Chapter 5

Frequency Domain Fluorometry: Theory and Application

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Abstract

Frequency domain fluorometry is a widely utilized tool in the physical, chemical, and biological sciences. This chapter focuses on the theory of the method and the practical aspects required to carry out intensity decay, i.e., lifetime measurements on a modern frequency domain fluorometer. Several chemical/biological systems are utilized to illustrate data acquisition protocols. Data analysis procedures and methodologies are also discussed.

Key words Frequency domain fluorometry, Multifrequency phase-modulation fluorometry, Ethidium bromide, tRNA, Phasor plot, Human serum albumin (HSA)

1 Introduction

Fluorescence spectroscopy has proven to be an extremely valuable tool in a wide variety of fields ranging from biology to chemistry as well as medical diagnostics and material sciences. Its usefulness stems from the detailed information available from multiple fluorescent parameters, i.e., emission spectra, excitation spectra, polarizations/anisotropies, lifetimes, and quantum yields. While knowledge of all these fluorescent properties is important to fully understand the processes being investigated, the focus of this review is on time-resolved measurements, in particular intensity decay or lifetime measurements.

The absorption of light generates an excited state within femtoseconds and deactivation typically occurs on the order of picoseconds to tens of nanoseconds depending on the fluorophore and its surroundings. The newly generated population of photoexcited molecules decays exponentially from the S_1 electronic excited state (usually, although some exceptions exist) through emission of a photon, though other non-radiative processes usually deactivate the excited state also. The time it takes for the number of excited molecules to decay to $1/e$ of the excited state population is defined

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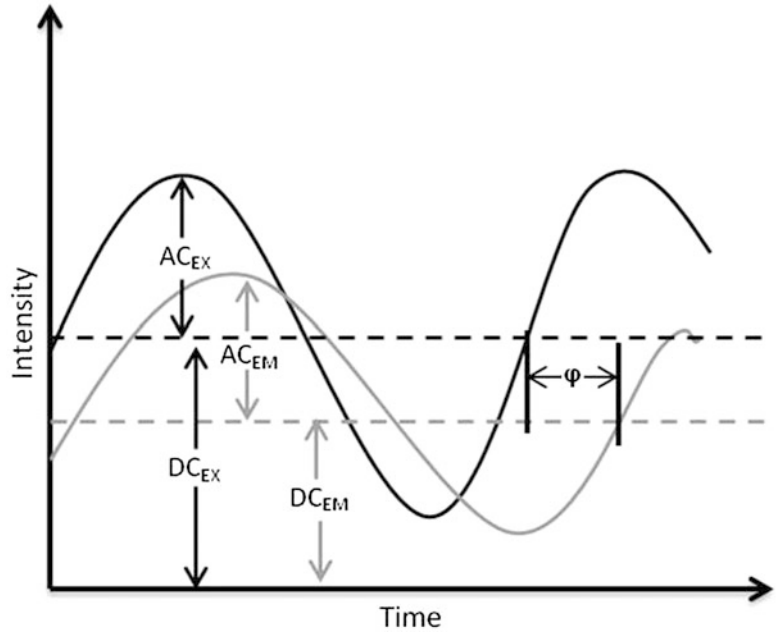

Fig. 1 Schematic representation of sinusoidally modulated exciting light (*black*) and the resulting sinusoidally modulated fluorescence (*grey*) delayed in phase and demodulated

as the excited state lifetime, τ, and is unique to a given molecule in a given environment [1]. This environmental sensitivity is one reason why the excited state lifetime is an important fluorescent property. Two principle methods, namely, frequency domain and time domain, are presently used to obtain time-resolved information. This chapter discusses the frequency domain method only and focuses specifically on data acquisition and analysis of intensity decay, i.e., lifetime data. We also describe practical aspects of the frequency domain method, also known as multifrequency phase and modulation fluorometry, by describing measurements on a simple fluorophore in solution (fluorescein), protein fluorescence (human albumin serum), and a ligand binding system (ethidium bromide-tRNA).

The first accurate frequency domain lifetime measurements were realized by Enrique Gaviola in 1926 [2, 3], while the mathematical theory behind frequency domain fluorometry was first described by Dushinsky in 1933 [4]. Dushinsky determined that a fluorophore, with a single exponential excited state decay time, τ, excited by light sinusoidally modulated at high frequencies, $E(t)$, emits light, $F(t)$, sinusoidally modulated at the same angular frequency, ω (equal to $2\pi f$, where f is the linear light modulation frequency), as the exciting light, but delayed in phase (φ) and demodulated as depicted in Fig. 1 and expressed as:

$$F(t) = F(0)[1 + M_F \cos(\omega\tau + \varphi) \tag{1}$$

The relative modulation of the emission, M, is given by the ratio of the AC/DC components for the exciting, M_E, and emitted light, M_F:

$$M_E = \left(\frac{AC_E}{DC_E}\right) \quad \text{and} \quad M_F = \left(\frac{AC_F}{DC_F}\right) \tag{2}$$

$$M = \left(\frac{M_F}{M_E}\right) \tag{3}$$

The correlation between the phase delay, modulation, and excited state lifetime is given by:

$$\tan\varphi = \omega\tau_p \tag{4}$$

$$M = \frac{1}{\sqrt{1 + (\omega\tau_m)^2}} \tag{5}$$

where τ_p and τ_m are independently determined and represent the phase and modulation lifetimes, respectively. For systems characterized by a single exponential decay, the phase and modulation lifetimes are identical and are independent of the light modulation frequency. For more complex systems with multiple fluorescing species, τ_p is less than τ_m and both are dependent upon the modulation frequency. In 1969, Weber and Spencer [5] described a frequency domain fluorometer which utilized the principle of cross-correlation, wherein the light source (in their case a xenon arc passing through a Debye-Sears light modulation tank) was modulated at one frequency, while the detector was modulated at a slightly different frequency. The "cross-correlation" frequency was typically low (about 40 Hz) such that a low-frequency signal could be isolated, which was far easier to work with than the original high frequencies. In this instrument only two light modulation frequencies were available. In 1983, Enrico Gratton introduced a true multifrequency fluorometer by passing laser light (originally an argon ion laser) through a Pockels cell [6]. Table 1 gives an example of τ_p and τ_m determined for a solution containing two components of 12.08 and 1.38 ns in the proportions of 53 and 47 % (fractional intensities, i.e., contributions to the total intensity), respectively. These differences between τ_p and τ_m, and their frequency dependence, permit one to extract the underlying lifetime components.

