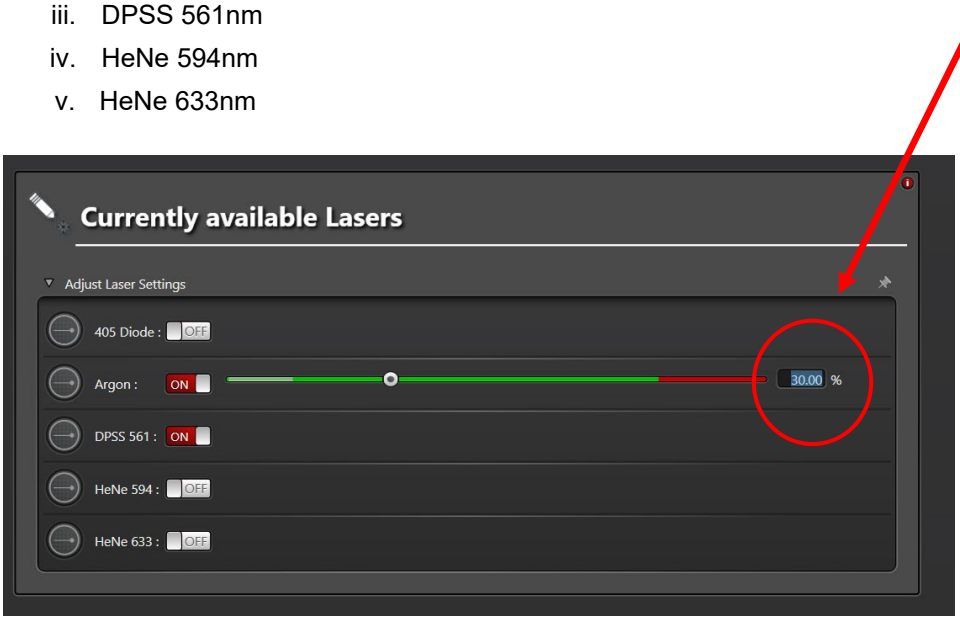
5. Open up **LAS X** program

6. In the **START-UP SCREEN**:
    a. Check the following setting → click OK



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

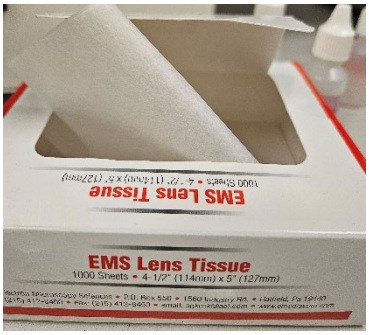
    a. **TURN ON <u>ALL</u> the lasers you need** for your experiment
        i. 405 Diode → use this for DAPI
        ii. Argon: 458, 476, 488, 496 and 514nm
            1. *<u>Note</u>: The default intensity for Argon laser is 0%. Start with 30% power and adjust according to signal intensity of your sample.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



