

Operation Guide for the Leica SP2 Confocal Microscope

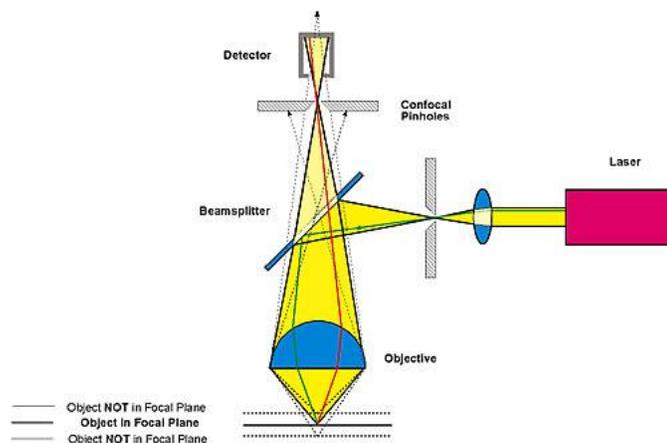
Bio-Imaging Facility

Hunter College

October 2009

Introduction of Fluorescence Confocal Microscopy

The first confocal microscope was invented by Princeton University researcher Marvin Minsky in 1957. "**Confocal**" is defined as "having the same focus." What this means in the microscope is that the final image has the same focus as or the focus corresponds to the point of focus in the object. The object and its image are "confocal." The microscope is able to filter out the out-of-focus light from above and below the point of focus in the object. Normally when an object is imaged in the fluorescence microscope, the signal produced is from the full thickness of the specimen which does not allow most of it to be in focus to the observer. The confocal microscope eliminates this out-of-focus information by means of a confocal "pinhole" situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged. Light from above and below the plane of focus of the object is eliminated from the final image. Thus, compared with conventional fluorescence microscope, the confocal microscope can obtain better images for thick samples. A diagram of the confocal principle is shown below:



While the image that is seen with confocal filtering is all in-focus information, this creates another problem. Compared to a normal fluorescence microscope, the amount of light that is seen in the final image is greatly reduced by the pinhole, sometimes up to 90-95%. To compensate for this loss of light somewhat, two components have been incorporated into modern confocal microscopes. First, lasers are used as light sources instead of the conventional mercury arc lamps because they produce extremely bright light at very specific wavelengths for

fluorochrome excitation. Second, highly sensitive photomultiplier-detectors (PMTs) are employed as imaging devices to pick up the reduced signal. A third technology that is incorporated into the confocal microscope is the modern microcomputer. The computer is used to control the microscope's scanner mirrors and motorized focusing mechanism as well as collect, store, and analyze the data. Data is stored in the form of digital images which may be observed on a computer video monitor or sent to a hardcopy output device such as a film graphics recorder or a video or digital color printer. The computer allows the system to scan sequential planes in the Z-direction, store them, and create overlays of all the in-focus Z sections. This information can also be used to create three dimensional images, or movie rotations of well stained specimens.

Starting-up procedure

- i) Turn on the following four units (Figure 1) in order:

Figure 1 Starting-up



1: Leica Mercury Lamp power switch: wait for 30 minutes ~ 1 hour for best performance.

2: Microscope main switch: ON

3: Ar/ArKr Laser Fan Button: ON

4: Scanner Power Button: ON

5: Ar/ArKr Laser key: ON (Wait for 30 minutes for best performance)

6: HeNe Laser Key: ON (Wait for 15 minutes)

7: Solid State Laser Key and Button: rotate the key from O to | ; press green button.

- ii) Log on to the computer using your ID and password. If you are a first time user, contact Zhong Wang for new account setup.
- iii) Start the LCS program: double click the program icon on the desktop of the computer.

