KNOW YOUR CONFOCAL SCAN HEAD

The confocal scan head functions to collect the appropriate emission wavelength of light from your sample. You must know the excitation (absorption) and emission (fluorescence) spectrum of your sample to utilize the scan head correctly.

A: The confocal aperture limits the amount of available light collected from your sample. The software will indicate which aperture is the best setting for your sample. You are free to change aperture sizes to best suit your needs.

Channel 1 (Ch1) = PMT 1
DETECTION (2° Dicroic Mirror)
   B) Leave in to have all wavelengths of light go to PMT 1
   B) Pull out one stop (< 570 nm to PMT 1, > 570 nm to PMT 2)
   B) Pull out two stops (< 630 nm to PMT 1, > 630 nm to PMT 2)

BARRIER FILTERS
   C) 510 nm Long Pass (in = all light > 510 nm to PMT 1)
   D) 505-525 nm Band Pass (in = light between 505-525 nm to PMT 1)

Channel 2 (Ch2) = PMT 2
   E) 560-600 Band Pass (in = wavelengths between 560-600 nm are collected)
   F) 660 Long Pass (in = only wavelength greater than 660 nm are collected)
Examples of fluorescence spectra from common dyes (Molecular Probes, Invitrogen)

Scan head settings for common dyes
(A = Absorption or Excitation peak; E = emission or fluorescence peak)

**Channel 1 (PMT 1)**
FITC/Fluorescein/Alexa 488 (A. ~ 492 nm, E. ~ 520)
  B, C and D in
  CY2 (A. ~ 492, E. ~ 510 nm)
    B & D in
    C out

**Channel 2 (PMT)**
TRITC, Cy3 (A. ~550 nm, E. ~ 570 nm)
  E in
  F out

Rhodamine RRX (A. ~ 570 nm, E. ~ 590 nm)
  E out
  F out

Texas Red (A. ~ 596 nm, E. ~ 620 nm)
  E out
  F out

Cy5 (A. ~ 650 nm, E. ~ 670 nm)
  E out
  F out
IMPORTANT: Prior to the use of the FV300 LSCM, you must receive formal training. All training sessions are arranged in accordance with the Laboratory Manager, Earl Ada.

STARTUP

Turn on the following items prior to use of the FV300 (the numbers on the images below correspond to the order in which the following components should be turned on):
1. Halogen lamp
2. Mercury lamp for widefield epifluorescence (see below)
3. Microscope Power

Lasers (turn on only those that you need)
4. Green Helium Neon (543 nm)
5. Red Helium Neon (643 nm)
6. Multi-Argon (457, 488, 514 nm)
7. Scan Unit
8. Computer

About the Mercury Lamp: The mercury lamp for epifluorescence is not necessary for confocal microscopy. For practical purposes, it is turned on to help locate specimens on slides that would otherwise be difficult to find under transmitted light, and to verify the fluorescence signal of your specimen. The mercury lamp should be left on while you are engaged in LSCM – even if you only need it to locate your specimen, etc. Turning the mercury lamp on and off over short a period of time (minutes to 1 hr) can damage the bulb.

About the Lasers: To turn them on, turn the key from the off position (O) to the on position (I). Only use those lasers that you require. All lasers need not be on if they are not of use to you.
The following sequence of events represents the simplest method for preparing your specimen for the microscope.

1. Click on the Fluoview Icon and the following screen will appear

You might not be able to see all of the control panel at first. The image on the right (Fluoview box) blocks its view. Click on the control panel window to bring up the entire window.

2. Place your slide on the microscope.
   Note that the microscope is automated and the objective lenses should only be changed using the Microscope Control Panel Window or using the Digital Controller on your left (see below). Always begin with the lowest magnification.
The window on the right shows the configuration of the microscope and the current light path. By clicking on the “Lamp” button on the lower right of the microscope, the lamp turns on and off. Notice that the current configuration allows light from the lamp to enter the binocular eyepieces. Clicking on the objective lens will change the objective lens. Clicking on a filter will change the filter. **DIC is the appropriate setting for viewing your specimens with transmitted light.**

3. Once you locate your specimen with transmitted light, change to the appropriate magnification using the controller unit or the control panel window.

4. To view your specimen under conventional epifluorescence, click on the appropriate filter (e.g., FITC) and click off the Lamp. Make sure the Light Path Selector Knob is open (pulled out; see right). See the examples below of incorrect and correct light path configurations.
Once you have your specimen in focus and at the appropriate magnification, you are ready to view your specimen with confocal imaging.

5. First, push in the light path selector knob on the microscope (see #4).

6. Click on the LSM button in the box at the top of the Microscope Control Panel Window (see arrow).

7. Click on the LSM button in the filter selector on the Microscope Control Panel (see arrow).

Which dye are you using? Make sure the setting on the confocal scan head is appropriate for Channel 1 (PMT 1) and Channel 2 (PMT 2).

8. Select the appropriate dye (wavelength). Drag and drop the dye for Channel 1 to the 1st dye box (PMT1 – black arrow); drag a 2nd dye to the 2nd dye box (PMT2).
After dye selection, you will prepare the PMT Channels *(red arrow)* for the appropriate dyes.

9. Click on the 1\textsuperscript{st} column (Channel 1) to collect a single wavelength (See Confocal Scan head for those emission spectra collected in PMT 1); add Channel 2 if you are collecting a 2\textsuperscript{nd} wavelength (See Confocal Scan head). Channel 3 is to collect a transmission image. If you are using a single dye, then you only require Channel 1.

The images for any channels can be manipulated by altering the PMT sensitivity, Gain and Offset of each individual channel.
To begin collecting LSCM images in the Z-axis (a Z projection):

A. Click on the XYZ button.

B. Click on Focus. An image should appear. You can focus the image using the arrow buttons in the window below.

C. Find a starting position to begin collection of your z-series, click “Set” on Start Z.

D. Find the stop position and click “Set” on Stop Z.

E. Enter the step size of the image slice you want to take. Be warned, the more image slices you take, the larger the file size and the longer the scan.

F. Check your Scan Size. Normally, 512x512 is sufficient, but a higher setting (1024x1024) might be appropriate. A higher setting will dramatically increase the scan time!

G. Click on Kalman (Kalman filter mode takes the average of a set number of images, i.e., it cleans each image up). A higher number provides a cleaner picture, but adds a significant amount of time to image collection. For most purposes, a Kalman of 5 to 10 works fine without adding too much time to your collection.

H. You may also consider scans of different speeds (fast, average, slow). Average works very well. Slow scans reveal more information in some cases, but can also photobleach your specimens much quicker.

I. Now, you are ready to collect a z-series. Click Start Scan and relax as your specimen is scanned.
J. Once complete, Click Series Done. You can now make a Z-projection of the image by clicking on the Z button (red arrow). Save your display under the File window, and make sure to save your experiment as well. Once an experiment is saved, you can import it into a variety of free software packages (e.g., ImageJ, Confocal Assistant) for making 3D images and video. Before logging off the system, burn your files onto a CD or jumpdrive.

Shutting Down the System – If you are the only user scheduled for the Confocal system that day, you are advised to shut down the system. If someone is scheduled after you, we advise you to log out of the software and leave the system on.

Shut Down: Log off the computer and shut it down. Turn off all instruments and the lasers. Be aware, after the Multi-line Argon laser is turned off, the fan will continue to run to cool it down. Cover the microscope with the blue cover and close the door to the room. Make sure to log your time into the Confocal Log Book.