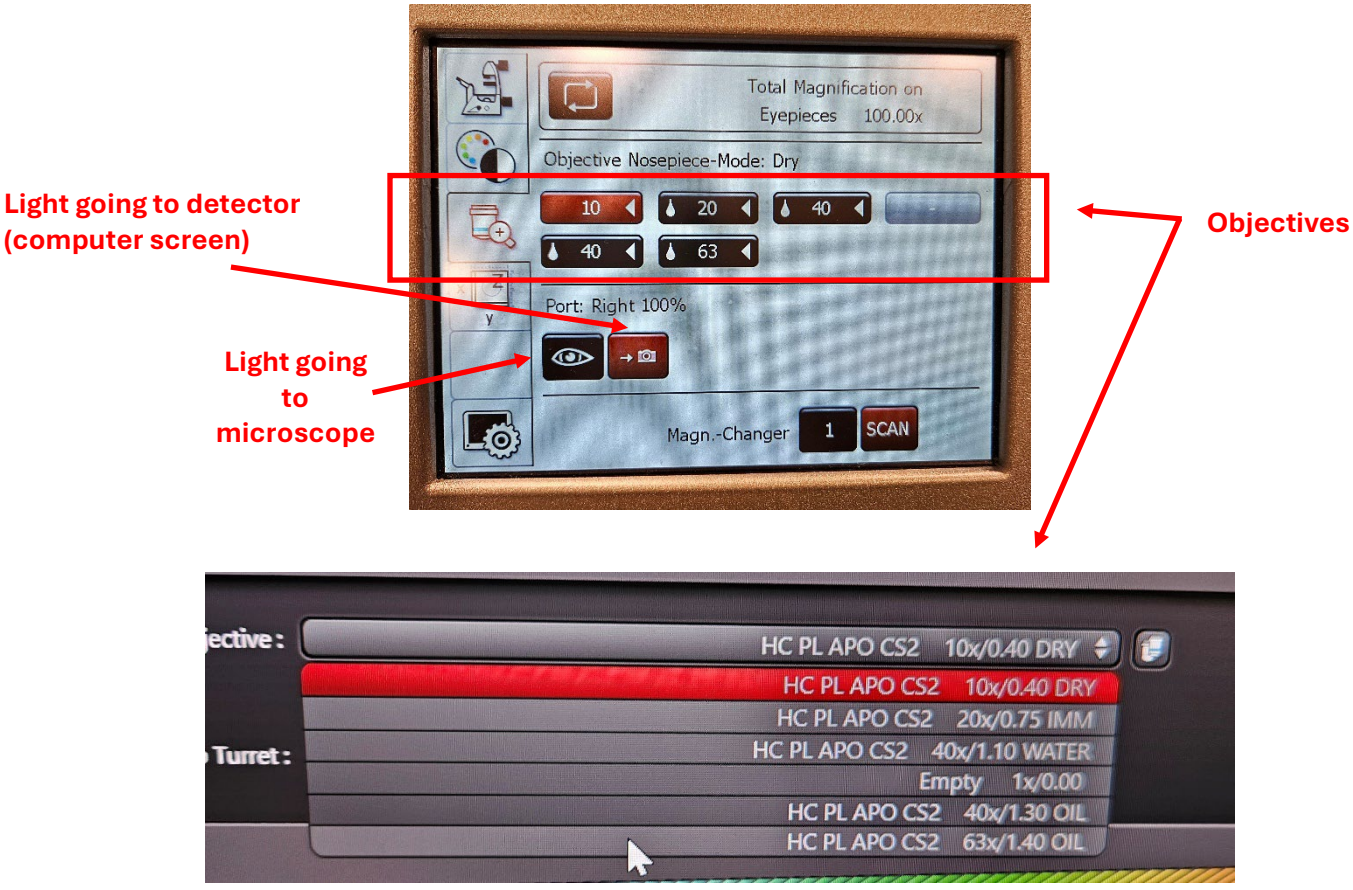
SAMPLE SET UP ON MICROSCOPE

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)



11. If using an **oil objective**, **put some oil** (2 drop size oil droplets) on the **sample** (on coverslip) **BEFORE** placing it on slide stage

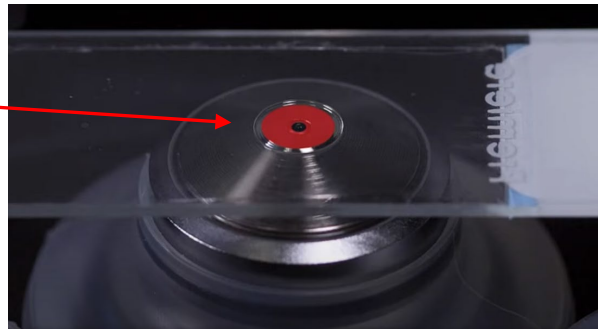
12. Load the slide onto slide stage **FACING DOWN** towards the objective lens
    a. Make sure objectives are lowered enough when loading slide onto the stage
    b. Make sure slide lays flat and fit snugly

13. Use **joystick dials** to position the sample slide close to the objective lens
    a. X dial: moves slide stage left and right
    b. Y dial: moves slide stage towards you or away from you
    c. Z dial: moves objective lens up and down
        i. Turn left (i.e. like loosening cap) → lens moves down
        ii. Turn right (i.e. like tightening cap) → lens moves up

    d. Keep the transmitted light arm tilted back so you can see the slide and lens movement
    e. 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
    f. Press **Z COARSE** button a few times then bring up the lens in Z direction
    g. 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