Weber [7] defined the terms S and G as

$$\varphi = \tan^{-1}(S/G) \tag{6}$$

$$M = (S^2 + G^2)^{-1/2} \tag{7}$$

Table 1
τ_p and τ_m values obtained for a fluorophore mixture (details in text)

Frequency (MHz)	τ_p (ns)	τ_m (ns)
5	6.76	10.24
10	6.02	9.70
30	3.17	6.87
70	1.93	4.27

where

$$S = \sum_i f_i M_i \sin \varphi_i \tag{8}$$

$$G = \sum_i f_i M_i \cos \varphi_i \tag{9}$$

Resolution of the underlying lifetime components in a mixture, and assignment of their relative contributions to the signal, requires fitting the frequency dependence of the phase delay and demodulation to a specific decay scheme (*see* **Note 1**). Two commercially available programs for analysis, Vinci Multidimensional Spectroscopy software and Globals for Spectroscopy, will be discussed later in the Analysis section.

The relationship between S and G provides another way to treat the data known as the phasor method [8]. This model-less approach offers immediate graphical evidence of lifetime heterogeneity as well as possible excited state reactions present in the sample. In recent years, the phasor method has gained in popularity as a tool used in fluorescence lifetime image microscopy (FLIM), but it is also useful in cuvette studies [9–11]. We should also note that this approach was introduced for FLIM studies by several groups who designated the graphs as phasor plots [12], AB plots [13], and polar plots [14]. A plot of S versus G values for a particular modulation frequency is characterized by a vector of length equal to M with an angle from the x-axis equal to φ as shown in Fig. 2. For a system with a single exponential lifetime, the frequency dependence of the vector forms a semicircle with a radius of ½ known as the universal circle. For systems that are multi-exponential, the phasor point is restricted to the inside of the universal circle [9, 10]. Thus, a phasor plot immediately indicates lifetime heterogeneity.

2 Materials

The frequency domain time-resolved measurements described in this chapter were carried out on a Chronos (ISS, Champaign, IL) frequency domain fluorometer. This instrument is designed to

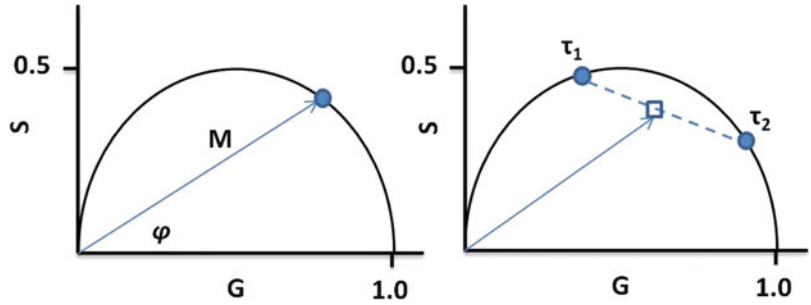

Fig. 2 Phasor plot illustrating the basic concept of a phasor point at one frequency for a system with a single exponential lifetime (*left*) and for a system with a double exponential lifetime (*right*)

accommodate light-emitting diodes (LEDs), laser diodes (LDs), as well as CW and mode locked lasers. Clearly, the sample under investigation, be it a protein or a simple fluorophore, must be as pure as possible. Equally important, though, is that the reagents used for the lifetime measurements, both sample and reference solvents, must be of the highest possible purity to eliminate errors due to background fluorescence. Solvent impurities should always be checked before measurements (*see* **Note 2**). Also, one must keep in mind that additional sample purification may be required, and one should not necessarily trust the purity of commercial compounds. It is important to follow proper protocol for storage, usage, and disposal for all reagents. It is also very important to know the optical transparency of the cuvettes being used. Plastic or glass cuvettes typically absorb light in the near-UV (<330 nm) wavelength range. For UV-visible range experiments, quartz cuvettes are recommended and were utilized for the lifetime experiments described here.

3 Methods

All multifrequency phase and modulation lifetime measurements involve choosing an excitation light source and optical filters, sample preparation, reference preparation, selecting an optimal frequency range, data acquisition, and analysis [8, 15, 16]. Below we have separated each of these required steps for additional discussion and to provide experimental details for the acquisition of lifetimes for three systems, namely, fluorescein in 0.01 M NaOH, intrinsic protein fluorescence (HSA), and EB-tRNA binding. Finally, the notes section provides additional information and nuances one learns with experience.

3.1 Experimental Considerations

One of the first tasks in any lifetime measurement is choosing the excitation wavelength. The probability of a species to absorb a photon of an appropriate energy to generate an excited state is proportional to the molecular absorption coefficient (extinction

