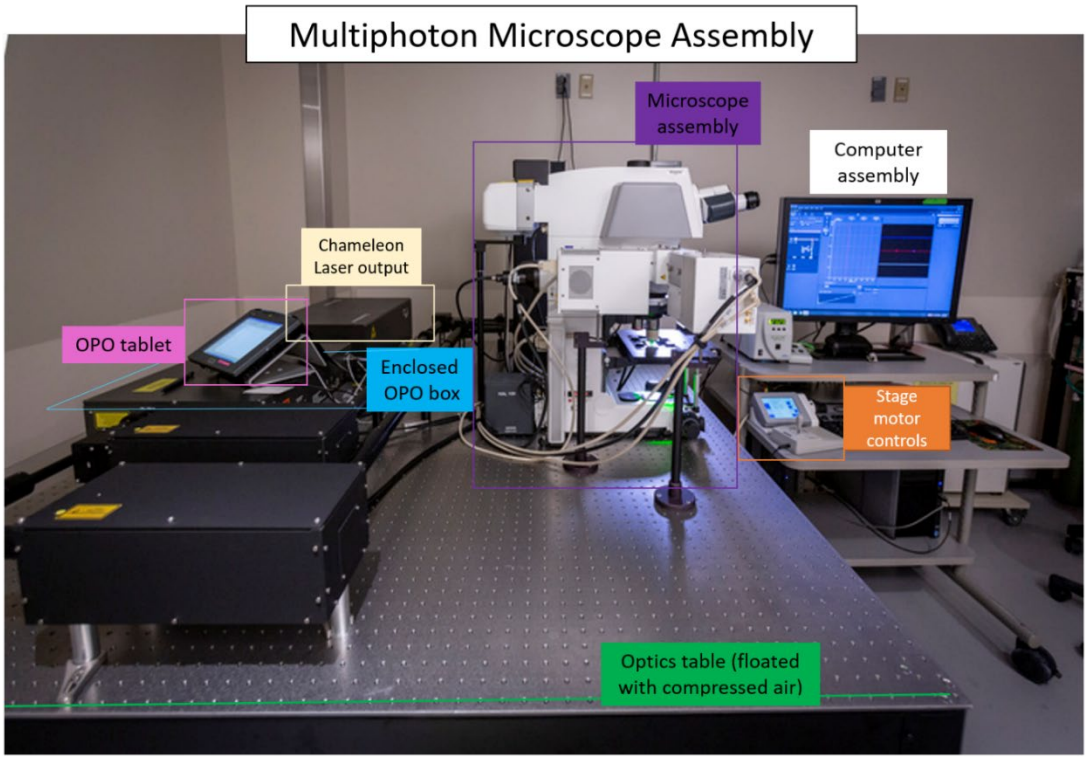
Two-photon fluorescence microscope basic training guide

This guide will outline how to perform basic operations with the two-photon microscope (2PM, also known as Multiphoton Microscope, MPM) in BE-154 within Comparative Medicine's animal vivarium.



This guide was prepared by Elena Carlson in Spring 2021. Contact x4021 or preclinicalimaging@fredhutch.org with any questions or comments.

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Maintenance and other miscellaneous topics like using a condenser and laser alignment are covered in the document "MaintenanceGuide.docx"

1. **Getting started:**
    a. Safety first!
        i. The chameleon is a very powerful, Class 4 laser. It can output up to 4 W power of *invisible* beams, which can easily blind a user. Be aware of where the beams are and practice safety while in the room and operating the microscope.
            1. Flip sign on front of door to indicate that the laser is in use.
            2. Move magnetic strip on inside of door to cover window.
            3. Put on safety googles
            4. Avoid leaning on the optics table, especially when the microscope is actively scanning. The table is floated, which means air is running to the table to ensure it is level and resistant to vibrations. Moving the table around also presents a safety risk to yourself and to the alignment of the laser-microscope system.
    b. Log into FOM to turn monitor on.
    c. Turn on all components
        i. To turn on the laser, flip key below table from "Standby" to "On". **Let this warm up for <u>at least</u> 10 minutes** before doing anything else.

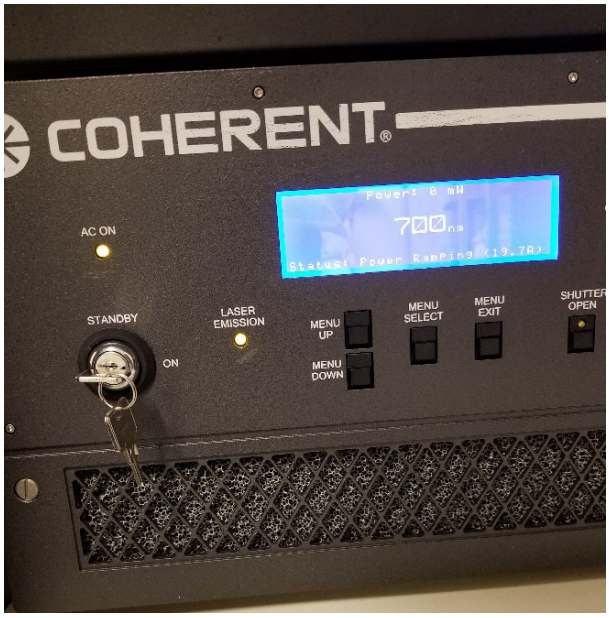

Figure 1: Coherent Laser Interface

        ii. Turn on system components (components → Systems PC → Main Power).

