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Single molecule studies of homologous recombination†

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Single molecule methods offer an unprecedented opportunity to examine complex macromolecular reactions that are obfuscated by ensemble averaging. The application of single molecule techniques to study DNA processing enzymes has revealed new mechanistic details that are unobtainable from bulk biochemical studies. Homologous DNA recombination is a multi-step pathway that is facilitated by numerous enzymes that must precisely and rapidly manipulate diverse DNA substrates to repair potentially lethal breaks in the DNA duplex. In this review, we present an overview of single molecule assays that have been developed to study key aspects of homologous recombination and discuss the unique information gleaned from these experiments.

1. Introduction

The development of single molecule techniques for studying biological processes has yielded insights into reaction mechanisms that were otherwise inaccessible to traditional biochemical methods. Large molecular assemblies affect complex biochemical transformations *via* a distribution of underlying intermediate states. Single molecule studies can directly access transient sub-populations that are obscured by ensemble averaging, an intrinsic property of bulk biochemical experiments. Observation of the time course of individual molecular trajectories elucidates the inter-conversion between these sub-populations and may offer crucial clues towards unraveling intricate molecular mechanisms. These advantages of single molecule techniques have been leveraged successfully to address diverse biological problems such as the dynamics of DNA replication and repair,[1–9] transcription,[10–12] translation,[13–16] ATP synthesis,[17–19] viral packaging,[20,21] and intracellular transport.[22–26]

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Single molecule studies of DNA–protein interactions have illuminated many aspects of DNA processing.[27–31] Early single molecule experiments probed the mechano-elastic response of individual DNA molecules under an applied force.[32] This work served as a basis for studies of DNA remodeling by helicases, nucleases, nucleosome packaging, and other remodeling and repair enzymes. Recently, single molecule experiments on DNA processing have been applied to complex multi-protein systems, such as the replisome.[1,2] Further work has extended studies of single molecule DNA processing to the level of a single cell.[33]

Single molecule methods are now being employed to study complex DNA transactions, such as those that occur during homologous DNA recombination (HR). HR is a high-fidelity mechanism that repairs double strand DNA breaks (DSBs) by employing complementary genetic information that is available at a homologous site in the genome. DNA recombination also enables crossover formation between paired chromosomes during meiosis to generate genetic diversity and ensure proper segregation during meiotic division. In addition, HR is crucial for repairing DSBs that arise as a result of exogenous damage such as chemical insults or ionizing radiation, during the collapse of replication forks at a single stranded nick or lesion, or as a result of recombination between linear viral DNA and the host genome. Multiple excellent reviews of this

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evolutionarily ubiquitous DNA metabolism pathway in both prokaryotes[34–36] and eukaryotes[37–39] are available, and a brief overview of HR in *Escherichia coli* is presented below.

A hallmark of HR is the generation of single stranded DNA (ssDNA) at the site of the DSB for use as a substrate in the homology recognition reaction. In *E. coli*, the tripartite RecBCD complex recognizes and loads onto the ends of DSBs. RecBCD processes double stranded DNA (dsDNA) to produce ssDNA overhangs that serve as a substrate for RecA. In cells lacking functional RecBCD, other exonucleases such as RecE process the DSB to yield suitable ssDNA overhangs. RecA rapidly polymerizes along the ssDNA, and initiates a genome-wide search for homologous DNA. Upon encountering a stretch of homology, RecA invades the intact duplex to form a displacement loop (D-loop) structure. RecA mediated branch migration extends the paired heteroduplex DNA along the region of homology and the exposed ssDNA is filled in by polymerases. The resultant Holliday junction (HJ) is resolved by the RuvABC complex and sealed with ligases to yield two intact dsDNA molecules without loss of genetic information.

This review summarizes single molecule experiments aimed at unraveling aspects of HR. Results from studies of RecBCD, RecA, Rad51, Rad54, Rdh54 and RuvAB molecular machines are presented in the Discussion. We conclude by describing recent developments that will drive progress towards obtaining a more complete picture of HR *via* single molecule techniques.

2. Discussion

Below, we present single molecule experiments on several enzymes that are central players in HR. In Section 2.1, we describe pioneering work on the processive and multifunctional molecular machine RecBCD.[3,40–44] Single molecule assays of various functions of the recombinase RecA and the eukaryotic homolog Rad51 are presented in Sections 2.2 and 2.3.[5,45–56] Observation of the eukaryotic Rad54 and Rdh54 translocation and DNA remodeling activity is discussed in Section 2.4.[6,7,57,58] Finally, Section 2.5 summarizes experiments on the HJ specific motor complex RuvAB.[59–62]

2.1 RecBCD helicase

In *E. coli*, the 330 kDa heterotrimeric RecBCD enzyme complex processes linear dsDNA for homologous recombination.[63] RecBCD is a helicase and nuclease that preferentially loads onto nearly blunt dsDNA termini.[64,65] The complex unwinds and nucleolytically cleaves the dsDNA as it translocates in a highly processive manner.

A crystal structure of the RecBCD–DNA complex presents a detailed picture of this enzyme.[66] RecB and RecD are members of the Superfamily 1 (SF1) type helicases with 3′–5′ (SF1a) and 5′–3′ (SF1b) directionality, respectively.[67] A central "pin" in the RecC unit bisects the duplex DNA and funnels the 3′ and 5′ ssDNA strands towards the RecB and RecD motors. As the ssDNA strands exit from their respective motor cavities, the ssDNA strands pass by an additional nucleolytic domain on RecB, where both strands are degraded, albeit at different rates.

