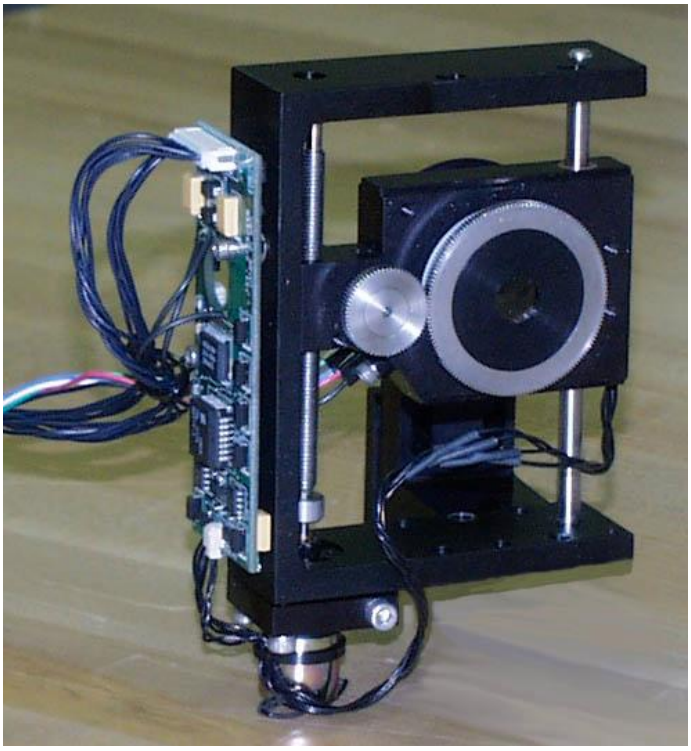
HORIBA Scientific

Polarizers with FluorEssence™

Operation Manual
Part number J81040 rev. F

FL-1044 and FL-1045 Polarizers for FluorEssence™ Software



Operation Manual rev. F

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HORIBA Scientific

April 2013

Part number J81040

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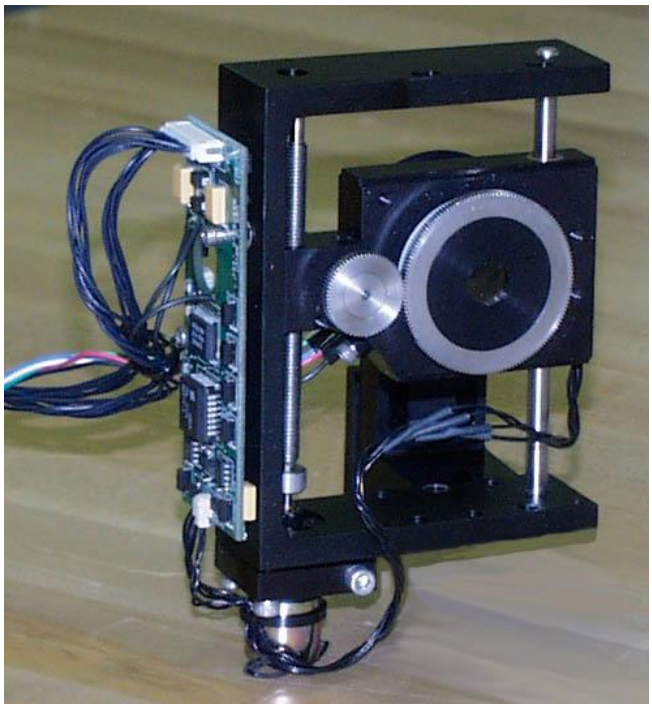
About the Polarizers



Polarizers for HORIBA Scientific spectrofluorometers add the ability to measure fluorescence polarization and anisotropy. Measurements of fluorescence-emission anisotropy provide information about the rotational behavior of molecules. Molecular size and shape, as well as the viscosity of the medium in which the fluorophore is rotating, may be studied with this technique.

HORIBA Scientific offers polarizers for FluoroMax, Fluorolog®, and Nanolog systems. Each of these systems employ Glan-Thompson polarizing prisms held in place on automated mounts. Each automated polarizer is controlled directly through FluorEssence™ software for Windows®. Polarizer accessories ordered with a new instrument are calibrated at the factory with that instrument. Polarizers ordered for instruments already in service should be checked for calibration as explained in the chapter “Alignment”.

Glan-Thompson polarizers are calcite-prism polarizers. They offer a transmission range from about 215 nm to above 2000 nm. Compared with film polarizers, Glan-Thompson prisms offer a much wider transmission range with more uniform transmission over that range. Glan-Thompson prisms provide a higher extinction ratio, needed for measurements of small changes in anisotropy, $\langle r \rangle$, or $\Delta\langle r \rangle$. Glan-Thompson polarizers will not bleach under prolonged UV radiation as film polarizers do.

Automated polarizers are set to their positions through software control of their stepper motors. Their calibration positions are physically set during calibration, using the collars that hold the polarizers. Users with FluorEssence™ may use the Polarizer Alignment option in the **Experiment Setup** window to automatically recalibrate their polarizers. The polarizer is calibrated using optical sensors on the polarizer mount that sense the home position (a small hole in the plate).

The Fluorolog®-3, and Nanolog use only automated polarizer assemblies. Their polarizers are automatically moved in and out of the optical path by the software. For this reason, never remove the polarizers from their mounts. They should not require manual realignment. If calibration must be performed, use the Polarizer Alignment software routine in the **Experiment Setup** window to calibrate the polarizers as described in "Alignment."

The FL-1044 is an L-format dual-polarizer setup. For T-format steady-state polarization or anisotropy-decay measurements, the FL-1045 Third Automated Polarizer Assembly is required in addition to the FL-1044.

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

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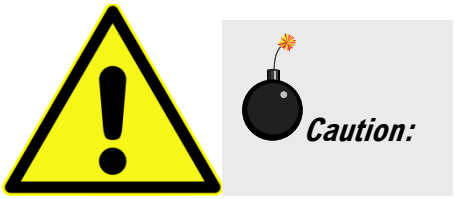

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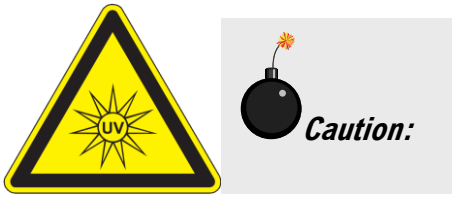
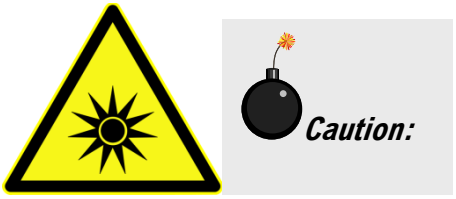
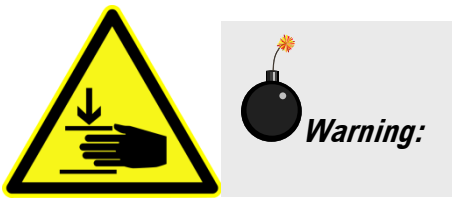


Ultraviolet light! Wear protective goggles, full face-shield, skin-protection clothing, and UV-blocking gloves. Do not stare into light.



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.



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.

Flammable! This symbol cautions the user that there is a risk of fire.

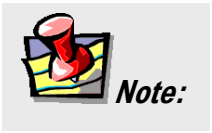
This symbol cautions the user that excessive humidity, if present, can damage certain equipment.

Read this manual before using or servicing the instrument.

Wear protective gloves.

Wear appropriate safety goggles to protect the eyes.

Wear an appropriate face-shield to protect the face.



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’Éclairage 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.

2 : Measurement of Fluorescence Polarization

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:

$$P = \frac{3\langle r \rangle}{2 + r} \tag{6}$$

$$\langle r \rangle = \frac{3P}{3 - P} \tag{7}$$

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

$$\langle r \rangle = 0.4 \langle P_2(\cos\alpha) \rangle \tag{8}$$

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

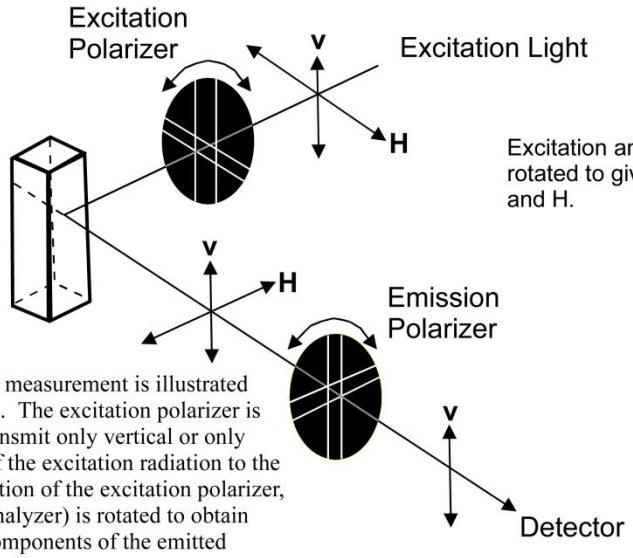
Polarization measurements are taken in two basic geometries:

- 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

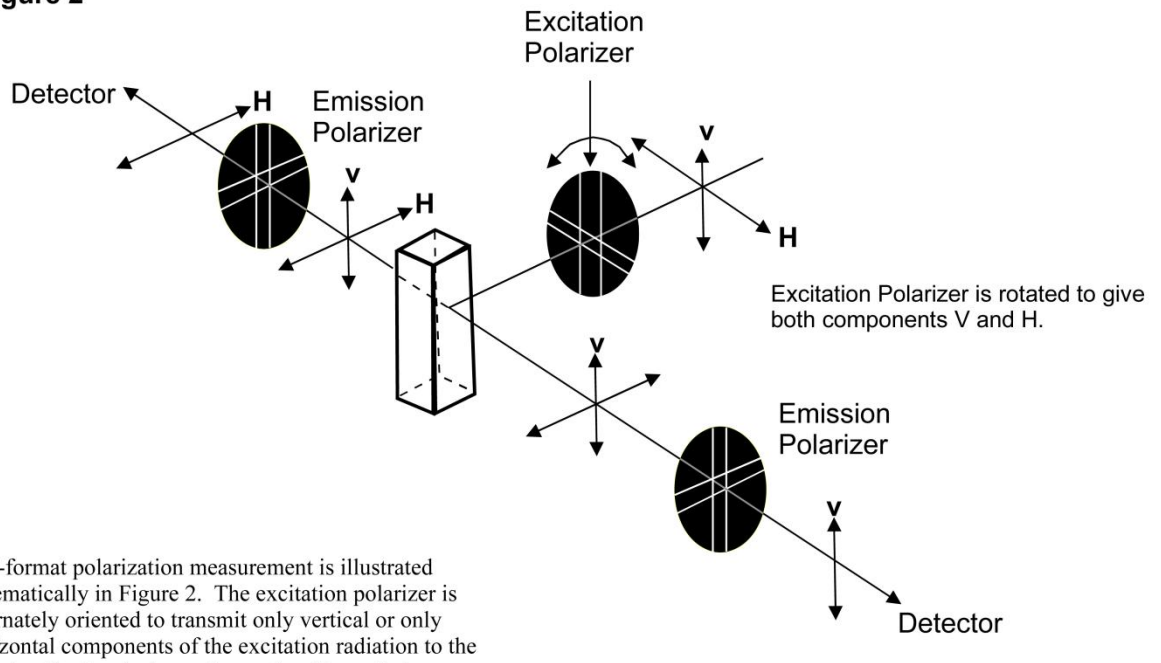
Figure 1



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.*

3: 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 and Nanolog 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 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.

4: 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:

$$\text{polarization ratio} = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \qquad (9)$$

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.

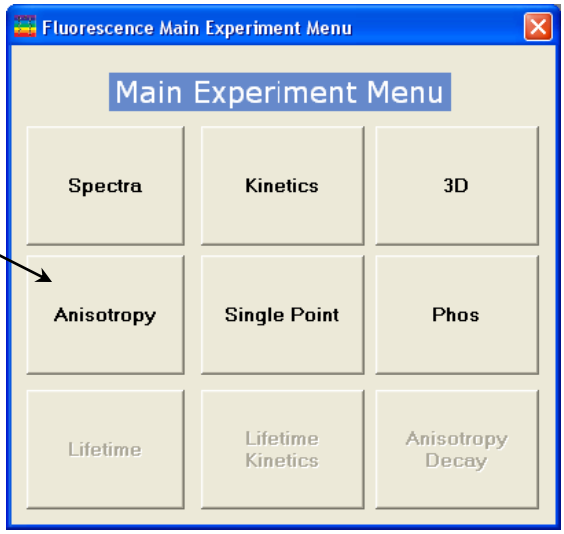
Note: *The polarization ratio can be lowered by using concentrated scatterer. Use only a* ***slight*** *amount of scatterer to align the system.*

Method

Checking alignment

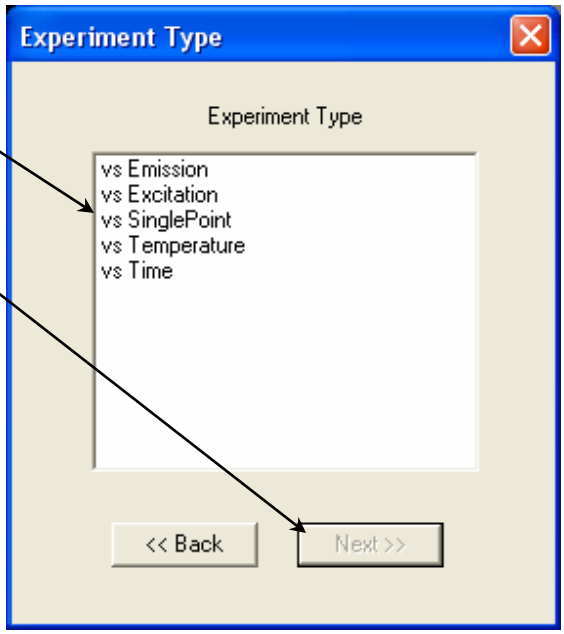
1. Place the scatterer in the sample-cuvette position.
2. Close the sample compartment's cover.

