Smith College – August 2010

Operating Checklist for using the Laser Scanning Confocal Microscope Leica TCS SP5.

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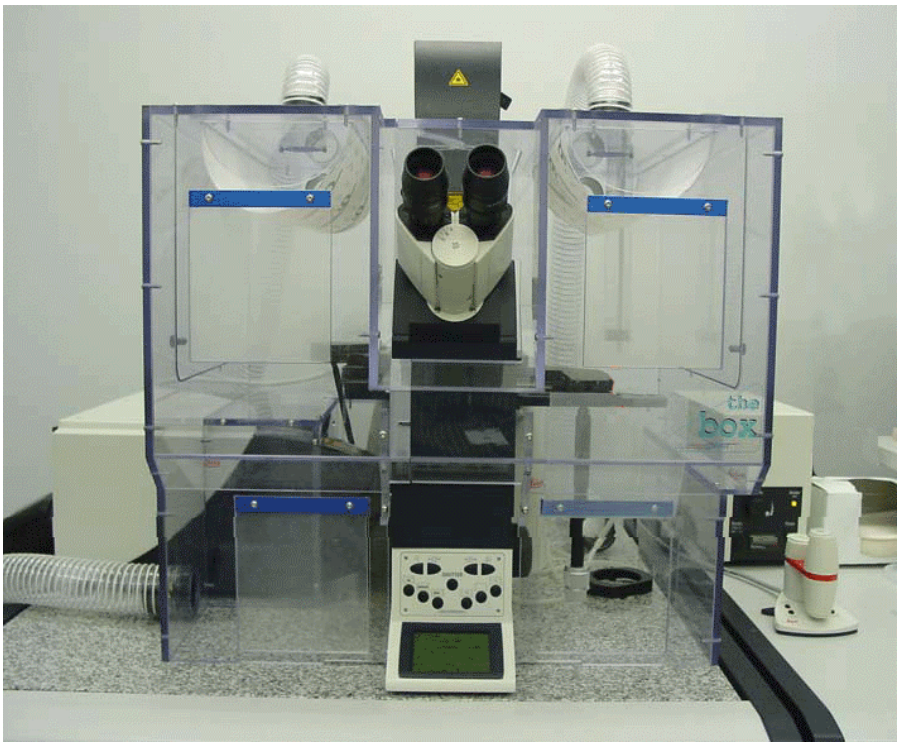
Leica TCS SP5 Laser Scanning Confocal Microscope.



NOTE: The PC running the Laser Scanning Confocal Microscope is a massive, data acquisition machine; it is not just a regular old PC. This means no email, no Internet surfing, no music, no games, etc. Do not add any software to this machine or make any changes in its operational parameters. To avoid a crash or possible data loss, do not do anything with the computer while acquiring data.

STARTUP:

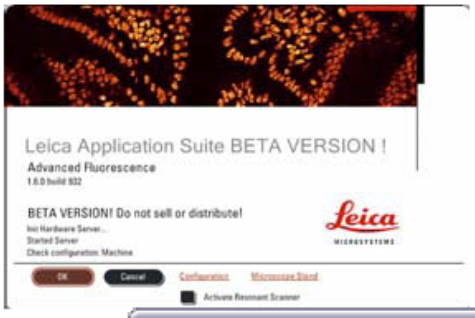
Initial set-up:

- **Sign the logbook**
- Turn on the room fan for HEPA filtered air and temperature stabilization, keeping the doors closed helps a lot.
- Turn on the N_2 tank for the vibration-dampening table.