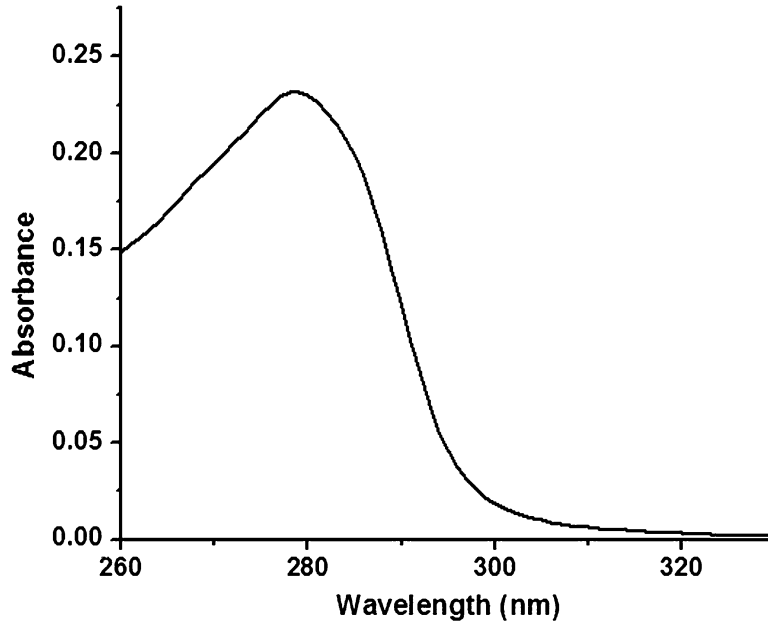

Fig. 3 UV-vis absorption spectrum of 15 μM HSA in 20 mM HEPES, pH 7.2

coefficient), and hence the absorption spectrum of the sample should be known. For instance, the absorption spectra of HSA in buffered solution displays a prominent absorption band centered near 278 nm, with absorption out to about 315 nm as shown in Fig. 3 (*see* **Note 3**). Therefore, an excitation source anywhere from 250 to 315 nm may theoretically be used (*see* **Note 4**). Fortunately, with the emergence of light-emitting diodes (LEDs) and laser diodes (LD), the choice of available wavelengths has increased and the costs have decreased. Together, LEDs and LDs provide a wavelength range that spans the entire UV and visible range of the spectrum (*see* **Note 5**). Unlike xenon arc lamps and CW lasers implemented in earlier instruments, the input voltage of the LEDs and LDs can be directly modulated [8, 15, 16]. While there are advantages to these types of light sources, LDs and LEDs often emit over a small range of wavelengths and require additional optical components to increase wavelength selectivity. The best types of optical filters to achieve a narrow excitation wavelength range of a few nanometers are interference filters. The narrow wavelength range of interference filters are specified by its wavelength plus the FWHM (full width at half max), i.e., a 300 nm interference filter with FWHM of 5 nm passes 50 % of the laser intensity at 295 and 305 nm as it does at 300 nm. The excitation wavelength, interference filter, and emission filter for the three systems are provided in Table 2 (*see* **Note 6**). The "magic angle" conditions [17, 18] for the excitation and emission polarizers are also summarized in Table 2 (*see* **Note 7**).

Table 2
Summary of the light source and instrument configuration for the three example systems

Sample	λ_{ex} (type/wavelength)	Filter type	Magic angle configuration (excitation/emission)	Frequency range (MHz)
Fl in 0.1 M NaOH	LED/471 nm	LP 515 nm	0°/55°	10–100
HSA 20 mM HEPES, pH 7	LD/300 nm	LP 305 nm	–/35°	10–150
EB-tRNA	LED/471 nm	LP 525 nm	0°/55°	1–100

Table 3
Summary of the sample preparations for the three example systems

Sample	Solvent	Temperature (°C)
500 nM Fl	0.01 aqueous NaOH	25
60 μM HSA	20 mM HEPES, pH 7.0	20
1 μM EB	20 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, pH 7.0	25
1 μM EB, 4.0 μM tRNA	20 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, pH 7.0	25

3.1.1 Sample Preparation

As already mentioned, optical grade solvents and highest purity compounds are required for reliable lifetime data. All experiments described here were performed in a 1 cm cuvette with a final volume of 2 mL. Fluorescein was purchased from Invitrogen and used without further purification. HSA was purchased from Sigma and purified according to the protocol described in the review by James et al. [10]. Yeast tRNA and ethidium bromide solution (10 mg/mL) were purchased from Invitrogen. Table 3 is a complete summary of the sample preparations.

3.1.2 Reference Compound

After the excitation wavelength has been determined and the sample prepared, a reference compound can be chosen. In the early days of phase-modulation fluorometry [5], a light-scattering solution, such as glycogen in water, was typically utilized to provide a reference signal from which to measure the phase shift and excitation light modulation. As higher frequencies became available, it was apparent that the PMTs being utilized could demonstrate considerable "color effects," which distorted the phase lifetime [19]. Even though better PMTs became available, the use of reference standards became common, i.e., lifetime standards with emission characteristics similar to those of the sample such that the PMT could always observe the same wavelength.

Table 4
Common fluorescence lifetime standards used in frequency domain spectroscopy: from refs. 1 and 20

Compound	Solvent	Lifetime (ns)	λ_{ex} (nm)	λ_{em} (nm)
2-aminopurine	Water	11.34	290	380
Anthracene	Methanol	5.1	295–360	375–442
Coumarin 153	Methanol	4.3	295–442	495–550
Dimethyl POPOP	Ethanol	1.45	300–400	390–560
Erythrosine B	Water	0.089	488–568	550–580
Fluorescein	NaOH/water	4.1	400	490–520
Indole	Water	4.49	290	360
NATA	Water	3.1	295–309	330–410
POPOP	Cyclohexane	1.12	295–360	380–450
p-Terphenyl	Ethanol	1.05	284–315	330–380
Rhodamine B	Water	1.74	488–575	560–630

Virtually, any fluorescent species with monoexponential decay can be deemed a standard; however, there are a few other qualities that make particular fluorophores preferred lifetime standards (*see* **Notes 8** and **9**) [20]. Over the years a detailed list of dependable fluorescence lifetime standards has been compiled. Some of those standards are summarized in Table 4 with the excitation wavelength, emission range, and lifetime.

One must choose a standard that displays sufficient emission intensity under the optimal instrument configuration designed for the sample, i.e., excitation wavelength, optical filters, and PMT voltage. With that being said, the reference emission intensity should be comparable to the sample intensity. Comparable counts on the detector will reduce inconsistencies in gain settings and signal to noise between the reference and sample which can be a source of error [1]. Table 5 lists the references utilized for the example systems.

3.1.3 Frequency Range

The frequency range of a multifrequency instrument should scale from kilohertz to hundreds of megahertz. The LD and LEDs available from ISS for the ChronosFD have a frequency response up to 600 MHz—although the frequency range of the various light sources differs. The idea, however, is not to utilize the entire frequency range of the instrument but rather to focus on the optimal range. The ideal multifrequency range for determining decay lifetimes by analysis of both phase delay and demodulation requires multiple data points before and after the optimal frequency. For a known lifetime, the optimal frequency is easy to establish, that is, when $\omega\tau = 1$, keeping in mind that $\omega = 2\pi f$ and

Table 5
Summary of the reference solutions used for each of the three example systems

Sample system	Reference	Solvent	Reference lifetime (ns)
Fl	Fluorescein	0.01 aqueous NaOH	4.1
HSA	p-Terphenyl	Ethanol	1.05
EB-tRNA	Ethidium bromide	DI H_2O	1.71

