SUPPLEMENTARY NOTE

Overview of Experimental Design

Figure s1 shows the schematic diagram of the experimental design. Here, we describe details for each component in the experimental setup:

1. Procedure to chemically functionalize two ends of DNA molecules, magnetic beads, and coverslips to form bead-DNA tethers in a flow cell.
2. Procedure to build a flow cell.
3. Technical details on the pressure-driven pump system.
4. A short note on the magnet set up.
5. Details on the through-objective dark-field microscopy.
6. Details on the image acquisition and image processing for Gaussian centroid determination of bead positions.
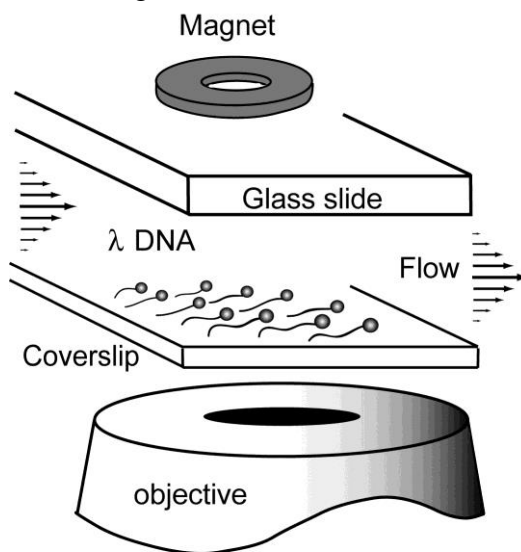

Figure s1 Schematic of a mounted flow cell in the experimental setup (not to scale).

7. Experimental details for nucleic acid enzymes shown in **Figure 2**.
8. Physical discussion of the forces exerted on DNA-tethered beads under shear flow.
9. Discussion of Brownian fluctuations of λ DNA-tethered beads and centroid determination error.

Construction of DNA template for single molecule assay

Linear λ phage DNA (48,502 base pairs, from New England Biolabs, Beverly, MA) was used as a DNA template. One end of the DNA molecules is labeled with biotin for coupling to the streptavidin-coated surface of the flow cell. The free end is labeled with digoxigenin and linked to antidigoxigenin-coated paramagnetic beads. For the orthogonal functionalization of DNA molecules, λ DNA was ligated to two modified oligonucleotides and one unmodified oligonucleotide (Integrated DNA Technologies, Coralville, IA), 5'-GGG CGG CGA CCT AAA AAA AAA AAA AAA AAA AA-biotin-3', 5'-digoxigenin- AAA AAA AAA AAA GGC TGC TCC TGC-3', 5'-AGG TCG CCG CCC GCA GGA GCA GCC–3', as shown in **Figure s2**[1]. By having the unmodified oligonucleotides (linker oligonucleotide) in the digoxigenin end of DNA, biotin and digoxigenin are labeled on the same strand of λ DNA. This allows for generating

ssDNA tethers by heat denaturation or enzymatic digestion by λ exonuclease. All the enzymes and buffers used for the functionalization are purchased from New England Biolabs.
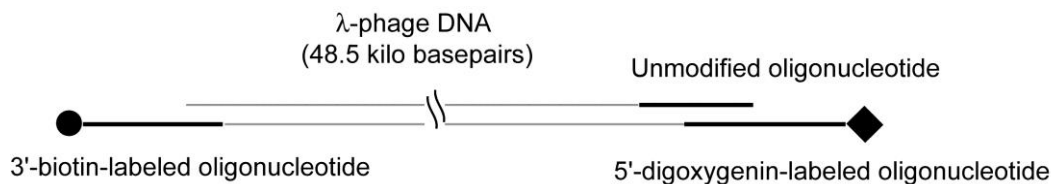

Figure s2 Arrangement of DNA oligonucleotides ligated to λ DNA. λ DNA (New England Biolabs) is orthogonally functionalized with biotin and digoxigenin through ligation with end-labeled oligonucleotides.

