Supporting Information For:

High-Throughput Universal DNA Curtain Arrays for Single-Molecule Fluorescence Imaging

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1. Supplemental Figures

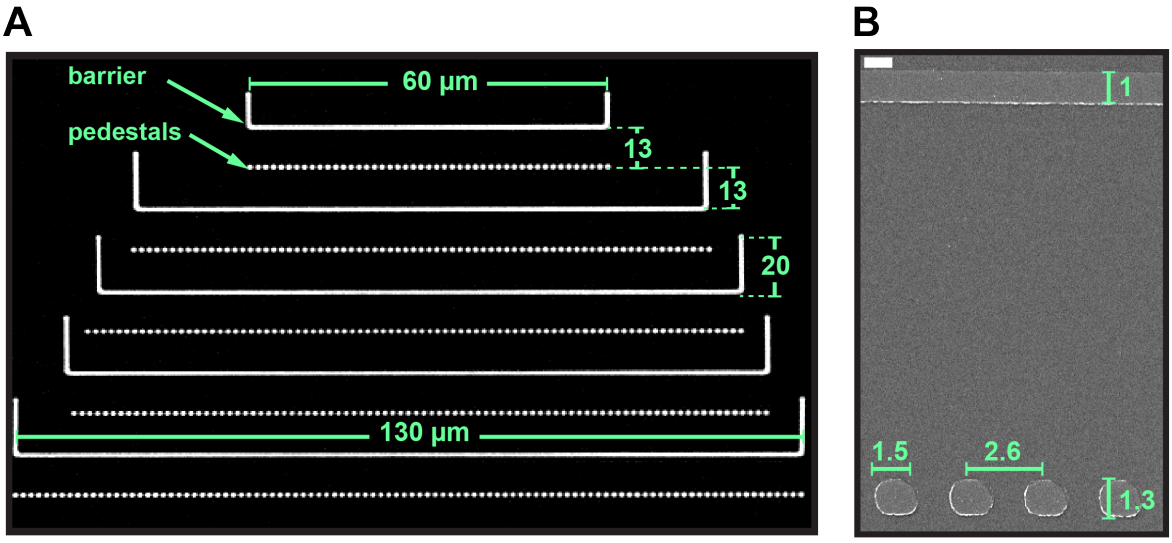

Figure S1. Barrier sets for double-tethered DNA curtains are illustrated in (A). There are five lines of alternating barriers and repeating 1.5 μm pedestals in each of the double-tethering barrier sets present on a single microfluidic chip. (B) Representative scanning electron microscope (SEM) image of a barrier and four pedestals. SEM images were taken by sputtering a 5 nm-thick layer of Iridium (99.95%) on the microfabricated slide and imaging in a Zeiss Supra 40V SEM. The barrier is 1 μm wide and each pedestal is a ~1 μm x 1.5 μm oval. The pedestals are separated by 2.6 μm. Scale bar: 1 μm. All distances in (A) and (B) are in micrometers.