Image acquisition and system experimental setup

Hereafter are general guide lines for system setup and image acquisition:

i) Slide mounting, and regular eyepiece fluorescent observation: check the following pictures for regular fluorescent observation:

Figure 2 Eyepiece Observation Setup



Fig 2-1: Put the sample slide on the stage properly (flat and even)

Fig 2-2: Push in the mode control lever (located at the left side of the microscope) completely

Fig 2-3: Adjust the filter wheel to position 2 or 3 for eyepiece observation. Position 2 is used for green illumination (Rhodamine B); and position 3 is used for blue illumination (FITC)

Fig 2-4: Adjust the illumination switch from 1 to 0 for illumination

Fig 2-5: Up/Down button 1 is used for raising & lowering stage; rotation wheel 2 is used for focus adjustment; button 3 is used to switch between coarse and fine adjustment.

ii) Confocal imaging: check the following pictures for confocal imaging setup:

Figure 3 Confocal Setup



Fig 3-1: Switch the microscope to confocal mode by fully pulling out the lever

Fig 3-2: Adjust the filter wheel to position 4, which is for confocal imaging

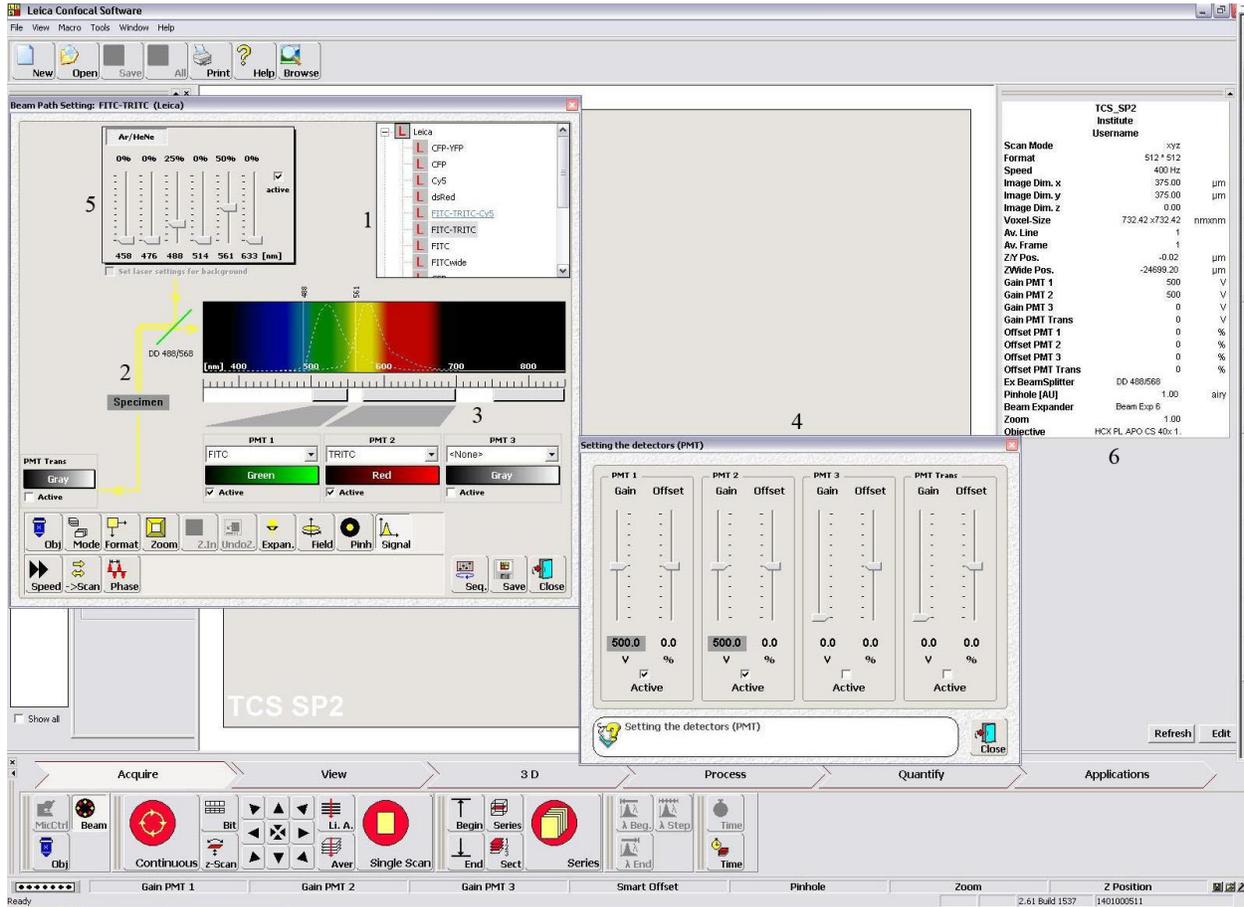
Fig 3-3: Up/Down button 1 is used for raising & lowering stage; rotation wheel 2 is used for focus adjustment; button 3 is used to switch between coarse and fine adjustment.

