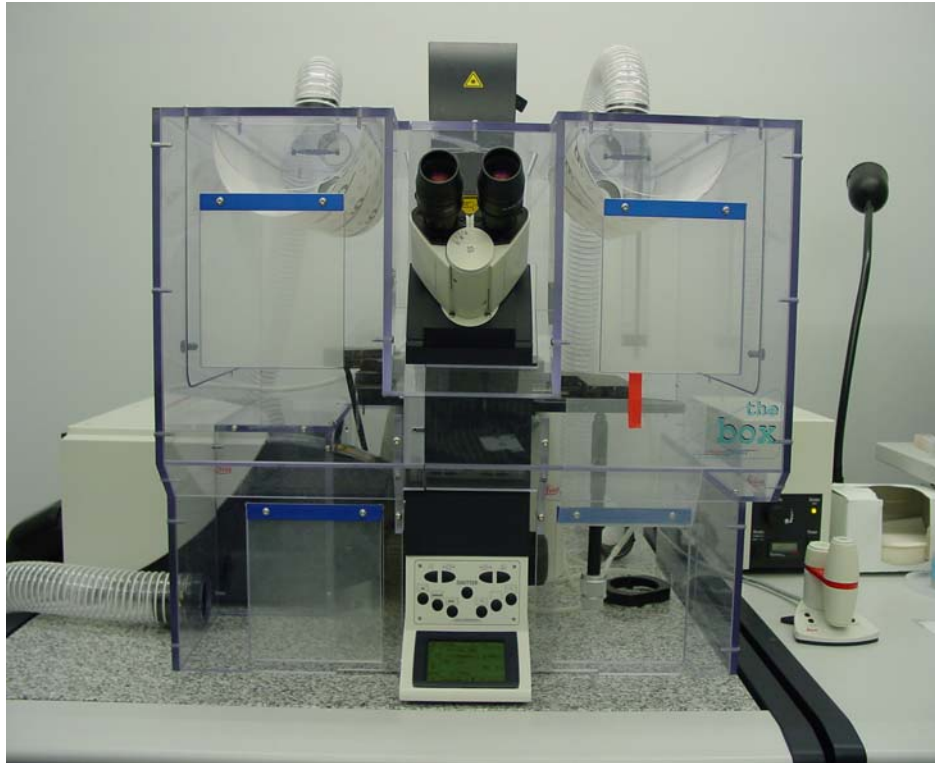


LASER SCANNING CONFOCAL MICROSCOPY WITH THE LEICA TCS SP5 INVERTED MICROSCOPE WITH ENVIRONMENTAL CHAMBER



The instrument will most likely be on and ready to operate when you arrive. If it is not, follow these steps to turn it on and begin your session:



1. Turn on the three green buttons, (right side of the table), beginning with the left button.
2. Turn the argon laser on by turning the key to the right and releasing it. The key will remain in that position and the yellow light should become illuminated.
3. Turn on the switch to the Excite light (mercury lamp) located to the right of the microscope.



Once the computer is running type in “**admin**” for the password.



If the LCS program is not running double click on the Leica icon on the desktop. Main menu will appear.

Click “**Start**”.

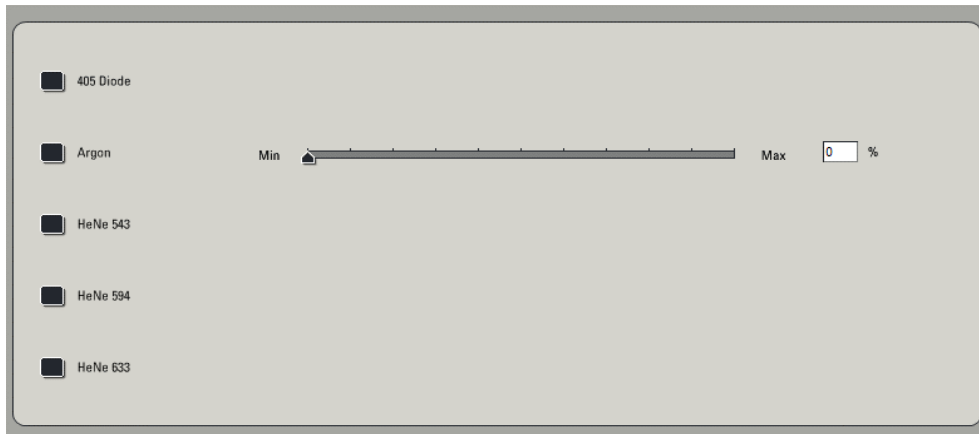
Click “**OK**” when asked, “Check Configuration of Machine?”

