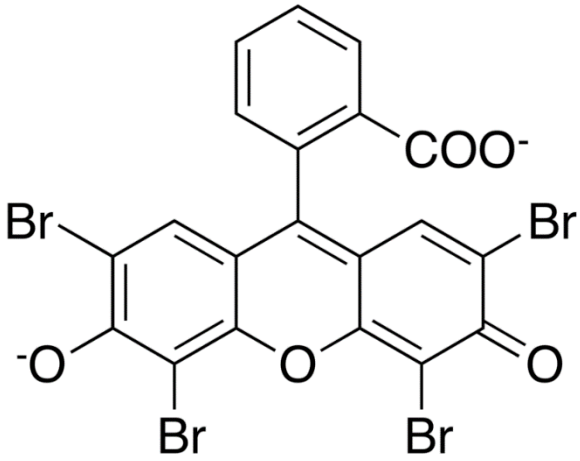
An example of two similar molecules with different lifetimes and quantum yields is shown in Figure 1. The differences in lifetime and quantum yield for eosin and erythrosine B are due to differences in non-radiative decay rates. Eosin and erythrosin B have essentially the same extinction coefficient



Eosin structure

and the same radiative decay rate (Equation 1). Heavy atoms such as iodine typically result in shorter lifetimes and lower quantum yields.

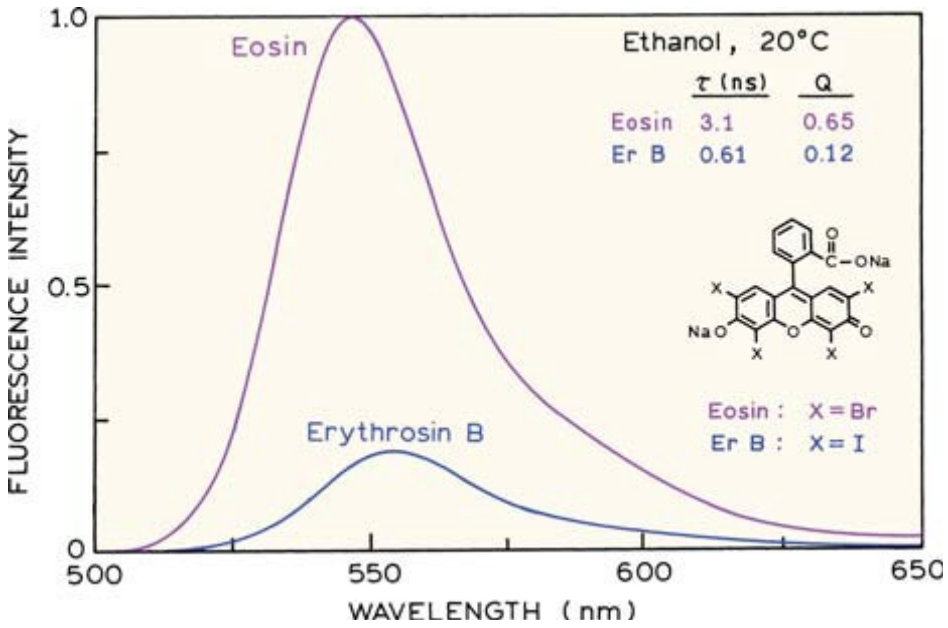


Figure 1. Emission spectra of eosin and erythrosin B (ErB).

In principle, the natural lifetime τ_n can be calculated from the absorption spectra, extinction coefficient, and emission spectra of the fluorophore. The radiative decay rate Γ can be calculated using

$$\Gamma \simeq 2.88\,X\,10^{9} n^{2} \frac{\int F(\bar{\upsilon})\,d\bar{\upsilon}}{\int F(\bar{\upsilon})\,d\bar{\upsilon}/v^{-3}} \int \frac{\mathcal{E}(\bar{\upsilon})}{\bar{\upsilon}}\,d\bar{\upsilon}$$

$$¿ 2.88\,X\,10^{9} n^{2} \langle \bar{\upsilon}^{-3} \rangle^{-1} \int \frac{\mathcal{E}(\bar{\upsilon})}{\bar{\upsilon}}\,d\bar{\upsilon} \quad \text{.....(1)}$$

where $F(\bar{\upsilon})$ is the emission spectrum plotted on the wavenumber (cm^{-1}) scale, $\mathcal{E}(\bar{\upsilon})$ is the absorption spectrum, and n is the refractive index of the medium. The integrals are calculated over the $S_0 \leftrightarrow S_1$ absorption and emission spectra. The fluorescence emission of aromatic substances containing $-NO_2$ groups are generally weak, primarily as a result of large k_{nr} values. The quantum yields of phosphorescence are extremely small in fluid solutions at room temperature. The triplet-to-singlet transition is forbidden by symmetry, and the rates of spontaneous emission are about 10^3 s^{-1} or smaller. Since k_{nr} values are near 10^9 s^{-1}, the quantum yields of phosphorescence are small at room temperature.

