

## Fluorescence Spectroscopy

Recall from the discussion of absorbance that:

$$\frac{-dI(\lambda)}{dt} = h\nu(N_a B_{ab} - N_b B_{ba})I(\lambda)$$

This suggests that molecules should continue a net absorption of light until  $N_a = N_b$ . This is illogical, since the molecules would be expected to be in the ground state in the absence of light. The implication is that molecules must have alternate methods of returning to the ground state other than by stimulated emission.

Based on statistical mechanics, in the absence of light the ratio of molecules in the ground to the excited states should be:

$$\frac{N_a}{N_b} = e^{-\frac{E_a - E_b}{kT}} = e^{+\frac{h\nu}{kT}}$$

Einstein pointed out that stimulated emission was not the only method for return to the ground state, and that the molecule could go from  $S_b$  to  $S_a$ , by spontaneous emission,  $A_{ba}$ . At equilibrium:

$$\frac{N_a}{N_b} = \frac{B_{ba}I(\lambda) + A_{ba}}{B_{ab}I(\lambda)}$$

If  $B_{ab} = B_{ba}$ , then: 
$$\frac{N_a}{N_b} = 1 + \frac{A_{ba}}{B_{ab}I(\lambda)}$$

The radiation density for a black body is: 
$$I(\lambda) = \frac{8\pi h\nu^3}{c^3 (e^{h\nu/kT} - 1)}$$

At equilibrium, the  $N_a / N_b$  ratios for the statistical mechanics equation and the spontaneous emission equations are equal. Then:

$$\frac{N_a}{N_b} = 1 + \frac{A_{ba}}{B_{ab}I(\lambda)} = \frac{A_{ba}c^3 (e^{h\nu/kT} - 1)}{B_{ab}8\pi h\nu^3}$$

and therefore (using the  $N_a / N_b$  ratios from the statistical mechanics equation:

$$A_{ba} = 8\pi h\nu^3 c^3 B_{ab}$$

Recall that: 
$$B_{ab} = \frac{2\pi}{3} \frac{D}{\hbar^2}$$

Thus,

$$A_{ba} = \frac{32\pi^3\nu^3}{3c^3\hbar} D$$

The dipole strength  $D$  and the frequency can be measured. In principle, therefore the rate of spontaneous emission should be:

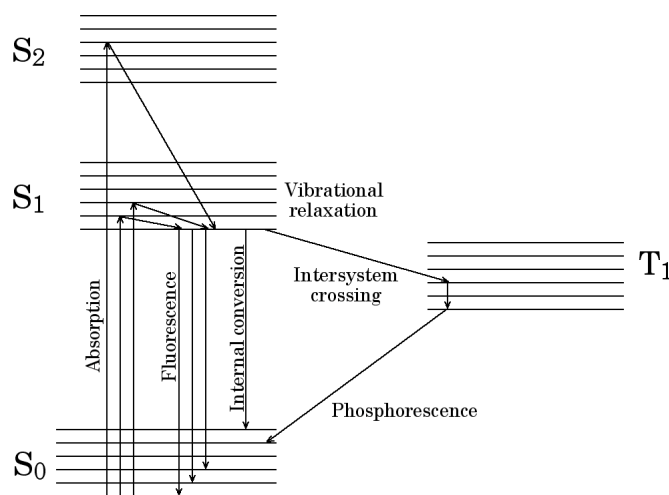
$$\frac{dN_b}{dt} = -A_{ba}N_b$$

Integrating gives:

$$N_b(t) = N_b(0)e^{-A_{ba}t}$$

The radiative lifetime of state  $S_b$  can therefore be defined as  $\tau_R = 1/A_{ba}$ . The relationship between  $D$  and  $A_{ba}$  states that molecules with higher extinction coefficients should have shorter excited state lifetimes (typical values for  $\tau_R$  are  $10^{-6}$  to  $10^{-9}$  seconds). This definition holds true only if the same initial excited state also emits the radiation.

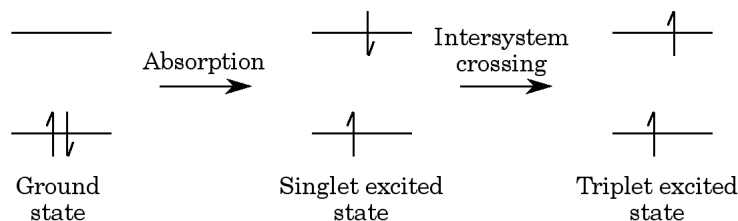
Some processes compete with fluorescence.



