REVIEW

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Visualizing one-dimensional diffusion of proteins along DNA

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The ability of proteins to locate specific target sequences or structures among a vast excess of nonspecific DNA is a fundamental property that affects virtually all aspects of biology. Despite this importance, experimental methods have lagged behind the establishment of theoretical principles describing potential target location mechanisms. However, recent advances in single-molecule detection now allow direct visual observation of proteins diffusing along DNA. Here we present an overview of these new observations and discuss the advantages, limitations and future prospects for imaging the motion of proteins along DNA.

As noted by Adam and Delbrück in 1968, reducing the dimensionality of diffusion-based reactions in biological systems can greatly increase the efficiency of bimolecular interactions[1]. Two years later the importance of this concept to protein–nucleic acid interactions came to light when Riggs and co-workers found that the *lac* repressor protein could locate its target site up to 1,000-fold faster than predicted by simple three-dimensional diffusion and random collision[2]. This startling discovery prompted a stream of studies investigating possible mechanisms proteins might use to travel along DNA. The seminal works of Peter von Hippel, Otto Berg and colleagues provided a thorough treatment of this phenomenon from both theoretical and experimental perspectives, leading to several diffusion-based models to explain the rapid protein movement on DNA[3–6]. These hypothetical mechanisms, which are not mutually exclusive, include (i) one-dimensional hopping, where the protein moves along the same molecule of DNA via a series of microscopic dissociation and rebinding events; (ii) jumping, where a protein moves over longer distances (or even between different DNA molecules) via dissociation and then rebinding at a distal location; (iii) one-dimensional diffusion or sliding, involving a random walk along the DNA without dissociation; and (iv) intersegmental transfer, involving movement from one site to another via a looped intermediate (**Fig. 1**). Collectively, these models laid the intellectual groundwork for all subsequent studies of target-search mechanisms.

Ensemble studies contributed tremendously to our understanding of target-search mechanisms and remain an extremely valuable tool for assessing how proteins move on DNA. However, the experimental achievements eventually reached an impasse; most studies could only address whether or not a protein might travel by facilitated diffusion, but they could not describe many important aspects of the motion itself. For example, kinetic assays revealed that association rates for EcoRI increased on substrates with longer nonspecific DNA flanking the target binding sequences[7]. Although these studies strongly implied that facilitated diffusion contributed to the reaction mechanisms, they could not distinguish between sliding, hopping and/or intersegmental transfer. This problem was overcome in a recent study of the restriction enzyme BbvCI, which recognizes and cleaves an asymmetrical target site[8]. In this elegant study, Halford and colleagues used DNA substrates with two sites separated by different distances and varying orientations relative to one another. These experiments revealed that, when the targets were separated by distances of <50 base pairs (bp), the two sites were more efficiently cleaved when they were in the same orientation. In contrast, when the sites were separated by distances >50 bp, there was no difference between the inverted sites relative to those oriented in the same direction. This outcome indicated that the enzyme could reverse orientation between cleavage of the first and second sites, implying that dissociation and rebinding had occurred when the protein traveled between the two sites[8]. These and similar experiments provided evidence supporting a sliding mechanism; however, all of these assays relied on indirect readouts of one-dimensional diffusion, such as cleaved DNA products, which drove the focus of many these experiments toward endonucleases[9–12].

Different approaches were necessary for proteins lacking nuclease activities. For example, both p53 and UL42 (a viral DNA replication–processivity factor) have been shown to dissociate from DNA by sliding off the free ends[13,14]. This was revealed in assays demonstrating that these proteins remained bound to DNA when the ends of the molecule were blocked with streptavidin. Several studies have also shown that proteins belonging to the MutS-family of DNA repair proteins show end-dependent dissociation that is restricted when the DNA ends are obscured with a protein block[15–19]. More recently, NMR techniques have been used to probe transient interactions between macromolecules, offering some of the most informative measurements of protein diffusion along DNA. Using these methods, Clore and colleagues have shown that the Hox D9 homeodomain both hops and slides along nonspecific DNA while searching for target sites in a 24-bp oligonucleotide[20,21]. Although all of these experiments

