The renaissance of fluorescence resonance energy transfer

Paul R. Selvin

Recent advances in fluorescence resonance energy transfer have led to qualitative and quantitative improvements in the technique, including increased spatial resolution, distance range, and sensitivity. These advances, due largely to new fluorescent dyes, but also to new optical methods and instrumentation, have opened up new biological applications.

Fluorescence resonance energy transfer (FRET) is a technique half a century old, yet, due to recent advances, it is undergoing a rebirth. FRET, which relies on the distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore, is one of the few tools available for measuring nanometer scale distances and changes in distances, both *in vitro* and *in vivo*. Recent advances in the technique have led to qualitative and quantitative improvements, including increased spatial resolution, distance range, and sensitivity. These advances, due largely to new fluorescent dyes, but also to new optical methods and instrumentation, have opened up new biological applications.

In FRET, a donor fluorophore is excited by incident light, and if an acceptor is in close proximity, the excited state energy from the donor can be transferred. This leads to a reduction in the donor's fluorescence intensity and excited state lifetime, and an increase in the acceptor's emission intensity. Förster[1] showed that the efficiency of this process (E) depends on the inverse sixth-distance between donor and acceptor: $E = 1 / \{1 + (R / R_o)^6\}$ where R_o is the distance at which half of the energy is transferred, and depends on the spectral characteristics of the dyes and their relative orientation. Styrer and Haugland[2] then showed that this could be used as a spectroscopic ruler — that is, by measuring E and knowing or calculating R_o, the distance could be inferred. Because R_o is typically 20–60 Å, distances on this order can be measured. In general FRET is better suited for detecting changes in distance (conformation) rather than absolute distances because E depends on the orientation of the dyes, which is often poorly measured, and because finite probe size and attachment methods cause uncertainty in probe position with respect to the biomolecular backbone.

New probes and labeling techniques

A central issue is site-specific attachment of the FRET probes. Genetically encoded dyes, such as green fluorescent protein (GFP) and its cousins (blue, cyan, and yellow), have revolutionized the ability to perform FRET *in vitro* and especially in living cells[3]. These proteins form FRET pairs with each other or with conventional organic dyes, and can be attached to many proteins of interest, usually at the N- or C-terminus. For example, Suzuki *et al.*[4] labeled the motor protein myosin at its N- and C-termini with BFP and GFP (Fig. 1) and showed, *in vitro*, that the distance between these probes changed as myosin underwent conformational changes associated with ATP binding and hydrolysis, thereby confirming what is known as the lever-arm

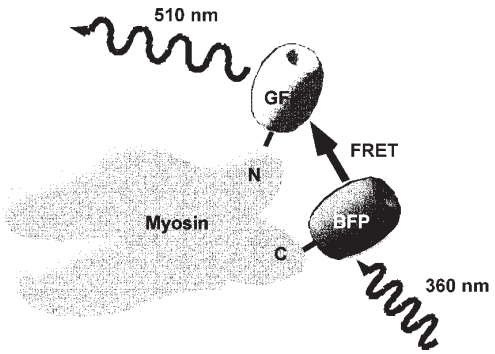

Fig. 1 GFP based FRET was used to measure conformational changes in myosin upon ATP binding and hydrolysis. Figure modified from ref. 4 with permission.

model of muscle contraction. Tsien *et al.*[5] and other workers have developed several GFP FRET constructs that are used to monitor the biochemical environment inside living cells. In these cases, the presence of some chemical, for example Ca^{2+}, alters the distance (or orientation) between donor and acceptor, causing the relative emission intensities at the donor and acceptor wavelengths to change (Fig. 2).

GFP-based constructs do, however, suffer from limited sensitivity, often precluding single cell analysis. Moreover, they are relatively large, thereby limiting spatial resolution. They should also be used with caution as it has been shown recently that GFP can undergo color changes upon irradiation due to photochemical changes that are independent of FRET[6]. In addition, GFP requires hours to assemble in its final fluorescent form and is thus limited in its ability to monitor kinetic phenomena before final assembly. Nevertheless, the importance of GFP is vast, and a new class of genetically encoded fluorescent proteins[7,8], especially those emitting in the red, indicates that its future potential is bright indeed.

