



*Microscopy
Division*

CONFOCAL SCANNING SYSTEM
Radiance

Operating Manual Appendix 1

Creating Methods

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Introduction

This Appendix is designed for users of the Bio-Rad MicroRadiance or Radiance 2000 model Confocal Imaging Systems, with Lasersharp 2000 software running on a computer with Windows NT.

If you are familiar with the main contents of the Operating Manual for your System, this Appendix will assist you in how to create a new "Method". It will also provide some background information on how Methods may be altered and used, and you will find a Glossary of definitions towards the end.

A Method is designed for imaging a sample which is prepared in a specific way; for example a reflective sample, or a biological sample with certain Fluorophores. Lasersharp 2000 configures itself to suitable predetermined preferences according to the currently selected Method.

The configuration of your Confocal Imaging System may differ considerably from the example used here, in terms of the Lasers and Detectors fitted for example.

The Tutorial section describes how to create a new Method **based on one specific configuration**, however the steps involve hints, tips and considerable discussion on the reasons why they are carried out, which are applicable to your system's configuration.

The section on 'Other Example Cases' will provide ideas to assist you in tailoring your own methods, to attain most efficient use of your Bio-Rad confocal imaging system.

The target of this document is to enable you to understand the procedures and rules for setting up Methods, enabling you to create your own Methods.

Tutorial: Method “Triple Labelling”

In the first tutorial, you will create a Method called “Triple Labelled”, for a sample which is marked using three specific single emission Fluorophores (FITC/TRITC/Cy5), each of which is excited by a separate Laser Line.

This example uses an R2000/AGR-3 CLSM, which has three Lasers, three PMT Detectors and a single transmission Detector. The Method will be designed to image all three Fluorophores simultaneously in the PMT Detectors.

The data from the three Fluorophores will be merged in different colours, to produce a fourth live image, the Merge Pane.

The same Method will also include a Sequential Setting for each Fluorophore, whereby only one Laser Line and one PMT is active at any time. Sequential acquisition reduces bleed through and can collect more of the emission spectrum because there is no need to split fluorescent light between different collection channels.


Step 1: The Methods Window

1. Follow the normal CLSM start-up procedure.
2. Run Laserssharp 2000 in live acquisition mode (not in ‘No hardware’ or ‘emulation’ mode)
3. Ensure that you are logged in as the correct User. You must have System or Read/Write access level to write Methods. See the System Manager if you are unsure of your user name, password or what access level you have. See the Glossary for an explanation of User access levels.
4. Select the ‘Methods’ pull down menu
5. Select ‘Edit’ to produce the Methods Window, shown in Fig. 1.

In this example case, a User called “Sam” has logged into Laserssharp 2000, and “Sam” is therefore the only User displayed in the Methods Window, Fig. 1. “Sam” has Read/Write access, shown by displaying “(Read/Write)” after his user name. The three existing methods are entitled “One Pane Reflection (Sam)”, “3p + M (Sam)” and “2p+m (Sam)”.

Note: The “(Sam)” at the end of the Method names indicates that these Methods are copies of the Methods associated with the System User, made when User “Sam” was created by the System User.

You are now going to create a new method called “Triple Labelled”. Since Methods are specific to Users (although they can be copied between users by a System User), once this Method is written, “Sam” will be the only non-System User who can use it.

6. Press the new method icon  (“Magic Wand”), on the top left of the Methods Window, to activate the Method Wizard.

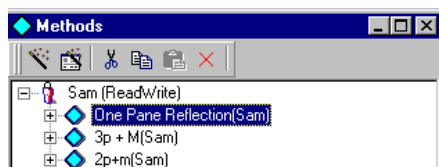


Figure 1 Methods Window

Step 2: The Method Wizard

1. In the 'Start method' window Fig. 2, enter a 'Method name' – in this case, "Triple Labelled". The name should be no longer than the length of the box into which it is written. Letters, numbers and hyphens may be used.
2. Enter an optional 'Description'
3. Click on 'Next' to continue

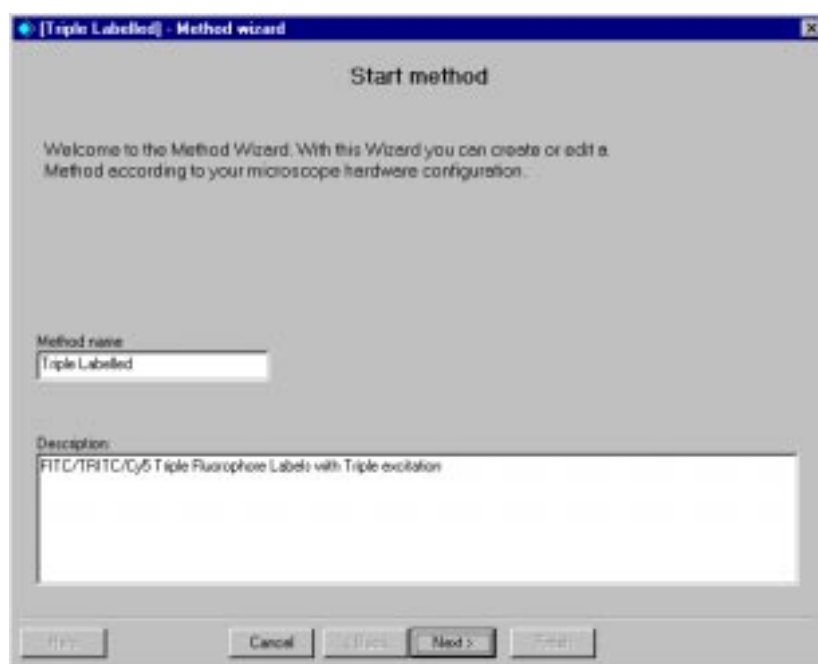


Figure 2 Start method window

Step 3: Screen Layout

In the 'Screen Layout' window Fig. 3, you will decide how many panes can be displayed during data acquisition, and if you would like a Merge Pane.

1. Select the total number of 'Panels', using the spin buttons, to four. **The maximum number is four.**

Note: If you select more than one pane here, when viewing your images later you can toggle between displaying only one pane or all panes by double clicking with the left mouse button over any pane

2. Select how you wish these panes to be arranged by altering the number of 'Rows'. If you wish to display all the panes together on your screen, you will need to consider how best this can be achieved.

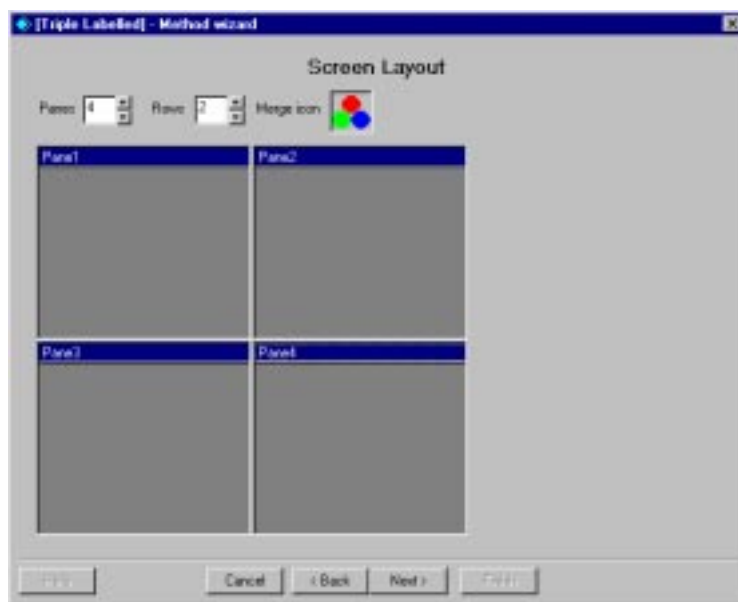


Figure 3 Screen Layout window

3. If you decide to have a merge pane (for this tutorial you will), move the mouse over the 'Merge icon'



, then hold down the left mouse button as you drag the icon into the dark blue bar above the **LAST** pane on display ('Pane 4' in this case). Release the mouse and you should see a miniature Merge icon in that pane, as in Fig. 4.



Figure 4 Pane 4 with Merge icon

Note: All panes without a Merge icon are known as Non-Merge Panes.

*There can only be a **maximum of one Merge pane**, which is updated by the computer at the same speed as the Non-Merge panes during live acquisition. If you have a Merge pane, you must also have **at least two Non-Merge panes**.*

You do not define which Non-Merge panes will be combined to make the Merge Pane at this stage, only that you wish to have a Merge Pane.

4. Click on 'Next' to continue.

Step 4: Setting Manager

You will now create settings for your method from the 'Setting Manager', Fig. 5.



Figure 5 Setting Manager window (before changes)


Step 4a: Simultaneous Setting

Click on 'New Simultaneous'.

You must create one simultaneous setting in a method. It is not possible to create more than one.

(i) Start Setting

1. In the 'Start setting' window, Fig. 6, enter a name for the simultaneous setting (max 4 characters) and an optional 'Description'. Use the name "Sim" here.

The Simultaneous Setting icon is fixed () and will be seen later on the Control Panel Simultaneous Setting button.

2. Click on 'Next' to continue.



Figure 6 Start setting window (simultaneous)

(ii) Optics Configuration

The 'Optics Configuration' will be shown in its default state, as Fig. 7. The purpose of this window is two-fold:

- a. To choose which Detectors and Lasers you will use (cannot be changed outside Method Wizard, see Table 1 for more details)
- b. To choose what optical configuration you intend to start imaging with (can be changed later, during data acquisition, and saved into the Method).

Note: Your hardware configuration may differ from that shown in Fig. 7.

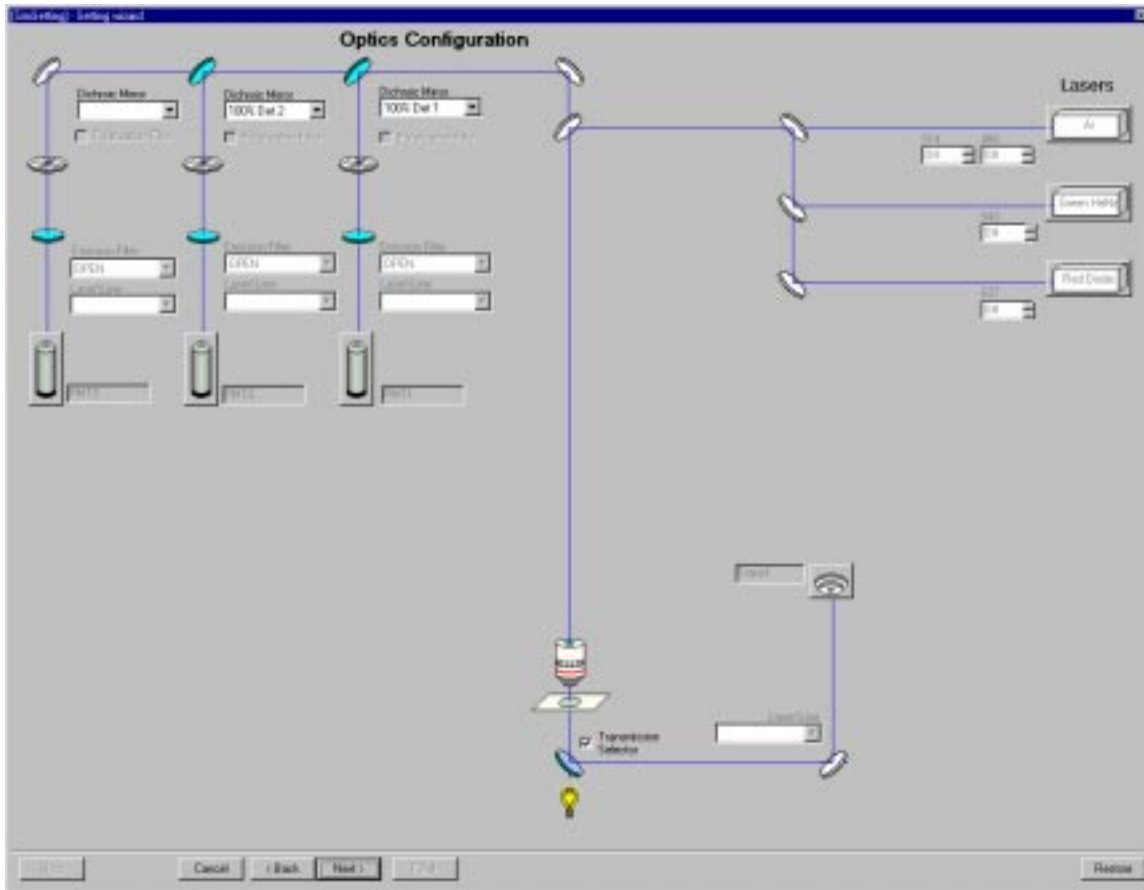




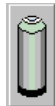
Figure 7 Optics Configuration window, before changes

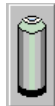
Note: The 'Restore' button in Fig. 7 is used to toggle the size of the displayed window, and does not affect the contents.

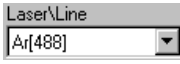
1. Select each of the Lasers to be used in the method by clicking on the Laser icons (e.g. ). The shading of these icons will change subtly, to show that they are highlighted. Use the 'Ar', 'Green HeNe' and 'Red Diode' Lasers.

2. Set the desired percentage power for each required Laser Line, next to the Laser icons (e.g. ). In this case set the powers to 488 : 3%, 543 : 20% and 637 : 5%.

Each highlighted laser must have at least one of the Laser Lines set to a value greater than 'Off'

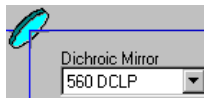


3. Highlight all of the PMT Detectors (e.g. ) which you will use in the method. Highlight one PMT Detector **for every Laser Line which is not set to 'Off'** (unless you really intend to use multiple Laser Lines with one Detector), so in this case highlight three PMT Detectors.

