Confocal 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.
- How to take time-lapse movies (optional – please ask before training if you'd like to include this session, which will take additional time).
- Saving personal settings & data.
- How to properly turn the system off.

Training Objective

By the end of this training session you will generate 5 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.

1. 10X Single
2. 20X Z-Stack projection
3. 40X (oil immersion objective) single.
4. 40X (oil immersion objective) Z-stack projection.
5. 63X (oil immersion objective) Z-stack projection.

Materials

Most materials will be provided for your during training, however you may want to bring the following:

- Thumb drive to save images
- Something to take notes with

Confocal Use Rules:

- No food or drinks are allowed in the microscope room.
- 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 users 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 in the confocal room to report usage and to record any problems you may encounter.

Turning the system on

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

Starting from Full Shutdown:

1. Green button – PC/Microscope
2. Green button – Scanner Power
3. Green button – Laser Power
4. Turn key to on (vertical position)
5. Flip on switch on STP 6000 (back right side)
6. Turn on power for mercury bulb

Starting from Standby:

1. Turn the laser key on
2. Login to the computer
3. Open LAS-AF software
4. Activate lasers (under Configuration tab, go to Lasers)
5. Take beautiful, publication quality images!

Logging into the computer:

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

Open Leica LAS AF software

- Hit **OK** to start

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**.

Turn on the lasers:

- Go to **CONFIGURATION** in the LAS AF software
  - Choose **LASERS**
  - Click the box next to each laser in order to turn it on. (you should hear the lasers turn on)
  - Turn the Argon laser to 20% power.

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 (currently labeled as the 2nd 63X position on the STP6000)
- Go to the **OBJECTIVE** icon on the STP6000
- Switch to 5X by touching the screen above the 5X icon.
  - The 5X icon will begin blinking. Touch it again for the objective to move into place.

Setting the Home and Focus position on the nosepiece:

- Go to the **BF/F/CS** icon and choose **BRIGHTFIELD (BF).**
  - 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**.

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 10X by touching the screen above the 10X icon.
- Go to the **STAGE** icon on the STP6000
- Hold down the **FOCUS** icon  until the nosepiece has moved all the way to the focus position.
- Using the fine focus knob, re-focus on the slide 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.

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.

Practice #1: Changing objectives

- Switch from the 10X objective to the 5X objective
- Summarized steps:
- Stage – Home
- Objective – 5X
- Stage – Focus
- Fine focus

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

- Switch from 5X Fluorescence to 10X Fluorescence and look at the sample with 2 filters.
- Summarized steps:
- Stage – Home
- Objective – 10X
- Stage – Focus
- BF/F/CS – Choose BF
- Fine focus
- BF/F/CS – Choose Fluo
- Open Shutter
- Choose filter

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.

In the LAS AF software select:

- Acquire Tab
- Acquisition Tab

Choosing Lasers:

- Click the UV icon to turn on the UV laser (yellow dot = ON)
- Click the Visible icon to turn on the Visible lasers (yellow dot = ON)
  - All visible lasers are set to 0% power.
  - Adjust the % power of the lasers that you want to use (start between 20 – 30% power)

Turning on the PMT's:

- Click: ACTIVE □ (ACTIVE ☑ = ON)
- Choose your laser setting (ex. Alexa488)
- Choose your preferred false color
- Make sure the spectral range of the PMT does not come within 8nm of lasers.
  - Double click on the gray spectral range bar to see the exact spectral range.
  - If your spectral range is within 8nm of a laser line, adjust the spectral range by moving the gray bar, or by typing in a new spectral range endpoint.

Choosing your dichroic mirror:

- Dichroic mirrors allow for incoming excitation light to pass to the sample, while outgoing emission light is directed to the detector (PMT). It is important to choose the correct dichroic mirror for the excitation/emission spectrum of the flourophores being used in your experiment.
  - RT 30/70: you can use it with any laser. 30% of the light is reflected, 70% is transmitted. Use it with 405nm laser and if you want to acquire in reflection mode.
  - RSP 500: good with 458, 476, and 488nm laser. Range 500-600nm. Reflection short-pass
  - Double Dichroic (DD) 458/514: good with 458 and 514 laser. Range 475-500 and 530-660nm, double band pass
  - Double Dichroic (DD) 458/543: good with 458 and 543 laser, Range 500-530 and 555-700nm, double band pass
  - Triple Dichroic (TD) 488/543/633: good with 488, 543 and 633nm laser, Range 500-535, 555-620 and 650-750, triple band pass
  - Substrate: Plain Glass

Practice #3: Turning on the lasers and PMT

- Turn on the 543 laser and PMT #2 with Alexa555
- Summarized steps:
- 20% power to laser 543
- PMT #2 – Active ☑
- Choose Alexa555 setting
- Choose False color
- Choose appropriate dichroic
- Adjust spectral range

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
- 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 Saving Data:

- Under the ACQUIRE Tab are two options: ACQUISITION & EXPERIMENT
- Choose the EXPERIMENT Tab
  - Each image you have captured is stored under EXPERIMENTS.
  - To Name files: 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 confocal 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 (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

Practice #4: Taking a single image

- Move the objective to 20X and capture a single image using Alexa488 & Alexa555 settings.
- Summarized steps:
  1) Stage – HOME
  2) Objective – 20X
  3) Stage – FOCUS
  4) BF/F/CS – Choose BF
  5) Fine focus
  6) Activate PMT #1
  7) Choose Alexa488
  8) Choose False color
  9) Adjust Spectral Range
  10) Choose AUTOGAIN
  11) Hit LIVE & then fine focus
  12) Choose AUTOGAIN again
  13) CAPTURE IMAGE

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.

and reducing the gain. If your gain is below 400 then reduce your laser power and increase your gain.

Taking 3-D Z-stacks:

- Switch objectives from 20X to 40X (moving from dry to oil)
  - On the STP6000, Select Stage & move to HOME position
  - Select OBJECTIVE and move to EMPTY OBJECTIVE (2nd 63X on STP6000)
  - Add oil to slide
  - Select 40X OBJECTIVE
  - Select Stage & move to FOCUS position
  - Fine focus
- 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.

Projecting 3-D Z-stacks:

- Click on the PROCESS TAB
- Click on TOOLS TAB
- Under VISUALIZATION (near the bottom), click on 3D PROJECTION
- 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.

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

- Move the objective from 40X to 63X, then take a Z-stack
- Summarized steps:
  1) Stage – HOME
  2) Objective – 63X (no need to add more oil)
  3) Stage – FOCUS
  4) BF/F/CS – Choose BF
  5) Fine focus
  6) BF/F/CS – Choose CS
  7) Choose AUTOGAIN
  8) Hit LIVE & then fine focus
  9) Choose AUTOGAIN again
  10) Focus up – Hit BEGIN
  11) Focus down – Hit END
  12) Click START
  13) Project the 3-D image

Properly Shutting down the confocal

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

- Put your objective in the Home position and remove your sample.
- Clean any used oil objectives with fresh lens paper (dab the objective with lens paper until you don't see any more oil 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 Key to OFF position
- 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):

- 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 Key to OFF position
- DO NOT TURN OFF THE LASER POWER BUTTON
- Turn off the Scanner Power button
- After the computer has shut down, Turn off the PC/Micro button
- Turn off the Mercury box
- Turn off the STP6000
- 5 Minutes after you have turned off the lasers (in the LAS AF software) you can turn off the Laser Power Button
  - 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 lasers!!
- Replace Microscope dust cover
- Record your end time in the log book.

Training Notes: