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Shimon Weiss, *et al.*
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REVIEW

Fluorescence Spectroscopy of Single Biomolecules

Shimon Weiss

Recent advances in single-molecule detection and single-molecule spectroscopy at room temperature by laser-induced fluorescence offer new tools for the study of individual macromolecules under physiological conditions. These tools relay conformational states, conformational dynamics, and activity of single biological molecules to physical observables, unmasked by ensemble averaging. Distributions and time trajectories of these observables can therefore be measured during a reaction without the impossible need to synchronize all the molecules in the ensemble. The progress in applying these tools to biological studies with the use of fluorophores that are site-specifically attached to macromolecules is reviewed.

Although it is becoming a routine practice in many laboratories, the ability to analyze individual molecules still amazes even the most zealous practitioners of this emerging field. As is often the case, the introduction of a novel technique generates enthusiasm among the converted but doubts among the skeptics: Are single-molecule methodologies going to teach us more than we can currently learn from ensemble measurements? Are we going to make new discoveries using these methods? What kinds of problems are best solved by single-molecule studies? How useful and widespread are these methodologies going to be?

In contrast with ensemble methods, single-molecule experiments provide information on distributions and time trajectories of observables that would otherwise be hidden. They allow one to examine individual members of a heterogeneous population and to identify, sort, and quantitatively compare their subpopulations. Ensemble measurements, on the other hand, yield information only on average properties. Single-molecule methods are also most suited to study fluctuating systems under equilibrium conditions and to measure time trajectories and reaction pathways of individual members in a nonequilibrated system. In particular, they can measure intermediates and follow time-dependent pathways of chemical reactions that are difficult or impossible to synchronize at the ensemble level.

The very rapid and remarkable development of fluorescence single-molecule detection (SMD) and single-molecule spectroscopy (SMS) will no doubt impact many scientific disciplines. This review is focused on biological applications. While it is too soon to answer many of the skeptics' questions, we will point out possible approaches and indicate what can or might be learned from single-molecule studies of fluorescently tagged biomolecules. Most importantly, we will show that single-molecule spectroscopy can probe biological macromolecules and provide information on their structure and function that is difficult, and sometimes impossible, to obtain by conventional techniques.

Materials Sciences and Physical Biosciences Divisions, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. E-mail: sweiss@lbl.gov

Historical Account

The quest for optical methods capable of detecting trace amounts of biologically important molecules under physiological conditions can be traced back to Hirschfeld, who demonstrated in the mid-70s the detection of a single antibody molecule tagged with 80 to 100 fluorophores (*1*). Later, together with his colleagues, he tried to develop a commercial instrument, the Virometer, designed to detect, size, and classify single viruses (*2*). His pioneering efforts realized many of the essential ingredients of SMD including reduced excitation volume, time-gated detection, and prephotobleaching of impurities (*2*, *3*) for discrimination against background. He also recognized that photobleaching is an inherent property and fundamental limit for the number of emitted photons of a single fluorophore (*4*). Two orders of magnitude short of SMD, he was still ahead of his time. During the '80s, Keller's group at Los Alamos was constantly improving their detection sensitivity

of molecules in hydrodynamically focused flows (*5*, *6*). By 1990, these efforts led to the first successful detection of a single fluorophore in a biologically relevant environment (*7*). Independently, Moerner's and Orrit's groups demonstrated the detection of single dopant molecules in a host molecular crystal at cryogenic temperatures (*8*), as reviewed elsewhere in this issue (*9*).

A major advance in the SMD field came about with the demonstrations of room temperature microscopy and spectroscopy of immobilized single fluorophores by near-field (*10*–*15*) and later far-field (*16*, *17*) scanning optical microscopies. Wide-field microscopy of single molecules with total-internal-reflection (TIR) and with epi-illumination excitations were demonstrated by improved charge-coupled device (CCD) cameras and thoroughly eliminating major sources of background. With these techniques, the first biological SMD applications of immobilized molecules were demonstrated. Individual adenosine 5′-triphosphate (ATP) turnovers by a single myosin molecule (*18*), individual actin filaments sliding over heavy meromyosin (*19*), the sliding motion of single kinesin molecules along microtubules (*20*), and the diffusion of partially immobilized molecules in lipid membranes (*21*) and in gels (*22*) were observed at video rate.

Detection methods for flowing and diffusing single molecules in solutions were also further developed. When a fluorophore traverses the laser excitation volume, a fluorescence photon-burst is generated. Such bursts can be analyzed for their brightness, duration, spectrum, and fluorescence lifetime, thereby providing molecular information on identity, size, diffusion coefficient, concentration, and electrophoretic drift. Because of the digital nature of burst analysis and the ability to tabulate such data in histograms, the technique allows separation of subpopulations from a heterogeneous ensemble and therefore is most suited to selecting, sorting, identifying, and sizing macromolecules in solution. When SMD is coupled with flow, it potentially offers one of the most intriguing, yet very challenging, ways to rapidly sequence DNA. This effort was pioneered by Keller *et al.* at Los Alamos (*23*, *24*) and is now joined by many other teams. Using closely related techniques, Rigler's group and others used fluorescence correlation spectroscopy to analyze, sort, and detect distributions of conformational states of single (or very few) molecules in the excitation volume (*25*, *26*). Nie *et al.* used a similar approach to show fluorescence saturation and diffusion of single molecules into and out of the probe volume (*27*). SMD by two-photon excitation (TPE) was shown to have a superior signal to background ratio (S/B) compared with single-photon excitation, but accelerated photodestruction dynamics (*28*–*30*). Single molecules were also detected in microscopic droplet streams (*31*). Although the field of single-molecule detection and spectroscopy is very young, there are already several excellent reviews (*24*, *32*–*37*).