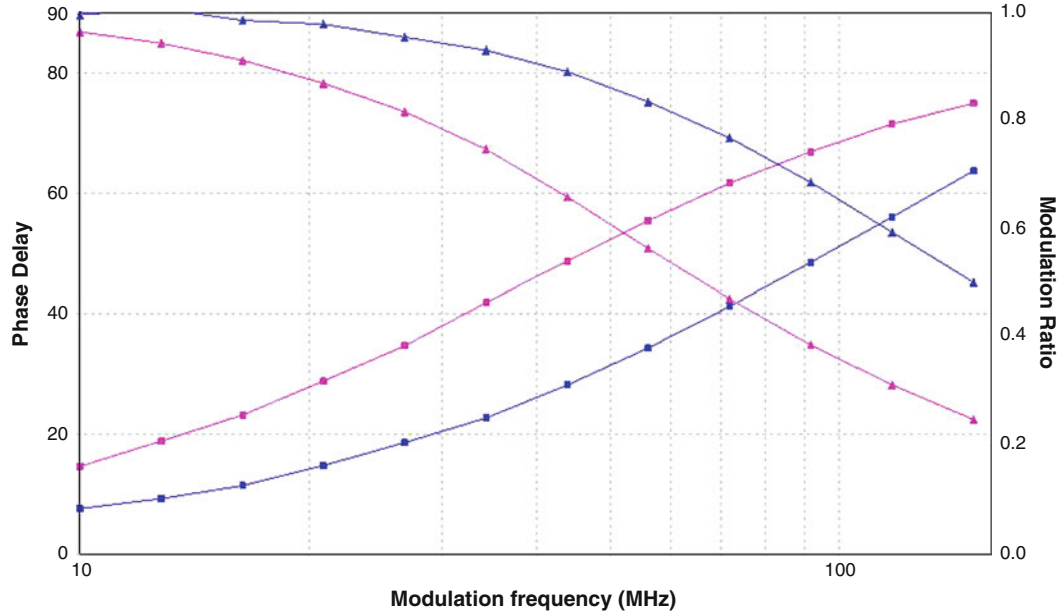

Fig. 4 Phase (*square*) and modulation (*triangle*) plot of fluorescein (*blue*) and ethidium bromide (*pink*)

$f = 1/2\pi\tau$, where τ is the excited state lifetime [1]. For example, the optimal frequency range for a lifetime like that of fluorescein (4.1 ns) is 10–100 MHz (optimal frequency of 40 MHz). On the other hand, that is not an optimal range for ethidium bromide (1.71 ns), which would have an optimal frequency of ~100 MHz, as shown in Fig. 4. We usually prefer to use a range which encompasses around 10–15° of phase shift up to around 70° of phase shift. More often than not, the general range of the experimental lifetime is known and choosing the frequency range becomes more apparent with experience. Table 2 presents the frequency range used for each of the example systems.

3.2 Data Analysis and Applications

Data Analysis is the most challenging aspect of any lifetime measurement. It involves selecting the appropriate decay scheme, evaluating the fit, and deciding whether or not the results are reasonable. Gregorio Weber solved the exact analytical solution to extract N lifetimes and fractional intensities from phase and

modulation data at N frequencies [7]. However, the algorithm became impractical for data with increasing number of frequencies due to the necessity for increased precision [8]. With development of true multifrequency instruments [6], lifetime values are extracted from phase and modulation data using a nonlinear least square method [8]. The goodness of fit is determined by reduced chi-squared (χ^2) value using

$$\chi^2 = \sum \left(\left[(P_c - P_m)/\sigma_p \right]^2 + \left[(M_c - M_m)/\sigma_m \right]^2 \right) / (2n - fp - 1) \tag{10}$$

where P and M are the phase and modulation data, the subscripts c and m indicate the calculated and measured values, and the σ_p and σ_m refer to the standard deviations. The number of frequencies is n, and fp is the number of free parameters. The sum in Eq. 10 is over n frequencies. Two commercial programs available for data analysis and phasor plots are Globals for Spectroscopy and Vinci2 (*see* **Note 10**). Analysis using these programs first requires the choice of a decay scheme (single, multi-exponential, discrete or distributions, etc.) followed by the selection of estimated parameters (lifetimes, fractional intensities, etc.). Satisfactory fits will have a reduced χ^2 value close to 1 [1]. The standard deviations generally used for phase shift and modulation ratio are typically 0.2° and 0.004, respectively, but of course those values may be changed to reflect the particular instrumentation and conditions. The data analysis for each of the three systems is described below.

3.2.1 Fluorescein, 0.01 M NaOH

To begin, data analysis for fluorescein in solution is demonstrated in Vinci2-Analysis as shown in Fig. 5a. The ISS data files can be opened in this program without any conversion of the file type (we note that with Globals the file type must be modified as discussed further below). Once the file is open, one selects the Fitting tab, and chooses lifetimes, which will open the options for the possible decay schemes. For fluorescein in basic solution, the decay scheme should be discrete monoexponential (in this case one will find that adding additional exponential terms does not improve the fit). After the scheme is chosen, the fit is carried out and the results, including residuals, are displayed (Fig. 5b). To view a complete summary of the results, select the Fit Report tab (Fig. 5c). Next, to convert data to a phasor plot, select View followed by phasor plot. The phasor plot of this sample is an ideal example of how one can immediately ascertain that the sample is single exponential, namely, all of the phasor points lie directly on the universal circle as predicted for a single exponential decay (Fig. 6).

3.2.2 HSA, 20 mM HEPES, pH 7

HSA demonstrates a slightly more complex system, namely, tryptophan fluorescence in a protein matrix rather than a single fluorophore free in an isotropic environment, i.e., in solution. The analysis described is performed in Globals for Spectroscopy. In order to use Globals for fitting ISS data files, they must be