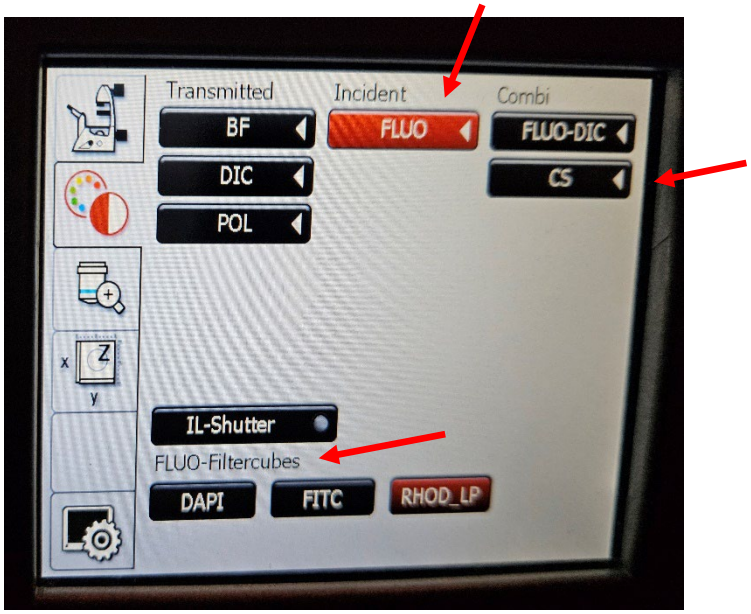
14. If using **immersion objectives**, lens **<u>has to make contact</u>** with the slide
    i. Use **coarse** movement to bring up the lens **first**
    ii. Once objective lens **<u>makes contact</u>** with the coverslip of the slide, **oil droplet will expand**
    iii. The minute the lens and slide make contact, change to **Precise** and **Fine** movement on joystick
    iv. Keep moving lens up slowly to a point where **oil droplet stops expanding** (this is the maximum closeness between the lens and the slide) → at this time, oil droplet <u>should cover the front part of the lens</u> (indicated in red circle below), but <u>not</u> the area beyond this point

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



15. Set the microscope with one fluorescent setting (i.e. **Fluo → DAPI,** etc.) on touchscreen → turn Z dial (still on precise and fine movements) **<u>counterclockwise</u>** to lower the lens → keep lowering lens until you find sample through eyepieces on microscope

