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DNA curtains shed light on complex molecular systems during homologous recombination

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DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOPHYSICS
650 W. 168th STREET, NEW YORK, NY 10032

New York, February 6, 2020

Dear Editors,

It is our pleasure to be invited to submit our manuscript entitled "**DNA curtains shed light on complex molecular systems during homologous recombination**" for consideration for the Collection '*Methods for Studying DNA Double Strand Break Repair and Its Impact on Genome Integrity*' at *Journal of Visualized Experiments*.

Since the inception of DNA curtains almost two decades ago, our lab has published regularly on its application in studying protein-DNA interactions in various DNA repair processes. Over the last decade, we have focused on applying the technology to study proteins involved in key intermediates of DNA double strand break repair. In this manuscript, we describe in detail the protocol for setting up single- or double-tethered dsDNA curtains, from nano-fabrication of chromium patterns to fluorescence imaging. In representative results, we provide the example of studying the yeast resection machinery Sgs1-Dna2 using single-tethered dsDNA curtains. Finally, we discuss critical steps in consistently assembling DNA curtains and highlight other practical considerations in the process. We envision that this protocol, accompanied by the unique visual nature of *JoVE* articles, will significantly benefit the readers in their experience understanding, setting up, and troubleshooting their own experiments.

Thank you for your consideration

Sincerely,

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TITLE:

DNA Curtains Shed Light on Complex Molecular Systems During Homologous Recombination

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KEYWORDS:

genome integrity, homologous recombination, DNA repair, double-stranded breaks, single molecule, DNA curtains

SUMMARY:

DNA curtains present a novel method for visualizing hundreds or even thousands of DNA-binding proteins in real-time as they interact with DNA molecules aligned on the surface of a microfluidic sample chamber.

ABSTRACT:

Homologous recombination (HR) is important for the repair of double-stranded DNA breaks (DSBs) and stalled replication forks in all organisms. Defects in HR are closely associated with a loss of genome integrity and oncogenic transformation in human cells. HR involves coordinated actions of a complex set of proteins, many of which remain poorly understood. The key aspect of the research described here is a technology called “DNA curtains”, a technique which allows for the assembly of aligned DNA molecules on the surface of a microfluidic sample chamber. They can then be visualized by total internal reflection fluorescence microscopy (TIRFM). DNA curtains was pioneered by our laboratory and allows for direct access to spatiotemporal information at millisecond time scales and nanometer scale resolution, which cannot be easily revealed through other methodologies. A major advantage of DNA curtains is that it simplifies the collection of statistically relevant data from single molecule experiments. This research continues to yield new insights into how cells regulate and preserve genome integrity.

INTRODUCTION:

The maintenance of genome integrity is crucial for proper functioning of all living cells[1]. Defects in genome integrity can lead to severe health conditions, including various types of cancer and

age-related degenerative diseases[2]. Homologous recombination (HR) uses template-dependent DNA synthesis to repair DNA double-stranded breaks (DSB), single-stranded DNA (ssDNA) gaps, and interstrand DNA crosslinks[3]. HR is also necessary for the recovery of stalled and collapsed replication forks[3,4]. Moreover, HR is essential for the accurate chromosome segregation during meiosis[5,6].

HR involves the coordinated actions of a complex set of proteins, many of which remain poorly understood[1]. Examples include replication protein A (RPA), Rad51, and Rad54, among many others[7]. HR reactions in both prokaryotic and eukaryotic cells involve an ssDNA intermediate, which is rapidly coated by ssDNA-binding proteins (SSB in prokaryotes and RPA in eukaryotes)[8]. These proteins protect the ssDNA from nucleases, eliminate secondary structure, and promote the recruitment of downstream factors[8,9]. Rad51 is a member of the highly conserved ATP-dependent Rad51/RecA family of DNA recombinases, which are present in all living organisms[1]. Rad51 promotes DNA strand invasion of the homologous dsDNA donor. Given its importance, Rad51 is highly regulated, and defects in these regulatory processes are commonly associated with a loss of genome integrity and oncogenic transformation[7]. Rad54 is a member of the Swi2/Snf2 family of dsDNA translocases and chromatin remodelers[10,11]. These proteins serve as essential Rad51 regulatory factors. Importantly, Rad54 removes Rad51 from the dsDNA product of strand invasion and is also necessary to prevent misaccumulation of Rad51 on chromatin[11]. Molecular activities for proteins involved in HR both in yeast and bacterial cells have shed light on their function in HR, but exactly how their activity contributes to HR remains poorly understood [12].

DNA curtains have emerged as a unique platform providing direct access to molecular mechanisms and macromolecular dynamics that would otherwise remain inaccessible[13,14]. To prepare DNA curtains, the surface of a microfluidic chamber is coated with a lipid bilayer, and DNA molecules are tethered to the bilayer through a biotin-streptavidin linkage. The bilayer renders the surface inert by mimicking natural cell membranes. Hydrodynamic force aligns the DNA along nanofabricated barriers, allowing visualization of hundreds of molecules in a single field-of-view by total internal reflection fluorescence microscopy (TIRFM). The barriers are made by electron-beam lithography, and variations in barrier design allow for precise control over the distribution and tethering geometry of the DNA. These approaches are readily applicable with either ssDNA or dsDNA[13-18]. Pedestals may be also nanofabricated (together with the barriers) to allow both ends of the DNA to be tethered to the sample chamber surface such that steady state experiments may be carried out in the absence of buffer flow.

