Leica Laser Scanning Confocal Microscope

THE MOST IMPORTANT THINGS YOU NEED TO KNOW:

NO ONE may use the confocal without reserving time with Dr. T. Budd or Jill.-

Unless you have been approved by T. Budd or Jill to use the confocal on your own, all use must be supervised. We are happy to help you with all aspects of your microscopy and we will also provide any level of training desired, including comprehensive training to be approved for independent use.

NEVER remove, substitute or add an objective lens. The only thing users can do with the objective lenses is to clean the oil of immersion from them. Use a Kimwipe soaked with Sparkle glass cleaner to wipe the metal surface of the lens. NEVER use a Kimwipe to clean the glass surface. Only use optical lens paper with Sparkle cleaner. Never wipe the glass lens unless it is hydrated or oiled.

Only use the approved oil of immersion provided. It has the correct (optimal) refractive index for the optical system at 21-23°C. [Note - It is also optimal to use # 1.5 coverslips for viewing your specimen.]

NEVER modify the confocal in any way. Do NOT add accessories without approval.

Do NOT remove the Z-stage without approval. Place your specimen (slide) on the Z-stage (coverslip facing down) WITH EXTREME CARE.

The confocal is a class IIIa laser device and contains class IIIb lasers. All users must receive laser safety training appropriate to the use of the instrument. NEVER attempt to access the laser compartment.

NEVER install computer software on the confocal computer.

NEVER store images on the C drive, save them to your own folder on the E drive.

If you don’t know what a control does, DON’T TOUCH IT.  
Start up:

DO NOT attempt this unless you have been approved to do so. If you have
signed up in advance as required, the system will usually be powered up and ready to use. If not:

Turn ON the Hg lamp power supply (on the floor to the left of the microscope), wait 1 minute (make sure that the computer and microscope power supply is off).

Turn ON the microscope power supply (on the floor just to the right of the Hg lamp supply).

Now go to the key switch power panel on the right side of the microscope display table.

Turn ON the laser power supply, listen for the cooling blower to come on.

Turn ON the computer using the red panel switch labeled “PC.” In a few moments you will be prompted to press Cont, Alt, Del and a login window will open. If you have been assigned login rights, enter your ID and Password.

Turn ON the scanner power supply, another cooling fan should come on.

Wait for about 2-3 minutes before turning on the lasers. Turn on only the lasers that are needed for your work. If you do not know which lasers that you need, you are not ready to use this instrument. Seek help.

Turn the Argon laser on by turning the key switch to the 1:00 o’clock position momentarily and then let the key return to the 12:00 o’clock position (much the same as starting a car engine). The Argon laser should always be turned on before the other lasers.

Turn the remaining lasers on by turning the key switch to the 12:00 o’clock position.

When you see the desktop, double left click the confocal icon and start the imaging software.

Adjust the potentiometer just to the left of the laser switches just barely off of the most counter-clockwise position. The YELLOW indicator lights will illuminate when the lasers are on.
It will take about 10 minutes for the electronics to “stabilize.” The room temperature should be held constant between 21-23°C. Turn the air conditioner ON if necessary.

You are now ready to place your specimen on the microscope and obtain an image using the Hg lamp and the appropriate filters. You must also set the parfocality of the lenses that you plan to use. This latter step is extremely important in order to avoid damaging the objective lenses and the Z-stage.

Using the inverted microscope:

The Leica microscope is unlike any other microscope that you have used. Do not assume that you can use it successfully even if you have used a standard fluorescence microscope. All users must be specifically trained.

Be absolutely sure that the coverslip is “fixed” to the slide. The mounting medium should be cured. Wet or fresh mounts will not work with the oil of immersion lenses since the coverslip will move by gravity or with any movement of the lens. The careful use of fingernail polish or fast curing epoxy glue can be used to “tac” a fluid coverslip in place but be sure that the polish/glue has cured completely. It is extremely difficult to remove the glue from the lens glass should one drag it through the uncured glue. Some workers choose to use an acrylocyanate glue (Krazy glue) but this should be avoided since removal of this material would require the use of solvents that could harm the lens coatings and mounting adhesives. The objectives cost several thousands of dollars. There is NO ROOM FOR ERROR in this regard.

Previewing your specimen:

If the confocal microscope is not turned on, use a standard fluorescence microscope to preview your specimen. The confocal microscope is very expensive to use so you should work out your specimen preparation protocols using the standard scopes. If the confocal is turned on and available, it may be used for previewing and when the emission of a fluor is not visible with the eye (e.g., far red).

It is common, especially with novice users, to use the 10x objective to preview your slide. This lens does not require oil of immersion and in fact, oil should
never be placed on this lens; it is not appropriately sealed and the oil will leak into the lens.

The 10x lens should be in place directly under the viewing position of the Z-stage. Always place this lens into the viewing position when you are finished using the microscope. If the 10x is not in place, use the black rocker switch on the left side of the microscope body to rotate it into position. Rock the switch downward to go to lower magnification lenses. The digital readout on the front of the microscope should read “10x” in the lower left part of the window. Be sure that the lens is lowered by using the black rocker switch on the right side of the microscope body.

