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 should hold true for a pair of molecules, considered as a large "supermolecule", if the excited state of one of these molecules (called the "donor") has a higher energy than that of the other one (called the "acceptor"). Relaxation will take place and transfer the excitation energy of the donor to excitation of the acceptor. The corresponding process is called *energy transfer*. It leads from a state with (electronically) excited donor and ground-state acceptor to the state with (electronically) excited acceptor and ground-state donor. The difference in electronic energy between those two states is dissipated in vibrations of the molecules. In the limit where the coupling between donor and acceptor is weak enough, i.e., weaker than the electronic energy difference between the molecules, and weaker than the energies of all vibrations involved, this process is known as fluorescence resonance 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 electromagnetic 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 molecule (the acceptor) inversely goes from its ground state to its own, energetically lower, excited state, while some part of the energy is released as vibrations or phonons. This process can be seen as the exchange of a virtual photon, as each electron remains localized on its respective molecule. Because the donor and acceptor are generally very close to each other, in practice less than 10 nm, each one of them lies in the near field of the other and their interaction is electrostatic, or Coulombic (retardation can be neglected).
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. Again, some vibrational

quanta are created to ensure energy conservation. The latter process, called *Dexter* mechanism, is particularly important in the case of energy transfer between triplet states, because the direct dipole-dipole process is then spin-forbidden.

The probability per unit time of FRET, called FRET rate, is given by Fermi's golden rule. It is proportional to the square of the coupling, and therefore varies very rapidly with distance R between donor and acceptor considered as points dipoles. This distance dependence is R^{-6} in the Förster case, and $e^{-4\alpha R}$ in the Dexter case; α is a typical decay coefficient of electronic wavefunctions ; the factor 4 arises from the square of the wavefunction and from the correlated exchange of two electrons in the Dexter process. Its strong dependence on distance makes Förster-FRET a very useful tool to measure and monitor distances between two dipoles in the range from 2 to 10 nm. The characteristic range of FRET depends on the donor (D)- acceptor (A) molecules, as explained below.

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 and at distances much shorter than the wavelength of light, dipole-dipole interaction varies as the classical electrostatic dipole-dipole interaction. It goes over into an inverse squared distance dependence at distances much larger than the wavelength (see Exercise 6.3), where light emission and absorption by dipoles are valid description of the energy transfer process. For usual donor-acceptor pairs, the distances are small (1-10 nm), 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:

$$V_{DA} = \frac{1}{4\pi\varepsilon_0 R^3} \vec{\mu}_A \left(1 - 3\hat{R}\hat{R}\right) \vec{\mu}_D$$

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 above formula supposes the dipoles to lie in vacuum; in solution or in condensed matter, the formula must be corrected for the index of refraction of the medium and for local fields due to nearby polarizable objects. 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). Thus, the dipole-dipole matrix element between initial and final states writes as :

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

where W_{DA} is the purely electronic dipole-dipole matrix element, involving only electronic wavefunctions and the other products are Franck-Condon amplitudes for donor and acceptor. W_{DA} varies as the inverse cube of the distance between donor and acceptor.

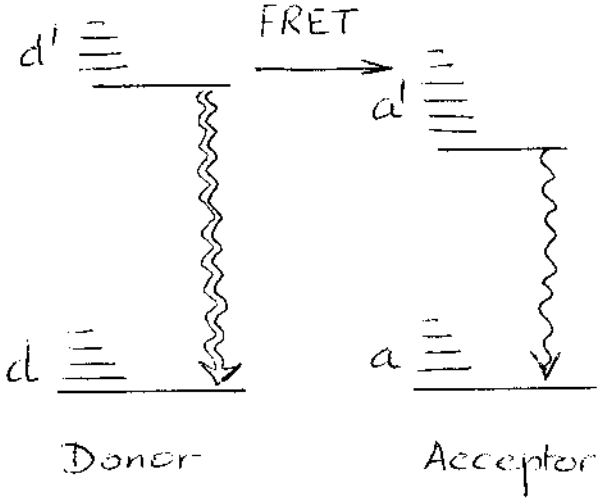

Figure 6.1 : Level diagram for energy transfer (FRET) from a donor to an acceptor. Fluorescence can arise from direct transitions from the excited donor, or from the excited acceptor after energy transfer.

