

6. Fluorescence resonance energy transfer (FRET)

6.1. Introduction

We have seen that the relaxation of a vibronic state (i.e. a state in which a molecule has both electronic and vibrational excitations), and that of an upper electronically excited state (higher than the first excited singlet) are very fast. It usually takes less than a picosecond to relax to the lowest vibronic state of the first excited singlet state. This means that the cooling of the molecule, i.e. the dissipation of excess vibrational energy is very fast. This also holds true for a pair of molecules, if the excited state of one (the donor) has higher energy than that of the other one (the acceptor). The corresponding process called *energy transfer* leads from the excited donor to the excited acceptor. In the limit where the coupling between donor and acceptor is weak enough (weaker than the energies of all vibrations involved, and weaker than the coupling energy between the molecules, see below), this process is known as fluorescence energy transfer, or FRET. There are two possible mechanisms for energy transfer.

- i) First, a dipole-dipole coupling mechanism called a *Förster* transfer. This process involves Coulomb (electrostatic) interactions between the charge distributions in the two molecules, so that the excited molecule (the donor) goes from its excited to its ground state, while the other one (the acceptor) inversely goes from its ground state to its own (energetically lower) excited state. This process can be seen as the exchange of a virtual photon, as the electrons remain localized on their respective molecules.
- ii) Second, a double electron exchange process. In that case, two electrons are exchanged in a correlated manner between the donor and acceptor, one between the HOMOs, and one between the LUMOs of these molecules. The latter process, called *Dexter* mechanism, is particularly important in the case of energy transfer between triplet states, because the direct process is then spin-forbidden.

The FRET rate (probability per unit time), as given by Fermi's golden rule, is proportional to the square of the coupling, and therefore varies very rapidly with distance R between donor and acceptor, as R^{-6} in the Förster case, and as $e^{-4\alpha R}$ in the Dexter case (α being a typical decay coefficient of the electronic wavefunctions ;

one of the factors 2 arises because of the correlated exchange of two electrons in a Dexter process). Its strong dependence on distance makes Förster-FRET a very useful tool to investigate distances in the range from 2 to 10 nm, depending on the donor (*D*)- acceptor (*A*) couple.

6.2. Model and calculation of the FRET rate

We consider the Förster process only, which applies to strongly allowed transitions of donor *D* and acceptor *A*. We introduce the vibrational levels *a*, *a'* (*d*, *d'*) of the acceptor (donor) in their ground and excited states respectively (see Figure 6.1). Donor and acceptor are coupled by dipole-dipole interaction. In the optical domain, dipole-dipole interaction varies as the classical electrostatic dipole-dipole interaction at distances much shorter than the wavelength of light, but goes over into an inverse squared distance dependence at distances much larger than the wavelength (see Exercise 6.1). For a donor-acceptor pair, the distances are small, and the corresponding interaction is obtained from the classical dipole-dipole interaction formula by replacing the classical dipole moments by quantum-mechanical operators (transition dipole moments) acting on the states of each molecule. Using a tensor notation, where \hat{R} is the unit vector along the axis joining *D* and *A*:

$$V_{AD} = \frac{1}{4\pi\epsilon_0 R^3} \vec{\mu}_A (1 - 3\hat{R}\hat{R}) \vec{\mu}_D$$

In this expression, only the *electrostatic* field of the dipole has been considered. The retarded field, which dominates at distances larger than the wavelength, has been neglected. This is valid since FRET is important only at short ranges (at long ranges, the process merges into emission of a « real » photon by the donor, followed by its absorption by the acceptor). Note also that the dipoles are supposed to lie in vacuum; in solution or in condensed matter, the formulas must be corrected for local fields and index of refraction. The initial and final states in FRET can be written:

$$|i\rangle = |D^* d'; a\rangle$$

$$|f\rangle = |d; A^* a'\rangle \quad ,$$

with Boltzmann populations

$$p(i) = p(d') \times p(a),$$

and where the star (*) indicates the excited electronic state of either molecule and the prime indicates vibrational states in the potential of an excited state. In the Born-Oppenheimer approximation, these states can be written as products of electronic and vibrational wavefunctions (note that the potentials of these vibrational wavefunctions differ in the ground and excited states, both for donor and acceptor) :

$$\langle i | V_{DA} | f \rangle = W_{DA} \langle d' | d \rangle \langle a | a' \rangle$$

where W_{DA} is the purely electronic dipole-dipole matrix element, involving only electronic wavefunctions.

