

## Introduction to Fluorescence Theory and Methods

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### 1. Luminescence

Luminescence is the emission of light from any substance and occurs from electronically excited states. Luminescence can be of two types: fluorescence and phosphorescence

#### 1.1. 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 forbidden and the emission rates are slow ( $10^3$ - $100\text{ s}^{-1}$ ), so phosphorescence lifetimes are typically milliseconds to seconds. Phosphorescence is usually not seen in fluid solutions at room temperature, but there are many deactivation processes that compete with emission, such as nonradiative decay and quenching processes.

#### 1.2. Fluorescence.

Fluorescence is emission light 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. This emission rates of fluorescence are typically  $10^8\text{ s}^{-1}$ , so that a typical fluorescence lifetime is near 10 ns. Fluorescence spectral data are generally presented as emission spectra. Emission spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved.

#### 1.3. Jablonski Diagram.

Processes which occur between the absorption and emission of light are usually illustrated by a Jablonski diagram. A typical Jablonski diagram is shown in Figure 1. The ground, first and second electronic states are depicted by  $S_0$ ,  $S_1$  and  $S_2$ , 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 about  $10^{-15}$  seconds, a time too short for significant displacement of nuclei. (Franck-Condon principle).

Following light absorption, several processes usually occur:

##### 1.3.1. Fluorescence.

A fluorophore is usually excited to some higher vibrational level of either  $S_1$  or  $S_2$ . With a few rare exceptions, molecules in condensed phases rapidly relax to the lowest vibrational level of  $S_1$ . This process, called internal conversion, is nonradiative and takes place in  $10^{-12}$  seconds or less. Return to the ground state occurs to a higher excited vibrational ground-state level, which then quickly reaches thermal equilibrium. An interesting consequence of emission to a higher 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.

##### 1.3.2. Intersystem crossing.

Molecules in the  $S_1$  state can also undergo a spin conversion to the first triplet state,  $T_1$ . Emission from  $T_1$  is termed phosphorescence and is generally shifted to longer wavelengths (lower energy) relative to fluorescence. Transition from the  $T_1$  to the singlet ground state is forbidden, and as a result, the rate constants for triplet emission are several orders of magnitude smaller than those for fluorescence.

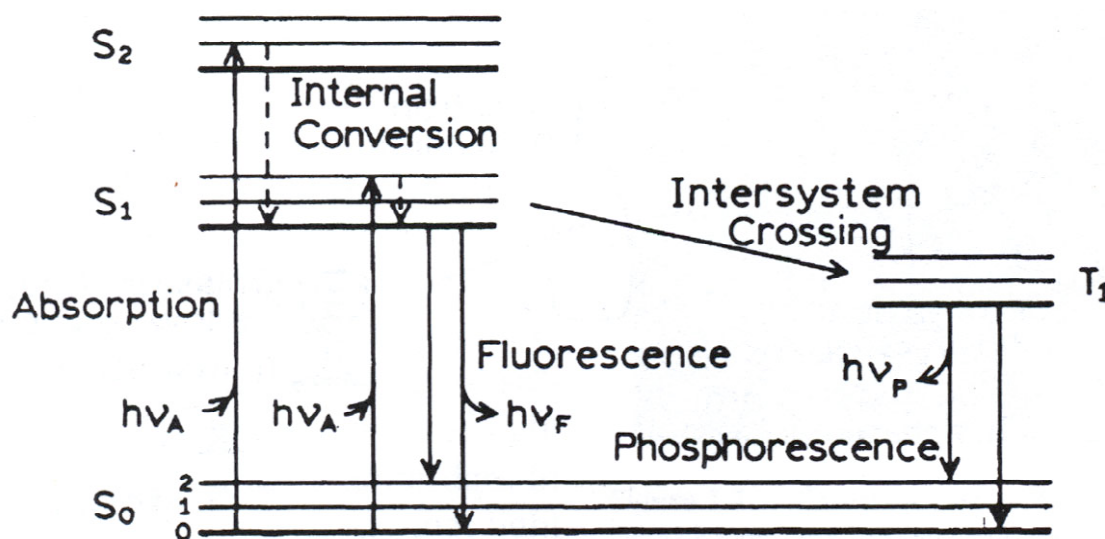


Figure 1. A simplified Jablonski diagram with absorbance, internal conversion, fluorescence, intersystem crossing, and phosphorescence. (Figure 1.5 from Lakowicz)

