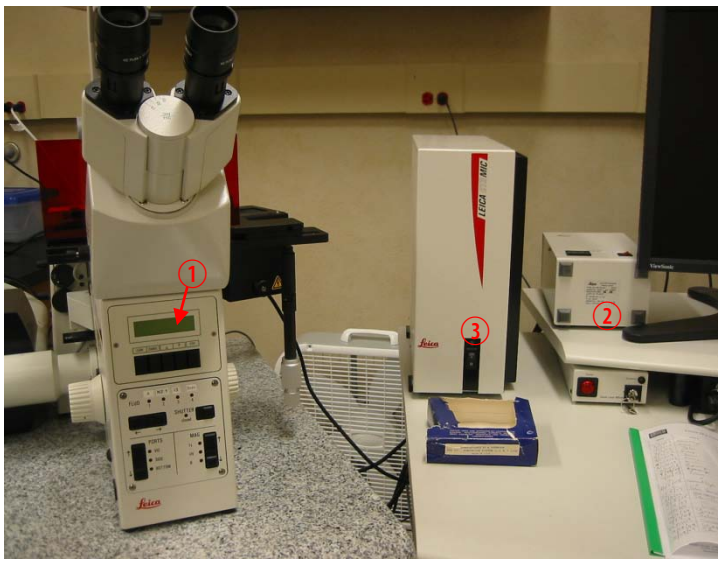
Manual for Leica SP2 Confocal Microscope

Enter you name, the date, the time, and the account number in the user log book.

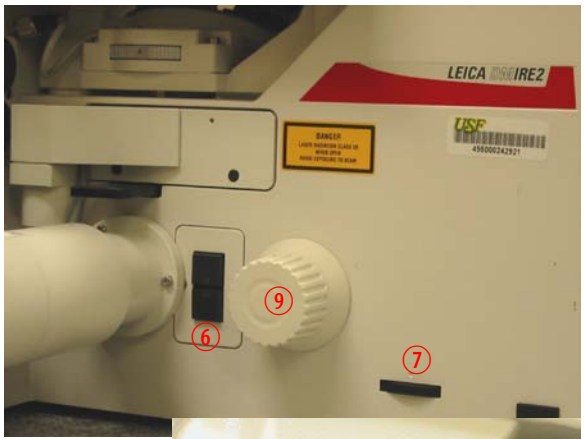
Things to check before start-up.

- Make sure that your sample slides are clean and sealed. Use Windex and cotton balls or Kimwipes to clean coverslips. Fixed samples need to be sealed with nail polish.
- Check the objectives. The 10x and 20x objectives are DRY objectives, and should NEVER have oil on them! There are 40x and 63x OIL immersion objectives, and a 63x WATER immersion objective also on this microscope. If oil immersion objectives have oil on them, wipe the lens gently only with lens paper. With the **microscope controller** ③ ON, you see the information of the objective in position on the **reading panel** ① at the front of the microscope. The order of the objective lenses in the lens turret is:10X, 20X, 40X Oil, 63X Oil, and 63X Water.



- The 63X objective has aperture collar adjustment rings that control the size of aperture opening. It should be wide open for most imaging condition. If your sample looks dim or the whole view field is not illuminated evenly when you look through the eyepieces, turn the collar to open the aperture.
- The field diaphragm and aperture diaphragm in the excitation path of the mercury lamp are controlled by round, black dials on the left side toward the back of the microscope, and control the fluorescence illumination from the mercury lamp. For all imaging with the mercury light source, these should be set wide open by turning all the way counter-clockwise.

Setting up for bright-field and fluorescence viewing.



Basic Operation of Leica DM IRE2 Microsocpe.

Turn on the **microscope controller** ③.

Turn on the **mercury arc lamp** ②.

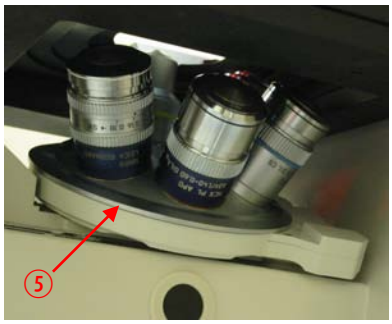
Bright-field viewing:

Place your sample slide with coverslip (preferably #1.5 thickness) facing down to the objective on the stage.

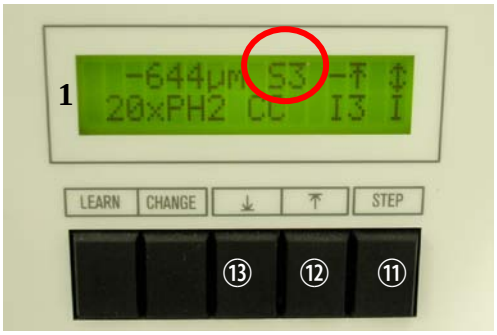
For bright-field viewing, turn on the microscope light by turning the wheel ⑦ toward you and turn the **VIS-SCAN switch** ⑧ to VIS position (Note: switch back to Scan position for confocal scanning, see below)

Start with low magnification objectives (10X or 20X) first to find and focus onto your specimen. The information of the selected objective and their z-

position can be read on the **readout panel** ① at the front of the scope. You can place the desired objective in position by turning **the lens turret** ⑤ by hand or by pressing the **objective change button** ⑥ on the left side of the microscope.



