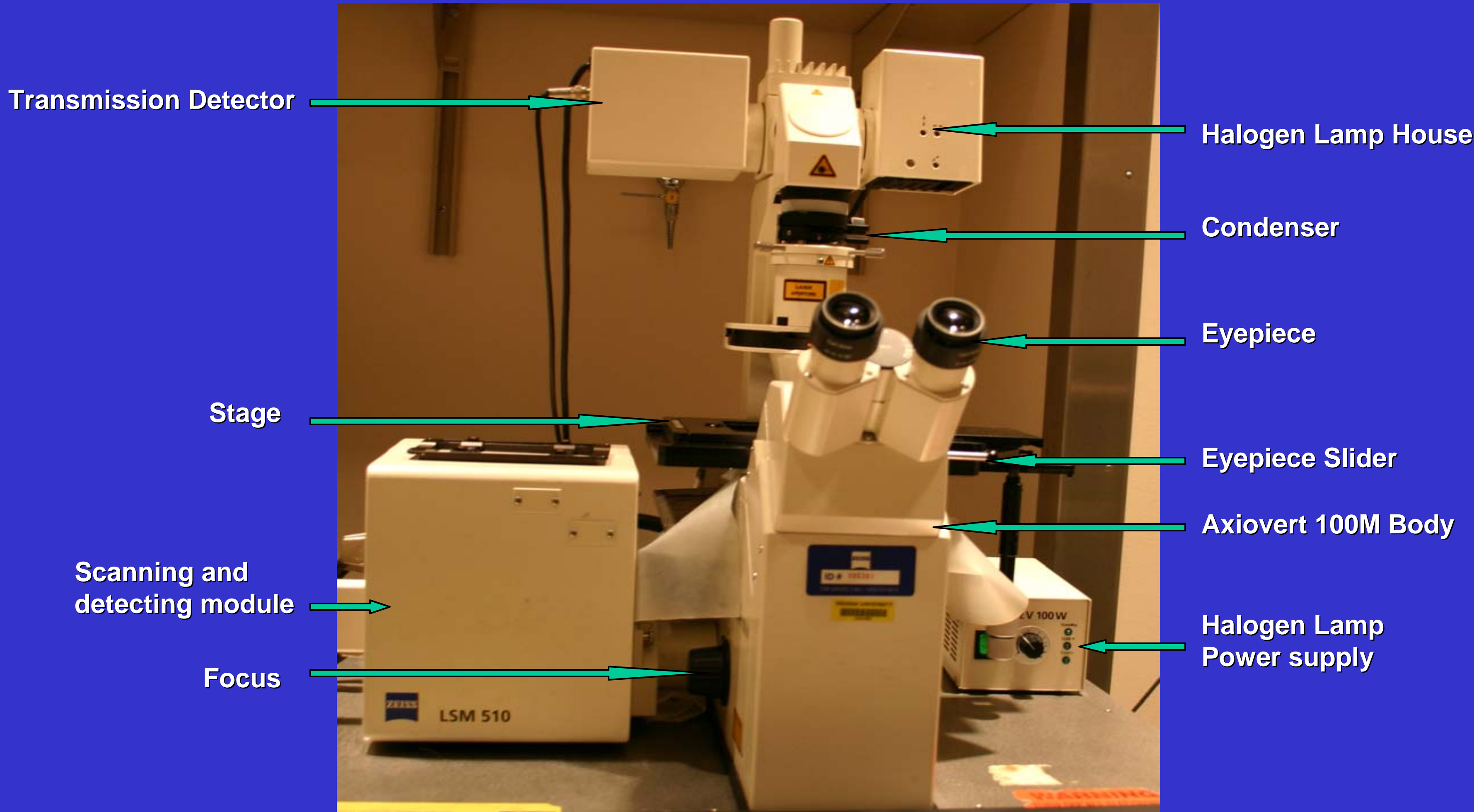
Indiana Center for Biological Microscopy

Zeiss LSM-510-UV Confocal Microscope

Microscope and the Attached Accessories



Xenon Lamp Power Supply

(This unit is located under the microscope table)

Laser and AOTF Controllers

Visible Lasers & Controller

UV Laser & Controller

UV Laser Power Supply

(NOTE: The Enterprise UV laser must be turned on PRIOR to launching the LSM software or it will not be recognized)

Starting Up The System

Skip to the next page if the system is already on, otherwise do the following:

1. **Turn on the Xenon Lamp power supply underneath the air table.**

2. **Power up the system's main switch (remote control) on the table located behind the scanning and detecting module.**

Starting Up The System

Skip to the next page if the system is already on, otherwise do the following:

3. **Turn on the RED TOGGLE SWITCH located between monitors to boot up the computer.**

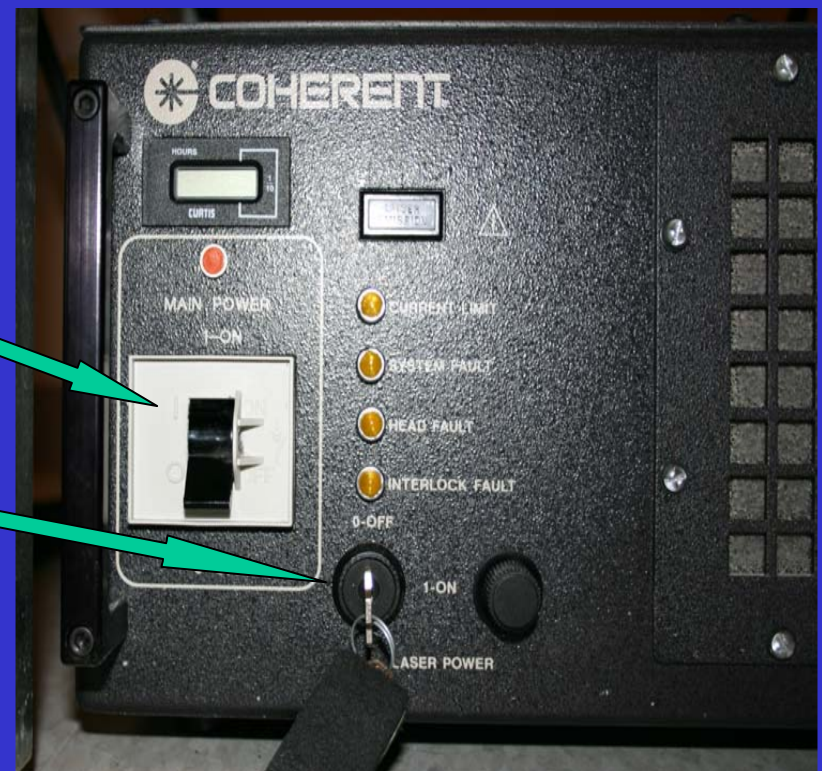
4. **ONLY IF NEEDED, turn on the UV power supply.**

   If you have dyes such as DAPI/Hoechst that require excitation by 364 nm line, you will need to use the UV laser. The UV laser also produces a line at 351nm, which is rarely used.

NOTE: The Enterprise UV laser must be turned on **PRIOR to launching the LSM software or it will not be recognized.*

To turn the UV laser power on, flip the main power switch up *(big black switch on the left).* This will turn on the water chiller.

Then TURN THE KEY clockwise so that the key is horizontal.



Using the LSM 510 Software Program

Using the LSM 510 Program

1. Logon to Nephrology Domain when prompted. Always use your own account.
   Do not share your account with anyone.
   Make sure to logoff when you are done.
2. Start the LSM510 program by double clicking on its icon on the desktop.
3. Select Scan New Images on the Switchboard window and click on Start Expert Mode to launch the program.

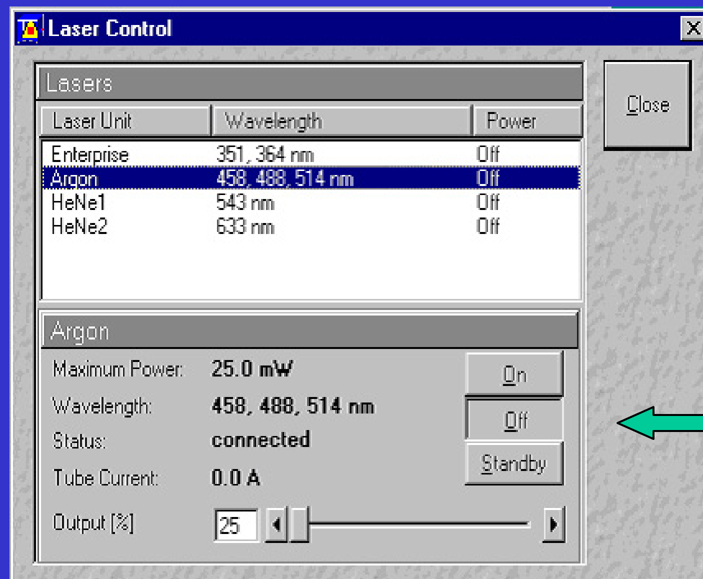
4. Click on the Acquire button on the LSM 510 window.
5. Then, click on the Laser icon on the LSM 510 window to start the Laser Control window.

Using the LSM 510 Program

6. Now, determine which laser lines you'll need.
7. Activate the appropriate lasers:

   To turn on the Argon UV laser *(named "Enterprise")* and Argon visible laser *(named "Argon")*, highlight the line and click on Standby. WAIT until the status becomes "Ready" before clicking the ON button.

   To turn on a HeNe laser, simply highlight the line and click the ON button.