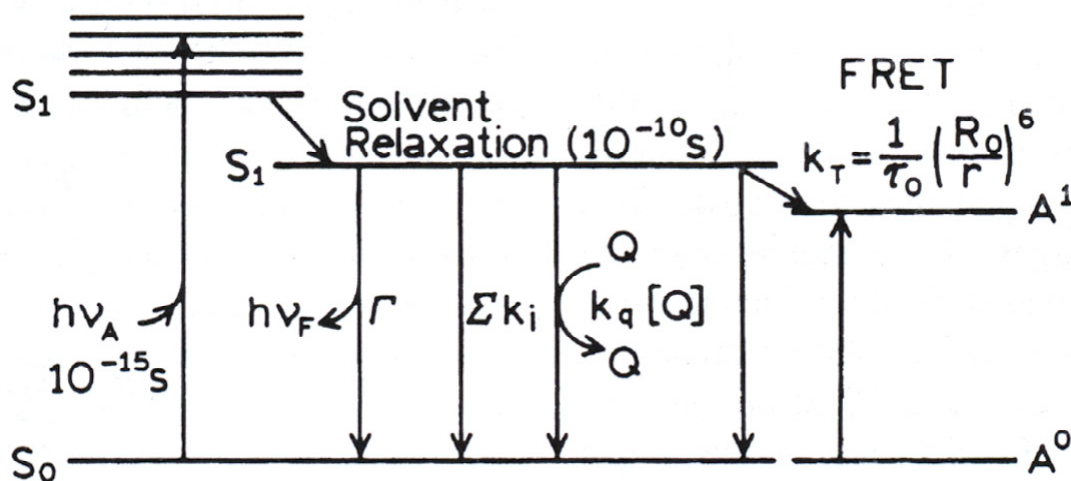


Figure 2. A more complex Jablonski diagram with fluorescence, collisional quenching and resonance energy transfer. The  $\Sigma k_i$  is used to represent all nonradiative paths to the ground state besides quenching and RET. (Figure 1.14 from Lakowicz.) We will discuss each of these processes further.

## 2. Characteristics of Fluorescence Emission.

### 2.1. 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:

- energy losses due to relaxation to ground vibrational states.
- Solvent effects
- Excited state reactions
- Complex formation
- Energy transfer

### 2.2. Solvent Reorientation

Rotational motions of small solvent molecules (like water) in fluid solution are rapid, typically occurring on a time scale of 40 ps or less. The relatively long timescale of fluorescence allows ample time for the solvent molecules to reorient around the excited state dipole, which can lower its energy and shift the emission to longer wavelengths. This process is called solvent relaxation and occurs in  $10^{-10}$  s in fluids. It is these differences between absorption and emission that result in the high sensitivity of emission spectra to solvent polarity and the smaller spectral changes seen in absorption spectra. Solvent relaxation can result in a substantial Stokes' shifts. In proteins, this is most recognized with the tryptophan residues, which absorbs light at 280-295 nm. The typical fluorescence emission 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.

### 2.3. Kasha's rule:

Emission spectra are typically independent of the excitation wavelength. Upon excitation to higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational level of  $S_1$ . It is from this position that the photon will be emitted.

