Fluorolog®

Tau-3 Lifetime System

Operation Manual

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0: Introduction

About the Fluorolog®-Tau-3 Lifetime System
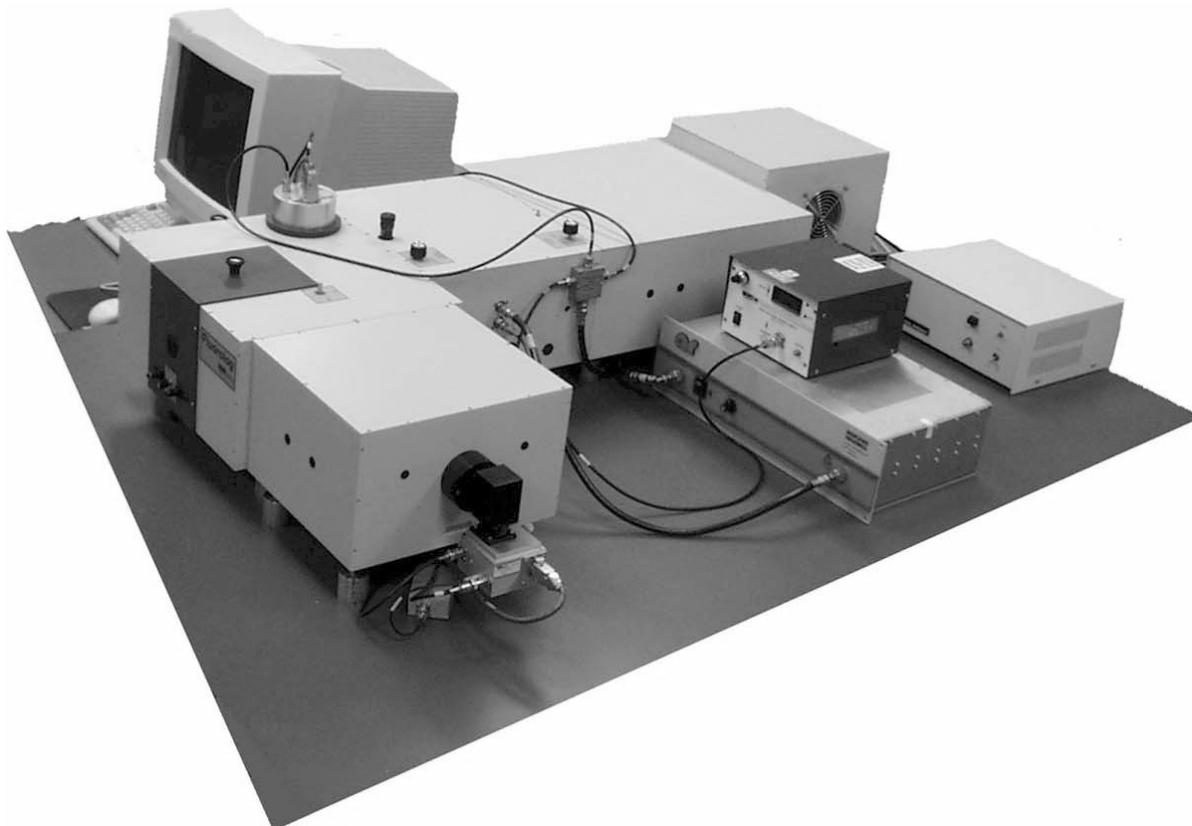
The main parts of the Fluorolog® Tau-3 Lifetime System are:

- a personal computer
- SpectrAcq controlling computer
- state-of-the-art optical components
- DataMax for Windows™, the driving software.

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

This manual explains how to operate and maintain a Fluorolog® Tau-3 Lifetime System. The manual also provides tutorials to learn how to operate the system. For a complete discussion of DataMax, refer to the *DataMax Data Collection Handbook* (contains data-acquisition information) and the *Grams/32® User's Guide* (contains post-processing instructions for data manipulation), which accompany the system. For polarization measurements, see the *Spex® Polarizers Manual*. Hardware information is given in the *Fluorolog®-3 Operation Manual*.

Chapter overview

Symbols used in this manual

Certain symbols are used throughout the text for special conditions when operating the instruments:

Warning: A hazardous condition exists, or danger exists that could damage the equipment. Jobin Yvon®, Inc., is not responsible for damage arising out of improper use of the equipment.

Note: General information is given concerning operation of the equipment.

1: Theory of Operation

Introduction

The Fluorolog® Tau-3 Lifetime System is used to measure the decay of fluorescence or anisotropy by a population of fluorescent molecules. The instrument design uses cross-correlation frequency-domain measurements of the phase shift and demodulation factors across a frequency range. The resulting data are fitted to solve for the respective decays found in the population of molecules.

This chapter explores measurements of fluorescence lifetimes and anisotropy decays, explains the theory behind data acquisition and fitting algorithms, and reviews the specific hardware used.

Fluorescence lifetime measurements

A *fluorescence lifetime* is the mean length of time between absorption of a photon, and emission of light by a population of molecules. Fluorescence lifetimes generally are about 10^{-9} s, although decays can occur as short as 10^{-11} s, and as long as 10^{-7} s. Phosphorescence decays, which occur via a different decay mechanism, may last from 10^{-6} s to > 1 s.

The processes of fluorescence and phosphorescence are illustrated in the *Jabłoński diagram* below. A molecule absorbs a photon, rising from a ground electronic state to an excited singlet state. In fluorescence, the molecule returns to the ground electronic state after a period of time called the fluorescence lifetime. In phosphorescence, the molecule undergoes intersystem crossing to a triplet state, and then decays after a longer time back to the ground state.
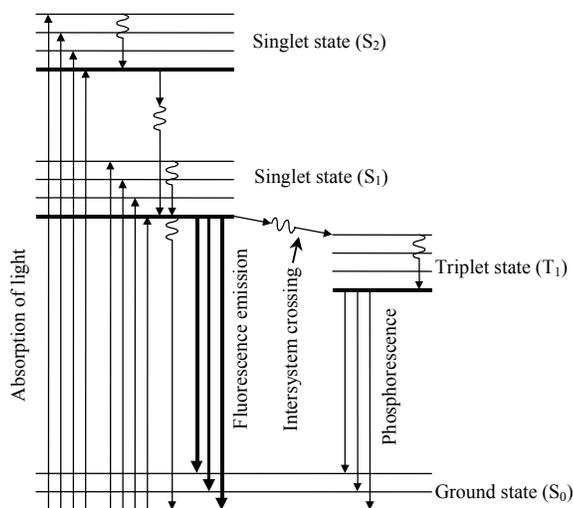
Jabłoński diagram.

There are three general methods for measuring fluorescence lifetimes. Two of them are time-domain, while the third is a frequency-domain technique:
- Pulse method
- Time-correlated single-photon counting
- Phase-modulation

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Pulse method

The pulse method uses a pulsed light source in concert with a detector, to measure the decay of a sample directly as it occurs. The detectors must possess an appropriate response time. This method, a time-domain technique, is that used by the Spex® Phosphorimeter. The system sweeps through the decay in fluorescence intensity by varying a time delay after the incident light pulse. The observed variation in intensity is used to construct the complete decay curve. After measurement, the pulse width, a number of instrumental parameters must be deconvolved (mathematically removed) from the data in order to fit the data properly:

- Incident pulse width
- Incident pulse shape
- Instrumental response function

Deconvolution is unnecessary if the pulse width and instrument response function are much shorter than the lifetime of the sample.

Time-correlated single-photon counting

Time-correlated single-photon counting is also a time-domain measurement used to construct a fluorescence-intensity decay curve for a given unknown. This method measures the statistical distribution of the intensity decay by recording the amount of time elapsed from the incident flash of light until the detector records the first emitted photon from the sample. The resultant histogram is analyzed with Poisson statistics to create the intensity decay curve of fluorescence for the sample. These systems usually require a high-performance flashlamp or pulsed-laser source, with suitable photon-counting electronics, in order to perform time-to-amplitude conversions.

Phase-modulation method

Like time-correlated single-photon counting, phase modulation does not measure fluorescence decay directly. Instead, the sample is irradiated with a sinusoidal continuous-wave excitation. The fluorescence response will be modulated, with constant phase-shift and reduction in signal compared to the excitation. The output beam's response function is measured and then fitted. Thus, this technique is a frequency-domain measurement. Below is a derivation of the frequency-domain lifetime measurements.

Theory of frequency-domain lifetime measurements

The responses of any system to an excitation impulse or a continuous sine wave may be interconverted via the *Fourier transform*. To put it non-mathematically, the response may be decomposed into a weighted series of harmonic components, called the *Fourier spectrum*. A Fourier spectrum also may be used to construct any arbitrary harmonic response.

Two examples of the Fourier transform, as applied to fluorescent systems, are shown in detail below:
- Multicomponent fluorescent decay
- Decay of fluorescent anisotropy for an isotropic (i.e., spherical) rotator with a single lifetime

Basic definitions

The complex Fourier transformation of an impulse response, $I(t)$, gives a real and an imaginary component, denoted R and I, respectively. R and I are defined by the equations

$$R = \int_0^\infty I(t)\cos\omega t\, dt \tag{1.1}$$

$$I = \int_0^\infty I(t)\sin\omega t\, dt \tag{1.2}$$

where $\sin\omega t$ and $\cos\omega t$ represent the harmonic excitation at a circular frequency, ω. Non-mathematically, the Fourier-transform components are the result of integrating the product of impulse response and harmonic excitation over all time, t, from 0 to infinity. The observable quantities (phase, ϕ, and amplitude, M) are derived from the transform components by the definitions

$$\phi = \arctan\left(\frac{I}{R}\right) \tag{1.3}$$

$$M = \sqrt{R^2 + I^2}$$
$$M^2 = R^2 + I^2 \tag{1.4}$$

Notice that the experimentally measured response function contains all components for all measured times. In practice, however, the instrument measures only for a limited time, truncating the data and harmonics. Therefore, the recorded data only can *approximate* the full harmonic response of the system.

Transformation of the Lifetime Equation

The decay response of a population of excited molecules with a single lifetime, τ, follows standard exponential decay:

$$I(t) = I_0 e^{-\frac{t}{\tau}} \tag{1.5}$$

where I_0 is the initial emission intensity. For an ensemble of non-interacting excited states with various lifetimes, the impulse response is a weighted sum of exponentials:

$$I(t) = \sum_i a_i e^{-\frac{t}{\tau}} \tag{1.6}$$

Here I_0 usually is set to unity. The pre-exponential weighting factors, a_i, are related to the observed fractional-intensity contributions, f_i, by the relation

$$f_i = a_i \tau_i \tag{1.7}$$

Real part of the transform

For a multiple-lifetime model, the real part R of the transform equation, (2.1), using the exponential-impulse model, is

$$\int_0^\infty I(t)\cos\omega t\, dt = \int_0^\infty \left(\sum_i a_i e^{-\frac{t}{\tau_i}} \right) \cos\omega t\, dt$$

$$= \sum_i \left(\int_0^\infty a e^{-\frac{t}{\tau_i}} \right) \cos\omega t\, dt \tag{1.8}$$

This corresponds to a sum of definite integrals of the general form

$$\int_0^\infty c e^{-ax} \cos bx\, dx = \frac{ca}{a^2 + b^2} \tag{1.9}$$

where $a = \tau_i^{-1}$, $b = \omega$, $c = a_i$, and $x = t$. We can write the real part of the transform, R, as

$$R = \sum_i \frac{a_i \tau_i^{-1}}{\left(\tau_i^{-1}\right)^2 + \omega} \tag{1.10}$$

We simplify this expression, by multiplying by $\dfrac{\tau_i^2}{\tau_i^2}$ to give the final form for R:

$$R = \sum_i \frac{a_i \tau_i}{1 + \omega^2 \tau_i^2} \tag{1.11}$$

Imaginary part of the transform

The derivation of the imaginary part, I, of the transform is similar to that for the real portion:

$$\int_0^\infty I(t)\sin\omega t\,\mathrm{d}t = \int_0^\infty \left(\sum_i a_i e^{-\frac{t}{\tau_i}}\right)\sin\omega t\,\mathrm{d}t$$

$$= \sum_i \left(\int_0^\infty a e^{-\frac{t}{\tau_i}}\right)\sin\omega t\,\mathrm{d}t \tag{1.12}$$

This last expression is a sum of definite integrals, of the form

$$\int_0^\infty c e^{-ax}\sin bx\,\mathrm{d}x = \frac{cb}{a^2 - b^2} \tag{1.13}$$

where $a = \tau_i^{-1}$, $b = \omega$, $c = a_i$, and $x = t$. As with the real part, we multiply this equation by a ratio equal to 1:

$$I = \sum_i \frac{a_i\omega_i}{\left(\tau_i^{-1}\right)^2 + \omega} \times \frac{\tau_i^2}{\tau_i^2}$$

$$= \sum_i \frac{a_i\tau_i}{1 + \omega^2\tau^2} \tag{1.14}$$

Again, as with the real part, we replace the pre-exponential factors a_i by the observable fractional intensities f_i. This gives:

$$R = \sum_i \frac{f_i\omega\tau_i}{1 + \omega\tau^2} \tag{1.15}$$

$$I = \sum_i \frac{f_i}{1 + \omega\tau^2} \tag{1.16}$$

Next, set R and I for one component, set the fractional intensity $f_i = 1$, and calculate the observables, ϕ and M, as defined in equations 1.3 and 1.4:

$$R = \sum_i \frac{1}{1 + \omega^2\tau^2}$$

$$I = \sum_i \frac{\omega\tau}{1 + \omega^2\tau^2} \tag{1.17}$$

Then the phase, ϕ, becomes

$$\phi = \arctan \frac{\left(\dfrac{\omega\tau}{1+\omega^2\tau^2}\right)}{\left(\dfrac{1}{1+\omega^2\tau^2}\right)}$$

$$= \arctan\omega\tau \tag{1.18}$$

and the phase lifetime, T_ϕ, becomes

$$T_\phi = \frac{\tan\phi}{\omega} \tag{1.19}$$

For the modulation, M, we rearrange the expression:

$$M^2 = \left(\frac{\omega\tau}{1+\omega^2\tau^2}\right)^2 + \left(\frac{1}{1+\omega^2\tau^2}\right)^2$$

$$= \frac{1+\omega^2\tau^2}{\left(1+\omega^2\tau^2\right)^2}$$

$$= \frac{1}{1+\omega^2\tau} \tag{1.20}$$

and the modulation lifetime is

$$T_M = \frac{1}{\omega}\sqrt{\frac{1}{m^2}-1} \tag{1.21}$$

Transformation of the time-resolved anisotropy equations

Derivation of the impulse expressions

Anisotropy, r, is the ratio of the difference in parallel and perpendicular intensities to the total emission intensity:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{tot}} \tag{1.22}$$

Here the total intensity, $I_{tot} = I_{\parallel} + 2I_{\perp}$. Further useful expressions are:

$$rI_{tot} = I_{\parallel} - I_{\perp} \tag{1.23}$$

$$I_{\parallel} = I_{tot} - 2I_{\perp} \tag{1.24}$$

Rearrangement of the basic definitions of anisotropy and total intensity gives an expression for the parallel component of the intensity, $I_{\parallel}$. Substitution of 1.24 into 1.23 gives:

$$rI_{tot} = I_{tot} - 3I_{\perp} \tag{1.25}$$

or

$$I_{\perp} = \frac{1}{3} I_{tot}(1-r) \tag{1.26}$$

Rearrangement of the definition of anisotropy gives an expression for the parallel component:

$$I_{\parallel} = rI_{tot} + I_{\perp} \tag{1.27}$$

Then we substitute in 1.26 for the perpendicular component:

$$I_{\parallel} = rI_{tot} + \frac{1}{3} I_{tot}(1-r) \tag{1.28}$$

$$= \frac{1}{3} I_{tot}(1+2r) \tag{1.29}$$

The Tau-3 measures a time-dependent property, so we convert the equations for $I_{\parallel}$ and $I_{\perp}$ into time-dependent forms:

$$I_{\perp}(t) = \frac{1}{3} I_{tot}(t)[1 - r(t)] \tag{1.30}$$

$$I_{\parallel}(t) = \frac{1}{3} I_{tot}(t)[1 + 2r(t)] \tag{1.31}$$

The impulse equation for the decay of the total intensity, $I_{tot}(t)$, is a standard exponential decay,

$$I_{tot}(t) = I_0 e^{-\frac{t}{\tau}}$$

The anisotropy decay, $r(t)$, has an analogous form,

$$r(t) = r_0 e^{-\frac{t}{\Theta}}$$

where the initial (limiting) anisotropy is r_0, and Θ is the rotational correlation time. For brevity, we use the symbol Γ for $1/\tau$, and $1/(6\Phi)$ for Θ. Then Φ corresponds to the rotational rate. Let us substitute these impulse equations into 1.30 and 1.31. The result is:

$$I_{\perp}(t) = \frac{1}{3} e^{-t\Gamma} \left(1 - r_0 e^{-6\Phi t}\right) \tag{1.32}$$

$$I_{\parallel}(t) = \frac{1}{3} e^{-t\Gamma} \left(1 + 2r_0 e^{-6\Phi t}\right) \tag{1.33}$$

The initial intensity, $I_0 = 0$, so is not written in the equations. Next, we transform these two equations into the frequency domain. The procedure is similar to that for the lifetime impulse equation. One difference is that we need the real and imaginary portions of the perpendicular and parallel components of the transform, $D_{\parallel}$, $N_{\perp}$, $D_{\perp}$, and $N_{\parallel}$. Four integrations are necessary; only the integration for the real component of the parallel emission is provided here in detail.

$$
\begin{aligned}
D_{\parallel} &= \int_0^{\infty} I_{\parallel}(t) \cos\omega_0 t \, dt \\
&= \int_0^{\infty} \left[\frac{1}{3} e^{-t\Gamma} \left(1 + 2r_0 e^{-6\Phi t}\right) \right] \\
&= \int_0^{\infty} \frac{1}{3} e^{-t\Gamma} \cos\omega t \, dt \\
&= \int_0^{\infty} \frac{2}{3} r_0 e^{-t(\Gamma + 6\Phi)} \cos\omega t \, dt \\
&= \frac{\dfrac{\Gamma}{3}}{\omega^2 + \Gamma^2} + \frac{\dfrac{2r_0(\Gamma + 6\Phi)}{3}}{\omega^2 + (\Gamma + 6\Phi)^2}
\end{aligned}
\tag{1.34}
$$

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Similar methods result in expressions for $N_\perp$, $D_\perp$, and $N_\parallel$:

$$D_\perp = \frac{\dfrac{\Gamma}{3}}{\omega^2 + \Gamma^2} + \frac{\dfrac{r_0(\Gamma + 6\Phi)}{3}}{\omega^2 + (\Gamma + 6\Phi)^2} \qquad (1.35)$$

$$N_\parallel = \frac{\dfrac{\omega}{3}}{\omega^2 + \Gamma^2} + \frac{\dfrac{2r_0\omega}{3}}{\omega^2 + (\Gamma + 6\Phi)^2} \qquad (1.36)$$

$$N_\perp = \frac{\dfrac{\omega}{3}}{\omega^2 + \Gamma^2} - \frac{\dfrac{r_0\omega}{3}}{\omega^2 + (\Gamma + 6\Phi)^2} \qquad (1.37)$$

One observable is the differential phase, $\Delta = \Phi_\perp - \Phi_\parallel$. Derivation of Δ is as follows. We start with 1.3,

$$\tan \Phi_\parallel = \frac{N_\parallel}{D_\parallel}$$
$$\tan \Phi_\perp = N_\perp \qquad (1.38)$$

Then

$$\tan \Delta = \tan(\Phi_\perp - \Phi_\parallel)$$
$$= \frac{\left[\tan(\Phi_\perp) - \tan(\Phi_\parallel)\right]}{1 + \tan(\Phi_\perp)\tan(\Phi_\parallel)} \qquad (1.39)$$

We substitute $N_\parallel/D_\parallel$ for $\tan(\Phi_\parallel)$ and $N_\perp/D_\perp$ for $\tan(\Phi_\perp)$, giving the equation for Δ:

$$\Delta = \arctan \frac{N_\perp D_\parallel - N_\parallel D_\perp}{D_\perp D_\parallel + N_\perp N_\parallel} \qquad (1.40)$$

But Δ is the square root of the ratio of modulated amplitudes, M:

$$\Delta = \sqrt{\frac{M_\parallel}{M_\perp}}$$
$$= \sqrt{\frac{D_\parallel^2 + N_\parallel^2}{D_\perp^2 + N_\perp^2}} \qquad (1.41)$$

Another observable is the ratio of modulated amplitudes, $\Lambda = M_\parallel/M_\perp$.

Unlike polarization, the average anisotropy of a mixture is a linear function of the individual anisotropies and their fractional intensity contribution to the total intensity:

$$r = \sum_i f_i r_i \tag{1.42}$$

Therefore, anisotropy is preferentially used for rotational analysis.

Anisotropic rotator with two correlation times

The decay of anisotropy over time, when more than one correlation time is involved, is a linear sum of weighted exponential components—expanded forms of the impulse equations for an isotropic rotator:

$$r(t) = r_0 \left(g_1 e^{-\frac{t}{\Theta_1}} - g_2 e^{-\frac{t}{\Theta_2}} \right) \tag{1.43}$$

The two different correlation times are denoted Θ_1 and Θ_2. The *g* parameter is analogous to fractional intensity: *g* assigns a fractional anisotropy loss to a particular correlation time. A linear expansion of 1.43 provides expressions for larger numbers of correlation times:

$$r(t) = r_0 \sum_i g_i e^{-\frac{t}{\Theta_1}}$$

$$\sum_i g_i = 1 \tag{1.44}$$

Hindered rotators with one correlation time

A hindered rotator is a rotator whose angular displacement is less than 360° (limited rotation). This yields a residual anisotropy value, r_∞, at times much longer than the fluorescence lifetime of the molecule. A simple model for the hindered decay of anisotropy is:

$$r(t) = (r_0 - r_\infty)e^{-\frac{t}{\Theta}} + r_\infty \tag{1.45}$$

An example of this type of rotational decay is found in probes attached to proteins or membranes. Expressions for the harmonic response of this model are derived by substituting the appropriate $r(t)$ into equations 1.30 and 1.31, followed by Fourier transformation into the frequency domain.

Fitting lifetime and anisotropy models to experimental data

The analysis of experimental multi-frequency data is an interative process that attempts to fit an appropriately chosen model to the data. The analysis varies the model's parameters (e.g., lifetime, fractional contributions, anisotropy, etc.) in a direction that minimizes the deviations of the model from the data. Decisions on how well the model suits the data are based on an examination of the statistics of the fit:

- a plot of the residual deviations with frequency
- the value of the reduced chi-squared, χ_r^2, a numerical value that reflects the overall goodness of fit.

Reduced chi-squared, χ_r^2

The reduced chi-squared for multiple-lifetime analysis is defined as:

$$\chi_r^2 = \frac{\chi^2}{\nu} \tag{1.46}$$

where ν is the degrees of freedom in the system. For lifetime analysis,

$$\nu = 2NQ - P \tag{1.47}$$

where N = number of frequencies, Q = number of wavelengths, and P = number of floating parameters.

In 1.46, the χ^2 parameter is given as:

$$\chi^2 = \sum_\omega \frac{1}{\sigma_{\Phi_\omega}^2} \left(\Phi_\omega - \Phi_{c\omega} \right)^2 + \sum_\omega \frac{1}{\sigma_{M_\omega}^2} \left(M_\omega - M_{c\omega} \right)^2 \tag{1.48}$$

Here, Φ_ω and M_ω are the observed values of the phase and modulation at a frequency ω. $\Phi_{c\omega}$ and $M_{c\omega}$ are the expected values of the phase and modulation at that frequency, based on the current values of the floating parameters (lifetimes and fractional intensities). $\Phi_{c\omega}$ and $M_{c\omega}$ are computed using equations 1.3, 1.4, 1.11, and 1.14 at each ω in the experimental data set. σ_{Φ_ω} and σ_{M_ω} are the estimated uncertainties in the experimental data. The floating parameters are varied in order to minimize χ_r^2. Each computation is halted when a minimum is found.

Reduced chi-squared for anisotropic multi-frequency data

Fitting of rotational models to multi-frequency anisotropy data uses the same technique with the inclusion of limiting anisotropy, r_0, correlation times, Θ_1, $\Theta_2,\ldots$, and fractional anisotropy losses, g, as fixed or floating parameters in the fitting algorithm. The reduced χ^2 then becomes

$$\chi_r^2 = \frac{1}{\nu}\left[\sum_\omega \frac{1}{\sigma_{\Delta_\omega}^2}\left(\Theta_\omega - \Theta_{c\omega}\right)^2 + \sum_\omega \frac{1}{\sigma_{\Lambda_\omega}^2}\left(M_\omega - M_{c\omega}\right)^2\right]$$

(1.49)

Essentially, the phase and modulation are replaced by differential phase and modulation ratio, along with their uncertainties. $\Delta_{c\omega}$ and $\Lambda_{c\omega}$ are computed according to the anisotropy model chosen for the fit.

Experimental setup

Major components of the Tau-3

Host computer
The top end of the Fluorolog® Tau-3 starts with DataMax, the software using the Windows™ platform. DataMax controls all aspects of measurement in both steady-state and dynamic modes. DataMax software resides in the host computer, which may be supplied by the user, or purchased from Jobin Yvon®.

SpectrAcq controller
The host computer is linked to the SpectrAcq controller via a serial port. The SpectrAcq accepts high-level commands from DataMax, and executes them to handle low-level control of the entire system. The SpectrAcq controller runs data acquisition and processing in the steady-state mode. It then communicates with the Lifetime Drawer via an Analog-to-Digital (A/D) board, in order to transfer instructions and data for lifetime measurements. The SpectrAcq is a PC-compatible system containing the appropriate power supplies and control cards to work with the Fluorolog® Tau-3. The DataMax boot disk, kept in the 3½"-floppy-disk drive of the SpectrAcq, contains all of the necessary drivers for your Tau-3 instrument's specific configuration.

The SpectrAcq directly controls:
- Power for the core spectrofluorometer
- Monochromators and sample-compartment accessories (e.g., slits, shutters, autopolarizers, and sample changers)
- Links to the detectors for steady-state signal processing
- High-voltage DC bias to the photomultiplier tubes
- Links to the Lifetime Drawer

Lifetime Drawer
The nickname for the electronics tray inside the electronics rack is the "Lifetime Drawer". The front plate of the Drawer houses the Lifetime and Steady-State LEDs to indicate the system's operating mode. The Drawer's functions are to
- set the radio frequency (RF) applied to the Pockels-cell modulator and photomultiplier-tube housings, and
- collect and process data from each detector channel during dynamic measurements.

In order to control the applied RF, the drawer connects to the dual-channel synthesizer, usually located just below the Lifetime Drawer in the electronics rack. For signal acqui-

sition, the Drawer connects to each lifetime-detector housing with a 9-pin–9-pin cable. At the other end, the Drawer connects to the SpectrAcq with two 37-pin cables—one for SpectrAcq input-output and control, and the other for data transfer.

Dual-channel synthesizer
The dual-channel synthesizer is a PTS dual 310-MHz synthesizer that provides two separate frequency-locked signal outputs (Main and Auxiliary). The frequency range for the dual-channel synthesizer is 100 kHz–310 MHz. Each channel is connected to an RF-amplifier. One channel applies RF signal to the Pockels-cell modulator; the other channel applies an RF signal 41 Hz higher to each detector housing. The 41-Hz beat frequency thus created allows cross-correlation measurements of the phase and demodulation values.

RF Amplifiers
Two Amplifier Research RF amplifiers take the attenuated RF signal from each channel of the synthesizer, and apply the appropriate voltage to the modulator or detector bases. The RF amplifier for the modulator is placed on the instrument table near the modulator, and delivers RF bias to the Pockels cell.

The photomultiplier-tube amplifier is mounted on the bottom of the electronics rack. This amplifier supplies RF voltage to a three-way splitter on the side of the modulator box. The splitter divides the bias into three equal components, which are then applied to each individual lifetime-detector housing. With only two detectors (1 reference and 1 emission), the extra output carries a 50-Ω dummy terminator load.

Each amplifier has a marked and preset output-power dial. This is set at the factory, and should be adjusted only by a Jobin Yvon® Service Engineer.

DC high-voltage bias supply
An external Bertan™ DC high-voltage supply is used to bias the Pockels cell at a level for the greatest possible depth of modulation for the system. The bias is mixed with RF signal as it enters the modulator box, passes across the Pockels cell, and is terminated with a 50-Ω load.

Pockels cell
The Pockels cell is the heart of the Fluorolog® Tau-3. The cell is a birefringent potassium dihydrogen phosphate (KDP) crystal, whose polarization is proportional to the bias applied along the longitudinal axis of the crystal. The crystal is enclosed in a fluid with a matching refractive index, to minimize losses in transmitted light. The Modulator Assembly includes the Pockels cell, crossed polarizers, collimating lenses, and bias electronics. The Modulator Assembly is an electro-optic modulator. This means that the light transmitted through the Pockels cell is modulated at the AC frequency applied to the cell.

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A highly collimated beam of light is gathered from the excitation monochromator (or user-supplied external laser), and directed through the first polarizer, allowing only the horizontally polarized component of the excitation beam to reach the Pockels cell. The polarized light passes through the Pockels cell, whose polarization is varied between 0 and 90°, depending on the applied AC signal. The emitted light's polarization is thus rotated by the current polarization state of the cell. The emitted light passes through a second polarizer on the exit side of the

Modulator Box

cell, which selects the vertical component of the beam. When the polarization is at 0 or 90°, no light is transmitted. As the polarization changes from 0 to 90°, so will the amount of transmitted light.
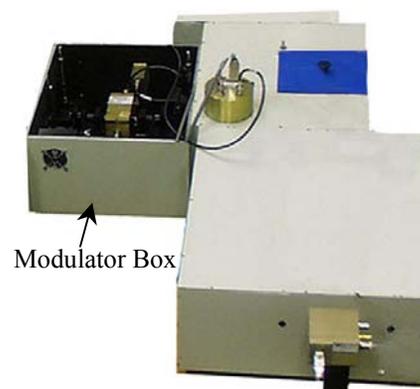
In summary, when an RF signal is applied to the Pockels cell, the polarization state of the cell changes at a rate equal to the applied frequency. Thus, the light emitted from the Modulator Assembly is modulated sinusoidally at the synthesizer's applied frequency. This emitted beam is directed onto the sample.

Detector housings
At least two lifetime-detector housings are available on each Tau-3 system:
- The reference photomultiplier-tube housing (between modulator and sample)
- The emission photomultiplier-tube housing (on the emission monochromator or the output port of the sample compartment)

Emission detector housing
The emission photomultiplier-tube housing monitors the sample's fluorescence response to the incident light. The AC signal, DC signal, and phase of the emission signal is sent to the Lifetime Drawer for processing. The photomultiplier tube requires a high-voltage DC bias for optimum sensitivity, and a cross-correlation bias (41 Hz higher than the RF bias to the modulator) to ease signal processing. (Processing a signal at 41 Hz is easier than at ~100 MHz.)

Reference detector housing
The reference detector helps remove instrumental variations from the measurement by providing a phase-lock on the measurement. The AC, DC, and phase of the modulated excitation beam are sent to the Lifetime Drawer for processing. The measured phase shift for the standard and unknown samples is the phase of the emission detector minus the phase of the reference detector.

2: Requirements & Installation

Selecting a location

Before unpacking the Fluorolog®-Tau-3, select its permanent location. The instrument should be placed on a stationary table or laboratory bench with ample room for the instrument, host computer and peripherals, power supplies, and any optional accessories. Try to leave room around the instrument on all sides, for maintenance and use.

The location should include room for:
- Instrument table
- The spectrofluorometer itself
- One RF amplifier
- High-voltage bias supply
- Xenon lamp
- Lifetime electronics rack (on the floor next to the spectrofluorometer)
  - Dual-channel synthesizer
  - I/O drawer
  - The other RF amplifier
- Host computer
- SpectrAcq controller

> **Note:** *The host computer and SpectrAcq should be located close to the spectrofluorometer and electronics rack.*

Jobin Yvon® recommends that you prepare the area where the instrument will be placed in advance:
- Select a proper location for the instrument.
- Have the selected spectrofluorometer table ready.
- Solve any electrical, plumbing, or space issues prior to installation.
- Set up the host computer, if already provided by the user.
- Have the equipment brought to the laboratory.

Surface requirements

Consider the following when setting aside space for a new instrument:
- Location of the host computer and its peripherals.
- Location of the SpectrAcq
- Room for optional accessories (e.g., external laser, temperature bath, etc.)
- Space for sample preparation

The lifetime rack must sit on the floor next to the spectrofluorometer table. The SpectrAcq should sit near both the spectrofluorometer table and the lifetime rack. The external power supplies (Pockels-cell RF amplifier and DC high-voltage bias) should be on the lab table next to the modulator compartment. The host computer may sit on top of the rack or on the lab table, if desired.

Warning: Do not place any power supply directly in front of the xenon lamp exhaust fan. This can cause the lamp to overheat.

Note: The instrument table should be able to support a mass of at least 240 lb (109 kg) for the instrument.

Note: Users who work with other RF devices (ESR, NMR, etc.) should place the lifetime system as far as possible away from these devices, to minimize any radio-frequency interference.

FL-3-11-Tau
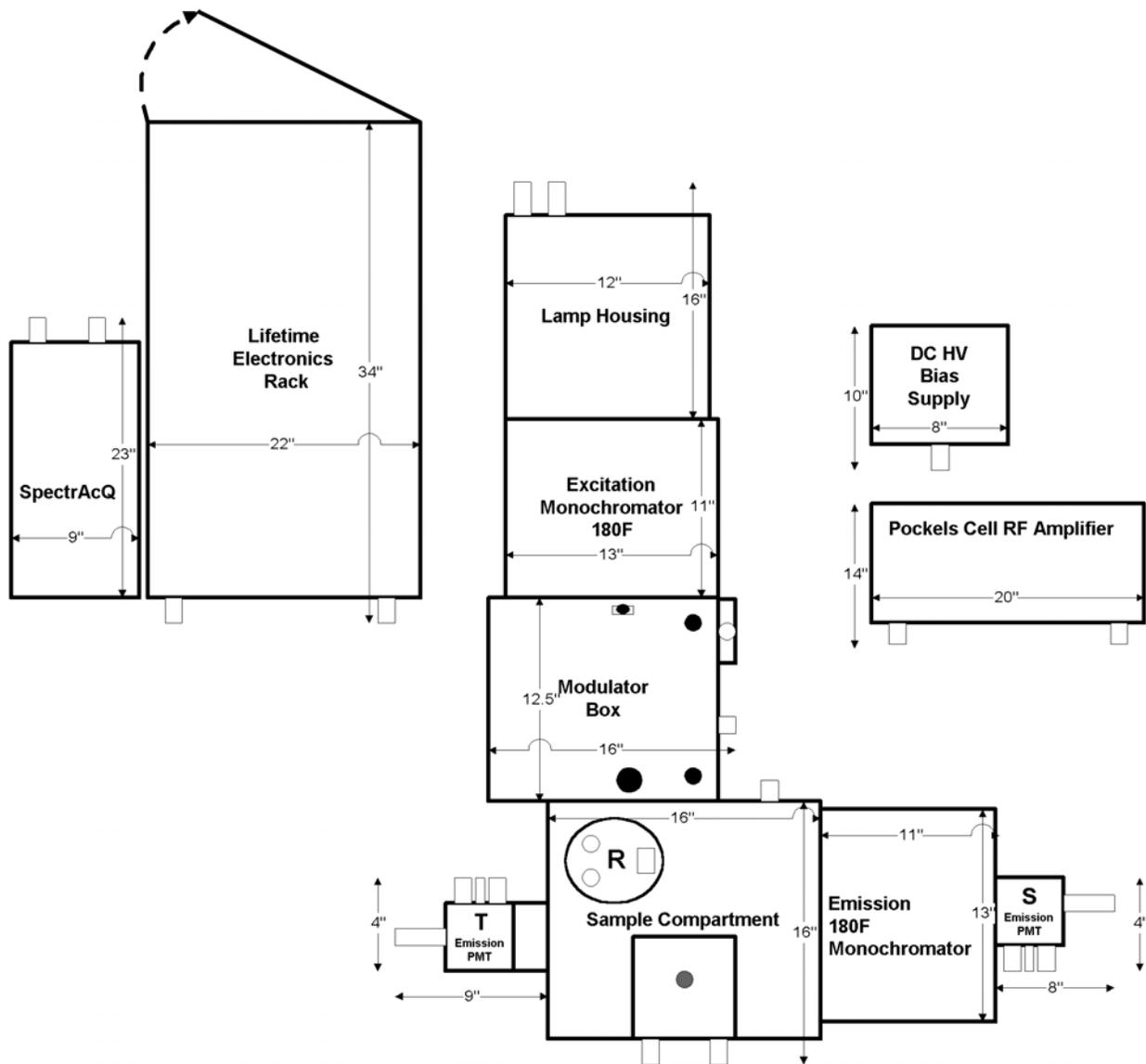
Single Excitation/Single Emission Lifetime Spectrofluorometer with T-side Detector

Recommended table size: 6 ft × 5 ft (1.83 m × 1.52 m)

FL-3-12-Tau
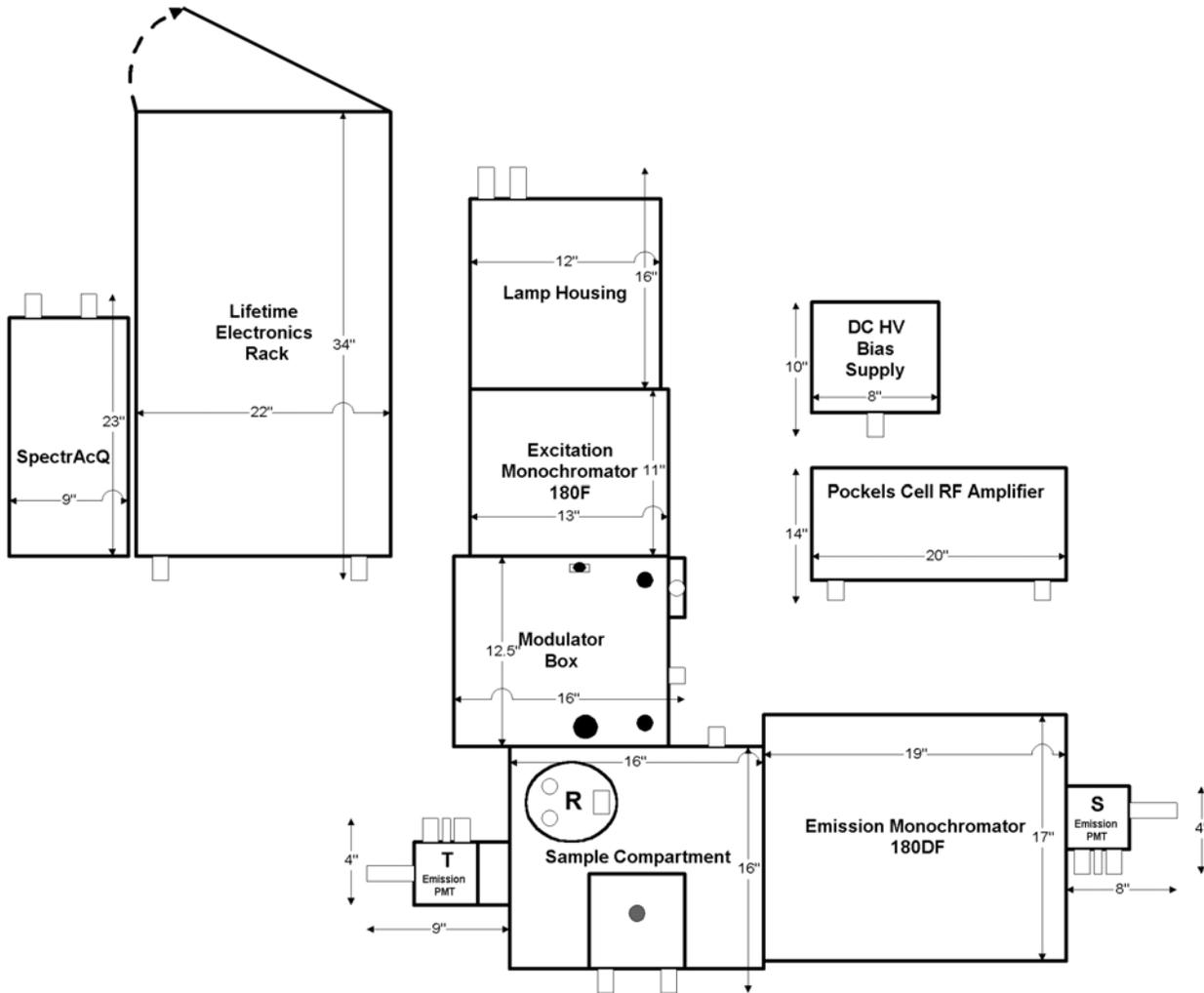
Single Excitation/Double Emission Lifetime Spectrofluorometer with T-side Detector

Recommended table size: 6 ft × 5 ft (1.83 m × 1.52 m)

FL-3-21-Tau
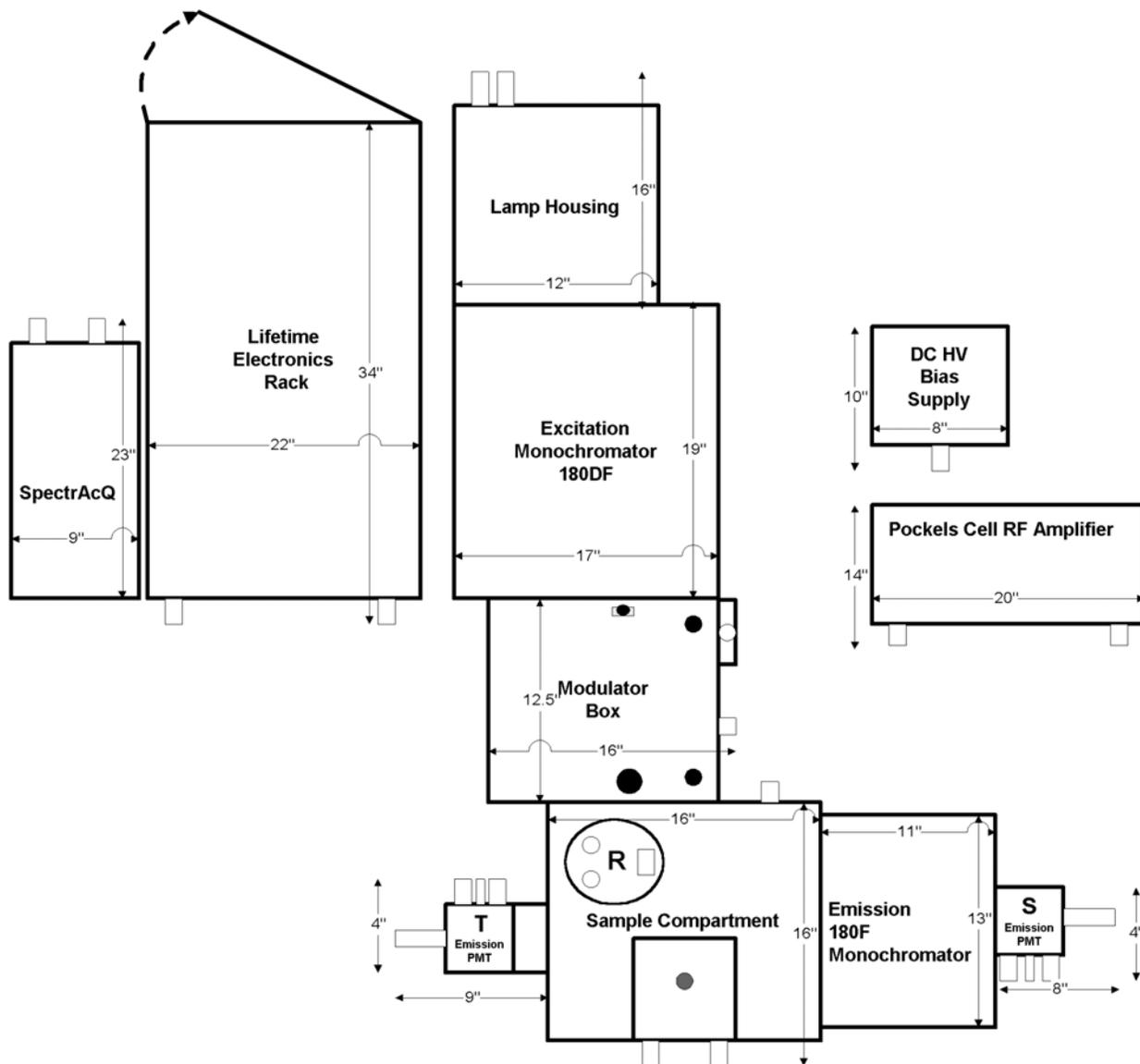
Double Excitation/Single Emission Lifetime Spectrofluorometer with T-side Detector

Recommended table size: 6.5 ft × 5 ft (1.98 m × 1.52 m)

FL-3-22-Tau
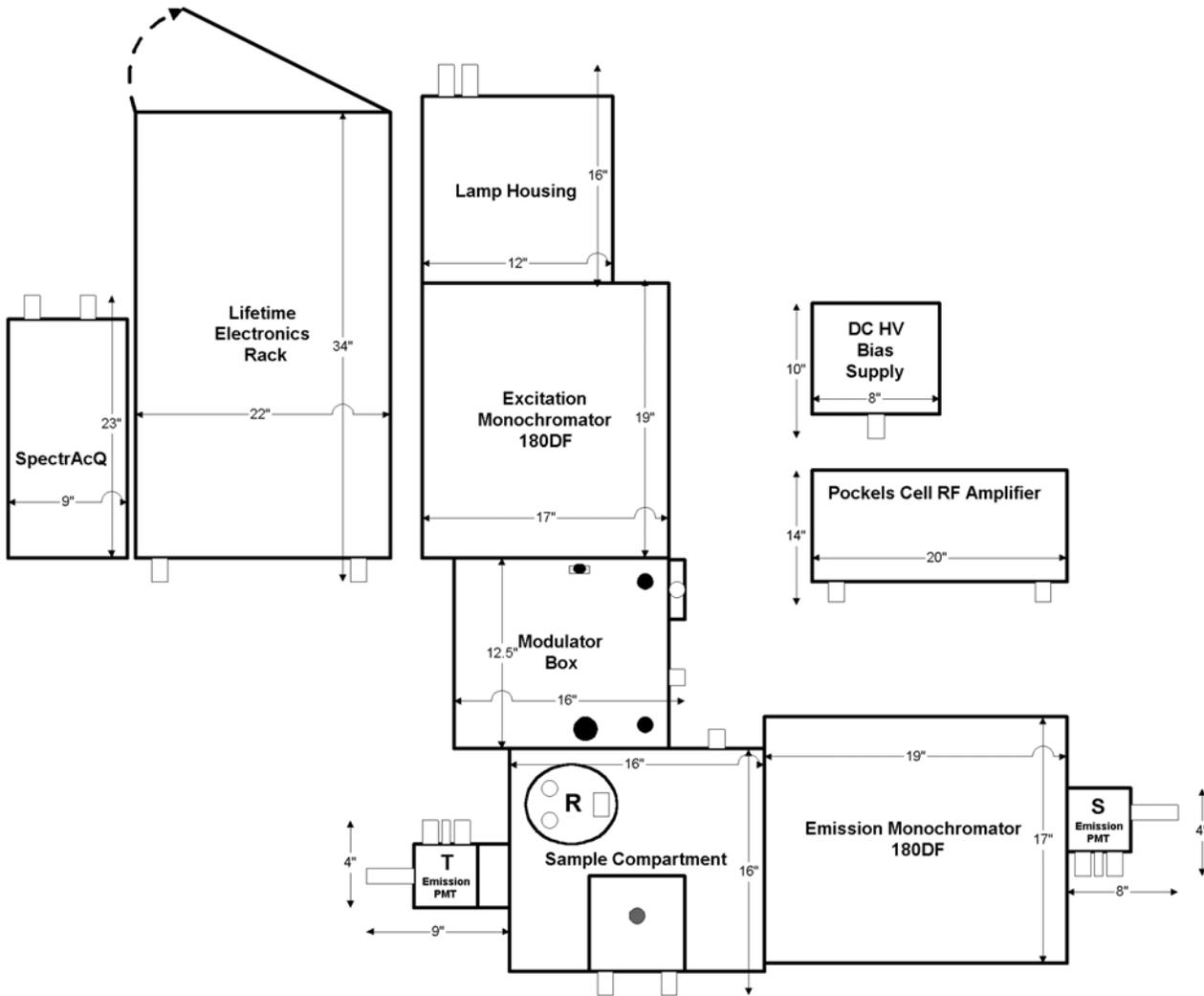
Double Excitation/Double Emission Lifetime Spectrofluorometer with T-side Detector

Recommended table size: 6.5 ft × 5 ft (1.98 m × 1.52 m)

FL-3-111-Tau
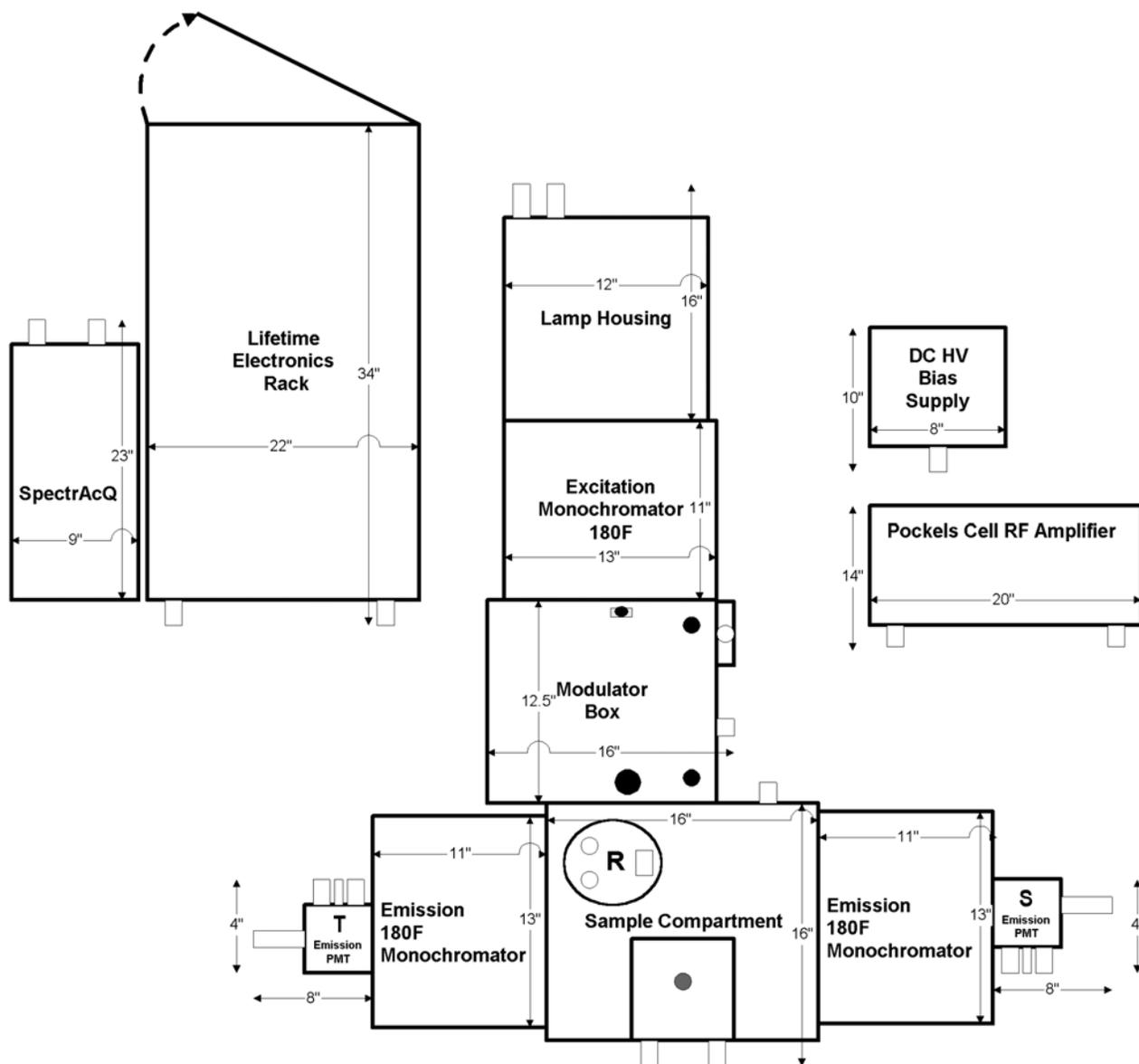
Single Excitation/Single Emission/Single 2nd Emission Lifetime Spectrofluorometer

Recommended table size: 6 ft × 5 ft (1.83 m × 1.52 m)

FL-3-222-Tau
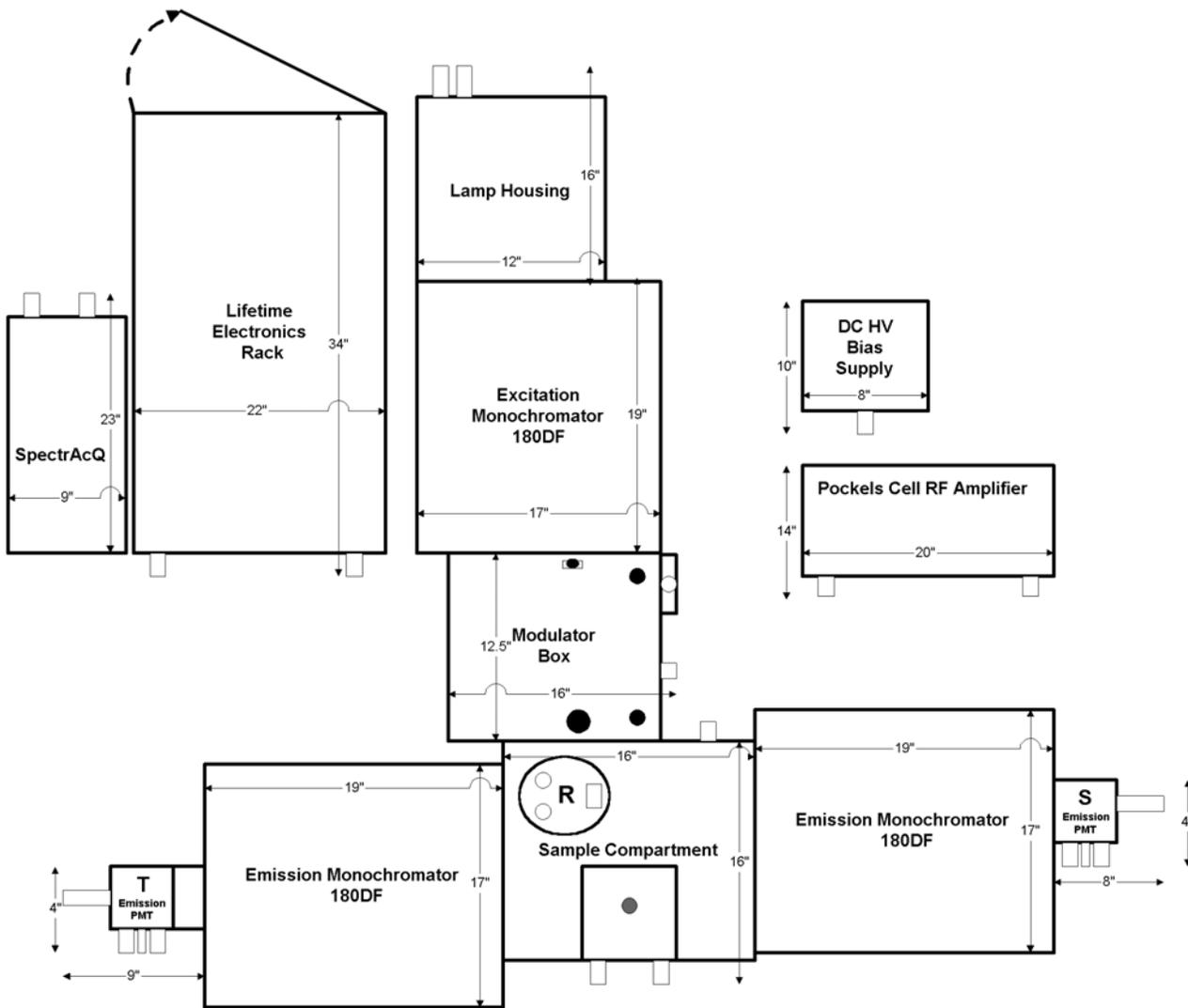
Double Excitation/Double Emission/Double 2nd Emission Lifetime Spectrofluorometer

Recommended table size: 6.5 ft × 6 ft (1.98 m × 1.83 m)

Environmental requirements

Maintaining a stable environment around the instrument is essential for good results. Changes in temperature and humidity can influence the performance of system electronics, the output of the source, the response of detectors, and the efficiency of optics, as well as affect the luminescence from your samples.

- Ambient temperature 15–30°C (59–86°F) ± 2°C.
- Ambient relative humidity <75%.
- Low dust levels and a clean laboratory environment.

Warning: Do not block exhaust inlets and outlets, or place electronic components in front of warm exhausts. These exhausts are found on:
- *Both sides of the xenon lamp housing*
- *Rear of electronics rack (double fans)*
- *Side of modulator compartment*
- *Rear of RF amplifiers*

Electrical requirements

Voltage

- Factory preset at 110 V ± 5%/60 Hz or 220 V ± 5%/50 Hz
- Three-conductor power cord connected to the system frame (earth) ground.
    This ground provides a return path for fault current from equipment malfunction or external faults.

> *Warning: For all instruments, ground continuity is required for safe operation. Any discontinuity in the ground line can make the instrument unsafe for use. Do not operate this system from an ungrounded source.*
>
> *Jobin Yvon®, Inc., is not liable for damage from line surges and voltage fluctuations. A surge protector is strongly recommended for minor power fluctuations. For more severe voltage fluctuations, a generator or an uninterruptible power supply is suggested. Improper line voltages can damage the equipment severely.*

Unpacking and installing

When the Fluorolog®-Tau-3 arrives

The Fluorolog®-Tau-3 Fluorescence Lifetime System is delivered disassembled. The shipment should include:

- optical modules
- lifetime electronics rack
- system controller
- optional equipment and accessories (e.g., host computer, if ordered)
- cables
- software
- manuals

Examine the shipping boxes carefully. If damage is evident, do not continue with the installation. Notify Jobin Yvon® and the shipper at once.

Note: Many public carriers do not recognize claims for concealed damage reported later than 15 days after delivery. For a shipping damage claim, inspection by the carrier agent is required. Therefore, the original packing material should be kept as evidence. While Jobin Yvon® is not responsible for damage occurring during transit, the company will extend every effort to aid and advise.

All Lifetime Systems require installation by a Fluorescence Service Engineer or local representative. When you receive your instrument, call Spex® Fluorescence Service or your local representative to set up an installation.

To avoid damage, unpack the equipment as close as possible to the selected location of the instrument. Jobin Yvon® suggests that you unpack the equipment in order to set the major components on the table, while also checking that all parts have arrived undamaged.

Warning: The Spex® Lifetime System is a delicate instrument with sensitive components. Mishandling may seriously damage the system.

Package contents

The spectrofluorometer and its accessories are contained in packing cartons. The Lifetime Electronics Rack is shipped in a crate with the feet bolted to a skid. If a host computer or any special accessories were included in the order, they accompany the instrument, unless specified by Jobin Yvon®, Inc. See the Performance Test Report or the sales order for a list of all items shipped or back-ordered.

General checklist for a basic Lifetime System

Quantity	Item	Part Number
1	Xenon lamp housing	FL-1007
1	450-W ozone-free xenon lamp	FL-450XOFR
1	Excitation monochromator	
	Single	FL-1004
	Double	FL-1006
1	Modulator compartment	FL-1021
1	Sample compartment	FL-1000
1	Emission monochromator	
	Single	FL-1004
	Double	FL-1006
1	SpectrAcq Controller	FL-1016
1	Lifetime rack	37180
	Dual-channel synthesizer	99150
	I/O Drawer	37385
2	RF amplifiers	99152
1	Tau-3 emission detector housing	400081
1	DM302 photon-counting module	DM302
4	6' (2 m) and/or 2' (0.6 m) RF-bias cables	37685 or 37686
2–3	RF bias cables for lifetime PMTs	37689
2–3	Lifetime signal cables	37195
1	I/O control cable	37186
1	DAS signal cable	37187
1	10-dB attenuator	90361
1	20-dB attenuator	90362
1	5-W coaxial terminator	90363
1	HV cable for external DC bias supply	37733
2	Laser targets	
	Vertical target	37293
	Pinhole target	37294
1	Monochromator control cable	400108
1	Reference detector cable	33979
2–3	HV cable for SpectrAcq HV supplies	34040
1	Null modem communication cable	400144 or 97133 & 97134
Many	Power cords (110 V)	98015
	(220 V)	98020
1	5/64" Balldriver	615017
1	Hex key set	53057
1	Fluorolog®-3 operation manual	81014
1	Fluorolog®-Tau-3 operation manual	81054
1	DataMax software and boot disk for SpectrAcq	DataMax or DataMax-STD

Unpacking instructions

1 Open the Fluorolog®-Tau-3 shipping carton containing the spectrofluorometer monochromators and compartments.

2 Remove the foam-injected top piece and any other shipping restraints in the carton.

3 With at least one helper, carefully lift the components

Warning: *Watch your fingers!*

from the carton and rest them on the table on which the system will rest.

4 Move the crate covering the Lifetime Electronics Rack into the laboratory where it will stay.

5 Remove the shipping crate enclosing the Lifetime Electronics Rack.

6 Unscrew the four posts underneath the skid attached to the base of the rack.

Replace these posts with the four legs shipped in one of the other shipping cartons.

7 Unpack all other boxes containing power supplies, detectors, cables, manuals and accessories.

Note: *Do not dispose of shipping cartons until after installation.*

Store all these items together in a safe location.

8 Inspect for previously hidden damage.

Notify the carrier and Jobin Yvon®, Inc. if any damage is found.

9   Check the packing list and sales order to verify
      that all components are present.

Suggested power connections

1   Connect the xenon lamp to an isolated 10-A circuit.

> *Warning: Do not use computers or other system electronics on this circuit. The xenon lamp is connected to a separate circuit, because the start pulse for xenon short-arc lamps requires ~25 kV. Some EMF from this pulse cannot be shielded, which can damage electronics on the same power circuit as the xenon-lamp power supply. Induced EMF also can travel through air to components located near the lamp power supply. (This is why the lamp must be started first, as explained in the next chapter).*

2   Plug all items inside the Lifetime Electronics Rack into the surge protector located below the fans.

Try to connect the external Pockels-cell RF amplifier and DC HV-bias supply to this surge protector. Having all lifetime components on the same circuit helps reduce effects of electronic noise on the system.

> *Note: All components may be left in the ON position and simply switched ON and OFF using the rocker switch on the front of the Lifetime Electronics Rack.*

3   Plug the surge protector on the electronics rack into a 10-A circuit shared with the computers and controllers.

4   Plug the SpectrAcq, host computer, and its peripherals into a surge protector.

Connect this surge protector to the same circuit as the Lifetime Electronics Rack. The SpectrAcq and host computer (with peripherals) are on the same surge protector, for they are always powered up last.

5   If there is an optional programmable tempera-
    ture bath, connect this to a separate (10 A) elec-
    trical circuit.

Note: *The Fluorolog®-3 spectrofluorometer and its inter-
nal accessories, including sample compartment, receive
power from the SpectrAcq.*

Computer installation

Set up the host computer reasonably close to where the Fluorolog®-Tau-3 system will reside. The limitation is the length of the null-modem communications cable. The recommended location is just to the left of the spectrofluorometer, on top of the electronics rack, or on a computer desk.

Follow the instructions with the host computer, to set up the computer system, including the CPU, monitor and related peripherals (keyboard, mouse, speakers, printers, etc.).

Software installation

The spectrofluorometer system is controlled by DataMax or DataMax-STD spectroscopy software operating within the Windows™ environment. If the computer and software were purchased from Jobin Yvon®, the software installation is complete.

If the computer is not from Jobin Yvon®, perform the installation. Contact a Jobin Yvon® Sales Representative for recommended specifications for a suitable host computer. Before the DataMax software can be installed, however, Windows™ must be installed already and operating properly. Refer to the Windows™ manual that came with the computer for installation instructions.

The DataMax software is supplied on one CD-ROM. The 3½" floppy disk is the DataMax Instrument Disk (or DataMax INI disk), which contains the spectrofluorometer's specific hardware configuration.