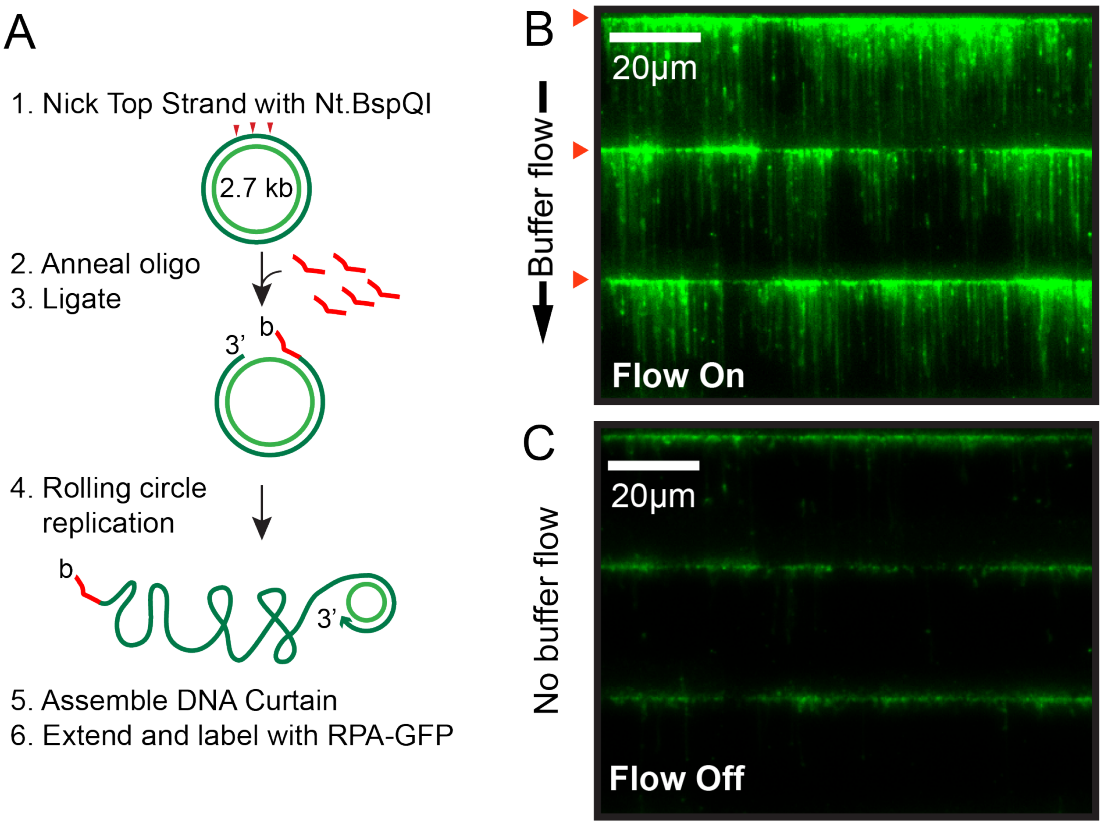

Figure S2. Single-stranded DNA curtains. (A) To create long ssDNA substrates, three closely spaced *Nt.BspQI* recognition sites (red ticks) were introduced into a 2.7 kb plasmid. After nicking with *Nt.BspQI*, a biotinylated oligonucleotide (red line) was annealed and ligated within the resulting ssDNA gap. The ssDNA was extended via rolling circle replication (RCR) with the highly processive phi29 DNA polymerase. RCR reactions were quenched with 75 mM EDTA and the resulting 5'-biotinylated ssDNA was used immediately for assembling DNA curtains. See supplemental methods for additional details. The ssDNA was organized at Cr diffusion barriers. To extend and image the ssDNA, the imaging buffer contained 0.2 nM human RPA-GFP. RPA is a heterotrimeric ssDNA-binding protein, and the RPA-GFP fusion retains full biochemical activities *in vitro*.[1] (B) ssDNA curtains were assembled within the UV-lithography fabricated flowcells and the resulting DNA curtains could be readily extended and imaged when the buffer flow was on. The red triangles represent three different Cr barriers. (C) In the absence of buffer flow, the ssDNA collapses, indicating that surface attachment occurs specifically via the biotin-streptavidin interaction.

