Fluorescence Theory

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Abbreviations

FRET	fluorescence resonance energy transfer
RET	resonance energy transfer

Fluorescence spectroscopy is a powerful method that can be applied to diverse biological problems. It relies on the intrinsic chromophores that many native protein sequences contain, for example, tryptophan side chains. Alternatively, nonnative chromophores can be site-specific introduced into both protein and DNA molecules. Fluorescence is a form of luminescence, which is defined as the emission of light from any substance. For electronically excited states, there are two types of luminescence: fluorescence and phosphorescence. This article will mainly discuss some basic fluorescence methods.

Phosphorescence

Phosphorescence is emission of light from triplet-excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are spin-forbidden, and the emission rates are relatively slow (10^3 to $100\,s^{-1}$). Therefore, phosphorescence lifetimes are typically milliseconds to seconds. Phosphorescence is usually not seen in fluid solutions at room temperature because there are many deactivation processes that have faster rate constants, such as nonradiative decay and quenching processes. These processes effectively compete with photon emission in liquid solutions, thus reducing phosphorescence.

Fluorescence

Fluorescence is defined as emission of photons from singlet-excited states, in which the electron in the excited orbital is paired (of opposite sign) to the second electron in the ground-state orbital. Return to the ground state is spin-allowed and occurs rapidly by emission of a photon. The emission rates of fluorescence are typically $10^8\,s^{-1}$, so that a typical fluorescence lifetime is near 10 ns. Basic fluorescence spectral data are generally presented as emission spectra. The intensities and wavelengths of these spectra can vary widely depending on the chemical structure of the fluorophore and the solvent in which it is dissolved.

Jablonski Diagram

Processes that occur between the absorption and emission of light are usually illustrated by a Jablonski diagram. Two typical Jablonski diagrams of differing complexities are shown in Figures 1 and 2. The ground and first electronic

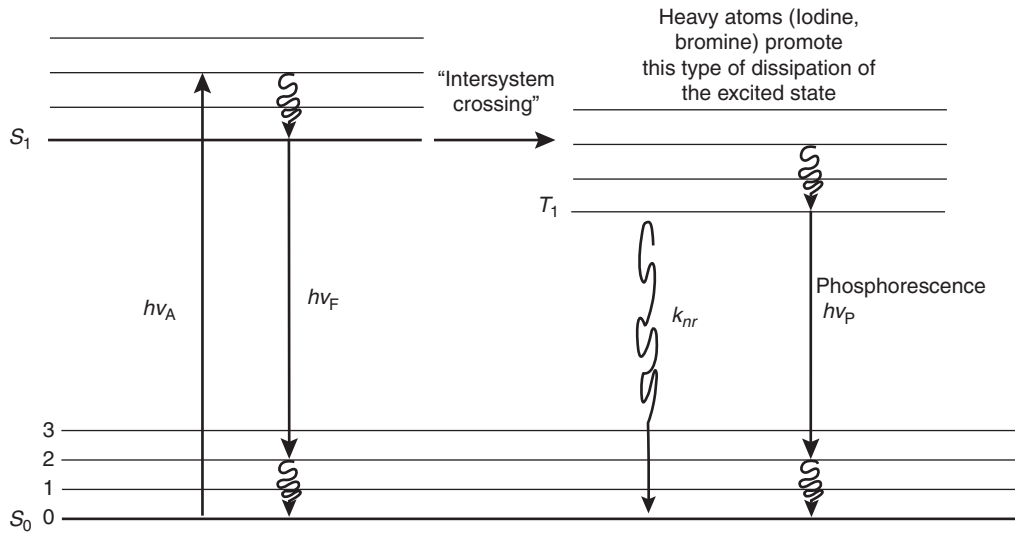

Figure 1 A simplified Jablonski diagram with absorbance, internal conversion, fluorescence, intersystem crossing, and phosphorescence.

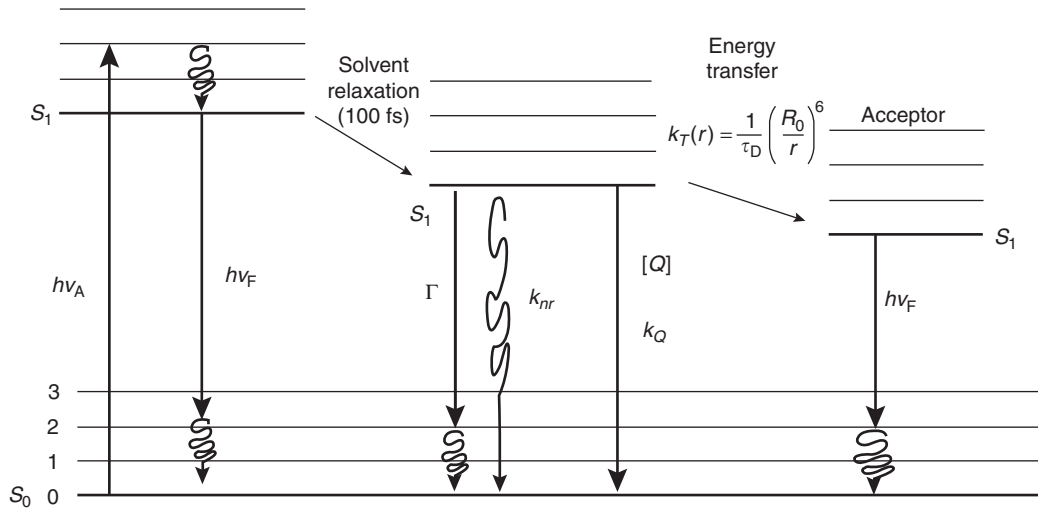

Figure 2 A more complex Jablonski diagram with fluorescence, collisional quenching, and resonance energy transfer (RET). The symbol k_{nr} is used to represent all nonradiative paths to the ground state besides quenching and RET.

states are depicted as S_0, and S_1, respectively. At each of these electronic energy levels, the fluorophores can exist in a number of vibrational energy levels (denoted by 0, 1, 2, etc.). Transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption. Transitions occur in approximately 10^{-15} s, a time too short for significant displacement of nuclei (Franck–Condon principle).

Following light absorption, several processes that usually occur are as follows:

- *Fluorescence* A fluorophore is usually excited to some higher vibrational level of the excited electronic state. Molecules in condensed phases rapidly relax to the lowest vibrational level of S_1. This process, called internal conversion, is nonradiative (does not emit a photon) and takes place in 10^{-12} s or less. Return to the ground electronic state also occurs to an excited vibrational level, which also quickly decays to reach thermal equilibrium. An interesting consequence of emission to an excited vibrational ground state is that the emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition.
- *Intersystem Crossing* Molecules in the S_1 state can undergo a spin conversion to the first excited triplet state, T_1 when the vibrational energies of the two excited states overlap. As discussed earlier, emission from T_1 is termed phosphorescence; these photons are generally shifted to longer wavelengths (lower energy) relative to fluorescence.

Characteristics of Fluorescence Emission

Stokes Shift

The energy of emission is typically less than that of absorption. Thus, fluorescence occurs at longer wavelengths, $h\nu_F < h\nu_A$. The phenomenon is known as the Stokes shift and can be caused by:

a. Energy losses due to relaxation to ground vibrational states
b. Solvent effects
c. Excited-state reactions
d. Complex formation
e. Energy transfer

Solvent Reorientation

Rotational motions of small solvent molecules (like water) in fluid solution are rapid, typically occurring on a timescale of 40 ps or less. The relatively long timescale of fluorescence (nanoseconds) allows ample time for the solvent molecules to reorient around the excited-state dipole. This has the effect of lowering the energy of the excited state; consequently the energy of the fluorescent photon is also lower, which shifts the emission to longer wavelengths. This process is called solvent relaxation and occurs in 10^{-10} s in fluids. The high sensitivity of emission spectra to solvent polarity originates from this process, and can result in a substantial Stokes shift. In proteins, this is most recognized with the tryptophan residues that absorb light in the range of 280–295 nm. The typical fluorescence emission of free tryptophan in solution occurs near 350 nm, but this can be shifted to lower or higher wavelengths depending on whether the tryptophan environment is apolar or polar, respectively.

