OPERATION OF THE IMAGING CENTER BIO-RAD MRC-1024 CONFOCAL LASER SCANNING IMAGING SYSTEM

*YOU MUST **SIGN UP** TO USE THE MICROSCOPE OR COMPUTER*
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Before attempting to use the microscope:

- you should read information on fixatives for biological material, on photobleaching and anti-fade agents, and on mounting media so that your samples are well-prepared and artifacts are minimized. See **Confocal Notebook** under the file "Preparation of Sample." It is best to grow specimens on **glass** slides or coverslips.
- you should also know the excitation and emission spectra for your particular fluorochromes so that you can select the proper filter blocks available in the microscope scan head. See **Confocal Notebook** under the file "Selection of Fluorochromes and Selection of Filters." Our Bio-Rad LSCM has **ONE** laser: a 15 mW Krypton/Argon ion laser with emission lines at 488nm, 568 nm and a 647 nm line, so triple labeling is possible.
- you should familiarize yourself with the choices of objectives on the microscope and the meanings of the markings on the objectives (e.g., the magnification, the numerical aperture (NA), the mechanical tube length, the coverslip thickness requirements
- An ***MRC-1024 LASER SCANNING CONFOCAL IMAGING SYSTEM OPERATING MANUAL*** is available on the book shelf to read for more detailed discussions of the microscope operation. Also available is the revised edition of ***HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY*** edited by James B. Pawley for reading on the theory of confocal imaging systems (a newer 2nd edition is available from the UofM Library).

A good introduction to light microscopy is ***OPTIMIZING LIGHT MICROSCOPY FOR BIOLOGICAL AND CLINICAL LABORATORIES*** by Barbara Foster available on book shelf (Sanders personal copy, so please return).

What users should <u>consider</u> purchasing:

Mounting Media

Fluoromount G #17984-25 (Electron Microscopy Sciences, P.O. Box 251, 321 Morris Road, Ft. Washington, PA 19034; Phone 215-646-1566; www.emsdiasum.com)

Vectashield #H-1000 (Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010; Phone 650-697-3600 (10 ml=$45.00 as of 8/2001)

SlowFade Antifade Kit # S-2828 (Molecular Probes, Eugene, OR)

Clear Fingernail Polish (Wet 'n' Wild Clear Nail Protector is recommended—other brands may work but shouldn't contain ethyl acetate/acetone which will lead to loss of fluorescence signal)
Glass Bottom 35 mm Dishes (MatTek Corporation, 200 Homer Ave., Ashland, MA 01721; Phone 508-881-6771; FAX 508-879-1532) (used for viewing live cells/living tissue in a fluid)
Chambered coverglasses (available from Applied Scientific at 650-244-9851 or http://www.appliedsci.com for part #'s AS-4851 one-chamber; AS-4852 two- chambers; AS-4854 four-chambers; AS-4858 eight-chambers) (used for viewing live cells)

NOTE: Plastic Petri dishes are NOT optically clear and can only be used for low magnification (10X, 20X) work. Standard chamber slides will NOT work as they have glass slide bottoms which are too thick to be imaged through.

BEFORE USING THE SYSTEM, YOU MUST CONTACT IMAGING CENTER STAFF FOR A TRAINING SESSION. YOU WILL BE ASSIGNED A USERNAME AND PASSWORD FOR LOGIN.

SWITCHING ON THE MACHINE TO ACQUIRE IMAGES

PRELIMINARY NOTE: *If you want to process (or view) images that you* ***have already acquired*** *in an earlier session, you need only to turn on the computer and monitor. Load LaserSharp program (see RUNNING LASERSHARP SOFTWARE AND LOGGING INTO THE SYSTEM below on this page), then click on Processing (red BioRad) icon. If you then decide to acquire images, turn off computer and monitor and proceed as follows*:

1) If using conventional epi-fluorescence (this is recommended to find areas of the sample of interest), **TURN ON MERCURY (Hg) LAMP FIRST** (SWITCH ON, then press START and hold for 2-3 seconds). The mercury lamp produces electrical surges that could damage the computer systems**. DO NOT TURN ON MERCURY LAMP** while computer is on. **NOTE**: Once starter is switched off, the Hg lamp should be allowed to cool at least 10 minutes before switching on again. Let Hg lamp stabilize (yellow stabilize light will be continuously ON).
2) On the Laser Power Supply to the right of the microscope, **SWITCH LASER POWER SUPPLY ON** by turning **THE KEY** on laser power supply **TO THE RIGHT** (**ON** position). Wait at least 10 minutes before switching on again once off. Do not switch off and on again quickly.
3) SWITCH ON COMPUTER AND MONITOR. Then wait until OS2 software loads and the Launcher window appears.
4) Once Launcher window is available - SWITCH ON SCAN HEAD CONTROLLER (on the floor between the scope and the confocal computer). If Tower Controller is switched off, leave it for least 10 seconds before switching on again.