Labeling Schemes and Physical Observables

Various properties of single fluorescent probes attached to macromolecules can be exploited to provide information on molecular interactions, enzymatic activity, reaction kinetics, conformational dynamics, molecular freedom of motion, and alterations in activity and in chemical and electrostatic environment. "Native" fluorescence probes such as fluorescing products (*38*–*40*) and fluorescing enzymes (*41*) were successfully and beautifully used to probe enzymatic turnovers of single molecules. Our discussion is focused, however, on the use of small dye molecules that are covalently and site-specifically attached to biomolecules. We note that another viable way to tag single proteins is through the fusion of green fluorescent protein (GFP), which was successfully used to monitor single motor proteins (*42*–*44*). On the ensemble level, GFPs were used to measure conformational changes (*45*, *46*) and local pH (*47*).

Molecular biology techniques such as site-directed mutagenesis and unnatural amino acid mutagenesis (*48*) can be used to introduce cysteine and ketone handles for specific and orthogonal dye labeling of proteins (*49*). Fluorescently labeled nucleotide analogs can be used to site-specifically label DNA and RNA. The large repertoire of molecular biology techniques and the ability to label many different sites on the macromolecule's surface offer great flexibility in dye labeling and thus in the generality and applicability of single-molecule experiments.

Several approaches to SMD and SMS studies of biomolecules can be classified by the labeling schemes and the physical observables that are used (Fig. 1).

A simple, but powerful, use of SMD localizes a single fluorophore with a few tens of nanometers precision (Fig. 1A). The dimensions of a dye molecule are much smaller than the wavelength of light it emits, and therefore it acts as a point source of light. The response of the optical system to this point source (the point-spread-function, PSF) is a spot of light, the center of which can be localized with great accuracy. This localization precision has been used to follow the motion of individual motor proteins, the diffusional trajectories of labeled lipid molecules in membranes, and the diffusion of molecules in gels, in solutions, and at the liquid-solid interface (*19*, *21*, *22*, *50*–*52*).

This positioning accuracy can be further exploited for colocalizing two (or more) different macromolecules (Fig. 1B). When two macromolecules are labeled with two noninteracting fluorophores that differ in their optical properties (absorption and emission spectra, fluorescence lifetime, dipole orientation), they can be colocalized with nanometer accuracy (*53*–*55*) and can report on association, binding, and enzymatic-turnover events (*18*, *20*, *56*).

Even higher colocalization accuracy can be obtained when the two fluorophores interact by fluorescence resonance energy transfer (FRET) (*57*–*59*). This technique, capable of measuring distances on the 2- to 8-nm scale, relies on the distance-dependent energy transfer between a donor fluorophore and acceptor fluorophore. The technique not only has superior static colocalization capabilities but can also report on dynamical changes in the distance or orientation between the two fluorophores for intramolecular (Fig. 1C) and intermolecular FRET (Fig. 1D). Since the first measurement of energy transfer between a single donor and a single acceptor (single-pair FRET, or spFRET) (*60*), it has been used to study ligand-receptor colocalization (*56*), to probe equilibrium protein structural fluctuations and enzyme-substrate interactions during catalysis (*61*, *62*), and to identify conformational states and subpopulations of individual diffusing molecules in solutions (*63*, *64*).

The absorption and emission transition dipoles of single fluorophores can be determined by using polarized excitation light or by analyzing the emission polarization, or both. The temporal variation in dipole orientation of a rigidly attached (Fig. 1E) or rotationally diffusing tethered probe (Fig. 1F) can report on the angular motion of the macromolecule or one of its subunits (*10*, *16*, *65*–*67*). Rigid attachment of probes to macromolecules can be achieved by the use of hydrophobic pockets in proteins and intercalating dyes in nucleic acids. Alternatively, a fluorophore with two reactive groups (bisfunctionalized) can be immobilized on a macromolecule by a two-site covalent attachment, as in the case of two properly spaced engineered cysteines in a protein (*68*).

Chemical and electrostatic activity could be studied by monitoring changes in the rotational freedom of motion of a tethered fluorophore (*69*) (Fig. 1F). The various interactions between the fluorophore, the macromolecule, and the surrounding solvent determine to what extent the probe is free to rotate around its tether. Changes in conformation, charge, potential, redox state, hydropathy, local pH, steric interactions, and stability may result in changes in the fluorophore's rotational diffusion.

Changes in local environment can bring about not only changes in rotational diffu-

sion but also changes in the fluorophore's emission properties (for example, spectrum, intersystem crossing rate, and triplet state lifetime) that can also be monitored on the single-molecule level. In fact, fluorescence indicators are optimized to do exactly that: Their emission properties are very sensitive to changes in charge, potential, pH, and ion concentration. Conjugation of such indicators to enzymes or ion channels, close to an active site or to the channel pore, could report on the biomolecule's function (Fig. 1G).

More elaborate labeling schemes could combine two (or more) different measurements for one construct. For example, an ion channel could be labeled with a donor-acceptor pair and an ion indicator (Fig. 1H). spFRET could report on conformational dynamics of the protein while the indicator would simultaneously monitor the ion flux.