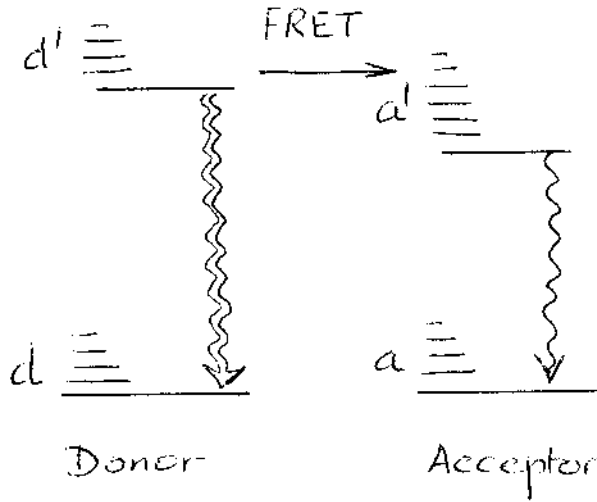


Figure 6.1 : Level diagram for energy transfer (FRET) from a donor to an acceptor. Fluorescence can arise from direct transitions from the excited donor, or from the excited acceptor after energy transfer.

Applying Fermi's golden rule, we may now write the FRET rate from D to A :

$$k_{DA} = \frac{2\pi}{\hbar} \sum_{i,f} p(i) |\langle i | V_{DA} | f \rangle|^2 \delta(E_i - E_f)$$

This expression can be rewritten using the auxiliary normalized functions of energy :

$$F_D(E) = \sum_{d,d'} p(d') |\langle d | d' \rangle|^2 \delta(E - E_D - E_{d'd})$$

$$A_A(E) = \sum_{a,a'} p(a) \left| \langle a | a' \rangle \right|^2 \delta(E - E_A - E_{a'a})$$

It can be checked easily that these functions are the normalized fluorescence of the donor and absorption of the acceptor, respectively, in the Born-Oppenheimer approximation. We therefore get the following relation:

$$k_{DA} = \frac{2\pi}{\hbar} |W_{DA}|^2 \int F_D(E) A_A(E) dE$$

The overlap integral, which has the dimension of the inverse of an energy, plays the role of a density of states, $\frac{1}{\Delta E} = \int F_D(E) A_A(E) dE$. The FRET rate therefore decreases as the sixth power of distance, and has an orientation dependence given by the angles θ_A , θ_D of the dipoles with the axis joining A and D, and φ the angle between the corresponding planes (see Figure 6.2). This angular dependence κ^2 is deduced from :

$$\kappa = -2 \cos \theta_D \cos \theta_A + \sin \theta_D \sin \theta_A \cos \varphi$$

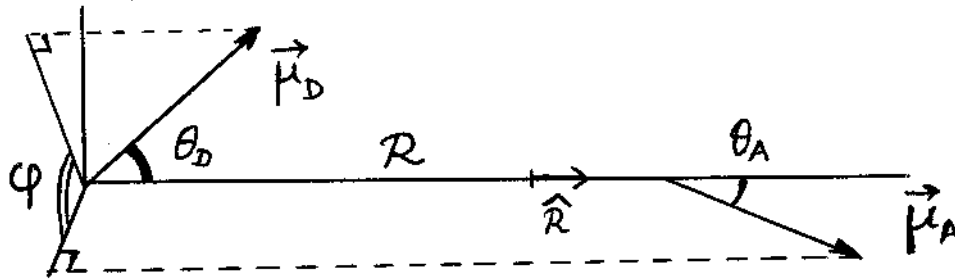


Figure 6.2 : The FRET rate depends on the orientations of the transition dipole moments of the donor and acceptor molecules, relative to the vector joining their centers, and relative to one another.

