Fluorolog®-3 with FluorEssence™

Operation Manual

www.HORIBA.com/scientific
rev. G

JOBIN YVON Technology

HORIBA Scientific

Copyright © 2002–2014 by HORIBA Instruments Incorporated.

All rights reserved. No part of this work may be reproduced, stored, in a retrieval system, or transmitted in any form by any means, including electronic or mechanical, photocopying and recording, without prior written permission from HORIBA Instruments Incorporated. Requests for permission should be requested in writing. Origin® is a registered trademark of OriginLab Corporation. Alconox® is a registered trademark of Alconox, Inc. Ludox® is a registered trademark of W.R. Grace and Co. Teflon® is a registered trademark of E.I. du Pont de Nemours and Company. Windows® and Excel® are registered trademarks of Microsoft Corporation.

Information in this manual is subject to change without notice, and does not represent a commitment on the part of the vendor.

May 2014

Part number J81014

Table of Contents

Chapter 0 : Introduction

About the Fluorolog®-3

The main parts of the Fluorolog®-3 spectrofluorometer system are:
• State-of-the-art optical components
• A personal computer
• FluorEssence™ for Windows®, the driving software.

This manual explains how to operate and maintain a Fluorolog®-3 spectrofluorometer. The manual also describes measurements and tests essential to obtain accurate data. For a complete discussion of the almost limitless power provided by FluorEssence™, refer to the *FluorEssence™ User's Guide* and on-line help, and the Origin® on-line help (contains post-processing instructions for data manipulation) which accompany the system.

The combination of time-tested, performance-proven hardware with the powerful data-acquisition and manipulation software yields a system suitable for a wide variety of applications. Equipped with expansion ports and slots, the Fluorolog®-3 can grow to meet the changing needs of the user, it will provide years of dedicated service, and can be upgraded easily with our TCSPC accessory for the Fluorolog® to provide lifetime measurements.

Note: *Keep this and the other reference manuals near the system.*

Chapter overview

1: Requirements & Installation	Power and environmental requirements; select the best spot for the instrument.
2: System Description	Various Fluorolog®-3 configurations; their features and benefits.
3: System Operation	Operation of the spectrofluorometer system, and calibration instructions.
4: Data Acquisition	How to use the special FluorEssence buttons to acquire and plot data; how to determine peaks in an unknown sample.
5: Optimizing Data	Hints for improving the signal-to-noise ratio, instructions for obtaining corrected data, and other information useful for optimizing data and ensuring reproducibility.
6: System Maintenance	Routine maintenance procedures such as replacing the lamp.
7: Troubleshooting	Potential sources of problems, their most probable causes, and possible solutions.
8: Producing Correction Factors	How to correct for variation in sensitivity across the spectral range.
9: Automated Polarizers	Installation, operation, and troubleshooting of the optional automated polarizers.
10: Phosphorimeter Operation	How to use and maintain the optional phosphorimeter.
11: Applications	Some interesting uses for the Fluorolog®-3.
12: Xenon Lamp Information & Record of Use Form	Information about the xenon lamp, and a form for recording the xenon-lamp usage.
13: Using iHR with the Fluorolog®-3	Special instructions on using an iHR imaging spectrometer with the Fluorolog®-3 system, including with a CCD detector.
14: Introduction to Lifetime Measurements	Determining the lifetime of a sample using the optional TCSPC accessory for Fluorolog®. The TCSPC accessory does not affect steady-state measurements.
15: Technical Specifications	Instrument specifications and computer requirements.
16: Components & Accessories	Description and application of the accessories available for the Fluorolog®-3.
17: Reassembly Instructions	How to reassemble the Fluorolog®-3 after it has been moved.
18: Glossary	A list of some useful technical terms related to fluorescence spectroscopy.
19: Bibliography	Important resources for more information.

Disclaimer

By setting up or starting to use any HORIBA Instruments Incorporated product, you are accepting the following terms:

You are responsible for understanding the information contained in this document. You should not rely on this information as absolute or all-encompassing; there may be local issues (in your environment) not addressed in this document that you may need to address, and there may be issues or procedures discussed that may not apply to your situation.

If you do not follow the instructions or procedures contained in this document, you are responsible for yourself and your actions and all resulting consequences. If you rely on the information contained in this document, you are responsible for:

- Adhering to safety procedures
- Following all precautions
- Referring to additional safety documentation, such as Material Safety Data Sheets (MSDS), when advised

As a condition of purchase, you agree to use safe operating procedures in the use of all products supplied by HORIBA Instruments Incorporated, including those specified in the MSDS provided with any chemicals and all warning and cautionary notices, and to use all safety devices and guards when operating equipment. You agree to indemnify and hold HORIBA Instruments Incorporated harmless from any liability or obligation arising from your use or misuse of any such products, including, without limitation, to persons injured directly or indirectly in connection with your use or operation of the products. The foregoing indemnification shall in no event be deemed to have expanded HORIBA Instruments Incorporated's liability for the products.

HORIBA Instruments Incorporated products are not intended for any general cosmetic, drug, food, or household application, but may be used for analytical measurements or research in these fields. A condition of HORIBA Instruments Incorporated's acceptance of a purchase order is that only qualified individuals, trained and familiar with procedures suitable for the products ordered, will handle them. Training and maintenance procedures may be purchased from HORIBA Instruments Incorporated at an additional cost. HORIBA Instruments Incorporated cannot be held responsible for actions your employer or contractor may take without proper training.

Due to HORIBA Instruments Incorporated's efforts to continuously improve our products, all specifications, dimensions, internal workings, and operating procedures are subject to change without notice. All specifications and measurements are approximate, based on a standard configuration; results may vary with the application and environment. Any software manufactured by HORIBA Instruments Incorporated is also under constant development and subject to change without notice.

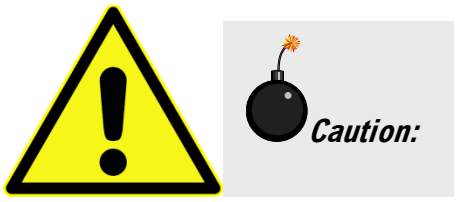
Any warranties and remedies with respect to our products are limited to those provided in writing as to a particular product. In no event shall HORIBA Instruments

Incorporated be held liable for any special, incidental, indirect or consequential damages of any kind, or any damages whatsoever resulting from loss of use, loss of data, or loss of profits, arising out of or in connection with our products or the use or possession thereof. HORIBA Instruments Incorporated is also in no event liable for damages on any theory of liability arising out of, or in connection with, the use or performance of our hardware or software, regardless of whether you have been advised of the possibility of damage.

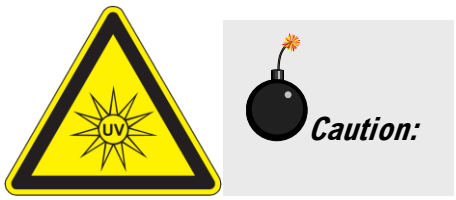
Safety summary

The following general safety precautions must be observed during all phases of operation of this instrument. Failure to comply with these precautions or with specific warnings elsewhere in this manual violates safety standards of design, manufacture and intended use of instrument. HORIBA Instruments Incorporated assumes no liability for the customer's failure to comply with these requirements. Certain symbols are used throughout the text for special conditions when operating the instruments:

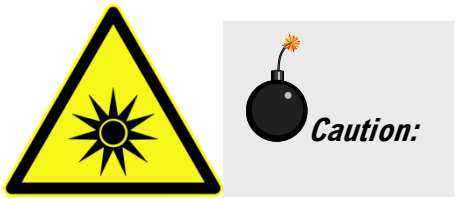
A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or similar that, if incorrectly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met. HORIBA Instruments Incorporated is not responsible for damage arising out of improper use of the equipment.



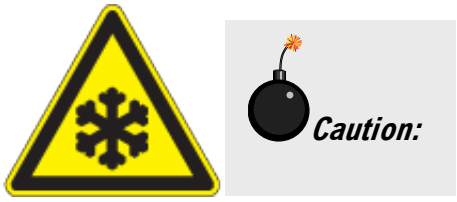
A CAUTION notice denotes a hazard. It calls attention to an operating procedure, practice, or similar that, if incorrectly performed or adhered to, could result in damage to the product. Do not proceed beyond a CAUTION notice until the indicated conditions are fully understood and met. HORIBA Instruments Incorporated is not responsible for damage arising out of improper use of the equipment.



Ultraviolet light! Wear protective goggles, full-face shield, skin-protection clothing, and UV-blocking gloves. Do not stare into light.



Intense ultraviolet, visible, or infrared light! Wear light-protective goggles, full-face shield, skin-protection clothing, and light-blocking gloves. Do not stare into light.



Extreme cold! Cryogenic materials must always be handled with care. Wear protective goggles, full-face shield, skin-protection clothing, and insulated gloves.

Explosion hazard! Wear explosion-proof goggles, full-face shield, skin-protection clothing, and protective gloves.

Risk of electric shock! This symbol warns the user that un-insulated voltage within the unit may have sufficient magnitude to cause electric shock.

Danger to fingers! This symbol warns the user that the equipment is heavy, and can crush or injure the hand if precautions are not taken.

This symbol cautions the user that excessive humidity, if present, can damage certain equipment.

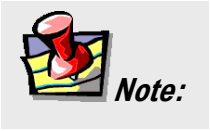
Hot! This symbol warns the user that hot equipment may be present, and could create a risk of fire or burns.

Wear protective gloves.

Wear appropriate safety goggles to protect the eyes.

Wear an appropriate face-shield to protect the face.

Read this manual before using or servicing the instrument.



General information is given concerning operation of the equipment.

Risks of ultraviolet exposure

Caution: *This instrument is used in conjunction with ultraviolet light. Exposure to these radiations, even reflected or diffused, can result in serious, and sometimes irreversible, eye and skin injuries.*

Overexposure to ultraviolet rays threatens human health by causing:

- *Immediate painful sunburn*
- *Skin cancer*
- *Eye damage*
- *Immune-system suppression*
- *Premature aging*

Do not aim the UV light at anyone. Do not look directly into the light. Always wear protective goggles, full-face shield and skin protection clothing and gloves when using the light source.

- Light is subdivided into visible light, ranging from 400 nm (violet) to 700 nm (red); longer infrared, "above red" or > 700nm, also called heat; and shorter ultraviolet radiation (UVR), "below violet" or < 400nm. UVR is further subdivided into UV-A or near-UV (320–400 nm), also called black (invisible) light; UV-B or mid-UV (290–320 nm), which is more skin penetrating; and UV-C or far-UV (< 290 nm).

- Health effects of exposure to UV light are familiar to anyone who has had sunburn. However, the UV light level around some UV equipment greatly exceeds the level found in nature. Acute (short-term) effects include redness or ulceration of the skin. At high levels of exposure, these burns can be serious. For chronic exposures, there is also a cumulative risk of harm. This risk depends upon the amount of exposure during your lifetime. The long-term risks for large cumulative exposure include premature aging of the skin, wrinkles and, most seriously, skin cancer and cataract.

- Damage to vision is likely following exposure to high-intensity UV radiation. In adults, more than 99% of UV radiation is absorbed by the anterior structures of the eye. UVR can contribute to the development of age-related cataract, pterygium, photodermatitis, and cancer of the skin around the eye. It may also contribute to age-related macular degeneration. Like the skin, the covering of the eye or the cornea, is epithelial tissue. The danger to the eye is enhanced by the fact that light can enter from all angles around the eye and not only in the direction of vision. This is especially true while working in a dark environment, as the pupil is wide open.

The lens can also be damaged, but because the cornea acts as a filter, the chances are reduced. This should not lessen the concern over lens damage however, because cataracts are the direct result of lens damage.

Burns to the eyes are usually more painful and serious than a burn to the skin. Make sure your eye protection is appropriate for this work. NORMAL EYEGLASSES OR CONTACTS OFFER VERY LIMITED PROTECTION!

Caution: *UV exposures are not immediately felt. The user may not realize the hazard until it is too late and the damage is done.*

Training

For the use of UV sources, new users must be trained by another member of the laboratory who, in the opinion of the member of staff in charge of the department, is sufficiently competent to give instruction on the correct procedure. Newly trained users should be overseen for some time by a competent person.

Additional risks of xenon lamps

Warning: *Xenon lamps are dangerous. Please read the following precautions.*

Among the dangers associated with xenon lamps are:

- Burns caused by contact with a hot xenon lamp.
- Fire ignited by hot xenon lamp.
- Interaction of other nearby chemicals with intense ultraviolet, visible, or infrared radiation.
- Damage caused to apparatus placed close to the xenon lamp.
- Explosion or mechanical failure of the xenon lamp.

Visible radiation

Any very bright visible light source will cause a human aversion response: we either blink or turn our head away. Although we may see a retinal afterimage (which can last for several minutes), the aversion response time (about 0.25 seconds) normally protects our vision. This aversion response should be trusted and obeyed. NEVER STARE AT ANY BRIGHT LIGHT-SOURCE FOR AN EXTENDED PERIOD. Overriding the aversion response by forcing yourself to look at a bright light-source may result in permanent injury to the retina. This type of injury can occur during a single prolonged exposure. Excessive exposure to visible light can result in skin and eye damage.

Visible light sources that are not bright enough to cause retinal burns are not necessarily safe to view for an extended period. In fact, any sufficiently bright visible light source viewed for an extended period will eventually cause degradation of both night and color vision. Appropriate protective filters are needed for any light source that causes viewing discomfort when viewed for an extended period of time. For these reasons, prolonged viewing of bright light sources should be limited by the use of appropriate filters.

The blue-light wavelengths (400–500 nm) present a unique hazard to the retina by causing photochemical effects similar to those found in UV-radiation exposure.

Infrared radiation

Infrared (or heat) radiation is defined as having a wavelength between 780 nm and 1 mm. Specific biological effectiveness “bands” have been defined by the CIE (Commission Internationale de l’Eclairage or International Commission on Illumination) as follows:

• IR-A (near IR) (780–1400 nm)
• IR-B (mid IR) (1400–3000 nm)
• IR-C (far IR) (3000 nm–1 mm)

The skin and eyes absorb infrared radiation (IR) as heat. Workers normally notice excessive exposure through heat sensation and pain. Infrared radiation in the IR-A that enters the human eye will reach (and can be focused upon) the sensitive cells of the retina. For high irradiance sources in the IR-A, the retina is the part of the eye that is at risk. For sources in the IR-B and IR-C, both the skin and the cornea may be at risk from "flash burns." In addition, the heat deposited in the cornea may be conducted to the lens of the eye. This heating of the lens is believed to be the cause of so called "glassblowers' " cataracts because the heat transfer may cause clouding of the lens.

- Retinal IR Hazards (780 to 1400 nm): possible retinal lesions from acute high irradiance exposures to small dimension sources.
- Lens IR Hazards (1400 to 1900 nm): possible cataract induction from chronic lower irradiance exposures.
- Corneal IR Hazards (1900 nm to 1 mm): possible flashburns from acute high irradiance exposures.

Who is likely to be injured? The user and anyone exposed to the radiation or xenon lamp shards as a result of faulty procedures. Injuries may be slight to severe.

Chapter 1 : Requirements & Installation

Surface requirements

- A sturdy table- or bench-top.
- Table size varies according to the system configuration; an average size of 38″ × 60″ (96.5 cm × 152.4 cm) is usually sufficient.

***Caution: Do not** split the system between two tables. Using two tables can cause instability, resulting in service requirements or erroneous data.*

Table size for standard systems*

	FL3-11	FL3-12	FL3-21	FL3-22	FL3-222
Length	102 cm	102 cm	123 cm	123 cm	132 cm
Width	91 cm	112 cm	104 cm	124 cm	183 cm
Height	43 cm	43 cm	43 cm	43 cm	43 cm

*Custom configurations are available. See the System Description chapter.

Environmental requirements

- Temperature 72 ± 5°F (22 ± 3°C)
- Humidity level ~70%
- No special ventilation.

Note: *The standard xenon lamp provided with the Fluorolog®-3 is ozone-free. The lamp housing contains an electrically powered fan that removes the heat.*

Electrical requirements

- 115 V, 20 A or 220 V, 20 A; factory-set.
- As an extra measure of caution, plug the xenon lamp into a circuit separate from the other components. This guarantees that the electrical surge from the lamp never will interfere with the computer or system.

Note: *For the computer, HORIBA Scientific recommends using a surge suppressor or an uninterruptible power supply (UPS) with a surge suppressor.*

Make sure enough AC outlets are available for the

- Host computer
- Printer (optional)
- Monitor
- Xenon lamp
- System controller (SpectrAcq)
- Any other accessories that require an outlet

Use three-prong plugs for proper grounding of the system. If a two-prong adapter is used, for the safety of the operator and to preserve the integrity of the system, the adapter must be attached to the wall outlet properly, according to the manufacturer's instructions. This provides a positive connection to the electrical ground (earth), ensuring that any stray or leakage current is directed to earth ground.

Warning: *A three-prong-to-two-prong adapter* ***is not recommended****.*

Installation

Schedule the initial installation of a Fluorolog®-3 by calling the Service Department at (732) 494-8660 × 8160. Customers outside the United States should contact a local representative. For up-to-the-minute information about products, services, upgrades, frequently-asked questions, etc., visit our web site:

http://www.horiba.com/scientific/products/fluorescence-spectroscopy/

Subsequent assembly because of relocation either can be performed by a HORIBA Scientific engineer for a specified fee, or by the user. Re-assembly instructions and diagrams are provided in *Reassembly Instructions*.

Chapter 2 : System Description

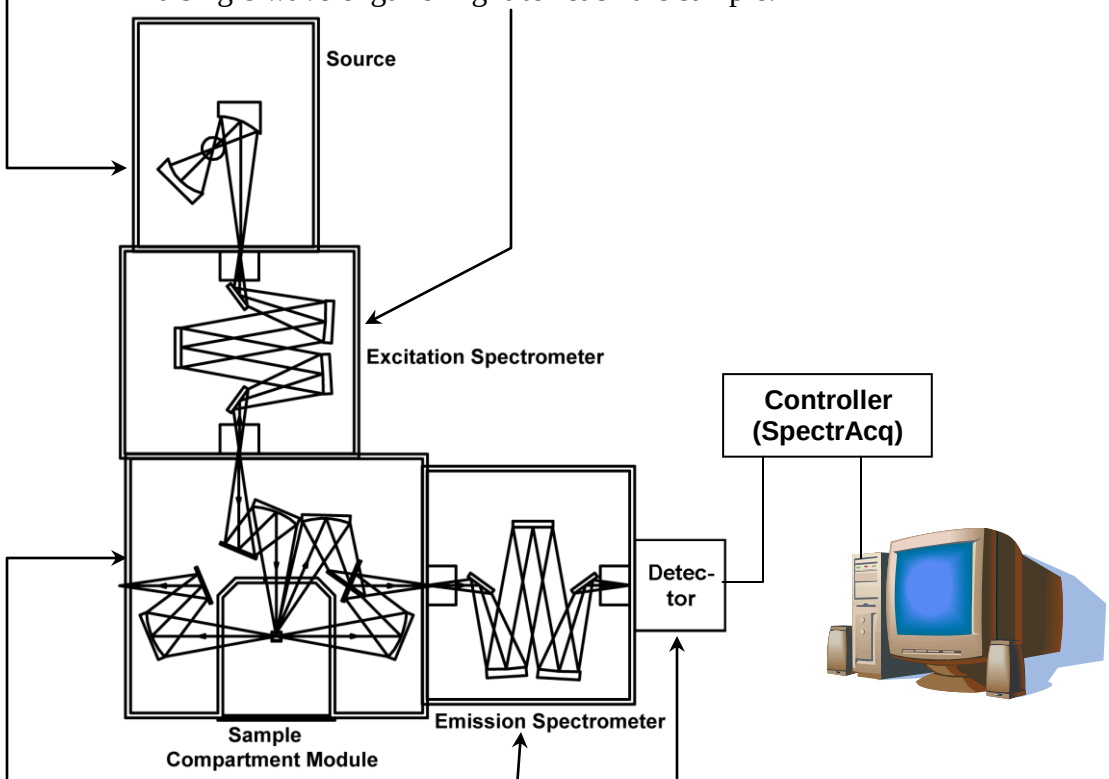
Overview

General operation

All Fluorolog®-3 spectrofluorometers have common features:

a A source of radiation produces photons.

b The beam of light is filtered by an excitation monochromator that allows a single wavelength of light to reach the sample.



c In the sample compartment, the sample responds to the incoming radiation.

d The resulting radiation is filtered by an emission monochromator that feeds the signal to a photomultiplier detector.

e By stepping either or both monochromators through a wavelength region, and recording the variation in intensity as a function of wavelength, a spectrum is produced.

f The spectrofluorometer components (monochromators, sample-compartment module, accessories) are connected to a controller (the "SpectrAcq"), which, in turn, transfers information to and from the host computer. The host computer may be attached to a printer or plotter.

Basic components

Monochromators

The Fluorolog®-3 comes equipped with either a single- or double-grating monochromator in the excitation and emission positions. Double-grating monochromators offer a significant increase in sensitivity, resolution, and stray-light rejection.

Sample compartment

The standard sample-compartment module is a *T-box*, which provides efficient throughput with a choice of standard right-angle emission collection or optional front-face emission collection. The sample-compartment module comes equipped with a silicon photodiode reference detector to monitor and compensate for variations in the xenon-lamp output.

Detector

The standard detector offered on the Fluorolog®-3 is the R928P photomultiplier tube, which provides sensitive spectral characterization in the UV through the visible.

Accessories

Fluorolog®-3 spectrofluorometers offer sampling accessories to increase flexibility, and extend their applications to techniques such as polarization measurements or phosphorescence lifetimes.

Configurations

The different configurations and various accessories available for the Fluorolog®-3 system allow you to customize a system specific for today's needs, while the interchangeability of the components and the inherent design enable the system to grow and change as new applications arise.

Standard systems

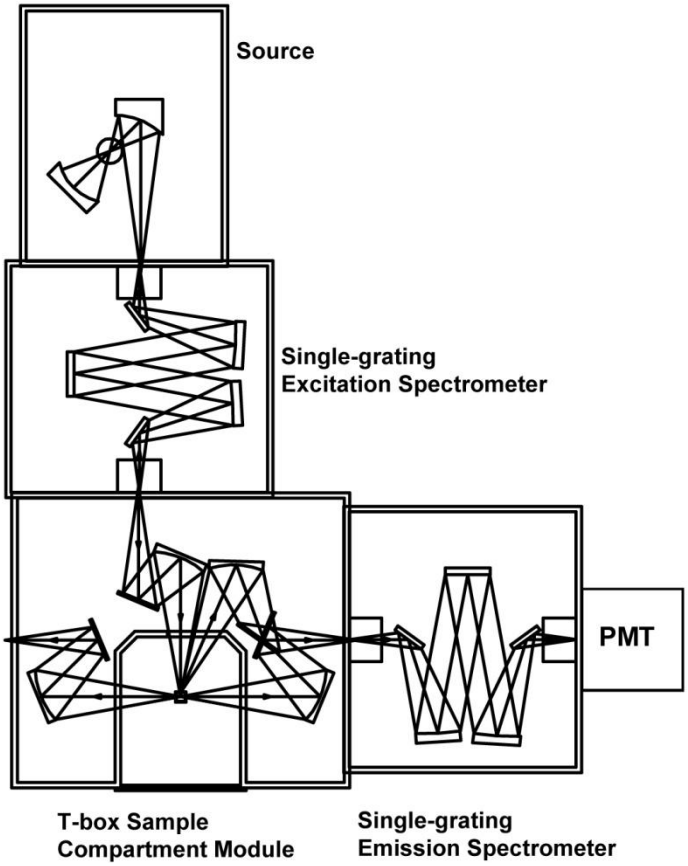
The standard Fluorolog®-3 systems include a single- or double-grating monochromator in the excitation and emission paths in an "L" configuration.

Standard systems available:

Model	Source	Excitation Monochromator	Sample Compartment Module	Emission Monochromator	Detector
FL3-11	450-W Xe	Single	T-Box	Single	PMT
FL3-12	450-W Xe	Single	T-Box	Double	PMT
FL3-21	450-W Xe	Double	T-Box	Single	PMT
FL3-22	450-W Xe	Double	T-Box	Double	PMT

Fluorolog®-3 Model FL3-11

The Fluorolog®-3 Model FL3-11 is an economical system designed for routine fluorescence measurements.

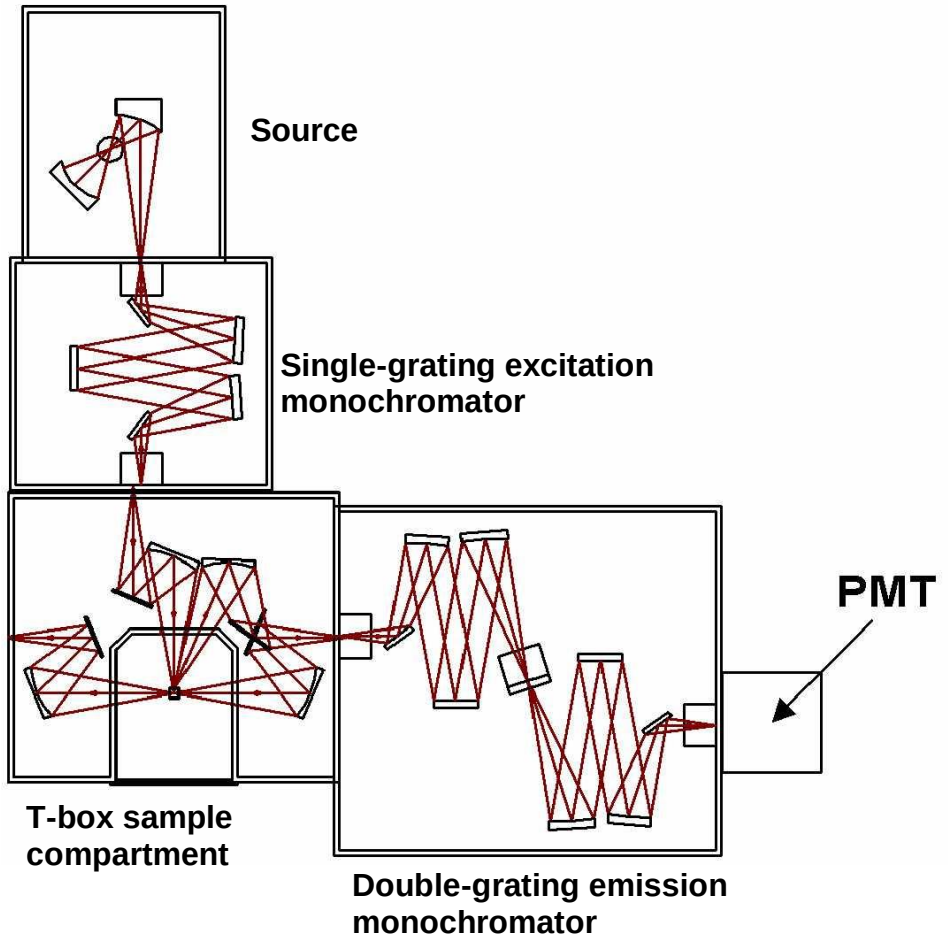


The standard model FL3-11 comes equipped with:

- 450-W light source
- Single-grating excitation monochromator
- Single-grating emission monochromator
- Automatic slits
- Room-temperature R928P detector

Fluorolog®-3 Model FL3-12

The Fluorolog®-3 model FL3-12 provides optimum performance for highly scattering samples such as proteins, membranes, and solid samples. Like the Model FL3-11, the Fluorolog®-3 Model FL3-12 has a single-grating excitation monochromator; but the optimum performance of the Model FL3-12 is as a result of a double-grating emission monochromator.

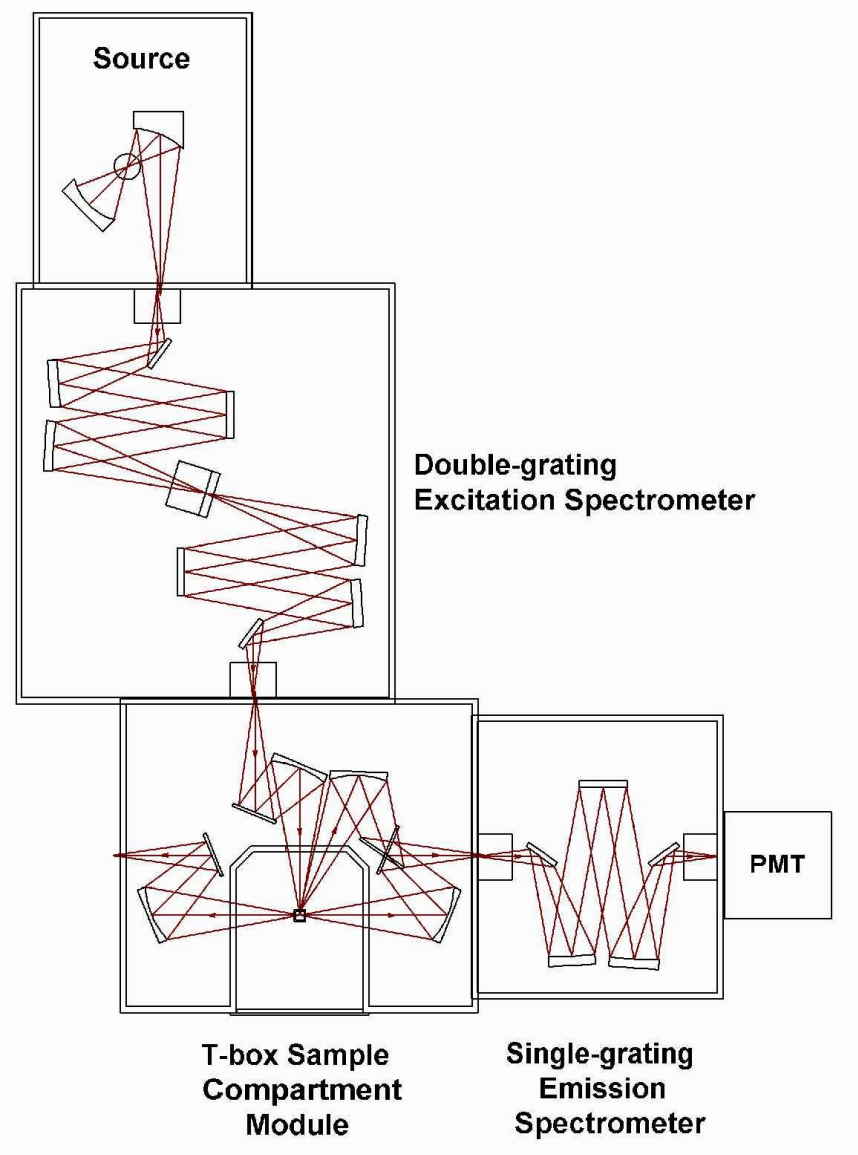


Features of the Model FL3-12 are:

- 450-W light source
- Single-grating excitation monochromator
- Double-grating emission monochromator
- Automatic slits
- Room-temperature R928P detector

Fluorolog®-3 Model FL3-21

The Fluorolog®-3 model FL3-21 includes a double-grating monochromator at the excitation position.

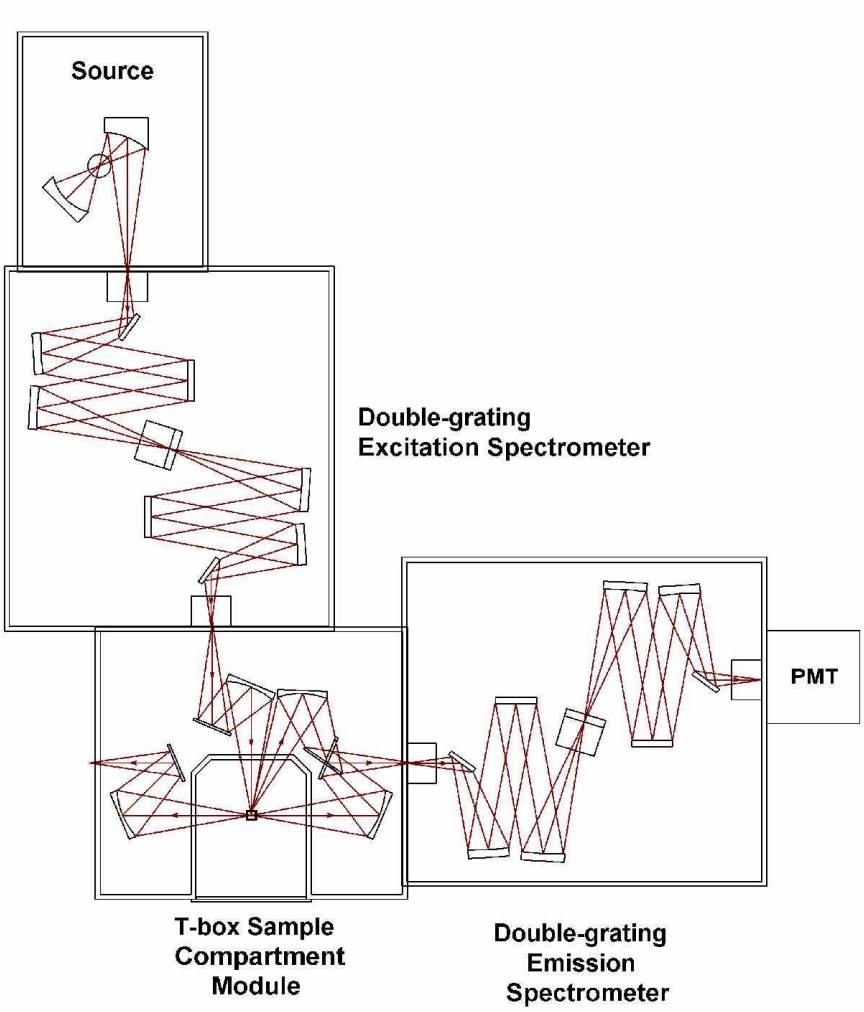


Features of the Model FL3-21 are:

- 450-W light source
- Double-grating excitation monochromator
- Single-grating emission monochromator
- Automatic slits
- Room-temperature R928P detector

Fluorolog®-3 Model FL3-22

Because of the double-grating excitation and emission monochromators, the Fluorolog®-3 model FL3-22 offers unsurpassed performance in resolution, sensitivity, and stray-light rejection. This system is perfect for highly scattering samples like lipids and proteins, or solids like powders, semiconductors, or phosphors.



Incorporating double-grating monochromators in both the excitation and emission positions places this model in a category by itself. Enhanced features, modular structure, and few external controls are just a few of the reasons to consider a Fluorolog®-3 model FL3-22:

- 450-W light source
- Double-grating excitation monochromator
- Double-grating emission monochromator
- Automatic slits
- Room-temperature R928P detector

Standard options

The previously described systems represent the standard configurations. Each system, however, can be customized by selecting different components. Available options are listed below. For additional information, or for a list of the most recently developed products, contact a HORIBA Scientific Sales Representative.

Sources

- Pulsed lamp, including our TCSPC upgrade
- HgXe
- Your laser

Detectors

- Cooled PMT
- IR
- CCD

Caution: *Always take appropriate protective measures against intense light from external lamps and lasers, including goggles or face-shield, and clothing that blocks the wavelengths that the source emits.*

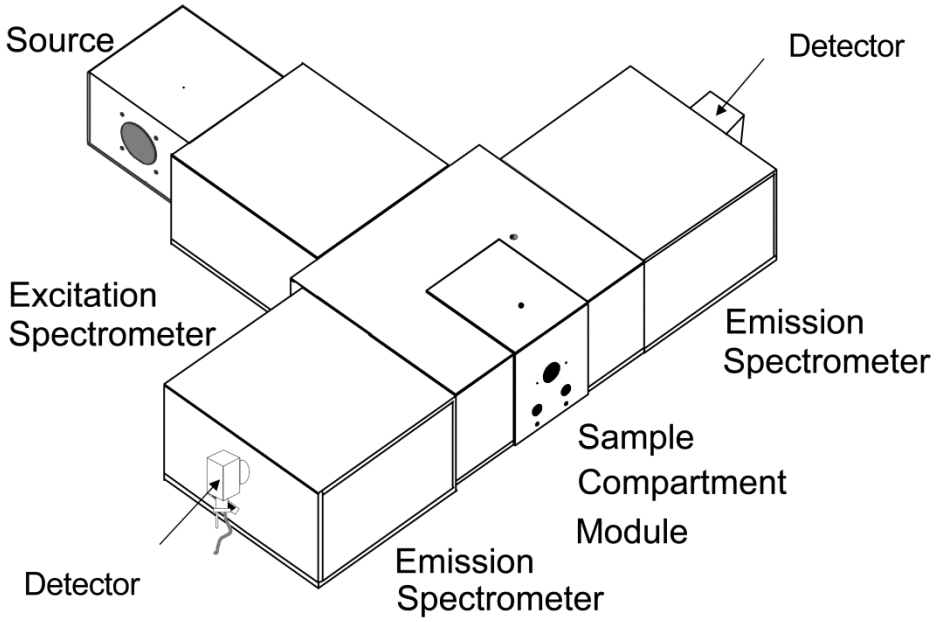
Refer to *Components and Accessories* for a list of accessories that will help you further tailor a system to your application needs.

Custom configurations

With custom configurations, you can change the layout of the system to a T-format or add an imaging spectrograph at the emission port of the system. Using single-grating, double-grating, or the imaging spectrometer, you can create a system for almost any application. Details on using an iHR imaging spectrometer are given in *Chapter 13: iHR Operation with the Fluorolog®-3*. Some of the more popular custom configurations are described on the following pages.

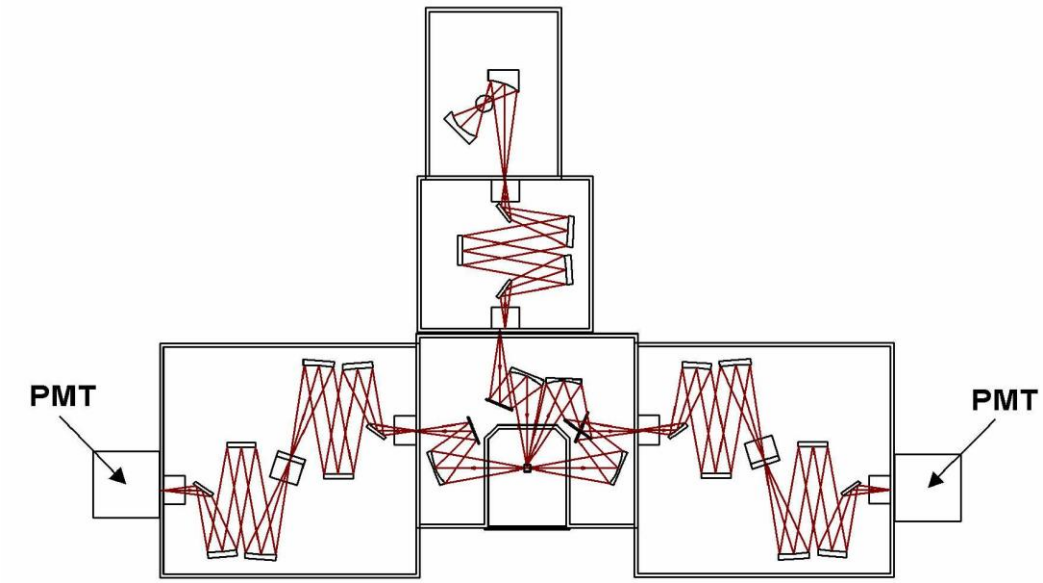
Fluorolog®-3 Model FL3-XXX

Systems with the T-configuration design were developed for T-format polarization or anisotropy and dual-emission spectroscopy. Models FL3-XXX (where the Xs are the type of spectrometer positioned at the excitation, first-emission, and second-emission positions, respectively) are available in numerous configurations.



Fluorolog®-3 model FL3-122

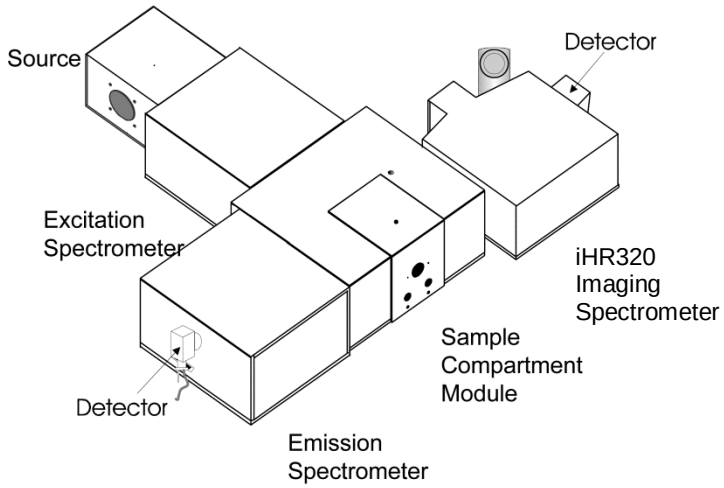
The T-configuration allows the Xe source and the signal detector to be positioned at right-angles to the sample, for a variety of experiments with results unequaled by other configurations. The optical configuration of the model FL3-122 is shown below.



Optical layout of the Fluorolog®-3 Model FL3-122.

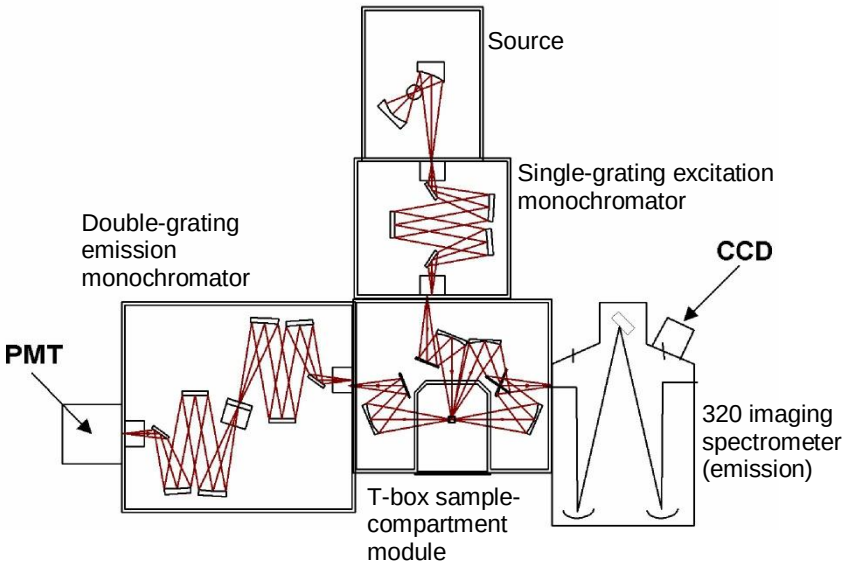
Fluorolog®-3 Model FL3-22iHR

The fully automated iHR320 imaging spectrograph can be a part of a custom Fluorolog®-3 configuration. The imaging spectrograph offers the latest advances in optical design and automation.



Fluorolog®-3 with a double-grating emission spectrometer and iHR320 imaging spectrograph.

The optical configuration of this system is shown below.



Custom optical layout. By switching the iHR320 and double-grating monochromator, both right-angle and front-face collection are possible.

The unique optical layout of the iHR320 eliminates rediffracted light. In addition, with the single-grating excitation spectrometer in place, the iHR320 imaging spectrograph can be placed on either side of the sample-compartment module. When the iHR320 imaging spectrometer is used as the first emission spectrometer, both front-face and right-angle collection are possible.

Custom options

Each system can be customized further by selecting different options. Available options are listed below. For additional information, or for a list of the most recently developed products, contact a HORIBA Scientific Sales Representative.

Sources

- Pulsed lamp, including our TCSPC upgrade
- HgXe
- Your laser

Detectors

- Cooled PMT
- IR
- CCD

Caution: *Always take appropriate protective measures against intense light from external lamps and lasers, including goggles or face-shield, and clothing that blocks the wavelengths that the source emits.*

Refer to *Components and Accessories* for a list of accessories that will help you further tailor a system to your application needs.

Chapter 3 : System Operation

Turning on the system

1 Start the lamp.

The lamp must be turned on prior to the Fluorolog®-3, accessories, and peripheral equipment.

a On the back of the lamp housing, turn on the switch marked POWER.

b Just above the POWER switch, turn on the MAIN LAMP switch.

Caution: *When the lamp is turned on, a large voltage is put across the lamp, during which a spike can feed back down the electrical line. This spike can cause damage to computer equipment if the equipment is operating at the time and on the same power circuit as the Fluorolog®-3, when the lamp is started.*

2 Start the fluorometer accessories.

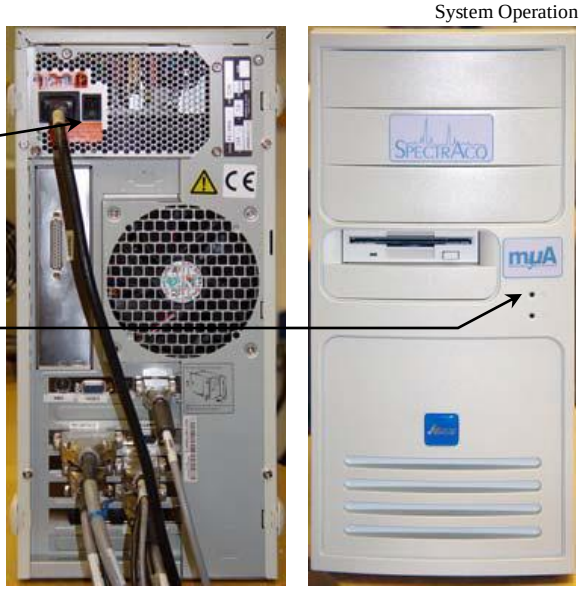
Turn on any automated accessories (e.g., Temperature Bath, MicroMax, etc.) used with the Fluorolog®-3. Let the system warm up for at least 60 min.

3 Start the SpectrAcq.

a For Fluorolog®s with serial numbers 1030 and lower, make sure the boot disk is in the floppy drive.

Caution: *For Fluorolog®s with serial numbers 1031 or higher, do NOT insert the 3¼" floppy disk into the SpectrAcq. The floppy disk is ONLY for backup purposes, NEVER for boot-up.*

b On the rear of the SpectrAcq, activate on the power switch to start. Immediately below the mμA logo on the front of the SpectrAcq, the LED indicator lamp should illuminate.



Note: *For Fluorolog®s that use the 3¼" floppy disk to boot up, the disk should access for ~ 60 s to load system drivers.*

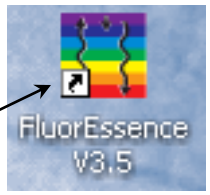
4 Start the peripheral devices.

Turn on all peripheral devices such as printers and plotters (i.e., all devices other than the computer).

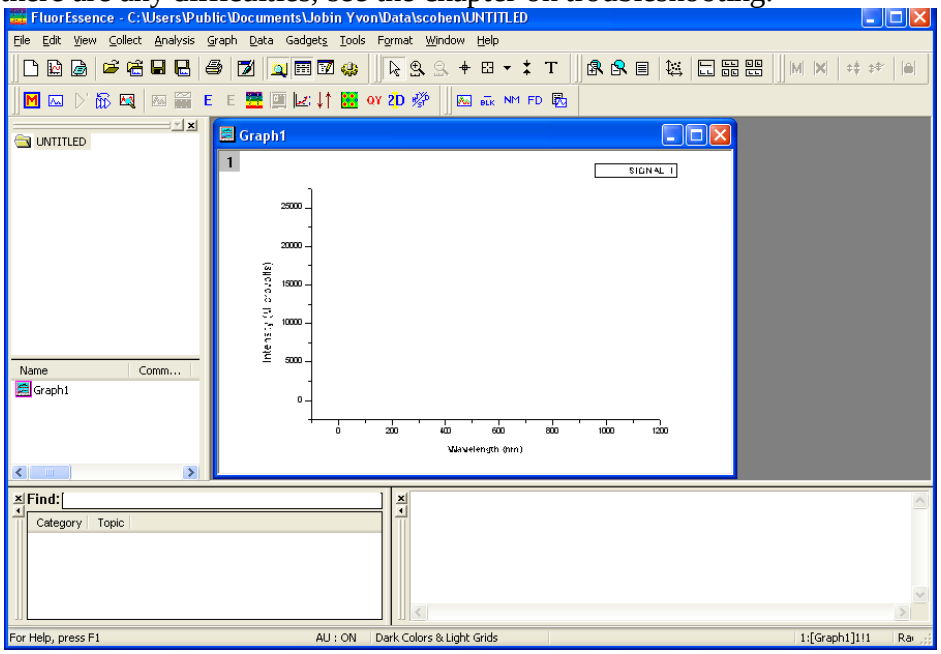
5 Start the host computer.

a Switch on the host computer.

b Click on the FluorEssence icon in Windows®.



The instrument initializes, then the **FluorEssence** window appears. If there are any difficulties, see the chapter on troubleshooting.



Checking system performance

Introduction

Upon installation and as part of routine maintenance checks, examine the performance of the Fluorolog®-3. HORIBA Scientific recommends checking the system calibration before each day of use with the system. Scans of the xenon-lamp output and the Raman scatter band of water are sufficient to verify system calibration, repeatability, and throughput.

- *Calibration* is the procedure whereby the drive of each spectrometer is referenced to a known spectral feature.
- *Repeatability* is the ability of the system to produce consistent spectra.
- *Throughput* is the amount of signal passing through and detected by the system. The throughput is correlated to the signal-to-noise ratio and sensitivity of the system.

The Fluorolog®-3 is an *autocalibrating* spectrofluorometer. This means the system initializes its monochromator's drives, locates the home position of the each drive, and assigns a wavelength value to this position from a calibration file. While the system usually maintains calibration by this method, it is wise to check the calibration prior to the day's session with the instrument. For the calibration checks detailed here, a single-sample mount or automated sample changer should be the only sample-compartment accessories used.

The scans shown herein are *examples*. A Performance Test Report for your new instrument is included with the documentation. Use the Performance Test Report to validate the spectral shape and relative intensity taken during the calibration checks.

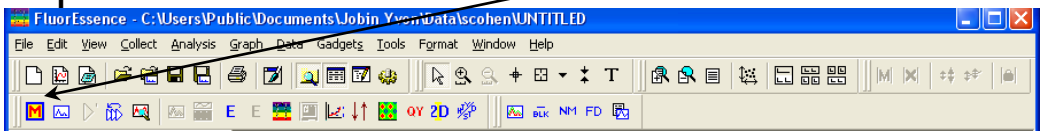
These scans are described for systems with the default 1200-grooves/mm gratings and an R928P PMT emission detector with coverage from the UV to high visible. If the monochromators contain gratings with groove densities other than 1200 grooves/mm, with different wavelength sensitivity, or with a different emission detector, please consult the Performance Test Report for appropriate scan parameters.

Excitation calibration check

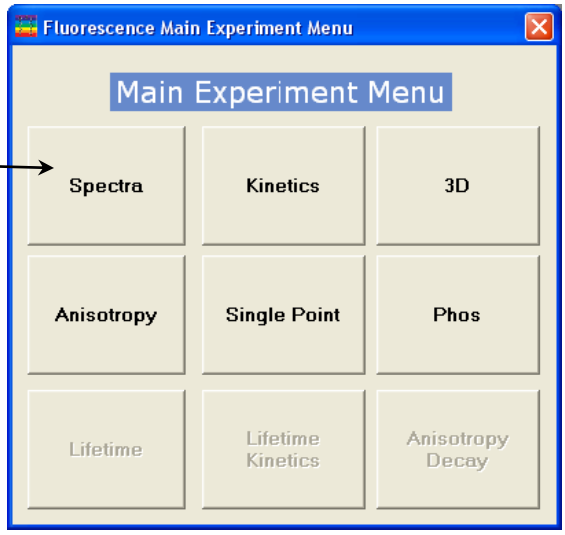
This calibration check verifies the wavelength calibration of your excitation monochromator, using the reference photodiode located before the sample compartment. It is an excitation scan of the xenon lamp's output, and should be the first check performed.

1 Close the lid of the sample compartment.

2 On the main FluorEssence toolbar, select the Experiment Menu button :

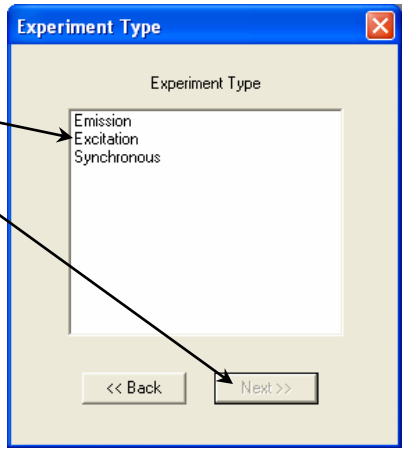


The **Fluoresence Main Experiment Menu** appears:



3 Choose the Spectra button.

The **Experiment Type** window appears:



4 Choose Excitation, then click the Next >> button.

The xenon-lamp scan experiment automatically loads.

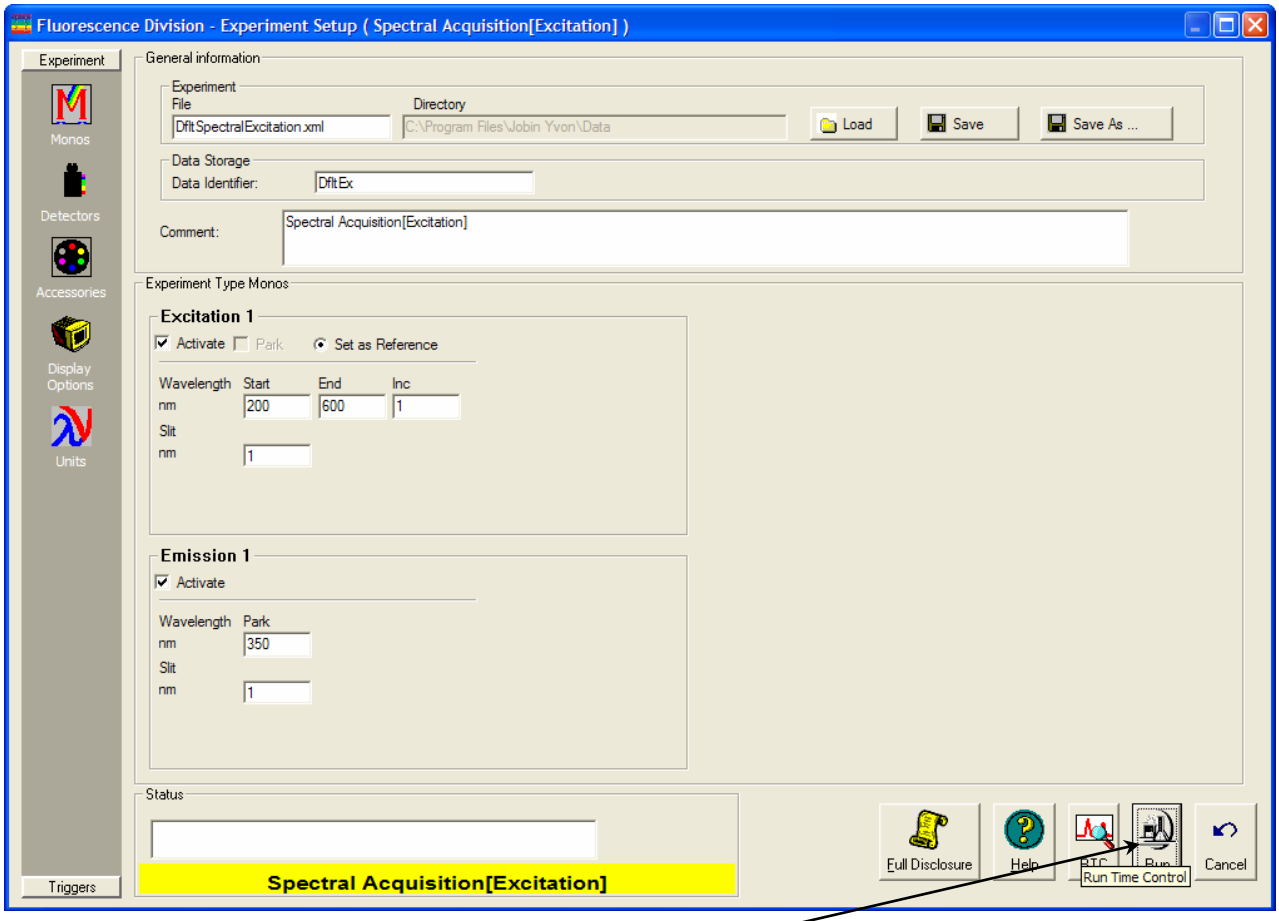
5 Use the default parameters or adjust them:

Default monochromator parameters for the xenon-lamp scan are:

Monochromator (1200 grooves/mm)	Initial wavelength	Final wavelength	Increment	Slits(bandpass)
Excitation	200 nm	600 nm	1 nm	1 nm
Emission	350 nm	--	--	1 nm

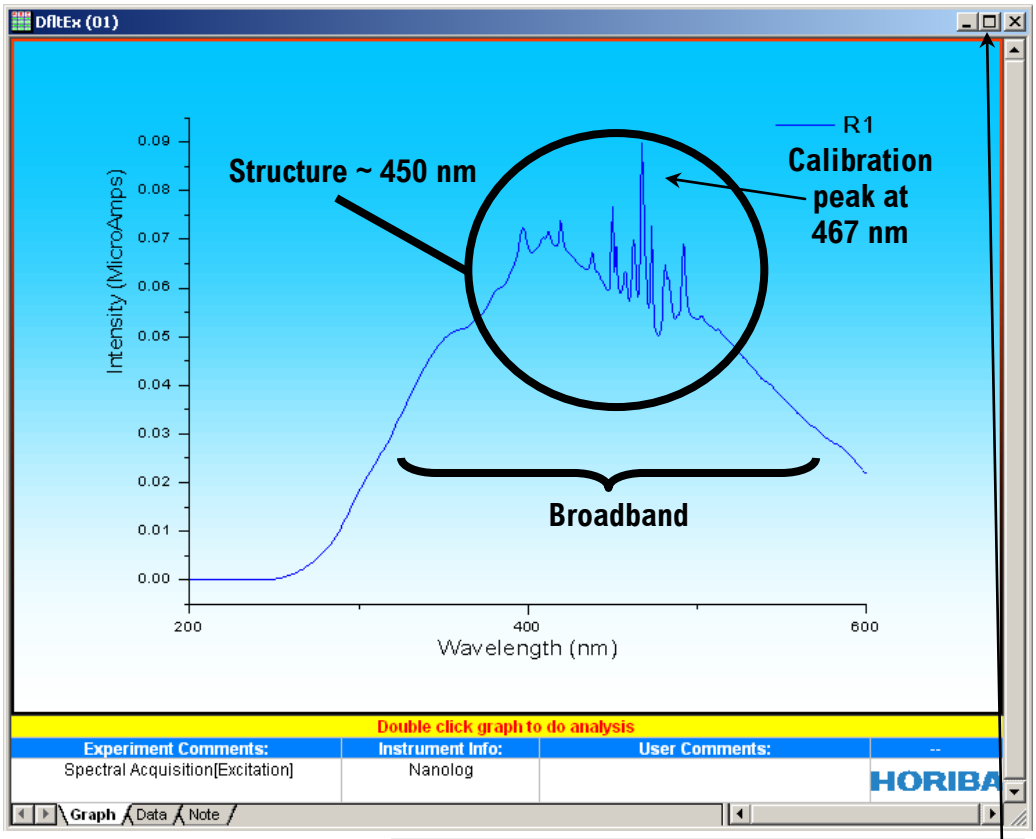
Default detector parameters for the xenon-lamp scan are:

Detector (Signal)	Integration time	Units
Signal (S1)	0.1 s	CPS
Reference (R1)	0.1 s	mA



6 Click the Run button.

The **Intermediate Display** opens. The xenon-lamp scan runs:



This is an uncalibrated FluoroMax lamp scan. The main peak ought to be at 467 nm, but here appears near 480 nm.

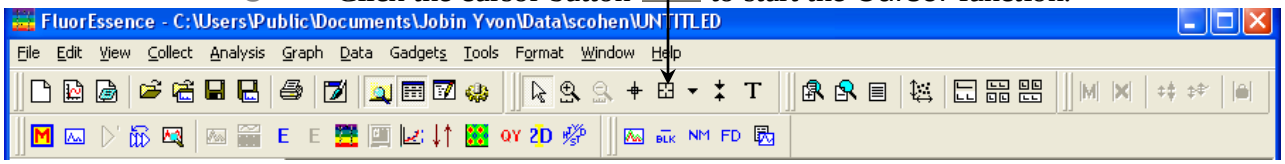
Note: *Your lamp scan may appear different, depending on the instrument configuration. See your Performance Test Report for a factory-run scan of your lamp.*

7 Calibrate the excitation monochromator, if required.

a Double-click on the graph to un-embed it from the workbook.

b Expand the plot by clicking the Expand button.

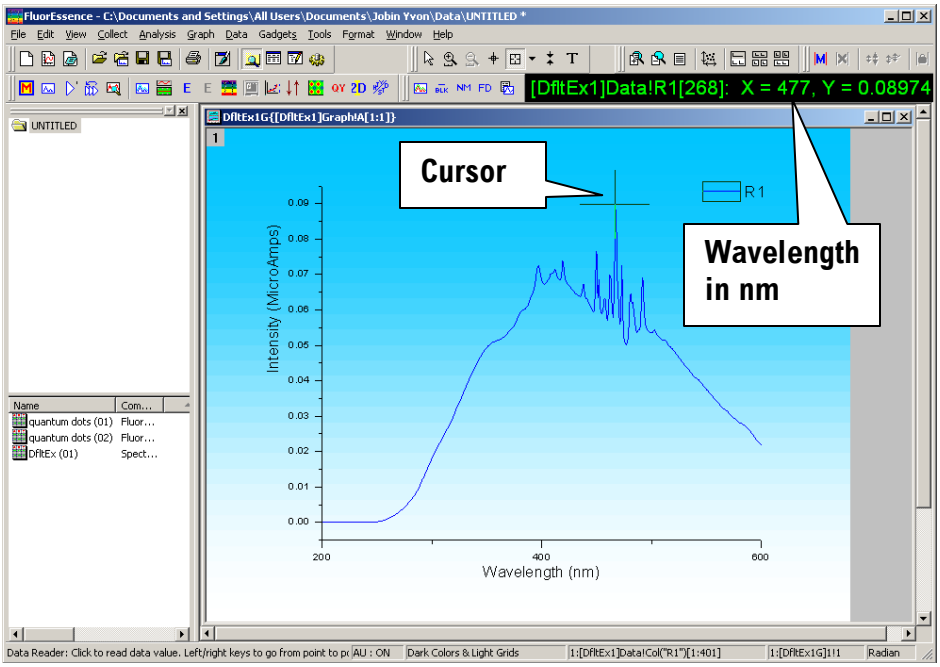
c Click the cursor button  to start the Cursor function.



d Click on the graph near the peak, to place the cursor on the graph.

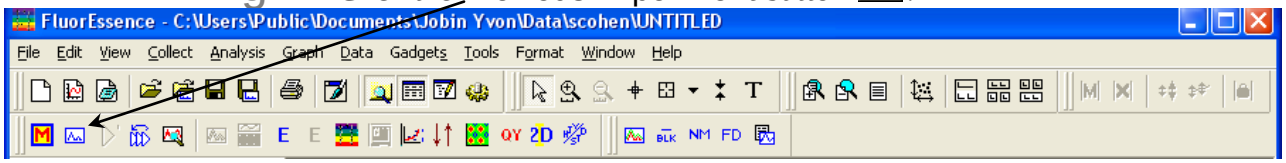
e Using the left and right arrows on the keyboard, move the cursor to the top of the peak.

f Read the *x*-value of this plot: this is the wavelength of the peak.

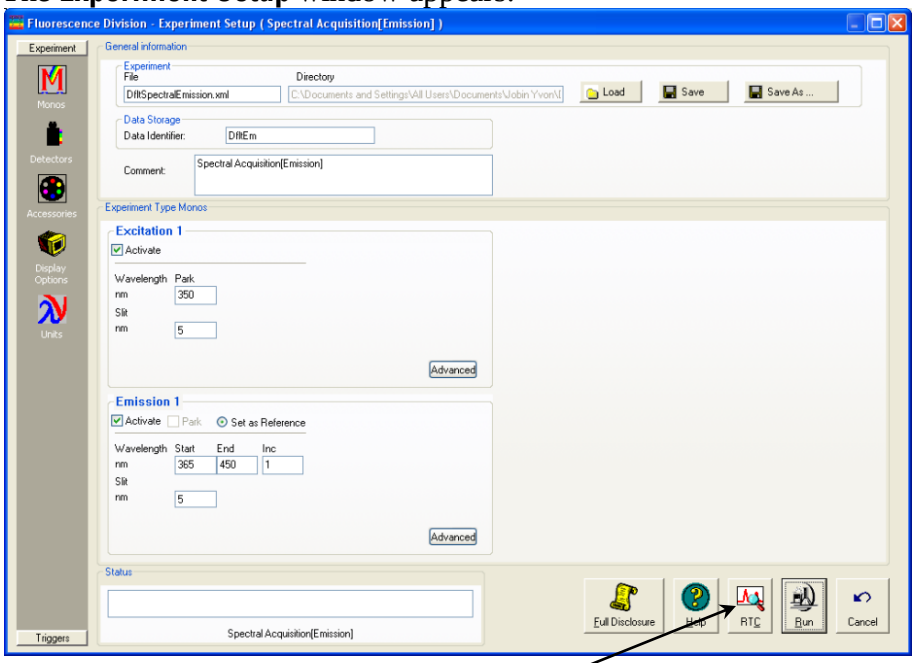


This example shows the peak actually at 477 nm, which is 10 nm too high. Therefore we must recalibrate the monochromator.

g Click the Previous Experiment button .

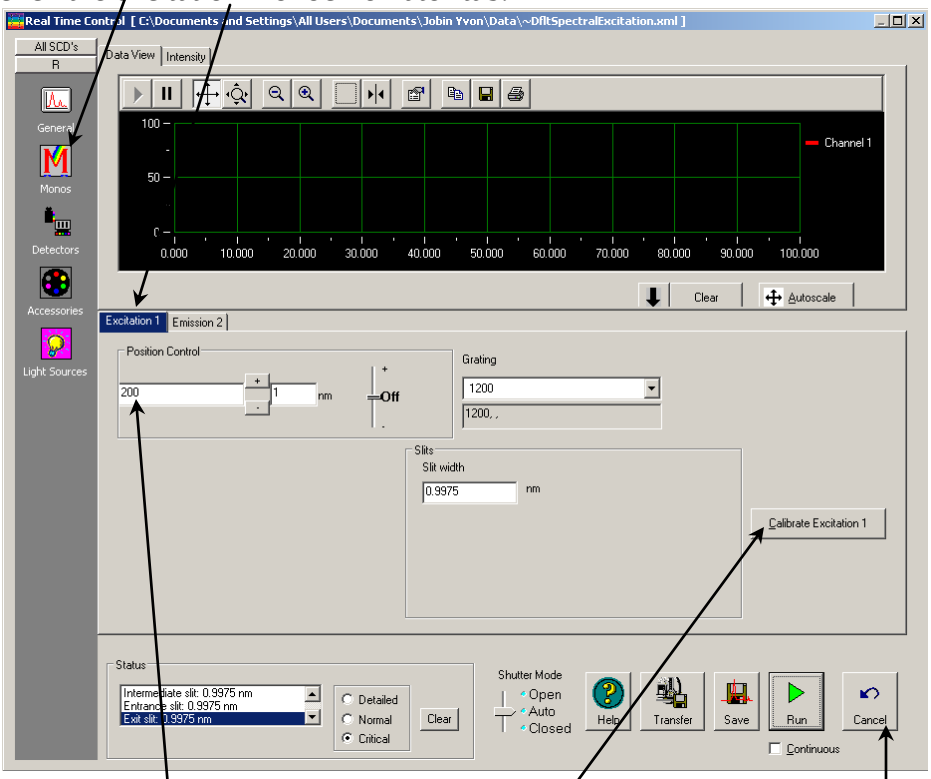


The **Experiment Setup** window appears:



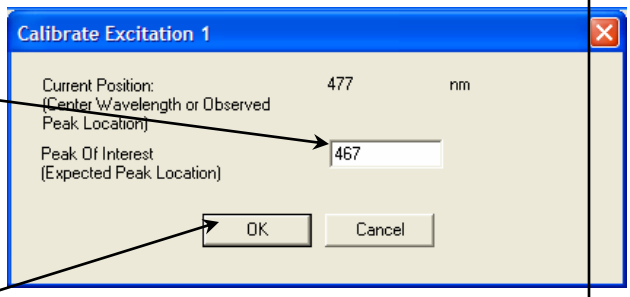
h Click the RTC button on the lower right.
The **Real Time Control** window opens:

i Click the Monos icon to view the monochromators' index card, then click the excitation monochromator tab.



j Enter the current, observed position of the peak in the Position Control (here, 477 nm).

k Click the Calibrate Excitation 1 button. The **Calibrate** window opens:

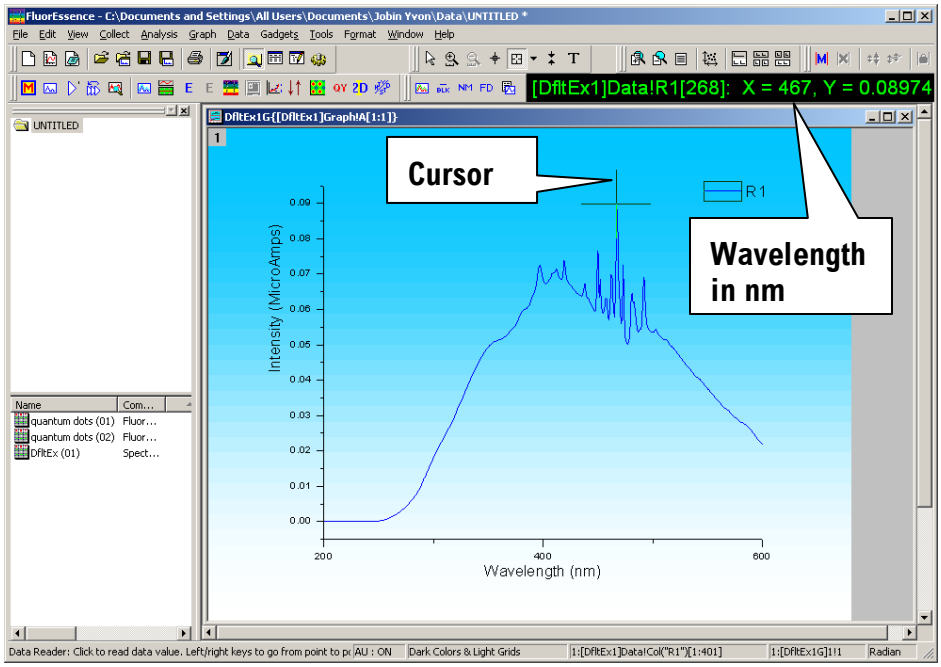


l In Peak Of Interest, enter the actual or expected position of the peak (it ought to be 467 nm).

m Click the OK button.

n At the bottom right of the **Real Time Control** window, click the Cancel button.

o In the **Experiment** window, click the Run button to confirm the correct peak position. A correct scan is shown below (peak is at 467 nm):


FluorEssence - C:\Documents and Settings\All Users\Documents\Jobin Yvon\Data\UNTITLED *
File Edit View Collect Analysis Graph Data Gadgets Tools Format Window Help
[DfltEx1]Data!R1[268]: X = 467, Y = 0.08974
UNTITLED
Name
Com...
quantum dots (01) Fluor...
quantum dots (02) Fluor...
DfltEx (01) Spect...
DfltEx1G:{[DfltEx1]Graph!A[1:1]}
Cursor
R1
Wavelength in nm
Intensity (MicroAmps)
Wavelength (nm)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
200
400
600
Data Reader: Click to read data value. Left/right keys to go from point to p
AU : ON
Dark Colors & Light Grids
1:[DfltEx1]Data!Col("R1")[1:401]
1:[DfltEx1G]1!1
Radian

Emission calibration check

Note: *The emission calibration of the instrument is directly affected by the calibration of the excitation monochromator.*

This calibration check verifies the wavelength calibration of the emission monochromator with the emission photomultiplier tube. It is an emission scan of the Raman-scatter band of water performed in right-angle mode. Perform this check after the xenon-lamp scan. When completed, the performance of the system has been verified.

Use research-quality, triple-distilled or deionized water. HPLC-grade (18-MΩ spec.) or equivalent water is suggested for the Raman scan. Impure samples of water will cause elevated background levels as well as distorted spectra with (perhaps) some unwelcome peaks.

Note: *Avoid glass or acrylic cuvettes: they may exhibit UV fluorescence or filtering effects.*

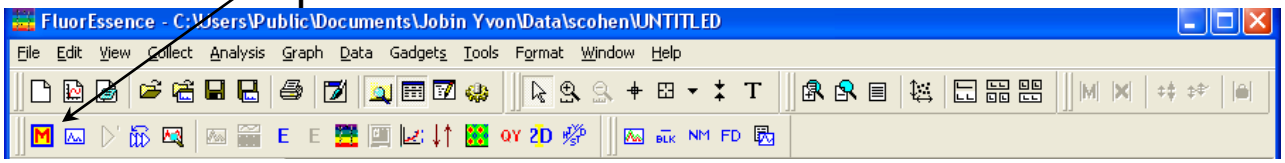
Use a 4-mL quartz cuvette to hold the water.

1 Insert the water sample into the sample compartment.

With an automated sample changer, note the position number in which the sample cell is placed.

2 Close the lid of the sample chamber.

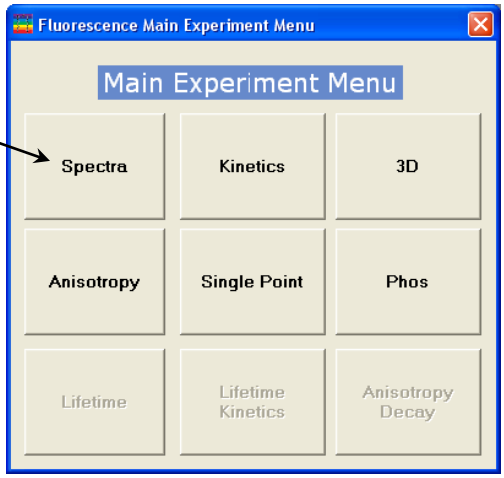
3 In the main FluorEssence toolbar, choose the Experiment Menu button .



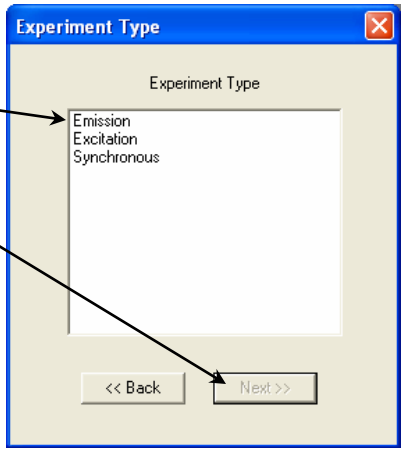
The **Fluorescence Main Experiment Window** opens.

4 Choose the Spectra button.

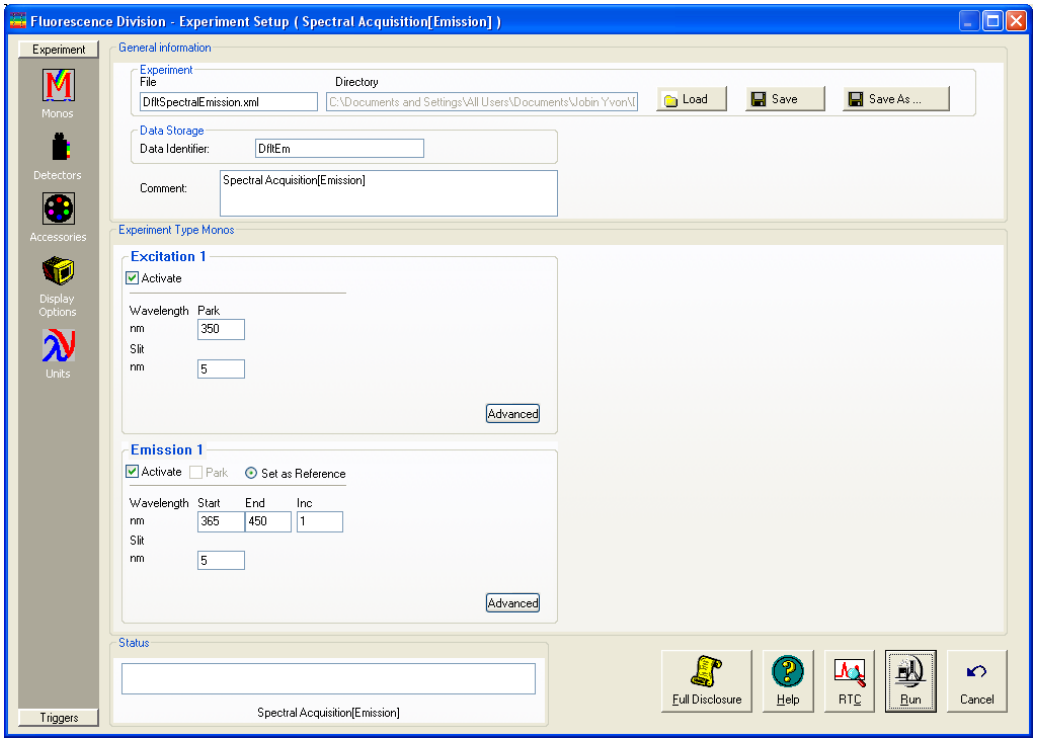
The **Experiment Type** window appears:



5 Choose Emission, then click the Next >> button.



The water-Raman experiment automatically loads.



6 Use the default parameters or adjust them.

Monochromator parameters for the water-Raman scan:

Monochromator (1200 grooves/mm)	Initial wavelength	Final wavelength	Increment	Slits (bandpass)
Excitation	350 nm	--	--	5 nm
Emission	365 nm	450 nm	1 nm	5 nm

Detector parameters for the water-Raman scan:

Detector (Signal)	Integration time	Units
Signal (S1)	0.1 s	CPS
Reference (R1)	0.1 s	mA

7 Click the Run button 

.

The **Intermediate Display** opens. The water-Raman scan runs.

A new Fluorolog®-3 should display a water-Raman peak intensity of at least 450 000 counts s^{-1}.

Note: *Observed throughput (and hence peak intensity) is affected by lamp age and alignment, slit settings, and sample purity. As the xenon lamp ages, the throughput of the system will decline slowly. Therefore, low water-Raman peak intensity may indicate a need to replace the xenon lamp.*

8 If the water-Raman peak is not at 397 nm, calibrate the emission monochromator as shown for the excitation-monochromator calibration on pages 3-6 to 3-9.

Calculation of water-Raman signal-to-noise ratio

Introduction

The water-Raman test is a good measure of relative sensitivity between different instruments, if the experimental conditions used to compare the systems are the same. Unfortunately, there are different ways of handling the data, all of which are valid but which will give quite different values. Therefore, it is important not only to know how the water-Raman *S*/*N* values are measured, but also how the data were treated. The water Raman *S*/*N* test method combines a value for system sensitivity (a signal) with a value for system noise (no signal) to show the overall performance of the instrument.

Definitions

At HORIBA Scientific, we define the *S*/*N* as the difference of peak and background signal, divided by the square root of the background signal.

– $\frac{\text{peak} - \text{background}}{\sqrt{\text{background}}}$ Our method

The peak signal is measured at the water-Raman peak (397 nm for 350 nm excitation) and the noise in a region (450 nm) where no Raman signal is present. An "ideal" system would give a signal value of zero at 450 nm. Thus the equation becomes

– $\frac{\text{peak}}{\text{rms noise}}$

Another commonly used method is to divide the difference (peak signal minus background signal) by the rms value of the noise on the background signal. This second method is used by a few other manufacturers:

– $\frac{\text{peak} - \text{background}}{\text{rms noise}}$ Other manufacturers' method

Example

Some actual data from a Fluorolog® FL3-11 system (a typical system, a few years old) serve to show the difference between the two methods. The experimental conditions were as follows:

Excitation	350 nm with 5 nm bandpass
Emission	360–450 nm with 5 nm bandpass
Increment	1 nm
Integration time	1 s

No smoothing of data points
Standard room-temperature, red-sensitive detector

Note: *Make sure the test is carried out with the actual detector you will be using. All HORIBA Scientific systems' parameters are specified with a R928P photomultiplier tube at room temperature.*

Results

The measurements provided the following data:

S_{peak}	peak signal at 397 nm	501 500 cps
$S_{background}$	background noise at 450 nm	10 500 cps
N_{p-p}	Peak-to-peak noise of background at 450 nm*	223 counts

*Measured with a separate kinetic scan

N_{p-p} gives an rms noise of the background signal of

$$\approx 44.6$$

The HORIBA Scientific method gives a water-Raman *S*/*N* of

The other method similarly gives a water-Raman *S*/*N* of

HORIBA Scientific feels that the first method is correct although it gives a lower *S*/*N*. The second method only takes into account the detector noise and the shot noise of the electronics. By using the background total intensity as a measure of noise, the HORIBA Scientific method is more representative of a real “live” experiment where noise is also influenced by factors like the quality of the optics and scattered light in the system. These additional factors influence the ability to measure a very low signal from a sample and should not be ignored.

Notes on excitation and emission calibration

- Two experiments, `lamp.exp` and `water.exp`, have been defined and saved. They can be run after the system is switched on each day to check the calibration and performance of the Fluorolog®-3.
- HORIBA Scientific recommends that the number of hours of xenon-lamp use be recorded in a log (see sample sheet in *Xenon Lamp Information & Record of Use Form*).
- Additionally, you may want to record the water-Raman intensity daily or weekly.
- The lamp is rated for 1800–2000 h, but if the Raman intensity starts to drop, you may wish to change the lamp sooner.

Warning: *To avoid explosion from lamp failure, do not allow xenon lamp to exceed rated lifetime. One clue to imminent failure may be extremely low water-Raman intensity. Please record the hours the xenon lamp has been used.*

Useful materials for characterizing system and samples

The following are materials that HORIBA Scientific has found useful in determining system sensitivity or as standards for lifetime measurements.

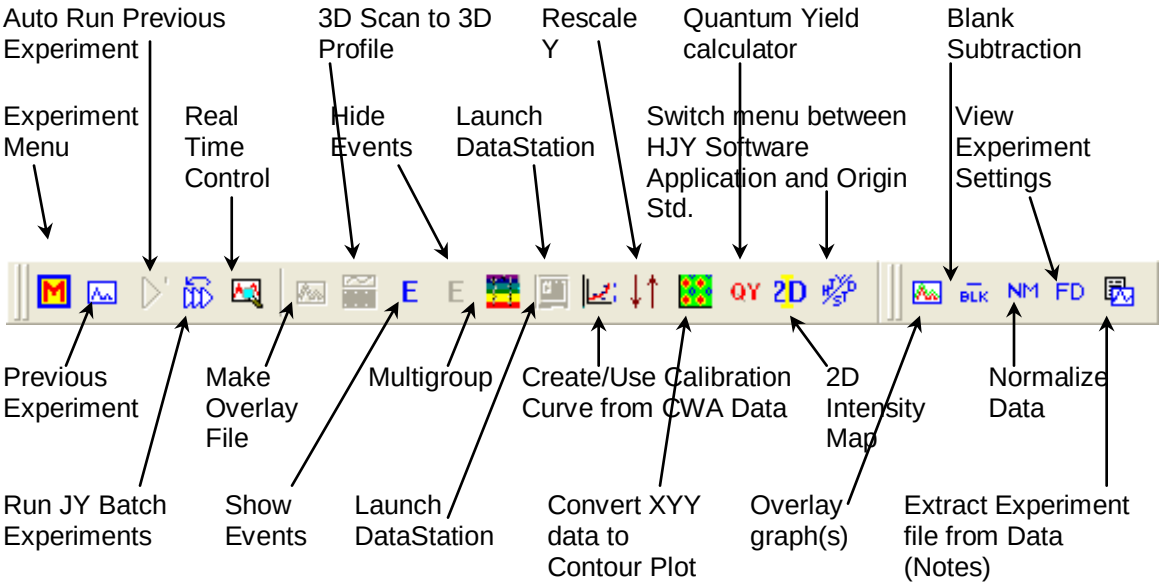
Substance	CAS Number	Purpose	Emission Wavelength Range (nm)	Lifetime (ns)
Anthracene (99+%, zone-refined)	120-12-7	Excitation and emission spectral characterization	380–480	4.1 (in MeOH)
Europium(III) chloride hexahydrate (99.9%)	13759-92-7	Phosphorescence emission and decay standard	580–700	1.40×10^5
Fluorescein (99%)	2321-07-5	Lifetime and sensitivity standard	490–630	4.02 (in pH ≥ 11)
D-glycogen	9005-79-2	Light-scattering standard		0
LDS 750, or Styryl 7	114720-33-1	Single-exponential lifetime standard	680–700	0.248 (in MeOH, λ_{exc} = 568 nm)
Ludox®, or colloidal silica	7631-86-9	Light-scattering standard		0
β-NADH (β-nicotinamide adenine dinucleotide)	606-68-8 or 104809-32-7	Single-exponential lifetime standard	390–600	0.38 ± 0.05 (in pH = 7.5)
POPOP (99+%), or 1,4-bis(5-phenyloxazol-2-yl)] benzene	1806-34-4	Single-exponential lifetime standard	370–540	1.32 (in MeOH)
PPD (97%), or 2,5-diphenyl-1,3,4-oxadiazole	725-12-2	Single-exponential lifetime standard	310–440	1.20 (in EtOH)
PPO (99%), or 2,5-diphenyloxazole	92-71-7	Single-exponential lifetime standard	330–480	1.40 (in EtOH)
Rose Bengal (90%), or 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein	632-69-9	Single-exponential lifetime standard	560–680	0.98 ± 0.10
p-Terphenyl (99+%)	92-94-4	Single-exponential lifetime standard	310–410	1.05 (in EtOH)
Water (18-MΩ, de-ionized, triple-distilled)	7732-18-5	water Raman sensitivity test		

Caution: *Read the Materials Safety Data Sheets that accompany these substances before using them.*

Chapter 4 : Data Acquisition

Introduction

This chapter presents an introduction to the special buttons used in FluorEssence™ to record and present data with the Fluorolog®-3. These buttons, located in FluorEssence™'s main window, are:



For a detailed description of these FluorEssence™ routines, see the *FluorEssence™ User's Guide* and on-line help.

In addition, two methods for determining best excitation and emission wavelengths are presented, in case these wavelengths are unknown for the sample.