Genetic engineering also has an enormous impact on the ability to place conventional dyes in a site-specific manner, sometimes in conjunction with GFP. 'Cysteine-light' proteins — proteins that contain a reactive Cys residue only at a desired position — allow the positioning of conventional fluorescent dyes to be attached at specific sites *in vitro*. Rice *et al.*[9], for example, made a series of kinesin mutants containing a GFP begin-

Loomis Lab of Physics, University of Illinois, 1110 W. Green St., Urbana, Illinois 61801, USA.

Correspondence should be addressed to P.R.S. *email: Selvin@uiuc.edu*

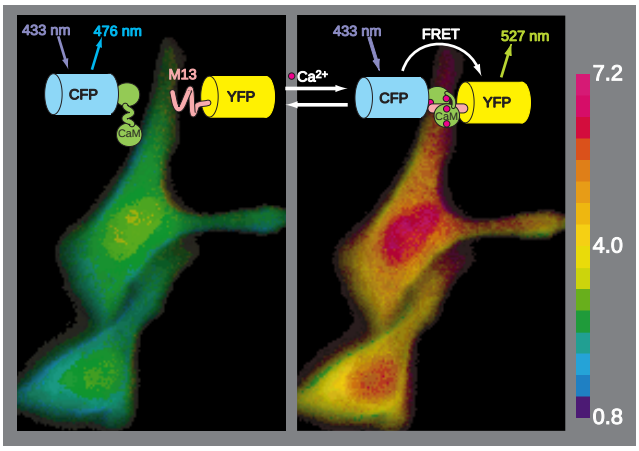

Fig. 2 FRET constructs for measuring intracellular calcium. Cyan fluorescence protein labeled calmodulin and yellow fluorescence protein labeled calmodulin binding peptide (M13-YFP) were coexpressed. High Ca^{2+} levels (right) lead to binding and FRET emission of YFP (pseudo color red); low Ca^{2+} levels (left) lead to little FRET and mostly blue emission (pseudocolor green). Figure from ref. 5 with permission.

ning at position 329 (Fig. 3) and individual unique Cys residues along the 'neck' region of the kinesin. Kinesin is a highly processive molecular motor involved in the transport of organelles towards one end of microtubule filaments. Using FRET and a number of other techniques, Rice and coworkers measured conformations of the kinesin neck linker in different nucleotide and microtubule binding states, enabling them to formulate a structural model of how kinesin moves along a microtubule. Recently, a cysteine-light myosin from *Dictyostelium*, a slime mold, has been created to monitor the conformational change in this motor molecule (J.A. Spudich, pers. comm.). For FRET, they introduced one unique Cys into the heavy chain of myosin and a second unique Cys into the light chain of myosin. Each chain could be separately labeled and then reconstituted to form an intact protein containing a FRET pair. The resulting system is similar to the GFP–BFP construct of Suzuki *et al.*[4] mentioned above, but with the advantages of having much smaller dyes and a fully intact protein. (A truncated protein was necessary in that case to introduce the fluorescent proteins.) Importantly, Dictyostelium containing this highly mutated myosin was still functional (J.A. Spudich, pers. comm.). This new family of cysteine-light motor proteins has recently been extended to include myosin from smooth muscle (A.L. Wells, L. Chen, Z. Yang, M. Xiao, P.R.S., & H.L. Sweeney, unpublished results). The number of cysteine-light proteins is likely to grow, and now includes voltage-gated and ligand-gated channels[10–12], Na-K ATPase pumps[13], and several DNA binding proteins[14,15].

