BIO-RAD *Cell Science Division*

LaserSharp2000 for the MRC1024

Software Tutorials

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1. PREFACE AND WARNINGS

1.1 LaserSharp2000 Software - limited use license conditions

The software described in this manual is supplied under a limited use license agreement.

1.2 Software copyright

The LaserSharp2000 software is the sole and exclusive property of Bio-Rad. The customer undertakes not to copy any part of the software without written permission from Bio-Rad except for a back-up copy for security purposes.

1.3 Software use

The software may only be used on the machine for which it was originally supplied.

1.4 Unauthorized use of software

The customer agrees to protect the software from unauthorized use.

1.5 Software support limitations

The LaserSharp2000 software will only be supported when run on a Bio-Rad recommended computer (at the time of purchase).

1.6 Other products referred to in this manual

Windows NT and Windows 2000 are registered trademarks of Microsoft Corporation.
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Contents:

These tutorials provide an introduction to the practical operation of the MRC-1024 system and describe the various basic manipulations needed to obtain images of high quality.

There is a software overview followed by an example tutorial based on the use of the Krypton/Argon laser with the MRC-1024 system.

The Tutorial Chapter is divided into 14 tutorials, each with exercises which should be worked through sequentially. It is designed to cover the topics considered to be most commonly required.

Please read the overview of LaserSharp software first.

The tutorial is not intended to introduce all the software, since that is covered comprehensively in the manual.

Once the Tutorial has been worked through, you should be confident in the use of the MRC-1024 and familiar with the general capabilities of the LaserSharp 2000 software.

Tutorial 1 – Software Overview

Session 1 Starting up the Confocal System

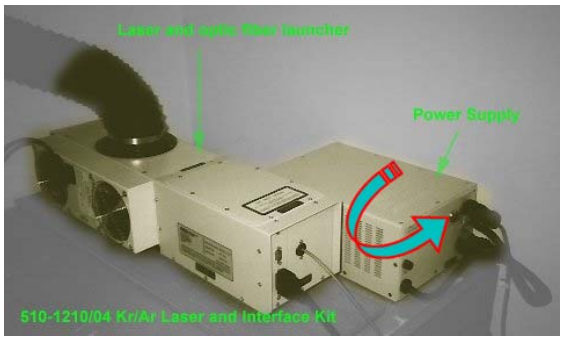
Exercise 1 Switch on.

Make sure that the mains supply is connected to the equipment and switched on

The sequence in which the various parts of the confocal system are switched on is important to the stability of its operation.

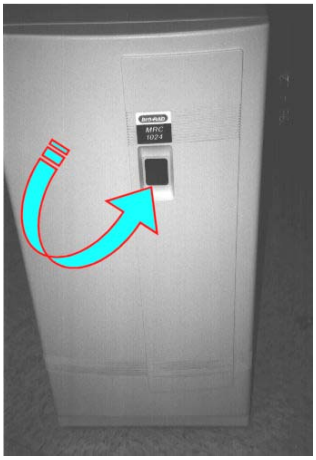
NOTE : If you are going to use epi-fluorescence, FIRST turn on the epi-fluorescence lamp, to prevent power surges.

AFTER the lamp is alight and stable, turn on the **computer and laser**. It does not matter in which order you turn these on. Also turn on the trans-illuminator lamp.



WAIT until the computer fully boots and the **WindowsNT** desktop is displayed.

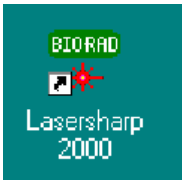
Turn on the **MRC1024 control tower**.



You may now start the Lasersharp2000 software.

Exercise 2 Starting the LaserSharp2000 software

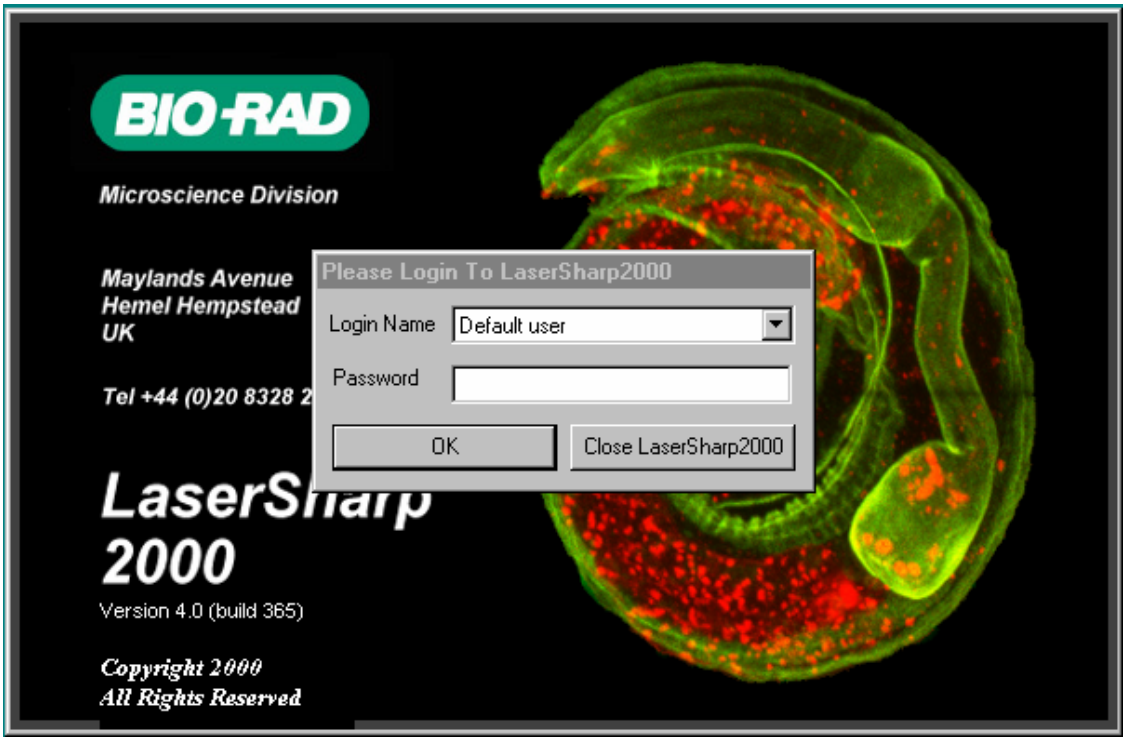
To start the software double click the ***LaserSharp2000*** icon.



USE THIS ICON TO START SYSTEM

Note: ***The Lasersharp 2K No Hardware icon is for emulation or workstation use only and CANNOT be used to acquire images. It is useful to practice software.***

The software will prompt you for your **Login name and password**



NOTE: Lasersharp2000 uses a login system so individual users can customize the system for their needs without reference to any other user. The login is not a secure process as picture files can be accessed from explorer

If are a new user your system administrator or manager will set up and advise you of your login name and password . A GUEST user account may be present for casual users

Once you have successfully logged in, the system will proceed with an initialization phase during which firmware is 'downloaded' to microprocessors throughout the system and system communications are checked. This process will be displayed with a check list with an OK . The final component to be loaded will be the visual subsystem. As a user this process requires no input from you and disregard any noise you hear as hardware initalises , this is normal.

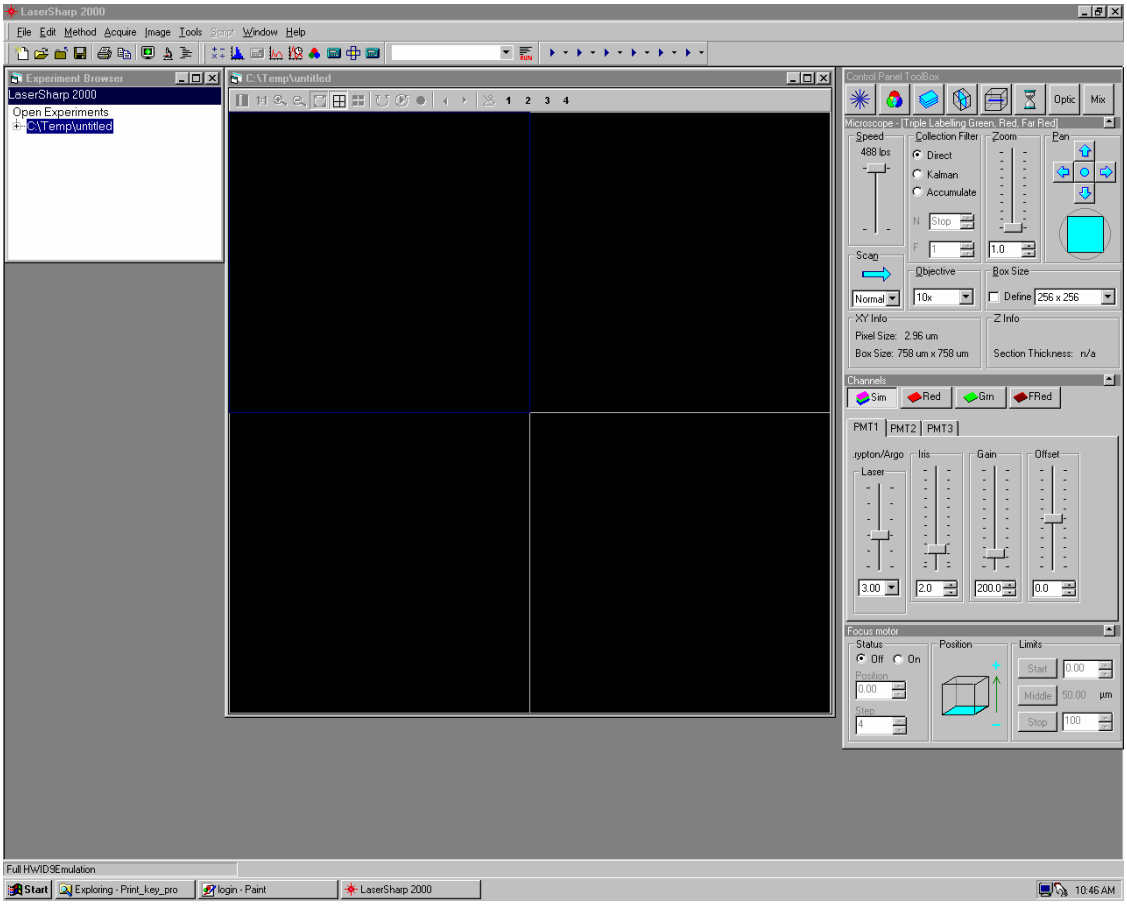
This Process may take some minutes to complete.

If any of the controls read 'failed' the Tower Controller has probably not been switched on. In this case exit LaserSharp, turn on the Tower Controller and start up LaserSharp again.

After this the "**YOUR USER INTERFACE**" will be displayed.

The software is mouse click operated with function from both the right and left mouse. Some features are keyboard driven and these follow the standard windows convention of using shift key and the underlined first letter of a menu command (ie File – shift on F)

Your user interface will be described in the next session.



Summary of Session 1

Session 1 has introduced you to the starting up of the confocal system, the initiation of the Lasersharp 2000NT software. In session 2 you will be shown the closing down procedure.

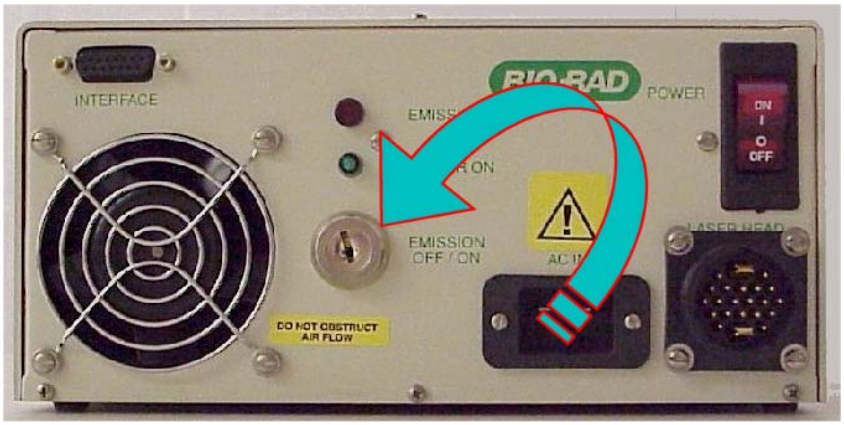
Session 2 Closing Down the System

Exercise 1 Closing Down

To switch the system off,

1. Close LaserSharp software by either selecting **FILE, --- EXIT** or by clicking the X in the top right corner of the program window.
2. **Wait** until the WindowsNT desktop appears (this may take some time), then switch off the system components. The order of switch off does not matter. Ensure the computer is shut down in the Windows NT **START MENU --- SHUTDOWN** etc.

Turn off the laser by the key switch **ONLY. DO NOT** switch it off at the wall or its circuit breaker. The cooling fan will continue to run for a few minutes and then shut down automatically. Only turn on again after the fan has stopped, indicating the laser is at RT.



ALC Power Supply

Summary of Session 2

Session 2 has introduced you to the closing down procedure of the system

Session 3 Test Samples

Three test samples are supplied with the system

1. A sample of paper stained with Safranin. This sample fluoresces over a very large range of wavelengths and also reflects light. It is therefore ideal for initial exploration into image collection
2. A slide bearing five circular areas. Each of the four outside regions contain beads which fluoresce either Blue, Green, Red or Far Red and are excited by UV, blue, green/yellow, and red light respectively. The central region contains a mixture of all four colours. **Note: The blue beads are only excited by UV or MP light and will not fluoresce in a standard system**.
3. A preparation of 210nm fluorescent beads. These are excited by blue light and fluoresce Green. These are close to the limit of resolution of light, and can be only properly imaged using a high NA immersion lens (ie 60X oil) and making use of the hardware zoom of the MRC1024. They provide a test for resolution and vibration.

You should use the paper sample throughout the tutorials and while gaining familiarity with the system. We suggest you use a low power 10X objective lens initially. Although it has a low NA and thicker optical section, it has a long working distance so will protect your slide against cover glass damage while in the early stage of familiarity of microscope use.

Session 4 AN INTRODUCTION TO THE SOFTWARE

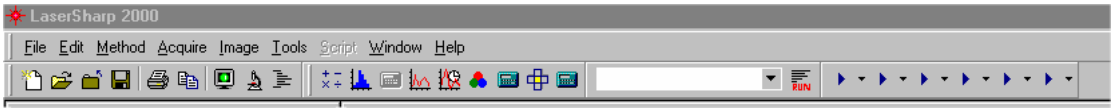
The LaserSharp software runs under the Windows NT4.0TM operating system. It controls image acquisition and provides functionality for 2D and 3D processing and analysis of images and data. There are many functions to the software, we will only discuss features in these tutorials that allow you basic understanding of image collection. The other many features you can explore and refer to in the main manual as you get more familiar

User Interface

The user interface requires a screen resolution of 1280 x 1024 pixels.(set up from within WindowsNT Properties) A feature of the software is that under Windows NT it is possible to run this application with dual monitors (provided that the appropriate graphics card is available).

MAIN MENU AND TOOL BARS

The main menu bar and tool bars will appear in the top left hand corner of the screen. This will allow access to other control menus from the various icons and pull downs. The row of arrow heads in the right hand part of the menu bar is for allowing icons for Visual Basic Script controls to be placed (Visual basic scripting will not be covered in these tutorials)



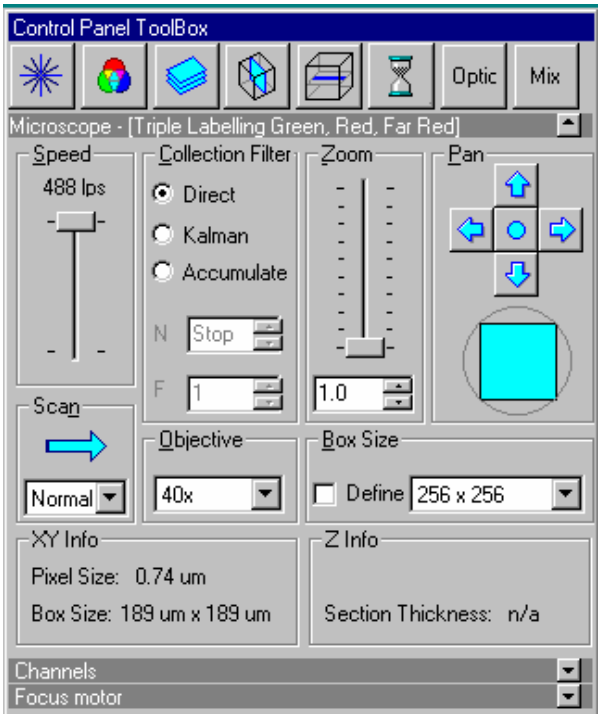
INSTRUMENT CONTROL PANEL

The instrument control panel will be displayed on the right hand side of the screen, but is collapsible and movable.

It is divided into three sections:

1. Top third for confocal instrument control fuctions
2. Middle third for control of laser, PMT and confocal aperture for each channel
3. Bottom third for control of Z stepper motor.

The top portion of the panel controls the scanning, zoom, collection filters, image size and advanced collection control.



The image collection icons are along the top of the panel

 Scan (simultaneous or single image)

 Sequential image collection

 XYZ/T series collection

 XZ collection (cross section)

 X (line) scan

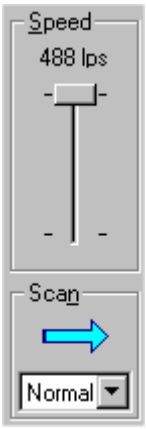
 timecourse.

Optical path set up

channel mixer controls

Scan Speed Control

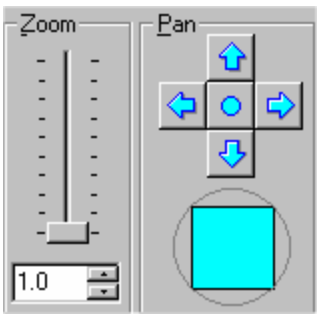
The scan speed selection allows scanning at 166 lines/second (l/s), also known as **slow scan**, 488 l/s **(normal**), 488 x 2 l/s (**aka F2 or fast**), 488 x 4 l/s **(aka F3 or fastest**). Scan arrow indicates scan direction (unidirectional raster scan) only



A Techie Bit: *: In a confocal optical system, the improved resolution of the system is achieved in part by illuminating your sample with a point of light rather then a field of light as in a standard microscope. This point has to be very small (to the limit of diffraction) and intense enough to excite the fluorescence and build an image. For this reason we use a laser as the light source. Since a point of light is 1 dimensional and to see an image we need least 2 dimensions, the light point must be moved or scanned across the field of view. This is achieved in this system (a point scanning microscope) by using two mirrors, one moving in x and the other in Y. The speed of the mirrors and therefore the scanning speed can be varied, hence the speed control. In normal scan the scan path (raster scan) overlaps one scan line with another as it fills the viewing area. The two basic scan speeds 488lps and 156lps are determined electronically by the mirror scan control circuits. Increase speed can be also gained by missing lines in this process. The X2 and X4 functions mean that only every 2nd or 4th line is imaged, giving a faster image rate but with lower resolution.*

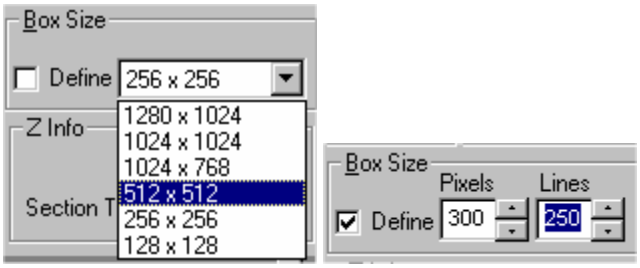
Zoom and Pan

Zoom and pan controls allow zoom on the slider up to 10 times or by back swiping the number below the slider, values up to 999 can be entered. A value of 999 effectively “parks” the beam. Pan control allows the imaged region to be moved over the sample without needing to disturb it. The central button returns the scan position to the center of the field.



BOX Size

Box size controls the size of the collected image display pane on the screen. All of the most popular sizes are represented. The sizes refer to pixel numbers ie 512 pixel X 512 pixel

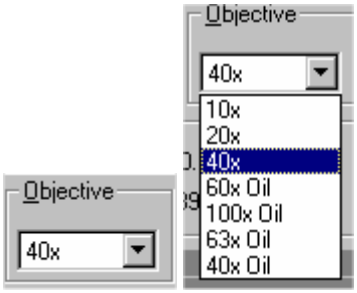


The user can define custom box sizes as well.

