

Fluorescence Workshop UMN Physics June 8-10, 2006

Fluorescence Lifetime + Polarization (2) Joachim Mueller

radiative lifetime, nonradiative processes, pulsed excitation, rotational depolarization, Perrin equation







Virtually all fluorescence data required for any research project will fall into one of the following categories.

- 1. The fluorescence emission spectrum
- 2. The excitation spectrum of the fluorescence
- 3. The quantum yield
- 4. The polarization (anisotropy) of the emission
- 5. The fluorescence lifetime

In these lectures, we examine each of these categories and briefly discuss historical developments, underlying concepts and practical considerations

Fluorescence Lifetime au



Note that the absorption process and vibrational relaxation (and internal conversion) processes happen on a much faster timescale than the fluorescence emission $(S_1 \rightarrow S_0)$. Thus the time it takes to return to the ground state S_0 only depends on the time spend in the lowest vibrational state of S_1 . We call this the fluorescence lifetime τ of the molecule.

Fluorescence Lifetime: Measure the average time it takes for a molecule after absorption to return to its ground state



Remember that a lifetime is also related to a rate coefficient:

$$\Gamma = \frac{1}{\tau}$$

Fluorescence Lifetime au

Although we often speak of the properties of fluorophores as if they are studied in isolation, such is not usually the case.

Absorption and emission processes are almost always studied on *populations* of molecules and the average properties of a molecule of the population are deduced from the macroscopic properties of the process.

In order to describe the behavior of the excited state population we define the following:

- n is the number of molecules in the ground state (•) at time t.
- n* is the number of excited molecules (•) at time t.
- Γ is the rate constant of emission. The dimensions of Γ are sec⁻¹ (transitions per molecule

per unit time).

 \cdot f(t) is an arbitrary function of the time, describing the time course of the excitation .

Illustration:

A laser beam passes through a solution containing fluorophores. At any given time some fluorophores will be excited, while the rest will be in its ground state.



The excited state population of fluorophores is described by a rate equation:

$$\frac{dn^*}{dt} = -n^* \Gamma + n f(t)$$

where $n + n^* = n_o$ (n_o describes the total number of molecules and is a constant)

Steady-state Illumination

The excitation intensity in a steady-state experiment is constant. In other words the function f(t) is constant.

The solution of the rate equation for a constant f(t) is given by a constant excited state population:



where $f(t) = I_{ex}$ describes the excitation intensity.



Note that the fluorescence intensity is directly proportional to the excited state population: $~{\bf I}_F \propto n^*$

The excited state population is initially directly proportional to the excitation intensity I_{ex} (linear regime), but saturates at higher excitation intensities (because one cannot drive more molecules in the excited state than are available).

Comment: We will always work under conditions where we are far from saturation. In other words we are in the linear regime, where $n^* \propto I_{ex}$.

Pulsed Excitation

Let's consider a very short pulse (much shorter than the lifetime of the fluorophore, say less than 10^{-12} s in duration) is applied to the sample at t = 0.



The solution of the rate equation is given by an exponential decay of the excited state population:

$$n^{*}(t) = n^{*}(0)e^{-\Gamma t}$$

If a population of fluorophores are 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:





The lifetime τ is equal to $\Gamma^{\text{-1}}$

In pictorial form:



Note, the excited state population $n^{*}(t)$ is proportional to the fluorescence intensity $I_{F}(t)$.

Strickler-Berg equation

Knowledge of a fluorophore's excited state lifetime is crucial for quantitative interpretation of numerous fluorescence measurements such as quenching, polarization and FRET.

In most cases of interest, it is virtually impossible to predict *a priori* the excited state lifetime of a fluorescent molecule. The true molecular lifetime, i.e., the lifetime one expects in the absence of any excited state deactivation processes – can be approximated by the Strickler-Berg equation (1962, J. Chem. Phys. 37:814).

Natural
radiative
lifetime
$$\tau_r$$
 $\tau_r^{-1} = 2.88 \times 10^{-9} n^2 \frac{\int F(\tilde{v}) d\tilde{v}}{\int \int_{\Delta v_a} F(\tilde{v}) \tilde{v}^{-3} d\tilde{v}} \int_{\Delta v_a} \varepsilon(\tilde{v}) d\ln \tilde{v}$

 τ_r is the natural radiative lifetime, n is the refractive index of the solvent, Δv_e and Δv_a correspond to the experimental limits of the absorption and emission bands (S₀ - S₁ transitions), ϵ is the extinction coefficient, v is the wavenumber and F(v) describes the spectral distribution of the emission in photons per wavelength interval.