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



The **Experiment Type** menu appears:

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

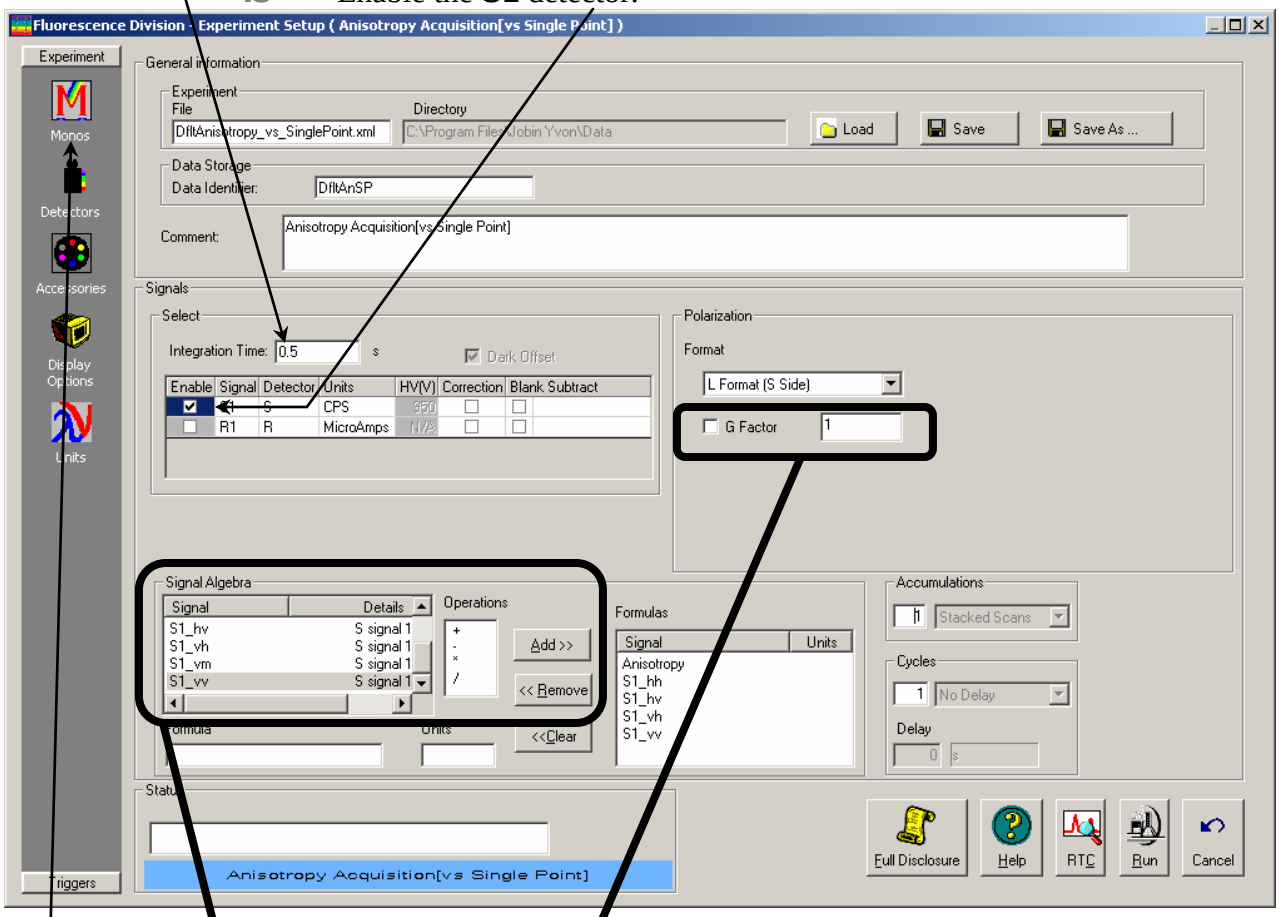


The **Experiment Setup** window opens:

5 Set up the anisotropy experiment.

a Enter an Integration Time of 0.5 s.

b Enable the S1 detector.



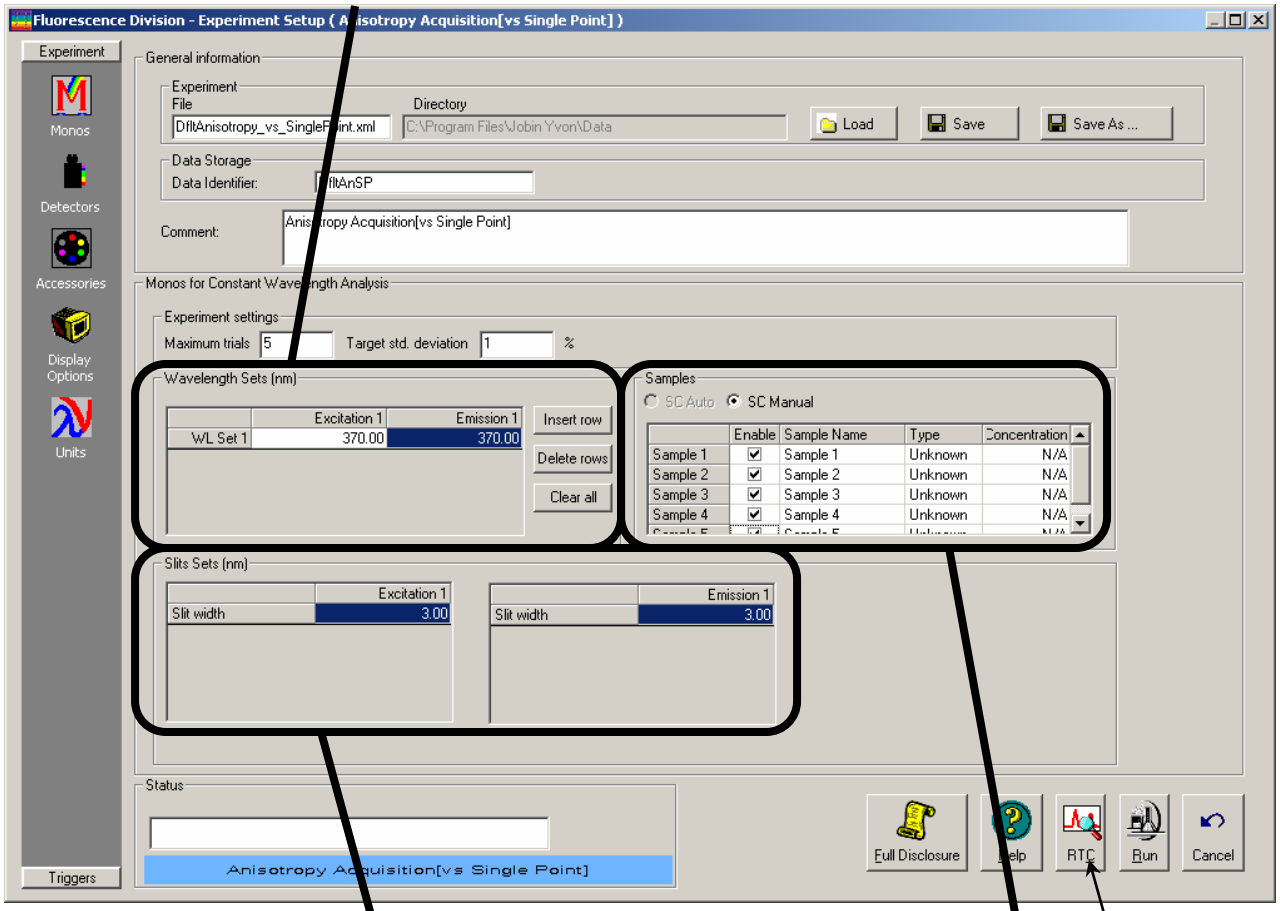
c Choose the *G* factor.
If you leave the G Factor checkbox unchecked, the instrument measures the *G* factor automatically. Or, enable the G Factor checkbox, and enter a *G* factor in the field.

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 `Enter`, and then hit `Tab`. Another row appears.

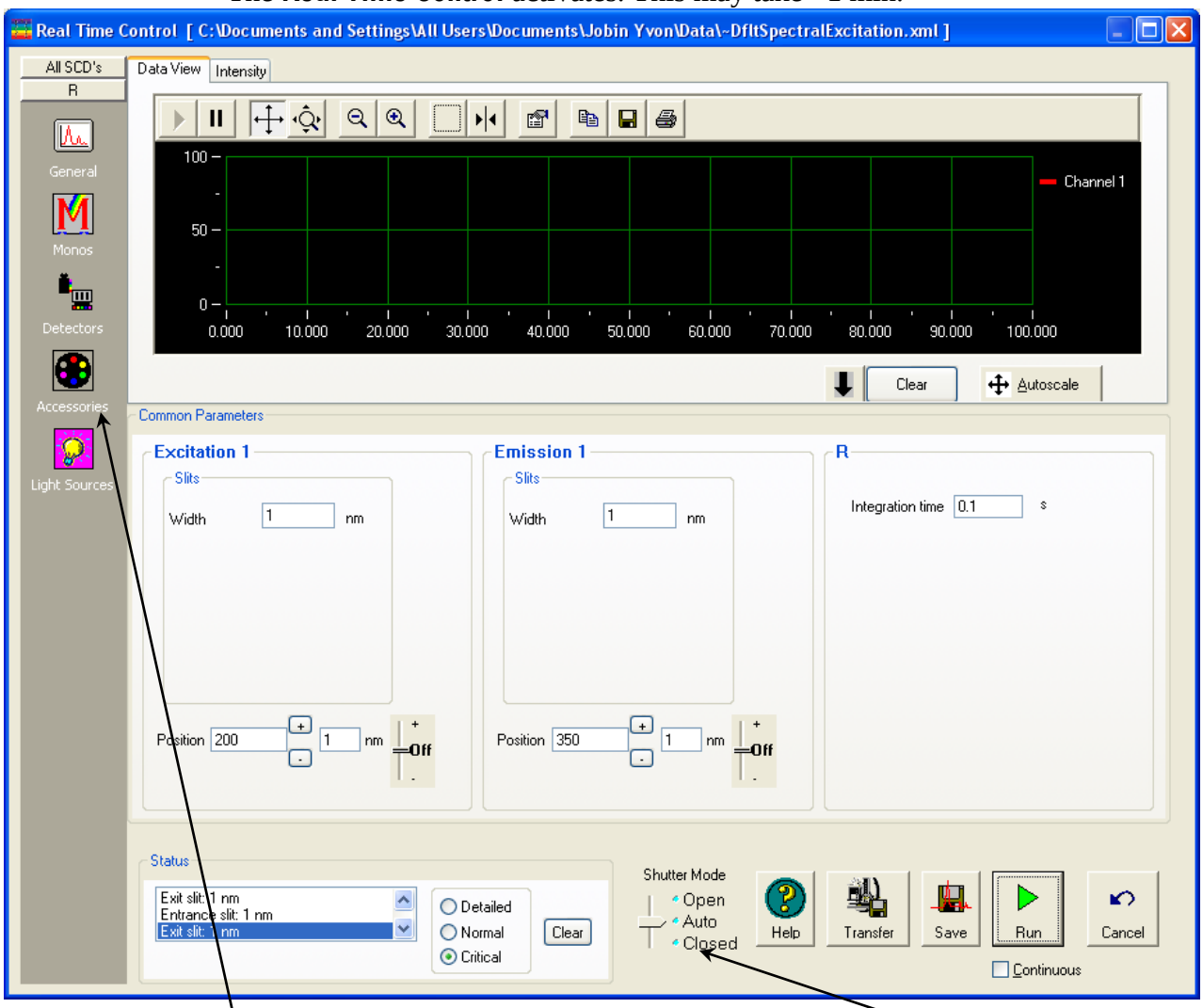
***Note:** If you don't add the rows, then you will get multiple spreadsheets with each sample on a separate spreadsheet.*

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).

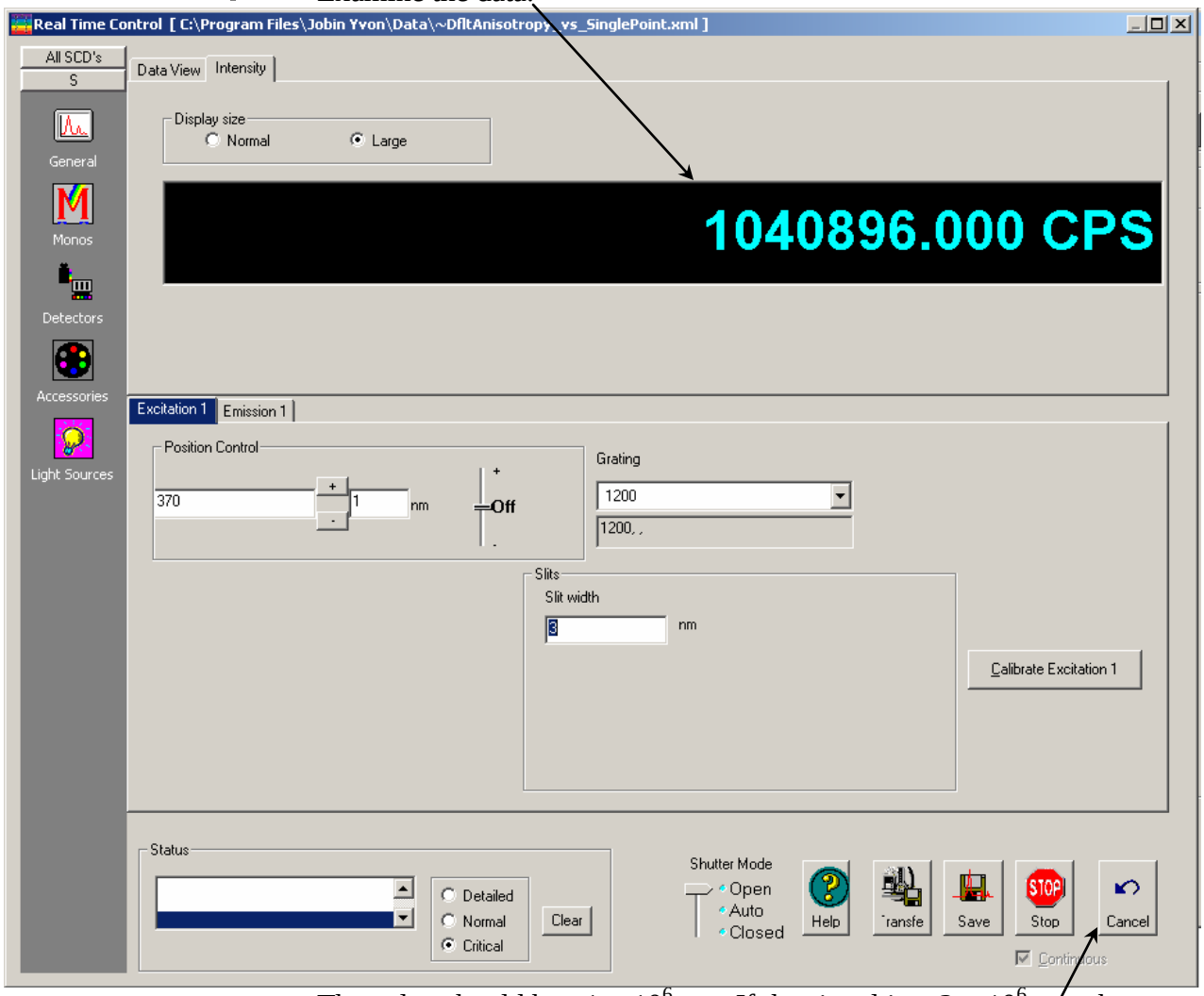
h Click the Monos icon, and review all the monochromator and slit settings.