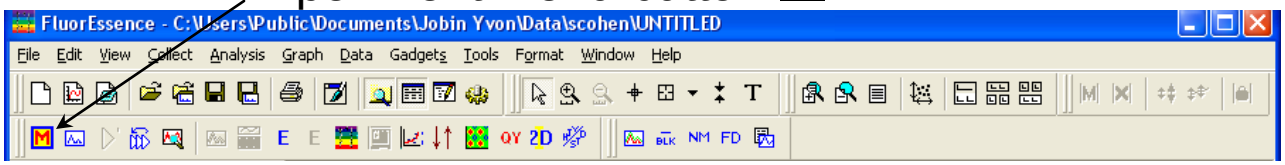
Experiment Menu button

The Experiment Menu button chooses an overall type of experiment to run, such as an emission scan, a phosphorimeter scan, a synchronous scan, etc., based on the instrument and connected accessories, such as a temperature bath, MicroMax, etc. Only those scans that can be run using the available hardware configuration are active; scans that cannot be taken are grayed out.

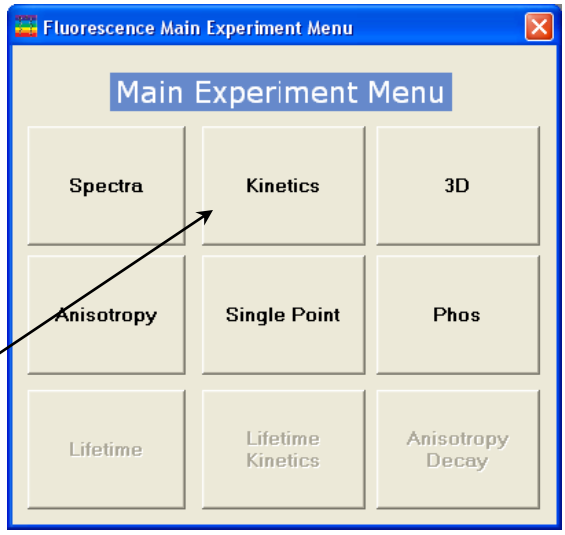
Calibration scans for the Fluorolog®-3 use default parameters:

- Excitation monochromator: Spectra/Excitation scan
- Emission monochromator: Spectra/Emission scan

1 To choose an experiment type, click the Experiment Menu button :



The **Fluorescence Main Experiment Menu** appears:



Note: *Those scan types not allowed with the instrument configuration are grayed out.*

2 Choose an experiment type.

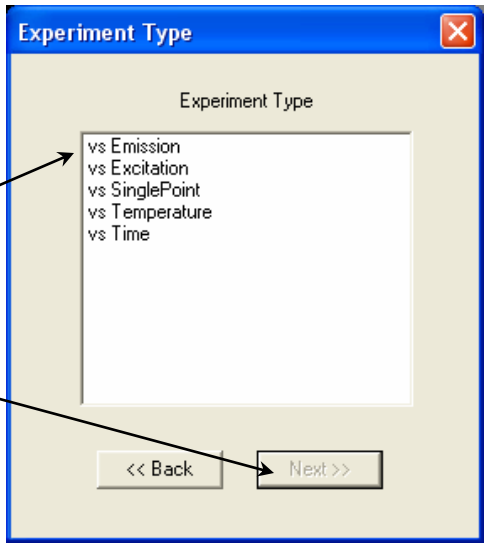
The **Experiment Type** menu appears.

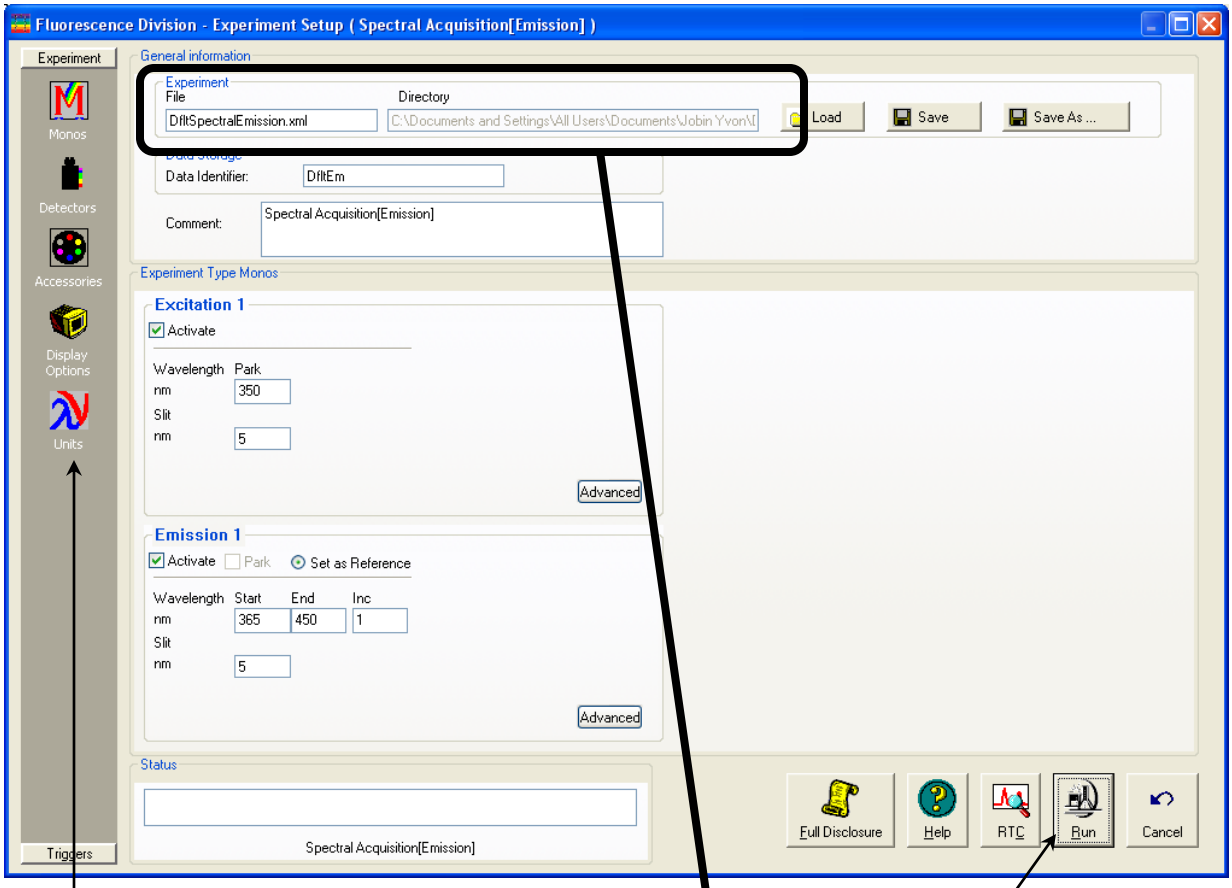
Note: *The list of subtypes depends upon the general type of experiment chosen.*

3 Choose a subtype of experiment.

4 Click the Next >> button.

The **Experiment Setup** window appears:





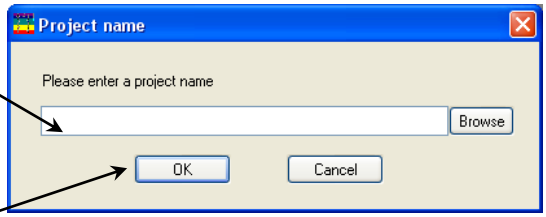
5 Click the Experiment File field, and enter a new file name or select a previously saved file.

6 Verify that experimental parameters are correct.
Be sure to check all parameters under all icons in the left-hand column.

7 Insert the sample into the sample compartment, and close the sample-compartment's cover.

8 Click the Run button 

.
The collected spectrum is displayed on the **Intermediate Display** screen. After all data are recorded, the **Intermediate Display** vanishes. For a new project, the **Project Name** window appears:



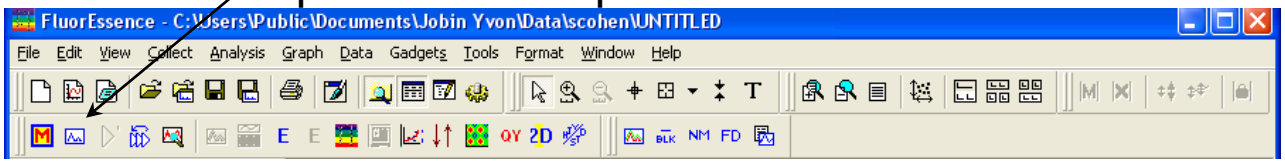
9 Enter a name for the entire project, or browse for an existing project name via the Browse button, then click the OK button.
All data are moved to Origin®'s graph window for post-processing.

Previous Experiment Setup button

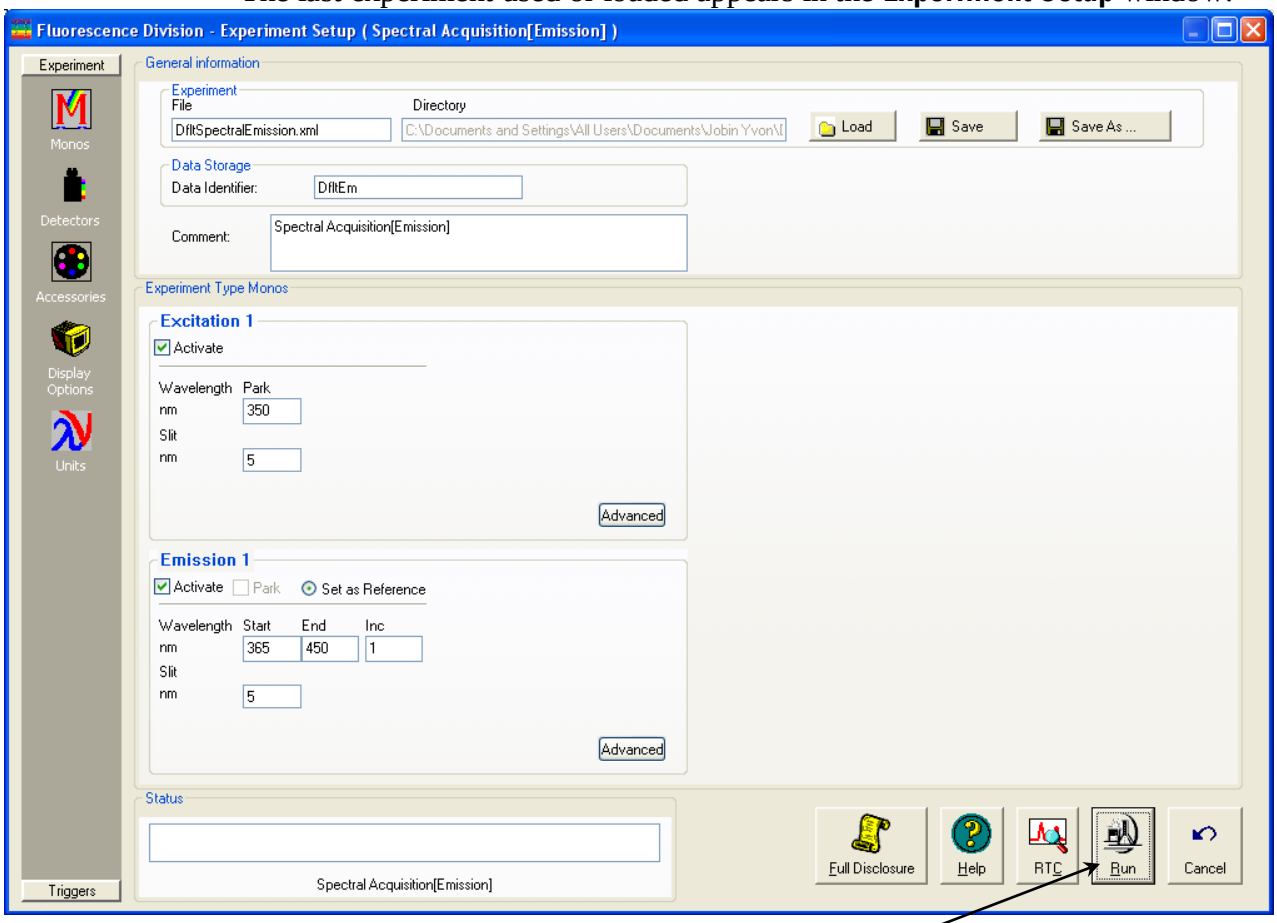
The Previous Experiment Setup button resets the experiment to the previous experiment used, with minor modifications to the hardware possible.

Note: *The* Previous Experiment Setup *button is active only after an experiment has already been loaded.*

1 After an experiment is loaded, click the Previous Experiment Setup button in the main toolbar.



The last experiment used or loaded appears in the **Experiment Setup** window:



2 Modify the parameters as required.

3 Click the Run button 

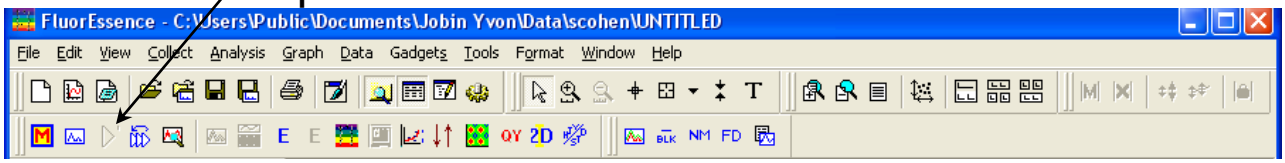
to run the experiment.

Auto Run Previous Experiment button

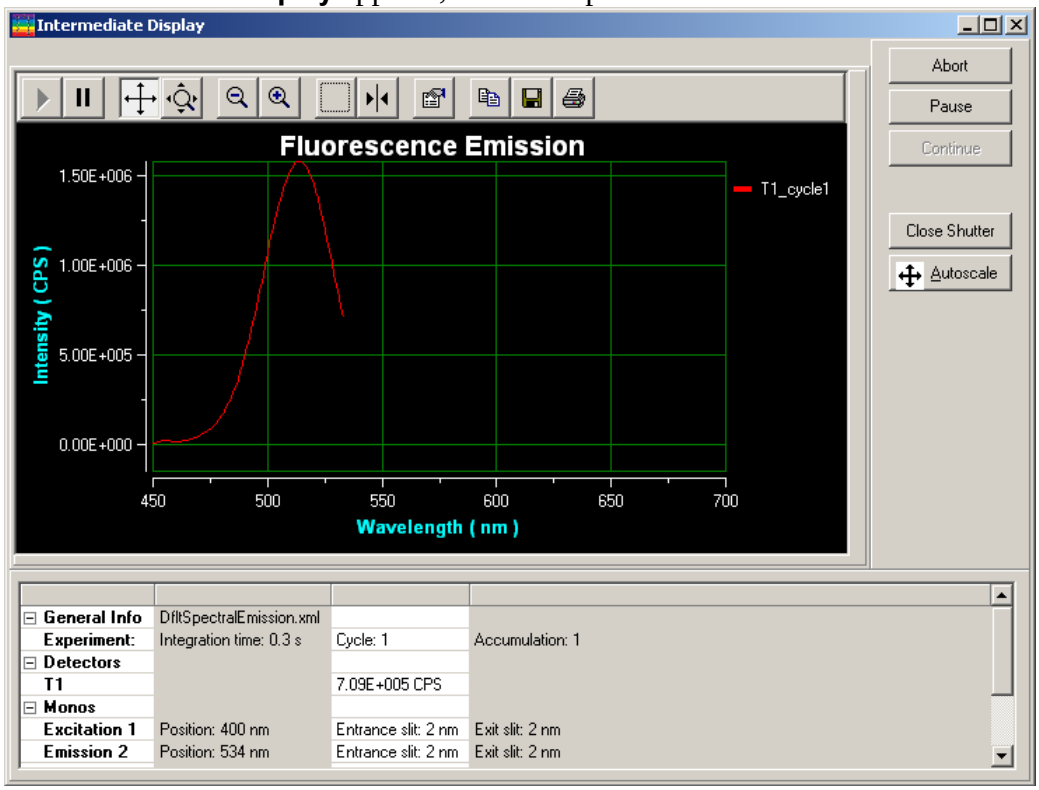
The Auto Run Previous Experiment button reruns the last experiment loaded without modifications.

Note: *The* Auto Run Previous Experiment *button is active only after an experiment has already been loaded and run.*

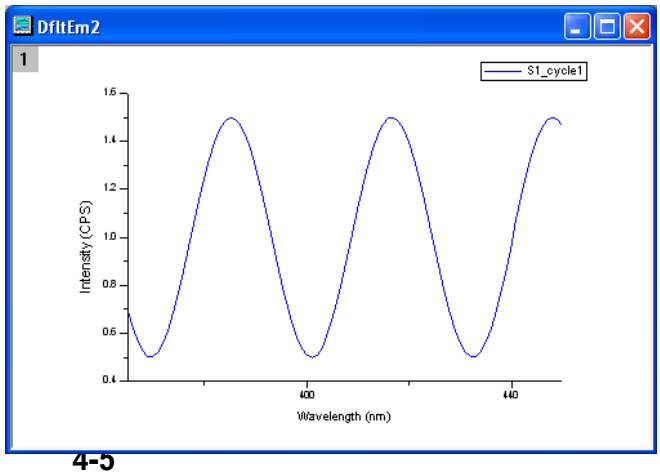
1 In the toolbar, click the Auto Run Previous Experiment button.



The **Intermediate Display** appears, and the experiment starts:



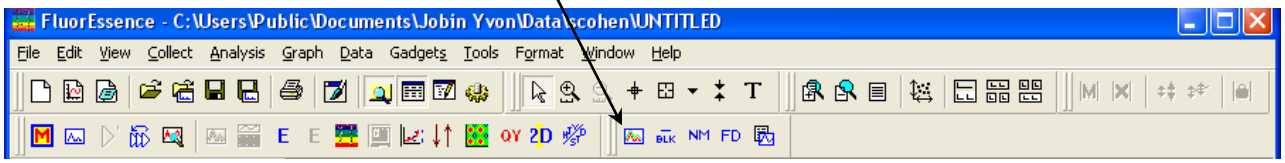
When the experiment is complete, the data are moved into a new Origin® graph window:



Make Overlay File button

The Make Overlay File button creates an `*.SPC` file for use as an overlay file. An overlay file can be used to compare data in real time. Inclusion of an overlay file is controlled in the Display Options icon in the **Experiment Setup** window. The overlay file appears in the **Intermediate Display** as data are acquired. Upon completion of the acquisition, both sets of data appear on the plot.

1 Click the Make Overlay File button in the toolbar.

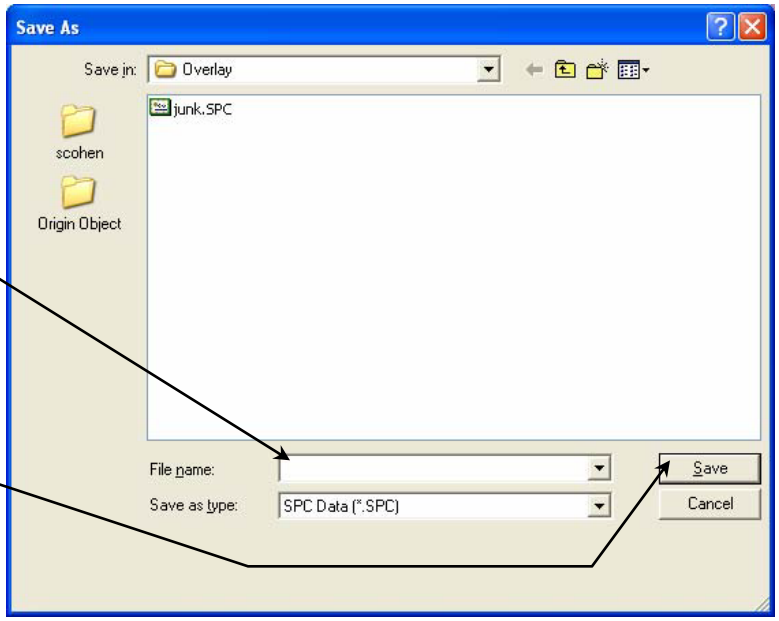


The **Save As** window appears.

2 Enter a file name in the File name field.

3 Click the Save button.

The overlay file is saved.

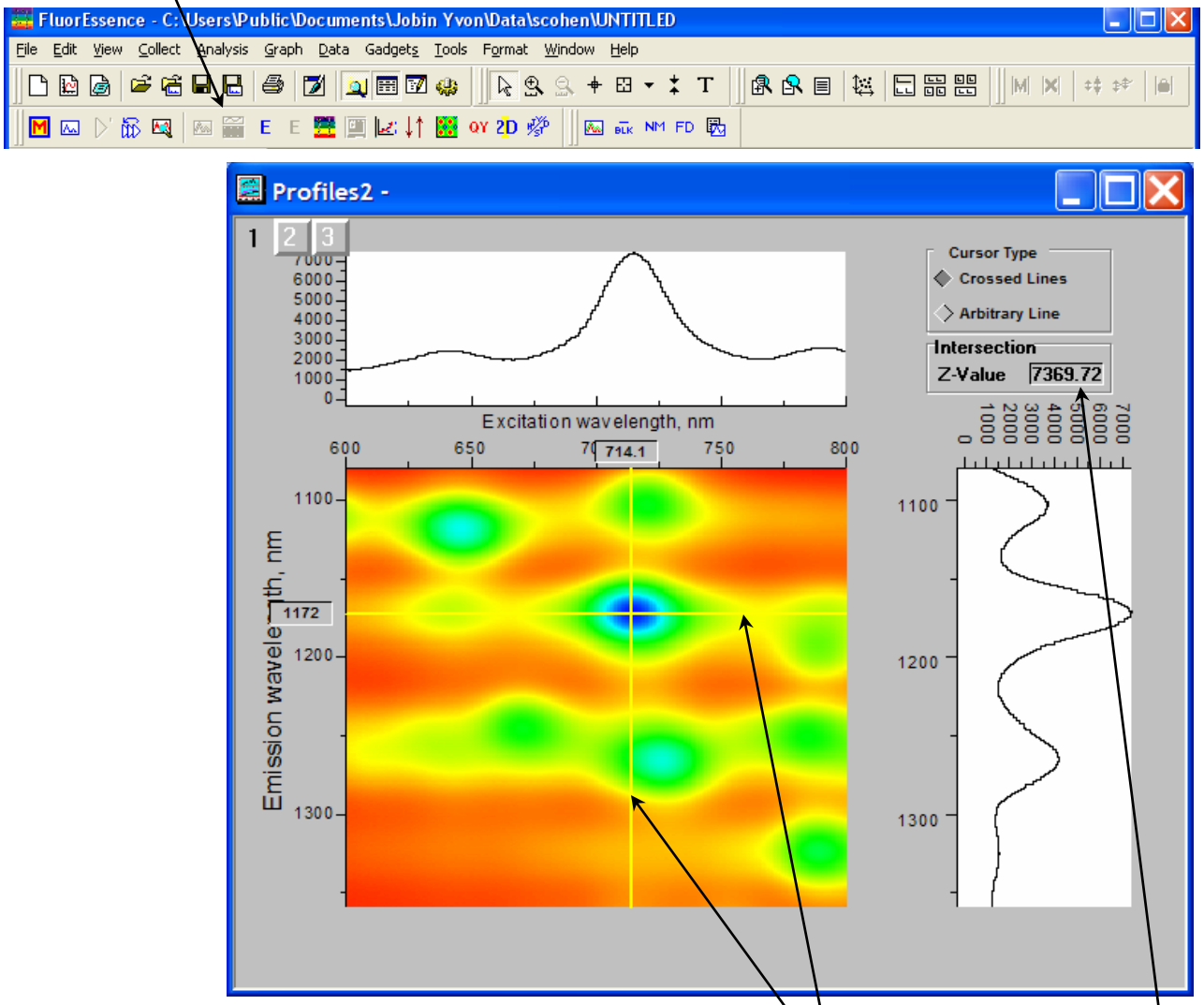


3D Scan to 3D Profile button

The 3D Scan to 3D Profile button extracts emission profiles from an excitation-emission matrix.

1 Open excitation-emission matrix data.

2 Click the 3D Scan to 3D Profile button in the toolbar.

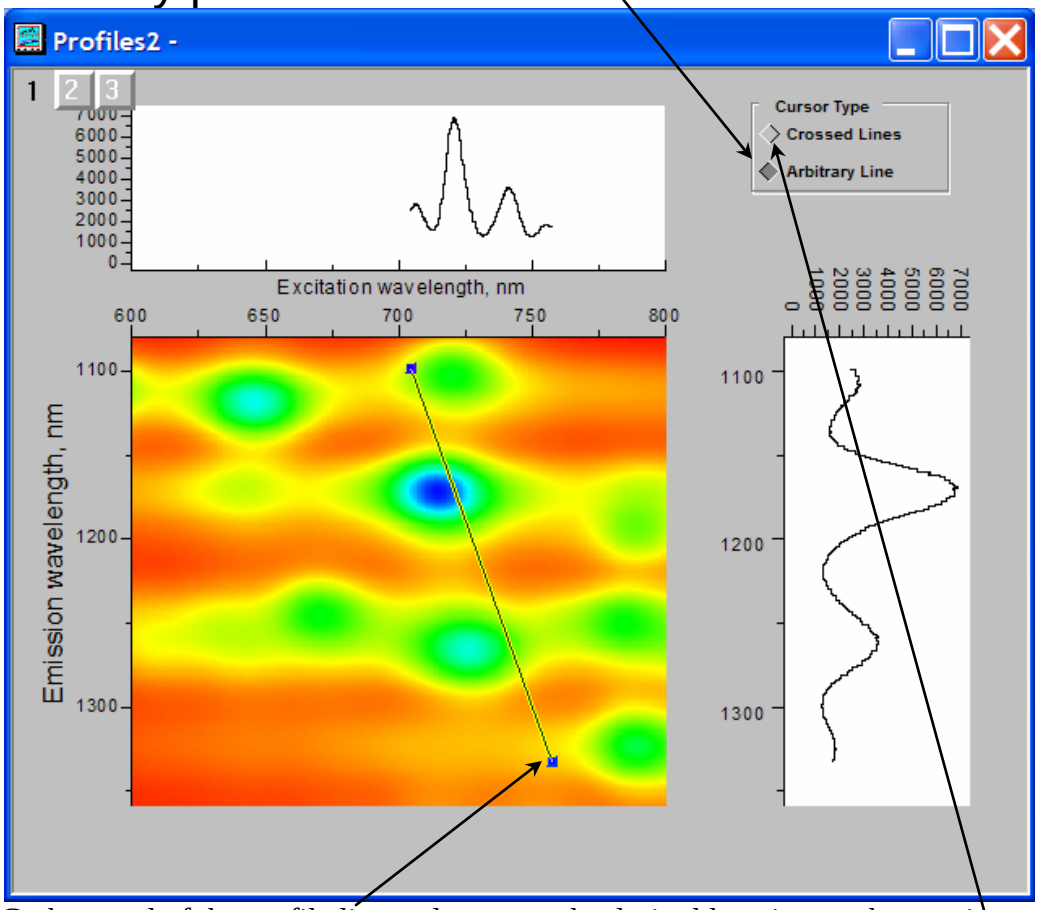


The **Profiles** window appears.

3 Grab and move the emission and excitation profile lines to see the profiles above and to the right of the data-matrix.

The Z-Value field shows the intensity where the excitation and emission profiles intersect.

4 Click the Arbitrary Line button to choose an arbitrary profile.



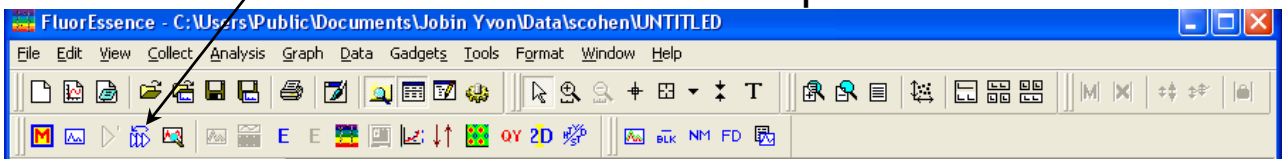
Grab an end of the profile line and move to the desired location on the matrix. The profiles are updated.

5 To return to perpendicular profiles, click the Crossed Lines button.

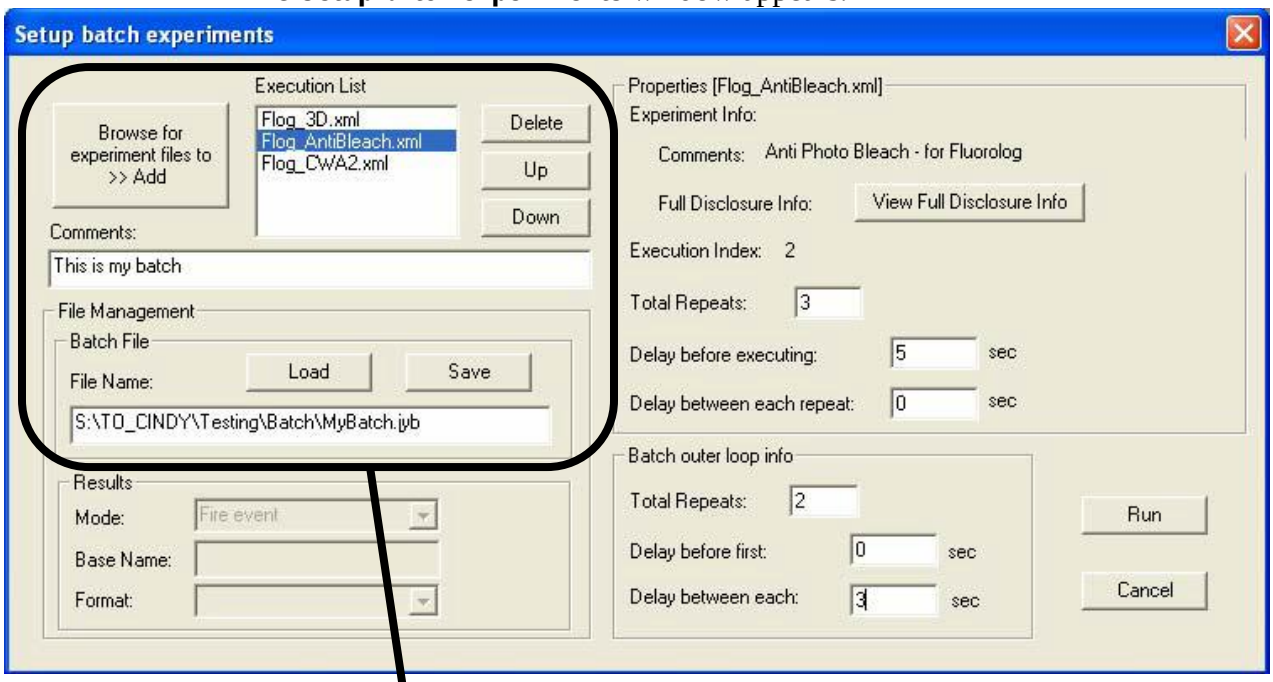
Run JY Batch Experiments button

The Run JY Batch Experiments button runs a series of automated experiments, including adjustable repeats and delays between experiments.

1 Click the Run JY Batch Experiments button.



The **Setup batch experiments** window appears.



2 Get the experiment files to create a batch job, or load a previous batch job.

a Load a previously created batch job using the Load button, or browse for experiment files (`.xml` format) using the Browse for experiment files to >> Add button.

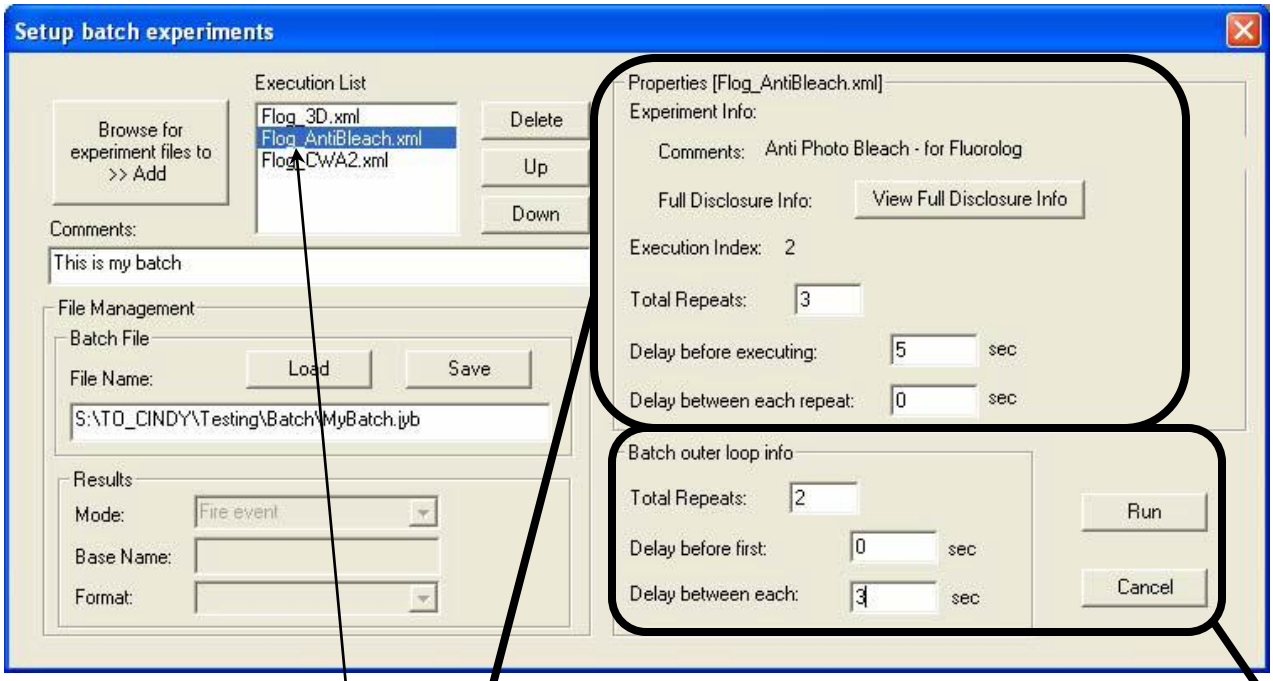
b Add each desired experiment file to the Execution List.

c Reorder or remove the files as necessary using the Delete button, the Up button, and the Down button.

d Add comments about the batch file in the Comments: field.

e Save the new batch job in the correct path, in the File Name: field, and click the Save button.
The file is saved in a `.jyb` format.

3 Set up each experiment in the batch job.



a Select an experiment from the Execution List.

b In the Total Repeats: field, enter the number of times that experiment should be repeated.

c In the Delay before executing: field, enter the number of seconds to wait before executing.

d In the Delay between each repeat list: field, enter the number of seconds to wait before repeating the experiment.

4 Set up an outer loop in the batch job, if desired.

a In the Total Repeats: field, enter the number of times to run the batch job.

b In the Delay before first: field, enter the number of seconds to wait before starting the batch job.

c In the Delay between each: field, enter the number of seconds to wait before rerunning the batch job.

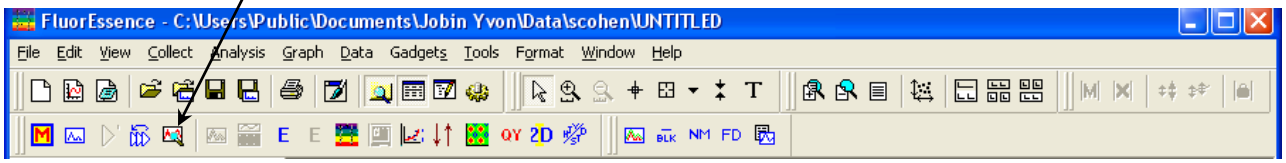
5 Click the Run button to start the batch job.

The batch job executes.

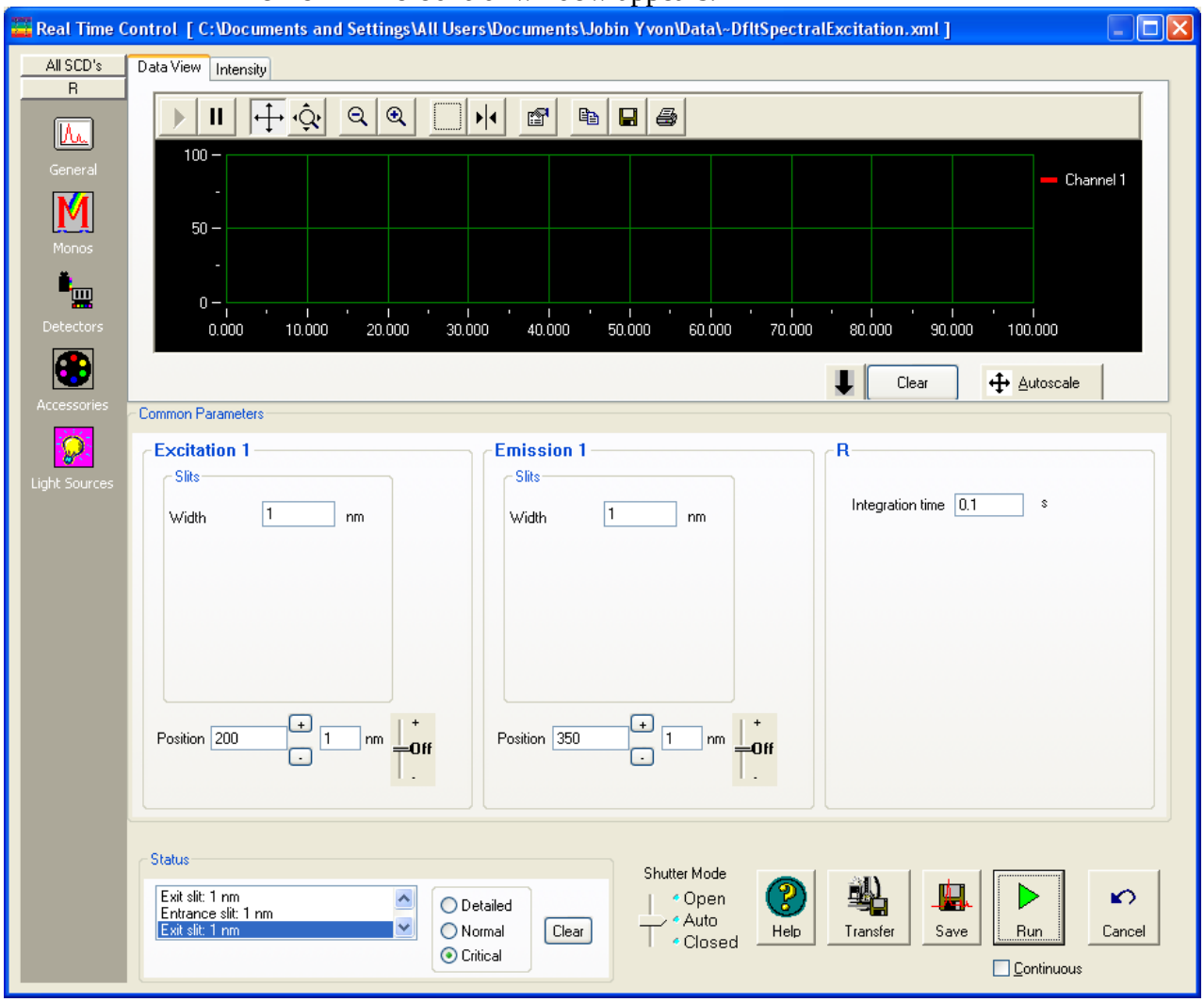
Real Time Control button 

The Real Time Control button opens the **Real Time Control** window directly, so that the user can adjust experimental parameters in real time.

1 In the toolbar, click the Real Time Control button  .



The **Real Time Control** window appears:



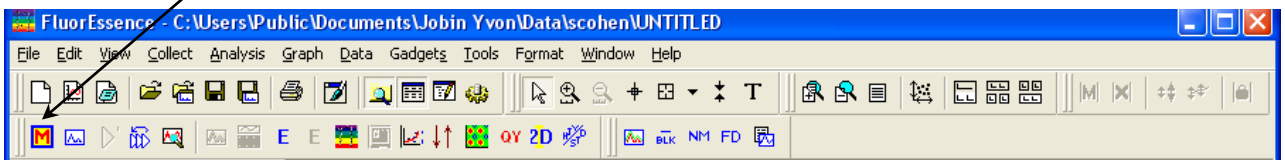
2 Adjust the desired instrumental parameters as necessary.

Create/Use Calibration Curve from CWA Data button

Note: *This button only operates if CWA data exist.*

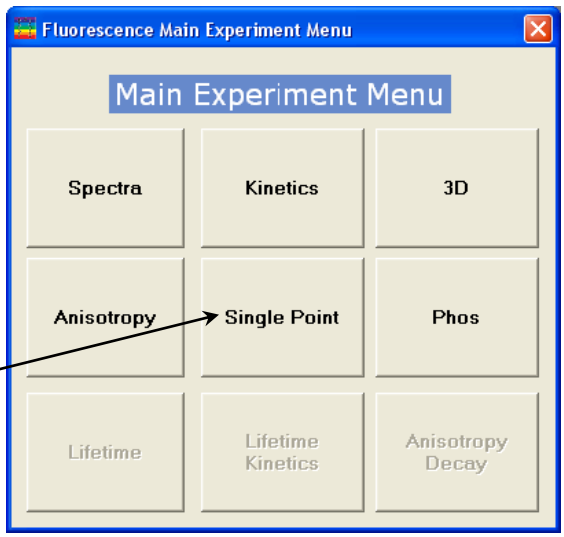
When the user is doing Single Point experiments (especially with the MicroMax or multiple-sample changers), the Create/Use Calibration Curve from CWA Data button creates a calibration curve for analytical measurements.

1 In the toolbar, click the Experiment Menu button :



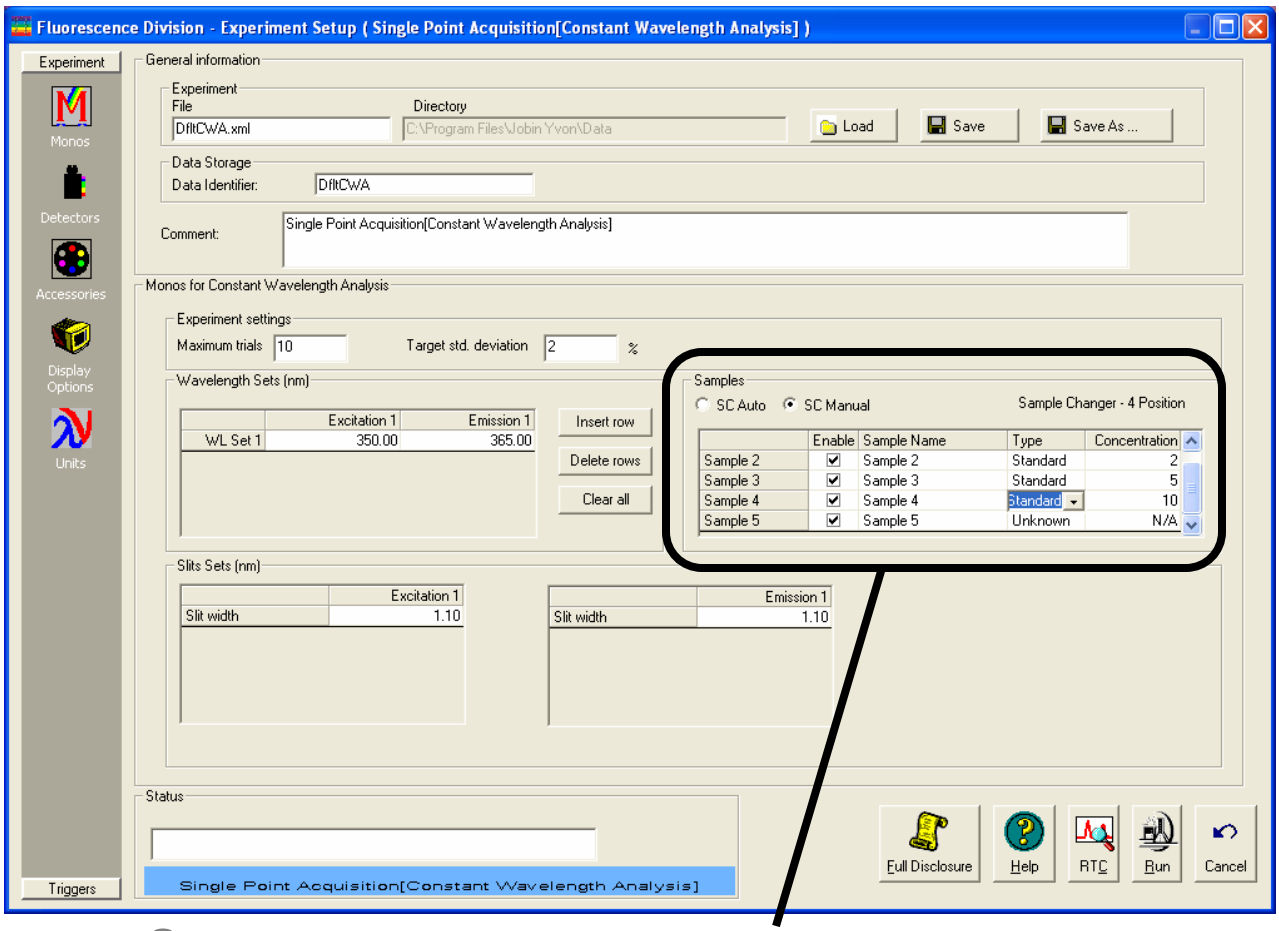
The **Fluorescence Main Experiment Menu** appears:

Note: *Those scan types not allowed with the instrument configuration are grayed out.*



2 Choose the Single Point button.

The **Experiment Setup** window appears:



3 Set up the standards and unknown(s).

a If you have an automatic sample changer, you can still choose to change the samples manually with the SC Manual radio button. Otherwise, the software changes the samples automatically.
This allows more than the default number of samples in the sample changer (i.e., more than 2 for the dual sample-changer or more than 4 for the four-sample-changer).

b To add more samples (if manually changed), place the cursor in the last sample-row's Concentration field, and press the `Enter` key twice.
A new sample row appears below the last sample row.

c Under the Type column, choose the type of sample from the drop-down menu: Standard, Unknown, Blank, or Empty.

d Enter the concentrations of the standards in the Concentration column. The unknowns retain the default "N/A" concentration.

e Click the Enable checkbox of all the samples to be measured.

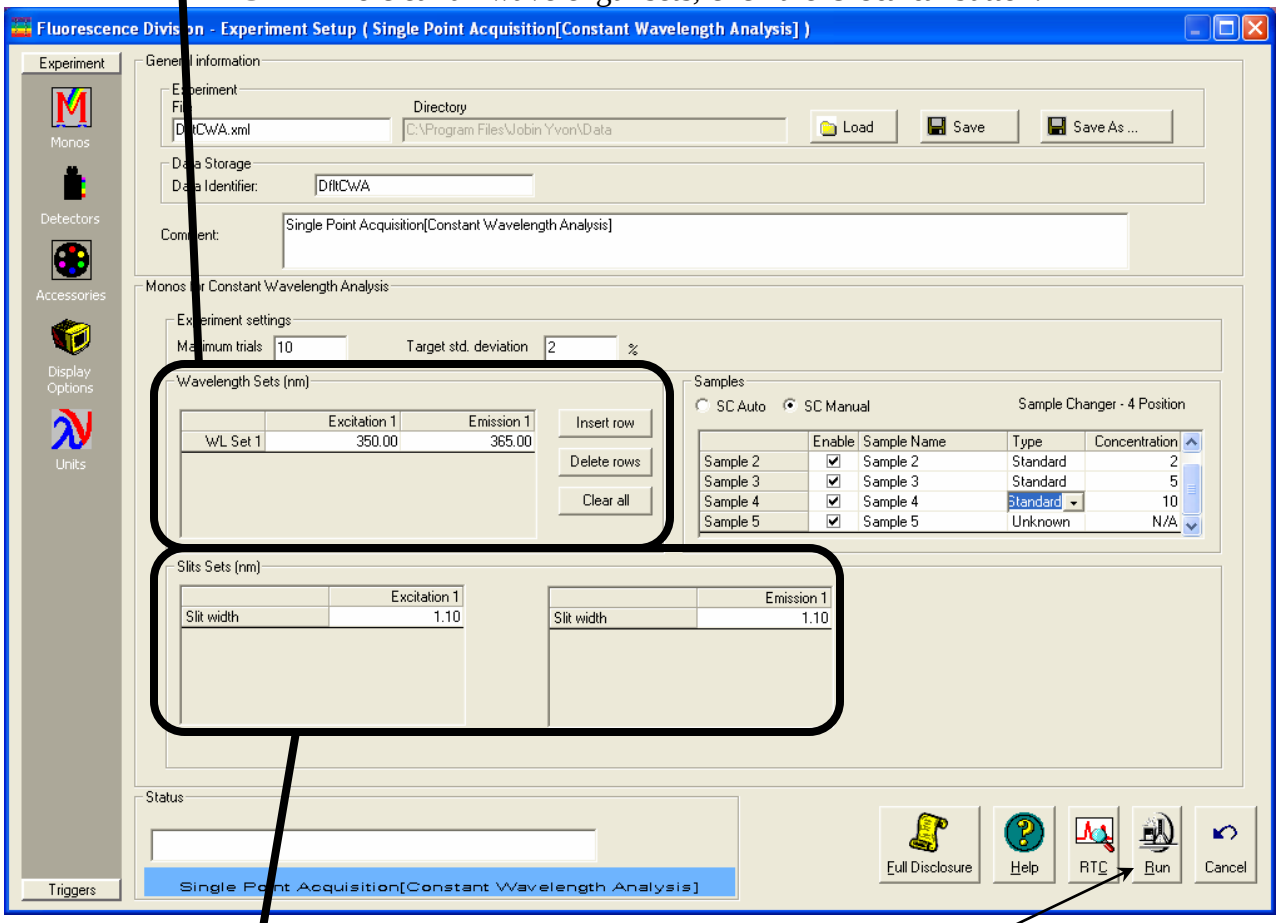
4 Set up the excitation and emission wavelength(s).

a Enter the appropriate excitation and emission wavelength set(s), one per row, in the Wavelength Sets area.

b To add a wavelength set, click the Insert row button.

c To delete a wavelength set, select the rows, and click the Delete rows button.

d To clear all wavelength sets, click the Clear all button.



5 Enter the excitation and emission slit-width(s) in the Slit Sets area.

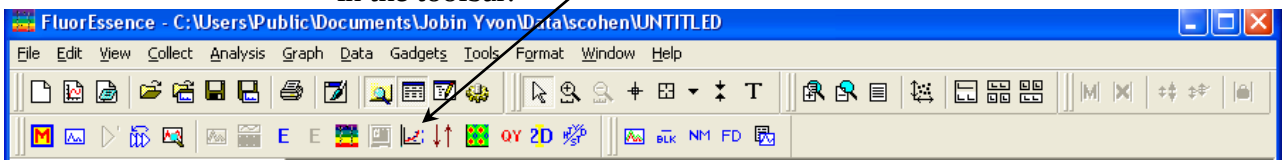
6 Click the Run button 

.

The experiment starts. FluorEssence™ prompts you to insert the sample, if you chose to change the samples manually. The dataset is recorded and appears on the screen.

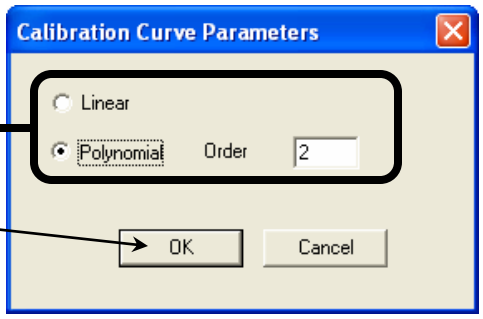
7 Create the calibration curve.

a Click the Create/Use Calibration Curve from CWA Data button in the toolbar.

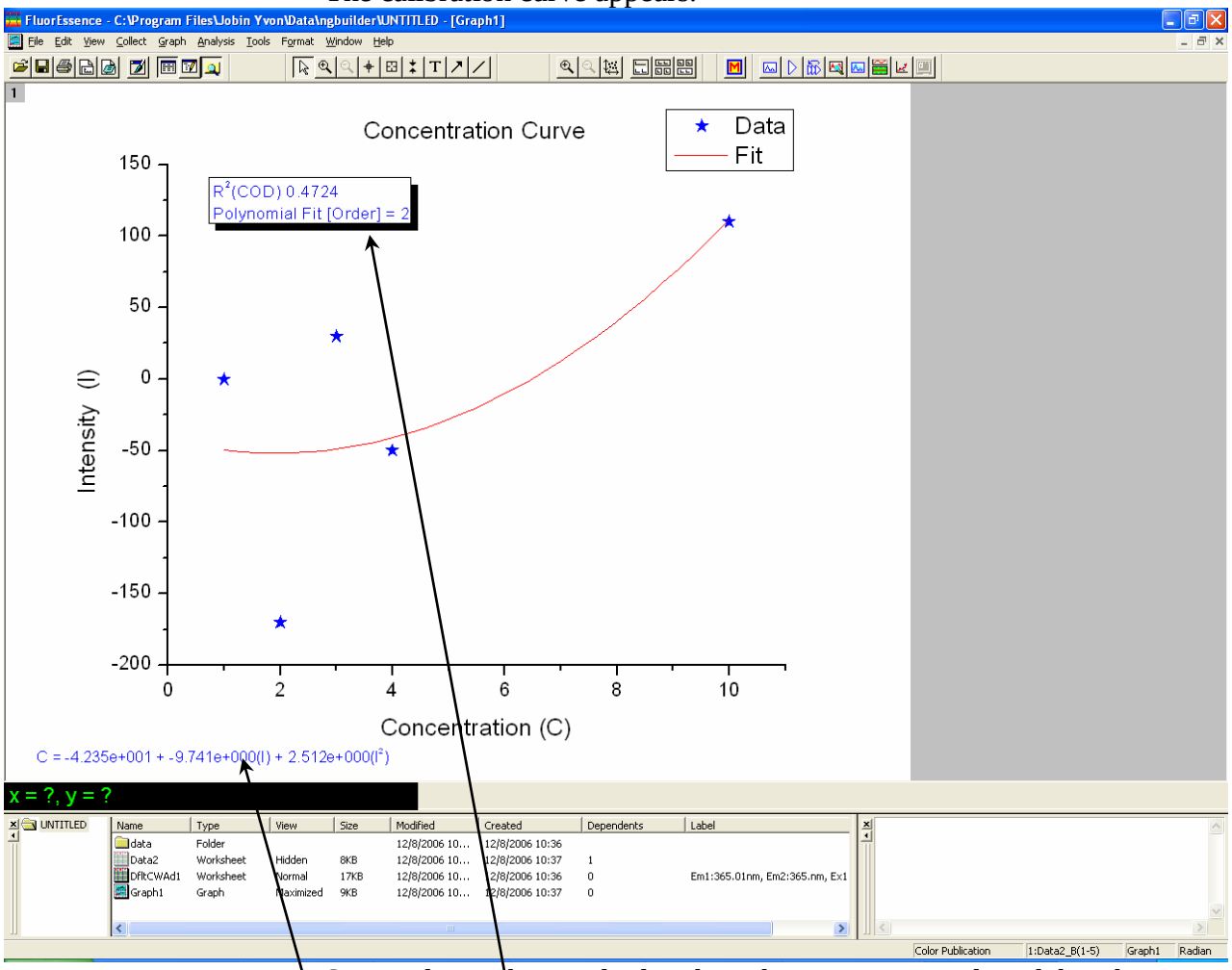


The **Calibration Curve Parameters** window appears.

b Choose a Linear fit radio button, or a Polynomial fit radio button with Order of the polynomial, and then the OK button.
The **Save Curve** window asks if you wish to save the curve.



c Answer yes or no.
The calibration curve appears.



Stars indicate the standards. The unknowns are not plotted, but their calculated concentrations are filled in on the dataset. The fit equation and correlation are displayed on the plot.

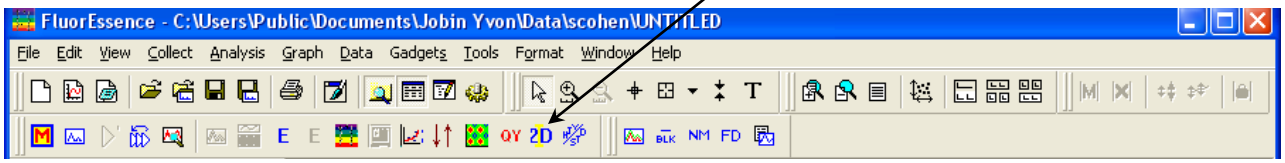
2D Intensity Map button 

This button creates a two-dimensional intensity map from the active data.

Note: *This button only operates if data are displayed, and is used for microscope mapping purposes.*

1 Be sure that data are open in FluorEssence™.

2 In the toolbar, click the 2D Intensity Map button  .

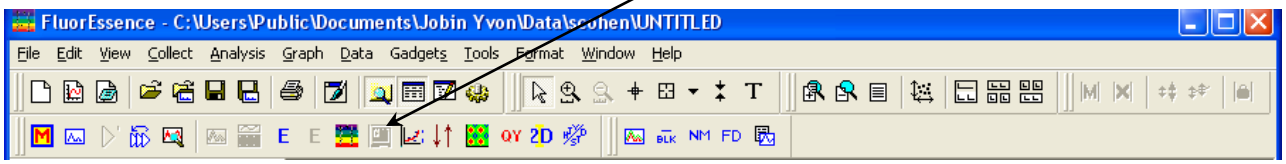


Launch DataStation button

The Launch DataStation button closes the FluorEssence™ software, and starts DataStation software.

Note: *This button only operates with TCSPC accessories.*

1 In the toolbar, click the Launch DataStation button.



FluorEssence™ shuts down. DataStation starts.

Running an unknown sample

Often a researcher will scan a sample whose spectral characteristics are unknown. For optimal spectra, the optimal excitation and emission wavelengths must be found.

The traditional method consists of running an emission scan to find the peak emission value. Then an excitation scan is run using the determined peak emission value.

1 Find the preliminary emission maximum.

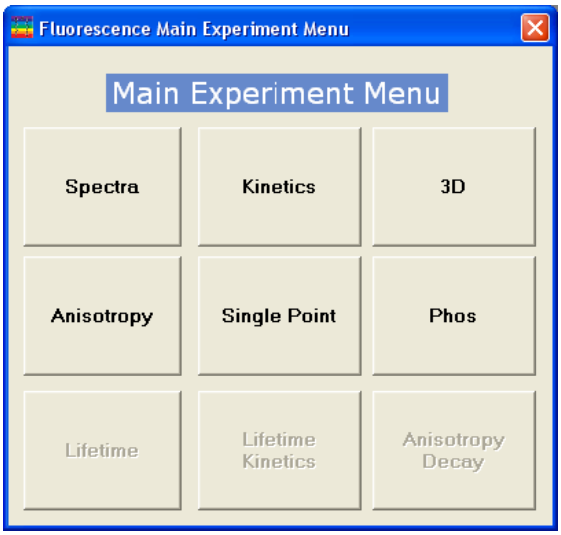
The object of this step is to acquire a preliminary emission scan, based on a "best-guess" excitation wavelength. Because the fluorescence emission of samples does not shift with excitation wavelength, the guessed excitation wavelength yields the emission peak, albeit perhaps at lower intensity.

a Be sure all system components are on, and the Fluorolog®-3 is calibrated as explained in Chapter 3.

b Set up the sample with the fiber-optic probe, with minimal stray-light interference.

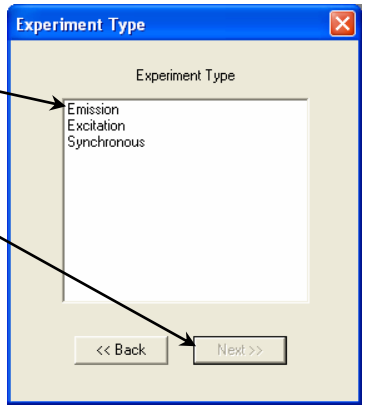
c In the toolbar, click the Experiment Menu button  :

The **Fluorescence Main Experiment Menu** appears:



d Choose the Spectra button.

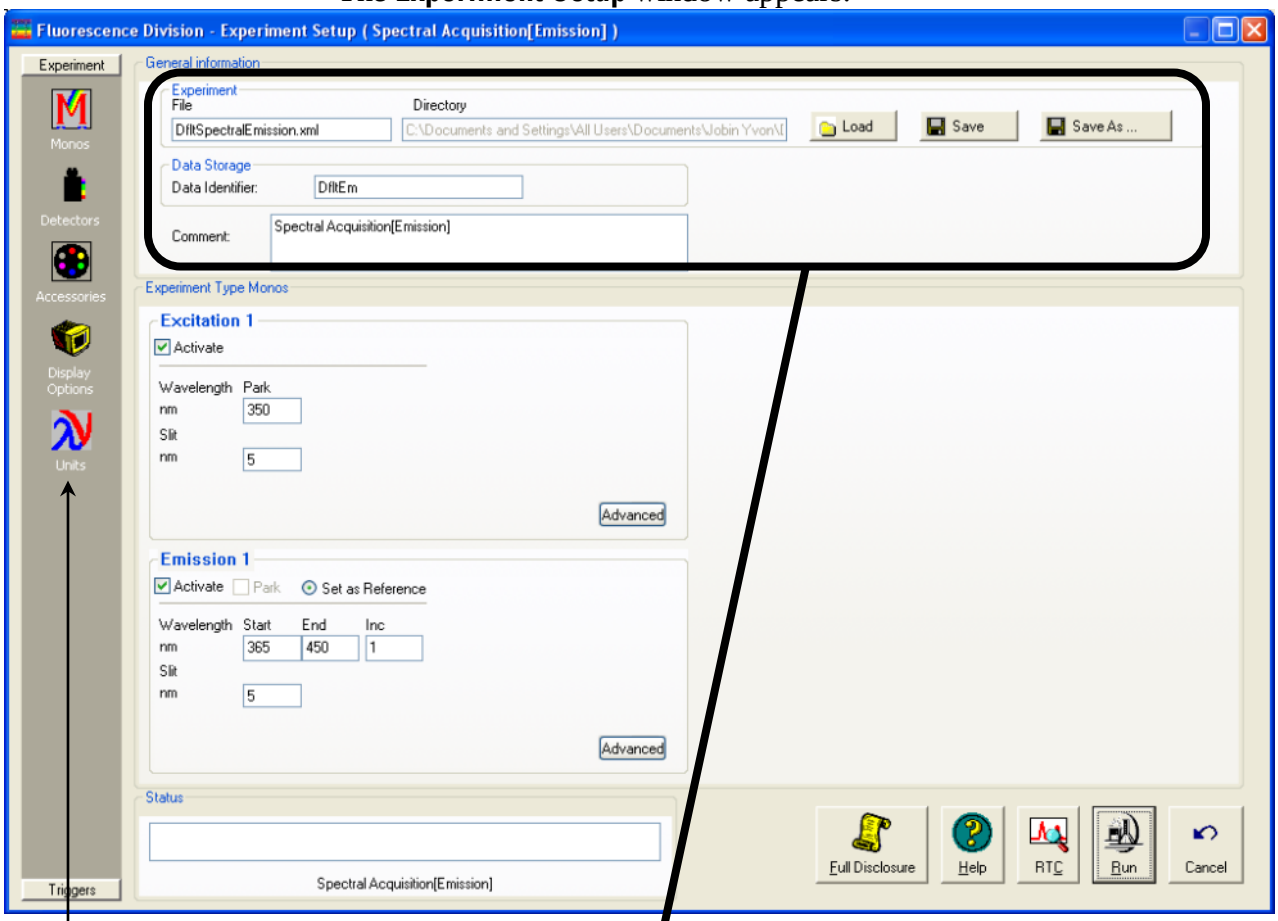
The **Experiment Type** menu appears:



e Choose the Emission button.

f Click the Next >> button.

The **Experiment Setup** window appears:



g Click the Experiment File field, and enter a new file name or select a previously saved file.

h Verify that experimental parameters are correct.
Be sure to check all parameters under all icons in the left-hand column.

i Set the scan parameters.
Most of these parameters are a trade-off between speed and precision. Choose integration time, increments, and number of scans judiciously, to give an accurate result without excessive time spent. HORIBA Scientific suggests an increment of 1.0 nm, an integration time of 0.1–0.5 s, and one scan. If unsure of an excitation wavelength, try 300 nm, at which many samples absorb light. Use S (signal detector) for the acquisition mode. Don't forget a data file name.

Note: *To minimize Rayleigh scatter, offset the start position by at least 15 nm from the excitation wavelength, with a bandpass of 5 nm. For example, for an excitation wavelength of 300 nm, use 315 nm as the start. Set the ending wavelength to 550 nm. Use an increment of 2 nm and an integration time of 0.1 s.*

j Insert the sample into the sample compartment, and close the sample compartment's cover.

k Click the Run button 

.
The scan starts.

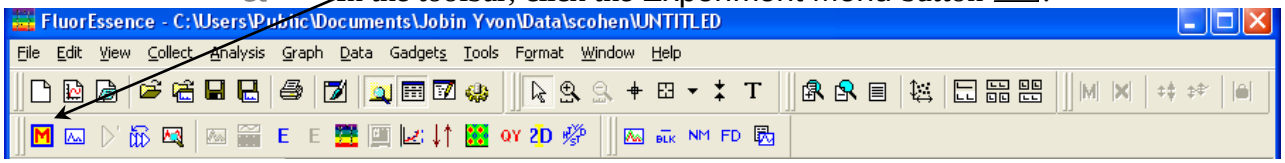
l With the spectrum on the screen, note the greatest intensity.
If the signal exceeds 12 µA, then the emission detector is saturating. Reduce the photomultiplier-tube bias by at least 100 V. If there is no obvious peak, increase the excitation wavelength, starting, and ending by 25 nm, and retry a scan.

m With an acceptable emission peak, record its wavelength.
This is the emission maximum.
Otherwise, repeat steps i through l until an obvious emission peak appears.

2 Find the optimal excitation wavelength.

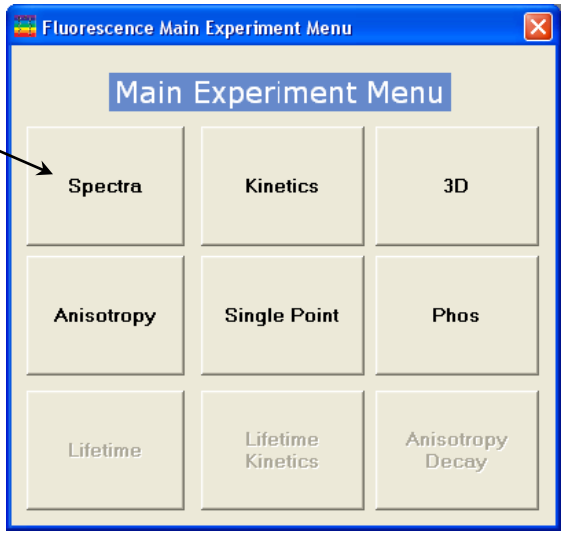
This procedure uses the emission maximum to determine the optimum excitation wavelength, and is similar to step 1.

a In the toolbar, click the Experiment Menu button  .



The **Fluorescence Main Experiment Menu** appears:

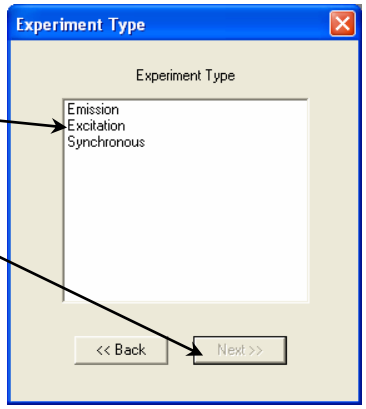
b Choose the Spectra button.



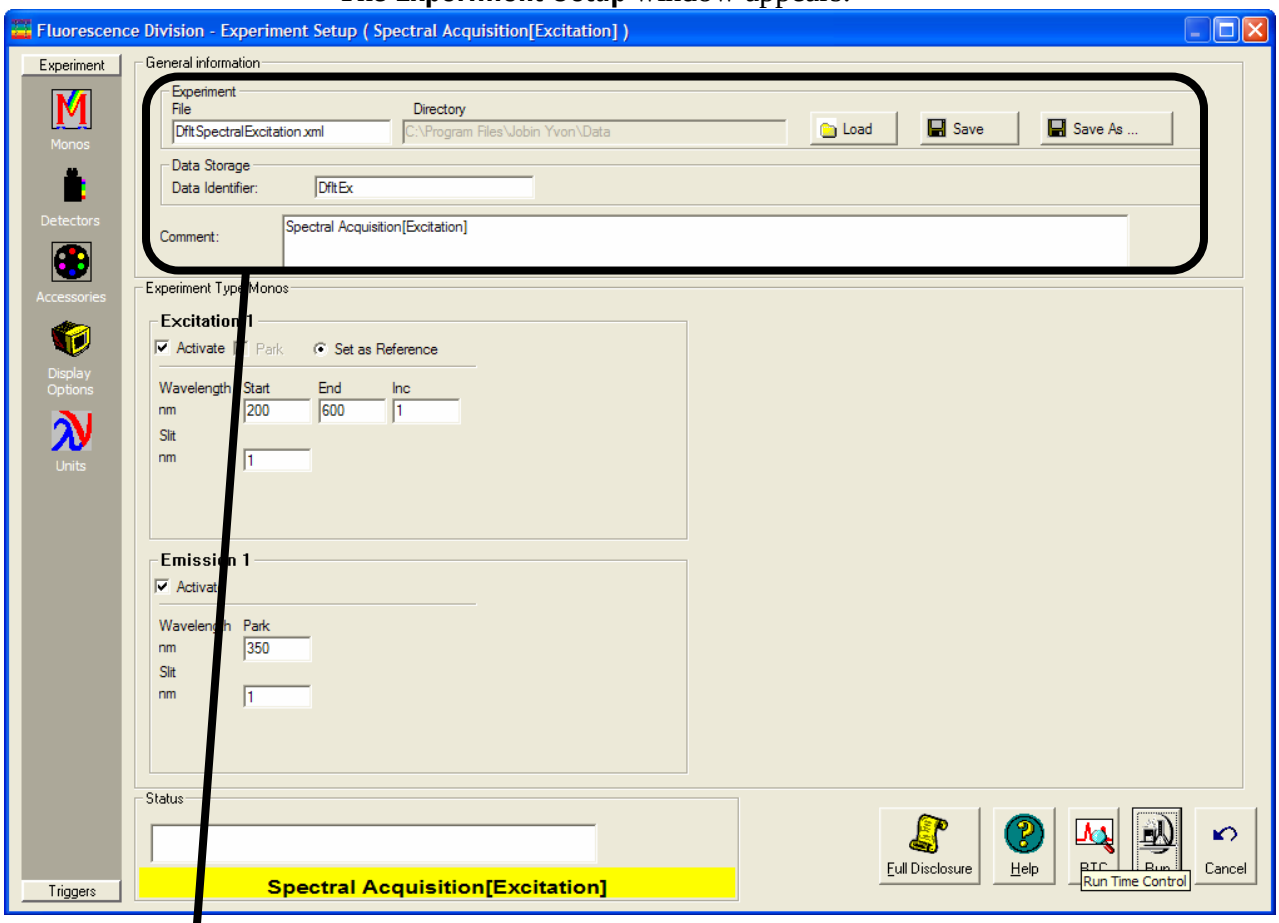
The **Experiment Type** menu appears:

c Choose Excitation.

d Click the Next >> button.



The **Experiment Setup** window appears:



e Set the scan parameters.
Use the emission maximum determined above for the excitation, use 250 nm for starting, enter the emission maximum minus 15 nm for the end of the scan, and select two acquisition modes, S and S/R. S collects raw signal from the emission detector, and S/R ratioes the signal to the reference detector.

f Set excitation and emission slits identical to the emission scan.
Be sure that the emission scan did not exceed 2×10^6 counts/s in the emission scan.

g Enter the Experiment File name.

h Click the Run button 

.
The scan starts.

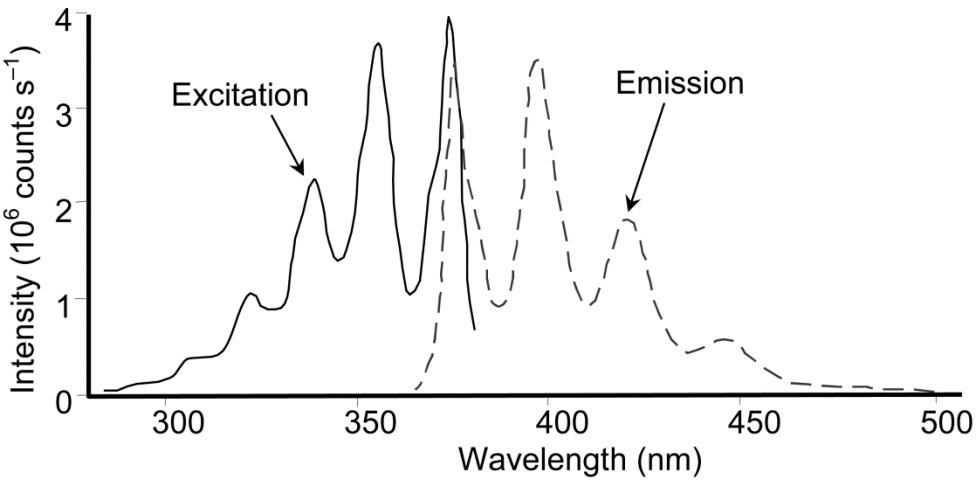
The resulting spectrum shows maximum excitation wavelength. If the raw S-channel signal > 2×10^6 counts/s, narrow the slits and rescan.

i Note the excitation peak.
This is the optimum excitation peak position.

3 Find the optimal emission peak.

a Use the optimum excitation wavelength determined in step 2.

b When complete, both excitation and emission peaks are found. Optimized excitation and emission spectra of a 1×10^{-8} *M* anthracene solution are shown below. Because the acquisition modes were different for the excitation and emission scans, the data intensity had to be normalized. After normalization, the excitation and emission scans are virtually mirror images of one another.



Normalized excitation and emission spectra of a 1×10^{-8}-*M* anthracene solution.

Using corrected signals in FluorEssence™

Introduction

Subtracting blanks, removing dark noise, and correcting for inhomogeneities in the instrument or detector response give more accurate spectra. Take special precautions to incorporate these functions properly into a FluorEssence™ experiment.

Method

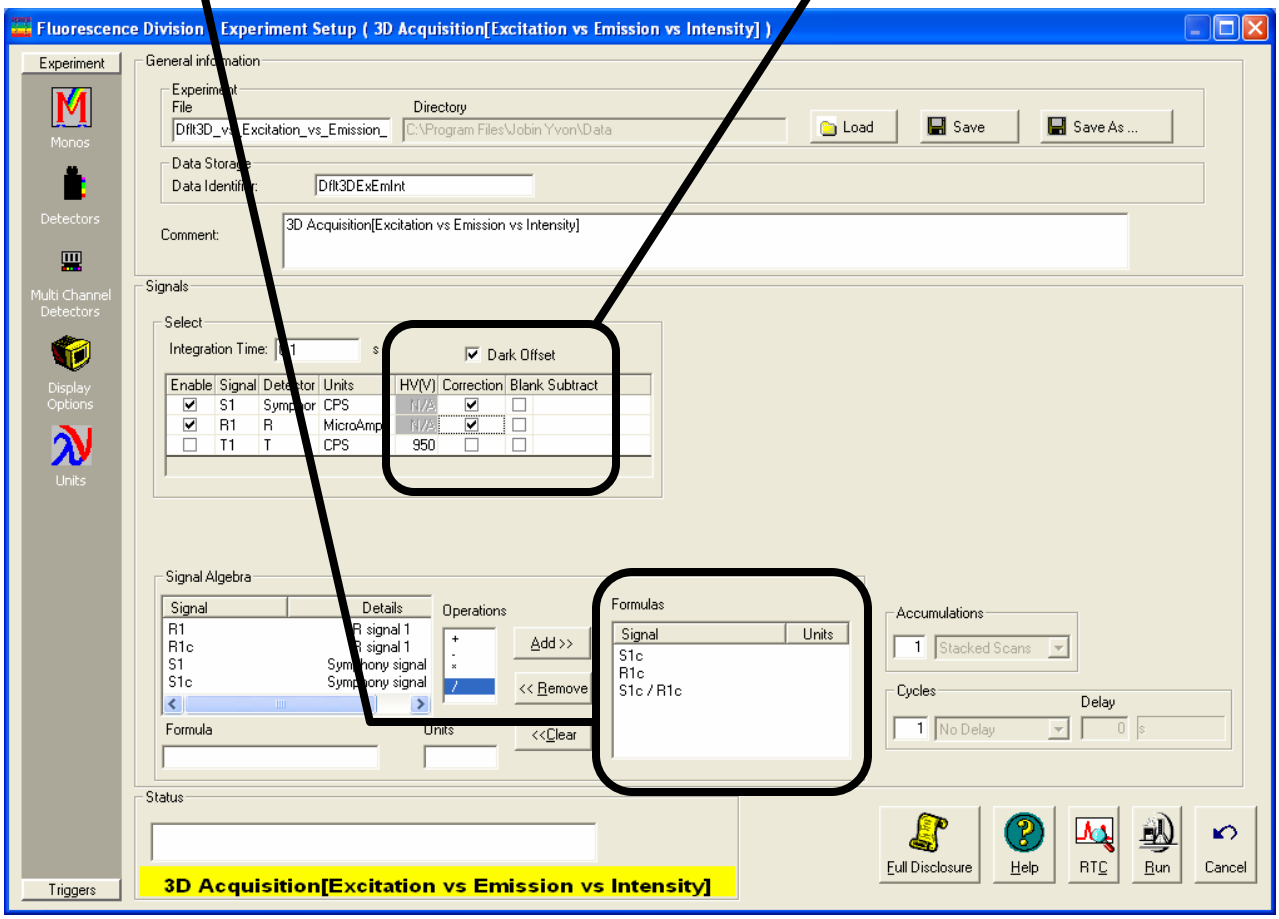
Any corrected signal (with a lower-case "c") or algebraic use of corrected signals must explicitly include all desired corrected signals in the Formulas list. Corrected signals include:

- Dark Offset
- Blank Subtraction
- Correction-factor file

Example

Note how the corrected signal, S1c, and corrected reference, R1c, along with their ratio, S1c/R1c, all must be included in the Formulas list in the Signal Algebra area.

Note: *Be sure all desired corrections are activated in their respective checkboxes.*



Chapter 5 : Optimizing Data

Spectra can be enhanced by optimization of data-acquisition. This chapter lists some methods of optimizing sample preparation, spectrofluorometer setup, and data-correction to get higher-quality data.

Cuvette preparation

Note: *Clean the sample cells thoroughly before use to minimize background contributions.*

1. Empty all contents from the cuvettes.
2. Fully immerse and soak the cuvettes for 24 h in 50% aqueous nitric acid.

This cleans the cuvettes' inner and outer surfaces.

Warning: *Nitric acid is a dangerous substance. When using nitric acid, wear safety goggles, face shield, and acid-resistant gloves. Certain compounds, such as glycerol, can form explosive materials when mixed with nitric acid. Refer to the Materials Safety Data Sheet (MSDS) for detailed information on nitric acid.*

3. Rinse with de-ionized water.
4. Clean the cuvettes in the cleaning solution with a test-tube brush.

Use Alconox® or equivalent detergent as a cleaning solution.

5. Rinse the cuvettes with de-ionized water.
6. Soak the cuvettes in concentrated nitric acid.
7. Rinse them with de-ionized water before use.

Caution: *Soaking the cuvettes for a long period causes etching of the cuvette surface, which results in light-scattering from the cuvettes.*

Sample preparation

Caution: *Always read the Materials Safety Data Sheet before using a sample or reagent.*

The typical fluorescence or phosphorescence sample is a solution analyzed in a standard cuvette. The cuvette itself may contain materials that fluoresce. To prevent interference, HORIBA Scientific recommends using non-fluorescing fused-silica cuvettes that have been cleaned as described above.

Small-volume samples

If only a small sample-volume is available, and the intensity of the fluorescence signal is sufficient, dilute the sample and analyze it in a 4-mL cuvette. If fluorescence is weak or if trace elements are to be determined, HORIBA Scientific recommends a 1-mL cell (5 mm × 5 mm cross-section), or our Microsense optical accessory for 1–5 µL applications.

Solid samples

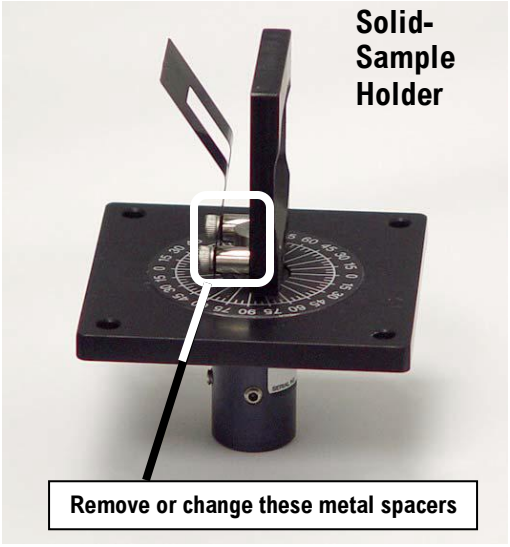
Solid samples usually are mounted in the J1933 Solid Sample Holder, with the fluorescence collected from the front surface of the sample. The mounting method depends on the form of the sample. See the section on "Highly opaque samples" for more information on sample arrangement in the sample compartment.

Note: *Avoid thick coverslips, because the excitation beam may not hit the sample directly with a thick coverslip. Microscope coverslips are useful, except that they are not quartz, and do not transmit UV light.*

- Thin films and cell monolayers on coverslips can be placed in the holder directly.
- Minerals, crystals, vitamins, paint chips, phosphors, and similar samples usually are ground into a homogeneous powder. The powder is packed into the depression of the Solid Sample Holder (see next page for diagram). For very fine powder, or powder that resists packing (and therefore falls out when the holder is put into its vertical position), the powder can be held in place with a thin quartz coverslip, or blended with potassium bromide for better cohesion.
- A single small crystal or odd-shaped solid sample (e.g., contact lens, paper) can be mounted with tape along its edges to the Solid Sample Holder. Be sure that the excitation beam directly hits the sample. To keep the excitation beam focused on the sample, it may be necessary to remove or change the thickness of the metal spacers separating the clip from the block.

Dissolved solids

Solid samples, such as crystals, sometimes are dissolved in a solvent and analyzed in solution. Solvents, however, may contain organic impurities that fluoresce and mask the signal of interest. Therefore, use high-quality, HPLC-grade solvents. If background fluorescence persists, recrystallize the sample to eliminate organic impurities, and then dissolve it in an appropriate solvent for analysis.



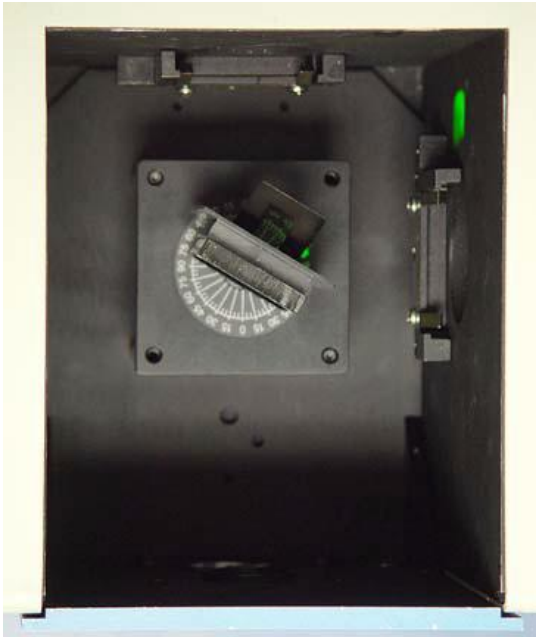
Biological samples

For reproducible results, some samples may require additional treatment. For example, proteins, cell membranes, and cells in solution need constant stirring to prevent settling. Other samples are temperature-sensitive and must be heated or cooled to ensure reproducibility in emission signals.

Running a scan on a sample

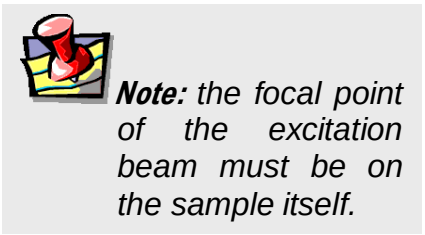
Precautions with the Solid-Sample Holder

Avoid placing the front face of the sample so that the excitation beam is reflected directly into the emission monochromator. If the sample is rotated at 45° from excitation, this may occur, increasing interference from stray light. Instead, set up the sample with a 30° or 60°-angle to the excitation, preventing the excitation beam from entering the emission slits. The photograph at right illustrates how a 60°-angle to the excitation keeps the incoming excitation light away from the emission monochromator's entrance.

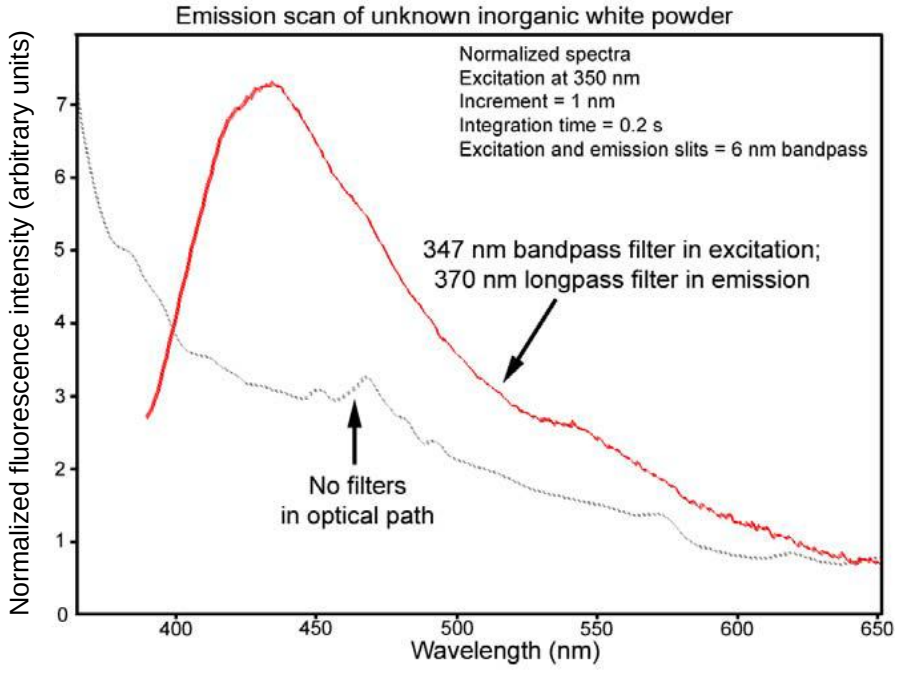


Use filters in the optical path.