16. 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

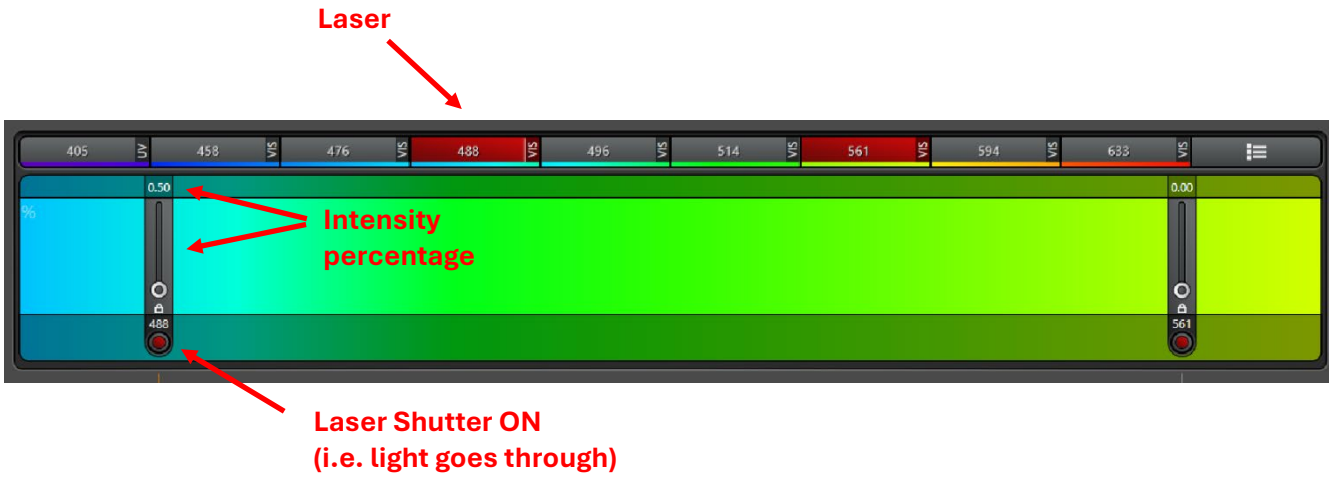


SOFTWARE SET UP

17. 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 1st Sequence

18. 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

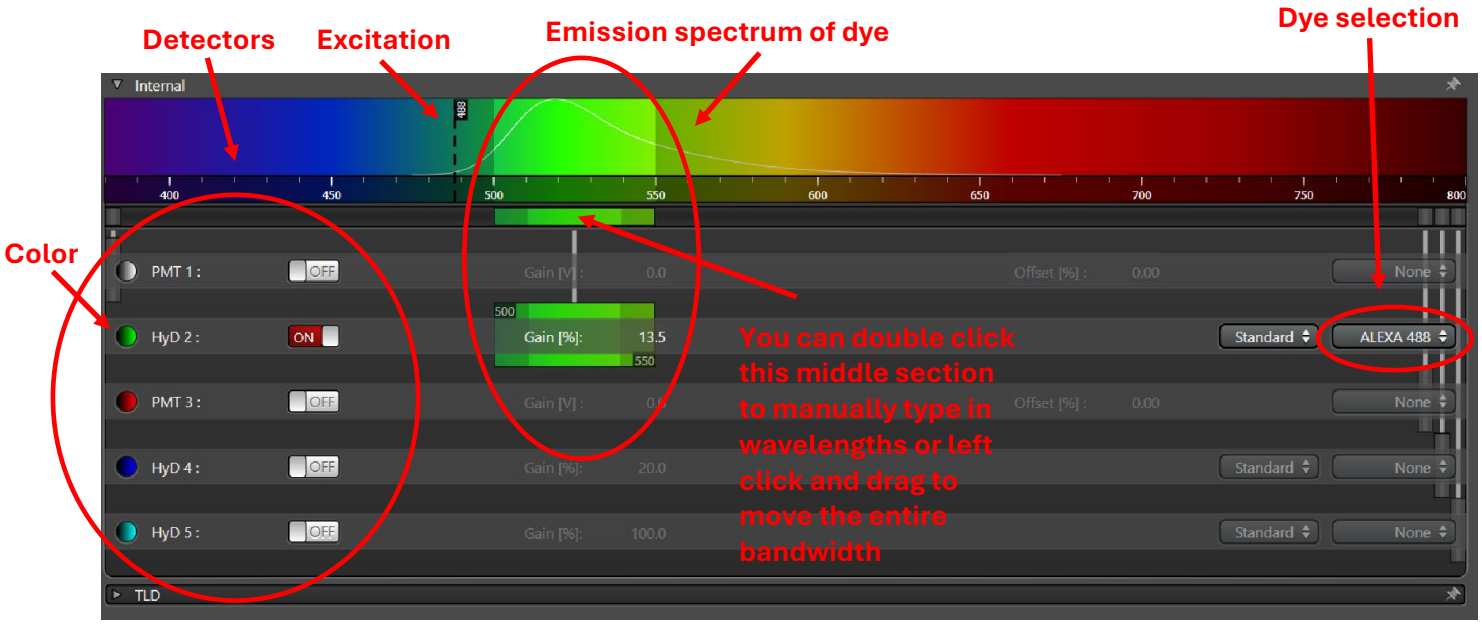


19. In the **detector section at the bottom**:
    a. Turn **ON** whichever detector you want to use
        i. Use **PMT** for **bright dye** like **DAPI**
            1. Gain is 0% so you have to increase it manually to see the image on computer screen when live imaging. You can change offset (i.e. 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.