RUNNING LASERSHARP SOFTWARE AND LOGGING INTO THE SYSTEM

1) To start the LaserSharp software, **DOUBLE CLICK blue BioRad LaserSharp icon** on the Launcher toolbar. System initialization will take about 60 seconds. After starting the software, a **Please Login** box appears**. LOGIN** with your name and password. Click **OK.**

NOTE: *If the system ever appears to have hung up, hold down CRTL and ESCAPE keys for 20 seconds. Click OK to stop application.*

ACQUIRING AN IMAGE

1. **Mount your slide coverslip DOWN** onto the microscope stage. The coverslip and objective lens should be dust and grime free. If you select an oil immersion lens (60x and 100X), place a drop of Nikon immersion oil onto the objective lens. At this point, make sure that your specimen is clean, the nailpolish is dry around the edges of the coverslip and free of oil. Make sure that the specimen has a coverslip. It is best to use a # 1.5 coverslip. If you need to clean the coverslip, use lens cleaner and cotton tipped applicators.
2. **Find a region of the sample for imaging using the conventional epi-fluorescence mercury (Hg) lamp.** There are two levers that allow light from the Hg lamp to reach the eyepieces and thus your eyes. (1) Slide the filter block assembly into the dovetailed slot below the objectives. The filter blocks available are for FITC (blue) and TRITC (green) excitation and emission. **DO NOT LOOK AT THIS LIGHT** for prolonged periods. (2) Move the optical path change-over lever located at the microscope base to the IN position (routes the light to the eyepieces). (3) move the shutter (located to the back of the microscope) for the mercury lamp in to open the light path to the filters. Look through the eyepieces and focus on the fluorescent specimen. Use the X-Y stage motion control knobs to move the microscope stage. For best results, do not expose specimens to the Hg lamp for long periods of time.

   Once an area of interest is chosen, **switch the light path for laser scanning** (e.g., 1) close the mercury lamp shutter, 2) move the lever at the microscope base to the OUT position, 3) completely remove the filter block assembly).

On the LaserSharp—Acquisition menu bar, go to **Methods**. The methods settings are combinations of excitation and detection parameters, each with its own name, and which when opened will set up the instrument for specific fluorochrome or imaging requirements. Click on the **Methods** heading and select one of the methods (e.g., Triple labeling). This will automatically set all the motorized filters appropriate for green/red imaging. If you wish to image any of the signals individually, select **Settings** and check **FITC.** The appropriate PMT and PMT controls for that color will then be displayed in the control panel under Filter Blocks. It is a good idea to start imaging with a low laser intensity (e.g., 3-30%).

1) In the Image Collection panel, set the speed to **Normal**, the collection filter to **Direct**, objective to **match objective used** (best to start with low power), **factor** to **1**, **N** to **0** and **Zoom 1**.

2) Choose **Settings - e.g., FITC** and set Black level to **0**, Gain to **1000** and Iris to **2.0**. Check that one image pane displays Mixer B for **FITC.** By default, the top left pane displays the red image, the top right the green image, and bottom left the blue (far red) image. The right-hand bottom pane shows all three colors simultaneously.

NOTE: ***Iris*** *diaphragm influences 3 properties (1) optical sectioning of Z resolution (2) x-y spatial resolution and (3) amount of light entering detector. A larger iris allows more light to enter the PMT but increase optical section thickness. Consult the chart mounted on the cabinet for the maximum iris opening for each objective.*

Gain *controls the output of the PMT by varying the high voltage input. Increasing the gain will produce brighter images but will also increase noise (noise can be reduced by Kalman averaging).*

Black Level *applies offset voltage to the PMT output (adjust level below which no signal is seen). When set correctly, only those parts of the image not emitting photons are displayed as true black. It is best to set the Black Level before adjusting Gain*.

3) Click on the **Scan** button (**Red Sun icon**) (or press the space bar on the keyboard). Focus to the brightest section of the sample. Adjust the **Gain** until the image is bright enough to see easily. The image will appear green because the Look Up Table (LUT) is representing FITC. When the image is acceptable, stop scanning by clicking on **Scan** button. Select **Settings/Save All**… to save the adjusted FITC setting.

