# PTI Fluorescence Master Systems

## *Felix32 Software User's Manual*

Version 1.21


300 Birmingham Road, P.O. Box 272, Birmingham, NJ 08011 Phone: (609) 894-4420 Fax: (609) 894-1579.
Website: www.pti-nj.com 

# PTI Standard Instrument Warranty

**Warranty Period and Extent**
Photon Technology International (PTI) warrants that its instruments will be delivered in a functional state and free from defect, and will meet stated specifications for a period of one (1) year. The warranty period will start on the date of shipment by PTI. In case of systems that include installation by PTI, the warranty will start from the date of installation or thirty (30) days after the shipping date, whichever is earlier.

This warranty is in lieu of all other warranties, expressed or implied, including, without limitation, the implied warranties of merchantability and fitness for a particular purpose, nor will PTI provide training on its use free of charge. PTI shall not be responsible for any liability, loss or damages, caused or alleged to be caused, by the system, as a result of use or operation including, without limitation, consequential damages and loss of profit.

**Specific Exclusions and Limitations**
1) It is recognized that the performance of consumable items will diminish as a function of use, and that it may be necessary to replace such items to restore the stated specifications. Consumable items (arc lamps, filters, cuvettes, lenses, etc.) are not covered by the warranty.
2) The original manufacturer's warranty will be maintained for major system components not manufactured by PTI (e.g. computers, printers, microscopes, cameras and components thereof).
3) Fiber optic bundles are not covered by the warranty.
4) The use of arc lamps not supplied by PTI (or approved in writing by PTI) will void PTI's warranty on all illuminator subsystem components.
5) If there is any evidence of physical contact with coated optics (e.g. fingerprints), the warranty on that item will be voided.
6) If the optical components are realigned by the customer without specific permission from PTI, the warranty will be voided. Please note that the customer is responsible for changing lamps and aligning the lamp after installation. Aligning the lamp will not void the warranty unless other exclusions are applicable (nos. 4 and 5).
7) Instrument systems that are not authorized to be installed by anyone other than PTI service personnel will not be warranted.
8) In case of systems that include installation as part of the original purchase, unpacking the instrument by anyone other than PTI personnel will void the warranty.
9) Moving systems to another site within a facility or to another location, without specific permission from PTI, will void the warranty.
10) Damage or loss caused by shipping is not covered by the warranty.
11) Damage caused by improper operation of the instrument will void the warranty.
12) Damage caused by equipment not purchased from PTI that is attached to the instrument is not covered by the warranty.
13) Warranty is valid only in the state, province or country of the original purchase.
14) Warranty is valid only on systems having a computer supplied by PTI.
15) Software upgrades performed on the PTI computer workstation (e.g., adding word processors, image editors, etc.) not authorized by PTI will void the warranty.
16) Hardware upgrades performed on the PTI computer workstation (e.g., adding network boards, sound cards, etc.) not authorized by PTI will void the warranty.

**Warranty Returns**
A Return Material Authorization (RMA) Number must be obtained from the PTI Service Department before any items can be shipped to the factory. Returned goods will not be accepted without an RMA Number. Customer will bear all shipping charges for warranty repairs. All goods returned to the factory for warranty repair should be properly packed to avoid damage and clearly marked with the RMA Number.

**Warranty Repairs**
Warranty repairs will be done either at the customer's site or at the PTI plant, at our option. All service rendered by PTI will be performed in a professional manner by qualified personnel.

**Software**
PTI makes no warranties regarding either the satisfactory performance of the software or the fitness of the software for any specific purpose. PTI shall not be responsible for any liability, loss or damages caused or alleged to be caused by our software as a result of its use, including, without limitation, consequential damages and loss of profit, nor will PTI provide training on its use free of charge.

# Contents

Chapter 1

# Introduction

## Welcome to the Family

Congratulations on your purchase of a fluorescence system from PTI. To help you get the most out of it and to safeguard your investment, please take the time to learn about your new instrument.

## About This User's Guide

**What it does**

This guide provides the following information:

1. Data acquisition for typical fluorescence techniques.
2. Data analysis functions for fluorescence data.
3. Saving and retrieving data.
4. Transferring data to and from other applications.
5. Basic hardware operation and maintenance.

**What it does not do**

1. Describe using Windows.
2. Explain the techniques of fluorescence spectroscopy.

For detailed information on fluorescence spectroscopy and time resolved measurements, please see the following references:

a) Guilbault, G. Practical Fluorescence, Marcel Dekker, New York (1990).

b) Lakowicz, J. Principles of Fluorescence Spectroscopy Second Edition, Plenum Press, New York (1999).

c) Rendell, D. Fluorescence and Phosphorescence Spectroscopy, Analytical Chemistry by Open Learning, John Wiley & Sons, New York (1987).

d) Valeur, B. Molecular Fluorescence: Principles and Applications, Weinheim Wiley, New York (2002)

**Guide Organization**

The guide has two main sections: Software and Hardware. The Software section applies to PTI's FeliX32™ for Windows. FeliX32 is the computer program used to control your instrument and to gather, analyze and store data. Since the operation of your instrument depends entirely on FeliX32, information on system startup and operation is included in the software section.

***Note:*** This manual is applicable only to FeliX32 version 1.00. Some PTI instruments may include other software. For information on those products, please refer to the manuals provided with them.

The Hardware section provides descriptions, operational settings, and maintenance procedures for the primary components and subsystems that are common to most PTI steady state fluorescence systems. For time resolved systems, please refer to appropriate hardware manuals.

***Note:*** Some systems may include options and accessories that are not covered in the hardware section of this manual. For information on those products, please refer to the materials provided with them. Conversely, there will be information on components that do not exist in your particular instrument configuration.

## PTI Fluorescence Instrument Lines Overview

All of PTI's fluorescence systems utilize an "open architecture" design that permits systems to be configured to best suit your needs. PTI has three open architecture fluorescence instrument lines:

***QuantaMaster™:*** Spectrofluorometers are used to measure steady state fluorescence and phosphorescence, as well phosphorescence lifetimes, luminescence, bioluminescence, and chemi-luminescence. FeliX32 is used with systems configured for steady state applications.

***TimeMaster™:*** Lifetime fluorometers measure fluorescence and phosphorescence lifetimes using PTI's patented strobe technique and gated detection. FeliX32 controls all acquisition modes and data analysis of the TimeMaster systems.

***MicroMaster™:*** Fluorescence microscopy systems. This comprehensive line of instruments can be subdivided into two categories that include photometry- [***RatioMaster™***] and imaging- [***ImageMaster™*** ] based systems. FeliX32 is a vital part of all RatioMaster systems as it features many powerful data collection and analysis tools designed expressly for ratio fluorescence work. Fluorescence and general imaging systems such as ImageMaster, measure intracellular ion concentrations and perform quantitative fluorescence microscopy and general imaging applications using a microscope and specialized imaging cameras.

For information on your system's hardware, refer to the *Hardware Overview* chapter.

**Chapter 2**

# Getting Started

Although we strongly recommend reading this manual before attempting to run your instrument, most users are anxious to get started. It is possible to learn as you go, but there are some basics to understand first. It should be noted the terms *trace* and *curve* are used synonymously throughout the manual.

**Important**: The system startup procedure has some critical steps that must become second nature to any system user. Otherwise, it is possible to damage the instrument as well as peripheral equipment attached to it.

## System Startup Procedure

**Warning**! When starting your instrument, if your system has an A1010B arc lamp, ***always ignite the lamp before turning on anything else!*** Otherwise, damage to the computer, the detector, or other sensitive subsystems could result. This point cannot be overemphasized.

1. On the LPS-220B Lamp Power Supply, set the LCD display control to Watts. Press the power button (it is illuminated when powered). Turn the current knob to vertical. Wait 10 seconds, then press and hold the Ignite button. You will hear an audible click as the lamp ignites. When the LCD display shows the wattage reading, the lamp has ignited. Release the Ignite button and allow the lamp to warm up for 15 minutes. Finally, adjust the Current control to display 75 watts. If the lamp does not ignite, see the Troubleshooting section at the end of this manual. It should also be noted that the LPS-220B has the ability to auto-ignite. By changing the switch on the back of the power supply the lamp will automatically ignite when the power button is pressed. All other steps must still be followed to maintain proper lamp operating conditions.

**Warning!** The ignition of an arc lamp requires a very high voltage pulse. A high voltage transient may be injected into the electrical system of the instrument. This transient can cause a read/write error or even damage your computer or other system components. PTI recommends that the LPS-220B be connected to a separate electrical line and that all other components (computer, motor driver, BryteBox, etc…) be connected to a surge suppressor on an isolated line. Contact PTI if you have any questions.

2. If you have other light sources than the arc lamp, please follow the appropriate procedures outlined elsewhere.

3. In no particular order, power up the Motor Drive Box, the BryteBox interface module, Shutter Controller, DeltaRam Power Module, Control Module, XenoFlash power supply, Delay Gate Generator, Nitrogen Laser, Peltier temperature controller & stirrer, and the Chopper Controller along with any other components your system may contain (as applicable).

4. Turn on the computer and launch FeliX32. You will be prompted to enter a user ID and a password to access the database. The default user name is *Administrator* and the default password is *pti* (password is case-sensitive).

5. To start collecting data, choose **Acquisition/Open Acquisition** to open a previously saved acquisition or **Acquisition/New Acquisition** to create a new acquisition. If selecting a new acquisition, a dialog box will open allowing you to select the type of acquisition you would like to perform (Emission Scan, Excitation Ratio, Fluorescence Decay, etc…). Selecting one of these options will open an acquisition window where you can choose the appropriate hardware configuration and input the experimental parameters. If you have just installed or upgraded FeliX32 and it was not configured for your hardware, you must create a hardware configuration that comprises the components in your system. Click on **Configure/New Hardware Configuration** to create a new hardware configuration. To modify an existing hardware configuration choose **Configure/Hardware Configuration.**

6. If you are using a steady state system, turn on the Photomultiplier Detector(s). They are powered from the Motor Driver Box or optional dedicated power supplies. The High Voltage control should be set to –1000 volts and in most cases should not need to be adjusted. The displayed voltage is the negative of the applied voltage.

7. You can now acquire data.

**Note.** You can analyze previously collected data ("off-line analysis") without starting the instrument. Remember to turn the computer off should you want to subsequently start the instrument and ignite the arc lamp. Data analysis can be performed on a different computer loaded with FeliX32.

Now that your system is up and running, it is time to learn FeliX32. As a bare minimum, we recommend reading the section *Getting the most out of FeliX32* in Chapter 3, and all of Chapter 4.

Chapter 3

# Software Overview

## What is FeliX32?

FeliX32 software is used for fluorescence data collection and analysis. The program runs on computers using Windows 98, 2000, or XP operating system. Since FeliX32 conforms to Windows conventions, the user interface is very similar to that of other Windows programs such as Word or Excel.

FeliX32 controls the instrument, collects the fluorescence data, and provides a number of tools needed for data analysis and presentation. The acquired data can be displayed in a variety of ways during and after acquisition. The versatility of Windows makes it easy to customize the display format. In time, you will develop your own preferences depending upon how you organize your experiments.

After the data is acquired, it can be analyzed in a variety of ways. The traces can be zoomed to look at a small region and/or mathematically manipulated and combined. Calibration curves can also be constructed.

Within FeliX32, data can be exported to other Windows programs for additional analysis or other processing. You can either use the Windows clipboard to transfer data from one open Windows application to another, or export your data in standard text file format for subsequent importation.

Windows handles printing and plotting from FeliX32, so you have a wide choice of output devices. Older or obsolete printers and plotters may not have Windows drivers. Contact the printer manufacturer to see if the correct Windows operating system drivers for your printer are available.

## Getting the most out of FeliX32

**Are you familiar with Windows?**

If you are familiar with Windows, you will be able to use FeliX32 immediately. If you are not familiar with Windows read through the Windows documentation. There are also online Windows tutorials. Once you acquire basic Windows skills you will quickly find what you need to get started and then pick up more detailed information as you go.

**Use the Help utility**

FeliX32 has an online Help utility that works similar to the Windows Help utility. Although FeliX32 came with this user's manual, the Help utility has more up-to-date information. There are also several sections in the Help utility that are not included in the user's manual, such as A Quick Review of Fluorescence Spectroscopy, Getting the Most from Your Instrument, and a detailed How To section.

 **Hint**. You can get a good feel for how FeliX32 works by reviewing some of the Help topics before using the program. You can do this without even running FeliX32 by double clicking on the FeliX32 Help icon that was added to the Windows Start Menu during installation. Explore FeliX32's menus and commands and learn what they do. Get to know the primary features of FeliX32 and some of the terminology used throughout the program. Most topics have several pop-up definitions and cross-references so you are never far away from the information you need.

**Help for FeliX32 is always available!**

You can instantly get on-line help several ways:

1.  Click on the Context Help button on the toolbar. The mouse cursor turns into a question mark that will bring up the Help topic for any item on the screen when you click on it.

2. Or press **F1** to see the Help topic for the currently active function. For example, if a dialog box is open, pressing F1 will bring up the Help topic for that dialog box. As a reminder, the message "For Help, press F1" is visible in the bottom left corner of the FeliX32 window, on the status bar.

3. Or click on the **Help** button that appears in most dialog boxes to bring up the Help topic for that dialog box.

In addition to the Help utility, FeliX32 provides brief descriptions of commands in two ways:

1. When you click and hold on a command in a menu, a message will appear on the status bar in place of the Help reminder. To avoid invoking an unwanted command, slide the cursor off the menu and then release the mouse button.

2. "Tool Tips" will pop up next to the cursor when it is positioned over most toolbar buttons. They will appear without having to press the mouse button.

**Keep Help handy**

For easy access to a specific Help topic while you are running FeliX32, select **Always On Top** in the Help menu of the Help utility window. This will keep the Help window visible while you work in FeliX32. You can resize and reposition the Help window to keep it out of your way.

To keep the screen less cluttered, you can toggle between the FeliX32 and Help windows. After opening Help from within FeliX32 and selecting the topic you want, press **Alt+Tab** simultaneously on the keyboard. Each time you press Alt+Tab, you will toggle between FeliX32 and Help (this only works if Help and FeliX32 are the only two windows opened, otherwise this will cycle through all opened windows). You can also make a hard copy of a Help topic by selecting **File/Print Topic** in the Help window.

For more information on using the Help utility, choose How to Use Help in the Help menu.

## Computer Requirements

For proper operation, the Photon Technology FeliX32 Software package requires:

1. PC compatible computer.

2. 500 MHz processor.

3. 128 Mb system RAM.

4. 50 Mb free hard drive space (need >50 Mb to store data).

5. Spare Ethernet connection.

6. Operating system: Windows 98SE, Windows 2000, or Windows XP.

**Note.** Some antivirus software packages may cause faults in the way FeliX32 communicates with the BryteBox. For more information, please contact your PTI Customer Service Representative.

## Installing FeliX32

1. Turn on your computer and wait for Windows to finish loading.

2. Insert the FeliX32 CD into the CD-ROM drive.

3. The installation should start automatically after Windows recognizes the CD. If not, on the task bar, select Start/Run. Type **D:\SETUP.exe** (or use the appropriate CD-ROM drive letter) in the entry field, and click OK.

4. Follow the onscreen instructions to install FeliX32.

    a) Enter the User Name (if different from displayed), of the registered user of the PTI FeliX32 Software. Then enter the Organization the registered user belongs to, if that Organization is the owner of the software. Under *Install this application for*, select *Anyone who uses this computer (all users)* if you wish this application to be available to any user that can log into the computer where the software is installed. Otherwise, select *Only for me (User)* if this application is to be available when the specified used (defined in "User Name" is logged on the computer.

    b) From the **Setup Type** window, most users will select *Typical* for a full install. You may choose *Minimum* if you will only be performing steady state measurements and do not need the Admin. Tool to administer users in the program database. Selecting *Custom* will give options on which components will be installed. Select Custom only if you are an advanced user of the PTI FeliX32 Software package.

    c) The default folder created for FeliX32 during software installation on the C: drive is **C:\Program Files\PTI\FeliX32**.

    d) Click *Install* to proceed with the installation after verifying the install options in the summary window. Upon completion, click *Finish* to exit the install wizard.

    e) You may terminate the installation program at any time by selecting **Cancel**.

**Note.** If you are updating FeliX32 with a new version, you must first remove the old FeliX32 software using **Add/Remove Programs** located in the Windows Control Panel. The database will not be removed. The new version of FeliX32 will be installed in the existing FeliX32 folder and it will use the existing FeliX32 database.

**Note.** For FeliX32 to communicate with the BryteBox your network card must be setup properly. The following is a general guide to setting up your network card.

1. From the Windows Control Panel select **Network and Dial-up Connections**.

2. Right click on **Local Area Connection** (LAC) and select *properties*. If you have more than one network card select the LAC connected to the BryteBox.

3. In the box *Components checked are used by this connection*, select **Internet Protocol (TCP/IP)** then click on properties.

4. Click on the radio button, *Use the following IP address* and enter the following IP address in the space provided: 192.168.030.2

5. In the *Subnet mask* field enter the values: 255.255.255.0. The field may fill automatically after entering the IP address.

6. Click OK to save the settings and exit from all the menus.

7. Restart the computer and BryteBox. FeliX32 should now be able to communicate with the BryteBox.

**Changing Screen Resolution**

FeliX32 is designed to work on most Windows-based computers at any screen resolution. However, for the best operation and appearance, we recommend running your computer monitor at 1024 by 768 (800 x 600 minimum) (16 color minimum, 256 color or more recommended) resolution.

To change the screen resolution, right-click anywhere on the desktop and choose Properties. Choose the Settings tab. Select the desired settings in the scroll-down boxes for color depth and screen resolution. Then restart the computer.

**Note.** If for some reason your monitor will not function with the new settings you selected, restart the computer in "Safe Mode" and restore the screen settings using the following procedure:

1. Press the computer Reset button or power down for 20-30 seconds and restore power.

2. When you see the message "Starting Windows," press F8.

3. Choose Safe Mode from the startup options.

Restore the screen settings and restart the computer again by choosing Start/Shut Down/Restart.

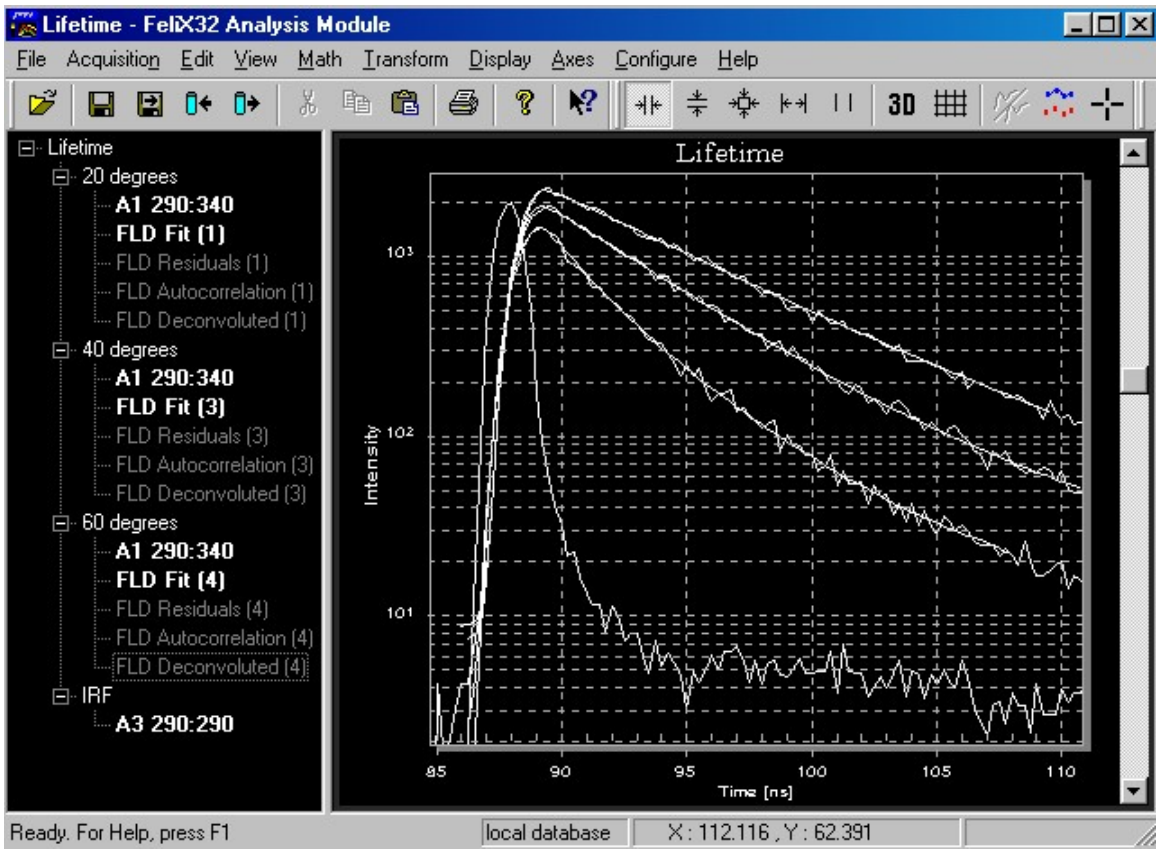
Chapter 4

# A Quick Tour of FeliX32

## Workspace

When FeliX32 is launched, the Workspace will be opened. Across the top, beneath the Title Bar, is the Menu Bar. Each heading in the Menu Bar represents a group of related commands. Below the Menu Bar is (optionally) the Toolbar. The buttons on the Toolbar provide instant access to a number of the most frequently used commands.

Beneath the Menu Bar and Toolbar is the space where datasets are opened for the viewing and processing of traces. Multiple datasets can be opened within the workspace although only one will be active at any give time. For more information see, Working with Windows in the FeliX32 Help utility.



## Menus

When you click on one of the menu items detailed below, a list of available commands will appear. You can see a brief description of any command on the status bar when you click and hold the mouse button on a command. (To avoid invoking an unwanted command, slide the cursor off the menu before releasing the mouse button.)

All of the commands are detailed in the following chapters.

**File** The File menu is used to open a new dataset, save data, import and export ASCII files and FeliX32 (along with FeliX 1.X and TimeMaster Pro) data. Printing is also accomplished from this menu. Some of these commands will appear as buttons on the Toolbar.

**Acquisition** From this menu, new acquisitions and macros can be created opened. Acquisition conditions are set up after selecting the technique for the acquisition. The fluorescence techniques available include excitation and emission ratio, excitation and emission scan, timebased and timebased polarization, multi-dyes, and synchronous scan. If you have a TimeMaster system, you may take advantage of additional acquisitions including, among others, fluorescence and phosphorescence decay and time-resolved spectra.

**Edit** The Edit menu has commands to cut, copy, or paste curves. Most of these commands utilize the Windows clipboard. Some of these commands will appear as buttons on the Toolbar.

**View** The commands in the View menu toggle the visibility of the various toolbars and TimeMaster control/output windows.

**Math** Acquired data can be processed mathematically through the commands in the Math menu. Two curves can be arithmetically combined and data can be fitted, smoothed, averaged, integrated, normalized, differentiated, etc… There is also a submenu for TimeMaster decay analysis functions that include but are not limited to 1-4 exponential fit, global analysis, MEM and ESM, and anisotropy decays.

**Transform** Transform is used to convert data into a concentration or polarization/ anisotropy values. You can also do post-acquisition emission and excitation correction.

**Display** The format of the active data Window is controlled from the Display menu. Some of these commands will appear as buttons on the Toolbar.

**Axes** The commands in the axes menu allow expansion or contraction of the axes for viewing and analyzing specific regions of a curve. The labels applied to the axes may also be altered. Some of these commands will appear as buttons on the Toolbar.

**Configure** The Configure menu is used to explicitly define the hardware components that are being used with FeliX32. It is critical that the hardware configuration be correct. The Configure menu is also used to modify user accounts and set system preferences.

**Help** The Help menu provides access to information on using Help and the Search function.

## Data Acquisition

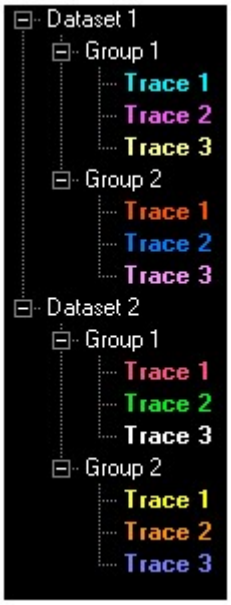
Fluorescence experiments are set up and run from the Acquisition menu. The acquisition menu items represent basic experimental techniques; for instance, excitation wavelength scans, emission wavelength scans, excitation ratios, fluorescence decays, phosphorescence decays, etc… Thus, only those instrument controls necessary for a given technique appear in an acquisition dialog box.

Additionally, common to all acquisition dialog boxes are controls to load/save Acquisition Setup files, to view acquired data in user defined modes, and to start/stop/pause an experiment.

Each of the acquisition menu items is introduced and its experimental applications and procedures are presented in the *Acquire Commands* chapter. Hardware control functions unique to these procedures are also detailed.

## Database Management

A dataset is comprised of multiple groups containing multiple curves. When acquiring data, you may be collecting several channels of information. This will result in several curves or traces. Using the options in the Display Setup dialog box, you can place them together in a single group or split them up into multiple groups. In either case, the curves will have a common acquisition and be placed within the same dataset.

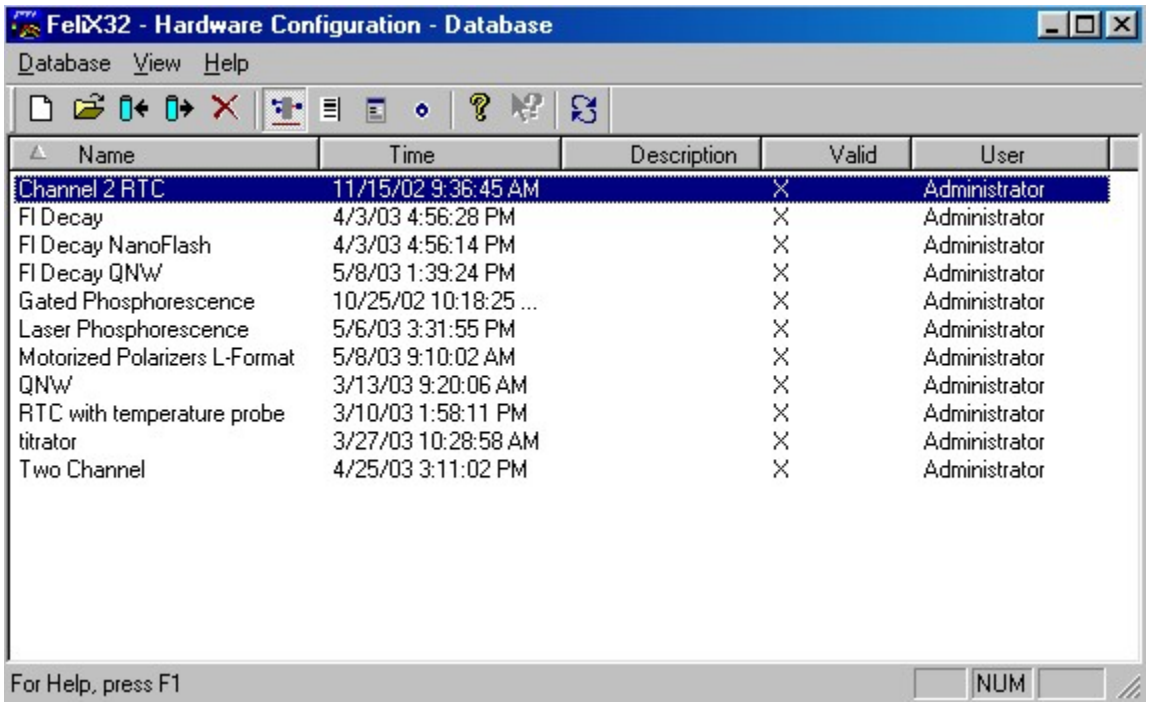



A dataset is what is saved to the database. The database is a new feature for FeliX32. Its purpose is to help save your data files, acquisition setup files, hardware configuration files, annotation files, and correction/transformation lookup curves in a consistent manner so that they can be retrieved when necessary with a minimum of effort.

Information in the database can be accessed via the **Database Control Window**. Categorical lists of *hardware configurations*, *acquisition scripts*, *macros*, and saved *acquisitions* make up the Database Control Window and allow for easy management of the database. Entries are stamped with the time and date, user name, and a brief description when saved. Hardware configurations contain an additional *Valid* field. A checkmark appearing in this column signifies that the hardware configuration, on a basic level, has been created properly. If there is no check, the configuration is invalid and must be edited before it can be used.

Selecting an entry gives one the opportunity to either open or delete the file. Hardware configurations and acquisition scripts cannot be removed from the database if they are referenced by saved macros or acquisitions.

Several methods exist to open the Database Control Window. From the main FeliX32 window select any of the following from the menu; *Acquisition/Open Acquisition; Acquisition/Open Macro; Configure/Hardware Configuration;* or *Configure/Script Configuration.* Or select *View/Database* or click on the database icon ( ) from an open hardware/script configuration, macro, or acquisition window. Both methods open the Database Control Window to the corresponding list. The other lists can be selected from the *View* menu or by clicking the appropriate toolbar icon.



File management options are located under **Database**. These include, with corresponding toolbar icons, opening (  ), deleting (  ), importing (  ), exporting (  ), and creating new entries (  ). A file must first be selected to enable export and delete commands. Only the selected file can be deleted or exported. Likewise, if using the toolbar icon to create a new entry, by default the new file will be of the same type as those in the currently displayed list. For example, if the macro Database Control Window is open, selecting the *new* toolbar icon will open a new Macro Command Editor dialog.

From the **View** menu, the user may select the database list they wish to display. The current open list will have a checkmark beside its name in the menu. Toolbar icons exist for each window list. They are; acquisition (  ); hardware configuration (  ); script configuration (  ); and acquisition macro (  ). The toolbar and status bar can be toggled on/off by selecting them in View. A checkmark will appear beside their names in the menu when they are turned on. *Refresh* (  ) updates the list to display any changes. The View menu also has links to acquisition *Preferences* and the S*tatus Window*. See later chapters for more information on these commands.

At some point you may want to distribute files or import and store files from colleagues. Most stored items in the database can be exported from their respective menus. Datasets and groups can be exported to another computer for data analysis by FeliX32 to allow the system to remain free for other experiments. Individual groups can be exported as text files for further analysis in an external software program. It is also possible to import setup files, datasets, and groups into the database. If importing text files they must have the appropriate structure.

| Extension | Contents |
|---|---|
| .ana | FeliX32 dataset |
| .ang | FeliX32 trace group |
| .acq | Acquisition setup |
| .hwc | Hardware Configuration |
| .txt | Text file (for importing or exporting ASCII data) |
| .wac | Macro Program |
| .res | TimeMaster results file |
| .fsc | Acquisition script |

## Macro Programming

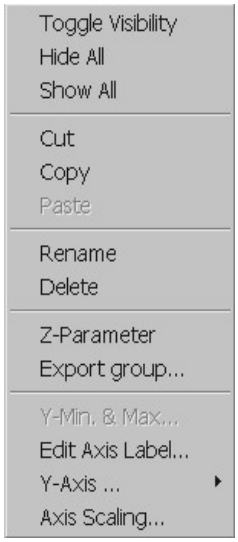
All data acquisition operations in FeliX32 can be automated using macro programs. You can create these custom programs easily with the FeliX32 Macro Program Editor. Creating macro programs requires no programming knowledge or experience. Virtually every acquisition command normally available in FeliX32 is featured in the macro editor, while some commands are exclusive to the macro command editor.

Details on using the Macro Command Editor are provided in **Chapter 7.**

## Legend Commands

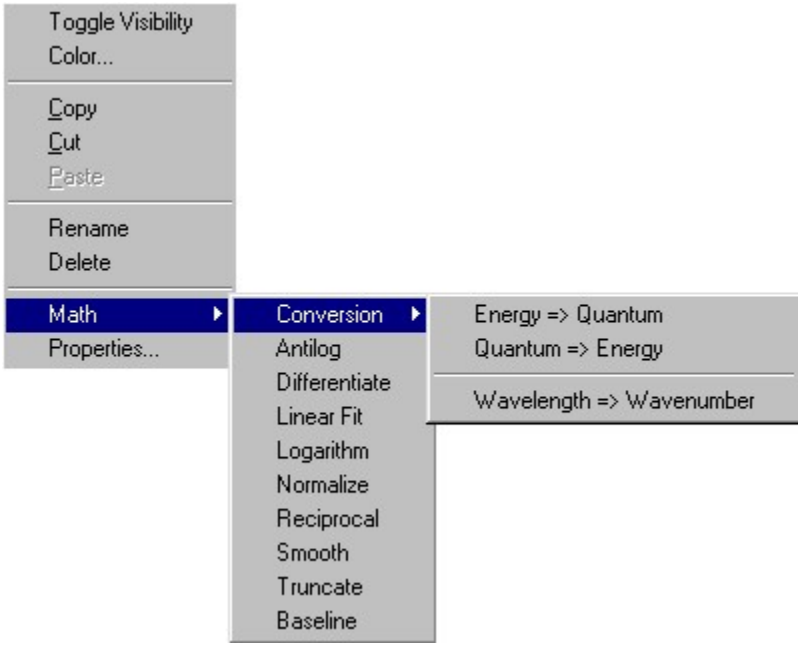
Right clicking on a curve, group, or dataset in the legend opens a menu with links to commands found in the main menus. The following comprises an overview of the listed commands found in each menu for right clicking on a curve, group, or dataset. Some options can only be accessed from these menus and they will be described in more detail. Information on the other commands can be found in later chapters.


**Dataset**: Select *New Group* to input a new group in the database. A dialog will open allowing the group to be titled. *Import Group* opens a Windows dialog to search for the group or file from the hard drive to import. Available files include .ang for groups, .spc for Grams files, and .txt for ASCII files. Datasets can only be exported as proprietary .ana files.




**Group**: Use *Toggle Visibility* to show/hide all the curves in the group. Curves that were hidden will become visible and curves that were visible will be hidden. To hide or show all of the traces within a given group select *Hide All* or *Show All* from the menu. *Export Group* will export all the traces within the group as an .ang file or as an ASCII .txt file. Select *Rename* to change the title of the group and *Z-Parameter* to assign a numerical Z-axis value to each curve in the group for 3D displays. The axis labels and units can be altered using *Edit Axis Labels*. Change the units to display the traces in the group on a different Y-axis. Select *Y-axis* to assign the group to a separate Y-axis than the rest of the acquisition. The new Y-axis will show to the right or left of the graph.

**Curve**: Right clicking on an individual curve allows the user to *Toggle Visibility* of the curve, change the *Color*, view acquisition *Properties*, and transform it with *Math* functions. Selecting properties opens a dialog displaying such information as; the name of the trace; any assigned Z-value; the function; the type of acquisition used to measure the trace; what corrections were used; the background employed; integration times; and any other acquisition setup commands. The math functions act as simplified versions of those found in the main **Math** menu. For example, selecting *Normalize* from the popup window automatically normalizes the trace at the peak to a value of one. The same normalize command located in the Math menu gives the user full control over how they wish to normalize the curve. Additional normalize commands include selecting the X-axis position and Y-axis value to which the trace will be normalized.



Chapter 5

# File Commands

## Open…

Use the Open command to open a group or acquisition stored in the database. Opening a dataset will automatically create a workspace and display all of the groups stored in the dataset within the window.

The File/Open command opens the recorded dataset dialog box, which provides detailed information about the data set and the curve(s) contained in each group. You can examine the information without opening the data set by clicking on the name in the description list.

The dialog box contains the following features:

**Description field**

Contains a list of all the data sets saved in the database. Each entry has the date and time of acquisition as well as identification of the user of the record. Click on each title to sort the datasets by that field. Sample and comment fields can have additional information about the experiment if the user chooses to fill these fields during the saving process.

**Show only my acquisition**

If this box is checked then only the records of the particular user logged onto the system will be displayed. Note that, in the administration part of the software, each user can be assigned different privileges.

**Open read only**

The administrator can set up various acquisition records that are in read only format and can be accessed by all users. This option is particularly useful for large groups where routine acquisitions are carried out.

**Groups**

When a data set is selected, all the groups in the record are displayed in this section and each group can be selected individually.

**Traces in selected group**

When a group is selected all the traces saved under that group are listed in this field.

**Shortcut:**  Use the toolbar button or press **Ctrl+O** on the keyboard.

## Import...

You can import FeliX 1.X and TimeMaster Pro as well as ASCII data files directly into FeliX32.

**File Name**

Type the name of the file to import.

**Look In**

Select the drive and the directory that contains the file(s) to import. Note that FeliX32 uses several file extensions:

.ana – analysis dataset files
.ang – analysis group files
.flx – FeliX files
.txt– FeliX text files
.spc – Grams spectral files
.tma – TimeMaster files
.res – TimeMaster results files
*.* – All files

ASCII data to import will be stored in .txt files. See the FeliX32 online help utility for information on acceptable ASCII file structure.

**Shortcut:**  Use the toolbar button.

## Insert New...

Allows one to add additional datasets into an open acquisition window. A dialog box opens prompting the user to name the new dataset by selecting this command.

## Save

This command to saves the contents of the dataset to its current name. When saving an untitled acquisition, FeliX32 presents the **Save As** dialog box so you can name and save the data. You will also be presented with a **Summary Information** dialog box that enables you to input the name of the sample and any special experimental conditions.

If you want to change the name and/or directory of an existing acquisition, open it and choose **File/Save As**.

**Shortcut**:  Use the toolbar button or press **Ctrl+S** on the keyboard.

## Save As...

Use this command to name and save the contents of the active window. Use it to save a new untitled dataset or to save an existing dataset under a different name.

To save a file with its existing name, use the **File/Save** command.

**Name**

Type a name in the field.

**Acquired by**

This field is filled automatically and it records the current user logged onto the system.

**On**

This field is defaulted to the date and time of saving.

**Sample**

Provide additional information for the particular acquisition being saved.

**Comments**

This field is provided for any particular information that is required for identification of a particular experiment by the user.

**Shortcut**:  Use the toolbar button.

## Export...

You can export the contents of a dataset or a group to a separate file on your hard drive or other storage device. Right click on a group within a dataset to export the contents of that group in a text (ASCII) format. Hardware configurations, acquisitions, and other FeliX32 components can also be exported for use on other systems.

The **Export** command opens a typical Windows save dialog box where you can name the file and select a directory to export the data. Refer to Windows documentation for details on using this dialog box.

**Shortcut**:  Use the toolbar button.

## Close and Close All

The **Close** command closes the active dataset and **Close All** closes all the open datasets. FeliX32 will alert you to save changes to your dataset before you close. If you close a dataset without saving it, you lose all changes made since the last time you saved it. Before closing an untitled dataset, FeliX32 displays the **Save As** dialog box so you can name and save the dataset to the database.

## Summary Info…

Summary Info attaches user-specified notations to the file in the active window. Use Summary Info to help you remember the sample, sample preparation details, etc… The information is stored with the file upon saving.

**Name**

Enter the name of the dataset.

**Acquired By**

This is set by FeliX32 and cannot be changed. The name in this field is dependent upon the user profile that acquired the data.

**On**

The date as defined by the computer that the dataset was acquired on. This parameter is set by FeliX32 and cannot be changed.

**Sample**

Enter the sample identification information up to 50 characters.

**Comments**

Enter special information about the sample, the experiment, etc…

## Print

Use this command to print the contents of the active workspace. This command opens a dialog box where you can specify the range of pages to be printed, the number of copies, the destination printer, and other printer options.

Refer to Windows documentation and online help for details on using this dialog box.

Shortcut:  Use the toolbar button or **Ctrl+P** on the keyboard.

## Print Preview

Use this command to display the active workspace as it would appear when printed. When you choose this command, the main window will be replaced with a print preview window.

Refer to Windows documentation and online help for details on using this dialog box.

## Print Setup...

Use this command to select a printer and a printer connection. The options allow you to select the destination printer, its connection, paper size and layout, and other printing related options.

Refer to Windows documentation and online help for details on using this dialog box.

## Exit

Use this command to end your FeliX32 session. FeliX32 will prompt you to save files that have not been saved.

**Shortcut:** Click the Window Close button (X) in the top right corner.

Chapter 6

# Edit Commands

## Undo

This command reverses the last performed edit operation.

## Cut

This command cuts the selected curve(s) from the active workspace and places the data on the Windows clipboard. Note that, unlike the **Delete** command, the selected data is not lost because it is transferred to the clipboard. However, any data that was previously on the clipboard will be lost. For more information on the Windows clipboard, refer to the Windows documentation.

**Shortcut:**  Click on the toolbar button or **Ctrl+X** on the keyboard.

## Copy

This command copies the selected curve(s) to the Windows clipboard. Any data that was previously on the clipboard will be lost. For more information on the Windows clipboard, refer to Windows documentation.

**Shortcut:**  Click on the toolbar button or **Ctrl+C** on the keyboard.

## Paste

This command places the contents of the Windows clipboard into the active workspace. For more information on the Windows clipboard, refer to Windows documentation.

**Shortcut:**  Click on the toolbar button or **Ctrl+V** on the keyboard.

## Paste as New Data

This command allows you to create a new dataset and place the contents of the clipboard into it. For example you can select one trace from each of the groups in your experiment and create a new dataset with one group containing all the selected curves. When you select this option a dialog box prompts you to name the new dataset.

## Delete

This command deletes the selected curve(s) and/or groups from the window. Note that, unlike the **Cut** command, this command does not copy the data to the Windows clipboard. Deleted curves are permanently removed from dataset and cannot be recovered.

**Shortcut:** Press the **Delete** key on the keyboard.

## Rename

This command changes the name of the selected curve, group, or dataset as it appears in the legend and also in the file when it is saved.

**Note**. Curve names are initially derived from the inputted parameters and labels assigned to the interface signal channels. You can change these labels in the Configure/Hardware Configuration dialog box to provide more descriptive curve names during acquisition.

**Shortcut:** Right click on the curve, group, or dataset name in the legend.

Chapter 7

# Acquisition Commands: Steady State

Experiments are invoked and data is acquired by selecting New Acquisition and the type of acquisition desired for the experiment from the resulting popup dialog box. Each acquisition command opens a data acquisition setup dialog box. The items in each box are organized according to the experiment type.

Because many of the items are common to all dialog boxes, they are presented together under the heading *Common Configuration Settings*. The descriptions for the configuration dialog boxes that follow provide details only for the settings that are unique to them.

All acquisition dialog boxes contain buttons that start, stop, and pause the data acquisition process, load and save setup files, etc… These are described under the heading *Acquisition Controls* in **Additional Acquisition Commands and Controls**. This portion of the chapter also has information on additional configuration and control dialog boxes that are accessed from within the configuration setup dialog boxes. These are presented under the headings *Additional Acquisition Setup Controls, Display Setup* and *Four Position Sample Turret* and *Data Collection Options*.

**Note.** The contents of any given dialog box may vary slightly depending on the particular hardware configuration of your instrument.

## Common Configuration Settings

The following acquisition controls appear in most dialog boxes.

**Acq. Background & Use Background**

Clicking the **Acq. Background** checkbox (at the top of the dialog box) will allow the acquisition of the background correction values. When the measurement is started using a reference or blank sample, the average of the acquired values is placed into the background buffer. When acquisition is restarted the second time, the background acquisition checkbox is automatically cleared and the just-acquired background values will be subtracted from the current measured values in real time. The background correction values will remain in effect for subsequent measurements until cleared in the Display Setup dialog box, or by removing the check mark in the **Use Background** checkbox located beside the Acq. Background check control, or by checking the Acq. Background checkbox, which will clear the previous value and force a new background to be acquired. Toggling Use Background keeps the background value in memory for future use. Care must be taken when using scanning dialogs since the background may change as a function of wavelength. Thus for these types of acquisitions a separate scan may be required of a blank sample that will be manually subtracted from the experimental sample to produce an accurate background measurement.

**Points/sec**

Enter the number of data points to be acquired each second. For fast processes, this parameter can be as high as 1000 points/second. The more data points that are collected per second, the greater the peak-to-peak noise associated with the signal. Conversely, the fewer data points are collected per second, the better signal-to-noise ratios obtained. The lowest value that FeliX32 will accept is 0.01 points/sec. For ratio experiments with a chopper, the chopper must be set to **Continuous** (see the *Additional Configuration Settings* section) for this setting to have effect.

There is no practical limit in FeliX32 to the total number of points that can be taken during an experiment. The real limit is the available space in your RAM. Avoid taking an excessive number of data points though, because the processing and analysis time takes longer.

**Integration**

A longer integration time will reduce the noise but extend the time of the measurement. For ratio experiments with a chopper, the chopper must be set to **Stepping** or **Stationary** (see the *Additional Configuration Settings* section) for this setting to have effect. FeliX32 will accept values from 0.0001 to 1000.

**Duration**

Data collection will continue until the time interval entered has elapsed. If the **Repeats** are set to a value greater than zero the duration is only the time of each repetition.

**Repeats**

Enter the number of times the experiment will be repeated. The time axis will continue to increment throughout the **Pauses** and subsequent **Repeats**, and all of the repeated experiments will be saved in the same file. By using **Repeat** and **Pause**, you can examine a dynamic sample over a long period of time. This is not available for wavelength scanning or temperature ramping experiments.

**Step Size**

For wavelength scan experiments, the step size value controls the scan rate. A smaller step size increases the resolution of the measurement, but also increases the time of the experiment. Allowable values depend on the monochromator used. The DeltaRam V can accept values as low as 0.5 nm, while the model 101M monochromator can accept 0.25 nm with a standard grating.

**Averages**

This is the number of times that the experiment will be repeated, over the course of which the intensities will be averaged on a point by point basis. The number of the experiment as it is running appears on the Status Bar. This is not available for timebased experiments.

**Pause**

Enter the time to pause between repeated experiments. This is not available for wavelength scanning or temperature ramping acquisitions. Pause only becomes available if the **Repeats** are greater than zero.

**View Window**

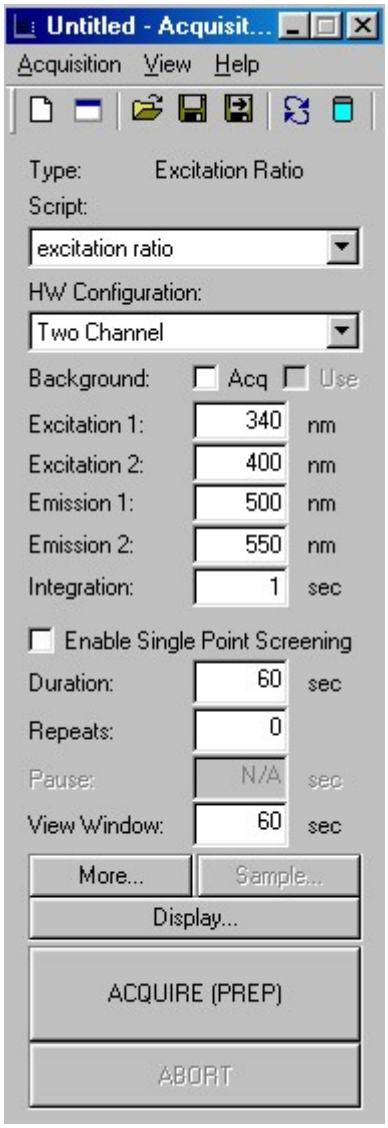
Enter the time segment that will be displayed on the X-axis. The **View Window** setting can be less than or equal to that of the **Duration**. If the **Repeats** are set to a value greater than zero, than the default View Window is equal to the sum of all repeated durations, plus **Pause** times. If a four position turret is used with more than one sample position, this is also in, but the turret slewing time is not factored into the default View Window. The data will be automatically shifted across the X-axis as each window is filled. Use a short view window for highest resolution of time.

**Single Point Screening**

When enabled, **Single Point Screening** will enter the values measured into a spreadsheet grid, one at a time, so that you can evaluate the progress of an experiment in detail. A value is measured and entered in the grid when the **Capture Value** button is pressed.

## Excitation Ratio

**Excitation Ratio** is used to set up and run experiments for intracellular ion determinations using excitation-shifted probes such as Fura-2 for calcium and BCECF for pH. In this experiment, the excitation source must alternate between two different excitation wavelengths that are characteristic of the probe. The emission intensity at both excitation wavelengths is measured at a longer emission wavelength and the ratio of these intensities is calculated. The ratio is proportional to the concentration of the ion under investigation.




**Excitation 1,2**

Enter the excitation wavelengths in the text boxes. Your instrument will automatically alternate between excitation wavelength 1 and excitation wavelength 2. The rate of alternation is dependent upon the illuminator type. The patented PTI DeltaRam V can provide up to 250 ratios/sec while the DeltaScan X can produce ultra-fast switching allowing for 650 ratios/sec. A model 101M monochromator must move from one excitation wavelength to the other at the slewing speed set in the hardware configuration.