4. For each highlighted PMT Detector select the 'Laser Line' (e.g. ) , which you wish to be associated with that Detector, from the pull down menu (this will be the Laser Line percentage power control which will appear on the Detector Tab in the Control Panel). These will be PMT 1 : Ar(488), PMT 2 : Green HeNe(543), PMT 3 : Red Diode(637).

The pull down menu will only show the Laser Lines which are not set to 'Off'. Each pull down menu has a default option, being the numerically first Laser Line which is not set to 'Off', Ar(488) in this case.

5. If you intended to image in a given PMT detector in reflection, you would click on the check box to install a Polarizer in the PMT paths (Polarization Filter) at this stage.




6. Select the required 'Dichroic Mirrors' (e.g. ) in order to split the emitted light between the PMT Detectors correctly. Some available options are:

500DCLP	500nm Dichroic Long Pass, allows light >500nm to pass through
560DCLP	560nm Dichroic Long Pass, allows light >560nm to pass through
605DCLP	605nm Dichroic Long Pass, allows light >605nm to pass through
650DCLP	650nm Dichroic Long Pass, allows light >650nm to pass through
880DCSP	880nm Dichroic Short Pass, allows light <880nm to pass through

In this case, set the first dichroic mirror to "560 DCLP" and the second to "650 DCLP".




7. Select the required 'Emission Filter' (e.g. ) for each highlighted PMT Detector. Some available options are:

HQ450/80	High Quality Band Pass, centered on 450nm with 80nm bandwidth
D488/10	Band Pass, centered on 488nm with 10nm bandwidth (for reflection imaging)
HQ500LP	High Quality Long Pass, allows light >500nm to pass through
HQ515/30	High Quality Band Pass, centered on 515nm with 30nm bandwidth
HQ525/150	High Quality Band Pass, centered on 525nm with 150nm bandwidth
HQ530/40	High Quality Band Pass, centered on 530nm with 40nm bandwidth
HQ530/60	High Quality Band Pass, centered on 530nm with 60nm bandwidth
HQ550/100	High Quality Band Pass, centered on 550nm with 100nm bandwidth
HQ570/40	High Quality Band Pass, centered on 570nm with 40nm bandwidth
E570LP	Extended Long Pass, allows light >570nm to pass through
E580LP	Extended Long Pass, allows light >580nm to pass through
HQ590/70	High Quality Band Pass, centered on 590nm with 70nm bandwidth
HQ600/40	High Quality Band Pass, centered on 600nm with 40nm bandwidth
HQ600/50	High Quality Band Pass, centered on 600nm with 50nm bandwidth
HQ640/40	High Quality Band Pass, centered on 640nm with 40nm bandwidth
HQ660LP	High Quality Long Pass, allows light >660nm to pass through

In this case, set the emission filters to PMT 1 : HQ515/30, PMT 2 : HQ600/50 and PMT 3 : HQ660LP.

8. If you were using a Multi-Photon system, you would select the required Infra-Red wavelengths 'Blocking Filter' for each highlighted PMT Detector at this point.

Note: This method will not use a Transmission Detector (), but see Example 2 for important information about this.

The 'Optics configuration' window is shown completed for the Simultaneous Setting in Fig. 8.

9. Click on 'Next' to continue.

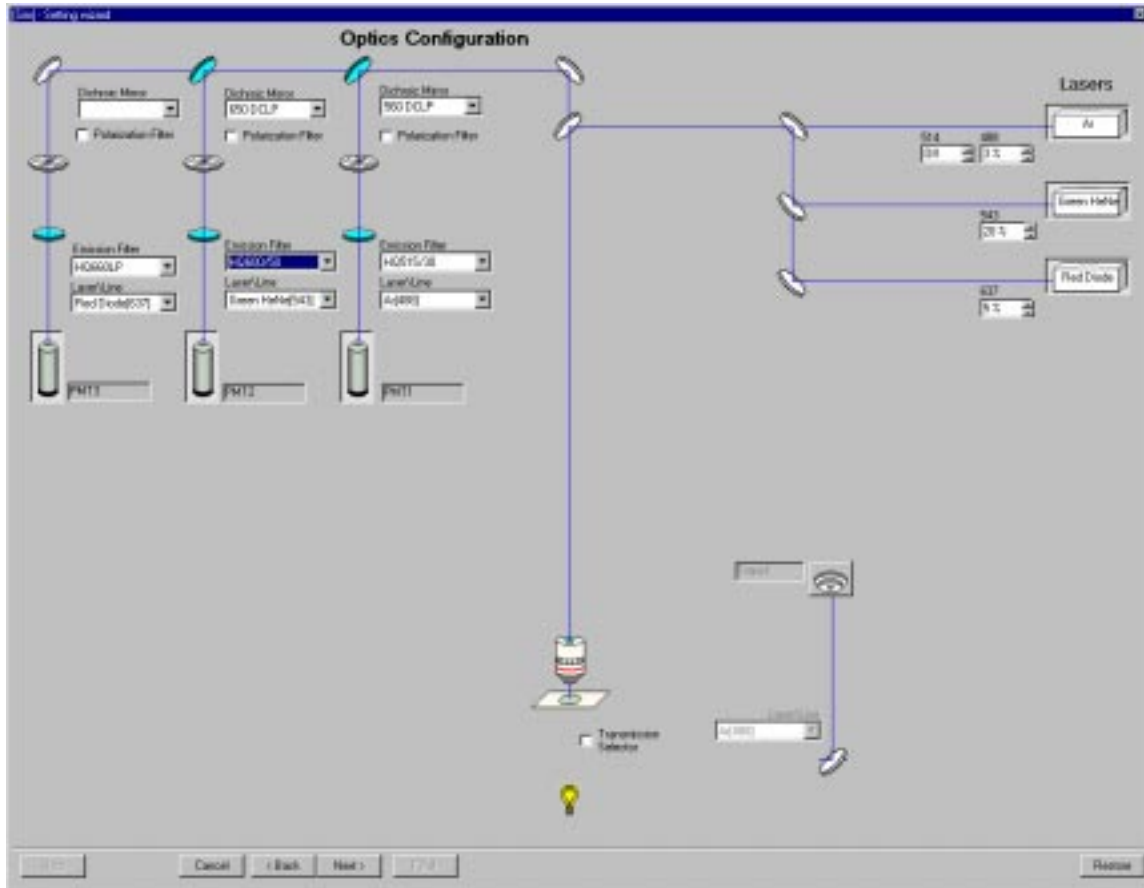


Figure 8: Optics Configuration window, after changes

[\(iii\) Assign detectors](#)

In the 'Assign detectors' window, you will assign each of the Detectors you nominated (by highlighting the PMT Detector icons in Step 4a (ii)) with the Non-Merge Panes you created in Step 3 (in this case Panes 1 to 3).

