

Single-Molecule Fluorescence Resonance Energy Transfer

Taekjip Ha¹

*Department of Physics and Center for Biophysics and Computational Biology,
University of Illinois at Urbana–Champaign, Urbana, Illinois 61801*

Fluorescent resonance energy transfer (FRET) is a powerful technique for studying conformational distribution and dynamics of biological molecules. Some conformational changes are difficult to synchronize or too rare to detect using ensemble FRET. FRET, detected at the single-molecule level, opens up new opportunities to probe the detailed kinetics of structural changes without the need for synchronization. Here, we discuss practical considerations for its implementation including experimental apparatus, fluorescent probe selection, surface immobilization, single-molecule FRET analysis schemes, and interpretation. © 2001 Academic Press

The study of single-molecules provides a number of advantages that make it attractive. For instance, in a heterogeneous population, it can reveal the population distribution most directly. More importantly, for complex biochemical reactions that cannot be synchronized, it provides a unique opportunity to determine the detailed kinetics. Technical advances made it possible to detect even a single dye molecule under biologically relevant conditions. Exciting developments in single-molecule fluorescence detection and spectroscopy for applications in physics, chemistry, and biology have been reviewed (1–4).

A single dye molecule can report on the host molecule to which it is attached to in a number of ways. First, molecules can be localized to certain spatial positions via fluorescence imaging (5). Fluorescence intensity fluctuation (6) and a single fluorophore's polarization response (7) can also be used to detect the motion and activities of biomolecules. Perhaps the most general approach is the use of two fluorophores rather than

one, in the form of fluorescence resonance energy transfer (FRET) or Förster transfer. Since the first demonstration of single-molecule FRET (8), there have been a number of experiments designed for biological applications (9–19). It has been shown that conformational fluctuations, folding pathways, and macromolecular interactions can be followed reliably at the single-molecule level and that new information can be obtained that can complement other existing assays. The ever-expanding list of biological systems that have been studied by single-molecule FRET includes DNA rulers (8, 10, 19), staphylococcal nuclease (9), biotin–streptavidin (17), GCN4 peptides (14), α -tropomyosin (15), S15 binding RNA junction (11), *Tetrahymena* ribozyme (12), calmodulin (13), Rep helicase (Ha *et al.*, unpublished data). There are already excellent reviews on existing single-molecule FRET works (1, 20). We focus on the methodology of single-molecule FRET based mostly on the practice in our laboratories.

In Section I, we discuss first fluorescence resonance energy transfer in general and, then, the motivations and advantages of performing FRET at the single-molecule level. In Section II, we discuss practical considerations for implementing single-molecule FRET using a question-and-answer format.

FRET AND ADVANTAGES OF SINGLE-MOLECULE FRET

1. Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer between two dyes, donor and acceptor, has proven to be a powerful spectroscopic technique for measuring distances in the range 10–75 Å (21, 22). Excitation energy of the donor

¹ To whom correspondence should be addressed. E-mail: tjha@uiuc.edu

is transferred to the acceptor via an induced dipole–induced dipole interaction. The efficiency of energy transfer, E , is given by

$$E = \frac{1}{1 + (R/R_0)^6}, \quad [1]$$

where R is the distance between the donor and acceptor and R_0 is the distance at which 50% of the energy is transferred and is a function of the properties of the dyes. R_0 contains a contribution from the relative orientation between the two dyes, called κ^2 . Without a priori knowledge of their orientations, $0 < \kappa^2 < 4$.

Energy transfer results in a decrease in fluorescence intensity and in excited-state lifetime of the donor and an increase in fluorescence of the acceptor. By quantification of these changes, E is determined. Distance, then, is extracted by inverting Eq. 1. It is often assumed that the dipole moments of donor and acceptor are free to rotate in all directions, on a time scale much faster than their radiative lifetime. In this case, a geometric averaging of the angles results in $\kappa^2 = 2/3$ (in many cases, however, the fluorophores interact with the macromolecule to which they are attached, thus restricting their motion and introducing uncertainty in the value of κ^2).

Because of its strong distance dependence, FRET can be used as a spectroscopic ruler (Fig. 1). For instance, a small change in distance between the two sites of a biological molecule where donor and acceptor are attached can result in a sizable change in E . One can easily imagine, then, how structural changes of biological molecules or relative motion and interaction between two different molecules can be detected by changes in FRET (Fig. 1). Selvin reviewed recent progress in FRET and luminescent energy transfer applied to a variety of biological systems at the ensemble level (23).

2. Advantages of Single-Molecule FRET

Distribution

FRET values can be determined from individual biomolecules and their histogram can directly give information on FRET value distribution (Fig. 2). Although powerful, this is not unique to single-molecule FRET. Let us consider a heterogeneous distribution with two populations that have different FRET values. A steady-state ensemble FRET measurement will give a single FRET value, the average of the two weighted by their relative populations. If homogeneous samples (with only one FRET value each) can be prepared and their

FRET values measured, the relative populations in a heterogeneous mixture can be determined. If homogeneous samples cannot be prepared, for example, because the different populations arise from spontaneous fluctuations occurring on a time scale faster than the measurement, time-resolved ensemble FRET can be used to determine the distribution. In time-resolved FRET, multiple donor lifetimes are interpreted as coming from multiple species with different FRET values.

