Fluorescence Lifetime

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Principles

The fluorescence lifetime is a measure of the time a fluorophore spends in the excited state before returning to the ground state by emitting a photon [1]. The lifetimes of fluorophores can range from picoseconds to hundreds of nanoseconds. A list of the some commonly used fluorophores and their fluorescence lifetimes are given in Table 1.

Fluorophore	Lifetime [ns]	Excitation Max [nm]	Emission Max [nm]	Solvent
ATTO 655	3.6	655	690	Water
Acridine Orange	2.0	500	530	PB pH7.8
Alexa Fluor 488	4.1	494	519	PB pH7.4
Alexa Fluor 647	1.0	651	672	Water
BODIPY FL	5.7	502	510	Methanol
Coumarin 6	2.5	460	505	Ethanol
CY3B	2.8	558	572	PBS
CY3	0.3	548	562	PBS
CY5	1.0	646	664	PBS
Fluorescein	4.0	495	517	PB pH7.5
Oregon Green 488	4.1	493	520	PB pH9
Ru(bpy)2(dcpby)[PF6]2	375	458	650	Water
Pyrene	> 100	341	376	Water
Indocyanine Green	0.52	780	820	Water
Rhodamine B	1.68	562	583	PB pH7.8

Table 1. Commonly used fluorophores and their fluorescence lifetimes.

If a population of fluorophores is excited, the lifetime is the time it takes for the number of excited molecules to decay to $1/e$ or 36.8% of the original population according to:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau} \qquad [1]$$

As shown in the intensity decay figure (Figure 1), the fluorescence lifetime, t, is the time at which the intensity has decayed to $1/e$ of the original value. The decay of the intensity as a function of time is given by:

$$I(t) = \alpha e^{-t/\tau} \qquad [2]$$

Where $I(t)$ is the intensity at time t, α is a normalization term (the pre-exponential factor) and τ is the lifetime. Knowledge of the excited state lifetime of a fluorophore is crucial for quantitative interpretations of numerous fluorescence measurements such as quenching, polarization and FRET.

Excited state lifetimes have traditionally been measured using the “time domain” method or the “frequency domain” method.

Time-domain method

In the time domain method, the sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally, these short light pulses were generated using flashlamps that had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter.

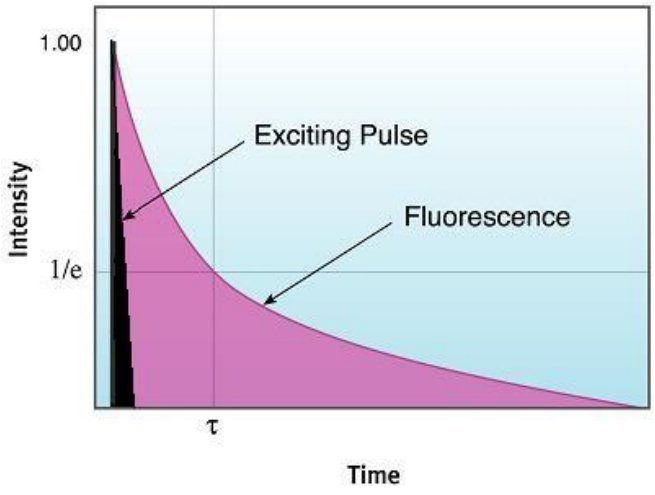

Figure 1. Representation of the fluorescence decay following excitation with a short pulse.

If the decay is a single exponential and the lifetime is long compared to the exciting light, then the lifetime can be determined directly from the slope of the curve. If the lifetime and the excitation pulse width are comparable, some type of deconvolution method must be used to extract the lifetime.

Great effort has been expended on developing mathematical methods to “deconvolve” the effect of the exciting pulse shape on the observed fluorescence decay (see, for example, many chapters in [2]). With the advent of very fast laser pulses these deconvolution procedures became less important for most lifetime measurements, although they are still required whenever the lifetime is of comparable duration to the light pulse.