Click “**YES**” when asked if you want to initialize the stage.

Click on “**Configuration**” tab at top of left screen.



Click on **Laser** button.



Turn on the lasers that you will be using by clicking in the box beside the appropriate laser. Set the slider for the argon laser to **30%**.

Click on **Acquire** tab to return to the imaging screen.

To view a sample through the microscope:

Tilt condenser back to access the stage.

Place slide with **coverslip side down**, or culture dish with glass coverslip bottom on the stage, return condenser to normal position.



Push the appropriate button for your photofluor located on the front of the scope, labeled **UV**, **green**, or **red**. Push the “**Shutter**” button in the center of the scope. You should see light visible through the objective.

The controls for the stage and focus are all controlled by the remote located to the right of the scope. The X & Y knobs located on the front of the remote control the movement of the stage. This movement can be adjusted from fast to precise by the two small buttons on the left side of the remote. The Z knob controls the focus of the microscope. The focus can be adjusted from fine to coarse with the two small buttons on the right side of the remote. A read out of the focus setting is also located on the lower right side of the microscope control panel window.



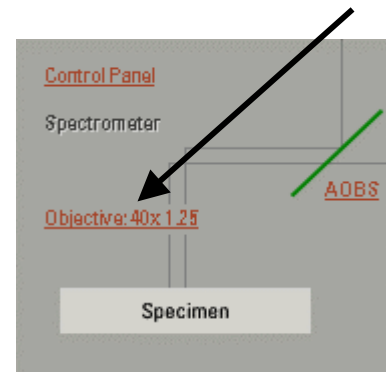
Objectives:

The SP5 has five objectives. They may be rotated by hand, but the preferred method is by clicking on the word “**objective**” in the middle area of the left screen. Choose the objective by clicking on it and it will automatically rotate into place.

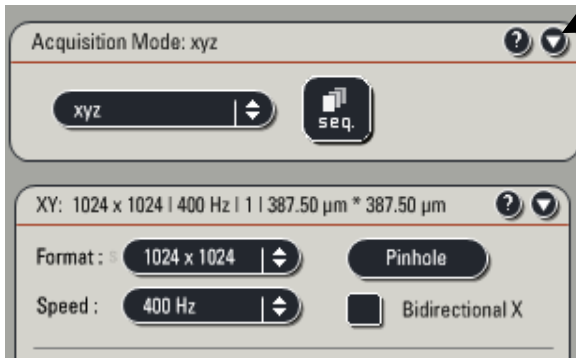
The available objectives are:

- Dry** 10x, 20x
- Glycerol** 63x (There is glycerol for 20 ° C & 37 ° C application.)
- Oil** 40x, 100x

NOTE: The 63x is periodically switched to 63x oil. Check with lab personnel to confirm.

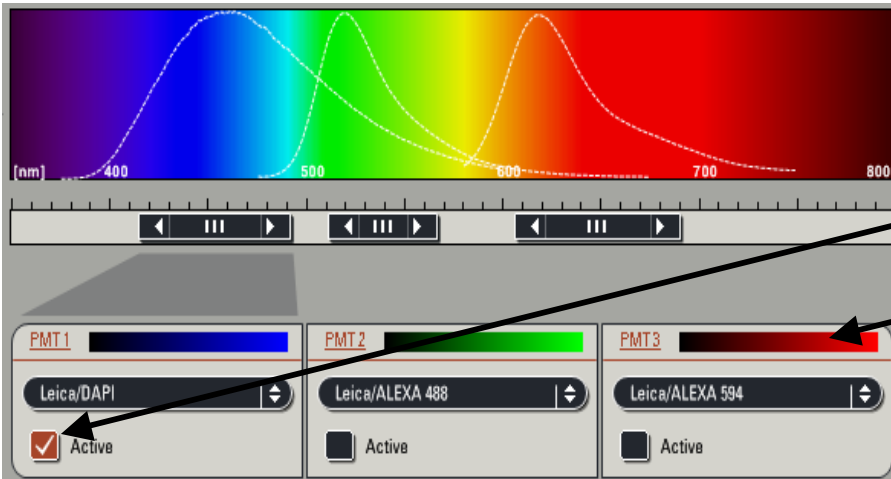
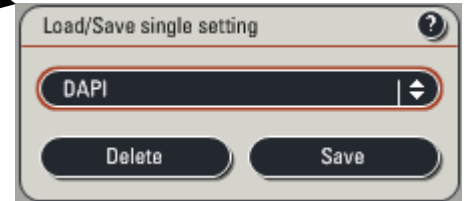


To scan with Confocal:



Under acquisition tab, expand the window by clicking on the arrowhead.
 Make sure the **Objective** reading on the screen matches the selected objective on the microscope.
 Change **Format** to 1024 x 1024 (bigger files but better resolution).
 Select the proper **Mode**, xyz is the normal mode for scanning.