Dynamics

Biochemical reactions can be monitored by temporal changes in ensemble FRET, hence giving their kinetic parameters. However, to study kinetics using ensemble FRET, the reactions have to be synchronized. Molecules have to be prepared in one state before initiating the reaction by an external trigger. For reactions that cannot be synchronized, single-molecule FRET still provides the opportunity to study the reaction kinetics. In addition, the study of single-molecules can detect very rare conformational transitions (Fig. 2) and nonaccumulating reaction intermediates that are difficult to detect

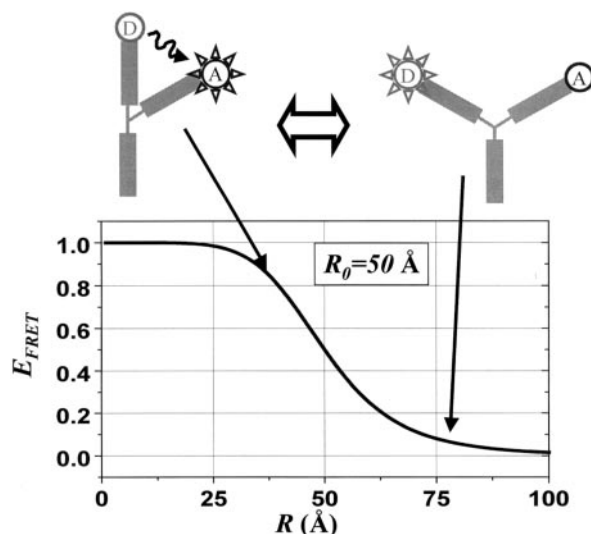


FIG. 1. Conformational changes of single biological molecules can be detected via fluorescence resonance energy transfer (FRET). Top: Two conformations of model RNA molecule (S15 protein binding three-way junction) with donor and acceptor attached to ends of two arms. In the open form (right side), two dyes are far from each other and there is little energy transfer. Therefore, donor excitation by the laser results in primarily donor's emission. In the closed form (left side), two dyes are close to each other and there is strong energy transfer. Then, donor emission is weak and acceptor emits strongly. Fluorescence intensity of donor and acceptor can easily be measured from a single pair of molecules, yielding information on the distance between the two and the conformation of the host molecule. Bottom: Energy transfer efficiency versus distance between the two dyes (E_{FRET} vs R) according to $E_{\text{FRET}} = [1 + (R/R_0)]^{-1}$ with $R_0 = 50 \text{ \AA}$.

using ensemble FRET. The dynamic aspects of single-molecule FRET are what really distinguish it from other approaches.

II. FREQUENTLY ASKED QUESTIONS ABOUT SINGLE-MOLECULE FRET

1. Experimental Setup

What Microscopic Tools Can Be Used?

There are two classes of fluorescence microscopic tools for single-molecule fluorescence studies. The first involves point detection with detectors with single elements (photomultiplier tube (PMT) or silicon avalanche photodiode (APD)) in the form of a confocal scanning optical microscope (CSOM) or near-field scanning optical microscope (24, 25). The second class uses wide-field

microscopy with two-dimensional detectors such as a CCD camera. One advantage of wide-field microscopy is that hundreds of single-molecules can be detected simultaneously, effectively performing hundreds of single-molecule experiments in parallel. This is especially useful for irreversible reactions or for very rare biological events. In addition, two-dimensional diffusion of molecules on a surface such as lipid bilayers can be directly visualized. However, because an arrayed detector has to be used, the time resolution and the sensitivity are not as good as those of point detection cases. Clearly, these two classes of microscopic tools are complementary.

CSOM became the method of choice for many laboratories since the pioneering works of Nie *et al.* (26) and Macklin *et al.* (27). In CSOM, laser excitation light is focused to a diffraction-limited spot using a high-numerical-aperture (NA) objective and the fluorescence coming from a single-molecule under the spot is collected using the same objective. A pinhole is used to block the out-of-focus autofluorescence signal to achieve single-molecule sensitivity. Unlike commercial CSOM that raster-scans the laser beam for high-speed imaging, single-molecule CSOM typically scans the sample because the imaging speed is limited by the photon counts rather than by scanning speed. Two detectors are needed to detect donor and acceptor emissions simultaneously after their separation using a dichroic beam splitter. Computer-controlled data acquisition allows the accumulation of a large quantity of single-molecule data by identifying individual molecules on the surface and taking the time records of single-molecule fluorescence signals.

Wide-field microscopy can be done either via epi-illumination or via evanescent field excitation. In epi-illumination, the excitation light is sent through the epi-illumination port of a conventional fluorescence microscope (28). Unlike in CSOM, autofluorescence generated from the microscope optics and sample cannot be removed, resulting in an inferior signal/noise ratio. In contrast, evanescent field excitation does not permit excitation light to propagate toward the detector and hence can reduce the autofluorescence to an undetectable level (29). Such an evanescent field excitation is generated by total internal reflection of the excitation light at the glass–water interface; therefore, we call this microscope a total internal reflection microscope (TIRM).