If Autorun is enabled:

1 Turn on the computer, and insert the DataMax CD-ROM.

The set-up window to install DataMax appears:

2 Click Install Datamax.
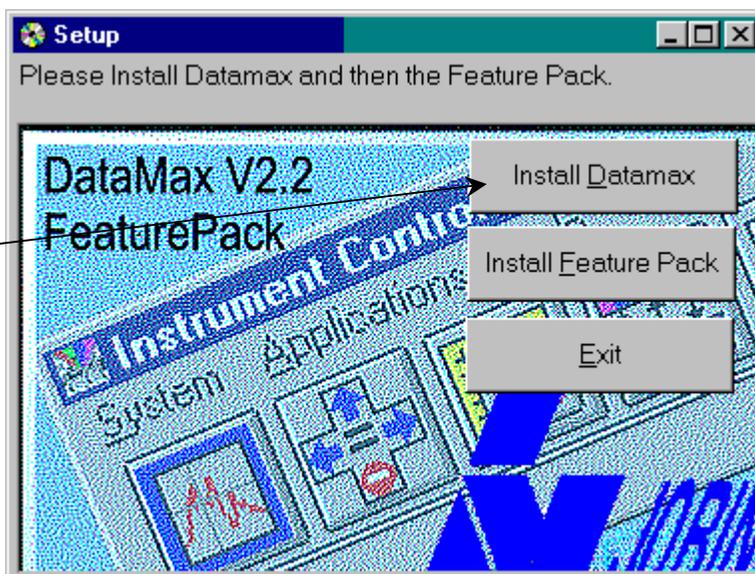
A warning appears, reminding you to install the Feature Pack after DataMax.

The computer automatically installs Data-Max.
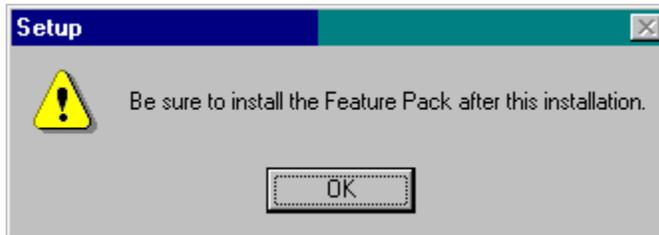
3 The computer asks for the instrument (INI) floppy disk.

Insert the floppy disk into drive A:.

4   Use the default options.

The computer installs the options, then returns to the set-up window:

5   Click
     Install
     Feature
     Pack.

Note: The Feature Pack is also on the DataMax CD-ROM.
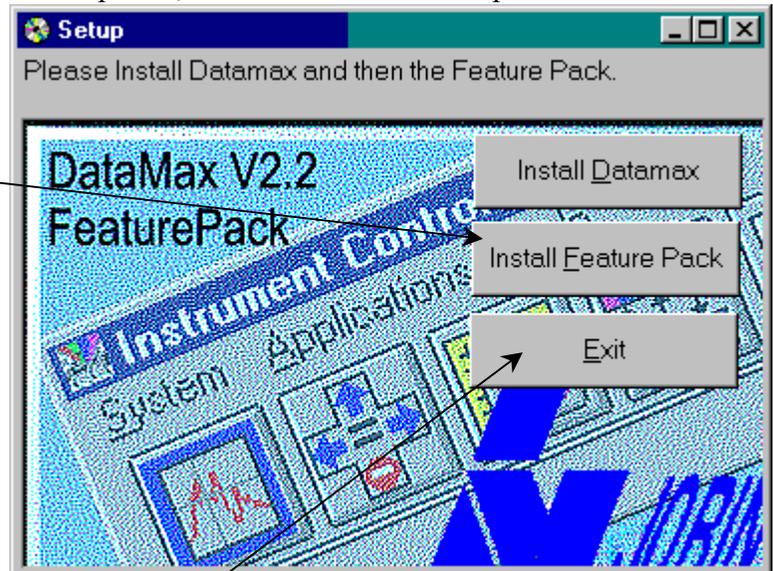
6   Accept the
     default
     options.

After installation, the computer returns to the set-up window.

7   Click Exit.

If Autorun is disabled, use the following—or other appropriate—method:

1   Insert the DataMax CD-ROM into the drive.

2   Go into Windows™ **Control Panel**.
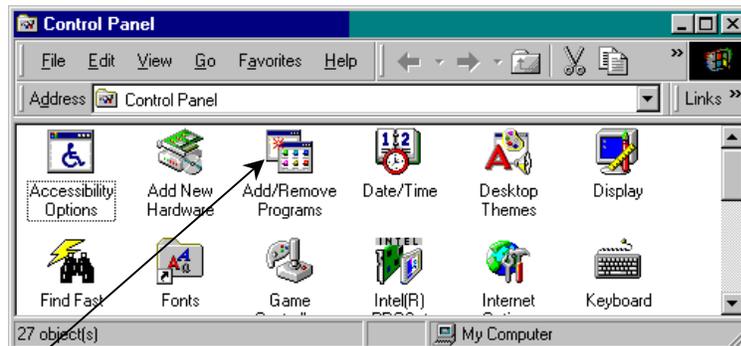
3   Click Add/Remove Programs.

This opens the **Add/Remove Programs Properties** dialog box:

4   Click Install…
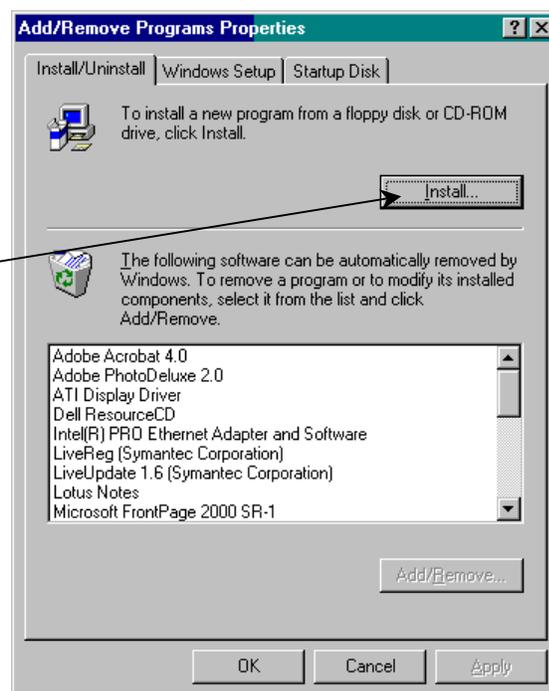
This opens the **Run Installation Program** window. The computer should find the SETUP.EXE file on the CD-ROM drive.

5   Click Finish to finish the installation.
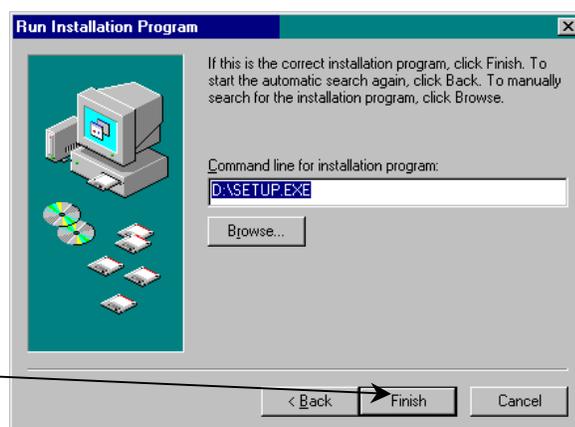
Users outside of the USA:

Users outside of the USA receive a softkey device that connects to the printer port of the host computer for software security. The softkey should be left in place on the host computer at all times.

Hardware emulation

1   Start DataMax.

2   Load a layout.

Layout Selection

Select configuration to work with:

FL3-22 Generic Layout
FL3-22 with 2-Position Sample Changer
FL3-22 with Autopolarizers
FL3-22 with 2-Position Sample Changer and Autopolarizers
Lifetime
Lifetime with Autopolarizers

OK   Cancel   Browse...

Click OK to continue.

3   The computer asks if DataMax should emulate hardware:

Click Yes to continue.

SpectrAcq

? Cannot connect to controller. Emulate?

Yes   No
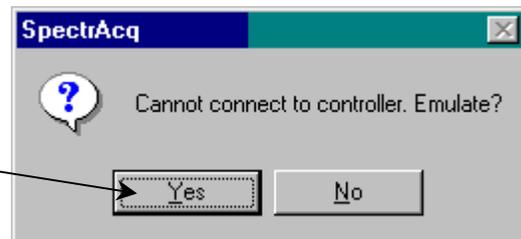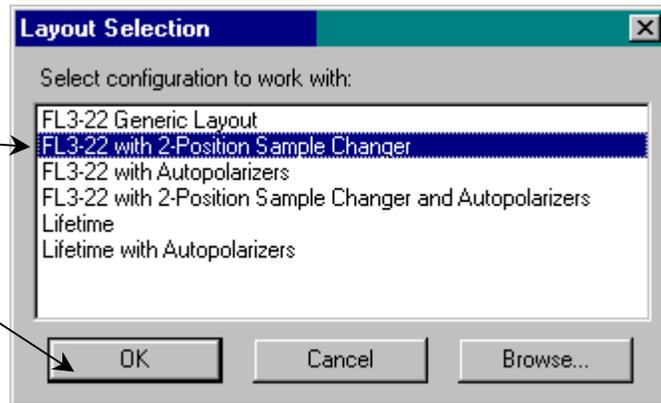
Browse through the menus to become familiar with the software layout before the system installation. For more detailed information about installing, launching, and operating DataMax for Windows™, see the *Datamax Software Manual*.

3: Getting Started

System startup

To ensure a smooth boot-up and no damage from the xenon-lamp ignition, follow the start-up sequence listed below.

1 Switch on xenon lamp.

a Switch on "power".   b   Ignite "main lamp".

2 Start the Lifetime Electronics Rack.

3 Start the SpectrAcq.

a Make sure the boot disk is in the floppy drive.

b Push in the power button to start. Immediately below the power button, the green LED indicator lamp should turn on.

4 Start the other lifetime power supplies (HV DC-bias, RF amplifier) not connected to rack.

5 Start automated accessories (temperature bath, MicroMax, etc.).

6 Switch on the host computer and peripherals.

The SpectrAcq takes ~ 1 min to load the drivers on the boot disk.

7 Click on the **DataMax** (**Instrument Control Center**) icon to run DataMax.

Load steady-state layout

1   The **Layout Se-
    lection** window
    appears:

    Choose a steady-state
    layout with no special
    accessories.

Layout Selection

Select configuration to work with:

FL3-22 Generic Layout
FL3-22 with 2-Position Sample Changer
FL3-22 with Autopolarizers
FL3-22 with 2-Position Sample Changer and Autopolarizers
Lifetime
Lifetime with Autopolarizers

[OK]   [Cancel]   [Browse...]
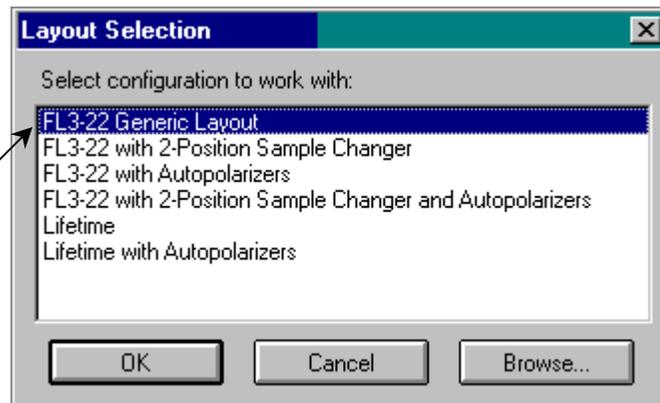
2   The Lifetime
    System spends ~ 1 min initializing the auto-
    mated monochromator drives and slit motors.

3   The **Instrument
    Control Center**
    appears:

Instrument Control Center - LAYOUT05.LAY

System   Applications   Help

Run CWA

4   Start one of the four available applications:

Run Experiment   **Real Time Display**

Visual Instrument Setup   **Constant Wavelength Analysis**

*Note: When initialized properly, these icons in the **Instrument Control
Center** will be multicolored. If they are gray, this means the system
has not been initialized, or the "emulation" mode has been se-
lected.*

Change from lifetime to steady-state mode

With the system initialized with a steady-state layout,

1 I/O drawer's Steady State LED is illuminated.

The I/O drawer's LEDs show the operating mode.
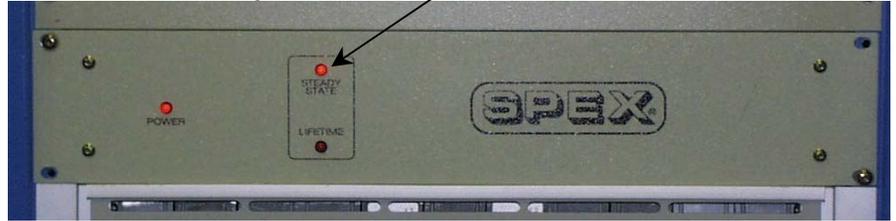
2 Turn the Modulator Position knob to Steady State:

The electro-optic modulator (Pockels cell) is now out of the light path.

3 Turn the Source Position knob to Xenon:
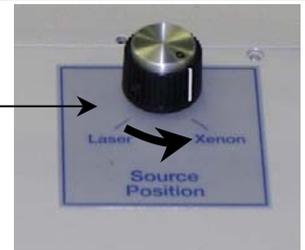
4 Open the iris lever fully.

(When facing the front of the sample compartment, maximum left.)
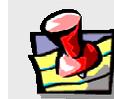
*Note: Some irises open to the **right**.*
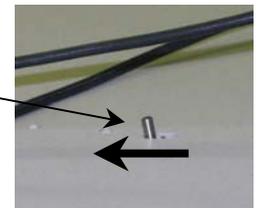
5 Set the manual slit to 7 mm (open wide).

The corresponding apertures around the modulator are open wide for steady-state measurements.

3-3
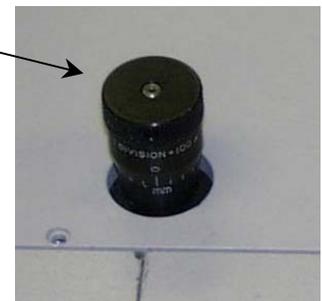
Verify that the system is in steady-state mode:

6   Open **Real Time Display**.

7   Check that the S detector units are cps (counts per second) and the R detector units are µA.
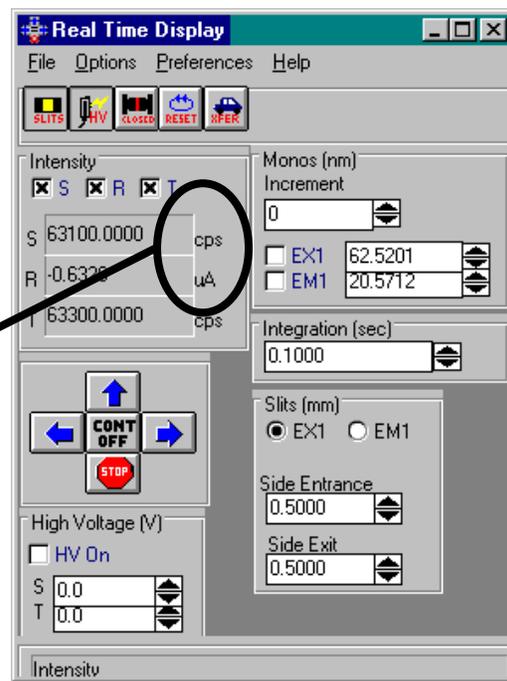
Steady-state system performance checks

As part of routine maintenance checks, first examine the steady-state performance of the Fluorolog®-Tau-3. We recommend a check of the steady-state system calibration before each day of use. Scans of the xenon-lamp output and the Raman scatter band of water are sufficient to verify system calibration and throughput.

- *Calibration* is the procedure whereby the drive of each spectrometer is referenced to a known spectral feature.
- *Throughput* is the amount of signal that passes through and detected from the system. The throughput relates to the signal-to-noise and sensitivity of system.

The Fluorolog®-Tau-3 is an autocalibrating spectrofluorometer. This means that the system initializes its monochromator's drives, locates the zero of each drive, and assigns a wavelength value to the low limit of each drive from a calibration file. It is wise to check the calibration prior to each day's session with the instrument.

The following procedure details the steady-state calibration checks and recalibration, if necessary, for the Fluorolog®-Tau-3. For further information on how to run experiments or use **Real Time Display** or **Visual Instrument Setup**, refer to the next chapter, *Lifetime Acquisition*, or the *DataMax Software Manual*.

For these calibration checks, a single sample mount or automated sample changer should be the only sample-compartment accessory used.

The scans shown in these instructions are examples. A Performance Test Report for the new instrument is included with the documentation. Use the Performance Test Report to validate the spectral shape and relative intensity observed during the calibration checks.

Excitation calibration check

This calibration check verifies the wavelength calibration of your excitation mono-chromator, 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   Secure the lid of the sample chamber in place.

2   On the **Run Experiment** toolbar, select the Experiment button
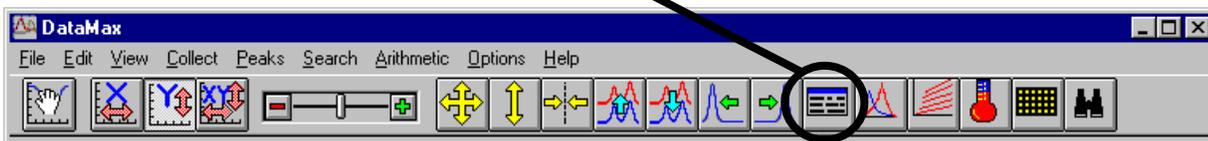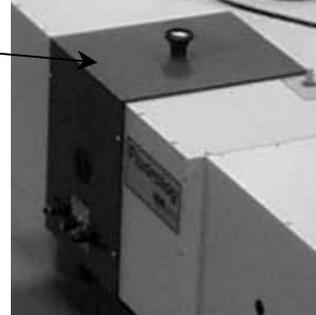
This opens the **Emission Acquisition** dialog box:
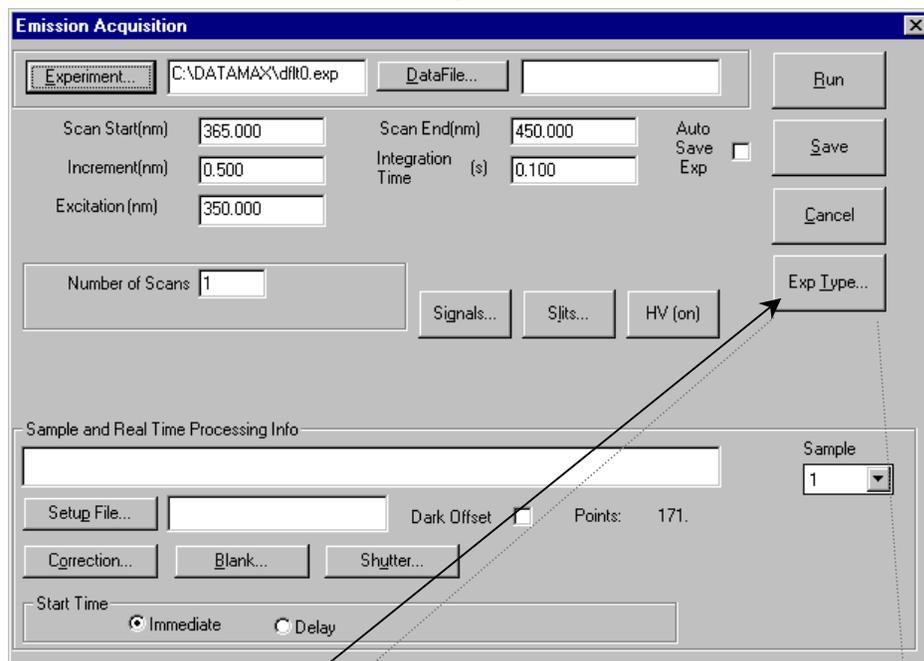
3   Click on the Exp Type button.

This opens the **Select Experiment Type** dialog box:

4   Choose Excitation
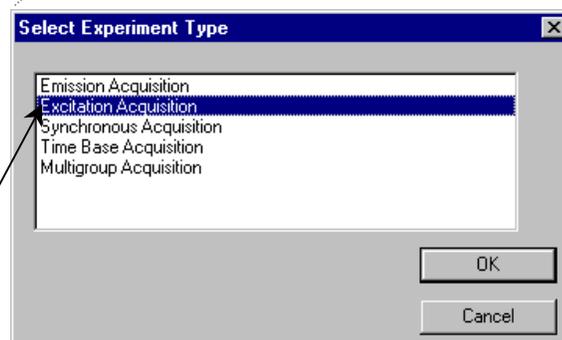
Acquisition.

Click OK to close the **Select Experiment Type** dialog box. The **Emission Acquisition** window converts to **Excitation Acquistion**. (For calibration and calibration verification, always adjust the excitation spectrometer first.)

5   Set the scan parameters for the xenon lamp scan:

a   In the **Excitation Acquisition** dialog box:

Name the experimental parameters lamp.exp,   Use a filename lamp.spc,
Start the scan at 220 nm,   End the scan at 600 nm,
Step the wavelength by 0.5 nm,   Use an Integration Time of 0.1 s.
Set the emission to 650 nm,
Perform 1 scan.
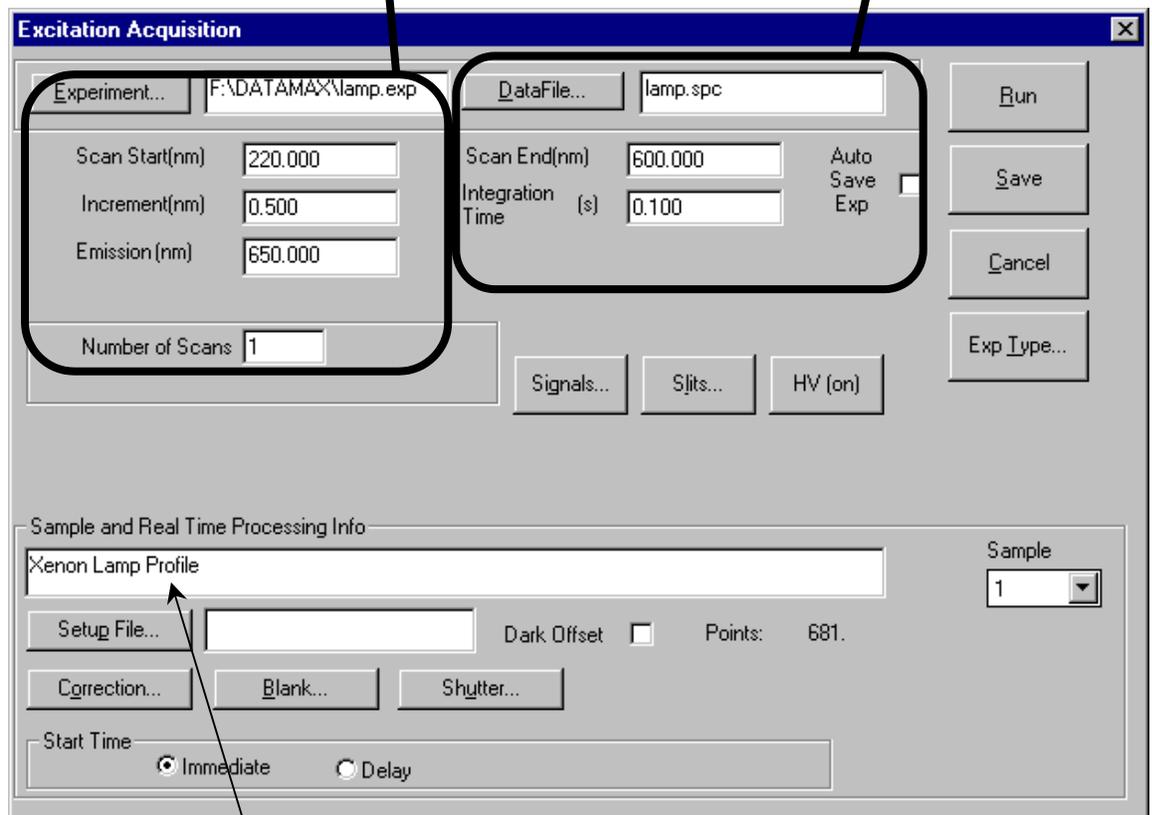
Describe the file as the spectral profile of the xenon lamp.

b   Click on Signals...

This opens the **Signals** dialog box.
Enter R (reference), then click OK to close the box.
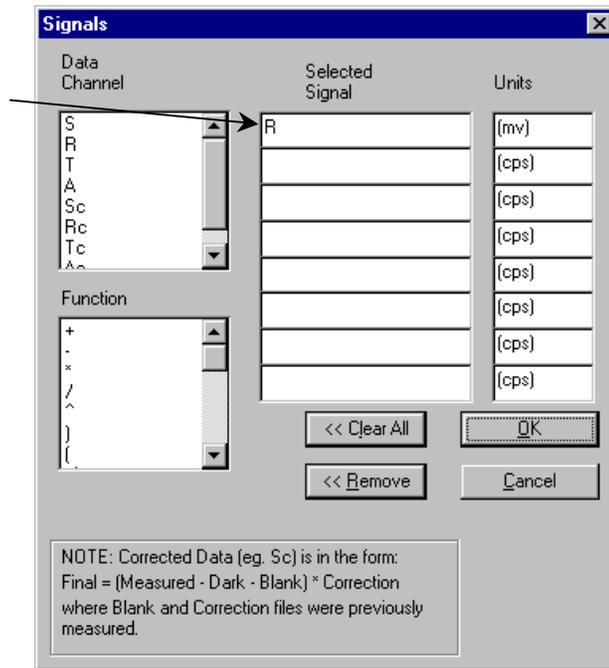
c   Click on Slits...

This opens the **Slits** dialog box.
Set the excitation entrance and exit slits to 1.5 nm.
Click OK to close the window.
If the slits' units are not in nanometers (nm), then open **Visual Instrument Setup**, select Options, then Units, and set the slits' units to nm. Use the reference (R) detector channel.
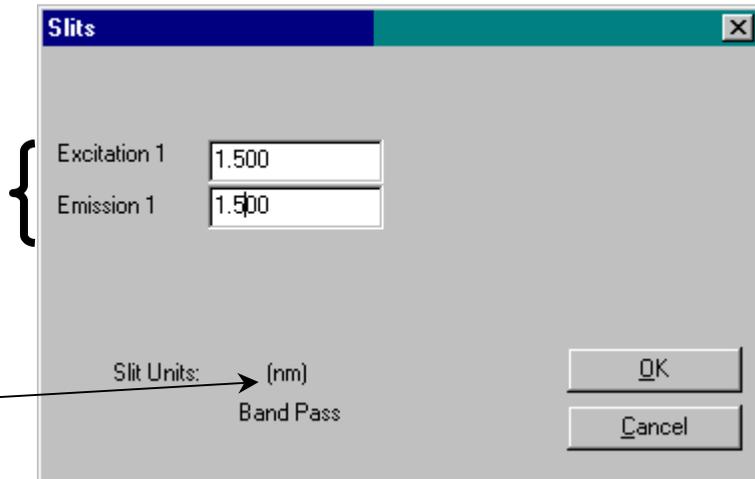
d   Click Run to execute the scan.

Lamp scan for single-monochromator systems:
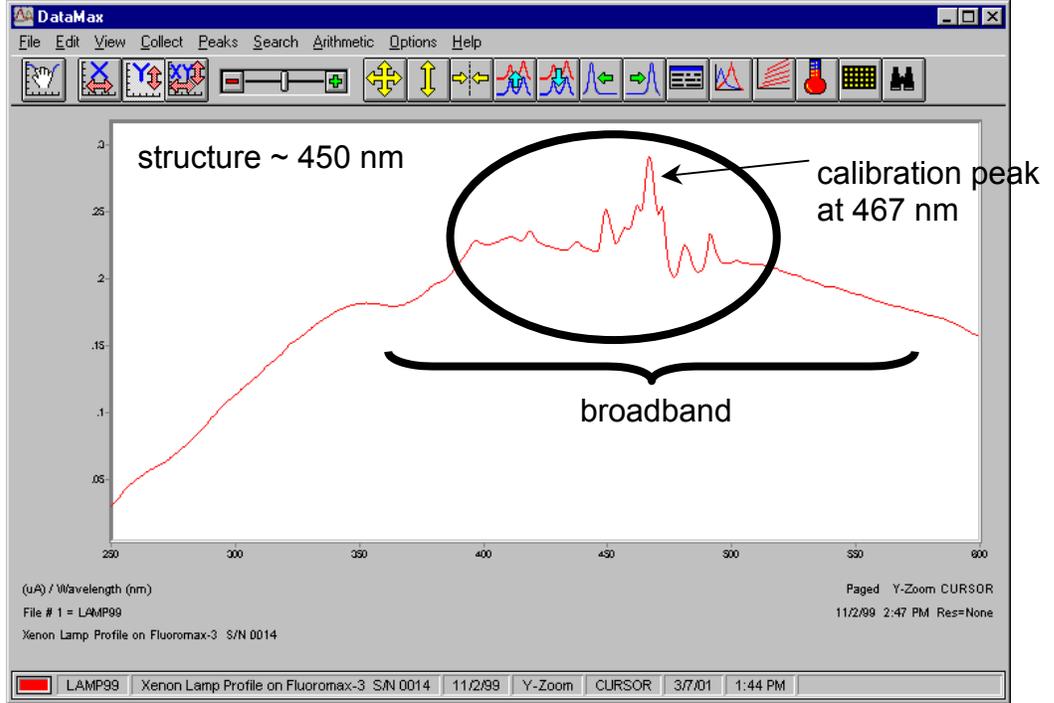
Xenon-lamp scan for an FL3-11 spectrofluorometer.

Lamp scan for double-monochromator systems:

On a double-monochromator system, the 467-nm line is no longer the most intense peak within the spectrum. Take care to isolate the 467-nm calibration line from other peaks.

Xenon-lamp scan for a double-mono-chromator spectro-fluorometer.
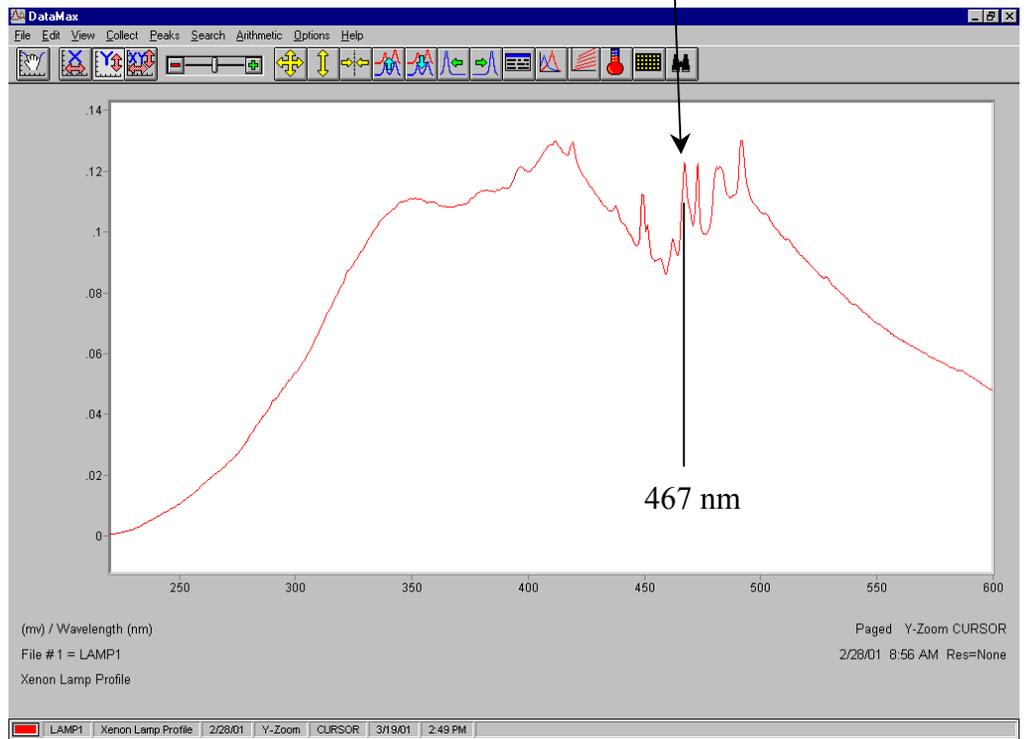
6   Find the calibration peak for the xenon-lamp spectrum.

The 467-nm peak is used for excitation calibration. The intensity of this spectrum should be noted for reference, although it is not used for instrument specifications.
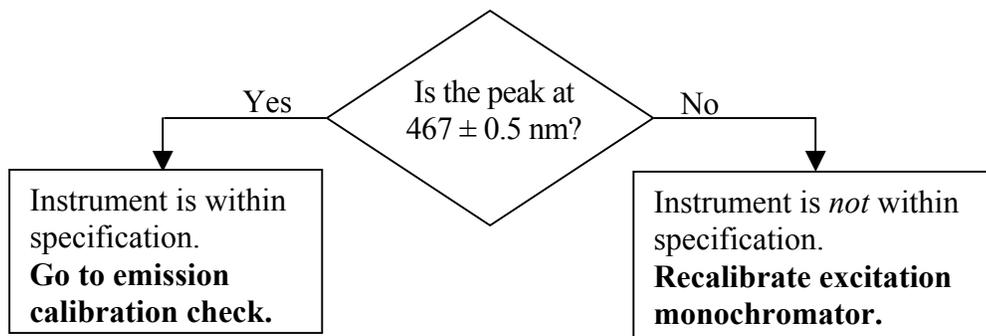
Excitation monochromator recalibration

Re-calibration of the Tau-3 is performed by moving to the position of the observed peak, going into **Visual Instrument Setup**, and telling the software the correct position at which this peak should be. The software will save this change in position to the calibration file.

1   Note the wavelength where the 467-nm peak was observed.

2   Open the **Real Time Display**.

3   Set the excitation monochromator to the position where the peak was observed.

Hit the Tab key on the keyboard to set the monochromator to the entered value.
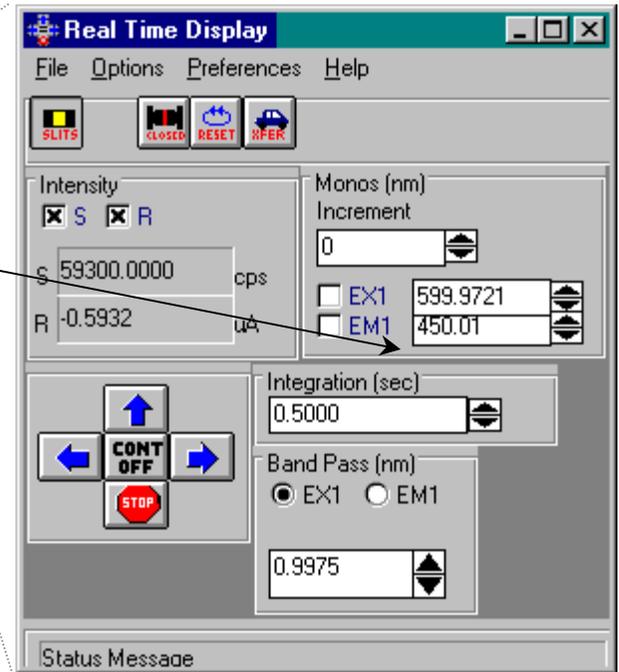
4   Close the **Real Time Display**.

5   Open the **Visual Instrument Setup** dialog box:

Note: The Visual Instrument Setup will vary, depending on the instrument configuration.

Visual Instrument Setup - DFLT.SET

File   View   Options   System   Help

350. nm

460.019 nm

Fluorolog - 3

For Help, press F1   Spex Instrument
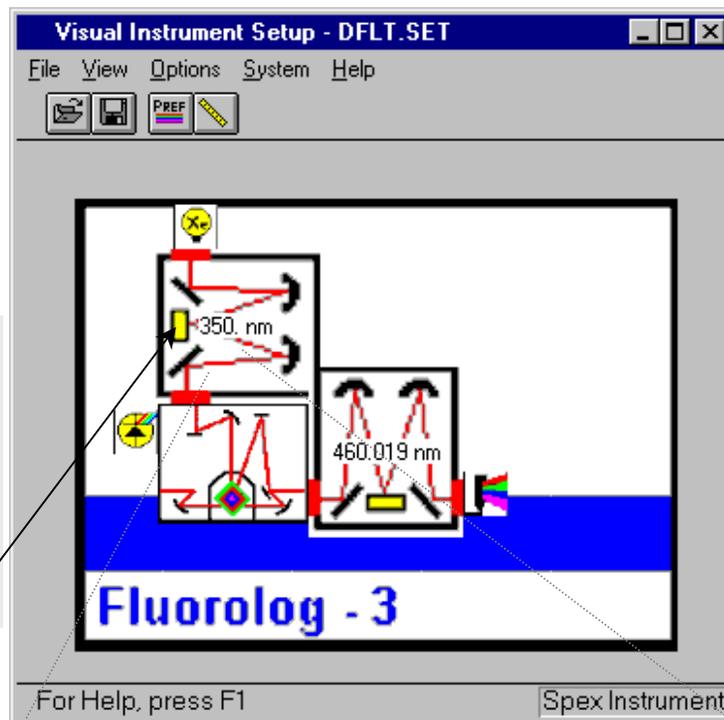
6   Click on the grating for the excitation monochromator.
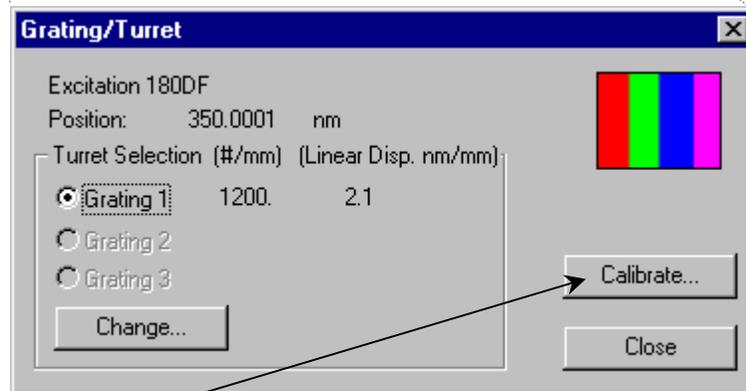
The **Grating/Turret** dialog box appears.

Grating/Turret

Excitation 180DF
Position: 350.0001   nm

Turret Selection (#/mm) (Linear Disp. nm/mm)

⦿ Grating 1   1200.   2.1
○ Grating 2
○ Grating 3   Calibrate...

Change...   Close

7   Click on the Calibrate... button.

This opens the **Enter Correct Position** dialog box:

Enter Correct Position

Excitation 180DF

Position   350.0001   nm

Calibration Position: 350.0001

OK   Cancel
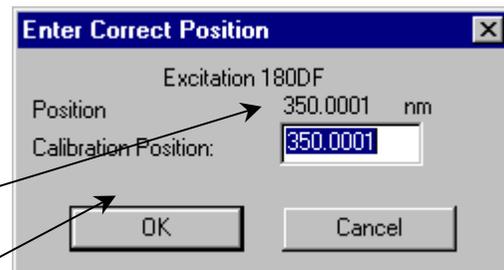
8   Enter the actual xenon-lamp peak, 467 nm.

9   Click OK.

The excitation monochromator should now be calibrated.

10 Click Close to close the **Grating/Turret** dialog box.

11 Close **Visual Instrument Setup**.

12 Confirm that the excitation monochromator is calibrated by running another lamp scan in **Run Experiment**.

This time the peak should occur at 467 ± 0.5 nm.

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. This check should be performed after the xenon-lamp scan. When completed, the performance of the system has been verified.

The water sample should be 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.

Use a 4-mL quartz cuvette.

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

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   Make sure the lid of the sample chamber is securely in place.

3   In the **Run Experiment** toolbar, choose the Experiment button.
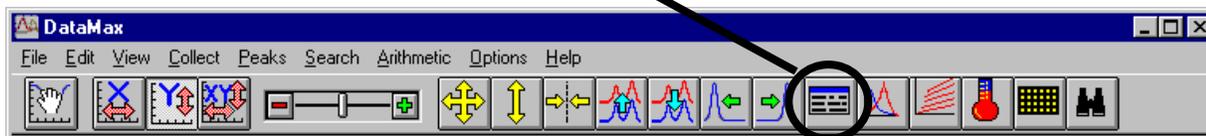
This opens the **Emission Acquisition** dialog box.

a   Click on Exp Type to open the **Select Experiment Type** dialog box:

Choose Emission Acquisition. Click OK to

Select Experiment Type

Emission Acquisition
Excitation Acquisition
Synchronous Acquisition
Time Base Acquisition
Multigroup Acquisition

OK
Cancel
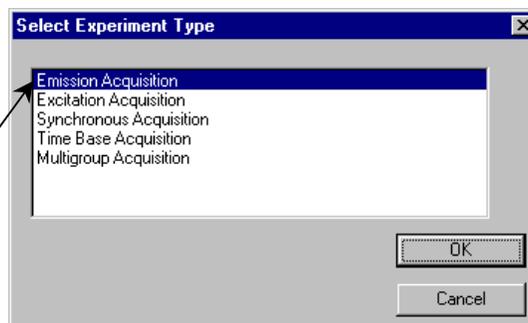
close the box.

b   In the **Emission Acquisition** dialog box:

Name the experimental setup water.exp,   Use a filename water.spc,
Start the scan at 365 nm,   End the scan at 450 nm,
Step the wavelength by 0.5 nm,   Use an Integration Time of 0.5 s.
Set the excitation to 350 nm,
Perform 1 scan.
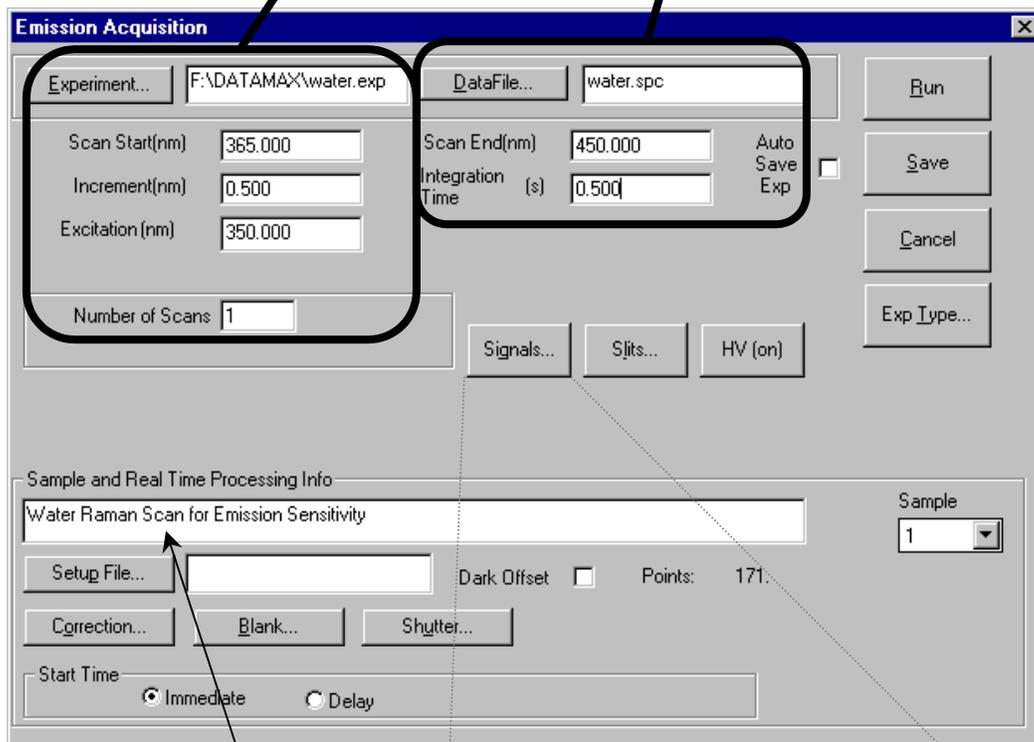
Describe the file as a water Raman-emission spectrum.

c   Click on the Signals...
        button to open the **Signals**
        dialog box:

Set the signal (S) detector.
Click OK to close the box.
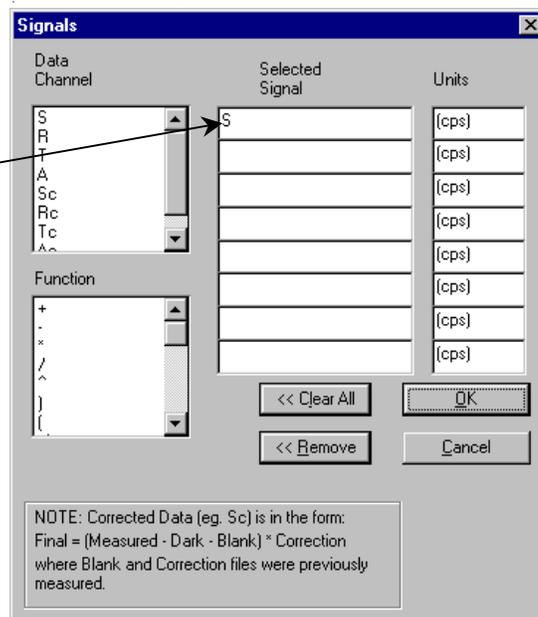
d   Click on the Slits... button
        to open the **Slits** dialog

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box:

Set the slits to 5 nm.
Click OK to close the
box.

If your slits' units are
not in bandpass units
(nm), then open
**Visual Instrument
Setup**, select
Options, then Units,
and set the slits' units
to nm. If you have a
Front-Face/Right-
Angle accessory, set it to the RA position.
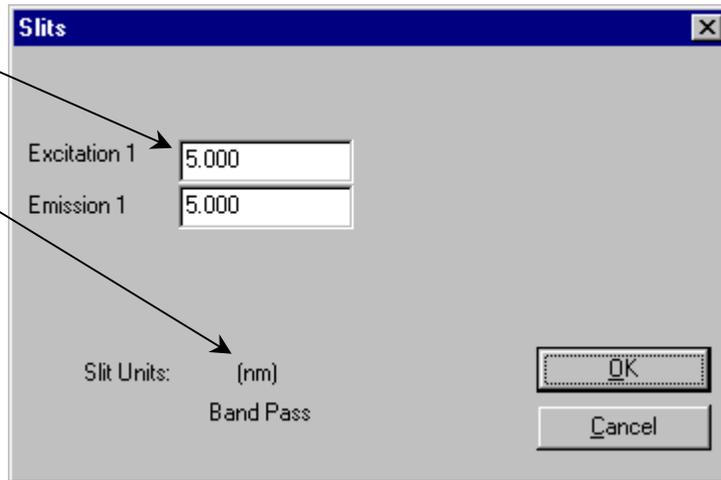
e   Click Run to execute the scan.

Your spectrum should resemble the following:
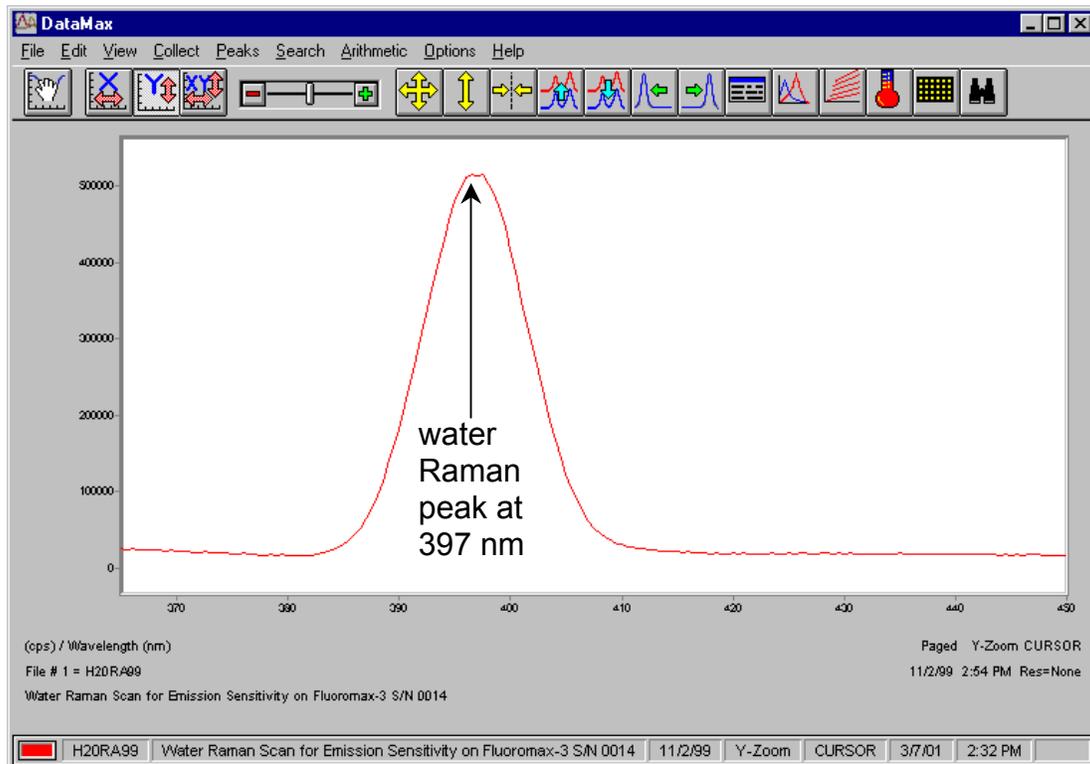
The Rayleigh scatter band or excitation band, which is about 10 times the intensity of the Raman band, occurs at 350 nm (the excitation wavelength). The scan begins at 365 in order to avoid detecting the Rayleigh band.

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.

Note: Be sure not to calibrate on the Rayleigh scatter band.

4 Find the peak of the water Raman band.

Is the peak at 397 ± 0.5 nm?

Yes → Instrument is within specification. **Calibration is complete.**

No → Instrument is *not* within specification.

Has the excitation monochromator been calibrated?

Yes → **Continue on to Emission re-calibration.**

No → **Go back to Excitation calibration.**
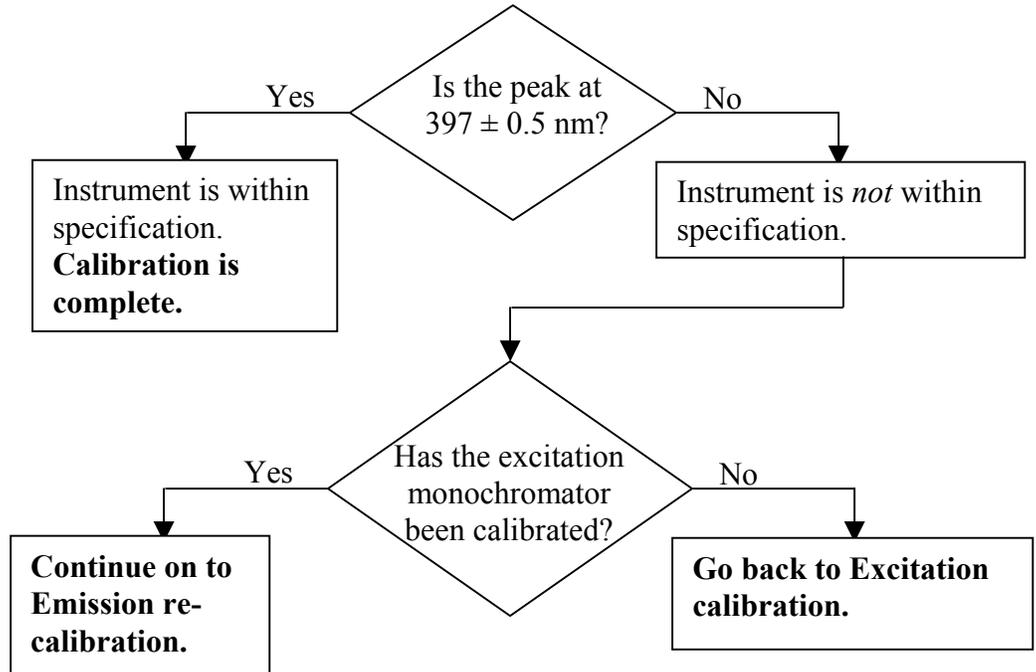
Emission recalibration

Calibration for the Tau-3 is performed by:
- Moving to the position of the observed peak,
- Going to the **Visual Instrument Setup** application, and
- Telling the software the correct position for this peak.

The software will save this change in position to the calibration file.

1   Note where the 397-nm peak appears.

2   Open the **Real Time Display**.

3   Reset the emission monochromator to the position where the peak was observed.

    Hit Tab on the keyboard to set the monochromator to the entered value.
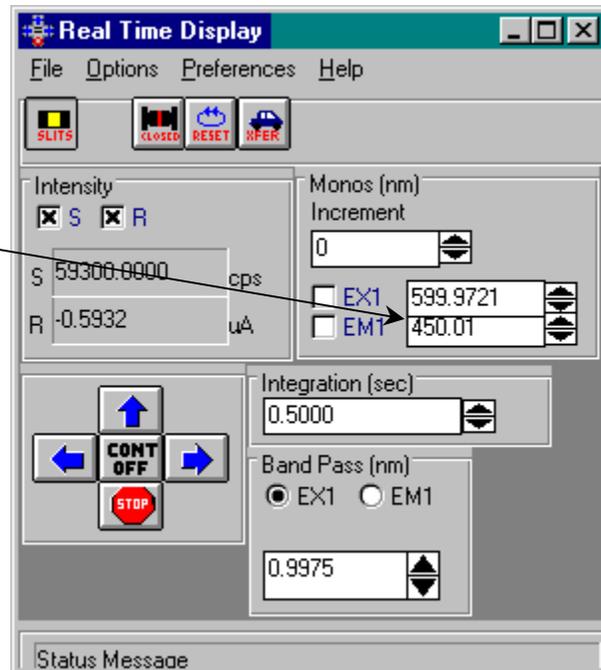
4   Close the **Real Time Display**.

5   Open the **Visual Instrument Setup** dialog box:

Note: The Visual Instrument Setup will vary, depending on the instrument configuration.
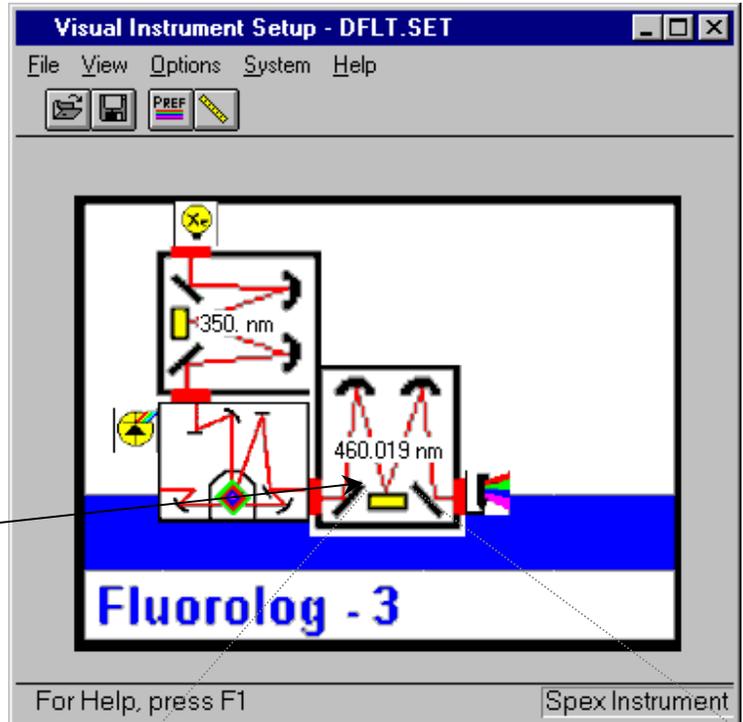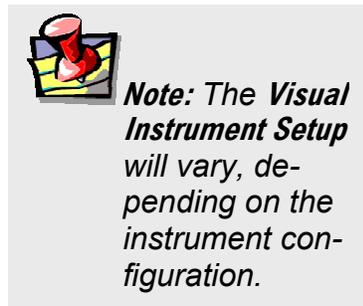
6   Click on the grating for the emission mono-chromator.

The **Grating/Turret** dialog box appears:

7   Click on the Calibrate... button.
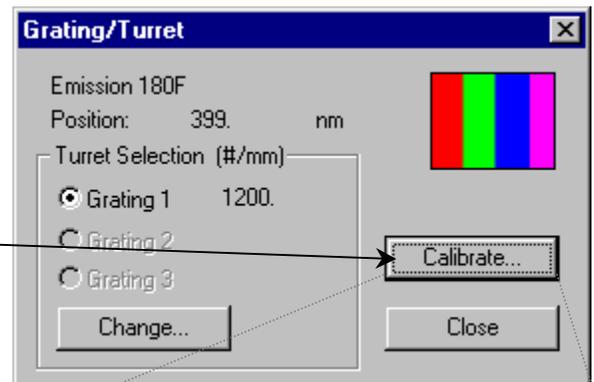
This opens the **Enter Correct Position** dialog box:

8   Enter 397 nm, the actual peak position using 350-nm excitation.
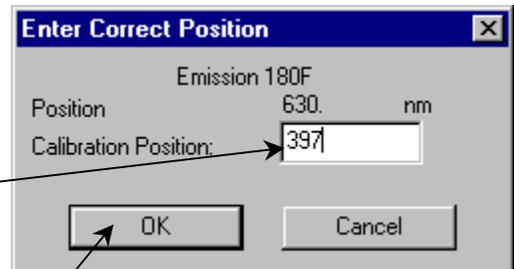
9   Click OK to close the dialog box.

The emission monochromator now should be calibrated.

10   Click OK to close the **Grating/Turret** dialog box.

11   Close **Visual Instrument Setup**.

12   Confirm that the emission monochromator is calibrated by running another water Raman scan in **Run Experiment**.

This time the peak should occur at 397 ± 0.5 nm.

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 Tau-3.
- Jobin Yvon recommends that the number of hours of xenon-lamp use be recorded in a log (see sample sheet in the *Fluorolog®-3 Operation Manual*, *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. The Raman intensity drops over time. When the signal-to-noise ratio becomes unacceptable, you may wish to change the lamp sooner.

Note: *Lamp output in the UV drops the fastest over time.*

Lifetime system performance checks

Do performance checks of the Lifetime components of the Fluorolog®-Tau-3 every 50 h (or sooner for questionable performance) of time-resolved use. The performance check examines the AC and DC signals, as well as depth of modulation, on a scatterer such as glycogen or Ludox®, then runs a lifetime acquisition of a standard fluorophore, POPOP, versus the scatterer.

Change from steady-state to lifetime (dynamic) mode

1   Load a lifetime layout.

(For the performance check, load a lifetime layout with no special accessories other than an optional automated sample changer).
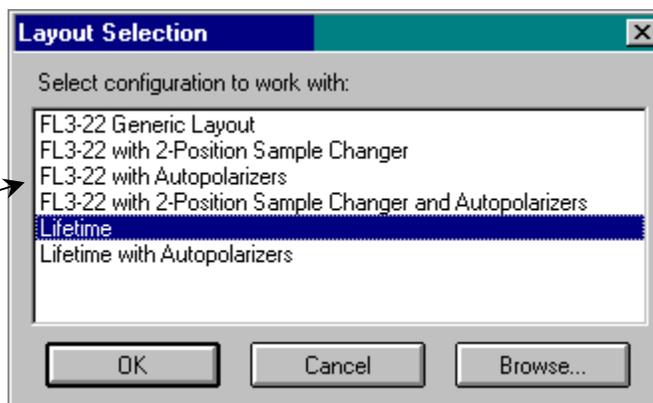
2   Turn the Modulator Position knob to Lifetime.

3   Turn the Source Position knob to Xenon.
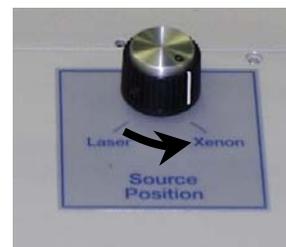
4   Close the iris most of the way.
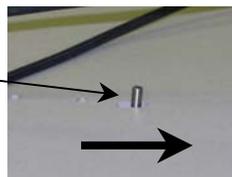
*Note: Some irises close to the **left**.*

(¾ of the way to the right)

5   Set the manual slit to narrow (0.75 mm).

When the layout is loaded, the DataMax icons should be colored in. (If they are in grayscale, then the instrument is running in emulation mode.) The LED on the I/O Drawer is now set to Lifetime mode for the detection system and electronics. You can also verify that the system is set for lifetime operation: Go to **Real Time Display** and note that the detector signals are in DC and AC volts, along with a display of the modulation and phase of the signal.

Check the AC, DC and modulation values for a scattering sample

The Fluorolog®-Tau-3 instrument should be switched on already and in *Lifetime* mode.

1   Prepare a dilute solution of glycogen or Ludox® in triple-distilled or de-ionized water.

2   Place the scattering solution in the active position of the sample changer.

3   Go to the **Real Time Display**.

Verify that your initial settings match those in the previous section.

4   Move mono-chromators:

Excitation: 370 nm
Emission(s): 370 nm

5   Set Integration time to 0.5 s.

6   Set instrument slits:

Excitation entrance: 6 mm
Excitation exit: 0.3 mm
Emission slits (optional): 6 mm
Intermediate 3rd slit (optional on double-grating monochromators): 6 mm

Note: Use mm units. To switch to mm, go to **Visual Instrument Setup** *or load default lifetime experiment.*

7   Set detector high-voltages:

Reference (R): 400 V
Signal (S): 800 V
T-Side (T): 600 V (if 2nd emission detector is present)
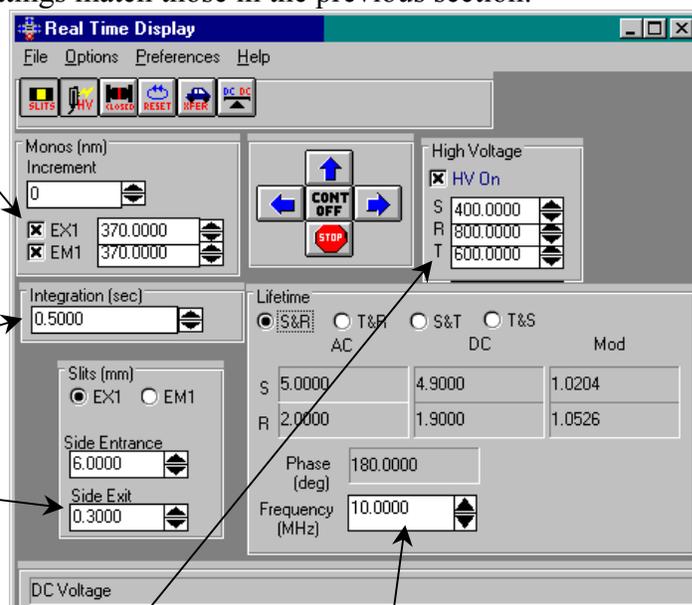
8   Set Frequency to 10 MHz.

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9   Adjust concentration of scatterer to yield 1.0 V < AC < 4.0 V.

10   Verify that the modulation (Mod) is ~1.0 on all lifetime detectors.

If the modulation is acceptable, then the basic lifetime performance check has been satisfied. To do a test scan, however, to check the performance of the lifetime system, follow the tutorial for a lifetime scan of POPOP versus glycogen in Chapter 8: *Tutorials* of this manual.

If the modulation is <<1.0, optimize some of the user-adjustable options on the system, and check that other settings have not been disturbed. Refer to Chapter 10: *Troubleshooting* of this manual for additional suggestions.

Adjustments to improve modulation or AC and DC signals

For good lifetime operation, a highly collimated beam must pass orthogonally through the faces of the Pockels cell. This yields strong AC and DC signals, along with a modulation ratio >1.0. The primary consideration is to have a narrow beam both before and just after the modulator compartment. Therefore, the slits and iris around the modulator are kept small during lifetime operation. The RF signals and external DC bias applied to the modulator and PMTs are set for optimal system performance. Variation of these settings will directly affect the signals and modulation seen from the system.

Verify that all system components are switched on. If a particular Lifetime controller or power supply is not receiving power, signals will be affected.

To improve low AC or DC signals:

1   Open excitation entrance and emission slits to 7 mm (wide).

2   Increase the high-voltage bias to the appropriate detectors.

The Lifetime detectors may be biased up to 1200 V. The normal range for the reference detector is 300–800 V, while the range for the S and T emission detectors is 600–1200 V. Emission detectors require different levels of bias depending on the concentration and fluorescence yield of the samples being measured.

3   Increase the scatterer's concentration.

For fluorophores, the recommended concentration levels are up to 0.1 A. You may use concentrations of up to 0.4 A, but artifacts may appear in the data.

To improve low AC or DC signals and change the modulation:

4   Open the manual slit.

This will diminish the modulation value as it increases the AC and DC signals to all detectors.

5   Open the iris.

As the iris opens, the signal levels should increase, with a corresponding drop in modulation.

6   Open the excitation exit slit.

This slit may be opened up, directly affecting the modulation ratio.

If modulation refuses to increase acceptably, or AC- and DC-signal levels are <<1.0 V

Unresolvable low modulation and signal levels point to poor optical alignment in the modulator compartment, or elsewhere in the system. Re-optimize the alignment of the Pockels cell in the modulator box. This procedure is explained in Chapter 11: *Maintenance*.

No modulation (< 0.01 at 10 MHz on a scattering sample) or no AC or DC signal (< 0.005 V at 10 MHz on a scattering sample) indicates that a major system component has no power, set incorrectly, or has failed. Consult Chapter 10: *Troubleshooting* for assistance in finding the cause of the absence of signal.

