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Bridging Conformational Dynamics and Function Using Single-Molecule Spectroscopy

Review

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In a typical structure-function relation study, the primary structure of proteins or nucleic acids is changed by mutagenesis and its functional effect is measured via biochemical means. Single-molecule spectroscopy has begun to give a whole new meaning to the "structure-function relation" by measuring the real-time conformational changes of individual biological macromolecules while they are functioning. This review discusses a few recent examples: untangling internal chemistry and conformational dynamics of a ribozyme, branch migration landscape of a Holliday junction at a single-step resolution, tRNA selection and dynamics in a ribosome, repetitive shuttling and snapback of a helicase, and discrete rotation of an ATP synthase.

There is compelling need for new tools to probe the relationship between structure and function of biological macromolecules. Whereas high-resolution structural tools such as X-ray crystallography and nuclear magnetic resonance provide snapshots at atomic detail, they do not always tell us what the molecule does and how it does it. Biochemical techniques provide valuable functional information, and in combination, these structural and biochemical tools have helped us deepen our understanding of living systems at the molecular level. Ideally, one would like to observe the structural dynamics and function simultaneously so that a direct correlation can be made between the two. Single-molecule fluorescence spectroscopy has proven to be up to this challenge by measuring the real-time conformational changes of individual molecules of DNA, RNA, and protein during their function.

Single-molecule studies have also revealed that nominally identical molecules can behave very differently from each other even under almost identical conditions. Such heterogeneity used to garner much interest and is fascinating in its own right, but for most cases its functional significance was unclear and strong proof that it was not a measurement artifact was lacking. Both technical improvements and closer ties to biology led to a flurry of recent studies which go well beyond the sort pronouncing that "the most interesting observation made in these first single-molecule experiments on System X is that single molecules are very heterogeneous!" This review is about a small collection of these studies that exhibit the virtue of single-molecule spectroscopy in providing previously unattainable data on biological mechanisms.

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In the first two sections on the hairpin ribozyme and Holliday junction, two-state fluctuations were observed due to docking/undocking reactions and stacking conformer dynamics, respectively. Interestingly, their "ticking" rates were not constant during the measurements but switched between two different sets of values. It is as though the molecule has two internal clocks that run at different speeds and it uses one clock for a while and then switches to the other and back, and it goes on. This so-called memory effect, where a molecule seems to remember which clock to use, was first reported in single-molecule fluorescence studies on the enzymatic turnover of cholesterol oxidase (Lu et al., 1998). In the new study reviewed here, the underlying mechanisms of switching between the clocks were unambiguously shown: cleavage and ligation reactions for the hairpin ribozyme (Nahas et al., 2004) and single-step branch migration for the Holliday junction (McKinney et al., 2005).

The third section reviews the tRNA dynamics in the ribosome where transient intermediates toward tRNA accommodation were clearly observed for the first time (Blanchard et al., 2004a, 2004b). The fourth section concerns the ATP-powered movement of a helicase protein on a single-stranded DNA (ssDNA). When the protein encountered an insurmountable obstacle, it was observed to employ a series of acrobatic moves to return to where it began and repeat the process over and over again, an unexpected finding which points to a potential new function of keeping ssDNA clear of unwanted proteins (Myong et al., 2005). The final section is about the direct measurement of discrete rotational steps of a protein complex called F_0F_1-ATP synthase during ATP synthesis (Diez et al., 2004; Zimmermann et al., 2005). Both the direction of rotation (clockwise versus anticlockwise) and the asymmetry in dwell times for three different rotor angles could be determined.

In all five studies, the primary signal was single-molecule fluorescence resonance energy transfer (FRET) (Ha, 2001), an excellent tool for measuring relative distance changes between two fluorophores, donor and acceptor, attached to specific sites on the macromolecules (Weiss, 1999). It should be noted, however, that there are other observables in single-molecule fluorescence measurements, notably intensity/lifetime (Laurence et al., 2005; Lu et al., 1998; Yang et al., 2003) and polarization response (Forkey et al., 2003; Nishizaka et al., 2004; Sosa et al., 2001).

Untangling Internal Chemistry and Conformational Dynamics of an RNA Enzyme

The hairpin ribozyme is an RNA enzyme that carries out site-specific self-cleavage and ligation of viral RNA. The ribozyme has modular architecture built of a four-way junction and two adjacent loops on the two arms of the junction (Figure 1A). The catalytic site is formed by docking the two loops onto each other, stabilized by divalent metal ions. The four-way junction is considered a folding enhancer because docking is accelerated 500-fold due to the junction-derived fluctuations within the undocked state that brings the two loops close to

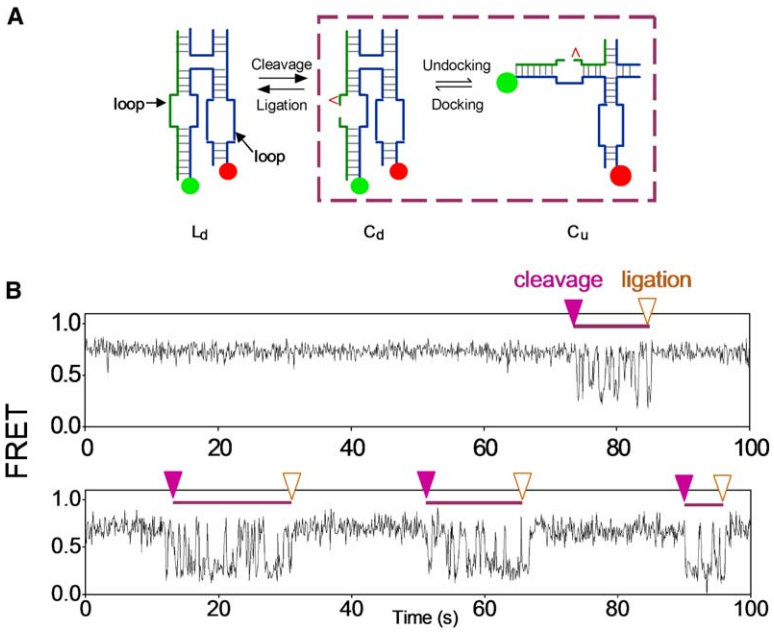

Figure 1. Single-Molecule FRET-Based Detection of Cleavage and Ligation Reactions of Hairpin Ribozyme

(A) Structure and labeling of the hairpin ribozyme. The hairpin ribozyme has a four-way junction and two loops. Fluorescent labels (donor in green and acceptor in red) are attached at the termini of the two arms with loops. The cleavage and ligation reactions do not lead to measurable change in FRET. Instead, they cause a large change in docking and undocking rates.