Fluorescence Quenching

The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in intensity are called

quenching. Quenching can occur by different mechanisms. Collisional quenching occurs when the excited-state fluorophore is deactivated upon contact with some other molecule in solution, which is called the quencher. Collisional quenching is illustrated on the modified Jablonski diagram in Figure 2. In this case the fluorophore is returned to the ground state during a diffusive encounter with the quencher. The molecules are not chemically altered in the process. For collisional quenching the decrease in intensity is described by the well-known Stern-Volmer equation:

$$\frac{F_\circ}{F}=1+K[Q]=1+k_q\tau_\circ[Q] \quad \text{.....(2)}$$

In this expression K is the Stern-Volmer quenching constant, k_q is the bimolecular quenching constant, $\tau_\circ$ is the unquenched lifetime, and $[Q]$ is the quencher concentration. The Stern-Volmer quenching constant K indicates the sensitivity of the fluorophore to a quencher.

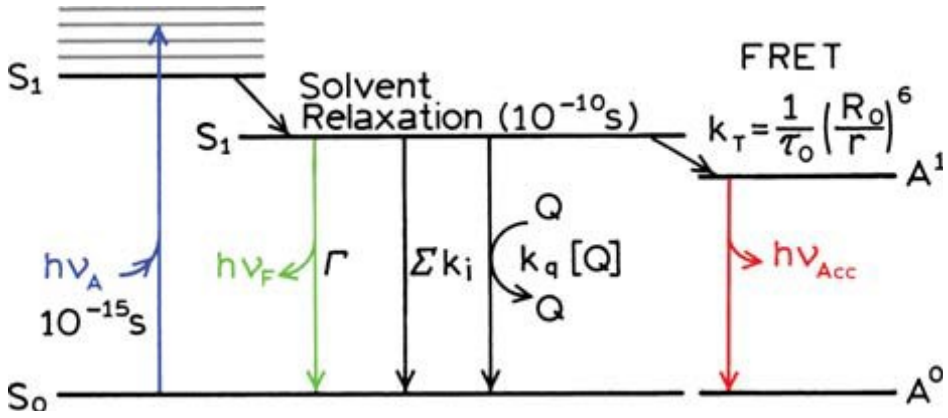


Figure 2. Jablonski diagram with collisional quenching and fluorescence resonance energy transfer (FRET). The term Σk_i is used to represent non-radiative paths to the ground state aside from quenching and FRET.

A wide variety of molecules can act as collisional quenchers. Examples include oxygen, halogens, amines, and electron-deficient molecules like acrylamide. The mechanism of quenching varies with the fluorophore– quencher pair. For instance, quenching of indole by acrylamide is probably due to electron transfer from indole to acrylamide, which does not occur in the ground state. Quenching by halogen and heavy atoms occurs due to spin–orbit coupling and intersystem crossing to the triplet state.

Aside from collisional quenching, fluorescence quenching can occur by a variety of other processes. Fluorophores can form nonfluorescent complexes with quenchers. This process is referred to as static quenching since it occurs in the ground state and does not rely on diffusion or molecular collisions.

According to the Franck-Condon principle, absorption occurs so fast that there is no time for molecular motion during the absorption process. Absorption occurs in the time it takes a photon to travel the length of a photon: in less than 10^{-15} s. As a result, absorption spectroscopy can only yield information on the average ground state of the molecules that absorb light. Only solvent molecules that are immediately adjacent to the absorbing species will affect its absorption spectrum. In contrast to absorption, emission occurs over a longer period of time. The length of time fluorescent molecules remain in the excited state provides an opportunity for interactions with other molecules in solution. Collisional quenching of fluorescence by molecular oxygen is an excellent example of the expansion of time and distance provided by the fluorescence lifetime. If a

fluorophore in the excited state collides with an oxygen molecule, then the fluorophore returns to the ground state without emission of a photon.

RESONANCE ENERGY TRANSFER

Another important process that occurs in the excited state is resonance energy transfer (RET). This process occurs whenever the emission spectrum of a fluorophore, called the donor, overlaps with the absorption spectrum of another molecule, called the acceptor. Such overlap is illustrated in Figure 3. The acceptor does not need to be fluorescent. It is important to understand that RET does not involve emission of light by the donor. RET is not the result of emission from the donor being absorbed by the acceptor. Such reabsorption processes are dependent on the overall concentration of the acceptor, and on non-molecular factors such as sample size, and thus are of less interest. There is no intermediate photon in RET. The donor and acceptor are coupled by a dipole–dipole interaction. For these reasons the term RET is preferred over the term fluorescence resonance energy transfer (FRET), which is also in common use.

