

"Leica Confocal" Software and Confocal Microscope 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 (351 and 363 nm). 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.

Turning on the lasers.

- 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. The water will flow later when the laser will warm up.
 - 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.
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- 5.) 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.

Starting the Leica Confocal program and preliminary adjustments of the image brightness

- 1.) Log into the computer by hitting cntl + alt + delete and then typing your user name and password.

- 2). To start the confocal program, double click on the 'Leica Confoc' icon. When the dialog window for the program will open, press the 'Start' button. It takes ~1 min to load this program.
- 3). Click on the "Obj" button 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**.
- 4). Click on the 'Beam' button. Choose the filter(s) to be used in your experiment by clicking on the appropriate routine under 'User' menu (for example, FITC, or Texas Red, or DAPI). Each routine may be changed and either saved under new name, or overwritten (Use the button 'Save'). For further instructions, please, ask the staff.

<u>Dye</u>	<u>Excitation (nm)</u>	<u>Emission Peak (nm)</u>
GFP	488	507
FITC	494 (use 488)	518
TRITC	544 (use 568)	572
Texas Red	595 (use 568)	615

- 5). Click in the "Mode" panel to ensure that it says "xyz". This is the default setting for basic applications. To leave it at xyz, click back in the mode panel and the menu will disappear.
- 6). Click in the "Format" panel and select the xy dimensions of your image. To avoid undersampling (poor resolution), one should use certain format and/or zoom, specific for the given objective. Minimal requirements for objectives are:

Objective	Format	Zoom
100X	1024x1024	2
63X	1024x1024	4
40X	1024x1024	16
25X	1024x1024	8

One may also use higher zooms for the same objectives.

However, zooming requires more laser power, therefore, to avoid bleaching sometimes you might prefer undersampling to zooming.

- 7). For "Speed" use the default: medium. Using a lower scan speed you get better signal-to-noise ratio.
- 8). For "Scan" use the default (unidirectional scan). Bidirectional scan works two times faster, however, recording images in bidirectional scan mode can result in a phase shift between the forward sweep and playback of the scanning beam. To address this problem you should click the "Phase" button to open a dialog window, which you can use to correct the shift.
- 9). Ignore the "Signal" button. Use the knobs of the panel box to modulate PMTs.
- 10). Check the pinhole size on the panel describing confocal settings. If necessary, change pinhole size to 1 (best confocal effect) with the help of pinhole knob on the panelbox. "Pinhole" button will give you the information about the absolute value of the pinhole, if you are really interested in that.
- 11). Use Zoom knob on the panelbox to increase the zoom gradually. Use "Zoom" button to go to appropriate Zoom level. Some objectives require zooming in to a certain level. See (6) above.

Also, you may use "Zoom In" button both during scan or between scans. Click on Zoom In button, point to the appropriate area and stretch the rectangle. Do it only once - you cannot correct or resize this rectangle. If you think that you zoomed the wrong area, go back to previous size and repeat the zooming.

- 12). Hit 'Continuous' scan button and adjust the PMTs and the offset observing the specimen in the Experiment window.

Always use 'Glow OverUnder' type of pseudocoloring to adjust the brightness correctly. If you need further instructions, please, ask the staff. Click on "Continuous" button again to stop the continuous imaging.

After you have adjusted the brightness, pinhole etc, and you are satisfied with the quality of image, use 'Save' button and save your routine. If you did not change the routine dramatically, save is under the same name.

Also, you may change the color of the image to appropriate (green, blue or red) after you adjusted everything. However, it is better to image everything in "Glow Over Under" mode, watching for saturation. You may change the colors later, after imaging.

Single and Sequential scans (imaging in multiple channels and z-sectioning)

1). If you want to obtain single focal plane image in the single channel, first click "Averg" button and select the number of images to be averaged. This will reduce noise in each focal plane. After that click "Single Scan". 'Accum' (accumulation) button does not work on this system.

2). If you want to acquire images in several channels sequentially and/or z-sectioning of your specimen, program sequential scan and after that use "Series" button.