Nongenetically encoded, but clever, FRET pairs can also be used to monitor cellular events in live cells. To overcome some of the limitations of GFP-based constructs, Tsien and coworkers developed several membrane permeant dyes useful for FRET. One construct contains a blue-emitting coumarin donor coupled *via* a cleavable linker to a green-emitting fluorescein derivative[16]. Because the intact linker is small, energy transfer is large (~95%) and emission is primarily green. The presence of lactamase in the cell cleaves the linker, eliminates FRET and results in primarily blue emission from the donor, with a 70-fold increase in the blue/green ratio. The importance of the technique is that gene expression activity can be monitored at the single-cell level by cotranslating the gene for β-lactamase with another gene of interest. Because one lactamase can cleave many FRET dye pairs, the signal is amplified and the sensitivity to low expression levels is excellent. Tsien and coworkers also developed another set of membrane permeant dyes that will likely be very useful in FRET. These are the arsenic based green (fluorescein)[17] and red (rhodal) derivatives (S. Adams, pers. comm.). They bind to a unique and rare sequence, CC-XX-CC (where C is cysteine and X is any amino acid), and, fortuitously, are fluorescent only when bound.

Another useful set of organic dyes includes those emitting in the far-red (for example >650 nm), particularly the cyanine dyes (Cy5, Cy5.5 and Cy7). The advantages of these are that background fluorescence is often much reduced and relatively large distances (expected to be >100 Å) can be measured. The latter is because the R_o values of these dyes are expected to be very large, primarily due to their excellent absorbance, and reasonable quantum yields. For example, R_o of the Cy5–Cy5.5 pair is expected to be >80 Å (ref. 18). Reasonably large R_o val-

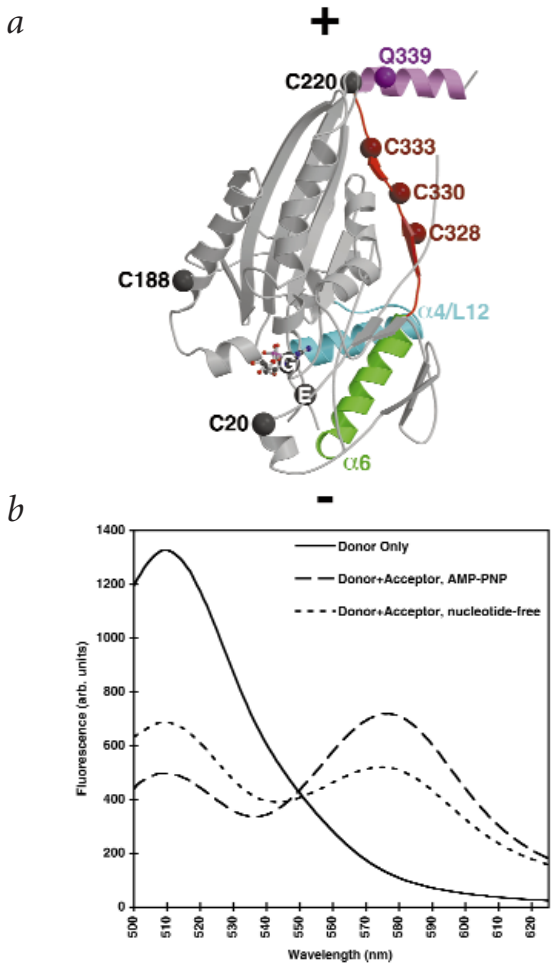

Fig. 3 Conformational changes in a cysteine-light GFP-tagged kinesin. ***a***, The kinesin monomer was modified to include a GFP (not shown) beginning at position 339 in the coiled coiled region (purple) and unique Cys residues were inserted for site specific labeling of extrinsic fluorophores in the neck region (red) and elsewhere. ***b***, FRET from a GFP donor to a tetramethylrhodamine acceptor at Cys 220 shows nucleotide dependent donor quenching and sensitized emission of acceptor. From ref. 9.

