S

Single-Molecule Fluorescence Resonance Energy Transfer

Alexander E. Johnson-Buck[1], Mario R. Blanco[2] and Nils G. Walter[1]
[1]Department of Chemistry, The University of Michigan, Ann Arbor, MI, USA
[2]Department of Cellular and Molecular Biology, The University of Michigan, Ann Arbor MI, USA

Synonyms

Fluorescence resonance energy transfer (FRET); Single-molecule Förster resonance energy transfer; Single-pair fluorescence resonance energy transfer

Definition

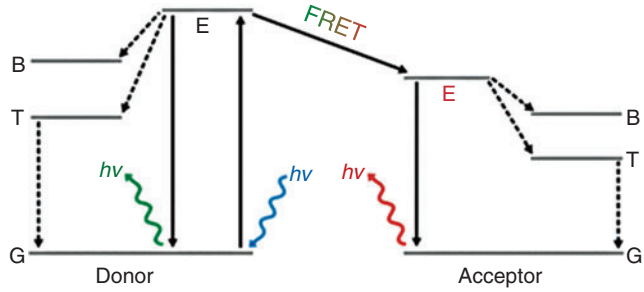
Single-molecule fluorescence resonance energy transfer (smFRET) is a technique used to measure nanometer-scale distances between specific sites on an individual molecule, usually as a function of time.

Introduction

In this entry, we review smFRET, a powerful technique for measuring distances and monitoring dynamics at the molecular scale. In this technique, the researcher monitors distances between two or more individual fluorescent labels by measuring how efficiently electronic energy is transferred between them, a phenomenon known as Förster resonance energy transfer (FRET). Its power lies in its ability to detect distance changes as small as ~0.3 nm in individual molecules (or pairs of molecules) within a heterogeneous population (Roy et al. 2008). Although it requires a specialized fluorescence microscope, smFRET is employed in many laboratories worldwide and has been used to address questions about topics ranging from intermolecular interactions to macromolecular folding and catalysis.

Although smFRET has only been technically possible since the mid-1990s, its theoretical foundations were laid much earlier through the pioneering work of Theodor Förster in the 1940s and through others who elucidated the distance dependence of FRET and its use as a molecular ruler (Stryer 1978). A separate branch of inquiry, the first single-molecule measurements of ion channels using the patch clamp technique, already established some of the core aspects of analyzing time-lapsed recordings from individual molecules in the 1970s and early 1980s (Sakmann and Neher 2009). By the mid-1990s, improvements in fluorescence detection brought about by total internal reflection fluorescence microscopy made it possible to measure the weak emission of single organic fluorophores under ambient conditions and, soon thereafter, smFRET was realized (Ha et al. 1996).

G. C. K. Roberts, A. Watts (eds.), *Encyclopedia of Biophysics*,
https://doi.org/10.1007/978-3-642-35943-9_492-1



Single-Molecule Fluorescence Resonance Energy Transfer, Fig. 1 *Simplified electronic diagram of FRET. Solid lines* represent rapid electronic transitions, and *dashed lines* represent slow transitions. A FRET donor can be excited from its ground state (G) to an excited state (E) by a photon of energy *hv* (*blue*). The excited donor can then return to the ground state, emitting a photon of lower energy (i.e., it can fluoresce, *green*); enter a nonfluorescent triplet state (T); permanently photobleach (B); or donate its energy to a nearby acceptor molecule by FRET. The excited acceptor can then similarly emit a photon (*red*), enter a triplet state, or photobleach

We begin with a discussion of the theory of FRET as it applies to single-molecule experiments, followed by a brief description of a typical smFRET experiment, including the necessary equipment and materials. We then discuss some of the major insights gained from smFRET, as well as some of its strengths and limitations. Finally, we conclude by commenting on recent and ongoing developments in the field.

