Single-molecule fluorescence of nucleic acids

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Less than a decade old, single-molecule fluorescence of nucleic acids has rapidly become an important tool in the arsenal of biological probes. A variety of novel approaches to investigate conformational dynamics, catalytic mechanisms, folding pathways and protein–nucleic-acid interactions have recently been devised for nucleic acids using this technique. Combined with biomechanical tools and ensemble measurements, single-molecule fluorescence methods extend our ability to observe and understand biomolecules and complex biological processes.

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Abbreviations

ds	double-stranded
FCS	fluorescence correlation spectroscopy
FRET	fluorescence resonance energy transfer
SMF	single-molecule fluorescence
ss	single-stranded

Introduction

Watching the flickering fluorescent light, or rather the wavering signal on the oscilloscope, produced by a single molecule, is an extraordinary and unforgettable experience for every first-time observer. Even more dramatic is the inevitable realization that the fluctuations of the signal reflect temporal fluctuations in molecular properties (i.e. valuable information that is lost in ensemble-averaged measurements). This detailed information can greatly enhance our understanding of the folding, dynamics and function of biological molecules.

Recent advances in single-molecule imaging and manipulation have enabled the observation of time trajectories for individual molecules and the direct measurement of distributions of molecular properties. In the short time since the first observations of single-molecule fluorescence (SMF) of biomolecules [1–3], a variety of mechanistic and kinetic approaches have been developed with this technique. Excellent reviews of the history and the technical aspects of SMF measurements [4–6] and their applications to biomolecules both *in vitro* and *in vivo* [5–8], and specifically to nucleic acids [9], have recently been published. This review focuses on the application of SMF methods to nucleic acids, reported within the past two years.

Two basic approaches to detection of SMF are used: fluorescence from individual molecules diffusing into (and out of) a small volume (Figure 1a) and fluorescence from single molecules restricted to a small volume (Figure 1b). In the former approach, the diffusing molecules are detectable only while they traverse the laser excitation volume. The short fluorescence bursts observed with this detection method provide valuable information on the distribution of molecular properties undisturbed by surface effects [6]. However, the short diffusion time precludes the recording of extended time trajectories for individual molecules. The second approach overcomes this limitation by spatially restricting the observed molecules. Restriction is achieved either by surface attachment through a specific interaction [10–12], or by trapping the molecule in a matrix [13] or optical trap [14]. Nucleic acids are readily amenable to surface attachment. When the attachment is properly designed, the structure and functional properties of the molecules are not affected by the surface. Spatial restriction offers the advantages of monitoring the response of individual molecules to rapid changes in buffer conditions and allows extended observation periods, during which time traces reflecting the fluctuations of the properties of an individual molecule can be recorded.

Conformational rearrangements and dynamics of nucleic acids

Since the first demonstration of fluorescence resonance energy transfer (FRET) [4,15] at the single-molecule level in 1996 [11], the technique has been widely used for studying protein and nucleic acid conformation, dynamics and folding (see Figure 1 for a brief description of FRET). In 1999, Ha *et al.* [10] first showed that conformational motions in nucleic acids can be followed by single-molecule FRET using a construct of the three-way helix junction of the 16S rRNA that contains the binding site for ribosomal protein S15. They observed conformational changes in surface-immobilized RNA junctions in response to binding of Mg^{2+} ions and/or ribosomal protein S15. The dissociation constants for S15 and Mg^{2+} and the rate constants for association and dissociation of S15 were determined. However, the fast dynamics of the junction in the presence of Mg^{2+} could not be analysed because of the limited time resolution of the experiment.

In an effort to overcome this limitation, Kim *et al.* [16•] combined FRET and fluorescence correlation spectroscopy (FCS, [17]) on surface-immobilized single molecules of the RNA junction. This approach allowed the dissection of the fast folding and opening dynamics of the RNA junction upon association of Mg^{2+} and Na^{+} ions. The results indicated that both the closing and opening rates for the junction are Mg^{2+}- and Na^{+}-dependent, and a model to account for these effects was presented. This work demonstrates that FCS applied to surface-immobilized single molecules can significantly improve the time resolution of the experiment allowing measurements over time scales ranging from microseconds to seconds.