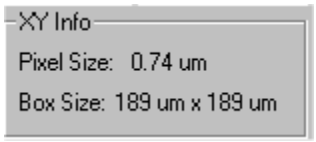
A Techie Bit: *You will notice that pixel sizes are limited say compared against what you may expect with a camera system. This is because in a microscope system there is a physical limit to resolution which is based on the NA of the objective. (ie NA 1.4 with 488nm light has a max res of 0.21um). In collecting the resolution information, we require the maximum pixel size to ideally be half this size of this value (Nyquist Theorem). Going beyond this gives no extra data. The box size and zoom can together allow you to match this physical value, without a data overload. It is easy to give larger display areas, but since they generally over-sample the data, you will end up with bigger files sizes and slower scan rates without any data advantage. Box sizes or display areas of 512 x 512 pixel are quite standard in microscopy. Unfortunately the software will not give you a calculation of the optimal pixel size for the lens resolution, however this information can be found in a table in tutorial 6 of this manual*

Objective Selection

Objective allows the choice of objective lenses as defined in the “tools” pull down menu. Each objective refers to an actual objective on the microscope. Ensure that this software setting matches the actual objective used otherwise magnification calculations and scale bar will be incorrect



XY information is displayed on the panel and is related to the objective used in terms of measurements shown.



This is automatically updated whenever a box size, objective or zoom is altered. The values are all calibrated at system installation and require no adjustments by users.

Collection Filters

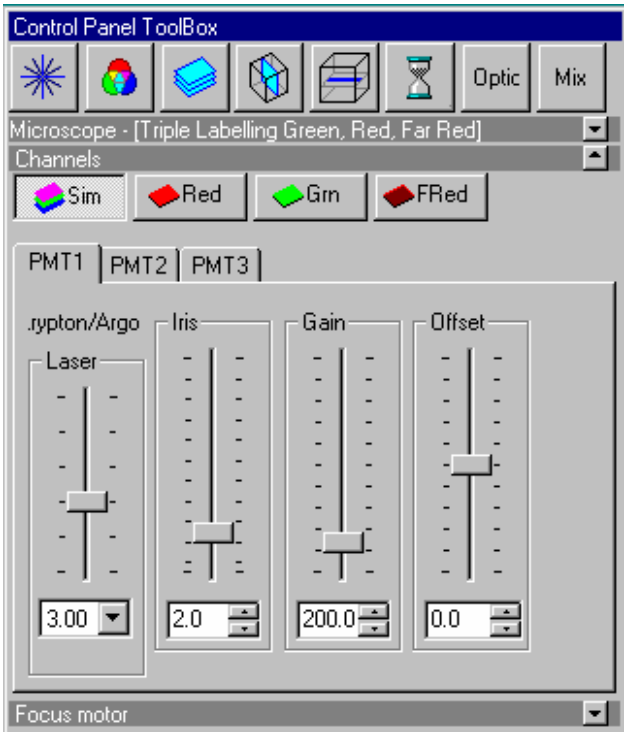
The collection filters are selected on the panel.

Direct is for a raw (un-filtered) display with the image refreshed with each scan cycle
Kalman is for average filtering to increase signal to noise and recommended for general use. N allows input of the number of scans for the kalman filter
Accumulate basically adds images increasing intensity but reducing noise. Used for low signal images

The F factor is not implemented in Lasersharp2000.

Laser, Iris and Detector Control

The center portion controls the **laser power and detector gain, black level (offset) and iris (pinhole) size.**



In Lasersharp 2000 each PMT channel has an independent set of controls for each of laser, iris and PMT functions. The user selects the individual channel tabs to set the image quality for each

channel. The initial settings are loaded with a **method** (see below**)** and in a multi-channel system you can have either **simultaneous** or **sequential** imaging processes

Laser powers can be set to 100%, 30%, 10%, 3%, 1%, 0.3% of full laser power.

A Techie Bit: *The laser with the MRC1024 is a Kr/Ar laser producing three individual wavelengths. 488nm, 568nm and 648nm. Each will produce a maximum power of about 4 mwatts each. The power to the sample is controlled by the use of neutral density filters that reduce the light intensity. In general use, it is important to control this as too much power from the laser will increase the bleach rate of the dye. In using a simultaneous imaging method, the laser power is set to the same for each line used. In sequential imaging the power for each laser can be set for each of the individual PMT detectors. Initial laser setting for imaging should be set to 1% laser power*

Iris settings range continuously from 0.7 mm to 8 mm. This is equivalent to pinhole sizes of 9 microns to about 200 microns.

A Techie Bit: *The iris or CONFOCAL APERATURE is the key component in allowing the confocal system to remove unwanted out of focus information coming back from the sample and thus form blurr free high resolution optical sections. In a confocal system an aperture is placde in the optical path at a point that represents the focus plane of the objective. If it is placed at this point then it will allow only light from the objective focal plane to pass through to the detector. Out of focus light comes from parts of the sample that are not in the focal plane and therefore will not have a focus at the confocal aperture and as such will not pass through to the detector. Since the focus and resolution of the objective will vary based on the wavelength of light, the aperture can be designed so that its size may be varied to optimize for each wavelength. Because each basic spectral group (green, red and far red) is viewed by a separate detector, an aperture is used for each detector. In this way the best resolution can be set for each wavelength emitted from the sample. Also a variable aperture will allow you to balance the colour (or signal) intensity from each channel. Fluorescent dye emissions are not equal, some are strong, some are weak, so by having a aperture for each detection channel , by varying the size of the aperture the intensity of the signal can be controlled and a better colour balance achieved.*

The controls for the iris (aperture) in the software allow control of the iris diameter to achieve these results.

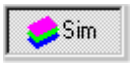
PMT Gain is a direct reading of PMT high tension (voltage) and ranges continuously from 0 to 1500 VDC. – More gain – more brightness but also MORE noise

Offset alters the black level (background) of the image. Increasing offset makes the background brighter, reducing it makes the background darker.

Sequential settings are selected by the sequential setting icons.

These are equivalent to a single fluorophore collection methods, and when selected, the pmt controls above , for that channel are the only controls displayed

Simultaneous setting icon selects simultaneous acquisition.



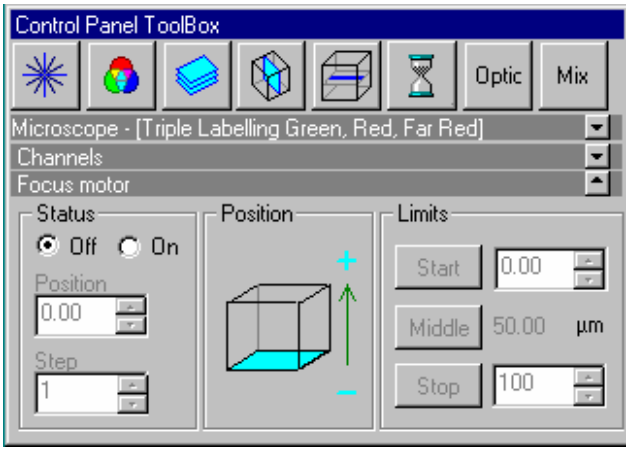
This setting will reveal all possible collection PMT controls at the same time. The control shown in

the main figure above is shown as a simultaneous setting. In this you can see all three possible PMT controls available for modification

The Focus Controls

The lower portion controls the operation of the focus motor.

The **focus motor**, also known as the Z drive couples to and rotates the fine focus knob of the microscope under computer control. The finest step size for the MRC1024 is 100 nm.



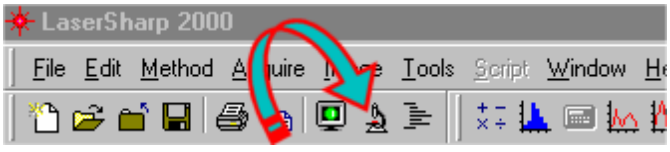
The **status** portion presents the focus motor ON/OFF switch**, Position** shows the current focus position in microns from an arbitrary zero point. **Step** is the distance in microns that the fine focus moves with each advance of the position.

Position icon shows a graphic representation of the focus point in the volume defined in the Limits portion.

The **Limits** portion allows the upper and lower limits (defines the volume) for automatic Z collection to be set. **Middle** allows a middle focus position to be selected to fine tune collection parameters before Z sectioning is started

Displaying the control Panel during imaging

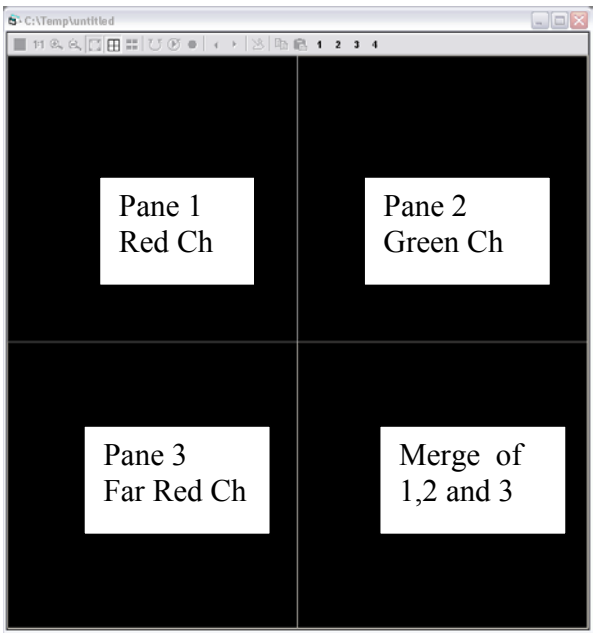
The control panel can be repositioned as desired by **drag and drop** and can be shrunk or expanded by clicking on the size arrows at the top right corner of each portion. The panel can be removed from the desktop by clicking **the microscope icon** on the tool bar. Clicking it again returns the control panel to full size.



To ensure that the window dragging behaves normally set the Display Properties option ‘Show window contents while dragging’ to unchecked. (**To access this dialog right click on the desktop and select Properties**.)

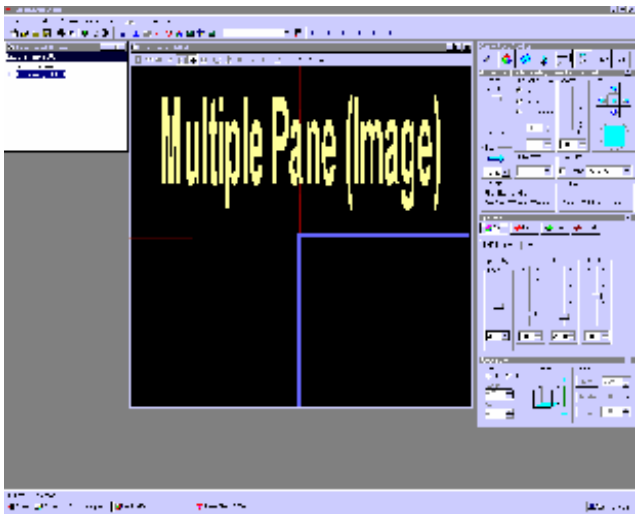
THE IMAGE DISPLAY WINDOWS

It is possible to display up to 4 image acquisition windows at a time. LaserSharp2000 is a unified application. This allows an acquired image to be immediately processed or operated upon.



In the screen shot above, each quadrant represents a display from the Red, Green and Far Red PMT's. The lower right pane represents the merged display of the other three channels. Note that this merge channel is not a "real" image but rather a computer generated image. This merge channel will have limited processing functions.

To display a single collection pane (image), left click once in the pane you want to display. This selects that pane as indicated by a **BLUE** border. Double click the selected pane to display it while hiding all other panes. To return to multiple pane display, double click anywhere in the image. A single pane can also be displayed by selection its pane number in the display box menu bar



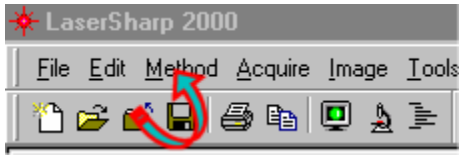
The Display pane's configurations are set up in each of the users methods and usually one pane is set up for each available channel and a another pane is reserved for the Merged or 24 bit image display area.

THE EXPERIMENTAL WORKFLOW IN LASERSHARP 2000

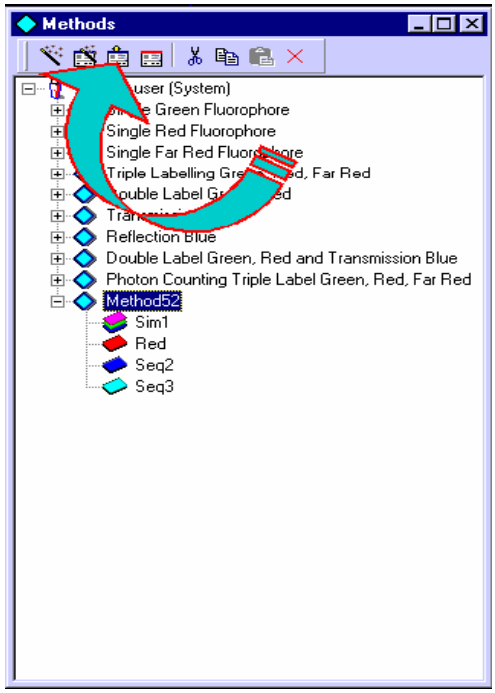
In Lasersharp 2000, the system workflow is based around user defined methods. A Method is a pre stored set up, which when selected will load a set of pre determined operating parameters to the system so that reproducible image conditions can be applied to similar samples. A basic set of general application methods are loaded for each new user. Methods can and are created by users to reflect specific sample requirements and then stored for future use.

Before you can collect an image, you must first load a METHOD.
The last method used in your previous session will be the default Method loaded on your next session

To load a new method ,from the tool bar, select **METHOD**.



Open the **METHODS** menu. Highlight the method you wish to use and **LOAD** it by clicking the load icon.

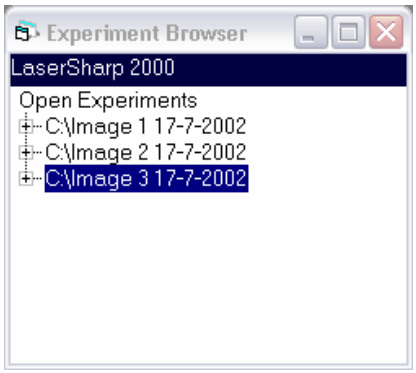


AFTER this you can start a new experiment.

The EXPERIMENT Folder and Browser

Lasersharp2000 users an individual folder to collect each image, image sets or other data for each collection session. In previous systems individual images are filed as individual images, in Lasersharp the folder – called **experiment folder** – will not only contain the collected image but all associated processes carried out on that image (ie measurements, projections, 3D rotations). All processes are automatically saved with the image so no data process is lost for latter review.

All of this data is stored in **the Experiment Folder and the collected folders are displayed in the experiment browser.** Clicking on any of the folder names will reload the image set and associated actions collected at this session for review.



To start a new experiment,(or collect a new image folder) click the "**New Experiment**" icon on the tool bar.

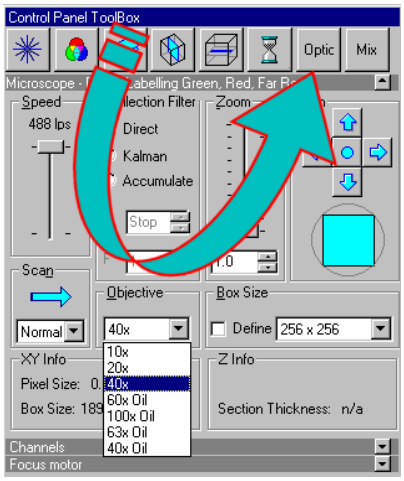
Alternatively, select **New Experiment** from the "**FILE**" pull down menu. This will create a new image display.

There is no need to name the experiment at this time. You will be prompted for an experiment name when you close the experiment**. Lasersharp makes it impossible to inadvertently close an experiment without a file name.**

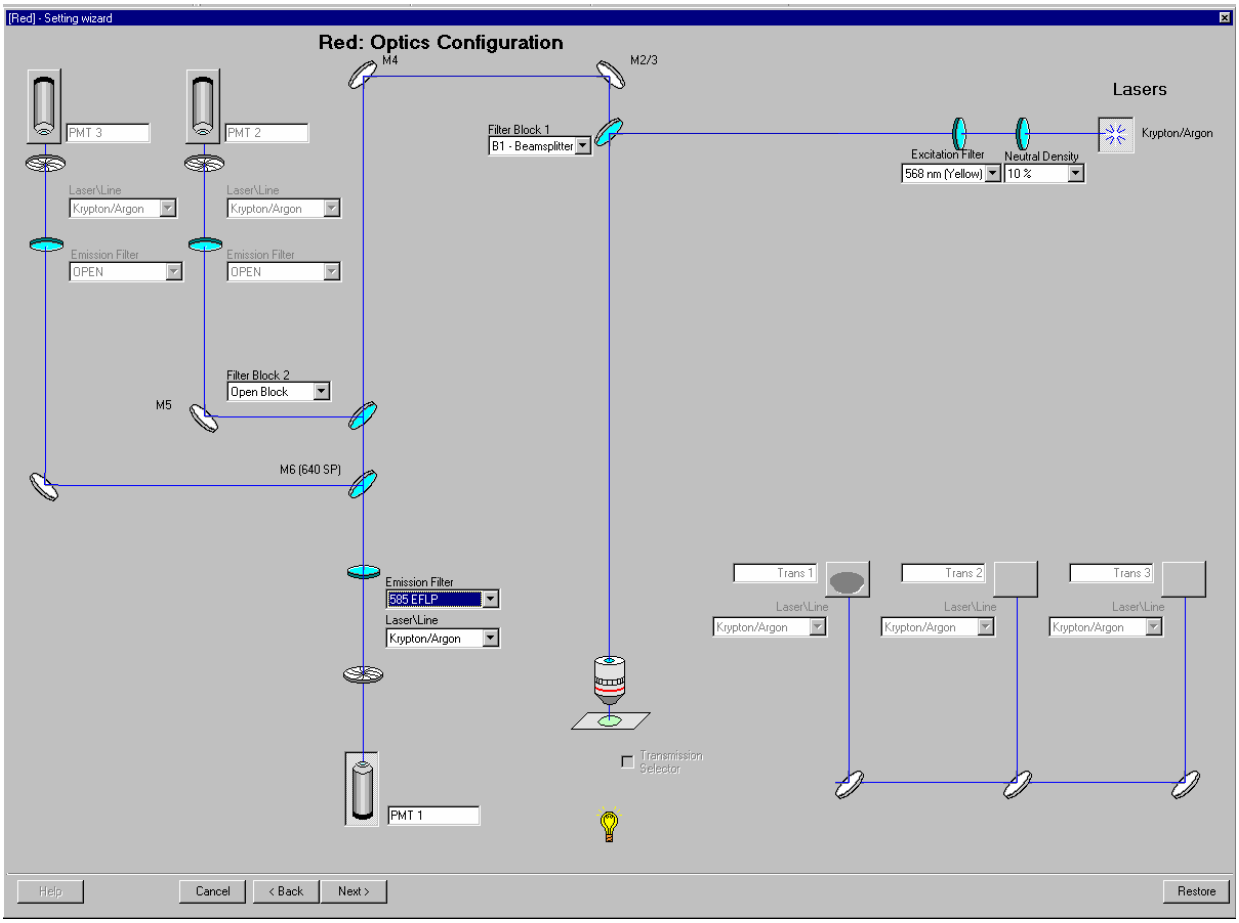
VIEWING AND CHANGING THE SYSTEM OPTICS

Control of the optical system set up is achieved through the **Optics/Filter** setup configuration screen.

To show this screen press the 'Optic' button in the Instrument control panel:



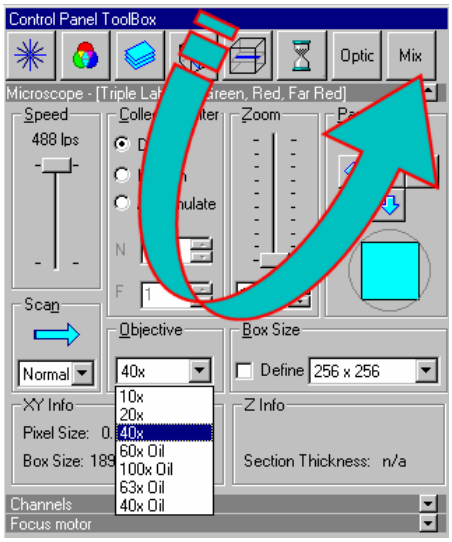
This screen gives a quick graphical view of the system's optical layout and allows you to alter emission filters and laser lines. To make an alteration, simply click the down arrow next to the item you wish to change. This will display the available choices for that item. Highlight the choice and double click. **Alterations in the optics panel can be made at any time, even while scanning**. Changes made to laser power, will be immediately passed to the laser slider in the control panel



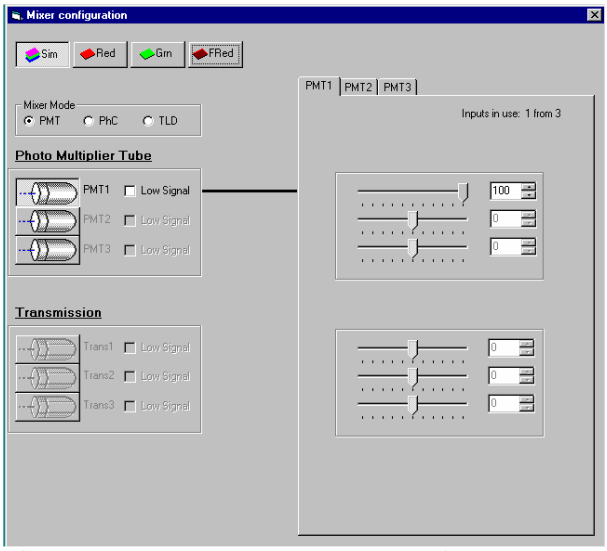
MIXERS (Real Time Spectral Correction)

The concept of allowing the mixing or un-mixing of individual PMT signals into the image display panels was first introduced in the MRC1000 CoMos software. It can also be applied to the Lasersharp2000 software. The mixer is used mainly to control fluorescent bleed through and help better resolve co-localisation signals. In simple terms, each PMT detector should display a “pure” signal into its respective display pane. Bleed through however can be looked at as an “impure” mixture of two signals in the one pane (ie some part of the green signal is being detected by the red PMT). With the mixer, any display pane can show any combination of signal from the available PMT, by adding the signals together in a single pane. In the case of bleed through, since this represents the natural adding of two signals in the one pane a “ pure” signal can be achieved by subtraction of the “unpure” component. The mixers are therefore Spectral analysis devices and can also be used to remove autofluorescence from a signal.

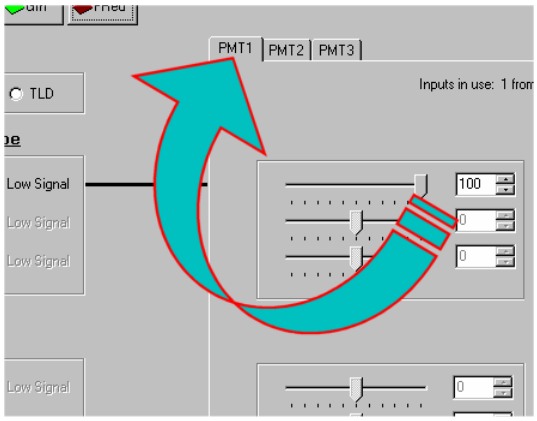
To display the Mixer control dialog press the ‘Mix’ button.



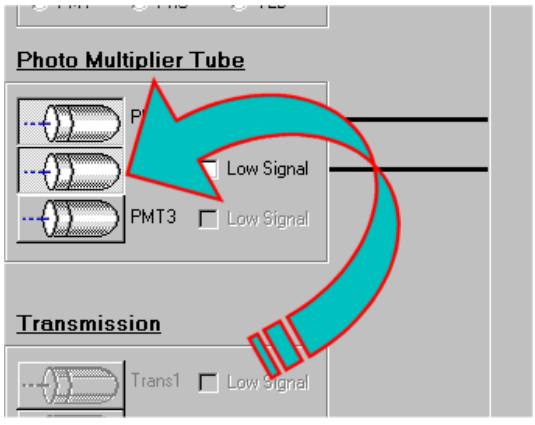
The Mixer controls enable the additive combination of multiple detectors into one data channel.



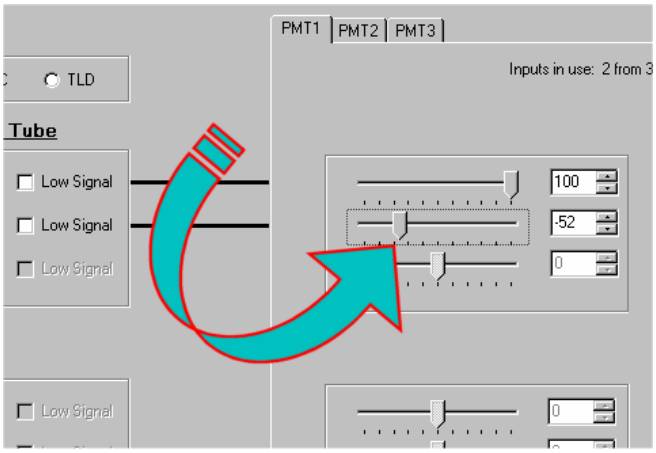
To unmix two images , first select the PMT you wish to modify by selecting the appropriate input.



Next select the PMT you wish to do the modifying.



Finally, adjust the level of interaction by the sliders in the mix panel.



You can observe the effects in the image as it is acquired.

Other features in the **MIX** panel are the switches for **Mixer mode** which selects **Normal PMT** operation, **Photon counting** and **Transmitted Light Detection**. The **Low Signal** switches are also found here. Low Signal is only effective with the Slow Scan Speed,

Summary of Tutorial 1

In this tutorial you have been introduced to the start up and close down procedure for the MRC1024 with Lasersharp 2000 NT software. You have also been introduced to the basic software controls needed to control the image collect parameters of **speed, digital filters, zoom** and **pan**. You have been introduced to the **control panel icon bar**. You have been introduced to the **laser, PMT and iris controls**, as well as the **simultaneous and sequential image** buttons. You have been introduced to the **focus motor**.

The image display window has been explained as well as an introduction to the selection of **methods, optics** and the **mixer.** This basic understanding will help you set up the system to acquire images.

Exercise : Explore all the software features described above to gain familiarity with the controls.

Note : you can explore the software by starting LaserSharp 2000 in EMULATION mode with the Lasersharp no hardware icon on your desktop. This will allow the software to operate with the confocal system being turned on.

You should carefully explore the Methods pulldown, and observe how changing the method changes the setup of the control panel.

Tutorial 2 Getting used to the Control Software

EXERCISE 1 - GETTING USED TO THE CONTROL SOFTWARE

Before collecting an image, familiarize yourself with the way in which the software and image display are controlled. Practice using the No-Hardware Lasersharp Icon.

1 After you log into LaserSharp and it has fully loaded go to the **method** pull down and select a **Triple labeling Method** form the list and select the load icon. When loaded you will see four 256x256-pixel window 'panes'. This is referred to as **'quad' mode**. Go to the Box Size area of the control panel and select **512X512 pixel**

2 Double click on one of the image panes with mouse button 1(left). Now, only the single 512x512 pane is visible.

3 Double-click on this pane and it will be displayed again in the quad mode. Practice this switch a few times.

4 The display is designed to fit the image into the default display size. To expand the display to fit the actual pixel size of the image disconnect the fit to window icon on the image menu bar.

If this button is disconnected then you may see slider bars on the edge of the image. This will allow exploration of the entire image if it is bigger then the display area.

5 Note the row of menu titles along the top of the Lasersharp 2000.

Click mouse button 1 once on a title to drop down the menu. Click on each title in turn (do not worry about menu items for the moment).

6 Do not execute any commands at the moment; a detailed description of all the menu commands is given in full Lasersharp manual or with the contact help information,

7 Place the mouse over the HELP pull down, go to help topics and review a section to give you practice with the online help.

EXERCISE 2 - PRACTICE WITH THE CONTROL PANELS

Using the mouse and the Control Panel Toolbox explore the controls. Try clicking on:

Speed – change the speed slider

Objective – select various objectives from the list.

Collection filter – select direct, kalman and accumulate. Increase the number of scan “N” when using Kalman or accumulate. Return to “STOP” for manual termination of scan

With the “ sim” button depressed, change the laser power slider in PMT 1,2,3

Do the same with the IRIS and GAIN control in each PMT

Select a single colour button, and again change the controls

With the “sim” button depressed carry out a false scan by pressing the scan button  – you will see a test pattern appear in each pane.

Now try with the sequential image button  and observe the result in the merge pane.

Go to the **OPTICS** button – the graphics that is displayed will show the optical path of the MRC1024, At certain points you will see selection boxes. These will allow selection of:

Neutral Density (laser power) – select 10% (if you look at the laser power on the control panel this will have also changed to the same power) – reduce to 3%

Excitation Filter – you will find all lines (default), and single line selection. – select a single colour setting from the top left hand graphics – did the laser line change? – change back to “sim” If you pull down the excitation filters you can see all the options!!

Filter block 1 – tells you what filter block you need for this method – usually T1, you will see for Filter block 2 the T2A again the usual block for this method. Remember you still need to physically insert the correct dichoric filter block into the scan head.

In front of the icon for each PMT you will see the emission filter selection list – look at the options in each pull down. If you select a new filter while imaging you will hear the filter wheel move. PMT 1 has the most filters as it’s the prime detector.

Close this box

Open the **Mix** button - the graphics that is displayed will show the PMT detectors available, on the left, and the panes they can display to on the right. In the “sim” mode you will see PMT 1 is connected to Pane 1 by a thick line. PMT2 is connected to the Pane2 pane etc. In each case 100% of the PMT is displayed in its associated pane. In any pane, any of the other PMT’s can be switched in with slider controlling the signal mix from these PMT’s from +100% to -100% of the signal.

A low signal switch is connected to each PMT to be used with slow scan to improve signal sensitivity.

The Mixer Mode buttons allows selection of the different detector types.

There is an optional non-confocal transmitted light detector (TLD) for brightfield, phase, DIC etc. imaging. When the Transmission button is pressed, the controls for the one or three colours of a transmission image are displayed.

Transmission 1 represents the red transmission.
Transmission 2 represents the green/yellow transmission.
Transmission 3 represents the blue transmission.

The input into each mixer can either be PMT or TLD or Phc.(photon counting) Different mixers can have different outputs, allowing a combination of PMT, TLD and Photon counting at the same time.

EXERCISE 3 - CONTROL PANEL SUMMARY

1 Click in the **Objective** box and select the **60X** lens. Click again and select the **10X**.

2 In the Focus Motor Panel, click on the motor and on the up arrow of the **Position** spin box.

3 Click in the **step box** and change the value to 3 (µm).

4 In the Mix display , ensure the **PMT** button is selected for all Channels
.
5 In the Optic display click on the arrow beside **All Lines** and select **488 (Blue)**. Click on the arrow again and select **All Lines** .

6 Change the PMT1 emission filters and return to 605DF32 (click on **OPEN** to reveal the options). Change the PMT2 emission filter to 522DF35, and PMT3 to 680DF32

7 Change the Laser Power to 30%, then 10%.

8 Select a **Gain** of 1000 for all three PMTs.

.9 Practice with the various different types of control.

Summary of Tutorial 2

This tutorial should enable you to feel comfortable when manipulating the main software components. The next tutorial will introduce you to the test samples provided, and will help you to perform image acquisition and image optimisation.

Tutorial 3 Image Acquisition.

PLEASE NOTE THE FOLLOWING:

 Different fluorochromes need different excitation lines and different emission filters. Different samples require different gain settings on the PMT to display the signal.

 The laser should always be used in 'Normal' mode (not 'Standby' or 'Low') when imaging. If a laser standby kit is fitted, the laser should be set to 'Low' and it will automatically switch to Normal when scanning is in progress.

 Use the lowest laser intensity, i.e. highest neutral density filter possible should be used for fluorescence imaging. (This is normally equivalent to 3% or 10% transmission).

 The filter blocks in the scan head must be appropriate to the sample. If a preset Method is selected, the correct filter block combination is displayed in the OPTIC graphic at position 1 and 2

 As a rule of thumb use the largest numerical aperture objective lens available at any given magnification to achieve the best spatial resolution and optical sectioning. There are special circumstances when this may not be possible due to the lens immersion medium and it's working distance.

 A larger iris (confocal aperture) allows more light to enter the photomultiplier, but reduces the optical sectioning (especially true with higher numerical aperture lenses which are capable of producing very thin optical sections).

 Try to match the refractive index of the specimen/mounting medium to that of the lens immersion medium. Failure to do so will cause distortion of specimen features due to spherical aberrations. If you use the Setup/User/Lens feature, you can enter all the Refractive Index information required for the software to make automatic corrections.

 Avoid saturation (large areas of peak brightness) when collecting an image.

 Always try to use the correct laser and PMT adjustments during image collection rather than process the digitised image afterwards.

 Do not move the laser/fibre assembly once it has been set up by the Bio-Rad installation engineer.

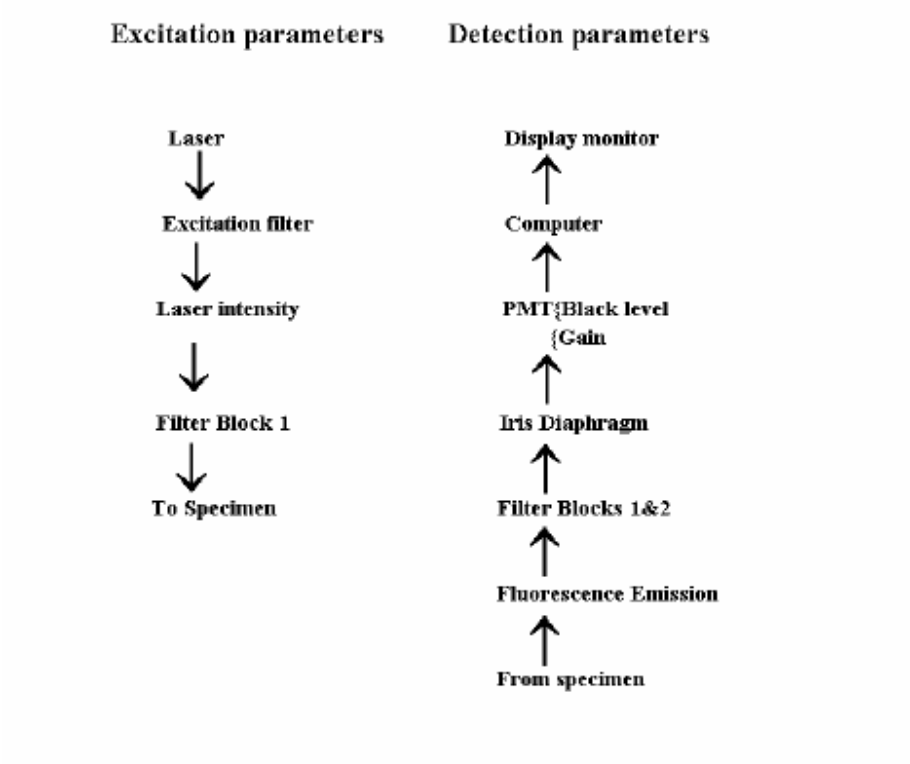
 Safety - ALWAYS stop scanning before removing a filter block from the scan head.
If, for some reason, it is necessary to align or change the filter blocks before use, you should first read the sections on changing filters and optical alignment in Chapter 4 of the MRC1024 Manual.

EXERCISE 1 - ACQUIRING AN IMAGE

1 Mount the paper sample under the microscope and select a low power objective lens, e.g. 10 x Plan Apo. The coverslip and objective lens should be dust and grease free.

2 Find a region of the paper for imaging using the microscope binocular eyepieces and conventional epifluorescence illumination. The epifluorescence image will be parfocal with the confocal fluorescence image. If not, then the scanhead has not been correctly positioned relative to the microscope.

Before starting confocal imaging, it is worthwhile consulting the flow diagram to become familiar with the factors which affect the amount of light incident upon the specimen, and the detection parameters which affect the brightness and confocal section thickness represented on the image screen. You have control over these factors and will be introduced to their manipulation during the course of this tutorial.



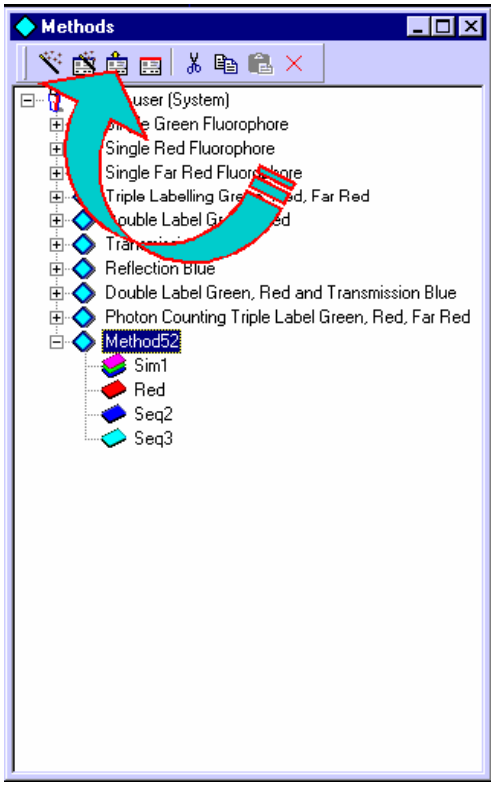
3 Opening the confocal light path. On some systems this is done by swinging the trinocular head to one side (Nikon Optiphot). On others a sliding prism is moved to one side using a push rod or circular click-stop knob (Nikon Diaphot, Zeiss, Leica, Olympus).

4 Make sure the microscope confocal laser light path is open.

5 Make sure the laser power switch (on the side of the laser housing) is in the 'normal' position. To maximize laser longevity, this switch should be left on 'Standby' or 'Low' if the machine is not in use.

Single-Section Acquisition

1. IF not already done, make certain the appropriate Method is loaded for your new experiment. From the method pull down, select a method from the list or open a more extensive list from **Methods….**



Select a triple label method from the method menu and load it. Select the simultaneous setting. Set the speed to 488 l/s and zoom to 1 (one). Select Direct Filter .Choose a 512 x 512 box size.

2. Generally a new experiment image window will open automatically with loading of the method, so in this case you should already see on the screen a box divided into four image panes. If this is not the case then….

3. Open a new image window, by clicking on the New Experiment button  or by selecting **File… New Experiment.**

4. An image window will open. To enlarge the window, grab any corner by clicking and holding with the mouse pointer, and drag the window corner until it is sufficiently large.

5. In the control panel toolbox, you will see three tabs at the top of the PMT controls, one for each of red, green and far red. The controls are located one behind the other. Select each tab in turn and observe each set of controls.

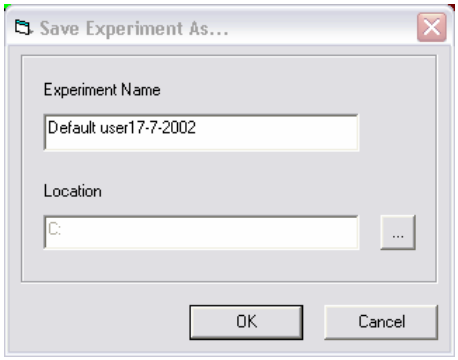
Set the laser power to 3% , set the iris for each colour to 2 mm as a nominal default setting to enhance the chance of getting an image from a average intensity stained sample, and set all gains to 0 (zero). Do not touch the offsets.

Note: If you have your aperture closed too much or even optimal for the wavelength, in a low intensity sample you may not see an image, alternatively having it too open on a brightly stained sample may flood the PMT with light, so 2mm is "reasonable starting point" until you can get a "feel" for the correct setting.

6. Start scanning, by clicking on the Laser button.  The laser will scan until you click the laser button again.

7. Increase the PMT 1 gain until you see an image appear on the monitor. Once achieved, repeat for the PMT 2 and PMT 3. You should now see the merged image in the lower right quadrant of the image pane. Once you stop scanning, the image can be saved by closing down the display box or using the save or save as menus in the file pull down.

A default save name will be supplied if no name is given



8. To collect another section, begin again at step 3.

Sequential Acquisition

1. Select each of the sequential settings in turn and optimize the image for each setting, by following a similar procedure to that in the above exercise. **Freely experiment** to get a feel for the relationship and effect of aperture size, gain and laser power. You will notice that to get a similar intensity result in each channel you may well have a different setting in the aperture size, gain and laser power. All this reflects on the nature of the sample and dye used.

2. Once each channel is set to give a good image Press the sequential acquisition icon. 

3. The images will be sequentially collected. As the last pane is scanned the merged image will appear.

EXERCISE 2 - OPTIMIZING IMAGE COLLECTION

To be performed immediately after Exercise 1.

A good image uses the full range of intensities between black and white (256 in an 8 bit image). The image on the screen will be made up of black, white and grey. In LaserSharp2000 each image pane will be displayed in a colour that represents the colour of the signal sampled, representing the range from BLACK to White. You can load a colour look-up table (LUT) called SETCOL to make sure that the full range of intensities are being employed in the image.