NOTE: *LUT is an electronic relationship between incoming information and information the computer puts out. It is frequently used for modifying contrast and for assigning colors in pseudo-images.*

OPTIMIZING IMAGE COLLECTION

1) A good image uses the full range of intensities between black and white. You can load an LUT called Load **SETCOL** in all panes to make sure that the full range of intensities are being employed in the image. It is best to avoid high laser intensities that will cause bleaching. Typically start with 3% to 30% before continuing, but if your sample can take it, there is nothing wrong with 100%.
2) Click on **Display/Load SETCOL in All Panes** and double-click on SETCOL.LUT. Now the parts which were black in the image will appear green, the parts which were peak white will appear red and the parts in between will be grey. This enables correct adjustments of microscope settings that prevent image saturation and enables full use of the available 256 grey levels.

NOTE*: THE RED AND GREEN COLOR CODING OF THIS LUT HAS NOTHING TO DO WITH THE FLUORESCENCE EMISSION COLOR, BUT IS SOLELY TO ASSIST THE CORRECT ADJUSTMENT OF THE GAIN AND BLACK LEVEL CONTROLS. IT WILL NOT SHOW UP ON THE FINAL IMAGE.*

3) Start scanning.

4) Adjust the **Gain** and **Black Level** controls one at a time, and notice the effect of each control on the amount of green and red displayed in the

image on the screen. Use the **Gain** to adjust the red and the **Black Level** to adjust the green. **Set these two controls until there is a very small amount of each color.** This is the optimum setting for the **Gain** and **Black Level** controls for the current sample. The **Black Level** setting will probably be between 0 and 5. Opening the **Iris** incrementally will send more light into the PMT, but this is because the optical section thickness is increasing. Each objective has an optimum **Iris** setting. Refer to the **Confocal Notebook** under "Objectives" (or refer to the sheet posted onto the lab bench) to determine the optimum **Iris** setting for the objective you are using.

5) **Stop scanning.**
6) If desired, turn off SETCOL by de-select **Display/Load RGB in All Panes**.
7) **Start scanning**. The image on the screen is now exploiting the full intensity range of 256 grey levels between black and white.
8) Clock on the **Kalman** filter in the **Image Collection** panel and notice how the signal/noise improves with each scan.
9) **Stop scanning.**
10) If you want to save the image, select **File/Save As**… and enter a filename for the green image. If you wish to save only the green image into this file, ensure that only Mixer B has a check beside it before clicking on OK. The FITC setting can now be saved by selecting **Settings/Save All**.

OPTICAL SECTIONING (ACQUIRING Z SERIES)

1) It is the size of the confocal iris that sets the limit of the confocal sectioning ability. The optimum setting for the iris diaphragm depends on the lens magnification and its numerical aperture (NA). For a Bio-Rad system mounted on a Nikon microscope, the theoretical optimal iris sizes are as follows:

Objective	NA	Iris (mm)
4x	0.20	1.0
10x	0.45	1.1
20x	0.75	1.4
40x	0.85	1.5
40x	1.3	1.6
60x	1.4	2.0
100x	1.3	3.2

1) **Focus on the sample by conventional epi-fluorescence and find an area of interest** (see Step 2 on page 3).
2) **Switch the microscope back to the confocal light path** (Step 3 on page 3).
3) If using a double labeled specimen, click on **Methods/Red Green double label/FITC**.
4) **Start scanning**. Adjust **Laser Power** (start with 3% or 10%) **Iris** (remember to set what objective you are using in Objective box), **Gain** and **Black Level** to achieve an image of reasonable brightness.
5) **Use the microscope focus** to raise and lower the sample stage. Use the focus motor to do this (focus motor panel), or, if you wish to focus manually, make sure that the motor is switched **OFF** first.
6) **Place the sample in the desired position and orientation on the screen**.
7) **Set the lens magnification** you are using by clicking on the arrows by the **Objective** entry in the **Image Collection** panel. YOU **MUST** SET THE CORRECT OBJECTIVE LENS BEFORE IMAGE COLLECTION.
8) **Check the depth of the interesting part of the specimen as follows:**
9) Start scanning. Focus manually above the top of the region of interest.
10) Turn the focus motor **ON** by clicking on the appropriate selection at the top of the **Focus Motor** panel. Notice that the z position of the current
11) Repeatedly click on the UP arrow of the **Position** adjustment. Watch the position indicator change by the z-step, and hear the motor turning as this occurs. Continue until the first z level of interest is visible on the image monitor.
12) Click on the **Z-START** button to record this position as the starting level.
13) Press the DOWN arrow of the **Position** indicator to advance through the sample. Notice that the indicated value changes.
14) Continue until the last focal level of interest is visible on the monitor, and click on the **Z-STOP** button to record this position as the stopping level. **Stop scanning** immediately.
15) Since all the gains and black levels have already been adjusted, you should now be ready to collect a z-series.
16) **Setting up for simultaneous RGB imaging**
17) Verify that the objective magnification is set correctly.
18) Make sure that the Focus motor is ON.
19) Select **Kalman** filtering and **N** (in the Image Collection panel) to 4-8, 6 is a good start.
20) Select **Collect/ Z Series/Simultaneous Series** from the menu bar. A dialog box will now appear which will show the chosen start and stop positions, the z-step, and the number of sections to be collected based on these values.