Double click on the desired photofluor(s) at the top of the left screen. If you wish to see the emission spectrum click on the drop down menu underneath the color bar. This will place an emission spectra curve on the spectrum in the middle of the screen.



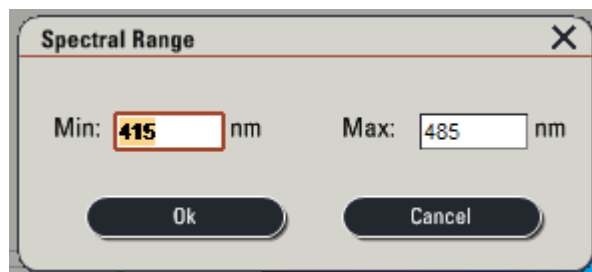
Alternatively, you may manually select the desired PMT by clicking on the “**Active**” box.
 The color of your image can be changed by double clicking on the PMT color bar for each PMT.

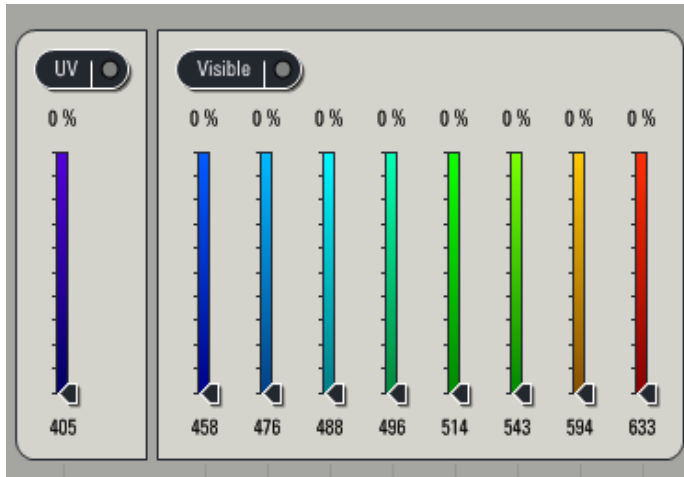
The emission wavelength can be adjusted in two ways:

Move the mouse arrow over one edge of the horizontal black boxes until it becomes a double arrow; then drag the edge in the appropriate direction.

or

Double click on the appropriate black slide bar; a dialogue box will appear and you can type in specific limits.

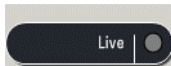


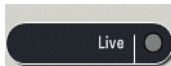



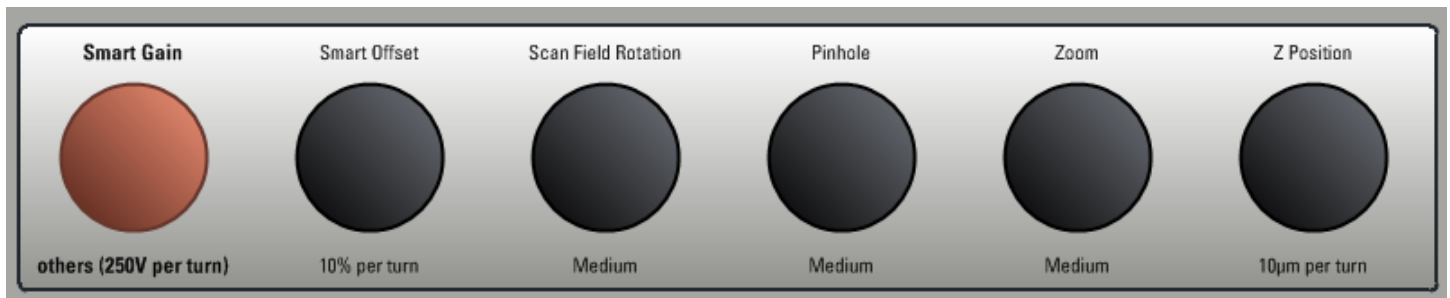
Click the “**UV**” button for the ultraviolet laser; click the “**Visible**” button for all other lasers, or both if you want both UV and visible light. The buttons will turn red when activated.