The biotin oligonucleotide and linker oligonucleotide are phosphorylated by T4 polynucleotide kinase for 2 hours at 37 °C. The λ DNA and biotin oligonucleotide are mixed at a 1:16 molar ratio in T4 DNA ligase buffer. These oligonucleotides are specifically annealed to λ DNA by cooling the mixture slowly from 60 ˚C to 30 ˚C, followed by a 3-hour ligation with T4 DNA ligase at 16 ˚C. Linker oligonucleotide and digoxigenin oligonucleotide (both 6-fold larger molar amount than biotin oligonucleotide) are then added to the ligation mixture. Next, the mixture is subjected to a similar slow annealing as described above and undergoes the same 3-hour ligation step. The concentration of the final DNA construct is 1 nM. Constructs are stored at –20 ˚C.

Preparation of functionalized beads

Anti-digoxigenin (Fab fragments from sheep, Roche Applied Science, Mannheim, Germany) is chemically linked to carboxylated supermagnetic beads (2.8 μm diameter, Dynabeads M-270 Carboxylic Acid carboxylate-modified; Invitrogen/Dynal Biotech, Carlsbad, CA). Here is the procedure. Perform reactions in ice from Step 4 to Step 8.

1. Take 3 mg of beads and wash with 10 mM NaOH, followed by water.
2. Dissolve 16 mg N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDAC, Sigma-Aldrich, Inc., Atlanta, GA) in 200 μL of 25 mM MES (2-(N-morpholino)ethanesulfonic acid, pH 5.0). Also, dissolve 10 mg NHS (N-Hydroxysuccinimide, Sigma-Aldrich) in 200 μl of 25 mM MES.
3. Add 100 μl of each EDAC and NHS to beads and slowly mix for 30 minutes at 22 ˚C. EDAC first reacts with the carboxyl group on the bead surface to form an amine-reactive intermediate. Next, NHS stabilizes the intermediate by forming an amide bond.
4. Wash beads with cold 25 mM MES and 100 mM carbonate buffer (pH 8.0) sequentially.
5. Add 200 μl of carbonate buffer to the washed beads.
6. Mix 0.5 mg anti-digoxigenin in 200 μl carbonate buffer and add to the beads (total volume

~400 µl)

7. Slowly mix for the coupling reaction for >2 hours at 4 ˚C.
8. Add 200 µl of 500 mM ethanolamine (pH 8.4) in order to quench free reactive groups and stop the reaction.
9. Slowly mix for 30 minutes at room temperature.
10. Wash beads with 800 µl of 'storage buffer' (50 mM phosphate (pH 7.0), 60 mM NaCl, 1.0 mg/ml BSA, 0.1 % Tween-20, 0.02 % NaN_3, 10 % glycerol, 1.0 mM EDTA). Dilute to 2 ml with the storage buffer.
11. Store beads at 4 ˚C in 50 µl aliquots. The beads are stable for several months in these storage conditions.

Preparation of biotin-PEG functionalized coverslips

Before functionalization with biotinylated polyethylene glycol (PEG), glass coverslips (24 mm x 60 mm, No.1 micro cover glass, VWR International Inc., Pittsburgh, PA) are cleaned by successive sonication in 1 M potassium hydroxide and ethanol for a total of 3 cycles each. The final ethanol sonication step is followed by brief sonication in acetone, and coverslips are incubated in 3-aminopropyl-triethoxysilane (2 % in acetone) for 5 minutes. The silanization reaction was quenched by pouring excess amount of water into the reaction mixture. Coverslips are then rinsed with copious amounts of water, and the water-rinsed coverslips are treated with 1 mg/ml biotin-PEG-NHS-5000 and 100 mg/ml mPEG-SPA-5000 (Nektar Therapeutics, Huntsville, AL) in 100 mM sodium bicarbonate buffer (pH 8.3). This reaction is allowed to proceed for at least 3 hours. Coverslips are carefully rinsed with distilled, deionized water and dried with nitrogen gas before storage in a vacuum desiccator for (up to) a few months.