Useful materials for characterizing system and samples

The following are materials that Jobin Yvon® 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)
9-CA (97%), or 9-Anthracenecarbonitrile	1210-12-4	Single-exponential life-time standard	380–500	11.8 (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 $\geq$ 11)
D-glycogen	9005-79-2	Light-scattering standard		0
LDS 750, or Styryl 7	114720-33-1	Single-exponential life-time standard	680–700	0.248 (in MeOH, λ_{exc} = 568 nm)
LUDOX®, or colloidal silica	7631-86-9	Light-scattering standard		0
(Me)$_2$POPOP, or 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene	3073-87-8	Single-exponential life-time standard	390–560	1.45 (in EtOH)
β-NADH (β-nicotinamide adenine dinucleotide)	606-68-8 or 104809-32-7	Single-exponential life-time 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 life-time standard	370–540	1.32 (in MeOH)
PPD (97%), or 2,5-diphenyl-1,3,4-oxadiazole	725-12-2	Single-exponential life-time standard	310–440	1.20 (in EtOH)
PPO (99%), or 2,5-diphenyloxazole	92-71-7	Single-exponential life-time 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 life-time standard	560–680	0.98 ± 0.10
p-Terphenyl (99+%)	92-94-4	Single-exponential life-time standard	310–410	1.05 (in EtOH)
Water (18-MΩ, de-ionized, triple-distilled)	7732-18-5	water Raman sensitivity test		

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4: Fluorescence Lifetime Measurements

This chapter deals with all aspects of the fluorescence lifetime acquisition, from the choice of standards, to pre-experiment settings, setting up the actual scans, and finally viewing the data collected, and modeling the results. Throughout, the chapter provides suggestions on how to optimize the system and the samples for best results.

Theory behind lifetime acquisitions

During a lifetime acquisition, the excitation light at a particular wavelength is modulated sinusoidally using the Pockels-cell electro-optic modulator. This modulated light is incident upon the sample minus a small percentage directed to a reference detector. The sample's response shows a demodulation (reduction in the AC component relative to the incident signal) and a phase-shift, determined by the lifetime of the excited state or other sample properties. The modulation and phase-shift are measured across an appropriate frequency range for the sample.

For the lifetime acquisition, a standard with a known single-exponential lifetime is used along with the unknown under study. Measurements are performed by alternately observing the standard and unknown at each frequency. The standard is used to remove the instrumental response from the measurement of the phase angles and demodulation values.

The results of the acquisition are saved in a datafile (`.DAT`) where they can be fitted using the modeling software provided with the instrument, or with an external package.

Performing lifetime acquisitions

Steps to completing a successful lifetime acquisition

- Determine the steady-state spectra of the unknown.
- Decide on a sample geometry.
- Choose an appropriate standard sample.
- Choose the instrument geometry.
- Set up the instrument for lifetime acquisitions.
- Balance the standard and unknown.
- Define the experiment scan parameters.
- Execute the acquisition and observe the data collection.
- Model the data properly and validate the fit.

1   Find steady-state spectra of the unknown.

The first requirement for running a good lifetime acquisition is to know the steady-state fluorescence characteristics of the unknown. The Fluorolog®-Tau-3 may be used in steady-state mode to measure these spectra for the unknown. Knowledge of the excitation and emission characteristics is necessary in order to choose the proper wavelengths for the lifetime acquisition, as well as to avoid artifacts (e.g., Rayleigh or Raman scatter) from introducing noise into the measurements.

Note: Run an excitation scan and emission scan, which show the fluorescence characteristics for the unknown, before attempting to measure fluorescence lifetimes. Having the entire scans available is best, in case no optimal standard for the measurement is possible.

2   Decide on sample geometry.

In what physical state will the sample be measured? The preferred state for measurement in the Fluorolog®-Tau-3 is an aqueous solution held in a 4-mL non-fluorescing quartz cell. Other sample geometries, however, are possible. Perhaps the sample is a powder, or pressed into a thin film or pellet. Other samples may be available only in small quantities, requiring a microcell. Some samples may require being heated to a specific temperature, or cooling well below 0°C using a liquid-nitrogen or liquid-helium cryostat.

Carefully consider the sample geometry necessary for a proper measurement before setting up the instrument for the lifetime acquisition. This dictates the types of standards (and their geometries) to use, as well as the scan options and measurement geometry that must be chosen to yield valid results. Verify that the chosen sample geometry for measurement will not change the fluorescence characteristics of the sample.

3   Choose the standard.

The choice of a good standard is perhaps the most important of all steps in this procedure. The standard is used to correlate the instrumental measurements of phase and modulation on an unknown with those on the known. Such comparison removes instrumental artifacts from the desired data. Therefore, choosing a poor standard, or inadequately measuring the standard's characteristics, can be detrimental to the quality of the results.

The standard must satisfy the following criteria for the best results:
- A single exponential lifetime or a scatterer
- The same geometry as the unknown
- Similar excitation and emission characteristics as the unknown

The standard must be a well-characterized fluorophore with a single exponential lifetime, or be a scattering material. The lifetime of the standard must be known accurately and entered in the experiment-definition window. A list of some suggested fluorophore standards is provided at the end of Chapter 3, with their peak excitation and emission wavelengths and room-temperature lifetimes.

Scattering samples are especially convenient, for most can be used over a wide excitation range, allowing for use with a multitude of unknowns, without worrying about the color effect. When using a scattering sample, set the lifetime to 0.000 ns (for there is no absorption). For the lifetime scan, use the same excitation wavelength for the scatterer as for the unknown. The scattering samples we recommend are listed with the fluorophores at the end of Chapter 3.

In most cases, a scattering sample is sufficient to obtain good results from the measurements. Use of a single exponential fluorophore with similar excitation and emission characteristics to the unknown is preferable, especially if the sample has a multi-exponential decay, and the lifetime of the standard is similar to the average lifetime of the unknown sample.

Use the same sample and measurement geometry for your standard as for the unknown. The more similar the excitation conditions for the standard and unknown, the more accurate the results. For example, to measure a thin film using the front-face option in the Fluorolog®-Tau-3, choose a fluorophore in a thin film or use a scattering block with front-face geometry.

The standard should have similar steady-state excitation and emission characteristics to the unknown. To minimize the color effect, find a standard to match the excitation and emission wavelengths within 20 nm. Ideally, these wavelength pairs are identical for the standard and unknown. A scattering sample of glycogen or Ludox® is particularly useful for matching the excitation wavelengths, and is recommended as a good general standard.

4   Choose instrument geometry.

Specify the instrument geometry to be employed for the lifetime acquisition. The instrument geometry options can include (but are not limited to):
- Right-angle versus front-face measurements
- T-side versus S-side detectors
- Xenon lamp versus external laser source
- Monochromators versus optical filters
- Sample-changers, single-cell mounts, solid-sample holders, fiber-optic platforms, cryostats, and other sample-related devices.
- Polarizers
- Temperature baths

Choose the best available instrument setup and accessories using an appropriate amount of time to yield the best results. A further discussion on the use of different instrument geometries to improve results can be found in the "optimizing results" section of this chapter.

5   Decide on instrument set-up for lifetime acquisitions.

Boot up the software in lifetime mode, to collect a lifetime acquisition with all lifetime controllers and power supplies turned on. Set the appropriate optical settings and instrument options manually to the proper positions for lifetime mode. Load the appropriate lifetime acquisition layout (depending on the accessories for the scan). While the software is loading, verify that the Pockels cell is in the light path, the iris aperture and intermediate slit are set for lifetime mode, and the laser mirror/xenon-lamp selector is in the proper position (see Chapter 3 for a detailed description of changing from steady-state to lifetime mode and back). When the software is running, verify that the lifetime rack indicates *Lifetime* mode.

6   Balance the standard and unknown samples.

It is essential to match the DC emission signal intensities for the unknown and standard samples, and to set up the system so that all of the signal levels are in the appropriate range.

Warning: *Failing to properly set the system for the measurement will lead to erroneous and noisy results.*

When setting signal levels and balancing the samples, use the **Real Time Display**. **Real Time Display** allows choice of detectors to be used for the acquisition. Windows in **Real Time Display** display the signal levels and high-voltage applied to each detector, along with Signal Balance (the selected excitation/emission wavelengths and sample-changer position for each sample). When setting signal levels and balancing is complete, the Transfer function allows the settings to be moved directly into the lifetime-experiment definition.

If, during this procedure, satisfactory signals or a good balance cannot be achieved, then reconsider the choice of standard sample, sample geometry, or instrument set-up.

7   Set the initial instrument settings.

The instrument should be set up with reasonable initial settings for lifetime measurements.

In lifetime (dynamic) mode, the instrument settings conform to different requirements than for steady-state. The Pockels cell (electro-optic modulator) in the light path requires the excitation light to be highly collimated for the most efficient modulation. Light slightly off-axis reduces the effective modulation ratio observed from the instrument. To achieve good modulation, the apertures at each end of the modulator box must be kept as small as possible. Therefore, set the slits *directly* adjacent to the modulator box to a small opening, and close down the iris aperture at the modulator box entrance.

The other slits in the system are used primarily to set an appropriate signal level. Therefore, by default, set the entrance slits (and intermediate slit, if applicable) in the excitation monochromator and the slits on the emission monochromator (if used) wide

Warning: *Settings on the RF amplifiers and the HV bias supply are optimized at the factory and during installation, and **should not** be adjusted by the user.*

in dynamic mode. The high voltages fixed in steady-state mode for photon-counting measurements may be adjusted in dynamic mode to optimize the signal levels. Set the monochromators to the wavelengths corresponding to the desired excitation and emission peak pair for the unknown sample. The standard sample should have similar characteristics to the unknown, to minimize the color-effect described earlier. If the Stokes shift between the excitation and emission peak is narrow, use good experimental prac-

tice to excite at a shorter wavelength than the excitation peak, and set the emission to a longer wavelength than the emission peak, to minimize scatter contribution to the measurement. The excitation and emission wavelengths can be adjusted in the balancing procedure to help match the DC signals.

Initially, set the system to a relatively low frequency, where there is ample modulation, in order to optimize the signal levels. Typically, we at Jobin Yvon choose 10 MHz as this frequency for setting the signals.

Settings and their limits
In the center column of the following table, the recommended initial instrument settings in **Real Time Display** to set and balance the signals are listed. On the right are the recommended extremes of adjustment during optimization and balancing.

Instrument parameter	Initial value	Limits for adjustment
Excitation and emission wavelengths	Dependent on steady state for unknown and standard	Dependent on steady state for unknown and standard
Detectors	S & R or T & R	
Excitation slits		
Entrance	6.0 mm	2–7 mm
Intermediate (if applicable)	6.0 mm	2–7 mm
Exit	0.3 mm	0.2–2 mm
Iris aperture	Vertical (midway between open and closed)	Full range
Modulator (Pockels cell)	In light path	
Source position	Xenon source	
Intermediate slit (manual bilateral slit)	0.5 mm	0.2–2 mm
Emission monochromator or filter		
Slits (entrance, intermediate, exit)	6.0 mm	2–7 mm
Cut-on filters	Appropriate cut-on filter(s) for excitation and emission wavelengths used for measurement	
Detector biases		
R	400 V	300–800 V
S or T with monochromator	800 V	400–1100 V
S or T with optical filters	600 V	400–1100 V
Modulation frequency	10 MHz	
Integration time	0.5 s	

Optimizing signal levels using the unknown sample
Optimizing the signal level on the unknown sample involves adjusting the slits, detector high-voltages, and other settings to obtain AC and DC signals on the emission detector at 1–5 V, and AC and DC signals on the reference detector to 1–5 V, with the

highest modulation available for both. Optimize the AC signals first on the emission detector, with the AC on the reference detector set later using its high-voltage setting.

Some instrument settings can be used to improve the AC without affecting the modulation, and other adjustments improve the AC signal at the expense of the modulation. Below are the recommended protocols for optimizing the levels on the unknown for each particular condition.

To increase AC and DC signals independent of modulation
1. Open emission slits wider (if using emission monochromator)
2. Open excitation entrance (and intermediate, if present) slits wider.
3. Increase high voltage to the detector.

To increase AC and DC signals at the expense of modulation
1. Open the intermediate slit in the modulator box wider.
2. Open the iris aperture wider.
3. Open the excitation exit slit wider.

To increase modulation while reducing AC and DC signals
1. Reduce the intermediate slit width in the modulator box.
2. Reduce the iris aperture.
3. Reduce the excitation exit slit width.

### To decrease AC and DC signals independent of modulation
1. Reduce emission slits' width (if using emission monochromator).
2. Reduce excitation entrance (and intermediate, if present) slit widths.
3. Decrease high voltage to the detector.

8   Balance signals for the standard and unknown.

Balance the DC signal intensity of the standard to the DC signal intensity of the unknown. The DC signals should be within 10–15% of each other, and both AC and DC signal levels should be at 1–5 V.

Real Time Display has a window, **Signal Balance**, for balancing the samples. Enter the excitation and emission wavelengths and sample-holder position for the standard and unknown. Click on the Set Standard and Set Unknown buttons to move the system to the appropriate settings for each.

The two adjustments to balance the samples are:
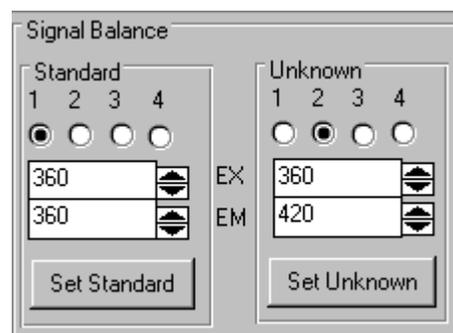
a   Change the concentration of the standard solution.

Reduce the concentration to lower the signal, or raise the concentration to increase the signal.

b   Move to shorter wavelengths on the excitation band, or longer wavelengths on the emission band, for the standard or unknown.

The further that the excitation or emission is moved from the peak, the lower the emission signal.

In some cases, balancing the samples using the two steps alone is impossible. The signal levels or concentration for the unknown then must be modified to allow the chosen standard to work effectively for the measurement. In cases where the balancing still cannot be accomplished, reconsider the standard and the entire experimental setup.

9   Set the lifetime-acquisition scan parameters.

a   Start the **Run Experiment** application.

b   Click on the Experiment button.

c   This opens the **Lifetime Acquistion** dialog box. There are eight different sections to adjust within this dialog box:

1   Load previously saved experiment and data files

2   Run and save the experiment

3   Adjust frequencies to be run

4   Choose wavelengths

5   Unknown-versus-standard parameters

6   Hardware parameters

7   Statistics parameters

8   Sample and real-time processing information

1   Load previously saved experiments and data files.

Click on Experiment to load a previously saved lifetime-acquisition experiment file. Use the experiment-file browser herein to search for a desired life-time-experiment file.

Click on DataFile to select a filename under which the data file is to be saved during an experiment. Enter the file-name and its path in the space provided. Lifetime acquisition datafiles are saved with a .DAT extension.
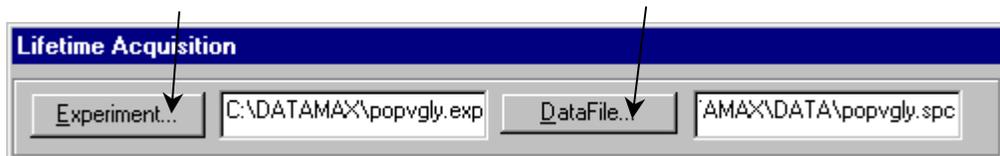
Lifetime Acquisition

| Experiment... | C:\DATAMAX\popvgly.exp | DataFile... | AMAX\DATA\popvgly.spc |

2   Run and save the experiment.

Click the Auto Save Exp checkbox to overwrite the experiment file automatically with your current settings at the start of an acquisition.

In Quick Scan, all phase and modulation data for each frequency point in the scan are taken for the standard first. Then the user is prompted to insert the unknown (with an automated sample changer, it is automatically moved into place), and the unknown data are taken.
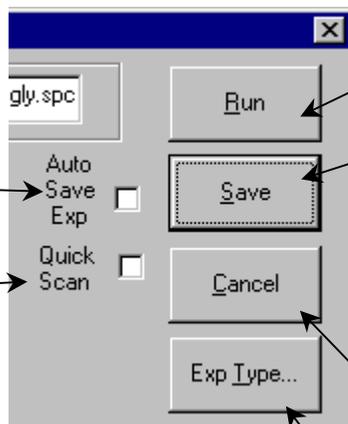
Click on Run to execute a lifetime acquisition.

Click Save to save the experiment to the file listed in the **Experiment** window. To save the experiment file to a different filename, type in the name and path in the **Experiment** window and click Save.

Click Cancel to go back from the lifetime acquisition to the main menu of the software application.

Hit the Exp Type… button to select the Lifetime Acquisition default experiment file when you are defining new lifetime acquisitions.

Note: Some error may be introduced in Quick Scan, because the data for each sample might be separated by a long period.

Use Quick Scan when the sample cannot be placed in an automated sample changer (e.g., low-temperature measurements or solids) to avoid the tedium of changing the standard and unknown at each frequency point.

3   Adjust frequencies to be run.

In Start Freq, enter the starting frequency, in MHz. Lifetime scans always progress from lower to higher frequency. The frequency range is 0.1–310 MHz.

In End Freq, enter the ending frequency, in MHz.

Enter the number of different frequencies to measure in Number of Freqs. Each frequency will give one data point in phase and modulation. The allowable number of frequencies is 3–99.

Integration Time is the time allotted for detection per data point during each average. The range is 1–99 seconds.

The Sequence specifies the frequencies used for the lifetime acquisition. By default, there are two allowable base settings for the sequence Linear and Log.

- Log sets the frequency range between the start and end frequency for even spacing on a logarithmic scale, depending on the number of frequencies specified to be measured.

- Linear sets the frequency range between the start and end frequency for even linear spacing of the number of frequencies specified.

Click on the Sequence button to display the frequencies to be measured. Before running an acquisition, customize the frequencies if desired.

To customize a frequency, choose the frequency to be changed in the list by clicking on it. Then enter the new frequency value.

4   Choose wavelengths for the standard and unknown.

Next to Std Ex (nm), enter the chosen excitation wavelength position (in nanometers) for the standard.

Next to Unknown Ex (nm), enter the chosen excitation wavelength position (in nanometers) for the unknown.

| Std Ex (nm) | 360 | Unknown Ex (nm) | 360 |
| Std Em(nm) | 360 | Unknown Em(nm) | 420 |

Next to Std Em (nm), enter the chosen emission wavelength position (in nanometers) for the standard.

Next to Unknown Em (nm), enter the chosen emission wavelength position (in nanometers) for the unknown.

5   Choose the data-correction algorithm.

Process by determines the algorithm for collecting the data for each individual average. There are two possible choices:

Discrete Pairs measures the phase-angle difference and demodulation of the standard, and then the unknown. From this, the computer saves the phase and modulation data and calculates lifetimes based on phase and modulation. Then, it repeats the procedure for the next average, measuring the standard, then the unknown, saving the data and calculating lifetimes. Thus, with discrete pairs, averages are non-shared measurements of the standard, followed by the unknown.

Note: When changing samples manually, use Discrete Pairs*.*

Interleaving measures the phase-angle difference and demodulation of the standard, and then the unknown. From this, the computer saves the phase and modulation data and calculates lifetimes based on the phase and modulation. Thereafter, the standard is again viewed, but a new average is measured, comparing with the previous unknown data. In this way, an average is generated each time the sample is changed. This is the recommended method when using an automated sample changer, for it saves time with no effect on experimental accuracy.

6   Adjust hardware parameters.

There are two possible signal choices that may be chosen in Signals… window: S & R or T & R (if available). The radio buttons show the detectors that may be selected for the lifetime scan. Choose the appropriate signal detector to be compared with the reference, R, detector for the lifetime acquisition.

Slits… leads to a window showing the available slits and their settings for the lifetime acquisition. Set the slits according to how the standard and unknown samples were balanced. Slits should be set in millimeters for lifetime acquisitions.

HV is the high-voltage bias setting for the lifetime detectors. The settings for each detector should have been determined during the balancing procedure. The limits for high voltage bias for R928P photomultiplier tubes in the lifetime detector housings are 300 to 1100 volts.

7   Decide on the statistics for measurement.

Averages are the number of measurements of the phase and modulation data for the standard versus the unknown for each frequency during a lifetime scan. The number of averages taken per frequency point depends on the minimum and maximum number of averages specified and the standard deviation for the measurement specified.

The *standard-deviation threshold* for the measured phase and modulation data is used to decide how reliable the data point is. If the standard deviation of the measurement is within the specified threshold after the minimum number of averages, then the experiment will move on the next frequency point. After the maximum number of averages, the experiment will move on the next frequency point automatically.

Min Avg is the minimum number of averages. The smallest minimum number of averages is 3, to calculate a standard deviation.

Set Pt. Std. Dev. (%) is the standard-deviation threshold for the measured phase and modulation data.

In Standard, enter the sample-changer position for the lifetime standard sample.

In Unknown, enter the sample-changer position for the unknown sample.

Max Avg is the maximum number of averages. The highest maximum number of averages allowed is 99.

In Std Lifetime (ns), enter the lifetime of the standard sample in nanoseconds. For scattering samples, enter 0.00 nanoseconds.

Note: If negative phase-angles or modulation values >> 1 are observed, the sample-changer number for standard and unknown are probably reversed.

8   Enter sample and real-time processing information

In this box, enter any comments to include with the datafile.   Shutter… is always set to
Auto Open.

Sample and Real Time Processing Info

Lifetime Acquisition of POPOP vs. Glycogen or LUDOX   Shutter…

Setup File…   Dark Offset ☐

Start Time
⊙ Immediate   ○ Delay   Estimated   0:14:00
                                          Time

In Setup File…   The Start Time   Dark Offset is not available for   Estimated
load a pre-defined   area allows the   lifetime scans, because the data   Time displays
set of positions   scan to start Im-   are compared with the reference   the estimated
for various acces-   mediately or De-   lifetime detector for all instru-   time to complete
sories.   lay a specified   mental corrections during the   this scan.
                      amount of time.   measurement.

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10 Click on Run to execute the lifetime acquisition.

The system measures the phase-angle differences and demodulation factors using data taken from the standard and unknown. The system takes repeated averages at each frequency point, until the specified standard deviation has been surpassed, or the maximum number of averages has been reached. The lifetime calculated from the phase angle and the demodulation factor at each frequency point, using the mean of the averages, is found with these mean values for phase and modulation. The scan always proceeds from the low- to the high-frequency point according the set-up sequence.

Data are displayed in a table:

The most recent data are displayed here.

Over-range indicator

Note: *If an over-range is detected, the over-range indicator turns red. Stop the scan and try to find the cause of the over-range. See the* Troubleshooting *section.*

Summary of all results so far.

The Summary Table shows:

#	(MHz)	φ	(±)	M	(±)	τ φ	(±)	τ M	(±)
1	10	16.16	0.14	0.96	0	4.6103	0.0428	4.642	0
2	14.7	22.79	0.06	0.917	0	4.5479	0.0127	4.7096	0
3	21.7	31.44	0.05	0.849	0	4.4846	0.0084	4.5647	0
4	31.9	42.16	0.05	0.733	0	4.5168	0.0074	4.63	0
5	47	52.88	0.04	0.594	0	4.4737	0.0067	4.5861	0
6	69.2	62.73	0.22	0.452	0	4.4619	0.0423	4.5389	0
7	101.9	71.4	0.24	0.329	0	4.6413	0.0651	4.4831	0
8	150	76.44	0.57	0.24	0	4.3975	0.1912	4.2918	0

A graph also is created in a separate window, showing the phase-angle difference and demodulation factor across the frequency range.
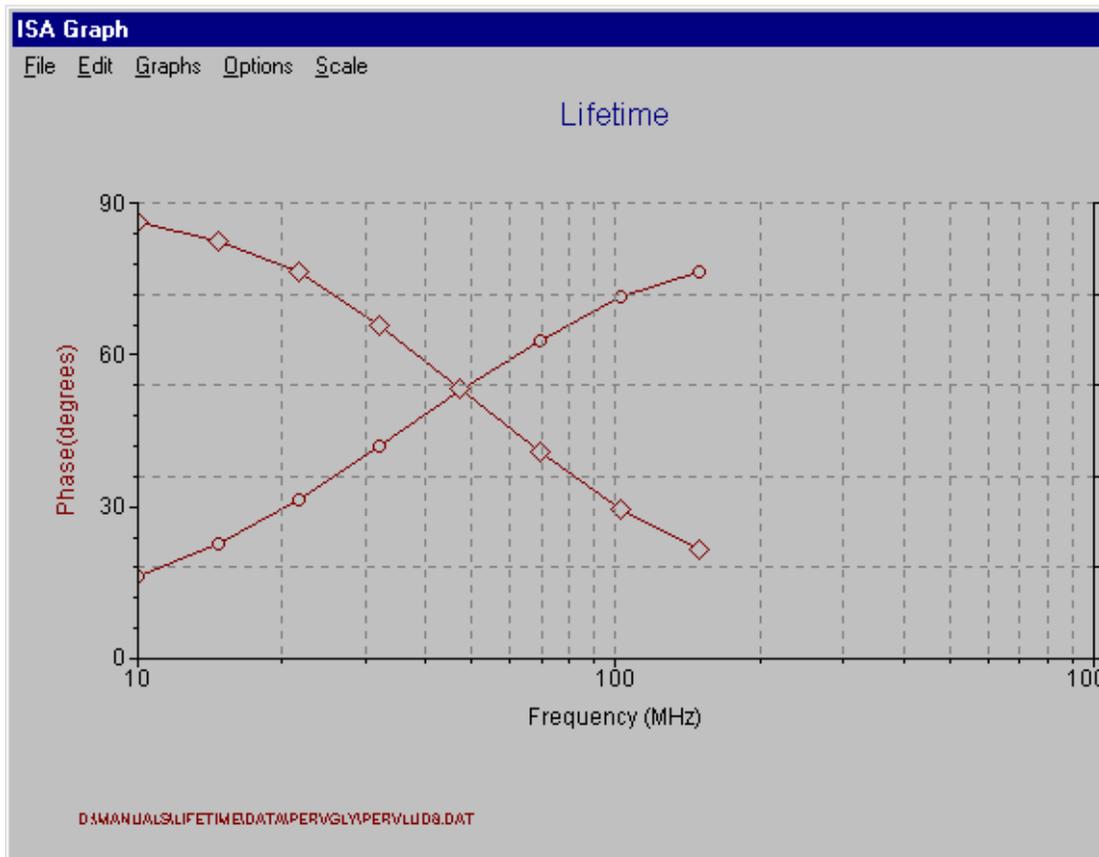
Model the data for final results

Modeling is the process by which the lifetime acquisition results are fitted, to predict the various lifetime components and fractional contribution of each component. The fit is accompanied by an error-function value to help validate the fit as an acceptable model.

The software includes a basic modeling software application, MODEL.EXE, which may be used to fit single- or multi-exponential lifetime models. More-complex models must employ the use of a more-advanced fitting program such as GLOBALS Unlimited™ software.

To start the modeling program in DataMax, click on the Run Lifetime Modeling software application button in the **Instrument Control Center**.
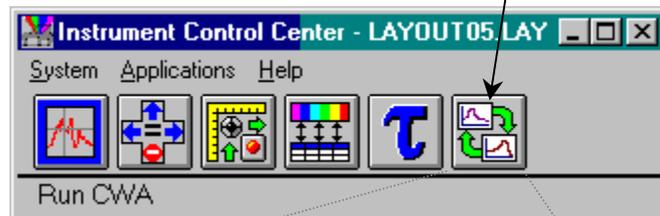
This brings up the **Model Windows Application** window. Choose Model. Under Model, click Lifetime.
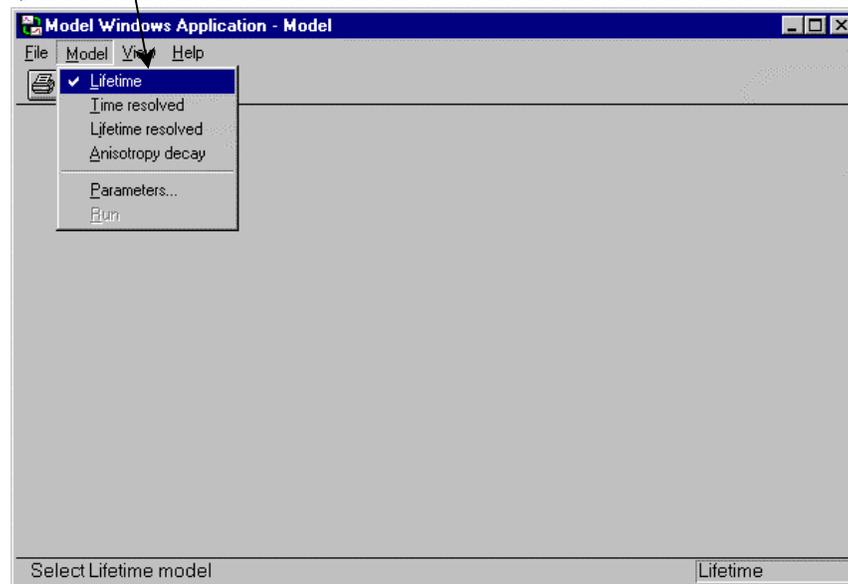
This calls up the **Lifetime Model** window specifically for modeling lifetime data.

Under Input file, choose the data file to be modeled.

Here, choose the type of standard deviation used for the model.

Choose the Number of components in the model here.

Here enter the Fractional Amplitudes for all components.

Here enter estimated Lifetimes for all components.

We recommend that novices use fixed deviations set at dPhase = 0.5° and dMod = 5 × 10^{-3} (i.e., 0.5%) modulation, as shown. Thus, the χ^2 value will reflect the validity of the fit versus these standard deviations, or error levels in the data. Therefore, $\chi^2 < 1$ indicates that the model has surpassed the error-threshold specified.

Single exponential decay

When fitting an unknown sample, we recommend starting with a single exponential decay, and then proceed to more complex models based on the χ^2 results and the residuals. Generally, the least-complex model which results in an acceptable χ^2 value is considered viable. Therefore, start with a model such as the one shown above, with a single exponential defined with a good estimate of the mean decay time. In the example above for perylene in glycerol, 10 ns was chosen.

Click on the Run Model option to fit the data using the selected model. The resulting decay time(s) are displayed on the parameters screen, with its(their) fractional contribution(s) (in this case, 1). A graph is shown with the actual phase-shift and demodulation-factor data points from the data file, along with solid lines showing the fit created by the model. At the top of the graph, the calculated values for the fractional contributions, lifetimes, and the χ^2 also are displayed. Below is an example of such a window.

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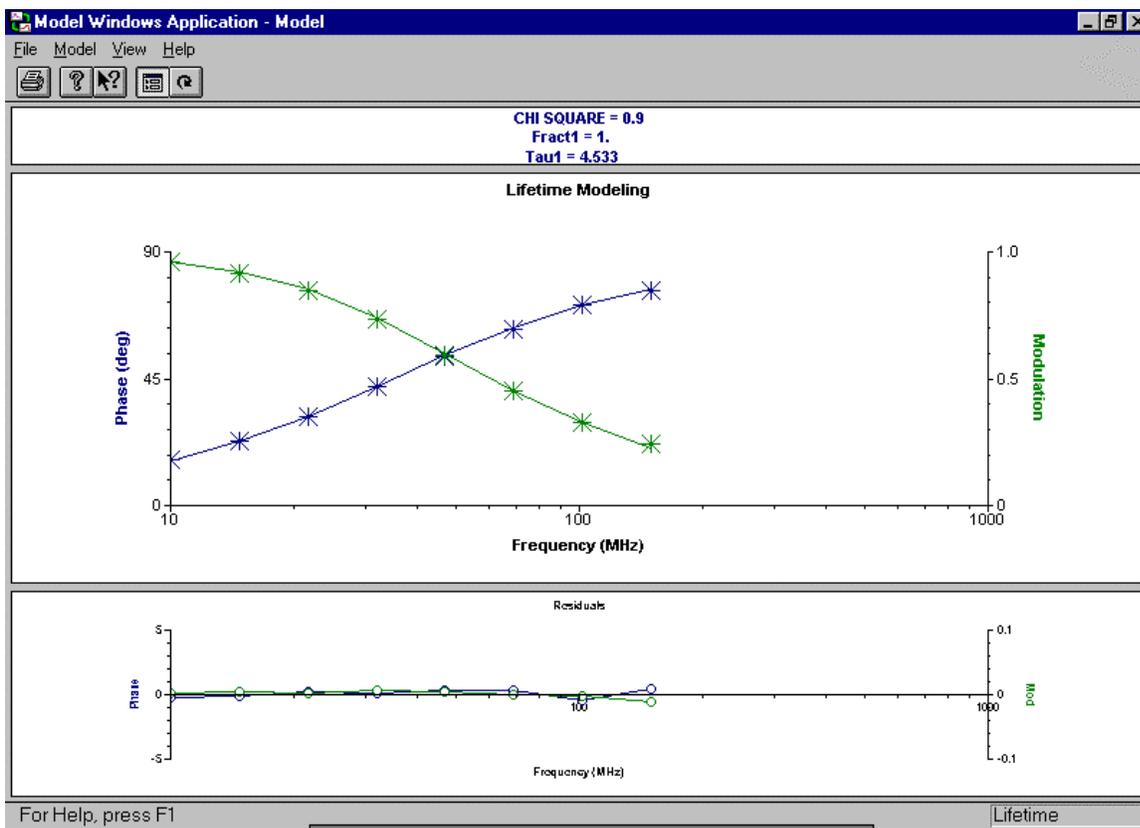
For the model shown above, the single exponential fit is satisfactory because of the low χ^2 value and the small residuals shown. This can also be demonstrated by attempting to fit this scan as a double-exponential.

Multi-exponential decay
To fit a multi-exponential decay,

1   Increase the Number of components to the number of desired exponential terms.

2   Enter the fractional amplitude and lifetime for each component of the decay.

3   Each fractional amplitude and lifetime has the option to be Fixed (in the check box) or held constant in the model.
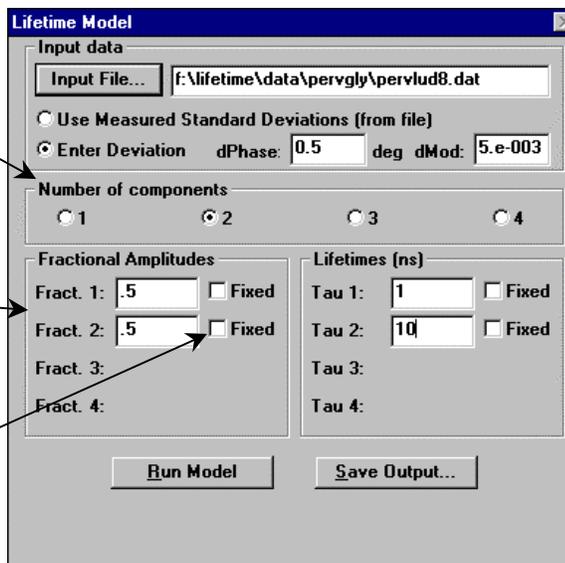
The results of using this particular double-exponential model are:

The model provides two decay times of 4.3 and 6.3 ns. The χ^2 value is low and the residuals *seem* acceptable. But because the χ^2 has improved only from 0.9 to 0.69, the less-complex fit of the *single* exponential should be accepted in the absence of other information. If the χ^2 had shown more dramatic improvement (e.g., 0.9 versus 0.5 or less), then the double-exponential fit would be a more-reasonable model.

Saving and outputting the results

There are options available in the parameters window for saving the fitted results for the data. Click on the Save Output button, and specify a proper filename and path to save the results.

Printing the results of the model is available in the graph window under the File menu. Use the standard Windows™ print window to define the desired printer from the host computer, and specify the available parameters.

5: Anisotropy Decay Measurements

Anisotropy decays, otherwise known as dynamic depolarization measurements, provide information into the rotational rates of molecules in the allowed directions. These rotational correlation times can provide great insight into molecular size, shape, structure and environment. This type of acquisition may be run on lifetime instruments with automated polarizer accessories.

Theory of anisotropy decay in the frequency domain

Measurements of anisotropy decays in the frequency domain involve acquiring the phase-angle and ratio of modulated amplitudes for an unknown sample across a specified frequency range.

Method

Light from the excitation source passes through the excitation monochromator, in which a specific wavelength is selected. This selected wavelength passes through the modulator box, in which the Pockels cell sinusoidally modulates the light at a chosen frequency. The light emitted from the Pockels cell is vertically polarized. This vertically polarized light enters the sample compartment, and hits the sample. The sample's response is measured using an emission polarizer that rotates between the vertical and horizontal position in order to examine both emission components from the sample.

For the measurement, the phase-angle difference (Δ) and the ratio of modulated amplitudes (RMA) between the vertical and the horizontal components of the sample's emission are recorded across the specified frequency range. Generally, the phase-angle difference increases from low to high frequencies. The RMA usually decreases as the modulation frequency is increased.

The *G* factor of the emission path, measured prior to experiment by the user, is applied to the measured AC-signal ratio, to calculate the RMA at each data point. The phase angle is measured against a reference detector specified by the user (either R or an emission detector with a polarizer set to H). A data file is created with the measured Δs and RMAs at each frequency point.

Calculations

The user models the resulting data file (`.DAT`) in order to fit for the rotation correlation time(s) and fractional contributions (β). The average lifetime of the sample and the limiting anisotropy must be known and fixed in the model, in order to reduce the degrees of freedom in the fitting algorithm. The modeling program outputs the fitted rota-

tional correlation times with their respective fractional contributions, along with the χ^2 value indicating the validity of the fit. Generally, the lower the χ^2 value, the more acceptable the fit.

Before the acquisition

Before starting an anisotropy-decay acquisition, the unknown sample must be well characterized, and the desired instrument setup carefully considered. Three parameters must be known for the experimental data to be valid and to minimize the degrees of freedom in the fitting algorithm. Other additional factors should be considered before defining and initiating an anisotropy decay acquisition. Parameters that must be known are:

- Steady-state *G* factor
- Limiting anisotropy (r_0)
- Average sample lifetime
- Sample concentration or geometry
- Steady-state spectrum
- Sample environment
- Instrument configuration

G factor

The *G* factor should be measured on the lifetime system just prior to running the acquisition. Find the *G* factor by loading a steady-state layout with polarization, proceeding to **Constant Wavelength Analysis** and measuring I_{HV} and I_{HH} for the sample at the excitation and emission wavelengths used for the decay measurement.

Limiting anisotropy

The *limiting anisotropy* should be approximately known prior to the experiment. The data-modeling software requires finding the rotational correlation times based on the experimental data. The limiting anisotropy may be found on the lifetime instrument by loading a steady-state layout with polarization, then measuring the anisotropy at an adequately low temperature for the sample.

Average sample lifetime

The *average lifetime* also must be known before the experiment, in order for the modeling software to fit the data properly. The value must be the average lifetime, or lifetime for the sample fitted as a single exponential decay. This value can be obtained by executing and fitting a lifetime acquisition on the sample being measured.

Sample concentration or geometry

The *sample concentration* should be below 0.1 OD for measurements made with right-angle geometry in a standard 4-mL cuvette. More-concentrated samples should be measured using a microcell in order to minimize inner filter effects. Front-face measurements of anisotropy decays are not supported by the Tau-3.

Steady-state spectra

Consult steady-state spectra before the acquisition in order to properly select the wavelengths for the acquisition. Run steady-state spectra first, when running a lifetime acquisition, to find the average lifetime of the sample.

Sample environment

The *environment* around the sample can have important effects on an anisotropy decay. Choose an appropriate solvent to allow the instrument to resolve the rotational correlation times of the molecule without adversely affecting the decay mechanism. Many samples inherently have anisotropy responses that are directly affected by temperature, pH, solvent, and other environmental conditions. Therefore, carefully choose the environment for the unknown in order to collect useful data. In many cases, the effect of the environment on the sample is the primary subject under study (e.g., protein unfolding during a temperature ramp). Automated data collection of several anisotropy decays, while varying these parameters, coupled with subsequent global analysis of the results, can provide great insight into the behavior and structure of the molecule of interest.

Instrumental configuration

Decide on the *instrumental configuration* used for the measurement before running the experiment. Changes in instrumental configuration for the measurement result from the need for more signal from a sample. Anisotropy decays may be made with two, one, or no emission monochromators, or using an appropriate optical filter in their places. The number of polarizers may be reduced for the scan. Three polarizers (excitation V, emission H to V, 2nd-emission H) may be used in the classical setup. Two or even only one polarizer (emission H to V) may be used for anisotropy decay measurements with good results.

> *Note: The G factor must be measured on the precise instrumental configuration used for the experiment, because the G factor is dependent on the polarization bias of the emission optics.*

Optimizing signal levels for the unknown before the measurement

Great care should be taken to optimize the signal levels for the emission channel(s) used for the anisotropy-decay acquisition. The amount of available signal and modulation across the frequency range of the instrument should be used to dictate many of the parameters for the scan.

For an anisotropy decay scan,
- No reference standard is required.
- Set the AC and DC signals to 1–5 V for the sample being measured.
- Verify these signal levels for both polarizer positions (H and V) of the primary emission channel as well as for referencing the emission channel.
  To achieve this signal level make all of the adjustments to the instrument that are recommended for the lifetime acquisition, including instrument slits, iris aperture, bias voltage to detectors, bias voltage to Pockels cell, and intermediate slit width. As with a lifetime acquisition, optimal AC signal level is most important, after which the best modulation (and optimal DC signal) should be achieved.

To determine the available range for scanning,
- Refer to the results of the lifetime acquisition for the sample.
  The lifetime components should indicate the frequency regions that can be scanned or must be scanned to provide useful data.
- Check the desired frequency endpoints for the scan to verify that there is enough AC signal (> 0.1 V).

> ***Note:*** *A signal < 0.1 V will result in larger error-bars in the data.*

Anisotropy-decay acquisition parameters

The **Anisotropy Decay Acquisition** dialog box
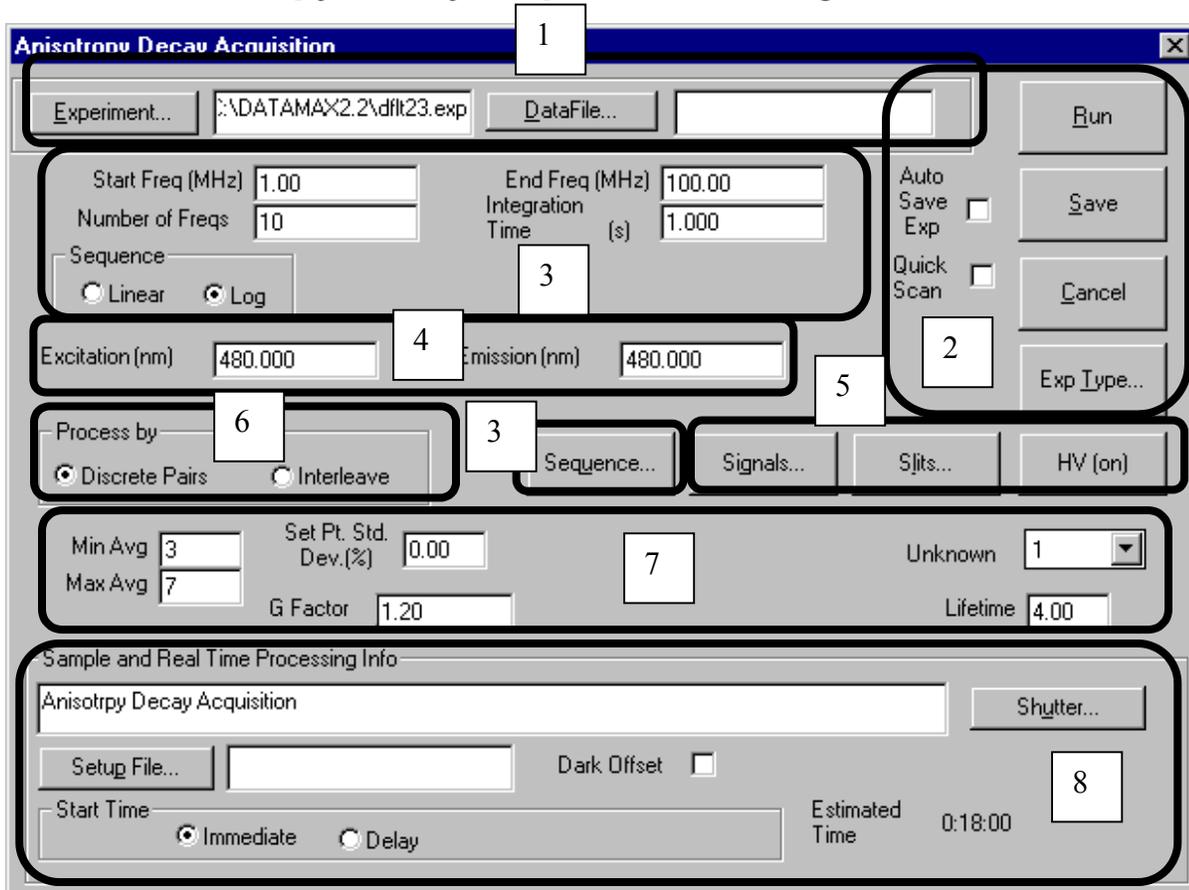
a   Load or save previously defined experiments or data files

b   Run or save experiments

c   Set the frequencies

d   Set the wavelengths

e   Set the hardware parameters

f   Process algorithm

g   Determine the statistics

h   Sample and real-time processing information

a   Load or save previously defined experiments or data files

Anisotropy Decay Acquisition

| Experiment... | :\DATAMAX2.2\dflt23.exp | DataFile... | |
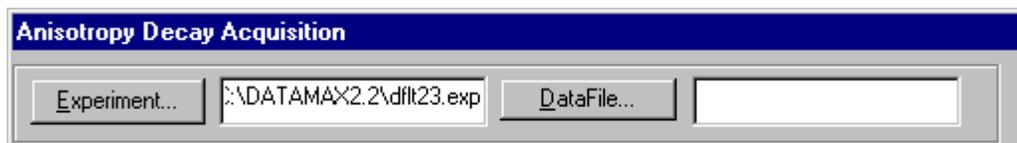
Click Experiment… to load a saved anisotropy-decay acquisition experiment file. The experiment file browser can also be reached using this button to search for a desired anisotropy-decay experiment file. You may also type in a desired experiment name (and path) to load in the space provided.

Click DataFile… to select a filename under which the data file is to be saved during a run. You may also enter the filename (and path) in the space provided. Anisotropy decay acquisition data files are saved with a .DAT extension.

b   Run or save experiments

Check the Auto Save Exp box to overwrite automatically the experiment file with your current settings at the start of an acquisition.

Quick Scan is not used for anisotropy decays.

Click Exp Type… to select the experiment type to define. For an anisotropy-decay acquisition, click Anisotropy Decay Acquisition.
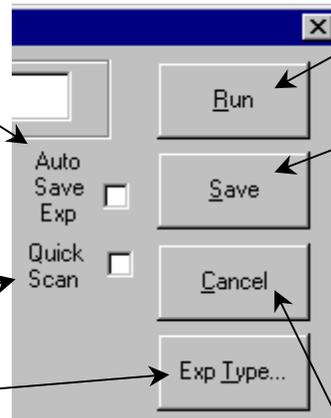
Click Run to execute a lifetime acquisition.

Click Save to save the experiment to the experiment file listed in the Experiment box. To save the experiment file to a different filename, type in the name and path in the Experiment box, and click the Save button.

Click Cancel to go back from the **Anisotropy Decay Acquisition** to the main menu.

C   Set the frequencies

For Start Freq (MHz), enter the starting frequency in MHz. Anisotropy-decay scans always progress from lower to higher frequency. The frequency range is 0.1–310 MHz.

In End Freq (MHz), enter the ending frequency in MHz.

In Number of Freqs, enter the number of frequencies to be measured for the anisotropy decay acquisition. Each frequency represents one data point for both phase and modulation measurements in the anisotropy decay. The range is 3–99.

Integration Time is the time allotted for detection of each average of the anisotropy decay. Maximum integration time is 99 s.

The Sequence specifies the frequencies used for the lifetime acquisition. There are two allowable base settings for the sequence, Linear and Log:

Log sets even spacing on a logarithmic scale depending on the number of frequencies specified.

Linear sets even linear spacing for the frequencies specified.

Click the Sequence button to display the frequencies to be measured. The frequencies may be customized before running an acquisition:

Choose the frequency to be changed by clicking on it.

Enter the new frequency value to measure.

d   Set the wavelengths

| Excitation (nm) | 480.000 | | Emission (nm) | 480.000 |

Enter the chosen Excitation wave-
length position (in nanometers) for
the acquisition.

Enter the chosen Emission wave-
length position (in nanometers) for
the acquisition.

e   Set the hardware parameters

Under Signals…, open the   Slits… leads to the **Slits**   Click on HV to change the
Signal Choice dialog box.   window.   voltage for the detectors.

Choose either S&T or T&S. In each, the first listed emission detector has its respective polarizer set to horizontal, while the second listed emission detector is set to vertical.

Slits shows the available slits and their settings for the anisotropy-decay acquisition. Set the slits according to how the unknown samples are balanced. The unit is millimeters for anisotropy decays.

High Voltage adjusts the high-voltage bias setting for the detectors. The high-voltage bias range for R928P PMT tubes in the lifetime detector housings is 300 to 1100 volts.

Note: The high-voltage setting for each detector should have been determined during the balancing procedure for the unknown.

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Process algorithm

Process by can be ignored for anisotropy-decay acquisitions.

f   Determine the statistics

Averages are the number of trials or measurements of the phase and modulation data, for the standard versus the unknown sample, at each frequency, during an anisotropy decay acquisition. The number of averages taken per frequency point depends on the minimum and maximum number of averages specified and the standard deviation for the measurement specified.

Min Avg is the minimum number of averages. The lowest minimum number of averages is 3, so that a standard deviation can be calculated.

Set Pt. Std. Dev.(%) is the standard deviation threshold for the measured phase and modulation data. If the standard deviation of the measurement is within the specified threshold after the minimum number of averages, then the experiment will move on to the next frequency point. After the maximum number of averages, the experiment will move on the next frequency point automatically.

Set the Unknown to the correct sample-holder position.

| Min Avg | 3 | Set Pt. Std. Dev.(%) | 0.00 | Unknown | 1 |
| Max Avg | 7 | G Factor | 1.20 | Lifetime | 4.00 |

Max Avg is the maximum number of averages. The highest maximum number of averages allowed is 99.

Enter the steady-state G Factor measured at the selected excitation and emission wavelengths (measured on the detector with a polarizer set to vertical for the acquisition), if this should not be done during the acquisition. The *G* factor is HV/HH.

Enter the average sample Lifetime, measured in nanoseconds.

g   Sample and real-time processing information

Enter any comments to include with the data   The Shutter is always set to Auto Open.
file on this line.

Setup File… loads a   In Start Time, set up the   Dark Offset is   Estimated Time
pre-defined set of po-   scan for Immediate   not available for   displays the esti-
sitions for various   starting, or Delay a   anisotropy de-   mated time to com-
accessories.   specified amount of time.   cays.   plete this scan.

Defining the anisotropy-decay experiment

While many of the parameters for an anisotropy-decay experiment are found from the steady-state results and from optimizing the signals in the previous section, there are additional parameters that may be set at the user's discretion:

- number of averages
- number of frequencies
- sequence
- integration time
- acceptable standard deviation

Set all of the above based on the complexity of the sample being measured, and the amount of signal observed while optimizing signals.

Samples with a single rotational correlation time that have acceptable signal will require the least time, frequencies and averages to acquire a good measurement. Samples with multiple correlation times or less available signal for the measurement will require more averages, frequency points and longer integration times to produce acceptable results.

	Recommendations	
Parameter	Range	Method
Frequency	6–99	More frequencies for more complex samples.
Averages	3–24	More averages when less signal is available.
Integration time	6–60 s	Higher integration time when less signal is available

Most anisotropy-decay experiments use a logarithmic frequency-sequence. Further options are the linear sequence, or custom frequencies or sequences.

The acceptable standard deviation specified for the measurement as Set Pt. Std. Dev.(%), specified the threshold of the observed standard deviation for averages that will allow the acquisition to move to the next frequency point. As a default, 0.5% is used. The user can specify higher or lower precision as dictated by the complexity of the sample.

Refer to *Chapter 8: Tutorial* for some examples of the experiment settings for an actual acquisition.

Running an anisotropy-decay experiment

When an anisotropy decay scan is running, the system collects the differential phase and ratio of modulated amplitudes (RMAs) across the frequency range specified. These values may be used later in non-linear least-squares analysis to solve for rational correlation times and fractional contributions for the sample.

The screenshot shown below shows the data output during an anisotropy-decay acquisition of perylene in glycerol.

The **Sequence Log** updates the data for each average on the active frequency point.

Over-range indicator

Note: *If an over-range is detected, the over-range indicator turns red. Stop the scan and check signal levels in* **Real Time Display***.*

The steady-state anisotropy is calculated by the software to verify the accuracy of the measurement for each data point.

The **Summary Table** shows the collected values for differential phase and RMA for the frequency data points in the scan.

A second window contains the graph of the differential phase-angles and RMAs across the frequency range. As data points are added, the window is updated for these new data.
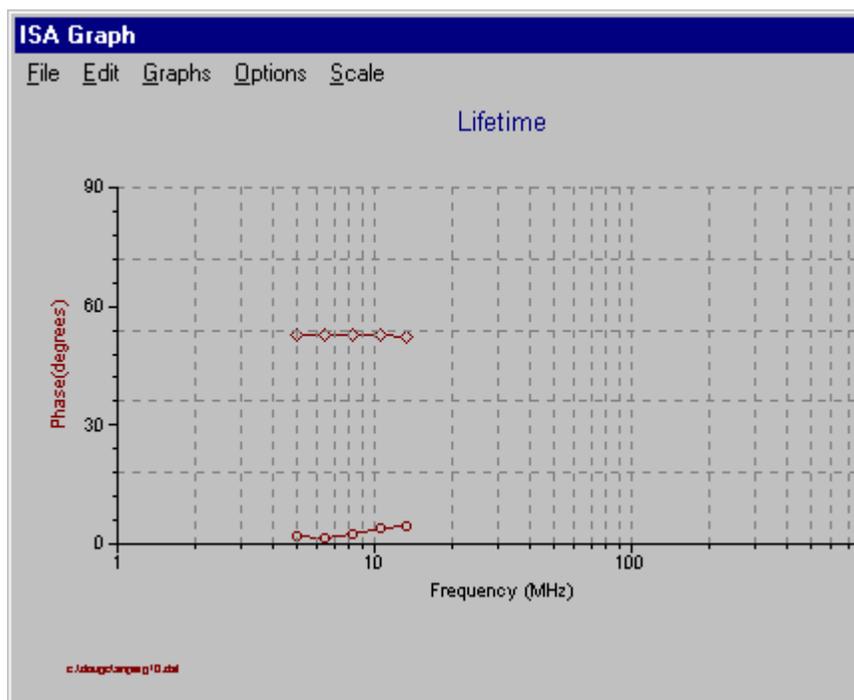
While the scan is progressing, the data values and shape of the fit can provide clues as to the nature of the decays observed. The figure shows a sample with a single exponential rotational correlation time. The differential phase-angle appears to be nearly a Lorentzian distribution, while the ratio of modulated amplitudes approaches the limiting anisotropy as it hits a maximum at the high-frequency end of the scan.

More-complex decays show a convolution of their individual components, therefore showing a departure from the basic shape for a single correlation time.

The data will indicate whether the measurement is progressing well, or if errors are becoming unacceptably high. Errors will increase as the scan progresses, because of the gradual reduction in AC signals. Unacceptably high errors, such as spurious data points or excessively high standard deviations for a data point, indicate that certain parameters for the scan require better optimization. In some cases, these data may indicate that the scan has been started at too low, or ended at too high a frequency.
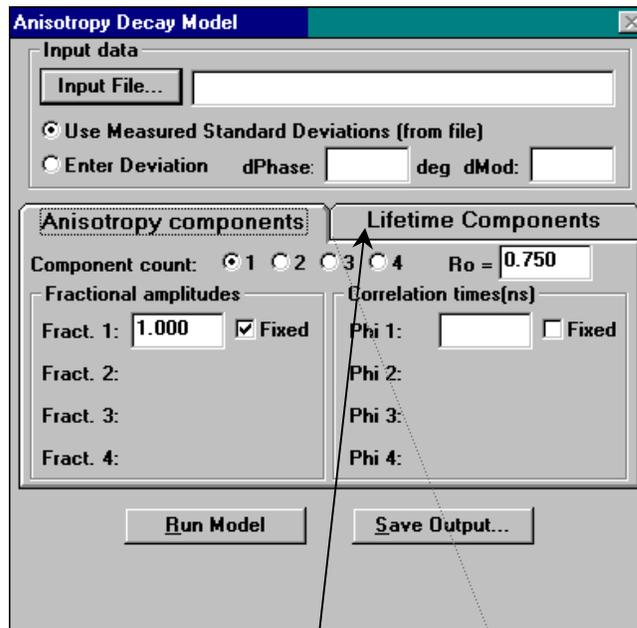
Modeling anisotropy-decay data

Anisotropy-decay data must be properly modeled in order to solve for the most acceptable resulting rotational correlation time(s) and fractional contributions. Modeling anisotropy-decay data may be performed using the **Model** software application in DataMax, or an external package such as Globals Unlimited™. In either case, there are several issues concerning properly fitting the anisotropy-decay data to yield acceptable results.

Choose the Model icon in the **Instrument Control Center**. This opens the **Model Windows Application** window:
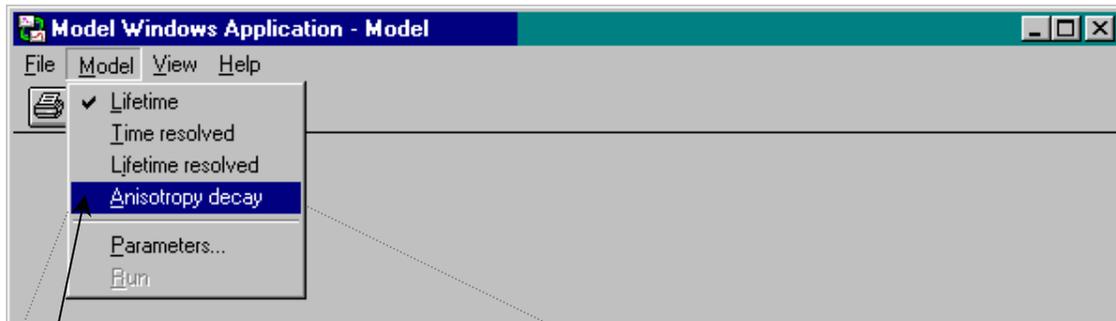
Choose Anisotropy decay to open the **Anisotropy Decay Model** window:

For the modeling, it is necessary to know the mean decay time (average lifetime) and the limiting anisotropy (r_0) for the unknown.

Choose the Anisotropy Components. Enter the r_0 value fixed. Take a fair guess at the fractional contribution of each rotational component and leave them variable. Always start the analysis by modeling as a single rotational component, and then add rotational components, as dictated by the residuals in the measurement and the reduced-χ^2 value.

Click on the index-card tab to adjust the Lifetime Components:

Fix the lifetime as a single exponential with the fractional amplitude set to 1.000,
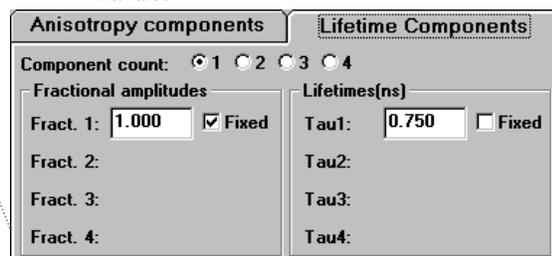
and the lifetime fixed at the known average lifetime.
Click Run Model to run the model with chosen parameters.

To save the model after running, click Save Output….

Users of the Globals Unlimited™ software have the option to perform global analysis on a group of several anisotropy decays linked across a temperature gradient, or some other variable parameter. Use of global analysis can allow the user to overdetermine the rotational components found in the sample of interest.

Optimizing anisotropy-decay acquisitions

Here are a few tips to obtaining the best possible results from an anisotropy-decay acquisition.

1   Know the lifetime and steady-state characteristics of the unknown before collecting the anisotropy-decay acquisition.

Ideally, try to understand the sample's steady-state excitation and emission characteristics, its lifetime components, and its response to various environments.

2   Use the polarizer on the exit end of the Pockels cell, removing the sample's excitation polarizer from the light path, to increase the excitation beam intensity.

To remove the sample's excitation polarizer, enter **Visual Instrument Setup**. Click on the excitation polarizer, which removes it from the optical path.

3   Use filters instead of monochromators to increase emission throughput to the detector.

- For solution chemistry, substitute the T-channel as a "reverse" S-side.
- For front-face or full T-format fluorescence measurements, substitute a filter for the S-channel monochromator.
  - ♦ Turn off the power to the entire system.
  - ♦ Remove the S-channel detector.
  - ♦ Remove the monochromator, and shift it not far from the instrument.
  - ♦ Install an FL-1031 filter-holder where the monochromator attaches to the sample compartment.
  - ♦ Secure the S-channel detector to the FL-1031 filter-holder.
  - ♦ With a #35154 25-pin-male–25-pin-female ribbon cable, electrically connect the sample compartment to the removed monochromator.
  - ♦ Restart the system.

> **Note:** Measure the G factor for filters before use in place of the monochromators. Do not assume that $G = 1$.

4   Open slits wider than with lifetime acquisitions, to increase the AC signal to ~ 1 V.

5   Increase the high-voltage bias slightly.

Adjust the Bertan™ HV-bias supply up to ~ 250 V.

> **Warning:** Do not increase the HV-bias > 300 V.

6   Integrate longer, up to ~ 30 s, per data point.

6: Lifetime Resolved Measurements

The Fluorolog®-Tau-3 Fluorescence Lifetime System has the capability to perform lifetime-resolved—otherwise known as phase-resolved—measurements. This type of measurement is useful in separating the steady-state spectra from heterogeneous species in a mixture of fluorophores. During a lifetime-resolved acquisition, the instrument measures the phase and modulation values for the unknown sample at a user-specified modulation frequency. The spectra for phase versus emission wavelength, and modulation versus emission wavelength, are saved in separate data files. After the scan, the data are modeled using a steady-state emission spectrum of the sample, to deconvolve the steady-state contributions of each lifetime component in the sample.

Prepare for a lifetime-resolved acquisition

Before a lifetime-resolved acquisition, acquire a steady-state emission spectrum for the sample across the same wavelength range. The lifetime of each component must be known to model the data. Choose a proper standard and modulation frequency for the measurement.

The standard sample must be a scattering solution of either Ludox® or glycogen. The standard is used as a zero-lifetime ($\tau = 0$) reference for the measurement, with its values for modulation and phase entered by the user in the experiment definition. In the experiment definition, the user has the option to enter the standard's phase and modulation values at the modulation frequency used for the measurement, or have the instrument measure these values at the beginning of the scans.

The modulation frequency should be selected at the point where the phase-angle difference between the desired lifetime components to be isolated is greatest from the other component(s). To find this point, compare modeled lifetime spectra for single exponential lifetime standards with similar lifetimes to the components. Try different modulation frequencies in order to find the best one for isolating the desired lifetime component(s).

Check signal levels in **Real Time Display**

Like other lifetime acquisitions, we recommend optimization of instrument settings for the sample in **Real Time Display**, before collecting a lifetime-resolved experiment. There are a few differences between setting signals for an optimization scan and a lifetime acquisition.

When measuring the standard initially, it does not have to be balanced with the unknown. A good phase and modulation value for the scattering sample for the experiment is important.

Note: *The standard must be measured under the same optical conditions, instrument settings, and modulation frequency as the unknown, in order to be valid.*

The instrument settings should be optimized to excite the unknown at an appropriate wavelength, with the emission and reference signals in the 1–4 V DC range for both standard and unknown, with as much modulation depth as possible. In order to maintain optical resolution, the high voltage on the detectors should be adjusted first to achieve acceptable signal levels. Thereafter, the slits should be adjusted to bring the signals up to a good level, if necessary.

Open the **Run Experiment** menu with **Real Time Display** still open, and choose Collect. Under Collect, select Experiment…:
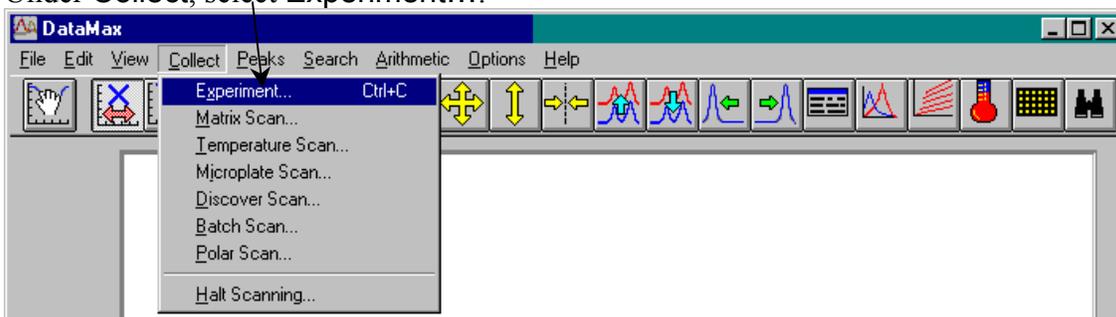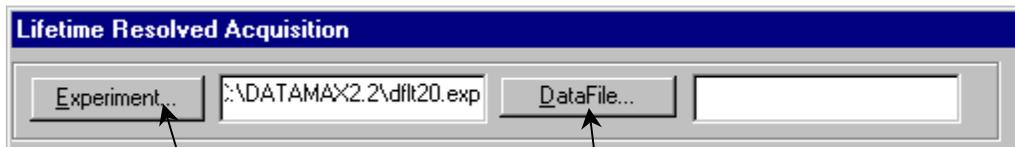

This opens the **Lifetime Resolved Experiment** window. Use the Transfer icon in **Real Time Display** to move the settings directly into the experiment definition.