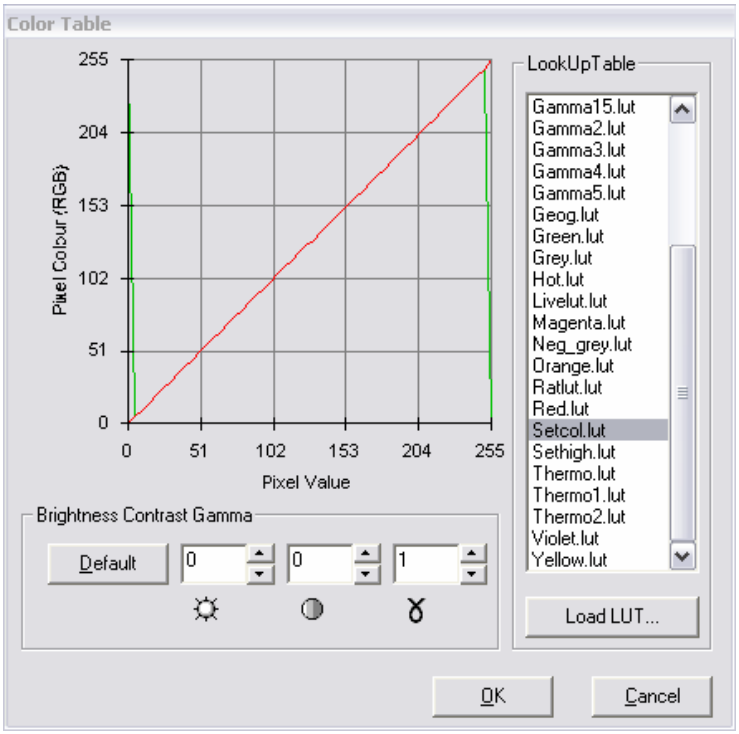
Laser intensity

The intensity of laser illumination will have a major effect on the brightness of the image and bleaching of the sample . It is always best to image with the lowest laser intensity possible with a given specimen. There are six laser intensity positions which are expressed as percentages of total power output and which are controlled by a motorised neutral density filter wheel. High laser intensities will cause stronger fluorescence but increased bleaching of the fluorescent specimen, and in general are not required for adequately prepared biological specimens. They are only used for deliberate photobleaching experiments. Whilst scanning, Move the laser Power slider for different laser intensities and observe the effect on the image. Return to a laser intensity maximum of either 3% or 10% before continuing.

1 Select the **Red Settings icon** in the control panel 

and start scanning. An image should appear in the Red Pane Stop scanning immediately.

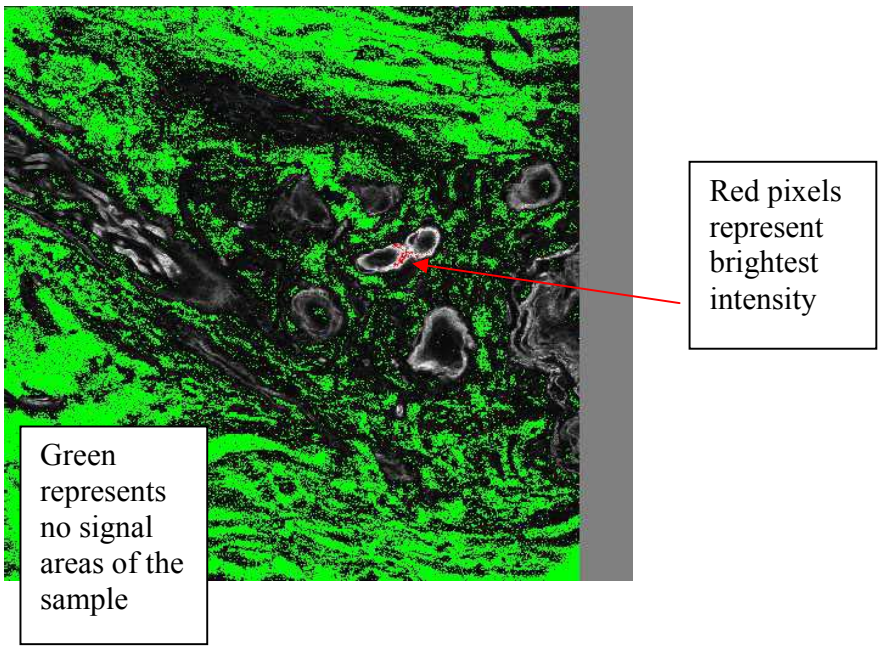
2 Click on **LUT icon** in the image display tool bar  This will open up the LUT selection graphic. From this select the LUT SETCOL and LOAD.



3 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.

Note
The red and green colour coding of this LUT is nothing to do with the fluorescence emission colour, but solely to assist the correct adjustment of the gain and black level controls.

A Techie Bit*: A look up table of LUT is a computer assigned colour table to show intensity as colour. In confocal imaging, colour as we see it is not detected, only fluorescent intensity can be collected. In a digital imaging system the intensity can be collected over the intensity range of the detector (for PMT in MRC1024 this is 8 bit or 256 gray levels) meaning that the intensity in each pixel can be measured from 0 for no intensity to 255 for maximum intensity, and a linear spread between these points. This therefore matches the darkest and brightest regions of the real sample and a ratio of intensities can be made between the two (the real sample and the digital representation) In the LUT Setcol, the table has been composed to allow the user to see pixels with no intensity green) and pixels with maximum intensity (red). Since the real sample will have defined areas of no intensity and maximum intensity, we can accurately defined these areas with the SetCol LUT and in doing so adjust the system to sample the full intensity range of the sample within the available 256 gray levels of the digital display. In practice the intensity can be adjusted by gain and confocal aperture, and the no intensity points selected by the use of the offset or "black level"*



4 Start scanning.

5 You can adjust the PMT controls incrementally

6 We suggest that you initially use the incremental adjustment to prevent the sensitive PMTs from sudden voltage increase.

7 Adjust the **Gain** and **Offset** 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.

8 Use the **Gain** to adjust the red and the **Offset** to adjust the green. Set these two controls until there is a very small amount of each colour within the focused paper fibres (note the empty gaps between them which should contain a lot of green anyway - note that with the fluorescent bead sample, the beads are more or less uniformly fluorescent, so there will not be any green showing within the beads, only between them). This is the optimum setting for the **Gain** and **Offset** controls for the current sample. The **Offset** setting will probably be between 0 and 5.

*N.B. You can enter intermediate **Gain** and **offset** settings by clicking on the integer window and entering another value, then pressing **<Enter>**.*

9 Open the **Iris** incrementally and notice that as it is opened, more light enters the PMT and the area of red pixel will increase, reducing the iris setting will increase the amount of green pixels as overall intensity of the image decreases. Set the iris to a point where only a few red pixels are observed.

***A Techie Bit**: In opening and closing the confocal aperture you are changing the confocal sectioning effect. In opening the iris, this is reducing the confocal effect which allows more out of focus information (light) to enter the detector and therefore the intensity will increase. Because the confocal effect is being diminished the resolution of the sample will also be decreasing. In closing the confocal aperture the image will get darker as less light is being collected from the focal plane and more out of focus information is being rejected. The result is that the image will show more resolution albeit at the expense of light intensity. Is there then an "ideal" setting for the confocal aperture? The answer is yes and this setting is related to a size that will just allow the full plane of focus of the lens to be collected. The Plane of focus or "optical plane or section" is related to the Numerical Aperture(NA) of the lens and the wavelength of the illumination. As a lens approaches the maximum NA then the optical plane gets thinner and thinner and the confocal aperture needs to be closed more to ensure rejection of the out of focus light outside of this plane. This is why a variable aperture is valuable in a confocal as it can adjust to the optical plane of each lens. So in each case there is an optimal aperture setting to ensure maximum out of focus rejection and hence maximum resolution at that wavelength.*

However not all samples are ideal, or staining reactions and fluorochrome emissions equal so in some samples setting the iris optimal may not produce a "bright" result, so opening the aperture more , reduces resolution but increase signal and allows a easier identification. At anytime the optimal setting can be set by pressing  *however the result may not give you what you need to see.*

10 Stop scanning.

11 To turn off SETCOL go back to the LUT Graphic and select RED from the list and LOAD.

12 Start scanning. The image on the screen is now exploiting the full intensity range of 256 gray levels.

13 Click on the **Kalman** filter in the Image Collection panel and notice how the signal/noise improves with each scan.

A Techie Bit: You will see your image improve at this step, because you are improving ***signal to noise*** *in the image. If you look a direct scan you will see that besides the image you see a speckle like background, this is noise. Fluorescent imaging is always low intensity imaging compared to bright field, so in any single short exposure not enough signal is being received to build a complete image. You can try to cure this by increasing the gain to collect more signal, but this will also increase the noise as well. Increasing the laser power will help but increases sample bleaching; opening the aperture will help but reduces the confocal resolution, so we increase image quality by collecting over time. You experience this with normal photography; a dull subject requires longer exposure. In a confocal if we collect over a longer time we average more signal and reduce the random noise. Kalman is an averaging filter that will collect a number of frames (based on time) and average the result which you see on the screen. Kalman is a "rolling" average so you see the result as you collect, the image improving the longer you sample.(within sensible limits of course 8-10 scans usually ample)*

14 Stop scanning. When the red image is satisfactory, you may wish to save it by going to FILE…Save Experiment AS…

16 Select **Green Icon** 

and repeat as above before.

17 Select **Far Red Icon** 

, start repeat as above.

18 The same process can be carried out for the "SIM" setting, but each display pane will need to be set to SETCOL individually.

Summary of Tutorial 3

In Tutorial 3, you have seen how to separately and simultaneously detect red, green and far red fluorescence by employing a pre-set-up 'Method' supplied with the software. By manipulating the gain and offset in each PMT and using the SETCOL LUT, the full dynamic range of gray scales can be exploited. Opening the iris increases the amount of light detected but reduces optical sectioning. Kalman averaging can be used to reduce random noise from the image.

The next tutorial will introduce you to the functions of the digital mixers, and enable you to perform on-line bleed through correction between detectors.

TUTORIAL 4 - Manipulating the Mixer

EXERCISE 1 - USING THE MIXER

1 Place the multicolour bead sample on the microscope stage, and using standard epifluorescence and 20 X lens, focus on the central multicolour bead region in a position where both red and green 15mm beads are in the field of view - carefully change to a 60 X oil immersion lens and refocus.

2 Select **Methods … Methods… and select a Triple Label method** In the control panel on the right hand side of the screen choose **Collection Filter** Direct, set n to STOP . In the **OPTIC** graphic recheck All **Lines** and 3% Laser Intensity.

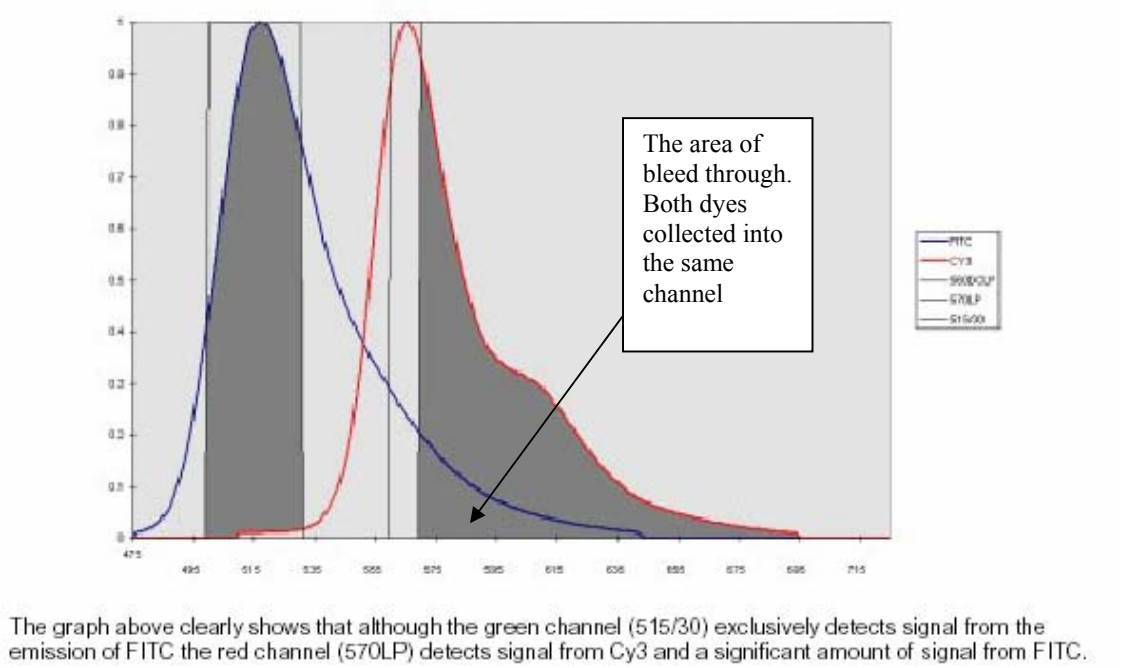
Set the PMT1 **Emission Filter** to 605DF32
Set the PMT2 **Emission Filter** to 522DF32
Set the PMT3 **Emission Filter** to 680DF32 (on 3-PMT systems).

If all checks close OPTIC graphic

4 Click on the **Mix** button in the Control Panel. Check that **PMT 1** shows 100% in Pane 1 **PMT 2** shows 100% Pane 2, **PMT 3** shows 100% Pane 3

5 Open the laser light path and start scanning. Refocus so the beads are round and at their brightest. (You may need to use some zoom to focus on the 1µm diameter far red-emitting beads in the 'blue' pane).

6 Click on the **PMT** button in the Control panel. If, having reduced PMT Gain, the images are still too saturated (there will be streaks across the screen), reduce the laser intensity to 1 % and increase the Gain as necessary. You will notice that the red beads also show up on the 'Blue' channel along with the small far red-emitting beads. This is because the emission spectra of the two bead types overlap. An analogy with real fluorochromes would be, for instance, FITC bleed through into the rhodamine or red detector.



The graph above clearly shows that although the green channel (515/30) exclusively detects signal from the emission of FITC the red channel (570LP) detects signal from Cy3 and a significant amount of signal from FITC.

7 Now, PMT 1 produces the red image, PMT 2 the green image and PMT 3 the Blue image.

8 Referring to the **Mix Graphic** again. N.B. You should still be scanning at this stage
PMT 1displays 100% PMT1 (red), PMT 2 100% PMT2 (green) and PMT 3
100% PMT 3 (blue). Looking at the Blue Pane, you can identify beads that are the same as in the Red Pane (the merged pane will show these beads as purple) Therefore, we can say that there is bleed through from PMT1 (red) into PMT3 (blue), and we need to remove the PMT1 signal from the PMT3 image.

9 While scanning. On Pane3 (select Pane 3 TAB), Turn on PMT 1 by clicking on the PMT Icon (a black line will connect PMT1 to the Pane3 display now showing connection between both PMT 1 and 3. The slider for PMT1 will have gone to 100%) reduce the PMT1 % initially back to ZERO (mid point) and then to more and more negative values until the bleed through is removed from the blue image. Note that the brightness of the small far red beads is not affected. In the merged pane the colours will become more discrete as red green and blue.

10 The same principle can be used for removal of background or autofluorescence. You will need to assess the colour of the unwanted fluorescence, that is, the PMT in which it is appearing, before you can perform the logical Mixer manipulation to remove it.

11 By default each Image Pane displays a different LUT. Pane 1 displays Red, Pane 2 Green and Pane 3 Blue. If desired, new LUTs can be used for imaging, selection from list of LUT's provided.

Summary of tutorial 4

You should now be familiar with the use of the digital mixers and how they can be manipulated to remove bleed through, or to add signals together. If not, continue to make logical changes and observe the effects on the image until you are confident you understand it clearly.

The next tutorial is intended to enable you to set up the instrument without using a preset Method, and then to save these customised settings for future use. Whilst the list of Methods provided is comprehensive, this exercise will permit personal customisation to suit specific samples. It has the added benefit of helping you to understand all the excitation and collection parameters involved, and encourages the development of a logical approach to sample imaging.

TUTORIAL 5 – Methods

EXERCISE 1 - CREATING A NEW METHOD FROM THE METHODS MENU

All operations within Lasersharp2000 are performed through methods.

Methods allow you to set up the system for immediate imaging. You can use methods in four ways.

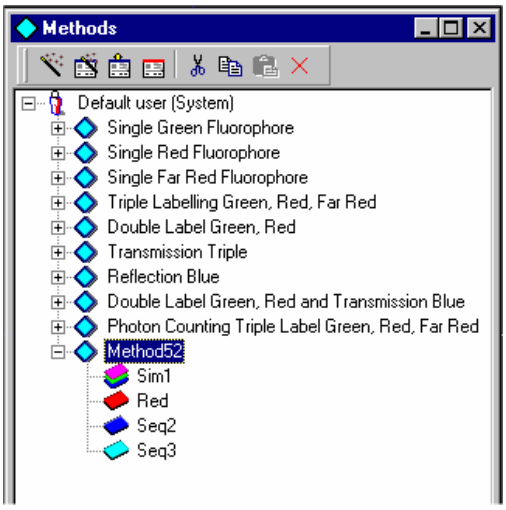
1. Use a factory default method
2. Modify the control panel whilst using a factory default method and resave these settings
3. Crete a unique new method
4. Edit a current method

Each new user is provided with a copy of the factory default methods, and any particular user methods are accessible only by that user. Only the system manager can globally modify users methods.

1. You may wish to create a new and unique method if you have a new procedure or want to have a method that standardises a procedure. In the Menu Bar select **METHOD….Method** This will open the Methods menu.

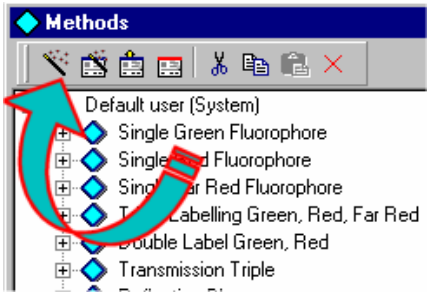
METHODS MENU
This menu contains entries to create a new method, edit existing methods, load methods for use, rename method files, cut and paste and delete methods.

Hint. Method wizards will **NOT** work on methods that are in use. If you wish to edit the method you are currently using, temporarily select any other method. Then editing can proceed as normal.

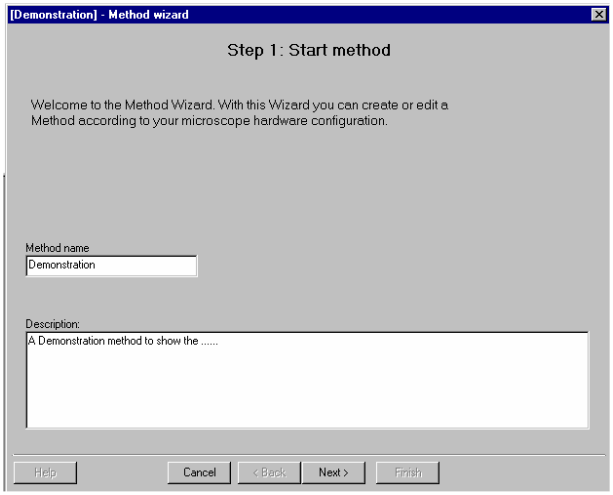


2 Use the METHOD WIZARD to create a new method

The wizard is used to create new methods.

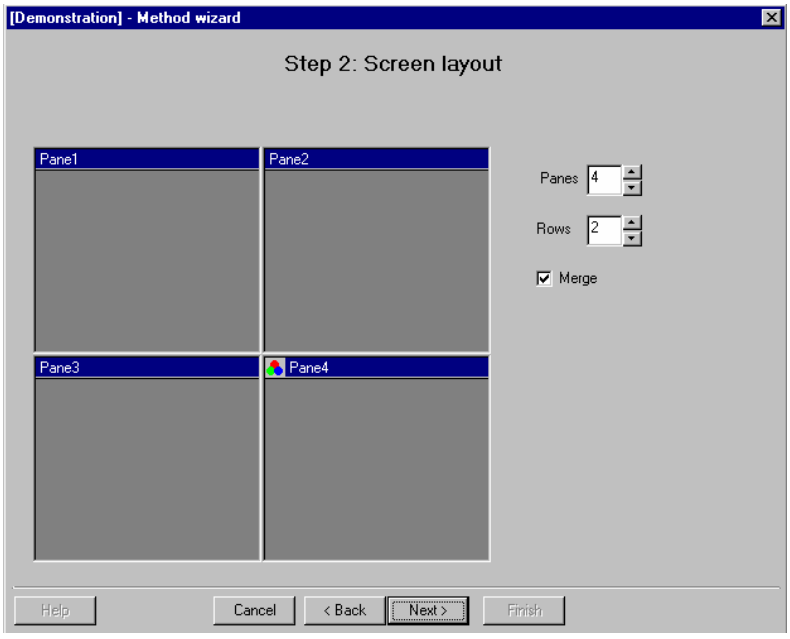


Step One
Enter your chosen Method name and description as desired.



Step Two

Set up the multi-pane layout by choosing the number of panes and the number of rows. Assign one pane only as the merge pane by clicking on the Merge radio button.