**Internal conversion** is a non-radiative process that allows the molecule to return to the ground state via collision with other molecules or via vibrational relaxation. Internal conversion is temperature dependent, which complicates the use of fluorescence to monitor temperature dependent processes (especially thermal unfolding of proteins). The rate constant for internal conversion is abbreviated  $k_{ic}$ .

**Quenching** is related to internal conversion; in quenching, solute molecules remove the extra energy from the excited state. Although quenching is (at least) a second order process, the high concentration of quenching agent required for most types of quenching allows a pseudo-first order analysis of the rate constant  $k_q$ .

A final method for leaving the excited singlet state is **intersystem crossing**, in which the excited state becomes a triplet state by inversion of the electron spin. The return to the ground state from a triplet excited state is forbidden, and resulting in a very long lifetime (milliseconds to seconds). The rate constant for intersystem crossing is  $k_{is}$ . In solution, phosphorescence is rarely observed unless oxygen (an effective quenching agent for both triplet and singlet states) is removed from the solution. In most biochemical experiments, only fluorescence is useful, because the fluorescent lifetime is short enough to allow fluorescence to occur before quenching.



The quantum yield is the fraction of absorbed light that is reemitted as fluorescent light:

$$\phi_F = \frac{k_F}{k_F + k_{ic} + k_{is} + k_q[Q]}$$

The kinetics of the return to the ground state is:

$$\frac{-d[S_b]}{dt} = (k_F + k_{ic} + k_{is} + k_q[Q])[S_b]$$

Integrating gives:

$$S_b(t) = S_b(0) e^{-\frac{t}{\tau_F}}$$

In this equation,  $\tau_F$  is the observed lifetime of the fluorescent state as a result of all of the processes that allow decay of the excited singlet state.

$$\tau_F = \frac{1}{k_F + k_{ic} + k_{is} + k_q[Q]}$$

The quantum yield is therefore:

$$\phi_F = \frac{\tau_F}{\tau_R}$$

### Fluorescence intensity

The number of excited molecules is proportional to the decrease in light intensity that occurs during passage of the light through the sample. This intensity change can be calculated from the Beer-Lambert law:

$$I = I_0 e^{-\ln(10)\epsilon\phi_c Cl}$$

where  $\epsilon(\lambda_c)$  is the extinction coefficient at the exciting wavelength used. For low absorbance values, this can be expanded to:

$$I = I_0[1 - \ln(10)\epsilon(\lambda_c)Cl]$$

The emission intensity  $F(\lambda)$  for one type of molecule at a given wavelength is a function of the quantum yield  $\phi_F$ , the fraction of emission that occurs at that wavelength  $f(\lambda)$ , and the fraction of the radiation that is actually collected by the detector,  $j$ :

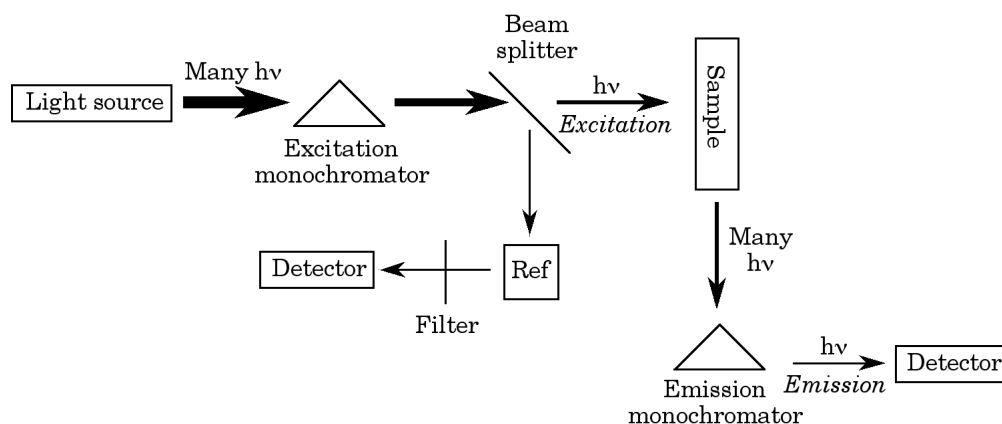
$$F(\lambda) = \ln(10) \epsilon(\lambda_c) C l I_0 \phi_F f(\lambda) j$$

Thus, the fluorescent intensity is related to the probability that the solution will absorb light of the wavelength used (from the  $\epsilon C l$  term) and the probability that the molecule will emit light at a particular wavelength.

