Unit of Cell Imaging
(By *Tadokoro, C.E.*)

Guidelines for "Prairie Ultima" Multi-photon Microscope

Three Laws of multi-photon users:

1) *"A user cannot damage the microscope or, through his/her inaction, allow somebody else to damage the microscope."*

2) *"A user must follow any given instructions from members of UIC, except if such instructions conflicts with the First Law."*

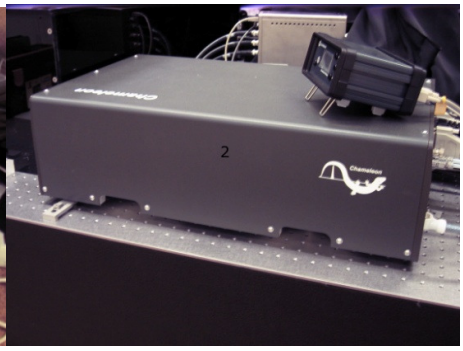
3) "A user must take care of her/his samples/data unless this conflicts with the First and Second Laws."

Don't break the microscope! Schedule a training session with someone from the UIC. Remember: only during day time you will have technical support for any unexpected problem. Since the microscope is a shared unit, we will work together to accommodate your samples to the correct use of the microscope.

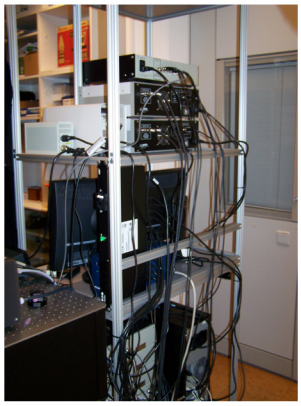
The beginning:

First, take some time to find these parts listed below in the microscope setup. After that, follow the instructions. For "first time users", we strongly suggest a "reading-and-identifying" step before "real" equipment turning on. Small notes will help you during the process and will also have important information about that specific step. Therefore, ***READ THESE NOTES*** *carefully before move to the next step!*

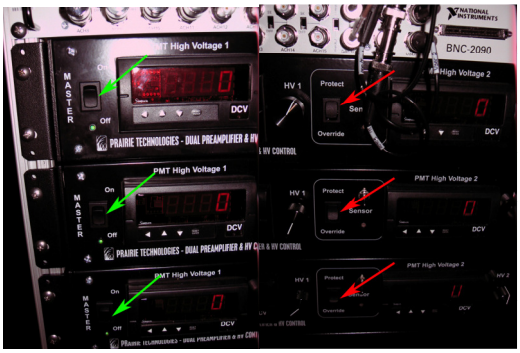
1. Cooler
2. Laser source
3. Main tower switch
4. Computer
5. Control switches for detectors ("PMTs")
6. Location of filter cubes and detectors
7. Microscope X-Y knobs to move the sample (x-y-stack positions)
8. Macrometer and micrometer knobs (z-stack positions)
9. Fluorescent and transmission lamps switchers
10. Microscope shutters and switchers
11. Microscope big knobs for X-Y-Z remote control



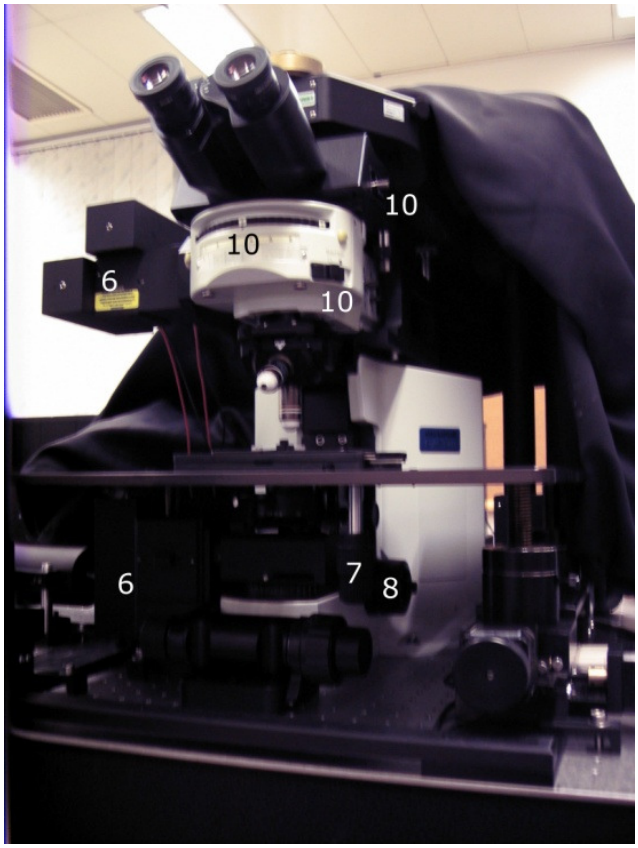
3. Main tower (frontal view)

