User Guide to the IBIF Leica TCS SP8 MP Confocal Microscope

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Introduction

The IBIF confocal microscope is made available on a fee-for-use-hour basis to all users who have been trained. Unlike our previous instrument, it has a number of hardware features, specifically the objectives, the stage, and the condensers, that can be added or removed by the user, with the permission of the Imaging Specialist. To minimize inconvenience to other users, and to reduce the risk of inadvertent damage to the equipment, we ask that users obey two rules:

1) Leave the confocal as you found it. Replace and/or reset everything that you modified.

2) Be aware that other users may have failed to obey rule 1. Be alert, especially in regard to mechanicals such as stage translation, condenser height, and objective rotation.

Thank you, and we wish you every success in your imaging. We are here to help.

The Confocal Microscope and Accessories

The instrument is a Leica TCS SP8 MP confocal microscope, equipped for multi-photon confocal microscopy. The components are the microscope body, two automated stages, the scan head, the electro-optical modulator (EOM), the monitor and computer, and the Spectra-Physics Mai-Tai tunable near-IR laser.

The items on the desk (along with the monitor, keyboard, and mouse) consist of a confocal controller, a microscope controller, a metal-halide power source (which performs the function of the Hg bulb in the previous system), and a controller for the Dodt contrast enhancement system (which replaces the Nomarski contrast enhancement method of the previous system).

The unlabeled switch is a shutter for the Mai Tai laser and should only be needed in emergencies in which the beam is exposed.
Two photomultiplier tube (PMT) detectors and two hybrid-PMT (HyD) detectors are available for conventional (single-photon) confocal microscopy.

Some cell-phone carriers use frequencies that interfere with the HyD detectors. Switch your phone off or leave it at the front of the room.

Two high-sensitivity Super HyD detectors are installed for non-descanned (multi-photon) confocal microscopy. These detectors are electrically- and liquid-cooled for decreased background. They should only be used in a darkened room and with the microscope covered by a dark room cloth (available).

The Super HyD detectors are also vulnerable to cell-phone interference.

Behind the air table at the rear are three boxes of instrumentation. The lower box is the Compact Supply Unit, which houses the visible light lasers. The smaller of the two boxes on top of the Compact Supply Unit is the power supply and cooling unit for the Super HyD non-descanned detectors.
The microscope has no manual focusing adjustment. Instead, it has two stages and two computer-controlled focusing mechanisms. The large X-Y stage performs lateral translation in all modes. The nosepiece has its own focusing drive, controlled from the microscope controller, or the confocal controller, or from the software.

The smaller Super Z Galvo stage rides on top of the large stage and includes the slide holder. It is used for stacks. The large translation stage can be used without the super X Galvo stage, primarily for whole animal work or specialized experimental chambers. Contact us for instructions on how to do this.
System Specification

Lasers
The system has four visible-light lasers: 405 nm, 488 nm, 514 nm, 552 nm, and 633 nm. The 405 nm should be used to excite DAPI or similar blue-emitting dyes, therefore use of the multi-photon laser will not be necessary. The 488 nm and 552 nm lasers are for FITC and rhodamine bands respectively. The 514 nm laser can be used to excite YFP, while the 633 nm laser is for longer wavelength dyes such as Cy5. All of the lasers are semiconductor types and can be switched on and off without special care.

**All of the lasers are more powerful than those in the previous system** (20 mW or better versus 1 mW for the 543 and 633 lasers of the previous system). Users will find that only a few percent excitation is required in most circumstances.