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

Using Polarizer Alignment

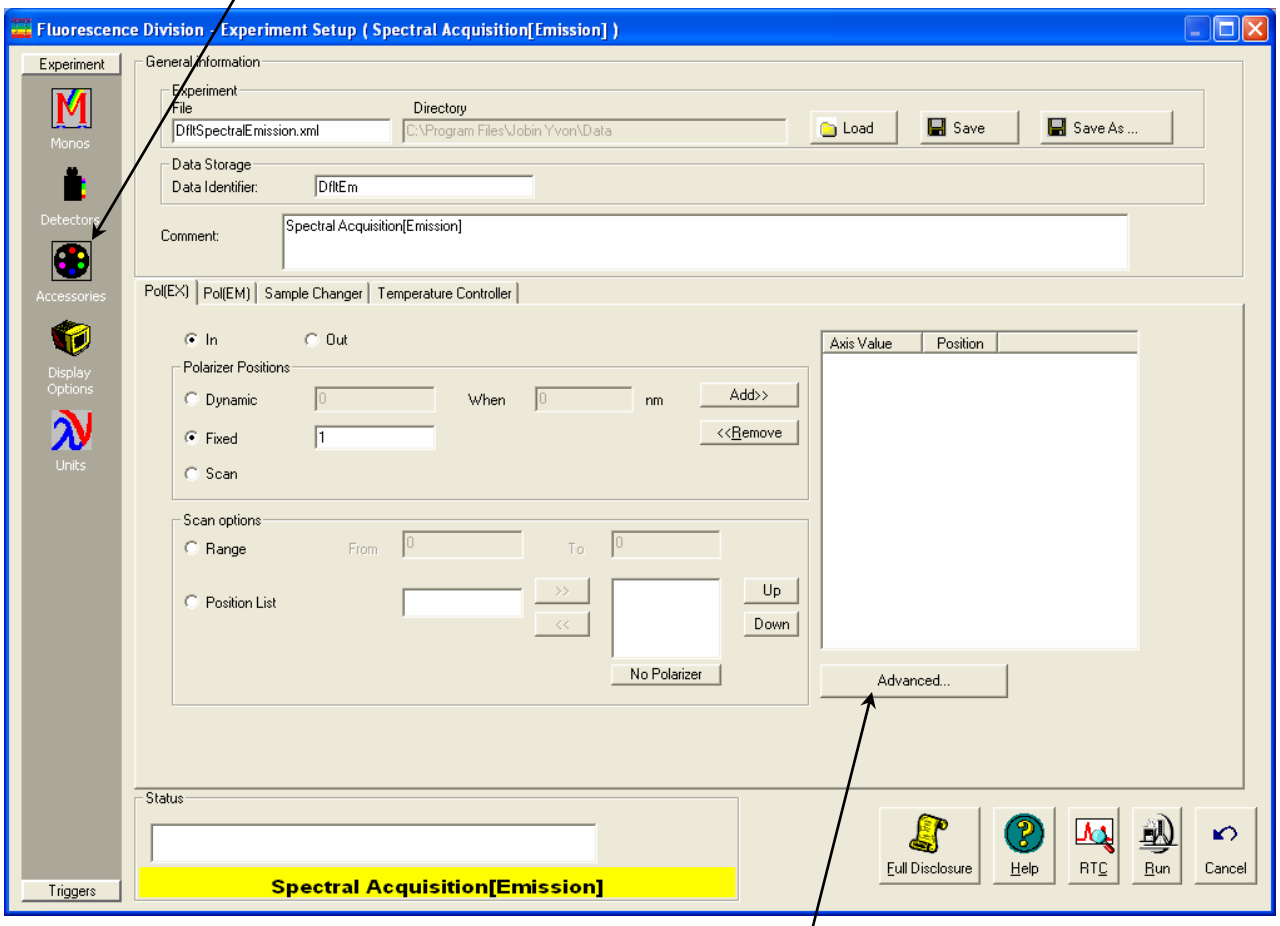
This routine automatically calibrates autopolarizers. Use a sample of Ludox® or glycogen to run the alignment routine. The software rotates the polarizers in 1° increments and locates the optimal positions for each autopolarizer. After completion, the anisotropy for the scattering solution is measured 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.

Automatic method

1 Start FluorEssence™.

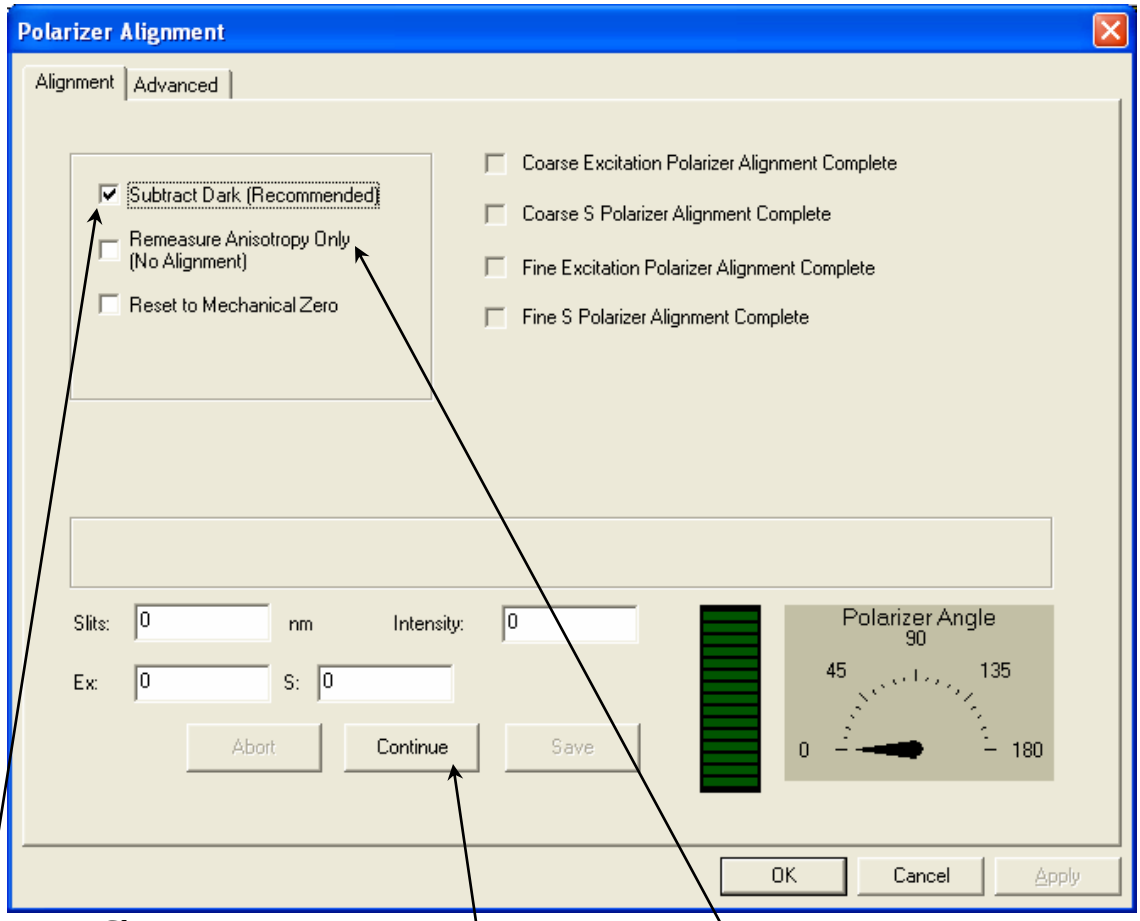
2 Open the **Experiment Setup** window.

3 Click the Accessories icon.



4 Click the Advanced... button.

5 This opens the **Polarizer Alignment** window:



Choose various options:

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

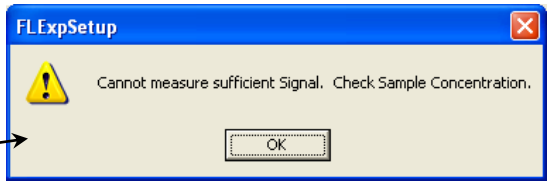
***Note: Do not** check the* Remeasure Anisotropy Only *checkbox.*

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

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

7 Click the Continue button.

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 To quit, hit the Cancel button at any time during the procedure.

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

Caution: *Do not save the new calibration settings if the polarization value < 0.98. Abort the procedure and call the Service Department.*

- Click the Save button to approve the measurement and overwrite previous calibration settings.
- Click the Abort button to quit the procedure and NOT overwrite previous calibration settings.

Physical alignment

Note: *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 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 occur at the calibration position of the polarizers.*

2 Loosen the screws holding 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.

They should not slip, but should allow easy manual rotation.

4 Set the polarizer crystals' position.

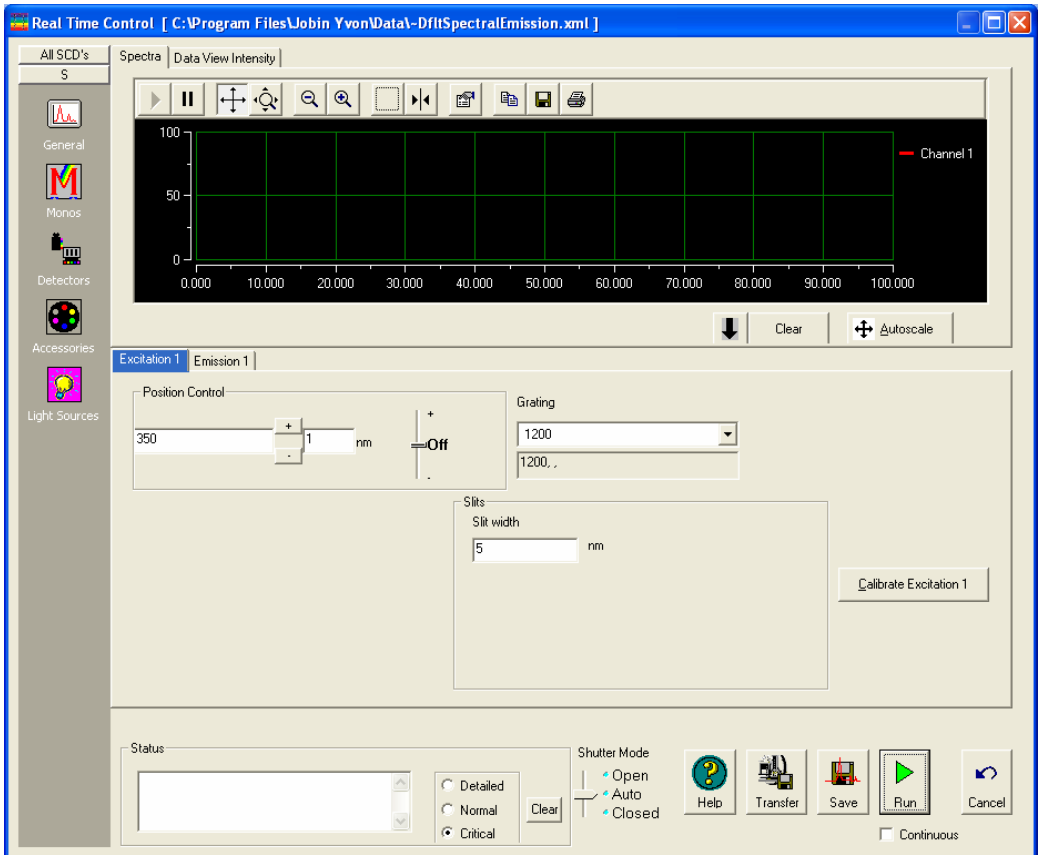
They should protrude from the mounts far enough (~¼″ or ~6 mm) to allow rotation.

5 Start the polarizers and accessory controller.

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

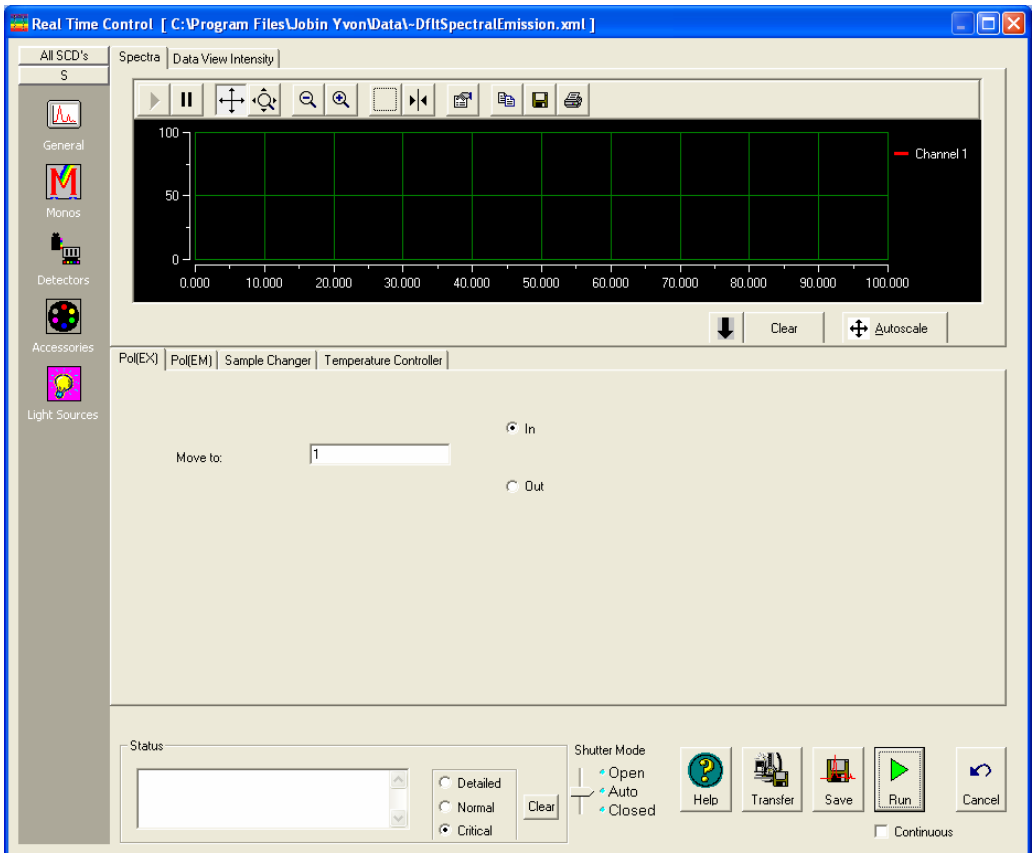
Caution: *Read the Materials Safety Data Sheets (MSDS) before using colloidal silica or glycogen.*

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

5: Using Automated Polarizers

Introduction

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™ User's Guide 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.*