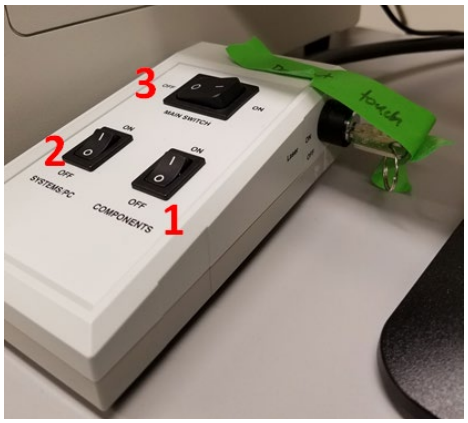

Figure 2: System Components

iii. Turn on PC monitor and login to LSM User account.
iv. Turn on X-cite lamp if you will need to view a fluorescent sample with the eyepiece.
v. Temporarily remove microscope cover
vi. Start Zen software (located on the desktop and task bar)
    1. Click “Start System” to setup for an imaging session.

2. Zen startup

a. First click the “acquisition” tab in top left box (1). Figure 3 is what you should see in the Acquisition tab upon initial start-up. The colored boxes and labels will be referenced in the following section for clarity.

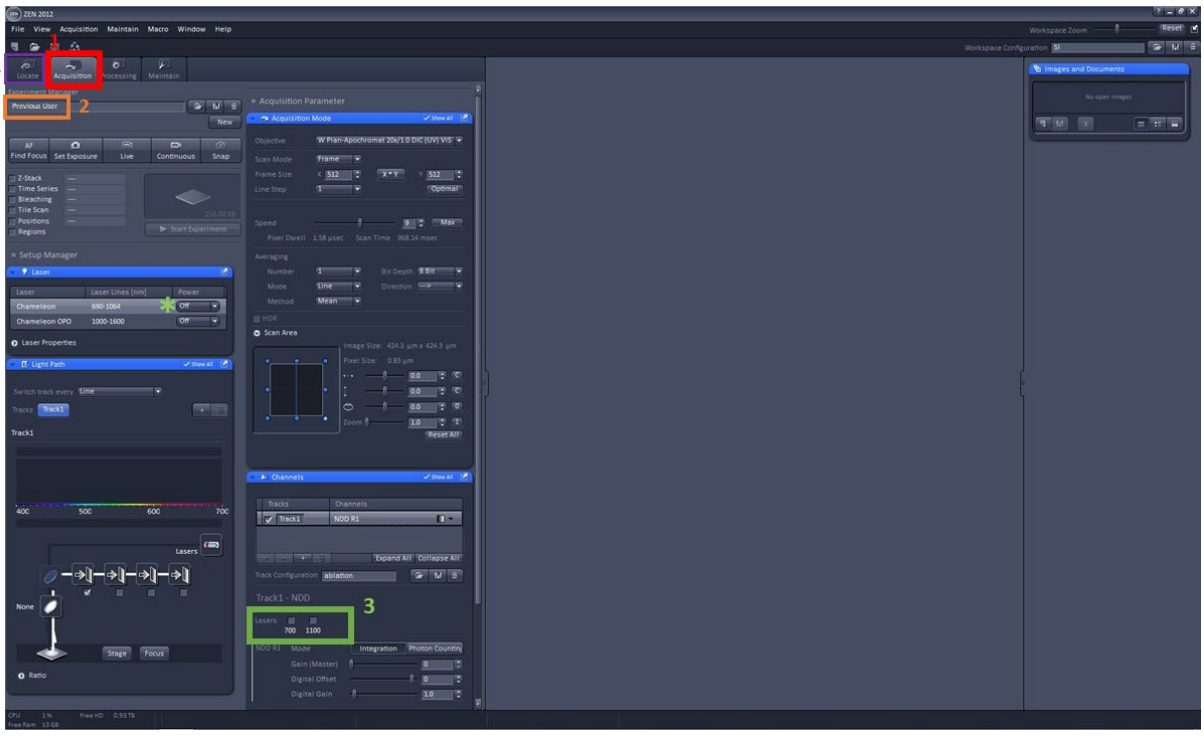

Figure 3: Zen Acquisition tab

b. Tuning the laser wavelength

i. The laser can be tuned to any wavelength in the range of 680 – 1080 nm. It should initially be set to an output wavelength of 700 nm. At the end of the day, it should be tuned back to 700 nm before turning everything off.

ii. If you would like to change to a different wavelength, in Zen you should **only move in steps of only 50 nm**, as this helps to prevent instability of the laser. (i.e. if you want to go to 850 nm, move from 700 nm→ 750 nm→ 800 nm→850 nm.).

iii. The Chameleon digital screen (Fig 1) confirms that the wavelength is set at 700 nm. Additionally, the laser is "off" in Zen (Figure 3 **box 3**).

iv. Click the 700 box in figure 3 above, **box 3** above to output the chameleon laser. You will receive a message asking if you want to turn the laser on. Click 'Yes'. The Chameleon box (by the green star) should turn to "On" once this has occurred. The bottom left half of the Acquisition tab should now look like Figure 4 below (**box 1**).