(B) At 1 mM magnesium, FRET traces show a stably docked state (high FRET) interspersed by rapidly fluctuating periods due to the cleaved state. Inverted triangles mark the moments of cleavage and ligation. Adapted from Nahas et al., 2004, Copyright 2004 Nature Publishing Group.

each other repeatedly (Tan et al., 2003). In the single-molecule studies, the ribozyme is labeled with a donor and an acceptor at the ends of the two loop-carrying arms so that the high FRET and low FRET signals would represent the docked and undocked states, respectively (Figure 1A). Cleavage and ligation would occur only within the high FRET docked state. In typical biochemical studies of the cleavage reaction, the product RNA piece once cleaved from the ribozyme no longer binds to the ribozyme tightly and quickly dissociates, effectively rendering the reaction irreversible. In order to observe multiple cleavage/ligation cycles, Nahas et al. (2004) lengthened the RNA piece, which allows the cleavage product to remain bound through 7 bp.

At 1 mM magnesium, most molecules were found in a stably docked state (high FRET) that was interspersed with bursts of rapid docking and undocking (Figure 1B). These bursts were a striking contrast to the steady high FRET signal obtained from the noncleavable mutant (Tan et al., 2003). From this comparison it was hypothesized that the stably docked form is the ligated ribozyme and that the rapidly fluctuating form corresponds to the cleaved ribozyme.

To test this hypothesis, ribozymes that had not been previously exposed to magnesium ions (and should be in the ligated form) were immobilized onto a quartz surface (Figure 2A). When magnesium ions were added via flow, the ribozymes went from an undocked state to a stably docked state (Figure 2B). Only later were rapid fluctuations observed, likely as a result of cleavage. Therefore, the stably docked form was assigned to the ligated ribozyme. In the second control experiment, ribozymes which initially had a short cleavage substrate were exposed to magnesium, resulting in a cleavage reaction and quick dissociation of the product. This left the ribozyme with an impaired loop structure which is unable to fold and hence exhibited a low FRET signal. These ribozymes were observed while a buffer containing a high concentration of long (7 bp) cleavage product was flowed in. When this long ligatable product strand bound to the ribozyme, a switch from low FRET to rapidly fluctuating behavior was observed followed by stable high FRET (Figures 2C and 2D). Because the ribozyme must be in the cleaved form immediately after the long product strand binds, the rapidly fluctuating FRET signal was assigned to the ribozyme in the cleaved form. Thus, it is now possible to record the exact moment when the cleavage or ligation reaction occurs via the changes in the docking/undocking kinetics (marked by inverted triangles in Figures 1 and 2). This opens up exciting new opportunities to carry out single-molecule enzymology in the most direct way, where the effects of mutations or solution conditions on the internal chemistry can be examined without complications arising from conformational degrees of freedom. For example, Nahas et al. determined the rates of internal cleavage and ligation reactions as a function of pH (Nahas et al., 2004), which provides support for general acid-base catalysis (Lilley, 2005).

Why is undocking accelerated so much upon cleavage? Nahas et al. (2004) speculated that rapid undocking after cleavage may be used to present the cleavage product for the next step in the replication of the viral RNA instead of allowing religation to occur. Surprisingly, such acceleration was not observed if the cleavage product contained a nonnatural terminus (Zhuang et al., 2002), a commonly used approach to approximate the cleaved ribozyme. Thus, it has to be recognized that alterations to the hairpin ribozyme (and possibly other ribozymes) can affect both folding and catalytic properties, and the ability to probe the internal chemistry directly as demonstrated by Nahas et al. (2004) should be valuable. The power of single-molecule FRET spectroscopy in RNA folding and catalysis studies has been demonstrated in a number of systems (Bokinsky et al., 2003; Ha et al., 1999; Hodak et al., 2005; Tan et al., 2003; Xie et al., 2004; Zhuang et al., 2000, 2002) and reviewed (Zhuang, 2005).

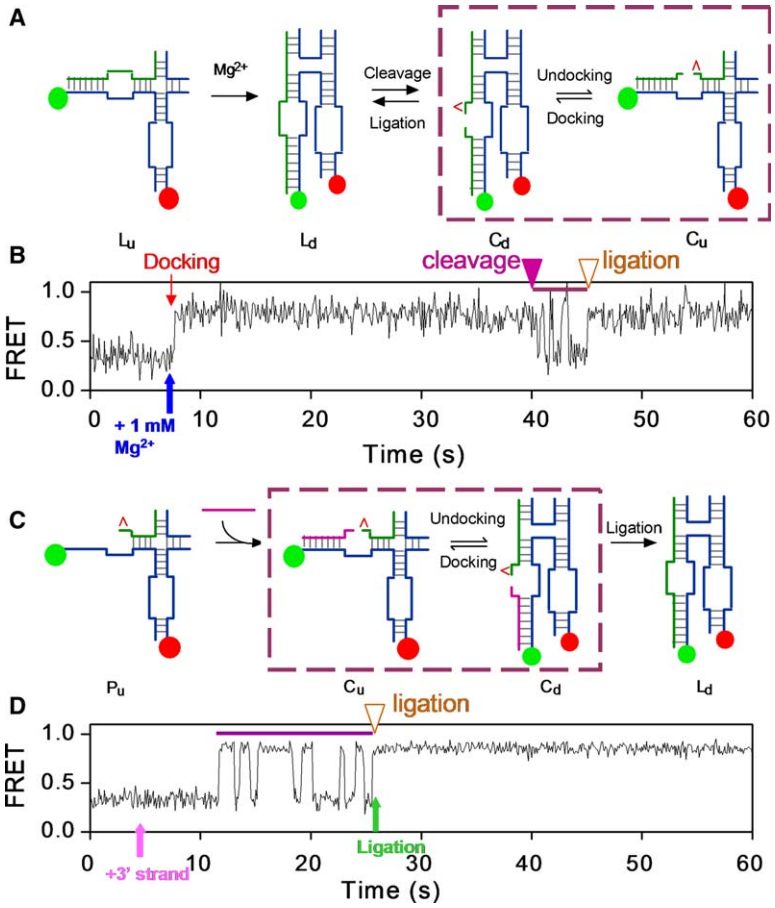

Figure 2. Assignments of Ligated and Cleaved Hairpin Ribozyme

(A and B) As magnesium ions are added to a ligated ribozyme, the ribozyme first changes into the stable high FRET state, indicating that the stably docked state represents the ligated form. At a later time, the molecule shows a brief period of rapid docking and undocking events due to the cleavage and ligation reactions.