Figure 1

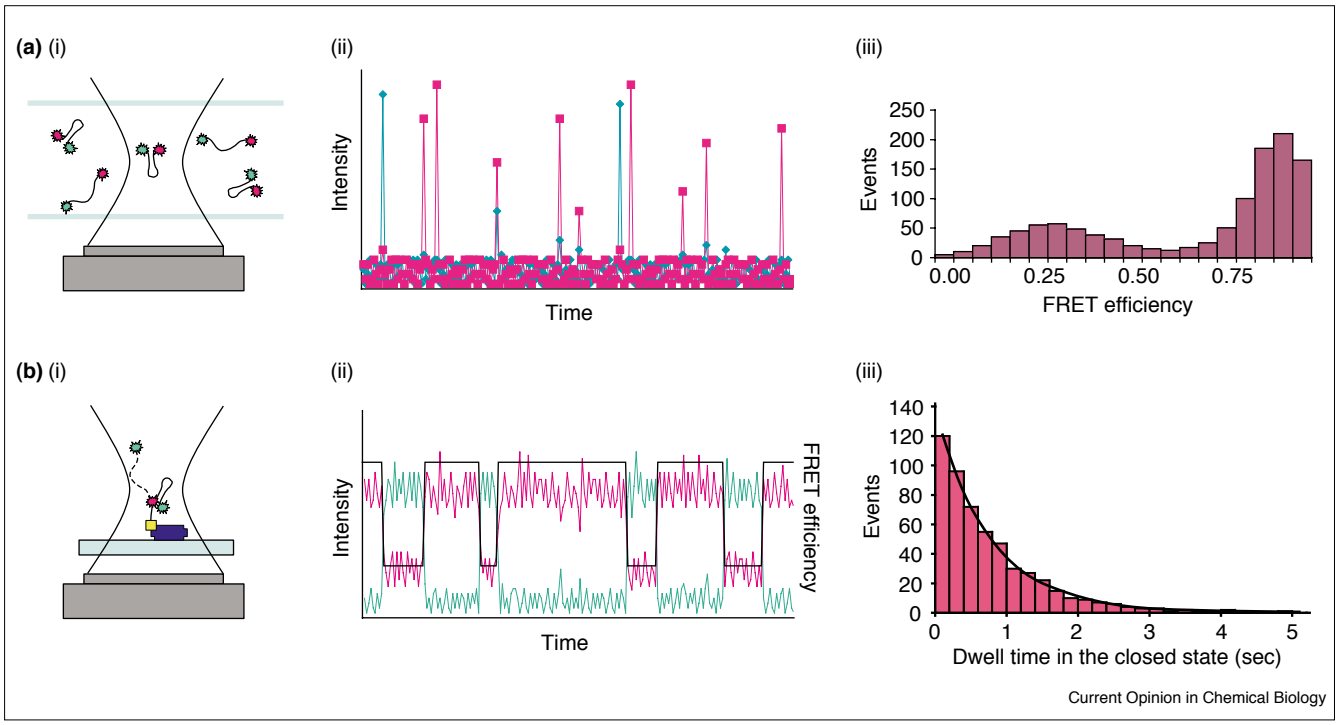


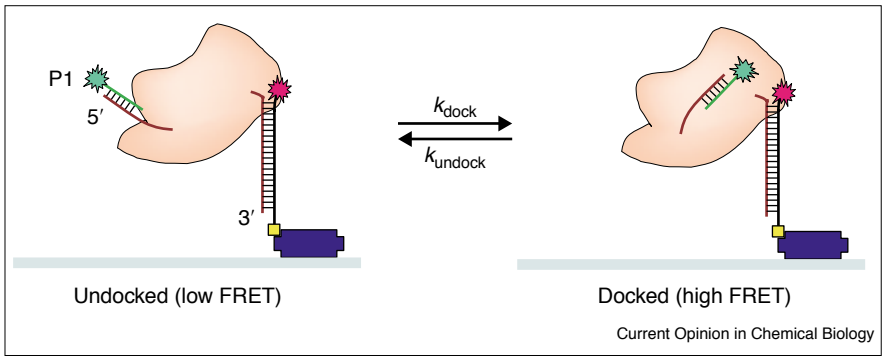
Illustration of the two basic configurations for observation of single-molecule fluorescence. A hypothetical hairpin-forming oligonucleotide molecule labeled at both ends with two different fluorescent dyes is used as an example. The observable monitored in this example is the FRET between the pair of dyes. FRET is the non-radiative transfer of electronic excitation energy from the donor dye molecule (cyan) to the acceptor dye molecule (magenta) via an induced dipole–dipole interaction. The efficiency of the transfer decreases with the distance R between the dyes as $1/[1+(R/R_0)^6]$. The parameter R_0 is a function of the properties of the dyes. The emission intensities of the donor dye and the acceptor dye are recorded simultaneously. The hypothetical hairpin molecules undergo transitions between open (low FRET ~0.3) and closed (high FRET ~0.9) states. **(a)** Fluorescence from freely diffusing molecules: (i) as a molecule traverses the laser excitation volume, a burst of fluorescence is recorded that appears as a peak on (ii) the time trace. Cyan and magenta peaks correspond to bursts of donor and acceptor fluorescence, respectively. The duration of the burst is proportional to the molecular diffusion rate. The intensity of the donor and acceptor peaks depends on, among other factors, the extent of FRET. FRET information extracted from the time trace is (iii) used to build a histogram. The peaks centred at FRET efficiency ~0.3 and ~0.9 reflect the distribution of hairpin molecules in the open and closed conformation, respectively. **(b)** Fluorescence from surface-attached single molecules: one of the ends of the oligonucleotide is extended and derivatized (yellow square) to provide a specific binding interaction with the coated glass surface (blue) (e.g. a biotinylated oligonucleotide specifically binds to a streptavidin-coated surface). (i) As the molecule undergoes transitions between closed (solid line) and