Pressing **upper objective change button** ⑥ once rotates in next higher objective into place and pressing **lower change button** ⑥ rotates in next lower objective into place.
However, pressing these buttons does not change between dry and oil objectives. To do this, first press ⇓⇑ **buttons** ⑫ ⑬ simultaneously, and when the readout panel reads "change objective I (or D by pressing again)", then push the **objective change button** ⑥.

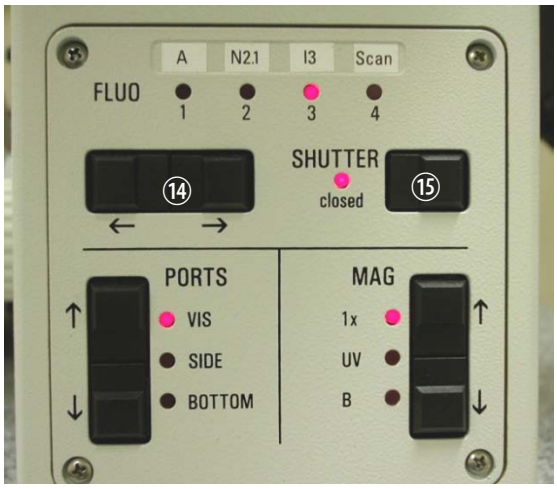


To focus, use **coarse movement button (⑩ upper one for bring lens up to a safety distance, "0" position and lower one for lowering lens down**) and **fine focus knob (9)** for focusing. The coarseness of focusing movement ("S0" (fine) to "S3" (coarse)) can be set by pressing "**STEP"** button ⑪**.**

For using oil or water immersion lens, lower the objective turret slightly, expose the lens by turning the turret half way, and place a drop of oil or water on the objective (be careful not to touch the lens directly with application tools!). Place the slide over the objective and raise the objective until the oil just spreads out as it contacts the slide. Use find focus knob to gradually bring your sample into focus. Check your slide occasionally to make sure that you are not pushing up the slide with the objective. This can damage the objective lens and your sample!

Please DO NOT press "LEARN" and "CHANGE" buttons.

<u>Fluorescent viewing:</u>
You can check your sample to see if your fluorescence labeling works by using the mercury lamp illumination and the appropriate filter set. The microscope has filter sets for viewing DAPI (A), Green fluorescence (I3) and red fluorescence (N2.1).



Press the **arrow horizontal button** ⑭ on the front of the scope to choose the filter set. "**Scan**" position has no filter set in place and is used for laser confocal scanning. The fluorescent light can be blocked (closed) or emitted by pressing **Shutter** ⑮ button. The "closed" light should be off for fluorescent viewing.

Turn the **VIS-SCAN switch** ⑧ to **Scan** position to block the transmitted bright-field light, if necessary.

Setting up for Confocal Imaging.

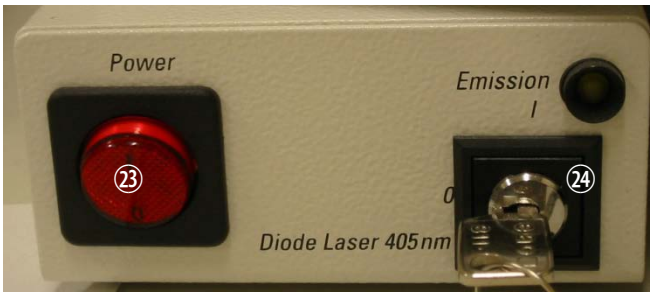
Turn on the **Scanner/Laser He/Ne** switch ⑰ (the scanner should be on for about 1 min before starting the LCS program).

Turn on the **PC/Stand** switch ⑯ and turn on the computer. Log onto using your User ID and password or TCS User.

Start the LCS program by double-clicking the LCS icon.

On the main menu, select the **Personal** that contains your setting for acquisition parameters you have created or **Company** for a default setting.

To start the laser(s);



(1) Turn on only the lasers you will use. They do not need more than a minute or so to warm up and stabilize, so it is not necessary to turn them on until you know you need them.

(2) Argon (for 458, 476, 488, and 514 nm laser): Turn on the red **Laser Ar/ArKr** switch ⑱ and turn the **Level Ar/ArKr** knob ⑲ to **MIN**. Then, turn the left **Ar/ArKr key** ⑳ to "ON", then push to "START" and release. Adjust the laser power with **LEVEL** knob (usually 9 o'clock position is sufficient).