Lifetime and Quantum Yield

The fluorescence lifetime and quantum yield are important characteristics of a fluorophore. The quantum yield is defined as the number of emitted photons relative

to the number of absorbed photons:

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \quad [1]$$

where Γ is the number of photons emitted and k_{nr} is all forms of nonradiative decay from the excited to the ground state. Nonradiative decay is any decay that does not involve the emission of a photon; one example is the dissipation of excited-state energy to vibrational and rotational modes of water. The quantum yield can be close to unity if the nonradiative decay rate is much smaller than the radiative decay rate, $k_{nr} \ll \Gamma$.

The lifetime of the excited state, τ, is defined by the average time the molecule spends in the excited state before returning to the ground state. Generally, fluorescence lifetimes are on the order of nanoseconds. The lifetime can be measured by an experiment diagrammed in **Figure 3** in which a very short, pulsed excitation is given followed by measurement of the time-dependent intensity. For a single exponential decay one can write

$$I(t) = I_0 \exp(-t/\tau) \quad [2]$$

where $I(t)$ and I_0 equal the intensities at time t and immediately after the pulse, and τ is the experimental lifetime. Thus, the lifetime is calculated from the slope of a plot of $\log I(t)$ *versus* t. Note that the observed lifetime is the inverse of the total decay rate, $(\Gamma + k_{nr})^{-1}$. The lifetime of the fluorophore in the absence of nonradiative processes is called the intrinsic lifetime and is given by $\tau_n = 1/\Gamma$.

It is important to keep in mind that fluorescence emission is a random process, and few molecules emit their photons precisely at $t = \tau$. This time is just the average lifetime of a fluorophore's excited state. Although not discussed in any detail here, time-domain measurements are an area of active research and can be used to distinguish fluorophores on the same molecule. Consider a protein with two tryptophan residues. Because of the spectral overlap of absorbance and emission, it is not usually possible in a steady-state experiment to resolve the emission spectra from the two residues. How is each of the tryptophan residues affected by interactions with the rest of the protein? Is one close to the binding site and the other far away? Are both affected or is only one affected? These questions can be addressed using a time-domain experiment.

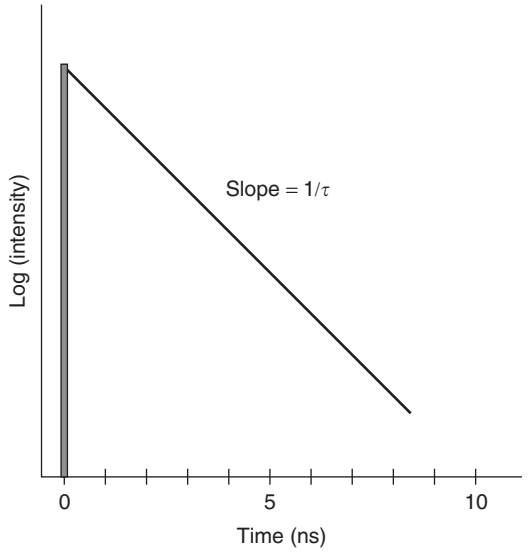

Figure 3 Collection of time-domain lifetime measurements. These are collected by an excitation pulse followed by monitoring the emission intensity as a function of time.

Fluorescence Quenching

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. There is a wide variety of quenching processes that include excited-state reactions, molecular rearrangements, ground-state complex formation, and energy transfer. Quenching experiments can be used to determine the accessibility of quencher to a fluorophore, to monitor conformational changes, and to monitor association reactions when the fluorescence of one of the reactants changes upon binding.

