

Leica Confocal Microscope and Leica TCSNT Instructions

No Food or Drink Permitted in the Microscope Room

CRITICAL: The microscope can be seriously damaged by improper care of either lenses or lasers. So review the following very carefully before turning on the microscope.

Lens care: Jarring a lens in any way can permanently destroy the alignment of the elements within it. Handle lenses very gingerly. Before examining a sample, make sure there is no foreign material on the sample that could contaminate the lens surface. After use, clean lenses while in the nosepiece and only with lens paper. Fold the paper several times and rub it back and forth over the lens surface. Use several pieces of lens paper until there is no longer evidence of oil on the paper. Avoid pressing the lens paper with your finger into the transparent part of the lens. If the lens becomes contaminated with any substance, contact the facilities manager for instructions on cleaning. Don't even think of applying any solvents to the lens surface.

All the lenses for this microscope are immersion-oil objectives, even 10X.

Laser care: The system should be shut down only by experienced users. Before switching off the lasers, please, run them for 5 min with the rheostat (black knob with a white dot) turned down. Once the lasers are turned off with the key switches, their cooling supply (whether air or water) should be left on for at least 15 min. This insures that the lasers cool sufficiently. This is done for the UV laser by leaving the cooling unit running and the water flowing (in the adjacent room). For the other lasers, the orange buttons controlling the fans are left on for 15 min after turning off the key switches. Before shutdown of the fans, check the hoses going to the exhaust line in the back corner of the room to see if they are near room temperature

Maximal laser power is 20 mW for 488 nm, 20 mW for 568 nm, 12 mW for 633 nm and 50 mW for UV laser. Approximately 75% of the laser power is lost on the way to the specimen. The beam radius is 0.2 mm for 63X and 100X objectives.

Turning on the microscope for standard fluorescence.

- 1.) Microscope switch (lower left) turns on the lamp for transmitted light and also turns on motors for the stage and nosepiece. Dial on lower left controls intensity of the transmitted light.
- 2.) To select a lens, use the pair of square black buttons on the left side of the microscope. These rotate the nosepiece clockwise or counterclockwise. The lens selected is shown on the display panel on the front of the scope.
- 3.) If you are using immersion oil, add it to the lens at this point. Avoid touching the clear, center part of the lens. Do not mix immersion oils. If your slide has oil from another bottle, clean it first. If there is any other contaminant on your slide, clean it, or do not use that slide. Contaminating the lens surface is a very serious problem.
- 4.) Place your sample on the stage and then carefully raise the objective using the upper black button from the pair of square black buttons on the right side of the microscope. If using an oil lens, raise the objective until it just flattens out the oil as it contacts your slide. Then observe the specimen through the oculars. You can use the focus knob now to gradually bring your specimen into focus. Check your slide periodically to make sure you are not over-focusing and pushing up the specimen with the lens. This can seriously damage the lens.
- 5.) Set up the optics for bright field viewing.
 - i.) Turn silver dial, left side, under nose piece, to one of the two unlabeled positions.
 - ii.) Turn silver dial, lower left side under nose piece to the "scan" position.
 - iii.) Turn silver dial, right side, lower level, to the "1x" position.
 - iv.) There are two plungers on the right side of the microscope. Both should be in.
 - v.) Intensity of bright-field light is controlled by turning black dial, left, bottom of microscope, toward you.
- 6.) Focus on your specimen by turning the focus knob away from you to move up the lens.

- 7.) To prepare for fluorescence, feel the housing around the mercury arc lamp on the back, bottom of the microscope. If this is warm, let it cool down before turning on the arc lamp. The switch for the mercury arc is on the power supply box sitting on the uv laser power supply (left of microscope).
- 8.) For fluorescence, turn off the transmitted light by turning the black dial away from you on the front, left, bottom of microscope. Turn away from you until the display panel on front of the microscope reads 0*V.
- 9.) Be certain the slider labeled ICT/P (left side of the microscope beneath the nosepiece) is in the out position.
- 10.) The lower silver dial on the left side of the microscope controls filters and dichroic mirrors. Choose position 3 for green dyes, 2 for red dyes and 1 for uv dyes.
- 11.) Plunger on the left side should be pulled into the out position to send excitation light up through the objective.
- 12.) You should see excitation light coming from the lens.