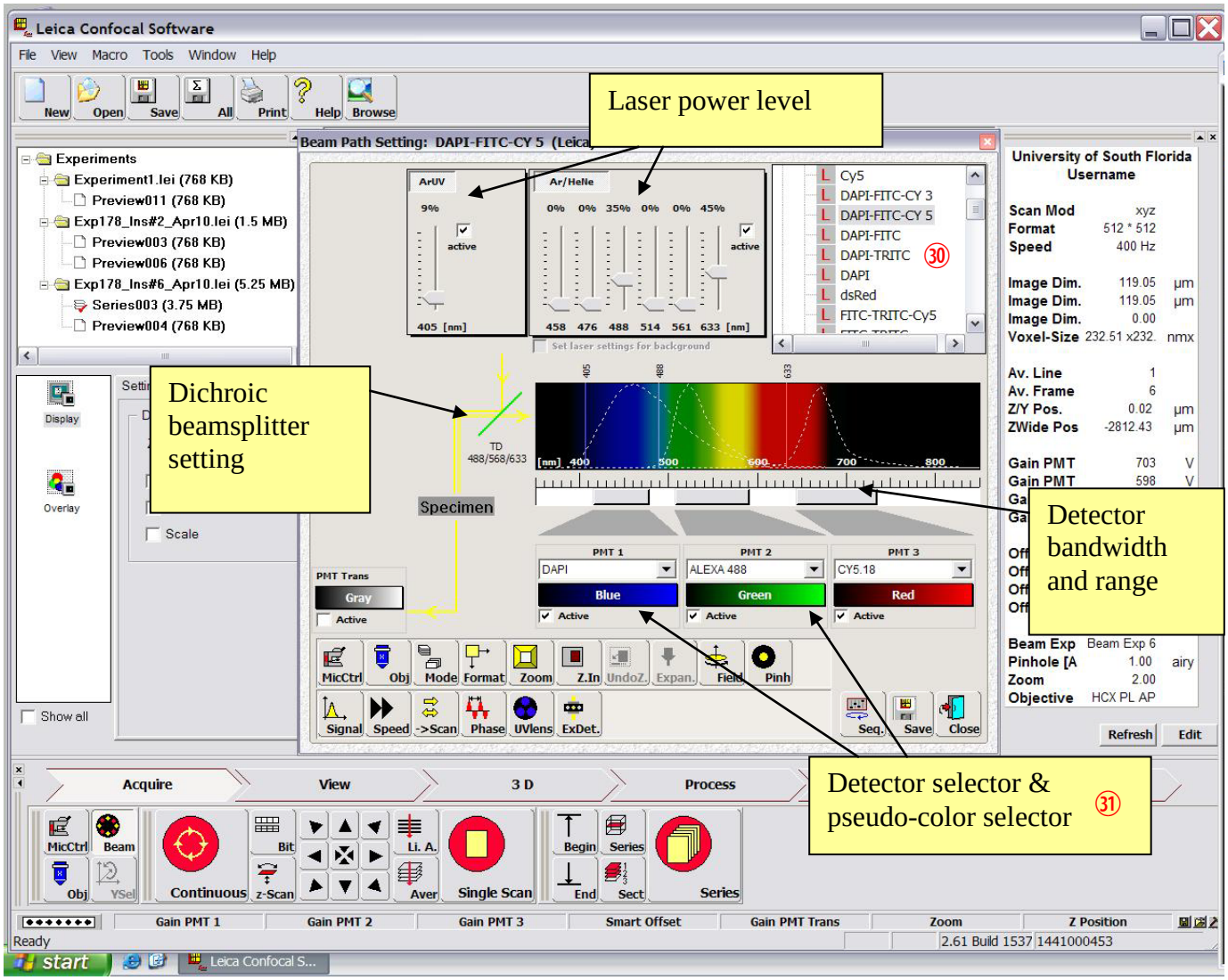
(3) Melles Griot yellow DPSS (561 nm) laser: push the **green button** ㉑.

(4) He/Ne (633 nm) laser: turn on the right **He/Ne 633 key**㉒.

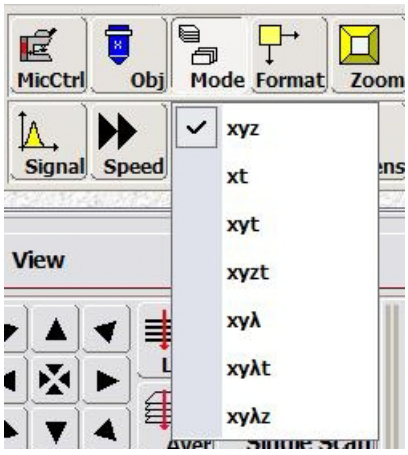
(5) Diode UV laser 405 nm: Turn on the **red power** button ㉓ and turn the key ㉔ to **I** position.

LCS Imaging program setting

Upon LCS program start, the basic image acquisition menu will appear. The "**Acquire**" button should appear pressed.



Click "**Beam**" button and select the appropriate detector setup for the fluorescent labels you used on your sample from the drop-down list ㉚. This will set up the emission detector band-width, activate the proper detector (PMTs), dichroic beamsplitter, and excitation laser wavelength and power. You can adjust the range and position of detector bandwidth and the laser power level as necessary.



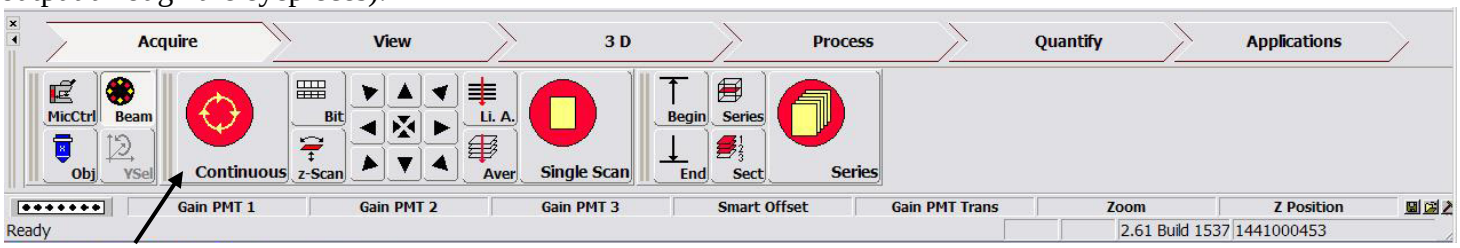
You also can change the color scheme of individual images into any color or grey scale by clicking **the pseudo-color selector** ㉛ associated with PMTs on the window screen (Note: the color information will be saved with image files when saved).