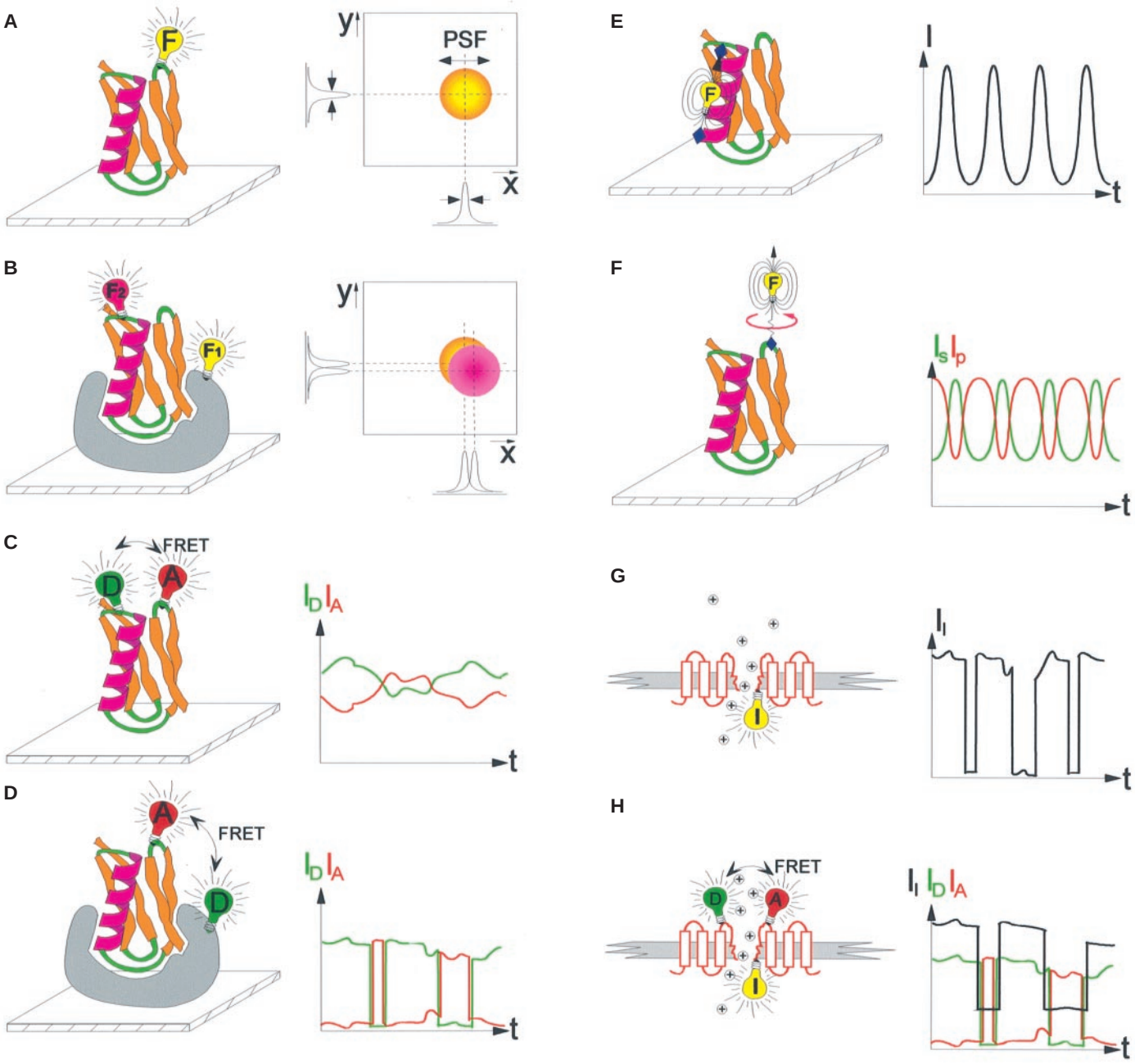

Fig. 1. Labeling schemes (left) and physical observables (right). (**A**) Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. (**B**) Colocalization of two macromolecules labeled with two noninteracting fluorophores, F_1 and F_2. Their distance can be measured by subtracting the center positions of the two PSFs. (**C**) Intramolecular detection of conformational changes by spFRET. D and A are donor and acceptor; I_D and I_A are donor and acceptor emission intensities; t is time. (**D**) Dynamic colocalization and detection of association or dissociation by intermolecular spFRET. Donor and acceptor intensities are anticorrelated both in (C) and (D). (**E**) The orientation of a single immobilized dipole can be determined by modulating the excitation polarization. The fluorescence emission follows the angle modulation. (**F**) The orientational freedom of motion of a tethered fluorophore can be measured by modulating the excitation polarization and analyzing the emission at orthogonal **s** and **p** polarization detectors. I_S and I_P are emission intensities of **s** and **p** detectors. (**G**) Ion channel labeled with a fluorescence indicator I. Fluctuations in its intensity I_I report on local ion concentration changes. (**H**) Combination of (C) and (G). D and A report on conformational changes whereas I reports on ion flux.

The challenges to realize such a construct are in the conjugation chemistry and in the choice of spectrally resolved fluorophores. It is currently very difficult to engineer two orthogonal chemistries for site-specific labeling of two fluorophores; adding a third will require major advances in protein chemistry.

Excitation and Detection Methods

The photons detected from single molecules must exceed the background noise. The background originates from Raman and Rayleigh scattering and from fluorescence as a result of impurities in the solvent, glass coverslips, and optical components, and from dark current in the detector. Single-molecule detection therefore calls for (i) a small excitation volume, to reduce the background; (ii) high-efficiency collection optics; (iii) the use of detectors with high quantum efficiency and low dark noise; and (iv) careful elimination of background fluorescence by various means such as a pinhole in the conjugate plane (reducing the detection volume), prebleaching of impurities in the solvent, and the use of very low-fluorescing optical materials. The two main implementations of these principles can be classified according to point detection and wide-field detection. Choosing one scheme over the other is very application-specific and needs careful consideration for optimal results.