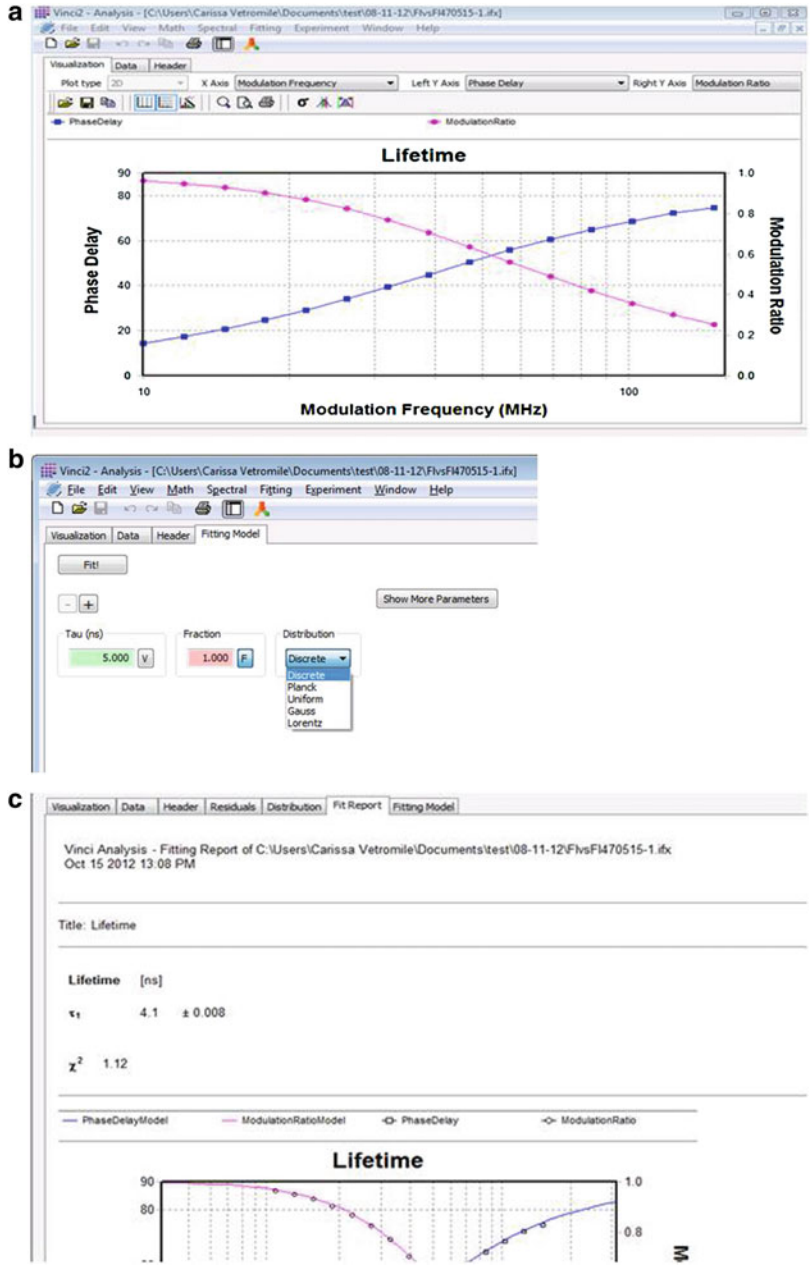

Fig. 5 Phase and modulation plot of fluorescein (**a**), page where one selects a decay model (**b**), and the fit report for a single exponential decay model (**c**) shown using Vinci2-Analysis (ISS)

converted to ISS Lif format. To do this one opens the file in Vinci2, then selects file, export, ISS Lif format (binary), and save. One then opens Globals and inserts the recently saved file. Before selecting the model, be sure to change Rec number to 1 as shown in Fig. 7. It is recommended that the reader refers to the tutorials on model selecting provided by Globals to become familiar with the program,

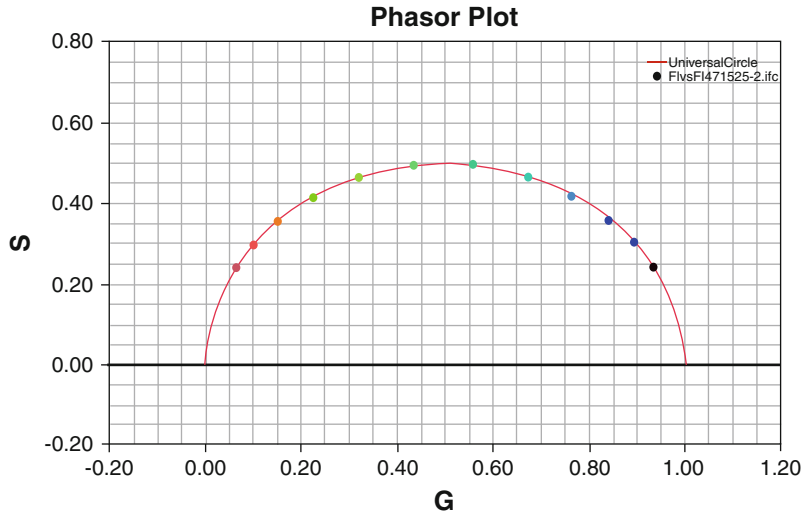

Fig. 6 Phasor plot of fluorescein in 0.01 M NaOH

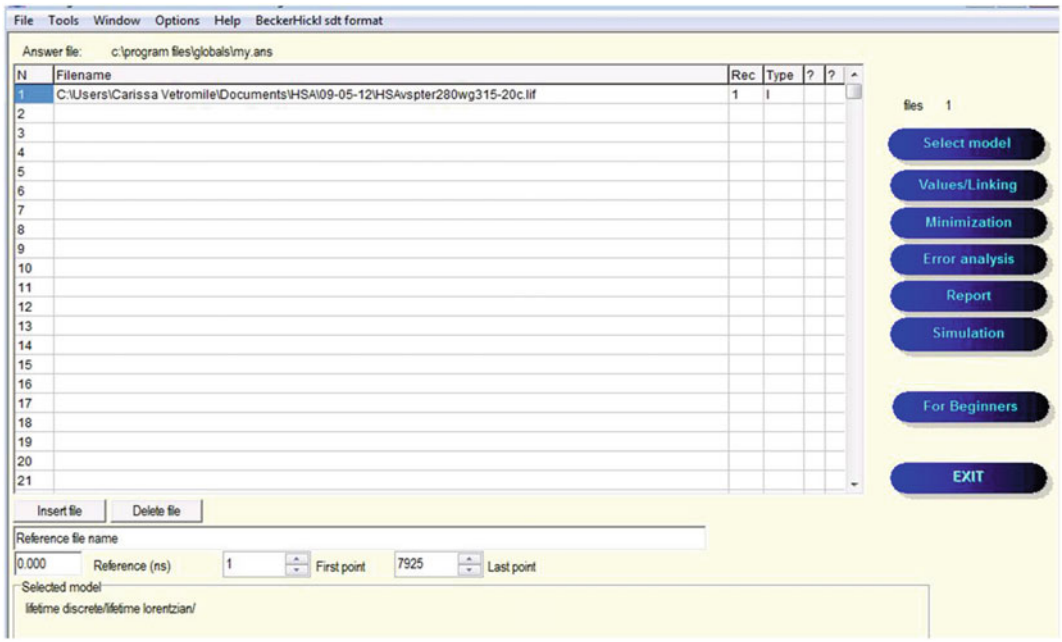

Fig. 7 Image of Globals for Spectroscopy workstation where one inserts and analyzes data files

which can be found under the help tab on the program or at http://www.lfd.uci.edu/globals/tutorials/.