Exceptions to this rule indicate a different geometric arrangement of nuclei in the excited state as compared to the ground state. Nuclear arrangements can occur prior to emission because of the relatively long lifetime of the  $S_1$  state, which allows time for

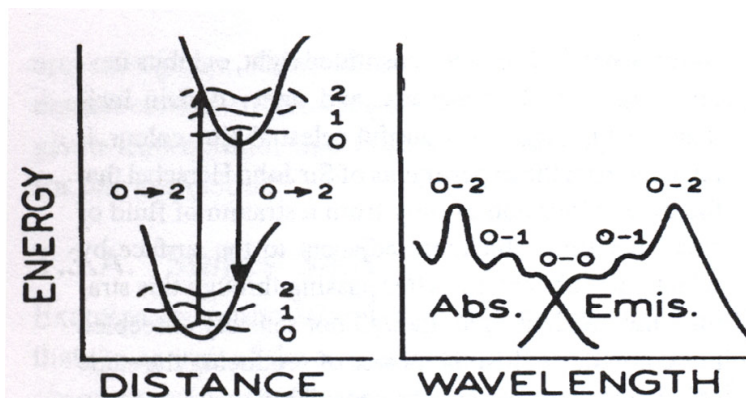


Figure 3. Mirror image rule and Franck-Condon Factors (Figure 1.8 from Lakowicz)

motion following the instantaneous process of absorption.

## 2.4. Mirror image rule.

The emission is the mirror image of the  $S_0 \rightarrow S_1$  absorption, not of the total absorption spectrum. This is a result of the same transitions being involved in both absorption and emission and the similarities of the vibrational levels of  $S_0$  and  $S_1$ . In many molecules these energy levels are not significantly altered by the different electronic distributions of  $S_0$  and  $S_1$ . If a particular transition probability between the zeroth and second vibrational levels is largest in absorption, the reciprocal transition is also most probable in emission. Figure 3 shows an example of this.

## 2.5. 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  $\Gamma$  is the number of photons emitted and  $k_{nr}$  is all forms of nonradiative

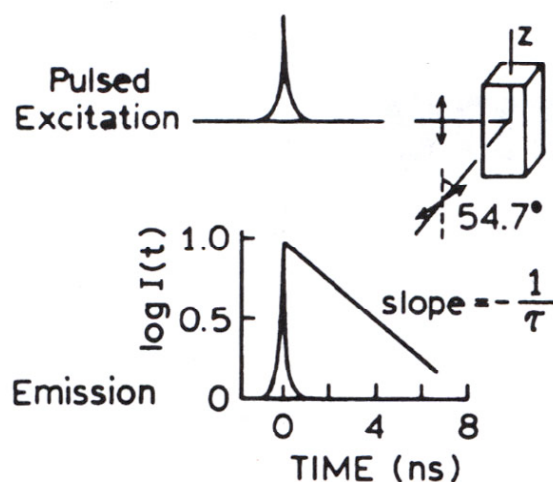


Figure 4. Time-domain lifetime measurements. The intensity decays are often measured through a polarizer oriented at  $54.7^\circ$  from the vertical z-axis in order to avoid the effects of anisotropy on the intensity decay. (Figure 4.1 from Lakowicz)

decay from the excited to the ground state. Nonradiative decay is any decay that does not involve the emission of a photon. The quantum yield can be close to unity if the radiationless decay rate is much smaller than the rate of radiative decay,  $k_{nr} \ll \Gamma$ .

The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetimes are on the order of nanoseconds. The lifetime can be measured by an experiment in which a very short, pulsed excitation is given followed by measurement of the time-dependent intensity. If we let  $n(t)$  equal the number of excited molecules at time,  $t$ , then the decay in this number is given by:

$$\frac{dn(t)}{dt} = -(\Gamma + k_{nr})n(t) \quad (2)$$

which can also be expressed as:

$$n(t) = n_0 \exp(-t/\tau) \quad (3)$$

The experimentalist actually observes intensity, but this is proportional to the number of photons, and so one can write:

$$I(t) = I_0 \exp(-t/\tau) \quad (4)$$

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  $\tau_n = 1/\Gamma$

One should remember 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. While we will not discuss them 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 2 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.