Building a flow cell

Flow cells are constructed in the following manner. First, a biotin-PEG functionalized coverslip is treated with 125 µg/ml streptavidin (Sigma Aldrich, Inc.) in 50 mM Tris/Tris HCl buffer for 15 minutes. The coverslip was then rinsed in distilled, deionized water and dried with nitrogen gas. A 120-µm-thick adhesive spacer (Grace Bio Labs, Inc., Bend, OR) was cut to have a 3 mm-wide channel in the middle and used to seal the functionalized coverslip to the microscope slide into which two holes were previously drilled at each end of 3-mm-wide channel. Polyethylene tubing (inner diameter 0.76 mm, outer diameter 1.22 mm, VWR International, Inc.) was inserted into these holes for fluid delivery within the flow cell channel. The assembled flow cell is further sealed with fast curing epoxy.

After the flow cell is assembled, it is rinsed with 50 mM Tris/Tris HCl buffer (pH 8.0) containing 0.1 mg/ml BSA. Functionalized DNA constructs in the same buffer were incubated at

a concentration of 1 pM in the flow cell for an hour. If ssDNA tethers were desired, 2 pM DNA were denatured to form ssDNA (described below in the section for HIV-1 RT primer-extension assay) and incubated for 40 minutes in flow cell. Before embarking on a single molecule enzyme experiment, the flow chambers are rinsed with 50 mM Tris/Tris HCl buffer (pH 8.0), and functionalized beads solution are infused to form DNA-tethered beads.

Fluctuation-free pump

The pressure-driven pump creates stable flow based on a large pressure drop (~340 kPa or ~50 pounds per square inch (psi)) that is insensitive to changes in ambient pressure in the laboratory. A syringe pump produced periodic flow fluctuations from rotary motion to push or pull a syringe. The absence of such an operational mechanism in the pressure-driven pump helps to remove fluctuations in the flow rate.

The pressure inside the tightly sealed container is regulated with high accuracy (Type 100 precision air pressure regulator, ControlAir Inc., Amherst, NH) and can be maintained and reproduced with 0.01 % accuracy by reading a digital gauge (Omega Engineering, Stamford, CT). PEEK tubing (>3 ft, 100-175 μm diameter) is purchased from Upchurch Scientific (Oak Harbor, WA) (**Supplementary Fig. 2**).

Fluctuations in the volumetric flow rate are <1 % over hours (data not shown), and we varied the rate between 0.5 ml/h and ~40 ml/h by changing the vessel pressure via the regulator and choosing PEEK tubing of different inner diameter (100-175 μm) and length (~3 ft) based on the Hagen-Poiseuille relation $Q = \dfrac{\pi \cdot \Delta P \cdot R^4}{8 \cdot \eta \cdot L}$, where Q = volumetric flow rate, ΔP = pressure drop between container and flow cell, R = inner radius of PEEK tubing, η = viscosity of water, and L = length of PEEK tubing.

Magnet set-up

We set up a rare earth magnet (NdFeB) with a linear translation stage (Newport 460A-X, Newport Corporation, Irvine, CA) to vary the relative distance of the magnet to the sample and consequently tune the magnetic force, in the rage of 0.5-3 pN perpendicular to the surface.

When operating the assay at different flow rates, it may be useful to vary the magnetic force to minimize bead-surface interactions. Here, we present a table as a guideline to choose magnetic force to maintain DNA-tethered beads ~200 nm above the surface at various flow rates. The method how to estimate it will follow later in this **Supplementary Notes**. For example, we used a larger magnetic force for the high flow rate (8 ml/h) ϕ29 DNA polymerase experiments (**Fig. 2a**).

