of the extent of binding. This effect (known informally as "Chip dip") is illustrated in Figs. 6 and 7 which concern the ethidium bromide/tRNA system.[41] In this case the free ethidium bromide has a lifetime of 1.86 nsec and a rotational relaxation time of 0.54 nsec. On binding the Phe-tRNA^{Phe} the lifetime increases to 26 nsec and the rotational relaxation increases to 136 nsec. A single rotating species cannot give rise to a negative value for the differential phase delay (assuming excitation at a wavelength which gives a positive P_0), and the negative excursion for this function shown in Fig. 6 is due to the presence of free ethidium bromide in equilibrium with bound material. Figure 7 shows the calculated effects for increasing amounts of bound material.

We should note that, given the difference in quantum yield between the free and bound probe, the fractional intensities utilized in Fig. 7 actually represent small percentages of bound probe on a molar basis. In fact, considering the accuracy of the differential phase measurement (better than 0.1°) one can detect, in this system, on the order of 0.1% bound probe. This phenomenon also occurs in time-domain measurements. Specifically, if one monitors the anisotropy decay of a system which displays multiple lifetimes associated with multiple rotational diffusion rates then one may observe a decline at short times of the anisotropy followed by a rise at latter times and subsequent decrease. This "dip and rise" effect has been observed by Millar and co-workers[22] in studies on protein–DNA interactions, specifically in the case of the interaction of a fluorescent DNA duplex with the Klenow fragment of DNA polymerase.

[13] Fluorescence Resonance Energy Transfer

By PAUL R. SELVIN

Introduction

Fluorescence resonance energy transfer (FRET) is a technique for measuring the distance between two points which are separated by approximately 10–75 Å. The technique is valuable because measurements can be made under physiological (or other) conditions with near angstrom

resolution and with the exquisite sensitivity of fluorescence measurements. For these reasons FRET has found wide use in polymer science, biochemistry, and structural biology. Reviews have appeared on FRET applied to actin structure,[1] nucleic acids,[2] phycobiliproteins,[3-5] cell surfaces with an emphasis on protein interaction,[6] *in situ* imaging,[7] diffusion,[8] and microscopy.[9-12] Among systems already studied in the literature are DNAs such as oligonucleotides[13-16] and Holliday junctions[14,17,18]; proteins such as oligopeptides,[19] rhodopsin,[8,20] myosin,[21] various calcium binders,[22] and major histocompatibility complexes[23]; RNA[24,25]; and nucleic

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acid–protein complexes such as nucleosomes,[26-29] chromatin[30,31] and pro-
tein–promoter interactions.[32] FRET has also been used to monitor dy-
namic processes such as actin assembly,[1,33] nucleosome assembly, and
human immunodeficiency virus (HIV) protease activity.[34] Van der Meer
and co-workers have recently written a book on FRET.[35] Excellent re-
views of the basics of FRET, which complement this chapter, have ap-
peared.[36-38]

The idea behind the technique is to label the two points of interest with
different dyes; one, which must be fluorescent, is called the donor, and
the other, which is not necessarily fluorescent but often is, is called the
acceptor. By choosing dyes with the appropriate spectral characteristics,
the donor, after being excited by light, can transfer energy to the acceptor.
The efficiency of energy transfer depends on the inverse sixth power of
the distance between the dyes. In general, the acceptor must be within
10–75 Å to get reasonable energy transfer, the exact range depending on
the dyes chosen.

If one measures the amount of energy transfer, it is therefore possible
to determine the distance between donor and acceptor. Qualitatively, the
farther apart the donor and acceptor, the less energy transfer. The extent
of energy transfer can be measured because the fluorescence of the donor
(both intensity and lifetime) decreases, or is quenched, and the acceptor,
if fluorescent, increases its fluorescence, or becomes "sensitized," with
energy transfer. These changes in fluorescence can be measured by com-
paring a complex labeled with both donor and acceptor to ones labeled
only with donor and only with acceptor. The experimental and theoretical
details are presented below.

Other Uses

Measuring the (static) distance between two points, although the main
use of FRET, is just one application. A number of workers have also used

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[36] C. R. Cantor and P. R. Schimmel, "Biophysical Chemistry," Vol. 2, pp. 448–455. Free-
man, San Francisco, California, 1980.

FRET to measure dynamic processes, including enzymatic activity such as HIV proteolysis[34] or molecular assembly such as actin assembly.[1,33] In these cases the distance between two points changes as a function of some dynamic process; the ends of a polypeptide separate after cleavage by HIV, for example. As long as the dynamic process is not too fast (long enough to acquire a reasonable fluorescent signal—roughly, a few minutes with ease or, with effort, subsecond), the process can be monitored by FRET.

Yet another application of FRET is to measure rates of diffusion and distances of closest approach. In these cases, one uses a long-lived donor (lifetime on the order of a microsecond to millisecond). During the excited state lifetime of the donor, the donor and/or acceptor can diffuse. If the distance which they can diffuse during the donor lifetime is on the order of the average distance separating the donor and acceptor, the amount of energy transfer will depend on the diffusion coefficients, and hence measurements of energy transfer can shed light on this quantity. If the average distance is considerably less than the diffusional distance, the donor and acceptor approach each other many times during the donor lifetime, and energy transfer will depend mostly on the distance of closest approach.

A third and relatively new application of FRET is the generation of new compound dyes with spectral characteristics that combine the best of both dyes. The idea is to attach covalently a donor and acceptor together in close proximity to one another. In the simplest case, where the absorption or emission properties of the individual dyes do not change, the absorption characteristic of the compound dye is the sum of the two individual dyes. At the same time, the emission is dominated by the acceptor since almost all of the energy absorbed by the donor is transferred to the acceptor. This results in dyes having potentially large Stokes shifts (the sum of the donor and acceptor Stokes shifts) and excellent quantum yields. So far, this work has mainly been applied to phycobiliproteins and DNA dyes.[39–42]

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Choice of Technique

Alternative techniques which give some of the same information as FRET include X-ray crystallography, nuclear magnetic resonance (NMR), cryoelectron microscopy, and biochemical methods such as gel-shift assays and cross-linking studies. Briefly, X-ray crystallography and NMR both produce potentially complete structural information but require large quantities of material. For *in vitro* studies, FRET is often used to get initial structural information, with the complete solution coming later from X-ray crystallography or NMR. X-Ray crystallography and NMR are limited to *in vitro* measurements and can analyze only relatively small molecules, restrictions which do not apply to FRET. In addition, in the case of X-ray crystallography, one is also faced with the difficult problem of crystallization and isomorphous replacement.

Cryoelectron microscopy achieves the high resolution of electron microscopy while minimizing some of the sample preparation artifacts associated with drying, staining, and interaction with the solid support. The technique has been shown to reproduce structural features of viruses, some protein crystals, and even more flexible samples such as DNA.[43] The technique is limited to using thin (100 nm) samples, contrast is fairly low, and aggregation can occur during the cooling period. A number of reviews on cryoelectron microscopy have appeared.[43–47]

Gel-shift assays, in which the mobility of a sample in a polyacrylamide gel is a function of the structure of the molecule (e.g., bent or straight, compact or extended) can supply some of the same information as FRET. This is because alterations in molecular shape can lead to changes in gel mobility and also changes in the distance (and hence energy transfer) between two site-specifically placed dyes (e.g., in DNA[17,48]). Sample preparation in the gel-shift techniques is generally easier, since no labels need be attached, but the technique has the disadvantage that the complex must be stable over the course of hours and structural changes must be inferred from changes in mobility, which are not well understood theoretically.

Cross-linking studies, like gel-shift assays, have the advantage that site-specific labels need not be introduced into the macromolecule. On the

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[44] R. H. Wade and D. Chretien, *J. Struct. Biol.* **110**, 1 (1993).
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other hand, it is not always clear where the cross-linking is taking place. Furthermore, if cross-linking does not occur, this may be because the sites are not in close proximity or because the sites are not chemically reactive. Finally, if flexible chemical linkers are used, the distance determination is limited to saying that the two points of interest are less than or equal to the maximal extension of the cross-linker. A number of reviews on cross-linking techniques have appeared.[49-52]

Problems

It is also important to understand the limitations of FRET. Although these are explored in some detail later, the most important drawback of FRET is its limited ability to measure absolute distances. It is quite good at measuring relative distances, namely, whether two points are closer together under condition A than condition B. The problem is that the efficiency of energy transfer depends not only on the distance between the donor and acceptor, but on the relative orientation of the dyes as well, a factor which is often not precisely known. Even when measuring relative distances one must take care to ensure that the orientation factor does not change between the two systems one is comparing. Unfortunately, this orientation factor can be significant, multiplying the fitted-distance anywhere from 0 to $4^{1/6} = 1.26$. Polarizaton measurements on the donor and acceptor can be made which constrain this factor, but rarely do they eliminate all uncertainty. In addition, FRET is limited in its ability to measure absolute distances because there is usually uncertainty in the exact position of the FRET dyes owing to flexibility in the linker arm used to attach the dyes. For these reasons FRET is more easily and reliably used as a measure of relative distance.

A second problem with FRET is the very sharp distance dependence. This has two drawbacks: (1) it is difficult to measure relatively long distances because the signal is very weak, and (2) the signal tends to be "all or none," that is, if the two points are less than a certain characteristic distance (this distance, known as R_0, is the distance at which 50% of the energy is transferred and is a function of the particular dyes chosen), almost all of the energy is transferred, but if greater than this distance, very little energy is transferred. It is therefore helpful to have some estimate of the distance of interest before a FRET measurement is undertaken.

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Underlying Principles: Förster's Theory of Dipole–Dipole Interaction

Here we present a brief review of the physical principles underlying fluorescence energy transfer. The theory was developed primarily by Förster and extended by Dexter.[53,54] Förster did some early experimental studies,[55] and Stryer and Haugland convincingly showed that fluorescence energy transfer could be used as a "molecular ruler" to measure distances.[19] Emphasis is on developing an intuitive feel for the important relevant parameters. Both a classical and a quantum mechanical approach are given.

In FRET, a fluorescent donor molecule transfers energy to an acceptor molecule, which is usually but not necessarily a fluorescent molecule. The mechanism is a nonradiative induced dipole–induced dipole interaction[36]: "nonradiative" because no photons are "passed" between the dye molecules; "dipole–dipole" because each dye molecule acts like a classical (or quantum mechanical) dipole antenna, emitting and absorbing energy; "induced" because the dipoles are not permanent but are a result of electric fields which create them. On energy transfer, the signal in a FRET experiment is a decrease in the fluorescence intensity and lifetime of the donor and, if the acceptor is also fluorescent, an increase in the fluorescence of the acceptor. The changes are measured by comparing the fluorescence of a complex containing both donor and acceptor to that of complexes containing only donor or only acceptor. The fluorescence of the donor decreases in the presence of acceptor because some of the energy goes to the acceptor instead of into the radiation (or photon) field. The lifetime of the donor also decreases because the energy transfer to the acceptor is another pathway for the excited state to decay to the ground state.