Important: If using more than one dye see “sequential scanning” before continuing to test for cross-talk.

The strength of the individual lasers can be adjusted by moving the appropriate vertical slider arrow from 0 to 100 %. A good starting point for this setting is 15-20%. **Settings that are much higher than this can cause photobleaching of your samples!**



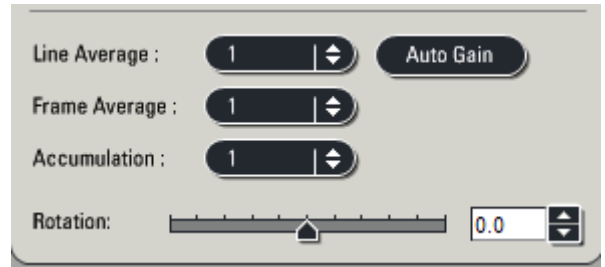
1. Click on  button at the bottom of the left monitor’s screen to begin scan. It will change to **Stop** and be highlighted. On the right side of the control box, turn the “**Z POS**” knob slightly in both directions to choose the brightest portion of the sample.
2. Click  button located on the left side of the right monitor’s screen. This is an aid in choosing the proper PMT settings for gain (brightness) and offset (contrast).
2. If you have more than one color, click on the image that you want to adjust. A dotted line should appear around that box.
3. Turn the **Gain** knob until bright blue pixels just begin to appear in the image. These pixels are saturated. Next, back the **Gain** knob off until the blue disappears.

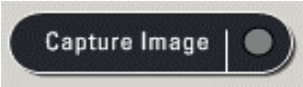


4. Turn the **Offset** knob until green pixels begin to appear in areas where there should be no fluorescence or tissue. **Note:** The **Offset** knob operates in reverse compared to most controls. Turning it to the right will give you less green, and turning it to the left will give you more green in the background.
5. Click  button again to get a gray-scale image. Click  button one more time to get back to the original color image.
6. Click “**Stop**” to stop the scan when all adjustments have been made.

Click on “**Line Average**” or “**Frame Average**” buttons to choose the number of sweeps by the laser to average out background noise. The greater the background; the more averaging should be done.

Clicking on “**Accumulation**” button allows you to stack scans on top of each other in order to boost a very weak signal.

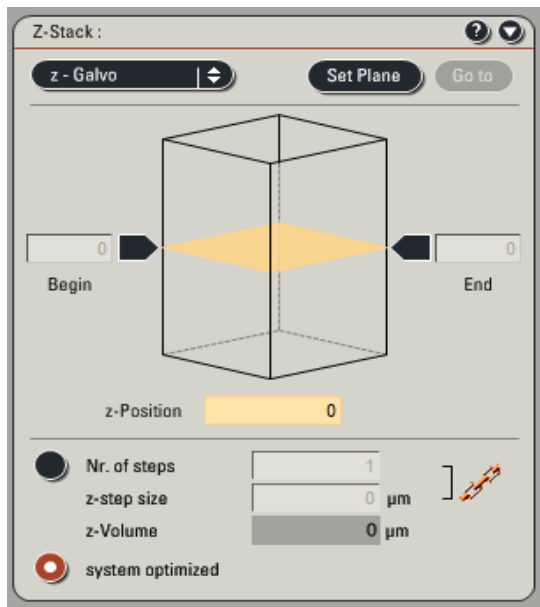


Clicking on  button at bottom of left monitor’s screen allows you to do a single scan.

Z Stack Operation:

Z stack allows for multiple optical sections to be cut through the structure of interest.

Open the **Z Stack** window on the left side of the screen. You should see a representation of a cube with two boxes labeled Begin and End.



Click the “**Live**” button. (It will change to stop and be highlighted.) Adjust the **Gain** and **Offset** if not done previously.

The **Z POS** knob is used to set the beginning and end positions of the area to be sectioned. Turn **Z POS** knob to the right until the fluorescence starts to disappear. This is the part of the sample closest to the coverslip.

Click the black box labeled **BEGIN**. Turn **Z POS** knob to the left to find the end of the sample, when fluorescence begins to fade again. This is the part of the sample closest to the slide. Click the **END** box.

Click the “**STOP**” button to stop the scan.

The software will suggest the optimum number of sections for your chosen volume of sample when the “**System Optimized**” button is highlighted. This will most often be more sections than you want.