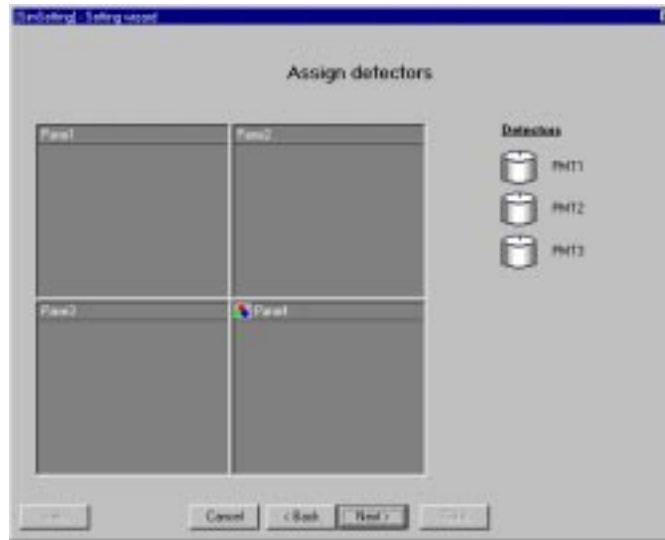



Figure 9 Assign detectors window, before changes

1. Drag each detector icon () for PMT 1, PMT 2 and PMT 3 (Fig. 9) into the Panes required. Ensure that every Non Merge Pane **has at least one Detector**, and a **maximum of three**, assigned to it.

It is conventional to assign the PMT Detector numbers so that they correspond with the Non Merge Pane numbers if possible. In this case therefore, drag PMT 1 into Pane1, PMT 2 into Pane2, and PMT 3 into Pane3.

Note: When acquiring data, the Non-Merge Panes are not automatically labelled with the corresponding Detectors, so it is easier to choose a memorable sequence.

2. Click on 'Next' to continue

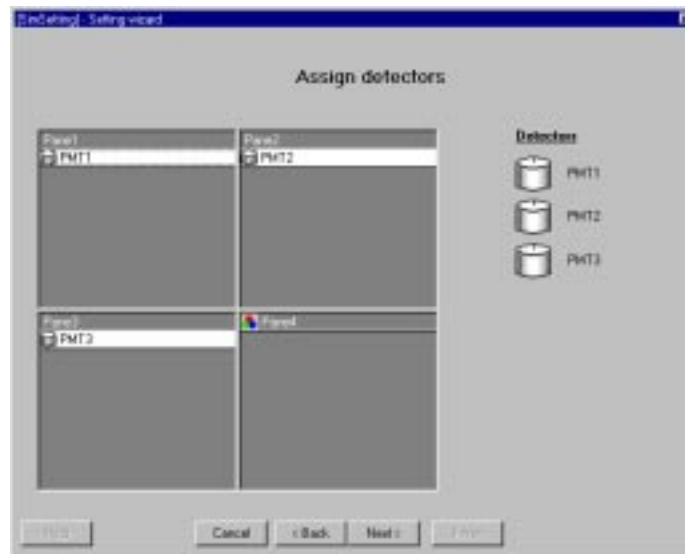


Figure 10 Assign detectors Window, after changes

[\(iv\) Finish Setting](#)

You have now completed the simultaneous setting. If you wish to edit the name or description, you may do so in the 'Finish setting' window, Fig .11.

If you wish to correct an error in the 'Assign detectors' or 'Optics configuration' windows, click on the 'Back' icons until you reach the correct window, correct it, and return to this point using 'Next'.

Click on 'Next' to continue.



Figure 11 Finish Setting window

You will now return to the 'Setting Manager' window, Fig. 12. The setting you have created has now appeared in the pull down Settings menu on the right, as "Sim".



Figure 12 Setting Manager window with Simultaneous Setting Completed

Step 4b: Sequential Settings

In this step, **you must create one Sequential Setting for every Non-Merge Pane**

In this example you will create a Sequential Setting for each of the three Fluorophores we are using. You may create a maximum of one Sequential Setting for each Non-Merge Pane.

Please first read items (i) to (iv) below, then follow these steps for each of the three Sequential Settings using the information given in item (v).

(i) Assign Setting to Pane

1. Click on 'New Sequential' from the Setting Manager, as in Fig. 12.
2. In the 'New sequential setting' window Fig. 13, you choose the pane where you wish this setting to be displayed, from the pull down menu. In this case, choose 'Pane1' for Sequential Setting 1, and so on.

Note that you will NOT be presented with an 'Assign detectors' window like the Simultaneous Setting, instead you make one choice for each setting in the New sequential setting window.

3. Click on 'OK' to continue

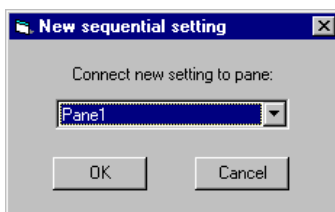


Figure 13 New sequential setting window

(ii) Start Setting

1. In the 'Start setting' window, Fig. 14, enter a name ("FITC", "TRITC" or "Cy5" in this case) for the Sequential Setting.

Note: The name should have a maximum of four characters in order to be fully visible in the Control Panel

2. Enter an optional 'Description'.
3. Select a colour for the Sequential Setting icon (e.g., green for green fluorescence). This will appear on the Control Panel.

Note: It is recommended that each Sequential Setting is assigned a different colour and name for ease of use.

4. Click on 'Next' to continue.

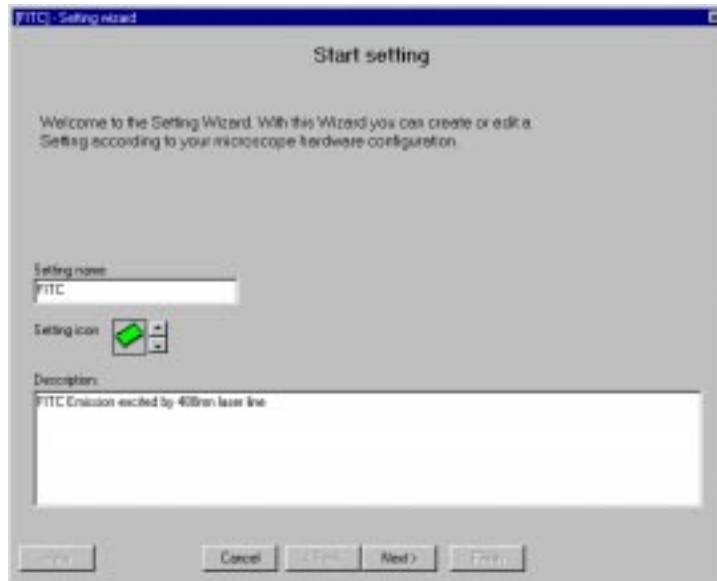


Figure 14 Start setting window (sequential)

[\(iii\) Optics Configuration](#)

In the 'Optics Configuration' window, you will:

1. Highlight one Laser
2. Select a Laser Line for that laser and choose the required percentage power.

Note: Some Lasers have more than one Laser Line

3. Highlight one Detector (the associated Laser Line will then be selected by default)
4. Choose the required optics: Dichroic Mirrors, Emission Filters, Blocking Filters, Polarizers

[\(iv\) Finish Sequential Setting](#)

Click on 'Next' to return to the Setting Manager, Fig. 12.