RecBCD dependent recombination is *cis* regulated by the DNA sequence Chi (crossover hotspot instigator, 5′-GCTGGTGG-3′).[35] Early genetic evidence and subsequent biochemical investigations revealed that Chi sequences are HR hotspots that are over-represented and dispersed uniformly at a frequency of approximately once per five kb throughout the *E. coli* genome.[35,68,69] Prior to encountering Chi, RecBCD degrades both strands of dsDNA. However, the nucleolytic behavior of RecBCD is altered upon encountering Chi.[63,70–72] Degradation of the 3′ tail is abolished, while translocation along the DNA and nucleolytic degradation of the 5′ ssDNA tail continue. The resulting 3′ ssDNA loop serves as the nucleation point for RecA and participates in downstream steps of HR.[73,74]

The RecBCD system has been studied intensively at the single molecule level.[3,40–44] In a series of groundbreaking papers, Kowalczykowski and co-workers detailed the translocation behavior and Chi-sequence mediated changes in RecBCD activity. The single molecule assay is schematically illustrated in Fig. 1a. A long dsDNA substrate with a single biotinylated end was conjugated to a streptavidin coated polystyrene bead (orange sphere). The bead was optically trapped at the focal point of a laser beam, and illuminated in an epi-fluorescence microscope. The DNA was labeled with the fluorescent intercalating dye YOYO1 (yellow stars) and extended in a laminar buffer flow. The bead-DNA conjugate

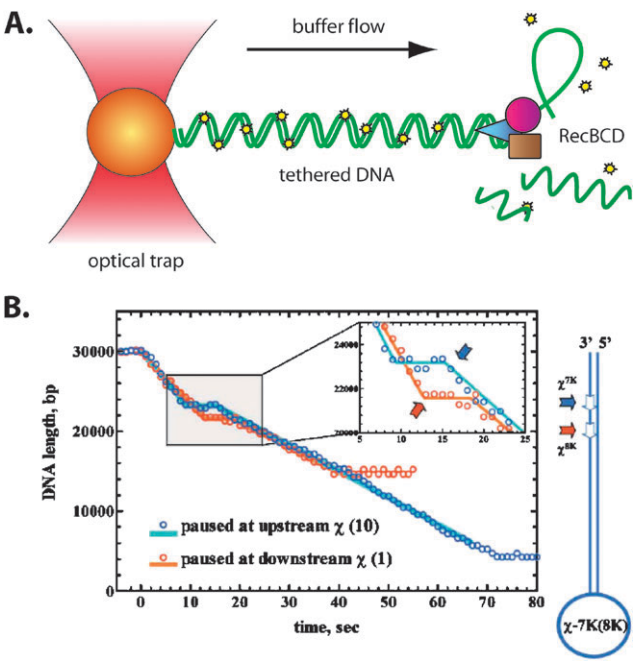

Fig. 1 (a) A schematic illustration of the RecBCD DNA unwinding assay. DNA is tethered at one end to an optically trapped bead and extended *via* buffer flow. The DNA is visualized by the intercalating dye YOYO1 (yellow stars). RecBCD activity liberates YOYO1 and is experimentally observed as a decrease in the length of the tethered DNA. (b) Chi mediated changes in RecBCD translocation for two representative single molecule traces. The observed change in DNA length (circles) and a piece-wise linear fit (lines) shows RecBCD pausing at a Chi locus. Location of Chi loci in the DNA substrate are depicted to the right of the graph. Reproduced with permission from ref. 44.

was incubated in the presence of RecBCD without ATP to form an initiation complex. Excess RecBCD was washed away and the flow buffer supplemented with ATP. Upon helicase unwinding, YOYO1 was displaced from the duplex DNA, which lead to a significant decrease in fluorescence.[75] Thus, the helicase/nuclease activity of RecBCD was directly observed as a decrease in length of the dsDNA as a function time.

In the absence of Chi, unwinding was rapid ($\sim$500 bp s^{-1}) and highly processive over tens of thousands of base pairs (bp).[3,44] DNA substrates containing several Chi loci were constructed to observe changes in RecBCD activity. Fig. 1b shows the model DNA substrate and typical trajectories of two RecBCD complexes both before and after Chi recognition.[41,44] Upon encountering Chi, RecBCD paused transiently for several seconds before resuming translocation at a rate that was reduced nearly two-fold ($\sim$300 bp s^{-1}).[44]

Several early bulk biochemical studies suggested that Chi sequence recognition triggered ejection of the RecD subunit and accounted for the changes in translocation and nucleolytic behavior.[76,77] Dohoney and Gelles directly tested this hypothesis at the single molecule level.[40] A RecD fusion product with a biotinylated peptide was co-expressed with both RecB and RecC, yielding a heterotrimeric complex that retained wild-type activity. The biotinylated RecBCD was conjugated to a large polystyrene bead and loaded onto a DNA substrate that was tethered to a glass surface at one end. Tethered particle motion analysis, pioneered by Landick and co-workers,[78] was used to observe RecBCD motion. As the enzyme digested the dsDNA, the decreasing DNA tether length was detected by analysis of the change in the bead Brownian motion; shortening of the DNA restricted the motion of the bead. When a DNA substrate containing a Chi locus was used, the RecBCD-bead conjugate continued moving along the DNA, definitively ruling out that RecD was ejected upon encountering Chi. This finding was later confirmed by Kowalczykowski and co-workers.[41]

Analysis of an ATP hydrolysis defective RecD mutant provided an elegant mechanistic explanation of the observed decrease in translocation velocity upon Chi recognition. The RecD mutant exhibited slow translocation rates, paused at Chi, but did not change velocity after the Chi locus.[43] Based on a series of single molecule experiments, the authors concluded that RecD is the faster, lead motor unit before Chi recognition. However, upon encountering Chi, RecB assumes the lead position and translocates at a reduced rate relative to RecD.[43]

Block and co-workers developed an optical trap with $\sim$6 bp spatial resolution to observe RecBCD motion with greater precision.[42] Biotinylated RecBCD was immobilized *via* streptavidin interaction on the passivated surface of a glass flow-cell. A polystyrene bead conjugated to a DNA substrate was reacted with the immobilized RecBCD and the reaction followed by observing displacement of the bead from the optical trap. The enzyme exhibited pauses that lasted for several seconds and force-dependent backsliding at moderate forces of 4–8 pN, suggesting that nucleolytic degradation did not occur immediately after the helicase unwinding of duplex DNA.

As indicated above, single molecule experiments have characterized RecBCD function with an unprecedented level of detail. RecBCD translocates along dsDNA in a highly processive manner with a step size less than 6 bp. Before Chi recognition, RecD is the lead motor unit. After encountering Chi, the enzyme pauses briefly to allow for a conformational transition that throttles RecD motion, abolishes degradation of the 3′ ssDNA tail, and makes the slower RecB enzyme the lead motor unit. Future work may capture RecBCD motion with single step size resolution[10,79] and may also extend the observation of RecBCD translocation in the presence of other DNA binding enzymes that are present during HR.

