BRUKER TWO PHOTON MICROSCOPY GUIDE

Intro:

This manual is meant to serve as a reference for best practices and to assist with basic troubleshooting. It does not replaced hands on training with an experienced user nor does it cover every situation/issue you may encounter. If you are uncomfortable with or unsure of something, ask for assistance!

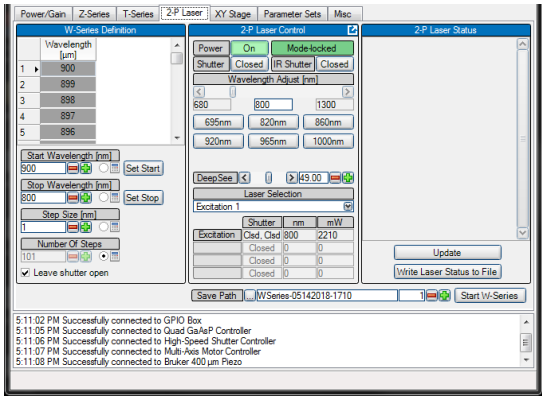
General Rules to follow:

- Lights must be off and the curtain to the dark box sealed before applying any PMT gain or increasing laser power. Conversely make sure to stop scanning and zero everything if you have to turn the lights back on or check your sample
- Always be aware of how much power you are using post objective! Refer to power curve posted on the wall. Otherwise you will bleach and/or damage your sample
- Always book the microscope in advance to avoid overlap and premature shutdowns
- Before imaging know your excitation/emission wavelengths and set the laser wavelength and filter cube accordingly

Section 1(a): Setting up for Imaging:

1. Turn on tower (Always do this first!)

2. Turn on computer and open Prairie View
3. Laser ON
4. Wait for mode lock



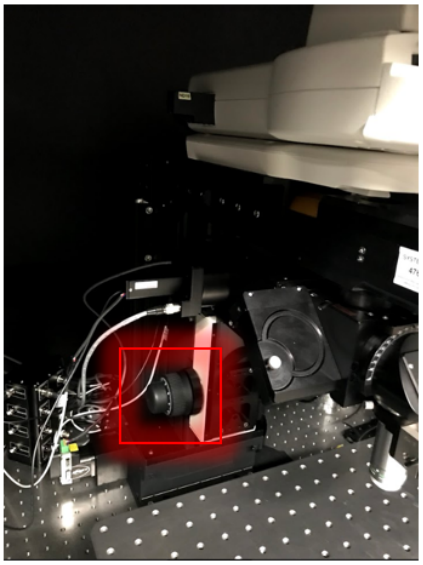
5. Laser shutter open
6. DON'T TOUCH IR SHUTTER, NOT FOR YOU
7. Select wavelength you'll use for imaging
8. Wait 20-30 minutes for pockel cell to warm-up
9. Just before putting your sample under, turn the lamp power on to allow it to briefly warm up (~1 min)

Section 1(b): Shutting Down:

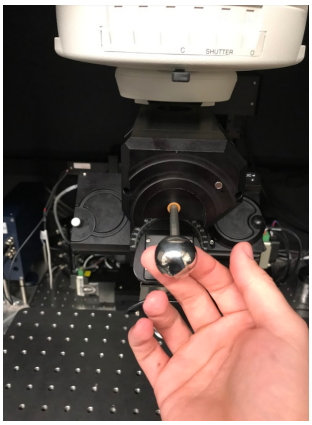
1. Always ensure proper shut down as the last user of they day! You can check the calendar to see if there are any other bookings
2. Ensure pockels/PMTs are zeroed (should be already after imaging is finished
3. Turn off and shutter laser
4. Exit Prairie View (If you have forgotten Step 3 it should prompt you about it
5. Ensure all your data is saved/transferred
6. Shutdown Computer (Always do this first!)
7. Shut off tower

Section 2: Focusing on your sample

1. Using the coarse focus knob, bring the objective slightly higher than it will need to be, this should be right around the center of the Z range marked by the orange arrows


Orange tape pictures here

2. Fix your sample under the objective, being very careful not to touch or bump the objective
3. Carefully bring the objective slightly closer than the working distance of the objective, judging carefully by eye and not allowing the objective to touch the sample
4. Using a transfer pipette, carefully add DI water between the sample and objective, making sure complete coverage and no bubble (Tip: Have the pipette tip just above to tip of the objective and allow the water to flow down the side of the objective)
5. Swap to Binocular focus/1P Illumination
    - Turn on lamp light to lowest power (one notch on the dial)
    - Open turret shutter
    - Pull out plunger gently to slide mirror into place(blocking laser)
    - Swap dichroic to mirror (Open latch,slide gently to desired setting, close latch)