Name of dye	Laser Line (nm)
DAPI, Hoechst	364 (UV laser)
Alexa488, Calcium green, Cy2, FITC, Fluorescein, fluo-4, GFP, Oregon green	488 (Argon)
Alexa543, Alexa568, Cy3, Rhodamine, Texas Red, DiI, propidium iodine	543 (HeNe1)
CFP	458 (Argon)
Alexa633, Alexa647, Cy5	633 (HeNe2)

Now that the laser lines are ready, you'll need to set up the right optics for your application.

Setting Up a Configuration That Fits Your Application…

Configuring Your Lasers/Dyes

1. To choose a configuration, click on ACQUIRE.

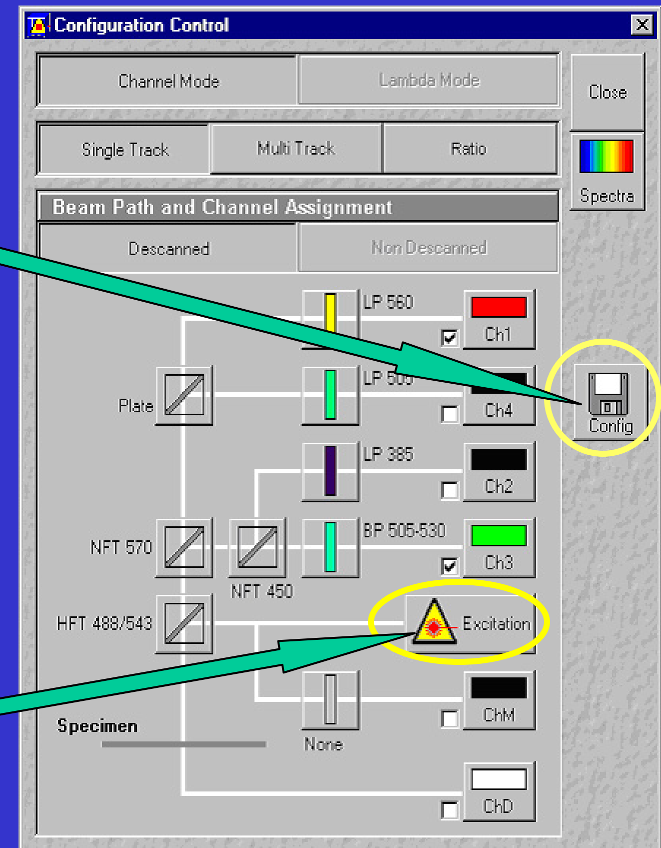
2. Select CONFIG.

3. Select the CONFIG button within the Configuration Control window to select the dyes for your experiment. There are a number of pre-programmed configurations that can be used for a variety of fluorochromes.

   For example, if you have FITC and Texas Red, you should then select FITC/Rhod.

   Please refer to the Laser/Dye table. You may also add your own configuration to the list, but make sure you save it under a different name from any existing configuration on that list.

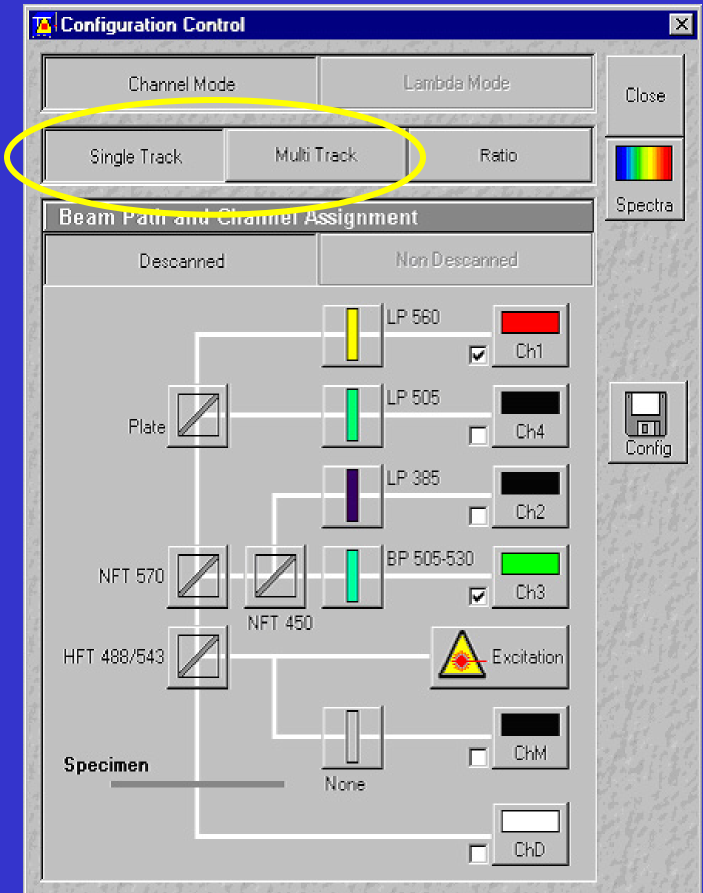
4. The Excitation button in the Configuration Control window shows which laser lines are currently activated for the present track.



Single Track vs. Multi-Track Mode

After selecting your dyes, you will then need to choose between **Single or Multi-Track mode.** There are 2 types of pre-programmed configurations:

1. Single Track
2. Multi-Track



Single Track

- Best used for single-labeled samples.
- May be used for multiple labels but because it excites both channels simultaneously, crosstalk can be more likely.
- Since the sample is illuminated by all laser lines simultaneously, single track mode is fast.

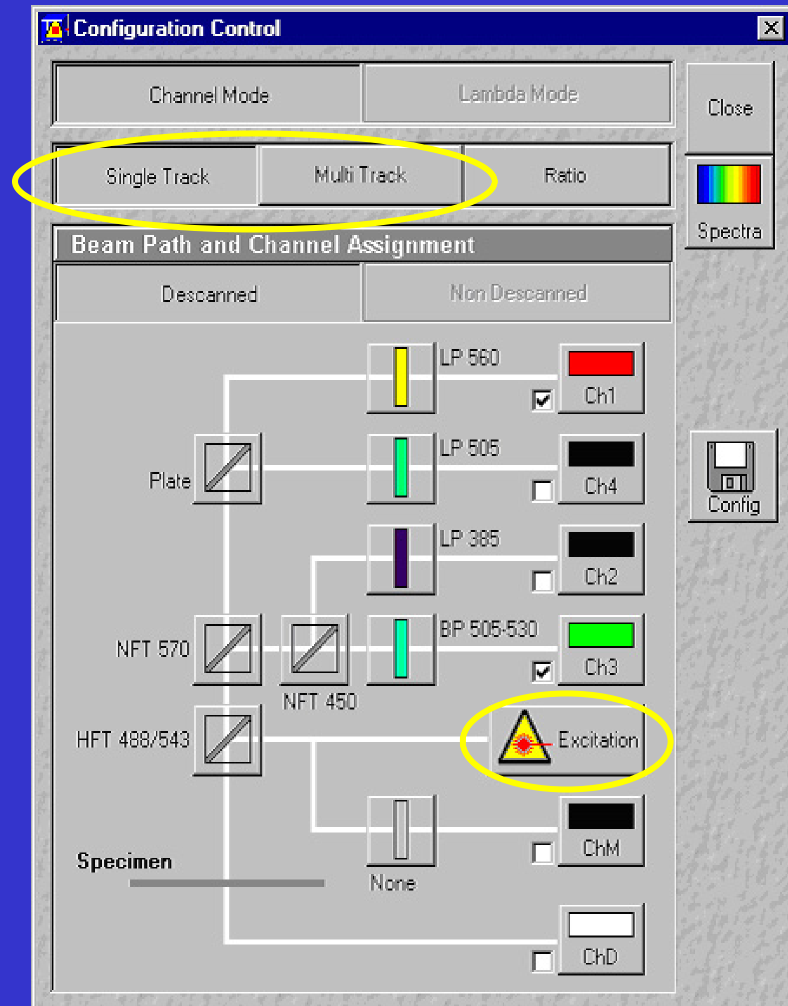
Multi-Track

- Best used for multi-labeled samples.
- Since the images are collected sequentially, switching between wavelengths, crosstalk is minimized from one channel to another.

Single Track vs. Multi-Track Mode

In the Single Track mode:

- The sample is illuminated by all laser lines simultaneously.
- The Excitation button will show which laser lines are currently activated.



In the Multi-Track mode:

- Sample is illuminated sequentially by the laser lines in different tracks.
- Only the channel(s) associated with the currently activated track will be collecting signal.
- There is reduced crosstalk from one channel to the other.

Configuring a Multi-Track Setup

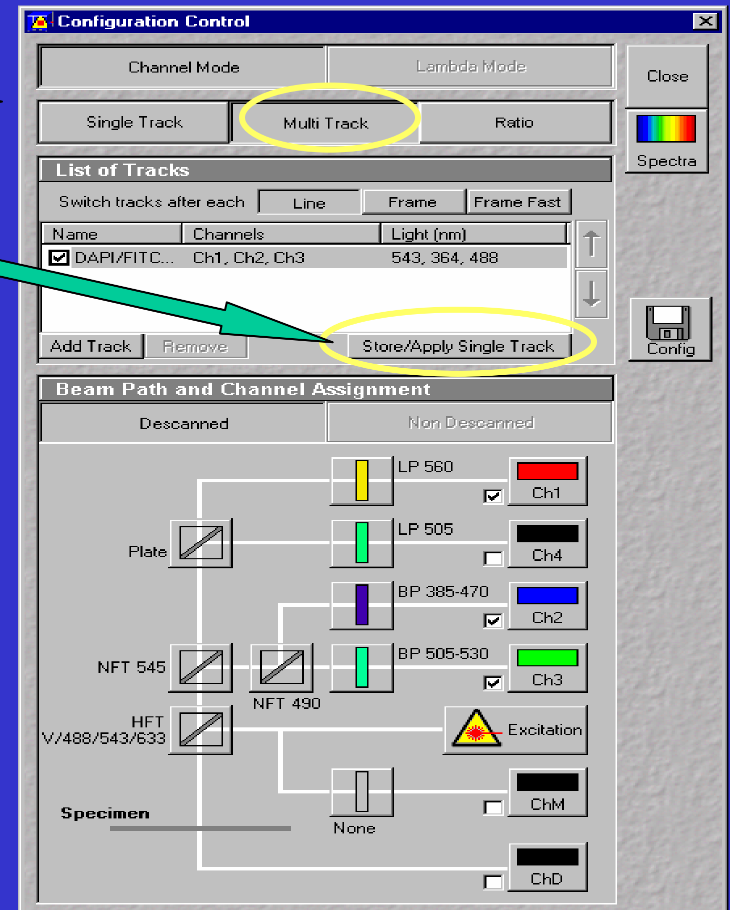
(DAPI/FITC/Rhod Example)

Setting Up the 1st Track:

1. Select the **Multi-Track button.**
2. Select the **Store/Apply Single Track button** to create a single track configuration for dyes being used, *(i.e. DAPI/FITC/Rhod).*
3. To create one activated channel for each track, you need to deactivate the two unwanted channels.
4. In this example, Ch1 is Rhodamine, Ch2 is DAPI and Ch 3 is FITC.