Setting up for confocal imaging.

- 1.) The PC should be left on, but if it is off, turn it on with the top orange button on the PC box.
- 2.) Turn on scan electronics (orange button on the small box).
- 3.) Turn on cooling units for lasers to be used. Select from below.
 - For fluorescein, GFP, other green dyes excited by laser lines at 488 nm, 512 nm , 476 nm. Depress orange button on argon laser.
 - For rhodamine, Texas Red, other red dyes excited by a laser line at 568 nm. Depress orange button on krypton laser
 - For Cy5, other far-red dyes excited by a laser line at 633 nm. (Controlled by scan electronics button, which should already be on).
 - For Dapi, Hoechst, other uv dyes excited by laser lines at 351 nm and 364 nm.
 - i.) Turn on water supply in next room. Push the handle about 30-45° from vertical. Water flow should be steady in the sink. **WATCH TO MAKE SURE THE SINK IS DRAINING AND NOT CLOGGED!**
 - ii.) Turn on LP20 cooler also in next room.
- 4.) Turn on lasers corresponding to those selected in #3.
 - For argon, krypton or helium/neon turn key to slightly beyond vertical and then release (like starting your car). Yellow laser light(s) should go on beneath the key(s). Increase laser power by turning black knobs (rheostats). For argon, turn the dial so that the white line is positioned around 9:00. For krypton, turn the dial so that the white line is positioned around 12:00. If necessary, these dials may be turned further for brighter images.
 - For UV, turn the black flip switch to the "1-ON" position on the power supply labeled Coherent (left of microscope). Then turn key to vertical position (this key does not turn past vertical). The laser will beep after about 1 min. indicating it is on. Then turn rheostat (black knob) at least one full turn or until "current limit" light turns off. For more power turn further, but no more than 3 full turns.
- 5.) Log into the computer by hitting ctrl + alt + delete and then typing TCS_User and return (no password is necessary yet).
- 6.) To start the confocal program, double click on the TCS NT icon. It takes ~1 min to load this program.

Compensation lenses for UV scans.

Compensation lenses are necessary to scan in the UV channel. We have compensation lenses for the 10x, 40x and 63x objectives. Those lenses are automatically put in place for the UV scan, if one has specified the objective correctly in the lens menu. If the objective was specified incorrectly, the wrong compensation lens will be in place, and the quality of the image will deteriorate. Always check that you specified the right objective. Unfortunately, for the 20x and 100x objectives the compensation

lens is never changed automatically, therefore whatever compensation lens is present in the light path will be used by default. For the 100x objective, the 63x-compensation lens is optimal. Therefore, perform a little trick. Before switching to an UV or DAPI routine, specify the 63x objective in the Lens panel. Then activate the UV or DAPI routine. You will typically see the warning that the 63x-compensation lens will be placed in the light path. Click OK. After the appropriate routine is activated, change the objective specification to 100X in the 'Lens' panel.

Acquiring a confocal image of a single focal plane.

