

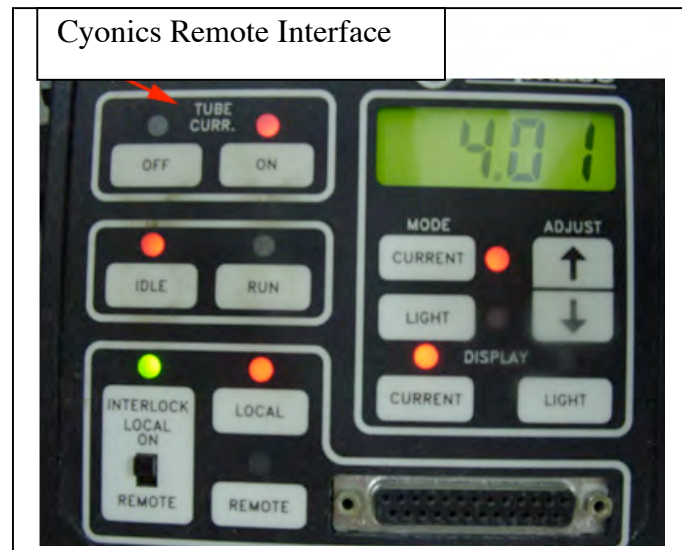
## Startup Procedure

1) **Turn on the Mercury source** (optional) at the OPTI-QUIP power supply. The lamp should be run for a minimum of 30 minutes before shut down and must cool to room temperature before it can be ignited again.

2) **Turn on PowerStrip.** This turns on the cooling fan for the Argon laser and the Argon Controller (aka Cyonics Remote Interface). Check that the cooling fan is running and that the exhaust is vented away from the microscope and other sensitive equipment. Do not go on to pump the laser if the fan is not working,

3) **Pump the Argon laser via Cyonics Remote Interface:** Press the ON button (TUBE CURR.) and wait until the laser ignites (once ignited the laser will run at about 4.02amps in the IDLE setting). Then set the laser to RUN and manually increase to power to ~6.2amps using the adjustment buttons. Do not run the laser at current settings higher than 7.0amps.

4) **Power up the computer, scope and monitors.** The power key on the computer, top left corner of the computer controls all of the devices. The key works like an ignition key for a vehicle, rotate the key clockwise past ON to START, then release. Press Cancel to login.



5) **Make sure PMT cooler is on at all times.** Never touch this switch and inform TA if light on switch is not on.

## Shutdown Procedure

1) **Shut down Argon Laser** via Remote interface: Switch the Argon Laser to IDLE and then to OFF via the Remote Interface and then allow the tube to cool to room temperature.

2) **Backup** your data to a CD, quit the Carl Zeiss program then choose shut down from the start menu

3) **Turn off the computer** via the Key switch on the front of the computer console

4) **Turn off the Mercury** source at the OPTI-QUIP power supply.

5) Once the Laser cooling fan is blowing cool room temp air (takes approximately 15 minutes), switch off the Argon Laser Cooling Fan and Argon controller by turning off **PowerStrip**

6) **Cover the scope.** Take care not the lay the cover on the Mercury housing.

## Viewing Sample through Oculars with Reflected or Transmitted Light

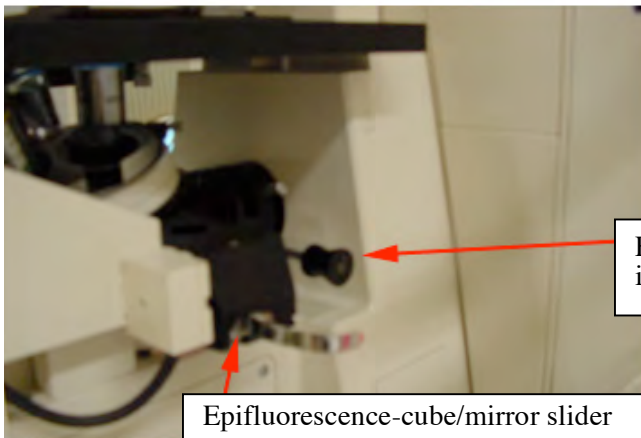
Generally, you will position your sample and locate your imaging region under the scope using transmission white light or epifluorescence. The stage can be moved using the x/y-axis position adjustment knob on the left side of the stage