[\(v\) Tutorial Exercise](#)

1. Create the first Sequential setting with the name "FITC". Select the Ar Laser 488nm Laser Line at 2.5% power. Use only PMT 1, associated with the Ar (488) Laser Line, with an E500LP emission filter. Set the first dichroic mirror to "100% Det 1" (there is no need to split fluorescent light into more than one colour, so all available light is best sent to the Detector). The Polarization Filter will not be needed.
2. Create the second Sequential setting with the name "TRITC". Select the Green HeNe Laser 543nm Laser Line at 18% power. Use only PMT 2, associated with the Green HeNe (543) Laser Line, with an HQ600/50 emission filter. Set the first dichroic mirror to "100% Det 2/3" and the second dichroic mirror to "100% Det 2". The Polarization Filter will not be needed.
3. Create the third Sequential setting with the name "Cy5". Select the Red Diode Laser 637nm Laser Line at 4.5% power. Use only PMT 3, associated with the Red Diode (637) Laser Line, with an E660LP emission filter. Set the first dichroic mirror to "100% Det 2/3" and the second dichroic mirror to "100% Det 3". The Polarization Filter will not be needed.

Completed Setting Manager

The Setting Manager should now display the Simultaneous Setting icon in all three panes, and also the three different Sequential Setting icons in the relevant panes.

If all the settings are correct, click 'Next' on the 'Setting Manager' as in Fig. 15



Figure 15 Setting Manager window, all settings completed

Editing or Deleting a Setting

If you wished to erase one of the Settings, select the setting name from the 'Settings' pull down menu in Fig. 15, then click on 'Delete'

If you wished to edit one of the Settings, select the setting name from the 'Settings' pull down menu in Fig. 15, then click on 'Edit'. If you are editing a Simultaneous Setting, return to Step 4a (i). If you are editing a Sequential Setting, return to Step 4b (ii).

Step 5: Define Merge

After the Setting Manager window, you will be presented with the 'Define Merge' window, Fig. 16.

Note: If you had not defined a Merge Pane in Step 3, you would proceed forward to Step 6 now.

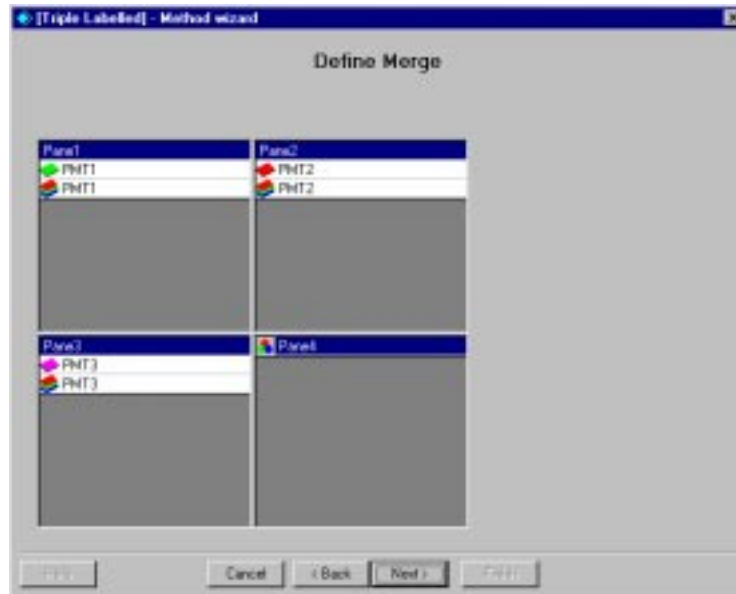


Figure 16 Define Merge window, before changes

1. Drag (one at a time) the Non-Merge Panes, which you want to merge together, into the Merge Pane. The panes are picked up by clicking in the blue title bars.

In the example above, you could choose Pane 1+Pane 2, Pane 2+Pane 3, Pane 1+Pane 3, or Pane 1+Pane 2+Pane 3. For this exercise, choose the latter. The end result is shown in Fig. 17.

2. Click 'Next' to continue.

NOTE: Do not press 'Next' without actually defining the merge, **as you must define which panes you wish to merge**. If you do press 'Next' inadvertently, return to this window using the 'Back' button, and you will find the Merge icon has disappeared from the Merge Pane. To solve this problem, press 'Back' until you return to the 'Screen Layout' window (Step 3), redefine the merge pane, and move forward using 'Next' to this point.



Figure 17 Define Merge window, after changes

Step 6: Choose Look-Up Tables

The 'Choose Look-Up Tables' window will appear, as Fig.18.

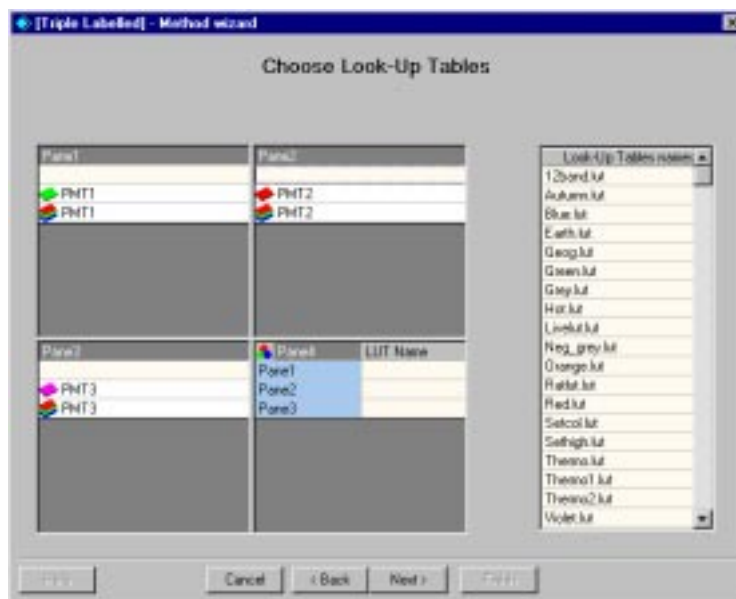



Figure 18 Choose Look-Up Tables, before changes

1. Drag the required Look-Up Table name from the 'Look-Up Tables names' list, to the cream areas in each Non Merge Pane (). In this example, choose "Grey.lut" for Pane1, Pane2 and Pane3. The outputs from the PMT Detectors will then be displayed as greyscale data. You could equally well choose to have different colours in each Non Merge Pane.
2. **OPTIONAL.** The data in the Non Merge Panes can be given different Look-Up Tables for their display in the Merge Pane together, than that used in the Non Merge Panes. If you do not perform this item, Laserssharp 2000 will simply use the same Look-Up Tables in the Merge Pane as in the Non Merge Panes.

Drag the required Look-Up Table names to the cream areas in the Merge Pane. For example in this case (Fig. 19), the merge pane will be created by applying "Green.lut", "Red .lut" and "Violet.lut" to the mixer outputs then combining them into the 24bit colour Merge Pane. You may prefer to display the Non-Merge Panes with these same colours also.

