Fluorescence Resonance Energy Transfer

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Fluorescence resonance energy transfer (FRET) is a valuable tool for determining intramolecular and intermolecular distances in the range 10–100 Å. It is particularly valuable for measuring changes in molecular distance such as conformational changes in proteins.

Secondary article

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Introduction

Fluorescence involves the absorption of light energy by a fluorophore molecule (**Figure 1**, light blue curve) and emission of that energy at a longer (lower energy) wavelength (dark blue curve). If this emission overlaps the absorption spectrum of an acceptor chromophore molecule (red curve), a radiationless transfer of energy emission will occur provided the two probes are within a few atomic diameters of each other. This property is called fluorescence resonance energy transfer (FRET), although some prefer the RET acronym arguing that it is the energy and not the fluorescence that is transferred.

The development of FRET began with Förster and Weber, who gave us the theoretical and practical tools to employ energy transfer measurements to 'measure' intermolecular and intramolecular distances. In 1967, Stryer and Haughland (Stryer and Haughland, 1967) metaphorically called FRET a molecular 'ruler', and today it is widely recognized for its ability to measure macromolecular distances in the range 10–100 Å. Arguably, FRET is best used to detect conformational changes as small as 1 Å, particularly in proteins.

FRET spectroscopy is more sensitive than most other methods of examining protein structure and therefore allows the use of more dilute (less than micromolar) solutions. Also, FRET can probe very large proteins (10^5–10^6 Da) using a wide range of solvent conditions. FRET experiments are relatively quick and fluorimeters are comparatively inexpensive. But before starting, some basic information is needed, data such as molecular weight, isoelectric point, whether there are reactive side-chains, and a known sequence. These can be found in protein database files by consulting www.rcsb.org/pdb/searchlite/http. Another excellent source of information on protein side-chain reactivity and corresponding fluorescent probes is available in the Molecular Probes Handbook.

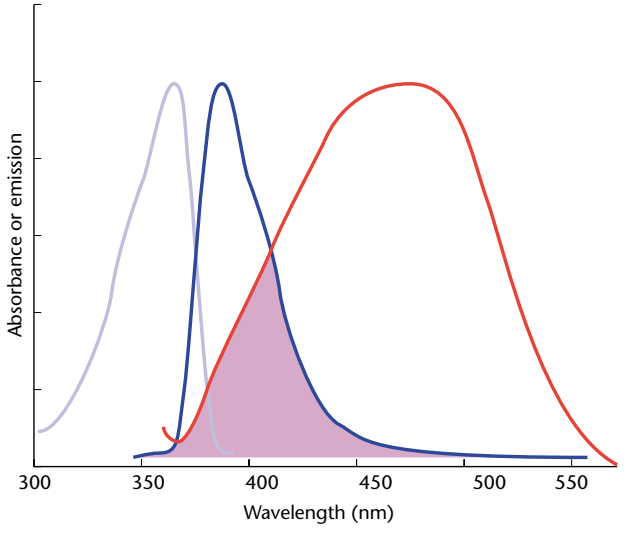

Figure 1 Relationship between the absorption spectrum (light blue) and emission spectrum (dark blue) of a donor probe and the absorption spectrum of an acceptor probe (red). The relationship between the emission spectrum of the donor and that of the acceptor has been arbitrarily set to unity. The spectral overlap of the donor and acceptor is shown by the purple hatched area.

Principles of FRET

FRET requires two probes: a donor, which must be fluorescent, and an acceptor, which need not be. Irradiation of the donor with light energy of an appropriate frequency effectively produces an oscillating dipole which resonates with a dipole of an acceptor probe in the near-field. This dipole–dipole interaction involves a radiationless transfer of energy from a donor fluorophore to an acceptor chromophore. For any given donor–acceptor pair, the energy lost by the donor is gained by the acceptor. The Jablonski diagram (**Figure 2**) illustrates the relationship between vibrational energy states, the fluorescent state and resonance energy transfer. A more detailed description of these processes can be found in Lakowicz (1999).