- 1.) Prepare the microscope optics for confocal scanning.
 - i.) On the left side of the microscope, push the plunger in to block the mercury arc excitation.
 - ii.) Turn the silver dial, lower left side to the "scan" position.
 - iii.) Turn the silver dial, right side, lower level, to the "scan" position.
 - iv.) There are two plungers on the right side of the microscope. Pull the upper one out.
- 2.) Click on "Acquire Images".
- 3.) A menu appears listing possible filter combinations. Choose the filter(s) to be used in your experiment. For example, when imaging a double-labeled specimen in red and green, choose FITC/TRITC (fluorescein/tetramethylrhodamine). This choice is not set in stone, and can be changed later if necessary without restarting the program.
- 4.) The "TCS_Tools" menu appears. In the lower panel, left corner, click on "Filters" to bring up the "TCS_Settings" window showing a rainbow spectrum with controls for filter settings and laser powers.
- 5.) Back in the "TCS_Tools" menu, click on the "Lens" panel (underneath the "Filter" panel) and select the lens in use. This will not automatically position the lens, but is important for setting the pinhole and pixel size. Also, see the section **Compensation lenses for UV scans**.
- 6.) Click in the "Mode" panel to insure that it says "xy". This is the default setting for basic applications. To leave it at xy, click back in the mode panel and the menu will disappear.
- 7.) Click in the "Format" panel and select the xy dimensions of your image. 512x512 or 256x256 are typical values.
- 8.) Ignore the "Scan" button for now, and click in the "Speed" button to select the rate of scanning. Medium is typical. A "2" at the end means the laser scans bidirectionally, which is faster but perhaps not as precise.
- 9.) Ignore the "Control" and "Pinhole" buttons. For initial scanning, set "Zoom" button at "1".
- 10.) Be certain that "Accum #" and "Sect. #" buttons are set at 1.
- 11.) Finally, click on the "Scan" button to open the shutter and acquire a laser scan of the specimen. You should hear the shutter click open, and see laser light shining up into your specimen. The "TCS_Image" window should appear on the second monitor, and an image of your specimen should appear within the window. (It is often convenient to zoom up the "TCS_Image" window to 1024x1024 using the appropriate button on the left side of the window).
- 12.) If no image is present, turn up the corresponding PMT voltage. Each of the first three dials controls a separate PMT. The dial for your image is the same position (#1,2 or 3) as the "stain" panel in use in the "TCS_Tools" menu. You may also increase brightness by turning up the laser power with the control bar in the "TCS_Tools" panel. Another option is to open the confocal pinhole (5th dial button). If you still see no image, check that the microscope is set up properly for confocal scanning (#1 above). You may also need to reexamine your specimen under conventional fluorescence to make sure it is still in focus.
- 13.) You may stop the scanning at any point by clicking on the "Stop" button (located where the "Scan" button was).
- 14.) Once you have obtained an image, adjust the PMT, pinhole and laser power to give a sufficiently bright image. Increased laser power yields a stronger signal, but causes faster bleaching. A higher PMT voltage also yields more signal, but at the expense of noise. Increasing the pinhole diameter leads to decreased confocality (i.e. lower contrast and less resolution in z).
- 15.) Once you have set the pinhole and laser power, optimize the PMT voltage by choosing the GlowOvUn color scale. This can be selected in the Clut (color lookup table) box in the "TCS_Tools" window under the "Stain" in question. With the GlowOvUn scale, the PMT should be set such that blue dots (due to saturation) just disappear. The offset of the PMT (fourth dial button) should be set such that green dots (due cut off at zero) also just disappear.

- 16.) To reduce noise in each focal plane, choose "Accum" in the "TCS_Tools" window and select the number of images to averaged. Click "Series" (not "Scan").

For a 3D confocal image

- 1.) Proceed through step #15 above.
- 2.) Identify the top and bottom of your region of interest. Do this using the z pos control (the 6th dial on the dial box). Turn this (clockwise or counterclockwise) until you find one end of your region of interest. Then in the "TCS_Tools" window click on the "Begin" button (it should turn white). This marks the beginning of your 3D image. Now use the z pos dial control to find the other end of your region of interest. Then click on the "End" button (it also turns white). This marks the end of your 3D image.
- 3.) Also in the "TCS_Tools" window click on the Sect # button, and choose the desired number of focal planes. If you click instead on "User defined", you may enter the focal plane spacing, and then the program will calculate the number of focal planes required to cover the distance between your endpoints marked by "Begin" and "End".
- 4.) To reduce noise in each focal plane, choose "Accum" in the "TCS_Tools" window and select the number of images to averaged.
- 5.) To acquire a 3D image, click on "Series".

Sequential scans.