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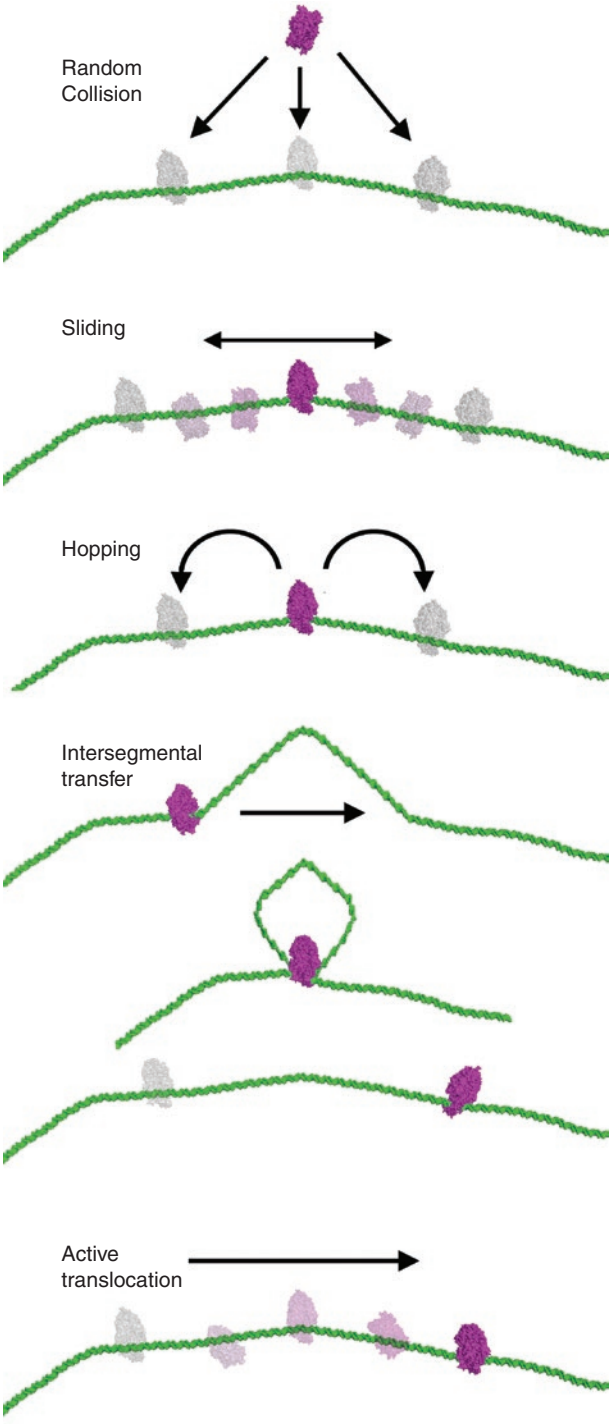

Figure 1 Potential modes of target-site location. Proteins can locate targets via several potential mechanisms, including random collision (please note that reiterative random collisions by the same protein can be referred to as 'jumps'), one-dimensional sliding, hopping, intersegmental transfer and active translocation.

expanded the repertoire of proteins that could be studied, they still failed to provide some detailed information regarding mechanisms.

As the experimental studies progressed, theoreticians continued building a more comprehensive framework for understanding how proteins might travel along DNA. For example, Michael Schurr noted that diffusion of a protein along DNA would be substantially slower if the protein had to rotate around the helix to track the phosphate backbone, as opposed to the much faster movement expected if lateral motion was not coupled to rotation[22,23]. In fact, for a typically sized protein, there is roughly a 1,000-fold difference in the predicted diffusion coefficient, depending upon whether or not the protein rotates around the helix to maintain register with the phosphate backbone. Further theoretical studies suggested that the most efficient search mechanisms would use a combination of one-dimensional and three-dimensional diffusion[24,25], and these predictions have been supported by the findings of several bulk studies[26]. This is because sliding along DNA occurs through a highly redundant random walk, which is inefficient for identifying a target located far away from the initial binding site. In addition, Slutsky and Mirny noted that the energy landscape of diffusion along DNA must be relatively smooth, with a roughness on the order of k_BT, otherwise the search becomes unreasonably slow[27]. They predicted that proteins would move only short distances before becoming stuck if the roughness of the landscape exceeded ~2 k_BT, and target-site specificity would be defined by the existence of traps considerably deeper than k_BT[27]. It was also proposed that, to reconcile a fast search with strong specific interactions, target binding may be coupled to conformational changes in the protein that further stabilize the specific complex, a concept supported by structural studies of proteins bound to specific versus nonspecific DNA substrates[20,28–30].