A large incident angle is required to achieve total internal reflection. For instance, an incident angle (relative to the surface normal) larger than 61° is needed for a glass–water interface. This angle can be achieved using a prism (prism-based TIRM) (29) or through the edge of a high-NA objective ($NA \geq 1.4$) (objective-type

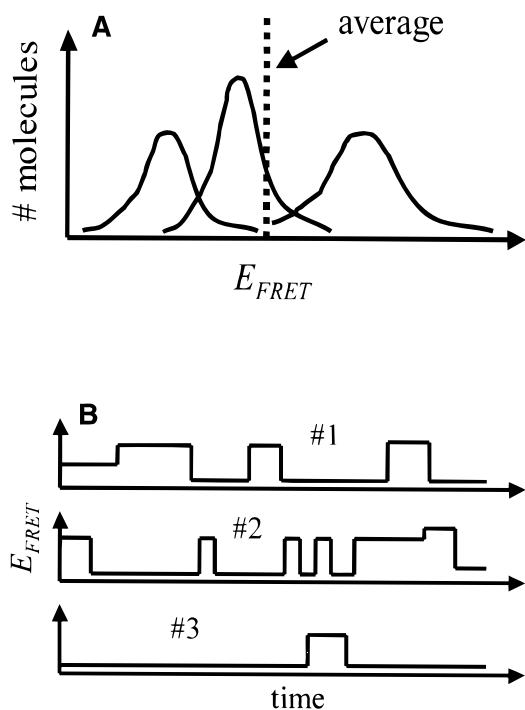


FIG. 2. Advantages of single-molecule FRET. (A) If there are subpopulations possessing different E_{FRET} values, single-molecule measurements can directly reveal the detailed distribution while steady-state ensemble FRET gives only the average value. (A) Cartoon of three peaks that have different FRET values in a histogram of single-molecule FRET efficiencies (E_{FRET}). The vertical dashed line is the average value of FRET that would be measured in a steady-state ensemble FRET. (B) Fluctuations of E_{FRET} value can be detected in real time from single-molecule measurements while the dynamic information is lost in ensemble FRET unless conformational changes of molecules are synchronized. The figure shows a diagram of three individual traces that fluctuate between multiple levels of FRET at random times.

TIRM) (30). To obtain donor and acceptor images simultaneously using one CCD camera, a dual-view scheme can be used (12, 31, 32). In a dual-view scheme, donor and acceptor images are projected to each half of the camera. Details on the scheme can be found in the references.

What Detectors Are Used?

For CSOM, the APD is favored over the PMT for a number of reasons. The APD has a higher detection quantum yield (>50%) for visible light, has a very low dark count rate (<50/s), and can essentially count photons. The PMT may be more useful for very blue or ultraviolet dyes. A limitation of the APD compared with the PMT is its small active area ($\sim 200 \mu\text{m}$), but this is an advantage for single-molecule detection rather than a disadvantage. Its small area can act as an additional pinhole to reject the out-of-focus autofluorescence and the single-fluorescence molecule acts as a point source that can be imaged entirely within the active area. For the APD, the signal/noise ratio is determined by shot noise (statistical photon number noise) rather than readout noise and, therefore, can exceed 20:1.

In contrast, readout noise is inevitable for digital CCD camera detection used for wide-field microscopy and becomes more significant if a high frame rate is desired. An intensified CCD camera amplifies the signal so that many electrons are generated per photon detected. Then, readout noise becomes negligible and a frame rate exceeding 30 Hz can be obtained. However, intrinsic noise coming from the intensifier dominates and signal/noise ratio for single-molecule experiments is typically limited to 5/1.

How Is the Decision on Excitation Source and Fluorescence Filters Made?

Because lasers are used as the excitation source for most single-molecule detection, selection criteria for fluorescence filters are different from those used for mercury or halogen lamp excitation. A spectrally narrow excitation source allows the use of fluorescence filters with a cutoff wavelength closer to the excitation wavelength. Therefore, more fluorescent photons are collected through the filters. It is always better to excite away from the absorption maximum to allow efficient collection of fluorescence rather than to excite at the absorption maximum and sacrifice the short-wavelength portion of fluorescence photons.

To optimize the resolution with which FRET efficiency can be determined, it is important to minimize the cross talk (donor emission leakage to the acceptor detector and acceptor emission leakage to the donor detector). The former is usually more significant because a fluorescence emission spectrum is asymmetric

with a long-wavelength tail. It is often advantageous to reduce the cross talk by adding bandpass filters even though the overall signal may decrease.

What Is the Typical Equipment for Single-Molecule FRET Apparatus?

Light source. Diode-pumped doubled YAG laser ($\lambda = 532 \text{ nm}$, 50 mW; GCL-050-M, Crystalaser, Reno, NV). This is a good color to excite dyes such as Cy3, tetramethylrhodamine, and Texas Red.

Inverted optical microscope. Olympus IX70 inverted microscope system with dual sideports (IX70, Olympus). Two side ports allow rapid exchange between two different experiments (for instance, CSOM or evanescent field microscopy).