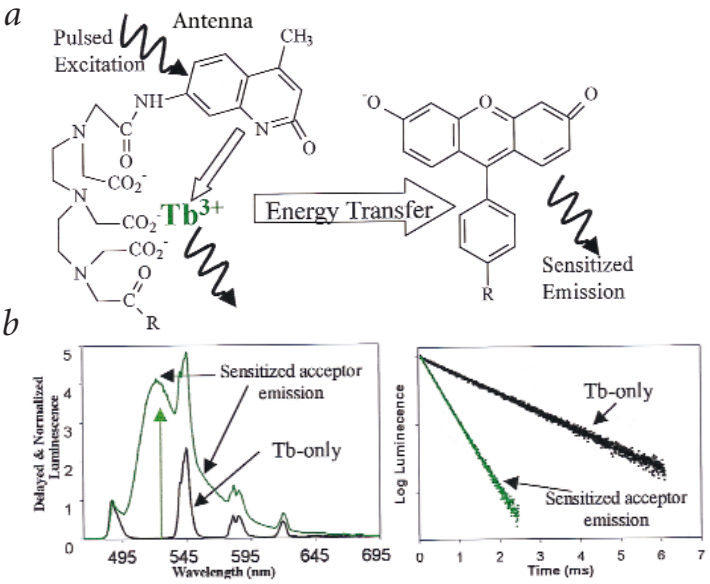

Fig. 4 Lanthanide-based resonance energy transfer. ***a***, Structure of a lanthanide (terbium) chelate and schematic of energy transfer to an organic fluorescent acceptor. ***b***, Terbium is excited *via* pulsed excitation through its antenna, and emits with sharply spiked emission spectra and a long lifetime. Emission from the acceptor due to energy transfer from the donor follows the long lived terbium lifetime and can be measured without interference from donor emission (for example at 520 nm) or direct acceptor emission, which lasts only nanoseconds (M. Xiao & P.R.S., unpublished results).

ues can also be achieved with donors and acceptors with well separated emission spectra. For example, R_o of the Cy3–Cy5 pair is >50 Å, with Cy3 emitting maximally at 570 nm and Cy5 maximally at 670 nm. Well-separated emission maxima allow the increase in acceptor emission from FRET to be measured without donor emission contamination. These advantages have made cyanine dyes (along with rhodamine-based dyes) the dyes of choice for single-molecule FRET studies[19]. FRET using the Cy3–Cy5 pair, for example, has recently been used to visualize the dimerization of single EGF receptors in a living cell[20]. (For further discussion of single-molecule FRET, see the review by Weiss[21], this issue; see also ref. 22) The lack of a commercial source for thiol-reactive cyanine dyes has limited the use of these important dyes, but recently a straightforward synthesis has been reported[23].

Finally, lanthanide (also called the rare-earth elements) atoms have been shown to offer many advantages as donors in FRET, for both basic[11,24] and applied studies[25]. In these cases, conventional organic dyes are used as acceptors. They offer many advantages including the ability to measure distances up to 100 Å with greatly improved accuracy and signal/background noise ratios compared to conventional FRET dyes, and insensitivity to incomplete donor or acceptor labeling of the sample. These advantages arise because of the highly unusual spectroscopic characteristics of the lanthanides. The emissions of terbium or europium, when placed in the appropriate chelate (Fig. 4), are sharply spiked in wavelength, have millisecond lifetimes following an excitation pulse, are unpolarized, and have high quantum yields. To highlight one attribute, the emission of the acceptor due only to energy transfer (called sensitized emission) can be measured with essentially no background contamination. Background fluorescence from the donor is eliminated spectrally by looking at wavelengths where the donor does not emit. Background fluorescence from the acceptor due to direct excitation is eliminated temporally because the lifetimes of organic dyes are typically in the nanosecond range, whereas the sensitized emission follows the lifetime of the donor, which is on microsecond to millisecond timescale. Because the delayed sensitized emission only arises from donor–acceptor pairs, any incomplete labeling (donor only or acceptor only labeled molecules) does not contribute to the FRET signals. Furthermore, both the sensitized emission lifetime, which is independent of absolute concentration, and the emission intensity can be measured. Because the terbium donor emission is unpolarized, the orientation dependence of FRET is greatly reduced, making distance determination more accurate.