The efficiency of energy transfer (E), which is defined as the fraction of donor molecules de-excited via energy transfer to the acceptor, therefore equals

$$E = (1 - I_{D_A}/I_D) = 1 - \tau_{D_A}/\tau_D \qquad (1)$$

where I_{D_A} and τ_{D_A} are the intensity and lifetime, respectively, of the donor in the presence of acceptor, and I_D and τ_D in the absence of acceptor. The efficiency of energy transfer can also be measured by looking at the increase in fluorescence of the acceptor:

$$E = (I_{A_D}/I_A - 1)(\varepsilon_A/\varepsilon_D) \qquad (2)$$

[53] T. Förster, *Mod. Quantum. Chem. Lect. Istanbul Int. Summer Sch.* (1965).
[54] D. L. Dexter, *J. Chem. Phys.* **21**, 836 (1953).
[55] T. Förster, *Z. Naturforsch. A: Astrophys. Phys. Phys. Chem.* **4**, 321 (1949).

where I_{A_D} is the emission of the acceptor in the presence of the donor (consisting of fluorescence arising from energy transfer and from direct excitation of the acceptor) and I_A is the fluorescence of the acceptor-only labeled sample (consisting of fluorescence arising from direct excitation only); ε_A and ε_D are the molar extinction coefficients of the acceptor and donor, respectively, at the wavelength of excitation. Equations (1) and (2) assume complete labeling; in other words, the doubly labeled complex is completely labeled with donor and acceptor. They can be readily modified to account for incomplete labeling.[56]

To get distance information from these experimental parameters, one needs to know how the efficiency of energy transfer depends on distance. Förster showed that

$$E = 1/(1 + R^6/R_0^6) \tag{3}$$

where R is the distance between the donor and acceptor and R_0 is a characteristic distance, typically 10–50 Å, related to properties of the donor and acceptor.[36,53] From Eq. (3) it is easy to see that R_0 is the distance at which 50% of the energy is transferred:

$$R_0 = (8.79 \times 10^{-5} Jq_D n^{-4} \kappa^2)^{1/6} \quad \text{(in Å)} \tag{4}$$

$$J = \int \varepsilon_A(\lambda) f_D(\lambda) \lambda^4 \, d\lambda \Big/ \int f_D(\lambda) \, d\lambda \quad \text{(in } M^{-1}\,\text{cm}^{-1}\,\text{nm}^4) \tag{5}$$

where J is the normalized spectral overlap of the donor emission (f_D) and acceptor absorption (ε_A), q_D is the quantum efficiency (or quantum yield) for donor emission in the absence of acceptor (q_D is the number of photons emitted divided by number of photons absorbed), n is the index of refraction (typically 1.3–1.4), and κ^2 is a geometric factor related to the relative angle of the two transition dipoles.

Figure 1 shows an example of the spectrum of a commonly used donor and acceptor and the intensity changes which occur with energy transfer. Figure 2 shows the experimental verification of the R^{-6} dependence of energy transfer using dansyl as a donor and naphthyl as an acceptor, separated by a series of rigid, polyproline linkers.

To generate an intuitive feeling for Eqs. (1)–(5), we first show how the form of Eq. (3) for the fraction of energy transferred is physically reasonable, then we show the sixth power dependence. Finally, we discuss the parameters which determine R_0 in Eqs. (4) and (5).

Form of Equation

We first show that the form of the energy transfer efficiency qualitatively looks like $1/[1 + f(r)]$ where $f(r)$ is some function of the distance,

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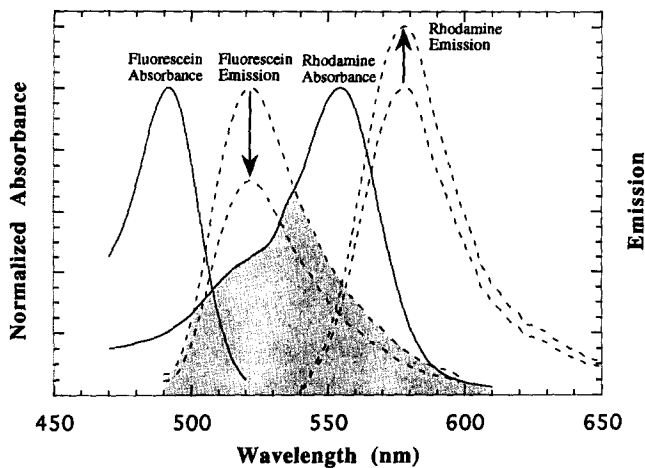
FIG. 1. Spectral characteristics and changes of the donor fluorescein and acceptor tetra-methylrhodamine undergoing energy transfer. The donor intensity decreases and the acceptor is sensitized with energy transfer. The spectral overlap which makes energy transfer possible is shown in gray. The absorbance and emission intensities are normalized for display purposes. The R_0 for the pair is approximately 45 Å.
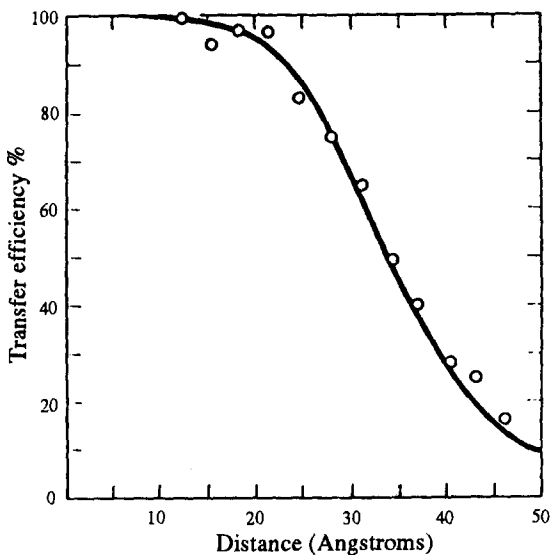

FIG. 2. Experimental verification of Förster's theory of fluorescence energy transfer. Energy transfer was studied with a series of end-labeled oligopeptides, dansyl-(polyproline)$_n$-naphthyl. The solid line is a fit to the data with Eq. (3) showing the R^{-6} dependence. (From Stryer and Haugland.[19]

r, between the donor and acceptor. Consider a donor which has been excited by light. The excited state will decay with rate $k_{nd} + k_{et}$, where k_{nd} is the sum of all distance-independent rates such as fluorescence (k_f) and heat (k_h) ($k_{nd} = k_f + k_h = \tau_D^{-1}$) and k_{et} is the distant-dependent rate of energy transfer to an acceptor.

The fraction of donor molecules giving energy to the acceptor is therefore just

$$E = k_{et}/(k_{et} + k_{nd}) = 1/(1 + k_{nd}/k_{et}) = 1/(1 + 1/\tau_D k_{et}) \qquad (6)$$

which is the form of Förster's equation [Eq. (3)] if k_{et} depends on R^{-6} and R_0 is related to constants in k_{et} and k_{nd}. In addition, the excited state donor lifetime decreases from $1/k_{nd}$ (τ_D) without the acceptor, to $1/(k_{nd} + k_{et})$ (τ_{D_A}) with the acceptor, directly leading to Eq. (1). When the acceptor is nearby, the energy transfer rate is fast compared to other decay pathways and most of the energy is transferred. If the acceptor is farther away this rate is less and the efficiency of energy transfer decreases. For later reference, we note that combining Eq. (3) and Eq. (6) yields

$$k_{et} = \tau_D^{-1}(R/R_0)^6 \qquad (7)$$

Why is the distance dependence of energy transfer R^{-6}? It comes about because the extent of energy transfer depends on the square of the electric field produced by the donor, and this field decays as R^{-3} at distances relevant in FRET. First we examine the donor electric field. The electric field of the donor arises because the incident excitation light induces electrons in the donor to oscillate (or, in quantum mechanics terms, induces transitions). This creates an induced, electric dipole moment in the donor, which creates its own, characteristic electric field. The dipole field of the donor has two parts, one which dies away like $1/R$, the other which decays like $1/R^3$. Far away from the molecule, the $1/R$ term dominates. This is called the radiation field and is what we see as fluorescent photons. (The energy carried away goes like the square of the electric field, and therefore drops off as $1/R^2$, as it must to conserve energy.) Close up, however, within a wavelength of light, the $1/R^3$ term dominates. This field is the one of interest in FRET. It does not carry away energy, that is, does not radiate, and so sometimes FRET is called nonradiative energy transfer. (In very rare instances, when using lanthanides as donors, for example, the donor may act partially like a magnetic dipole[57] or, in the solid phase, even like an electric quadrupole[58-60] instead of, or in addition

[57] J.-C. G. Bunzli and G. R. Choppin (eds.), "Lanthanide Probes in Life, Chemical and Earth Sciences: Theory and Practice," Elsevier, New York, 1989.
[58] R. Reisfeld and L. Boehm, *J. Solid State Chem.* **4**, 417 (1972).
[59] E. Nakazawa and S. Shionoya, *J. Chem. Phys.* **47**, 3211 (1967).
[60] R. Reisfeld, E. Greenberg, and R. Velapoldi, *J. Chem. Phys.* **56**, 1698 (1972).

to, acting like an electric dipole. Such cases are covered theoretically by Dexter in a seminal paper.[54])

To understand why energy transfer is proportional to the square of the R^{-3} field of the donor, we must understand how the acceptor interacts with (takes energy from) the donor electric field. If an acceptor molecule is in this close-up electric field, its electrons will be induced to oscillate, creating an induced dipole moment, $\mathbf{p}_A$, in the acceptor (just as an induced dipole was formed in the donor by the incident electric field of the exciting light). The size of the dipole is related to the size of the electric field, E_D, creating it: $p_A = \alpha_A E_D$, where α_A is the polarizability of the acceptor and is a measure of how easily the electrons can be made to oscillate.

What is the fraction (or efficiency) of energy transferred? It is simply the energy absorbed by the acceptor from the donor, divided by the energy absorbed by the donor from the excitation light. The latter term is independent of any distances of interest and so is irrelevant here. Moreover, the amount of energy absorbed by the acceptor is just $p_A \cdot E_D = \alpha_A E_D^2$. Consequently, because E_D decays as R^{-3}, the amount of energy absorbed by the acceptor is a function of R^{-6} between donor and acceptor.