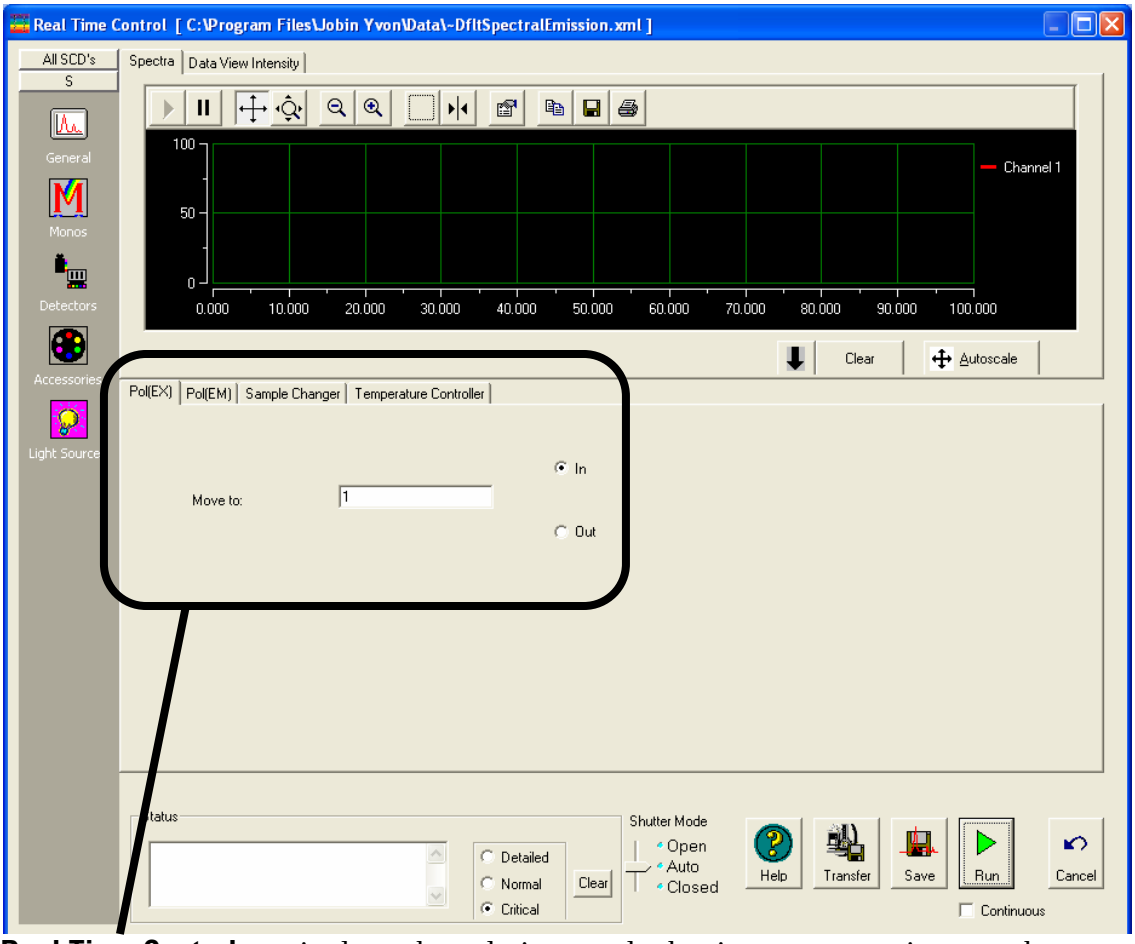
Applications for polarizers

- 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

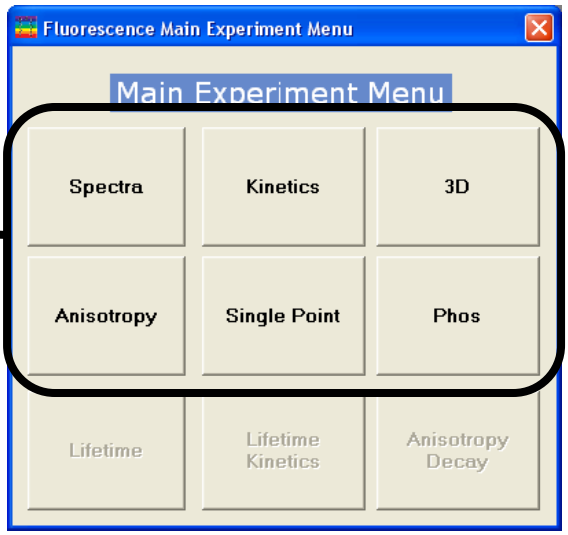


Real Time Control manipulates the polarizers and other instrument settings, to observe and optimize the spectrofluorometer in real time. Under the Accessories icon, each polarizer may be set independently into or out of the optical path under its own index-card tab. A custom angle may be set from 0–180°,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

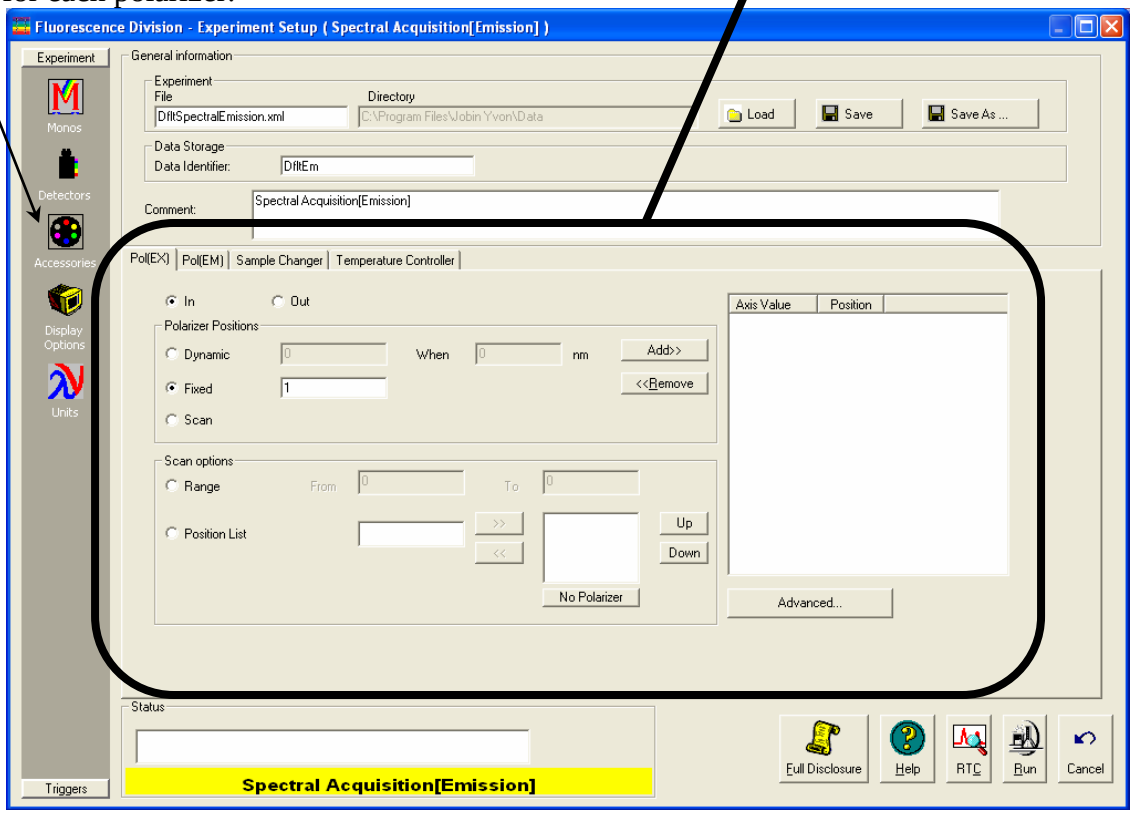
***Note: Real Time Control** is only intended for real-time setup of a scan. Use the **Experiment Setup** window to work at fixed wavelengths.*

Experiment Setup window

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

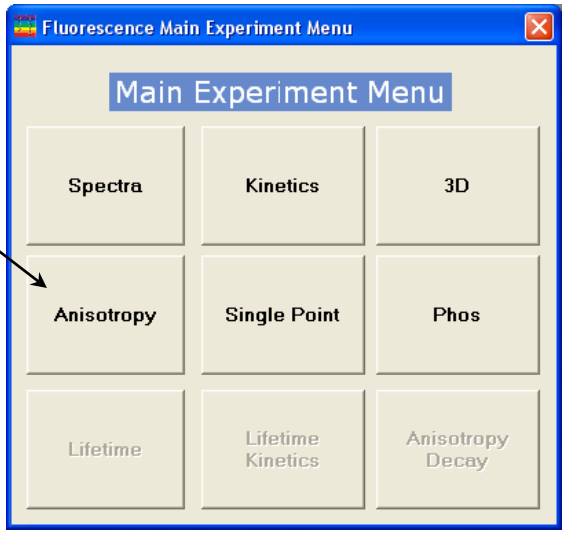


The **Experiment Setup** window appears.
Adjust polarizer parameters under the Accessories icon. One index-card tab appears for each polarizer.

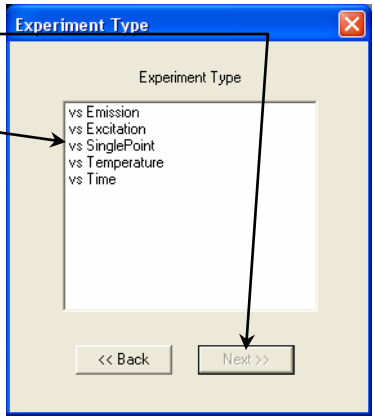


Constant Wavelength Analysis

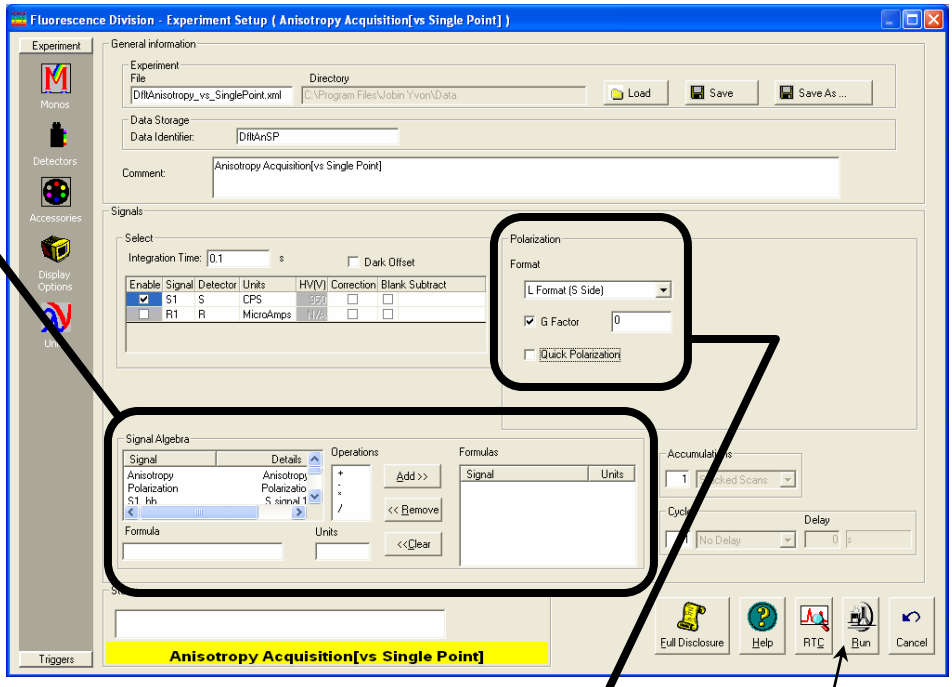
To run a constant-wavelength analysis experiment, that is, to take polarization acquisitions at fixed excitation/emission wavelength-pairs, click the Anisotropy button in the **Fluorescence Main Experiment Menu**.



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.

6: 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 and Nanolog 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 it dry.

Caution: *Refer to the Materials Safety Data Sheet (MSDS) for detailed information on methanol.*

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 provides a fast check that the instrument system is ready to perform measurements.

7: 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 ×160, 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.

8: Tutorial

Introduction

When plane-polarized light is used to excite a fluorophore, and linearly polarized components of the emission are detected, information can be obtained about the size, shape, and flexibility of proteins or other macromolecules. Fluorescent polarization techniques can be used to monitor the binding of small molecules to proteins and other macromolecules, to study conformational changes in proteins, and to study the self-association of peptides and proteins.

This tutorial provides an example of how the polarizers work in a HORIBA Scientific spectrofluorometer. The tutorial examines the polarized fluorescence of Coumarin 153, a fluorescent laser dye, in glycerol solution.

Materials

- Coumarin 153 (laser grade) [CAS # 53518-18-6], can be acquired from Acrōs Organics (Geel, Belgium)
- Reagent-grade glycerol [CAS # 56-81-5]
- 1-cm quartz cuvette
- UV-Visible spectrophotometer (optional)

Caution: *Refer to the Materials Safety Data Sheets (MSDS) for more detailed information on glycerol and Coumarin 153. Wear safety glasses and gloves, and work in a well-ventilated area.*

Prepare the sample.

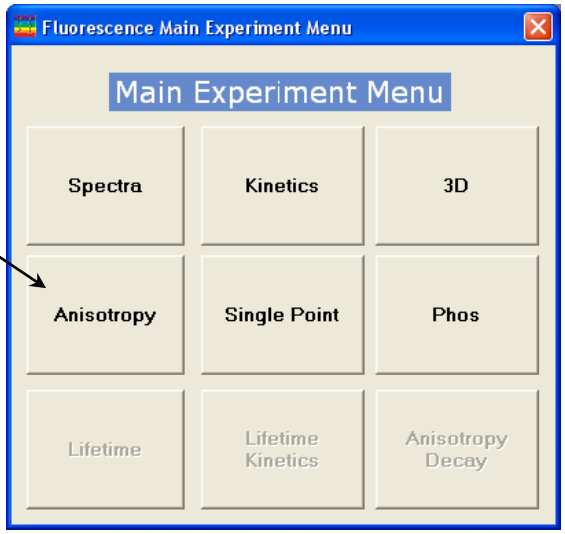
1. Dissolve a small amount of Coumarin 153 in reagent-grade glycerol.
2. Transfer the solution to a 1-cm quartz cuvette.
3. If a UV-Visible spectrophotometer is available, obtain the absorption spectrum of the Coumarin 153 in glycerol.
   Aim for $A \sim 0.1$ at $\lambda_{peak} = 430$ nm. Dilute with more glycerol or add Coumarin 153 as necessary to obtain this absorbance.

Calibrate the instrument.

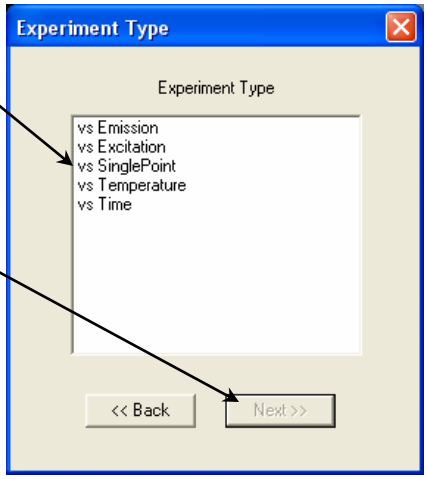
Measure steady-state anisotropy

Multiple and single-point measurements at specified excitation and emission wavelengths of anisotropy, polarization, components of anisotropy (I_{VV}, I_{VH}, I_{HV}, I_{HH}, and I_{VM}) can be displayed in a table format.

1. Put the sample cuvette in the sample compartment, and close the sample compartment's lid.
2. Start FluorEssence™.
   a. Load an instrument configuration with the polarizers.
   b. In the **Fluorescence Main Experiment Menu**, choose the Anisotropy scan-type.



   c. In the **Experiment Type** window, choose the vs SinglePoint sub-type, then click the Next >> button.