**Emission**

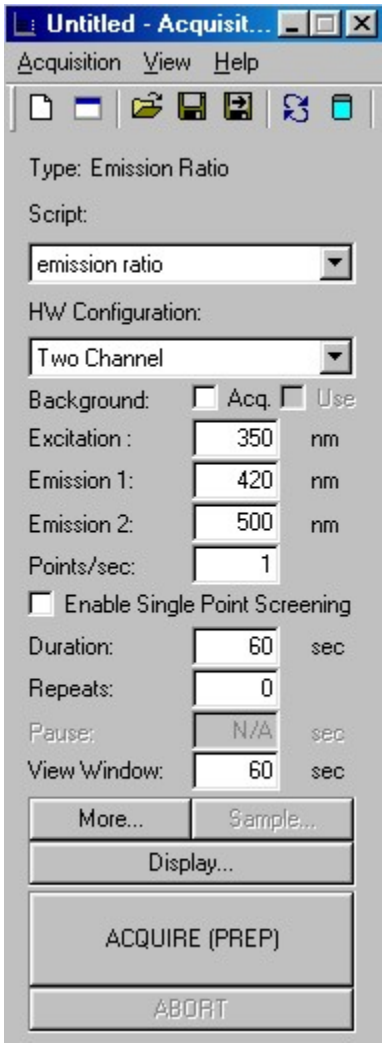
Enter the emission wavelength in the text box. If your instrument has two emission monochromators, FeliX32 will ask for two emission wavelengths. The wavelengths you enter will be the wavelengths to which the monochromators will automatically move prior to data acquisition. If your system uses filters for wavelength selection, simply enter the peak wavelengths of the filters in these boxes.

**Enable Single Point Screening**

This is used to collect single data points into a spreadsheet display.

## Emission Ratio

**Emission Ratio** is used to set up and run experiments for intracellular ion determinations using emission-shifted probes such as Indo-1 for calcium and SNAFL for pH. In this experiment, a constant excitation wavelength is used and two emission wavelengths must be selected. This is normally done with two monochromators in a cuvette system, but one monochromator can be utilized. In a microscope-based system, the two emission wavelengths are selected using a dichroic assembly in the photometer. The emission intensity at both emission wavelengths is measured and the ratio of these intensities is calculated. The ratio is proportional to the concentration of the ion being determined.




**Excitation**

Enter the excitation wavelength in the text box. The wavelengths you enter will be the wavelengths to which the monochromators will automatically move prior to data acquisition. If your system uses filters for wavelength selection, simply enter the peak wavelengths of the filters in these boxes.

**Emission 1,2**

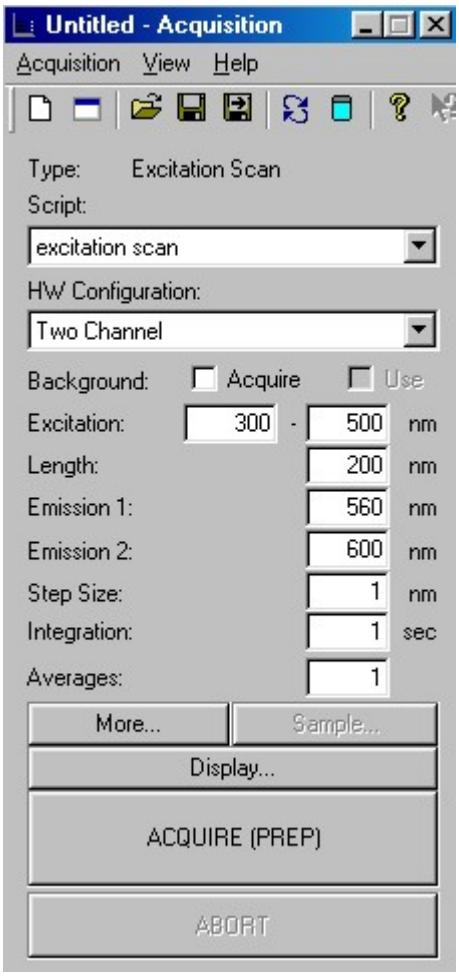
Enter the emission wavelengths in the text boxes. Your instrument will automatically alternate between wavelength 1 and wavelength 2. The rate of alternation is dependent upon the configuration. Dual emission systems provide up to 1000 ratios per second. Single monochromator emission systems slew between the wavelengths to provide up to 1 ratio per second. A model 101M monochromator must move from one excitation wavelength to the other at the slewing speed set in the hardware configuration.

**Enable Single Point Screening**

This is used to collect single data points into a spreadsheet display.

## Excitation Scan

In an **Excitation Scan**, the excitation monochromator is scanned between two wavelengths while the emission monochromator is fixed. The emission intensity is measured as a function of excitation wavelength. Due to the nature of fluorescence, the emission wavelength is set at a wavelength that is longer than the excitation wavelength range (red-shifted).




**Start and Stop**

Enter the initial excitation wavelength and the final excitation wavelength for the scan in these text boxes.

**Emission**

Enter the emission wavelength in the text box. If your instrument has two emission monochromators, FeliX32 will ask for two emission wavelengths.

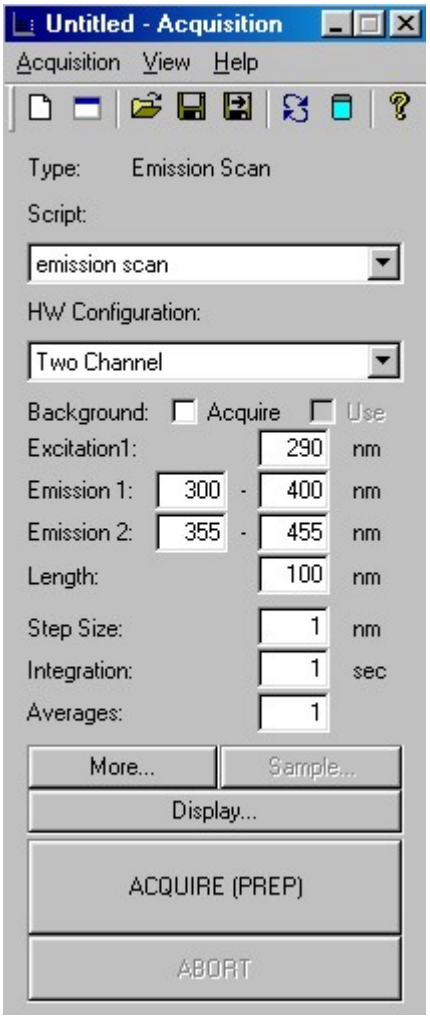
**Length**

This shows the length of the scan that will be run. If the starting wavelength and the length are entered, FeliX32 will calculate the ending wavelength corresponding to these parameters.

***Warning!*** FeliX32 does not prevent you from scanning the excitation across the emission wavelength. This should be avoided as it allows the excitation light to be reflected or scattered to the detector, resulting in possible damage to the PMT. As a rule, the emission wavelength should be at least 5 nm greater than the excitation stop wavelength (more if the bandwidth is greater than 5 nm).

## Emission Scan

In an **Emission Scan**, the emission wavelength is scanned between two wavelengths while the excitation monochromator is fixed. The emission intensity is measured as a function of excitation wavelength. Due to the nature of fluorescence, the excitation wavelength is set at a shorter wavelength than the emission wavelength range.




**Excitation**

Enter the excitation wavelength in the text box.

**Start and End wavelength**

Enter the emission wavelength scanning range in the *Emission 1* text boxes. If the system is equipped with two emission monochromators, FeliX32 will request two wavelength ranges.

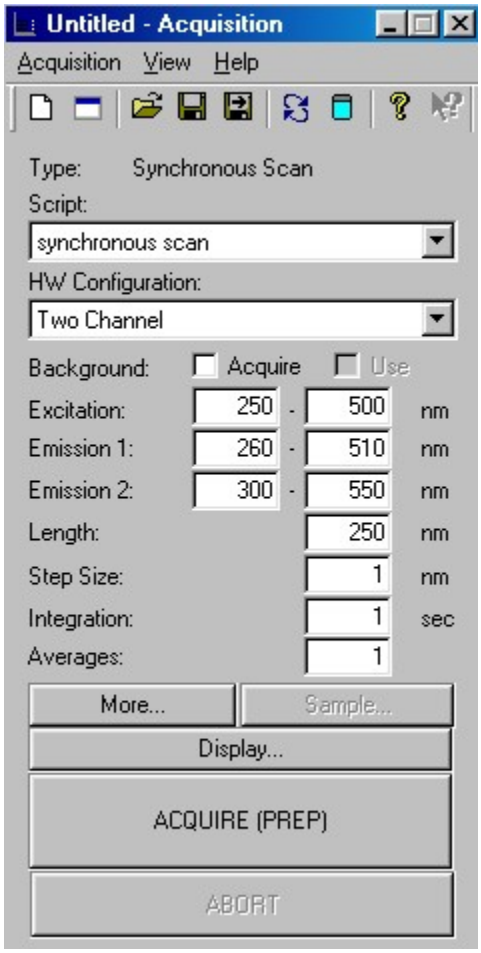
**Length**

This shows the length of the scan that will be run. If the starting wavelength and the length are entered, FeliX32 will calculate the ending wavelength corresponding to these parameters. For dual emission systems, the length of the scan will be the identical for both emission channels. FeliX32 will adjust the emission ranges to ensure they both have the same length.

***Warning!*** FeliX32 does not prevent you from scanning the emission across the excitation wavelength. This should be avoided as it allows the excitation light to be reflected or scattered to the detector, resulting in possible damage to the PMT. As a rule, the excitation wavelength should be at least 5 nm lower than the emission wavelength (more if the bandwidth is greater than 5 nm).

## Synchronous Scan

In a **Synchronous Scan**, the excitation and emission monochromators are scanned simultaneously at identical scan rates with a constant wavelength difference between them. A synchronous scan often results in the simplification of complex excitation or emission scans.




**Excitation Range**

Enter the excitation wavelength range in the text boxes.

**Emission Range(s)**

Enter the emission wavelength range in the text boxes. If the system is equipped with two emission monochromators, FeliX32 will request two wavelength ranges.

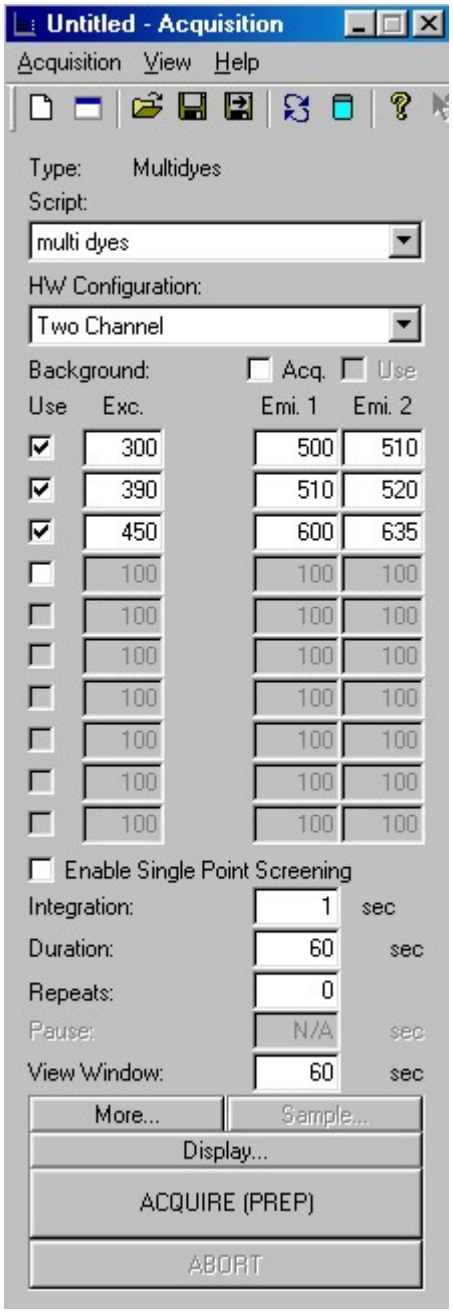
**Length**

Enter the scan range in nanometers. This value will be calculated for you if a range is entered. If starting wavelengths and length is entered, FeliX32 will calculate the ending wavelengths based upon these parameters. The length for all monochromators, excitation and emission, will be identical. FeliX32 will adjust the range values to ensure that the length is the same.

***Warning!*** FeliX32 does not prevent you from entering the same excitation and emission start wavelengths. This should be avoided as it allows the excitation scatter light to pass directly through to the detector, resulting in possible damage to the PMT. As a rule, the emission start wavelength should be at least 5 nm higher than the excitation start wavelength (more if the bandwidth is greater than 5 nm).

## Multiple Dyes

The **Multiple Dyes** function is used to set up and run experiments for intracellular ion determinations using several indicators in combination, such as Fura-2 for calcium and BCECF for pH. In this experiment, the excitation light source must alternate between four different excitation wavelengths that are characteristic of the two probes (e.g. 340, 380, 440, 490-nm). In addition the isosbestic wavelength for Fura-2 is frequently monitored at 361 nm to obtain a calcium-independent signal.




The emission intensity resulting from excitation at the above five wavelengths is measured at longer emission wavelengths (510 and 525 nm, respectively) and the ratio of these intensities is calculated. The ratio is proportional to the concentration of the ion under investigation. Any combination of up to 10 excitation and 10 emission wavelengths may be defined to accommodate the simultaneous measurement of both excitation- and emission-shifted dyes.

**Use**

Use the checkboxes to select the number of wavelength pairs for the experiment.

**Ex**

Enter the excitation wavelengths in these text boxes.

**Emi. 1 and Emi. 2**

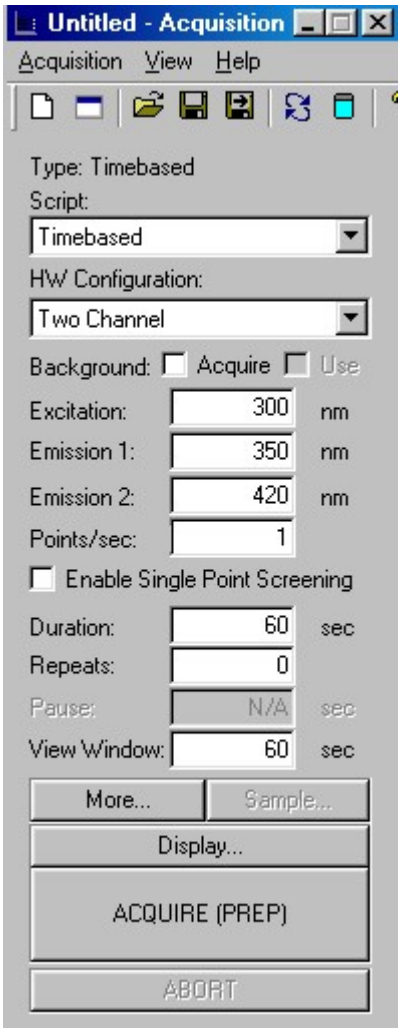
Enter the emission wavelengths in these text boxes. If your system only has a single emission channel, the system will only display a single column for entering emission wavelengths.

**Enable Single Point Screening**

This is used to collect single data points into a spreadsheet display.

## Timebased

In a **Timebased** experiment, the excitation and emission wavelengths remain fixed throughout the experiment. The emission intensity is measured as a function of time. Timebased experiments typically involve kinetic measurements.




**Excitation**

Enter the excitation wavelength in the text box.

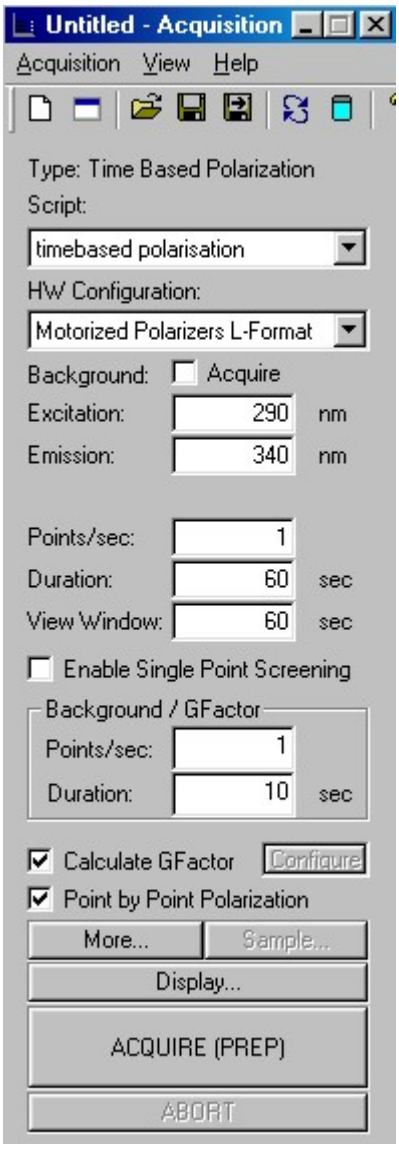
**Emission**

Enter the emission wavelength in the text box. If your instrument has two emission channels, FeliX32 will ask for two emission wavelengths. The wavelengths you enter will be the wavelengths to which the monochromators will automatically move prior to data acquisition. If your system uses filters for wavelength selection, simply enter the peak wavelengths of the filters in these boxes.

**Enable Single Point Screening**

This is used to collect single data points into a spreadsheet display.

## Timebased Polarization




Polarization measurements can be done in several ways in FeliX32. Some methods include using a timebased experiment or excitation/emission scans. One of the easier ways to perform polarization is to use the **Timebased Polarization** acquisition, which is useful for performing the polarization measurements under complete FeliX32 automation, including measuring the G-factor. Anisotropy and polarization will be calculated in real time and displayed in the workspace. This experiment is mainly intended for systems with a single emission channel using motorized polarizers, although manual polarizers may be used as well. The various parameters have much in common with the other experiments.

**Excitation**

Enter the excitation wavelength in the text box.

**Emission**

Set the emission wavelength for the experiment in the text box.

**Enable Single Point Screening**

This is used to collect single data points into a spreadsheet display. It is useful for measuring the polarization of a number of samples. The two intensities, polarization, and anisotropy for the sample will be determined.

**Background/G-Factor**

The system can perform background subtractions and G-Factor calculations automatically. The parameters for these measurements may be set to different values than the above section, which contains the information to run the polarization experiment on the sample. The measurement of G-Factor and background may be controlled using either the Calculate G-Factor or Acquire Background checkbox.

**Calculate G-Factor**

Toggling this control determines if the G-Factor is calculated during the experiment, or whether a previously acquired G-Factor is used. If a pre-determined G-Factor is used, it

is set in the *Configure* menu found beside this checkbox. Filter based systems do not require the calculation of a G-Factor. To cancel the acquisition of a G-Factor, enter the *Configure* menu and set the G-Factor to a value of 1.

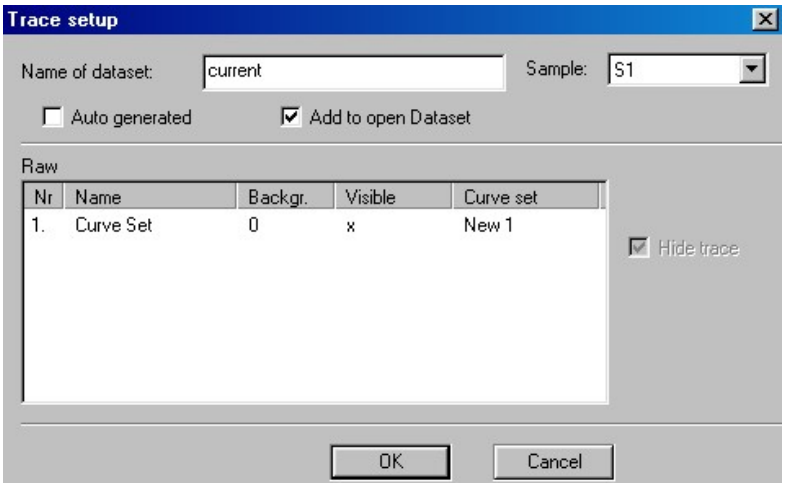
**Configure G-Factor**

Entering this menu allows the selection of a pre-determined G-Factor. There are two options that may be followed. You can enter a known G-Factor into the G-Factor text box or you may use a G-Factor lookup table. Please refer to **Chapter 10** for more information on lookup tables.

**Note.** The value of the G-Factor is wavelength dependent, therefore remember to use an appropriate G-Factor for the experiment.

**Point by Point Polarization**

When selected, this feature forces FeliX32 to rotate the emission polarizer between vertical and horizontal at a rate determined by the points/second. The polarization and/or anisotropy will be determined one point at a time. If toggled off, one full measurement for the set duration will be acquired for each polarizer orientation.




**Display**

The *Trace Setup* menu for Timebased Polarizations is different than that for the other acquisitions. The *Curve Set* in the *Raw* data window represents all the curves that will be generated during the experiment including G-Factor measurements, raw data, and anisotropy and polarization traces. If a four cuvette turret is used, it is possible to toggle the visibility of individual curve sets by selecting *Hide Trace* for each sample that you wish to hide. The rest of the *Trace Setup* features remain identical in nature to the other acquisitions.

**Sample…**

The **Four Cuvette Turret** section allows the ability to run up to four samples for measurement within a single experimental setup. The series of check boxes listed under Sample Position determine which samples are going to be measured. The radio buttons listed under **Single Point Screening** sample position determines which turret position is going to be used for the single point screening measurement. No data from other samples will be collected while using this option. The radio buttons listed under the *Background Position* allow the user to select which sample position will hold the background sample.

# Acquisition Commands: TimeMaster

Experiments are invoked and data is acquired by selecting New Acquisition and the type of acquisition desired for the experiment from the resulting popup dialog box. Each acquisition command opens a data acquisition setup dialog box. The items in each box are organized according to the experiment type. There are three fundamentally different experiment types.

**Decay:** In decay mode, the excitation and emission wavelengths remain fixed throughout the experiment while the delay is scanned in time. Decay experiments are used to measure the lifetimes of samples.

**Time Resolved Spectra (gated emission/excitation spectra):** In time resolved spectral mode, the delay remains fixed throughout the experiment while either the emission wavelength is scanned and the excitation wavelength is held fixed or, for xenon lamp systems, vice versa. Time resolved spectra are used to investigate the spectral properties of various decay mechanisms in samples with complex decays.

**Timebased:** In timebased mode, the excitation wavelength, the emission wavelength and the delay remain fixed throughout the experiment. The emission intensity is measured as a function of time. Timebased experiments ususally involve kinetic measurements but are also useful in maximizing intensity when adjustments are being made to the instrument.

FeliX32 is able to run a whole family of lifetime measuring instruments. These may be categorized by the range of lifetimes they can measure and by the light source they employ. Thus, there are fluorescence systems employing laser or nanosecond flash lamp sources and phosphorescence systems employing laser or xenon flash lamp sources. The controls required for each member of the family are slightly different and different acquisition setup features appear according to the component specifications in the hardware configuration.

The decay, timebased and time resolved spectra acquisition dialog boxes for a phosphorescence system will be labeled Phosphorescence Decay, Phophorescence Time-based and Phosphorescence Excitation/Emission Scan respectively. For a fluorescence system, similar dialog boxes will be labeled Fluorescence Decay, Fluorescence Time-based and Fluorescence Time Resolved Spectera. In addition, the Fluorescence Decay dialog box, which appears with a laser system is slightly different from that which appears with a nanosecond lamp system.

For any particular system, only those acquisition dialog boxes that are appropriate to the specific system will be available. Systems that can be run with more than one hardware configuration can have only one configuration selected at any time. Only those acquisition dialog boxes that are appropriate to the current configuration will be available.

All acquisition dialog boxes contain buttons that start, stop, and pause the data acquistion process, load and save setup files, etc… These are described in **Additional Acquisition Commands and Controls** under the heading *Acquisition Controls*.

Lastly, there are additional configuration and control dialog boxes that are accessed from within the acquisition setup dialog boxes by pressing the buttons *more…*, *display…*, *sample…*, and *options…* These are presented under the headings *Additional Acquisition Setup Controls*, *Display Setup, Four Position Turret* and *Data Collection Options* respectively.

**Note.** The contents of any given dialog box may vary slightly depending on the particular hardware configuration of your instrument. For example, systems with two monochromators, two detectors, or two motorized emission polarizers have extra entries in the dialog boxes. The function of these extra entries should be obvious from the description below.

## Decay Acquisition

**Fluorescence Decay** or **Phosphorescence Decay** is used to setup and run experiments that determine the fluorescence or phosphorescence lifetime of a sample. The most obvious difference between the Fluorescence Decay and the Phosphorescence Decay dialog boxes is that the start and end delays are in nanoseconds and microseconds respectively reflecting the basic difference in time scale associated with the two phenomena. The selections common to all decay systems are described below.

### Background

Check the **Acq** box to acquire the background when the acquisition is started. On subsequent scans the background acquisition checkbox is automatically cleared and the just-acquired background values will be subtracted from the current measured values in real time. The background correction values will remain in effect for subsequent measurements until cleared in the Display Setup dialog box, or by removing the check mark in the **Use Background** checkbox located beside the Acq. Background check control, or by checking the Acq. Background checkbox, which will clear the previous value and force a new background to be acquired. Toggling Use Background keeps the background value in memory for future use. It is important to measure the background during the first scan, otherwise the signal may be distorted. This function only measures the electrical background on the signal integrator, i.e. it measures the pre-acquisition signal before the light source is fired. It does not account for an optical background due to stray light, solvent, etc… It is important to re-measure the background every time the integration time is changed.

### Excitation

Enter the excitation wavelength (nm) in the text box. If your instrument has an excitation monochromator, this will be the wavelength used for the decay.

If your instrument has a dye laser but no excitation monochromator, enter the reading on the dye laser counter (half this value with a frequency doubler). This will have no effect on the hardware but allows the excitation wavelength to be recorded with the decay.

If your instrument uses filters to select the excitation wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the excitation wavelength to be recorded with the decay.

**Emission**

Enter the emission wavelength (nm) in the text box. If your instrument has an emission monochromator, this will be the wavelength used for the decay.

If your instrument uses filters to select the emission wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the emission wavelength to be recorded with the decay.

**Scatterer**

This selection is only available when a four position turret is installed. Enter the scatterer wavelength (nm) in the text box. Usually this is the same as the excitation wavelength. For systems with a single sample holder, the scatterer is acquired by substituting the sample for scatterer and changing the emission wavelength to the same as the excitation wavelength.

**Start/End Delay**

Enter the delays at which data collection will start and end. For fluorescence modes, these are measured in nanoseconds while for phosphorescence modes they are measured in microseconds. The excitation pulse is typically situated 50 to 100 ns for fluorescence and at approximately 100 μs for phosphorescence, after the beginning (0) of the delay allowing data to be collected before the excitation pulse to establish a baseline. The exact position of the pulse must be found from a scattering experiment. The start delay is normally chosen a little before the excitation and the end delay is some 5 to 10 lifetimes after the excitation.

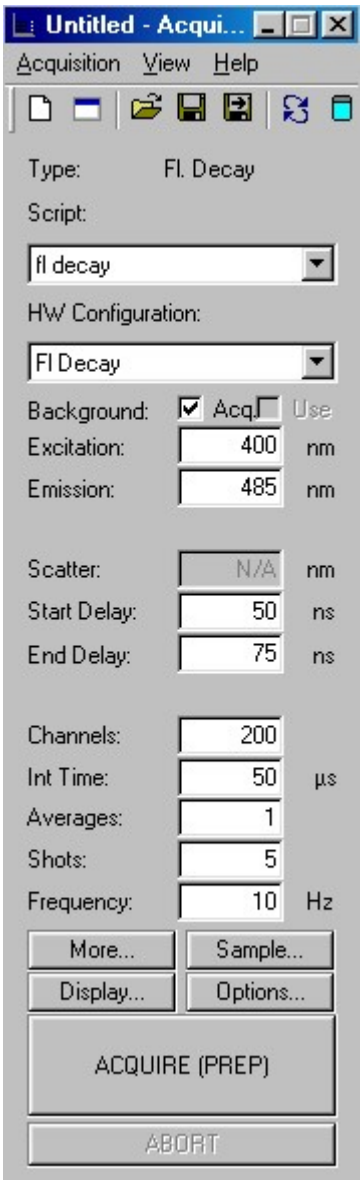
**Channels**

Enter the number of data points to be collected for each scan. Although the limit imposed by the software is very large, the time taken to collect and analyze decays may become excessive for large numbers of points. A maximum of 1000 points is reasonable.

**Averages**

Enter the number of complete scans to be averaged to give the final decay curve.

## Fluorescence Decay: Laser




**Int Time**

This is the time window in microseconds within which the signal is integrated for each laser pulse. The window should be long enough so that the emission signal is fully contained within it. Set this parameter to 50 µs.

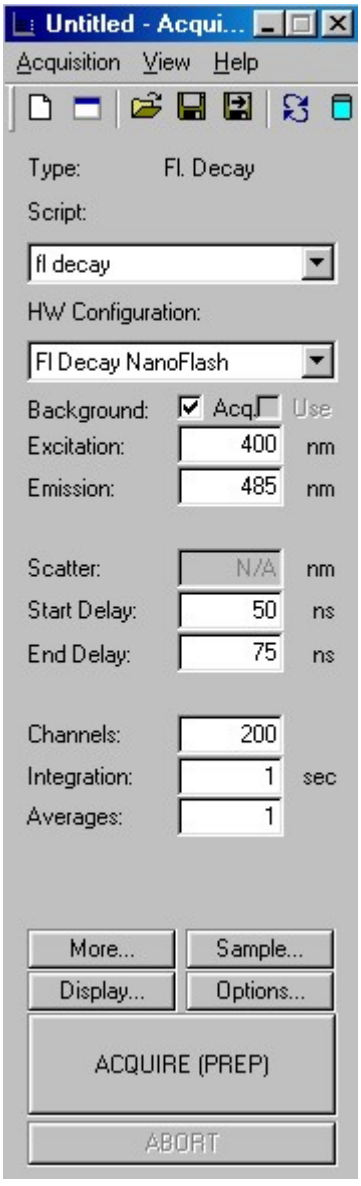
**Shots**

Enter the number of laser shots to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time. For statistical reasons, it is generally preferable to average over several scans than over more shots on a single scan. Thus averaging three scans with five shots each scan is better than one scan with fifteen shots.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire decay data. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

## Fluorescence Decay: Nanosecond Flash Lamp

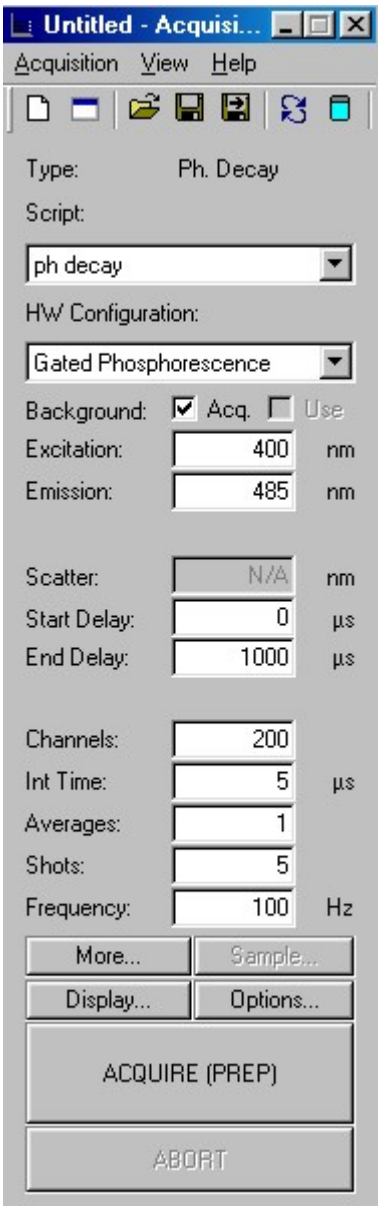



For this system the frequency of lamp pulses is set in the hardware configuration to be 18 to 20 kHz. The electronics convert this to an essentially DC signal from the detector.

**Integration**

Enter the time in seconds over which the signal will be averaged for each point of each scan. Extra integration time will improve the signal to noise ratio at the expense of additional acquisition time.

## Phosphorescence Decay: Xenon Flash Lamp




**Int Time**

This is the width of the integration window for each lamp pulse. Since, in this case, the observation window is defined by the integration time, it is normal to choose the integration time to be comparable to the channel spacing. Choosing an integration time of 1000 µs when the channel spacing is only 1 µs loses time resolution while choosing an integration time of 1 µs when the channel spacing is 100 µs loses sensitivity.

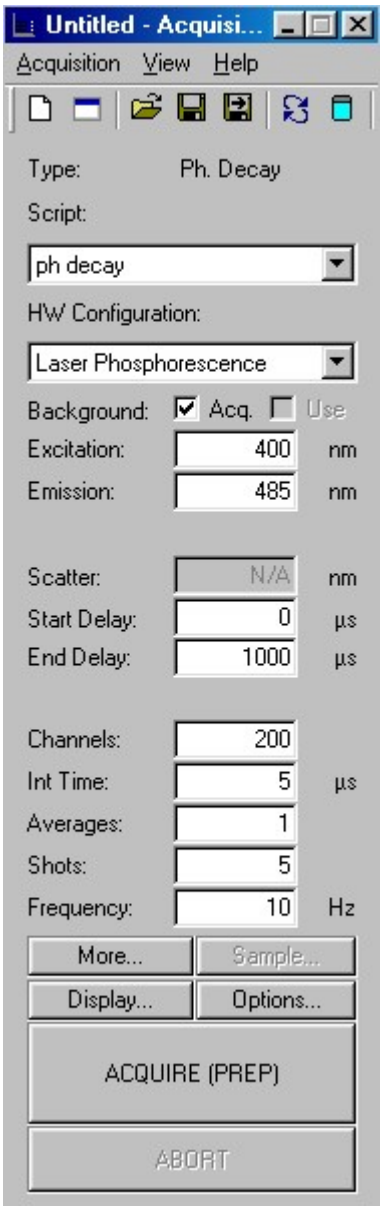
**Shots**

Enter the number of lamp pulses to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time. For a XenoFlash, 20 shots is an acceptable number.

**Frequency**

The lamp frequency can be set up to 100 Hz. For very long-lived samples, the phosphorescence from one pulse may not have completely decayed before the next pulse arrives. At least ten sample lifetimes should be allowed between each lamp pulse. Thus a lamp frequency of 100 Hz may be used for samples whose lifetimes are shorter than 1000 µs.

## Phosphorescence Decay: Laser




**Int Time**

This is the width of the integration window for each laser pulse. Since, in this case, the observation window is defined by the integration time it is normal to choose the integration time to be comparable to the channel spacing. Choosing an integration time of 100 µs when the channel spacing is only 1 µs loses time resolution while choosing an integration time of 1 µs when the channel spacing is only 100 µs loses sensitivity.

**Shots**

Enter the number of laser shots to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time. For statistical reasons, it is generally preferable to average over several scans than over more shots on a single scan. Thus averaging three scans with five shots each scan is better than one scan with fifteen shots.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire decay data. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

## Time Resolved Spectra/Gated Scans

In time resolved spectra, the delay and the position of one monochromator are held fixed while the other monochromator is scanned between two wavelengths. Selecting different decay times along the decay curve can be useful for exciting individual species within a sample mixture. For laser systems (fluorescent or phosphorescent) and for nanosecond flash lamp systems, only emission spectra are supported. For xenon flash lamp systems, gated emission and gated excitation scans are supported. The selections common to all systems are listed below.

**Background**

Check the **Acq** box to acquire the background when the acquisition is started. On subsequent scans the background acquisition checkbox is automatically cleared and the just-acquired background values will be subtracted from the current measured values in real time. The background correction values will remain in effect for subsequent measurements until cleared in the Display Setup dialog box, or by removing the check mark in the **Use Background** checkbox located beside the Acq. Background check control, or by checking the Acq. Background checkbox, which will clear the previous value and force a new background to be acquired. Toggling Use Background keeps the background value in memory for future use. It is important to measure the background during the first scan, otherwise the signal may be distorted. This function only measures the electrical background on the signal integrator, i.e. it measures the pre-acquisition signal before the light source is fired. It does not account for an optical background due to stray light, solvent, etc… It is important to re-measure the background every time the integration time is changed.

**Excitation**

Enter the excitation wavelength (nm) in the text box. If your instrument has an excitation monochromator, this will be the wavelength used for the spectrum.

If your instrument has a dye laser but no excitation monochromator, enter the reading on the dye laser counter (half this value with a frequency doubler). This will have no effect on the hardware but allows the excitation wavelength to be recorded with the spectrum.

If your instrument uses filters to select the excitation wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the excitation wavelength to be recorded with the spectrum.

**Emission**

This option is only used for gated excitation scans (Xenon flash lamp). Enter the emission wavelength (nm) in the text box.

If your instrument has an emission monochromator, this will be the wavelength used for the spectrum.

If your instrument uses filters to select the emission wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the emission wavelength to be recorded with the spectrum.

**Start/Stop**

Enter the wavelengths (nm) between which the spectrum will be run. These will be excitation wavelengths for gated excitation scans (Xenon flash lamp) and emission wavelengths for all other time resolved scans.

***Warning!*** TimeMaster does not prevent you from scanning the emission monochromator across the excitation wavelength. This should be avoided since it allows the excitation light to be reflected or scattered to the detector. Although damage to the PMT is unlikely because pulses are so short, the signal will very likely saturate. As a rule, the emission wavelength should be at least 5 nm greater than the excitation stop wavelength. For bandwidths greater than 5 nm the emission start wavelength should be increased accordingly.

**Step Size**

Enter the step size (nm) to be used in recording the spectra. The minimum value is 0.25 nm with a standard grating monochromator.

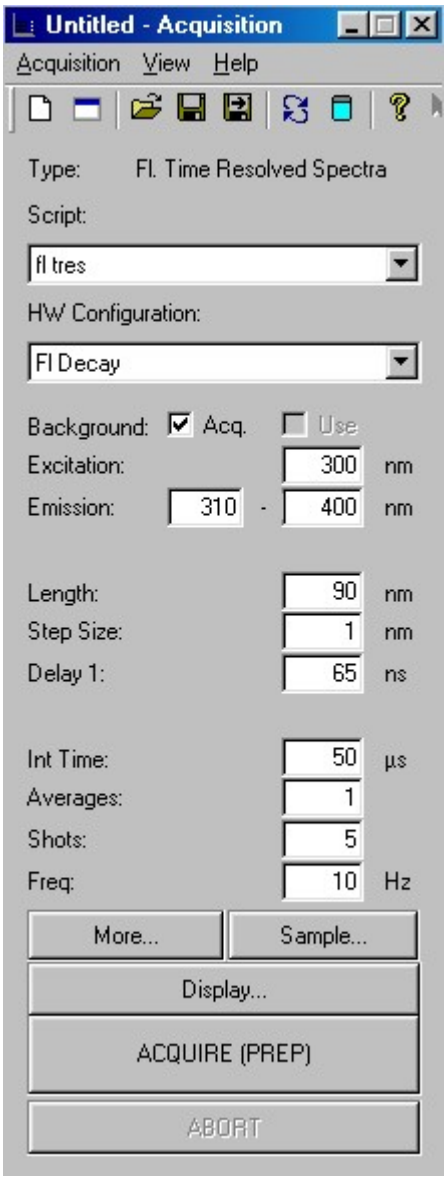
**Delay**

Enter the delay at which the detection window will be opened. For fluorescence systems this is in nanoseconds. For phosphorescence systems this is in microseconds. Phosphorescence systems may be used to acquire fluorescence and phosphorescence spectra by choosing the appropriate delay and integration time. See the discussion under Int Time for details.

**Averages**

Enter the number of complete scans to be averaged to give the final spectrum.

## Fluorescence Time Resolved Spectra: Laser




**Int Time**

This is the time window in microseconds within which the signal is integrated for each pulse. The window should be long enough so that the emission signal is fully contained within. Set this parameter to 50 µs.

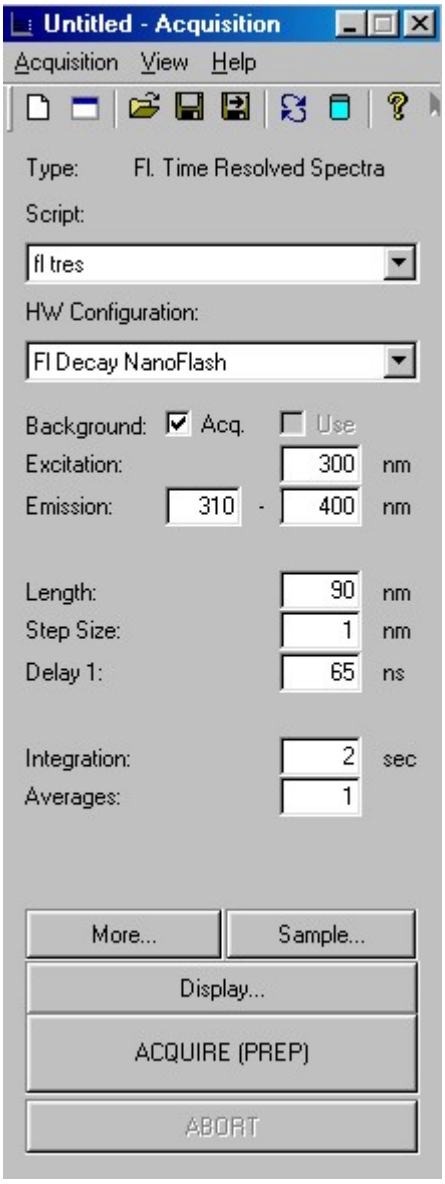
**Shots**

Enter the number of laser shots to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ration at the expense of additional acquisition time. For statistical reasons, it is generally preferable to average over several scans than over more shots on a single scan. Thus averaging three scans with five shots each scan is better than one scan with fifteen shots.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire decay data. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

# Fluorescence Time Resolved Spectra: Nanosecond Flash Lamp

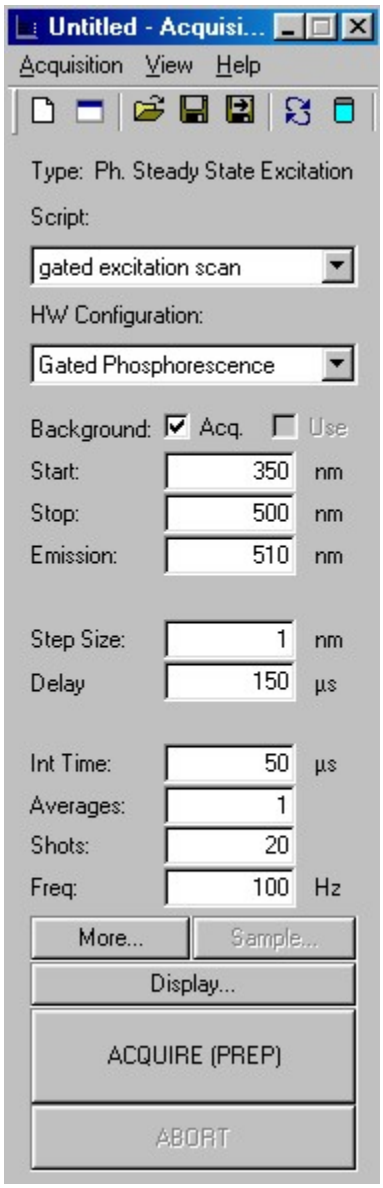



For this system the frequency of lamp pulses is set in the hardware configuration to be 18 to 20 kHz. The electronics convert this to an essentially DC signal from the detector.

**Integration**

Enter the time is seconds over which the signal will be averaged for each point of each scan. Extra integration time will improve the signal to noise ratio at the expense of additional acquisition time.

## Phosphorescence Steady State Excitation/Emission Scans: Xenon Flash Lamp (Gated Excitation/Emission Scans)




**Int Time**

This is the time in microseconds for which the integration window is open for each lamp pulse. Since, in this case, the observation window is defined by the integration time, increasing the integration time will increase the signal at the expense of lifetime resolution while decreasing the integration time will increase the lifetime resolution at the expense of signal strength. In particular, when the instrument is used to separate fluorescence spectra from phosphorescence spectra, care must be used in selecting the integration time. Since fluorescence is essentially over in the first 5 to 10 µs after the excitation pulse, the delay should be set to the excitation peak and the integration time to 5 to 10 µs. Longer integration times will contaminate the fluorescence with phosphorescence. When collecting phosphorescence, the delay should be set 5 to 10 µs after the excitation pulse and the integration time chosen to be larger to maximize sensitivity.

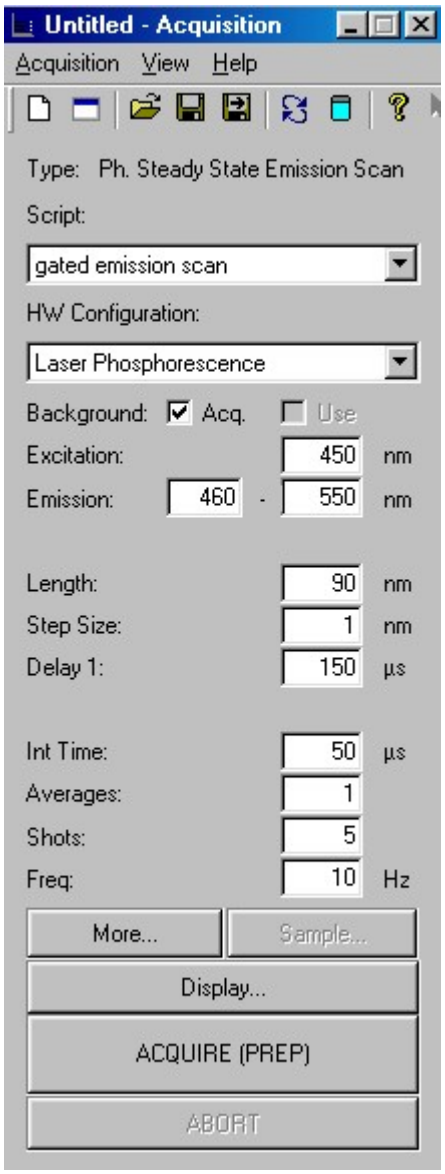
**Shots**

Enter the number of lamp pulses to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time.

**Frequency**

The lamp frequency can be set up to 100 Hz. For very long-lived samples, the phosphorescence from one pulse may not have completely decayed before the next pulse arrives. At least ten sample lifetimes should be allowed between each lamp pulse. Thus a lamp frequency of 100 Hz may be used for samples whose lifetimes are shorter than 1000 µs.

## Phosphorescence Steady State Emission Scan: Laser (Gated Emission Scan)




**Int Time**

This is the width of the integration window for each laser pulse. Since, in this case, the observation window is defined by the integration time, increasing the integration time will increase the signal at the expense of lifetime resolution while decreasing the integration time will increase the lifetime resolution at the expense of signal strength. In particular, when the instrument is being used to separate fluorescence spectra from phosphorescence spectra, care must be used in selecting the integration time. Since fluorescence is essentially over in the first 5 to 10 µs after the excitation pulse, the delay should be set to the excitation peak and the integration time to 5 to 10 µs. Longer integration times will contaminate the fluorescence with phosphorescence. When collecting phosphorescence, the delay should be set 5 to 10 µs after the excitation pulse and the integration time chosen to be larger to maximize sensitivity.

**Shots**

Enter the number of laser shots to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ration at the expense of additional acquisition time. For statistical reasons, it is generally preferable to average over several scans than over more shots on a single scan. Thus averaging three scans with five shots each scan is better than one scan with fifteen shots.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire decay data. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

## Timebased Acquisition

In a timebased experiment, the excitation wavelength, the emission wavelength, and the delay remain fixed throughout the experiment. The emission intensity is measured as a function of time. The timebased experiment usually involves a kinetic measurement but it is also useful in maximizing intensity when adjustments are being made to the instrument. The selections common to all systems are listed below.

**Background**

Check the **Acq** box to acquire the background when the acquisition is started. On subsequent scans the background acquisition checkbox is automatically cleared and the just-acquired background values will be subtracted from the current measured values in real time. The background correction values will remain in effect for subsequent measurements until cleared in the Display Setup dialog box, or by removing the check mark in the **Use Background** checkbox located beside the Acq. Background check control, or by checking the Acq. Background checkbox, which will clear the previous value and force a new background to be acquired. Toggling Use Background keeps the background value in memory for future use. It is important to measure the background during the first scan, otherwise the signal may be distorted. This function only measures the electrical background on the signal integrator, i.e. it measures the pre-acquisition signal before the light source is fired. It does not account for an optical background due to stray light, solvent, etc… It is important to re-measure the background every time the integration time is changed.

**Excitation**

Enter the excitation wavelength (nm) in the text box.

If your instrument has an excitation monochromator, this will be the wavelength used for the acquisition.

If your instrument has a dye laser but no excitation monochromator, enter the reading on the dye laser counter (half this value with a frequency doubler). This will have no effect on the hardware but allows the excitation wavelength to be recorded with the acquisition.

If your instrument uses filters to select the excitation wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the excitation wavelength to be recorded with the acquisition.

**Emission**

Enter the emission wavelength (nm) in the text box.

If your instrument has an emission monochromator, this will be the wavelength used for the acquisition.

If your instrument uses filters to select the emission wavelength, enter the filter's center wavelength. This will have no effect on the hardware but allows the emission wavelength to be recorded with the acquisition.

***Warning!*** Excitation and emission bandwidths should not overlap as damage to the PMT may result.

**Delay**

Enter the delay at which the detection window will be opened. For fluorescence systems this is in nanoseconds. For phosphorescence systems this is in microseconds. Phosphorescence systems may be used to acquire fluorescence and phosphorescence spectra by choosing the appropriate delay and integration time. See the discussion under Int Time for details.

**Duration**

Enter the length of time in seconds that the acquisition will be run.