Frequency-domain method

In frequency-domain the excitation light is described by:

$$E(t) = E_0\left[1 + M_E \sin \omega t\right] \qquad [3]$$

where $E(t)$ and E_0 are the intensities at time t and time 0, M_E is the modulation factor and $\omega = 2\pi\nu$ is the modulation frequency of the light beam. Figure 2 displays schematically the excitation intensity against time and the fluorescence against time.

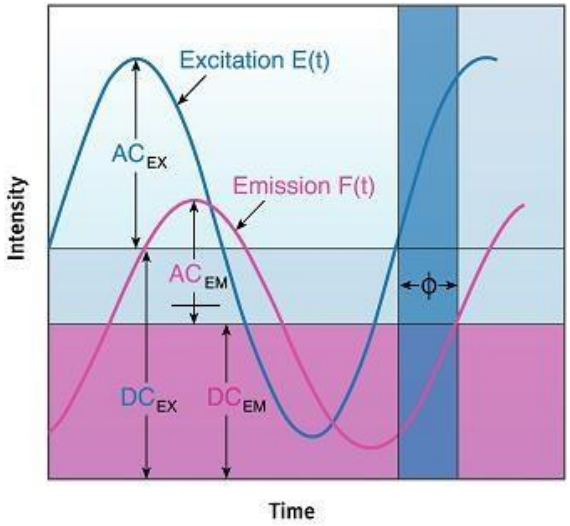

Figure 2. The excitation light is in blue; the fluorescence, in red, displays a demodulation and a phase shift with respect to the excitation light.

The fluorescence is demodulated and it is phase-sgifted with respect to the excitation beam. The relative demodulation, M, of the emission is then:

$$M = \frac{\left(\frac{AC}{DC}\right)_{EM}}{\left(\frac{AC}{DC}\right)_{EX}} = \frac{1}{\sqrt{1+(\omega\tau)^2}} \qquad [4]$$

And the phase shift is:

$$\varphi = tg^{-1}(\omega\tau) \qquad [5]$$

Both relationships are related to the decay time τ. The instrumentation measures the demodulation and the phase shift at different modulation frequencies (typically 15-20); the data are fitted against a theretical model.

Thus using the phase shift and relative modulation one can determine a phase lifetime τ_P and a modulation lifetime τ_M. If the fluorescence decay is a single exponential, then τ_P and τ_M will depend upon the modulation frequency, i.e.,

$$\tau_P(\omega_1) < \tau_P(\omega_2) \qquad \omega_1 < \omega_2 \qquad [6]$$

The differences between τ_P and τ_M and their frequency dependence form the basis of the methods used to analyze for lifetime heterogeneity, i.e., the component lifetimes and amplitudes.

One must be careful to distinguish the term fractional contribution to the total intensity f from α, the pre-exponential term referred to earlier in the time domain. The relation between these two terms is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \qquad [7]$$

where j represents the sum of all components, α their pre-exponential factors and τ are the lifetimes of these components.

Analysis

Multifrequency phase and modulation data are usually analyzed using a non-linear least squares methods in which the actual phase and modulation ratio data (not the lifetime values) are fitted to different models such as single or multiple exponential decays. The quality of the fit is then judged by the reduced chi-square value (χ^2):

$$\chi^2 = \frac{1}{\nu}\left\{\sum_{j=1}^{N}\left[\frac{\varphi^j(\omega)-\varphi^C(\omega)}{\sigma_\varphi}\right]^2 + \sum_{j=1}^{N}\left[\frac{m^j(\omega)-m^C(\omega)}{\sigma_M}\right]^2\right\} \qquad [8]$$

where:

N	Total number of frequencies
ν	Number of degrees of freedom of the system. Since the number of data points is twice the number of frequencies, ν = 2N – p where p is the number of variables.
σ_ϕ , σ_M	Uncertainties used in the phase and modulation values. It was found that the experimental result is not strongly dependent on σ_ϕ and σ_M .
$\phi^j(\omega)$	Measured frequency-dependent values of phase angle
$m^j(\omega)$	Measured frequency-dependent values of demodulation
$\phi^C(\omega)$	Calculated frequency-dependent values of phase angle
$m^C(\omega)$	Calculated frequency-dependent values of demodulation

In addition to decay analysis using discrete exponential decay models, one may also choose to fit the data to distribution models. In this case, it is assumed that the excited state decay characteristics of the emitting species actually results in a large number of lifetime components. Shown below is a typical lifetime distribution plot for the case of a single tryptophan containing protein – Human Serum Albumin.