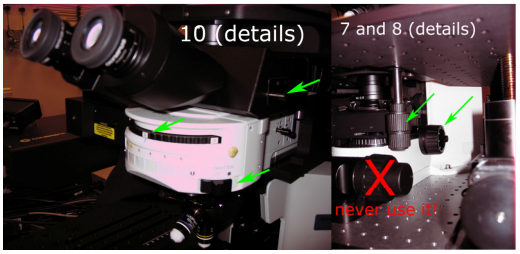


3. Main tower (rear view)



These pictures show the 3 big knobs to control the x, y, and z axes from the main tower and the beam expander (20x obj.). The detector switches (green arrows) and protection switches (red arrows) of channels 1, 2, and dodt are also shown. The switches for channels 3 and 4 are localized on the highest part of the main tower.





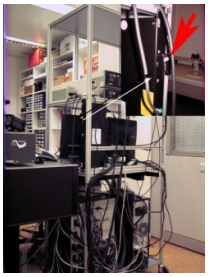
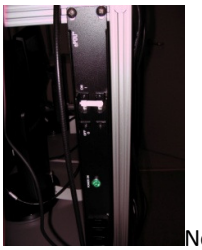
Turning on the microscope:

1) Check the cooler. The temperature should be around 20°C.

2) Switch the laser from “stand by” to “on” by clockwise turning the key (turn the key to the right).

Note: You should hear the sound of a shutter opening.

3) Turn on the main power switch (white switch on the side of two-photon main tower).

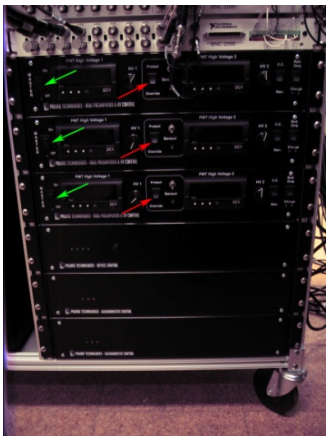
Note: You should hear the sound of some shutters opening/closing.

4) Turn on the computer.

5) Turn on the detectors that you will use.

6) Override the detectors protection.

Note: Switch the detectors protection to the “override” position. Then, put them back in the “protection” position. This step is important to preserve the detectors, also called “PMTs”. In this picture, switches are shown in green and red.

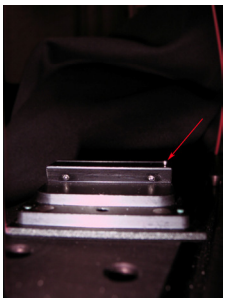


7) Check the filter cubes that you will use and put them in the right positions.

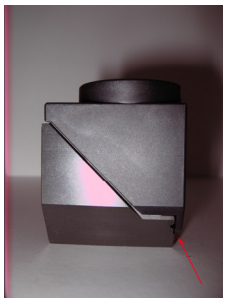
Note: This will be determined according to the wavelengths ("colors") that you want to separate in each PMT. You will probably need the help of UIC members to set up the best filters to your conditions.