Visualizing the movement of single proteins in real time

It had finally become apparent that new experimental methods would be required to elucidate facilitated target location mechanisms[24]. Significant advances have recently been made using optical techniques, in particular total internal reflection fluorescent microscopy (TIRFM), to visualize single proteins moving along individual DNA molecules. For TIRFM, a laser is reflected off the interface between an aqueous sample and a microscope slide (**Fig. 2**). This illumination geometry generates an evanescent field, which decays exponentially into the aqueous sample. Only molecules confined within a few hundred nanometers of the surface are illuminated, and this spatially selective excitation is the key element necessary for detecting single fluorophores. TIRFM combined with methods for confining long DNA molecules within the evanescent field has resulted in a surge of direct visual evidence demonstrating lateral diffusion along individual DNA molecules by proteins involved in a range of biological processes.

Gene expression and facilitated diffusion. RNA polymerase (RNAP) is thought to locate promoters through facilitated diffusion. Indeed, several early biochemical and single-molecule studies were used to examine the movement of RNAP along nonspecific DNA while seeking out promotor sequences[31–34]. In fact, the earliest single-molecule optical microscopy experiments with *Escherichia coli* RNAP were a remarkable technological *tour de force*, and revealed the very first images of proteins interacting with individual molecules of DNA[33,34]. For example, Shimamoto and co-workers used a new assay with stretched 'belts' of λ-DNA to show that fluorescently tagged RNAP could slide along the DNA[34]. Several years later, Harada *et al.* used TIRFM in combination with a dual optical trap to detect binding and one-dimensional sliding of RNAP[33]. Most recently the ability of T7 RNAP to slide along nonspecific DNA was demonstrated using TIRFM assays in which λ-DNA was adhered at nonspecific sites to the surface of a flow cell through molecular combing or attached at a single end and flow-stretched as in the experimental setups described below[35]. T7 RNAP, labeled with rhodamine, was observed diffusing along the DNA molecules in a manner consistent with a sliding mechanism over long

periods of time (dissociation was not observed on the timescales of the observations) and showed diffusion coefficients ranging from 6.1×10^{-3} up to 4.3×10^{-1} $\mu m^2 s^{-1}$. The diffusion coefficients were not dependent upon NaCl concentration, ruling out the possibility that hopping contributed to the observed motion (see below) and confirming that RNAP can travel along DNA by diffusion.

Transcription factors such as the *lac* repressor present another classical example of an entire class of proteins that must locate specific targets to fulfill their biological roles. The *lac* repressor (LacI) binds a specific operator sequence (*lacO*), preventing RNAP from transcribing genes involved in lactose metabolism. Wang *et al.* used TIRFM to show that one-dimensional sliding contributes to this mechanism[36]. For these experiments, λ-DNA containing 256 tandem *lacO* sites was incubated briefly with green flourescent protein (GFP)-tagged LacI, and the proteins bound to *lacO* sites served as anchors through nonspecific adsorption to the fused silica surface of the slide, whereas DNA located between the protein anchor points was freely suspended, as confirmed by transverse fluctuations of the molecule. Additional molecules of GFP-LacI bound the freely suspended DNA and were observed diffusing on the helical axis of the molecules. Images of GFP-LacI particles were recorded for up to 5 s before photobleaching, and analysis of 15 trajectories revealed a broad distribution of one-dimensional diffusion coefficients ranging from 2.3×10^{-4} to 1.3×10^{-1} $\mu m^2 s^{-1}$ as the protein spanned distances of 120–2,920 nm (~350–8,600 bp). This observation confirmed that the *lac* repressor can travel along DNA via a sliding mechanism, finally providing a direct experimental explanation for the observation of Riggs *et al.*[2] that the protein could locate its target site much faster than predicted by three-dimensional diffusion.

One-dimensional diffusion of DNA repair proteins. One of the first TIRFM studies visualized one-dimensional sliding of the eukaryotic recombinase Rad51, a member of the RecA family of DNA recombinases composed of ATP-dependent DNA binding proteins essential for homologous recombination[37]. These experiments relied upon λ-DNA molecules (48,502 bp, ~16 μm), which must be maintained in an extended configuration parallel to the surface of the microscope slide to allow visualization along their full contour length. Two strategies were used for confining the λ-DNA within the evanescent field. In the first, DNA molecules were anchored by one end to a lipid bilayer, then aligned along a mechanical barrier and extended parallel to the surface with hydrodynamic force[37]. Rad51 complexes, fluorescently labeled with Alexa-fluor 555, were observed moving along DNA molecules. Members of the RecA family are known to form extended helical filaments, compressed filaments and oligomeric rings comprised of six to eight subunits, and it is likely that the proteins traveled as either octameric rings or possibly as small sections of compressed filaments. The buffer flow used to extend the DNA was found to bias the direction of Rad51 movement, pushing the proteins toward the free ends of the DNA. To confirm that the motion was one-dimensional diffusion, DNA molecules were held in an extended conformation by anchoring both ends to the slide surface, eliminating the need for continuous buffer flow. This confirmed that the motion was unbiased, bidirectional and did not require ATP hydrolysis. For diffusive motion, a plot of mean-squared displacement (MSD) versus time interval is expected to yield a linear graph whose slope can be used to calculate a one-dimensional diffusion coefficient[37,38]. This MSD analysis demonstrated that the movement of Rad51 was an unbiased random walk, fully consistent with the mathematical predictions for one-dimensional diffusion, and yielded diffusion coefficients ranging from 1.0×10^{-3} to 2.1×10^{-1} $\mu m^2 s^{-1}$.