Microscope objectives. (1) Plan APO 60X oil immersion objective, NA = 1.4, working distance (WD) = 0.15 mm (PLAPO60XO, Olympus) for CSOM and objective-type TIRM. (2) U Plan APO 60X water immersion objective, NA = 1.2, WD = 0.25 mm (UPLAPO60XW, Olympus) for prism-based TIRM.

Intensified CCD camera. 512EFT intensified digital CCD camera system, Gen IV intensifier, 512×512 -pixel CCD, 5-MHz A/D converter (IPentaMAX-512EFT, Roper Scientific, Trenton, NJ) for TIRM.

Sample scanning stage. XYZ piezo flexure stage $200 \times 200 \times 20 \mu\text{m}$ with digital piezo-controller (P-527.3CL and E-710.3CL, Polytec PI, Germany) for sample scanning in CSOM. This can also be used for single-molecule manipulation experiments that require precise movement of the stage.

Fluorescence filters. Long-pass filter at 550 nm (E550LP) to reject laser scattered light (532 nm), dichroic mirror at 645 nm with reflection range 550–630 nm (645DCXR), and laser line filter at 532 nm (D532) for rejecting spontaneous emission from the laser. All filters are from Chroma Technology, Brattleboro, Vermont.

Prism. Small pellin broca prism (PLBC-5.0-79.5-SS, CVI laser) for prism-based TIRM.

Quartz microscope slides. Quartz microscope slides 1 in. \times 3 in. \times 1 mm (Finkenbeiner, Waltham) for prism-based TIRM.

Silicon avalanche photodiodes. Silicon avalanche photodiode photon counting unit (SPCM-AQ-14, Perkin-Elmer Optoelectronic Canada) for CSOM.

Counting board. Counter/Timer Board (PCI-6602, National Instruments). This computer board can count pulses from Perkin-Elmer detectors directly.

2. Biological Constructs, Probes, and Immobilizations

How Are Dye Molecules Attached to Nucleic Acids?

Since DNA or RNA oligonucleotides can be synthesized, it is relatively straightforward to attach dyes to them. A number of dyes are available in phosphoramidite form and can be readily incorporated during nucleic acid synthesis. Alternatively, amine-reactive dyes can be attached to an amino group introduced during synthesis. Then, dye-labeled nucleic acids can be purified from free dyes and unlabeled nucleic acids by using size-exclusion gel filtration, HPLC, or gel electrophoresis.

How Are Dye Molecules Attached to Protein?

It is commonplace to label proteins and antibodies with amine-reactive dyes because lysine residues are frequently found on protein surfaces. But, they are not practical for specific labeling of most proteins because there are too many lysine residues on their surface. Cysteine residues are much less common and “cysteine-light” proteins—proteins that contain reactive cysteines only at a desired position or positions—allow the attachment of thiol-reactive fluorophores to specific sites. Because introducing a second modification (chemically orthogonal to cysteine as far as dye labeling is concerned) to the protein is very difficult, a more practical approach is to have two reactive cysteine residues and label them with an equal mixture of donor and acceptor molecules. Single-molecule measurement should be able to distinguish donor–acceptor-labeled complex from other complexes labeled with donor–donor or acceptor–acceptor using their spectroscopic signatures. Two-step labeling by donor and then by acceptor, each step followed by the purification of unlabeled, singly labeled, and doubly labeled proteins, may be applicable to proteins that can withstand the purification procedures (33).

What Dyes Should Be Used as Donor and Acceptor?

Ideal dyes for single-molecule fluorescence studies have to possess as many as possible of the following characteristics. They have to (1) be photostable; (2) be bright (high extinction coefficient and quantum yield of emission); (3) show little intensity fluctuation, at least on the time scale of interesting biological events under study; (4) be excitable and emitting in the visible wavelength; (5) be relatively small so that they introduce minimum perturbation to the host molecule; and (6) be commercially available in a form that can be conjugated to biomolecules. In addition, the ideal pair of dyes for single-molecule FRET study would have (1) large spectral separation to minimize donor emission leakage into the spectral range of acceptor emission and to reduce the amount of direct excitation of the

acceptor by the laser, and (2) comparable emission quantum yield for donor and acceptor. The latter is useful because it guarantees clearly anticorrelated intensity changes of donor and acceptor when there are FRET changes.

Cy3 and Cy5 are a useful pair for single-molecule FRET because (1) their spectral separation is large (~100 nm), (2) they are both photostable in an oxygen-free environment, and (3) the quantum yields (~0.2) are comparable. Unfortunately, they are not commercially available in thiol-reactive form and it is necessary to perform chemical synthesis to label cysteine residues of proteins with Cy3 or Cy5. When Cy5 is used as the acceptor, even when other assays indicate the donor is very close to Cy5 ($R \ll R_0$) with 1:1 stoichiometry, there is a fraction of donor–acceptor complex that shows only donor emission. This is due presumably to the inactive Cy5, likely caused by prebleaching, and accounts for 15–55% of the total population depending on the batch of Cy5 used. Fortunately, for most experiments we could easily identify and disregard them because truly zero FRET values were not seen when two dyes were on the same host molecule. Nevertheless, it is an inconvenience and limits the distance resolution in the low FRET range.