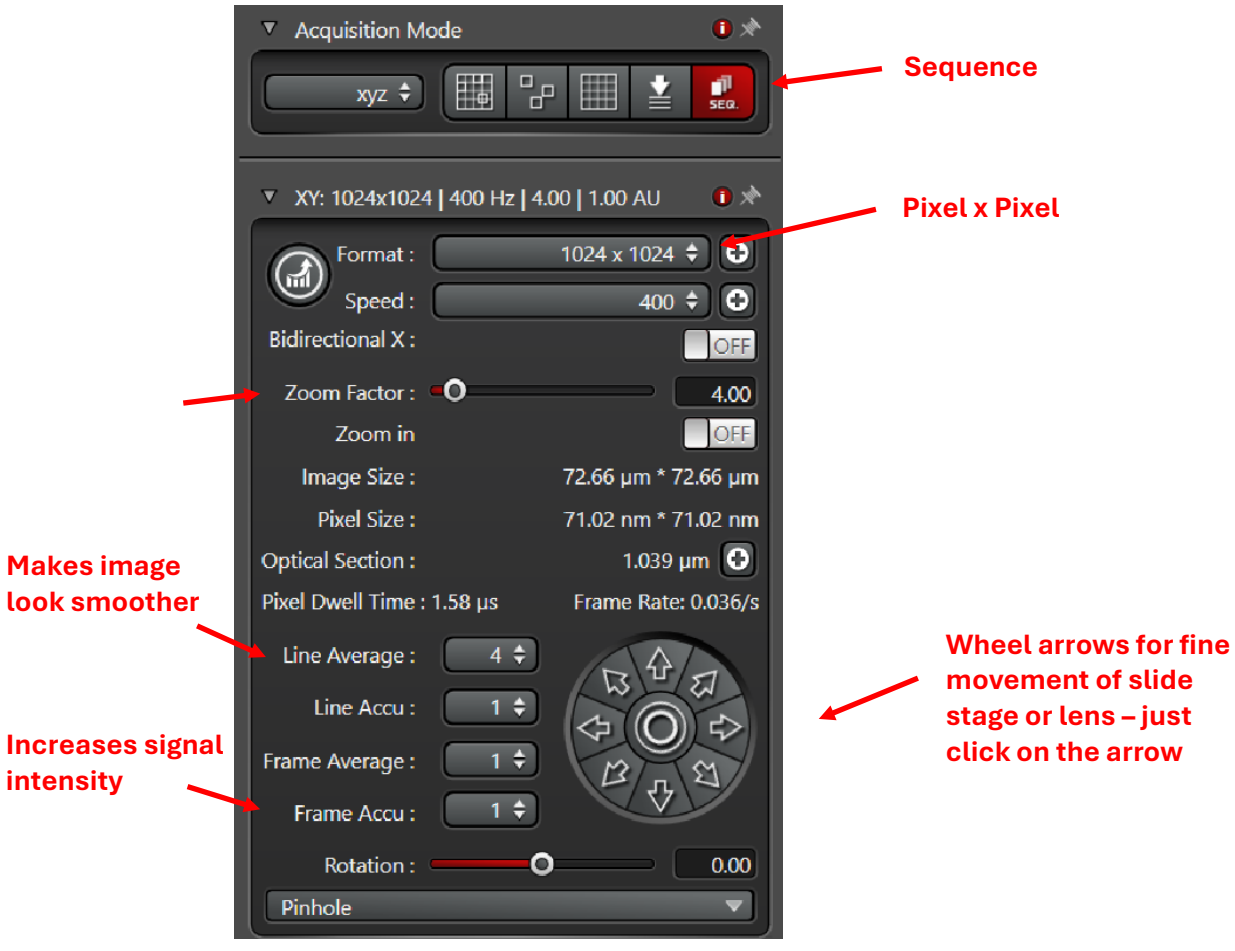
    d. Assign a **color** to the detector → this is only to visualize your images on screen (not necessarily the same as fluorescent signal color of your sample)



20. 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 (1st icon on the top left)
        i. Blue: saturation
        ii. Green: background
        iii. Get image in dynamic range (i.e. from low to high signals)