Note: If you have previously used our Laserssharp software for OS/2, you may have been used to seeing Pane1 as red, Pane2 as green and Pane3 as blue.

Note: Look-Up Tables can be changed during data acquisition in Non-Merge Panes, but not in a Merge Pane.

3. Click on 'Next' to continue



Figure 19 Choose Look-Up Tables, after changes

Step 7: Finish Method

1. In the 'Finish method' window, check the 'Method name' and 'Description' you entered earlier is correct, edit if desired.
2. Click on 'Finish'



Figure 20 Finish method window

3. The information box, Fig. 21, will appear. Read this then press 'OK'.



Figure 21 Method Create/Edit completed

Step 8: Using the Method

Using the Method Window

Bring up the Methods Window as before. The new Method will be shown on the list as in Fig. 22. Click on the cross icon next to the name “Triple Labelled” to view the Settings, which together comprise the Method.

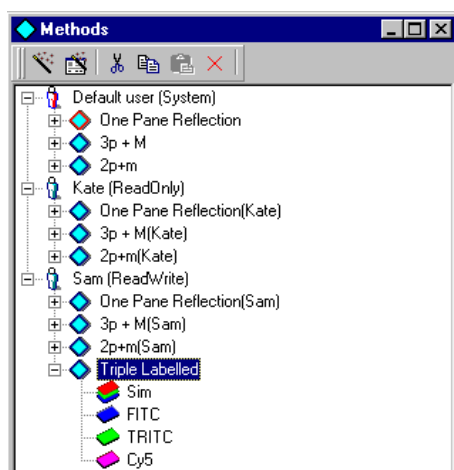


Figure 22 Methods Window, after Method created

Note that Method “Triple Labelled” is the only Method not followed by “Sam”. See Step 1, point 5 for an explanation.

Selecting the Method

Select the method you have just created from the ‘Methods’ pull down menu (if the method is not listed, click on the ‘More’ option at the foot of the list to view all available methods in a separate window list and select it from here instead).

The currently selected Method name will appear in the title of the Control Panel, Fig. 23 (“Triple Labelled”)

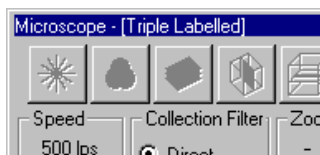


Figure 23 Control Panel Title

Note: If you are presented with an error message at this point, please read the section “Editing and Deleting Methods” under “Other Information about Methods”.

The Settings will appear as buttons in the ‘Channels’ section of the Control Panel. In Fig. 24, the Simultaneous Setting button is selected and the three PMT Detector Tabs are shown beneath. PMT1 is the Detector Tab currently displayed, on which you will see the Laser Line power control for the Ar Laser, 488nm Laser Line.

Note: the Laser Line power control shown is defined by the ‘Laser Line’ pull down menu in the ‘Optics configuration’ window of the Method Wizard. The name of the Laser is written above this control, in this case “Ar Laser”.

In the case of multi-line Lasers the actual Laser Line is not shown, although adjusting the control only adjusts one Laser Line. You can find the Laser Line concerned (and also change any emission filters or dichroic filters subsequently during data acquisition), by pressing the ‘Optic’ button in the Control Panel.

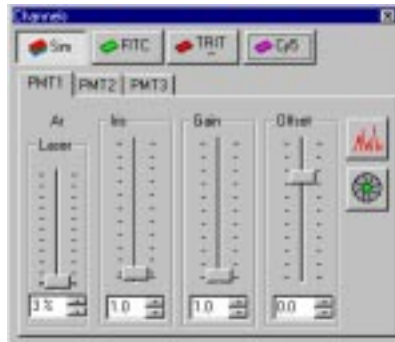


Figure 24 Channels window of Control Panel, Simultaneous Setting button depressed

In Fig. 25, the last Sequential Setting is selected, “Cy5”. There is only one “PMT1” Detector Tab, controlling the associated Laser Line (Red Diode 637nm). Although all three Non Merge Panes will still be visible in the experiment, only the relevant Non-Merge Pane (Pane 3) will be updated during scanning.

By pressing the ‘Multi-Channel Exploration’ button on the Control Panel, Laserssharp 2000 will automatically cycle through the three Sequential settings, scanning each associated Non-Merge Pane in turn, and also updating the Merge pane after completing each cycle.



Figure 25 Channels window of Control Panel, Third Sequential Setting button depressed

Fine Tuning of Method

Now commence your imaging and adjust parameters to suit. This may include the iris, gain, offset of the Detectors, the Laser Line percentage powers, the box size, scan speed, collection filter and so on. It may also include adjusting the Mixer (press ‘Mix’ button on Control Panel) to balance out cross-channel bleedthrough in the Simultaneous Setting.

At any subsequent time you may select ‘Save Method’ from the ‘Methods’ pull down menu. **When you do this, the parameters summarized in Table 1, for the currently selected method, are permanently saved.**

Some of the parameters can be altered only from the Method Wizard (whether creating or editing a Method). Some of the parameters can be altered outside the Method Wizard. Please refer to Table 1 for full details.

Other Example Cases

This section presents some sample ideas for Methods with different CLSM configurations than that used in the Tutorial.

Multiple Fluorophores with a Single PMT Detector

Sequential Settings are useful with, for example, a dual Laser and single PMT Detector MicroRadiance CLSM. In this case a Sequential Setting could be created for each of two Fluorophores, but using the same PMT. This allows dual emission imaging in separate Panes, as long as the images are collected sequentially.

By pressing the 'Multi-Channel Exploration' button on the Control Panel, Lasersharp 2000 will automatically cycle between the three Sequential settings, scanning each associated Non-Merge Pane in turn, and also updating the Merge pane as it progresses.

Using the Mixer to Correct for Bleed Through between PMT Detectors

If you intend to use on-line bleed through correction (where, for example, a small amount of the red signal could be subtracted from the green signal, and a small amount of the green signal subtracted from the red signal), you only need to define **one** PMT Detector for each Non Merge Pane in step 4a (iii).

You may then use the Mixer (press the 'Mix' button on the Control Panel) to activate another PMT (click on the PMT icon to create an association shown by a black line) and set it to a negative percentage, e.g. -10%. When the Mixer is set up correctly for each Non Merge Pane, select 'Save Method' from the 'Method' pull down menu.

Note: If you subsequently edit such a Method with the Method wizard, the Mixer settings will be lost, and will need to be re-entered.

Methods Involving a Transmission Detector

Method Using Transmission Detector Only

To create a Method with **only** the Transmission Detector, you would need to create a Simultaneous and a Sequential Setting, however both of these would be identical, single pane settings.

In the Optics configuration window, you would activate one Laser Line (and set the percentage power to a value greater than “Off”), and also the Transmission Detector icon, Fig. 26.