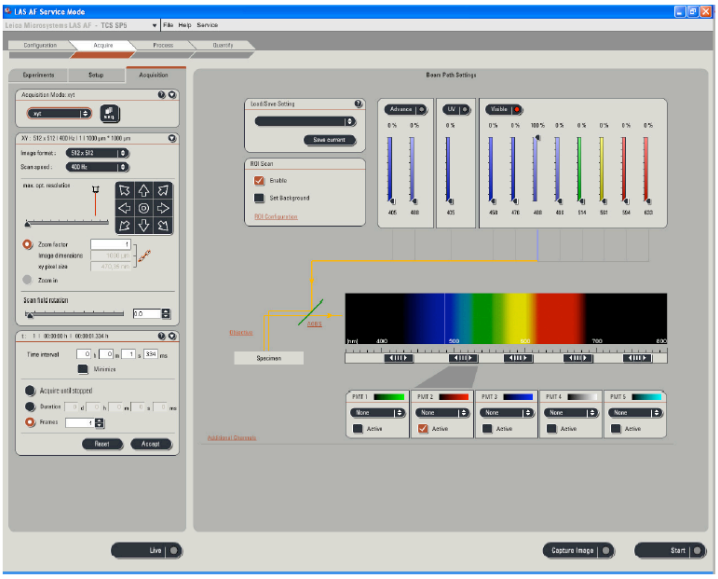
- If working with live material, consider turning ON the regulator for chamber temperature using the toggle switch on the blue box (on the floor to the left of microscope). Do this two hours before imaging.
- Tilt the head of the microscope back.
- Turn the metal halide lamp Power ON (on table to the right of monitor) only if you need fluorescence to find your specimen through the eyepieces; intensity should be low.
- Open shutter (press black button, the yellow light goes ON).
- Turn ON the green PC-MICROSCOPE toggle switch. This will turn on the computer, monitors and microscope (Note: microscope stage moves around).
- Turn ON the green LASER POWER toggle switch. This will turn on the laser fan.
- Turn the LASER EMISSION key to ON (emission warning indicator lamp goes ON).
- Log on (Requires lab name (User) and password)
- Wait until you see the icons on the monitors.
- Turn ON the green SCANNER POWER toggle switch.
- WAIT 30 seconds.

Software:

- Select LAS AF icon to start the software.
- After the Leica application suite window opens. Go to CONFIGURATIONS and make sure MACHINE is selected.
- Make sure the "Activate Resonant Scanner" box is NOT checked UNLESS you need it for very fast scans.
- Click OK (wait … this is the initialization step for the system and takes a minute or two).
- After the MICROSCOPE STAND window opens (it should be set at "Initialize Stage: DMI 6000"), check to be sure that the head of the microscope is tilted back and then select NO to initialize the stage. [If you are going to be doing mosaic, stage automation, etc. then check YES; the stage will buzz around to find its center.]



- Wait until software opens and both monitors are active, showing the main view of LAS AF.



- On the left monitor, click on CONFIGURATION (upper left, left monitor).
- Select the "Laser" icon.

- Turn ON the lasers you need (405Diode, ARGON, HeNe543, HeNe594 and/or HeNe633). Check the boxes; fans will come on.
- Make sure the argon laser is set to 20%.
- Select the "Dyes" icon to see a list of fluorophores and their spectral data, if necessary.
- Go to the ACQUIRE (again, top left, left monitor) screen and select the 10X objective in the Beam Path Settings window (if not already selected).

NOTE: ALWAYS START WITH THE 10X OBJECTIVE.

- Insert a slide (bottom up, specimen down); for consistency, place the slide in the right rear corner of the stage.
- Use joystick, center specimen over the objective (front knobs, top and bottom, are X and Y coordinates - select fast or precise to control rate of movement)
- Tilt microscope head forward to the upright position.

<u>Microscope - Specimen observation through the eyepieces</u>:

Start by finding and focusing the specimen through the microscope itself, using the 10X objective:

- For brightfield viewing, push TL/IL button to turn on the light (left side of microscope base).
- Press the upper or lower INT button to increase or decrease illumination.
- Focus (Z) on the sample using the joystick (knob in the back, select Z-coarse or Z-fine)

OR

- For fluorescence viewing (used primarily to find things), on the front panel of the microscope, select a filter-cube (A=UV; N2.1=green excitation, red emission; I3=blue excitation, green emission).
- Open the shutter to see fluorescence signal (push buttons on the front of microscope).
- Switch between filter cubes if needed.

