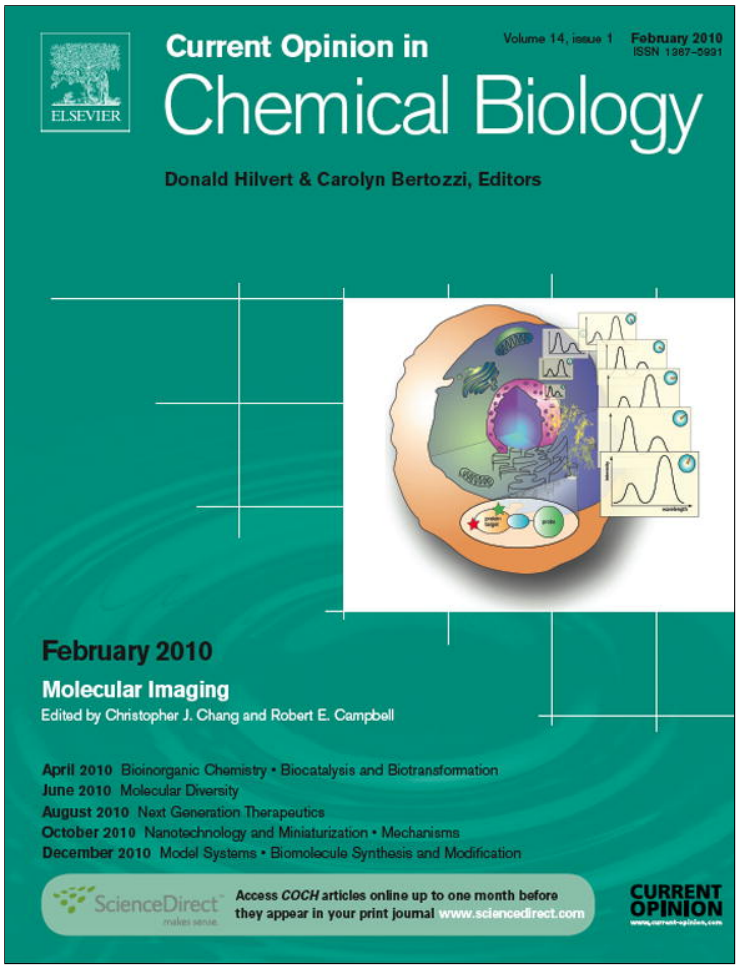

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Visualizing protein–DNA interactions at the single-molecule level

Jovencio Hilario[1,2] and Stephen C Kowalczykowski[1,2]

Recent advancements in single-molecule methods have allowed researchers to directly observe proteins acting on their DNA targets in real-time. Single-molecule imaging of protein–DNA interactions permits detection of the dynamic behavior of individual complexes that otherwise would be obscured in ensemble experiments. The kinetics of these processes can be monitored directly, permitting identification of unique subpopulations or novel reaction intermediates. Innovative techniques have been developed to isolate and manipulate individual DNA or protein molecules, and to visualize their interactions. The actions of proteins that have been visualized include: duplex DNA unwinding, DNA degradation, DNA packaging, translocation on DNA, sliding, superhelical twisting, and DNA bending, extension, and condensation. These single-molecule studies have provided new insights into nearly all aspects of DNA metabolism. Here we focus primarily on recent advances in fluorescence imaging and mechanical detection of individual protein–DNA complexes, with emphasis on selected proteins involved in DNA recombination: DNA helicases, DNA translocases, and DNA strand exchange proteins.

Addresses
[1] Department of Microbiology, University of California, Davis, CA 95616-8665, USA
[2] Department of Molecular and Cellular Biology, University of California, Davis, CA 95616-8665, USA

Corresponding author: Hilario, Jovencio (jhilario@ucdavis.edu) and Kowalczykowski, Stephen C (sckowalczykowski@ucdavis.edu)

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Introduction

Single-molecule biochemistry has emerged as a powerful tool for the study of complex biological systems, and an indispensable complement to traditional bulk-phase experiments. While classical biochemical methods yield parameters that are ensemble averaged, single-molecule experiments offer a way to investigate the individual behavior, stochastic fluctuations, and the molecular heterogeneity of biomolecules. This approach allows subpopulations and new intermediates of biochemical reactions to be identified. Single-molecule methods are nondestructive to biological molecules allowing them to be studied under thermodynamic equilibrium *in vitro* and *in vivo*, or under nonequilibrium conditions. Lastly, they offer high spatial (nm) and temporal (ms) resolution, providing precise information about reaction rates, molecular motions, and forces generated.