TIRFM has also revealed that the human oxoguanine glycosylase protein (OGG1), which initiates base-excision repair of mutagenic 8-oxoguanine lesions arising from oxidative stress, can travel along DNA by one-dimensional diffusion[38]. OGG1 was labeled with Cy3B and observed on flow-stretched λ-DNA anchored to a flow-cell surface by a single end. Movement of the proteins was unbiased, despite the buffer flow required to extend the DNA, probably because of the relatively small size of OGG1. The protein showed a mean diffusion coefficient of $4.8 \pm 1.1 \times 10^6$ $bp^2 s^{-1}$ (0.55 ± 0.13 $\mu m^2 s^{-1}$), and analysis of the salt dependence of the reaction verified that sliding, not hopping, was the mechanism of the observed diffusion. On the basis of the shorter residence times observed near physiological salt concentrations, the authors also concluded that a three-dimensional mechanism must also contribute to the overall search process (see below). Importantly, this work addressed several theoretical issues previously raised for one-dimensional diffusion. For instance, the one-dimensional diffusion coefficients obtained in this study were consistent with a model in which the protein rotated about the helical axis of the DNA as it slid[22]. By calculating the upper limit of diffusion coefficients for this rotational model with estimated hydrodynamic values for the protein size, the authors determined that the mean activation energy for sliding from one position to the next was ~1 k_BT, consistent with theoretical predictions[27]. Finally, the authors found that the energy landscape for diffusion was dependent upon pH, and they could adjust the activation barriers by mutating a single histidine residue within the protein.

Post-replicative mismatch repair proteins belonging to the MutS family are involved in the repair of DNA replication errors[39,40]. These proteins present a interesting case in that they must identify two different targets: first, they must find mispaired bases to initiate repair; second, they must locate strand-discrimination signals (for example, hemimethylated dGATC sites in *E. coli*), which can reside at distances greater than 1 kb away from the mispaired base[39,40]. Several studies suggested that MutS family members could travel along DNA by sliding[15,17,41], and indeed the structures of the proteins resemble a clamp that completely encircles DNA molecules[42–44]. Using TIRFM, Gorman *et al.* demonstrated that the mismatch repair complex Msh2–Msh6 (MutS homologs) could travel along λ-DNA by one-dimensional sliding[45]. The experimental system was analogous to that of Graneli *et al.*[37], but here the protein complex was fluorescently tagged with a quantum dot. The observed movement of Msh2–Msh6 was consistent with one-dimensional sliding with a mean diffusion coefficient of $1.2 \pm 1.8 \times 10^{-2}$ $\mu m^2 s^{-1}$. Multicolor experiments

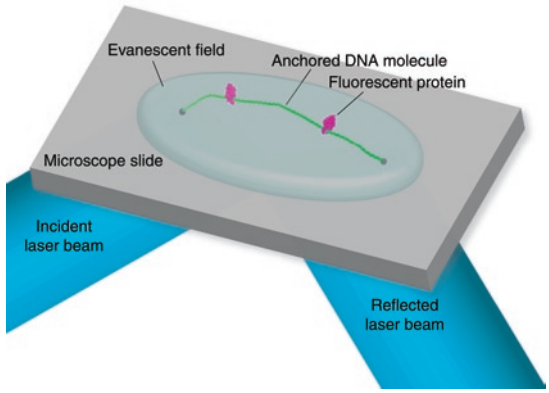

Figure 2 Using TIRFM to visualize protein one-dimensional diffusion. The evanescent field is created by reflecting a laser off the interface between a microscope slide surface and a buffer containing the sample. The penetration depth of the field is typically 100–200 nm, and any fluorescent molecules that are to be visualized must be confined within this region. Therefore, long DNA molecules must be stretched along the surface to visualize their entire contour length.