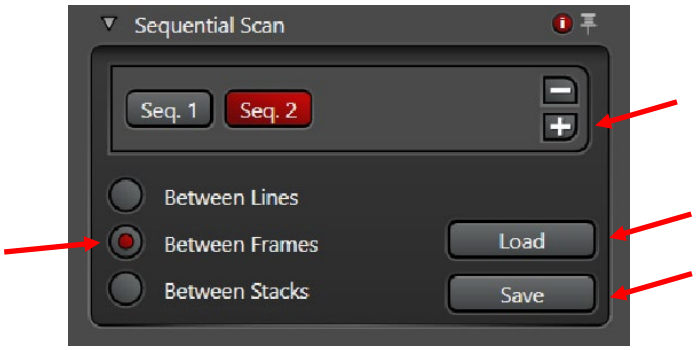
    b. **WAYS TO ADJUST SIGNAL INTENSITY**
        i. **Gain / Offset on dial controller**
            1. **Gain: adjusts signal intensity**
            2. **Offset: adjusts background**
        ii. **Laser power**
        iii. **Line / Frame accumulation**
        iv. **Argon laser power if being used**

    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

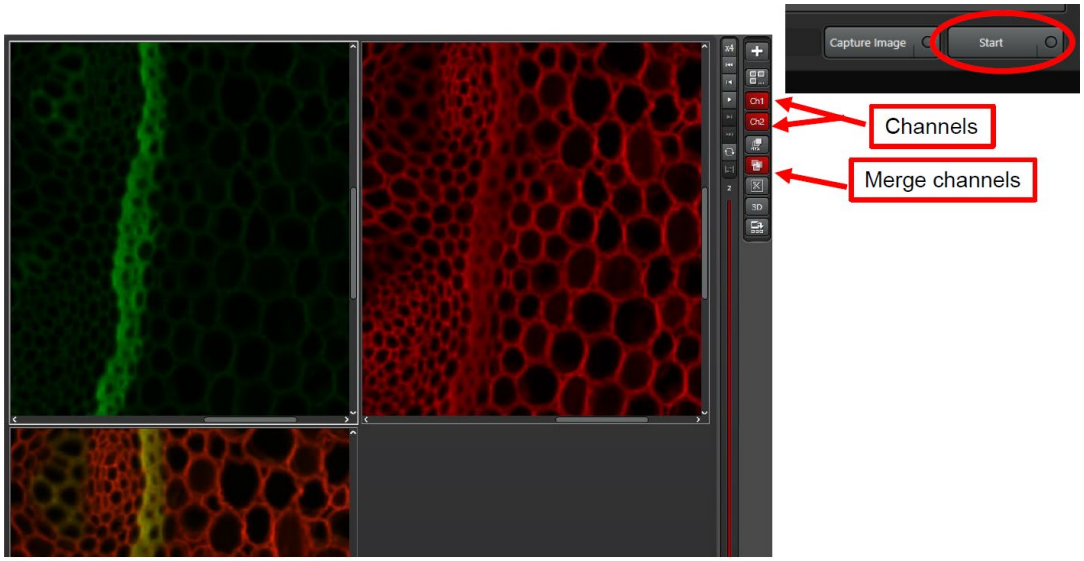
21. When you see a good quality image (i.e. all the settings are optimized) in the first channel, this is Seq. 1 by default → click on **SEQ.** icon at the top under **Acquisition Mode** panel first then Sequential Scan panel appears at the bottom of screen → click on **+** icon to create Seq. 2. This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



22. **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

23. **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.

24. 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

25. 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**

26. 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)

27. When you Start, Leica will take images in all channels with all Z-stack information

28. 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

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

30. 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

31. Bring your USB drive
32. Go to **Open projects** tab
33. **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**

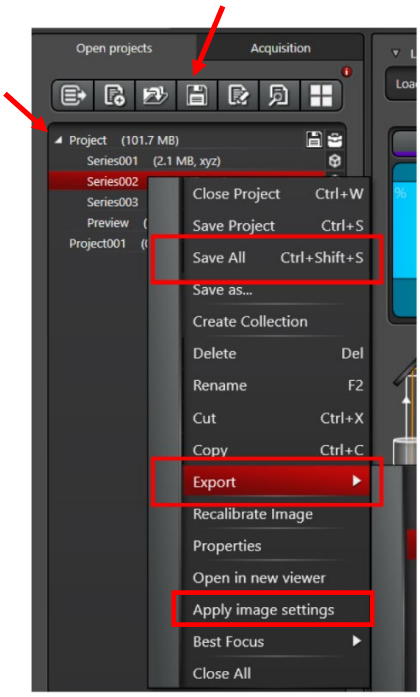
34. **Right click on Project → Save All**