Absorption of light by a donor causes excitation from the ground singlet state (S_0) to a higher state. A very rapid (ps) decay from the S_4 to the lowest vibrational level in this state (S_1) is followed by a slower (ns) return to the S_0

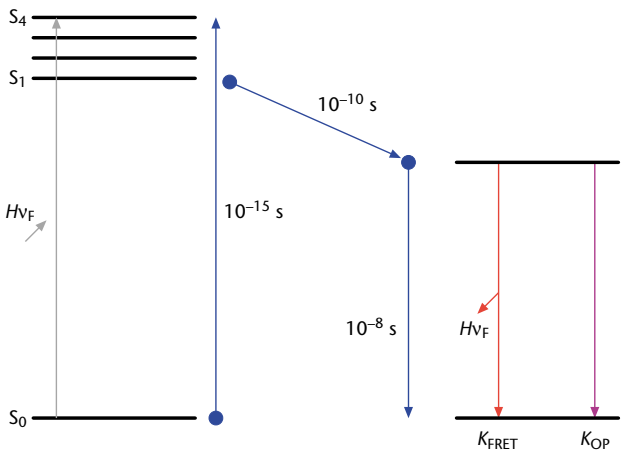

Figure 2 Modified Jablonski diagram indicating the donor energy levels at ground state (S_0) and in the excited state (S_{1-4}). A radiationless transfer of energy from the donor to an acceptor probe will reduce the fluorescence of the donor probe.

ground state, and the process is accompanied by the emission of a photon ($H\nu_F$). Return to the ground state generally occurs with a lifetime of about 10^{-8} s.

Fluorescence emission is only one of several potential pathways for the loss of excitation energy. Excited fluorophores can be deactivated by a number of competing pathways whose rate constants include: (1) the fundamental photon emissive rate (k_F) of fluorescence; (2) the rate of loss as heat by internal conversion (k_{IC}); (3) the rate of transfer to a quencher (k_Q); (4) the rate of photodestruction or photobleaching (k_{PB}); (5) the rate of triplet state formation through intersystem crossing (k_{ISC}); and (6) the rate of resonance energy transfer (k_{FRET}).

Fluorescence requires the emission of photons from an excited donor molecule, whereas FRET involves the radiationless transfer (coupling) of the donor energy to another chromophore (the acceptor) rather than the emission of photons. All six processes are interrelated and can occur in parallel such that measurement of one rate constant can be used to determine the rate constants of the others. The most common procedure is to determine k_{FRET} by observing k_F in the presence and absence of an acceptor, but it is possible to observe the transfer process by monitoring other processes such as the rate of photobleaching (k_{PB}).

There is a reciprocal relationship between k_{FRET} and donor fluorescence lifetime (τ):

$$1/\tau = k_{FRET} + k_{OP} \qquad [1]$$

where k_{OP} is the rate constant for all other processes. From this relationship, an increase in k_{FRET} will decrease the donor lifetime (τ). Clearly, a significant energy loss via parallel processes (k_{OP}) other than the k_{FRET} will introduce significant errors into the calculation of the donor–acceptor distance.

The Förster Equation

The Förster equation defines the essential elements needed to calculate donor–acceptor distance:

$$E = 1/(1 + R^6/R_0^6) \qquad [2]$$

R_0 is the so-called Förster distance at which the efficiency of transfer is 50% (**Figure 3**). It is defined as:

$$R_0^6 = (8.79 \cdot 10^{23})\kappa^2 n^{-4} \Phi_d J_{da} \qquad [3]$$

where R_0 is expressed in angstroms; κ^2 is the orientation factor; n is the refractive index of the solvent; Φ_d is the quantum efficiency of the donor probe attached to the protein; and J_{da} is the overlap of the donor emission spectrum (see **Figure 1**) with the absorption spectrum of the acceptor. The constant in eqn [3] depends on the units employed, thus where R_0 is expressed in angstroms, and the units of the absorbance/emission overlap, integral J_{da} are in $M^{-1}\,cm^3$.

The orientation factor, κ^2

For years, FRET distance determinations were challenged over concerns about the value of κ^2. This factor cannot be determined experimentally. It describes the relative orientations of the donor and acceptor oscillating dipoles. Theoretically, values of κ^2 can range between 0 and 4, but in practice these extremes may be disregarded. The range of donor–acceptor distances can be estimated from anisotropy measurements, but it is usually so large as to render the distance values meaningless. If the probes precess freely during the lifetime of the donor, $\kappa^2 = 2/3$. This problem is discussed in detail elsewhere (dos Remedios and Moens, 1999). Virtually all reported FRET distances have assumed $\kappa^2 = 2/3$, and the calculated distances generally agree with the available crystallographic distances suggesting that the assumption is reasonable. The probable reasons for this are that probes have some mobility, they are attached to protein side-chains which are also mobile, and they usually have more than one absorption and emission dipole. These factors cause κ^2 to collapse towards 2/3. Two precautions should be taken: (1) where possible, switch the locations of the donor and acceptor; and (2) measure fluorescence lifetimes rather than intensities.

Refractive index, n

The refractive index of the solvent is the least contentious element in eqn [3]. Values fall within a narrow range around the refractive index of water ($n = 1.33$). For proteins in solution, $n = 1.39$.