In principle, the fluorescence intensity equation could be used to calculate the quantum yield for a molecule. In practice, however, relative quantum yields are measured. The method involves using standards such as quinine sulfate in 0.5 M  $H_2SO_4$  ( $\phi_F = 0.7$ ) or fluorescein in 0.1 M NaOH ( $\phi_F = 0.93$ ), at concentrations such that  $\epsilon C l$  is the same for the standard and the unknown.

## Spectrofluorometers

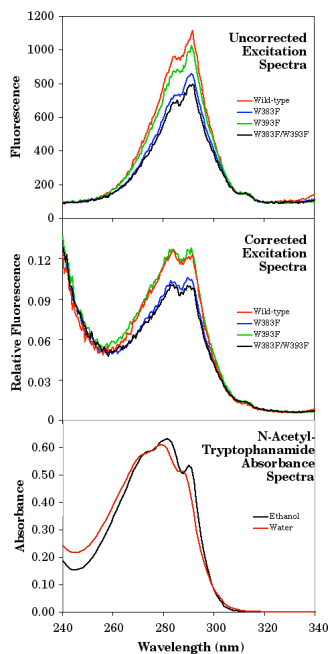
In practice, the measurements are more complex than the relatively simple emission intensity equation implies. To see why, it is necessary to look at a simple diagram for a spectrofluorometer.



The spectrofluorometer consists of a light source, an excitation monochromator, a sample holder, an emission monochromator, and a detector.

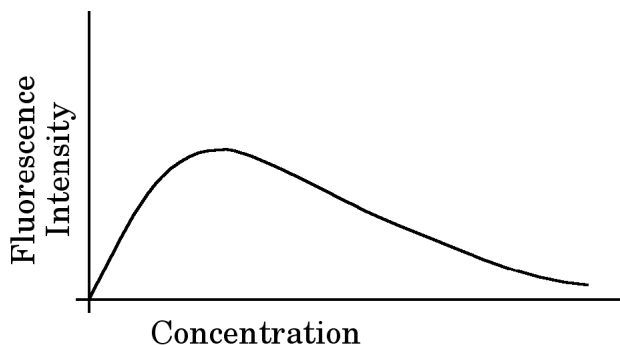
Most spectrofluorometers also have a reference sample. The reference is necessary to correct for lamp output, especially when varying the excitation wavelength, and to correct for differences in detector sensitivity. The graphs below show excitation spectra for proteins containing different amounts of tryptophan. The spectra

uncorrected for lamp output are distorted, while the spectra corrected for lamp output are much more similar to the absorption spectrum of N-acetyl-tryptophanamide.



### Inner filter effect

The fluorescent intensity of a sample is proportional to concentration at low concentrations, levels off, and then decreases. This is because, as the absorbance of the sample increases the light intensity experienced by some of the fluorescent molecules is lower than that experienced by others. When excitation intensity decreases, so does fluorescent emission intensity. It is generally necessary to use concentrations that result in absorbance values of 0.1 or lower to observe concentration dependent emission.



### Emission spectra for single fluorophores

In most cases, the emission occurs at longer wavelengths than are required for excitation. This difference, the **Stokes shift**, is due to a variety of factors; some of these factors are intrinsic to the fluorophore, and some are due to interactions of the fluorophore with its environment.

Absorption occurs on a very short time scale ( $\sim 10^{-15}$  seconds). Several processes then may occur. If the molecule is excited to a higher electronic state (*e.g.*, the  $S_2$  state), it will usually rapidly lose energy via internal conversion, to reach the lowest energy singlet excited state,  $S_1$ , in a process that takes  $\sim 10^{-12}$  seconds. In most cases, the excited state will vibrationally relax to reach the lowest energy state within the excited state.

If the fluorophore has a larger dipole moment in the excited state (which is usually the case, especially for polar fluorophores), the solvent will rearrange to stabilize the greater charge separation. Solvent rearrangement takes  $10^{-11}$  to  $10^{-10}$  seconds. The solvent rearrangement effect is too slow to occur during absorption processes, and therefore absorption tends to be much less sensitive to solvent effects than is fluorescence.