Considering the dipole's electric field, one sees that κ^2 can vary between 0 and 4. For isotropic distributions of D and A, and provided the orientation fluctuations are slower than the transfer itself, one finds for the average value of κ^2 the value $\frac{2}{3}$. For random orientations of donor and acceptor, the probability distribution of κ^2 presents

a divergence at $\kappa^2 = 0$: there is a very large probability density of finding donor and acceptor with nearly perpendicular dipole moments.

6.3. FRET efficiency and Förster radius

FRET opens a non-radiative channel for the donor molecule. We can define the FRET efficiency E as the yield of transfer to the acceptor as compared to the local dissipation channels on the donor in the absence of the acceptor: fluorescence and non-radiative relaxation.

$$E = \frac{k_{DA}}{k_{DA} + k_{fD}}$$

k_{fD} being the fluorescence decay rate of the donor (including radiative and non-radiative channels). The Förster radius R_0 is the distance at which, for isotropic distributions, the FRET efficiency is 50%. If we suppose that the non-radiative decay is negligible, which is a good approximation for many dyes, the Förster radius can be expressed by using the radiative fluorescence rate of the donor:

$$k_{fD} = \frac{4}{3} \frac{\mu_D^2}{4\pi\epsilon_0\hbar} \left(\frac{\omega}{c} \right)^3$$

$$R_0 = \left[\pi \frac{\mu_A^2}{4\pi\epsilon_0\Delta E} \left(\frac{\lambda}{2\pi} \right)^3 \right]^{\frac{1}{6}}$$

For typical dyes, transition dipole moments are of the order of 1 electron-Angstrom (i.e. several Debye). For a large value of the overlap integral of $1/[200 \text{ cm}^{-1}]$, corresponding to a large overlap, the maximum Förster radius is about 8 nm (case of Cy5-Cy5.5, with a very good overlap between donor emission and acceptor absorption). More typical values of the Förster radius are in the range 5-8 nm (5 nm for Cy3-Cy5), corresponding to lower overlap integrals. The FRET efficiency is related to the Förster radius and to the distance (for isotropic distributions) by:

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

The transfer efficiency can also be deduced from lifetime measurements of the donor, in the absence and in the presence of the acceptor, according to:

$$E = 1 - \frac{\tau_D(A)}{\tau_D(0)}.$$

6.4. Single-pair FRET

In order to observe significant FRET, we need donor and acceptor molecules within about one Förster radius from each other. This can be achieved in biomolecules by labeling the same molecule (e.g. a protein) with the two dyes (cf. S. Weiss, Science 283 (1999) 1676). FRET is well adapted to typical sizes of protein molecules. The transfer will appear as acceptor fluorescence when the donor only is excited (due to the breadth of absorption bands, acceptor molecules are always partially excited too; the effect of direct acceptor absorption has to be corrected for).

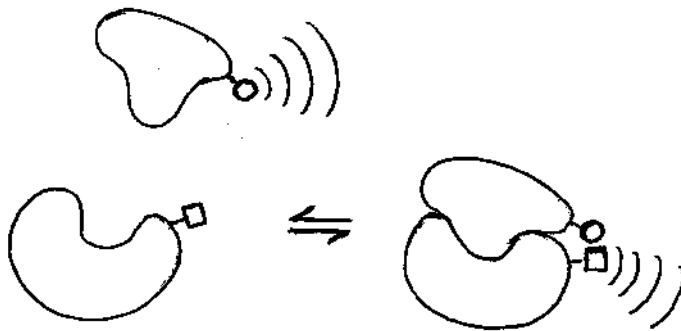


Figure 6.3 : Two biomolecules labelled with donor and acceptor fluorophores respectively, may associate. In the bound form, FRET is efficient and only the acceptor fluoresces.