open (dotted line) conformation, the emission intensities of the donor (green line) and acceptor (magenta line) dyes alternate between two levels. High acceptor and low donor fluorescence correspond to the closed conformation whereas low acceptor and high donor fluorescence correspond to the open conformation. (ii) A time trajectory for the emission intensities is recorded, from which a time trajectory for the FRET efficiency can be calculated (black line). The dwell times of the molecule in the high FRET (closed conformation) and the low FRET (open conformation) can be measured from this trajectory. (iii) The histogram plots the distribution of the dwell times in the closed state. A fit to a single-exponential curve allows the determination of the rate constant for opening of the hairpin from this histogram.

The conformational dynamics of short and long single-stranded (ss) and double-stranded (ds) DNA has also been studied with SMF methods. Grunwell *et al.* [12] observed conformational fluctuations of DNA hairpins by single-molecule FRET and determined the lifetimes of the closed and open states and their dependence on the loop and stem characteristics. Visualization of individual long ss and ds DNA chains by fluorescence videomicroscopy has provided detailed information about polymer dynamics. In a recent study of DNA dynamics in shear flow, Doyle *et al.* [18] showed that the deformation of tethered chains is partially governed by the thermal fluctuations of the chain transverse to the flow direction. Sewer and Hayes [13] observed partially condensed conformations for bacteriophage G DNA and followed the formation and dynamics of globules (partially condensed DNA segments) along an elongated DNA molecule. Using real-time fluorescence microscopy, Ladoux *et al.* [19•] were able to follow the early events associated with chromatin assembly and to measure directly the compaction rate. The length of the DNA molecule decreased sigmoidally, suggesting a three-step mechanism of nucleosome formation.