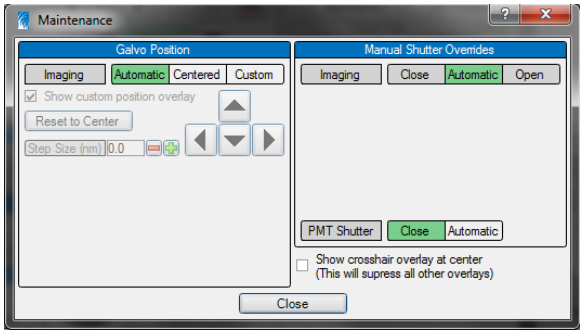


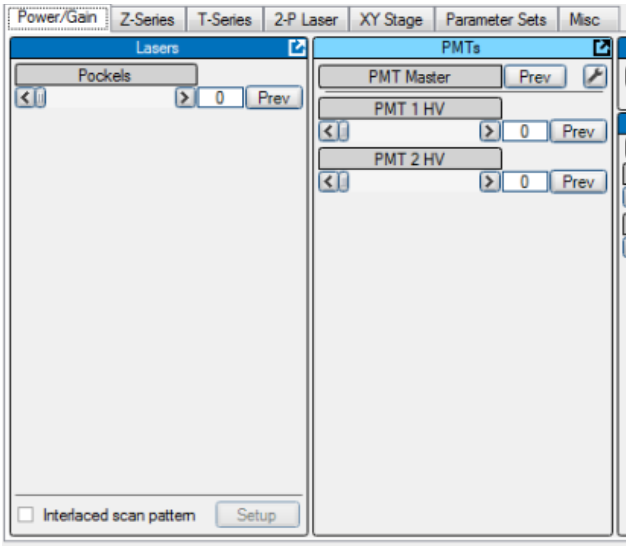

mirror/dichroic here

6. Slowly move objective up and away(Rotate coarse knob away from you) from the sample, which should move you toward your focus
7. While adjusting, monitor the image through the binoculars, looking for your sample to come into focus
8. Once you're happy with what you see, swap back to scanning configuration by:
    - Turn lamp dial to off
    - Close turret shutter
    - Push plunger gently back into place
    - Swap from mirror back to dichroic
9. Close curtain and seal using the magnetic strips
10. TURN OFF LIGHTS AND CLOSE DOOR CURTAIN
11. If not already done, swap door sign to red/active

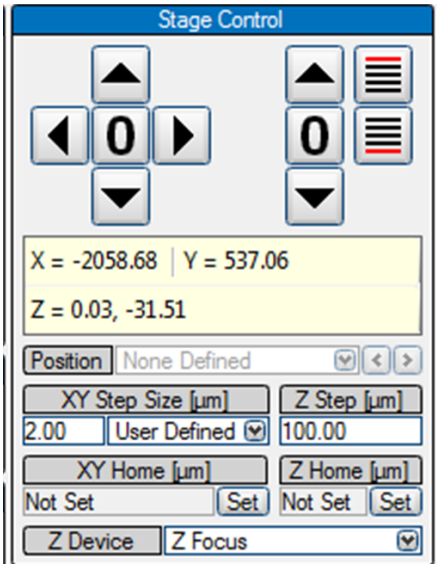
Section 3: Normal Scanning

1. Use Section 2 to prepare for scanning your sample
2. Double check shutter (Imaging & PMT) under Tools-> Maintenance , both should be set to Automatic (PMT default is closed)



3. Set PMT to a reasonable value (I prefer 750) in correct Channel (Channel 1 is Red, Channel 2 is Green for our scope)
4. 

5. Start live scan
6. Slowly (increments of 10) bring up pockels value until you can see your image, **never surpass safe power** (consult power curve) even if you don't see your image. If you are not seeing anything, refer to you LUT to see if you are getting any signal, or try moving in ~10 micron increments up and down
7. Once you have an image, you can use the manual controller (set to fine adjustment!) or the stage control in Prairie View to move in x/y/z to find your ROI. **Always be aware of your step size**