All of these factors mean that the wavelength of the fluorescent radiation is usually longer than that of the absorbed radiation. Because the excited state that decays to the ground state is the same, the fluorescent spectrum is usually independent of the excitation wavelength. This is useful for molecules that contain more than one fluorophore; choosing an excitation wavelength that excites one fluorophore without affecting the other will give an excitation spectrum similar to any other wavelength that would excite the fluorophore if that fluorophore were alone. For example, proteins contain tryptophan and tyrosine. Tyrosine has little absorbance at 295 nm; although this is not the absorbance peak of tryptophan, excitation at this wavelength will result in tryptophan fluorescence unaffected by tyrosine contributions.

### Solvent rearrangement

The solvent rearrangement effect depends on the solvent, with more polar solvents typically exhibiting greater effects. The Lippert equation describes the energy change that results from solvent effects.

$$\bar{\nu}_A - \bar{\nu}_F = \frac{2}{hc} \left[ \frac{d - 1}{2d + 1} - \frac{n^2 - 1}{2n^2 + 1} \right] \frac{(\mu_E - \mu_G)^2}{a^3} + \text{constant}$$

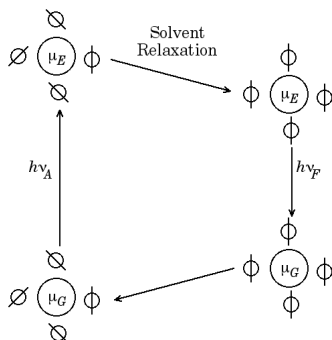
where  $\bar{\nu}_A$  and  $\bar{\nu}_F$  are the frequencies of absorption and emission in wavenumbers ( $\text{cm}^{-1}$ ); the difference for these terms is the Stokes shift;  $d$  is the solvent dielectric constant,  $n$  is the index of refraction,  $\mu_E$  and  $\mu_G$  are the dipole moments of the excited and ground states, and  $a^3$  is the volume of the cavity surrounding the fluorophore. The “constant” reflects the Stokes shift that results from the vibrational relaxation and internal conversion effects mentioned earlier. The term within the brackets is the orientation polarizability of the solvent,  $\Delta f$ :

$$\Delta f = \frac{d - 1}{2d + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

The Lippert equation derivation makes several assumptions. It assumes a spherical fluorophore. It assumes that the dipole vectors for the excited and ground states are

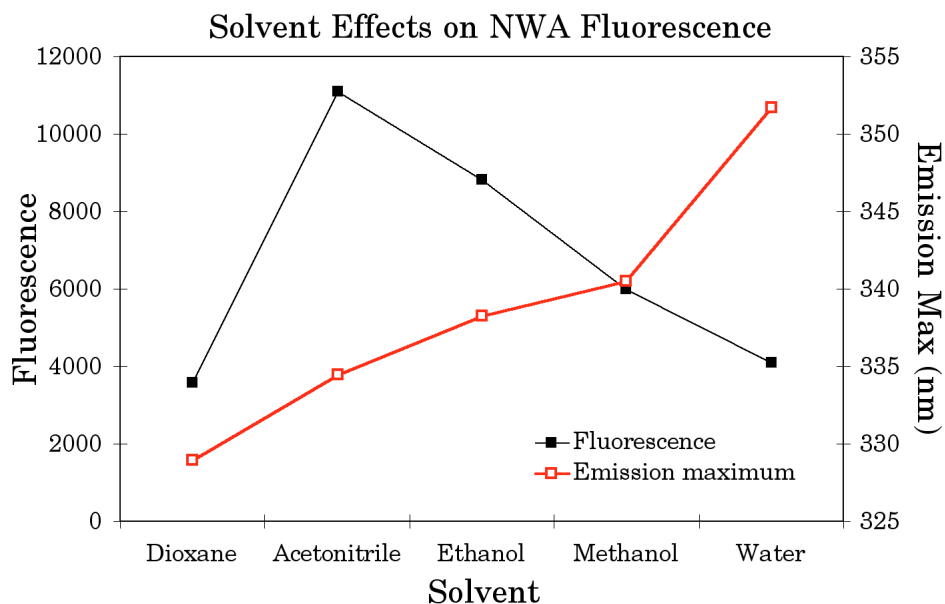
similar (this is a reasonable assumption for most fluorophores). Finally, it ignores specific solvent interactions, such as hydrogen bonds that stabilize the excited state.