Upper filter/detectors chambers

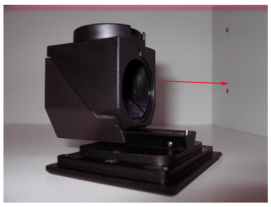
1. This picture shows the 2 upper detectors (PMTs) and the upper filter slot. The filter slot is fixed by magnets.



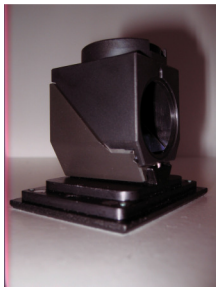
2. Keep the filter slot with the little pin at the right side (the red arrow shows the pin).



3. Every filter cube has a notch. Keep it also at the right side.



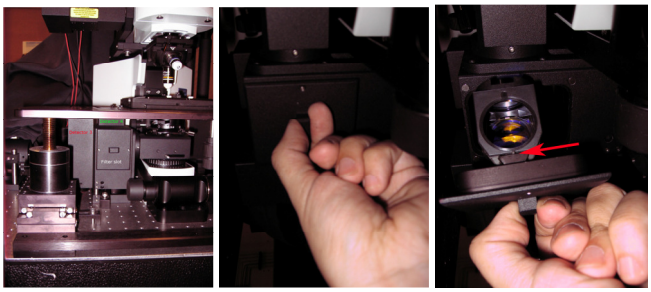
4. Mount the filter cube on top of the filter slot by sliding it over the rail. Note that the pin (filter slot) and notch (cube) are both at the right side!



5. This should be the final assembly of your filter on top of the filter slot. Any doubts, contact UIC.

6. Put the whole piece back.
Remember: notch and pin to the right side!

Lower filter/detectors chambers

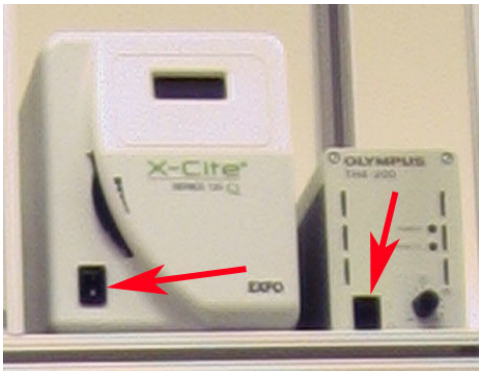


The same mounting steps are required here. Note the red arrow is showing the pin and notch regions. Pin and notch facing the top side!

8) Turn on the fluorescent lamp.

9) Turn on the transmission light, if you will need it.

Note: This will allow you to visualize your sample at the eye piece and select the region of interest. Lamp switchers are indicated by the red arrows. For the transmission lamp, there is also this small green button closer to the big multi-photon knobs, in the main tower.

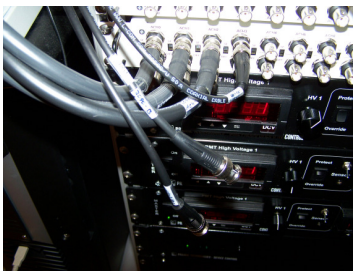


10) Change the beam expander switcher to the objective set that you are using.

Note: This will allow the photons coming from the 2-photon laser to fill the back of your objective, optimizing the amount of photons to reach it. Basically, you will switch between 20x or 40/60x.

11) Check the cable attached to the 4th connector. Change it according to your setup.

There are 3 different cables that can be attached to this connector. By default, the cable for the 4th channel upper detector should be attached.



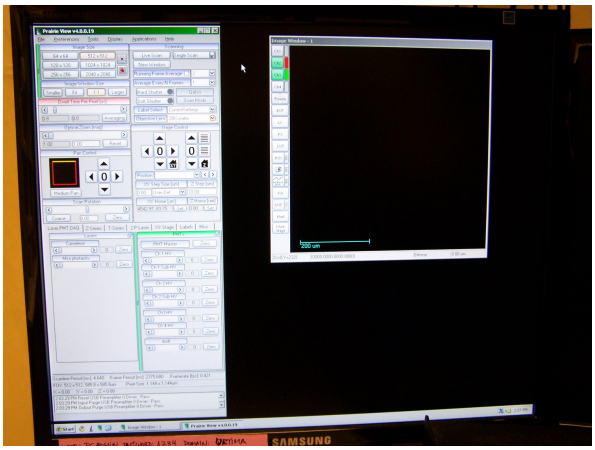
Now the microscope is ready to be used. The next step is turning on the software to control it.