There are two basic types of quenching: static and dynamic (collisional). Both types require an interaction between the fluorophore and the quencher. In the case of dynamic quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. On contact, the fluorophore returns to the ground state without emission of a photon. In the case of static quenching, a complex forms between the ground-state fluorophore and the quencher, and this complex is nonfluorescent. The formation of this static quenching complex does not rely on population of the excited state. They will be considered independently.

Collisional Quenching

Collisional quenching occurs when the excited-state fluorophore is deactivated by contact with some other molecule in solution, which is called the quencher. The molecules are not chemically altered in the process. For collisional quenching, the decrease in intensity is described by the ratio of the fluorescence in the absence of quenching to the presence of quencher by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K[Q] = 1 + k_q \tau_0 [Q] \quad [3]$$

where F_0 and F are the observed fluorescence in the

absence and presence of quencher, K is the Stern–Volmer quenching constant, k_q is the bimolecular quenching constant, τ_0 is the lifetime in the absence of quencher, and $[Q]$ is the quencher concentration. The Stern–Volmer constant is sometimes abbreviated as K_{SV} or even as K_D. The use of the K_D abbreviation seems very unwise, since it could lead to confusion. Thus, the reader should be aware of the context in which this term is being used.

Mechanisms of Quenching

The accessibility of fluorophores to quenchers can be used to determine the location of fluorescent probes on macromolecules, and a wide variety of molecules can act as collisional quenchers. The mechanism varies with the fluorophore–quencher pair, but they must all be able to interact with the fluorophore, which gives some information about its accessibility. Common substances used for collisional quenching are halides: bromide and iodide (I^-), oxygen, and acrylamide.

Mechanisms of quenching are subject to debate. In the case of oxygen, which is paramagnetic, it is thought that it may cause the fluorophore to undergo intersystem crossing to the triplet state. In fluid solutions, these long-lived triplet states decay by nonradiative means before phosphorescence can occur. Halides such as iodine and bromine are also thought to cause intersystem crossing to the excited triplet state, promoted by spin–orbit coupling of the excited-state fluorophore and the halogen. Other quenchers, such as Cu^{2+}, Pb^{2+}, Cd^{2+}, and Mn^{2+}, are thought to cause the donation of an electron from the fluorophore in the excited state.

Stern–Volmer Plot

Because of the linear dependence, quenching data are usually presented as plots of F_0/F versus $[Q]$. These plots should yield an intercept of unity on the y-axis and a slope equal to K_{SV}. It is useful to note that $1/K_{SV}$ is the quencher concentration at which $F_0/F = 2$, or 50% of the intensity is quenched. A linear Stern–Volmer plot is generally indicative of a single class of fluorophores that are all equally accessible to the quencher. If two fluorophore populations are present, and one class is not accessible to the quencher, then the Stern–Volmer plots deviate from linearity toward the x-axis (downward). This result is frequently found for the quenching of tryptophan fluorescence in proteins by polar or charged quenchers.

Static Quenching

Static quenching involves the formation of a complex between the quencher and the fluorophore that does not rely on diffusion in the excited state. The dependence of the fluorescence intensity on quencher concentration for static quenching is derived from consideration of the association constant for complex formation:

$$K_S = \frac{[FQ]}{[F][Q]} \qquad [4]$$

where K_S is the fluorophore–quencher association constant, $[FQ]$ is the concentration of the complex, $[F]$ is the concentration of the uncomplexed fluorophore, and $[Q]$ is the concentration of quencher. Since the total concentration of the fluorophore, $[F]_T$, is given by $[F]_T = [F] + [FQ]$, the static quenching constant can be written as follows:

$$K_S = \frac{[F]_T - [F]}{[F][Q]} = \frac{[F]_T}{[F][Q]} - \frac{1}{[Q]} \qquad [5]$$

which rearranges to

$$\frac{[F]_T}{[F]} = 1 + K_S[Q] \qquad [6]$$

By recognizing the fluorescence signal in the absence of quencher, F_0 would correspond to the total concentration of fluorophore; one can substitute the fluorescence intensities F_0 and F for the total and free concentrations $[F]_T$ and $[F]$, respectively, to obtain

$$\frac{F_0}{F} = 1 + K_S[Q] \qquad [7]$$

which will be recognized is exactly the same linear equation that is used for dynamic quenching.