Single-pair FRET is detected by measuring for each doubly-labeled molecule the fluorescence intensities of donor and acceptor. The molecular fluorescence is split by a dichroic filter into signals from the donor F_D , and from the acceptor F_A . These quantities must be corrected for non-radiative rates (fluorescence quantum yields) and for direct acceptor absorption. The ratio

$$E = \frac{F_A}{F_A + F_D},$$

is then the FRET efficiency, and directly gives the D-A distance if the angular distribution of the molecules is isotropic. In the case of fixed donor and acceptor orientations, the angular factor is much more difficult to evaluate. It not only depends on the respective orientation of the dipole moments, which could be measured by polarization microscopy, but also on the radius vector, which is usually unknown. Lifetime measurement also provide access to the FRET efficiency. When possible, this measurement in the time domain is more direct and reliable than intensity measurements, which are subject to cross-talk and background artefacts.

In bulk experiments, it is very important to ensure a high purity of the doubly-labeled molecules, because only average intensities are measured. For example, a fraction of the molecules labeled with the donor only would lead one to underestimate the transfer efficiency. In single-molecule experiments, one can measure the fluorescence signals of each molecule separately by exciting at two different wavelengths. The corresponding method is called Alternating Laser Excitation (ALEX, Kapanidis) or Pulse-Interleaved Excitation (PIE, Lamb) when the two lasers deliver consecutive pulses of two colors. In this way, the “stoichiometry” of the constructs (a number characterizing the presence of both donor and acceptor in the construct) can be verified: bursts of complete constructs should give fluorescence under either donor or acceptor excitation. Simply-labeled constructs with the donor alone give no fluorescence upon acceptor excitation, while constructs with acceptor alone give only the cross-talk of acceptor fluorescence upon donor excitation. These incomplete constructs will appear in the corners of a stoichiometry-FRET efficiency scatter plot. Incomplete constructs can thus be easily eliminated from the statistics, either in a simple histogram (see Fig. 6.4) or in a scatter plot.

The easiest measurement in FRET is that of the distributions of efficiencies in immobilized molecules, or in slowly diffusing molecules if the signal is sufficient. In liquid solution, one often assumes isotropy, but this may not be valid if the labels are interacting strongly with the molecules to which they are bound, for example protein chains.

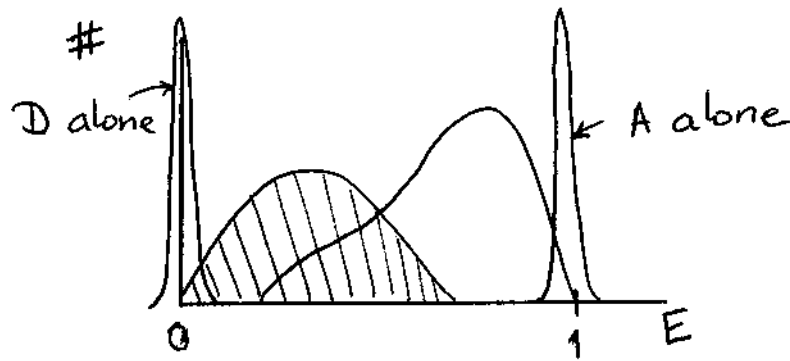


Figure 6.4 : A histogram of the FRET efficiencies measured for a large number of donor-acceptor pairs may reveal several distributions. Peaks at 0 and 1 may represent unbound molecules, or pairs in which either the acceptor or the donor have been photobleached. The other distributions represent two conformations of the pair, with a distributions of distances and/or of angles.

A more difficult measurement is that of the dynamical fluctuations of the FRET efficiency. If the donor-acceptor distance fluctuates, while donor and acceptor are still randomly exploring isotropic distributions, anti-correlated fluctuations will appear either directly in the intensity traces of D and A fluorescence for immobilized molecules (Fig. 6.5), or as a negative cross-correlations in a FCS experiment in solution.