How to program the z-sectioning

a). 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 counterclockwise until you find top end of your region of interest. click on the "Begin" button (it should turn white). This marks the beginning of your 3D image. Turn z-control clockwise until you find bottom end of your region of interest. Then click on the "End" button (it should turn white). This marks the end of your 3D image.

b). 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".

c). To reduce noise in each focal plane, choose "Averg" and select the number of images to averaged.

d). To acquire a 3D image, click on large button "Series".

Small button "Series" located to the left of large "Series" button will show you exactly where each focal plane lays during the scan (yellow rectangle).

How to program the sequential scan.

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.

Click on "Sequential Scan" button and the Sequential Scan dialog window will appear. Select routines that you saved and "add" them to the list of routines. Use the checked defaults. Check "Frame averaging" if you specified different number of frames averaged for each routine. Under 'change seq. method' the default is 'between frames'. 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.

Click 'Series' to execute the sequential scan.

3). FRAP and TimeLapse

Select the Time (TimeLapse) button. Define Lapse 1.

Click the arrow expander button found on the right side of the time lapse dialog window.

Define a suitable preview image, check that current mode is in XYZ.

Select Lapse 2 at the bottom of the Lapse 1 to move on to the next Lapse button.

Click D in the Lapse 1 series dialog box to define the settings as the first lapse.

Set up the bleach with Zoom In and define Lapse 2

Zoom in on a region of interest to bleach. Increase the AOTF setting to 100% laser power

Define amount of time for bleach by setting the time in the interval-pause window.

Select Lapse 3 at the bottom of the Lapse 2 column to move on to the next lapse sequence

Click D to define the settings.

You may alternatively set up the Spot bleach (not to be used with Zoom IN)

Select the bleach button icon and define the bleach points by clicking each position to bleach.

Each position will be listed in the bleach dialog box.

Select the duration of the bleach in the bleach dialog box. Be sure that the bleach flag is checked

Do not change Repeat from 1 in the Lapse 2 column, if the duration has been defined in the bleach dialog.

Select Lapse 3 at the bottom of the Lapse 2 column.

Click D to define the settings.

Set up the Recovery in Lapse 3.

Select XYT mode for a single scan or XYZT for a series scan.

Define a time sequence for review of recovery. Select # of frames and change in time suitable for the experiment and apply the changes.

Do not set a repeat of this sequence, as this will ultimately generate a new series of scans.

Click on Lapse 1 button to begin the time lapse/ bleach sequence.

Save/View/Quantify the Recovery

The last series in this example is the Recovery series. To save this, click the series and export the file as .avi file

Go to Quantify and select the Pr. (Z) profile Z.

Select a drawing tool such as Poly to draw around the structure of interest to quantify the recovery.

If you click STOP in the Lapse dialog window only the currently running lapse sequence will be stopped rather than complete series of lapse.

A for Apply will reset all parameters in the system to the conditions of the lapse.

All conditions of the system including averaging, pinhole, accumulate etc. will be used when you define a lapse, therefore it is very important to configure all settings properly before clicking D to define a Lapse.

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.

Saving your data.

Save your images in your folder on the D drive. **DO NOT SAVE YOUR DATA ON C DRIVE, IT WILL INTERFERE WITH PROGRAM WORK.** In case of emergency when D drive is full inform staff about this problem and save images on J drive. After the end of the session transfer your data to SC-B41-SSA_SSA_41_G1_SERVER/SSA_G1 /LRBGEIMAGE/YourFolder.

The second workstation is available in the facility to view and quantify your data.

Later one can retrieve their data from the server on their own computer. This server will be automatically mounted on all the computers of the facility. If you will not see this server mapped on your personal computer, 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. Check "Reconnect at Logon" in the dialog window. For the users that are not on Novell network the FTP option and HTTP option is available.

Images collected in the same window are kept in RAM as a set called "Experiment #...". You may run several Experiments during each session. To start a new experiment click "New" button in the toolbar and a new window will open. Within Experiment set you may right-click on any file and use the command "Delete" from the pop-out menu to delete some images. To save your data, select the name of the directory ("Experiment #...") and click on "Save' or "Save as.." button on the toolbar.