The effects of the index of refraction differ from those of the dielectric constant. The index of refraction is a property of the electrons of the solvent molecules, while the dielectric effects require both electronic and molecular orientation effects. The difference means that the index of refraction affects both the excitation and emission properties, while the solvent dielectric affects only the state that results in emission.



The Lippert equation works fairly well for non-protic solvents; protic solvents (such as water and ethanol) tend to form hydrogen bonds, and tend to result in greater than predicted Stokes shifts. In some cases, the specific solvent effect can be corrected for by using solvent mixtures. For example, both ethanol and water have the ability to form hydrogen bonds, but these solvents have ~3-fold differences in dielectric constant.

Observations of Stokes shift can be used to assess environment polarity. The solvent also affects the quantum yield by differential effects on the rate constants for fluorescent emission relative to other relaxation mechanisms. This property can also be used to assess changes in environment. However, the change in quantum yield does not change systematically with polarity. An example of this is shown in the graph at right, which considers the fluorescence of N-acetyl tryptophanamide in different solvents. Note that while the position of the peak emission varies with solvent polarity, the relative quantum yield varies in a non-regular fashion.



## Quenching

Quenching is any process that decreases the fluorescence intensity, and as such, may be the result of a variety of processes. Some of these processes are uninteresting, such as concentration changes or inner filter effects due to excessive concentration or due to light scattering. Photobleaching, in which high radiation intensity may damage the fluorophores, may also cause an apparent quenching; the time-dependent nature of the process differentiates photobleaching from normal quenching effects.

One experimentally useful type of quenching is due to collisions between quenching agents and fluorophores, and is called collisional or **dynamic quenching**. A second type of quenching, sometimes confused with dynamic quenching, is **static quenching**, in which the quenching agent forms a non-fluorescent complex with the quenching agent. A final type of quenching, discussed below, is resonance energy transfer.

Static and dynamic quenching require direct contact between the fluorophore and the quencher. For dynamic quenching, the result of this contact is loss of the fluorescence pathway for return to the ground state, although the mechanism can vary significantly between quenchers. Some quenchers act by inducing intersystem crossing (oxygen and iodide are thought to quench by this method). Others, such as aromatic amines, appear to donate electrons to the excited state.

In the case of dynamic quenching, contact must occur while the fluorophore is in excited state. Dynamic quenching exhibits a concentration-dependence that is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q]$$

where  $\tau_0$  is the lifetime of the fluorescent state in the absence of the quenching agent. If the quenching is not known to be due to dynamic quenching,  $K_D$  is replaced by  $K_{SV}$ .

For dynamic quenching,

$$\frac{F_0}{F} = \frac{\tau_0}{\tau}$$

because the quenching agent decreases the lifetime of the excited state. Dynamic quenching increases with temperature, because temperature increases diffusion rates.

The term  $k_q$  is the second order rate constant that describes the quenching process. It is proportional to the effectiveness of the quencher and the accessibility of the fluorophore to collisions with the quencher. The quenching rate constant is actually comprised of two terms:

$$k_q = f_Q k_0$$

where  $f_Q$  is the fraction of collisions that result in quenching, and  $k_0$  is the diffusion-controlled bimolecular rate constant:

$$k_0 = \frac{4\pi N_0}{1000 \frac{\text{cm}^3}{\text{L}}} (r_f + r_q)(D_f + D_q)$$

where  $N_0$  is Avogadro's number,  $r_f$  and  $r_q$  are the radii of the fluorophore and quencher, and  $D_f$  and  $D_q$  are the diffusion coefficients of the fluorophore and quencher.

Typical values of  $k_0$  for free fluorophores and free quenchers are  $\sim 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ . If the fluorophore is bound to the surface of a protein, the  $k_0$  will be roughly half of this value due available surface occupied by the protein. If the fluorophore is buried within the protein, the value of  $k_0$  will be even smaller, depending on the accessibility of the fluorophore.