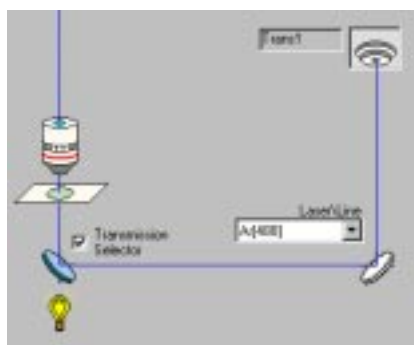



Figure 26 'Trans1' Detector activated in Optics configuration window

In the Assign detectors window, you would drag the Trans 1 icon () into the Non Merge Pane.

When operating the CLSM, the Laser Line power control **will not appear** on the Transmission Detector icon. However, it is easy to alter the Laser Line power by pressing 'Optic' on the Control Panel and using the Laser Line control spin box here.

Any Method with a Transmission Detector and other Detector(s)

If you wish to write a Method involving the Transmission Detector and one or more other Detectors (e.g. PMT Detectors), proceed in a similar way to the “Method Using Transmission Detector” example above, but there is one important issue to be aware of.

If the Transmission Detector Pane is set with a “Grey.lut” Look-Up Table, and other detectors are set, for example, to “Green.lut”, “Red.lut” or “Blue.lut”, then the Transmission Detector image will appear pixellated, as though at a lower resolution than the other panes.

To prevent this from happening, in Step 6 (Choose Look-Up Tables window) of the Method wizard, **do not define a Look-Up Table for the Transmission Detector Non Merge Pane**. When you later start imaging, that pane will be displayed as grey scale data anyway, by default.

This problem does not affect you if you intend to use a Look-Up Table other than “grey.lut” for the Transmission Detector Non Merge Pane.

When operating the CLSM, the Laser Line power control **will not appear** on the Transmission Detector icon. However, it is easy to alter the Laser Line power by pressing 'Optic' on the Control Panel and using the Laser Line control spin box here. Alternatively, you may operate the Laser Line control on one of the other Detector tabs.

Methods Involving More than Three Detectors

It is only possible to display three live Non-Merge Panes simultaneously. However, if you wish to use more than three Detectors in your method this is also possible.

For example, if you had highlighted three PMT Detectors and the Transmission Detector in the Optics configuration window (Step 8), then in the Assign Detectors window (Fig. 10) you may drag the

Transmission Detector icon () into Panes **1, 2 AND 3**.

Each pane would then have one PMT Detector, and “Trans1”, displayed in it.

When acquiring data, you may press ‘Mix’ on the Control Panel and assign each Non-Merge Pane (remember, a Mixer is essentially the same as a Non-Merge Pane) to be allocated to either 100% Transmission Detector, or 100% PMT *N*.

In this manner, although you can only acquire three Detector channels together, its is straightforward to look at the image from the Transmission Detector, without having to go to a different Method.

Other Information about Methods

Back Up Methods

Factory Methods

When a Confocal Imaging System is supplied new, Bio-Rad provides a set of Methods tailored to each system, to cover the most common range of imaging techniques. If at any stage you wish to return to this "default" set of Methods, please contact your local Bio-Rad Representative or email: confocal_support@bio-rad.com.

Backing Up Your Own Methods

If you wish, you may make a back up copy of all the Methods in use with Laserssharp 2000. This would be useful if, for example, some Methods were deleted unintentionally or altered by a less experienced User. To make a back up of **all** the Methods, proceed as follows:

1. Close Laserssharp 2000
2. Create a directory for your backup, e.g. C:\LaserssharpNT\backup\[today's date]
3. Copy the file c:\LaserssharpNT\database\lsmessages.mdb into your backup directory

To return to the back up copy at any time, proceed as follows:

1. Close Laserssharp 2000
2. Copy the file lsmessages.mdb from your backup directory into C:\LaserssharpNT\database, overwriting the existing file.
3. Run Laserssharp 2000

Note: This file may be too large to fit onto a floppy disc. To make the file fit onto a floppy disc without corrupting it, run the utility C:\LaserSharpNT\CompactDBWizard.exe . This will compact the existing database (LsMessages.mdb) , and also make a back up copy with a different name which includes the date. The compacted database may still be used with LaserSharp as normal.

Copying Methods

Methods may be copied between **Users provided that this is done whilst logged in to Laserssharp 2000 as the System User.**

When logged in as the System User “Default user”, all Users can be seen in the Methods Window.

In Fig. 27, all three Users with the three different User access levels are shown in the Methods Window – “Default user”, “Kate” and “Sam” (c.f. Fig. 1, when User “Sam” was logged in).

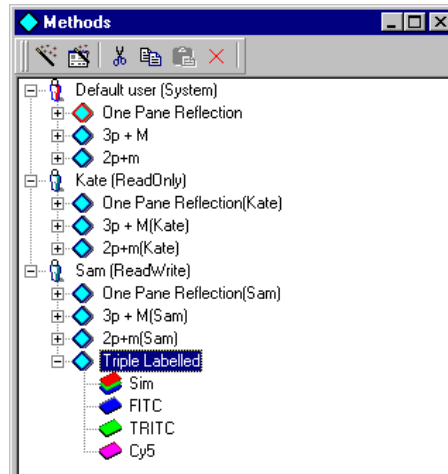


Figure 27 Methods Window, logged in as System User

To copy Method “Triple Labelled” from User “Sam” to User “Kate”, proceed as follows:

1. Highlight the Method as shown in Fig. 27

2. Click on the Cut icon ()

3. Highlight “Kate”

4. Click on the Paste icon ()

Editing Methods


You may edit a Method using the Method wizard in exactly the same way that you created it originally.

Editing a Faulty Method

If, upon selecting a Method for the first time, an error message appears reporting "...Method cannot fit to hardware configuration...", proceed as follows:

1. Press 'OK' when prompted if you wish to view the method errors log. The information given in the log file should help you to find the problem with the Method.
2. You will then need to edit the method you just created, but as it is not permitted to edit the currently selected method, at this point you will need to select a different method from the 'Methods' pull down menu.
3. Once this other Method is loaded, the 'Edit' icon in the Methods Window will no longer be greyed out.
4. Proceed with the 'Editing a Method' section, following, **ensuring that you select every Simultaneous Setting and Sequential Setting** from the Settings Manager window, press 'Edit' and then click on 'Next' until you reach the Settings Manager window again.

Editing a Method

1. Select the Method which requires editing from the Methods Window
2. Press the 'Edit' icon ().
3. Use the Method wizard in exactly the same way as in the Tutorial, to make any changes needed
4. Click on 'Next' until you reach the 'Finish method' window. Click on 'Finish'.
5. Select the method you have just created from the 'Methods' pull down menu.

Renaming Methods

It is not possible to directly rename a Method. Instead, proceed as follows to rename "*Method Old*" to "*Method New*":



1. Highlight "*Method Old*" in the Methods Window
2. Press the 'Edit' icon ().
3. In the 'Start method' window, change the name from "*Method Old*" to "*Method New*"
4. Click on 'Next' until you reach the 'Finish method' window. Click on 'Finish'.
5. You will now see "*Method New*" appearing in the Methods Window, in addition to "*Method Old*"
6. Highlight "*Method Old*" in the Methods Window
7. Press the 'Delete' icon ().