Optics

The available objectives (illustrated above, from left to right) are as follows:

<table>
<thead>
<tr>
<th>Position</th>
<th>Objective</th>
<th>Magnification</th>
<th>n.a.</th>
<th>Working distance (mm)</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HC PL Fluotar</td>
<td>10x</td>
<td>0.3</td>
<td>11.0</td>
<td>air</td>
</tr>
<tr>
<td>2</td>
<td>HC PL Apo</td>
<td>20x</td>
<td>0.7</td>
<td>0.59</td>
<td>air, coverglass</td>
</tr>
<tr>
<td>3*</td>
<td>IRAPO</td>
<td>25x</td>
<td>0.95</td>
<td>2.5</td>
<td>water</td>
</tr>
<tr>
<td>4</td>
<td>HC PL Apo</td>
<td>40x</td>
<td>1.30</td>
<td>0.24</td>
<td>oil</td>
</tr>
<tr>
<td>5*</td>
<td>IRAPO</td>
<td>40x</td>
<td>1.10 Corr</td>
<td>0.65</td>
<td>water</td>
</tr>
<tr>
<td>6</td>
<td>HC PL Apo</td>
<td>63x</td>
<td>1.40</td>
<td>0.14</td>
<td>oil</td>
</tr>
</tbody>
</table>

*available on request

The air and oil objectives (1, 2, 4, and 6) are similar to those you have used in the past. The two water objectives are especially good for deep-tissue imaging and are corrected for multi-photon excitation. **Note: the 40x and 63x objectives have spring-loaded front ends that can be inadvertently twisted and locked in a withdrawn position. Handle them with care.**

Disk Drives
The computer has two drives, a 120 Gb solid-state drive (C:) and a 1.8 Tb disk drive (D:). All data should be saved to the D: drive.
Condenser

A transmitted light condenser is available if high-quality visualization of unstained tissue or cells is required. However, there is enough unfocussed light available in transmitted light mode to enable simple localization of specimens without a condenser. The Imaging Specialist will demonstrate the user of the condenser if requested.

The condenser has a short working distance (1.0 mm) and very few options. Condenser centering is via two screws, as in the Zeiss system. The condenser focus knob is to the left side. The condenser cannot normally be brought into focus, but as long as it is properly centered it will function satisfactorily for the acquisition of transmitted light images. The Dodt contrast system does not use the condenser.

If you have brought the condenser near to focus (for example, if trying to visualize unstained cells), please remember to lower it before using the large X-Y stage, otherwise damage to the condenser may result. For the same reason, install the condenser after the large X-Y stage has been initialized. The condenser must be removed after use.
The Microscope Controller

Control of Stage Movement

Manual x, y and z stage control is via the knobs attached to the microscope controller, or by the manual X-Y stage control on the stage itself. The microscope controller moves the large X-Y stage only. The knobs allow much finer control than the manual X-Y stage control.

Touch Screen Control of Microscope Functions

The touch screens on the microscope controller are as follows:

Screen 1: Status and Illumination. Use to adjust illumination intensity, and the aperture and field stops in transmitted light (TL). These are in the microscope body, not the optional condenser.

Screen 2: Illumination Method. Select TL or RL (Reflected Light, i.e., epifluorescence). Open and close the shutters to either path. In RL, select the filter cubes (labeled GFP, RFP and DAPI) for green, red, or blue emissions.

Screen 3: Objective selection. Some selections will pause to allow time for oil or water application or removal. The operation is completed by pressing the selection again.
Screen 4: Nosepiece (i.e., objective) Z-control or stage X-Y control.

**Z-mode:** The Home position is the highest point to which the nosepiece can currently be raised. It is usually set at the upper limit of travel of the nosepiece. The Focus Stop position is the approximate focus position (actually, a little above) for the 10x objective. Both positions are indicated by – marks. The current position of the nosepiece is indicated by the = mark and its distance from the Focus Stop is indicated in mm on the display.

Press and hold the Home button to raise the nosepiece to the Home position. Press and hold the up/down button to move the nosepiece to any position. *Note that this button will allow the nose piece to travel below the focus position. Careless use of it may drive the objectives into the specimen.*

The Home and Focus Stop positions should not normally be modified. See the section below for a procedure to set or re-set them.