Press **Mode** button to select Scan Mode (default is XYZ).

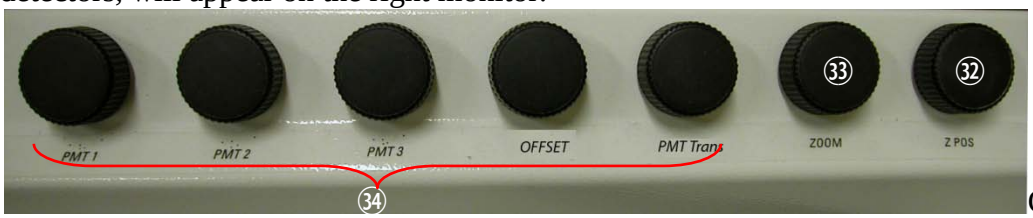
Press **Format**, **Speed**, and **Bit** buttons to select these parameters (defaults are 512x512 format, 400Hz scan speed, and 8 bit image).

Press **Z-scan** button and select **z-Wide** in order to use **Z POS** knob ㉜ to move the objective for focusing (Default is z-Galvo, which is used with Galvo stage adaptor).

Click **MicCtrl** button to switch to "**Visual"** mode, in which you can examine your sample visually at the microscope. Switch "**MicCtrl**" to "**Scan"** mode for laser scanning (it will automatically shut off visual output through the eyepieces).



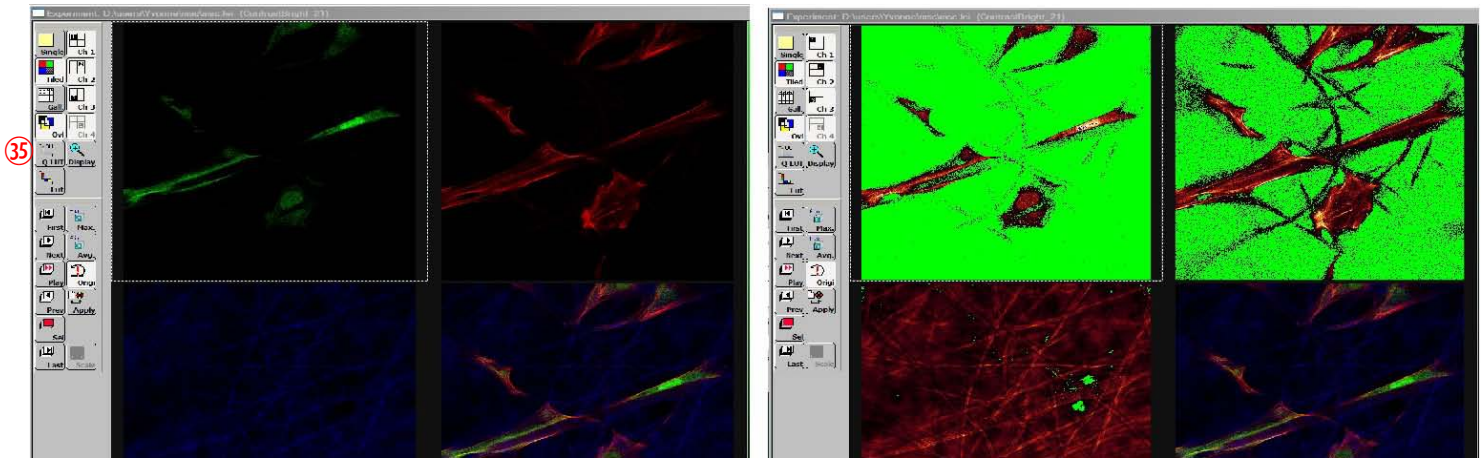
Press "**Continuous"** button to start scanning. An image or images, depending on the number of active detectors, will appear on the right monitor.



Control Panel

Optimize the images by adjusting parameters including;

- The z-position (focusing) within the specimen (**Z-POS** knob㉜).
- Zoom factor (**ZOOM** knob ㉝): Default is 1. Increasing zoom will magnify the image but also will bleach the sample faster!
- **PMT** and **Smart Offset** knobs ㉞ **:** Turn **PMT knobs** to increase the signal intensity of each fluorescent and transmission image channel. To decrease background signal, click the mouse over the image channel to select it and turn **Offset** knob counterclockwise. By activating **Q LUT** button ㉟, a full dynamic range of the PMT can be obtained. In this mode, pixels saturating the PMT will appear blue and pixels which are black (0 value) appear green. Set the PMT gain so that the brightest pixels are just under the saturation and set the PMT offset such that the darkest pixels are just above the zero value.