Figure 4: Laser Control Panel in Zen

v. There is now a box that you can type the wavelength you want to move to (Figure 4 above, **box 1**). If we want to move our laser wavelength to 800 nm, we should first type in 750 and press enter. Then wait for the red bar above to disappear, then type in 800 nm. The laser interface (below the table, Figure 1) should read 800 nm (it will display "Status: OK" when stable)

c. Filter determination: We need to select the desired filters imaging. It is good practice upon first starting an experiment to check all the small boxes in **box 2** in Figure 4

i. In Zen, the order of detection channels is Red, Far Red, Blue, Green from left to right

ii. These corresponding detection bands are 570-610nm (red), 645-710 nm (far red), <485 nm (blue), and 500-550 nm (green)

iii. Click on each filter to change the color. It is best to color these (in above boxes with arrow/filter symbol) as red, far red, blue, and green for clarity, so we know which channel we are be looking at.

iv. We will fine tune this and determine which filters work for our experiment later.

3. Using the OPO (If not using the OPO skip to part 4)
   a. The OPO can only operate when the Chameleon laser is outputting certain wavelengths. See below chart in Figure 5.

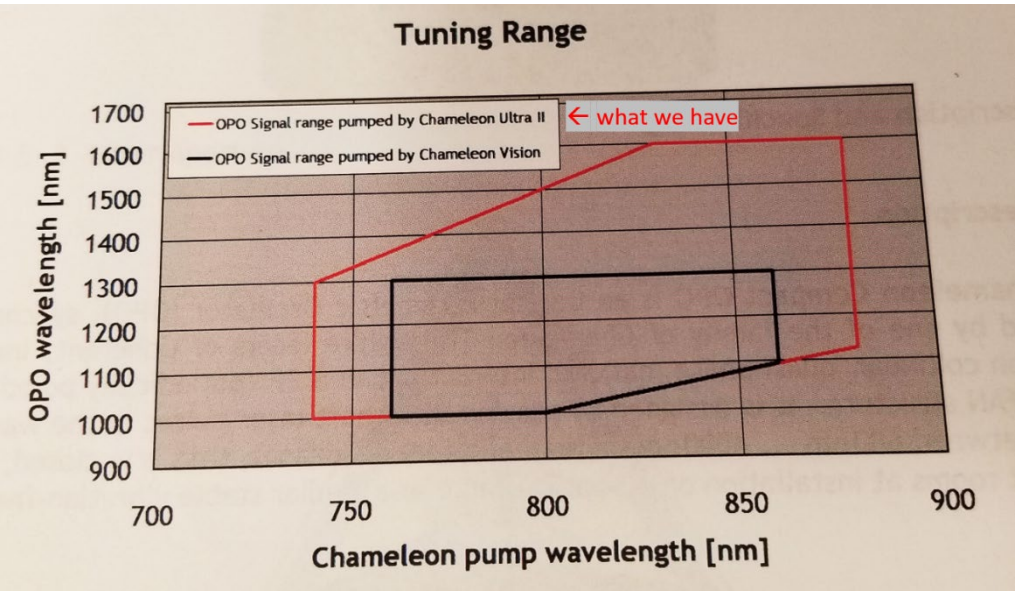

Figure 5: OPO Tuning Range Chart

   b. When system is not running, the OPO tablet (on laser table) will indicate the pump laser is 'not available' (Figure 6 below). When the chameleon laser is outputting a beam (done via Zen software) without the OPO on, the OPO tablet status will change to "bypass" and the tablet will display a peak at the wavelength that the chameleon laser output is set to.