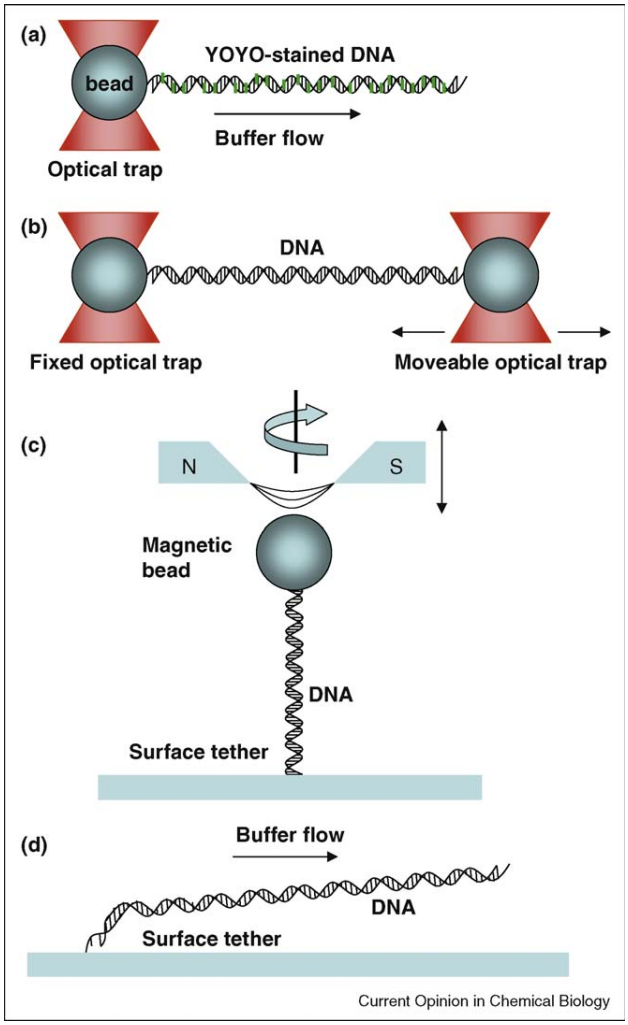
The single-molecule toolbox encompasses a wide range of techniques including atomic force microscopy (AFM), fluorescence/Förster resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), total internal reflection fluorescence microscopy (TIRFM), and microscopies involving optically or magnetically trapped particles. It is outside the scope of this review to discuss all varieties of single-molecule experimentation, and readers are encouraged to examine selected review articles that cover both specific and broad applications [1–5]. Here we will focus primarily on recent experiments that utilize fluorescence imaging of protein–DNA interactions. We will give only a brief overview of the relevant methods and, instead, discuss the mechanistic details learned from these single-molecule studies. Special emphasis will be given to biochemical reactions involved in DNA recombination, the process by which broken DNA is repaired and genetic diversity is created [6].

Manipulating and detecting individual DNA or protein molecules

Optical tweezers

Originally designed for trapping super-cooled atoms, optical tweezers were adapted to isolate, visualize, and handle single cells [7]. When a tightly focused infrared laser strikes a particle, both scattered and gradient forces are produced. When appropriately balanced, these forces can be used to trap the particle in three dimensions. In this way, a single molecule of DNA from phage λ (48 502 bp, 16.5 μm length) was captured by attaching it to a μm-sized polystyrene bead and trapping the bead in an optical tweezer [8]. Visualization was achieved by binding a highly fluorescent dye to the DNA and extending the DNA using fluid flow (Figure 1a). The kinetics of DNA relaxation upon flow cessation could therefore be watched in real-time [8]. Shortly thereafter, dual-beam optical tweezers were used to study the dynamics of single DNA molecules held in a partially extended state via attachment of beads to both ends of the DNA (Figure 1b) [9]. Alternatively, a single molecule of DNA with attached beads was studied by holding it with a single optical trap and a micropipette [3,5]. Here, DNA was not directly visualized, but rather the forces needed to extend the DNA were measured. In this way, the

Figure 1



Schematic representations of methods for manipulating DNA at the single-molecule level. **(a)** Single optical tweezer. Biotinylated DNA is attached to a μm-sized, streptavidin-coated, polystyrene bead. The DNA–bead complex is captured by the optical trap. DNA can be visualized by bound fluorescent dye. **(b)** Dual optical tweezers. DNA is attached at both ends to beads. Tension on the DNA can be created by either moving the traps apart, or protein-dependent changes in DNA conformation. **(c)** Magnetic tweezer. DNA is tethered at one end to a glass slide and the other end is bound to a paramagnetic bead. Magnets can be moved in the vertical direction to control DNA tension or rotated in the horizontal plane to change superhelicity. **(d)** Surface attachment and TIRFM. DNA can be tethered to a surface and extended by buffer flow. An array of hundreds of DNA molecules can be created to form 'DNA curtains'. The fluorescent DNA or bound proteins are visualized by TIRFM.

actions of proteins acting on DNA can be detected as movements of, or changes in forces acting on, the trapped bead.