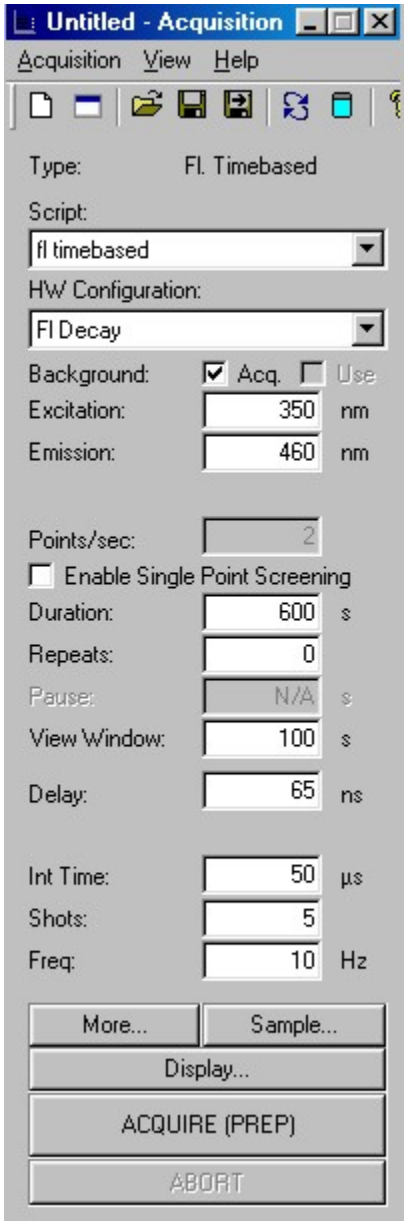
**Repeats**

Enter the number of times the experiment is to be repeated. The repeats will be added on at the end of the current time period.

**View Window**

Enter the time segment in seconds that will be displayed on the X-axis. The **View Window** setting can be less than or equal to that of the **Duration**. The data will be automatically shifted along the X-axis as each window is filled. Use a short view window for highest screen resolution of time.

## Fluorescence Timebased: Laser




**Points/sec**

The value in this box is completely defined by the choice of shots and frequency and cannot be chosen directly.

**Int Time**

This is the time window in microseconds within which the signal is integrated for each laser pulse. The window should be long enough so that the emission signal is fully contained within. Set this parameter to 50 µs.

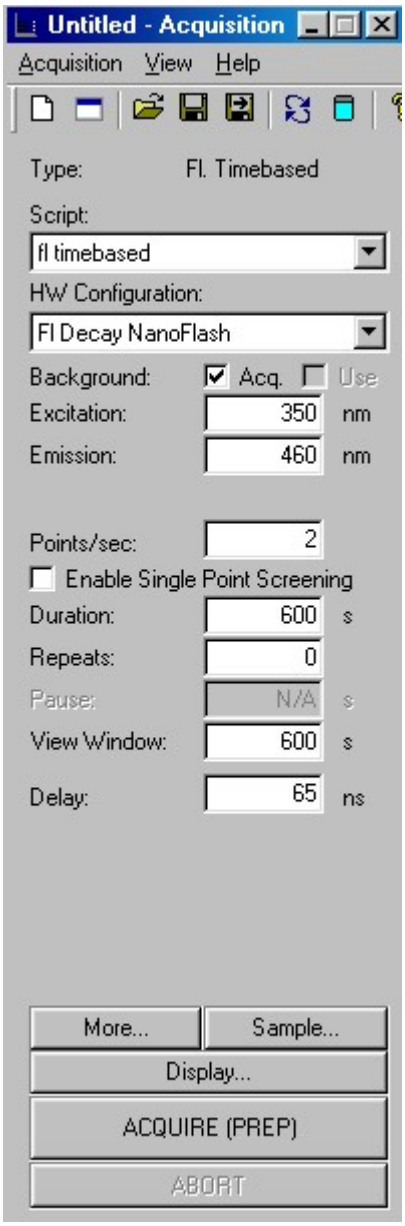
**Shots**

Enter the number of laser shots to be collected and averaged for each point for each scan. Extra shots will improve the signal to noise ratio at the expense of time resolution. When using a timebased experiment to adjust the instrument hardware this value is set rather low so that the effects of adjustments can be seen quickly.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire data and can improve time resolution. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

## Fluorescence Timebased: Nanosecond Flash Lamp

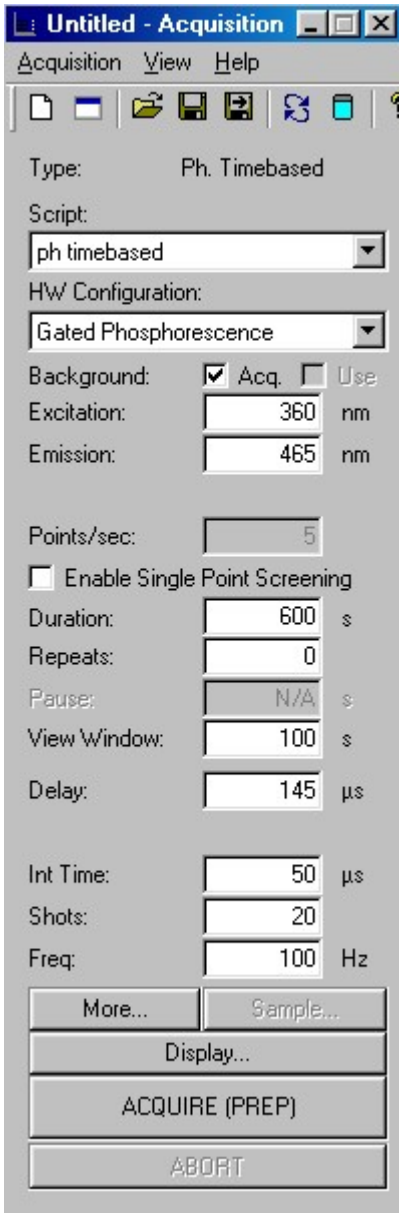



For this system the frequency of lamp pulses is set in hardware to be 18 to 20 kHz. This gives rise to an essentially DC signal at the detector.

**Points/sec**

Enter the number of points per second to record. The integration time for each point decreases inversely as the number of points increase. Time resolution can be increased by increasing the number of points per second at the expense of signal to noise. The choice depends on the time scale of the kinetics to be studied. For use in adjusting the instrument, values between 5 and 20 are found to give adequate response.

## Phosphorescence Timebased: Xenon Flash Lamp




**Points/sec**

The value in this box is completely defined by the choice of shots and frequency and cannot be chosen directly.

**Int Time**

This is the width of the integration window for each lamp pulse. Since, in this case, the observation window is defined by the integration time, increasing the integration time will increase the signal at the expense of lifetime resolution while decreasing the integration time will increase the lifetime resolution at the expense of signal strength. In particular, when the instrument is being used to separate fluorescence spectra from phosphorescence spectra, care must be used in selecting the integration time. Since fluorescence is essentially over in the first 5 to 10 µs after the excitation pulse, the delay should be set to the excitation peak and the integration time to 5 to 10 µs. Longer integration times will contaminate the fluorescence with phosphorescence. When collecting phosphorescence, the delay should be set 5 to 10 µs after the excitation pulse and the integration time chosen to be larger to maximize sensitivity.

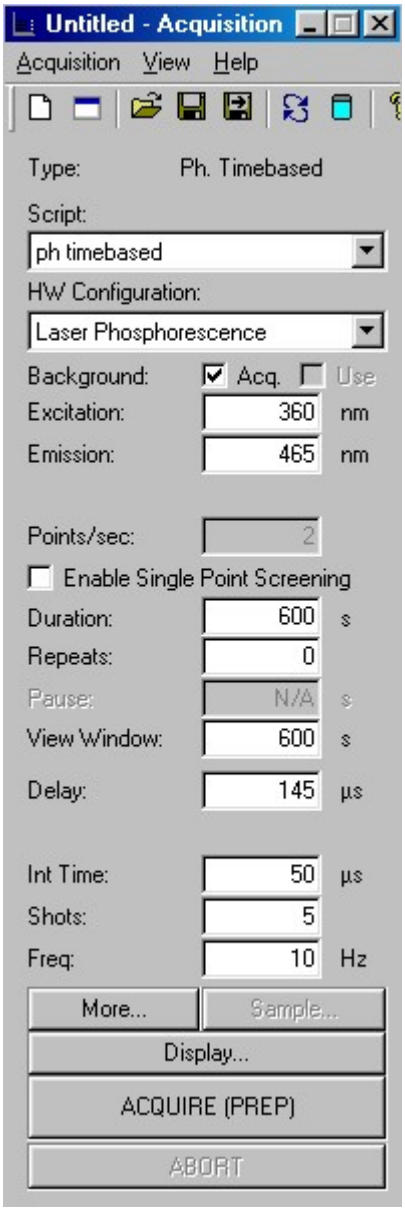
**Shots**

Enter the number of lamp pulses to be collected and averaged at each point for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time. When using a timebased experiment to adjust the instrument hardware this value is set rather low so that the effects of adjustments can be seen quickly.

**Frequency**

The lamp frequency can be set up to 100 Hz. For very long-lived samples, the phosphorescence from one pulse may not have completely decayed before the next pulse arrives. At least ten sample lifetimes should be allowed between each lamp pulse. Thus a lamp frequency of 100 Hz may be used for samples whose lifetimes are shorter than 1000 µs. Smaller frequencies may be useful when very long timebases are run, otherwise extremely large amounts of data will be collected.

## Phosphorescence Timebased: Laser




**Points/sec**

The value in this box is completely defined by the choice of shots and frequency and cannot be chosen directly.

**Int Time**

This is the width of the integration window for each laser pulse. Since, in this case, the observation window is defined by the integration time, increasing the integration time will increase the signal at the expense of lifetime resolution while decreasing the integration time will increase the lifetime resolution at the expense of signal strength. In particular, when the instrument is being used to separate fluorescence spectra from phosphorescence spectra, care must be used in selecting the integration time. Since fluorescence is essentially over in the first 5 to 10 µs after the excitation pulse, the delay should be set to the excitation peak and the integration time to 5 to 10 µs. Longer integration times will contaminate the fluorescence with phosphorescence. When collecting phosphorescence, the delay should be set 5 to 10 µs after the excitation pulse and the integration time chosen to be larger to maximize sensitivity.

**Shots**

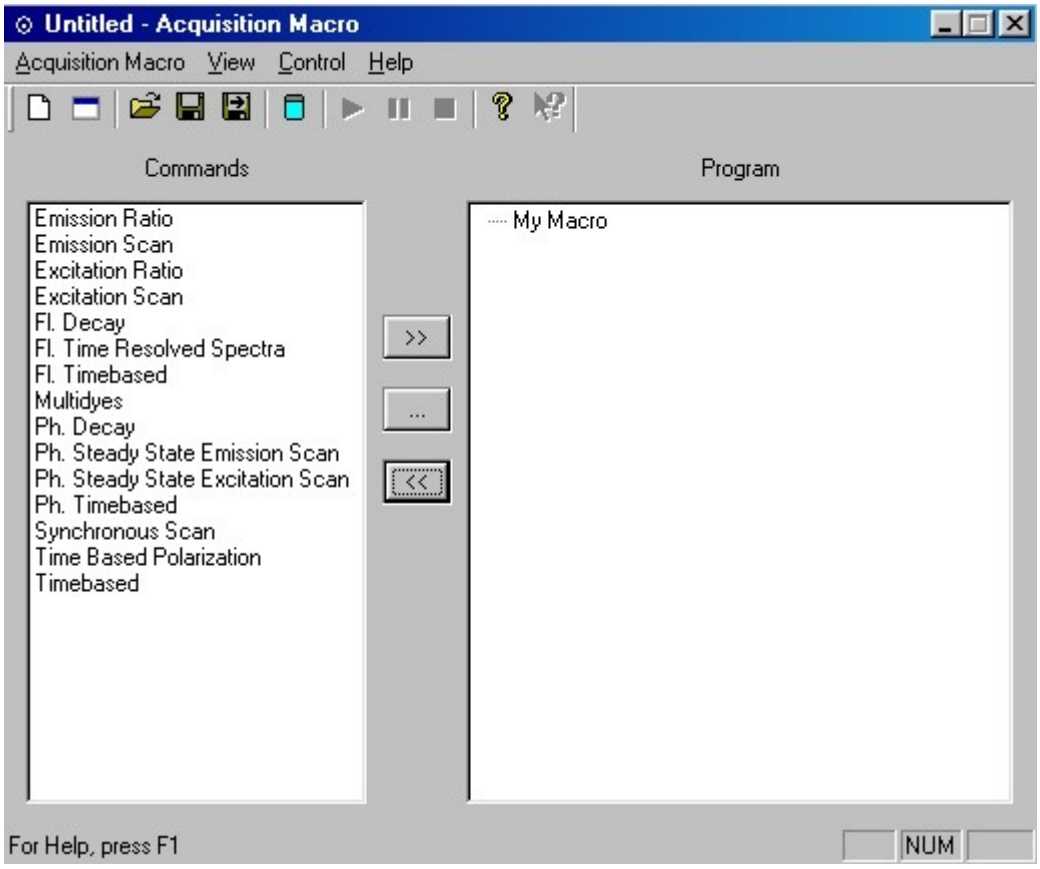
Enter the number of laser pulses to be collected and averaged at each point for each scan. Extra shots will improve the signal to noise ration at the expense of time resolution. When using a timebased experiment to adjust the instrument hardware this value is set rather low so that the effects of adjustments can be seen quickly.

**Frequency**

This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies can improve time resolution. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Smaller frequencies may be useful when very long timebases are run, otherwise extremely large amounts of data will be collected.

# Acquisition Commands: Macro Command Editor

The **Macro Command Editor** is a utility for creating command sequences to be executed automatically. To use the Macro Command Editor, highlight a command from the list box on the left, and click the **double right arrow** button. The command will appear in the Program list box to the right. Commands can be removed individually with the **Delete** or **double left arrow** button.




Most commands require the user to supply an argument such as a numerical value or a choice of options. A pop-up dialog box will solicit arguments when you add a command to the program. Arguments can be changed after the command is added to the program by highlighting the command and clicking the **Modify** button (three dots in a row) found between the double arrow buttons.

Save your macro program to the database by clicking the **Save As** button on the toolbar and assigning the macro a name. Load an existing macro program by clicking the **Open** button on the toolbar. If you modify an existing macro program, click the **Save** button to update the file.

After creating a macro program, run it by clicking the **RUN** (  ) button. **PAUSE** (  ) will temporarily suspend the execution of a running macro. **STOP** (  ) halts a macro program and subsequently clicking on **RUN** will start the macro program from the beginning.

**Note.** The available macro commands depend on the system configuration and acquisition mode. If the Macro Program Editor encounters an inappropriate command or argument, an error message will be displayed.

***Hint.*** A macro command can be inserted into the program by double clicking on its name in the Command list box. A command's argument can be modified by double clicking on the command in the Program list box.

The following is a list of all possible Macro Commands in alphabetical order. Details on the commands and macro hierarchy/structure can be found in the online Help utility.

| | | |
|---|---|---|
| Acquire Background | Set Delay | Set Points/Second |
| Calculate G-Factor | Set Duration | Set Background Points/Second |
| Gated Detectors | Set Background Duration | Set Shots |
| Increment Emission End | Set Emission End | Set Start Delay |
| Increment Emission Start | Set Emission Start | Set Step Size |
| Increment Emission Wavelength | Set Emission Wavelength | Set View Window |
| Increment Excitation End | Set End Delay | Temperature Control |
| Increment Excitation Start | Set Excitation End | Titrator |
| Increment Excitation Wavelength | Set Excitation Start | Trigger (TTL In) |
| Manual Polarizer | Set Excitation Wavelength | TTL Out |
| Motorized Polarizer | Set Frequency | Use Background |
| Real Time Correction | Set Integration | Use Dye |
| Set Averages | Set Integration Time | Wait Before Start |
| Set Channels | Set Pause | |

# Additional Acquisition Commands and Controls

## Acquisition Control Menu

The following commands are common to all of the acquisition dialog boxes. They can be found under *Acquisition* or *View* in the control menu or on the Acquisition toolbar.

**New Acquisition** 

Replaces the current active acquisition window with a new acquisition dialog box. Any type of acquisition in the database can be selected. The user is given the option to save the current acquisition prior to closing.

**New Window** 

Opens a new acquisition dialog box in a separate window. Any type of acquisition in the database can be selected.

**Open** 

Loads a previously saved acquisition dialog from the database. The opened acquisition will replace the currently active acquisition window. The user is given the option to save the current acquisition prior to closing.

**Close**

Closes the active acquisition dialog window. The user is given the option to save the acquisition prior to closing.

**Save** 

This command saves the active acquisition to the database under the current name. A dialog box will open enabling the user to input a name for the acquisition if it has not yet been titled. There is also a space for a brief description of the parameters. The button labeled Show Database will list all the saved acquisitions to date to help the user select an appropriate name for the saved acquisition.

**Save As** 

This command saves the acquisition to a new name so that any parameter changes can be saved as a separate acquisition without overwriting the previous acquisition. A dialog box will open enabling the user to input a new name for the acquisition. There is also a space for a brief description of the parameters. The button labeled Show Database will

list all the saved acquisitions to date to help the user select an appropriate name for the saved acquisition.

**Database** 

Opens the **Database Control Window** where the user can open/view/modify/create acquisition dialogs, acquisition scripts, hardware configurations, and macros.

**Status Window**

Opens a window that monitors all functions of the acquisition. The window will display temperature settings and other relative information about the active acquisition. It also contains a clock to measure experimental duration.

**Preferences**

Opens a dialog window where additional acquisition preferences can be selected. Please see **Acquisition Preferences** for more information.

**Bandpass/Bandwidth Calculator**

Opens a dialog to calculate bandpass or bandwidth.

**Toolbar**

Toggles the visibility of the acquisition window toolbar. If displayed, a check mark will appear beside Toolbar in the View menu.

**Status Bar**

Toggles the visibility of the status bar at the bottom of the acquisition window. The status bar provides contact sensitive help for functions in the acquisition dialog. If displayed, a check mark will appear beside Status Bar in the View menu.

**Refresh** 

Refreshes the acquisition script and the hardware configuration.

**Always On Top**

If selected, the acquisition window will remain in the foreground. A check mark will appear in the View menu if this option is chosen.

## Acquisition Controls

The following commands are common to all of the acquisition dialog boxes.

**More…**

This button opens the **Additional Acquisition Setup Controls** dialog box. Advanced data acquisition parameters are entered in this box, including shutter/TTL Out trigger programming, set temperature control, real time correction control, and access to titrator operations. There are also settings for additional hardware devices, such as motorized slits, polarizers, cryostat settings and analog devices, for example. This dialog box is described in more detail in the following section.

**Sample…**

If your system is equipped with a **Four Position Cuvette Turret**, clicking on this button opens the Four Position Cuvette Turret dialog box. This dialog box is described in the following section.

**Display…**

Opens the **Display Setup** dialog box, which allows you to control where and how the acquired data will be displayed. You may also define and display derived data traces here, such as ratios, transformations and concentrations that will then be calculated and displayed in real time during the experiment. This dialog box is described in the following section.

**Grating…**

This button opens the Grating Setup (Scan) or Grating Setup (Timebased/States) for your Acton Mono (spectrograph). For most applications this button is either permanently dimmed or non-existent. Please see the online help files for the details appropriate for your system configuration.

**Options**

Opens the **Data Collection Options** dialog box. This button is available only for decay mode acquisitions and is described in the following section.

**ACQUIRE (PREP)**

This button prepares the system for the acquisition of data. When clicked, the monochromators, computer controlled slits and detector gains are reset to begin the experiment. After this is done, the button label is changed to *START*. When *START* is clicked, data acquisition begins and the label changes to *PAUSE*. If a temperature control device is installed and selected, the system will go to the desired temperature as measured by the device or sample probe (if installed), before the *START* label appears.

**PAUSE/CONTINUE**

This button temporarily suspends data acquisition. After activation the button label changes to *CONTINUE*. Acquisition resumes when it is clicked again.

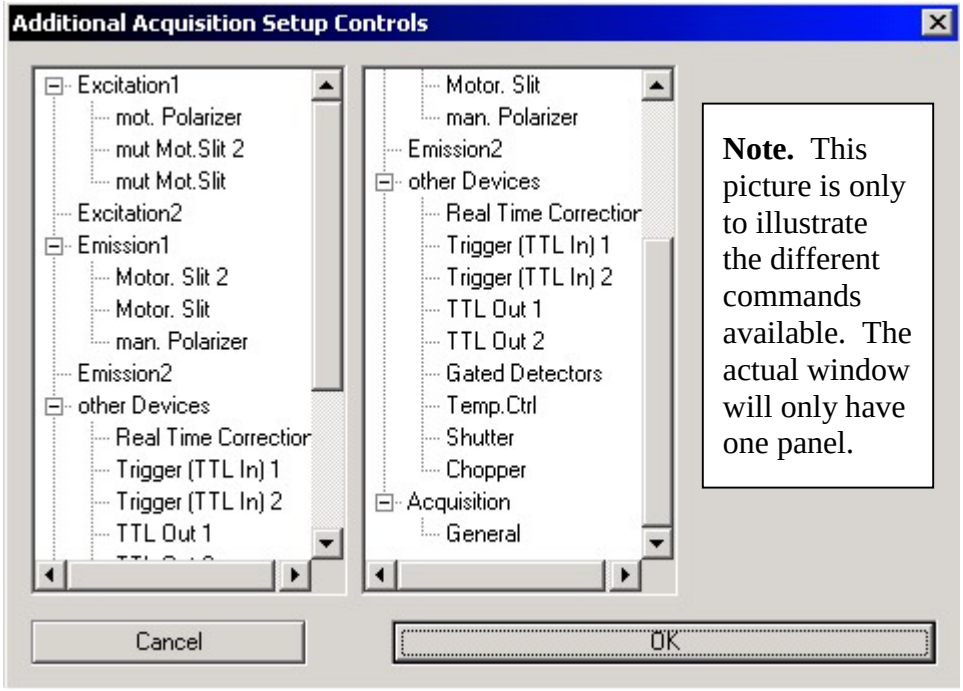
**ABORT/STOP**

Click *ABORT* to cancel the current acquisition preparation and return the system to standby mode. The *ABORT* button changes to a *STOP* button once data acquisition via the *START* button has begun.

**Note.** If **Enable Single Point Screening** has been selected the *PAUSE* button will be replaced with *CAPTURE VALUE*. An acquisition using this option can only be stopped not paused.

## Additional Acquisition Setup Controls

The additional Acquisition Setup Controls dialog box controls real time spectral correction, temperature control, and the shutters and chopper where applicable. Motorized polarizers and slits are also controlled based on the settings in this dialog. If your instrument has the temperature control option, you may set the temperature conditions for your samples here. It is accessed using the **More** button on the acquisition.




**Real Time Correction**

Allows excitation and/or emission correction as defined in the Hardware Configuration. This is applicable for correction of timebased and wavelength spectra in cuvette-based systems. A system must have an installed reference detector accessory and an excitation correction curve in the *Lookup Table* for excitation correction and an emission correction curve in the *Lookup Table* for emission correction.

Selecting **Enabled** turns on real time corrections. Excitation and emission corrections can be individually toggled on/off using the checkboxes beside their labels. Clicking on **Reference Source Gain** sets the gain of the reference detector RCQC. It may need to be adjusted depending on the experimental parameters, primarily the excitation intensity, which varies according to bandpass.

**Note.** The reference detector RCQC is an analog device and thus may have a non-zero offset when no light is incident upon it. The user should evaluate the offset (run an

acquisition with no light incident on the RCQC) to ensure background subtraction for effect is done appropriately.

**Polarizer**

The radio buttons in this section are used to set the polarizers for all experiments except for Timebased Polarization. The angles may be set to vertical (normally 0°) or horizontal (normally 90°) by selecting the desired option. Selecting the *Angle* option, and entering a value into the edit box will allow other angles. If motorized polarizers are installed FeliX32 will automatically rotate the polarizer to the set angle. If manual polarizers are used a popup will appear prior to acquisition start to remind you to adjust the polarizer angle.

**Note.** If you have changed the angles used for V and H in the hardware configuration Polarizer Setup these non-standard angles will be used instead of the standard V = 0° and H = 90°!

**Trigger (TTL In)**

A Trigger In signal is used to synchronize data acquisition with an external device such as a stopped flow module for fast kinetics measurement. The signal from the external device is input at the Trigger In BNC on the front pane of the BryteBox computer interface. Data Acquisition will commence upon receipt of a TTL signal at Trigger In.

**Note.** Trigger In signals must conform to TTL standards, with a low value of 0 volts and a high value of +5 volts. The pulse length of the signal must be between 30 and 50 milliseconds.

If the *Use Trigger* box is checked, then pressing the acquisition *START* button will cause acquisition to wait for a TTL signal as characterized by the Trigger Mode radio buttons. A message “wait for trigger” will appear in the status window.

**Note.** If the state of the TTL In signal line is the same as the chosen Trigger Mode, then acquisition will start immediately. For example, if the signal is already *low* and a *low* trigger mode is chosen, then acquisition will commence immediately upon pressing *START*.

**TTL Out**

TTL Out is controlled in the same manner as a shutter. Please see below for more information.

**Gated Detectors**

If the *Use: Configurations* settings is enabled under *Gain Settings*, then the gain(s) will be the value set in the Hardware Configuration (default). If it is disabled, then you can select a gain setting that is appropriate for your experimental conditions. Selecting the *Reference Source Gain* button opens the *Gain Settings* dialog box allowing you to change values for the gain of the reference detector RCQC and the emission detectors Emission 1 and Emission 2. All values are selectable between 0 and 100 percent.

If *Use Default Settings* is checked under *Delay Time Settings*, then the RCQC delay time and integration time will be the defaults that are set in the *Delay Timing* dialog. Disabling this allows the defaults for the gated detectors to be changed.

**Shutters**

This defines the way that the shutters interact with the data acquisition process. If a single excitation shutter is installed in your system, it is controlled by either the Shutter 1 or 2 of the connectors on the back of the SC-500 Shutter Controller or by a signal from TTL OUT 1 or 2. Additional shutters may be added, if desired. The FeliX32 software allows control of up to four (4) shutters. Special provisions are available to extend this range.

***Manual:*** The shutter is opened and closed by the user by selecting a shutter or TTL button that appears on the workspace.

***Automatic:*** When selected, the acquisition scripts will be used to control the shutter behavior. These shutter commands will be written into an acquisition itself, either by the user or by PTI, upon request.

***Program:*** Program is used to provide a TTL signal to open and close the shutter at preset time intervals. When Program is selected, the Program pushbutton is highlighted. Clicking on this button opens the Programmable Shutter dialog box.

***Open:*** When this option is checked, the shutter will open automatically at the beginning of the acquisition, remain open for the entire experiment and automatically close when the experiment finishes.

***Close:*** If selected, this option maintains the shutter in a closed position throughout the experiment.

***Pause Control:*** This determines whether pressing the *PAUSE* button on the acquisition dialog will close the shutter or leave it untouched. Pressing *CONTINUE* will reverse the action of the *PAUSE* button for the shutter (unless PAUSE leaves the shutter untouched).

## Chopper

If your system is equipped with a chopper (the DeltaScan and PowerFilter illuminators have choppers), you have a choice of chopper modes.

***Continuous:*** The chopper revolves continuously (OC-4000 and OC-4000D). Use this setting for standard excitation ratio acquisitions, where each excitation monochromator (DeltaScan) or filter (PowerFilter) is set to a different wavelength, and the chopper alternates rapidly between them.

***Stepped:*** The chopper steps between Positions 1 and 2 (OC-4000D only). This is the digital mode of chopper operations, used in special applications.

***Stationary:*** The chopper is maintained in a fixed position at either Position 1 or Position 2. The OC-4000D Chopper Controller is required for automatic position control. If you only have an OC-4000 than you must manually position the chopper after it has stopped moving. This setting is enabled when only a single monochromator or channel is desired (typically, the bottom monochromator in a DeltaScan X is chosen), for experiments involving a single excitation wavelength or for excitation wavelength scanning.

## Titrator

If your system is equipped with a Hamilton ML500B/C Series Dispenser/Diluter, you can access the Hamilton Dispenser/Diluter Operations Dialog to execute manually commands from the list of operations. Please see the online Help utility for further information.

## Temperature Control

In this section a temperature may be defined for an experiment if your hardware configuration comprises a temperature controller. You can choose to either *Set* the temperature to a certain value or, in the case of steady state *Timebased* acquisitions, ramp the temperature over a wide range. For more information please see the **Temperature Control** section.

**Motorized Slits**

Use this menu to set the width or bandpass of the motorized slits. The default value is that which is set in the hardware configuration. In order to change the slit width, you must first unselect *Use Configuration Settings*. Now you can enter the bandpass into the text box in nanometers. A conversion to millimeters is shown in the space below. To enter the width in millimeters with a nanometer conversion, simply select mm(W) from the drop down menu located next to the text box. If non-standard gratings are used or dual excitation/emission monochromators employed, the *reciprocal linear dispersion* factor may need to be changed. Each slit must be set individually.

**General**

***Keyboard Event Mark:*** Enabling this command allows the user to mark an event in the trace by pressing the space bar at the desired moment. A vertical line will appear to mark the X-axis value where the event occurred. Event markers are stored as a separate trace that can be specifically labeled by changing the text in the *TraceName* dialog. This option is not available to scanning or lifetime acquisitions.

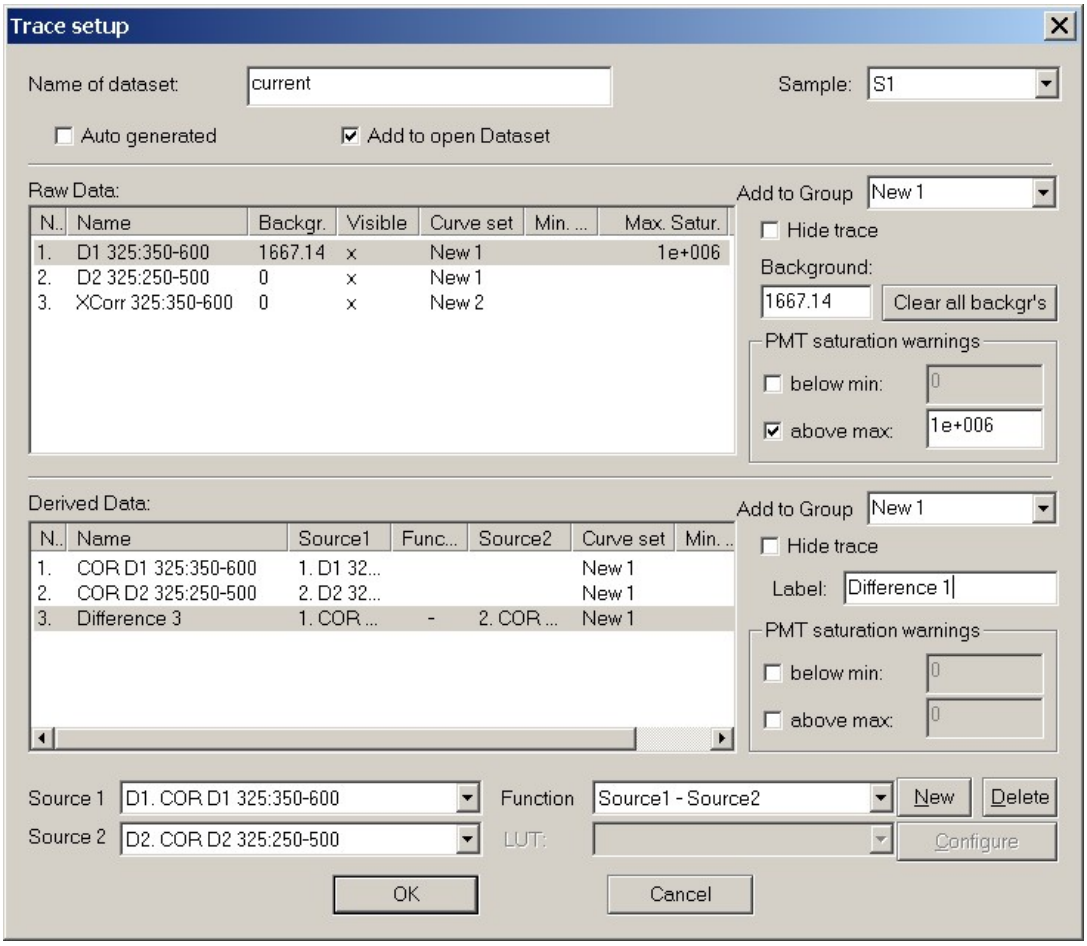
***Hardware Initialize:*** Initializes the hardware to ensure devices are ready for acquisition. If an accessory has auto calibrate activated than it will align itself to the proper position.

***Background:*** Changes the number of data points that are collected and averaged for the background when *Acq. Background* is in use.

***Disable PMT saturation warnings***: Checking this box disables the PMT saturation warnings set up in the Display Setup dialog.

## Display Setup

The data that is displayed during acquisition is dependent upon the type of fluorescence experiment being performed. In many experiments, the intensity is measured and displayed as a function of wavelength or time. If you use real time corrections, have more than one detector, or ratio dual wavelength probes, each acquisition will generate several curves. You can choose to display these in the same or separate groups.




Controls in the Display Setup dialog box allow you to specify where each portion of the data you acquire is displayed and ultimately how it will be saved.

**Note.** Displaying and saving are performed independently of each other. The settings in Display Setup are used only to organize the acquired data in the dataset legend. After acquisition is complete, saving the data to files is performed in a separate operation.

The top portion of the Trace Setup dialog box is used in the naming of the dataset. Enter your preferred name for the dataset into the text box. When the acquisition is run, all curves that are produced will go into this dataset. If *Add to Open Dataset* is checked than subsequent acquisitions will also go into this dataset. If not, new trace groups will be placed into a new dataset with the same name. All of this can be overridden by selecting an *Auto Generated* name. In this case, FeliX32 will name your dataset with the type of acquisition and date and time. Additional acquisitions will be placed into their own dataset, each with unique names based upon the time at which they were acquired.

**Raw Data**

The raw data that is collected during acquisition is shown in the list box. Select a curve by clicking on it.

**Note.** Remember that the term "curve" (and/or trace) in FeliX32 refers to a single contiguous group of data points collected during an experiment. Depending on the type of experiment, there may be one or several curves listed. For example, in a Fura-2 experiment the intensity at 340 nm is one curve and the intensity at 380 nm is another. If your instrument is equipped with an excitation correction accessory, the reference signal will also be listed as a curve if correction has been enabled. The corrected curves will also be listed in this box.

**Note.** The term Curve Set in the Raw Data title bar refers to the trace Group. The name that appears in this column is the name of the group the curve will be placed into.

**Note.** If a *Four Position Turret* is installed you can see the raw data for each sample by selecting which sample to display from the *Sample* drop-down box in the top right corner of the dialog.

**Derived Data**

Derived Data is data that is mathematically generated from the raw data during acquisition. Select *New* to configure a new derived curve. A derived trace can be removed by selecting the trace and clicking *Delete*.

Use the *Source 1* and *Source 2* drop-down boxes to select the curves used in the mathematical derivation. Choose the type of operation to be performed from the *Function* box. Possible math calculations include subtraction, addition, division, multiplication, anisotropy and polarization, and concentration equations.

**Note.** *Source 1* refers to the numerator (VV trace for polarization) and *Source 2* the denominator (VH for polarization) where applicable.

*Lookup Tables* are also available for ratio-to-concentration, ratio-to-pH, and intensity-to-concentration operations. The proper Lookup Table can be selected using the drop-down box below *Function* or configured using the *Configure* button. In the case of polarization

and anisotropy measurements you must select Configure to enter a pre-determined G-Factor or to select a G-Factor Lookup Table.

A default trace name will appear when the type of function is selected. You may change the name by entering a new one into the *Label* text field.

**Add to Group**

The drop-down list box will contain a list of default group titles from *New 1* through *New 9*. You can create your own title be clicking in the text box and entering a new group name.

You can add the data you are about to acquire to an existing group by selecting its name in the list box. In this way, you can accumulate several runs of the same experiment into a single group. You can also split the raw data into different groups. For example, you can place intensity curves together in one group and the ratios in a separate group.

Groups need not be present prior to data acquisition. FeliX32 will automatically create them as needed and organize the groups in the manner you specify. If no settings are made in the *Display Setup* dialog box, FeliX32 will place all acquired data together as a single group within a dataset. If you want to create separate groups in a new experiment, select *New 1*, *New 2*, etc… from the list box or create your own name.

The Group status of each curve is displayed in the Raw Data list box in the Curve Set column.

**Hide Trace**

Selecting a trace, either a raw or derived trace, and checking this box will toggle the visibility of the trace during acquisition. Visibility status is displayed in the Raw Data list box with an X if visible.

**Background**

Enter a background value that will be subtracted from each raw data point of the selected curve. This box will also show the current background in memory if a background has been collected using the *Acq. Background* command. The current background for each trace is displayed in the Raw Data list box. *Clear all backgr's* will set all background values to 0.0.

**PMT saturation warnings**

Selecting a trace, either raw or derived, and checking the *below min.* or *above max.* boxes will cause a beep to sound when a data point is outside the specified limits and a message will appear in the Acquisition Status Window. The data will not be affected. The PMT saturation warnings can be disabled in the Additional Acquisition Setup Controls dialog.

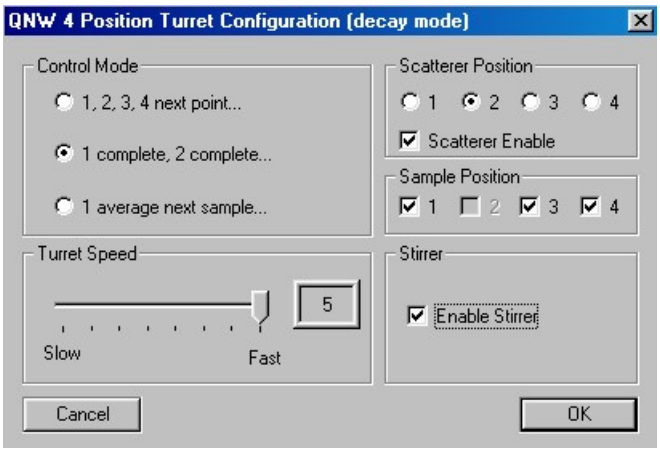
## Four Position Sample Turret

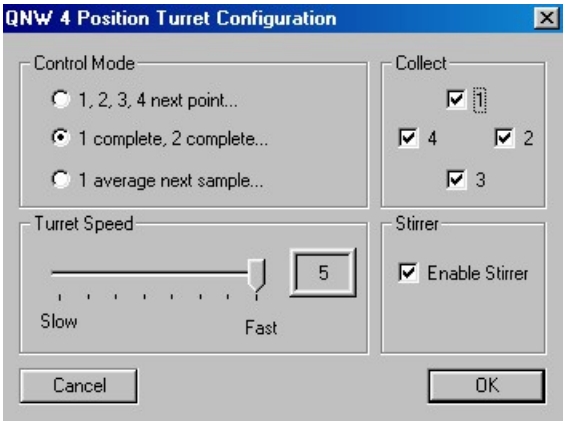
The **Sample** dialog box controls the mode of automatic data acquisition of multiple samples (1-4) for instruments having a four position sample turret accessory. Data is acquired with the parameters established in the acquisition setup.

Using the **Display Setup** dialog box, specify how the curves will be displayed. Samples are identified as *S1*, *S2*, *S3*, and *S4*. The curves produced by each sample may be displayed in separate groups or they may all be displayed together in one group.

**Note.** Controls within this dialog may differ depending upon the model of four position turret in your instrument and upon the acquisition type.

**Note.** When a background is collected using Acq. Background during timebased acquisitions, FeliX32 will acquire the background under the same conditions as those outlined in the experimental parameters. Background traces will even be produced although only the average value for each sample will be used as the background the next time the acquisition is started. To acquire a background faster simply reduce your duration during the background acquisition.



**Control Mode**

***1, 2, 3, 4, next point…:*** Allows you to acquire data from multiple samples concurrently. One data point is acquired for Sample 1 then one data point is acquired for Sample 2, etc…

***1 complete, 2 complete…:*** Allows you to acquire data from multiple samples sequentially. A complete curve set is acquired for Sample 1 then another is acquired for Sample 2, etc…

***1 average next sample…:*** A complete curve set is acquired for one scan of Sample 1 then another is acquired for Sample 2, etc… The whole process is then repeated for as many scans as required by the average parameter in the acquisition dialog box. This is not available during timebased acquisitions.

**Collect**

Appears for spectral scans and timebased acquisitions and allows you to specify which samples will be used in an experiment. Check the positions to activate. Those not checked will be skipped during an acquisition.

**Sample Position**

For decay mode, select the appropriate positions for the samples you wish to run.

**Scatterer Position**

For decay mode, the position of the scatterer (1-4) is selected by activating the appropriate radio button. If you do not wish scatterer data to be collected, toggle the *Scatterer Enabled* checkbox off. This is useful for samples with long lifetimes when the IRF is not required or when you are running multiple samples and do not wish to repeat the scatterer for each subsequent acquisition.

**Turret Speed**

For QNW rapid temperature control four position turrets you can set the speed at which the turret rotates from slow (250) to fast (5).

**Enable Stirrer**

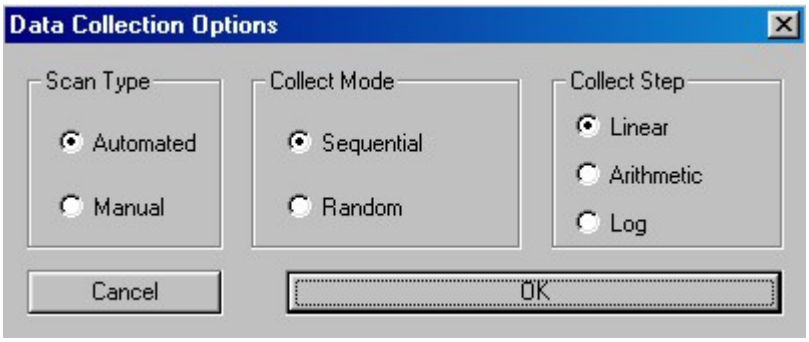
For QNW rapid temperature control four position turrets you can toggle the four position magnetic stirrer on or off.

**Background Position**

For Timebased Polarizations, you can select the position of the turret from which the background measurement will be acquired.

## Data Collection Options

This dialog box controls the several aspects of how decay data are collected. These controls are available only for fluorescence and phosphorescence decay acquisitions.




**Scan Type**

Radio buttons allow the selection of *Automated* or *Manual* when a four position turret is installed.

***Automated:*** Collects data for the selected positions of the four position turret without pausing between samples. This option is useful when all the samples can be run with the same instrument settings.

***Manual:*** Pauses data collection after each change in the four position turret's position. This allows the user to change neutral density filters etc between samples. Acquisition is continued by pressing the continue button.

**Collect Mode**

Radio buttons allow the selection of *Sequential* or *Random*, which controls the order in which data points are collected.

***Sequential:*** Causes the data to be collected in "conventional" order, i.e. from the shortest delay to the longest delay.

***Random:*** Causes the data to be collected in random order. This can be useful in situations where photochemical reactions are suspected of producing systematic effects on sample lifetimes.

**Collect Step**

Radio buttons allow the selection of *Linear*, *Arithmetic* or *Logarithmic*, which controls the spacing between consecutive time delays.

***Linear:*** The conventional choice and divides the time between the start delay and end delay into equal time increments.

***Arithmetic:*** Adds a constant time increment on to the previous time step to obtain the next time step. Thus the time between data points increases as the delay increases.

***Logarithmic:*** Multiplies the previous time step by a constant factor to obtain the next time step. With this option, time between data points increases even faster than it does with the Arithmetic option.

The Arithmetic and Logarithmic options are particularly useful when the sample decays with several very different lifetimes. In such cases, it may be necessary to have good data at both short and long time delays. Good data at short time delays could be obtained by choosing Linear and a small time increment. However, this would require many channels for this small time increment to be extended to long delays. Choosing Arithmetic or Logarithmic concentrates the points in the short delay region but still gives coverage in the long delay region.

## Temperature Control

Rapid temperature control options include a Linkam single cuvette holder and a QNW 4-position sample turret. If your instrument has a temperature control device setup in the hardware configuration it is possible to perform temperature-based experiments, either by setting the temperature or ramping the temperature. There are several models of controllers and temperature probe supported by FeliX32. They all must be setup properly in the hardware configuration via the RTC icon or else loss of function will occur. This includes the QNW rapid temperature control turret, which requires configuration of two icons, one for the turret and one for the temperature controller.

Typical temperature values can range from $-20^{o}$C to $100^{o}$C. Refer to your specific hardware manual for an exact range.

There are three modes of temperature control, *Set Temp.*, *Ramp Temp.*, and *Increment Temp.* with the latter only available during macros and ramping only during timebased acquisitions. Once a mode is selected you need to configure the experimental parameters. Additional temperature based controls can be found in Acquisition Preferences. Here you may select the temperature delta (how close the sample temperature must approach the set temperature before the set temperature is reached) and the units of temperature.

**Set Temperature**

Use this set of commands to bring the sample to a specific temperature.

***Set temp.:*** Defines the experimental temperature.

***Temp. rate:*** Rate at which the desired temperature will be attained. The range can be selected anywhere from 0.1°/min to 20°/min. If the value is between 0.1 and 9.9°/min, the change may be entered in 1/10 degree increments. If the value is between 10 and 20°/min, only whole numbers should be entered.

***Settle time:*** The time that transpires to allow the temperature to equilibrate to the set value. The settle time starts counting as soon as the temperature (probe temperature if installed) is within the delta range. Data acquisition is allowed to start once the settle time is complete.

***Hold temp after acquisition:*** When checked, this forces the temperature controller to maintain control over the temperature to keep it at the set temperature. If unchecked, the sample temperature will naturally equilibrate to room temperature.

**Ramp Temperature**

The following parameters allow you to ramp the temperature over a user defined range and speed.

***Start temp.:*** Defines the starting temperature for the forward ramp.

***End temp.:*** Defines the end temperature for the forward ramp.

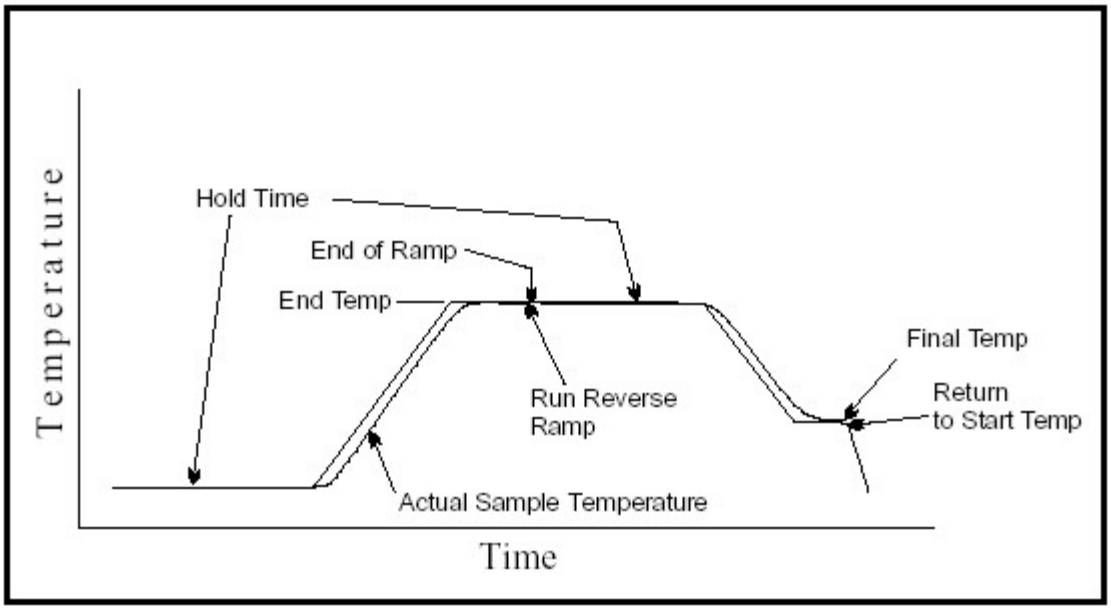
***Run Reverse Ramp:*** Select to run a reverse ramp after the end temperature of the forward ramp has been achieved.

***Final temp.:*** Only used if a reverse ramp is selected. Defines the end temperature for the reverse ramp.

***Hold Time:*** The time that transpires to allow the temperature to equilibrate to the initial set value. The hold time starts counting as soon as the temperature (probe or controller) is within the delta range of the controller. There is also a hold time between forward and reverse ramps in order to equilibrate the temperature at the midpoint.

***Temp. rate:*** Rate at which the temperature will be ramped. The range can be selected anywhere from 0.1°/min to 20°/min. If the value is between 0.1 and 9.9°/min, the change may be entered in 1/10 degree increments. If the value is between 10 and 20°/min, only whole numbers should be entered. The temperature rate and temperature range will determine the duration of the experiment.