   If you decide to use the first track for Rhod, then Ch1 will remain activated, but Ch2 and Ch3 will need to be **deactivated.**

   Notice that the activated channels are those with a checked box.



continued…

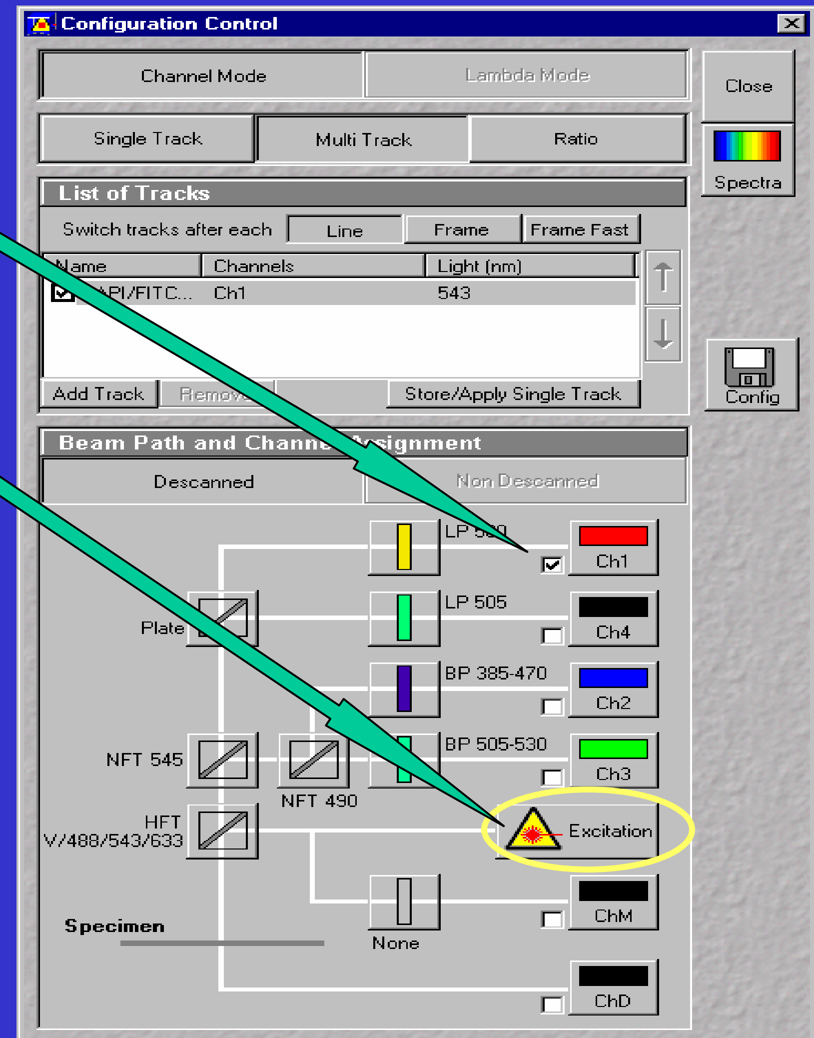
Configuring a Multi-Track Setup

(DAPI/FITC/Rhod Example)

Setting Up the 1st Track:

5. To deactivate a channel(s), uncheck it's respective box.
6. To deactivate unwanted laser lines, click on the Excitation button in the configuration window and then uncheck the unwanted lines.
7. In this example, the only line that now remains activated is the 543 nm.
8. This completes the First Track for Ch1.

Since this example has three dyes, two more dyes/laser lines need to be configured.



Configuring a Multi-Track Setup

(DAPI/FITC/Rhod Example)

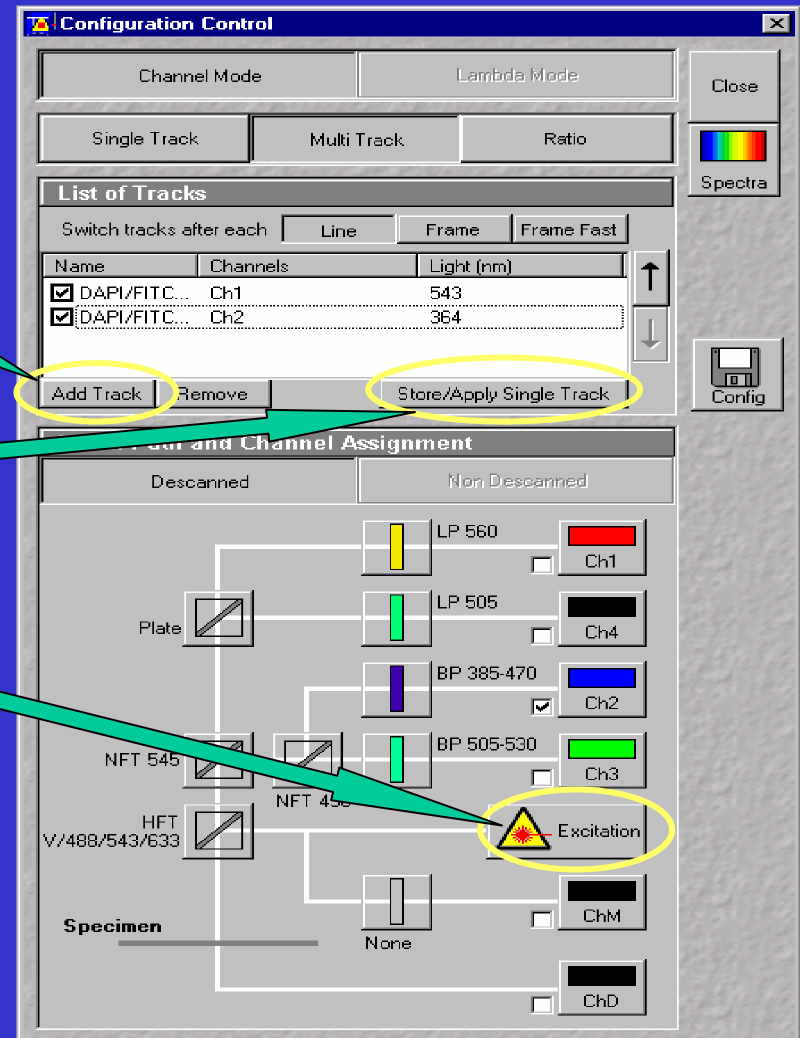
Setting Up the <u>2nd Track</u>:

1. Select Add Track.

2. Select the Store/Apply Single Track button and reassign the triad as done for the first track. *(i.e. DAPI/FITC/Rhod).*

3. To configure for Ch2, deactivate the other two detection channels (Ch1 and Ch3).

4. To deactivate unwanted laser lines, click on the Excitation button in the configuration window and then uncheck the unwanted lines.

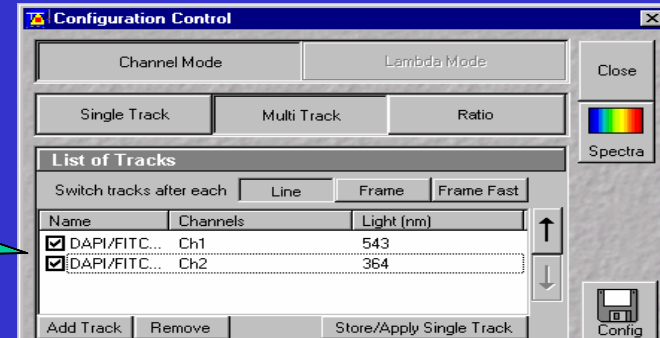


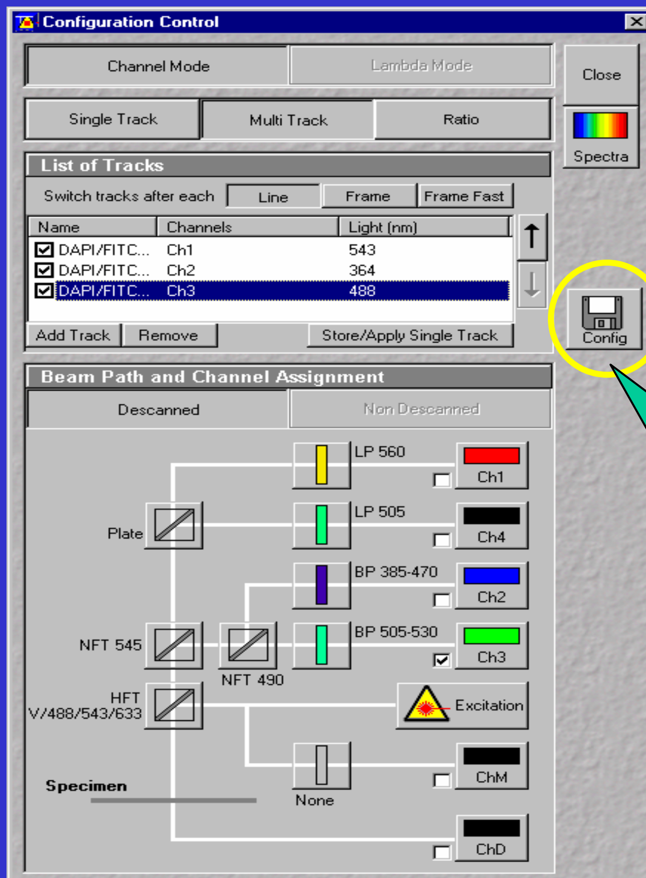
Configuring a Multi-Track Setup

(DAPI/FITC/Rhod Example)