Quantum yield, Z_d

Quantum yield or quantum efficiency (Φ_d) is defined as the ratio of light quanta emitted by a donor divided by the number of quanta absorbed by it. The theoretical maximum value of $\Phi_d = 1.0$ can be achieved, but it may also be so small (< 0.01) that it is beyond the detection of modern instruments. Inclusion of this parameter in the Förster equation takes into account the many other pathways that lead to deactivation of the excitation energy, other than the emission of a photon. Most steady-state fluorimeters deal with this parameter by recording the fluorescence intensity relative to a known reference standard such as quinine sulfate or rhodamine. For an extensive listing of Φ_d values, see Van Der Meer *et al.* (1994). Also, Molecular Probes Incorporated provides a useful list of absorption and emission spectra ranked using the product of the quantum yield and extinction coefficient of each probe (www.probes.com). With these data it is easy to select probes that have large quantum yields and a good overlap of the donor emission and acceptor absorption spectra. In addition, this company has developed several new probes (e.g. the Alexa range of dyes) that resist photobleaching, have good Φ_d values and improved pH sensitivity.

Spectral overlap, J_{da}

J_{da} refers to the normalization of the donor emission and acceptor spectra and is defined as:

$$J_{da} = \int F_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda / \int F_D(\lambda) d\lambda \; (M^{-1}\, cm^3) \quad [4]$$

where $F_D(\lambda)$ is the donor fluorescence per unit wavelength interval and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at wavelength λ. If FRET is measured using steady state (rather than lifetime) methods, the spectral overlap of the donor emission and acceptor absorption spectra (J_{da}) must be determined. The fluorescence emission spectrum is integrated using the digital output of the fluorimeter and the integral is set to unity. The peak absorption spectrum, downloaded from a digital spectrophotometer, is then set to one mole and the wavelength scales (nm) are aligned, thereby allowing the spectral overlap to be integrated.

Precision and the measurement of R and R_0

The relationship between FRET efficiency (E) and donor–acceptor distance (R) is given by:

$$E = R_0^6/(R_0^6 + R^6) \quad [5]$$

which shows that maximum efficiency is approached more abruptly than its minimum (**Figure 3**, dotted lines). Virtual extinguishment of fluorescence intensity occurs at short R values. However, longer distances can be determined slightly more accurately than shorter distances. Where the precision of intensity or lifetime measurements is 1–3%, the donor–acceptor distances is limited to $\pm$ 50% of R_0. Thus, for a donor–acceptor probe pair with an R_0 of 30 Å, values of R will range from 15 to 45 Å. However, if this precision is improved (for example, by increasing the signal-to-noise ratio) then distances of about 55 Å can be measured (where the R_0 is 30 Å). This level of precision has been achieved with acceptors such as Tb(III) and Eu(III) whose sharp, sensitized emission peaks occur in a low noise region of the spectrum and so can be determined with great precision.

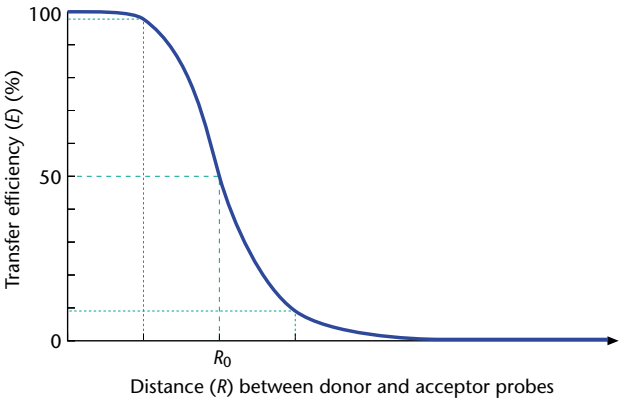

Figure 3 Graphical representation of the relationship between fluorescence resonance energy transfer (FRET) efficiency and the distance between the donor and acceptor probe. R_0 is defined by the dashed line. The practical limits of the donor–acceptor distances are indicated by the dotted lines.