Why and How Are Biological Molecules Immobilized?

The most exciting promise of single-molecule FRET is the observation of conformational dynamics of biological molecules (1). This requires a long observation time and hence some form of immobilization of the molecules. If performed improperly, immobilization can perturb the integrity of the molecule.

Biomolecules can be immobilized nonspecifically. For instance, DNA can be attached to a charged surface such as an aminopropylsilane-coated surface via electrostatic interaction (8). Even though this method avoids DNA aggregation and works in water, it is unlikely that the properties of DNA and its interaction with other molecules can be studied reliably under these conditions. We found that dyes on DNA immobilized in this way often display polarized emission, meaning they momentarily stick to the surface (34). This complicates the interpretation of FRET signal changes. Another nonspecific immobilization method successfully used for single-molecule fluorescence study is trapping molecules inside the pores formed in polyacrylamide (35) or agarose gel (6, 36). While gel immobilization has the merit of not requiring any special modification of the biomolecule, it has some disadvantages. First, the concentration of other small molecules such as enzyme substrates and ions is difficult to change in a short time. Sudden changes in the buffer conditions

are necessary for a certain type of single-molecule studies. Second, because of limited molecular diffusion, it is not easy to study interactions between macromolecules in gel.

Specific immobilization requires a well-defined modification of the biological molecule. For instance, a biotin or a digoxigenin can be attached to nucleic acids or protein to immobilize them to streptavidin- or antidigoxigenin-coated surfaces respectively (11, 12). Or, histidine tags that are typically introduced to help the purification of recombination proteins can be used to immobilize the protein on a Ni-NTA-coated surface (37). Specificity of biomolecule immobilization can be tested in a variety of ways. For instance, to check the specific immobilization of biotinylated DNA to a streptavidin-coated surface, control experiments can be performed (1) with the same DNA but without biotin, (2) without streptavidin, or (3) by presaturating streptavidin surface with free biotins. Below we describe a detailed procedure for preparing a mini-flow cell to immobilize biotinylated nucleic acids.

Two pieces of double-sided tape (3M) are attached to a bottom coverslip (24×30 mm) with a 5 mm gap between them. Another coverslip (18×18 mm) is attached to the tapes from above and thoroughly pressed. This defines a flow channel that is 5 mm wide, 18 mm long, and $100 \mu\text{m}$ tall. Both coverslips were cleaned thoroughly (see below) prior to the assembly. The channel is filled with 1 mg/ml biotinylated bovine serum albumin (BSA) (Sigma) solution in buffer A (Tris 10 mM, NaCl 50 mM, pH 8). BSA immediately adsorbs to the glass surfaces and makes a dense coating. After 10 min incubation, BSA solution is washed out by flowing through $100 \mu\text{l}$ of buffer A. Then, 0.2 mg/ml streptavidin (Molecular Probes, Eugene, OR) solution ($40 \mu\text{l}$) in buffer A is introduced, incubated for 10 min and washed out in the same manner. Finally, $40 \mu\text{l}$ of 50 pM biotinylated DNA or RNA solution in an appropriate buffer is added. This protocol allows the stepwise deposition of reagents without drying of the sample and typically results in a surface concentration of nucleic acids suitable for single-molecule imaging. Specificity of the immobilization was better than 500:1 as determined by control experiments described above. We have not found any evidence that biological integrity of nucleic acids is compromised on this surface.

Unfortunately, a BSA-based surface was not suitable for protein studies. Small proteins were easily denatured when attached to this surface (X. Zhuang, personal communication) and large proteins such as DNA helicases stick to the surface with high affinity. A working solution was found where the surface was densely coated by polyethylene glycol (PEG). PEG is known to reject protein adsorption to a surface if it forms a dense

coating. We used bifunctional PEG to immobilize nucleic acids specifically to a surface while rejecting protein adsorption. This approach works very well for the case of helicase–DNA interactions and S15 protein–RNA interactions and reproduces the bulk kinetics measured in solution studies (Ha *et al.*, unpublished data).

How Are Slides and Coverslips Cleaned?

Cleaning serves two purposes. First, fluorescent impurities on the glass/quartz surface are removed. Second, the surface is well primed for uniform affinity for immobilization of single-molecules. Impurities on the surface can give rise to fluorescent backgrounds and can present nonuniform local environments to biological molecules. The general protocol we used to clean slides or coverslips (called substrates from now on) follows:

Sonicate in (1) 30% detergent solution for 1 h, (2) distilled water for 5 min, (3) acetone for 15 min, (4) distilled water for 5 min, (5) 1 M KOH for 15 min, (6) ethanol for 15 min, (7) 1 M KOH for 15 min, and (8) distilled water for 15 min. Between each step, rinse substrates thoroughly with distilled water. Heating the substrates with a propane torch for a few seconds afterward can help destroy any organic impurities left over from the previous experiment if substrates are recycled.

Do Dyes Affect Biological Molecules?