Magnetic tweezers

Instead of using optically generated forces, other instruments have been constructed that rely on magnetic gradient forces [10]. In such cases, DNA is tethered at one end to a specially prepared surface, and is attached to a magnetic bead at the other end (Figure 1c). The magnetic field produces forces that pull vertically on the magnetic bead, thereby extending the DNA. By varying the height of the magnets, forces can be varied to produce a desired DNA extension. Furthermore, rotation of the magnets in the x–y plane provides a convenient way to control the superhelicity of the anchored DNA molecule (provided that both strands are anchored at both ends) and to investigate the effect of enzymes which alter DNA supercoiling. Neither DNA nor protein is visualized in a magnetic tweezer experiment; rather, the changes in vertical displacement of the attached bead are observed by bright-field microscopy to reveal the mechanical consequences of protein action on the DNA.

Surface attachment and TIRFM

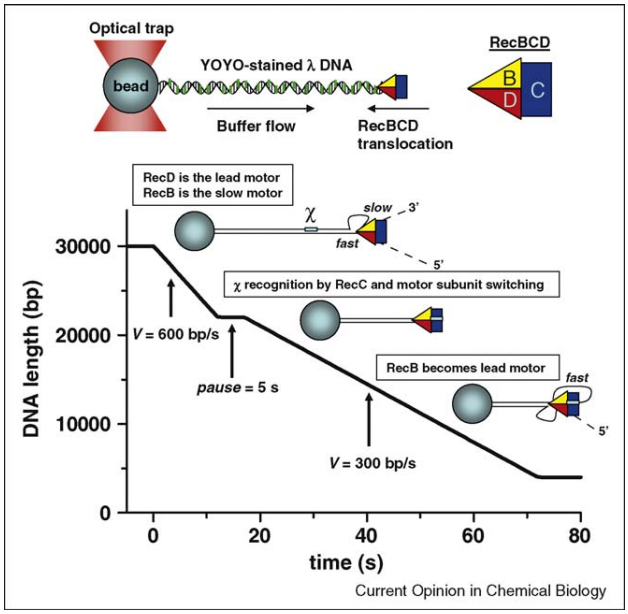
Instead of confining the particles in three dimensions, DNA or proteins can be immobilized on a glass surface, and their actions can be watched using either TIRFM (Figure 1d) or FRET [2]. Recently, the advantages of multiplexing were achieved using 'DNA curtains' or zero-mode waveguides (ZMWs) [11,12]. DNA curtains permit hundreds of molecules to be surface-tethered and aligned in a linear array within a flow cell. ZMWs are aluminum nanostructures deposited on a glass surface that create an array of tiny wells. As exemplified recently, individual molecules of DNA polymerase can be immobilized in each well, and DNA sequencing conducted in real-time using fluorescent nucleotides [13].

Mechanistic insights into enzyme function from single-molecule fluorescence visualization and DNA nano-manipulation

Single-stranded DNA translocases: RecBCD helicase/nuclease

RecBCD is a heterotrimeric enzyme involved in the processing of broken double-stranded DNA (dsDNA) during recombinational DNA repair in *E. coli* [14]. It functions by unwinding the DNA, and then nucleolytically degrading the single-stranded DNA (ssDNA) as it is produced [6,14]. A single optical trap combined with epifluorescence microscopy was used to directly observe the behavior of individual molecules of RecBCD [15,16,17••]. In these experiments, an individual molecule of RecBCD was bound to the end of a single λ DNA molecule that was attached to a polystyrene bead (Figure 2). The DNA was stained with YOYO-1 dye, whose fluorescence intensity increases upon intercalation between base pairs. Using a microfluidic, multichannel flow cell [15,18], a single RecBCD–DNA–bead complex was optically trapped in one channel of the flow cell in a solution that lacked ATP, which is essential for translocation. The flow cell consists of two or more parallel channels that contain different solutions; initially physically separated, the solutions flow