(C and D) Ligation is directly observed as a long ligatable substrate binds to the cleaved ribozyme missing the cleavage product. The molecule starts in the low FRET state, it starts to dock and undock rapidly as the substrate is added, and then docks stably. The data confirm that the rapid docking/undocking phase is due to the cleavage ribozyme. Adapted from Nahas et al., 2004, Copyright 2004 Nature Publishing Group.

Single-Step Branch Migration of a Holliday Junction

In this study (McKinney et al., 2005), the DNA four-way (Holliday) junction was observed as it underwent spontaneous branch migration. The Holliday junction is formed when two DNA molecules exchange strands during homologous recombination, an important process in maintaining genome stability and diversity. These junctions are capable of spontaneous branch migration because of sequence homology, but almost all structural studies have so far focused on nonhomologous junctions whose sequences forbid branch migration. In the absence of metal ions, the junction adopts an "open structure," where the four helices are pointing to four corners of a square. In the presence of divalent metal ions, the junction folds into a more compact "stacked X-structure" (Murchie et al., 1989), where two possible conformers are in continual exchange through the open intermediate (Joo et al., 2004; McKinney et al., 2003; Miick et al., 1997; Overmars and Altona, 1997). This junction folding also inhibits spontaneous branch migration of homologous junctions, implicating the open structure as an intermediate for branch migration (Panyutin and Hsieh, 1994). Prior to the current study, it was not known whether structural knowledge obtained from junctions with a fixed branch point would apply to homologous junctions. That is, would a homologous junction behave just like nonhomologous junctions while it is residing in a single branch point?

To address this question, McKinney et al. (2005) designed the monomigratable junction, whose sequence allows only a single step of branch migration between two branch points termed U and M (Figure 3A). The strands were labeled with two fluorophores attached to the ends of the two adjacent arms that would show low and high FRET differentiating two stacking conformations, *isoI* and *isoII*, respectively (Figure 3A).

Single-molecule traces displayed fluctuations between four states which could be grouped into two phases, slow- and fast-fluctuating (Figure 3B). Single molecules were observed to switch between the two distinct phases, and the switching was interpreted as the moment branch migration occurred (vertical arrows in Figure 3B) because such behavior was never observed in junctions with a fixed branch point. Hydroxyl radical cleavage probing confirmed this interpretation and assigned the two phases to definite locations of the branch point (slow phase for U and fast phase for M; Figures 3A and 3B).

Adopting a physical metaphor, the junction can be thought of as a pendulum with a defined frequency, and branch migration as a spring linking two pendulums. If one pendulum is induced to swing, the energy eventually flows into the second pendulum and back and so on. If the coupling is weak, each pendulum swings multiple times, more or less behaving as an independent pendulum, until eventually energy sloshes to the other one. It turns out that the monomigratable junctions studied here retained within each branch point the hallmark behavior of nonhomologous junctions: two-state fluctuations whose overall rates, but not their relative

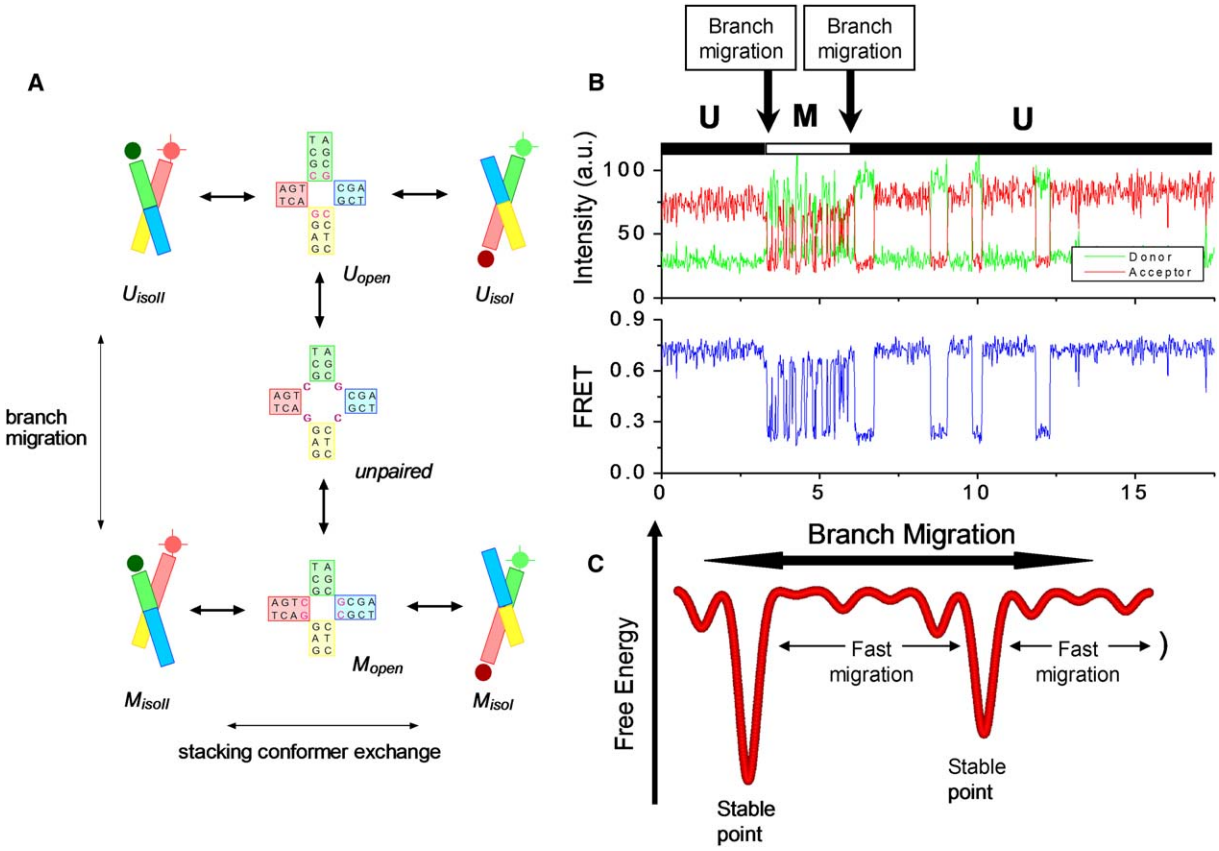

Figure 3. Observation of Holliday Junction Branch Migration

(A) A monomigratable junction was designed to allow a single step of branch migration. Donor and acceptor dyes are attached to the ends of two adjacent arms of the junction where low FRET and high FRET would differentiate *isoI* and *isoII*, respectively.