21) In the dialog box, click on **Save As…** You will now see an option to save the image produced by Mixers A, B and C. At least one of them must be checked before selecting OK. If you are interested in the green image, choose Mixer B and remove the ticks from Mixers A and C**. Click in the filename box and enter a name.** It is a good idea to relate the name of the z-series file to the sample type, but prefix it with Z.
22) **Click on OK**. You will be returned to the Preview dialog box**; click on OK.** When you are returned to the Z-series dialog box, **click on OK.**
23) **The z-series will now start and be saved automatically**. A Collection Information panel will inform you of the progress of the z-series and will disappear on completion. The sections will be collected and the motor will advance the image plane by the specified z step after each image.

NOTE: A rule of thumb is that not more than 20 sections are generally required for a successful 3-D reconstruction.

VIEWING THE Z-SERIES

The z-series has now been stored on the D:\LS_USER\ yourname directory under the filename that you previously specified. If you want to look at it:

- Click on **3-D** icon to display the LaserSharp Processing menu. The current image will be transferred directly to the screen. If not, you can open a saved file by selecting **File/Open** from the menu bar.
- Double click on the filename you wish to open. The specified set of images will be loaded into memory and appear on the screen. To perform 3D reconstructions or image processing measurements and analysis, see Tutorial 10 in the ***MRC-1024 LASER SCANNING CONFOCAL IMAGING SYSTEM OPERATING MANUAL.***

EXPORTING FILES

This option allows you to save image files in formats other than Bio-Rad's proprietary '.pic' format. Please be aware that files saved in other 'standard' file formats will not contain important calibration and system information that is an integral part of the '.pic' file format**. It is advised that you keep a '.pic' original of any file that you export for future reference.** This option is only available if you already have an image file open.

- Select **Export**… from the **File** menu.
- To export the PIC file in a different format, click on the **File Extension** drop-down menu and select the required format. This can be one of many formats

including BMP, TIF, GIF, or PCX. All files with the chosen extension will appear in the box underneath. The chosen extension also appears in the **Save As Filename**: field. TIF is suggested.

- Click at the beginning of the window and enter a filename for the image.
- Select the sections that you wish to export.
- Press **OK.** To get back to the Acquisition screen, double-click on the "Red Sun" icon in the extreme upper right of the screen.

SHUTTING DOWN

DON'T JUST SWITCH THE COMPUTER OFF.

1) **Always first close the program** by selecting **File/Exit.** Then click **CLOSE LASERSHARP**.
2) When the LaserSharp program has closed, the OS/2 desktop will be displayed. Choose **System Shutdown** from the Launchpad.
3) **Wait until the shutdown confirmation message appears ("SHUTDOWN HAS COMPLETED..IT IS NOW SAFE TO TURN OFF YOUR COMPUTER").**
4) Turn **OFF Tower Controller**.
5) Turn **OFF COMPUTER and MONITOR from the #3 powerstrip**
6) Turn **OFF Laser using keyswitch #2** (turn key counterclockwise to the left). Turn **OFF** Hg lamp.
7) **CLEAN microscope lenses with <u>LENS PAPER</u> only**. If you have used an oil objective, take a piece of lens paper and squirt a drop of Sparkle Glass Cleaner in the center. Place the lens paper on the objective on the wet section, pull the flat lens paper across the objective, moving to a clean (dry) section of the paper. Do NOT scrunch up the lens paper and scrub the objective as this may scratch the objective.
8) Clean up work space. Use Kim Wipes to clean the stage and any other parts of the microscope that might have oil on them.
9) Transfer your files to the Transfer server. Remove them from the local drive.
10) Contact the Imaging Center Staff with any problems or questions.