Multiplexed single-molecule assay for enzymatic activity on flow-stretched DNA

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We report a single-molecule assay for nucleic-acid enzymes on flow-stretched DNA templates. To facilitate the detection of slow or intermittent enzymatic activities, we developed the assay with 15-nm spatial resolution at a frame rate of 1 Hz and ~10 nm mechanical stability over the timescale of hours. With multiplexed data collection, we applied the assay to ϕ29 DNA polymerase, HIV-1 reverse transcriptase, λ exonuclease and *Escherichia coli* RNA polymerase.

The study of protein-DNA interactions and processive nucleic-acid enzymes *in vitro* has been greatly facilitated by a variety of single-molecule techniques for manipulating individual DNA molecules (see ref. 1 for review). The use of hydrodynamic flow to stretch DNA molecules in a microchannel is a relatively simple approach that is easily implemented without specialized equipment[2] and allows for multiplexed data collection from uniform manipulation of many DNA molecules spread across a wide area (~1 mm^2; ref. 3). Parallelization of single-molecule measurements reduces the number of experiments required to accrue enough data to draw statistically sound conclusions, and is advantageous when a low-efficiency step is involved in experimental preparation (for example, formation of DNA tethers and durability of single-stranded DNA tethers at high stretching force) or actual biological processes under investigation (for example, assembly of multi-protein complexes). A multiplexed magnetic tweezing technique has been previously demonstrated with spatial resolution of ~500 nm (ref. 4). Here we report a multiplexed flow-stretching technique that has high spatial resolution (~10 nm) with stable long-term mechanical properties and minimal flow fluctuations. We demonstrate high spatial accuracy for detection of several nucleic-acid enzymes, including ϕ29 DNA polymerase, HIV-1 reverse transcriptase, λ exonuclease and *E. coli* RNA polymerase.

The DNA extension assay for nucleic-acid enzyme activity is based on the intrinsic difference in elasticity between single-stranded and double-stranded DNA under applied tension[5], or on the translocase activity of the enzyme[6]. At stretching forces below 6 pN, ssDNA is more compact than its rigid dsDNA counterpart because of secondary-structure formation and a shorter persistence length. At high stretching forces, however, ssDNA exhibits larger extension than dsDNA because of its longer contour length for an equivalent number of bases (**Fig. 1a** for λ DNA of 48.5 kilobases). Therefore, enzymatic conversion between ssDNA and dsDNA can be followed in real time by monitoring the extension of tethered DNA molecules at a constant stretching force as the instantaneous extension of DNA corresponds to a