**X/Y Mode:** When X/Y mode is selected, the sensitivity of the X-Y control knobs can be selected (coarse or fine). Positions in the X-Y plane can be stored for future use. Maintained touch in the screen will cause the stage to translate to that position. *If the condenser is installed, be sure to lower it first.*

Screen 5: Allows programming of any of the black buttons for specific functions or combinations of functions. *See us before you program any of them.*

Screen 6: Not normally needed by users.
Setting and Restoring Home and Focus Positions (Screen 5).

In some situations, for example when using the large X-Y stage in a lowered position, it may be desirable to adjust the Home and Focus positions. Here’s how to do it.

First, select Screen 3 on the microscope controller display and select the 10x objective. Then select Screen 4 and select Z-mode (top right).

**Setting the Home position**

Using the controls on the microscope controller, raise the nosepiece all the way to the top, or to a level that allows you to change your sample without interference from the objectives. Note the Set/Clear Limits button (bottom left) which has two parts, the Up/Down symbol and the word Home.

On the Set/Clear Limits button, push the Home button on the Set/Clear Limits button (it will turn red). The button will now be labelled Set/Clear Home Position.

To set the Home position, hit the Set button (it will turn red). The Home position indicator will appear.

To clear the Home position, hit the Clear button. It will also turn red. The Home button will go gray.
**Setting the Focus Stop position**

Raise the nosepiece and set a glass slide specimen on the stage. If you do not have a suitable slide, there are some H & E stained slides in the top drawer of the desk. The slides are autofluorescent to GFP or YFP excitation, so you may use fluorescence as an alternative to transmitted light.

Manually lower the nosepiece until the specimen is in focus through the eyepieces with the 10X lens. Raise it a small amount.

On the Set/Clear Limits button, push the Up/Down button (it will turn red). The button will now be labelled Set/Clear Focus Position.

To set the current nosepiece position as the Focus Stop position, hit the Set button (it will also turn red). To clear the Focus Stop position, hit the Clear button. It will also turn red. The Focus Stop position indicator will appear.

If you have changed either setting, remember to restore the Home and Focus positions for the next user.
Positioning a Slide

Raise the objectives using the microscope controller. Displace the stage to the left manually or using the stage X-Y manual control. For extra room, rotate the objectives manually so that the 10x is over the stage. Be sure that the clips are positioned at the edges of the slide recess. If the clips are hard to move, use the hi-tech tool provided.

Place the slide in the slide recess. Usually, placing the right side first is the most effective method.

Place the clips over the slide, using the tool if necessary.

Manually reposition the stage under the objectives.

Note: dishes can also be accommodated, subject to the limitations of objective size and working distance. Ask us for details.
Confocal Operation without Multi-photon

Users will not need multi-photon microscopy for DAPI excitation because of the availability of the 405 nm laser.

Metal-Halide Power Supply

Be sure that the metal halide power supply shutter control button is set to remote (out).

Application Start

Turn on the computer, monitor, confocal power strip, and metal-halide power supply, in any order. Double-click on the desktop LAS AF icon. Do not start LAS AF if the confocal power strip is not on.

There are two questions to be answered during startup.

Optional selection of multi-photon laser. Select MP_LASER_OFF.

Initialization of the large X-Y stage. Usually, select No.

Initialization of this stage is not necessary unless you anticipate using the stage for tile scans. The manual X-Y controls can still be used for specimen positioning.

If tile scans are to be used, protect the large X-Y stage if necessary (raise the objectives, lower the condenser if present), then select Yes. The large X-Y stage will execute a series of X and Y movements before returning to its home position.
LAS AF Application

The application screen will start up in the Acquire pane and will look like this:

Buttons and Sliders

Most functions are controlled by sliders. Click on the slider button to change the slider value. Drag the button or, for more precise adjustment, use the mouse wheel. Some functions also allow keyboard data entry.