Both Static and Dynamic Quenching Can Have a Linear Stern–Volmer Plot

Since both static and dynamic quenching result in a linear Stern–Volmer plot, they cannot be distinguished by a single experiment; however, they are distinguished from each other by their differing dependences on temperature, viscosity, and/or lifetime measurements. For example, an increase in temperature leads to an increase in the diffusion constant of the quencher and will generally lead to an increase in collisional quenching. In contrast, an increase in temperature will usually lead to a decrease in the binding constant of the quencher for fluorophore and will result in a decrease in quenching for a static quencher.

Resonance Energy Transfer

Resonance energy transfer (RET) is the transfer of the excited-state energy from an initially excited fluorophore, called the donor, to a second molecule, called an acceptor. This reduces the fluorescence of the donor and

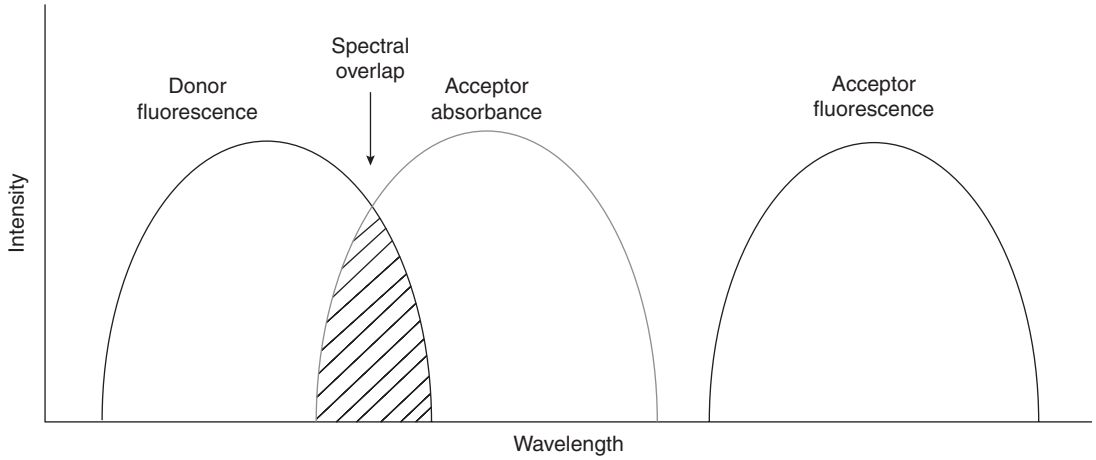

Figure 4 Resonance energy transfer occurs when the acceptor absorption energies overlap with the donor emission energies.

can be considered as a quenching process. The transfer of energy from the donor to the acceptor occurs when the emission spectrum of a donor overlaps with the absorption spectrum of the acceptor as shown in **Figure 4**. Importantly, the acceptor does not need to be fluorescent for energy transfer. A property of RET is that it is nonradiative: it does not involve emission of a photon by the donor in the classical sense. Rather, the energy transfer occurs when the donor and acceptor chromophores are electronically coupled by a long-range dipole–dipole interaction. For these reasons, the term RET is preferred to the term fluorescence resonance energy transfer (FRET), which is also commonly used.

The extent of RET is determined by the distance between the donor and the acceptor (**Figures 5 and 6**) as well as the extent of spectral overlap (**Figure 4**). For convenience, the spectral overlap is described in terms of the Förster distance, R_0, which is defined as the distance at which the RET is 50% efficient (**Figure 6**). The rate of energy transfer between the donor–acceptor pair, $k_T(r)$, is given by

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \quad [8]$$

where r is the distance between the donor and the acceptor and τ_D is the lifetime of the donor in the absence of energy transfer. **Figure 6** shows that the transfer rates are very fast at short distances. At the Förster distance, the donor emission would be decreased to one-half of its intensity in the absence of acceptor because the rate of transfer equals the decay rate of the donor in the absence of the acceptor. At a fixed distance, the efficiency of energy transfer for a single donor–acceptor pair at a fixed distance equals:

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad [9]$$

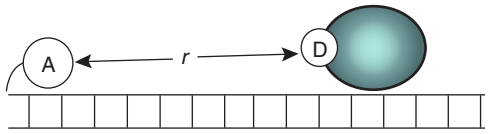

Figure 5 Energy transfer rate depends inversely on the separation distance, *r*, between the donor and the acceptor.