2.2 RecA recombinase

In *E. coli*, RecA executes the key step of HR: homology search and strand invasion into a complementary duplex sequence.[34,80–83] Studies of RecA function continue to serve as a keystone for understanding multiple aspects of HR. In addition, RecA is involved in multiple DNA metabolism pathways, such as induction of the SOS response and translesion DNA synthesis.[84–86] Homologs of RecA participate in HR in organisms ranging from bacteriophage to man, and the RecA fold is itself ancient and pervasive throughout all forms of life.[87]

Upon processing of a DSB by an exonuclease/helicase such as RecBCD, ssDNA is generated. RecA is a DNA-dependent ATPase that polymerizes onto the ssDNA to form a nucleoprotein filament. The RecA-ssDNA nucleoprotein filament rapidly scans for a homologous sequence amidst a large pool of heterologous DNA. Once homology between the site of the DSB and intact dsDNA is found, the RecA filament invades the duplex DNA to form a D-loop. Subsequent repair enzymes synthesize missing DNA using the undamaged complementary template and process the resulting HJs.[82,83]

Despite nearly fifty years of intense research into the structure and function of RecA and related homologs, several aspects of RecA catalyzed reactions remain poorly understood. Bulk biochemical methods do not directly or fully address the mechanism of RecA polymerization on DNA, homology search, strand exchange reactions, and the role ATP hydrolysis plays in each of these processes. However, single molecule methods are now being used to observe RecA nucleoprotein filament formation,[5,51,52,56,88,89] study the interaction of RecA with single stranded DNA binding protein (SSB),[5] and to follow the strand invasion reaction.[55,90]

Biochemical evidence, largely gleaned from careful monitoring of DNA-dependent RecA ATPase activity, suggested that nucleation of a RecA cluster consisting of several monomers onto ssDNA was a relatively slow step that was followed by a rapid, unidirectional filament extension in the 3′ direction.[81,83,84] Under certain *in vitro* conditions, RecA polymerizes on dsDNA.[91] Several early single molecule studies characterized the mechanical properties and rates of RecA filament assembly and disassembly on dsDNA stretched in an optical tweezers apparatus.[92,93] These studies concluded that RecA-dsDNA filaments remained relatively flexible and dynamic when ATP is used as a cofactor but adopted a rigid structure when the slowly hydrolysable analog ATPγS was used.[92,93]

Kowalczykowski and co-workers directly observed RecA nucleation and filament extension on dsDNA.[52] An optically trapped bead conjugated to a single 48 kb dsDNA molecule was incubated in a laminar flow channel containing fluorescent

RecA, followed by observation *via* epi-fluorescence in a second laminar flow channel free of RecA to afford better signal to background discrimination. Although this 'dipping and observation' approach precluded real-time monitoring of filament formation, snapshots of filament extension were obtained. Appropriate buffer conditions to facilitate RecA loading onto dsDNA had to be chosen, since this nucleation is not observed under physiological conditions.[91,94] The authors concluded that nucleation was highly dependent on solution conditions and thus most likely the key regulatory mechanism of RecA function *in vivo*. In contrast, filament extension appeared to be rapid and largely independent of reaction conditions. This observation was in accord with biochemical data that implicate multiple protein complexes such as RecBCD and RecFOR in facilitating nucleation of RecA onto ssDNA.[82]

A seminal study of RecA nucleation and filament extension on short oligonucleotide substrates mimicking HR intermediates utilized changes in fluorescence resonance energy transfer (FRET) to detect RecA polymerization on ssDNA with single protein monomer resolution.[5] A schematic of the experimental design is presented in Fig. 2a. A short DNA with a biotinylated dsDNA region (18 bp) and a long poly-dT ssDNA tail was immobilized *via* biotin–streptavidin interaction on the surface of a quartz slide and viewed with a total internal reflection fluorescence microscope (TIRFM).

Single-pair FRET was used as a readout of the distance between two dyes incorporated into the DNA substrate.[95] The

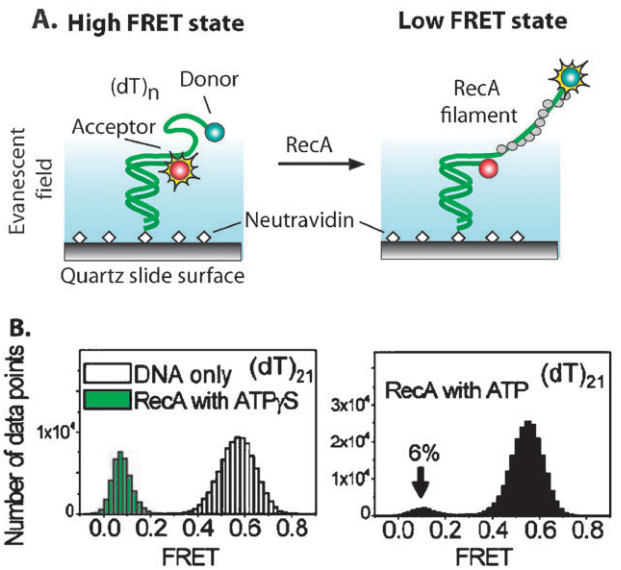

Fig. 2 A FRET-based assay for observing RecA nucleoprotein filament formation. (a) A DNA substrate with a poly(dT) ssDNA tail is labeled at two positions with a FRET dye pair (green and red spheres), immobilized on the surface of a flow-cell, and is illuminated *via* an evanescent field (blue gradient). Naked ssDNA is highly flexible, bringing the dye pair sufficiently close to observe a high FRET value (left panel), whereas a rigid RecA nucleoprotein filament separates the dyes, yielding a low FRET value (right panel). (b) A histogram of measured FRET values for naked ssDNA and RecA nucleoprotein filaments in the presence of various nucleotides. RecA–ATP filaments are highly dynamic and unstable. Reproduced with permission from ref. 5.

DNA was functionalized at one end with a Cy3 fluorophore (FRET donor, green sphere Fig. 2a) and at the dsDNA–ssDNA junction with Cy5 (FRET acceptor, red sphere Fig. 2a). Upon laser excitation of the donor dye, the highly flexible ssDNA brought the two fluorophores sufficiently close together to observe a high FRET signal (Fig. 2b, left panel). Introduction of RecA and ATPγS into the flow-cell initiated formation of the nucleoprotein filament, extended the ssDNA, and shifted the FRET signal to lower values. When ATP was used as a cofactor, a bimodal distribution of FRET values with both filament-like and naked ssDNA characteristics was observed (Fig. 2b, right panel). This data suggested that in the presence of ATP, RecA forms a highly dynamic nucleoprotein filament that is constantly undergoing assembly and disassembly.