Turning on the software and imaging acquisition:

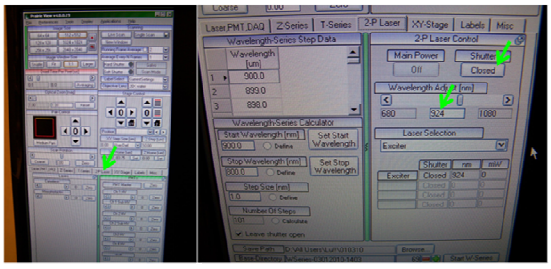
All the procedures from now on will be done only at the software level. Do not change anything else! Switching buttons, tuning dials, etc. will mess up the microscope configuration, making its use unavailable for you and all the other UIC users until fixing by one of UIC members!

In most of the next pictures, you will find arrows indicating the place to find the command.

1) Start the “Prairie View” software (“Prairie View.exe” icon).

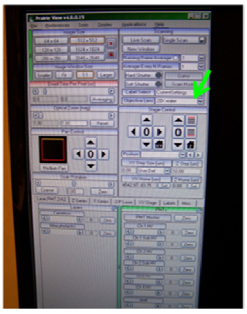


2) Find the “2-P Laser” tab and click on “closed” underneath “shutter” box. Then, type the target wavelength and click “enter”.



This pictures show the location of “2-P Laser” tab, the “close” button that you will need to click and the place to type the wavelength that you will use.

Note: This step will tune the laser to the wavelength needed for your fluorophore. At this point, the laser path will be available to reach the sample, but it will not hit the specimen yet.



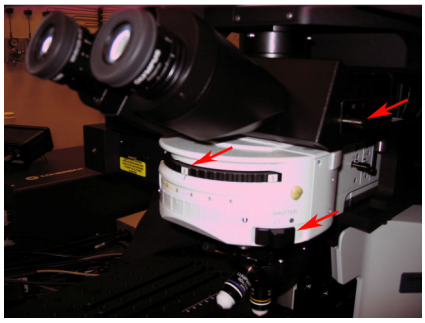
<u>Very important note</u>: The software does not select/change automatically for every single objective that you are using! You have to select it by clicking on the objective field at Prairie View software, indicated by the green arrow.

4) Objective installation: by default, the 20x objective will be installed. This objective has a numerical aperture of 1. However, you can use the 40x or 60x objectives despite their numerical aperture of 0.8 and 0.9, respectively. In this case, please ask first to someone at UIC to teach you how to perform this exchange.

***<u>Very important note</u>*: please, KEEP ALL THE MATERIAL NOT IN USE INSIDE THE "4 CHANNEL BOX"! This minimizes the risk of damaging the objectives, dichroics, filters, and also avoid loss of time to find the material for the next users!**

5) Increase the light intensity of the fluorescent lamp, select the correct filter in the fluorescent filter wheel (position 3 for GFP, CFP, etc; position 4 for RFP, Rhodamine, etc) and find your region of interest. To observe the sample at the eye piece, three shutters/switchers have to be opened:

The fluorescent lamp is already turned on, you will now adjust the light intensity.



These arrows show exactly how you microscope should be to allow the specimen visualization by the binocular. Put them according to the next instructions to have it ready for laser scanning.

a) Objective shutter in the open circle position.

b) Fluorescent filter at position 3 or 4 (GFP or RFP).

c) Ocular switch pushed in ("BI" position).

At the end of your search for the region of interest:

a) Objective shutter in the closed circle position.

b) Decrease the intensity of the fluorescent lamp (very important!)

c) Fluorescent filter at position 1.

d) Ocular switch pulled out ("LSM" position).