How well do these equations actually work? Not very well – usually off by factors of 2 - 5 fold.

Natural Radiative Lifetime



only radiative decay possible

radiative decay competes with nonradiative decay

Experimentally measured rate coefficient:

 $\Gamma = k_r$

 $\Gamma = k_r + k_{nr}$

Quantum yield:

Φ=1



Analogy: Empty a full room of (excited-state) people



Experimentally we can only observe the radiative (fluorescent) decay. Nonradiative processes are alternative paths for the molecule to return to its ground state. These additional paths lead to a faster decay (emptying of the room) of the excited state population. Thus we observe a faster decay of the fluorescence intensity. The lifetime and quantum yield for a given fluorophore is often dramatically affected by its environment.

Examples of this fact would be NADH, which in water has a lifetime of ~0.4 ns but bound to dehydrogenases can be a long as 9 ns.







ANS in water is ~ 100 picoseconds but can be 8 – 10 ns bound to proteins Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA

The lifetime of tryptophan in proteins ranges from ~0.1 ns up to ~8 ns

Qualitative Explanation:



ANS in water is ~100 ps but can be 8 – 10 ns bound to proteins

Experimentally measured rate coefficient:

Quantum yield:



The rate coefficient k_r for radiative decay is approximately the same in both cases. However, the rate of non-radiatve decay depends often strongly on the environment. In water (polar solvent) the nonradiatve decay dominates ($k_{nr,H20} \gg k_r$), while in the apolar protein environment the non-radiatve decay rate decreases ($k_{nr,H20} \gg k_{nr,protein}$).

>>

$$\Phi = \frac{k_r}{k_r + k_{nr, protein}}$$



Fluoresence Lifetime Measurement

Excited state lifetimes have traditionally been measured using either the *impulse* response or the *harmonic* response method. In principle both methods have the same information content. These methods are also referred to as either the "time domain" method or the "frequency domain" method.

In the *impulse* (or pulse) 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* which 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.

In the harmonic method (also known as the phase and modulation or frequency domain method) a continuous light source is utilized, such as a laser or xenon arc, and the intensity of this light source is modulated sinusoidally at high frequency.

We briefly discuss the impulse method ...





Time-Domain Lifetime Measurement



As shown in the intensity decay figure, the *fluorescence* lifetime τ is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation: $I_{+} = \alpha e^{-t/\tau}$

Where I_{t} is the intensity at time t, α is a normalization term (the preexponential factor) and τ is the lifetime. It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.



Note, an exponential decay leads to a straight line in the logarithmic plot

If the decay is multiexponential, the relation between the intensity and time after excitation is given by:

$$I(t) = \sum_{i} \alpha_{i} e^{-t/t}$$

One may then observe data such as those sketched below:

Here we can discern at least two lifetime components indicated as t_1 and t_2 . This presentation is oversimplified but illustrates the point.



Note the logarithmic plot shows a curved decay (thus there has to be more than one lifetime present)

Here are pulse decay data on anthracene in cyclohexane taken on an IBH 5000U Time-correlated single photon counting instrument equipped with an LED short pulse diode excitation source.



Polarization Part II: Rotational Depolarization

So far we assumed that the molecule is not rotating during the lifetime of the excited state (for example if the fluorophores are embedded in a highly viscous or frozen medium).

This allowed us to determine the intrinsic polarization P_o .

the equation describing the intrinsic polarization (or anisotropy) is given by:

$$P_{o} = \frac{1 + 3\cos(2\beta)}{7 + \cos(2\beta)}$$
 $r_{o} = \frac{2}{5} \left(\frac{3\cos^{2}\beta - 1}{2}\right)$

where β is the angle between absorption and emission dipoles.

Example



We may now consider the case where the fluorophore is permitted to rotate during the excited state lifetime.



Absorption dipole



Ground state population





Photoselection



Average direction

Excited state population just after absorption (t = 0)



No preferred direction

Excited state population some time after absorption (+ > 0)

Thus, the polarization value is time dependent !!!