Förster distances are comparable in size to biological macromolecules, 20–90 Å, and energy transfer is, therefore, convenient for studies of biological macromolecules. Anything that affects the donor–acceptor distance will affect the rate of energy transfer. As a consequence, energy transfer has been used as a 'spectroscopic ruler' for measurements of distance between sites on macromolecules and the effects of conformational changes on these distances. Binding reactions can also be measured using RET, since it is only possible when two reactants are close together.

Fluorescence Anisotropy

Anisotropy measurements provide information on the size and shape of proteins or the rigidity of various molecular environments. These are based on the principle of photoselective excitation of fluorophores by polarized light. In an isotropic solution, the fluorophores are oriented randomly. Excitation with polarized light will result in a selective excitation of those fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation as shown in **Figure 7**. This selective excitation results in a partially oriented population of polarized fluorescence emission. Emission also occurs with the light polarized along a fixed axis in the fluorophore. The relative angle between these moments determines the maximum measured anisotropy in the absence of other mole-

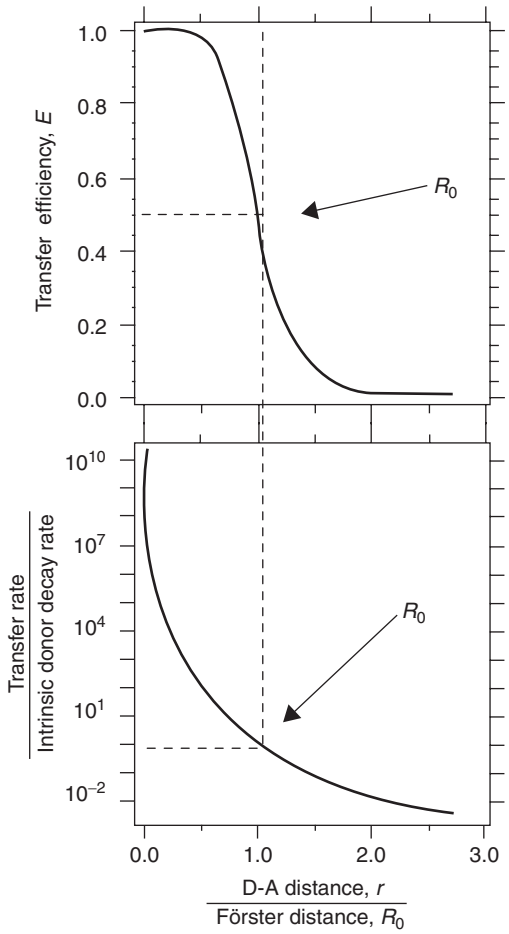

Figure 6 The dependence of energy transfer on the distance between the donor and the acceptor fluorophores. The top panel shows the efficiency of transfer, which is 50% at the Förster distance. The bottom panel shows the rate of transfer, which is equal to the intrinsic donor decay rate at the Förster distance.

cular rearrangements. The fluorescence anisotropy, R, and polarization, P are defined by:

$$R = \frac{I_{||} - I_{\mathrm{Per}}}{I_{||} + 2I_{\mathrm{Per}}} \quad [10]$$

and

$$P = \frac{I_{||} - I_{\mathrm{Per}}}{I_{||} + I_{\mathrm{Per}}} \quad [11]$$

where $I_{||}$ and I_{Per} are the fluorescence intensities of the vertically and horizontally polarized emission when the sample is excited with vertically polarized light. The anisotropy is a dimensionless quantity that is independent of the total intensity of the sample.