Setting Up the 2nd Track:

5. This completes the configuration of two tracks, (Ch1 and Ch2).





Setting Up the 3rd Track:

1. Repeat all steps as in the 1st and 2nd tracks.

   The final list might look something like this…

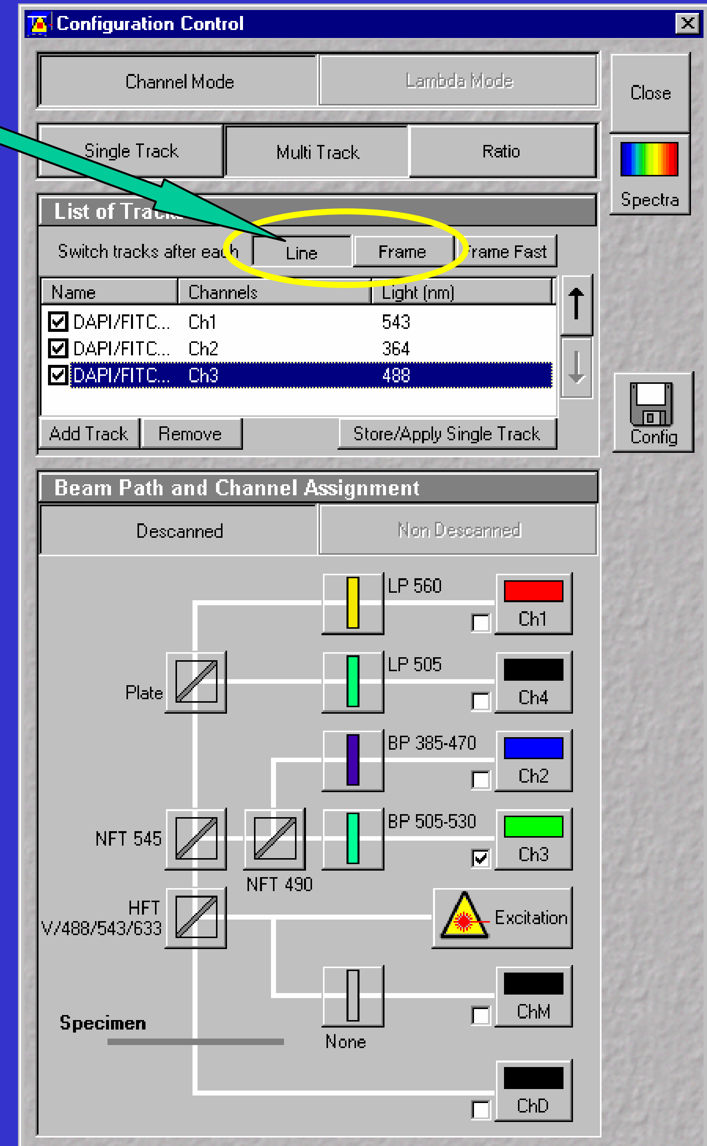
   This configuration can be saved by selecting the Config button and naming it for future use.

   To access it, click on the Config button, highlight the name of the configuration and click apply.

Line Switching vs. Frame Switching
in the Multi-Track Mode

Multi-Track mode offers two different forms of switching between tracks:

1. **Line Switching *(most commonly used)***
2. **Frame Switching**



Line Switching:

- The system scans line by line, one laser at a time, switching tracks sequentially.
- Good for imaging live cells to avoid registration problems between channels.
- NOTE: *No dichroic beam splitter or emission filter movement will be allowed in this mode. This will restrict the flexibility of coexistence of certain tracks, which requires the movement of dichroic mirrors and emission filters.*

Frame Switching:

- The system scans each track and channel sequentially, frame by frame.
- Not a good mode for kinetics as there is a time lag between the two collections.

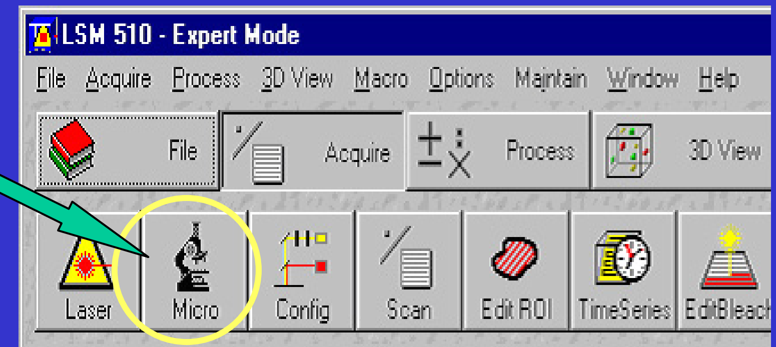
Visualizing a Sample with Transmitted & Epi-Fluorescent Light

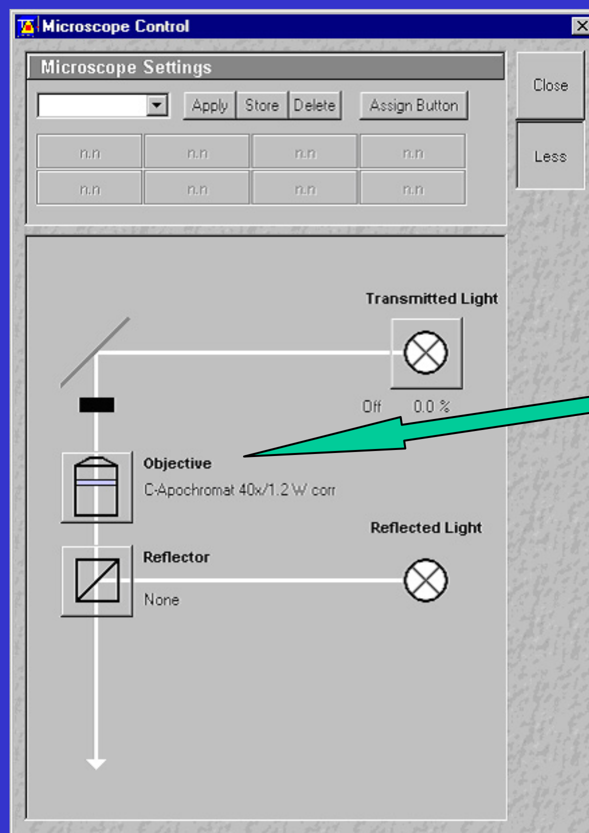
Visualizing a Sample through the Eyepiece

As part of preparation for image acquisition, you should view your sample using transilluminated light or epi-fluorescence before collecting images with the laser.

The following instruction will guide you step by step through the procedures:

1. Open the Microscope control window by clicking on the Micro icon on the LSM510 window.





2. Select an objective from the Microscope Control window by clicking on the icon of the intended objective from the list.

The 4 objectives on the turret are:

3 - WATER immersion objectives
(10X, 40X, and 63X)

1 - OIL immersion objective
(100x)

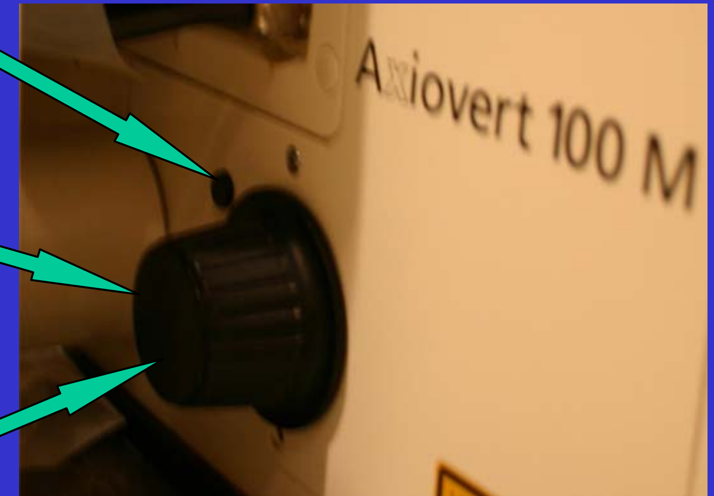
Visualizing a Sample through the Eyepiece

3. On the Axiovert 100 microscope, the small black button on the left side of the scope next to the focus knob alternates between coarse and fine focus.

4. To lower the stage, turn the focus towards you and apply a drop of the appropriate medium (water or oil) on the lens to be used.

5. Place the sample on the stage adapter. Make sure your slide or dish is fixed to the stage adapter.

6. To raise the stage, turn the course focus away from you until the medium on the lens has made contact with the cover slip.