Time-dependent changes in individual protein-nucleic acid complexes are revealed by the inspection of real-time videos and are represented in print using kymographs, which present the changing position of proteins on DNA over time. An important aspect of the DNA curtains approach is that it does not necessarily require *a priori* models or assumptions about molecular mechanisms, because the behavior of individual reaction components can be observed in real-time. This allows the direct observation of molecular behaviors. Herein, this protocol describes how to prepare DNA curtains with dsDNA substrates as well as its application to study intermediates in homologous recombination.

PROTOCOL:

1. Preparation of lipid stock

1.1. Dissolve 1 g of 18:1 (Δ9-Cis) PC, 100 mg of 18:1 PEG2000 PE, and 5 mg of 18:1 biotinyl cap PE in 10 mL of chloroform.

NOTE: Both DOPC and DPPE are well-characterized and (along with PEG modification) selected for their ability to minimize non-specific adsorption to the surface of the flow cell. Dissolved lipid master mix can be aliquoted and stored at -20 ˚C up to 12 months.

1.2. Use chloroform to clean an organic-solvent glass syringe, then transfer 200 µL (10% of the final desired volume) of the lipid master mix to the glass vial.

1.3. Evaporate the chloroform from the glass vial using a very gentle stream of compressed nitrogen. Slightly increase the pressure towards the end of the evaporation to ensure that all traces of chloroform are removed.

NOTE: Tilt and rotate the vial during the evaporation process so that the lipids dry as a uniform layer of white film on the wall.

1.4. Place the vial uncapped under vacuum overnight.

1.5. Add 2 mL of lipid buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl) to the dried lipid film and cap the vial. Incubate at room temperature (RT) for at least 1 h and vortex until dissolved.

1.6. Transfer the solution to a 5 mL polystyrene round-bottom tube and sonicate on ice using a microtip with the following parameters: amplitude = 50, process time = 1.5 min, pulse = 15 s, time off = 2 min. The solution will be clear after sonication (total energy = 1,500–2,000 J).

1.7. Filter the solution through a 0.22 µm PVDF syringe filter and store the lipid stock at 4 ˚C for up to 1 month.

2. Preparation of dsDNA substrate

2.1. Warm the λ-DNA stock (500 µg/mL) to 65 ˚C.

2.2. Prepare annealing/ligation mix containing: 1.6 pM of λ-DNA, 100x molar excess (160 pM) of each oligonucleotide (BioL: 5'Phos-AGG TCG CCG CCC-3Bio and DigR: 5'Phos-GGG CGG CGA CCT-3Dig_N) and 60 µL of 10x T4 ligase reaction buffer (in a total volume of 600 µL).

NOTE: BioL and DigR oligonucleotides are biotinylated (Bio) and modified with digoxigenin (Dig), respectively. Biotinylation allows the attachment of DNA molecules to biotinylated lipids via

streptavidin linkages. The Dig modification enables double tethering through binding of anti-Dig antibodies that are coated on pedestals to Dig moieties.

2.3. Anneal the oligonucleotides to the λ-DNA cos-ends by incubating the reaction at 65 ˚C for 10 min, then allow it to slowly cool to RT.

2.4. Add 5 µL of T4 DNA ligase and incubate at RT overnight.

NOTE: Annealing and ligation steps may also be carried out in a thermal cycler.

2.5. Precipitate the λ-DNA by adding 300 µL of PEG-8000 solution (30% w/v PEG-8000 and 10 mM $MgCl_2$). Incubate at 4 ˚C for at least 1 h.

2.6. Centrifuge at 14,000 x *g* for 5 min at RT. Carefully remove the supernatant without disturbing the pellet. Optionally, wash the pellet 1x with 500 µL of chilled 70% ethanol.

2.7. Resuspend the pellet in 40 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

3. Nanofabrication of chromium patterns

3.1. Drill two holes at the center of a 1" x 3" quartz microscope slide (~3 cm apart) using a diamond-coated 1.4 mm drill bit on a table-top drill press (see **Figure 1** for geometry of the drilling position).

3.2. Clean the drilled slides by submerging them into 200 mL of Piranha solution (75% sulfuric acid [97%] and 25% hydrogen peroxide). Incubate for 30 min.

CAUTION: Extreme care must be taken to avoid all contact with exposed skin. Prepare the solution by very slowly mixing cold hydrogen peroxide into sulfuric acid. Dispose of Piranha waste properly in a designated waste container.

3.3. Rinse slides by submerging in 200 mL of double-distilled water. Repeat 2x to eliminate any remaining Piranha. Dry the slides with a gentle stream of compressed nitrogen.

3.4. Using a spin coater (4,000 rpm for 45 s, ramp rate of 300 rpm/s), coat the clean slides with photoresist and conductive polymer as follows:

3.4.1. Coat slides with a layer of 3% (w/v) polymethylmethacrylate (PMMA; 24.3K MW; dissolved in anisole).

3.4.2. Coat slides with a layer of 1.5% (w/v) polymethylmethacrylate (PMMA; 495K MW; dissolved in anisole).

3.4.3. Coat slides with a layer of antistatic agent for electron beam lithography.

3.5. Write custom patterns for barriers and pedestals on the coated slides using electron beam lithography with a scanning electron microscope equipped with nanopattern generation system software.