(B) The fast fluctuating phase represents the branch migrated state as confirmed by hydroxyl radical cleavage assay.

(C) Energy landscape for spontaneous branch migration is illustrated as having rapidly branch migratable regions bounded by very stable branch points. Adapted from McKinney et al., 2005, Copyright 2005 National Academy of Sciences, U.S.A.

populations, depend on magnesium concentrations. Thus, we can conclude that "migrability" is a weak perturbation. On average, the junctions displayed approximately ten oscillations before branch migration but the exact number depended on whether GC versus AT base pairs are broken during branch migration.

One surprise was that the different branch points could have up to 30-fold variations in their lifetimes, as illustrated in Figure 3C. Thus, spontaneous branch migration may occur very rapidly in a local segment bounded by very stable branch points. A similar conclusion was also reached by a recent single-molecule study of junctions that can migrate over several different branch points (Karymov et al., 2005). In vivo, junctions can quickly find stable branch points if they are transiently free of protein, possibly affecting the outcome of homologous recombination. The new knowledge on the dynamic properties of the naked junction should help design and interpret future studies of enzymes that recognize and process the Holliday junction.

tRNA Dynamics in Ribosomes

Ribosomes make protein using mRNA as a template. In bacteria, the ribosome is made of two subunits, 30S and 50S (Figure 4). The mRNA binds to the 30S such that the codon in mRNA is recognized by base pairing to the anticodon in the tRNA that brings an amino acid to be linked to the growing peptide. Using single-molecule FRET between tRNA molecules in the aminoacyl (A) and peptidyl (P) sites (Figure 4), Blanchard et al. (2004a) probed the mechanism for the very high translation fidelity which cannot be explained solely by codon/anticodon binding energy differences between the cognate and noncognate tRNA (Rodnina and Wintermeyer, 2001).

Ribosome complexes that contain a donor-labeled tRNA at the P site were formed on biotinylated mRNA (Blanchard et al., 2004b) that had been specifically bound to a passivated, PEG-covered surface (Ha et al., 2002; Rasnik et al., 2004; Figure 4). Then, ternary complexes containing an acceptor-labeled aminoacyl tRNA, EF-Tu (a GTPase), and GTP were flowed into the sample as single-molecule FRET trajectories were being recorded during the accommodation of the ternary complex at the A site. Because the moment of the ternary complex binding varied between single-molecule traces, a postsynchronization technique was employed wherein many FRET traces were synchronized at the first time FRET efficiency went above an established noise threshold of 0.25 (Figure 5A; Blanchard et al., 2004a).

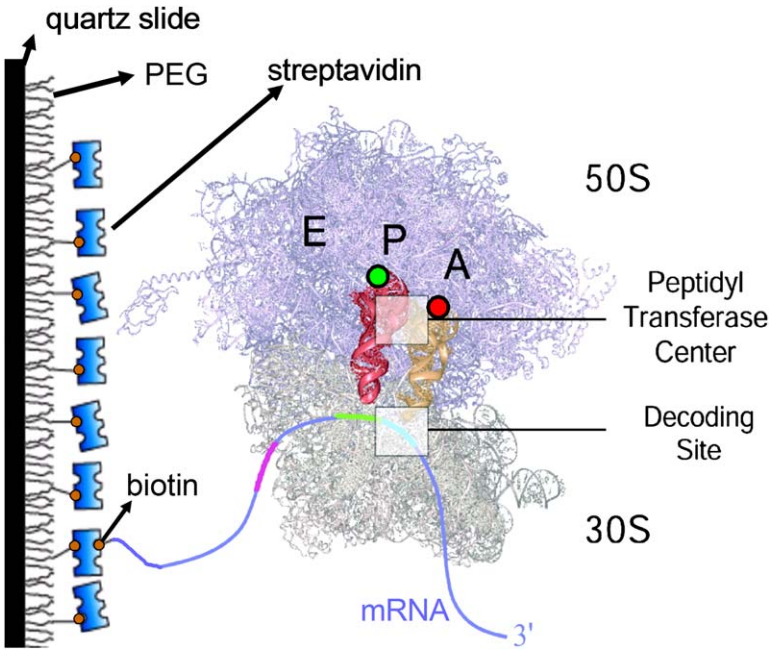

Figure 4. Ribosome with tRNA and mRNA for Single-Molecule Measurements

The 70S ribosome is bound to 5′-biotinylated mRNA and is shown with tRNAs. Fluorescence labels on the tRNAs are marked approximately with green (donor) and red (acceptor) circles. The 50S and 30S subunits are purple and gray, respectively. Regions of the deacylated-tRNA (E), peptidyl-tRNA (P), and aa-tRNA binding sites (A) are also shown. The whole construct is tethered to a PEGylated quartz surface via biotin-streptavidin binding. Adapted from Blanchard et al., 2004b, Copyright 2004 National Academy of Sciences, U.S.A.

For cognate tRNAs, single-molecule traces transited through low and intermediate FRET (0.3–0.5) to high FRET (0.6 and above) as the ternary complex accommodated into the A site. The antibiotic tetracycline was found to allow initial binding and codon recognition, but not full accommodation; a short-lived FRET state of 0.35 was observed. Replacing GTP with a nonhydrolyzable analog GDPNP stalled the complex in a longer lived (~8 s) 0.5 FRET state, indicating the formation of more contacts between the ternary complex and the ribosome. Cleavage of the sarcin-ricin loop, part of the GTPase activating center (GAC), known to play a role in tRNA selection, also stalled the reaction at the 0.5 FRET state. Near-cognate (one-base mismatch) tRNAs tend to dissociate from the ribosome from the low and intermediate FRET states rather than progressing further in accommodation. The ratio of probabilities with which cognate and near-cognate tRNA progressed from low to intermediate FRET and then intermediate to high FRET was compared to assess the fidelity of the system. This analysis revealed that the two previously established proofreading steps in tRNA selection corresponded to the branch points in the FRET data; cognate tRNA tends to progress through these branch points, whereas near-cognate tRNA tends to dissociate from the ribosome. Thus, initial selection and proofreading steps could be directly related to FRET transitions and therefore tRNA movements within the A site. These data were used to estimate the fidelity of tRNA selection at roughly 1 miscoding error in 5000.