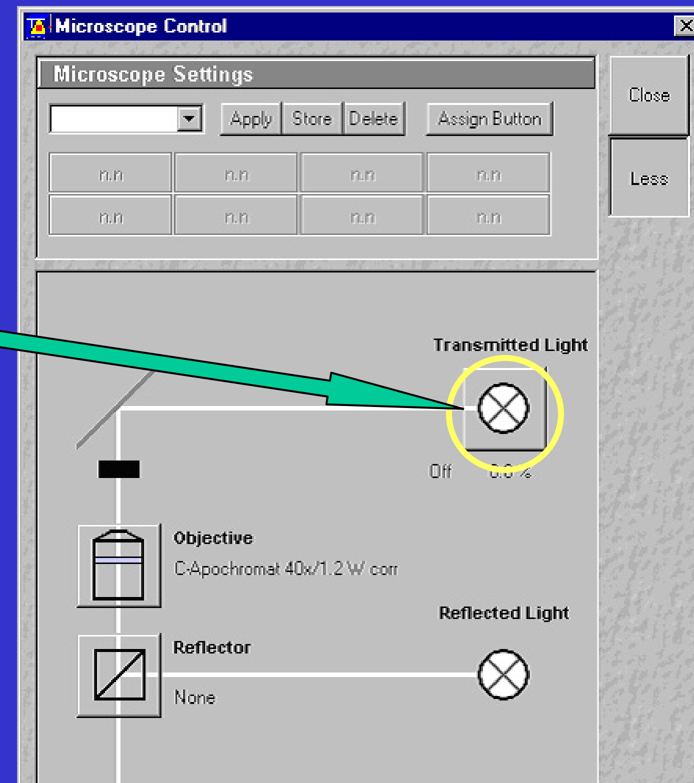


Visualizing a Sample Using Transmitted Light

1. There are two slider bars located on the right side of the microscope. Push the upper slider bar IN and pull the lower slider bar OUT.

IN

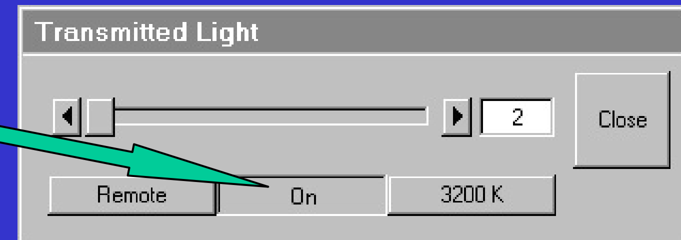
OUT



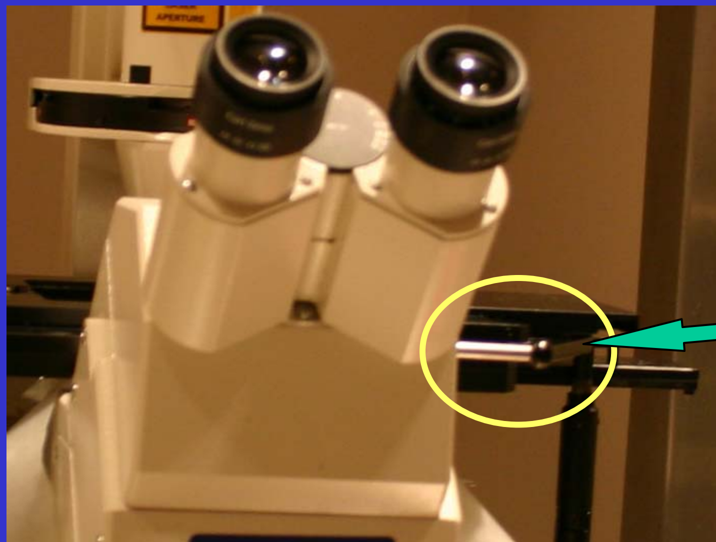
2. Under the Microscope Control window, click on the Transmitted Light button.

Visualizing a Sample Using Transmitted Light

3. Click the **ON button** in the Transmitted Light sub-window.



4. There is a bug in the program that prevents you from changing the intensity of the transmitted light using the scroll bar in the Transmitted Light window, but you can avoid this problem by using the output adjustment knob on the **Halogen Lamp power supply,** located on the table to the right of the microscope.



5. Make sure that the eyepiece slider is pulled **OUT.**

6. Adjust the focus until you begin to sharpen the image.

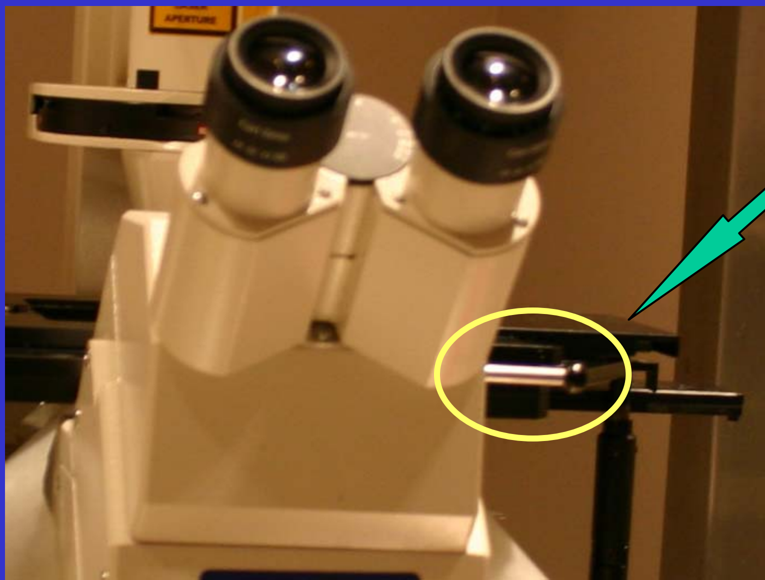
Visualizing a Sample Using Epi-Fluorescence

IN

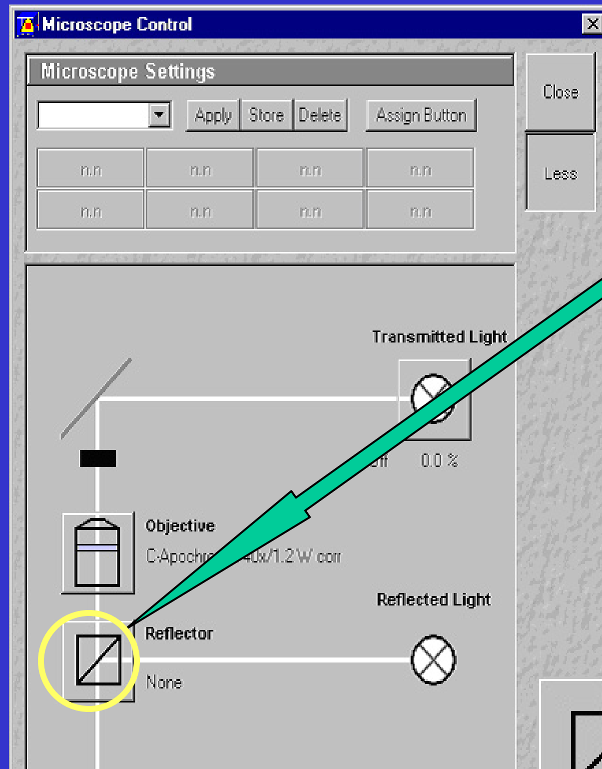
OUT

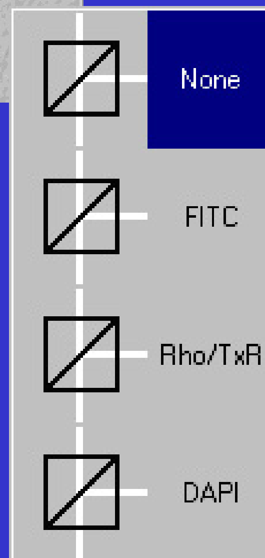
1. Push the upper slider IN and pull the lower slider OUT *(same as transmitted light).*

2. Pull the eyepiece slider OUT.



Visualizing a Sample Using Epi-Fluorescence





3. Under the Microscope Control window click on the Reflector button.

4. Select the a dichroic mirror by clicking on its name from the drop-down menu.

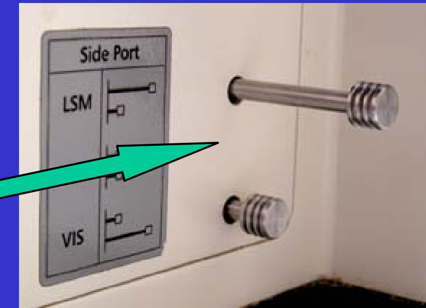
You may view fluorescence of DAPI, FITC, Rhodamine (Texas Red) one at a time from the eyepiece.

You may need to use the combination of the XY stage motion control and the focus to get the best field and the sharpest image as you would with the transmitted light.

Keep the observation time brief since the sample may get bleached rapidly.