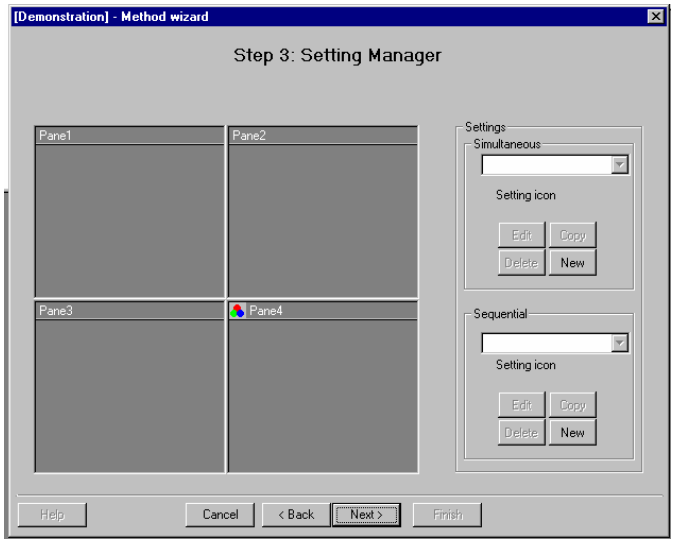
Step Three

The setting manager allows you to create your Simultaneous and Sequential settings (**the number of settings must equal either zero or the number of collection panes. For example, in the figure below, there are 3 collection panes and 1 merge pane therefore, either 0 or 3 sequential settings can be created**) and at least one Simultaneous setting.

FIRST create at least one simultaneous setting.

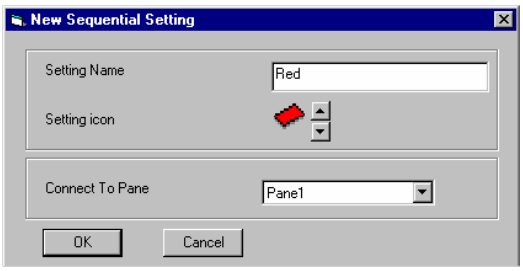
When you press the New Simultaneous button you will be prompted to name the setting and choose an icon for it.

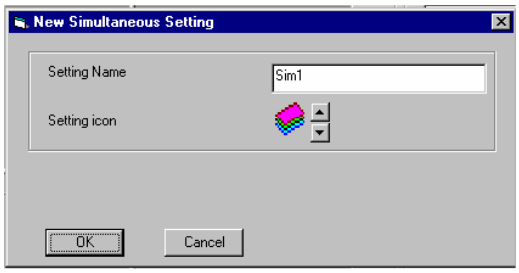
The following steps apply to both simultaneous and sequential setting creation.



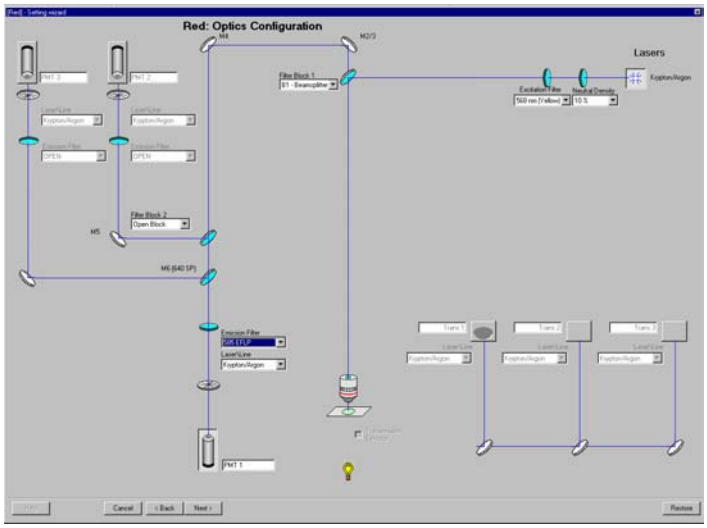
Step Four

Use the Setting Wizard to define the setting name and the colour of the icon that will appear in the control panel.

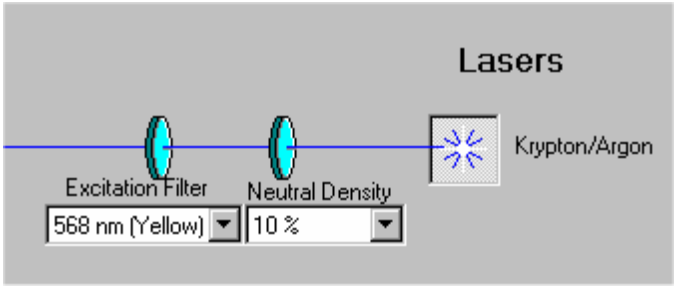




Step Five
Set up the instrument appropriately for the setting you desire in the following sequence:

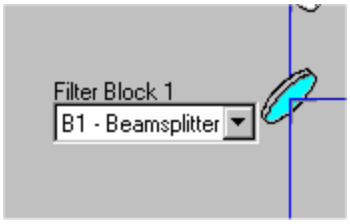


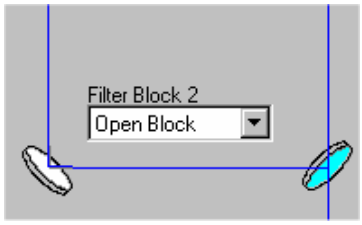
1. Turn on the laser(s) to excite the fluorophore.



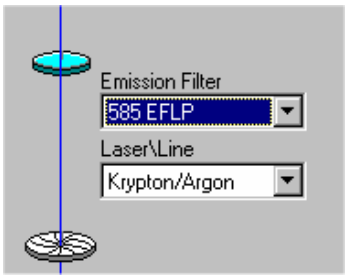
2. Set an appropriate power. This can be altered later. **Hint**, do **not** use 0.3%, choosing 3% or 10% is best. Select an appropriate excitation filter.

3. Select an appropriate filter block pair to reflect the excitation light to the sample and the emission signal(s) into the chosen PMT(s) and turn on that PMT. Edit the name of the PMT as desired.



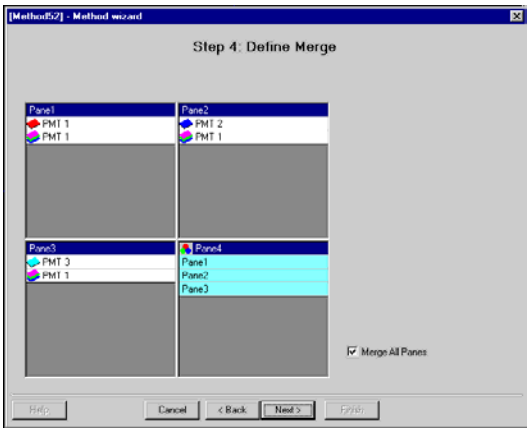


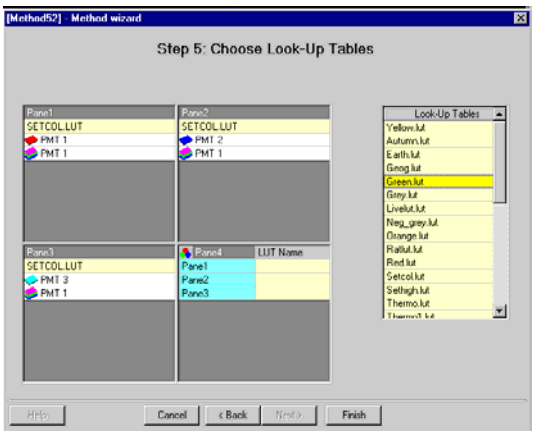
4. Select a suitable emission filter for each PMT fom the drop down.



For each PMT select the laser line which is causing the fluorescence from the drop down.
This operation determines which laser control slider appears in the control panel together with
the gain, iris and offset sliders.

Step Six
To define the contents of the Merge pane drag and drop the chosen panes into the Merge pane or simply click the **Merge all Panes** radio button and then, by drag and drop, apply the look up tables (**LUT**s) to the individual panes and to the merge pane entries.



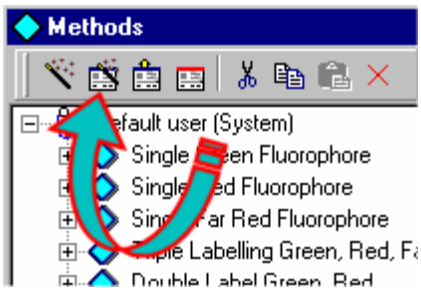


Step Seven
Finish.

Your new method will now appear in the Methods menu and is ready for use. To edit the method use the **EDIT** Wizard and step through to the appropriate stage to make the change.

Edit a pre-existing method

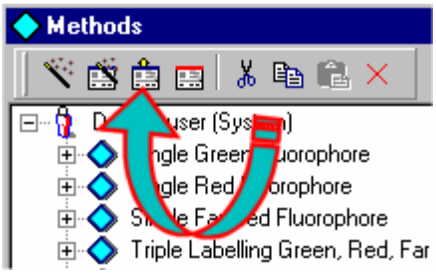
The Edit Methods dialog shows a graphical representation of the methods and their settings on a per user basis. To edit an existing method select the method name in the list and press the **Method Editor Wizard** button.



The Edit Wizard will follow a step process like the new method wizard, in the edit case follow through each screen and make changes to the original method. When finished, the newly edited version will be saved over the original version.

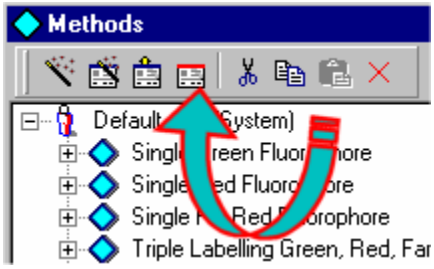
Load (Method)

Load method will load the selected method into the control panel and open a new experiment.



Rename (Method)

Rename opens a dialogue box that allows the renaming of a method file.



Exercise – Save a method

1. Open the Method menu and select a suitable method from the list.
2. Make modification to the laser power, Gain and Iris controls in the control panel created by the method.
3. Go the Method Menu in the Menu Bar of LaserSharp 2000 and select SAVE..
4. Click on SAVE – all control settings and changes will now be saved as part of this method, so on reopening the new control settings will be re-installed.

Note: A reference set of Default methods are kept in the Administrator (manager) file. Each new user gets a copy of this set, but can modify and save over them within the users folder. The managers set can not be generally accessed so can be used as backup if a new default set is required

Summary of Tutorial 5

You should now be able to set up the microscope in an optimal way for your sample, and to be able to open a method that best fits the best imaging for the sample. You can create new methods and edit current methods. In the next tutorial you will learn how to perform a z series.

TUTORIAL 6 - Optical Sectioning

INTRODUCTION

The thickness of an optical section will depend on several factors. Firstly, if the objective lens is low power, e.g. 10 X, it tends to have quite a low numerical aperture, e.g. 0.5. This limits the confocal sectioning ability of the system. However, with a high numerical aperture 60 X 1.4NA lens which has much better resolving power, 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 Optiphot with both epi-fluorescence and DIC attachments, the theoretical optimal iris sizes are as follows:

Objective	NA	Iris (mm)
10 X	0.45	1.1
40 X	1.3	1.6
60 X	1.4	2.0
100 X	1.4	3.4

A Techie Bit: *The optimal iris size is based on a relation between, wavelength, NA and lens magnification and represents the size of the diffraction limited point of light formed at the focus of the lens.*

$$Diameter = \frac{73.2 \times \lambda \times mag_{obj}}{N.A.}$$

This is the point where the depth of focus of the individual lens is optimized by the confocal aperture giving the best optical section for that particular lens with a particular illumination wavelength. In Lasersharp this calculation is made by the use of the  *button. As has been previously mentioned, opening the aperture greater then this will introduce more out of focus light and reduce resolution (especially in the Z direction), closing it will reduce the light intensity but will not increase resolution. In optical sectioning we are trying to collect a continuous volume or depth set, so ideally we want to set the focus step size so it matches the optimal optical section thickness. In this way each section will join continuously to optical section above and below If the iris is set small for best sectioning but the z-step is set very large, there may be empty spaces in the data set; this should be avoided In LaserSharp 2000 at installation the lens parameters will have been set up to give the best Z step based on the NA of the lens. When you choose a lens you will notice the step number change in the Focus Motor control panel.*

EXCERCISE 1 - OPTICAL SECTIONING

You can now investigate the optical sectioning property of the confocal. To do this:

1 Use the fluorescent paper sample and focus on it by conventional epifluorescence.

2 Open the microscope confocal light path, but do not start scanning.

3 Click on **Methods and select a Triple Label method**.

4 It is easier to work in single-pane mode for this. If you are in quad mode, double-click on the top left pane. You should now only see the red image.

5 Start scanning. Adjust the **Gain** to achieve an image of reasonable brightness.

6 Use the microscope focus to raise and lower the sample stage (for the upright microscopes) or the objective turret (inverted microscopes). 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. As the stage is lowered, the image will change and then go dark. At this point all regions of the sample are outside (below) the narrow depth of focus of the optical section. As the sample is raised again, first the top most regions are imaged and then, as the focal plane moves into in the sample, deeper optical sections are visualised.

7 At some level, image quality and intensity, deteriorate partly due to absorption of light in the upper sample regions, but also due to aberrations. The maximum depth in the sample at which images can be produced will vary from sample to sample and with the objective lens used. It is not unusual to section a distance greater than 100 microns into the sample. The maximum depth at which imaging is feasible will increase with good refractive index matching between the lens immersion medium and the sample, as well as the use of a suitable sample 'clearing' agent. It is imperative that you do not try to focus deeper into the sample that is allowed by the working distance of the lens.

8 Take time to explore the various options on the **Image Collection panel of the control panel toolbox** . These control the scan speed, zoom factor and filtering mode. The slow scan speed is approximately 3 seconds per 512 x 512 box and the Normal, 1 second per 512 x512 box. Try changing the scan speed. Very fast collection rates are also available for Live Cell imaging.

9 The zoom factor can be varied from 1 (no zoom) to 10 either in small steps by clicking on the arrows by the zoom slider, by entering a chosen zoom number into this box or by using the mouse to continuously slide the zoom. At higher zoom factors, a smaller area of the sample is illuminated with the same laser intensity. Therefore, bleaching will be greater at high zoom than at low zoom (it increases as the square of the zoom factor). Try changing the zoom factor. Return to zoom 1.0 or 1.5. If you use high illumination intensity and high zoom, you will bleach out a rectangle on your sample. You can visualize this by returning to low zoom.
Each lens has a different resolving power. As the zoom is increased, the resolution can be improved up to a point, but it is only magnification (rather than resolution) which is increased. This is termed 'Empty Magnification'. For the optimum zoom factor for each lens, refer to the following table

Maximum zoom for different lenses

Objective	Pixel size at Zoom 1, Box 1	Theroretical lateral (XY) resolution (488/2*NA)	Maximum useful Zoom
Nikon			
Fluor 10 X/0.5	1.38	0.49	5.6
Fluor 20 X/0.75	0.690	0.33	4.2
Fluor 40 X/0.85	0.345	0.287	2.4
Fluor 40 X/1.3 oil	0.345	0.188	3.6
Fluor 100 X/1.3 oil	0.138	0.188	1.5
Plan Apo 10 X/0.45	1.38	0.54	5.0
Plan Apo 20 X/0.75	0.690	0.33	4.2
Plan Apo 40 X/0.95	0.345	0.26	2.6
Plan Apo 60 X/0.95	0.230	0.26	1.8
Plan Apo 40 X/1.0 oil	0.345	0.24	2.8
Plan Apo 60 X/1.4 oil	0.230	0.17	2.6
Leica			
25 X/0.6 water	0.522	0.41	2.8
50 X/1.0 water	0.276	0.24	2.2

10 If the zoom factor is greater than 1.0, the image may be panned electronically by clicking on the four arrows under the label **Pan** on the **Image Collection** panel(arrow and enter key on Keyboard). This enables the region of interest to be positioned on the screen with more precision than is possible using the manual controls of the microscope stage. Try panning the image. If you are using the slow scan speed, you will have to wait a few seconds before seeing the results.

Notice that the field of view diagram is redrawn to show the position of the image within the objective field. Zooming out to a setting of 1.0, or clicking on the square at the center of the **Pan** arrows, forces the scan to be centered in the field of the instrument. Return to zoom 1.0.

Even if the emitted fluorescence from the sample is dim, the image can be made suitably bright by increasing the gain. In this case the image may be noisy due to statistical variations in intensity from one image point (pixel) to the next. The **Image Collection** panel provides several choices of filters to overcome this random noise by averaging t the signal collected over several frames.

The various averaging modes are described in more detail in MANUAL . You should experiment with them on this simple sample. In general, the best and most useful mode is Kalman filtering . Also, superb low noise images can be accumulated using **Fast photon counting** or **Slow** scan in combination with the **Low SIG mode**.(Found in the Mix graphic)

11 When a satisfactory image is obtained stop scanning.

12 The **Image Collection** panel allows you to limit the total number of scans averaged by any filter. This is the factor **N-scans**. If this restriction is not required, set **N** to STOP and stop scanning when each image is satisfactory.

Exercise 1.1 - Measuring the depth of the sample

Before attempting to image your own sample, use conventional epifluorescence microscopy to make sure that it is in fact fluorescent at a wavelength suited to the laser you have.
To measure the depth of the sample (or the depth through which data collection is desired) proceed as follows:

1 Load **Methods…Triple labeling** and start scanning.

2 Ensure that the image is displayed in quad mode. If not, double-click on the central image to revert to quad mode. Ensure that the box size (from the **Box** menu) is set to 512x512.

3 Select the **Normal** scan speed.

4 Start scanning.

5 Place the sample in the desired position and orientation on the screen.

6 Stop scanning.

7 Set the lens magnification you are using by clicking on the arrows by the **Objective** entry in the **Image Collection** panel. If you are using a non-standard objective, your System Manager will need to add it to the setup configuration if other people are also to have access to it. If this is not a requirement, you can add your own user setup. The magnification value is necessary for subsequent length, area measurements to be correct.

YOU MUST SET THE CORRECT OBJECTIVE LENS BEFORE IMAGE COLLECTION .

In addition, you will have chosen a z-step based on the objective lens numerical aperture. (The recommendations are actually based on the z-sectioning ability of the objective, which is positively correlated with its numerical aperture. The Z-step value should be e.g. about 0.2µm for a 60 X 1.4NA lens and about 5µm for a 10 X 0.5NA lens. The z-step appears in the **Focus Motor** section, of the **Image Collection** panel. Check the depth of the interesting part of the specimen as follows:

Note: *the optimal Z resolution of the objective is a physical value based on wavelength and NA and expressed by the rather complicated expression*

$$R_{ax} = \frac{2\lambda}{n(\sin 2\theta)} = \frac{2\lambda}{n(\sin 2\sin^{-1}(N.A./n))}$$

						Default Z Step, um		
Lens	**Real Mag**	**Immersion Ref Index**	**Coverglass Ref Index**	**Sample Ref index**	**NA**	**488**	**568**	**647**
x1		1	1	1.333	0.05	170	200	255
x4		1	1	1.333	0.2	10	12	14
x10 plan		1	1.525	1.333	0.3	5	6	7
x20 plan apo		1	1.525	1.333	0.75	0.4	0.5	0.6
x40 dry NCG		1	1	1.333	0.95	0.2	0.25	0.3
x40 water		1.333	1	1.333	1.15	0.2	0.23	0.26
x40 oil		1.515	1,525	1.333	1.3	0.2	0.24	0.27
x60 oil		1.515	1,525	1.333	1.4	0.17	0.19	0.22
x60 dry NCG		1	1	1.333	0.85	0.32	0.38	0.48
x63 oil		1.515	1.525	1.333	1.4	0.17	0.19	0.22
x100 oil		1.515	1.525	1.333	1.4	0.17	0.19	0.22

The above table lists suggested default values for the Z step based on the lens type

1 Start scanning. Focus manually above the top of the region of interest.

2 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 imaging plane is now defined to be 0 microns.

3 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.

4 Click on the **START button in the LIMITS BOX** button to record this position as the starting level. Notice that a new diagram showing a stylised representation of a data set now appears in the Position icon box.

5 Press the down arrow of the **Position** indicator to advance through the sample. Notice that the indicated value changes, and at the same time a blue platform will be seen to move within the diagram, indicating the relative depth within the sample.

6 Continue until the last focal level of interest is visible on the monitor, and click on the **STOP** button to record this position as the stopping level. Notice that the diagram is redrawn. Stop scanning immediately.

7 Since all the gains and offsets have already been adjusted, you should now be ready to collect a z-series.

Exercise 1.2 - Performing a z-series

Note
If you anticipate doing many experiments in which a series of optical sections is collected, it is advisable to have a high capacity mass storage device (typically an optical disk), because the hard disk (even with 9 Gbyte) will eventually become full. **Wherever possible, use the hard disk for work space only. Archive images to mass storage devices.**

At this stage it is appropriate to observe the effect of altering the diameter of the iris (confocal aperture).

We began this tutorial with the iris set at a normal default setting from the method . As the iris is opened, the amount of light entering the PMT is increased, but the optical sections become thicker. As the iris is closed, light is collected from thinner and thinner sections of the sample.

1 Start scanning.

2 Gradually open the iris by clicking on the **Iris** slider. The image now becomes brighter, but the optical sections are thicker.

3 Gradually close down the iris in one of the PMTs.

4 You will notice that the image on the screen becomes darker and darker. Adjust the z position now to find the brightest part of the sample.