1. On the microscope: Manually select the objective. For epifluorescence, move the epifluorescence-cube/mirror slider to the middle (green filter) or leftmost (red filter) position and move the epifluorescence intensity slider into open position.
2. Within the software, choose the objective in the scroll-down list to match the objective selected manually on the microscope.  
In the Control Panel, under Conv (conventional): Select FL (epifluorescence) or T (transmitted white light).
3. Position your slide on the stand and focus on your specimen.
4. Slide the epifluorescence intensity slider back into closed position to avoid bleaching the sample.

## Troubleshooting: Why can't I see anything through the oculars?



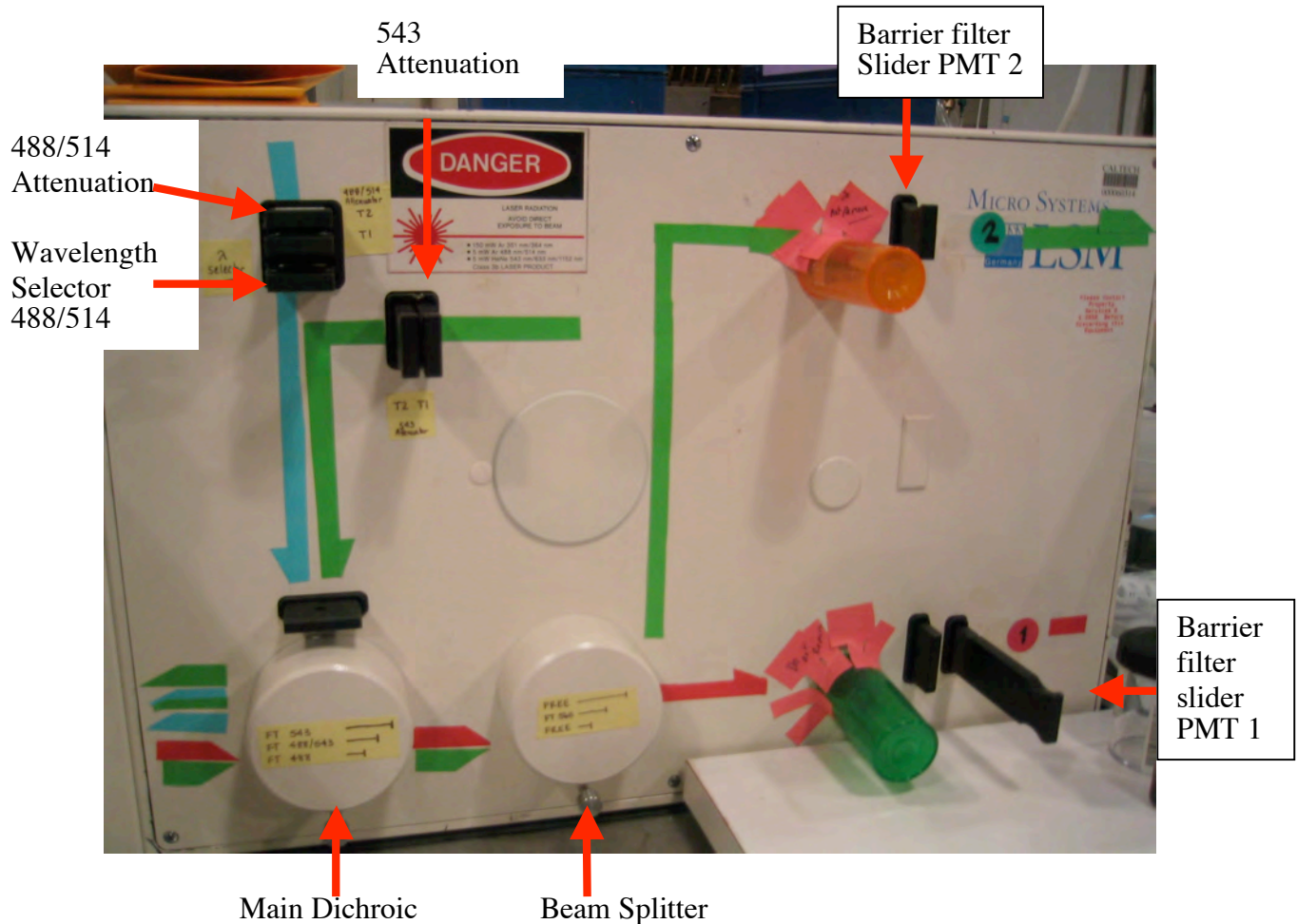
**A) Check Eye Shutter:** Directly below the eyepieces, on the right of the scope, there is a blackout slider for added protection during laser scanning. An internal, automatic shutter (that is activated when the LSM light path is selected) duplicates this slider.