Acquiring Images

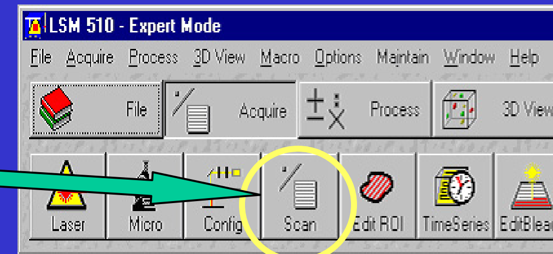
Acquiring Images



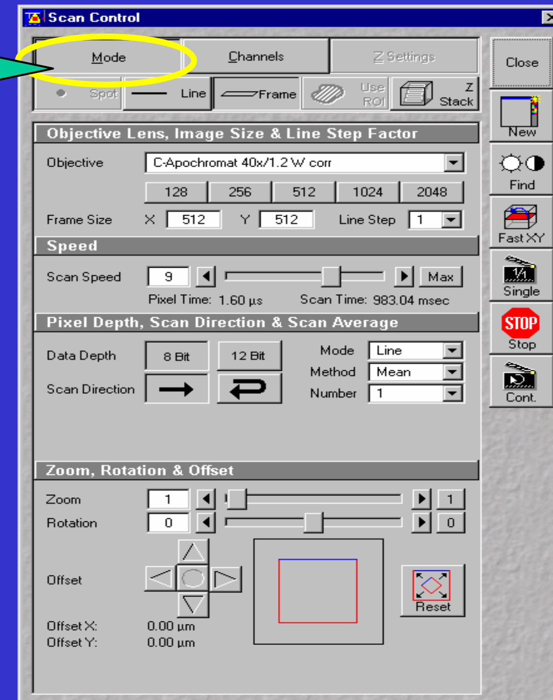
Initial Setup:

- Focus sample *(using either transmitted/epi-fluorescent light).*
- Select or set up a configuration.
- Set the slider bars to the LSM mode by pulling the upper slider OUT and pushing the lower slider IN.

How to Acquire an Image:



1. Select the Scan button in the LSM 510 Expert window *(make sure the "Acquire" button has been pressed first).*



2. When the Mode button is selected in the Scan Control window, you will be able to do the following:

   a. Select the frame size

   b. Select scan speed

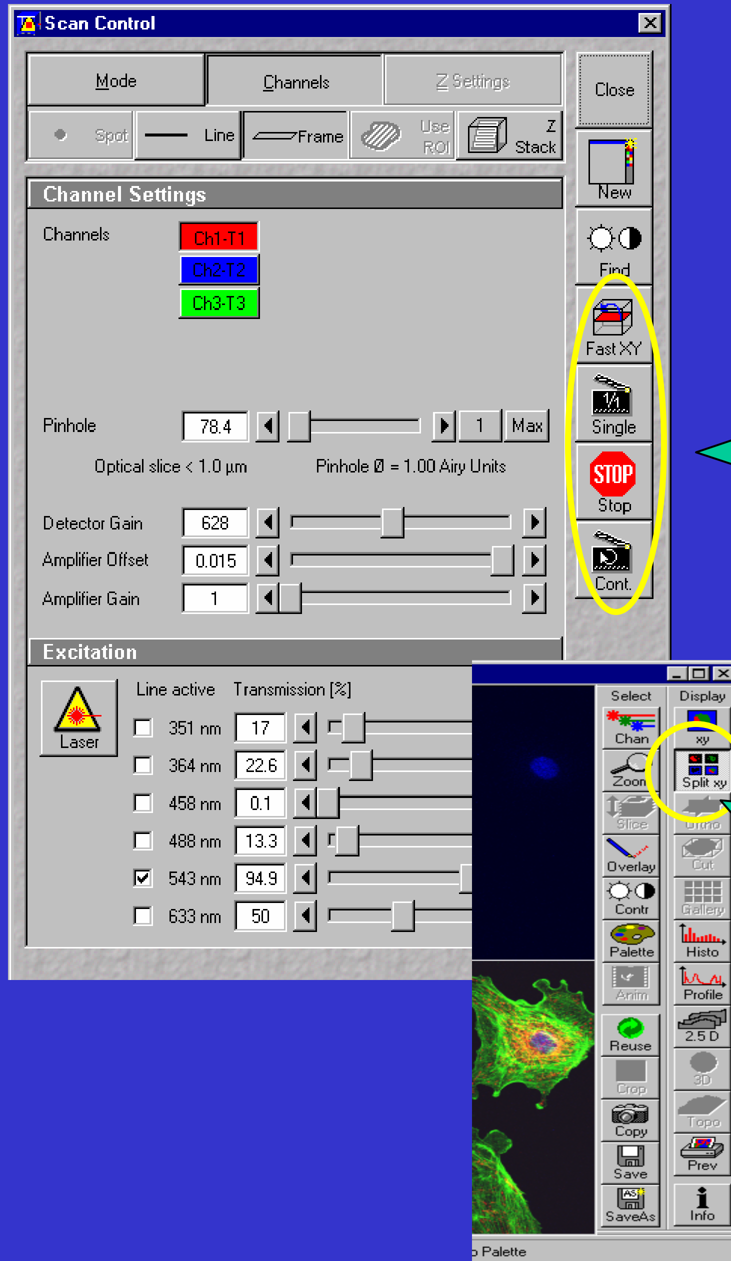
   c. Image depth (8 bit or 12 bit)

   d. Select the number of scans to average

   e. Set zoom

   f. Select methods of scan (line or frame), etc.

Acquiring Images



3. The system will start to scan when any of the following three buttons are selected:

   1. **Fast XY:**
      A continuous XY scan at optimum speed, ignoring settings from MODE.

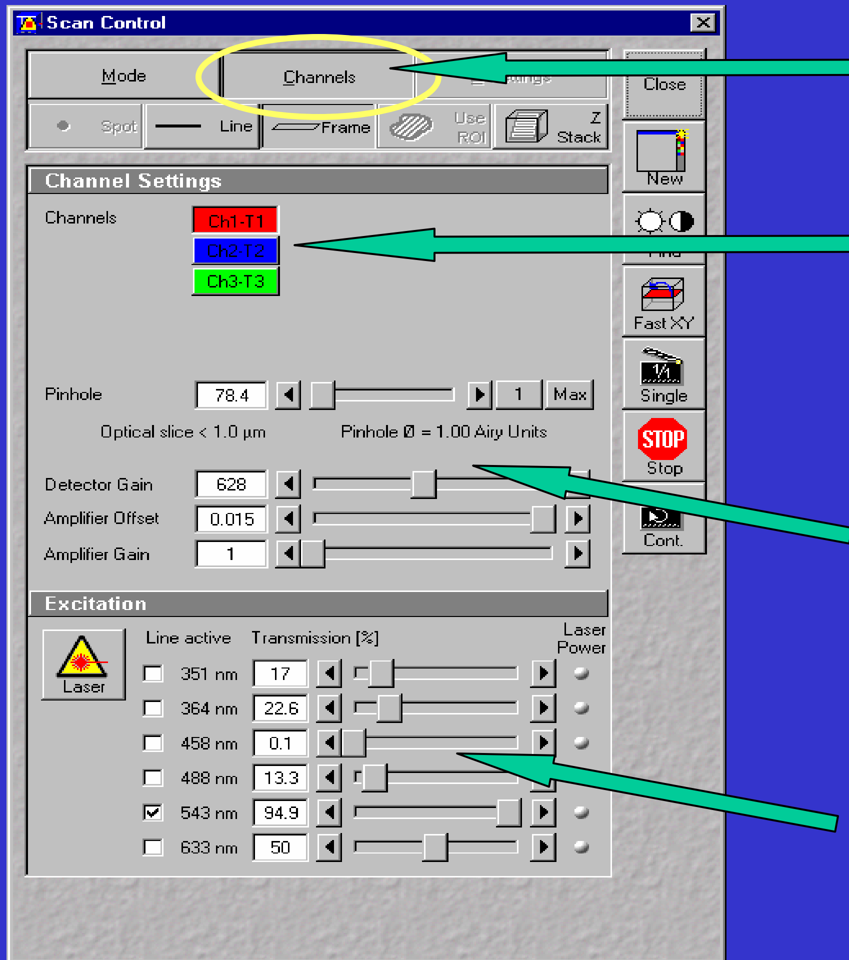
   2. **Single:**
      Takes a single XY scan, using settings from MODE.

   3. **Continuous:**
      A continuous XY scan, using settings from MODE.

   4. **Stop:**
      Stops the current scan.

   5. **Split XY:**
      Displays each channel separately, as well as, an overlay of the channels.

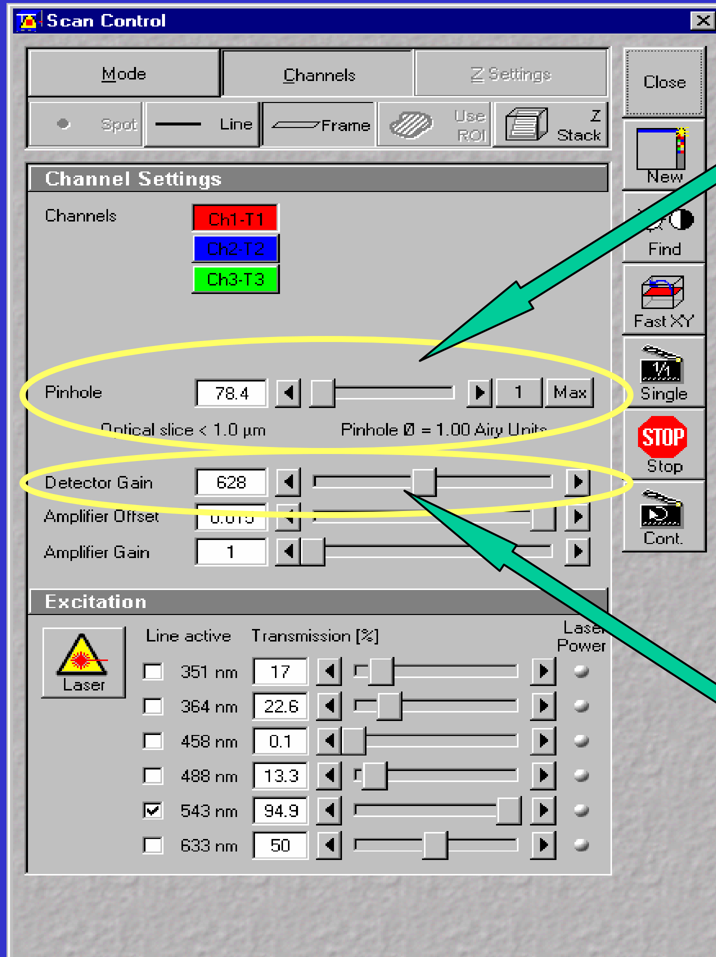
Optimizing Image Quality



1. By selecting the Channels button, you can apply settings to each individual channel to get the best quality image.
2. To select a channel, click on its respective button in the Scan Control window. The parameters that are associated with the selected channel will appear below.
3. Each channel has its own control over the following several parameters:
   a. Pinhole diameter
   b. Detector gain
   c. Amplifier offset
   d. Amplifier gain
4. Laser line selection and intensity are controlled by the Acousto-Optical Tunable Filter (AOTF), shown here.