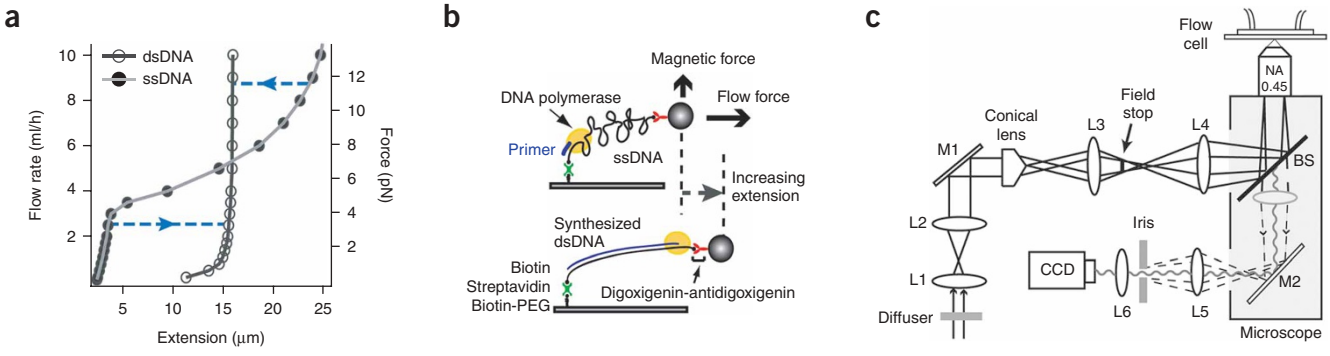

Figure 1 | Single-molecule assay for nucleic-acid enzymes using flow-stretched DNA templates. (**a**) Force-extension curve of λ-phage ssDNA and dsDNA measured in the HIV-1 reverse transcriptase reaction buffer. The dsDNA data were fitted with the wormlike chain model[5]; the ssDNA data are shown with a smoothed curve. Arrows illustrate DNA extension or shortening as a result of DNA polymerization at a low or high stretching force, respectively. (**b**) Schematic for DNA extension owing to primer-extension activity of a polymerase at a low stretching force (not to scale). (**c**) Optics for mechanically stable through-objective darkfield illumination (L1-L6 optical lenses; M1-M2 mirrors; BS 50:50 beam splitter). Field stop and iris are positioned at the equivalent back focal plane. The reflected light and scattered light in the detection pathway are shown as dashed and curved lines, respectively.

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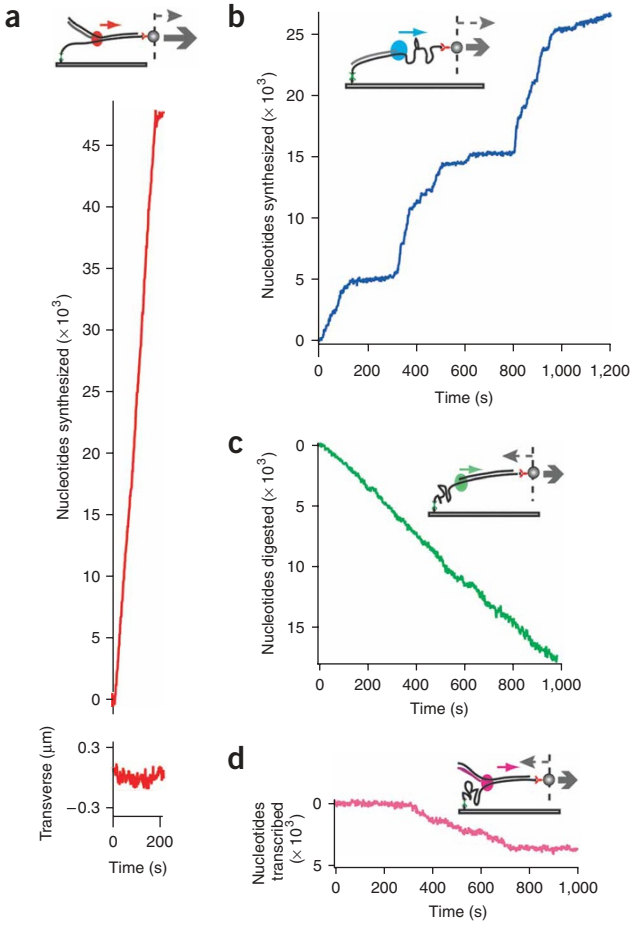

Figure 2 | Single-molecule traces obtained with the low-noise assay. (**a**) Strand displacement by ϕ29 DNA polymerase at a stretching force of 12 pN. The transverse displacement trace confirms that the DNA-tethered bead exhibits Brownian motion and does not interact with the surface during the polymerization event. (**b**) Primer extension by HIV-1 reverse transcriptase at a stretching force of 3.7 pN. (**c**) λ exonuclease activity at 3.7 pN. (**d**) Transcription by *E. coli* RNA polymerase at 3.7 pN. The RNA transcript is shown in purple. Trajectories from beads moving against the flow direction are illustrated in the downward direction. Raw experimental traces are shown in all cases. Dotted arrows show bead movement, and the solid gray arrows show hydrodynamic force.

unique number of single-stranded and double-stranded bases in the DNA tether.

