FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

General methodology:

Fluorescence energy transfer can be used to determine molecular distances in biological macromolecules the range of 10-100 Å. Although the distances are quite rough in comparison with those determined by X-ray crystallography or NMR, this technique is helpful in characterizing major conformational changes in macromolecules. A very clear review of the technique is presented by Lupert Stryer (*Ann. Rev. Biochem.* **47**, 819-846 (1978).) The technique is based on the theories of Förster who proposed that electronic excitation energy can be efficiently transferred through dipole-dipole interactions from a donor fluorophore to an acceptor fluorophore in a distance dependent manner. Several factors determine how efficiently the energy is transferred. For a given donor-acceptor pair the distance at which energy transfer is 50% (R_o) is governed by the terms in the following equation.

$$R_o = 9.78 \times 10^3 (n^{-4} f_d k^2 J)^{1/6} \text{ Å}$$

Where n is the refractive index of the sample, usually assumed to be 1.4; f_d is the quantum yield of the donor, J is the overlap integral which is the area of overlap between the donor emission spectrum and the acceptor absorbance spectrum, and finally k^2 is known as the orientation factor. The k^2 term is effectively a function of how the dipole moment of the donor is angularly oriented with respect to the dipole moment of the acceptor. This is the only term in the Förster equation which cannot readily be determined experimentally and is often assumed to be 2/3. Two-thirds is the theoretically appropriate value if both the donor and the acceptor rotate freely, on a time scale which is short relative to the excited state lifetime of the donor.

The R_o value is specific to a given set of donor and acceptor molecules. When these fluorophores are placed at specific sites in the macromolecule of interest, the distance between them is quantified by measuring the efficiency of energy transfer (%E) which then relates to the distance between the probes (r) by the following equation:

$$\%E = R_o^6/(R_o^6 + r^6)$$

So if the molecular distance was R_o, the equation shows that the %E would be 50%, which is how R_o is defined.

The %E can be measured by three slightly different approaches. The first two monitor the quenching of the donor and the third monitors the enhanced fluorescence of the acceptor.

1. Decrease in the fluorescence intensity of the donor. Where F_{da} is the intensity of the donor in the presence of the acceptor and F_d is the intensity of the donor in the absence of the acceptor. ($\%E = 1 - F_{da}/F_a$)

2. Decrease in the excited state lifetime of the donor. Where t_{da} is the lifetime of the donor in the presence of the acceptor and t_d is the lifetime of the donor in the absence of the acceptor. $\%E = 1 - t_{da}/t_d$

3. An increase in the fluorescence of the acceptor, if the acceptor happens to be fluorescent. (A fluorescent acceptor is not necessarily a requirement for energy transfer; the acceptor must only be able to absorb the light given off by the donor. There are several different, and rather complex formulations for quantifying %E by this method.

In looking for systems which are amenable to this technique, one should first consider if it is possible to place the donor and acceptor chromophores at unique sites in the macromolecule. Some proteins contain single tryptophan residues which can serve as a donor. Extrinsic probes (for use as donors or acceptors) can be attached through cysteine residues or amino groups. Other considerations include determining the relative efficiency of donor and acceptor molecular labeling, and determining if the introduction of the label has dramatically perturbed the biological molecule of interest.