Of course the proper treatment of energy transfer is via quantum mechanics. The analysis is very straightforward, and an excellent outline is presented by Cantor and Schimmel.[36] The excitation light induces transitions in the donor to an excited (singlet) state. This decays rapidly to the lowest excited state. The donor can then relax either via fluorescence, nonradiative processess, or interaction with the acceptor via a dipole–dipole interaction (see Fig. 3). The Hamiltonian or energy of interaction between the donor and acceptor is

$$H = (\mu_D \cdot \mu_A)/R^3 + (\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})/R^5 \qquad (8)$$

where $\mu_D(\mu_A)$ is the transition dipole moment of the donor (acceptor) and $\mathbf{R}$ is the vector separating their centers. According to Fermi's rule, the rate of inducing transitions is proportional to the square of the Hamiltonian matrix element between final and initial states:

$$k_{et} \propto [\langle D^*|\langle A|[(\mu_D \cdot \mu_A)/R^3 - 3(\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})/R^5 |D\rangle|A^*\rangle]^2 \qquad (9)$$

where the initial state is the product of the excited state of the donor ($\langle D^*|$) and the ground state of the acceptor ($\langle A|$) and the final state is the product of the donor ground state ($|D\rangle$) and acceptor excited state ($|A^*\rangle$). We can write the wave function as this simple product because we assume the coupling between donor and acceptor is weak, and so the individual wave functions are not much perturbed. (If this is not the case, one gets into exciton coupling where the absorption spectra of the individual dyes change in the donor–acceptor complex). Only those donor and acceptor

wave functions with nearly the same energy will significantly contribute to the rate; this is the resonance condition of FRET. The dot products can be explicitly written and Eq. (9) separated into those quantities depending on the donor wave functions and those on the acceptor wave functions, and those depending on the relative orientation:

$$k_{et} \propto R^{-6} \langle D^*|\mu_D|D\rangle^2 \langle A|\mu_A|A^*\rangle^2 [\langle D^*\langle A|(\cos\theta_{DA} - 3\cos\theta_D\cos\theta_A)|D\rangle|A^*\rangle]^2 \tag{10}$$

The rate is therefore proportional to the square of the transition dipole moments of the acceptor and donor, which can be related to the absorption and emission properties of each dye, respectively.[36] The rate also depends on a geometric factor.

Combining Eq. (10) and Eq. (7) yields Förster's equation [Eq. (3)] where R_0 is a function of the acceptor absorption cross section, the donor emission efficiency, and also the relative angles of the donor and acceptor dipoles [see Eqs. (4) and (5)].

Parameters in R_0

We can now understand the parameters which enter into R_0 [Eq. (4)]. R_0 is a measure of how well the donor and acceptor can transfer energy to one another, where a large R_0 indicates that the donor and acceptor
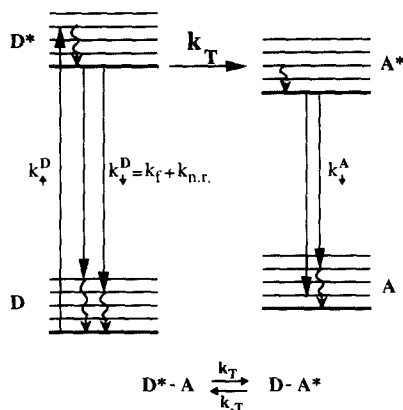

FIG. 3. Jablonski energy level diagram. The donor is excited and rapidly drops to the lowest vibrational level of the excited state, where it can radiatively (primarily via fluorescence) or nonradiatively decay to the group state, or transfer energy to the acceptor. Only those levels of the donor and acceptor with similar energies contribute significantly to the transfer rate. Once the acceptor is excited, rapid vibrational relaxation prevents back transfer. The acceptor then decays to the ground state via fluorescence or heat.

can transfer energy efficiently even if they are relatively far apart. To transfer energy from donor to acceptor, the electric field produced by the donor must be at a frequency (or wavelength) that can induce transitions in the acceptor (or, classically, a frequency which can efficiently drive the electrons in the acceptor). Consequently, R_0 depends on the spectral overlap term, J, which is a measure of how well the donor fluorescence frequency (or wavelength) and acceptor absorbance overlap in wavelength. Why is there a λ dependence in the J term? The answer is that the electric field dies off as $(\lambda/r)^3$. If λ is large (further to the red) the electric field drops off more slowly, and energy transfer can occur at farther distances. Mathematically, the λ^4 power comes about because μ_A^2 is proportional to $\varepsilon_A\lambda$ and μ_D^2 is proportional to $\lambda^3 q_D/\tau_D$.[36] Note that the dependence of μ_D^2 on τ_D^{-1} eliminates the donor lifetime dependence from R_0. This is expected because, although the rate depends on the lifetime, a longer lifetime means a slower rate integrated over a longer time. The index of refraction enters into R_0 because the size of the electric field produced by the donor is modified by the polarizability of the medium, which is directly related to the index of refraction. Finally, the quantum yield of the donor, q_D, enters because it is a measure of how well the donor converts the energy it has absorbed into an electric field (as opposed to converting the energy to phonons, or heat). (A donor with a high quantum yield efficiently creates a large electric near-field, which is relevant for FRET, as well as a large electric far-field, the latter being fluorescent photons.)

The last and most troubling term in R_0 is κ^2, which arises because the efficiency of energy transfer depends on the relative orientation of the two dyes (the $\mu_D \cdot \mu_A$ term) and the relative orientation in space [the $3(\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})$ term]. The expression for κ^2 is

$$\kappa^2 = (\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A)^2 \qquad (11)$$

where θ_{DA} is the angle between the donor and acceptor transition dipole moments and θ_D (θ_A) is the angle between the donor (acceptor) transition dipole moment and the **R** vector joining the two dyes.

One can immediately see that κ^2 can vary from 0 if all angles are perpendicular to 4 if all angles are parallel. If the orientation of the dipoles is random, because they are moving rapidly (within the donor lifetime) then $\kappa^2 = 2/3$. This assumption is often made, even if not strictly true, and accounts for much of the uncertainty in FRET measurements. If just the donor or just the acceptor is randomized, then $1/3 < \kappa^2 < 4/3$. In this case the uncertainty in measured distance is approximately $\pm 11\%$.[38] In reality, what usually happens is that the dyes undergo fast, restricted motion such that κ^2 approaches 2/3, with some uncertainty remaining.

Dale and Eisinger have analyzed the effect of rotational mobility[61]; Stryer presents an analysis of the errors introduced by assuming $\kappa^2 = 2/3$[38]; and van der Meer et al. present a treatment on the effects of restricted rotational and translational diffusion.[62] Experimentally, one determines the rotational mobility of the dyes by a steady-state or time-resolved fluorescence depolarization experiment.[63]

In practice, R_0 is often measured from a model system (see, e.g., Fig. 2) and assumed to apply to the system of interest, or R_0 is calculated from Eqs. (4) and (5), assuming a value $\kappa^2 = 2/3$.

Labeling

Perhaps the most difficult aspect of FRET is the problem of labeling the sites of interest with the appropriate dyes. One must choose dyes that are spectrally compatible and that can be site specifically labeled without significantly perturbing the original structure of the molecule of interest. In general it is also important that the sample be completely labeled with both donor and acceptor, or at least that the extent of labeling be known. Analyses have appeared which take into account incomplete labeling.[2,64] Waggoner ([15] in this volume) reviews fluorescent labeling, and Brinkley has reviewed techniques for labeling proteins with dyes, haptens, and cross-linking reagents.[51]

Naturally Occurring Fluorophores

The ideal situation is if the biological molecule is fluorescent, or can be made so with slight modification. In proteins, tryptophan is a naturally occurring amino acid which makes a reasonable donor. Tyrosine is also fluorescent but is rarely a good donor to an external acceptor because it is often quenched by tryptophans in the protein.[36] Beardsley and Cantor have used the fluorescent Y-base associated with yeast phenylalanine transfer RNA.[25] For DNA and RNA there are a number of fluorescent nucleotide analogs.[38,42] Yet another possibility is if the protein binds a ligand which is either fluorescent or can be made so. In metal-binding proteins, zinc can be replaced with cobalt, which can act as a good acceptor because of wide visible absorbance.[22,65] Terbium, another metal, can act

[61] R. E. Dale, J. Eisinger, and W. E. Blumberg, *Biophys. J.* **26**, 161 (1979).
[62] B. W. van der Meer, M. A. Raymer, S. L. Wagoner, R. L. Hackney, J. M. Beechem, and E. Gratton, *Biophys. J.* **64**, 1243 (1993).
[63] J. R. Lakowicz, "Principles of Fluorescence Spectroscopy." Plenum, New York, 1983.
[64] J. R. Lakowicz, I. Gryczynski, W. Wiczk, J. Kusba, and M. L. Johnson, *Anal. Biochem.* **195**, 243 (1991).
[65] S. A. Latt, D. S. Auld, and B. L. Vallee, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1383 (1970).

as an excellent donor especially if there is a tryptophan nearby. (The tryptophan, in effect, increases the absorption cross section of the terbium by absorbing light and passing energy to the terbium, which by itself, has very weak absorbance.)[66] Terbium and europium have also been used as isomorphous replacements for calcium[22] and in metal-binding engineered proteins.[67,68]

Fluorescent Dyes

There is a large number of dyes to choose from, many of which are listed in the Molecular Probes (Eugene, OR) catalog.[42] Ideally, one picks a pair with R_0 equal to the distance to be measured, since small changes in distance around R_0 lead to large changes in signal. However, because the distance is generally unknown, or only approximately known, it is wise to pick a donor–acceptor pair with a large R_0, equal to or larger than the distance to be measured. If necessary, R_0 can be decreased by adding reagents such as iodine that reduce the donor quantum yield or by choosing an acceptor with poorer spectral overlap to the donor.

To achieve a large R_0 one wants a donor with a high quantum yield of long-wavelength emission and an acceptor with a large absorbance at the donor fluorescence wavelengths. Unfortunately, as the emission becomes more red, the quantum yield tends to drop, although there are some promising new dyes with reasonable quantum yields in the red, such as La Jolla Blue,[69] CY-5,[70,71] and Bodipy[42] as well as nucleic acid stains such as BOBO and POPO,[42] and also the phycobiliproteins.[42]

Increasing R_0 and thereby getting a large signal is only part of the strategy. Minimizing background is also important. When measuring donor quenching or sensitized emission, it is desirable to have little spectral overlap between donor and acceptor fluorescence. For sensitized emission, it is also desirable for the acceptor not to be excited directly by the excitation light. This requires the acceptor absorbance to be small where the donor absorbance is large. Finally, to maximize the sensitized emission signal, it is desirable for the acceptor to have a good quantum yield.