5 You will notice that although the image is darker, it shows better optical sectioning. Check this by focusing up and down through the sample. Stop scanning.

6 Load the SETCOL LUT

7 Start scanning.

8 Adjust the **Offset and Gain** controls to their optimum as before (the gain will require a different value now that the iris has been closed down).

9 Stop scanning.

10 Remove the SETCOL LUT as before to restore the image to its default colour.

11 Make sure that the binocular head or prism slide is in the correct position for laser scanning.

12 Verify that the objective magnification is set correctly.

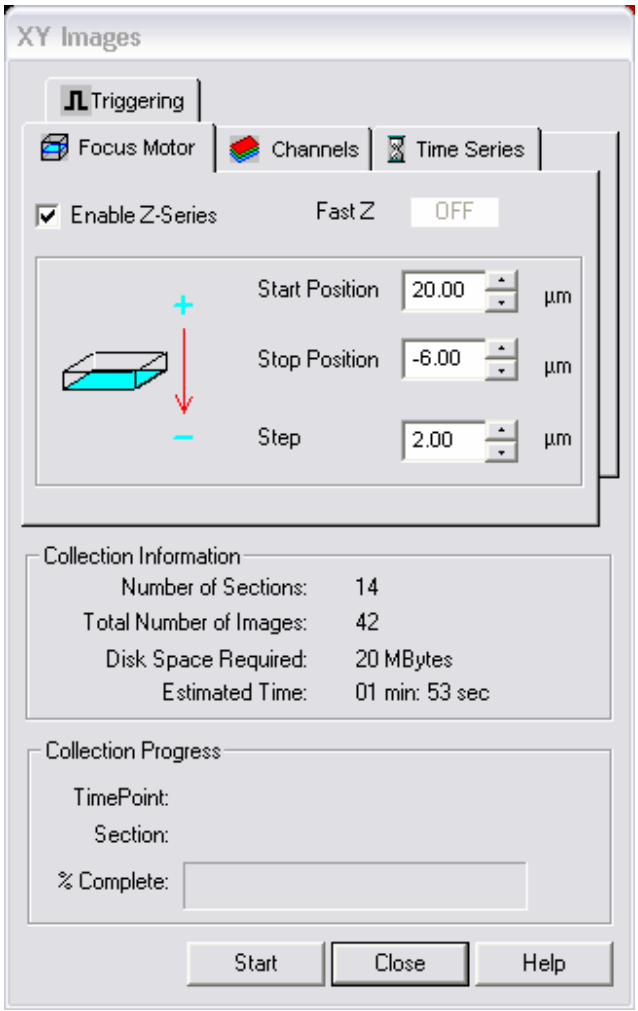
13 Make sure that the Focus motor is ON.

14 Ideally, the images should look quite bright all the way through the specimen. However, sometimes the deeper images are less bright than the surface. If the images are

reasonably bright, or if processing is likely to be required, it may be a good idea to Kalman average each image for, say six scans. So, select **Kalman** filtering and set **N** to 6.

15 Click the Z-series button**,** to open the Z-series control window. 

16 Click the **Enable Z-series** check box. Notice that the **Start** and **Stop** values are automatically entered. From the settings you made in the control panel previously



Note that this box shows the details about the Z series you will collect. In this case you will collect a total of 14 optical sections, and since you are collecting three channels this will make 14x3 =42 images. These will take up 20Mbytes of disk space and take 1min 53 sec to complete

17. Recheck the appropriate **Step**. The finer the step size, more optical sections will be collected. (indicated below the step window).

18. Click **Start** to begin the acquisition.

19 The z-series will now start and be saved automatically. A % complete 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. As the series is collected you will be able to see each image on the screen. If you have set N to 6, the Kalman filter will continue to average each image for six scans.

Click CLOSE to return to the acquisition mode.

When deciding on the number of images to be collected in the z direction, bear in mind that a single 512x512 image requires 266Kb of disk space. The smallest step size likely to be used will be 0.2 or 0.3 microns **(** *based on your default Z step for the lens used*), and then only when using an objective lens of approximately NA 1.4. A rule of thumb is that not more than 20 sections are generally required for a successful 3D reconstruction. There are exceptions to this rule such as when imaging very large neurons which may be as deep as 100 microns. In these circumstances, it is not unheard of to take more than 100 sections. Again, don't forget to check on hard disk space. (The computer will tell you if you don't have enough)

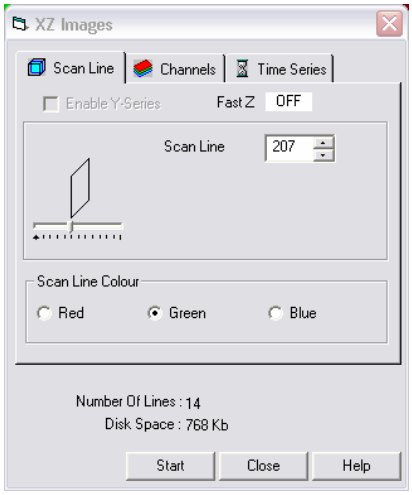
Note : When collecting images, you can collect images into boxes which are a fraction of the size of the full image display screen. If you made a mistake when you were setting up the z-series, or during series collection, you can interrupt the procedure by pressing* STOP *on the Collection Information panel.(The START button changes to STOP when collecting images) All images collected up to this point will have been saved. If you want to write over the abandoned z-series, just close the image display box and select EXIT, and then start again

20 If there is insufficient disk space for the number of sections chosen, a message will appear stating how much disk space you have and how much is required for the z-series you have chosen. If this happens, it is necessary to delete some files or copy them to another storage device to make room for new data. There is a Low disk space warning which occurs during LaserSharp startup which if not seen too, will not let Lasersharp load

Exercise 1.3 - Collecting vertical sections

A vertical section (or XZ section) allows you to choose a line through a feature of interest from your normal XY image. That line is scanned repeatedly as the focus motor is moving thus producing a cross-section profile through the feature. A vertical section can be accomplished by setting **Start** to a focal plane above the feature of interest and setting **Stop** to below the feature of interest.- same procedure as for a Z series. This ensures that the entire feature is sectioned. Kalman averaging can be set in exactly the same way as for a z-series.

1. Set up the image as in Z series collection.
2. Click the XZ acquisition icon. 
3. A control panel will appear.

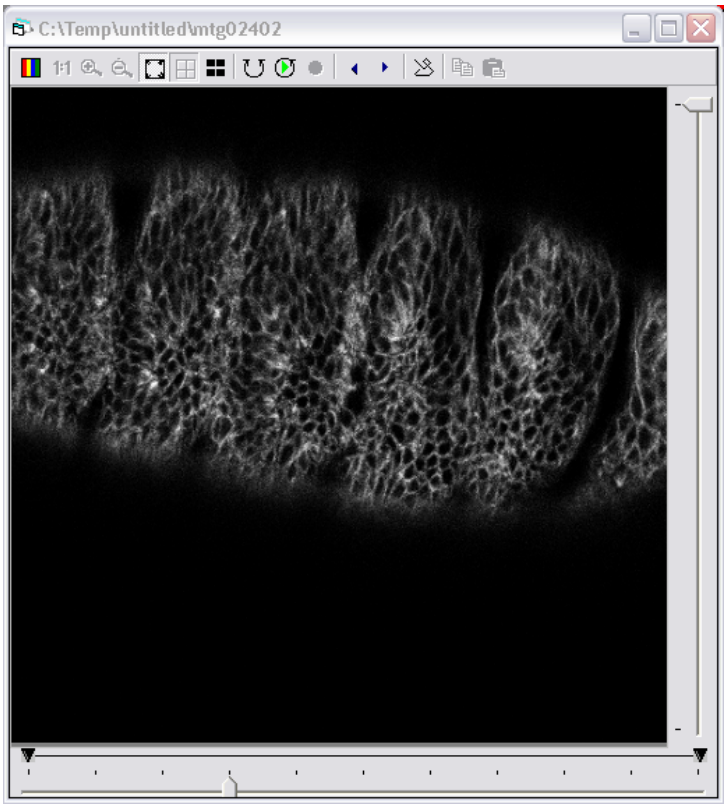


4. Press the up down arrows or move the slider to select which scan line is to be examined.
5. Press START. The image will be automatically collected.

The size of the iris diaphragm is just as important with xz sections as with xy sections.

Exercise 1.4 - Viewing the z-series

Once a z-series has now been collected into memory you can view it



1 Each of the optical sections is stored in the display window, and the slide bar at the bottom of the window shows the position of each section. Each can be displayed in turn by moving the slider to a different position mark.

2 The computer can be used to file through the series by selecting the rotate or loop icon . This will continue until the stop button is pressed. The speed of display can be controlled by the speed setting

Exercise 1.5 – Time Series

Most time course studies will be carried out using the included Time Course Software.
However, it is possible to perform a time series with the standard LaserSharp software.
For very fast imaging, choose the Fastest scan speed and a small box size (or a user defined box). The speed of collection is directly related to the number of lines (in the Y direction) which are collected.

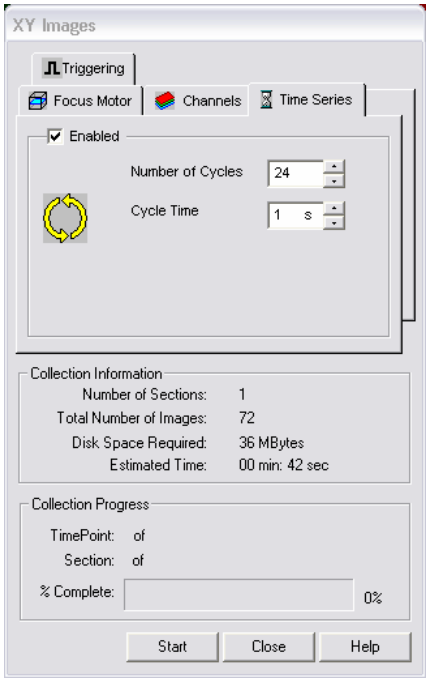
Fast imaging will improve temporal resolution but will severely impair spatial resolution. Signal averaging is normally only suitable for very slow changes.

Focus on the part of the sample you are interested in using conventional means (brightfield or epifluorescence) after loading the physiological probe .

Make sure that the Focus Motor is OFF.

1. Choose **Z series/Time series Icon**

2. Select Time Series in the Display Box and enable the time series (ensure that the focus motor is disabled)



3. Enter the number of cycles or number of time points required.

4. Enter the cycle time for each time point (includes imaging and wait time)
For the fastest imaging, do not set any cycle time. If the changes are very slow, then you do not need such a small box or fast scan speed, and can set the cycle time between images in seconds.

5. Click START to start the time collection, again the button will change to STOP for stopping the process at any time (needs to complete the next cycle)

6. Once complete the Time Series are displayed in a similar way to the Z series, except each image represents a time point. The time series can be played by using the same controls. The series can be saved to you experiment folder by closing down the set and following the instructions

If both a time series and a Z series are carried out together then you will collect a 4D series which will represent Z series sections sampled through time, (XYZT series) These images are displayed in a similar fashion to both a z series or a time series.

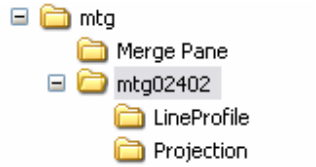
Summary of tutorial 6

In this session you have learned how to perform a z series and a vertical section and a time series.

TUTORIAL 7 - Saving and retrieving images

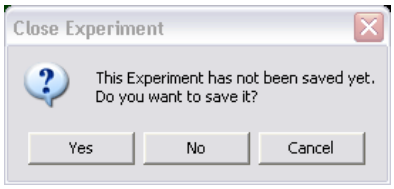
EXERCISE 1 - SAVING AN INDIVIDUAL IMAGE .

You have looked at image saving earlier. In Lasersharp 2000 images are saved into an individual experimental folder which not only contains the image file (.pic) but other associated processes carried out on the image.



In this case the example folder MTG, contains the actual image set, the merge image as well the results of a projection image and a line measurement.

You cannot close down an image display without electing to save or not save.



You can save from a close down or from the **FILE… Save Experiment AS…** menu

1 Click on or select **File…Save Experiment As** from the menu bar of the LaserSharp screen. A dialog box will prompt you for a file name.

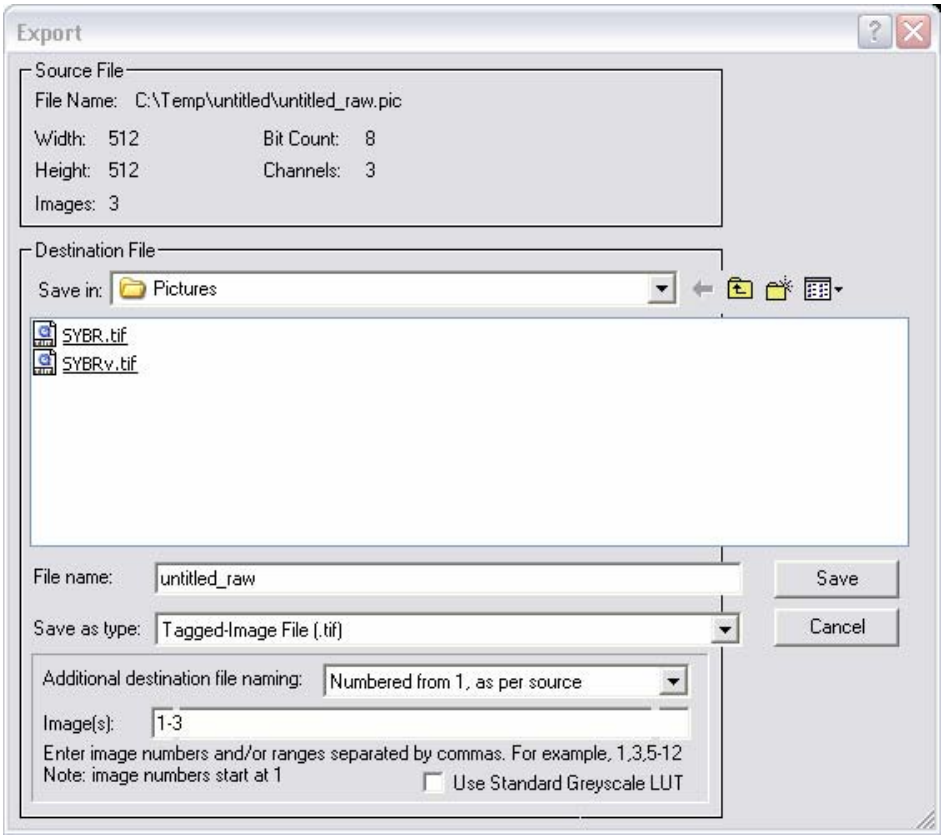
2 Make the filename appropriate to the sample so that you will be able to identify it quickly

3 Click on **OK**.

Images can also be saved to floppy disk or optical disk by using the correct drive and path names.

EXERCISE 2 - EXPORTING A SECTION OR SERIES IN A DIFFERENT FILE FORMAT

1 Click on the image to be exported in the display area with the right mouse click, a menu will appear select EXPORT. Left Click and the Export box will appear.



a whole range of different file formats: PIC, TIF, BMP, AVI, TGA, GIF and PCX and many others are available.

.

2 Select **the destination file**
3 Choose a filename extension from the **File extension** box.(save as type)
4 Choose how to Export the file
5 Click on **SAVE**

Only PIC files can be read in by the Bio-Rad software; you will need other graphics software to read in the other formats.

Hint.

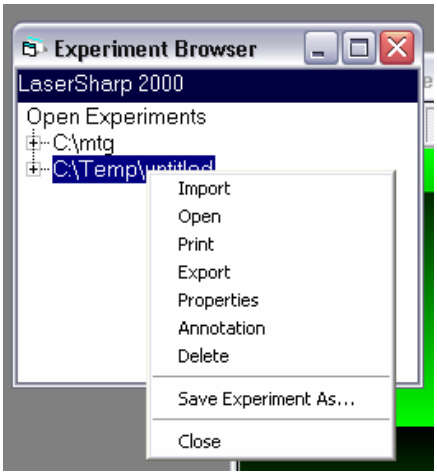
• It is a good idea to create a folder called **TIFs**. Store all your new TIF images in this folder. It will make it easier to find them later. You can create a folder in the same window you use to give the image a new filename by clicking on the new folder button.

• It is also a good habit to create this folder in either your personal folder, or, if you're planning on making several TIFs from the same experiment, in the experiment folder.

- If you're working with a multi-section series, LaserSharp will ask you whether you want to create TIFs from the entire series. Respond either **Yes** or **No**. If you respond yes, LaserSharp will append a 01, 02, 03… suffix to the filename of each TIF it creates.
- In the file menu you will find **BATCH EXPORT**. This allows you to export multiple files to a desired format in a single action.

*EXERCISE 3 - RETRIEVING A SINGLE *.PIC IMAGE*

1 Go to the **Experiment Browse**r and right click on a folder position, This will bring up a menu bar



2 Click on or select **Import** from the menu bar. The files directory will be listed.

3 Click on the desired filename

4 The image will now appear on the image screen. (only works with pic files)

Summary of tutorial 7

In this session, you have learnt how to save images to the hard disk and also to retrieve them. In the next session, you will learn how to produce a 2-D projection of this 3-D data and how to build and animate rotations.

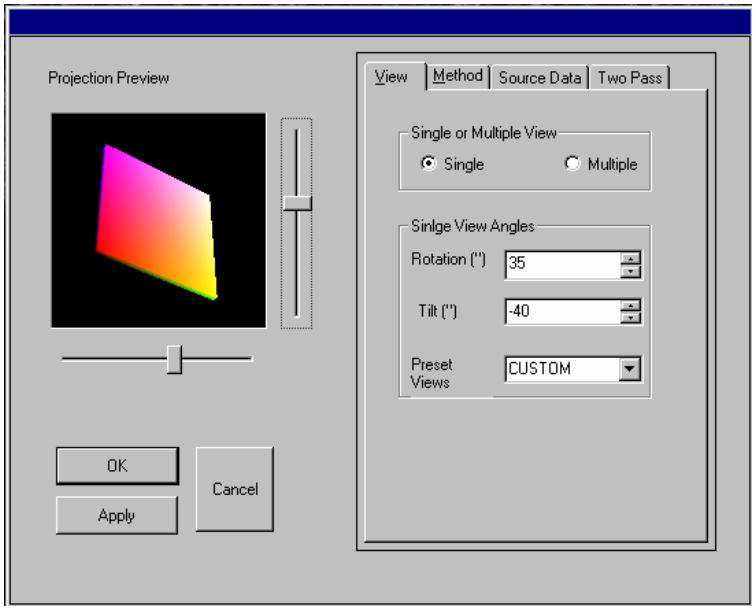
TUTORIAL 8 3D Processing

Exercise 1 Generating a 2D projection

1. Use a newly collected Z series or import a z-series image file.

2. Right-click on one of the individual channel image panes (not the merge pane) in the image window.

3. Select **New… Projection** from the pop-up menu.



This control box will allow all function to generate a 2D or 3D rotation image.

4. Select the View page and select the Single View button.

5. Enter values into the rotation and tilt roll boxes or move sliders beside the image ICON and see how the icon image moves. Preset views can be selected from the choice box. Select Front View.

6. Select **OK or Apply (both are the same) to initiate the BUILDING** of the projected image.

Note: In this process the computer will build a projection of all the individual optical sections added together to give a through focus image of the complete structure. This process is sometimes referred to as an extended depth of focus series. If an angle of view has been introduced then the projection will show the image as if observed from this angle

7. Explore the other tabs but leave the settings at default. These settings are for more advanced processing, and can be experimented with latter.