To understand the complex kinetics of the observed FRET transitions, the authors adapted a statistical approach based on hidden Markov modeling to interpret the results in an unbiased manner.[96] A detailed analysis of multiple FRET time courses taken with the Cy3–Cy5 dye pair at various locations on the DNA substrate led the authors to the following conclusions regarding the mechanism of RecA nucleation and filament extension: (1) RecA polymerizes in the net 3′ direction with a minimum nucleation cluster of ~5 monomers, (2) filament extension and dissociation occur *via* single RecA monomer units, (3) given a nucleation site, extending RecA filaments readily displace SSB from ssDNA.[5] These results provided the first direct observation of crucial parameters such as the filament extension unit size as well as binding and dissociation rates at both 5′ and 3′ ends of the nucleoprotein filament.

Recently, strand exchange between single DNA molecules was monitored in real time using magnetic tweezers (Fig. 3a).[55] A 10 kb duplex DNA substrate was conjugated to a magnetic bead (orange sphere, Fig. 3a) at one end. The other end of the DNA was attached to a glass slide surface. Both ends were tethered *via* multiple anchor points to prevent free rotation of the DNA. Once a bead was captured in the magnetic field, the multiple attachment sites on both ends of the DNA allowed the introduction of supercoils by rotating the magnet. RecA–ssDNA nucleoprotein filaments were introduced into the flow-cell. In the presence of ATPγS, a RecA mediated three-strand structure was formed over the region of homology and experimentally observed as an increase in the DNA tether length (upper left panel, Fig. 3b). The change in DNA tether length increased linearly with the homologous overlap length. By measuring the DNA extension as the supercoils were unraveled, the authors observed a homology length dependent change in the number of dsDNA supercoils. Negative control experiments with nonhomologous ssDNA and dsDNA substrates confirmed that the reaction was dependent on sequence complementarity between the substrates (upper right panel, Fig. 3b).

In the presence of ATP, the dsDNA extension behavior differed markedly from ATPγS. Upon introducing the protein filament, the dsDNA extended, plateaued, and contracted back to the original length (lower left panel, Fig. 3b). This behavior was independent of the length of homologous overlap and was not observed between heterologous nucleoprotein filaments and dsDNA (lower right panel, Fig. 3b). In addition, there was no detectable change in supercoiling upon reaction

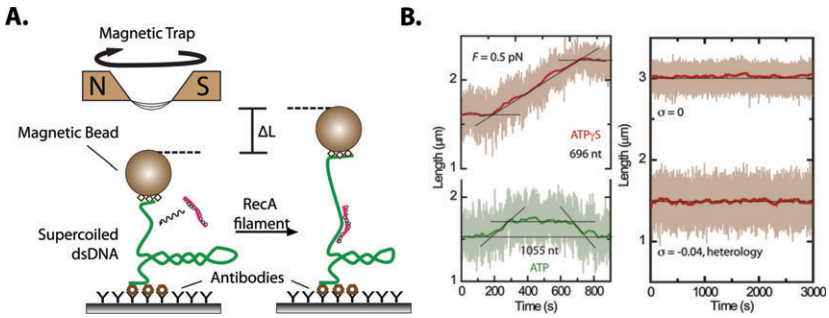

Fig. 3 (a) Schematic of a magnetic tweezers assay for observing HR. DNA is immobilized between a glass slide and a magnetic bead held in a magnetic trap. The DNA is supercoiled by rotating the magnet. RecA nucleoprotein filaments and nucleotides are introduced. RecA mediated D-loop formation is observed as an elongation of the DNA substrate. (b) Representative traces of DNA elongation in the presence of ATPγS and ATP and a homologous RecA filament (left panel) or a non-homologous filament (right panel). Reproduced with permission from ref. 55.

with RecA. This peculiar signature of the ATP mediated reaction led the authors to conjecture that RecA had catalyzed D-loop formation and that the protein dissociated from the duplex product.

Restriction enzyme mapping was used to define the DNA structure of the RecA–ATP dependent reaction product. An EcoRI site was introduced into the dsDNA tether region that was homologous to the ssDNA nucleoprotein filament. EcoRI incubation before the RecA reaction releases the magnetic bead by severing the dsDNA. After the RecA–ATP reaction, EcoRI digestion increased the distance between the bead and the surface, but did not result in complete bead separation from the DNA tether. Further overwinding of the DNA did not introduce any additional plectonemic coils, proving that a ssDNA patch was introduced by RecA at the EcoRI site.

The magnetic tweezers single molecule assay described above presented an unprecedented view of the steps governing RecA mediated strand exchange. In the presence of ATPγS, a three-stranded DNA–RecA structure was formed over the entire length of the homologous DNA region, but the protein could not disassemble after the DNA molecules were paired. With ATP, a RecA mediated structure was formed, but synapsis occurred over a limited ($\sim$80 bp) region and propagated as a traveling wave along the length of homology before RecA was completely disassembled from the resulting D-loop.

Numerous aspects of RecA function are still poorly understood and future single molecule studies may help shed light on these reactions. For example, assays similar to the ones described above may be used to probe RecA mediated synapsis and strand exchange between DNA strands with limited homology or heterologous inserts.[97] Several biochemical studies have suggested that RecA is a motor protein capable of using ATP hydrolysis to unwind dsDNA that is a few hundred bp ahead of the nucleoprotein filament.[83] This 'indirect helicase' activity may be probed at the single molecule level and could explain the mechanism of heterology bypass in RecA mediated strand exchange reactions. In addition, further work is necessary to characterize the interaction between RecA and other HR proteins such as RecBCD, RecFOR, and RecX.[82]

2.3 Rad51 recombinase

Rad51 is a eukaryotic RecA homolog that catalyzes homology search and strand invasion during HR. As in the case of RecA, Rad51 nucleoprotein filaments assemble onto exposed ssDNA tails and initiate the search for complementary sequences throughout the genome.[98] Although the key function of Rad51 is similar to RecA, there are notable differences between the biochemical properties of the two proteins. For example, Rad51 binds both ssDNA and dsDNA readily,[99] Rad51 requires many additional regulatory proteins,[100,101] and Rad51 does not appear to exhibit the rich array of auxiliary functions that have been observed in RecA reactions.[98]

In contrast to RecA, polymerization of Rad51 onto dsDNA is rapid and occurs with a slightly higher affinity than for ssDNA.[99] Fig. 4a describes a single molecule "DNA curtain" assay to probe the key elements of Rad51 assembly and disassembly on dsDNA.[28,45,48,53,102] A fluid lipid bilayer doped with biotinylated lipids was assembled on the surface of a quartz slide. DNA molecules were tethered directly to the bilayer *via* a biotin–neutravidin interaction, leaving the DNA free to diffuse within the bilayer. The application of hydrodynamic force (buffer flow) collects the tethered DNA molecules along edges of microscale diffusion barriers and extends the DNA to near full-length along the surface. The DNA is stained with YOYO1 and visualized *via* TIRFM. This approach allows the simultaneous observation of hundreds of physically aligned DNA molecules in real time within a single field-of-view (Fig. 4b). To monitor filament formation, Rad51 is flushed into a flow cell with a pre-assembled DNA curtain and the Rad51 assembly dynamics are observed as an increase in the DNA length. Thus, both the rate of DNA extension and final DNA–nucleoprotein filament lengths are obtained.