Confocal Controller

The confocal controller has six knobs that control acquisition functions and two buttons that toggle the current image/detector combination. The knobs and buttons can be operated while acquiring in Live mode. Modifications to them will be reflected in the Acquire pane. From left to right they are:

- Left button – move the current detector/image one step to the left.
- Right button - move the current detector/image one step to the right.
- Smart Gain – adjust the gain of the current detector.
- Smart Offset - adjust the offset of the current detector (if it is a PMT).
- Scan Field Rotation - self-explanatory.
- Pinhole Diameter (in Airy units) – use sparingly.
- Zoom – a large range is available (0.7 to 40). An audible warning will sound when the usable zoom has been exceeded.
- Z position (in µm) – particularly useful when setting up stacks.
**Program Panes**

The program has four panes – Configuration, Acquire, Process, and Quantify, selected by the tabs at the top left of the screen. Many Configuration pane options can be selected from the Acquisition pane, which reduces the need to use the configuration window.

**Configuration Pane**

The Configuration Pane allows setting of multiple hardware parameters. Most parameters can also be set or modified from dialogs in the Acquisition Pane, so users do not need to use its features often.

The Laser Configuration panel may be used to turn on or off lasers. The lasers may also be set to Standby mode if not required for a period.

The Hardware dialog is useful to select the acquisition bit depth. The default is 8-bit, which is adequate for image acquisition. If quantitative analyses are to be performed, we recommend 12-bit acquisition. 16-bit acquisition is available, but only for PMT channels.

The Configuration Pane also has a comprehensive dye data base that is valuable when setting up novel experiments.

The Objectives panel presents a window with useful information about the objectives, such as numerical aperture and working distance. It cannot be used to change the working objective.

The Super-Z button allows selection of the working range of the Super –Z Galvo stage. We recommend leaving it at 0.5 mm.
**Acquisition Pane**

**Selection of Configuration**

Sixteen standard configurations are available to all users from the pull-down menu above. Please don’t modify them! You can also create your own configurations and store them.

**Acquisition Mode**

The acquisition mode defaults to xyz, with \( z = 1 \), that is, a single frame. Other modes are discussed later in this document.
**Acquisition Rate**

The acquisition rate window selects the acquisition scan parameters, including the number of pixels in the image and the scan speed, via pull-down menus. We recommend 400 Hz as an initial rate.

The image scan may be zoomed down to 0.7 and up to 20.

Averaging may be by line or by frame, using pull-down menus – we recommend line averaging and no more than 4 averages.

The scan orientation may be controlled using the rotation slider. The image area may also be translated in the directions indicated by the rosette arrows. Large lateral translations at low zoom will move the scan range off axis and are therefore not recommended. Move the stage instead.

**Pinhole**

There is only one pinhole. Its diameter is determined by the longest emission band and defaults to 1 Airy disk. It may be modified by clicking and dragging on the pinhole button.

**Lasers**

The visible light lasers must be enabled by clicking on the ON/OFF button. The lasers may be selected using the laser menu in the Configuration Plane, or by clicking on the ‘+’ sign. Click and drag on the button to adjust power level.
Objectives
Objectives may be selected by the pull-down menu. The objectives will be raised and lowered as part of the rotation. The multi-function plate (MFP) should be set to substrate for most applications. Use Automatic determination of the beamsplitter.

Selecting the Fluorescence Detectors
Up to four detectors may be selected simultaneously. The detector type (HyD or PMT) is indicated in the top left of each detector window. The detector is selected by clicking on the on/off button. The color of its display output is selected from a pull-down menu next to the type indicator. HyD detectors have a gain selected by clicking on its slider button. PMT detectors have both gain and offset functions, controlled by clickable sliders. Leica recommends a starting offset of -0.3%.

Modifying the Detector Bandwidth
The detector bandwidth may be modified in either direction by simply clicking on and dragging its edges. The minimum bandwith is 5 nm. Adjacent detector bands may not overlap.

Selecting the Transmitted Light Detector
The TL detector is selected by clicking on its ON/OFF box. It is a PMT and is responsive up to 900 nm. Like the other PMTs it has gain and offset controls. Proper centering of the condenser aperture is required for optimum effectiveness. The condenser need not be focused but it should be close to the specimen.
Use the Dodt controller to modulate the contrast. The optimum Dodt setting when viewed with the eyepieces is not necessarily the same as the optimum setting when using confocal.