Organic dyes may affect the biological activity of the host molecules. So far in our single-molecule fluorescence studies of small RNA junction, large RNA enzyme, and DNA unwinding by helicases (11, 12) (Ha *et al.*, unpublished data), we have not found evidence that dyes attached to nucleic acids affect the molecular reaction kinetics significantly. Recent single-molecule FRET experiments on folding–unfolding equilibrium distribution of chymotrypsin inhibitor 2 (18) are very encouraging because even for a small single-domain protein, attaching two bulky fluorophores did not compromise stability. In general, several alternative sites for dye attachment should be developed in parallel and the biological activity of the labeled molecules should be measured to choose the labeling scheme with minimum perturbation.

How Is Photobleaching Avoided?

Photobleaching is an inherent property of organic dyes and effectively limits the number of meaningful FRET value measurements on a single molecule. For instance, more than 10^5 photons can be detected from single molecules of Cy3 or Cy5 before bleaching. Since 100 photons are enough to give a signal/noise ratio of 10/1, adequate for most applications, 10^3 data points

can be obtained before photobleaching. This is enough for measuring multiple conformational changes. For very slow reactions, we can block the laser light periodically (for instance, one exposure of 100 ms every 5 s) to lengthen the observation time. Photobleaching is known to occur as a result of excited-state (probably from triplet state) reaction with highly reactive species in solution. Singlet oxygen is the prime culprit and, indeed, removing oxygen molecules from solution with an enzymatic oxygen scavenging system [we typically use 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 1% 2-mercaptoethanol, and 3% (w/w) glucose] can lengthen the photobleaching lifetime of several dyes (so far, the biggest changes have been from Cy5, where an up to 30-fold increase in photostability is seen; smaller but significant effects have been observed for Cy3 and tetramethylrhodamine; we found that tight sealing of the sample chamber from the ambient air is important). But, this is not general, and for other dyes, removing oxygen has only small effect on photostability (Texas Red and Alexa 488) or an adverse effect (fluorescence intensity is significantly reduced for fluorescein).

3. Analysis

How Is Single-Molecule FRET Efficiency Determined?

In a typical single-molecule FRET experiment, the single-molecule emission intensities at the donor detector and acceptor detector are measured simultaneously (I_{D_0} and I_{A_0}). These raw intensities (I_{D_0} and I_{A_0}) are integrated in time according to the desired time resolution. For diffusing molecules in solution, all the photons emitted in a burst while the molecule is diffusing through the excitation volume are summed to obtain I_{D_0} and I_{A_0} . To distinguish a real molecular signal from noise, a threshold level for the total intensity is set. Only when the signal level is above the threshold level, is it included in the subsequent analysis. No further selection of molecules is needed for distribution analysis. For dynamic analysis, a fraction of molecules that show photobleaching of donor or acceptor before real dynamics occur are not analyzed. However, even these molecules are included for calculating the frequency of rare conformational changes.

To calculate FRET efficiency, raw intensities are processed in the following way. I_{A_0} contains the leakage of donor emission into the donor detector and also the acceptor emission due to the direct excitation by the laser. The latter is negligibly small for the case of the Cy3–Cy5 pair, but the former can be between 8 and 15% of I_{D_0} depending on the fluorescence filters used. Its ratio to I_{D_0} , β , is easily measured from the constructs

that contain only donors. Then, corrected donor and acceptor intensities, I_D and I_A , are determined using $I_D = (1 + \beta)I_{D_0}$ and $I_A = I_{A_0} - \beta I_{D_0}$. A general expression for FRET efficiency E is $[1 + I_D^0 \phi_A / I_A^0 \phi_D]^{-1}$, where I_D^0 and I_A^0 are the true donor intensity and the sensitized emission intensity of the acceptor in the presence of energy transfer and ϕ_D and ϕ_A are quantum yields of donor and acceptor emission, respectively. I_D and I_A are reduced from I_D^0 and I_A^0 by factors of η_D and η_A because of overall instrument detection efficiencies. Even though ϕ_D , ϕ_A , η_D , and η_A can all be measured experimentally, we chose to set $\eta_A \phi_A / \eta_D \phi_D = 1$; hence $E = [1 + I_D / I_A]^{-1}$, for convenience. Single-molecule FRET studies concern mostly relative changes so this approximation does not affect most of the data interpretation. Furthermore, errors introduced in this approximation will likely have a smaller effect on the absolute distance estimation than potential errors associated with the determination of R_0 .

How Significant Are the Orientational Effects of the Dyes?

Since the orientation factor κ^2 is difficult to determine experimentally, the absolute distance determination by FRET can be hindered. In general, single-molecule FRET focuses more on the dynamic aspects, that is, relative distance changes. Nevertheless, a concern remains regarding the origin of the changes in FRET measured in single-molecule experiments because it is, in principle, possible that FRET signal change arises purely from the changes in the way dyes are interacting with the host molecule. There are two ways to address this issue.

In the first (“physical control”), one can measure fluorescence anisotropy at the ensemble level in solution. If anisotropy value is low, it can be concluded that the dye does not interact strongly with the host molecule and rotates relatively freely around it. However, a transient interaction between the dye and the host may not be detected by ensemble study and surface immobilization may introduce additional rigidity to the dye’s rotation relative to the host or the surface. Therefore, it is important to measure the polarization response of individual dye molecules when the host molecules are immobilized. When all these tests indicate that dyes do not stick to the host molecule or surface even transiently under the condition of single-molecule FRET measurement, it is reasonable to assume that observed FRET changes are not due to purely orientational effects of the dyes.