FRET efficiency, E

The relationship between FRET efficiency and fluorescence intensity (I) is given by:

$$E = (1 - I_{DA}/I_D) \quad [6]$$

where I_{DA} and I_D are the fluorescence intensities of the donor in the presence and absence of acceptor, respectively. Measurement of fluorescence intensity does not require complex equipment but is sensitive to light scattering (therefore sample OD should always be < 0.05). A major advantage of fluorescence lifetime measurements is that they are essentially uncomplicated by either scattering effects or secondary absorption at high concentrations of fluorophores. Traditionally lifetime measurements were made using pulsed instruments, but this method can be used only with fluorophores that have τ values greater than a few nanoseconds, which is the duration of the shortest available pulses. The development of the cross-correlation phase fluorometer led the way to the current fluorometers. Early single-phase determina-

tions had the potential to create serious artefacts, but the modern multiple-phase instruments have overcome these problems. The relationship between fluorescence lifetime and FRET efficiency is:

$$E = (I - \tau_{DA}/\tau_D) \quad [7]$$

where τ_{DA} and τ_D are the fluorescence lifetimes of the donor in the presence and absence of acceptor, respectively.

FRET Determination of Distances in Proteins

Range of R_0 values available for probing proteins

Van Der Meer *et al*. (1994) have compiled Förster distances for about 270 donor–acceptor probe pairs; most are

Table 1 Ranking of donor–acceptor pairs based on their Förster (R_0) distances

Förster distance (R_0) (Å)	Donor probe (locus)	Acceptor probe (locus)	Donor quantum yield (Φ)	Overlap integral (J) (10^{14} M^{-1} cm^{-1} nm^4)
5	ANT-AMP (nucleotide)	Tb(III) (Ca site)	0.12	0.0014
7.7	Tb(III) (Ca site)	Pr(III) (Ca site)	0.49	0.00024
10.0–11.0	Eu(III)ε(Ca site)	Nd(III) (Ca site)	0.39–0.70	0.0014
11	EMal (Cys)	CPM (Cys)	0.15	1.10
9.2–11.4	ε-ADP (nucleotide)	Co(II) (metal ion)	0.40–0.47	0.00291–0.00307
21.9	DCl (Lys)	DDPM (Cys)	0.12	0.60
27.5–31.1	ε-ADP/ATP (nucleotide)	DDPM (Cys)	0.35–0.50	0.82–0.98
29	IAEDANS (Cys)	DDPM (Cys)	0.63	0.62
30.5	IAEDANS (Cys)	TNP (Lys)	0.64	0.821
32	FITC (Lys)	TNP-ATP (nucleotide)	0.4	1.76
33	MANT-dGDP (nucleotide)	sNBD (Lys)	0.29	2.319
34.9	ANS	DABMI (Cys)	0.25	4.65
36–40.0	CPM (Cys)	5-IAF (Cys)	0.14	10.2–15.6
37.5–45.5	ε-ADP (nucleotide)	5-IAF (Cys)	0.35–0.71	5.22–8.30
38.9–43.8	IAEDANS (Cys)	DABMI (Cys)	0.53–0.63	5.02–8.35
40.3	IAEDANS (Cys)	TNP-ADP (nucleotide)	0.48	5.87
40.7–61.7	FITC (Lys)	TRITC (Lys)	0.34	29
40.9–48.1	IAEDANS (Cys)	FITC (Lys)	0.33–0.44	9.30–18.40
43.8–51	ε-ADP (nucleotide)	TNP-ADP (nucleotide)	0.63–0.80	6.15–14
40.3	BFP	GFP	0.38	–
45	CPM (Cys)	EMal (Cys)	0.42	10
46.2	IAEDANS (Cys)	IANBD (Cys)	0.63	1.01
48	CPM (Cys)	FMal (Cys)	0.42	17
51	ε-ADP (nucleotide)	TNP-ADP (nucleotide)	0.8	14
52	IAEDANS (Cys)	5-IAF (Cys)	0.63	17.3
53.8	IAEDANS (Cys)	5-IAF (Cys)	0.48–0.63	1.05–30.1
54	FITC (Lys)	EITC (Lys)	0.48	33.9
56	FITC (Lys)	RITC (Lys)	1.0	–
56.1	IAEDANS (Cys)	IAE (Cys)	–	–
57	FMal (Cys)	EMal (Cys)	0.61	37
58	Tb(III)-DTPA cs124 (Cys)	TMR (Cys)	0.7 (1.0 in D_2O)	–
58	5-IAF (Cys)	ErITC (Lys)	0.4	46
62	FITC (Lys)	ErITC (Lys)	0.4	46

continued

Table 1 – *continued*

Förster distance (R_0) (Å)	Donor probe (locus)	Acceptor probe (locus)	Donor quantum yield (Φ)	Overlap integral (J) (10^{14} M^{-1} cm^{-1} nm^4)
64 in H_2O 70 in D_2O	Tb-DTPA-cs124 (Cys)	Cy3	–	–
65.7	Tb(III)-cryptate	RB	1.0	4.51
90	TBP-Eu(III)	APC	0.3	490