3.6. Develop the slides by first rinsing off the antistatic agent for the electron beam lithography with double-distilled water. Then, place them into 50 mL tubes containing the developing solution (75% methyl isobutyl ketone [MIBK] and 25% isopropanol; keep chilled at -20 ˚C) and sonicate in an ice bath on low power for 1 min.

3.7. Rinse off the developing solution with isopropanol and dry with a gentle stream of compressed nitrogen.

3.8. Using an electron beam evaporator, deposit a 250 Å thick layer of chrome onto the patterned surface at 0.5 Å/s.

3.9. Lift off the chrome and remaining PMMA using acetone from a squirt bottle, followed by sonicating the slides in an acetone bath for 10 min. Wash again with acetone from a squirt bottle.

3.10. To prevent any deposits from the remaining acetone, rinse the slides with isopropyl alcohol and dry with a gentle stream of compressed nitrogen.

4. Assembly of flow cell

4.1. Place a 35 mm x 5 mm rectangular paper template (covering the chrome pattern as well as the predrilled inlet/outlet holes) over the quartz slide, with the patterned side facing up (**Figure 1A**).

4.2. Apply a piece of double-sided tape (1 mm thickness) over the quartz slide, covering the paper template, then use a razor blade to cut out the rectangular paper template (**Figure 1A**).

4.3. Place a glass coverslip over the double-sided tape and apply gentle pressure to seal the coverslip to the tape (**Figure 1A**). The space between the coverslip and slide, sealed by double-sided tape, forms the microfluidic chamber.

NOTE: It is crucial that the seal between the cover slip and slide is sufficiently secure to prevent leakage during the experiment.

4.4. Place the coverslip-slide sandwich in between glass slides and attach binder clips on all four sides to distribute pressure evenly. Place the assembly in a vacuum oven for 60 min at 135 ˚C to melt the double-sided tape and seal the chamber.

4.5. Remove it from the oven, then release the binder clips and glass slides.

4.6. Attach microfluidic ports to the predrilled inlet/outlet holes with a hot glue gun by placing the glued assembly onto a heating block (220 ˚C) for 1 min, taking it off, and carefully applying gentle pressure to properly seal the microfluidic ports. Allow glue to cure completely before proceeding.

NOTE: Assembled flow cells may be placed in 50 mL tubes and stored under vacuum.

5. Assembly of dsDNA curtain

5.1. Attach inlet/outlet tubing to the flow cell microfluidic ports and wash the chamber with 20 mL of double-distilled water using two 10 mL syringes.

5.2. With 10 mL syringe filled with double-distilled water connected to both sides of the flow cell, apply force to push/pull the water between the inlet and outlet syringes. This step removes all air bubbles from the flow cell.

5.3. Wash the chamber with 3 mL of lipid buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl).

NOTE: Make sure all connections to flow cells are drop-to-drop to avoid introducing air bubbles into the flow cell.

5.4. Mix 40 µL of liposome solution with 1 mL of lipid buffer. Inject 300 µL of the lipid solution into the flow cell and incubate for 5 min. Repeat the injection and incubation 2x.

NOTE: Alternate the inlet/outlet side for injection each time a syringe is connected to the flow cell.

5.5. Wash the chamber with 3 mL of lipid buffer and incubate for 15 min.

NOTE: This incubation period can vary from 5 min to 2 h, depending on the scheduling of experiments, without noticeable differences in final curtain assembly.

5.6. Mix 40 µL of 1 mg/mL anti-digoxigenin stock solution with 200 µL of lipid buffer. Inject 100 µL of the solution and incubate for 10 min. Repeat the injection and incubation 1x before washing with 3 mL of lipid buffer.

NOTE: This step is needed only for double-tethered dsDNA curtains. Skip this step for single-tethered dsDNA curtains.

5.7. Wash the chamber with 3 mL of BSA buffer (40 mM Tris-HCl [pH 8.0], 2 mM $MgCl_2$, 1 mM DTT, and 0.2 mg/mL BSA).

5.8. Mix 10 µL of 1 mg/mL streptavidin stock solution with 990 µL of BSA buffer. Inject as two separate 400 µL injections with a 10 min incubation after each injection.

5.9. Wash the chamber with 3 mL of BSA buffer to remove any excess streptavidin.

5.10. Dilute 25 ng of dsDNA substrate stock in 1 mL BSA buffer. Slowly inject the DNA solution 200 µL at a time, followed by a 5 min incubation after each injection. The amount of DNA needed may be optimized for specific types of experiments.

5.11. Mount the flow cell onto the microscope stage and adjust the focus as necessary.

5.12. Connect the input and output ports to a sample injection system comprised of a syringe pump and high-pressure switch valve.

5.13. Double-tether the DNA to the pedestals (12 µm tether length) by continuously washing the chamber with BSA buffer at 0.8 mL/min for 5 min.

NOTE: It may be necessary to adjust flow rates and times to optimize for different double-tether lengths. This step is needed only for double-tethered DNA curtains. Skip this step for single-tethered dsDNA curtains.

5.14. The curtains are now ready for experiments.

6. Recycling of flow cells

6.1. Submerge the slides in ethanol for at least 30 min before using a razor blade to carefully remove the microfluidic ports and coverslip. Properly dispose of the coverslip and clean any remaining glue off the microfluidic ports so they can be reused. Scrub clean any glue residue on the slide.