Acquisition Start Buttons
At the base of the pane are four action buttons. At left, the Autofocus button should not be used. The Live button initiates scanning at the indicated rate and format until it is pressed again.
At right, the *Capture Image* button is used to acquire a single image. The *Start* button is used to initiate acquisition of a stack or a sequence.

**Display**

The displayed image is rotated 90° anti-clockwise with respect to the image seen in the objectives. Thus x-axis stage translation moves the image vertically on the screen, and y-axis stage translation moves the image horizontally.

**Displaying Results**

The look-up table LUT used to display the results may be changed using the button at the top left of the display window. The first click selects a range-finding LUT for the active window that can be used to set gains and offsets. The second click changes the display in all active windows. A third click returns the LUT to the normal type.

The range-finding LUT is different to the one used by LSM. The LSM range-finding LUT used blue for below range, red for above range, and white for within range.

<table>
<thead>
<tr>
<th>below range</th>
<th>within range</th>
<th>above range</th>
</tr>
</thead>
</table>

LAS AF uses green for below range, blue for above range, and red for within range.

| below range | within range | above range |
Programming a Stack

Programming a stack works similarly to the Mark First/Last mechanism used in Zeiss LSM. It requires the following steps:

1. With Live acquisition going, adjust the focus to the top of the stack. Use the Confocal Controller, or the microscope controller fine focusing knob. Fine adjustments may be made using the clickable wheel.
2. Click on Set Start.
3. Re-adjust the focus to the bottom of the stack.
4. Click on Set End.

The number of steps and the step size will be automatically computed based on the longest wavelength emission band. It can be modified by clicking on Nr. of Steps or z-step size.

The diagram indicates the size and spacing of the stack and the current stage position. It may be re-scaled for better viewing using the slider provided.

Clicking on the double-vertical arrow button at the top moves the stage to the middle of the stack.

Clicking on the garbage can icon will delete the stack. This should be performed before creating a new stack.

Use of the z-Galvo stage or the nosepiece for vertical movement is controlled by the pull-down menu. The z-Galvo stage is more precise.

Leave Galvo Flow off.

Acquiring from a Region of Interest

Unlike in Zeiss LSM, images can be acquired from a region of interest (ROI) alone, or from an ROI with different conditions for an ROI and the background.
Click on the ROI button to set the foreground conditions. Acquisition conditions within the ROI will be as specified in the Detectors panel.

Draw the ROI using the tools above the image window. In this example, a square ROI has been selected.

To set the background conditions, click on the Background button.
Alternate Acquisition Modes – Sequential
Click on the right-most icon in the Acquisition Mode menu.

The Sequential Mode menu appears at the end of the acquisition parameters window. Click ‘+’ to add sequential events, ‘-’ to remove them. Up to six sequential acquisition events are possible. Events may be any of the available modes, e.g., xy, xyz, xyt, xyλ. Events may be acquired line-by-line (indicated by arrow). This is the preferred method, because it involves less switching of the scan components. However, if the detector bandwidth or the average or accumulation number changes between events, use “Between Frames”. If one or more event is a stack, use “Between Stacks”.
Alternate Acquisition Modes - Line Scan
**Alternate Acquisition Modes - Spectral**

Select $xy\lambda$ or similar acquisition mode.

The laser and detector section of the acquisition pane will appear as shown here.

The number of bands and the bandwidth is set in the lambda-Scan Range Properties window. The maximum spectral range is 380 nm to 785 nm. The shortest-wavelength laser is 405 nm, so the shortest practical wavelength is 420 nm, unless multi-photon excitation is used. The minimum bandwidth is 5 nm. The minimum step between bands can be less than 5 nm. At 5 nm spacing, the maximum number of bands is 81. Any one of the four descanned or the two non-descanned detectors may be selected as the detector.
Alternate Acquisition Modes - Time Series

Select xyt or similar acquisition mode.
Alternate Acquisition Modes - Tile Scan

Select Tile Scan in the Acquisition Mode menu. The Tile Scan window will appear below in the Acquisition window.