The ideal situation is therefore when both donor and acceptor have high quantum yields and large Stokes shifts. Unfortunately a large Stokes

[66] P. Cioni, G. B. Strambini, and P. Degan, *J. Photochem. Photobiol. B* **13**, 289 (1992).

[67] J. P. MacManus, C. W. Hogue, B. J. Marsden, M. Sikorska, and A. G. Szabo, *J. Biol. Chem.* **265**, 10358 (1990).

[68] I. D. Clark, J. P. MacManus, D. Banville, and A. G. Szabo, *Anal. Biochem.* **210**, 1 (1993).

[69] R. Devlin, R. M. Studholme, W. B. Dandliker, E. Fahy, K. Blumeyer, and S. S. Ghosh, *Clin. Chem.* **39**, 1939 (1993).

[70] H. Yu, L. Ernst, M. Wagner, and A. Waggoner, *Nucleic Acids Res.* **20**, 83 (1992).

[71] R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, and A. S. Waggoner, *Bioconjugate Chem.* **4**, 105 (1993).

shift generally implies a small quantum yield, so there is a trade-off in these properties. (In the section on future directions, we discuss an energy transfer scheme using lanthanides as donors and organic chromophores as acceptors which gets around these problems.)

Perhaps the most popular donor is fluorescein, which often has a quantum yield exceeding 0.5. It can be used with eosin (R_0 = 50–54 Å[14]), chlorofluorescein ($\approx$50Å), tetramethylrhodamine (R_0 = 45 Å[16]), tetraethylrhodamine (R_0 = 40 Å[37]), or Texas Red (R_0 not reported but $\ll$40 Å). (All R_0 values are approximate because they depend on the donor quantum yield and emission spectra which can change depending on solvent conditions.) In this series, as R_0 decreases, the spectral separation increases. Greater spectral separation is especially helpful if one is measuring the sensitized emission of the acceptor (see measurement section below) or if only a relatively small percentage of molecules have both donor and acceptor bound or are capable of transferring energy. (See the FRET work of McConnell and co-workers on conformational changes in high mobility group (HMG) proteins for an example of such a case.[23]

Tetramethylrhodamine is perhaps the most popular acceptor with fluorescein because of its large R_0 and because the acceptor fluorescence is somewhat separated from donor fluorescence. Fluorescein fluorescence can be monitored from 500 to 525 nm with no contamination from acceptor fluorescence. A new carbocyanine dye, CY-3, is spectrally very similar to tetramethylrhodamine but is reported to have somewhat higher maximum absorbance.[71–73] Eosin as acceptor has a somewhat higher R_0 than tetramethylrhodamine, but its emission strongly overlaps that of fluorescein, such that there is not even a separable maximum. Fluorescein as donor does have the disadvantage that it may be quenched approximately 50% in proteins,[42] and it has a short, multiexponential (main component of 3 nsec) lifetime which makes donor lifetime measurements difficult, of limited accuracy, and especially problematic if R is significantly greater than R_0. The quantum yield of fluorescein is also a strong function of pH below pH 8 and decreases with increasing Na^+, Cl^-, and Mg^{2+}.[18]

Other dyes such as dansyl and AEDANS are popular donors in protein studies, in part because of the relatively long lifetimes (13–20 nsec), large (150 nm) Stokes shifts, and reasonable quantum yields (0.1–0.5).[42] If steric hindrance is not a problem and a large-sized donor or acceptor can be used, the multichromophoric phycobiliproteins (molecular weight of 104,000 for B- or R-phycoerythrin; 240,000 for allophycocyanin) make excellent donors or acceptors, having extinction coefficients which can exceed

[72] "Biological Detection Catalog." Biological Detections Inc., Pittsburgh, Pennsylvania, 1993.
[73] "Research Organics Inc., Catalog." Cleveland, Ohio, 1993.

2×10^6 and quantum yields of 0.68–0.98. They are also available with reactive groups.[42] Fairclough and Cantor present a useful list of donor–acceptor pairs, including spectra and R_0 values,[37] and van der Meer and co-workers[35] are compiling an extensive list.

Site-Specific Attachment

Fortunately there is a wide variety of dyes that are available with reactive groups. For attachment to amines, the most common reactive groups are succinimdyl esters and isothiocyanates. The former have generally better coupling efficiency if the coupling can be done in an organic phase. For attachment to synthetic DNA, an amine-modified base can be introduced via commercially available phosphoramidite, either internally or on the 3' or 5' ends.[74–76] Direct attachment to the DNA backbone has also been achieved.[15] Relatively short polypeptides can often be readily labeled, especially if they contain a unique cysteine or amine-containing amino acid.[19,34,77] For proteins, lysines (and to a significantly lesser extent arginines) are available for labeling.[78]

With proteins the problem is generally that there are too many reactive sites. Site-directed mutagenesis can sometimes be used to introduce a free sulfhydryl group which can then be coupled to a dye via an iodoacetamide or a maleimide. An extensive list of proteins which have been labeled with iodoacetamides has been tabulated.[42] Site-directed mutagenesis can also introduce a tryptophan (as donor) if one is not already present. ANS or AEDANS are good acceptors for tryptophan, both yielding an R_0 of 22 Å. Proteins with N-terminal serine or threonine can also be specifically labeled.[37,38] Objects which can be biotinylated can be labeled with fluorescent avidin or streptavidin. More generally, fluorescently labeled antibodies can be used to bind to a wide variety of biological substrates.

The reactive groups are often attached to the dyes via a *n*-carbon linker, where *n* typically ranges from 2 to 12. The linker often allows relatively free rotation of the dye, which minimizes uncertainty in κ^2. It can also minimize quenching of the dye, especially if the dye is quenched by a hydrophobic environment. The linker, however, has the disadvantage of adding uncertainty to the exact position of the dye. In general, the

[74] "Clonetech Catalog." Palo Alto, California, 1993.
[75] "Glen Research Catalog." Sterling, Virginia, 1993.
[76] "Peninsula Laboratories Catalog." Belmont, California, 1993.
[77] Y. Pouny, D. Rapaport, A. Mor, P. Nicolas, and Y. Shai, *Biochemistry* 31, 12416 (1992).
[78] T. E. Creighton, "Proteins: Structures and Molecular Properties," 2nd Ed., Freeman, New York, 1993.

minimal length that allows free rotation of the dye and does not cause quenching is desirable. A six-carbon linker is a good starting point.

Testing for Altered Structures

It is important to check whether the introduction of fluorescent labels alters the macromolecular structure. For DNA, testing the hybridization melting temperature is a crude measure. For proteins, comparing the function (enzymatic activity, binding constant, etc.) of the labeled versus unlabeled molecule is an excellent check.

How to Measure Energy Transfer

There are several ways to measure the amount of energy transfer: (1) decrease in donor intensity or quantum yield; (2) increase in intensity of acceptor emission (sensitized emission); (3) decrease in lifetime of donor; (4) decrease in photobleaching of donor; and (5) change in lifetime of sensitized emission (discussed in the section on future directions below).

Donor Intensity

The simplest way to measure energy transfer is to measure the decrease in fluorescence of the donor in the presence and absence of acceptor. The fractional decrease in the donor fluorescence with the acceptor present is equal to the efficiency of energy transfer [see Eq. (1)]. The only instrumentation necessary is a steady-state fluorimeter. Besides simplicity, the steady-state measurement has the advantage that even a relatively small amount of energy transfer can be measured. If care is taken in measuring concentrations and in the spectroscopy, a 5% decrease in fluorescence is measurable: this corresponds to a distance of $1.6 R_0$. One caution is that the optical density (OD) of the sample must be kept sufficiently low that no appreciable absorption of the donor fluorescence takes place; less than 0.02 OD is recommended. Another caution is that the donor and acceptors should be chosen so that there is a region of donor-only fluorescence. Although it is not necessary to have an acceptor which is fluorescent in this experiment, it is important confirmation that energy transfer is taking place because an increase in acceptor emission can arise only from energy transfer (assuming the optical density is low enough), whereas donor quenching can arise from several trivial sources. With a fluorescent acceptor, one can also measure the polarization of the acceptor emission, which tells about its rigidity and hence limits the uncertainty in κ^2.

In addition to measuring the decrease in donor fluorescence, if one wants to calculate R_0, it is necessary to measure the quantum yield of

the donor in the absence of acceptor, and also to measure the steady-state or time-resolved polarization of both donor and acceptor. Quantum yield measurements are generally done by comparing total fluorescence of the sample to a reference with known quantum yield.[36,79,80] Polarization measurements are important to limit uncertainty in κ^2, and they are covered by H. van Amerongen and W. S. Struve (this volume [11]) as well as in a book by Lakowicz on fluorescence.[63]

Sensitized Emission

With the same steady-state fluorimeter, one can also measure the sensitized emission of the acceptor. The efficiency of energy transferred calculated via donor quenching should agree with that calculated by sensitized emission. Sensitized emission can also be particularly useful when measuring long distances or when the sample is inhomogeneous and only a small fraction of the sample contributes to energy transfer.[23] In either case it is helpful if the fluorescence of the acceptor is well separated from that of the donor. In principle, one can measure the energy transfer via sensitized emission based on Eq. (2). In reality, there are experimental difficulties, discussed by Epe et al.[56] In particular, for distances much beyond R_0, the ratio I_{A_D}/I_A approaches unity, and even small errors in measurements lead to large errors in the calculated energy transfer.

A number of workers have attempted to make sensitized emission (and donor quenching) measurements more robust. In general these techniques attempt to reduce the number of independent samples (donor–acceptor, donor only, acceptor only) which must be compared. Fairclough and Cantor cover standard methods.[37] Epe and co-workers[56] developed a technique where a donor–acceptor labeled sample is measured and then enzymatically digested, thereby separating the donor from the acceptor and eliminating energy transfer. Donor quenching or sensitized emission can therefore be made on one sample. Clegg and co-workers have developed an analysis of acceptor emission which yields reproducible results, even when measuring samples with small energy transfer and under significantly different conditions.[2,17,18] They applied the analysis to DNA samples labeled with fluorescein and tetramethylrhodamine, but it should be generally applicable to other FRET pairs. Clegg has outlined the many advantages of the technique.[2] We present a brief outline of the technique.

