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Fluid-driven DNA stretching for single-molecule studies on chromatin-associated proteins

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ABSTRACT

There have been many attempts to understand the central principle of life mediated by DNA-protein interactions surrounding complex environments. Still, the mechanistic insight of individual protein functions has been lacking in traditional ensemble assays. Thus, techniques visualizing a single molecule have emerged to uncover the discrete roles of DNA-protein interactions and their biophysical properties. This paper will review the advances in single-molecule tools imaging long genomic DNA and their applications in studying dynamic protein interactions. We focus on the three representative techniques, including molecular combing, nanochannel confinement, and DNA curtain assays, which use fluid-driven force to elongate the individual DNA. We provide an integrated perspective and a direction for future use to those who want to observe single DNA molecules along with their cellular factor of interest and employ them for dissecting protein function.

1. Introduction

Numerous efforts to understand the source of life faced many difficulties in finding the exact role of the DNA and DNA-associated proteins. As it is a very complex environment within the nucleus where DNA exists, it blurred the discrete function of the participants [1]. Moreover, the natural coiled state of DNA itself makes investigations and manipulations difficult (Fig. 1A). Furthermore, investigating such heterogeneous behaviors of proteins on DNA entails blended data that could overlook a critical intermediate state. In this regard, strategies to obtain and observe single DNA molecules on a solid surface are essential for studying individual roles of DNA-binding proteins obscured under the ensemble study.

Early single-molecule techniques to stretch individual DNAs have utilized hydrodynamic forces, electrophoresis, agarose gel embedment, optical trapping, and magnetic tweezer [2–6]. Although these early methods could observe an elongated DNA, there were disadvantages that the throughput of DNA assay was low and the directions of spread DNA were irregular. Also, additional biochemical modifications at the DNA extremities were necessary. Afterwards new technologies have been developed to overcome the drawbacks, which allows us to observe high-throughput DNA molecules and investigate the dynamic functions of various genome-associated proteins. This review focused on the advances in fluid-driven methods, including molecular combing, nanochannel confinement, and DNA curtain platforms which facilitate the analyses in genomic locations, biophysical DNA properties, and dynamic protein interactions on DNA (Fig. 1B–D).

2. Molecular combing: immobilized genomic DNA

2.1. Methodology and principle

In 1994, A. Bensimon et al. developed a facile DNA stretching method called *molecular combing*, a process that attach DNA molecules to a solid surface in an extended form by using air-water interface (meniscus) without any modifications at the DNA ends [7]. It provides a simple and convenient procedure with reasonable reproducibility for studying large-scale, ample DNAs [8].

The combing principle comprises three steps: adsorption, stretching, and relaxation. (Fig. 2A). In the adsorption step, the

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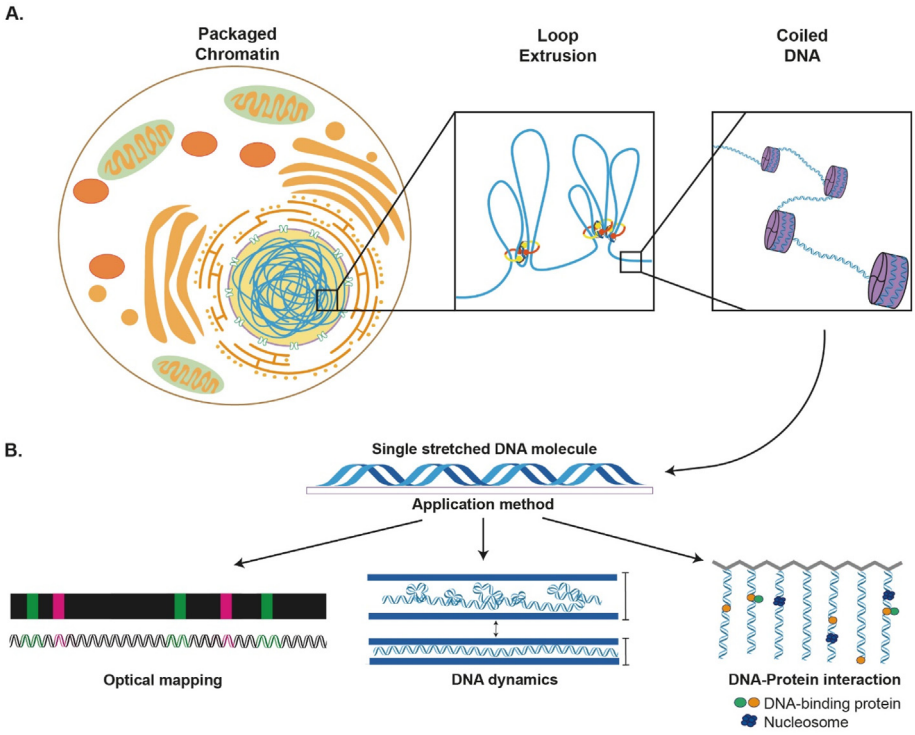