Stray light from the excitation beam can interfere with the emission from the sample. To reduce the deleterious effects of stray light, place a filter that removes excitation wavelengths from the emission beam in the emission optical path. Here is an example of scans with and without filters on a FluoroMax®-3, using an unknown white powder as the sample. A 347-nm band-pass filter rejects higher-order wavelengths from reaching the sample, while a long-pass filter in the emission side lets only fluorescence, and



Note: *the focal point of the excitation beam must be on the sample itself.*

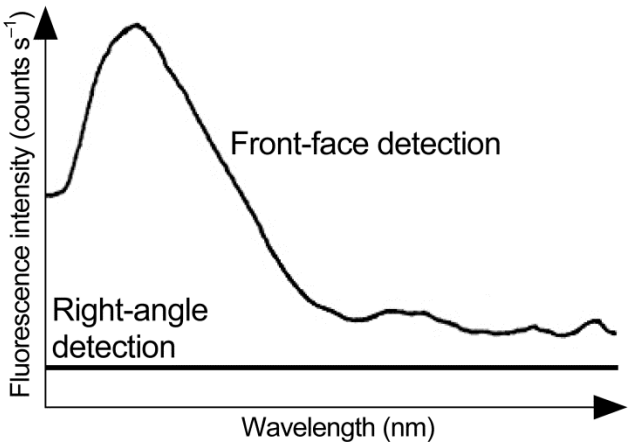


not stray excitation, into the detector. Notice how the shape of the spectrum changes drastically when filters are added.

Highly opaque samples

Highly concentrated and opaque liquids often have problems with self-absorption or complete attenuation of the beam. Intensity measurements with the excitation beam at 90° to the emission beam may not be reproducible or detectable, and the excitation or emission spectra may appear distorted. Try front-face detection: the excitation light is focused to the front surface of the sample, and fluorescence emission is collected from this region at an angle that minimizes reflected and scattered light. With front-face detection, set the front face of the sample at 30° or 60° to the excitation beam.

An example at right shows the difference between detection at right-angles versus front-face.



Comparison of fluorescence emission signal from sickle-cell hemoglobin using right-angle versus front-face detection. The β-37 tryptophan is primarily responsible for this fluorescence.

Data collection techniques

Select the collection method

The two basic collection methods are right-angle and front-face. In right-angle detection, primarily for clear solutions, the fluorescence is collected at 90° to the incident exciting beam. Front-face detection is used for optically dense solutions and for solid samples. In front-face detection, fluorescence is collected off the front surface of the cuvette or the solid sample. Inner-filter and re-absorption characteristics of opaque samples preclude right-angle detection. The optional front-face accessory for cuvettes is required for solutions. For front-face collection on solids, use the J1933 Solid Sample Holder.

Determine optimal wavelengths

The optimum excitation and emission wavelengths are known for many samples. If you are running a sample whose wavelength positions are unknown, you must determine these wavelengths to obtain the best possible results when you run the sample.

To determine the excitation and emission wavelengths, first run an emission scan and observe the peak emission value. Once this value has been obtained, conduct an excitation scan using the peak emission value determined by running an emission scan. See the section entitled “Running an unknown sample”, in Chapter 4, for more details.

Measure the *G* factor

Note: *Measure the G factor before a polarization experiment.*

The grating factor, or *G* factor, ought to be included anytime polarization measurements are taken. The *G* factor corrects for variations in polarization wavelength-response for the emission optics and detectors. A pre-calculated *G* factor may be used when all other experimental parameters are constant. In other cases, the system can measure the *G* factor automatically before an experimental run. See Chapter 9 for more details.

Improve the signal-to-noise ratio

Because of various hardware or software conditions, occasionally it is necessary to optimize the results of an experiment.

The quality of acquired data is largely determined by the signal-to-noise ratio (*S*/*N*). This is true especially for weakly fluorescing samples with low quantum yields. The signal-to-noise ratio can be improved by:

- Increasing the integration time,
- Scanning a region several times and averaging the results,
- Changing the bandpass by adjusting the slit-widths, and
- Mathematically smoothing the data.

The sections that follow discuss the alternatives for improving the *S*/*N* and the advantages and disadvantages for each.

Determine the optimum integration time

The length of time during which photons are counted and averaged for each data point is called the integration time. An unwanted portion of the signal comes from noise and dark counts (distortion inherent in the signal detector and its electronics when high-voltage is applied). By increasing the integration time, the signal is averaged longer, resulting in a better *S*/*N*. The ratio is enhanced by a factor of $t^{½}$, where *t* is the multiplicative increase in integration time. For example, doubling the integration time from 1 s to 2 s increases the *S*/*N* by over 40%, as shown below:

For an integration time of 1 second, $\frac{S}{N} = t^{½}$

$= 1^{½}$

$= 1$

For an integration time of 2 seconds, $\frac{S}{N} = t^{½}$

$= 2^{½}$

≈ 1.414

or an approximately 41% improvement. Because *S*/*N* determines the noise level in a spectrum, using the appropriate integration time is important for high-quality results. To discover the appropriate integration time for your experiment:

a Find the maximum fluorescence intensity by acquiring a preliminary scan, with an integration time of 0.1 s and a bandpass of 5 nm.

b From this preliminary scan, note the maximum intensity, and select the appropriate integration time from the table below.

Signal intensity (counts per second)	Estimated integration time (seconds)
1000 to 5000	2.0
5001 to 50 000	1.0
50 001 to 500 000	0.1
500 001 to 4 000 000	0.05

Note: *This table is only a* ***guide****. Optimum integration time for other scans, e.g., time-base, polarization, phosphorescence, and anisotropy, may be different.*

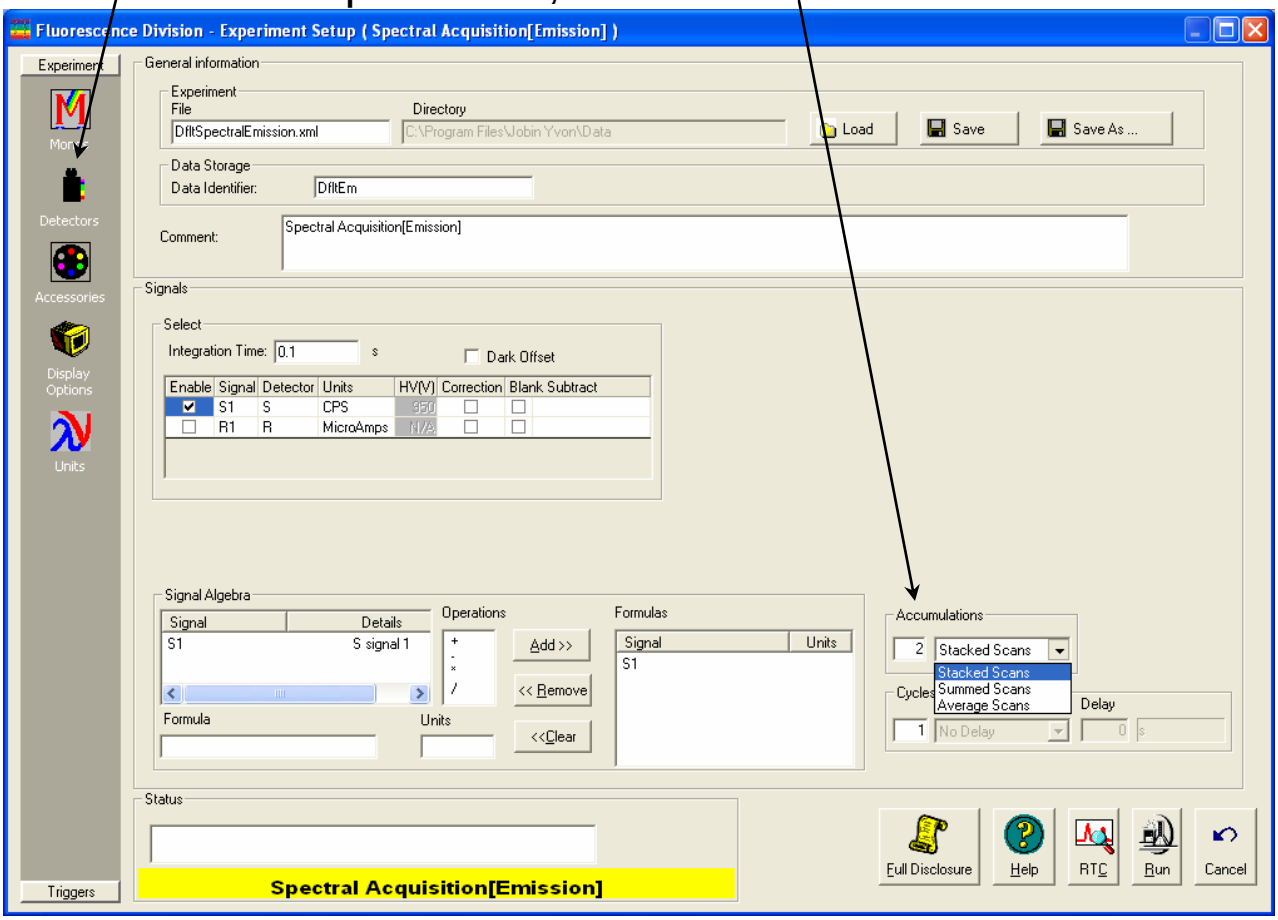
Set the integration time either through the **Experiment Setup** window (under the Detectors icon) for a specific experiment, or **Real Time Control** to view the effects of different integration times in real time. See the on-line FluorEssence™ help for more information about the integration time.

Scan a sample multiple times

Scanning a sample more than once and averaging the scans together enhances the *S/N*. In general, the *S/N* improves by $n^{½}$, where *n* is the number of scans.

To scan a sample multiple times,

1. Open the **Experiment Setup** window.
2. Choose the Detectors icon.
3. Specify the number of scans, and how to handle multiple scans, in the Accumulations field.



See FluorEssence™ on-line help for detailed instructions regarding the data-entry fields.

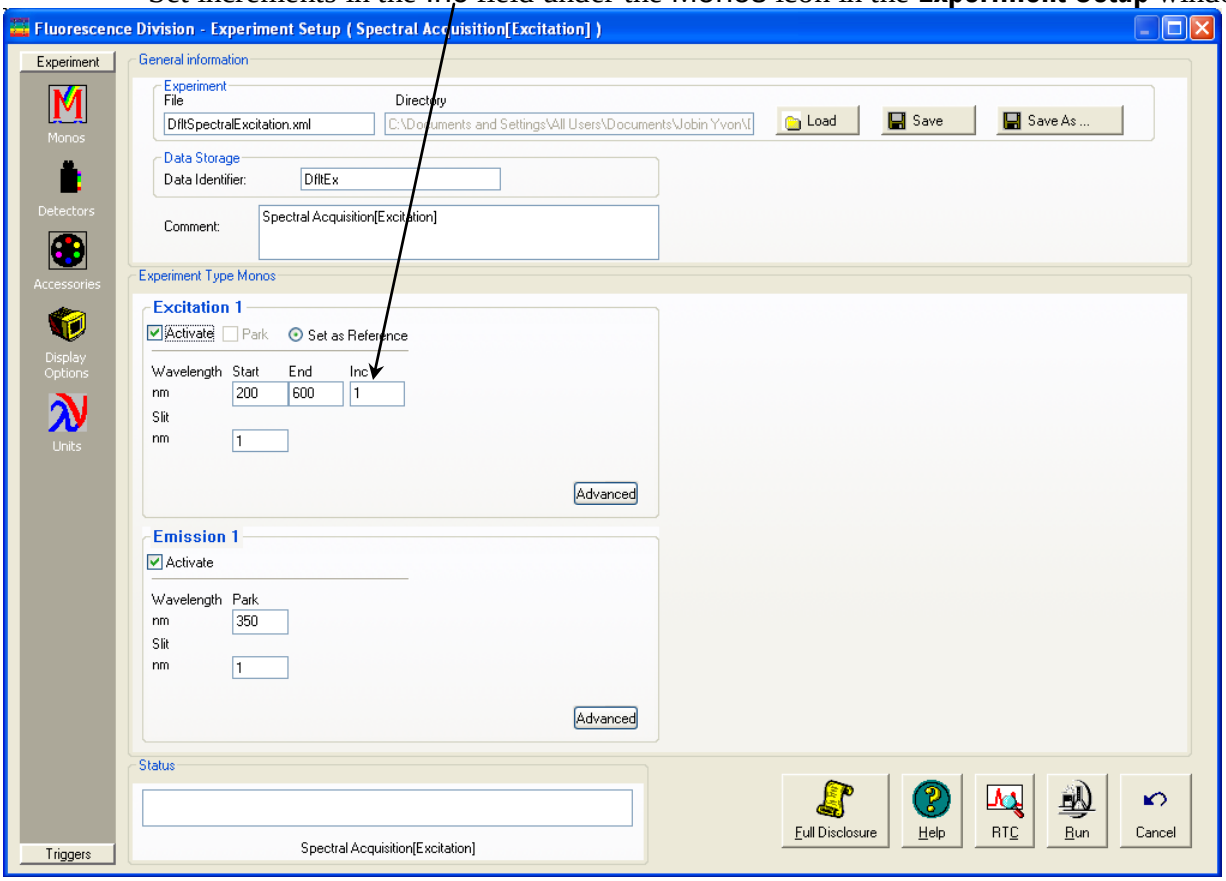
Use the appropriate wavelength increment

The increment in a wavelength scan is the spacing, in nm, between adjacent data points. The spacing between the data points affects the resolution of the spectrum and total time for acquisition. Consider the required resolution, time needed, and concerns about photobleaching of the sample. Most samples under fluorescence analysis display relatively broad-band emissions with a Lorentzian distribution, so they do not require a tiny increment.

Common increments range from 0.05–10 nm, depending on the sample and slit-size. A first try might be 0.5–1 nm increment. After acquiring the initial spectrum, examine the results. If two adjacent peaks are not resolved (i.e., separated) satisfactorily, reduce the increment. If the spectrum is described by an excessive number of data points, increase the increment to save time and lamp exposure. A scan taken using an increment of 0.1 nm, with a peak at full-width at half-maximum (FWHM) of 20 nm, should be run with a 1-nm increment instead.

For time-based scans, the increment is the spacing in s or ms between data points. Here, the consideration is the necessary time resolution. The time-increment dictates the total time per data point and for the scan in general. Set this value to resolve any changes in the luminescence of samples as they react or degrade. Time increments often range from 0.1–20 s.

Set increments in the Inc field under the Monos icon in the **Experiment Setup** window.



See the FluorEssence™ on-line help for more information.

Select the appropriate bandpass

The bandpass (wavelength spread) affects the resolution of spectra. If the bandpass is too broad, narrow peaks separated by a small change in wavelength may be unresolved. For example, for two 2 nm peaks 5 nm apart, and a bandpass of 10 nm, one broad peak, instead of two well-defined ones, is visible.

By adjusting the slit widths, you can control the intensity and bandpass of the light. The slits of the excitation monochromator determine the amount of light that passes through the excitation monochromator to the sample. The emission spectrometer's slits control the amount of luminescence recorded by the signal detector.

Calculate the bandpass using the following formula:

bandpass (nm) = slit-width (mm) × dispersion (nm/mm)

The dispersion of the Fluorolog®-3 spectrofluorometer system depends upon the type of monochromator and spectrometer, and the groove-spacing of the gratings. For example, a Fluorolog®-3 with a single-grating monochromator and 1200 groove/mm grating has a dispersion of 4.0 nm/mm, while a Fluorolog®-3 with a double-grating monochromator and 1200 groove/mm gratings has a dispersion of 2.0 nm/mm. Below is a table listing various monochromators, installed gratings, and their respective dispersions.

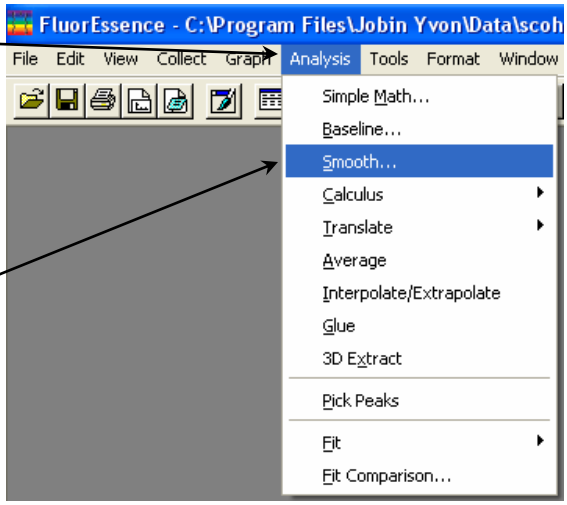
Monochromator	Grating groove-density (grooves/mm)	Monochromator's dispersion
Double	1200	2.1
	600	4.2
	300	8.4
Single	1200	4.2
	600	8.4
	300	16.8

Smooth the data

Smoothing the data improves the appearance of the spectrum. Smoothing, as are most post-processing features, is handled by Origin® software.

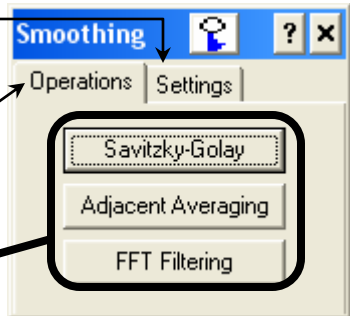
1 Choose Analysis from the main FluorEssence™ menu.

A drop-down menu appears.



2 Choose Smooth....

The **Smoothing** dialog box opens:



3 Under the Settings tab, choose the appropriate parameters.

4 Under the Operations tab, choose the type of smoothing:

- FFT Filtering button (fast-Fourier transform)
- Adjacent Averaging button
- Savitsky-Golay button.

See the on-line Origin® help for additional information regarding smoothing data.

In general, start with a 9- or 11-point smooth for a time-base measurement. To select the proper number of points for wavelength-scan types, first locate the area that requires smoothing—usually this is a peak. Determine the number of data points used to make up the peak, and then smooth the data using the number of points closest to this number. To avoid artificially enhancing the data, use the appropriate number of points to smooth the data. For example, selection of too large a number results in the background being smoothed into the peak.

Correcting data

Introduction

Collecting accurate information about the luminescent properties of a sample depends upon several factors:

- Equipment specifications,
- Sample characteristics, and
- Timing considerations.

To ensure that the spectra collected indicate the actual properties of the sample and not external conditions, data often must be corrected. To correct data means to subtract information not directly related to the properties of the sample. Gratings, detectors, and monochromator or spectrometer components have response characteristics that vary as a function of wavelength. These characteristics may be superimposed on spectra, thereby yielding a potentially misleading trace. For accurate intensity comparisons, such as those required for quantum-yield calculations, response characteristics must be eliminated. Supplied with the instrument are sets of excitation and emission correction factors to eliminate response characteristics. These files (we ignore their 3-letter extensions), `xcorrect` and `mcorrect`, are included with the software; copy them to your hard disk.

Items that may be convoluted into a spectrum	Ways to remove these artifacts
Fluctuations caused by the light source	Monitoring lamp-output using the reference detector R, and using the signal ratio S/R to correct lamp-profile or temporal fluctuations
Influence of the sample holder	Running a blank scan (which is later subtracted from the sample scan)
System hardware (e.g., optics, detectors)	Using radiometric correction factors

Note: *The excitation range is 240–600 nm and the emission range is 290–850 nm for the reference detector.*

To use radiometric correction factors, either:

- Select the ones supplied with the software, or
- Select a set generated at your facility during or after data-acquisition, discussed in the following section. Acquiring radiometric correction factors is explained in the chapter *Producing Correction Factors*.

Blank Subtraction and Dark Offset functions are described in the on-line FluorEssence™ help files.

Correcting data during acquisition

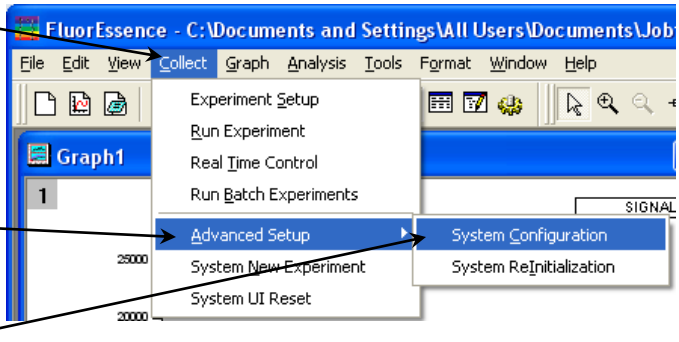
Data can be acquired either as raw data or as corrected data. A spectrum composed of raw data exhibits the effects of system parameters, while a corrected spectrum displays only the properties related to the sample.

Caution: *The* `mcorrect`, `xcorrect`, *and* `tcorrect` *files are custom-generated at the factory for each particular instrument, and cannot be swapped.*

1 Be sure the instrument configuration has a layout that includes a correction file associated with the appropriate detector.

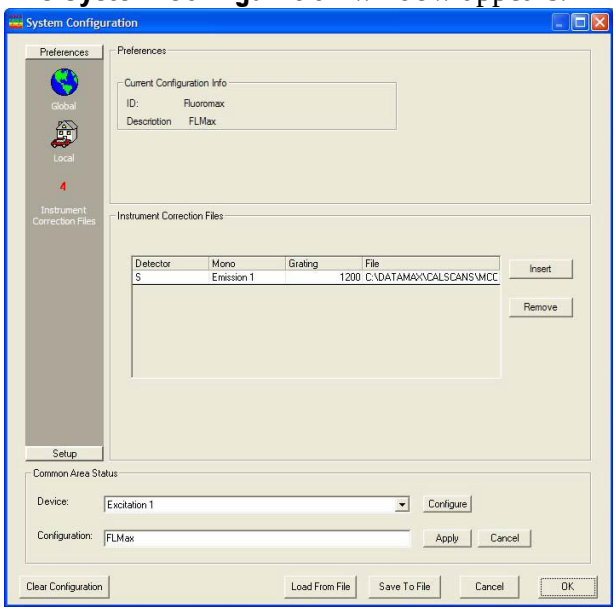
a Choose Collect in the toolbar. A drop-down menu appears.

b Choose Advanced Setup, then System Configuration.

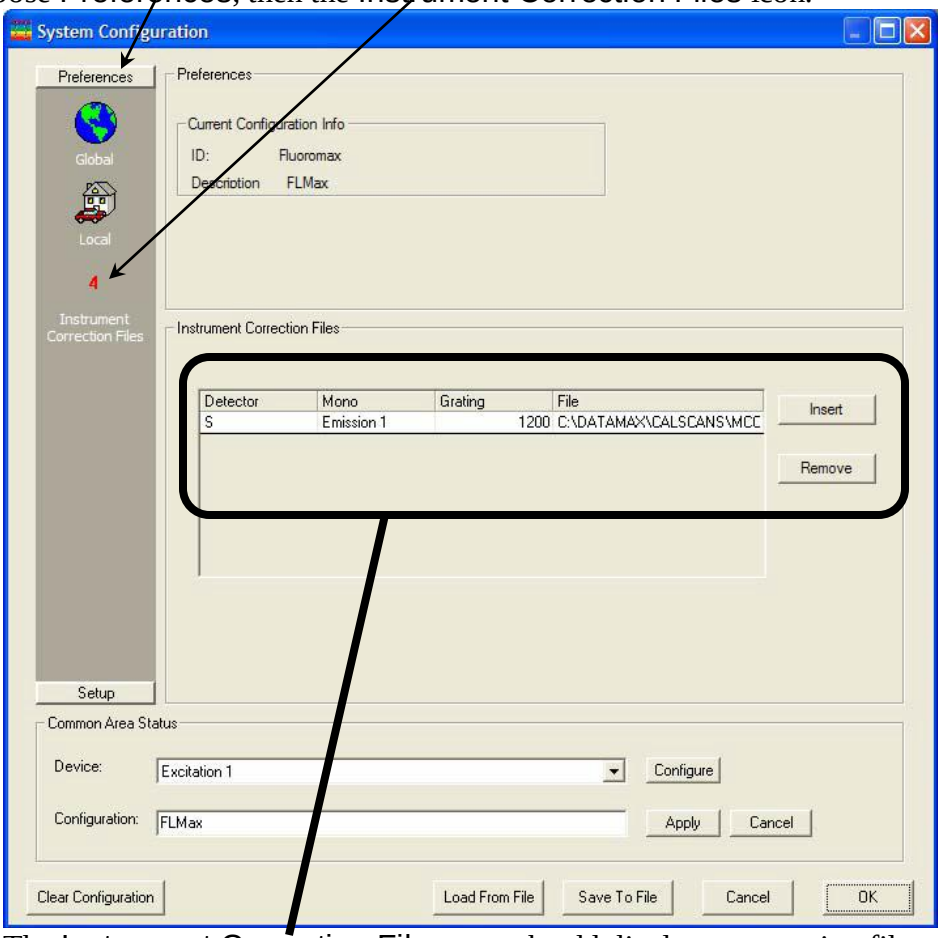


If there is more than one hardware configuration available, the **Select Hardware Configuration** menu appears. Choose the desired hardware configuration for the correction-factor file.

The **System Configuration** window appears:



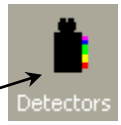
c Choose Preferences, then the Instrument Correction Files icon.



The Instrument Correction Files area should display a correction file for the Detector (S or R). If not, click the Insert button, and browse for the desired correction file.

Detector	Correction-factor file name
S	`mcorrect`
R	`xcorrect`
T (if used)	`tcorrect`

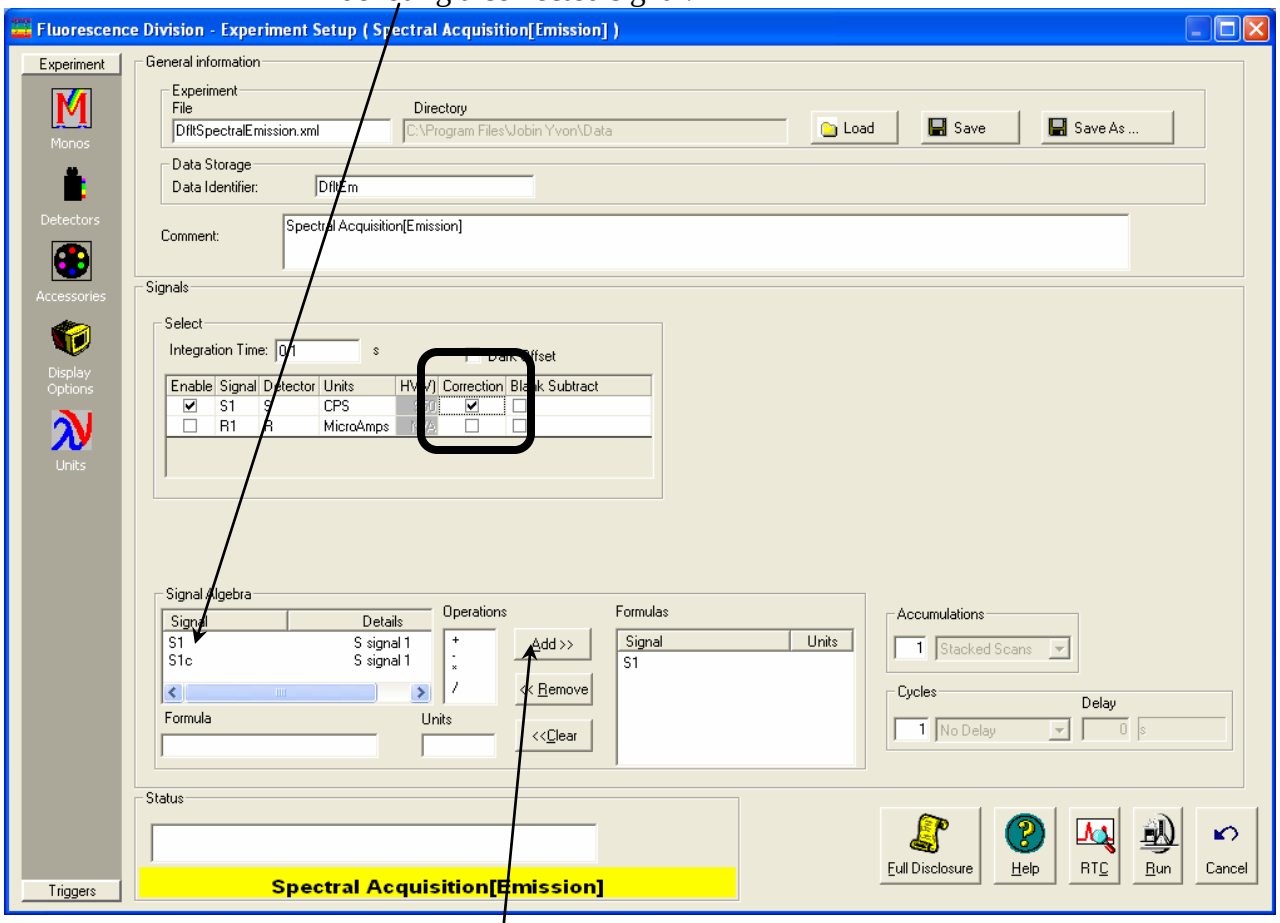
2 Choose correction when setting up the experiment.



a In the **Experiment Setup** window, choose the Detectors icon to display the detectors' parameters.

b Click the Correction checkbox for the detector you want corrected.

In the Signal Algebra area, a signal with appended "c" appears, denoting a corrected signal:

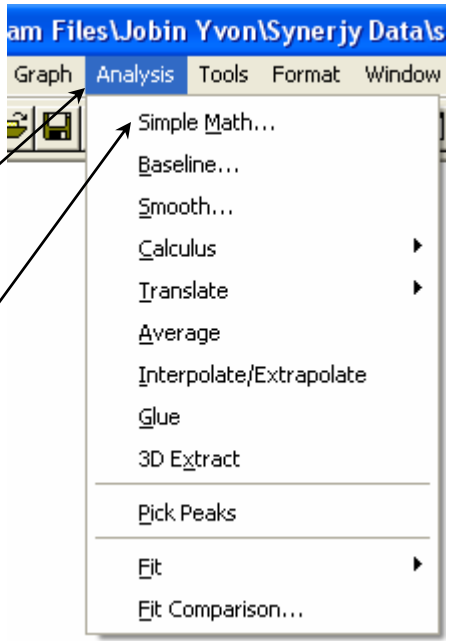


c Click the Add >> button to add the corrected signal to the Formulas table.
The corrected signal appears in the Formulas table.

3 Run the experiment with the corrected signal.

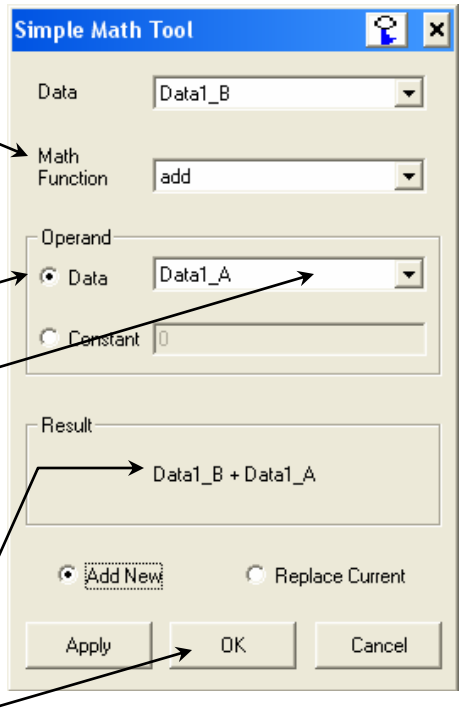
After acquisition

To apply the correction factors after the data have been acquired, multiply the data file by the appropriate correction factor file (`mcorrect` for the S detector, `xcorrect` for the R detector, or `tcorrect` for the T detector).



1. Make sure the graph is open and trace to be corrected is active in the main **FluorEssence** window.
2. Choose Analysis.
   A drop-down menu appears.
3. Select Simple Math....

   This opens the **Math Functions** dialog box. The chosen trace should appear in the Data field. If not, browse for it with the down arrow.



4. In the Math Function drop-down menu, select multiply.
5. Choose the Data radio button in the Operand area.
6. Browse for the appropriate correction-factor file (`mcorrect` or `xcorrect`).
   The proper formula should appear in the Result area.
7. Click the OK button.
   The trace that appears on the screen is a result of the mathematical operation, giving a corrected spectrum.

Chapter 6 : System Maintenance

External case

The Fluorolog®-3 spectrofluorometer requires very little maintenance. The outside panels may be wiped with a damp cloth to remove dust and fingerprints. Blow pressurized air onto the exhaust filters periodically to clean away dust.

Lamp-housing exhaust filters

To clean away dust:

1. Remove the filters.
2. Clean the filters under tap water.
3. Let the filters dry before reinstalling.

Caution: *Not removing dust from the exhaust filters can cause the xenon lamp's power supply to fail from overheating.*

Xenon lamp

The lamp is the only component that has to be replaced routinely. Regular examination of lamp and water-Raman spectra serves as an early indicator of the system's integrity. (These two tests are described in Chapter 3, *System Operation*.)

Obtaining good spectral results depends upon the xenon lamp. After 1700–2000 hours of use for the 450-W xenon lamp, the lamp output decreases significantly, indicating that the lamp should be replaced. Replacing the lamp within the specified time may prevent system failure. HORIBA Scientific advises to keep a laboratory notebook near the Fluorolog®-3 to record lamp usage. Each time the lamp is turned on, it constitutes one full hour of use; therefore, HORIBA Scientific suggests leaving the lamp on between brief periods of inactivity. Record the hours of use on the form in Chapter 12: *Xenon Lamp Information and Record of Use Form*.

Required tools

Tool	Size	Purpose
Allen wrench	5/32″	Screws for lamp power cables
Allen wrench	5/64″	Height adjustment; centering adjustment
Allen wrench	7/64″	Lamp support arm
Phillips screwdriver	Medium	Screws for top cover of housing

Replacement

The replacement xenon lamp is packed in the manufacturer's box and must be installed in the lamp housing. Read all the packing material including instructions and precautions before attempting to insert the lamp into the lamp housing.

Caution: *Do not remove the protective cover from the replacement xenon lamp until instructed to do so.*

Warning: *Xenon lamps are an explosion hazard. Make sure the power is off and all AC power is disconnected from the system. Read and follow the cautions presented below.*

Hazards

- Xenon-arc lamps are an explosion hazard. Wear explosion-proof face-shield and protective clothing when opening the lamp housing and handling the lamp.

- Disconnect the lamp power supply from the AC power line (mains) while handling lamp leads. Lethal high voltages may exist.
- The lamp remains extremely hot for approximately one-half hour after it has been turned off. Do not touch the lamp or the metal unit until the lamp has cooled.
- Never look directly at the xenon arc or its reflection. Intense radiation can permanently damage eyes.
- Do not touch the focusing lens, back-scatter mirror, or the surface of the lamp. Fingerprints will be burned onto these surfaces when the lamp is ignited.

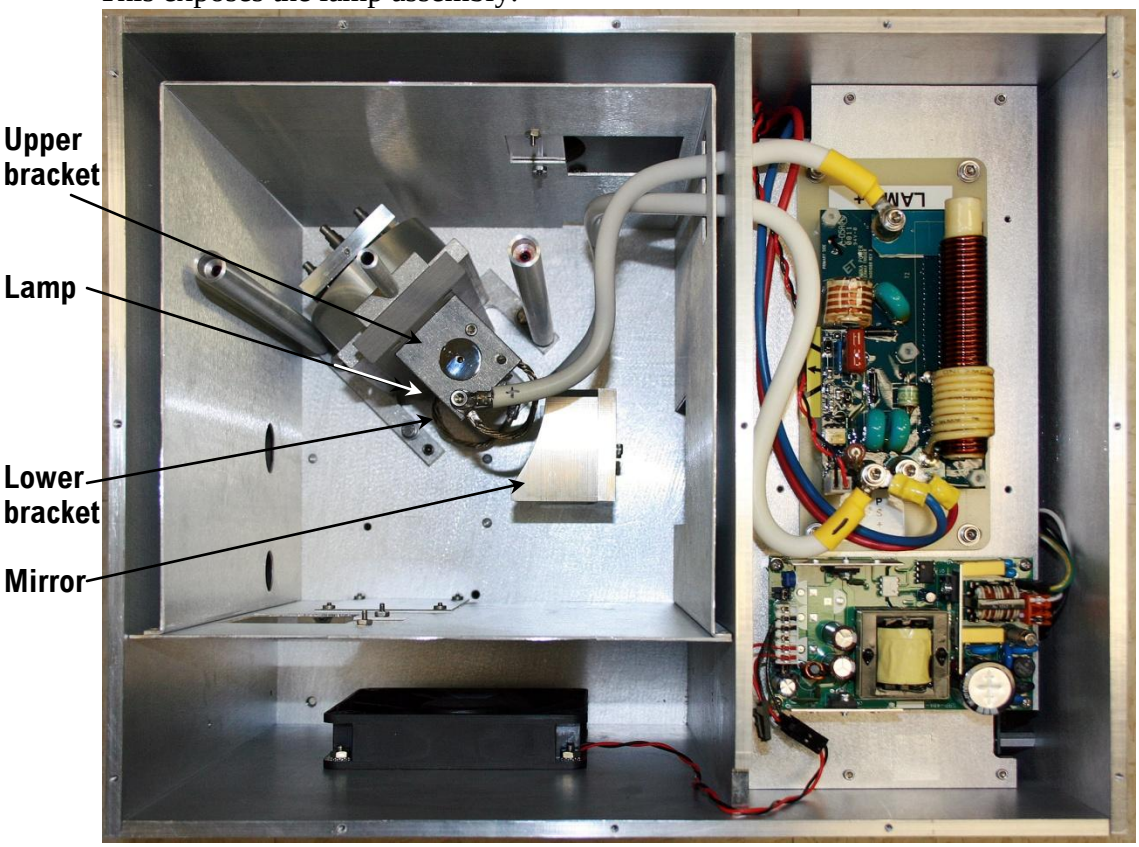
The following instructions are divided into three distinct procedures—each of which may be performed as a stand-alone operation:

- Open the lamp housing
- Remove the existing xenon lamp
- Install a new xenon lamp

To open the lamp housing:

1. Remove the 13 Phillips-head screws on top of the lamp housing.
2. Lift off the cover.

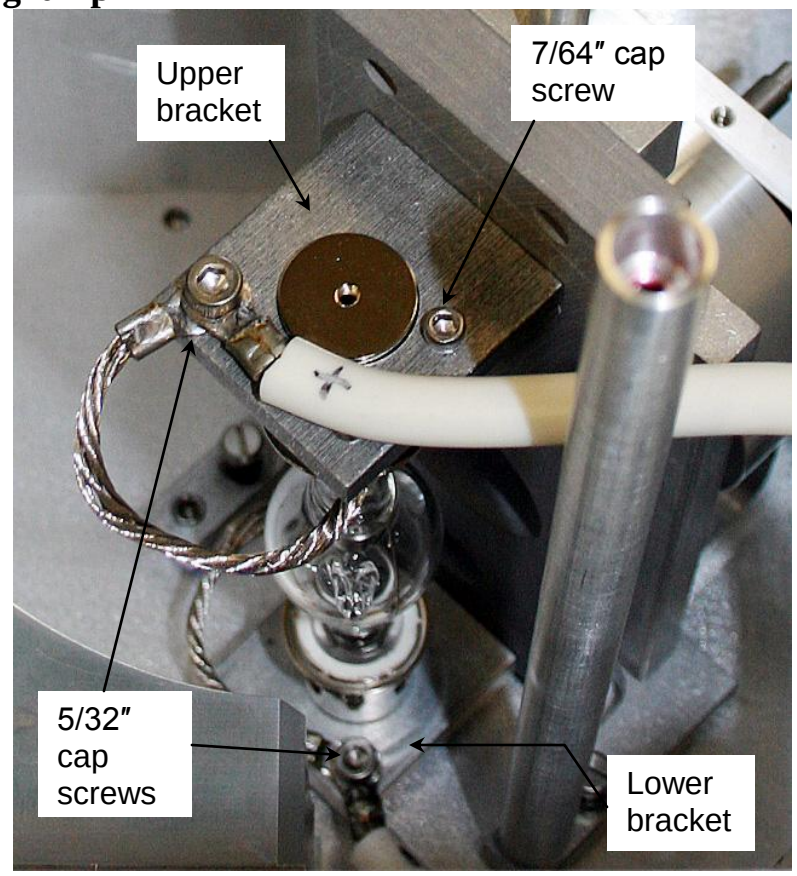
This exposes the lamp assembly:



Top view of the lamp housing with cover removed.

To remove an existing lamp

1 Remove the 5/32″ cap screws from the upper and lower brackets, freeing the positive and negative power leads.

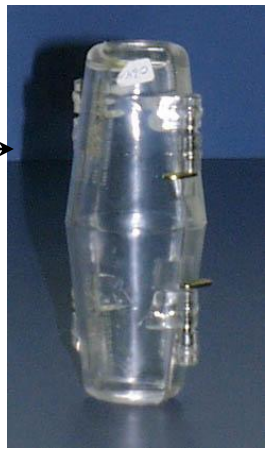


2 Remove the 7/64″ cap screw from the upper bracket.

3 Remove the upper bracket.

4 Remove the lamp.

5 Place the protective cover around the xenon lamp.



6 Lock the cover into place.

Dispose of the spent xenon lamp, following all safety precautions and regulations.

To insert a xenon lamp

Note: *HORIBA Scientific provides new bulbs with leads and connectors of the proper length and size. Other manufacturers' lamps may require extra adjustment before installation.*

1 Be sure the cover of the lamp housing is removed.

2 Pay attention to the polarity of the xenon lamp.

Orient the lamp as shown in this diagram, so that the cathode is above the anode.

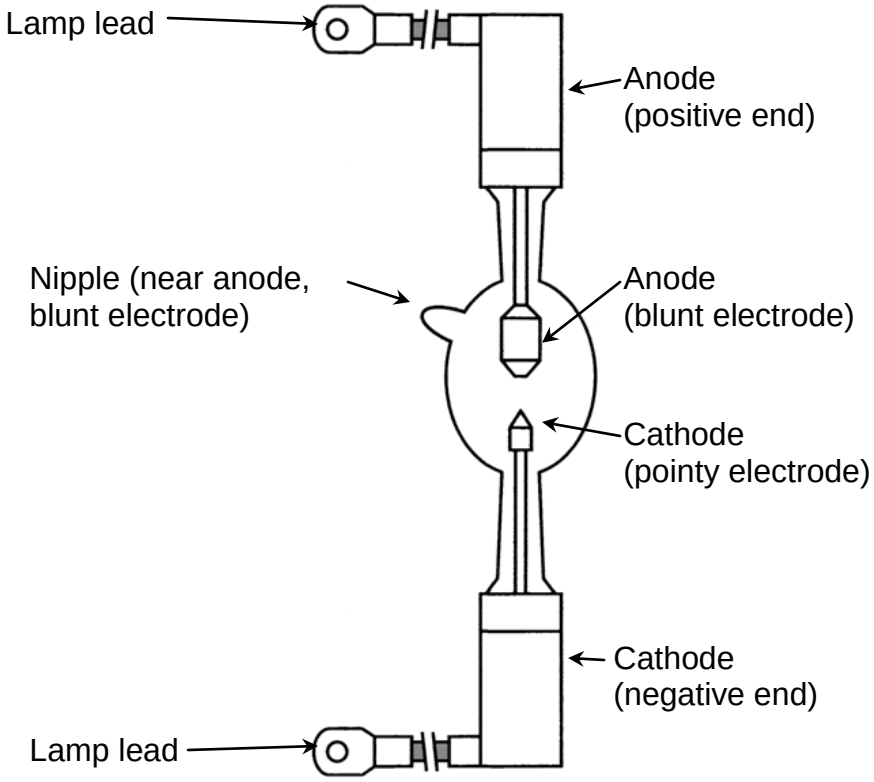
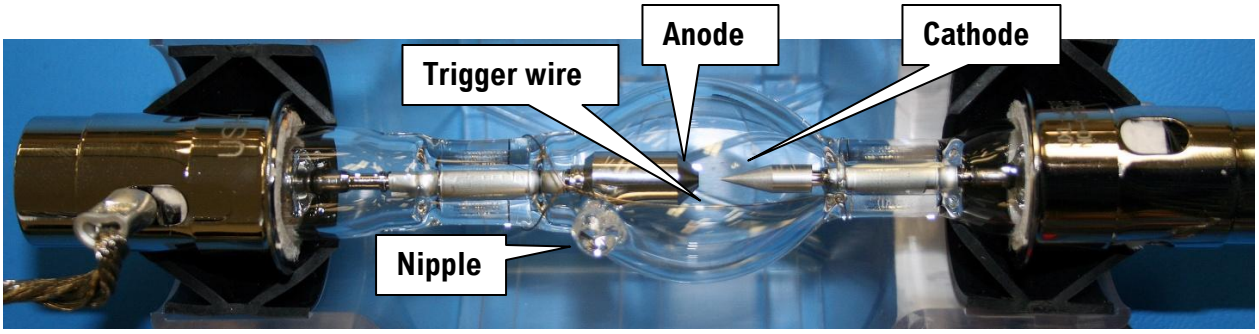


Diagram of lamp emphasizing polarity.

3 Note the correct location of the trigger wire.

If the wire is not correctly oriented, carefully rotate it around the bulb using a small wooden dowel or cotton swab.



Warning: *Never touch the bulb with bare hands. Always wear cotton gloves.*

4 Seat the xenon lamp in the bottom bracket.

Make sure that the lamp is inserted all the way into the aluminum bracket. The negative end should be downward.

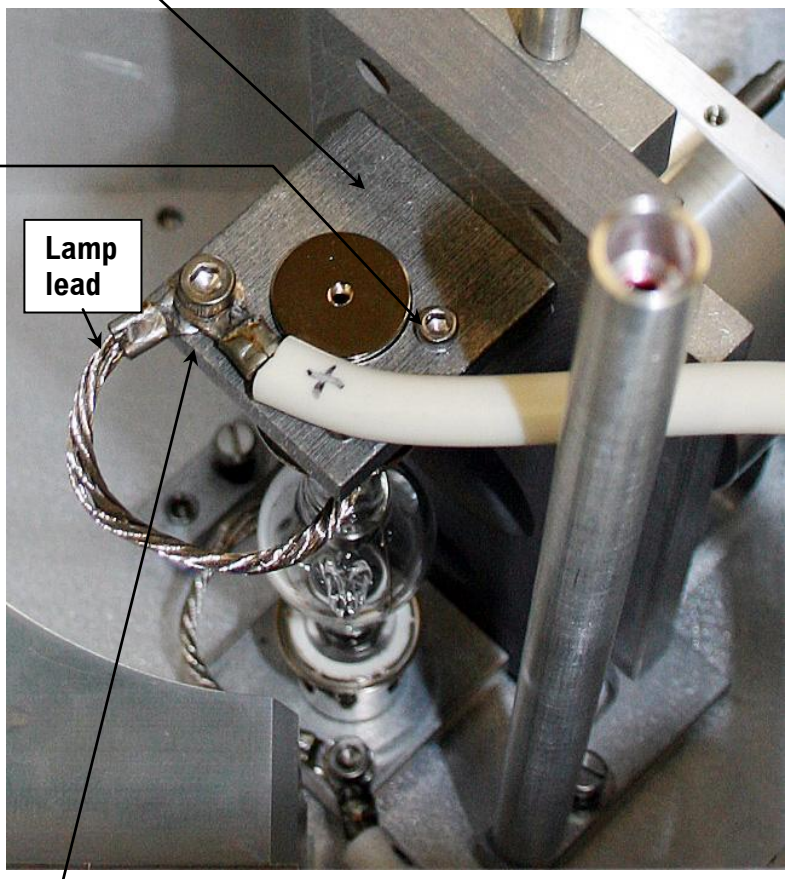
Note: *Position the nipple and trigger wire so they are not in the light path from the backscatter mirror to the turning mirror.*

5 Place the square aluminum upper bracket over the anode terminal of the bulb.

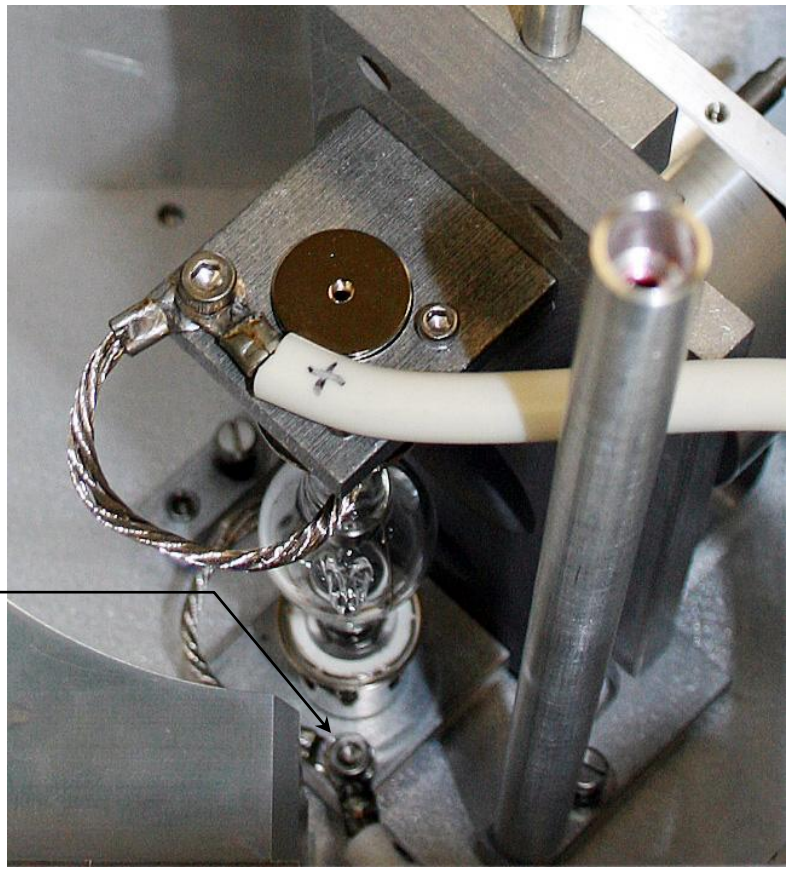
The positive end should be pointing upward.

6 Insert and tighten the 7/64″ cap screw on the upper bracket.

7 Secure the upper lamp lead and upper power cable to the upper bracket, using a 5/32″ cap screw.

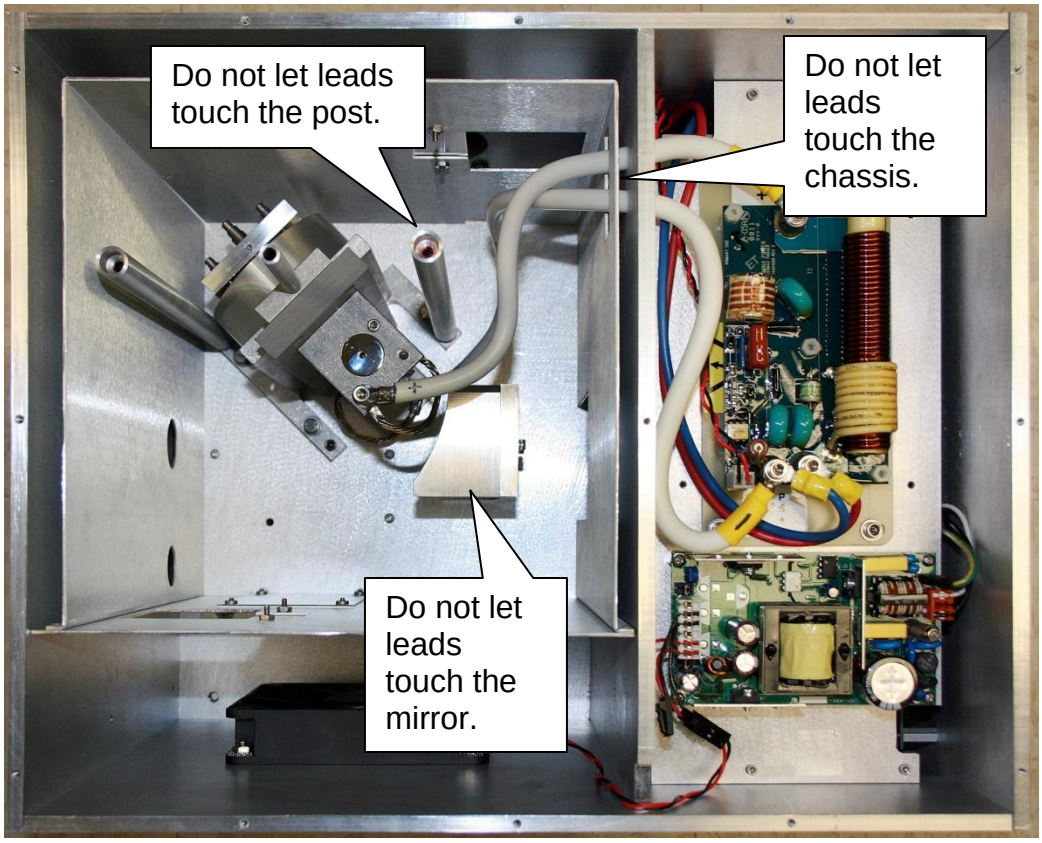


8 Secure the lower lamp lead and lower power cable to the lower bracket, using a 5/32″ cap screw.



9 Tighten these two 5/32″ cap screws on the upper and lower brackets.

Caution: *Both lamp leads are at a potential with respect to the ground (earth). Do not let bare lamp leads touch any part of the chassis, which is grounded (earthed). You can severely damage the power supply if they do.*



10 Replace the cover of the lamp housing and secure with the 13 Phillips-head screws.

11 Plug the power cord from the lamp power-supply into a grounded (earthed) outlet with the proper line voltage.

Warning: ***DO NOT*** *operate this system from an ungrounded AC power source.*

Note: *After installation, the lamp should burn in for 24 hours. After the burn-in, the lamp's position may be adjusted to optimize the signal intensity.*

Adjustment

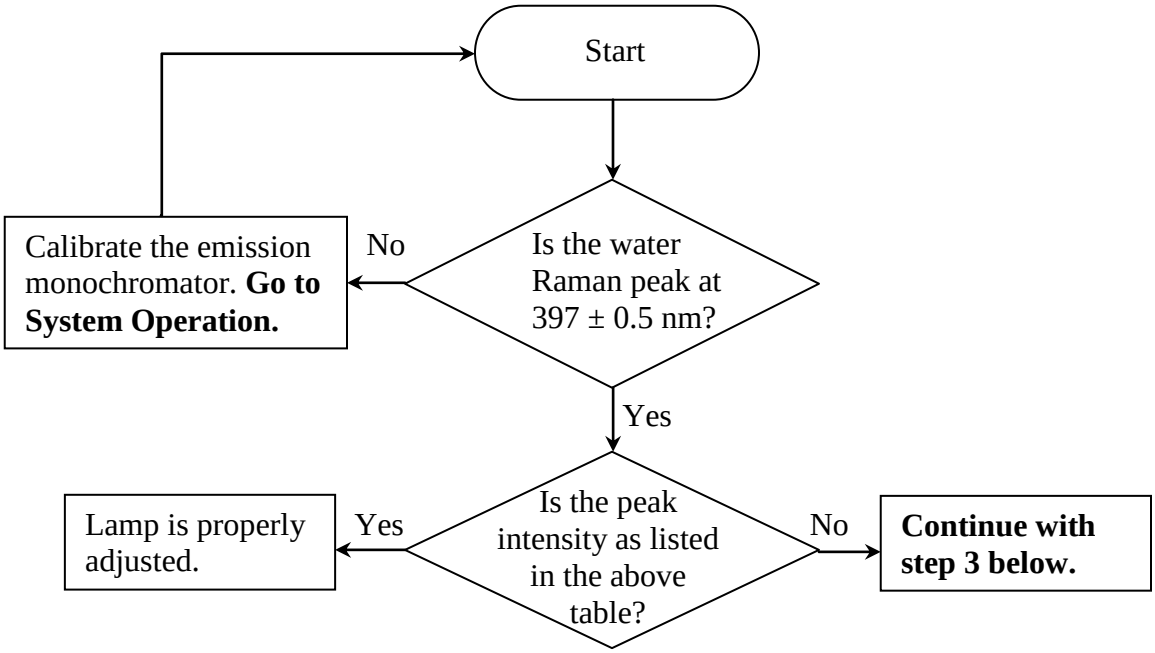
Once the lamp is installed (and after a 24-hour burn-in period), it may need an adjustment to maximize the sensitivity of the Fluorolog®-3. To do this, HORIBA Scientific recommends running a water Raman scan to check wavelength accuracy, and then monitoring the peak intensity while adjusting the vertical height of the lamp:

1 Insert a sealed water-filled cuvette in the sample compartment.

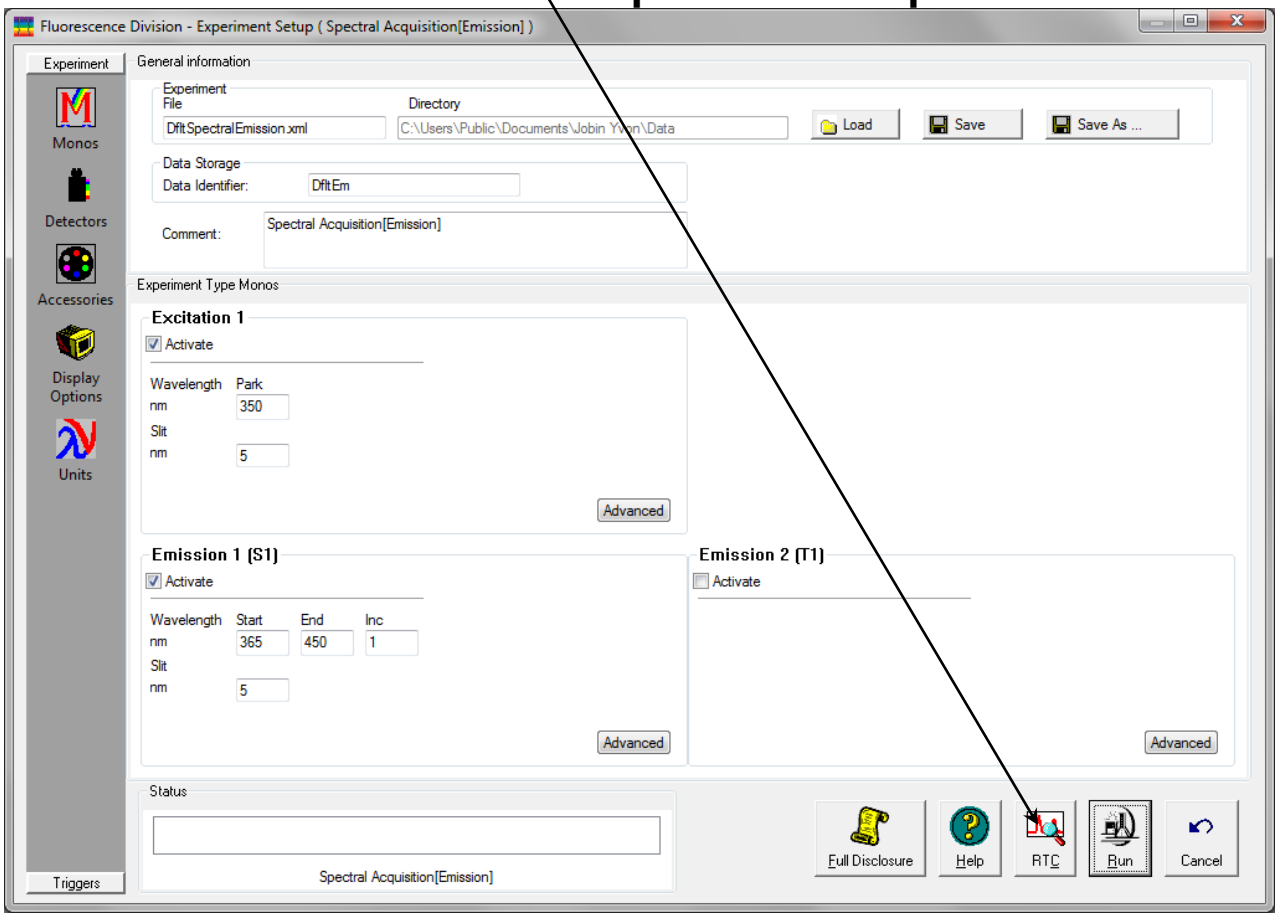
2 Take a water-Raman scan with the following parameters:

Emission wavelength	350 nm
Excitation scan range	365 nm to 450 nm
Increment	1 nm
Integration time	0.1 s
Slits	5 nm bandpass
Acquisition mode	S1

The expected water Raman peak intensity for the Fluorolog®-3 spectrofluorometer systems is > 450 000 cps:

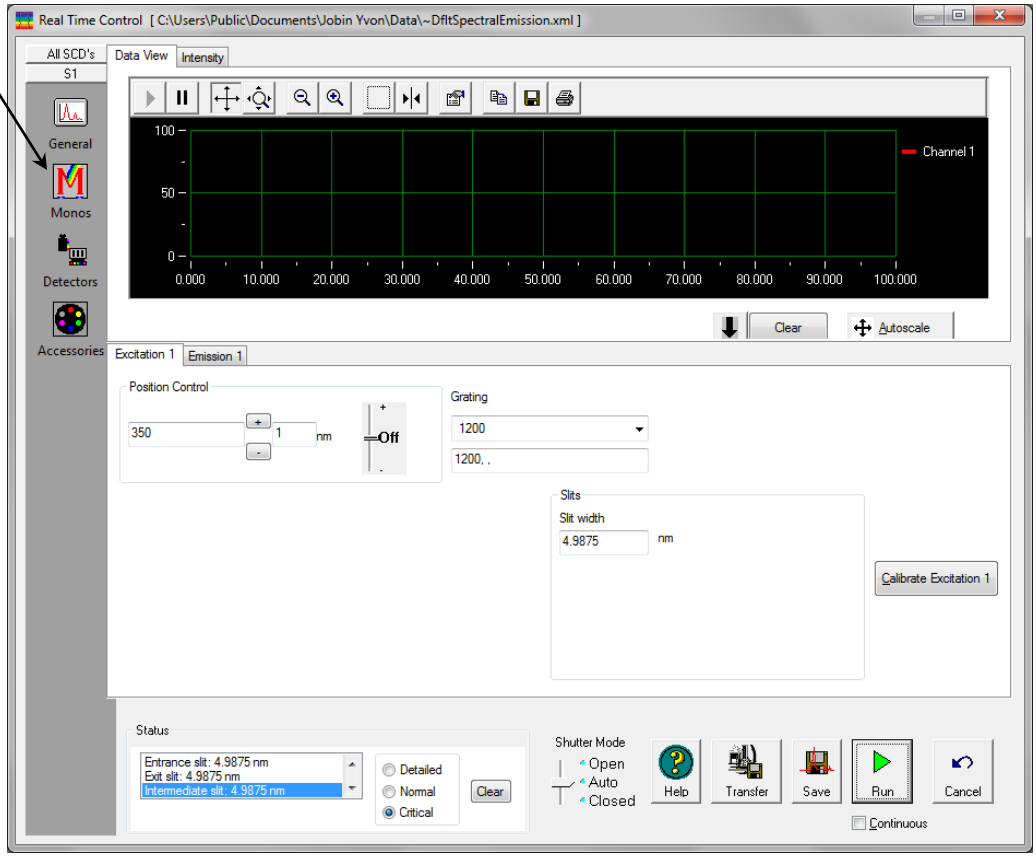


3 Click the RTC button to enter the **Real Time Control** from the **Experiment Setup** window.

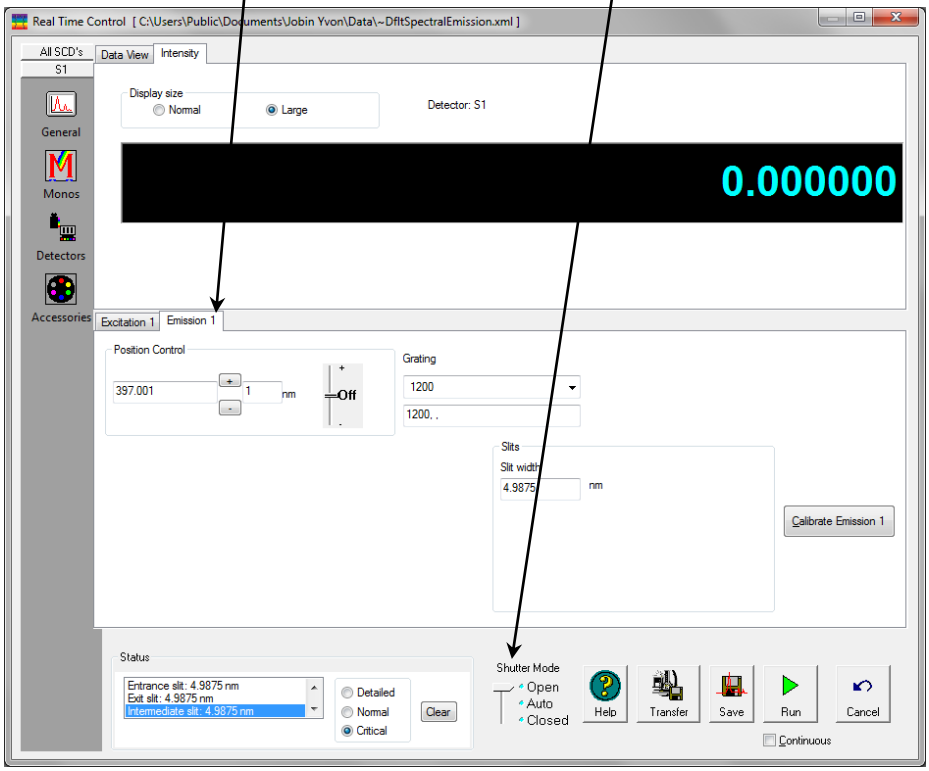


The **Real Time Control** opens.

4 Click the Monos icon:

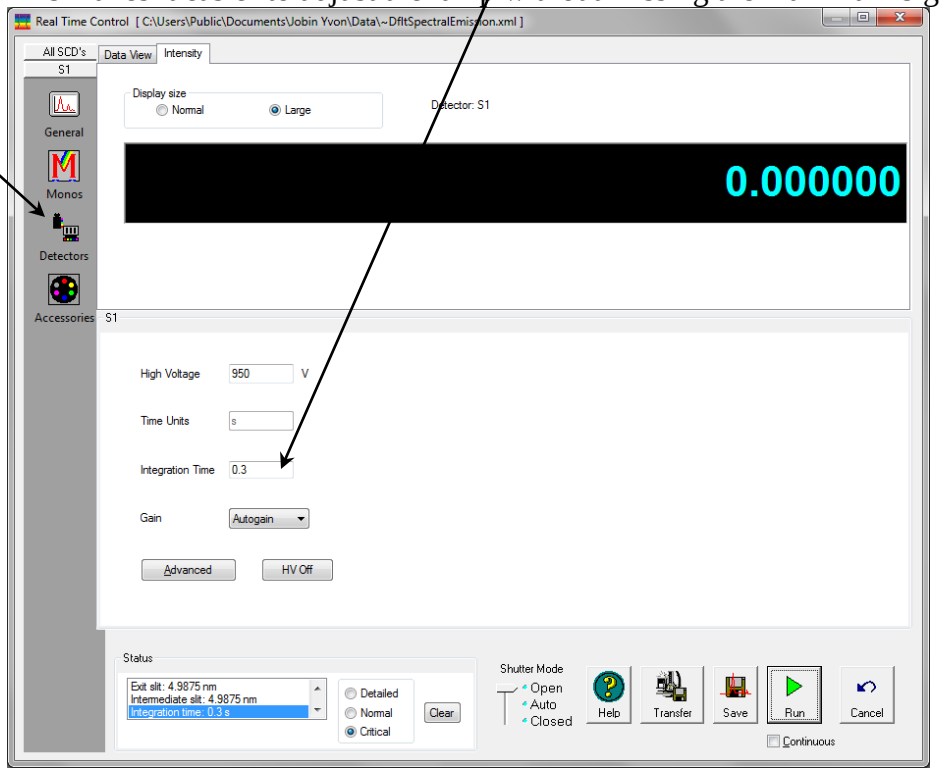


5 In the Emission 1 tab, enter 397.00 in the Position Control, then slide the Shutter Mode switch to Open.

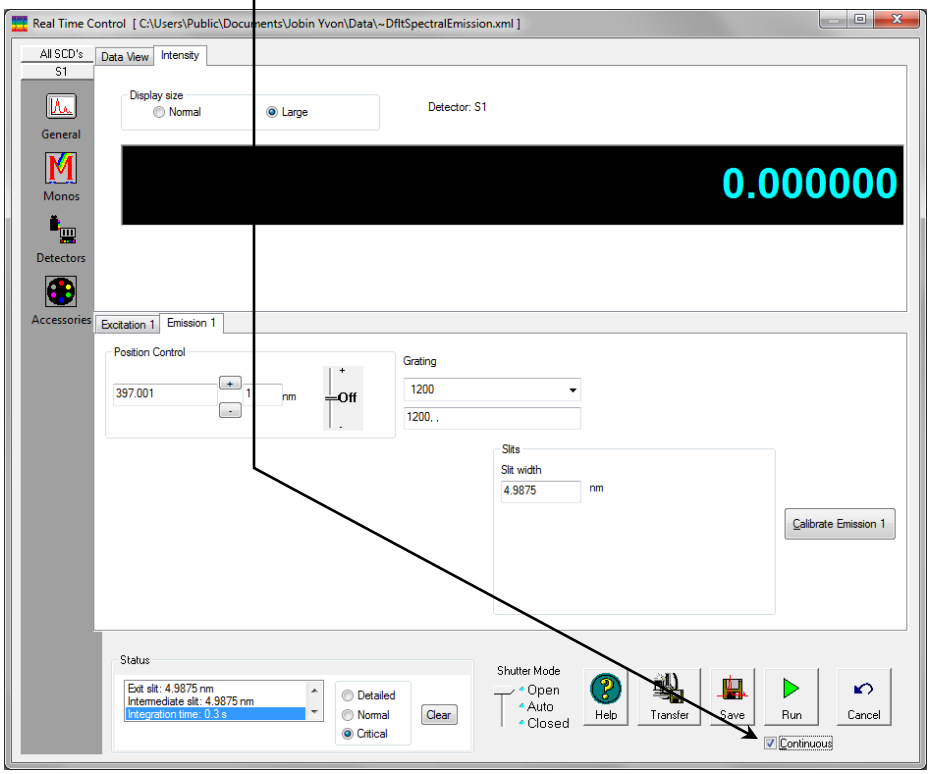


6 Click the Detectors icon, and slow the Integration Time to about 0.3–0.5 s.

This makes it easier to adjust the lamp without missing the maximum signal.



7 Click the Continuous checkbox.



8 Click the Run button.

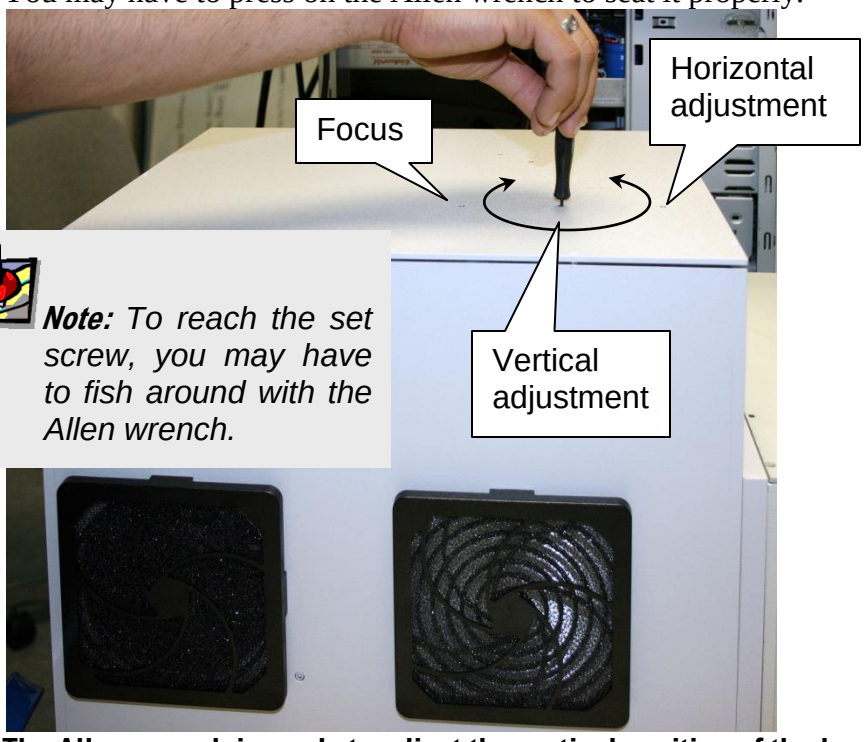
9 View the incoming data in acquisition mode S (signal detector).

Watch the signal intensity as you adjust the vertical position of the lamp. Therefore, position the monitor to see the changes while standing by the lamp housing.

Note: *If the lamp is not at the best vertical position, correctly positioning the lamp will increase the signal significantly.*

10 Insert a 5/64″ Allen wrench inside the middle port on the top of the lamp housing.

You may have to press on the Allen wrench to seat it properly.



Note: *To reach the set screw, you may have to fish around with the Allen wrench.*

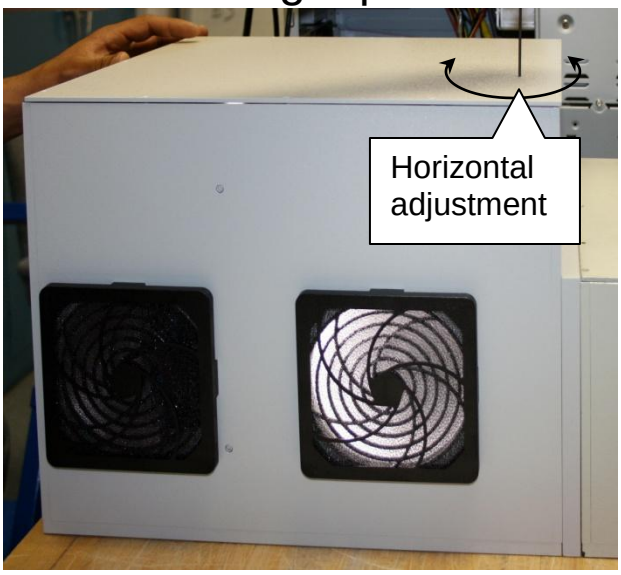
The Allen wrench is ready to adjust the vertical position of the lamp.

11 View the trace and make vertical adjustments by rotating the wrench either left or right to increase the signal.

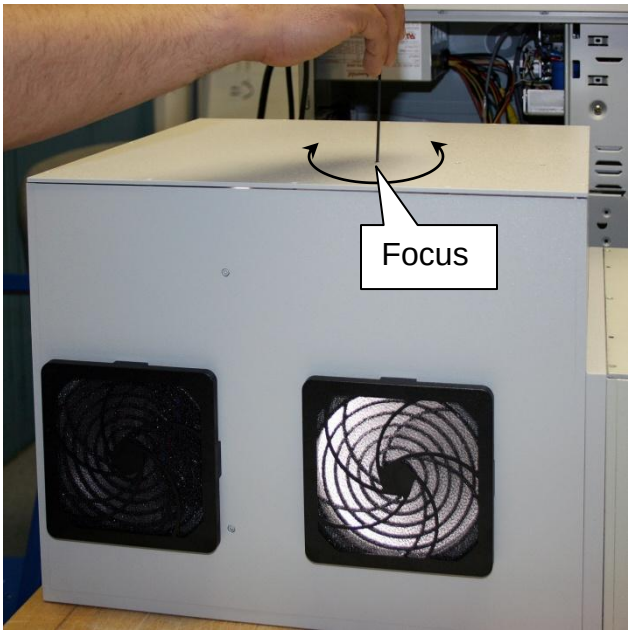
To raise the lamp, rotate the vertical adjustment clockwise.
To lower the lamp, rotate the vertical adjustment counterclockwise, and give the Allen wrench a light tap.

12 Insert the Allen wrench into the right port.

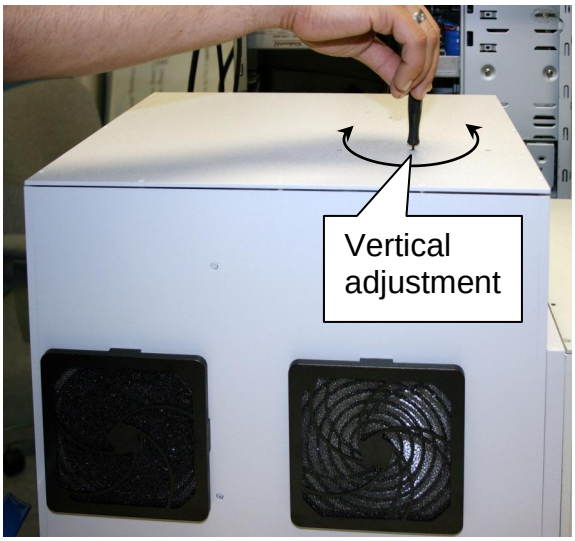
This action adjusts the horizontal position of the lamp. Rotate the wrench left and right to maximize the signal.



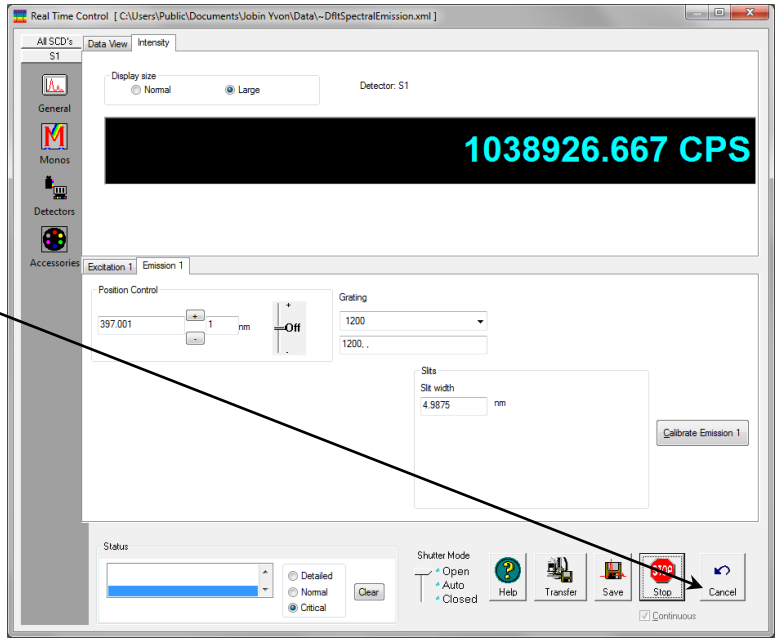
13 Insert the Allen wrench into the left port.



14 Return to the central port and make a final adjustment of the vertical position to maximize the signal.



15 Close the **Real Time Control** by clicking the Cancel button.



16 Record the date that the new lamp was installed, as well as its maximum intensity.

17 Run another water-Raman spectrum, to compare its quality with the previous lamp.

18 Save the protective cover of the new lamp, for when the lamp must be replaced in the future.

Installing an optional new photomultiplier tube

If your Fluorolog®-3 has an optional R928P (usually on the T-side of the sample compartment), and you want to change the detector, this section is applicable. When the R928P photomultiplier is replaced with a different detector, the emission correction factors must be updated (see Chapter 8: *Producing Correction Factors*).

Warning: *Lethal high voltages are applied to the photomultiplier cathode. Turn off all instrument power, especially for the SpectrAcq, before installing or removing a photomultiplier. Never expose a photomultiplier tube to room light when the high voltage is turned on.*

1. Turn off power to the instrument and SpectrAcq.
2. Be sure that a liquid-N_2-cooled PMT is warmed to room temperature.
3. Remove cables and wires attached to the PMT.

Note: *Fingerprints on the glass of the photomultiplier increase the dark counts of the tube. Immediately remove fingerprints from the photomultiplier by rinsing with methanol and wiping with lens tissue.*

Caution: *Read the Materials Safety Data Sheet (MSDS) before using methanol.*

4. Carefully lift the tube from its base socket.
5. Remove the protective cap from the new PMT.
6. Plug the photomultiplier into the base socket.
   Make sure the photomultiplier is positioned all the way down on the socket.
7. Carefully slide the photomultiplier into the base socket.
8. Connect the cables as per Chapter 17.

Reference signal detector

The reference signal detector is a state-of-the art silicon diode that requires no routine maintenance.

Gratings

The standard excitation and emission monochromator gratings are 1200 grooves/mm, and are blazed at 330 nm and 500 nm, respectively. If an application requires that the system be optimized for a particular region, the gratings can be changed by following a simple procedure.

Required tools

Tool	Size	Purpose
Phillips screwdriver	medium	Remove top cover.
Lid support	--	Supports the lid in an open position.

Replacement

Caution: *Never touch the diffraction surface of a grating.*

Gratings require no routine maintenance. To use a grating with different specifications than those installed in the system, replace the existing ones.

Mirrors in the spectrometers have been aligned at the factory. If mirror realignment is necessary, contact the Service Department. Typically, mirror realignment requires special targets and fixtures, and a laser.

Caution: *Do not loosen the mirror mounts.*

Caution: *HORIBA Scientific recommends that you never attempt to clean off any dust on the gratings and mirrors. The effect of cleaning may cause more problems than the dust. The surface is easily marred and cannot be cleaned the way a mirror can.*

Remove the existing grating

1. Close the slits in the emission spectrometer.
2. Turn off all instrument power.

3 Remove the Phillips-head screws holding the lid on the monochromator.

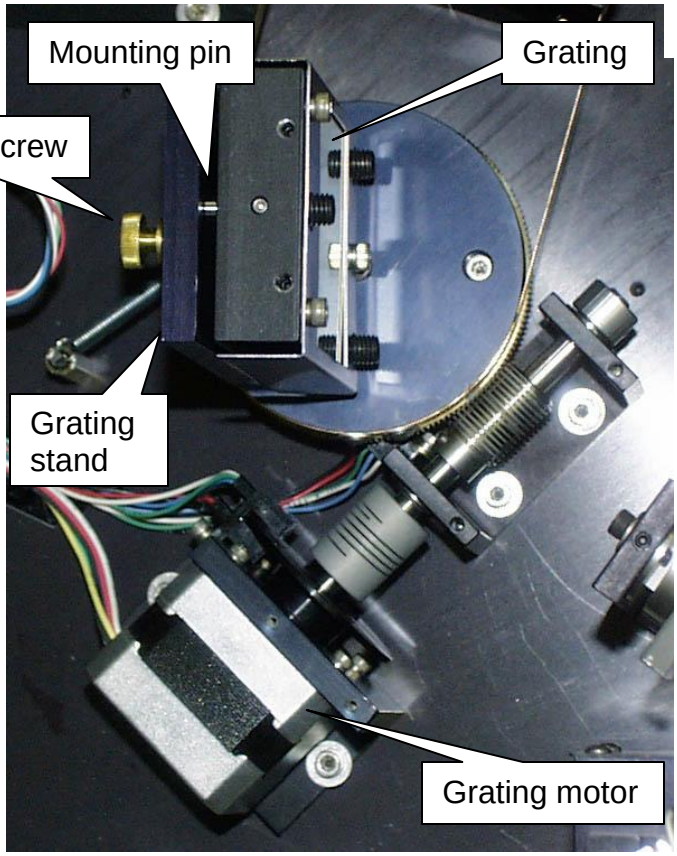
4 Lift the lid slowly until you feel resistance.

This is the maximum length of the cables attached to the lid.

Caution: *A circuit board and critical connections are attached to the lid of the spectrometer. DO NOT attempt to lift the lid completely off.*

5 Insert the lid support at the rear of the monochromator.

6 Place the lid in the support.

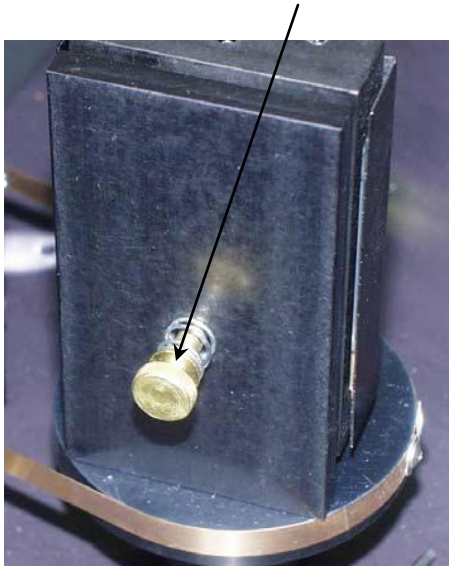


Mounting screws for baffle

Overhead view of the grating assembly.

7 Release the grating from the stand.

a Loosen the thumbwheel screw securing the grating to the grating stand.



Back view of grating assembly.

b Pull the grating away from the stand.

To insert a new grating,

1. Place the 3 mounting pins on the back of the grating into the matching mounting slots on the grating stand.
2. Tighten the thumbscrew on the back of the grating stand.
3. Lift the lid of the monochromator off of the lid mount.
4. Remove the lid mount.
5. Place the lid on the monochromator.
6. Secure the lid with the Phillips screws.

No further adjustments or alignments are necessary.

Mirrors

Mirrors are aligned at the factory and usually do not need realignment.

Caution: *Adjust the mirrors* ***only if a laser is available*** *to check the alignment. Misalignment will destroy the similarity of the right-angle and front-face light paths in the single-beam sampling module. (Front-face capability is an option in the sample compartment.)*

Improperly moving any of the mirrors in the monochromator reduces resolution; refocusing will be required. If the system becomes misaligned, contact the Service Department to arrange for a service visit.

Automated 4-position turret

If a circulating bath is used to regulate the turret's temperature, periodically replace the fluids in the bath.

Note: *Over time, bacteria can grow in the temperature bath, or the water can become hard.*

Chapter 7 : Troubleshooting

The Fluorolog®-3 spectrofluorometer system has been designed to operate reliably and predictably. Should a problem occur, examine the chart below, and try the steps listed on the following pages.

Problem	Possible Cause	Remedy
Light is not reaching the sample.	Excitation shutter closed.	Using the software, open the shutter.
	Slits are not open to the proper width.	Adjust the slits.
	Lamp is not turned on	Turn on lamp by pressing lamp rocker switch, and then the Start button on the xenon-lamp power-supply front panel.
	Excitation monochromator is miscalibrated.	Check and recalibrate excitation monochromator.
	Sample turret is not in correct position.	Using the software, set the position and open the cover to verify the position.
Signal intensity is low.	Lamp is not aligned or focused.	Align and focus the lamp.
	Slits are not open to proper width.	Adjust the slits.
	Shutter(s) is(are) not completely open.	Open the shutter(s).
	Lamp power supply is set to the wrong current rating.	Call the Service Department. (450 W Xe lamp current = 25 A.)
	High voltage is improperly set.	Enter proper voltage: Default HV1 = 950 V
	Polarizer is in the light path.	Move the polarizer out of the light path.
	Lamp is too old.	Replace lamp. (450 W lamp has lifetime 1500–2000 h.)
Signal intensity is at least 10 times lower than normal.	Polarizer is in the light path.	In **Real Time Control**, move the polarizer out of the light path.
No change in signal intensity.	Monochromators are set to wrong wavelength.	Select appropriate wavelength based on excitation and emission of sample.
	Detectors are saturated.	Adjust slits. (Signal detector is linear to 2×10^6 cps in photon-counting mode. Reference detector saturates at 12 μA.)
	Optical density effects and self-absorption.	Sample is too concentrated. Dilute sample by a factor of 10 or 100 and retry experiment.
No change in signal intensity.	Shutter(s) closed.	Open all shutters.
	Detectors are saturated.	