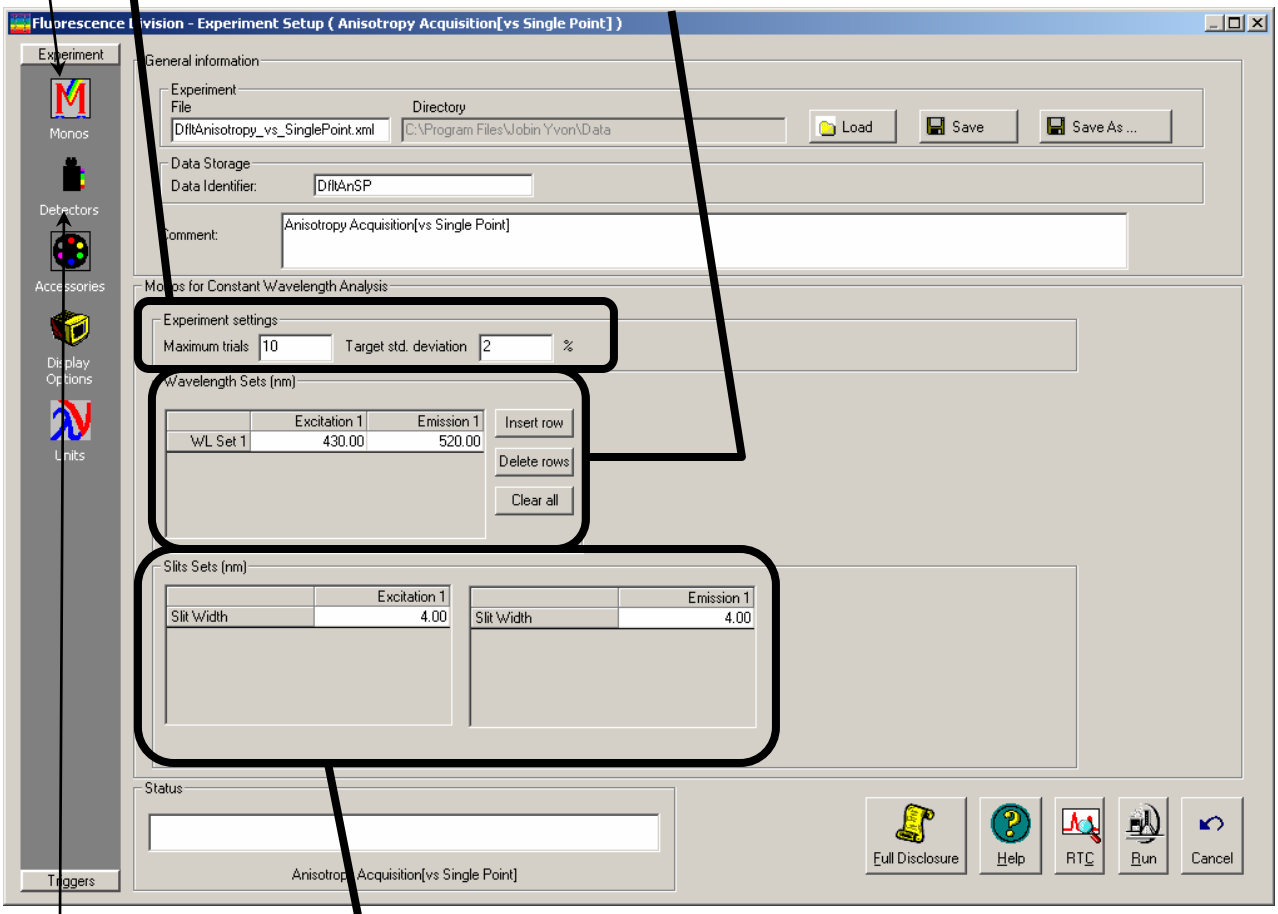
3 Set up the monochromators.

a Click the Monos icon 

.

b In the Experiment settings area, set the Maximum trials to `10` and Target std. deviation to `2`%.

c In the Wavelength Sets area, set the Excitation 1 column to `430` nm and the Emission 1 column to `520` nm.



d In the Slits Sets area, set both Excitation 1 and Emission 1 slit-widths to `4` nm.

Note: *You may add more* Wavelength Sets *by clicking the* Insert row *button.*

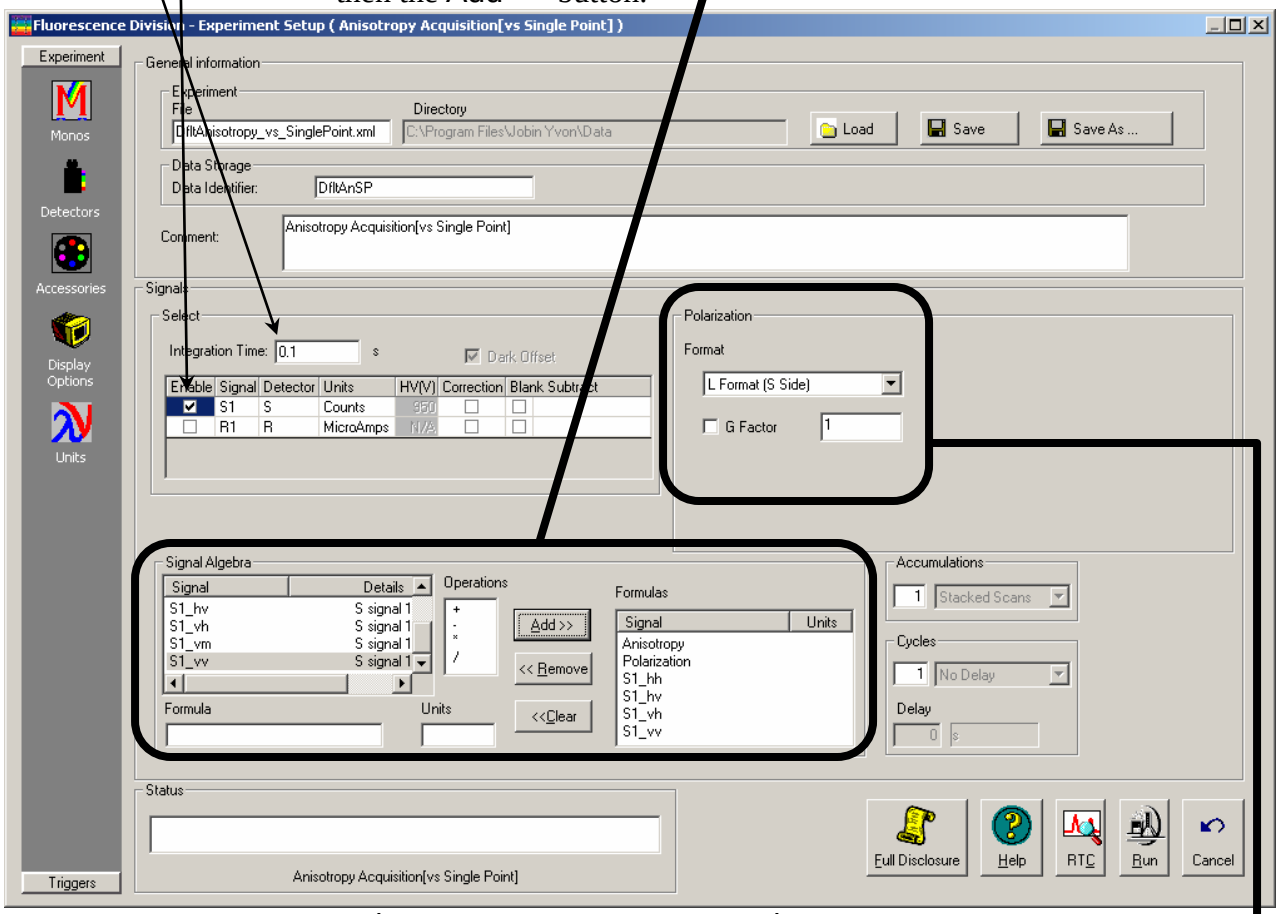
4 Set up the detectors.

a Click the Detectors icon.

b Enter 0.1 s Integration Time.

c Be sure the Enable checkbox next to the S1 detector is active.

d In the Signal Algebra area, in the Signal column, click Anisotropy, then the Add >> button.



Anisotropy appears in the Formulas box.

e Add Polarization, S1_hh, S1_hv, S1_vh, and S1_vv as described in steps b and c.
We will examine all six observables in this tutorial.

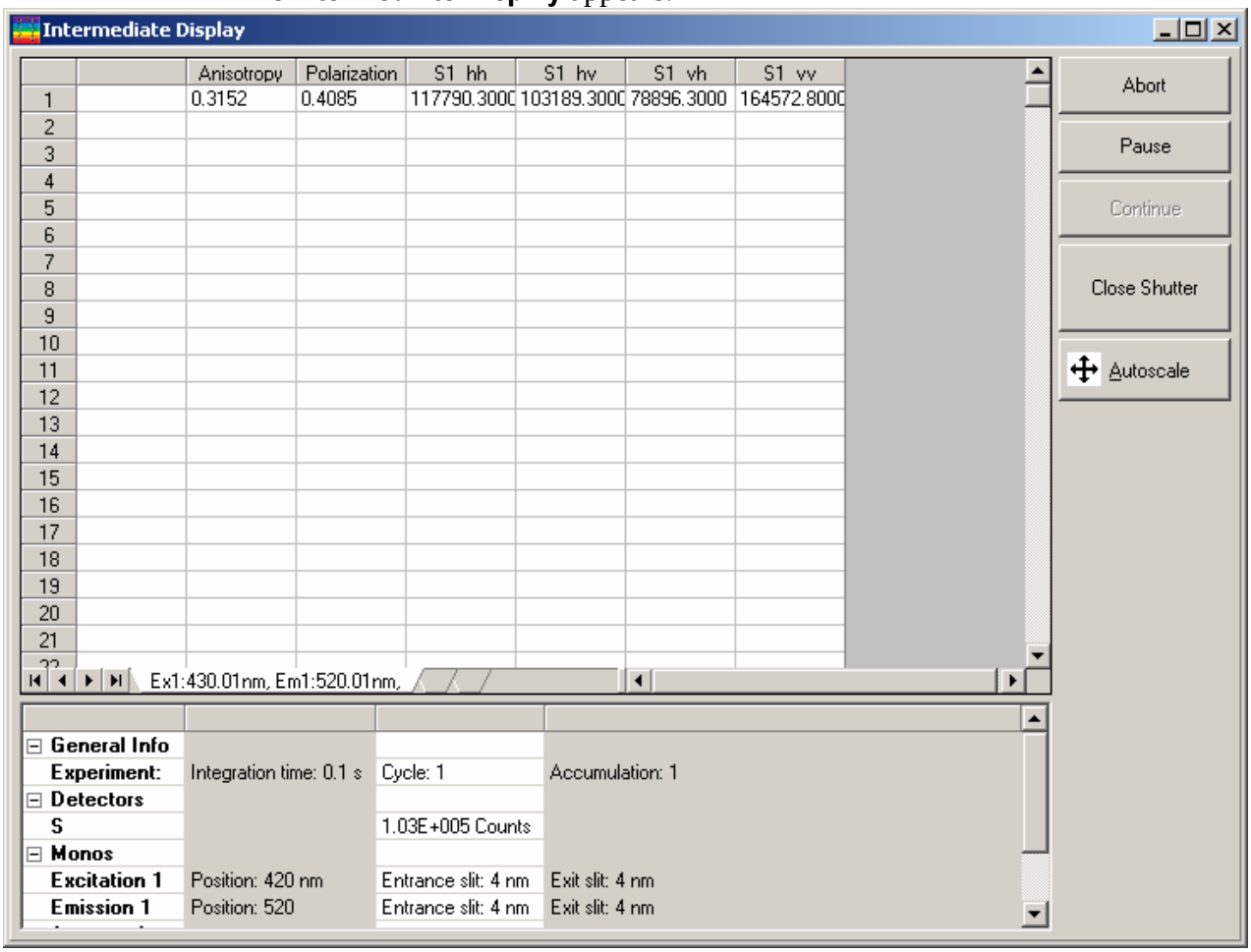
Note: *Vertical is defined as 0°; horizontal is defined as 90°.*

f In the Polarization area, choose the correct format from the drop-down menu.

g Leave the G Factor checkbox inactive.
The instrument will measure the *G* factor automatically.

5 Click the Run button 

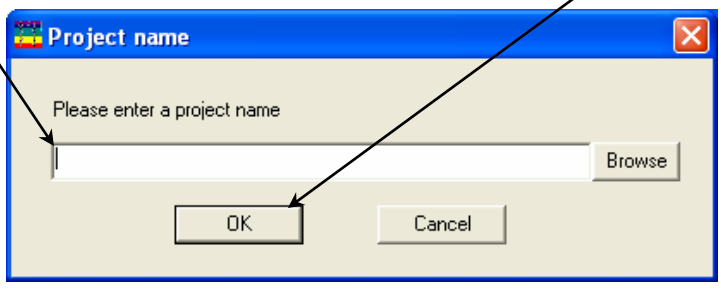
The **Intermediate Display** appears.



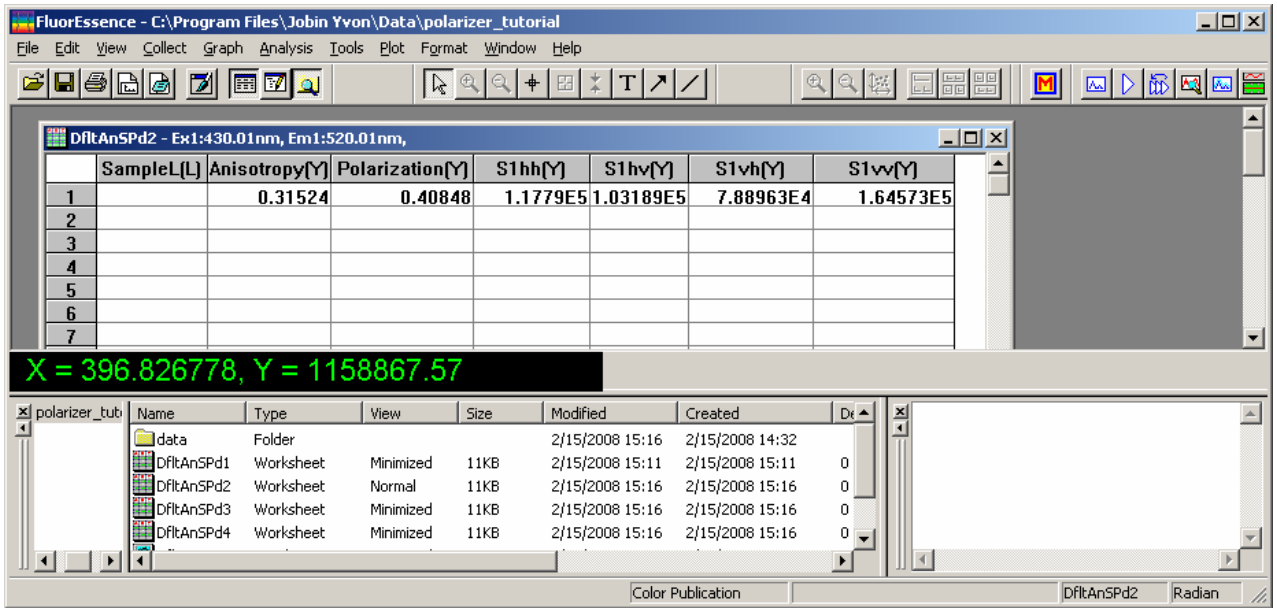
The **Experiment Paused** window may appear. If the sample is not in the sample chamber, insert it and close the lid, then click the OK button.

When the experiment is complete, the **Project Name** window appears.