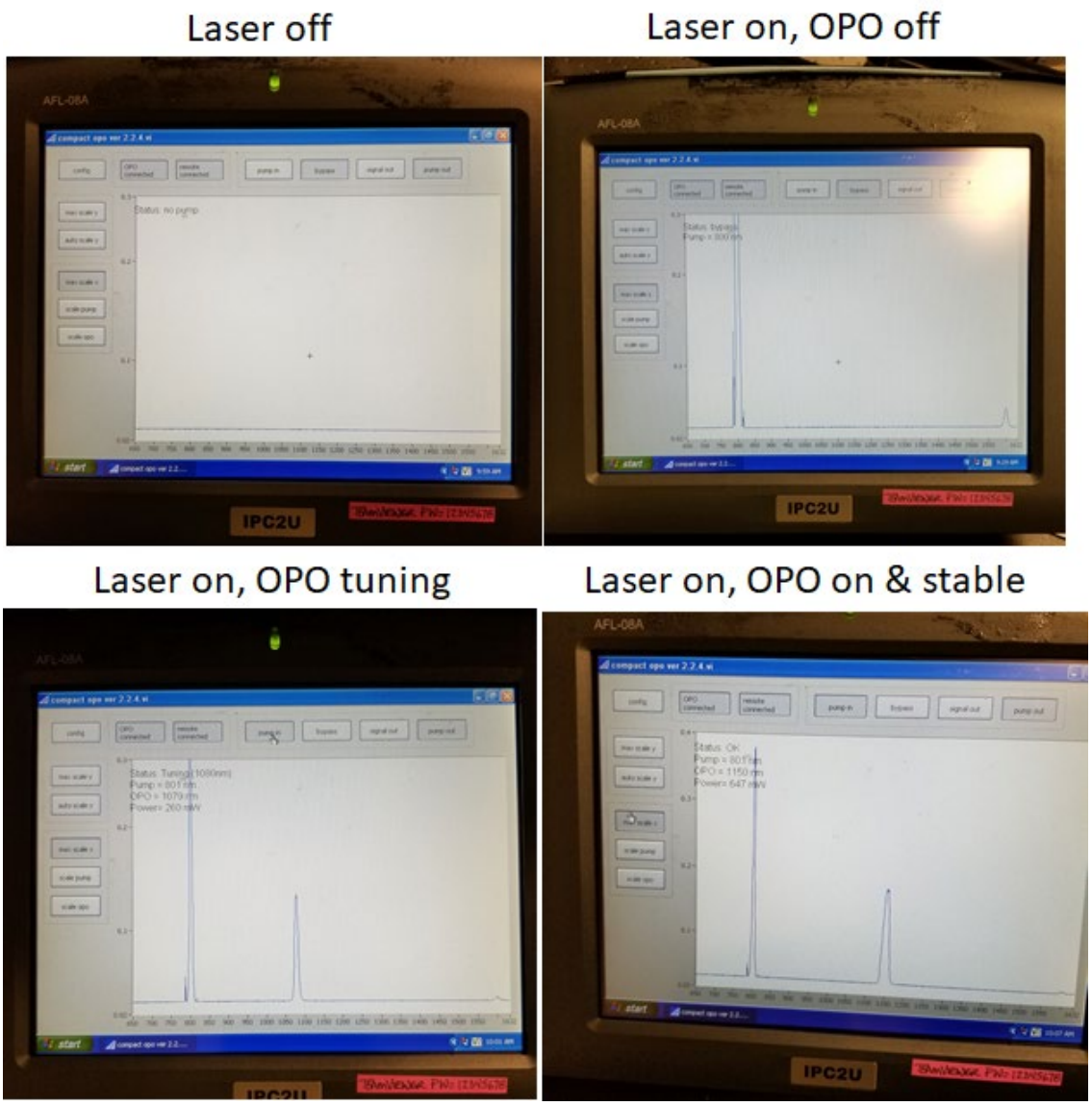

Figure 6: OPO interface

c. Ensure chameleon laser is set to an appropriate wavelength. Reference the red tuning curve in the below chart
d. On tablet, select “bypass”. This will turn the OPO on. Make sure the “Signal Out” box is selected for 100% of the OPO signal power to be output. If you want 20% pump (chameleon laser) power output and 80% OPO wavelength power output, select both “Signal Out” and “Pump out”.
e. The OPO wavelength initially outputs 1080 nm. The tablet will display that it is “tuning”. Wait until it says “Status = OK” to change the OPO wavelength in Zen (similar to the method described in step **2e**).
f. Once Status=OK on tablet, in Zen click the OPO box (right check box within green area in Figure 3) and adjust the wavelength <40 nm at a time in the same manner as is done for the chameleon laser.
g. Wait until tablet indicates the Status is OK to move the wavelength in Zen again

i. NOTE that when OPO is on, the Chameleon laser power will drop by about 25% of its original power level. The power can be adjusted accordingly (with the % Transmission bar mentioned in section 4.b below

h. See section **7.b** for instructions on turning off the OPO

troubleshooting

- IF OPO wavelength is UNSTABLE, retune the chameleon first. This will force the OPO to reinitialize.
- If tablet is not syncing with Zen software, reboot. Follow this procedure:
    - Ensure OPO is OFF in Zen desktop interface (on the main computer)
    - Press the red X at the top right corner of 'compact OPO ver 2.2.4' software
    - Press green start button at bottom left hand corner and select Shut Down > Restart
    - After tablet has restarted, select User 'OPO user"
    - Open compact OPO software
    - Once opened, select 'remote connected' and 'OPO connect' buttons

4. Adjust powers for imaging.
    a. This is a good point at which to turn off the laser lights to protect the detectors from damage.
        i. **VERY IMPORTANT NOTE ABOUT Detectors**:
        ii. LSM 7 is equipped with two LIGHT-SENSITIVE BiG non-descanned detectors
        iii. DO NOT OPERATE MICROSCOPE WITH LIGHTS ON!! Room light can damage detectors irreparably.
        iv. You can still see monitor with lights off. The monitor display settings are optimized for use in darkness. Please do not try to adjust monitor display settings.

b. After steps **2.f.iii**, the Zen interface should now look like what is shown in figure 7 below (except that the transmission power bar will be near 0)