The Rad51 filament assembly reaction is sensitive to the type of nucleotide cofactor used. Fig. 4c presents representative filament assembly trajectories for ATP, ADP, and several nonhydrolyzable analogs. Although DNA extension is not observed when ADP is used, bulk gel-shift experiments indicate that the protein is associated with DNA, suggesting that Rad51 still assembles on dsDNA, but assumes a filament structure that does not extend the dsDNA significantly.[103]

The interdependence between Rad51 filament formation and nucleotide binding was gleaned from filament extension studies of Rad51 ATPase deficient mutants (right panel, Fig. 4c). The mutant K133R binds ATP and supports elevated levels of *in vitro* HR but has a significantly reduced hydrolysis

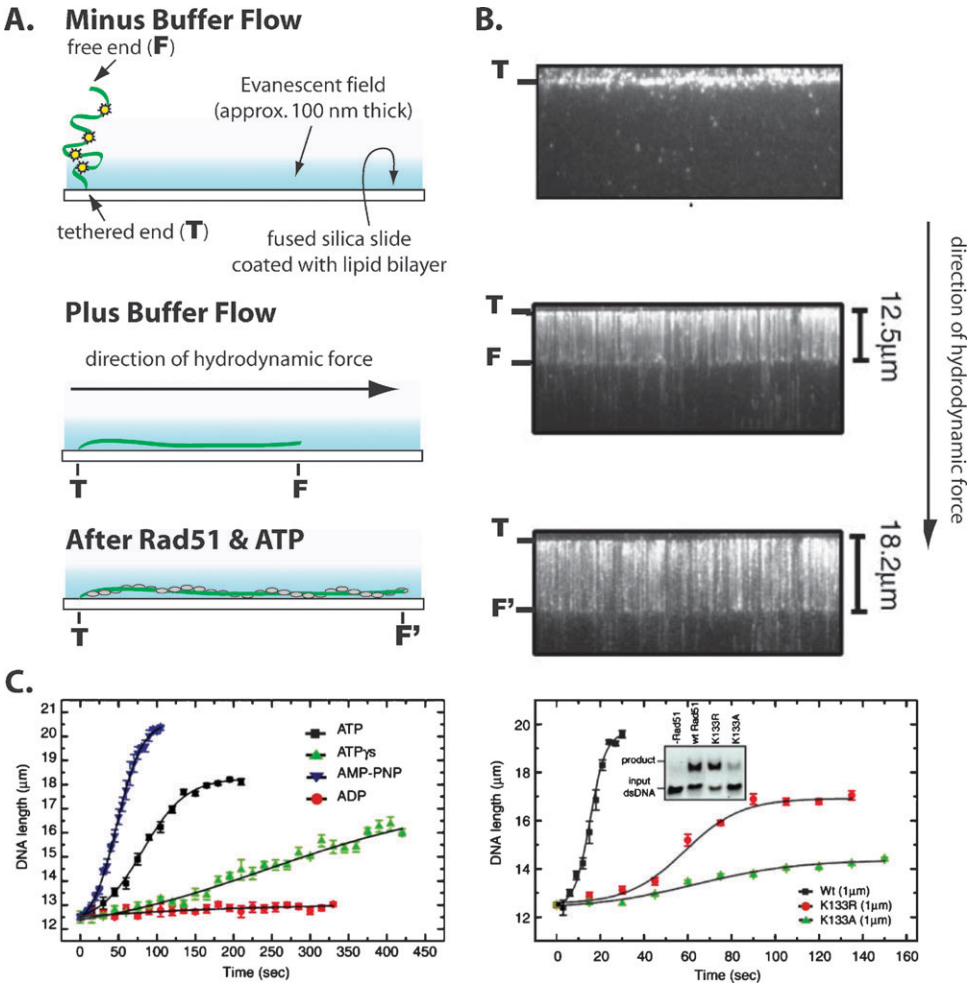

Fig. 4 A high throughput system for studying Rad51–DNA interactions. (a) A cartoon depiction of the components that make up the DNA curtain. DNA is tethered at one end to a lipid bilayer, labeled with YOYO1, and extended by buffer flow within an evanescent wave. Rad51 nucleoprotein filament formation is experimentally observed as an increase in the DNA length. (b) Shows an example of a DNA curtain at a scratch (top panel) that is extended into the evanescent wave (middle panel) and elongated by Rad51 nucleoprotein filament assembly (bottom panel). (c) Rad51 filament assembly on single DNA molecules as a function of nucleotide cofactor and mutations in the Rad51 ATP binding site. The inset demonstrates the strand exchange activity of wtRad51 and both ATPase deficient mutants. In all panels, (T) denotes the tethered end, (F) is the flow-extended free DNA end, and (F′) is the flow-extended DNA end after Rad51 nucleoprotein filament formation. Used with permission from ref. 48.

activity, whereas K133A binds dsDNA but is deficient for recombination (inset, Fig. 4c).[104] Single molecule DNA extension curves for both mutants were compared to the wild-type (wt) protein. Mutant protein filament assembly rates were significantly reduced and the resulting filaments were shorter for the ATPase mutants than for wtRad51. These results indicated that both mutant proteins either assembled as incomplete patches that left much of the dsDNA exposed or formed structurally distinct filaments.

Wyman and co-workers observed the disassembly of a fully functional fluorescent Rad51 from dsDNA in real time.[49] A Rad51 nucleoprotein filament was assembled on λ-DNA in the presence of Ca^{2+} to inhibit ATP hydrolysis, immobilized on the surface of a flow-cell, and visualized *via* TIRFM. Nucleoprotein filament disassembly was activated by exchanging the Ca^{2+} ions for Mg^{2+} to initiate ATP hydrolysis. The loss of fluorescence intensity from one end of the nucleoprotein filament offered the first direct observation of Rad51 disassembly from dsDNA in an ATP-hydrolysis dependent manner.