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

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

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

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

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

It can be checked easily that $F_D(E)$ is the normalized fluorescence spectrum of the donor and $A_A(E)$ is the normalized absorption spectrum of the acceptor in the Born-Oppenheimer approximation. The normalization is done so that the integral of these functions over the whole energy spectrum is unity. We therefore get the following relation:

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

The overlap integral, which has the dimension of the inverse of an energy, plays the role of a density of states, $\rho(E) = \frac{1}{\Delta E} = \int F_D(E) A_A(E) \mathrm{d}E$. We thus note that, from the molecule's point of view, the emission and absorption of a photon corresponds to

the same process, whether the field is created by a faraway source such as a laser, or by a nearby dipole such as the donor. The overlap integral thus involves the same absorption spectrum measured by excitation with a plane wave and spontaneous emission spectrum towards vacuum modes measured in standard ensemble experiments. Finally, note that the FRET rate decreases as the *sixth* power of distance between donor and acceptor.

The orientation dependence of the FRET rate is given by three angles defining the geometry of the donor-acceptor system: θ_A, θ_D are the angles of the dipoles with the axis joining D and A, and φ is the angle between the two planes containing respectively the DA axis and the donor dipole on one hand, and the DA axis and the acceptor dipole on the other hand (see Figure 6.2). This angular dependence of the rate amounts to multiplication by a factor κ^2 deduced from :

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

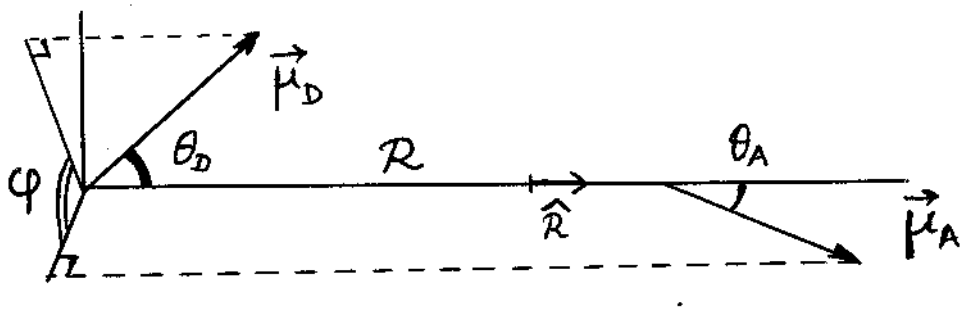

Figure 6.2 : The FRET rate depends on the orientations of the transition dipole moments of the donor and acceptor molecules, relative to the DA vector joining their centers, and on the angle φ between the planes defined by DA axis and dipole moments.

Considering the dipole's electrostatic 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, the average transfer rate is proportional to the average value of κ^2, $\frac{2}{3}$ (see Ex. 6.1). For random orientations of donor and acceptor, the probability distribution of κ^2 presents a divergence at $\kappa^2 = 0$ (see Ex. 6.5): there is a relatively large probability density of finding donor and acceptor with dipole moments oriented so that no FRET takes place, essentially because one of the dipole

moments has a large probability to be nearly perpendicular to the field created by the other.

6.3. FRET efficiency and Förster radius

FRET opens up a non-radiative channel for the donor molecule. We can define the efficiency of this channel as the FRET efficiency *E* as the yield of transfer to the acceptor as compared to the local dissipation channels of 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 donor's 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, itself a function of the donor's transition dipole moment:

$$k_{fD} = \frac{4}{3}\frac{{\mu_D}^2}{4\pi\varepsilon_0\hbar}\left(\frac{\omega}{c}\right)^3$$

$$R_0 = \left[\pi\frac{{\mu_A}^2}{4\pi\varepsilon_0\Delta E}\left(\frac{\lambda}{2\pi}\right)^3\right]^{\frac{1}{6}}$$

For typical dyes, transition dipole moments are of the order of 1 electron-Angstrom (i.e., 4.8 Debye). For a large value of the overlap integral of 1/[200 cm^{-1}], corresponding to a strong spectral overlap, the Förster radius is large, about 8 nm in the case of the couple Cy5-Cy5.5, which presents strongly allowed transitions with a very good overlap between donor emission and acceptor absorption. More typical values of the Förster radius for strong transitions are in the range 5-8 nm, e.g., 5 nm for Cy3-Cy5, corresponding to a lower overlap integral. For isotropic angular distributions of the dipoles, the FRET efficiency is related to the Förster radius and to the distance by:

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

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

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

Lifetime-based measurements of the FRET efficiency are often preferred to those based on fluorescence intensities, as lifetime measurements are less sensitive to experimental conditions than intensity measurements.

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 DNA strand, or 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. However, due to the breadth of absorption bands, acceptor molecules are always excited to some extent by the laser exciting the donor; in quantitative FRET measurments, where absolute distances are sought, the effect of direct acceptor absorption has to be corrected for.