Static quenching is the result of the formation of a non-fluorescent complex between the fluorophore and the quencher. The association constant for the quencher-fluorophore complex describes the effectiveness of a static quencher:

$$K_s = \frac{[FQ]}{[F][Q]}$$

where  $[FQ]$  is the complex concentration, and  $[F]$  and  $[Q]$  are the concentrations of free quencher and free fluorophore. Because the total fluorophore concentration,  $[F]_0 = [F] + [FQ]$ ,

$$K_s = \frac{[F]_0 - [F]}{[F][Q]}$$

which rearranges to:

$$K_s = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]}$$

and therefore:

$$\frac{[F]_0}{[F]} = 1 + K_s[Q]$$

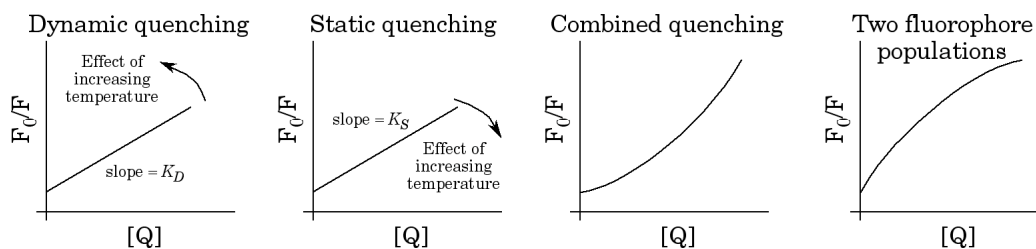
If you assume that the all of the decrease in observed fluorescence is due to complex formation, the equation becomes:

$$\frac{F_0}{F} = 1 + K_s[Q]$$

which is identical in form to the Stern-Volmer equation for dynamic quenching. Static and dynamic quenching can be distinguished by lifetime measurements (which require instruments capable of time resolutions in the nanosecond range), because dynamic quenching reduces the apparent fluorescent lifetime, while static quenching merely reduces the apparent concentration of the fluorophore. Alternatively, temperature effects can be used to distinguish the two forms of quenching. Diffusion rates, and therefore dynamic quenching rates, increase with higher temperature. In contrast, complex formation strength tends to be inversely proportional to temperature, and therefore static quenching tends to be higher at lower temperatures.

In some cases, the effect of the quencher is due to a combination of static and dynamic quenching. This results in a modified equation:

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) = 1 + (K_D + K_S)[Q] + K_D K_S [Q]^2$$



At high quencher concentrations, a dynamic quencher will appear to exhibit combined quenching. This is thought to be due to the fact that, at high concentrations, a significant amount of the quencher molecules are already in close proximity to the fluorophore. Assuming that any quencher within a sphere surrounding the fluorophore will quench the fluorescence, a modified Stern-Volmer equation can be derived:

$$\frac{F_0}{F} = (1 + K_D[Q])e^{\frac{[Q]VN_0}{1000}}$$

in which  $V$  is the volume of the sphere ( $V$  is usually slightly larger than would be predicted for the sum of the quencher and fluorophore radii).

In proteins, more than one population of fluorophore may be present. This is especially true for tryptophan residues, where some may be readily solvent accessible, and others may be buried. Stern-Volmer plots for these proteins frequently curve downward, reflecting the quenching of the accessible fluorophore. Assuming the buried fluorophore is not quenched, fluorescence will be:

$$F = \frac{F_{0a}}{1 + K_a[Q]} + F_{0b}$$

where  $K_a$  is the Stern-Volmer constant for the accessible quencher. In practice, it is likely that the buried fluorophore will exhibit some quenching also, and therefore the curve would be expected to be more complex than is described by this equation.

In quenching experiments with proteins, the quenching agent may interact with the protein in ways that alter the protein structure or that affect the degree of quenching observed. One method for examining this is the use of several quenchers with different properties. Commonly used quenchers include iodide, which is negatively charged, cesium, which is positively charged, and acrylamide, which is neutral. Cesium is a fairly poor quencher due to its low quenching efficiency. Acrylamide, due to its non-polar character, tends to exhibit static quenching (or dynamic quenching masked by its binding to proteins near fluorophores).

When ionic quenchers are used, it is usually necessary to maintain a constant ionic strength in the solution by adding KCl or other non-quenching salt. In addition, for iodide, addition of a reducing agent, such as sodium thiosulfate is necessary to prevent formation of  $I_2$ . Iodide absorbs below 290 nm, and therefore it is advisable to use excitation at 295 nm to prevent artifacts due to low excitation intensities.

Quenching agents are used to probe the environment around fluorophores. Differential quenching by positively and negatively charged quenchers suggests a charged environment. Small  $K_D$  values typically reflect steric hindrance of quencher-fluorophore collisions.

Quenching studies may allow isolation of signals from different fluorophores. They may also allow characterization of conformational changes that alter the accessibility of the fluorophore to the quenching agent.