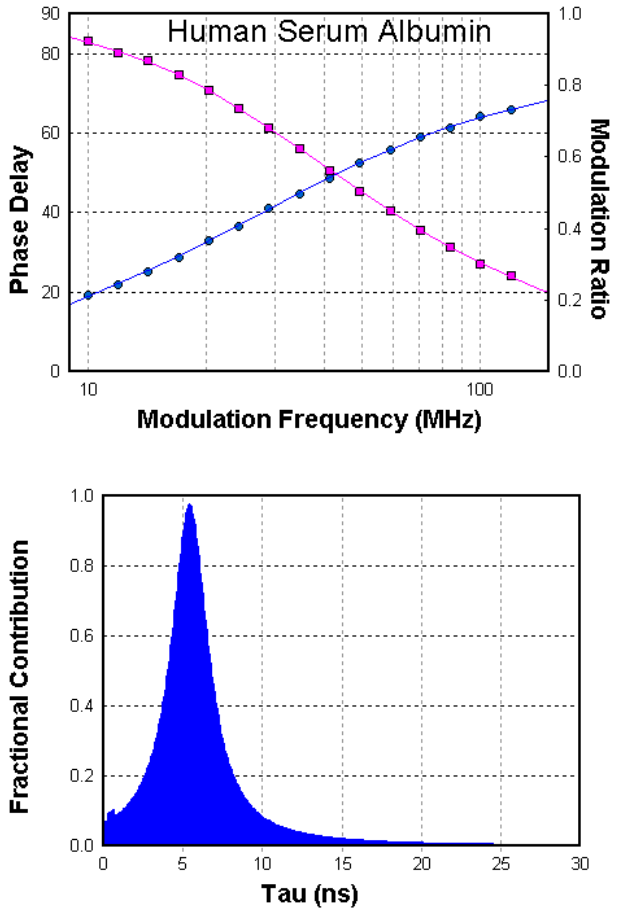

Figure 3. The plots show the frequency response curves (phase and modulation vs. modulation frequency) for Human Serum Albumin (left). The excitation source was a 300-nm UV-LED; the emission was collected through a WG320 high-pass filter at a temperature of 20℃. Lifetime analysis was performed using a Lorentzian distribution (center at 5.4 ns, width = 2.9 ns, fractional distribution = 98%) and a second discrete component (t = 0.51 ns and fractional contribution = 0.02%). For a review of HSA lifetime studies see [3].

The distribution shown here is Lorentzian, but depending on the decay kinetics of the system, different types of distributions, e.g., Gaussian, or asymmetric distributions (Planck), may be utilized. This approach to lifetime analysis is described in [4].

Applications

Fluorescence Lifetime Assays

The fluorescence lifetime (FLT) has been widely utilized for the characterization of fluorescence species and in biophysical studies of proteins, e.g. the distances between particular amino-acid residues by Foerster Resonance Energy-Transfer (FRET). FLT is a parameter that is mostly unaffected by inner filter effects, static quenching and variations in the fluorophore concentration. For this reason FLT can be considered as one of the most robust fluorescence parameters, and therefore it is advantageous in clinical and high throughput screening (HTS) applications where it is necessary to discriminate against the high background fluorescence from biological samples. Also FLT offers more leverage with regards to multiplexing. The ability to discriminate between two fluorophores with similar spectra but different lifetimes is another way to increase the number of parameters to be measured (see, for example [5]).