Several studies have taken advantage of these attributes. Heyduk *et al.*[26], for example, measured distances up to 100 Å in protein–DNA complexes that contained a heterogeneous mixture of labeled biomolecules. Root[27] measured the interaction between antibody-labeled dystrophin and actin inside a cell. In my laboratory, lanthanide-based FRET has been used to measure relatively long distances in myosin[28] and to detect conformational changes in voltage-gated ion channels[11]. In the latter case, Shaker potassium ion channels containing a unique Cys residue on each of their four identical subunits were expressed in *Xenopus* oocytes (Fig. 5). The channels were labeled with a mixture of donor and acceptor probes, with the donor in excess to ensure that most channels contained at most only one acceptor. (Channels containing all donors do not contribute to the measured signal and can be ignored.) A donor therefore sees an acceptor on a contiguous subunit (distance R_{SC}) or on a subunit across the pore (distance R_{SA}). The sensitized emission lifetime is therefore bi-exponential, with the shorter lifetime corresponding to the greater transfer efficiency and thus the shorter distance. Importantly, the two distances are in excellent agreement with those derived from the Pythagorean relationship based on the tetrameric symmetry of the channel. As the voltage across the membrane changes, the distances between subunits change correspondingly, producing a model indicating that the so-called voltage sensing region of the channel likely undergoes a rotation rather than a large translation, as had been proposed[11].

New optical methods

New technical advances in optical instrumentation and FRET measurement methodology — sometimes combined with new dyes — have led to new applications. The use of lasers and sensitive detectors, for example, has led to the ability to measure energy transfer between a single donor and single accep-

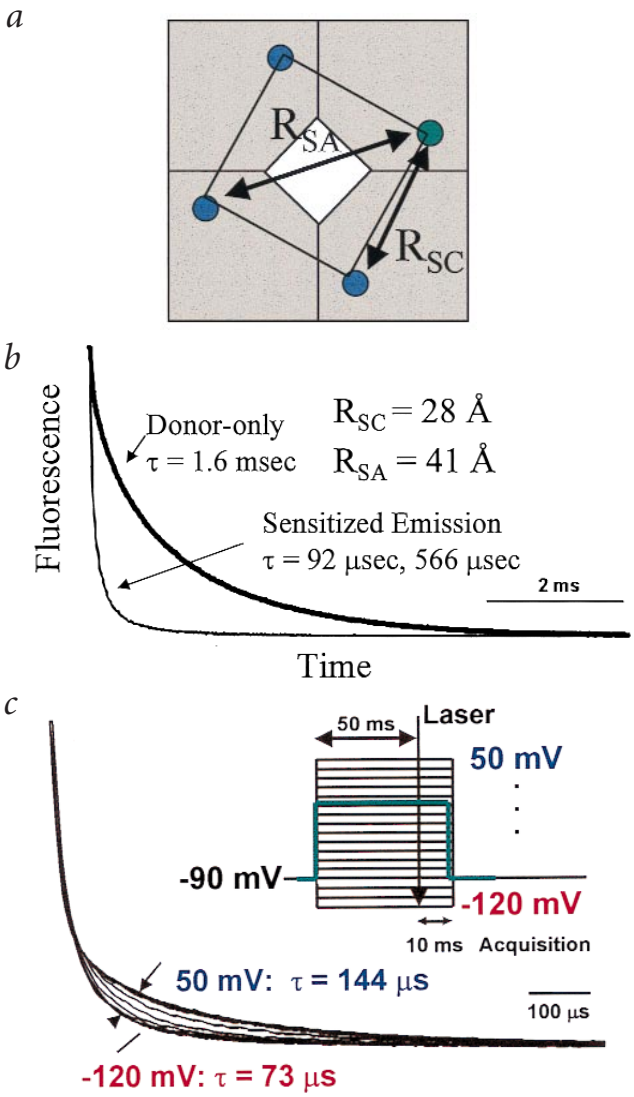

Fig. 5 Structure and lanthanide-based resonance energy transfer measurements of a voltage-controlled ion channel. ***a***, The tetrameric structure of the Shaker potassium channel labeled with donors (blue) and acceptor (green). ***b***, Donor only and sensitized emission lifetimes, the latter displaying two lifetimes corresponding to distances R_{SA} and R_{SC} (see text for discussion). ***c***, A step change in voltage (inset) causes the donor–acceptor distance and hence sensitized emission lifetimes to change. Figure from ref. 11 with permission.