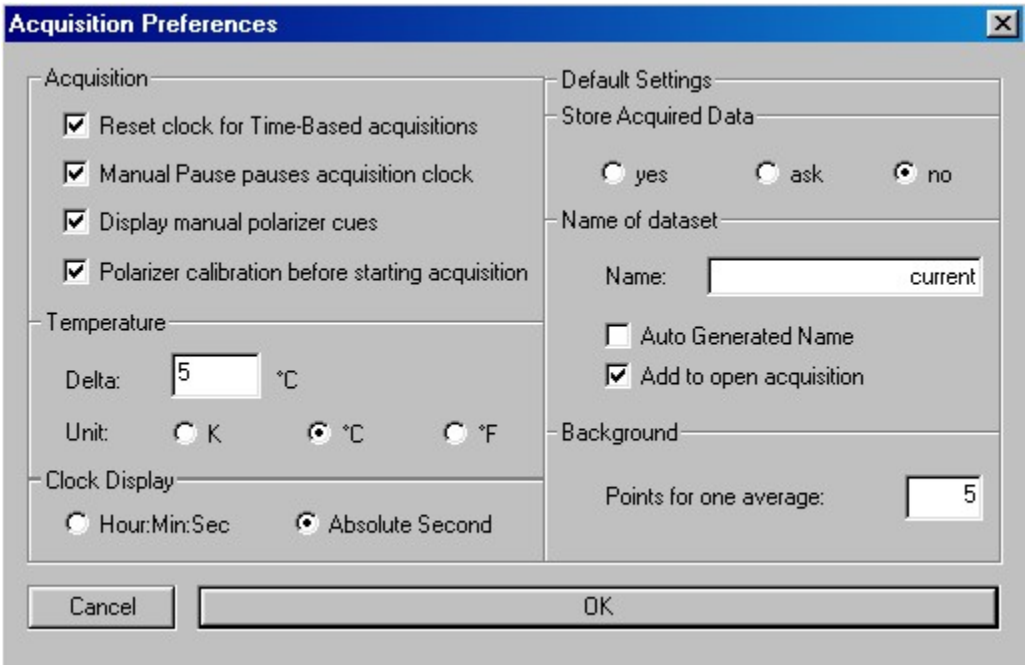
***Temperature after acquisition:*** After the scan is finished, this controls whether the temperature should be 1) uncontrolled, in which case it will tend towards the ambient temperature; 2) return to the temperature at the start of the forward ramp; or 3) hold at the final temperature.




**Note.** The QNW RTC four position turret has additional controls that may affect the rate of a temperature based experiment. These parameters are located in the RTC icon in the hardware configuration. The QNW performs an internal check to determine if it has reached the set temperature prior to releasing temperature information and checking the delta range with FeliX32. The check requires the temperature of the controller to get and remain within $0.02^{o}$ of the set temperature for a period of 5 minutes. If the temperature varies out of this range then the clock will be reset. This produces very accurate temperature measurements but at the price of long experiment times. As such, FeliX32 allows you to change the QNW internal temperature range check and settle time. You can enter a new temperature window within which the temperature must reside for a period of your choosing prior to delta confirmation with FeliX32.

## Acquisition Preferences

Several aspects of the way FeliX32 looks and behaves can be adjusted to suit the user. All changes made in this menu are automatically applied to all acquisitions. Selecting *View* then *Preferences* in an *Acquisition dialog* will access the dialog box.




**Acquisition**

***Reset clock for Time-Based acquisitions:*** Selecting this option will reset the time counter in the status window to zero at the beginning of each acquisition cycle.

***Manual Pause pauses acquisition clock:*** If pause is selected from the acquisition dialog window during an experiment the clock will also be paused. The counter will resume upon selecting continue.

***Display manual polarizer cues:*** If the hardware configuration used for the acquisition contains manual polarizers this option will create pop up windows to inform the user when and by what degree to rotate the polarizers during the experiment.

***Polarizer calibration before starting acquisition:*** This forces the motorized polarizers to confirm their angle against the optical encoder prior to starting an experiment. This feature adds to the time before the experiment is actually run.

**Temperature**

***Delta:*** This function is utilized during temperature controlled experiments. Once the temperature controller is within this range, FeliX32 will allow the user to start the acquisition. The controller temperature will continue towards its set temperature. If an external temperature probe is used such as the DP41, the acquisition will be allowed to start once the temperature of the probe is within the delta range of the controller temperature. The controller temperature must first reach its set temperature before FeliX32 looks for agreement with the temperature of the probe. In both cases, a smaller delta will produce greater precision of temperature. However, due to heat transfer, especially at the extremes, the sample temperature may never reach the set temperature. If the delta is set too small, it may take an excessive amount of time for the range to be breached. Testing with a dummy sample is encouraged to determine the best tradeoff between waiting on the experiment and temperature accuracy.

***Units:*** Sets the default units for temperature control. The user can select Kelvin (K), Celsius ($^{o}$C), or Fahrenheit ($^{o}$F).

**Clock Display**

Select hours:minutes:seconds or absolute seconds to be displayed in the acquisition status window.

**Default Settings**

***Store Acquired Data:*** Select yes, no, or ask.

***Name of Dataset:*** Enter the default name of new acquisition datasets. This name will be used if *Auto Generated Name* is not selected. If it is selected, the database will be titled with the type of acquisition and the date and time. *Add to Open Acquisition* will force acquisitions with the same dataset name (entered in the **Display Setup** menu) to have their trace groups inserted into the open dataset. If not selected, a new dataset with the same name will be created. This option is irrelevant if *Auto Generated Name* is selected because the name constantly changes with the time.

**Background**

Select the number of data points to be acquired that will be averaged to form the background value.

Chapter 8

# View Commands

The view menu contains commands for displaying different toolbar options.

1. **Main Toolbar:** Hides or sets main toolbar from menu.

2. **Annotation Toolbar:** Hides or sets annotation toolbar from menu, which is used for inserting comments in graphs.

3. **Graph Toolbar:** Hides or sets graph toolbar buttons from menu, which is used for zooming and selecting regions.

4. **Status Bar:** Hides or sets the main status bar at the bottom of menu, which lists brief help for commands that the mouse floats over.

5. **Output:** Opens TimeMaster output options menu, which displays current decay curve fittings.

6. **TM Result:** Displays previously saved TimeMaster decay results. The files can be opened and edited.

## TimeMaster Output

When doing any kind of Data Analysis (except for DAS/TRES) a notepad window named TimeMaster Outputs pops up containing identification information, fitted parameters and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window.

**File menu commands**

open: Opens text file (for example result file from previous performed analysis).

save: Saves text in the TimeMaster Output Window as text file (default extension .txt).

print: Opens standard windows print dialog box.

**Fonts menu commands**

font: Opens standard windows dialog box for font selection.

**Edit menu commands**

undo: Cancels the last operation and restores previous situation.

cut: Cuts selected text area out of the text.

copy: Copies selected text area.

paste: Pastes from clipboard (for example text area which was cut / copied).

clear: Deletes all text in editor window.

The information displayed in TimeMaster Outputs is listed below with a brief description of the parameter.

**Identification Information**

***Analysis Function:*** Type of analysis.

***Curves:*** Curve names the analysis is based on.

***Time Range:*** Characterized by Start Time and End Time.

***Start Parameters:*** Fixed or floating start values of the used parameters.

**Statistic Results**

***Fitted Curve:*** Curve generated by the fitting procedure.

***Residuals:*** Curve displaying the difference between the calculated fit and the real data.

***Autocorrelation:*** Autocorrelation curve.

***Deconvoluted:*** Deconvoluted curve.

***Chi2:*** Chi Square Statistic for testing correlation.

***Durbin Watson:*** Durbin-Watson parameter for testing correlation.

***Z:*** Parameter expressing the result of a Runs Test.

***Pre-exponential:*** Defined as $a_i$ in the equation $I(t) = \sum[(a_i)\exp(-t/\tau_i)]$, where t is time and $\tau_i$ is the lifetime.

***Lifetime:*** Defined as $\tau_i$ in the equation $I(t) = \sum [(a_i)\exp(-t/\tau_i)]$, where t is time and $a_i$ is the pre-exponential factor.

***F1:*** Relative integrated intensities defined as $Fi= [(a_i)(\tau_i)]/[\sum(a_i)(\tau_i)]$, where $a_i$ and $\tau_i$ are the pre-exponential factors and lifetimes, respectively.

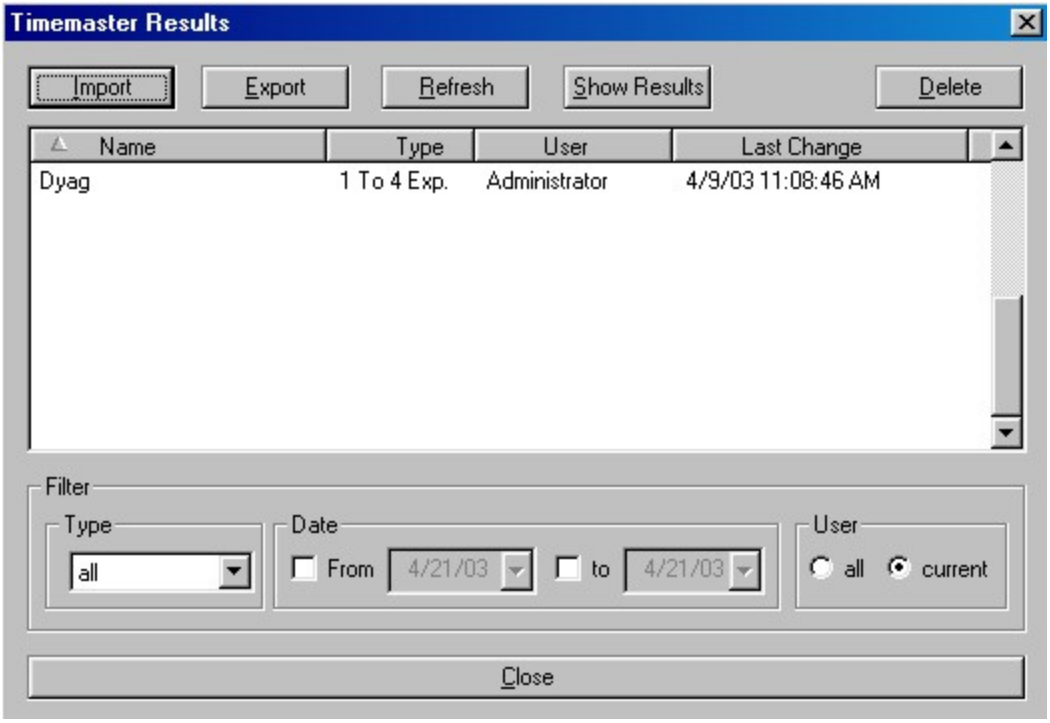
***Tau-av1:*** Steady state average lifetime defined as $Tau\text{-}av1= \sum[(a_i)(\tau_i)^2]/\sum[(a_i)(\tau_i)]$, where $a_i$ and $\tau_i$ are the pre-exponential factors and lifetimes, respectively.

***Tau-av2:*** Amplitude average lifetime defined as $Tau\text{-}av2= \sum[(a_i)(\tau_i)]/\sum(a_i)$, where $a_i$ and $\tau_i$ are the pre-exponential factors and lifetimes, respectively.

***Fitted Parameters***: Values and deviations of the curve parameters resulting from the fit.

## TimeMaster Results

TimeMaster decay fits that are saved using the Save Results button can be opened and viewed from this menu. The output results are listed in alphabetical order in the display window along with the fitting procedure, user and last date modified. Once a file has been selected, the user can view and export the data.




***Import:*** TimeMaster results from other FeliX programs or workstations can be imported by selecting this button. A typical Windows open file window will appear allowing the appropriate file to be selected. Files of extension .res, .exp, .mex, .glo, .ast, .mkn, .esm, .mem, and .nex can be imported into Felix32.

***Export:*** Opens a typical Windows file save window. Results will be saved as TimeMaster data with the extension .res.

***Refresh:*** Updates the window to show the influence of any changes to the listed files.

***Show Results:*** Opens a TimeMaster Output window where the data can be viewed and exported as a text file.

***Delete:*** Erases the file from the database.

***Filter:*** Can be used to sort the TimeMaster result files based on fitting procedure, date, and/or the user.

***Close:*** Closes the TimeMaster Results window.

Chapter 9

# Math Commands

The results of a fluorescence experiment are usually fluorescence emission intensity values that have been measured at specific wavelength or time increments. A contiguous group of data points is a curve, and the curve(s) resulting from an experiment are displayed as a group in a window.

The commands in the Math menu allow specific mathematical functions to be carried out on single curves or selected regions of a curve. Many of the math dialog boxes can be left open so that multiple operations can be performed.

Settings and controls that are common to all dialog boxes are presented later in the chapter under the heading Common Math Controls. The descriptions for the configuration dialog boxes that follow provide details on the specific math function as well as settings and controls that are unique to them.

**Note.** Some math functions are performed on a selected region of a curve (a subset of the X values). To select this region, first choose the target curve by clicking on its name in the legend. Then select the **Mark Region** icon (  ) from the graphing toolbar and use the mouse to click and drag within the graph display along the desired region of the curve. For more precise control, you can then enter *Low X* and *High X* values into the text boxes provided. The selected region will be highlighted, and the desired math value will be displayed. The math function dialog box can be left open while different regions are selected, and math values, when displayed, will change dynamically.

## Common Math Controls

**Create New Data**

If checked, a new curve will be created. The original (source) data will be preserved.

**Replace Old Data**

If checked, the original curve will be permanently lost, as it will be replaced by the new data.

**Label**

Type the name of the new curve in the text box. If no label is specified, the new curve will be listed in the legend with a name comprised of a generic math function descriptor (e.g., Smooth, or Logarithm) added to the source curve's original name.

**Execute**

Carries out the operation. If you type in new values to select an X-axis region, **Execute** is required to perform the new calculation.

**Close**

Closes the math function dialog box.

## Mark Region

This command, which is only found in the *Graph Toolbar*, is used extensively when performing math functions to select the region of interest. It can also be used as a highlighting tool to focus on an area of interest. To use this function select the trace from the legend, click on the Mark Region icon in the toolbar, then click and drag the mouse over the desired region on the graph to highlight it.

**Shortcut:**  Use the toolbar icon.

## Antilog

Calculates the antilogarithm of the selected curve.

## Average

Calculates the average value of the Y-axis parameter on a selected region of a curve. The average value is the sum of the values divided by the number of points.

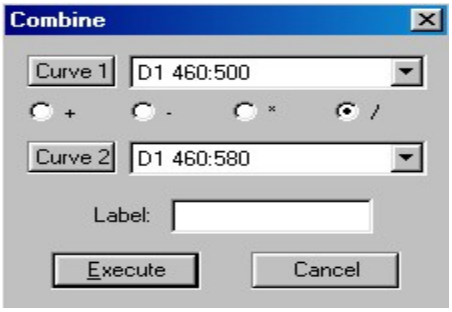
The standard deviation is also determined using the equation:

$$\sigma = \sqrt{\frac{\sum_i x_i^2 - (\sum_i x_i)^2 / n}{n-1}}$$

Where $x_i$ is a data point and $n$ is the total number of data points in the portion of the data trace being averaged.

## Combine

The combine command allows you to add one curve to another, subtract a curve from another, multiply a curve by another, or divide a curve by another. The math is performed in a point-by-point fashion. Only the portions of the curves that overlap are combined.




**Curve 1, Curve 2**

Select curves for the operation by clicking on their names in the drop-down list boxes. Alternatively, select a curve from the legend and click on the Curve 1 or Curve 2 button.

**Operation**

Check an operator to add (+), subtract (-), multiply (x), or divide (/) Curve 1 by Curve 2.

## Combine Constant

This command allows you to apply an arithmetic operation between a curve and a constant.

**Curve 1**

Select a curve for the operation by clicking on its name in the drop-down list box. Alternatively, select a curve from the legend and click on the Curve 1 button.

**Operation**

Check an operator: add (+), subtract (-), multiply (x), or divide (/).

**Constant**

Enter a numerical value. Exponential notation is allowed.

## XY Combine

This feature allows the user to construct a new data trace, using the X values of one trace, and the Y values of another trace. In this way, complex data, such as time-dependent temperature ramps and correlated data, can be converted into new traces that have compatible X axes to simplify the display and treatment of the data.

**Source trace with X data**

Use the drop-down menu to choose the trace from which to create the X data. Alternatively, select a curve from the legend and click on the Pick icon beneath the Source trace with X data header.

**Source trace with Y data**

Use the drop-down menu to choose the trace from which to create the Y data. Alternatively, select a curve from the legend and click on the Pick icon beneath the Source trace with Y data header.

## Differentiate

Differentiate takes the derivative of the selected curve. Subsequent application of the differentiate command results in the second derivative, etc… Differentiation is done using the 5 point Savitzky-Golay algorithm, which provides a smoothed derivative.

## Integrate

This function integrates within the range of the selected region of a curve. The Total Area is the integral of the data above the absolute X-axis. The Peak Area is used to integrate a peak within a curve.

**Total Area**

Displays the total integrated area within the selected range. If there is negative data, then the total integrated area may also be negative.

**Peak Area**

Displays the integral of the peak above the background. FeliX32 projects a line between the points where the boundaries of the range intersect the curve. Peak Area is the integrated area above that line. If most of the curve data lies below this line, then the Peak Area will be a negative number.

## Linear Fit

Calculates and overlays a linear fit to the selected region of a curve. The slope, intercept, and correlation coefficient are displayed.

## Linear Scale

The Linear Scale is used to shift a curve or a selected region of a curve on either the X- or the Y-axis. The curve can be shifted on the Y-axis by a multiplier, divisor, or an addend. The curve can be shifted on the X-axis by an addend only.

**Y and X Value**

***Multiplier:*** Multiplies all Y values in the curve by the specified multiplier.

***Divisor:*** Divides all Y values in the curve by the specified divisor.

***Offset:*** Adds the specified value to each X or Y point in the curve.

**Select Range**

Applies the transformation only within the region selected by the user. The range is selected using the **Mark Region** toolbar icon and clicking and dragging the mouse over the desired area in the workspace.

## Logarithm

Calculates the logarithm of the selected curve.

## Normalize

Normalizes a curve to a set value. The normalization function reference may be either a peak or a specified point.

**Reference**

Select ***Peak*** or ***Specified Point***. Enter the X value of the specified point in the text boxes.

**Normalize to:**

Enter the value to which the curve will be normalized.

## Reciprocal

Calculates the reciprocal (1/Y) of the Y-axis data in the selected curve.

## Smooth

This function performs a Savitzky-Golay smoothing of the selected curve.

**Buffer Size**

Select a *7*, *15*, or *21*-point buffer. A higher buffer results in greater smoothing.

## Truncate

Truncate is used to reduce the X-axis range on the selected curve. The selected region of the curve is preserved and all X values above and below this region are permanently deleted. The region may also be selected using the **Mark Region** icon in the toolbar and clicking and dragging the mouse over the desired range in the workspace.

## Baseline

Baseline suppression causes a selected region of a curve to be set to a constant Y value (commonly zero). The region is selected as described in the introduction to this chapter. The chosen Y-value is entered into the text box and the function is performed by pressing the execute button. This function is useful when noise in the baseline of the scatterer affects the lifetime of the sample. This happens because the IRF (scatterer) is convoluted with the sample lifetimes to give the observed decay. Thus noise in the scatterer is also convoluted and becomes a major problem for long-lived samples when observations are recorded out to many sample lifetimes. The convoluted noise has the same effect as small light pulses long after the real light pulse has ended. The derived lifetimes therefore appear to be smaller than they really are. See the **Analysis** chapter for further details.

## Peak Finder

This function finds the global peak as the highest Y-value and local peaks as being higher than immediate left and right neighboring points.

**X-range limits**

Displays the low and high limits set by Mark Region.

**Mark peak on graph**

Shows a crosshair at the peak position on the graph.

**Global peak**

The peak within the selected range with the highest Y-axis value.

**Local peak to right/left**

Click on **Execute** to find the next peak to the right or left.

## Analysis

The various analysis programs are accessed through a drop-down menu. Only users with the correct Customer Access Code can access them (see **Configure, Preferences** in Chapter 13). These programs are discussed in **Data Analysis,** Chapter 15.

## Conversion: Energy to Quantum

Converts the selected spectrum from energy units to quantum units proportional to the number of photons per second.

## Conversion: Quantum to Energy

Converts the selected spectrum from quantum units, expressed as the number of photons detected at a given wavelength (or wavenumber), to energy units proportional to the number of photons detected at a given wavelength (or wavenumber) multiplied by the photon energy.

## Conversion: Wavelength to Wavenumber

Converts the selected trace from units of wavelength (nm) to wavenumber (1/cm). This command will also convert the trace to wavelength from wavenumber. Selection of which direction to convert is performed automatically.

# Fluorescence Resonance Energy Transfer (FRET)

**Theory**

The Fluorescence Resonance Energy Transfer (FRET) takes place between an excited donor molecule (D) and the ground-state acceptor molecule (A) over a range of distances, typically 10-100 Å. FRET is a non-radiative process (i.e. there is no photon emitted or absorbed during the energy exchange). The efficiency of FRET is strongly dependent on the D-A distance and is characterized by the Förster critical radius $R_o$, a unique parameter for each D-A pair. When the D-A distance is $R_o$, the efficiency of energy transfer is 50%. Once $R_o$ is known, the D-A pair can be used as a molecular ruler to determine the distance between sites labeled by D and A.

There are two basic methods to determine the efficiency of FRET: a) by measuring a decrease of fluorescence intensity of D in the presence of A, and b) by measuring the fluorescence lifetime of D, which becomes shortened as a result of FRET. In some cases, one can also monitor an enhancement of the acceptor fluorescence or the acceptor lifetime, if it is much shorter than the donor lifetime. *PTI* provides specialized systems for both steady state and time-resolved FRET techniques, which can be used for a variety of FRET applications.

If any quantitative information is expected from a FRET experiment, it is imperative that the $R_o$ value is known. The $R_o$ value can be calculated as follows:

$$R_o = 0.2108\ (\kappa^2\ \Phi_D\ n^{-4}\ J_{DA})^{1/6} \qquad \text{Eq. 1}$$

where $\kappa^2$ is the orientation factor, $\Phi_D$ is the quantum yield of D in the absence of A, n is the refraction index of the medium, and $J_{DA}$ is the spectral overlap integral between the excitation spectrum of A and the emission spectrum of D. The overlap integral $J_{DA}$ can be calculated if the absorption (excitation) spectrum of A and the fluorescence emission spectrum of D are known, i.e.

$$J_{DA} = C\int_0^\infty I_D(\lambda)E_A(\lambda)\lambda^4 d\lambda \qquad \text{Eq. 2}$$

where $I_D$ is the emission spectrum of D, $E_A$ is the absorption (excitation) spectrum of A and C is the normalization factor defined as:

$$C = \frac{\varepsilon(\lambda_{\max})}{E_A(\lambda_{\max})\int_0^\infty I_D(\lambda)d\lambda} \qquad \text{Eq. 3}$$

where $\varepsilon(\lambda_{max})$ is the molar extinction coefficient of A at the absorption (excitation) maximum.

The value of $\kappa^2$ depends on a relative orientation of D and A transition moments. If the transition moments have fixed orientations, $\kappa^2$ will vary from 0 (transition moments perpendicular) to 4 (transition moments collinear). For parallel transition moments $\kappa^2 = 1$. When A molecules are randomly distributed about D in a rigid medium, $\kappa^2 = 0.476$. If D and A undergo a rotational motion, which is faster than the decay time of D, $\kappa^2 = 2/3$.

The energy transfer efficiency E can be calculated from either fluorescence intensity or lifetime measurements for D alone and D in the presence of A.

$$E = 1 - \frac{I_{DA}}{I_D} \qquad \text{Eq. 4}$$

where $I_D$ and $I_{DA}$ are fluorescence intensities of D in the absence and presence of A, respectively, or

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \qquad \text{Eq. 5}$$

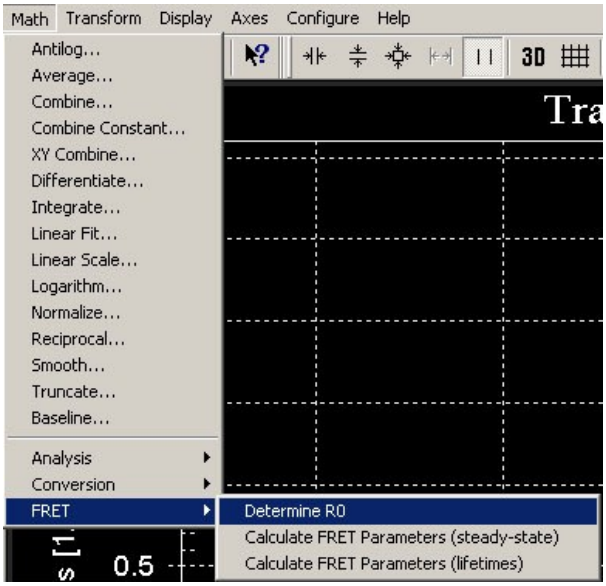
where $\tau_D$ and $\tau_{DA}$ are fluorescence lifetimes of D in the absence and presence of A, respectively.

Once $R_o$ and E are know, the distance r between D and A can be calculated:

$$r = \left(\frac{1}{E} - 1\right)^{1/6} R_o \qquad \text{Eq. 6}$$

If the lifetime of D is known, the FRET rate constant can also be calculated:

$$k_{ET} = \frac{1}{\tau_D}\left[\frac{R_o}{r}\right]^6 \qquad \text{Eq. 7}$$




**Using the FRET Calculator**

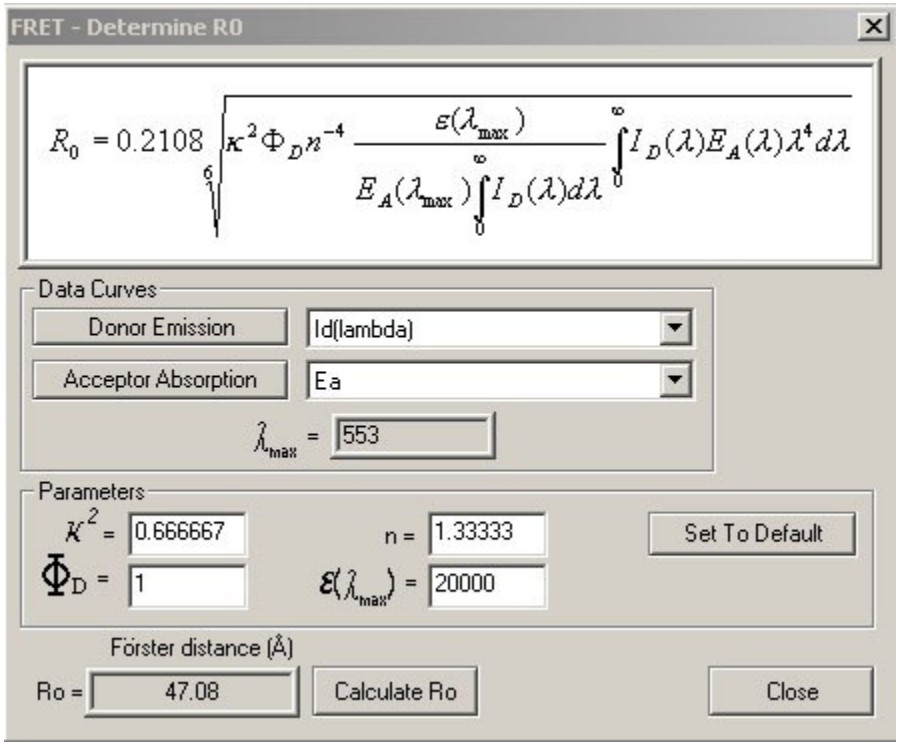
Requires the correct Customer Access Code (see **Configure, Preferences** in Chapter 13).

Clicking on Math and then on FRET can access the FRET Calculator. The FRET drop down menu gives three choices: *Determine Ro*, *Calculate FRET Parameters (steady-state)* and *Calculate FRET Parameters (lifetimes).*

**Determine $R_o$**

The ***Donor Emission*** button selects the curve to be used as donor emission spectrum. Select a curve by clicking on its name at the left side of the FeliX32 screen and then click on the ***Donor Emission*** button. The name of the selected curve will appear on the box beside the button.

The ***Acceptor Absorption*** button selects the curve to be used as acceptor absorption (excitation) spectrum. Select a curve by clicking on its name at the left side of the FeliX32 screen and then click on the ***Acceptor Absorption*** button. The name of the selected curve will appear on the box beside the button.




Once the curves have been defined, the acceptor absorption (excitation) maximum wavelength will be displayed in the $\lambda_{max}$ box.

Enter the value for the orientation factor in the $\kappa^2$ box or leave the default value of 2/3 for the fast rotation limit.

Enter the value for the donor emission quantum yield in the $\Phi_D$ box (the default value is 1).

Enter the value for the index of refraction in the ***n*** box (the default value is 1.33333 for water).

Enter the value for the molar extinction coefficient for the acceptor at the absorption (excitation) maximum in the $\boldsymbol{\varepsilon(\lambda_{max})}$ box (the default value is 20000).

Click on the ***Calculate $R_o$*** button and the Förster distance $R_o$ will be displayed in the $\boldsymbol{R_o}$ box.

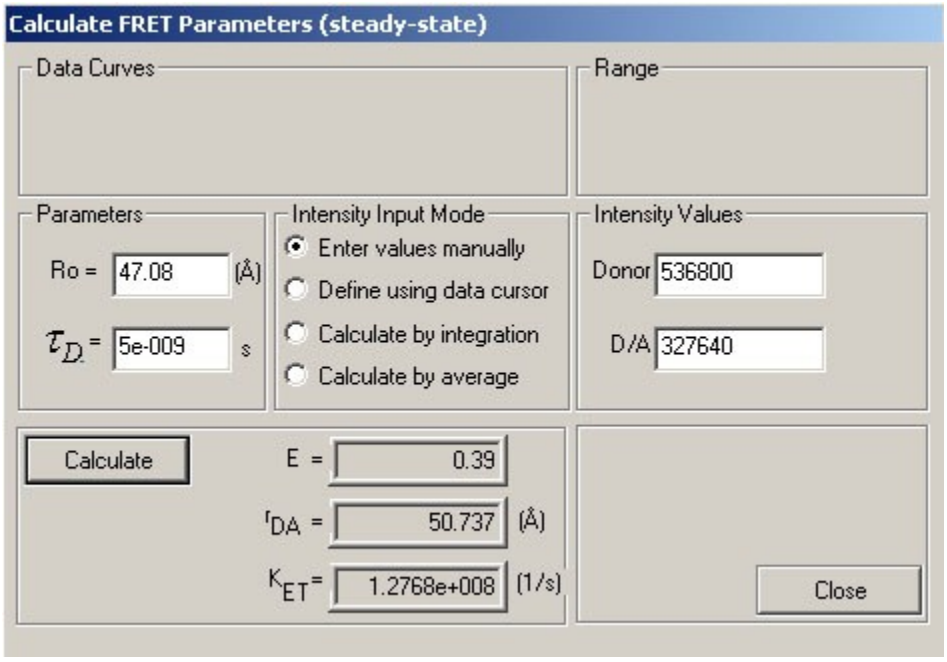
Click on the ***Set To Default*** button to reset all the parameters to default values.

**Calculate FRET Parameters (steady-state)**

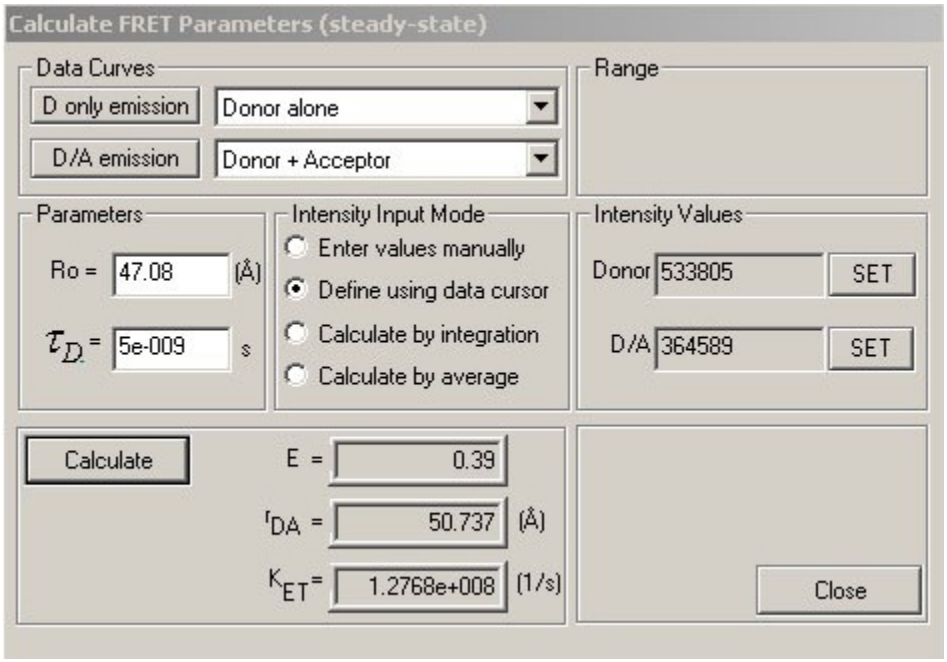
In the ***Parameters*** box, either enter the value of $\boldsymbol{R_o}$ or retain the value calculated in the *Determine $R_o$* option.

In the ***Parameters*** box, enter the lifetime of the donor $\boldsymbol{\tau_D}$ if you want the FRET rate constant to be calculated.

- To enter intensity values manually, click on the ***Enter values manually*** radio button in the *Intensity Input Mode* box. Then type in intensity values for donor alone in the ***Donor*** box and for donor in the presence of acceptor in the ***D/A*** box. Click on ***Calculate*** and the FRET efficiency ***E***, donor-acceptor distance ($\boldsymbol{r_{DA}}$) and FRET rate constant $\boldsymbol{k_{ET}}$ will be displayed. The $\boldsymbol{k_{ET}}$ value will only have any meaning if the correct $\boldsymbol{\tau_D}$ has been entered, otherwise it should be ignored.

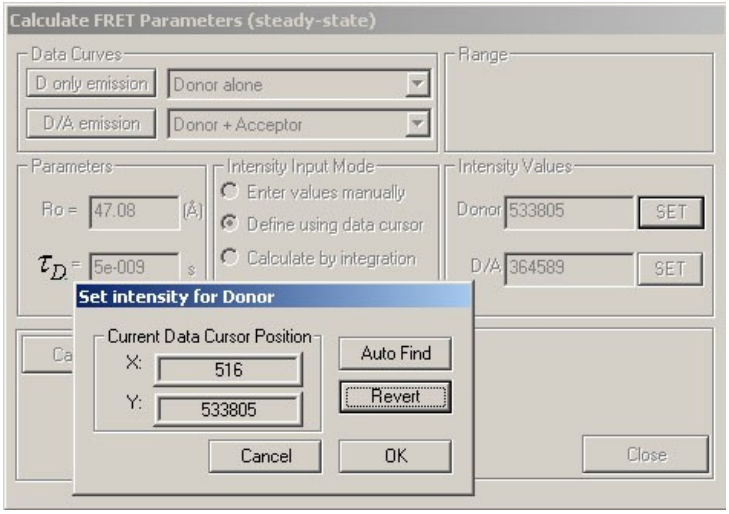


- To enter donor intensities using the data cursor, click on the ***Define using data cursor*** radio button. The *Data Curves* box becomes available.




The ***D only emission*** button selects the donor emission curve. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D only emission*** button.

The ***D/A emission*** button selects the donor emission curve measured in the presence of acceptor. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D/A emission*** button.

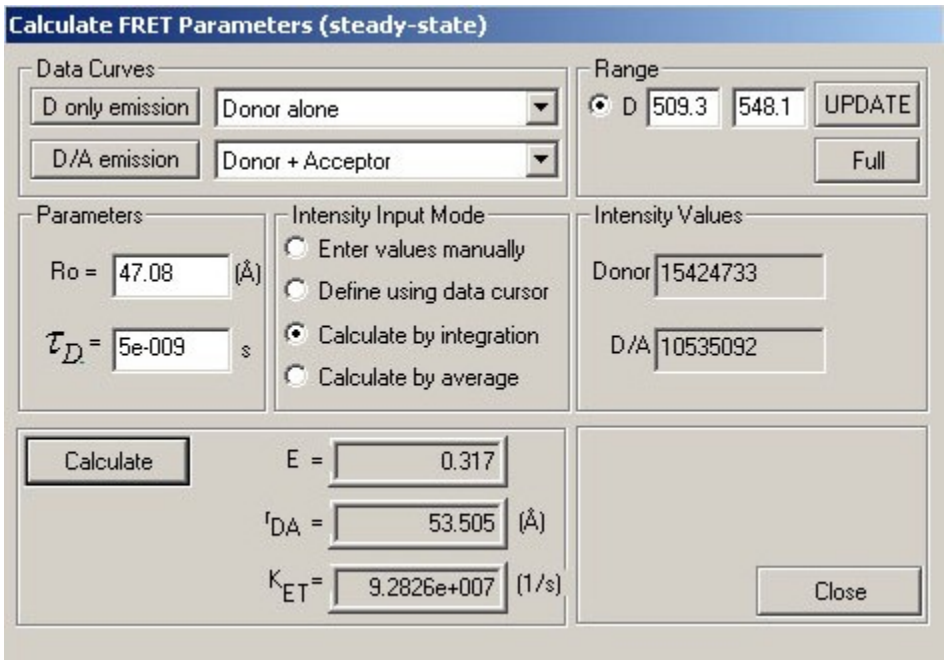



In the ***Intensity Values*** box click on the ***SET*** button next to the *Donor* box, which opens the ***Set Intensity for Donor*** box. Using the left and right arrow keys move the cursor to the desired position for the intensity readout and click OK. Alternatively, position the mouse cursor at the desired point on the curve and left click. The intensity of the *Donor* will be captured. Use ***Auto Find*** to automatically position the cursor at the maximum of the donor curve. Click on the ***Revert*** button to re-position the cursor at the beginning of scale. Click on the ***Revert*** button whenever the cursor becomes unresponsive.

In the ***Intensity Values*** box click on the ***SET*** button next to the *D/A* box, which opens the ***Set Intensity for D/A*** box. Using the left or right arrow keys move the cursor to the desired position for the intensity readout and click OK. Alternatively, position the mouse cursor at the desired point on the curve and left click. The intensity of the *D/A* will be captured. Use ***Auto Find*** to automatically position the cursor at the maximum of the *D/A* curve. Click on the ***Revert*** button to re-position the cursor at the beginning of scale. Click on the ***Revert*** button whenever the cursor becomes unresponsive.

Click on the ***Calculate*** button and the FRET efficiency ***E***, donor-acceptor distance ($r_{DA}$) and FRET rate constant $k_{ET}$ will be displayed. The $k_{ET}$ value will only have any meaning if the correct $\tau_D$ has been entered, otherwise it should be ignored.

- To calculate donor and D/A intensities by integration, click on the ***Calculate by integration*** radio button. The *Data Curves* box becomes available.




The ***D only emission*** button selects the donor emission curve. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D only emission*** button.

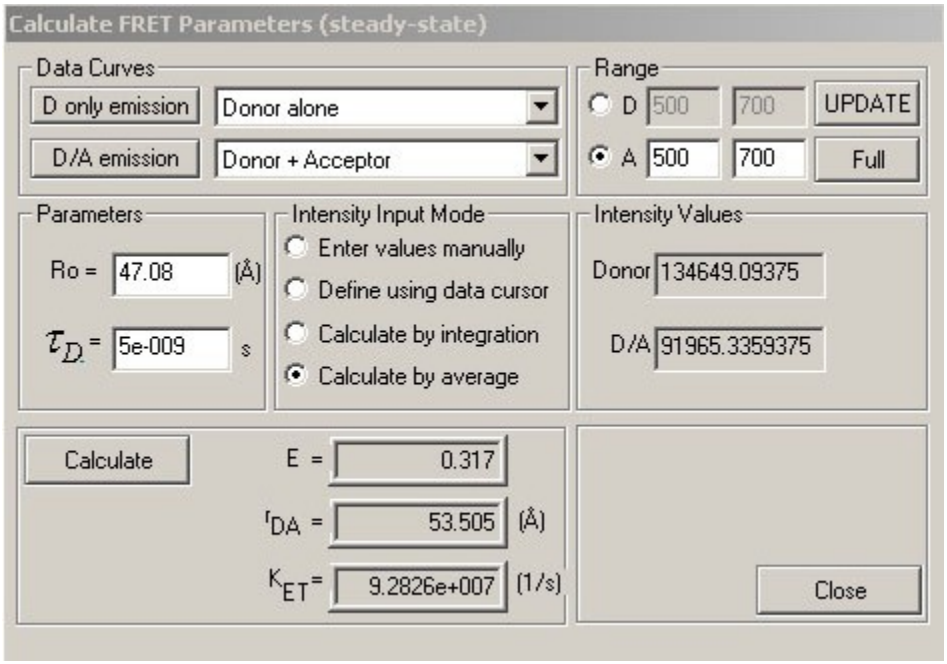
The ***D/A emission*** button selects the donor emission curve measured in the presence of acceptor. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D/A emission*** button.

***Range:*** To select the integration range, position the mouse pointer at the desired start of the integration, click and hold down the left mouse button, drag the mouse to the desired end of the integration and release the button. The integrated intensity values will be displayed in the *Intensity Values* box. Alternatively, click on the ***Range*** radio button, type in the start and end integration values and click on the ***UPDATE*** button.

The intensities will be displayed in the *Intensity values* box. If the integration is to be carried out over the entire range, just click on FULL and the intensities will be captured and displayed.

Click on the ***Calculate*** button and the FRET efficiency ***E***, donor-acceptor distance ($r_{DA}$) and FRET rate constant $k_{ET}$ will be displayed. The $k_{ET}$ value will only have any meaning if the correct $\tau_D$ has been entered, otherwise it should be ignored.

- To calculate donor and D/A intensities by average, click on the ***Calculate by average*** box. The *Data Curves* box becomes available.




The ***D only emission*** button selects the donor emission curve. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D only emission*** button.

The ***D/A emission*** button selects the donor emission curve measured in the presence of acceptor. Select a curve by clicking on its name at the left side of the FeliX32 screen, then click on the ***D/A emission*** button.

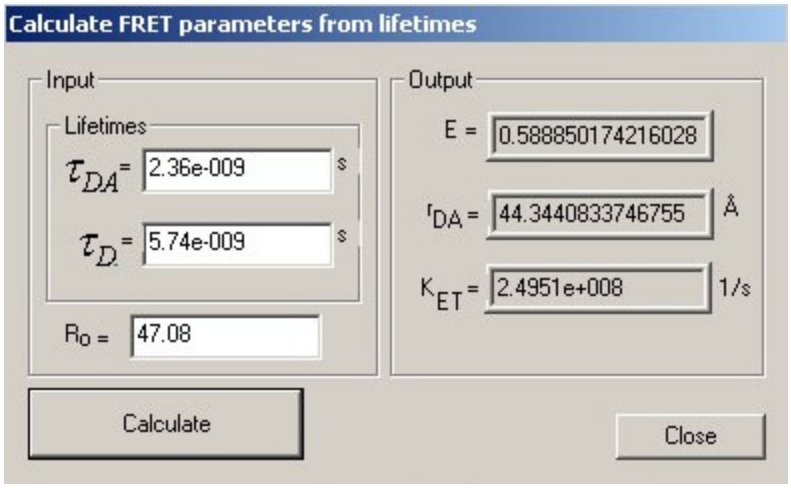
***Range for D:*** To select the averaging range for the donor alone, click on the ***D*** radio button in the *Range* box, position the mouse pointer at the desired start of the integration, click and hold down the left mouse button, drag the mouse to the desired end of the range and release the button. The average intensity value for *D* will be displayed in the *Intensity Values* box. Alternatively, type in the start and end values for the range and click on the ***UPDATE*** button. The average *D* intensity will be displayed in the *Intensity values* box. If the averaging is to be carried out over the

entire range, just click on the ***FULL*** button and the intensity will be captured and displayed.

***Range for D/A:*** To select the averaging range for the donor in the presence of acceptor, click on the ***A*** radio button in the *Range* box, position the mouse pointer at the desired start of the integration, click and hold down the left mouse button, drag the mouse to the desired end of the range and release the button. The average intensity value for *D/A* will be displayed in the *Intensity Values* box. Alternatively, type in the start and end values for the range and click on the ***UPDATE*** button. The average *D/A* intensity will be displayed in the *Intensity values* box. If the averaging is to be carried out over the entire range, just click on the ***FULL*** button and the intensity will be captured and displayed.

Click on the ***Calculate*** button and the FRET efficiency ***E***, donor-acceptor distance ($\boldsymbol{r_{DA}}$) and FRET rate constant $\boldsymbol{k_{ET}}$ will be displayed. The $\boldsymbol{k_{ET}}$ value will only have any meaning if the correct $\boldsymbol{\tau_D}$ has been entered, otherwise it should be ignored.

**Calculate FRET Parameters (lifetimes)**




In the ***Input*** box, enter the lifetime value of donor in the presence of acceptor ($\boldsymbol{\tau_{DA}}$) and the donor alone ($\boldsymbol{\tau_D}$). At the bottom of the *Input* box, either enter the value of $\boldsymbol{R_o}$ or retain the value calculated in the *Determine* $R_o$ option.

Click on the ***Calculate*** button and the FRET efficiency ***E***, donor-acceptor distance ($\boldsymbol{r_{DA}}$) and FRET rate constant $\boldsymbol{k_{ET}}$ will be displayed.

Chapter 10

# Transform Commands

Settings and controls that are common to all dialog boxes are presented together at the end of the chapter under the heading Common Transform Controls. The descriptions for the configuration dialog boxes that follow provide details on the specific math functions as well as settings and controls that are unique to them. For further information on commands in the Transform menu please see the online Help utility.

## Concen. Map...

Concentration mapping is used to convert saved experimental data to concentration. The experimental data may be intensity or the ratio of two intensities.

**Lookup Tables**

Lookup tables can be constructed to calculate the concentration in several different ways.

***Intensity to Concentration:*** For most steady state experiments, the intensity is related directly to concentration.

***Ratio to Concentration:*** For most ratio fluorescence experiments, the ratio of two intensities is related to concentration.

***Ratio to pH:*** Converts ratio values to pH.

**Formula**

The concentration of intracellular ions can be calculated directly from the ratio of intensities through the equation from Grynkiewicz, Poenie, and Tsien.

Select the radio button for the appropriate conversion and click Edit/Select to choose/modify/create a Lookup Table (or Formula) that contains the calibration curve. For more information in regards to constructing Lookup Tables and Concentration Equations please see the **Configure Lookup Table** and **Configure Concentration Equation** sections below.

## Correct…

Correction is used in post-acquisition mode to correct fluorescence emission spectra and fluorescence excitation spectra. This is done to compensate for the wavelength dependence of detector sensitivity and excitation light source output, respectively. The correction files are enabled in the *Configure Correction* dialog box.

**Configure Correction**

In this menu you are given the option to choose excitation and emission corrections for post-acquisition analysis. Double click in the boxes next to Excitation and Emission to enable their corrections factors. An X appears in the box to signify that the correction will be used. You must select the appropriate correction curve for excitation and emission by double clicking on the line directly below the label excitation and the label emission. In each case you will be presented with a list of available correction curves for that type of correction. You may have several correction files depending upon your instrument configuration.

**Note.** Your database must contain the correction curves in the Lookup Table dataset. Your system will be delivered with the correction curves specific to your instrument. It is potentially harmful to FeliX32 if you alter the Lookup Table Dataset.

**Note.** Post-acquisition excitation correction is not valid for all circumstances. Use this feature with care!

Once the corrections are selected they will become the default values for post-acquisition analysis. Click *Okay* to return to the main *Correction* menu.

## Polarization…

This function is used for post-acquisition calculation of polarization or anisotropy from saved experimental data. Use the radio buttons to select the operation to perform anisotropy or polarization.

**Curve 1 (VV)**

Select from the drop-down box the curve with polarizers in the vertical excitation and vertical emission orientation (parallel). Alternatively, select the curve from the legend and click on the *Curve 1 (VV)* button.

**Curve 2 (VH)**

Select from the drop-down box the curve with polarizers in the vertical excitation and horizontal emission orientation (perpendicular or crossed). Alternatively, select the curve from the legend and click on the *Curve 2 (VH)* button.

**Config. G-Factor**

The G Factor is used in calculating polarization or anisotropy. It is the ratio of the relative transmission efficiencies of the emission channel for horizontally and vertically polarized light. The G Factor can be measured with any sample. The excitation polarizer is rotated to the horizontal position. Emission is measured with the emission polarizer in the horizontal and vertical positions.

$$G = I(HV)/I(HH)$$

The G Factor is wavelength-dependent, and is different between gratings. If the grating is changed, the G-factor will need to be measured, even if the same wavelength is studied.

The G Factor can be entered manually in the text box or it can be captured directly from a G Factor curve. The G Factor curve is calculated by running the I(HV) curve and the I(HH) curve and taking the ratio. For a Timebased curve, use **Mark Region** and click and drag the mouse over the desired range of data. If the selected region contains more than one data point, the data points are averaged. Click Capture. For an acquisition in which the wavelength was scanned, select only one wavelength.

***G-Factor:*** Enter a pre-determined G-Factor manually or highlight a region of a G-Factor curve (or select a curve) using *Mark Region* and select *Capture*. The average Y value over the selected range will be entered into the G-Factor text box. Prior to clicking Capture you can see the value that will be captured in the *Capture Value* text box. If you enter **HV** and **HH** values into their text boxes, the G-Factor will be calculated automatically.

***HV:*** Select the curve from the legend having polarizers with horizontal excitation and vertical emission orientation. Click on *Capture* to enter the average value of the selected curve. Alternatively, enter an HV value manually or select a region of a curve using *Mark Region* and click Capture to acquire the region's average value into the text box. Prior to clicking Capture you can see the value that will be captured in the *Capture Value* text box.