NOTE: BE CAREFUL ABOUT BLEACHING YOUR SAMPLE. CLOSE SHUTTER WHENEVER POSSIBLE!

- You can change the intensity with the INT button, and the size of the field illuminated with the FD button; this may be helpful in preventing bleaching.
- Focus (Z) on the sample using the joystick (knob in the back, select Z-coarse or Z-fine).
- When finished with fluorescence viewing, press SHUTTER button again to close it.
- Push the TL/IL button again to go to brightfield mode.

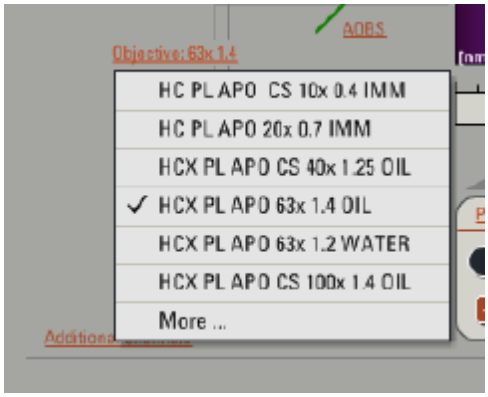
NOTE: THERE ARE FOUR BUTTONS ON THE LEFT SIDE OF THE MICROSCOPE, BEHIND THE FOCUS KNOB:

CHG-TL	Toggles between TL-BF (Bright Field), TL-DIC (Differential Interference Contrast), and TL-POL (Polarized)
CHG-FLUO	Switches to Fluorescence mode
CUBE	Switches filter cubes
CHANGE CS	???

- If you want to use an objective of a different power at this time:
  - Select the ACQUIRE tab.
  - Select OBJECTIVE in the BEAM PATH SETTING window if you want one other than 10X.

NOTE: WHEN CHANGING OBJECTIVES FROM 10X TO SOMETHING ELSE WILL REQUIRE THE ADDITION OF AN IMMERSION FLUID (SEE NEXT PAGE).

- On the OBJECTIVE screen, highlight desired objective.
- Select YES in the new window. The objective turret is automated and will move the desired one into position. You should not switch objectives by hand, as most of them have adjustment rings on them that may be rotated inadvertently.



- Objective choices:

10X	Dry
20X	Water, glycerol or oil
40X	Oil
63X	Oil
xxxxxx	
63X	Water
xxxxxx	

NOTE: ONLY USE LEICA IMMERSION OIL, TYPE F; Cat. # 11 513 859 AND BE CAREFUL NOT TO USE OIL WITH EITHER THE 10X OBJECTIVE OR THE 63X WATER OBJECTIVE.

- To apply oil:
  - Close shutter if open
  - Tilt head back
  - Remove slide - be careful stage doesn't rotate or you may lose your orientation
  - Place SMALL AMOUNT of oil on objective or slide
  - Replace slide
  - Tilt head forward and refocus (Only use Z-FINE setting):
    a. Fluorescence: select filter cube again, open shutter, refocus, close shutter
    b. Bright field: push TL/IL button for bright field, refocus, push again to return to fluorescence, close shutter.

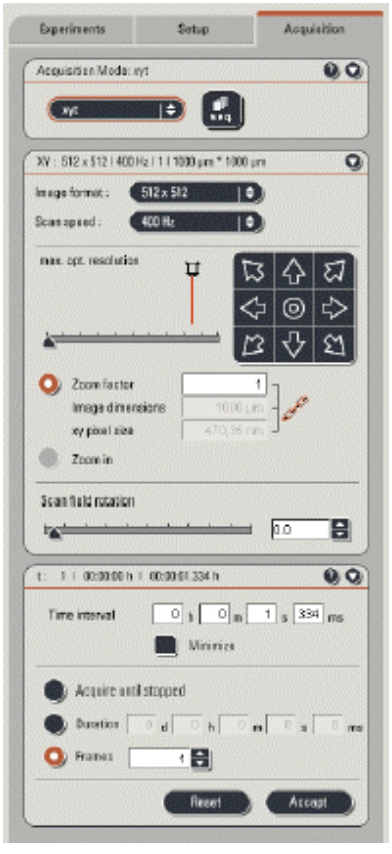
Kohler Illumination (necessary for brightfield and DIC):