RNA folding

In the living cell, RNA performs a variety of functions that are determined by its complex and specific three-dimensional structure. However, our understanding of the process of formation of a stable RNA structure is still very limited

Figure 2



Cartoon representation of the docking transition of the P1 helix of *Tetrahymena* ribozyme. The construct shown here was used in most of the SMF studies of *Tetrahymena* ribozyme discussed in the text. The peach-coloured area represents the ribozyme with its 5′- and the 3′-ends shown as brown lines. The oligonucleotide substrate (green line) with the donor dye attached to it (cyan circle) is hybridized to the 5′-end sequence of the ribozyme. The 3′-end of the ribozyme is extended and a biotinylated tether oligonucleotide (black) is hybridized to the extension. The acceptor molecule (magenta) is attached to the 3′ end of the tether. The ribozyme molecule is anchored to the streptavidin (blue) coated surface through the biotin-streptavidin interaction. In the folded ribozyme, the P1 helix undergoes a transition between a docked state, characterized by specific tertiary interactions between P1 and the core of the ribozyme, and an open state in which no known tertiary interactions with the rest of the molecule are formed. In the SMF experiment the closed and open states produce high and low FRET level, respectively.

and, thus, RNA folding is a subject of intense research efforts. Single-molecule techniques are particularly useful for studying the folding of biological macromolecules as they can facilitate the observation of multiple pathways and the detection of specific intermediates, including intermediates that do not accumulate. The potential of SMF spectroscopy in folding studies of macromolecules has been promptly recognized, and the technique has been applied successfully to both proteins [20,21] and nucleic acids [22••,23•].

The first observation of the folding of a large structured RNA by SMF was made by Zhuang *et al.* [22••]. Folding of surface-immobilized molecules of the *Tetrahymena* ribozyme was followed by FRET. A donor fluorescent dye was attached to the oligonucleotide substrate that hybridizes to the 5′-end of the ribozyme molecule forming helix P1 (Figure 2). The acceptor dye was covalently linked to the biotinylated DNA tether that anchored the 3′-end of the molecule to a streptavidin-coated glass surface. Extensive controls indicated that surface immobilization did not perturb the catalytic activity of the ribozyme. During the transition from unfolded to folded state, the distance between the two dyes changed, resulting in an increase in the FRET efficiency of the dye pair. This setup allowed the exploration of both local folding events and overall folding of the ribozyme.

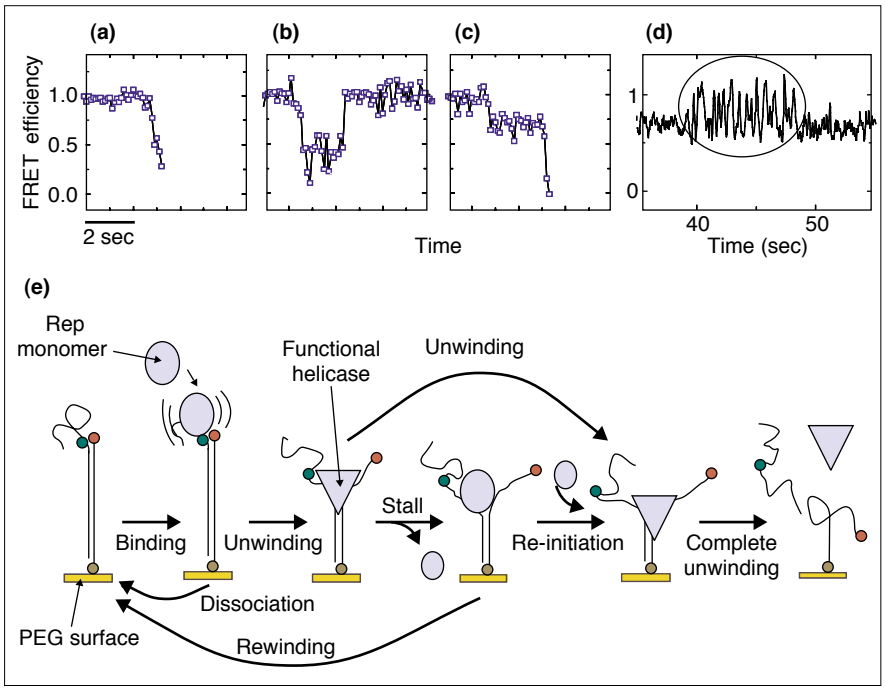
Docking of the P1 helix (Figure 2) into the active core is a relatively simple local folding transition that also represents the first step in the catalytic mechanism of the *Tetrahymena* ribozyme. Zhuang *et al.* [22••] measured directly and independently the equilibrium constant for docking and the rate constants for docking and undocking. The authors compared the docking parameters for an RNA substrate and a substrate with a modification that disrupts a specific tertiary contact formed upon docking that greatly destabilizes the docked state. Whereas the docking rate constant was unaffected by the modification, the undocking rate constant was increased 50-fold, suggesting that the specific tertiary contact was formed after the transition state for docking.