Confocal microscopy and the detection of flowing molecules in a stream use a very small excitation volume (subfemtoliters for confocal, picoliters for flow experiments), a point detector such as an avalanche photodiode (APD) or a photomultiplier tube, and a pinhole to reject out-of-focus background light. Point detection methods have the advantage of high S/B ratio, microsecond temporal resolution (0.1-ns fluorescence lifetime resolution), and the ability to perform complicated spectroscopies on immobile molecules. However, they lack the ability to observe several mobile molecules at once.

Excitation by wide-field epi-illumination or TIR combined with CCD detection, on the other hand, use a somewhat larger excitation volume per resolvable spot and have lower (millisecond) temporal resolution but allow the parallel detection of many mobile molecules in the field of view. Wide-field epi-illumination allows for greater flexibility and handling of various samples but suffers from reduced S/B ratio because of the larger detection volume. TIR excitation has a better S/B ratio compared with the epi-illumination scheme, but only molecules within a few tens of nanometers to the glass surface can be detected (*70*).

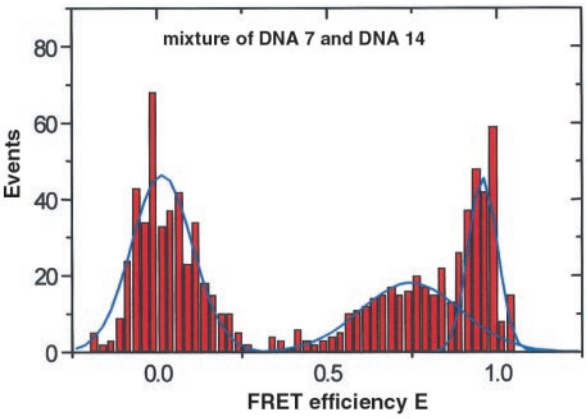

Fig. 2. FRET Histogram of a sample containing a 1:1 mixture of two different double-stranded DNA molecules with 7– and 14–base pair (bp) separation between donor and acceptor. The peak around zero results from faster photobleaching of acceptors (compared with that of donors), leaving donor-only–labeled molecules; the two peaks at energy transfer efficiency $E \sim 0.7$ (14-bp separation) and $E \sim 1$ (7-bp separation) demonstrate the ability to identify subpopulations according to their conformational states.

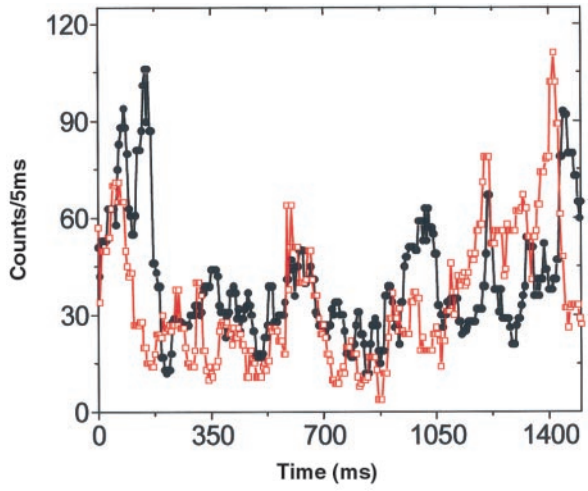

Fig. 3. Donor (red) and acceptor (black) emission time-traces of a doubly labeled SNase molecule with tetramethylrhodamine (TMR, donor) and Cy5 (acceptor) immobilized on glass in buffer. The large and gradual fluctuations in I_d and I_a report on protein structural fluctuations on the millisecond time scale.

Reaction Conditions

Another classification of SMD and SMS experiments is based on reaction conditions. Molecules can be freely diffusing or flowing in solutions or can be immobilized on surfaces or in gels. Also, the observation can be under equilibrium or nonequilibrium conditions.

Diffusing or flowing molecules, *equilibrium*. Samples consist of a small amount of analyte molecules in a liquid or flow cell. The laser excitation spot is focused in the solution, and photon bursts are collected when molecules traverse the beam. These bursts are too short to provide information on conformational dynamics. They can, however, provide invaluable knowledge of the distributions of molecular properties of interest, undisturbed by surface effects. Most importantly, subpopulations of analyte molecules in heterogeneous ensemble can be identified (*71*, *72*).

Most single-molecule burst studies have been limited to measuring distributions in burst size or in fluorescence lifetime (or both). With the help of two photon–counting detection channels, other molecular properties such as distributions in intramolecular distances (conformations), in spectral peak position, and in rotational degrees of freedom can be interrogated. Recently, Deniz *et al.* have demonstrated the ability to identify conformational states and subpopulations of individual macromolecules in a heterogeneous solution by ratiometric burst methods. They extended spFRET to measure distributions in energy-transfer efficiency of freely diffusing single molecules (*63*, *64*). A series of donor-acceptor DNA constructs with varying intramolecular fluorophore distances were used to measure the mean and the distribution width of FRET efficiencies as functions of distance. It was shown that subpopulations could be identified according to their conformational states (Fig. 2). Moreover, these results imply that single-molecule FRET measurements could be performed even when the sample is not purified. This is in contrast to conventional ensemble FRET studies, where great care must be taken to purify the complexes labeled with both donor and acceptor. Similar ratiometric burst methods have been used to measure distributions of polarization anisotropy and spectral-peak position of freely diffusing single molecules (*64*, *73*).