- Push CHG-TL button (left side of microscope, behind focus knob) until display panel shows 'TL-BF'
- Adjust brightness with the INT buttons.
- Focus on specimen.
- With AP (aperture/condenser diaphragm) button, open the diaphragm all the way (AP 24 on panel)
- With the illumination or field diaphragm slider (inside box, just below lamp housing on the head), manually close the diaphragm all the way (slide to right).
- Looking through the eyepieces, focus the edges of the illumination/field diaphragm sharply using the silver knob on the left side of the illuminator (condenser); if image is not centered, call for assistance.
- Manually open the illumination/field diaphragm slider all the way.
- Carefully remove one of the oculars (don't set it on the bench where it might roll off or be knocked off), look down the tube and adjust the aperture/condenser diaphragm (AP button), closing it to occlude the perimeter of the field of view.
- Reinsert the ocular.

NOTE: THIS MUST BE REPEATED FOR EACH CHANGE OF OBJECTIVES.

ACQUIRING IMAGES:

- Go to: Acquire tab (Acquisition tab).
- The left side of the screen is Acquisition Mode; here you configure image acquisition parameters.

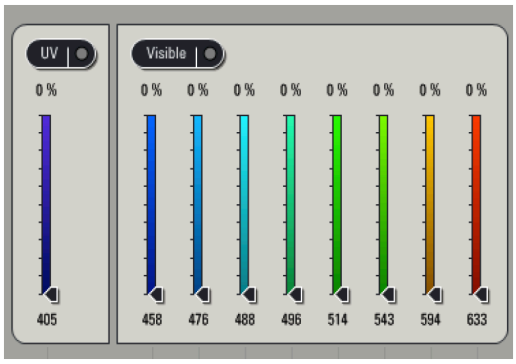


- Some of the acquisition parameters:

Acquisition type - box for Sequential (see instructions below)	XYZ is standard
Image size (pixels)	512 x 512 is standard
Acquisition speed	400 Hz is standard
Bidirectional and Phase slider	Check for faster scanning (see below)
Zoom factor	Typically not useful above 2X
Line average; frame average	Used to reduce noise
Rotation (do not use with DIC)	
Pinhole size	Bigger pinhole = brighter image, reduced resolution
Z-Stack (see instructions below)	

- XY panel - default Image Format is 512x512; Scan Speed is 400 Hz. Start with these settings.

- In BEAM PATH SETTINGS window (above the sliders), activate UV (405) and/or VISIBLE (458, 476, 488, 496, 514, 543, 594, 633). Red dot means ON; these will show up as a line on the spectrum below.



- Increase the intensity of the UV and/or selected lasers by using the sliders - start with 20-30%.
- You can open the Load/Save Single Setting drop down menu list to select stored settings if appropriate.
- Activate PMT's (check Active box in each frame).
- Set filter sliders to collect the correct wavelengths (emissions).

- You can use drop-down menus in each PMT box to select the appropriate fluorophores - click on NONE and then select fluorophore - and you will see emission wavelengths on the spectrum. Then move sliders to the left or right, or extend their length, as necessary to configure range of detection to match the emissions of the fluorophore. Make sure the detection range doesn't include the laser lines.
- For help on what wavelengths to collect:
    - On the left monitor, click on CONFIGURATION (upper left, left monitor) and select the "Dyes" icon to see a list of fluorophores and their spectral data.