Figure 2



Visualization of DNA unwinding and χ recognition by a single molecule of the RecBCD helicase/nuclease. Translocation on ssDNA and resultant unwinding of dsDNA by RecBCD can be monitored by either displacing YOYO-1 from the DNA or labeling the enzyme with a fluorescent nanoparticle (not shown). The RecBCD–DNA–bead complex is captured by an optical trap, and translocation is initiated by movement to an ATP-containing buffer channel in a multichannel flow cell. Single-molecule visualization can detect a brief pause by the enzyme at the χ sequence followed by a resumption of translocation at approximately one-half of the rate. Single-stranded DNA loops are created before and after χ recognition because of the different translocation velocities of the RecB and RecD subunits. Adapted from [16].

into a region without channel separators but they remain separate (except for diffusion) due to laminar flow [18]. In the case of the RecBCD experiments, the trapped complex was moved (by translating the microscope stage) from the first flow channel to the second channel into a solution that contained ATP, to trigger DNA unwinding by the enzyme. As a result of the displacement of dye upon DNA unwinding, the translocation of RecBCD was visualized in real-time as a decrease in observed dsDNA length. This initial study [15] revealed that RecBCD unwound DNA at speeds of ~1000 bp/s without pausing. Furthermore, it was seen that RecBCD was highly processive, unwinding an average of 30 000 bp per binding event.

RecBCD is also regulated by a specific DNA sequence called Chi (*C*rossover *h*otspot *i*nstigator, χ, 5′-GCTGGTGG-3′) [6,14]. When χ is recognized, homologous recombination is stimulated. Stimulation results from an attenuation of nuclease activity, a switch in the polarity of degradation, and the direct loading of RecA protein onto the χ-containing ssDNA. However, the effect of χ on DNA unwinding by RecBCD could not be determined from ensemble studies because of its rapid unwinding rates, broad distribution of velocities, and lack of molecular synchrony [15]. These limitations were overcome by directly imaging the translocation of single RecBCD molecules on χ-containing λ DNA [16]. This study literally showed that RecBCD paused at the χ site for a few seconds, and then continued DNA unwinding at a rate that was, on average, one-half of the pre-χ rate (Figure 2) [16].

In a follow-up study, RecBCD translocation and χ recognition were investigated by directly watching the enzyme instead of monitoring changes in the DNA caused by fluorescent dye displacement [19]. The RecBCD enzyme was detected by attaching a streptavidin-coated, fluorescent nanoparticle to the RecD subunit which had been biotinylated. This experiment was also designed to test whether the χ-induced changes described above result from loss of the RecD motor. The RecBCD-nanoparticle was clearly seen to pause upon recognizing χ and to continue translocating at the reduced velocity without ejection of the RecD motor and the bound fluorescent nanoparticle.

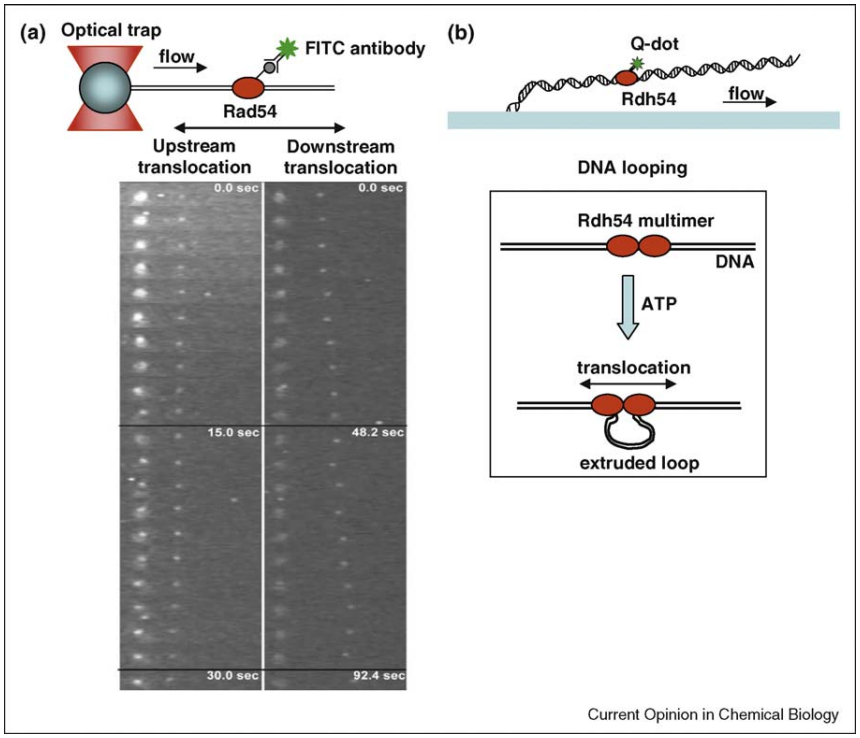
Finally, the most recent study in this series revealed the molecular basis by which χ regulates the translocation behavior of RecBCD [17••]. RecBCD is unusual among helicases in that it has two motor subunits which are arranged in a bipolar manner [20,21]. Combining single-molecule analysis with biochemical genetics revealed that the reduction in unwinding velocity at χ was the result of a change in motor subunit usage: before χ, the faster RecD motor subunit is the lead (i.e. helicase) motor but, after χ, the slower RecB motor assumes the role of the lead subunit (Figure 2) [17••]. This reduction in velocity was proposed to be important for the subsequent step of the process, namely the loading of RecA onto the χ-containing ssDNA.