***HH:*** Select the curve from the legend having polarizers with horizontal excitation and horizontal emission orientation. Click on *Capture* to enter the average value of the selected curve. Alternatively, enter an HH value manually or select a region of a curve using *Mark Region* and click Capture to acquire the region's average value into the text box. Prior to clicking Capture you can see the value that will be captured in the *Capture Value* text box.

***Use Lookup Table:*** Lookup Tables can be constructed to correct for the G-Factor over a range of wavelengths. Select this option to use/create/modify a G-Factor Lookup Table. For more information on Lookup Table properties and construction please see the **Configure Lookup Table** section below.

## Common Transform Controls

**Create New Data**

If checked, a new curve will be created. The original (source) data will be preserved.

**Replace Old Data**

If checked, the source curve will be permanently lost. It will be replaced by the new data.

**Label**

Type the name of the new curve in the text box. If no label is specified, the new curve will be listed in the legend with a name comprised of a generic transform descriptor (e.g. Concentration) added to the source curve's original name.

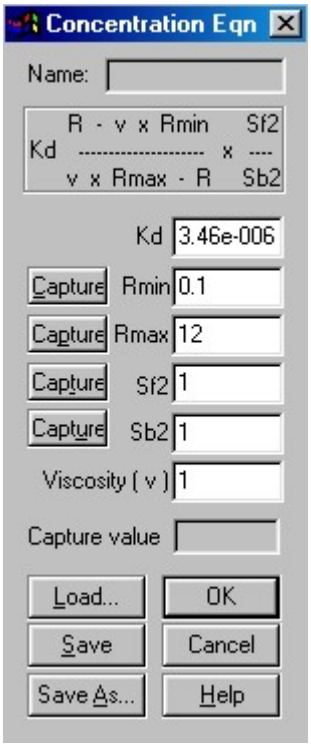
**Execute**

Carries out the operation.

**Close**

Closes the transform dialog box.

## Configure Concentration Equation




The Concentration Equation establishes the conditions for converting intensity ratios directly to intracellular ion concentrations using the equation from Grynkiewicz, Poenie, and Tsien (J. Biological Chemistry, 260, 3440-3450(1985)) in the calculation of concentrations from ratio fluorescence data.

The dialog box displays the concentration equation, and has text boxes to enter the following values:

***Kd:*** Enter this value manually.

***Rmin:*** Enter this value manually or capture it from the selected region of a curve. Highlight a selected region using *Mark Region*, and click *Capture*.

***Rmax:*** Enter this value manually or capture it from the selected region of a curve using. Highlight a selected region using *Mark Region*, and click *Capture*.

***Sf2:*** Enter this value manually or capture it from the selected region of a curve. Highlight a selected region using *Mark Region*, and click *Capture*.

***Sb2:*** Enter this value manually or capture it from the selected region of a curve. Highlight a selected region using *Mark Region*, and click *Capture*.

***Viscosity (v):*** Enter this value manually.

**Note.** You can see the value that will be captured prior to selecting capture in the *Capture Value* text box.

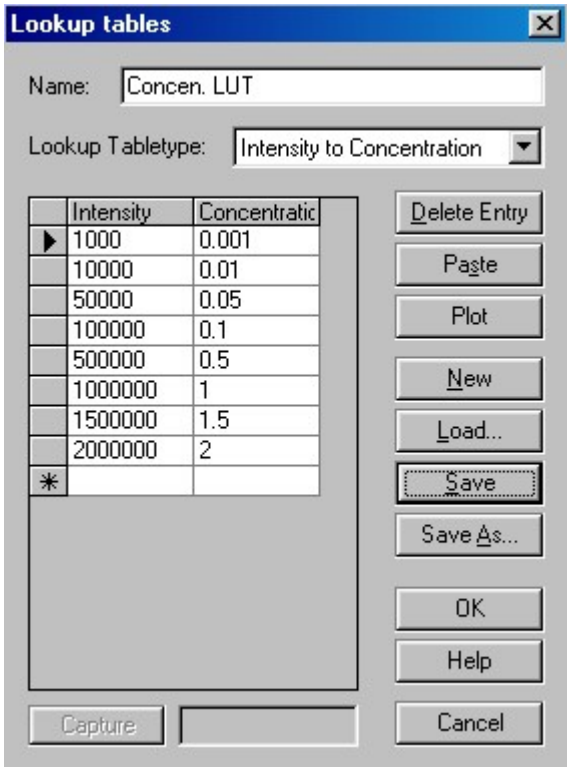
***Load:*** Opens a Lookup Table containing a saved equation.

***Save:*** Stores any changes to the current values to the database as a Lookup Table. The current Lookup Table name appears on the title bar of the dialog box.

***Save As:*** Saves the current values to a new Lookup Table in the database.

***OK:*** Selecting OK exits from the menu without saving any modifications. You must save the equation prior to exiting if you wish to use it.

## Configure Lookup Table




This command is used to construct a lookup table to relate intensities or ratios of intensities to concentration. A lookup table is a plot of intensity (or an intensity ratio) versus concentration or pH. The concentration of an unknown sample is calculated by measuring the intensity and interpolating between values on the lookup table to calculate the concentration.

**Note.** This dialog box only constructs the lookup table. The lookup table (or LUT) is implemented by *selecting Inten.-Concen. LUT, Ratio-Concen. LUT,* or *Ratio-pH LUT* under *Function* in the **Display Setup** dialog box or by selecting the same functions under **Transform/Concentration Map**

An LUT can also be used for calculating *G-Factor* in anisotropy and polarization experiments. The G-Factor LUT can similarly be reached under Function in the *Display Setup* dialog box or by selecting *Configure G-Factor* in **Transform/Polarization** or in a **Timebased Polarization** acquisition.

To prepare the lookup table, first collect data from a series of standard samples that bracket the concentrations you expect to encounter. You may save these standards as individual curves or in one curve set. Data acquisition parameters must be identical for standards and samples. To put it more explicitly: the calibration of ratio-based data will be valid only if the samples are measured with the same slit widths as the standards. The Configure Lookup Table can be left open while working with other features.

To construct the lookup table:

1. In the drop-down list box, specify whether you are converting Intensity to Concentration, Ratio to Concentration, or Ratio to pH. Polarization is the default for G-Factor.

2. Enter the intensity/ratio and concentration/pH for each standard, or wavelength and G-Factor, into the table below the list box. The table functions like a spreadsheet. Click on a cell to enter the value manually.

You can also capture the value directly from a selected region in a curve. Select a curve from the legend. Identify the intensity to be captured by clicking and dragging the mouse over the desired range of data using the **Mark Region** function. If the selected region contains more than one data point, the data points are averaged. Click on Capture to place the intensity into the highlighted cell in the table. The concentration value is entered manually.

To place an entire curve into the LUT (especially useful for G-Factor), simply select the curve in the legend and select **Edit/Copy** from the main menu. In the LUT menu, select **Paste** and all data points in the trace will be pasted into the cells with X values in the first column and Y values in the second.

***Hint:*** To aid in capturing data for this process, choose **Display/Connect Points** (or use the toolbar button) to display curves as data points only. Select the region containing the wanted data using **Custom X-Zoom** (or use the toolbar button). You can also scroll along the X-axis using the left and right arrow keys.

The Lookup Table dialog box has these additional controls:

***Delete Entry:*** Select an entry in the Lookup Table and click Delete Entry to have the data point stricken from the LUT. Deleting in this manner is irreversible.

***OK:*** Selecting OK exits from the menu without saving any modifications. You must save the Lookup Table prior to exiting if you wish to use it. You will be prompted to save the Lookup Table if it is not already saved.

***Paste:*** Enters all values from a curve. To use this feature, select a curve, and choose *Edit/Copy*. Then click on *Paste*.

***Plot:*** Displays a graphical representation of the values in the table.

***Load:*** Opens a saved file.

***Save:*** Saves any changes to the current values. The current Lookup Table name appears on the title bar of the dialog box.

***Save As:*** Saves the current values to a new Lookup Table in the database.

***New:*** Clears any unsaved information from the cells so that a new lookup table can be created.

***Cancel:*** Exits from the menu without saving. You will not be prompted to save any modifications.

**Note.** Real time correction curves for excitation and emission are not created using this technique. They must be physically placed into the appropriate groups in the Lookup Table dataset in the database. Opening the Lookup Table dataset is **not recommended** as you may **damage** system files.

Chapter 11

# Display Commands

The display of curves in the FeliX32 workspace is controlled by commands in the **Display** and **Axes** menus.

## Normal View

Changes the display mode to a graphical plot of X and Y values. Curve(s) will be presented graphically. The X and Y scales can be adjusted in order to best display data.

## 3D View

FeliX32 has been developed to provide scientists with a software package that aids the data plotting and visualization and helps present your work in its best light. And when you think of a new, better, or different way to present the data, FeliX32 gives you full control over 2D and 3D plot parameters. FeliX32 is a complete package that contains plotting software with extensive 2D and 3D capabilities for visualizing data from analyses, and experiments. Whether you're doing scientific analyses, or experiments, FeliX32 allows you to explore the data, produce informative 2D and 3D views and create presentation-quality plots and animations.

Here is how you can use FeliX32 to generate your 3D plots.

1. Ensure that your data are all under one group and that all these traces have the same length.

2. Select the group and right-click the mouse button. This opens a dialog box with a series of options.

3. Select the *z-parameter* option and a new window will open enabling the definition of the z-axis.

4. Choose excitation wavelength as the z-axis or create your own with the user defined option. The *First* and *Step* boxes allow for user defined z-parameters to be inputted, First representing the minimum z value to be used.

5. Now that the parameters have been entered, selecting 3D mode from the graph toolbar will plot the graph.

**Shortcut:** 

Click on the toolbar icon. This icon when clicked will toggle the view between *normal view* and *3D view*.

Having generated the 3D plot you have a variety of options to manipulate the plot to generate a visually stimulating presentation. You may change the way the 3D display looks within the workspace by right clicking anywhere within the graph screen, which will show the 3D View chart options.

***Viewing Style:*** Gives one the options of color and monochrome.

***Font Size:*** You can select three different (small, medium, large) sizes for plot features such as title, axes titles.

***Numeric Precision:*** Allows one to select the number of decimal places to plot the data to on all the axes.

***Grid Lines:*** You can display grid lines on both axes, one individually or not at all.

***Show Bounding Box:*** This option encloses the 3D plot in a cube which allows one a better appreciation of the depth being displayed. There are three choices under this selection; 1) *While Rotating* will display the bounding box only when the image is being rotated; 2) *Always* will display at all times; and 3) *Never* will disable this option.

***Rotation Animation:*** By selecting this option the 3D image is put in an animated environment where it rotates clockwise through a 360° angle in increments.

***Rotation Increment:*** This option allows one to choose a particular angle rotation for the selected image. The following angles of rotation are available through selecting this option (15, 10, 5, 2, 1, -1, -2, -5, -10, -15).

***Rotation Detail:*** This option lets you set how much detail is shown during graph rotation.

***Plotting Method:*** This option gives the following choices for plotting the data: wireframe, surface, surface with shading, surface with contouring, and pixels.

***Shading Style:*** There are white and various color types of shading available under this option.

***2D Contour:*** The Contour option performs the calculations on the data allowing the representation to be projected onto either equal angle or equal area stereograms. The contour option allows the user to set contour lines on top or bottom as well as color or black and white contours.

***Maximize:*** Maximize viewing area for plot.

***Customization Dialog:*** This dialog box provides the user with more options in customizing the looks of the generated plot. This menu has submenus that set other plot parameters, such as font style, plot style, color, etc…

***Export Dialog:*** Allows the 3D plot and key areas to be exported as Windows ordinary and enhanced metafiles (ex. Bitmap). These can be imported into many applications including CorelDraw, Word etc…

## Grid View

This is a toggle command. It is used to change the display mode of the active window. Clicking on Grid View will change the display to a spreadsheet format. The spreadsheet looks and behaves as those in other applications. You can select ranges of cells, individual curves, or multiple curves for copying and exporting. Clicking on Grid View will change the display to a graphical plot of X and Y values. The X and Y scales can be adjusted in order to best display the data.

**Shortcut:**  Use the toolbar button. It will toggle between the two modes.

## Grid Lines

This toggle command is used to display or hide the grid lines in the active workspace. When a checkmark appears next to the command in the menu, the grid lines of the active workspace will appear. To control which grid lines are shown, right click on the graph area and choose *Grid Lines*.

**Shortcut:** Press **Ctrl+G** on the keyboard.

## Data Cursor

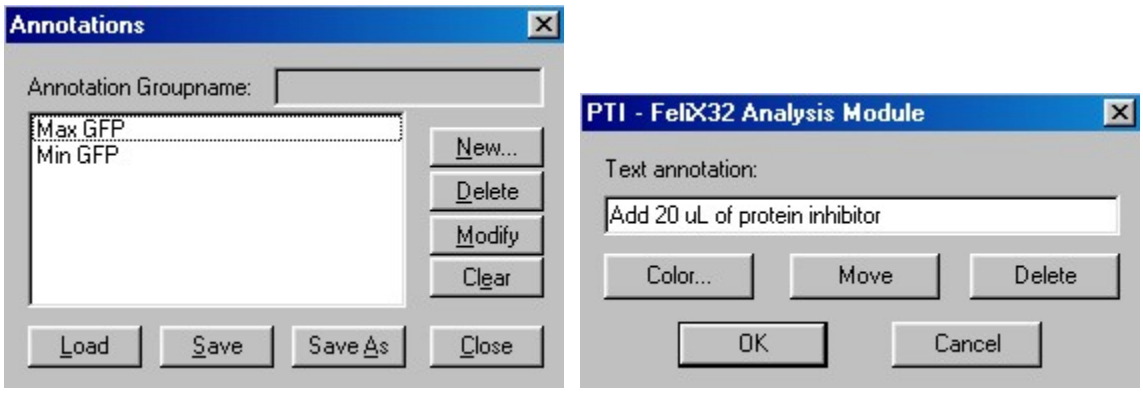
This command toggles the Data Cursor on and off. A checkmark will appear next to the command in the menu when the Data Cursor is active. The data cursor is used to interrogate the values of individual data points displayed in the active dataset. The left and right arrow keys on the keyboard move the Data Cursor along the X-axis of a curve. The X and Y values at the Data Cursor are displayed at the top left of the active workspace, next to the legend. If the window contains multiple curves, use the up and down arrow keys to move the Data Cursor from one visible curve to the next.

**Note.** Move the mouse cursor to a data point on a curve. When the cursor changes to a hand, left click the mouse. The data pointer jumps to that point.

**Shortcut:**  Use the toolbar icon or press **Ctrl+D** on the keyboard to toggle the Data Cursor on/off.

## Annotations

Use Annotation to add boxes, data pointers and text directly to the experimental output. These annotations are attached to the X-Y coordinates of the dataset and are disabled in Grid View and 3D View. If the particular X-Y coordinates of an annotation are off the display then so will be the annotation.




***Load:*** Load a previously saved annotation.

***Save:*** Saves the current annotations to the database, overwriting the previously saved annotations of the same name.

***Save As:*** Opens a dialog to save these annotations to the database under one name.

***Close:*** Closes the annotation dialog box.

***New:*** Shows a list of available annotation types.

> ***Box:*** Using the mouse cursor, left-click to place the upper left corner of the box and drag the lower right corner of the box to the desired size.
>
> **Shortcut:**  Use the toolbar icon.
>
> ***Datapointer:*** Using the mouse cursor select a data point in the graph window. Left-click the mouse. After selecting a data point, the text annotation dialog box will open and allow you to append text to the end of the data pointer arrow (see text entry below). Use the color button to change the color of the data pointer and arrow. Do not use the *Move* button as this will cancel the annotation.
>
> **Shortcut:**  Use the toolbar icon.

***Text:*** Point with the mouse cursor in the graph window to where the text should be placed then left-click the mouse. This will open the text annotation dialog box. Use the color button to change the color of the text. Do not use the *Move* button as this will cancel the annotation.

**Shortcut:**  Use the toolbar icon.

***Delete:*** Deletes the selected annotation.

***Modify:*** Use Modify to alter an already created annotation.

***Clear:*** Erases all the annotations in the group.

## Connect Acquire

Toggles on/off the connection between the analysis module and the BryteBox. This option allows you to initiate an acquisition file from the analysis part of the software.

## Show Acquisition Status

This window displays the status of the current acquisition. The display shows the parameters of the current acquisition and its status as well as the devices in use and their status.

**Note.** The Acquisition Status window created by the Acquisition module (in the View menu) also has a clock and temperature display in addition to what is shown in this Acquisition Status window.

## Toggle Visibility

Use this toolbar command to toggle the display of the selected curve(s). When a curve is visible in the workspace, the trace name will be in bold color in the legend. When the curve is hidden, the name will appear as plain gray text. Multiple curves (hidden, visible, and mixed sets) can be toggled at one time. Selecting a group or multiple groups enables the user to *Hide All* curves, *Show All* curves, or toggle the visibility of all curves within the group(s). *Hide All* and *Show All* commands are located in a user menu that can be found by right clicking on one of the selected group names.

**Shortcut:**  Use the toolbar button to toggle between hidden and visible or right click with the mouse on the selected curves or groups and choose either Toggle Visibility, Hide All, or Show All from the pop-up menu.

## Toggle Plotting Mode Lines/Points

Use this toolbar command to change the way curves are plotted in graph mode. The traces can be displayed as individual data points or as lines. The default display for TimeMaster acquisitions is data points.

**Shortcut:**  Use the toolbar button to toggle between points and lines.

## Additional Display Options

Additional plotting methods and display options are available by right clicking anywhere on the graph area. A menu dialog will appear with the following commands.

***Viewing Style:*** Gives one the options of color and monochrome.

***Font Size:*** You can select three different (small, medium, large) sizes for plot features such as title and axes values and titles.

***Numeric Precision:*** Allows one to select the number of decimal places to plot the data to on all the axes.

***Plotting Method:*** Select the method for which FeliX32 will plot the data. Options include point, line, area, stick, points + best fit line, points + best fit curve, points and line, points and spline, and spline.

***Data Shadows:*** Shadows can be selected as normal shadows, 3D, or toggled off.

***Grid Lines:*** You can display grid lines on both axes, one individually or not at all.

***Grid in Front:*** Toggle to overlay or underlay the grid lines on the graph.

***Mark Data Points:*** When toggled on, this command will display the data points in the plots marking them clearly visible with all plotting methods.

***Show Annotations:*** Toggles the visibility of the annotations.

***Undo Zoom:*** Selecting this command when zoomed in on an area of the graph will re-expand the plot to the set Y and X-axes values (depends on the axes settings for example, Full Autoscale, Autoscale from 0, Fixed Y- Min & Max, etc…).

***Customization Dialog:*** This dialog box provides the user with more options in customizing the looks of the generated plot. This menu has submenus that set other plot parameters, such as font style, plot style, color, axis range, etc…

***Export Dialog:*** Allows the 3D plot and key areas to be exported as Windows ordinary and enhanced metafiles (example: Bitmap and JPEG). These can be imported into many applications including CorelDraw, Word, etc… Select the export destination (clipboard, folder, or printer) and the image size. Click **Export** to complete the operation if the destination is either the clipboard or the printer. Click **Save** in the Windows save dialog box to export the image to a folder on the hard drive or disk drive.

Chapter 12

# Axes Commands

The display of datasets in the workspace is controlled by commands in the **Display** and **Axes** menus.

## Full Autoscale

Scales the X and Y-axis to provide maximum space for the displayed curves in the workspace. A checkmark will appear next to this command when this scaling mode is in effect.

**Hint:**  Use the toolbar icon to re-scale the axes after zoom features are used.

## Autoscale From 0

Scales the Y-axis to provide maximum space, starting at 0, for the displayed curves in the workspace. A checkmark will appear next to this command when this scaling mode is in effect.

**Hint:**  Use the toolbar icon to re-scale the axes after zoom features are used.

## Fixed Y-Min. & Max...

Assigns a minimum and a maximum value to the Y-axis. The Y-scale will remain fixed within this range even when the X-axis is zoomed in or out. A symbol will appear next to this command when this scaling mode is in effect.

**Shortcut:**  Use the toolbar icon to re-scale the axes after zoom features are used.

## Logarithmic Y-Scale

Makes the Y-axis logarithmic. The default log scale is automatic decade selection. If there are zero or negative values in the displayed curve(s) the automatic log scale may not be optimized. You can change the number of decades to display the data over using the **Visible log Decades** command in the *Axes* menu.

## Visible log Decades

Use this menu to define the number of log decades to be displayed on the screen. The default will automatically scale the Y-axis using an appropriate number of decades. If there are negative values in the displayed curve(s), automatic selection of log decades may not be optimal for the display. The user can select from two to eight log decades to display the data over. To view the displayed curves in log scale select **Logarithmic Y-Scale** from the *Axes* menu.

## Custom Y-Zoom…

Custom Y-Zoom causes a selected region to fill the entire Y-axis in the window. The toolbar shortcut, the only location to use this function, can be used to expand the region of interest. Select the icon then click and hold on the graph in the active workspace and vertically drag the mouse. Releasing the mouse button will expand the desired region.

**Note.** After a region has been expanded, you can use the Left and Right arrow keys on the keyboard to scroll up or down along the X-axis.

**Shortcut:**  Use the toolbar icon to select the region on the graph using the mouse.

## 2x X-Zoom In

Expands the X-axis by factors of 2, beginning at the center of the display.

## 2x X-Zoom Out

Contracts the X-axis by factors of 2, beginning at the center of the display.

## Custom X-Zoom…

Causes the selected region to fill the X-axis. Enter a minimum and maximum X value into the available text boxes.

**Note.** When the X-axis is zoomed you can use the Left and Right arrow keys (see *Configure/Preferences* for reference on X-shift keys) on the keyboard to scroll up or down along the X-axis. The Y-axis will automatically re-scale to display the expanded region optimally unless the Y-axis scale mode is set to **Fixed Y-Min & Max.**

**Shortcut:**  Select the toolbar icon then click and drag over the desired region in the workspace using the mouse.

## Custom X and Y-Zoom

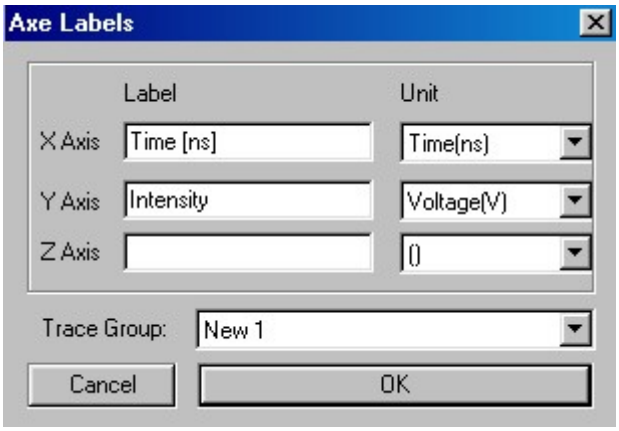
Custom X and Y-Zoom causes a selected region to fill the entire window. The toolbar shortcut, the only location to use this function, can be used to expand the region of interest. Select the icon then click and hold on the graph in the active workspace and drag the mouse. Releasing the mouse button will expand the desired region.

**Note.** After a region has been expanded, you can use the Left and Right arrow keys (see *Configure/Preferences* for reference on X-shift keys) on the keyboard to scroll up or down along the X-axis.

**Shortcut:**  Use the toolbar icon as described above.

## Edit Axes Labels

This window lets the user rename the axes labels and change the units with which the curves are plotted against. The text in the *Label* box will appear on the screen. The text under *Units* is only used to signify the type of unit. In the figure below for example, changing only the X-axis Units to Time(us) will not alter the displayed units to (us) from (ns).




To change the axes label, first select the trace group that you wish to rename from the bottom drop down menu. Type the *Axis Label* into the appropriate text space and select the corresponding units. If a trace group in a dataset has different Y-axis units than other groups, the curves will be plotted against different Y-axes (left or right). The axis to which the trace groups are plotted will alternate from left to right beginning on the left-hand side. It is only possible to have one X-axis however. All data will be plotted on the same scale although they may have different units and X-axis labels.

**Note.** Axis labels can be quickly changed by double clicking on the label in the workspace.

## Axes Scaling

This function allows the Y-axis scale to be displayed as hundreds or thousands or millions of units etc… The number the user enters represents a factoring of $10^X$.

Chapter 13

# Configure Commands

The **Configure** menu has several entries including *Preferences, Hardware and Script Configuration,* and *Administrator Tool*. Use *New* and *Open* commands to either create a new hardware or script configuration or modify an existing one. Each of the entries is described in a separate section below. Due to the importance of **Hardware Configurations** it will be the first area that is explained.

## Hardware Configuration

In order to ensure proper operation, it is critical that FeliX32 is configured to exactly match the hardware components of your instrument. If a PTI Service Engineer installed your computer or instrument then your system already has the proper hardware configurations. If you received an upgrade for an existing version of FeliX32, you may still have to perform hardware configuration, because configuration files saved in previous versions of FeliX32 may not be compatible.

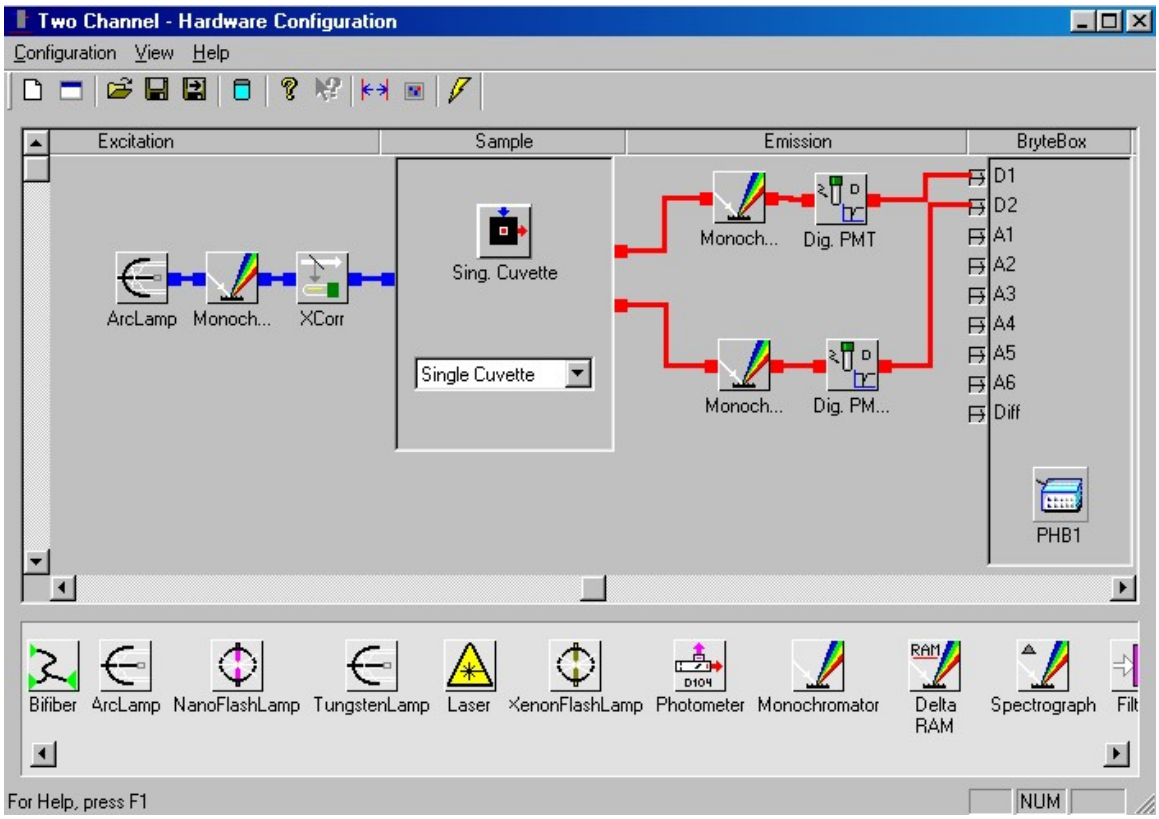
There are two steps in hardware configuration: *Hardware Configuration* and *Component Setup*. Each step is described in detail below.

**Important!** All steps of hardware configuration must be completed before the system will operate properly.

After both configuration steps are completed, save the settings to the database by clicking the *Save As* button in the Hardware Configuration dialog box and entering a name. Once your system is properly configured and the configuration information is saved, you can easily select it from an acquisition window. Appropriate hardware configurations for the selected acquisition will be available in the *Hardware Configuration* drop down menu for you to choose from. There is no need to change the configuration unless you add or remove a component.

## Step 1. Hardware Configuration

First, from the FeliX32 workspace, open either an existing configuration (Configure/Hardware Configuration) or create a new one (Configure/New Hardware Configuration). If your instrument is a custom design, you can start with a similar existing configuration by selecting it in the Database Control Window and modifying it, or creating the complete configuration yourself.




Icons representing various components (monochromator, arc lamp, etc.) are arranged across the bottom of the hardware configuration workspace. Depending on your specific hardware controller devices, some or all of the following icons will be present:

**Mono:** Monochromator. Used in the excitation and/or emission channel(s).

**DeltaRAM V™:** DeltaRAM V high-speed multi-wavelength illuminator.

**ArcLamp:** PowerArc Xenon Arc Lamp.

**Tungsten Lamp:** Tungsten Lamp.

**Laser:** PTI Nitrogen Laser.

**NanoFlash:** NanoFlash nanosecond flashlamp.

**XeFlash:** Xenon flash lamp.

**Xcorr:** Excitation Correction Accessory for steady-state systems.

**Gated Xcorr**: Excitation Correction Accessory for XenoFlash systems.

**Chopper:** Optical chopper in the PowerFilter or DeltaScan for high-speed measurements of excitation-shifted probes.

**Shutter:** Computer-controlled shutter. May be on the excitation or emission side.


**Photometer:** D104 Microscope Photometer, single or dual channel.


**DigPMT:** Model 810 or 814 Photon Counting Photomultiplier Detector.


**AnaPMT:** Model 812 or 814 Analog Photomultiplier Detector.


**FL Detector:** Fluorescence Lifetime Detector.


**PH Detector:** Phosphorescence Detector.

**BiFiber:** Bifurcated fiber optic cable, which can have either 2 inputs/1 output or 1 input/2 outputs.

**Man. Polarizer:** Manual Polarizer. May be on the excitation or emission side.

**Mot. Polarizer:** Motorized Polarizer (computer controlled) may be on the excitation or emission side.


**RTC:** Rapid Temperature Controller, a Peltier device for controlling sample temperature.

**Titrator:** Hamilton Titrator/Diluter Controller.

**BlackBox:** A component unique to your system, such as a peripheral device that generates an analog signal you want to capture. For example, a temperature monitor, an edge detector, or an electrophysiological instrument would be identified as a Black Box and connected to one of the Analog In signal channels.

**Filter:** Filter. Include wherever primary excitation/emission filters appear in your system. For example, a PowerFilter illuminator would have two. Also include filters located in a sample compartment or a microscope photometer.

**Motorized Slits:** Motorized slits (computer controlled, two types).

**Spectrograph:** Acton Monochromator. Used in one emission channel.

The icons are used to configure your instrument by "building" it within the workspace of the dialog box, under the appropriate classifications of Excitation, Sample, and Emission. The icons are added to the workspace by dragging and dropping. Click and hold on the icon you want, drag it onto the workspace and release the mouse button to position the icon. For example, place the cursor on the ArcLamp icon, click and hold, and drag the icon to the left side of the workspace under Excitation. Release the mouse button and the icon will remain where you placed it. Then, drag the Mono icon into position just to the right of the arc lamp icon. You can also click once on an icon in the icon list, move to the position in the workspace where you want to build the icon and click again to place the icon. Move to another spot in the workspace, click again and a second copy of the icon will be placed there. When you want to stop placing this icon click once more on the icon in the icon list.

Notice that the icons in the Excitation area now have blue squares on one or both sides. These are connection points that are used to create the light path from one component to the next. To connect the ArcLamp to the Mono, place the mouse cursor on the blue square of the ArcLamp icon, click and hold, and drag the cursor to the blue square on the Mono icon. You will see a circle with crosshair behind the cursor; if not, you missed the connection point. Release the mouse button on the target connection point to form the light path. When you are successful, a solid blue line will connect the two icons.

To remove an icon from the workspace, simply right-click on the icon and select delete, or left-click and press the Delete key. To disconnect two components, click and hold on the connection point, move the mouse cursor away from the connection point and release the mouse button.

**Note.** Components connected with bifurcated fiber optic cables (such as dual-excitation systems) must be configured the way they are physically assembled using the BiFiber icon. However, components connected by single fiber optic cables are not configured using a fiber cable icon. Simply connect them the way all of the other components are connected. Double-click on the BiFiber icon to change its orientation, that is, 2-in/1-out or 1-in/2-out.

Next, choose the sample device from the pull-down list. There are four sample device options:

**Single Cuvette**: Standard PTI single 1-cm cuvette holder.

**Microscope:** Inverted fluorescence microscope.

**More Pos. Turret:** Optional PTI four-position cuvette turret.

**QNW 4 Position Turret:** Optional four-position rapid temperature control turret. There are two menus that require setup for this device. One can be located by double clicking on the QNW icon, the other is found by double clicking on the RTC icon.

Continue to build your system by connecting the illumination section to the sample section, choosing the appropriate sample hardware, and completing the detection section. The light path connections in the detection section will be red instead of blue.

Finally, connect your detector hardware to the computer interface signal channels, indicated along the right edge of the workspace by small arrow symbols.

**Note.** Icons must be connected by light paths and from detectors to signal channels. The only exception is that a black box does not need to be connected by a light path.

**Note.** Some components such as the DeltaScan X™ cannot be setup with an individual icon. For more information on this and additional setup commands for other systems please refer to the online Help utility.

## Step 2. Component Setup

Before you can use your instrument, you must setup each component. Do this by double-clicking on each component icon in the workspace (clicking on the icons along the bottom of the dialog box will not work). Double clicking on a component icon will open a dialog box to request specific information on that component. You must do this for every component in your system, including the computer interface signal channels and arc lamp. To configure interface signal channels, double-click on the arrow symbols at their connection points. Further information regarding specifics of setup for each component can be found in the online Help utility under **Configuration Setup**.

Every dialog box has a **Label** text box. The default label is a generic name that may be changed to a more descriptive one if desired. In several dialog boxes this is the only option.

Several dialog boxes (monochromator, motorized polarizer, four position turret) require the choice of a **Motor Driver Channel**. A drop down menu allows the choice of channels 1-6. The selections are arbitrary but the selection must correspond to the physical wiring of the motor driver. If your instrument has two motor driver units (MD-5020), individual units can be selected by choosing either CIF-1 or CIF-2 in the setup dialog box. CIF-1 and –2 refer to the MD-5020 plugged into Controller Interface I and II respectively on the back of the BryteBox.

When setting up monochromators make sure to select **Auto-Calibration Installed**. When you initialize the hardware configuration, or select calibrate, the monochromators will slew to their default position and a **Monochromator Position Check** text box will open asking for the auto-calibrated monochromator position. If the displayed position does not agree with that on the monochromator counter, enter the wavelength that appears on the monochromator control dial(s) and click **OK**. Your monochromators are now calibrated. They will move to their auto-calibrated position when you invoke an Acquire command.

Remember that the wavelength indicated on the monochromator control dial is always correct (differently ruled gratings may require a multiplication factor be applied to the value on the dial to correspond correctly). Any discrepancy with the wavelength reported by FeliX32 must be corrected in the software before proceeding.

It should be noted that if your system lacks monochromators you must use a *Filter* icon for wavelength selection. If you do not have a wavelength selection device the Hardware Configuration will be invalid.

Additional setup features can be found by double clicking on the *PHB1* icon, which will display the *BryteBox Configuration* window. In this menu you can setup *Ethernet Communications, PMT/Electrometer Calibration, Delay Gate models, Real Time Correction Lookup Tables,* and *Serial Interface Devices.* For the most part, you will not need to change any of the default settings. However, you may wish to setup *Configure Corrections* or, if you are using TimeMaster acquisitions, you may need to select a *delay gate generator*.

*Configure Correction* is used to activate excitation and/or emission and to specify the correction Lookup Tables to be used to correct for 1) spectral response of the reference detector and 2) emission correction. Up to two emission correction files are allowed to accommodate T-format configurations that employ two emission monochromators. When **Real Time Correction** is enabled in the **Additional Acquisition Setup Controls** dialog box, corrections are applied as defined in this dialog box. If spectral corrections are not enabled, no correction is applied to the data during acquisition. Real Time correction is necessary to acquire XCorr data for excitation correction. Emission correction may be applied in post-acquisition analysis. The Lookup Table traces for Real Time Corrections are located in the Lookuptables dataset. If you specify a new correction trace it must go within the appropriate group in Lookuptables for it to be visible to select in *Configure Correction.* The default traces for excitation and emission correction are labeled excorr and emcorri/emcorii for excitation and emission (possible T-format) respectively.

**Warning!** Altering any of the traces/groups in Lookuptables may cause system failures.

## Script Configuration

Photon Technology currently does not offer any support on this feature. It is recommended that you do not attempt to alter or edit any of the supplied acquisition scripts as loss of hardware control may occur.

## Preferences

Several aspects of the way FeliX32 looks and behaves can be adjusted to suit the user. The dialog box contains these features:

**Acquire Application**

***Host:*** This is the name of the computer that is acquiring data. If you want to connect to a remote acquisition computer via a network you must enter the name of that computer here.

***Port:*** TCP/IP setting. Leave as 9999.

**Background**

You can set the legend and graph display background to either white or black. This can also be changed in the Customization Dialog.

**Trace Color**

***Custom:*** Click on Custom to enable the Trace Colors… dialog to be opened. Otherwise a fixed default cycle of trace colors will be used to display the traces in the workspace.

***Trace Colors:*** This dialog box contains a color palette used to change the default curve colors. Click on the drop-down box to select a curve number, then click on the color you want. The number represents the order in which the curves are created. The color changes made here become the default. You can also change individual colors by right clicking on a curve, then choosing Color.

**Acquire Data**

***Clear All Curves Before Starting Acquisition****:* When checked, all curve(s) in the active dataset will be cleared when a new acquisition is initiated. **WARNING!** If you have not saved the old curve(s), they will be lost! This option is intended for method development and sample evaluation. If your instrument has multiple users, be sure they all understand this option before it is used

If you're not sure you may use ***Hide All Curves*** before starting acquisition instead. When checked, all curve(s) in the active Graph will be hidden.

**X-Shift Keys**

When the data cursor is displayed you can choose whether the left/right keys are used for moving the data cursor and Ctrl-left/right keys are used to scroll along the X-axis or vice versa.

**Auto Save**

The dataset and all curves will be saved in memory in case of unwarranted shutdown of FeliX32. This does not save the dataset permanently. It is only an emergency backup. The dataset must be saved in normal fashion on manual exiting of FeliX32 or else loss of data will occur. Set the *Interval* to 0 for the auto save to put the dataset in temporary memory only before and after acquisitions and on opening and importing. Set the *Interval* to another integer for an auto save every X minutes.

**Customer Access Code**

The Customer Access Code must be entered to access FRET and TimeMaster data analysis functions. Click on the button and enter the Customer Access Code provided to you by PTI.

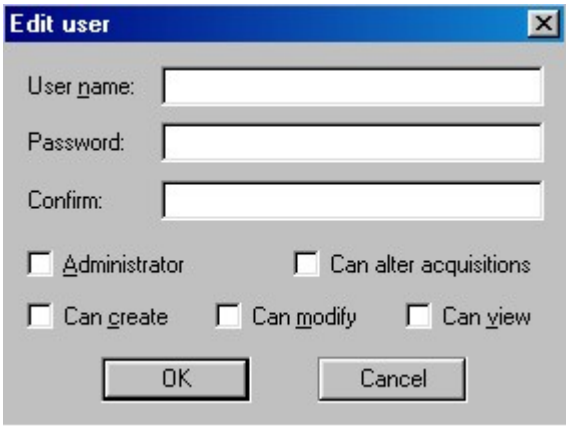
## Administrator Tool

One new feature in FeliX32 is the multi-user environment. This allows more than one user to "log in" to FeliX32 and perform their experiments in their own pre-selected environment. An administrator sets the privilege level for each user as to what hardware/software operations they are allowed to perform.

By clicking on Configure/Administrator Tool a password activated login appears. Supplying the correct password will give the user Administrator level privileges, which is essentially unlimited. After login an Administrator screen pops up listing all the users currently saved to the database and their security level.

The dialog box allows administration of many individual users privilege levels that are set in a menu described below. If a highlighted user clicks on Log On from the File menu, they are sent into the Acquisition and Analysis portions of FeliX32.

The User drop down menu allows the Administrator to Add, Delete or Change the privilege level of a user or users of the experimental system. The active tools can be activated from either the Status Bar or the Toolbar, which has icons immediately below the Status Bar.




When the Administrator enters the edit user menu, he/she can create a new user or alter the privilege level of an existing user by setting checkboxes in the menus. A user-activated password is also set from this menu for user security purposes. The Administrator can set 5 separate privileges:

1. Administrator Level- essentially all privileges set.

2. Alter acquisitions- allowing changes in hardware settings.

3. Create- can create new acquisition/analysis macros.

4. Modify- can modify previously existing acquisition/analysis macros.

5. View- can only view and use existing acquisition macros.

These levels allow an administrator to have users with varying levels of instrumental experience run and analyze their experiments without harming the instrumentation.

Chapter 14

# Help Commands

## Help Topics

This command opens the FeliX32 Help utility. The Table of Contents is in the left panel and a menu containing a general overview of FeliX32 opens in the right panel. Selecting any of the labels from the Table of Contents will make that topic appear in the right panel. You can also use the Help Index and Search functions, which are listed as tab menus above the Table of Contents. The online Help utility contains information not presented in the manual. It is suggested to look over the Help file if the answer to your question is not found within the manual.

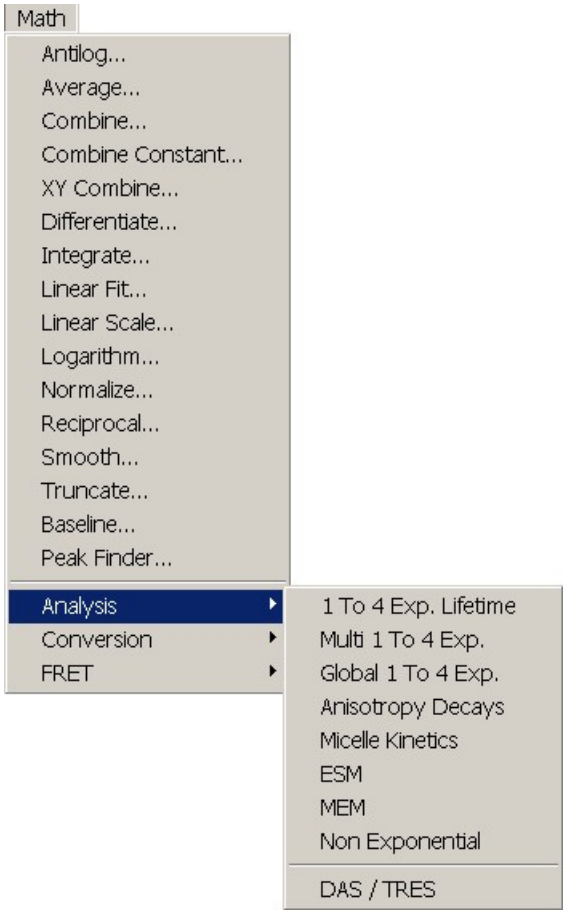
## About FeliX32 Analysis...

Use this command to display the copyright notice and version number of your copy of FeliX32.

## Chapter 15

# Data Analysis

Perhaps the most important aspect of the TimeMaster portion of the FeliX32 software after data collection is data analysis. This chapter is devoted to this very important topic.




The various methods of data analysis are found under Math Analysis. They are:

1. **1 To 4 Exp. Lifetime**
2. **Multi 1 To 4 Exp.**
3. **Global 1 To 4 Exp.**
4. **Anisotropy Decays**
5. **Micelle Kinetics**
6. **ESM**, **MEM**
7. **Non Exponential**
8. **DAS/TRES**

These methods are covered in separate sections that are independent of each other. Thus, only the section of interest needs to be read. However, it is recommended that the **General Introduction** is read first as most of the concepts and topics used in the other sections are introduced there.

# General Introduction

**Fitting function**

Every method of lifetime analysis depends on a model or fitting function for the decay of luminescence intensity. This may be as simple as a single exponential decay or as complicated as schemes for micelle kinetics including quenchers and distributions of micelle sizes etc. The various methods of analysis presented here differ mostly in the model they employ. The fitting function (explicitly dependent on time) is denoted in what follows as D(t) and can be thought of as the time dependent luminescence excited by a delta function (infinitely short) excitation pulse.

**Convolution**

In any pulsed excitation fluorescence lifetime instrument the finite width of the excitation pulse will distort the free decay of fluorescence as described by D(t). This distortion is known as convolution and the mathematical description is given by *Equation 1*:

$$I(t) = \int_0^t L(t-s)D(s)ds$$

Eq. 1

where L(t) is the instrument response function (IRF) also known as the excitation pulse curve, and I(t) is the experimentally determined decay intensity at time t. The meaning of Equation 1 is that the intensity of the decay at time t is determined by both the continuous re-pumping of the fluorescence excitation during the emission from the nanosecond flash lamp or laser and the decay of fluorescence emission that has occurred up to time t.

The convolution distortion cannot be removed experimentally. Instead, the IRF L(t) is measured by using a scattering solution in a companion measurement prior to determining the fluorescence decay of the sample. This experimental L(t) is then used to actually determine I(t) from Equation 1 by the procedure known as iterative reconvolution.

**Curve Fitting Procedure**

The fitting procedure uses an iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969) where the experimental data are compared to a model decay based on Equation 1. Deviations from the best fit are characterized by the reduced chi-square statistic, $\chi^2$, as shown in *Equation 2*:

$$\chi^2 = \frac{1}{N-n-1} \sum_{1}^{N} \frac{(I(i)_{calc} - I(i)_{exp})^2}{s(i)^2}$$

Eq. 2

where N is the number of data channels, n is the number of fitting parameters, and s is the standard deviation (see below). The best fit is determined when chi-square is minimized. If the standard deviations are estimated correctly, a perfect fit to the data will produce a chi-square close to 1.0. Good results typically produce $\chi^2$'s of 0.9 to 1.2.

It is necessary to incorporate an estimate of the data precision when using a statistical fitting procedure. For the case of the stroboscopic optical boxcar the standard deviation is determined from within the decay by actually measuring the "noise" at time t and applying a special procedure developed for this type of experimental data. (James *et al*, 1991)

**Artifacts**

There are several well-known artifacts due to the intrinsic nature of photomultiplier tubes, etc… which must be accounted for during the analysis procedure.

***Color Shift Artifact Correction*:** Photomultiplier tubes do not respond identically at all wavelengths of incident light. This is due to the fact that the photoelectrons ejected from the photocathode will have excess kinetic energy when the incident photon is more energetic, i.e., bluer. This effect manifests itself primarily, although not exactly, as a zero-time shift in the excitation position relative to the decay. This time shift can be approximated by a single parameter $\delta$ as shown in *Equation 3*:

$$I(t) = a \exp\left(\frac{-(t+\delta)}{\tau}\right)$$

Eq. 3

This parameter may be either determined experimentally or incorporated in the fitting procedure as a variable parameter. Both options are available in this program. The fitting procedure for using the variable time shift parameter is based on that from *Time-Correlated Single Photon Counting* by O'Connor and Phillips.

***Analog Baseline Offset:*** Since the stroboscopic technique is based on an analog measurement, there will always be a small difference between the measured baseline for the IRF and that for the fluorescence decay, typically on the parts per thousand level. This offset can cause inaccuracies to the determination of fluorescence lifetimes since it will be treated as due to a true convolution effect. The effects of this offset can be removed (James *et al*, 1991) by determining the offset during the data analysis by assigning a non-convolved constant, c, to the decay intensity as shown in *Equation 4*:

$$I(t)_{corrected} = I(t)_{uncorrected} + c$$

Eq. 4

**Useful Statistical Parameters**

A variety of statistical parameters have been developed to assist in determining the quality of the analysis. First among these is the reduced $\chi^2$ parameter as discussed above. Others are:

***Randomness of the Residual Pattern:*** The residual $R_i$ is the difference between the calculated fit and the real data at time $t_i$. Weighted residuals $r_i$ are the ratio of $R_i$ to $s_I$, i.e. $r_i = R_i/s_i$ and should range from about -3.3 to 3.3.

A plot of these residuals should produce a flat pattern randomly distributed about zero with no features. Periodic oscillations or other deviations indicate a poor fit. This is a simple and reliable test for the goodness of the fit.

***Autocorrelation Function of the Weighted Residuals:*** This function is calculated from *Equation 5* (Grinvald and Steinberg, 1974):

$$Cr_j = \frac{\frac{1}{m}\sum_{i=n_1}^{n_1+m-1} r_i\, r_{i+j}}{\frac{1}{n_3}\sum_{i=n_1}^{n_2} [r_i]^2}$$

Eq. 5

where $n_3=n_2-n_1+1$, $n_1$ and $n_2$ are the first and last channels chosen to do the calculation over. An upper limit is set at $j = n_3/2$ to allow for maximal testing of a finite data set.