with Msh2–Msh6 differentially labeled using either green or red quantum dots revealed that the sliding proteins could not bypass one another when bound to the same substrate despite frequent collisions, suggesting that hopping was not involved in the lateral motion. This conclusion was supported by experiments demonstrating that the diffusion coefficients showed no dependence on the ionic concentrations. Although the DNA used in these experiments did not contain any mismatches, over longer periods of time many of the Msh2–Msh6 complexes entered transiently immobile states corresponding to energetic traps in the diffusion landscape, and the authors speculated that these immobile proteins mimicked damage-recognition complexes. Interestingly, rapid exchange of ADP for ATP released Msh2–Msh6 from the immobile state, possibly reflecting a conformational change in the protein, which then continued sliding along the DNA molecule. These observations suggested an overall pathway for the repair mechanism involving some degree of lateral movement of the protein along DNA to locate lesions whereupon Msh2–Msh6 enters a nondiffusive state that may provide time for the recruitment of other repair factors and, finally, ATP hydrolysis provokes reentry into the diffusive state, possibly allowing the proteins to continue the search for the strand-discrimination signals[45].

Is diffusion coupled to rotation around the helix?

As indicated above, the predicted diffusion coefficients are drastically different depending on whether or not the protein in question rotates around the DNA as it slides back and forth[22,23,38,45]. So far, all of the studies that have visualized one-dimensional diffusion reported diffusion coefficients that were remarkably consistent with the predictions of the Schurr rotation model[35–38,45]. Intuitively, this makes sense, because many proteins make nonspecific contacts with DNA through electrostatic interactions with the phosphate backbone. Consequently, lateral movement from one position to the next would require rotation for a protein to maintain correct register with the DNA. Maintaining continuous contact with the DNA during sliding could be advantageous, because it would prevent a protein from hopping over and potentially missing a target site[10]. However, rotational motion coupled to lateral sliding has not been directly visualized for any diffusing protein. Therefore, it is formally possible that the agreement between the experimentally observed diffusion coefficients and the Schurr model is purely coincidental. For example, the proteins could be sampling nondiffusive states that are too short lived to detect directly with current charge-coupled device (CCD) cameras. Thus, the observed diffusion coefficients could represent a combination of diffusive and nondiffusive states.

Although rotation has not yet been directly observed during one-dimensional diffusion, Sakata-Sogawa and Shimamoto have shown that *E. coli* RNAP tracks along the groove of DNA[46]. In this study, a streptavidin-coated bead was asymmetrically labeled through the attachment of a smaller fluorescent sphere. A DNA molecule was anchored to the bead through multiple contact points, such that any rotation of the DNA would in turn convey a torque to the bead. An optical trap was used to slowly drag the DNA past the RNAP-coated coverslip, which in turn caused rotation of the bead. This led to the conclusion that the RNAP tracked the helical pitch of the DNA. Sakata-Sogawa and Shimamoto have taken an important step toward validating the Schurr model; however, the data were not collected under freely sliding conditions, and it remains to be seen whether this is a general concept that can be applied to other DNA binding proteins.

Does facilitated diffusion contribute to reactions *in vivo*?

All of the *in vitro* TIRFM studies relied on naked DNA molecules that were stretched in an extended configuration, which is necessary to experimentally visualize motion of proteins along the DNA. These substrates are very different from what is encountered *in vivo*. For example, eukaryotic chromosomes are highly condensed structures bound by many proteins, such as nucleosomes, which could pose as obstacles to facilitated diffusion[25,47]. How might proteins overcome these obstructions during target searches? Proteins capable of hopping or intersegmental transfer could readily bypass these barriers. Sliding proteins could also bypass obstacles if the sliding mechanism did not require them to continually track the phosphate backbone (also referred to as two-dimensional diffusion)[25]. However, proteins that track the phosphate backbone would not be able to bypass immobile proteins on the DNA. There are several potential solutions to this problem: (i) combined use of one-dimensional sliding and three-dimensional diffusion; (ii) one-dimensional sliding combined with other modes of transport that permit bypass (that is, hopping and/or intersegmental transfer); (iii) restricting the search to regions of the genome that are relatively free of proteins (that is, euchromatin); or (iv) coupling the sliding to a second protein capable of actively clearing DNA of bound proteins (for example, a replication fork or transcribing polymerase). The combined use of one-dimensional and three-dimensional searches is well accepted[8,24,48], and probably contributes to many *in vivo* searches, but the last three mechanisms remain to be tested.