NOTE: Take care not to scratch the patterned area in the center of the slide.

6.2. Thoroughly rinse the slides with double-distilled water and submerge in 2% alkaline liquid quartz cleaning concentrate overnight, with stirring.

6.3. Thoroughly rinse the slides with double-distilled water and submerge in 2% alkaline liquid quartz cleaning concentrate solution for 1 h with stirring at 80 ˚C, followed by sonicating for 15 min at 45 ˚C.

6.4. Thoroughly rinse the slides with double-distilled water and submerge in 1 M NaOH for 1 h, with stirring.

6.5. Thoroughly rinse the slides with double-distilled water, then submerge in isopropyl alcohol and sonicate for 15 min at 45 ˚C.

6.6. Thoroughly rinse the slides with ethanol and submerge in ethanol for 1 h, with stirring.

6.7. Place the slides in a vacuum oven for 1 h to dry at 135 ˚C. The slides are now ready to be reassembled into flow cells.

REPRESENTATIVE RESULTS:

Described above are the preparation, assembly, and imaging of single- and double-tethered dsDNA curtains in the context of studying protein-DNA interactions in DNA repair intermediates. **Figure 1A** shows all components in a flow cell, layered in the order that they are assembled. **Figure 1B** depicts the schematic of a single- or double-tethered DNA curtain. A lipid bilayer is used to passivate the surface of the flow cell. The DNA curtain consists of a parallel array of DNA molecules tethered at one end to the lipid and aligned at the chromium barrier, oriented perpendicular to the direction of flow. For double-tethered DNA curtains, the other end of DNA is tethered to the pedestal through Digoxigenin and anti-Digoxigenin interactions, such that imaging can be carried out in the absence of flow while DNA remains extended. dsDNA can be stained with fluorescent dye YoYo1 and visualized by TIRF microscopy. **Figure 2A** shows a representative wide-field image of YoYo1-stained double-tethered DNA curtain.

Time-lapse image series collected from DNA curtain experiments are typically analyzed by first generating a kymograph, which plots the position along the DNA molecule on the vertical axis vs. time on the horizontal axis for each DNA molecule of interest in ImageJ. **Figure 2B** are representative kymographs showing the resection of YoYo1-stained single-tethered λ-DNA (green) by the yeast resection machineries GFP-Sgs1 (not visible), Top3-Rmi1, and Dna2 in the presence of single-stranded DNA binding protein RPA-mCherry (magenta) and ATP. YoYo1 signal is lost over time as dsDNA is resected from the free end by Sgs1-Dna2. Simultaneously, mCherry signal colocalizes with DNA ends at which ssDNA is being generated as a result of the resection. Biophysically relevant characteristics of the resection, such as velocity and processivity, can be extracted from by quantifying the slopes of the YoYo1 signal loss trajectories. **Figure 2C**,**D** shows the distribution of velocities and processivity, respectively, of resection by Sgs1-Dna2.

FIGURE LEGENDS:

Figure 1: Schematics of flow cell assembly and DNA curtains. (**A**) Stepwise illustration for flow cell assembly. Double-sided tape is placed on top of the quartz slide, and a paper template is used to cut out a rectangular channel from the center, which is sealed with a cover slip on top to form a chamber. (**B)** Schematics of fully assembled DNA curtains. Middle panel presents a single-tethered flow cell, while the bottom panel presents a double-tethered flow cell.

Figure 2: Single molecule DNA curtain visualization and analysis. (**A**) Representative wide-field image of a double-tethered λ-DNA curtain stained with YoYo1 (green). (**B**) Representative kymographs showing the resection of YoYo1-stained dsDNA (green) in the presence of pre-

bound GFP-Sgs1 (not visible) when chased with Dna2, in the presence of RPA-mCherry (magenta) and 2 mM ATP. Velocity distribution (**C**) and processivity plot (**D**) of dsDNA resection by Sgs1-Dna2, obtained by quantifying the rate and extent of the YoYo1 signal loss over time. Panels (B), (C), and (D) are adapted from Xue et al.[19].

DISCUSSION:

DNA curtains has proven to be a versatile platform for studying various DNA repair processes[13-16]. DNA molecules that remain extended near the surface throughout the experiment, through either continuous buffer flow or double-tethering to the pedestal, allow for TIRFM imaging of protein-DNA interactions on repair intermediates at the single molecule level. The method provides improvement in experimental throughput and predictability, since hundreds of DNA molecules are orderly aligned at nanofabricated barriers rather than non-specifically attached to the surface, which further benefits processing of large data sets.

Careful analysis of the resulting time-lapse image series provides quantitative measurements of protein-DNA interactions (i.e., binding lifetimes, positions, stoichiometry, dissociation kinetics, translocation rates, processivities of motor proteins, and colocalizations and interactions between proteins and/or DNA). Compared to other single molecule imaging techniques that either tether DNA molecules directly to the surface or combine confocal imaging with force manipulation in commercial instruments, the major trade-off of DNA curtains for higher throughput is establishing the techniques necessary for initial set-up[13-16]. Once established, however, the ability to collect data on hundreds of molecules simultaneously in one flow cell leads to significant savings in time spent on data collection.