[continued on next page]

No signal.	Lamp is not on.	Replace bad xenon lamp.
	Detectors are saturated.	Reduce slit settings.
	High voltage is off.	Turn on high voltage through the software.
Lamp unstable.	Erratic signal.	Let lamp warm up 20 min before use.
	Light leaks.	Check dark value to determine.
Raman band superimposed on fluorescence scan.	Aqueous solutions and solvents have Raman bands.	Change excitation wavelength to move Raman band away from fluorescence peak, or run a blank scan of the solvent and subtract it from the fluorescence spectrum.
Large off-scale peak at twice the excitation wavelength.	2^{nd}-order effects from the spectrometer.	Use cut-on filters to eliminate 2^{nd}-order peak.
Stray light in emission scan (also see example in this chapter).	Scattered light off the excitation wavelength.	Place bandpass filters in excitation light path. Decrease emission-monchromator slit widths.
Corrected excitation spectrum curves upward ~240–270 nm.	Dark count is divided by low reference signal.	Use Dark Offset checkbox; retry scan.
Noisy spectrum with magnetic stirrer.	Stirring speed is too fast.	Use slower stirring speed.
Communication problems between computer and instrument.	Software was initialized before hardware	Wait for beeps from SpectrAcq before initializing the software.
	Cables are improperly connected.	Check communications cables' connections.
	Computer's or SpectrAcq's I/O-controller is failing.	Replace I/O controller: Call Service Department.
`Hardware Init. Error` appears.	Broken IR sensor in monochromator.	Replace IR sensor: Call Service Department.
`Data file does not exist` or `file read error` message appears.	User is not logged into Windows® as administrator or power user.	Log into Windows® as administrator or power user, and restart FluorEssence™.
Sample turret is not operating.	Software is not enabled.	Check status.
	Cables are improperly connected.	Check cable connections

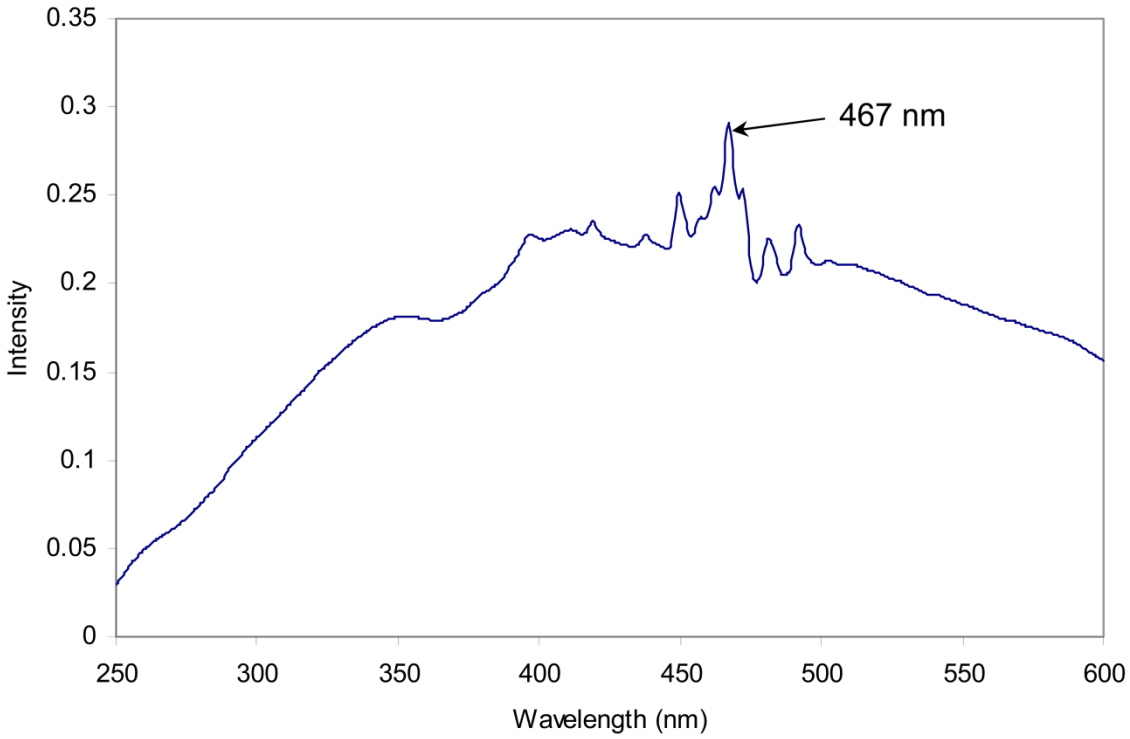
Using diagnostic spectra

Often the spectrum reveals information regarding the hardware or software parameters that should be adjusted. The following spectra occur with explanations regarding problems leading to their appearance.

Note: *Not all spectra shown in this section were produced using the Fluorolog®-3. The spectra are presented to show different possible system or sample problems, and may not reflect the superior performance of the Fluorolog®-3.*

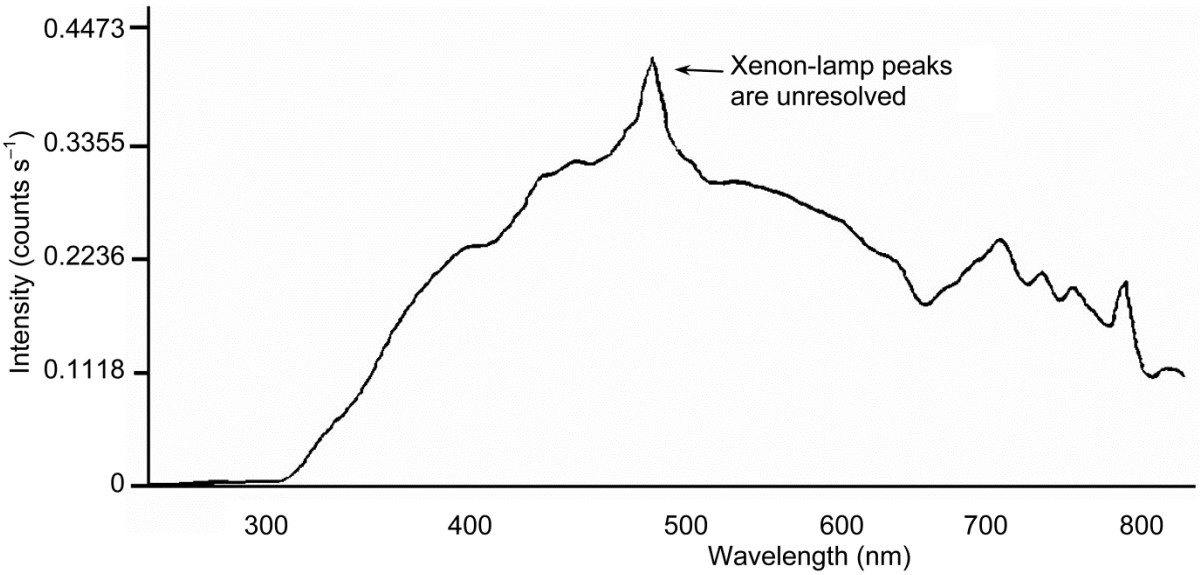
Lamp scan

Running a lamp scan verifies system integrity and indicates whether the correct parameters for the best possible trace are being used. The following spectrum shows the trace resulting from a lamp scan run with a known good lamp.



Scan of good quality 450 W xenon lamp in Fluorolog-3 with single excitation monochromator.

The following lamp scan spectrum shows poor resolution in the area around the peak.



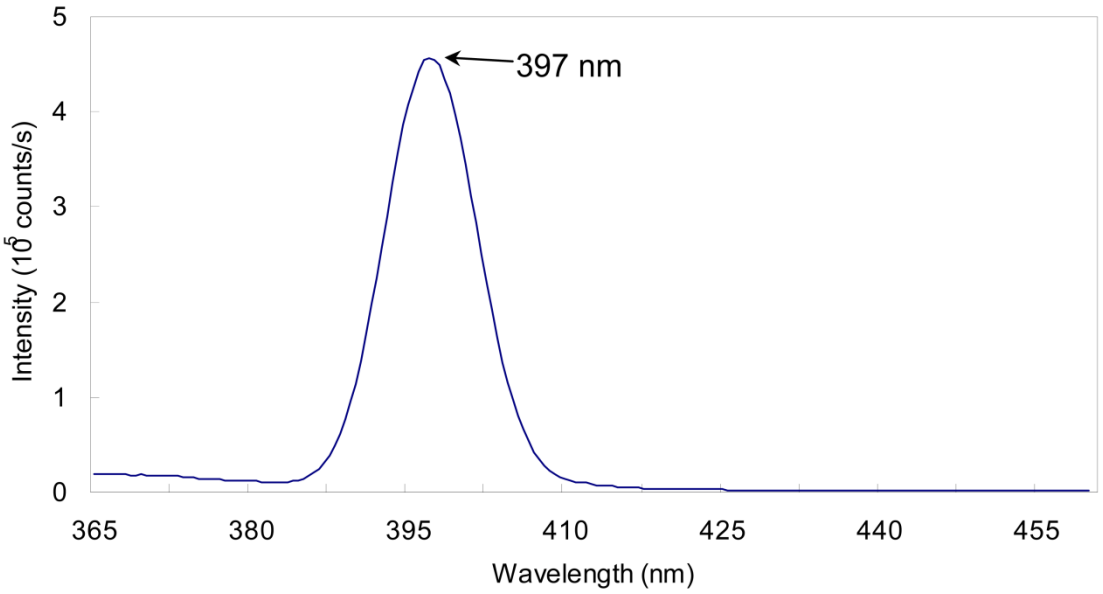
Lamp scan of 150-W Xe lamp. Note poor resolution in the area near the 467-nm peak.

This lack of spectral resolution appears because the slit widths are set too wide. To resolve this problem, narrow the slit widths.

Water-Raman spectra

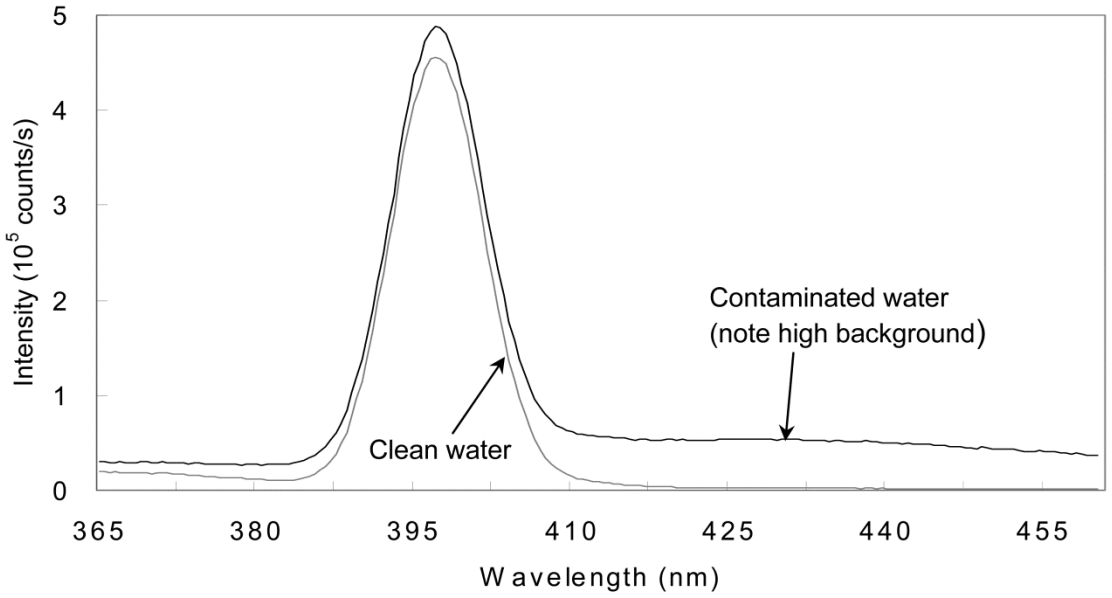
Contaminated water

Running a water-Raman scan helps identify abnormalities as a result of accessory problems or miscalibration. The following spectrum is normal:



Clean water Raman scan.

Below is a normal water Raman spectrum superimposed on one that exhibits a problem. In this instance, the water was contaminated, resulting in a high background.



Contaminated water in a water Raman scan.

If a spectrum similar to this is obtained after running a water Raman scan,

1 Rotate the cuvette 90° and rerun the scan.

If the problem goes away, then the problem was caused by the cuvette surface. Clean the cuvette, or use a different cuvette.

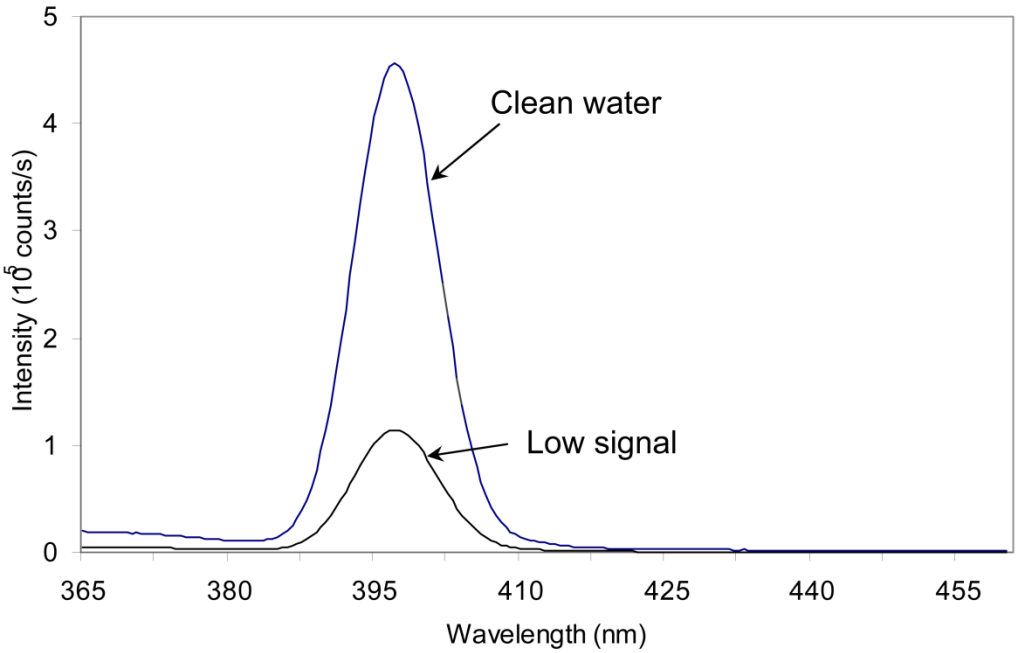
Or

1. Clean the cuvette.
2. Fill with fresh, double-distilled, deionized water.

   If the problem goes away, then the problem was caused by contaminated water.

Light not striking cuvette

The following graph shows a normal water-Raman scan with a superimposed problem scan.



Low intensity during a Raman scan.

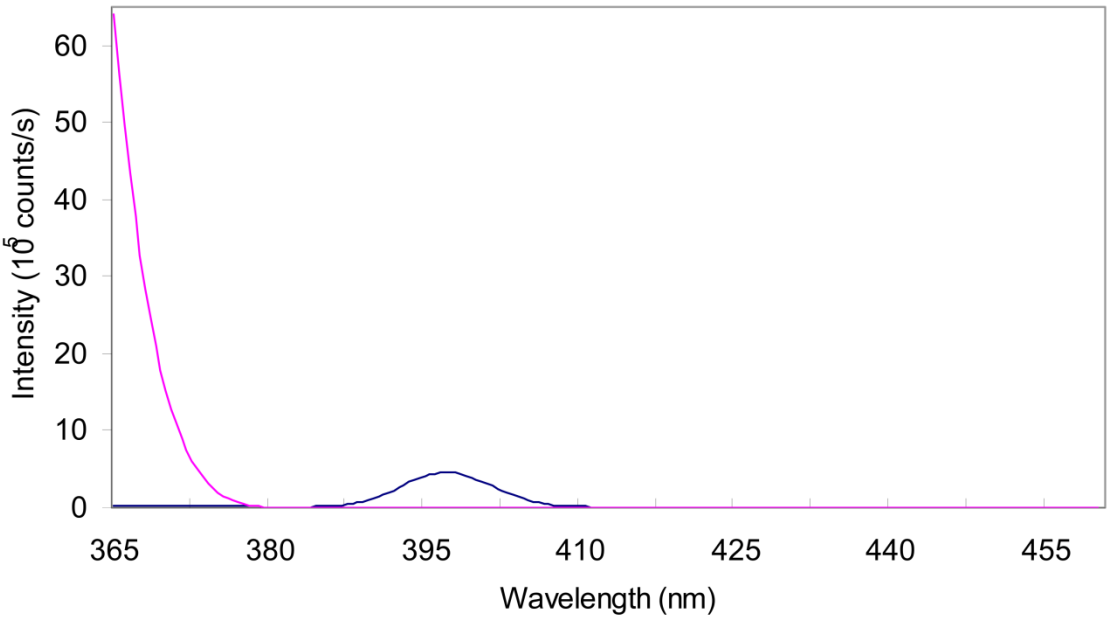
Here the problem is low intensity of the water signal when compared with the superimposed typical water-Raman scan. To resolve this problem:

1. Make sure the cuvette is filled to the proper level.

   Light should fall on the sample, and the meniscus should not be in the light path.
2. Make sure that the excitation and emission slits are set to the proper widths.
3. Verify that the detector is set to the proper voltage.

Stray light

In the following diagram, notice the high level of stray-light below 380 nm in the water-Raman spectrum.



High stray light in a water Raman scan.

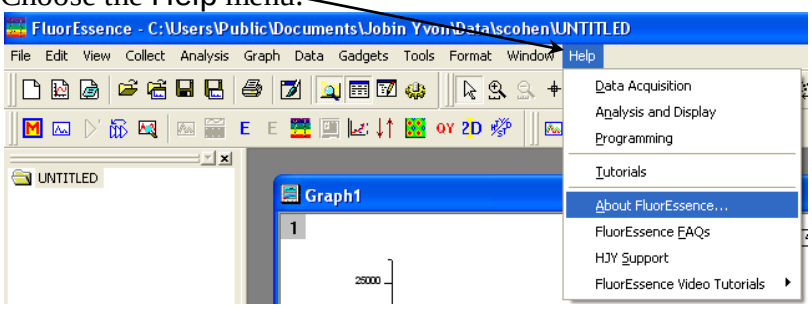
To correct this problem,

1. Inspect the cuvette surface for fingerprints and scratches.
2. Clean the cuvette or use a new one.
3. Verify that the excitation and emission slits are set correctly for a water-Raman scan.
4. Verify that the excitation monochromator is at the correct position.

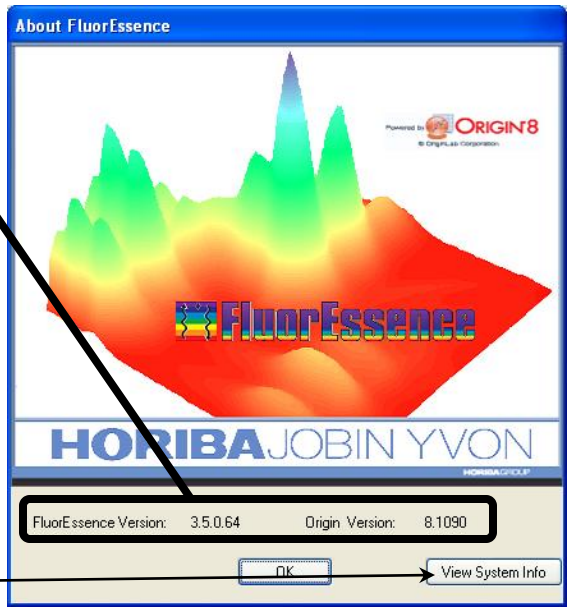
Further assistance...

Read all software and accessory manuals before contacting the Service Department. Often the manuals show the problem's cause and a method of solution. Technical support is available for both hardware and software troubleshooting. Before contacting the service department, however, complete the following steps.

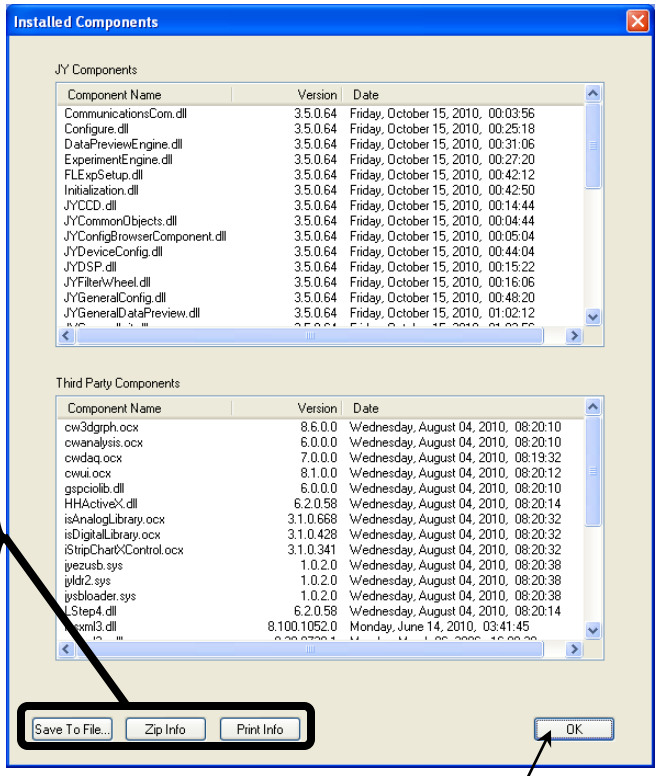
1. If this is the first time the problem has occurred, try turning off the system and accessories.
   After a cool-down period, turn everything back on.
2. Make sure all accessories are properly configured and turned on as needed.
3. Following the instructions in *System Operation*, run a xenon-lamp scan to make sure the system is properly calibrated.
   Print the spectrum and note the peak intensities.
4. Check this chapter to see if the problem is discussed.
5. Visit our web site at
   http://www.horiba.com/scientific/products/fluorescence-spectroscopy/
   to see if the question is addressed on the site.
6. Try to duplicate the problem and write down the steps required to do so.
   The service engineers will try to do the same with a test system. Depending on the problem, a service visit may not be required.
7. If an error message appears in FluorEssence™, write down the exact error displayed.
8. Determine FluorEssence™'s version number.
   a. Choose the Help menu.



b Choose About FluorEssence....
The **About FluorEssence** window opens. Near the bottom are the FluorEssence™ and Origin® version numbers.



c Click the View System Info button.
The **Installed Components** window appears, displaying all the software required for FluorEssence™.



d Record the information by clicking the:

- Save To File... button, which saves the information to a file;
- Zip Info button, which compresses the information while saving it;
- Print Info button, which prints out the software information.

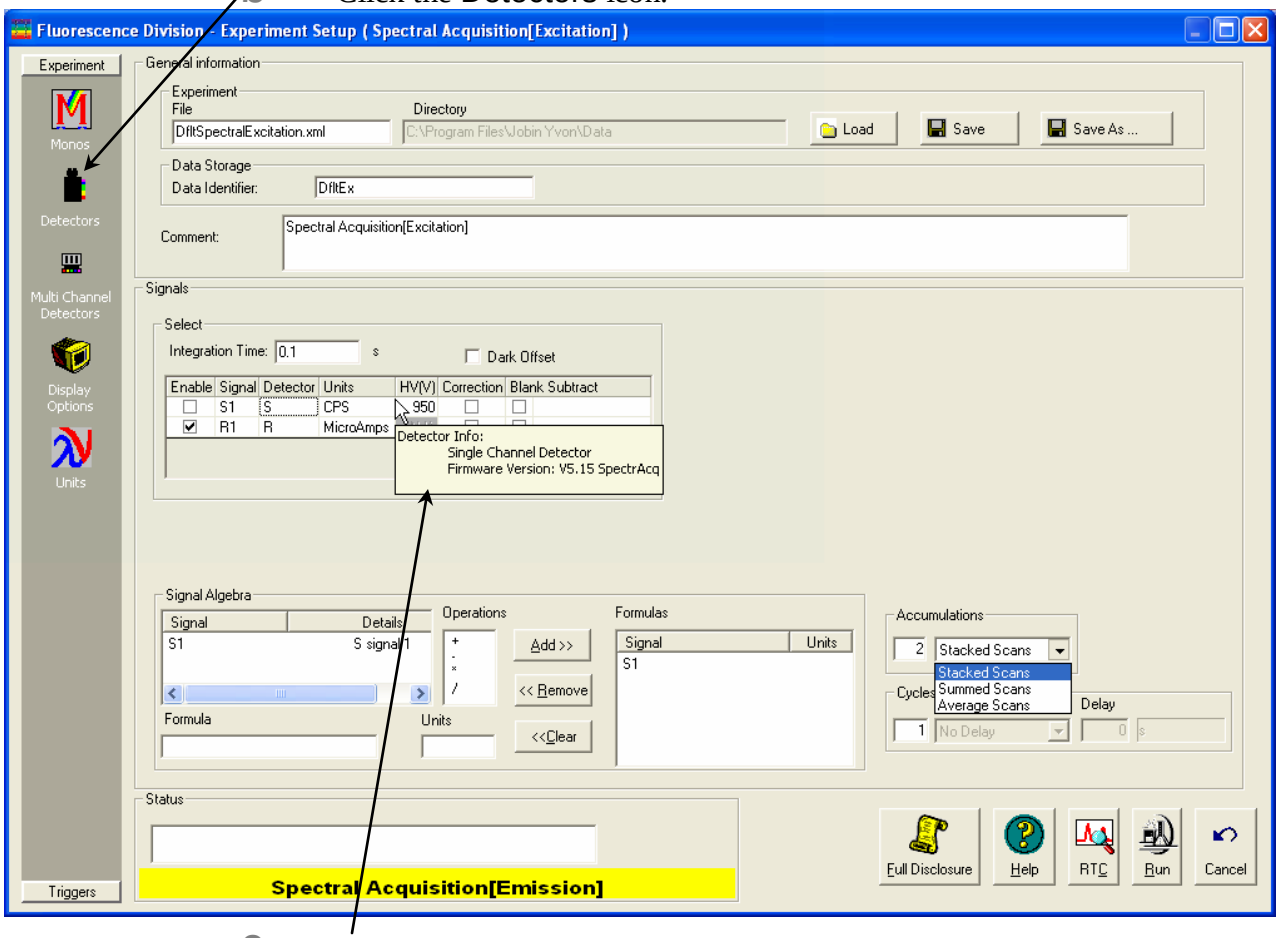
e Click the OK button to close the **Installed Components** window.

f Click the OK button to close the **About FluorEssence** window.

9 Determine the SpectrAcq firmware version.

a Open the **Experiment Setup** window:

b Click the Detectors icon.



c Move the mouse over the detectors' table in the Select area.
The SpectrAcq firmware version appears in a small pop-up window.

10 Write down the software's version numbers, along with the purchase dates, model numbers, system configuration, and serial numbers of the instrument and its accessories.

11 Call the Service Department at (732) 494-8660 × 8160.

Be prepared to describe the malfunction and the attempts, if any, to correct it. Have serial and version numbers of all software and equipment handy, along with all relevant spectra (sample, polarization ratio, xenon-lamp scan, emission calibration, etc.).

Chapter 8 : Producing Correction Factors

Introduction

Gratings, detectors and other monochromator components have response characteristics that are functions of wavelength. These characteristics are superimposed on spectra, and may yield a potentially misleading trace. For accurate intensity comparisons, such as those required for quantum-yield determinations, monochromator-response characteristics must be eliminated. Corrections are made for each of these potential problems by using radiometric correction factors.

Supplied with the Fluorolog®-3 are sets of excitation and emission correction factors designed to eliminate response characteristics. These files, `xcorrect.spc` and `mcorrect.spc` (and `tcorrect.spc` for T-format instruments), are included with the software and should be copied to the hard disk. The excitation correction range is from 240–1000 nm, and the correction range for emission spectra is from 290–850 nm (for an instrument with a R928P photomultiplier tube; the wavelength range varies for different detectors).

Emission correction factors should be updated periodically or whenever different gratings or signal detectors are installed. The correction factors can be updated either at the user's location, or by a representative from the Service Department. To arrange for a visit and a fee estimate, call the Service Department. To update the correction factors without a service visit, follow the instructions below.

One way to generate correction factors for the instrument is to scan the spectrum of a standard lamp. Because the actual irradiance values of the standard lamp as a function of wavelength are known, dividing the irradiance values by the lamp spectrum results in a set of relative correction values. These values can then be applied to the raw fluorescence data. The emission correction factor file `mcorrect.spc` was acquired in this manner.

Note: *Generate a new correction-factor file only when the gratings or detectors have been replaced with those of different specifications than the original hardware.*

Types of Correction-Factor Kits

To generate emission correction factors, several items are needed: a standard lamp, appropriate holders, and a scatter assembly. HORIBA Scientific offers two methods. You may also use your own standard lamp.

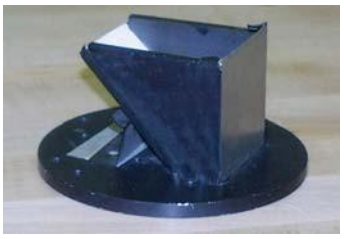
1908 Accessory

Note: *Contact the Service Department for use of the 1908 correction-factor kit.*

The 1908 is a complete correction-factor kit, while the 1908MOD Scatter Assembly is provided for users who already have a calibrated standard lamp and a constant-current source.

The 1908 Standard Lamp Assembly is a complete correction factor kit, which includes the following items:

- 200-watt quartz tungsten-halogen filament lamp with irradiance values
- Constant-current power supply with lamp holder
- 1908MOD scatter assembly



The 1908MOD scatter assembly includes:

- Lamp-mount assembly and mask with square center
- Scatter block with neutral-density filter and reflectance plate

The F-3026 Correction Factor Kit

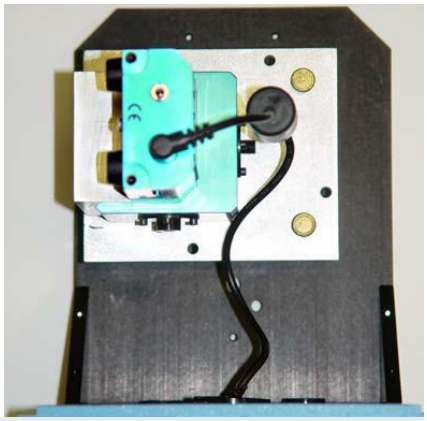
The F-3026 is a small module that sits within the instrument's sample compartment. It is composed of:

- Tungsten-halogen source, combined with an
- Integrating sphere
- 12 V DC power supply
- Special sample-compartment cover

Generating emission correction factors with the F-3026 Correction Factor Kit

Note: *You also may use your own kit, or contact the Service Department for use of our 1908 calibrated-lamp kit.*

1 Set up the accessory.

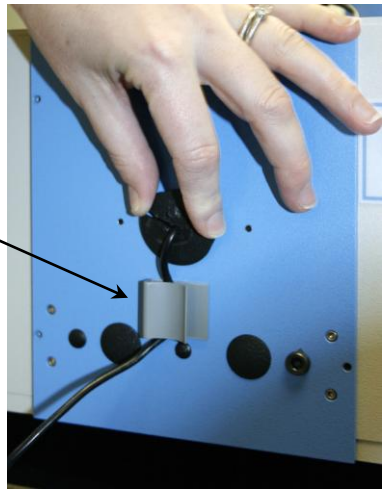


a Remove the standard sample drawer from the sample compartment.

b Attach the correction-factor light-source to its gap-bed with the brass thumbscrews.
Use this orientation. Be sure the power cable doesn't block optical apertures.

Note: *This example (see image above) was set up for measuring the S-channel side of a Fluorolog®-3. To set up using the T-channel, the lamp faces the opposite emission port (180° to the left).*

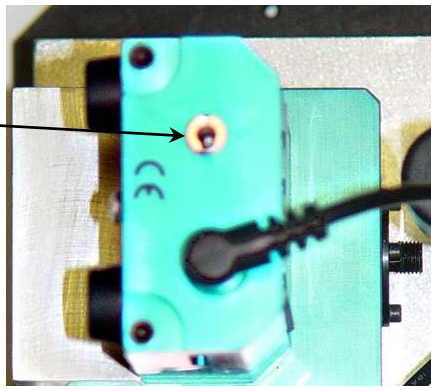
Note: *The source/integrating sphere module may be pre-mounted atop the special gap-bed.*



c Feed the wire through the hole on the front of the sample compartment, and seal with a black plastic plug.
Clip the wire into its clamp.

d Insert the sample drawer for the correction factor kit.
Make sure all filter-holders are removed from the sample compartment. Fix the gap-bed firmly to the sample compartment with screws through the front panel.

2 Run the emission scan.



a

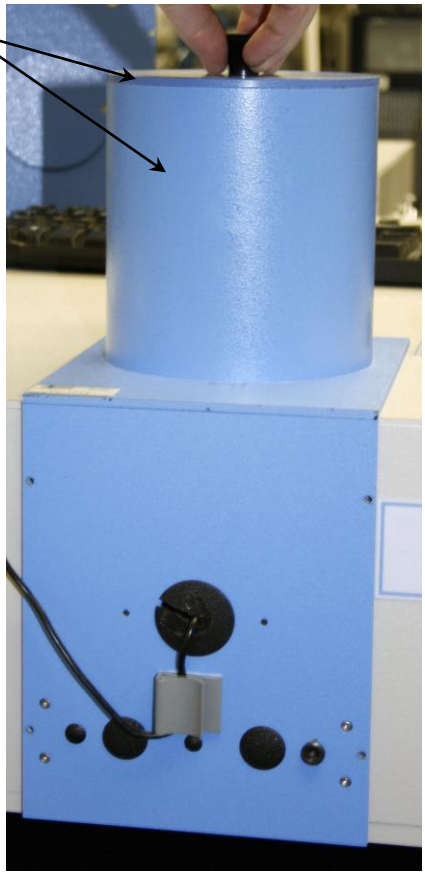
Switch on the F-3026 and let it warm up for 15 min.

Caution: *Wear appropriate eye-protection against UV, visible, and IR when the tungsten-halogen lamp is on and the sample-compartment is uncovered.*

Note: *We recommend keeping a log of the amount of time the source is on. Calibration is necessary after every 50 h of use.*

b

Place the special lid onto the sample compartment.
Make sure it fits securely, and there is no light leak into the sample compartment.



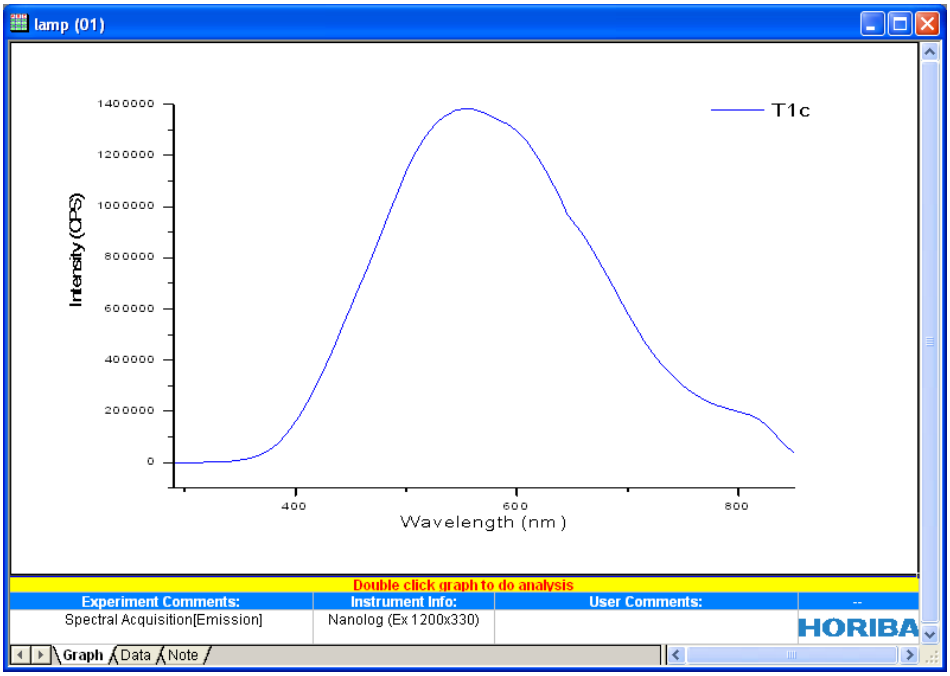
Caution: *Do not operate the accessory without the lid completely covering the sample compartment.*

c

Start FluorEssence™ and initialize the Fluorolog®-3.
The xenon lamp should be turned OFF.

d Collect an emission scan of S1c only (or S2c, T1c, etc. depending on the detector used) using the following scan parameters:
Range = 300 to 850 nm (depending on the range of the detector and the standard lamp used)
Increment = 5 nm
Emission slit = 5 nm
Integration time = 1 s
Dark Offset on

HORIBA Scientific recommends detecting ~10^6 cps at 520 nm in the **Real Time Control**. If not near this value, open or close the slit to obtain this value. Your emission scan should look something like this, using an R928P detector:

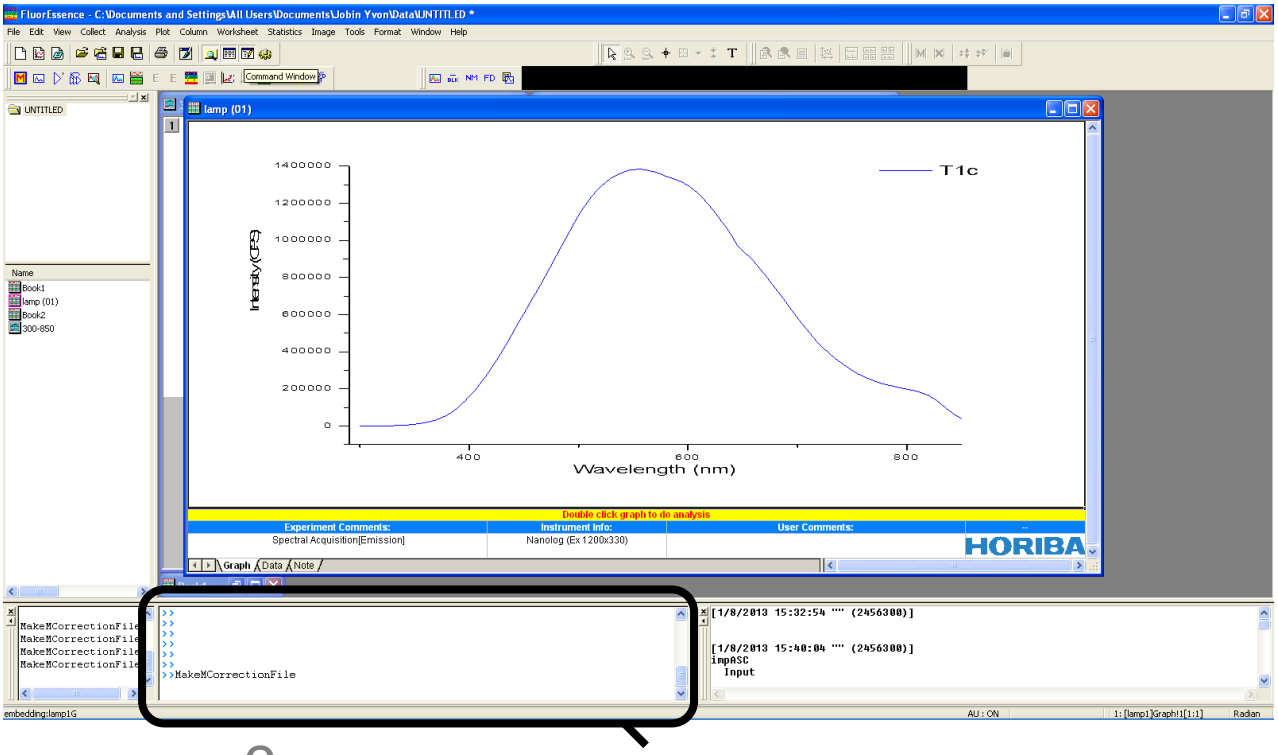


3 Create the correction-factor file.

a Insert the DVD with the correction factors into the host computer.

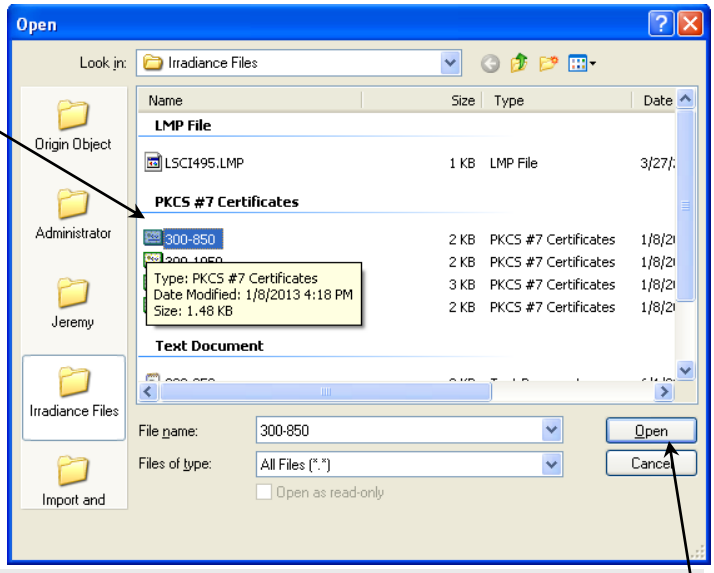
b In the main FluorEssence window, click the Command Window button  .

The **Command Window** opens:



c Type `MakeMCorrectionFile`, then the `Enter` key.
The **Open** window appears.

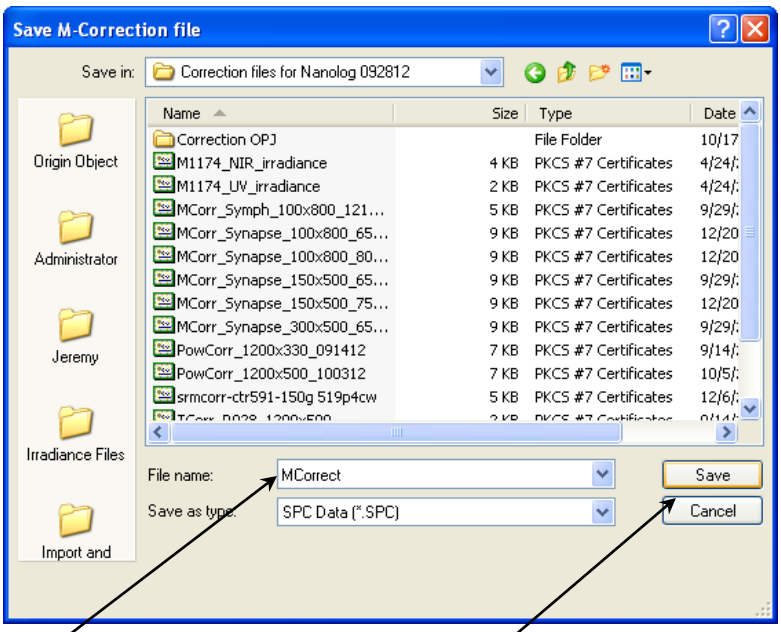
d Browse for the appropriate irradiance data file (in `.SPC` format) on the DVD.



Note: *Be sure to choose the correct irradiance file associated with the desired detector.*

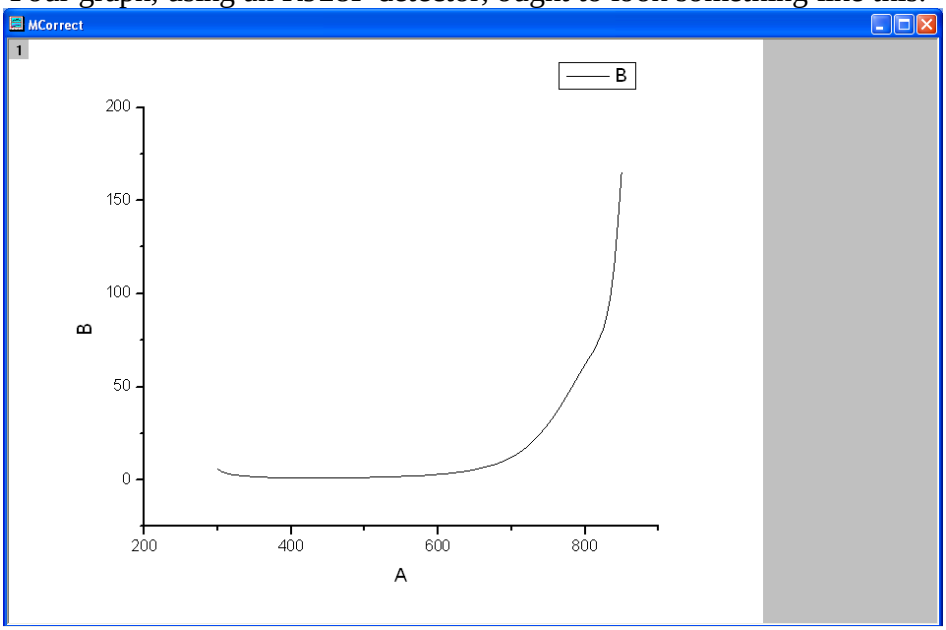
e Click the Open button.
The **Save M-Correction File** window appears:

f Browse to save the correction file in the desired folder.



Give the file a name something like `mcorrect`.

g Click the **Save** button to save the file in `.SPC` format.
The `mcorrect` graph appears, with data normalized automatically.
Your graph, using an R928P detector, ought to look something like this:



4 Load the correction factors into the instrument configuration as explained in “Using correction-factor files” (p. 8-17).

Calculating emission correction factors

For more information about the theory and application of radiometric correction, consult *Accuracy in Spectrophotometry and Luminescence Measurements*, Mavrodineau, Schultz, and Menis, NBS Spec. Publ. 378 (1973), especially p. 137, "Absolute Spectrofluorometry," by W.H. Melhuish.

Irradiance values for a standard lamp, packaged with the lamp, usually are expressed in 10^{-6} W·cm^{-2}·nm. With photon-counting systems like the Fluorolog®-3 spectrofluorometer, however, data usually are collected in units of photons·s^{-1}·cm^{-2}·nm. To convert the units, multiply each irradiance value by the wavelength at which it is valid. (The data will still be off by a factor of *c*, but normalizing the correction factors compensates for this.) Such a mathematical procedure can be done in a spreadsheet program.

The `MakeMCorrectionFile` macro used in the previous section does this automatically, but the mathematics is the following: The irradiance (in W·cm^{-2}) is divided by the lamp spectrum. This result is then normalized by the minimum value. The resulting graph is the correction-factor curve, and this is exported in `.SPC` format.

Calculating excitation correction factors

The photodiode reference detector handles the bulk of excitation correction from 240–1000 nm when a ratio-acquisition mode is selected (i.e., *S*/*R*). More accurate measurements require that compensation be applied for the difference in optical path between the detector and the sample. This can be accomplished by a simple excitation scan with rhodamine-B placed in the sample position.

Note: *To calibrate the reference detector out to 1000 nm, use HORIBA Scientific's calibrated photodiode accessory.*

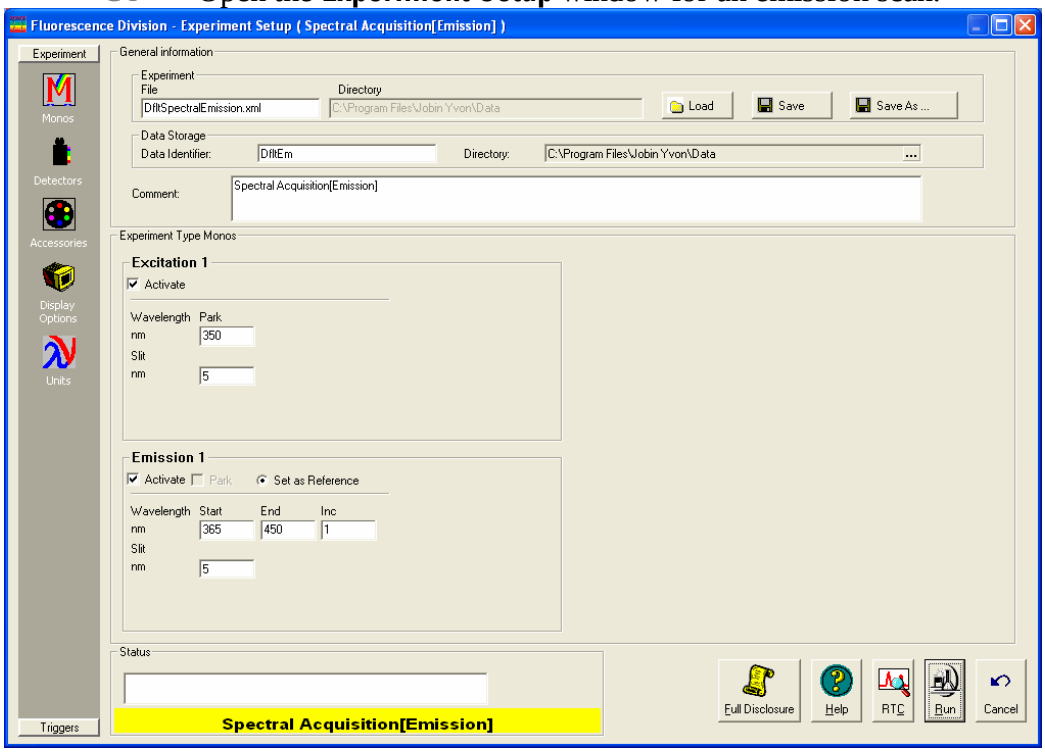
1 Fill a cuvette with a solution of rhodamine-B.

Use 8 g L^{-1} of laser-grade rhodamine-B in 1,2-propanediol.

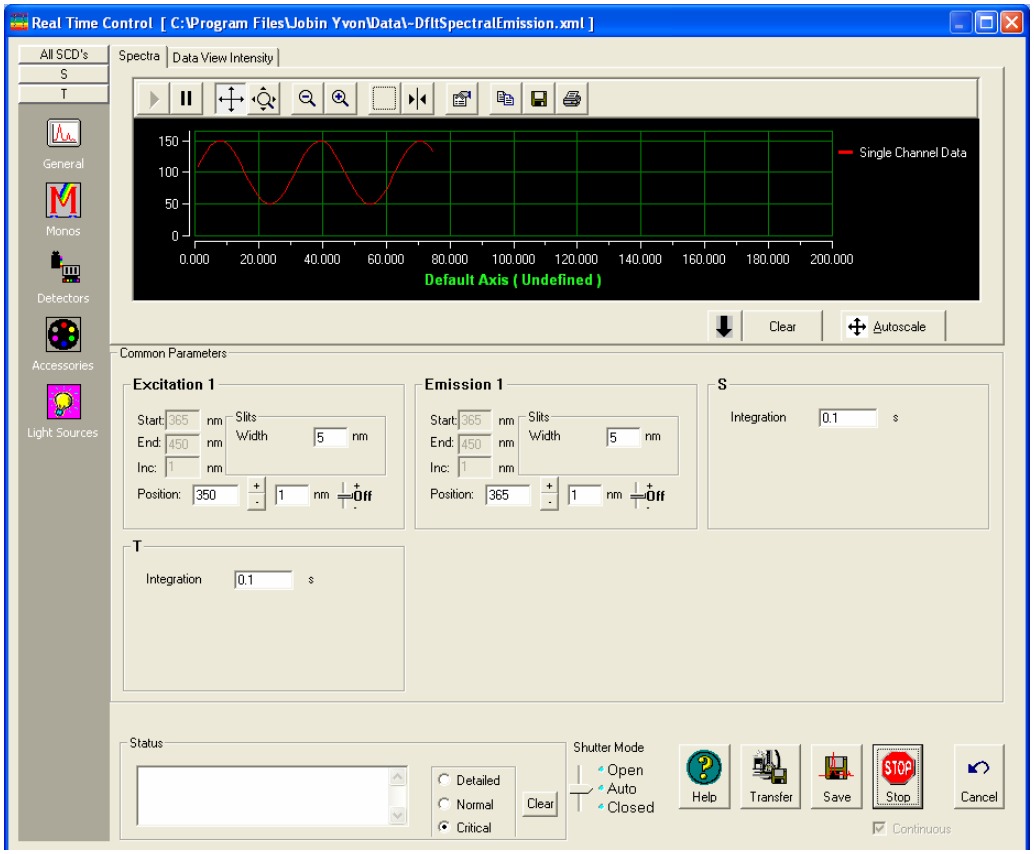
2 Place the cuvette in the sample compartment.

3 Check the hardware to be sure that you do not over-range the detector.

a Open the **Experiment Setup** window for an emission scan.



b Open the **Real Time Control**.



c Set the excitation and emission monochromators to 467 nm and 630 nm, respectively.
The largest lamp peak occurs at 467 nm.

d Set the slit on the excitation monochromator to 0.5 mm.

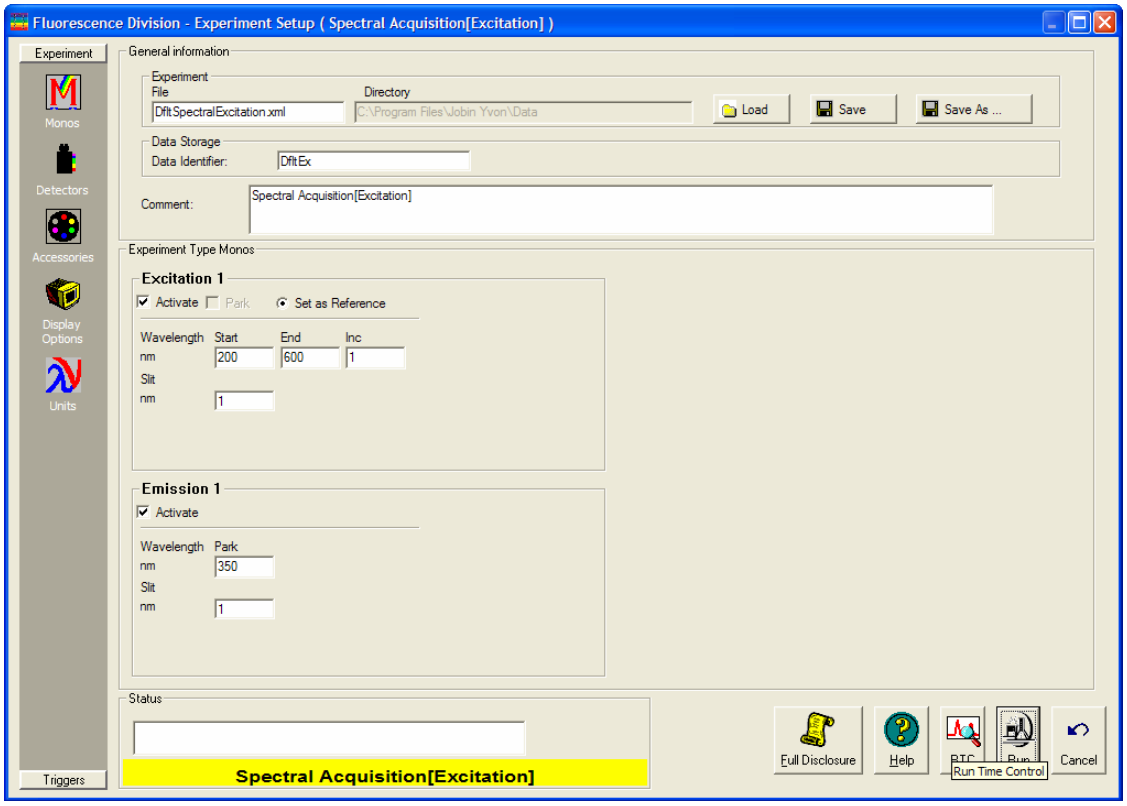
e Make sure the shutter is open.

f Adjust the slit on the emission monochromator to obtain a signal intensity of $(1 \pm 0.1) \times 10^6$ cps.
Note the slit-width: the slit-width discovered in this step will be used to run the scan.

g Close the **Real Time Control** and revert to the **Experiment Setup** window.

h Close the dialog box and open the **Experiment Setup** window for an excitation scan instead.

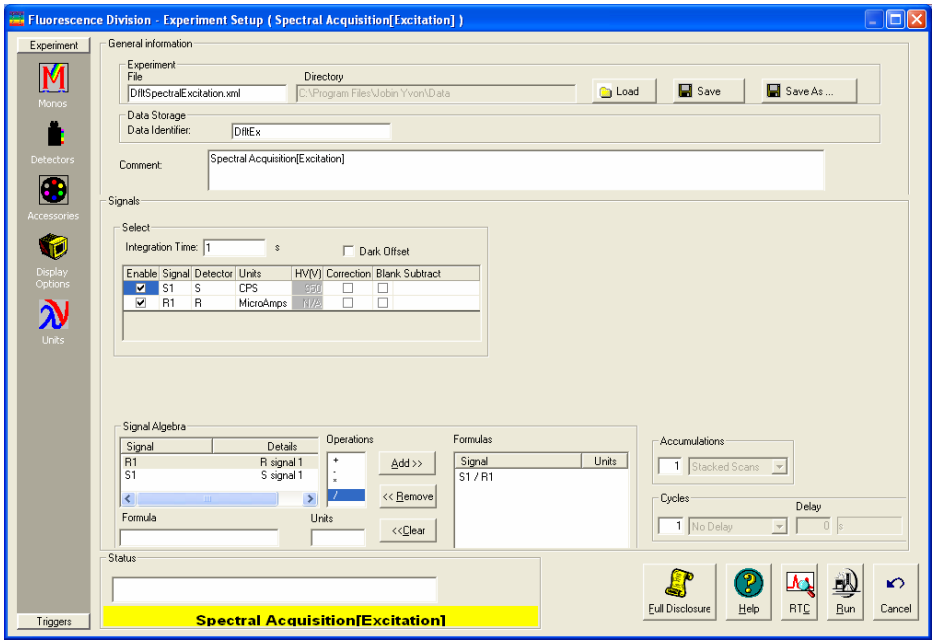
4 Set up the excitation-correction scan.



a Enter the scan parameters:

Excitation Wavelength Start (nm)	240
Excitation Wavelength End (nm)	600
Inc (nm)	5
Emission Wavelength Park	650
Excitation Slit (nm)	0.5
Emission Slit (nm)	Use value determined in step 3.

b Click the Detectors icon .



c Enable both S1 and R1 detectors.
The codes S1 and R1 appear in the Formulas table.

d Remove both S1 and R1 from the Formulas table.

e In the Signal column, click S1. In the Operations column, click the division sign, /. In the Signal column, click R1.
In the Formula box, S1/R1 appears.

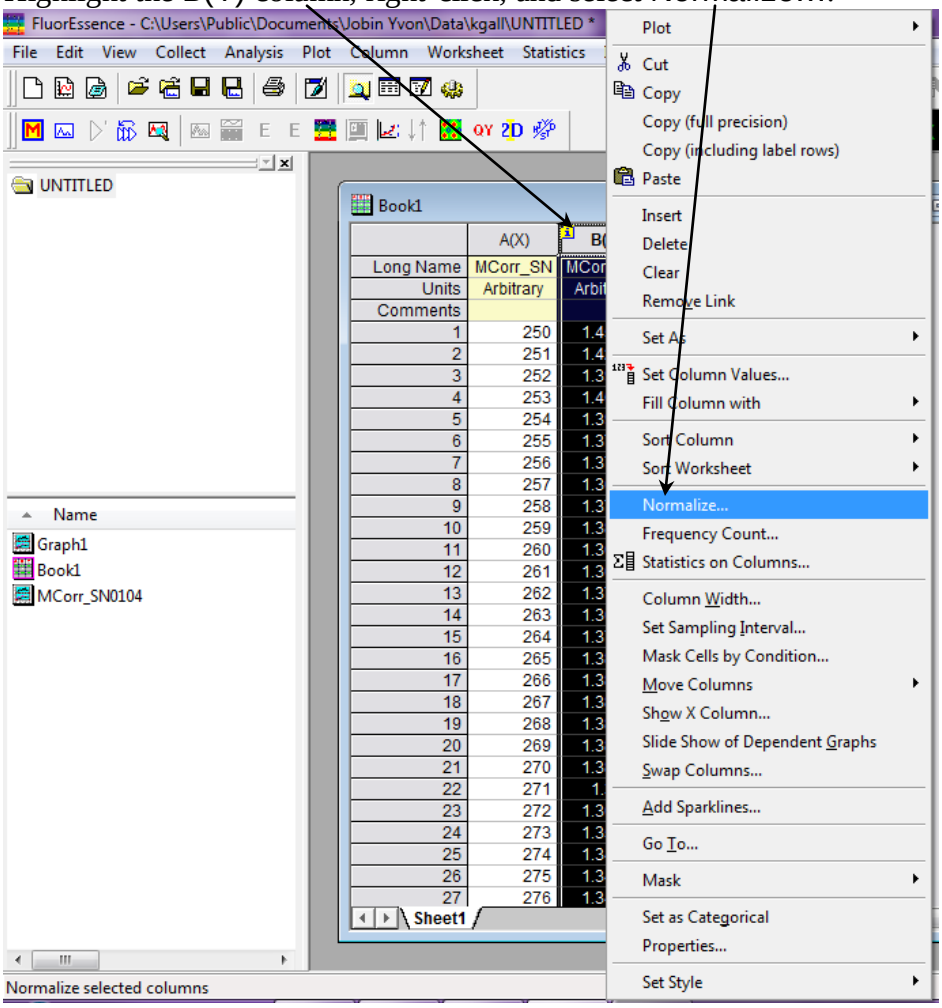
f Click the Add >> button to add this formula to the Formulas table.

g Enter an Integration Time of 1 second.

5 Click the Run button to execute the scan.

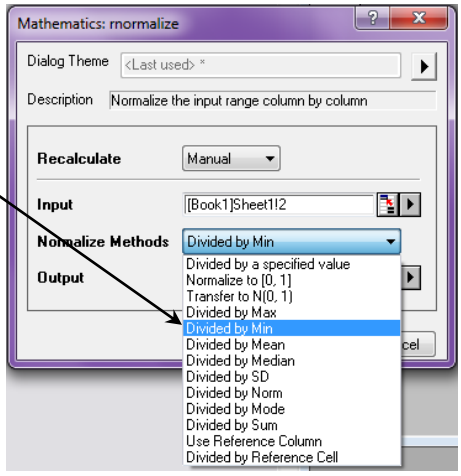
6 Normalize the graph as follows:

a Highlight the B(Y) column, right-click, and select Normalize….



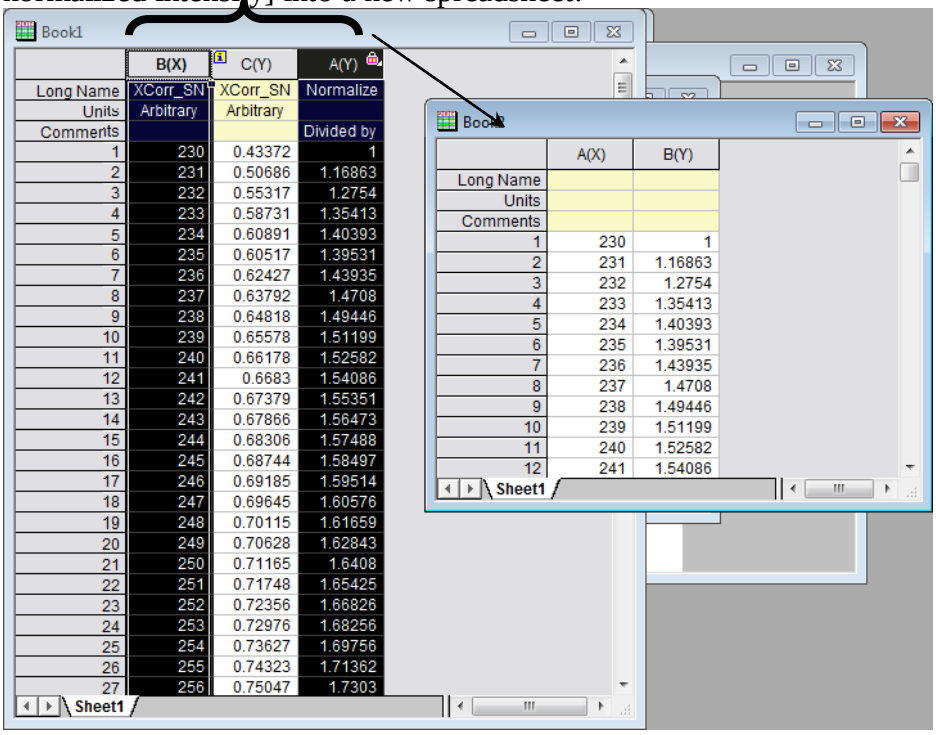
The **Mathematics rnormalize** window opens:

b In the Normalize Methods drop-down menu, choose Divided by Min.

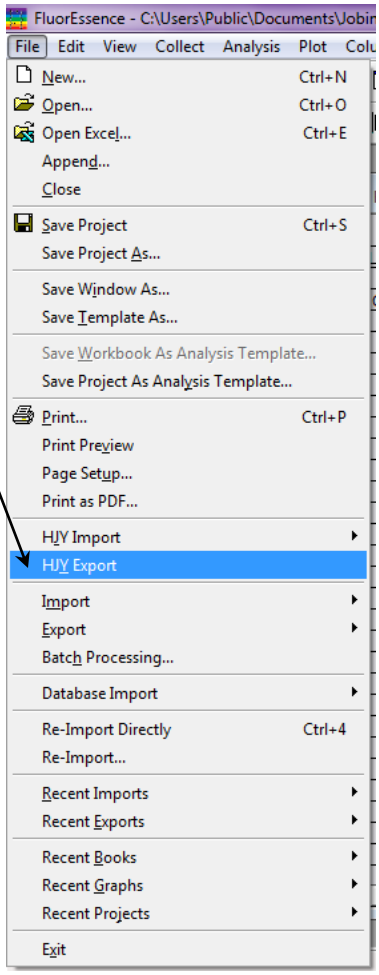


The normalized data appear as the third column in the spreadsheet.

c Copy the B(X) column [the wavelengths] and the A(Y) column [the normalized intensity] into a new spreadsheet:

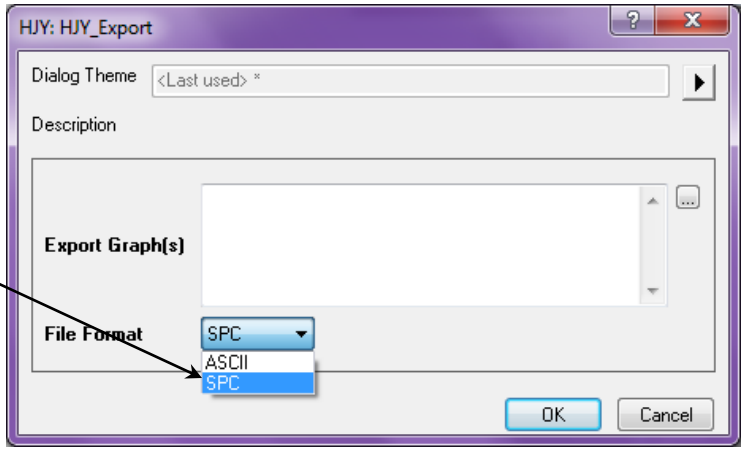


d In the File menu, choose HJY Export:

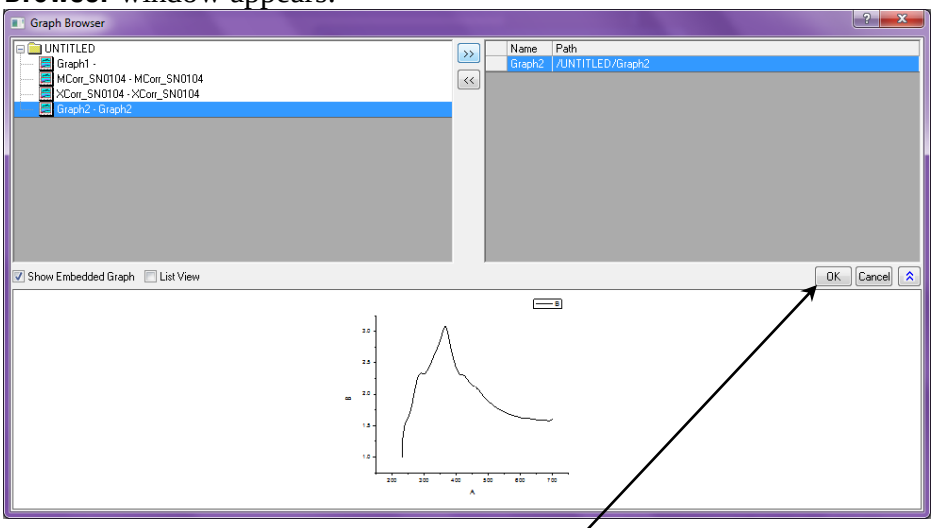


The **HJY_Export** window appears:

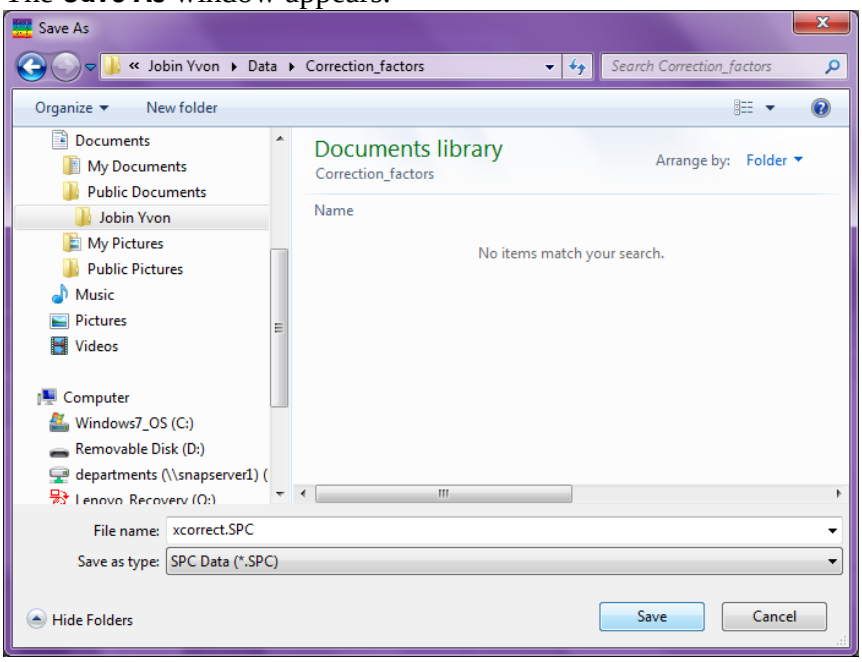
e In the File Format drop-down menu, choose SPC.



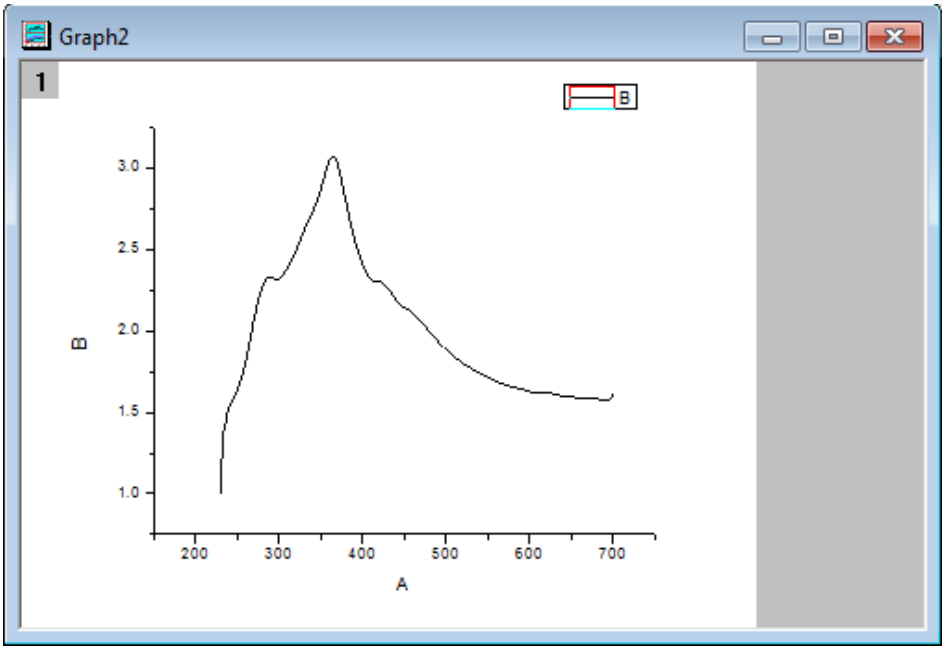
f Click the … button to browse for the graph. The **Graph Browser** window appears:



g Browse to select the graph, then click the OK button. The **Save As** window appears:



h Choose the desired file path in which to save the file, then click the Save button.



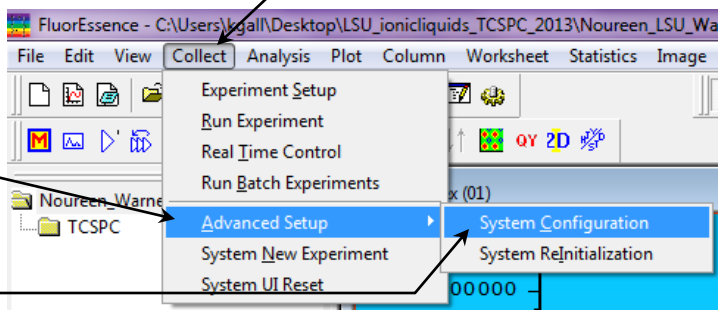
Using correction-factor files

To use the newly acquired `xcorrect` and `mcorrect` files, you must tell FluorEssence™ use them when the Correction check box is activated in the **Experiment Setup** window.

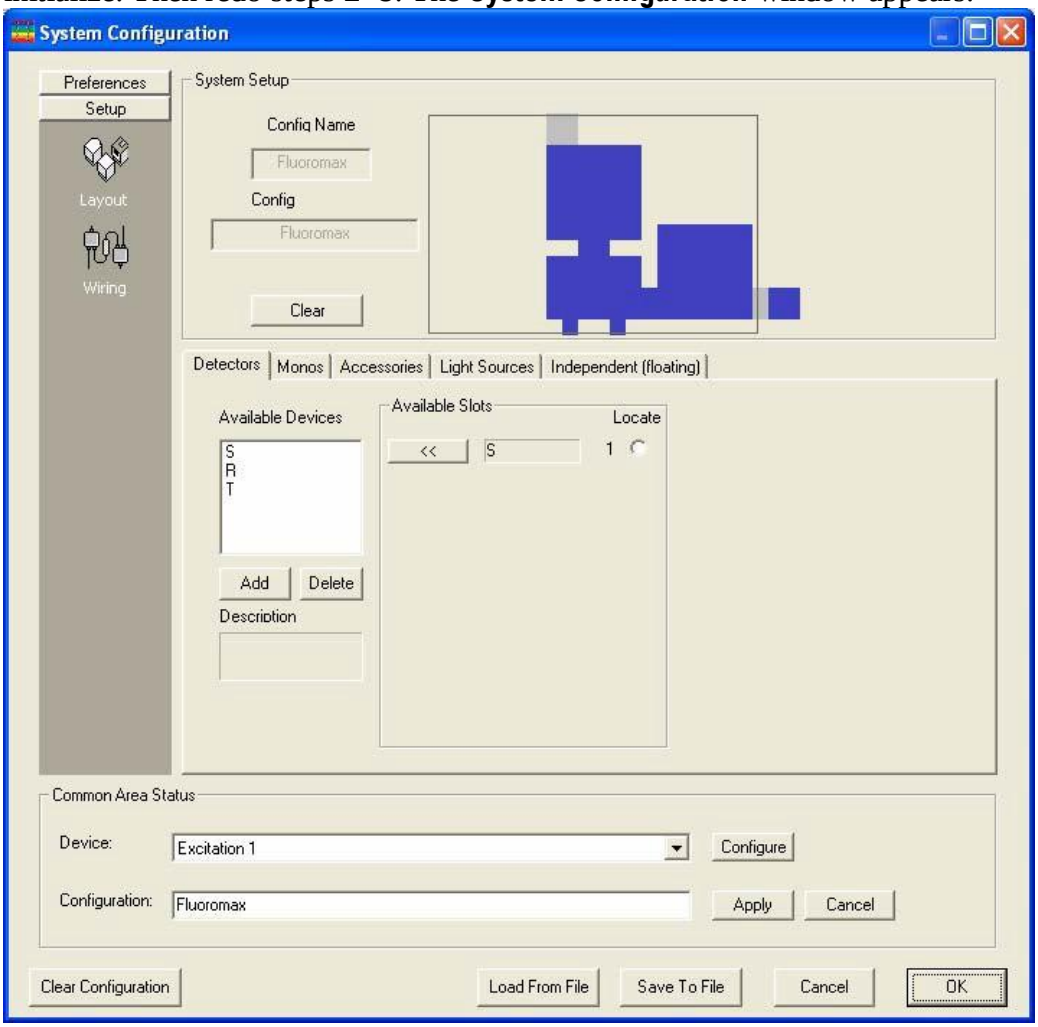
1 In the FluorEssence toolbar, choose Collect.
A drop-down menu appears.

2 Choose Advanced Setup.
A sub-menu appears.

3 Click System Configuration.



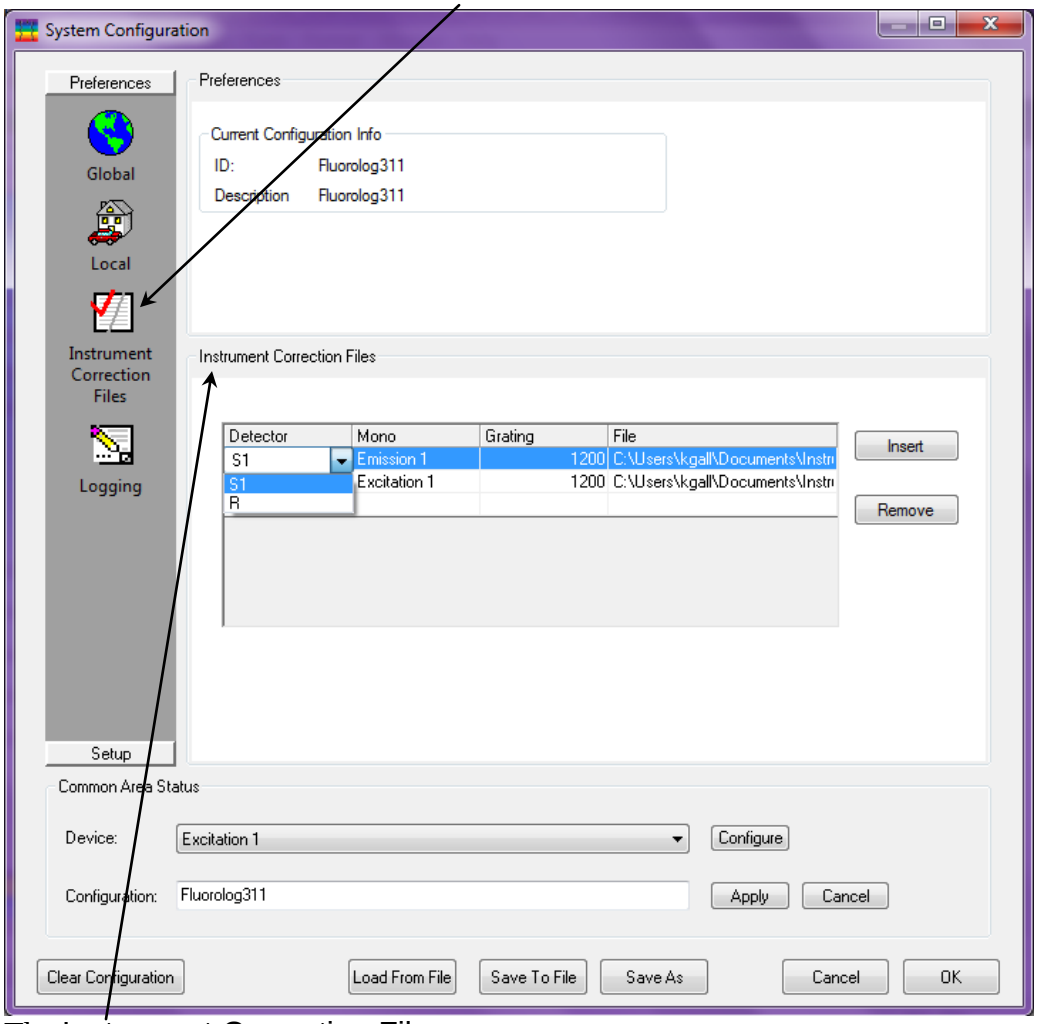
If the system has not been initialized, choose the instrument, and let the system initialize. Then redo steps 1–3. The **System Configuration** window appears:



4 Choose Preferences.

The Preferences area appears.

5 Choose the Instrument Correction Files icon.



The Instrument Correction Files area appears.

6 If there are no active fields in the Instrument Correction Files area, click the Insert button.

7 In sequence,

a Choose the Detector column, and select the appropriate detector from the drop-down list,

b The Mono column, and the correct monochromator from the drop-down list,

Note: *The signal detector, S, uses only the* `mcorrect` *file; the reference detector, R, uses only the* `xcorrect` *file; the optional T-side detector, T, uses only the* `tcorrect` *file.*

c The Grating column, the correct grating from the drop-down list,

d Then, in the File column, browse for the appropriate correction-factor file in `.SPC` format.

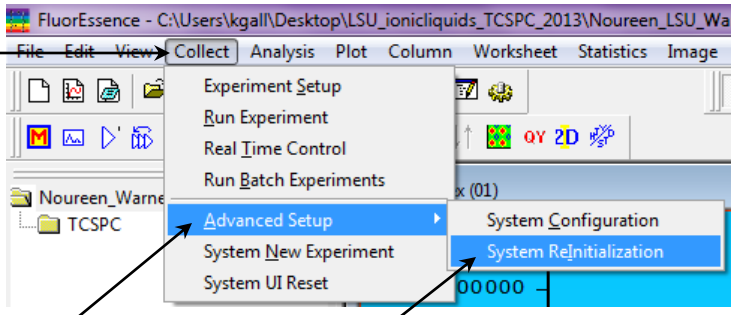
8 When all necessary detectors have an associated correction-factor file, click the OK button.

The **System Configuration** window closes.

Before the correction-factor files are loaded into the FluorEssence™ software configuration, the software needs to be re-initialized.

9 Re-initialize FluorEssence™.

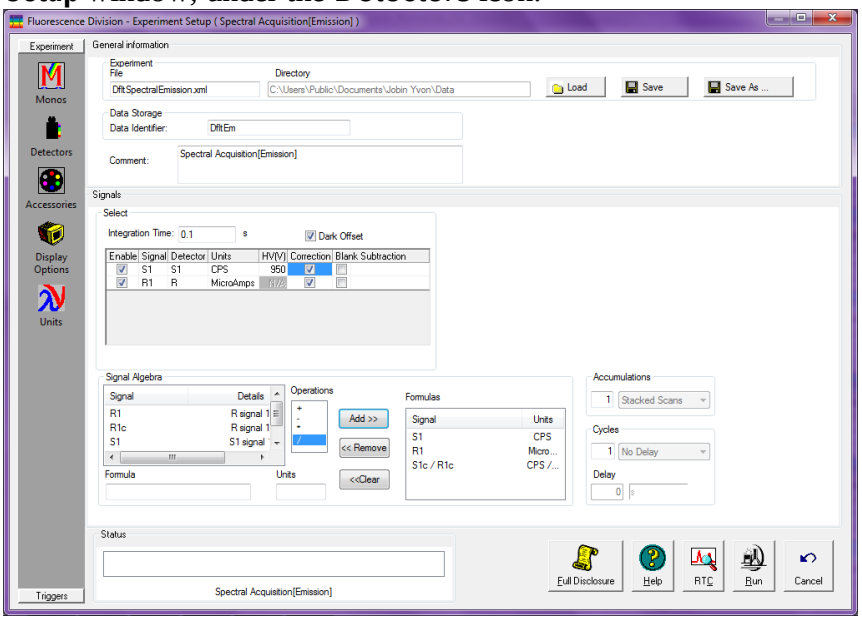
a In the toolbar, choose Collect. A drop-down menu appears.



b Choose Advanced Setup. Another submenu appears.

c Choose System ReInitialization, and select the appropriate software configuration to reinitialize.

The new correction-factor files are now ready to be activated in the **Experiment Setup** window, under the Detectors icon:



Chapter 9 : Automated Polarizers

Introduction

Theory

The measurement of polarized emission of fluorescence allows the observation of rotational motions in fluorophores during the lifetime of the excited state. Because the rotation of macromolecules depends on their size, shape, and local environment (i.e., solvent), several kinds of information may be extracted. Polarized-emission measurements often are used to detect small changes in molecular size (*viz.*, aggregation, binding, cleavage) as well as environmental changes (local viscosity, membrane microheterogeneity, and phase transitions).

The first step in these measurements is the *excitation* of a selected group of fluorophores, a fraction of the total ensemble of molecules. This process is known as photoselection. Vertically polarized light typically is used to excite a population of molecules whose absorption dipole is oriented in the vertical direction. For photoselection, vertically polarized exciting light usually is produced using a polarizer in the excitation path. A laser whose emission is V-oriented also may be used.

The second step is molecular *rotation*. The molecule, once excited, may rotate during the lifetime of the excited state, typically ~10^{-9} s. Such rotation will depolarize the fluorescence emission. Measurement of the polarized emission components allows calculation of the type and extent of rotational motions of the molecule.

The third step is measurement of *emission*. The polarized components of fluorescence emission are measured using polarizer(s) in the emission path(s). Measurements of polarization or anisotropy are derived from the intensities of the vertically and horizontally polarized components of the fluorescence emission.

The last step is *calculation*. From the magnitude of the V and H emission components, the extent and type of rotational behavior may be calculated. Both polarization and anisotropy are used to express the rotational behavior. Polarization is a ratio, defined as the linearly polarized component's intensity divided by the natural-light component's intensity. Anisotropy is also a ratio, defined as the linearly polarized component's intensity divided by the total light intensity. Anisotropy is the preferred expression, because it is additive. Polarization is not additive, but often appears in earlier literature. The measurement is performed in exactly the same manner, differing only in the calculations.