Please add the oil media (**Use Leica Oil Media Only**) on the cover slip when you need to use oil immersion lens.

iii) System parameter adjustment and Signal improvement:

To set up the system parameters, the beam path setting window (shown in Figure 4 below) can be activated by clicking the **Beam** button in the **Acquire** tab.

Figure 4 Beam Path Setting



- 1: Select proper dye or dye combinations to correctly set up the optical components.
- 2: Use this schematic drawing to make sure that the correct optical components are applied for the experiment.
- 3: Use these PMT spectral controls to modify the receiving band of the PMTs to match your application need.
- 4: This PMT gain/offset control menu is activated by clicking the **Signal** button in the Beam Path Setting window. Use these controls to adjust the PMT gain and offset in order to obtain the best image quality. **Please note: the PMT gain should be less than 750.**
- 5: Use these laser controls to modify the laser emission intensity for obtaining higher signal output.
- 6: All the current system parameters can be viewed in this table.

Shutdown procedure

- i) When you finish your experiments, stop scanning to terminate the image acquisition and restore all the parameters to original settings. Then quit the Leica application software.
- ii) Make sure you save all the image data sets in the local hard drive or network, then shut down the following four units in order as shown in Figure 5:

Figure 5 Shutdown



- 1: Ar/ArKr Laser Key: OFF (rotate from | to O)
 - 2: HeNe Laser Key: OFF. **Wait 5~10 minutes for the laser cooling process, then move to 3**
 - 3: Laser Fan button: OFF
 - 4: Scanner Power Button: OFF
 - 5: Solid State Laser: press red button and then turn key from | to O
 - 6: Microscope Main Power Switch: OFF
 - 7: Leica Mercury Lamp Switch: OFF. Check if someone is using this system after you. If that is the case leave switch on, otherwise turn it off.
- iii) Log off the computer
 - iv) Wait 5 minutes for mercury lamp to cool , clean the lens, cover the microscope to prevent dust.

System maintenance and lens cleaning

i) Sample preparation: before the slide is mounted on the stage, make sure that the cover slip is completely sealed by nail polishing or other materials. This prevents mixing of the immersion oil and embedding materials, movement of the cover slip when moving the stage.

ii) Setting restoration: after experiments, restore all the parameters to the original settings

iii) Immersion oil: immersion oil is provided by the bio-imaging facility, immersion oil from other resource is not allowed in Leica confocal system.

iv) Lens cleaning: When the experiment is completed, clean the lens by lens cleaning liquid and the Edmund lens tissue. Other cleaning chemicals and tissue are prohibited to avoid damage.

Specifications of the available Lenses

See the following table for the available lenses:

Magnification Power	Immersion Type	Numerical Aperture	
10x	Dry	0.4	
40x	Oil	1.25	
63x	Oil	1.4	
100x	Oil	1.4	

Available Laser lines and excitation wavelengths of the popular dyes

Laser emission wavelength (microns)	Popular dyes
458	DIA
476	Fluo 3
488	FITC,Alex488,cy2,Dio, GFP, YoYo-1
561	Alexa 568
633	Cy5, Alex633