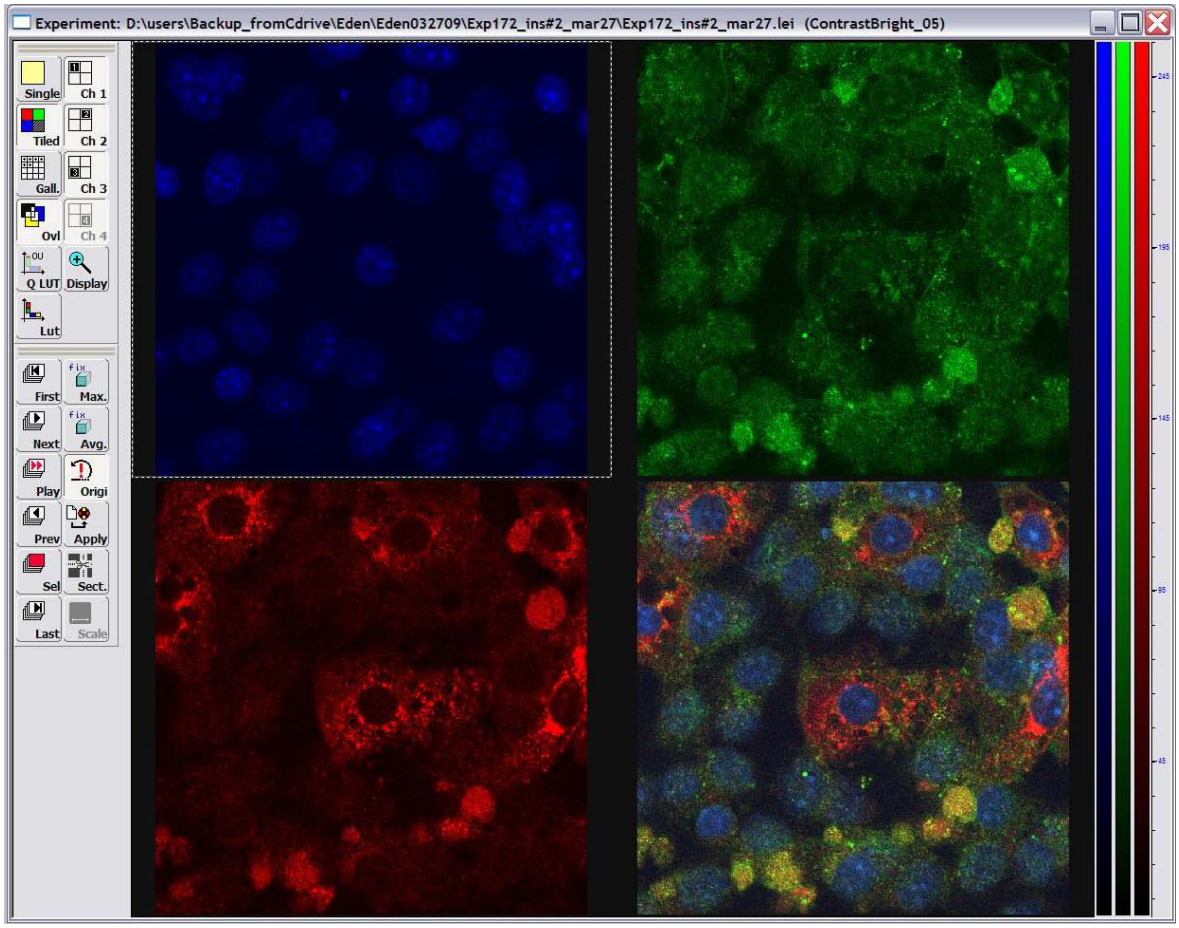


- **Laser power:** adjust with dragging the laser power bar on the menu window if necessary.

Click the **Continuous** button again to stop scanning.
You can save the current parameter setting with **Save** button for later use.

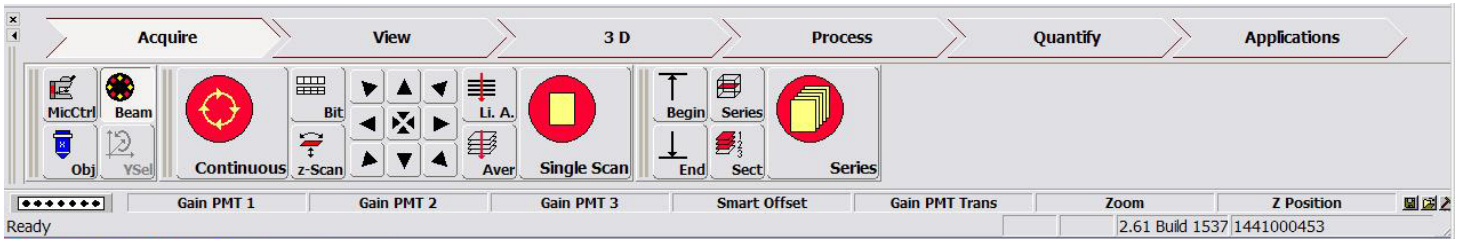
Working with Images.

Single button– puts up full screen of one fluorescent channel.
Ch1, 2, 3, 4 buttons– activate the channel on the screen.
Tiled button– views up to 3 channels simultaneously.
Ovl button – overlays (merge) multiple channels into one.
Lut button- open color look up table.



Acquiring Single Optical Section Image.

Make sure to switch "**MicCtrl**" to Scan mode.
Press "**Single Scan**" button to acquire an image by a single laser scanning at a scanning speed (normally 400Hz). The resulting image may display background noise. Image averaging is a process to decrease this noise and improve the signal-to-noise ratio.
To use averaging, click either "**Aver"** (frame averaging) or "**Li.A"**. (line averaging) button and select the number of frames (or lines) to be averaged. Then, click "**Single Scan"** button.