Ideally, polarization (P) and anisotropy ($\langle r \rangle$) are measured using only the vertically polarized excitation with the horizontal and vertical emission components. These

measurements are designated I_{VV} and I_{VH}, respectively, where the first subscript indicates the position of the excitation polarizer, and the second, the emission polarizer. Vertically oriented polarizers (V) are said to be at 0° with respect to normal, and horizontally oriented polarizers (H) are said to be at 90°. Polarization and anisotropy are expressed as follows:

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \quad (1)$$

$$\langle r \rangle = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \quad (2)$$

In a real optical system, the *G*, or grating factor, must be included to correct for the wavelength-response to polarization of the emission optics and detectors. The *G* factor is defined as:

$$G = G(\lambda_{EM})\frac{I_{HV}}{I_{HH}} \quad (3)$$

Note: *In some literature, the G factor is defined as the inverse of Equation 3. Therefore, some equations derived in this manual may differ from other sources.*

The *G* factor is primarily a function of the wavelength of the emission spectrometer. The spectral bandpass of the emission also affects *G*. Thus, a pre-calculated *G* factor can be applied to experiments in which instrumental factors (emission wavelength and emission bandpass) are kept constant throughout the entire experiment. In experiments where constant emission wavelength and bandpass are impractical, such as in emission anisotropy spectra, the *G* factor must be measured by recording I_{HH} and I_{HV} during the experiment at each emission wavelength.

Polarization in a spectrofluorometer is defined as:

$$P = \frac{I_{VV} - G * I_{VH}}{I_{VV} + G * I_{VH}} = \frac{\frac{I_{VV} * I_{HH}}{I_{VH} * I_{HV}} - 1}{\frac{I_{VV} * I_{HH}}{I_{VH} * I_{HV}} + 1} \quad (4)$$

Anisotropy in a spectrofluorometer is defined as:

$$\langle r \rangle = \frac{I_{VV} - G * I_{VH}}{I_{VV} + 2G * I_{VH}} = \frac{\frac{I_{VV} * I_{HH}}{I_{VH} * I_{HV}} - 1}{\frac{I_{VV} * I_{HH}}{I_{VH} * I_{HV}} + 2} \quad (5)$$

Polarization and anisotropy can be interconverted using these two equations:

$$P = \frac{3\langle r \rangle}{2 + r} \quad (6)$$

$$\langle r \rangle = \frac{2P}{3 - P} \quad (7)$$

For single-photon excitation, the allowed values for the emission anisotropy are governed by:

$$\langle r \rangle = 0.4\langle P_2(\cos\alpha) \rangle \quad (8)$$

where $P_2(x) = \frac{3x^2-1}{2}$ is the second Legendre polynomial, and α is the angle between the molecule's absorption and emission dipoles. The angle α may vary from 0 to 90°. Thus the allowed values for $\langle r \rangle$ and P are:

Parameter	α = 0°	α = 90°
P	+0.5	0.333
$\langle r \rangle$	+0.4	0.2

Values of $\langle r \rangle > 0.4$ indicate scattered light is present in the measurement of $\langle r \rangle$. Values of $\langle r \rangle < 0.2$ indicate the rotation correlation time to be faster than the luminescence lifetime of the sample. If the sample is excited with depolarized light—a less common technique—the measured value of P ranges from –1/7 to +⅓ (and $\langle r \rangle$ from –1/11 to +¼). The individual intensity components (I_{HH}, I_{HV}, I_{VH}, I_{VV}) are also referred to as *raw polarization*.

Experimentalists often multiply polarization units by 1000 to yield *millipolarization units*, m*P*, for very small changes in the polarization.

Polarization geometries

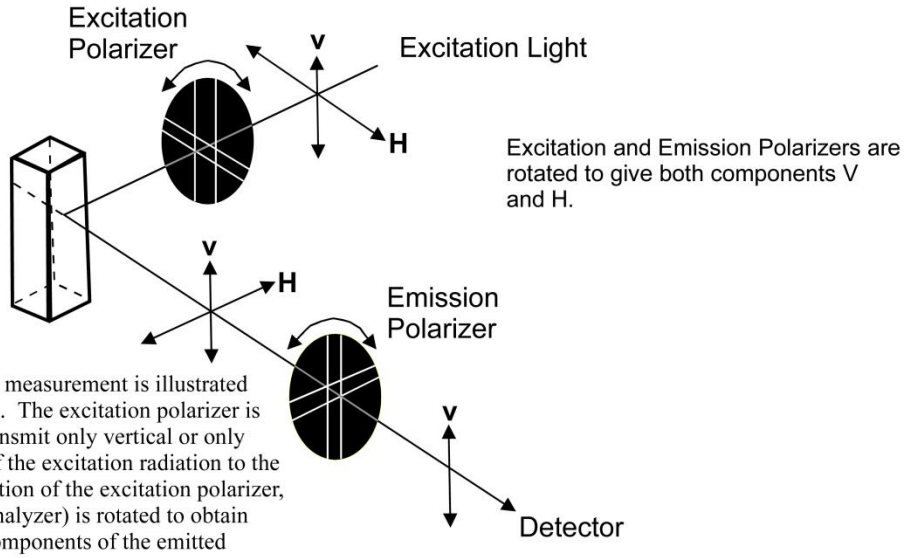
Polarization measurements are taken in two basic geometries:

- L-format uses two polarizers, which are both rotated between horizontal and vertical positions for measurements. If the *G* factor is determined beforehand, only two measurements are required: the VV and VH components, found by rotating only the emission polarizer.
- T-format uses one excitation and two emission polarizers. The excitation polarizer is rotated between horizontal and vertical for measurements, while the emission polarizers are fixed—one horizontal and the other vertical. If the *G* factor is determined beforehand, it is possible to obtain the anisotropy or polarization in one measurement cycle, for the VV and VH components are available simultaneously on the two emission detectors. Note that the *G* factor is measured differently in the T-format technique.

Schematic diagrams of both polarizer geometries are shown on the following page:

L-Format Polarization

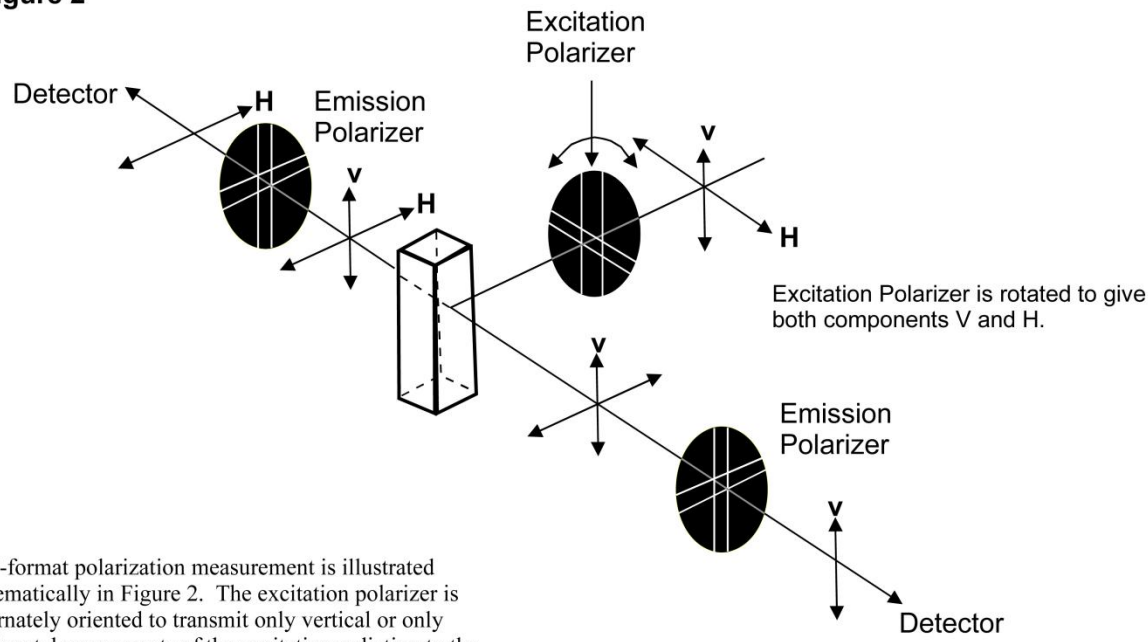
Figure 1



An L-format polarization measurement is illustrated schematically in Figure 1. The excitation polarizer is alternately oriented to transmit only vertical or only horizontal components of the excitation radiation to the sample. For each orientation of the excitation polarizer, the emission polarizer (analyzer) is rotated to obtain vertical and horizontal components of the emitted luminescence.

T-Format Polarization

Figure 2



A T-format polarization measurement is illustrated schematically in Figure 2. The excitation polarizer is alternately oriented to transmit only vertical or only horizontal components of the excitation radiation to the sample. Dual emission paths, each with a polarizer, permit simultaneous measurement of the vertical and horizontal components of the emitted luminescence.

Magic-angle conditions

Some fluorescent compounds exhibit molecular rotations on the same time-scale as their fluorescent lifetimes. This can cause a spectral distortion if the excitation and emission channels of a spectrofluorometer show some polarization bias. Specifically, when the rotational correlation time of a fluorophore is similar to the fluorescence lifetime, the effect can be significant. To record spectra that are free of rotational artifacts, use polarized photoselection conditions that cause the anisotropy to be zero. These polarization angles are called *magic-angle conditions*.

The two magic-angle conditions are:

- Use a single polarizer oriented at 35° in the excitation path with a scrambler plate,

or

- Use two polarizers, with excitation at 0° and emission at 55°.

We recommend using the two-polarizer method, exciting with vertically polarized light, and measuring spectra with the emission polarizer set to 55°. The reason for this is scrambler plates do not offer complete depolarization of the light beam at all wavelengths, and thus are not suitable for all experiments.

To use magic-angle conditions during data collection, set the excitation polarizers to V (0°) and the emission polarizer to magic-angle V (55°) using the Accessories icon in the **Experiment Setup** window. Collect spectra in the normal manner. To use magic-angle conditions for corrected spectra, measure an additional set of correction factors with the polarizers held at the chosen magic-angle settings.

Note: *The majority of samples do not exhibit an appreciable change in their spectrum when they are measured under magic-angle conditions. Thus, magic angles need not be used for most samples.*

Installation

HORIBA Scientific polarizers are made for easy installation and removal of the crystals from the light path. All HORIBA Scientific polarizers use pinned collars to hold the polarizers in their mounts and maintain calibration when the polarizers are removed.

New instrument and complete-polarizer orders are shipped with pre-aligned polarizers marked for excitation (“X”) or emission (“M”), and are locked in their collars.

Caution: *Do not remove polarizers from their collars, or else the polarizers must be realigned.*

Store the polarizer crystals in a dust-free environment, in a cabinet or drawer.

The Fluorolog®-3 autopolarizers are located within the sample compartment and cannot be seen with the instrument cover on. The FL-1044 is the dual-autopolarizer for the excitation and first emission optical paths. The FL-1045 is the third autopolarizer for the optional T-side optical path. These autopolarizers have an automated mount that automatically positions the polarizers in or out of the light path depending on the instrument configuration loaded in FluorEssence™. Therefore, after proper unpacking and setup by a HORIBA Scientific service engineer, these autopolarizers are permanently installed within the system. Be sure that the SpectrAcq software is version 4.13 or higher, and that FluorEssence™ is installed.

To use the instrument with its autopolarizers, load the desired instrument configuration with autopolarizers. Proceed to the *Alignment* chapter in this manual to verify alignment of the polarizers.

Alignment

Introduction

Polarizer alignment is verified by measuring the anisotropy of a dilute scattering solution. Scattered light is highly polarized, and this allows a simple check of the crystal alignment in the instrument. We recommend using a very dilute solution of glycogen or Ludox® (colloidal silica) as the scattering sample. The Ludox® we use as the reference is Aldrich 420859-1L, Ludox® TMA Colloidal Silica, 34 wt. % suspension in water, de-ionized.

Caution: *Refer to your Material Safety Data Sheets (MSDS) for hazards regarding the use of glycogen, colloidal silica, or other scatterers.*

The alignment test may be a measurement of the polarization or anisotropy within the software using the Anisotropy scan-type, or use of the Remeasure Anisotropy Only utility (click Advanced..., and the **Polarizer Alignment** window opens). The test also may be performed manually using the **Real Time Control** application. One measures the polarization, anisotropy, or the polarization ratio of scattered light (typically, the excitation and emission monochromators are both set to 370 nm for the measurement). To calculate the polarization ratio, use the definition:

$$\text{polarization ratio} \tag{9}$$

Alignment is satisfactory when the polarization ratio ≥ 100, or $P \geq 0.98$, or $\langle r \rangle \geq 0.97$.

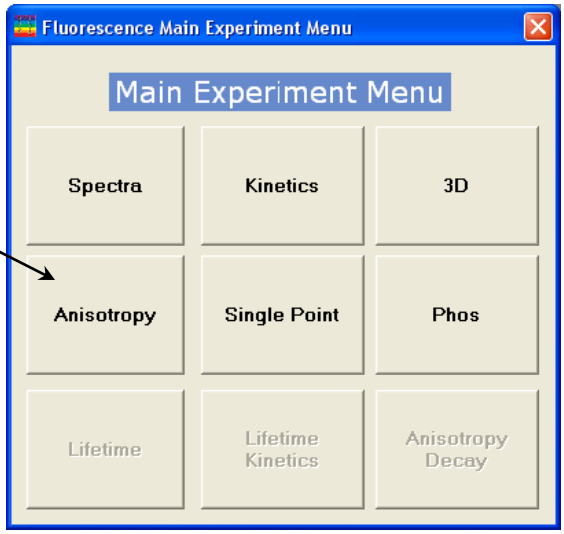
The check below assumes a sample of Ludox® or glycogen is used.

Note: *The polarization ratio can be lowered by using concentrated scatterer. Use only a* ***slight*** *amount of scatterer to align the system.*

Checking polarizer alignment

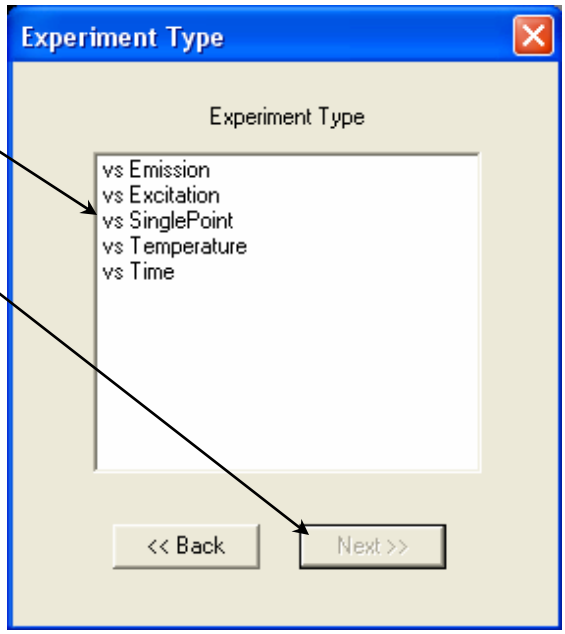
1. Place the scatterer in the sample-cuvette position.
2. Close the sample compartment's cover.

3 In the **Fluorescence Main Experiment Menu**, choose the Anisotropy button.



The **Experiment Type** window appears:

4 Choose vs SinglePoint, then click the Next >> button.

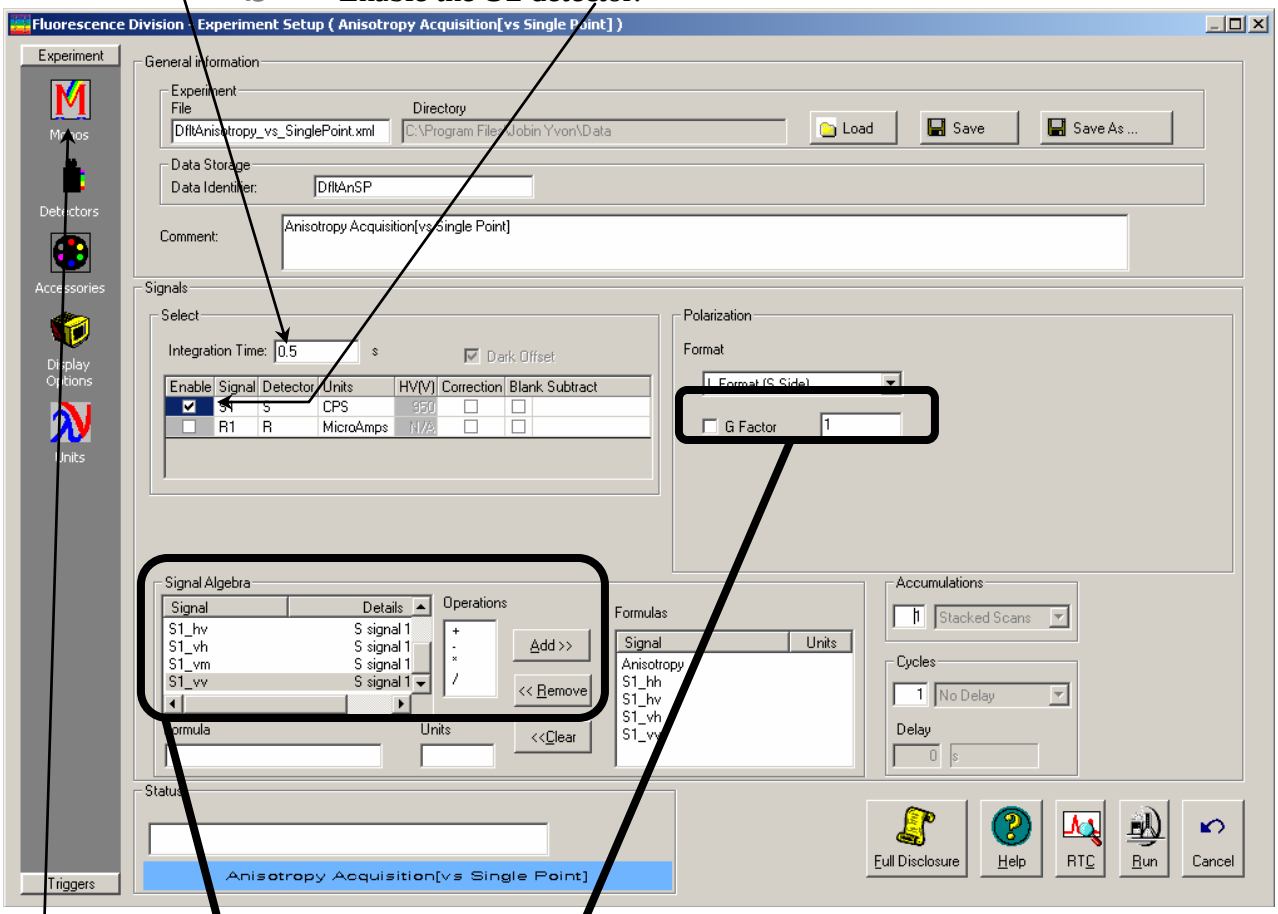


The **Experiment Setup** window opens:

5 Set up the anisotropy experiment.

a Enter an Integration Time of 0.5 s.

b Enable the S1 detector.



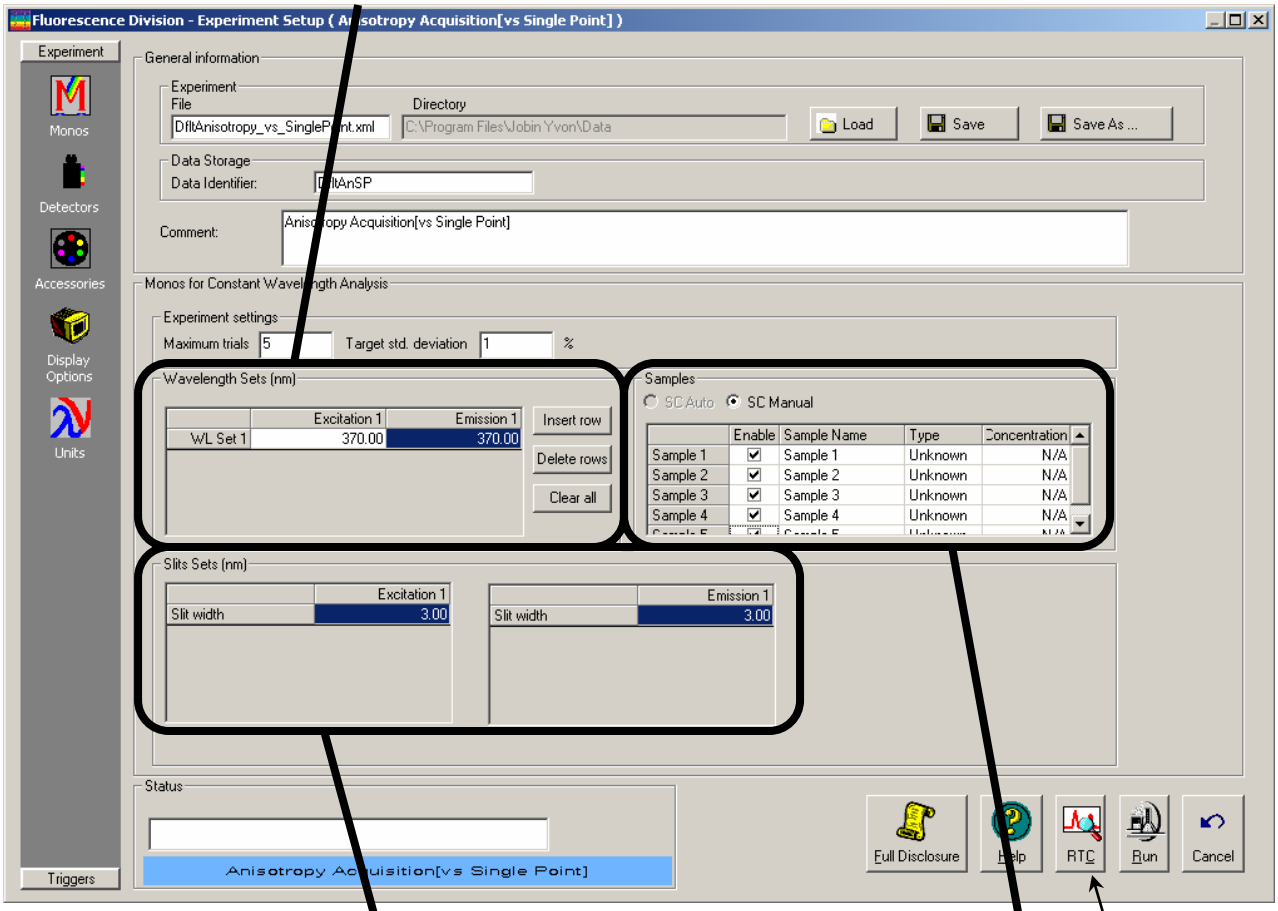
c Choose the *G* factor.
If you leave the G Factor checkbox unchecked, the instrument measures the *G* factor automatically. Or, enable the G Factor checkbox, and enter a *G* factor in the field.

d In the Signal Algebra area, choose the Anisotropy signal(s) to detect, and click the Add >> button.
If you wish to view the individual raw values, you may also add the S1_hh, S1_hv, S1_vv, and S1_vh signals.

The signal(s) appear(s) in the Formulas column.

e Click the Monos icon.

f In the Wavelength Sets area, in the Wl. Set 1 row, enter `370` under Excitation 1, and `370` under Emission 1.
This sets both monochromators to 370 nm.



g In the Slits Sets area, set both Excitation 1 and Emission 1 slits to 3 nm.

h If you wish to perform multiple measurements and keep all values on a single final spreadsheet, then right-click on the N/A in the Concentration column, hit the `Enter` key, and then hit the `Tab` key. Another row appears.

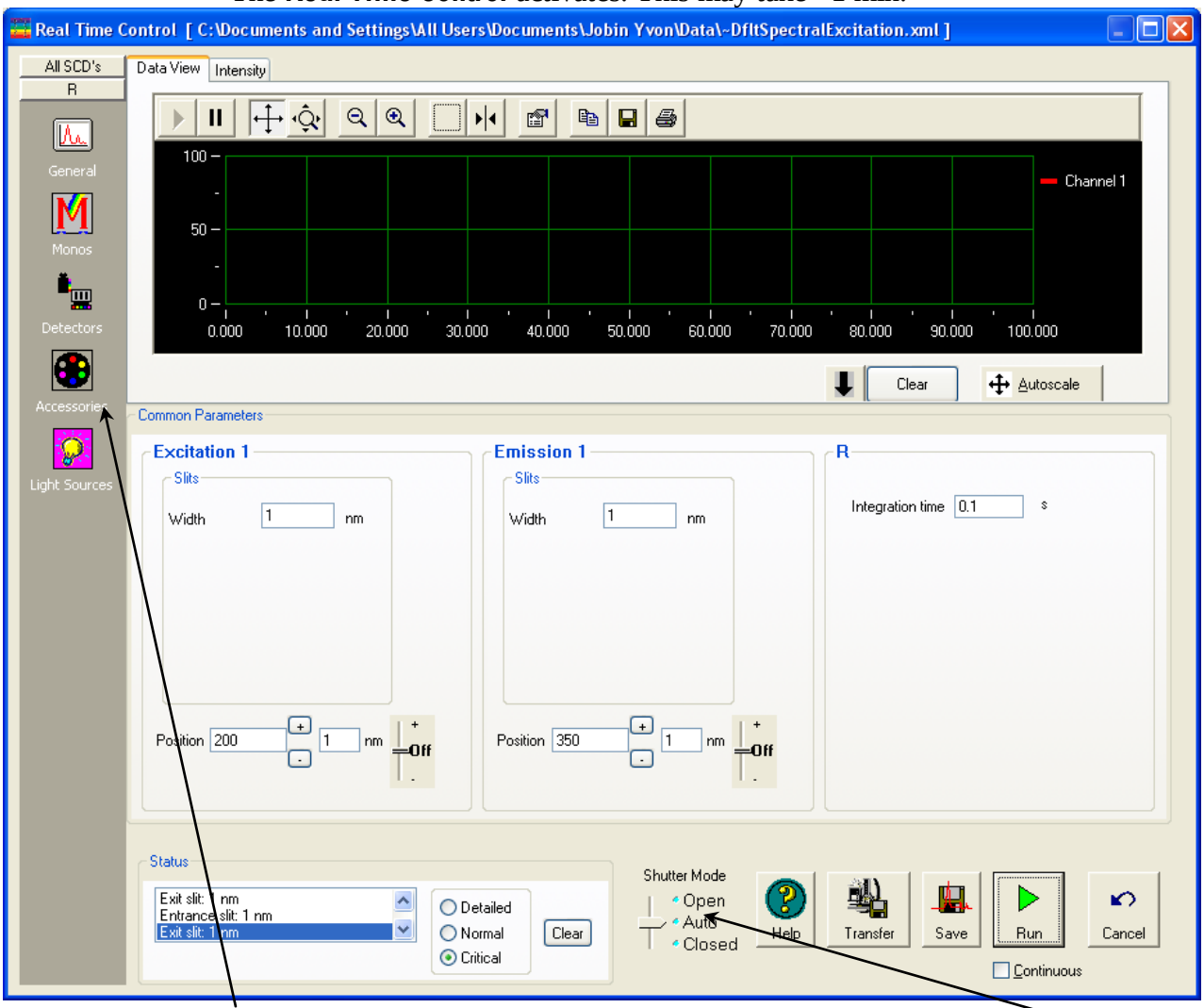
***Note:** If you don't add the rows, then you will get multiple spreadsheets with each sample on a separate spreadsheet.*

i Click each Enable checkbox to enable all of these samples.

6 Click the RTC button 

to go to the **Real ~~Time~~ Control**.

The **Real Time Control** activates. This may take ~1 min.



a Click the Accessories icon.

b Choose the Pol(ex) tab for the excitation polarizer.

c Click the In button to place the polarizer in the optical path.

d Enter 0 for the rotational value (corresponds to vertical).

e Choose the Pol(em) tab for the emission polarizer.

f Click the In button to place the polarizer in the optical path.

g Enter 0 for the rotational value (corresponds to vertical).

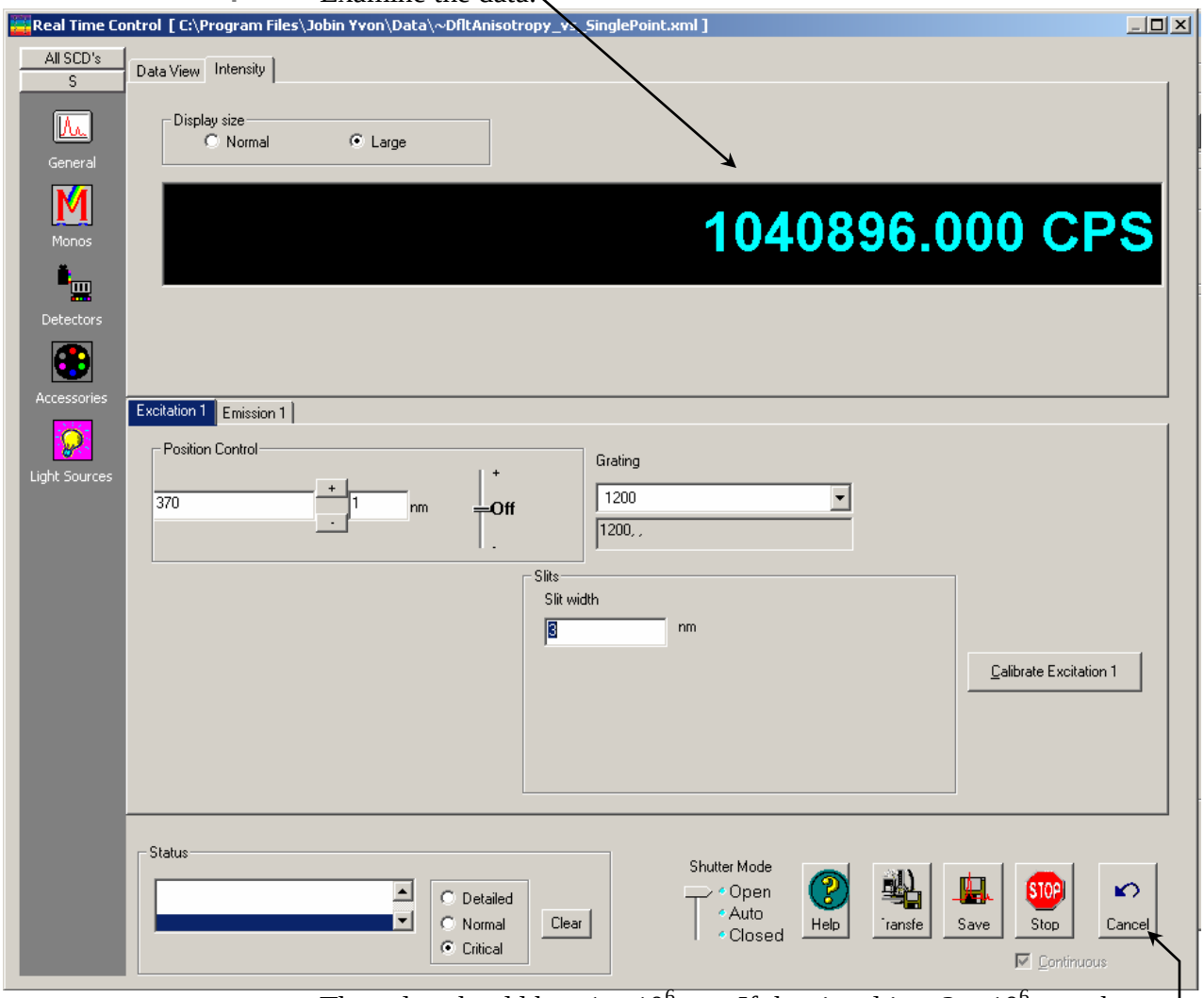
h Click the Monos icon, and review all the monochromator and slit settings.

i Move the Shutter Mode slider to Open. This opens the shutter.

j Click the View Intensity tab to see the numerical values of the data.

k Check the Continuous checkbox, and click the Run button.

l Examine the data.



The value should be ~1 × 10^6 cps. If the signal is > 2 × 10^6 cps, then dilute your scatterer. If the signal is < 2.5 × 10^5 cps, add more scatterer to increase its concentration. You may also adjust slits, but keep them between 3–5 nm for best results.

m Click the Cancel button to leave the **Real Time Control**. The **Experiment Setup** window reappears.

7 Click the Run button  .

The **Intermediate Display** appears. The **Experiment Paused** window may appear.

If the sample is not in the sample chamber, insert it and close the lid, then click the OK button.

When the automatic measurement is complete, the final spreadsheet appears.

The Anisotropy value in the spreadsheet ought to be > 0.97. If the Anisotropy ≤ 0.97, contact the Service Department, or re-align the polarizers as explained below in "Re-alignment of Polarizers".

8 With T-format polarizers, repeat the calibration check for the T-polarizer versus the excitation polarizer.

To be aligned, $P \geq 0.98$ or Anisotropy, $\langle r \rangle \geq 0.97$.

Re-alignment of polarizers

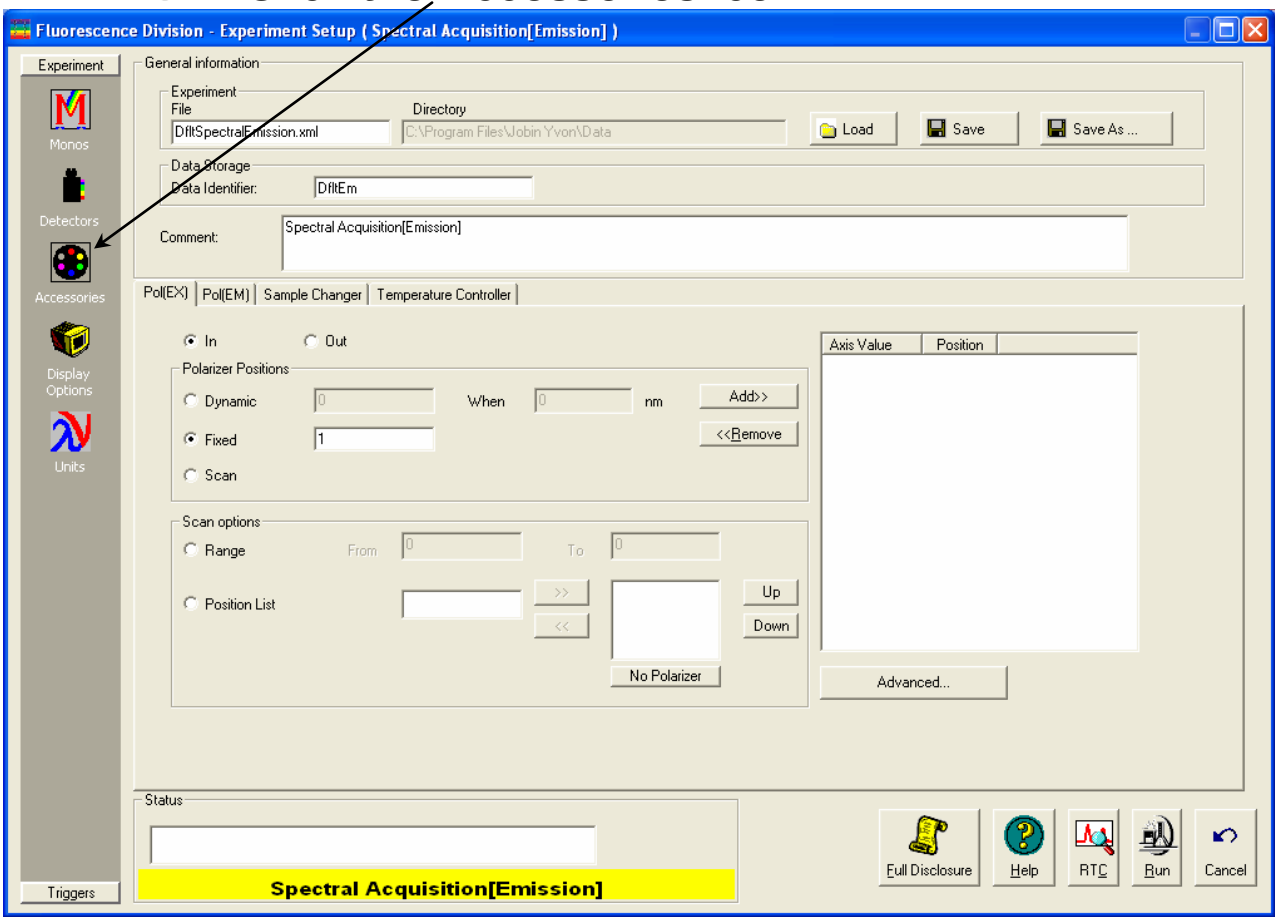
Fluorolog®-3 autopolarizers may be aligned using a software routine called Polarizer Alignment in the **Experiment Setup** window.

Using Polarizer Alignment

This routine automatically calibrates autopolarizers. Use a sample of Ludox® or glycogen to run the alignment routine. The software rotates the polarizers in 1° increments and locates the optimal positions for each autopolarizer. After completion, the anisotropy for the scattering solution is measured and displayed for user approval of the alignment. If approved, the new calibration positions are saved in the sample-compartment initialization file, and a log file, `POLAR.LOG`, is saved with the results of the calibration procedure. Otherwise, the previous calibration positions are still used.

Caution: *Refer to your Material Safety Data Sheets (MSDS) for hazards regarding the use of glycogen, colloidal silica, or other scatterers.*

1. Start FluorEssence™.
2. Open the **Experiment Setup** window.
3. Click the Accessories icon.

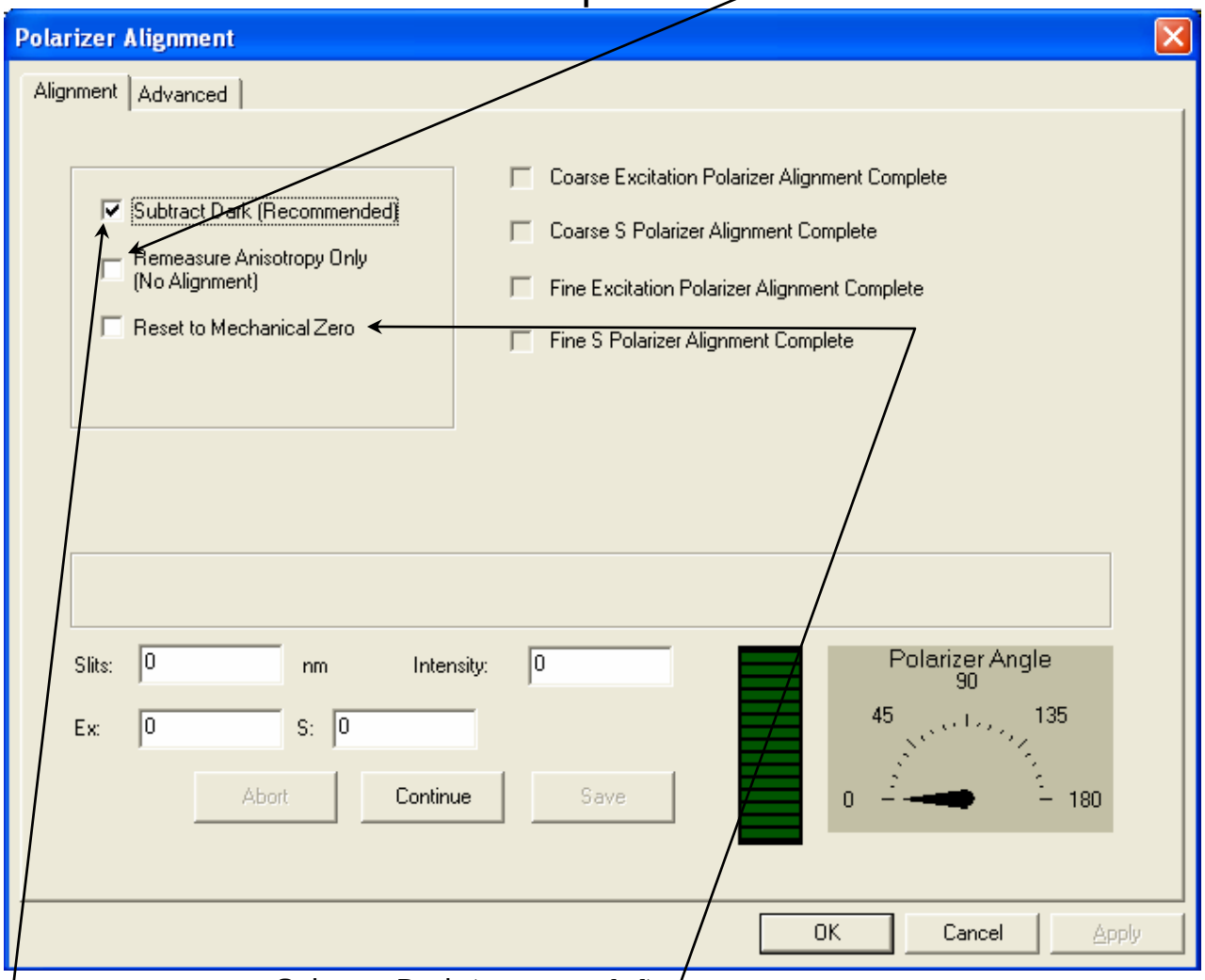


4 Click the Advanced... button.

This opens the **Polarizer Alignment** window:

Note: *Do* ***not*** *check the* Remeasure Anisotropy Only *checkbox.*

5 Choose various options:

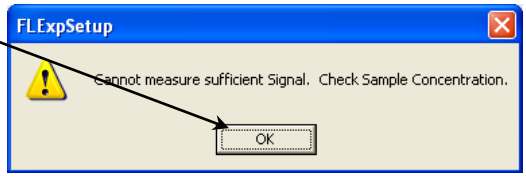


- Subtract Dark (recommended)
- Reset to Mechanical Zero—only if the polarizers are definitely miscalibrated. This deletes the previous calibration.

6 Place the Ludox® or glycogen in the sample holder.

7 Click Continue.

The system rotates through the polarizers as shown in the checklist on the window. As each phase is completed, the checkboxes are updated. If the sample is too concentrated or dilute, the software prompts you to correct this.



When complete, the software routine displays the measured anisotropy for each emission channel (S or T).

8 Approve or retry the measurement based on satisfaction with the result.

9 To quit, hit Cancel any time during the procedure.

Physical alignment

Caution: *Adjust the polarizers with the room lights off or the instrument covered with a tarpaulin. Stray light can have a deleterious effect on the photomultiplier tube, or make optimization of the alignment more difficult.*

Caution: *Never attempt to realign the FL-1044 or FL-1045 polarizers manually.*

1 Turn off power to the polarizers and 1976 Accessory Controller.

Note: *If the motor rotates during alignment of automated polarizers, immediately stop the procedure. Secure the polarizers in their collars, then re-initialize the polarizers. Otherwise, the alignment may not be performed at the calibration position of the polarizers.*

2 Loosen the screws that hold the polarizers inside their collars.

Do not loosen the set screw holding the collar in the mount. FL-1044 and FL-1045 autopolarizers have three set screws: two on one side of the collar, and one on the other side of the collar.

3 Set the tension on these set screws so that they do not slip, but allow easy manual rotation.

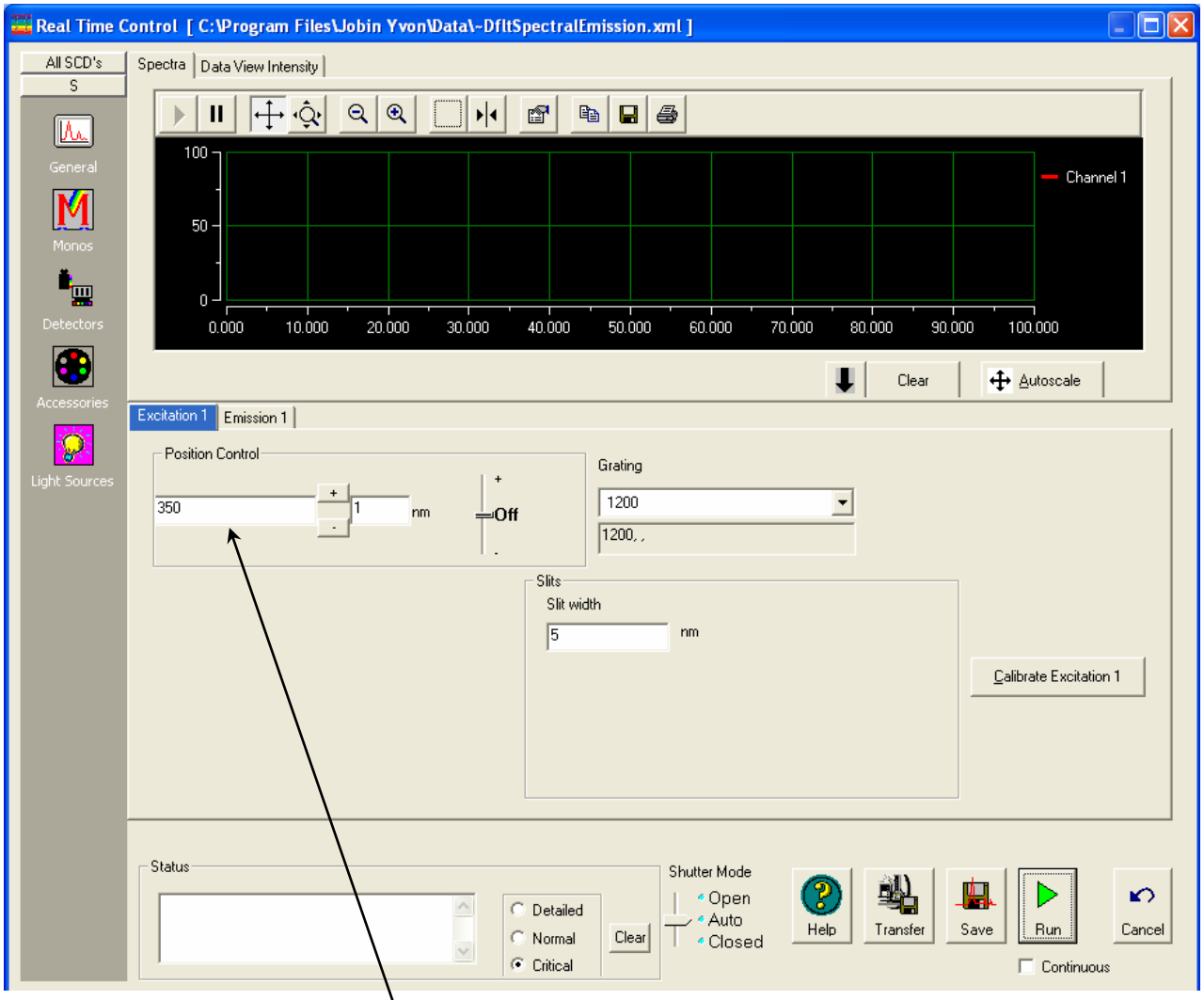
4 Set the polarizer crystals so that they protrude from the mounts far enough (~1/4″ or ~6 mm) to allow rotation.

5 Start the polarizers and accessory controller.

6 Insert the Ludox® or glycogen sample into the sample holder.

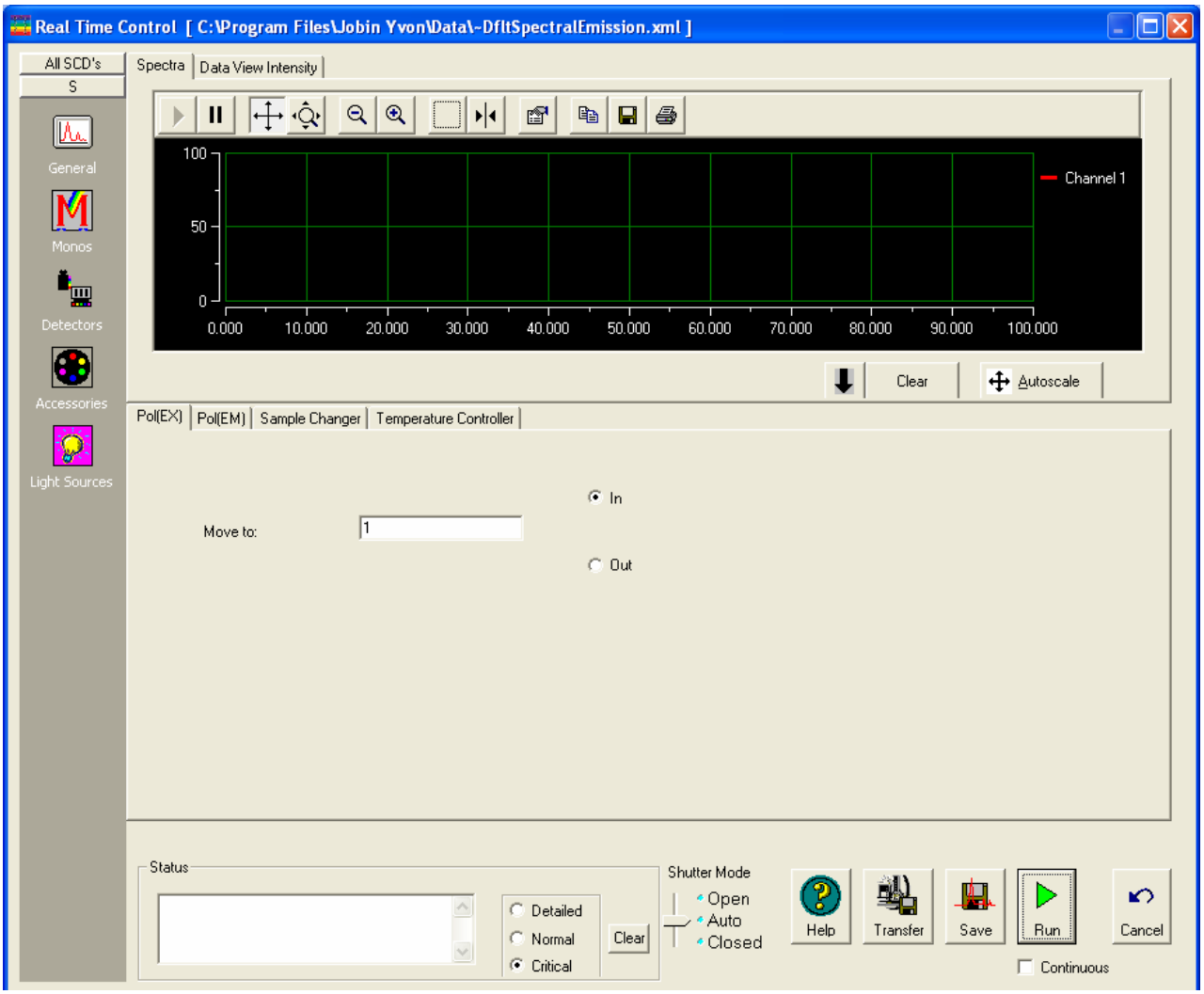
Caution: *Read the Materials Safety Data Sheets (MSDS) before using colloidal silica or glycogen.*

7 Start the software (if not yet running) and go to **Real Time Control**:



8 Set all monochromators to 400 nm under the Monos icon.

9 Set polarizers to VV (0°, 0°) under the Accessories icon:



10 Open the excitation shutter (if applicable).

11 Turn on high voltage and set appropriately for S channel (950 V for R928P; 1050 V for R1527).

12 Set slits to 5-nm bandpass for all monochromators.

13 Set scatterer concentration to give 1–1.5 × 10^6 cps on S.

14 Rotate the excitation polarizer to a rough maximum.

15 Set the polarizers to HV (90°, 0°) and rotate the excitation polarizer for the minimum signal on S.

16 Set the polarizers to VH (0°, 90°) and rotate the emission polarizer for the minimum signal on S.

17 Set polarizers to VV. Reset slits for 1–1.5 × 10^6 cps on S channel.

18 Measure polarization ratio (Equation 9). If the polarization ratio > 100, then the alignment is acceptable. Otherwise, repeat steps 15–18.

19 Secure the polarizers in their collars.

20 Verify that all polarizers are properly labeled for their locations in the system:
X = excitation
M = S-side emission

Using automated polarizers

FluorEssence™ software with HORIBA Scientific polarizers provides many choices for polarization measurements. Depending on the accessories, the opportunity exists to remove polarization effects from the sample, measure the polarization characteristics, or analyze the decay of anisotropy using frequency-domain techniques. For further software information, refer to the FluorEssence™ and Origin® on-line help.

Note: *HORIBA Scientific Polarizers are useful only at wavelengths longer than 280 nm. Index-matching material between the crystals absorbs at wavelengths shorter than 280 nm.*

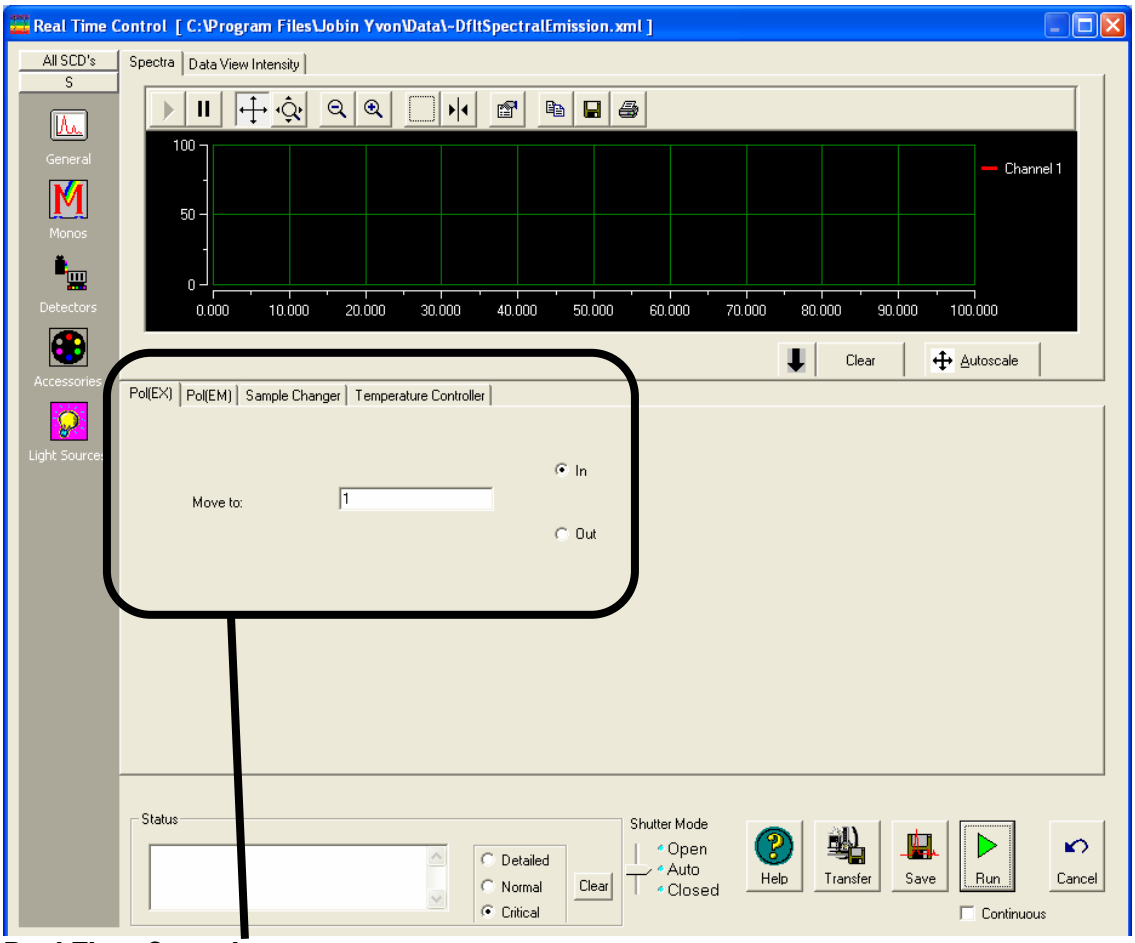
Applications for polarizers

- Measurement of emission anisotropy or polarization at fixed wavelengths. This is used for binding assays, kinetics of molecular size- or shape-change, temperature effects on rotational motion of fluorophores (e.g., phase transition of phospholipid bilayers).
- Measurement of excitation and emission spectra using magic angles. This helps to eliminate spectral artifacts.
- Measurement of a principal polarization or excitation anisotropy spectrum, using an excitation scan with polarization or the `POLAR.AB` macro acquisition. This provides information about rotational sensitivity of the excitation spectrum by measuring $\langle r \rangle$ versus λ_{exc} (with λ_{em} constant). Examine relative molecular dipole-angles at cryogenic temperatures in a viscous solvent.

Using FluorEssence™

To use the autopolarizers, load an instrument configuration with autopolarizers.

Real Time Control

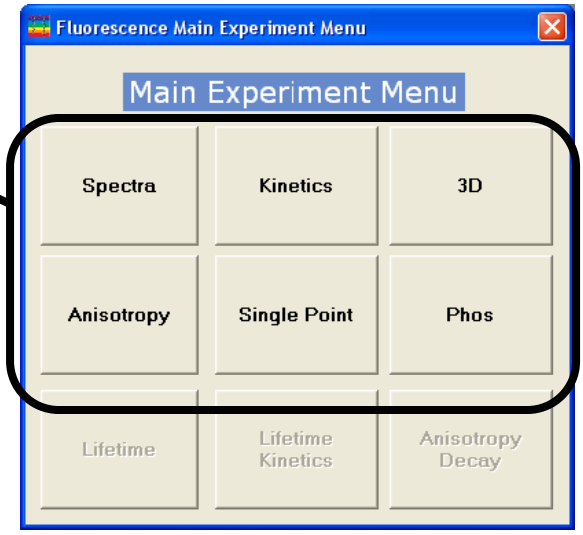


Real Time Control manipulates the polarizers and other instrument settings, to observe and optimize the spectrofluorometer in real time. Under the Accessories icon in the **Experiment Setup** window, each polarizer may be set independently into or out of the optical path under its own index-card tab. A custom angle may be set from 0–180°, in the field provided.

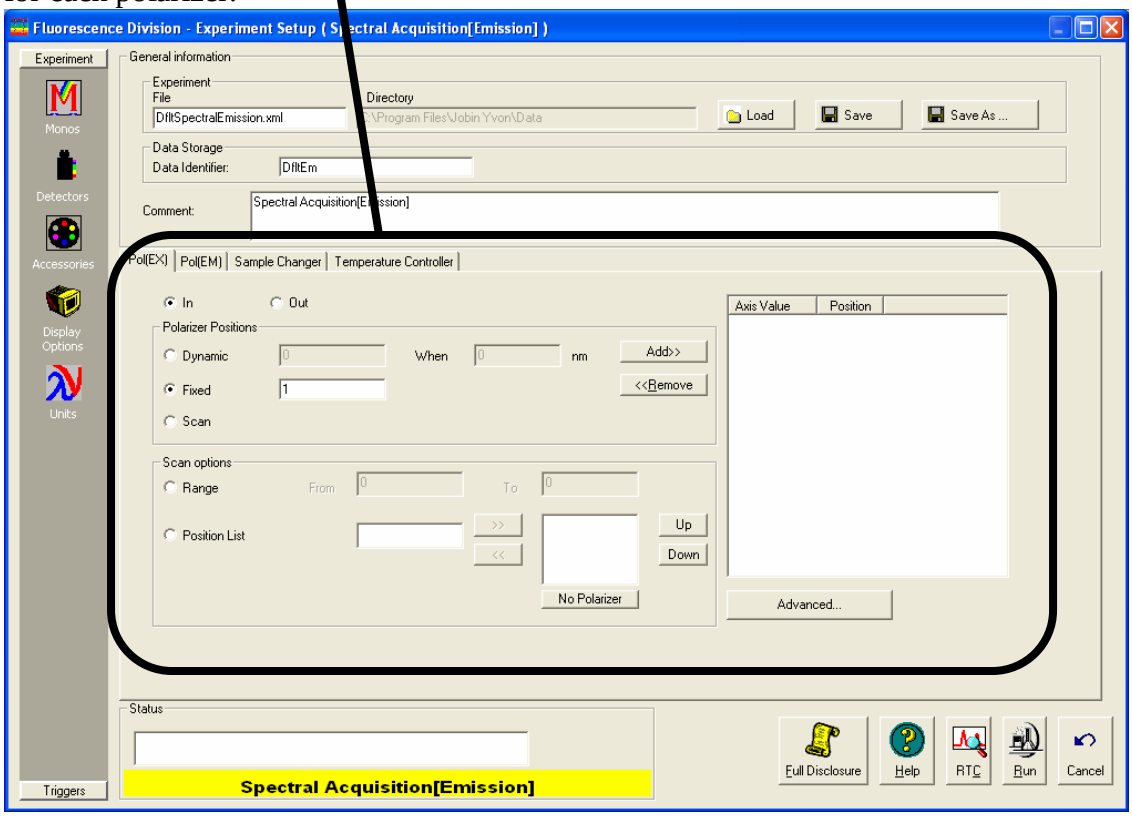
Note: **Real Time Control** *is only intended for real-time setup of a scan. Use the* **Experiment Setup** *window to work at fixed wavelengths.*

Experiment Setup

The **Experiment Setup** window runs all scanning options for the autopolarizers. First choose the type of scan using polarizers in the **Fluorescence Main Experiment Menu**:

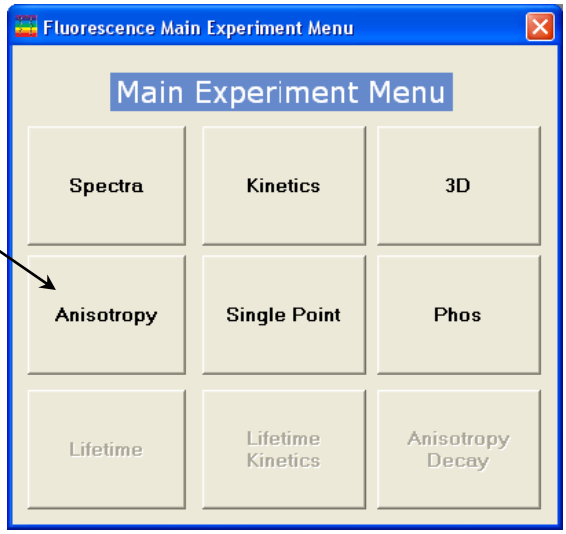


The **Experiment Setup** window appears. Adjust polarizer parameters under the Accessories icon. One index-card tab appears for each polarizer.

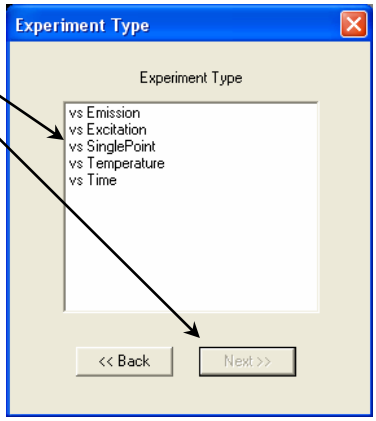


Constant Wavelength Analysis

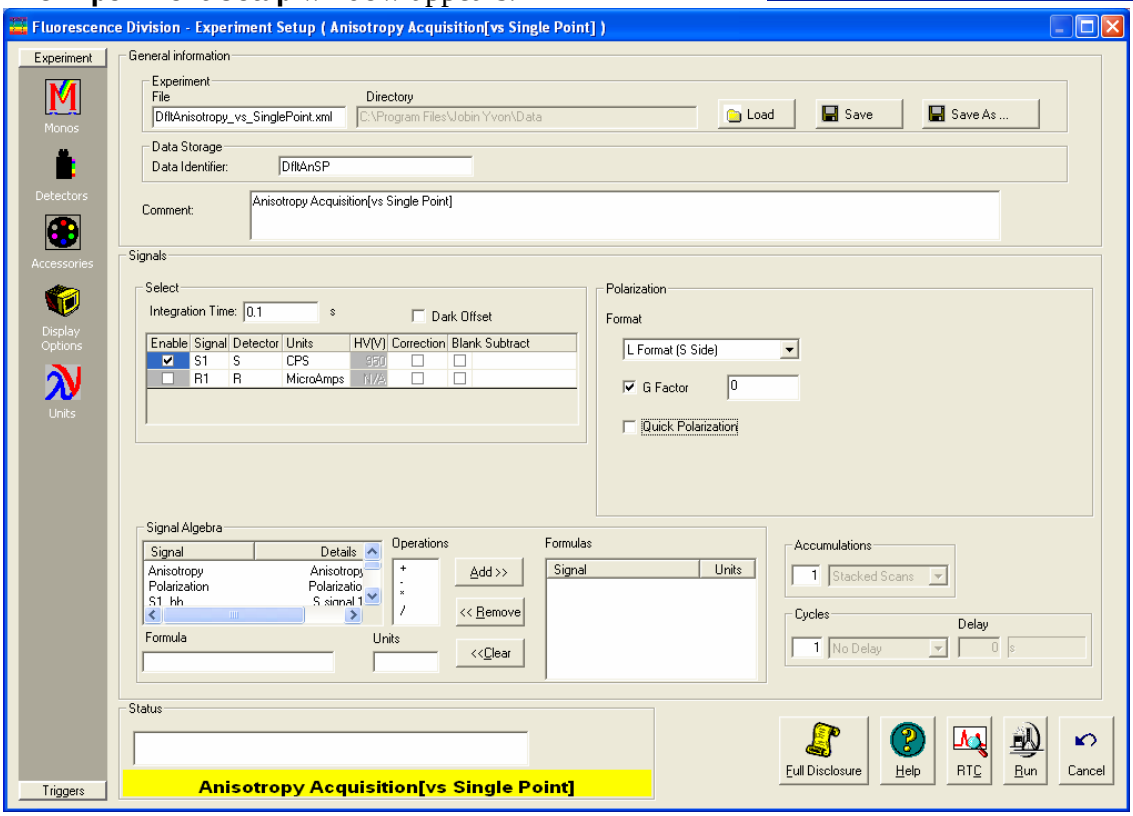
To do a constant-wavelength analysis experiment, that is, to take polarization acquisitions at fixed excitation/emission wavelength-pairs, choose Anisotropy from the **Fluorescence Main Experiment Menu**.



The **Experiment Type** window opens. Choose vs SinglePoint, then click the Next >> button.



The **Experiment Setup** window appears:



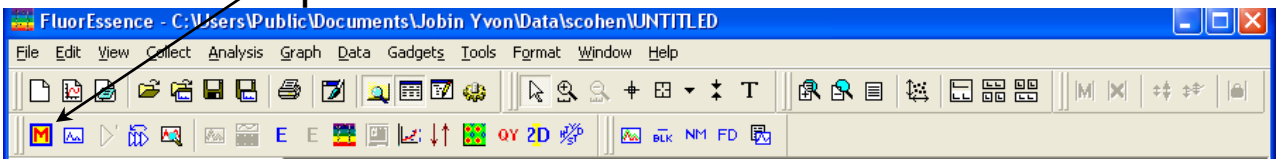
Use the appropriate Signal in the Signal Algebra area. Add >> it to the Formulas table. To cause the instrument to measure any *G* factor(s), disable the G factor checkbox during the scan. To specify *G* factor(s) beforehand, enter the *G* factor in the field.

Click the Run button when ready to run the experiment.

Measuring the *G* factor

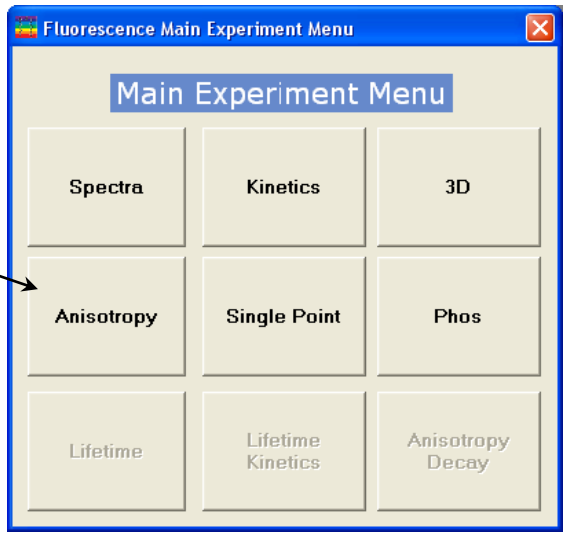
Include the grating factor, or *G* factor, whenever polarization measurements are taken. The *G* factor corrects for variations in polarization wavelength-response for the emission optics and detectors. A pre-calculated *G* factor may be used when all other experimental parameters are constant. In other cases, the system can measure the *G* factor automatically before an experimental run. *G* factors are incorporated into the Anisotropy scan-type:

1 In the FluorEssence main window, click the Experiment Menu button .



The **Fluorescence Main Experiment Menu** appears.

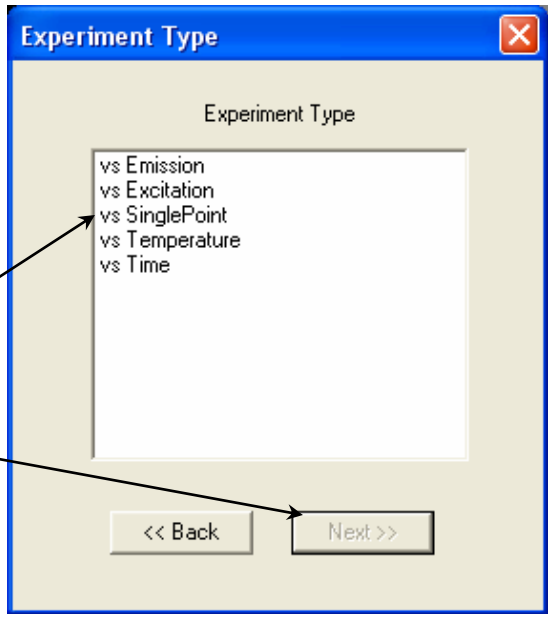
2 Click the Anisotropy button.



The **Experiment Type** window appears.

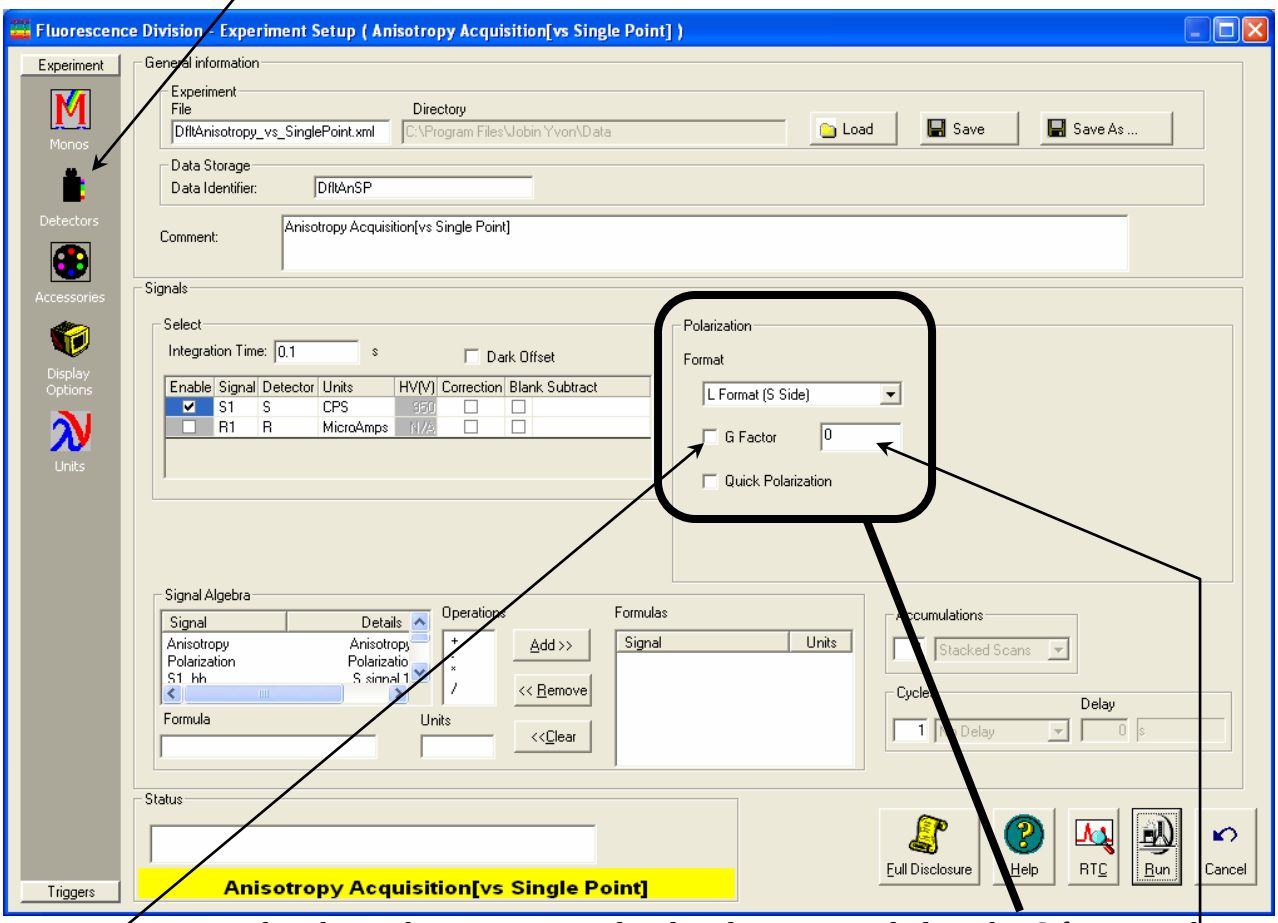
3 Choose the type of Anisotropy experiment.

For example, to determine polarization at particular excitation/emission wavelengthpairs, choose the vs SinglePoint button, then click the Next >> button.



The **Experiment Setup** window opens:

4 In the **Experiment Setup** window, click the Detectors icon.



This shows the parameters related to detectors, including the *G* factor, in the Polarization area.

5 Click the G Factor checkbox to include a *G* factor in your measurements.

6 Enter a value for the *G* factor in the field, if you want to use a pre-determined value.

To automatically measure the *G* factor, use the default value of 0.

Note: *For weak signals, enter the G factor, rather than measure it automatically. This may improve the S/N.*

Maintenance

Like all optics, polarizers should be handled with care and stored properly. With proper care, a polarizer should last for many years. Aside from installation, removal, and storage, there is no routine maintenance necessary for a polarizer. Polarizers should be removed and stored when not in use. (Fluorolog®-3 polarizers, however, remain within the sample compartment.) Store the polarizers in their collars to maintain calibration, in a drawer or cabinet. Wrap the polarizers in lens tissue—to keep them dust-free and for protection—and then place them in a plastic bag. The automated accessories should also be stored in a dust-free environment.

Should the polarizer windows need cleaning, apply a mild solution of methanol, and blow them dry.

Caution: *Always read the Materials Safety Data Sheets (MSDS) before using methanol or any scatterers.*

We recommend measuring the anisotropy of scatter (to verify the alignment of the crystals) before any critical experiment. In addition to the standard xenon-lamp spectrum and water Raman spectra, which serve to verify the wavelength calibration, measurement of the anisotropy of scatter will provide a fast check that the instrument system is ready to perform measurements.

Troubleshooting

For difficulties with polarizers, consult the table below to see if your question is answered here. Otherwise, reach the Service Department at HORIBA Scientific by phone, fax, or e-mail. Before contacting us, please follow the instructions below:

1 Note the problem and record any error messages.

2 See if the problem is listed on the following pages.

If so, try the suggested solutions. Be sure to note carefully the steps taken to remedy the problem and the result. Refer to the appropriate section of this manual (and the software manuals, if necessary).

3 If the problem persists, or is not listed, call the Service Department by phone at (732) 494-8660 ×8160, or fax at (732) 494-8796.

Outside the United States, call the local distributor. You may also reach us by e-mail at service.jyus@horiba.com.

When you contact the Service Department, have the purchase date, serial number, system configuration, and software version available. Be prepared to describe the malfunction and the attempts, if any, to correct it. Note any error messages observed and have any relevant spectra (sample, polarization ratio, xenon-lamp scan, water-Raman scan) ready for us to assist you.

Problem	Cause	Possible Remedy
Poor polarization data	Improper sample concentration	Adjust sample concentration.
	Photomultiplier saturated; slits improperly set	Check that sample signals are in linear region ($< 2 \times 10^6$ cps on S or T, < 10 μA on R). Reset slits.
	Dirty cuvette	Clean the cuvette.
	Polarizer misaligned	Check polarizer alignment.
	System misaligned	Check system alignment in a generic layout. Run lamp scan and water Raman scan to check calibration.
Low polarization ratio	Highly concentrated standard	Check Ludox® or glycogen concentration: higher concentrations can cause inner-filter effect, lowering ratio.
	Improperly set slits	Set slits for ~ 1×10^6 cps in VV. Signals much less than this give excessive contribution from dark noise, while signals $> 2 \times 10^6$ cps are in non-linear region.
	System misaligned	Check system alignment in generic layout. Run lamp scan and water Raman scan to check calibration.
Autopolarizers do not initialize (they do not move during initialization).	Wrong instrument configuration is loaded	Check that a configuration with autopolarizers is loaded.
	Bad cable connections	With the system power off, recheck cable connections.
Software failure initializing autopolarizers	Wrong instrument configuration is loaded	Check that a configuration with autopolarizers is loaded.
	Bad cable connections	With the system's power off, recheck cable connections.
	Computer hang-up	Exit the software, and reboot the system and host computer.

Chapter 10 : Phosphorimeter

Introduction

The FL-1040A phosphorimeter, used with the Fluorolog®-3 and Nanolog® spectrofluorometers, allows measurement of long-lived luminescence from samples, especially that caused by phosphorescence. The phosphorimeter includes a pulsed xenon lamp, plus gating electronics to control the size and temporal displacement of the detection window. The full-width half-maximum of each pulse is 3 μs, so that lamp interference during acquisition of decay curves is minimized. The tail of the pulse extends to 45 μs, allowing phosphorescence decay to be monitored an order of magnitude faster than with systems that have mechanical choppers.

The phosphorimeter is a part of a dual-lamp housing FL-1042 that contains both pulsed and continuous xenon lamps, plus all electronics and cables. Switching between pulsed and continuous lamps is controlled entirely by FluorEssence™ software.

Theory of operation

A second source of illumination, a pulsed xenon lamp, is used for phosphorescence measurements. Samples are excited with pulsed light; the emitted phosphorescence is measured using a photon-counting detector with a variable delay and open window between pulse and detection.

Sequence of data-acquisition

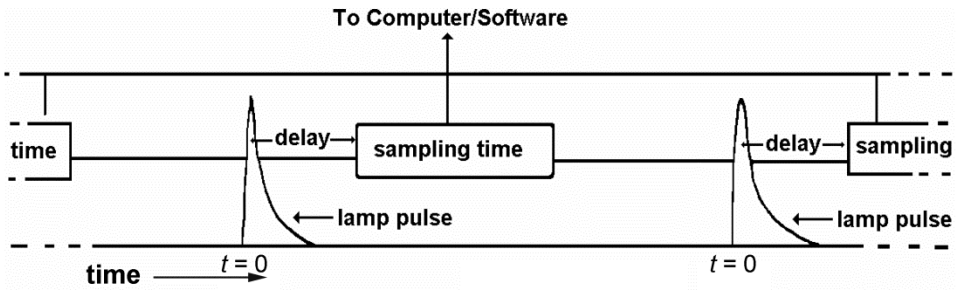
The illuminator housing, or flash lamp, operates at up to 25 Hz. The control module triggers each lamp pulse. When the start of the light output is detected, a signal is sent to the control module for timing purposes. The control module houses the signal-gating circuitry that intercepts the signal from the pulse-counting emission photomultiplier tube, collects a selected, time-delimited portion of the signal, and later passes it to the software. The maximum signal detectable per flash varies with the integration time (the "sample window"):

Sample window	Maximum signal (counts) per flash	
	R928P, TBX, R2658	H10330, H5509
100 µs	200	100
1 ms	2000	1000
10 ms	20 000	10 000
100 ms	200 000	100 000
1 s	2 000 000	1 000 000

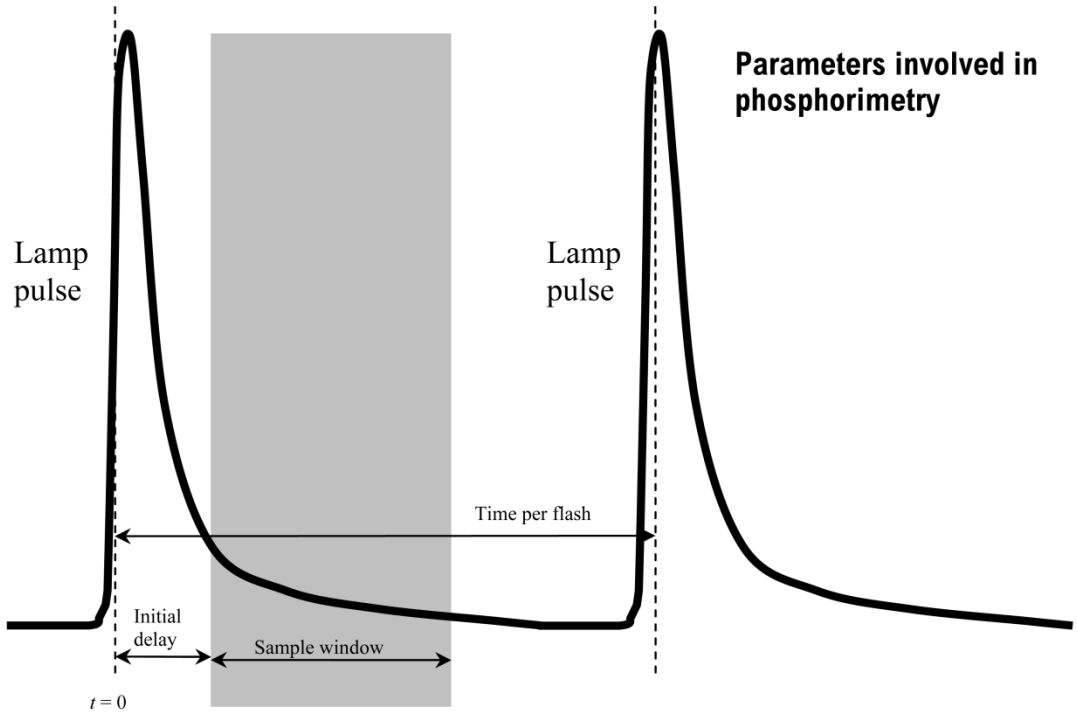
With, for example, an R928P detector and the counts integrated over 10 ms, the maximum detectable signal for 50 flashes is:

Maximum linear count-rate × Sample Window × Flash Count =	maximum detectable linear signal in a phosphorimeter experiment
2 000 000 counts/flash × 0.01 s × 50 flashes =	1 000 000 counts

A typical sequence of data-acquisition starts with a flash from the pulsed lamp, sensed by the control module as time $t = 0$. The light enters the excitation monochromator, where it is dispersed. Monochromatic light from the monochromator excites the sample. Luminescence emission from the sample then passes through the emission monochromator to the photomultiplier-tube detector. The control module includes a gate-and-delay generator, allowing the signal at the detector to be integrated only during a specific period after the flash (the Initial Delay),

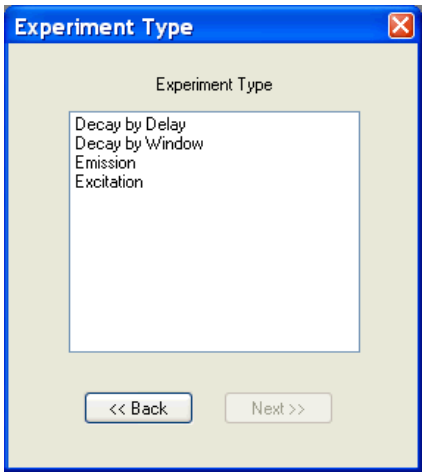


for a pre-determined length of time (the Sample Window). Any signal arriving before or after the gating is ignored. To increase or decrease the signal, you can adjust the time per flash and the sample window.



This sequence of excitation, delay, and sampling, is repeated for each lamp flash. The signal is accumulated for a predetermined number of excitation pulses, then FluorEssence™ collects the total signal. After collection, FluorEssence™ displays the intensity of the luminescence as a function of time or wavelength. The *x*-axis is based on one of the four scan options:

- Decay by Delay
- Decay by Window
- Emission
- Excitation



Phosphorimeter parameters

Four FluorEssence™ parameters govern the sequence in a phosphorimeter experiment. These parameters automatically appear on the phosphorimeter experiment-acquisition window.

Initial Delay

Sets the time, typically in ms, between the start of the lamp flash and the onset of data-acquisition (opening of the Sample Window). Initial Delay can range from 0–10 000 ms, in increments of 0.001 ms. Accuracy of Initial Delay is better than ± 0.001 ms.

Set Initial Delay long enough so that fluorescence emission and lamp decay are complete, so that the resulting spectrum represents phosphorescence only. The full-width at half-maximum lamp-pulse width is 3 μs, but there is a long decay time for the light output. Lamp intensity falls to less than 1% of peak output after 45 μs. Setting the delay to > 0.05 ms effectively removes any interference from the lamp.

Initial Delay can be varied with time to yield a decay curve. Spectra can be scanned to isolate different phosphorescing components based on the lifetime of the luminescent species in the sample. Together, these two techniques can be used to create three-dimensional plots. For example, successive scans with varying delay times can be plotted.

To record both fluorescence and phosphorescence emission, set Initial Delay to zero.

Sample Window

Sets the duration of signal acquisition, typically in ms. The Sample Window opens when the Initial Delay ends. When the Sample Window opens, the signal is counted and integrated. After the Sample Window closes, any signal is ignored.

The Sample Window may be set from 0.01 to 10 000 ms. If the lifetime of the phosphorescence is known, set the Sample Window to 5–10 times the lifetime. If the phosphorescence lifetime is unknown, make the Sample Window a small fraction of the anticipated lifetime, and then increase it until acceptable results are observed.

If the Sample Window is too long, the detector will record spurious background signal. If the Sample Window is too short, components of the lifetime decay may be missed. With two or more species decaying simultaneously, try varying the Initial Delay and the Sample Window.

Time per flash

Sets the total cycle length per flash, including on time, decay time, and dead time between flashes. The Time per flash is the reciprocal of the repetition rate of the lamp pulses. The allowable repetition rate is 0.03–25 Hz. The Time per flash must be slow enough to let the Sample Window close before another flash begins. Accuracy of the repetition rate is ± 1 ms.