Assessing the actual modes of target search *in vivo* remains a challenge, but the advent of GFP tagging has lead to new approaches for observing protein behavior in living cells. Fluorescence recovery after photobleaching (FRAP) is one extremely powerful approach for probing the diffusive properties of GFP-tagged proteins *in vivo* and is helping to reveal the patterns of protein mobility in the nucleus[47,49]. Most of these studies suggest a three-dimensional diffusion component is involved in target-search mechanisms for DNA binding proteins, but, interestingly, diffusion coefficients measured by FRAP are often appreciably (10-fold to 100-fold) smaller than expected based solely on the mass of the proteins under observation. This finding is taken as a reflection of interactions between the fluorescently tagged proteins and other, less mobile, cellular components[47,49]. In the case of DNA binding proteins, this potentially includes interactions with nonspecific DNA, even though these interactions may be very transient in nature (see below). For example, in one elegant study, Misteli and colleagues used FRAP to quantitatively evaluate the transient DNA binding behavior of a spectrum of nearly 20 nuclear proteins and showed that, at any given time, most of the proteins were bound to DNA but showed mean residence times typically on the order of just a few seconds[50]. Although this may seem like rapid dissociation, it is important to realize that one-dimensional movement can also be fast, and even with a residence time of just 1 s a typical protein (assuming a one-dimensional diffusion coefficient on the order of 0.01–0.1 $\mu m^2 s^{-1}$) could potentially scan ~300–1,000 bp[45,51]. Thus, although three-dimensional diffusion may permit rapid transit throughout the nucleus, one-dimensional movement can still contribute substantially to local events (see below). Nevertheless, the precise mechanistic nature of any potential nonspecific interactions is extremely difficult to deconvolve based solely on *in vivo* FRAP measurements, because they do not reveal microscopic events that have a role in local interactions[47,52].

Although direct observation of one-dimensional diffusion or careful distinction between other forms of facilitated diffusion *in vivo* remains beyond current technological capabilities, Elf *et al.* have taken the heroic first steps in pursuit of this effort by taking data from *in vitro* single-molecule, one-dimensional sliding studies and combining it with *in vivo* measurements to disentangle the contributions of three-dimensional and one-dimensional components of DNA target location within a cell[53]. For these studies, a yellow fluorescent protein (YFP)-fused LacI protein was expressed at low levels (~seven monomers) in

E. coli, allowing individual proteins to be visualized by fluorescence microscopy. Induction with IPTG removed LacI-YFP from its specific operator site. The target search was initiated by flushing IPTG from the media and replacing it with 2-nitrophenyl-β-D-fucoside (ONPF), a competitor that prevented LacI from rebinding any residual IPTG. From these experiments, the authors deduced a search time of ~270 s, with an apparent diffusion coefficient of 4×10^{-1} μm^2 s^{-1}, approximately 7.5-fold lower than the diffusion coefficient of a LacI-YFP mutant defective in DNA binding. These results were then compared to *in vitro* experiments using flow-stretched λ-DNA, yielding a one-dimensional diffusion coefficient for LacI-YFP of 4.6×10^{-2} μm^2 s^{-1}. Importantly, the apparent *in vivo* diffusion coefficient represents a composite of the time that the protein spent diffusing in three dimensions and the time bound to nonspecific DNA, and by inference sliding along the helix. Interpreting the effective diffusion observed in the cell as a combination of the one-dimensional and three-dimensional diffusion coefficients led the authors to conclude that the protein spends ~90% of its time diffusing along nonspecific DNA, with a mean residence time on the order of <5 ms. This important finding, along with the FRAP studies cited above, illuminates an important principle: that is, even though nonspecific binding constants may be orders of magnitude weaker than the binding constants for a specific site, the vast majority of the DNA inside a cell is actually nonspecific, and as a consequence site- or structure-specific DNA binding proteins are expected to spend most of their time interacting with nonspecific sequences rather than diffusing freely in an unbound state.

Active mechanisms of target location

Although the focus of this Review is search mechanisms involving passive diffusion, it is worth mentioning that there are many examples of proteins that locate their targets by actively translocating along DNA using energy derived from ATP hydrolysis (**Fig. 1**). Some examples in this category include (i) the RecBCD exonuclease that locates χ-sites in DNA during recombination in *E. coli*[54]; (ii) FtsK, which participates in chromosomal segregation in *E. coli* and must identify specific consensus sequences to know which way to travel along the DNA[55]; (iii) type I restriction enzymes such as EcoR124I, which must first bind a specific sequence and then translocate along the flanking DNA to locate potential cleavage sites[56]; and finally (iv) chromatin-remodeling proteins such as RSC, Swi/Snf, Rad54 and Rdh54 (ref. 57), all of which must be delivered to specific regions of the genome and then must translocate along DNA to exert forces on specific nucleoprotein targets, such as nucleosomes.