Based on these data, a model of tRNA selection was proposed (Blanchard et al., 2004a; Figure 5C). In step 0, the P site is occupied by peptidyl-tRNA. In step 1, the ternary complex approaches the ribosome and FRET is 0. In step 2, the ternary complex interacts with mRNA; this gives 0.35 FRET, representing the codon-anticodon recognition event. In step 3, the ternary complex moves the aa-tRNA closer to the P site (0.5 FRET) and is ready for GTP hydrolysis. In step 4, GTP is hydrolyzed by the interaction of EF-Tu with the GAC. In step 5, inorganic phosphate is released and EF-Tu has changed to the GDP-bound form. In step 6, the 3′ end of the aa-tRNA has accommodated at the peptidyl transferase center and FRET increases to 0.75. In step 7, peptide bond formation occurs.

Blanchard et al. also analyzed what happens *after* proper accommodation of the A site tRNA (Blanchard et al., 2004b). Interestingly, they observed FRET fluctuations between two values, 0.74 and 0.45 (Figure 5B; note the time scale change from Figure 5A). The 0.74 FRET peak was attributed to the "classical state" with both the anticodons and the 3′ ends of tRNAs in the A and P sites. The 0.45 FRET state was assigned to the "hybrid state" where the anticodons stay in the A and P sites on 30S but the 3′ ends move to the P and E (exit) sites. After accommodation at the A site, fluctuation between classical and hybrid states occurred with roughly similar populations in each state. However, the lifetime of the classical state was reduced 6-fold by peptide bond formation which therefore increases the probability of the ribosome being in the hybrid state and readying the ribosome for the next step of ribosome translocation.

The ribosome is one of the most complex systems studied so far by single-molecule spectroscopy, and there is great potential to look at even more details by combining new structural information and biochemical approaches. For example, the ribosome itself can be labeled by inserting loops in the ribosomal RNA for hybridization of a fluorescently labeled oligomer (Dorywalska et al., 2005) or by attaching fluorophores to specific locations on the recombinant ribosomal proteins and reconstituting the functional ribosome (Majumdar et al., 2005). It may not be too far in the future when we are treated to real-time movies of single ribosomes in the process of protein synthesis.

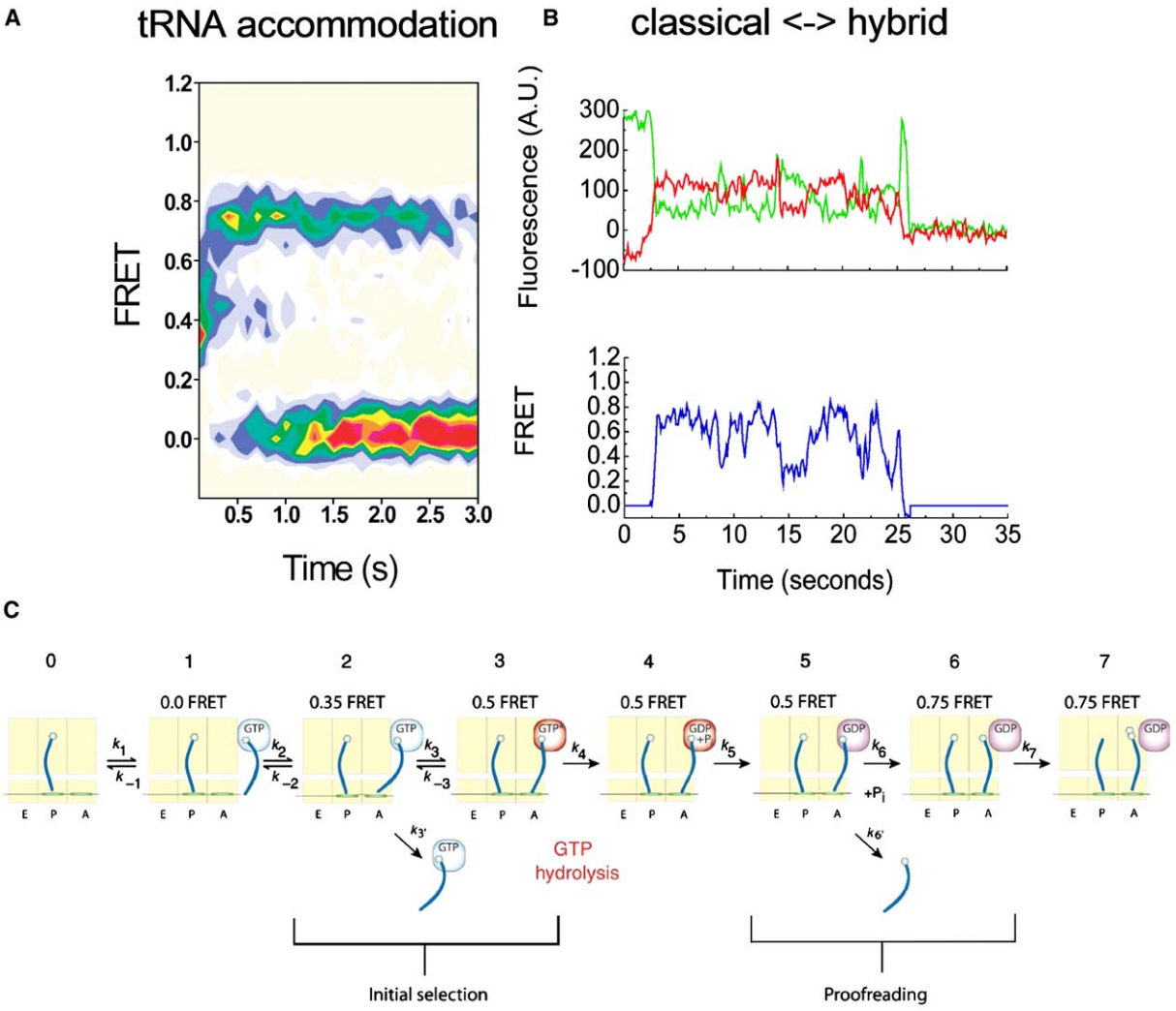

Figure 5. Kinetic Dissection of tRNA Accommodation and Classical-Hybrid Transitions

(A) Postsynchronized single-molecule traces are combined into a contour plot of FRET populations versus time. Cognate aa-tRNAs undergo a selection step at 0.35 FRET before accommodation.

