
Multi-Photon Training

Patterson Microscope Center

University of Texas at Austin

Patterson Labs, Room 334

471-2443

<http://w3.biosci.utexas.edu/pmc/>

Overview

This training will take approximately 4 hours. The first 2 hours will be spent learning one-on-one with your trainer, while the following 2 hours will be your opportunity to practice what you have just learned on your own, but with help readily available for you if you should need it.

Training Outline

Learning to use the confocal will require multiple steps. It is critical that you understand both how to use the microscope and how to use the software.

- Turning the system on.
- Using the STP6000: Control of the motorized stage and nosepiece
- Using the Leica LAS AF software
- How to take single images and 3-D Z-stacks.
- Saving personal settings & data.
- How to properly turn the system off.
- Optional Training (by request):
 - How to take time lapse movies
 - How to take Region of Interest (ROI) images
 - Using the FRAP & FRET wizards

Training Objective

By the end of this training session you will generate 4 images in order to show your understanding of the microscope and software. In order to streamline the training process you will be using a standard Leica slide for all training purposes. Once you have completed your training and are given access to the microscope you will be able to schedule time to image your own experiments.

- 10X Single
- 25X Z-Stack projection
- 40X single.
- 63X Z-stack projection.

2-Photon Use Rules:

- No food or drinks are allowed in the microscope room.
- You must wear a lab coat (provided by you) if working with wet mounts or non-fixed slides
- You must provide eye protection for all work requiring it.
- No chemicals are to be stored in the facility.
- No biohazardous or chemical waste is to be disposed of in the facility.
- You must complete OH 207 safety training.
- All data must be removed from the microscope computer via an external drive (the computer is never hooked up to the internet). It is the user's responsibility to remove all data on the day it is collected. Data left on the microscope computer can be deleted and/or lost at any time.
- Users must reserve time on the microscope using the on-line Google calendar. Users may cancel time within 24 hours of use in order to avoid charges.
- Users must always sign-in on the user logbook.

Turning the system on

The components of the multi-photon microscope must be turned on in the correct order for the system to work properly.

- **(#1)** Turn Laser Key to ON position on Chameleon laser Display will read “POWER RAMPING (XX%)”. This can take a few minutes. Do not continue until the screen displays “OK”.
- **(#2)** Turn on the EOM power strip
- **(#3)** Turn on the mercury bulb
- **(#4)** Flip on the Green PC/Microscope switch
- **(#5)** Flip on the Green Scanner Power switch
- **(#6)** Flip on the Green Laser Power switch
- **(#7)** Turn the laser key to ON (vertical position)
- **(#8)** Flip switch on STP6000 (back right side)

The computer will start up automatically:

- Create user login:
Login: _____
Password: _____
- Record your start time in the logbook.

Open Leica LAS AF software

- Hit **OK** to start
- If you get an error message reading: “NO MACHINE SELECTED”, click on CONFIGURATION, then select MP-LASER ON-STAGE.
- If you want increased scanning speed click the box to activate the RESONANT SCANNER.

Initializing the motorized stage

- The software will *sometimes* ask you if you want to initialize the stage. Check to make sure that the stage can move from left to right without hitting an objective or the condenser.
- Hit **YES** if you are going to be doing **TILE SCANNING**
- Hit **NO** if you are not going to be doing **TILE SCANNING**.


Using the STP6000: Controlling the motorized stage & nosepiece

The Leica STP6000 controls the X & Y-axis of the stage and also the Z-axis of the nosepiece using rotating knobs. It also allows you to switch objectives, and switch between Brightfield, Fluorescence, and Confocal imaging using a touch-screen panel.

Choosing an objective:

- You should find the nosepiece placed in the empty objective position
- Go to the **OBJECTIVE** icon on the STP6000
- Switch to 10X by touching the screen above the 10X icon, or by manually moving the objective nosepiece.