**Carefully** tilt the condenser tower back out of the way and place your slide (coverslip down) onto the Z-stage. There is no need to secure the slide with the clips at this time. Use the stage controls to center the coverslip over the lens. Tilt the condenser tower back into the vertical position.

Although this microscope has brightfield, phase contrast and differential interference contrast optical capabilities (depending on the lens selected), the 10x only does brightfield imaging. If you want to initially view your specimen using brightfield illumination (in order to find the correct focal plane) you will need to:

Turn on the tungsten light source using the thumb wheel on the left side of the microscope body to give appropriate illumination.

Adjust the condenser iris diaphragm to give contrast to the image (almost closed). The upper (optical) turret should be set on the “H” setting.

Move the 10x objective lens UP using the black rocker switch on the right side of the microscope body while viewing through the ocular lenses. You do not have to worry about “hitting” the slide due to the long working distance of this lens.

If you do not see the image come into focus you probably need to use the manual focus knob (matching knobs on each side of the microscope body). Use the black, right most button on the front of the microscope body to give a digital reading of “S3” in the read out window. This is the coarse focus setting and will allow you to search for your correct focal plane. Once you have found the focal plane, you are ready to view under fluorescence settings to determine whether your preparation has been done correctly.

Note – it is usually easier to find the focal plane under fluorescence viewing
settings. The filter turret is fitted with the appropriate filters for viewing fluorescine (FITC) and rhodamine (TRITC) or fluors with similar excitation and emission spectra. Position 3 is for TRITC and 2 is for FITC. The other positions on this turret are “Scan” for laser scanning of the specimen and 1 which is open for using the transmitted light photomultiplier tube (Trans-PMT).

To view the fluorescence image, rotate the turret to the appropriate filter (3 or 2), be sure that the black rocker switch on the front left of the microscope is set to “Vis” (red LED), then pull the Hg lamp slider (shutter) out (open). You should see the colored light in the specimen area (green for TRITC, turret position 3 or blue for FITC, turret position 2). Raise the 10x objective using the right side rocker switch as described above while viewing through the ocular lenses. If your specimen is fluorescing, you should at least see a glow of illumination. Use the manual focus knob (S3, coarse focus) to focus the image. You may now view the entire specimen to determine what you wish to view at a higher magnification. At this point you will also know whether your specimen preparation was as expected and whether you should proceed with advanced imaging.

You may also use the 10x lens (along with the others lenses) with the confocal imaging system. The confocal system is controlled by the computer. You will need to select the appropriate parameters to accomplish this. You will also need to set the parfocality for all objective lenses to be used. The following table will help you select the appropriate objective lenses.

<table>
<thead>
<tr>
<th>MAG</th>
<th>NA</th>
<th>TYPE</th>
<th>AREA OF VIEW</th>
<th>WD*</th>
<th>LIGHT OPTICS**</th>
<th>OPTICAL TURRET</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 0</td>
<td>.3</td>
<td>DRY</td>
<td>1.5 X 1.5 mm</td>
<td>11 mm</td>
<td>BF</td>
<td>H</td>
</tr>
<tr>
<td>2 0</td>
<td>.7</td>
<td>OIL</td>
<td>750 x 750 µ</td>
<td>250 µ</td>
<td>BF</td>
<td>C</td>
</tr>
<tr>
<td>4 0</td>
<td>1.25</td>
<td>OIL</td>
<td>375 x 375 µ</td>
<td>100 µ</td>
<td>BF or PhC</td>
<td>H (BF) or E (PC)</td>
</tr>
<tr>
<td>6 3</td>
<td>1.32</td>
<td>OIL</td>
<td>230 x 230 µ</td>
<td>70 µ</td>
<td>BF or DIC</td>
<td>H (BF) or D (DIC)</td>
</tr>
<tr>
<td>1 0 0</td>
<td>1.4</td>
<td>OIL</td>
<td>150 x 150 µ</td>
<td>90 µ</td>
<td>BF or DIC</td>
<td>H (BF) or D (DIC)</td>
</tr>
</tbody>
</table>

* Working Distance -remember to include the thickness of the coverslip in the WD.

** BF = bright field, PhC = Phase contrast, DIC = differential interference contrast.
Using the confocal software:

If the confocal software is not open, do so now.

First, you will need to select the objective lens you wish to (initially) use and find (and set) the focal plane of your specimen. To do this, you will need to place oil of immersion on the lens (unless you are going to use the 10x lens). If a specimen slide is mounted on the Z-stage, it will need to be removed. Carefully tilt the condenser tower back out of the way. Gently slide the securing clips to the side, grasp the slide on each end using both hands and pull the slide towards you and lift the slide off the stage. Place it out of the way.