Perhaps more straightforward is the second method (“biological control”). One can demonstrate the biological origin of the observed FRET change by changing the biological parameters and observing how FRET signal

changes accordingly. For instance, to establish that FRET signal change of RNA with a protein binding motif is due to protein binding and dissociation, a titration study can be performed by varying the protein concentration in solution (11). In general, it often does not matter whether FRET signal change is due purely to distance change or has an orientational effect as long as the biological origin of the change can be identified.

What Type of Photophysical Effects Can Complicate the Interpretations?

Dyes are not ideal emitters and are known to show intermittence, that is, transitions between bright and dark periods (25, 38). The “on” time, the average time for which the emission is on between successive on and off transitions, is dependent on excitation intensity. In single-molecule FRET, the dark period of donor can be simply disregarded since it will result in the total annihilation of emission during which no information about the host molecule can be obtained. In contrast, the dark period of acceptor will result in an increase in donor emission and can be mistaken as a large conformational change that brings two dyes completely out of FRET range (39). Here, an excitation intensity dependence study can distinguish photoinduced transitions of the acceptor from spontaneous changes of the host molecule. A more direct approach uses another laser source to excite the acceptor directly to test if the acceptor is fluorescently active.

How Small a Distance Change Can Be Detected?

Because of uncertainties in determining R_0 , FRET is more powerful in detecting relative changes in distance than in determining absolute distance. Then, how small a distance change can be detected using single-molecule FRET? A simple estimate is given below based on signal/noise ratio arguments alone.

Because the distance dependence of FRET is the strongest when $R \sim R_0$, the best sensitivity can be obtained near $E = 0.5$ (Eq. [1] and Fig. 1). The precision with which we can determine R depends on the signal/noise ratio (S/N) for I_A and I_D . Often, the dominant noise source is the statistical noise due the finite number of photons used. In this case, S/N can exceed 20. Then, error propagation analysis of the inverted form of Eq. [1] and $E = I_A/(I_A + I_D)$, a good approximation for E (see above), yield 1 Å uncertainty for R when $R \sim R_0$ assuming $R_0 = 50$ Å. S/N drops to 5 if an intensified CCD camera is used because the intensifier adds noise. Even in this case, distance changes of 5 Å, in principle, can be detected from single molecules if they occur around R_0 . Whether such small changes can be detected experimentally remains to be seen. Also, this analysis ignores the intrinsic noise from the dye molecules such

as changes in quantum yield or spectrum. Only very large distance changes will be detected in the flatter part of Eq. [1]. A detailed analysis of S/N versus distance resolution is found in an earlier publication (40).

What Is The Time Resolution?

The time resolution of a single-molecule FRET study is determined by factors that limit the photon emission rate. Fluorescence signal reaches saturation due to the shelving of the molecule onto the triplet state. Triplet-state lifetime is in the microsecond range in water. However, if oxygen molecules are removed to extend the photobleaching lifetime of the dyes, the triplet-state lifetime increases up to 1 ms. Therefore, under high excitation intensity under oxygen-deficient conditions, single-molecule emission becomes intermittent (41, 42) and it becomes unsuitable for measuring biological events occurring on a similar or faster time scales. Therefore, the best time resolution obtainable from single-molecule FRET study is about 1 ms unless a new chemical reagent is discovered that quenches the triplet state specifically without changing the photobleaching properties and the emission properties of the dyes.

ACKNOWLEDGMENTS

We thank Shimon Weiss and Tom Kerppola for helpful comments on the manuscript and past and current collaborators for their roles in formulating the ideas of single-molecule fluorescence experiments and performing them.

REFERENCES

- Weiss, S. (1999) *Science* 283, 1676–1683.
- Moerner, W. E., and Orrit, M. (1999) *Science* 283, 1670.
- Nie, S. M., and Zare, R. N. (1997) *Ann. Rev. Biophys. Biomol. Struct.* 26, 567–596.
- Tamarat, P., Maali, A., Lounis, B., and Orrit, M. (2000) *J. Phys. Chem.* 104, 1–16.
- Schmidt, T., Schutz, G. J., Baumgartner, W., Gruber, H. J., and Schindler, H. (1995) *J. Phys. Chem.* 99, 17662–17668.
- Lu, H. P., Xun, L. Y., and Xie, X. S. (1998) *Science* 282, 1877–1882.
- Ha, T., Laurence, T. A., Chemla, D. S., and Weiss, S. (1999) *J. Phys. Chem. B* 103, 6839–6850.
- Ha, T., Enderle, T., Ogletree, D. F., Chemla, D. S., Selvin, P. R., and Weiss, S. (1996) *Proc. Natl. Acad. Sci. USA* 93, 6264–6268.
- Ha, T. J., Ting, A. Y., Liang, J., Caldwell, W. B., Deniz, A. A., Chemla, D. S., Schultz, P. G., and Weiss, S. (1999) *Proc. Natl. Acad. Sci. USA* 96, 893–898.
- Deniz, A. A., Dahan, M., Grunwell, J. R., Ha, T. J., Faulhaber, A. E., Chemla, D. S., Weiss, S., and Schultz, P. G. (1999) *Proc. Natl. Acad. Sci. USA* 96, 3670–3675.
- Ha, T., Zhuang, X. W., Kim, H. D., Orr, J. W., Williamson, J. R., and Chu, S. (1999) *Proc. Natl. Acad. Sci. USA* 96, 9077–9082.