In cases where the lifetime is unknown, we prefer to begin the analysis with a simple single exponential decay scheme. From Fig. 8, it is clear that HSA tryptophan fluorescence does not fit to a single exponential, as is also evident from the phasor plot in Fig. 8 ($\chi^2 = 63.09$). One nice feature of Globals is that the phasor plot

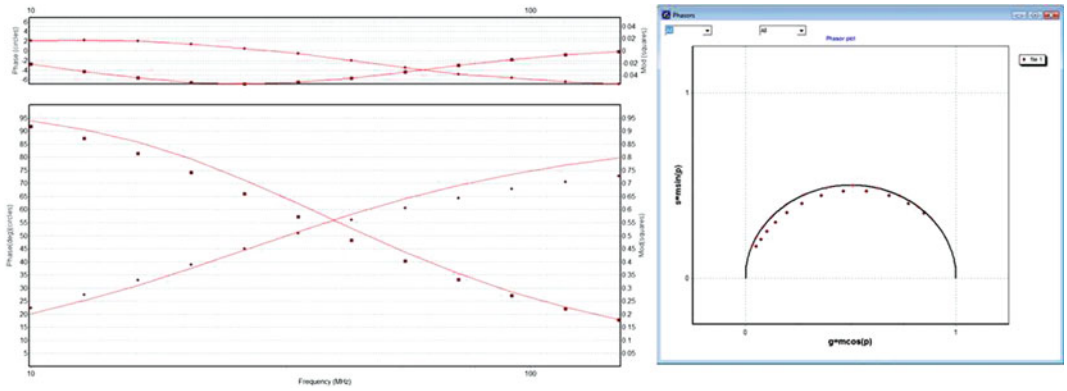

Fig. 8 Phase and modulation plot of HSA in 20 mM HEPES, pH 7.2 fit to a single exponential decay model (*left panel*) and the resulting phasor plot (*right panel*). $\chi^2 = 63.03$

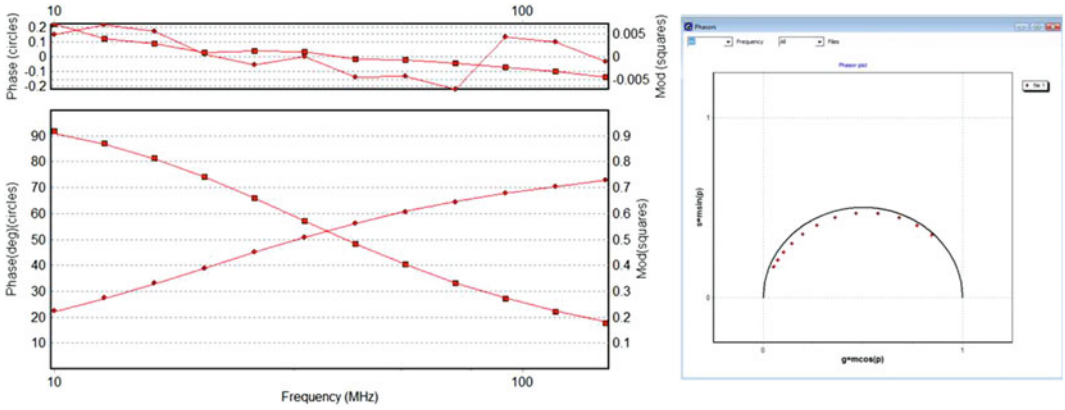

Fig. 9 Phase and modulation plot of HSA in 20 mM HEPES, pH 7.2 fit to heterogeneous decay model described by a fast discrete exponential lifetime and a longer lifetime with a Lorentzian distribution (*left panel*) and the resulting phasor plot (*right panel*). $\chi^2 = 0.61$

also displays the fit on the plot (+). When dealing with a sample that is unknown, it can be beneficial to check the phasor plot first before fitting the data. If the phasor plot reveals the lifetime is not single exponential, then the next step is to systematically try fitting the phase and modulation data to different decay schemes, i.e., a single lifetime distribution, two discrete lifetimes, one discrete on distribution, and so on.

Fluorescence lifetime measurements of the single tryptophan in HSA have been carried out by many labs, and the general agreement is that the tryptophan exhibits a heterogeneous lifetime [21]. We found that under our conditions the data fits best to two components, a fast discrete lifetime (1.03 ns) accompanied with a longer lifetime (6.73 ns) with a Lorentzian distribution (width = 1.03) as shown in Fig. 9 ($\chi^2 = 0.63$). Lifetime distribution models for protein fluorescence were discussed in detail by Alcala et al. [22–24].

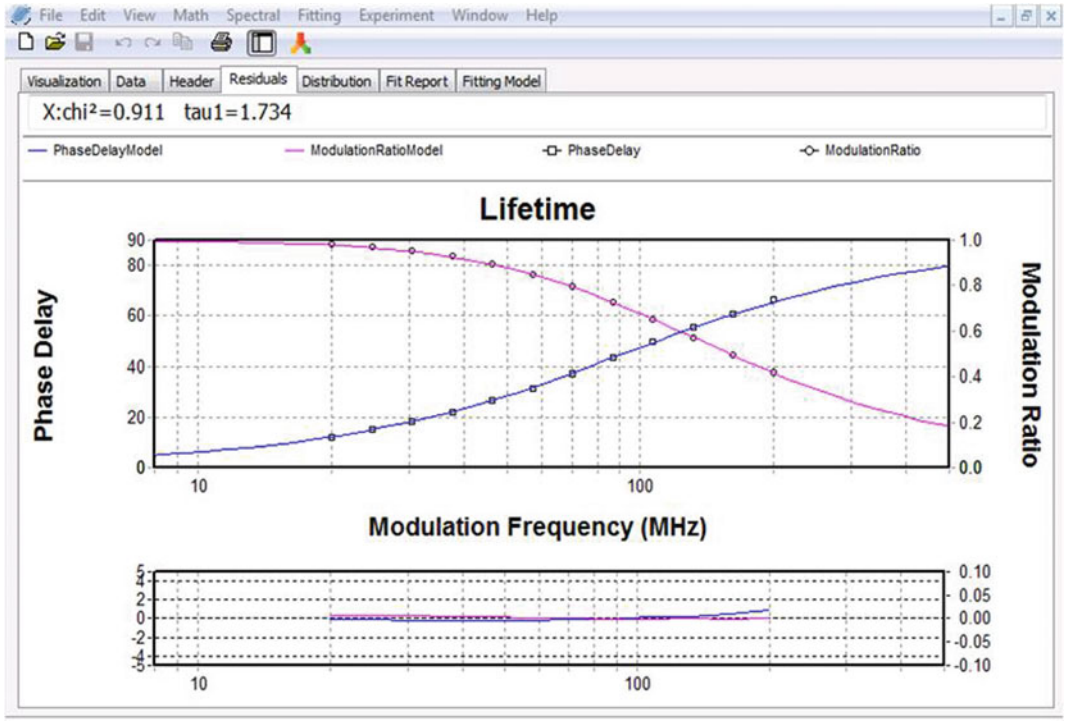

Fig. 10 Phase and modulation plot of ethidium bromide fit to a single exponential decay model

3.2.3 EB-tRNA

Another application of frequency domain fluorometry is toward the study of ligand binding. The final example system, ethidium bromide binding to yeast tRNA, is a great instance of this due to the significant increase in the fluorescence lifetime of ethidium bromide upon binding to tRNA. Previously reported fluorescence lifetime measurements of ethidium bromide binding to tRNA suggest the existence of a strong binding site along with some weaker binding sites [27]. To keep this exercise from becoming too complex, we will investigate ethidium bromide in the absence and presence of excess tRNA, to ensure only the strong binding site is occupied.