As with many other single molecule imaging methods, the ability to prepare a clean surface largely dictates the user's ability to reliably assemble DNA curtains. In this case, clean surfaces require not only starting with a clean slide but also making sure not to introduce microscopic air bubbles into the chamber while making any of the fluidic connections. First, regarding cleanliness of the slides, the steps detailed above in section 6 should suffice as a routine cleaning process. Use of filtered solutions and buffers is also recommended. Second, making drop-to-drop connections during attachment of the flow cell to the buffer injection system is essential to avoid introducing bubbles. It may be helpful to degas the double-distilled water used for making buffers. It is also recommended to carefully observe any injection process to ensure that no bubbles are present. Should a microscopic bubble be introduced before it reaches the center patterned area, it may be remedied by injecting buffer from the microfluidic port on the opposite side to push the bubble back out.

Another important point of consideration in any fluorescence-based experiments is signal-to-noise. A key to observing single fluorescent proteins (i.e., GFP) also resides in clean surfaces. After extended usage, protein aggregates typically accumulate at the chrome barriers and pedestals. Therefore, if a strong fluorescence signal is observed at the barriers prior to the addition of fluorescent proteins, it is advised to treat slides with a 10 min submersion in Piranha

solution. The time period is kept short to avoid eroding the chrome features. The Piranha wash should be followed by the regular cleaning procedure.

Nanofabrication using electron beam lithography can be challenging, due to its requirements of equipment and facilities as well as existing expertise in material science. As an alternative, UV lithography-based method for fabrication of chromium features for DNA curtains has been developed[20]. Also provided with this protocol is a basic pattern for double-tethering lambda DNA at 12 μm (see supplemental information). Several aspects of the design of this pattern may be adjusted to suit various experimental needs. These include the distance between barriers and pedestals, spacing between adjacent wells in the barrier to control DNA density, and the shape and size of pedestals to optimize double-tethering efficiency and minimize uncertainty in double-tethering lengths. Other practical challenges may include choice of fluorescent labeling strategies for proteins, minimizing bias in manual data analysis, and development of scripts for more streamlined data processing.

While the protocol described here involves dsDNA substrates, DNA curtains based on ssDNA have also been used extensively[13-16]. Preparation of ssDNA substrates utilizes rolling circle replication of the M13 ssDNA plasmid with biotinylated primers[13]. Assembly of the ssDNA curtain also requires the use of urea and ssDNA-binding protein RPA to eliminate secondary structure and extend the ssDNA substrate[13]. It may also be possible to extend DNA curtains to using RNA or DNA/RNA hybrids for studies of RNA-interacting proteins. As it currently stands, the DNA curtain platform can benefit from automation in the assembly process as detailed in section 5, most of which is repetitive injections of a standard set of buffers. This may lead to improved efficiency in carrying out multiple experiments simultaneously. In addition, a unified and potentially machine learning-based suite of analysis scripts would further quicken data processing with minimal biasing.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