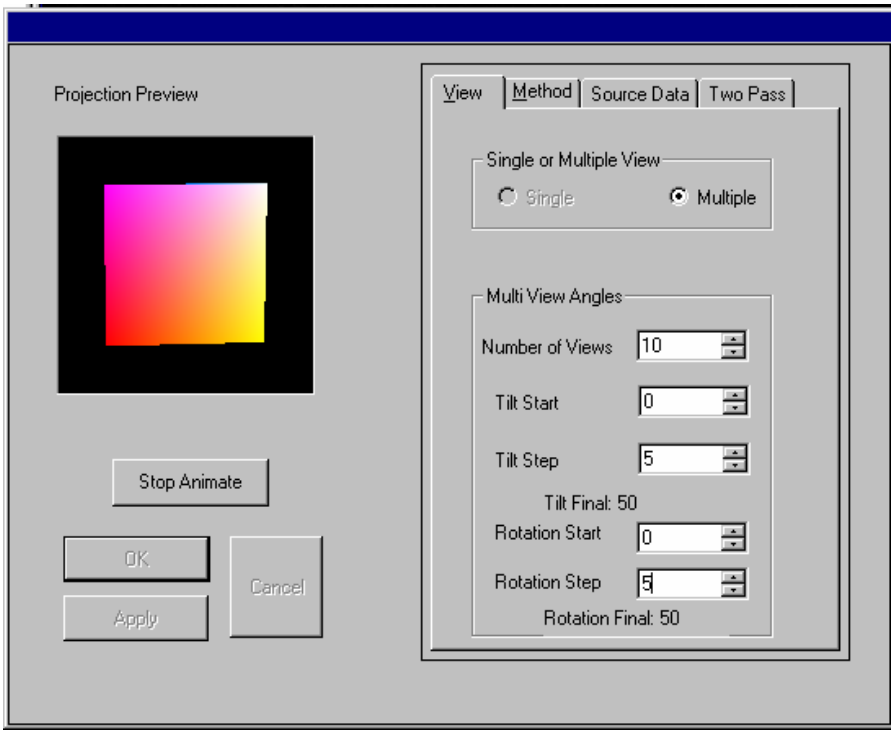
You have now generated a 2D Projection. Experiment with this at different angles, Observe how image resolution changes with angle. – Why should the image quality reduce when looked at in the Z direction ? The answer is in physical optics not the instrumentation,

Exercise 1 Generating a 3D projection and Rotation

1. Use a newly collect Z series or import a z-series image file.

2. Right-click on one of the individual channel image panes (not the merge pane) in the image window.

3. Select **New… Projection** from the pop-up menu.



4. Select the View page and select the Multiple View button.

5. First enter the **number of views** that you want to create and project to build the 3D rotation. These views will be like frames of a movie, each representing a different projection at a different angle of tilt and /or rotation. The more the number of views you can have the smoother will be the rotation, and the greater the rotation degree. Increasing the number of views will however increase computing time.

Tilt Start – this is the starting tilt angle for your rotation – 0 degrees or front view is default – you can go –or +

Tilt Step will the angle between each of the views in relation to tilt. Again the smaller the angle of difference between views the smoother the rotation.

The same will apply for the **Rotation start and steps**

Once all these values are entered you will see that the image icon will rotate showing you the selected 3D rotation of the final image. Changing any of the above values will alter the movement.

6. Select **Stop Animate** when the desired rotation is achieved. Then click **OK or Apply** to BUILD the 3D projection series.

7. Once finished (progress shown by a scale bar) you can start the rotation by using the loop and rock features of the display window previously described.

8. Explore the other tabs but leave the settings at default. These settings are for more advanced processing, and can be experimented with latter.

You have now generated a 3D Projection and rotation. Experiment with different angles, Observe how image resolution changes with 3D rotation angle –is it better than the static 2 D image?

Summary of Tutorial 8

In this session you have learnt the rudiments of 3D reconstruction and animation.

TUTORIAL 9 - Displaying Merged images

INTRODUCTION

Imaging using more than one PMT is most useful with a biological preparation which has been stained with two or more different fluorochromes. These are usually a combination of a Fluorescein-like dye, a rhodamine-like dye and a far red emitting dye, e.g. Fluorescein/Texas Red and CY5. In many cases, the fluorochromes will have been bound to specific antibodies directed against antigens present within the preparation. Therefore, you will be interested in the positional distribution of the fluorochromes in relation to one another.

In order to visualise this, it is useful to merge the images together.

If an image has been saved with the “as a 24-bit, merged pane” ,then **File…Open Experiment** function will open it as such and no merging will be required.

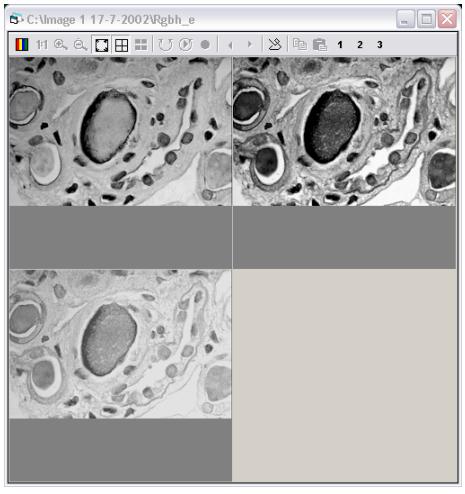
Merge can also be used to differentially colour images taken at particular depths in the specimen or with projected images made from a z-series Merge can also be used to differentiate one morphological component from another, e.g. after a seed fill operation

Note Images or multi-image files can only be merged if they are exactly the same size.

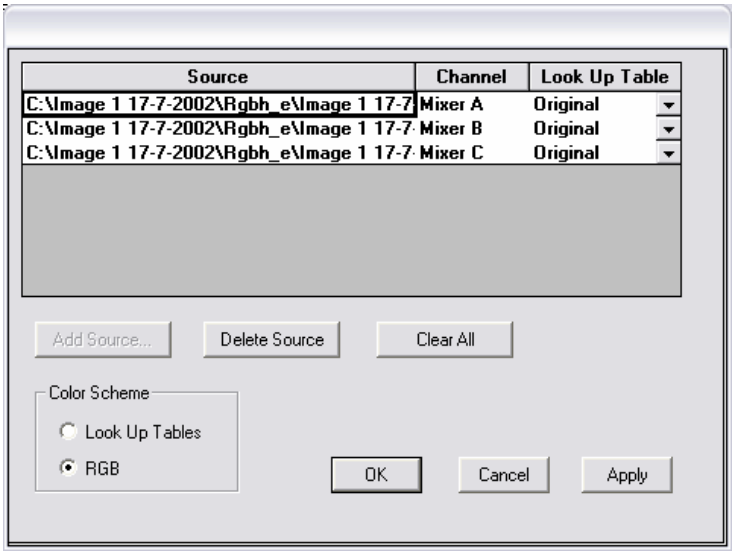
EXERCISE 1 - DIFFERENT MERGES

To merge imported images collected in the multi-channels, collected on earlier systems such as the MRC1024 OS/2

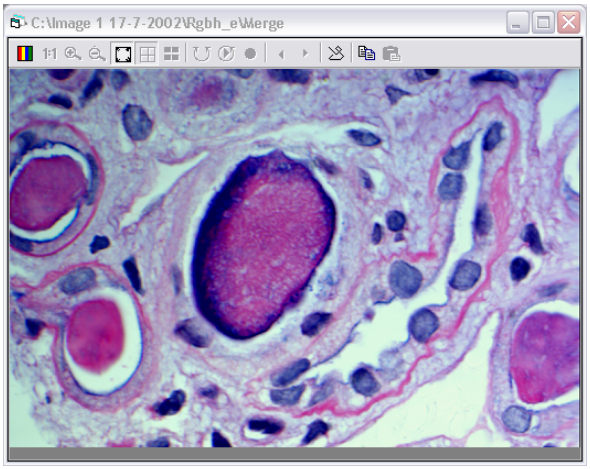
1. Import the file into LaserSharp 2000 using the import function in the Experiment Browser



2 Right click any image of the set and select **NEW…Merge**. This displays the merge control box.



The files should have been automatically loaded into the merge control box. If each image represents a simple R,G.B image, then leave RGB selected, and then click OK
This will then generate a separate merged 24bit image file. This can be saved



If windows contain multi-image files (e.g. z series), they must all be of exactly the same size in order to be merged.

This same procedure can be followed for projections and 3D rotations previous generated with the projection methods.

Note: An automatic merge file is not generated with projections or 3D projections, only the collected files from the individual channels will be shown.

Summary of tutorial 9

Here you have learned how to merge single images from a file and from open windows.
You have also learned how to merge multi-image files.

TUTORIAL 10 - Making measurements on images

EXERCISE 1 - MEASURING LENGTHS

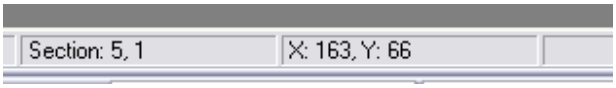
Note
***It is very important that the correct lens magnification was entered when the images were collected in order for the measurements to be correct The lenses specific to your system should already have been entered under* Setup**

1 Import a z-series into the experiment browser e.g. mgt02402

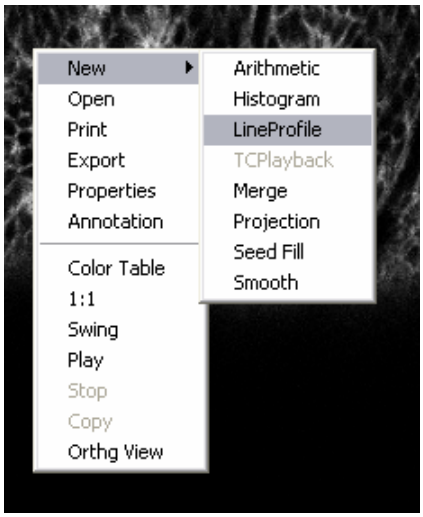
2 Move the series slider to “leaf” through the sections

3 Find a section which contains an interesting structure at which you want to start your measurement .

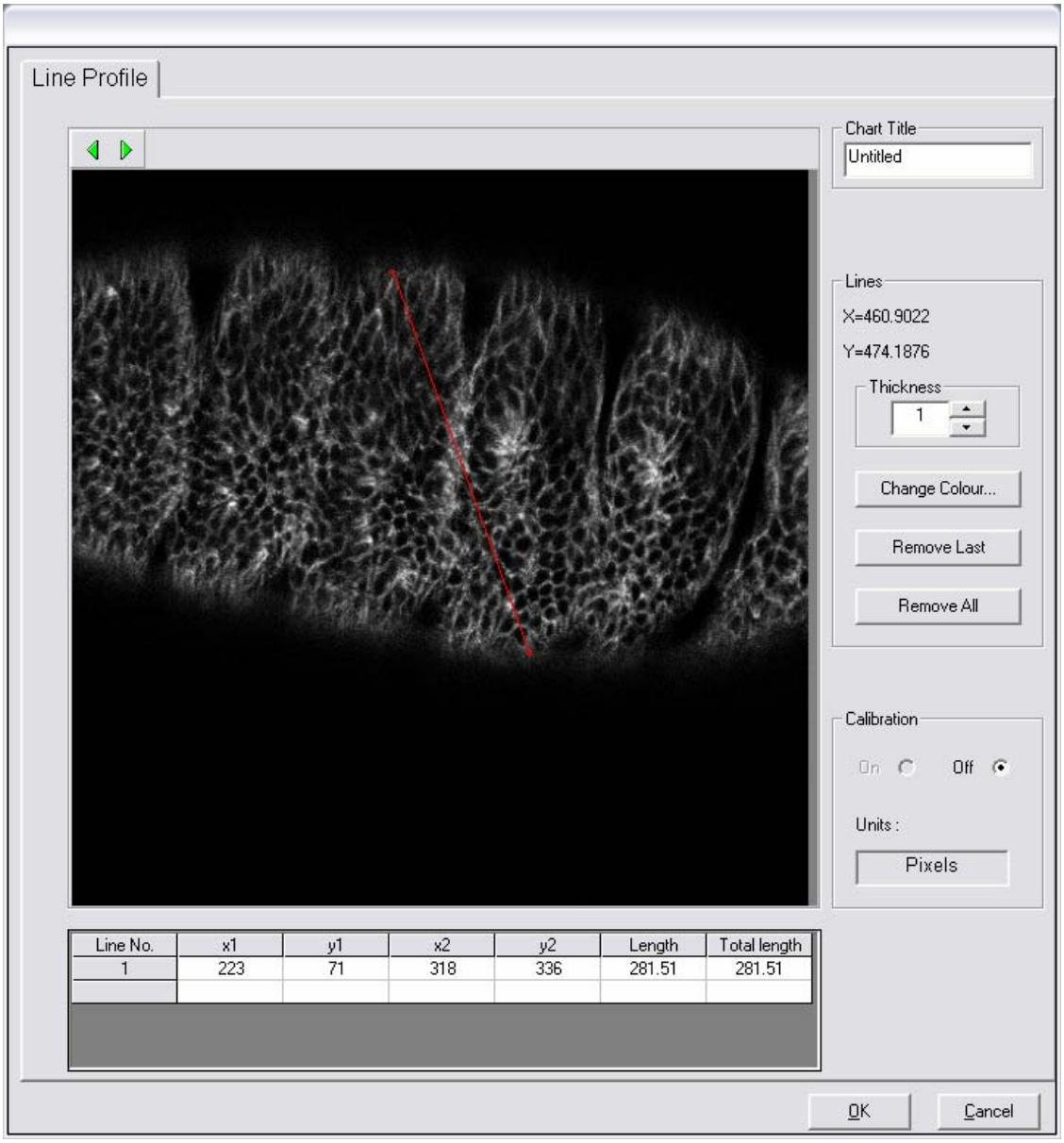
6 Before performing a measurement, it is often useful to see the cursor position as an X,Y coordinate, and the intensity at that position. This is because structures can often be defined by their intensity in relation to their surroundings. To see X,Y,Intensity, As the mouse pointer is moved across the image, the X,Y,Intensity values are displayed in the status bar at the bottom of the screen.



7 Right click on the selected image and select **NEW… LineProfile**



This will open the line profile box. On the image select the two points (point mouse and left click) A line will be drawn between these two points and the length details will be displayed.



Calibration between pixels and microns can be selected in the Calibration area. Line thickness can be varied (default 1 pixel) Increasing line thickness reduces noise. Line colour can be changed as well as the chart title.

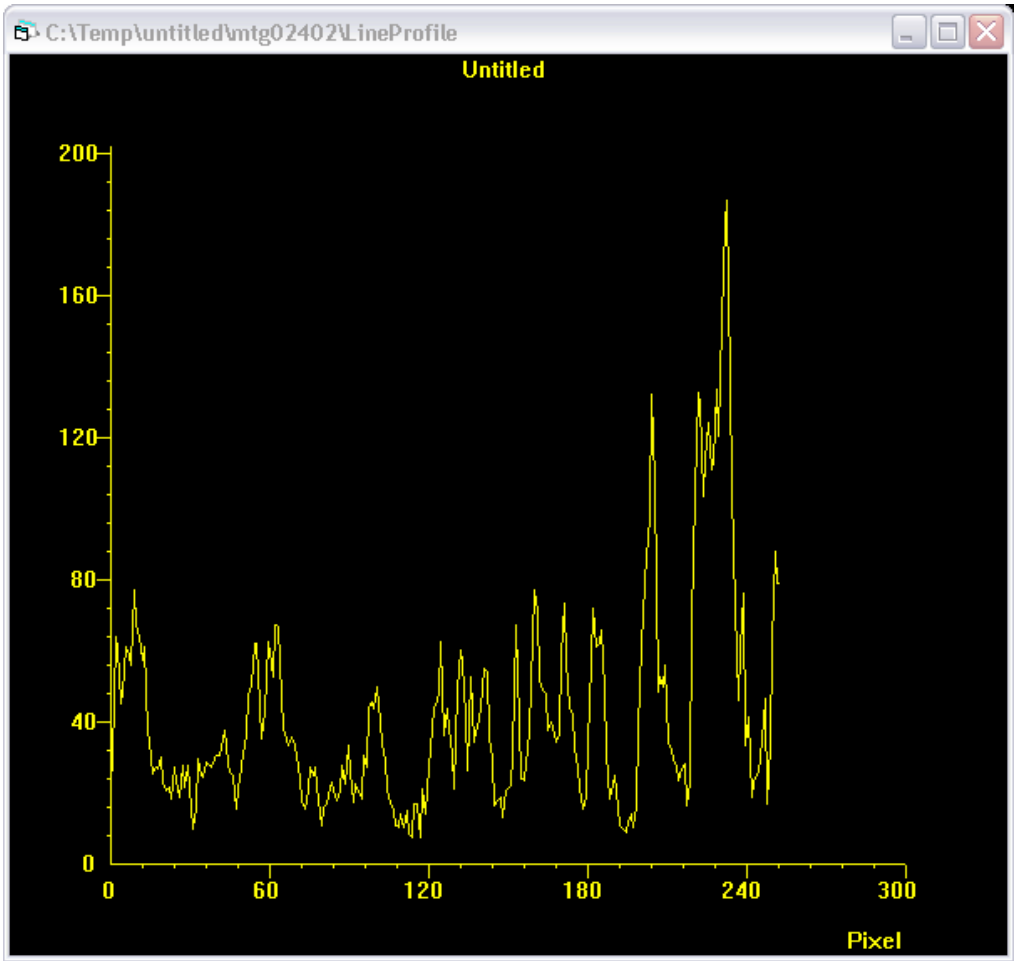
Extra line can be connected by clicking extra measurement points.

Clicking the line anchor point will allow the line to be dragged to another position for a new measurement.

8 If the structure continues into the next optical section, press the  arrow keys on “the top left hand corner” to move backwards and forwards through sections, Note that when you go backwards through the sections, the overlay remains on the image as a visual indication of what you have measured.

9 To view the line definition data from the length measurement, look at the data table which should appear by default. This shows the length of each segment measured, as well as the total.

10 To view an intensity profile through the length measured, select **OK** A graph will appear showing positions along the length in microns (x-axis) and the intensity (y-axis).

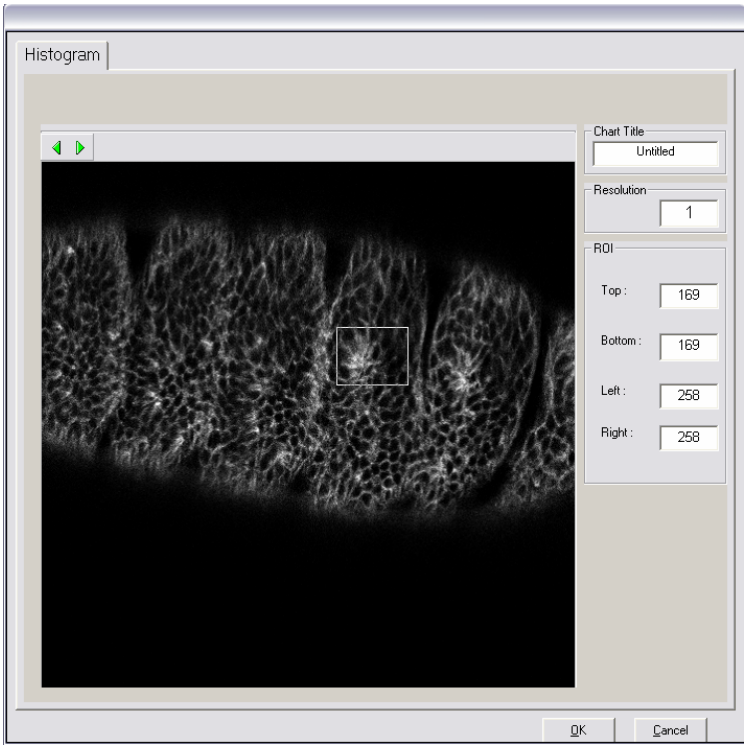


Move the mouse cross hair in the graph. As you move the mouse along the graph, its position is shown by a cross. The two figures at the top of the graph update constantly to show the distance along the line in µm and the intensity.

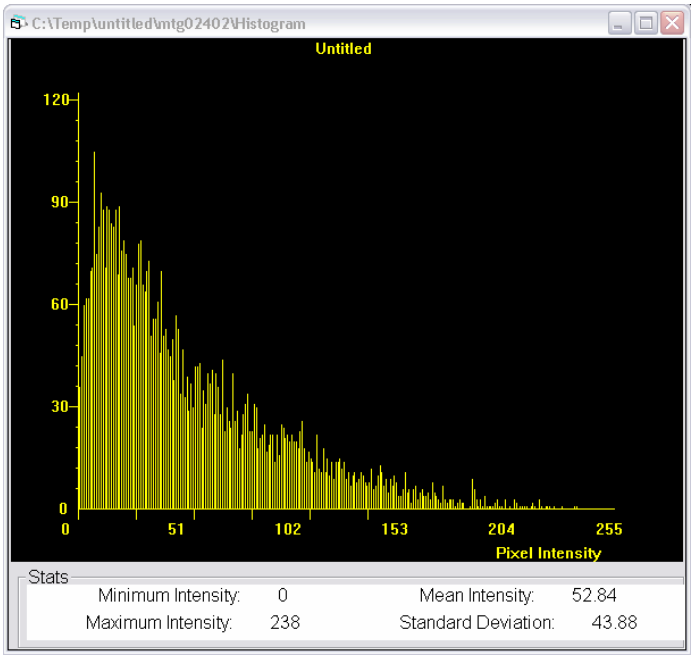
11 To view a histogram of the number of pixels at different intensities, right click in image and select

New…Histogram

A new display will be open to allow the drawing of a rectangle to measure the Histogram



Click on OK to generate the Histogram…



Summary of tutorial 10

In this tutorial you have learned how to make measurements on images.

TUTORIAL 11 - Photon counting

Fast photon counting is a facility which can be used for gaining high-quality images from samples with a very low fluorescence signal. Moreover, the result can be truly quantitative.

EXERCISE 1 - PERFORMING FAST PHOTON COUNTING

1 Choose a sample which has structure but very weak fluorescence. For example, you could remove some of your cheek cells with a swab on to a slide. Add a small drop of water and a coverslip.