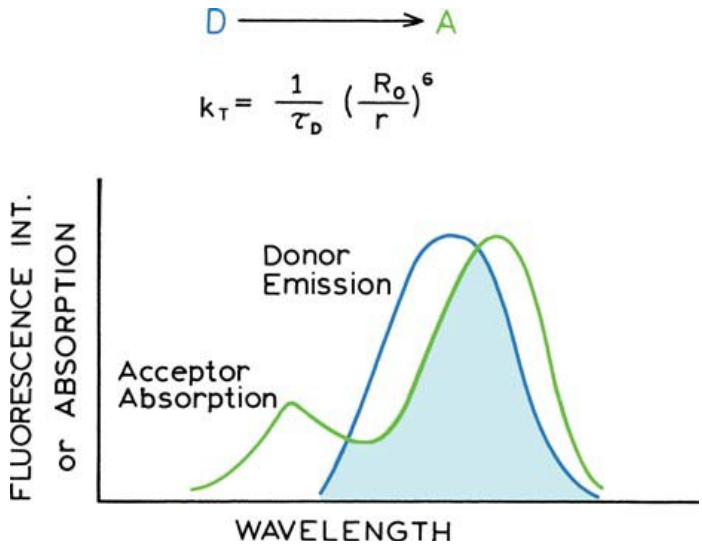


Figure 3. Spectral overlap for fluorescence resonance energy transfer (RET).

The extent of 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 in terms of the Förster distance (R_0). The rate of energy transfer $k_T(r)$ is given by equation 3

$$K_T(r) = \frac{1}{\tau_D}\left(\frac{R_\circ}{r}\right)^6 \quad \text{..... (3)}$$

where r is the distance between the donor (D) and acceptor (A) and τ_D is the lifetime of the donor in the absence of energy transfer. The efficiency of energy transfer for a single donor–acceptor pair at a fixed distance is

$$E = \frac{R_\circ^6}{R_\circ^6 + r^6} \qquad \text{.....(4)}$$

Hence the extent of transfer depends on distance (r). Fortunately, the Förster distances are comparable in size to biological macromolecules: 30 to 60 Å. For this reason energy

transfer has been used as a "spectroscopic ruler" for measurements of distance between sites on proteins. The value of R_0 for energy transfer should not be confused with the fundamental anisotropies (r_0). The field of RET is large and complex. The theory is different for donors and acceptors that are covalently linked, free in solution, or contained in the restricted geometries of membranes or DNA. Additionally, depending on donor lifetime, diffusion can increase the extent of energy transfer beyond that predicted by eq. 4.

MOLECULAR INFORMATION FROM FLUORESCENCE, Emission Spectra and the Stokes Shift

The most dramatic aspect of fluorescence is its occurrence at wavelengths longer than those at which absorption occurs. These Stokes shifts, which are most dramatic for polar fluorophores in polar solvents, are due to interactions between the fluorophore and its immediate environment. The indole group of tryptophan residues in proteins is one such solvent-sensitive fluorophore, and the emission spectra of indole can reveal the location of tryptophan residues in proteins. The emission from an exposed surface residue will occur at longer wavelengths than that from a tryptophan residue in the protein's interior, which shows a shift in the spectrum of a tryptophan residue upon unfolding of a protein and the subsequent exposure of the tryptophan residue to the aqueous phase. Prior to unfolding, the residue is shielded from the

solvent by the folded protein. For example, one of the widely used probes for such studies is 6-(p-toluidinyl) naphthalene-2-sulfonate (TNS), which displays the favorable property of being very weakly fluorescent in water (Figure 4). The green emission of TNS in the absence of protein is barely visible in the photographs. Weak fluorescence in water and strong fluorescence when bound to a biomolecule is a convenient property shared by other widely used probes, including many DNA stains. The protein apomyoglobin contains a hydrophobic pocket that binds the heme group. This pocket can also bind other nonpolar molecules. Upon the addition of apomyoglobin to a solution of TNS, there is a large increase in fluorescence intensity, as well as a shift of the emission spectrum to shorter wavelengths.

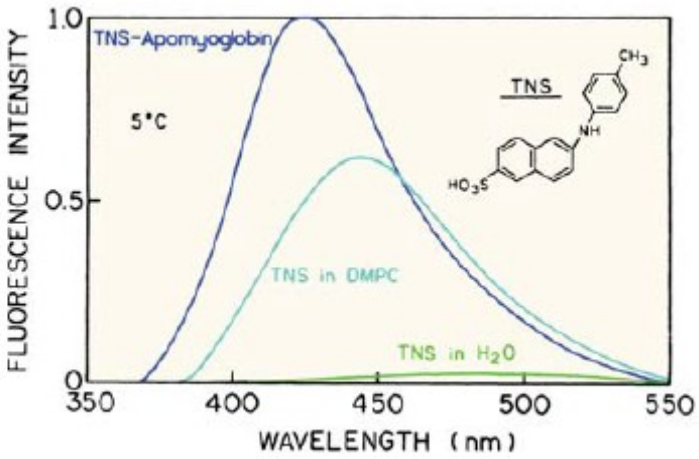


Figure 4. Emission spectra of TNS in water, bound to apomyoglobin, and bound to lipid vesicles