Data in this table were extracted from Van Der Meer *et al*. (1994) and supplemented from more recent publications. Note that the same probe pair can be associated with different R_0 values, and that the quantum yields and spectral overlaps of the donors differ because the donor is attached to different proteins. Abbreviations: -ADP, 1-*N*(6)-ethenoadenosine-5'-diphosphate (in some references the triphosphate was used); APC, allophycocyanin; ANS, 1-anilinonaphthalene-8-sulfonic acid; ANT-AMP, antranyloyl-2'-adenosine-5'-monophosphate; BFP, blue fluorescent protein; CPM, 7-diethylamino-3-(4'-maleimidophenyl)-4-methyl coumarin; Cy3 is the commercial name for Amersham's carbocyanine dye; DABMI, 4-dimethylaminophenylazophenyl-4'-maleimide; DCl, dansyl chloride; DDPM, *N*-(4-(dimethylamino)-3,5-dinitrophenyl)-maleimide; EITC, eosin isothiocyanate; EMal, eosin-5-maleimide; ErITC, erythrosin-5'-isothiocyanate; FITC, fluorescein-5'-isothiocyanate; FMal, fluorescein-5-maleimide; GFP, green fluorescent protein; IAE, 5-(iodoacetamido)eosin; IAEDANS, 5-((2-(iodoacetyl)amino)ethyl)amino-naphthalene-1-sulfonic acid; 5-IAF, 5-(iodoacetamido)fluorescein; IANBD, 4-(*N*-(iodoacetoxy)ethyl-*N*-methylamino)-7-nitrobenz-2,1,3-oxadiazol; MANT-dGTP, 2'(3')-*O*-(*N*-methylanthrananilyoyl)deoxyguanosine-5' triphosphate; RB, rhodamine B; RITC, rhodamine isothiocyanate; Ru(III), tris(2,2'-bipyridyl)ruthenium; sNBD, succinimidyl-6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate; TBP-Eu(III), a complex of Tb(III) with tribipyridine diamine; TNP, the reaction product of trinitrobenzene sulfonate; Tb-DTPA-cs124 (Cys), a chelate of Tb(III) with diethylenetriaminepentaacetic acid, which is attached covalently to the organic chromophore carbostyril 124; TNP-ADP, 2',3'-*O*-(1,4,6-trinitrocyclohexadienylidine)adenosine-5'-diphosphate; TRITC, tetramethyl-rhodamine 150 thiocynate. Atomic symbols are used without abbreviation and their valence is shown in parentheses.

directly applicable to proteins, and **Table 1** should be viewed as a supplement. Using these ranked Förster distances, it is possible to select a value of R that is close to a distance you wish to measure and then select the best probe pair to attach to the protein. R_0 can range from 3.4 to 90 Å. No distances greater than 100 Å have been reported using FRET spectroscopy.

Strategies for measuring short distances

If you intend to measure conformational changes in proteins or ligand–protein interactions, generally short (intramolecular) distances (< 20 Å) are involved. Measurements such as these are limited by two factors: (1) finding a probe pair with a short R_0, and (2) the accurate and precise determinations of FRET efficiencies. Measurement of short distances is logically done by selecting probe pairs with short R_0 values. These can involve using lanthanide ions that probe Ca^{2+}-binding sites. For example, using Tb(III) as the donor and Pr(III) as the acceptor, the R_0 is 7.7 Å, and distances between Trp and Tb(III) have an R_0 value of 3.4 Å. Here the problem of κ^2 disappears because both probes are completely randomized during the long lifetime (ms) of the Tb(III) donor.

Donor–acceptor distance can also be minimized by selecting parameters other than the donor–acceptor overlap integral. Donors with low quantum yields, and/or acceptors with small extinction coefficients, can be selected. For nonlanthanide donor–acceptor pairs, R_0 values tend to be a little longer, for example 11 Å for eosin-5-maleimide (donor) and 7-diethylamino-3-(4′-maleimidophenyl)-4-methyl coumarin (acceptor), both of which attach to Cys side-chains.

Except for fluorescent ions, fluorescent donors and acceptors themselves tend to be bulky molecules. Thus, when short distances (< 20 Å) need to be measured, the size of the probes themselves becomes a significant factor. Clearly, if both probes contain oscillating dipoles of dimensions 10–15 Å, a 20-Å measurement would be inaccurate even though it might still be able to sense changes in the donor–acceptor distance because of the high precision of the measurement. **Figure 4** illustrates three common donor and acceptor probes, and makes the point that they represent a significant proportion (12–17 Å) of FRET distances, particularly short distances.