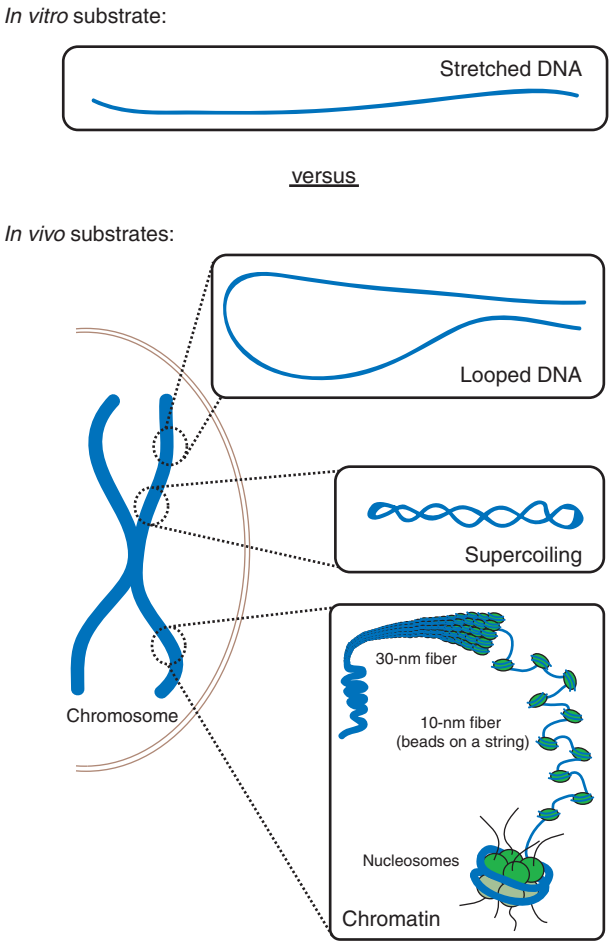

Figure 3 Comparison of *in vitro* versus *in vivo* DNA substrates. Above, a schematic of a stretched DNA molecule, currently the only substrate compatible with TIRFM single-molecule imaging (also refer to **Fig. 2**). Below, schematic illustrations of the substrates that are more likely to be encountered *in vivo*: looped DNA structures in which distal sites can be brought close together; supercoiled DNA; and higher-order chromatin structures. All of these substrates are vastly different from the stretched DNA molecules that can currently be imaged by TIRFM, and future experiments must overcome this technical hurdle and incorporate these more realistic substrates to establish more complete views of protein mobility on DNA.

Current limitations and future challenges

Single proteins undergoing one-dimensional diffusion have now been visualized with TIRFM, opening a new area of analysis that can continue to contribute to our understanding of the fundamental nature of protein–nucleic acid interactions. However, these new studies also highlight the numerous technical limitations in this burgeoning field. For example, the spatial and temporal resolution of current detection systems are limited relative to the scale of molecular events that must occur during facilitated diffusion. The precision in locating the centroid of fluorescent molecules depends on the number of captured photons, shot noise and pixel size, and with current technology spatial resolution can theoretically approach 1 nm. However, Brownian fluctuations of the DNA molecules themselves decrease the spatial resolution of these measurements by at least one to two orders of magnitude above the theoretical limitations of the detection optics. Temporal resolution is limited by the readout rate of the CCD cameras used for image collection and the emission intensity of the individual fluorophores, so in practice most data are collected in the range of 10 frames per second. This limited temporal resolution can be contrasted with the anticipated individual step frequencies that occur during diffusion, which are on the order of 10^5–10^7 steps per second[38,45]. Photobleaching and photodamage are always significant problems in single-molecule fluorescence measurements. Even under the best conditions, most fluorophores remain visible for only a few seconds, and intercalating dyes used to stain DNA also cause extensive DNA damage when illuminated. These problems can be partially mitigated by inclusion of an oxygen-scavenging system; however, the components of the scavenging system must first be carefully tested in bulk assays, because they are not compatible with all proteins. Quantum dots offer one solution to these problems because they are extremely bright and photostable; however, they are large compared to organic fluorophores, and the potential for attachment of multiple proteins to each quantum dot is also a concern[45].