Diffusing or flowing molecules, *nonequilibrium*. Nonequilibrium conditions imply a heterogeneous population of reacting molecules. Identification of subpopulations according to molecular properties could therefore be used to follow the kinetics of biochemical reactions. For example, enzymatic conversion of substrate to product involves the time-dependent deple-

tion of one subpopulation and increase in the other. In a protein-folding reaction, unfolded molecules are converted into their native folds. In binding assays, the population of free ligands is reduced whereas the population of ligand-bound receptors is increased in time. In contrast to ensemble kinetics measurements, ratiometric burst techniques could monitor not only the average time behavior of the whole ensemble but also the full kinetics of each of the subpopulations. Moreover, reaction intermediates that are very difficult to detect in ensembles because of inhomogeneity and lack of synchronization could, in principle, be identified in single-molecule experiments, provided that their lifetime exceeds the time resolution of the technique.

Immobilized molecules, *equilibrium*. Distributions of molecular properties (for example, intramolecular distance, dipole orientation, orientational freedom of motion, and spectra) can also be measured for immobilized molecules under equilibrium conditions. In contrast to diffusing molecules, the same molecules could be probed several times in alternating environments (limited by photobleaching). This can be done by changing solvents, temperature, or potential, letting the molecules equilibrate after each change and acquiring spectroscopic data in each new environment. In this manner, a reaction could be followed at fixed equilibrium points along its pathway. A folding reaction, for example, could be followed by measuring changes in the FRET distribution as a function of denaturation conditions. Similarly, spectroscopic images of immobile molecules can probe the initial and final states of reactions such as binding and hybridization (*56*).

Single-molecule equilibrium measurements can also unravel dynamical information. Using intramolecular spFRET measurements on single staphylococcal nuclease (SNase) protein molecules, Ha *et al.* observed gradual fluctuations in FRET efficiencies. A combination of single-molecule polarization measurements, spectral fluctuation measurements, and simulations showed that the observed FRET-efficiency fluctuations originated from the conformational dynamics of the proteins themselves (*61*, *62*). Anticorrelated fluctuations in donor and acceptor emission time-traces reflected the protein's conformational dynamics (Fig. 3). They also studied protein-inhibitor binding by single-molecule polarization and spFRET and showed that these methodologies are sensitive enough to distinguish between the ligand-free and inhibitor-bound states of the enzyme (Fig. 4) (*61*). Such observations cannot be made in conventional ensemble studies; the fluctuations would be averaged out because of the lack of synchronization among molecules.

Immobilized molecules, *nonequilibrium*. When biomolecules are immobilized on surfaces or in gels, not only equilibrium fluctuations but also full time-trajectories of single-molecule reactions can be measured. Lu *et al.* monitored the enzymatic turnovers of flavinenzyme molecules, immobilized in an agarose gel, in real time by detecting changes in the native fluorescence from the active site of the enzyme (*41*). A more general approach might be the use of fluorescent tags to report on reaction trajectories. Energy transfer, dipole orientation, and emission spectrum of the fluorophores could then be used to observe single enzymes at work, single protein molecules unfolding, or ion channel pores opening and closing.

For example, spFRET can be used to measure single-enzyme catalysis (Fig. 5). If a nuclease (an enzyme that digests DNA and RNA) is labeled with a donor and acceptor, its catalytic activity can be monitored by measuring its intramolecular conformational changes (Fig. 5A). When the catalytic activity is not diffusion-limited (high substrate concentration), we expect to measure anticorrelated and quasi-periodic time-traces for donor and acceptor emissions, with a period corresponding to a single catalytic cycle. Such measurements could provide us with information on the catalytic rate, processivity, and turnover statistics of individual enzymes. Moreover, by repeating such measurements for many individual enzymes, it should be possible to obtain information on the distributions of time trajectories (*41*). In an intermolecular (enzyme-substrate) spFRET catalysis experiment (Fig. 5B), the enzyme is labeled with a donor molecule and the substrate is labeled with one (or multiple) acceptor molecule (or molecules). During catalysis we again expect to measure anticorrelated time-traces for donor and acceptor emissions. In the case of equally spaced acceptor molecules on a "DNA ruler," these time traces will be quasi-periodic. In addition to catalytic rate, processivity, and turnover statistics, these measurements can furnish data on association and dissociation rates of the substrate or product molecules. Intermolecular spFRET was recently used to study the interactions between single immobilized SNase proteins and single-strand DNA substrate molecules (*61*).

Immobilization of molecules affords not only the observation of spontaneous time-trajectories, as in diffusion-limited catalysis, but also the observation of the reaction pathway in response to a rapid, induced change. Stopped-flow, laser-induced temperature jump and photoactivation of caged molecules by flash photolysis can all be used to promptly trigger single-molecule reactions.

Future Improvements

Fluorescence spectroscopy of single biomolecules is here to stay. However, major improvements in bioconjugation chemistry, dye chemistry and photophysical properties, instrumentation and methodologies are still needed to perform better single-molecule experiments.

Conjugation chemistry. Many interesting protein-protein and protein-DNA interaction experiments can be constructed with existing conjugation chemistries. However, to take full advantage of single-molecule fluorescence techniques, it will be necessary to develop easier and more accessible chemistries for multiple, site-specific labeling. It was previously shown that unnatural amino acid mutagenesis can be used to selectively label proteins (*49*). A cysteine point mutation provides a thiol handle for conjugating to maleimides. A second, orthogonal, chemistry is provided by an unnatural keto-containing amino acid that can be introduced during in vitro protein synthesis with chemically aminoacylated suppressor tRNAs. The ketone handle can be labeled in high yield with hydrazide-containing fluorophores with no cross-reactivity (*49*). Other alternative approaches such as separately labeling two split inteins and then splicing them together (*74*) or differentiation of conjugation conditions for proteins containing two cysteines

