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Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer

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Abstract

Classical structural biology can only provide static snapshots of biomacromolecules. Single-molecule Förster resonance energy transfer (smFRET) paved the way for studying dynamics in macromolecular structures under biologically relevant conditions. Since its first implementation in 1996, smFRET experiments have confirmed previously hypothesized mechanisms and provided new insights into many fundamental biological processes, such as DNA maintenance and repair, transcription, translation, and membrane transport. We review 22 years of contributions of smFRET to our understanding of basic mechanisms in biochemistry, molecular biology, and structural biology. Additionally, building on current state-of-the-art implementations of smFRET, we highlight possible future directions for smFRET in applications such as biosensing, high-throughput screening, and molecular diagnostics.

Until the mid-1990s, insights in structural biology came mainly from static macro-molecular structures obtained by x-ray crystallography (1,2). Nuclear magnetic resonance (NMR) spectroscopy allowed identification of many of the different structures associated with a single conformation of a biomolecule (1). However, a biomolecule can adopt many different conformations. Cryo-electron microscopy (cryo-EM) has recently complemented this toolkit, facilitating the determination of multiple conformations of macromolecular structures in the ensemble with near-atomic resolution (3,4). Molecular mechanisms can be inferred from such static structural “snapshots” and validated using biochemical and biophysical assays [e.g., (5)]. Although structural snapshots can identify distinct conformational states that macromolecules explore at equilibrium (e.g., ligand-bound or unbound, folded or unfolded), they lack information on the interconversion dynamics

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between these states. Understanding the functional roles of these structures requires a full dynamic picture (6–8).

NMR, electron paramagnetic resonance (EPR) (9), and double electron-electron resonance (DEER) (10) spectroscopies, as well as fluorescence-based techniques such as fluorescence anisotropy (11), ensemble Förster resonance energy transfer (FRET, Fig. 1) (12), or photo-induced electron transfer (13), can provide access to dynamic information about biomolecular interactions and macromolecular conformations. The interpretation of experimental results from these techniques is, however, highly model-dependent (14,15). Even for two-state systems in equilibrium (e.g., transitions between open and closed conformations of a protein, or bound and unbound states of interacting molecules; Fig. 2, A and B, respectively), ensemble methods yield limited insight into structural and mechanistic details. This is because molecules in an ensemble undergo changes between conformational states asynchronously (Fig. 2C). This results in averaged-out signals (Fig. 2C), so that the underlying dynamical information can be retrieved by model fitting only, and only in the simplest cases (16,17). One way of solving the problem of asynchronicity is by measuring one molecule at a time and retrieving the underlying conformational states and dynamics directly (Fig. 2, D and E).

Following the development of single-molecule detection techniques (18–24), the first demonstration of FRET at the single-molecule level was published in 1996 (25). It suggested that single-molecule FRET (smFRET) could be used to study dynamic processes and identify transient conformations and interactions between macromolecules labeled with a donor-acceptor dye pair. Schütz *et al.* used smFRET to monitor binding of ligands to streptavidin immobilized on phospholipid membranes (26), opening the way for similar experiments in live cells. In another pioneering application of smFRET, Ha *et al.* characterized the intricate conformational and substrate-binding dynamics of the staphylococcal nuclease enzyme (27). Further smFRET studies of conformational changes in other enzymes and in RNA molecules followed, using either diffusing molecules (Figs. 2D and 3A) (28,29) or immobilized molecules (Figs. 2E and 3C) (6, 27, 30, 31). In one example, Deniz *et al.* showed how information on distance-related distributions can be derived from smFRET measurements of double-stranded DNA (dsDNA; Fig. 3B) (28). Additionally, the combination of total internal reflection (TIR; Fig. 3, C and D) illumination with immobilized single molecules allowed Zhuang *et al.* to follow the conformation of individual RNA enzyme molecules and measure their folding dynamics at equilibrium (Fig. 3D) (6). In the two following decades, smFRET has matured into a toolkit to explore dynamic structural biology. This article reviews achievements in the use of smFRET to establish structure-function relationships and outlines challenges and prospects for the future.

A brief historical overview of single-molecule FRET in biochemistry and molecular biology

FRET was first identified in the 1920s by Cario, Franck, and Perrin. In the late 1940s, Förster and Oppenheimer independently formulated a quantitative theory of the energy

transfer between a pair of point dipoles. Stryer and Haugland verified this theory in the late 1960s and coined the term “spectroscopic ruler” for FRET. Around the same time that the effect of heterogeneity on FRET was taken into account in ensemble measurements (32), Hirschfeld pioneered single-molecule fluorescence detection (33). The first observations of individual fluorescent molecules in the late 1980s and early 1990s (34–38) were followed by an explosion of studies, including imaging of complex biological systems such as molecular motors (39). Since its first demonstration in 1996, smFRET has been used to provide mechanistic answers in diverse areas of biological research. These studies unraveled molecular mechanisms of helicases and topoisomerases (40), DNA replication, DNA repair (41), transcription (42–44), translation (42,45,46), enzymatic function (47–49), molecular motors (50), membrane proteins (51), protein folding (52, 53), nucleic acids (54, 55), RNA folding (54, 56, 57), and ribozyme catalysis (58, 59). Because a short review cannot do justice to the large number and diversity of smFRET studies, we will discuss a few representative examples. The theory describing FRET is given in Box 1.