Measuring long distances

The maximum distance that can be measured between a donor and an acceptor also depends on elements in the Förster equation. Thus, the spectral overlap parameter (J) should be maximal and the quantum yield (Φ) should be large, ideally 1.0. The luminescent lanthanide ion donors tend to have long R_0 values and their FRET efficiencies can be measured accurately. This seems to suggest that it may be possible to measure distances greater than 100 Å. While there is nothing in the Förster equation that precludes the determination of 100–150 Å, empirically, the longest recorded FRET distance is approximately 100 Å. It is

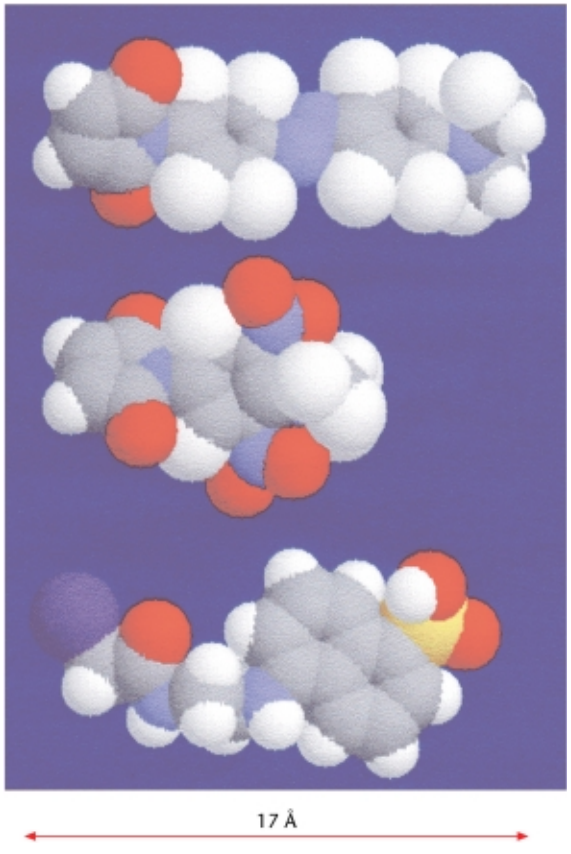

Figure 4 Space-filled models of a commonly used fluorescence resonance energy transfer (FRET) donor (IAEDANS) and nonfluorescent acceptors, DDPM and DABMI (top to bottom). The size of the probes is indicated by the scale bar. (See **Table 1** footnote for abbreviations.)

likely that the problem of detecting distances in the 100–500-Å range will not be solved using FRET. New techniques such as fluorescence correlation spectroscopy are under development that may fill this gap in the biological 'ruler'.

Comparing FRET with crystallographic distances

In his celebrated 1978 review, Stryer showed that distances determined by both FRET and by X-ray diffraction using transfer ribonucleic acid essentially agreed. FRET distances have been used to assist the determination of the crystallographic structure of actin where the initial X-ray diffraction resolution was not sufficient to follow the peptide backbone unambiguously. We also obtained good agreement between FRET distances both within actin and between actin and myosin (Miki *et al*., 1992).

FRET distances have also been used to constrain molecular models of proteins for which there is no known structure, particularly the long fibrous proteins. Knowledge of only a few molecular distances can greatly constrain models of proteins from thousands of possible structures to a couple of dozen structure families. Such an approach was used in the determination of the first atomic structure of actin.

Location of FRET probes

With intrinsic fluorophores such as Trp and Tyr, the delocalized electrons reside in their indole and phenyl rings, and these can often be precisely determined from high-resolution structures. However, identifying the precise location of an extrinsic probe can be more problematic. The problem is usually solved by knowing which side-chains react with the probe of interest, by limiting the ratio of label per protein, by limiting the duration of the reaction, and by controlling the pH. A limited proteolysis can be performed in which the sizes of the resulting peptides are estimated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or mass spectrometry to identify the labelled peptides. Algorithms are available (e.g. www.angis.usyd.edu.au) that predict the patterns of peptides generated by limited proteolysis from proteins with a known sequence, although it is usually a straightforward process to identify the labelled side-chain.

Single-molecule FRET

Detector technology has improved so much in recent years that FRET can now be performed between single molecules. Single subfragments of myosin (subfragment 1) can be observed as they hydrolyse single molecules of adenosine triphosphate (ATP) in real time. In this case, photodestruction (rather than fluorescence) was used to measure FRET efficiency (see Principles of FRET above, and eqn [1]) by observing the changes in fluorescence of a single tetramethylrhodamine isothiocyanate donor and a single Texas red-labelled acceptor. Photobleaching of the acceptor coincided with instant jumps in the fluorescence intensity of the rhodamine donor probe. The combination of single-molecule FRET with single-molecule force measurement will be a powerful tool in investigations of motor proteins.