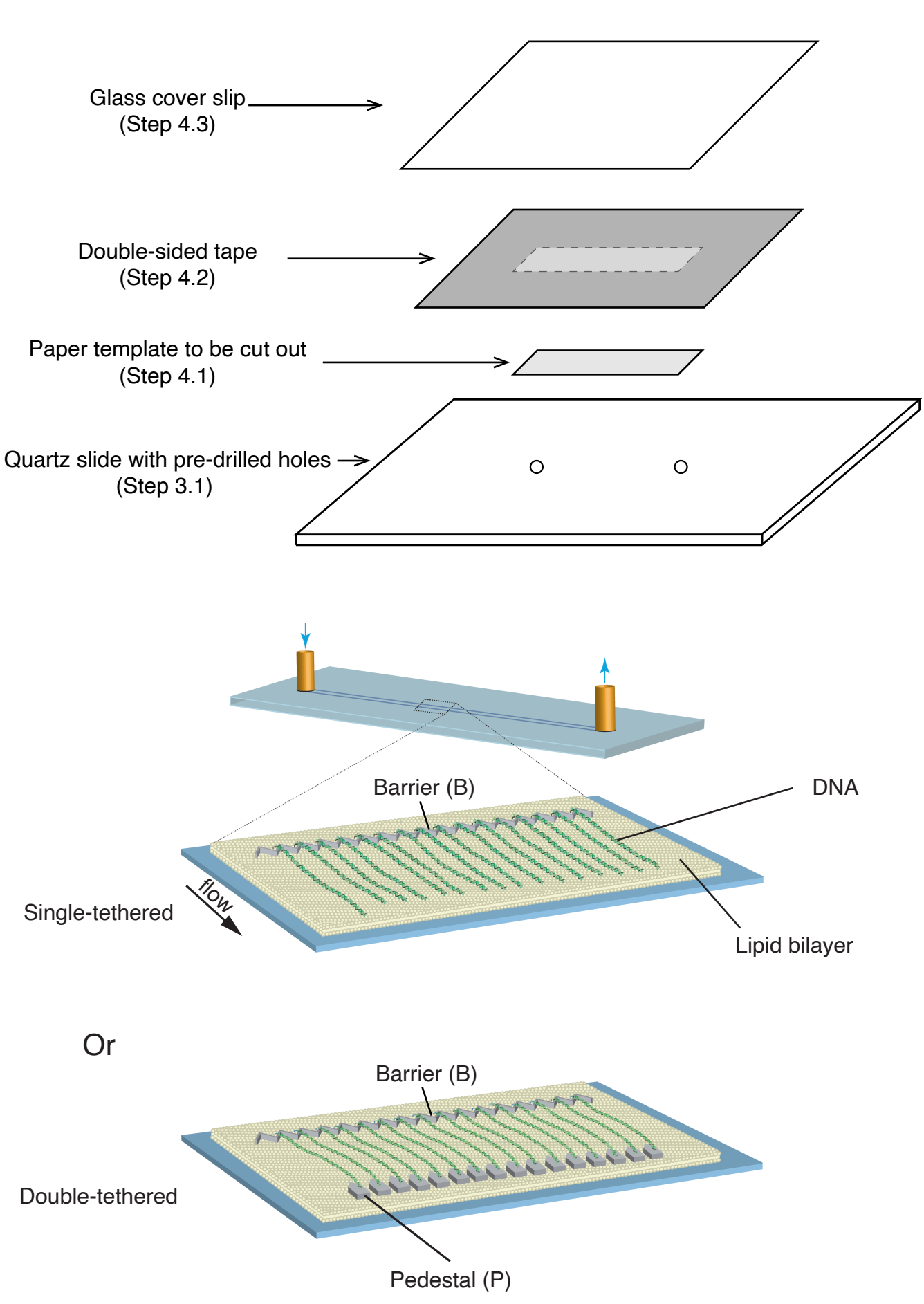
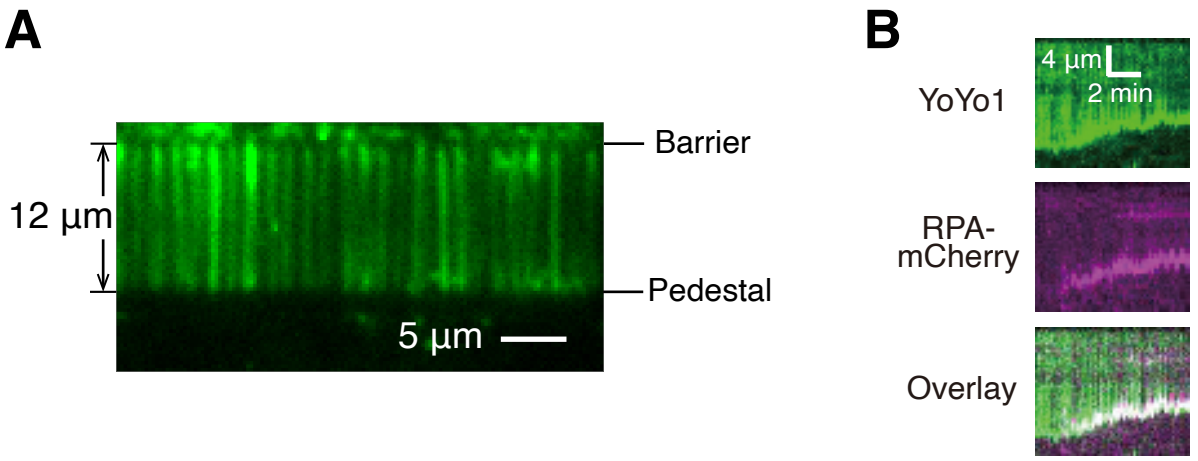
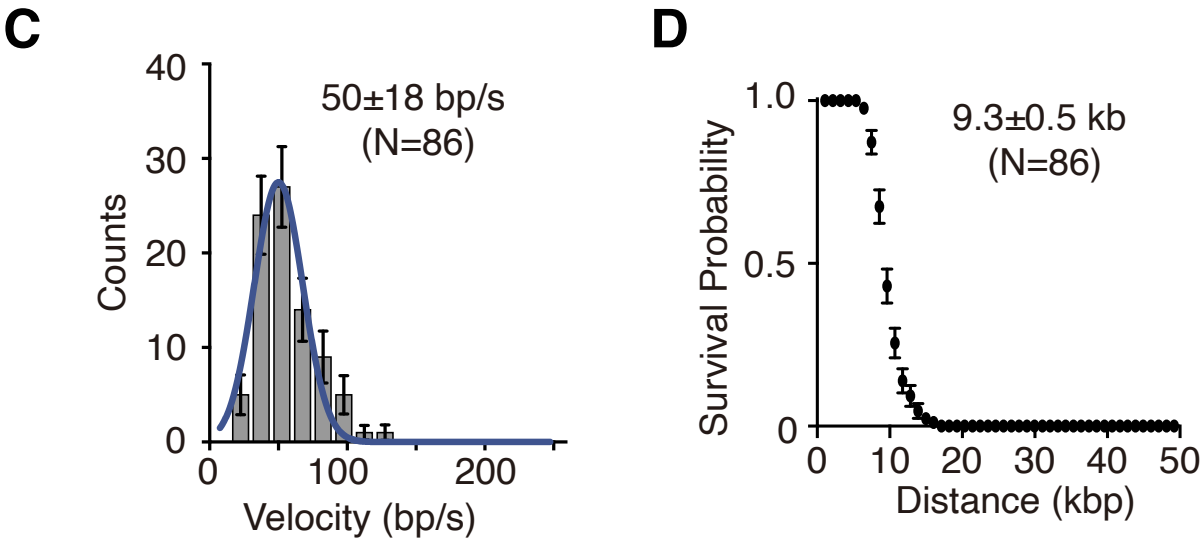