35. **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

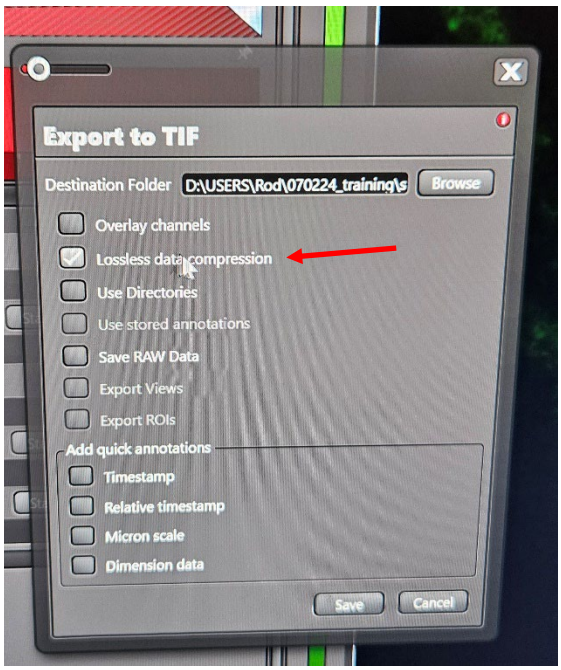
    b. To save **Overlay** channels (merged), select:
        i. **Overlay channels**
        ii. **Lossless data compression**
        iii. **Micron scale**

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

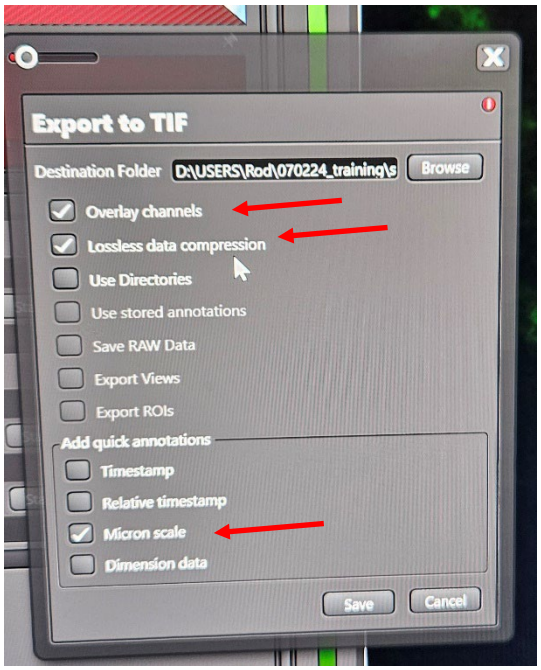
36. Once you have set up an exp. setting, you can load it from an image that you acquired with this setting → open the image first then click on the image → click **Apply image settings**
    a. Contains gain, offset, averaging, accumulation, and laser power values



Saving INDIVIDUAL images



Saving MERGED images



SHUT DOWN

37. **CONFIGURATION** tab → turn **OFF** all lasers
38. **Turn LASER KEY** under computer to **OFF** position → do NOT turn off the 3 green buttons yet
39. Turn **OFF** Mercury lamp

40. **LOWER** the objective lens
41. Get your **SLIDE** out
42. **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

43. Turn the **OBJECTIVE to 10x, DRY** (use the touchscreen or software)
44. **CLOSE** LAS X
45. Transfer data onto **USB**
46. **EJECT** your USB

47. **SHUT DOWN COMPUTER COMPLETELY** (wait until all computer and monitor lights are turned off)
48. End session in **iLab**

49. **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)
50. Put the microscope **COVER** back on
51. Fill out the **log book**