Double-stranded DNA translocases: Rad54/Tid1

Rad54 and its homolog, Tid1 (Rdh54), belong to a family of ATP-dependent, chromatin remodeling enzymes. Rad54 functions in all stages of recombination [22]. It uses the energy derived from ATP hydrolysis to translocate on dsDNA. Rad54 can displace Rad51 protein from dsDNA, reposition nucleosomes, and stimulate DNA strand exchange. Recent single-molecule studies have peered into the behavior of both of these motor proteins [23–26].

The purification of recombinant Rad54 as a GST-fusion protein afforded the ability to fluorescently label the protein via the GST tag. The translocation of yeast Rad54 and Tid1 was visualized by labeling the proteins with fluorescein-labeled, anti-GST antibody (Figure 3a) [23,24]. Single complexes of Rad54–DNA–bead (or, instead, Tid1) were optically trapped in a flow channel

Figure 3



Visualization of translocation on duplex DNA by a single Rad54/Rdh54. **(a)** Still frames and graphs of upstream and downstream translocation by Rad54 on single dsDNA molecules. Rad54 is fluorescently tagged with a fluorescein-labeled antibody. The Rad54–DNA–bead complex is captured by a single optical trap and translocation is initiated by movement to an ATP-containing buffer channel in a multichannel flow cell. Reprinted from [23] with permission from Elsevier. **(b)** Diagram of DNA surface attachment used to visualize Rdh54 translocation by TIRFM. Translocation of Rdh54 multimers on DNA can extrude DNA loops. Adapted from [25] with permission from Elsevier.

lacking ATP, and then moved to a separate channel with ATP to initiate translocation. The position of the fluorescent antibody signal could be measured to nanometer precision using two-dimensional Gaussian fitting of the point spread function [4]. Rad54 was seen to translocate rapidly with a mean velocity of 300 bp/s. Rad54 movement was generally unidirectional along the DNA, but complex motions, such as pauses and reversals of direction were also observed — such complex translocation behavior would not easily be detected in ensemble experiments. Translocation was highly processive with an average translocation distance of 11 000 bp, and with some Rad54 molecules traveling upwards of 30 000 bp. The translocation speed and distances obtained were surprising, especially in light of its rather slow chromatin remodeling activity [27]. These findings suggested that Rad54 rapidly travels along naked DNA until it arrives at a nucleosome where its movement is limited by nucleosome sliding or displacement. It was speculated that under a Rad54-generated force, the histone–DNA complex can be destabilized and repositioned. The Rad54 homolog, Tid1, showed similar processivity but a four-fold slower translocation velocity [24]. By combining single-molecule results with biochemical assays, it was concluded that Rad54/Tid1 translocation functions to remodel various protein–DNA complexes and DNA intermediates produced during homologous recombination.

