Video Article

Visualization of Surface-tethered Large DNA Molecules with a Fluorescent Protein DNA Binding Peptide

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Abstract

Large DNA molecules tethered on the functionalized glass surface have been utilized in polymer physics and biochemistry particularly for investigating interactions between DNA and its binding proteins. Here, we report a method that uses fluorescent microscopy for visualizing large DNA molecules tethered on the surface. First, glass coverslips are biotinylated and passivated by coating with biotinylated polyethylene glycol, which specifically binds biotinylated DNA via avidin protein linkers and significantly reduces undesirable binding from non-specific interactions of proteins or DNA molecules on the surface. Second, the DNA molecules are biotinylated by two different methods depending on their terminals. The blunt ended DNA is tagged with biotinylated dUTP at its 3' hydroxyl terminus, by terminal transferase, while the sticky ended DNA is hybridized with biotinylated complementary oligonucleotides by DNA ligase. Finally, a microfluidic shear flow makes single DNA molecules stretch to their full contour lengths after being stained with fluorescent protein-DNA binding peptide (FP-DBP).

Video Link

The video component of this article can be found at <http://www.jove.com/video/54141/>

Introduction

Visualization of large DNA molecules tethered on glass or bead surfaces has been utilized for investigating DNA-protein interactions, protein dynamics on DNA substrate,^{1,2} and polymer physics.^{3,4} A platform for single-tethered large DNA molecules has a few distinct advantages compared to other DNA immobilization methods.⁵ First, a large DNA molecule tethered on the surface has a natural random-coil conformation without a shear flow, which is critically important for a DNA-binding protein to recognize its binding site. Second, it is very easy to change the chemical environment around DNA molecules for a series of enzymatic reactions in a flow chamber. Third, a microfluidic shear flow induces DNA molecular stretching up to 100% of the full contour length, which is very difficult to achieve using alternative DNA elongation approaches such as surface immobilization⁶ and nanochannel confinement.⁷ A fully stretched DNA molecule also provides positional information that can be useful for monitoring enzymatic movements on the genomic map.

Nevertheless, the DNA tethering approach has a critical shortcoming in that the intercalating dye such as YOYO-1 generally causes tethered DNA molecules to be readily broken by fluorescence excitation light. Generally, large DNA molecules have to be stained with a fluorescent dye for visualization under a fluorescent microscope. For this purpose, YOYO-1 or other TOTO series dyes are primarily used because these dyes fluoresce only when they intercalate double-stranded DNA.⁸ However, it is well-known that bis-intercalating dye causes light-induced DNA photo-cleavage because of the intercalation of fluorophores.⁹ Further, fluorescently stained DNA molecules tethered on the surface are more fragile since shear flows can exert breaking forces on freely moving DNA molecules. Therefore, we developed FP-DBP as a novel DNA-staining protein dye for imaging large DNA molecules tethered on the surface. The advantage of using FP-DBP is that it does not cause photo-cleavage of the DNA molecules to which it binds.¹⁰ In addition, FP-DBP does not increase the contour length of DNA, while bis-intercalating dyes increase the contour length by about 33%.

This video method introduces the experimental approach for tethering large DNA molecules to a PEG-biotin surface. **Figure 1** illustrates different approaches of tethering DNA with blunt ends and sticky ends. Thus, this staining method can be applied to any type of DNA molecule. **Figure 2** depicts a schematic representation of the flow chamber assembly that can be controlled by a syringe pump to generate shear flows to stretch DNA molecules as well as to load chemical and enzyme solutions. **Figure 3** demonstrates micrographs of fully stretched DNA molecules tethered on the PEGylated surface¹¹ and stained with FP-DBP.

Protocol

1. DNA Biotinylation

1. Biotinylation of blunt ended DNA using Terminal Transferase (TdT)

Note: Use T4 DNA (166 kbp), which is a blunt ended DNA.

1. Add 5 μ l of 2.5 mM CoCl_2 , 5 μ l of 10 $\times$ reaction buffer, 0.5 μ l of 10 mM biotin-11-dUTP, 0.5 μ l of terminal transferase (10 units), and 0.5 μ l of T4 DNA (0.5 μ g/ μ l) to the reaction mixture. Make to a final volume of 50 μ l by adding 38.5 μ l of water.
2. Incubate the reaction mixture at 37 $^{\circ}\text{C}$ for 1 hr.
Note: Extended reaction time may yield double-tethered DNA, *i.e.*, it is possible that biotins are tagged at both ends.
3. Stop the reaction by adding 5 μ l of 0.5 M EDTA, pH 8.
4. Keep the tube at 4 $^{\circ}\text{C}$.