### **Resonance energy transfer**

A separate type of quenching is resonance energy transfer (sometimes called fluorescence resonance energy transfer), in which the energy from an excited state is transferred to an acceptor molecule. This transfer occurs *without* photon emission, but the process is related to absorbance in some respects. The rate of transfer depends on:

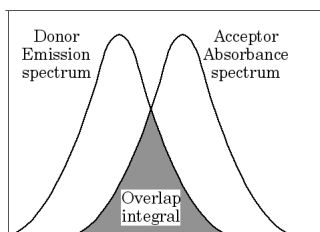
- 1) the spectral overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor;
- 2) the quantum yield of the donor;
- 3) the relative orientation of the transition dipoles of the donor and acceptor;
- 4) the distance between the donor and acceptor.

Resonance energy transfer is critical during photosynthesis; the light antenna molecules use resonance energy transfer to donate the collected energy to the photosynthetic reaction center. In studying proteins, resonance energy transfer is commonly used to measure distances within or between molecules. These studies are either carried out with single donor and acceptor functions, or by the use of instruments capable of time-resolved measurements.

The derivation of the transfer rate is complex, and I will only comment on a few points that directly affect the phenomenon. The rate of transfer for a given distance between donor and acceptor is:

$$k_T(r) = \frac{\phi_D \tau_D^2}{\tau_D r^6} \frac{9000 \ln(10)}{128 \pi^5 N_0 n^4} \int_0^\infty F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda$$

where  $\phi_D$  is the donor quantum yield in the absence of the acceptor,  $\tau_D$  is the lifetime in the absence of the acceptor,  $n$  is the medium index of refraction,  $N_0$  is Avogadro's number, and  $\lambda^2$  describes the relative orientation of the donor and acceptor. The  $\lambda^2$  term is important; if the transition dipoles of the donor and acceptor are perpendicular,  $\lambda^2 = 0$  and no transfer will occur. Although  $\lambda^2$  can vary between 0 and 4, in most cases,  $\lambda^2$  is assumed to be 2/3, which assumes some randomization between the dipoles during the lifetime of the donor excited state. The overlap integral (see the graph below) describes the area of overlap between the emission spectrum of the donor (with  $F_D(\lambda)$  being the fluorescence intensity at wavelength  $\lambda$ ), and the absorption spectrum of the acceptor, (with  $\epsilon_A(\lambda)$  being the extinction coefficient of the acceptor at that wavelength).



For most experiments, it is more convenient to use a standard transfer distance, the Förster distance  $R_0$ .  $R_0$  is calculated for the distance  $r$  at which the rate of transfer is equal to the lifetime;  $R_0$  is thus the distance at which the fluorescent intensity will be 50% of the intensity in the absence of the acceptor.

$$R_0^6 = \frac{\phi_D \tau_D^2 9000 \ln(10)}{128 \pi^5 N_0 n^4} \int_0^\infty F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda$$

If the overlap integral (abbreviated  $J(\lambda)$ ) is in units of  $M^{-1}cm^{-1}nm^4$ , then the Förster distance will be:

$$R_0 = (0.211) \sqrt[6]{\frac{\lambda^2 \phi_D}{n^4} J(\lambda)}$$

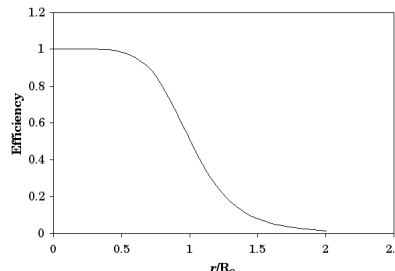
If the  $R_0$  is known, either from calculations, from experiment, or (most commonly) using published values of the  $R_0$  for a particular donor-acceptor pair, the rate of transfer can be readily calculated:

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

where  $\tau_D$  is the donor lifetime in the absence of the acceptor,  $r$  is the distance between the donor and acceptor, and  $R_0$  is the Förster distance, the distance at which transfer is 50% efficient. The  $1/r^6$  distance dependence means that only distances less than  $2R_0$  are typically measurable.

The efficiency of transfer is:

$$E = \frac{k_T}{\frac{1}{\tau_D} + k_T} = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{F}{F_0}$$



The equations given above allow distance measurements for donor-acceptor pairs that do not move relative to one another during the measurement. In proteins, this can be a good assumption; in solution or in membranes, the distances tend to change, and the measured value will be an average distance weighted toward minimum distances.