Photophysical Basis of smFRET

In 1948, Förster developed the theory governing the non-radiative transfer of energy from one electronically excited molecule to another nearby molecule. When a fluorescent molecule (fluorophore) is excited by a photon of the proper energy, it returns to the ground state via one of several possible pathways: It can dissipate its energy by emitting a photon, by transferring energy to other molecules without emitting a photon (non-radiatively), or by reacting chemically (Fig. 1). In FRET, one form of non-radiative energy transfer, the excited "donor" fluorophore transfers its excitation energy to a nearby "acceptor" fluorophore through an electronic dipole–dipole interaction. The excited acceptor may then return to its ground state via one of various pathways, including by emitting a photon (fluorescence).

For FRET to occur efficiently, the fluorescence emission spectrum of the donor must overlap considerably with the absorption spectrum of the acceptor, i.e., the fluorophores must be in resonance (Fig. 2), the two molecules must be within a certain distance of one another (typically <10 nm), and their transition dipole moments must be in (partial) alignment. At the same time, their absorption spectra should be separate enough that the donor can be excited with high specificity, and their emission spectra sufficiently separate to ensure specific detection of both donor and acceptor.

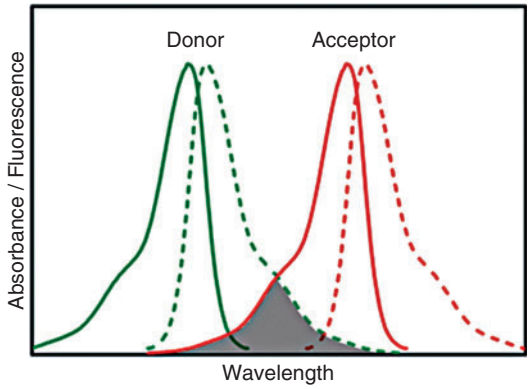
Since FRET is a dipole–dipole interaction, its efficiency E depends on the sixth power of the separation r between the donor and acceptor as:

$$E = r^{-6}/\left(r^{-6} + R_0^{-6}\right) \tag{1}$$

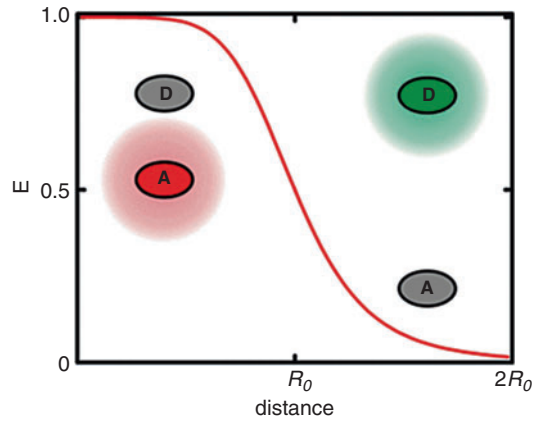
where the Förster radius R_0, corresponding to the separation at which energy transfer is 50% efficient, is:

$$R_0 = \left(J\kappa^2 Q_0 n^{-4}\right)^{1/6} \times 9.7 \times 10^3 \text{ Å} \tag{2}$$

The value E, which can be calculated from the relative fluorescence of the donor and acceptor (see "Data Processing and Analysis," below), thus reports on the distance between the two fluorophores. The value of R_0 depends on the overlap integral J between the donor emission



Single-Molecule Fluorescence Resonance Energy Transfer, Fig. 2 *Desirable spectral properties for a FRET donor–acceptor pair.* For specific fluorescent excitation and detection, the absorption spectra (*solid curves*) of the donor and acceptor should overlap little, as should their emission spectra (*dashed curves*). However, for efficient FRET to occur, the emission spectrum of the donor should overlap considerably with the absorption spectrum of the acceptor