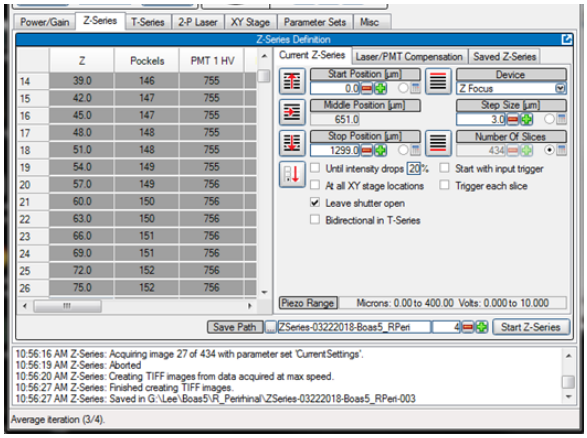


8. Things to consider when imaging:
    - Resolution/FOV/Pixel Size: Higher resolution will result in higher quality images but slower frame rates. Make sure your pixel size is smaller enough to resolve what you're imaging
    - Dwell Time: Increases the time spent per pixel. Increases will tend to give you more signal at the cost of imaging speed and risk of photobleaching/damage
    - Optical Zoom: Restricts the FOV you scan over while keeping the same resolution
    - Frame Averaging: Can increase signal to background at the cost of imaging speed. *In-vivo* if you are imaging anything dynamic (Calcium activity/RBC flux) you should not be averaging

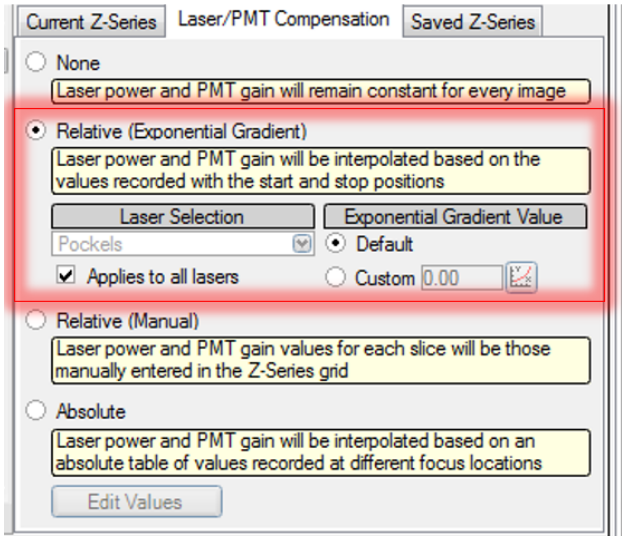
9. It's good practice to:
    - Take a single image or your ROI for your notes later, especially if you ever want to return to a certain region
    - Stop the live scan if you are not actively adjusting your imaging setting or looking for some non-static event. There's no need to be running the galvos to look at the same image.
    - Use the Zero feature of the stage control once in your starting ROI, especially in Z so you can track your stage movement

Section 4: Z series:

1. Refer to the above sections for setting up your sample and find your ROI. In a Z-series your starting ROI should typically be at the top of your sample
2. Once at the top of your ROI move over to the Z-series tab and set this as the top of your Z-series, this will save the Z coordinates as well as the pockels and PMT settings



3. **IMPORTANT:** Go to Laser/PMT Compensation sub-tab in the Z-series tab and select relative instead of none, this will ensure your pockels/PMT will be adjust during the Z-series as well as if you use the shortcuts to move to top/middle/bottom of series



4. Move down in Z, typically 50-100 micron steps *in-vivo* (Note this will show Z position to be increasing). As your go deeper, your image will get darker, you can slowly increase the pockels/PMT gain to keep your image bright, be careful not to use too much power!
5. Once you are at your desired depth, make any final tweaks to your pockels/PMT gain and set your bottom (Be careful to hit the set button not the move to button!). This will save your Z depth and your pockels/PMT at that depth. **Double check you set the compensation to relative**. You should see a chart on the left that matches pockels/PMT gain to depth showing a slow increase in both with Z.
6. Check your middle! The compensation interpolates between the top and bottom settings you selected. Hit the move to middle button to traverse to the middle position. Your image brightness should be about as good as the top and bottom. You can use pseudocolor to check saturation at all three positions. Once you're happy you can stop live scanning and perform the last steps.
7. Set your step size
8. Set your frame averaging

9. Double check any other important parameters including resolution, dwell time,etc. You can also estimate your total acquisition time as Frame Period * Number of slices (This does not include time for averaging and saving which will make the total time longer)
10. Set your save path
11. Start Z series- It will automatically traverse to the starting position and set the pockel/PMT gain settings you gave it
12. Once the stack completes use the playback to look over the stack for quality
13. **IMPORTANT:** During playback you will not be able to adjust anything in the software, and the pockel and PMT will still be set to imaging values, do not turn on room light until you have exited playback and zeroed everything)