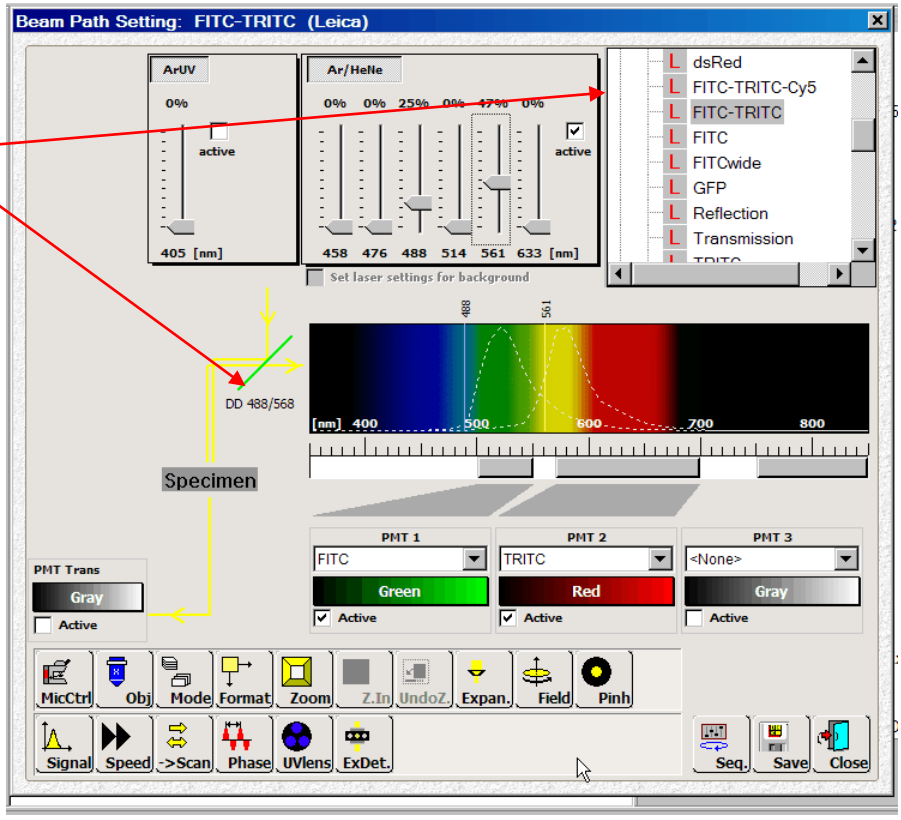


Sequential Scanning Mode

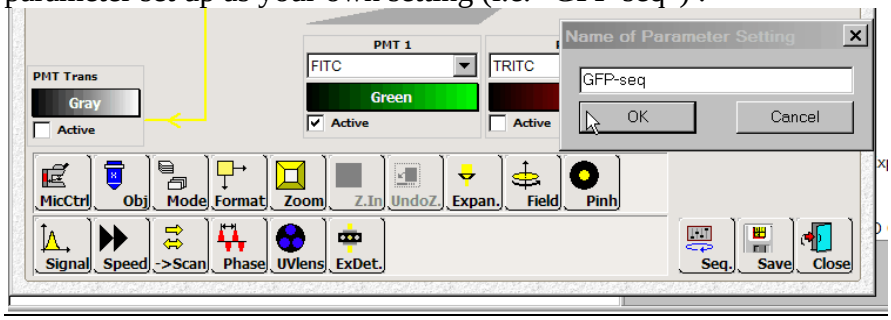
This confocal microscopy can detect up to four fluorophores simultaneously as long as all the excitation and emission spectra of fluorophores are well separated. Imaging of samples stained with different dyes thus simply requires manipulation of laser power and adjustment of the detector bandwidth. However, often excitation of one fluorophore may cause emission into the range of another (bleed-through) or can be induced by neighboring laser lines (cross-talk), which produce false signals. To avoid these, the sample can be scanned sequentially by collecting one fluorophore signal at any given time.

To scan sequentially:

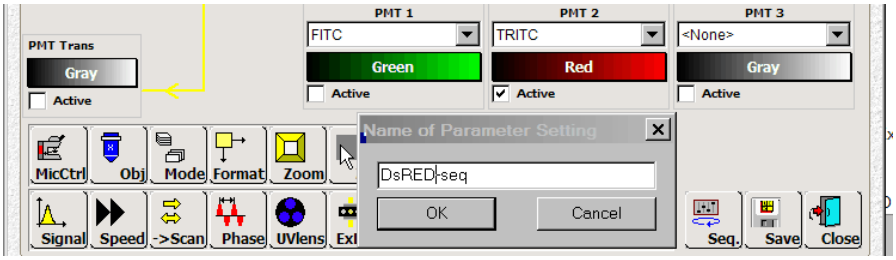
- Choose the beam setting parameter for your fluorophores (i.e. GFP and DsRED) by selecting laser lines, dichroic mirror, and PMTs.



- Set up a condition for one fluorophore by only activating and adjusting laser level and PMTs. Save this parameter set up as your own setting (i.e. “GFP-seq”) .