Bartley *et al.* have further explored the transition state for docking by applying this approach to all eight known tertiary contacts formed upon P1 docking (L Bartley, X Zhuang, R Das, S Chu, D Herschlag, personal communication). Only small effects of less than three-fold on the rate constant for docking were observed, whereas the rate constant for undocking varied over two orders of magnitude. These results strongly suggest that the transition state for this folding transition forms early, bearing little resemblance to the docked state. As the docked state is rarely populated for many of the modified substrates employed, the kinetic constants for docking and undocking could not have been measured by ensemble methods.

Zhuang *et al.* [22••] also observed overall folding of the *Tetrahymena* ribozyme from an unfolded state with only secondary structure. Folding was initiated by the addition of Mg^{2+}-containing buffer. A new fast-folding pathway was discovered that was accessible only in the presence of the oligonucleotide substrate (a necessary component of this particular FRET application). Each pathway proceeded through an early intermediate characterized by a FRET level of ~0.3 (compared with ~0.1 for the unfolded state and ~0.9 for the native state). Thus, the results also demonstrate the ability to identify (and partially characterize) folding intermediates by SMF. The intermediate with a FRET level of ~0.3 appears to be an early collapsed species [24].

Russell *et al.* [23•] explored further the folding landscape of the *Tetrahymena* ribozyme. Folding of individual molecules was monitored by single-molecule FRET, starting from distinct regions of the folding landscape, which were accessed by preincubation with different Na^+ concentrations.

Figure 3



DNA unwinding by *E. coli* Rep helicase observed with SMF. An 18 bp or 40 bp DNA duplex with a 3′-$(dT)_{20}$ ssDNA tail was used in the experiments. An acceptor fluorophore (Cy5, red circle in (e)) was incorporated in phosphoramidite form at the 5′-end of the shorter DNA strand, near the ss/dsDNA junction, whereas the donor fluorophore (Cy3, green circle in (e)) was incorporated in the longer strand either at the ss/dsDNA junction (a–c) or at the 3′-end of the tail (d). **(a–d)**. Time traces of the FRET efficiency of the fluorophore pair illustrating (a) complete unwinding of 18 bp, (b) stall and DNA rewinding of 40 bp, (c) stall and unwinding re-initiation of 40 bp, and (d) conformational fluctuations in the 18 bp DNA–enzyme compex. **(e)** Model for DNA unwinding by Rep helicase proposed by Ha *et al.* [33•] (see discussion in the text). Reprinted by permission from Nature [33•]. Copyright 2002 Macmillan Publishers Ltd.

Folding was initiated by rapidly exchanging the buffer with a standard Mg^{2+}-containing solution. The results suggested that at least three different conformational ensembles of starting structures were monitored in these experiments. To differentiate between two possibilities, either that the three starting states result in three independent folding pathways or that they are intermediates along a single pathway, the authors performed small-angle X-ray scattering measurements, which provided strong evidence in favour of independent folding pathways. Structural features responsible for correct folding and fast folding were further identified by dimethyl sulfate footprinting of wild type and mutant ribozymes. In this study, single-molecule FRET was instrumental in the identification and characterization of the distinct pathways by providing the tools for direct and accurate measurement of folding kinetics and population distributions. This work also demonstrates the requirement to combine single-molecule and ensemble methods to understand the structural and functional behaviour of biological molecules.

Nucleic acid enzymology

The ability to look beyond ensemble averages is opening up new possibilities for studying enzyme mechanisms [25]. It makes possible the determination of kinetic parameters that cannot be obtained by conventional biochemical methods and the capture of transient intermediates that are undetectable in ensemble measurements.