To begin one examines ethidium bromide in the absence of tRNA under the conditions described in Table 3. The measured phase and modulation data for ethidium bromide fits to a single exponential decay scheme with a fluorescence lifetime of 1.73 ns as shown in Fig. 10. Next, to investigate the effects of the strong binding site, one must fit the phase and modulation data of ethidium bromide with excess tRNA. Using conditions described above, the data should also fit to a single exponential decay model (all ethidium bromide molecules bound) but with a significantly longer lifetime (Fig. 11). Once again, the phasor plot of both ethidium bromide in the absence and presence of excess tRNA provides another excellent example of how one can instantly observe the differences between samples and distinguish the heterogeneity of an unknown sample (Fig. 12).

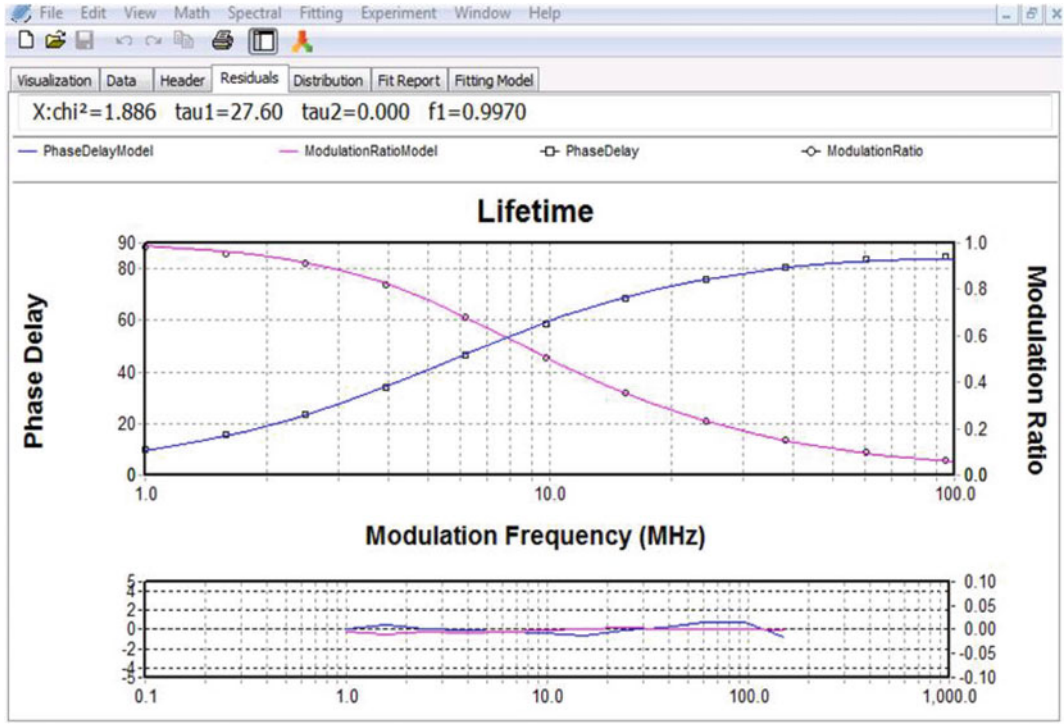

Fig. 11 Phase and modulation plot of ethidium bromide in the presence of excess yeast tRNA fit to a single exponential decay model

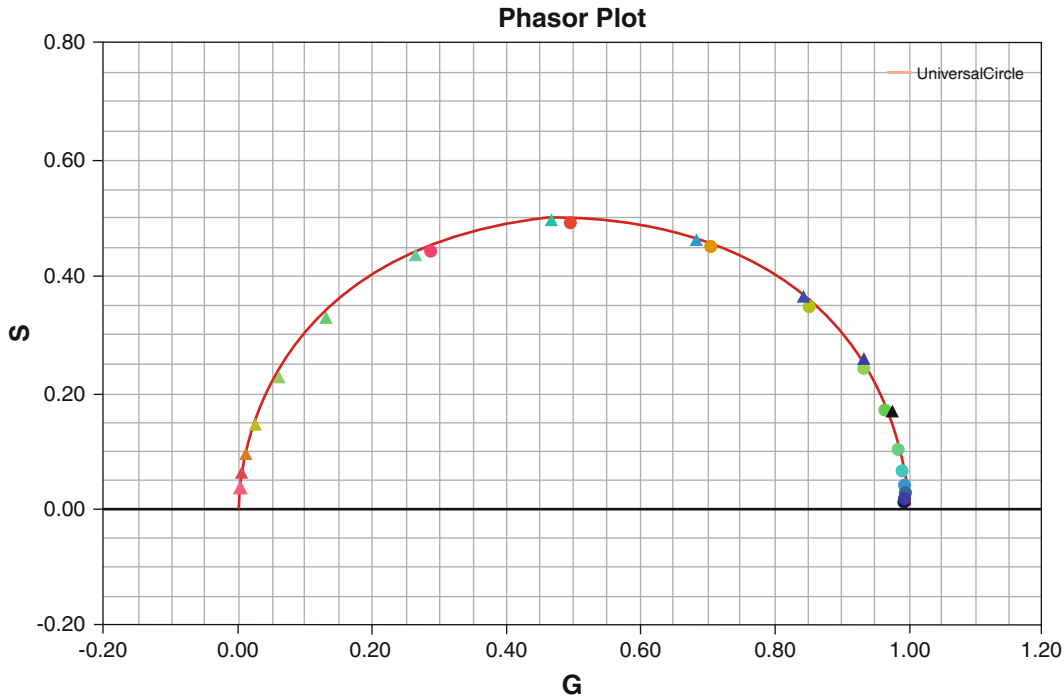

Fig. 12 Phasor plot of ethidium bromide in the absence (*circles*) and presence (*triangles*) of excess yeast tRNA