Fig. 1. The need to use single-molecule methods for genome research. (A) The complex environment of the cell, packaged chromatin, and coiled DNA disenabled investigations and manipulations to study the exact role of the DNA and DNA-associated protein. (B) The applications of single-molecule study using the stretched DNA to visualize specific DNA sites, physical dynamics of DNA, and functions of diverse DNA-binding proteins.

extremities of floating coiled DNA adsorb to a solid substrate. Following the attachment, the DNA ends are denatured by the functional pH range between 5.0 and 5.6 [9]. The hydrophobic bases in the denatured DNA ends are exposed and allow interactions with the hydrophobic surfaces. In the stretching step, a constant receding speed at air-water meniscus exerts a constant stretching force that is perpendicularly applied to DNA [10]. After the stretching force ends, the extended DNA molecule relaxes to its final length.

2.2. Challenges and alternative methods for DNA combing

Although molecular combing seems to be an efficient way for stretching single DNA molecules, there have been several challenges. Firstly, the outcome of the molecular combing displayed an irregular distribution and random attachment [7]. To improve this issue by aligning the DNA, Guan et al. has developed a method using a PDMS stamp to transfer DNA as aligned arrays [11]. Another study have created PDMS stamp with 'DNA trap' arrays that are transferred onto the APTMS-coated surface for the DNA combing [12].

The second issue was overstretching of the combed DNA, which makes the contour length ~1.7 times longer and is called S-form DNA [7,13]. This phenomenon renders the hydrophobic part of DNA exposed and triggers hydrophobic interaction on the combing surface [14]. Moreover, the overstretched DNA could compromise biological activities. For example, a single-molecule study of DNA replication revealed that the rate of DNA replication depends strongly on the stretching force applied to the template [15]. Furthermore, as both B form and S form coexist along the overstretched DNA, the S-form domains could suppress an enzymatic activity [16]. Overstretching also can induce DNA breakage and generate single-stranded DNA that is altered in the chemical and physical properties [17]. A recent study reported 'DNA bridge' using UV-lithography to resolve the S-form problem, which DNA can partially attach to the pre-designed surface and no longer get overstretched (Fig. 2B) [18].

Lastly, the combing surface sterically block enzymatic molecules to the DNA backbone and hinders its enzymatic activities. For example, the efficiency of incorporation of single fluorochromes onto the surface-adsorbed DNAs diminished to approximately twenty percent of the original one [19]. Since molecular combing is mainly used in genomic mapping and profiling technologies, it requires ample steric space to attach fluorochromes at a specific DNA locus. Righini et al. suggested a gel-based approach to trap and suspend the DNA molecules as 'bridges', which allows the DNA to be more accessible to enzymes (Fig. 2B) [18]. Another study proposed a dual-functionalizing surface that provides alternating weak and strong adhesion to DNA, which increased the enzyme accessibility [20].

2.3. Applications of molecular combing for DNA mapping

Essentially, DNA mapping requires a labeling process with fluorescent probes to visualize the spatial location of the DNA. Fluorescence in situ hybridization (FISH) has been widely used to map desirable sites optically (Fig. 2C). FISH is a cytogenetic technique developed in early 1980s and uses fluorescent DNA to target specific chromosomal loci [21]. Combining molecular combing with FISH has become a powerful tool to facilitate genomic analysis such as measuring the size of overlapped or gapped regions and detecting significant structural variants [20].