You can click the button, “**Nr. of Steps**”, and enter the number of sections that you want to scan or enter the desired section size in “**z-step size**” and the software will calculate the number of sections needed.

Remember to set scan for **Line Average**, **Frame Average**, or **Accumulations** if you need these.

Click on the “**Start**” button to begin a Z stack series.

To view the series of sections click on the  button on the right side of the working window (right monitor).

Z stack scan is finished when the “Start” button reappears.

SEQUENTIAL SCANNING

Testing for Cross-Talk


Load the two dye parameters you are interested in simultaneously and adjust the **Gain** and **Offset** for each. Click “Live” to start the scan. In the beam path setting box, pull down the slider for the first dye to 0%. The screen should go to black. If you are still seeing color then there is cross-talk. Conversely, raise the slider for dye number 1 back up and lower the slider for dye 2. The signal for dye 2 should disappear. If it doesn’t, then again you have cross-talk. If there is cross-talk in either case you should use sequential scanning.

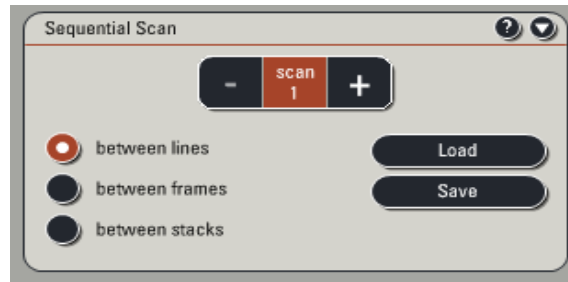
To Scan Sequentially (By Lines)

Stop any current scan


1. Beginning on the left, spread the slider underneath each PMT that you will be using to correspond to the emission spectra of the corresponding dye. You can place an emission curve on the spectrum for most fluorophores by clicking on the drop down list beneath the PMT color bar. **Once these sliders have been set to the desired position, do not adjust them anymore during setup!**



2. Click the  button. Scan 1 should be highlighted.
3. Select “between lines”.
4. Set up Scan 1 by making sure that the UV or **Visible** button is active, the correct **PMT** is active, and the slider for laser intensity is raised to 20%.
5. Click the “+” button in the **Sequential Scan** box to move to Scan 2. **Do not touch any buttons after setting scan 1 before you hit the “+” button to move to scan 2!!**
6. Set up parameters for Scan 2 as in step 5.
7. Click the “+” in the **Sequential Scan** box to move to Scan 3 if necessary and adjust settings as before.
8. When you have successfully made all of the adjustments to do the number of scans you wish to execute, leave the **Sequential Scan** box expanded. If you need to go back to a previous scan click on the corresponding number. **Do not click the “minus (-)“ button unless you wish to delete scan settings!!**
9. Click “Live”. Adjust **Z POS** knob to find the brightest portion of your sample.
10. Since you are scanning by lines you will have all three scans on the screen. Adjust the gain and offset for each scan. You can switch from one scan to another by clicking on the image of the scan you wish to adjust. **NOTE:** If you need to adjust the laser intensity for a particular channel click the number of the scan to return to that scan and raise or lower the slider for laser intensity. Click “Stop”.
11. If you wish to optically section, click “Live”, and determine the beginning & end of your sample volume as described in **Z Stack Operation**.
12. Choose the number of sections using either the **System Optimized** or **Nr. Steps** buttons.
13. Adjust for line averaging if necessary.
14. Click “Start” to begin sequential scan.





15. Click on the “” button to view the images as they are scanned.
16. Scan will be finished when “**Start**” button reappears.

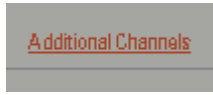
This sequential setting can be saved by clicking “**Save**” in the sequential scan box. When needed for future use, click “**Load**” to recall the setting.

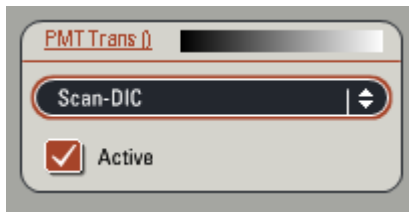
NOTE: The procedure for scanning between frames or stacks is the same except only the active channel will appear on the screen thus you must adjust the gain and offset individually.

Nomarski (DIC) Imaging:

Nomarski or Differential Interference Contrast Imaging is a light microscopy technique that will enable you to see a bright field image of your sample with some contrast of depth depending on the thickness of your sample. This is very useful to see where your cells actually are in relationship to the fluorescent tags that you are imaging with the confocal scanner.