**B) Check Epifluorescence Intensity Slider**  
Directly behind the epifluorescence filter cube is a slider that controls if and how much light from the mercury source reaches the stage. When the slider is pulled to the far right it blocks all light, the middle and left positions are empty allowing light to pass through.

**C) Make sure software is in FL or T mode**

## Hardware and Software Setup for Confocal Laser Scanning Microscopy



1) On the microscope: Manually choose an objective and find your sample visually (see instructions above). When done, move the epifluorescence-cube/mirror slider to the rightmost position. The guiding tracks are worn so it must be moved smoothly. Finally, move the epifluorescence intensity slider to the empty positions.

2) On the LSM box: Choose a laser line appropriate for your dye by setting the intensity with the attenuators by selecting the proper dichroic filter. Set the wavelength selector of the Argon laser to 488 nm. If using channel 2 (green), set beam splitter to FT 560, otherwise slide it to an empty position. Choose the proper emission filter by adjusting the barrier sliders in front of the PMTs.

3) Within the software, in the Control Panel: Select the channel corresponding to the PMT to be used. Via the Lens menu, choose the objective in the scroll-down list to match the objective selected manually on the microscope. Enter the proper attenuation value “At” and scanning time “t”. Select R/FL1 under LSM for single channel fluorescence imaging. Ensure that Function is set to “Frame”, that the “Laser” line(s) match the settings on the LSM box. Choose an image Size and select “Confoc” (confocal) in the “Extras” box.

3) Within the Software: Click on the Start button in the Scan box. Then, click the AUTOBC button in the upper-left corner of the main LSM screen to gather signal intensity data and automatically select contrast and brightness settings.