Set the lifetime-resolved-acquisition scan parameters
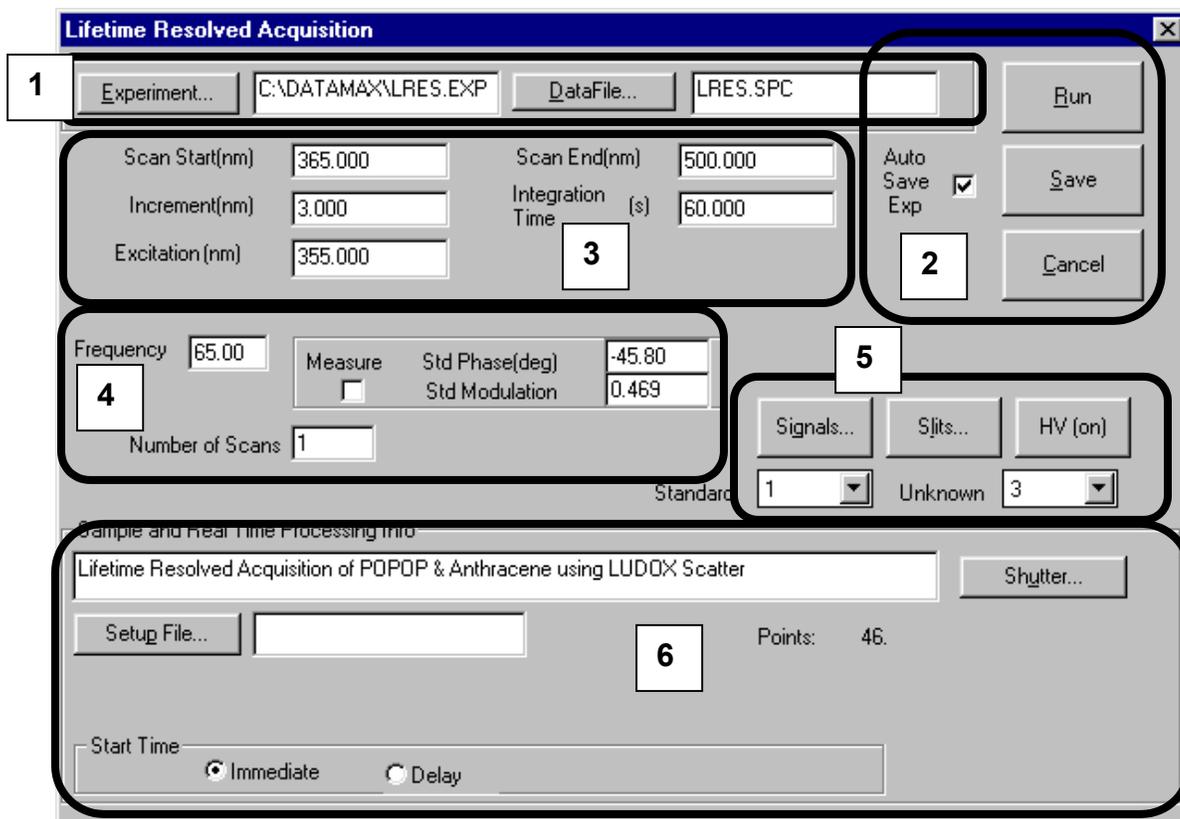
1   Load previously saved experiment and data files

2   Run or save the experiment

3   Set the wavelengths

4   Set the frequencies

5   Set the hardware parameters

6   Sample and real-time processing information

1   Load previously saved experiment and data files


Click on Experiment… to load a saved
lifetime-resolved acquisition experiment
file. The experiment file browser can also
be reached using this button to search for a
desired lifetime experiment file. You may
also type in a desired experiment name
(and path) to load in the space provided.

Click on DataFile… to go to the file
browser to select a prefix-filename for the
data files saved during an experiment run.
You may also enter the filename (and
path) in the space provided. The lifetime-
resolved acquisition saves two data files
with an `.SPC` extension, one for modula-
tion versus emission wavelength, and one
for phase versus emission wavelength.

2   How to run or save the experiment

Click the Auto Save Exp checkbox to overwrite the experiment file automatically with your current settings at the start of an acquisition.
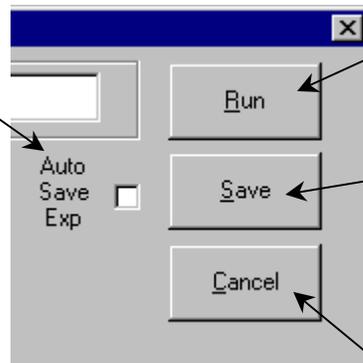
Click on Run to execute a lifetime acquisition.

Click Save to save the experiment to the file listed in the **Experiment** window. To save the experiment file to a different filename, type in the name and path in the **Experiment** window and click Save.

Click Cancel to go back from the lifetime acquisition to the main menu of the software application.

3   Set the wavelengths

In Scan Start(nm), enter the start-ing emission wavelength (in nano-meters).

In Scan End(nm), enter the ending emission wavelength (in nanome-ters).

In Increment (nm), enter the wavelength increment between emission data points.

Scan Start(nm)	300.000	Scan End(nm)	400.000
Increment(nm)	1.0000	Integration Time (s)	1.000
Excitation (nm)	350.000		

For Excitation (nm), enter the cho-sen excitation wavelength position (in nanometers).

The Integration Time is the amount of time the emission detector collects signal for each data point. The rec-ommended range integration times for a lifetime resolved acquisition is 6–99 seconds.

4   Set the frequencies

Enter the Frequency in MHz de-
sired for the lifetime-resolved ac-
quisition. Choose the frequency so
that the phase-angle difference be-
tween the desired component and
the undesired component(s) are
greatest.

In Measure, enter the standard's phase and modula-
tion values or specify the excitation and emission
wavelengths to measure these values before the scan. If
checked, then prompts appear for the Excitation and
Emission wavelengths to measure for the standard. If
not checked, then prompts appear for the Std
Phase(deg) and the Std Modulation measured al-
ready at the modulation frequency used for the scan.
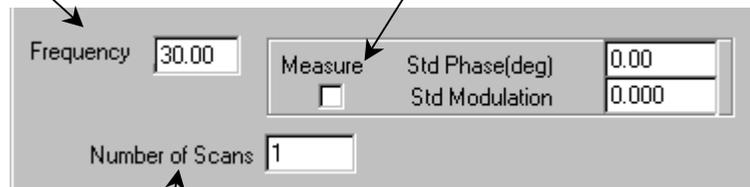
Frequency 30.00   Measure   Std Phase(deg)   0.00
                          □   Std Modulation   0.000

        Number of Scans 1

Enter the Number of Scans (repetitions) to run during the acquisition.

5   Set the hardware parameters

Enter the sample-changer position for the Standard.

Enter the sample changer position for the Unknown.

Signals… opens the **Signal Choice** window.

Slits… leads to **Slits**, showing the available slits and their settings.

HV is the high-voltage bias setting for the lifetime detectors.

There are two available signal choices in **Signal Choice**, S&R or T&R (if available). The radio buttons show the detectors that may be selected for the lifetime scan. Choose the appropriate signal detector to be compared with the reference, R, detector for the lifetime acquisition.

Set the slits according to how the standard and unknown are balanced. Slits should be set in millimeters for lifetime-resolved acquisitions.

In **High Voltage**, the high-voltage settings for each detector should have been determined during the balancing procedure for the standard and unknown. The limits for high-voltage bias for R928P PMT tubes in the lifetime detector housings are 300 to 1100 volts.

Note: If negative phase-angles or modulation values much greater than one are observed, the standard and unknown are probably reversed.

6   Sample and real-time processing information

Enter any comments to include with the data file on this line.   Shutter is always set to Auto Open.

Sample and Real Time Processing Info

Lifetime Resolved Acquisition   Shutter...

Setup File...   Dark Offset ☐   Points: 101.

Start Time
⦿ Immediate   ○ Delay

In Setup File…, load a pre-defined set of positions of various accessories.

In Start Time, set up the scan for Immediate starting, or Delay a specified amount of time.

Dark Offset is not available for lifetime scans, because the data are compared with the reference lifetime detector for all instrumental corrections during the measurement.

Points: shows the number of data points to be taken.

Run the lifetime-resolved acquisition

When the experiment runs, the system either measures the standard's phase and modulation values, or proceeds directly to the unknown, depending on the selection in the **Measure** checkbox.

The experiment measures the phaseangle difference and demodulation value for the unknown, and displays them on the same graph, plotted versus the emission wavelength. An example from the POPOP-and-anthracene-in-methanol acquisition in the tutorial chapter is shown below.
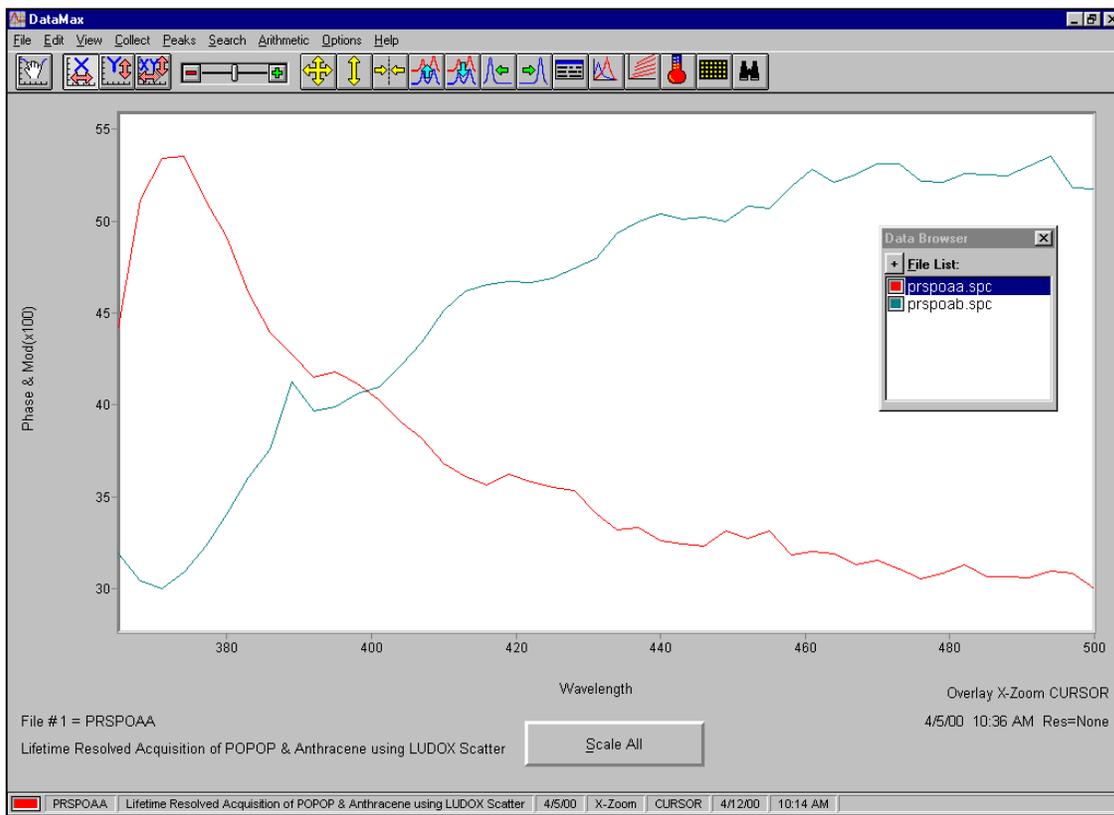
The data shown have been scaled (after the experiment) to fit on the screen together. In practice, usually there is only one trace visible during data collection. The phase spectrum is given an "a" suffix while the modulation spectrum has a "b" suffix.

Modeling lifetime-resolved acquisitions

In the **Instrument Control Center**, choose the Lifetime Modeling icon.
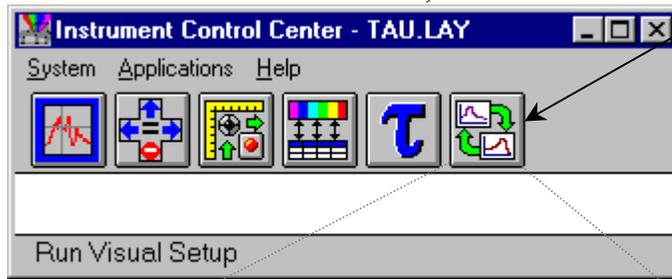
This opens the **Lifetime Resolved Model** dialog box:
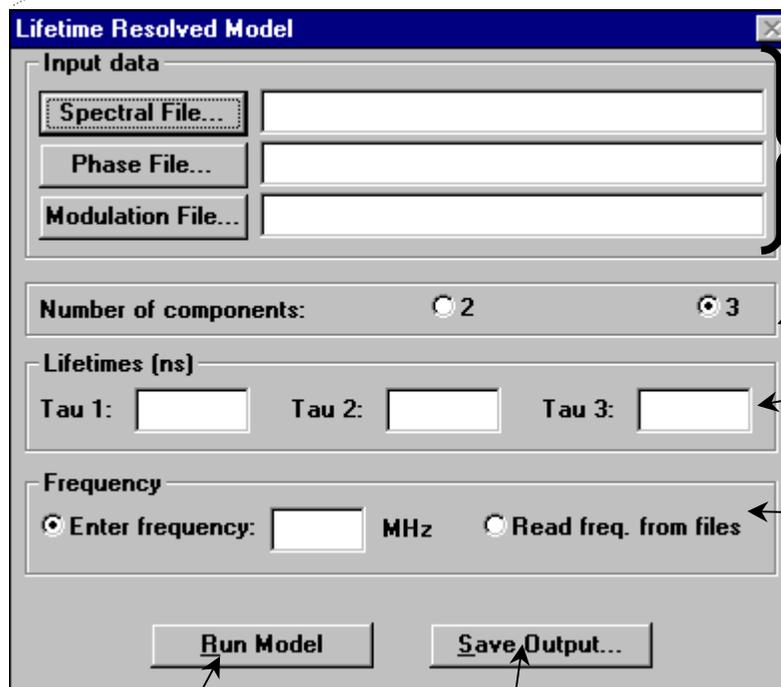
Enter or find the Spectral File, Phase File, and Modulation File in this area.

Choose the Number of components in the model.

Set the Lifetimes (in nanoseconds) for each component.

Here, for the modulation frequency, either Enter frequency, or have the software Read freq. from files.

Click Run Model to start the fitting proce-dure.

The Save Output… button is used to save the data in a text file that can be used by various applications for later processing.

The **Lifetime Modeling** software can model a lifetime-resolved acquisition for up to three discrete lifetime components. The application requires a steady-state spectrum of the mixture, the phase and modulation emission spectral files with the modulation fre-quency used, and the lifetime of each component in the mixture. (Mixtures of only two lifetime components do not use the modulation-emission spectral file to model the data.) The phase used for the measurement can be read directly from the phase and modulation emission spectral files. The model uses data files and lifetime values to fit the steady-state spectral contributions of each lifetime component. These spectral files are shown as the output.

The example of POPOP and anthracene in methanol is shown below.

Parameters for lifetime-resolved acquisition of POPOP and anthracene in MeOH.
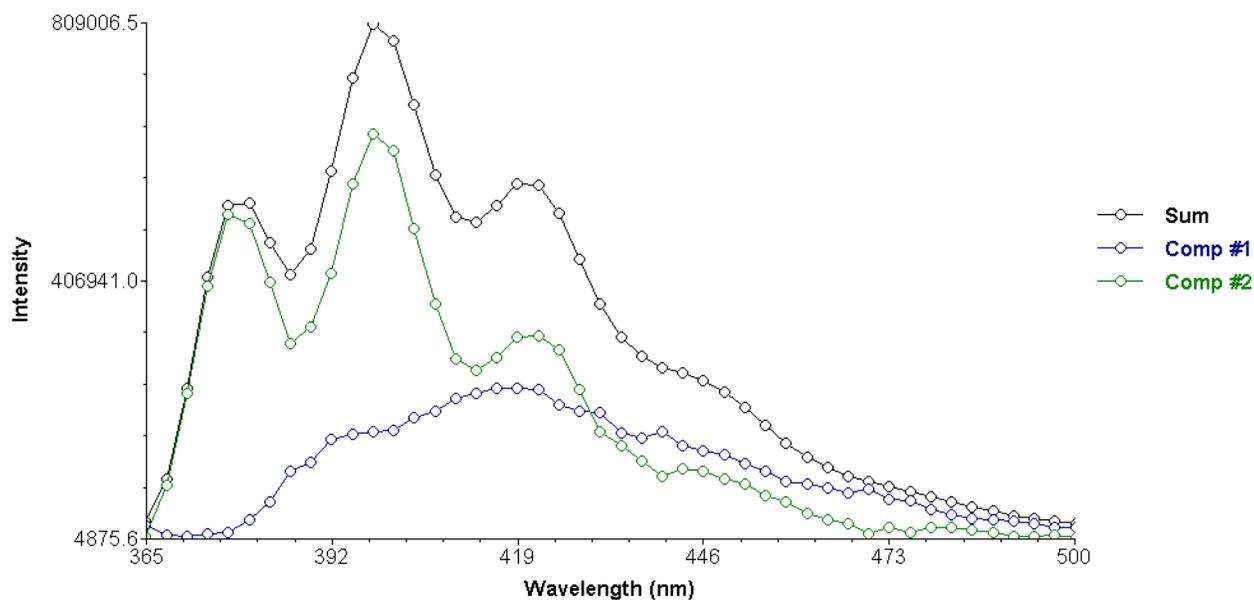
Deconvolution of POPOP emission spectrum (Comp #1) and anthracene emission spectrum (Comp #2) from the total spectrum (Sum).

6-12

Optimizing lifetime-resolved acquisitions

Here are a few tips to obtaining the best possible results from a lifetime-resolved acquisition.

1   Know the lifetime and steady-state characteristics of the unknown before collecting the lifetime resolved acquisition.
Ideally, you should understand the sample's steady-state excitation and emission characteristics, its lifetime components, and its response to various environments.

2   Use a new scattering-solution, and with the appropriate excitation wavelength.
The sample measurements are only as good as the phase and modulation standard. Glycogen solutions do not last more than a day or two before becoming contaminated. Ludox® is more stable, but cannot be used below 320 nm.

3   Match the optical settings for the steady-state emission spectrum as closely as possible to the settings required for the lifetime-resolved acquisition.
The lifetime-resolved acquisition usually requires different slit-settings from a typical steady-state emission scan. Keep the slits around the Pockels cell narrow for optimal modulation ratios, while open other slits on the excitation and emission monochromators wide to achieve acceptable signal levels. Make the steady-state scan with slits similar to the lifetime acquisition, so that similar optical conditions are used, and the modeling algorithm works optimally.

4   Avoid using relatively narrow slits.
The lifetime modeling program will still model a steady-state spectrum with relatively narrow slits, although there may be some error introduced because of the differences in optical resolution between the steady-state and lifetime-resolved scans.

5   Choose the modulation frequency carefully.
The chosen modulation frequency inherently determines the resolving power of the lifetime-resolved acquisition.

6   Try to find the modulation frequency with a large difference between the lifetime components in the unknown.
If a modulation frequency is chosen where only a few degrees separate the phase-shift of the various lifetime components, then the acquisition will not separate the components acceptably. Use response curves for single exponential decays that resemble the lifetime components in the unknown, to find the greatest difference in phase angles. For example, look at the differences between POPOP and perylene shown below. The maximum phase-difference would occur roughly in the 60–80 MHz range.
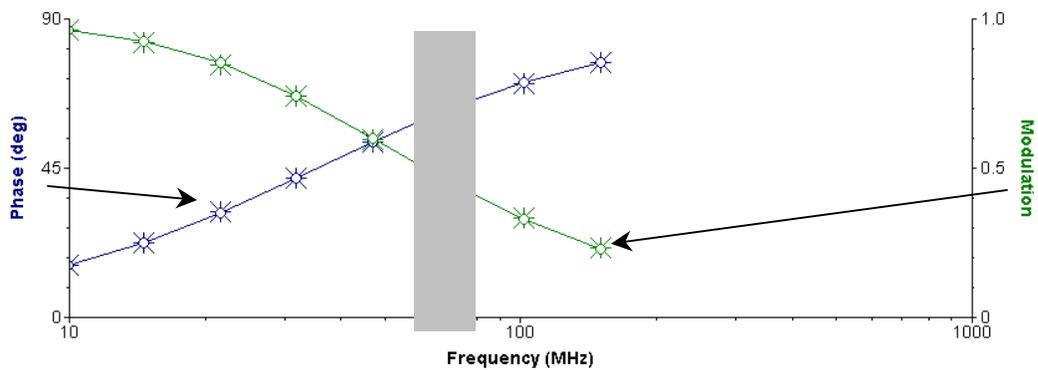
Lifetime response for perylene in methanol (4.4-ns lifetime)
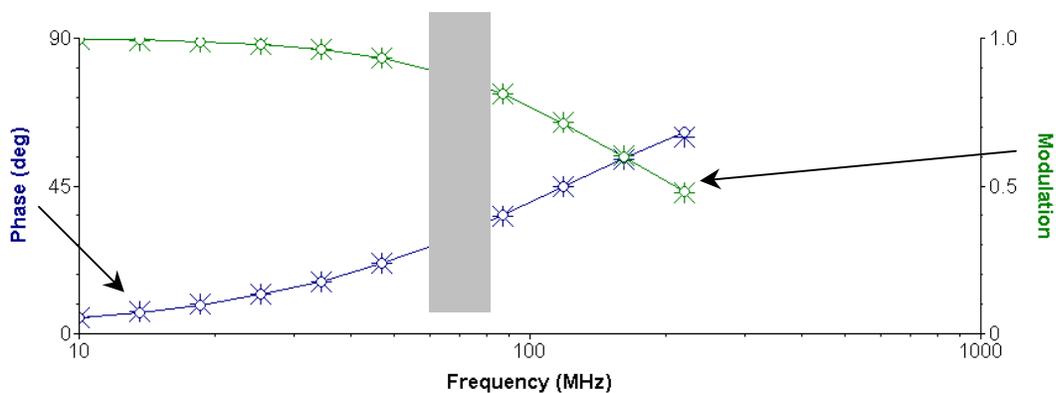
Lifetime response for POPOP in methanol (1.3-ns lifetime)

7: Time-Resolved Acquisitions

The time-resolved acquisition for the Fluorolog®-Tau-3 Fluorescence Lifetime System can fit time-resolved emission spectra (TRES) using the frequency-domain method. The acquisition collects individual lifetime spectra across a user-defined emission wavelength range. The modeling software fits these spectra with the specified decay components and their lifetime contributions to a specified steady-state emission spectrum. The results are a series of emission spectra covering the lifetime of the excited states. TRES data are useful for analyzing excited-state reactions, energy transfer, and solvent effects.

Setting up the time-resolved acquisition

Know the steady-state characteristics of the unknown being studied. The excitation and emission maxima, as well as the range to be covered for the emission, should be available. We recommend that you run a lifetime acquisition for the unknown at a central emission wavelength, in order to know the number of components and decay times for the sample, before collecting the entire time-resolved acquisition. We recommend collection of a steady-state emission spectrum (to be used for modeling the data) after collection of the time-resolved acquisition. In this way, the steady-state slit settings can be adjusted to match reasonably with the settings used for the time-resolved acquisition.

Instrument settings and balancing for the time-resolved acquisition

As with an individual lifetime acquisition, system settings must be optimized. A standard is used as a reference to correct for any instrumental bias affecting the unknown's response. Optimize signals and balance samples in **Real Time Display**, with the resulting high-voltage, slit, wavelength, and other settings transferred into the time-resolved experiment definition. Adjust the reference detector to an appropriate level for the experiment. See *Chapter 4: Lifetime Acquisitions* for more information on balancing samples, and setting up the system for appropriate signal levels.

Balance the standard and unknown as much as possible, with the best signal levels across the wavelength range used for the time-resolved acquisition, in order to obtain the most reliable results. This can be difficult, for a time-resolved emission scan usually will cross wavelength regions of low fluorescence-emission from the unknown, along with those wavelengths where the peak fluorescence is observed. In many cases, a rough balancing of the samples across these regions of low emission is satisfactory to fit the data properly after the experiment. In cases where a rough balancing is not sufficient, consider collecting the lifetime scans at each emission wavelength one by one, using the lifetime acquisition. In this way, each scan can have the signal levels optimized, with the samples meticulously balanced, and the data still can be modeled using the time-resolved fitting algorithm in the modeling application.

> **Note:** *To acquire the scans separately:*
> - *Do NOT change the slit settings, aperture settings, or excitation wavelength between each lifetime acquisition that will make up the time-resolved acquisition. Only adjust high-voltage biases, the emission wavelength, and the reference.*
> - *Name the filenames with a short prefix (e.g.,* `TRESx.DAT`*), where x is an incremented numerical suffix (e.g., 1, 2, 3, etc.) corresponding to increasing wavelength. This is the easiest way to select these files from a large series of data for later modeling.*

The standard can be specified to use a single excitation and emission wavelength-pair for the entire time-resolved acquisition, or it may be specified to cover the same emission range as the unknown being measured. This choice is available in the **Mono Positions** dialog box in the time-resolved experiment definition.

Time-resolved acquisition scan parameters

1   Load previously-saved data or experiments

2   Run and save experiments

3   Choose the frequencies for the experiment

4   Set up monochromators and processing

5   Set hardware parameters

6   Choose statistical parameters

7   Sample and real-time processing information

1   Load previously-saved data or experiments

Time Resolved Acquisition

| Experiment... | :\DATAMAX2.2\dflt22.exp | DataFile... | |
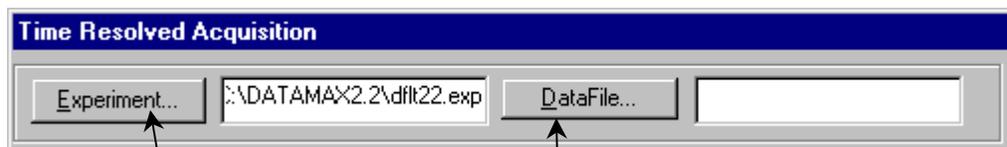
Click on Experiment… to load a
saved lifetime acquisition experiment
file. The experiment file browser can
also be reached using this button to
search for a desired lifetime experi-
ment file. Type in a desired experi-
ment name (and path) to load in the
space provided.

Click on DataFile… to go to the
file browser to select a filename for
the data file to be saved during an
experiment run. Enter the filename
(and path) in the space provided.
Lifetime acquisition data files are
saved with a .DAT extension.

2   Run and save experiments.

Click the Auto Save Exp checkbox to overwrite auto-matically the experiment file with its current settings at the start of an acquisition.

Click Quick Scan to change the acquisition algorithm. All phase and modulation data for each frequency point in the scan are taken first for the standard. Then the user is prompted to insert the un-known (with an automated sample changer, it is auto-matically moved into place), and the unknown data are taken. Some error may be in-troduced, because the data for each sample can be taken separated by a long time.
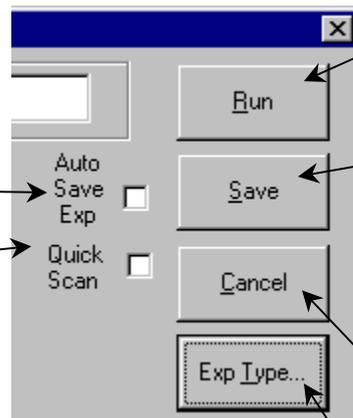
Click Run to execute a lifetime acquisition.

Click Save to save the experi-ment to the experiment file listed in the Experiment win-dow. To save the experiment file to a different filename, type in the name and path in the Ex-periment window and click the Save button.

Click Cancel to go back from the lifetime acquisition to the main menu.

Click Exp Type… to select the Experiment type to define. For a lifetime acquisition, click Lifetime Acquisition.

Note: Use Quick Scan *when the sample cannot be placed in an automated sample changer (e.g., low-temperature measurements or solids) to save the tedium of changing the standard and unknown samples at each frequency point.*

3   Choose the frequencies for the experiment

In Start Freq (MHz), enter the starting frequency, in MHz. Lifetime scans always progress from lower to higher frequency. The frequency range is 0.1–310 MHz.

In End Freq (MHz), enter the ending frequency, in MHz.

In Number of Freqs, enter the number of frequencies to be measured. Each frequency represents one data point for both phase and modulation measurements. The allowable number of frequencies is from 3 to 99.

Integration Time is the time allotted for detection of each sample during each average of the lifetime acquisition. Maximum integration time is 99 s.
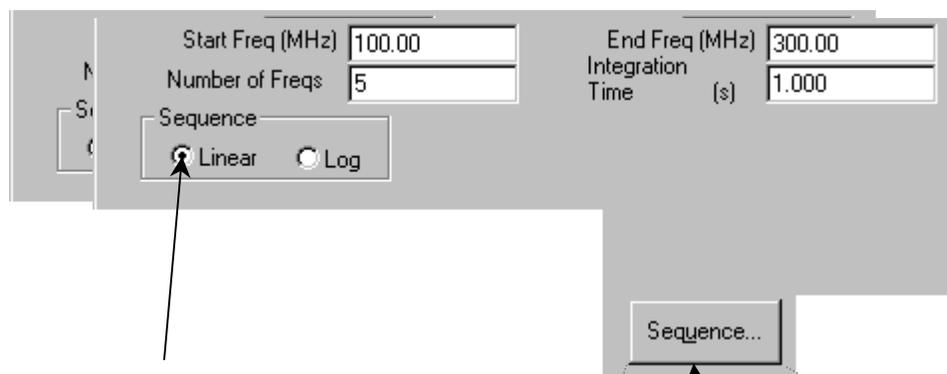
The Sequence specifies the spacing of frequencies used for the lifetime acquisition. There are two settings for the sequence, Linear and Log.

Clicking on the Sequence button displays the **Frequencies** dialog box:

- Log sets even spacing on a logarithmic scale, depending on the number of frequencies specified to be measured.
- Linear sets even linear spacing for the number of frequencies specified.
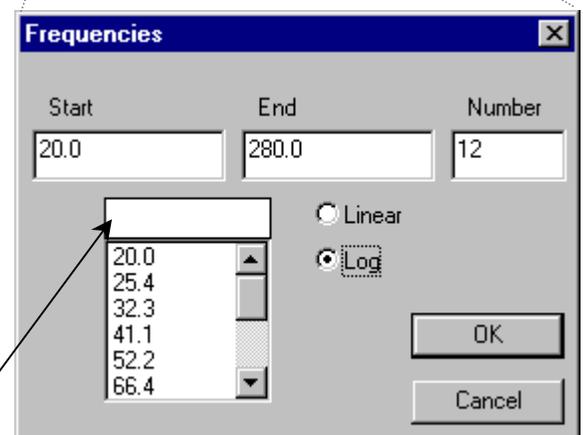
To customize a frequency, choose the frequency to be changed in the list of frequency points by clicking on it. Then enter the new frequency value to measure instead.

4   Set up monochromators and processing

Mono Positions… opens the **Mono Positions** dialog box:

Process by determines the algorithm for collecting the data for each individual average. Choose from Discrete Pairs and Interleave.

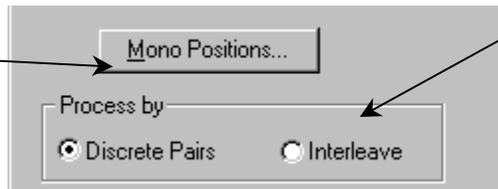Enter the monochromator values (in nm) here. These values should have been determined in the balancing procedure for the unknown and standard.

In Std Emission Start, enter the starting emission wavelength for the standard.

In Unk Emission Start, enter the starting emission wavelength for the unknown.

For Std Excitation, enter the chosen excitation wavelength position (in nanometers) for the standard.

For Unk Excitation, enter the chosen excitation wavelength position (in nanometers) for the unknown.

Std Emission End, enter the ending emission wavelength for the standard.

In Unk Emission End, enter the ending emission wavelength for the unknown.

Enter the number of lifetime Scans to be run. The software determines the wavelengths to be used, by interpolating between the starting and ending emission wavelengths for the desired number of scans.

Discrete Pairs measures the phase-angle difference and demodulation of the standard, then the unknown. From this, the phase and modulation data are saved and lifetimes are calculated based on phase and modulation. The procedure for the next average is repeated: measuring the standard, then the unknown, saving the data, and calculating lifetimes. Thus, with the discrete pair averages are non-shared measurements of the standard, followed by the unknown. Use discrete pairs when changing samples manually.

Interleave measures the phase-angle difference and demodulation of the standard, then the unknown. From this, the phase and modulation data are saved and lifetimes are calculated based on the phase and modulation. The standard is again viewed, but a new average is measured, comparing with the previous unknown data. In this way, an average is generated each time the sample is changed. This is the recommended acquisition when using an automated sample changer since it saves time with no effect on experimental accuracy.
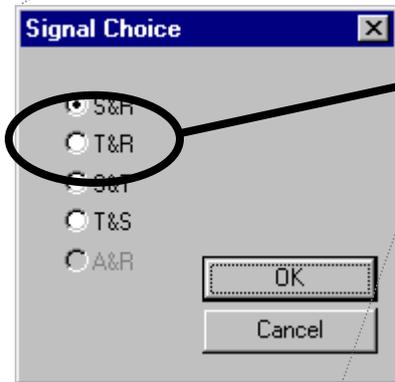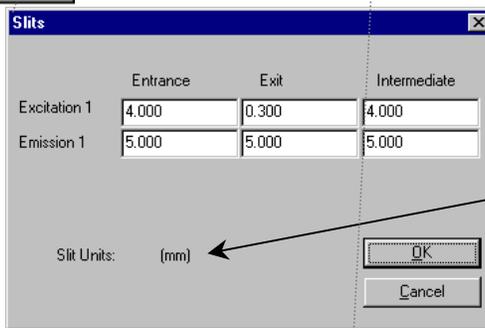
5   Set hardware parameters

| Signals... | Slits... | HV (off) |

Click on Signals… to open the **Signal Choice** dialog box.

Slits… opens the **Slits** dialog box.

HV opens the **High Voltage** dialog box.

Signal Choice

- ○ S&R
- ○ T&R
- ○ S&T
- ○ T&S
- ○ A&R

OK

Cancel

There are two available signal choices in this window, S&R or T&R (if available). The radio buttons show the detectors that may be selected for the lifetime scan. Choose the appropriate signal detector to be compared with the reference detector, R.

Slits

	Entrance	Exit	Intermediate
Excitation 1	4.000	0.300	4.000
Emission 1	5.000	5.000	5.000

Slit Units: (mm)

OK

Cancel

Set the slits according to how the standard and unknown were balanced. Slits should be set in millimeters for lifetime acquisitions.

The settings for each detector should have been determined during the balancing procedure for the standard and unknown. The limit for high-voltage bias for R928P PMT tubes is 300 to 1100 volts.

High Voltage

Detector:
- S: 850.000
- R: 450.000
- T: 0.000

OK

Cancel
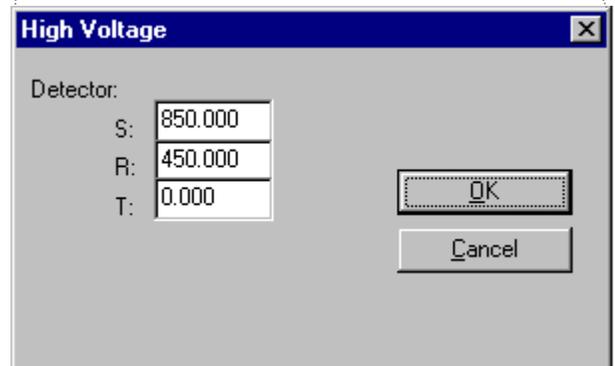
6   Choose statistical parameters

Averages are the number of measurements of the phase and modulation data for the standard versus the unknown at each frequency during a scan. The number of averages taken per frequency point depends on the minimum and maximum number of averages specified, and the standard deviation for the measurement specified.

Min Avg is the minimum number of averages for the lifetime scan. The lowest minimum number of averages is 3, so that a standard deviation can be calculated.

Set Pt. Std. Dev.(%) is the standard deviation threshold for the measured phase and modulation data. If the standard deviation of the measurement is within the specified threshold after the minimum number of averages, then the experiment moves on the next frequency point. After the maximum number of averages, the experiment moves on the next frequency point automatically.

Unknown: Enter the sample changer position for the unknown.

Min Avg [3]   Set Pt. Std. Dev.[%] [0.50]   Standard [1 ▾]   Unknown [2 ▾]
Max Avg [7]

Max Avg is the maximum number of averages for the lifetime scan. The highest maximum number of averages is 99.

Standard: Enter the sample-changer position for the lifetime standard.

Note: *If negative phase angles or modulation values much greater than one are observed, the sample-changer positions of the standard and unknown are probably reversed.*

7   Sample and real-time processing information

Enter any comments to include with the data file on   Shutter… is always set to
this line.   Auto Open.

Sample and Real Time Processing Info

First lifetime time resolved acquisition screen   Shutter...

Setup File...   Dark Offset ☐

Start Time
  ◉ Immediate   ○ Delay   Estimated   0:08:10
                                                  Time

In Setup File…,   In Start Time, set   Dark Offset is not available   Estimated Time
load a pre-defined   up the scan for   for lifetime scans, because   displays the esti-
set of accessory   Immediate start-   the data are compared with   mated time to
positions.   ing, or Delay a   the reference detector for all   completion for
                       specified amount of   instrumental corrections dur-   this scan.
                       time.   ing the measurement.

Running the time-resolved acquisition

The time-resolved acquisition usually will take a long time, for it is essentially an array of lifetime acquisitions with varying emission wavelength. As the acquisition progresses, one can observe the individual lifetime scans in progress, as the frequency-domain phase and demodulation curves are collected across the specified emission range. This is simply a series of lifetime acquisitions collected sequentially with an incremented filename for the data. For reference information on the feedback provided during the collection of lifetime data, refer to Chapter 4: *Lifetime Acqusitions*. The modeling algorithm for TRES acquisitions requires:

- A series of lifetime acquisitions collected during the time-resolved acquisition
- Steady-state emission spectra to be used for modeling, and
- The lifetimes of each decay component.

Modeling time-resolved acquisitions

In **Instrument Control Center**, click on the Model icon:

This opens the **Time Resolved Model** dialog box:
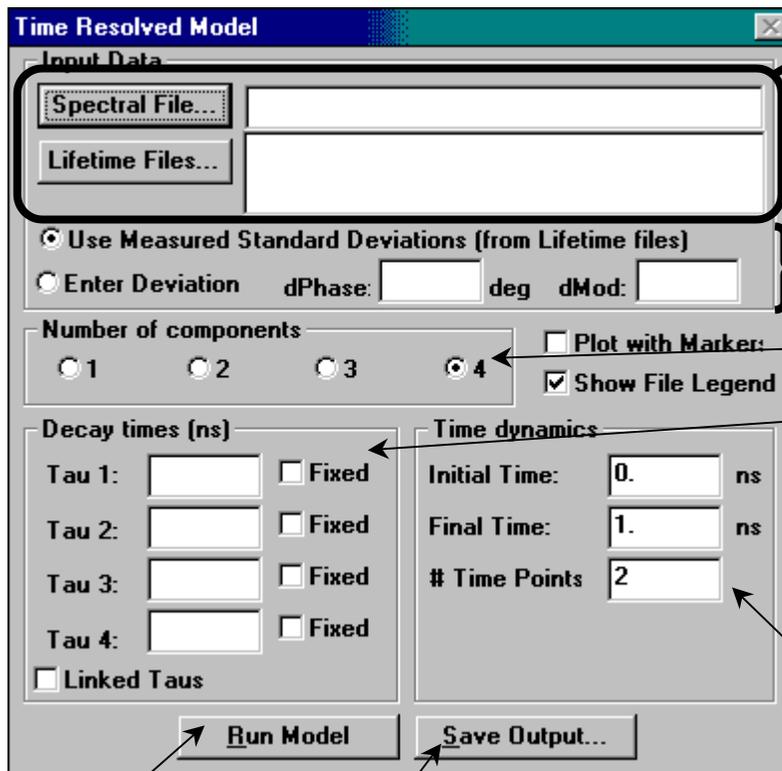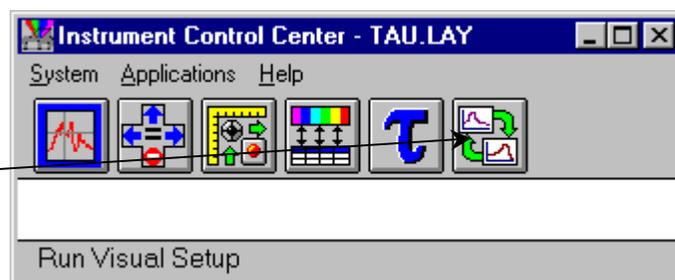
Define the data to use for the fit.

Select Measured Standard Deviations or fixed errors for the fitting algorithm.
Choose the Number of components in the decay model.
Enter the Decay times (in ns) for the components, and if each is to be fixed in the model. Check Linked Taus to link decay times across the series of lifetime measurements.
Choose the Time dynamics for the model. Enter initial and ending times for the TRES data, and the number of spectra to generate.
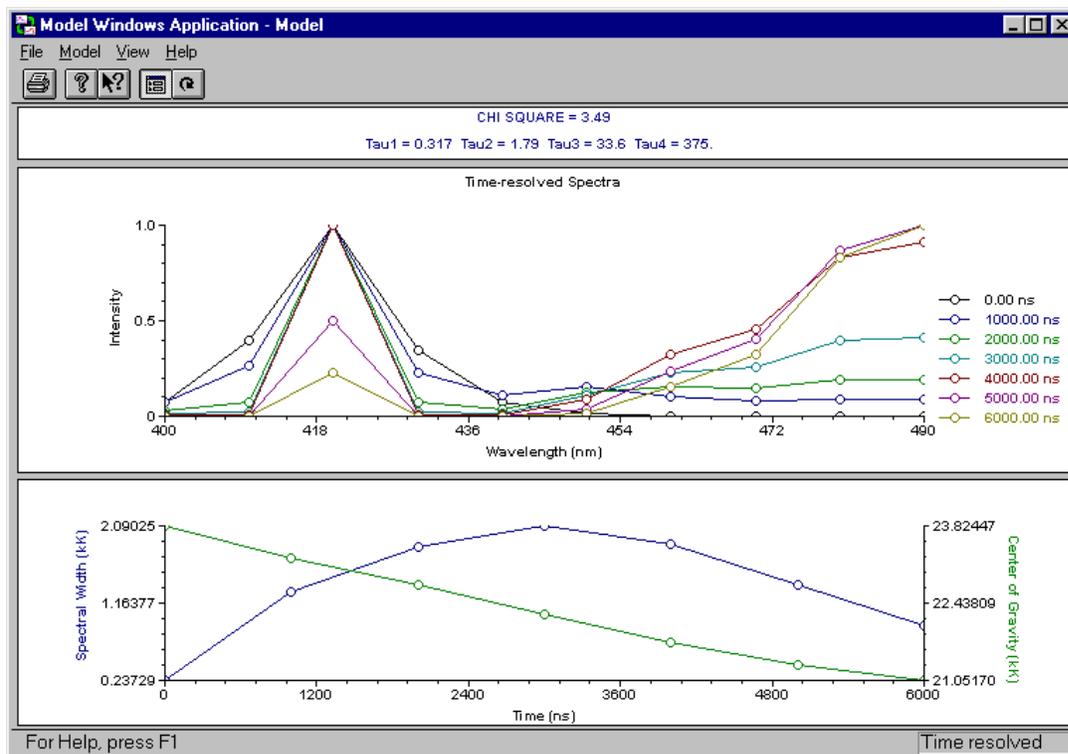
Run the model.

Save the Output… opens the **Save Modeling Output** dialog box:
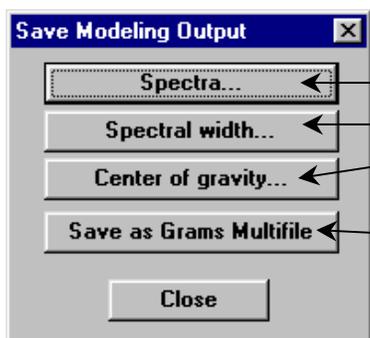
In Spectra…, save the spectra generated by the model.
In Spectral width…, save the widths generated by the model.
In Center of gravity…, save the center of gravity information the
model generated.
Click Save as Grams Multifile to save the entire model.

Below is an example of the output actually generated with the modeling software:

Example of TRES data modeled for time-resolved acquisition.

The outputs of the model in the upper graph are the TRES spectra calculated at each decay-time point. The lower graph contains the calculated spectral width and center of gravity with respect to decay time. The spectral width assists in identifying the type of process that gives rise to the changes in the emission spectra during the lifetime of the excited state. For example, in the data above, a two-state process seems to be occurring because of the broadening of the spectral width centered at about 3 µs. In other cases, the spectral width may remain constant throughout the lifetime of the excited state, indicating a continuous relaxation process. The center of gravity helps to indicate the shift of the fluorescence emission to higher (or lower) wavelengths at longer decay times.

8: Tutorial

Introduction

This chapter provides a set of experiments as a tutorial to help the user learn the main acquisitions available.

Tutorial 1: POPOP versus Ludox®

This tutorial is a basic performance-validation lifetime scan of POPOP, also known as 1,4-bis[5-phenyl-2-oxazolyl]benzene, or 2,2′-*p*-phenylene-bis-[5-phenyloxazole], versus LUDOX® (colloidal silica in suspension). This information also may be used as a reference procedure to test the performance of the lifetime spectrofluorometer.

Tutorial 2: 9-anthracenecarbonitrile versus Ludox®

This acquisition demonstrates the use of a long-lifetime compound, "9-CA", versus a Ludox® standard.

Tutorial 3: Lifetime acquisition of a solid sample versus Ludox®

Solid samples present special mounting concerns in the sample chamber. This tutorial shows how to set up a solid sample, and how to model a multi-exponential fit.

Tutorial 4: Time-resolved acquisition of an organic solid

Time-resolved acquisitions may be taken automatically, or, more commonly, by running a set of acquisitions, one per wavelength-pair. This tutorial demonstrates the method of time-resolved acquisition, plus some hints in adjusting plots to get the best view.

Tutorial 5: Anisotropy-decay acquisition of perylene in glycerol

This tutorial shows how to do an anisotropy-decay acquisition using a large, flat molecule (perylene) dissolved in glycerol. No reference is required. A comparison of one anisotropy component versus two components in the fit is provided.

Lifetime acquisition of POPOP vs Ludox®

Introduction

This lifetime acquisition is the standard scan used to verify system performance for the Spex® Fluorolog®-Tau-3 in lifetime mode. The procedure consists of running a lifetime standard (POPOP) versus a scattering reference (Ludox®).

Ludox® is colloidal silica in suspension, and is a useful inorganic scattering solution for excitation wavelengths above 340 nm. Below 340 nm, Jobin Yvon® recommends using a scattering solution of glycogen (e.g., type II from Oyster) in triple-distilled and deion-ized water.

POPOP, or 1,4-bis[5-phenyl-2-oxazolyl]benzene, or 2,2′-*p*-phenylene-bis-[5-phenyloxazole], is a single-exponential fluorescence lifetime standard with an expected lifetime of 1.32 ± 0.03 ns in research-grade methanol. The excitation spectrum consists of a broad absorption band centered at ~358 nm with shoulders on each side. (There is also a large absorption band below 300 nm not used for this experiment. The emission is broad and centered at 418 nm, with shoulders on each side, as well.)

If desired, start the system in a steady-state layout, and run the excitation and emission spectra for POPOP before starting this experiment. Refer to the *Fluorolog®-3 Opera-tion Manual* and *DataMax Software Manual* for more information on running these scans in DataMax.

Equipment required:

POPOP [CAS 1805-34-4], may be purchased from Aldrich
Ludox® [CAS 7631-86-9], may be purchased from Aldrich
Methanol (research grade)
De-ionized or distilled H_2O
Two 1-cm quartz cuvettes
400-nm cut-on filter, 1" × 2" (with 4-position sample changer), or 1" × 2" or 2" × 2" (with 2-position sample changer), if desired.

Start-up

1   Be sure that the SpectrAcq, host computer, and main electronics rack are switched off.

This prevents possible damage to these devices when the xenon lamp is ignited.

2   Start the xenon lamp.

a   Turn on the power.

b   Allow the fan to run for about 45 seconds.

c   Turn on the Main Lamp switch to ignite the xenon lamp.

The internal supply will repeatedly strike the lamp until it arcs. If the lamp does not ignite within 10 seconds, then wait another 45 seconds and try again.

3 Make sure that the SpectrAcq has the proper boot-up floppy disk in the floppy-disk drive.

4 Start the main electronics rack, SpectrAcq, host computer, and any accessories to use.

The external RF amplifier for the Pockels cell and DC high-voltage supply are often separate from the electronics rack (usually next to the Tau-3 modulator-compartment), so they may have to be switched on using their respective ON/OFF switches on the front panel of each device.

5 Start DataMax software.

Click on the DataMax icon on the desktop, or by selecting the **Instrument Control Center** within the DataMax folder in Start/Programs.

6 The **Instrument Control Center** window appears.

7 Select a lifetime layout from the layout list in the **System/Load User Layout** drop-down menu.

Select the lifetime software layout without other accessories (usually just Lifetime) when not using a programmable water bath or polarizers. Other lifetime layouts will indicate support for certain automated accessories.

8 The instrument runs a calibration sequence for ~1 min.

> *Note: One of the last steps of the calibration is finding the home position for the 2-cell or 4-cell sample holder, so keep hands away from the sample compartment during this time.*

Jobin Yvon® recommends allowing the system to warm-up for about 45 minutes before taking measurements.

Unknown and standard preparation

> *Note: Frequency-domain measurements are based on a differential technique in which a standard and unknown are measured alternately throughout the experiment. The accuracy of experiments critically depends on the quality of the standard.*

1 Prepare a sample of POPOP in methanol.

The concentration of the POPOP should be sufficient to yield about 0.050 A at 358 nm in a 1-cm quartz cuvette. (In general, keep the absorbance below 0.1 A for right-angle measurements with a 1-cm fluorescence cell.)

2 Prepare the standard sample of LUDOX® in de-ionized water in the other 1-cm quartz cuvette.

Prepare a stock solution of Ludox® (at somewhat higher concentration than expected to use for the experiment), a few transfer pipettes, a separate flask of the de-ionized water, and a waste beaker. These items will be used later to balance the standard emission signal to the unknown.

3 Place the unknown and standard cuvettes in positions 1 and 2 for the 2-cell sample-holder (or 1 and 3 for the 4-cell sample-holder).

4 Decide whether to use the emission monochromator, or the spatial filter-holder (FL-1031) on the T-side of the instrument.

For the T-side, place a 400-nm cut-on filter on the T side. Place the filter in the spring-clip holder on the sample changer, next to the unknown. The filter must be positioned at the unknown so that it automatically moves out of the emission path when the scattering standard is measured.

5 Check that the unknown and standard cuvettes are filled high enough to prevent the excitation beam from scattering off the top of the solution.

Such unwanted scattering prevents an accurate measurement of the modulation and phase.

Selecting and adjusting lifetime hardware

In addition to choosing one lifetime software layout, select and adjust up to six hardware components to complete the instrument conversion to lifetime operation. The hardware components include: the entrance and exit slits of the excitation monochromator (the 180DF double-grating monochromator may have an intermediate slit), the iris at the entrance to the Pockels-cell assembly, the Pockels-cell position, and the manual bilateral slit located at the exit of the Pockels cell assembly.

Note: *The PMT base is automatically switched to lifetime operation when any lifetime software layout is selected.*

1   The monochromator slit units should be in mm.

To change units, go to **Visual Instrument Setup**, enter the Options menu, select millimeters from the drop-down list, then click Apply. The actual slit settings for the excitation monochromator are discussed in the next section.

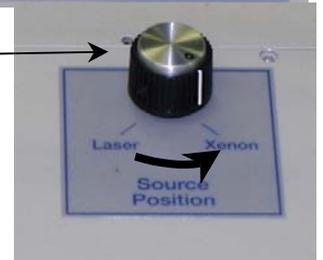## 2   Position the iris lever to vertical, midway between left and right limits.
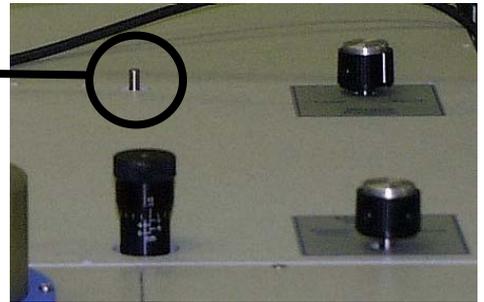
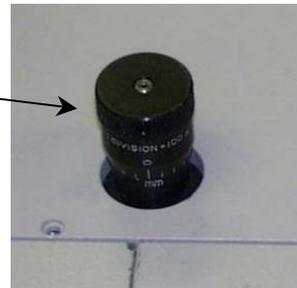## 3   Turn the Modulator Position knob to Lifetime.

This brings the Pockels-cell light modulator into the excitation light path. A full stop indicates that the modulator is properly positioned in the light path.
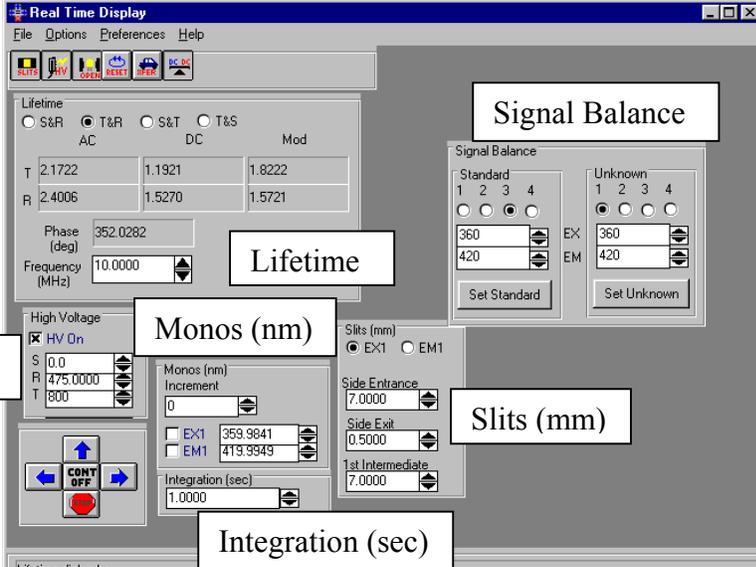
4   Turn the Source Position knob to Xenon.

5   Set the manual slit to 0.5 mm.

Defining the experiment parameters

1   Click on the Tau icon   🇹   (Run Life-
    time) to activate the lifetime functions and open
    the two lifetime data-display windows, **ISA Graph**
    and **Lifetime Summary**.

2   Select Run
    Experiment to open   *Note: Do not make any*
    the **Lifetime Acquisition**   *entries yet.*
    dialog box.

3   In the **Instrument Control Center**,
    click the Real Time Display icon.

4   Make sure that the following dialog boxes within
   the **Real Time Display** window are visible:

*Note: If a dialog box is missing, click on the appropriate toolbar
icon to open the dialog box, or re-arrange overlapping boxes by
dragging them with the mouse.*

Signal adjustment consists of two main steps: (1) unknown signal optimization and (2) standard DC balance.

5   Click the EX1 radio button.

This selects the excitation monochromator in the **Slits** dialog box. Enter 6.0 mm for the Side Entrance and 0.5 mm for the Side Exit. If there is an intermediate slit, set it to 6.0 mm. Wait after each adjustment to allow the slit change to finish, before adjusting the next.
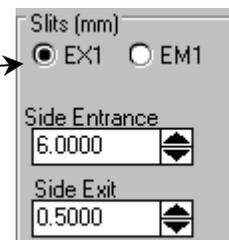
6   Click the EM1 radio button.

This adjusts the emission monochromator on the S-channel. Enter 6.0 mm for the entrance and 6.0 mm for the exit slit. (Set the optional intermediate slit to 6.0 mm.) Go to step 9.
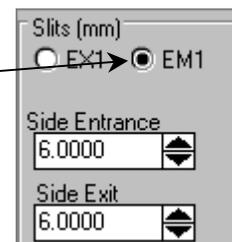
7   With a 400-nm cut-on filter on the T-channel (left side of T-box), the filter should be in place on the appropriate side of the POPOP sample, on the sample changer's filter mount.

There are no slits to adjust on the emission T-channel with an optical filter. Here is a two-position sample compartment with the scatterer in the active position and the unknown inactive. The excitation light enters from the right, and the T-side emission is at the top of the picture. Note the 1" × 2" cut-on filter placed by the unknown, for collection on the T-side (rotate 180° to active position).
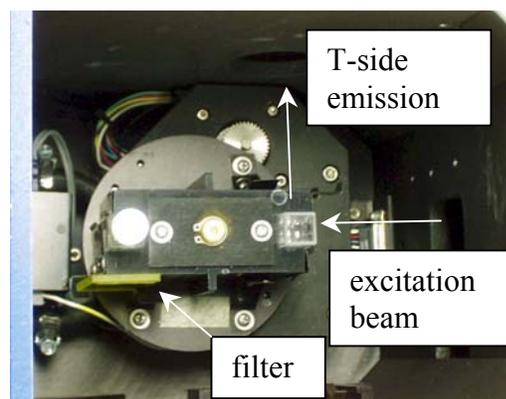
8   Click S&R (for S-channel detector on the mono-chromator) or T&R (for filter on the T-channel).
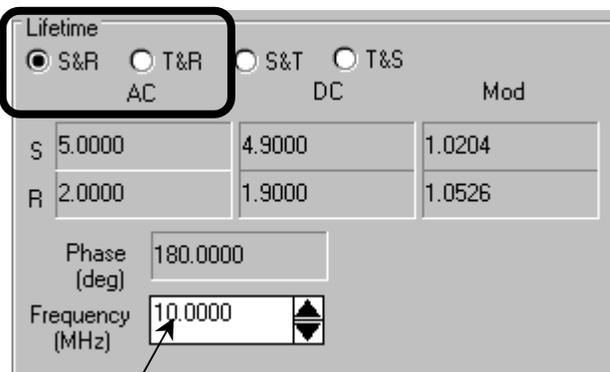
9   Enter 10 in the Frequency (MHz) field.

Press ENTER after entering frequency values.

10 Set up the **Signal Balance** dialog box.

a   Store the optimal excitation and emission wavelengths, if necessary, and the sample-changer's position for the two samples.

b   Reset the instrument by clicking the Set Standard or Set Unknown button.

- Set the standard EXcitation and EMission for 360 nm and 360 nm with Ludox®.
- Set the unknown EXcitation and EMission to 360 nm and 420 nm, respectively, for POPOP.
- Set the POPOP EMission to 360 nm if using filters on the T-side.
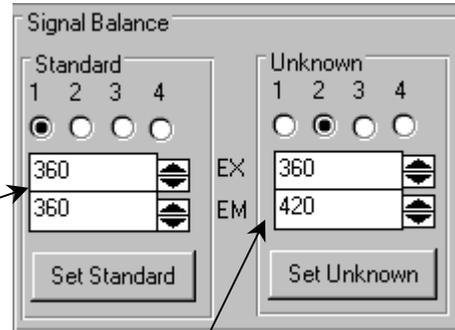
Note: If not using the emission monochromator, set the unknown to the same emission wavelength as the standard, to avoid waiting for the emission monochromator to move unnecessarily during an experiment in which the emission monochromator is not used.
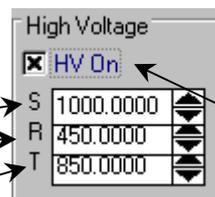
c   Set the integration time to 1.0 second.

d   Set the PMT high voltages:

S = 1000 if used, otherwise leave at 0
R = 450
T = 850 if used, otherwise leave at 0
If the HV On box is not checked, click HV On to send voltage to the PMTs.

e   Click on the shutter icon in the toolbar to open the shutter.

When changing a parameter's value, always double-check the status of the shutter. It will automatically close while some of the parameters change.

f   Watch the signal levels reported in the **Lifetime** box.

Note: The settling time for the AC signals can be quite long—up to ~ 1 min—so be patient.

Look only at the AC and DC levels at this step; don't worry about modulation ratio or phase angle. Only values shown in black

updated at the integration time should be considered. Signal values shown in red, or that do not update, are not reliable data; allow the system more time to settle signals.

g   After the signal stabilizes, adjust the slits, iris and high voltage to obtain an AC level on T (or S) of ~ 1.5 volts.

- The recommended maximum AC or DC level is about 5 volts.
- An AC or DC level of 10 indicates an over-range of signal level. You must re-duce the high-voltage slits or the iris to bring the signal on scale before proceeding.

Note: Do not lower the high voltage below 300 volts for any channel.

- Once the unknown's signal is optimized, only adjust the R-channel high-voltage and standard's concentration to bring the R-channel AC- and DC-levels to an acceptable level (i.e., 1–5 V AC and DC).

One possible procedure to adjust signal levels:
- If AC and DC are low, increase the slit-width on the emission monochroma-tor (if using S channel). Go to 7.0 mm as necessary.
- Adjust slits and high-voltages in tandem so that no one parameter is at its maximum, while others are far from their maxima. For example, to increase the unknown AC signal from 0.1 to 1.5 volts then, starting from above:
  - Increase high voltage from 750 V to 900 V
  - Increase excitation-monochromator exit slit from 0.3 to 0.5 mm
  - Increase manual slit from 0.5 to 0.7 mm
  - Increase iris aperture by rotating counterclockwise 5°.
- If AC is still below 1.5 volts, then increment the parameters in the same se-quence and about the same magnitude. Maximum high voltage is 1200 V. Watch the R level as slits are opened. If R over-ranges, then reduce the R-channel high-voltage.

Note: When running at the maximum voltage on T-channel, take extra care not to leave sample chamber lid open for longer than necessary to insert sample cuvettes and filters.

h   Once the unknown AC is ~1.5 V, check that the DC level is < 5 volts.

If DC > 5 V, try reducing the manual slit and iris while increasing high-voltage, to reach an AC-to-DC ratio (modulation ratio) of ~1.0 or higher. If you can't achieve modulation ratios of ~1, or AC levels of 1.5 V, you can still expect use-ful results, although precision will decrease. Expect accurate results for simple decays of several nanoseconds even if with a starting AC intensity < 0.5 V (be-cause of low concentration or quantum yield).

i   Click on the Set Standard button of the **Signal Balance** dialog box.

This places the standard in the measurement position. Balancing involves set-ting the DC signal on the emission detector for the standard (Ludox®) roughly equal to the unknown (POPOP). Adjust the Ludox® signal by changing concen-tration (add deionized water to decrease or stock Ludox® to increase concentra-tion, or use neutral-density filters so that the DC level of the Ludox® standard is within about 10–15% of the POPOP).

> *Note: Do not adjust slits, iris, or high voltages for the T-channel (or S-channel) for this step, for this will change the unknown's optimized signal.*

j   Adjust the R-channel high-voltage to keep the R AC at 1.5–2.0 V.

Do not reduce the high voltage below 300 V. If required, keep R on scale by re-ducing one or more slit widths, and redo the unknown signal by changing only high voltage (do not forget to re-balance the standard sample signal to match the unknown).

k   Click on the transfer icon   in the toolbar to preset the following parameters:

- Lifetime channel selection (S&R or T&R)
- Monochromator slits
- Excitation and emission wavelengths
- High voltages

> *Note: The transfer function will work only when the **Lifetime Acquisition** window is open (Step 3 in Defining Experiment Parameters).*

l   Close the **Real Time Display** window.

8-10

Experiment setup and measurement

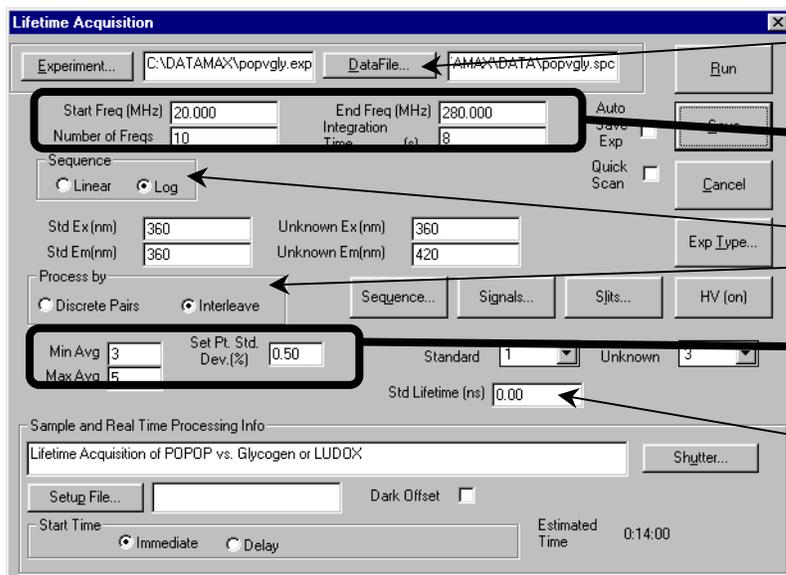## 1 Activate the **Lifetime Acquisition** dialog box.