These results, in addition to other single molecule studies of Rad51 disassembly, are beginning to yield a picture of Rad51 filament dynamics on DNA.[46] The avid association of Rad51 with dsDNA that has been directly observed *in vitro* poses an enigma for *in vivo* function—Rad51–dsDNA filaments that can potentially form *in vivo* will sequester the cellular pool of available protein and may lead to undesired recombination reactions. The molecular motors Rad54 and Rdh54 (see below) have been implicated in clearing Rad51 from dsDNA.[105–107] Single molecule approaches offer the possibility to directly observe the mechanism of Rad51 removal from dsDNA by these enzymes. In addition, future work will address the interaction of Rad51 filaments with an ever-growing list of protein mediators such as Hop2-Mnd1 and BRCA2.[39]

2.4 Rad54/Rdh54 translocases

Rad54 and Rdh54 are members of the Snf2 family of DNA remodeling proteins.[108,109] Snf2 family proteins are ubiquitous in nature and have been implicated in multiple DNA processing roles, including chromatin remodeling, DNA replication, transcription, translation, and DNA repair.[108–110] Several biochemical activities have been demonstrated for the Rad54 DNA-dependent ATPase. Rad54 introduces supercoils in closed circular DNA,[111,112] stimulates DNA pairing, heteroduplex extension,[111,113] Rad51-heteroduplex disassembly,[105] and chromatin remodeling.[114,115] These findings, along with others,[116,117] led to the conclusion that Rad54 binds DNA as a multimeric complex and is capable of processive translocation. Rdh54 is another member of the Rad54-like Snf2 subfamily, and is closely related to Rad54 (37% sequence identity and 55% similarity). Rdh54 and Rad54 appear to be somewhat functionally redundant, and *rdh54 rad54* double mutants are more sensitive to DNA damage that either single mutant.[118] Single molecule investigations of both proteins have the potential to offer a unique vantage point on the diverse DNA processing capabilities of these molecular machines.

Kowalczykowski and co-workers reported the first direct observation of fluorescently labeled Rad54 translocation along dsDNA.[7] Biotinylated DNA was conjugated to a polystyrene bead, captured in an optical trap, and extended in the focal plane of an epifluorescence microscope *via* laminar buffer flow. *S. cerevisiae* Rad54 was isolated as a glutathione S-transferase (GST) fusion product and bound to a fluorescein labeled anti-GST antibody. The protein was loaded onto the dsDNA and motion of the Rad54-oligomeric complex was followed as a translocating bright spot along the extended dsDNA. Multiple fluorescent dyes per antibody and the oligomeric nature of Rad54 allowed the researchers to observe motion over hundreds of seconds.

Initial binding of Rad54 to dsDNA was sequence independent. Translocation occurred both upstream (against buffer flow) and downstream (with buffer flow) with a wide distribution of rates and frequent pauses and direction reversals. The motion was highly processive, spanning tens of thousands of base pairs. The authors attribute the heterogeneous rates of translocation and observed pauses and direction reversals due to engagement of different DNA binding domains of Rad54 with DNA.[7]

Bianco and co-workers adapted a similar experimental approach using a dual optical trap system.[57] Two beads, each with a single dsDNA molecule were trapped in two independent optical traps and brought into close contact. The dsDNA was labeled with the intercalating fluorescent dye YOYO1. Changes in DNA length and DNA co-aggregation could be followed with the native protein. Although the location and number of Rad54 clusters was not observed, this study offered indirect evidence that DNA aggregation is mediated by Rad54 in a protein concentration dependent manner.[57]

Greene and co-workers applied the high-throughput 'DNA curtain' assay to observe Rdh54-mediated DNA remodeling events (Fig. 5a).[6] A curtain of dsDNA was assembled at a lipid diffusion barrier on the surface of a flow-cell (see Section 2.3 for description). Rdh54 was expressed as a GST fusion product and conjugated with anti-GST antibodies covalently linked to fluorescent quantum dots (QDs). Quantum dots are highly fluorescent semiconductor nano-particles that are not prone to photobleaching, allowing observation of Rdh54

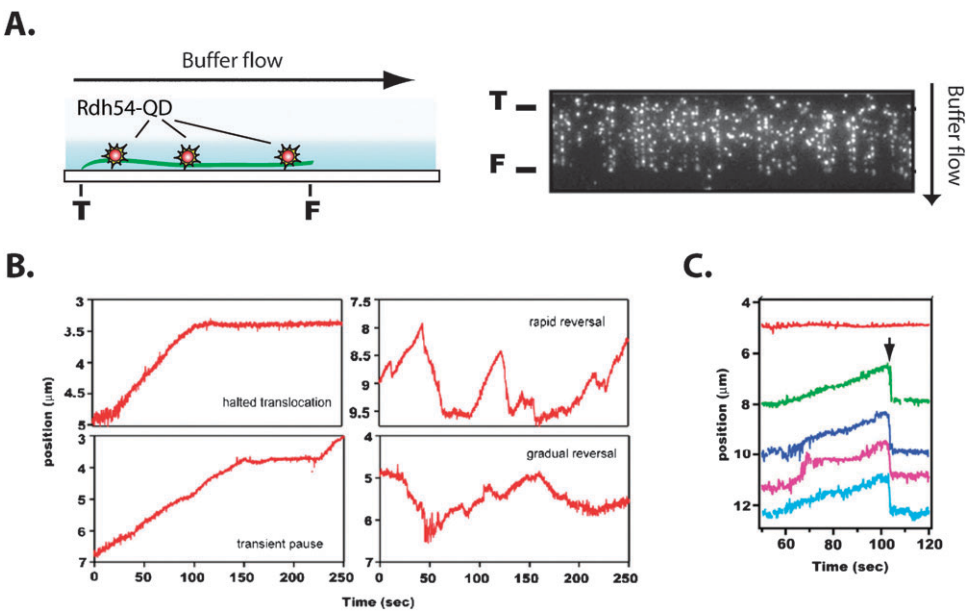

Fig. 5 (a) A schematic illustration and representative image of the Rdh54 translocation assay. In the image, the DNA is not fluorescently labeled and the Rdh54–QD complexes form a punctate pattern on the DNA curtain. (b) Representative trajectories of Rdh54 motion on DNA. Rdh54 exhibits complex translocation behavior that is characterized by transient pausing, and changes in velocity and direction. (c) A signature of Rdh54 looping activity is the simultaneous release of multiple QD complexes on the same single DNA molecule in the direction of flow (highlighted by arrow). In this example, an upstream complex released a DNA loop that was observed as a sudden jump in the locations of the downstream molecules. Used with permission from ref. 6.

translocation for many minutes without reduction in signal intensity. A representative frame of a DNA curtain (unlabeled) bound by Rdh54-QD conjugates is presented in the left panel of Fig. 5a.