DNA replication is tightly regulated by the replication machinery in terms of the location and the initiation timing on the genome and combing up to hundreds of DNA molecules allows the statistically significant measurements [22]. This approach allows

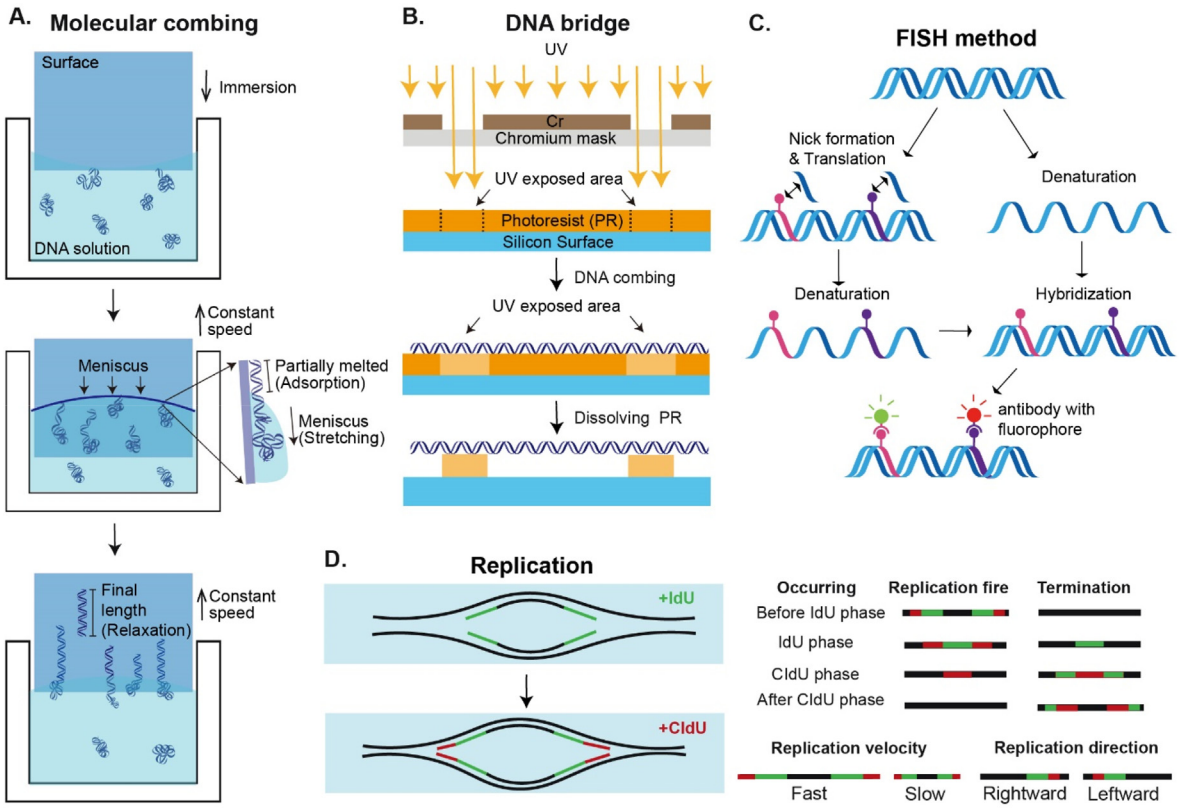

Fig. 2. Molecular combing in single-molecule experiments. (A) Overall scheme of molecular combing. DNA end adsorbs and gets stretched by meniscus caused by constantly receding surface. Molecular combing allows a large amount of stretched DNA irregularly. (B) Overall scheme of DNA bridge. Silicon surface coated by photoresist illuminated by UV through a Cr-coated mask. The cross-linked DNA is selectively left and enables enzymatic accesses. (C) Overall scheme of FISH method. (D) Molecular-combing based replication study. DNA labeled with IdU and CldU. A pattern of color and length of labeled DNA provides the information of replicated DNA direction, pattern, and replication speed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

visualizing an individual replication origin and its activity such as Ori to Ori distance or fork elongation speed (Fig. 2D) [23]. For example, Stanojcic et al. has studied the relationship between DNA replication features and ploidy patterns by characterizing their inter-origin distances and fork velocities in three species harboring different ploidy [24]. Also, DNA combing with mouse embryonic stem cells displayed the two-fold increased origins spaced at half distance compared to its somatic cells, suggesting fundamental differences during development [25].

Besides DNA, the combing materials can be fibril molecules that capillary tensile force can apply. Akhtar et al. has compared the morphology of fibrillin microfibrils derived from diabetic or healthy tissues by atomic force microscopy along with molecular combing, which suggested the fragmented and weakened structure in the diabetic one [26]. Another report has shown that poly n-nonyl acrylate can be molecularly combed on mica by a simple-casting method, demonstrating the diversity of polymers for molecular coming [27].