In fluorophores with small Stokes shifts, the resonance energy transfer between identical fluorophores can be significant. In proteins, tryptophans with blue-shifted emission spectra may transfer energy to other tryptophans.

### Anisotropy

Absorbance transitions are most favorable when the electric field vector aligns with the chromophore dipole. In many fluorophores, the emitted photon is also polarized. The use of polarizing filters may allow the selective collection of specific fluorescent signals. In addition, polarizing filters can be used to look at rotational motions of molecules.

Polarizing filters and controls for changing the polarization angle are only found in moderately sophisticated instruments. For such instruments, anisotropy is measured by comparing observed emission intensities using excitation polarizers set in one direction and emission polarizers set parallel and perpendicular to the excitation setting:

$$Anisotropy = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

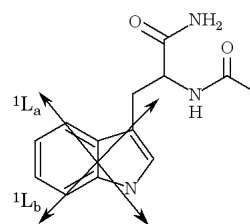
### Fluorescence studies in proteins

Proteins contain three fluorescent amino acids: phenylalanine, tyrosine, and tryptophan. Because of its low extinction coefficient ( $\sim 200 \text{ M}^{-1}\text{cm}^{-1}$ ) and low quantum yield ( $\sim 3\%$ ), phenylalanine fluorescence is rarely used. The quantum yields of tyrosine and tryptophan in proteins are quite variable, with average values of  $\sim 15\%$ ; however, the tyrosine fluorescent emission maximum observed at 303 nm in water has sufficient overlap with the tryptophan absorption spectrum to result in resonance energy transfer. Thus, in most proteins, only the tryptophan fluorescence

is observed. Proteins that do not contain tryptophan do exhibit tyrosine fluorescence, although in many cases, the tyrosine emission intensity is small, with a maximum near 350 nm. This is due to dissociation of the hydroxyl proton; the excited state has a pK of  $\sim 4$  (compared to 10 for the ground state), and the deprotonated form has a much lower quantum yield and a red-shifted spectrum compared to the protonated form of tyrosine.

Incident light at 295 to 300 nm results in selective excitation of tryptophan. Blue-shifted tryptophan may transfer energy to other tryptophans. In addition, tryptophan fluorescence is very sensitive to local environment and is quenched by many other side-chains. Tryptophan quantum yield and spectral properties in proteins can vary dramatically. This can lead to considerable amounts of useful information.

The main tryptophan absorbance band is due to two transitions ( $^1L_a$  and  $^1L_b$ ), which have similar energy, but different dipole moments and different directions. (The dipole vectors for the two transitions are shown at right.) The difference in dipole moment results in differential solvent effects; the different direction means that tryptophan absorption and fluorescence is anisotropic, and the anisotropy varies depending on the transition. The  $^1L_a$  state is more common in protein (especially for excitation at or above 295 nm); it has a single approximately gaussian emission spectrum, and emits photons polarized at  $90^\circ$  from the incident photons. The  $^1L_b$  state is normally only seen in non-polar solvents (such as cyclohexane); its emission spectrum has multiple peaks with very small Stokes shifts.



N-Acetyl-Tryptophanamide

The  $^1L_a$  state has a strong dipole ( $\sim 6$  debye), and in consequence, its energy is sensitive to the solvent polarity. Non-polar environments result in blue-shifted emission spectra, while exposure to aqueous environments result in red-shifted spectra.

In experiments using free amino acids, a neutral form (especially N-acetyl tryptophanamide) is used to prevent interactions between the ionized functions and the indole. N-acetyl tryptophanamide is a reasonable analog of a tryptophan within a protein.

In multi-tryptophan proteins, the observed spectrum is the result of the contributions of all of the tryptophans. These spectra can still be useful, although detecting specific changes due to a single tryptophan can be difficult. One approach that can be useful is site-directed mutagenesis to reduce the number of tryptophans in the protein. Fortunately, tryptophan is relatively rare ( $\sim 1$  in 100 in most proteins), and therefore most proteins have relatively small numbers of tryptophans.

When performing fluorescence experiments, subtracting background is much less necessary than for absorbance or CD. This is because most buffers totally lack fluorescent signals. However, the baseline does show non-fluorescent effects: note

the water Raman peak at ~330 nm and the monochromator artifact at 590 nm (twice the 295 nm excitation wavelength). While the background signals are fairly small, careful measurements, especially for samples with small fluorescence intensities require correcting for these artifacts.