By definition $Cr(0)=1$. For the remaining points $Cr_j$ should form a flat band of high frequency low amplitude noise about zero. Any pattern indicates a lack of fit. The autocorrelation function is very sensitive to any radio-frequency (RF) noise.

***Durbin-Watson Parameter:*** This parameter was introduced by Durbin and Watson (Biometrika, 1950, 1951) to test for correlations. The parameter DW is defined in *Equation 6* as:

$$DW = \frac{\sum_{i=n_1+1}^{n_2} [r_i - r_{i-1}]^2}{\sum_{i=n_1}^{n_2} [r_i]^2}$$

Eq. 6

where the other parameters are defined above.

This parameter may be interpreted as follows; the fit is likely satisfactory if the value of DW is greater than 1.7, 1.75 and 1.8 for single, double and triple exponential fits respectively (O'Connor and Phillips, 1984).

***Runs Test:*** The runs test determines the number of positive and negative groups or runs of the residuals as defined in *Equation 7*:

$$z = \frac{zn}{\sqrt{(zd)}}$$

Eq. 7

where

$$zn = (nn + np) - \frac{(2 \times nvn \times nvp)}{(nvn + nvp)} + 1$$

$$zd = \frac{2 \times nvn \times nvp \times (2 \times nvn \times nvp - nvn - nvp)}{(nvn - nvp)^2 \times (nvn + nvp - 1)}$$

and np is the number of positive transitions, nn is the number of negative transitions, nvn is the number of negative residuals and nvp is the number of positive residuals.

A value of -1.96<Z indicates a satisfactory fit at the 95% confidence level (Hamburg, 1985).

The general structure of each analysis program is the same, so there is considerable similarity in running the programs. For each method the data to be analyzed and time range over which to analyze must be selected. Then the initial model parameters must be selected, perhaps holding some of them at pre-selected values. Finally, the fit is run and the results interpreted.

# 1 To 4 Exponential Lifetime

**Theory**

This is the simplest and arguably the most generally useful of the fitting procedures. It is suitable for the analysis of fluorescence decays consisting of up to 4 exponentials and associated pre-exponentials.

**Fitting Function**

This analysis program can fit up to a 4 exponential decay that follows the fitting law:

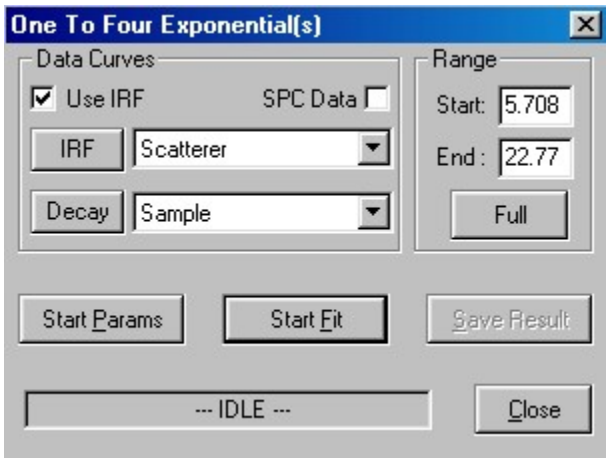
$$D(t) = \sum a_i \exp\left\{\frac{-t}{\tau_i}\right\}$$

Eq. 1

where D(t) is the delta function generated decay at time t. This fitting function allows for negative $a_i$'s so that risetimes can also be determined with this program.

**Using the Program**

The initial **One To Four Exponential(s)** dialog box is shown below.




**Data Curves**

The ***Use IRF*** check box selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** check box is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the ***IRF*** button. The name of the selected curve will appear in the box beside the button.

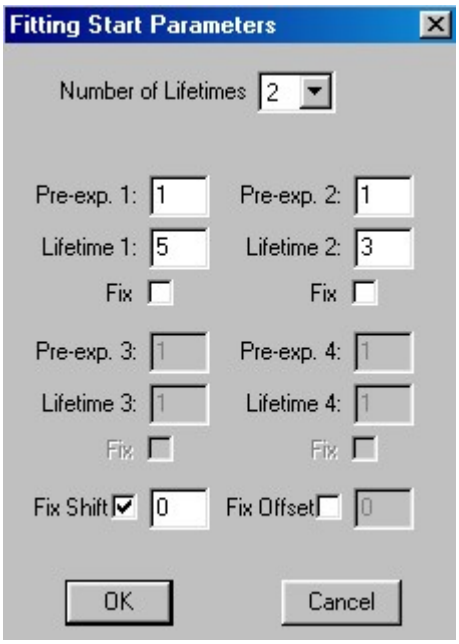
The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the ***Decay*** button. The name of the selected curve will appear in the box beside the button.

***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Start Parameters** dialog box for the **1 To 4 Exp**. **Lifetime** method is shown at left.

The ***Number of Lifetimes*** text box selects the number of different lifetimes used to analyze the decay curve. Select a number between 1 and 4. Normally, for the first fit of a new sample, the number one is chosen.

***Pre-exp., Lifetime, Fix:*** For each of the lifetimes to be used in the fit an initial guess for the lifetime and the pre-exponential factor (*Pre-exp*) must be given. Only the relative values of the pre-exponential factors are relevant here so that for a single exponential fit the value 1 is normally used. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

Study of the portion of the sample and scatterer curves before the laser pulse may indicate whether an offset is required. However, it is often better to adjust the sample and scatterer curves to average zero before the pulse (using math functions provided in FeliX32) than to trust the fit. Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the analysis.

**Results**

The results of the analysis are displayed in two forms.

1. The names of the fitted curve, the residuals, the autocorrelation function and the deconvoluted decay i.e. D(t) appear on the left of the screen.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

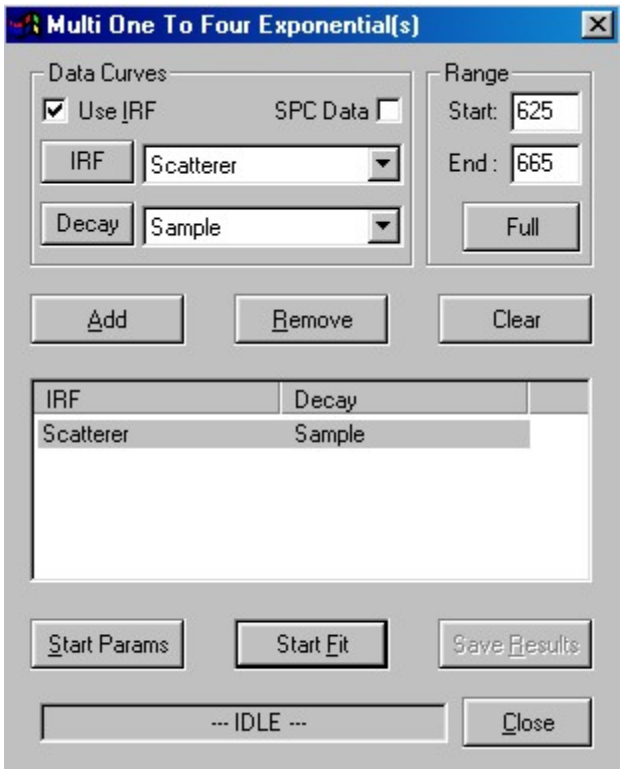
# Multi 1 To 4 Exponential

The multiple file one to four exponential lifetime method, as its name implies, allows the analysis of multiple scatterer/sample pairs at the same time. Each pair will be separately analyzed over the same range with the same number of exponentials and the same options. The analysis results in a set of parameters (lifetimes and pre-exponential factors) for each data pair. The theory for this method is exactly the same as that for the **1 To 4 Exp. Lifetime** method.

This type of analysis is useful when a series of otherwise identical decay curves has been collected as a function of some parameter (temperature, composition or wavelength for example). Trends in the values of the lifetime parameters may then be recognized rather easily.

**Using the Program**

The initial dialog box for **Multi One To Four Exponential(s)** is shown below.




**Data Curves**

The ***Use IRF*** check box selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** check box is used only when single photon counting data has been imported.

Multiple scatterer/sample pairs are selected by first choosing a single pair.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

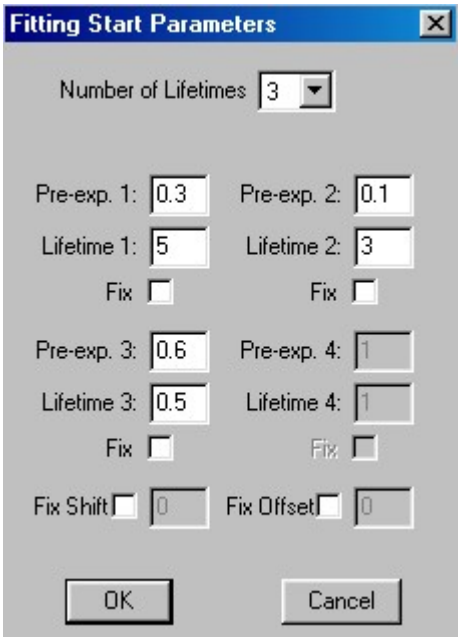
Enter this data pair into the analysis by clicking the *Add* button at which point their names appear in the text window. Data pairs may be deleted by clicking on the appropriate line in the text window to highlight the line then clicking on the *Remove* button. All sample pairs may be removed by clicking on the *Clear* button.

***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The ***Start Parameters*** dialog box for the **Multi 1 To 4 Exp**. method is shown at left.

The ***Number of Lifetimes*** text box selects the number of different lifetimes used to analyze the decay curves. Select a number between 1 and 4. Normally, for the first fit of a new sample, the number one is chosen.

***Pre-exp., Lifetime, Fix:*** For each of the lifetimes to be used in the fit an initial guess for the lifetime and the pre-exponential factor (*Pre-exp*) must be given. Only the relative values of the pre-exponential factors are relevant here so that for a single exponential fit the value 1 is normally used. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

Study of the portion of the sample and scatterer curves before the laser pulse may indicate whether an offset is required. However, it is often better to adjust the sample and scatterer curves to average zero before the pulse (using math functions provided in FeliX32) than to trust the fit.

Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis.

**Results**

The results of the analysis are displayed in two forms.

1. The names of the fitted curve, the residuals, the autocorrelation function and the deconvoluted decay i.e. D(t) appear on the left of the screen.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

## Global 1 To 4 Exponential

### Theory

This analysis program provides for the analysis of up to 4 exponential decays for a number of data files simultaneously. The global analysis assumes that the lifetimes are linked among the data files but that the associated pre-exponentials are free to vary.

### Fitting Function

The analysis program can fit up to a 4 exponential decay, which follows the fitting law:

$$D(t) = \sum a_i \exp\left\{\frac{-t}{\tau_i}\right\}$$

Eq. 1

where D(t) is the delta function generated decay at time t. This fitting function allows for negative $a_i$'s so that risetimes can also be determined with this program.

### Global Analysis

Global analysis is a procedure whereby several data sets, which have parameters in common may be analyzed simultaneously (Knutson, Beechem and Brand, 1983). This program assumes that the lifetimes are linked among the data files, i.e., the lifetimes are the same for all decays. This is accomplished by using a matrix mapping of the fitting parameters whereby the pre-exponentials are unique for each decay curve while the lifetimes are mapped to the same value for each decay.

For example, two linked lifetimes with 2 unique pre-exponentials each and 4 decay curves map as:

$$\begin{vmatrix} a(1,1) & a(1,2) & a(1,3) & a(1,4) \\ \tau(1,1) & \tau(1,1) & \tau(1,1) & \tau(1,1) \\ a(2,1) & a(2,2) & a(2,3) & a(2,4) \\ \tau(2,1) & \tau(2,1) & \tau(2,1) & \tau(2,1) \end{vmatrix}$$

Eq. 2

Least squares data analysis using the Marquardt algorithm is done on all data files simultaneously using the map to substitute parameters appropriately while minimizing the global $\chi^2_g$:

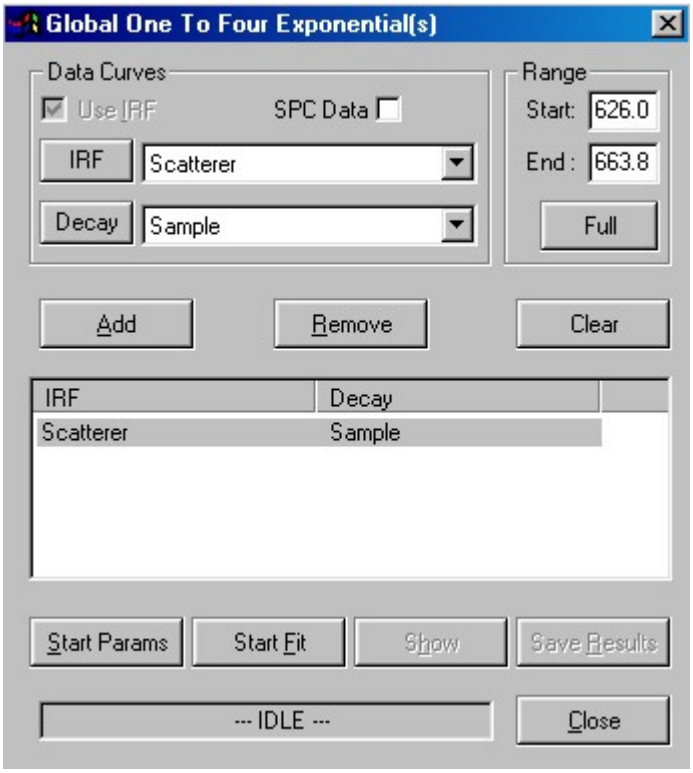
$$\chi^2_g = \sum_j \chi^2_i$$

Eq. 3

where $\chi^2_i$ is given by Equation 2 of the **General Introduction**. Refer to the **General Introduction** for a discussion of fitting procedures and statistical parameters. This type of analysis is useful when a series of otherwise identical decay curves has been collected as a function of some parameter, which alters the relative amounts of two or more fluorophores without altering their lifetimes. For example, this form of analysis could be used for various mixtures of non-interacting fluorescent compounds.

**Using The Program**

The initial dialog box for **Global One To Four Exponential(s)** is shown below.




**Data Curves**

The ***Use IRF*** check box selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** check box is used only when single photon counting data has been imported.

Multiple scatterer/sample pairs are selected by first choosing a single pair.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

Enter this data pair into the analysis by clicking the *Add* button at which point their names appear in the text window. Data pairs may be deleted by clicking on the appropriate line in the text window to highlight the line then clicking on the *Remove* button. All sample pairs may be removed by clicking on the *Clear* button.

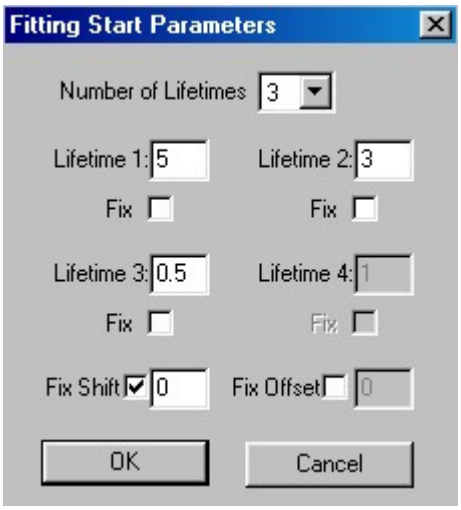
***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box, which allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Show:*** In order to avoid screen congestion, only selected analysis curves will be displayed. Data pairs are selected by clicking on the appropriate line in the text window to highlight the line. Clicking on the *Show* button will then display the fitted curve, the residuals, autocorrelation, and deconvoluted curves associated with this data pair.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Start Parameters** dialog box for the **Global 1 To 4 Exp**. method is shown at left.

The ***Number of Lifetimes*** text box selects the number of different lifetimes used to analyze the decay curves. Select a number between 1 and 4. Normally, for the first fit of a new sample, the number one is chosen.

***Lifetime, Fix:*** For each of the lifetimes to be used in the fit an initial guess for the lifetime must be given. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

Study of the portion of the sample and scatterer curves before the laser pulse may indicate whether an offset is required. However, it is often better to adjust the sample and scatterer curves to average zero before the pulse (using math functions provided in FeliX32) than to trust the fit.

Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis.

**Results**

The results of the analysis are displayed in two forms.

1. The results may be displayed in graphical form. However, in order to avoid screen congestion, only selected analysis curves will be displayed. Data pairs are selected by clicking on the appropriate line in the text window of the **Global One To Four Exponential(s)** dialog box to highlight the line. Clicking on the **Show** button will then display the fitted curve, the residuals, autocorrelation and deconvoluted curves associated with this data pair.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

# Anisotropy Decays

**Theory**

This program allows for the calculation of up to four rotational correlation times plus a residual anisotropy term. The program first allows the user to calculate the fluorescence lifetime(s) from the parallel and perpendicularly polarized emission intensities. The user can then calculate the rotational correlation time(s).

**Fitting Function for Fluorescence Lifetimes from Polarized Emissions**

The analysis program can fit up to a 4 exponential decay, which follows the decay law:

$$D(t) = \sum a_i \exp\left\{\frac{-t}{\tau_i}\right\}$$

Eq. 1

where D(t) is the delta pulse excited decay function at time t. This fitting function allows for negative $a_i$'s so that risetimes can also be determined with this program.

For polarized light, F(t) may be calculated from the raw data:

$$F(t) = I(t)_{par} + 2 \times G \times I(t)_{per}$$

Eq. 2

where $I(t)_{par}$ is the intensity of light detected with a vertical excitation polarizer and a vertical emission polarizer (*i.e.*, the polarizers are parallel to each other), $I(t)_{per}$ is the intensity of light detected with a vertical excitation polarizer and a horizontal emission polarizer (*i.e.*, the polarizers are perpendicular to each other), and G is the correction term for the relative throughput of each polarization through the emission optics.

**Convolution**

Refer to the **General Introduction** for information on convolution.

**Decay of Anisotropy**

Anisotropy, r(t), is defined as:

$$r(t) = \frac{I(par) - G \times I(per)}{I(par) + 2 \times G \times I(per)}$$

Eq. 3

where I(par) and I(per) are as defined above and the time dependence is assumed. This function is known to decay with a multi-exponential decay law (Phillips *et al*, 1985):

$$r(t) = \sum_{i=1}^{5} b_i \exp\left(\frac{-t}{\phi_i}\right) + b_\infty$$

Eq. 4

Although the sum can run to 5 terms for completely anisotropic rotational motion, at lower precision levels and with relatively symmetric rotors, equation 4 will only yield in practice 1 or 2 terms. The $b_\infty$ term refers to residual anisotropy remaining after all the transient terms have decayed and is commonly interpreted to imply restricted motion of the rotor.

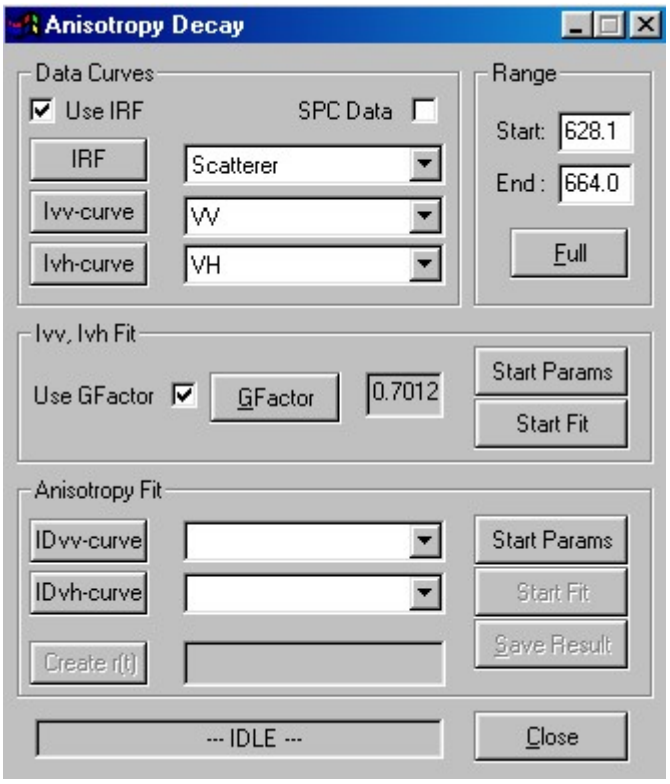
**Curve Fitting Procedure**

Refer to the **General Introduction** for information on fitting procedures and fitting statistics. Numerical analysis of anisotropy decay data is non-trivial. For this software, we chose to perform fits on the raw data files, $I_{vv}$ and $I_{vh}$, without manipulating the curves prior to analysis. In the first step, the $I_{vv}$ and $I_{vh}$ curves are analyzed simultaneously by the global multi-exponential program. The fitted deconvolved curves, $ID_{vv}$ and $ID_{vh}$ are then used to create the anisotropy function r(t) according to Equation 3. The so-constructed r(t) is free of any convolution effects and can be directly fitted to Equation 4. It must be remembered that r(t), being constructed from fitted curves, contains no experimental noise and therefore typical fit criteria like the $\chi^2$ value, randomness of residuals, D-W parameters etc… do not apply. Instead, the guiding criterion should be the minimum value of the sum of the least squares obtained with different kinetic models, *i.e.*, different numbers of parameters.

The interpretation of the $b_j$'s is that $b_j$ at t=0 is the initial polarization of the molecule (for single exponential decays, often known as r(0) and $b_\infty$ is the residual polarization, often known as $r_\infty$.

## Using the Program

The initial **Anisotropy Decay** dialog box is shown below.




### Data Curves

The ***Use IRF*** checkbox selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** checkbox is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

The ***Ivv-curve*** and ***Ivh-curve*** buttons select the two curves to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Ivv-curve* or *Ivh-curve* button. The name of the selected curve will appear in the box beside the button.

***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

### Ivv, Ivh Fit

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box, which allows these values to be entered (see below).

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

The user has the option of using a G factor in the analysis or not. Toggle this option on or off by clicking on ***Use GFactor***. Clicking on the *GFactor* button opens a dialog box that allows the G factor to be entered in several different ways (see below).

**Anisotropy Fit**

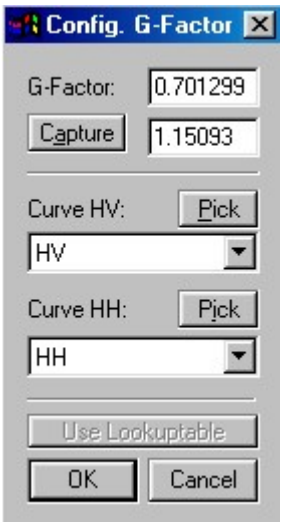
The ***IDvv-curve*** and ***IDvh-curve*** buttons select the two deconvolved curves to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IDvv-curve* or *IDvh-curve* button. The name of the selected curve will appear in the box beside the button.

The ***Create r(t)*** button calculates r(t) from the $ID_{vv}$ and $ID_{vh}$ curves.

***Start Params****:* The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box, which allows these values to be entered (see below).

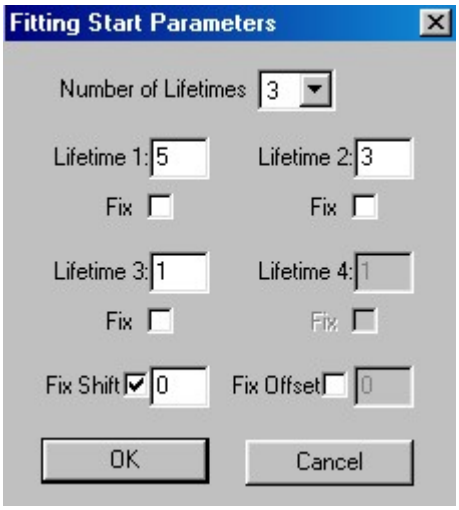
***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The ***Configure G-Factor*** dialog box is shown at left. The G-factor may be entered directly into the *G-factor* text box or captured from HV and HH decays. To capture the G-factor select the HV curve in the left legend and click on *the Curve HV* ***Pick*** button. Select the HH curve in the left legend and click on the *Curve HH* ***Pick*** button. Select the region of the curves to be used in calculating the G-factor in the normal manner (usually this is the whole decay curve). The ratio of the integrals under the HV and HH curves is displayed in the **Capture** text box. Click on **Capture** to accept this value for the G-factor. It will be displayed in the **G-Factor** text box. Click **OK** to return to the previous dialog box.

The **Start Parameters** dialog box for the **Ivv, Ivh Fit** is shown below.

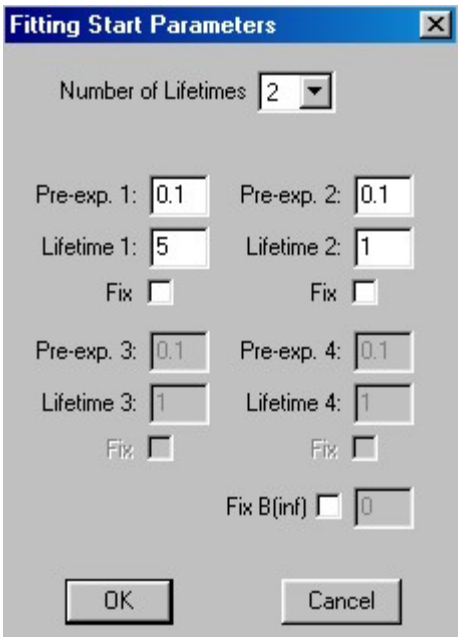



The ***Number of Lifetimes*** text box selects the number of different lifetimes used to analyze the decay curve. Select a number between 1 and 4.

***Lifetime, Fix:*** For each of the lifetimes to be used in the fit an initial guess for the lifetime must be given. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.




Study of the portion of the sample and scatterer curves before the laser pulse may indicate whether an offset is required. However, it is often better to adjust the sample and scatterer curves to average zero before the pulse (using math functions provided in FeliX32) than to trust the fit. Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis.

The **Anisotropy Fit** start parameters dialog box is shown at left.

The ***Number of Lifetimes*** text box selects the number of different lifetimes used to analyze the r(t) curve. Select a number between 1 and 4.

***Lifetime, Fix:*** For each of the lifetimes to be used in the fit an initial guess for the lifetime must be

given. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix B(inf):*** $B_\infty$ is the long time residual polarization and may be included as a parameter in the analysis. The parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis of r(t).

**Results**

The results of the analysis are displayed in two forms.

3. The names of the fitted curve, the residuals, the autocorrelation function, the deconvoluted decay curves i.e. D(t) or IDvv for example, the anisotropy, etc… appear on the left of the screen.

4. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

# Micelle Kinetics

## Theory

This program allows for the analysis of quenching processes in micelles.

## Fitting Function

The analysis program uses the "stretched exponential" fitting function (Rogers *et al*, 1978). This function can be used to describe the quenching in micelles when quencher molecules are Poisson distributed among the micelles. The fitting function is:

$$D(t) = a_1 \exp\{-a_2 t - a_3[1 - \exp(-a_4 t)]\}$$

Eq. 1

For the case of quenching in micelles these parameters can be interpreted as:

$a_1$ = scale factor for the fitting function
$a_2 = 1/\tau$, the reciprocal of the unquenched fluorophore lifetime
$a_3$ = aggregation number
$a_4 = 1/k_q$, the reciprocal of the quenching rate constant

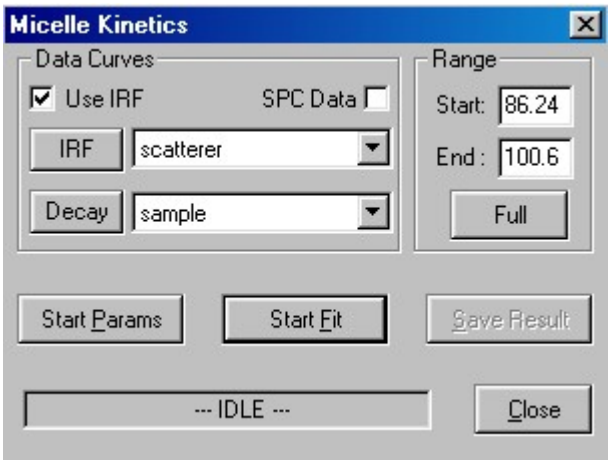
providing that the quenching process is fast relative to exchange of species.

Equation 1 implies that the fluorescence decay can be represented by a set of exponential decays with Poisson distributed amplitudes and discretely spaced lifetimes, which means that this type of data can also be analyzed with the PTI Maximum Entropy Method program (Siemiarczuk and Ware, 1990).

Please see the **General Introduction** for a discussion of the fitting procedures and statistics.

**Using The Program**

The initial dialog box for **Micelle Kinetics** is shown below.




**Data Curves**

The ***Use IRF*** checkbox selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** check box is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

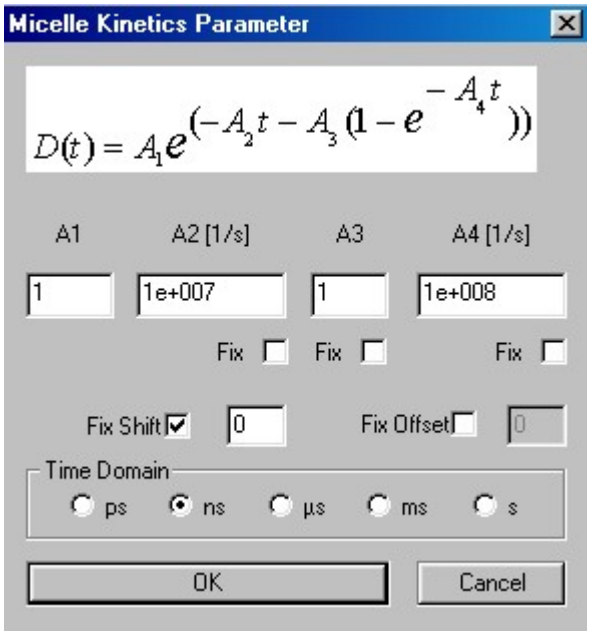
The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Parameters** dialog box for the **Micelle Kinetics** method is shown at left.

The fitting function is shown in a text box as a reminder of what the various parameters are.

***A1-A4:*** For each of the parameters to be used in the fit an initial guess must be given. Each of the parameters chosen for the analysis may be fixed at the input value (except **A1**) or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

***Time Domain:*** This method can be used to analyze fluorescence or phosphorescence data and imported data. The units used on the time axis may be different for each of these cases. For PTI instruments the units are nanoseconds for fluorescence and microseconds for phosphorescence.

**Results**

The results of the analysis are displayed in two forms.

1. The names of the fitted curve, the residuals, the autocorrelation function, and the deconvoluted decay curves i.e. D(t) appear on the left of the screen.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

# Non-Exponential Decay

**Theory**

This program allows for the analysis of data by a general fitting function consisting of two exponentials multiplied together each with variable exponents of time. The exponents can be either varied or fixed which provides a powerful general function for models such as Förster energy transfer and time-dependent quenching.

**Fitting Function**

The fitting function is:

$$D(t) = a_1 \exp(-a_2 t^n) \exp(-a_3 t^m)$$

Eq. 1

The parameters are:

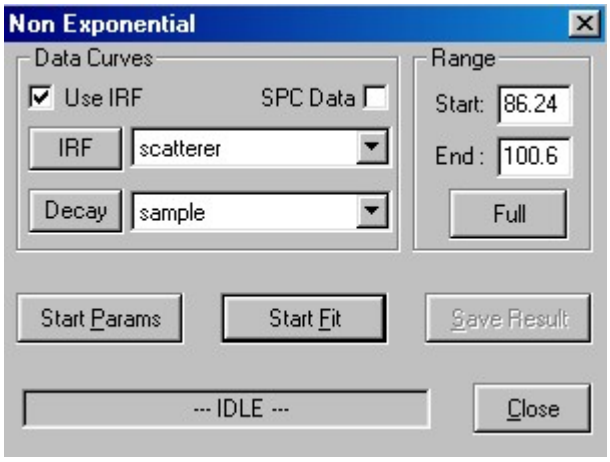
$a_1$ = scale factor for the fitting function
$a_2 = 1/\tau$, the reciprocal of the "slow" decay component
$a_3 = 1/\tau$, the reciprocal of the "fast" decay component
n = exponent of the "fast" component
m = exponent of the "slow" component if n>m.

The exponents can be held constant or found as parameters of the fit. For example, by setting n=1 and m=0.5, this fitting function is suitable for Förster energy transfer kinetics (Förster, 1949, Birks, 1948, Steinberg *et al*, 1983) or time-dependent quenching (Ware and Andre, 1983). Any other decay law, which can be modeled by two exponentials multiplied together, can be analyzed by this program.

Refer to the **General Introduction** for a discussion of the fitting procedures and statistical parameters.

**Using the Program**

The initial dialog box for **Non-exponential Decay** is shown below.




**Data Curves**

The ***Use IRF*** check box selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** check box is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

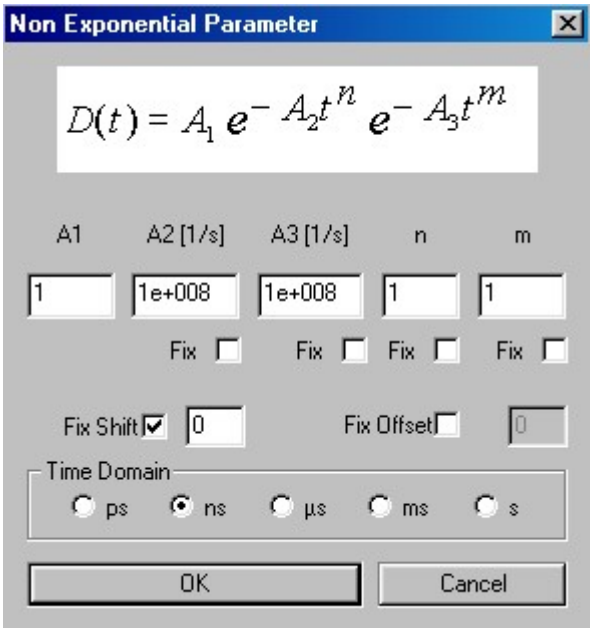
The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Parameters** dialog box for the **Non-exponential Decay** method is shown at left.

The fitting function is shown in a text box as a reminder of what the various parameters are.

***A1-A3, m, n:*** For each of the parameters to be used in the fit an initial guess must be given. Each of the parameters chosen for the analysis may be fixed at the input value (except **A1**) or allowed to float in the fit. Toggle this option on or off by clicking on it. Occasionally, the fit will not succeed if the starting values are very poor. If this occurs, try changing the starting values.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

***Fix Offset:*** Because of difficulties in establishing a noise free baseline there may be a small intensity offset for a decay curve. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the offset is allowed to float, a value of 0.0 is used as the initial guess.

***Time Domain:*** This method can be used to analyze fluorescence or phosphorescence data and imported data. The units used on the time axis may be different for each of these cases. For PTI instruments the units are nanoseconds for fluorescence and microseconds for phosphorescence.

**Results**

The results of the analysis are displayed in two forms.

1. The names of the fitted curve, the residuals, the autocorrelation function, and the deconvoluted decay curves i.e. D(t) appear on the left of the screen.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

# ESM – Exponential Series Method

**Theory**

Fluorescence lifetime measurements often result in complex decays requiring a more sophisticated approach than a single- or double-exponential fitting function (James and Ware, 1986, Siemiarczuk *et al*., 1990). This applies especially to the emission originating in such intrinsically complex systems as:

- bichromophoric molecules exhibiting distributions of conformers in the excited state
- fluorophores adsorbed on surfaces
- fluorophores attached to polymers
- fluorescent probes in micelles and liposomes
- fluorescent probes in biomembranes and other biological systems
- fluorophores in monolayers
- intrinsic fluorescence from proteins
- systems undergoing Förster-type energy transfer
- and many others…

Even intuitive considerations would lead one to expect distributions of lifetimes in these systems. Quite often, however, especially for low precision data, a good fit can be obtained with a double- or triple-exponential function for a system, which in fact represents a continuous distribution of lifetimes. In general, however, the parameters recovered from such a fit have no physical meaning.

The Exponential Series Method (ESM) is designed to recover lifetime distributions without any *a priori* assumptions about their shapes. This method uses a series of exponentials (up to 200 terms) as a probe function with fixed, logarithmically-spaced lifetimes and variable pre-exponentials. This allows covering a lifetime range of several orders of magnitude. In many situations the ESM is capable of differentiating between continuous distributions and discrete, multi-exponentials decays.

**Fitting Procedure**

The fluorescence decay is approximated by the exponential series:

$$F(t) = \sum_{i=1}^{N} a_i \exp\left(\frac{-t}{\tau_i}\right)$$

Eq. 1

where $a_i$ are the variable amplitudes, $\tau_i$ are the lifetimes that are fixed and logarithmically-spaced, and N is the number of terms. Initially all $a_i$ are set equal. In order to recover amplitudes $a_i$ the ESM uses an iterative procedure to minimize the chi-square function that is defined as follows:

$$C = \left(\frac{1}{n}\right) \sum_{k=1}^{n} \frac{\left(Y_k - \sum_{i=1}^{N} D_{ki}\, a_i\right)^2}{\sigma_k^2} \approx 1.0$$

Eq. 2

where $Y_k$ represents the fluorescence intensity (e.g. number of photons) in the kth channel, $\sigma_k$ is the standard deviation in the $k^{th}$ channel, n is the number of channels, $D_{ki}$ is the convolution matrix:

$$D_{ki} = \int_0^{t_k} L(t_k - t) \exp\left(\frac{-t}{\tau_i}\right) dt$$

Eq. 3

where L(t) comprises the excitation pulse profile and the instrument response function.

**Distribution Moments and Related Parameters**

The program, if requested, calculates five central distribution moments and related parameters useful in describing the shape of a distribution.

The central distribution moments are defined as follows:

$$M_i = \sum_{k=n_1}^{n_2} a_k \, (\tau_k - \mu)^i \, \frac{1}{M_o}, \; i = 1,...,5$$

Eq. 4

where $\mu$ represents the mean of the distribution:

$$\mu = \sum_{k=n_1}^{n_2} a_k \left( \frac{\tau_k}{M_o} \right)$$

$$M_o = \sum_{k=n_1}^{n_2} a_k$$

Eq. 5

and $n_1$ and $n_2$ are the indices determining the lifetime integration range. Note that by definition $M_1$=0 and $M_2$ represents the variance of the distribution. The standard deviation sigma is calculated as square root of the variance.

The parameters skewness and kurtosis are useful in describing the shape of distributions. They are defined as follows:

$$\text{skew} = 0.5 \, M_3/\text{sigma}^3$$

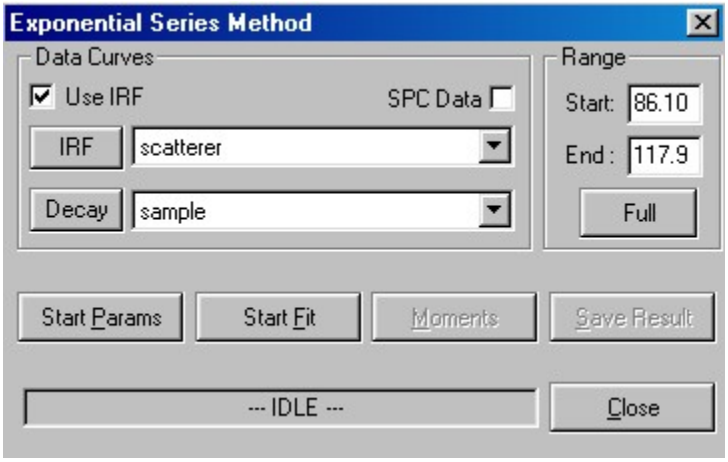
$$\text{kurt} = M_4/\text{sigma}^4 - 3$$

Skewness is a measure of the degree of asymmetry of a distribution. For a symmetrical distribution, skew = 0. Negative skewness indicates a tail at short lifetimes while positive skewness is observed when tailing occurs at long lifetimes.

Kurtosis is a measure of the flatness of a distribution. For the normal distribution, kurt = 0. Negative kurtosis indicates that a distribution is flatter than the normal.

**Using the Program**

The initial **Exponential Series Method** dialog box is shown below.




**Data Curves**

The ***Use IRF*** checkbox selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** checkbox is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

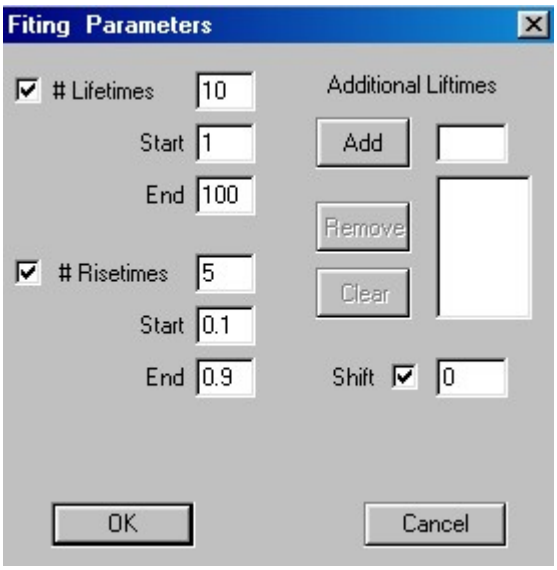
***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Moments:*** Clicking the *Moments* button calculates the moments for a selected portion of the lifetime distribution curve and displays them in the notepad window. See the **Results** section for more details.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Fitting Parameters** dialog box for the **Exponential Series** method is shown at left.

The ***# Lifetimes*** check box and text box select the number of different lifetimes used in the analysis of the decay curve. These are distributed in a logarithmic manner between the *Start* lifetime and the *End* lifetime.

The ***# Risetimes*** check box and text box select the number of different risetimes used in the analysis of the decay curve. These are distributed in a logarithmic manner between the *Start* lifetime and the *End* lifetime.

**Additional Lifetimes**

Additional fixed lifetimes may be entered one at a time in the text box. Clicking on the **Add** button enters this value on the lower text window. Lifetimes may be deleted by clicking on the appropriate line in the text window to highlight the line then clicking on the **Remove** button. All lifetimes may be removed by clicking on the **Clear** button. This option is useful when there are some lifetimes lying far outside the range of the distribution. Extending the range of the lifetime distribution to include these would be very wasteful since most of the lifetimes would lie in regions with zero amplitude.

*Fix* ***Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis.

**Results**

The results of the analyses are displayed in several forms.

1. The names of the fitted curve, lifetime distribution, the residuals, the autocorrelation function and the deconvoluted decay i.e. D(t) appear on the left of the screen. Initially, only the fitted curve and the lifetime distribution are displayed the others being hidden to avoid clutter. The lifetime distribution curve contains most of the information from this analysis. Commonly, all other files must be hidden to see this curve since the Y scale is much smaller than most data curves. The numerical values associated with the distribution are not included in the notepad window since they are, typically, very numerous. The numerical values can be viewed by hiding all other curves except the distribution curve and using the **Grid View** button to display a spreadsheet of the results.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

3. Various moments of selected portions of the lifetime distribution curve may be calculated. First hide all curves except the lifetime distribution. Select a portion of the curve by clicking and dragging the mouse across the area of interest. Clicking on the **Moments** button calculates the moments of the selected region and enters them into the notepad window along with some identification information.

4. While the fit is executing a **Fit Status** window displays the current lifetime distribution and residuals on a logarithmic time scale. Should the user wish to capture this window, this can be done by making **Fit Status** the active window (click on title line), saving the active window to the clipboard (**Alt+ Print Screen**), opening a graphics program e.g. Paint and pasting the clipboard into the program (**Ctrl+V**).

# MEM – Maximum Entropy Method

**Theory**

Fluorescence lifetime measurements often result in complex decays requiring a more sophisticated approach than a single- or double-exponential fitting function (James and Ware, 1986, Siemiarczuk *et al*, 1990). This applies especially to the emission originating in such intrinsically complex systems as:

- bichromophoric molecules exhibiting distributions of conformers in the excited state
- fluorophores adsorbed on surfaces
- fluorophores attached to polymers
- fluorescent probes in micelles and liposomes
- fluorescent probes in biomembranes and other biological systems
- fluorophores in monolayers
- intrinsic fluorescence from proteins
- systems undergoing Förster-type energy transfer
- and many others…

Even intuitive considerations would lead one to expect distributions of lifetimes in these systems. Quite often, however, especially for low precision data, a good fit can be obtained with a double- or triple-exponential function for a system, which in fact represents a continuous distribution of lifetimes. In general, however, the parameters recovered from such a fit have no physical meaning.

The Maximum Entropy Method (MEM) is designed to recover lifetime distributions without any *a priori* assumptions about their shapes (Skilling and Bryan 1989, Smith and Grady, 1985). This method uses a series of exponentials (up to 200 terms) as a probe function with fixed, logarithmically-spaced lifetimes and variable pre-exponentials. This allows covering a lifetime range of several orders of magnitude. In many situations the MEM is capable of differentiating between continuous distributions and discrete, multi-exponentials decays.

**Fitting Procedure**

The fluorescence decay is approximated by the exponential series:

$$F(t) = \sum_{i=1}^{N} a_i \exp\left(\frac{-t}{\tau_i}\right)$$

Eq. 1

where $a_i$ are the variable amplitudes, $\tau_i$ are the lifetimes that are fixed and logarithmically-spaced, and N is the number of terms. Initially all $a_i$ are set equal. The MEM theory utilizes the Shannon-Jaynes entropy function:

$$S = -\sum_{i=1}^{N} a_i \log\left(\frac{a_i}{\sum_{i=1}^{N} a_i}\right)$$

Eq. 2

which has to be maximized in order to recover the least biased set of amplitudes $\{a_i\}$ out of all feasible solutions. On the other hand, to ensure that the recovered solution is in agreement with the experimental decay, the following constraint based on the chi-square statistics is implemented:

$$C = \left(\frac{1}{n}\right) \sum_{k=1}^{n} \frac{\left(Y_k - \sum_{i=1}^{N} D_{ki}\, a_i\right)^2}{\sigma_k^2} \approx 1.0$$

Eq. 3

where $Y_k$ represents the fluorescence intensity (e.g. number of photons) in the kth channel, $\sigma_k$ is the standard deviation in the kth channel, n is the number of channels, $D_{ki}$ is the convolution matrix:

$$D_{ki} = \int_0^{t_k} L(t_k - t) \exp\left(\frac{-t}{\tau_i}\right) dt$$

Eq. 4

where L(t) comprises the excitation pulse profile and the instrument response function. Conditions (2) and (3) can be combined in one function:

$$Q = \alpha S - C$$

Eq. 5

where $\alpha$ is a Lagrange multiplier. Q is then maximized by an iterative procedure thus ensuring simultaneous maximization of S and minimization of C until constraint (3) is satisfied. After a target value of chi-square is reached, the program keeps maximizing S with C kept constant until the entropy test parameter:

$$0.5\left[\left(\frac{\text{gradC}}{|\text{gradC}|}\right) - \left(\frac{\text{gradS}}{|\text{gradS}|}\right)\right] < 0.1$$

Eq. 6

This condition ensures that the global maximum of Q has been reached.

**Distribution Moments and Related Parameters**

The program, if requested, calculates five central distribution moments and related parameters useful in describing the shape of a distribution.

The central distribution moments are defined as follows:

$$M_i = \sum_{k=n_1}^{n_2} a_k \ (\tau_k - \mu)^i \ \frac{1}{M_o}, \ i = 1,...,5$$

Eq. 7

where $\mu$ represents the mean of the distribution:

$$\mu = \sum_{k=n_1}^{n_2} a_k \left(\frac{\tau_k}{M_o}\right)$$

$$M_o = \sum_{k=n_1}^{n_2} a_k$$

Eq. 8

and $n_1$ and $n_2$ are the indices determining the lifetime integration range. Note that by definition $M_1$=0 and $M_2$ represents the variance of the distribution. The standard deviation sigma is calculated as square root of the variance.

The parameters skewness and kurtosis are useful in describing the shape of distributions. They are defined as follows:

$$\text{skew} = 0.5\ M_3/\text{sigma}^3$$

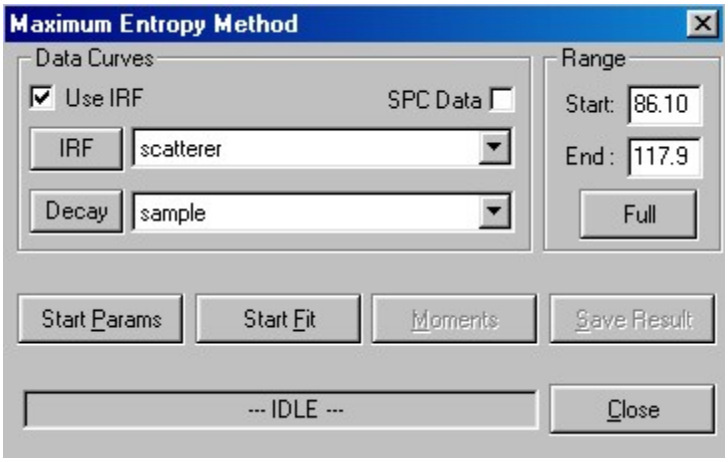
$$\text{kurt} = M_4/\text{sigma}^4 - 3$$

Skewness is a measure of the degree of asymmetry of a distribution. For a symmetrical distribution, skew = 0. Negative skewness indicates a tail at short lifetimes while positive skewness is observed when tailing occurs at long lifetimes.

Kurtosis is a measure of the flatness of a distribution. For the normal distribution, kurt = 0. Negative kurtosis indicates that a distribution is flatter than the normal.