Setting the Home and Focus position on the nosepiece:

- Go to the **Color Wheel** icon and choose **BRIGHTFIELD (BF)**.
- There is a shutter for the brightfield light below the condenser. Make sure this is in the open position.
- You can adjust the intensity of the light using the **MICROSCOPE** icon on the STP6000
- Using the fine and course focus knobs on the STP6000, focus on the slide sample.
- Go to **STAGE** on the STP6000
- Click on the  icon below **SET/CLEAR**, which will switch to **SET/CLEAR FOCUS**. Then hit **SET**.
- This position will serve as a reference for you to find your focal position on other par-focal objectives. (Useful when using a multi-objective nosepiece)

Changing objectives:

- Hold down the **HOME** icon until the nosepiece has moved all the way to the home position.
- Go to the **OBJECTIVE** icon on the STP6000
- Switch to 25X by touching the screen above the 25X icon or you can move the objectives by hand.
- Move the new objective into the water (this is the lowest point you will ever need to go – the focal position will be UP from this point)

Go to the **STAGE** icon on the STP6000

- Hold down the **FOCUS** icon until the nosepiece has moved all the way up to the focal position.
- Using the fine focus knob, re-focus on the sample.

****It is critical that you move the nosepiece to the HOME position each time you switch objectives.** This practice will eliminate the risk of accidentally getting oil on a dry objective, which could ruin the objective.**

Switching the microscope from BRIGHTFIELD (BF), to FLUORESCENCE (FLUO):

- Go to the **BF/F/CS** icon and switch from **BRIGHTFIELD (BF)** to **FLUORESCENCE (FLUO)** by touching the screen above **FLUO**.
- Open the shutter by touching the **SHUTTER** icon (gray circle will turn yellow)
- Select a filter cube:
 - I3 = Green (488)
 - N21 = Red (555)
 - A = UV (DAPI)
- You can adjust the brightness of the fluorescence by touching the **MICROSCOPE** icon and changing the fluorescent intensity.

Practice #1: Changing objectives

Switch from the 25X objective to the 10X objective

Summarized steps:

1. Stage – Home
2. Objective – 10X
3. Move the objective into the water
4. Stage – Focus
5. Fine focus

Practice #2: Changing objectives and switching from Fluorescence and Brightfield.

Switch from 10X Fluorescence to 25X Fluorescence and look at the sample with 2 filters.

Summarized steps:

1. Stage – Home
2. Objective – 10X
3. Move the objective into the water
4. Stage – Focus
5. BF/F/CS – Choose BF
6. Fine focus
7. BF/F/CS – Choose Fluo
8. Open Shutter & choose filter

****Open the shutter for as short a time as possible to avoid bleaching/quenching the fluorescence in your sample.****

Your introduction to the STP6000 is now complete! Let's move on to the LAS AF software.

Using the Leica LAS AF Software

The Leica LAS AF software will control all aspects of your confocal imaging including; lasers, PMT, capturing single images, capturing 3-D Z-stack images, and all the fine-tuning that goes into taking great confocal images.

Taking an image:

- Acquire Tab
- Acquisition Tab

First √: BEAM SPLITTER/DICHROIC ⇔ SUBSTRATE

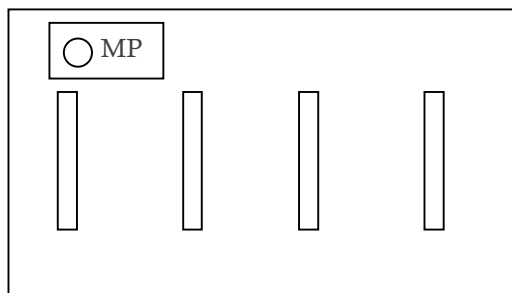
Second √: MFP ⇔ SP 665

Third √: Open PINHOLE all the way

Activating the MP Laser:

- The MP laser is controlled by 4 different safety features, which must ALL be activated before you can use the laser.
- Shut the curtain and connect the magnetic safety interlock.
- Go to the **CONFIGURATION** tab in the LAS AF software
- Choose **LASERS**
- Click the box next to the MP laser
- Click the box next to the HeNe3 laser if you will be using it.
- Go to the **ACQUIRE** tab in the LAS AF software
- Click on the MP LASER icon to activate the power controls of the MP laser (red = ON)
- Click on the words MP LASER. A new window will open. Hold down the MP-SHUTTER for 2 seconds – you will hear the shutter open. Choose the wavelength that you need to excite your fluorophore (MP excitation is different from single photon excitation – use the table!!)