In a Decay by Delay type of scan, the Time per flash is governed by

Time per Flash ≥ Maximum Delay + Sample Window + 20 ms

In a Decay by Window type of scan, the Time per Flash is governed by

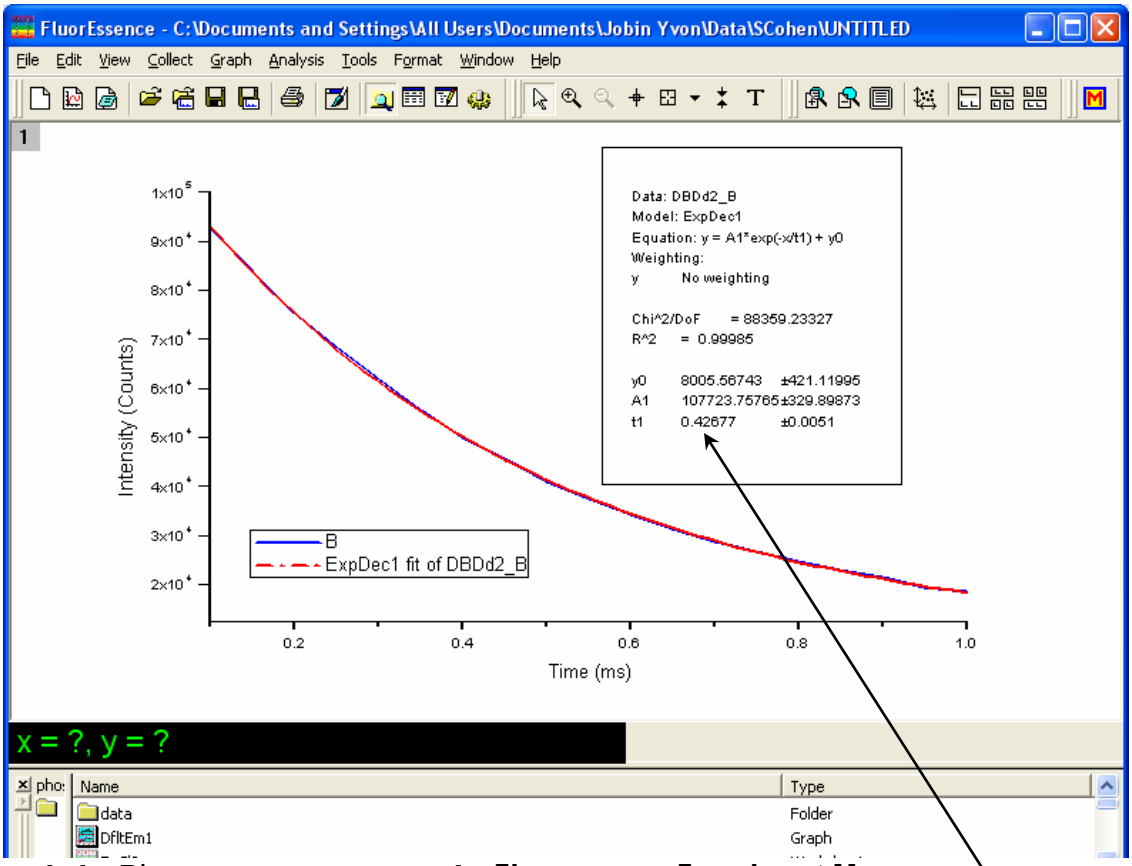
Time per Flash ≥ Initial Delay + Maximum Sample Window + 20 ms

Flash count	Sets the number of lamp pulses that contribute to each data point. The range for Flash count is 1 to 999. The signal collected per flash is integrated over the total Flash counts before FluorEssence™ stores it. The more flashes accumulated, the higher the signal-to-noise ratio becomes.

For more information about FluorEssence™ phosphorimeter commands, consult the FluorEssence™ on-line help and the Phosphorimeter manual.

Applications for the phosphorimeter

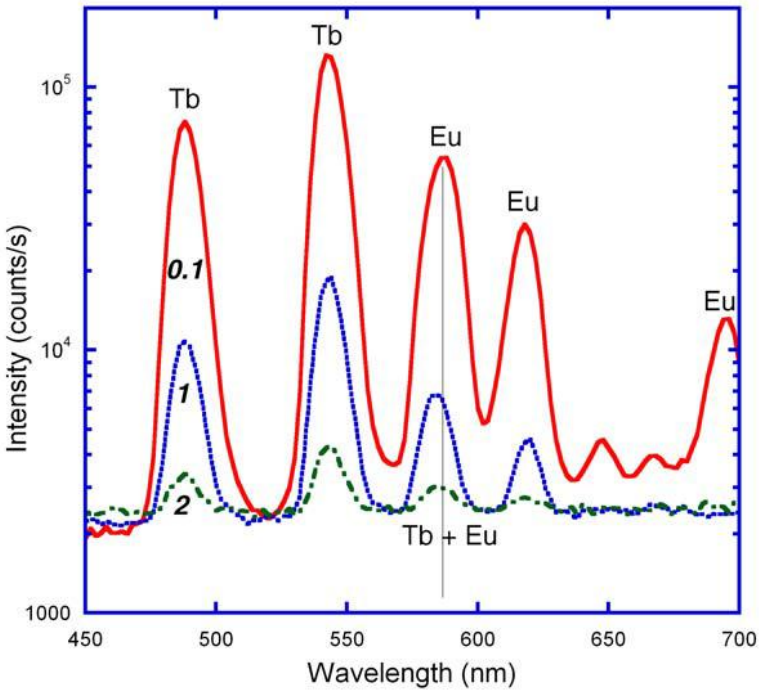
Phosphorescence decay curve



With the Phos experiment type in the **Fluorescence Experiment Menu**, create a phosphorescence-decay curve, as in the screenshot from FluorEssence™ shown above. This is an example using data from $TbCl_3$(*aq*), fitted to a single-exponential curve-fit in Origin®. All parameters are automatically displayed, including the fitted lifetime of 427 µs.

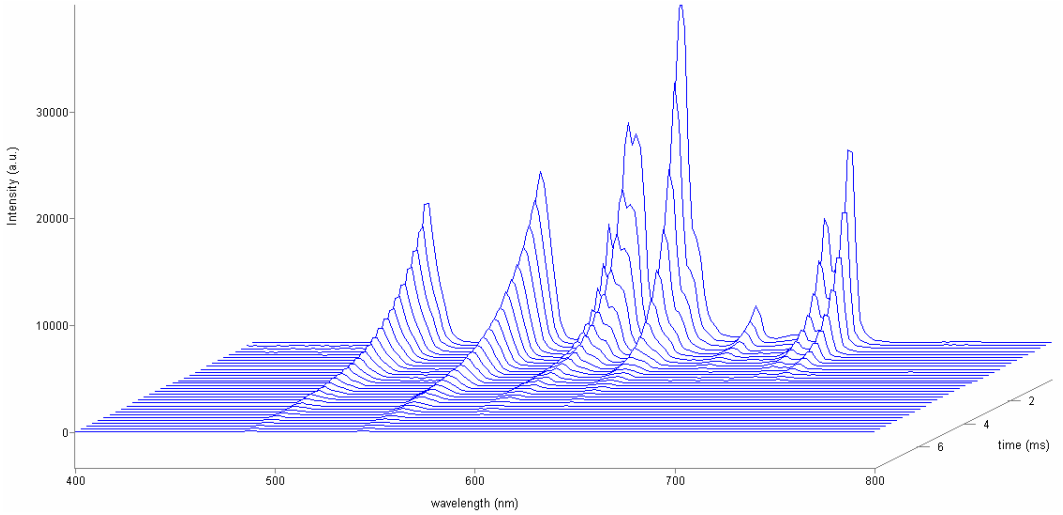
Isolate components in a mixture based on lifetimes

To the right are three scans of an aqueous mixture of terbium and europium chlorides that isolate different phosphorescent components based on their lifetimes. For example, a sample containing two phosphorescent species with different lifetimes (Tb = 421 µs; Eu = 113 µs) can be resolved into its components by varying the Initial Delay parameter, here shown as bold italic numerals, ***0.1*** ms, ***1*** ms, and ***2*** ms initial delay. Notice the wavelength shift in the 593 nm peak (marked with a gray line) from initially mostly europium fluorescence (short lifetime) to a mixture of terbium (longer lifetime) and europium fluorescence at later delays. Also notice the Eu peak near 690 nm at 0.1 ms that vanishes at later times. This experiment was performed using the Emission subtype of Phos experiment.



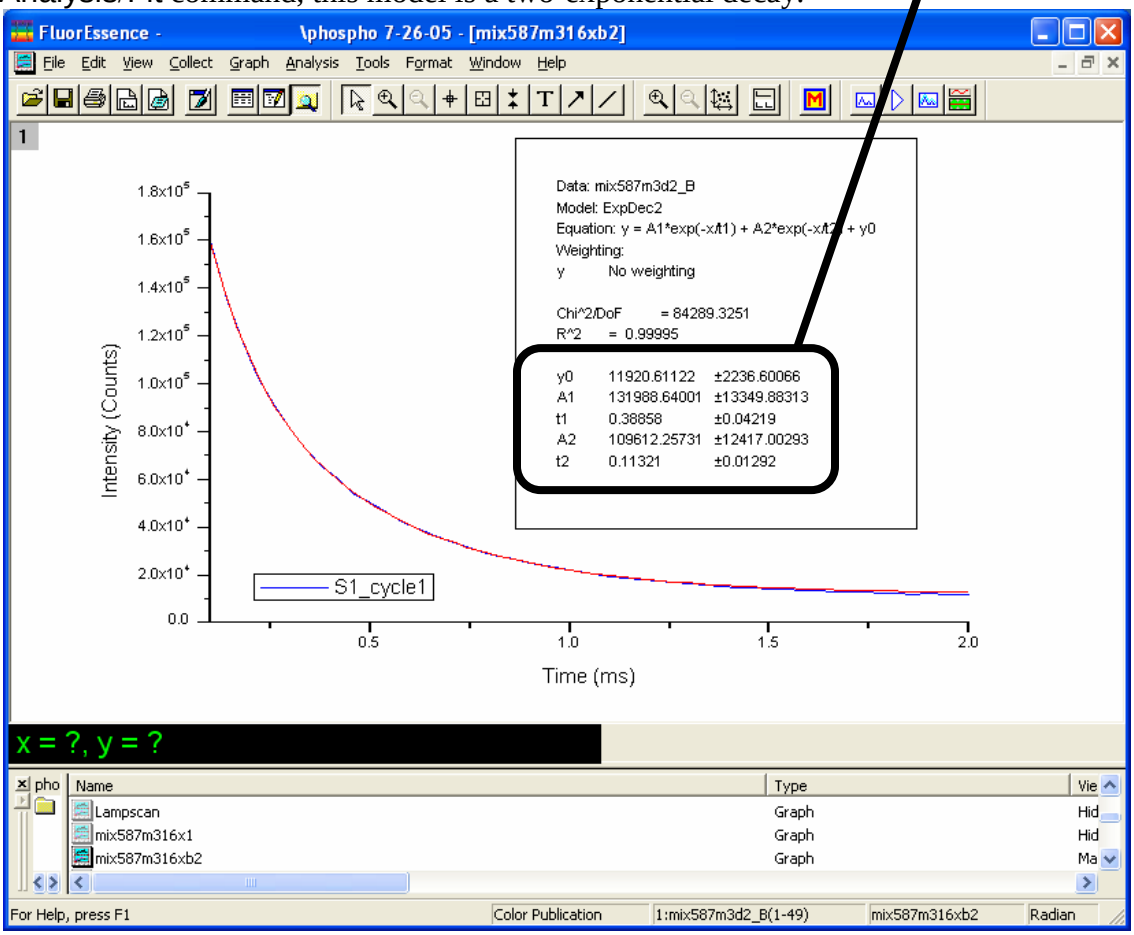
Three-dimensional plots

Varying the Initial Delay with the Phos vs Emission technique, a three-dimensional graph can be created, as shown below. This graph shows successive scans of a mixture of Tb-L and Eu-L (where L = benzophenone antenna chromophore) with varying time delays, segregating species into contours representing a particular slice of time.



Kinetic analysis of mixtures

Often a sample containing a mixture of components can be analyzed through fitting its phosphorescence-decay curve. Here is a phosphorescence decay of an aqueous mixture of $EuCl_3$ and $TbCl_3$, whose different lifetimes have been extracted by FluorEssence™'s dual-exponential fit. Curve-fitting merely involves choosing the analytical model. From Analysis/Fit command, this model is a two-exponential decay.



Installation

Warning: *Untrained personnel should not attempt to install or calibrate this accessory. High voltage exists inside the lamp housing.*

The phosphorimeter must be installed and calibrated by a HORIBA Scientific Service Engineer.

Operation of the phosphorimeter

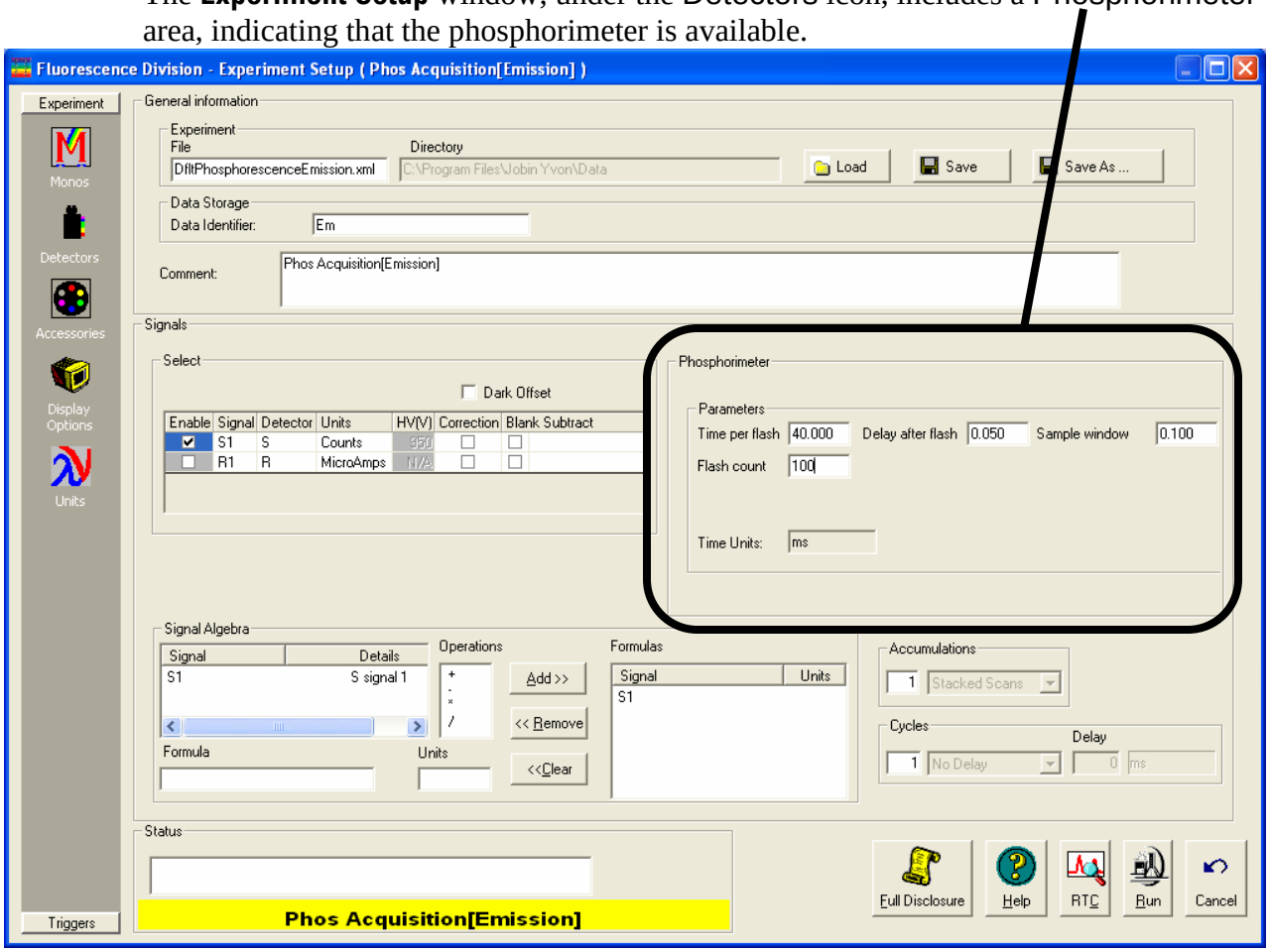
Start-up

Load an appropriate instrument configuration that includes the phosphorimeter.

FluorEssence™ features

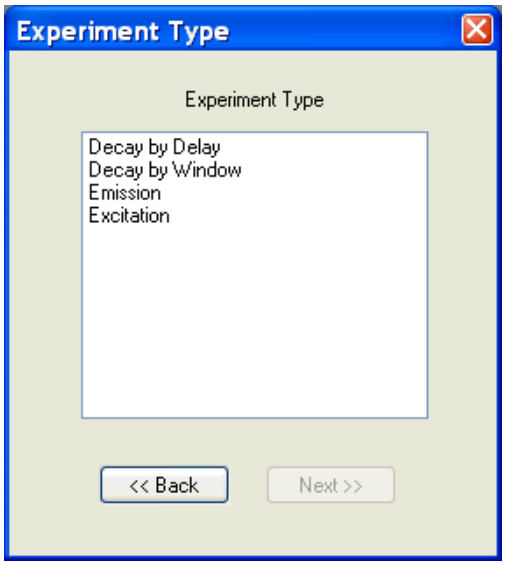
Experiment Setup window

The **Experiment Setup** window, under the Detectors icon, includes a Phosphorimeter area, indicating that the phosphorimeter is available.



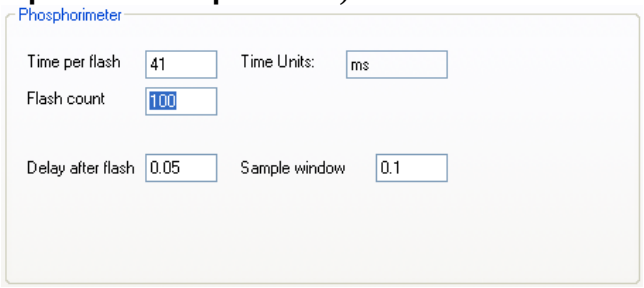
Experiment Type window

After choosing the Phos experiment type in the **Fluorescence Main Experiment Menu**, up to nine experiment types are available for the phosphorimeter, depending on the instrument configuration:



Excitation
Emission

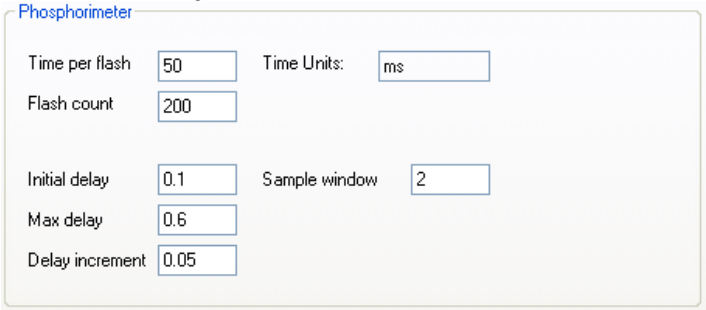
These are similar to the standard excitation, and emission scans. Among the changes are that the Integration Time field is removed, and instead are data-entry fields for Sample window, Delay after flash, Time per flash, and Flash count. These four fields are described above, in the Theory of Operation section, and shown below (extracted from the **Experiment Setup** window).



Above is a Phosphorimeter area with a typical set of parameters for the emission of $EuCl_3$. Use an excitation monochromator set to 393 nm; the emission monochromator should start at 570 nm, end at 750 nm, with an increment of 1 nm.

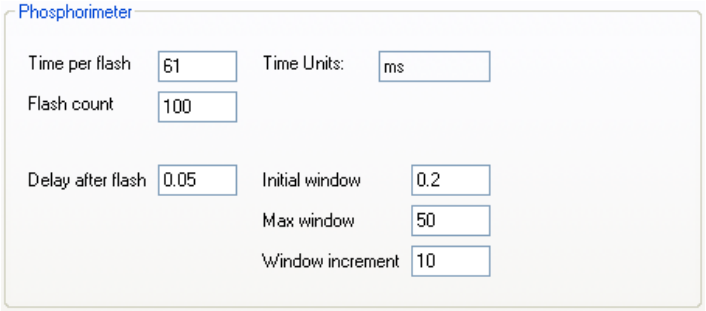
Decay by Delay

These produce a decay of phosphorescence over time. Decay by Delay varies the Delay after flash in order to construct the decay curve:



Decay by Window

Decay by Window varies the length of the Window increment with constant Delay after flash while taking data, to construct the decay curve:

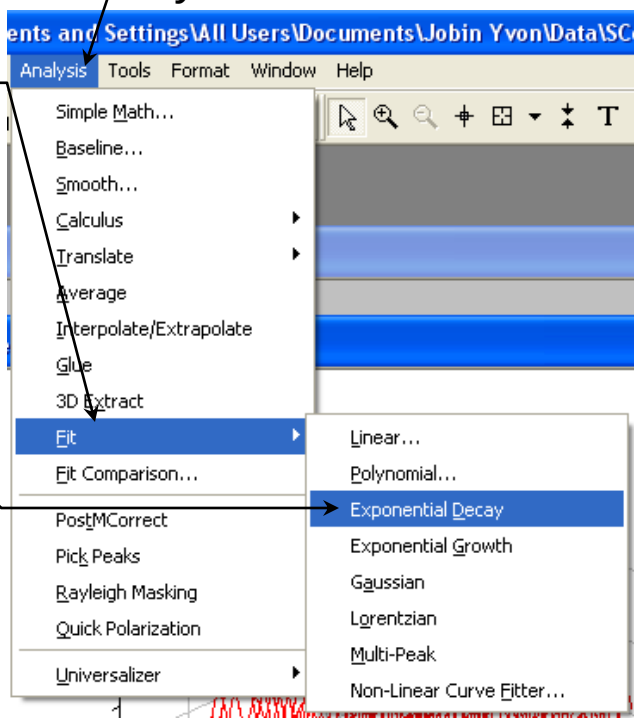


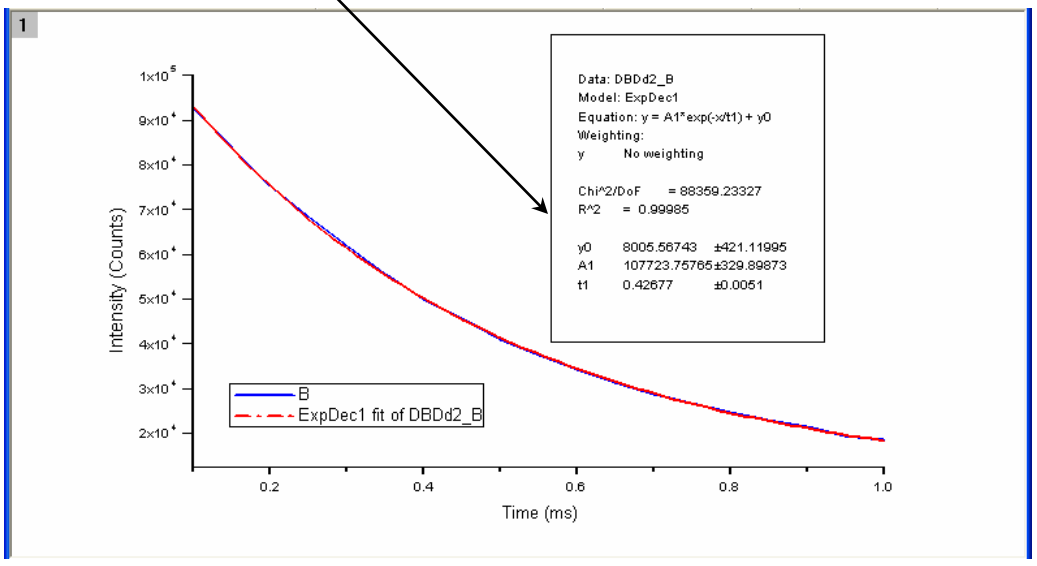
Above is a Phosphorimeter area showing typical parameters for the phosphorimeter decay of $EuCl_3$. Set the emission monochromator to 590 nm and the excitation monochromator to 393 nm.

Anisotropy phosphorimeter scans run the automated polarizers as well.

Processing phosphorimeter data

1. Open the graph to be processed.
2. Click on the data points to be processed.
3. In the toolbar, choose Analysis.
   A drop-down menu appears.
4. Choose Fit.
5. From the submenu, choose the type of analytical curve to use.
6. Follow the instructions for that type of curve.
   The fit plus parameters appear on the graph.





Lamp replacement

The xenon flash lamp (part # FL-1035) typically has a half-intensity life of at least 10 million flashes. If you hear an abnormal click with each flash or missed flash, this is an indication that the flash lamp is failing. Replace the flash lamp as soon as possible. Follow the procedure below for replacement and alignment.

Warning: *High voltage exists within the FL-1040 chassis. To avoid fatal shocks, follow these precautions: Before removing the lamp cover, unplug the power cords, and wait at least 1 min while the internal capacitors discharge.* ***Never operate the lamp with the cover removed!***

Warning: *Intense ultraviolet, visible, or infrared light may be present when the instrument is open, so wear eye and skin-protection, such as light-protective goggles and light-blocking clothing.*

Warning: *Xenon lamps are an explosion hazard. Be sure that the power is off, and all AC (mains) power is disconnected from the system. Read and follow all the cautions below:*

Hazards

- Xenon-arc lamps are an explosion hazard. Wear explosion-proof face-shield and protective clothing when opening the lamp housing and handling the lamp.
- Disconnect the lamp power supply from the AC power line (mains) while handling lamp leads. Lethal high voltages may exist.
- The CW lamp remains extremely hot for approximately one-half hour after it has been turned off. Do not touch the lamp or the metal unit until the lamp has cooled.
- Never look directly at the xenon arc or its reflection. Intense radiation can permanently damage eyes.
- Do not touch the focusing lens, back-scatter mirror, or the surface of the lamp.
- Fingerprints will be burned onto these surfaces when the lamp is ignited.

1 In FluorEssence™, be sure the phosphorimeter instrument configuration is loaded.

The flip-mirror automatically rotates to the flash lamp, giving you more room to work.

Caution: *Never rotate the flip mirror inside the lamp housing manually. This can strip the gears in the gearbox.*

2 Switch off and prepare the lamp housing.

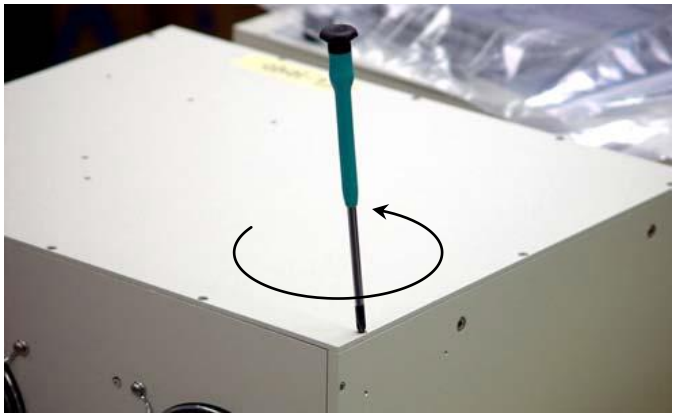
a Be sure that the SpectrAcq controller itself, and the lamp-housing's two power switches, are all turned off.

Warning: *High voltage exists within the lamp housing. To avoid fatal shocks, follow these precautions: Before removing the lamp cover, unplug the power cords, and wait at least 1 min while the internal capacitors discharge.* ***Never operate the lamp with the cover removed!***

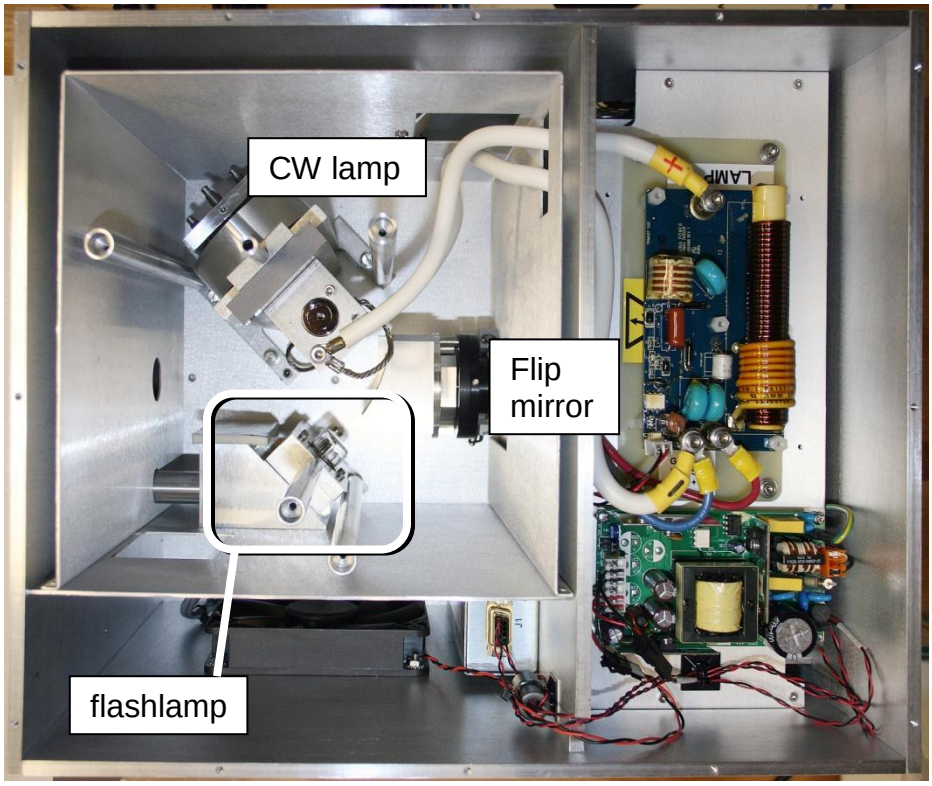
b Remove the AC (mains) power cord from the SpectrAcq controller and the lamp housing.

3 Remove the cover of the lamp housing.

Remove the 13 Phillips-head screws.



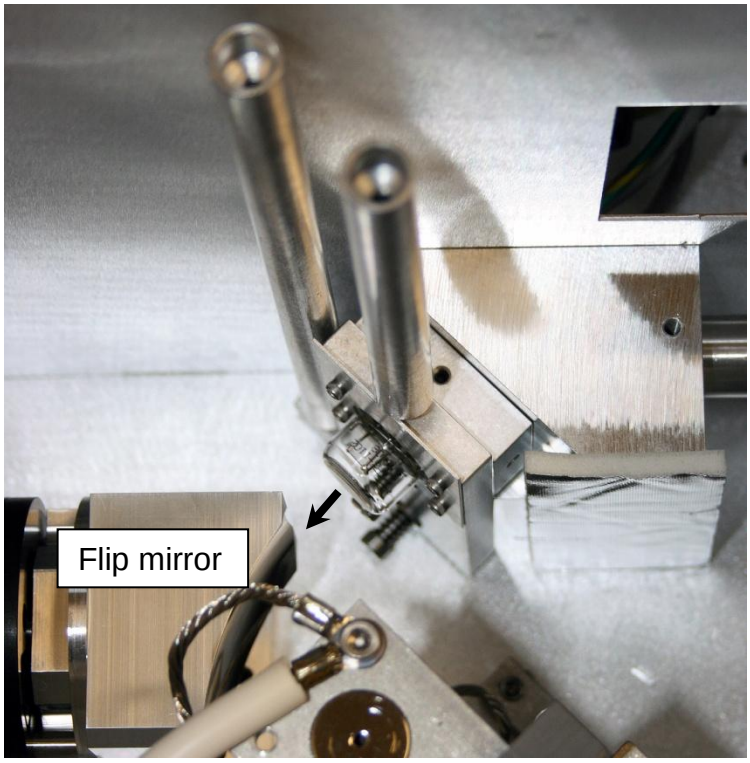
Here is the internal layout of the FL-1042 lamp housing:



4 Remove the flash lamp.

a Follow all safety precautions on the new lamp's box.

b Pull the old flash lamp out with a steady motion.



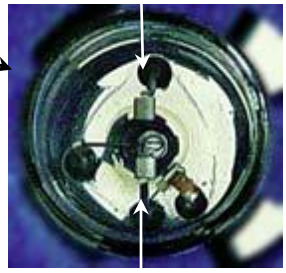
Caution: *Be sure not to hit the flip mirror with the flash lamp.*

c Discard the old flash lamp in a safe and appropriate manner.

5 Insert the new flash lamp.

Warning: *Never touch the flash lamp's glass bulb with bare hands. The oils from your hands can weaken the bulb and cause catastrophic failure. Handle the flash lamp with tissues, cloth gloves, or soft cloths only.*

a Align the pins with the lamp socket.
The cathode and anode should be vertical, as shown here.



b Push the lamp in firmly until it is seated securely and properly.

Note: *If the lamp tilts during insertion, check the pins—they should be straight. If they are not straight, re-align the pins before insertion.*

6 Replace the lamp-housing cover.

Re-attach the 9 Phillips-head screws.

7 Reconnect all cables.

Troubleshooting

The phosphorimeter accessory has been designed to operate reliably and predictably. If there is a problem, examine the chart below. If all else fails try the steps in the Troubleshooting chapter, in the "Further Assistance..." section.

Warning: *High voltage exists within the lamp housing. To avoid fatal shocks, follow these precautions: Before removing the lamp cover, unplug the power cords, and wait at least 1 min while the internal capacitors discharge.* ***Never operate the lamp with the cover removed!***

Problem	Possible Cause	Remedy
No signal.	Flash lamp is broken.	Replace flash lamp.
	Cables are improperly connected, or disconnected.	Check cable connections.
	Communication problems between computer and accessory.	Shut down entire system, let sit for 1 min., then restart and re-initialize the system.
No single flash is heard during initialization of a phosphorimeter layout, followed by no signal during an experiment.	Bad flash lamp. Faulty circuitry in phosphorimeter.	Shut off phosphorimeter "power" switch and disconnect power cable to phosphorimeter. Remove phosphorimeter lid, and inspect position of the flip-mirror. Call the Service Department.
	Flip-mirror is not rotated toward flash lamp.	
An abnormal click occurs with each flash.	Flash lamp is failing.	Replace flash lamp.
Occasional missed clicks.	Flash lamp is failing.	Replace flash lamp

Phosphorimeter trigger

Introduction

There are two important pins to consider: Pin 5 is for the flash lamp, which provides a 12.8-µs pulse, active LOW. The acquisition delay starts on the HIGH→LOW transition (< 10 ns jitter). Pin 4 is the phosphorimeter trigger input, which requires a minimum pulse-width of 200 ns. Acquisition begins at 250 to 350 ns after the HIGH→LOW transition. Any pulses received when the board is not ready for them are ignored. The time between pulses should be at least 1 µs longer than the interval between pulses set in the FluorEssence™ software.

Pin connections

Below is a table of connector pins and their use.

Caution: *Do not connect pin 1 to pin 3.*

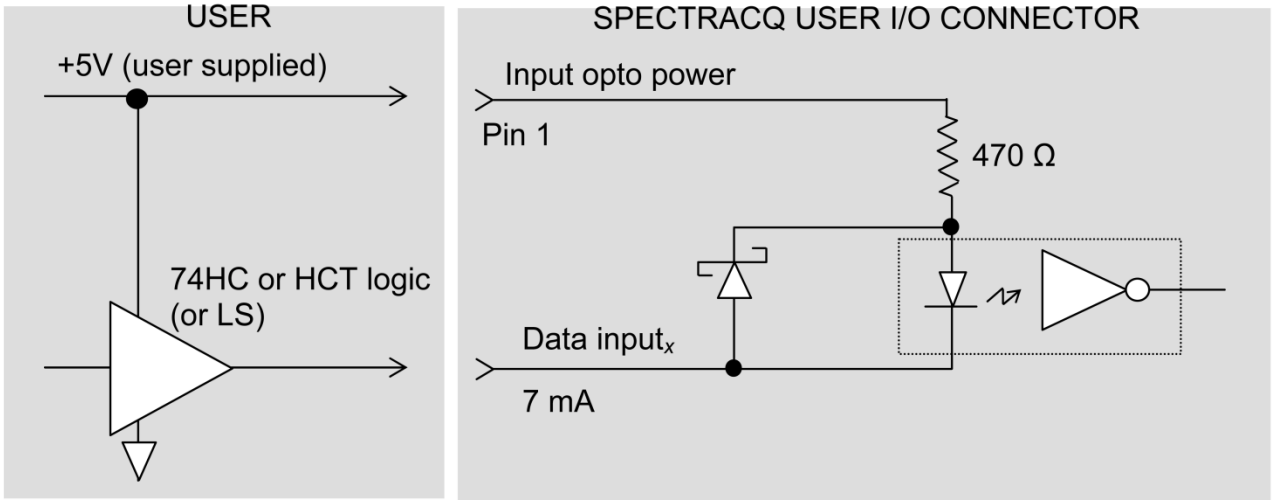
Pin #	Signal	I/O
1	Output opto power	(5 V)
6	Disable CW xenon lamp (active LOW)	O
2	Enable phosphorimeter-mirror position (active LOW)	O
7	Valid phosphorimeter-mirror position (active LOW)	I
3	Input opto power	(5 V)
8	Status 1 (reserved for future use)	I
4	Trigger phosphorimeter cycle (on HIGH→LOW transition)	I
9	Status 2 (reserved for future use)	I
5	Flash phosphorimeter lamp (on HIGH→LOW transition)	O

Note: *All inputs are active low; all digital outputs are active low.*

Typical circuitry

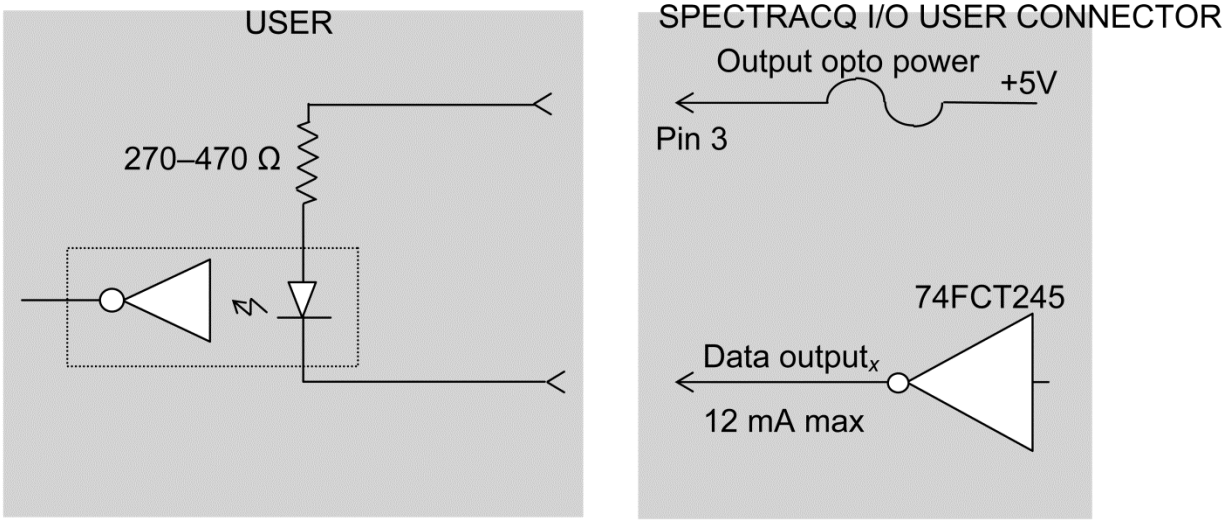
Input

A typical input circuit to the user trigger might look like this:



Output

A typical output circuit to the user trigger might look like this:



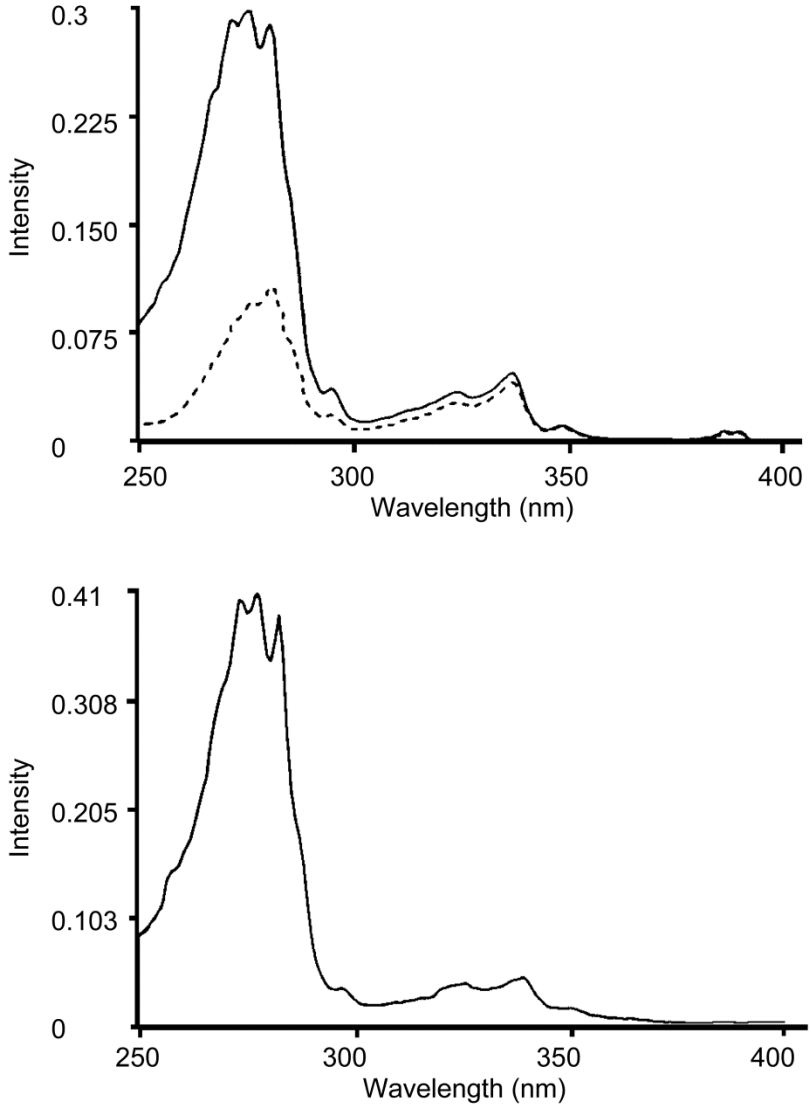
Chapter 11 : Applications

Introduction

HORIBA Scientific realizes the importance of accurate and reproducible data. Therefore, all systems are calibrated and tested prior to shipment to a customer site and then again after installation. Performance spectra indicate the operational parameters and ensure that the system is operating within specifications. Fluorolog®-3 systems employ the most sophisticated data-correction techniques to yield accurate and reproducible fluorescence spectra.

For example, fluorescence emission spectra are affected by the response characteristics of the monochromators and optical components such as gratings and detectors. To compensate for these responses, radiometric correction factors are individually determined for each Fluorolog®-3 system and are supplied with the FluorEssence™ software. The user can update these correction factors periodically if necessary. Simple point-and-click operations or user-controllable default system parameters allow real-time or post-processing correction of spectral data.

To correct for variations in the intensity of the excitation source, Fluorolog®-3 spectrofluorometers also monitor the excitation beam with a wavelength-independent reference detector. The raw emission data can then be automatically ratioed to intensity information generated by the reference detector. The effect of this correction can be seen in the upper plot on the next page.



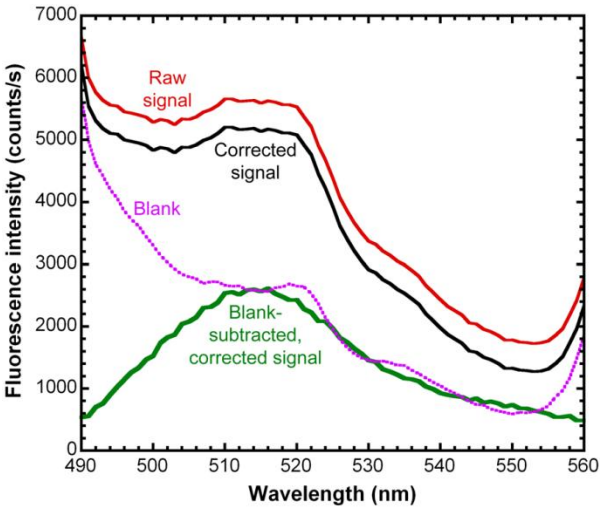
Spectra of azulene. Upper plot: corrected (solid line) and uncorrected (broken line) excitation spectra. Lower plot: an absorption spectrum.

The broken line in the upper plot traces the uncorrected excitation spectrum of an azulene sample acquired on a Fluorolog® system without reference-detector ratioing. The solid line represents the same spectrum automatically corrected for the wavelength-dependence of excitation-source intensity. The lower plot shows the absorption spectrum of azulene, acquired on the same spectrofluorometer system. The corrected excitation spectrum shows strong similarity and fine structure compared with the corresponding absorption spectrum.

HORIBA Scientific spectrofluorometers have carved a niche in the scientific community by consistently demonstrating the capability to perform extraordinary tasks. Some of the remarkable features of the system are outlined below.

Detecting sub-picomolar concentrations of fluorescein

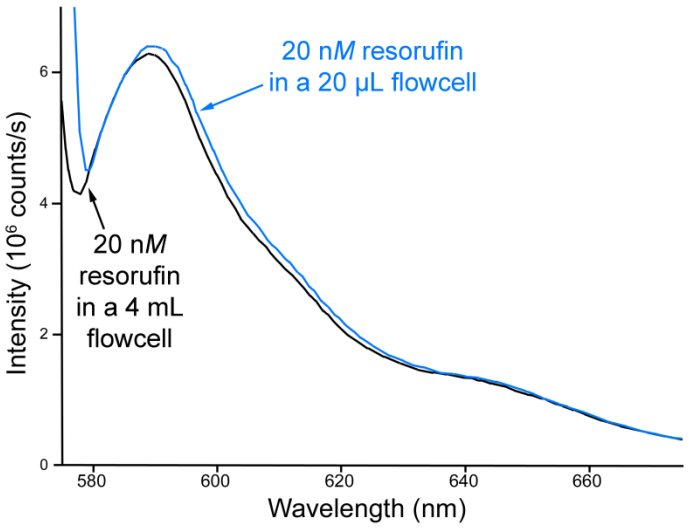
Instrument sensitivity is often expressed in terms of the limit of detection of a standard substance. The superior sensitivity of Fluorolog®-3 systems is demonstrated by their ability to detect sub-picomolar concentrations of standard substances such as fluorescein. Often, other instruments perform this task by extrapolating from much higher concentrations. The Fluorolog®-3, however, not only detects femtomolar concentrations of fluorescein, but directly measures the true emission spectrum of 50-femtomolar fluorescein using an integration time of only 10 seconds—a clear demonstration of superior performance.



Single-scan emission (no smoothing) from 50 f*M* fluorescein in 0.01 *N* NaOH. Note the H-O-H Raman bending mode at 521 nm in both blank and sample.

Reduced-volume samples

Because the samples required to produce spectral data may be expensive or obtained only in limited quantities, the precise imaging quality and photon-counting sensitivity of the Fluorolog®-3 spectrofluorometers are invaluable assets. The following diagram compares the fluorescence emission of 20-n*M* resorufin acquired using the 20-µL and 4-mL cells. Notice that the signal-level is maintained with either cell.



Comparison of the fluorescence emissions of 20-n*M* resorufin using a 20 µL cell and a 4 mL cell.

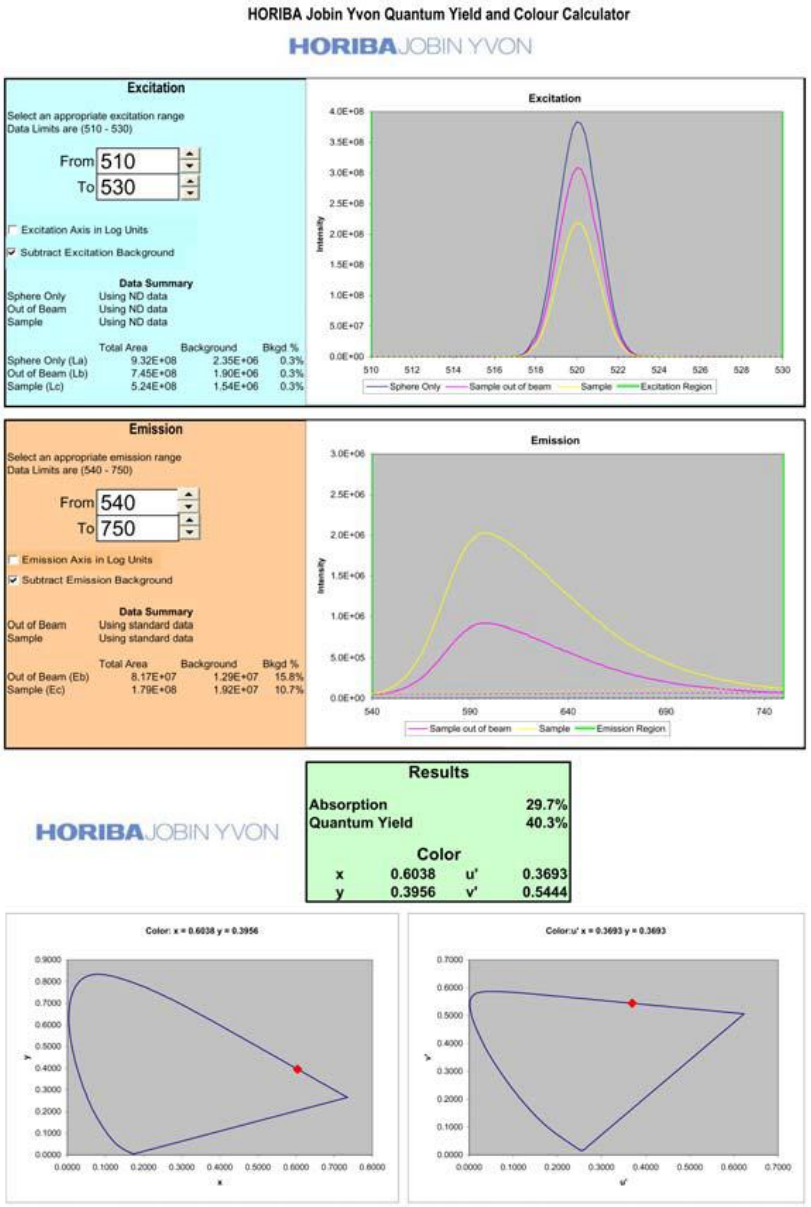
Fluorescence detection of highly scattering samples

Qualitative and quantitative determinations normally are difficult to ascertain from highly scattering samples. Typically, fluorescence signals are dwarfed by stray or scattered light from the sample. The flexibility of the Fluorolog®-3 systems, however, allows the introduction of a double-grating emission spectrometer, thereby improving the system's stray-light rejection. With a single-grating emission spectrometer, scattered light from the sample often finds its way through the exit along with the selected band of emitted light. However, when the band is re-dispersed by the double-grating spectrometer, most stray light is stripped away as the band passes through the exit slit to the emission detector.

Quantum-yield calculations

Among the many features unique to Fluorolog®-3 systems is the capacity to measure fluorescence-related parameters required to calculate the quantum yield for samples in solution, using the F-3029 integrating sphere accessory, the Quanta-φ. This includes recording excitation-source intensity and calculating the area under the corrected emission spectra.

Results from HORIBA Scientific's exclusive quantum-yield software include absorption, quantum yield, and CIE color values in both 1931 and 1976 coordinates.

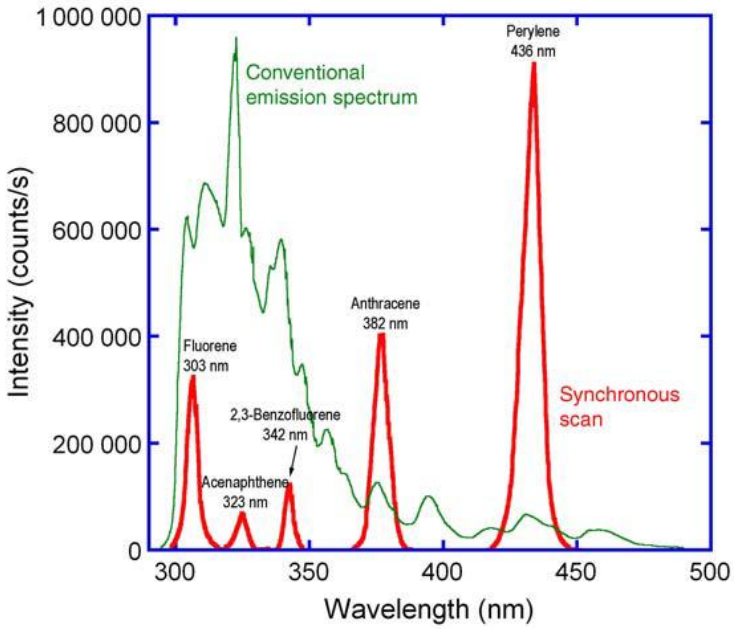


Characterizing complex mixtures via synchronous scanning

The fluorescence spectrum of a complex mixture often contains overlapping spectral features representative of the mixture and revealing no indications of the contents of the sample. A spectrum of this nature is all but useless. *Synchronous scanning* offers a solution to this problem.

Simultaneous scanning of the excitation and emission spectrometers with a constant offset between them yields an intensity proportional to the product of the emission and excitation intensities. This resulting spectrum often can be analyzed readily.

On the right are an emission scan and synchronous scan of a mixture of polynuclear aromatic hydrocarbons.

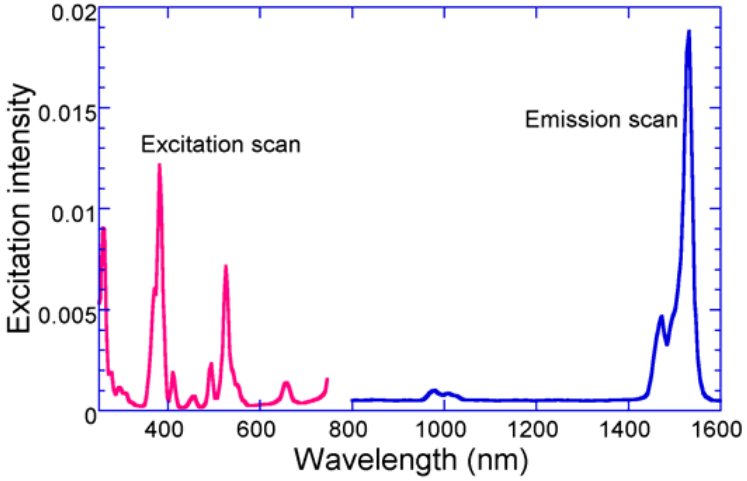


Emission (solid line) and synchronous (separate peaks) scans of a mixture of polynuclear aromatic hydrocarbons.

The solid line in the figure above is the emission spectrum acquired on a Fluorolog® system (single-grating monochromators) with constant wavelength excitation. As shown by the broken line, when the sample is scanned synchronously, five individual components are resolved into unique, sharp peaks indicative of the individual compounds.

Operating in the IR region

With optional near-IR detectors and arrays, the Fluorolog®-3 spectrofluorometer operates in the infrared region of the spectrum, thereby opening up totally new areas of applications for fluorescence spectroscopy. Typically, an IR

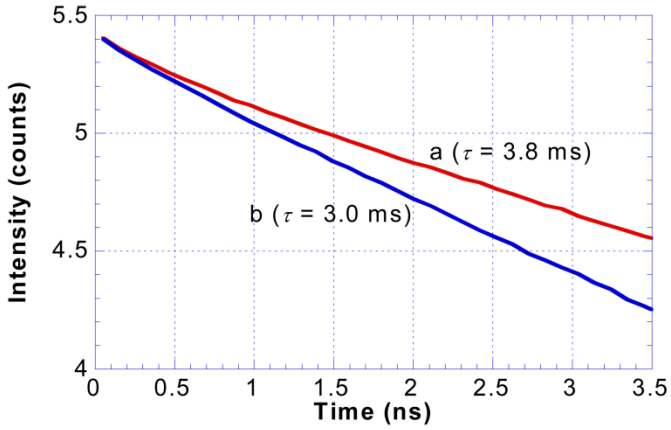


Visible and near-IR spectra of erbium-doped laser-glass acquired on a Fluorolog®-3.

spectrofluorometer requires components significantly different from those found in conventional instruments. Because the most widely used photomultiplier detector is insensitive above 860 nm, an IR spectrofluorometer must be equipped with a red-sensitive photomultiplier, or a solid-state detector whose response is effective far into the IR region. With a photomultiplier sensitive to 1 µm, only minimal system modification is necessary. A phase-sensitive lock-in amplifier and a light chopper are required.

Phosphorescence from time-resolved data

Both fluorescence and phosphorescence spectra are photon emissions that occur when molecules return from an excited electronic state to the ground state. The nature of the excited state distinguishes the two: fluorescence is associated with relaxation from a singlet excited state, while phosphorescence is associated with relaxation from a triplet excited state. Fluorescence usually occurs within a few nanoseconds after excitation. Because triplet transitions are "forbidden" quantum-mechanically, the average phosphorescence decay times are generally longer, ranging from a few microseconds to several seconds. Thus, phosphorescence offers a longer observation period for monitoring reactions, looking at environmental effects on a sample, or following changes in the hydrodynamic characteristics of macromolecular systems.



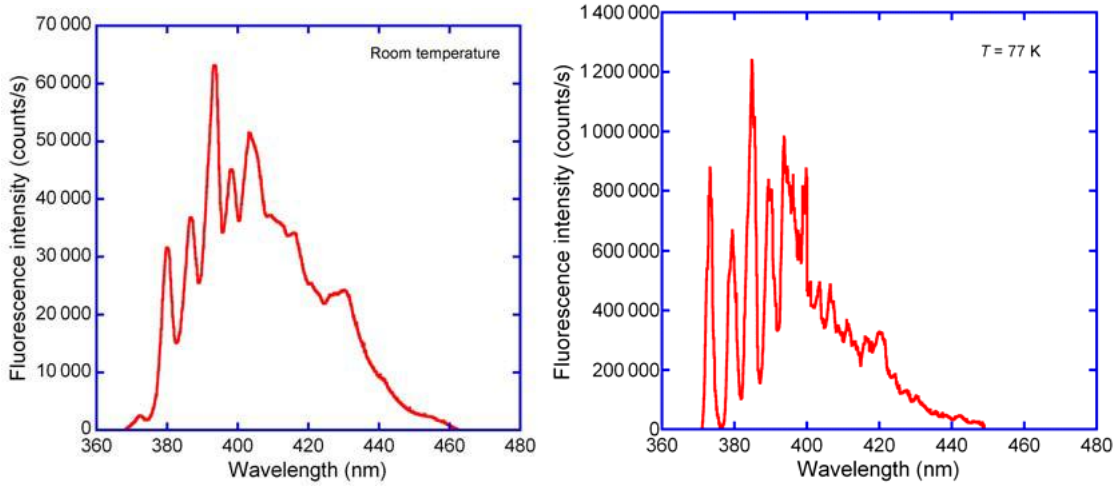
Phosphorescence decay from (a) Tb-peptide complex, and (b) Tb-peptide + fluorescein.

In phosphorescence experiments using the Fluorolog®-3 and the FL-1040 Phosphorimeter Assembly, the sample is excited by a pulsed light source. Acquisition of the emission signal is synchronized to the pulse, with user-specified delay and sampling times, to produce time-resolved spectral data. With an appropriate choice of delay time, the user may select only the luminescence of interest.

Time-resolved data-acquisition also makes it possible to acquire phosphorescence decay curves and compute lifetimes of lanthanides such as europium and terbium, as well as the biological probe eosin.

Low-temperature scans

One way to protect a sample from molecular collisions that can quench luminescence is by isolating the sample in a rigid matrix. Thus, cooling with liquid nitrogen enhances the phenomenon of fluorescence, even for seemingly dormant samples. In addition, the superior resolution of a Fluorolog®-3 double-grating-monochromator system optimizes measurements under these conditions.



A sample of pyrene at room temperature (left) and at liquid-nitrogen temperature (right). Note the sharper peaks at lower temperature.

Monitoring kinetic reactions using time-based fluorescence

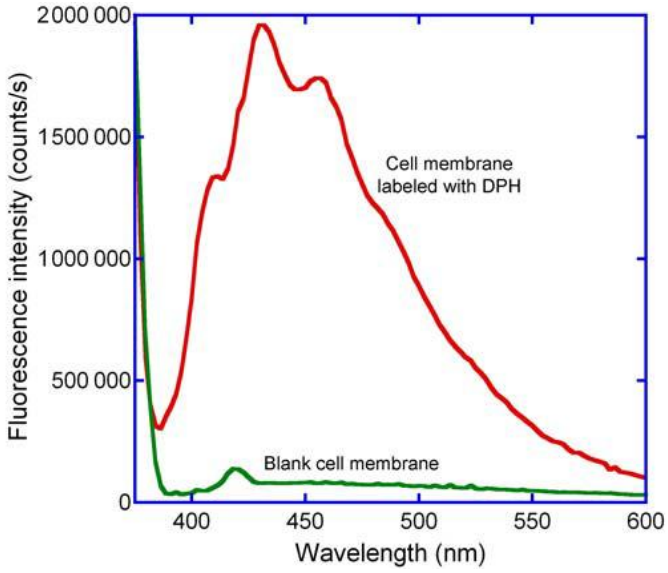
By setting the wavelengths at the excitation and emission peaks of a sample, the Fluorolog®-3 systems can monitor fluorescence as a function of time. This permits the use of Fluorolog®-3 systems in reaction-rate determinations, which monitor the formation or breakdown of a fluorescing species. Reaction-rate determinations are highly selective. Because only changes in intensity are considered, the method is not affected by interference from continuous background signals or steady-state scatter.

Front-face detection to enhance data collection for absorbent or solid samples

Fluorescence typically is collected at right angles (90°) from transmitted or scattered light. Yet right-angle viewing is inappropriate for some samples. Imprinted paper, for example, reflects light, which interferes with accurate data collection. In highly absorbent samples like hemoglobin or milk, most of the emitted light is reabsorbed before the fluorescence can be measured. A significant design feature of the Fluorolog®-3 spectrofluorometers is that they offer a choice between conventional right-angle or front-face fluorescence detection. Front-face viewing is ideal for solid, turbid, or highly absorbent samples such as pellets, powders, and monolayers on microscope slides. A swing-away mirror is positioned to allow collection of sample luminescence at 90° to the excitation beam, or front-face at 22.5°. In front-face viewing, the fluorescence is collected from the sample's surface.

Polarization to detect rotation correlation times of biological molecules

Used in conjunction with the large number of fluorescent dyes suitable for biological research, fluorescence spectroscopy has greatly expanded our understanding of metabolic processes on the molecular level. The Fluorolog®-3's design offers unparalleled sensitivity for such work. The cell membrane tagged with 1 µM DPH is detected easily in this graph.



The cell membrane tagged with 1 µ*M* DPH is detected easily in this graph.

Fluorescence polarization offers a safe, sensitive immunoassay technique—fluoroimmunoassay (FIA). This method has none of the licensing and waste-disposal problems associated with radioimmunoassay (RIA). Immunoassay methods, which are based on competitive antibody-binding reactions, require the ability to distinguish between bound and unbound species. This selectivity is inherent in fluorescence polarization techniques.

In fluorescence polarization, the excitation beam is passed through a polarizing prism, and the emitted luminescence is analyzed with another polarizer alternately oriented parallel and perpendicular to the excitation polarization. The measured polarization depends on the rotation of the molecules between absorption and emission. Because the measurement reflects changes in rotation, polarization can be used to distinguish between free molecules and the larger, slower antibody-bound molecules in immunoassays. The use of polarizer prisms instead of film polarizers ensures that the researcher is guaranteed full spectral coverage from the ultraviolet to the visible. Over time, film polarizers tend to become photobleached, especially if exposed to UV light.

The applications for the Fluorolog®-3 spectrofluorometer are almost endless. By simply changing accessories, adding or removing a hardware component, or accessing the proper software controls, the user can ensure that the system continues to grow or change as application needs change. The modular construction and interchangeable accessories make the Fluorolog®-3 even more attractive to most industries.

Chapter 12 : Xenon Lamp Information & Record of Use Form

Introduction

Xenon lamps typically are used in fluorescence instruments because they provide a continuous output from 240 nm to 600 nm. In the Fluorolog®-3 spectrofluorometers, the standard xenon lamp is ozone-free. The xenon-lamp spectrum exhibits a characteristic peak around 467 nm, which can be used to indicate whether the excitation spectrometer is properly calibrated.

As the xenon lamp ages, water Raman spectra have a progressively lower peak intensity. During its lifetime, the lamp stabilizes at approximately 60% of its original intensity. Keep a record of the time the lamp is in use on the form provided on the following page. From this record, you will be able to determine when the lamp is near the end of its lifetime. The maximum lifetime of the 450 W lamp is 1500 hours.

Warning: *To avoid explosion from lamp failure, do not allow xenon lamp to exceed rated lifetime. One clue to imminent failure may be extremely low water-Raman intensity. Please note the hours the xenon lamp has been used.*

Note: *Each time the lamp is ignited counts for 1 hour of lamp usage.*

Xenon Lamp Record of Use

Page _____ of _______

In-Service Date	Operator	Current Date	Time On	Time Off	Total Time (Hours/Minutes)
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
		/ /			/
			Total Hours		/

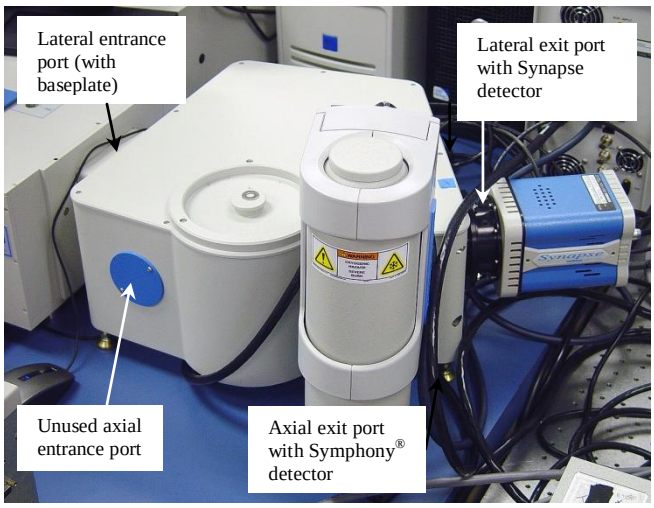
Chapter 13 : iHR operation with the Fluorolog®-3

Introduction

Using an iHR imaging spectrometer on the emission side of the sample mount offers the option of detection with a CCD, to create an image of the dispersed fluorescence for subsequent analysis. This chapter discusses special hardware connections and software operation specific to iHR users, especially with regard to CCD detectors. On most iHR spectrometers, there are movable grating-turrets and multiple ports with mirrors to choose; therefore extra information is necessary for operation.

Differences between the iHR and standard monochromators

- Four ports generally are available, rather than two on a standard monochromator. Most iHR spectrometers for the Fluorolog® have two entrance ports ("lateral", i.e., on the side, and "axial", i.e., on the back) and two exit ports (lateral and axial). Usually the lateral entrance port is attached to the sample compartment.
- A FluorEssence™-controlled flip mirror is used to choose between entrance ports and exit ports. Be sure the flip mirror is set to the proper position before running an experiment.
- Three gratings can be mounted on the rotatable grating turret. FluorEssence™ controls which grating to use.



Fluorolog® users typically choose the lateral entrance port as the path for luminescence from the sample compartment into the iHR. To attach the iHR to the sample compartment, you must insert a baseplate adapter between the sample compartment and the iHR. This baseplate is automatically included with all Fluorolog® instruments factory-built with an iHR spectrometer.

Hardware

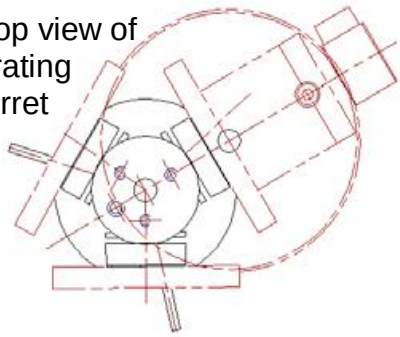
Electrical power

Have a free AC (mains) outlet available for the iHR. A 24 V adapter and power supply plugs into the AC circuit, and connects to the back of the iHR. The power supply contains a voltage converter that automatically adjusts to the input AC voltage.

Caution: *Some users may wish to remove the monochromator and replace it with a iHR. Always switch off the power to the monochromator and iHR before removal of these accessories from or addition to a system. Failure to shut off the power to the iHR and monochromator can damage the SpectrAcq.*

Internal workings

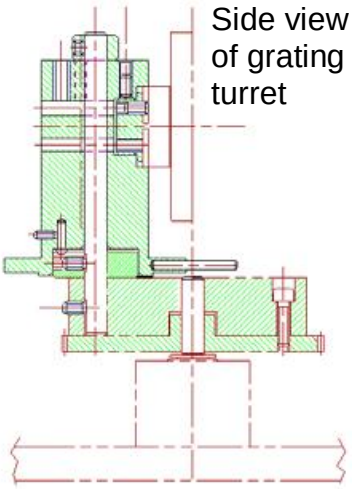
Top view of grating turret



Multiple gratings

Generally, gratings with different groove-densities are installed, but gratings with different blaze-angles are also possible for custom applications. Rotation to a new grating is discussed in **Real Time Control** below. After installing a new grating on the turret, its groove density must be entered in System Configuration, and its calibration must be performed in **Real Time Control**. Both of these procedures are described later in this section.

Side view of grating turret



Turret removal

1. Grasp turret by the thumbscrew on top.
2. Lift the turret up and out of the instrument.
3. Leave one shim-washer on the shaft.

Caution: *Never touch the face of a grating!*

Turret installation

4 Reverse steps 1 to 3.

The turret is self-indexing, so the initial position does not matter.

5 Rotate turret until it rests in a depression.

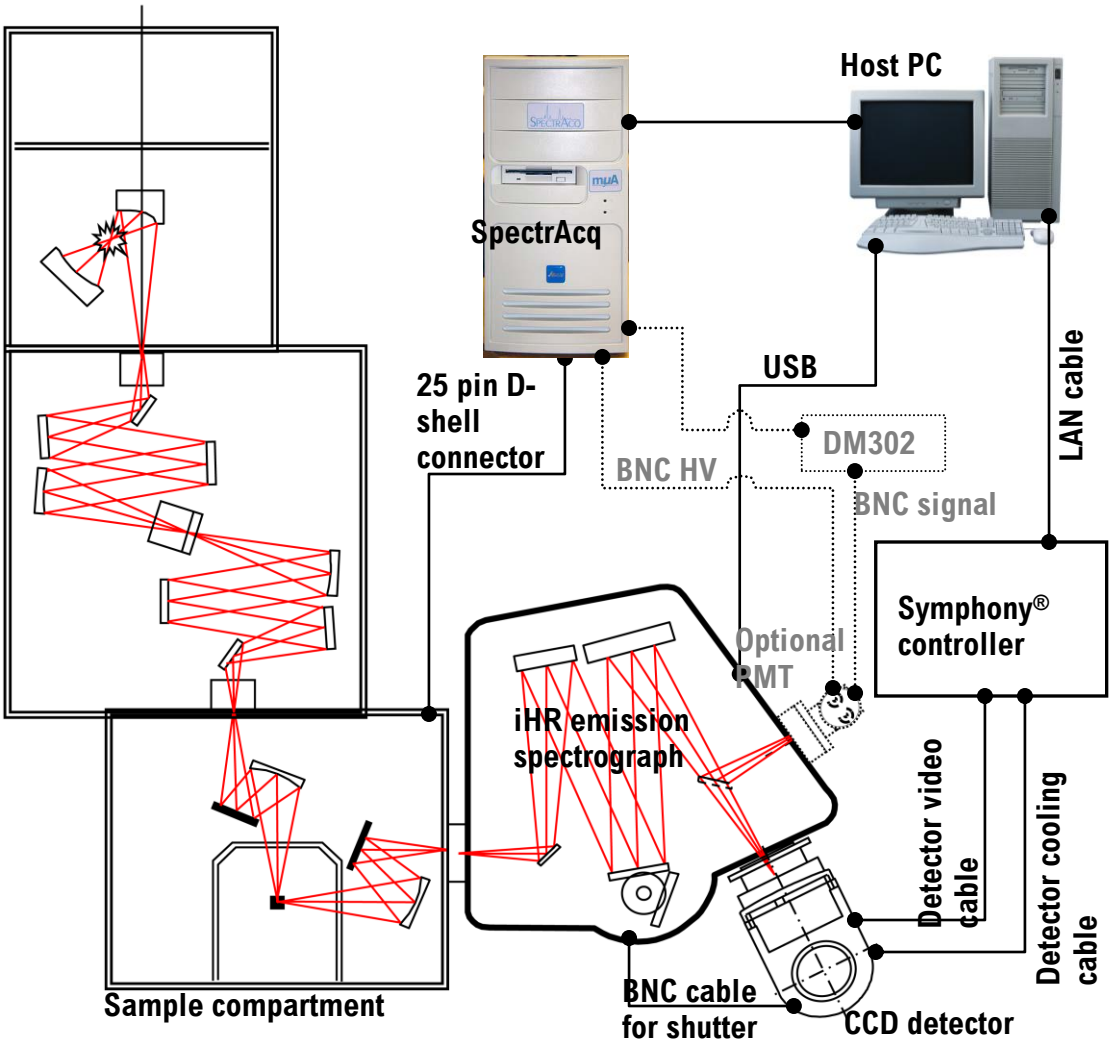
When switched on, the turret rotates to the correct position.

Automated slits

The iHR has automated adjustable slits at the entrance (2 mm maximum) and exit (7 mm maximum) ports. The slit width may be adjusted in steps of 0.0125 mm. For a 0.6 mm slit on the iHR320, dispersion is 1.58 mm.

Connections to system

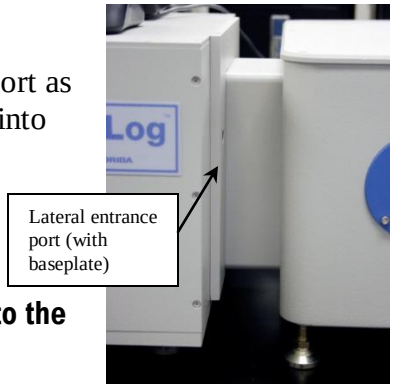
Below is a sketch of the special cable connections required between an iHR spectrometer, the CCD detector, and the system. Details of these connections are given on the following pages. Other Fluorolog®-3 connections are not shown. An optional second photomultiplier tube detector with its connections is shown in dotted lines.



Note: *Check your custom configuration with supplied documents.*

Attachment to sample compartment

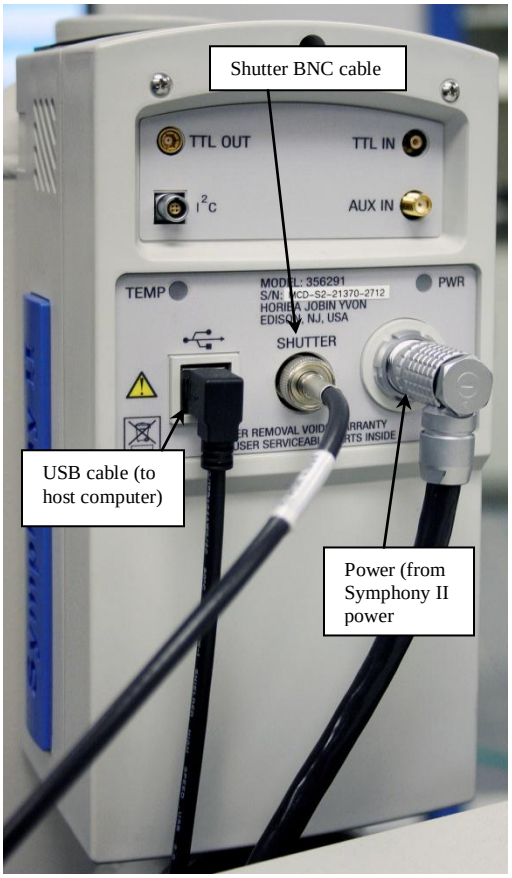
Fluorolog®-3 users typically choose the lateral entrance port as the path for luminescence from the sample compartment into the iHR. To attach the iHR to the sample compartment, a baseplate adapter must be inserted between the sample compartment and the iHR.



Note the baseplate attached to the lateral entrance port.

Cables to CCD detector

A coaxial cable runs from the terminal on the back of the iHR to the CCD. This controls the CCD shutter. The two large cables carry power and information between the Symphony® II and the host computer.

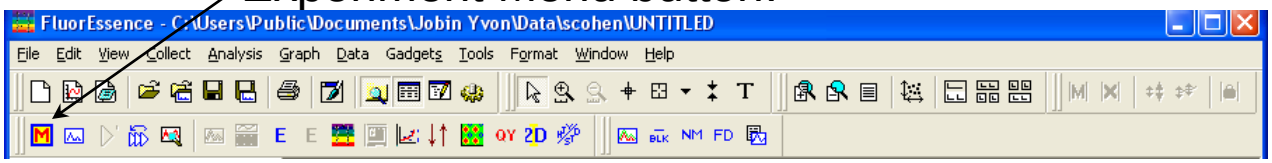


Software

Experiment Setup

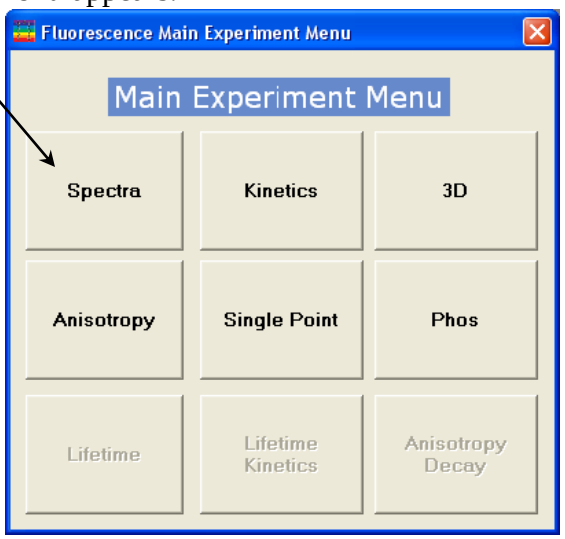
To run a CCD acquisition with an instrument layout that includes an iHR,

1 On the main FluorEssence toolbar, select the Experiment Menu button:



The **Fluoresence Main Experiment Menu** appears:

2 Choose the Spectra button.

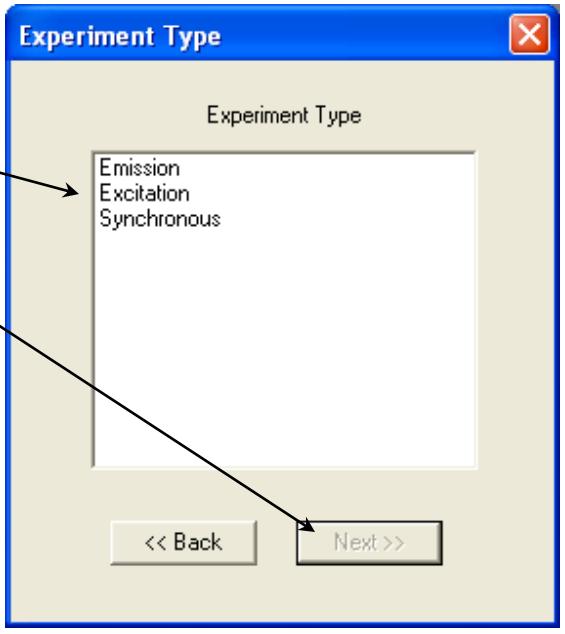


Note: *Certain types of experiments are grayed out.*

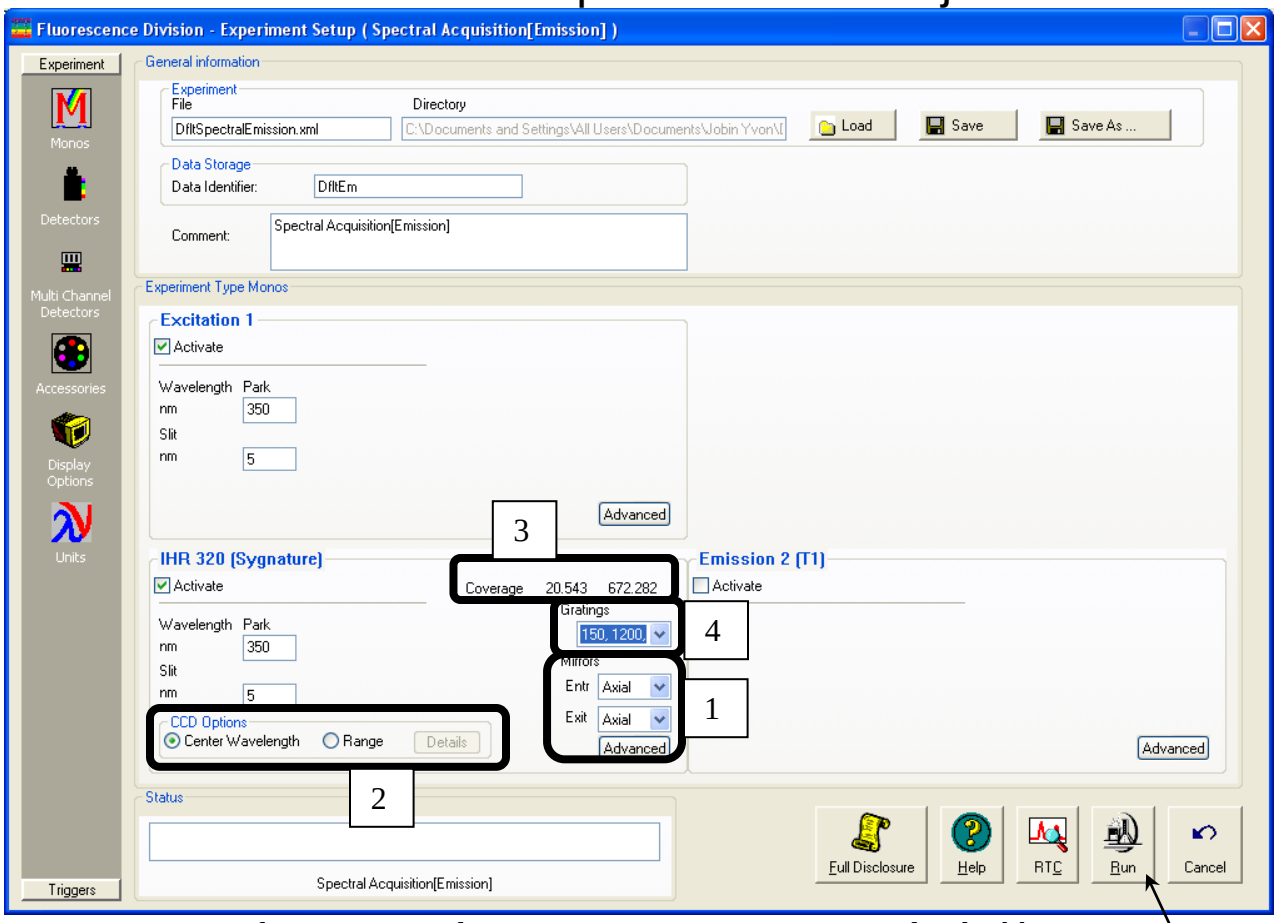
The **Experiment Type** window appears:

3 Choose the experiment type, then click the Next >> button.

The experiment automatically loads with the **Experiment Setup** window:



4 Use the default parameters or adjust them.



This is an example using an emission scan-type. Under the Monos icon, functions specific to the iHR are available, such as:

1 Mirrors
2 CCD Options
3 Coverage information
4 Gratings

These and other parameters are discussed on the following pages.

5 Complete all required parameters.

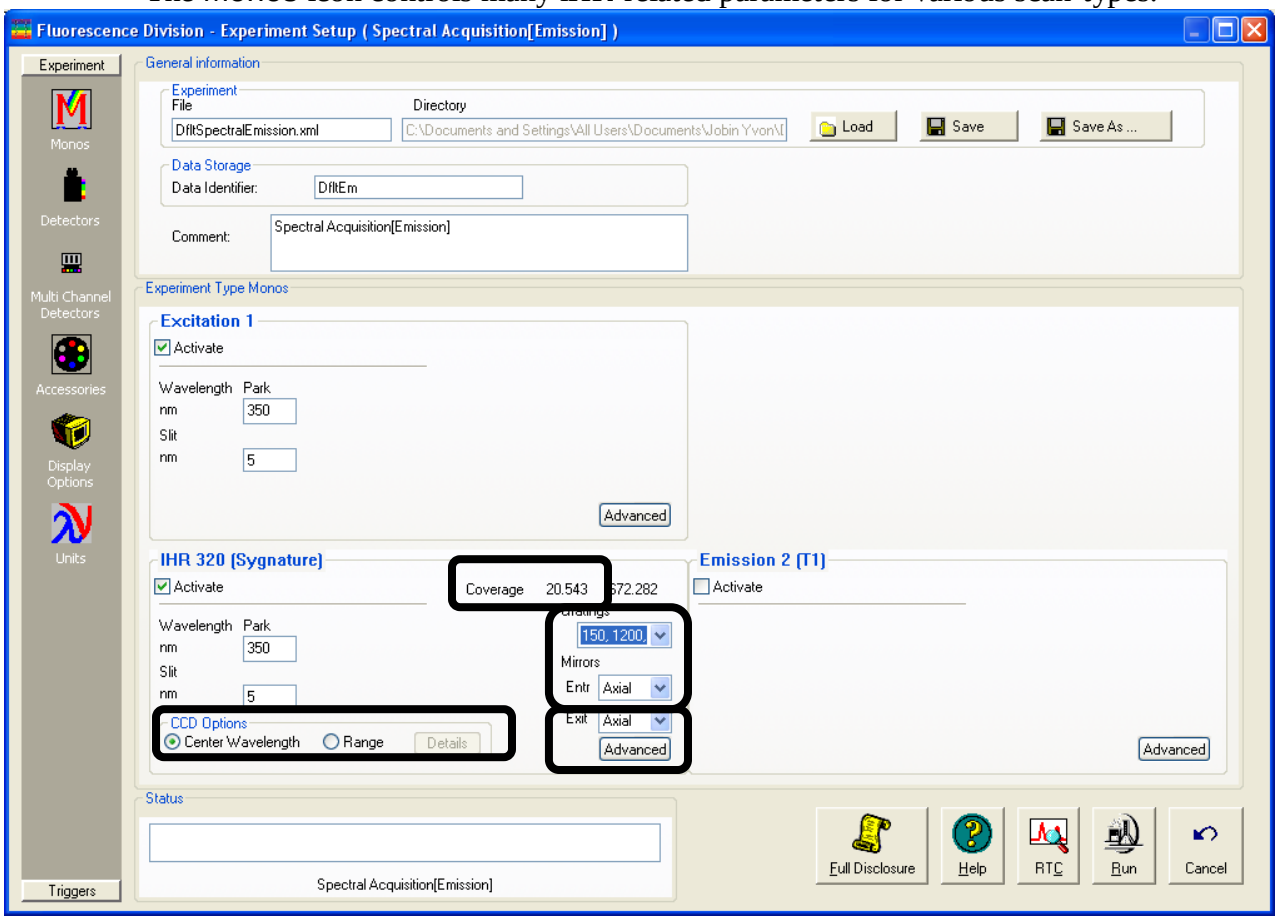
6 Include also detector-related parameters under the Detectors or Multi Channel Detectors icons.

7 Click the Run button.

The experiment begins.

Monos icon 

The Monos icon controls many iHR-related parameters for various scan-types.