2 Focus on the cells with normal transmitted light and view the image with conventional epifluorescence to assess the autofluorescent signal level. It is probably very low compared with a stained sample.

3 The colour of the fluorescence will depend on which PMT you use for photon counting. If the fluorescence is green or yellow/green, use the filter block pair **B1 and Open**.in the scan head to send all the signal to pmt1

4 Select **Methods… Single RED (uses PMT1)**

Check Optic Graphic in the control panel – change excitation to 488nm, ND 10%, Filter block 1 to B1, Filter block 2 to OPEN, Emission filter on PMT 1 to OG515. Close Optic

5 Select **Mix** and choose **mixer mode PhC** (Photon Counting) from the **Mix Mode** list. Close Mix

14 Select **Accumulate** from the Collection filter panel.

15 Start scanning. The image may not be visible at first, but it will eventually appear. Stop scanning when the image is of requisite intensity.

16 Save the image.

17 Set Mix Mode back to PMT, keeping all other settings the same.

18 Select the Accumulate Collection Filter and start scanning, then stop when the image is bright.

19 Save the image.

20 Compare the two photon counting images with the non-photon counting image

21 Having designed a suitable setting for photon counting with a particular sample type, you can, of course, save this as you can any microscope setting by selecting **Methods…Save Settings, or make a new method using the method wizard.**

Summary of tutorial 11

You have learned how to take advantage of the Photon Counting facility on the MRC-1024 and seen that noise is discriminated against and not accumulated in the image.
In the next session, you will learn how to perform reflection imaging.

TUTORIAL 12 - Reflection imaging

Confocal reflection imaging can be performed either separately, simultaneously with confocal fluorescence imaging, or simultaneously with non-confocal transmission imaging or all 3 at once.

Many materials, such as metal, glass and integrated circuits, reflect light extremely well. Some biological materials also reflect light, for instance muscle cells and the corneal epithelium. However, normally, biological materials are stained by a process which frequently uses metal enhancement, such as Golgi Staining for neurons or metal-enhanced DAB reaction product, or immunostaining with immunogold particles. With reflection imaging, it is best not to have the structures of interest too close to the coverslip, since interfering reflections from the surfaces of the coverslip and the glass slide could obscure the image.

EXERCISE 1 - PERFORMING REFLECTION IMAGING

1 Focus on a suitably stained sample using conventional transmitted light, and open the laser light path.

2 Make sure that filter blocks B1 and T2A (or supplementary reflection block option) are in positions 1 and 2 respectively of the scanhead.

3 Select **Methods…Reflection Blue**.

4 Start scanning and adjust the PMT2 Gain until the reflection image is visible. Refocus to find the best part of the sample.

5 Select the Kalman Collection Filter and average until the image is of good quality.

6 If the reflection signal is very weak, you may see a small central spot, resulting from a reflection from the eyepiece in the scanhead. If you collect an image showing the spot, but with the sample completely out of focus (i.e. black), you can save this image and later subtract it from the sample image to remove the spot.

Summary of tutorial 12

You have now learned how to perform blue reflection imaging. In the next tutorial, the use of the transmitted Light Detector (TLD) will be introduced.

TUTORIAL 13 - Transmitted Light Detector (TLD)

The TLD is sited near the conventional transmission lamp. The lever on the top of the TLD is moved to determine whether you see a conventional transmission image down the eyepieces (lever straight) or a non-confocal diode image from the TLD (lever to the left).

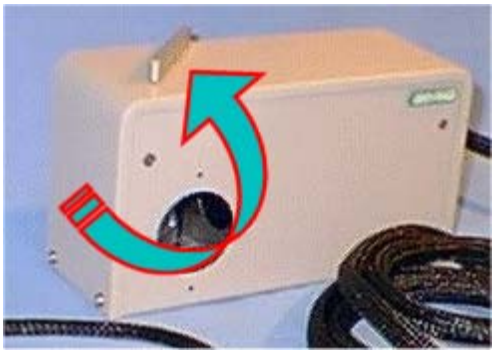
The TLD can be used with phase contrast or DIC lenses and accessories. You may find that when DIC has been optimized, quite a high laser intensity is required to obtain a reasonable image brightness.

The TLDs can be in one of two forms:

- Containing a single diode for gray scale imaging, or

- Containing three diodes for multi-colour imaging.

Depending on which type is present, you will see either one or three TLD controls in the Control Panel. The TLD picks up laser light which passes through the specimen. This means that only when All laser lines are in use can all three controls (i.e. Gain and Black level for the three diodes, RGB) be used.



EXERCISE 1 - USING THE TRANSMITTED LIGHT DETECTOR

1 Choose a stained sample (e.g. Haematoxylin and Eosin).

2 Focus on the sample by conventional means.

3 Move the TLD lever to the left.

4 Select **Methods…Transmission**.**Triple**

5 Open the laser light path and start scanning.

6 Using the Sim button for a three channel detector you can adjust the Gain and offset (or all three Gain and offset levels) to optimize the brightness and contrast.

If you have a three-colour TLD,you can adjust the % from TLD1 (red), TLD2 (green) and TLD3 (blue) displayed in the **Mix** graphic until the result is satisfactory. The merged pane will display a combination of the diode outputs.

7 Use the Kalman Collection Filter to average the signal.

For a single-channel transmission detector

1 Optimise the signal for each of the sequential buttons using the individual gains and offsets to get the best image in each of the display boxes

2 Click on the Sequential scan button  to collect each channel in turn, A merged colour image will be displayed in the merge pane

It may be preferable to collect the transmission images and confocal images sequentially rather than simultaneously, since some DIC components may affect the quality of the confocal image.

Summary of tutorial 13

Here you have learned how to aquire an image using the optional non confocal TLD. You have seen how to alter the contrast, brightness and colour balance using the Black level, offset and Mixer controls.

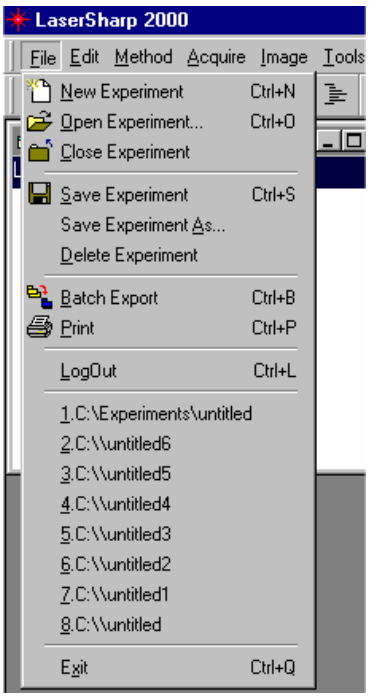
TUTORIAL 14 – Software Overview

14.1 Menu Bar

This tutorial provides a reference to the LaserSharp2000 menu bar.

Menu bar

14.1.1 FILE MENU



14.1.1.1 New Experiment 

Select this item from the menu or use the button on the application toolbar to create a new experiment. An image display window to suit the currently selected Method will be created.

14.1.1.2 Open Experiment 

Opens an existing experiment from disk. The experiment will appear in the experiment browser and the desired data can be opened from there. To open a particular component of the Experiment simply double click the item in the Experiment Browser.

14.1.1.3 Close Experiment 

Closes the experiment - shuts all windows in the experiment.

14.1.1.4 Save Experiment 

Saves the current open experiment. If you wish to rename an Experiment you can do this using the Windows Explorer, but you must ensure that the Experiment has been closed in LaserSharp

first.

14.1.1.5 Save Experiment As…
Saves the currently open experiment with the name and location of your choice.

14.1.1.6 Batch Export
Exports the experiments of your choice in the format of your choice.

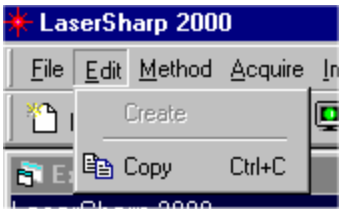
14.1.1.6 Print 
Prints the selected pane from the currently highlighted image. LaserSharp2000 prints to the default printer as determined by Windows NT. For details on setting the default printer see Windows NT Help.

14.1.1.7 Log Out
Logs out the current user and displays the Login dialog ready for the next user.

14.1.1.8 A list of recently opened Experiments
The eight most recently used experiments are shown for rapid access.

14.1.1.9 Exit the application
Exits LaserSharp2000.

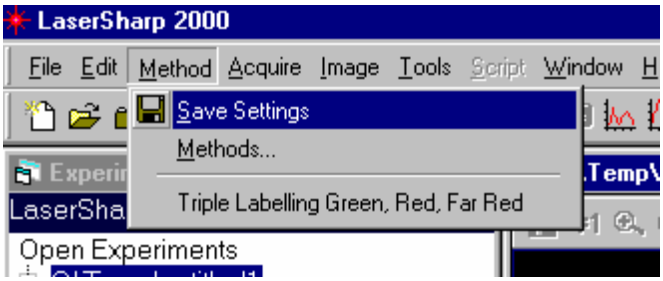
14.1.2 EDIT MENU



A single entry, copies the currently selected image to the clipboard for transfer to other applications.

14.1.3 METHODS MENU

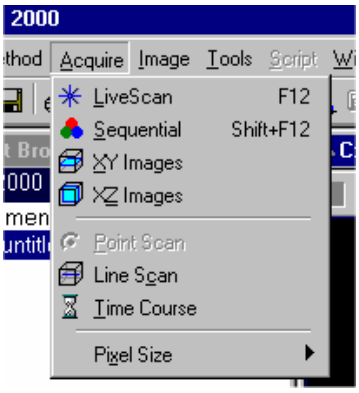
This menu contains entries to Save a modified method and to edit an existing method or to create a new method.



14.1.3.1 Save Settings
Saves the currently loaded method with any modifications to the optics, control or mixer settings.

14.1.3.2 Methods… This opens the Methods menu

14.1.4 Acquire Menu



14.1.4.1 Live Scan

On pressing the live scan button the system will start scanning and acquiring an image. Whilst the system is scanning almost all system parameters can be changed. For example, detector gain and iris, image zoom, Setting etc.

14.1.4.2 Sequential live scan

The sequential live scan button automatically cycles through the sequential settings and updates the merge pane (if you have configured one) frame by frame. This mode of scanning is useful when imaging samples which can only be imaged in sequential mode due to bleedthrough.

14.1.4.3 XY (Z-Series) collection

On pressing this button the XY dialog box is shown; Z-Start, Z-Stop and the Z-Step can be reset.
The Channels page is used to select Simultaneous or Sequential acquisition.
The Time Series page allows either an XY-T series or an XYZ-T series to be acquired.

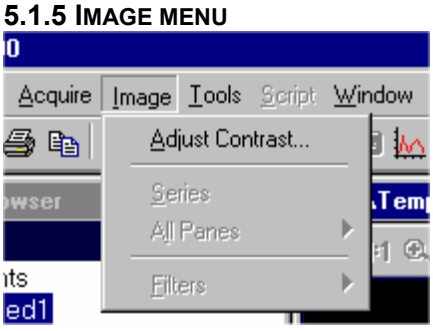
14.1.4.4 X-Z (vertical section) collection

Pressing the XZ button causes the XZ dialog to be shown. The scan line (Y position) is selected using the spin buttons.
The Channels and Time Series pages function in the same way as they do for XY images.

14.1.4.5 X-T (line scan) collection

XT data collection runs in two distinct modes and will allow collection of up to 1024 lines.

5.1.5 IMAGE MENU



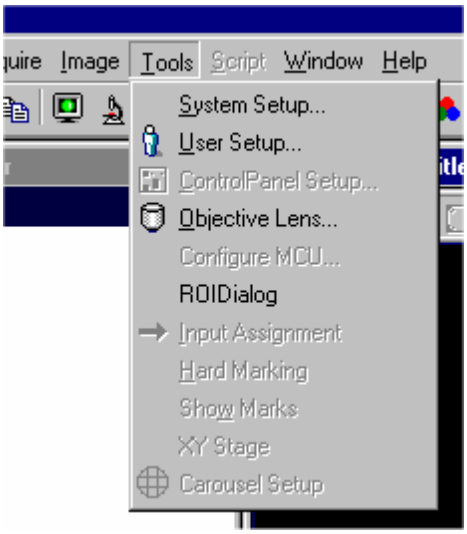
The image menu currently only has one entry - Adjust contrast.

14.1.5.1 Adjust contrast

The adjust contrast dialog allows you to change the brightness, contrast and gamma settings for the current image. You can also load different look up tables from here.

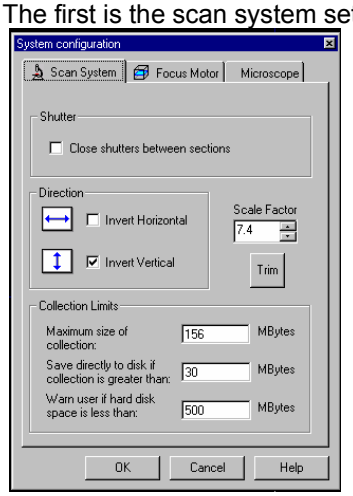
14.1.6 TOOLS MENU

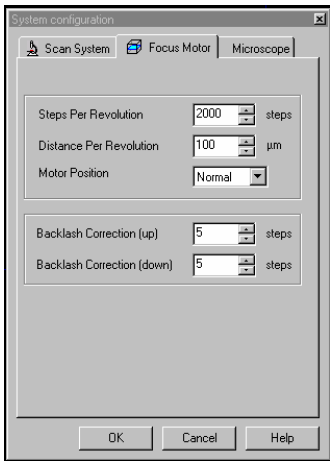
The tools menu contains options for setting up the system configuration, user login details and access rights.

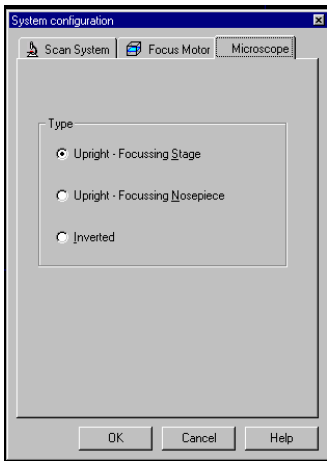


14.1.6.1 System Setup

The system setup dialog consists of three pages.
The first is the scan system setup:







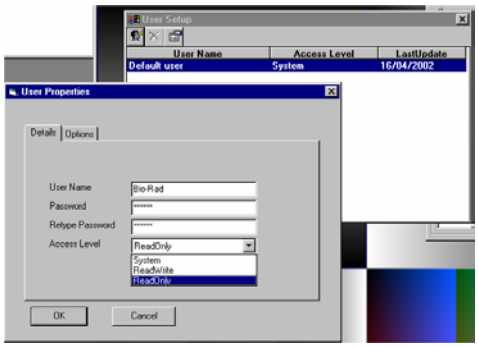
Check the ‘Close shutter between sections’ to ensure that the beam is turned off between sections. Leave this option unchecked to achieve the fastest possible series acquisition.
The Direction controls allow you to invert the direction of scan in both axes to orient the image on the screen to be the same as that seen down the eyepieces. The Trim button is for engineering purposes and should not be touched.

The scale factor determines the XY calibration of the system and will be set up at the time of installation. **Changing this will render measurements incorrect.** The ‘Maximum size of collection’ value should be set to a size such that the real memory and virtual memory of the PC cannot be exceeded. ‘Use memory pool if collection is greater than’ should be set to a value that is lower than the amount of real memory remaining available. The second page is for set up of the focus motor: Again, all of these settings will be made at installation and will only need changing if you change the microscope to which your system is attached.

The Focus Motor page is used to set the calibration and motor drive directions. These will be set up during equipment installation.

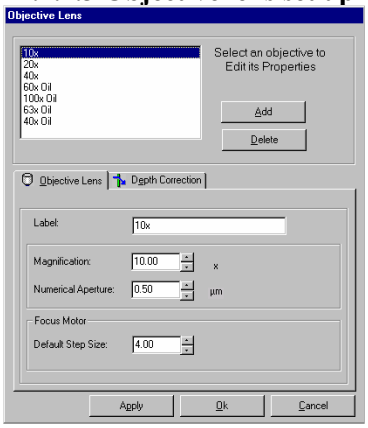
The Microscope page sets the type of microscope that is connected to the confocal. This will be set up during installation.

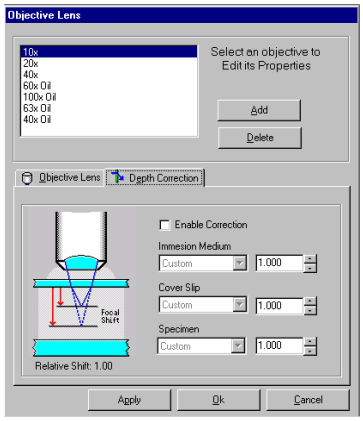
14.1.6.2 User set up



The User setup dialog allows the manager to add or delete other users or to edit their access rights. There are three access levels; System, Read/Write and Read Only. It is recommended that the appointed system manager has System rights, all authorised users should have Read/Write access (so that key system configurations cannot be accidentally modified) and that unauthorised users are give Read Only access. Only users with System access can create, delete or edit other users or edit their access level.
A default working directory can be assigned for each user so that their data is automatically saved in a known folder on the system.

14.1.7.3 Objective lens set up

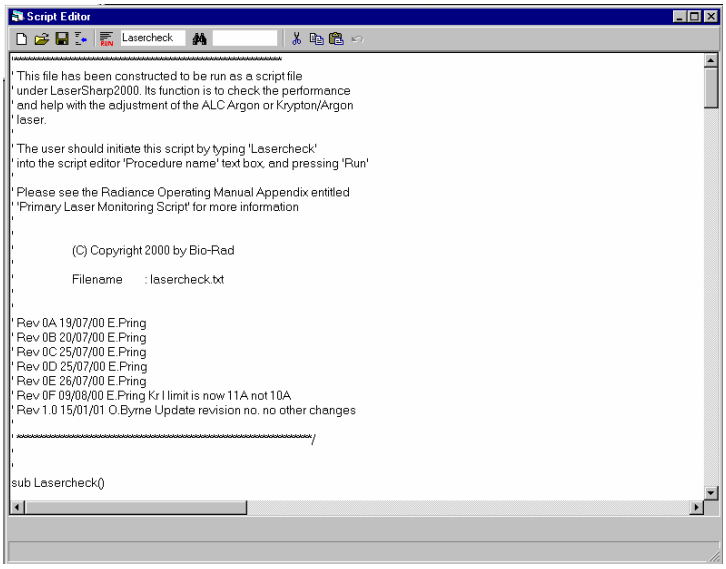




The Objective lens page of this dialog allows you to add, edit or delete lens descriptions from

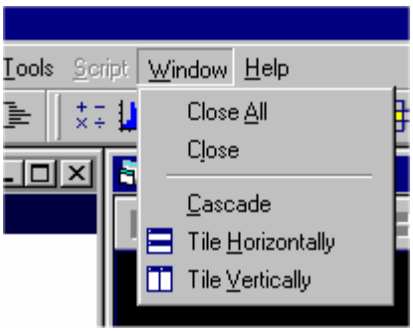
the list which appears in the control panel. The Magnification value is used for calibration of measurements. The default focus motor step size should be set to a convenient value for each lens. The Depth Correction page activates a correction algorithm to correct for axial geometric distortion caused by mismatching of refractive indices above and below the cover slip.

14.1.8 SCRIPT MENU



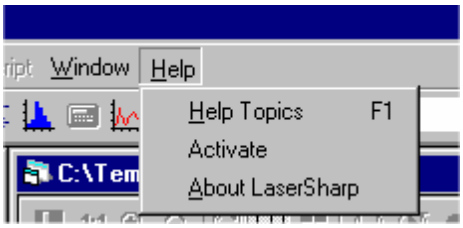
The script menu can be accessed by pressing the  button and contains entries for editing and running scripts. These scripts are standard Microsoft Visual Basic Scripts (VB Script) and their documentation should be consulted for details of this programming language.

14.1.9 Window Menu

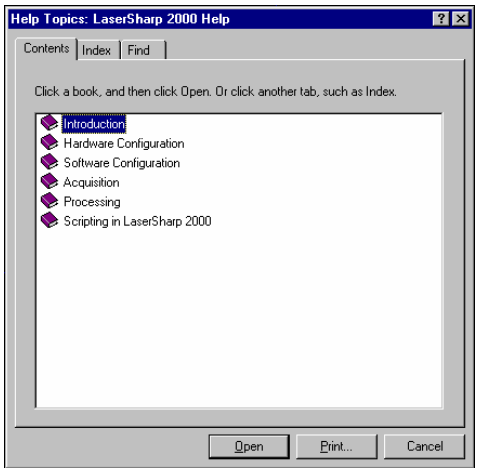


The window menu allows the usual Windows layout options of close, tile and cascade.

14.1.10 Help Menu



The help menu has three pages. The first page is the help manual.



The second page is the Activate page which is not available for the MRC1024.

The last LASERSHARP page is the **ABOUT** page showing the version and build number.