(B) Single-molecule FRET time traces show the accommodation of the acceptor-labeled tRNA into the A site, followed by FRET fluctuations attributed to transitions between classical and hybrid states.

(C) A model for tRNA selection, proofreading, and movement into the accommodated (high FRET) state, as described in the text. Adapted from Blanchard et al., 2004b, Copyright 2004 National Academy of Sciences, U.S.A. and Blanchard et al., 2004a, Copyright 2004 Nature Publishing Group.

Repetitive Shuttling of a Helicase on ssDNA

Helicases, first discovered as "DNA unzipping enzymes," participate in virtually all cellular processes involving nucleic acids. The engine underlying their various functions is the ability to move (or translocate) on single-stranded DNA or RNA (von Hippel, 2004). For example, Rep is an *Escherichia coli* helicase that undergoes ATP-powered ssDNA translocation in the 3′ to 5′ direction as a monomer (Brendza et al., 2005) even though a monomer alone cannot unwind DNA (Cheng et al., 2001; Ha et al., 2002). In order to probe ssDNA translocation directly, Myong et al. (2005) engineered single-cysteine mutants of Rep (Rasnik et al., 2004) and labeled them with the donor fluorophore Cy3, and their movement on an acceptor (Cy5)-labeled DNA was detected via single-molecule FRET. 3′-tailed partial duplex DNA with an acceptor at the junction was attached to a polymer-coated surface at the duplex end and data were obtained with 300 pM of Cy3-labeled Rep and ATP in solution with 15 ms time resolution (Figure 6A).

When the labeled Rep bound the DNA, the donor fluorescence signal rose abruptly, combined with a weak acceptor signal (Figure 6B). This was followed by a gradual increase in FRET indicated by a decrease in donor signal and a concomitant gradual increase in acceptor signal, consistent with ssDNA translocation in the 3′ to 5′ direction toward the junction. In contrast to expected dissociation at the junction, however, there was an instantaneous FRET decrease to near the initial value (Figure 6B), which is succeeded by further cycles of gradual increase and sudden decrease in FRET. This sawtooth-like pattern was interpreted as repeated cycles of ssDNA translocation followed by snapback of a Rep monomer, and termed "repetitive shuttling."

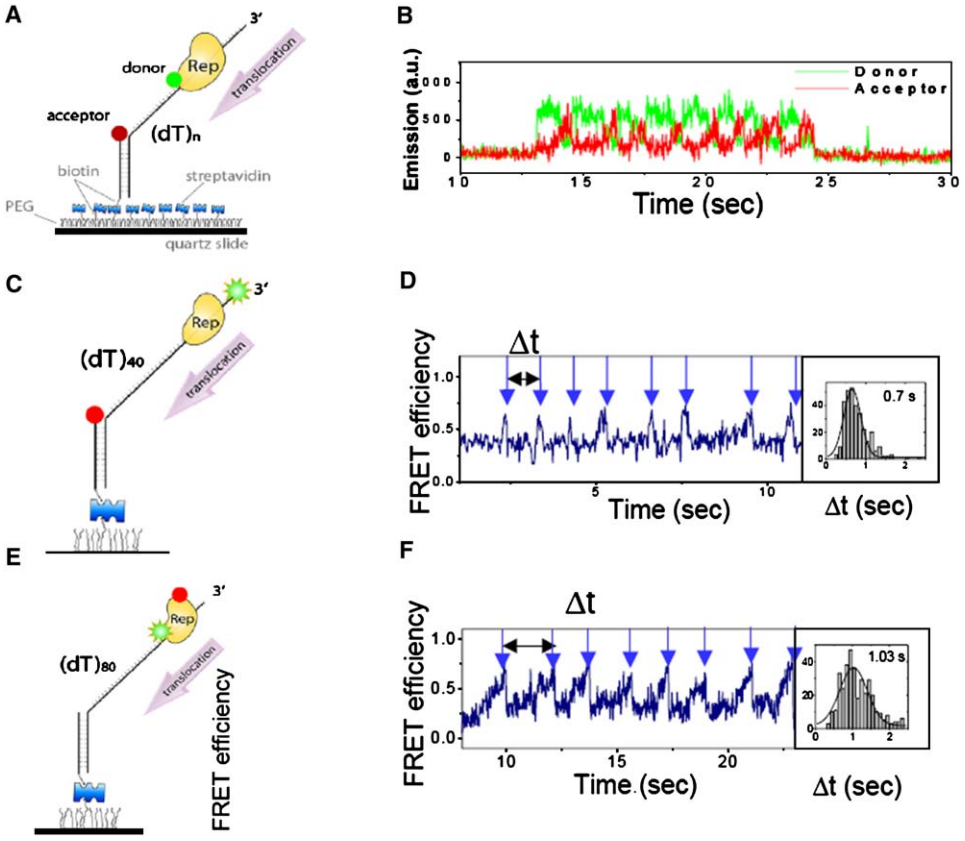

Figure 6. Repetition of Translocation Performed by Rep Helicase on ssDNA

(A) Rep helicase was labeled with donor at a single site and applied to acceptor-labeled partial duplex DNA molecules immobilized on a PEG surface.

(B) Fluorescence emission intensity time traces indicate that there is an expected 3′ to 5′ translocation (gradual increase in FRET) followed by an unexpected snapping back from the 5′ to 3′ end of DNA, followed by several cycles of translocation and snapback.

(C) Unlabeled Rep was added to a partially duplex DNA labeled at both extremities of the ssDNA tail.

(D) FRET trace displays low FRET interrupted by high FRET spikes in regular intervals, indicating the formation of a temporary ssDNA loop.

(E) Rep helicase was labeled at two sites where high FRET and low FRET would represent closed and open conformations, respectively.

(F) FRET trace shows a gradual increase followed by an abrupt decrease, which corresponds to gradual opening as Rep approaches the junction followed by an immediate closing as it snaps back. Adapted from Myong et al., 2005, Copyright 2005 Nature Publishing Group.