Q (ml/h)	F_{mag} (pN)
1.5	0.27
2	0.35
3	0.52
5	0.85
7	1.18
9	1.50
11	1.84
13	2.17
15	2.49

Table s1 Magnetic force (F_{mag}) required to maintain a vertical bead separation of ~200 nm from the coverslip surface as a function of volumetric flow rates (Q) for 2.8 micron diameter beads and for the microfluidic channels described in the previous section. The magnetic force can be varied by changing the position of the magnet from the sample

Through-objective dark-field illumination

As depicted in **Figure 1c**, a collimated laser beam (Coherent Innova Argon Laser 0.5 W at 514 nm) is first expanded to a radius (r) of 5 mm. Next, 160-cone angle axicon (Del Mar Ventures, San Diego, CA) generates collimated annulus beam. A field stop at the equivalent back focal plane cleans the interior of the annulus beam. After passing a 50:50 beam splitter, the annulus (r = 6.0 mm) focuses at the back focal plane of the objective (10x NA 0.45 Plan-Apochromat, Carl Zeiss microimaging, Inc., Thornwood, NY). Light scattered by beads is collected through the same objective and 50:50 beam splitter together with an annulus of illumination light reflected from the air-glass interface. This reflected light is eliminated by an iris in the detection path yielding a high-contrast dark-field image at the CCD.

Due to the coherent properties of laser light, an interference pattern (or speckle pattern) was observed in the background of scattered image. To mitigate the effects of the coherent laser light and to detect beads with a uniform illumination, a rotating diffuser was added to the laser beam. A piece of ground glass (grid #600, ThorLabs, Inc., Newton, NJ) was mounted on a DC-micromotor (2224U006SR, MicroMo Electronics, Inc., Clearwater, FL) and rotated at ~150 rpm. The diffuser helped to average speckle patterns within the image acquisition time and resulted in a uniform illumination in ~1 mm x 1 mm field of view.

Image acquisition and processing

The images from a CCD camera (CoolSNAP ES, 1392 x 1040 pixels, Photometrics, Tucson, AZ) are acquired with MetaVue (Molecular Devices, Downingtown, PA) software. The time trajectories are obtained by tracking bead positions using DiaTrack (Semasopht, North Epping, Australia). DiaTrack fits a 2-D Gaussian to the scattered image of beads to obtain the centroid bead position.

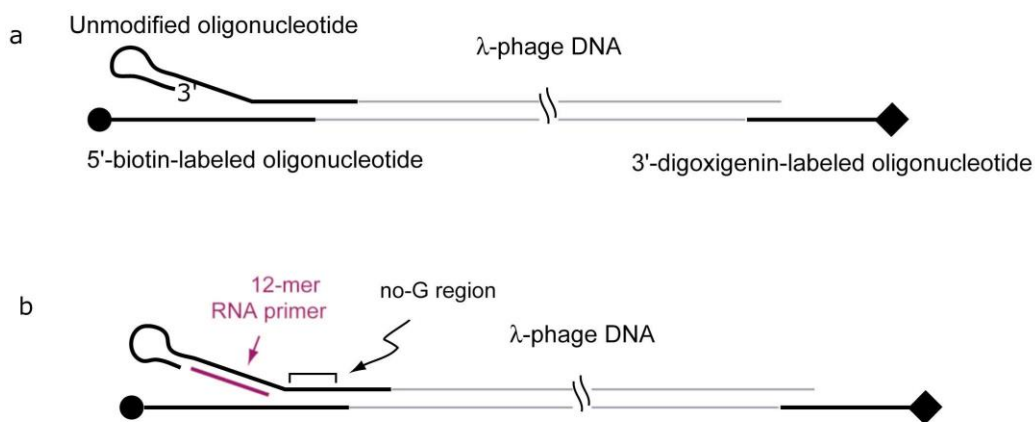**ϕ29 DNA polymerase strand displacement assay**

Figure s3 DNA construct used for (a) ϕ29 DNA polymerase strand displacement assay and (b) *E. coli* RNA polymerase transcription assay.