A good example of the power of smFRET to explore heterogeneous mixtures and distinguish subpopulations of conformers can be taken from the field of bacterial transcription. Here, the molecular mechanism of the long-known but poorly understood abortive transcription initiation was deciphered by two concerted single-molecule experiments (60, 61), one of which was based on smFRET (60). Both studies showed that RNA polymerase (RNAP) repeatedly and unsuccessfully attempts to reel the downstream DNA into its active site (using a mechanism called “DNA scrunching”) before clearing the promoter and proceeding to transcript elongation (Fig. 4A) (60). By using distinct labeling schemes, the FRET study ruled out other proposed mechanisms (“inchworming” and “transient excursion”; Fig. 4A). When the acceptor (A, red dot) labeled the promoter sequence and the donor (D, green dot) labeled the RNAP’s leading edge, no difference was observed between the FRET histograms of the RNAP-promoter open complex (RP_o) and the RNAP-promoter complex transcribing up to seven bases ($RP_{itc, 7}$; Fig. 4A, left). This excluded the inchworming model. When the acceptor labeled the DNA upstream of the promoter sequence and the donor labeled the RNAP’s trailing edge, again no difference could be observed between the FRET histograms of RP_o and $RP_{itc, 7}$ (Fig. 4A, center). This excluded the transient excursion model. In a third experiment, the acceptor and donor labeled the DNA downstream and upstream relative to the promoter sequence, respectively (Fig. 4A, right). In this case, smFRET showed an increase in the long-distance fraction (small apparent FRET efficiency, E^*) upon addition of nucleotides permitting transcription initiation. This data unambiguously supported the scrunching mechanism, where DNA is reeled into the active site by RNAP during the initial stages of transcription, resulting in an increase in the size of the transcription bubble.

Another good example of smFRET’s ability to disentangle conformational subpopulations is the study of the enzyme adenylate kinase (62, 63). Previous ensemble time-resolved FRET measurements had suggested a single conformational state characterized by a broad distance distribution for the enzyme in the absence of its substrates, adenosine monophosphate and Mg-adenosine triphosphate (64). smFRET measurements showed, however, that at least two distinct dynamically interconverting conformations were present in the absence of substrates: an apo conformation and an active-like conformation (Fig. 4B) (62,63). These

and similar studies (47,48) have shed light on how enzymes exist in different precatalytic conformations and how substrate binding can stabilize one of these conformations.

Another fruitful area of smFRET investigations is protein folding. The function of a protein is encoded in its three-dimensional (3D) structure. Although deduction of a protein's tertiary structure (its native conformation) from its primary sequence has been revolutionized by computational techniques (65), smFRET experiments have provided many additional insights into the process of folding—whether into the correct structure or into incorrect structures (“misfolding”)—and characterization of possible folding intermediates (66, 67). Measurements involving a variety of denaturing agents have yielded evidence of a monotonic shift in the mean FRET value of the unfolded subpopulation as a function of denaturant concentration. These observations have been interpreted as a manifestation of rapid interconversion between the unfolded state and folding intermediates (29,68), which would imply the existence of folding intermediates stabilized by non-native contacts (52,53). Such studies have been expanded by many groups, and fast microfluidic mixers have enabled the extension of research from equilibrium to nonequilibrium regimes (69). The relevance of these in vitro folding studies to in vivo chaperone-assisted protein folding or to cotranslational protein folding is a topic of current investigation (70).

A related area of investigation to benefit from smFRET is the conformation of intrinsically disordered proteins (IDPs). IDPs are often stabilized in a folded state upon ligand binding (i.e., cofolding). For example, α -synuclein (α Syn), a major determinant in Parkinson's disease, is an IDP that co-folds upon binding to membranes. Deniz and co-workers studied the conformational changes of α Syn upon co-folding with different ligands and characterized its associated rapid conformational dynamics (71). They found that α Syn gains different α -helical structures after binding to lipid-mimetic agents with varying surface curvature.

Similarly, smFRET helped to elucidate the conformational dynamics, folding mechanisms, and function of RNA molecules (54–59). Not all genes code for proteins. These RNAs become functional upon folding into specific structures. Many such RNA molecules serve as ribozymes (RNA-based enzymes) or regulate various cellular processes such as gene expression and ribosome translation (54). The complexity of folding scales with the structural complexity of the RNA. The folding of RNA molecules goes through multiple free energy local minima separated by barriers of various heights (72). Therefore, it is easy for ribozymes to become trapped in long-lived, nonfunctional states (73). Even hairpin ribozymes, previously presumed to be “simple,” exhibit multiple intermediates and multiple pathways during folding (74, 75).

These examples illustrate how smFRET can be used to study the conformational dynamics and function of biological macromolecules. Next, we consider different kinds of smFRET measurements, the type of data and analyses associated with them, and examples of the dynamics these methods are capable of exploring.

Conformational states and their dynamics

smFRET can be used to characterize distinct conformational states in macromolecules and the dynamics of their interconversion. However, transitions between states can only be measured if they occur over a time scale comparable to the technique's temporal resolution. Transition time scales are proportional to the height of the activation barrier between states (Fig. 5A). Separation by a low barrier means rapid interconversion between states, which results in averaged-out smFRET data and indistinguishable states. Transitions occurring over time scales much longer than the typical observation time will, of course, not be detected either. The temporal resolution of a smFRET experiment depends on several parameters. One of the most important is whether the experiment involves freely diffusing molecules (Figs. 2D and 5B, left) or immobilized molecules (Figs. 2E and 5B, right). In both cases, FRET efficiency is calculated for each individual molecule over short, finite time intervals. For freely diffusing molecules, this observation period is set by the transit time of the diffusing molecule through the observation volume. These rare events generate a "burst" of fluorescence photons with a typical duration on the order of 1 ms. A given molecule may or may not be detected again subsequently, depending on its random diffusion path. For immobilized molecules, observation can last for several seconds or even minutes, generating time traces of fluorescence (or FRET efficiency, once processed) with a temporal resolution set by a combination of detector readout rate and signal level (a few milliseconds at best; Fig. 5B, right). Although improved organic dyes have been developed (76, 77), dye photobleaching remains the main constraint on the maximal observation time and temporal resolution (78). Analysis of burst (freely diffusing) or time-binned (immobilized) data allows identification of distinct conformational subpopulations, their FRET efficiency, and, in favorable cases, their interconversion rates.