    OR

    - Go to the Load/Save Settings drop down menu to automatically adjust the settings for the fluorophores you select. Wait for the scope to adjust itself here.
- To turn on confocal detection of brightfield or DIC:
    - Go to the Beam Path Settings window.
    - Check the ADDITIONAL CHANNELS box.
    - Check Active Box in the new PMT.
    - Select SCAN-BF or SCAN-DIC in the detector drop-down menu.
    - Make sure the ARGON laser is turned on and the 488 line is active.

- Select color of the image you want to generate by clicking on the color bar in each selected PMT box.

- To see your images, select LIVE to start continuous imaging. In this configuration, all the images are collected simultaneously. You now see what the PMT's are detecting - typically weak or no signal.
- To optimize the signal, turn up the SMART GAIN knob on the PANEL BOX. Your image should appear.

- For each frame (selected by left push button on PANEL BOX or mouse click on desired frame on right monitor):
    - Select GLOW OVER UNDER (aka false color or QLUT in the left margin of right monitor).

    - Use SMART GAIN and SMART OFFSET on the PANEL BOX to adjust for brightness and contrast in the selected channel on the right monitor.
    - Be sure you are at a good Z position - perhaps try moving up or down.
    - Blue pixels signify saturation. Adjust with SMART GAIN until there are a few blue dots. Orange/yellow pixels are good.
    - Green pixels signify black background (no signal) and so less noise. Adjust with SMART OFFSET until there are just a few green dots.

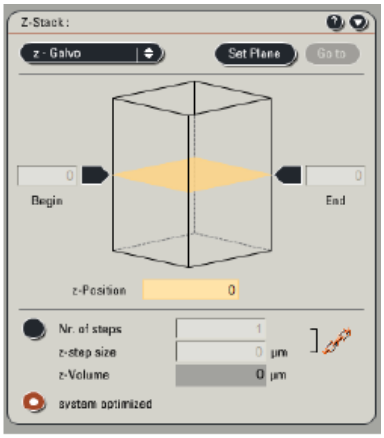
    NOTE: A smart gain value lower than 400 V would mean that you can lower the laser power and increase the smart gain to about 900-1000 V. A smart gain between 1100-1250 would suggest increasing the laser power. BUT, by enhancing the laser power you will expose your sample to more laser, hence your sample ***will bleach faster***. On the other hand, enhancing the gain won't expose your sample to more laser light, and it will protect your sample from too much light. Thus, in order to protect your sample signal, it is better to first adjust your gain, and then if not enough signal is present, carefully enhance laser output.
- At this point you may need to adjust the laser power, the PMT sliders, and/or the Z position (focus) to get the best image.

- To prevent overexposure of sample, click STOP whenever possible.
- Click GLOW OVER UNDER twice to see the image in the selected colors (click once to see black/white, the intensity values).
- On the right margin of right monitor, click on the various frames (1, 2, 3, etc.) to see the individual channels or the OVERLAY image to see the overlay of the selected channels.

- Double click on any frame to make it full screen; double click again to return.
- ONCE YOU HAVE YOUR IMAGE, you can make additional changes to perhaps improve it. On the X/Y settings drop-down window (ACQUIRE, Acquisitions, xy), adjust the following:
    - FORMAT - changes size in pixels of the image. Must STOP, change setting, then go to LIVE
    - SPEED - rate of scan. A slower setting might improve your image. If rate is set too high it will change the area scanned. Must STOP, change setting, then go to LIVE
    - BIDIRECTIONAL - checking this box alters the scan pattern so it scans back and forth, thereby making the scan twice as fast. To get the scans in synchrony with each other, use the Phase slider to eliminate any jagged edges (try the zoom to see these better).
    - ZOOM FACTOR - set with slider or click Zoom box and draw square around selected area of image or use Zoom knob on Panel Box - this can be done while acquiring image. Typically a zoom greater than 2X suggests going to the next higher objective to take advantage of the better NA.
    - LINE AVERAGE/FRAME AVERAGE - number of scans to be averaged to reduce background noise and enhance quality of image. Must STOP, change setting (click on line or frame averaging and select a number), then go to LIVE. The appropriate number for your sample has to be determined empirically. Generally, for live imaging where the specimen moves, use line averaging. For fixed imaging, use line or frame averaging.
    - ACCUMULATING - a function for weakly fluorescent samples
    - ROTATION - rotates image, duh - this can be done while acquiring image. Do not rotate if collecting a DIC image.
- Once your image is what you want, click on CAPTURE IMAGE for single image acquisition or START if you are doing an image sequence (such as a Z-STACK, see below).