2. Biotinylation of sticky ended DNA Using DNA Ligase

Note: Use λ DNA (48.5 kbp), which is a sticky-end DNA.

1. Add 1 μ l of T4 DNA ligase, 5 μ l of 10 $\times$ ligation buffer, 1 μ l of 25 ng/ μ l λ phage DNA, and add 43 μ l of water to make a final volume of 50 μ l.
2. Keep the tube at 4 $^{\circ}\text{C}$.

2. Functionalized Surface Derivatization

Note: In order to tether an end of a DNA molecule on glass surface, a primary amine group is silanized on a coverslip followed by biotin-PEG coating as shown in **Figure 1**. This PEGylation process is important for single-molecule DNA imaging because it can significantly decrease random noise created by attachment of undesired molecules on the surface.

1. Piranha Cleaning

Note: Piranha solutions react vigorously with organic materials, therefore it should be handled with care, following proper safety guidelines.

1. Place coverslips on a polytetrafluoroethylene (PTFE) rack, and hold them by PTFE thread seal tape lengthwise, half on the edge of the surfaces and half on the rack. After wrapping, leave a long piece ($\sim$ 5 cm) of tape for handling the rack during the cleaning procedure.
2. Fill a 1 L glass beaker with 350 ml of H_2SO_4 and 150 ml of H_2O_2 to make piranha solution in a fume hood. Place the rack in piranha solution for 2 hr.
3. Empty piranha solution from the beaker, and rinse coverslips thoroughly with deionized water until the pH of water in the beaker reaches neutral. Use pH paper strips.
4. Sonicate the racks of cover slips in the beaker containing water, for 30 min. Empty water from the beaker just before amino silanization. Use 75 W of sonication power to clean and derivatize the glass substrates.

2. Aminosilanization on Glass Surface

1. Add 2 ml of *N*-[3-(trimethoxysilyl)propyl]ethylenediamine and 10 ml of glacial acetic acid to 200 ml of methyl alcohol in a clean polypropylene container for preparing the aminosilanization solution.
2. Place cleaned coverslips in the polypropylene container. Shake them at 100 rpm and room temperature for 30 min.
3. Sonicate the beaker with derivatized cover slips for 15 min at 75 W. Shake them at 100 rpm and room temperature for at least 30 min.
4. Empty the solution from the beaker, and rinse coverslips carefully, once with methyl alcohol, and twice with ethyl alcohol. Store coverslips in ethyl alcohol and use them within two weeks.

3. PEGylation of the coverslip

1. Make 10 ml of 0.1 M sodium bicarbonate (NaHCO_3). Filter the solution with a 0.22- μ m syringe filter.
2. Dissolve 2 mg of biotin-PEG-succinimidyl carbonate (biotin-PEG-SC) and 80 mg of PEG-succinimidyl valerate (mPEG-SVG) in 350 μ l of NaHCO_3 . Use a light protection tube.
3. Vortex the tube vigorously for 10 sec, and centrifuge it at 10,000 $\times$ g for 1 min to remove bubbles.
4. Rinse a glass slide with acetone, followed by rinsing with ethyl alcohol. Allow the slide to air dry completely.
5. Place a drop (50 μ l) of PEG solution on the clean glass slide. Cover the droplet gently with an amino silanized cover slip, without generating any bubbles.
6. Place slides for 3 hr to overnight in a dark, well-leveled humid chamber at room temperature. Use an empty pipette tip holder as the chamber, with water at the bottom. Seal residual PEG solution in the tube and keep it at 4 $^{\circ}\text{C}$.
7. Rinse the PEGylated coverslip thoroughly with deionized water. Store it in a dark and dry place until use.

3. Assembling a Flow Chamber

1. Fabricate a punched acrylic holder, as in **Figure 2**. Ensure that the diameter of the tubing insert is 0.762 mm, and designate one hole is an inlet and the other is an outlet (**Figure 2**).
2. Place double-sided sticky tape strips (width 5–6 mm) on an acrylic holder (**Figure 2**), aligning perpendicularly to an inlet and outlet hole, not perturbing any channel holes. Use these tape strips as multi-channel walls to make chambers. Scrub the tape using a pipet tip.
3. Place a PEGylated coverslip on the top to make flow chambers, with the PEGylated side facedown.
4. To prevent any solution from leaking, press the top of a coverslip over the area where double-sided tapes are placed. Add quick-dry epoxy glue to close the edges of the chamber at the top and at the bottom (yellow color in **Figure 2**).