12. Zhuang, X. W., Bartley, L. E., Babcock, H. P., Russell, R., Ha, T. J., Herschlag, D., and Chu, S. (2000) *Science* 288, 2048.
13. Brasselet, S., Peterman, E. J. G., Miyawaki, A., and Moerner, W. E. (2000) *J. Phys. Chem. B* 104, 3676–3682.
14. Jia, Y. W., Talaga, D. S., Lau, W. L., Lu, H. S. M., DeGrado, W. F., and Hochstrasser, R. M. (1999) *Chem. Phys.* 247, 69–83.
15. Ishii, Y., Yoshida, T., Funatsu, T., Wazawa, T., and Yanagida, T. (1999) *Chem. Phys.* 247, 163–173.
16. Sako, Y., Minoghchi, S., and Yanagida, T. (2000) *Nat. Cell Biol.* 2, 168–172.
17. Schutz, G. J., Trabesinger, W., and Schmidt, T. (1998) *Biophys. J.* 74, 2223–2226.
18. Deniz, A. A., Laurence, T. A., Beligere, G. S., Dahan, M., Martin, A. B., Chemla, D. S., Dawson, P. E., Schultz, P. G., and Weiss, S. (2000) *Proc. Natl. Acad. Sci. USA* 97, 5179–5184.
19. Ying, L. M., Wallace, M. I., Balasubramanian, S., and Klenerman, D. (2000) *J. Phys. Chem. B* 104, 5171–5178.
20. Weiss, S. (2000) *Nat. Struct. Biol.* 7, 724–729.
21. Forster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.), pp. 93–137, Academic Press, New York.
22. Stryer, L., and Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. USA* 58, 719–726.
23. Selvin, P. R. (2000) *Nat. Struct. Biol.* 7, 730–734.
24. Betzig, E. (1993) *Science* 262, 1422.
25. Ambrose, W. P., Goodwin, P. M., Martin, J. C., and Keller, R. A. (1994) *Phys. Rev. Lett.* 72, 160–163.
26. Nie, S. M., Chiu, D. T., and Zare, R. N. (1994) *Science* 266, 1018–1021.
27. Macklin, J. J., Trautman, J. K., Harris, T. D., and Brus, L. E. (1996) *Science* 272, 255–258.
28. Sase, I., Miyata, H., Corrie, J. E. T., Craik, J. S., and Kinosita, K. (1995) *Biophys. J.* 69, 323–328.
29. Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., and Yanagida, T. (1995) *Nature* 374, 555–559.
30. Tokunaga, M., Kitamura, K., Saito, K., Iwane, A. H., and Yanagida, T. (1997) *Biochem. Biophys. Res. Commun.* 235, 47–53.
31. Suzuki, K., Tanaka, Y., Nakajima, Y., Hirano, K., Itoh, H., Miyata, H., Hayakawa, T., and Kinosita, K. (1995) *Biophys. J.* 68, 739–748.
32. Sase, I., Miyata, H., Ishiwata, S., and Kinosita, K. (1997) *Proc. Natl. Acad. Sci. USA* 94, 5646–5650.
33. Lillo, M. P., Beechem, J. M., Szpikowska, B. K., Sherman, M. A., and Mas, M. T. (1997) *Biochemistry* 36, 11261–11272.
34. Ha, T., Glass, J., Enderle, T., Chemla, D. S., and Weiss, S. (1998) *Phys. Rev. Lett.* 80, 2093–2096.
35. Dickson, R. M., Norris, D. J., Tzeng, Y. L., and Moerner, W. E. (1996) *Science* 274, 966–969.
36. Dickson, R. M., Cubitt, A. B., Tsien, R. Y., and Moerner, W. E. (1997) *Nature* 388, 355–358.
37. Noji, H., Hasler, K., Junge, W., Kinosita, K., Yoshida, M., and Engelbrecht, S. (1999) *Biochem. Biophys. Res. Commun.* 260, 597–599.
38. Xie, X. S., and Dunn, R. C. (1994) *Science* 265, 361–364.
39. Ha, T. J., Ting, A. Y., Liang, J., Deniz, A. A., Chemla, D. S., Schultz, P. G., and Weiss, S. (1999) *Chem. Phys.* 247, 107–118.
40. Dahan, M., Deniz, A. A., Ha, T. J., Chemla, D. S., Schultz, P. G., and Weiss, S. (1999) *Chem. Phys.* 247, 85–106.
41. Ha, T., Enderle, T., Chemla, D. S., Selvin, P. R., and Weiss, S. (1997) *Chem. Phys. Lett.* 271, 1–5.
42. Veerman, J. A., Garcia-Parajo, M. F., Kuipers, L., and van Hulst, N. F. (1999) *Phys. Rev. Lett.* 83, 2155–2158.