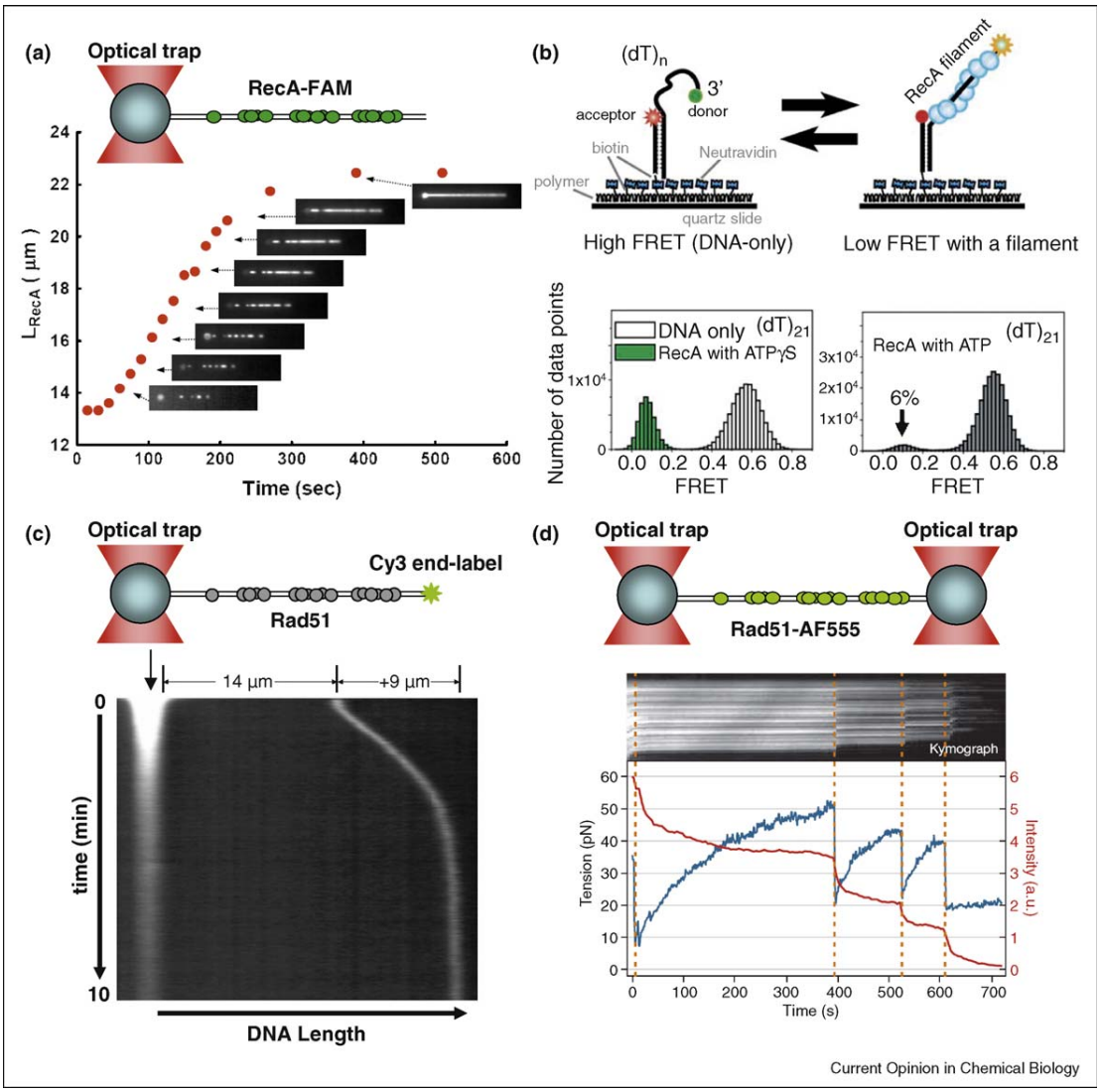
If Rad54/Rdh54 binds DNA as a multimeric assembly, then potentially, DNA loops may be extruded as a result of different orientations or translocation activities of the individual motor units. While the ability to form DNA loops was inferred from several papers, recent imaging of Rdh54 fused to thioredoxin and labeled with antibody-coupled quantum dots permitted description of their characteristics (Figure 3b) [25]. Here, the fluorescent Rdh54 molecules were bound to DNA that was arranged as a linear curtain in a flow cell. Translocation velocities and processivities similar to the optical trapping experiments were observed, but DNA looping was readily detected from the correlated movement of several fluorescent Rdh54 molecules on the same DNA. The average size of loops was 6000 bp, but loops of 15–20 kbp were occasionally generated.

DNA strand exchange proteins: assembly and disassembly of RecA nucleoprotein filaments

The RecA/Rad51-family of DNA strand exchange proteins is central to the recombinational repair of DNA [28]. These proteins polymerize on DNA, forming nucleoprotein filaments, and catalyze the exchange of strands between homologous DNA. While early single-molecule force microscopy experiments measured the assembly on individual DNA molecules and established various mechanical properties of RecA-DNA filaments [29–32], recent advances permitted direct visualization of the real-time assembly and disassembly of these nucleoprotein filaments [33–36].