Fig. 5b shows several typical traces of Rdh54 translocation along single DNA molecules. These studies found that Rdh54 could translocates at a rate of approximately 80 bp s^{-1}. The motion was highly heterogeneous and included transient pauses and translocation direction reversals, in agreement with similar studies on Rad54.[7,57] In addition, DNA loop release events were observed occasionally. When multiple complexes were loaded onto the same dsDNA molecule, rapid and correlated translocation direction reversals would simultaneously appear at several Rdh54 foci (Fig. 5c, black arrow). This ATP dependent behavior was explained as the formation and release of DNA loops by an upstream Rdh54 complex. Recent work in the lab has extended the single-molecule curtain assay by introducing a second color quantum dot at the free, flow extended terminus of the dsDNA. This provides a simultaneous readout of Rdh54 translocation and looping products by independently monitoring the changes in the DNA length (*unpublished*).

Thus, single molecule assays have revealed the translocation and DNA looping behavior of Rad54 and Rdh54. Current single molecule studies of Rad54/Rdh54 translocation have utilized naked dsDNA substrates. In light of the diverse DNA remodeling roles attributed to Rad54, it will be of particular interest to observe what happens when these motor proteins encounter molecular roadblocks such as Rad51 filaments, nucleosomes, and chromatin fibers.

2.5 RuvAB resolvase

Holliday junctions are key intermediates of the RecA/Rad51-mediated homology search and strand invasion.[34,37] In *E. coli*, HJs are recognized by the RuvA protein, which preferentially binds at the junction with >1000 fold specificity relative to linear dsDNA.[61] A tetrameric RuvA assembly at each side of the HJ recruits the molecular motor RuvB, which forms a hexameric ring around RuvA.[119,120] The RuvAB complex catalyzes branch migration of the HJ in a highly processive, ATP dependent fashion.[61] The RuvC homodimer completes HJ resolution by endonucleolytically cleaving the junction at the consensus sequence 5′-(A/T)TT′(G/C)-3′.[121–123]

Structural, biochemical, and mechanistic analysis of the RuvAB complex suggests that the RuvB motor pumps DNA through the RuvA core, inducing a rotation of the helical dsDNA.[120] To directly observe the rotation of dsDNA that is being processed by RuvAB, Han and co-workers developed the simultaneous magnetic tweezers and fluorescence microscope system shown in Fig. 6a.[62] A cruciform DNA was constructed with long (4.7 kB, 1.7 μm) vertical arms and short horizontal arms. One end of the long arms was decorated with multiple biotins and the other with multiple digoxigenins. The cruciform DNA was immobilized on the glass surface of a flowcell *via* digoxigen–anti digoxigenin interactions. The other end of the cruciform DNA was reacted with a large (0.7 μm) streptavidin coated magnetic particle (brown sphere, Fig. 6a) that had a small (20 nm) fluorescent bead (red sphere, Fig. 6a) affixed asymmetrically to the large bead. The cruciform DNA was extended orthogonally to the surface in a magnetic trap.

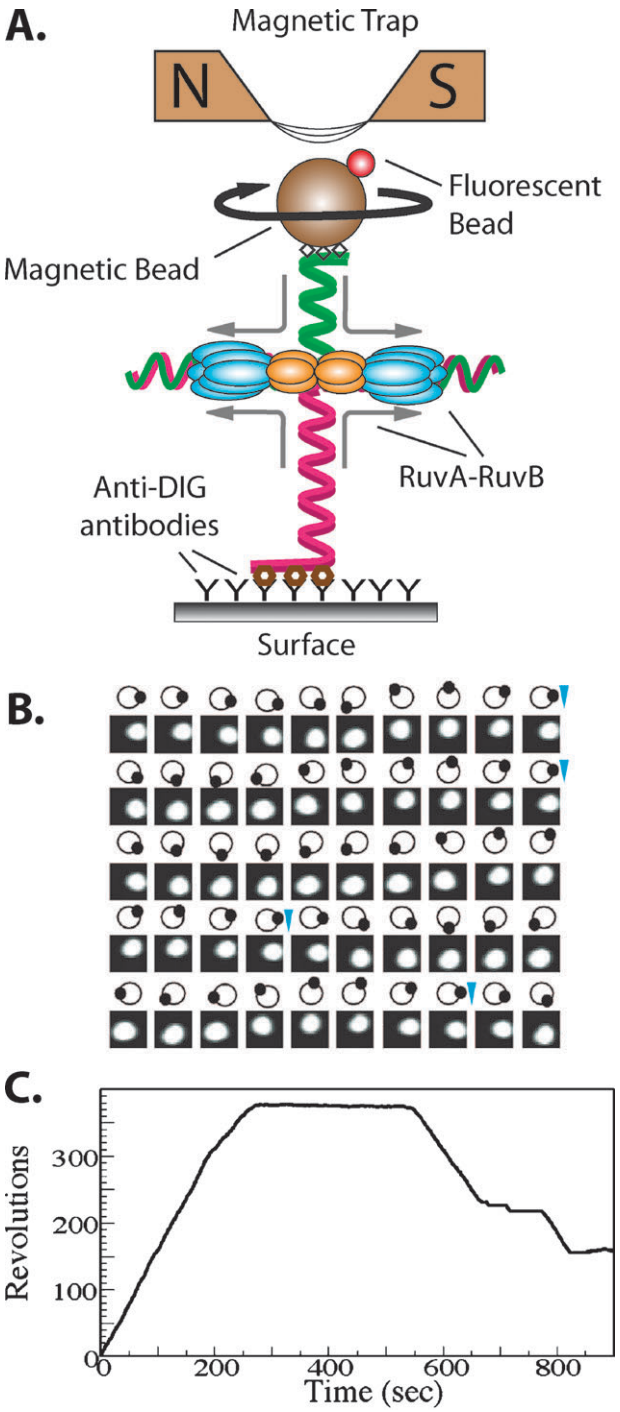

Fig. 6 (a) A cartoon of the experimental setup used to observe RuvAB Holliday junction migration. A cruciform DNA is trapped between a flow cell surface and a streptavidin-coated magnetic bead (brown sphere) in magnetic tweezers. The magnetic bead is decorated with a fluorescent polystyrene sphere (red sphere). (b) RuvAB processes the Holliday junction, which leads to a rotation of the fluorescent sphere. Blue triangles represent a complete revolution of the fluorescent particle. (c) A representative trace of RuvAB-mediated processing of a Holliday junction. The reaction is characterized by transient pausing and occasional direction reversals. Panels (b) and (c) used with permission from ref. 62.