Studying slow conformational dynamics (from 0.1 to 10 s) requires long observation times and is mostly done with immobilized molecules (Figs. 2E and 3C). Here, the different durations (dwell times) spent by a molecule in each state are analyzed, and energy transfer efficiencies are either directly extracted or obtained via hidden Markov modeling or Bayesian statistical analyses (79,80) (Fig. 5B, right). Results from many individual molecules observed in parallel are pooled to obtain statistically meaningful information. This approach has been used to study the dynamics of nucleic acid-processing enzymes such as helicases (40), the complex molecular mechanism of translocation of the ribosome (81), and HIV reverse transcriptase initiation (82), among many others. Extraction of these dynamical parameters would be very difficult using ensemble techniques.

Faster conformational dynamics (10 μ s to 0.1 s) are typically best studied with diffusion-based smFRET (Figs. 2D and 3A). Here, dynamics can be extracted from fluctuations in FRET efficiency within single-molecule bursts or between consecutive bursts of the same molecules (moving in and out of the observation volume several times) with accessible time scales in the range of 0.1 to 10 ms. If diffusing molecules change conformation during transit through the observation volume, the time-averaged FRET efficiency within each burst is of little use, although it could hint at the presence of faster dynamics (83,84). Analytical methods to investigate such dynamics have been developed in recent years (85, 86). For instance, Torella *et al.* examined the short time scale variance of FRET efficiency within

individual single-molecule bursts [burst variance analysis (BVA)] (86) (Fig. 5C, left). FRET variance exceeding that expected from photon-counting statistics (“shot noise”) was used to detect millisecond–time scale dynamics in complexes of the Klenow fragment of DNA polymerase. Using the same approach, Robb *et al.* showed that in transcription initiation, the transcription bubble (the DNA region opened up by RNAP) exhibits conformational dynamics on the submillisecond time scale (Fig. 5C, left) (87).

Single molecules freely diffusing in 3D may reenter the observation volume several times before diffusing away permanently. This results in a series of consecutive single-molecule bursts, between which the molecule may change its conformation. This opens the possibility of analyzing conformational dynamics by recurrence analysis of single particles (RASP; Fig. 5C, center) (88). Analyzing the succession of FRET efficiencies of consecutive bursts separated by variable recurrence times enabled quantification of the folding relaxation times of small proteins such as cold shock protein (Csp), spectrin R15, and the B domain of protein A (BdpA), revealing time scales of 250 ms, 32 ms, and 0.7 ms, respectively (88).

Faster conformational changes (<0.1 ms) yield single-molecule bursts with averaged-out FRET values. Approaches that do not rely on the analysis of separate single-molecule bursts, but rather on photon statistics within bursts, are therefore called for. In addition, because such rapid conformational changes include multiple transitions within each single-molecule burst, the variance of the FRET efficiency becomes noisy (in a way that resembles shot noise). In this limit, techniques that resolve FRET dynamics through variance analysis (such as BVA) cannot resolve faster FRET dynamics. For this regime, fluorescence correlation spectroscopy (FCS) methods applied to smFRET (FRET-FCS) are the most straightforward to implement, even if demanding in terms of statistics (Fig. 5C, right). For instance, Nettels *et al.* performed diffusion-based smFRET measurements on Csp, acquiring data in order to compute correlation curves down to the picosecond time scale. Using this approach, they showed that the unfolded state of Csp undergoes structural reconfiguration within ~ 40 ns (89). The additional information attained from fluorescence lifetimes has also been used in the analysis of rapid FRET dynamics. Fluorescence lifetime analysis (using pulsed laser excitation) can also be used to unravel fast dynamics. Wo niak *et al.* used time-correlated single photon counting (TCSPC) to explore the bending dynamics of short dsDNA (90). Dolino *et al.* observed submillisecond dynamics in the ligand-binding domain of the N-methyl-D-aspartate receptor (91). Using alternating laser excitation on the nano-second time scale (nsALEX; see Box 1), Laurence *et al.* analyzed fluorescence decays of specific FRET subpopulations to infer an effective distance distribution for the folded and unfolded chemotrypsin inhibitor 2 (CI2) (92).

Although powerful, these fast conformational dynamics methods usually do not provide information on the exact number of conformational states (and their mean FRET efficiencies) involved in the identified dynamics. Recently, Pirchi *et al.* reported an analytical method to extract the values of these parameters by performing a photon-by-photon hidden Markov modeling analysis of smFRET experiments (H2MM) (93), as previously suggested by Gopich and Szabo (94). They were able to extract rate constants (ranging from ~ 10 μ s to ~ 1 s) and the mean FRET efficiencies of the corresponding states. We are therefore on a path toward full characterization of fast conformational dynamics of

macromolecules: the number of states, their FRET values, and the interconversion rate constants.

All of the methods mentioned above, although powerful, rely on a single reaction coordinate (the distance between a single donor-acceptor pair) and therefore provide a limited perspective on the underlying dynamics. We next discuss how multiple reaction coordinates can be simultaneously measured to untangle complex conformational dynamics.

Toward multiple reaction coordinates

A single smFRET measurement reports on a single distance within a macromolecular structure, projecting a complex 3D structure onto a single 1D reaction coordinate (Fig. 5A). In some macromolecules, domains or subunits may be approximated as rigid bodies linked by flexible linkers or interacting through well-defined binding interfaces. In other cases, allosteric ligand binding to one part of a macromolecule can cause conformational changes in other parts of the same macromolecule. In these cases, a single reaction coordinate may not be enough to report coordinated motions. Additionally, regardless of the presence or absence of allosteric binding and coordinated motion, some smFRET-derived single distances may be insensitive to conformational changes (e.g., structural change occurs tangential to the monitored distance or occurs in another part of the macromolecule). For all these reasons, it is generally desirable to study conformational changes with more than one set of positions for a pair of dyes.