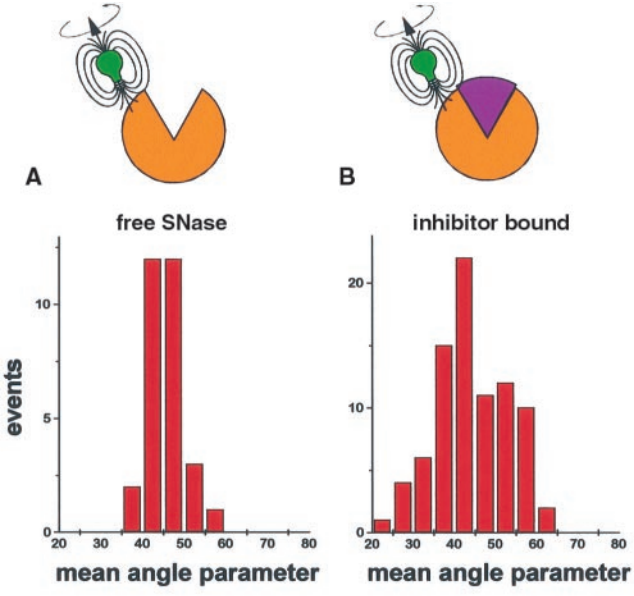

Fig. 4. Histograms of the angle parameter, an observable that reports on the degree of rotational diffusion of the fluorophore, for immobilized TMR-labeled SNase enzymes without (**A**) and with (**B**) inhibitor (dissociation constant K_d = 100 nM). Each data point in the histogram represents the time-averaged angle parameter for one molecule. (A) The narrow distribution is centered at 45° for uninhibited SNase, indicative of free and rapid rotation of the attached fluorophore. (B) A broader distribution for the inhibitor-bound SNase is indicative of hindered and fluctuating rotational behavior.

might also be sought.

Fluorophore photophysics and photochemistry. The ability to measure the biological activity of individual macromolecules by single-molecule fluorescence methods is greatly limited by the nonideal emission properties of the fluorophores. SMD and SMS techniques suffer from "fluorophore noise." A single fluorescent molecule can act as a very sensitive probe of its immediate local environment. However, uncontrolled or unknown changes in the environment can cause spectral diffusion, spectral jumps, changes in quantum efficiency, long-lived triplet states, "blinking" or long-lived dark states, rotational jumps, or quenching. Extreme care must therefore be taken to separate out fluorophore dynamics from the biological dynamics of interest.

Another main limitation is the finite duration of emission as a result of photobleaching. Photodestruction of fluorophores is one of the most important yet least understood processes that affect the application of fluorescence in biology. It often depends on the presence of molecular oxygen, which shortens the fluorophore's dark triplet excited state by quenching, producing the highly reactive singlet oxygen that then attacks the fluorophore and bleaches it. Removing oxygen will prolong the fluorophore lifetime with respect to photobleaching but at the same time will increase the triplet-state lifetime. Ideally, a triplet quencher reagent that does not affect photobleaching should be sought.

The development of better probes and the full photophysical characterization, on the single-molecule level, of existing dyes are crucial. A systematic study that will identify the best fluorophores for each specific set of (physiological) conditions, both regarding biological activity and emission properties, is greatly needed.

Methodologies and instrumentation. Obviously, this is a wide-open area. Various instrument designs, data acquisition, and corresponding analysis schemes are being explored by many single-molecule laboratories. Here we discuss two examples currently being pursued in our laboratory.

In spFRET measurements, the relative motion between the donor site and the acceptor site entails rotation and translation, both affecting the signal. Dipole rotation changes the amount of direct excitation by the laser and affects the orientational factor κ^2, thus altering the energy transfer efficiency. Translation alters both the total signal and the energy transfer efficiency attributable to the distance dependence (R^{-6}) of the dipole-dipole interaction. For many applications, it will be useful to distinguish rotation from translation. This is especially true when the fluorophores are rigidly attached to the macromolecule. The best strategy will be to combine polarization measurements with spFRET by separating the donor-acceptor pair emission both in polarization and in emission color and simultaneously recording the signals on four detectors.

A major disadvantage of single-molecule spectroscopy by point detection is that only one molecule can be observed at a time. For certain applications it would be very useful to monitor many macromolecules simultaneously and in parallel, by using wide-field methods. The time resolution afforded by current CCD technology, however, does not match that of APD point detectors. The development of high–quantum yield, low-noise cameras with faster read-out rate would be of great use.

More generally, the development of single-molecule commercial instruments that are user-friendly and automated, and utilize robust methodologies and easy-to-use data analysis algorithms, should make the field accessible to many interested researchers.

Outlook

With the aid of single-molecule manipulation techniques such as patch-clamp, atomic force microscopy, and optical and magnetic tweezers, ionic current fluctuations in individual ion channels have been measured; overstretching and supercoiling of single DNA molecules have been studied; forces and displacements generated during single molecular motor reactions have been observed, and proteins were mechanically unfolded. Some of these manipulation methods are reviewed elsewhere in this issue (*75*, *76*). These techniques are ideally suited for the study of mechanical, chemical, and electrical properties and for molecular mechanisms and functions of macromolecules. They do not, however, provide local, dynamical structural information.

By performing single-molecule fluorescence measurements together with one (or more) of the manipulation techniques, it will be possible to simultaneously monitor several different observables of biochemical reactions. It should be possible to correlate, for example, conformational changes with ionic-current fluctuations in a single ion channel, conformational changes and force production of motor proteins with ATP hydrolysis, and conformational changes and displacements with polymerization or digestion of various processive nucleic acid enzymes such as DNA and RNA polymerases and nucleases.