[79] G. Weber and F. W. J. Teale, Trans. Faraday Soc. 53, 646 (1957).
[80] J. B. Birks, "Photophysics of Aromatic Molecules." Wiley (Interscience), New York, 1970.

Steady-state emission spectra of a donor–acceptor labeled sample and a donor-only labeled sample are taken. The donor emission is removed from the donor–acceptor emission spectrum by subtracting the normalized donor-only emission spectrum. This leaves the fluorescence of the acceptor due to direct excitation and due to energy transfer (see Fig. 4). Clegg and co-workers call this the "extracted acceptor emission" spectrum, $F_{em}{}^A$. Note that this process does not require the concentration of donor-only sample to be the same as the donor–acceptor sample—only the shape of the donor spectrum is used. This spectrum is divided by a fluorescence value (often the maximum) of an emission spectrum taken on the donor–acceptor complex excited at a wavelength where only the acceptor absorbs (565 nm for fluorescein–tetramethylrhodamine). Alternatively, one can divide by the maximum of the excitation spectrum of the donor–acceptor complex (excitation at 400–590 nm, emission in the range 580–600 nm, for fluorescein–rhodamine). In either case, the resultant ratio spectrum, "(ratio)$_A$," is normalized for quantum yield of acceptor, for concentration of total molecules, and for incomplete acceptor labeling.
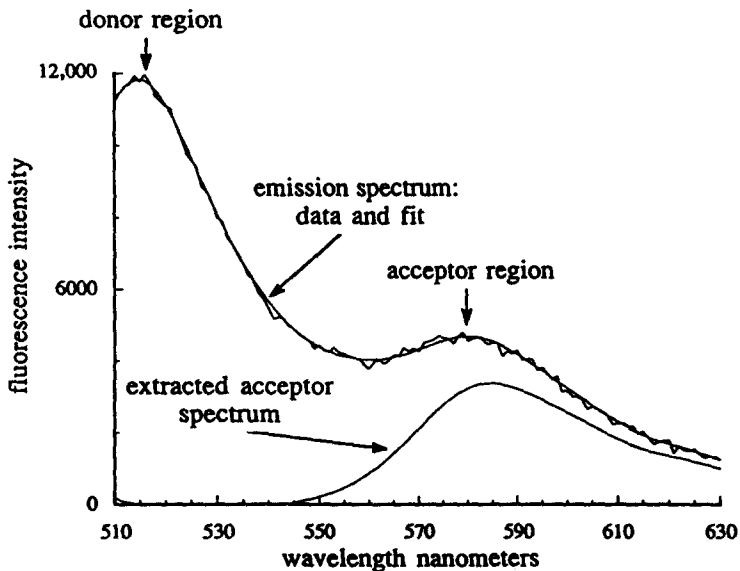

Fig. 4. Emission spectrum of fluorescein–tetramethylrhodamine labeled DNA oligomer. The region where only the donor emits (500–530 nm) is fit to a fluorescein-only spectrum and then subtracted from the entire spectrum, leaving the "extracted acceptor spectrum" consisting of acceptor fluorescence arising from energy transfer and from direct fluorescence. This spectrum is then fit using Eq. (4). The Raman background is subtracted from each fluorescence spectrum. (From R. M. Clegg, this series, Vol. 221, p. 372.)

Clegg and co-workers[2,18] have shown this ratio to be

$$(\text{ratio})_A = F_{em}^A(\nu_1,\nu')/F_{em}^A(\nu_2,\nu'')$$
$$= \{E \cdot \varepsilon^D(\nu')/\varepsilon^A(\nu'') + \varepsilon^A(\nu')/\varepsilon^A(\nu'')\}[\phi(\nu_1)/\phi(\nu_2)] \qquad (12)$$

where superscripts D and A refer to donor and acceptor, ε is the molar extinction coefficient, E is the efficiency of energy transferred, ν' and ν_1 are the excitation and emission wavelengths, respectively, for the FRET measurement, ν'' is the excitation wavelength where the acceptor alone absorbs (560 nm for rhodamine), and ν_2 is the wavelength(s) where the acceptor emission is measured. $\phi(\nu_i)$ is an "emission spectrum shape function" of the acceptor, where the integral over ν is proportional to the quantum yield. It is simply the fluorescence of the acceptor-only sample at wavelength ν_i. [Because $\phi(\nu_i)$ enters only as a ratio, the concentration of acceptor is unimportant.] For the fluorescein–tetramethylrhodamine pair, ν_1 and ν_2 are both 585 nm. The second term within the braces, $\varepsilon^A(\nu')/\varepsilon^A(\nu'')$ can be measured on the acceptor-only sample via absorbance or via an excitation spectrum.

In the scheme developed by Clegg and co-workers,[2,17,18] it is not necessary to compare the intensities of a donor-only or an acceptor-only sample with the doubly-labeled sample; hence, errors in concentration between samples do not lead to errors in measured energy transfer. When comparing samples under different solvent conditions, it is necessary to measure the quantum yield of donor in each case since this parameter enters into R_0. More general equations can be found which include the effect of incomplete labeling, and for the case when the donor and acceptor absorbances and emissions overlap significantly.[2]

Donor Lifetime

Measurement of the donor lifetime, which typically is 2–25 nsec, requires adequate time resolution. Two techniques, time-correlated single-photon counting and frequency-domain fluorimetry modulation, can be used (see A. R. Holzwarth, this volume [14]). Excellent books have been written which include discussion of each technique,[63,81] and Lakowicz and co-workers have discussed advances in frequency-domain instrumentation and applications to FRET.[82] Donor lifetime measurements, unlike steady-state measurements, are capable of detecting multiple donor–acceptor transfer efficiencies in the sample. These lead to multiexponential decays. Donor lifetime measurements are also not affected by an inner-filter effect

[81] D. V. O'Connor and D. Phillips, "Time-Correlated Single Photon Counting." Academic Press, London, 1984.
[82] K. W. Berndt, J. R. Lakowicz, and I. Gryczynski, *Anal. Biochem.* **192**, 131 (1991).

where the donor fluorescence is absorbed by the acceptor. Even more significantly, donor lifetime is not sensitive to concentration; therefore, differences in concentration between the donor-only sample and the donor–acceptor sample do not lead to errors in the measurement of energy transfer.

Donor Photobleaching

A fourth way to measure energy transfer is based on changes in photobleachability of the donor. The technique, pioneered by the Jovin group, is particularly well suited to FRET in a microscope where the high light intensities necessary are readily accessible and where other FRET techniques have yielded only qualitative results.[10-12] (The discussion here is adopted from Ref. 10.) The idea is that the donor photobleaches more slowly if energy transfer to an acceptor is occurring since energy transfer is an alternative pathway for the excited state to give up energy. It can be shown that the fractional change in the photobleaching time constant caused by energy transfer is the same as the fractional change in the fluorescence lifetime of the donor. Thus, in the simplest case, the efficiency of energy transfer is just $1 - \tau_{bl}/\tau_{bl}'$ where τ_{bl} (τ_{bl}') is the bleaching time constant in the absence (presence) of acceptor. The rate of photobleaching can be easily measured in solution or in a microscope.

A second, related way of measuring energy transfer is based on changes in quantum yield. First a low-light level image is taken of a sample labeled only with donor. The fluorescent intensity at any spot is proportional to the quantum yield and total number of fluorophores. To normalize by the total number of fluorophores (in a manner independent of quantum yield), a high-light level image is taken, and all the fluorescent photons are counted (integrated) until complete photodestruction has occurred. The ratio of the low-light image to the integrated, high-light image is proportional to the quantum yield. The procedure is repeated with a donor–acceptor label sample, and the energy transfer is just the usual 1 minus the ratio of quantum yields [analogous to Eq. (1)]. If a linear camera [e.g., one based on a charge-coupled device (CCD) sensor] is used as the detector, a pixel-by-pixel energy transfer image can be obtained. A number of workers have applied photobleaching-FRET to epitope mapping in T cell lines,[83] visualizing receptor aggregation on the surface of living mast cells,[84] and

[83] G. Szaba, Jr., P. S. Pine, J. L. Weaver, M. Kasari, and A. Aszalos, *Biophys. J.* **61**, 661 (1992).
[84] U. Kubitscheck, R. Schweitzer-Stenner, D. J. Arndt-Jovin, T. M. Jovin, and I. Pecht, *Biophys. J.* **64**, 110 (1993).

studying the binding of haptens to monoclonal immunoglobulins on cell surface receptors.[85]

Controls

In all of the above techniques it is important to subtract background arising from Raman, specular, or other sources. The best way to do this is to prepare a sample identical to the fluorescence samples, but without the attached dyes. It is also important to assure that the energy transfer is arising only from intramolecular energy transfer, and not from diffusional contact or aggregation. To control for this, one can mix a donor-only labeled sample and an acceptor-only labeled sample under conditions where they will not form a donor–acceptor complex. Noncomplementary DNA strands, for example, can be mixed together, or a donor-only labeled protein can be mixed with an acceptor-only labeled protein, etc. FRET measurements should also be made with magic angle settings (analyzer set to 54°) to assure no polarization artifacts.

Examples and Application of Fluorescence Resonance Energy Transfer

Human Immunodeficiency Virus Protease

Matayoshi and co-workers have developed a simple and effective means for measuring HIV protease activity using FRET.[34] This is an example of measuring dynamics with FRET. The idea is to use a relatively short polypeptide that HIV can cleave into two and attach a donor on one end and an acceptor on the other end (see Fig. 5A). If the polypeptide is intact, the donor is highly quenched. On cleavage, the energy transfer is eliminated and the donor fluoresces. By making the polypeptide with an end-to-end distance less than R_0, the increase in signal on cleavage was 40-fold. They used EDANS as donor and DABCYL, a nonfluorescent dye, as acceptor (see Fig. 5B).