There is no magic setting that works for all. You should understand what each parameter does in order to optimize the best setting for your experiment.

Defining the Parameters



- **Pinhole:**
  The size of the pinhole affects the amount of fluorescence signal getting to the detector (photomultiplier tube, PMT).

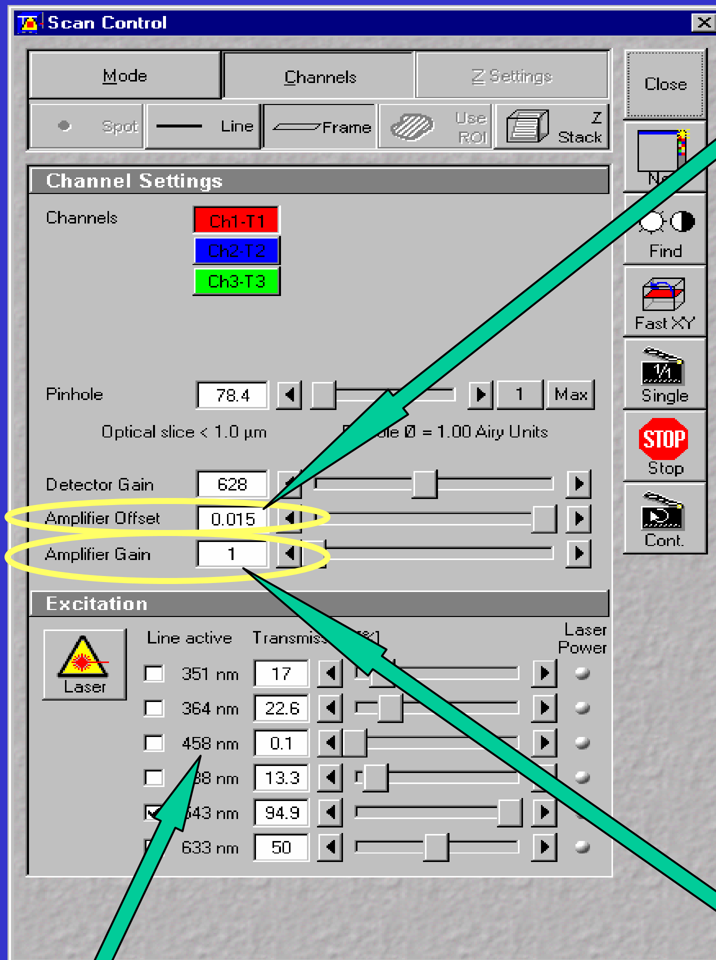
  A larger pinhole diameter will increase the light intensity reaching the detector, since it allows more out-of-focus signal to get through it. There is always a trade off between the confocality and overall signal intensity.

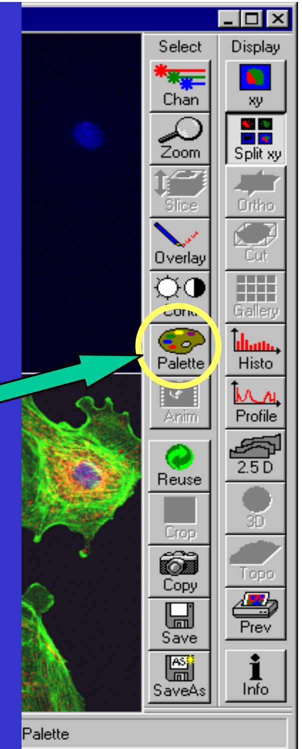
  A pinhole value of **1 airy unit** is a good place to start, but often times, you may need to increase the size in order to gain more signal. **It is important to assign the SAME PINHOLE SIZE for EACH CHANNEL, or as close as possible.**

- **Detector Gain:**
  The gain actually regulates the high voltage of the PMT (i.e. image brightness).

  Your image may start to become very noisy when a gain value exceeds 800, especially on ch3 and ch4.

Defining the Parameters





- **Amplifier Offset:**
  This parameter determines the electronic offset *(i.e. the background intensity).*

  To adjust this, click on the **Palette button** on the image window, which will appear after the scan starts.

  Select the **Range Indicator button** in the Palette panel. In the **Range Indicator,** red indicates that the PMT is saturated and bright blue indicates that the pixel value is below the threshold of the detector **(i.e. black).** Change the offset value by using the slider on the scan window, while the image is being scanned. Adjust it until only a trace of blue dots can be seen in the dark region of the image.

- **Amplifier gain:**
  This sets the amplification factor.

- **Laser Line Intensity:**
  The intensity of the laser line will affect the **intensity and the quality** (signal to noise ratio) of an image, as well as, the photobleaching rate of the sample.

Optimizing Image Quality

More Helpful Hints

Scanning a Live Image:

The best way to optimize settings is to adjust the parameters when a live image is being scanned.

1. Click on the Continuous button in the Scan control window.
2. Adjust each of the those parameters mentioned to see the varying effects.

You may also go to check the following link to learn how different parameters affect the outcome of an image using a confocal microscope:

http://microscopy.fsu.edu/primer/virtual/confocal/index.html

Signal Saturation:

In digital imaging, the maximum pixel values for an 8 bit image is 255 and for a 12 bit image, 4095. Signal (digital) saturation simply means that the maximum value of a pixel has been reached.

When you use the Range Indicator button, the saturated pixels will be shown in red. When pixels are saturated, their values are no longer trustworthy.

Collecting Z-Stacks

Collecting Z-Stacks

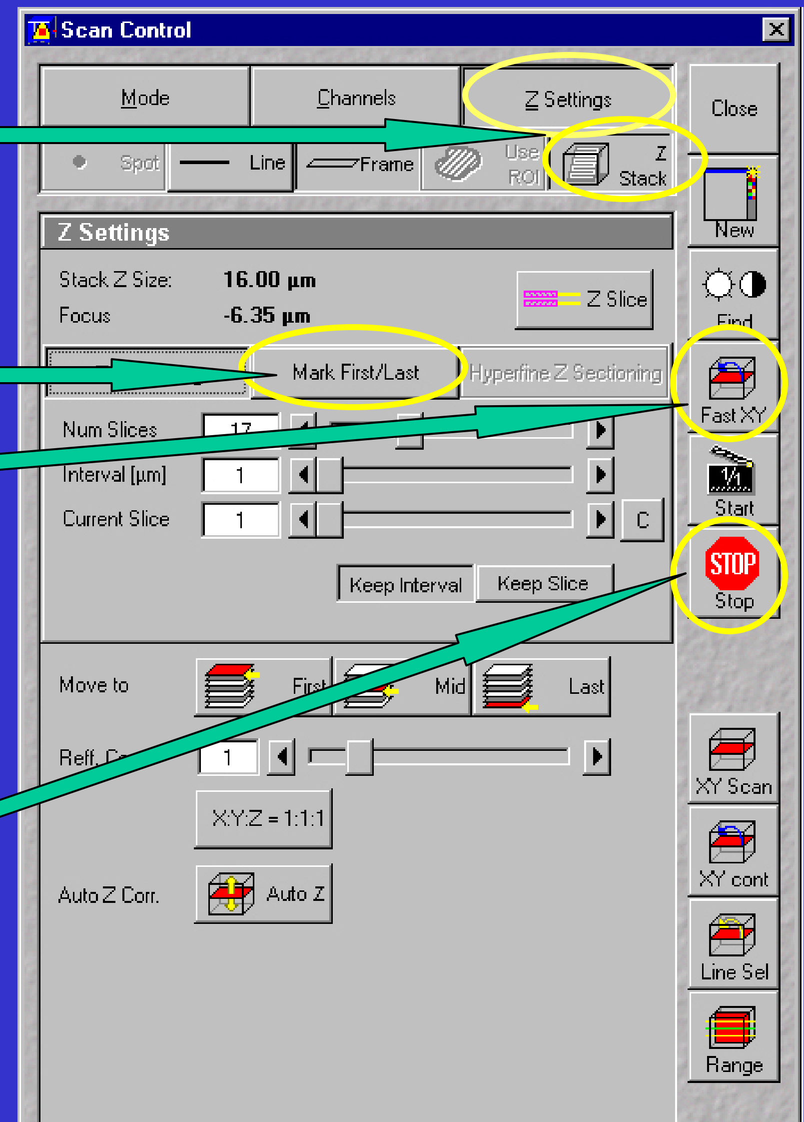
1. After optimizing an image of a representative slice, select Z-Settings, as well as, Z-Stack from the Scan Control window.

2. To set the top and bottom of the stack (Z-axis), select MARK FIRST/LAST.

3. Start fast scanning by selecting FAST XY.

4. Using the fine focus on the microscope, move to one end (top or bottom) of the object and select MARK FIRST/LAST button.