6 Enter a name for the project, and click the OK button.



The final values appear in a spreadsheet. If you scan more than one wavelength set, FluorEssence™ displays one spreadsheet per signal:


FluorEssence - C:\Program Files\Jobin Yvon\Data\polarizer_tutorial
File Edit View Collect Graph Analysis Tools Plot Format Window Help
DfltAnSPd2 - Ex1:430.01nm, Em1:520.01nm,
SampleL(L) Anisotropy(Y) Polarization(Y) S1hh(Y) S1hv(Y) S1vh(Y) S1vv(Y)
1 0.31524 0.40848 1.1779E5 1.03189E5 7.88963E4 1.64573E5
2
3
4
5
6
7
X = 396.826778, Y = 1158867.57
polarizer_tut
Name Type View Size Modified Created
data Folder 2/15/2008 15:16 2/15/2008 14:32
DfltAnSPd1 Worksheet Minimized 11KB 2/15/2008 15:11 2/15/2008 15:11 0
DfltAnSPd2 Worksheet Normal 11KB 2/15/2008 15:16 2/15/2008 15:16 0
DfltAnSPd3 Worksheet Minimized 11KB 2/15/2008 15:16 2/15/2008 15:16 0
DfltAnSPd4 Worksheet Minimized 11KB 2/15/2008 15:16 2/15/2008 15:16 0
Color Publication
DfltAnSPd2
Radian

Measure polarization scan

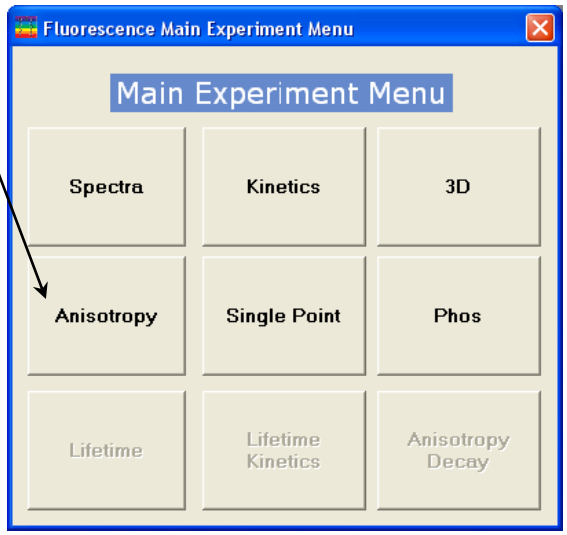
A polarization scan records the anisotropy versus wavelength. This tutorial will record data incrementally by wavelength for the four different polarizer orientations, using the same Coumarin 153 sample.

1 Put the sample cuvette in the sample compartment, and close the sample compartment's lid.

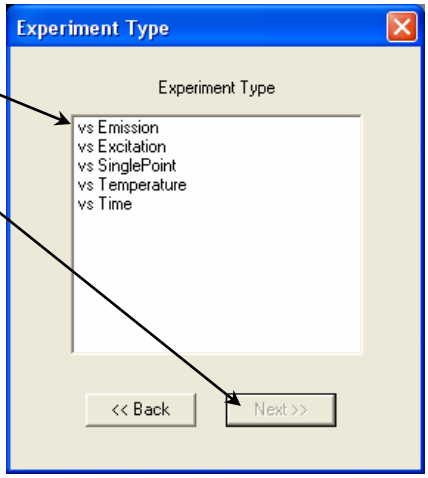
2 Start FluorEssence™.

a Load an instrument configuration with the polarizers.

b In the **Fluorescence Main Experiment Menu**, choose the Anisotropy scan-type.



c In the **Experiment Type** window, choose the vs Emission sub-type, then click the Next >> button.



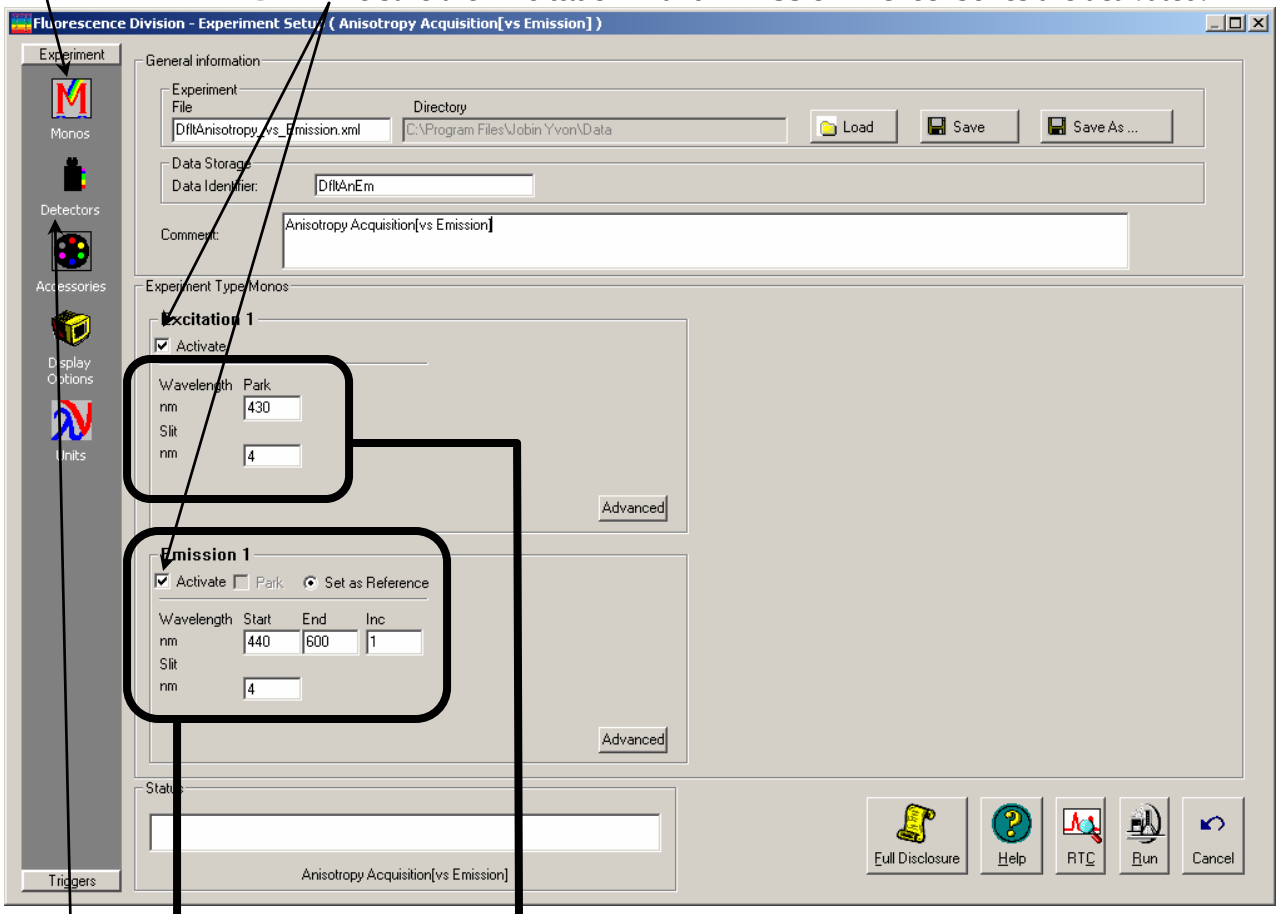
The **Experiment Setup** window appears:

3 Set up the instrument.

a Click the Monos icon 

.

b Be sure the Excitation 1 and Emission 1 checkboxes are activated.



c Set the excitation monochromator to Park at 430 nm, with 4 nm Slit.

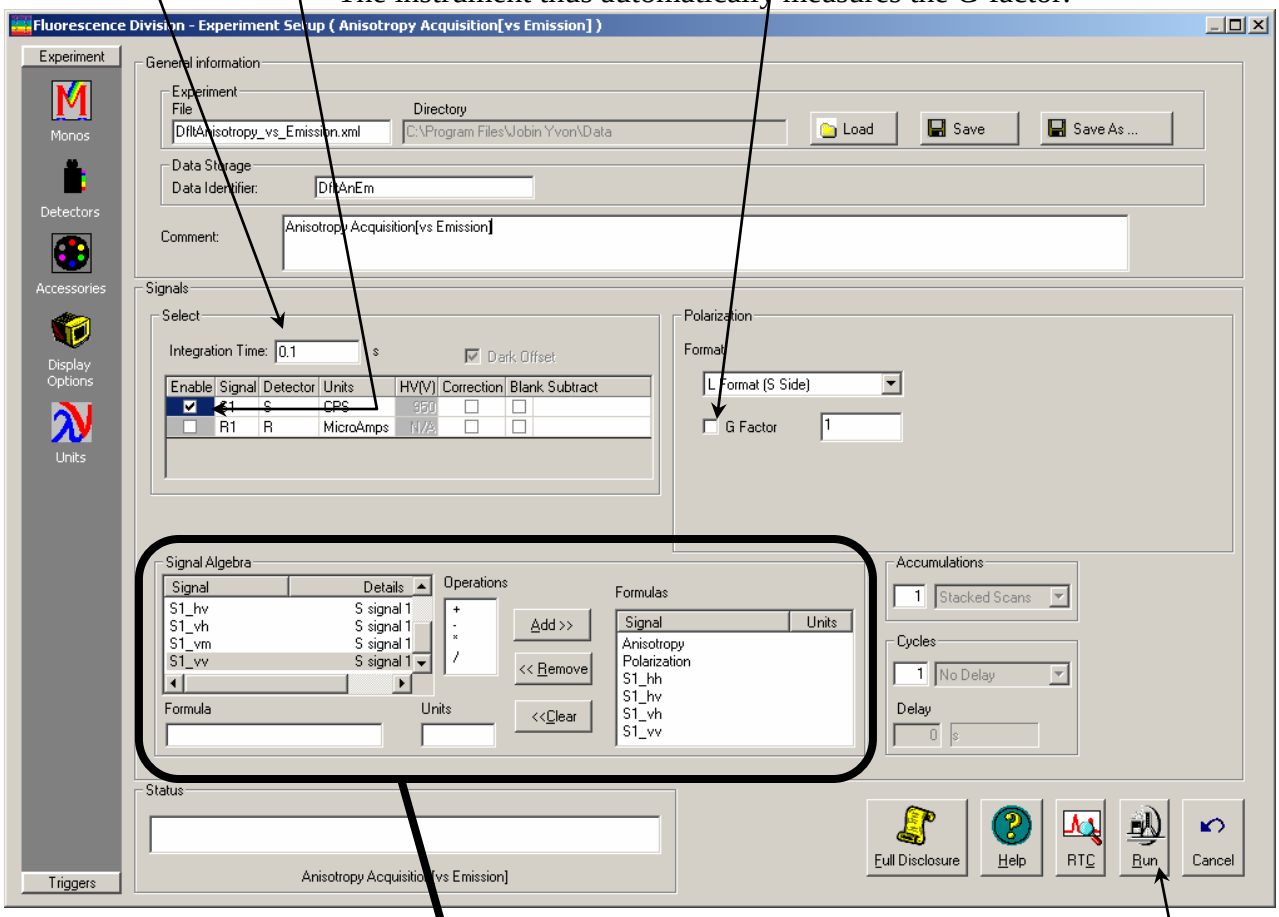
d Set the emission monochromator's Start at 440 nm, its End to 600 nm, the Inc (increment) to 1 nm, and the Slit to 4 nm.

e Click the Detectors icon.

f Set the Integration Time to `0.1` s.

g Be sure the S1 signal Enable checkbox is checked.

h Deactivate the G Factor checkbox.
The instrument thus automatically measures the *G* factor.

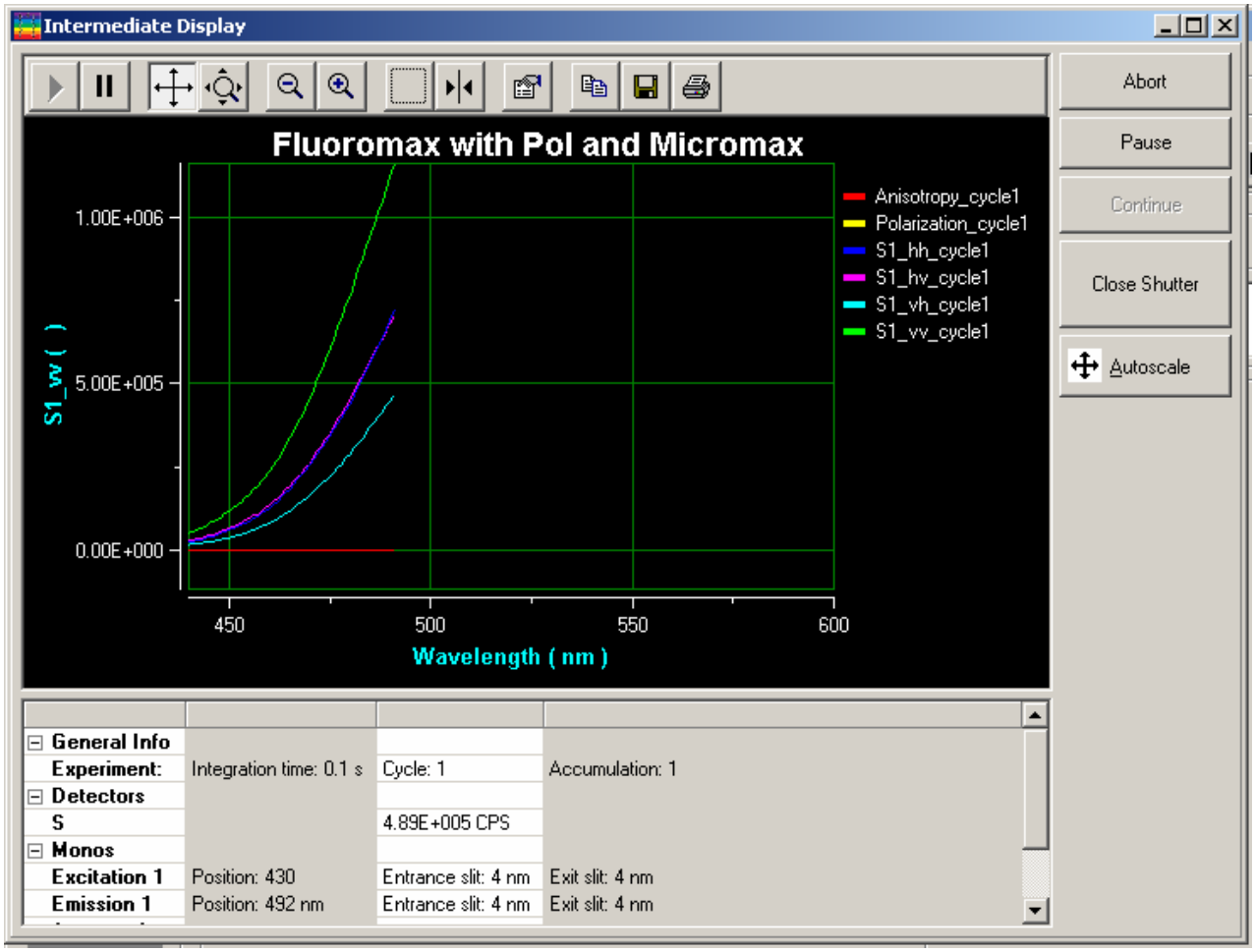


i In the Signal Algebra area, click each signal and then the Add >> button: Anisotropy, Polarization, S1_hh, S1_hv, S1_vh, and S1_vv. This adds each signal to the Formulas table. We will examine all six observables in this tutorial.

Note: *In the* Accessories *icon, verify that the polarizers are automatically into the optical path for the scan. They cannot be removed, except using the* RTC *button. Vertical is defined as 0°; horizontal is defined as 90°.*

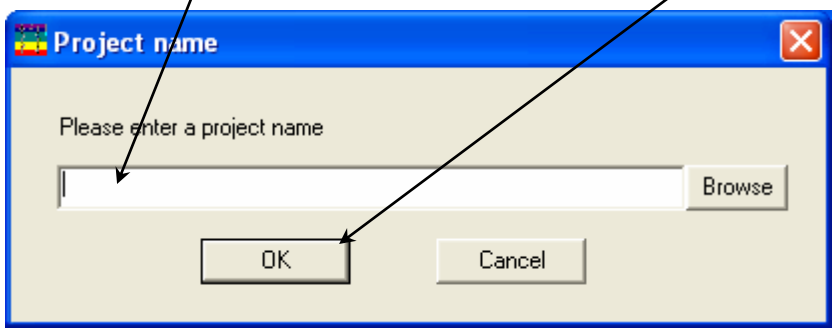
4 Click the Run button  .