For RecA, visualization was made possible by labeling the protein specifically at the N-terminus with fluorescein (Figure 4a) [34]. Using a three-channel flow cell, individual bead–DNA complexes, stained with YOYO-1 to

Figure 4



Direct observation of individual RecA/Rad51 filament dynamics by single-molecule fluorescence. **(a)** Assembly of fluorescent RecA (RecA–FAM) protein onto a single dsDNA molecule. DNA length increases by ~60%. Data points and still frames from time-dependent 'dipping' in a flow channel containing fluorescent RecA show nucleation followed by extensive growth. Reprinted by permission from Macmillan Publishers Ltd: Nature, [34], 2006. **(b)** Single-molecule FRET assay of RecA binding to ssDNA. The high FRET state exists in the absence of RecA, when the FRET-pair fluorophores are in proximity. The low FRET state occurs upon RecA binding and DNA extension when the FRET pair is beyond their Förster distance. Reprinted from [35] with permission from Elsevier. **(c)** Rad51 binding to a single dsDNA molecule that is fluorescently labeled at one end. Filament formation extends the DNA by 65% as seen by the movement of the fluorescent end-label, Cy3. Adapted from [44••]. **(d)** Dissociation of fluorescent Rad51 filaments (Rad51–AF555) from DNA held in a dual optical trap. ATP hydrolysis by Rad51 creates tension that stalls the disassembly process. Disassembly is resumed upon tension release. Reprinted by permission from Macmillan Publishers Ltd: Nature, 2009 [45••].

permit selection, were isolated in one channel, destained in the second, and incubated or 'dipped' in the third channel containing fluorescent RecA. Direct imaging using epifluorescence microscopy showed that RecA binding stretched the DNA by 50–60%. Examining the time-dependence of RecA filament assembly allowed many aspects of this process to be determined. Most importantly, it was found that four to five RecA monomers were needed to form a stable nucleus on dsDNA, from which bidirectional growth occurred indefinitely to create extended filaments. The nucleation rate was found to depend on many solution variables (e.g. the type of nucleotide cofactor), but growth rate was less sensitive, or not at all, highlighting the potential to regulate filament formation by controlling nucleation. This inference was confirmed using a RecA that was fused to monomeric red fluorescent protein (RecA–RFP), instead of being chemically labeled. The assembly of RecA–RFP nucleoprotein filaments was visualized and found to be attenuated [36]; however, the RecA–RFP also displayed reduced activities both *in vivo* and *in vitro*. This reduction in activities was found to result from a three-fold lower rate of nucleation onto DNA, quantitatively demonstrating the important role that appropriate rates of filament nucleation play in biological function.

A separate study used FRET-based single-molecule assays to study RecA dynamics (Figure 4b) [35]. This work revealed that about five RecA monomers were needed for a stable nucleus, independently validating the interpretations from the epifluorescent approach. The high sensitivity of the FRET signals also permitted the detection of intermediate FRET states which allowed the authors to conclude that RecA filaments grow or shrink by one monomer at a time. Binding and dissociation occurred at both ends of the filament but at different rates, thereby explaining the previously defined polarity of filament growth. Additionally, it was demonstrated that a growing RecA filament can displace ssDNA binding protein.

Magnetic tweezer experiments have also yielded valuable information about the nature of protein–DNA interactions, some of which are unattainable by current fluorescence methods [37,38]. For example, studies with RecA showed that polymerization on DNA is affected by the extent of supercoiling [37]. In addition, a RecA–ssDNA nucleoprotein filament invades the duplex at multiple sites and, during the course of DNA strand exchange, tension is produced in the target dsDNA molecule [38].

DNA strand exchange proteins: assembly and disassembly of Rad51 nucleoprotein filaments

Studies with the eukaryotic Rad51 proteins have had a similar focus [39–42,43••,44••,45••,46,47]. Initial work, using fluorescently modified Rad51, showed that Rad51 could form rings that could laterally diffuse along DNA [39]. A fluorescent variant of Rad51, labeled at a single genetically engineered cysteine residue, showed that Rad51 formed many nuclei along DNA [41]. The work also showed that Rad51 disassembly is a relatively slow process taking over 45 min to occur.