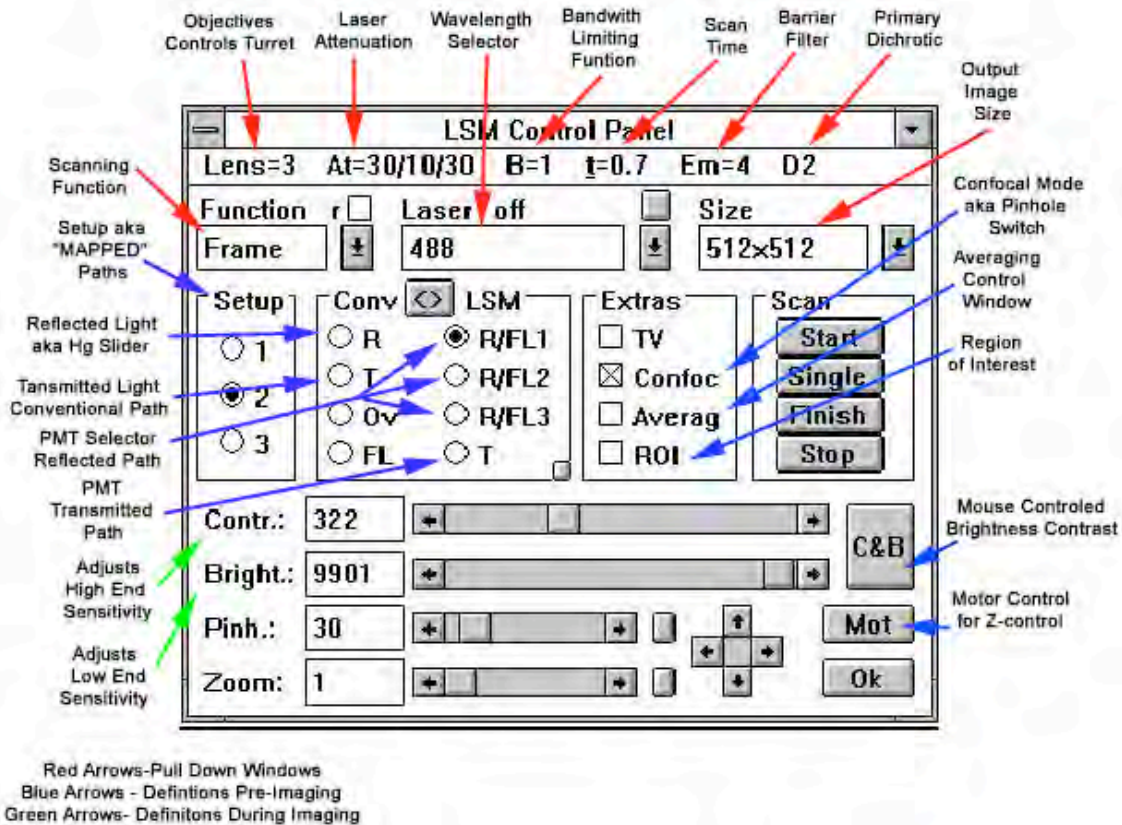
4) Fine-tune the quality of your imaging with the Contr (contrast) and Bright (brightness) controls. Contrast corresponds to the PMT gain and brightness to the intensity offset.

5) Check for pixel saturation, which indicates a loss of image quality, by repeatedly clicking on the Color button in the separate Display Control window different color tables can be selected. This applies the Range Indicator LUT (look-up-table, a pseudo color palette), in which red pixels mark high-end saturation and blue pixels mark low-end saturation. Adjust the contrast/brightness settings until all or most pixels are in a displayable range.

6) Clicking on the Color button again cycles to a Glow scale LUT, and one more click returns you to the original image (grayscale LUT). These LUT can also be accessed through the IMAGE tab of the main software menu under Color Tables.

7) Save your images with File->Store Image... from the main LSM window. The Save dialog box allows creation of new folders. Store your data in directories on the D:, E:, or F: drives.

## The Menu Bar on the Control panel



**Lens:** The 410's lens carriage is rotated mechanically. The turret will drop rotate and return to the same plane of focus as before. The lens setting must be correct in the software for accurate measurement (i.e., microns per pixel). Therefore, choose the objective in the scroll-down list to match the objective selected manually on the microscope.



**At (attenuation):** The Attenuation on the 410 is manually controlled. The sliders on the top left of the imaging box use the following nomenclature. T 1= no attenuation, T 0.1= 1:10 attenuation, etc. There are two sliders per laser line. The horizontal oriented sliders attenuate the 488/514 and the vertical sliders attenuate the 543. Suggested starting point is 1:100 attenuation. Values of 1:100 or 1:10 generally yield good results depending on the strength of your fluorescence staining or expression. Lower attenuation (1:3 or 1:1) increases signal at the expense of more photobleaching.