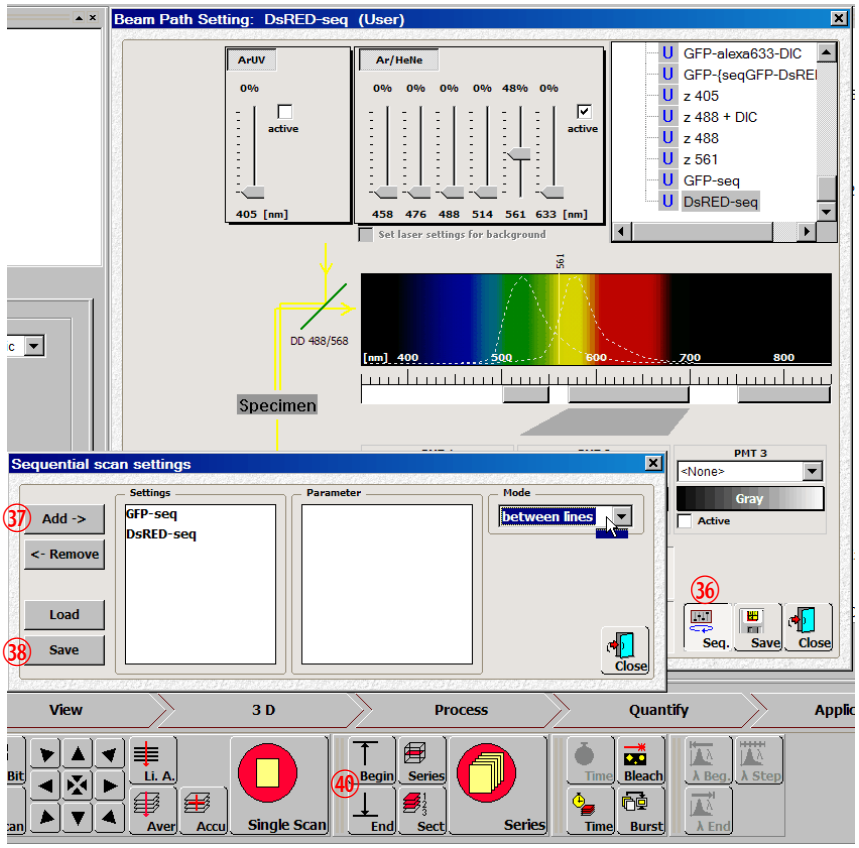
- Repeat the same process for the other fluorophore and save the condition as another setting (i.e. “DsRED-seq”).



- Click the **Seq** button㊱ in the beam window. Add the fluorophore acquisition setting one at a time by selecting them in the beam window and then click **Add** button㊲ in the Sequential scan settings window. Keep the scan mode at "between lines"
  Do not close the settings window.

  You can save these sequential acquisition settings by **Save** ㊳ button.

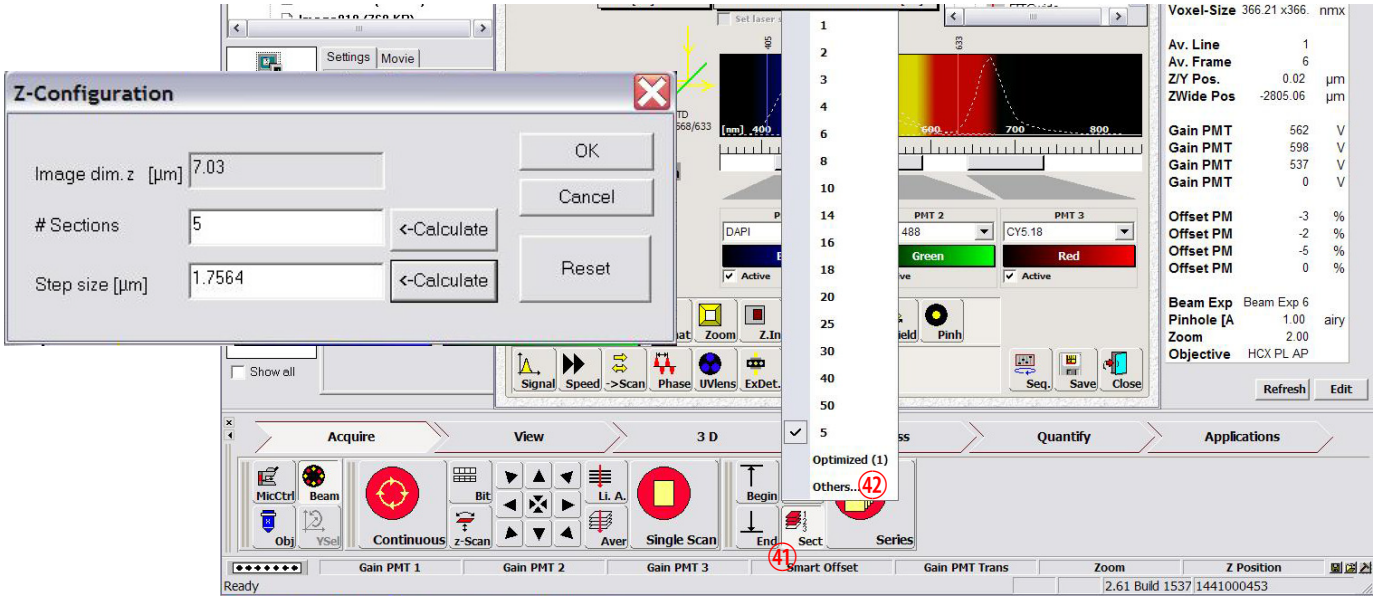
- Start the acquisition by clicking **Single Scan** or **Series** button depending on your imaging condition.



Acquiring Z-series images.

Z-series allows obtaining optical sections through the certain volume of your sample that can be used for making 3D images.