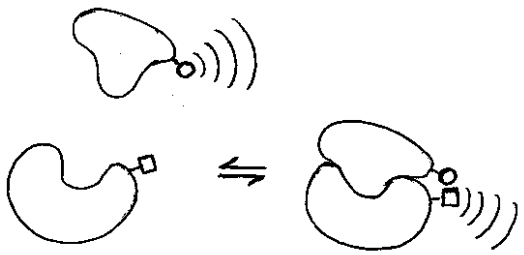

Figure 6.3 : Two biomolecules labelled with donor and acceptor fluorophores respectively, may associate. In the bound form, FRET is efficient and the acceptor fluoresces more strongly than in the unbound state.

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 measurements 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, however, 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, i.e., a number characterizing the presence of both donor and acceptor in the construct can be verified: bursts of complete constructs should give fluorescence both under donor and 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 of stoichiometry versus efficiency.

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 proteins.

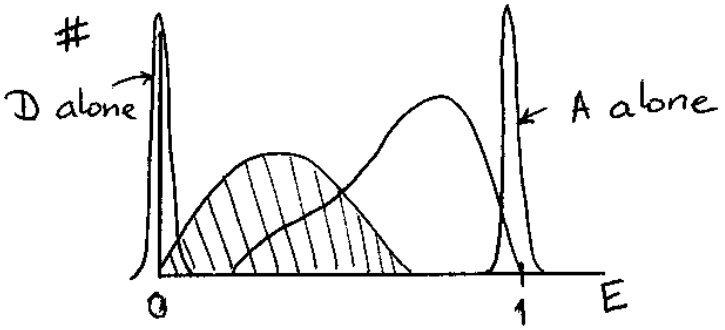

Figure 6.4 : A histogram of the FRET efficiencies measured for a large number of donor-acceptor pairs may reveal several distributions. Peaks at 0 and 1 may represent unbound molecules, or pairs in which either the acceptor or the donor have been photobleached. The other distributions represent two conformations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

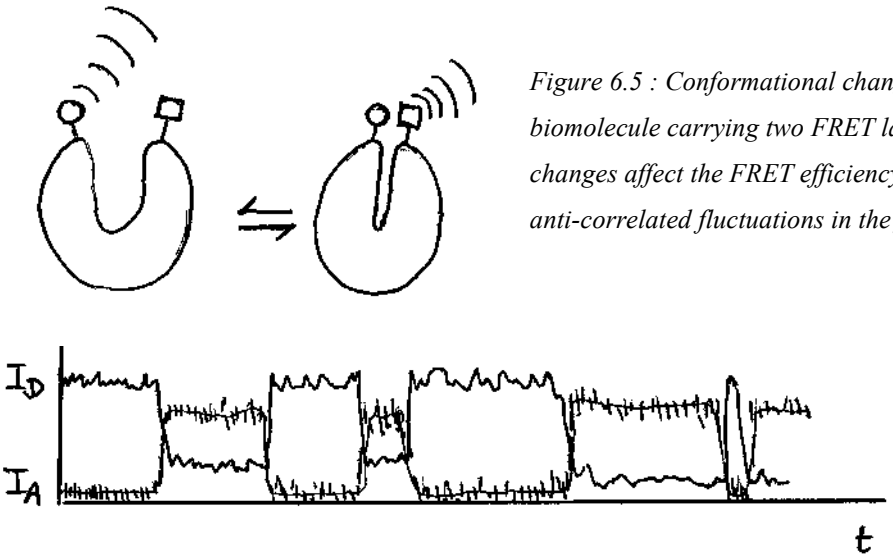

Figure 6.5 : Conformational changes of a biomolecule carrying two FRET labels. Because these changes affect the FRET efficiency, they appear as anti-correlated fluctuations in the fluorescence traces of donor and acceptor.

Exercise 6.1: Show by integration over angles that, for an isotropic distribution of donor and acceptor, the average angular factor $\left\langle \kappa^2 \right\rangle = \frac{2}{3}$.

Exercise 6.2: We consider energy transfer from a single donor molecule to a single molecular layer of acceptors (e.g., a Langmuir film, or a graphene sheet).
We assume the donor to be at distance h from the acceptor layer, and we disregard the angular dependence of the transfer rate. Integrate the donor-acceptor transfer rate over the whole acceptor layer. Show that the FRET rate varies as h^{-4}.