### 3. Fluorescence Quenching

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. There are 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, monitor conformational changes, monitor association reactions of 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 quencher. In the case of dynamic quenching the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact the fluorophore returns to the ground state without emission of a photon. In the case of state quenching a complex forms between the fluorophore and the quencher, and this complex is non-fluorescent. The formation of this complex does not rely upon population of the excited state. We will consider them independently.

#### 3.1. 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:

$$\frac{F_0}{F} = 1 + K[Q] = 1 + k_q \tau_0 [Q] \quad (5)$$

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,  $\tau_0$  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 in my opinion, since it could lead to confusion. Thus, the reader should be aware of the context in which this term is being used.

### 3.2. 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, and the mechanism varies with the fluorophore-quencher pair. Common substances used for 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 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.

### 3.3. Distance dependence of collisional quenching

To get a sense for how efficient collisional quenching can be, it is informative to consider the root mean square distance over which a quencher can diffuse during the lifetime of the excited state. This distance is given by:

$$\sqrt{\Delta x^2} = \sqrt{2D\tau} \quad (6)$$

where  $D$  is the diffusion coefficient of the quencher. Consider an oxygen molecule at 25°C. The diffusion coefficient in water is  $2.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ . In a typical tryptophan lifetime (4ns) the oxygen molecule can travel 44Å. If the lifetime is longer, then the average distances are longer.

### 3.4. Stern-Volmer Plot

Because of the linear dependence quenching data are usually presented as plots of  $F_0/F$  versus  $[Q]$ . This plot should yield an intercept of unity on the y-axis and a slope equal to  $K_{sv}$ . An example of such a plot is shown in Figure 5. 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 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.

### 3.5. Static quenching

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

$$K_s = \frac{[FQ]}{[F][Q]} \quad (7)$$

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:

$$K_s = \frac{[F]_T - [F]}{[F][Q]} = \frac{[F]_T}{[F][Q]} - \frac{1}{[Q]} \quad (8)$$

which rearranges to:

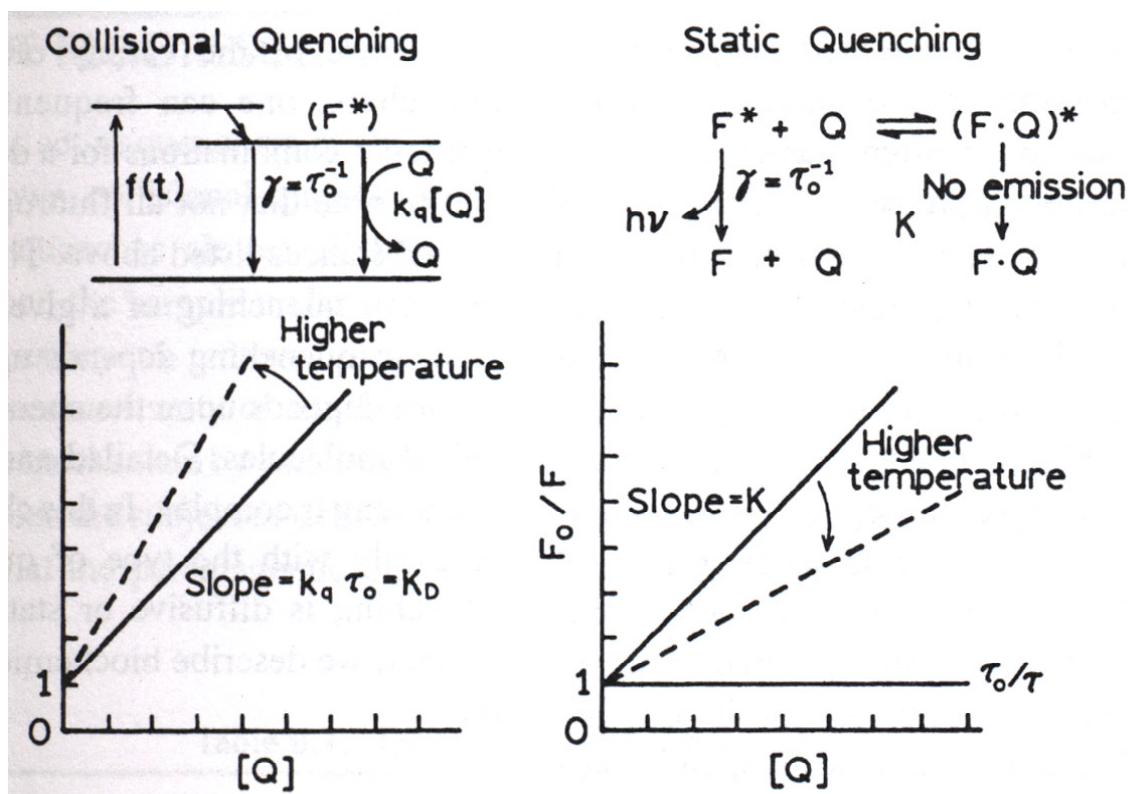


Figure 5: Quenching can be static or collisional. For a single fluorophore and a single type of quenching the Stern-Volmer plot will be linear in both cases.



$$\frac{[F]_T}{[F]} = 1 + K_s[Q] \quad (9)$$

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:

$$\frac{F_0}{F} = 1 + K_s[Q] \quad (10)$$

which you will recognize is exactly the same linear equation we used for dynamic quenching.

### 3.6. Both static and dynamic quenching can have a linear Stern Volmer Plot

Static quenching also gives a linear Stern-Volmer plot and so static and dynamic are distinguished from each other by their differing dependences on temperature, viscosity or lifetime measurements. An increase in temperature leads to an increase in the diffusion constant of quencher and will generally lead to an increase in collisional quenching. In contrast, an increase in temperature will generally lead to a decrease in the binding constant of quencher for fluorophore and will result in a decrease in quenching for a static quencher.

### 3.7. A Molecular Example of Quenching: Binding of e-ADP to DnaB helicase hexamer – page 257 Lackowitz

Jezewska MJ and Bujalowski W (1997) *Biophys. Chem.* **64**:253-269.

In this paper, the use of a quencher allowed measurement of a binding reaction that would have otherwise been difficult to measure. The fluorescence of e-ADP displayed only a small increase upon binding to the protein. Collisional quenching was used to induce a larger change in fluorescence upon binding. Acrylamide is an efficient quencher of e-ADP, and the authors reasoned that the fluorescence of e-ADP should be quenched more strongly in solution than when bound to the DnaB helicase. Thus, the addition of acrylamide should lead to a larger change in the e-ADP fluorescence upon binding.

Solutions of e-ADP were titrated with the helicase. In the absence of acrylamide, there was little change in the e-ADP fluorescence. The titrations were performed again in solutions of increasing

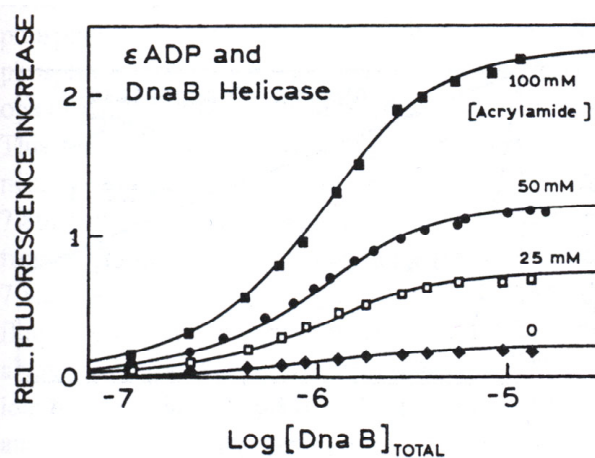


Figure 6. Fluorescence titration of e-ADP binding to DnaB helicase



acrylamide, and under these conditions, the e-ADP showed a much larger increase in fluorescence upon binding.