An obvious solution is to label the macromolecule with more than two dyes. Multicolor smFRET techniques (95) can indeed provide a wealth of information (Fig. 6A). Ha and co-workers (96) and Person *et al.* (97) used three-color smFRET to study Holliday junctions, which spontaneously switch between two distinct conformations. They simultaneously determined three distances unambiguously and specified the correlated movement of the junction's hairpin structure. Multicolor smFRET techniques provide high information content but are challenging to implement. They require multiple orthogonal and efficient site-specific labeling chemistries, elaborate optics, and data analysis techniques. Some of these difficulties can be mitigated by using a dark quencher as one of the (three) dyes. Because the dark quencher accepts excitation energy through FRET but does not emit photons, there is no need for detection of emission, thereby simplifying data collection. Kapanidis and co-workers (98) used this approach to monitor the binding and unbinding of a DNA polymerase to its substrate in real time, without the need for three-color detection for simultaneous detection of protein binding and associated conformational changes.

It is, however, also possible to monitor more than one distance using multiple identical dyes in a two-color excitation and detection scheme, using some photophysical tricks. In biomolecular complexes with more than one donor and acceptor of the same kind, fluorophore interactions via FRET are highly complex, and the relation of FRET efficiency E to inter-dye distances R is generally nontrivial because of multiple energy transfer pathways. For immobilized molecules, this multiplicity can be removed by using chemically induced stochastic blinking of the acceptor fluorophores (99,100), leaving only one active acceptor per molecule for a brief period of time. Using this "photoswitchable FRET"

approach, Uphoff *et al.* measured the distances between DNA and two residues on the catabolite activator protein (CAP), as well as the strand exchange dynamics in Holliday junctions (100). Because the acceptor blinks randomly, smFRET time traces exhibit different values over time, allowing measurement of multiple distances from a single donor to multiple acceptor fluorophores (Fig. 6B).

Another alternative to multicolor smFRET—which requires as many detection channels as there are different dyes—is using simple two-color smFRET with other independent observables (translational diffusion, fluorescence anisotropy, brightness, etc.) (101). For example, by using an alternating laser excitation (ALEX) scheme (see Box 1), Kapanidis *et al.* were able to simultaneously report FRET values E for each molecule as well as the “stoichiometry” S , defined as the ratio between fluorescence originating from donor excitation and that originating from both donor and acceptor excitations (102). Changes in mean S values report changes in the ratio of donor and acceptor brightnesses. ALEX can therefore distinguish molecules with different numbers of donor and acceptor dyes, or molecules with altered dye fluorescence quantum yields. In a recent implementation of ALEX to simultaneously report two distances, smFRET was combined with protein-induced fluorescence enhancement (PIFE) (103,104). One distance was between the dyes and within the FRET distance range (~3 to 9 nm); the other, between one dye and a bound protein, was in the shorter PIFE distance range (<3 nm). PIFE-FRET thus provided direct evidence for molecular coordination in the open transcription bubble (Fig. 6C) (104). Similarly, smFRET was combined with photo-induced electron transfer (PET) (105), where the donor dye was quenched by a nearby tryptophan moiety. However, the steep dependence of PET on dye-quencher distance (angstroms) results in a binary output (contact/no contact) rather than a quantitative distance measurement. Finally, because both multicolor smFRET and ALEX achieve high information content, combining the two techniques doubles the information content (both FRET and brightness ratio for each dye pair permutation). Lee *et al.* used three-color ALEX to monitor the translocation of bacterial RNAP on DNA on two distinct reaction coordinates (106). Such high information content can be used for multiplexed sorting in molecular diagnostics. Yim *et al.* have shown the capability to sort and quantify multiple different biomarkers in a four-color ALEX experiment (107).

In each of these techniques, macromolecule labeling with fluorophores (or quenchers) is important. Although high-purity site-specific dye-labeled nucleic acids are now commercially available, preparation of site-specifically labeled proteins at multiple residues is far more challenging. Advances in this field [reviewed in (108,109)] will allow the study of multiple reaction coordinates and coordinated motions in single subunit proteins. As an alternative [already suggested in 1999 (110)], smFRET can be combined with other single-molecule techniques not involving fluorescence. These include patch-clamp (111) and single-molecule manipulation methods such as optical (112) and magnetic (113) tweezers, atomic force microscopy (114), and microfluidics and drag forces (115). These hybrid approaches are very powerful and simultaneously measure multiple orthogonal reaction coordinates. A detailed account of these methods is outside the scope of this review and can be found elsewhere (95).

Solving 3D structures with smFRET

If properly calibrated, FRET efficiency E carries information on the precise distance between donor and acceptor dyes. Can this information be extracted and used for 3D structure determination? X-ray crystallography, NMR, and cryo-EM are currently the gold standards for obtaining atomic-resolution 3D structures of complex macromolecules. However, crystallization conditions may preferentially stabilize one conformation over others (62); in extreme cases, crystallization may even induce a structure never observed in solution, as detected by comparison with structures solved by solution-based techniques (116). Thus, structural characterization of macromolecules in solution and at ambient temperature is desirable.