In “At” menu, set the left column to 1 and the right column to the number that matches the total laser line attenuation that results from the chosen attenuator positions (see tables below).

**Table 1. Total attenuation as a function of T1 and T2**

T1/T2	1	0.01=1/100	0.0032=1/300	0.001=1/1000
1	1	1:100	1:300	1:1,000
0.32=1/3	1:3	1:300	1:1,000	1:3,000
0.1=1/10	1:10	1:1,000	1:3,000	1:10,000
0.032=1/30	1:30	1:3,000	1:10,000	1:30,000

**Table 2. Possible combinations settings for T1 and T2 to achieve a given attenuation**

Attenuation	T1	T2	T1(alt)	T2(alt)	T1(alt)	T2(alt)
1	1	1				
1:3	0.32	1				
1:10	0.1	1				
1:30	0.032	1				
1:100	1	0.01				
1:300	0.32	0.01	1	0.0032		
1:1000	1	0.001	0.1	0.01	0.32	0.0032
1:3000	0.032	0.01	0.1	0.0032	0.32	0.001
1:10,000	0.1	0.001	0.032	0.0032		
1:30,000	0.032	0.001				

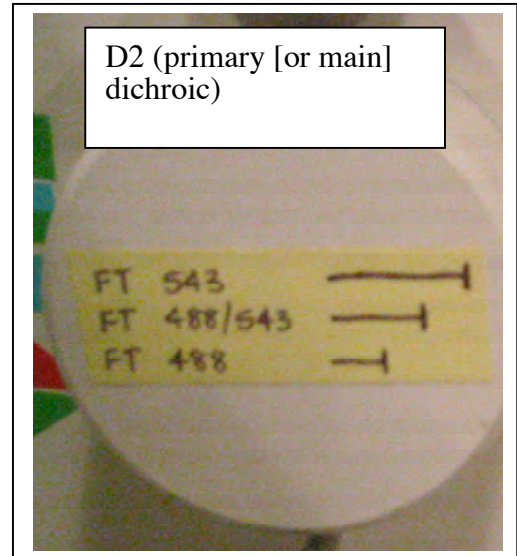
**B (bandpass):** This is a low-pass filter that will limit the bandwidth of the incoming signal. For most applications it should be left at 0 or 1. Increase B value when scanning at fast speeds.

**t (time):** The frame dwell time of the laser; the slower the laser scans, the more fluorophores are potentially excited (beware of bleaching). Lower it ( $t \sim 1$ ) to increase scan speed, and raise it ( $t \sim 8$ ) to increase picture quality.

NOTE: Search for your sample or find focus with fast scans. Then move to 4-8s (or longer) scans for optimization and image collection.

**Em (emission filter a.k.a. barrier filter):** These are the filters in front of each photomultiplier. Select one appropriate for the emission of your dye. LP = long pass; BP=band pass

**D2 (primary [or main] dichroic):** The first dichroic FT 488 is for use with 488 excitation, green channel emission. The second FT488/543, is for multichannel green red imaging. The final dichroic, FT 543 is for use with 543nm excitation and the red emission channel.



## Hardware Settings Suggestions

### Single Channel

Main Dichroic FT 488 or FT 543 based on needed excitation for fluorophore.  
 Beam Splitter: FT 560 for red dyes.  
 Barrier Filters: Based on dye emission.

### Dual Channel

Main Dichroic; FT 488/543  
 Beam Splitter 560  
 Barrier Filters: Based on dye emission

To image with a green and red channel (for instance, FITC and rhodamine), the FT560 filter would be used at Beam Splitter (Middle Position). Wavelengths longer than 560nm (red light) will pass through the dichroic to R/FL1. Remaining (green) light below 560nm will be reflected up to R/FL2 (see schematic below).

The General rule: Light spectra are passed to the photomultipliers in order from longest to shortest. R/FL1 will always receive the longest wavelength. Shorter wavelengths are reflected up to R/FL2.