Ishijima *et al.* have already demonstrated the marriage between fluorescence imaging and force measurements. They observed ATP hydrolysis simultanously with the mechanical response of single myosin molecules during force generation (*77*). Their experiment points to the main difficulty in the single-molecule fluorescence-manipulation marriage—the incompatibility in time scales. A single molecule can be manipulated and studied for hours. Fluorescence tags, however, bleach in a few seconds. Automation and rapid sample exchange need to be developed to allow this marriage to flourish.

To give an outlook and flavor for what might be achievable in the future, a few examples based on previous single-molecule manipulation work are presented in cartoons (Fig. 6). DNA stretching by optical tweezers (*78*) could be combined with spFRET (Fig. 6A). Simultaneous

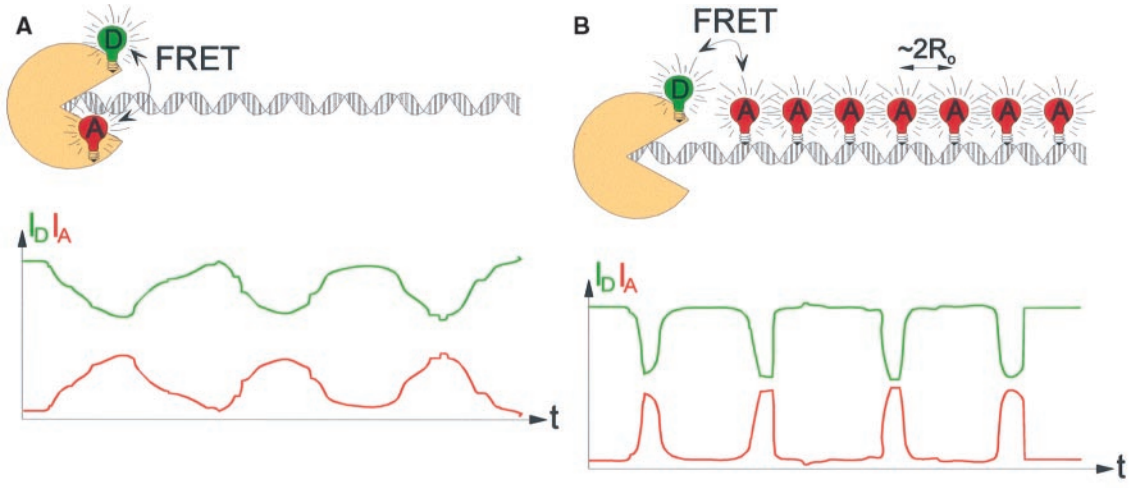

Fig. 5. A cartoon illustrating (**A**) intramolecular and (**B**) intermolecular spFRET nuclease-DNA interactions. Intramolecular spFRET measures conformational dynamics of the enzyme during catalysis. Intermolecular spFRET measures association, catalysis, and dissociation of substrate molecules. Multiple acceptors at equal distances on the DNA act as a "ruler." R_0 is the Förster radius (distance at which 50% of the energy is transferred). This scheme can be generalized to many other protein-DNA interactions.