Several mechanisms can be utilized for the development of lifetime-based assays. There are the simple binding assays, where binding of 2 components (one being fluorescently labeled) is accompanied by a FLT-change. Another

scenario would be a quench-release type assay where the quenched species has low but finite fluorescence but is initially present in large excess. If the fluorescence compound is released (binding to a complementary DNA strand (Molecular Beacon) or by an enzymatic reaction) the lifetime of the system increases. Finally, FLT is a powerful method to measure energy transfer efficiency in FRET (fluorescence resonance energy transfer) assays, circumventing the issue of spectral cross talk between donor and acceptor, by using a non-fluorescent acceptor.

Fluorescence Lifetime Sensing

Most of the fluorescence sensors and assays that are in use today are based on intensity measurements. Though these methods are easier to implement they lack robustness and they require frequent calibration [6]. Many difficulties that are associated with intensity-based measurements can be circumvented using lifetime-based measurements. Lifetime-based measurements have the advantage that they are independent of the fluorescence intensity. In past 10 years many probes that exhibit analyte-sensitive fluorescence lifetime changes have been identified and characterized. Some of these probes are listed in Table 2. For a detailed discussion on lifetime-based sensing we refer you to the book chapter “Lifetime-based Sensing” in [6].

Fluorescence Lifetime Imaging

Fluorescence lifetimes also offer opportunities in fluorescence microscopy where the local probe concentration cannot be controlled. FLIM allows image contrast to be created based on the fluorescence lifetime of a probe at each point of the image. Typical examples are the mapping of cell parameters such as pH, ion concentrations or oxygen saturation by fluorescence quenching, fluorescence resonance energy transfer (FRET), or photon-induced energy transfer (PET). Examples of biological applications of lifetime imaging technology include scanning of tissue surfaces, photodynamic therapy, DNA chip analysis, skin imaging and others (see, for example [7]).

Fluorescent Probes	Mean Lifetime [ns]		Absorption Max [nm]		Emission Max [nm]	
	free	bound	free	bound	free	bound
a) Calcium Probes						
Fura-2	1.09	1.68	362	335	500	503
Indo-1	1.4	1.66	349	331	482	398
Ca-Green	0.92	3.66	506	506	534	534
Ca-Orange	1.20	2.31	555	555	576	576
Ca-Crimson	2.55	4.11	588	588	610	612
Quin-2	1.35	11.6	356	336	500	503
b) Magnesium Probes						
Mg-Quin-2	0.84	8.16	353	337	487	493
Mg-Green	1.21	3.63	506	506	532	532
c) Potassium Probe						
PBFI	0.52	0.59	350	344	546	504

d) Sodium Probe						
Sodium Green	1.13	2.39	507	507	532	532
e) pH Probes						
SNAFL-1	1.19	3.74	539	510	616	542
Carboxy-SNAFL-1	1.11	3.67	540	508	623	543
Carboxy-SNAFL-2	0.94	4.60	547	514	623	545
Carboxy-SNARF-1	1.51	0.52	576	549	638	585
Carboxy-SNARF-2	1.55	0.33	579	552	633	583
Carboxy-SNARF-6	1.03	4.51	557	524	635	559
Carboxy-SNARF-X	2.59	1.79	575	570	630	600
Resorufin	2.92	0.45	571	484	587	578
BCECF	4.49	3.17	503	484	528	514

Table II. Spectral properties (absorption and emission maxima) and mean lifetime for ion probes

Books and Book Chapters related to Fluorescence Lifetime

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3. Herman B. (1998). *Fluorescence Microscopy, 2nd Edition, Springer-Verlag, New York.*
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5. Jameson, D. M. and Hazlett, T.L. (1991). *Time-Resolved Fluorescence in Biology and Biochemistry, in Biophysical and Biochemical Aspects of Fluorescence Spectroscopy (Dewey, Ed.) Plenum Press, New York.*

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3. Helms, M.K., Petersen, C.E., Bhagavan, N.V., Jameson, D.M.; *Time-resolved fluorescence studies on side-directed mutants of human serum albumin.* FEBS letters, 408, 67-70 (1997).
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