Properties of Probes

Probes should not perturb the native protein structure. They fall into two categories, intrinsic and extrinsic. Intrinsic probes include Trps, metal ions (such as Cu) or fluorescent analogues of natural ligands (e.g. ε-ATP, Cy3-ATP). Unfortunately, these are not always accessible or applicable. Whenever a specific distance is required, it is wise to try to attach a donor or acceptor probe to a

particular locus in a particular protein, to determine the distance, and then to swap the positions of the probes.

The properties of probes that determine their suitability for FRET include: (1) high quantum yield, (2) lack of absorption interference from other fluorophores that may be present, (3) insensitivity to solvent environment, and (4) resistance to photobleaching. Quantum yield is difficult to predict because its value depends on the environment of the probes when bound to a protein. For example, 1,5-IAEDANS (5-((2-(iodoacetyl)amino)ethyl)amino-naphthalene-1-sulfonic acid) is the most commonly used fluorescent probe in the FRET literature and its quantum yield can reportedly vary by a factor of two or more. Some of this variation is due to different environments in different proteins, and some is due to the fact that other ligands may bind close to the IAEDANS site and alter its quantum yield. Thus, unless the exact replication of a published experiment is wanted, it is wise to redetermine the value of Φ for any particular donor.

Although it is usual, it is not essential to determine the quantum yield of the donor in order to calculate a donor–acceptor distance. FRET efficiency from the fluorescence emission of the acceptor probe can be used to calculate interprobe distances, and in this case it depends only on the acceptor quantum yield, not on the donor quantum yield.

pH resistance

The emission spectra of some fluorophores are highly sensitive to pH (and indeed are used as pH sensors), whereas others are comparatively less so. Emission of fluorescein is sensitive in the pH range 5–8. Its quantum yield increases by about 50% when going from pH 6.5 to pH 8, and some fluorescein derivatives used to sense intracellular pH are even more sensitive to pH. Rhodamine is comparatively insensitive to pH. Molecular Probes Incorporated now sell fluorescein-like probes called Alexa dyes which are effectively insensitive to solvent pH and come in a range of excitation and emission maxima.

Sensitivity to solvent polarity

Pyrene iodoacetamide reacts with a specific Cys residue (Cys374) in actin. Pyrene-labelled monomeric actin is very weakly fluorescent but the quantum yield increases more than 20-fold when actin polymerizes. As a consequence it has become a well established tool for following the assembly of actin into filaments.

Resistance to photobleaching

Another 'good' property of probes is their resistance to photobleaching. In this respect, rhodamine is better than fluorescein. All fluorophores can be destroyed by photodestruction. Some, such as Trp, are very susceptible and can be excited only a few times before they photobleach. Others (e.g. fluorescein) can be excited about 10 000 times, and yet others several hundred thousand times. Photobleaching competes with other processes during the deactivation of a probe. For example, photobleaching was used to identify energy transfer between a single donor and a single acceptor. A FRET measurement can be made by observing photobleaching of either the donor or the acceptor probe.

Temperature sensitivity of probes

In general it is preferable to carry out FRET experiments at temperatures lower than room temperature, and in any case it is always wise to maintain a constant temperature. There are two reasons for this. Fluorescence intensity is higher at lower temperatures because at higher temperatures the chances of other processes (k_{IC}, k_{ISC}, etc.) is increased. In addition, proteins are more susceptible to contaminating proteases and so will be more rapidly digested at raised temperatures. Most fluorometers provide a capacity for temperature control, and it is wise to stir the sample gently to maintain an even temperature as well as to minimize photobleaching.

Water solubility of probes

Most fluorescent probes are soluble in nonpolar solvents and are usually dissolved at high (mmol L^{-1}) concentrations in solvents such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF). Also, water-soluble probes can be unstable and break down with prolonged storage. **Figure 4** illustrates the structure of one of the most commonly used probes (1,5-IAEDANS) as well as two nonfluorescent acceptor probes, DABMI and DDPM (see **Table 1** footnote for abbreviations).

Nucleotide probes

The number of available fluorescent nucleotide analogues has increased steadily over the past 25 years. Two classes of covalent modifications have been produced: those on the purine ring (e.g. ε-ATP) and modifications of the ribose moiety (e.g. TNP-ATP). An ε-ATP donor can be coupled with divalent cation acceptors such as Ni(II) and Co(II) with R_0 values of 11 and 12 Å, respectively, or with Cys-directed probes such as IANBD ($R_0 = 41$ Å). Other useful nucleotide probes include MANT-ATP, Cy3-ATP and Cy5-ATP or their ADP/AMP forms (for a review see dos Remedios *et al.*, 1987; see **Table 1** footnote for abbreviations).