**Using the Program**

The initial **Maximum Entry** method dialog box is shown below.




**Data Curves**

The ***Use IRF*** checkbox selects whether an instrument response function (scatterer) will be used in the analysis or not. Normally, an IRF is used. However, if the lifetime of the sample is long compared to the width of the excitation pulse or the range of data to be analyzed starts at a delay long compared to the width of the excitation pulse an IRF is not required.

The ***SPC Data*** checkbox is used only when single photon counting data has been imported.

The ***IRF*** button selects the curve to be used as scatterer. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *IRF* button. The name of the selected curve will appear in the box beside the button.

The ***Decay*** button selects the curve to be analyzed. Select a curve by clicking on its name at the left side of the FeliX32 screen then click on the *Decay* button. The name of the selected curve will appear in the box beside the button.

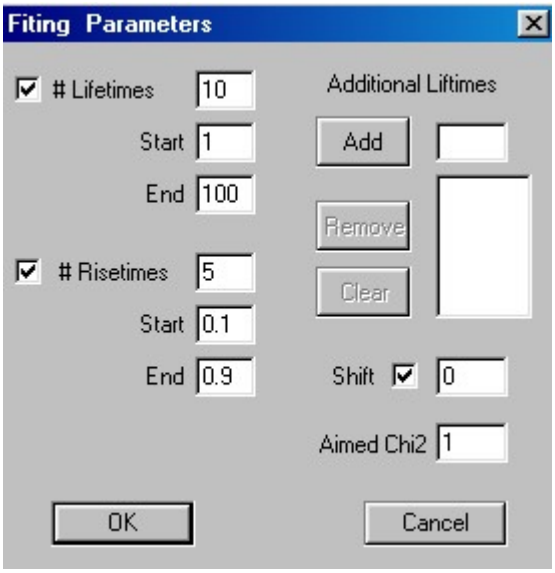
***Range:*** The *Start* delay and *End* delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard. Alternatively, select the *Mark Region* function from the graph toolbar and position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be highlighted on the screen. If the entire range is to be used in the analysis, click the *Full button*.

***Start Params:*** The non-linear-least-squares fit used in the data analysis requires estimated starting values for the various parameters. Clicking on the Start Params button opens a dialog box that allows these values to be entered.

***Start Fit:*** Clicking the *Start Fit* button starts the analysis program. The box showing *IDLE* in the above picture changes to show the progress of the fit. The *Close* button in the above picture changes to a *Stop Fit* button. Clicking the *Stop Fit* button immediately aborts the analysis.

***Moments:*** Clicking the *Moments* button calculates the moments for a selected portion of the lifetime distribution curve and displays them in the notepad window. See the **Results** section for more details.

***Save Results:*** The *Save Results* button opens a dialog box that allows the *TimeMaster Output* results for that fitting to be saved to the database. The data can be viewed by opening *TM Result* in the **View** drop down menu, selecting the appropriate result from the list and clicking *Show Results*.




The **Fitting Parameters** dialog box for the **Maximum Entropy Method** is shown at left.

The ***# Lifetimes*** check box and text box select the number of different lifetimes used in the analysis of the decay curve. These are distributed in a logarithmic manner between the *Start* lifetime and the *End* lifetime.

The ***# Risetimes*** check box and text box select the number of different risetimes used in the analysis of the decay curve. These are distributed in a logarithmic manner between the *Start* lifetime and the *End* lifetime.

**Additional Lifetimes**

Additional fixed lifetimes may be entered one at a time in the text box. Clicking on the **Add** button enters this value on the lower text window. Lifetimes may be deleted by clicking on the appropriate line in the text window to highlight the line then clicking on the **Remove** button. All lifetimes may be removed by clicking on the **Clear** button. This option is useful when there are some lifetimes lying far outside the range of the distribution. Extending the range of the lifetime distribution to include these would be very wasteful since most of the lifetimes would lie in regions with zero amplitude.

***Aimed Chi2:*** Enter the target value of $\chi^2$.

***Fix Shift:*** There may be a small time shift between the sample and the scatterer decay curves (see the **General Introduction** for details). This shift may be included as a parameter in the analysis. The shift parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on or off by clicking on it. If the shift is allowed to float, a value of 0.0 is used as the initial guess.

Once all parameters have been set, click the *OK* button to return to the previous dialog box and then **Start Fit** to start the Analysis.

**Results**

The results of the analyses are displayed in several forms.

1. The names of the fitted curve, lifetime distribution, the residuals, the autocorrelation function and the deconvoluted decay i.e. D(t) appear on the left of the screen. Initially, only the fitted curve and the lifetime distribution are displayed the others being hidden to avoid clutter. The lifetime distribution curve contains most of the information from this analysis. Commonly, all other files must be hidden to see this curve since the Y scale is much smaller than most data curves. The numerical values associated with the distribution are not included in the notepad window since they are, typically, very numerous. The numerical values can be viewed by hiding all other curves except the distribution curve and using the **Grid View** button to display a spreadsheet of the results.

2. A notepad window named **TimeMaster Output** pops up containing identification information, the lifetimes and pre-exponential factors and various statistics associated with the fit. Since this is a Notepad window, the text may be edited, saved or printed as the user desires. The results are not deleted from this window when another analysis is run. This feature allows the results of several analyses to be combined. However, this feature may also lead to very long files if many trial analyses are run without clearing the window. To clear the window select *Edit* in the TimeMaster Output window and clear from the drop-down menu or press *Ctrl+D*. Another option is to use the **Save Results** button after fitting which saves the TimeMaster Output for the specific fit to a separate section of the database. Opening the **View/TM Output** window and selecting **Show Results** will then later retrieve the data.

3. Various moments of selected portions of the lifetime distribution curve may be calculated. First hide all curves except the lifetime distribution. Select a portion of the curve by clicking and dragging the mouse across the area of interest. Clicking on the **Moments** button calculates the moments of the selected region and enters them into the notepad window along with some identification information.

4. While the fit is executing a **Fit Status** window displays the current lifetime distribution and residuals on a logarithmic time scale. Should the user wish to capture this window, this can be done by making **Fit Status** the active window (click on title line), saving the active window to the clipboard (**Alt+ Print Screen**), opening a graphics program e.g. Paint and pasting the clipboard into the program (**Ctrl+V**).

## DAS / TRES

As discussed in the General Introduction, the analysis of time domain data acquired using a pulsed light source is complicated by convolution with the intensity profile of the light source. This is true both for decays and for time resolved spectra and is particularly serious at delay times short compared to the width of the exciting pulse. FeliX32 allows the direct acquisition of time resolved spectra (called gated spectra for phosphorescence modes) but it must be remembered that these must suffer to some extent from convolution caused distortion. In many cases, the convenience of the direct acquisition of time resolved spectra far outweighs the effect of distortion at short time scales particularly when only qualitative comparisons are required.

In cases where a more quantitative analysis is required, deconvoluted spectra may be calculated using the **DAS / TRES** method. The **DAS / TRES** method, is unique among the analysis tools in that it is a secondary method requiring the results of a previous **Global 1 To 4 Exp** or a **Multi 1 To 4 Exp** fit in order to construct *Decay Associated Spectra* or deconvoluted *Time Resolved Emission Spectra*. The experimental data required consists of decay curves collected at a series of wavelengths (a scatterer curve or curves will also be necessary). Every sample decay must be collected with the same experimental parameters (except wavelength). In particular, slits and/or filters must not be changed between sample decays. Each scatterer curve must also be collected with the same experimental parameters but these may be different from those used for the sample decays. The series of wavelength selected decays is entered into a **Global 1 To 4 Exp** or a **Multi 1 To 4 Exp** fit (see the appropriate section). The results of the analysis are saved by clicking on the **Save Results** button in the **Multi One To Four Exponential(s)** or the **Global One To Four Exponential(s)** dialog box.

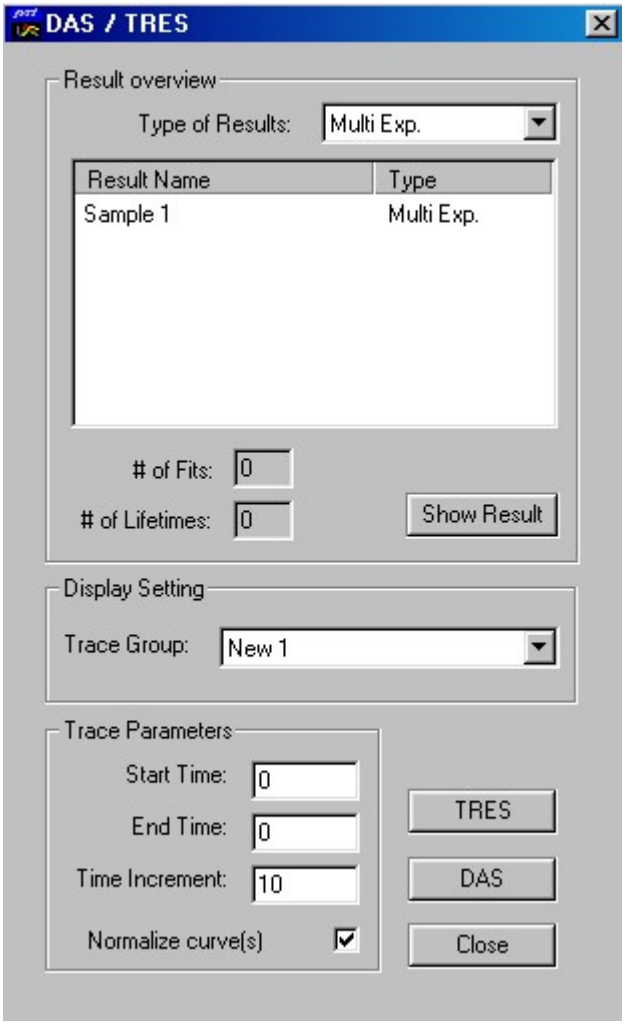
The results of the preceding analysis are a series of wavelength selected D(t) functions (deconvoluted decays) defined mathematically as a sum of up to four exponentials multiplied by their associated pre-exponential factors (see equation 1 in the **1 To 4 Exp. Lifetime** section).

The **TRES** program calculates the D(t) at a series of selected time delays for all the wavelengths and displays the results as a series of spectral curves each at a selected time delay. These curves constitute the deconvoluted TRES.

The **DAS** program takes this analysis further. Each of the terms in equation 1 of the **1 To 4 Exp. Lifetime** section (corresponding to a different lifetime) is calculated for every selected time delay and wavelength. Pseudo-spectra are displayed for each lifetime and each selected delay time. In the case of **Multi 1 To 4 Exp** fits the lifetimes found at each wavelength will not be exactly the same. The **DAS** program deals with this problem by displaying the shortest lifetime at each wavelength in one curve the second shortest in another etc… If the lifetimes displayed in a single curve vary wildly, the information contained in a DAS analysis may be of limited utility.

Both DAS and TRES analysis are useful in the analysis of complicated decay kinetics where various physical effects have different wavelength dependence.

**Using the Program**




The initial dialog box for **DAS /TRES** is shown on the left.

The file containing the results of a global or multi-exp. fit is selected from the list of files of the appropriate type. The listed type may be selected as **Global Exp.** or **Multi Exp.**.

Once a file has been selected, *the **# of Fits*** box and the ***# of Lifetimes*** box will display the values associated with the selected file.

Clicking on the ***Show Results*** button brings up a Notepad window with the results contained in the selected file.

The time delays at which the TRES or DAS will be calculated are selected by entering the start, end and interval times in the ***Start Time***, ***End Time*** and ***Time Increment*** boxes respectively.

Clicking on the **TRES** or the **DAS** button starts the selected calculation.

**Results**

The results of the calculation are displayed on screen as a number of spectra. For a TRES calculation there will be one spectrum for each delay time selected while for a DAS calculation there will be one spectrum for each lifetime at each selected delay time. Since the values displayed in the calculated spectra are often rather small, it may be necessary to hide all other curves and to select **Axes** from the menu bar and **Full Autoscale** from the drop down menu in order to see the results.

Chapter 16

# FeliX32 at Work

Perhaps the best way to understand how all of the features of FeliX32 and your instrument go together is to walk through some examples of fluorescence analyses on samples that are easily reproduced.

As one exercise, we will measure the Raman scatter of water, which can be used to determine the sensitivity of your instrument. The second exercise is a titration of the calcium indicator Fura-2 with calcium.

## Raman Scatter of Water

The Raman scatter of water can be used as a quick check of an instrument's overall functional integrity, and also to measure its sensitivity. The peak in the spectrum of water is not due to fluorescence; it is Raman scattering that gives rise to the fluorescence-like response of water. It simulates fluorescence nicely in that the scattered light is observed at a longer wavelength than excitation. The signal is of low intensity, making it an appropriate test for the sensitivity of a fluorescence spectrometer.

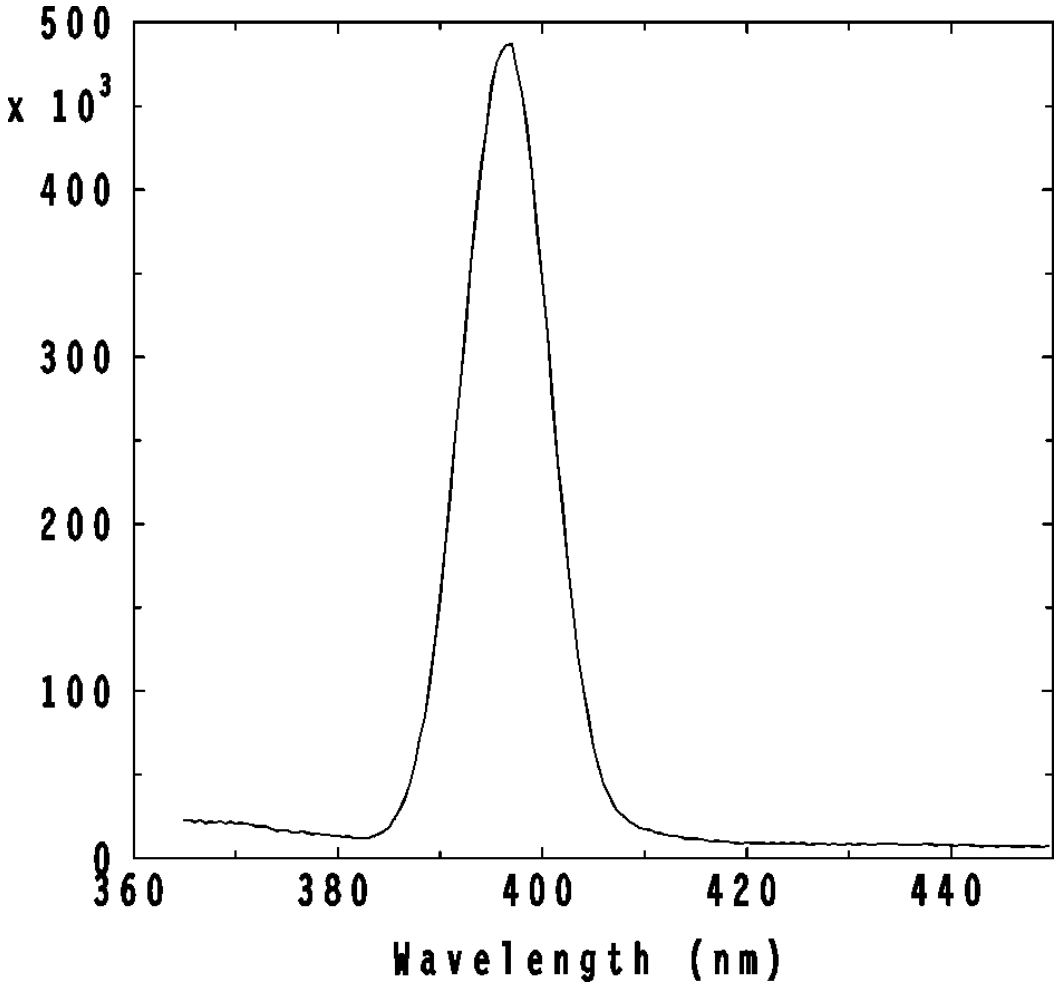
The wavelength maximum of the Raman band of water is dependent on the excitation wavelength. The scatter peak is always red-shifted (toward longer wavelengths) 3382 $cm^{-1}$ from the excitation wavelength. If your excitation monochromator is set at 360 nm, for example, the peak will be at 410 nm.

Choose New Acquisition/Emission Spectra and set it up as follows:

| | |
|---|---|
| Acquire: | Emission Scan |
| Excitation: | 350 nm |
| Start: | 365 nm |
| Stop: | 450 nm |
| Length: | 85 nm |
| Step Size: | 0.5 nm |
| Integration: | 1 second |
| Bandpass: | 5 nm* |

*for the entrance and exits sides of both the excitation and emission monochromators (2.5 turns of the slit micrometers for a model 101M monochromator and the DeltaRAM V, 0.5 turns for a DeltaRAM).

Fill a clean, 1 cm, quartz cuvette with distilled water, tap it to displace any bubbles adhering to the walls, and place it in the sample compartment. Click **ACQUIRE (PREP)**, then **START**. The Raman band should appear as shown with the peak at 397 nm. The intensity at the peak should be between 300,000 and 800,000 counts per second (cps) and the data on the baseline should be relatively noise-free. The number of counts apply for a model 101M monochromator with standard gratings, other systems may give differing results.




**Signal-to-Noise Ratio**

For details on the measurement of the signal-to-noise ratio of the Raman band of water, ask for the PTI Technical Note, "The Measurement of Sensitivity in Fluorescence Spectroscopy," see American Laboratory, September 1994, page 32G, or visit our website at www.pti-nj.com.

## Titration of Fura-2 with Calcium

This section outlines a procedure for calibration of Fura-2 experiments for the measurement of calcium. A Fura-2 titration is carried out with known concentrations of free $Ca^{++}$ that are controlled by $Ca^{++}$/EGTA buffers. The resulting data are used to determine the dissociation constant, Kd, of the $Ca^{++}$/Fura-2 complex. Essentially, this procedure reproduces the results in figure 3 of the original work by G. Grynkiewicz, M. Poenie, and R.Y. Tsien, "A New Generation of $Ca^{++}$ Indicators with Greatly Improved Fluorescence Properties", Journal of Biological Chemistry, **260**, 3340 (1985).

This is approximately a three hour exercise that is meant to acquaint you with the operation of FeliX32 and your instrument. Although a more rigorous calibration procedure may be needed in some cases, this exercise will provide valuable experience with steady state ratio fluorescence measurements and result in data that will unequivocally indicate your mastery of the technique and the thorough understanding of FeliX32.

Obtaining a satisfactory set of titration curves, which yield a dissociation constant comparable to the literature value will also confirm the performance of the instrument and the condition of the reagents.

The calibration equation is:

$$[Ca^{++}] = K_d \times ((R - R_{min})/(R_{max} - R)) \times Sf2/Sb2$$

R = F1/F2, the ratio of fluorescence intensities obtained with excitation at $\lambda_1$ = 340 nm and $\lambda_2$ = 380 nm.

$R_{min}$, $R_{max}$ = F1/F2 ratios of the calcium-free and calcium-saturated Fura-2 sample, respectively.

Sf2 = F2 of the calcium-free Fura-2 sample.

Sb2 = F2 of the calcium-bound Fura-2 sample.

$K_d$ is the effective dissociation constant for the $Ca^{++}$/Fura-2 complex.

Although not applicable to this experiment, other factors, such as viscosity, may affect the measurement of calcium.

In the following, 1 will refer to 340 nm and 2 will refer to 380 nm. These wavelengths are appropriate for Fura-2. It should be understood that other wavelengths may be chosen and that different indicators will have different wavelength pairs that should be selected for the calibration equation.

**Preparation for Measurement**

Prepare two solutions of pH 7.0, 10 mM EGTA buffers containing 100 mM KCl and 10 mM K-MOPS. One of the buffers will contain 10 mM $Ca^{++}$ (use a 1 M $CaCl_2$ stock solution), the other will contain no $Ca^{++}$. They will be called CaEGTA and EGTA buffers, respectively.

Fura-2 will be added to both buffers during the exercise. If 1 mM Fura-2 stock solutions are used, a thousand-fold dilution of the stock would yield 1 μM final Fura-2. We have found it convenient to store Fura-2 frozen in 50-μl quantities.

During the exercise, you will prepare a range of free calcium concentrations by removing specific volumes of EGTA buffer and replacing them with CaEGTA buffer.

To a washed and dried test tube, add 8.991 ml of CaEGTA buffer and 9 μl of the 1 mM Fura-2 stock solution to obtain a final Fura-2 concentration of 1 μM. Mix the contents of the test tube thoroughly.

**Excitation Scan Measurements**

This section describes the preparation of a range of calcium-Fura-2 solutions by serial exchange of reagents. An excitation scan is performed for each solution. The excitation scans will be used to calculate the dissociation constant.

In FeliX32, select *Excitation Scan* from the **New Acquisition** menu. Enter the following parameters:

| | |
|---|---|
| Start: | 300 nm |
| Stop: | 450 nm |
| Emission: | 510 nm |
| Step Size: | 0.5 nm |
| Integration Time: | 0.25 sec. |
| Averages: | 1 |

Set all slits to 3 nm bandpass (1.5 turns of the slit micrometers for a model 101M monochromator and DeltaRAM V with standard gratings, ¾ turns for a DeltaRam, and 3 turns for a model 201M monochromator).

You will be making several volume exchanges in the cuvette and measuring the excitation spectrum of each. Choose *More* to reach the **Additional Acquisition Setup Controls** dialog box. Set the parameters as appropriate for your instrument. For information on each parameter, select Help.

Add 2.997 ml of EGTA buffer to a clean, dry cuvette (use a 1 ml digital pipette set to 0.999 ml) that is optically transparent above 300 nm. A quartz cuvette is the best choice. Use caution with plastic cuvettes. A plastic cuvette may be opaque to 340 nm light.

Select **AQUIRE(PREP)** and then **START** to verify that you have a relatively flat baseline with no fluorescence due to contaminants. Your instrument is very sensitive, so you may observe a peak at about 435 nm from the Raman band of water. This will not affect the measurement since it is beyond the wavelength of interest.

Introduce 3 µl of Fura-2 into the 2.997 ml EGTA buffer directly into the cuvette using a 10 µl adjustable pipette. This results in a 1 µM final concentration of Fura-2. Using a 1 ml digital pipette set to 1 ml, carefully siphon and subsequently release the sample in the cuvette to ensure thorough mixing. 4-5 such cycles should suffice. (If 3 µl cannot be delivered with precision to the cuvette, add 9 µl of Fura-2 to 2.991 ml of the EGTA buffer in the cuvette and remove 2 ml after mixing. With a fresh pipette tip, dilute the remaining 1 ml in the sample cuvette with 2.0 ml of EGTA buffer. Repeat 4-5 cycles of mixing.)

Take the fluorescence excitation spectrum of the sample containing 1 µM Fura-2 by scanning from 300 to 450 nm. Make sure that a peak is observed at about 370 nm. If the maximum wavelength is much shorter, calcium may have been introduced at some point or the cuvette was not calcium-free when you added Fura-2. In that case, the procedure must be repeated from the beginning.

You may save the spectrum of the sample with no calcium by using the File/Save As command. Remove 300 µl of the sample with the digital pipette, discard and replace with 300 µl of the CaEGTA buffer preparation containing Fura-2. Take the excitation spectrum again and save it with File/Save.

Remove 333 µl of the sample from the cuvette and replace it with 333 µl of CaEGTA buffer containing Fura-2. Measure the excitation spectrum and save it.

See the Table on the following page. Continue to exchange the volumes in the first column and measure the excitation scan. These serial exchanges take you through a series of measurements of a solution containing 9 mM EGTA and 1 mM CaEGTA, 8 mM EGTA and 2 mM CaEGTA, etc… The CaEGTA concentration is increased by 1 mM and the EGTA concentration is concurrently decreased by 1 mM at each subsequent step by replacing a volume of 3/(11–n) ml, where n is the number of iterations.

The Table also tabulates the respective free $Ca^{++}$ concentrations that are controlled by the two buffers, assuming an apparent dissociation constant for the $Ca^{++}$ EGTA complex of 380 nm at pH 7.0 in 100 mM KCl at 20°C. Note that the temperature dependence of this dissociation constant may mean that this value is only appropriate for data gathered at 20°C and conversely, that $K_d$ values measured at 20°C may not be correct for analyzing experimental data gathered at other temperatures. Thus:

$$Kd = [Ca^{++}][EGTA]/[CaEGTA]$$

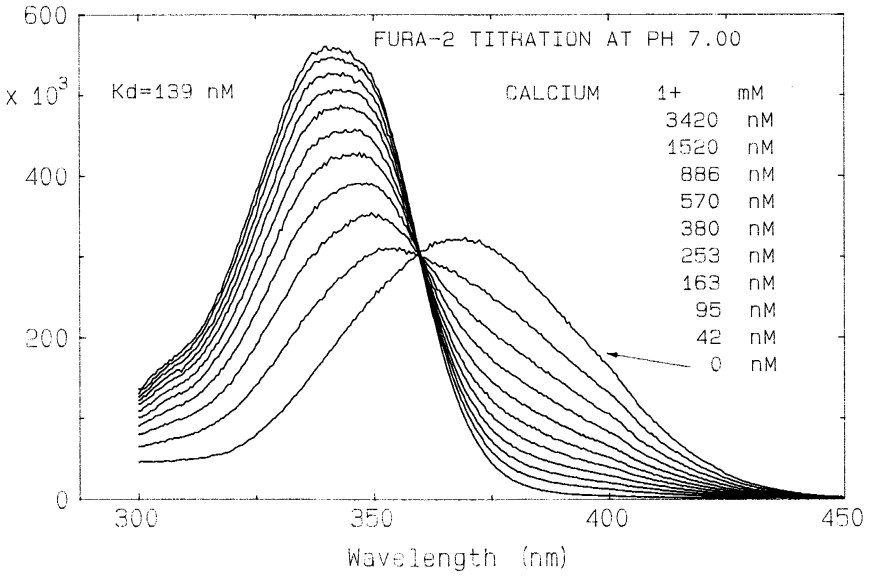
$$[Ca^{++}] = K[CaEGTA]/[EGTA]$$

$$= 380 \times 1/9,\ 380 \times 2/8,\ 380 \times 3/7,$$
$$380 \times 4/6,\ 380 \times 5/5,\ ...380 \times 9/1.$$

| **CALCULATION WORKSHEET** | | | | |
|---|---|---|---|---|
| Volume Exchange, ml | $[Ca^{++}]$ nm | R | $R - R_{min}$ | $\frac{R - R_{min}}{[Ca^{++}]}$ |
| 0 | 0 | .61776 | — | — |
| 0.3 | 42.2 | 1.11306 | 0.4953 | 0.01174 |
| 0.333 | 95 | 1.73439 | 1.11663 | 0.01175 |
| 0.375 | 162.85 | 2.44889 | 1.83113 | 0.01124 |
| 0.429 | 253.33 | 3.31391 | 2.69615 | 0.01064 |
| 0.5 | 380 | 4.53287 | 3.91511 | 0.01030 |
| 0.6 | 570 | 5.94783 | 5.33007 | 0.00935 |
| 0.75 | 880 | 7.81845 | 7.20069 | 0.00813 |
| 1.0 | 1520 | 10.60804 | 9.99028 | 0.00657 |
| 1.5 | 3420 | 15.20347 | 14.58571 | 0.00426 |
| 2.990 | >0.1-mM | 22.15208 | — | — |

Note that the first value for R, 0.61776, becomes $R_{min}$. Following the last measurement, 30 µl of 100 mM $CaCl_2$ solution are added to bring the free calcium concentration above 1 mM and saturate Fura-2, and another spectrum is taken. That value becomes $R_{max}$.

**Isosbestic Point**




The superimposed excitation spectra all intersect at a single point, the isosbestic point, indicating that the spectra are linear combinations of two components and reflect an equilibrium between these two components. It is very critical to perform the above dilution series as carefully as possible. Otherwise, the isosbestic point will not be well defined, and the ratios obtained from the spectra may not reflect the true concentrations. (The isosbestic point does not depend on the tabulated values of dilutions; any haphazard value of exchange volume will result in the curves intersecting at the isosbestic point as long as the exchanged volumes are precisely identical, e.g., 1.23456 ml exchanged for 1.23456 ml. However, the calcium concentration in the table will obviously change accordingly.)

**Ratio Determination**

Using the Data Cursor (Display/Data Cursor), measure and note the intensity values at 340 and 380 nm for each excitation spectrum. Calculate the ratio R = F(340)/F(380) and enter it into a separate column next to the corresponding calcium concentration. Calculate the difference $R - R_{min}$ and the ratio $(R - R_{min})/[Ca^{++}]$ and enter these values into a separate column as well.

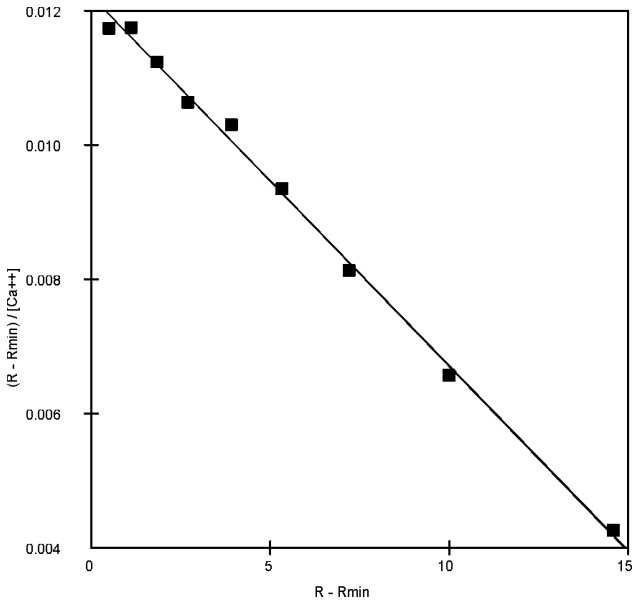
## $K_d$ Determination

Plot the values $(R - R_{min})/[Ca^{++}]$ as a function of $R - R_{min}$ and obtain a linear fit. Note that the final measurement of R is not used because of uncertainty in the calcium concentration. The slope of the fitted line is m and thus $K_d = -1/(m \times Sf2/Sb2)$ where Sf2 is the fluorescence intensity at 380-nm of the Ca-free sample and Sb2 is the fluorescence intensity at 380 nm of the Ca-bound sample. Linearization of the calibration equation is given at the end of this section. Data from the family of excitation scans yield Sf2/Sb2 = 12.917.

## Linear Fit of Data from Worksheet

Calculating: $K_d = -(1/(m \times (Sf2/Sb2)))$

Yields: $K_d = 139$ nm



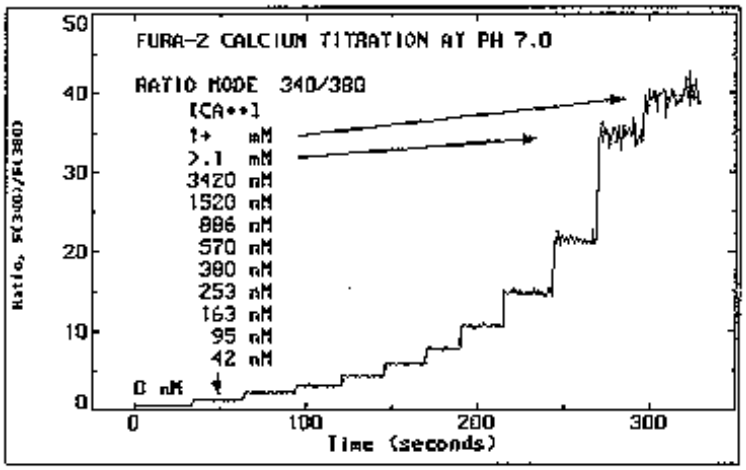
**Excitation Ratio Mode Measurement**

PTI RatioMaster systems are capable of direct measurement of R values. Instead of measuring the excitation spectra of Fura-2 at various calcium concentrations and subsequently determining the F(340)/F(380) ratios from the spectra, the ratios can be directly measured in Excitation Ratio mode.

Select **New Acquisition/Excitation Ratio**. Enter the following parameters:

| | |
|---|---|
| Excitation 1: | 340 nm |
| Excitation 2: | 380 nm |
| Emission: | 510 nm |
| Points/sec: | 15 (DeltaScan- and PowerFilter-based systems) |
| Integration: | 0.1 sec (monochromator-based systems |
| Duration: | 650 sec |
| Repeats: | 1 |
| Pause: | Not applicable |
| View Window: | 650 sec |

Choose **Display** to bring up the **Display Setup** dialog box. Under **Derived Data**, select the 340 nm trace for **Source 1** and the 380 nm trace for **Source 2** on the drop-down list box to and select **Source1/Source2** from the function box to calculate and display the ratio. We recommend that the ratio and intensity be displayed in separate groups. To do this, click on the **Add to Group** drop-down list box under **Derived Data** and select **New2**.

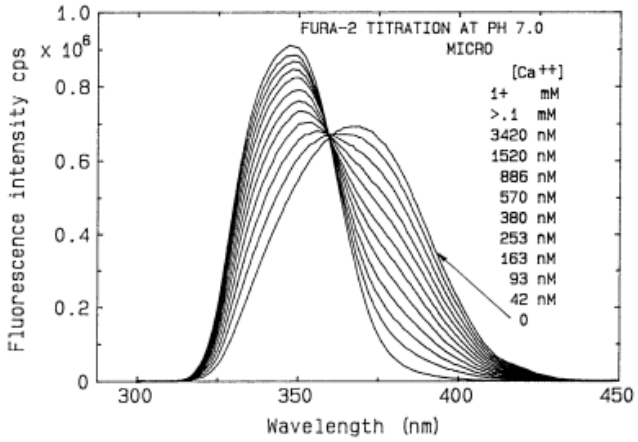


The titration proceeds exactly as before. The excitation ratio is paused between sample dilutions: Click **PAUSE** on the **Acquisition Setup** dialog box after 50 seconds of data acquisition to pause for sample manipulation, then click on **CONTINUE** to resume data acquisition. Select **Status Window** from the **View** menu in the Acquisition Setup dialog box to display a clock timer. Pressing the **space bar** will deposit an event marker (if enabled in the **More** menu under the *General* tab). The ratio values are determined from the stepwise titration curve by taking the average value of each step segment. Subsequent calculations are the same as above.

**Fura-2 Titration with a Microscope-Based System**

When the titration is done in a tissue chamber on a microscope stage, the fluorescence excitation spectra of Fura-2 will differ from those obtained in a cuvette-based system.

The light transmission properties of the optical path of the microscope are responsible for a significant reduction in UV intensity. Therefore, the excitation peak of the Ca-saturated form of the dye will be less than twice as intense as the Ca-free form. Also, the excitation peak position will tend to be shifted towards the longer wavelengths. The extent of this observed shift is strongly dependent on the quality of the UV optics in the microscope objective that is employed. An example of this effect is shown in the figure below. The background was subtracted from all traces prior to display.




The calculations presented in the preceding section will still be valid and correct $K_d$ values may be obtained from this family of curves as well. It is understood, however, that the experimental values to be tabulated for the calculations will be different.

## Using Look-Up Tables

**Concentration Calibration for FURA-2**

In the sample procedure presented below, data from a calcium titration of FURA-2 is used to construct a lookup table (LUT) to relate measured ratio values to actual concentrations. The data is gathered in from a stored file saved following the actual experiment.

1. Select **File/Open,** highlight the dataset of interest, and click **OK**.

2. If multiple curves are present, select the curve of interest.

3. Select **Transform/Concentration Map**. You may need to move the dialog box in order to see the whole curve. Click and drag the title bar of the dialog box to move it.

4. In the dialog box, choose **Ratio to Concentration** then **Edit/Select**.

5. Check the **Capture Value** option at the bottom of the dialog box. If this option is dimmed, make sure that the curve is selected (the name will be highlighted in the legend).

6. Highlight a selected region of the curve using the **Mark Region** icon and clicking and dragging the mouse in the workspace. The selected region will be highlighted, and the average value of the selected data points will be displayed next to the **Capture** button.

7. Click the **Capture** button to place the value in the lookup table.

8. Type the corresponding concentration value into the open cell next to the captured value.

9. Repeat steps 6, 7 and 8 for each value to be entered.

At least two values must be entered to constitute a valid calibration LUT. Each ratio value is the value of the range in the titration curve where data was gathered under constant conditions over a period of seconds to sample the experimental signal and its associated noise. The Y value for each ratio is therefore chosen to represent the mean of the data values within the single-concentration plateau.

After the last value has been entered, save the LUT by clicking on the Save As button and entering a filename for the LUT. Then click on OK. LUT values saved to the database can be recalled, edited and resaved. You can create as many different LUT files as you need.

**Ratio-to-Concentration Transformations**

The following procedures illustrate how ratio data extracted from a raw data file may be transformed into concentrations using the variety of formalisms provided.

1. Open the Lookup Table to be used. Select **Transform/Concentration Map** then the appropriate LUT type and finally click **Edit/Select**. In the Lookup Table dialog box, click **Load**, highlight the filename, and click **OK**. This LUT will be used in all subsequent ratio-to-concentration transformations.

2. Open the dataset containing the curve(s) to be transformed, and select a curve (a checkmark should appear next to its name in the legend).

3. Select **Transform/Concen. Map**. Choose the Ratio to Concentration lookup table. Click Execute.

To view the concentration curve, hide all other curve(s) by selecting them from the legend (press and hold the **Ctrl** key to select more than one), right click on the curve and choose **Hide All**.

Alternatively, the concentration equation can be enabled by selecting the formula in the **Concen. Map** dialog box. Once the transform (lookup table or formula) has been defined, you can close the dialog box by clicking **Close**.

Chapter 17

# Advanced Experiments

FeliX32 is a very powerful program, and many of the basic experiments can be extended for powerful measurement and analysis. For example, either polarization or ratio experiments may be done while ramping the temperature. This chapter is intended to help users make better use of the FeliX32 software.

## Polarization and Anisotropy

The easiest way to perform most polarization experiments is to use the **Timebased Polarization/Anisotropy** experiment. However, there are times when a user may wish to do things themselves.

In all polarization experiments, the first step is to determine the G-Factor. The G-Factor measures the emission channel's efficiency at detecting horizontally polarized light in reference to vertically polarized light. The major sources of instrument polarization are from mirrors and monochromator gratings.

**Estimate of G-Factor**

The G-Factor is given by the equation $G = I_{HV}/I_{HH}$, and is wavelength dependent. In this equation, the first subscript refers to the position of the excitation polarizer, while the second subscript describes the emission polarizer position. PTI systems come with both manual and motorized polarizers. In our systems, vertical polarization is defined as 0° while horizontal polarization is 90°. Manual polarizers must be set by hand, while the position of the motorized polarizers is set in the **Additional Acquisition Setup Controls** dialog. In a system with two emission polarizers, one may be set horizontal and the other set vertical to allow $I_{HV}$ and $I_{HH}$ to be measured at the same time. If a system has only a single emission channel, the two intensities need to be measured in two runs with the emission polarizer reset between the runs.

The G-factor may be measured as a timebased experiment. In this case, the wavelength devices are set to the correct wavelength for the experiment prior to the measurement. This is generally the preferred method to measure the G-factor.

The G-Factor may be measured as a wavelength experiment as well. In this case, an excitation or emission scan is done with the polarizers set as described in the timebased experiment. The advantage of this method is that a number of wavelengths are measured in a single experiment, if there is a need to change wavelengths. The measurement is done once, and the G-factor may be determined from the wavelength traces.

The G-factor is then calculated using the **Configure/Polarization** dialog box.

### As Timebased Experiments

Polarization experiments can be easily measured in timebased mode. The first step is to generate the G-Factor as described above. Then set the Excitation polarizer to the vertical position. If the system has polarizers on two emission channels, set one horizontal and the other to the vertical position. Inside of the **Display Setup** dialog, select **New Derived Trace**. The curve represented as VV (0°) should be in **Source 1**, while the horizontally polarized channel (VH) should be in **Source 2**. Select "Polarization" from the **Function** list box. The G-Factor used in the polarization calculation is the one measured as described earlier. To enter this value click on the **Configure** button.

If the system has only a single emission channel, then two time based experiments need to be done. The excitation polarizer will be set to 0° (vertical) for both runs. One experiment is done with the emission polarizer horizontal, and the other run is done with it set vertical. The **Transform** menu item is used to calculate the polarization. Curve VV is the trace run with the vertical emission polarizer, while Curve VH should be the trace run with the horizontal emission polarizer. Click on **Configure G-Factor** to enter the predetermined value.

### As Temperature Ramp Experiments

Temperature ramp polarization experiments are run in the same fashion as for timebased experiments. In fact, temperature ramping is setup within the timebased acquisition. The only difference is that two emission polarizers are needed, since the temperature ramp experiment may not be reproducible using the same sample on repeated ramps.

**Note.** It is recommended that excitation corrections not be used during polarization experiments due to the nature of the beam-splitter used on the RCQC unit. The lens is biased to vertically oriented light.

## Ratiometric Measurements

In general, the easiest way to perform a ratiometric experiment is to use either the **Excitation Ratio** or **Emission Ratio** dialog. These handle wavelength selection and calculations, leaving the user free to concentrate on the data.

### As Timebased Experiments

Excitation ratios can only be done as two separate runs with the excitation wavelength device set to different wavelengths. Likewise, a system with a single emission channel needs two runs with the emission wavelength device set to the different wavelengths. If two runs are needed, the traces may be ratioed using the **Math/Combine** menu item.

However, the monochromators can slew between two wavelengths in which case only one trace would be needed regardless of excitation or the number of emission monochromators. In this case, or if you have a dual emission channel system, the **Display Setup** dialog allows the ratio to be calculated. Select **New Derived**, the trace number selected into **Source 1** is the numerator, and **Source 2** contains the denominator. From the **Function** list box, select *Source1/Source2*.

**As a Multiple Dye Experiment**

Multiple dyes allow a user to measure several wavelengths on either the emission or excitation in a single experiment. This experiment uses only a single emission channel. To perform the calculations, open the **Display Setup** dialog and select **New Derived Trace**. Select the trace number for the numerator into **Source 1**, and the trace number for the denominator into **Source 2**. Select *Souce1/Source2* from the **Function** list box. Several calculations may be performed if there are other source traces.

**As Temperature Ramp Experiments**

First, select the timebased acquisition and the appropriate hardware configuration. The temperature ramping conditions must be setup in the **More** menu under Temperature Control first. Use the **Display Setup** dialog to cause the ratio to be calculated. Click **New Derived Trace**. Select the trace number into **Source 1** for the numerator, and the trace for the denominator into **Source 2**. Then select *Source1/Source2* in the **Function** list box to calculate the ratio. Close the **Display Setup** dialog, and run the experiment**.**

## Corrected Data

Correction compensates for intensity variations due to either the instrument or the light source. Some corrections are done in real-time, others are done post-acquisition. Corrections compensate for the excitation portion of the instrument (EXCORR) or the Emission side (EMCORR).

The corrections are performed by multiplying or dividing the raw data by a curve generated during the initial system calibration. In addition, the EXCORR correction is based on the actual lamp output, so variations based on the amount of light impinging on the sample can be reduced.

**Excitation Correction (EXCORR)**

This correction is done during sample acquisition, although both the corrected and raw data are available to the user. The excitation correction curve is generated by measuring the actual illuminator output with a photodiode. This lamp emission data is then divided into the raw emission data to obtain an intermediate result. The intermediate result, which is not displayed, is divided by the EXCORR file provided by PTI to generate the final excitation corrected trace.

To use EXCORR, the XCorr icon must be present in the hardware configuration. Double click on this icon, then uncheck the box for Rhodamine Quantum Counter. The **Excitation Correction** check box must be enabled in the dialog from the hardware configuration menu under PHB1 **Configure Corrections**, and the proper correction lookup table must be selected. In most cases, this is the lookup table provided by PTI.

**Note.** The EXCORR curve provided by PTI is fully valid at the 5-nm slit at which it was measured. If narrower slit widths are used, the EXCORR curve may need to be measured again at the slit width required for your measurement. Please call PTI for details.

In addition, **Real Time Correction** must be enabled in the **Additional Acquisition Setup Controls** dialog (opened by clicking the **More** button in an experiment dialog). The gain should be set correctly as well. This is set by clicking the **Reference Source Gain** button to display the **XCorr Gain Control** dialog. Enter the Excitation Wavelength into the edit box, and press the **Goto** button. The excitation wavelength is generally the excitation wavelength that gives the largest emission value (for scans), or the wavelength for the timebased experiment. Move the slider control until the **Signal** measures ~1 volt. Press **Ok** to save the changes.

The program will display the corrected curve, the raw data, and the raw photodiode output upon acquiring data.

During scanning, EXCORR will correct for fluctuations in illumination output due to the lamp power envelope and variations in the arc itself

**Emission Correction (EMCORR)**

The emission correction is much simpler than excitation correction. The data trace, which may already be excitation corrected, is multiplied by the correction file provided by PTI. This correction is intended to compensate in wavelength dependant variations in the system on the emission channel. Each emission channel grating has its own EMCORR file. The EMCORR file is generated by comparison of the emission channel response to the spectrum of a NIST traceable tungsten light.

**Note.** If the grating, PMT voltage, or PMT is changed, the Emission Correction should be re-calibrated for best results. The emission channels will also undergo changes as they age, and the Emission Correction should also be re-evaluated periodically. Please contact PTI for details.

To use the EMCORR file in real-time, the **Emission Correction** check box must be enabled in the dialog from the hardware configuration menu under PHB1 **Configure Corrections**, and the proper correction lookup table must be selected. This lookup table is provided by PTI, generally with the names emcorri (for channel 1) or emcorrii (for channel 2). These must be matched with the proper channel, and are properly configured by PTI personnel during installation. Next, turn on the **Real Time Correction** in the **Additional Acquisition Setup Controls** dialog (opened by clicking the **More** button in an experiment dialog) and selecting **Emission**. The raw and corrected traces will be displayed.

Chapter 18

# Hardware Overview

## Instrument Subsystems

Each PTI fluorescence system is comprised of three subsystems: Illumination, Sample Handling, and Detection. Since these subsystems are shared across product lines, the following information is organized according to the subsystem type, rather than the instrument line.

## Illumination

Your PTI instrument will have one of seven illumination subsystems:

1. The standard illuminator combination is comprised of a compact arc lamp housing coupled to a Model 101M computer-controlled QuadraScopic monochromator. This assembly will be coupled either to a sample compartment (direct attachment) or to a microscope via a fiber optic cable. The monochromator may optionally include a shutter. With various gratings, illumination can be generated from 200 to 1000 nanometers. Continuously variable micrometer- adjusted slits provide bandpass control. When used with the standard 1200 lines/mm grating, the bandpass is 4 nm/mm. Model 102M and 201M monochromators will have different bandpass factors (2 nm/mm for example).

   **Note.** Because the Model 101M monochromator can accommodate up to two light sources, it may have another lamp housing attached in a customized system. For example, a XenoFlash Xenon Flash Lamp may be added for phosphorescence applications. Selection between two sources is via a manual flipping mirror on the entrance side of the monochromator.

2. The **PowerFilter** is comprised of a compact arc lamp housing coupled to a filter-based high-speed dual-wavelength unit (containing a shutter, an IR filter, an optical chopper and two bandpass filters). This assembly is coupled either to a sample compartment or a microscope via a bifurcated fiber optic cable. The illumination produced by the arc lamp first passes through a continuously variable iris, which permits adjustment of the overall illumination intensity. The light then encounters an IR cold mirror that serves to prevent overheating of the optics by reducing the unwanted wavelengths above 550 nm.

The light reflected from the cold mirror passes through a standard broadband UV filter to further reduce stray light, followed by UV neutral density filters for further control of sample illumination (these filters are removed for measurements of pH using BCECF). Light then strikes a rotating chopper disk, which alternately presents open and reflecting segments to the incident beam, passing it through directly to one bandpass filter or redirecting the beam to another bandpass filter.

3. The **DeltaRAM V™** is comprised of a compact arc lamp housing coupled to a patented high-speed random wavelength monochromator. This assembly is either attached directly to a sample compartment, or coupled to a microscope via a fiber optic cable. The monochromator may optionally include a shutter. Wavelength selection is achieved by computer control, and any wavelength can be selected at random within 2 milliseconds or less. With a standard grating, the bandpass is 4 nm/mm.

4. The **DeltaScan X™** is comprised of a compact arc lamp housing coupled to a patented monochromator-based high-speed dual-wavelength unit (containing a shutter, an optical chopper and two computer-controlled monochromators). This assembly is coupled either to a sample compartment or a microscope via a bifurcated fiber optic cable. Alternating wavelength exposure/integration times can be varied from approximately one millisecond to hundreds of seconds, with minimal crosstalk between the two channels. Continuously variable micrometer-adjusted slits provide bandpass control of both monochromators. The high-grade quartz fiber optic bundle provides ease of optical alignment, freedom from vibration and maximum flexibility in laboratory space utilization.

   The illumination produced by the arc lamp first encounters a rotating chopper disk, which alternately presents reflecting and transmitting segments to the incident beam, passing it through directly to the lower monochromator or redirecting the beam to the upper monochromator via a focusing mirror. For maximum throughput with minimal crosstalk, PTI's patented design positions the chopper before the monochromators to ensure that the effective duty cycle is at a maximum, determined by the beam-forming optics within the arc lamp housing, with minimum variation as the monochromator slit (bandpass) settings are changed. The ends of a bifurcated quartz fiber optic cable collect the two separate monochromator outputs.