For strand displacement assay we used a λ DNA template that is slightly different than what was described previously (**Fig. s1**). The three oligonucleotides differ in the following way although we followed the same ligation procedure as described in the previous section.

Biotin oligonucleotide has biotin at the 5' end, and digoxigenin oligonucleotide is functionalized at the 3' end. Unmodified 'linker' oligonucleotide is hybridized on the biotin end (**Fig. s3**). This linker oligo does not hybridized completely to the biotin oligonucleotide but is partially off to create a 'fork' configuration. Also this oligonucleotide has a hairpin structure to yield a free 3'-end as a primer. Having a primer in a hairpin configuration avoids a chance of primer denaturation when the DNA is stretched at high force.

Here are sequences of the oligonucleotides, written from 5' to 3':

Biotin oligonucleotide: Biotin-AAAAAAAAAAAAAAAAAAAACAGCCATATCAGTTACACC GAATAGGAGGTGAG
Digoxigenin oligonucleotide: GGGCGGCGACCT-digoxigenin
Unmodified linker oligonucleotide (with hairpin primer): AGGTCGCCGCCCCTCACCTCCTAT TCTTCGCCGTGTCCATCGCTGTGTCGGTCGGCTACTTTCCTTTTTGGAAAGTAG

ϕ29 DNA polymerase and 10x reaction buffer were purchased from New England Biolabs. For 8 ml/h flow, 150-μm inner diameter PEEK tubing was used with 28 psi. 1.5 nM enzyme solution was used during experiment shown in **Figure 2a**.

HIV-1 reverse transcriptase (RT) primer-extension assay

We generated ssDNA template by heating end-functionalized λ-phage dsDNA molecules (**Fig. s1**) to 99 ˚C in 2 mM NaOH[2] and quickly quenching the reaction with excess amount of 4 ˚C buffer. After ssDNA tethers were formed in the flow cell, a 21-mer primer (250 nM primer and 200 μM dNTP (New England Biolabs) in the enzyme buffer) was infused to hybridize at the surface-end of DNA. The sequence of the primer is 5'-AGG TCG CCG CCC CGT AAC CTG-3' (Integrated DNA Technologies).

HIV-1 RT was purchased from Worthington Biochemical Corporation, and 50 mM Tris buffer pH 8.3 (40 mM KCl, 10 mM MgCl$_2$, 1 mM DTT, 100 μg/ml BSA) was used. 11 nM enzyme solution was infused at the flow rate of 3 ml/h during an experiment shown in **Figure 2b**. To generate this flow rate, 4-ft-long 100-μm inner diameter PEEK tubing was used and pressure was set at 50 psi in the regulator.

λ exonuclease assay

The enzyme and 10x reaction buffer were purchased from New England Biolabs. DsDNA template (**Fig. s1**) was incubated in a flow cell and was tethered to beads. 1 μM enzyme in the buffer was infused at 3 ml/h by the pressure-driven pump while the images were taken at 1 Hz. The resulting ssDNA tethers can also be used to DNA polymerase primer-extension assay.

E. coli RNA polymerase transcription assay

We used the same DNA construct described in the φ29 DNA polymerase assay section for *E. coli* transcription assay. As shown in **Figure s3b**, 12-mer RNA primer (GGACACGGCGAA; Integrated DNA Technologies) was designed to anneal on the unmodified oligonucleotide. Consequently, the DNA strand having the unmodified oligonucleotide becomes template strand for RNA transcription. By design, there is a region in the unmodified oligonucleotide where there is no G in sequence (see **Fig. s3b**). Therefore, if rCTP is omitted in the transcription reaction, the elongation complex can be stalled. We employed this feature to make a stalled complex in a test tube and to trigger synchronized transcription by adding all rNTPs with *E. coli* RNA polymerase in the flow cell.