Note: most of the time, users cannot see the fluorescence on their tissue because they forget to put these tree parts (a, b, and c) in the positions described above.

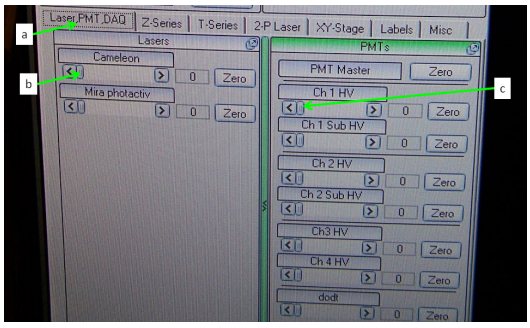
6) Now you will need to increase the electrical power (voltage) in the pocket cell and the detectors.

***Very important observation*: the PMTs are very sensitive and the room light will burn them. Little by little, if the room lights are turned on at the same time than PMTs, they will lose their capacity to detect dim fluorescence and only very bright signals will be visible. This type of action goes against the 2-photon laws! Therefore, BEFORE turn on the room lights, ALWAYS turn off the PMTs by decreasing the voltage to zero!**

TURN OFF the room lights!

(work in the dark, use the lantern, if needed)

Generally a power range among 20% to 30% (cameleon range goes from 0 to 500, where 500 corresponds to 100%) in the pocket cell is more than enough (the higher is the power in the pocket cell, the higher is the probability to cause photodamage!). Usually, the detector power is used at the range of 600 to 800.



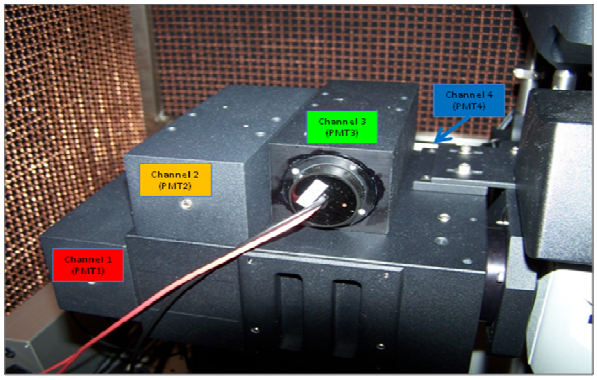
a) Go to "Laser, PMT, DAQ" tab;

b) Increase the power in your "cameleon" box on the screen;

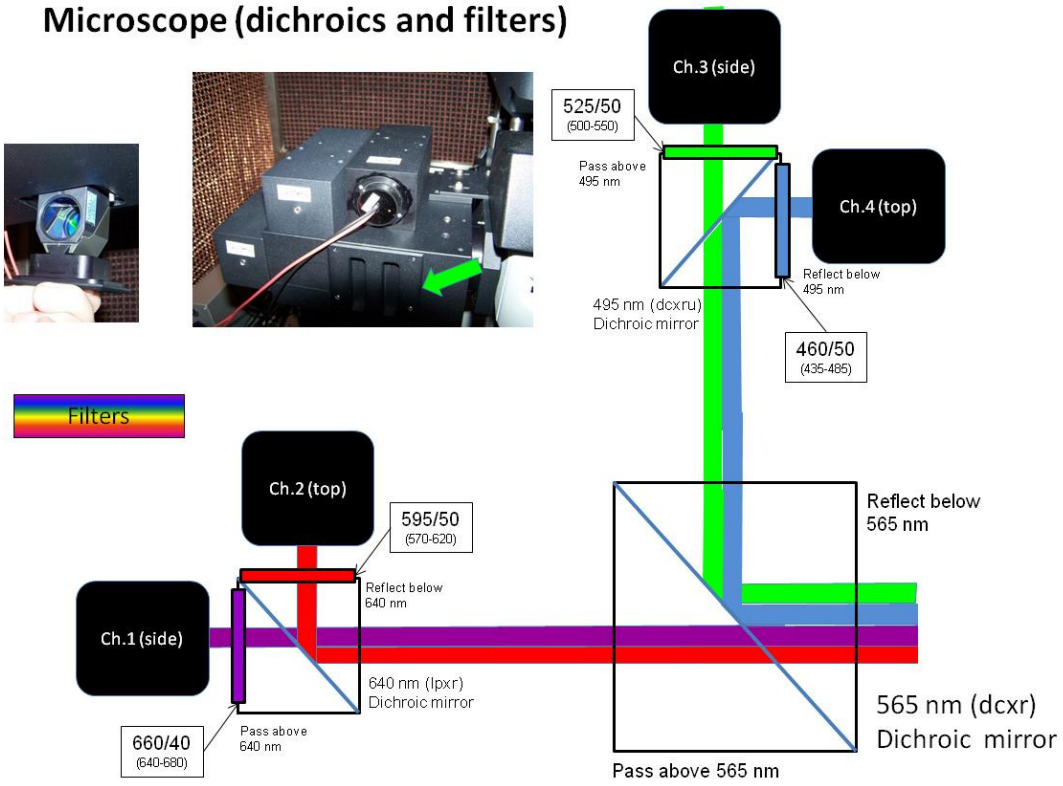
c) Increase the power in each Channel (e.g. "Ch1 HV") in the "PMT" screen region.