*Note: Tile Scan should not be combined with image rotation or erroneous results will be obtained.*

Tile Scan Window

The Tile Scan window features an X-Y map showing the current position of the stage. The scale is in mm, and the X-axis is vertical, as for the image display. The map scale can be expanded or contracted using the button and slider below the map.

Above the map are four buttons. The left-most button selects the current position for storage. Multiple positions can be stored. The current position number is reflected below the map. The second button deletes the current stored position, as reflected by the position number below the map. The third button deletes all currently-stored positions. Clicking on the right-most button opens a stage configuration menu.

The simplest method is to select Auto Stitching, with Smooth off.

Stage Configuration Menu

Note that in the default condition, the X- and Y- axes are inverted in sign (corresponding to the inverted microscope image), and that the X and Y axes are exchanged.

When Auto Stitching is selected, the percent of overlap is selected in this menu. We find that 10% overlap works satisfactorily without requiring excessive sampling or computation time.
Setting up a Tile Scan

The approach to setting up a tile scan is different from that in Zeiss LSM.

Step 1: Click on the Select Position button to select the current position as Position 1.

Step 2: Move the stage to a position at one extreme of the area to be tiled. Use the microscope controller X-Y controls to move to the new position, while observing through the eyepieces or observing the display in Live mode. Click on the Select Position button to select the current position as Position 2. The map will display a map indicating the number of tiles to be scanned. In this example, a 2 x 1 tile map is to be acquired.

Stage 3: To expand the tiled area, move the stage to a new position at an orthogonal extreme, as before. Click on the Select Position button to select the current position as Position 3. The map will display a revised map indicating the expanded number of tiles to be scanned. In this example, a 3 x 3 tile map is now to be acquired.

Click on ‘Start’ to begin acquisition of the tiled scan. If ‘Auto Stitching’ is selected, after acquisition is completed, the images will be automatically stitched together to form a composite image.

Combining Tiling with Stacks

Stacks may be combined with tiles and with averaging. Be sure to check that the stack dimensions are appropriate at all locations of the tile scan.
Alternate Acquisition Modes – Mark and Find

Mark and Find enables you to store multiple locations on your specimen so that you can return to them individually. It functions much as the way you store locations in the X-Y plane using Screen 4 of the Microscope Controller. However, it has the advantage that the stored locations can be employed in a sequential acquisition.

Setting Up a mark and Find List

The window shows an X-Y map, as in Tile Scan. Remember that the X-axis is vertical, as in the Display. Move the stage using the Microscope Controller X-Y controls, observing either via the eyepieces or in Live mode.

At each position, find the appropriate focus. Click on Select Position to store the position. The Position Number window will increment. You may delete the current position using Delete Current Position, or delete all currently-defined positions using the trash can. You may also re-define the current position.

A family of positions can be named and saved using the floppy-disc icon (second from left). The left-most icon enables you to recall a saved list of positions.

Use the pull-down list of position numbers to select a new position. The stage will move to that position and the focus position will be adjusted accordingly.

Combining Tiling with Sequences

When defining a sequence involving changing X-Y position, remember to select the appropriate position number when defining the sequence event.

Combining Tiling with Stacks

A stack acquisition may be defined in a sequence acquisition. The stack may be different for each position in a sequence. If the stack is to be the same, check the Same Stack for All box at the bottom of this window.
Storing Experiments

Click on the Experiments tab to show a list of completed experiments.

Double-click on an experiment to inspect the result or analyze it. Right-click on an experiment or sub-experiment to change its name.

Use the Save button below in the Experiments window to save experiments. You will also be prompted to save unsaved experiments on shutdown.
Using the Experiment Metadata

If you desire to repeat the acquisition parameters of a previous sub-experiment, you can do so by two different methods.

If you are confident that the sub-experiment represents the correct parameters
Load the experiment if it is not already loaded. Click on the sub-experiment.

Select the Apply button at the base of the Experiments window to apply the parameters.

