## **Quenching Processes**

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 result in chemical destruction of 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**. The dynamic quenching agent provides a non-radiative route for loss of the excited state energy.



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.

$$Fluor + Q \implies Fluor \bullet Q$$
(Quenched)

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 is 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 diffusioncontrolled bimolecular rate constant:

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

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 ~10<sup>10</sup> M<sup>-1</sup>sec<sup>-1</sup>. 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 quencherfluorophore 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:

$$F = \frac{F_{0_a}}{1 + K_a[Q]} + F_{0_b}$$

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 poorly 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.



Wavelength

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:

$$k_T(r) = \frac{\phi_D \kappa^2}{\tau_D r^6} \left( \frac{9000 \ln(10)}{128\pi^5 N_0 n^4} \right)_0^\infty F_{D,\lambda} \varepsilon_{A,\lambda} \lambda^4 d\lambda$$

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  $\kappa^2$  describes the relative orientation of the donor and acceptor. The  $\kappa^2$  term is important; if the transition dipoles of the donor and acceptor are perpendicular,  $\kappa^2 = 0$  and no transfer will occur. Although  $\kappa^2$  can vary between 0 and 4, in most cases,  $\kappa^2$  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  $\varepsilon_{A,\lambda}$  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.

$$R_0^6 = \frac{\phi_D \kappa^2 9000 \ln(10)}{128 \pi^5 N_0 n^4} \int_0^\infty F_{D,\lambda} \varepsilon_{A,\lambda} \lambda^4 d\lambda$$

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

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

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  $\tau_{\rm D}$  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:





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.