tor[19,21,22]. Samples with heterogeneous distribution of energy transfer pairs can be analyzed with such single-molecule studies[29]. FRET measured on an area detector (for example, a charged-coupled device, CCD) has led to the ability to measure FRET on a pixel by pixel basis, yielding a picture of molecular interactions, such as receptor–ligand interactions or dimerization, throughout a cell. New ways of measuring energy transfer have increased the versatility and accuracy of conventional FRET experiments.

The conventional method for determining FRET is to compare the donor intensity of the donor–acceptor sample to that of the donor only sample, and possibly to compare the acceptor intensity of the donor–acceptor sample to that of the acceptor only sample. The problem is that this requires matching concentrations of different samples, which is difficult to do accurately. Lifetime measurements, as opposed to simple intensity measurements, have the advantage of being concentration independent and, if multiple lifetimes can be resolved, of being able to differentiate subpopulations with different amounts of energy transfer. FRET can be measured because it leads to a shortening of the donor lifetime.

Clegg, Jovin and others have pioneered a method to measure donor lifetimes on a point by point basis in the image of a cell (or a microscopic sample); this is called fluorescence lifetime imaging microscopy (FLIM)[30] (Fig. 6). The essence of the idea is that excitation light is modulated rapidly on the order of the lifetime of the donor's excited state, which is in the nanosecond timescale for organic dyes. For dyes with lifetimes significantly faster than the modulation time, the emission intensity will simply follow excitation intensity, with little or no phase lag, and a large modulation amplitude. Dyes that have a much longer lifetime cannot follow the rapidly changing excitation intensity and will have very little modulation. For dyes on the order of the modulation time, their emission will have intermediate modulation amplitude and a significant phase lag. A CCD with a modulatable image intensifier is used to detect this phase and modulation at every pixel — that is, in a spatially resolved manner.

Gadella and Jovin used this technique to detect epidermal growth factor receptor (EGFR) dimerization and its conformational state[31]. Fluorescently labeled EGF molecules with the donor (fluorescein) and acceptor (rhodamine) were allowed to bind EGFR; the presence of FRET indicated receptor dimerization. They concluded that the 'high affinity' EGFR exists as a dimer, even in the absence of EGF ligand. They concluded that the 'high affinity' EGFR exists as a dimer, even in the absence of EGF ligand. Some models had postulated that EGF binding promotes dimerization and thereby, indirect activation of EGFR. Rather, the authors postulated that EGF binding causes a conformational change in the preformed dimer, indirectly activating a latent tyrosine kinase on its intracellular side[20,31]. More recently, Ng *et al.*[32] used FLIM to measure activation of protein kinase C (PKC) in fixed, living and pathological cells (Fig. 6). For example, they found that activation of PKC was increased in about half of human breast tumors tested, whereas total PKC content was not correlated with the pathology.

The main drawback of FLIM is that the instrumentation is relatively sophisticated. Jovin and colleagues have pioneered an alternative way of measuring FRET *via* donor photobleaching[31]. The idea and technology are quite simple. Photobleaching, which involves light-induced destruction of a dye while in the excited state, is proportional to the excited state lifetime of the dye. Because FRET reduces the lifetime of the donor's excited state, its photobleaching rate decreases proportionally. Thus, the experiment is simply to shine a continuous beam onto the sample and monitor the fluorescence intensity of the donor as a function of time. Because the photobleaching rate is on the order of seconds, the instrumentation is simple; the technique is also sensitive because each donor yields the maximum number of photons it can emit. The main disadvantage of the technique is that it requires comparing separate samples, one containing donor only and one containing donor and acceptor, and, because the fluorophores are destroyed, multiple measurements on the same sample cannot be made. Gadella and Jovin used this methodology in their EGFR studies mentioned above as a complement to FLIM. More recently, Glauner *et al.*[10] used a