Several phenomena can decrease the measured anisotropy to values lower than the maximum theoretical values. The most common cause is rotational diffusion of a macromolecule to which the fluorophore is attached. Such rotation diffusion occurs during the lifetime of the excited state and displaces the emission dipole of the fluorophore. Conveniently, rotation correlation times for macromolecules are on the order of nanoseconds. For example, the rotational correlation time for human serum albumin is approximately 50 ns. Anisotropy is especially useful for measuring binding; when a macromolecule binds a ligand, the complex will be bigger and will have a longer rotational correlation time. This can be observed as a change in the anisotropy of the complex with respect to the unliganded macromolecule.

In addition to binding, there can be segmental motion of the fluorophore about its bonds, which happens on the picoseconds timescale. In this case, the fluorescent molecules can rotate many times during the 1–10-ns excited-state lifetime, and the orientation of the polar-

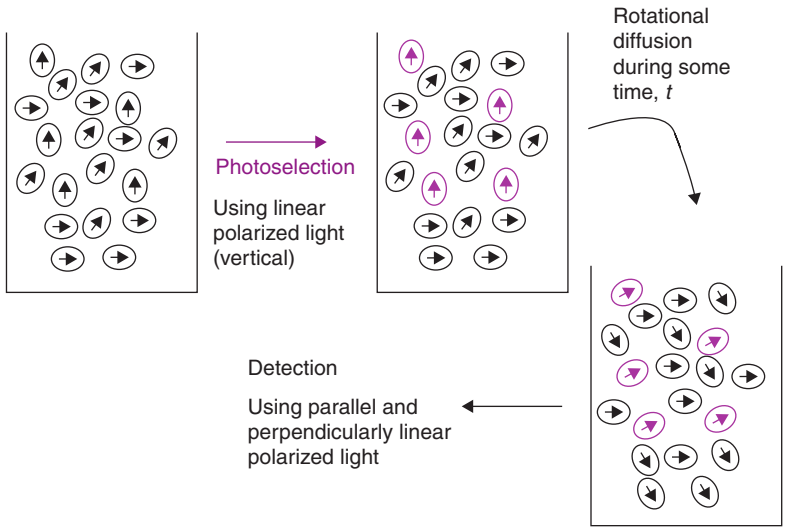

Figure 7 Principle of the fluorescence anisotropy experiment.

ized emission can be randomized. When this occurs, there is a diminished ability to observe any changes in the rotational correlation time of the molecule to which the fluorophore is attached, but this can be countered if the fluorophore becomes immobilized when the macromolecule binds a ligand.

When bound to a macromolecule and assuming no other processes result in loss of anisotropy, the expected anisotropy is given by the Perrin equation:

$$r = \frac{r_0}{1 + (\tau/\theta)} \quad [12]$$

where r_0 is the anisotropy that would be measured in the absence of rotational diffusion and θ is the rotational correlation time for the diffusion process. For a sphere

$$\theta = \frac{\eta V}{RT} \quad [13]$$

where η is the viscosity and V is the molecular volume equal to $M(\bar{v} + h)$, where M is the molecular weight, $\bar{v}$ is the partial specific volume, and h is the hydration of the molecule. In this case, the binding of the probe has slowed the probes' rate of rotational motion.

See also: Biochemical Applications of Fluorescence Spectroscopy, Fluorescence Microscopy, Applications, Fluorescence Polarization and Anisotropy, Fluorescent Molecular Probes, Organic Chemistry Applications of Fluorescence Spectroscopy, UV-Visible Fluorescence Spectrometers.

Further Reading

Lakowicz JR (2006) *Principles of Fluorescence Spectroscopy*, 3rd edn. New York, NY: Springer Science + Business Media.

Miller JN (ed.) (1981) *Techniques in Visible and Ultraviolet Spectrometry, vol. 2: Standards in Fluorescence Spectrometry*. London: Chapman & Hall.