Section 5: PSF Measurements

1. This measurement is done by taking the FWHM of a Z-series for a 1 micron fluorescent bead. It will be very similar to Section 4 but with a few additional steps
2. Set up your bead slide as described in Section 2, when you bring the beads into focus it will looks like a bunch of small green dots scattered across the FOV
3. Begin scanning, Pick a single bead to image, use optical zoom until bead takes up almost entire FOV (Tip: Use "Mark stage" and click on bead to center it in the image window)
4. Choose a spot for imaging, using 1 micron steps move up and down to try and find bead Z center (Where it appears brightest/largest, I like to zero the Z here)
5. Define z ranges(top/bottom by adjusting z values to until you can no longer see the bead. Note: Top and bottom should be ~5 microns above/below bead center
6. Set step size 0.3, frame averaging to 1
7. Start Z series
8. Check Z-series by observing bead coming into and then completely out of focus
9. Exit playback **and zero pockels/PMT**
10. Open Fiji
11. Drag-and-drop .XML file from you Z-series to import stack
12. Choose appropriate channel(Should only be one anyways)
13. Move to middle of z
14. Image -> properties
    - Adjust voxel depth to z step size
15. Create line in X across desired point, centered in the bead
16. Image -> stacks -> dynamic reslice
    - If this doesn't work, Press control H for Orthogonal Slice/View
17. create line in z center along bead profile
18. Plugins -> FWHM
19. Want smooth Gaussian
20. Want: Less than 5 microns (less than 4 without piezo attached)

Section 6: PLIM:

1. Check Filter Set for the Dye you are using, diagrams are posted on the inside of the light box
2. Refer to sections 1-3 for setting up and finding your ROI
   IMPORTANT: Ptp is fluorescent in the green while Oxphor-2p(PtG) is not, you can use the green channel to see PtP easily. With PtG you will either need something else that is fluorescent in channel 2 (ie FITC). In both cases bring the LUT almost all the way down and be very stingy with power *in-vivo* as exciting the dye creates phototoxicity.
3. PLIM is very slow, therefore you should use point scans to acquire the data you need
4. Once you have a reference galvo scanned image of your ROI, change the Acquisition Mode Galvo->FLIM (Tip: Always zero your Z before doing this)
5. Set FLIM mode to acquire phosphorescence data: Tools->FLIM-> Acquire Phosphorescence Data
6. Select Point Scan button on the imaging window and new window should open up
7. Select your desired points
8. To scan multiple points in one acquisition (most likely case) drag and highlight the desired points in the PLIM window and select create group. Highlight the new group on the list. (Note: Moving any current point will cause that point(and only that point) to be highlighted for acquisition)
9. Adjust acquisition parameters
    - Cycles Per Point: Number of times to pulse and measure a single point before moving on to the next in the group. Cycles are summed to create a single decay curve
    - Repetitions: Number of times to repeat the point scan (either single or group), Repetitions are summed as a single measurement and saved to a single .sdt file (can be parsed out of necessary though)
    - Iterations: Number of times to repeat scan, saved as individual measurement in separate .sdt files
10. Set pockels/PMT gain- Rule of thumb is to keep laser power under 10 mW from the surface, always try to use as little power as possible while having good SNR in your decays. Over exciting will results in significant consumption of local O2 that will confound your measurements in addition to phototoxicity
11. Start Acquisition- If you do not see any decays or poor signal after the first repetition/iteration, begin troubleshooting/debugging
12. Once the acquisition is complete swap back a doing a short galvo scan to double check your points are still aligned in your ROI. When you swap back a galvo point scan window will pop up, close it before scanning

Troubleshooting questions(Not exhaustive):

- Are you using the correct wavelength?
- Has the pockel cell been allowed to heat up? What's your extinction ratio? Post objective power at 0 pockels(background excitation)?
- Is your filter set correct?

- Are you using Channel 1 PMT? Check the PLIM window to make sure Channel 1 is active
- Are you in PLIM mode (FLIM acquisition mode, acquire PLIM under preferences)
- Can you see the dye sufficiently in fluorescence with appropriate power (Channel 2, Ptp ONLY)?
- Is the dye fresh/prepared correctly? How much did you inject?
- Is the objective heated to correct temperature?
- Is the Z stage jumping on you/did your ROI move (*in-vivo*)?

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