In a test tube, 5 pM DNA construct was mixed with 10 nM RNA primer, 16 nM *E. coli* core RNA polymerase (Epicentre Biotechnologies, Madison, WI), rATP, rGTP, and rUTP (10 μM each) in a reaction buffer (40 mM Tris pH 8.0, 10 mM MgCl$_2$, 200 mM KCl, and 50 μg/ml BSA; total reaction volume 100 μl). The reaction went for 10 minutes at 37 ˚C, and it was infused into a flow cell to form tethers.

To trigger the elongation again, a flow solution was prepared with all 4 rNTPs (100 μM each),

90 pM RNA polymerase, and 5 mM DTT in the reaction buffer. This solution was infused using the pressure-driven pump at ~3 ml/h while the images of DNA-tethered beads were taken at 1 Hz.

Microscopic view of forces on DNA-tethered beads under shear flow

In this section, we provide a quantitative physical model of a flow-stretched DNA template.
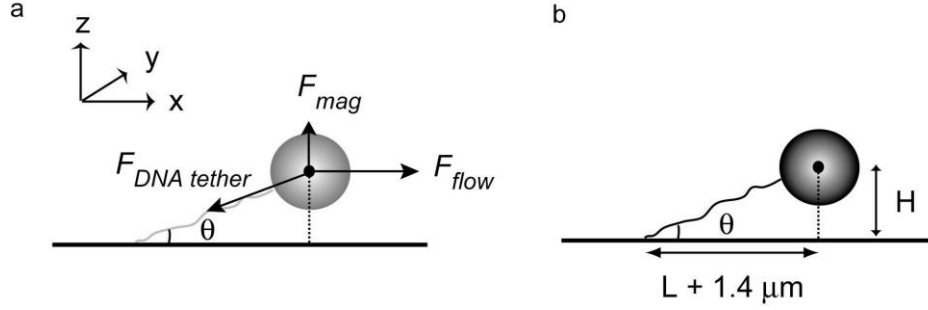

Figure s4 Quantitative physical model of a flow-stretched DNA template. (a) Schematic of a steady state force balance on a DNA-tethered bead under flow. (F_{mag} = magnetic force; $F_{DNA\ tether}$ = restoring force of DNA; F_{flow} = hydrodynamic force). (b) Schematic of the angular geometry of a flow-stretched DNA molecule. (L = projected extension length of DNA; H = vertical height of bead centroid position; the bead radius = 1.4 μm)

Figure s4a shows a steady state force balance on a DNA-tethered bead under flow. The force balance is:

$$\mathbf{F}_{mag} + \mathbf{F}_{flow} + \mathbf{F}_{tether} + \mathbf{F}_{Br} = \mathbf{0} \tag{1}$$

where F_{mag} is the magnetic force, F_{flow} is the hydrodynamic force, F_{tether} is the entropic restoring force of the DNA chain, F_{Br} is the random Brownian force and boldface denotes a vector quantity. We average Equation (1) and make use of the isotropic nature of Brownian forces such that $\langle \mathbf{F}_{Br} \rangle = \mathbf{0}$, where an ensemble average is noted by brackets $\langle \rangle$. We define the flow direction to be the x-direction and surface normal direction as the z-direction. The force balance in the flow (x) direction becomes:

$$\zeta U - \cos(\theta)\ F_{tether} = 0 \tag{2}$$

where F_{flow} is given as the product of bead resistivity ($\zeta = 6\pi\eta R$ from Stokes drag for a bead with R radius) with bulk fluid velocity (U) and θ is the angle between the DNA tether and the surface ($\cos\theta = \dfrac{x}{\sqrt{x^2 + z^2}}$, where x = projected DNA extension and z = vertical bead height from the

surface). In the vicinity of the surface, fluid velocity varies linearly with vertical distance (z) from the surface, giving $U = cz$, where c is the constant shear rate. The force balance in the surface normal (z) direction is:

$$F_{mag} - \sin(\theta)\, F_{tether} = 0 \qquad\qquad (3)$$

For double stranded DNA, we use the worm-like chain (Marko-Siggia) force relation for the entropic restoring force F_{tether}.