The scan starts and the **Intermediate Display** appears; the data are updated in real time as the scan progresses:

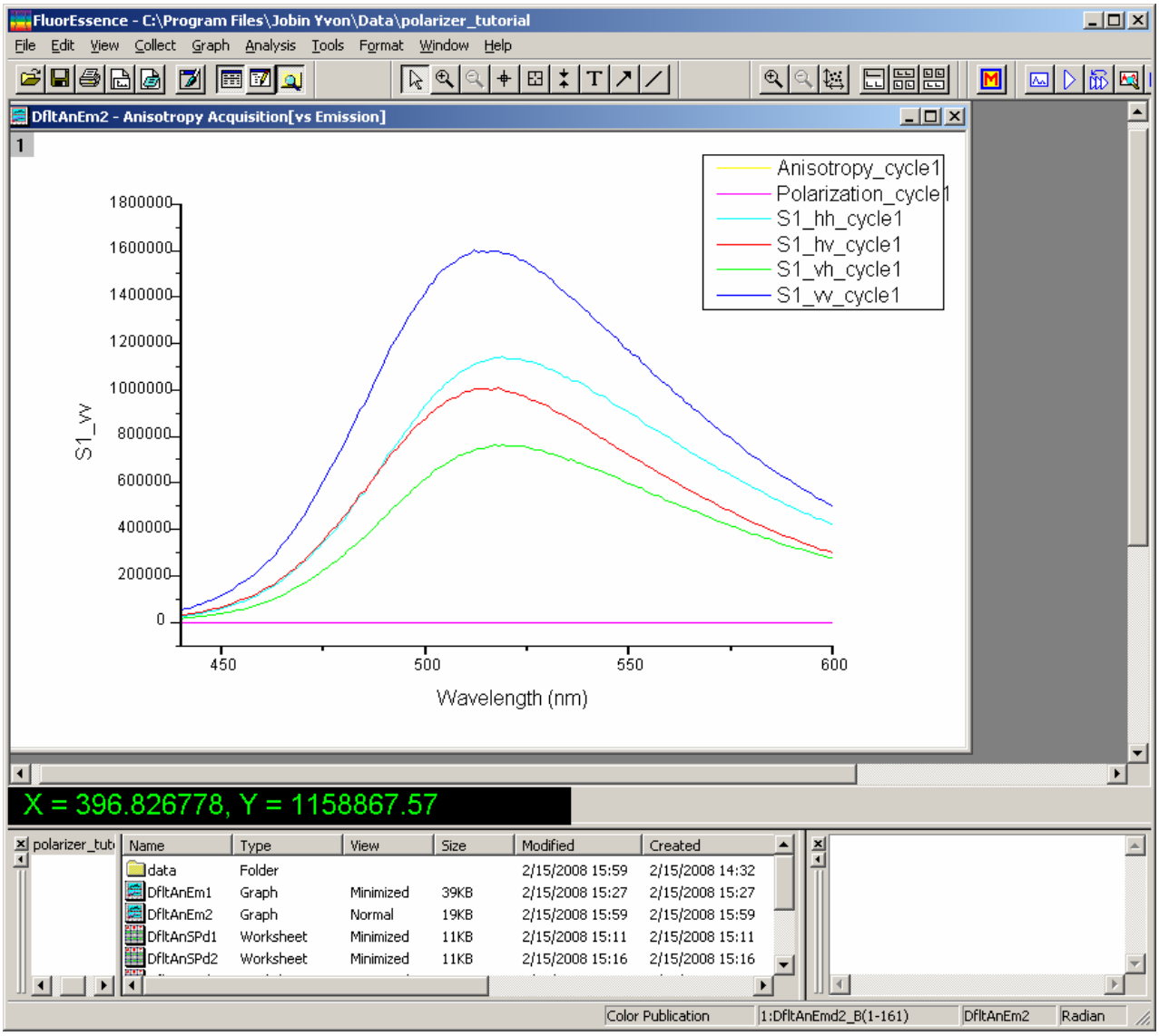


The software takes four scans, one per polarizer-pair orientation, plus FluorEssence ™ calculates the anisotropy and polarization values. When complete, the **Project Name** window appears:

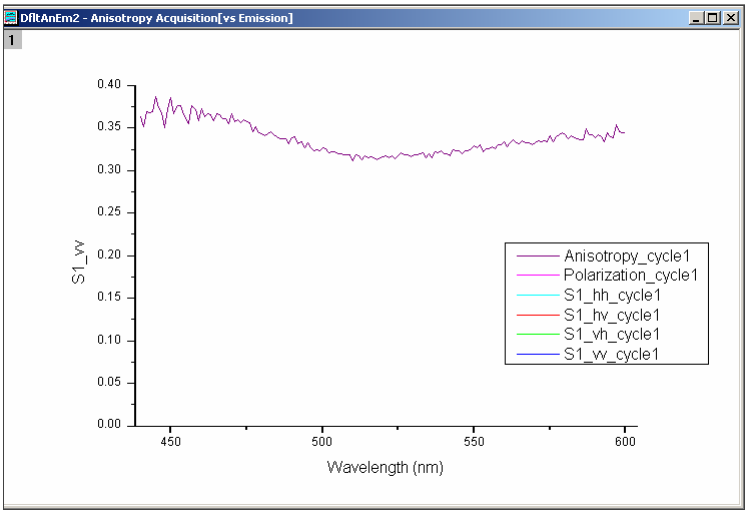
5 Enter a name for the project, and click the OK button.



The results appear:



We can expand the *y*-axis to better see the anisotropy values:



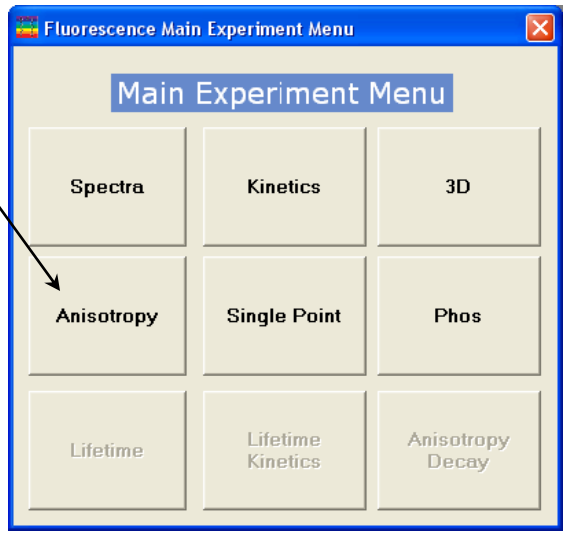
Now we examine the 515 nm peak from the VV spectrum:

6 Set up the experiment.

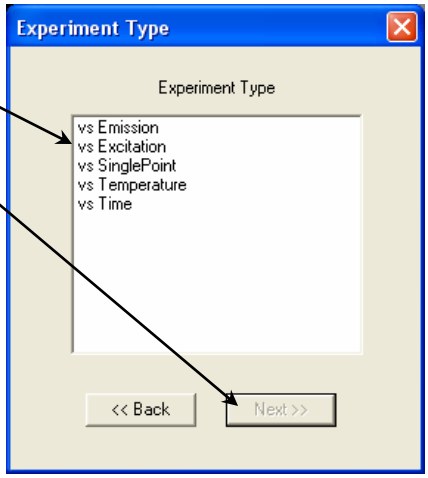
a Click the Experiment Menu button  .

b The **Fluorescence Main Experiment Menu** appears.

c Choose the Anisotropy scan-type.



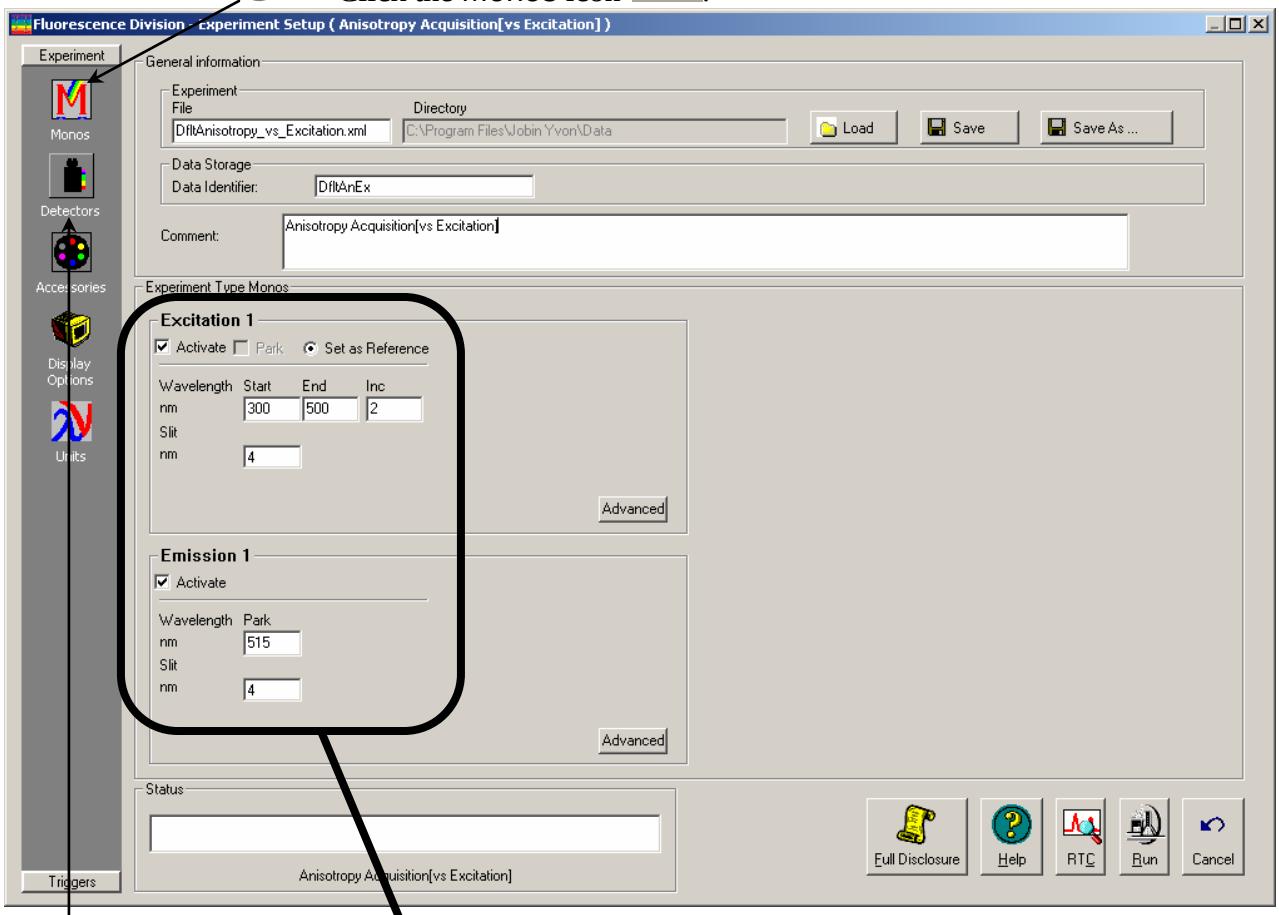
d In the **Experiment Type** window, choose the vs Excitation subtype, then click the Next >> button.



The **Experiment Setup** window appears:

e Click the Monos icon 

.



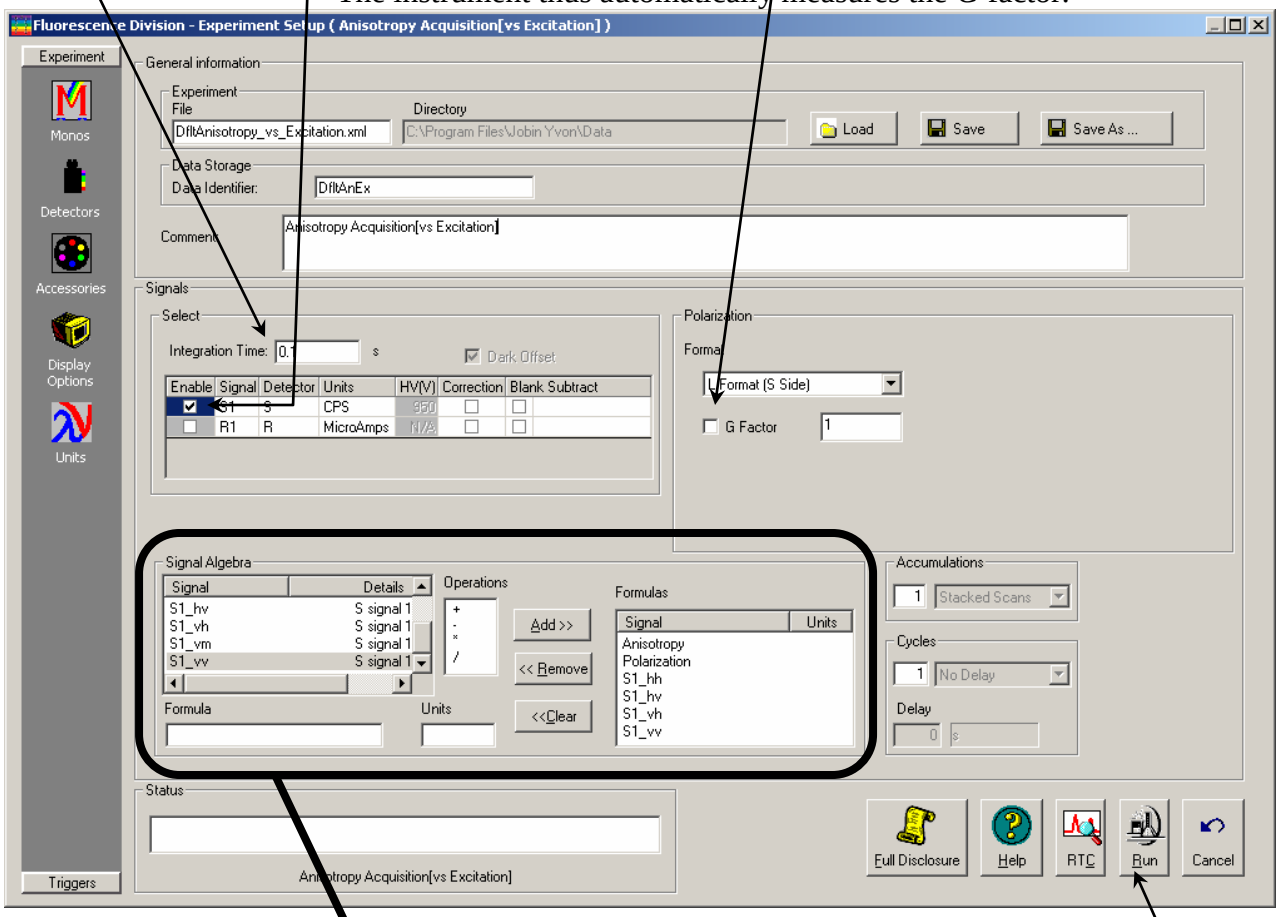
f Set up the monochromators as shown above:
Excitation monochromator Start at 300, End at 500, Inc at 2, Slit at 4.
Emission monochromator Park at 525, Slit at 4.

g Click the Detectors icon.

h Set the Integration Time to `0.1` s.

i Be sure the S1 signal Enable checkbox is checked.