To collect the images in three channels, for instance, FITC, TRITC and DAPI, or to alternate between UV laser and Argon/Krypton laser one should choose the sequential scan. Adjust the settings in each channel separately and save them by clicking on Save button under the listing of available routines in the Filters window. You may wish to save them as your personal routines if you are going to use them frequently. Alternatively, just resave the existing routine with corrected settings. Click on 'Seq' button and the Sequential Scan dialog window will appear. Select routines that you saved and add them to the list of routines. The first channel will be assigned green color, the second channel - red color, the third channel will be assigned blue color. This is the default that cannot be changed. Therefore, first add the argon laser routine, then the krypton laser routine, and then the UV laser routine. After that check all the boxes under "Activate stored method parameters", especially "filter wheels". If you do not check them, then the program will use the previous non-optimized settings. Checking 'filter wheels' is especially important, because without this the band pass will not be changed and there will be no UV image. Under 'change seq. method' select 'between frames'. Then the filters will be changed for each focal plane of the z-scan, rather than scanning all the z-sections in one channel and then repeating the scan in the next channel. You may save and afterwards load sequences that you generated using 'Save' and 'Load' buttons. Click 'Activate sequential scan', then 'Close', and then 'Series' to execute the sequential scan.

Saving your data.

Save images in your folder on the D drive. In case of emergency when D drive is full inform Tatiana about this problem and save images on J drive. After the end of the session transfer your data to NCI SSA1 server (see **Shutting down the system** section).

To save your image, or a series of images click on the file menu, and choose "Save as". A window will appear allowing you to name your file. It can be saved in "Scanner File" format, which is the standard format that Leica uses for its images. Always save your image in "Scanner file" format so that you will be able to open it in TCS NT later if necessary. After saving your file in 'Scanner file' format you may also save a second version of the same file in a different format.

Scanner file images may be opened in other programs (Corel Photo Paint or Adobe Photoshop). Therefore you may use this format for exporting single images. However, if a stack of images was saved as a "Scanner file", Corel Photo Paint or Adobe Photoshop will open only the first slice of the stack. For instance, if you collected image in three channels, you will be able to see image in only one channel. To view images in all three channels or multiple time points save your image in "TCS export" format. This command generates a folder containing your images as separate TIFF files, which can be opened by Corel

Photo Paint or Adobe Photoshop. EXP file is also generated. This file is necessary for opening in TCS NT the separate images saved in "TCS Export" format. For saving overlays and projections use "Save selected" command (for details see next section).

Some basic tips for viewing and quantifying data.