3. Nanochannel confinement: DNA stretching evading immobilization

Typical DNA stretching methods require DNA attachment to a modified surface, which could restrict its dynamic movement. Following the demand for DNA stretching technique devoid of surface immobilization, scientists started exploiting nanoscale-channels [28,29]. Han Cao et al. initially achieved the successful DNA stretching in such tiny space of 50-nm x 10-nm (width and depth) channels [29]. The physical confinement elongating DNA molecules follows the polymer physics theory of Odijk or de Gennes regimes, which are defined by the relationship between the polymer's persistence length (P) and the diameter (D) of channels (Fig. 3A) [28,30]. Persistence length defines the length that the polymer does not bend and maintains straightness like a rod [31]. In the de Gennes regime, in which $D \gg P$, the polymer chain is confined weakly and has a series of blobs of polymer [28]. In contrast, in the Odijk regime, in which $D < P$, the confined polymer can no longer coil (Fig. 4A). If the polymer moves from de Gennes to the Odijk regime, it implies that the polymer chain becomes more confined due to smaller space, and a slightly compressed coil progresses to a highly elongated polymer [28]. Based on these fundamentals, researchers have developed various forms the channels and addressed many open questions.

3.1. Diversity in nanochannel design

One of the significant merits of nanochannel confinement is that we can design and obtain various shapes of channels depending on each experimental demand. For example, to study the effect of a specific component on the DNA conformation, it must exchange the buffer solution inside the channels. A study reported a cross-shaped channel in which a DNA molecule is accommodated within a microchannel while exchanging the buffer environment through the intersection connecting the small nanochannel (Fig. 3B) [32]. Also, Riehn et al. has designed a platform that enables controlling the diffusion of different reagent by electrophoresis and

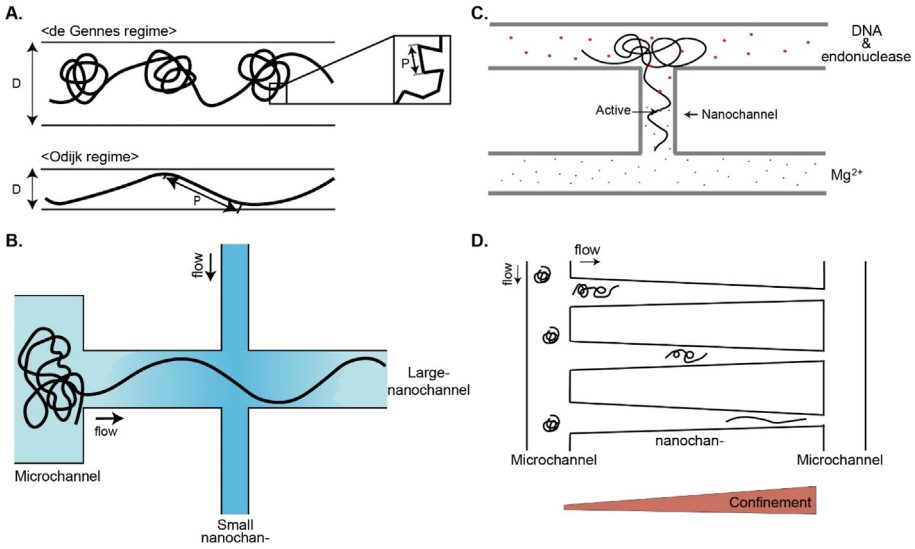

Fig. 3. Nanochannel confinement in single-molecule experiments. (A) Biophysical regimes of confined polymers. De Gennens regime ($D \gg P$) has a series of blobs of polymer, and the Odijk regime ($D < P$) stretches polymer linearly. D: channel dimension. P: persistence length. (B) Efficient buffer exchange in cross-shaped nanochannels. The solution containing DNA enter through the large-nanochannel and a different reaction buffer is injected through the small-nanochannel. The buffer can be replaced at the intersecting point. (C) Controlling enzymatic activity in a nanochannel. DNA and endonuclease are injected through the upper microchannel, and $Mg2^{+}$ is injected through the lower microchannel. Substances entering different channels meet in a connected nanochannel and the reaction is activated. (D) One-set visualization of varied confinements. DNA in the left microchannel moves toward the right microchannel through the funnel-shaped nanochannels. As DNA moves along the flow in the nanochannel, the DNA confinement increases as the DNA get extended.

temporally regulate each enzymatic reaction through the channel geometry (Fig. 3C) [33]. Another device that controls the direction of current passing through a nanochannel allowed DNA molecules to be specifically positioned in the channels and could be reused by moving the samples back to the observation position [34].