Upon addition of RuvAB in the presence of both Mg^{2+} and ATP, branch migration was expected to cause a rotation of the DNA strands. Multiple anchor points of the cruciform DNA between the magnetic particle on one end and the flow-cell surface on the opposite end ensured that the DNA rotation was transmitted to the magnetic bead.

Fig. 6b presents sequential images of a rotating fluorescent sphere due to RuvAB dependent branch migration of a single DNA cruciform in and ATP dependent reaction. Loading of RuvAB onto the sides of the cruciform orthogonal to the tethered ends causes counter-clockwise rotation and subsequent shortening of the vertical dsDNA length until ultimately the bead irreversibly associates with the glass surface (see Fig. 6a). If the RuvAB complex assembles along the vertical (long) arms of the cruciform DNA, clock-wise bead rotation is observed, and the observed DNA length will increase until all available cruciform DNA is processed into two disjoined dsDNA strands (observed as a release of the untethered magnetic bead).

Individual traces of hundreds of revolutions as a function of time (Fig. 6c) revealed that RuvAB complexes are highly processive, exhibit long pauses, and undergo frequent reversals in the revolution direction. The authors interpret the observed pauses and revolution direction reversals as disassembly of the RuvAB complex at one side of the DNA junction and reassembly of a different complex at the opposite sides of the junction. The torque exerted by the RuvAB molecular motor was approximated as at least $\sim$10 pN by estimating the force necessary to rotate a $\sim$0.7 μm bead in water.[62]

Stavans and co-workers employed a similar magnetic tweezers approach to study RuvAB-mediated branch migration.[59] A magnetic particle was tethered to a flow-cell surface *via* a cruciform DNA with two long vertical arms. The branch migration reaction was followed by a change in length of the vertical arms of the cruciform DNA by observing a changing diffraction pattern of the magnetic particle as the bead approaches or recedes away from the surface. The authors observed that RuvAB-catalyzed branch migration was processive ($>$1000 bp), marked by pauses and transitions between elongation and shortening of the cruciform tether, and exhibited a complex distribution of branch migration rates. Again, the pauses were interpreted as multiple unloading and reassembly events of the RuvAB complex on opposing sides of the Holliday junction. The DNA migration rate distribution showed multiple well defined peaks that remained largely independent of force up to $F \sim 15$ pN and at saturating ATP concentrations. The discrete HJ branch migration rate distribution supports the view that the hexameric RuvB assembly consists of distinct dimer or trimer subunits.[59]

Using a similar magnetic tweezers and cruciform DNA assay, Heslot and co-workers probed the dependence of branch migration rates on applied force, enzyme concentrations, and DNA sequence.[60] The authors confirmed that the branch migration rate remains largely independent of applied force below several pN and exhibited pausing and direction reversal behavior. The rate of pausing and branch migration restart was found to depend strongly on RuvB, but not RuvA concentration, confirming that RuvA assembly at the junction is not the rate limiting step, and that free RuvA in solution does not sequester RuvB away from the DNA. Finally, the branch migration rate did not exhibit any DNA sequence dependence.

Biochemical studies have demonstrated that RuvAB can bypass heterologous regions.[124] RuvAB-mediated branch migration in the context of Holliday junctions with heterologous sequences was investigated by both bulk and single molecule methods.[61] Bulk biochemical experiments confirmed that RuvAB translocation was impeded by heterologous sequences and a single molecule magnetic tweezer assay demonstrated that although migration is generally stalled, infrequent translocation events past the heterology can be observed.[61]

Thus, single molecule assays have began to unravel the mechanism of HJ processing by RuvAB, but future studies may reveal even more detailed aspects of these reactions. For example, the precise nature of the mechanical transactions that must occur during branch migration remain unknown. Although it is clear that DNA rotation occurs during branch migration, is not known how this rotation and translation movement of the DNA is coupled to structural transitions in the protein complex. Further work may also address the function of RuvAB in complex with RuvC, an important player in HJ resolution. A more complete picture of RuvAB function in the context of HR will continue to emerge as these studies are conducted in the presence of other enzymes that are present during the final steps of recombination.

3. Future perspectives

Single molecule studies on DNA remodeling enzymes have yielded new information that was unobtainable *via* traditional bulk biochemical methods. Observation of RecBCD translocation have directly demonstrated the highly processive motion of this enzyme along DNA and have uncovered conformational changes upon recognition of the recombinase hotspot Chi. The first steps of recombinase mediated strand invasion—nucleation on DNA and filament extension—have both been observed for RecA and Rad51. RecA mediated strand invasion and D-loop formation was captured on single DNA molecules. The translocation and DNA loop extrusion of Rad54 and Rdh54 have been reported. Finally, the RuvAB mediated Holliday junction branch migration and DNA rotation have been observed directly.

Rapid development of the single molecule field will continue to transform our understanding of HR. Ultrasensitive microscopes and optical tweezers assays have permitted the tracking of enzymes along DNA with ever increasing resolution.[10,125,126] Nanofrabrication of birefringent microparticles for optical tweezer experiments has recently allowed the simultaneous application of force and torque to generate and measure length changes in supercoiled DNA with millisecond time resolution.[127] By combining fluorescence techniques with optical and magnetic tweezers, the manipulation and simultaneous direct observation of HR reactions is rapidly becoming feasible.[128]

Improvements in fluorescent markers will continue to expand the capabilities of single molecule techniques. The development of long-lived organic fluorophores with distinct absorption and emission spectra has spurred the development

of three color FRET.[129] Observing FRET between three substrates may reveal the precise time course of events that must occur during RecA and Rad51 mediated homology search, strand invasion and filament disassembly reactions. Recent progress in minimizing the size of quantum dots promises to extend our mechanistic understanding of individual recombination enzymes as well as multi-protein complexes *in vitro* and will begin to transfer that knowledge to an intracellular context.[130,131]

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