DNA Structure

One of the more recent applications of FRET is in the study of DNA structure. Clegg and co-workers have published much in this field[16–18] including a 1992 review.[2] They have measured the end-to-end distances of a series of DNA oligonucleotides, ranging from 8 to 20 base pairs (bp)

[85] U. Kubitscheck, M. Kircheis, R. Schweitzer-Stenner, W. Dreybrodt, T. M. Jovin, and I. Pecht, *Biophy. J.* **60**, 307 (1991).
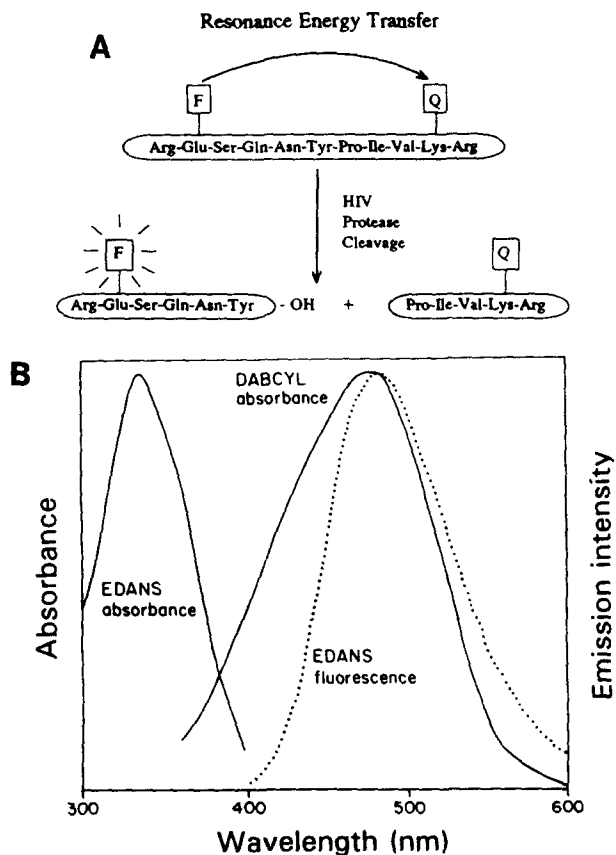
Fig. 5. (A) Use of FRET technique for measuring HIV protease activity. The polypeptide was Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys(DABCYL)-Arg. Because a simple system which could be commercialized was desired (fluorescence means HIV activity, no fluorescence means no HIV activity), the nonfluorescent acceptor was beneficial because it does not contribute signal after cleavage. This system also has the attribute that detailed information about the dyes and their energy transfer properties are not needed. (B) Good overlap between donor emission (maximum at 490 nm, excitation at 340 nm) and acceptor absorbance (maximum of 28,000 M^{-1} cm^{-1}). (From Molecular Probes Catalog.[42])

in length (see Fig. 6). Although the structure of short oligonucleotides is well understood, their detailed study showed conclusively that FRET can be used to study DNA structures, despite some early confusion.[14] The helical repeat of the DNA in solution was observed and the enthalpy of strand hybridization calculated. The helical repeat can be seen in the modulation of the R^{-6} energy transfer as a function of number of base pairs separating donor and acceptor.
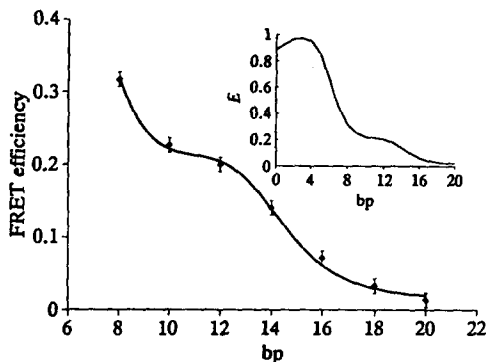
FIG. 6. Fluorescence energy transfer on a series of DNA oligomers differing in length from 8 to 20 bp. The modulation in the energy transfer decay curve (compare with Fig. 2) arises from the helical geometry of the DNA. Inset shows the theoretical FRET signal calculated from Eq. (12) for 0–20 bp. (From Clegg et al.[16])

Clegg and co-workers used fluorescein as donor and tetramethylrhodamine as acceptor (R_0 = 45 Å), attaching the dyes to the 5' ends of complementary DNA strands. Others have also used fluorescein–eosin.[14,15] The ability to measure the relatively long distances of a 20-mer (end-to-end distance of approximately 72 Å plus linker lengths) required careful measurement and analysis of the sensitized emission (discussed above in the section on measurement). They took care to ensure that the local environment around the dyes was constant for all samples. They also adjusted the dye linker length to ensure that at least one of the dyes was rotationally mobile. (The fluorescein had a low steady-state anisotropy of 0.07; the rhodamine was less mobile, with an anisotropy of 0.25. In this case, the distance error should be less than 10%.)

Cardullo has also used FRET to study the hybridization of DNA oligonucleotides. Donor (fluorescein) and acceptor (tetramethylrhodamine) were placed on the 5' ends of single-stranded DNA oligomers. On hybridization, energy transfer took place. (This is quite analogous to the HIV study cited above, where here the quantity of interest is hybridization rather than cleavage.) FRET has the advantage that hybridization can be measured at quite low concentration (<100 nM), in contrast to standard absorbance melting studies which require micromolar quantities. Measurement of hybridization is just a specific example of the more general problem of measuring binding constants. FRET can be useful in this regard because measurements can be made at low concentrations (in contrast to NMR), which is necessary for measuring large binding constants.
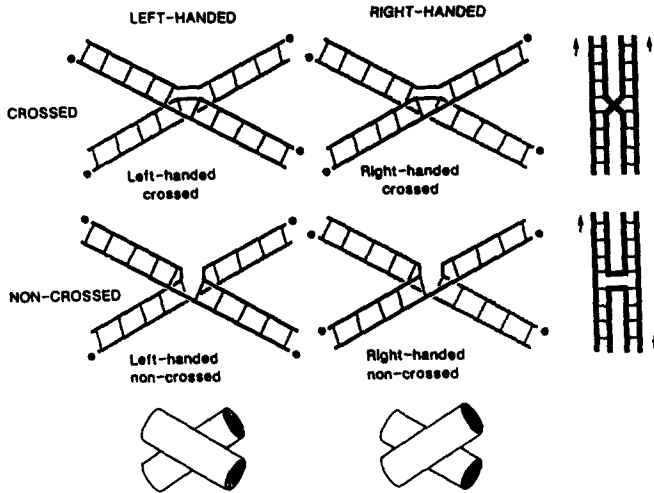
FIG. 7. Use of FRET to show that the overall geometry of the four-way DNA junctions is a right-handed noncrossed structure. The 5' end of the DNA strands are labeled with filled circles. Noncrossed and crossed structures generate antiparallel or parallel alignment of DNA sequences, shown by the arrows at right. The six possible end-to-end distances were measured by labeling the appropriate 5' ends with fluorescein (donor) or tetramethylrhodamine (acceptor) and monitoring energy transfer. (From Murchie et al.[17])

A more sophisticated example of FRET is its application to DNA four-way junctions, also called Holliday junctions. These are believed to be important intermediates in homologous recombination where genetic material is swapped between chromosomes.[86] The geometry of the junction presumably facilitates this swapping process, in combination with enzymes such as resolvase. Lilley and co-workers examined the three-dimensional structure of the junction by several methods, including gel electrophoresis and FRET.[17,18,87] Hagerman and co-workers have also studied the problem.[14,88] Crudely speaking, the junction looks like a nonplanar X (Fig. 7). Gel electrophoresis measurements indicated that the X structure involved strands which did not cross, but this conclusion was based on poorly understood assumptions about DNA mobility in gels. In contrast, the same information could be determined using FRET, where the assumptions are minimal. In addition, the junctions, because they are nonplanar, had a handedness to them (right- or left-handed). FRET could be used to distin-

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[87] D. R. Duckett, A. I. H. Murchie, S. Dickmann, E. von Kitzing, B. Kemper, and D. M. J. Lilley, Cell (Cambridge, Mass.) 55, 79 (1988).
[88] J. P. Cooper and P. Hagerman, J. Mol. Biol. 198, 711 (1987).

guish the crossed from noncrossed structure and right-handed from left-handed because the different proposed structures had different end-to-end distances, which could be measured by end-labeling them with donor and acceptor dyes. Fortunately, measurement of absolute distances was not required to differentiate between the possible structures. Crossed versus noncrossed models, for example, gave different predictions about which two of the six end-to-end distances were closest. Handedness of a junction could be determined by changing the length of one arm of the X (in effect, walking the end-labeled dye around the DNA helix of the arm): left-handed versus right-handed models gave different predictions for when the acceptor and donor would be closest or farthest away from one another. Lilley and co-workers concluded that four-way DNA junctions are right-handed noncrossed structures.[17]

Diffusion-Enhanced Fluorescence Resonance Energy Transfer

FRET has been used to measure diffusional rates and to examine the accessibility of certain sites to collisional quenching. Stryer *et al.* have presented an excellent review,[8] and Thomas and co-workers put the technique on a firm experimental foundation.[89] Three time regimes can be distinguished in FRET. If D is the sum of the donor and acceptor diffusion coefficients, s is the average separation, and τ is the donor lifetime, then the static limit, where the donor and acceptor do not appreciably move during the donor lifetime, is when $6D\tau \ll s^2$. The intermediate region, which is useful for measuring diffusion constants, is when $6D\tau \approx s^2$. The rapid diffusion, where donor and acceptor collide many times and energy transfer is a sensitive function of the closest distance, is when $6D\tau \gg s^2$. If the acceptor (or donor) is free to diffuse and has a diffusion constant of approximately 10^{-6} cm^2/sec (a typical value for a small dye) and the donor has a lifetime of a millisecond, then the rapid diffusion regime can occur if the acceptor concentration is on the order of 1 μM or more. The intermediate regime, which is less often used, has been achieved using naphthalene, which has a lifetime of approximately 100 nsec.[90]

To achieve the rapid diffusion limit requires a very long-lived donor. Chelates of terbium, which typically have lifetimes of 1.5–2.2 msec, are frequently used.[8] Fluorescein and rhodamine make excellent acceptors for terbium. Chelates of europium also have long lifetimes, 0.5–2.3 msec,[91] although these appear not to have been used. (Cronce and Horrocks have used europium in a calcium-binding site as a donor in a rapid diffusion

[89] D. D. Thomas, W. F. Carlsen, and L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5746 (1979).

[90] Y. Elkana, J. Feitelson, and E. Katchalski, *J. Chem. Phys.* **48**, 2399 (1968).

[91] C. C. Bryden and C. N. Reilley, *Anal. Chem.* **54**, 610 (1982).

experiment.[22] The chelates are often polyaminocarboxylic, such as EDTA. By modifying the chelator to alter its charge and measuring distances of closest approach, one can deduce the local charge surrounding the acceptor. Replacing one or two carboxyl groups of $EDTA^{4-}$ with alcohol groups to make HED3A or BED2A, respectively, yields chelates with one or two fewer negative charges. In addition, by attaching an organic chromophore to the chelator, the extremely weak absorbance of the terbium or europium ($\sim 1 \ M^{-1} \ cm^{-1}$ or less) can be increased several thousandfold.[8,92–94] By operating in D_2O, the quantum yield of the donor is often assumed to be unity (H_2O partially quenches lanthanide luminescence[95,96]), although the exact value is difficult to measure.