7) Choosing the right channels.

Below you have the location of each channel and the configuration map of all dichroics and filters installed. Using this map you can figure out what will be the channels that you need. As usual, help form UIC members is available.



PMTs and corresponding channels



The table below also can help you to select your configuration.

Channel	Ch.1	Ch.2	Ch.3	Ch.4
Range (nm)	(640-680)	(570-620)	(500-550)	(435-485)
Fluorophores (emission)	Cy5 (665)	RFP (584)	eGFP (508)	CFP (475/504)
	7-AAD (645)	mCherry (610)	YFP (530)	Collagen (410)*
		CMRA (575)	CFSE (521)	DAPI (460)
		Rhodamine B (590)	FITC (519)	

*At 800 nm excitation

8) Now it is time for an important check-point:

a) Is the shutter of 2-P Laser opened? (Details on step 2)

b) Is the right objective selected? (Details at the end of step 2)

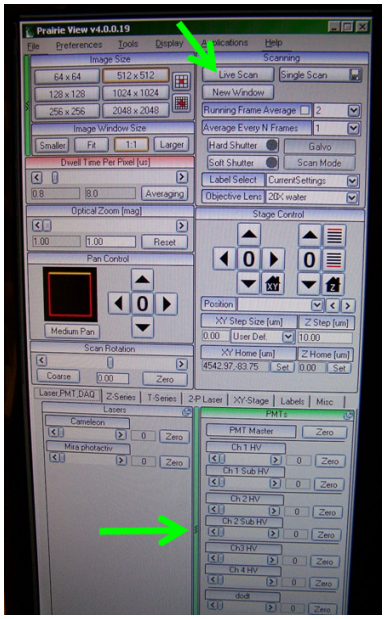
c) Is the shutter wheel closed? (Details on step 5)

d) Are the shutters and buttons on the "microscope head" in the right imaging position? (Details on step 5)

e) Did you turn on (increase voltage) in the right channels? (Details on step 6. Note that there are sub-channels 1 and 2! They are not Channel 1 and 2!)

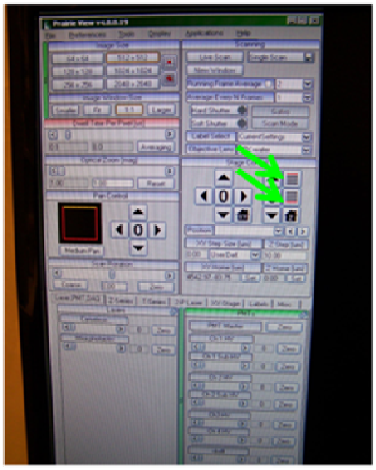
After this check-point, go to the next steps.

9) Start the live scan of your sample.