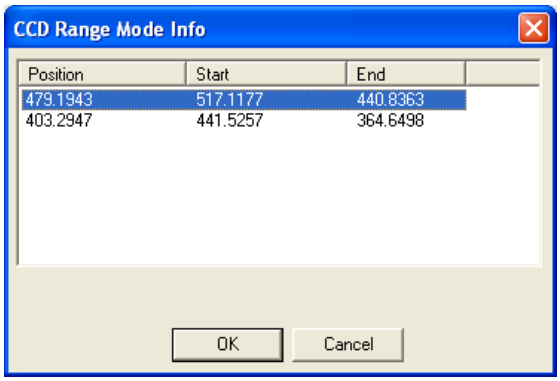


Mirrors

The Mirrors area controls the entrance and exit flip mirrors' positions. Choose Axial or Lateral positions for each mirror, from drop-down menus.

CCD Options

In the CCD Options area, choose a Range of wavelengths to detect, or pick a Center Wavelength, with the radio buttons. With the Center Wavelength option, the iHR shows a wavelength to Park the detector, instead of Start and End wavelengths. Click the Details button to reveal the **CCD Range Mode Info** window. This shows the exact range of wavelengths the detector will detect.



Coverage

Coverage shows how many wavelength ranges are used on the CCD detector.

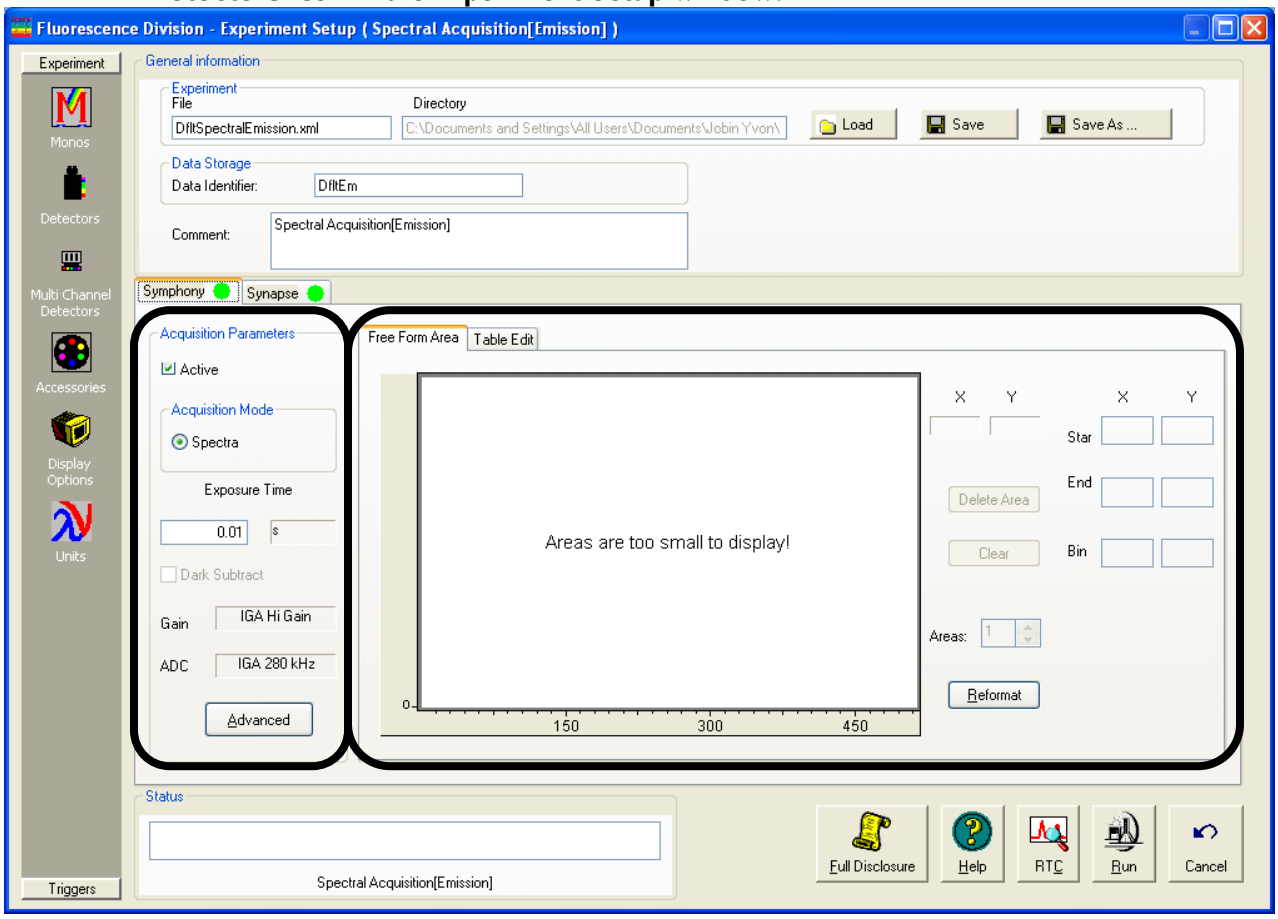
Gratings

The Gratings area allows you to choose from a drop-down menu which grating to use.

Note: *To choose a grating, the gratings must have been previously entered using* System Configuration. *The* Turrets *checkbox in* System Configuration *also must be enabled to allow the turret to rotate.*

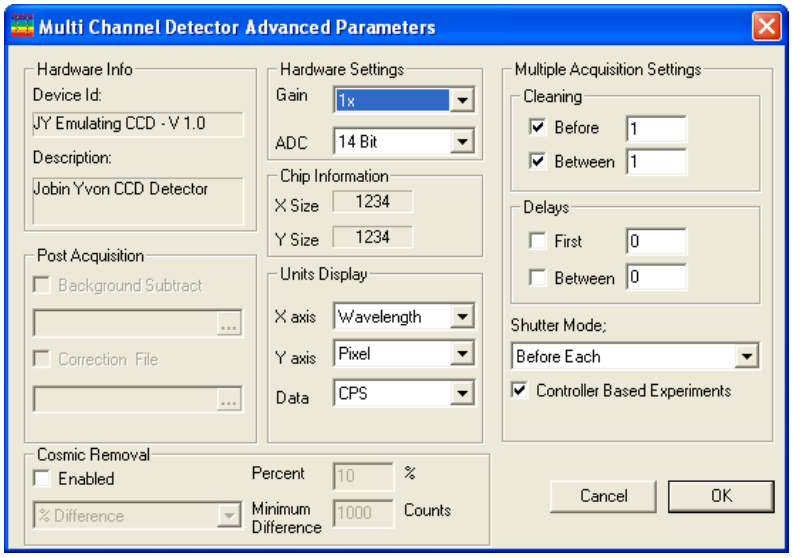
Multi Channel Detectors icon

To adjust parameters related to the CCD detector, click on the Multi Channel Detectors icon in the **Experiment Setup** window.

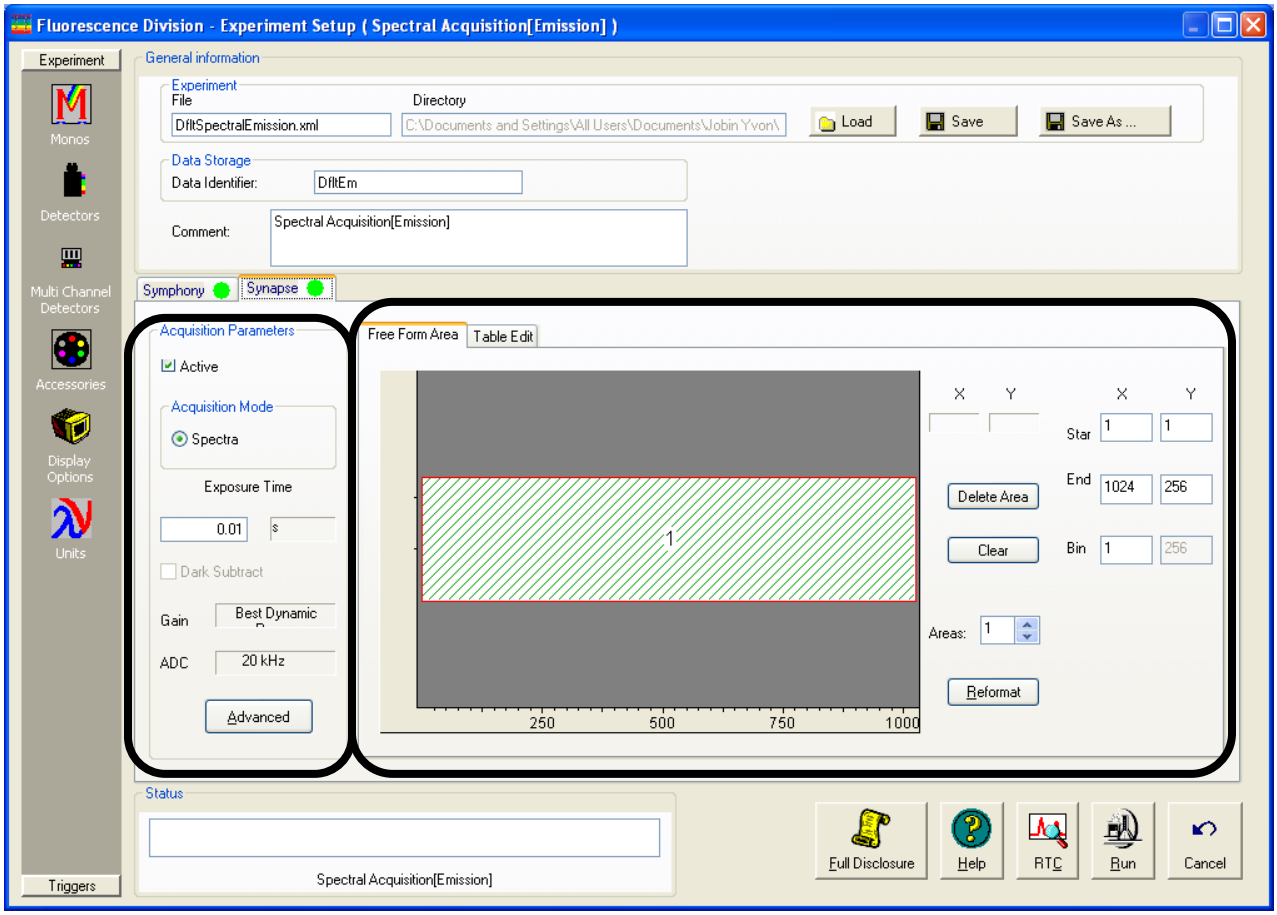


Acquisition Parameters

Under Acquisition Parameters, change the Exposure Time, and view the Gain, and the precision of Analog-to-Digital Conversion (ADC). Clicking the Advanced button opens the **Multi Channel Detector Advanced Parameters** window, to adjust Gain, Units Display, Cosmic Removal, ADC, and other parameters.



Below is the window for a Synapse with CCD detector:

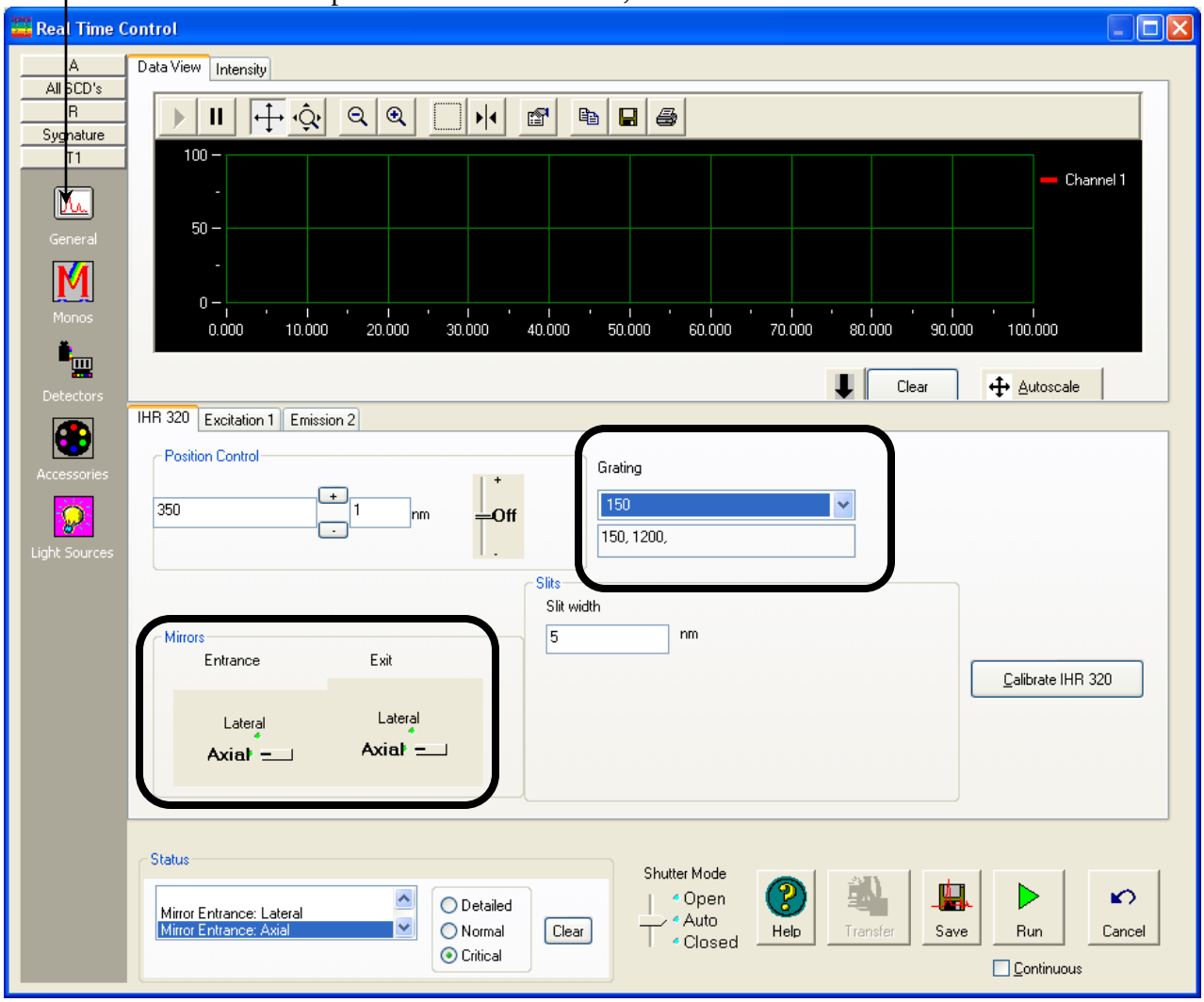


Pixel editing

There are two methods of choosing which pixels are used on the CCD: graphically or tabular. To choose the active pixel areas graphically, click the Free Form Area tab, and draw with the cursor or enter the coordinates under the X and Y columns. Choose the number of active areas with the Areas field. To enter the active areas as a table, choose the Table Edit tab. In both methods, click the Reformat button to clear the existing description and start over.

Real Time Control

In **Real Time Control**, some iHR parameters can be adjusted in real time. To gain access to the iHR in the **Real Time Control**, click the Monos icon. The iHR, when used as an emission spectrometer in L-format, is listed under the Emission 1 tab.



Grating

Rotate the turret in real time by clicking on the Grating area, and choosing the desired grating (listed as grooves/mm) from the drop-down menu.

Mirrors

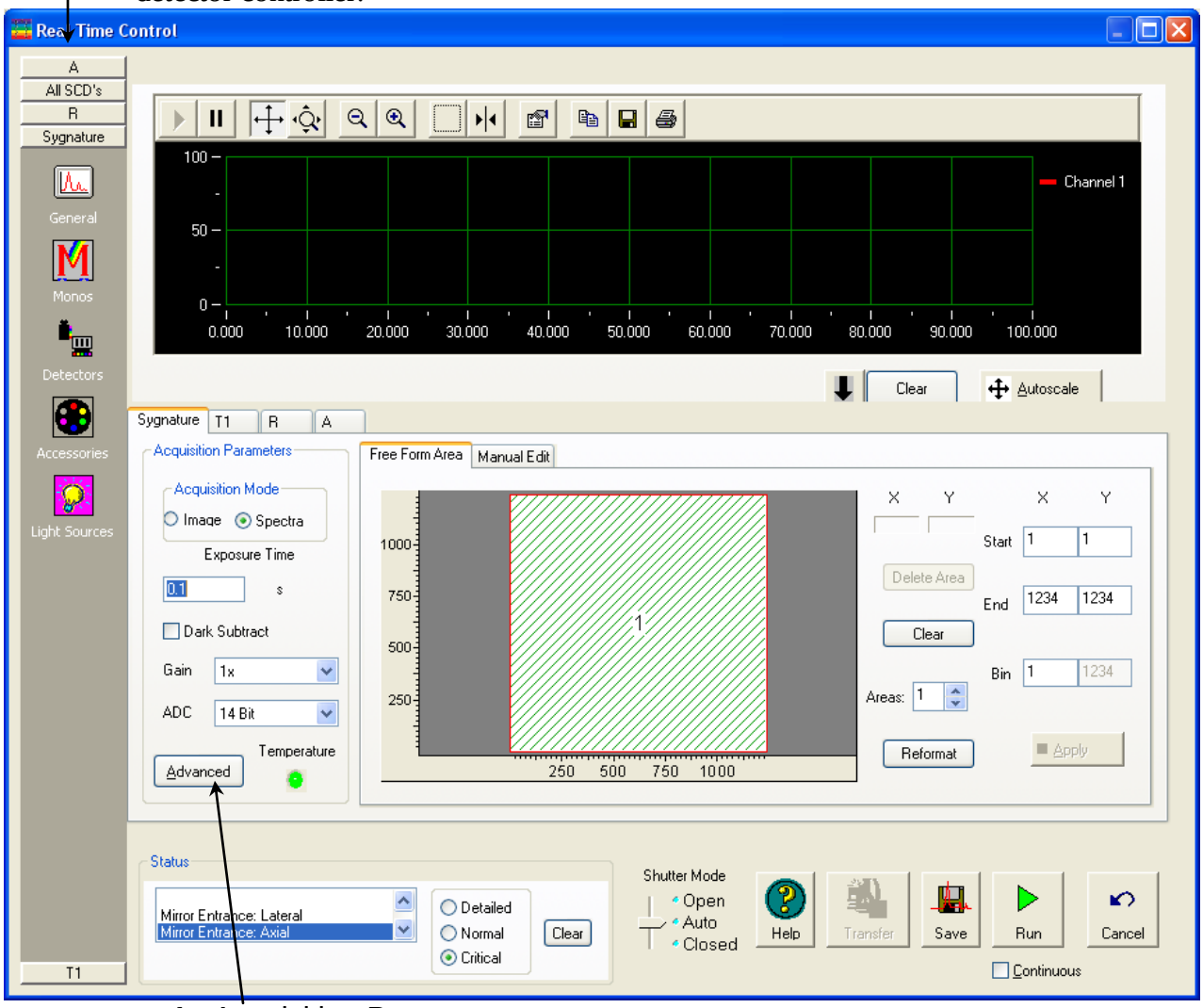
Switch the Entrance and Exit mirrors by clicking on the knob underneath each label. The knob will automatically change between Lateral and Axial. The current position is shown in bold type.

Note: *To choose a grating, the gratings must have been previously entered using* System Configuration. *The* Turrets *checkbox in* System Configuration *also must be enabled to allow the turret to rotate.*

Use this function to re-align the iHR if the peaks' wavelengths are correctly assigned near the center of the CCD, but are incorrect near the edges.

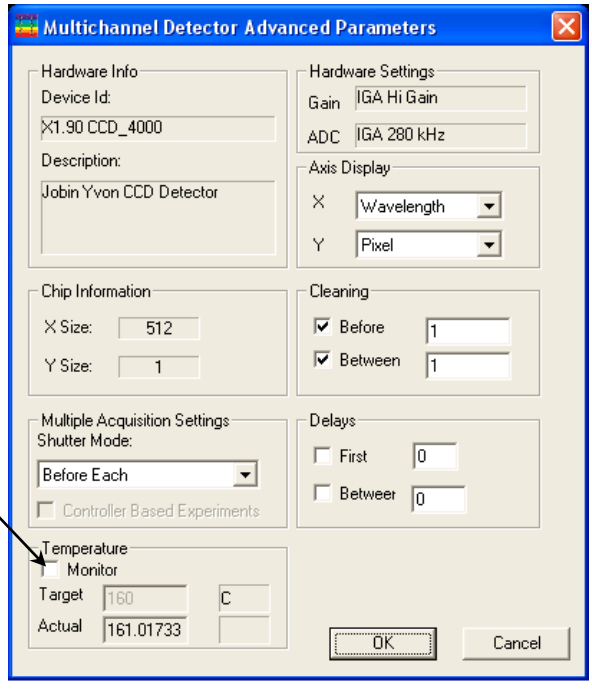
Monitoring temperature of the detector

Click the Synapse or Symphony button to access the functions for the multichannel-detector controller.



In the Acquisition Parameters area, click the Advanced button to open the **Multichannel Detector Advanced Parameters** window.

Click the Monitor checkbox in the Temperature area to monitor the detector's temperature.



System Configuration window

To set the cooled detector's temperature, use the **System Configuration** window.

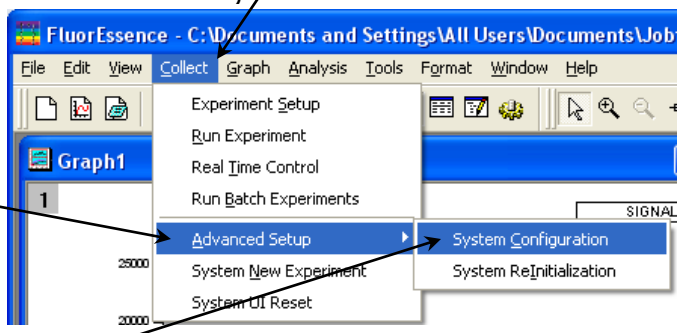
1 In the FluorEssence toolbar, choose Collect.

A drop-down menu appears.

2 Choose Advanced Setup.

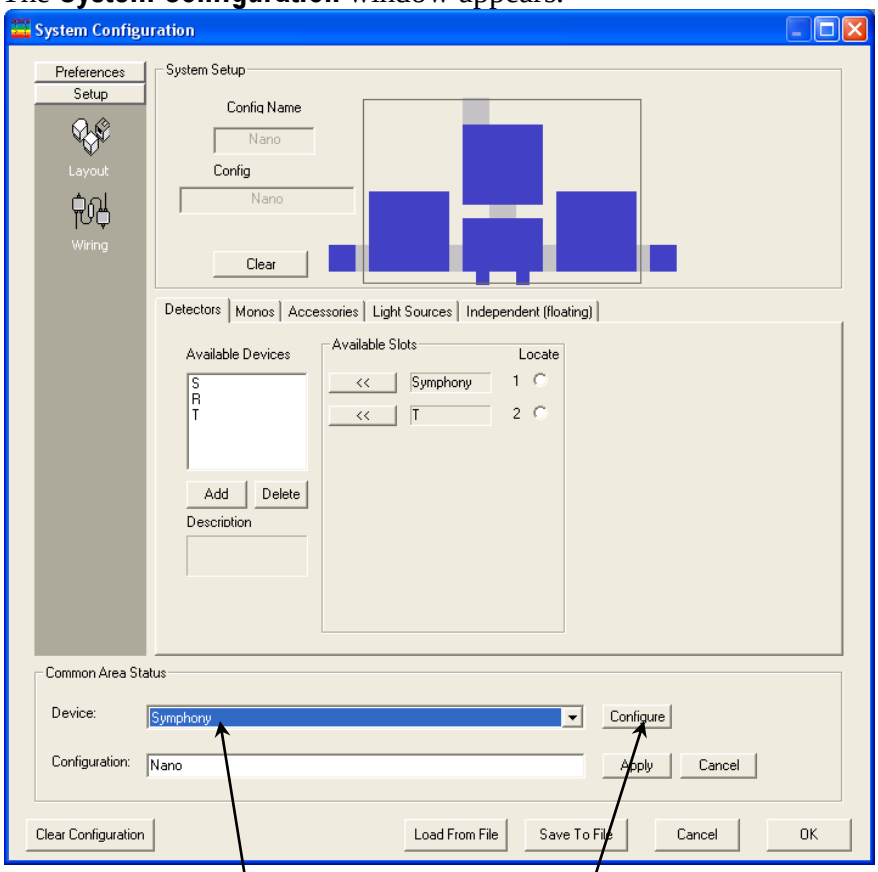
A sub-menu appears.

3 Click System Configuration.



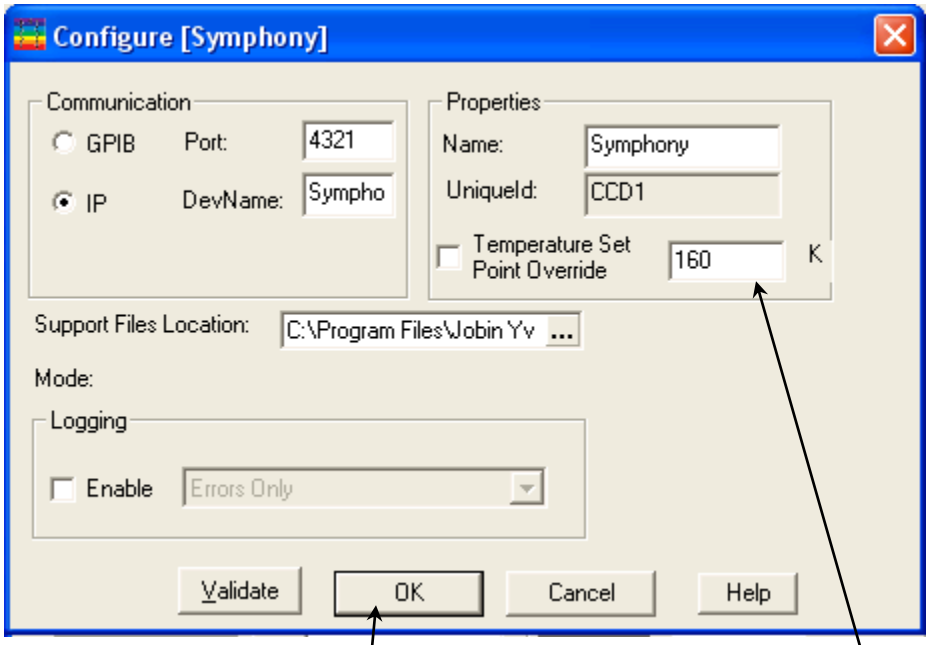
If the system has not been initialized, choose the instrument, and let the system initialize. Then redo steps 1–3.

The **System Configuration** window appears.



4 From the Device drop-down menu, choose Symphony, then click the Configure button.

The **Configure [Symphony]** window appears:



5 Enter the desired temperature for the detector, then click the OK button.

The **Configure [Symphony]** window closes.

6 Click the OK button to confirm and close the **System Configuration** window.

Correcting data with the iHR

Introduction

General comments about data correction are found in *Chapter 5: Optimizing Data*. This section deals with the procedures that are different when using the iHR.

Correction-factor file names for multiple gratings

When the Fluorolog®-3 uses an iHR (which contains multiple gratings) or multiple detectors, multiple correction-factor files are required. Each of these has a unique file name, which needs deciphering. Following are rules to guide the correct choice of correction-factor files.

Note: *Upper- or lowercase characters are not important.*

1 The first two characters show the type of correction-factor file.

`MC` = emission correction-factor file
`XC` = excitation correction-factor file

2 After `MC`, the next several characters determine the appropriate detector:

Note: *Ignore this rule for `XC`… files.*

`2658` = near-IR detector
`928` = R928P photomultiplier tube
`IGA` = InGaAs array
`CD` = CCD array
`DSS` = InGaAs detector

3 The next set of characters gives the grating's groove-density:

`100` = 100 grooves/mm
`15` = 150 grooves/mm
`12` = 1200 grooves/mm
`6` = 600 grooves/mm
`3` = 300 grooves/mm

4 Then may come a separator, `x`.

5 Finally, the last characters show the blaze of the grating:

`300` = 300 nm
`330` = 330 nm
`500` = 500 nm
`750` = 750 nm

etc.

Here are some examples of deciphering the emission correction-factor file:

`Mcorrect.spc`	The default emission correction-factor file for an instrument, with no iHR, with only one emission grating and detector.
`MC9286x300.spc`	An emission correction-factor file for an R928P PMT, with a grating of 600 grooves/mm and a blaze at 300 mm.
`MCCD12x500.spc`	An emission correction-factor file for a CCD array, with a grating of 1200 grooves/mm and a blaze at 500 nm.
`MC26586x300.spc`	An emission correction-factor file for an IR detector, with a grating of 600 grooves/mm and a blaze at 300 nm.
`Xcorrect.spc`	The default excitation correction-factor file for an instrument, with no iHR, with only one excitation grating.
`XC12x330.spc`	An excitation correction-factor file for a grating with 1200 grooves/mm and a blaze at 330 nm.

Caution: *For CCD arrays, these correction-factor files are only valid under the same conditions, i.e., when used at the same central wavelength as performed in the factory. The files may give invalid corrections when used at other central wavelengths on the CCD array.*

To determine the center wavelength for a particular correction-factor file when used with a CCD array, see the Performance Test Report that accompanies the instrument.

Correcting data during acquisition

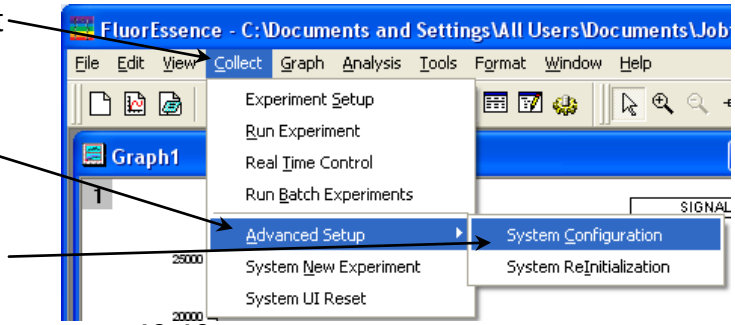
Data can be acquired either as raw data or as corrected data. A spectrum composed of raw data exhibits the effects of system parameters, while a corrected spectrum displays only the properties related to the sample.

Caution: *The iHR's* `mc...`, `xc...`, *and* `tc...` *files are custom-generated at the factory for each particular instrument, and cannot be swapped.*

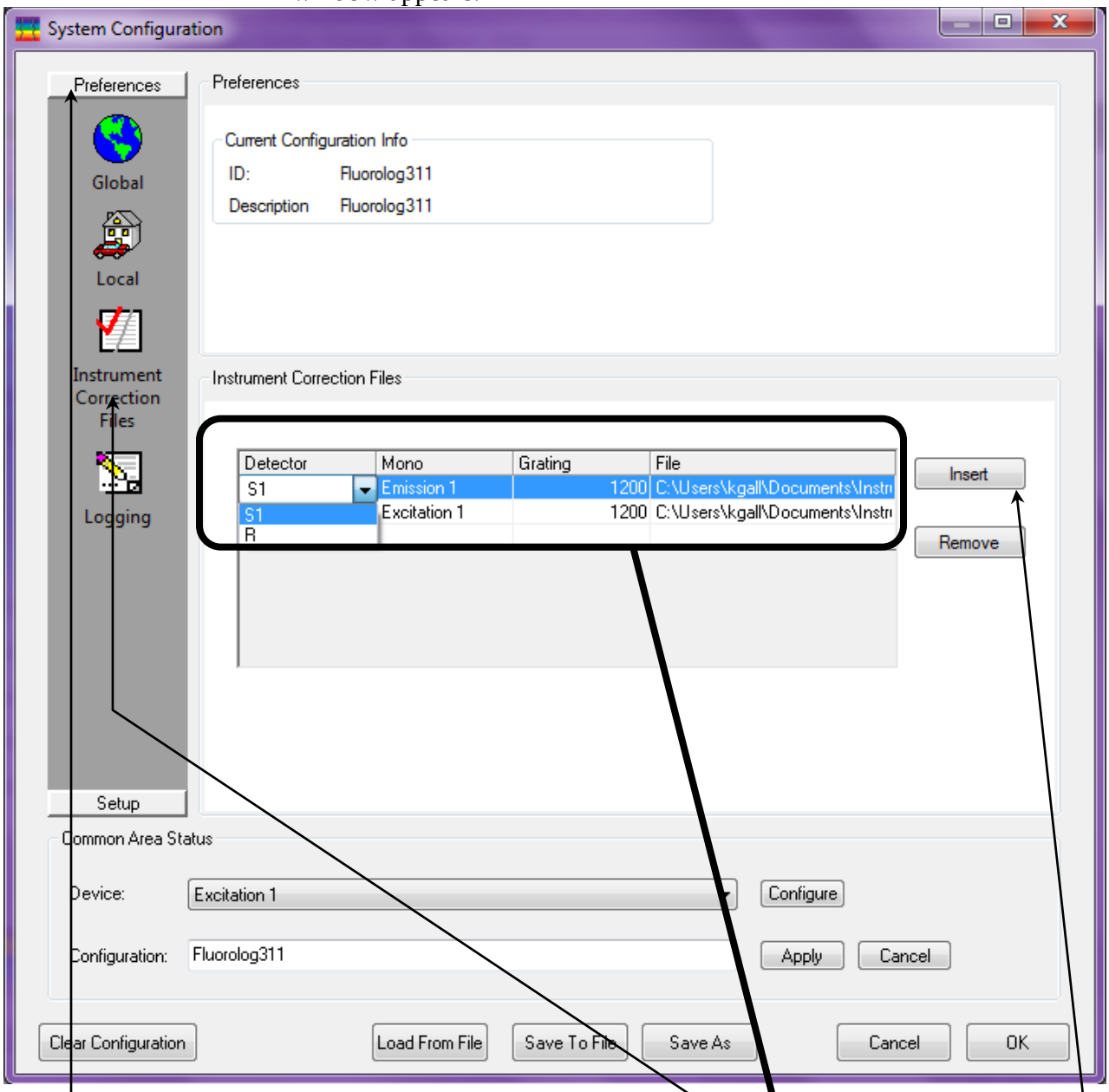
1 Be sure the instrument configuration has a layout that includes a correction file associated with the iHR.

a Choose Collect in the toolbar.

b Choose Advanced Setup, then

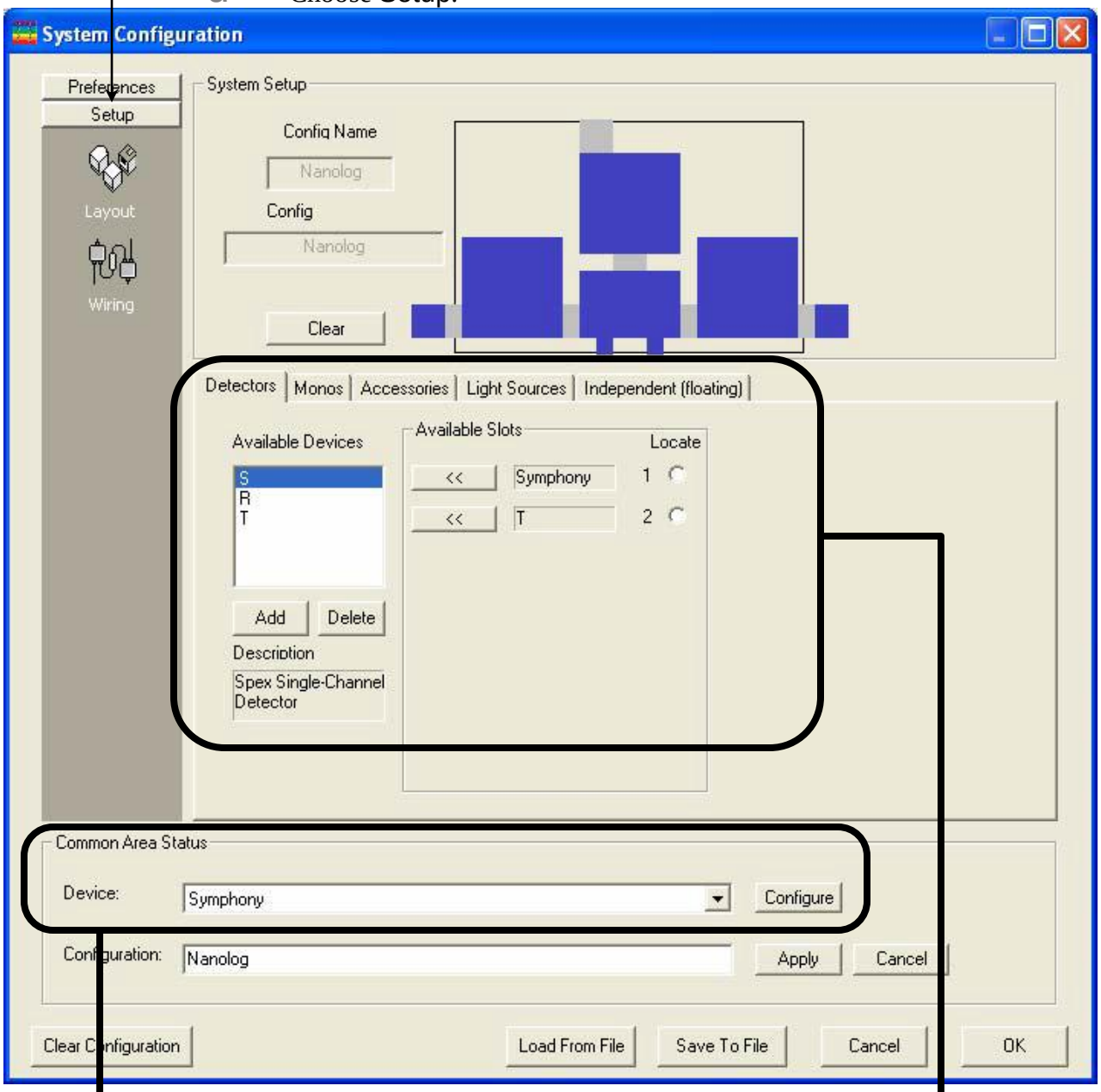


System Configuration.
If the system has not been initialized, choose the instrument, and let the system initialize. Then redo steps 1–3.The **System Configuration** window appears.



C Choose Preferences, then the Instrument Correction Files icon. The Instrument Correction Files area should display a correction file for the Detector (Symphony or other special iHR-related controller and detector).
If there are no active fields in the Instrument Correction Files area, click the Insert button, and browse for the desired correction file (mc… generally, as discussed above).

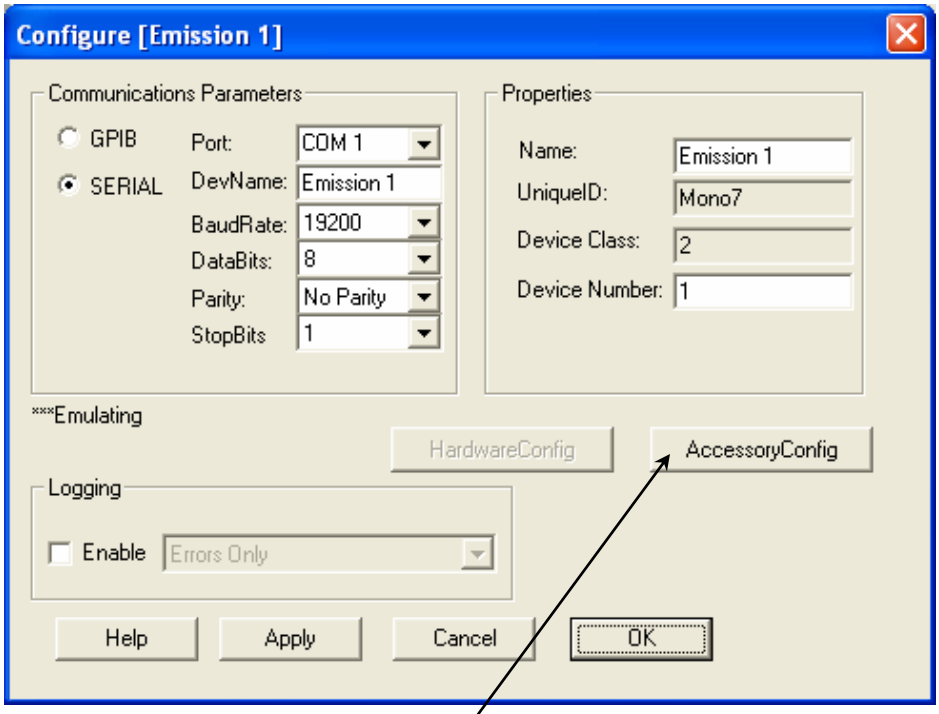
d Choose Setup.



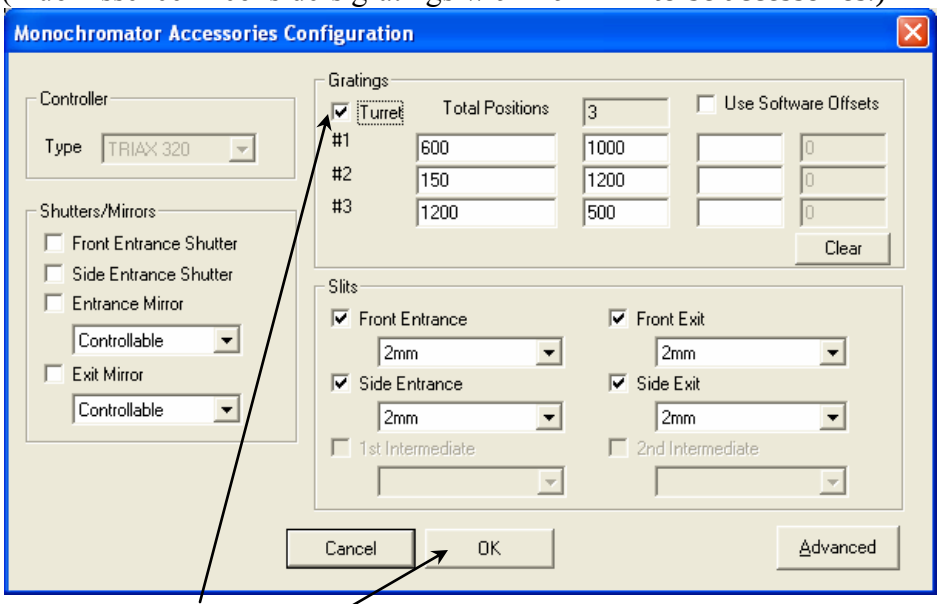
e Choose the Detectors tab, S, from the Available Devices area, and confirm that the Symphony® or other special detector/controller combination is correct in the Available Slots area.

2 Be sure the instrument configuration has a layout that includes an active turret within the iHR.

a In the Common Area Status area, choose Emission 1 (that is, the iHR) from the Device drop-down menu, then click the Configure button.
The **Configure [Emission 1]** window appears:



b Click the AccessoryConfig button.
The **Monochromator Accessories Configuration** window opens.
(FluorEssence™ considers gratings within an iHR to be accessories.)



c Check the Turret checkbox to activate the grating turret in the iHR, then click the OK button.
The **Monochromator Accessories Configuration** window closes.

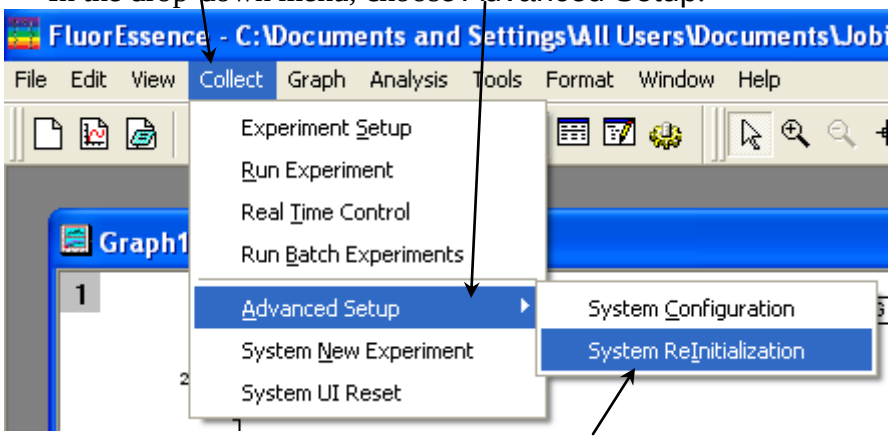
d Click the OK button to close the **Configure [Emission 1]** window.

e Click the OK button to close the **System Configuration** window.
Before the correction-factor files are loaded into the FluorEssence™ software configuration, the software needs to be re-initialized.

f Reinitialize the instrument and software.

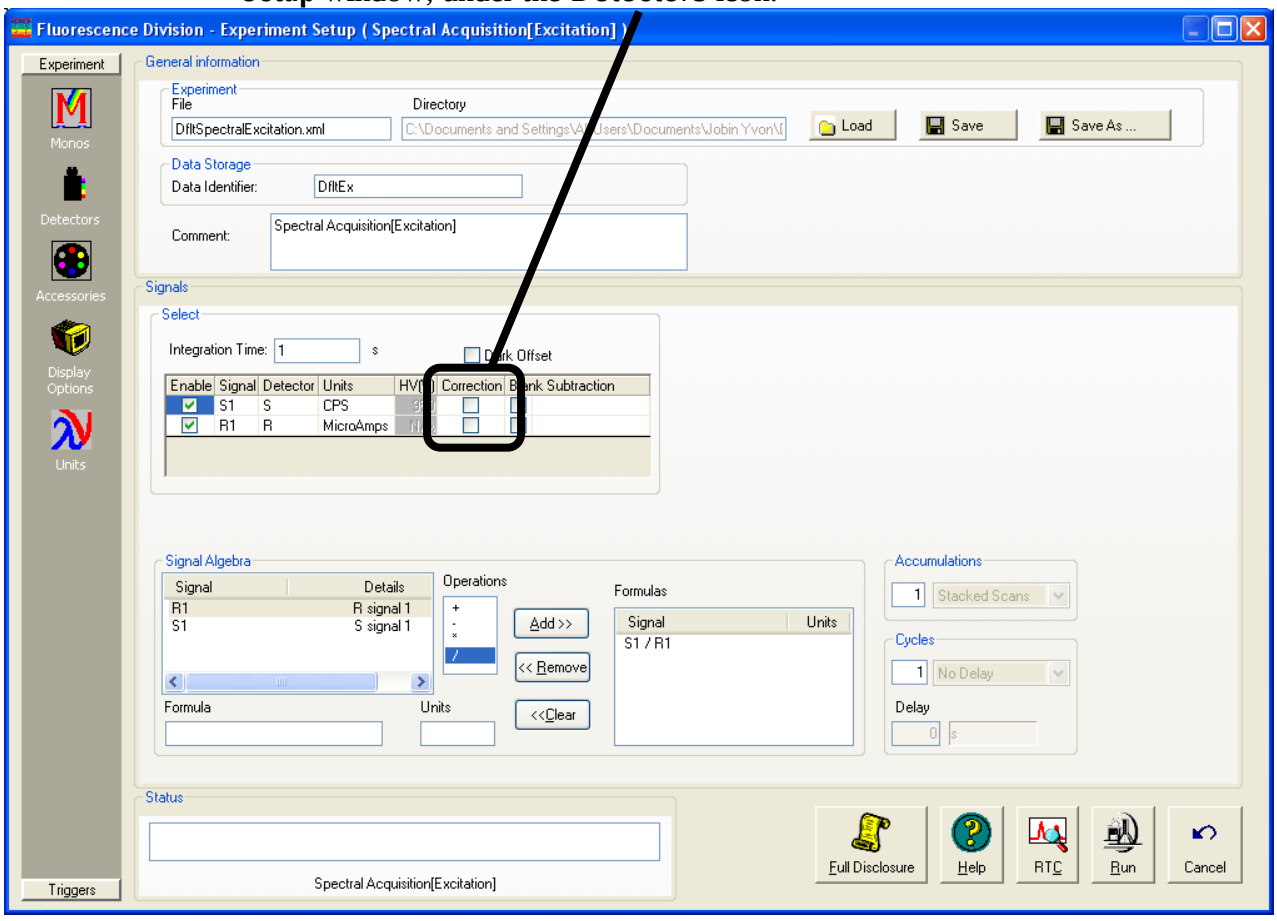
In the FluorEssence™ toolbar, choose Collect.

- In the drop-down menu, choose Advanced Setup.



- In the drop-down menu, choose System ReInitialization.

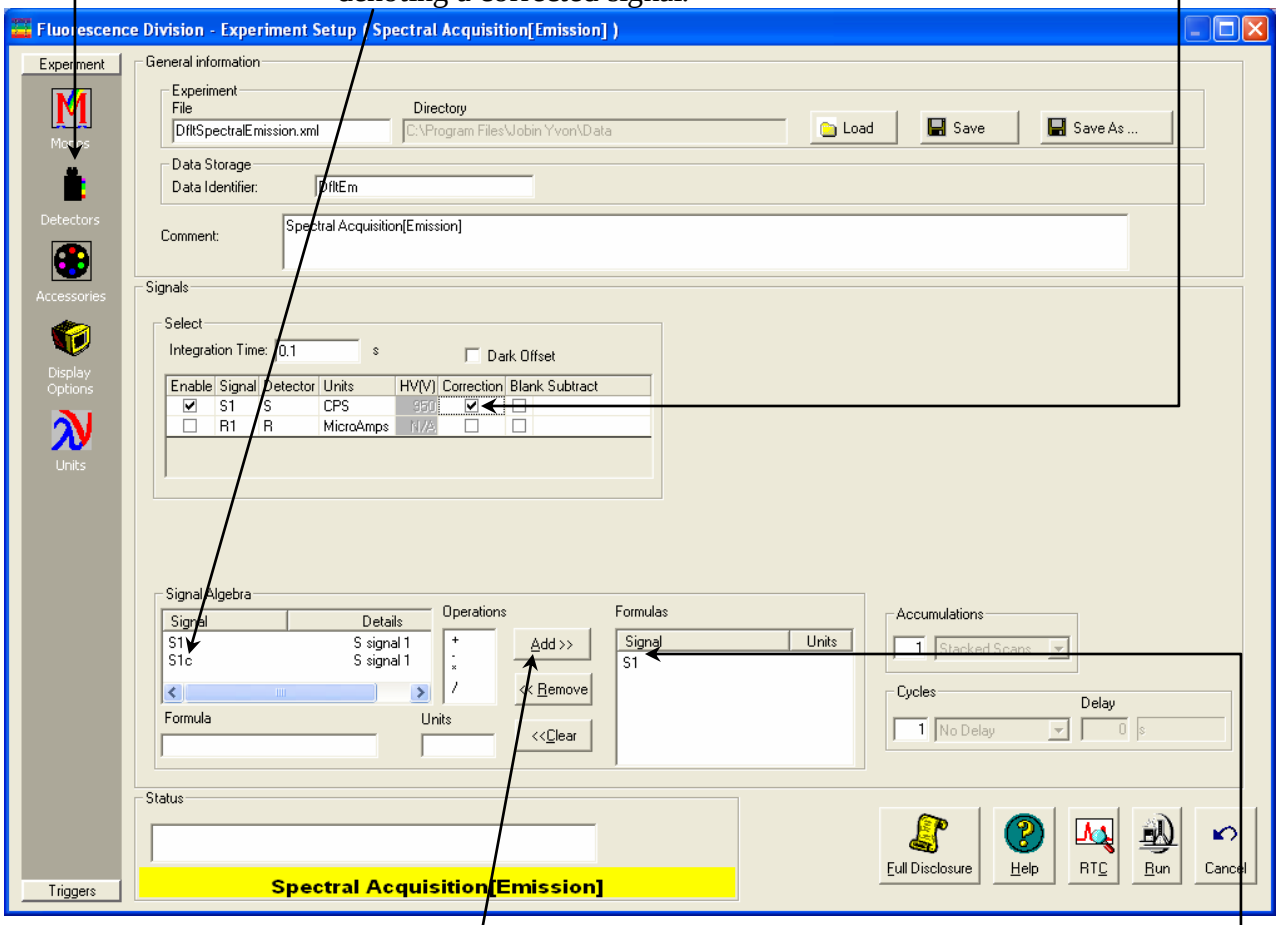
The new correction-factor files are now ready to be activated in the **Experiment Setup** window, under the Detectors icon:



3 Choose correction when setting up the experiment.

a In the **Experiment Setup** window, choose the Detectors icon to display the detectors' parameters.

b Click the Correction checkbox for the detector you want corrected. In the Signal Algebra area, a signal with appended "c" appears, denoting a corrected signal.



c Click the Add >> button to add the corrected signal to the Formulas table.
The corrected signal appears in the Formulas table.

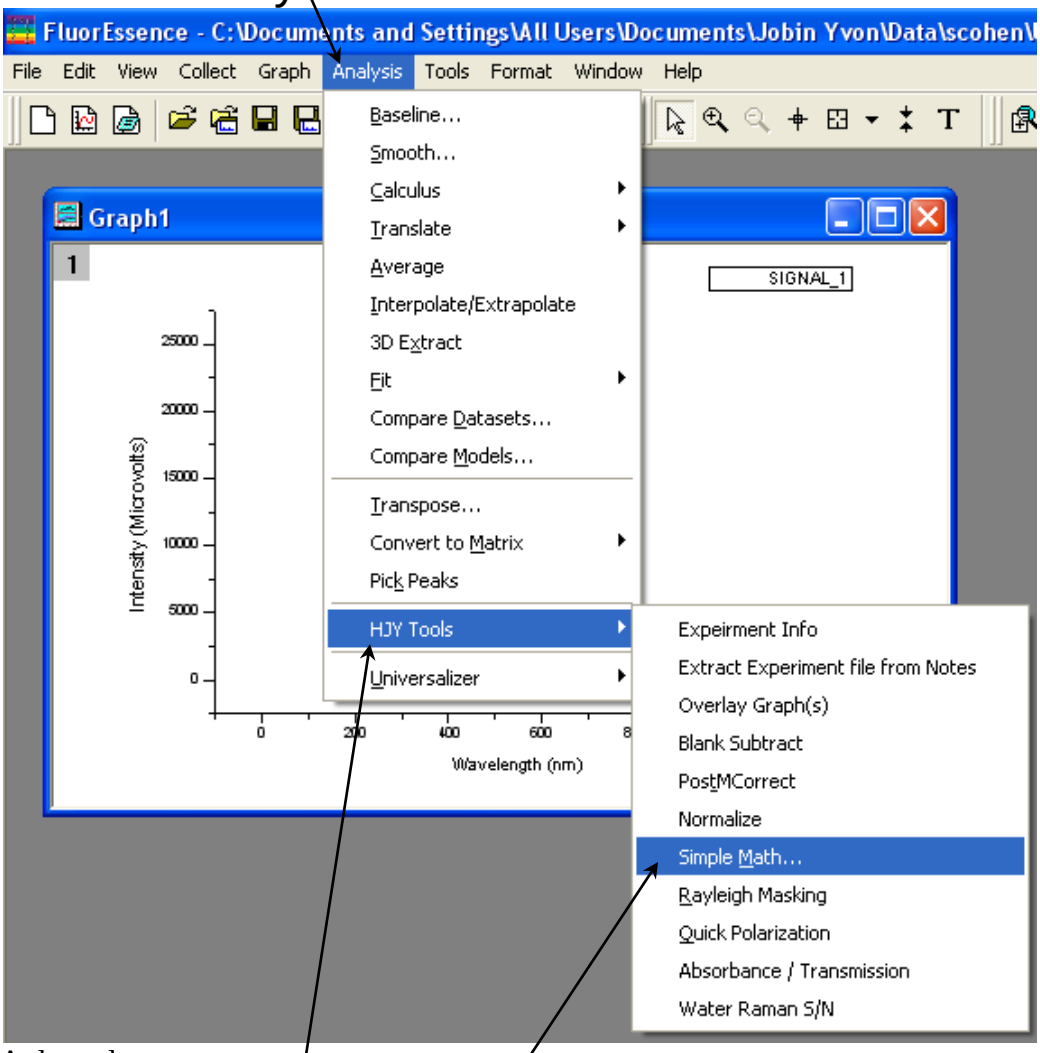
4 Run the experiment with the corrected signal.

Correcting data after acquisition

To apply the correction factors after the data have been acquired, multiply the data file by the appropriate correction factor file (`mc`... for the S detector, `xc`... for the R detector, or `tc`... for the T detector).

1 Make sure the graph is open, and the trace to be corrected is active in the main **FluorEssence** window.

2 Choose Analysis.



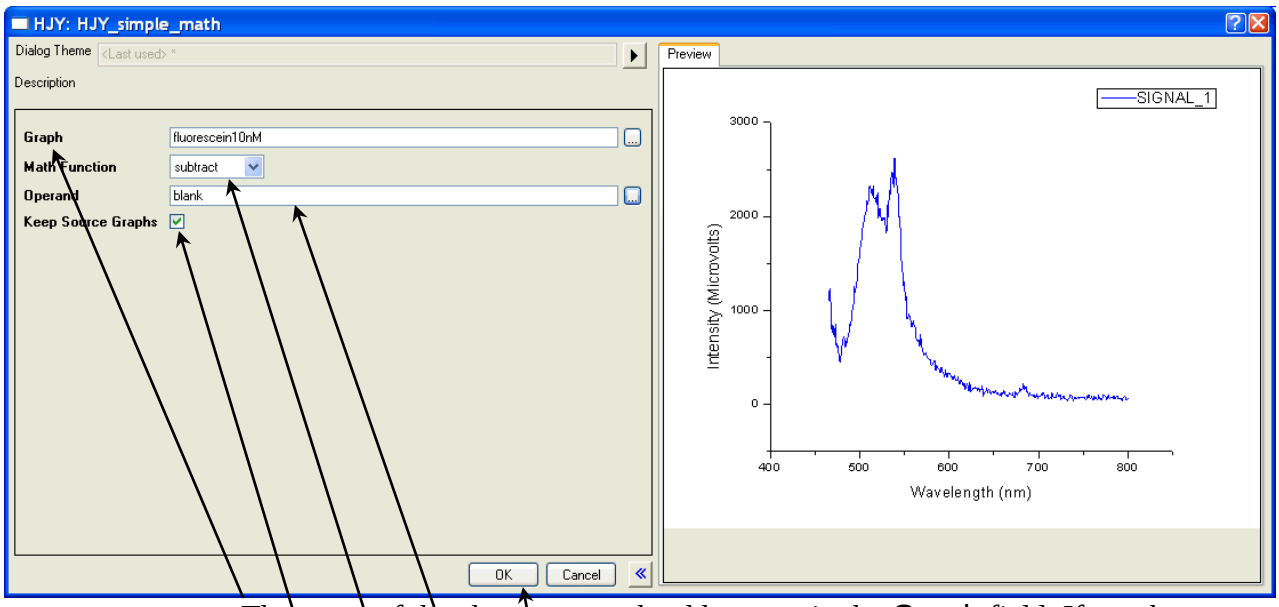
A drop-down menu appears.

3 Select HJY Tools.

Another drop-down menu appears.

4 Select Simple Math....

The **HJY_simple_math window** opens:



The name of the chosen trace should appear in the Graph field. If not, browse for it with the drop-down menu.

5 From Math Function, select multiply from the drop-down menu.

6 Browse for the appropriate correction-factor file in the Operand field (`mcorrect` or `xcorrect`).

You may also enter a numerical constant if you choose.

7 If you want to create a new graph, activate the Keep Source Graphs checkbox.

If the Keep Source Graphs checkbox is inactive, the data in the current graph are overwritten.

8 Click the OK button.

The trace that appears on the screen is a result of the mathematical operation, giving a corrected spectrum.

iHR320 Specifications

Focal length	0.32 m
Entrance-aperture ratio	*f*/4.1
Grating size	68 mm × 68 mm
Image magnification at exit	1.1
Scanning range	0–1500 nm (with 1200-grooves/mm grating)
Multichannel coverage	62 nm over 26.7-mm array width (with 1200-grooves/mm grating)
Focal plane	30 mm wide × 12 mm high
Spectral dispersion	2.31 nm/mm nominal (with 1200-grooves/mm grating)
Spectral resolution	0.06 nm nominal (with 1200-grooves/mm grating), when scanning as a monochromator with 10 µm slit and single-channel detector
Wavelength accuracy	±0.20 nm
Wavelength repeatability	±0.075 nm
Step size	0.002 nm (with 1200-grooves/mm grating)
Dimensions	16.4″ long × 16.6″ wide × 7.56″ high 41.7 cm long × 42.2 cm wide × 19.2 cm high
Weight	45 lbs. (20 kg)

Note: *For more information on the iHR spectrometer, see the System Performance chapter in the iHR Operation Manual.*

Troubleshooting

Should there be a problem with the iHR spectrograph, check the following chart for possible problems. Try the remedies listed on these pages before contacting the Service Department. See the Troubleshooting chapter for information on how to contact the Service Department.

Problem	Possible Cause	Remedy
iHR does not respond to any commands	iHR is switched off.	Check AC outlet (mains), power switch, and fuse.
	Cables are improperly connected.	Check external cable connections.
	Software is inappropriate.	Check that system hardware matches the software
	Problem with USB port	Check USB cable and ports.
	Software is inappropriate.	Check that system software matches the hardware.
iHR responds to some commands, but not all	Improper parameters used with correct command.	Check that the failing command is valid, and that parameters are within correct limits for that function.
	Software is inappropriate.	Exit FluorEssence™. Check that the software matches the hardware, especially device configuration.
iHR wavelength-drive or accessories do not move	Grating may not be working properly.	With the power off, remove the turret cover of the iHR and gently move the turret partially through its range of motion. When the system is powered, the device should return to its home position.
	Unconnected cables	Be sure that all cables are connected.
	Improper software or parameters	Check that the failing command is valid, and that parameters are within correct limits for that function.
Slit and wavelength drives and accessories do not move	Bad electrical connection.	Test turret as follows: 1 Exit FluorEssence™. 2 Disconnect power. 3 Remove top cover of iHR. 4 Gently move turret partway through its range of movement. 5 Reconnect power. 6 Turret should automatically return to home position. 7 Check control-cable connections and interface.

Background signal is too high	Light leak.	Make sure all covers are in place.
	 ***Note:*** *To prevent damage to the knife-edges, the slits never close completely. Therefore some light always enters.*	Be sure that the area between the sample and the entrance slit is enclosed and light-tight. Test by blocking the entrance slit.
		Check detector mounting and housing for light-leaks. Test for stray light: 1 Start at detector, close exit and entrance slits and shutters in order. 2 Be sure all openings and screw holes are plugged. 3 Check that the cover, side, and baseplate fit tightly. 4 In a dark room, shine small flashlight at a suspicious part or joint, and observe detected signal levels.
Signal is too noisy	Signal is too weak.	Try to increase signal strength at the detector.
	Light leak.	Follow directions above for detecting light-leaks.
	Improper grounding.	1 Exit FluorEssence™. 2 Turn off iHR. 3 Rearrange power connections so that spectrograph, source, and detector are connected to the same ground (earth), and if possible, the same power circuits. 4 Add redundant grounds (earths) to various points in the system.
	 ***Note:*** *Ground loops often are difficult to diagnose and cure. Experiment patiently. Keep ground wires short, make tight connections, and avoid coated surfaces.*	Consider a "star" ground (earth) of wires radiating from a single central location, e.g., a grounded metal table surface under the system.
	Electromagnetic interference from lasers or other high-energy apparatus	Construct a faraday cage or shield.
"Warning! Connect Failed (7)" error message appears	CCD-3000 or -3500 controller is off.	Exit FluorEssence™, turn on CCD controller, and restart FluorEssence™.

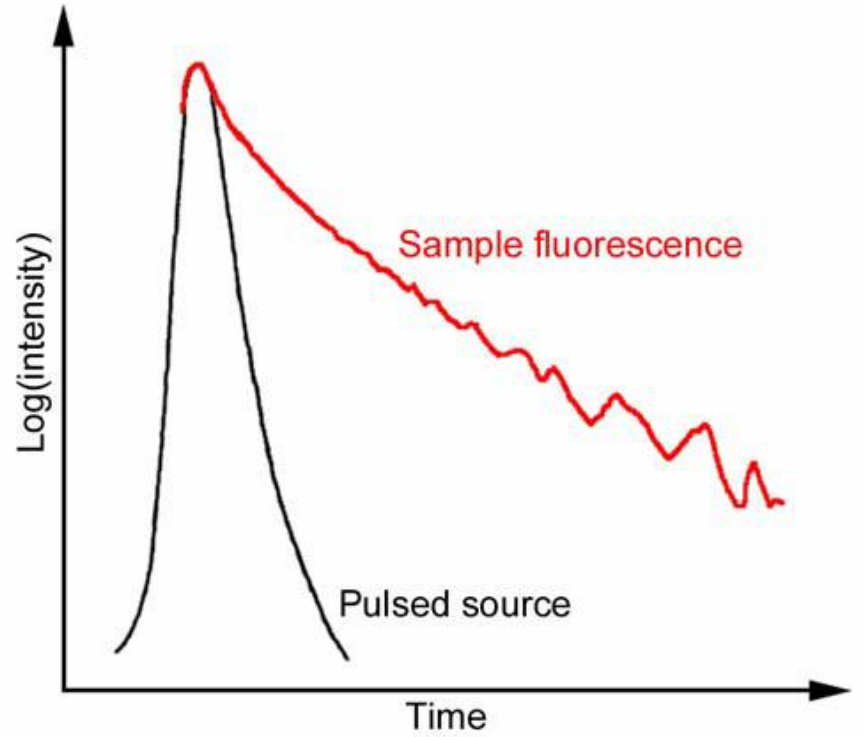
Chapter 14 : Introduction to Lifetime Measurements

Introduction

The standard Fluorolog®-3 spectroscopy system is designed to perform steady-state measurements. HORIBA Scientific offers you a method of upgrading your Fluorolog®-3 system to lifetime capability, the TCSPC on Fluorolog® accessory, which uses the time domain for lifetime detection.

Lifetime measurements

The TCSPC on Fluorolog®'s software is based on acquisition of time-domain lifetime measurements, known as time-correlated single-photon counting, or TCSPC. In this technique, the excitation light is pulsed, ideally with a pulse-width shorter than the luminescent lifetime. The emission from the sample is collected from a series of repeated pulses, with the time of arrival of these photons binned into channels. The fluorescence from the series of excitation pulses builds up a decay curve, from which the lifetimes of all sample components are calculated. Reconvolution techniques handle the finite duration of the excitation pulse. Note that the entire decay curve is examined at once, giving a super-fast experimental method. The following figure shows how the time domain for lifetime measurements works.



Schematic of time-domain lifetime measurements.

Types of lifetime scans

Introduction

The type of scan defines which measurement will be acquired. In lifetime operation, four scan types are available:

- Lifetime
- Lifetime-resolved
- Dynamic depolarization or anisotropy-decay
- Time-resolved.

Recalling an experiment allows the user to retrieve the experimental parameters. Each scan type is defined below.

Lifetime acquisition

The lifetime acquisition type of scan determines accurate lifetimes from simple single-component systems as well as complex heterogeneous systems.

Lifetime-resolved acquisition

A lifetime-resolved acquisition scan resolves up to three components of overlapping spectra based on differences in the fluorescence lifetimes. More complex systems can result in improved resolution of one or more spectra but complete resolution requires additional manipulation of data-acquisition parameters such as excitation wavelength. An application using lifetime-resolved-acquisition scans can spectrally resolve tyrosine and tryptophan emission spectra from a protein containing both residues. To improve resolution, simply measure the lifetime and obtain the spectral characteristics of the individual components prior to conducting a lifetime-resolved acquisition scan. Generally, this technique works best if a factor of at least 1.5 exists between the lifetimes being resolved spectrally.

Anisotropy-decay acquisition

An anisotropy-decay acquisition experiment choice allows the study of rotational properties of fluorescent molecules and probes. As the fluorophore rotates, a change in the polarization occurs. Monitoring this change provides information about the excited state properties of the sample. The anisotropy is affected by Brownian rotation, energy-transfer, re-absorption, re-emission and light scattering. Applications involve studying asymmetric complex molecules, environmental perturbations, binding, hindered-rotation phase-transitions, and internal viscosities of bilayers.

Time-resolved acquisition

Time-resolved acquisition scans measure the change in the spectral characteristics of the sample during the lifetime of the excited state. The measurement consists of determining the frequency response of the sample over a specified emission range. Applications involve solvent relaxation of the excited state and excimer formation.

Chapter 15 : Technical Specifications

Each Fluorolog®-3 system consists of:

- An excitation source
- An excitation monochromator
- A sample compartment with reference detector
- At least one emission monochromator
- At least one emission detector.

Each system is controlled by an IBM-PC-compatible computer, and may include a printer for hard-copy documentation.

The details and specifications for each component of the Fluorolog®-3 series of spectrofluorometers follow.

Spectrofluorometer system

The Fluorolog®-3 spectrofluorometer consists of modules and components controlled by the specialized software. Although the system can be configured in various ways for a variety of applications, the basic (standard) Fluorolog®-3 spectrofluorometer system consists of the following components:

Excitation source

450 W xenon short arc, mounted vertically in an air-cooled housing. Light collection and focusing by an off-axis mirror for maximum efficiency at all wavelengths.

Optional pulsed lamp or laser port interface available.

Monochromators

Single-grating monochromators (standard). Monochromators are *f*/3.6 Czerny-Turner design with kinematic classically-ruled gratings and all-reflective optics. Optional double-grating units are available for highest stray-light rejection and sensitivity.

The following specifications are based on 1200-grooves/mm gratings:
Resolution 0.3 nm
Accuracy ±0.5 nm
Step size 0.0625 nm min. to 100 nm max.
Wavelength repeatability 0.3 nm
Slit settings 0–30 nm continuously adjustable via computer
Speed 150 nm/s
Range 0–950 nm (physical)
Gratings*
Excitation 330-nm blaze (220–600 nm range)
Emission 500-nm blaze (290–850 nm range)
*Other gratings available for wavelengths > 1000 nm.

Sample compartment

T-format sample compartment with excitation reference detector R and signal detector S. The T-format design allows a second emission-detection channel T to be incorporated. The sample compartment also has a removable gap-bed assembly for sampling accessory replacement.

Optional front-face collection assembly available.

Detectors

Reference photodiode for excitation correction from 200–980 nm, selected for stability.

Emission detector is a side-on R928P for high sensitivity in photon-counting mode (180–850 nm). Linearity to 2×10^6 cps; < 1000 dark counts/second

Other PMTs to 1100 nm, with thermoelectrically cooled option.

Solid-state detectors for higher wavelength emissions. CCD multichannel detector for instant emission spectra and sample spatial information.

Time-domain lifetime option	Lifetime range: 200 picoseconds to 0.1 milliseconds Minimum resolution: < 7 picoseconds/channel
High voltage	S or T detectors, ≤ 1200 V for R928P
Excitation shutter (standard)	Computer-controlled
Integration time	1 ms to 160 s
Sensitivity	Double-distilled deionized ICP-grade water Raman scan 4000:1 *S/N* minimum at 397 nm, 5-nm bandpass, 1 s integration time, background noise 1st standard deviation at 450 nm.

Spectrofluorometer dimensions (not including computers or electronics)

3-11	40″ long × 36″ wide × 13.5″ high 102 cm long × 91 cm wide × 34.3 cm high
3-12	40″ long × 44″ wide × 13.5″ high 102 cm long × 112 cm wide × 34.3 cm high
3-21	48″ long × 41″ wide × 13.5″ high 123 cm long × 104 cm wide × 34.3 cm high
3-22	48″ long × 49″ wide × 13.5″ high 123 cm long × 124 cm wide × 34.3 cm high
3-111	43″ long × 54″ wide × 13.5″ high 109 cm long × 137 cm wide × 34.3 cm high
3-222	52″ long × 72″ wide × 13.5″ high 132 cm long × 183 cm wide × 34.3 cm high

Dimensions (sample compartment)	5.5″ long × 7″ wide × 7″ high 14 cm long × 17.8 cm wide × 17.8 cm high
Ambient temperature range	15–30°C
Relative humidity maximum	75%
Power requirements	1500 W 50/60 Hz single-phase

Minimum computer requirements

Microprocessor	Pentium IV or higher recommended
Operating system and environment	Windows® 2000, XP Pro, Windows® 7 (in compatibility mode), or Windows® Vista
Hard disk	1 GB minimum
DVD-ROM drive	Required
Memory	1 GB RAM
Video display	Video resolution of at least 1024 × 768
Other ports	One USB port available One serial port capable of 115 kilobaud for the SpectrAcq One serial port for optional plotter or mouse One available Ethernet Network Interface Card (NIC) connection (no hubs)

Software

FluorEssence™ software for data-acquisition and manipulation through the Windows® environment.

Chapter 16 : Components & Accessories

The Fluorolog®-3 can be configured to obtain optimum results for a variety of applications. The basic system, regardless of configuration, consists of slits, detectors, a xenon lamp with power supply, and a sampling module. These items can be combined in different ways to provide maximum benefits.

The following list represents all the accessories and components (including those mentioned above), in alphabetical order, available for the Fluorolog®-3 spectrofluorometers. A brief description of each is included in the following sections. Like the list presented below, the descriptions that follow are alphabetized, except where logical order dictates otherwise.

For additional information or product literature on any of these items, contact your local HORIBA Scientific Sales Representative.

Itemized list

FL-1013 Liquid Nitrogen Dewar Assembly

Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of cryogenic materials such as liquid nitrogen.*

For phosphorescence or delayed fluorescence measurements, samples are often frozen at liquid-nitrogen temperature (77 K) to preserve the fragile triplet state. The sample is placed in the quartz cell and slowly immersed in the liquid-nitrogen-filled Dewar flask. The white Teflon® cone in the bottom of the Dewar flask keeps the quartz sample-tube centered in the Dewar flask. The Teflon® cover on the top of the Dewar flask holds any excess liquid nitrogen that bubbled out of the assembly. A pedestal holds the Dewar flask in the sampling module. A special stove-pipe sample cover replaces the standard sample lid, so that liquid nitrogen can be added to the Dewar flask as needed. The Dewar flask holds liquid nitrogen for at least 30 min with minimal outside condensation and bubbling.

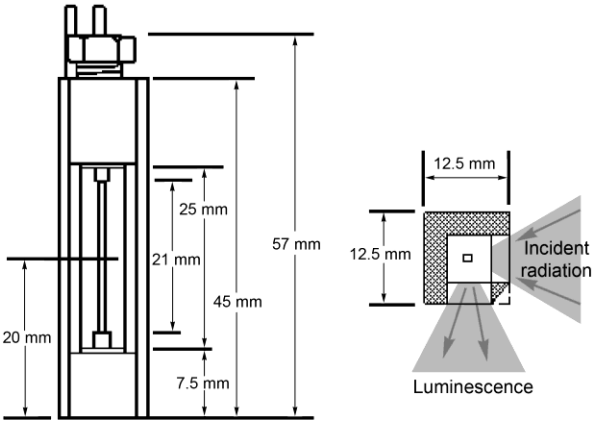
Included in the FL-1013 Liquid Nitrogen Dewar Assembly, the Dewar flask can be purchased as a spare. The bottom portion, which sits directly in the light path, is constructed of fused silica.

Note: *If condensation appears on the outside of the Dewar flask, it must be re-evacuated.*

Sample Cells

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

J1955 HPLC Flow Cell



With a sample capacity of 20 µL, this non-fluorescing fused silica cell is ideal for on-line monitoring of fluorescent samples. The cell maintains high sensitivity because it has a large aperture for collecting the excitation light to the sample and fluorescence emission from the sample. The flat sides allow maximum throughput while keeping the scattering of the incident radiation to a minimum. The cell fits in a standard cell holder.

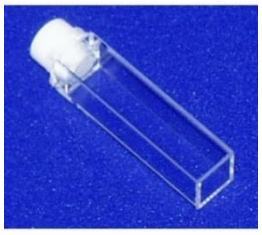
F-3011 Micro Cell Adapter

This is the adapter to hold the F-3012 Micro Cell in the Sample Holder.

F-3012 Micro Cell

This non-fluorescing fused silica cylindrical cell holds 250 µL. A magnetic stirrer cannot be used with this cell.

1925 Quartz Cuvette



With a 4-mL volume, this cell measures 10 mm × 10 mm in cross-section, and comes with a Teflon® stopper to contain volatile liquids.

1920 Sample Cell

This 2-mL to 4-mL non-fluorescing fused silica cell, capable of accepting a magnetic stirrer, has a 10-mm path length and includes a white Teflon® cap that prevents sample evaporation.

J650518 Reduced-Volume Sample Cell

This non-fluorescing fused silica cell is selected for samples with a maximum volume of 500 µL. The square cross-section of the sample cavity is 5 mm. The precise imaging capability of the excitation light focused onto the sample allows for high sensitivity. The adapter (J650519) and a “flea” magnetic stirrer are included.

Microsense microliter sample cell

To measure fluorescence of ultra-small samples with volumes less than 10 µL, HORIBA Scientific offers the Microsense. This microliter sample cell allows you to add your sample via a pipette, without dilution for easy recovery. The Microsense provides accurate, repeatable fluorescence measurements.

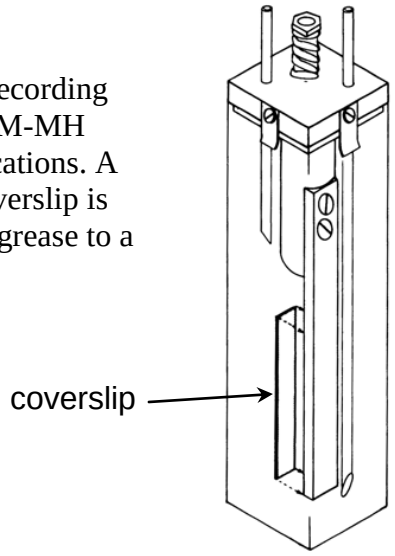
F-3026 Correction Factor Kit

The F-3026 Correction Factor Kit is an NIST-traceable accessory for the FluoroMax® and Fluorolog® spectrofluorometers for calibrating the relative spectral response of the spectrofluorometer, and includes a Spectralon® integrating sphere as a sampling optic. Each Correction Factor Kit includes a regulated 12 VDC power supply, a calibration certificate, and a CD with data files for use with FluorEssence™ software. The Correction Factor Kit provides 50 hours of operation before recalibration is necessary.

Caution: *Tungsten-halogen lamps can be dangerous. They pose a risk of explosion, and are a source of potentially hazardous bright light.*

CM-MH Monolayer coverslip

The CM-MH system is designed to assist researchers in recording cation measurements of live cells grown on the special CM-MH monolayer coverslip, or other multiple-wavelength applications. A diagram of the CM-MH accessory is shown here. The coverslip is approximately 5 mm × 15 mm, and affixed with silicone grease to a polished stainless-steel jig within the CM-MH.



F-3023 Janis cryostat

Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of cryogenic materials such as liquid nitrogen.*

The F-3023 Janis cryostat is a liquid-nitrogen variable-temperature cryostat with the sample located in flowing vapor. Ideal for experiments with samples that are difficult to thermally anchor, e.g., liquids or powders, the F-3023 features a top-loading sample chamber for rapid sample-exchange, and four-way f = 1.0 optical access to the sample chamber. Among the cryostats features are:

- Quick refill with included funnel assembly.
- High-quality bellows-sealed evacuation valve and a built-in cryopump for maintaining high vacuum.
- Safety pressure-relief valves protecting all independent spaces.
- Variable cooling system which places the sample in flowing N_2 vapor (ideal for low thermal conduction samples), providing excellent cooling power and temperature control.
- Light-weight sample positioner/mount assembly, with multi-pin electrical-feed-through access to the window region, offering rotation and translation around the cryostat's axis.
- Two silicon diodes installed on the vaporizer (heat exchanger) and sample mount, for controlling and monitoring the system temperature.

CCD Detectors

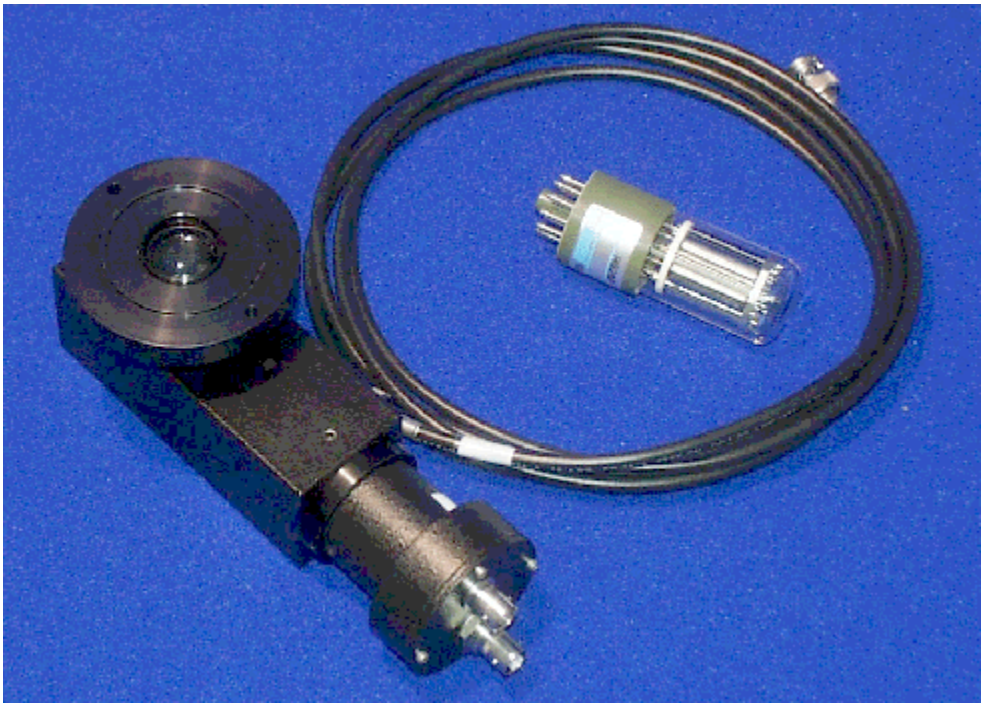
For multichannel spectral acquisition, many charge-coupled devices are available to suit the researcher's needs. Both air-cooled and liquid-nitrogen-cooled CCDs can be inserted into the Fluorolog®-3. Available choices include extended-UV and near-IR detection, various pixel sizes and arrays, and maximum-coverage options. Contact a local Sales Representative for details and specific model number.

Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of cryogenic materials such as liquid nitrogen.*

Symphony® II array for detection in the near-IR

FL-1073 Room-Temperature Signal Detector

The Fluorolog®-3 includes a room temperature R928P emission signal detector. This detector is mounted to the emission monochromator and operated in photon-counting mode. The dark count is specified at <1000 counts/second, and the R928P delivers useful output from 190 nm to 860 nm.



FL-1048 Thermoelectrically Cooled Signal Detector

As a rule, cooling a detector improves the *S*/*N* by reducing the inherent dark counts or noise. For the standard signal detector, FL-1048 (described above), cooling reduces the dark counts from 1000 cps to 20 cps; the useful wavelength band (190–860 nm) remains the same.

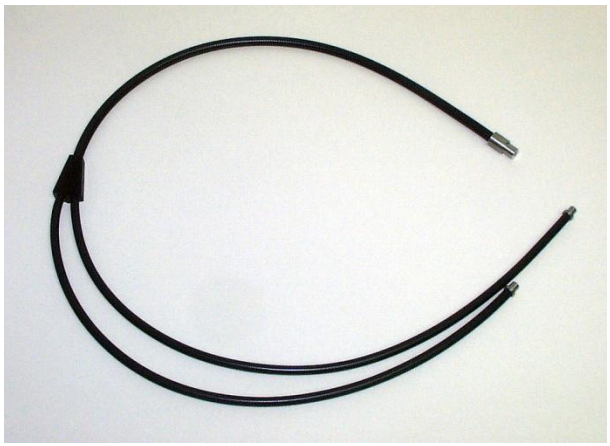
The Thermoelectrically Cooled Signal Detector consists of the housing, power supply, Model 1630 Field Lens Adapter, a black flange, and a silver adapter plate. To cool, a water line and drain are attached to the tubing extending from the housing, and room-temperature water is circulated through the housing.

FL-1030 Thermoelectrically Cooled Near-IR Photomultiplier Tube

For spectral measurements extending into the near-infrared, the FL-1030 Thermoelectrically Cooled PMT is perfect. Included in the FL-1030 is the thermoelectrically cooled housing. The InGaAs detector has a spectral range from 250 nm all the way to 1050 nm. Required for this item is the DM302 Photon Counting Module.

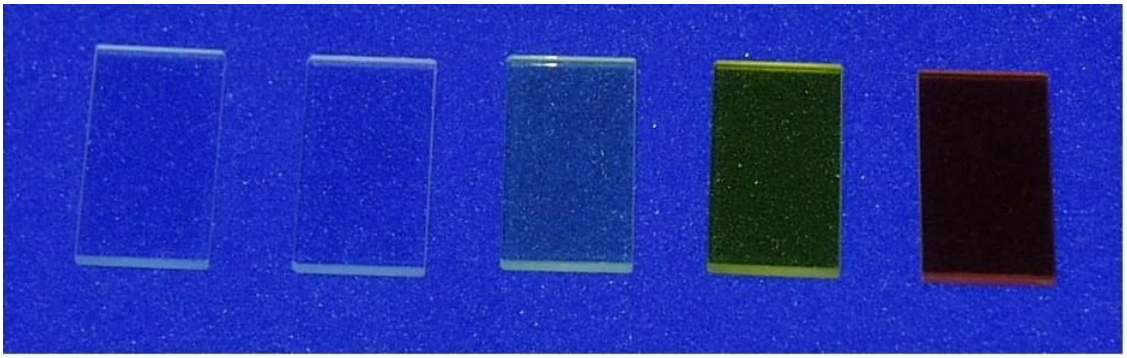
FL-3000 Fiber-Optic Mount and 1950 Fiber-Optic Bundles

Now you can study marine environments, skin and hair, or other large samples in situ! For those users who want to examine samples unable to be inserted into the sample compartment, the FL-3000 Fiber-Optic Mount (plus fiber-optic bundles) allows remote sensing of fluorescence. The FL-3000 couples to the sample compartment; light is focused from the excitation spectrometer onto the fiber-optic bundle, and then directed to the sample. Fluorescence emission from the sample is directed back through the bundle and into the front-face collection port in the sample compartment. Randomized fiber-optic bundles (model 1950) ranging in length from 1 meter to 5 meters are available. Contact the local Sales Representative for details.