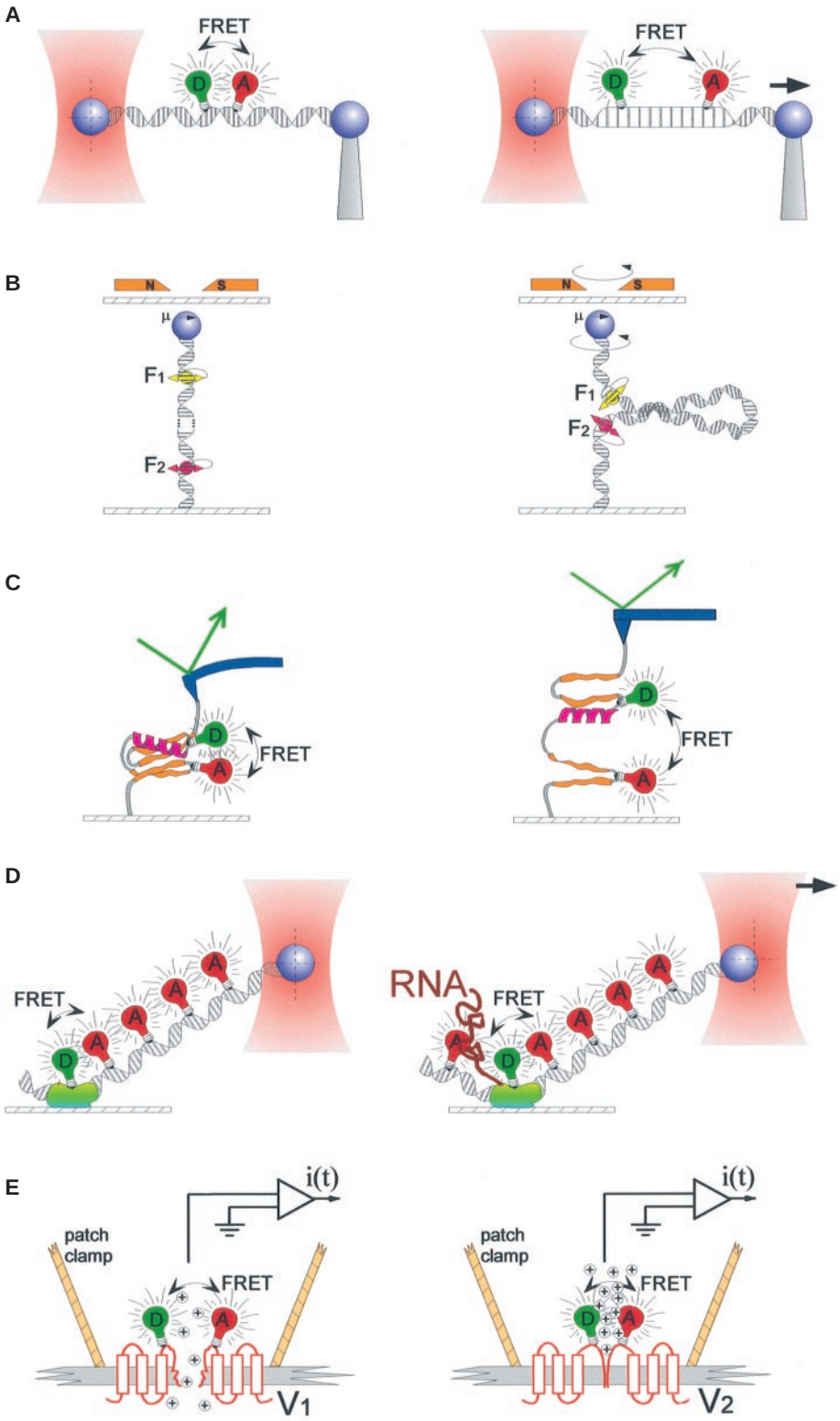

Fig. 6. An outlook to possible future experiments that combine single-molecule manipulation and single-molecule fluorescence spectroscopy techniques. Such measurements will allow correlation of local structural changes with global macromolecule function or response to an external stimulus. Left and right panels show two different time points in the experiment. (**A**) DNA mechanical stretching together with spFRET. (**B**) DNA mechanical coiling together with dipole orientation measurement. (**C**) Protein mechanical unfolding together with spFRET. (**D**) Monitoring movements and forces during transcription by laser tweezers and spFRET. (**E**) Single-channel recording by patch-clamp together with spFRET. The ionic current measured by the patch-clamp is represented by $i(t)$.

force-extension curves and spFRET time traces will allow the correlation of the global overstretching response with local conformational changes. Similarly, dipole orientation measurements could be used to correlate the local twisting of a DNA molecule with its global response to coiling (*79*). Two different-colored tethered intercalating dyes could be used to measure the relative rotation between two sites and possibly the formation of denaturation bubbles in response to mechanical coiling of DNA (Fig. 6B). Combining mechanical unfolding of proteins (*80*–*82*) with spFRET will allow the correlation of the global stretching response with local structural changes (Fig. 6C). The use of optical tweezers and accurate bead positioning in the study of molecular motors and other linear mechanoenzymes was pioneered by the Block group (*83*). Tweezer measurements not only provide information on the molecular basis for movement but are also ideal for studying processive nucleic acid and amino acid enzymes including gyrases, polymerases, nucleases, topoisomerases, and ribosomes. A combined optical tweezers and spFRET "DNA ruler" experiment could be conceived (Fig. 6D). The movements and possibly the forces generated during transcription are DNA sequence–dependent and might be relevant to the control of initiation, elongation, and termination. spFRET can complement force measurements and correlate local, sequence-dependent information with pauses in transcription. Lastly, single-molecule fluorescence techniques may allow researchers to correlate conformational changes accompanying the activation of an ion channel (*84*) with ionic flux measurements by the patch-clamp technique (Fig. 6E).

Single-molecule fluorescence detection and spectroscopy holds great promise for enhancing our understanding of biological macromolecules and their structure-function relations. The biggest challenge, however, will be to tame these methodologies for the study of individual and rare biological processes in the living cell. Current dye-based fluorescent technologies do not stand up to the challenge. The development of new fluorescent probes with superior photophysical properties is needed. The recently developed semiconductor nanocrystal probes (*85*, *86*) might measure up to the venture. In a few years, fluorescence SMD and SMS will probably find their way not only into cutting-edge biological research, but also into the biotechnology and analytical chemistry industries (*87*).

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87. Two drug-discovery companies are already promoting fluorescence correlation spectroscopy (FCS) and single-molecule detection as a means for high-throughput screening (Evotec and Molecular Machines).

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REVIEW

Nanoscale Science of Single Molecules Using Local Probes

James K. Gimzewski[1]* and Christian Joachim[2]

Experiments on individual molecules using scanning probe microscopies have demonstrated an exciting diversity of physical, chemical, mechanical, and electronic phenomena. They have permitted deeper insight into the quantum electronics of molecular systems and have provided unique information on their conformational and mechanical properties. Concomitant developments in experimentation and theory have allowed a diverse range of molecules to be studied, varying in complexity from simple diatomics to biomolecular systems. At the level of an individual molecule, the interplays of mechanical and electronical behavior and chemical properties manifest themselves in an unusually clear manner. In revealing the crucial role of thermal, stochastic, and quantum-tunneling processes, they suggest that dynamics is inescapable and may play a decisive role in the evolution of nanotechnology.

In 1952, Erwin Schrödinger wrote that we would never experiment with just one electron, atom, or molecule (*1*). Eight years later, Richard P. Feynman told us that there are no physical limitations to arranging atoms the way we want (*2*). By the early 1980s, scanning tunneling microscopy (STM) (*3*) radically changed the ways we interacted with and even regarded single atoms and molecules. The very nature of proximal probe methods encourages exploration of the nanoworld beyond conventional microscopic imaging. Scanned probes now allow us to perform "engineering" operations on single molecules, atoms, and bonds, thereby providing a tool that operates at the ultimate limits of fabrication. They have also enabled exploration of molecular properties on an individual nonstatistical basis.

Molecules represent an amazingly diverse range of structures and associated properties. Their complexity increases through the fields