Z-STACK IMAGING: (this can be combined with sequential imaging).

- Go to Acquisition and select the xyz mode.
- Open the Z-STACK drop down box.
- Turn on LIVE and define depth of specimen to be scanned:
    - Move through the sample (Z position knob) or knob on PANEL BOX.
    - Turn CCW to desired level (such as when signal disappears).
    - Click BEGIN in the Z series box (turns brown).
    - Use the Z position knob to move through the sample the other direction (CW) to the end
    - Click END

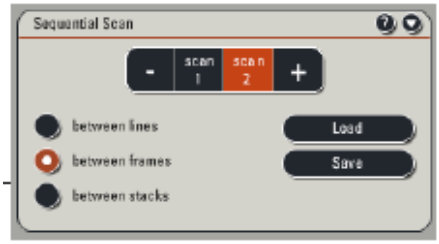


- Click on STOP.
- Select the number or size of steps (or accept the instruments suggestion- check by clicking on System Optimization).
- Select the number of scans set in the xy panel - set line or frame averaging
- To acquire a stack, click START, NOT LIVE
- Wait quietly and watch progress on the scale at bottom of the screen
- When scan is finished, play through the stack in various ways using the buttons on the right margin of the right monitor.

SEQUENTIAL IMAGING: This eliminates the common problem of cross talk, where there is overlap of emitted signals. Better separation of the fluorescent signals is achieved by acquiring data one channel at a time (sequentially). This is often very important if one is performing co-localization studies. This can also be combined with Z-series imaging).

- Select the ACQUIRE tab and click on the SEQ button in the Acquisition Mode frame to open the Sequential Scanning box below.

- Configure the PMT and laser for setting 1 (this means turning OFF the other PMT's and lasers):
    - Select LIVE.
    - Adjust Smart Gain and Smart Offset.
    - Select STOP.
- Click the + button to add a second setting option.
- Configure the laser and PMT for setting 2:
    - Select LIVE.
    - Adjust Smart Gain and Smart Offset.
    - Select STOP
- Continue until all needed laser/PMT settings have been configured.



NOTE: IF YOU SELECT PMT SLIDER POSITION THAT OVERLAP EACH OTHER, YOU MUST SCAN BETWEEN FRAMES OR STACKS.

- Click on START (NOT LIVE) to generate images or CAPTURE IMAGE to, duh, capture a single image (not a series).
- When finished, click on the SEQ button again to turn it off and return to prior settings.

DIFFERENTIAL INTERFERENCE CONTRAST (DIC): (Cannot do DIC at 10X)

- Set Kohler illumination for BF.
- Open condenser aperture fully with AP button (AP 24 on scope panel).
- Push CHG-TL button to get TL-POL on panel.
- Adjust the small silver wire (move forward and back) to maximum darkness (top right of condenser).
- Push CHG-TL button to get TL-DIC on panel.
- Adjust Wollaston prism for maximum view (wheel, below stage, left side, toward the rear).
- TL/IL button toggles between fluorescence and DIC (make sure to press the shutter if you want to see the fluorescence image).