Consider a single fluorophore:



Immediately after excitation the polarization is given by the intrinsic polarization P_0 :

The fluorophore rotates on average through an angle ω during its fluorescence lifetime τ . This leads to additional depolarization:

$$P_{o} = \frac{1 + 3\cos(2\beta)}{7 + \cos(2\beta)}$$

 $r_{o} = \frac{2}{5} \left(\frac{3\cos^{2}\beta - 1}{2} \right)$

 $\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right) \left(\frac{2}{3\cos^2 \omega - 1}\right) \qquad r = r_o \left(\frac{3\cos^2 \omega - 1}{2}\right)$

So the total polarization is determined by an intrinsic factor (P_o or r_o) and an extrinsic factor ω .

Perrin Equation

F. Perrin related the observed polarization to the excited state lifetime and the rotational diffusion of a fluorophore: *Perrin, F. 1926. Polarisation de la Lumiere de Fluorescence. Vie Moyene des Molecules Fluorescentes. J. Physique. 7:390-401.*

Specifically:
$$\frac{r_o}{r} = \left(1 + \frac{3\tau}{\rho}\right)$$
 or $\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right)$

where ρ is the Debye rotational relaxation time which is the time for a given orientation to rotate through an angle of 68.42° (= arccos(e⁻¹)).

For a spherical molecule:

$$\rho = \frac{3\eta V}{RT}$$

where V is the molar volume of the rotating unit, R is the universal gas constant, T the absolute temperature, η the viscosity and τ the excited state lifetime.

For a spherical protein, it follows that:



Where M is the molecular weight, v is the partial specific volume and h the degree of hydration.

If the molecule is not spherical then the relevant term is the harmonic mean of the rotational relaxation times (ρ_h) about the principle rotational axes

$$\rho_{h}^{-1} = \left(\frac{\rho_{1}^{-1} + \rho_{2}^{-1} + \rho_{3}^{-1}}{3}\right)$$

* Rotational relaxation time versus rotational correlation time.

We should note that it is not uncommon to see the term "rotational correlation time", often denoted as τ_c , used in place of the Debye rotational relaxation time. The information content of these terms is similar since $\rho = 3\tau_c$ but we have observed that some people become rather fervently attached to the use of one term or the other.

In the original development of the theories of rotational motion of fluorophores Perrin and others used the rotational relaxation time, as originally defined by Debye in his studies on dielectric phenomena. Only later (in the 1950's) during the development of nuclear magnetic resonance was the term rotational correlation time used by Bloch. It thus seems reasonable for fluorescence practitioners to use ρ but certainly adoption of either term should not lead to confusion. In terms of anisotropy and rotational correlation times, then, the Perrin equation would be:

$$\frac{r_{o}}{r} = \left(1 + \frac{\tau}{\tau_{c}}\right)$$

Perrin-Weber Plot

A plot of 1/P - 1/3 (or r) versus T/η predicts a straight line, the intercept and slope of which permit determination of P_o and the molar volume (if the lifetime is known). Shown below is such a plot (termed a Perrin-Weber plot) for protoporphyrin IX associated with apohorseradish peroxidase - the viscosity of the solvent is varied by addition of sucrose.



Fig. 3. Perrin plot for HRP(desFe) at 22°C; viscosity was varied by sucrose addition. Excitation wavelength was 514 nm; emission was observed through a Corion LL 600 cuton filter which passed $\lambda > 600$ nm.

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right)$$
$$\rho = \frac{3\eta M(\upsilon + h)}{RT}$$

The polarization observed in buffer alone was 0.151 while the limiting polarization obtained from the intercept on the Y-axis was 0.225, which is the same value one obtains for upon excitation of protoporphyrin IX in glycerol at low temperatures. From the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right)$$



Fig. 3. Perrin plot for HRP(desFe) at 22°C; viscosity was varied by sucrose addition. Excitation wavelength was 514 nm; emission was observed through a Corion LL 600 cuton filter which passed $\lambda > 600$ nm.

and knowing the lifetime of 16.9 ns, one can calculate a rotational relaxation time of 96 ns for the protein-porphyrin complex:

$$\left| \frac{1}{0.151} - \frac{1}{3} = \left(\frac{1}{0.225} - \frac{1}{3} \right) \left(1 + \frac{3x16.9 \text{ns}}{\rho} \right) \right|$$

 $= 96 \, \text{ns}$

 $\rho = \frac{3\eta M(\upsilon + h)}{PT}$

For a spherical protein of 44,000 daltons and assuming a partial specific volume of 0.74 and 0.3 ml/mg for the hydration, one can then calculate:

 $\rho = (3)(0.01)(44000)(0.74+0.3)/(8.31x10^7)(293) = ~56$ ns

Thus it appears as if this protein is non-spherical

Local Motion

In the case of fluorescence probes associated noncovalently with proteins, (for example porphryins, FAD, NADH or ANS to give but a few systems), the probe is held to the protein matrix by several points of attachment and hence its "local" mobility, that is, its ability to rotate independent of the overall "global" motion of the protein, is very restricted.