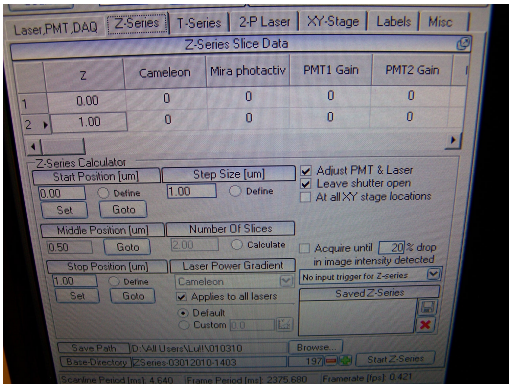
Note: You should see the image in the screen. To fine tune your image, adjust the pockel cell and PMTs voltages. You can also change the dwell time. The higher is the dwell time, the better contrast you will have. However, the catch here is that you will also increase the tissue photo damage probability. You can also adjust your gain and offset by clicking on the green tab in the middle of the PMTs tab (in this case, no photo damage problems because you are not dealing with the amount of laser that goes to your sample but improving your image quality only). You can also increase the image resolution by changing the pixelation (for example, from 512x512 to 1024x1024). But, again, the higher is the image resolution, the slower is the image acquisition (and the final file will be very big).

10) Define your “z-starting” and “z-ending” positions (“how deep you will go”).



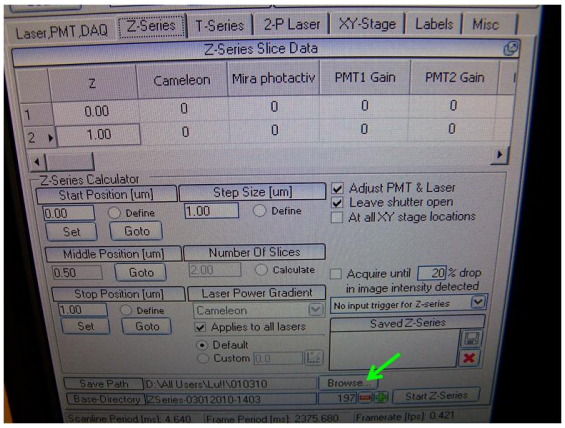
Note: To define your z-starting position, first find the 3 big knobs on the multi-photon command tower that remotely control the X-Y-Z position of your sample. Turn the Z-knob until you reach the first imaging plane. After that, on the software screen, click the button with black lines followed by one red line. You just select one of your ends. Then, using again the Z-knob, go to the other limit of your z-stack. Once there, click on the button with one red line followed by black lines. You selected the other end of your z-stack.

11) Acquiring a “z-stack” series (“3D image of your sample”)



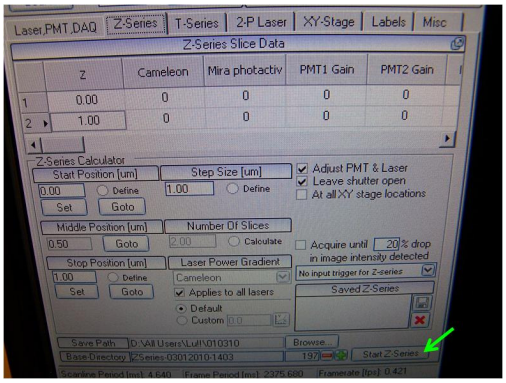
Note: Select the “Z-Series” tab on your software. There you will find 4 parameters: first step, last step, step size, and number of steps. You will see that you can change up to 3 of these parameters. The other will be automatically selected by the software. Usually, you will only have to change the step size or the number of steps. This is determined according to your previous experience with your samples. Remember: too many step sizes, your file will be huge. Too little, you can lose tracking details between z-stacks.