An array of funnel-shaped nanochannels is another interesting device, which allows to observe the confinement effect on dynamic changes in DNA conformation in a single experiment (Fig. 3D) [35]. As the size of the nanochannel decreases, the confinement and DNA elongation increase. However, when the nanochannel inner space decreases, the energy required to insert DNA into the channel rises [30]. Thus, it would be useful to narrow the channel dimension after introducing DNA molecules. Researchers could achieve this task by squeezing an elastomeric channel device after DNA molecule is introduced [36].

3.2. Applications to the dynamic study of a single DNA molecule

The major advantage of the nanochannel confinement is the lack of DNA modification and maintaining its motional freedom under various conditions. To mimic the cellular environment and its effect on DNA conformation, researchers often use dextran as a crowding reagent that influences spatial confinement. The extent of DNA elongation increases under a threshold volume fraction of the agent, while the higher volume of dextran results in DNA compaction in the same nanochannel geometry [37]. In addition, Y. Kim et al. could obtain fully stretched DNA molecules by reducing the ionic strength as low as possible in an optimized channel size [38]. They also showed that DNA was more readily compressed by divalent magnesium ions than monovalent sodium ions [38]. Similarly, higher concentration of TBE buffer in the nanochannel decreased the degree of DNA extension [39].

Researchers have also investigated the role of diverse DNA-associated proteins by using nanochannel-confined DNA. The proteins organizing DNA folding in prokaryotic cells or eukaryotic nucleus have been of great interest. For example, the heat-stable nucleoid-structuring protein (H-NS) known to form a filament and pack the bacterial DNA has been characterized in 200 nm × 250 nm channels to show the significant effect of divalent ionic strength and protein concentration on DNA compaction [40]. Matsuoka et al. has developed an elastomeric nanochannel that can be squeezed to narrow the geometry and applied it to obtain a DNA map presenting the methylated histone H3 or acetylated histone H4 [36].

4. DNA curtain: tethering DNA along the embedded metal barrier

4.1. Principle and feature

In 2006, Graneil et al. reported a new method tethering lambda genomic DNA to the surface that has been rendered inert by the supported mobile lipid bilayer [41]. This platform is called a 'DNA curtain' because hundreds of DNA molecules get aligned at the diffusion barriers (Fig. 4A). By embedding the diffusion barriers on the surface and pushing the DNA via hydrodynamic force, researchers visualized a large number of aligned single DNA molecules within the field of view of a microscope [42]. DNA curtain could minimize the steric hindrance and readily control the exchange of biochemical reactions occurring on the DNA, allowing the real-time observation of DNA-protein interactions at the single-molecule level in a high-throughput manner.

4.2. Challenge and advances

The diffusion barriers used in the initial report were at random positions without defined loci of DNA assembly. Later, a nano-engineering technique has been integrated with the DNA curtain for assembling thousands of individual DNA at the defined barriers, which has been advanced and diversified. To obtain even distribution of DNA, a saw-tooth pattern barrier has been fabricated by e-beam or nanoimprint lithography [42]. In order to catch the other