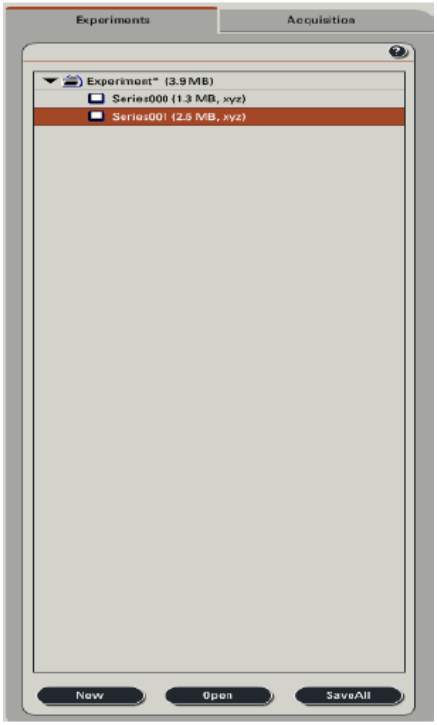
- For confocal detection of the DIC image, go to the Beam Path Settings window, check the ADDITIONAL CHANNELS box, check Active Box in the new PMT and select SCAN-DIC in the detector drop-down menu. Be sure the ARGON laser is turned on and the 488 line is active.

BRIGHTFIELD:

- Set Kohler illumination for BF.
- For confocal detection of the BRIGHTFIELD image:
    - Go to the Beam Path Settings window.
    - Check the ADDITIONAL CHANNELS box.
    - Check Active Box in the new PMT.
    - Select SCAN-BF in the detector drop-down menu.
    - Be sure the ARGON laser is turned on and the 488 line is active.

RESONANCE SCANNING:

- If you are going to do Resonance Scanning, at start up, after selecting the LAS AF icon and the Leica application suite window opens, make sure the "Activate Resonant Scanner" box is checked.
- If you want to go to Resonance Scanning from conventional scanning:
    - Turn off the software
    - Open LAS AF
    - Check the "Activate Resonant Scanner" box, click OK
    - Configure lasers and detectors in Beam Path Settings
    - To start, scan XY; 512X 512, 8000 Hz



SAVING DATA:

- Save image/experiment data:
    - Go to EXPERIMENTS (Acquisition tab in ACQUIRE).
    - Highlight and right click.
- If you want to save a merged image, right click on the image and send it to the experiments, then save.

SHUTDOWN: *FOLLOW THIS SEQUENCE EXACTLY*

- Go to CONFIGURATION and select the LASER icon.
- Turn all the lasers OFF (uncheck box).
- Check that the argon laser goes to STANDBY.
- SAVE your experiments if not already done so. Then download or transfer your data files; BE SURE ALL DATA ARE REMOVED FROM THE PC AT THE END OF YOUR SESSION.

- Exit LAS AF software (close windows by clicking the red X in the top right corner of the left monitor OR select File: Exit).
- Turn LASER key to OFF (emission warning indicator should go out).
- Set timer for 10 minutes.
- Turn OFF the scanner by flipping the green SCANNER POWER toggle switch.
- On the monitor, select START (bottom left, left monitor) followed by Shut Down and wait for the Shut Down window to show up.
- Select Shut Down and click OK to end the computer session.
- Lower the microscope stage by pressing the Z-button (the lower black push button, lower right side on microscope).
- TILT HEAD BACK
- Remove slide and clean objectives. Use LENS PAPER ONLY.
- If necessary, turn objective turret by hand by using the silver nosepiece wheel, not by grabbing an objective.
- Return to 10X objective by rotating the nosepiece by hand (again, try not to grab an objective). You can read which objective is in place on the front screen of microscope.
- Approximately center stage (X and Y).
- Tilt head forward.
- Turn OFF the microscope, computer and monitors by flipping the green PC-MICROSCOPE toggle switch.
- Close the SHUTTER on the metal halide lamp power supply.
- Turn OFF the lamp (light indicator should go out).
- Turn temperature regulator OFF (switch on blue box).
-Turn off the LN_2 to the table.
- After 10 minutes … turn OFF the laser fan by flipping the green LASER POWER toggle switch.
- Be sure to turn off the room fan.
- SIGN OUT IN THE LOGBOOK.
- Take your specimens with you.
- Leave room clean and tidy.