Exercise 6.3: 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* $\vec{E}=\frac{\mu_0}{4\pi}\left[\omega^2+c^2\vec{\nabla}\left(\vec{\nabla}\cdot\right)\right]\vec{\mu}\frac{\exp(i\omega r/c)}{r}$ *and deduce the following expression of the field, including radiated field and near field:*
$\vec{E}=\frac{\exp(i\omega r/c)}{4\pi\varepsilon_0}\left[\frac{\omega^2}{c^2 r}\left(1-\frac{\vec{r}\vec{r}}{r^2}\right)+\frac{1}{r^3}\left(1-\frac{i\omega r}{c}\right)\left(\frac{3\vec{r}\vec{r}}{r^2}-1\right)\right]\vec{\mu}$.

Exercise 6.4: Energy transfer from a single donor to a spatially random distribution of acceptors (Förster 1949).
We suppose the acceptors to be immobile during each donor fluorescence decay, but to diffuse fast enough that all configurations are sampled when the statistics of the fluorescence decay are acquired. For simplicity, we assume the donor's fluorescence lifetime to be very long.

i) Express the probability $p(t)$ *of still finding the donor excited after time t, starting from the excited donor at time zero,* $p(0)=1$. *Consider the donor as interacting with N acceptors* A_k *at distances* R_k.

We average this probability over all possible configurations of acceptors within a large sphere of radius R_M.

ii) The acceptors being identical and uncorrelated, show that the average obeys: $\langle p(t)\rangle = [J(t)]^N$, *with* $J(t) = \langle \exp(-k(R)t)\rangle$, *and* $k(R) = k_0 [R/R_0]^{-6}$ *is the Förster transfer rate as a function of distance and Förster radius* R_0.

iii) Argue that $J(t)$ *is always very close to 1 for a large integration sphere.*

Expanding to first order, $J(t) = 1 - \lambda(t)$, *derive the following expression:*

$\lambda(t) = \sqrt{\pi k_0 t}\,(R_0 / R_M)^3$ *(Hint: in the expression of* $\lambda(t)$, *extend the integration to infinity, use an integration by parts, and* $\int_0^\infty x^{-1/2} e^{-x} dx = \sqrt{\pi}$*).*

iv) Conclude that the averaged decay is of the form: $\langle p(t)\rangle = \exp\left(-bc_A\sqrt{k_0 t}\right)$, *where* c_A *is the volume concentration of acceptors and b is a constant. Physically discuss the origin of this unusual analytical form.*

<u>*Exercise 6.5:*</u> *Probability distribution function of* κ^2, *the angular factor in FRET. One often averages this angular factor to 2/3, assuming the angular distributions of donor and acceptor to be isotropic. However, it is important to realize that the distribution function is very spread between 0 and 4, with a divergence for 0. Here, we calculate this distribution function following Dale et al., Biophys. J.* ***26*** *(1979) 161.*

i) Write the angular factor κ^2 *as a function of the donor angle* θ_D *and of the angle* ω *between the acceptor's dipole and the donor's field at the acceptor position. The result is:* $\kappa^2 = \left(1 + 3\cos^2\theta_D\right)\cos^2\omega$.

Because donor and acceptor have uncorrelated random orientations, an isotropic distribution of acceptor dipole is an isotropic distribution of the acceptor dipole with respect to the donor's field as well, for every donor orientation.

ii) Integration over the two angles through the variables $x = \cos\theta_D$ *and* $y = \cos\omega$ *leads to the following expression of the probability density* $p(z)$ *of* $z = \kappa^2$ *:*

$$p(z) = \int_0^1 dx \int_0^1 dy\, \delta\left[z - y^2\left(1 + 3x^2\right)\right],$$

where delta is the Dirac function. Argue why this expression gives the probability density and prove that it is normalized to unity.

Use the theorem $\delta\left[f(x)\right] = \delta(x - x_0) / \left|\frac{df}{dx}\right|_{x_0}$, x_0 *being the zero of the function in the integration domain (supposing there is one at most) to perform the integral on x. Note that not all values of y are allowed to get possible values of* x_0 *falling inside the integration domain.*

The result is:

For $0 \le z \le 1$ $\qquad p(z) = \int_{\frac{1}{2}\sqrt{z}}^{\sqrt{z}} \frac{dy}{y\sqrt{z - y^2}} = \frac{\ln\left(2 + \sqrt{3}\right)}{2\sqrt{3z}}$

For $1 \le z \le 4$ $\qquad p(z) = \frac{1}{2\sqrt{3}} \int_{\frac{1}{2}\sqrt{z}}^{1} \frac{dy}{y\sqrt{z - y^2}} = \frac{1}{2\sqrt{3z}} \ln\left(\frac{2 + \sqrt{3}}{\sqrt{z} + \sqrt{z - 1}} \right)$

Plot the function p(z) between 0 and 4 and explain why the value 2/3 is not particularly probable in an experiment where donor and acceptor have fixed orientations.