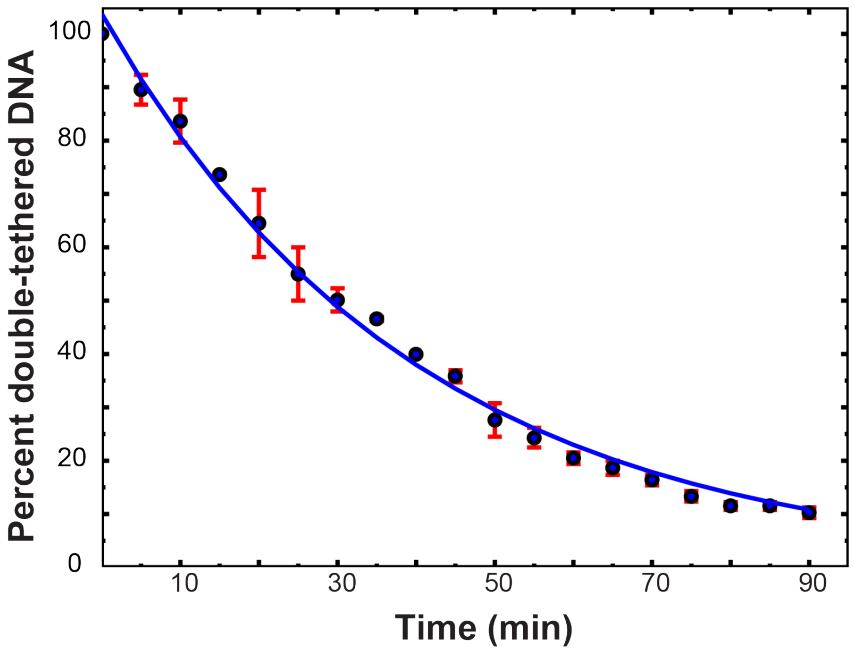

Figure S3. The lifetime of double-tethered DNA molecules in the absence of buffer flow. The DNA was stained with YOYO-1 and a single frame (1 sec exposure) was acquired once every 5 minutes. When data was not being acquired, the laser was blocked via a computer-controlled shutter (LS6, 6 mm, Unistable Shutter; Vincent Associates). We confirmed that at this frame rate, we did not see appreciable DNA photo-cleavage over the entire 90-minute observation time. The data was fit to a single exponential of the form $y = Ae^{-bt}$ where A is the normalized amplitude and b is the lifetime using a custom fitting routine written in MATLAB (blue curve). The lifetime of double-tethered DNA was 29±0.2 minutes (half-life±standard error; N=163). The error bars (red) indicate the standard deviation of at least three replicates.