The ability to identify distinct conformational subpopulations can help in structure determination, because relevant subpopulations can be identified and selected for further processing. Structure determination requires the preparation and measurement of multiple donor-acceptor variants labeling different pairs of positions on the macromolecule. The nontrivial transformations from uncorrected FRET efficiency E^* to corrected FRET efficiency E , and then to inter-dye distance R and to inter-residue distance r , require additional preparations and measurements of control mutants, modeling and simulations, structural convergence procedures, and control and validation of refined structures. Several studies (117–119) have followed this route, reporting successful structure determination (120–122). The information retrieved from smFRET measurements of multiple distances can be used to directly triangulate a structure of a whole or part of a macromolecule, or can be used as experimental constraints for structural simulations (121). In the latter approach, each iteration produces a structural snapshot. After assessing the dyes' accessible volumes via the "nanopositioning system" (NPS) approach (117), the computationally derived mean inter-dye distances are compared with the experimentally derived ones for all measured constructs, and the sum of all deviations (cost function) is computed. This process, performed on a large library of simulated structural snapshots, should result in a subset of candidate conformations selected by minimization of the cost function. Using this approach, we recently identified two conformations of the transcription bubble in the bacterial RNAP-promoter open (RPo) complex (123). The set of distances of one conformation agreed with the crystal structure of bacterial RPo (124), while the other did not. The latter conformation had characteristics of a scrunched transcription bubble, where a few bases from the duplex downstream to the bubble were reeled into the active site of RNAP and increased the size of the transcription bubble.

Although successful structural determinations by smFRET have been reported, single-particle cryo-EM has also gained the ability to resolve several (up to three) conformational states in the frozen ensemble (4,125,126). Nonetheless, single-particle cryo-EM fundamentally lacks what smFRET readily provides: the ability to detect the dynamics of transitions between conformations. We anticipate that FRET-derived macromolecular structures or distance constraints will also be accepted in the future as entries in the Protein Data Bank. However, different laboratories currently use different measurement and analysis techniques, different protocols, and different types of data files. Therefore, smFRET

experiments—and more important, the control experiments and data analysis procedures required for obtaining exact distances—have to be standardized, as outlined below.

Standardizing smFRET measurements

Because of the challenges of smFRET data calibration, it is important to strive for reproducibility across laboratories by establishing standard protocols and data-sharing practices. Such a standardization effort, led by the Hugel and Seidel groups, was recently initiated through the wwPDB Hybrid/Integrative Methods Task Force (127). Equally important to this effort is a standard set of recommended practices that could be verified by peer review. These include (i) avoiding subjective selection of data sets (e.g., time traces in surface-immobilized experiments), (ii) requiring the donor-acceptor fraction of the labeled sample to be larger than 10%, (iii) using different excitation powers to assess photophysics effects, (iv) requiring fluorescence anisotropy measurements to characterize fluorophore rotational freedom, and (v) comparison with ensemble assays (denaturation curve, enzymatic assay, secondary structure content, thermal stability, ligand binding affinity, etc.) of the labeled macromolecule with its unlabeled counterpart to verify its activity and the relevance of the smFRET measurement.

We also recommend that every smFRET experiment, including experiments with surface-immobilized molecules, should begin with a “control” solution-based smFRET assay, so as to determine (i) the quality of labeling, (ii) the number of states or biochemical species resolved as distinct FRET subpopulations in the sample, (iii) the mean FRET efficiencies of the resolved subpopulations, and (iv) interconversion rate constants between subpopulations. With this information, analysis of smFRET time trajectories from surface-immobilized molecules can be guided by, and compared to, a statistically robust diffusion-based analysis. Moreover, this two-step process will allow assessing whether biomolecule-surface interactions are present and perturb the system under study—in particular, measured FRET efficiencies, population frequencies, and time constants.

Finally, to improve cross-checking and reproducibility, standardized data analysis protocols should be used and preferably based on open-source software. For instance, the FRET Bursts open-source package provides a starting point for diffusion-based analysis (128), and similar packages are available for surface-immobilized smFRET (129,130). The use of standardized file formats such as Photon-HDF5 (131) is a prerequisite to making the raw data freely available and preserved for the long term for independent validations and future reanalysis with new methods. Depositing the raw data, analysis tools, and pipelines in public repositories such as Dryad, Data-verse, Zenodo, Fig share, or Github (128) will allow different groups to cross-validate results and accelerate the development of new analysis tools.

So far, we have discussed past and present applications of smFRET in biophysics, biochemistry, molecular biology, and structural biology. Future technological advances striving to overcome the current limitations of smFRET measurements could further extend the power of smFRET. Several areas of improvement can be envisioned: temporal resolution,

extension to more in vivo and in vitro experimental formats, simplification, and higher throughput compatible with biopharmaceutical applications.

What is next for smFRET?

smFRET has become the accepted method for dynamic structural biology but is still almost entirely used in the context of in vitro experiments. In vivo smFRET, which has recently emerged as a promising methodology requiring further development (43), may allow explorations of conformational dynamics and heterogeneity in the living cell—an approach so far limited to bulk “in-cell NMR” (132) and “in-cell FCS” (133). By removing the artificial constraints of in vitro experiments, in vivo smFRET promises to shed light on outstanding questions in biology by monitoring smFRET as a function of location, diffusivity, and interactions with other partners, thereby illuminating the long-sought link between conformational states and dynamics of biomolecules. Some of the challenges of in vivo smFRET measurements are the generally low signal-to-noise (S/N) and signal-to-background (S/B) ratios and poor photostability of fluorescence proteins. Organic fluorophores are the probes of choice, but their use in live cells requires specific delivery and tagging protocols, which generally introduce larger perturbations and uncertainties than conventional molecular biology techniques. Using microinjection in cultured cells, Sakon and Weninger were the first to track folding of individual SNARE proteins (134). Recently, Schuler and co-workers also used microinjection and smFRET to probe the submicrosecond dynamics of individual freely diffusing, intrinsically disordered proteins in different cellular compartments (Fig. 7, lower center) (70). The ability to distinguish between subpopulations while also detecting fast dynamics allows identification of different folding behaviors in the cytosol and the nucleus. While successfully used in these examples, microinjection relies on high-precision and low-throughput procedures. Techniques for internalization of labeled molecules, such as electroporation in bacteria and in yeast (135) or permeabilization using pore-forming reagents in mammalian cells (136), are more feasible. For instance, several studies have probed the conformations and localizations of doubly labeled oligonucleotides after microinjection in eukaryotic cells (137) or electroporation in bacteria (Fig. 7, lower left) (135). Simpler, robust delivery strategies for better labeling yields and high cell viability are needed.