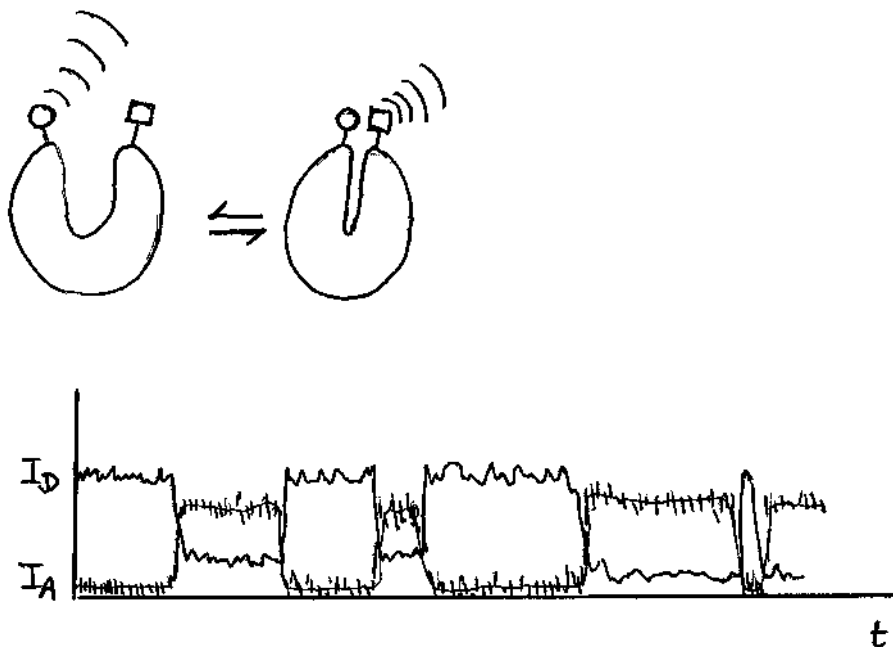


Figure 6.5 : Conformational changes of a biomolecule carrying two FRET labels. Because these changes affect the FRET efficiency, they appear as anti-correlated fluctuations in the fluorescence traces of donor and acceptor.

Exercise 6.1: We want to derive the field created by a classical oscillating electric dipole $\vec{\mu} \exp(i\omega t)$. Apply Maxwell's equations and use the scalar (V) and vector

(\vec{A}) potentials satisfying Lorentz's gauge ($\vec{\nabla} \cdot \vec{A} + \frac{1}{c^2} \frac{\partial V}{\partial t} = 0$) to derive Helmholtz's

equation for the vector potential: $\frac{1}{c^2} \frac{\partial^2 \vec{A}}{\partial t^2} - \vec{\Delta} \vec{A} = \mu_0 \vec{j}$, where the source term stems

from the current density \vec{j} . For an oscillating dipole, show that this source term

becomes $\vec{j} = i\omega \delta(\vec{r}) \exp(i\omega t) \vec{\mu}$ and that the retarded potential solution

$\vec{A} = \frac{\mu_0}{4\pi} i\omega \vec{\mu} \cdot \frac{1}{r} \exp\left(\frac{i\omega r}{c}\right)$ solves the Helmholtz equation. Using the electric field

expression $\vec{E} = -\vec{\nabla} V - \frac{\partial \vec{A}}{\partial t}$, show that $\vec{E} = \frac{\mu_0}{4\pi} \left[\omega^2 + c^2 \vec{\nabla} (\vec{\nabla} \cdot) \right] \vec{\mu} \frac{\exp(i\omega r / c)}{r}$ and

deduce the following expression of the field, including radiated field and near field:

$$\vec{E} = \frac{\exp(i\omega r / c)}{4\pi\epsilon_0} \left[\frac{\omega^2}{c^2 r} \left(1 - \frac{\vec{r}\vec{r}}{r^2} \right) + \frac{1}{r^3} \left(1 - \frac{i\omega r}{c} \right) \left(\frac{3\vec{r}\vec{r}}{r^2} - 1 \right) \right] \vec{\mu}.$$

Exercise 6.2: Show by integration over angles that, for an isotropic distribution of

donor and acceptor, the average angular factor $\langle \kappa^2 \rangle = \frac{2}{3}$.