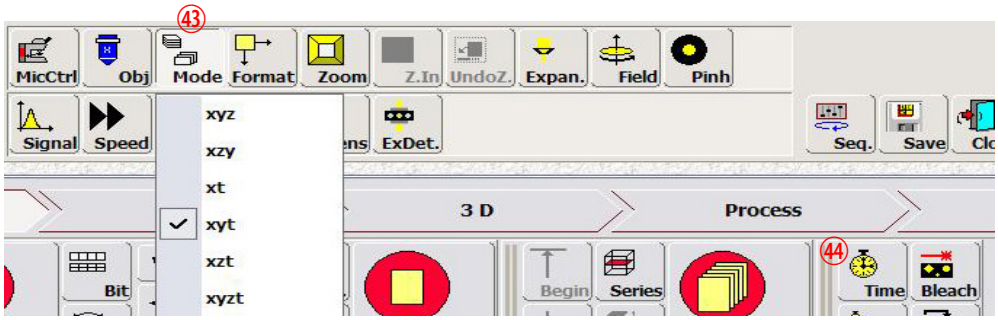
To set the z range, press "**Continuous"** button (it will be highlighted with "**Stop"**), turn **Z-POS** knob㊴ in a direction to focus to a specific z position of your sample and click **Begin** button ㊵ (it will appear depressed). Turn **Z-POS** in opposite direction to reach a desired position and click **End** button ㊵.
Click **Stop** button to stop scanning.
Click **Series** button to check the thickness of the z-series and determine the number of optical sections.



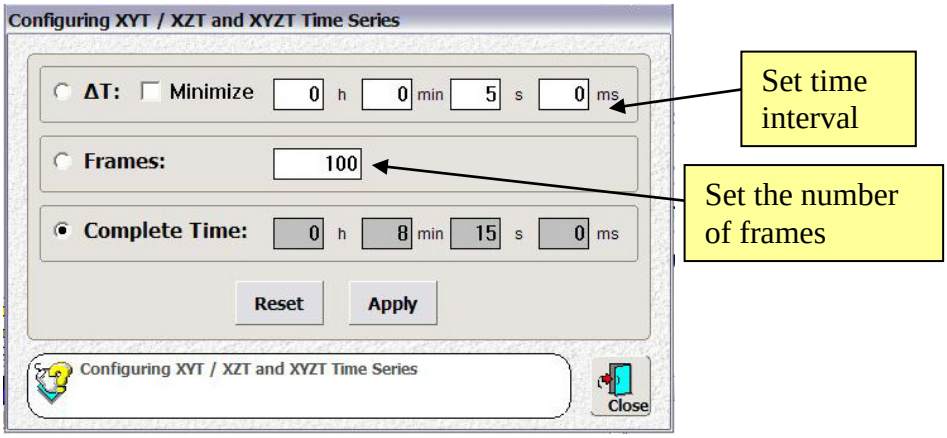
Click **Sect** button ㊶ to select the predetermined number of optical section, or to select **Others** ㊷…
It brings up a z-configuration window to determine the number of sections and spacing (step size) between sections. Press OK.
Choose the number of averaging using "**Aver"** button and click "**Series"** button to begin Z-series stack imaging.

Acquiring Time Series images.

Time series allows time-lapse imaging of live samples to study the changes and dynamics of your object of interest. The movie can be saved as multiple Tiff files and AVI files for playing in movie programs.



To set obtain time-series images, select scan **Mode** ㊸ as **xyt** (or other modes containing't'). It will activate "**Time**" button ㊹.
Click "**Time**" button to open the time-lapse setting dialogue and set the parameters such as the time interval between frames and the number of frames. Hit Enter key on keyboard, which will calculate the complete time. Click "**Apply**".



(Option) Click on **Aver** or **Li.A.** button to set the number of averaging per frame. Note: If your object of interest changes or move faster than scanning speed, averaging will generate distorted or ghost images. To increase the signal, use slower scan speed.
Click "**Series**" button to start the time series imaging.

Saving Images.

This is a very important for your effort and data. Computer crash or power outage will result in loss of unsaved data! SAVE FREQUENTLY!!

To save the imaging data files, click "**Save**" button at the top of the window. Select **D** drive and **users** folder. Create your folder, rename the Experiment# and save.

Since the storage memory in the computer will be filled up quickly and this lack of hard drive space prevents image acquisition, all user files will be deleted after two weeks. Be sure to copy your files to a flash memory or CD/DVD disks.

Shut-down Procedure

Please enter your time of use in the log book and note any problems and suggestions during your time.

1. Clean the oil from the objectives only with lens paper (not Kimwipe). Clean the microscope and stage.
2. Close the LCS program.
3. Copy your data files to a flash drive or CD. Users are responsible for their own files, as there is no automatic backup at present.
4. Log off the computer (not turning off the computer) and turn off the microscope control box.
5. If the next user won't be ready within 1 hour: Turn off the mercury lamp.
6. If you are the last user of the day:
   a. Turn off the mercury lamp.
   b. Minimize the power of the Ar for >3min before turning it off.
   c. For the Ar laser, turn the key to "off" and after 10 min turn off the red button switch.
   d. Turn the Melles Griot DPSS laser off by pushing the OFF button.
   e. For the He/Ne, turn the key to off position.
   f. Exit windows and shut down the power for PC stand, Scanner/Laser HeNe, and Laser Ar/Kr with the red buttons.