Figure 2





Name of Material/ Equipment	Company
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1 mL Syringe, Slip Tip	BD
1.4 mm Diamon Coated Drill Burs	Shor International
10 mL Syringe, Luer-Lok Tip	BD
10X T4 DNA Ligase Reaction Buffer	NEB
15 x 45 mm Screw Thread Vials, Clear	Thermo Scientific National
18:1 (Δ9-Cis) PC (DOPC)	Avanti Polar Lipids
18:1 Biotinyl Cap PE	Avanti Polar Lipids
18:1 PEG2000 PE	Avanti Polar Lipids
3 mL Syringe, Luer-Lok Tip	BD
495 PMMA A3	MicroChem
5 mL Polystyrene Round-Bottom Tube	BD Falcon
Alkaline Liquid Quartz Cleaning Concentrate	Sigma-Aldrich
Anisole	Sigma-Aldrich
Anti-Digoxigenin, Fab fragments	Roche
Antistatic Agent for Electron Beam Lithography	Mitsubishi Rayon Co., Ltd.
BioL Custom Oligonucleotide	IDT
Bovine Serum Albumin (BSA)	Sigma-Aldrich
BSA Buffer	N/A
Chloroform	Supelco
DigR Custom Oligonucleotide	IDT
Electron Beam Evaporator	Angstrom Engineering
Glass coverslip	Fisher Scientific
Hydrogen Peroxide, 30%	Fisher Chemical
ImageJ	NIH
Lambda DNA	NEB
Lipid Buffer	N/A
MIBK	MicroChem
Microfluidic Ports	IDEX

MX Series II 2 Position/6 Port PEEK Switching Valve	IDEX
nano pattern generation system software	JC Nabity Lithography Systems
PFA Tubing, Natural 1/16" OD x .020" ID	IDEX
Poly(Methyl Methacrylate), Atactic (24.3K MW)	Polymer Source Inc.
Quartz Microscope Slide	G. Finkenbeiner Inc.
Scanning Electron Microscope	FEI
Scotch Double Sided Tape, 3/4 x 300 inches	3M
Sonicator	Misonix
Sonicator	Branson
Streptavidin from Streptomyces avidinii	Sigma-Aldrich
Sulfuric Acid	Avantor - J.T.Baker
Syringe Pump	KD Scientific
Syringe, 500 µL, Model 750 RN SYR, Large Removable NDL, 22 ga, 2 in, point style 2	Hamilton
T4 DNA Ligase (400,000 units/ml)	NEB

Catalog Number	Comments/Description
09-720-3	For filtering lipid stock after sonication
309659	
DIT-211	
302995	
B0202S	
C4051-1	
850375P-1g	1,2-dioleoyl-sn-glycero-3-phosphocholine
870273C-25mg	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)
880130P-25mg	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)
309657	
M130003 0500L	
1GL	
352058	
Z805939	Hellmanex
123225-1L	
11214667001	Dissolved in 1X PBS to a final concentration of 1 mg/mL
aquaSAVE-53za	
N/A	5’Phos-AGG TCG CCG CCC-3Bio
A7030	
N/A	40 mM Tris-HCl [pH 8.0], 2 mM $MgCl_2$, 1 mM DTT, and 0.2 mg/mL BSA
1.02445.1000	Reserve for use only with lipids
N/A	5’Phos-GGG CGG CGA CCT-3Dig_N
N/A	EvoVac Multi-Process Thin Film Deposition System
12-548-5P	
H325-500	
N/A	Image processing software
N3011S	
N/A	10 mM Tris-HCl [pH 8.0], 100 mM NaCl
M080100 40001	
LPE	
N-333	NanoPorts

MXP9900-000	
N/A	NPGS
1512L	
P9790-MMA	
N/A	1" x 3" x 1 mm thick
N/A	Nova Nano SEM 450
N/A	
S-4000	For preparation of lipids
1800	For sonicating slides
S4762	Dissolved in 1X PBS to a final concentration of 1 mg/mL
9681-01	
78-0200	
80830	Reserve for use only with lipids
M0202S	

Dear Editor,

Attached please find our manuscript titled:" DNA curtains shed light on complex molecular systems during homologous recombination" which is submitted for the second time and we will be grateful if it can be reconsidered for publication in JoVE. We have fully addressed all the editorial comments on the manuscript, all changes were formed using track changes. Two reviewers have reviewed the manuscript previously, the first reviewer have asked to better describe the lithography process involved with assembling flow cells. We expanded the discussion and described in better details the lithography used for assembling a flow cell. Furthermore, we added a pattern used for lithography to form a double-tethered flow cell. Besides that all the reviewer's minor spellings/typos comments were corrected. As for the second reviewer most of his/her comments were as for refining several issues within the manuscript, all being better addressed now in. Overall, all of the reviewers comments of the previous manuscript have been fully addressed and revised. We would like to stress, that the significance of this manuscript is to describe in details a novel approach for single-molecule experiments which are carried out using flow-cells experiments. We believe this manuscript describes our method in its most minor details, making it easily available for the wide audience.

We have addressed all of the reviewers comments below, and hope that with this revision, the manuscript will be found suitable for publication.