The signals, slits, high voltage, standard wavelengths, unknown wavelengths, and sample changer positions were transferred from **Real Time Display**.

*Note: If you did not select the transfer option from the **Real Time Display** toolbar, either manually enter these parameters, or open the **Real Time Display** and click the transfer icon.*

2 Close **Real Time Display**.

3 Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Process By
h. Min Avg
i. Max Avg
j. Set Pt. Std Dev (%)
k. Std Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a file name in the desired subdirectory. Or, type a file name directly in the DataFile field. Allow the system to append the proper extension (`.dat`) to the file name.

b   Set integration from 8–15 s.

Note: The detected signals in lifetime experiments are weaker than in steady-state measurements, so we recommend longer integration times and averaging of several readings (see step f below).

c   Define the frequency sequence used to find the frequency response of the POPOP sample.

Enter the starting frequency, ending frequency, and number of frequencies. Choose logarithmic (preferred) or linear. The following parameters assume that at least one lifetime is between 5 and 20 ns. For samples with shorter lifetimes, raise the End Freq. For samples with longer lifetimes, lower the Start Freq. For systems more complex than a bi-exponential, add more frequencies to improve separation of the individual decays.

- Start Freq (MHz): 20
- End Freq (MHz): 280
- Number of Freqs: 10

d   Sequence: Log

e   Process by: Interleave.

Note: Interleave is a faster way of averaging data pairs (standard & unknown).

f   Min Avg: 3

g   Max Avg: 5

h   Set Pt. Std Dev (%): 0.5

The values entered in steps f through h work together to obtain a defined error-level in a minimum time. The minimum of 3 averages is calculated and the resulting standard deviation is compared to the set-point standard deviation. If the set-point standard deviation is less than the calculated standard deviation, then the next measurement is completed and averaged. This continues until the set-point standard deviation is equal to or greater than the calculated average, or until the maximum number of averages is reached.

i   Dark Offset: Do not use

j   Std Lifetime (ns): Enter 0 ns for the Ludox® scattering standard. (Enter the known lifetime, in nanoseconds, for a fluorophore reference.)

k   As desired, enter one line of text in the Sample and Real Time Processing Info field.

Note: Do not change the default values of any other field in this section.
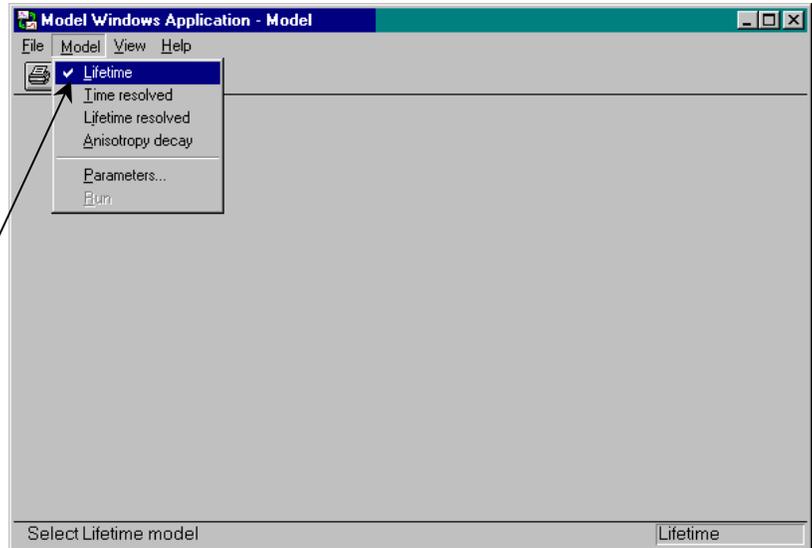
l   Click Run to start the experiment.

Following along with data-collection and modeling

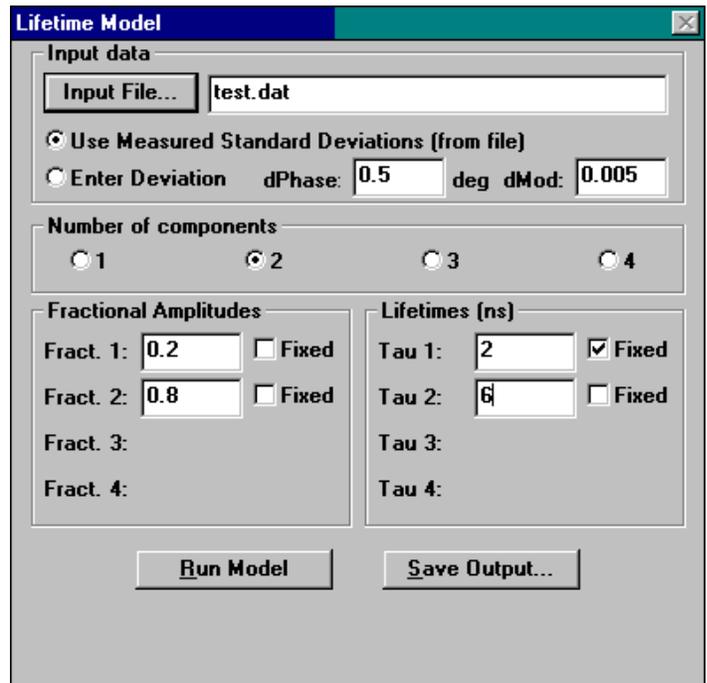1   Select the **Run Lifetime** program from the **Instrument Control Center**.

This opens the **Model Windows Application – Model** window:

2   Select Lifetime.

This opens the **Lifetime Model** window:

3   Enter the data
    file name of
    the completed
    experiment.

    Click Input File to use
    the standard
    Windows™ file dialog

4   Select Enter
    Deviation.

5   Enter dPhase
    (phase error)
    = 0.5 and
    dMod (modulation error) = 0.005.

Lifetime Model

Input data

Input File...   test.dat

● Use Measured Standard Deviations (from file)
○ Enter Deviation   dPhase: 0.5   deg dMod: 0.005

Number of components
○ 1   ● 2   ○ 3   ○ 4

Fractional Amplitudes   Lifetimes (ns)

Fract. 1: 0.2   ☐ Fixed   Tau 1: 2   ☑ Fixed

Fract. 2: 0.8   ☐ Fixed   Tau 2: 6   ☐ Fixed

Fract. 3:   Tau 3:

Fract. 4:   Tau 4:

Run Model   Save Output...

6   Enter the expected number of components.

    Choose from 1 to 4 by selecting the appropriate radio button.

7   Enter the active fractional amplitudes and life-
    times (in ns).

    Fill in for all selected components (enter a non-zero value in every open field).

8   Click Run Model.

    The results include reduced χ^2, best fits for fractional contributions and life-
    times for all selected species, and a graph of measured data (symbols) and
    model data (solid lines). A second graph reveals the difference between meas-
    ured and model for phase and modulation. A reduced $\chi^2 \sim 1$ with the residual
    scattered evenly about zero suggests that a good description of the unknown
    system's fluorescence decay was found.

9   Click on the printer icon in the toolbar to gener-
    ate a printout.

10 Close the **Lifetime Model** window.

    Return to the lifetime window for further experiments.

Lifetime acquisition of 9-anthracenecarbonitrile versus Ludox$^{®}$

Introduction

This lifetime acquisition examines a single-exponential-lifetime compound in lifetime mode. The procedure consists of running a 9-anthracenecarbonitrile (also called 9-cyanoanthracene and 9-CA) versus a scattering reference (Ludox$^{®}$), toward the blue end of the visible spectrum. 9-CA lifetime is relatively long, about 11.8 ns, which is a factor of 10 larger than POPOP, thus illustrates how to deal with acquisitions of long-lived fluorescence.

If desired, start the system in a steady-state layout, and run the excitation and emission spectra for 9-CA before starting this experiment. Refer to the *Fluorolog$^{®}$-3 Operation Manual* and *DataMax Software Manual* for more information on running these scans in DataMax.

Equipment required:

9-anthracenecarbonitrile [CAS 1210-12-4], may be purchased from Alfa Aesar
Ludox$^{®}$ [CAS 7631-86-9], may be purchased from Aldrich
Methanol (research grade)
De-ionized or distilled H_2O
Two 1-cm quartz cuvettes
400-nm cut-on filter, 1" × 2" (4-position sample changer), or 1" × 2" or 2" × 2" (2-position sample changer), if desired.

Start-up

Follow the procedure listed in steps 1–8 of the POPOP-versus-Ludox$^{®}$ tutorial on pages 8-2 through 8-3.

Unknown and standard preparation

> *Note:* *Frequency-domain measurements are based on a differential technique in which a standard and unknown are measured alternately throughout the experiment. The accuracy of experiments critically depends on the quality of the standard.*

1   Prepare a sample of 9-CA in methanol.

The concentration of the 9-CA should be sufficient to yield about 0.050 A at 382 nm in a 1-cm quartz cuvette. (In general, keep the absorbance below 0.1 A for right-angle measurements with a 1-cm fluorescence cell.)

2   Prepare the standard sample of Ludox® in de-ionized water in the other 1-cm quartz cuvette.

Prepare a stock solution of Ludox® (at somewhat higher concentration than expected to use for the experiment), a few transfer pipettes, a separate flask of the de-ionized water, and a waste beaker. These items will be used later to balance the standard emission signal to the unknown.

3   Place the unknown and standard cuvettes in positions 1 and 2 for the 2-cell sample-holder (or 1 and 3 for the 4-cell sample-holder).

4   Decide whether to use the emission monochromator, or the spatial filter-holder (FL-1031) on the T-side of the instrument.

For the T-side, place a 400-nm cut-on filter on the T side. Place the filter in the spring-clip holder on the sample changer, next to the unknown. The filter must be positioned at the unknown so that it automatically moves out of the emission path when the scattering standard is measured.

5   Check that the unknown and standard cuvettes are filled high enough to prevent the excitation beam from scattering off the top of the solution.

Such unwanted scattering prevents an accurate measurement of the modulation and phase.

Selecting and adjusting lifetime hardware
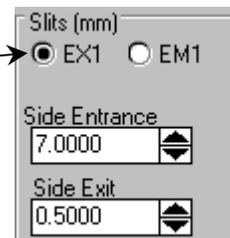
Follow the directions listed in steps 1–5 on page 8-5.

Defining the experiment parameters

1   Follow the directions on page 8-6 in the POPOP-versus-Ludox® tutorial.

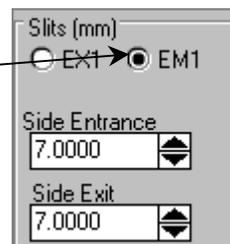Signal adjustment consists of two main steps: (1) unknown signal optimization and (2) standard DC balance.

2   Click the EX1 radio button.

This selects the excitation monochromator in the **Slits** dialog box. Enter 7.0 mm for the Side Entrance and 0.5 mm for the Side Exit. Set the optional intermediate slit to 7.0 mm. Wait after each adjustment to allow the slit change, before adjusting the next.

3   Click the EM1 radio button.

This adjusts the S-channel emission monochromator. Enter 7.0 mm for the entrance and 7.0 mm for the exit slit. Set the optional intermediate slit to 7.0 mm. Go to step 8.
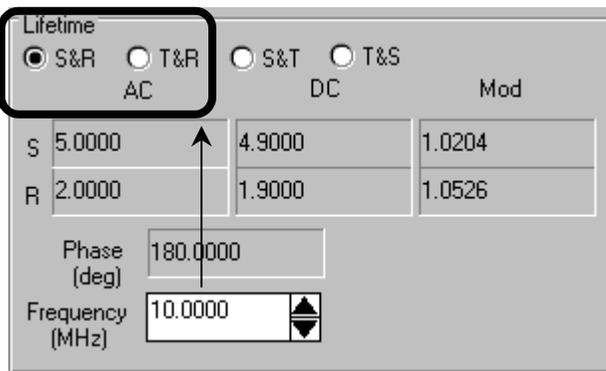

4   Place a 400-nm cut-on filter on the external T-channel box, and a filter on the appropriate side of the 9-CA sample, on the sample changer.

With an optical filter, there are no slits to adjust on the emission T-channel. Here is a two-position sample compartment with the scatterer in the active position and the unknown inactive. The excitation light enters from the top, and the T-side emission is at the left. Note the 1" × 2" cut-on filter placed by the 9-CA, for collection on the T-side (when rotated 180° to active position). No filter is used for Ludox® because the excitation and emission wavelengths are identical.

excitation beam

T-side emission

Ludox®

unknown

filter

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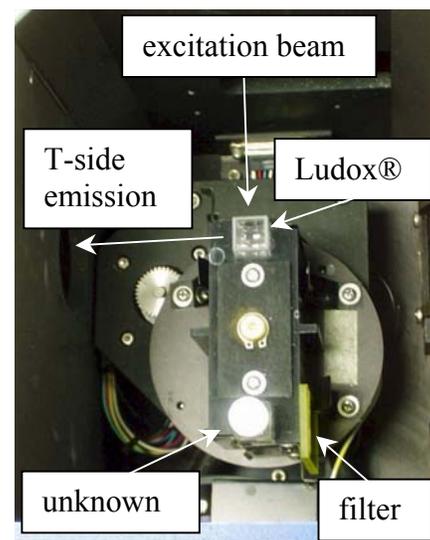
5   Click S&R (for S-
channel detector
on the mono-
chromator) or
T&R (for filter on
the T-channel).

6   Enter 10 in the Frequency (MHz) field.

Press ENTER after entering frequency values.

7   Set up the **Signal Balance** dialog box.

     a   Store the optimal excitation and emission wavelengths, if necessary, and
the sample-changer's position for the two samples.

     b   Reset the instrument by clicking
the Set Standard or Set
Unknown button.

- Set the standard EXcitation and
EMission for 382 nm and 382
nm with Ludox®.
- Set the unknown EXcitation
and EMission to 382 nm and
442 nm, respectively, for 9-CA.
- Set the 9-CA EMission to 382 nm if using filters on the T-side.
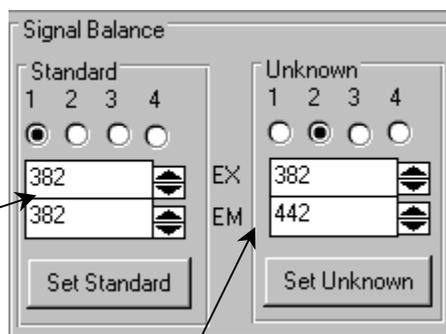
*Note: If not using the emission monochromator, set the unknown to the
same emission wavelength as the standard, to avoid waiting for the emis-
sion monochromator to move unnecessarily during an experiment in which
the emission monochromator is not used.*

     c   Set the integration time to 0.5 second.
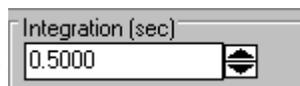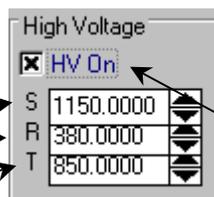

     d   Set the PMT high voltages:

S = 1150 if used, otherwise leave at 0
R = 380
T = 950 if used, otherwise leave at 0
If the HV On box is not checked, click HV On to send voltage to the PMTs.

e   Click on the shutter icon in the toolbar to open the shutter.

When changing a parameter's value, always double-check the status of the shutter. It will automatically close while some of the parameters change.

f   Watch the signal levels reported in the **Lifetime** box.

Note: The settling time for the AC signals can be quite long—up to ~ 1 min—so be patient.

Look only at the AC and DC levels at this step; don't worry about modulation ratio or phase angle. Only values shown in black updated at the integration time should be considered. Signal values shown in red, or that do not update, are not reliable data; allow the system more time to settle signals.

g   After the signal stabilizes, adjust the slits, iris and high voltage to obtain an AC level on T (or S) of ~ 1.5 volts. Follow the directions listed in steps g and h on page 8-9 of the POPOP-versus-Ludox® tutorial.

h   Click on the Set Standard button of the **Signal Balance** dialog box.

This places the standard in the measurement position. Balancing involves setting the DC signal on the emission detector for the standard (Ludox®) roughly equal to the unknown (9-CA). Adjust the Ludox® signal by changing concentration (add de-ionized water to decrease or stock Ludox® to increase concentration, or use neutral-density filters so that the DC level of the Ludox® standard is within about 10–15% of the 9-CA).

Note: Do not adjust slits, iris, or high voltages for the T-channel (or S-channel) for this step, for this will change the unknown's optimized signal.

i   Adjust the R-channel high-voltage to keep the R AC at 1.5–2.0 V.

Do not reduce the high voltage below 300 V. If required, keep R on scale by reducing one or more slit widths, and redo the unknown signal by changing only high voltage (do not forget to re-balance the standard sample signal to match the unknown).

j   Click on the transfer icon in the toolbar to preset the following parameters:

- Lifetime channel selection (S&R or T&R)
- Monochromator slits
- Excitation and emission wavelengths

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- High voltages

> **Note:** *The transfer function will work only when the **Lifetime Acquisition** window is open (Step 3 in Defining Experiment Parameters).*

k   Close the **Real Time Display** window.

Experiment setup and measurement

1   Activate the **Lifetime Acquisition** dialog box.

The signals, slits, high voltage, standard wavelengths, unknown wavelengths, and sample changer positions were transferred from **Real Time Display**.

*Note: If you did not select the transfer option from the **Real Time Display** toolbar, either manually enter these parameters, or open the **Real Time Display** and click the transfer icon.*

2   Close **Real Time Display**.

3   Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Process By
h. Min Avg
i. Max Avg
j. Set Pt. Std Dev (%)
k. Std Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a file name in the desired subdirectory. Or, type a file name directly in the DataFile field. Let the system append the proper extension (.dat) to the file name.

Note: The detected signals in lifetime experiments are weaker than in steady-state measurements, so we recommend longer integration times and averaging of several readings (see step f below).

b   Set integration from 8–15 s.

c   Define the frequency sequence used to find the frequency response of the 9-CA sample.

Enter the starting frequency, ending frequency, and number of frequencies. Choose logarithmic (preferred) or linear. The following parameters assume that at least one lifetime is between 5 and 20 ns. For samples with shorter lifetimes, raise the End Freq. For samples with longer lifetimes, lower the Start Freq. For systems more complex than a bi-exponential, add more frequencies to improve separation of the individual decays.

- Start Freq (MHz) : 1
- End Freq (MHz) : 150
- Number of Freqs: 16

Note: For 9-CA, the AC signal drops by 150 MHZ, so there is no need to go to higher frequencies.

d   Sequence: Log

e   Process By: Interleave.

Note: Interleave is a faster way of averaging data pairs (standard & unknown).

f   Min Avg: 3

g   Max Avg: 5

h   Set Pt. Std Dev (%): 0.5

The values entered in steps f through h work together to obtain a defined error-level in a minimum time. The minimum of 3 averages is calculated and the resulting standard deviation is compared to the set-point standard deviation. If the set-point standard deviation is less than the calculated standard deviation, then the next measurement is completed and averaged. This continues until the set-point standard deviation is equal to or greater than the calculated average, or until the maximum number of averages is reached.

i   Dark Offset: Do not use

j   Std Lifetime (ns): Enter 0 ns for the Ludox® scattering standard. (Enter the known lifetime, in nanoseconds, for a fluorophore reference.)

k   As desired, enter one line of text in the Sample and Real Time Processing Info field.

Note: Do not change the default values of any other field in this section.

l   Click Run to start the experiment.

Following along with data-collection and modeling

1   Follow steps 1 and 2 on page 8-13 of the
    POPOP-versus-Ludox® tutorial.

2   Enter the data
    file name of
    the completed
    experiment.

    Click Input File to use
    the standard
    Windows™ file dialog.

3   Select Enter
    Deviation.

4   Enter dPhase
    (phase error)
    = 0.5 and
    dMod (modulation error) = 0.005.

5   Choose the expected number of components.

    Here we choose 1, because 9-CA follows *single*-exponential-lifetime decay.

6   Enter the active fractional amplitude and lifetime
    (in ns).

    Enter a non-zero value in every open field. We guess ~ 10 ns for the lifetime,
    and the fractional amplitude must be 1, because there is only one lifetime to
    model.

7   Click Run Model.

    The results include reduced χ^2, best fits for fractional contributions and life-
    times for all selected species, and a graph of measured data (symbols) and
    model data (solid lines). A second graph reveals the difference between meas-
    ured and model for phase and modulation. A reduced $\chi^2 \sim 1$, with the residuals
    scattered evenly about zero, suggests that a good description of the unknown
    system's fluorescence decay was found.

For 9-CA, we found that the signal was extremely low at 150 MHz (from its longer lifetime) causing a large residual, so we opened the `.dat` file in **Notepad**, deleted that data point from the `.dat` file, and reran the model.

Below is the result from our model of 9-CA, after removal of the 150-MHz data. Note the lifetime calculated as 11.81 ns.
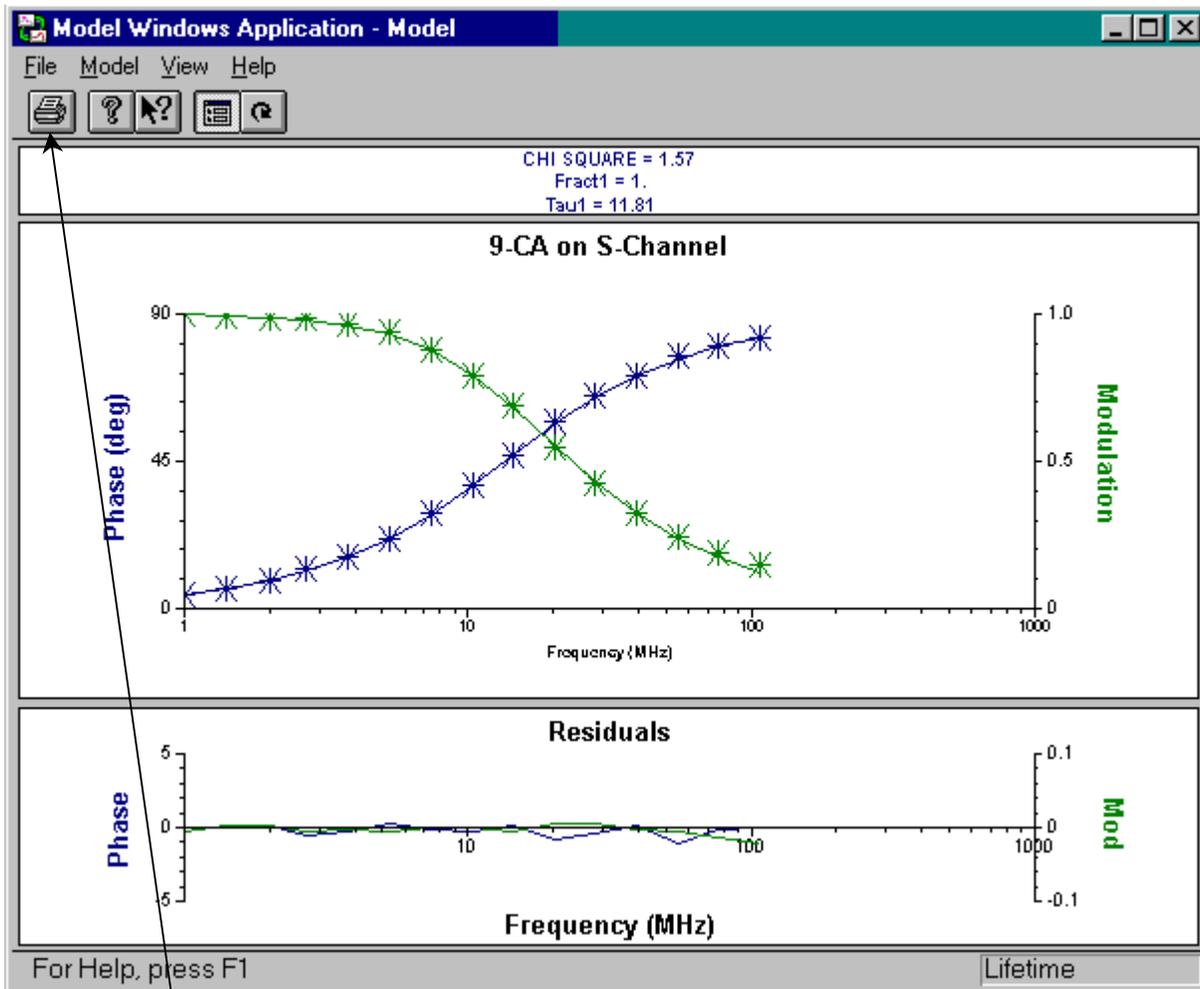
8   Click on the printer icon in the toolbar to generate a printout.

9   Close the **Lifetime Model** window.

Return to the lifetime window for further experiments.

Lifetime acquisition of a solid sample

Introduction

This lifetime acquisition examines an unknown solid sample in lifetime mode. The procedure consists of running the solid unknown versus a scattering reference (Ludox®), toward the blue end of the visible spectrum. This tutorial illustrates the use of a solid sample, and how to model multi-exponential fits.

If desired, start the system in a steady-state layout, and run the excitation and emission spectra for the solid sample before starting this experiment. Refer to the *Fluorolog®-3 Operation Manual* and *DataMax Software Manual* for more information on running these scans in DataMax.

Equipment required:

Solid unknown sample
Ludox® [CAS 7631-86-9], may be purchased from Aldrich
Methanol (research grade)
De-ionized or distilled H_2O
One 1-cm quartz cuvette
400-nm cut-on filter, 1" × 2" (4-position sample changer), or 1" × 2" or 2" × 2" (2-position sample changer), if desired.

Start-up

Follow the procedure listed in steps 1–8 of the POPOP-versus-Ludox® tutorial on pages 8-2 through 8-3.

Unknown and standard preparation

Note: Frequency-domain measurements are based on a differential technique in which a standard and unknown are measured alternately throughout the experiment. The accuracy of experiments critically depends on the quality of the standard.
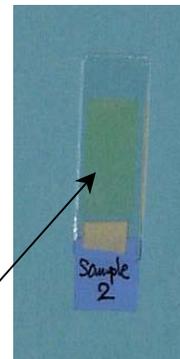
1   Choose an appropriate solid unknown.

The unknown used in this tutorial is a thin film on a glass slide:

2   Prepare the Ludox® standard in de-ionized water in a 1-cm quartz cuvette.

Prepare a stock solution of Ludox® (at somewhat higher concentration than expected to use for the experiment), a few transfer pipettes, a separate flask of the de-ionized water, and a waste beaker. These items will be used later to balance the standard emission signal to the unknown.

3   Place the standard cuvette in position 1 for the 2-cell sample-holder.

4   Mount the solid sample at a right angle to the Ludox®.
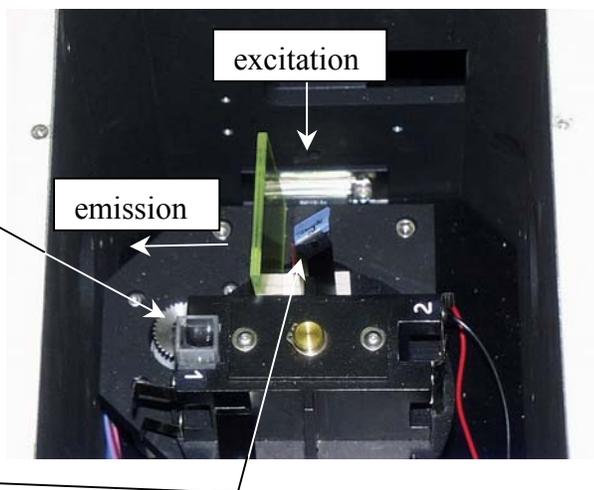
Notice how the slide is tilted at 45° between the excitation and emission beams.

5   Decide whether to use the emission monochromator, or the spatial filter-holder (FL-1031) on the T-side of the instrument.

For the T-side, place a 400-nm cut-on filter on the T side. Place the filter in the spring-clip holder on the sample changer, next to the unknown. The filter must

be positioned at the unknown so that it automatically moves out of the emission path when the scattering standard is measured.

6   Check that the standard cuvette is filled high enough to prevent the excitation beam from scattering off the top of the solution.

Such unwanted scattering prevents an accurate measurement of the modulation and phase.

Selecting and adjusting lifetime hardware

Follow the directions listed in steps 1–5 on page 8-5.

Defining the experiment parameters

1 Follow the directions on page 8-6 in the POPOP-versus-Ludox® tutorial.

Signal adjustment consists of two main steps: (1) unknown signal optimization and (2) standard DC balance.

2 Click the EX1 radio button.

This selects the excitation monochromator in the **Slits** dialog box. Enter 7.0 mm for the Side Entrance and 0.4 mm for the Side Exit. Set the optional intermediate slit to 7.0 mm. Wait after each adjustment to allow the slit change, before adjusting the next.
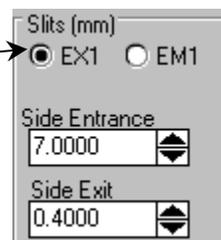
3 Click the EM1 radio button.

This adjusts the S-channel emission monochromator. Enter 7.0 mm for the entrance and 7.0 mm for the exit slit. Set the optional intermediate slit to 7.0 mm. Go to step 5.
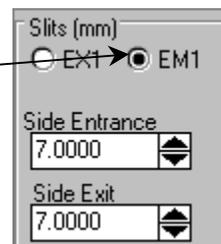
4 Place a 400-nm cut-on filter on the external T-channel box, and a filter on the appropriate side of the solid sample, on the sample changer.
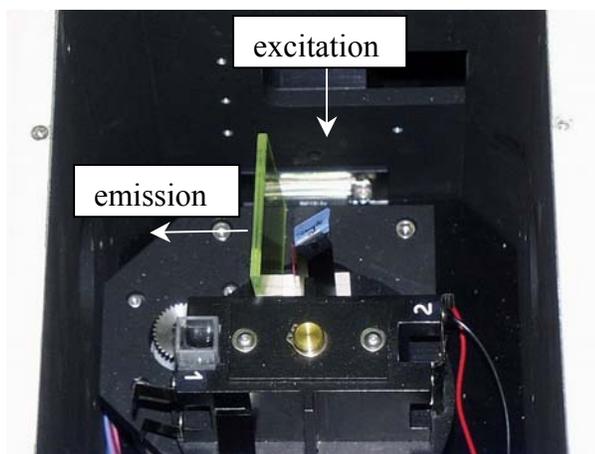
With an optical filter, there are no slits to adjust on the emission T-channel. Here is a two-position sample compartment with the unknown in the active position and the Ludox® (position 1) inactive. The excitation light enters from the top, and the T-side emission is at the left. Note the 2″ × 2″ cut-on filter placed by the solid sample, for collection on the T-side. No filter is used for Ludox® because the excitation and emission wavelengths are identical.

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5   Click S&R (for S-
     channel detector
     on the mono-
     chromator) or
     T&R (for filter on
     the T-channel).

Lifetime			
○ S&R	⊙ T&R	○ S&T	○ T&S
	AC	DC	Mod
T	2.1866	1.2598	1.7357
R	3.5044	1.3251	2.6446

Phase (deg)   -13.1546

Frequency (MHz)   10.0000

6   Enter 10 in the Frequency (MHz) field.

Press ENTER after entering frequency values.

7   Set up the **Signal Balance** dialog box.

a   Store the optimal excitation and emission wavelengths, if necessary, and
     the sample-changer's position for the two samples.

b   Reset the instrument by clicking
     the Set Standard or Set
     Unknown button.
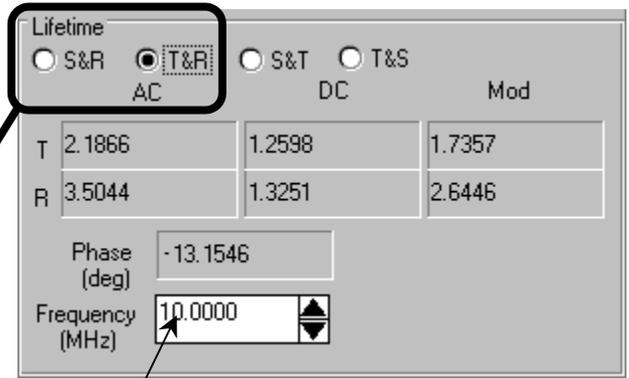
     • Set the standard EXcitation
       and EMission for 380 nm and
       380 nm with Ludox®.
     • Set the unknown EXcitation
       and EMission to 380 nm and
       520 nm, respectively, for the unknown.
     • Set the unknown EMission to 380 nm if using filters on the T-side.

Signal Balance			
Standard		Unknown	
1 2		1 2	
⊙ ○		○ ⊙	
380	EX	380	
380	EM	520	
Set Standard		Set Unknown	

*Note: If not using the emission monochromator, set the unknown to the
same emission wavelength as the standard, to avoid waiting for the emis-
sion monochromator to move unnecessarily during an experiment in which
the emission monochromator is not used.*

c   Set the integration time to 0.2 seconds.

Integration (sec)   0.2000

d   Set the PMT high voltages:

High Voltage
☒ HV On
S 0.0
R 550.0000
T 1100.0000

     S = 1150 if used, otherwise leave at 0
     R = 550
     T = 1100 if used, otherwise leave at 0
     If the HV On box is not checked, click HV On to send voltage to the PMTs.

e   Click on the shutter icon ![] in the toolbar to open the shutter.

When changing a parameter's value, always double-check the status of the shutter. It will automatically close while some of the parameters change.

f   Watch the signal levels reported in the **Lifetime** box.

> *Note: The settling time for the AC signals can be quite long—up to ~ 1 min—so be patient.*

Look only at the AC and DC levels at this step; don't worry about modulation ratio or phase angle. Only values shown in black updated at the integration time should be considered. Signal values shown in red, or that do not update, are not reliable data; allow the system more time to settle signals.

g   After the signal stabilizes, adjust the slits, iris and high voltage to obtain an AC level on T (or S) of ~ 1.5 volts. Follow the directions listed in steps g and h on page 8-9 of the POPOP-versus-Ludox® tutorial.

h   Click on the Set Standard button of the **Signal Balance** dialog box.

This places the standard in the measurement position. Balancing involves setting the DC signal on the emission detector for the standard (Ludox®) roughly equal to the unknown. Adjust the Ludox® signal by changing concentration (add de-ionized water to decrease or stock Ludox® to increase concentration, or use neutral-density filters so that the DC level of the Ludox® standard is within about 10–15% of the unknown).

> *Note: Do not adjust slits, iris, or high voltages for the T-channel (or S-channel) for this step, for this will change the unknown's optimized signal.*

i   Adjust the R-channel high-voltage to keep the R AC at 1.5–2.0 V.

Do not reduce the high voltage below 300 V. If required, keep R on scale by reducing one or more slit widths, and redo the unknown signal by changing only high voltage (do not forget to re-balance the standard sample signal to match the unknown).

j   Click on the transfer icon ![]   in the toolbar to preset the following parameters:

• Lifetime channel selection (S&R or T&R)
• Monochromator slits
• Excitation and emission wavelengths

- High voltages

>
> *Note: The transfer function will work only when the **Lifetime Acquisition** window is open (Step 3 in Defining Experiment Parameters).*

k   Close the **Real Time Display** window.

8   Determine the frequency range

To decide what frequencies create an adequate signal, this step tries various frequencies and tests the ensuing signals. Such a test provides a range of frequencies for the experiment definition.

a   At 10 MHz, a mid-range frequency, we already have the following signal on the T-channel:

b   For a high-frequency limit, try 200 MHz:

Note how the T-channel AC signal has dropped drastically. This indicates that 200 MHz is too high.

c   Lower the Frequency to 150 MHz:

The T-channel signal is nearly triple that at 200 MHz. Therefore use 150 MHz as the upper limit.

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d   Try two low frequencies: 1 MHz, and 0.5 MHz:

Lifetime		
○ S&R ◉ T&R ○ S&T ○ T&S		
AC	DC	Mod
T 2.4909	1.3101	1.9012
R 3.8666	1.4090	2.7442
Phase (deg) -0.6020		
Frequency (MHz) 1.0000		

Lifetime		
○ S&R ◉ T&R ○ S&T ○ T&S		
AC	DC	Mod
T 2.2983	1.2976	1.7712
R 3.6298	1.3907	2.6101
Phase (deg) -0.1486		
Frequency (MHz) 0.5000		

There is little difference between the two T-channel signals, the phase, and the modulation. Thus there is no advantage to including 0.5 MHz in the experiment, and the lower frequency limit is set to 1 MHz.

Experiment setup and measurement

1   Activate the **Lifetime Acquisition** dialog box.

The signals, slits, high voltage, standard wavelengths, unknown wavelengths, and sample changer positions were transferred from **Real Time Display**.

> *Note: If you did not select the transfer option from the **Real Time Display** toolbar, either manually enter these parameters, or open the **Real Time Display** and click the transfer icon.*

2   Close **Real Time Display**.

3   Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Process By
h. Min Avg
i. Max Avg
j. Set Pt. Std Dev (%)
k. Std Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a file name in the desired subdirectory. Or, type a file name directly in the DataFile field. Let the system append the proper extension (`.dat`) to the file name.

b   Set integration to 12 s.

c   Define the frequency sequence used to find the frequency response of the solid sample.

> *Note: The detected signals in lifetime experiments are weaker than in steady-state measurements, so we recommend longer integration times and averaging of several readings (see step f below).*

Enter the starting frequency, ending frequency, and number of frequencies. Choose logarithmic (preferred) or linear. The following parameters assume that at least one lifetime is between 5 and 20 ns. For samples with shorter lifetimes, raise the End Freq. For samples with longer lifetimes, lower the Start Freq. For systems more complex than a bi-exponential, add more frequencies to improve separation of the individual decays.

- Start Freq (MHz): 1
- End Freq (MHz): 150
- Number of Freqs: 15

Note: For this unknown, the AC signal drops by 150 MHZ, so there is no need to go to higher frequencies.

d   Sequence: Log

e   Process By: Interleave.

Note: Interleave is a faster way of averaging data pairs (standard & unknown).

f   Min Avg: 3

g   Max Avg: 5

h   Set Pt. Std Dev (%): 0.5

The values entered in steps f through h work together to obtain a defined error-level in a minimum time. The minimum of 3 averages is calculated and the resulting standard deviation is compared to the set-point standard deviation. If the set-point standard deviation is less than the calculated standard deviation, then the next measurement is completed and averaged. This continues until the set-point standard deviation is equal to or greater than the calculated average, or until the maximum number of averages is reached.

i   Dark Offset: Do not use

j   Std Lifetime (ns): Enter 0 ns for the Ludox® scattering standard. (Enter the known lifetime, in nanoseconds, for a fluorophore reference.)

k   As desired, enter one line of text in the Sample and Real Time Processing Info field.

Note: Do not change the default values of any other field in this section.

l   Click Run to start the experiment.

Following along with data-collection and modeling

1   Follow steps 1 and 2 on page 8-13 of the
     POPOP-versus-Ludox® tutorial.

2   Enter the data
     file name of
     the completed
     experiment.

     Click Input File to use
     the standard
     Windows™ file dialog.

3   Select Enter
     Deviation.

4   Enter dPhase
     (phase error)
     = 0.5 and
     dMod (modulation error) = 0.005.

5   Choose the expected number of components.

     First try 1.

6   Enter the active fractional amplitude and lifetime
     (in ns).

     Enter a non-zero value in every open field. We guess ~ 10 ns for the lifetime.

7   Click Run Model.

     The results include reduced χ^2, best fits for fractional contributions and life-
     times for all selected species, and a graph of measured data (symbols) and
     model data (solid lines). A second graph reveals the difference between meas-
     ured and model for phase and modulation. A reduced $\chi^2 \sim 1$, with the residuals
     scattered evenly about zero, suggests that a good description of the unknown
     system's fluorescence decay was found.

8-35
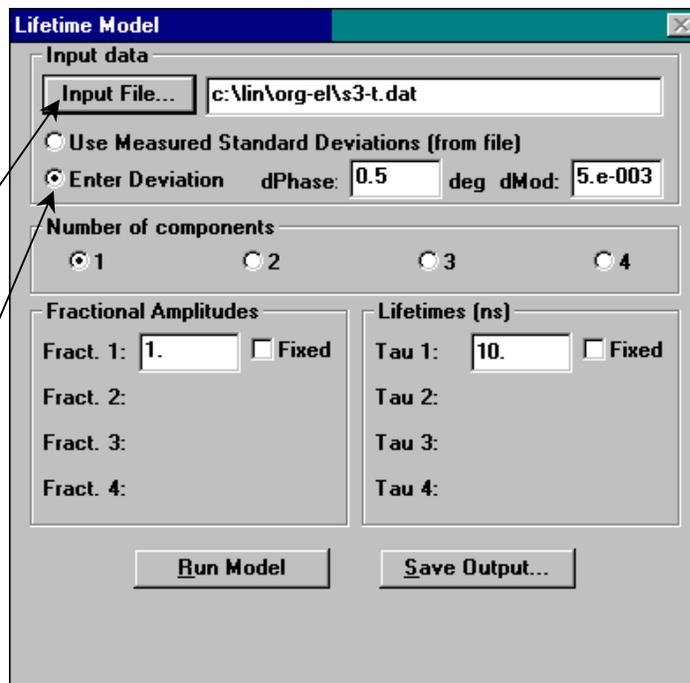
Below is the result from our model of the unknown. Note the lifetime calculated as 3.749 ns. The mid-frequency range is not fitted well, supported by a large χ^2 = 5.85.
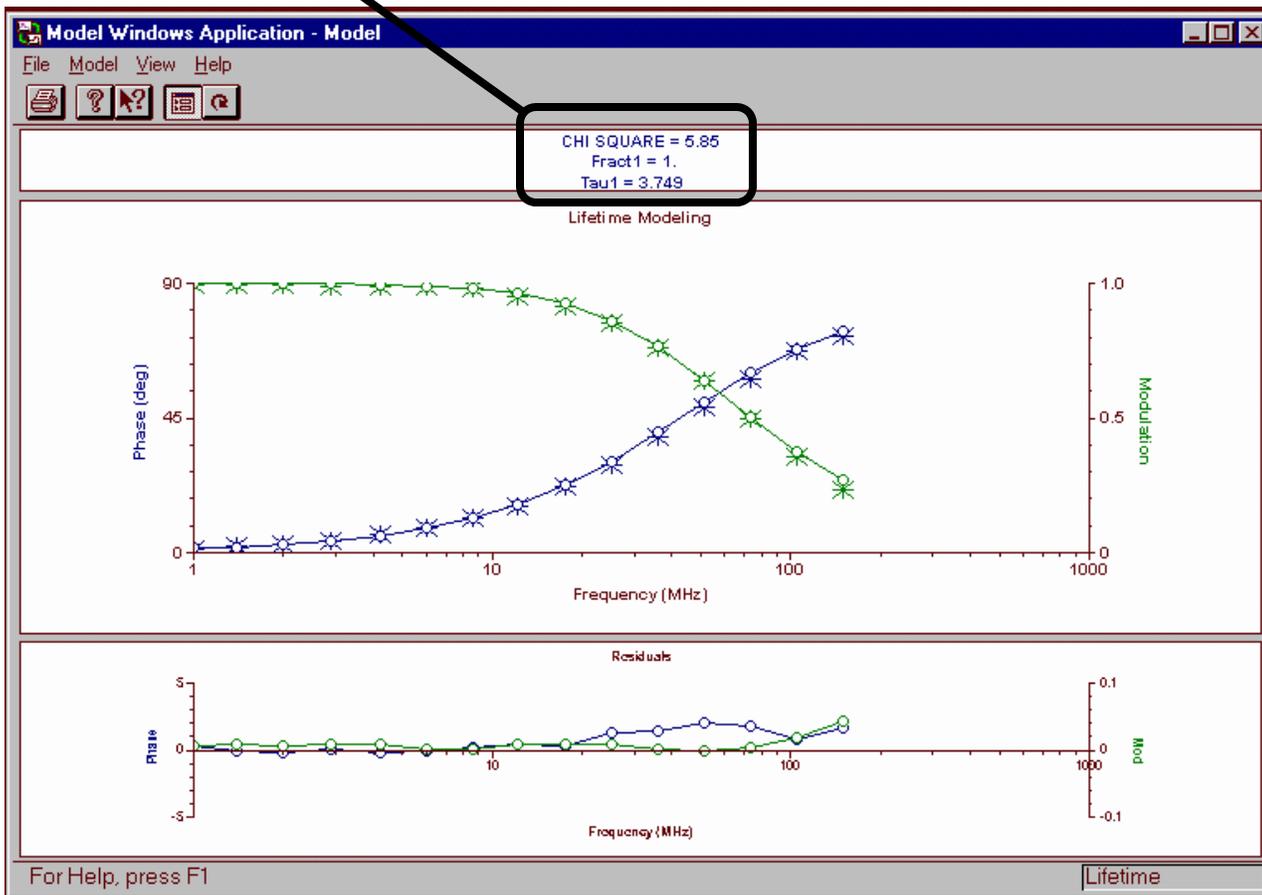
8   Retry the fit with 2 components:

Click the radio button for 2 components, and guess at the fractional amplitudes and possible lifetimes.

9   Click Run Model.

The χ^2 is only a bit smaller, 5.02, and the mid-frequency fit is somewhat improved. The two lifetimes found are 3.7 ns (98.98%) and a long 341.7 ns (1.02%). But can we do better?
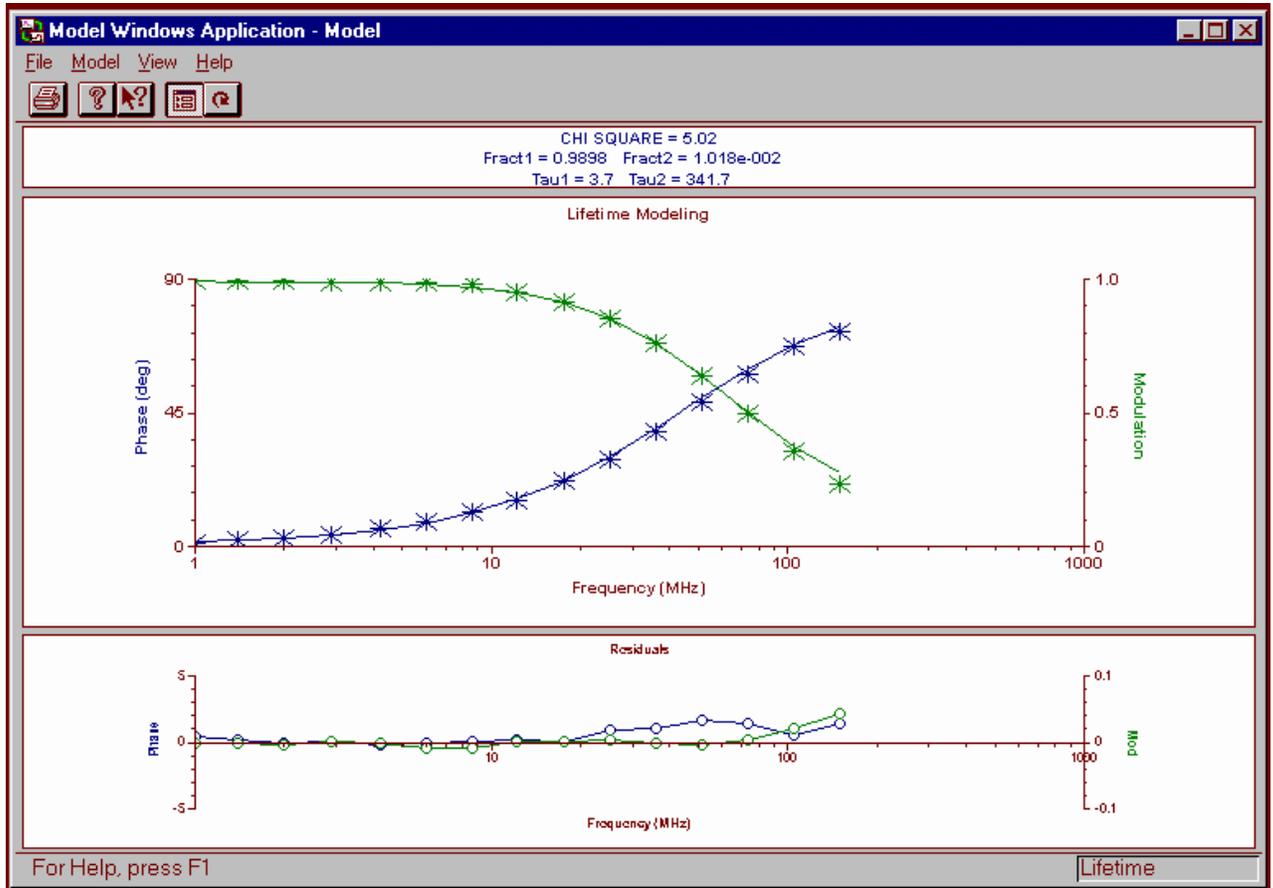
10   Retry the fit with 3 components:

Click the radio button for 3 components, and guess at the fractional amplitudes and possible lifetimes.

11 Click Run Model.
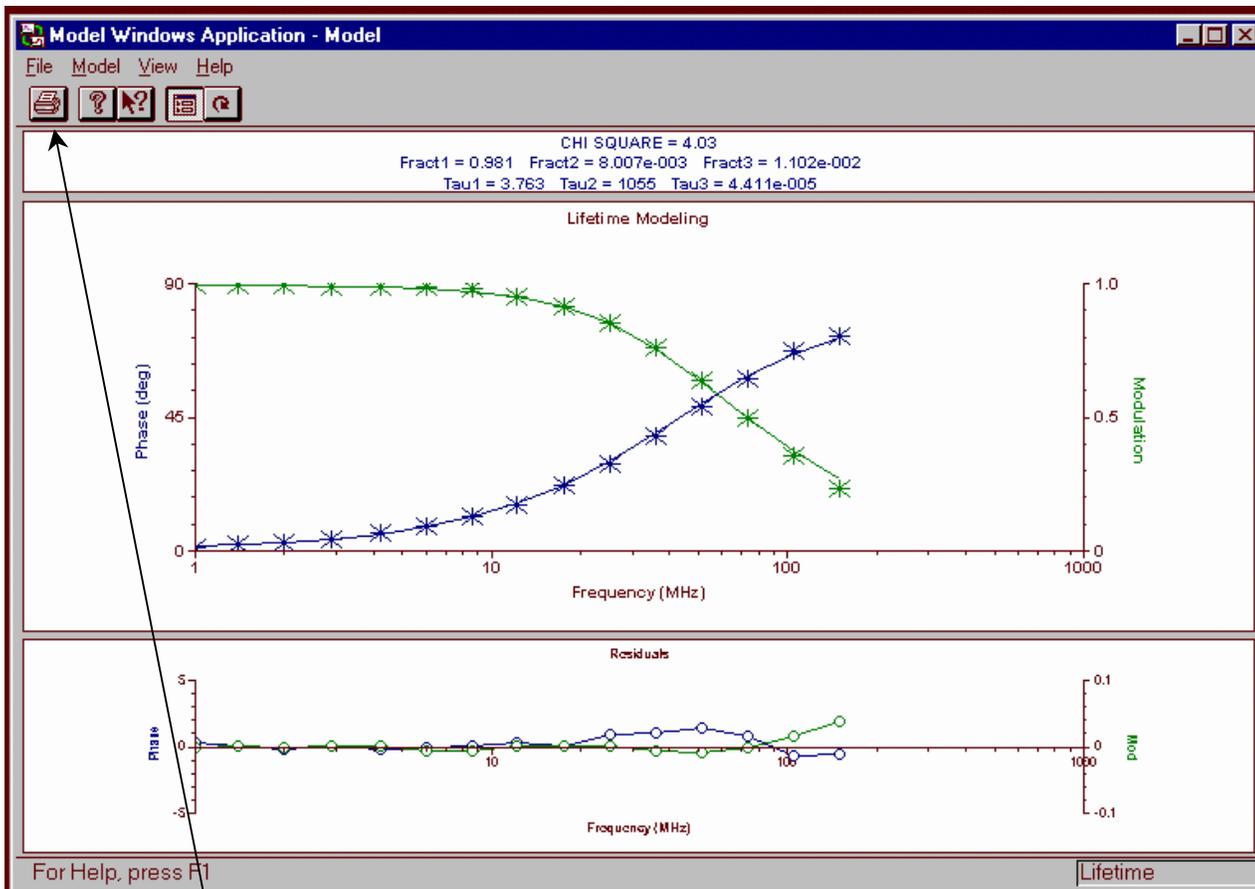
The χ^2 is yet smaller, 4.03, and the mid-frequency fit is further improved. The three lifetimes found are 3.763 ns (98.1%), 1.055 μs (1.1%), and a short 4.4 × 10^{-5} ns (1.1%). We conclude our modeling.

12 Click on the printer icon in the toolbar to generate a printout.

13 Close the **Lifetime Model** window.

Return to the lifetime window for further experiments.

Time-resolved acquisition of an organic compound

Introduction

This lifetime acquisition examines an unknown solid organic sample in time-resolved mode. The procedure consists of running the organic unknown versus a scattering reference (POPOP). This tutorial illustrates the use of time-resolved mode, and how to model multi-exponential fits.

If desired, start the system in a steady-state layout, and run the excitation and emission spectra for the solid sample before starting this experiment. Refer to the *Fluorolog®-3 Operation Manual* and *DataMax Software Manual* for more information on running these scans in DataMax.

Equipment required:

Solid unknown organic sample
POPOP [CAS 1805-34-4], may be purchased from Aldrich
Methanol (research grade)
One 1-cm quartz cuvette
Sample changer
Front-face accessory

Start-up

Follow the procedure listed in steps 1–8 of the POPOP-versus-Ludox® tutorial on pages 8-2 through 8-3.

Unknown and standard preparation

1   ## Prepare a sample of POPOP in methanol.

The concentration of the POPOP should be sufficient to yield about 0.050 A at 358 nm in a 1-cm quartz cuvette. (In general, keep the absorbance below 0.1 A for right-angle measurements with a 1-cm fluorescence cell.)

2   ## Choose an appropriate solid unknown.

The unknown used in this tutorial is an organic thin film.

3   ## Place the standard cuvette in position 1 for the 2-cell sample-holder.

4   ## Mount the solid sample on the front-face acces-sory, at a right angle to the POPOP.
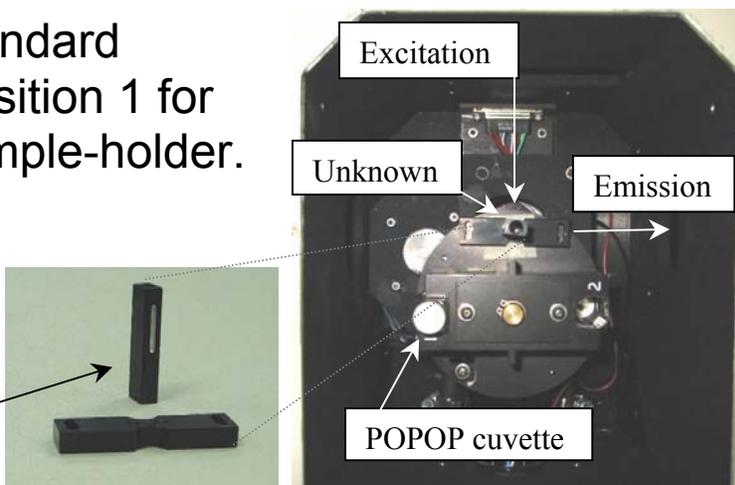
5   ## Switch the instrument to front-face acquisition.

Rotate the knob on the sample compartment to FF.

6   ## Check that the standard cuvette is filled high enough to prevent the excitation beam from scattering off the top of the solution.

Such unwanted scattering prevents an accurate measurement of the modulation and phase.

Selecting and adjusting lifetime hardware

Follow the directions listed in steps 1–5 on page 8-5.

Defining the experiment parameters

1   Follow the directions on pages 8-6 through 8-7
     in the POPOP-versus-Ludox® tutorial.

2   Set up the **Signal Balance** dialog box.

Note: To run the full time-resolved acquisition across all emission and excitation wavelengths automatically, the standard's signal must match the unknown's signal to within ~20% across the entire desired wavelength range. The wavelength range for the standard need not be the same as for the unknown, nor need the interval between wavelengths be the same. The standard's signal need only match the unknown's signal.

*If this signal requirement is not met, **do not balance the signal at one wavelength and continue automatically**. Instead, balance the signal at each wavelength-pair, run a lifetime-acquisition at that wavelength-pair, and rebalance the signal before acquiring at the next wavelength. Do the procedure listed here (steps 2 and 3), then skip to "Manual experiment setup and measurement".*

a   Store the optimal excitation and emission wavelengths, if necessary, and the sample-changer's position for the two samples. A previous steady-state determination of the unknown gave an absorption peak at 375 nm, and an emission peak at 530 nm.

b   Reset the instrument by clicking the Set Standard or Set Unknown button.

- Set the standard EXcitation to 375 nm and EMission to 530 nm with POPOP.
- Set the unknown EXcitation and EMission to 375 nm and 530 nm, respectively, for the unknown.

Note: If not using the emission monochromator, set the unknown to the same emission wavelength as the standard, to avoid waiting for the emission monochromator to move unnecessarily during an experiment in which the emission monochromator is not used.

8-41

c   Set the integration time to 0.2 seconds.

d   Set the PMT high voltages:

S = 1150 if used, otherwise leave at 0
R = 550
T = 1100 if used, otherwise leave at 0
If the HV On box is not checked, click HV On to send voltage to the PMTs.

e   Click on the shutter icon in the toolbar to open the shutter.

When changing a parameter's value, always double-check the status of the shutter. It will automatically close while some of the parameters change.

f   Watch the signal levels reported in the **Lifetime** box.

Note: The settling time for the AC signals can be quite long—up to ~ 1 min—so be patient.

Look only at the AC and DC levels at this step; don't worry about modulation ratio or phase angle. Only values shown in black updated at the integration time should be considered. Signal values shown in red, or that do not update, are not reliable data; allow the system more time to settle signals.

g   Continue balancing the POPOP standard and the organic unknown as on pages 8-30 through 8-31, steps g through k.

3   Determine the frequency range as shown on pages 8-31 to 8-32, step 8.

For this sample, the range 5–250 MHz was chosen, based on the signal and modulation.

Automatic experiment setup and measurement

1   Open the **Time Resolved Acquisition** dialog box.

The signals, slits, high voltage, standard wavelengths, unknown wavelengths, and sample changer positions were transferred from **Real Time Display**.

Note: If you did not select the transfer option from the **Real Time Display** toolbar, either manually enter these parameters, or open the **Real Time Display** and click the transfer icon.

2   Close **Real Time Display**.

3   Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Mono Positions…
h. Process By
i. Min Avg
j. Max Avg
k. Set Pt. Std Dev (%)
l. Std Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a file name in the desired subdirectory. Or, type a file name directly in the DataFile field. Let the system append the proper extension (`.dat`) to the file name.

b   Set integration to 12 s.

c   Define the frequency sequence to scan the unknown.

Enter the starting frequency, ending frequency, and number of frequencies. Choose logarithmic (preferred) or linear.
• Start Freq (MHz): 5

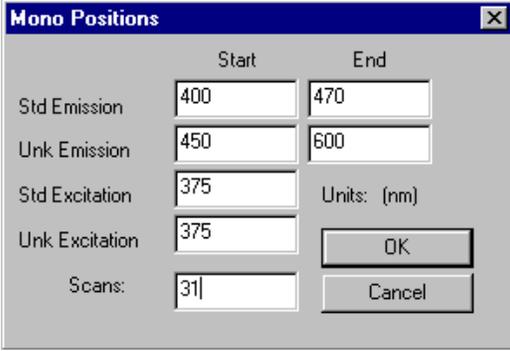- End Freq (MHz): 250
- Number of Freqs: 12

d   Sequence: Log

e   Mono Positions…: Fill in the dialog box to the right.

f   Process By: Interleave.

g   Min Avg: 3

h   Max Avg: 5

i   Set Pt. Std Dev (%): 0.5

The values entered in steps g through i work together to obtain a defined error-level in a minimum time. The minimum of 3 averages is calculated and the resulting standard deviation is compared to the set-point standard deviation. If the set-point standard deviation is less than the calculated standard deviation, then the next measurement is completed and averaged. This continues until the set-point standard deviation is equal to or greater than the calculated average, or until the maximum number of averages is reached.

j   Dark Offset: Do not use

k   Std Lifetime (ns): Enter 1.32 ns for the POPOP standard.

l   As desired, enter one line of text in the Sample and Real Time Processing Info field.

Note: Do not change the default values of any other field in this section.

m   Click Run to start the experiment.

Note: For automatic time-resolved acquisition, skip to "Following along with data-collection and modeling".

Manual experiment setup and measurement

1   Activate the **Lifetime Acquisition** dialog box.

The signals, slits, high voltage, standard wavelengths, unknown wavelengths, and sample changer positions were transferred from **Real Time Display**.

*Note: If you did not select the transfer option from the **Real Time Display** toolbar, either manually enter these parameters, or open the **Real Time Display** and click the transfer icon.*

2   Close **Real Time Display**.

3   Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Set Monos
h. Process By
i. Min Avg
j. Max Avg
k. Set Pt. Std Dev (%)
l. Std Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a file name in the desired subdirectory. Or, type a file name directly in the DataFile field. Let the system append the proper extension (`.dat`) to the file name.

b   Set integration to 12 s.

c   Define the frequency sequence to scan the unknown.

- Start Freq (MHz): 5
- End Freq (MHz): 250
- Number of Freqs: 12

d Sequence: Log

e Set the monochromators:

STanDard EXCitation = 375 nm
STanDard EMission = 400 nm
UNKnown EXCitation = 375 nm
UNKnown EMission = 450 nm

f Process By: Interleave.

g Min Avg: 3

h Max Avg: 5

i Set Pt. Std Dev (%): 0.5

j Std Lifetime (ns): Enter 1.32 ns for the POPOP standard.

k As desired, enter one line of text in the Sample and Real Time Processing Info field.

> **Note:** Do not change the default values of any other field in this section.

l Click Run to start the experiment.

4 Move to the next wavelength range.

5 Rebalance the signals as in steps b to g, pages 8-41 to 8-42.

6 Take another lifetime acquisition as in steps 1-3 of this section, pages 8-45 to 8-46.

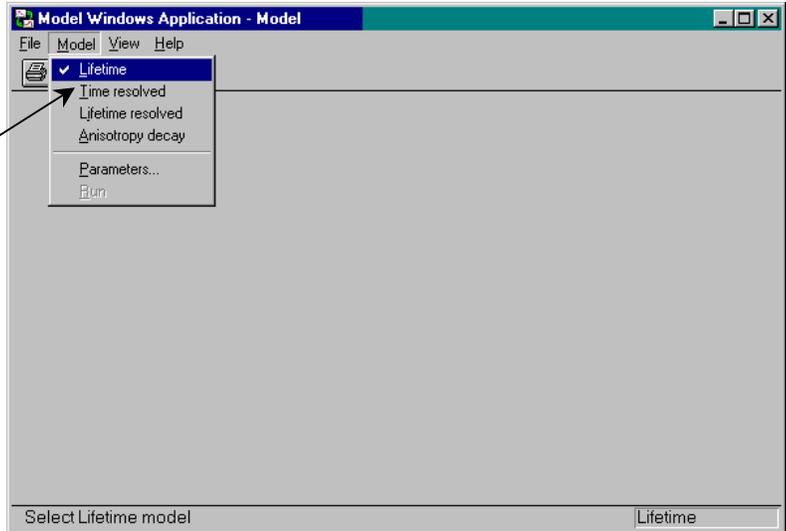…and so on, till the complete wavelength range is covered.