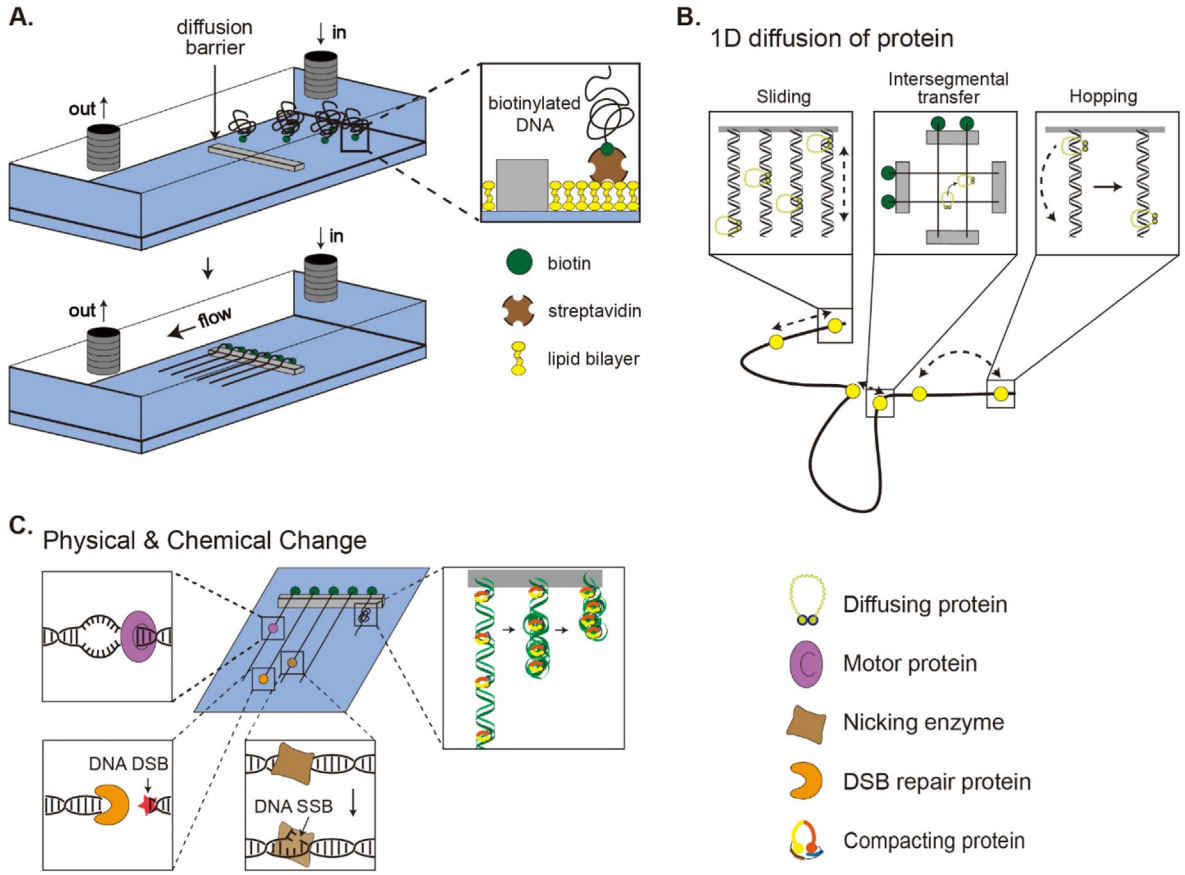

Fig. 4. Schematics of DNA curtain in single-molecule experiments. (A) DNA molecules tethered via biotin-streptavidin interaction to a mobile lipid bilayer. DNA is pushed by hydrodynamic force (buffer flow) and caught at the barrier. (B) Direct visualization of one-dimensional (1D) movements of DNA-binding proteins on DNA curtain. (C) Chemical and physical change of DNA molecules induced by diverse protein functions. Fluorescent imaging of DNA curtain assays allows single-molecule observation of DNA molecules that are chemically altered or degraded by each enzyme's activity. Physical change of DNA molecules with an example of protein-mediated DNA compaction on the curtain platform.

DNA end, double-tethered DNA curtain has been designed by introducing the secondary patterns of pentagons that antibodies can readily adsorb. This platform allows observing protein movement on DNA without buffer flow in the chamber [43]. It took many attempts to improve the quality of the barrier. For example, a recent study enhance durability of the barrier by improving cleaning process compared to classical lithography that often showed degradation and protein aggregates remaining at the barriers [44]. Also, Gibb et al. have investigated critical biological reactions involving single-stranded DNA binding proteins by using single-stranded DNA curtain generated by the rolling circle replication method [45]. On the other hand, Robinson et al. described a strategy for a multichannel PDMS-based microfluidic system, which provides a high-throughput DNA imaging platform along with a multilinear generator that controls five different reaction conditions [46].