In the case of a probe attached covalently to a protein, via a linkage through an amine or sulfhydryl groups for example, or in the case of tryptophan or tyrosine sidechains, considerable "local" motion of the fluorophore can occur. In addition, the protein may consist of flexible domains which can rotate independent of the overall "global" protein rotation. This type of mobility hierarchy is illustrated on the right for the case of a probe covalently attached to the dimeric protein L7/L12



In such a system one would see a downward curvature in the Perrin-Weber plots as illustrated below:



A detailed analysis of the rotational modalities in such a system requires time-resolved measurements, which is beyond the scope of this workshop.

Polarization and Protein Aggregation

Polarization methods are ideally suited to study the aggregation state of a protein. Consider, for example the case of a protein dimer - monomer equilibrium:



Following either intrinsic protein fluorescence (if possible) or by labeling the protein with a suitable probe one would expect the polarization of the system to decrease upon dissociation of the dimer into monomers since the smaller monomers will rotate more rapidly than the dimers (during the excited state lifetime).



Hence for a given probe lifetime the polarization (or anisotropy) of the monomer will be less than that of the dimer

Polarization of a Mixture of Species

In the concentration range near the dimer/monomer equilibrium constant, one expects to observe a polarization intermediate between that associated with either dimer or monomer. One can relate the observed polarization to the fraction of dimer or monomer using the additivity of polarizations first described by Weber (1952) namely:

Polarization and Anisotropy of a mixture:

$$\left(\frac{1}{\langle \mathsf{P} \rangle} - \frac{1}{3}\right)^{-1} = \sum f_i \left(\frac{1}{\mathsf{P}_i} - \frac{1}{3}\right)^{-1} \qquad \text{or} \qquad \langle \mathsf{r} \rangle = \sum f_i \mathsf{r}_i$$

where $\langle P \rangle$ ($\langle r \rangle$) is the observed polarization (observed anisotropy) of the mixture, f_i is the fractional intensity contributed by the i-th component and P_i (r_i) is the polarization (anisotropy) of the i-th component. One must then relate the fractional intensity contributions to molar quantities which means that one must take into account any change in the quantum yield of the fluorophore associated with either species.

Note that the anisotropy function is directly additive

So to determine the dissociation constant, one can dilute the protein and observe the polarization (or anisotropy) as a function of protein concentration as shown below.



A typical plot of polarization versus ligand/protein ratio is shown below:



In this experiment, 1 micromolar mant-GTP γ S (a fluorescent, non-hydrolyzable GTP analog) was present and the concentration of the GTP-binding protein, dynamin, was varied by starting at high concentrations followed by dilution. The binding curve was fit to the anisotropy equation (in this case the yield of the fluorophore increased about 2 fold upon binding). A K_d of 8.3 μ M was found

Another example of the utility of polarization/anisotropy data is shown here for the case of cyanine analogs of ADP binding to myosin subfragment. The 3'-isomer shows increased intensity upon binding while the 2'-isomer does not. But anisotropy data indicate binding of both isomers (from Oiwa et al 2003 Biophys. J. 84:634)



FPIA – Fluorescence Polarization ImmunoAssay

Among the first commercial instruments designed to use a fluorescence polarization immunoassay for clinical diagnostic purposes was the Abbott TDx – introduced in 1981.



The basic principle of a polarization immunoassay is to:
(1) Add a fluorescent analog of a target molecule – e.g., a drug – to a solution containing antibody to the target molecule

(2) Measure the fluorescence polarization, which corresponds to the fluorophore bound to the antibody

(3) Add the appropriate biological fluid, e.g., blood, urine, etc., and measure the decrease in polarization as the target molecules in the sample fluid bind to the antibodies, displacing the fluoroescent analogs.



Time dependent spectral relaxations

Solvent dipolar orientation relaxation



As the relaxation proceeds, the energy of the excited state decreases and the emission moves toward the red



The emission spectrum moves toward the red with time



Wavelength

time

Time resolved spectra



Time resolved spectra of TNS in a Viscous solvent and in a protein