Energy transfer is typically measured by a decrease in the donor lifetime and, in the rapid diffusion limit, is a sensitive function of closest approach. Energy transfer based on a Förster dipole–dipole mechanism is proportional to a^{-4} in three dimensions and a^{-3} in two dimensions (e.g., on membranes), where a is the distance of closest approach.[8] If the distance of closest approach is less than 10 or 11 Å, energy transfer is dominated by a Dexter type exchange mechanism owing to overlapping wave functions between the donor and acceptor.[8,54] The exact distance of closest approach then becomes difficult to measure.

Lerho et al. have used FRET in the rapid diffusion limit to measure the accessibility of H5 histone in chromatin as a function of salt.[30] They labeled the H5 histone with fluorescein-labeled antibody and used TbHED3A and $TbEDTA^-$ as the freely diffusing donor. They found that the fluorescein becomes less accessible to the chelates as ionic strength increased. In the presence of DNA, they found H5 to be already folded at low ionic strength and the fluorescein inaccessible to the donor chelates. Thomas and co-workers have performed a number of experiments measuring the position of retinal in membranes by its ability to quench freely diffusing terbium chelates.[8,20,97] Retinal is the chromophore in rhodopsin, which acts as a signal transducer in vision, and in bacteriorhodopsin, which acts as a light-driven proton pump. They conclude that retinal in both bacteriorhodopsin and in bovine rhodopsin is buried with respect to both inner and outer membrane surfaces. In the case of rhodopsin, they measure a distance of 22 Å from the inner surface and 28 Å from the outer

[92] A. Canfi, M. P. Bailey, and B. F. Rocks, Analyst 114, 1405 (1989).
[93] T. Ando, T. Yamamoto, N. Kobayashi, and E. Munekata, Biochim. Biophys. Acta 1102, 186 (1992).
[94] A. K. Saha et al., J. Am. Chem. Soc. 115, 11032 (1993).
[95] W. D. Horrocks, Jr., and D. R. Sudnick, Acc. Chem. Res. 14, 384 (1981).
[96] W. D. Horrocks, Jr., and D. R. Sudnick, J. Am. Chem. Soc. 101, 334 (1979).
[97] D. D. Thomas and L. Stryer, J. Mol. Biol. 154, 145 (1982).

surface[97]; for bacteriorhodopsin they measure the retinal to be approximately 10 Å from the periplasmic surface.

Generating New Dyes

An application of FRET which is still in its infancy but shows great promise is the production of new heterodimeric dyes. One dye serves as the energy donor, the other as the energy acceptor. Over 10 years ago, Glazer and Stryer applied this technique to fluorescent phycobiliproteins,[39] covalently attaching phycoerythrin to allophycocyanin via a disulfide cross-link. The extent of energy transfer was 90%. The complex had the intense absorption of phycoerythrin around 545 nm, with the fluorescence maximum of allophycocyanin (660 nm), a Stokes shift of 115 nm. Glazer and co-workers have applied this technique to DNA dyes.[40,41] Again, the extent of energy transfer is approximately 90%, and they have created a series of dyes which have the same or similar absorption characteristics but with differing emission wavelengths (see Fig. 8). This allows simultaneous excitation with one light source and independent detection of the emission. In addition, the linker used to join the donor and acceptor is positively charged to enhance binding to the DNA and is of such a length to promote intercalation. Because the dyes intercalate and are therefore separated by a DNA base, there does not appear to be excitonic coupling, which would alter the spectral characteristics of the individual dye. The energy transfer mechanism is therefore presumed to be Förster type, and the spectral characteristics of the compound dye are therefore very similar to the sum of those of the individual dyes. Furthermore, a large number of such dyes is possible because the spectral overlap between donor and acceptor need not be large since they are so close together that efficient energy transfer takes place. By choosing one dye (donor) with large absorbance (the quantum yield need not be large) and the other dye (acceptor) with good quantum yield, unusually bright dyes should be possible (see A. Waggoner, this volume [15]). Molecular Probes has generated a series of DNA dyes based on homodimeric and heterodimeric compounds which span a wide wavelength range.[42] Application to molecules other than DNA will depend on the ability to link dyes without creating significant excitonic coupling.

Problems and Future Directions: Luminescence Resonance Energy
    Transfer Using Lanthanide Chelates

Despite the numerous successful applications of FRET, the technique has a number of drawbacks. First, the maximum distance which can be measured is less than optimal for many biological applications. Second,
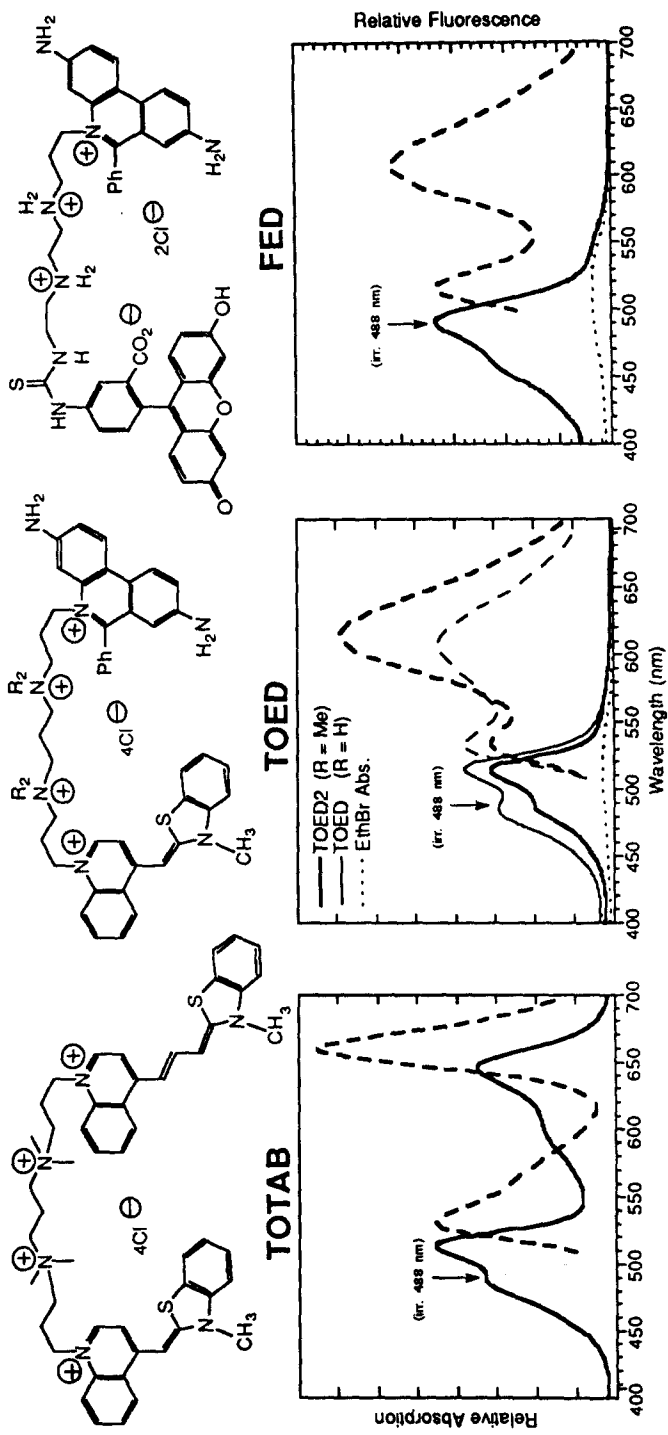
FIG. 8. Use of FRET to generate new DNA dyes. TO stands for thioazole orange, AB for Azure Blue, F for fluorescein, and ED for ethidium bromide. The dyes bind tightly to DNA such that they are stable even during electrophoresis. Solid lines represent absorption; dashed lines, emission. (From Benson et al.[40,41])

the lifetime of commonly used donor fluorophores are short (typically a few nanoseconds) and are often multiexponential, making lifetime measurements difficult and of limited accuracy. An accuracy of 10% limits measurements of quenching of more than 10% and hence less than $1.45R_0$. For R_0 values of 40–55 Å, the largest yet attained for small dyes, the maximum measurable distance via donor quenching is therefore 58–72 Å. Third, when measuring the sensitized emission of acceptor the signal-to-background ratio is poor, typically on the order of 1:1. The background arises from interfering fluorescence from the donor and from direct excitation of the acceptor by the laser or excitation light. The poor signal-to-background ratio limits the maximum measurable distance and also makes measurement of the lifetime of the sensitized emission not feasible. The large background also severely inhibits the use of FRET on biological systems that are impure (where, e.g., only a small percentage of donor–acceptor complexes form). Fourth, distances are difficult to determine precisely because of the uncertainty in κ^2.

We have developed an energy transfer system which overcomes these difficulties. We use a luminescent lanthanide chelate as donor and an organic dye such as fluorescein, rhodamine, or CY-5 as acceptor. A number of workers have noted that the luminescent lanthanide elements terbium and europium are attractive donors because they have multiple transition dipole moments such that they act as randomized donors even in the absence of any rotational motion. This limits κ^2 ($1/3 < \kappa^2 < 4/3$) even if the acceptor is stationary. Furthermore, the lifetimes are extremely long (0.6–2.3 msec) and single exponential, and thus are easy to measure. The quantum yields of the donors are also likely to be large (approaching 1 in D_2O), although the exact value is difficult to measure. The lanthanides can be relatively easily attached to macromolecules via the chelator, and, by covalently coupling an organic chromophore onto the chelator, the lanthanides can also be easily excited.[92–94,98,99] In addition, the spectral overlap is large when using terbium as donor and fluorescein or rhodamine as acceptor, or when using europium as donor and CY-5 as acceptor. The net result is unusually large R_0 values exceeding 50 Å (depending on whether the experiment is performed in H_2O or D_2O, what quantum yield is assumed, and which donor–acceptor pair is used). With terbium and rhodamine, for example, we calculate an R_0 of 65 Å, assuming the quantum yield of terbium is 1 in D_2O, and find that energy transfer experiments are consistent with this value[98] (see below). Using europium and CY-5 in D_2O we calculate an unusually large R_0 of 70 Å and, again, find that experiments are consistent with this value.[99] Using a europium cryptate