5. Connect a short piece (2.5 cm) of tubing (outer diameter, OD, 0.042") to a gas-tight syringe and seal the joint with epoxy glue.
6. Connect a long flexible tubing (OD: 0.03") to the tubing linked to the syringe and seal the joint with epoxy glue.
7. Fill the tubing linked to the syringe with DI water. Make sure there is no air bubble.
8. Insert the tubing into the hole of the flow chamber sealed with epoxy glue.
9. Place a yellow tip (200 μ l tip) on the other hole as a reservoir.

4. Sample Loading into Flow Chamber

Note: Neutravidin can be replaced with other avidin proteins such as streptavidin. All of the reactions can be performed at room temperature, unless it is mentioned. Take sample solution in a yellow tip, and install the tip with the solution onto the hole of acrylic holder (**Figure 2b**).

1. Set the flow rate of the syringe pump at 50 μ l/min. Load 20 μ l of avidin protein (25 μ g/ml in T50 solution, Tris 10 mM, NaCl 50 mM, pH 8), and keep it for 10 min.
2. Load 20 μ l of biotinylated oligodeoxynucleotides (100 μ M in 1 $\times$ TE), and keep it for 10 min. If terminal transferase is used, skip the loading of oligodeoxynucleotides.
3. Load 20 μ l of DNA solution from Step 1 into the flow chamber at a flow rate of 10 μ l/min, and keep it for 30 min.
4. Wash the flow chamber with 1 $\times$ TE, and load 40 μ l of FP-DBP¹⁰ (~80 nM).
5. Observe DNA under a fluorescent microscope with continuous flow of staining molecules in 1 $\times$ TE on a 60X objective lens. Use 488 nm solid state laser for excitation of FP(eGFP)-DBP. For full stretch of DNA molecules, apply different flow rates according to the DNA lengths used. For example, apply 50 μ l/min for 48.5 kbp of λ DNA, and 100 μ l/min for 166 kbp of T4 DNA.
 1. For recycling of the acrylic holder, soak assembled flow chambers in a household detergent solution¹². Remove tapes and epoxy with a razor blade and rub with hands to completely take them off. Unclog holes with syringe needles. Keep the holders in deionized water until further use.

Representative Results

Figure 1 shows two different DNA tethering methods depending on terminal structures of the DNA molecule. **Figure 1a** illustrates how the sticky ended DNA molecules are hybridized with complementary biotinylated oligonucleotides, which are immobilized on the avidin-coated PEG surface. **Figure 1b** shows the addition of biotinylated ddNTP or dNTP to the 3' hydroxyl group of a blunt ended DNA by terminal transferase. We added flexible linkers between DNA molecules and biotin. This increased the ligation and avidin-biotin binding efficiency to provide sufficient space for the reactions. **Figure 1c** demonstrates the overall schematic representation for DNA tethering on the avidin-coated PEG surface.

Figure 2 depicts the assembly of the flow chamber along with an acrylic holder. Compared to glass/quartz slide glasses, a custom-made acrylic holder simplifies the preparation of the flow chamber for epi-fluorescent microscopy.¹² The flow chamber enables instant changing of buffer conditions for enzyme reactions,⁶ and stretching DNA molecules.

Figure 3 demonstrates single-tethered DNA and T4 λ DNA stained with FP-DBP. Microfluidic shear flows elongated single DNA molecules up to full contour lengths such as 16.5 μ m (48.5 kb λ DNA $\times$ 0.34 nm) and 56.4 μ m (166 kb T4 DNA $\times$ 0.34 nm). We were able to repeat DNA stretching and relaxation multiple times during an extended period (e.g., half-hour), since we utilized FP-DBP that does not cause photo-cleavage.

Figure 1. Schematic illustration of DNA tethering. **a)** A sticky ended DNA is hybridized with a biotinylated complementary oligonucleotide. Triethylene glycol is a flexible linker between the oligonucleotide and biotin. **b)** Biotinylated dNTP is added to a blunt end of double stranded DNA, by terminal transferase. Polycarbon spacer (11-atoms) is a flexible linker between dNTP and biotin. **c)** Biotinylated DNA is anchored to an avidin protein, which is linked to a biotinylated PEG covalently bonded to the glass surface. The ratio of biotinylated PEG to normal PEG was 0.025 (1:40). [Please click here to view a larger version of this figure.](#)

Figure 2. Flow chamber. **a)** Flow chamber consisting of an acrylic acid resin holder, strips of double-sided tapes and a PEGylated cover slip. The acrylic holder of dimensions 76 mm x 26 mm x 5 mm (L x W x H), was fabricated with laser cutting. **b)** Side view of the Flow chamber: punched holes are an inlet port on which a yellow tip is installed for a buffer reservoir, and an outlet port that is connected to a tubing controlled by a syringe-pump. [Please click here to view a larger version of this figure.](#)