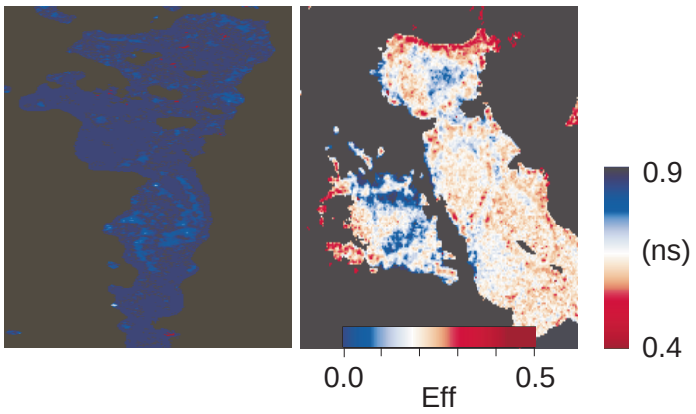

Fig. 6 Fluorescence lifetime imaging FRET can be used to localize the position of phosphorylated biomolecules in a cell. Individual phospholipid kinase C (PKC) molecules in fixed cells were stained with either donor (Cy3) only (left panel) or with donor and acceptor (Cy5) (right panel). The acceptor labeling was through an antibody specific for the phosphorylated form of PKC. Only doubly labeled PKCs — phosphorylated PKC molecules — resulted in FRET and a reduction in donor lifetime. Figure from ref. 32 with permission.

variant of photobleaching FRET to measure conformational changes in the Shaker potassium voltage-gated ion channel, an experiment complementary to the lanthanide based FRET experiment by Cha *et al.*[28] mentioned above. Siegel *et al.*[33] recently combined photobleaching FRET and GFP–YFP labeling to measure self-association of a cell surface protein Fas, which is involved in apotosis. It was previously believed that ligand-induced trimerization of the Fas receptor led to a signaling cascade resulting in cell death, but Siegel *et al.*[33] showed that such trimers existed without ligand, suggesting that such signaling may be regulated at the level of receptor assembly.

Clegg and coworkers have also developed a relatively simple and robust method for measuring FRET in solution using conventional steady-state fluorescence[34]. The main advantage is that FRET measurements are made on a single donor–acceptor labeled sample. The sample is excited at two wavelengths — one predominantly excites the donor, and the other predominantly excites the acceptor. Comparisons to donor only and acceptor only samples are then made based on spectral shape, which can be accurately measured, but not on absolute intensities. The method has been used to show that four-way junctions involved in DNA recombination are right-handed crosses of antiparallel strands[35]; it has also be used to measure the helical handedness of DNA in solution[36,37]. Using this technique, differences in energy transfer efficiencies as small as one percent between samples can be measured. These examples represent just a few of the many recent applications of FRET on DNA and DNA–protein complexes. Other examples include generating a molecular model of the three-dimensional structure of a hammerhead ribozyme by Tuschl *et al.*[38], and characterization of the binding orientation of the Fos–Jun heterodimer protein complex on DNA by Leonard and Kerppola[15]. The increase in the use of FRET for studying DNA or DNA–protein complexes has arisen largely because labeling and synthesis of the DNA samples using phosphoramidite chemistry is now routine.

Perspective

The recent advances in developing new fluorescent probes, instrumentation and methodologies have greatly increased the utility and scope of FRET. The next round of development will undoubtedly lead to new applications for this technique. Be on the lookout for further probe development, including new methods for their attachment to samples, smaller and more photostable fluorophores, probes with a wide range of intrinsic excited state lifetimes, probes based on inorganic materials, and a wider variety of genetic fluorescent proteins, particularly ones that are smaller than GFP. Commercialization of some of the more advanced fluorescent instrumentation will also make today's sophisticated techniques tomorrow's routine measurements. The future of FRET is indeed bright.

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