4 Notes

1. Traditionally frequency domain practitioners report the fractional intensity, f_i, of each lifetime component, i.e., the contribution of the ith component to the total fluorescence intensity. The sum of all fractional intensities equals one. In contrast, time domain practitioners generally report pre-exponential terms, α_i, for each lifetime component. These α_i values are related to the number of emitting molecules, not directly their contributions to the observed emission intensity. Assuming the quantum yields for each fluorescing species are proportional to their lifetimes, fractional intensities and pre-exponential factors can be converted using

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \tag{11}$$

   For instance, using the example in the text, one can calculate the ratio of the pre-exponential components by

$$0.53 = \frac{12.08\alpha_1}{12.08\alpha_1 + 1.38\alpha_2}$$

$$\frac{\alpha_1}{\alpha_2} = 0.13 \cong 1/8$$

   Thus, for this example there are ~8 times as many molecules with a lifetime of 1.38 ns as there are molecules with a lifetime of 12.08 ns.

2. After the instrument is set up for the measurements, i.e., exciting wavelength and filters are in position, a cuvette with sample is placed in the sample holder and the PMT voltages and/or gain settings are adjusted to give an appropriate signal. Then, this sample cuvette should be momentarily replaced by a cuvette containing only the solvent. The shutters to the light source and detectors are opened using the Vinci2 software and the solvent signal is checked. This signal should be the same as (or at least very close to) the dark configuration (detector shutter open but light source shutter closed); if not then the solvent may be contaminated. In our experience, for example, ethanol frequently contains contaminants that exhibit fluorescence upon UV or mid-UV excitation. Even if the ethanol is only being used for the reference compound, spurious fluorescence will be problematic since the lifetime of this background fluorescence will be convoluted in with the reference lifetime. We note that even a very clean solvent can give rise to a Raman peak, which may pass the emission filter, but usually the Raman peak is very weak and is seldom a problem with lifetime measurements.

3. It is not always necessary to excite at the maximum absorption band since the emission of a photon is from the lowest excited

electronic state, regardless of which excited state is initially populated (there are a few exceptions). That being said, the more probable the transition, the larger the population of excited state molecules, and the greater the florescence intensity. In addition to the excitation wavelength, the sample concentration is another way to adjust the observed fluorescence intensity; however, one must be careful not to introduce inner filter effects to the sample. Inner filter effects can occur in samples sufficiently concentrated such that reabsorption and reemission of photons can occur [1]. It is best to work with solutions having optical densities less than 0.1 at the excitation wavelength.

4. This choice of 280 versus 300 nm excitation is always in question when investigating intrinsic protein fluorescence [23]. Intrinsic protein fluorescence originates from the three aromatic amino acids: tryptophan, tyrosine, and phenylalanine. In 1957, Weber and Teale published the first photophysical characterization of these aromatic amino acids and influenced the foundation for investigating proteins with fluorescence techniques [24]. Tryptophan, tyrosine, and phenylalanine have different molar extinction coefficients and quantum yields [15]; nevertheless, excitation at 280 nm results in significant emission from all tyrosine and tryptophan (for a more detailed discussion of this topic, *see* ref. 15). The contribution to the total intensity from each is not equal, with tryptophan fluorescence typically dominating. It was also established by Gregorio Weber that energy transfer from tyrosine to tryptophan can occur in proteins, a process which clearly complicates the excited state decay kinetics. Fortunately, tryptophan is the only aromatic amino acid with significant absorption at 300 nm, and excitation at this wavelength can be used to remove the effects of energy transfer from tyrosine and promote tryptophan selectivity [25, 26]. Unfortunately, in the vast majority of single tryptophan protein studies, the excited state lifetime is not characterized by a single exponential decay. The complexity of tryptophan fluorescence in proteins is beyond the scope of this chapter and readers are referred to ref 15 and references therein for more information.
5. The ISS website provides a list of available light sources for ChronosFD. LED wavelengths range from 280 to 520 nm, while LD wavelengths range from 405 to 830 nm.
6. Optical filters are often used in lifetime measurements to ensure that the detected light is from the fluorophore under investigation and not parasitic or scattered light. There are many types of optical filters including bandpass, interference, and longpass (cut-on) filters. Both bandpass and interference filters allow a specific range of wavelengths to pass through. The wavelength range of bandpass filters are generally 50–100 nm, while

interference filters have a more narrow range, typically 5–10 nm. Longpass filters block light at lower wavelengths until a specific range is reached and then allow longer wavelengths to pass. Before running a measurement, it is always a good idea to test whether a chosen emission filter is blocking all the excitation light. To test the filter, it should be placed in the excitation path with the emission and excitation shutters opened. If the filter is good, the observed signal should be on the same order as the dark signal.

7. When fluorescence is monitored at 90° to the excitation, as in most instruments, the vertically polarized molecules are favored over the horizontally polarized molecules since one of the horizontally polarized populations cannot be observed and as a result the measured lifetime is affected if the emission is polarized. To reduce this effect, "magic angle" configurations of the polarizers in the excitation and emission pathway can be used [17]. There are four configurations which yield equivalent results: (1) 35° excitation, no emission polarizer; (2) no excitation polarizer, 35° emission polarizer; (3) 55° excitation polarizer, 0° emission polarizer; and (4) 0° excitation polarizer, 55° emission polarizer. The configurations can be interchanged depending on what works best for the users' instrument and sample.
8. Nine laboratories tested the quality of multiple fluorescence standards; before starting they summarized a list of characteristics all reliable standards should possess: (1) display single exponential decay regardless of excitation or emission wavelength, (2) good quantum yield, (3) considerable Stokes shift, (4) characteristic lifetime should be on the same order as sample, and (5) must be chemically stable and photostable [20].
9. Lifetime standards may also be used to test for systematic errors. It is a good rule of thumb to test the accuracy of the instrument using a well-known fluorescence standard as a sample and reference before starting an unknown sample to insure the instrument configuration will yield accurate results.
10. Globals for Spectroscopy was developed by the Laboratory for Fluorescence Dynamics (LFD). The software is an extremely valuable tool for fluorescence data analysis including a number of methods not mentioned here. Globals can be downloaded from the LFD website at http://www.lfd.uci.edu/globals/. In addition, tutorials can be found on the LFD website to assist in using the program.

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