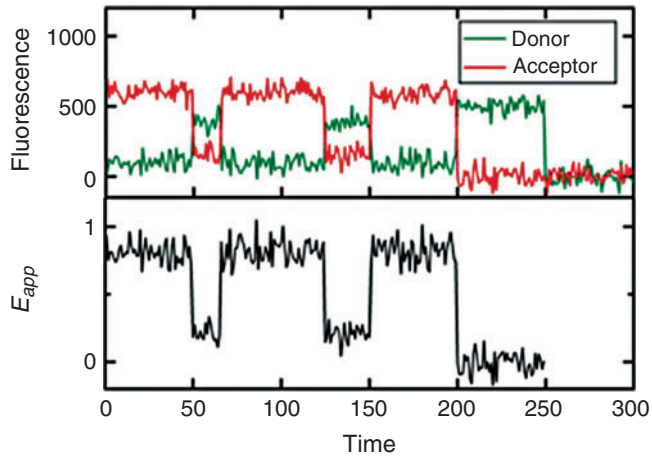
Single-Molecule Fluorescence Resonance Energy Transfer, Fig. 3 *Distance dependence of FRET.* Efficiency of energy transfer (E) is a steep function of distance at values near R_0

and acceptor excitation spectra, the so-called orientation factor κ^2 describing the relative orientations of the donor and acceptor, the quantum yield Q_0 of donor fluorescence in the absence of acceptor, and the refractive index n of the medium in which the interaction takes place. If the fluorophores are freely rotating at a rate faster than the excited state lifetime of the donor, $\kappa^2 = 2/3$, and R_0 is constant for a given donor–acceptor pair in aqueous solution. This can be confirmed for a particular system by measuring the fluorescence anisotropy of both fluorophores.

Experimental Design and Data Acquisition

In order to use smFRET to measure conformational changes or interactions between molecules, the molecule(s) of interest are labeled, usually at specific sites, with donor and acceptor fluorophores that report on a distance of interest. The ideal fluorophore is stable under high photon flux, has high molar absorptivity and fluorescence quantum yield, and undergoes minimal "blinking" (spontaneous excursions into nonfluorescent states). The fluorophores used are generally small (<1 nm) organic molecules, the two most common being the cyanine dyes Cy3 and Cy5, though the Alexa Fluor and ATTO series of dyes appear comparable. Quantum dots and fluorescent proteins can also be used in smFRET, but their use has been more difficult to implement because they are larger and, in the case of fluorescent proteins, less photostable (Roy et al. 2008). Positions of the fluorophores should ideally be chosen such that the distance r between them is close to the Förster radius since that is where FRET efficiency is most sensitive to changes in r (Fig. 3).

All organic fluorophores eventually photobleach, permanently losing their fluorescence properties through reaction with molecular oxygen (Figs. 1 and 4). This is useful because the instantaneous loss of fluorescence signal upon bleaching is evidence that the fluorescence originates from a single molecule. However, because photobleaching also reduces the time window of observation, it is often desirable to delay it as much as possible. So-called oxygen scavenging systems reduce the concentration of oxygen in solution by catalyzing its reaction with substrates other than the FRET donor and acceptor. The most common systems use either: (1) the enzymes glucose oxidase and catalase in combination with the substrate glucose or (2) the enzyme



Single-Molecule Fluorescence Resonance Energy Transfer, Fig. 4 *Simulated typical FRET time trace of a single molecule*. The fluorescence intensity counts of the donor and acceptor change in a discrete, anticorrelated fashion, reporting on underlying molecular distance changes that are detected as transitions between high and low FRET efficiency (E_{app}). Upon photobleaching, the donor and acceptor intensity counts instantaneously fall to ~0, evidence that the signal originated from a single FRET pair

protocatechuate dehydrogenase and its substrate, protocatechuic acid. A related problem is blinking, a term used for temporary non-fluorescence caused by excursions to kinetically trapped triplet states (Fig. 1). When oxygen, a good triplet state quencher, is removed from solution using a scavenger system, other additives (Trolox, β-mercaptoethanol) are often employed to dramatically reduce blinking.