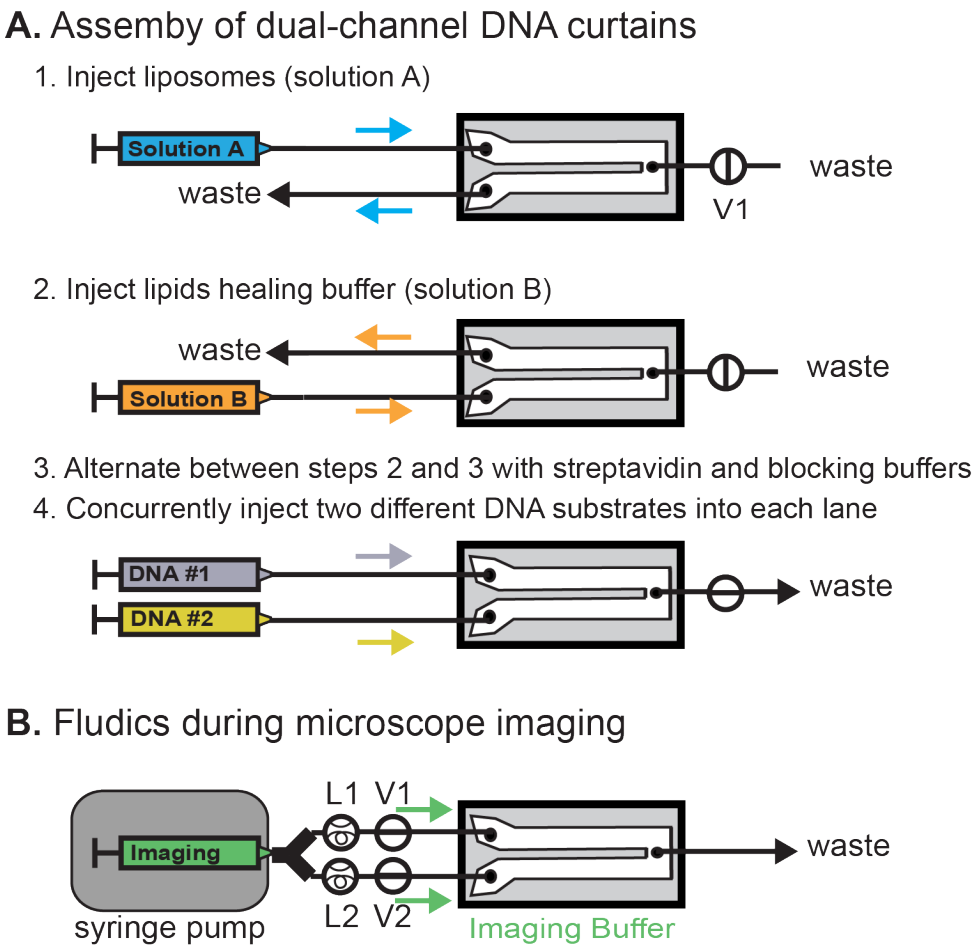

Figure S4. Assembly and imaging of a dual-lane microfluidic flowcell. (A) Lipid bilayers and blocking buffers were injected through the inlet ports of both lanes keeping the single outlet port of the flow-cell closed with valve V1 (IDEX P-732A). To assemble the lipid bilayers, solutions were sequentially flushed through inlet ports #1 or #2. To inject different DNA substrates into each lane, the outlet port was opened and the two DNA solutions were injected concurrently through the two inlet ports. Both lanes were flushed out through the same waste port. (B) Schematic of the fluidics configuration during flowcell imaging. A syringe pump (KD Scientific Legato 210P) provides imaging buffer to both flowcell lanes. Loops L1 and L2 (Rheodyne MXP9900) permit the injection of proteins and valves V1 and V2 are used to stop buffer flow in lanes 1 and 2, respectively. Nucleosomes were fluorescently labeled by injecting a QD-conjugated anti-Flag antibody (monoclonal M2, Sigma) via loop L1. To fluorescently stain the DNA, the imaging buffer had 0.2 nM of YOYO-1.

2. Supplemental Methods

DNA Substrates for Single Molecule Experiments. For single-tethered DNA curtains, λ–phage DNA (New England Biolabs) was annealed to biotinylated oligo IF7. Briefly, ~4 nM λ–DNA was heated to 65˚C, combined with ~10 μM IF7, and allowed to slowly cool to room temperature. After cooling, the reaction was supplemented with 1 mM ATP, T4 DNA ligase (2000 units; New England Biolabs) and incubated overnight at room temperature. To avoid adding nicks, the ligase was salt inactivated by supplementing the reaction with 100 μl of 5M NaCl to (final concentration: 1 M NaCl). Excess proteins and oligonucleotides were removed by passing the reaction over an S-1000 gel filtration column (GE) in TE150 buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 150 mM NaCl) and the ligated DNA stored at 4˚C. For double tethering experiments, the same protocol was followed with oligos IF7 and IF9.