Following along with data-collection and modeling

1   Select the **Run Lifetime** program from the **Instrument Control Center**.
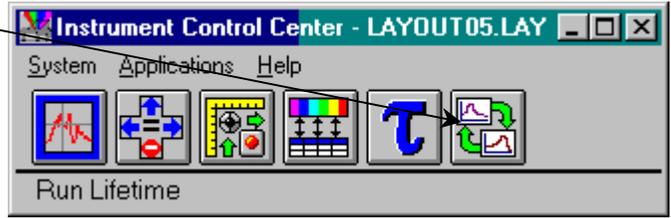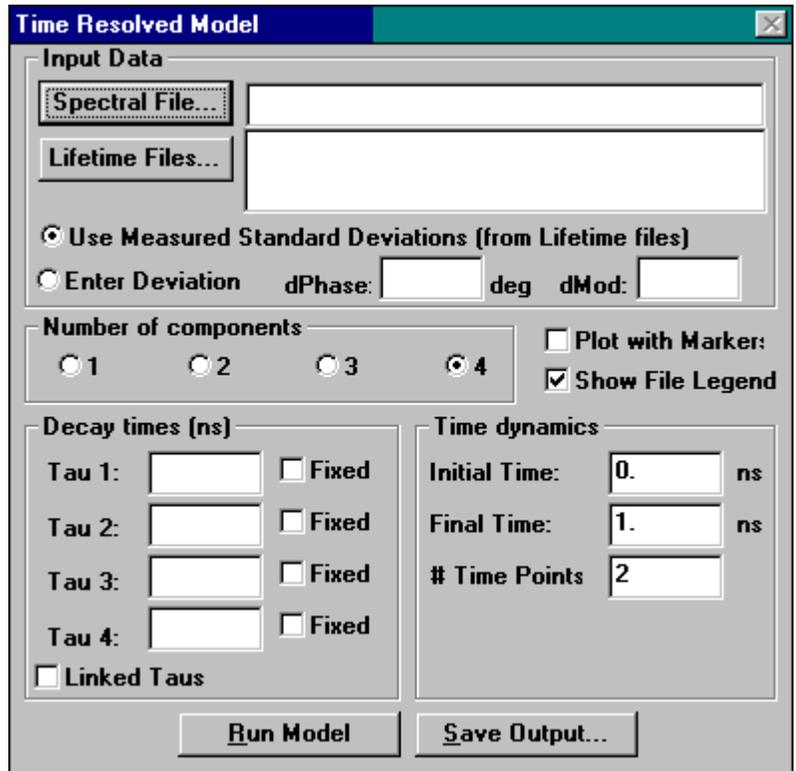

This opens the **Model Windows Application – Model** window:

2   Select Time Resolved.

This opens the **Time Resolved Model** window:

3   Enter the data file name of the completed experiment.

Time Resolved Model

Input Data
Spectral File...   c:\lin\taiwan\ff-x375.spc
Lifetime Files...   c:\lin\taiwan\2tres01.dat
c:\lin\taiwan\2tres02.dat
c:\lin\taiwan\2tres03.dat

○ Use Measured Standard Deviations (from Lifetime files)
● Enter Deviation   dPhase: 5.e-003 deg   dMod: .005

Number of components
○ 1   ● 2   ○ 3   ○ 4
☐ Plot with Markers
☑ Show File Legend

Decay times (ns)
Tau 1: 0.8   ☐ Fixed
Tau 2: 2.   ☐ Fixed
Tau 3:
Tau 4:
☐ Linked Taus

Time dynamics
Initial Time: 0.   ns
Final Time: 0.1   ns
Time Points 20

Run Model   Save Output...

   a   In Lifetime Files…, clicking on a file name automatically opens a series of files.

   b   For a set of manually acquired files, click Lifetime Files…, and select each file individually.

4   Select Enter Deviation.

5   Enter dPhase (phase error) = 0.5 and dMod (modulation error) = 0.005.

6   Choose the expected number of components.

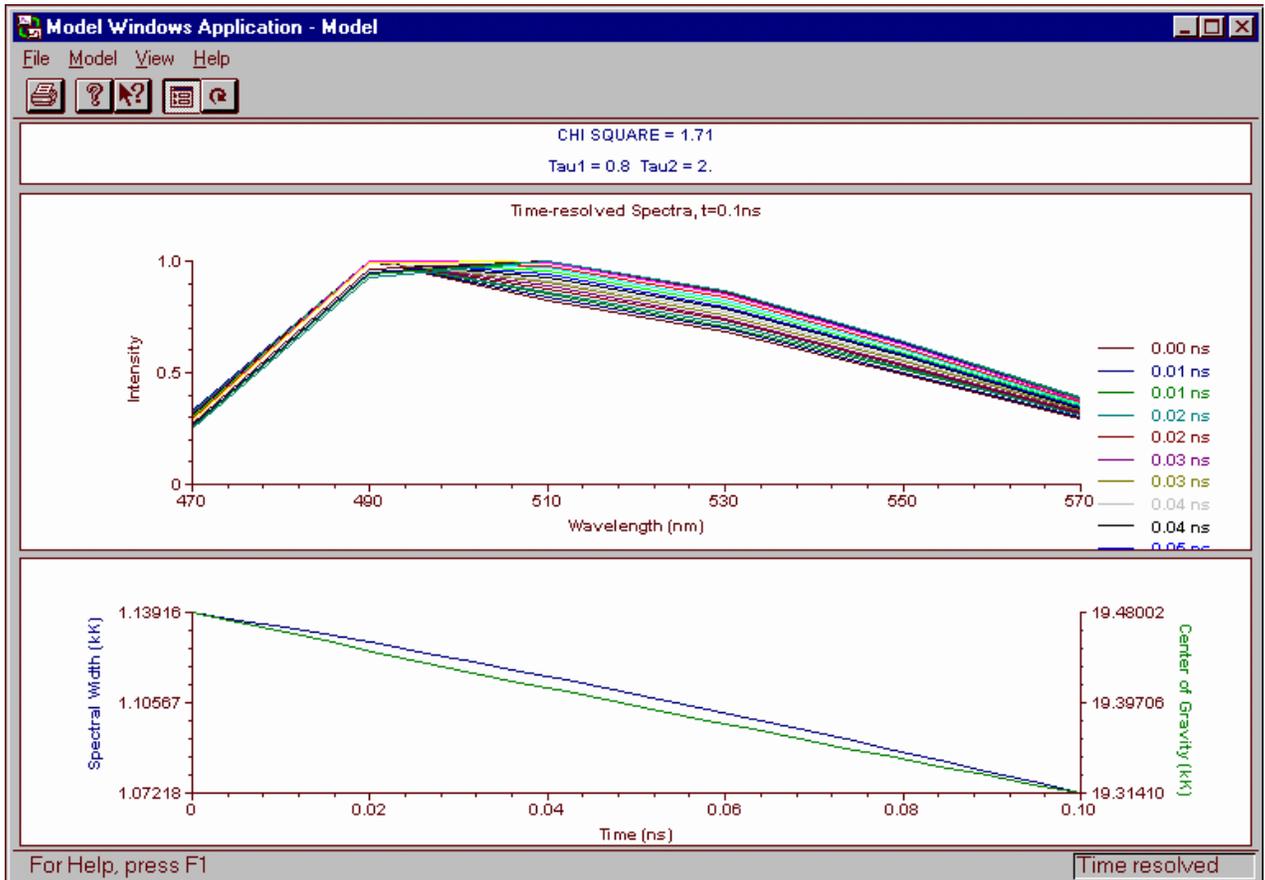Here we try 2.

7   Enter the decay times (in ns).

Enter a non-zero value in every open field. We guess 0.8 ns for the first life-time, and 2 ns for the second lifetime.

8   Enter the time dynamics for which the model is to be run.

Use 0 ns to start, 0.1 ns to finish, and 20 time points between the start and end.
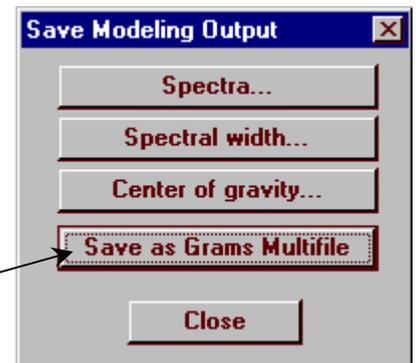
9   Click Run Model.
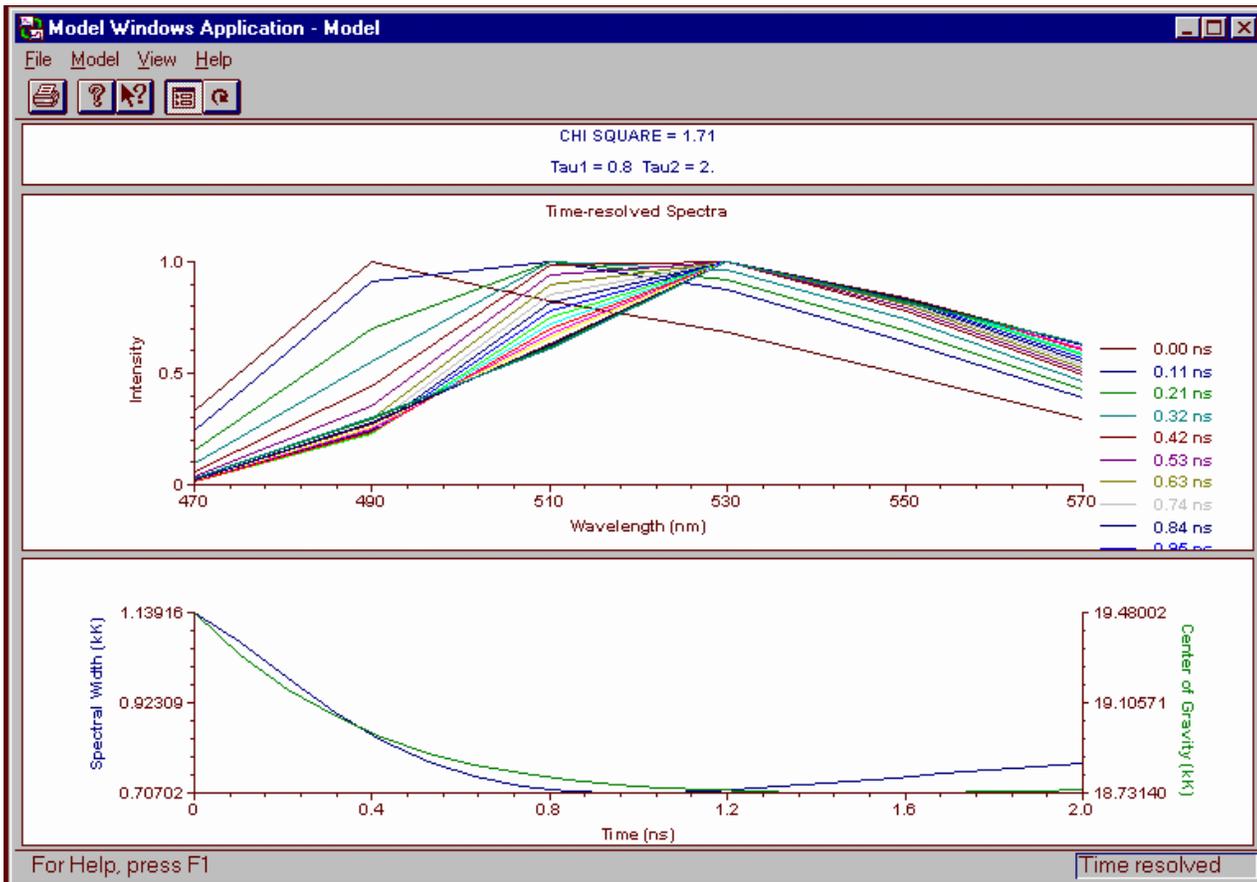
Below is the result from the unknown organic sample.

10 Click Save Output… to save the graph for later processing.

The **Save Modeling Output** window opens. Choose Save as Grams Multifile to save the fit.

11 Rerun the **Time Resolved Model** window, with time dynamics from 0 to 2 ns.

The model appears below:

Note the difference between 0.1 and 2 ns.

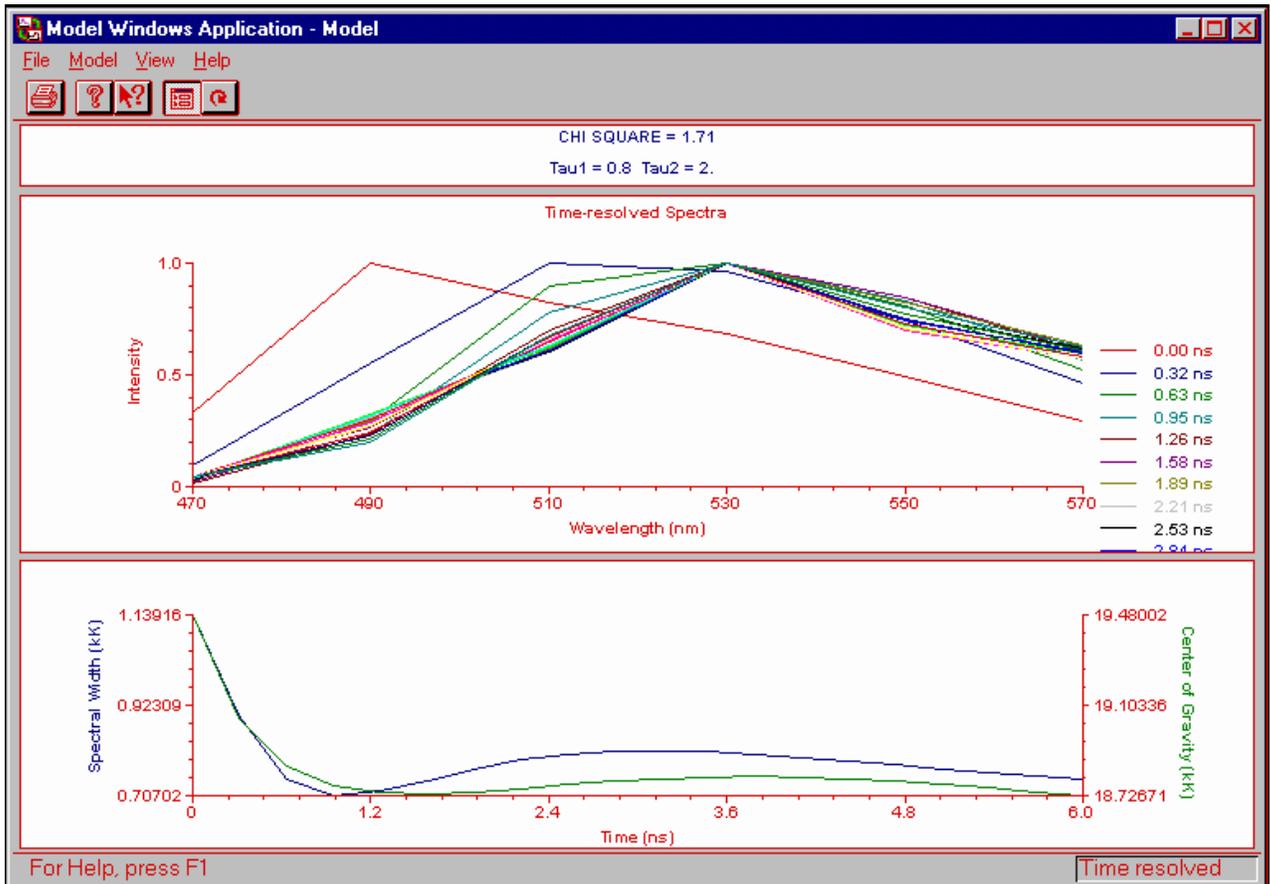## 12 Click Save Output… to save the graph for later processing.

The **Save Modeling Output** window opens.
As before, choose Save as Grams Multifile to save the fit.
Let us try another length of time to model.

13 Rerun the **Time Resolved Model** window, with time dynamics from 0 to 6 ns.

The model appears below:

This time, compare this graph with the 0.1-ns and 2-ns plots.

14 Click Save Output… to save the graph for later processing.

The **Save Modeling Output** window opens.
As before, choose Save as Grams Multifile to save the fit.

15 Close the **Model Applications** window.

16 Open the **Run Experiment** window.

17 In the main menu, choose File…
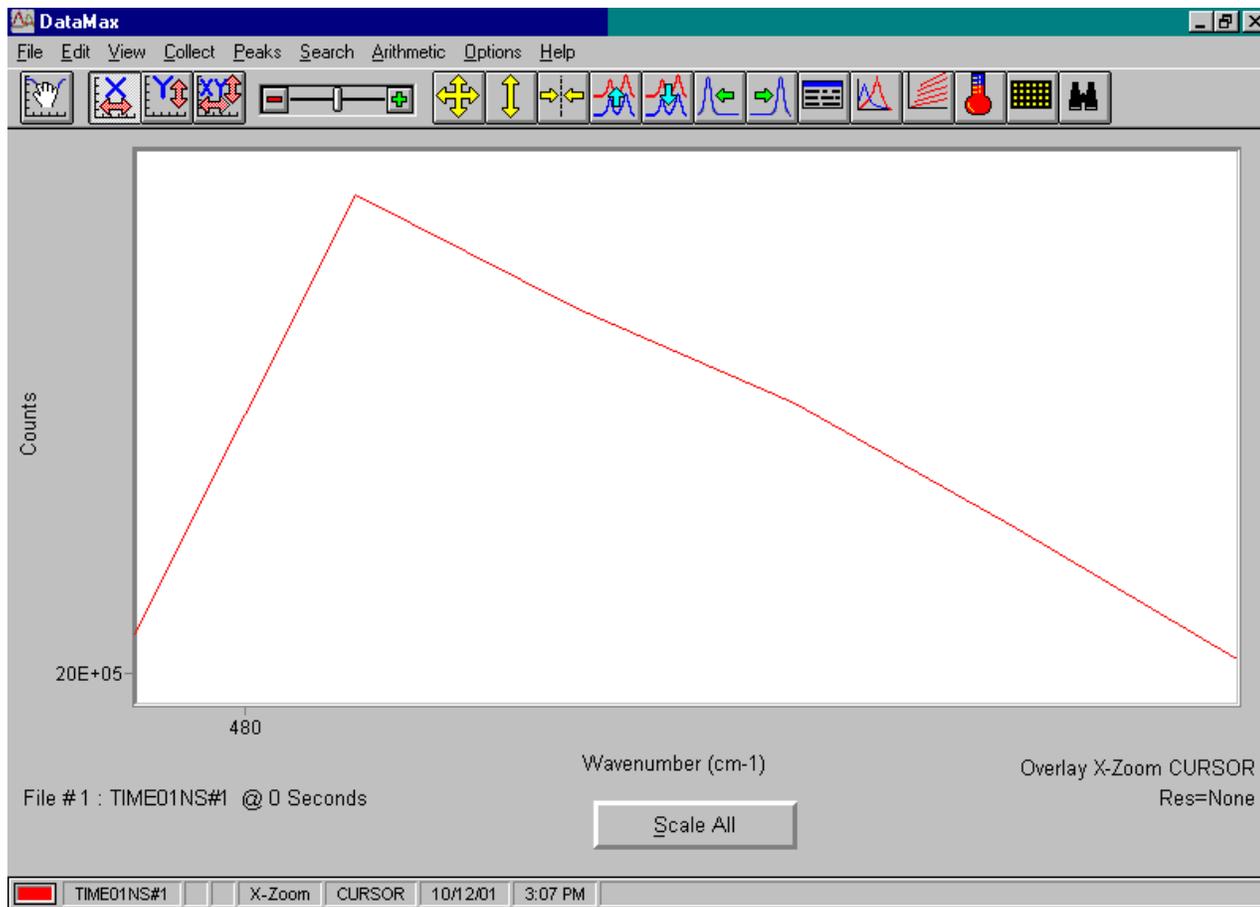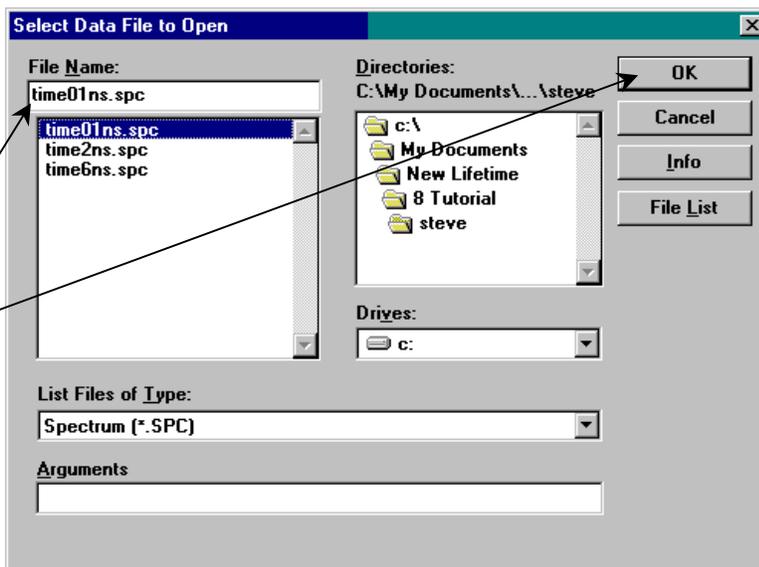
18 In the drop-down File menu, choose Open…

The **Select Data File to Open** dialog box appears.

19 Enter the name of the file to plot in the **Select Data File to Open** dialog box.
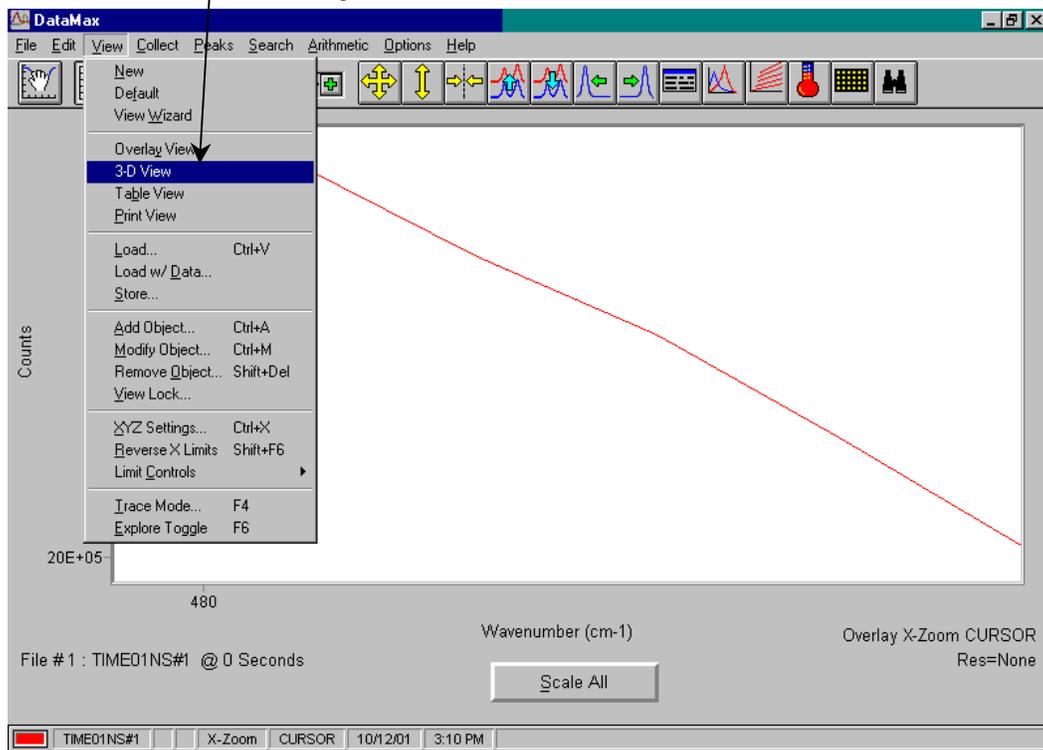
The spectral data file has an extension of `.SPC`. First, we choose to plot the data from the 0.1-ns model.
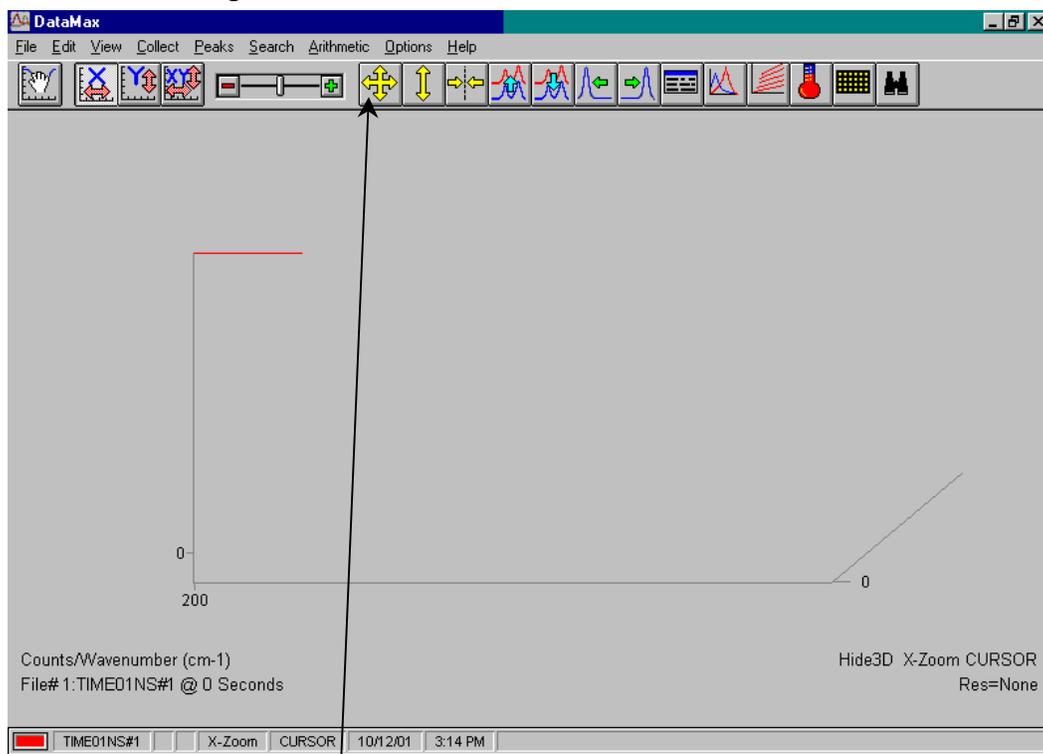
20 Click OK.

A crude spectrum appears on the blank central area in the **Run Experiment** window:
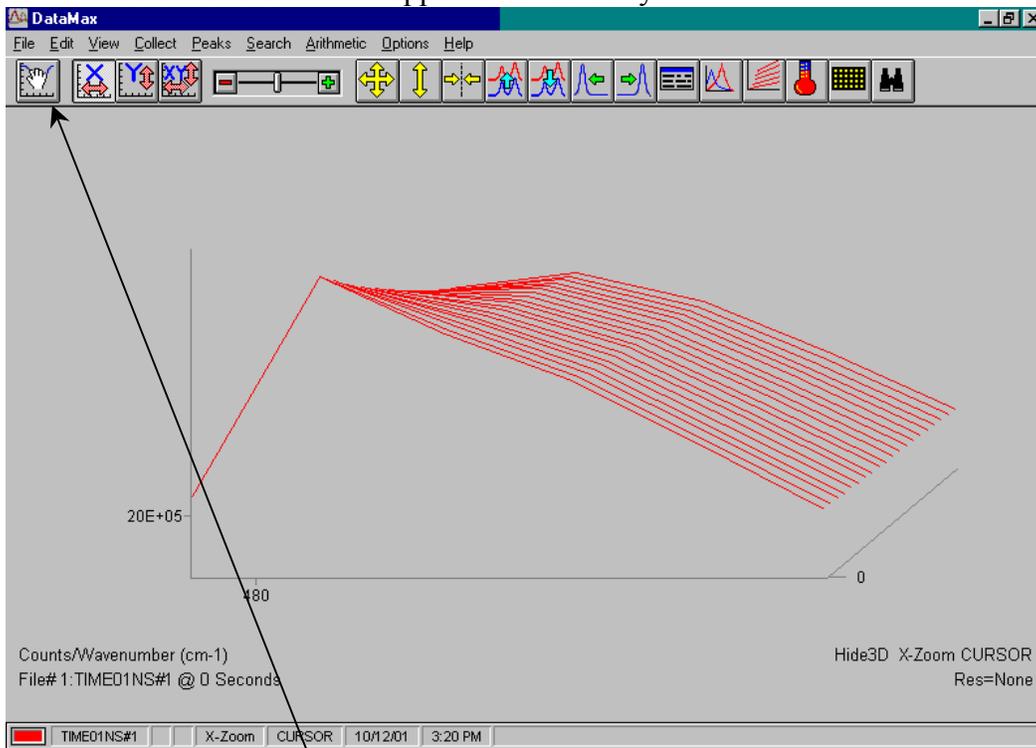
21 In the View drop-down menu, choose 3-D View:

The screen changes to a "3-D View":

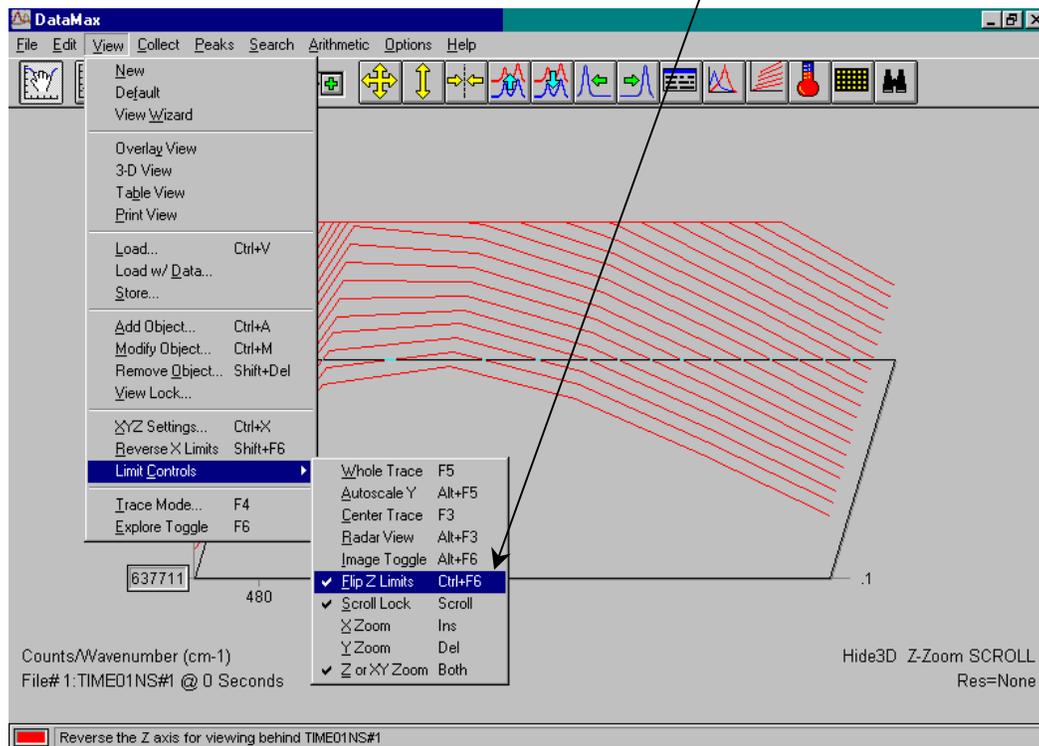## 22 Click the Scale All Axes button.

A three-dimensional view appears more clearly:

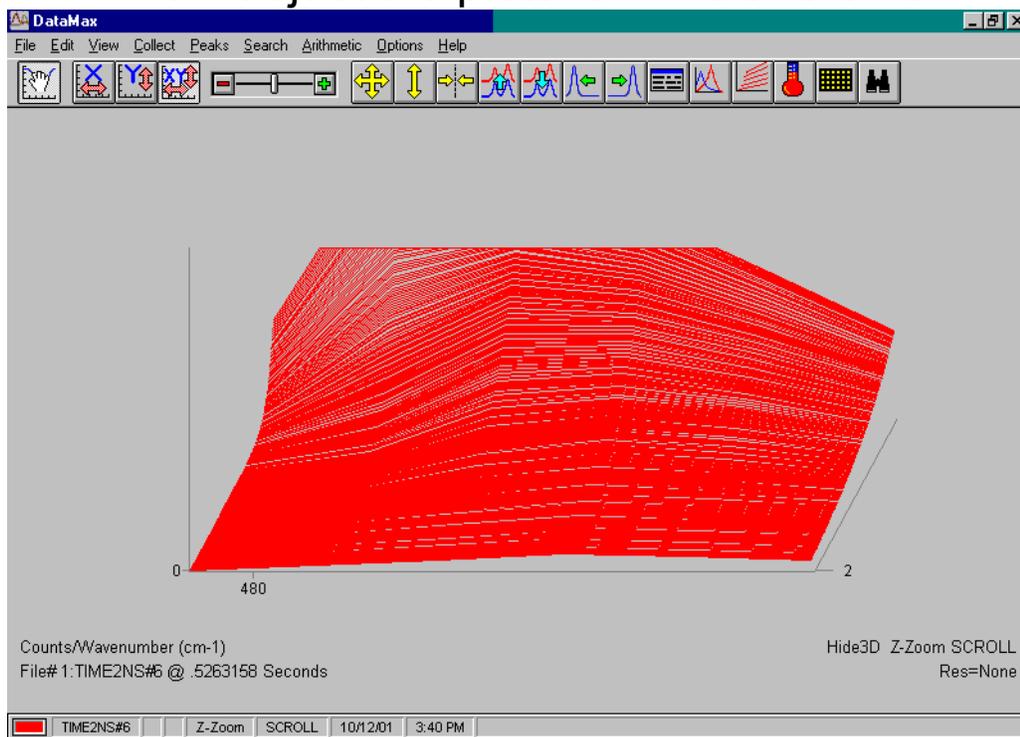

23 Click the hand icon, and place it on the plot.

With the left mouse-button down, change the axis angle.
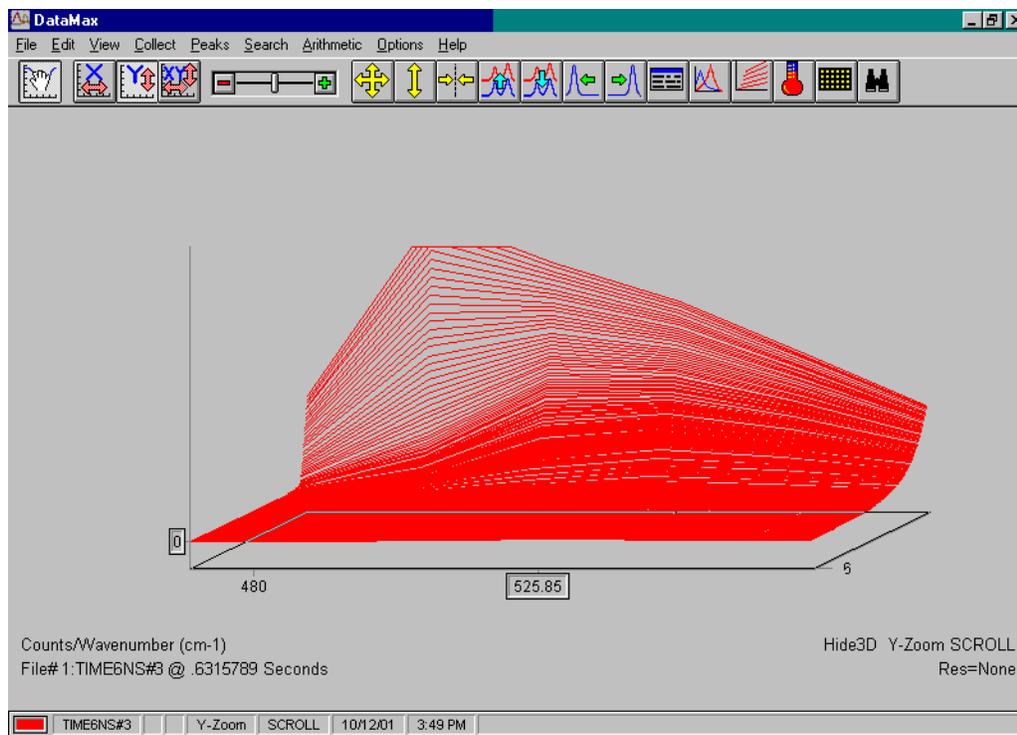
24 Choose View, Limit Controls, Flip Z Limits.

This may aid viewing the time-resolved peaks in the fit.

25 Now adjust the plots for 2 ns and 6 ns.

Lifetime-resolved spectrum for 2-ns model. *Z*-interpolation = 512.

Lifetime-resolved spectrum for 6-ns model. *Z*-interpolation = 512.

Anisotropy-decay acquisition of perylene in glycerol

Introduction

This anisotropy-decay acquisition examines perylene, a yellowish solid organic compound, dissolved in glycerol.

If desired, start the system in a steady-state layout, and run the excitation and emission spectra for perylene before starting this experiment. Refer to the *Fluorolog®-3 Operation Manual* and *DataMax Software Manual* for more information on running these scans in DataMax.

Equipment required:

Perylene (99+% Gold Label) [CAS # 198-55-0], can be acquired from Aldrich
Reagent-grade methanol [CAS # 67-56-1]
Reagent-grade glycerol [CAS # 56-81-5]
125-mL round-bottom flask
Laboratory hotplate with magnetic stirrer
UV-Visible spectrophotometer (optional)
One 1-cm quartz cuvette
Temperature bath or Peltier device for Tau system

Warning: Perylene is a carcinogen. Other reagents may be harmful. Read all Material Safety Data Sheets and take all appropriate precautions. Wear safety goggles and protective gloves when handling these substances.

Start-up

Follow the procedure listed in steps 1–8 of the POPOP-versus-Ludox® tutorial on pages 8-2 through 8-3.

Sample preparation

1 In a 125-mL round-bottom flask, dissolve < 1 mg of perylene in approximately 1 mL of reagent-grade methanol.

2 Evaporate off the methanol, leaving a thin film of perylene on the bottom of the flask.

3 Add 100 ml of reagent-grade glycerol to the flask with the perylene thin film. Gently heat (~50°C) and stir on a laboratory hotplate overnight.

Warning: Take appropriate precautions with unattended laboratory equipment.

4 Let the glycerol solution cool.

5 Transfer the solution to a 1-cm quartz cuvette.

6 Measure the emission spectrum of this solution.

a Use 5-nm excitation and 5-nm emission bandpass slits; excite at 440 nm.

b Scan the emission from 455 to 500 nm.

c If the emission signal (without polarizers in place) is > 4 million cps, adjust the slits to smaller bandpass or dilute the sample with more glycerol.

d Optional: If a UV-Visible spectrophotometer is available, obtain the absorption spectrum of the perylene in glycerol. The OD should not exceed 0.05.

Selecting and adjusting lifetime hardware

Follow the directions listed in steps 1–5 on page 8-5.

Defining the experiment parameters

1 Follow the directions on page 8-6 in the POPOP-versus-Ludox® tutorial.

2 Set the temperature controller to 30°C.

3 Click the EX1 radio button.

This selects the excitation monochromator in the **Slits** dialog box. Enter 6.0 for the Side Entrance and 0.5 mm for the Side Exit. Set the optional intermediate slit to 6.0 mm. Wait after each adjustment to allow the slit to change,

before adjusting the next.
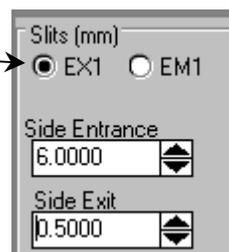
4 Click the EM1 radio button.

This adjusts the S-channel emission monochromator. Enter 7.0 mm for the entrance and 7.0 mm for the exit slit. Set optional intermediate slit to 7.0 mm.
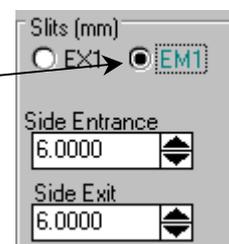
5 Place a 400-nm cut-on filter on the external T-channel box, and a filter on the appropriate side of the cuvette, on the sample changer.
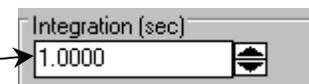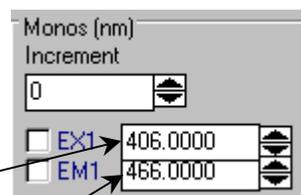
With an optical filter, there are no slits to adjust on the emission T-channel.

6 Set the system's parameters.

a Set the excitation monochromator to 406 nm.

b Set the emission monochromator to 466 nm.

c Set the integration time to 1.0 s.

d   Set the PMT high
     voltages:

     High Voltage
     ☒ HV On

     S 0.0
     R 425.0000
     T 900.0000

     • S = 0 (not used)
     • R = 425
     • T = 900
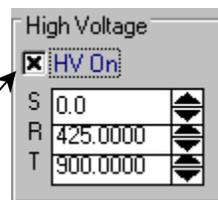
If the HV On box is not checked, click HV On to send voltage to the detectors.

e   Follow steps e through h on pages 8-8 and 8-9.

f   Follow steps j through l on page 8-9.
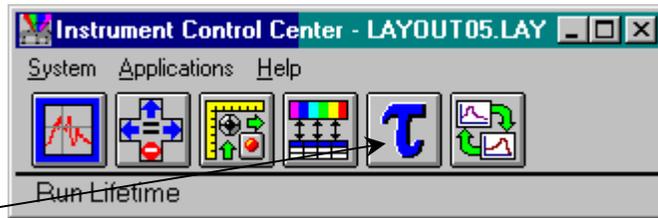
Experiment setup and measurement

1   Activate the **Anisotropy Decay Acquisition** dialog box.

In **Instrument Control Center**,

a   Click the Tau button.

The **Lifetime** and **ISA Graph** windows open.

b   In the **Lifetime** window, choose Collect.

A drop-down menu appears.

c   Click Experiment.
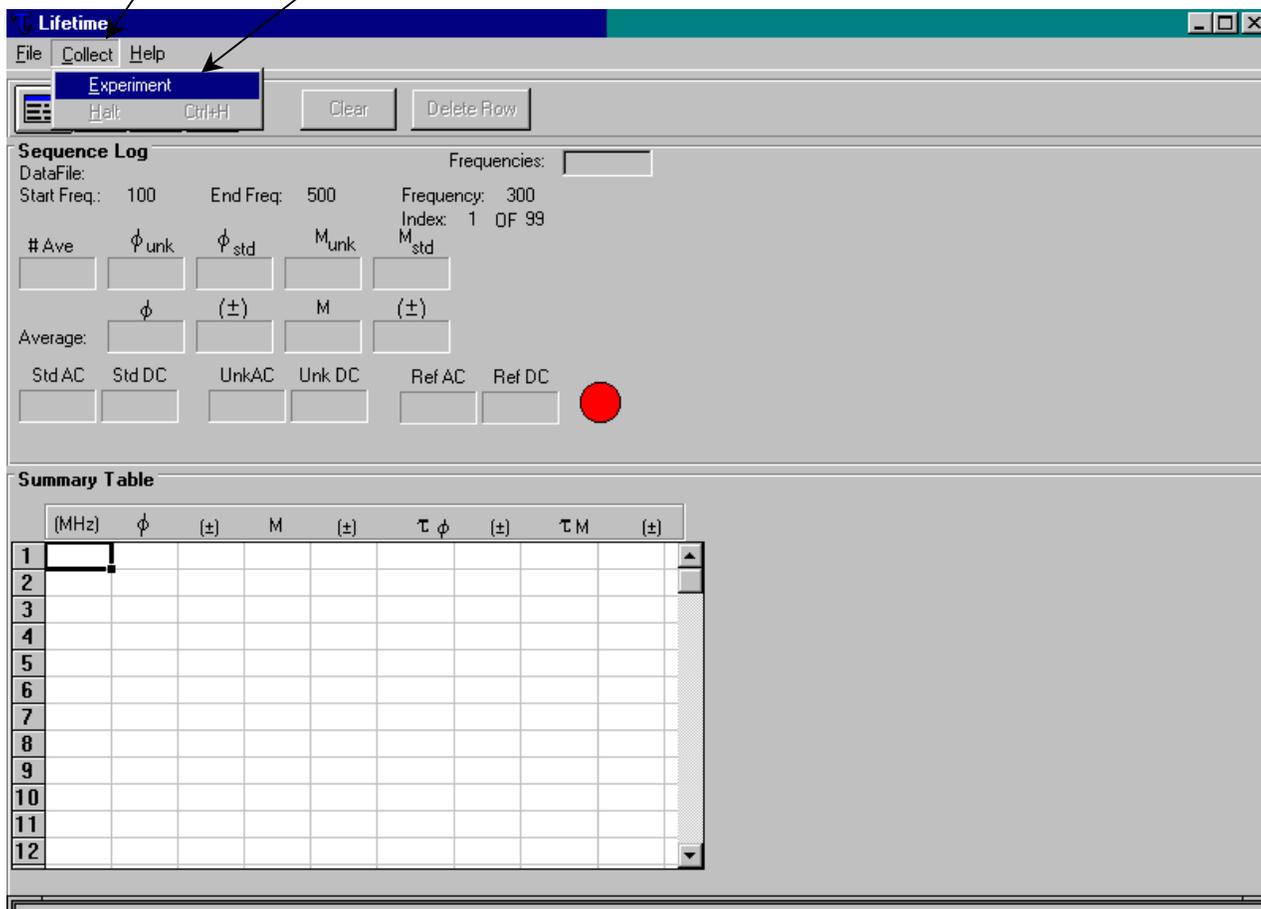
The **Lifetime Acquisition** dialog box opens.
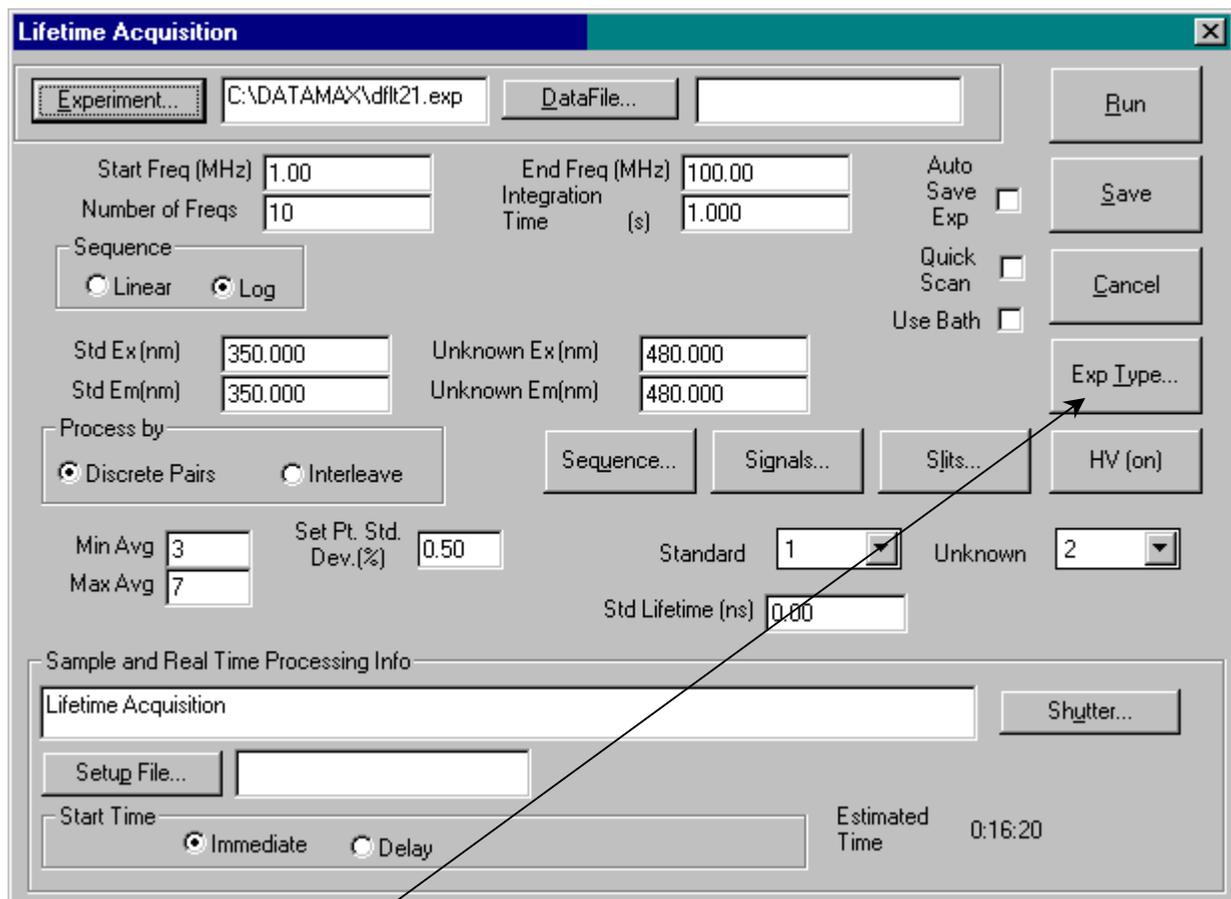
d   Click Exp Type….

The **Select Experiment Type** dialog box opens:

e   Click the Polarization checkbox.

New experiment types
appear in the list.

f   Choose Anisot-
ropy Decay.

g   Click OK.
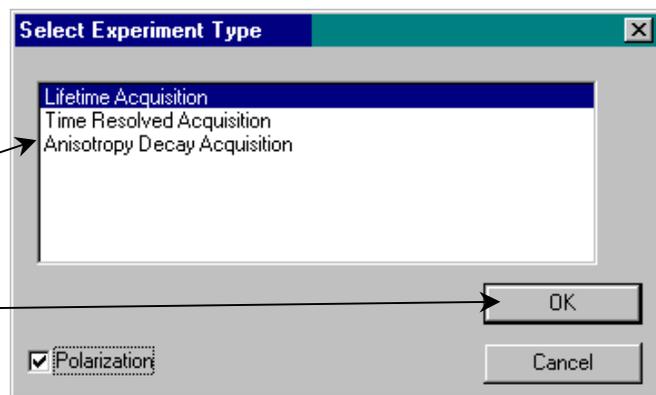
The **Select Experiment
Type** dialog box closes;
the **Acquisition** dialog box changes into an **Anisotropy Decay Acquisition** dialog
box.

2   Set the following parameter fields:

a. DataFile
b. Integration Time
c. Start Freq (MHz)
d. End Freq (MHz)
e. Number of Freqs
f. Sequence
g. Set Monos
h. Process by
i. Min Avg
j. Max Avg
k. Set Pt. Std. Dev.(%)
l. Steady State G Factor
m. Lifetime

a   Click the DataFile… button to open the dialog box.

Enter a name in the desired subdirectory. Or, type a file name directly in the DataFile field. Let the system append the proper extension (.dat) to the file name.

b   Set the integration time to 8 s.

c   Define the frequency sequence to scan the unknown.

- Start Freq (MHz): 5
- End Freq (MHz): 180
- Number of Freqs: 24

d   Sequence: Log

e   Set the monochromators:

- Excitation = 406
- Emission = 466

f   Process by: Interleave

g   Steady State G Factor: Enter the predetermined *G* factor; for this system, we found *G* = 1.002.

> **Note:** *With filters, do not use G = 1.000 exactly, for a distorted spectrum can result.*

h   Min Avg: 3

i   Max Avg: 5

j   Set Pt. Std. Dev.(%): 0.5

k   Lifetime: Enter 4.75 (units are ns) for perylene in glycerol. This was found from a steady-state experiment.

l   As desired, enter one line of text in the Sample and Real Time Processing Info field.

> **Note:** *Do not change the default values of any other field in this section.*

m   Choose the correct signals to record.

- Click Signals….
  The **Signal Choice** window opens
- Choose T&R.
  This is reverse L-format.
- Click OK.
  The **Signal Choice** window closes.
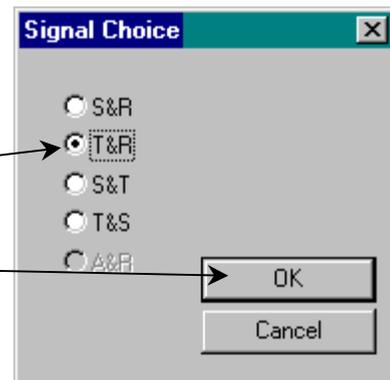
n   Choose the slit settings.

- Click Slits….
  The **Slits** dialog box opens.
- Set the slits as shown.
- Click OK.
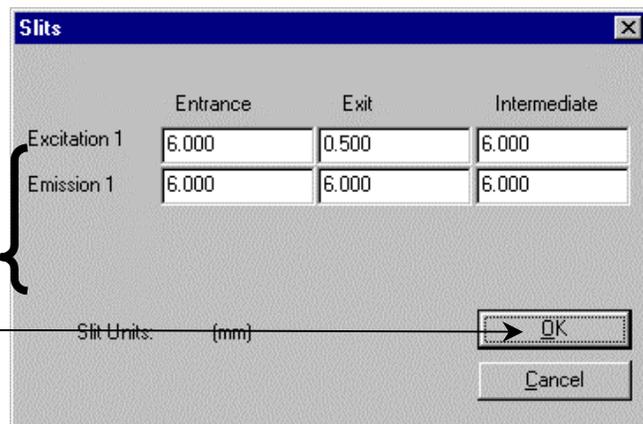
o   Click Run to start the experiment.

As set up, the run time is ~ 30 min.

Modeling the data

1   Select the **Run Lifetime** program from the **Instrument Control Center**.
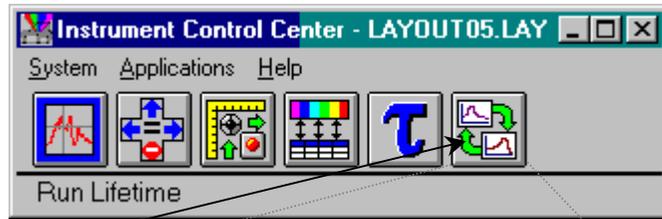
This opens the **Model Windows Application – Model** window:

2   Select
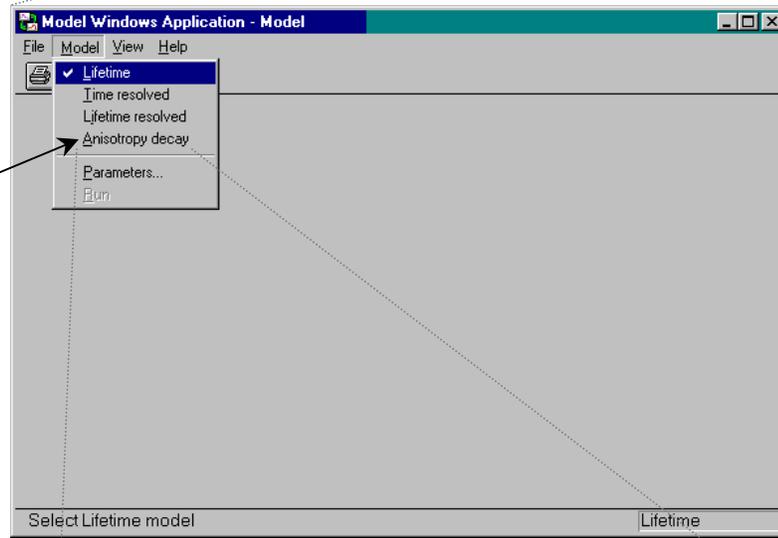
Anisotropy decay.

This opens the **Lifetime Model** window:

3   Guess one rotational component exists.

   a   Choose one lifetime.

   b   Set the fractional amplitude to 1,

8-64
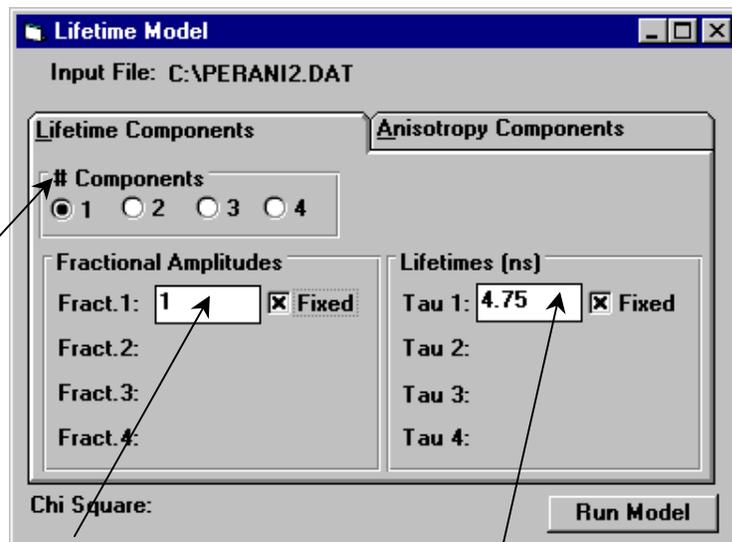
and make it fixed.

c Set the previously determined lifetime fixed to 4.75 ns.

d Click the Anisotropy Components tab.

e Choose 1 anisotropy component.

f Try R_0 = 0.4.

g Set the fractional amplitude to 1, but not fixed.

h Guess a correlation time = 5 ns, not fixed.

i Click Run Model.
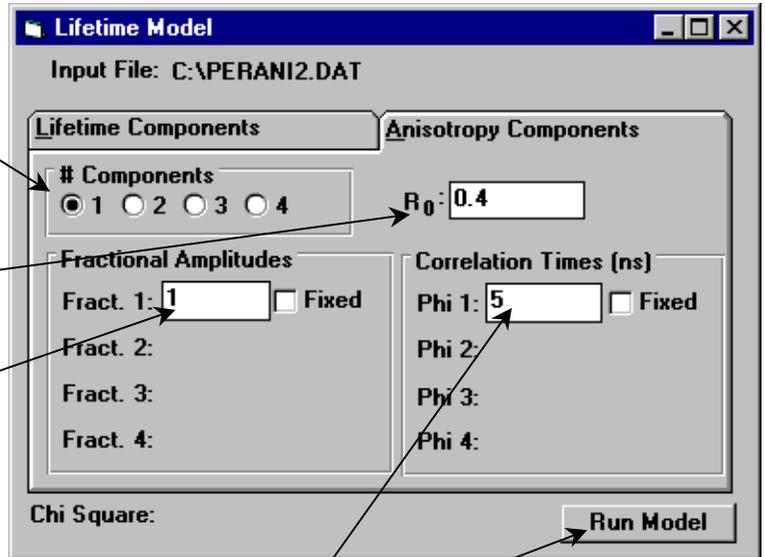
Below is the result, using 45 data points from 1 to 180 MHz:

Note the odd fractional amplitude, which is > 1.
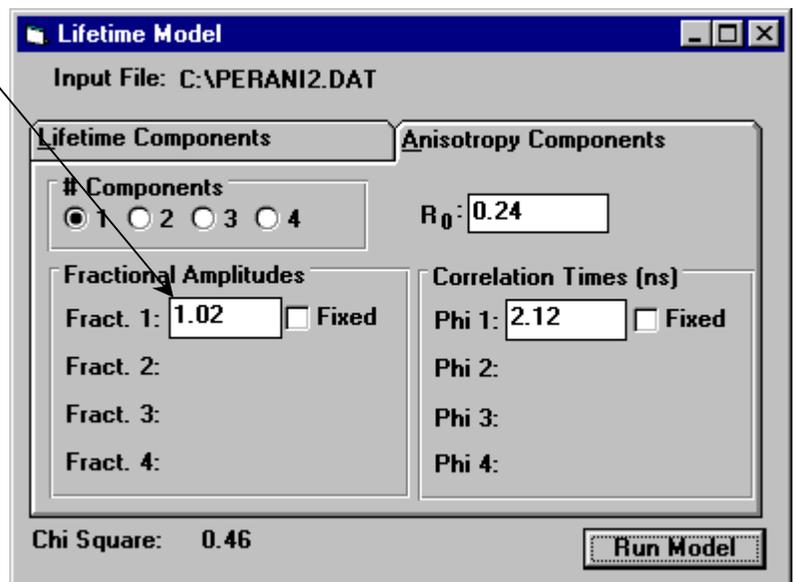
On the next page is the fit:

8-65
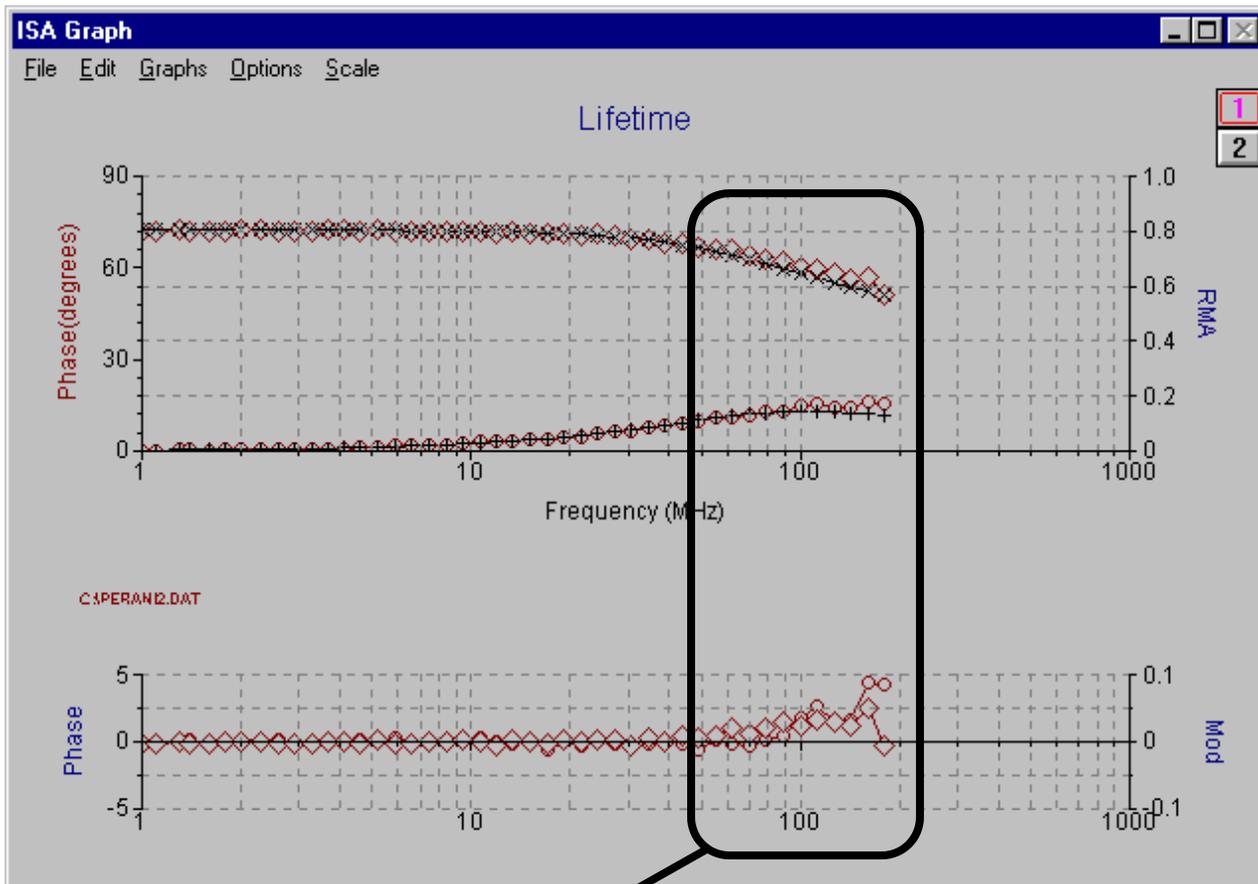
Note the systematic deviations > 50 MHz, indicating an improper fit, and perhaps a missing shorter component. Thus, the single-rotor model is inadequate to describe perylene dissolved in glycerol.

4   Guess two rotational components exist.

a   Leave the lifetime components set to 1.

b   Select two components.

c   Try two fractional amplitudes, 0.3 and 0.7, both not fixed.

d   Try two correlation times, 0.5 ns and 4.0 ns, both unfixed.

8-66

e   Click Run Model.