[98] P. R. Selvin and J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10024 (1994).
[99] P. R. Selvin, T. M. Rana, and J. E. Hearst, *J. Am. Chem. Soc.* **116**, 6029 (1994).
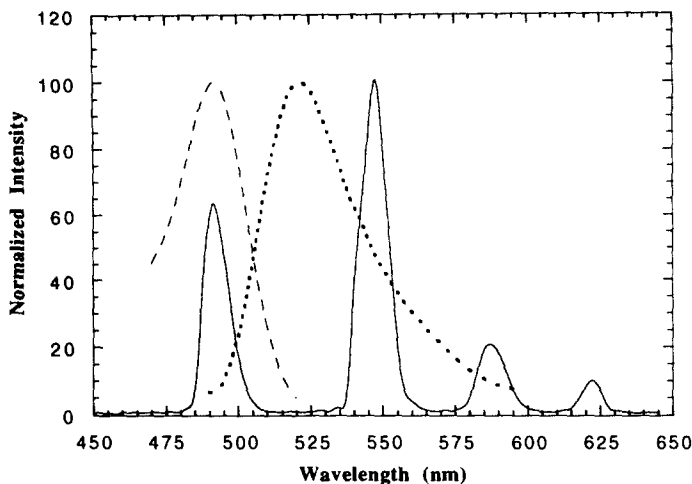
FIG. 9. Emission spectrum of a terbium chelate (structure shown in Fig. 10), used as a donor (solid line), along with absorbance (dotted line) and emission (dashed line) spectra of fluorescein, used as acceptor. Note that emission of the donor is silent around 520 nm, where the acceptor emission is maximal. Also note the excellent overlap between the 492 nm donor emission line and fluorescein absorbance, leading to a large R_0 ($\cong 52 \text{Å}$). By using a pulsed excitation source and monitoring at 520 nm, any signal arises only from sensitized emission, that is, fluorescein fluorescence due only to energy transfer (see Fig. 11).

as donor and allophycocyanin as acceptor, Mathis reports an exceptionally large R_0 of 90 Å.[100]

For all of these reasons, lanthanide donors and organic acceptors have been used in diffusion-enhanced energy transfer experiments. We find, however, that they can be used quite effectively in static FRET as well, as long as care is taken to ensure that no intermolecular energy transfer takes place. Furthermore, the most powerful aspect of these donor–acceptor pairs appears not to have been recognized until relatively recently: one can measure the sensitized emission of the acceptor without any interfering background.[98,99,100] This is in contrast to most donor–acceptor pairs, where the sensitized emission is much less than the background. Such a dark-background sensitized emission experiment has a number of advantages. First, like all sensitized emission experiments, it is less susceptible to artifacts than donor quenching. Second, because even a small amount of energy transfer yields fluorescence much above background, distances well beyond R_0 can be measured. Third, it is possible to measure the lifetime of the sensitized emission. (We measure not the nanosecond lifetime of each acceptor molecule, but the millisecond decay of the ensemble of acceptors.) By measuring the lifetime of the sensitized emission, studies are insensitive to concentration effects, to quantum

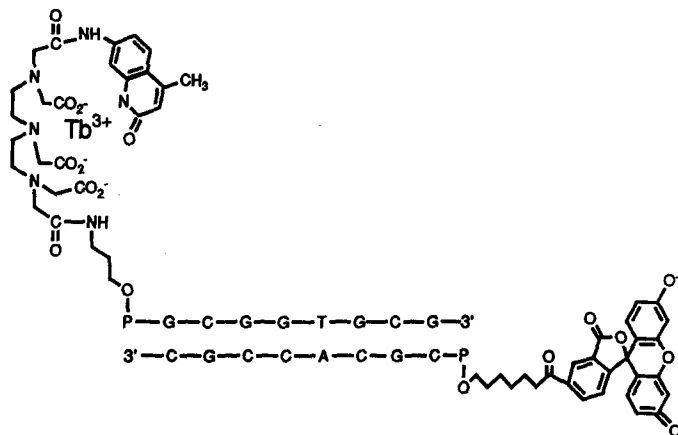[100] G. Mathis, *Clin. Chem.* **39**, 1953 (1993).

FIG. 10. Structure or terbium chelate, namely, terbium diethylenetriaminepentaacetic acid coupled to carbostyril 124. The carbostyril absorbs light (maximally at 327 nm) and transfers energy to the terbium. The net result is an increase in the effective absorbance of the terbium by several thousandfold. The DTPA chelate shields the terbium from the quenching effects of water and allows for easy attachment to macromolecules. Here the macromolecule is an 8-mer DNA oligomer modified with a primary amine on the 5' end. The acceptor, fluorescein, is attached to the 5' end of a complementary DNA oligomer.
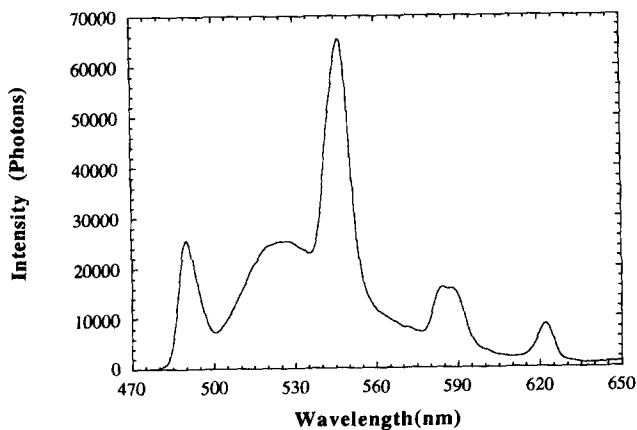

FIG. 11. Emission spectrum showing energy transfer after excitation at 337 nm with a 2 nsec pulse, with the signal collected after a 80 μsec delay with a 7 msec gate. Each point (taken every 2 nm) is the average of 160 pulses. The sensitized emission signal-to-background ratio at 520 nm is approximately 400 : 1. The efficiency of energy transfer is 70%.
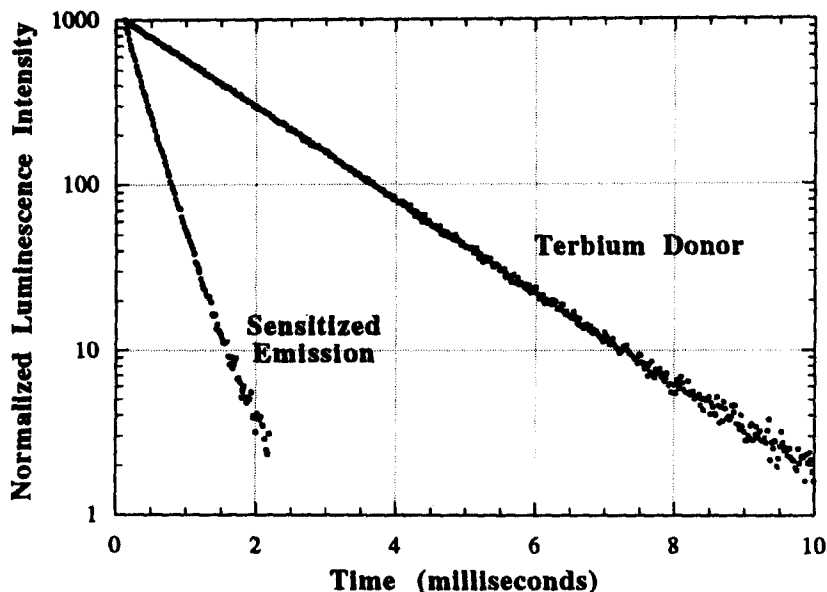
FIG. 12. Lifetime of 1.56 msec for unquenched terbium donor and 270 μsec for the fluorescein sensitized emission of donor–acceptor complex from Fig. 11. The sensitized emission lifetime indicates 83% quenching. Only completely labeled donor–acceptor complexes contribute to the sensitized emission signal. The somewhat higher energy transfer efficiency measured via lifetime (83%) versus intensities (70%, Fig. 11) is due to some donor-only species which contributes signal to intensity but not lifetime measurements.

yields (except as they affect R_0), and to incomplete labeling; only those species that are labeled with both donor and acceptor contribute to the signal.

We achieve a dark-background, sensitized emission experiment by eliminating the two usual sources of background. The donor fluorescence can be eliminated because the lanthanide luminescence is highly spiked and has regions of darkness (see Fig. 9). Terbium, for example, is silent at 520 nm. The fluorescence of the acceptor arising from direct excitation can be eliminated by using pulsed excitation and gating the detector off for a brief period, during which time the acceptor fluorescence dies away (lifetime typically a few nanoseconds) while the donor remains excited (lifetime of 1.5 msec in H_2O, 2.2 msec in D_2O for terbium). As a result, any fluorescence striking the detector in the donor dark region after a few microseconds is due only to energy transfer.

In Figures 11 and 12 we show an experiment where a terbium chelate (terbium diethylenetriaminepentaacetic acid coupled to carbostyril 124) transfers energy to fluorescein. The donor and acceptor are separated

by an 8-mer DNA duplex oligomer (Fig. 10). The sensitized emission is measured with no background at around 520 nm (Fig. 11).

The 8-mer DNA oligomer is used to separate rigidly the donor and acceptor, and the complex is immersed in a viscous sucrose solution to eliminate intermolecular interactions. (We find that intermolecular interactions do not significantly contribute to energy transfer for this system even without the sucrose, presumably because of charge repulsion of the DNA oligomers and because the diffusional rate of energy transfer is much smaller than that energy transfer arising from the acceptor fixed to the same DNA oligomer as the donor.) The efficiency of energy transfer based on both donor quenching and the integrated sensitized emission area is 70%. Figure 12 shows measurement of the unquenched donor lifetime (1.5 msec) in the absence of acceptor and the sensitized emission lifetime in the donor–acceptor complex (lifetime 270 μsec). The lifetimes indicate a quenching of 83%. (The discrepancy between 70 and 83% appears to be due to a small fraction of terbiums which cannot transfer energy, presumably arising from some donor-only complex. This lessens the percent quenching as measured by donor intensity quenching but does not affect the percent quenching as measured by the sensitized emission lifetime.) Note that the sensitized emission lifetime measurement is completely insensitive to incomplete labeling and to absolute concentrations.

The ability to measure intensities and lifetimes of both donor and acceptor emission with high accuracy and excellent signal-to-background, coupled with the unusually large R_0s, makes luminescence resonance energy transfer a potentially powerful technique for measuring distances in biological systems.

[14] Time-Resolved Fluorescence Spectroscopy

By ALFRED R. HOLZWARTH

Introduction

Fluorescence, that is, the phenomenon of light emission from an electronically excited state of a molecule, has found numerous and still rapidly growing applications for studies in the life sciences. The principal advan-