5. Next, focus through to the opposite end of the specimen and select MARK FIRST/LAST button, then click STOP.

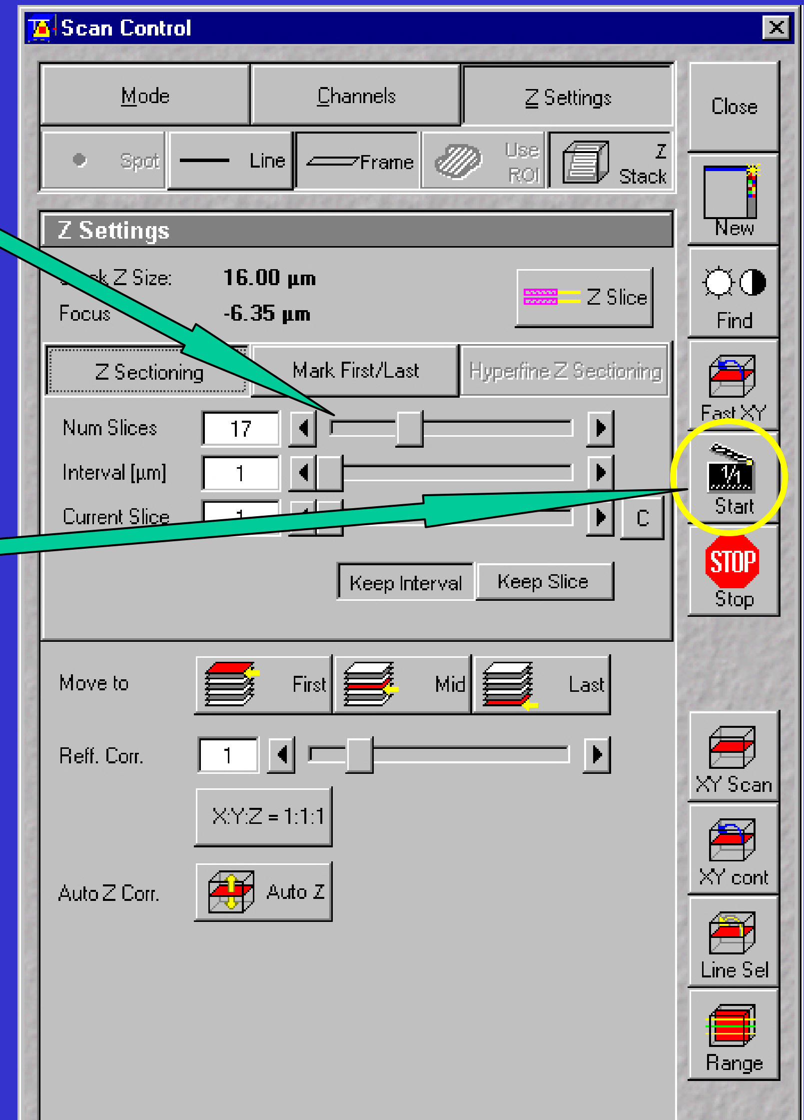


Collecting Z-Stacks

6. Use the **SLIDER** to indicate the number of slices and the interval.

   Please Note:
   If you are going to use the VOXX software, 70-120 slices must be collected.

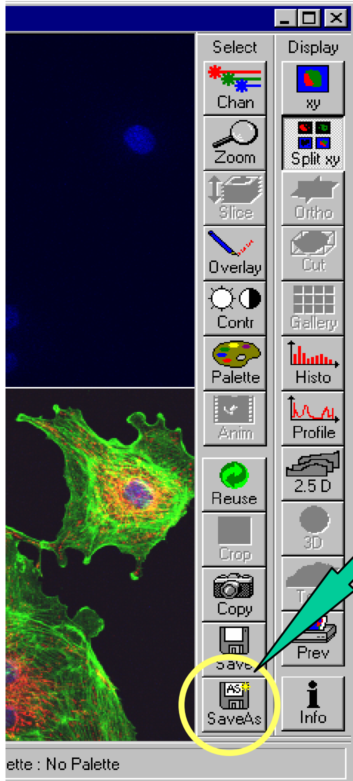
7. To collect a Z-Stack, click on **START** in the Scan Control window.

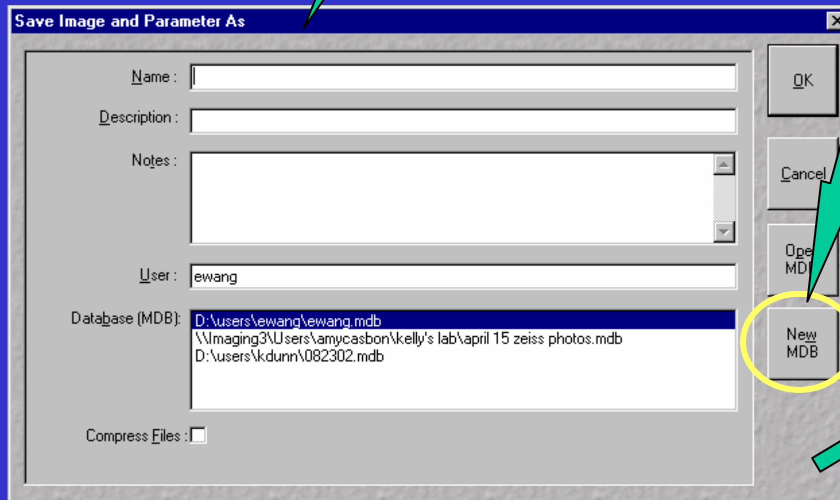


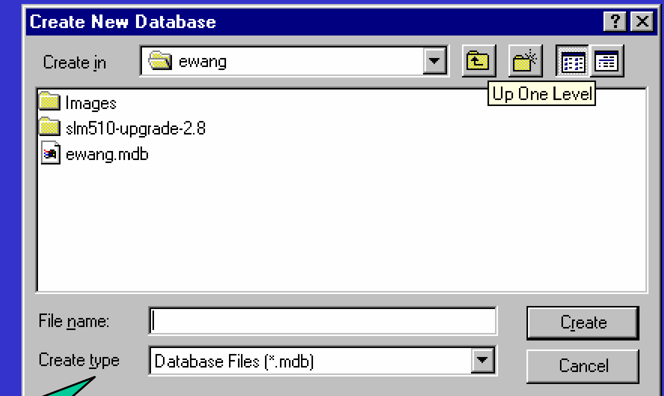
Saving Images

To save an image:

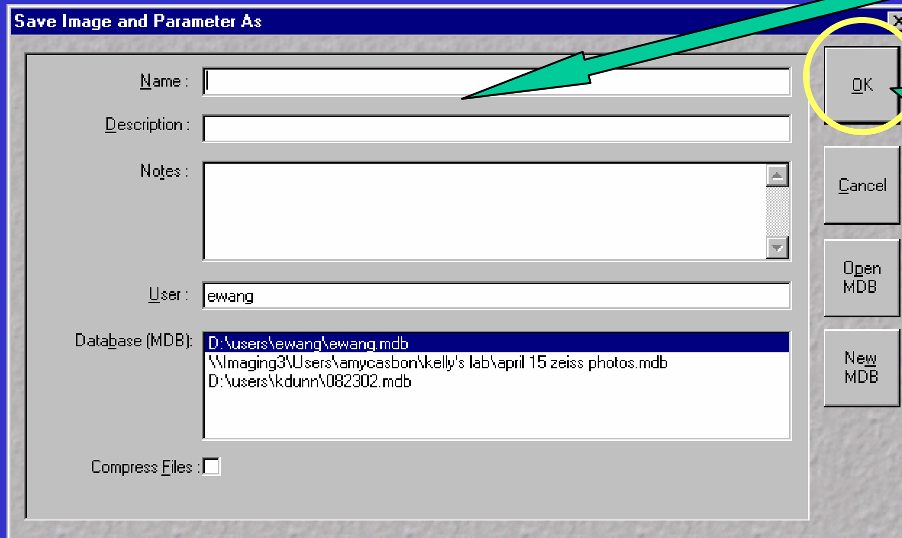
1. Click on **SAVE AS** in the Select menu of the image.
2. This will open the **Save Image and Parameter window.**
3. Click on **NEW MDB** to create an **MDB (multiple data base)** file, in which to save your images. Images cannot be saved unless a database is created. Images can be saved in a pre-existing MDB. Some prefer to have MDB's for each experiment. It's your preference.







Saving Images



4. Enter the Filename and any helpful notes for future reference.

5. Click OK. The image will be saved on either the local hard drive or a remote computer, depending on where the database file is located.

LOGOFF Procedure - *NOT THE LAST User of the Day*

When your experiment is completed:

1. Click on the LASER icon.
2. Select the Argon Laser and click STANDBY.
3. Select the Enterprise Argon UV Laser and click STANDBY.
4. Leave the HeNe Lasers ON.
5. Select the Windows START button *(bottom left)* and LOGOFF USERNAME.

SHUTDOWN Procedure - *LAST User of the Day*

1. To turn lasers off, select each laser and click the OFF button.
2. You must WAIT 5 MINUTES BEFORE TURNING OFF THE REMOTE CONTROL SWITCH *(The fan will stop running when the lasers have cooled).*
3. Exit the LSM510 software by selecting the "X" *(upper right hand corner of the software).*
4. Select the Windows START button *(bottom left)* and select SHUTDOWN.
5. Once the fan has stopped running *(5 minutes)* and the computer has been shutdown, it is now safe to turn OFF the REMOTE CONTROL SWITCH on the table, as well as, the RED TOGGLE SWITCH located in between the monitors.
6. Turn OFF XENON LAMP *(As a courtesy, please check the schedule to see if anyone is signed up following your session before turning Xenon Lamp off. Once this lamp has been turned off, it should NOT be turned on again for 30 minutes).*
7. Turn OFF the Enterprise UV Laser power supply:
    1. Turn key to OFF.
    2. Turn OFF main power switch on the Enterprise power supply.