Caution: *Intense ultraviolet, visible, or infrared light may be present when the sample compartment is open. Do not aim fiber-optic bundles onto the skin or eyes. Use extreme caution with the fiber-optic probes.*

J1938 and J1939 Cut-On UV-Visible Filters

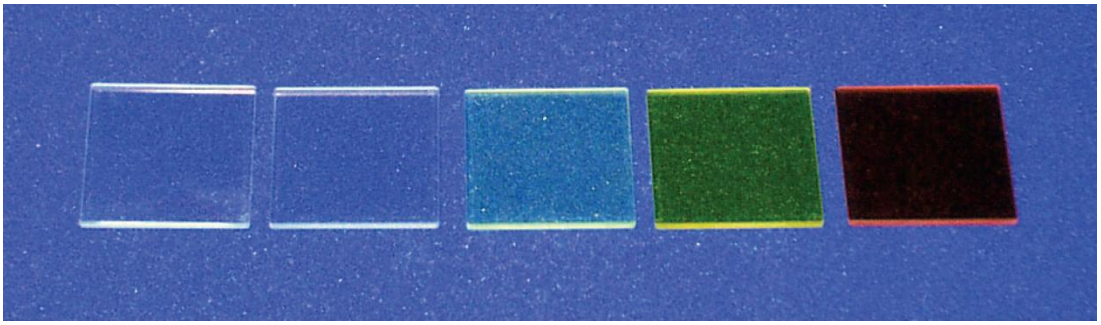


The J1938 Cut-On Filter Set consists of 5 filters with dimensions of 1″ × 2″ (2.5 cm × 5 cm). To properly position the filter, the FL-1010 Filter Holder is required.

Cut-on filters are used to eliminate second-order effects of the gratings. For example, if sample excitation is at 300 nm, a second-order peak occurs at 600 nm. If the emission spectrum extends from 400 nm to 650 nm, a sharp spike occurs at 600 nm. This peak is the second-order peak of the excitation monochromator. To remove this unwanted peak in the emission spectrum, place a 350-nm filter in the emission slot. Cut-on filters typically are used for phosphorescence measurements, where second-order effects are likely to be found.

The sample compartment has three slots that can hold the FL-1010 filter holder. One slot is in the excitation light path and the other two are the emission light-path positions. To eliminate second-order effects from an excitation spectrum, install the filter-holder and the appropriate cut-on filter in the excitation light path.

The J1939 Cut-On Filter set consists of five 2″ × 2″ (5 cm × 5 cm) filters with cut-on wavelengths of 350 nm, 399 nm, 450 nm, 500 nm, and 550 nm. To properly position the filter, the FL-1010 Filter Holder is required.

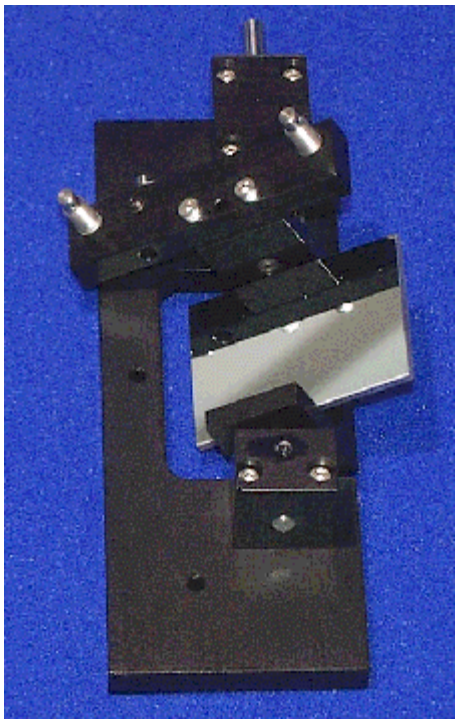


FL-1010 Cut-On Filter Holder

Cut-on filters are used to eliminate second-order effects of the gratings. The sample compartment has three slots that can hold the FL-1010 Filter Holder. Refer to J1938 or J1939 Cut-On Filters for a detailed description of the placement of the filter holder and the interaction of the cut-on filters and the holder.

FL-1001 Front-Face Viewing Option

Designed to examine fluorescence from the surface of solid samples, the FL-1001 Front-Face Viewing Option includes a swing-away mirror. This allows the researcher to change from front-face and right-angle data collection instantly. In the front-face collection mode, the viewing angle is 22.5°. The FL-1001 is ideal for such samples as pellets, powders, inks, monolayers, dyes, turbid materials, highly concentrated solutions, and various solids.



Gratings

Many gratings are available to replace the standard model in the Fluorolog®-3. Rulings with the following specifications are available:

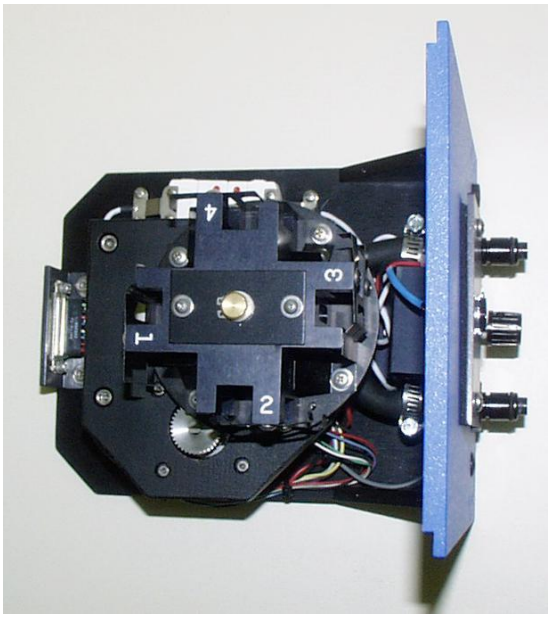
- 300 grooves/mm
- 600 grooves/mm
- 1200 grooves/mm

In addition, a number of different blazes can be purchased:

- 250 nm
- 330 nm
- 500 nm
- 750 nm
- 1000 nm

All gratings are classically ruled, and measure 50 mm × 50 mm. For details and model numbers, contact a HORIBA Scientific Sales Representative.

FL-1011 Four-Position Thermostatted Cell Holder



Introduction

The FL-1011 Four-Position Thermostatted Cell Holder keeps a sample at a constant temperature from –20°C to +80°C. The temperature is maintained by an ethylene-glycol–water mixture pumped through from an external circulating temperature bath (not included). The holder also includes a magnetic stirrer, for mixing turbid or viscous samples.

Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of an ethylene-glycol–water mixture.*

Installation

1. Remove the compartment gap-bed.
2. Position the FL-1011 gap-bed drawer.
3. Tighten with four screws.
4. Attach the ¼″ tubing to the brass inlets on the bottom of the holder.

Caution: *Failure to clamp these hoses securely may result in flooding and damage to the optics and electronics of the instrument.*

Use

1. Place the sample in a 10 mm × 10 mm cuvette and insert a magnetic stirring bar.
   The stirring bar is available from Bel-Art Products, Pequannock, NJ
2. Place a cuvette in each holder.
3. Allow the samples to reach the desired temperature.

Note: *While the four-position model maintains the temperature of all four samples, only one sample is mixed at a time.*

4. Turn on the magnetic stirrer.
5. Select the appropriate mixing speed.
   The speed at which the sample should be mixed depends on the viscosity of the sample.

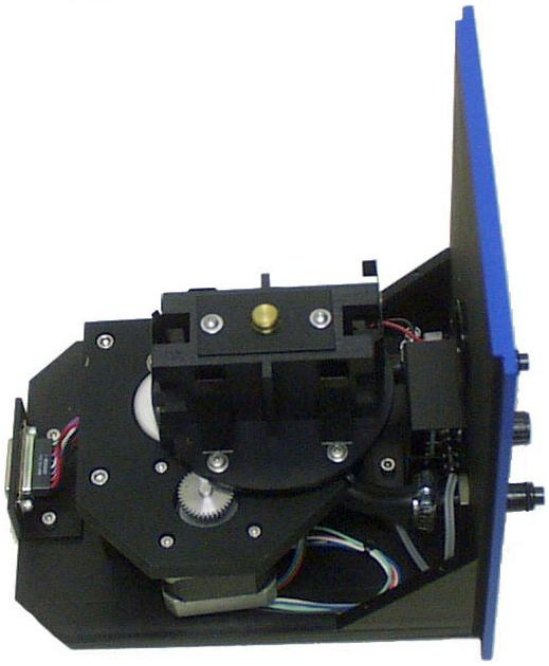
Note: *Selecting too high a speed may create a vortex, which could affect the reproducibility of the measurement.*

6. Run your experiment as usual.
   Either right-angle or front-face detection can be used.
7. Place the next cuvette in the sample position by lifting up the knob and rotating the holder.
   Be sure to press down, to lock the cuvette into the proper position.

FL-1012 Dual-Position Thermostatted Cell Holder

Introduction

The FL-1012 Dual-Position Thermostatted Cell Holder keeps a sample at a constant temperature from –20°C to +80°C. The temperature is maintained by an ethylene-glycol–water mixture pumped through from an external circulating temperature bath (not included). The holder also includes a magnetic stirrer, for mixing turbid or viscous samples.



Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of an ethylene-glycol–water mixture.*

Installation

1. Remove the compartment gap-bed.
2. Position the FL-1012 gap-bed drawer.
3. Tighten with four screws.
4. Attach the ¼″ tubing to the brass inlets on the bottom of the holder.

Caution: *Failure to clamp these hoses securely may result in flooding and damage to the optics and electronics of the instrument.*

Use

1. Place the sample in a 10 mm × 10 mm cuvette and insert a magnetic stirring bar.
   The stirring bar is available from Bel-Art Products, Pequannock, NJ
2. Place a cuvette in each holder.
3. Allow the samples to reach the desired temperature.

Note: *While the two-position model maintains the temperature of both samples, only one sample is mixed at a time.*

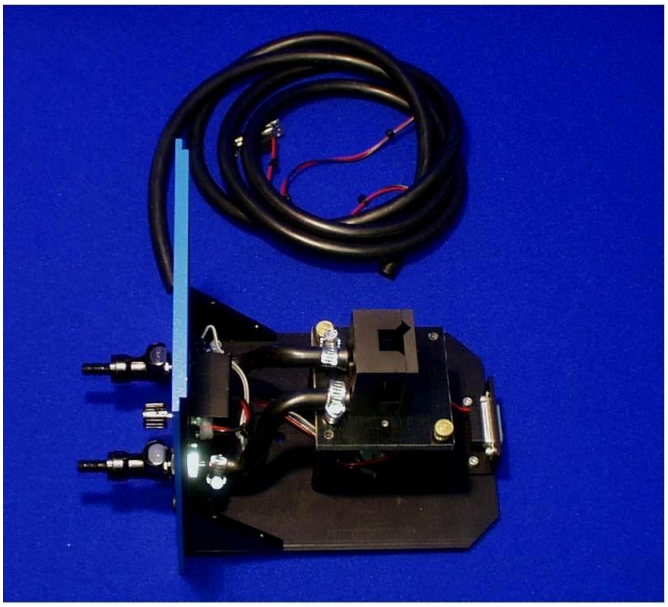
4. Turn on the magnetic stirrer.
5. Select the appropriate mixing speed.
   The speed at which the sample should be mixed depends on the viscosity of the sample.

Note: *Selecting too high a speed may create a vortex, which could affect the reproducibility of the measurement.*

6. Run your experiment as usual.
   Either right-angle or front-face detection can be used.

FL-1027 Single-Position Thermostatted Cell Holder

Introduction



The FL-1027 Single-Position Thermostatted Cell Holder keeps a sample at a constant temperature from –20°C to +80°C. The temperature is maintained by an ethylene-glycol–water mixture pumped through from an external circulating temperature bath (not included). The holder also includes a magnetic stirrer, for mixing turbid or viscous samples.

Caution: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of an ethylene-glycol–water mixture.*

Installation

1. Remove the existing holder from the posts.
2. Replace with the FL-1027.
3. Tighten the two thumbscrews.
4. Attach the ¼″ tubing to the brass inlets on the bottom of the holder.

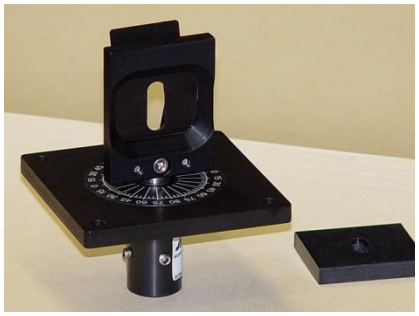
Caution: *Failure to clamp these hoses securely may result in flooding and damage to the optics and electronics of the instrument.*

Use

1. Place the sample in a 10 mm × 10 mm cuvette and insert a magnetic stirring bar.
   The stirring bar is available from Bel-Art Products, Pequannock, NJ
2. Place a cuvette in the holder.
3. Allow the sample to reach the desired temperature.
4. Turn on the magnetic stirrer.
5. Select the appropriate mixing speed.
   The speed at which the sample should be mixed depends on the viscosity of the sample.
6. Run your experiment as usual.

Note: *Selecting too high a speed may create a vortex, which could affect the reproducibility of the measurement.*

J1933 Solid Sample Holder



Introduction

The J1933 Solid Sample Holder is designed for samples such as thin films, powders, pellets, microscope slides, and fibers. The holder consists of a base with graduated dial, upon which a bracket, a spring clip, and a sample block rest.

Installation

1. Remove the present holder.
2. Position the base on the posts.
3. Tighten the two thumbscrews.

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

Use with pellets, crystals, creams, gels, powders, and similar materials:

1. Fill the well of the block.
2. Place a quartz coverslip or Teflon® film over the well.
   This holds the sample in place when vertically positioned.
3. Carefully insert the block between the bracket and spring clip, so that the sample is perpendicular to the excitation light.

Note: *When the sample is perpendicular the light is collected at an angle of 22.5°. This orientation minimizes stray and reflected light off the surface of the sample.*

Use with samples such as thin films, microscope slides, fibers, or other materials:

1. Place the material on the block on the side opposite that of the well.
2. Insert the block between the bracket and spring clip.

   The sample should be perpendicular to the excitation light and fluorescence collected using front-face detection.
3. Select front-face detection by turning the knob on the top panel of the sample compartment to FF (front-face).

Note: *In the sample compartment, the 1692M Selection Mirror is an option. If front-face measurements are to be acquired, this mirror must be installed in the sample compartment.*

Note: *Before scanning a solid sample, HORIBA Scientific recommends running a water-Raman scan in a cuvette with front-face detection. This ensures accurate alignment of the 1692M Selection Mirror.*

FL-1039A Xenon Lamp Housing

The FL-1039A is the standard lamp housing for the 450-W xenon lamp. The power supply is included internally.

FL-1039A Lamp Housing.

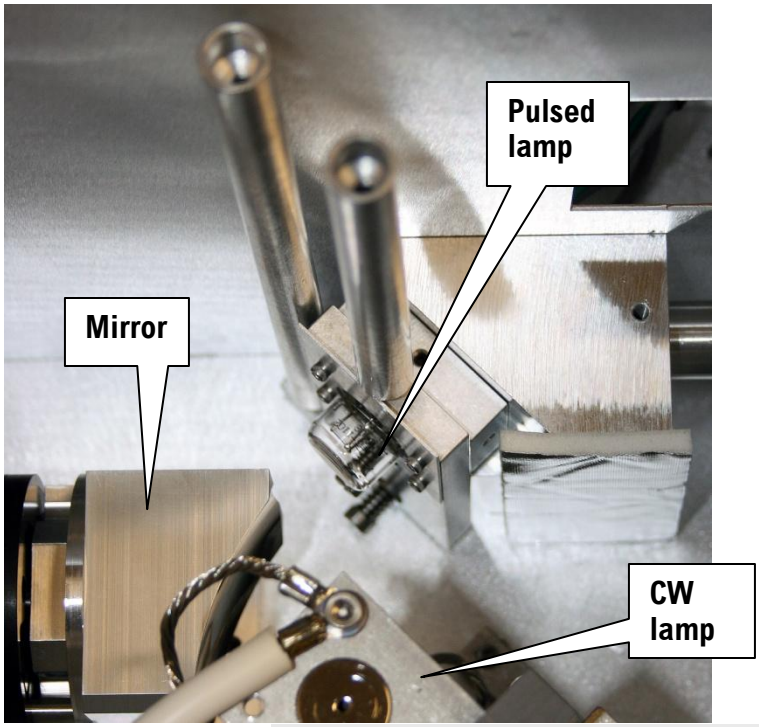
Caution: *This lamp emits intense light and heat, and contains xenon gas under pressure. Understand all safety precautions before handling or using this xenon-arc lamp.*

FL-1040A Dual Lamp Housing

The FL-1040A Dual Lamp Housing contains both the standard continuous 450-W xenon lamp and a UV xenon flash tube. A swing mirror selects which of the two sources excites the sample. This housing with pulsed lamp is useful for studies of phosphorescence lifetimes and decay analysis. The power supply is internal to the housing.

The phosphorimeter adds a programmable, pulsed excitation source and selectable signal gating from the signal photomultiplier tube. This provides time-discrimination capability to sort out the lifetimes of simultaneous, competing luminescence emissions. Because the duration of each exciting pulse from the phosphorimeter is very short (~3 µs), lamp interference during acquisition of decay curves is minimized. This allows the researcher to follow the decay of samples an order-of-magnitude faster than can be achieved with conventional systems that depend on mechanical choppers.

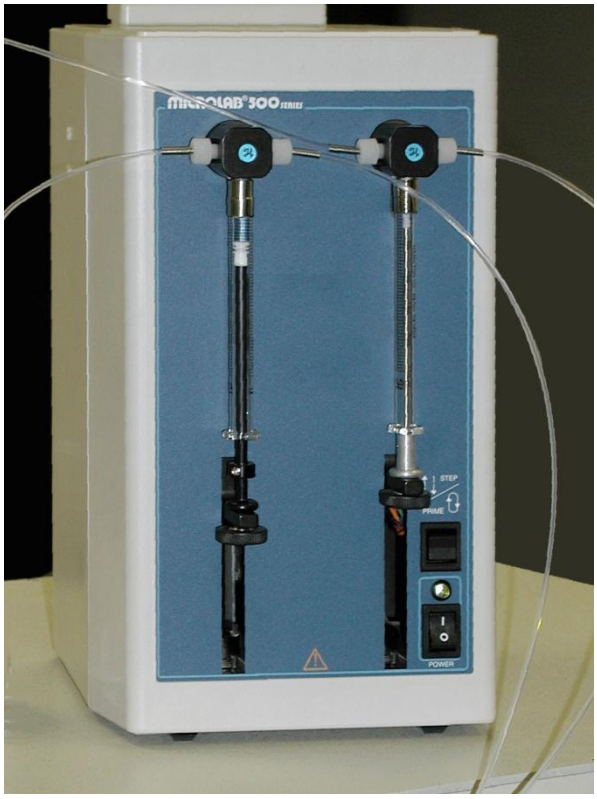
To run phosphorescence experiments, the FL-1040A dual-lamp housing is required. To use the phosphorimeter, direct the FluorEssence™ software to run a phosphorescence experiment. This action rotates a mirror inside, directing light from the flash tube to the sample:



Caution: *This lamp emits intense light and heat, and contains xenon gas under pressure. Understand all safety precautions before handling or using this xenon-arc lamp.*

F-3005/6 Autotitration Injector

For controlled, automatic injection of aliquots into the sample of your choice, the F-3005/6 Autotitration Injector is just the thing, available in both 110 V (F-3005) and 220 V (F-3006) models. The F-3005/6 comes with dual syringes, for complete control over dispensing and aspirating volumes of liquids into and out of the sample cell. A mix function is included. With the injector come 18-gauge Teflon® tubing and two syringes (1 mL and 250 µL). The syringes are interchangeable; aliquot size is controllable to 0.1% of total syringe volume.

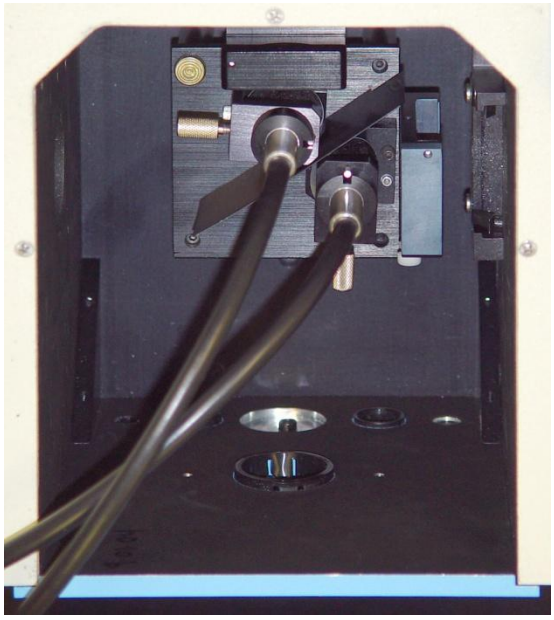


Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

Microscope Interface

The Microscope Interface eases the use of the Fluorolog®-3 system for fluorescence-microscopy measurements. The accessory includes fiber-optics to bring excitation light to the microscope's stage and emission light to the emission monochromator, plus a sample-compartment adapter to direct light in and out of the Fluorolog®-3.

Microscope Interface.



Fiber-optics in sample compartment

Caution: *Intense ultraviolet, visible, or infrared light may be present when the sample compartment is open, so avoid looking at the beam or its reflections. Do not aim fiber-optic bundles onto the skin or eyes. Use extreme caution with the fiber-optic probes.*

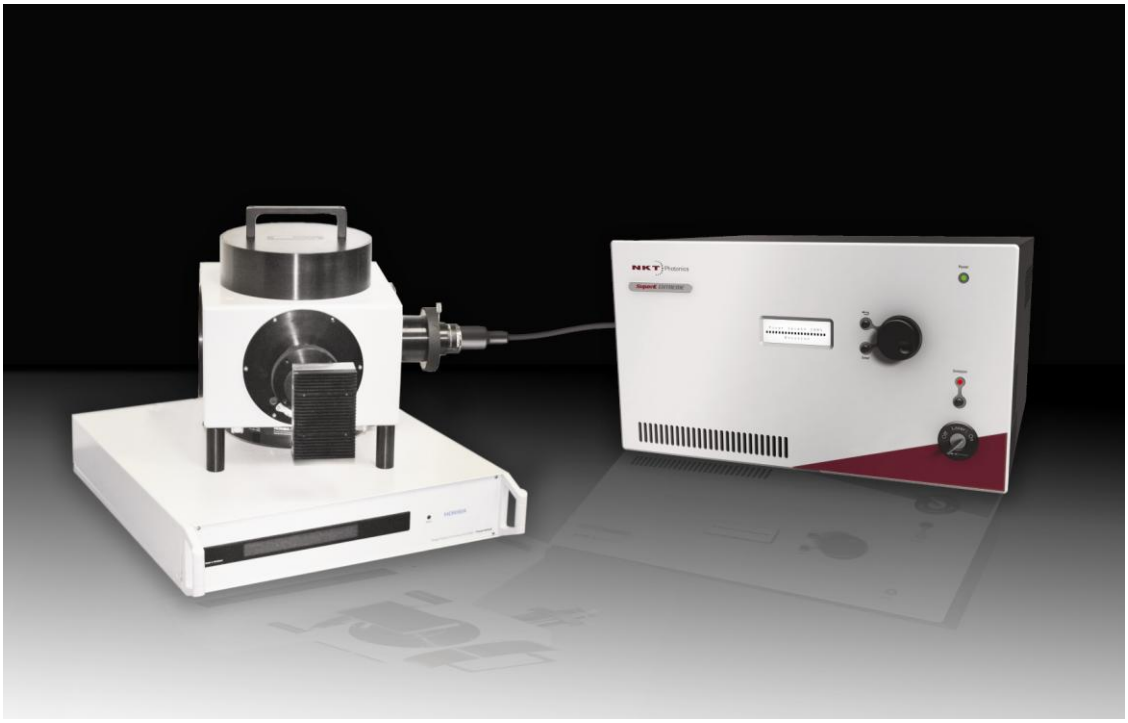
FL-450XOFR 450-W Xenon Lamp

The FL-450XOFR 450-W xenon lamp delivers light from 240 nm to 850 nm for sample excitation. The lamp has an approximate life of 2000 hours, and is ozone-free. The lamp is designed to fit into the FL-1039 Xenon Lamp Housing and the FL-1040 Dual Lamp Housing.

450-W xenon lamp, removed from the protective case.

Caution: *This lamp emits intense light and heat, and contains xenon gas under pressure. Understand all safety precautions before handling or using this xenon-arc lamp.*

Fluorolog® EXTREME Supercontinuum Laser Fluorometer

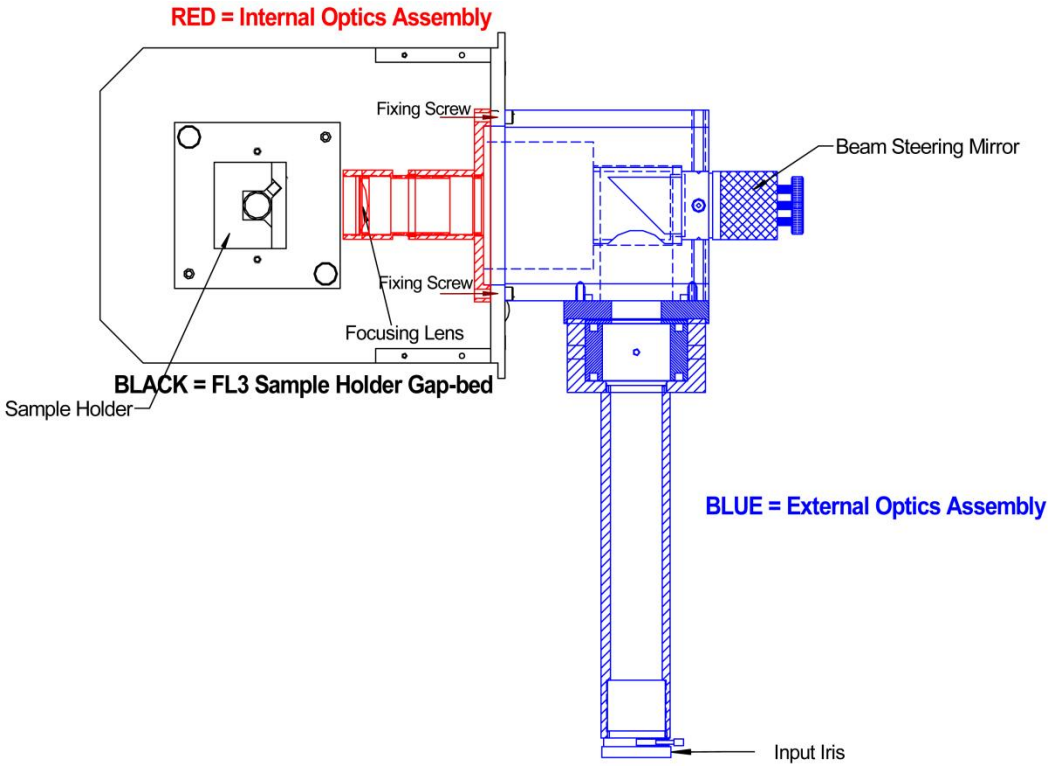


The Fluorolog® EXTREME is the world's first fully integrated supercontinuum laser-powered spectrofluorometer.

Optimized optomechanical coupling and complete software integration mean plug-and-play simplicity. The Fluorolog® EXTREME offers exceptional excitation power for steady-state measurements, especially in the near-IR. This solution represents the first time the high intensity and broad wavelength-range of a supercontinuum laser have been seamlessly integrated into a commercial spectrofluorometer.

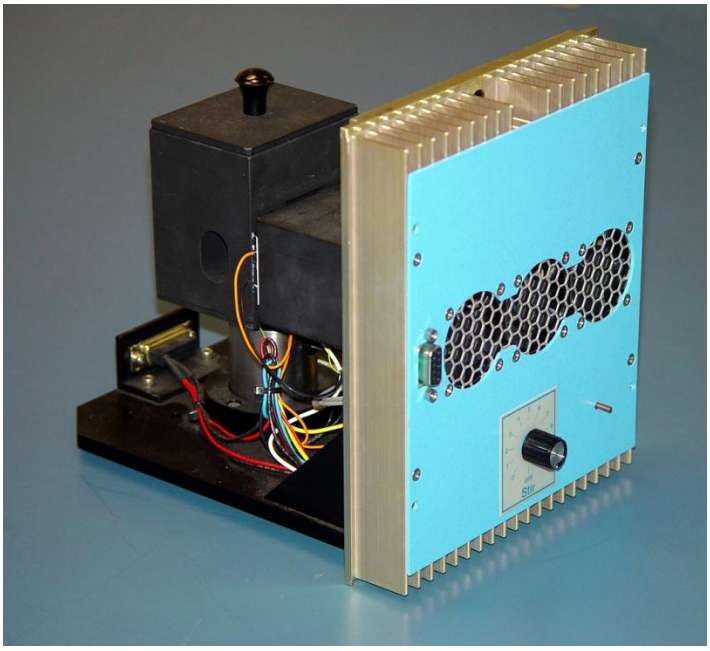
FC-OP-LIO1 and FC-OP-LIO2 Laser Input Optics accessories

For research and analysis using your own external mode-locked laser, such as a Ti:sapphire, add the Laser Input Optics to your Fluorolog®-3. The standard FC-PLIO1 accessory includes input iris and tube to the Sample Compartment to exclude ambient light, a beam-steering mirror, a polarizer mount (polarizer not included), filter and lens mount for 1″ (2.5 cm) optics, beam-sampling plate, photodiator and discriminator for optical synchronization, and more. The anisotropy input accessory FC-OPLIO2 enables you to perform anisotropy and polarization measurements, with the FCOP-LIO1. It includes a Babinet-Soleil compensator, and two extra prism polarizers in mounts. Contact your HORIBA Scientific Sales Representative for details. Note that the laser itself is not included in these accessories.

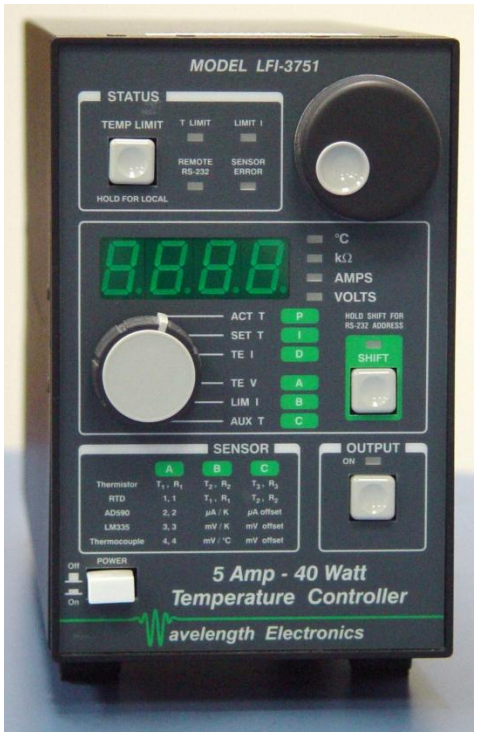


F-3004 Sample Heater/Cooler Peltier Thermocouple Drive

For rapid control of the sample's temperature in the Fluorolog®-3's sample compartment, choose the F-3004 Peltier Drive. Instead of messy fluids, the Peltier device heats and cools the sample thermoelectrically and fast! The temperature range is –10°C to +120°C. To prevent condensation of moisture on chilled cuvettes, an injection port for dry nitrogen gas is provided. All software is included, along with a controller and stirring mechanism.



F-3004 Sample Heater/Cooler Peltier Thermocouple Drive.



Peltier controller.

MicroMax 384 Microwell Plate Reader

The MicroMax 384 Microwell Titer-Plate Reader allows multiple samples to be scanned in one experiment. The MicroMax 384 is controlled through FluorEssence™ software via a serial port to the host computer. The titer plate moves beneath a stationary optical beam, and fluorescence measurements are collected with top-reading geometry. Thus, any titer plates—even disposable ones—may be used. Plates with 96 and 384 wells may be inserted into the MicroMax 384, with a scan speed < 1 min for all plates.

Various scan types are possible:

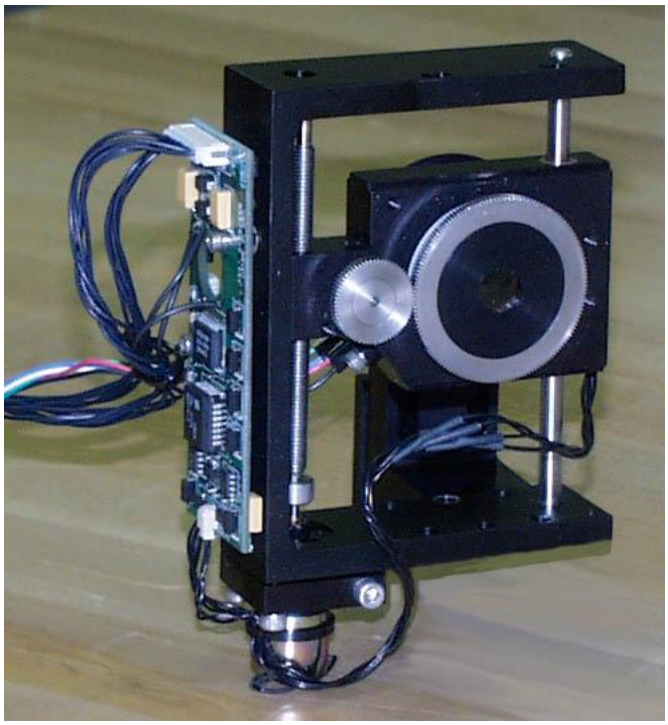
- Single-Point Analysis
- Excitation
- Emission
- Time-Base
- Synchronous
- Multigroup

Signals are transmitted between the Fluorolog®-3 and the MicroMax 384 via fiber-optic bundles.

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

FL-1044 L-Format Polarizer & FL-1045 T-Format Polarizer

For L-format spectrofluorometers, the FL-1044 dual polarizer is ideal. The kit includes two polarizers, to be placed at the entrance and the exit of the sample compartment. The polarizers are fully automated, and are adjustable to within 1° rotation. Insertion and removal from the optical path is controlled by the computer. For T-format spectroscopy, researchers should order the FL-1045 third polarization unit in addition to then FL-1044.



Caution: *Intense ultraviolet, visible, or infrared light may be present when the sample compartment is open, so avoid looking at the beam or its reflections. Do not aim fiber-optic bundles onto the skin or eyes. Use extreme caution with the fiber-optic probes.*

FL-1015 Injector Port

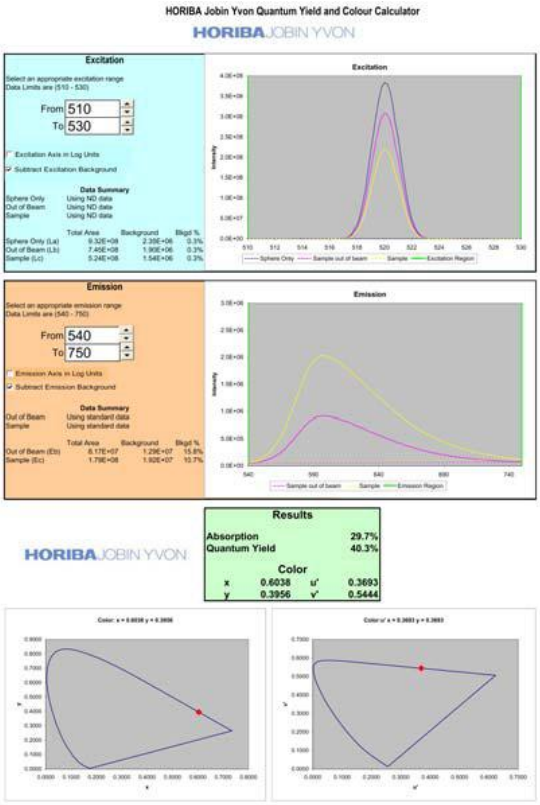
For the study of reaction kinetics, such as Ca^{2+} measurements, the FL-1015 Injector Port is ideal. This accessory allows additions of small volumes via a syringe or pipette to the sample cell without removing the lid of the sample compartment. With the injector in place, a lock-tight seal is achieved, prevented both light and air from reaching the sample. The Injector Port will accommodate most pipettes and syringes, with an injection hole diameter of 0.125″ (3.2 mm). A cap is included to cover the port when not in use.

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

Quanta-φ Quantum-Yield accessory

The F-3018 Quanta-φ integrating sphere is used in the Fluorolog®-3 spectrofluorometer to study fluorescence from solid and liquid samples. The sphere has an internal diameter of 6″ (15 cm). Of special interest is the measurement of photoluminescence quantum yields of such materials, especially for thin solid films. Measurement of quantum yields of solids requires more complicated apparatus when an integrated sphere is unavailable. The integrating sphere's base mounts directly on the gap-bed, and is inserted into the spectrofluorometer's sample compartment. Exclusive HORIBA Scientific software is included for automatic calculation of quantum yields, absorptions, and CIE (1931 and 1976) color values.

Quantum-yield accessory.

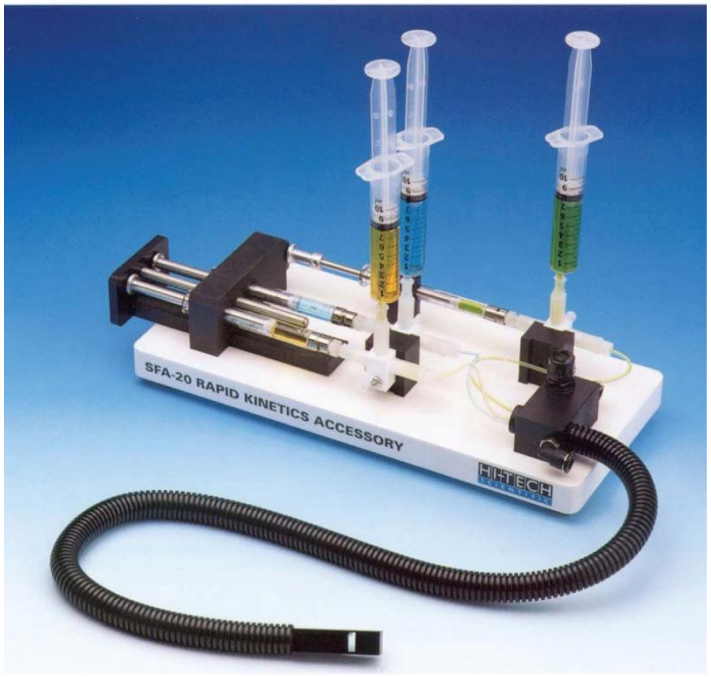


Quantum Yield and Color Calculator software.

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

Stopped-flow accessory F-3025

The stopped-flow rapid-kinetics accessories F-3025 and F-3025M offer versatility for spectroscopic monitoring of fast reactions in solution.



In addition to the conventional two-syringe mixing system, there is also a three-syringe version with two sequential mixers in the cell, giving you an option to do double-mixing. Further choices include a micro-volume version, reducing the volumes of reagents required to load the instrument, and thus improve sample economy.

The stopped-flow accessory permits observation of the reaction rate of two reactants forced through a mixing chamber, and into an observation cell. The reactant solutions are contained in drive syringes whose pistons simultaneously are driven. After leaving the observation cell, the reactants advance a stop syringe, triggering data-acquisition by the spectrofluorometer.

This accessory has been designed to suit the particular needs of Fluorolog® spectrofluorometers. The optical cell matches the beam geometry of this instrument. A cable is supplied so data-acquisition can be externally triggered at stopping, providing a reproducible time-zero registration for all traces, and allowing accurate overlay and averaging.

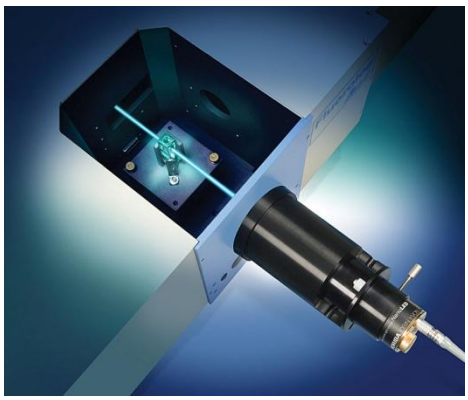
Model F-3025 includes 600 mm of tubing, while F-3025M has 300 mm of tubing.

Caution: *Always read the Material Safety Data Sheet (MSDS) to understand the hazards of handling your sample.*

TCSPC upgrades FL-1054, FL-1057, FL-1065, and FL-1066

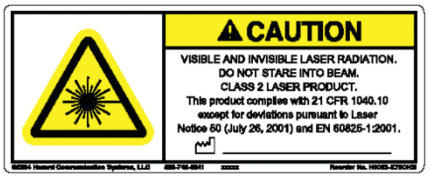
Now you can have The World's Most Sensitive Spectrofluorometer you've come to expect in the Fluorolog®-3, with the bonus of pico- and nanosecond lifetime capability. Time-correlated single-photon counting, or TCSPC, is perfect for dynamic anisotropy, TRES, and virtually any application requiring time-resolution, all with the ultimate, unrivaled sensitivity of digital photon-counting that strips away the noise, rather than adding noise to your signal the way an analog system does.

The pulsed sources used in the TCSPC upgrades are our NanoLED and SpectraLED solid-state pulsed diodes—which can be ordered from a full spectrum of wavelengths ranging from deep-UV to near-IR—our broad-band coaxial nanosecond flashlamp, and submicrosecond xenon spark-lamp. The Triple-Illuminator version (FL-1054) includes all steady-state or pulsed sources, switchable with the turn of a knob, attached to a special module. The economical NanoLED version (FL-1057) is a sample-drawer with pulsed NanoLED mounted on the sample compartment itself.



The FL-1065 is a new FL-1057 with a thermostatted sampling module, while the FL-1066 is the thermostatted upgrade to an existing FL-1057.

The TCSPC upgrades for the Fluorolog®-3 include all electronics, a special sample compartment (for the FL-1057), and your choice of NanoLED or SpectraLED.



Caution: *Intense ultraviolet, visible, or infrared light may be present when the sample compartment is open. Do not aim fiber-optic bundles onto the skin or eyes. Use extreme caution with the fiber-optic probes.*

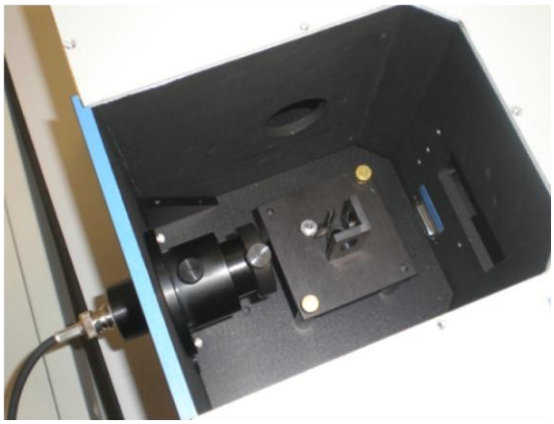
F-3030 Temperature Bath

For studies of samples whose properties are temperature-dependent, use the F-3030 Temperature Bath. The controller circulates fluids externally, with tubes leading to the sample chamber. The temperature range is from –25°C to +150°C. Sensor and all cables are included with the F-3030. The Temperature Bath is available in a 110 V (F-3030) and 220 V (F-3030-220) version.

Warning: *Refer to your Material Safety Data Sheet (MSDS) for information on the hazards of an ethylene-glycol–water mixture.*

This instrument uses high-temperature fluids, which can cause severe burns.

F-3031 Transmission Accessory



The F-3031 Transmission Accessory allows you to collect absorbance (transmittance) spectra of fluorescence samples. Such spectra can be used to verify that sample concentrations are appropriate, or even to correct for inner-filter effects. It comes assembled as a complete gap-bed, ready to insert into the Fluorolog®'s sample compartment.

The spectral range is 220–1000 nm. The F-3031 includes a DM303 electronics module and required cables. When used, it is configured as an "A" (auxiliary) detector in the instrument configuration.

FL-1024 Windows on the Fluorolog®-3 sample compartment

Introduction

The FL-1024 windows are useful when the sample compartment contains a sample chilled with liquid-nitrogen, in order to prevent condensation on the optics. With the windows installed, the sample compartment can be purged with dry nitrogen gas.

Installing the windows

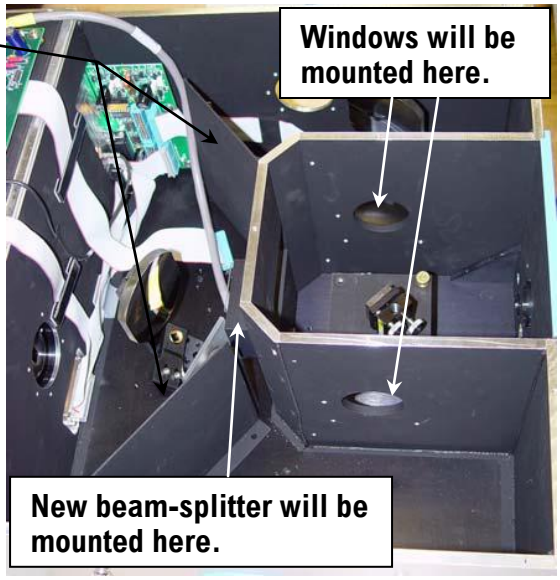
1 Prepare the instrument.

Caution: *Never touch any optical surfaces of the gratings, mirrors, etc.*

a Remove the instrument cover.

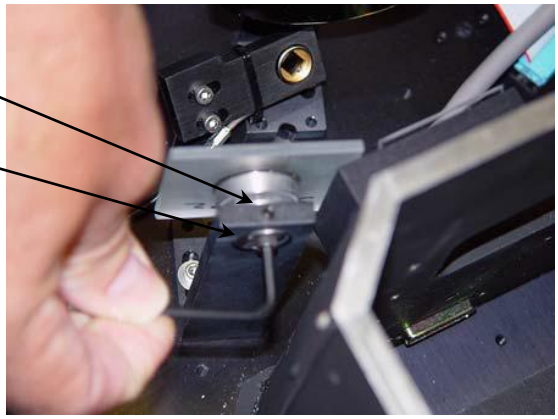
b Remove baffles.
Remove 2 cap screws holding each baffle in place.

Note: *Remove the baffles for easier access.*

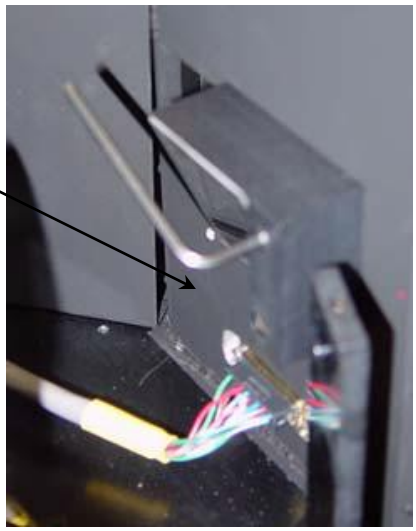


c Remove mirror from its mount.
Loosen set screw on top of mirror.
Remove cap screw from back of mirror.

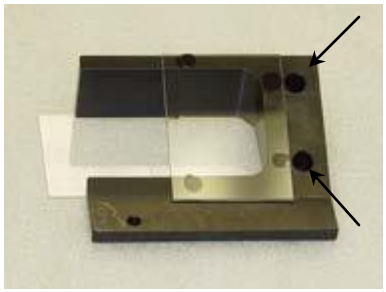
Note: *Remove the mirror temporarily because it is close to the beam-splitter.*



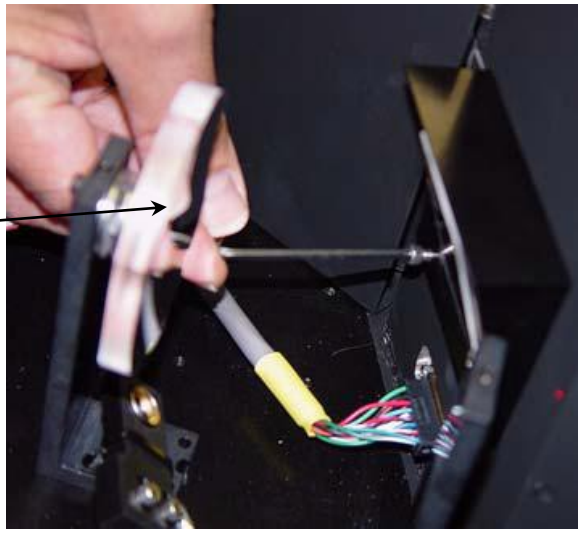
2 Replace the beam-splitter assembly.



a Remove old beam-splitter assembly from external wall of sample compartment.



b Insert two 6-32 × ⅜″ cap screws in the side holes of the new beam-splitter assembly.



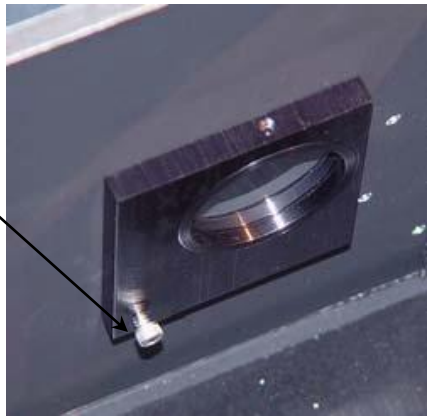
c Mount new assembly on external wall of sample compartment.

d Add third 6-32 × ⅜″ cap screw to mount assembly on sample compartment.

e Tighten all 3 cap screws.

3 Insert window assembly.

a Snap one window assembly onto an external side-wall of sample compartment.



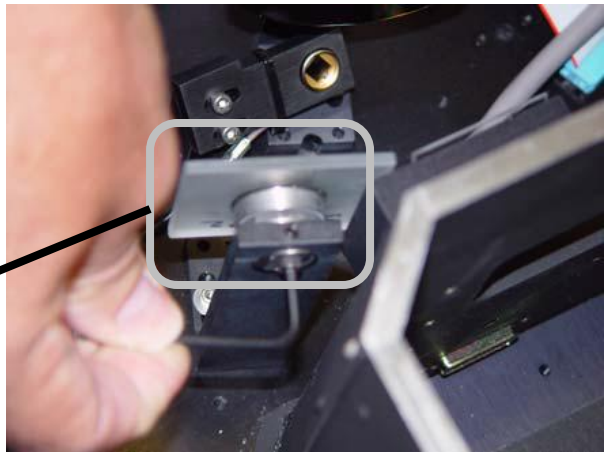
b Insert a 6-32 × ⅜″ cap screw.

c Tighten the cap screw.

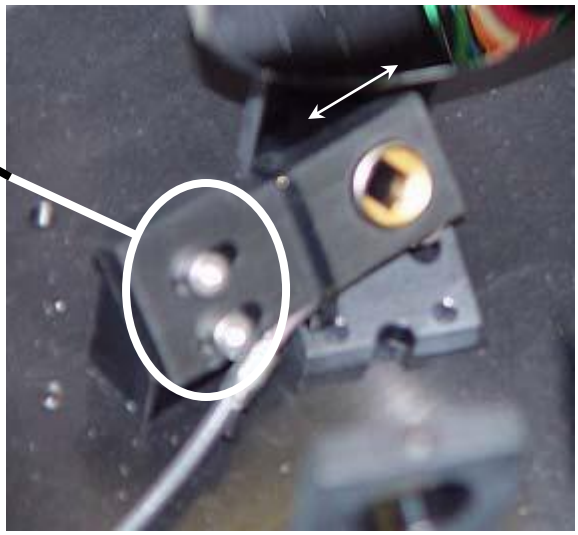
d Repeat steps (a) through (c) for the other window assembly.

4 Replace mirror and baffles.

a Replace mirror on mount.
Replace cap screw on back of mirror.
Tighten set screw on top of mirror.



b Center the excitation beam's image on photodiode.
Loosen two cap screws.
Slide photodiode sideways in order to center image on it.
Tighten the two cap screws.

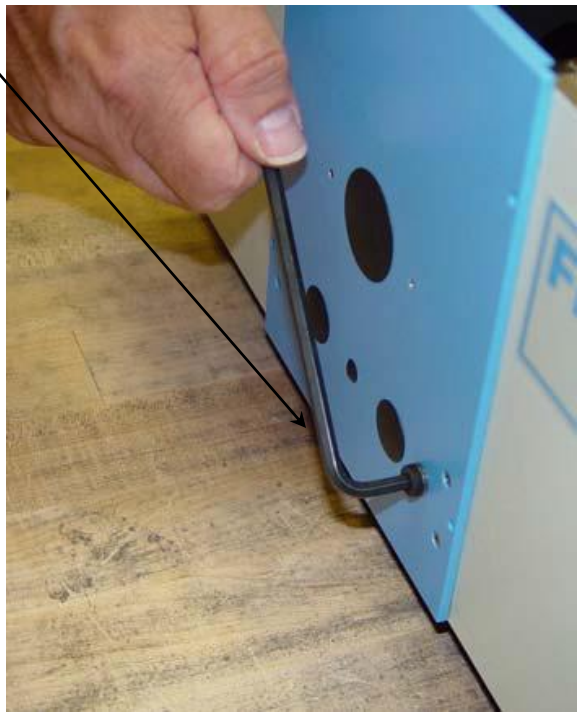


c Replace baffles using two cap screws for each.

5 Install purge port.

a Remove plastic original plug, using a 3/16″ Allen key.

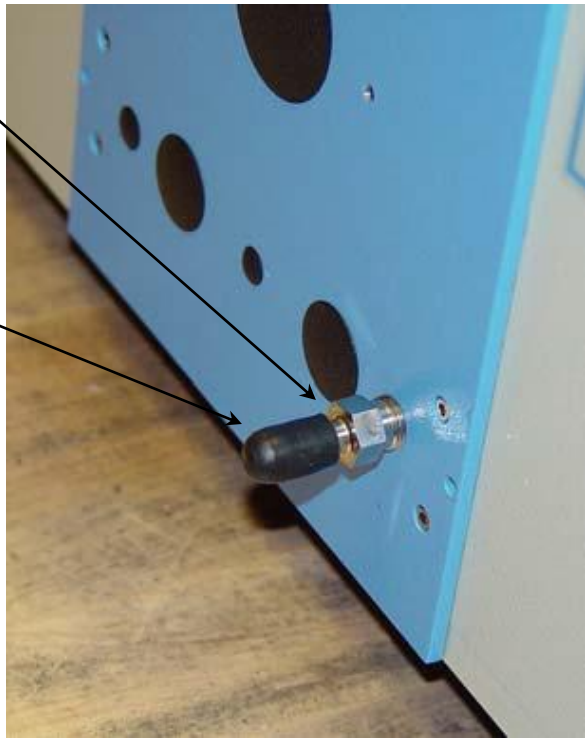
New purge port.



b Screw in manually the new metal purge port.

c Tighten with a 7/16″ wrench.

d Remove the plastic cap whenever a hose is to be attached to the new purge port.



Note: *The plastic cap also prevents light from entering the sample compartment.*

6 Close the instrument's cover.

Chapter 17 : Reassembly Instructions

Caution: *HORIBA Scientific does not recommend reassembly by the user. Contact the Service Department for reassembly.* ***Moving the Fluorolog®-3 system may degrade performance, and invalidates all warranties by HORIBA Instruments Incorporated.***

Introduction

The Fluorolog®-3 system consists of four main components:

- Personal computer
- Color monitor
- Expanded keyboard
- Fluorolog®-3 spectrofluorometer

An optional printer may also be included. A Fluorolog®-3 spectrofluorometer is a combination of discrete modules and components. These components include an excitation monochromator, an emission spectrometer, sample compartment, xenon light source and a System Controller (SpectrAcq).

Occasionally, because of relocating the instrument, reassembling the system is necessary. The following instructions are provided solely as a guide.

Host computer

The host computer must be set-up and operating before the spectrofluorometer system can be connected.

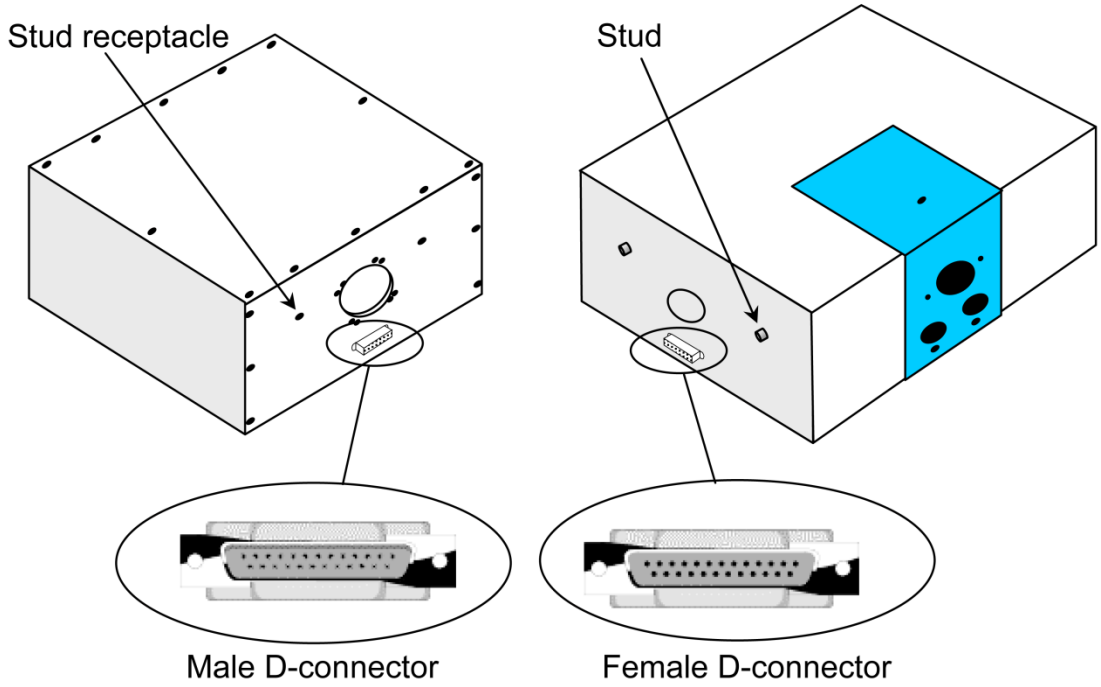
Note: *The computer's COM1 port will be connected to the System Controller in a later step. Make sure this port is available.*

1. Connect the host computer's components according to the instructions supplied with the computer.
2. Plug the power cables from the monitor, the main unit (CPU), and the printer or plotter into outlets with the proper line voltage.

Caution: *Do not turn on the computer or the peripherals.*

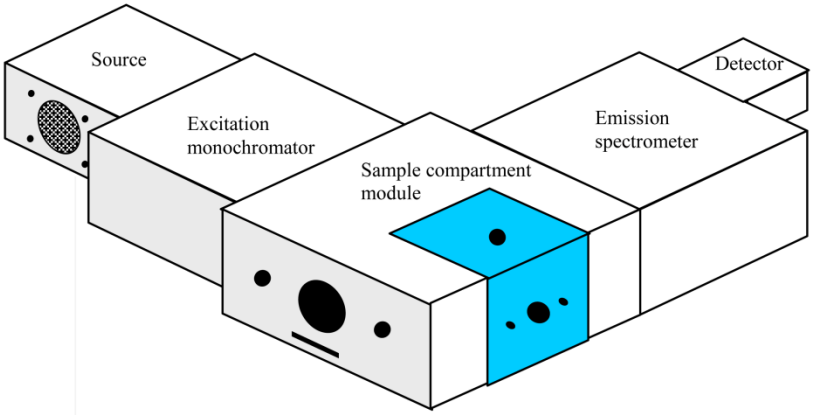
Spectrofluorometer assembly

After the host computer has been assembled, the external components of the Fluorolog®-3 spectrofluorometer must be connected. The modules of the Fluorolog®-3 fit together in a seamless configuration. Each module and the lamp housing have alignment studs, alignment receptacles, and D-connectors on the outside of the housing:



1 Assemble the spectrofluorometer system's modules into the proper configuration.

That is, attach the monochromators to the sample compartment, and the lamp housing to the excitation monochromator.

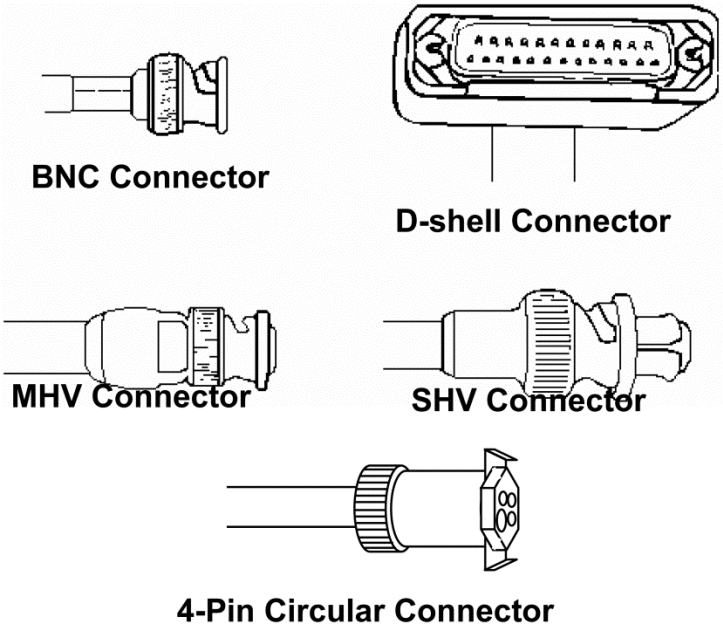


Example of a Fluorolog®-3 configuration.

Note: *See Chapter 2, System Description, for configurations. Concerning the lamp housing, xenon sources, and cautions, see Chapter 6, System Maintenance.*

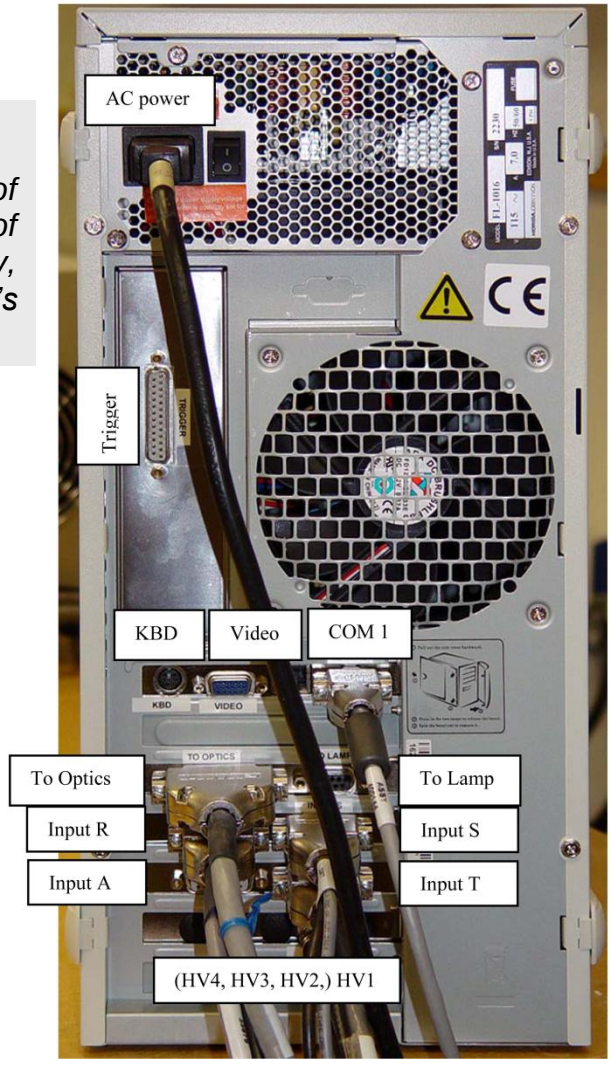
Cable connections

These cables connect the spectrofluorometer components with the SpectrAcq (SAC) and the computer system.

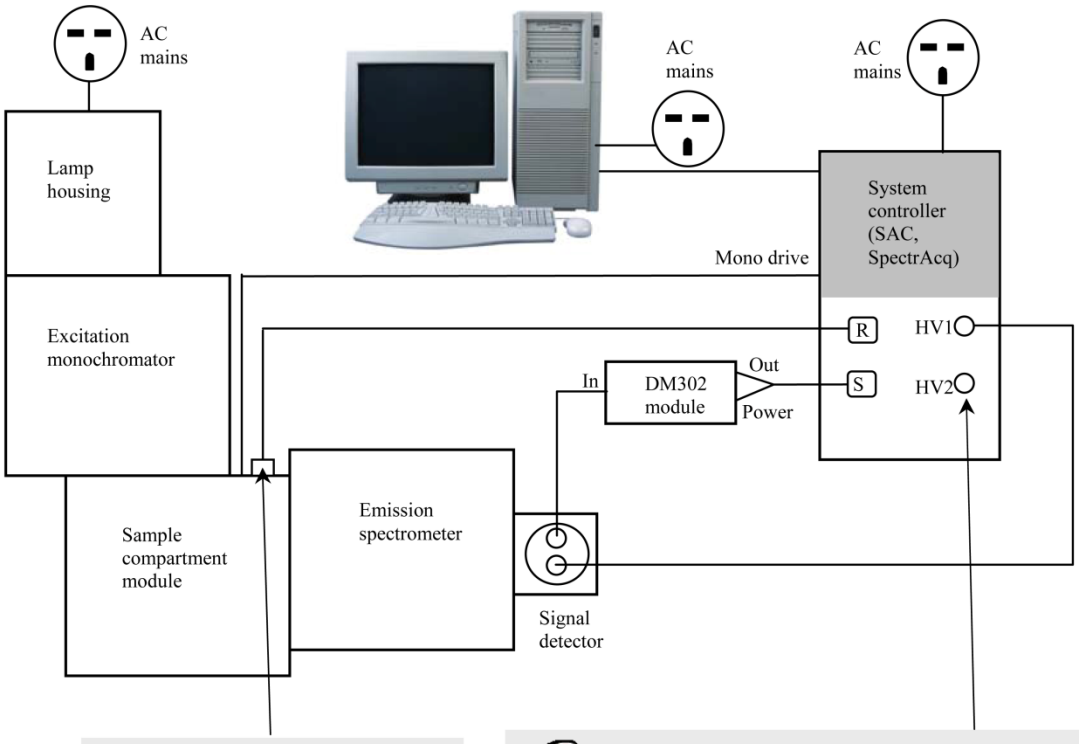


The jacks on the rear of the SpectrAcq are shown to the right:

Note: *The actual layout of connections on the rear of the SpectrAcq may vary, depending on the system's configuration.*



The cable diagram below shows the path of each cable. During installation, refer to the schematic to ensure proper system interconnections.

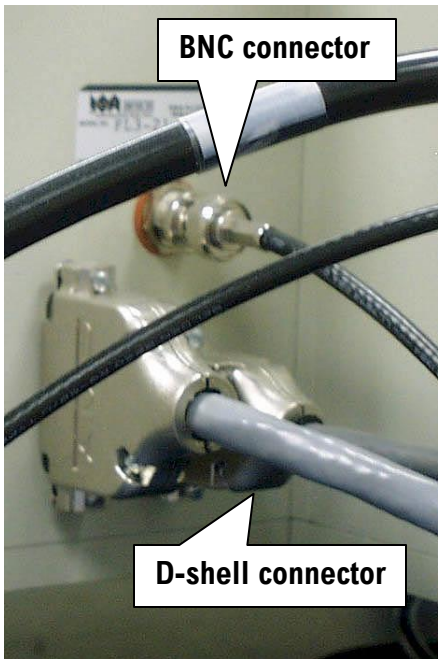


Note: *May be a single- or double-ended cable.*

Note: *Use HV2 for the second emission spectrometer.*

Connecting the reference detector to the SpectrAcq

The Fluorolog®-3 spectrofluorometer system comes standard with a silicon-photodiode reference detector inside the sample-compartment module. The connections to the reference detector are on the outside front of the sample-compartment module. The sample-compartment module (reference detector) is joined with the SpectrAcq via a split cable; that is, a cable with three ends.



1. Find the double-headed end of the split cable J33979A.
2. Plug the end with the 7-pin D-shell connector into the D-shell jack on the front of the sample compartment module.
3. Plug the BNC plug into the BNC jack next to the D-shell jack.
4. Plug the remaining D-shell end of the cable into the INPUT R connector on the back panel of the SpectrAcq.

DM302 PC module and signal-detector connections

1. Plug the end of the high-voltage cable J34040 with the MHV connector into the signal detector.
2. Connect the other end to the HV1 SHV connector on the SpectrAcq.
3. The DM302 PC module has three plugs labeled SIGNAL OUT, POWER and IN. Plug the short cable J30645 with the BNC connector from the signal detector to the IN connector on the DM302.

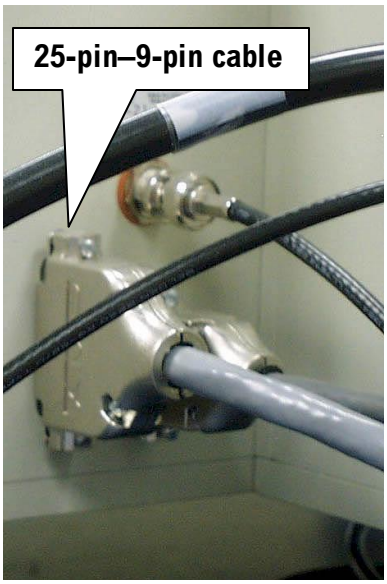
   The DM302 is joined to the SpectrAcq via the split cable J33977.

4 Take the bifurcated end of the J33977 split cable.

5 Plug the end with the 4-pin circular connector into the POWER jack of the DM302, and the BNC plug into the OUT jack of the DM302.

6 Plug the remaining end of the J33977 cable into the INPUT S connector on the back panel of the SpectrAcq.

Sample compartment module



1 Insert the 25-pin–9-pin cable J400108 between the sample compartment and the Mono Drive jack on the SpectrAcq.

Xenon-lamp power supply

The xenon lamp's power supply is integrated into the lamp housing. No additional connections are required.

Host computer to SpectrAcq

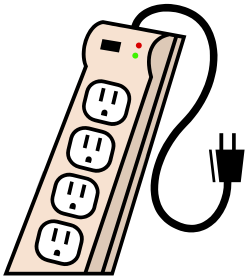
The spectrofluorometer system is controlled via the SpectrAcq through the host computer.

1 Connect the 9-pin–9-pin connector cable between COM1 of the host computer and COM1 of the SpectrAcq.

Connecting power cables

Several components in the system have AC power cables. These items include:

- SpectrAcq
- CCD-detector power supply
- Host computer (and peripherals)
- Xenon lamp
- Accessories (such as MicroMax, temperature bath, etc.)



1 Plug the AC components into a properly rated receptacle or power strip.

Calibrate the system before conducting an experiment.

Refer to the appropriate chapter for calibration instructions.

Note: *To properly operate the equipment, have a complete understanding of the software. Read the* FluorEssence User's Guide *to gain an understanding of the system interaction, and to discover the best methods to unleash the power of FluorEssence™.*

Chapter 18 : Glossary

3D excitation/emission display This maps a specified emission-scan wavelength range using various excitation wavelengths.

Absorption Transition, when a photon enters a molecule, from the ground state to the excited singlet state. This process typically occurs in ~10^{-15} s.

Absorbance The extent of absorption by a substance. Absorbance, A, is –log T, where T is the transmittance of the sample. Absorbance is also synonymous with optical density (OD). Absorbance can be calculated using the Beer-Lambert Law:

$$\text{OD} = A = \varepsilon cl = -\log T$$

where ε = the extinction coefficient (M^{-1} cm^{-1}); c = sample concentration (M); l = path length (cm)

Bandpass The wavelength range of light passing through the excitation and emission spectrometers. The wider the bandpass, the higher the signal intensity.

Bandpass filter Optical element that selectively transmits a narrow range of optical wavelengths.

Bioluminescence Emission of light originating from a chemical reaction in a living organism.

Blaze wavelength Wavelength at which a grating is optimized for efficiency. Generally the gratings are efficient to ⅔ before the blaze wavelength to twice the blaze wavelength. The excitation and emission gratings are blazed in the UV and visible respectively.

Chemiluminescence Emission of light originating from a chemical reaction.

Color effect for pulse technique Time-dependent wavelength distribution of the lamp pulse.

Color effect for phase-modulation technique Phase of the excitation and the degree to which it is modulated.

Corrected emission scan An emission scan corrected for the wavelength characteristics of the emission monochromator and the response of the signal detector. To obtain a corrected emission scan, an emission spectrum is multiplied by the emission correction factors. An emission correction-factor file is supplied with the instrument and stored as `mcorrect.spt` on the software disks. (Use `tcorrect.spt` for the T-side detector.)

Corrected excitation An excitation scan corrected for the wavelength characteristics of the xenon lamp, the aging of the xenon lamp, and the gratings in the

scan excitation monochromator. To obtain a corrected excitation scan, the detector signal is ratioed to the reference signal (e.g., S1/R1), which provides 90% of the correction. To obtain a completely correct scan, the excitation scan acquired in the manner described above is multiplied by correction factors. An excitation correction-factor file, `xcorrect.spt`, is included with the software.

Correction factors Compensates for the wavelength-dependent components of the system, like the xenon lamp, gratings, and signal detector. Emission and excitation correction-factor files are included with the software and are titled `xcorrect.spt` and `mcorrect.spt` (`tcorrect.spt` for the T-side detector).

Cut-on filter Optical component that passes light of a higher wavelength.

Cut-off filter Optical component that passes light of a lower wavelength.

Dark counts Inherent background signal of the photomultiplier when high voltage is applied. Cooling the detector decreases the dark counts.

Emission scan Shows the spectral distribution of light emitted by the sample. During an emission scan, the excitation monochromator remains at a fixed wavelength while the emission monochromator scans a selected region.

Excitation scan Shows the spectral distribution of light absorbed by the sample. To acquire an excitation scan, the excitation monochromator scans a selected spectral region while the emission monochromator remains at a fixed wavelength.

Extrinsic fluorescence Inherent fluorescence of probes used to study non-fluorescent molecules.

Flash lamp A lamp that provides pulsed-light output to excite a sample. Can be either “free running” or “gated.”

Fluorescence The emission of light or other electromagnetic radiation during the transition of electrons from the excited singlet state to the ground state. Fluorescence typically occurs within about ~10^{-9} s.

Fluorescence lifetime The average length of time that a molecule remains in the excited state before returning to the ground state.

Front-face detection A mode of detection in which fluorescence is collected off the front surface of the sample. Front-face detection usually is selected for samples such as powders, thin films, pellets, cells on a cover-slip, and solids.

Grating Optical element in the monochromator, consisting of finely scribed grooves that disperse white light into a spectrum.

Inner-filter effect The absorption of the excitation beam or fluorescence emission from a concentrated sample by components in the sample. Note there are Primary and Secondary inner-filter-effects (IFEs). IFEs reduce the signal intensity from the sample creating artifacts in the spectra. For this reason, we recommend using concentrations of <0.05 OD in a 1-cm-pathlength cell. The Aqualog IFE tool can automatically correct most samples for IFE. IFE correction requires the sample concentration be in the linear Beer-Lambert region.

Intrinsic fluorescence The natural fluorescent properties of molecules.

Laser A monochromatic light source that provides high intensity of excitation.

Mercury lamp Light source that emits discrete, narrow lines as opposed to a continuum. A mercury lamp can be used to check the monochromator's calibration.

Mirror-image rule When the emission profile appears to be the mirror image of the absorption spectrum.

Monochromator The component in a spectrofluorometer that is scanned to provide the excitation and emission spectra. Monochromators are chosen for stray-light rejection, resolution, and throughput.

Optical density A synonym of *Absorbance*. See *Absorbance*.

Optical-density effects Fluorescence intensities are proportional to the concentration over a limited range of optical densities. High optical densities can distort the emission spectra as well as the apparent intensities.

Phosphorescence The emission of light or other electromagnetic radiation during the transition of electrons from the triplet state to the ground state. Phosphorescence is generally red-shifted relative to fluorescence and occurs within ~10^{-6} s to several seconds. To enhance phosphorescence detection, samples are often chilled to liquid-nitrogen temperature (77 K).

Pulse-sampling method A technique for measuring fluorescence lifetimes, in which an initial population of fluorophores is excited by infinitely short pulses of light. An advantage of this technique is the direct recording of time-resolved emission spectra.

Raman scattering Scattering caused by vibrational and rotational transitions. Raman bands generally appear red-shifted relative to the incident electromagnetic radiation. The primary characteristic of Raman scatter is that the difference in energy between the Raman peak and the incident radiation is constant in energy units (cm^{-1}).

Rayleigh scattering Light scattering from particles whose dimensions are much smaller than the wavelength of incident light. The scattered light is of the same

	energy as the incident light. Rayleigh scatter shows scatter radiation intensity inversely proportional to the 4^{th} power of the wavelength of incident radiation.
Rayleigh-Tyndall scattering	Combination of Rayleigh and Tyndall scatter. These two scattering phenomena cannot be separated. If the molecule's Stokes shift is small, Rayleigh-Tyndall scatter will limit the ultimate resolution.
Red-sensitive photomultiplier	A photomultiplier-tube detector that extends fluorescence detection to 850 nm.
Reference photodiode	Solid-state detector used to monitor the output of the xenon lamp.
Resolution	The ability to separate two closely spaced peaks. Resolution can be improved by decreasing the bandpass and the increment (step size).
Resonance energy transfer	The transfer of the excited energy from a donor to an acceptor. The transfer occurs without the appearance of a photon and is primarily a result of dipole-dipole interactions between the donor and acceptor.
Right-angle detection	Collection of fluorescence at 90° to the incident radiation. Right-angle detection typically is selected for dilute and clear solutions.
Scatter	A combination of Raman, Rayleigh, and Rayleigh-Tyndall scattering, which can distort fluorescence spectra with respect to intensities and wavelengths.
Signal photomultiplier	Detector commonly used to measure excitation and fluorescence from the sample. It is operated in photon-counting mode to provide high sensitivity. Different detectors cover different wavelength regions.
Singlet state	The spin-paired ground or excited state. The process of absorption generally produces the first excited singlet state that emits fluorescence, or undergoes intersystem crossing to form a triplet state.
Stokes shift	Generally, the energy-difference between the absorption peak of lowest energy and the fluorescence peak of maximum energy.
Synchronous scan	Scan that characterizes the overlap between the excitation and emission. The excitation and emission monochromators are scanned simultaneously, with a constant offset specified as either nanometers (wavelength units) or in cm^{-1} (energy units).
TCSPC	Time-correlated single-photon counting. Technique in which the sample is excited by a pulsed source, and the sample's fluorescence is collected over the course of many pulses. The arriving photons are timed after the excitation. Gradually a decay curve is built up and the sample's fluorescent lifetime is calculated.
Time-based scan	Scan in which the sample signal is monitored while both the excitation and the emission monochromators remain at fixed wavelengths. Time-based data are used to monitor enzyme kinetics, dual wavelength

measurements, and determine the reaction rate constant.

Time-resolved emission scan Scan in which the emission spectra are acquired at various times after the excitation pulse. Provides insight into excited-state reactions, charge-transfer-complex formation, solvent dipolar relaxation and other experiments.

Triplet state (T_1) The spin-paired ground or excited state formed from the excited singlet state when paired electrons become unpaired. The triplet state emits phosphorescence.

Tyndall scattering Scatter that occurs from small particles in colloidal suspensions.

Xenon lamp Lamp that produces a continuum of light from the ultraviolet to the near-infrared for sample excitation.

Xenon-lamp scan A profile of the lamp output as a function of wavelength. The lamp scan is acquired with the reference detector while scanning the excitation spectrometer. The maximum xenon-lamp peak at 467 nm can be used to determine proper calibration of the excitation spectrometer.

Chapter 19 : Bibliography

P.M. Bayley and R.E. Dale, *Spectroscopy and the Dynamics of Molecular Biological Systems*, Academic Press, London, 1985.

R.S. Becker, *Theory and Interpretation of Fluorescence and Phosphorescence*, Wiley-Interscience, New York, 1969.

I.B. Berlman, *Handbook of Fluorescence Spectra in Aromatic Molecules*, 2nd ed., Vols. I & II, Academic Press, New York, 1971.

C.R. Cantor and P.R. Schimmel, *Biophysical Chemistry: Techniques for the Study of Biological Structure and Function*, Vols. 1 & 2, W.H. Freeman, New York, 1980 & 1990.

M. Chalfie and S. Kain, *Green Fluorescent Protein: Properties, Applications, and Protocols (Methods of Biochemical Analysis)*, 2nd ed., Jossey-Bass, New York, 2005.

R.F. Chen, *et al.*, *Biochemical Fluorescence: Concepts*, Vol. I & II, 1964 & 1970.

J.N. Demas, *Excited State Lifetime Measurements*, Academic Press, New York, 1983.

Enrico Gratton, David M. Jameson, and Robert D. Hall, "Multifrequency Phase and Modulation Fluorometry," *Ann. Rev. Biophys. Bioeng.* **13**, 105–124 (1984).

G.G. Guilbault, ed., *Fluorescence—Theory, Instrumentation and Practice*, Marcel Dekker, New York, 1967.

_____, *Practical Fluorescence: Theory, Methods and Techniques*, 2nd ed., Marcel Dekker, 1990.

_____, "Molecular Fluorescence Spectroscopy," *Anal. Chem.* 8, 71–205 (1977).

D.M. Hercules, ed., *Fluorescence and Phosphorescence Analysis*, Wiley-Interscience, New York, 1966.

J.D. Ingle and S.R. Courch, *Spectrochemical Analysis*, Prentice-Hall, Englewood Cliffs, NJ, 1988.

F.H. Johnson, *The Luminescence of Biological Systems*, Amer. Assoc. Adv. Sci., Washington, D.C., 1955.

S.V. Konev, *Fluorescence and Phosphorescence of Proteins and Nucleic Acids*, Plenum Press, New York, 1967.

M.A. Konstantinova-Schlezinger, ed. *Fluorometric Analysis*, Davis Publishing Co., New York, 1965.

Joseph R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, New York, 2006.

_____, ed., *Topics in Fluorescence Spectroscopy*, Vols. 1–5, Plenum Press, New York, 1991–1998.

_____, Badri P. Melinal, Enrico Gratton, "Recent Developments in Frequency-Domain Fluorometry," *Anal. Instr.*, **14** (314), 193–223 (1985).

_____, S. Soper, and R. Thompson, *Advances in Fluorescence Sensing Technology IV*, SPIE Proc. Series, Vol. 3602 (1999).

W.T. Mason, ed., *Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide to Technology for Quantitative Real-Time Analysis*, 2nd ed., Academic Press–Harcourt Brace & Co., 1999.

W.H. Melhuish and M. Zander, "Nomenclature, Symbols, Units and Their Usage in Spectrochemical Analysis VI: Molecular Luminescence Spectroscopy," *Pure App. Chem.*, **53**, 1953 (1981).

J.N. Miller, ed., *Standardization & Fluorescence Spectrometry: Techniques in Visible and Ultraviolet Spectrometry*, Vol. 2, Chapman and Hall, 1981.

W.G. Richards and P.R. Scott, *Structure and Spectra of Molecules*, John Wiley & Sons, 1985.

A. Schillen, et al., *Luminescence of Organic Substances*, Hellwege Verlag, Berlin, 1967.

S.G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications*, Vols. 1–3, Wiley-Interscience, New York, 1985–1993.

A. Sharma and S. Schulman, *Introduction to Fluorescence Spectroscopy*, Wiley Interscience, New York, 1999.

D.A. Skoog, F.J. Holler, and T.A. Nieman, *Principles of Instrumental Analysis*, 5th ed., Brooks Cole, New York, 1998.

N.J. Turro, V. Ramamurthy, and J.C. Scaiano, *Modern Molecular Photochemistry of Organic Molecules*, University Science Books, New York, 2006.

K. Van Dyke, *Bioluminescence and Chemiluminescence: Instruments and Applications*, Vol. 1, CRC Press, Boca Raton, FL, 1985.

T. Vo-Dinh, *Room Temperature Phosphorimetry for Chemical Analysis*, Wiley-Interscience, New York, 1984.

I.M. Warner and L.B. McGown, ed., *Advances in Multidimensional Luminescence*, Vols. 1 & 2, JAI Press, Greenwich, CT, 1991–1993.

E.L. Wehry, ed., *Modern Fluorescence Spectroscopy*, Vol. 1–4, Plenum Press, New York, 1975–1981.

C.E. White and R.J. Argauer, *Fluorescence Analysis: A Practical Approach*, Marcel Dekker, New York, 1970.

J.D. Winefordner, S.G. Schulman, and T.C. O'Haver, *Luminescence Spectrometry in Analytical Chemistry*, Wiley-Interscience, New York, 1972.

In addition, the following journals may prove useful:
Analytical Chemistry
Biophysics and Biochemistry
Journal of Fluorescence
Nanotechnology Letters

Chapter 20 : CE Compliance Information

Declaration of Conformity

Manufacturer:	HORIBA Instruments Incorporated
Address:	3880 Park Avenue Edison, NJ 08820 USA
Product Name:	Fluorolog-3
Product Model Numbers:	FL3-11, FL3-12, FL3-21, FL3-22 FL3C-11, FL3C-12, FL3C-21, FL3C-22
Consisting of Combinations of the Following:	FL-1016 SpectrAcq Controller FL-1039A / FL-1040A Illuminator Box FL-1005 Double Monochromator FL-1004 Single Monochromator FL-1073 Uncooled PMT Assembly FL-1048 / FL-1030 Cooled PMT Assemblies FL-1000 Sample Compartment FL-1011, FL-1012, FL-1041, 400313 Sample Drawers FL-1014 Sample Compartment Electronics DM302 Photon-Counting Module DM303 / DM303M Current & Voltage-Input Modules FL-1083-XXX, FL-1081, FL-1092 NIR PMTs FL-1096 / 220ASH Shutter Assemblies FL-1044 / FL-1045 Auto Polarizers DSS-IGAXXXX DSS Detectors DSS-15V-TEP DSS Controller

Conforms to the following Standards:

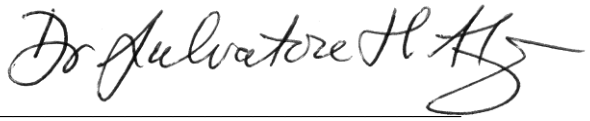
Safety:	EN 61010-1: 2001 EN 61010-1: 2001/AC: 2002
EMC:	EN 61326-1: 2006 (Emissions & Immunity)

Supplementary Information

The product herewith complies with the requirements of the Low Voltage Directive 2006/95/EEC and the EMC Directive 2004/108/EC.

The CE marking has been affixed on the device according to Article 8 of the EMC Directive 2004/108/EC.

The technical file and documentation are on file with HORIBA Instruments Incorporated.



Sal Atzeni
Vice-President, Retail Engineering, and CTO

HORIBA Scientific
Edison, NJ 08820
USA
April 20, 2012

Applicable CE Compliance Tests and Standards

Test	Standards
Emissions, Radiated/Conducted	EN 55011: 2006
Radiated Immunity	IEC 61000-4-3: 2006
Conducted Immunity	IEC 61000-4-6: 2008
Electrical Fast Transients	IEC 61000-4-4: 2004
Electrostatic Discharge	IEC 61000-4-2: 2008
Voltage Interruptions	IEC 61000-4-11: 2004
Surge Immunity	IEC 61000-4-5: 2005
Magnetic Field Immunity	IEC 61000-4-8: 2009
Harmonics	IEC 61000-3-2: 2006
Flicker	IEC 61000-3-3: 2008
Safety	EN 61010-1: 2001
	EN 61010-1: 2001/AC: 2002