Controlling the Laser Power:

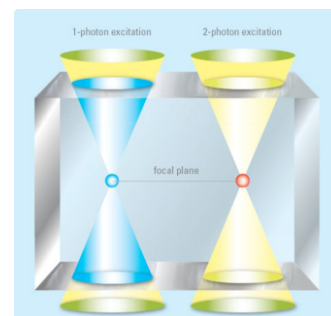


Practice #3: Turning on the lasers and PMT

Turn on the MP laser

Summarized steps:

1. Click on MP laser icon
2. Click on MP Laser
3. Hold down MP Shutter
4. Choose MP excitation wavelength
5. Make sure the curtain interlock is closed



Practice #4: Taking a single image

Move the objective to 10X and capture a single image using the RLD PMT's.

Summarized steps:

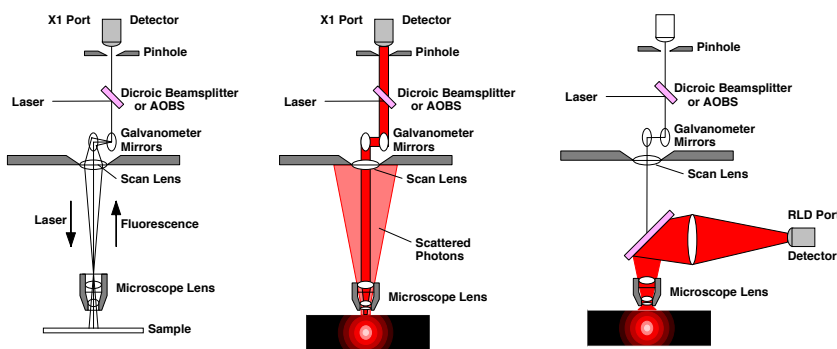
- 1) Turn off the MP Power controller (red to grey dot)
- 2) Close the MP Shutter
- 3) Disconnect the curtain safety interlock & open the curtain
- 4) Stage – HOME
- 5) Objective – 10X
- 6) Stage – FOCUS
- 7) BF/F/CS – Choose BF
- 8) Fine focus
- 9) Close the curtain & connect the interlock
- 10) Turn on the MP Power controller.
- 11) Open the MP shutter
- 12) Choose AUTOGAIN
- 13) Hit LIVE & then fine focus
- 14) Choose AUTOGAIN again
- 15) CAPTURE IMAGE

Activating the HeNe3 Laser:

- Click the Visible icon to turn on the Visible laser (yellow dot = ON)
- The visible laser is set to 0% power.
- Adjust the % power of the laser to 20-30% to start & adjust depending on the fluorescent strength of your sample

Turning on the PMT for use with the Visible laser (HeNe3):

- Click: ACTIVE (ACTIVE = ON)
- Choose your preferred false color
- Make sure the spectral range of the PMT does not come within 8nm of the HeNe3 laser line.
 - Double click on the gray spectral range bar to see the exact spectral range.
 - If your spectral range is within 8nm of the HeNe3 laser line, adjust the spectral range by moving the gray bar, or by typing in a new spectral range endpoint.