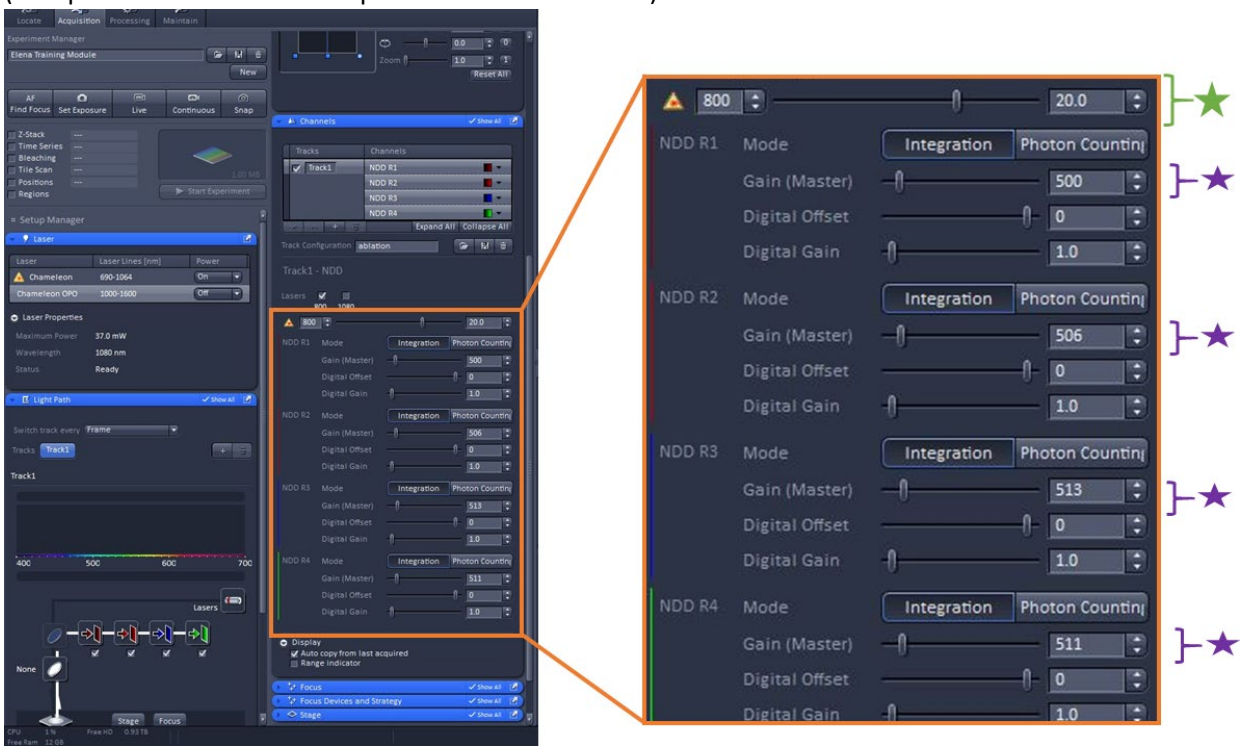

Figure 7: Power and Gain adjustment

i. Move power bar to desired transmission percentage or type in desired number in box on right. (**green star in Figure 7** above, **orange inset**)
    1. With a setting of 1% transmission, approximately 1% of the power listed ON THE DISPLAY will be delivered to the focus
        a. Example: if at 800 nm the laser digital interface (Figure 1) indicates there is 3124 mW power, a power setting of 1% transmission will deliver ~ 3 mW power to the sample. These powers are very dependent on wavelength and vary daily. Thus it is essential to check the power output listed on the Chameleon digital input daily before imaging your sample.

ii. Detector gain adjusted with "Gain (Master)" bar (**purple stars, Figure 7**). There is one Gain (Master) section for each channel that you are detecting.
    1. Start with this AS LOW AS POSSIBLE. Increasing Gain increases noise in your images. Therefore, it is better to increase power to your approved limit (not too high or you will damage sample or burn animal) THEN increase detector gain.

troubleshooting

- Cannot verify that the laser is on
    - Turn lights off in room. Cover tablet interface (it gives off a lot of light). *Carefully* place IR card under objective without touching objective, we do not want to scratch it. put microscope cover back over microscope. In Zen press "Live" button in Acquisition tab. You

will hear microscope start to scan. You should see light on IR card (Figure 8 below). You may need to move card closer or further to get the light focused on the card correctly.

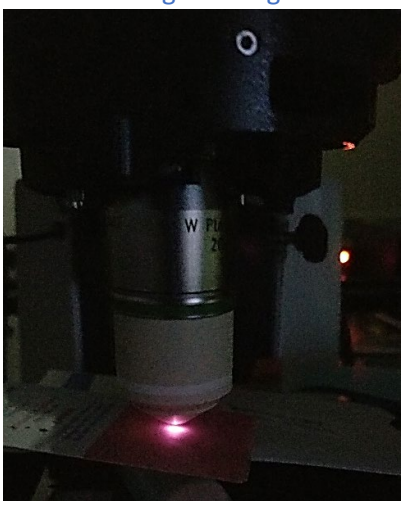

Figure 8: Verifying laser scanning

- If still no light, increase power in Zen.
- If still no light, verify that the box "Shutter Open" has yellow indicator light on (reference Figure 1, p.2). If it does not, press the Shutter Open button.
- If still no light, contact Elena Carlson (x4021, ecarlson@fredhutch.org or preclinicalimaging@fredhucth.org)

5. **Focusing the microscope onto your sample:**
   - **a.** Choose your objective. We have two:
     i. 5x Air with 75 mm objective adapter
     ii. 20x, 1.0 NA water immersion
         1. You can rotate their position on the microscope mount, but do not attempt to remove the objectives from the microscope and rearrange them. Leave these in their designated positions on the nosepiece. They are expensive!
   - **b.** Prep your necessary samples to put on microscope stage. Consider stage height. You may need to adjust the height of the stage to make your objective reach the sample and to focus on it. Your objective should be positioned ~2 mm from the surface of your sample.
     i. **Stage adjustment**, if necessary. This is best done with 2 people; the stage is heavy.