We used a nicking enzyme-based cut-and-replace strategy for creating long ssDNA substrates for DNA curtains.[2,3] A 2.7kb plasmid (pIF100) was constructed by inverse PCR mutagenesis of pHSG298 (Clontech) using primers KA10 and KA11. These primers introduce three *Nt.BspQI* nicking sites that are each separated by 12 nucleotides. pIF100 was transformed into DH5α cells (New England Biolabs) and purified using a MaxiPrep plasmid purification kit (Qiagen). A biotinylated flap was inserted into pIF100 by combining 185 μg of the plasmid with 1,000 units of *Nt.BspQI* (New England Biolabs) in a total restriction digest reaction volume of 792 μl. After nicking the plamsid for 1 hr at 55˚C, 8 μl of 800 units/ml Proteinase K (New England Biolabs) was added and the reaction was incubated for another hour at 55˚C. The reaction was further heated to 80˚C for fifteen minutes and supplemented with the biotinylated flap oligo AdlT7 to a final concentration of 5 μM. The reaction was slowly cooled to 37˚C and supplemented with ATP to 1 mM, 40,000 units of T4 ligase (New England Biolabs), and ligated overnight at room temperature. The ligase was heat-inactivated and the plasmid purified away from the oligo insert via gel filtration over HiPrep 16/60 Sephacryl S-300 HR column with TE150 running buffer (GE). Plasmid-containing fractions were pooled and stored in small aliquots in -20˚C. The ssDNA substrates were made using RCR by combining 100 nM phi29 DNA polymerase with 0.15 nM plasmid in 1 ml of RCR buffer (10 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 10 mM ammonium sulfate and 200 μM deoxyribonucleoside triphosphates, dNTPs). The reaction was incubated 30 minutes at 30°C and quenched with 75 mM EDTA. The resulting ssDNA was injected into the flow cell as indicated below.

Flowcell preparation. We followed previously-described protocols for assembling DNA curtains.[4] Briefly, nano-fabricated flowcells were pre-equilibrated in buffer L (10 mM Tris-HCl, pH 7.8, 100 mM NaCl) and covered with a ternary lipid bilayer composed of a mixture of DOPC (97.7 mol %), DOPE-biotin (0.3 mol %) and DOPE-mPEG2K (2 mol %; Avanti Lipids). Flowcells were next incubated in imaging buffer (40 mM Tris-HCl, pH 8, 2 mM DTT, 1 mM $MgCl_2$, 0.2 mg ml^{-1} bovine serum albumin [BSA; fraction VI, Sigma-Aldrich]) for 10 minutes.

Next, streptavidin (Life Technologies, 0.1 mg ml^{-1} in imaging buffer) was injected into the flow cell. Finally, biotinylated λ-DNA (or ssDNA) was injected into the flowcell and the flowcell was mounted on a custom-machined microscope stage for immediate fluorescent observation.

3. Supplemental Table

Table S1. Oligonucleotides used in this study

Name	Sequence
IF7	[p]AGG TCG CCG CCC[Bio]
IF9	[p]GGG CGG CGA CCT[Dig]
KA10	[p] TTC CCA TGG TGC GAT CGC TCT TCG GGA TTT AAA TAT TCG TAA TCA TGT CAT AGC TGT TTC
KA11	GAG CGG CCG CAT GCA TGA AGA GCT TTA TTT AAA TTT CGA GCT CGG TAC CCG
AdlT7	[Bio] TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TCT TCC CTT GGT GCG ATC GCT CTT CG

4. Supplemental References

(1) Modesti, M. Fluorescent Labeling of Proteins. *Methods Mol. Biol.* **2011**, *783*, 101–120.

(2) Luzzietti, N.; Knappe, S.; Richter, I.; Seidel, R. Nicking Enzyme-Based Internal Labeling of DNA at Multiple Loci. *Nat. Protoc.* **2012**, *7* (4), 643–653.

(3) Luzzietti, N.; Brutzer, H.; Klaue, D.; Schwarz, F. W.; Staroske, W.; Clausing, S.; Seidel, R. Efficient Preparation of Internally Modified Single-Molecule Constructs Using Nicking Enzymes. *Nucleic Acids Res.* **2011**, *39* (3), e15–e15.

(4) Finkelstein, I. J.; Greene, E. C. Supported Lipid Bilayers and DNA Curtains for High-Throughput Single-Molecule Studies. In *DNA Recombination*; Tsubouchi, H., Ed.; Methods in Molecular Biology; Humana Press, 2011; pp 447–461.