Here is the result, using 45 data points from 1 to 180 MHz:

Note that Chi Square is reduced by a factor of three.
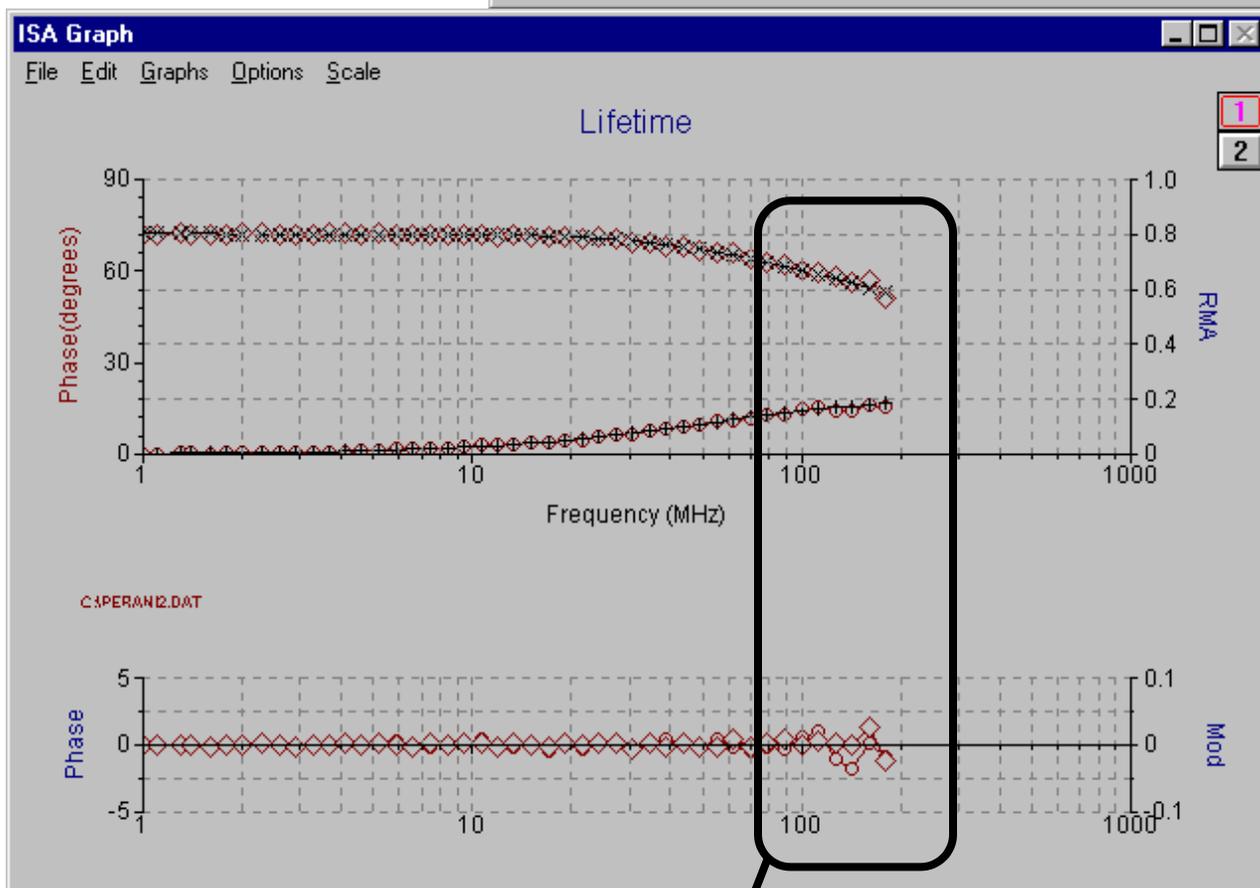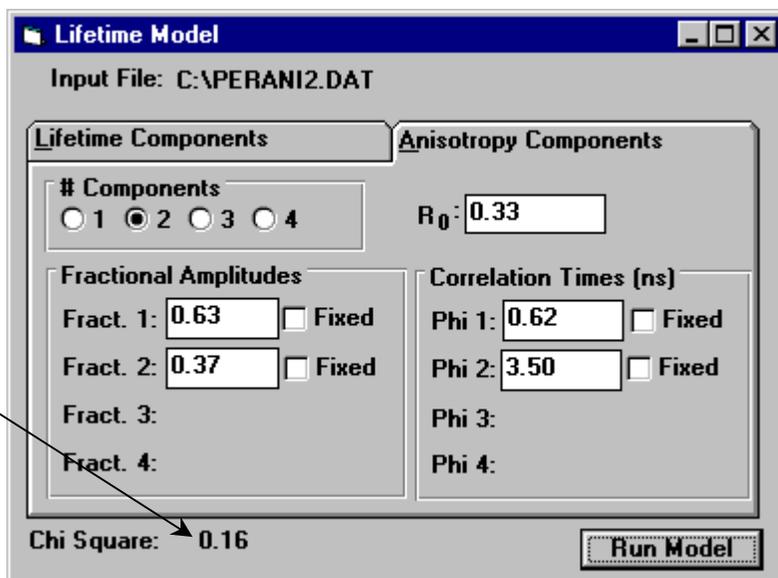
Note the improved fit and the small random errors > 100 MHz. We conclude that there are two types of perylene rotation: in-plane (0.62 ns) and out-of-plane (3.50 ns). The in-plane rotation is faster, because it displaces fewer solvent molecules. Adding a third component to the fit does not improve the fit.

8-67

9: Accessories

Tau-3 systems may be used in steady-state mode with any accessory for the Fluorolog®-3 system. This chapter focuses on dynamic (lifetime) accessories for the Fluorolog®-Tau-3 only. Refer to the *Fluorolog®-3 Operation Manual* for specific information on accessories used for steady-state measurements.

For up-to-date information on all available accessories, including new accessories released after the publication date of this manual, or to find out more information about a particular accessory, contact your local representative or go to our web page, http://www.jyhoriba.com/fluor.

Fluorolog®-Tau-3 Accessories for Lifetime Applications

Item	Model	Page
22.5° front-face viewing accessory	FL-1001	9-2
Dual xenon lamp housing (CW and flash lamps)	FL-1040	9-3
Emission filter holder and spatial filter	FL-1031	9-4
Emission PMT detector, for lifetime measurements	FL-1029	9-5
Filter holders, for excitation and emission light-paths	FL-1010	9-6
Filter set, cut-on, 1" × 2" (370, 399, 450, 500, 550 nm)	1938	9-7
Four-position, automated thermostatted cell holder	FL-1011	9-8
Globals Unlimited™ Software		9-9
Liquid-nitrogen Dewar accessory	FL-1013	9-10
Liquid-nitrogen automated dual Dewar accessory	FL-1041	9-11
Phosphorimeter accessory	FL-1042	9-12
Polarizer, dual auto	FL-1044	9-13
Polarizer, third for T-format	FL-1045	9-14
Sample heater/cooler Peltier thermocouple drive	F-3004	9-15
Solid sample holder	1933	9-16
T-channel optics for 2nd emission channel	FL-1002	9-17
Temperature bath, programmable	F-1000 or F-1001	9-18
Two-position, automated thermostatted cell holder	FL-1012	9-19

Model FL-1001 22.5° front-face viewing accessory

Optically dense samples, films, and solids cannot be measured using the traditional right-angle geometry. Rotating the sample to 45° can be used to measure these samples, but this introduces a large amount of scattered light into the measurement. The front-face accessory provides a choice of collection positions—either right-angle or front-face. In front-face mode, light is collected at 22.5° from the sample and directed to the emission monochromator. This accessory may be used only on the S side of the instrument.

When performing lifetime measurements on an unknown in front-face mode, the standard should be a fluorophore reference standard either in the unknown's format or in a cuvette. Scattering samples may not be used as a standard for front-face lifetime measurements.

Model FL-1040 dual xenon lamp housing
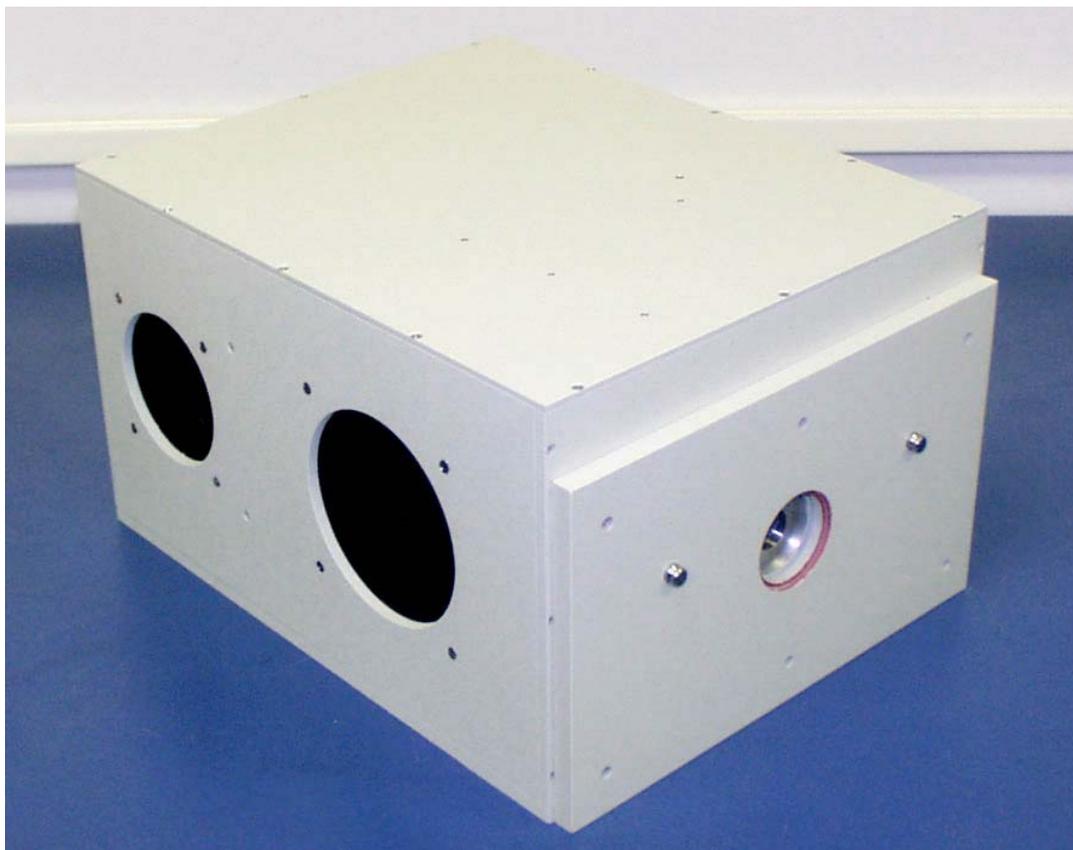
The FL-1040 is a lamp housing that contains both a continuous-wave (CW) xenon source and a xenon flash lamp. Selecting between the two sources is as easy as a turn of a knob, which changes the orientation of the collection mirror from one source to the other. The FL-1040 contains the power supplies and power connections for both lamps internal to the lamp housing. Also included are the modules and controller for phosphorescence measurements.

For fluorescence lifetime measurements, the CW xenon source (or an external laser provided by the user) may be used. For delayed fluorescence or phosphorescence measurements on the microsecond-to-second timescale, the xenon flash lamp and phosphorimeter may be used. Measurements of decays with the phosphorimeter are done using the pulsed method. Refer to the Model FL-1042 phosphorimeter accessory below for more information.

Model FL-1031 emission filter holder and spatial filter

This emission filter-holder and spatial filter allows an emission lifetime detector to be mounted directly to the sample compartment without an emission monochromator in the light path. As the name implies, this accessory contains a filter-holder for 2" × 2" or 1" × 2" filters, and sets the emission detector at the appropriate focal length for the emission beam from the sample. The accessory also has a manual shutter to protect the detector from saturation.

This accessory is a basic tool for lifetime measurements when filters are used to select the appropriate emissions from the standard and unknown (either in the sample compartment or on this accessory), rather than an emission monochromator. Advantages of this method are:
- The narrow bandpass of a monochromator is generally not required for most lifetime measurements.
- Properly chosen filters exhibit much higher throughput than a monochromator.

Choose this accessory when more signal is required from the sample in order to make measurements.

Model FL-1029 emission PMT detector for lifetime measurements

The FL-1029 is the emission photomultiplier-tube detector for lifetime measurements. A second emission-PMT detector for lifetime measurements is added to a lifetime system primarily for T-format collection of anisotropy decays. This is the best method for anisotropy decay measurements, for it corrects for most of the artifacts that can affect the data through simultaneous collection of VH and VV for the decay. Other uses for a second emission lifetime PMT are when different monochromator configurations or grating types are used on an instrument, requiring more than one detector.

The FL-1029 includes the PMT housing and an R928P PMT selected for photon-counting measurements. This detector requires FL-1002 T-Channel optics for the 2nd emission channel, used on the T-side of the instrument, and the FL-1031 emission filter holder and spatial filter, if used on the T-side without an emission monochromator on the T-side.

Model FL-1010 filter holders for the excitation and emission light paths
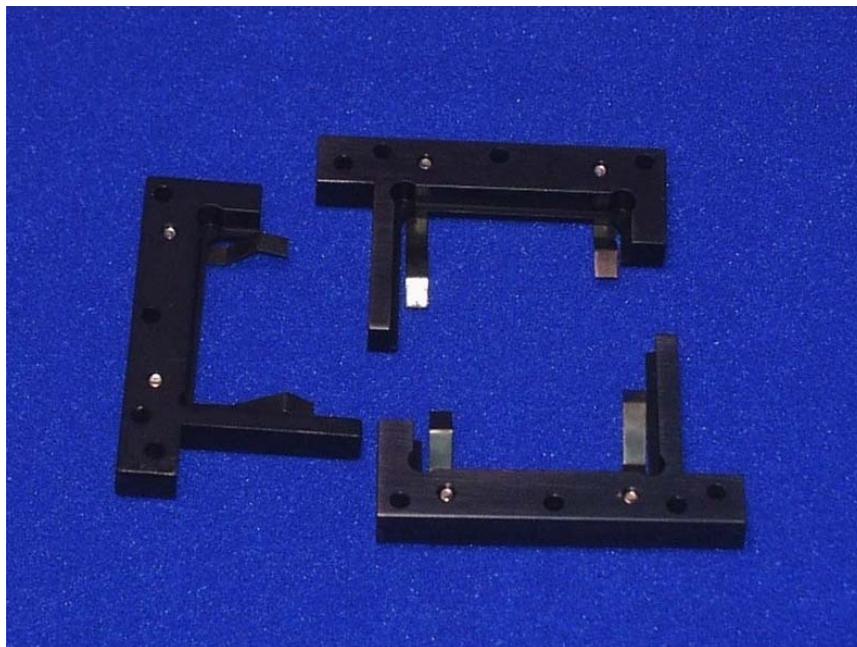
These manual filter holders may be used to mount 1" × 2" or 2"-square filters in the excitation or emission light paths. Several accessories contain filter holders with the accessory (the FL-1031, FL-1011, and FL-1012). The FL-1010 may be used to hold filters for anisotropy-decay scans and lifetime-resolved scans, in which only the unknown is being measured. For lifetime scans and time-resolved scans, in which a standard and unknown are being measured, consider geometry and samples when deciding where to place filters for the acquisition. For example, when using a two-position sample-changer with two samples with different excitation and emission characteristics, use the filter holders on the sample changer itself.

The FL-1010 contains three filter-holders, one for the excitation light path, and two for both emission light paths (S & T).

Model 1938 cut-on filter set
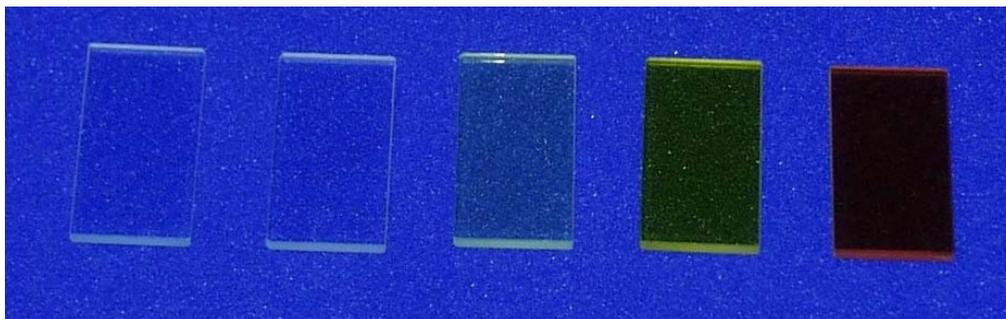
The Model 1938 cut-on filter set consists of five 1" × 2" filters with different cut-on wavelengths: 370, 399, 450, 500, and 550 nm. These filters are recommended for use with the FL-1011 and FL-1012 multi-position cell-holders in lifetime systems for lifetime acquisitions. When these holders are used, the filters are placed on the cell-holder at the sample positions to select the proper emission from the standard and unknown.

Jobin Yvon Inc. also offers a Model 1939 filter set with 2-inch square filters with the same cut-on wavelengths. These filters are too large to be used with the FL-1011 or FL-1012, but may be used with manual filter-holders or with the FL-1031 filter-holder.

Use these filters so that the emission detectors see only the emission from the sample for lifetime measurements. An example of this is POPOP versus glycogen (a scattering material). In this case, a 400-nm cut-on filter is placed on the POPOP sample position to select the 420-nm emission peak, while removing the Rayleigh band. No filter is placed at the glycogen position, because the Rayleigh band is the scatter signal of interest.

Cut-on filters are also used to eliminate second-order effects of the gratings. For example, if sample excitation is at 300 nm, a second-order peak will occur at 600 nm. If the emission spectrum extends from 400 nm to 650 nm, a sharp spike will occur at 600 nm. This peak is the second-order peak of the excitation spectrometer. To remove this unwanted peak in the emission spectrum, place a 370-nm filter in the emission slot.

Model FL-1011 automated four-position thermostatted cell holder
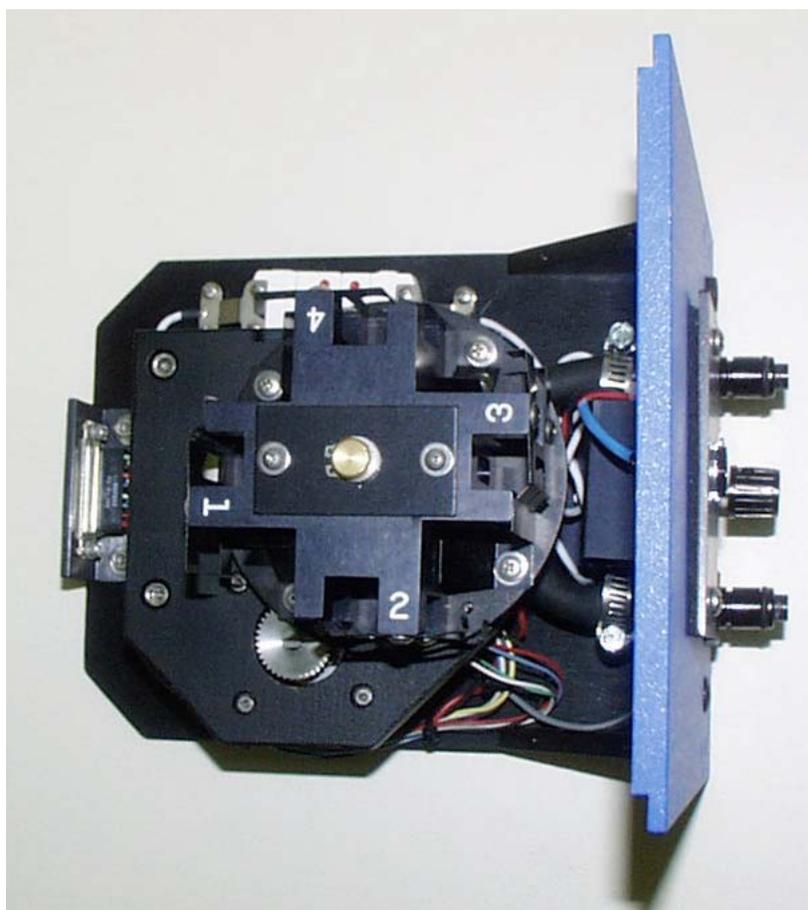
The Model FL-1011 Automated Four-Position Thermostatted Cell Holder provides four standard cuvette positions on an automated sample changer. The active cell is thermo-statted to keep a sample at a constant temperature from $-10°C$ to $+80°C$. The temperature of the sample is maintained by an external circulating temperature bath, such as the F-1000, purchased separately.

The holder also includes a magnetic stirrer, enabling mixing a turbid or a viscous sample while positioned in the light beam. Control for the stirrer is conveniently located on the front of this sample mount.

The FL-1011 (and the FL-1012 described later in this chapter) is a mainstay for lifetime systems, which inherently require a standard and an unknown for lifetime measure-ments. The lifetime and time-resolved scan modes are set up to take advantage of an automated cell changer, to reduce the amount of user interaction required during an ac-quisition. The FL-1011 and the FL-1012 include filter holders for each cell position, so that specific filters may be applied to certain samples during a measurement.

Globals™ Unlimited software

This software package, written by the Laboratory for Fluorescence Dynamics Group at the University of Illinois–Urbana, contains algorithms to fit advanced decay models. Use Globals™ to model energy transfer, non-discrete decays, quenching, and other complex decay mechanisms not supported with the modeling software in DataMax.

Model FL-1013 liquid nitrogen Dewar accessory

In some experiments, samples are frozen at liquid-nitrogen temperature (77 K) to observe fluorescence, delayed fluorescence, or phosphorescence. The liquid-nitrogen Dewar accessory is used to freeze and maintain the temperature of the sample for this type of measurement. For the Fluorolog®-3, the Dewar flask is placed on a pedestal within the sampling module of the spectrofluorometer. The sample is placed in a quartz cell and slowly immersed in the liquid-nitrogen-filled Dewar flask. A white Teflon® cone in the bottom of the Dewar flask keeps the quartz sample-tube centered in the Dewar flask, while a Teflon® cover on the top of the Dewar flask aids in centering the quartz sample tube, and holds excess liquid nitrogen consumed during the experiment.

A special sample cover replaces the standard sample lid, so that liquid nitrogen can be added conveniently to the Dewar flask as needed. The Dewar flask holds liquid nitrogen for at least 30 minutes with minimal outside condensation and bubbling. A dry-nitrogen purge port for the sample compartment is included to reduce water condensation.

Included in the FL-1013 Liquid Nitrogen Dewar Assembly are the quartz Dewar flask, sample tube, pedestal, and light-tight lid.

Lifetime acquisitions performed with this accessory generally use the Quick Scan feature in DataMax, which only requires one swap of sample (or sample mount) during the lifetime run, as opposed to swapping at every frequency point.

Model FL-1041 liquid nitrogen automated dual Dewar accessory

Designed specifically to automate lifetime measurements on samples at low temperatures, this accessory consists of two liquid-nitrogen-Dewar accessories mounted on a two-position sample-changer base. A special sample cover is also included to replace the standard sample lid, to allow the addition of liquid nitrogen to the Dewar flask as needed.

Dewar flasks may be selected automatically within the software to move to the active position in the sample compartment. During lifetime measurements, these Dewar flasks are swapped to a two-position automated cell-holder, to switch between the standard and the unknown. An appropriate standard is chosen that can be measured in a Dewar flask at liquid-nitrogen temperature (77 K) in order to minimize artifacts caused by this sample geometry. Using this accessory can help to minimize the errors and time required by using the Quick Scan feature and swapping sample mounts for this type of measurement.

Please refer to the description for the FL-1013 liquid nitrogen Dewar accessory below for more information on the two Dewar flasks included on the FL-1041.

Model FL-1042 phosphorimeter accessory

The phosphorimeter adds the ability to perform phosphorescence or delayed fluorescence measurements using a programmable pulsed-excitation source and selectable signal gating from the signal photomultiplier tube. This provides time-discrimination capability to remove competing luminescence emissions based on their lifetimes. The FL-1042 consists of a dedicated xenon flash-lamp housing, acquisition controller, and related electronics. The FL-1040 dual-lamp housing is required.

The duration of each exciting pulse from the phosphorimeter is relatively short (about 3 microseconds), making this accessory useful for measurements of phosphorescence lifetimes on the microsecond-to-second timescales.

Model FL-1044 dual auto polarizers
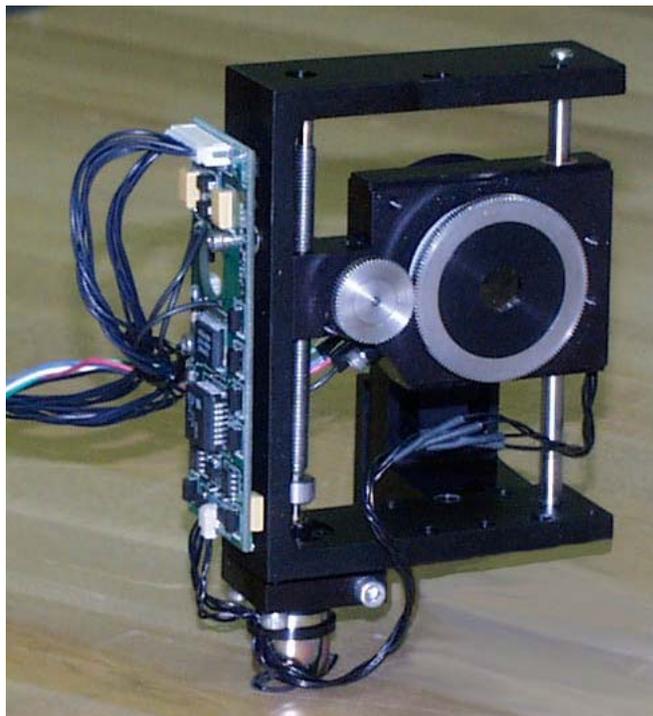
Dual autopolarizers on the Fluorolog®-Tau-3 add the capability for the measurement of anisotropy decays, otherwise known as dynamic depolarization. Measurements of time-resolved emission anisotropy (TREA) provide information about the rotational behavior of molecules. Molecular size and shape, as well as the viscosity of the medium in which the fluorophore is rotating, may be studied with this technique.

Each FL-1044 polarization kit includes two Glan-Thompson UV polarizers mounted in fully-automated computer-controlled mounts. Polarizers can be positioned horizontally, vertically, at the magic-angle settings of 35° and 55°, or at any other user-defined position. Glan-Thompson polarizers offer a much wider transmission range and greater stability than film polarizers.

The polarizers are completely controlled through the DataMax software, from insertion into the light path, to running calibration on the crystals, to conducting complex acquisitions of anisotropy decays.

Model FL-1045 automated third polarizer for T-format

A third polarizer may be added when the T-box sample-compartment module is configured with both emission paths active. This allows simultaneous measurement of vertical and horizontal emissions during anisotropy-decay measurements, reducing the amount of time required for these acquisitions, and removing artifacts that may be created by not taking VH and VV simultaneously.

F-3004 Sample Heater/Cooler Peltier Thermocouple Drive

For rapid control of the sample's temperature in the Tau-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.
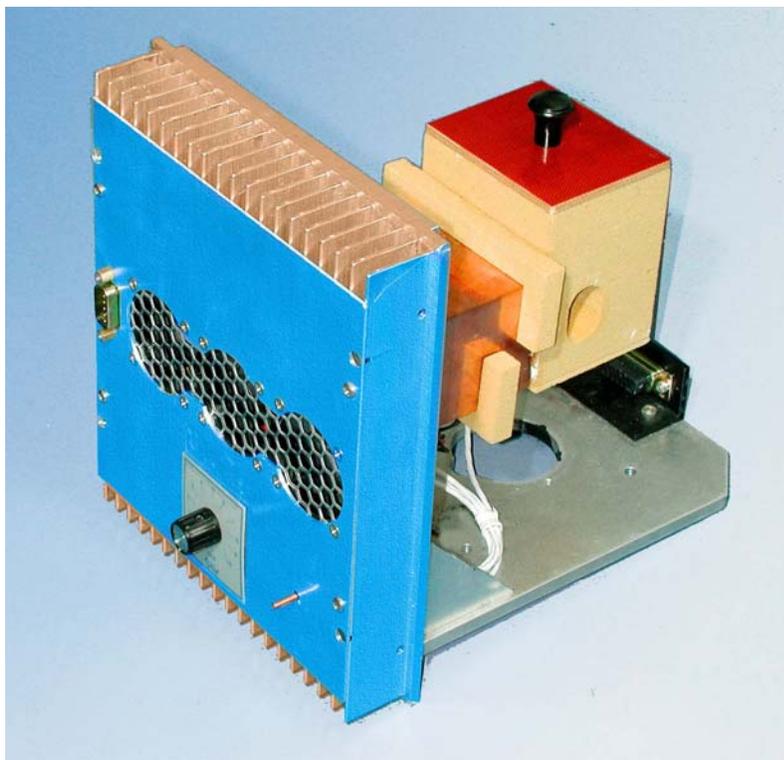
F-3004 Sample Heater/Cooler Peltier Thermocouple Drive.

Model 1933 solid sample holder

The Model 1933 solid sample holder is designed for samples such as thin films, pow-ders, pellets, microscope slides, and fibers. The holder consists of a base, upon which a bracket, spring clip, and sample block rest. The user may rotate the holder for optimal alignment of samples for measurements. Although this accessory can be rotated to 45° for measurements, it is recommended that this accessory be used in conjunction with the FL-1001 front-face viewing accessory to view the sample mounted on the Model 1933 at 22.5°.

Model FL-1002 T-channel optics for 2nd emission channel
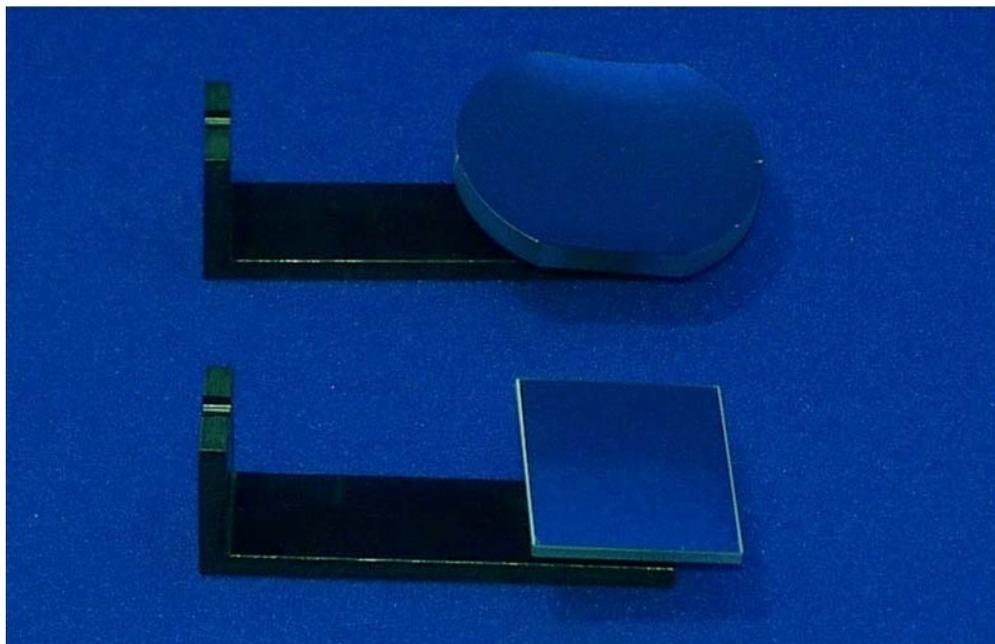
This accessory includes the necessary optics and electronics to complete the T-channel 2nd emission for use with a detector. The only items not included in this accessory are the emission detector, and the emission monochromator or spatial filter holder used in this emission path.

# Models F-1000 and F-1001 program-mable temperature bath

The F-1000/1 is offered as an accessory for maintaining sample temperature as well as for automating the measurement of temperature curves and other heating- or cooling-related assays. The bath is controlled by the host computer via a single serial (COM port) link. The bath may be used with water or a solution of ethylene glycol to control sample temperature from about –15 to +80°C. The F-1000 requires 110 V/60 Hz and the F-1001 uses 220 V/60 Hz line voltage.

A sensor mounted in the thermostatted cell-holder or sample-changer may be used to monitor the temperature at the sample. Monitoring the temperature in the water bath is also possible. Ask Jobin Yvon Inc.'s Fluorescence Applications Group about custom sensors for monitoring temperature within the sample.

For lifetime systems, the automated temperature bath may be used to generate temperature curves for global analysis of lifetime or anisotropy-decay data. The bath is fully controlled by DataMax software.

Model FL-1012 automated two-position thermostatted cell holder
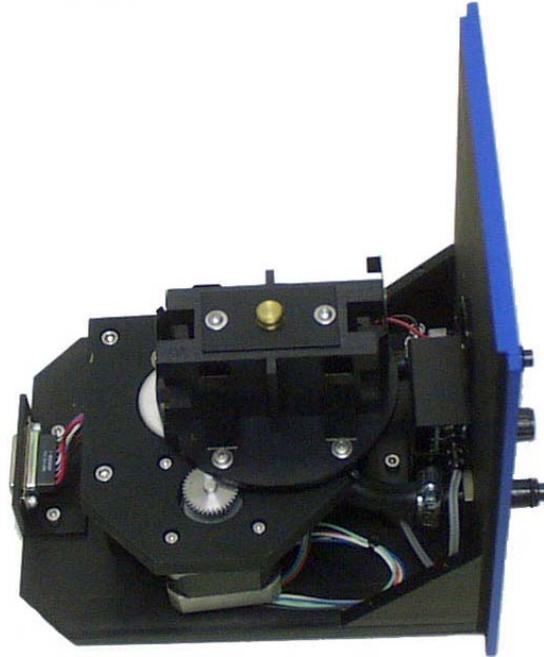
This accessory is identical to the FL-1011 automated four-position thermostatted cell holder except it has two cell positions 180° apart. Refer to the description of the FL-1011 for more information on the use of this accessory with lifetime systems.

10: Troubleshooting

Because the lifetime system has many user settings and adjustable power supplies, many apparent system failures are simply problems with the experimental setup, sample geometry, or choice of sample. If the symptoms indicate that a problem may be a system failure, contact the Fluorescence Service Department, or your local distributor.

Refer to the *Fluorolog®-3 Operation Manual* and the *DataMax Operation Manual* for help with troubleshooting problems in steady-state operation. This chapter is intended to isolate and solve problems regarding lifetime (dynamic) operation only.

Basic troubleshooting steps

When an apparent problem is first observed, follow the basic steps and suggestions outlined below to troubleshoot the initial problem. In many cases, shutting down the system, carefully restarting, and redoing an experiment helps to remove an unseen or outside issue that was causing the apparent problem.

If the suggestions that follow do not help to remove the problem, note the steps that led up to the problem, and proceed to the next section.

1   Reboot.

If this is the first time the problem has occurred, try restarting the system. Turn off all components and accessories. After a cool-down period, turn the system on and see if the failure persists. Many apparent software and hardware problems are actually conflicts with other software or peripheral devices currently active on the system. For information on the proper start-up and shutdown sequence, please refer to *Chapter 3: Getting Started*.

2   Check the connections.

With the power to all components and accessories turned off, make sure all accessories and cables are attached and configured properly. Verify that line cords provide the proper input AC voltage, and that all appropriate accessories and power supplies are powered ON during use of the system.

3   Verify system performance.

Following the instructions in *Chapter 3: Getting Started*, verify that the system is properly calibrated in steady-state mode. Check that the system operates properly in lifetime mode by running a scan of a standard fluorophore versus a scatterer. Print out each spectrum, and note the peak intensities and wavelength positions for the steady-state scans, and the lifetime and reduced χ^2 for the lifetime measurement.

4   Duplicate the error.

Try to duplicate the problem, and write down the steps required to do so. Our service engineers will attempt the same with a test system. Depending on the nature of the problem, a service visit may not be required.

Problems and solutions

Each problem is explained in a later section of the chapter, with suggestions for how to solve it and how to avoid it in the future.

Communications problems	Loose connections on cables
	Lifetime rack turned off
	Improper power-up sequence
	Windows™ conflicts
	Improper software layout loaded
No signal or very low signal	Loose connections on cables
	Lifetime rack or one of its components turned off
	HV Bias or RF amplifier switched off or improperly adjusted
	No HV supplied to emission detector
	Improper start-up sequence
	Improper software layout selected
	Pockels cell is not fully in light path or not aligned properly.
	Lamp is not on
	Improper experiment setup (slits closed, shutter closed, wrong monochromator position, etc.)
	Improper frequency setting
	Poor choice of filters for sample being measured
Detector is really saturated, but displays very low signal	Terminator missing on RF splitter when using only two lifetime detectors
Saturated signal	High voltage applied to detector is too high
	Slits or iris open too wide
	Loose cable connections between detector and splitter, or detector and lifetime drawer
No modulation	Lifetime rack not turned on
	RF amplifier or HV bias not turned on or improperly adjusted
	Pockels cell out of position or in need of adjustment
	Improper frequency setting
	No signal or low signal
Modulation >1 at low frequencies	Sample errors
	Pockels cell out of alignment
Noisy or erratic phase or modulation data	Improper experiment setup (short integration time, low averages, low number of frequencies, wrong wavelengths, improper slits, etc.)
	Poor choice of standard sample
	Low signals or modulation
Poor lifetime results	Poor choice of standard sample
	Improper experiment setup
	Low signals or modulation
	Noisy or erratic phase or modulation data
	Poor measurement geometry or choice of filters
	Solvent effects
	Improper modeling of data
Poor anisotropy-decay results	Improper experiment setup
	Poor measurement geometry or choice of filters
	Polarizers out of alignment
	Noisy or erratic phase or modulation data
	Solvent effects

Improper modeling of data

Communications problems

Problems with communication between the host computer and the SpectrAcq, or the SpectrAcq and the lifetime-system components, can be common, especially when a new host computer is set up for the system. Any changes in the configuration of the host computer, or the addition of new components such as fax modems, network cards, or new operating systems, can cause communications problems between the host computer and the SpectrAcq controller.

Communications problems can be broken down into two types of difficulties:
•   Failure to establish communication
•   Intermittent communication failures during operation

1   Failure to establish communications is, by far, the most common problem.

Determine if anything has changed the host computer configuration. If so, this may be the cause of the communications problem.

2   Restart the entire system, including the SpectrAcq and the host computer.

While the system is shut down, verify that all connections are secure, especially communcations cables and power cords. Restart the system in the proper sequence (see Chapter 3: *Getting Started* for the procedure), restart the software, and often communication will be restored. Usually the cause of the problem is a loose connection, a power switch in the OFF position, or a Windows™ I/O conflict that was corrected during the reboot.

Note: While booting up the system, watch the floppy-drive-access light on the SpectrAcq. The SpectrAcq should access the disk for 30 s to 1 min, loading drivers. If the drive light turns on and off quickly (< 10 s) with no further access, or the light stays on continuously, then there may be a problem with the boot disk itself.)

3   Try a different communications port on the host computer for communication with the SpectrAcq.

To change the desired COM port, change the file SAQ.INI in the ISA_INI directory of DataMax™, to point to the appropriate serial port. Move the communications cable to the appropriate port on the host PC itself.

Intermittent communications problems are usually caused by one of two issues:

4   A loose connection between the instrument, the SpectrAcq and the host computer.

5   A Windows™ I/O conflict that has arisen during the current session.

Both of these issues can be solved by switching off the system, checking cable connections, and rebooting the entire system.

No signal or very low signal

We often hear from our customers that no signal or very low signal is observed on the instrument. Our first suggestion to solve this problem is to go back to a standard sample, or preferably a scattering sample, to isolate the problem as peculiar to the instrument, the particular sample, or experiment setup.

Instrumental problems
Instrumental problems usually are caused by not starting one of the lifetime components such as the lamp, lifetime rack, RF amplifiers, or HV bias. In other cases, faulty instrument setup usually is to blame.

1   Carefully review the instrument settings:

high-voltage bias to detectors, excitation and emission wavelengths, slit settings, selected modulation frequency, shutter positions, etc.

2   Check that the system is set to the desired mode:

dynamic or steady-state.

3   Verify the optical set-up:

Check that the Pockels cell is fully in or out of the light path, the laser mirror or collimating lens is in or out of the light path, and the iris is in the proper position.

4   Check that the measurement setup is correct:

Verify that a proper sample, filters, and software layout have been selected for your desired measurement.

5   Switch off the complete system.

Carefully check cable connections, and reboot the system. A loose connection or a software conflict can mimic a hardware failure.

Other tips for troubleshooting signal problems:

1   Make sure that the experiment settings match the **Real Time Display** settings.

This may sound silly, but it does happen. Verify that the settings optimized in **Real Time Display** before the experiment are properly transferred to the experiment settings. Use the Transfer button in **Real Time Display** with **Run Experiment** open.

2 A saturated PMT may display low or zero signal levels.

Reduce the HV bias to the detector to see if there is really too much signal.

3 A concentrated sample may be absorbing the excitation before the focus.

This is the inner filter effect. Try using a dilute scattering sample to verify instrument performance, then check that the unknown is not optically dense, precluding a measurement in right-angle mode.

4 A 50-Ω terminator may be missing on the RF splitter.

This concerns users with one lifetime signal detector and one reference detector. If switching between one and two lifetime emission detectors (S and T), make sure that there are always three loads on the RF splitter. A 50-Ω terminator is placed on the third output when only one lifetime emission detector is used.

5 The RF settings have been changed.

The RF amplifiers and the HV bias are set at the factory, and optimized during installation for best system performance (with the source we provide). Adjustment of the output settings on these devices can have a detrimental effect on signal. Contact Fluorescence Service if there is a suspicion that the RF settings have been changed.

Saturated (or very high) signal

Reduce the amount of light
Adjustments of the slit widths, apertures around the modulator box, high voltage to detectors, and even sample concentration or excitation/emission wavelengths can solve this problem.

Slits and high-voltage levels
These are the easiest adjustments to make.

1 Narrow the excitation entrance slit and emission slits.

Do this as desired to reduce the signal levels.

2 Reduce high-voltage bias levels.

3 Adjust slits and iris around the modulator box.

These are saved for last, for these usually are very narrow to begin with for lifetime measurements.

The sample

1 Use a neutral-density filter or screen to reduce the emission signal from a sample.

Be sure the filter doesn't fluoresce.

2 Lower the concentration of the sample.

3 Change the excitation or emission wavelength of the sample.

This moves down the absorption or emission edge, to change the effective output level while not changing the lifetime components.

4 Check hardware.

Hardware problems can mimic saturated signal. Switch off the system, check all RF and signal cable connections, and then reboot the system with the appropriate software layout.

No modulation

A lack of modulation indicates that the AC component of the signal is missing. The first question is whether the problem resides in the instrument or the sample. We suggest using a scattering sample or a standard fluorophore to check the instrument, before troubleshooting with the unknown. Depth of modulation is created by the lifetime rack and its RF components. All components on the lifetime system must be on for the system to work properly.

1 Check that the RF amplifiers and the external DC high-voltage bias supply are ON.

2 The system must be set appropriately in lifetime (dynamic) mode for good modulation.

Check that the Pockels cell is fully in the light path, the iris is closed down, and slits are set properly for dynamic mode (especially the excitation entrance and intermediate slits around the modulator box).

3 Be sure that an appropriate frequency has been selected in the software.

Higher frequencies result in lower modulation values, and longer lifetimes result in lower modulation values at a discrete frequency. As an example, a sample of fluorescein will show very little modulation at 250 MHz, because it has a lifetime of about 4.1 ns.

4 Lack of AC or DC signals may cause poor modulation values.

Please see the troubleshooting sections above for further assistance.

Modulation > 1 during acquisition

Modulation here refers to the demodulation factor measured during the lifetime acqui-sition, not the modulation value observed in **Real Time Display**. The demodulation fac-tor, labeled "modulation" in the experiment display, indicates the measured modulation value for the unknown divided by the modulation measured on the standard, and then is corrected to indicate demodulation as compared with a 0 ps lifetime. Therefore, a de-modulation factor during the experiment greater than 1 should be impossible (because it corresponds to a lifetime of < 0 ps).

There are two causes for this type of error.

Standard and unknown switched

If the phase angle > 90° throughout the experiment, then the standard and unknown po-sitions in the experiment are probably mixed up in the experiment definition.

> **Note:** *An improper standard (e.g., wrong lifetime) or a poorly prepared unknown can cause this as well.*

Misaligned Pockels cell

Finally, if this problem is still observed even after running a standard fluorophore ver-sus scatter (see Chapter 3: *Getting Started*), then the culprit is probably misalignment of the Pockels cell. See Chapter 11: *Maintenance* for suggestions on optimizing the Pockels-cell alignment.

Noisy or erratic phase or modulation data

High error-bars in the phase or modulation data are usually caused by poor experiment setup, poor measurement geometry, or a weakly fluorescing sample.

Poor experiment setup

1 Use an appropriate integration time for the measurement.

(usually between 6–24 seconds, depending on signal and complexity of the sample).

2 Check that an appropriate number of frequency points, averages, and standard-deviation tolerance has been set.

Set the experiment for 6 or more frequency points, depending on the complexity of the unknown. Averages are usually set between 3 and 24, with a standard deviation around 0.5–1%.

3 Check other settings in the experiment definition.

They must match the settings found during balancing in **Real Time Display**.

Low signals or modulation levels
These are often the primary cause of erratic phase or modulation values. See the sections above for more troubleshooting information.

Choice of standard
The precision and accuracy of a measurement will only be as good as the standard. Erratic phase or modulation (and corresponding lifetime results) is directly affected by the quality and appropriate choice of a standard. Be sure that the standard is the correct lifetime, at the correction excitation and emission wavelengths, and useful over the desired frequency range. Refer to the lifetime chapter for help in selecting appropriate standard.

Poor lifetime results

A poor lifetime result is:
- Incorrect lifetime value(s), wrong number of lifetimes, or inappropriate fractional contributions
- High χ^2 value
- Poor residuals

Each of these factors will be considered with respect to their effects on the measurement parameters:
- The samples and measurement geometry
- Standard
- Experiment setup
- Modeling the results

Incorrect lifetime value

Lifetime results are only as good as the samples and the measurement geometry selected for the experiment.

1   Outside knowledge of the unknown is essential.

The excitation and emission characteristics of the sample can be measured on the Fluorolog®-Tau-3. The concentration should be set to < 0.1 A, if possible, for right-angle measurements through a 1-cm^2 cuvette. Know the sample's solubility characteristics, pH, photostability, and other appropriate information before a measurement, to choose the best geometry and the standard. Consider the lifetime stability of the sample in different environments, such as varying temperature, pressure, and pH.

2   Consider the solvent used for the unknown.

The sample must dissolve easily into (ideally) a homogeneous solution, and the solvent should not contribute any artifacts (certainly no fluorescence emission) to a measurement.

3   The measurement geometry should be appropriate.

Chapter 4: *Lifetime Acquisitions* considers different types of samples and geometries. A poor measurement geometry will lead to sub-optimal lifetime results. For questions or concerns in selecting a particular setup for a measurement, please contact our Applications Group.

4   A poor standard may cause poor lifetime results.

For lifetime measurements, the standard is the basis for correcting instrumental artifacts. Therefore, know the standard's exact lifetime and its fluorescence characteristics. The standard should be a well-characterized fluorophore with a

single exponential decay or a scattering sample (0 ns lifetime). The standard should be used only at appropriate excitation and emission wavelengths, across its useful frequency range, and in an appropriate measurement geometry. For example, the Applications Group frequently uses Ludox® as a standard. As a scatterer, Ludox® has a 0.0-ns lifetime, and is appropriate for right-angle-cuvette-based measurements in de-ionized water. Ludox®, however, is known to fluoresce in the deep UV, therefore, for measurements with excitation below 350 nm, we choose glycogen as a scattering standard, based on its fluorescence characteristics.

5   The experimental setup can cause poor lifetime results.

Usually, a poor setup reveals itself in poor signal levels, poor modulation values, or erratic phase and modulation data. Review the suggestions from the sections above if you observe these problems, before proceeding further in this section.

Check the instrumental setup, to make sure that the components are set in dynamic mode (slits, iris, Pockels cell, laser mirror, supplies, etc.). Review the experiment definition to verify that the entire scan definition is appropriate.

> *Note: Make sure the samples are in the sample-changer positions indicated in the scan definition, otherwise unexpected results may occur. Be sure that the correct standard lifetime is defined in the scan.*

High χ^2 value

Modeling the data is the last step in a lifetime measurement. The results (and the χ^2) are only as good as the model defined by the user and the method used for the model. The modeling program supplied with DataMax performs non-linear least-squares analysis on frequency-domain lifetime data with the option of using fixed or variable errors. The user may choose up to three discrete lifetime components and their associated fractional contributions for the decay scheme.

When modeling data, start from the least-complex model and move to more-complex models only when a significant improvement in the reduced χ^2 value is observed. This means that, unless outside information on a sample is known, a less-complicated decay scheme is more plausible than a more-complicated one, given the same reduced χ^2 value.

During modeling, non-linear least squares analysis moves across the error surface looking for a statistical local minimum in the reduced χ^2 value. In some cases, the local minimum may not be the best fit across the entire surface. Therefore, try the model with different initial conditions (i.e., lifetime and SAS values) to certify that the best fit

has been found. Be careful of the values that are fixed for the model. These components will not be adjusted during actual modeling of the data. Therefore, with fixed errors, enter realistic values. Typically, we use ~ 0.5° phase-shift and 0.5% modulation for fixed errors.

Complex samples may require global analysis to measure properly their decay characteristics. Global modeling consists of overdetermining the lifetimes while varying outside environmental conditions such as temperature, pressure, and pH on repeated lifetime acquisitions. Modeling these acquisitions together (i.e., globally) with their lifetime values linked, allows more accurate determination of decay characteristics.

High residuals

Scatter in the emission also can cause incorrect lifetime results, by weighing down the high-frequency phase and demodulation values with essentially a 0.0-ns lifetime. This can be removed during modeling. To add a scatter component to the measurement, add a separate decay with a 1-ps lifetime (fixed) and ~ 0.5% fractional contribution (variable). Generally, with a good experimental setup and measurement geometry, the amount of scatter seen from the unknown can be kept to a minimum.

Lastly, if the sample's decay mechanism is more complex than a single or multi-exponential decay, then use a more comprehensive algorithm to model it properly. A software program from the LFDG called Globals provides for other decay mechanisms such as quenching, energy transfer, and other processes not properly modeled in DataMax.

Poor anisotropy-decay results

In essence, anisotropy decays follow a similar procedure to lifetime measurements, with only two significant differences:
- no standard, and
- autopolarizers are used.

Therefore, the discussions on how to set up a lifetime scan, and how to troubleshoot poor lifetime results, may help to correct poor anisotropy-decay results.

The indications of poor anisotropy-decay results are:
- High reduced-χ^2 value
- Incorrect rotational correlation times, fractional contributions, and limiting anisotropy values
- Poor residuals

The components of an anisotropy-decay that can cause poor results are:
- The sample itself and the chosen measurement geometry
- Polarizer alignment
- Experiment setup
- Modeling the results

As with lifetime acquisitions, knowledge of the unknown and the choice of an appropriate measurement geometry are essential for good results. In addition to the sample characteristics that must be known for a lifetime acquisition, the anisotropy-decay acquisition requires that the mean decay time of the unknown be known accurately. This usually means that the sample's lifetime has been measured on the Fluorolog®-Tau-3 before measuring the anisotropy decay. In addition, the steady-state G factor for the instrument at the wavelengths used for the acquisition must be measured *before* the decay acquisition, even if filters are employed. Errors in the G factor can ruin the measurement of RMA values during the acquisition.

Autopolarizers
Naturally, the measurement geometry for the anisotropy-decay acquisition must include autopolarizers. There are a few different options for the number of polarizers in the light path, involving a trade-off between accuracy and the amount of signal available for the measurement. At minimum, an autopolarizer must be located in the emission light path that will be changed from H to V throughout an L-format measurement. An excitation polarizer is not essential because the output from the Pockels cell is vertically polarized. Other options include two polarizers or three polarizers in the classic T-format measurement geometry. Improperly aligned polarizers affect the results. See the autopolarizer manual for instructions on verifying polarizer alignment, and using the automated alignment procedure in **Visual Instrument Setup**, if necessary.

Experimental setup
A proper experimental setup is just as critical for the anisotropy decay as it is for a lifetime scan. The steady-state G factor is an additional parameter to be specified properly for good results with anisotropy-decay measurements. The G factor should be calcu-

lated as HV/HH for DataMax; do not enter the inverse of the *G* factor! The mean fluorescence lifetime must be entered in the experiment setup. An inaccurate mean fluorescence lifetime also can lead to poor data in both phase and RMA values. Refer to the poor-lifetime-results troubleshooting section for more information on properly setting up the other experiment parameters.

> ***Note:*** *To verify that the acquisition is proceeding well, compare the steady-state anisotropy calculated during the experiment with a value measured in steady-state mode. The software uses the steady-state* G *factor specified in the experiment definition and the measured VH and VV DC signals, to compute steady-state anisotropy at each frequency point.*

Modeling the results

When modeling the results of an anisotropy-decay scan, the mean lifetime decay should be entered as a fixed value, because it should be known before the experiment. Always start from a simpler model with a single rotational correlation time, and work towards a more-complex model based on the resulting reduced-χ^2 values. Another parameter that can be used to indicate the validity of the model and fit for an anisotropy-decay is the limiting anisotropy (r_0) value. This value can be measured in steady-state mode for the sample by cooling the sample to a temperature that effectively restricts rotational diffusion. If the value for r_0 calculated while fitting the data agrees with the value measured previously, it should serve as an endorsement of the fit, along with an acceptable reduced-χ^2 value.

Further assistance...

Read all software and accessory manuals before contacting the Jobin Yvon® Fluorescence Service Department. Often the manuals show the problem's cause and a method of solution. Before contacting the service department, however, complete the following steps.

1   Find the serial number for the core instrument, on the back of the sample compartment. Collect the serial numbers for the host computer and all automated accessories (e.g., temperature bath).

2   Note the system configuration with all accessories.

3   Determine the brand name and type of host computer, along with processor speed, free RAM, and hard-disk space, and whether the computer was purchased from Jobin Yvon®.

4   Write down the experimental configuration and what samples are under study. Be prepared to fully describe the samples and their spectral characteristics, as well as the specific instrument setup and sample geometry.

5   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.

6   If an error dialog box appears in DataMax, write down the exact error displayed. Note whether the error was in GRAMS or Windows™.

7   In DataMax, in the **Instrument Control Center** toolbar, choose Help. Under Help, choose About Instrument Control Center. This opens the **About Instrument Control Center** window. The version of the software is listed here.
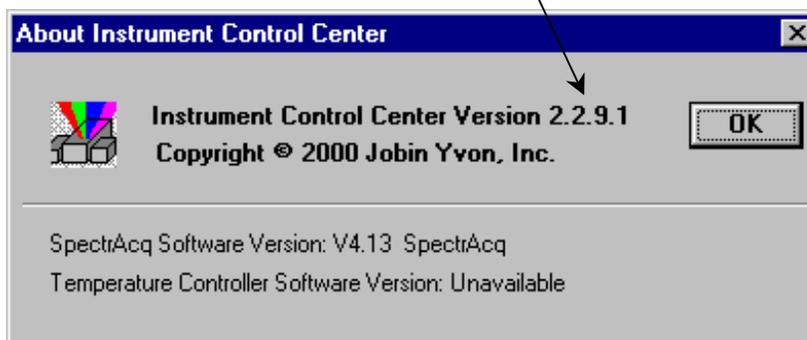
8   In Run Experiment toolbar, open the **About DataMax** window. Make a note of the software's and instrument's serial numbers, and instrument configuration, including all accessories.
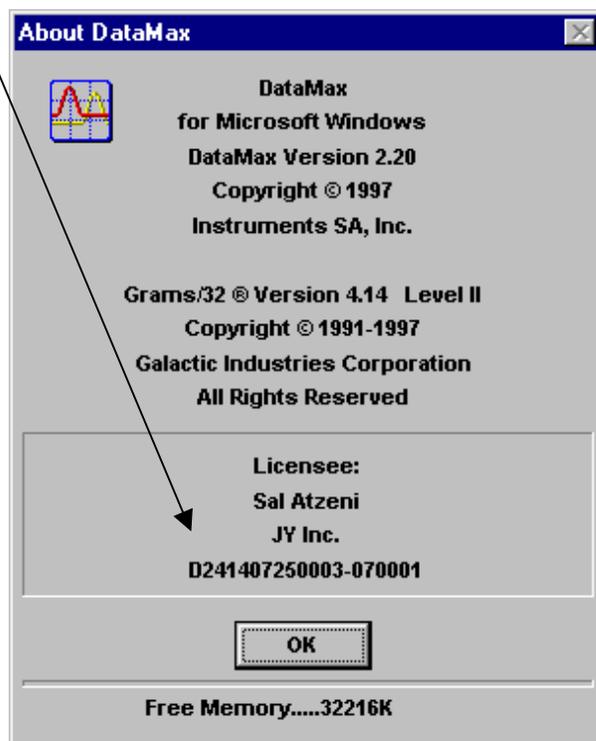
If the problem persists or is unlisted, call the Fluorescence Service Department at (732) 494-8660 × 160, fax us at (732) 549-5157, or e-mail us at

fluorescence_service@jyhoriba.com.

11: Maintenance

Proper maintenance of the Fluorolog®-Tau-3 system will ensure good performance for years to come. Maintenance of the fluorescence lifetime system consists of two basic components:

- Maintenance of the steady-state system
- Maintenance of the lifetime portion of the system.

Basic maintenance for the steady-state system (e.g., lamp replacement) is covered in the *Fluorolog®-3 Operation Manual*. For dynamic operation, there are a few additional recommended maintenance procedures to follow. These include:

- Realignment of the Pockels cell and modulator box
- Setting optimal levels for the HV bias
- Setting up an optional external laser source for use with the lifetime system.

Pockels-cell alignment

Introduction

Optimizing the Pockels-cell alignment is required to maximize the depth of modulation, giving the highest precision to lifetime measurements. This procedure involves setting the pitch and yaw for the cell in the modulator box, so that the cell is centered precisely on the optical axis. Realignment of the cell may be required periodically, if a drop in the modulation depth is noted, or if the overall performance of the lifetime system appears to drop off.

If poor alignment is suspected, then, before realigning the Pockels-cell modulator, perform a test of system performance in both steady-state and dynamic modes of operation. The water Raman scan and xenon-lamp scan will verify system calibration and signal-to-noise in steady state mode. In dynamic mode, a lifetime scan of POPOP versus Ludox® serves as a good indicator of system performance. Instructions for performing these scans are found in Chapter 3: *Getting Started*. Typical signs from this scan that indicate a need to optimize the Pockels-cell alignment are:
- Demodulation values > 1.0 during a scan at the lower-frequency points on the scan
- A systematic split between the phase and modulation data
- A systematic error in the data in the higher-frequency portion of the scan.

Procedure

Note: *A visual inspection of the isogyre pattern is described in Chapter 17:* Visual Inspection of the Isogyre Pattern*. Do not inspect the isogyre pattern without permission of the Fluorescence Applications Department.*

1   Optimize Pockels-cell alignment on a scatterer.

Note: *Proper alignment of the Pockels cell is essential for good results.*

Optimize the alignment of the modulator for the maximum depth of modulation using a scattering standard of glycogen or Ludox®.

Make very gradual adjustments on the Pockels cell. Adjust to the center position of the peak in modulation.

a   System setup

Monochromators:

Excitation	370 nm
Emission	370 nm

Slits:

Excitation entrance and intermediate (if applicable):	6 mm
Excitation exit:	0.3 mm
Iris setting:	2/3 closed
Manual slit at end of modulator:	0.5 mm
Emission slits (if applicable):	6 mm

High voltage:

Reference detector:	400 V
Emission detector (monochromator side):	900 V
Emission detector (filter side):	750 V
Bias voltage to Pockels cell:	See System Performance Report.

Sample:

Dilute Ludox® in de-ionized water (same sample as for the lifetime scan of POPOP versus Ludox®).

b   Remove the knobs and lid of the modulator box. The Pockels cell should be shifted to the right (lifetime position) as shown here:
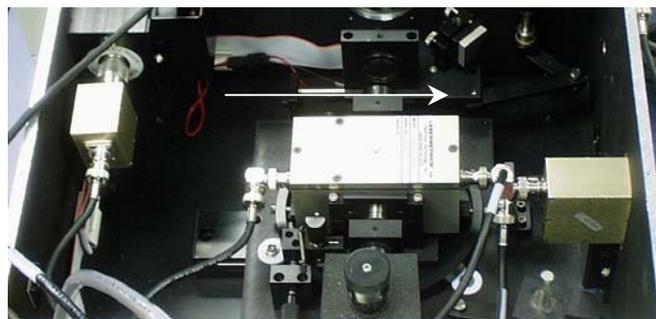
C   Adjustment
Gently fine-tune both adjustments for the Pockels-cell mount, while observing the signal channels in **Real Time Display**, for the maximum signal on the AC channel and modulation.

- The vertical knob, typically a coarser adjustment, controls the yaw for the Pockels cell.

- The horizontal knob, generally a finer adjustment, controls the pitch of the modulator.

Note: If there are 2 detection channels (S and T) re-optimize for that channel after switching to that channel.
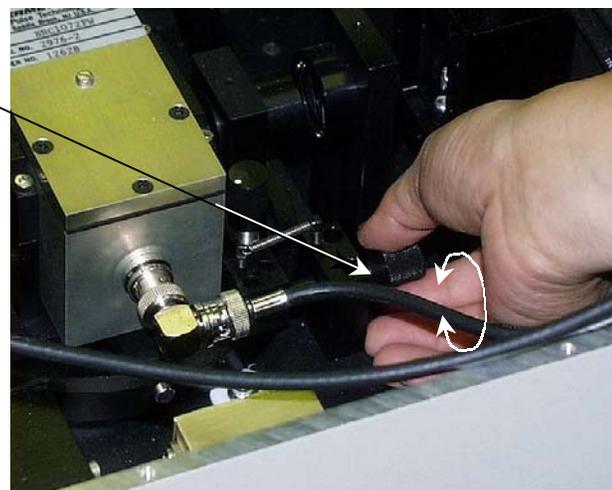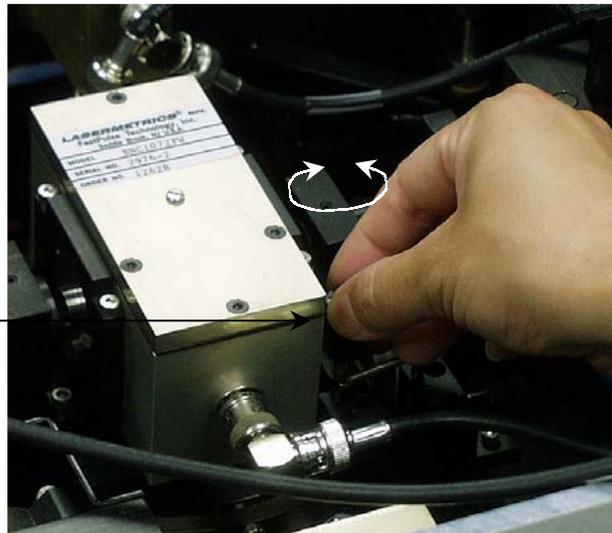
When complete, the modulation should be >1.0 for a xenon-lamp source, and >>2.0 for an external laser source.

2   Set the high-voltage DC bias to the Pockels cell.

Optimize the high-voltage bias in order to provide the system with the highest depth of modulation available or to help increase the signal levels observed through the system. Only if:

- An external laser is turned into the light path, or
- The Pockels cell needs to be adjusted, or
- The signal is extremely low,

should this bias level be optimized.

Note: Normally, the high-voltage DC bias on the external Bertan™ power supply should be kept at the factory-set value. Keep the dial for adjusting this setting in the locked position.

Use a scattering sample of glycogen or Ludox® in this procedure. No other tools or targets are required.

a   Enter **Real Time Display**, and optimize the system settings for the measurement of the scattering sample at 370 nm. (See Chapter 3: *Getting Started* for more information on settings).

When properly set up, 1–4 V DC and AC should be seen on the R and S (or T) channels.

b   Unlock the dial on the Bertan™ power supply, and gradually adjust the setting until the greatest modulation value is achieved.

c   If a detector's signal falls to < 1 V, increase the respective PMT's high-voltage bias in **Real Time Display**.

In cases where low fluorescence-yields from an unknown mean that the DC signal is more important than modulation depth, the high-voltage DC bias to the

Warning: Do not increase the HV DC bias level to the Pockels cell above 350 V. Higher voltages can damage the Pockels-cell electronics.

Pockels cell may be increased to improve the AC and DC levels, although this lowers the modulation.

3   Set up an external laser source for lifetime measurements

Warning: Strictly adhere to all required safety precautions related to working with laser sources while using a laser with the lifetime system.

A portion of the laser beam directed through the beamsplitter to the photodiode can be reflected off the outer coating of the photodiode and out of the top of the sample chamber. Avoid exposure to this reflection (and any other portions) of the laser beam when working with samples to be measured.

Introduction

An external continuous-wave laser source can be helpful for measuring life-times and anisotropy-decays for weakly fluorescing samples. Laser choice depends on the particular excitation wavelength(s) required. The modulator box includes a turning mirror to bring the external laser beam into the system and direct it through the Pockels cell on the same optical axis used for the xenon source.

In this example, a multi-wavelength argon laser (457, 488, 514 nm) is set up.

The beam is set up to enter the modulator box with horizontal polarization (90°). Most lasers emit vertically po-larized light. Therefore, the polarization of the laser must be rotated, for exam-ple, with two 45°-mirrors, rotated
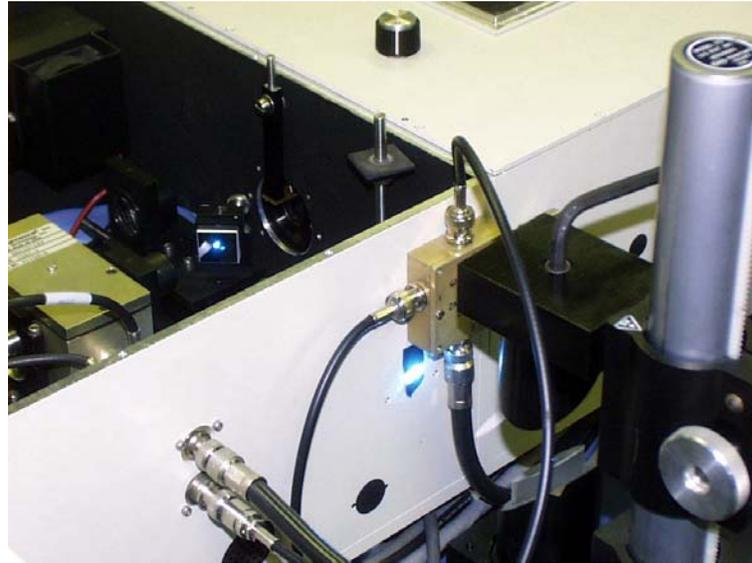
90° apart, just outside the laser port on the modulator port. This procedure assumes the beam is polarized at 90°, and aligns this horizontally polarized beam entering the laser port on the modulator box.