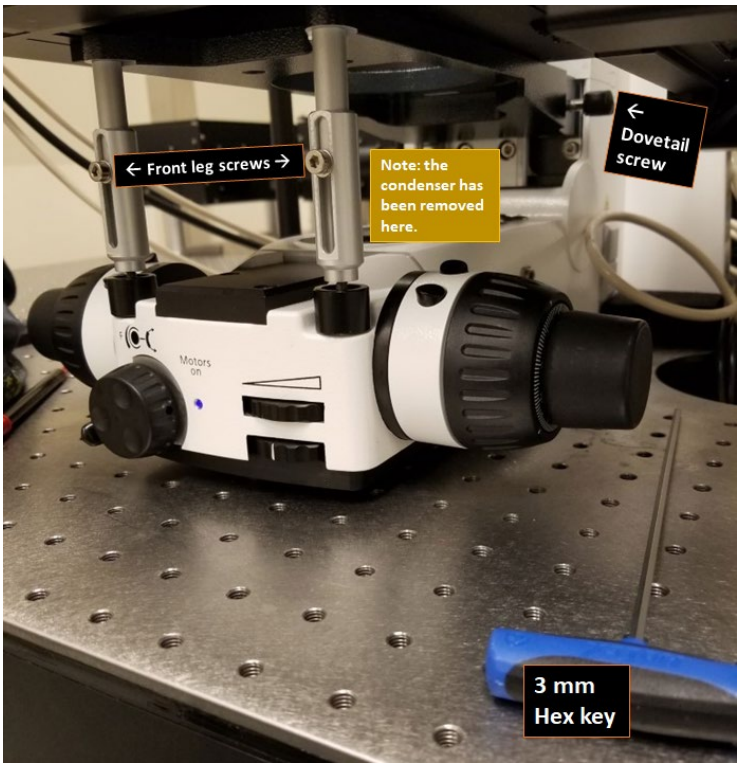

Figure 9: Stage Adjustment

1. Use 3mm Hex key to loosen screws securing the front legs (stored in black cloth holder within plastic boxes on wire rack)
2. Loosen dovetail screw in back of stage
3. Adjust height, remove stage by pulling and rotating
4. Tighten dovetail screw and front legs
5. Verify stage is level (use a phone bubble app or actual level)

ii. Put sample on. It is recommended you put a dye or test calibration sample on to test the system performance before proceeding with any animal imaging.

iii. Animal stage positioning

1. We recommend you develop your own lab-specific training guide on how to set up your lab's stereotax configuration for your study.

c. Confirm laser is not scanning.

d. Remove eyepiece cover.

e. Remove dichroic.

f. Finding focus

i. There are many ways to find focus. Here we will describe a manual method for finding focus on a calibration sample and one example of finding focus for animal imaging samples. Your method may vary, especially for your research animals based on your research application, but just be extra careful when working with the objective as we do not want to damage it. If you have never done this before, we recommend following this guide or asking another experienced 2PM user for guidance.

ii. Making test sample slide & finding focus (see Figure 10 below for step-by-step instructions)