Table 1: Summary of Method Save Options

Property	Alterable and saved by Method Wizard	Alterable outside Method Wizard	Saved from 'Save Method' on 'Method' pull down menu
Detectors Used	Y	N	--
Number of Panes	Y	N	--
Merge Panes	Y	N	--
Lasers Used	Y	N	--
Laser Line transmission powers	Y	Y	Y
Dichroic Filters	Y	Y	Y
Emission Filters	Y	Y	Y
Blocking Filters	Y	Y	Y
LUT	Y	Y	N
Scan Rotate	N	Y	N
Scan Pan	N	Y	N
Scan Speed (x1, x2 or x4)	N	Y	N
Objective Lens	N	Y	N
Focus Motor Start/Stop/Interval	N	Y	N
Iris	N	Y	Y
Zoom	N	Y	Y
Gain	N	Y	Y
Offset	N	Y	Y
Photon Counting button	N	Y	Y
Box Size (pixels)	N	Y	Y
Scan Speed (lps)	N	Y	Y
Mixer Settings	N	Y	Y
Collection Filter	N	Y	Y

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Glossary

The following technical terms have specific meanings in this guide:

CLSM	Confocal Laser Scanning Microscope (a.k.a. Confocal Imaging System). The Instrument Control Unit (ICU) is built to a specific configuration; e.g. Lasers, Detectors, dichroic and emission filters, AOTF and excitation filter options etc.						
Laser	A laser unit, which may have single (e.g. Green Helium-Neon, 543nm) or multiple (e.g. Krypton/Argon, 488nm and 568nm) Laser Lines						
Laser Line	A single wavelength (<i>N</i> nm) of light emitted from a Laser						
Fluorophore	Fluorescent chemical for marking specific structures in a biological specimen; e.g. FITC, GFP, Chromomycin, auto-fluorescence. Excited by a specific Laser Line. Note that one Laser Line may be used to excite more than one Fluorophore.						
Lasersharp 2000	The software used to operate the CLSM						
User	A person who uses Lasersharp 2000 may be assigned a user name and password. Once logged in, they have one of three access levels: <table><tr><td>System</td><td>One User (only) may be classified as the "System Manager" or "System User", normally being the person who is most familiar with the CLSM. May create, edit or delete other Users and change access levels May create, edit or delete the set of Methods assigned to the "System User". May edit or delete Methods created by other Users, or copy Methods between Users May acquire data from the CLSM The default user name for the System User is "Default user"</td></tr><tr><td>Read/Write</td><td>Inherits a copy of the Methods which are associated with the System User (at the time the User was created). May edit or delete these copies. May create, edit or delete personal Methods May acquire data from the CLSM</td></tr><tr><td>Read Only</td><td>May not access the 'Methods' pull down menu May not acquire data from the CLSM</td></tr></table>	System	One User (only) may be classified as the "System Manager" or "System User", normally being the person who is most familiar with the CLSM. May create, edit or delete other Users and change access levels May create, edit or delete the set of Methods assigned to the "System User". May edit or delete Methods created by other Users, or copy Methods between Users May acquire data from the CLSM The default user name for the System User is "Default user"	Read/Write	Inherits a copy of the Methods which are associated with the System User (at the time the User was created). May edit or delete these copies. May create, edit or delete personal Methods May acquire data from the CLSM	Read Only	May not access the 'Methods' pull down menu May not acquire data from the CLSM
System	One User (only) may be classified as the "System Manager" or "System User", normally being the person who is most familiar with the CLSM. May create, edit or delete other Users and change access levels May create, edit or delete the set of Methods assigned to the "System User". May edit or delete Methods created by other Users, or copy Methods between Users May acquire data from the CLSM The default user name for the System User is "Default user"						
Read/Write	Inherits a copy of the Methods which are associated with the System User (at the time the User was created). May edit or delete these copies. May create, edit or delete personal Methods May acquire data from the CLSM						
Read Only	May not access the 'Methods' pull down menu May not acquire data from the CLSM						

Glossary (ctd)

Method	<p>A Method is designed for imaging a sample which is prepared in a specific way; for example a reflective sample, or a biological sample with certain Fluorophores.</p> <p>Laserssharp 2000 configures itself to suitable predetermined preferences according to the currently selected Method.</p> <p>When first logging in, the Method which was last used is selected. Methods comprise of one Simultaneous Setting and Sequential Settings.</p> <p>Methods are specific to users but can be copied or made available to other users.</p> <p><u>Methods are specific to the CLSM.</u></p>
Method Window	<p>Allows Methods to be created and edited. If logged in as System User, allows Methods to be copied and pasted.</p> <p>Accessed by clicking on 'Methods' pull down menu in Laserssharp 2000, and selecting 'Edit'</p>
Methods Wizard	<p>Wizard for writing new, or editing existing, Methods. Activated from the Method Window.</p>
Simultaneous Setting	<p>Every Method contains one Simultaneous Setting. Normally, every Laser Line and Detector used in the method will be active in this setting.</p>
Sequential Setting	<p>A Method contains a number of Sequential Settings. Each involves only one Laser Line, and one Mixer and Non-Merge Pane for displaying images.</p>
Detector	<p>A single channel physical detector module in the CLSM, which could be a transmitted light photo-diode, a transmitted light photomultiplier tube (PMT), an epi-PMT (confocal), an external detector PMT (Multi-Photon CLSM), and so on. Outputs a grey scale intensity to the Mixer.</p>
Control Panel	<p>Window containing numerous user alterable CLSM parameters on the right hand side of the Laserssharp 2000 screen, set up depending on the Method and Setting in use.</p>
Detector Tab	<p>On the Channels section of the Control Panel, each Detector which is activated in a Setting has a Detector Tab. On this tab can be found controls for the Laser Line transmission intensity, black level, gain, and sometimes other functions.</p>
Mixer	<p>There are three mixers available on the Radiance control unit. Each mixer can add or subtract three inputs from Detectors to any combination in real time, and produces a single output which is then passed to the computer for displaying in a Non-Merge Pane</p>
Non-Merge Pane	<p>Displays live image output from a single Mixer in a box on the screen. Can subsequently have software (processing) operators applied to it. The currently selected Non-Merge Pane is shown with a thin green line around it.</p>
Merge Pane	<p>Displays live image output from a combination of more than one Mixer</p>
Methods Database	<p>All Methods available when Laserssharp 2000 is in operation are stored in a single Microsoft Access format database file, C:\LaserssharpNT\Database\lsmessages.mdb. This database may not be altered except using Laserssharp 2000. It may be backed-up elsewhere for security. When a system is supplied new, a set of Methods is supplied which will allow the most commonly used fluorophores to be imaged.</p>
Look-Up Table	<p>A false colour algorithm which converts grey scale data into one or many colours dependant on the intensity of each individual pixel component.</p>