Lower hole is for alignment of Tau-3

Equipment

Two laser targets, as shown at right:
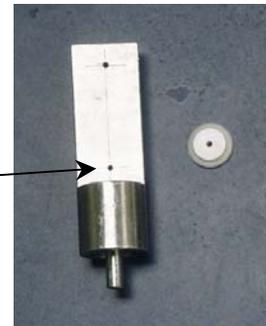
Procedure

a Insert the circular pinhole target into the laser entry port just below the RF-splitter module.

b Insert the vertical target into the alignment hole on the laser mirror mount.

c Align the laser through these two targets.

d Remove the vertical target and replace the laser turning mirror.

e Adjust the laser turning mirror carefully, directing the beam
   - Through the center of the Pockels cell,

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- Out of the modulator box,
- Through the center of the manual slit, and
- Into the sample compartment.

f   Double-check the alignment: the beam should be centered on a Ludox$^{®}$ sample.

g   Go into **Real Time Display** in the DataMax software.

h   Adjust the settings in **Real Time Display** to yield 1–4 V AC on both the R and S channels. Use a narrow manual slit setting ($\leq$ 0.5 mm).

i   Monitoring the AC signal and modulation on the R & S channels, adjust the Pockels cell for the highest AC and modulation possible.

If the AC levels increase beyond 6 V AC, then adjust the bias to that particular detector, before continuing with the alignment of the Pockels cell.

j   When properly aligned, high modulation values (> 2.0) should be observed, because of a highly collimated laser beam, with small spot size, propagating directly through the center of the Pockels cell.

k   Set the DC high-voltage bias to the Pockels cell for the highest modulation on S&R. This nearly always involves reducing the DC HV bias.

Note: The Pockels cell has different optimal positions between the laser and the xenon lamp because of beam-collimation differences between these sources. Therefore, when switching between measurements with the laser and xenon lamp, always realign the Pockels cell on a scattering sample, and re-optimize the DC HV bias.

Experimental settings with an external laser as the source are very similar to those with the xenon lamp, with the following exceptions:
- Keep the excitation slits and iris closed when using a laser entering the modulator box.
- Reduce high-voltage biases to the PMTs to ~ 1 V DC.

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12: Technical specifications

Each Fluorolog®-3 system consists of an excitation source, an excitation spectrometer, a sampling module with reference detector, and at least one emission spectrometer and emission detector. Each system is controlled by an IBM PC-compatible computer and may include a printer.

Important

The Fluorolog®-3 spectrofluorometer system is designed to comply with the requirements of the Low Voltage Directive 73/23/EEC and the EMC Directive 89/336/EEC and, as of 1 January 1997, carries the CE marking accordingly. The system was tested using standard (JY-authorized) components, cables, etc.

Spectrofluorometer system

The Fluorolog®-Tau-3 spectrofluorometer consists of the steady-state Fluorolog®-3 spectrofluorometer plus additional components for lifetime measurements. The entire system is controlled by DataMax software for Windows™.

Excitation Source   450-W xenon short arc, mounted vertically in an air-cooled housing. Light collection and focusing by off-axis mirror for maximum efficiency at all wavelengths.

Spectrometers   Single-grating excitation and emission spectrometers (standard). Spectrometers are Czerny-Turner design with kinematic gratings and all-reflective optics.

The following specifications are based on 1200-groove/mm gratings:

Resolution	0.2 nm
Accuracy	0.5 nm
Speed	150 nm/s
Range	0–1300 nm
Gratings*	
Excitation	*330-nm blaze (200–700 nm range)*
Emission	*500-nm blaze (300–1000 nm range)*

Optional double-grating units are available for highest stray-light rejection and sensitivity.

*Other gratings are available for wavelengths > 1000 nm.

Sample module   T-format sample module with excitation reference detector. The T-box design allows a second emission detection channel to be incorporated. The sample module also has a removable gap-bed assembly for sampling accessory replacement.

Optional front-face collection assembly available.

Detectors   Photodiode for excitation correction between 240 and 1000 nm. Emission detector is an R928P for high sensitivity in photon-counting mode (240–850 nm).

Lifetime   Lifetime Range: 10 picoseconds to 10 microseconds

Frequency Range: 0.1 to 310 MHz

Sensitivity   Water Raman 4000:1 *S/N* at 397 nm, 5-nm bandpass. Background noise 1st standard deviation at 450 nm.

Optics	All reflective optics for focusing at all wavelengths and precise imaging for microsamples
Dispersion	2.1 nm/mm (with 1200-groove/mm gratings)
Spectrometers	f/3.6 plane-grating Czerny-Turner
Excitation wavelength range	0–950 nm (physical); 220–600 nm (optically) 1200-groove/mm grating optimized for 330 nm
Emission wavelength range	0–950 nm (physical); 290–850 nm (optically) 1200-groove/mm grating optimized for 500 nm
Wavelength accuracy	0.5 nm
Minimum step size	0.0625 nm
Wavelength repeatability	0.3 nm
Resolution	0.3 nm
Slit settings	0–30 nm continuously adjustable from computer
Signal detector	Side-on R928P PMT, 180–850 nm response. Linearity to 2×10^6 cps; < 1000 dark counts/second
Reference detector	Photodiode 200–980 nm range selected for stability
Signal-to-noise ratio	A minimum of 4000:1 on water Raman using 397 nm peak, excitation = 350 nm, bandpass = 5 nm; integration time = 1 s. [signal-to-background-noise (1st standard deviation)]
High voltage	S or T detectors, ≤ 1200 V for R928P
Excitation shutter (standard)	Computer-controlled
Integration time	1 ms to 160 s
Step size	0.0625–100 nm
Maximum Scan Speed	200 nm/s
Dimensions (not including computers or electronics)	
311-Tau, 312-Tau	55.5" long × 44" wide × 13.5" high 141 cm long × 112 cm wide × 34.3 cm high

3-21-Tau	63.5" long × 44" wide × 13.5" high
	161 cm long × 112 cm wide × 34.3 cm high
3-111-Tau	55.5" long × 54" wide × 13.5" high
	141 cm long × 137 cm wide × 34.3 cm high
3-222-Tau	65.5" long × 71" wide × 13.5" high
	166 cm long × 180 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

Computer

Although JY offers state-of-the-art computer systems and loads all software prior to shipment, you have the option of providing your own computer and installing the software. Make sure that the computer meets the following minimum requirements:

Microprocessor/clock speed	Pentium or higher recommended.
Operating system and environment	Windows™ 95 or 98, NT 4.0 or greater.
Floppy drive	A 3½" floppy-disk drive.
Fixed disk	A hard disk with at least 60 megabytes of free storage.
Memory	16 MB RAM.
Graphics adapter	SVGA monitor and interface card.
Keyboard	A 10- or 12-function keyboard.
Available ports	One parallel port for a dot-matrix printer One serial port capable of 115 kilobaud for connecting to the SpectrAcq One serial port for optional plotter or mouse

13: Quick Guide to Anisotropy-Decay Measurements

Start-up

1   Make sure that the SpectrAcq, host computer, and main electronics rack are switched off.
    This prevents possible damage to these devices when the xenon lamp is ignited.

2   Start the xenon lamp.

    a   Switch on "power".

    b   Ignite "main lamp".

3   Start the Lifetime Electronics Rack.

4   Start the SpectrAcq.

    a   Put the boot disk in the floppy drive.

    b   Push in the power button. The green LED indicator should glow.

5   Start other lifetime power supplies not connected to rack.

6   Start all automated accessories.

7   Switch on the host computer and peripherals.
    The SpectrAcq takes ~ 1 min to load the drivers on the boot disk.

8   Click on the DataMax icon.
    The **Instrument Control Center** appears after the DataMax boot sequence.

9   The **Layout Selection** dialog box appears.
    Select one lifetime layout. Select the lifetime software layout without other

Layout Selection

Select configuration to work with:

FL3-22 Generic Layout
FL3-22 with 2-Position Sample Changer
FL3-22 with Autopolarizers
FL3-22 with 2-Position Sample Changer and Autopolarizers
Lifetime
Lifetime with Autopolarizers

OK   Cancel   Browse...
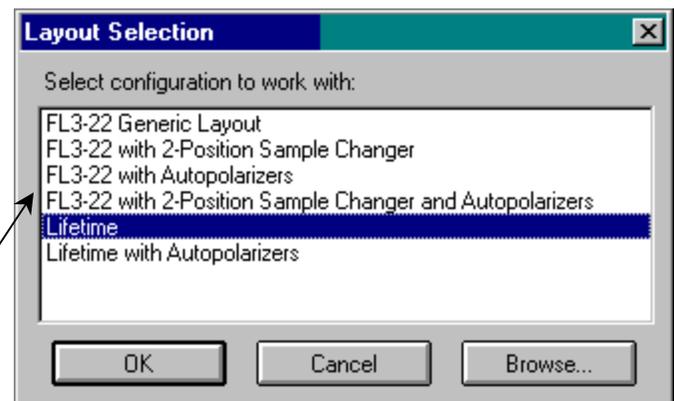
accessories (usually plain Lifetime) when not using a programmable water-bath or polarizers. Other lifetime layouts indicate support for automated accessories.

10 The instrument performs a calibration sequence for ~ 1 min.

> *Note:* *One of the last steps of the calibration is to find the home position for the 2-cell or 4-cell sample holder, so keep hands away from the holder during calibration.*

11 Let the system warm up for ~ 45 minutes before taking measurements.

Unknown and standard preparation

When preparing the unknown, keep the absorbance below 0.1 A for right-angle measurements in a 1-cm cuvette. Higher absorbances tend to generate artifacts by the inner-filter effect. For samples with an absorbance greater than ~ 0.1 A, use a microcell. For very-high-absorbance solutions or solids, use the Spex® front-face option.

> **Note:** *Frequency-domain measurements are based on a differential technique in which a standard and unknown are measured alternately throughout the experiment. The accuracy of experiments depends critically on the quality of the standard.*

1 Prepare the standard.

The standard can be a dilute solution of a scattering material such as Ludox® (colloidal silica suspension) or glycogen. A well-characterized fluorophore can be substituted for the scatterer. A fluorophore standard must exhibit a single exponential fluorescence-decay constant over temperature and excitation and emission range. Prepare a stock solution of the standard (at somewhat higher concentration than expected for the experiment), a few transfer pipettes, waste beaker, and a separate flask of the solvent used to prepare the standard.

2 Place the unknown and standard cuvettes in the sample holder.

Use positions 1 and 2 for the 2-position sample holder, or 1 and 3 for the 4-cell sample-holder.

3 Place the optical filter in the spring-clip holder on the sample changer next to the unknown.

An optical filter for emission-wavelength selection should be on the T side. The filter must be positioned at the unknown, so that it automatically moves from the emission path when the scattering standard is measured. This is unnecessary when a fluorescent standard with emission characteristics similar to the unknown is used.

4 Place one filter in the external filter-holder (FL-1031) between the PMT housing and T-box.

5 Check that the unknown and standard cuvettes are filled high enough to prevent the excitation beam from scattering off the top of the solution.

This unwanted scattering prevents an accurate measurement of the modulation and phase.

Selecting and adjusting lifetime hardware

Select and adjust up to six hardware components to complete the instrument conversion to lifetime operation. The hardware components include the entrance and exit slits of the excitation monochromator (the 180DF double-grating monochromator also may have an optional intermediate slit), an iris at the entrance to the Pockels-cell assembly, Pockels-cell position, and the manual bilateral slit at the exit of the Pockels-cell assembly. The procedure requires positioning all the hardware and then fine-tuning the settings (see the next section, "Defining the experiment parameters").

Note: The PMT base is automatically switched to lifetime operation when any lifetime software layout is selected.

1 The monochromator slit units should be in mm.

To change units, go to **Visual Instrument Setup**, enter the Options menu, select millimeters from the drop-down list, then click Apply. The actual slit settings for the excitation monochromator are discussed in the next section.

2 Set the iris adjustment lever to vertical.
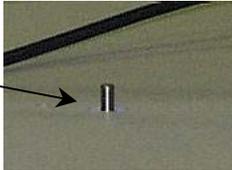
3 Rotate the Modulator Position knob to Lifetime.

This brings the Pockels-cell light modulator into the excitation light path. A full stop indicates that the modulator is properly positioned in the light path.

4 The Source Position knob should point to Xenon.
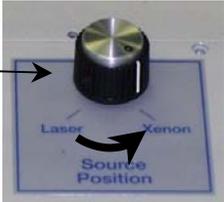
5 Set the manual slit to 0.5 mm.

Defining the experiment parameters

1 In **Instrument Control Center**, click once on the **Tau** icon.
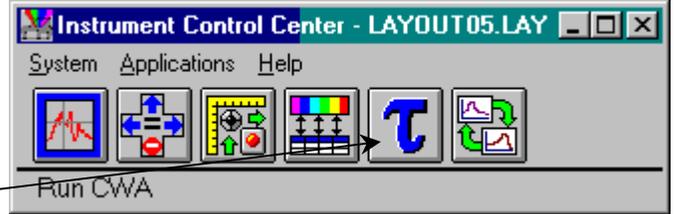
This opens the two data display windows, **ISA Graph** and **Lifetime**.

2 In the **Lifetime** window, choose Experiment.
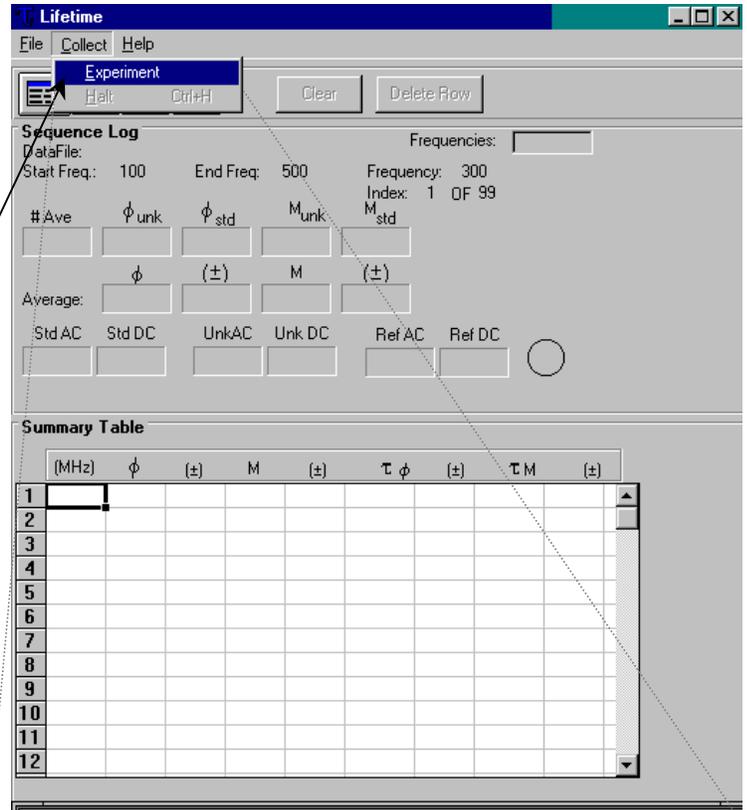
This opens the **Lifetime Acquisition** window. Don't make any entries yet.
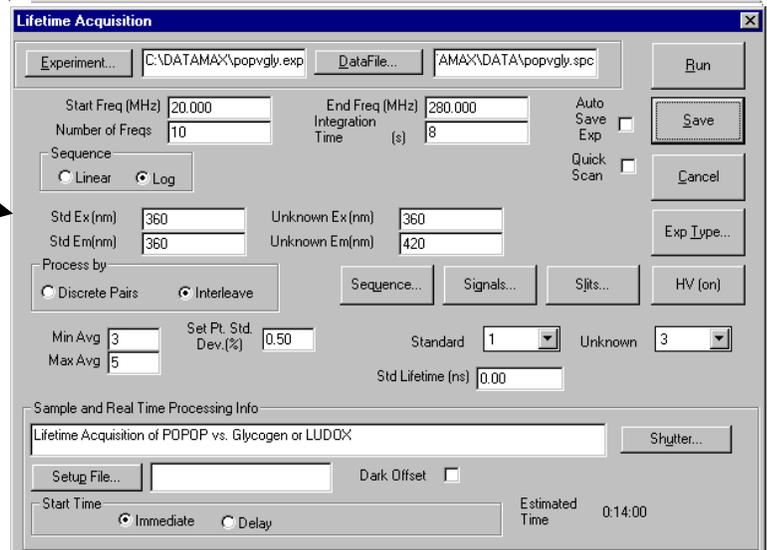
3   Click on the
    **Real Time
    Display** icon in
    **Instrument Con-
    trol Center**.

    Run CWA
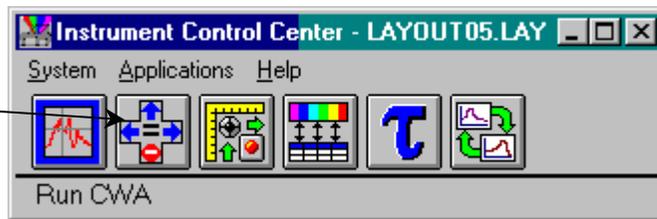
    This opens the **Real Time Display**.

4   Make sure that these dialog boxes are visible in
    Real Time Display:

    Slits (mm)
    Integration (sec)
    High Voltage
    Lifetime
    Monos (nm)
    Signal Balance

    Note: If a dialog box is missing, click on the appropriate toolbar icon to open the dialog box, or rearrange overlapping boxes by dragging them with the mouse.

Signal adjustment consists of two main steps:
    (1) unknown signal optimization, and
    (2) standard DC balance.

5   Click on the EX1 radio button in the
    Slits dialog box.

    Slits (mm)
    ◉ EX1 ○ EM1

    This selects the excitation monochromator.

    Side Entrance
    7.0000
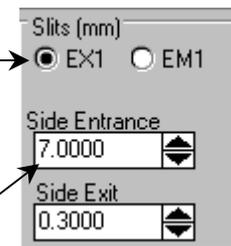
    Side Exit
    0.3000

6   Enter 7.0 for the Side Entrance and
    0.3 for the Side Exit.

    Set an optional intermediate slit to 7.0 mm. Wait after each adjustment to allow
    the slit change to finish before adjusting the next.

7   Select the EM1 radio button.

    Slits (mm)
    ○ EX1 ◉ EM1

    This adjusts the emission monochromator on the S-channel.

8   Enter 5.0 mm for the Side Entrance
    and 5.0 mm for the Side Exit.

    Side Entrance
    5.0000

    Side Exit
    5.0000

    An optional intermediate slit should be 5.0 mm. Go to step
    11.
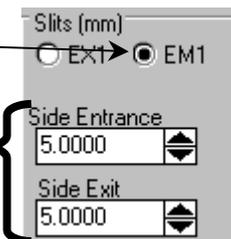
9   For an optical filter on the T-channel (left side of
    T-box), make sure that the filter is in place.

    There are no slits to adjust on the T-channel when using an optical filter.

10 Find the **Lifetime** dialog box.

a   Click either the S&R radio button for the S-channel detector on the monochromator, or the T&R radio button if using a filter on the T-channel.

b   Enter 10 in the Frequency (MHz) field.

c   Press Enter after entering frequency values.

11 Set up the **Signal Balance** dialog box.

Store the optimal excitation and emission wavelengths and the sample-changer position for the two samples. Then click the Set Standard or Set Unknown button to reset the instrument.

12 Set Integration time to 1.0 s.

13 Set the PMT high voltages:

a   Set R to 350

b   Set S to 900 if used, otherwise leave at 0

c   Set T to 750 if used, otherwise leave at 0

d   If the HV On box is not checked, click on the box to send current to the PMTs.

14 Click on the shutter icon in the toolbar to open the shutter.

When changing a parameter's value, always double-check the status of the shutter. The shutter will close automatically when changing some of the parameters.

15 Monitor the signal levels in the **Lifetime** box.

Look only at the AC and DC levels at this step; don't worry about the modulation ratio or phase**Error! Bookmark not**

16 Let the signal stabilize.

Note: The settling time for the AC signals can be quite long, up to ~1 min, so be patient.

17 Adjust the slits, iris, and high voltage to obtain an AC-level on T (or S) of ~1.5 volts.

Note: The recommended maximum AC or DC is ~5 volts. An AC or DC level of 10 indicates an over-range of signal level. Reduce the high voltage slits or iris to bring the signal on-scale before proceeding.

Once the unknown sample's signal is optimized, only adjust the R-channel high-voltage and concentration of the standard.

Warning: Do not reduce the high voltage below 280 volts for any channel!

Here are a few suggestions for adjusting the signal level:

a   If AC and DC are low, increase the slit width (up to 7.0 mm) on the emission monochromator (if using S channel).

b   Adjust slits and high voltages in tandem, so that no one parameter is at its upper limit, while others are far from their maxima.

For example, to increase the unknown's AC from 0.1 to 1.5 volts, then, starting from the above:

- Increase high voltage from 750 to 900.
- Increase excitation-monochromator exit-slit from 0.3 to 0.5 mm.
- Increase manual slit from 0.5 to 0.7 mm.
- Increase iris by rotating counterclockwise 5°.

c   If AC is still below 1.5 volts, then re-increment the parameters in the same sequence and about the same magnitude.

Note: Maximum high voltage is 1200 V. When running at the maximum voltage on the T-channel, do not leave the sample chamber lid open for longer than required to insert sample cuvettes and filters.

Watch the R level as the slits are opened. If R over-ranges, then reduce the R-channel high-voltage.

18 Check that the DC level is below 5 volts.

a   If DC is > 5 V, try reducing the manual slit and iris while increasing high voltage, to reach an AC to DC ratio (modulation ratio) of ~ 1.0 or higher.

b   If you can't achieve modulation ratios of about 1, or AC levels of 1.5 volts, you can still expect useful results, although precision will decrease. Accurate results for simple decays of several nanoseconds can be obtained, even with a starting AC-intensity lower than 0.5 (because of low concentration or quantum yield).

19 Click on the Set Standard button in **Signal Balance**.

This places the standard in the measurement position.
Adjust the standard's signal by changing concentration or using neutral-density filters, so that the DC level of the standard is within about 10–15% of the unknown.
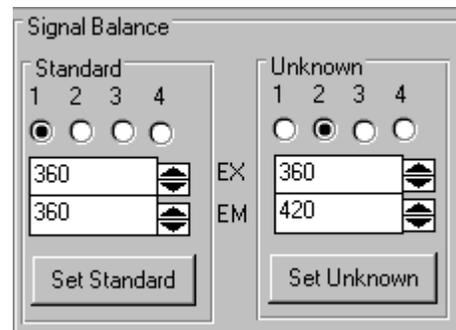
Note: Do not adjust slits, iris, or high voltages for the T-channel (or S-channel), for this will change the optimized, unknown sample signal.

20 Adjust the R-channel high-voltage to keep the R AC between 1.5 and 2.0 V.

• Do not reduce the high voltage below 280 V.
• As necessary, keep R on-scale by reducing one or more slit widths, and redo the unknown signal by changing only high voltage. Do not forget to re-balance the standard's signal to match the unknown.

21 Click on the transfer icon [icon] in the toolbar to pre-set the following parameters:

Lifetime channel selection (S&R or T&R)
Monochromator slits
Excitation and emission wavelengths
High voltages

*Note: The transfer icon will work only when the **Lifetime Acquisition** window is open.*

22 Close the **Real Time Display** window.

Experiment setup and measurement

Note: *If, in the previous section, you did not select the transfer option from the **Real Time Display** toolbar, you can either manually enter these parameters or open the **Real Time Display** and click the transfer icon.*

1   Activate the **Lifetime Acquisition** dialog window:

Lifetime Acquisition   ✕

| Experiment... | C:\DATAMAX\popvgly.exp | DataFile... | 'AMAX\DATA\popvgly.spc | **Run** |

Start Freq (MHz) `20.000`   End Freq (MHz) `280.000`   Auto Save Exp ☐   **Save**

Number of Freqs `10`   Integration Time (s) `8`

Quick Scan ☐   **Cancel**

Sequence
- ○ Linear   ⊙ Log

Std Ex (nm) `360`   Unknown Ex (nm) `360`

Std Em(nm) `360`   Unknown Em(nm) `420`   **Exp Type...**

Process by
- ○ Discrete Pairs   ⊙ Interleave

Sequence...   Signals...   Slits...   HV (on)

Min Avg `3`   Set Pt. Std. Dev.(%) `0.50`   Standard `1` ▾   Unknown `3` ▾

Max Avg `5`

Std Lifetime (ns) `0.00`

Sample and Real Time Processing Info

`Lifetime Acquisition of POPOP vs. Glycogen or LUDOX`   Shutter...

Setup File... `____`   Dark Offset ☐

Start Time
- ⊙ Immediate   ○ Delay   Estimated Time   0:14:00

---

2   Close **Real Time Display**.

3   Set the following parameter fields:

a   Click DataFile to open the **Windows file** dialog box.

Enter a file name in the desired subdirectory, or, type a file name directly into the DataFile field. Let the system to append the proper extension (.dat) to the file name.

b   Integration Time: Set between 8 and 15 seconds.

c   Define the frequency sequence to find the frequency response of the unknown.

Note: The detected signals in lifetime experiments are always of much-lower intensity than steady-state measurements, so we recommend longer integration times and averaging of several readings (see step e below).

This sequence assumes one or more lifetimes of 5 to 20 ns. For samples with

Start Freq (MHz) 20.000
Number of Freqs 10
Sequence
  Linear   Log
End Freq (MHz) 280.000
Integration Time (s) 8

shorter lifetimes, extend the End Freq. For samples with longer lifetimes, lower the Start Freq. If the system is more complex than a bi-exponential, then add to the Number of Freqs to improve resolution of the individual decays.

Start Freq (MHz): 10
Sequence: Log
End Freq (MHz): 200
Number of Freqs: 10

d   Process by: Set to Interleave.

Process by
  Discrete Pairs   Interleave

Note: Interleaving is a much faster way of averaging data pairs, i.e., the standard and the unknown.

e   Min Avg: Set to 3.

f   Max Avg: Set to 5.

g   Set Pt. Std Dev(%): Set to 0.5.

The values entered in steps e through g work together to obtain a defined error-level in a minimum time. The minimum of 3 averages is calculated and the resultant standard deviation is compared to the set-point standard deviation. If the set-point standard deviation is less than the calculated standard deviation, then the next measurement is completed and averaged. This continues until the set-point standard deviation is equal to or greater than the calculated average, or until the maximum number of averages is reached.

h   Dark Offset: Do not use

i   Std Lifetime (ns)

Enter 0 for a light-scattering standard. Enter the known lifetime, in nanoseconds, for a fluorophore reference.

j   Enter one line of
      text in the note
      field in the Sam-
      ple and Real
      Time
      Processing Info field.

Note: Do not change the default values of any other field in this section.

4   Click Run to launch the experiment.

Now sit back and watch the action.

Following the data collection and modeling

You can follow the course of the experiment as the raw data are measured, corrected for instrument bias, and tabulated. At the completion of the run, you will activate the modeling program to determine the number of species, contribution to the total fluorescence signal, and each lifetime.

1   From the **Instrument Control Center**, click the **Run Lifetime Modeling** icon.
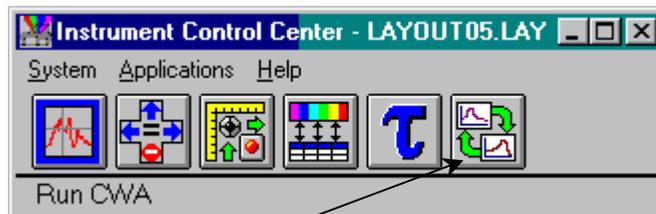
This opens the **Model Windows Application – Model** window:
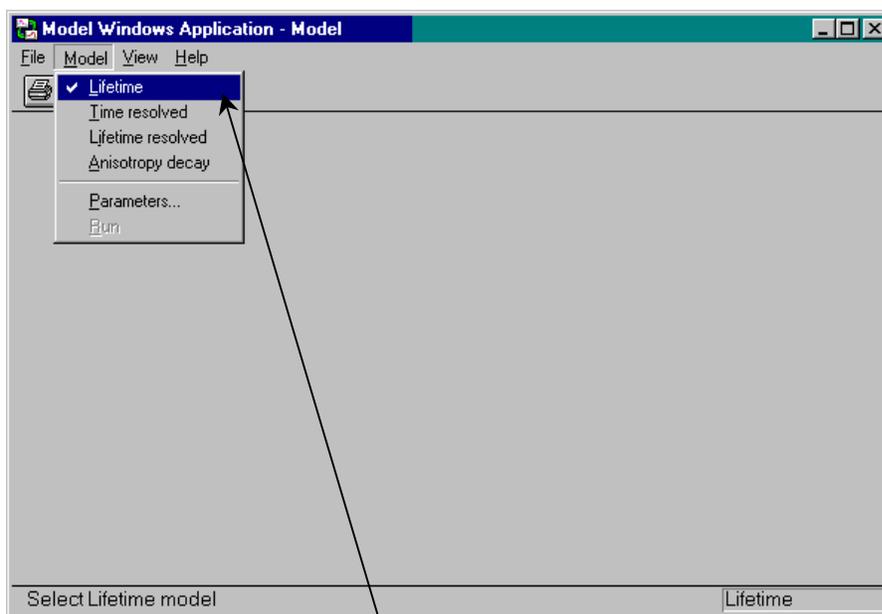
2   Under Model, select Lifetime.

This opens the **Lifetime Model** window:

3   Input the data file's name of your completed experiment in the field.

Click Input File to find the file.

Lifetime Model

Input data

Input File...   test.dat

⦿ Use Measured Standard Deviations (from file)

○ Enter Deviation   dPhase: 0.5   deg dMod: 0.005

Number of components

○ 1   ⦿ 2   ○ 3   ○ 4

Fractional Amplitudes   **Lifetimes (ns)**

Fract. 1: 0.2   ☐ Fixed   Tau 1: 2   ☑ Fixed

Fract. 2: 0.8   ☐ Fixed   Tau 2: 6   ☐ Fixed

Fract. 3:   Tau 3:

Fract. 4:   Tau 4:

Run Model   **Save Output...**

4   Click the Enter Deviation radio button.

Enter a phase error of 0.5 and modulation error of 0.005 in dPhase and dMod fields respectively.

5   Enter the expected number of components (from 1 to 4)

6   Fill in the active Fractional Amplitude fields and Lifetimes (ns)

Fill in for all selected components. Enter a non-zero value in every open field.

7   Click Run Model to start modeling.

The results include reduced-χ^2, best fits for fractional contributions, lifetimes for all selected species, and a graph of measured data (symbols) compared to the model (solid lines). A second graph reveals the difference between measured and model for phase and modulation. A reduced-χ^2 ~1 with residual scattered evenly near 0 suggests that a good description of the unknown's fluorescence-decay is found.

8   Click on the printer icon in the toolbar to generate a printout.

9   Close the modeling window.

14: Glossary

Absorption

The electronic transition from the ground state to the excited singlet state, by absorption of a photon of a particular energy. This process typically occurs in $\sim 10^{-15}$ s.

Absorbance

The extent of light absorption by a substance, $-\log T$, where T is the transmittance of the sample. Absorbance is also synonymous with optical density, OD. Absorbance is defined as

$$A = \varepsilon cl = OD = -\log T$$

where ε is the extinction coefficient (M^{-1} cm^{-1}), C is the sample concentration (M), and l is the path length (cm).

Acquisition modes (R, S, T, A channels)

The logical input channels used on the spectrofluorometer to input collected signal from the various detectors present on the system. Commonly the detectors are assigned as the reference detector connected to channel R, the first emission connected to channel S, the second emission connected to channel T, and finally, an auxiliary input available as channel A. These logical channel names are used in the collection of data in most applications of the software. The user may create algebraic expression on these input channels when defining experiments in DataMax (e.g., S/R).

Anisotropy (<r>)

A measurement of the fluorescence polarization of a samples, defined as the linear-polarizer's component's intensity divided by the total light intensity. The measurement of anisotropy can provide insight into molecular size and shape, as well as the environment that surrounds it.

Autopolarizers

An automated device used to hold and precisely rotate a set of polarizers to acquire anisotropy (or polarization) measurements. FluoroMax®-3 systems with autopolarizers contain two automated polarzer mounts, one for the excitation polarizer and one for the emission polarizer. Both of these are located between the sample compartment and their respective monochromators. Their calibration is maintained by optical sensors that are offset in the software. Autopolarizers on the FluoroMax®-3 may be inserted into and out of the light path in DataMax, in the **Visual Instrument Setup** software application. Automated realignment of the polarizers may also be performed in **Visual Instrument Setup**.

Bandpass

The range of wavelengths of light passing through the ex-

citation and emission spectrometers, usually expressed in nanometers (nm). This value is dependent on the size of all slits in the monochromator, as well as the dispersion of the monochromator. For spectrofluorometers, both slits on the monochromator should be set equal to properly set the bandpass, if this is not done automatically through the software. When adjusting the bandpass for samples, the wider the bandpass, the higher the signal intensity, with a trade-off in lower resolution.

Bandpass filter	An optical element which selectively transmits a range of wavelengths, while absorbing light of higher and lower wavelengths.
Bioluminescence	Emission of light originating from a chemical reaction in a living organism.
Blank subtraction	The removal of the spectral response of the solvent (and sample container) from the sample's spectral response. To accomplish this, an identical scan is run on the solvent just before running the actual sample. Proper use of a blank can remove solvent luminscence artifacts, scattering events, and any artifacts from the sample cuvette or container. In the experiment portion of the software, click on the Blank button to recall a previously acquired blank spectrum.
Blaze wavelength (of gratings)	The wavelength at which a grating is optimized for peak efficiency. As a rule of thumb, gratings are usable from 2/3 of the blaze wavelength to twice the blaze wavelength. The excitation and emission gratings for the FluoroMax®-3 are blazed for efficiency in the UV and visible.
Chemiluminescence	Emission of light originating from a chemical reaction.
Concentration determination	A function of the **Constant Wavelength Analysis** software application, that calculates an unknown sample's concentration. The user runs known samples and enters the concentration in order to calibrate the routine. Then an assay may be completed with the measurements based on concentration.
Constant wavelength analysis (CWA)	The DataMax software application designed for performing single-point measurements at discrete wavelength pairs. The data are acquired as single points at a user-defined set of excitation/emission wavelength pairs for a user-defined number of samples. These data are displayed in either spreadsheet format, or in a plot. This application is perfect for MicroMax or FluoroMax®-3 users who routinely perform assays on a large number of samples. Additional options such as Variable Time Kinetics make this application even more flexible.

Corrected emission scan   An emission scan that has been corrected for the wavelength response of the emission monochromator and the signal detector. To obtain a corrected emission scan, an emission spectrum is multiplied by the appropriate emission correction factor file. A set of emission correction factors is supplied with your instrument and stored under the name MCORRECT.SPC on the software disks.

Corrected excitation scan   An excitation scan corrected for the wavelength-characteristics of the xenon lamp, the aging of the xenon lamp, and the gratings in the excitation spectrometer. To obtain a first-order correction of the excitation scan, the emission detector signal is ratioed to the reference signal (i.e., *S/R*). This provides correction for the lamp and excitation-monochromator spectral response, which is ~95% of the required correction. To obtain a completely correct scan, the excitation scan acquired in the *S/R* acquisition mode is multiplied by excitation correction factors. A set of excitation correction factors (XCORRECT.SPC) is included on the software disks.

Correction factors   A spectral set of multiplicative factors used to compensate for the instrumental wavelength-response from detectors and optics. These correction factors are specific to a particular optical configuration for the instrument, and must be run for each detector in the spectrofluorometer system. Excitation and emission correction factors are included by default with each new spectrofluorometer. Their default filenames and XCORRECT.SPC and MCORRECT.SPC, respectively.

CTI card   This acquisition and control card, located in the rear of the FluoroMax®-3, is the *counter-timer-integrator* board. It handles all spectrofluorometer control, timing, and data acquisition for measurements on the system. The boards carry by default two acquisition channels, and are linked to all monochromator and accessory control boards on the underside of the instrument. The CTI card fits into an ISA slot in the motherboard on the rear of the FluoroMax®-3.

Current input module (DM303)   The current input module collects the current signal from the reference photodiode, digitizes the data, and sends it to the CTI card for data-processing. This module is located directly behind the reference photodiode. It has linear response from 0–10 μA.

Dark counts   Inherent background signal measured in counts/second (cps) observed on the photomultiplier tube when high voltage is applied. Cooling the detector in a Peltier housing can be used to decrease the dark counts on a Fluorolog®-3 sys-

tem. Typically, the R928P photomultiplier tube used for the FluoroMax®-3 system has dark counts of < 1000 cps.

Dark offset The software correction used to subtract dark counts (or dark signal) on a detector from a spectral acquisition. This option usually appears as a checkbox in the DataMax software. Use a corrected signal channel for the acquisition (e.g., S) in order to run the dark offset correction.

Datafile A file used to store spectral data, constant-wavelength analysis data, or other recorded data. In DataMax, the most common datafile is the spectral file (.SPC). This is the file-type that contains spectra acquired from a scan run from the **Run Experiment** menu (e.g., emission scan, time base scan, multigroup, etc.). Datafiles contain all of the information regarding the instrument setup for a scan as well as the spectra (see *File Information* in the *DataMax Software Manual*). Multifiles are three-dimensional datafiles that contain an array of spectral files.

Dispersion The range of wavelengths of light across the field of view of the entrance and exit apertures. Dispersion depends on the focal length of the monochromator, the groove density of the optics, and the *f*-number (or speed) of the monochromator. Dispersion is usually expressed in nanometers of spectral coverage per millimeters of slit width (nm/mm).

Emission monochromator The monochromator located after the sample compartment used to isolate discrete wavelength components of the sample's fluorescence, and may be used to scan the emission from a sample. The emission monochromator on the FluoroMax®-3 is a 0.18-m single monochromator with a Czerny-Turner design: the monochromator includes a collimating mirror, the reflection grating (blazed at 500 nm), and a focusing mirror, with slit apertures at the entrance and exit. The emission-photomultiplier detector is connected to the exit of this monchromator to measure the fluorescence emission.

Emission scan An acquisition that shows the spectral distribution of light emitted by a sample. During an emission scan, the excitation spectrometer remains at a fixed wavelength while the emission spectrometer scans a user-selected region.

Energy transfer The inter-molecular or intra-molecular 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.

Excitation/emission ma- A three-dimensional plot showing the total luminescence

trix (EEM) from a sample across all useful wavelengths. The FL-3D instrument can acquire EEMs with excitation from 250–550 nm and emission from 250–750 nm. Total luminescence spectroscopy is devoted to measurements of these EEMs for various materials. *See also:* **Multifile**, **Total Luminescence Spectroscopy**

Excitation monochromator The monochromator, located between the xenon lamp and the sample compartment, used to isolate discrete wavelength components of the excitation beam. This beam is directed to the sample, during which the excitation monochromator may be used to scan the excitatioin spectrum from a sample. The excitation monochromator on the FluoroMax$^{®}$-3 is an 0.18-m single monochromator with Czerny-Turner design. This means that the monochromator includes a collimating mirror, the reflection grating (blazed at 330 nm), and a focusing mirror, with slit apertures at the entrance and exit. An excitation shutter is located directly after the excitation exit slit to protect the sample from photobleaching. The reference detector looks at a fraction of the light exiting the excitation monochromator to correct for the lamp response, if desired.

Excitation scan A scan that reveals the spectral distribution of light absorbed by the sample. The scan is collected by rotating the excitation grating while holding the emission monochromator fixed. For non-ratiometric acquisitions, acquire the scan using *S/R* to correct for the the spectral response of the lamp.

Excited state (S_1) The energy level to which an electron in the ground level of a molecule is elevated after the absorption of a photon of a particular wavelength. Subsequently, fluorescence will occur if the molecule returns to the ground state via a radiative transfer from the S_1 state to the ground state.

Experiment file A file that contains specific information on the experimental setup for an acqusition defined in **Run Experiment**.This file is saved with a default `*.EXP` extension. In addition to basic scan parameters, this file saves system defaults (such as slit units), and some accessory settings for the acquisition. Each acquisition type in the **Run Experiment** menu has its own default experiment file (e.g., `DFLT0.EXP` is the default emission-scan definition). These are the default experiments that appear in the Experiment menu on startup. Use experiment files to archive scan settings for acquisiitions that are performed on a routine basis.

Extrinsic fluorescence Inherent fluorescence of fluorescent probes added to a sys-

tem to study non-fluorescent molecules. These probes have gained acceptance in a variety of applications.

Filter

An optical element that is used to select certain wavelengths of light. Types of filters include high pass, low pass, bandpass, and neutral density.

Flash lamp

A source that provides pulsed light to excite a sample for phosphorescence or fluorescence measurements. The repetition rates and time response of the source determine the useful range of the source for these measurements. The lamps can be used in either "free running" or "gated" modes depending on the support electronics.

Fluorescence

The emission of light during the transition of electrons from the excited singlet state to the ground state from molecules originally excited by the absorption of light. Fluorescence typically occurs within $\sim 10^{-9}$ seconds.

Fluorescence lifetime

(Symbol is τ.) The average length of time that a molecule remains in the excited state before returning to the ground state.

Fluorophore (fluorescent probe)

A molecule or compound that has a known fluorescence response. These probes comes with various sensitive areas depending on the peak exictation and emission wavelengths and their fluorescence lifetimes. Fluorophores are used to provide information on concentration, size, shape, and binding, in a particular medium. Good fluorophores are stable over wide pH and temperature ranges.

Frequency-domain fluorescence lifetime measurements

A technique for measuring fluorescence lifetimes, in which a sample is excited with light whose intensity is modulated sinusoidally. The emission is an easily-detected forced response to the excitation. Fluorescence lifetimes are calculated from both the phase-angle difference between the sample and a standard, and the demodulation factor for sample and standard.

Front-face detection

A mode of detection in which fluorescence is collected off the front surface of the sample. Front-face detection is usually selected for turbid samples in solution (e.g., blood), samples of high concentration, or solid samples such as powders, thin films, pellets, and cells on a coverslip. Front-face detection collects fluorescence off the sample at a 22.5° angle to minimize reflections and scattering.

Grating

An optical element in a monochromator that uses finely-etched vertical grooves to disperse incident light into its constituent wavelengths. Reflection gratings (grooves etched on a highly reflective surface) are used in the FluoroMax®-3. Gratings are scanned by rotating their opti-

cal centers about the optical axis of the instrument, with the incident angle of the entrance beam determining the wavelength of light directed to the exit aperture. Gratings come in a variety of formats and are commonly made by physically *ruling* (scribing) the grating, or holographically *etching* the grating grooves.

Ground state (S_0)
The lowest energy level in a molecule. For fluorescence to occur a molecule absorbs a photon of light, thereby exciting it to the S_1 level. A fluorescence emission occurs during a transition from an excited state S_1 to the ground state S_0.

High-pass filter
Optical component which passes light of a higher wavelength.

Increment
The spacing between adjacent measurement points in an acquisition, as specified in the experiment definition. Typically, increments are in the form of wavelength (nm) or time (s or ms).

Inner filter effect
The scattering of the excitation or emission beam from a concentrated sample by the individual molecules in the sample. This reduces the apparent signal intensity from the sample creating an artifact in the data. For this reason, we recommend using concentrations of <0.05 OD in a 1-cm-pathlength cell. Samples measured in higher concentrations should be measured in a reduced-pathlength cell, or in front-face mode.

Integration time
The amount of time that each datapoint is collected from the detector(s), specified in either seconds or milliseconds. Longer integration times can help improve the signal-to-noise ratio for a measurement, while shorter integration times reduce the amount of time required for a scan.

Internal conversion
Electronic transitions within an excited molecule that do not result in emission. Also called a "non-radiative transition", this usually involves changes in vibrational levels.

Intersystem crossing
The electronic transition from the excited singlet state to the excited triplet state prior to returning to the ground state. This transition involves a change of spin that is quantum-mechanically forbidden, giving a much longer time-scale than fluorescence. This transition causes phosphorescence on the timescale of microseconds to seconds.

Intrinsic fluorescence
The natural fluorescent properties of molecules.

Jabłonski (energy) diagram
A diagram that illustrates various energy levels and electronic transitions available in a particular molecule. Possible paths for fluorescence, phosphorescence, and non-radiative transfers are shown on this diagram, along with

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the various vibrational sub-levels available around each energy level.

Laser   A monochromatic light source which provides high excitation intensity.

Linearity   (1) Signal response; the desired response from a light detector is a linear relationship. For example, when detector response is linear, if the light intensity doubles, the detected signal also doubles. Most detectors exhibit non-linear behavior near saturation. On the FluoroMax®-3, the emission PMT is linear up to 2–4 million counts per second. Above this, pulse pileup occurs on the photon-counting module (when multiple photons are counted as one). This results in a non-linear response, and the detector efficiency drops. (2) Spectral positioning accuracy or tracking error of a spectrometer drive mechanism. *See* **Spectral Calibration**.

Low-pass filter   Optical component which passes light of a lower wavelength.

Luminescence   The emission of light from matter excited from a variety of processes, resulting in an electronic transition within the molecule to a lower energy state. *See also*: **Bioluminescence**, **Chemiluminesence**, **Fluorescence**.

MCD shutter   **M**ulti-**c**hannel **d**evice shutter. The Uniblitz shutter is used for its rapid cycle time.

Mercury lamp   A light source which offers discrete narrow lines as opposed to a broadband radiation (e.g., xenon). Mercury lamps are often used to verify the accuracy and resolution of a spectrometer or spectrograph.

Mirror-image rule   A sample that absorbs light for certain energy-level transitions usually exhibits an emission profile that appears to be the mirror image of the absorption spectrum. The reason is that the same energy-level transitions are used for excitation and emission, with the transitions returning to the ground state as the complement to the those in the excited state.

Molar extinction coefficient (ε)   The absorptivity of a particular substance, in M^{-1} cm^{-1}.

Multifile   The three-dimensional acquisition datafiles collected by the software using matrix scans or temperature scans, stored as an array of datafiles. A multifile is still stored with an `.SPC` extension. Multifiles may be used in their entirety in DataMax as 3D files, or they may be split up into individual two-dimensional spectra using multifile

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utilities.

Multigroup scan   This experiment type allows a time-base scan to be acquired across more than one excitation/emission pair. Up to 16 different wavelength pairs may be entered for a multigroup scan. The spectrofluorometer will cycle through each pair, integrating for the specified time, before moving on to the next point. Use the multigroup scan for measuring ratiometric probes (such as Fura-2 or BCECF).

Neutral-density filter   An optical element which absorbs a significant fraction of the incident light. These filters usually are characterized by their optical density. For example, a filter with OD = 1 transmits 10% of the incident light. Ideally, these filters absorb all wavelengths equally. *See also* **Absorbance**.

Optical-density effects (Inner filter effect)   Fluorescence intensities are proportional to the concentration over a limited range of optical densities. High optical densities can distort the emission spectra along with apparent intensities. For fluorescence measurements in a 1-cm-pathlength cell, samples should have an OD of 0.05 or less. *See also*: **Inner filter effect**.

Phase-modulation method   *See* **Frequency-domain lifetime method**.

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 $\sim 10^{-6}$ to ~ 1 second. To enhance phosphorescence, samples are often frozen at liquid-nitrogen temperature (77 K).

Photobleaching   The reduction in fluorescence from a photosensitive sample overly exposed to excitation light. Not all samples photobleach, but if so, take great care to keep the sample out of room light, and to use the excitation shutter and its photobleach modes on the spectrofluorometer to protect the sample from excessive exposure.

Photoelectron   An electron released through the interaction of a photon with the active element of a detector. The photoelectron may be released either from a junction to the conduction band of a solid-state detector, or from the photocathode to the vacuum in a PMT. A photoelectron is indistinguishable from other electrons in any electrical circuit.

Photon-counting detection   A method of detection used primarily with photomultiplier tubes, in which discrete current pulses from the tube are integrated and "counted up". With this method, noise inherent to the detector can be minimized, resulting in much more sensitive detection than used in traditional current- or voltage-detection modules. A limit to photon-counting is

when pulse pileup occurs, that is, when two counts occur too fast for the module to count them individually. This creates non-linearity in the detector at high signal-levels.

Polarization (*P*)

A measurement of the fluorescence polarization of a sample defined as the linear polarizer's component's intensity divided by the natural light intensity. The measurement of polarization provides insight into molecular size, shape, and the environment surrounding the molecule. Another unit, called millipolarization (m*P*), is used to monitor small changes in polarization. $P = mP \times 1000$.

Quantum yield (Fluorescence quantum yield)

The efficiency of the absorption of a photon to be emitted (fluoresced). Quantum yields are typically expressed as percents. The fluorescence quantum yield is the percentage of photons absorbed that actually lead to fluorescence. This number is reduced by scattering, quenching, internal conversion, and non-radiative effects, along with several other specialized processes. Measurements of quantum yields usually require the comparison of a sample with a known fluorophore such as Rhodamine-B or $Ru(BPY)_3$.

Quenching

Reduction in the fluorescence intensity of a sample by a variety of chemical or environmental influences. Quenching may be static, dynamic, or collisional in nature.

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. Rayleigh-scattered light is of the same energy as the incident light. The scattered radiation's intensity is inversely proportional to the 4th power of the wavelength of incident radiation.

Real Time Display

The DataMax software application that gives the user full control of the system in real-time in order to optimize the system setup for a particular measurement. Use **Real Time Display** to find the optimal slit widths for sample measurements, or to check that the excitation beam is striking the sample properly.

Reference detector

The detector used to monitor the output of the xenon lamp. A silicon photodiode with enhanced-UV response is used for the FluoroMax®-3, and is connected to input channel R. Use S/R to correct for the xenon-lamp response during an excitation scan.

Resolution

The ability of a spectrometer or spectrograph to separate two closely-spaced peaks. Resolution can be improved by decreasing the number of pixels binned together or the slit widths in the instrument.

Right-angle detection

Collection of fluorescence at 90° to the incident radiation. Right angle detection is typically selected for dilute and clear solutions in order to minimize the scatter component in the detected emission.

Sample changer (automated)

An automated accessory that automatically positions up to four cuvette samples held in the sample compartment. Use this accessory to run up to four samples at one time for a small assay, or to run blanks with your samples simultaneously. Automated sample changers are thermostatted and possess magnetic stirrers.

Saturation

The effect of having too much signal incident on a particular detector. Saturated detectors give an erroneous result and no longer show any response for small changes in signal. In some cases, saturation can be damaging to a detector's performance, so avoid saturation whenever possible. The R928P PMT used on the FluoroMax®-3 saturates at 1×10^7 cps.

Signal channel

See: **Acquisition modes**.

Signal photomultiplier

Detector used to measure excitation and fluorescence from the sample, operated in photon-counting mode to provide the highest sensitivity. Different detectors can be used to optimize different wavelength regions.

Signal-to-noise (S/N) ratio

The measurement of the signal observed divided by the noise component seen in that signal. Generally, the better the S/N ratio, the better the measurement. This is accomplished by using photon-counting detection with the proper high-voltage bias for improved sensitivity during fluorescence measurements. The user then optimizes the sample signal to the higher area of the linear range for the detector, typically between 100 000 and 2 000 000 cps. Next, dark offsets or blank subtraction may be used to improve the S/N. Finally, increasing the integration time or repeating the same scan several times can improve the signal to noise. For specifications, signal-to-noise may be represented as signal to peak-to-peak noise, or signal to noise at first standard deviation (FSD).

Singlet state

The spin-paired ground or excited state. The process of absorption generally produces the first excited singlet state, which takes time to fluoresce, and may undergo intersystem crossing to form a triplet state.

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Spectral calibration   The accuracy of a monochromator with respect to its wave-
                              length alignment. This is a measure of the monochromator
                              being at the correct wavelength when it is set there. Mono-
                              chromators are traditionally calibrated using line-spectra
                              sources, such as mercury lamps. Spectrofluorometers may
                              be calibrated by performing two scans, one of the source,
                              and one of a standard (such as water) to calibrate all of the
                              monochromators. For Spex® spectrofluorometers, the xe-
                              non-lamp scan is performed on the excitation with the
                              467.1-nm peak assigned as such in the software. The water
                              Raman band is scanned with 350-nm excitation, and the
                              397-nm peak is assigned as such in the software for the
                              emission monochromators.

Spectral correction   The removal of the wavelength sensitivity of detectors, op-
                              tics, sources, and backgrounds from the spectrum taken on
                              a sample. When spectral correction has been properly per-
                              formed, the true theoretical spectra from a sample should
                              be all the remains. Spectral correction is accomplished
                              with a variety of options on Spex® spectrofluorometers.
                              Excitation and emission correction factor files are provided
                              to remove the wavelength sensitivity of detectors and their
                              optics. The reference detector is present to remove the
                              lamp and excitation optics response. Blank subtraction and
                              dark offset are used to remove background levels and re-
                              sponses.

Spectral response   Most detectors have a higher sensitivity to some wave-
                              lengths than to others. The spectral response of a detector
                              is often expressed graphically in a plot of responsivity ver-
                              sus wavelength.

Spectrofluorometer   An analytical instrument used to measure the fluorescence
                              properties of a molecule or substance. The device consists
                              of at least two monochromators, a source, sample com-
                              partment and detection electronics. The instruments may
                              be scanned on the excitation, emission or both to provide
                              insight on the characteristics of the sample being studied.
                              Newer spectrofluorometers provide many more automated
                              options, including polarization, temperature, titer plates,
                              pressure, and many more. Today, these instruments are
                              computer-controlled, allowing easy control of assays and
                              complex experiments.

Stokes shift   The energy difference between the absorption peak of low-
                              est energy and the fluorescence peak of maximum energy.

Synchronous scan   Scan type which characterizes the overlap between the ex-
                              citation and emission. The excitation and emission spec-
                              trometers are scanned at the same time, with a constant

offset specified in either nanometers (wavelength units) or in cm^{-1} (energy units).

Technical spectrum
A spectrum acquired on research instrumentation with instrumental bias in the measurement. This spectrum must undergo proper spectral correction in order to match up with the theoretical spectrum. Spex® spectrofluorometers offer various methods for such correction, including spectral correction, dark offset, blank subtraction and others.

Temperature scan
A DataMax 3D scan-defintion that consists of a particular scan made across a user-defined temperature range. This scan may be used to monitor a sample's temperature response, or, more specifically, to perform a melting curve for a sample. Temperature scans require an automated bath compatible with DataMax to be attached to the spectrofluorometer system along with a thermostattable sample mount.

Throughput
The amount of light that passes through the spectrofluorometer for a particular measurement. The throughput is usually measured as the counts per second measured on the water Raman band at 350-nm excitation with 5-nm bandpass. As bandpass increases, so will the throughput. Like bandpass, throughput has an inverse relationship with resolution. When the throughput is increased, the resolution will decrease.

Time-based scan
Scan type in which the sample signal is monitored as a function of time, while both the excitation and the emission spectrometers remain at fixed wavelengths. Time-based data are used to monitor enzyme kinetics, dual-wavelength measurements, and determine reaction-rate constants.

Total luminescence spectroscopy (TLS)
Spectroscopy devoted to monitoring changes to the entire excitation/emission matrix of luminescence on a sample. This discipline is best applied to fast kinetics measurements of samples during reactions, temperature curves, or changes in other parameters.

Transmission
Light that passes through a sample without being absorbed, scattered, or reflected. Transmission is usually measured as a percentage of the incident light at a certain wavelength.

Triplet state (T_1)
The spin-paired ground or excited state formed from the excited singlet state, in which electrons are unpaired. The triplet state gives rise to phosphorescence.

Tyndall scattering
Scatter that occurs from small particles in colloidal suspensions.

Variable time kinetics
A special measurement menu in the **Constant Wavelength Analysis** software application. The user defines measure-

ments that occur at specific times, for specific durations, and with different integration times. Those measuring assays can do the actual measurements at the desired times. Refer to the DataMax™ software manual for more information.

Vibrational states   Sublevels within an electronic energy level that are the result of various types of motion of the atoms in a molecule. Transition between these states at a particular energy level does not involve a large change in energy, and typically is a non-radiative transition. In larger electronic transitions such as fluorescence, a molecule drops from the lowest vibrational level of the excited state to the highest vibrational level of the ground state. This emission is termed the Stokes shift between the S_1 and ground states.

Xenon lamp   A high-intensity lamp that produces a continuum of light from the ultraviolet to the near-infrared for sample excitation. A xenon lamp is classified as a broad-band source.

Xenon-lamp scan   A profile of the lamp output as a function of wavelength. The lamp scan is acquired using 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.

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In addition, the following journals may prove useful:
Analytical Chemistry
Biophysics and Biochemistry
Fluorescence

16: DataMax Initialization Files

This chapter contains printouts of the main initialization files for the Fluorolog®-Tau-3 in the ISA_INI directory for the lifetime system. These printouts are included for reference purposes only. The files should not be modified unless directed to do so by a Fluorescence Service Engineer.

HARDWARE.INI

This file specifies the type of communication, controller device and DataMax virtual instrument information. The default controller is the CCD-3000 using IEEE-488 communication.

```
; SAQ.ini file which defines the structures and the
; number of ports available
;   SERIAL=1
;   IEEE488=2
;   SCSI=3
[INFO]
NAME=CCD_LOCAL

[COMMUNICATION]
TYPE=2
CHAN_NUM=5

; Specifies the controller type
;   HC135=509 (Photon counter - Hamamatsu)
;   LOCKIN=508 (SR510, SR810)
;   SAQ_TRIAX=507 (Direct Drive)
;   DS_TRIAX=506 (Direct Drive)
;   CCD 3000=505 (IEEE-488 addr 5 only)
;   TempBath=504
;   CCD 2000=503 (see also [ISA CCD Driver] entry in SYSTEM.INI)
;   SPECTRAQ=502
;   DATASCAN=501 (DS1010 or CCD3000 External Input)
[CONTROLLER]
TYPE=505

; 2 CTI cards, 2 HV cards and a CCD controller
[PORTS]
NUM_MOT_CHANNEL_PORTS=8
NUM_ACQ_CHANNEL_PORTS=5
NUM_HV_CHANNELS=2
NUM_CONTROL_CHANNELS=0
NUM_LIGHT_CHANNELS=0
; Number of ports is max(8,5,3) --> 8
; mc_ports==> 1..8
; acq_ports==> 1..5
; hv_ports==> 1..2
;
; In api, would create 8 port objects with command channels
; equal to 0..7
;  mc_command_channels  0..7
;  acq_command_channels 0..4
;  hv_commmand_channels 0..1
```

LIFETIME.INI

This file defines the CCD-chip size, pixel-spacing and linearization parameters for the Spex-3D. This file is instrument-specific because linearization is performed on each new instrument.

```
[CCD]
XPixels=512
YPixels=512
XSpacing=240
YSpacing=240
;BorderLeftX=0
;BorderRightX=0
;BorderTopY=100
;BorderBottomY=100

[Instrument]
SPC_CenterX=500
SPC_CenterY=500
SPC_RangeX=400
SPC_RangeY=250
PolynomialXC1=-0.00002
PolynomialXC2=0.9877
PolynomialXC3=243.18
PolynomialYC1=0.000008
PolynomialYC2=1.2875
PolynomialYC3=124.57
```

CCDLOAD.INI

This file contains specific information about the CCD chip: the chip size, pixel spacing, and signal collection. The file also has limits set for shutter opening (exposure time) and allowable temperature range for the chip.

```
        ; file: CCDLOAD.INI
        ; CCD hardware (chip and controller) parameters
1       ; CCD Number

768     ; Base address of RISC board (300 hex = 768 dec)

        ; Number of pixels
512     ; total_active_x_pixels (in the horizontal direction)
512     ; total_active_y_pixels (in the vertical direction)
15      ; number_serial_pxls_before_active_area
15      ; number_serial_pxls_after_active_area
0       ; number_parallel_rows_before_active
0       ; number_parallel_rows_after_active

3       ; readout register location and direction
        ; add one from each of the following:
        ;       register location =     0       left
        ;                               1       top
        ;                               2       right
        ;                               3       bottom
        ;
        ; direction is relative to our standard configuration
        ; with the readout register in the left position
        ;       direction        =      0       top to bottom
        ;                               4       bottom to top
        ;
        ;
        ;   ^                                                                        */
        ;   |
        ;   *_____        *******->      _____         _____
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *  0   |       |  1   |       |  2   *        |  3   |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *_____|       |_____|       |_____*        <-*******
        ;                                   |
        ;                                   V
        ;
        ;
        ;                                   ^
        ;                                   |
        ;   _____        <-*******        _____    *    _____
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
        ;   *  4   |       |  5   |       |  6   *        |  7   |
        ;   *      |       |      |       |      *        |      |
        ;   *      |       |      |       |      *        |      |
```

```
;     *      |            |      |            |      *          |      |
;     *_____|            |_____|            |_____*          *******->
;     |
;     V
;
;
; Limit checking for driver calls
0        ; Minimum temperature in degrees K
300      ; Maximum temperature in degrees K

1                ; Minimum shutter time in milliseconds
400000000        ; Maximum shutter time in milliseconds

0        ; Minimum Gain
4        ; Maximum Gain

         ; Returned in driver call. Currently not used by the driver.
         ; pixel spacing/size in tenths of um
240      ;        horizontally
240      ;        vertically
```

17: Visual Inspection of the Isogyre Pattern

Introduction

This test examines the *isogyre* pattern created by the Pockels cell itself. The orientation and location of this pattern on the manual slit are primary indicators that the system is in near-perfect alignment. A dental mirror (diameter < 2.5 cm) is recommended. For a routine adjustment of the Pockels cell, skip this chapter; instead do step 1 in Chapter 11: *Maintenance*.

> ***Warning:*** *Do not attempt to do this procedure without permission of the Fluorescence Applications Department. The telephone number is (732) 494-8660 × 144.*

Procedure

1   Set the excitation monochromator to 540 nm.

2   Open all excitation slits to 4 mm.

3   Open the iris to its maximum aperture size.
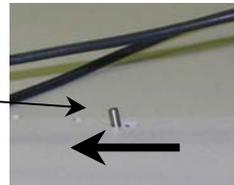
*Note: Some irises open to the **right**.*

4   Remove the lid to the modulator compartment.

5   Turn OFF the external high-voltage DC bias to the Pockels cell.

6   Using a 1/16" Allen wrench, remove the mask over the manual slit.

Note: Remove three of the screws holding the mask on, loosen the fourth one turn, and rotate the mask out of the way without removing it completely.

In this way, the isogyre pattern can be observed.

7   Remove the Pockels-cell mount from the light path.

8   Check that all detector HV-biases are OFF.

9   Close the manual slit.

10   Open the excitation shutter.

Green light should be visible passing through the modulator compartment, and centered on the blades of the manual slit. If

Dental mirror
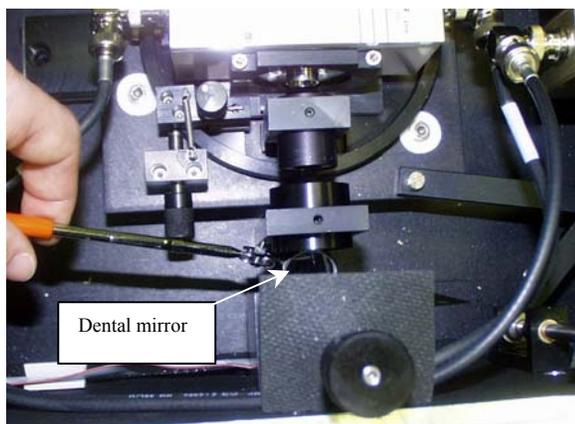
this image is not centered on the slit, there is an optical misalignment in the excitation monochromator, lamp housing, or the modulator compartment lenses.

11 Slowly close the iris while observing the isogyre image.

As the iris is closed, the image of the isogyre should shrink until only the crosshairs are visible. If the crosshairs are cut off, then the height of the Pockels cell needs adjustment.

Isogyre pattern on metal target in sample compartment.
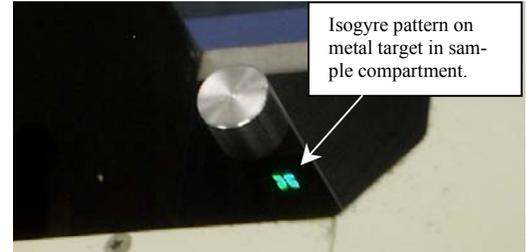
12 Move the Pockels-cell mount into the light path.

- The green light now should pass through the Pockels cell with an isogyre pattern visible on the blades of the manual slit.
- The crosshairs ought to be perfectly horizontal and vertical, crossing in the center of the slit. Crosshairs that are perfectly horizontal and vertical, but not centered, indicate a need to realign the Pockels cell. Move the two adjustments on the Pockels cell to center the isogyre pattern on the manual slit.

Good alignment of Pockels cell.

Requires height adjustment.

Requires lateral adjustment.

Call Fluorescence Service (polarizers or collimating lenses need realignment).

Adjustment of the horizontal knob.

Adjustment of the vertical knob.

13 Turn on the external high-voltage DC-bias sup-
ply **for a moment** to see how the isogyre pat-
tern rotates with this bias applied.

Properly aligned Pockels
cell

Rotation: poor polarizer
alignment around Pockels
cell

14 Turn off the external HV DC-bias supply to con-
tinue alignment.

> *Note: If you have any questions or difficul-
> ties with this procedure, call Fluorescence
> Applications at (732) 494-8660 × 144.*

18: Index

Key to the entries:

Times New Roman font	subject or keyword
Arial font	command, menu choice, or data-entry field
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