For the hydrodynamic flow-stretching assay, we orthogonally functionalized two ends of λ-phage DNA molecules with biotin and digoxigenin, which allowed us to tether the DNA termini to the streptavidin-coated surface of a flow cell and 2.8-μm magnetic beads functionalized with anti-digoxigenin, respectively (**Fig. 1b** and **Supplementary Note** online). We applied hydrodynamic flow to the microchannel to uniformly stretch template DNA molecules by the frictional force exerted by the fluid on the magnetic beads. To minimize nonspecific interactions between the beads and the surface, we placed above the sample a small rare earth magnet (NdFeB), which exerts 0.5–3 pN perpendicular to the surface. We obtained images of >100 DNA-tethered beads formed by scattered light in a darkfield microscope with a high-resolution charge-coupled device (CCD) camera at 0.5–2 Hz (**Supplementary Fig. 1** online) and generated spatial trajectories of the beads by Gaussian centroid determination. The mean-square displacement in the transverse direction, $\langle \delta y^2 \rangle$, is separately measured at 100 Hz to calibrate the stretching force F, based on the equipartition theorem, $F = k_B T l / \langle \delta y^2 \rangle$, where k_B is the Boltzmann constant, T is the absolute temperature and l is the length of DNA molecule[7]. We performed all the experiments at 22 °C.

Previous versions of flow-stretching assay exhibited long-term instability owing to flow fluctuations and mechanical drifts. We removed the contribution of these drifts in enzyme activity traces by subtracting control traces from those for bead-tethered DNA molecules that did not undergo enzymatic conversion[3]. The correction, however, is imperfect, and control traces are not available for every experiment type. Therefore, our goal was to develop an assay with minimal flow and mechanical drift rather than to rely on the subtraction method.

The slow fluctuations in the flow rate arose from a syringe pump functioning in series with an air buffer, which was sensitive to changes in ambient pressure in the laboratory. We designed a fluctuation-free flow source driven by high pressure. Pressurized nitrogen gas (regulated to 0.01%) pushes enzyme solution from a tightly sealed container to the flow cell through polyetheretherketone (PEEK) tubing. The pressure drop in the PEEK tubing is relatively large (~344 kPa) so that the volumetric flow rate becomes insensitive to changes in ambient pressure at the flow cell outlet (**Supplementary Note** and **Supplementary Fig. 2** online).

The original assay was susceptible to mechanical drift of a flow cell on the stage owing to loose mounting by flexible stage clips and movement of the nosepiece in our inverted microscope, resulting in substantial defocusing of bead images over a timescale of hours. We partially reduced the long-timescale mechanical drift by mounting the flow cell with double-stick tape onto a nosepiece-mounted stage (Olympus IX2-NPS), which fixed the relative distance between the microscope stage and the objective.

Compared with other wide-field microscopy techniques, darkfield microscopy provides a uniformly illuminated field of view for magnetic beads, is simple to implement and yields high-contrast, centro-symmetric bead images for precise Gaussian centroid determination. A traditional darkfield microscope setup, however, introduces several sources of mechanical drift. The configurable illumination equipment on a commercial microscope is generally not as mechanically stable as optics fixed on an optical table, and its movement relative to the sample and detection path translates into shifts of the illumination beam, yielding drift of the bead images on the CCD camera. The combination of these sources of drift resulted in micrometer-scale mechanical drift over a timescale of hours.

We achieved a major improvement in the image drift by replacing traditional darkfield illumination with through-objective darkfield illumination[8]. Here the objective lens functions as a condenser, and the darkfield effect is created by (i) sample illumination by an annulus-shaped laser beam and (ii) elimination of the reflected annulus light from the scattered light by an iris diaphragm in the detection path (**Fig. 1c** and **Supplementary Note**). This new geometry provides precise alignment of optics and superior stability compared to a commercial epi-darkfield apparatus.

The combination of these improvements yielded ~10 nm of mechanical drift on the timescale of hours. Accuracy for determining DNA-tethered bead motion along the flow direction was 15 nm at the frame rate of 1 Hz. A high-numerical-aperture objective

helped to reduce centroid determination error for fixed beads to <5 nm. The magnitude of Brownian fluctuations of λ DNA–tethered beads under 3 ml/h flow rate is ~10 nm in flow direction, estimated by the equipartition theorem $\langle \Delta z^2 \rangle = k_B T / (dF/dz)$, where dF/dz is the slope in the force-extension curve[7]. Such Brownian fluctuations occur at a characteristic corner frequency of 240 Hz (**Supplementary Note**). Therefore, the frame rate of 1 Hz during our enzyme experiment is sufficiently slow for the bead to fully sample its harmonic potential well in the longitudinal direction, and equilibrated position of the bead is measured. In general, with increasing illumination power, the accuracy of determining DNA extension under the same experimental conditions also increases as more photons are collected and error in centroid determination decreases. Finally, this new illumination scheme frees the space above the flow cell and simplifies positioning of a magnet and a temperature-controlling device over the flow cell. Temperature control of the flow cell is eased with respect to the old arrangement by elimination of an immersion-type objective, which acted as a powerful heat sink.