Δt, the time between two successive snapbacks, was measured for various ssDNA tail lengths and showed longer times for longer lengths, suggesting that the gradual FRET increase corresponds to ssDNA translocation. It was reasoned that the initiation site of translocation is likely to be localized at the 3′ end of DNA because of the remarkable regularity of the sawtooth pattern and narrowly peaked Δt histograms that shifted linearly with increasing tail length.

What would be the mechanism of repetitive shuttling? While it is very likely that Rep possesses two different DNA binding sites in order to prevent dissociation during snapback, Rep does not maintain contact with the 3′ end of ssDNA during translocation given that there was a clear FRET decrease when Cy5-labeled Rep moved away from the 3′ end of ssDNA labeled with Cy5 (Myong et al., 2005). Instead, it was suggested that an encounter with a physical blockade stimulates Rep to bind the 3′ end of ssDNA during snapback. Experimental evidence for this model was obtained by measuring the conformation of the ssDNA tail during repetitive shuttling of Rep, which indicated the formation of a transient DNA loop with the same frequency as repetitive shuttling (Figures 6C and 6D). This is plausible because ssDNA in solution is highly flexible and its conformational fluctuation is much faster than the time resolution used (Murphy et al., 2004).

The next experiment was performed to inquire how Rep senses the presence of a blockade. The crystal structure of Rep and its homologs showed the existence of two very different conformations, open and closed (Korolev et al., 1997; Velankar et al., 1999), and the closed form is favored when bound to a partial duplex DNA (Rasnik et al., 2004; Velankar et al., 1999), suggesting the blockade encounter may induce the closed form. To test this, Myong et al. (2005) carried out a translocation assay with Rep labeled at two sites so that high FRET is observed for the closed form and low FRET for the open (Figure 6E). The representative time trace in Figure 6F indicates that Rep gradually closes as it approaches the duplex junction and abruptly opens up when it snaps back to restart another round of translocation.

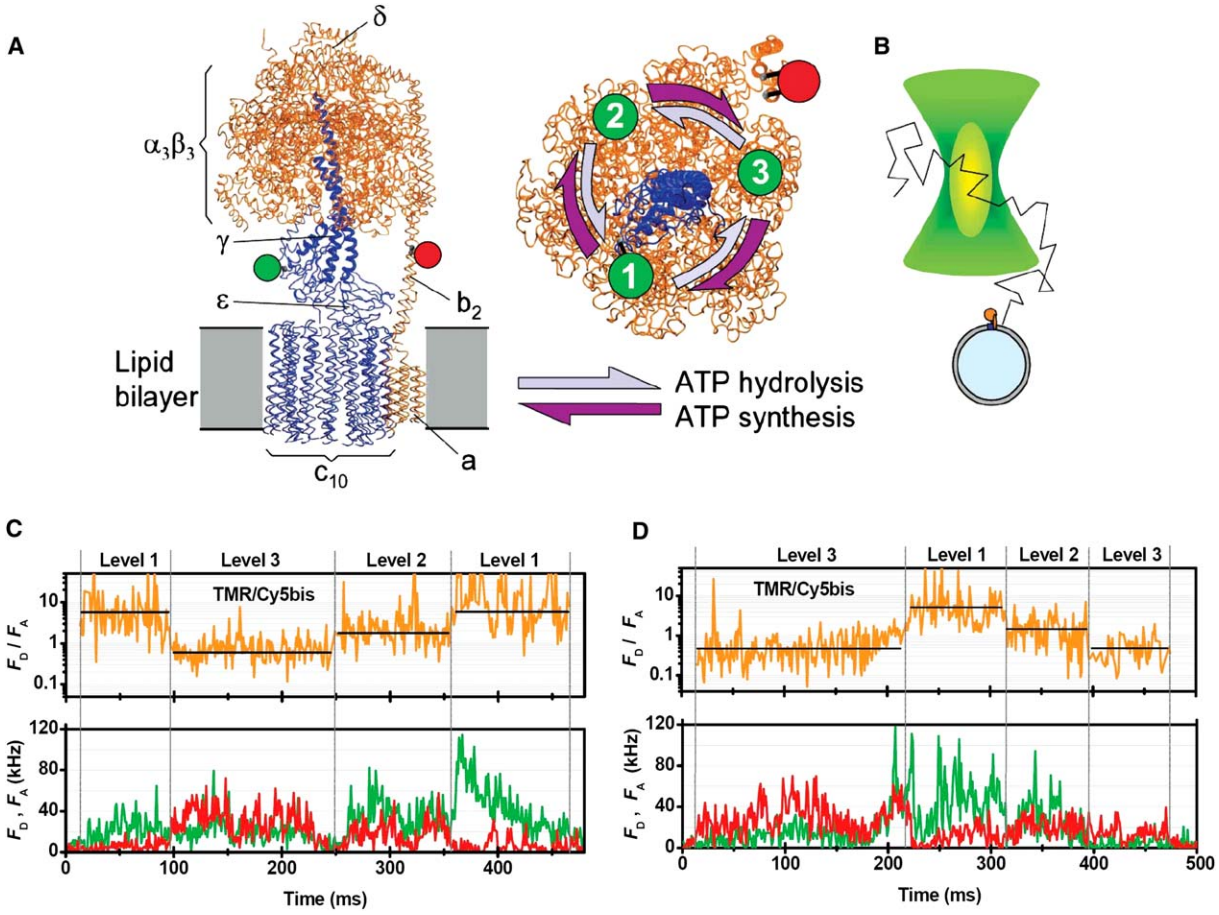

Figure 7. Stepwise Rotation of F_0F_1-ATP Synthase during ATP Hydrolysis and Synthesis

(A) Side view of a donor- and acceptor-labeled F_0F_1-ATP synthase embedded in a lipid bilayer. The rotor subunits are blue and the stator subunits are orange. Rotation of the rotor changes distances between the dyes (top view of a cross-section on the right panel), resulting in three distinct FRET levels.

(B) The F_0F_1-ATP synthase-bearing liposome diffuses through a diffraction-limited excitation volume.

(C) Single-molecule time traces of donor/acceptor intensity ratio (top panel) and fluorescence intensities of donor and acceptor (green and red curves in the bottom panel) during ATP hydrolysis.

(D) Same as in (C) except that the data were obtained during ATP synthesis. Note the opposite direction of rotation from hydrolysis. Adapted from Diez et al., 2004, Copyright 2004 Nature Publishing Group.

Blockade-induced closing of Rep may coincide with an increase in affinity toward the 3′ end of ssDNA, which would then allow it to snap back.