- 1.) The "TCS_Image" window provides some **simple viewing tools**. Single permits viewing of one focal plane filling the whole window. Tiled includes images from up to three different channels (e.g. green and red). Gallery shows the entire sequence of images, in postage stamp size. Overlay produces a combined two or three color image in the fourth panel.
- 2.) Some **3D viewing tools** are available by clicking on "View" in the "TCS_Tools" menu. Step through your image using the "Next" and "Prev" buttons. "First" and "Last" brings you to these positions in the image. "Contrast" and "Gamma" can be used to enhance brightness and/or eliminate background. "Project" generates a projection view of all focal planes collected.
- 3.) Other 3D viewing tools are available by clicking on "Pseudo3D". The standard setting here is an orthogonal slicer which displays **xy, xz and yz views** of your image. Placing your cursor within any view allows you to step through the alternate views.
- 4.) Tools for **quantification** are available by clicking "Quantify" in the "TCS_Tools" menu. By clicking "Line" you can draw a line across a region of interest in an image shown in the "TCS_Image" window. Then click on "Profile" to see a graph of intensities along the line. "Rect" allows you to draw a rectangle over a region of interest. Following this with "Hist" yields the histogram of the pixels enclosed within the rectangle. Alternatively, "Rect" followed by "Stack" yields a profile of the average value of the pixels within the rectangle as a function of focal plane.
- 5.) **Saving overlays and projections so that they can be readable by other programs.** The overlay or the projection should be the only image that appears in the View window, therefore, click on Single button on a toolbar to the left of the window. Use Save Select command and save the overlay or projection as a scanner file. The following file should open in Adobe Photoshop and Corel Draw.
- 6.) You may also export an overlay or a projection as Corel Draw file (or Photoshop file) if Adobe Photoshop or Corel Draw is installed on the same computer where TCS NT runs. Therefore, you cannot specify Corel Draw or Photoshop on the confocal computer. Open your Overlay or Projection as a Single image. Snap. You will see a minimized TCS Annotation window. Restore it. Click on the Annotate button on the main toolbar at the bottom of the screen. Go to Preferences and specify the path to Corel Draw or Adobe Photoshop. Click on Export. Corel Draw or Photoshop will be opened and your projection or overlay will be displayed.
- 7.) **Color of the overlays and images.** If one wants to change the color of the image or of the projection made from this image, one has to click on the red/green stripe next to the image. The table will appear from which one may choose appropriate pseudo color scheme and then save the new image using "Save Selected" command. Unfortunately, one cannot change colors of the overlay after the data are collected. The image collected in the first channel will always be assigned green in the overlay. The image collected in the second channel will always be assigned red in the overlay. The image collected in the third channel will always be assigned blue in the overlay. Therefore, if one is imaging only in two channels and wishes the first channel image to be green, and the second to be blue in overlay, one should insert into a sequential scan a mock channel between two channels for the real imaging.
- 8.) Images, collected separately, may be **merged into one multi channel file** for the purpose of creating overlays. For that use TCS Merge program and view and overlay the merged images in TCSNT afterwards.

Shutting down the system

(If the system will be used within four hours, then only wipe oil from the lenses and turn down the laser rheostats (black knobs with white dots). Leave everything else on.)

- 1) Move your files to the SC-B41-SSA_SSA_41_G1_SERVER/SSA_G1 /LRBGEIMAGE/YourFolder. This server will be automatically mounted on all the computers of the facility. If you will not see this server mapped on your personal computers, map it yourself. Find red N in the lower right corner of the computer screen, right-click on it. Select Novell Map Network Drive and for the "network path to resource" field, use the following syntax
\\lrbgeimage.nci.nih.gov\ssa_41_g1\lrbgeimage.
- 2) Turn the laser rheostats (black knobs) counterclockwise to their minimum position. Continue to run the lasers for 5 min.
- 3) Turn back the laser key(s) counterclockwise to a "quarter-before-the-hour position." For the uv laser, also turn the flip switch to the off position.
- 4) DO NOT SHUT OFF ORANGE BUTTONS ON Ar, Kr, and HeNe LASER(s)!
- 5) Exit from the confocal program by first going to "Main", and then using "Exit Program" far right in the menu options.
- 6) Shut down the microscope.
- 7) Tilt back condenser arm.
- 8) Bring down the lens to its lowest position holding down the bottom black button on right.
- 9) Clean this lens with lens paper if oil was used (See procedure at the beginning!)
- 10) Use the black buttons on the left to rotate the nosepiece and examine each lens for oil.
- 11) Clean oil from any other lenses used with lens paper.
- 12) Turn off the mercury arc lamp (orange button).
- 13) Turn off the microscope (flip switch at bottom left).
- 14) Bring condenser forward to upright position.
- 15) Remove all slides, lens tissue from table area.
- 16) Wipe down the tables with a towel moistened with cleaning liquid.
- 17) Cover microscope with plastic cover. Avoid contact of the plastic with the arc lamp housing, which will melt it.
- 18) Sign the logbook and note any problems.
- 19) Check now if lasers have cooled down. (Read procedure at the beginning!) When cool, turn off orange buttons. For uv laser, also turn off water flow in adjacent room by returning the lever to the vertical position. Turn off the cooling unit in adjacent room with the orange button. In the microscope room, flip the black switch on the Coherent power supply to the "off" position.
- 20) Turn off the scan electronics (orange button).
- 21) Turn off the room lights, and check once more if any microscope-associated lights remain on.