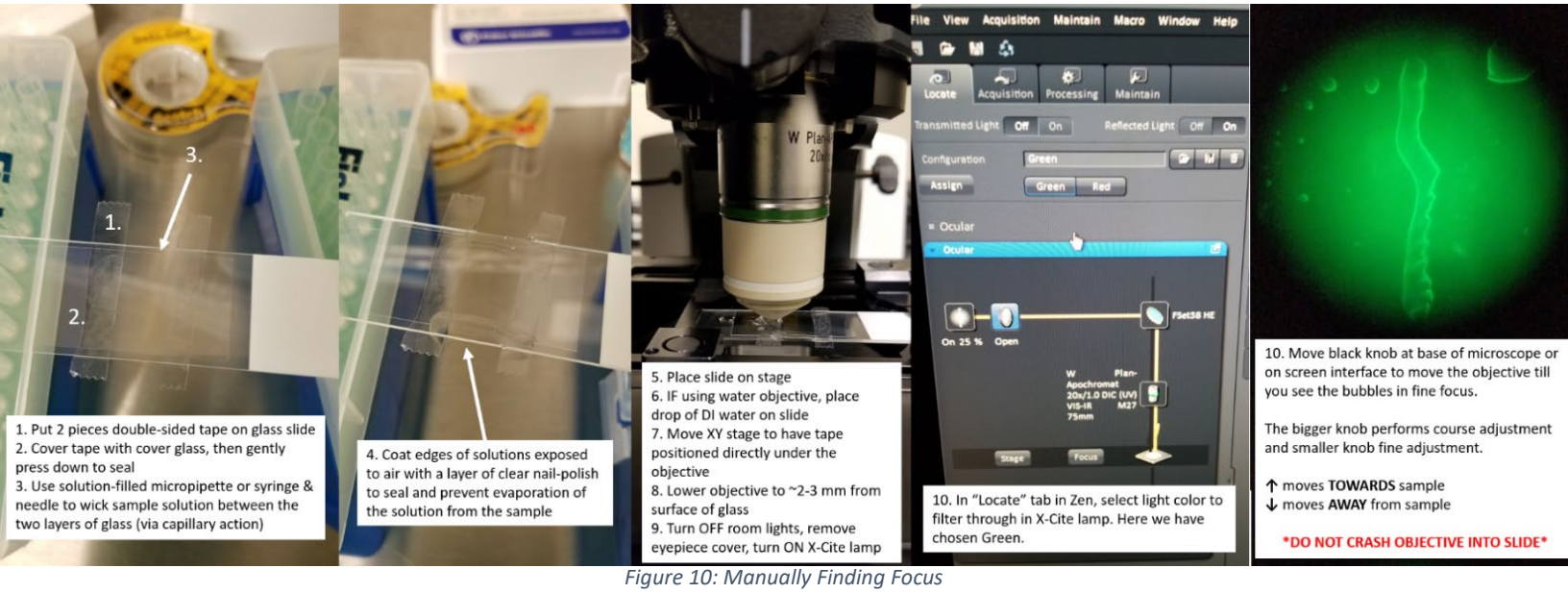

Figure 10: Manually Finding Focus

iii. Once you have found focus on bubbles, move XY stage to position objective over the CENTER of the slide where it will be focused in the midst of the test solution.

iv. Finding focus on animal samples is similar but trickier as you may not have something as clearly defined as a bubble to visualize. You will need to look for features such as blood vessels to visualize. This will likely require a lot of trial and error to find focus here, and we recommend consulting with lab members for advice on this. As always, please be mindful of the objective's position while doing this, being careful to avoid running it into the sample and damaging it.

g. Place dichroic back in microscope slot

h. Turn off X-cite lamp.

i. Replace eyepiece cover and microscope cover.

j. Proceed with imaging.

6. Controlling the microscope in Zen:

a. We will now go between the acquisition tab and locate tab in Zen to determine which filter settings are needed for imaging your specific samples, and to acquire some images.

b. Configure correct filters and save settings.

i. Put sample of interest on

ii. Click on the "Locate" tab.

iii. With lights off, press "live" to acquire live images. In the main box on the right in Figure 11 below you can see all 4 channels coming in. The Merge channel at the top right is also displayed

c. Each new image/result will pop up in a new tab (**red box 1** in Figure 11 below). On the right side of the screen you can add more notes about the image, do some initial data analysis, and adjust the display settings.

1. The reason there are 4 separate channels is because in section **2.c** we selected all 4 filters to be checked.

2. The gain settings for each channel are below the power transmission bar, one set of parameters for each channel (in a different color)
3. Adjusting the gain for channel should yield a signal intensity change for each channel. Be careful not to saturate the detector!
4. If this is the first time working with a new substrate, here you may want to do a wavelength scan, depending on your application, to probe your fluorophore's response to different probe wavelengths and the effect on different channels.
    a. You likely won't need to collect 4 channels; most people only need 1 or 2 channels for their research.
    b. If you do not see a substantial change when varying your wavelength in a given channel, or if a channel gives no signal except noise at the wavelength you have chosen, you should 'deselect' that channel by unchecking the box

d. Once you have decided which filters, channels, and wavelengths are necessary for your experiment, you can save these settings in Zen by clicking the "save" button (near the **orange box** back in Figure 3) and save the experimental settings under your name or an experiment name so that you can re-open these settings for when you repeat this experiment. Save these parameters in an appropriate file location.

e. Set the parameters in Zen for scanning.

i. In left-most tab area, collapse the blue laser bar and open Acquisition Mode (under "Acquisition Parameters", see Figure 11 below). Here you can adjust the number of pixels acquired per frame (2), the **scan speed (3)**, the **number of times to average an image (4)** (to increase the SNR), **the bit depth (5),** and **how zoomed in you are in your field of view (6)**. (see fig. 11 for colored box references)

1. It is recommended that you collect data at 12-bit depth or greater if: you plan to publish this data, if you need quantitative data, or if imaging in low signal regimes. However, also consider that a greater bit depth and greater number of pixels will slow down your scan speed.