Observation of the weak fluorescence signal from single molecules requires: (1) high-powered illumination, (2) means of reducing or rejecting background fluorescence that would otherwise greatly diminish the signal-to-noise ratio, and (3) sensitive detection. To meet requirement (1), the illumination is almost always provided by the high-powered, monochromatic light of lasers. Requirement (2), the reduction of background fluorescence, is usually achieved by exciting only a small volume of the sample by means of one of the following illumination schemes: total internal reflection fluorescence (TIRF), confocal, highly inclined, and laminated optical sheet (HILO), near-field scanning optical (NSOM), or zero-mode waveguides (Walter et al. 2008). Most common of these illumination schemes is total internal reflection fluorescence (TIRF), which reduces background fluorescence by illuminating only that part of the sample that is within ~100 nm of the surface of the microscope slide or coverslip. This requires immobilizing the molecules of interest at the illuminated interface, which is usually achieved using specific, high-affinity binding such as the streptavidin–biotin interaction. To resolve single molecules, they must be immobilized from a very dilute (~100 pM) solution, resulting in a surface density no larger than about 0.2 μm^{-2}. Surface immobilization has the added benefit of allowing one to observe the same molecule over several seconds, minutes, or even hours. Finally, requirement (3), sensitive detection, is usually provided by an electron multiplying charge-coupled device (EMCCD) camera in the case of wide-field illumination, such as TIRF, or avalanche photodiodes in the case of point-detection schemes like confocal microscopy.

An smFRET experiment also requires optics for filtering out stray excitation light and directing fluorescence from the donor and acceptor into separate detection channels. Scattered excitation light is removed from the detection path using high-optical density filters that only transmit certain frequency bands. Separation of donor and acceptor signals is accomplished using dichromatic (dichroic) mirrors, which reflect specific frequencies of light and transmit others.

Additional mirrors are then used to direct the emission signal onto the detector(s) so that the donor and acceptor emission can be measured simultaneously.

Data Processing and Analysis

Processing of smFRET data from raw camera movies involves: (1) locating and matching corresponding donor and acceptor signals in the field of view, (2) matching each donor signal with its acceptor signal (channel registration), and (3) determining the FRET efficiency as a function of time for each molecule throughout the movie (Fig. 4). Since the donor and acceptor channels of the CCD image do not typically have a linear correspondence, they are generally mapped using a higher-order polynomial transformation to ensure correct assignment of donor–acceptor pairs. Usually so-called fiduciary markers (such as fluorescent beads) that are visible in both channels are used to establish this mapping.

The apparent FRET efficiency E_{app} can be calculated as:

$$E_{app} = \frac{I_A}{I_A + I_D \times \gamma} \tag{3}$$

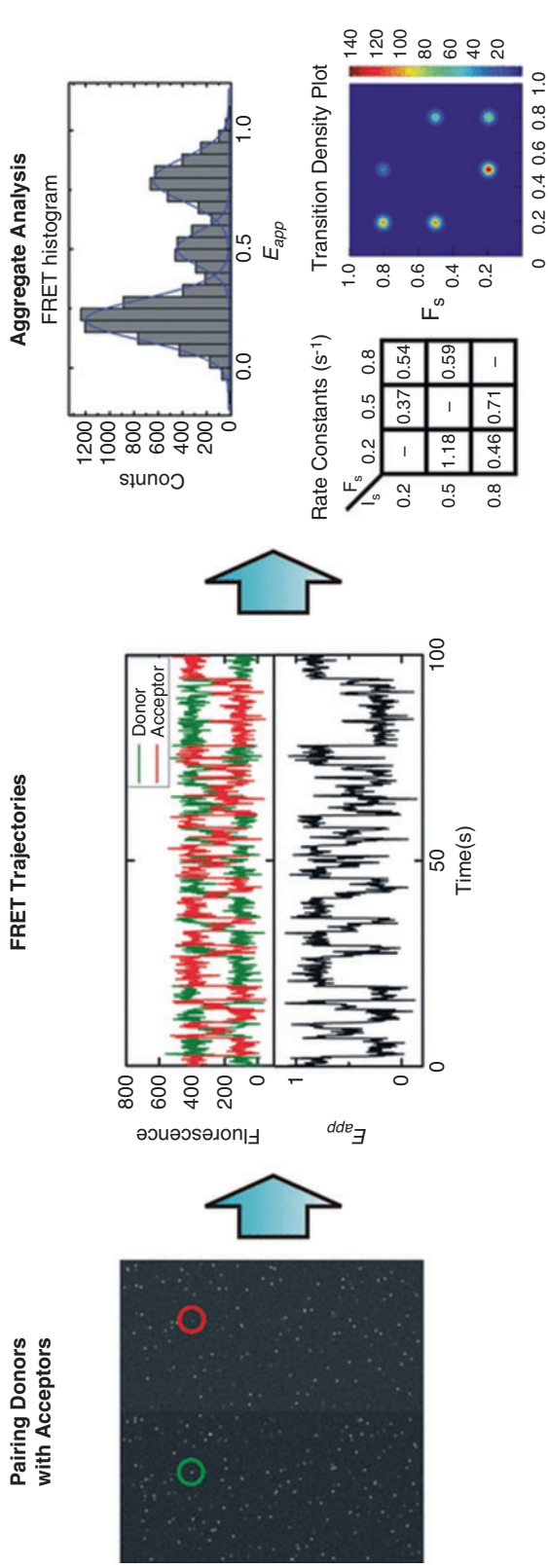
where I_D and I_A are the total number of photons emitted per movie frame by the donor and acceptor, respectively. The parameter γ, which depends on the relative quantum yields and detection efficiencies of the donor and acceptor, can be calculated as $\gamma = \left|\frac{\Delta I_A}{\Delta I_D}\right|$, where ΔI_A and ΔI_D are the changes in intensity of the acceptor and donor intensity upon photobleaching of the acceptor. If the Förster radius is known and if anisotropy of both fluorophores can be ruled out (see "Insights, Strengths, and Limitations," below), E_{app} can be related to absolute distances according to Eq. (1). If not, E_{app} still gives an estimate as to the relative distances between donor and acceptor in different FRET states (Roy et al. 2008).

Analysis of the E_{app} versus time data varies greatly depending on the behavior of the sample. The typically low signal-to-noise ratios of single-molecule detection often make it challenging to determine the number and values of different FRET states in a given molecule. To facilitate the assignment of FRET states, hidden Markov modeling (HMM) or nonlinear filters may be applied. Histograms of FRET efficiency are usually generated from the time traces of hundreds of molecules under the same conditions and can often be fit with multiple Gaussian distributions to estimate the number of states and their values of E_{app} (Fig. 5), thus providing information about the equilibrium properties of the system (Roy et al. 2008).

If the molecules show transitions between discrete E_{app} values over time (i.e., dynamics), either HMM or analysis of the time spent in each state (dwell times) can be used to estimate the underlying kinetics of the system (Fig. 5). Genuine FRET transitions are characterized by inversely proportional changes in acceptor and donor signal intensity, or anticorrelation (Figs. 4 and 5), a property that can be used to filter out spurious transitions due to other photophysical phenomena such as changes in the local environment of one of the fluorophores. Transition density plots, which simultaneously display the probability of all possible transitions between different FRET states, are often compiled based on HMM to provide a global view of the observed dynamics. If certain transitions have slow kinetics or appear only infrequently in time traces, evidence of them may be suppressed by faster (more frequent) transitions; in this case, the probability that a transition occurs *at least once* within a given molecule may instead be plotted (Blanco and Walter 2010).

Insights, Strengths, and Limitations

Single-molecule FRET has several strengths. First, the steep distance dependence of E_{app} near R_0 and specificity of the energy transfer between donor and acceptor make it possible to monitor very specific interactions and events. Second, the ratiometric property of smFRET renders it one of the most sensitive and robust single-molecule techniques. Third, when using appropriate oxygen scavengers, blinking suppressants, and