Commonly used illumination geometries such as TIR or confocal imaging are not always ideal. In TIR excitation mode, only a thin layer (~100 nm) of the cell above the cover glass is illuminated by an evanescent field (Fig. 7, upper left). Confocal excitation (Fig. 7, upper center) allows observation deeper into the cell while maintaining good S/N and S/B, but the diffraction-limited sampling volume requires raster scanning for image formation, which competes with continuous recording at each location. Traditional wide-field epi- or trans-illumination is unsuitable for single-molecule detection because of low S/N and S/B and high levels of photobleaching and phototoxicity. Light-sheet or single-plane illumination microscopy (SPIM) is more complex but enables 3D sectioning with high background rejection, limited phototoxicity, and bleaching, and has been successfully extended to single-molecule imaging (138). The use of SPIM for in vivo smFRET measurements could therefore provide new opportunities, as suggested by recent work using a simplified version (139). Combined with fast detectors [scientific-CMOS (sCMOS) cameras or single-photon

avalanche diode (SPAD) arrays], probing fast biological events such as protein binding and conformational dynamics in live cells may become feasible (Fig. 7, right).

smFRET on immobilized molecules allows continuous monitoring of conformations or binding events with fairly good temporal resolution. A potential drawback is the introduction of artificial perturbations due to the surface proximity and immobilization chemistry. One way to bypass this problem is to entrap individual molecules in immobilized lipid vesicles (140,141). However, this technique limits the ability to modulate the local environment (for instance, by buffer exchange). Another solution, the anti-Brownian electrokinetic (ABEL) trap (142), counteracts the Brownian diffusion of a single molecule in solution by active modulations of an external electric field, but this requires observing one molecule at a time and results in very low throughput. We note that in some cases, proteins and DNA molecules may gain different structures or activities under such conditions. To overcome the need for immobilizing or trapping molecules, we envision confinement within a thin chamber (<100 nm) limiting the diffusion along the *z* axis. Combined with fast detectors such as sCMOS cameras or SPAD arrays, this would enable tracking of multiple molecules for extended periods of time during their quasi-2D diffusion with reduced surface-interaction artifacts.

A major drawback of diffusion-based smFRET measurements is the long acquisition time (several minutes) needed to accumulate a large number of single-molecule bursts. Acquisition times on the order of a few seconds would enable an entirely new class of applications such as diffusion-based smFRET kinetic studies, high-throughput (HT) drug screening, and diagnostic assays (Fig. 7, lower right). Throughput can be multiplied by parallel acquisition from multiple excitation spots using SPAD arrays for detection (Fig. 7, right). This multispot approach provides a reduction in acquisition time proportional to the number of spots (143), which could potentially reach up to 1000 pixels for next-generation SPAD arrays suitable for single-molecule detection (144). Although the excitation geometry can be multispot as well, more scalable excitation schemes include zero-mode waveguides or light-sheet illumination. Such schemes will eliminate the tedious task of aligning the excitation pattern to the detector pixels. Additionally, as noted above, such 2D illuminations allow the use of fast sCMOS cameras (>100 Hz full frame, >1 kHz partial frame) (145), which may be sufficient for some applications such as high-throughput screening that currently relies on SPADs or live-cell smFRET imaging (Fig. 7, lower right) (135,139).

Whereas ensemble kinetics can identify kinetic processes that are well separated in time, non-equilibrium smFRET kinetic studies can identify multiple conformations or binding states and their associated transitions. Non-equilibrium smFRET kinetic studies rely on rapid exchange or mixing of reagents to initiate a perturbation in the system under study. In experiments on immobilized molecules, rapid exchange of conditions initiates the reaction, which is then recorded in as many time trajectories as there are molecules. Time-dependent FRET efficiency histograms that describe the reaction are computed by aligning all the smFRET trajectories (145). Non-equilibrium smFRET kinetic studies performed on diffusing molecules are more challenging because molecules randomly enter the observation volume, preventing continuous time traces to be acquired. Ingargiola *et al.* recently measured the kinetics of transcription initiation (promoter escape) using a multispot setup (143). They directly followed the kinetics by monitoring the conformation of the

transcription bubble at the single-molecule level with 30-s temporal resolution, limited only by the number of single-molecule bursts detected across the eight spots (Fig. 7). A 48-spot system currently in development (146) should improve the temporal resolution accordingly. However, as the temporal resolution of the measurement is improved, faster mixing is required. A combination of a continuous-flow microfluidic mixer (147) together with multispot detection could provide the solution for fast non-equilibrium smFRET kinetic studies of diffusing molecules (Fig. 7, lower right).

Drug discovery using drug-ligand interactions measurements relies on high-throughput ensemble techniques to rapidly screen large libraries of small molecules for identification of interactions and quantification of affinities. Various screening methods differ in the range of affinities they can measure, their throughput, sample consumption, accuracy, measurement modality (kinetic, steady-state), possible requirement for immobilization, lowest binding stoichiometry, etc. Many of the techniques that allow high-throughput screening of more than 10^4 molecules per day report either quantitatively on low affinity ranges in bulk, or on interactions with immobilized small molecules measured by surface plasmon resonance (148). Diffusion-based smFRET assays could enable probing such interactions with minimal sample consumption. However, until recently, such measurements required long acquisition times. Additionally, such screening requires an automated system that can rapidly exchange conditions. Kim *et al.* used a microfluidic mixing device to automate titration from many input channels and perform serial smFRET measurements at different conditions (149). Multispot and multicolor smFRET in combination with an automated mixing device would allow highly multiplexed smFRET measurements, suitable for high-throughput screening (Fig. 7). As an example, a 1024-spot system would allow measurements lasting ~250 ms, translating to ~350,000 assays per day (assuming that the microfluidic chip enables dispensing of as many samples at this frequency). The same approach could be used to titrate binding components to produce affinity curves for each ligand down to picomolar concentrations and in varying conditions. Alternatively, a multispot setup could be used in conjunction with a titer plate and scanning stage, where many different conditions in each well can be tested at a much higher rate than with a single-spot excitation. Lastly, using multispot smFRET acquisition in a stopped-flow format would allow measuring association and dissociation rate constants and extraction of molecular affinities (Fig. 7, right).