Zhuang *et al.* [22••] first demonstrated direct measurement of RNA catalytic activity by applying SMF microscopy to surface-immobilized molecules of the *Tetrahymena* ribozyme. A fluorophore was attached to the 3′-end of the oligonucleotide substrate. After cleavage of the substrate by the ribozyme, the 3′-end product was released, leading to loss of signal. The kinetic constants of individual steps in the catalytic mechanism of the ribozyme were determined from time traces collected for a large number of molecules.

Exploring the technique further, Zhuang *et al.* [26••] examined the correlation between structural dynamics and function of the hairpin ribozyme using a single-molecule FRET assay. The heterogeneous reaction kinetics observed in ensemble measurements was confirmed. By measuring the docking and undocking rate constants the existence of four distinct docked states was established. Three of these states are characterized by fast undocking rate constants and are not significantly populated, making them virtually impossible to detect by ensemble methods. Time trajectories of individual molecules showed also a pronounced ‘memory’ effect [27,28]: for long periods of time, the molecules returned to the same docked state before switching to a different one. Memory effects have been previously observed and, indeed, are common for RNA [29,30]. Single-molecule approaches will provide a uniquely powerful tool for dissecting the origin of these effects. A model was proposed involving slow transitions between different structural configurations of loops A and B of the hairpin ribozyme in the undocked state that lead to different conformations in the docked state. The combination of multiple undocking rates and slow conformational transitions results in the overall heterogeneous cleavage kinetics observed in bulk experiments.

DNA enzymes

Single-molecule techniques have begun to help researchers take a closer look at the molecular mechanisms of enzymes that process DNA substrates. A unifying feature in all experimental approaches published to date is the detection of changes introduced in fluorescently labeled individual DNA molecules by the action of the DNA enzymes. Digestion of single fluorescently stained DNA molecules by λ exonuclease was observed by Matsuura *et al.* [31]. The digestion rate was measured directly by monitoring in real time the shortening of individual DNA molecules straightened in an electric field, in the presence of the enzyme. DNA rotation during transcription by RNA polymerase was observed in a single-molecule assay combining fluorescence microscopy and magnetic tweezers [32•]. The authors were able to estimate the torque produced by RNA polymerase and established that the enzyme rotates DNA by tracking along its right-handed helix. These and other examples [13,14] illustrate the exciting opportunities for combining fluorescence methods with other single-molecule manipulation tools such as optical and magnetic tweezers, atomic force microscopy, and controlled DNA stretching in electrical field or hydrodynamic flow.

A high-resolution SMF method for observation of DNA unwinding by *Escherichia coli* Rep helicase has recently been developed by Ha and co-workers [33•]. The method uses FRET and can detect the unwinding of <10 base pairs. Complete DNA unwinding, stalls, re-initiation of unwinding and conformational fluctuations in the DNA–enzyme complex were observed (Figure 3). The data suggest a model for DNA unwinding according to which a Rep monomer binds to ssDNA and uses ATP hydrolysis to translocate along ssDNA and position itself at the ss/dsDNA junction. However, a Rep monomer alone can neither initiate nor restart DNA unwinding of stalled complexes unless it forms a functional complex with at least one other Rep monomer.

Conclusions

Constantly improving methodology and instrumentation has driven the rapid progress of SMF of biomolecules. Nucleic acids offer the great advantage of being easily labeled with fluorescent probes and manipulated in single-molecule experiments. The development of new fluorophores that minimally disturb the structure and function of nucleic acid molecules (e.g. highly fluorescent nucleoside analogues [34]) is of great importance for the future advancement of SMF of nucleic acids. Combining SMF with other single-molecule and ensemble methods will further broaden the scope of mechanistic questions that can be addressed *in vitro* and, ultimately, *in vivo*. SMF has already offered a glimpse into cellular processes such as the uptake of individual DNA molecules by the nucleus [35] and the invasion of living cells by single viruses [36], raising our hopes that an ambitious goal in biological research — the observation *in vivo* of the function and dynamics of single molecules and functional molecular complexes — will be successfully achieved in the near future.

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