To view a DIC image in single image or sequential scanning format click on  on the left screen and choose Scan-DIC.



This will open up an additional window alongside the color windows that you are using for your confocal images. This image can then be adjusted for “**Gain**” & “**Offset**” in the standard fashion.

Customizing & Manipulating Images:

Right side of confocal image screen:



– These buttons allow you to turn different channels of a sequential scan off and on.



– Overlays all colors to form one image.



– Takes the multiple images from a **Z Stack** and combines the brightest pixels into one image.



– Shows the intensity along a chosen x & y axis through each section of a stack.



- Permits the user to see each image through a stack while the instrument is optically sectioning.




- Advance the images of a stack one at a time, all the way to the beginning or end, or play them in sequence as a movie.

Left side of confocal image screen:




- Expands the options so that you have access to all of the tools.



- Highlights items to be moved on the screen or highlight and then click the  button to erase from the image.



- This button highlights all of the annotations that have been added onto an image. They may then be removed by clicking the  button.



- Highlighting items and then clicking this button removes the item from the image.



- Define areas of interest by drawing a line, placing ovals, or squares.



- Place arrows to define points of emphasis.



- Type in text by hand or place the date & time onto an image.



- Places a micron bar for size reference on an image. To place a micron bar of a certain length, highlight it after it is on the image, right click, and choose properties. You may then enter in the length that you want the bar to be. You may also reposition the bar by clicking and dragging it to another location.



- Define ROI with numbers.



- Zoom in or out on a captured image.



- Makes images the correct size to avoid being pixilated when saved and printed.



Saving Files:

The images in the experiment window are temporary. A power surge or computer crash could cause you to lose your data. **SAVE FREQUENTLY!!** Files should be saved to the hard drive as Leica files and also exported as tiff files after every session. The tiff images can be copied and taken with you.

ALL FILES LEFT ON THE HARD DRIVE WILL BE DELETED AFTER ONE WEEK!!

1. Open **“Experiments”** window.
2. Right click on **Experiment** and choose **Save Experiment As**.
3. **“Save As”** box will open.
4. Open drop down box and select **(D:)**.
5. If you do not have a folder already, click on **“new folder”** icon and create one in your name.
6. Double click on your folder so that it appears next to **“save in”**.
7. Next to **“file name”** type in identifying information for your particular experiment. **Note:** You will have to create a new subfolder for each slide or experiment in order to keep your data organized.

8. Click **save**.
9. You must also export your file as a tiff file in order to open it in Photoshop. Right click on your experiment name, choose export experiment, then export as a tiff image. Save the tiff images in your folder along with the Leica files.

Saving Max Projections, Overlays, Etc:

When you do a stack your **“series”** is automatically placed in the Experiment folder or your assigned file name in the Experiments window.

To rename the series right click on the series, and choose rename series. You may then type in your desired name.

Any max projection, overlays, or image w/ annotations must be saved individually.

1. Click on the image that you wish to save. The box around it should be highlighted.
2. Double click on the image to make it full screen size.
3. This is the time to add annotations to your image with the tool buttons.
4. Right click on the image, choose snapshot.
5. A file named snapshot will be placed in your experiment folder.
6. Rename the file by right clicking on it. Choose rename snapshot and rename it.

Snapshot All

To use snapshot all, right click on the highlighted image on the screen, and then choose snapshot all. This will save whatever is on the screen at the time, regardless of what is highlighted. This is useful if you want to save tiled images or the whole gallery in one picture.

Note: Nothing that is placed in your Experiment file is saved permanently until you tell the software to save it. You can save the entire file by right clicking on the experiment file and choosing “**Save As**”. Remember that any additional images captured after the initial save process will also have to be saved. You can do this by clicking the “**Save All**” button in the Experiment window. Finally, you must right click on the experiment name and export the file as a tiff before your session is finished.

To start a new experiment click on the “**New**” button at the bottom of the Experiment window. It is advisable that you not put a large number of experiments in one file as several series with their accompanying snapshots can be confusing.

Saving to a CD:

1. Open NeroStartSmart
2. Choose Make Data CD
3. Click Add
4. Select File or Folder you wish to copy.
5. Click Add
6. Click Finished
7. Click Next
8. Click Next
9. Click Burn



When you are finished return the argon laser power to 0%, & clean the objectives with lens tissue paper.