Your images will be saved in a directory under the name you chose. Single images, z-scans, multi channel images will be saved as a series of TIFF images that can be viewed in other programs (Corel Photo Paint or Adobe Photoshop).

To view these images in Leica Confocal software you click "Open". You will see only the name of the appropriate experiment in the dialog window. Select it and the whole experiment will show up in the left panel as a directory with all the files belonging to this experiment.

You may save Overlays and Projections by right-clicking on them. In the pop-out menu select "Send to"/"Experiment" and then "selection raw". Overlay or Projection will be added to the set of files in RAM belonging to the appropriate Experiment. After that "Save" the whole Experiment.

You may save a single image with a scale bar or the whole set of images (three channels and overlay) with or w/o scalebar. To get scale bar go to View/Viewer Options/Display and select scale bar. Right-click on image, choose "Send to"/"Experiment"/"Snap " You will get a single image with a scale bar. Right-click on image, choose "Send to"/"Experiment"/"Snap all" You will get a single image containing everything that was displayed in imaging window (images in three channels and overlay).

You may save files as 3D stacks (only data, but no overlays may be saved in this way). Right-click on file name and choose "Export". You may select two formats, including .AVI.

Some basic tips for viewing data.

Saving overlays and projections. You may save Overlays and Projections by right-clicking on them. In the pop-out menu select "Send to" and then "selection raw". Overlay or Projection will be added to the set of files in RAM belonging to the appropriate Experiment. After that "Save" the whole Experiment.

Color of the overlays and images. If one wants to change the color of the image or of the overlay made from this image, one has to right-click and select "LUT" on the pop-out menu. A colored stripe will appear to the left of the image window. Double-click on this stripe and select any color you wish. The color of this image on the overlay will automatically change too.

Scale Bar. To get scale bar, go to View/Viewer Options/Display and select scale bar. To save the image with the scale bar, right-click on image. In the pop-out menu select "Send to" and then "All".

Adjusting brightness, contrast and gamma. Imaging Tool, Amplitude, Brightness, Contrast and Gamma-correction.

Quantification. Go to Quantify menu. Click on "Area" button. Then you will be able to draw Regions of Interest (ROI). If you will activate Polygon or Rectangle tool and also activate Wizard, then, if you will click within the region of interest, Wizard will automatically draw the appropriate ROI. ROI may be selected and resized if Sel button is activated.

When ROI is selected, hit Histogram. The intensity histogram and info about this ROI (size in pixels and mm², mean intensity and statistics) will be displayed in the separate window. Hit Qexp and export the data to Excel. Graph can be saved in a manner similar to saving of overlays and projections. Right-click on the graph itself and use "Send to" to add graph to the set of files in RAM belonging to the appropriate Experiment. When Experiment will be saved, the graph will be saved too.

If you want to analyze stack, you will have to step through the slices of the stack and hit Qexp for each slice.

Use "Profile" to analyze the profile along the selected line. This line may be moved around the image and resized (Sel).

Info (Legend). The info about image size, resolution and zoom may be displayed if you right-click on the image and select Legend.

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.
- 6). Shut down the microscope.
 - i.) Tilt back condenser arm.
 - ii.) Bring down the lens to its lowest position holding down the bottom black button on right.
 - iii.) Clean this lens with lens paper if oil was used (See procedure at the beginning!)
 - iv.) Use the black buttons on the left to rotate the nosepiece and examine each lens for oil.
 - v.) Clean oil from any other lenses used with lens paper.
 - vi.) Turn off the mercury arc lamp (orange button).
 - vii.) Turn off the microscope (flip switch at bottom left).
 - viii.) Bring condenser forward to upright position.
 - ix.) Remove all slides, lens tissue from table area.
 - x.) Wipe down the tables with a towel moistened with cleaning liquid.
 - xi.) Cover microscope with plastic cover. Avoid contact of the plastic with the arc lamp housing, which will melt it.
 - xii.) Sign the logbook and note any problems.
- 7). 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.
- 8). Turn off the scan electronics (orange button).
- 9). Turn off the room lights, and check once more if any microscope-associated lights remain on.