We tested the performance of the low-noise, high-stability assay using ϕ29 bacteriophage DNA polymerase, which has not yet been assayed by single-molecule techniques[9,10]. The dsDNA molecule lengthens at 12 pN upon conversion to ssDNA by strand-displacement activity of ϕ29 DNA polymerase (**Fig. 2a** and **Supplementary Note**). ϕ29 DNA polymerase polymerizes full-length λ DNA within a few minutes, a timescale in which slow, large-amplitude drifts of the previous flow setup were not significant.

The benefits of the improved stability and accuracy are evident for slower and less processive DNA polymerases, such as HIV-1 reverse transcriptase, which performs DNA-dependent DNA polymerization with low processivity (~1–300 nucleotides depending on the template sequence) at a rate of ~30 bases/s at 37 °C (ref. 11). Owing to its weak polymerization activity, no single-molecule experiments have yet been attempted using ssDNA templates as long as the HIV viral genome (9.7 kilobases). When the previous flow setup was used to assay primer extension activity of HIV-1 reverse transcriptase on λ DNA, the enzymatic conversion of DNA tethers was indistinguishable from the long-term drifts of the setup. However, HIV-1 reverse transcriptase polymerization activity is easily revealed with the new assay (**Fig. 2b**). The average rate of polymerization is ~30 bases/s at a stretching force of 3.7 pN, and enzyme dissociation or pausing events are visible as plateaus in the raw trace. The improved stability provides sufficient spatial precision and time resolution to locate major pause sites without filtering the time traces.

Application of the assay is not restricted to DNA polymerases. We observed DNA tethers shorten at 3.7 pN as λ exonuclease processively degrades one strand of dsDNA in the 5′ to 3′ direction (**Fig. 2c**). Furthermore, we assayed transcription elongation by *E. coli* RNA polymerase by allowing the enzyme to elongate a 12-mer RNA primer hybridized to the template strand of dsDNA. The polymerase transcribes from the 3′ terminus of the primer in a strand-displacement manner producing an RNA-DNA heteroduplex and ejects the nontemplate strand of the dsDNA as ssDNA[12], yielding DNA tether shortening at 3.7 pN (**Fig. 2d** and **Supplementary Note**). The transcription elongation rate was 10–15 bases/s, and the efficiency of enzymatically active tethers was relatively low compared to that in DNA polymerase experiments (only 3 active traces from ~100 DNA tethers in one experiment). Therefore, the reduced noise and multiplexing capability in the assay were crucial to observation of *E. coli* RNA polymerase activity in the flow-stretching assay.

DNA translocases may also be studied using the flow-stretching assay if the enzyme is anchored to the surface of flow cell and only one end of DNA is functionalized for bead attachment. In this arrangement, the DNA-tethered bead would show unidirectional movement as the enzyme translocates the DNA molecule. RNA or DNA hairpins can be designed in the DNA template to study the enzymatic action of helicases. Finally, DNA-binding proteins that trigger a change in DNA length by >10 nm upon binding may be studied at the single-binding event level. For example, DNA bending upon the specific binding of a restriction endonuclease may be monitored in real time at the single-molecule level.

In summary, we developed a flow-based single-molecule assay with high long-term stability while maintaining massive multiplexing and 15-nm spatial resolution. We demonstrate that this technique is easily implemented and is suitable for high-accuracy single-molecule measurement of nucleic-acid enzymes. We anticipate that the assay will be generally useful to study enzymatic activity over long time periods and DNA-binding proteins on single DNA templates.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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