Single-Molecule Fluorescence Resonance Energy Transfer, Fig. 5 *Schematic workflow of smFRET analysis for a simulated three-state system.* Each acceptor (one of which is encircled in *red*) is paired with its corresponding donor molecule (*circled in green*) in the CCD image (*left*). Using the intensity of donor and acceptor in each movie frame, the apparent FRET efficiency E_{app} is calculated in each frame and plotted as a function of time (*middle*). An aggregate analysis of typically hundreds of molecules yields thermodynamic information, such as a histogram of the population distribution of the various FRET states, as well as first-order rate constants and a probability plot of transitions from each FRET state (I_S) to each other state (F_S) (*right*)

acquisition hardware, smFRET can be used to monitor molecular events occurring on timescales ranging from ~1 ms to hours. Because of these properties, smFRET is particularly well suited to monitoring conformational changes in single immobilized molecules over long periods of time, but has been used to study phenomena ranging from the dynamics of motor proteins and RNA enzymes to structural transitions in DNA nanomachines and transient intermolecular interactions.

Perhaps the greatest advantage of smFRET, as with ▶ "Single-Molecule Spectroscopy" in general, lies in its ability to discern heterogeneous behavior within a population of molecules at equilibrium without the need for rapid mixing or other synchronization. In studies of conformational dynamics in protein and RNA enzymes, smFRET has repeatedly revealed heterogeneous behavior, that is, the kinetics of conformational transitions varies over time or from molecule-to-molecule. Some molecules even appear to have multiple native states with the same activity but subtly different structures that do not interconvert. This stands in contrast with the classical view of macromolecules as having a unique native state with a single, well-defined structure and behavior (Hwang et al. 2009).

There are also several limitations to consider with smFRET. First, and perhaps most fundamentally, this technique generally reports on only a single dimension of interest, though strides have been made toward extending it to two or more dimensions (see "Recent Developments and Extensions of smFRET," below). Due to this fact, data from smFRET experiments must be carefully interpreted in light of all available structural and functional information about the system, such as that obtained by X-ray crystallography, mutational studies, and structural footprinting. Second, the millisecond time resolution is too slow to detect some important molecular dynamics. Third, only $\sim 10^5$ photons can be collected from even very stable organic fluorophores before photobleaching occurs so that a finite number of observations can be made on a given molecule. Furthermore, even with the addition of antiblinking agents such as Trolox, the acceptor may occasionally enter a dark state, giving the appearance of an excursion to a state with $E_{app} = 0$; such dark states should be excluded from analysis or their influence quantified using controls (Benítez et al. 2010).

Another concern is that rotational constraint of the donor or acceptor fluorophore can interfere with accurate distance measurements; for instance, common organic fluorophores have been shown to stack at the ends of nucleic acid duplexes, influencing the apparent FRET efficiency. To reduce the likelihood of such interactions, a short flexible organic linker (generally an alkyl moiety) can usually be added between the molecule of interest and each fluorophore. Still, if accurate absolute distance information is required, fluorescence anisotropy measurements are needed to ensure that the anisotropy values of the donor and acceptor are acceptably low (generally <0.2). If only relative distance information is needed, higher anisotropy values are tolerable since E_{app} is still generally a monotonic function of distance (Roy et al. 2008). Finally, it is important to verify that the surface immobilization, if used, does not perturb the behavior of interest; this can be done by comparing results with those of ensemble FRET assays in solution, conducting single-molecule activity assays, or comparing results using different immobilization strategies.

Recent Developments and Extensions of smFRET

As the complexity and heterogeneity of molecular dynamics becomes more apparent, efforts are in progress to extend the capabilities of smFRET and combine it with other techniques for a more comprehensive real-time picture of molecular events. For instance, three-color smFRET has been developed to simultaneously monitor the distances between a single donor and two distinct acceptor fluorophores (Roy et al. 2008). A challenge in extending such approaches to measure a larger number of distances is finding multiple acceptors whose emission spectra are sufficiently distinct to resolve their signals. To partially bypass this issue, switchable smFRET was developed to

monitor energy transfer from one donor to multiple acceptors by photochemically switching each acceptor on and off in succession (Uphoff et al. 2010).