Protein diffusion on DNA: Because DNA curtains can tether stretched genomic DNA with minimal contacts on the surface, it is useful to observe one-dimensional (1D) diffusion of proteins on DNA. The diffusion types can be classified into 1D diffusion (sliding, hopping), 3D diffusion, and intersegmental transfer (Fig. 4B). For instance, Silverstein et al. visualized the protein movement of two different DNA translocases, RecBCD and FtsK, which participate in homologous and site-specific DNA recombination, using crossed DNA curtains to visualize inter-site transfer of 90° turns in the diffusion trajectories at intersections [47]. Brown et al. visualized mismatch repair complex, Msh2-Msh3 and Msh2-Msh6, that hops over nucleosome and other protein roadblocks but usually maintains sufficient contact with DNA to recognize a single DNA lesion [48]. A recent single-molecule study of MRN complex, a participant of eukaryotic homologous recombination (HR), visualized how MRN searches for free DNA ends by facilitated diffusion and EXO1 resects the 3' end of free DNA to recruit next participants including RPA and RAD51 [49]. Interestingly, a recent study using the double-tethered DNA curtains have revealed that intrinsically disordered regions (IDRs) of Mlh1-Pms1 play a critical role in promoting the facilitated 1D diffusion for target search and are essential for DNA repair *in vivo* [50].

Chemical and physical changes in DNA: Enzymatic processes often accompany certain types of chemical changes on DNA and the DNA curtain assays allow direct visualization of each protein's function (Fig. 4C). Roy et al. conducted research using ssDNA curtain assay to understand the critical steps in HR executed by RecA/Rad51 recombinases and revealed that Rad51 paralogs are required for HR and DSB repair in a single-molecule scale [51]. Gibb et al. showed that RPA remains bound to ssDNA when free protein is absent, and then when free RPA is present, rapid exchanges between free and bound state is observed, which indicates that RPA undergoes constant microscopic dissociation under all conditions but is only manifested as macroscopic dissociation when free proteins are present [52]. A single-molecule study of bloom helicase (BLM), which helps dismantle potentially harmful HR intermediates, showed that RPA and RAD51 can block the BLM translocation on ssDNA or dsDNA [53].

DNA compaction is the process in which a large DNA molecule undergoes a transition from an elongated conformation to a very condensed state, which plays a significant biological role in DNA synthesis and transcription regulation [54]. A recent single-molecule study reported direct evidence that human cohesin complex extrudes a DNA loop and compact nucleosome-DNA in an ATP-dependent manner [55]. Another ssDNA curtain study for Abo1, the family of histone chaperones that regulate nucleosome density and chromatin dynamics, have shown its activity in histone H3–H4 deposition onto DNA in an ATP-hydrolysis-dependent manner [56]. Especially, DNA condensation is related to phase separation, which is separating a phase into two or more new phases. Recently, biomolecular condensate formation in the complex biological environment has been highlighted [57]. Several studies conducted single molecule approaches using DNA curtains to study phase separation. Larson et al. observed phase separation that unmodified and phosphorylated HP1α forms smaller and fewer nuclear puncta *in vitro* and the observation suggest that heterochromatin-mediated gene silencing may occur in part through sequestration of compacted chromatin in phase-separated HP1 droplets [58]. Also, Zuo et al. confirmed that EWS-FLI1 and FUS/EWS/TAF15 (FET) fusion form biomolecular condensates at their target binding loci, and these condensates could enhance gene transcription by recruiting RNA polymerase II [59].

4.3. Perspective

This review describes three state-of-the-art DNA stretching methods for studying DNA and DNA-protein interactions at the single-molecule level. These techniques allow examining discrete individual DNA and protein blurred by ensemble studies and complex environment in the nucleus.

We anticipate several new directions beyond the traditional usage of the above methods. Since most studies are limited to lambda DNA, utilizing various eukaryotic DNA including human or yeast as the curtain templates will be useful to uncover the obscured biological phenomena in the study using genomic DNA. Novel epigenetic factors including DNA methylation, histone variants, and ncRNA can be integrated to study the dynamic behavior of their associated proteins.

On the other hand, obtaining the lithography-integrated DNA curtain platform costs high for the imaging system. Thus, we propose a new curtain platform capable of detecting protein behavior through sensing electronic signals. Aligned and stretched DNAs can be used as a molecular wire sensor reading the single-molecule electronic signals [60]. Other than the solid nanoscale materials such as carbon nanotubes and silicon nanowires, the biopolymers are precisely engineerable at a single DNA base level for biofunctionalizations. Combining with the fluid-driven DNA stretching methods, parallelly aligned DNA sensors can be mass-produced with a low-cost and simple manufacturing process.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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