Use the left side black rocker switch to select the appropriate objective lens into viewing position. The lens should be at its lowest vertical position. If it is not, lower it using the rocker switch on the right side of the microscope body. Carefully place a small drop of oil on the glass surface without actually touching the lens surface with the applicator. Only allow the oil to contact the lens. Be careful not to put too much oil on the lens otherwise the excess may run down the side of the lens body. That would not be good.

Replace your slide (coverslip down) and secure it with the clips. If you did not move the stage x – y control, you will return to the same general field of view of your specimen. Carefully raise the objective using the right side black rocker switch just until the oil makes contact with the coverslip. This is easily seen if you have the Hg lamp illumination hitting the slide. You will need this setting (the same one used if you previewed with the 10x lens as explained above, filter turret to 3 or 2, Vis, Hg slider out) so that you can find the focal plane. Using the manual focus (S3=coarse, S2= medium, etc.), find the focal plane. [NOTE: Be very careful doing this operation. It is very easy to run the objective up into the coverslip and to “push” the z-stage upward. This must be avoided since the glass lens would be in contact with the glass coverslip and could damage the lens and it also puts stress on the Z-stage that could affect its operation.] Once you find the approximate plane, you can use finer focus settings (S0 – S2, right most front black rocker switch, digital readout) to precisely focus and search for a desirable field to observe.

NOW you need to set the focal plane into the computer memory so that the computer knows where the lens is in the vertical plane. To do this, use the second rocker switch from the right on the front switch panel. Depress this switch
momentarily. The digital readout window will display “Delete”. The display will then switch to read “Set?” Depress this switch again to set the computer. Do this same routine for any other lens you anticipate using during your session. **This ensures parfocality between all of the lenses.** Once this is done, the lens may be lowered and raised repeatedly to allow different specimens to be viewed or to change to a different lens without fear of running the lens into the slide. This step is **very critical** to avoid damage to the lenses and to the Z-stage. **Make absolutely sure that you do this correctly.**

**Setting the imaging parameters of the software:**

Set the microscope to allow laser scanning. Close the Hg lamp shutter (slider) by pushing it IN. Rotate the filter turret to “Scan” and set the front port toggle to “Side”. The microscope will now basically be a robot of the software.

Many of the confocal viewing settings are fairly standard and are set as default settings for the software. We will only discuss the few settings that are important for obtaining optimal results. The left hand monitor is called the **profile screen** (where you will set the imaging parameters) and the right monitor display is called the **template screen** (where the image will be displayed).

Open the “Beam” window by left clicking the button at the bottom left of the template screen. Drag this window so that it is centered adjacent to the parameters menu (blue background). From the beam window, you may select a factory set beam template based on commonly used fluorochromes, or you may create and save customized beam settings.

There are 3 different lasers available to provide 6 different excitation wavelengths as follows:

<table>
<thead>
<tr>
<th>LASER</th>
<th>WAVELENGTHS (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon</td>
<td>458, 476, 488, and 514</td>
</tr>
<tr>
<td>HeNe 1</td>
<td>5 4 3</td>
</tr>
<tr>
<td>HeNe 2</td>
<td>6 3 3</td>
</tr>
</tbody>
</table>

There are factory templates that will select several common combinations of these “bands” (based on common fluorochromes available) but only 3 bands can be used at one time because there are only 3 PMTs to simultaneously collect emission data. Each PMT
is “gated” such that the fluoresced light that is directed towards it is passed through a prism, thus broken down into the wavelengths present in the emission. The gates control which wavelengths are “seen” by each of the PMTs. This literally turns the PMTs into spectrophotometers, thus allowing qualitative and quantitative data to be collected. The gate (i.e., the wavelengths to be collected) is set in the beam window just below the emission spectrum displayed for each fluor selected by the template.

Select or design a custom template based on the fluorochromes in your specimen. If you selected a factory template, the potentiometer control bar settings are selected for you. These may be changed by clicking the buttons at the bottom of the template screen.

The largest buttons at the bottom of the template screen are “Continuous”, “Single Scan”, and “Series”. The Continuous button is basically a preview program. When this button is clicked, the scan head begins to scan the specimen with the selected laser wavelengths (bands). You may then adjust the PMT gain to “see” the “image” of the fluorochrome emission(s). At this point you may adjust the PMT gates and laser intensity (strength) in the beam window. The only other setting that you might want to change at this time is the “Format” setting which controls the resolution (i.e., pixel size). To change this setting you must first click the Continuous button which is now labeled “Stop” (the buttons toggle back and forth between their function and “Stop”). Click on the Format button and select the desired resolution. This setting will affect the size of the image file and will make the image display more dim (you can increase the PMT gain to compensate for this latter effect). A 512 x 512 setting (points x lines) will yield a .25 megabyte file while a 4096 x 4096 setting will yield a 16 megabyte file. Since you are collecting more data (pixels) as you increase resolution, this will slow down the scan time. Use a 512 x 512 or lower setting for previewing and then increase to a higher setting for collecting a high quality image using the Single Scan program (button).