If you wish to check the parameters first
Select the file, and right-click on it. A panel will appear displaying the acquisition parameters. You may scroll down the list of parameters to inspect them.

Click on the “Apply Settings” button at the bottom left of the window to apply them.
Note - Not all parameters will be applied.

These parameters are applied from an image file:

- Laser status, including multiphoton (assuming multiphoton laser is on)
- Multiphoton gain and offset
- All detector settings (including acquisition type for HyDs - Standard vs Photon-Counting)
- Pinhole diameter

These parameters are NOT applied:

- Bit depth (important)
- Format (pixel-by-pixel size)
- Acquisition speed
- Zoom
- Number and type of average
- Stage position
- Z-axis position
- Stack parameters
- Tiling parameters
Process Pane
Quantify Plane
Confocal Operation with Multi-photon

Detector Filters and Barrier Filter

The Super HyD detectors have a 620 nm barrier filter in front of them to protect against reflected IR light. For bandwidth selection, you will need a suitable filter in front of the detector or detectors. Ask us how to set this up.

Start Up

It is not necessary to power down the computer or the microscope when switching from MP_LASER_OFF to MP_LASER_ON operation. However, certain steps must be followed for the correct sequence.

Switch on the Electro-Optical Modulator.

The modulator is ready for use immediately, but does not achieve full modulation depth for about 15 minutes. Switch it on first.

Switch off the Compact Supply Unit at the back of the table.

Switch on the Super-HyD detectors power and cooling unit.

Switch the Compact Supply Unit back on.

Application Start - Select MP_LASER_ON.
**Pinhole**
The pinhole is not in the emission path for the non-descanned detectors, so it can be ignored. Operation of the confocal with multi-photon excitation and descanned detection is ineffective and should be avoided.

**Enabling the MP Laser**
Unlike the semiconductor lasers, the MP laser must be turned on in the *Configuration* pane.

**Setting up the MP Laser**
In the *Acquire* pane, first click MP on. Then click on the ‘+’ button to open the MP laser window.

**The MP Laser Window**
Select the laser wavelength. The laser will take 2-3 minutes to go from ‘CW’, with little or no power, to ‘pulsing’, with full power (2-3 W). To open the shutter, press and hold the shutter button.
Dispersion Compensation

Dispersion compensation of the near-IR laser is set in the GDD pull-down panel. Select the appropriate objective form the table. The default (zero GDD) is adequate for multi-photon operation. The fine tuning slider can be adjusted for special circumstances (e.g., operation in deep tissue).

Setting the NDD Detectors

Starting up in MP_LASER_ON mode will enable the NDD Super HyD detectors. These are controlled using a single gain slider. The NDD detectors are very sensitive. Use low gain and low laser power initially. Operate only in a darkened room. A darkroom cloth is available to mask stray light.

Managing MP Laser Power

The EOM window, which controls MP laser power, has four slider controls.

On the right, the EOM slider must be set to 1 to permit near-IR excitation to reach the specimen. The offset parameter is set by the application as a function of wavelength and should not be modified.
On the left of the window are two controls that are misleadingly labeled. They are, effectively, coarse (left) and fine (right) control of laser intensity at the specimen. The number at the base of the left slider is the excitation wavelength, in nm.

Best practice is initially to set the coarse slider to zero and the fine slider to a low value. Using the Live mode, increment the coarse slider slowly until an image is obtained. Adjust the fine slider to obtain the optimum image.
Shut Down

The order is not important.

Switch off the EOM controller and the Super HyD power supply, if used.
Close the application – you will be prompted to save the files that you want to save. Save to folders on drive D:
Log off the computer.
Switch off the confocal at the power strip.

Don’t forget to restore the Home and Focus levels if you have changed them. Replace the condenser and any non-standard objectives in the drawer.

Ejecting Storage Devices

When ejecting your stage device, be careful not eject the F: drive. This drive is the license dongle that permits operation of the LAS AF software. Ejecting it will prevent other users from using LAS AF.