12) Select/create the folder to save your data



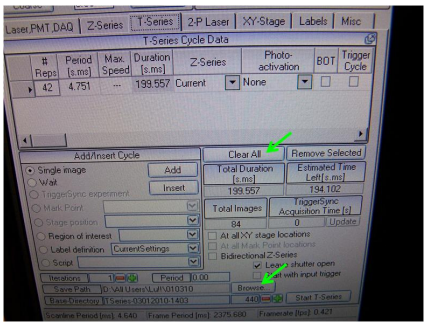
Note: After determining the number of z-stacks or the step size between your z-stacks, browse the right folder to save your image. Select the folder or create a new one. IMPORTANT: Always use “D:\All Users” to create your user folder where all the next acquisitions have to be saved.

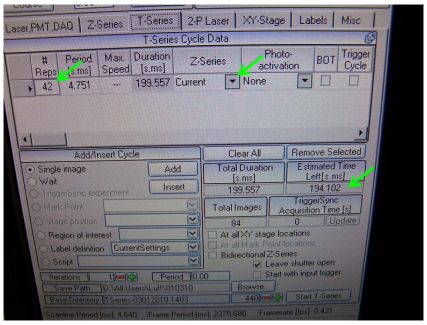
13) Acquire z-stack image



Note: Now you are ready to acquire a static image of your sample (if you don't want to do this, go directly to the next step). Otherwise, just press the button "Start Z-Series" at the lower right side of this tab. The software will acquire all pre-determined z-stacks in this tissue and open a new window with them for your examination. It's already saved on that specified folder and you can exit this new window (now showing your recent acquisition) anytime that you want by selecting "exit" button.

14) Acquiring a "t-series" image ("how to mount a time-lapse movie of your sample")





Note: If you want to acquire images of your tissue at different time-points, then go to the "T-Series" tab. There, erase all previous program lines already determined by the previous user by clicking on "delete all lines" button. Now, add a new line. The next steps are all related to a "t-series" image and you will find all the places to add your new information in this tab.

a) In this new line, you will find a cell column labeled "Z-Series". It will be selected with "none". Change it for "Current". Now the time for the acquisition of one slice will appear in the time column.

b) Since that you know how long one entire z-stack will take (indicated by "Period"), you can calculate the amount of time that you will need for a 15 min movie, 30 min movie, etc. Every time that you scan your tissue will become one time frame on your final movie. You can also extend the time for each z-stack and then the extra-time will be a time interval between frames.

c) Now you know how many times you will need to scan your z-stacks to acquire the movie with the length that you want. Then, go to the first column "# Reps" (number of repetitions") and change it for the number of times that you want. Now the "Duration" of your acquisition should contain all seconds needed for the total amount of time. Click on the "start t-series" button on this tab and the software will start to acquire the images.

d) At the end, the software will open a new window and it will show all the z-stacks and t-series in a "movie" format. It's already saved on that pre-determined folder that you selected/created. You can exit this window by selecting "exit" button.

In summary, these are the steps described above:

a) Define in the new line of “T-Series” tab: “current” in the “Z-Series” column

b) Calculate the number of repetitions of this line needed for your movie

c) Add this number of repetitions in the “# Reps” column

d) At the end of the acquisition, the software will open a new window with all your data

15) Once you finish your images acquisition, exit the Prairie software and transfer your files to your xserver02 folder, external HD, pendrive, or DVD.

Important Note: It’s strictly forbidden to keep the files in the computer HD since it will go against the 2-photon computer laws (after all, the software will run slower, the next user can have problems to save his/her files, and the final outcome is “virtual damage to the software function”). The files will be deleted immediately by UIC members, with no further notice, every Friday.

16) Finally, to turn off the whole equipment, you will procedure backwards to the turning on procedure:

a) Zero all PMTs in the “Laser, PMT, DAQ” tab

b) Turn off the PMTs switches on the lower part of the 2-photon command tower

c) Turn on the room lights (if you need them)

d) Clean your objective by passing a proper objective Kleenex paper without touching it.

e) Turn off the computer

f) Turn off the 2-photon command tower by the white switch

Note: you will hear the sound of all shutters closing.

g) Turn the key position (on the laser box) to “stand by”

Note: It will start to decrease the laser power and you will hear a noise during this process.

Good luck in your next step, the imaging analysis. ;-)