Thank you for you kind consideration

Sincerely yours

Eric Greene

Editorial comments:
Changes to be made by the Author(s):
-Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Author's reply: Manuscript was well proofread.

-Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Author's reply: All of the above were implemented in the manuscript.

-Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

Author's reply: The abstract is 163 words.

-Please revise the manuscript text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author's reply: This was double checked and all personal pronouns were revised.

-JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: aquaSAVE, Milli-Q, FEI Nova NanoSEM, EvoVac, NanoPorts, Hellmanex, etc,

Author's reply: This was double checked and all commercial language were revised.

-Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Author's reply: This was double checked and revised.

-The Protocol should contain only action items that direct the reader to do something.

Author's reply: This was double checked and revised.

-Please ensure you answer the "how" question, i.e., how is the step performed?

Author's reply: This was double checked and revised.

There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Author's reply: This manuscript is in the correct range of length.

-Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Author's reply: Chaoyou Xue and Eric C. Greene are first and corresponding authors on the PNAS paper from which panels in Figure 2 are adapted. According to PNAS policy authors need not obtain permission for use their original figures or tables in their future works. This can be also verified in the link: https://www.pnas.org/page/about/rights-permissions.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Author's reply: Discussion was revised according to all reviewers' comments and we believe that now it addresses all of the above.

Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Author's reply: all trademarks were removed from the manuscript.

Reviewer #1:
This methods paper from the Greene lab describes the DNA curtains protocol that they have developed and refined over the years. It can have broad utility in single molecule experiments and is not as widely adopted as it could be, in part due to technical challenges. There are clear text descriptions here that will be helpful to labs in establishing the technique. The area that is not really discussed and which is critical to the technique is the design and implementation of the lithography to make chrome barriers and pedestals. It would be useful to expand on this section to give further tips as otherwise it is very vague at the moment. In the interests of open science, the CAD (?) designs used in this paper could be supplied as supplementary information. Otherwise I have only minor typos/comments.

Author's reply: thank you for this comment, we extended our discussion section and broadly discussed the lithography process including the pattern used for writing slides.

-Page 1, lines 68 and 72: "nanofabricated" vs "nano-fabricated"

Author's reply: Corrected.

-Page 1, line 78: "a priori"

Author's reply: Corrected.

-Page 3, line 129: Piranha solution is quite nasty and it might be a good idea to describe exactly - how this is safely made and disposed of.

Author's reply: we better described the preparation of the Piranha solution and disposal to ensure safety usage.

-page 3, line 147: "icy water" reads a little colloquial.

Author's reply: Corrected to ice bath.

-Page 4, line 164: thickness of tape? Since that gives flow cell dimensions.

Author's reply: Corrected, 1mm thickness was added.

Reviewer #2:
Manuscript Summary:

In the manuscript entitled "DNA curtains shed light on complex molecular systems during homologous recombination", Meir et. al describe detailed experimental procedures and knowhows for the DNA curtain assay. The manuscript is well organized and provides useful information for each step of the protocols. It will be greatly beneficial for the related research communities. The manuscript can further be improved by considering following issues:

Major Concerns:
None

Minor Concerns:
-On page 2 (Protocol 1.1), the authors used 18:1 (D9-Cis) PC and 18:1 PEG2000 PE. Can they provide reasons why they chose these lipids?

Author's reply: Both 18:1 (D9-Cis) PC and PEG2000 PE are well characterized lipids which help to reduce non-specific absorption to the surface of the flow cell. This was added to the protocol as:" Both DOPC and DPPE are well characterized and, along with PEG modification, are selected for their ability to minimize non-specific adsorption to the surface of the flow cell."

-On page 2 (protocol 1.1), the authors described that the aliquots can be stored at -20dC. How long can the aliquots be stored?

Author's reply: Thank you for this comment, we added to the manuscript that the aliquots can be stored in -20dC up to 12 months.

-It would be easier for the readers to follow the protocol if the buffers used in the protocol is summarized with their compositions at the end of the manuscript. (ex. Lipid buffer and BSA buffer, etc)

Author's reply: We added the buffers at the materials table

-On page 2 (protocol 1.6), the unit of amplitude should be provided so that the readers can estimate how much power they have to set when a sonicator from another company is used.

Author's reply: We added to the manuscript that the total sonication energy results in 1,500-2,000 J.

-On page 3 (protocol 3.1), it would be helpful if they refer Fig. 1B to show the geometry of the drilling positions.

Author's reply: Figure 1B was revised and now refers to step 3.1.

-Figure 1A is somewhat difficult to understand. At a first glance, the double-sided tape and the paper template seemed to be embedded within the cover slip. It became clear only after reading the text that the cover slip, double-sided tape and the paper were stacked on top of each other. The confusion comes mainly from the drawing that the thickness of the cover slip, tape, paper, quartz slide is not illustrated. Please consider revising the drawing for easier understanding.

Author's reply: Thank you for this comment, we redesigned the figure and added references to which steps of the protocol each stage is related.

-The list of material/Equipment provided at the end of the manuscript does not seem to be finished. Each material/Equipment is better listed with following information: 1) company, 2) catalogue(model) number, 3) commercial name and the name used in the protocol, and 4) the place mentioned in the protocol.

Author's reply: Thank you for this comment. It was a excel file formatting issue, it was corrected.

Supplemental Figure

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Supplemental Coding Files

180117. Double Tether - 12um(1).dxf