#### 4. Resonance energy transfer, RET

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 process occurs when the emission spectrum of a donor overlaps with the absorption spectrum of the acceptor. The acceptor does not need to be fluorescent in order for energy transfer to occur. An important property of RET is that it does not involve emission of a photon by the donor in the classical sense. The

energy transfer occurs when the donor and acceptor are 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 resonance energy transfer is determined by the distance between the donor and acceptor and the extent of spectral overlap. For convenience the spectral overlap is described as in terms of the Förster distance,  $R_0$ , which is defined as the distance at which the RET is 50% efficient (Fig. 9). The rate of energy transfer between the donor-acceptor pair,  $k_T(r)$  is given by:

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 \quad (11)$$

where  $r$  is the distance between the donor and the acceptor, and  $\tau_D$  is the lifetime of the donor in the absence of energy transfer. Hence the rate of transfer is equal to the decay rate of the donor in the absence of the acceptor. At the Förster distance, the donor emission would be decreased to one-half of its intensity in the absence of acceptor. The efficiency of energy transfer for a single donor-acceptor pair at a fixed distance is:

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (12)$$

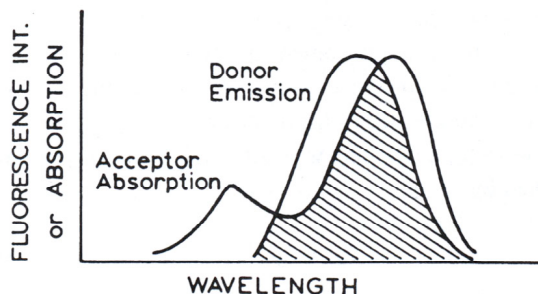


Figure 7: Resonance energy transfer occurs when the acceptor absorption overlaps with the donor emission.

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 = \text{TRANSFER RATE}$$

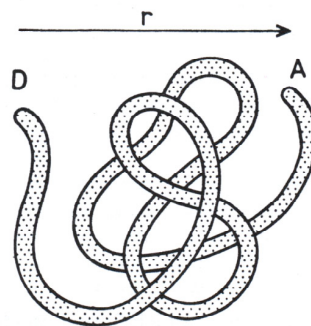


Figure 8. The transfer rate depends inversely on the separation distance between the donor and acceptor.

Thus, the extent of transfer depends on distance,  $r$ . 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.

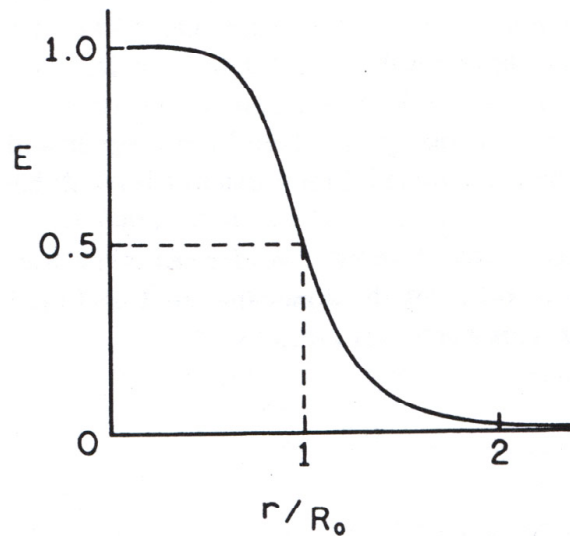


Figure 9. The dependence of the efficiency of energy transfer as a function of distance between fluorophores.

### 5. **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 photo selective 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. 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 molecular rearrangements. The fluorescence anisotropy,  $R$ , and polarization,  $P$  are defined by:

$$R = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (13)$$

and

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (14)$$

where  $I_{\parallel}$  and  $I_{\perp}$  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 albumen is around 50 ns. 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. Thus, anisotropy is often used to measure binding reactions.

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

$$r = \frac{r_0}{1 + (\tau/\theta)} \quad (15)$$

where  $r_0$  is the anisotropy which would be measured in the absence of rotational diffusion and  $\theta$  is the rotational correlation time for the diffusion process. For a sphere:

$$\theta = \frac{\eta V}{RT} \quad (16)$$

where  $\eta$  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.