What might be the biological role of repetitive shuttling of Rep? DNA damage can cause a stall in replication which needs to be restarted after damage has been repaired. Rep was known to be involved in replication restart (Sandler, 2000) and to bind extremely tightly to a DNA structure found in a stalled replication fork (Marians, 2004). Indeed, Myong et al. (2005) observed repetitive shuttling of Rep on ssDNA bounded by the stalled replication fork and an Okazaki fragment analog. In addition, Rep was shown to interfere with the filament formation of the recombination protein RecA on ssDNA. Because uncontrolled recombination that starts from ssDNA coated with RecA filament can be toxic to the cell, it was suggested that repetitive shuttling of Rep in vivo may keep the ssDNA clear of unwanted proteins which can cause toxicity in cells (Myong et al., 2005). This function does not require the canonical function of helicases, DNA unwinding, and may in fact be representative of the mode of operation for a certain class of helicases in the cell.

Rotational Stepwise Movement of ATP Synthase

ATP is synthesized by F-type ATP synthases in membranes of mitochondria, bacteria, and chloroplasts. A proton gradient is used to make conformational changes in the synthase that alter the binding affinities of substrates (ADP and P_i) and allow for covalent bond formation and release of synthesized ATP (Boyer, 1998).

The F_0F_1-ATP synthase has two main parts, F_0 and F_1. The hydrophobic F_0 part is integrated into the membrane and consists of subunits a, b_2, and c_{10} in *E. coli* (Figure 7A; subscripts denote the number of each subunit). The hydrophilic F_1 part is made up of three α, three β, γ, δ, and ε subunits. The β subunits contain the catalytic binding sites for ADP and P_i; they, along with the α, δ, a, and b dimer subunits, make up the stator part of the motor. The γ, ε, and c_{10} subunits comprise the rotor; for

synthesis, protons move through the rotor, causing it to rotate relative to the stator. Additionally, the ε subunit is involved in switching between the synthase's active and inactive states.

The famous experiment by Noji et al. (1997) demonstrated that F_1-ATPase, which is F_0F_1-ATP synthase minus F_0, is a rotary motor powered by ATP hydrolysis. The $\alpha_3\beta_3\gamma$ subunits were immobilized to the glass surface and an actin filament attached to the γ subunit was observed to rotate in three discrete steps of 120° (Noji et al., 1997; Yasuda et al., 1998). This elegant approach greatly refined our knowledge of this remarkable stepper rotatory motor through subsequent improvement in time resolution and combination with other imaging and manipulation techniques, including FRET (Borsch et al., 2002; Yasuda et al., 2003). However, the observation of rotation during ATP synthesis proved much more challenging because the whole complex, including F_0, has to be studied in the context of a membrane under proton gradient. In an amazing technical feat, Diez et al. (2004) achieved just that.

Diez et al. (2004) set out to do three things: observe γ subunit rotation during ATP synthesis, determine whether the rotor moves in the same or opposite direction during hydrolysis and synthesis, and find out whether γ subunit rotation is stepwise or continuous.

ATP synthase mutants were specifically labeled with donor and acceptor at engineered cysteines. The γ subunit was labeled with the donor and a bifunctional acceptor crosslinked the two b subunits (Figure 7A). The labeled enzyme complex was reconstituted into liposomes and imaged on a confocal microscope (Figure 7B). The large size of the liposomes slowed the diffusion substantially, allowing data collection for a few hundred milliseconds for each liposome. The resulting photon bursts varied strongly in total intensity, but FRET efficiency was constant while the donor-acceptor distance was constant (Figures 7C and 7D). Rapid mixing of liposome solution with a solution of different pH could be used to establish a proton gradient that drove ATP synthesis for a few minutes.

To observe γ subunit rotation during ATP hydrolysis, 1 mM ATP was added to reconstituted F_0F_1-ATP synthase. Three constant FRET levels, termed 1, 2, and 3, were observed with abrupt (<1 ms) switching between levels. In over 70% of traces, the order of switching followed the sequence $1 \rightarrow 3 \rightarrow 2 \rightarrow 1$….

A similar stepwise γ subunit rotation was observed during ATP synthesis induced by introducing ADP, P_i, and pH and electric potential differences across the membrane. There were again three levels of FRET but they switched in the reverse direction ($1 \rightarrow 2 \rightarrow 3 \rightarrow 1$…) for over 80% of traces. This result confirms the expectation that ATP synthesis and hydrolysis drive the γ subunit in opposite directions. Additional support for this idea comes from an independent study of F_1-ATPase which showed that mechanically forced rotation of the rotor in the reverse direction results in ATP synthesis (Itoh et al., 2004).

The dwell times of the FRET states during catalysis agree with the average turnover time for the synthesis or hydrolysis of ATP in bulk experiments, with a hydrolysis dwell time of 19 ms and a synthesis dwell time of 51 ms. This shows that the switching between the FRET states is correlated with the catalytic event. Distances between fluorophores, as calculated from FRET, agreed with those predicted by a structural model.

In a more recent study, Zimmerman et al. probed the relative movement of the ε subunit of the rotor relative to the stator (Zimmermann et al., 2005). Again, opposite rotational directions were observed during synthesis and hydrolysis. Interestingly, various degrees of asymmetries were observed between the three FRET states in terms of dwell times and overall populations. While such asymmetry was never observed in the F_1-ATPase studies, it is possible that the presence of the peripheral stalk, absent in the F_1-ATPase, may break the symmetry by contacting one of the three $\alpha\beta$ units. In addition, the FRET values were significantly different when the enzyme complex was inactive, suggesting that the ε subunit may undergo a conformational change during activation.

Conclusion

In this review, we have highlighted several recent examples that clearly show how single-molecule fluorescence spectroscopy can be used to explore the relationship between structure and function in biological macromolecules. These studies go beyond merely reporting molecular heterogeneity to provide specific and illuminating details about the motion of one part relative to another, and thus provide the necessary link between, for example, a crystallographic study delineating the structure of a molecule and a biochemical assay determining its function. These single-molecule experiments may also complement ensemble studies by uncovering signatures for new functions that had not been observed previously. Such an endeavor would also be assisted by additional technical developments, including alternative excitation of donor and acceptor (Kapanidis et al., 2004), microfluidic mixer (Lipman et al., 2003), and three-color FRET (Clamme and Deniz, 2005; Hohng et al., 2004).

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