Limitations are also imposed by the buffer conditions necessary for these experiments, highlighted by the fact that only a small fraction of the published data have been obtained under salt conditions that would

be considered physiologically relevant. Reduced salt concentrations are necessary to promote DNA binding at the low concentrations of proteins required for detection of single fluorophores, because higher protein concentrations create an insurmountably large background signal due to unbound fluorescent molecules. These low salt concentrations also promote longer diffusion trajectories, which make it possible to calculate one-dimensional diffusion coefficients. One-dimensional diffusion coefficients for pure sliding mechanisms are independent of salt concentration; however, the observed diffusion coefficients are expected to increase as a function of salt concentration if the mode of travel involves a hopping component, because higher salt concentrations will promote more rapid dissociation from the DNA[38]. Therefore, analysis of the diffusion coefficients at different salt concentrations can be used to distinguish sliding from hopping. Although one-dimensional diffusion coefficients are independent of salt concentration and low salt can be used to facilitate the measurements[38,45], these conditions do not reflect the *in vivo* environment, and the length of the trajectories observed *in vitro* may not represent the actual distances traveled *in vivo*. In addition, many of these proteins participate in additional interactions or functions that may be compromised under these conditions. Thus, although conditions of lower ionic concentrations allow longer lifetimes for which current temporal resolution is suited, further investigation into the protein's properties may suffer.

Another important consideration of the current *in vitro* TIRFM studies is that the DNA molecules must be stretched out along a surface to confine them within the detection volume defined by the penetration depth of the evanescent field and at the same time enable observation along their entire contour length. These stretched molecules of simple naked DNA do not accurately reflect the complex nature of native DNA substrates that exist *in vivo* (**Fig. 3**)[24,25]. For example, any DNA molecule longer than a few persistence lengths (~150 bp) behaves as a random coil, which can decrease the effective distance between two distal sites on the DNA chain as they are transiently brought closer in three-dimensional space. Similarly, native DNA is typically supercoiled, which also facilitates juxtaposition of distal sites along the DNA chain. Finally, native DNA is highly compacted into higher-order chromatin structures, which can both bring distal sites into close proximity, at the same time providing stationary roadblocks in the form of nucleosomes that may impede the movement of other proteins along the DNA. All of these factors can influence the mechanisms proteins use to travel along DNA, but none of these more realistic scenarios can be mimicked in the current single-molecule TIRFM assays, largely because the DNA molecules must be stretched for observation. Therefore, future experiments must attempt to tackle these issues by establishing methods compatible with these much more complex DNA substrates.

Despite the limitations discussed above, a number of new challenges are within reach using the current generation of tools, which will allow experimentalists to continue testing theoretical predictions as well as expand the scope of biochemical systems studied. For example, whereas one-dimensional sliding has been demonstrated, other types of facilitated diffusion, such as hopping or intersegmental transfer, have yet to be directly observed in single-molecule experiments. The Schurr model for rotation coupled to lateral diffusion remains to be confirmed, and proteins showing one-dimensional diffusion coefficients consistent with the absence of a rotational component also have yet to be identified. Importantly, none of the published studies where one-dimensional diffusion was visualized used DNA substrates that actually contained specific target sites for the proteins being studied, so diffusion and target binding still remain to be seen in the same reaction trajectory. Finally, as indicated above, most of the new studies have been limited to relatively simple, single-protein systems with naked DNA molecules. But many of the most interesting biological problems related to DNA metabolism involve numerous protein components, multiple reaction steps and chromatin substrates. Potentially exciting examples of more complex reactions involving lateral movement of proteins along DNA might include regulation of transcription complexes[58], the deposition and movement of cohesin[59], and the repositioning of nucleosomes within a chromatin fiber[60], to name only a few. Thus, another remaining challenge is to begin looking at more complex biological systems to understand how lateral movements along DNA might be coordinated among different proteins participating in these reactions.

Summary

The ability of proteins to travel along DNA while seeking out their respective targets has long been recognized as an important mode of interaction that allows many different types of proteins to efficiently fulfill their biological functions. Recent advances have made it feasible to visualize proteins as they slide along DNA and obtain quantitative information regarding mechanisms involved in the movement. Although numerous challenges remain, this burgeoning field is opening new possibilities for how protein-DNA interactions can be studied and offers the potential for moving beyond simple measurements of model systems to begin answering important questions regarding the implications of this movement for various biological problems.

Note added in proof: During preparation of this Review, two additional studies detailing the one-dimensional diffusion of p53 and EcoRV were published, highlighting the current escalation in TIRFM studies on facilitated diffusion[61,62]. Both articles report diffusion coefficients consistent with the Schurr rotational model. Additionally, the EcoRV study revealed that sliding of the protein was interrupted by large, sudden movements, suggesting a jumping or hopping mechanism.

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