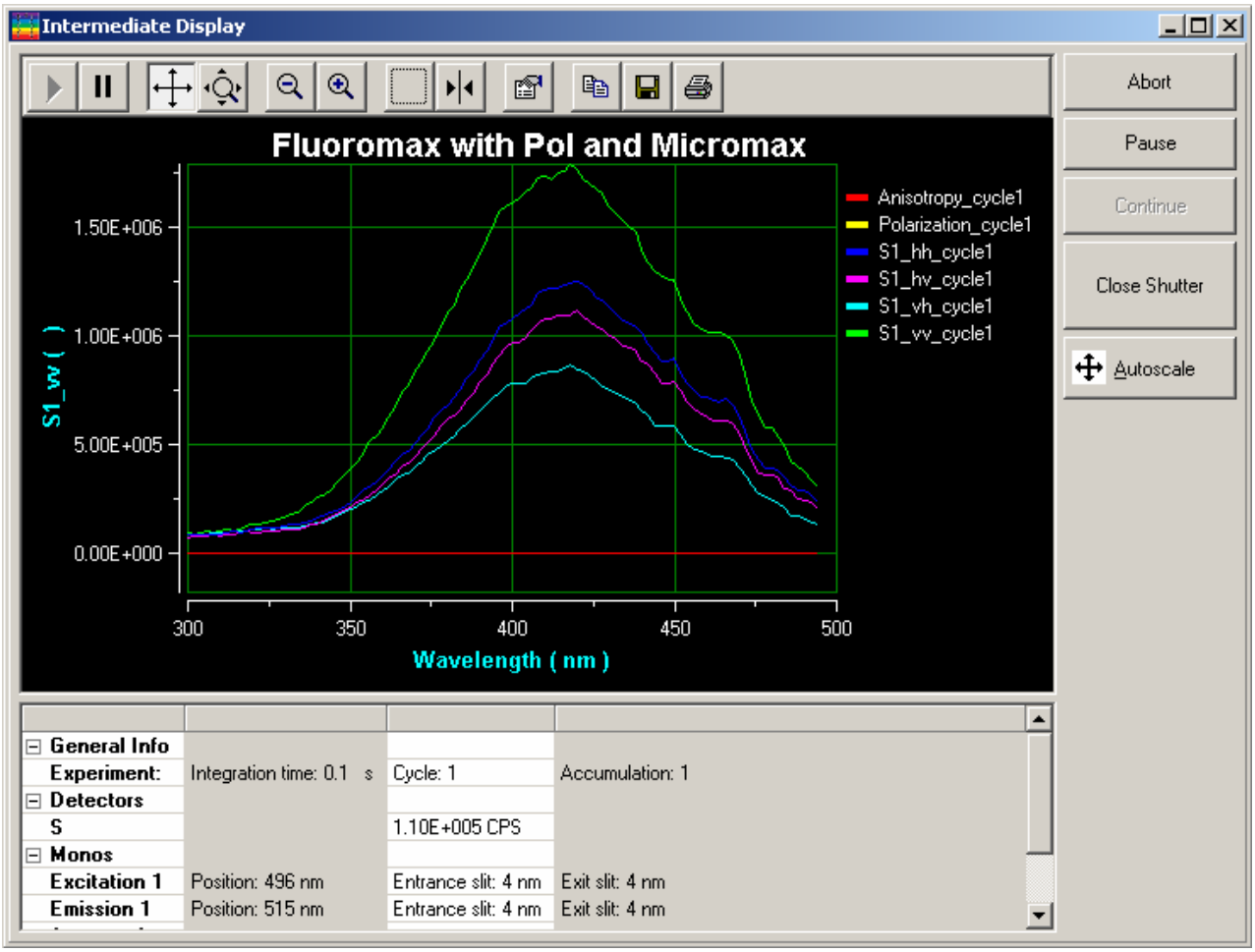
j Deactivate the G Factor checkbox.
The instrument thus automatically measures the *G* factor.



k In the Signal Algebra area, click each signal and then the Add >> button: Anisotropy, Polarization, S1_hh, S1_hv, S1_vh, and S1_vv. This adds each signal to the Formulas table. We will examine all six observables in this tutorial.

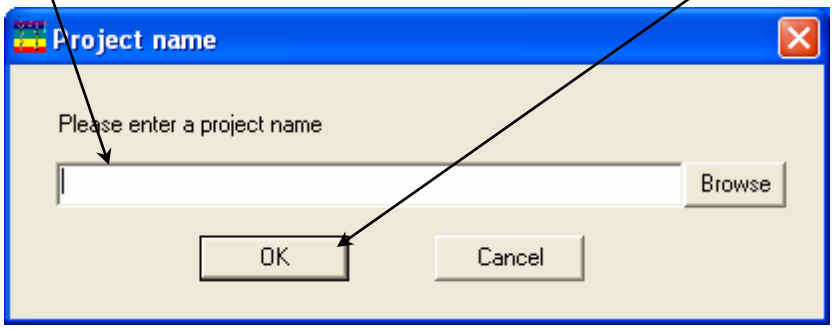
7 Click the Run button  to start the experiment.

The scan starts and the **Intermediate Display** appears; the data are updated in real time as the scan progresses:

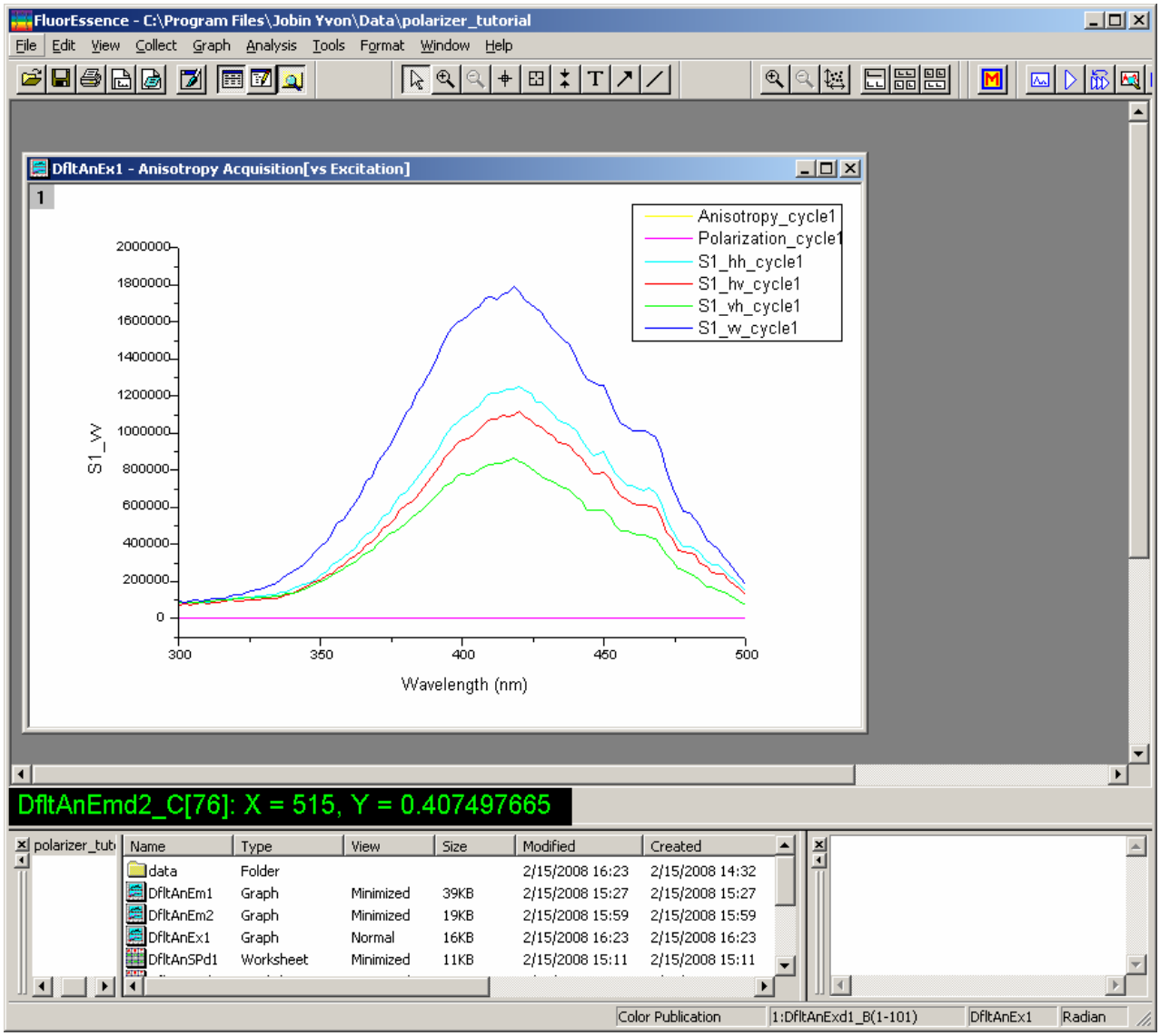


The software takes four scans, one per polarizer-pair orientation, plus FluorEssence ™ calculates the anisotropy and polarization values. When complete, the **Project Name** window appears:

8 Enter a name for the project, and click the OK button.



The results appear:


FluorEssence - C:\Program Files\Jobin Yvon\Data\polarizer_tutorial
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Anisotropy_cycle1
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9: Glossary

Anisotropy ($\langle r$) — The directional nature of fluorescence that is additive across different populations of fluorophores. The linearly polarized component's intensity divided by the total fluorescence intensity. For a fluorophore, the allowed anisotropy range is $+0.4 \geq \langle r \geq -0.02$.

Anisotropy decay — The change in anisotropy during the lifetime of the excited state. Anisotropy decay provides insight into the rotational properties and environment of fluorescent molecules. To measure anisotropy decay, record the phase and ratio of modulated amplitudes across a frequency range. The values are fitted with the mean decay time.

Autopolarizer — A device containing a motorized mounted polarizer in order to rotate the polarizer automatically. HORIBA Scientific autopolarizers are computer-controlled, and may be set to any angle from 0–360°.

Dynamic depolarization — see **Anisotropy decay**

Film polarizer — A polarizer made of stretched film. These polarizers generally have poor performance in the UV, and exhibit poor stability with age, because of photodegradation.

Fluorescence — The emission of light by a substance because of absorption of a shorter-wavelength radiation. The emission occurs during the transition of electrons form the excited singlet state down to the ground state. Fluorescence usually occurs on the nanosecond time-scale. Lifetimes range from 10^{-15}–10^{-6} s.

Fluorescence lifetime — The average length of time that a molecule remains in the excited state.

Fluorophore — A molecule that exhibits fluorescence emission.

Glan-Thompson polarizer — A rugged, high-performance polarizer consisting of a cemented prism with its cut side parallel to the optical axis. The Glan-Thompson polarizers used in HORIBA Scientific polarizer accessories exhibit very high extinction ratios, have relatively wide acceptance angles, and transmit light from 215 nm to beyond 2000 nm.

Intrinsic anisotropy (r_0) — Anisotropy observed for a molecule without rotational motion. Usually measured with the fluorophore completely restricted by holding at cryogenic temperatures. Theoretically, $r_0 = +0.4$, reduced by orientations, not considering molecular orientations.

L-format polarization — Polarization measurement that uses one excitation and one emission polarizer. The emission polarizer must be rotated from V to H to collect polarization data. The excitation polarizer also must be rotated, unless the *G* factor is entered in the experiment definition.

Limiting anisotropy (r_∞) A limit to the decay of the anisotropy that results from the presence of a hindering fluorophore. The hindered motion restricts the angular range of movement of a fluorescent molecule.

Magic angle Position of the excitation and emission polarizers that removes the polarization bias of a sample from a measurement. There are two magic-angle setups for polarizers:

- Excitation polarizer = 35°, with scrambler plate in, and no emission polarizer.
- Excitation polarizer = 0° and the emission polarizer = 55°.

Millipolarization (m*P*) 1/1000 of a polarization unit, or 1 *P* = 1000 m*P*. Commonly used for small changes in polarization.

Monochromator The component in a spectrometer system that is scanned to collect the excitation and emission spectra. Important factors in the choice of a monochromator are: stray-light rejection, resolution, and throughput.

Photoselection Excitation of a subpopulation of fluorophores with their transition excitation oscillators aligned along the excitation polarization vector of the incident beam.

Polarization The linearly polarized component's intensity divided by the natural fluorescence component's intensity. Allowable range for a fluorophore is $+0.5 \geq P \geq -0.33$.

Polarization ratio Defined as polarization ratio = ———, the polarization ratio may be used to verify the alignment and performance of autopolarizers. The polarization ratio may be used to determine the polarization, *P*, or anisotropy, ⟨*r*, of a sample.

Raman scatter Scattering caused by vibrational and rotational transitions. Raman bands are generally red-shifted relative to the incident radiation. The difference in energy between the Raman peak and the incident radiation is constant in energy units (cm^{-1}). For polarization measurements, Raman scatter appears with an anisotropy or polarization of ~1.

Raw polarization The individual intensities, I_{VV}, I_{HH}, I_{VH}, and I_{HV}, measured with the different permutations of the polarizer positions. These are used to calculate anisotropy, polarization, or the polarization ratio.

- I_{VV} is intensity with both polarizers at 0°;
- I_{HH} is intensity with both polarizers at 90°;
- I_{VH} is intensity with polarizers crossed (excitation = vertical; emission = horizontal), used to find *G* factor;
- I_{HV} is intensity with polarizers crossed (excitation = horizontal; emission = vertical), used to find *G* factor.

Rayleigh scatter Scattering by particles much smaller than the wavelength of the incident light. Rayleigh-scattered light is the same energy as the incident light. The scattered intensity is inversely proportional to the 4^{th} power of the incident wavelength. For polarization measurements, Rayleigh scatter appears with an anisotropy or polarization of ~1.

Spectrofluorometer Instrument consisting of a broadband light source, at least two scanning monochromators (excitation and emission), sample compartment, and detectors dedicated to measuring fluorescence spectra from samples. HORIBA Scientific spectrofluorometers are available in several configurations. These systems allow a wide range of fully automatic scans of samples in a variety of measurement geometries.

T-format polarization Polarization measurements that employ three polarizers—one excitation and two emission. The emission polarizers are set to V and H, while the excitation is set to V. This allows fast acquisition of polarization measurements when the *G* factor is known, because the polarizers need not be rotated.

10: CE Compliance Information

Declaration of Conformity

Manufacturer:	HORIBA Instruments Incorporated
Address:	3880 Park Avenue Edison, NJ 08820 USA
Product Name:	Automated Polarizers
Product Model Number:	FL-1044 and FL-1045

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

11: Index

Key to the entries:

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Arial font	command, menu choice, or data-entry field
Arial Condensed Bold font	dialog box
`Courier New` font	file name or extension

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Scientific

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www.horiba.com/scientific

[Design Concept]

The HORIBA Group application images are collaged in the overall design.
Beginning from a nano size element, the scale of the story develops all the way to the Earth with a gentle flow of the water.