1. Equations (2) and (3) may be solved to calculate bead "trajectories" – that is, steady state bead (x,z) positions as functions of magnetic and flow forces. Using Equations (2) and (3) and the measured DNA extension (or, equivalently, the flow force) and the magnetic force, we can estimate the vertical bead separation to be ~250 nm at typical experimental conditions with a magnetic force of 0.6 pN and flow rate of 3.0 ml/h (corresponding to flow force of 3.7 pN).

2. Equations (2) and (3) may also be solved to estimate F_{mag} for a given flow rate in order to estimate the magnetic force required to maintain DNA-tethered beads ~200 nm above the surface at various flow rates. Results are presented in **Table s1**. A larger magnetic force is generally required for higher flow rates to oppose the increased vertical component of F_{tether}, which tends to push the bead to the surface.

3. **Figure s4b** shows the angular geometry of a flow-stretched DNA molecule. Experimentally, we measure projected DNA extension, not actual DNA tether length. Here, we justify by geometrical arguments that projected DNA extension is a quantitative measure of actual DNA extension within a small error. Therefore, we verify that a correction is not required for the measured projected DNA lengths. In the low stretching force region, e.g. 3 ml/h, the rate for most of our enzyme experiments, L (projected extension) is 4.4 μm for ssDNA and 15.1 μm for dsDNA. Defining the vertical height of bead center (H) to be the bead radius plus 250 nm, the error due to projection, $(1 - \cos\theta) \times 100\%$, is 3.8 % for the ssDNA (max) and 0.5 % for the dsDNA. Additionally, the force extension curves for ssDNA and dsDNA, which are our basis for estimating the enzymatic conversion of DNA, are also based on the same geometrical consideration, and we are interested in the change in the bead position during enzyme experiments rather than the actual DNA extension length. Therefore, the projection does not yield a significant error in our results.

Brownian motion of DNA-tethered bead

Power spectral densities (PSDs) of Brownian fluctuations (in units of displacement^2/Hz) can be fit with a Lorentzian function with a corner frequency, $f_c = \dfrac{\kappa}{2\pi\gamma}$, where κ is the stiffness of the force clamp and γ is the Stokes drag coefficient of the bead. Using water viscosity (η) and 2.8 µm bead diameter (d) we estimate γ (= $3\pi\eta d$), and κ is given by the derivative of force extension curve of dsDNA (= $\partial F/\partial x$), yielding $\kappa = 0.04$ pN/nm at 3 ml/h. The above formula yields a corner frequency of 240 Hz for longitudinal Brownian fluctuation of dsDNA tethers under 3 ml/h flow.

Centroid determination error

We used the following equation to estimate centroid determination error:

$$\sigma_x = \sqrt{\frac{s_x^{\,2}}{N} + \frac{a^2}{12N} + \frac{8\pi s_x^{\,4} b^2}{a^2 N^2}}$$

where N is the number of photons, a is the pixel size, b is the standard deviation of the background, and s_x is the standard deviation of the point spread function[3,4]. For a 2.8 µm diameter bead that are unspecifically stuck on the surface, we experimentally measured $N = 230{,}000$ photons, $a = 2.06$ µm, $b = 130$, and $s_x = 1.35$ µm in the through-objective dark-field illumination mode of operation with a 10x NA 0.45 objective. Hence, the error in centroid determination is ~4 nm.