Figure 3. Fluorescent microscopic images of stretched DNA molecules stained with FP-DBP. **a)** Bacteriophage λ DNA (48.5 kbp) tethered on the surface after ligation with biotinylated complementary oligonucleotides. **b)** Bacteriophage T4 DNA (166 kbp) tethered on the surface after adding biotin with terminal transferase. Indicated arrows show DNA molecules stretched up to their full contour lengths such as 16.5 μm for λ DNA and 56.4 μm for T4 DNA. Scale bars: 10 μm . [Please click here to view a larger version of this figure.](#)

Discussion

Here we present a platform for visualizing long DNA molecules biotinylated for anchoring on surfaces. We have reported an approach for DNA molecules tethered on an avidin protein coated surface with biotinylated bovine serum albumin.⁶ In the earlier approach, we found a crucial issue of DNA photo-cleavage caused by bis-intercalation dyes that stain DNA molecules tethered on the surface. As these continually excited fluorophores have a high probability to attack a DNA phosphate backbone,⁹ the excitation light power had to be minimized to avoid photo-cleavage.

In order to overcome this inconvenience, we developed genetically engineered fluorescent proteins with DNA-binding capability (FP-DBP) for staining DNA without photo-cleavage.¹⁰ We observed reliable DNA staining on avidin-coated protein surfaces. Nonetheless, this platform had another issue of undesirable aggregation of fluorescent proteins on the protein surface, resulting in increased background noise. It is essential to minimize this random noise for single-molecule DNA analysis to enhance the contrast of DNA and proteins from the background. The use of FP-DBP therefore requires surface passivation to prevent the fluorescent protein from being adsorbed on the surface. PEG could be a powerful candidate for this purpose.¹² Hence, we used biotinylated-PEG, which dramatically enhanced signal-to-noise ratio for imaging FP-DBP stained DNA molecules tethered on the surface.

There are several critical steps and notes in this method for higher yields. In order to tether an end of a DNA molecule on a glass surface, a primary amine group is silanized on a coverslip followed by biotin-PEG coating as shown in **Figure 1**. This surface passivation process is critical for single-molecule DNA imaging because it can significantly decrease noise-generating random adsorption on the surfaces. Therefore, overnight aminosilanization and PEGylation are strongly recommended. In addition, cleaning the glass slide as well as the cover glass with piranha solution can further reduce background noise.

A biotinylated oligonucleotide must have a phosphate group at 5' end in order to link it to 3' hydroxyl group of a large DNA molecule. The sequence of biotinylated oligonucleotide is 5'-p-GGGCGGCGACCT-TEG-Biotin-3' for λ DNA tethering. The λ DNA solution has to be loaded shortly after the preparation of λ DNA solution (described in 1.1), because λ DNA often forms concatemers with long-time storage. For blunt-ended DNA such as T4 DNA, terminal transferase can add biotinylated dUTPs at both ends of DNA without any specificity. Thus, terminal transferase reaction time has to be carefully adjusted around an hour, otherwise extended reaction may yield double-labeled DNA (*i.e.*, both ends are labeled with biotin). In addition to viral DNA such as λ and T4, any types of DNA can be utilized in this platform. However, it is recommended to use fragments from gigantic genomic DNA after digestion by rare-cutter restriction enzymes such as *SwaI* or *NotI*.

Different flow rates are applied for different lengths of DNA. Usually, longer DNA molecules require faster flow rates to fully stretch the DNA molecules, to match the exact contour length. For example, λ DNA can stretch up to 16.5 μm , which corresponds to the calculated value of 0.34 nm $\times$ 48,502 bp.

It is quite important to freshly prepare all materials, particularly all of proteins and PEGs. For example, Neutravidin readily loses its function of binding biotins if it is stored as a diluted solution. For PEG solutions, pH is critical to conjugate functional groups of a primary amine and *n*-hydroxysuccinimide. Therefore, it is recommended to freshly prepare all materials at least every week.

Visualization of large DNA molecules tethered on the surface is a versatile platform to be applied for a variety of applications such as genomics, epigenomics, biochemical studies for DNA and protein interaction, and polymer physics studies. However, this approach is currently limited only to model systems using relatively simple and well-known DNA molecules such as the viral genome. To be extended to the human genome and epigenome, it is critically important to establish a robust, reliable, and simple platform for practical use. By overcoming light-induced DNA photo-cleavage and preventing random adsorption of fluorescent protein on the surface, this platform therefore provides clear and high-contrast images for DNA elongated with shear flows.

Disclosures

The authors have nothing to disclose.

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