Likewise, molecular diagnostics could benefit from high-throughput smFRET capabilities. Such applications require highly specific and sensitive molecular recognition of low-abundance molecular markers (proteins, self-antibodies, microRNAs, freely circulating DNA, etc.) in a small volume of bodily fluids, ideally without any amplification (107,150). Here again, fast acquisition coupled with automation of liquid handling is required. A combination of multispot smFRET with multicolor ALEX capabilities and a microfluidic chip could provide a powerful molecular diagnostics platform.

Conclusion

Two decades after its introduction, the promise of smFRET has largely materialized; several variants have now reached maturity to form a robust and mainstream toolkit available to biochemists, molecular biologists, and biophysicists. Commercial systems implementing

smFRET have been introduced in recent years. We anticipate further development of such systems into turnkey and, eventually, fully automated devices based on open-source and validated data analysis algorithms, which will lower the barrier of entry to this powerful technology and further help to disseminate the method.

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Box 1.**FRET as a spectroscopic ruler for macromolecular distances.**

FRET is a “spectroscopic ruler” with a distance-dependent efficiency E given by Förster theory (151) (Fig. 1). If R is the distance between two point dipoles [representing the center of the donor (D) and acceptor (A) fluorophores; Fig. 1A], E depends on the sixth power of the distance (Fig. 1B), assuming a “frozen” molecule (Eq. 1):

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

$$R_0^6 = \frac{9 \ln(10)}{128 \pi^5 N_A} \frac{\kappa^2 \phi_D}{n^4} \int f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (2)$$

The Förster radius, R_0 , is the R at which $E = 50\%$. R_0 depends on parameters indicated in Eq. 2: N_A is Avogadro's number, n is the refractive index in the medium between the donor and acceptor, ϕ_D is the donor fluorescence quantum yield in the absence of acceptor, f_D is the donor emission spectrum with its area normalized to 1, ε_A is the spectrum of molar extinction coefficient of the acceptor, and κ^2 is the orientation factor of the dyes. The range of distances that can be accurately measured with FRET is $0.5R_0$ to $1.5R_0$ (for commonly used smFRET dye pairs, this translates into a dynamic range of ~3 to 9 nm). The parameters in Eq. 2 (R , ϕ_D , κ^2 , n) may dynamically change and therefore complicate the interpretation of smFRET distance measurements. Careful control experiments are therefore required. Note that E can be transformed into R if the dyes can be approximated by point dipoles. This approximation holds if the dye sizes are much smaller than R . Although smFRET has been demonstrated using quantum dots (152), they are too large to be approximated by point dipoles. Similarly, genetically encoded fluorescent proteins that are frequently used to monitor binding events and conformational changes in vivo (153) have chromophore groups that are bound deep inside their cores, complicating the transformation of E to R . Small and bright organic fluorophores are therefore the emitters of choice for smFRET measurements.

The average E can be measured experimentally using several approaches. The most straightforward way uses the donor mean fluorescence lifetimes (Eq. 3):

$$\langle E \rangle = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_{DO} \rangle} \quad (3)$$

where $\langle \tau_{DA} \rangle$ and $\langle \tau_{DO} \rangle$ are the donor mean fluorescence lifetimes in the presence or absence of an acceptor, respectively. Knowing the value of R_0 of the dye pair, it is possible to deduce the mean distance between dyes using Eq. 1. $\langle \tau_{DA} \rangle$ and $\langle \tau_{DO} \rangle$ can be

retrieved in a single measurement via nanosecond alternating laser excitation (nsALEX) (92) [also known as pulsed-interleaved excitation (PIE) (103)], in which donor and acceptor are alternately excited with pulsed lasers and fluorescence photons are collected using time-correlated single photon counting (TCSPC).

A simpler approach extracts E from the donor and acceptor fluorescence intensities recorded using continuous-wave donor excitation, or with alternated donor and acceptor excitations [microsecond ALEX (103)] (Fig. 2, D and E; Eqs. 4 and 5):

$$\langle E \rangle = \frac{F_{\text{FRET}}}{\gamma F_{\text{D}}^{\text{D}} + F_{\text{FRET}}} \quad (4)$$

$$F_{\text{FRET}} = F_{\text{D}}^{\text{A}} - lk F_{\text{D}}^{\text{D}} - dir F_{\text{A}}^{\text{A}} \quad (5)$$

where F_{D}^{D} and F_{D}^{A} are the background-corrected fluorescence intensities of the donor and the acceptor, respectively, measured during donor excitation; in the case of ALEX, F_{A}^{A} is the background-corrected acceptor fluorescence intensity during acceptor excitation, lk is the donor fluorescence leakage into the acceptor detection channel, dir is the acceptor fluorescence when directly excited by the donor excitation laser, and γ is the ratio between acceptor and donor fluorescence quantum yields and detection efficiencies.