Labelling specific residues

The presence of a single cysteinyl in a protein does not guarantee specific labelling by a Cys-directed probe such as 1,5-IAEDANS. γ-SH side-chains may be partially or completely inaccessible and/or the added fluorophore leaving group may react with a Lys, His, Met or Tyr. Some of this lack of specificity can be avoided by using a maleimide derivative, which is less reactive with other side-chains unless the pH is raised above 8 when it reacts with amino (Lys) groups. Some maleimides (e.g. pyrene maleimide) are essentially nonfluorescent until they react. When labelling proteins, it is wise to take some precautions: (1) dissolve the probe at high concentrations (200 times the protein concentration) in DMSO; (2) add the label using a fine, positive, displacement syringe and add it over an extended (2–3 min) period with constant stirring; (3) check the final pH; (4) the labelling of Cys residues can be most effectively stopped by sedimenting the protein or by adding a 10-fold excess of *N*-acetylcysteine; and (5) always protect the labelled protein from light.

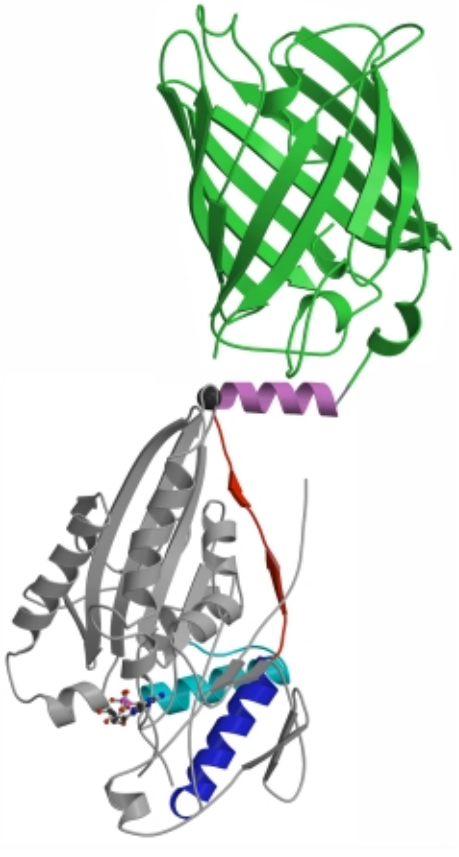

Figure 5 Molecular model of green fluorescent protein (GFP) covalently attached to single-headed kinesin (a motor protein). The GFP is connected via a linker peptide to the motor domain of kinesin.

Green Fluorescent Protein

Green fluorescent protein (GFP) is a large (238 residues, 27.3 kDa) and barrel-shaped (see **Figure 5**) ($27 \times 27 \times 42$ Å) highly stable molecule, commonly used as a marker of gene expression because it can easily be localized in cells. Mutant GFPs with different emission wavelengths and improved quantum yields have been produced. GFPs are particularly good for specific tagging of expressed proteins in live cells without having to introduce a probe. They can be detected at very low concentrations by means of confocal microscopy. The wild-type GFP has two excitation peaks (λ_{ex} at 395 and 475 nm) and an emission maximum (λ_{em}) at 508 nm, but is only weakly fluorescent. In addition, it dimerizes, photobleaches and has a number of idiosyncratic features, all of which have prompted a search for mutants with improved spectral properties. One mutant, the blue fluorescent protein (BFP), is blue-shifted ($\lambda_{ex} = 432$ and 453 nm; $\lambda_{em} = 480$ nm; $\Phi = 0.72$), whereas others are slightly red-shifted ($\lambda_{ex} = 396$ and 504 nm; $\lambda_{em} = 514$ nm; $\Phi = 0.54$), which has opened the way for FRET between GFP mutants (for more details see Sulivan and Kay, 1999).

A clever example of the use of a GFP was reported in 1999 by Sarah Rice (Rice *et al*., 1999) from University of California in San Francisco. Rice and her colleagues expressed a mutant GFP to tag a recombinant kinesin fragment. They used FRET to assess the stiffness of a linker peptide between the GFP and the catalytic region of the motor protein, kinesin.

Summary

FRET is a valuable tool for determining intramolecular and intermolecular distances in the range 10–100 Å. It is particularly valuable for measuring changes in molecular distance such as conformational changes in proteins. A brief, theoretical analysis of FRET spectroscopy is provided and practical advice is offered to help guide the choice of probes for FRET spectroscopy.

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