REFERENCES

1. van Oijen, A.M. *et al. Science* **301,** 1235-1238 (2003).
2. Maier, B., Bensimon, D. & Croquette, V. *PNAS* **97**, 12002-12007 (2000).
3. Thompson, R.E., Larson, D.R. & Webb, W.W. *Biophys. J.* **82**, 2775-2783 (2002).
4. Yildiz, A. *et al. Science* **300**, 2061-2065 (2003).

Supplementary Figure 1
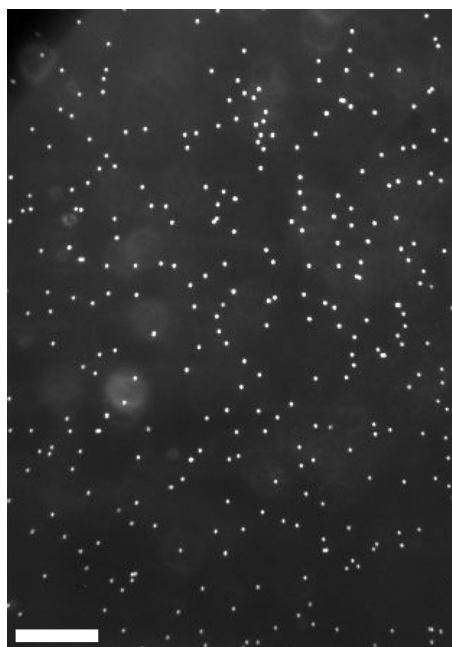
Typical dark-field microscope image of ~180 DNA-tethered beads in 0.6 x 0.8 mm^2 field of view. The image was acquired using CCD camera (frame rate 1 Hz, pixel size 1.87 μm) as described in the Image Processing section of **Supplementary Notes**. The scale bar indicates 100 μm.

Supplementary Figure 2
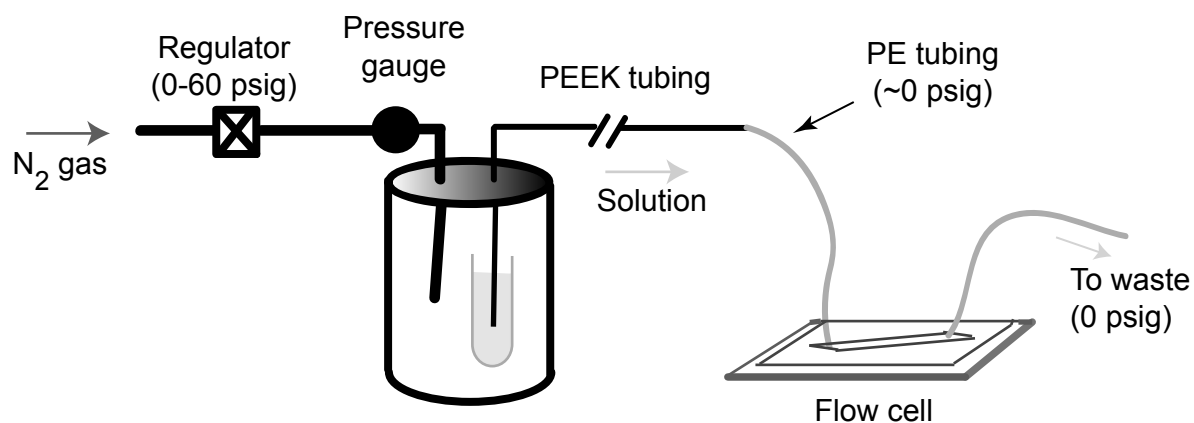
Schematic of a pressure-driven pump (pressures specified as gauge pressures in pounds per square inch (psig = psi (absolute) - atmospheric pressure); PEEK, polyetheretherketone; PE, polyethylene). Nitrogen gas pressurizes a tightly sealed vessel that contains enzyme solution. The enzyme solution is pushed through >3-ft-long PEEK tubing to the flow cell. The pressure drop in the PEEK tubing is ~50 psi (i.e. ~340 kPa). See **Supplementary Notes** for more details.