Recently, visualization utilizing both fluorescently end-labeled DNA (Figure 4c) and fluorescently tagged Rad51 revealed that the nucleoprotein filaments nucleated readily but that, in contrast to RecA, growth did not continue indefinitely. Minimally, two to three Rad51 monomers were required to form a stable nucleus from which limited filament growth for only a few kilobase pairs occurred [44••]. Additionally, Rad51 filament disassembly was found to occur by two distinct kinetic steps: firstly, a fast kinetic phase resulting from ATP hydrolysis which caused rapid shrinking of the extended filament to a compressed ADP-bound form; and secondly, a slow phase of Rad51 release from these compressed filaments. This conclusion is in accord with results from length measurements of Rad51 nucleoprotein filaments obtained from both magnetic tweezer experiments [43••] and imaging using DNA end-labeled with a quantum dot [46].

The novel combination of dual optical trapping with fluorescence visualization allowed researchers to provide further insights into the Rad51 dissociation process (Figure 4d) [45••]. By applying tension to an ATP-hydrolyzing filament, Rad51 filament disassembly could be blocked. Relaxation of the tension resulted in large bursts of Rad51 loss from the filament ends. Their data suggested that Rad51 disassembly results from a combination of ATP hydrolysis, which produces ADP and lowers the binding affinity, and the release of stored filament tension due to changes in DNA conformation.

Magnetic tweezer studies examining Rad51 assembly on single-stranded and double-stranded DNA found nucleation events dominate the binding process [42,43••]. The average filament lengths were calculated to be ~20–40 monomers — much smaller than resolvable by typical fluorescence visualization [42]. In these studies, ~5 Rad51 monomers were determined to comprise the nucleus. Additionally, it was shown that even though Rad51 assembled faster on ssDNA than dsDNA, this propensity was offset by the slower disassembly of Rad51 from dsDNA [43••].

Regulation of the assembly and disassembly of RecA/Rad51 nucleoprotein filaments

Now that the dynamic behavior of RecA and Rad51 nucleoprotein filaments can be quantified at the single-molecule level, it is possible to assess the roles of mediator proteins involved in recombination. These mediators are accessory proteins akin to the protein factors found to regulate actin and tubulin dynamics. They can nucleate and/or stabilize nucleoprotein filaments, or

can act to prevent undesirable filament formation [48]. *In vivo*, the function of Rad51 is regulated by the breast cancer associated protein, BRCA2, among others [49]. This is achieved through protein–protein interactions with the unique amino acid sequences within BRCA2, known as the BRC repeats. Recently, single-molecule imaging, together with ensemble analysis, demonstrated that the DNA binding selectivity of Rad51 is regulated by interactions with the BRC repeats of BRCA2 [50••]. Biochemical assays showed that a single BRC repeat, as well as a domain comprising all eight repeat units, can enhance Rad51 binding to ssDNA; complementary single-molecule measurements showed that the BRC repeats blocked Rad51 nucleation on dsDNA but did not disassemble preformed filaments. Together, these ensemble and single-molecule studies revealed that the BRC repeats modulate the specificity of Rad51 binding to ssDNA and dsDNA, and that they also control ATP hydrolysis by Rad51.

Conclusions

Single-molecule imaging has provided detailed information on the activities of many proteins involved in DNA metabolism. These mechanistic insights were uniquely derived from the analyses of complexes of single molecules that were studied in real-time. Studies of DNA translocating enzymes revealed that they can be highly processive, travel at fast velocities, pause, reverse direction, and can be controlled by specific DNA sequences. The studies of DNA strand exchange proteins provided a means for determining the size of the nucleating species, the rates of filament nucleation and growth, and the mechanistic consequences of ATP hydrolysis. Additionally, it is now possible to study protein and small molecule factors that potentially enhance or block the interactions between these proteins and DNA.

In general, these techniques are applicable to a wide range of other DNA or RNA translocases, such as chromatin remodeling motor proteins. Furthermore, the dynamic real-time behavior of regulatory proteins and complexes that bind to DNA or RNA can be studied. In fact, potentially all DNA and RNA metabolic processes involving the actions of proteins can be detected and visualized at the single-molecule level.

While most current studies typically involve a single fluorescent protein and its actions on labeled DNA, future advancements will come from the use of different fluorophores on multiple proteins with simultaneous multiplexed detection involving multiple traps and as many flow channels as needed. This will allow imaging multiple DNA transactions or protein–protein interactions simultaneously in real-time. Innovative micromanipulation and microfluidic control will permit researchers to build biochemical reactions of ever-increasing complexity. It is not too optimistic to think that, one day, complete reconstitution of a complex biochemical pathway, such as homologous recombination, will be realized at the single-molecule level.

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