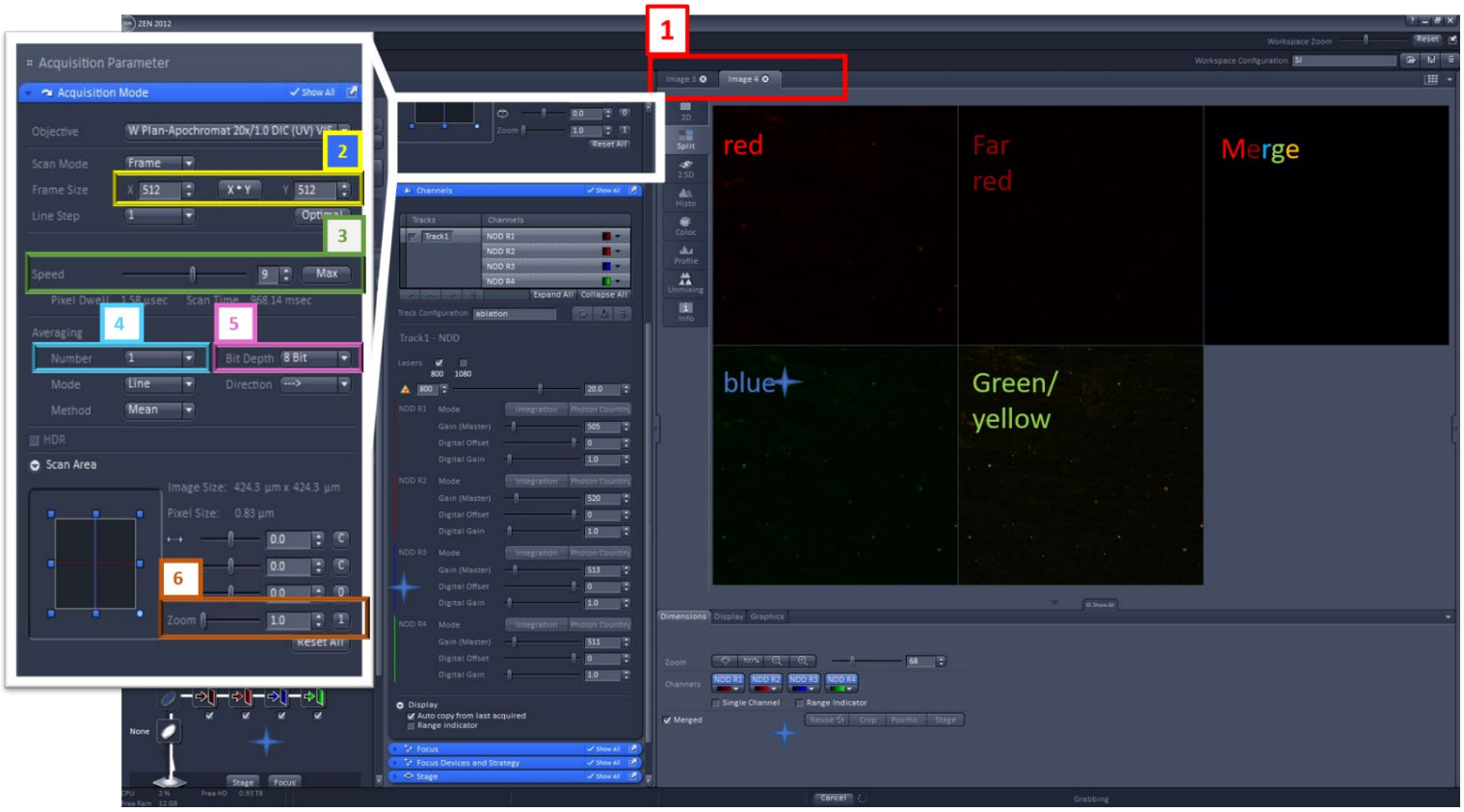

Figure 11: Image Acquisition Parameters

f. Acquire and view images by selecting “Set Exposure”, “Live”, “Continuous”, or “Snap”. ***Room lights MUST be off before selecting any of these below***

- **i.** **Set exposure** pre-adjusts the detector gain and offset for you.
- **ii.** **Live** does not *save* data, but it provides a quicker, live look at your specimen. This is useful for changing the focus and moving to a different area of the sample.
- **iii.** **Continuous** continuously scans at the selected scan speed
- **iv.** **Snap** records a single image
- **v.** **Stop** stops the current scan procedure

g. Save each image as it comes in by clicking file>Save with the image tab of interest open.

- **i.** Bring a CLEAN USB to transfer data. It MUST be a clean USB that has been scanned for viruses (be aware that even brand-new USBs can have viruses on them depending on the security of where/how they are manufactured). You must scan for viruses before plugging into the LSM computer, no exceptions.
- **ii.** Note: LSM 5 file format is the native Zeiss LSM image data format and will contain the hardware settings and all the extra information saved

7. Turning off the system

a. Always check that you have saved your data before closing the software! (see 6.f and 6.g above)

b. IF using the OPO:

- **i.** IN ZEN: Set OPO to off

ii. ON TABLET: set to bypass mode
iii. Leave tablet on

c. Turn off laser
   i. First in ZEN: turn OFF chameleon
   ii. Turn laser key to standby
d. Quit ZEN software
e. Turn off X-Cite lamp
f. Power off system controls (Fig. 1, p.3), remote switches Main Switch →Systems PC→Components.
g. Leave PC on
h. Log out of FOM so you aren't charged for further time

8. Further resources for more advanced topics

To perform more advanced topics like z stacks, time series, tile scans, we strongly recommend watching these two webinars from Zeiss (the manufacturer of our microscope):

Getting started with ZEN blue | Basic Training Part I

- *How to acquire an image step by step*
- *Introduction of the most common modules for Life Sciences*
- *How to set up a Multichannel Experiment*

Generating Multimodal Acquisition Experiments | Basic Training Part II

- *Complimentary to Basic Training I*
- *How to set up a Tile experiment*
- *Combine the different modules (Z-Stack, Multichannel, Time Lapse, Tiles & Position, Autofocus) with the Experiment Designer to generate a multimodal acquisition*

You can find these webinar trainings by searching for "Software Training / From Basic to Advanced" in the search engine at zeiss.com or by going to https://www.zeiss.com/microscopy/int/cmp/afs/21/webinar-series-software-training-from-basic-to-advanced.html?vaURL=www.zeiss.com%2Fsoftware-series

Note that these webinars are tailored to work with Zen Blue (we have Zen Black) but this is still very similar to the Zen Black software and setup that we have. These should be helpful to get an idea of how to perform these more advanced topics.