In ensemble FRET, the measured $\langle E \rangle$ reports on all molecules in all conformations; by contrast, in smFRET, $\langle E \rangle$ values (diffusing or immobilized formats, Fig. 5B) represent time-averaged FRET values over limited duration and/or limited number of molecules or events. During a single-molecule burst or time trace, the molecule might not visit all the states that define the system. The average of all $\langle E \rangle$ values for many single molecules and over a long enough observation will equal the ensemble-averaged $\langle E \rangle$. smFRET can distinguish between distinct subpopulations of $\langle E \rangle$ values, and each subpopulation may represent a distinct conformational state. However, if interconversion between conformational states takes place on time scales faster than the method's temporal resolution, $\langle E \rangle$ subpopulations may only represent time averages of these interconverting states.

Fig. 1. The concept of FRET.

(A and B) An electromagnetic transmitter-receiver (A) is a macroscopic analog for the molecular dipole-dipole coulombic interaction between donor and acceptor (D and A) fluorophores (B). The dependence of the efficiency of energy transfer from D to A on their distance provides a molecular ruler with a high dynamic range on the 3- to 9-nm scale.

Fig. 2. Principle and use of FRET for elucidating biomolecular reaction mechanisms and structural dynamics.

(A to C) Principle of intra-molecular (A) and intermolecular (B) FRET assays and their readout (C) in single-molecule and bulk fluorescence (Fl.) experiments. The bulk experiments always show an average value [i.e., donor (D) and acceptor (A) intensity of, e.g., hypothetical 50/50], whereas smFRET can determine (dynamically interconverting) states directly. The crystal structure overlay of substrate-binding domains of an ABC transporter in (A) shows open (red) and closed (green) conformations. (D and E) smFRET with diffusing molecules (D) or immobilized molecules (E) including accessible biophysical parameters (i.e., conformational states and dynamical changes). For characterization of conformational states, histograms of FRET efficiency E with frequency n are used; dynamics are directly seen via temporal evolution of E obtained via ratio of acceptor (A) fluorescence to fluorescence from both donor (D) and acceptor after donor excitation. [(A) and (D) adapted, with permission, from (154)]

Fig. 3. Pioneering implementations of smFRET.

(A) Schematic of a confocal microscope setup used for the acquisition of diffusion-based smFRET data; F(D) and F(A) indicate the donor and acceptor detection channels, respectively. (B) Example of data obtained with such a setup. The different histograms show the FRET efficiency distributions obtained for DNA samples differing by the distance between donor and acceptor labels; bp, base pairs. [Adapted, with permission, from (28)] (C) Schematic of a total internal reflection fluorescence (TIRF) setup allowing the study of smFRET on surface-immobilized molecules. (D) Example of data obtained with such a setup, showing the real-time dynamics of RNA catalysis and folding. FRET trajectories were retrieved for individual RNA molecules (right) and histograms of dwell times reported on the time scale of the dynamics (lower left). [Adapted from (6)]

Fig. 4. Typical examples of smFRET studies.

(A) Transcription initiation involves a DNA scrunching mechanism. The results of three experiments differing by the location of the donor and acceptor dyes are shown (see text). The cartoons indicate which model is or is not compatible with the results. [Adapted from (60)] (B) Intrinsic domain motions between conformations in adenylate kinase (AK). The experiment tracks the distance between substrate-binding domains (donor and acceptor dyes as green and red stars, respectively) in the AK enzyme in apo form (left histogram) and when bound to the substrate-mimicking inhibitor Ap₅A (right histogram). FRET efficiency histograms (left) and single-molecule time traces (right) show that in apo conformation, AK dynamically switches between two conformations, one of which is similar to the substrate-bound state. [Adapted, with permission, from (62)]

Fig. 5. Biomolecular dynamics accessible by smFRET.

(A) Hypothetical energy landscape with Gibbs free energy projected onto a single reaction coordinate r showing different local minima (states) separated by energy barriers of different heights, giving rise to conformational transitions over different time scales. (B) smFRET data from diffusing molecules (bursts, left) and immobilized molecules (time traces, right) can be analyzed by various methods with differing temporal resolutions to study conformational transitions over different time scales. Conformational dynamics slower than ~ 0.1 s can be studied by analysis of single-molecule traces and dwell times in each FRET-associated state. (C) Examples of data analysis techniques using details of burst properties and photon statistics: Burst variance analysis (BVA) identifies bursts with variance of the FRET efficiency larger than expected from shot noise; recurrence analysis (RASP) identifies whether the FRET efficiency has changed between consecutive bursts of the same molecule; and correlation techniques identify time scales (including <100 μ s) at which fluorescence-related processes occur, including changes in FRET efficiency. [Reproduced, with permission, from (87, 88)]

Fig. 6. smFRET-based approaches to study molecular coordination.

(A) Multicolor smFRET studying coordinated movement of a Holliday junction via proximity ratio PR: donor-transmitter D-T (green trace), transmitter-acceptor T-A (black), and donor-acceptor D-A (red). [Adapted, with permission, from (97)] (B) Photoswitchable FRET relies on temporal separation of donor-acceptor interactions via photoswitching and isolation of molecular species with one distinct donor-acceptor pair at any given time point. [Adapted, with permission, from (100)] (C) PIFE-FRET uses a standard two-color assay with donor and acceptor (D-A) but adds information on protein binding via use of an

environmentally sensitive donor (Cy3; Cy3B is used as the control dye that is insensitive to changes in the environment). [Adapted, with permission, from (104)]

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Fig. 7. Emerging applications and future directions of smFRET.

Top rows show current detector and excitation formats for smFRET; the bottom row shows emerging developments that go beyond existing capabilities. smFRET measurements have been demonstrated in live bacteria using TIRF with probes internalized via electroporation [left; adapted from (135)] and in eukaryotic cells using confocal excitation and microinjected molecules [center; after (70)]. Multipixel SPADs (right) allow fast detection schemes and will allow retrieval of FRET trajectories of single molecules in vivo (scanning different z-layers via light-sheet microscopy) and in vitro (nonequilibrium kinetics via smFRET using mixers or continuous-flow microfluidic devices) [adapted from (143)].