5. The **Nitrogen Laser** can be coupled to a **Dye Laser** and **Frequency Doubler** for continuous excitation from 235 to 990 nm. The laser attaches to the sample compartment or microscope through a 350 micron diameter optical fiber providing 440 KW peak power at 5 Hz with a pulse width of 1 nanosecond. The range of measurable lifetimes varies from 100 picoseconds to several milliseconds (detector dependent) from concentrations as small as 10 pM.

6. The **NanoFlash** is a thyratron-gated flash lamp capable of measuring fluorescence time-resolved acquisitions at a flash rate of 25 kHz. This assembly is either attached directly to a sample compartment, or coupled to a microscope via a fiber optic cable. The monochromator may optionally include a shutter. Wavelength selection is achieved by computer control (gas dependent). The range of measurable lifetimes varies from 100 picoseconds to ~20 microseconds.

7. The **XenoFlash** is a pulsed light source capable of measuring steady state and phosphorescence time-resolved acquisitions. It is comprised of a compact arc lamp housing coupled to a Model 101M computer-controlled QuadraScopic monochromator. This assembly will be coupled either to a sample compartment (direct attachment) or to a microscope via a fiber optic cable. The monochromator may optionally include a shutter. With various gratings, illumination can be generated from 200 to 1000 nanometers. Continuously variable micrometer- adjusted slits provide bandpass control. When used with the standard 1200 lines/mm grating, the bandpass is 4 nm/mm.

## Sample Handling

Your PTI instrument will have one of two sample handling subsystems:

1. The **Model MP-1 QuadraCentric Sample Compartment** is a versatile, spacious chamber designed to accept up to two excitation and two emission subsystems. It is easily configured for L- or T-format. The MP-1 uses high-grade quartz lenses to focus the excitation beam on the sample and to collect emitted light. It features filter holders on all ports, and mechanical lid-activated shutter(s) on the emission port(s).

   The MP-1 can be fitted with various accessories, including but not limited to a variable-speed microstirrer, a rapid temperature control four-position sample holder that can be rotated under software control, sheet and Glan-Thompson polarizers, microcuvette holders, solid and powdered sample holders, a second detection channel for dual wavelength studies or T-format measurements, and a solid state Peltier Controlled sample heating and cooling cuvette holder. Contact your PTI Sales Representative for more information.

2. A **fluorescence microscope** is the second possible sample handling subsystem. PTI illumination and detection subsystems are compatible with nearly every popular inverted fluorescence microscope on the market today. Please refer to the documentation provided with your microscope for information on its capabilities and use.

## Detection

Your PTI instrument will have one of four detection subsystems:

1. A **Model 814 Analog/Photon-Counting Photomultiplier Detector** is a compact unit that may be attached directly to a sample compartment or fitted to a Model 101M monochromator that is attached to a sample compartment. The 814 may be substituted with a Model 810, which works only in photon counting mode. Also, the Model 101M monochromator may be substituted with a Model 102M (dual-grating) or Model 201M (double monochromator).

2. A **Stroboscopic Detector** has built-in avalanche and stripline detector circuits. It may be attached directly to a sample compartment or fitted to a Model 101M monochromator that is attached to a sample compartment. The detector is used for fluorescence time-resolved acquisition systems from the TimeMaster lineup or instruments with optional Nitrogen laser or NanoFlash illumination sources.

3. A **VCI Detector** is a compact unit that may be attached directly to a sample compartment or fitted to a Model 101M monochromator that is attached to a sample compartment. The VCI detector is used for phosphorescence time-resolved acquisition systems from the TimeMaster lineup or instruments with optional Nitrogen laser or XenoFlash illumination source.

**Note.** As part of a detection subsystem, a monochromator can accommodate up to two detection devices, and so it may have another unit attached. For example, a gated detector may be added for phosphorescence applications. Selection between two detectors is made with a manual flipping mirror on the exit side of the monochromator.

4. A **Model D-104B Microscope Photometer** can be fitted to virtually any microscope through a camera port. It features a bilateral adjustable iris to control the field of view, a viewing eyepiece, and either one or two Model 810 photon-counting PMT detector(s), depending on the application. The 810 detector(s) may also be substituted with Model 814 analog/photon-counting detector(s). Stroboscopic and VCI detectors may also be substituted. A video edge detector can be attached to the photometer and the signal(s) from this fed to analog channels on the BryteBox.

PTI fluorescence systems are designed to accept up to two digital (photon-counting) data channels and up to six analog data channels. The analog channels are provided so that you can gather data from other devices used in experiments concurrently with the digital information. Analog accessories that can be interfaced with your system may include muscle strip measurement units, patch clamps, stimulus probes, and more. In addition, your PTI system can be programmed to activate external devices with a trigger pulse. Refer to the software section for information on interfacing these devices with your system. An intensified or cooled CCD camera may also be used as a detection subsystem for imaging applications. FeliX32 software is not used for image acquisition.

Chapter 19

# Illumination Subsystems

The use and maintenance of the components that make up some of the various illumination subsystems is broken out and detailed in this section. For light sources other than arc lamps, please refer to appropriate hardware manuals.

## Arc Lamp Housing

Common to virtually all illumination subsystems is a compact arc lamp housing. It is designed to accommodate a variety of Xenon, Mercury and Mercury-Xenon high-pressure short arc lamps with power ratings of 75 to 200 watts. The housing features an f/4.5 elliptical reflector that collects and focuses 67% of the emitted light. The unit is completely sealed and requires no ozone venting, and no water-cooling is required for 75-watt lamps.

**Compact Arc Lamps**

High pressure gas discharge lamps having an arc length, which is small compared with the size of the electrodes, are referred to as short arc or compact arc lamps. These lamps have the highest luminance and radiance of any continuously operating light source and are the closest approach to a true point source.

Xenon compact arc lamps are filled with several atmospheres of xenon gas. They reach 100% of final output within ten minutes or less of starting. The spectrum is continuous in the visible range and extends far into the ultraviolet. A Xenon lamp exhibits strong lines in the near infrared between 800 and 1000 nm and some weak lines in the blue portion of the spectrum.

A Mercury-Xenon lamp contains a specific amount of mercury and a small amount of xenon added at a pressure exceeding one atmosphere. The xenon is necessary to facilitate starting and to sustain the arc until the mercury is fully vaporized; it also reduces the warm-up period. Normal warm-up time is 10 to 15 minutes. The output in the visible range consists of the mercury lines superimposed on the xenon continuum.

Mercury-xenon lamps are excellent excitation sources for emission scans and fluorescence lifetime measurements because of the intensity of the mercury lines. Mercury-xenon lamps are not recommended in experiments where the excitation wavelength is scanned.

## Lamp Stability

Short-term stability is measured over seconds, while long term stability is measured over minutes, hours, or even days.

Arc wander, flare, and flutter affect short-term stability. Arc wander is the movement of the attachment point of the arc on the cathode surface. Typically, the arc moves around the conical cathode tip in a circular fashion, taking several seconds to move a full circle. Arc flare refers to the momentary change in brightness as the arc moves to an area on the cathode having a preferential emissive quality over the previous attachment point. Arc flutter is the rapid side-to-side displacement of the arc column as it is buffeted by convection currents in the xenon gas, which are caused as the gas is heated by the arc, and cooled by the envelope walls.

Arc wander and flare can sometimes be reduced by a slight decrease in the operating current. For example, a 75 watt xenon lamp rated at 5.4 amps may be operated at 4.5 amps for the first one or two minutes of operation, after which the current should be brought up to the specified normal operating level. Alternatively, operating at 6.0 amps for the first few minutes may lead to more stable operation. Both methods are used to establish a preferred location for the arc on the surface of the electrodes.

## Lamp Life

The useful life of compact arc lamps is determined primarily by the decrease of luminous flux caused by the deposition of evaporated electrode material on the inner wall of the envelope. Frequent ignition accelerates electrode wear and hastens the blackening of the envelope. The average life of the 75-watt xenon lamp ranges between approximately 500 and 700 hours. Some lamps have lasted as long as 1,000 hours or more.

As the lamp ages, the operating voltage will increase. Lamp current should be decreased to maintain output until the minimum operating current is reached. At this time the lamp should be replaced.

The end of the lamp life is the point at which the UV output has decreased by approximately 25%, the arc instability has increased beyond 10%, or the lamp has ceased to operate under specified conditions. Lamps should be replaced when the average lamp life has been exceeded by 25%.

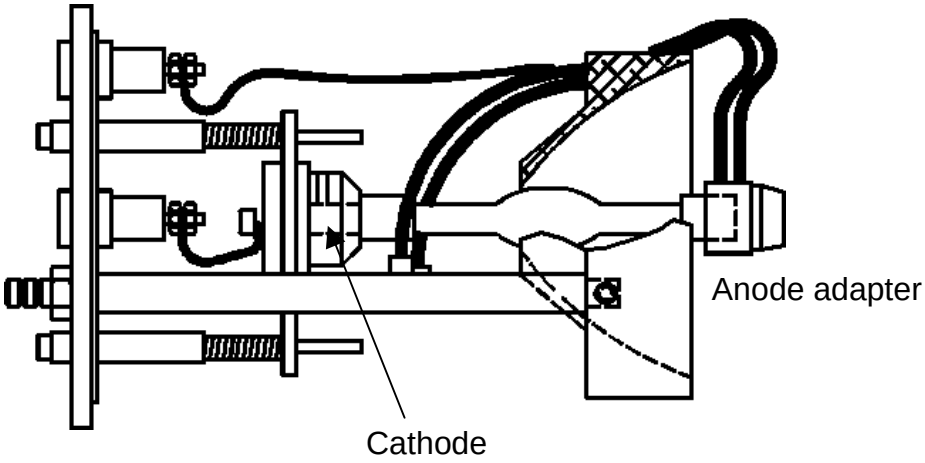
## Arc lamp installation/replacement

**Important!** PTI systems are always shipped with the lamp removed from the lamp housing. The lamp must be re-installed prior to system use. Remove the lamp from the housing if the system is moved or shipped to avoid the possibility of explosion. Tie the anode adapter back onto the threaded mounting posts so that it does not move around and damage the reflector. Make sure the there are no kinks in the rubber hoses when the anode adapter is tied back.

**Warning!** Before servicing the lamp housing, be sure to disconnect the electric lines and completely drain the cooling system where applicable. Pressurized air applied at the water inlet is recommended for removal of any remaining water. Make certain the lamp is at room temperature. Also remember to wear eye protection when working around arc lamps.

1. Remove the LPS-221B igniter from atop the lamp housing by unfastening the large slotted screw.

2. Remove the six phillips-head screws, four on the back and two on the top plate of the lamp housing, and slide the lamp assembly out. (On DeltaScan illuminators, the lamp housing access plate must be removed first.)




3. Loosen the anode adapter set screw and slide the adapter off the lamp.

**Important!** Note the orientation of the anode cooling tubes and the evacuation bubble on the lamp. They are both aligned together at a 45° angle, which corresponds to the insulating panel inside the corner of the lamp housing.

4. Loosen the two cathode adapter stand set screws and remove the lamp. There is only one cathode adapter set screw for 75 W lamps, two cathode adapter stand set screws for 150 W lamps. Be sure to discard the old lamp properly and safely.

5. Insert the lamp cathode (–) in the cathode adapter and tighten the two setscrews. Be sure to orient the evacuation bubble the same way as the old lamp.

**Important!** Be careful when inserting a 150 W lamp. They can be easily oriented backwards by mistake.

6. Slip the anode adapter over the lamp anode (+) and tighten the setscrew.

**Important!** Be sure to orient the cooling tubes at the same angle as they were originally.

**Warning!** Compact arc lamps contain highly pressurized gas, and present an explosion hazard, even when cold. Wear face protection, such as a welder's helmet, whenever handling lamps.

**Caution!** The anode adapter must not put any mechanical stress on the lamp. It may be necessary to bend the wire to the adapter to relieve any stress on the lamp.

7. Be sure all water lines are snug. (75-watt Xenon lamps do not require cooling, so the water lines may be omitted.) Connect the water lines to the supply, start water flow and check all internal connections for leaks.

8. Insert the lamp assembly into the housing. Note that the anode cooling tubes are oriented toward the corner of the lamp housing having an insulating panel. Tighten the six screws securely. (On DeltaScan illuminators, replace the lamp housing access plate.)

**Lamp Alignment**

Lamp alignment should always be performed after lamp servicing or replacement.

**Note.** User realignment of any optical components other than the lamp housing may void the Warranty.

**Warning!** Use protective eyewear to prevent the possibility of permanent eye damage. A welder's helmet is recommended when focusing the lamp.

The lamp housing will be part of some of the possible illumination subsystems, and certain steps in the procedure will vary slightly. However, the overall lamp alignment procedure is the same:

1. Obtain access to the illuminator entrance to observe the focused spot of light generated by the lamp.

   The standard, *DeltaRAM V™*, and *XenoFlash* illuminators: Remove the lamp adapter access plate.

   *PowerFilter* illuminators: Remove the illuminator lid; remove the excitation filters; power on the chopper and operate it manually at approximately 50 Hz.

   *DeltaScan* illuminators: Remove the illuminator lid and the lamp access plate; power on the chopper and operate it manually at approximately 50 Hz.

2. Ignite the lamp and allow it to warm up for at least 5 minutes. Adjust the lamp for a spot that is properly positioned and the smallest possible size. Using welder's goggles to observe the spot, adjust the arc lamp housing as necessary by progressively making ±1/4-turns of each of the three focus adjustment knobs.

**Warning!** Do not turn one adjustment screw without doing likewise equally to the other two, and do not make turns larger than 1/4-turn. Lamp failure could occur as a result of undue stress.

Standard, DeltaRAM V™, and XenoFlash illuminators: The spot should be 3-4 mm diameter centered on the entrance slit jaws.

PowerFilter illuminators: The spot should be 3-4 mm diameter centered on each of the two fiber optic ferrules. (If after centering the spot on one ferrule the spot is no longer centered on the other, then the chopper head may need adjustment. ***Contact PTI for assistance before making any other adjustments.***)

DeltaScan illuminators: The spot should be 3-4 mm diameter centered on the lower monochromator slit jaws, and 4-5 mm diameter centered on the upper monochromator slit jaws. (If after centering the spot on the lower slit jaws the spot is no longer centered on the upper slit jaws, then the upper focusing mirror may need adjustment. ***Contact PTI for assistance before making any other adjustments.***)

3. After closing all access plates and housing lids, and replacing filters as necessary, power up and run the instrument to perform illuminator maximization.

**Illuminator Maximization**

Illuminator maximization is most effectively accomplished by observing the Raman band of water over time. Set the emission wavelength to the peak and acquire data using the following instrument parameters:

| | |
|---|---|
| Excitation | 350 nm |
| Emission | 397 nm |
| Counts/sec | 20 point/second |
| Duration | 300 sec |
| View Window | 30 sec |

While acquiring data, progressively turn each arc lamp housing focus adjustment knob 1/4-turn to maximize the signal rate.

**Warning!** Do not turn one adjustment screw without doing likewise equally to the other two, and do not make turns larger than 1/4-turn. Lamp failure could occur as a result of undue stress.

When the signal rate begins to decrease, make progressive 1/16-turns in the opposite direction to return the signal to maximum.

When you are satisfied with the operation of a new lamp, it is helpful to enter the date that the lamp was changed in the Arc Lamp dialog box in the Hardware Configuration.

## Dual Wavelength Optics

Unlike the standard monochromator and DeltaRAM illuminators, witch use a single monochromator that slews between two positions, the DeltaScan and PowerFilter illuminators generate alternating wavelengths of light through the use of optical components, most notably an optical chopper. The following section deals with the maintenance of these systems.

**Channel Balancing**

**Note.** Most of the adjustments detailed in this section require that eye protection, such as welder's black goggles, be worn. Do not use ordinary UV goggles when viewing focused illumination.

The following steps should be taken to correct channel imbalance:

1. Adjust or replace the arc lamp.
2. Adjust the chopper phase.
3. Check the light focused the upper monochromator slits.
4. Adjust the height aperture.
5. Adjust the slits.
6. Check the fiber optic bundle.

The steps listed above are detailed in the following text.

1. Adjust or replace the arc lamp.

After an arc lamp is used in excess of 500 hours, it can become unstable. This instability could be affecting the balance of the C1 and C2 channels. After the lamp is replaced, it must be aligned using the following procedure.

Set up a Timebased experiment and position the excitation monochromators on the peak maximum for the $R_{min}$ solution of Fura-2 being used. Use a 10 data point per second time resolution and a scan duration of 1000 seconds with a time window of 100 seconds. In the **Display Setup** dialog box, establish a window for the signals from each monochromator. For microscope-based systems, keep the slit settings at 2 nm bandpass and the photometer aperture at 2 mm. For cuvette-based systems, keep all slit settings at 5 nm bandpass and the emission wavelength at 510 nm.

After starting the experiment, the respective count rates for the two monochromator channels will appear on the screen. While observing the channel for the top monochromator, turn each of the three focusing knobs on the arc lamp housing either clockwise or counterclockwise.

**Warning!** Do not turn one adjustment screw without doing likewise equally to the other two, and do not make turns larger than 1/4-turn. Lamp failure could occur as a result of undue stress.

If the peak intensity drops, reverse directions on the focusing knobs. The intensity of the signal should increase with each move. When the count rate begins to fall, the optimal focus has been passed. Reverse directions on the focusing knobs to reach the maximum signal.

Now observe the relationship between the two channels. If they are within 10% of one another, then the instrument is properly adjusted. If not, then only one of the three focusing knobs on the back of the arc lamp housing may have to be adjusted to better balance the signals. Remember not to turn any one knob more than 1/4-turn. Turn the upper left or upper right knob slightly (less than 1/8-turn) and observe the relationship between the two signals. If they are still not within 10% of each other, then refer to other diagnostics within this section.

2. Adjust the chopper phase.

If the phase of the chopper is significantly out of adjustment, crosstalk between the two channels can occur which will cause channel imbalance. To adjust the chopper phase, refer to the next section.

3. Check the light focused on the upper monochromator slits

Using welder's black goggles only, observe the light spot on the upper monochromator slits. A small bright spot should be centered on the slits. If the spot is not focused and centered, the chopper and/or upper focusing mirror may need to be adjusted. Contact PTI for assistance before making any other adjustments.

4. Adjust the slit height aperture.

**Note.** Use the other techniques outlined earlier in this section before adjusting the slit height.

At each of the exit slits of the two monochromators in the illuminator is a height aperture adjustment slider. (This slider is a long, thin metal rectangle with a V opening.) As the slider is moved from sided to side, it will change the height of the illumination beam leaving the monochromator. As the height of the image is changed, the subsequent intensity of the light leaving that monochromator will be changed.

Use the timebased experiment outlined in the arc lamp alignment procedure, observe which channel has the higher count rate. On the corresponding monochromator, move the slider until the count rate appears to be equal to that of the other channel.

5. Adjust the monochromator slits.

**Note.** Use the other techniques outlined earlier in this section before adjusting the slit widths.

An imbalance between the two channels may be compensated by setting a different slit width on one of the excitation monochromators. Use the timebased experiment outlined in the arc lamp alignment procedure and observe which channel has the higher count rate. Close the entrance and exit slits to the corresponding monochromator equal amounts until the two channels are equal.

6. Check the fiber optic cable.

For proper operation, the fiber optic cable ends must be seated properly in their respective receptors on the illuminator. Ensure that each fiber optic cable is pushed all the way in to the receptor. Further, the ends of the fiber cable have rectangular optical grid networks. Ensure that the rectangular grids are oriented parallel to the edges of the slit jaws on the monochromators.

The opposite end of the fiber optic cable connecting to either the microscope or the sample chamber must also be properly seated. On microscope-based systems, the fiber cable has an optical coupler composed of lenses. The fiber must be inserted into the coupler as far possible. On cuvette-based systems, the optical fiber should be seated so that it comes in contact with the retaining setscrew in the flange barrel on the sample chamber.

**Note.** If channel imbalance is still evident after attempting the adjustments as described above, call PTI for assistance.

### Chopper Phase

Data acquisition follows the position of the rotating chopper disk, which alternates the channel delivering excitation illumination to the sample. The voltage level of the chopper's trigger output signal indicates which source channel is currently selected, and the transitions (edges) produce interrupts in the computer at each half-cycle. The timing for data gathering is always synchronized with the start of each chopper half-cycle and the signal's level provides an absolute calibration of which half-cycle is in progress.

Following an interrupt from the chopper indicating that the source illumination has changed and a new data measurement should be started, a gate signal simultaneously enables counting at each of the signal inputs for a precisely defined number of microseconds. Completion of the gate interval stops the data counting, and the accumulated count values are then read from the interface board and stored in the

computer's memory. Data accumulation remains stopped until the chopper advances to the next source channel, as signified by the next chopper interrupt.

The relative timing between the incident illumination and the position of the data-acquisition gate windows is adjusted by means of the PHASE control (front panel knob) on the Chopper Controller. The chopper phase advances or retards the trigger output waveform with respect to the actual alternating illumination passed by the chopper.

The system's internal timing protocol ensures that no measurable crosstalk will be present in the data when the phase is correctly set. Use the following procedure to check and set the chopper phase:

1. Set the system for timebased data collection, display both excitation channels, a data rate of 20 points/second for a total of 500 seconds with a display window of 20 seconds, so that the behavior of the signals can easily be tracked as the instrument is adjusted.

2. The two excitation wavelengths should be adjusted for different strength signals on the two channels; both signal levels should be appreciably above the no-light background level.

3. Start data acquisition. Change the phase setting on the Chopper Controller, and observe how the two signals respond. As the phase is moved through its adjustment range, the two signals can be made to separate, draw together and even invert in respective intensity level.

4. Set the phase such that the channel 2 signal appears maximized. Remove all light from channel 1. For the DeltaScan, block the exit aperture of the upper monochromator with the slider. For the PowerFilter, remove the Channel 1 fiber from the illuminator.

5. If blocking the channel 1 signal changes the other signal, however slightly, this is evidence of crosstalk and signifies that the phase is not adjusted properly. When the phase is correctly adjusted, blocking and unblocking one signal should have no observable effect on the intensity of the other signal.

As various phase settings are explored, you may notice that the best results are obtained over a range of phase angles, perhaps up to several degrees in extent, rather than at a single sharply defined value. This is because the data read interval is restricted to about 85% of each chopper half-cycle to ensure that data is never taken during those transitional parts of the chopper cycle when illumination from both sources is falling on the sample.

Note that the phase setting is not dependent on the data rate. Once the phase is properly set, any data rate will function properly.

## Monochromators

PTI's Model 101M monochromator is an f/4 0.2-meter Czerny-Turner configuration and is used for both excitation and emission wavelength selection. When the monochromator is fitted with the standard 1200 lines/mm grating, the bandpass is 4 nm/mm, and the display dial reads the actual wavelength. The entrance and exit slits are continuously adjustable from 0 to 6 mm with micrometers. Each turn of the micrometer is equal to 2 nm in bandpass. If stray light is a concern, the Model 121M Double Monochromator is a solution. It can be used for excitation and/or emission.

The DeltaRam monochromator is used for excitation wavelength selection and is connected to the system with a light guide. The bandwidth adjustment is performed with a micrometer where each turn of the micrometer represents 4 nm bandpass.

The Acton monochromator is an f/4 0.2-meter Czerny-Turner configuration with motorized grating selection, shutters, and exit port selection. It provides a flat-field output suitable for photodiode array or CCD arrays.

### Grating Selection

PTI has exercised great care in the choice of monochromator gratings. The standard grating(s) supplied are optimized for use in the UV region for the excitation monochromator (blazed at 300 nm) and the visible for the emission monochromator (blazed at 400 nm).

If very broad wavelength ranges are required which are beyond the range of the standard grating, PTI's Model 102M monochromator has two different gratings on a rotating turret. To upgrade from the Model 101M to the Model 102M, your instrument must be returned to the factory for installation of the double-grating turret. In the DeltaScan double-monochromator illuminator, only one monochromator can have a double-grating turret.

### General Grating Properties

Maximum throughput generally increases as the number of grooves per millimeter (lines/mm) decreases. Optimum wavelength resolution always increases as lines/mm increases, i.e., reciprocal linear dispersion decreases. Standard ruled gratings usually offer the highest efficiency throughput (sometimes over 80%) of any grating when used near their blaze wavelength. However, they are prone to imperfections such as ghosts, which arise from periodic miss-rulings. The ghosts may cause this type of grating to produce stray light. If this problem exists, it can often be remedied using bandpass or cutoff filters.

Holographic gratings offer very low stray light levels because of fewer imperfections in their manufacture. Therefore, they may be more suited to applications that are very susceptible to stray light interference. Holographic gratings generally have lower throughput efficiency (typically 20%) but it is usually relatively constant over its complete useful range. They also tend to introduce more polarization anomalies than ruled gratings. This should be considered when making polarization/ anisotropy measurements. Polarization effects can be practically eliminated by passing the illumination beam through a depolarizer, certain types of diffusers, and fiber optics.

**Determining Wavelength Bandpass**

The bandpass is the range of wavelengths that the monochromator emits about a central wavelength setting. This depends on the grating lines/mm, slit widths, and somewhat upon the wavelength. For most applications the variation with wavelength can be ignored and the bandpass (BP) is simply the product of the slit width (W) times the reciprocal linear dispersion (Rld):

$$Rld = 10^6/(n \times L \times F) \quad \text{and} \quad BP = Rld \times W$$

where:
Rld = Reciprocal Linear Dispersion (nm/mm)
BP = Bandpass (nm)
W = Slit Width (mm)
L = Grating (lines/mm)
F = Focal Length of Monochromator (200 mm)
n = Order Integer (1,2,3, ... ; use n=1 for highest throughput)

Therefore, the calculated reciprocal linear dispersion values for the most common grating types (lines/mm) are 2 nm/mm at 2400 lines/mm, 4 nm/mm at 1200 lines/mm, 8 nm/mm at 600 lines/mm, etc… When the bandpass must be precisely known, refer to the article Optimizing Grating Based Systems by J.M. Lerner and A. Thevenon in *Lasers and Applications*, Jan. 1984.

The optimum resolution of the monochromators is, in practical terms, the minimum useful bandpass. This depends on the dispersion, slit width, and optical image quality. The resolution using 2400 lines/mm gratings is approximately 0.25 nm (slit width = 0.125 mm). The optimum resolution at 1200 lines/mm is twice as large, and so on, for other gratings.

**Setting Wavelength Bandpass**

The bandpass is set by adjusting the slit width. When increased light intensity is required, the slits should be opened wider. This is only appropriate if wavelength resolution can be sacrificed.

When optimum and true bandpass readings are needed, both the entrance and exit slits of a particular monochromator must have the same setting. This will also reduce stray light. Note that the slit height adjustment sliders (if present) should be fully opened when maximum throughput is required. However, partly closing them can sometimes help to increase resolution.

At zero order, the grating acts like an ordinary mirror and white light exits from the monochromator. Zero order is selected by specifying a wavelength of 0 nm. The zero order setting of a monochromator is useful when broadband excitation is required for maximum light throughput. Setting a monochromator to 0 nm is also a quick way to confirm that it is calibrated.

Chapter 20

# Sample Handling/Detection Subsystems

## Model MP-1 QuadraCentric Sample Compartment

The Model MP-1 Sample Compartment uses quartz lenses for both collection and emission optical paths, is very simple in its design, and can be easily aligned by the user. It also includes the variable temperature sample holder for a single cuvette as well as the sample stirrer feature.

The sample compartment is fitted with filter holders that accept any standard 1- or 2-inch bandpass, order sorting or light attenuating filters. They are located on the interior sample compartment walls at the excitation/emission port openings. A lid-activated shutter is included to protect the sensitive PMT detector from excessive light.

PTI's photomultiplier detector housings may be attached directly to the sample compartment. Using bandpass filters, one or two fixed emission wavelengths may be detected. The same photomultiplier housing(s) may be attached to monochromator(s) for variable/scanning wavelength detection.

The standard sample holder is designed to accommodate 1-cm path length cuvettes and has provision for sample stirring and for variable temperature measurements. The stirrer utilizes a small, variable speed DC motor, which spins a magnet below the sample cuvette. If a small magnet bar is placed on the bottom of the cuvette, it will rotate at a speed determined by the speed of the spinning motor. The excitation beam is located in the center of the cuvette and is sufficiently high that the stirrer bar is not illuminated and will not scatter energy, which could interfere with the normal measurement process. The speed is set by a control on the front panel of the Motor Driver Box, or by a small, separate power supply/controller.

A small micro-cuvette adapter/holder is also available and can be used when a smaller sample volume is required. The size of the excitation beam passing through the sample is sufficiently small to accommodate this micro cell, with little loss of overall sensitivity.

Variable temperature operation can be accomplished by flowing heated or chilled fluid through the sample holder, or by using a rapid temperature control Peltier device. The circulator/bath is not normally provided but is optionally available. Any small heater/chiller/circulator can be used over the temperature range of -30°C to +100°C. Dry nitrogen may have to be blown over the sample cuvette in order to minimize condensation at low temperatures in a humid environment.

The sample compartment may be fitted with a four position cuvette turret and/or polarizers. The sample turret holds up to four standard cuvettes and may be heated and/or cooled by an optional circulating liquid bath. It is under complete computer control and may be positioned manually (through the keyboard) or automatically during a programmed experimental procedure.

For polarization and anisotropy measurements, polarizers are fitted directly adjacent to the wall of the sample compartment. Each manual polarizer has detents for 0, 45, 54.75, and 90 degrees.

## Model D-104B Microscope Photometer

Illumination is delivered via a quartz fiber optic bundle to the epi-fluorescence microscope via the epi-illumination port. Fluorescence emission is collected by the microscope optics and directed to the D-104B/C Microscope Photometer, which is attached directly to the side camera port of the microscope.

In the photometer, the collected fluorescence first passes through a bilateral, continuously variable aperture. Four control knobs manually adjust the region of interest (ROI), which represents that portion of the field of view from which fluorescence emission will be measured. In this way, single cells or regions of cells can be selected for analysis.

Following the ROI aperture, the light encounters a movable mirror. In the View position, this mirror redirects the emission beam up to a parfocal eyepiece for viewing by the user. In this mode, the user may directly observe the emission image to adjust the ROI. In the Measure position, the mirror is removed from the optical path, and, in a single-channel photometer, the emission light then passes through a 1-inch bandpass filter holder and finally strikes the photon-counting photomultiplier tube in the detector housing.

In a simultaneous dual-channel photometer, the light encounters a dichroic cube with two barrier emission filters. The dichroic cube assembly splits the fluorescence light into two different wavelength regions and delivers them to two separate detectors. Dichroic cubes are available from PTI for Indo-1 and SNARF.

**Filters and Dichroics**

Selection of filters is critical to the performance of your microscope-based system. The filters that come with your system have been carefully selected after rigorous testing to deliver the best possible results for the study of ratio fluorescence probes such as Fura-2. They provide the mapping of $R_{min}$ and $R_{max}$ values over the widest dynamic range. Filters not provided by PTI may also be used. However, they may not possess the same stray light or bandpass characteristics and hence may not provide the same results.

For single-channel detection, a 1-inch filter holder is provided directly before the PMT housing for an optional emission barrier filter. Note that an emission barrier filter is not required in this holder if one is used in the microscope dichroic cube assembly directly beneath the sample. For dual-channel detection, an Indo-1 dichroic cube assembly may be installed, which comprises a dichroic mirror (455 nm), and two bandpass filters (485/10 and 405/10 nm).

Selection of your microscope dichroic cube is also very important. For any single- or dual-excitation wavelength probe, the microscope dichroic must reflect the excitation wavelengths required for illumination, and transmit the fluorescence emission to be observed. Further, an appropriate emission barrier filter must be installed either in the microscope or the photometer.

For Fura-2 studies, a microscope dichroic cube with 400 nm dichroic mirror and a 510/20 nm emission barrier filter is recommended. Indo-1 studies require the dual-channel detector and microscope dichroic cube with a 380 nm dichroic mirror. No emission filters are required in the microscope dichroic cube. Consult your PTI Sales Representative for questions and information about other available filter sets and dichroic cube assemblies.

## Photomultiplier Detection Systems

The Model 814 PMT Photon-Counting Detector can be installed directly on a sample compartment, a monochromator, or a microscope photometer. Photon-counting PMT detection was chosen as the standard detection technique because its high sensitivity and fast time resolution make it ideal for rapid kinetics observation and low-level fluorescence measurements. Further, this system has a dynamic range of five orders of magnitude, and requires no high-voltage adjustments or zeroing associated with other detection technologies.

If maximum detection sensitivity is not required, analog detection may be implemented with the Model 814 PMT Detection System. Since both the high voltage and the gain are adjustable, an analog system can have a dynamic range of 6-7 orders of magnitude. The unit can be switched between digital (photon counting) and analog detection. In analog detection mode, the Model 814 voltage display can be switched to provide a signal level reading.

**Replacing the Photomultiplier Tube (PMT)**

**Caution!** To avoid possible damage to the sensitive photomultiplier tube, it is suggested that the tube be kept in darkness as much as possible until it is installed in its socket and the housing is closed. It is best to work in a darkened room, as a light shock will increase the background count of a PMT.

Turn the power switch off and disconnect the power supply and signal lines.

1. Loosen completely, but do not remove the four semi-captive screws from the housing cover and take the lid off.

2. Remove the PMT shroud and carefully pull the tube downward out of its socket.

3. Plug the new tube into the socket.

4. Replace the shroud and close the housing lid tightly. Check that the connectors for the cables that run from the PC board to the housing lid are secure before closing the unit.

5. Reconnect the power supply and signal lines.

**Caution!** To avoid possible damage to the sensitive photomultiplier tube, it is suggested that the tube be kept in darkness as much as possible until it is installed in its socket and the housing is closed.

## Other PTI Systems

Information about other Photon Technology systems can be found in appropriate hardware manuals.

Chapter 21

# Troubleshooting

This chapter describes simple troubleshooting procedures for PTI steady state systems. For other systems, please refer to appropriate hardware manuals. Before calling PTI for service, please read through this section and follow the suggestions, as applicable for your hardware configuration.

## Lamp Severely Out of Focus

A lamp severely out of focus can cause loss of fluorescence intensity and a distortion of the spectrum. On a microscope system, a spectrum may be seen that is entirely due to stray light ("auto-fluorescence").

1. Check the focus of the lamp on the entrance slit.

**Note.** This requires welder's grade 5 (or higher) goggles to protect your eyes against intense UV light (Footnote: welder's grade 5 refers to the attenuation of the UV light. The amount of visible light transmitted by such goggles is not specified and may still be too intense to look at).

2. Check the quality of the image on the entrance focusing mirror in the excitation monochromator (standard monochromator, DeltaRAM or DeltaRAM V). You should see a well defined "doughnut" of light that fills 80 – 100 % of the horizontal diameter of the mirror and a hole in the doughnut that is about 20 % of the diameter. The doughnut hole should be circular and reasonably sharp. You may even be able to see one or two radial lines, which are the shadow of the anode cooler and the ignition wire.

   If the doughnut hole is very different than the above description or very misshapen (even showing more than one shadow area) then slightly move each of the lamp adjustment thumbscrews no more than ± 90° while observing the entrance mirror for improvement in the image. Often an adjustment of only 10° can dramatically alter the shape of the image.

   If the doughnut hole is too large, then clockwise adjustments of the thumbscrews usually improves the hole's appearance. If the hole is non-existent and there is just a (circular) patch of light in the mirror, then counterclockwise adjustments of the thumbscrews usually bring about a hole. In this case the hole may start small, just a millimeter in diameter when first seen. Adjust each thumbscrew in turn to gradually enlarge the hole.

   Viewing the image is best done at wide slits, 5 nm or more. The image should be centered. Once an optimum shape is seen, narrow the slits to 1 nm. The whole image

should still be seen, although dimmer. If either side of the image is cut off, then the reflector may be out of alignment.

3. Once the lamp is focused by the above method (coarse adjustment), then adjust the lamp focus while observing a fluorescence signal intensity.

## Lamp Will Not Ignite

Turn off power switch on front panel of LPS-220B power supply.

1. Remove the AC cord from both the wall plug and the rear panel.
2. Check all electrical connections.
3. Check the Line (Mains) Fuse for visual defects and/or test with an ohmmeter. Replace as necessary (see below "Replacing The Fuses").
4. Check the condition of the lamp and replace as necessary.
5. Reconnect the LPS-220B AC cord to both the wall plug and the rear panel.
6. Turn on power switch on front panel.
7. Verify the volts/watts/amps settings as specified in the Operation:
8. If the lamp still fails to ignite, call PTI for assistance.

**If the front panel LCD display is blank while power is on:**

1. Turn off power switch on front panel.
2. Remove the LPS-220B AC cord from both the wall plug and the rear panel.
3. Check the Overload Protection Fuse for visual defects and/or test with an ohm-meter. Replace as necessary (see below "Replacing The Fuses").
4. Reconnect the LPS-220B AC cord to both the wall plug and the rear panel.
5. Turn on power switch on front panel.
6. If the LCD display remains blank, call PTI for assistance.

***Warning!*** Any fuse that fails repeatedly is potentially indicating a problem of a serious nature. In the event that a fuse fails shortly after, or upon replacement, contact PTI for assistance.

## Replacing The Fuses

**Line (Mains) Fuse:**

1. Turn off power then remove the AC cord from the wall plug and also from the rear panel.
2. Slide the plastic fuse cover to the left to expose the fuse and its removal lever.
3. Pry the lever labeled “FUSE PULL” outward and to the left to remove the fuse.
4. Move the fuse lever back into place **before** replacing the fuse.
5. Replace with the appropriate fuse from the following table:

| AC Voltage | Fuse Specification |
|---|---|
| 105 - 132 (110) VAC | 3AB, 4 amps, slow blow |
| 210 - 264 (220) VAC | 3AB, 2 amps, slow blow |

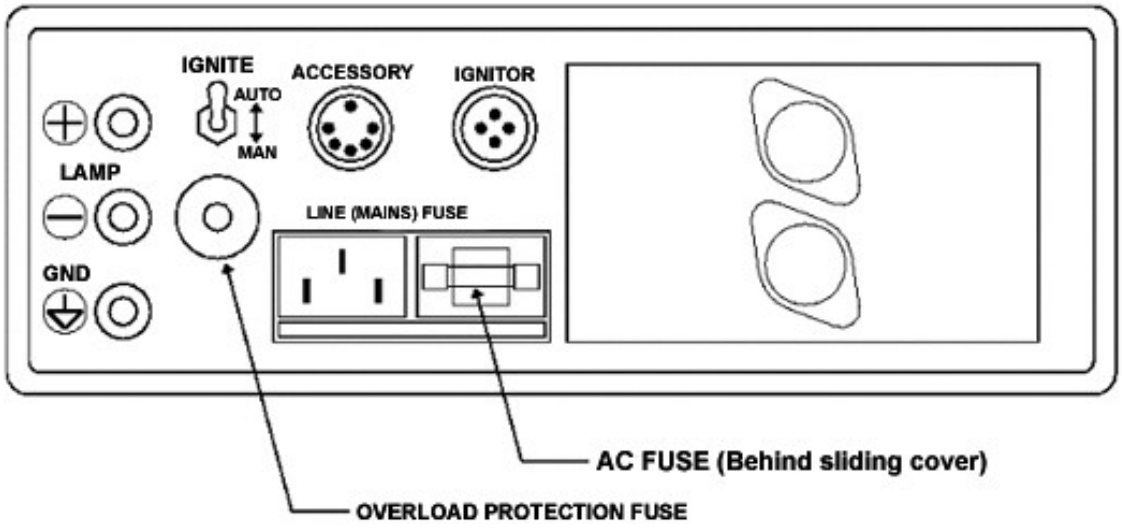
6. Slide the plastic fuse cover to the right and reconnect the AC cord.

Please refer to the diagram on the following page.

**Overload Protection Fuse:**

1. Turn off power then remove the AC cord from the wall plug.
2. Gently push black fuse holder in, while turning counter-clockwise.
3. Remove the fuse.
4. Replace with 10 amp 250V fuse.
5. Push fuse holder in and rotate clockwise until seated.
6. Reconnect the AC cord.

Please refer to the diagram on the following page.




## Lamp Will Still Not Ignite

1. Check the position of the Igniter switch. Older power supplies have an Auto setting. If the switch is set to the Auto position, automatic ignition attempts will cease after 20 to 30 seconds and will not be attempted again until the power supply is turned off. The supply must remain off for at least 30 seconds (one minute is recommended) before being turned back on. Ignition may be attempted again immediately by setting the Igniter switch to Manual, then pressing and holding the Start button. PTI recommends leaving the Ignition switch set to Manual.

2. Check for ignition attempts. When the Ignite button is pressed, a series of audible clicks will be heard as the lamp attempts to ignite. The lamp may flash briefly with a burst of light during a failed attempt. These indicate that the igniter system is functioning properly. Raise the current level (turn the current control knob clockwise slightly) and attempt ignition again.

3. Inspect the lamp. If the power supply indicators are lit, the lamp flashes during ignition attempts, and the current control is 5 amps or greater, then the lamp must be inspected and possibly replaced.

## Insufficient Illumination Intensity

The illumination intensity is ultimately limited by the properties of the components. This section deals only with light loss resulting from improper function and its correction.

**Note.** Some of the adjustments detailed in this section require that eye protection, such as welder's goggles, be worn. Do not use ordinary UV goggles when viewing focused illumination.

1. Check the slits and/or slit height sliders. Inspect all slits and height sliders on both monochromators to see if one or more have been inadvertently closed or narrowed, resulting in lowered throughput.

2. Check the illuminator iris (if present). This is located between the lamp housing and the optical chopper inside the illuminator. Ensure that the iris is open completely.

3. Illumination system may be out of alignment. Check the lamp focus on the monochromator entrance slits. The spot size, when viewed with welder's goggles, should be approx. 3 to 4 mm in diameter.

4. Lamp instability. Lamp instability due to lamp age or other factors is remedied by lamp replacement in most cases. If the lamp voltage is fluctuating, an unstable arc position may be the cause and lamp replacement may be necessary. The power supply can also become unstable if it has been damaged.

5. Excessive scratches, dust or fingerprints on the optical elements, especially the chopper disk, upper monochromator focusing mirror, or the collimating mirrors. Dust can be removed by pressurized inert gas but any other damage usually necessitates replacement. Call PTI for service. When using an aerosol duster, be sure to hold the container upright to avoid frosting the mirrors.

## Wavelength adjustment and scanning errors

1. The wavelength setting accuracy of the monochromators is 1 nm. If slippage after a scan or adjustment is greater than 1 nm the drive may need tightening. Loss of calibration can occur by manual adjustment and exceeding the mechanical scanning range. Call PTI for service to correct any of these problems.

2. Verify that the monochromator has not exceeded the upper or lower limit and is not jammed at either end. Turn the Motor Driver Box power off and adjust the wavelength dial by hand to 500 nm to free the lead screw that may have been jammed due to improper position/autocalibration setup.

## No signal or low signal

1. Check BNC cable connections.

2. Check ribbon cables between interface and computer.

3. Check the slits and/or slit height sliders.

4. Check front face/right angle mirror for proper position.

5. Check shutters to be sure they are fully open.

6. Check lamp and supply for proper current and voltage.

7. Check cooled detector window (if applicable) for moisture or frosting.

8. Perform Throughput Optimization (detailed at the end of this section).

## Monochromators not moving

1. Check stepper motor cable connections.

2. Verify that the monochromator has not exceeded the upper or lower limit and is not jammed at either end. Turn system power off and adjust the wavelength dial by hand to free the jammed lead screw.

## Excessively Noisy Signals.

First ensure that it is not due to the observed sample. Then check if it is present in only one detection channel in dual channel detection systems. If this proves to be the case, pay special attention to points 1, 2, and 3 below.

1. Chopper may be out of phase (DeltaScan and PowerFilter only). Refer to the chopper phase adjustment section.

2. Chopper may be unstable. Chopper instability may be detected by observing a significantly noisier signal for channel 1 relative to channel 2 in the dual channel mode when the phase is properly set. If this is the case, check the chopper disk for excessive dust, scratches, or fingerprints. Damaged disks must be replaced. If the chopper disk appears undamaged, the chopper motor may need replacement. Higher chopper speed may minimize the instability. Call PTI for any chopper related servicing.

3. Strong and/or fluctuating electric and magnetic fields, including power surges, may be present. These factors can contribute to instability in the detector and interface electronics, which are both required to measure signals. Using shorter cables (especially for the computer and interface) can help reduce interference.

4. Detector may be unstable. Use single channel illumination at data acquisition frequencies of between 50 and 250 Hz. You should measure a sample of constant luminance. Significant noise in the signal at count rates above 5000 cps indicates detector instability. Detectors having dark counts above 1000 cps should be suspect. Detector noise can be caused by interference from strong electrical or magnetic fields in the vicinity of the system. Call PTI for servicing if the detector is damaged.

5. Lamp may be unstable. Instability due to age or other factors is remedied by lamp replacement in most cases. If the lamp voltage is fluctuating, an unstable arc position may be the cause, and lamp replacement may be necessary. The power supply can also become unstable if it has been damaged. Verify that the detector is stable before changing the lamp.

6. Stray room light may be leaking into the system, especially when using a microscope. Turn off room lights to check. When no light should be reaching the detector the digital dark count for a R1527 PMT should be less than 100 counts per second and a R928 PMT should have a dark count less than 1000 counts per second.

## Excitation Correction Signal is Noisy or Correction is Poor

For the most part, potential causes, diagnosis and correction are the same as for photon-counting detection problems.

1. Gain is set too high or low.

2. The reference detector should not be used outside its useful range of approximately 250-600 nm (200-800 nm optional).

3. Stray light is interfering with the signal-to-noise levels.

4. Excessive scratching, dust or fingerprints appear on the optical elements, especially the chopper disk, upper monochromator focusing mirror, or the collimating mirrors. Damaged optics must be replaced. Call PTI for service.

5. Illumination system may be out of alignment. Check the lamp focus. The lamp can be realigned by the user. Otherwise, call PTI for service.

## High Stray Light (Microscopes).

High numerical aperture objectives can accept significant stray light from outside the detection diaphragm. Some dichroic mirror/microscope assemblies can exhibit significant backscatter, which is often manifested in the observation of ghost images. This can be removed by using a better dichroic mirror or by adding additional filtering. Call PTI for assistance.

## Photon-Counting Saturation occurs below 1,000,000 cps.

1. Inner filter effect is quenching and limiting the fluorescence output. Reduce fluorophore concentration.

2. Amplifier/discriminator is damaged. Call PTI for assistance.

3. Power supply to the PMT and/or amplifier/discriminator is unstable or low. Call PTI for assistance.

4. Computer interface board is not functioning properly. Call PTI for assistance.

## Service Calls to PTI

Before calling for service, review the Troubleshooting Section. The FeliX32 software should not crash or exhibit anomalous performance. Any such behavior, however minor, may indicate a potential error and should be called to the attention of PTI Service. To aid our engineers in discussing your questions, as well as to aid in the timely solution of any problems, please assemble as much as possible of the following information before calling PTI:

1. Your instrument type, hardware configuration, master serial number, and name of the purchaser or principal investigator.

2. The date on which your instrument was installed.

3. The version number of the FeliX32 software that you are using. Click on Help/About to see the software version number.

4. As much detail as possible on the particular chain of events or circumstances that led to the problem. This information should include the complete instrument status and data gathering protocol.

5. If possible, be prepared to send sample data and hardware and acquisition setup files as email attachments to PTI service personnel.

## Optimizing Fluorescence Intensity

Fluorescence observation, particularly from living cells, depends on a variety of factors. The intensity increases at excitation nearer 336 nm (for Fura-2), with increasing path length (cell thickness), with greater dye incorporation, with decreasing viscosity, and with greater intracellular calcium at 336 nm excitation. Fluorescence quenching due to heavy ion contamination should be minimized using specialized intracellular chelators, if necessary. Obviously, many of these factors are either difficult to control or must have

experimentally expedient values which do not maximize the fluorescence intensity. When fluorescence is too faint for needed visualization, increased throughput efficiency is required in the illumination and observation path.

The following methods should increase the combined system efficiency for improved fluorescence visualization. Most of them can be performed quickly and easily. In fact, many of the conditions can be employed temporarily when visual determinations are needed and then replaced quickly by other conditions that may be required for a particular application.

The following methods can be used to enhance the visual observation of fluorescence:

1. Increase all monochromator slit widths. Generally, throughput is maximized at bandpasses of 4 to 8 nm.

2. Open the sliders on the monochromator slits completely.

3. Set both illumination channels to the excitation maximal wavelength.

4. Avoid microscope configurations that require beam splitting.

5. Optimize the transmission and/or reflection characteristics of the dichroic mirror and emission filter.

6. Remove any polarizers, diffusers, or order-sorting filters from the light path, if possible.

7. Use objectives with the best combination of high numerical aperture (N.A.) and low magnification (M). Recall that the gathered fluorescence is proportional to $(N.A.)^4$ and $(M)^{-2}$.

After optimizing the throughput on the system, run a sample. For example, run the excitation spectra of the Fura-2 from 300 nm to 400 nm. The counts per second should be in the range of 500,000 to 1 million for 1 μM Fura-2 using a 40-power objective and a 5 nm bandpass on the monochromators. Measure both the bound and unbound forms of the dye and compare the results to the traces below. Using the sample compartment, similar (or higher) counts can be achieved with similar settings on the monochromators.

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