Recently, smFRET has also been used in conjunction with other single-molecule techniques, as proposed by Shimon Weiss (Weiss 1999). For example, a combination of smFRET and electrical recording was used to monitor dimerization of single ion channels, thus simultaneously providing structural and functional information. Optical or magnetic trapping has been used to manipulate single molecules while monitoring their dynamics by smFRET (Hwang et al. 2009).

Finally, efforts are in progress to perform smFRET measurements in vivo to study the behavior of molecules in their native environment. Such efforts are complicated by the significant background autofluorescence within the cell, as well as the limited ability to control the photophysics of fluorophores in vivo, as is done in vitro through oxygen scavenging and additives. Nevertheless, smFRET has been employed to study a small number of intracellular systems, including protein–protein interactions at the cell membrane, where TIRF illumination can significantly reduce background fluorescence. A step in that direction can also be the immunoprecipitation from a cell extract of a biomolecular assembly of interest in which two fluorophores are judiciously placed, a technique that has been coined single-molecule pulldown FRET or SiMPull-FRET (Krishnan et al. 2013).

Summary

Single-molecule FRET is a powerful technique with the unique ability to monitor dynamic processes in single molecules over distances of <10 nm and timescales of milliseconds to hours. It exploits the steep distance dependence ($\sim r^6$) of Förster energy transfer and high sensitivity of ratiometric fluorescence detection to measure the distance between specific molecular sites over time in single molecules. Although it requires some specialized equipment, smFRET is now widely used and has revealed kinetics, mechanistic details such as transiently visited states, and heterogeneous behaviors that are masked in traditional assays by ensemble averaging.

Cross-References

- CNS (Crystallography and NMR System)
- Fluorescence and FRET in Membranes
- Fluorescence Labeling of Nucleic Acids
- Helicases
- Hidden Markov Modeling in Single-Molecule Biophysics
- Magnetic Tweezers
- Optical Tweezers
- Patch-Clamp Recording of Single Channel Activity: Acquisition and Analysis
- Protein Fluorescent Dye Labeling
- Single Fluorophore Blinking
- Single Fluorophores Photobleaching
- Single-Molecule Methods
- Single-Molecule Spectroscopy
- Total Internal Reflection Fluorescence Microscopy for Single-Molecule Studies

References

Benítez JJ, Keller AM, Chen P (2010) Nanovesicle trapping for studying weak protein interactions by single-molecule FRET. Methods Enzymol 472:41–60

Blanco M, Walter NG (2010) Analysis of complex single molecule FRET time trajectories. Methods Enzymol 472:153–178

Ha T, Enderle T, Ogletree DF, Chemla DS, Selvin PR, Weiss PR (1996) Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. Proc Natl Acad Sci U S A 93:6264–6268

Hwang LC, Hohlbein J, Holden SJ, Kapanidis AN (2009) Single-molecule FRET: methods and biological applications. In: Hinterdorfer P, van Oijen A (eds) Handbook of single-molecule biophysics, vol 1. Springer, Dordrecht, pp 129–163

Krishnan R, Blanco MR, Kahlscheuer ML, Abelson J, Guthrie C, Walter NG (2013) Biased Brownian ratcheting leads to pre-mRNA remodeling and capture prior to first-step splicing. Nat Struct Mol Biol 20:1450–1457

Roy R, Hohng S, Ha T (2008) A practical guide to single-molecule FRET. Nat Methods 5:507–516

Sakmann BA, Neher E (eds) (2009) Single channel recording, 2nd edn. Springer, New York

Stryer L (1978) Fluorescence energy transfer as a spectroscopic ruler. Annu Rev Biochem 47:819–846

Uphoff S, Holden SJ, Le Reste L, Periz J, van de Linde S, Heilemann M, Kapanidis AN (2010) Monitoring multiple distances within a single molecule using switchable FRET. Nat Methods 7:831–836

Walter NG, Huang C-Y, Manzo AJ, Sobhy MA (2008) Do-it-yourself guide: how to use the modern single-molecule toolkit. Nat Methods 5:475–489

Weiss S (1999) Fluorescence spectroscopy of single biomolecules. Science 283:1676–1683