Turning on the NDD detectors for use with the MP laser:

- Click: ACTIVE (ACTIVE = ON)
- Choose your preferred false color
- RLD (Reflected Light Detector - Top NDD detector) = NDD PMT #1 & #2 (corresponds to filter cube inserted into the RLD NDD box)
- TLD (Transmitted Light Detector – Bottom NDD detector = NDD PMT #3 & #4 (corresponds to filter cube inserted into the TLD NDD box)
 - To use the TLD detector the shutter to the condenser must be open (same shutter used for Brightfield)
 - The microscope must be collared in order to use the TLD

How to take single images and 3-D Z-stacks

Single images are a snapshot of data in a single XY plane. 3-D Z-stacks add the 3rd dimension to your image by taking a series of images at varying focal planes (varying Z positions), which can then be assembled into a 3-D image projection.

Taking a single image:

- Open the XY toolbar by clicking on the arrow
- The default FORMAT for your image is set to 512 x 512.
- The default PINHOLE is set automatically via the AIRY1 setting

- MP imaging does not require the use of a pinhole! Open the pinhole all the way.
- The default scan SPEED is set to 400Hz
 - Leica suggests that you leave all settings at their default position to start with and then adjust later as you get closer to taking a publication quality image.
- Click on AUTOGAIN
- Click on CAPTURE IMAGE

Organizing and Naming Data:

- Under the ACQUIRE Tab are two options: ACQUISITION & EXPERIMENT
 - Choose the EXPERIMENT Tab
 - Each image you have captured is stored under EXPERIMENTS.
 - Right click on files to rename them with meaningful names or to delete files you don't want to keep.
 - To Save files: right click on individual Files or the entire Experiment & choose export as .tiff.
 - To save your data choose to export your data directly onto your thumb-drive.
 - When you click export as .tiff a window will open with the option to export an overlay of all of your individual channels.
 - ★ Overlay = Exports 1 image for each active PMT
 - ★ Overlay = Exports a single image of all your data merged into 1 picture

Ways to enhance your images:

- Click BIDIRECTIONAL - Your image will be scanned in both directions. This will decrease the time it takes to capture an image, but may increase noise slightly.
- Adjust the FORMAT to a higher resolution setting
- Adjust the PINHOLE (for HeNe3 laser images) - Making the pinhole smaller reduces detection of light from out-of-focus planes, but making the pinhole too small will also reduce detection of light from the in-focus plane)
- Adjust the scan SPEED (slower = better signal: noise ratio, but more bleaching due to longer laser exposure)
- Average your images: Choose Line Averaging
- Adjusting the Gain vs. adjusting the laser power strength – Increasing the laser power can result in bleaching, however reduced laser power can result in increased noise (if the Gain is set too high).
- Optimal gain is between 400 – 900. If your gain is above 1,000, then Leica suggests increasing your laser power and reducing the gain. If your gain is below 400 then decrease your laser power and decrease your gain.
- Look-up Table (LUT) – Green pixels show you what part of your image is below detection and blue pixels show you what part of your image is above detection. Leica suggests having a few blue & green pixels in each image so you are accessing the full range of detectable signal.

Practice #5: Enhancing your image

Take about 20 minutes to play around with the many setting options available to you to optimize your confocal image.

Capture multiple images of the same section using various settings and compare the image quality.

Practice #6: Taking a 3-D Z-stack

Move to the 40X objective and then take a Z-stack

Summarized steps:

- 1) Turn off the MP Power controller
- 2) Close the MP Shutter
- 3) Open the curtain
- 4) Stage – HOME
- 5) Objective – 40X
- 6) Move the 40X objective into the water
- 7) Stage – FOCUS
- 8) BF/F/CS – Choose BF
- 9) Fine focus
- 10) Close the curtain
- 11) Turn on the MP Power controller
- 12) Open the MP Shutter
- 13) Choose AUTOGAIN
- 14) Hit LIVE & then fine focus
- 15) Choose AUTOGAIN again
- 16) Focus up – Hit BEGIN
- 17) Focus down – Hit END
- 18) Click START
- 19) Project the 3-D image

Taking 3-D Z-stacks:

- Switch objectives from 25X to 40X
 - Turn off the MP Power controller
 - Close the MP Shutter
 - Open the curtain (disconnecting the MP safety interlock)
 - On the STP6000, Select Stage & move to HOME position
 - Select OBJECTIVE and move to 40X OBJECTIVE (or move to the 40X objective by hand)
 - Select Stage & move to FOCUS position
 - Fine focus
 - Close the curtain
 - Turn on the MP Power controller
 - Open the MP Shutter
- Optimize the settings for your sample (as described above) or select AUTOGAIN.
- Open the Z-STACK window
- Hit LIVE
- Focus up, & hit BEGIN
- Focus down & hit END
- Click LIVE again to stop scanning
- Hit START
 - CAPTURE IMAGE takes a single image, but START takes a series of images, aka, a Z-stack.

Processing your 3-D Z-stacks:

- **Projecting your z-stack:**
 - Click on the PROCESS TAB
 - Click on TOOLS TAB
 - Under VISUALIZATION (near the bottom), click on 3D PROJECTION
 - Click APPLY
- **Cropping your z-stack:**
 - Click on the PROCESS TAB
 - Click on TOOLS TAB
 - Under EDIT (at the very top), click on CROP
 - Select a STARTING POINT and an ENDING POINT
 - Click APPLY

Saving personal settings

Saving Personal Settings:

- Saving Laser settings:
 - Under the ACQUIRE TAB click on the ACQUISITION TAB

- Next to the Laser settings is a window with a SAVE button, select SAVE and give your settings a name.
- Your settings will be saved under your computer login only
- Saving Control Panel settings:
 - Under the CONFIGURATION TAB click on CONTROL PANEL
 - Select SAVE and give your control panel settings a name
 - Control panel settings will be saved under your computer login.

Properly Shutting down the MP Microscope

Standby - If someone is signed up within an hour after you:

- Turn off the MP Power controller
- Close the MP Shutter
- Open the curtain (disconnecting the MP safety interlock)
- Put your objective in the Home position and remove your sample.
- Clean any used objectives with fresh lens paper (dab the objective with lens paper until you don't see any more liquid on the lens paper – do not rub the lens paper on the objective)
- Move the objective nosepiece to the empty position
- In the LAS AF software go the CONFIGURATION TAB
- Click LASERS
- De-select each laser in order to turn it off
ACTIVE = OFF
ACTIVE = ON
- Close LAS AF software
- Log-out of computer
- Turn HeNe3 laser key to OFF position (**key labeled #7**)
- Turn MP laser key to OFF position (**key labeled #1**)
- Remove your sample and clean up any mess
- Remember to take everything with you when you leave (do not store materials at PMC)

Complete Shutdown - If no one is signed up after you (or is signed up more than an hour after you):

- Turn off the MP Power controller
- Close the MP Shutter
- Open the curtain (disconnecting the MP safety interlock)
- Put your objective in Home position and remove your sample.
- Clean any used oil objectives with fresh lens paper
- Move the objective nosepiece to the empty position
- In the LAS AF software go the CONFIGURATION TAB
- Click LASERS
- De-select each laser in order to turn it off
 - ACTIVE = OFF
 - ACTIVE = ON
- Close LAS AF software
- Log-out of computer & shut the computer down
- Turn off the STP6000 (**#8**)
- Turn HeNe3 laser key to OFF position (**key labeled #7**)
- **DO NOT TURN OFF THE LASER POWER BUTTON (#6)**
- Turn off the Scanner Power button (**#5**)
- After the computer has shut down, Turn off the PC/Micro button

- (#4)
- Turn off the Mercury box (#3)
 - Turn off EOM/NDD power strip (#2)
 - Turn MP laser key to STANDBY position (**key labeled #1**)
 - 5 Minutes after you have turned off the HeNe laser (in the LAS AF software) you can turn off the Laser Power Button (#6)
 - The laser power button provides power to the fan and must be left on for 5 minutes in order to cool down the lasers. Turning off the power (and the fan) too early will burn up our laser!!
 - Record your end time in the log book.

Training Notes: