Materials and Methods

Preparation of DNA construct. To specifically couple one end of a single λ phage DNA molecule (48,502 base pairs) to the glass surface of a flow cell and the other end to a polystyrene bead, we functionalized (as shown in Figure S1A) the ends of λ phage DNA (New England Biolabs, Beverly, MA) with biotin and anti-digoxigenin using three oligonucleotides (Oligos Etc. Inc., Wilsonville, OR): A 3' biotin-modified oligo (‘Oligo 1’; 5'-Agg TCg CCg CCC AAA AAA AAA AAA AAA AAA AA-biotin-3’), a 5' digoxigenin-modified oligo (‘Oligo3’; 5'-digoxigenin-AA AAA AAA AAA AAA Atg gTC CAA CTT gCT gTC C-3’), and an unmodified oligo (‘Oligo 3’; 5'-ggg Cgg CgA CCT ggA CAg CAA gTT ggA CCA-3’). The unmodified oligo allows the attachment of biotin and digoxigenin to opposite ends of the same strand. Removal of the complementary strand yields a single-stranded template of 48,555 base pairs.

The oligonucleotides and the λ phage DNA are first phosphorylated by means of T4 polynucleotide kinase (New England Biolabs). The λ phage DNA (10⁻⁸ M, 50 µL) and Oligo 1 (10⁻⁷ M) are hybridized (cooled from 55 °C over 1 h) and then ligated (1 Weiss Unit of T4 ligase, New England Biolabs, 16 °C, 2 h). The resulting construct is hybridized (as before) with Oligos 2 and 3 (both 10⁻⁶ M) and ligated (as before). The construct can be stored at 4 °C for several weeks. The presence of T4 ligase in the solution (1 Weiss Unit) minimizes nicking of the DNA.

Binding of antibodies to beads. Anti-digoxigenin (Fab fragments from sheep, Roche Molecular Biochemicals, Mannheim, Germany) is covalently bound to fluorescent beads
(2 µm diameter, absorption 535 nm, emission 575 nm, carboxylate-modified; Molecular Probes, Eugene, OR) by means of a cross-linking agent (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Molecular Probes) that is reactive towards both the carboxyl groups on the bead and the amine groups on the antibody. First, the beads are activated by washing them extensively in 50 mM MES (2-(N-morpholino)ethanesulfonic acid, pH 6.0). After reacting with the carbodiimide (1:1 weight ratio with beads, 15 minutes) the beads are washed in 0.2 M borate buffer (pH 8.5) and incubated for 4 hours with anti-digoxigenin (3 times excess for monolayer). Finally, the reaction is quenched by washing the beads once with 50 mM Tris (pH 8.0). The antibody-coated beads are then washed with and stored in PBS (pH 7.4) with 0.5% BSA and 0.1% Tween-20.

**Preparation of flow cells.** After thorough cleaning with acetone, ethanol and 1 M sodium hydroxide, 170 µm-thick (#1) borosilicate microscope slips are amine-functionalized by treatment with 3-aminopropyl-triethoxysilane (2% in dry acetone for 2 minutes). After rinsing with demineralized water and curing at 110°C for 30 minutes, the slips are incubated for 4 hours at pH 8.2 with a solution of amine-reactive poly(ethylene glycol) (100 mg/ml mPEG-SPA (MW = 5,000, Shearwater Corporation, Huntsville, AL) and 1 mg/ml biotin-PEG-CO2NHS (MW = 3,400, Shearwater Corporation) in 100 mM NaHCO₃). The biotinylated slips are then treated with 0.2 mg/ml streptavidin (Sigma, St. Louis, MO) for 30 minutes to provide binding sites for the biotin-modified DNA.

Flow cells were prepared by carving out a 2 mm-wide channel in 100 µm thick adhesive spacers (Grace Bio Labs, Inc., Bend, OR) that are sandwiched between the functionalized cover slip and a microscope slide (Figure S1C). The slide has two holes which accommodate the polyethylene tubing used for delivery of solutions. The
completed flow cell is infused with blocking buffer (10 mM Tris (pH 8.0), 2 mM EDTA, 10 mM NaCl, 0.1% Tween-20, 3 mM NaN₃, 1.0 mg/ml BSA, 1.0 mg/ml tRNA) and left for 1 hour to block nonspecific interaction sites on the flow cell’s inner surfaces. Subsequently, the cell is incubated for 30 minutes with the biotin- and digoxigenin-modified DNA construct at a concentration of $10^{-12}$ M in 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 10% blocking buffer. Finally, the anti-digoxigenin-modified beads are infused at a concentration of $10^{-12}$ M (in PBS buffer (pH 7.4) with 10% blocking buffer) and allowed to link with the digoxigenin-modified ends of the DNA over a period of about two hours. At this point, the unbound beads can be flushed out of the flow cell with λ exonuclease buffer (67 mM glycine, 2.5 mM MgCl₂, 50 μg/ml BSA, pH 9.4) at a flow speed of 1 mL/hour. A continuous and laminar flow, necessary for the high spatial resolution desired for the single-molecule experiments was provided by means of an automated syringe pump (Harvard Apparatus, PhD 2000, Holliston, MA). The irregularities in the flow rate caused by the syringe pump were removed by a buffering compression vessel between the pump and the flow cell (Figure S1C).

**Optical microscopy.** An inverted fluorescence microscope (Olympus IX70, Olympus America Inc., Melville, NY) was used for wide-field imaging of fluorescent polystyrene beads attached to the individual DNA strands (Figure S1C). The output of a continuous-wave, diode-pumped, frequency-doubled Nd:YAG laser (BWT-50-E; wavelength 532 nm, maximum output 50 mW; B&W Tek Inc., Newark, DE) is focused into the back aperture of the microscope objective (PlanApo; N.A.=1.45; Olympus) to create a collimated beam, illuminating a circular area with a diameter of 150 μm at the focal plane. The fluorescence of the beads is collected by the same objective and, after passing
appropriate filters to remove scattered laser light (dichroic mirror 540DCLP, emission filter D570/60; Chroma Technology Corp., Brattleboro, VT), focused onto an intensified CCD camera (Pentamax; Roper Scientific, Trenton, NJ).

It is important to note that the experimental results described in this study could easily have been obtained with a low numerical-aperture objective and a moderately sensitive video-rate CCD. The aforementioned apparatus is chosen to also allow for the simultaneous observation of the fluorescence of individual chromophores, which requires higher sensitivity.

\( \lambda \) *exonuclease assay*. The ratio of the number of DNA molecules immobilized in the flow cell to the number of beads is optimized such that beads are either bound to one DNA or to none at all. To ensure that beads are tethered to single DNA molecules, we compared the flow-extension data with calculated force-extension curves for one and multiple DNA molecules obtained with the Worm-Like Chain model. The amplitude of the transversal (perpendicular to the flow, parallel to the surface) Brownian noise provided a means to determine the stretching force \( F \). According to the equipartition theorem, \( F \) is related to the bead’s mean-square displacements \( \langle \Delta x^2 \rangle \) perpendicular to and \( \langle \Delta z^2 \rangle \) along the stretched DNA) by \( \langle \Delta x^2 \rangle = k_B T l / F \) and \( \langle \Delta z^2 \rangle = k_B T (\partial F / \partial z) \) where \( k_B \) is the Boltzman constant, \( T \) is the temperature, and \( l \) is the length of the DNA (1). By simultaneously monitoring DNAs that did not undergo digestion, artifacts such as irregularities in the flow speed could be detected and such trajectories discarded. As a further control, the stability of the setup and experimental conditions was verified by monitoring the bead positions for both ds and ssDNA, excluding any artifacts that are only apparent for one of the two types of DNA. Nonspecific interactions of the bead with
the surface of the flow cell could lead to artifacts that can appear as fluctuations in the digestion rate. These ‘sticky’ beads, however, were clearly recognizable by sudden reductions of amplitudes of their transversal motion. Figure S2 shows the Brownian noise in transversal bead position (i.e., perpendicular to the flow) for a ‘sticky’ bead and for a ‘non-sticky’ one. All the traces used for the final analyses in our study have a variance consistent with the free Brownian motion of a particle in a harmonic well, $<\Delta x^2> = k_BTl/F$. As is clearly visible in the lower trace, the variance decreases as the DNA shortens during the digestion process. Sticky behavior of the beads is clearly recognizable as a large, sudden decrease of the variance of the signal (top trace). We did not observe events where the standard deviation of the signal was decreased by less than a factor of three, indicating that all sticky behavior gives rise to an easily detectable change in the transversal noise.

Nonspecific interactions of the $\lambda$ exonuclease with the surface could also lead to apparent fluctuations in the turnover rate. The functionalization of the flow cell surface with PEG minimizes these nonspecific interactions as is confirmed by incubating the flowcell with fluorescently labeled proteins and monitoring the fraction of nonspecifically immobilized protein. Nonspecific enzyme-surface interaction is further minimized by the treatment of the flowcell with blocking buffer.

To observe the enzymatic activity of individual $\lambda$ exonuclease enzymes, a 500 nM (trimer concentration) solution of $E. coli$ $\lambda$ exonuclease (New England Biolabs; homogeneity verified by SDS-PAGE) in $\lambda$ exonuclease buffer was introduced into the flow cell. To measure enzyme processivity, the solution was replaced by a similar buffer,
lacking only the $\lambda$-exonuclease, after enzymatic digestion of the DNA was observed to begin.

**Data Analysis.** Wide-field fluorescence images typically showing about ten tethered beads were acquired at a frame rate of 1 Hz or 2 Hz (see Movie S1). The positions of the beads were obtained by fitting their images with a 2D Gaussian, a process with a precision of $\sim$1 nm, much smaller than the experimental uncertainty introduced by Brownian motion, flow irregularities and mechanical drift.

The free energy variations as a function of template position were determined by, as a function of template position $n$, summing the free energy of hydrogen bonding of base $n$ in the $\lambda$ phage sequence (GenBank code NC_001416) and the free energy of base stacking between $n$ and $n+1$. The hydrogen-bonding component was obtained by simply multiplying the free energy of a single hydrogen bond (0.25 kcal/mol, from (2)) by two for every AT pair and by three for every CG pair. The stacking component for every possible neighboring base combination is obtained from (3). Note that the stacking free energy is dependent on the directionality of the strand. For example, melting of the 5' guanine in Figure 3A involves breaking the hydrogen bonding with the complementary cytosine, associated with a free energy change of 0.75 kcal/mol, and disrupting the base stacking with the neighboring adenine, with a free energy change of 0.62 kcal/mol.

Assuming base melting is the rate-limiting step, the digestion rate $k$ at every template position $n$ can be expressed as $k(n) = A \cdot \exp(-\Delta G^\lambda_{melt}(n)/k_BT)$, where $\Delta G^\lambda_{melt}(n)$ is the sequence-dependent activation barrier for the rate-limiting step (see Figure S3), the melting of the 5' base from the DNA. For small changes in $\Delta G^\lambda_{melt}$, the concomitant
change in $k$ is $\frac{\Delta k(n)}{k(n)} \approx -\frac{1}{k_B T} \cdot \Delta \Delta G^\ddagger_{\text{melt}}(n)$, where $\Delta \Delta G^\ddagger_{\text{melt}}(n)$ is the fluctuation of $\Delta G^\ddagger_{\text{melt}}(n)$ around its mean. $\Delta \Delta G^\ddagger_{\text{melt}}(n)$ is in general linearly related to the fluctuation of the free energy of the reaction, $\Delta \Delta G^0_{\text{melt}}(n)$, with a proportionality constant $\beta$, the Brønsted value (4). It follows that $\frac{\Delta k(n)}{k(n)} \approx -\frac{1}{k_B T} \cdot \beta \cdot \Delta \Delta G^0_{\text{melt}}(n)$, linking the known thermodynamic properties of DNA to the rate fluctuations along the DNA.

**Supporting data**

The sequence-dependent digestion rate is also related to processivity. Figure S4B shows the spatial distribution of dissociation events on λ DNA. Since the enzyme-containing solution had to be replaced by an enzyme-free buffer after the onset of digestion to accurately determine the processivity, digestion events of less than 5,000 nucleotides were not considered here. The nonrandom distribution rules out nicks in the DNA as the cause of dissociation. Comparison with the averaged experimental rate trace (Figure S4A) shows that dissociations take place at positions where the digestion rate is low and the residence time is consequently long. In addition, we note that the majority of the observed dissociation events occur at template positions between 10,000 and 15,000 bp, the region in λ DNA containing the highest concentration of $\chi$-like sites, which are known hotspots for λ phage recombination (5).
Captions

Figure S1. A) Attachment of λ DNA to the glass surface and a polymer bead. B) Flow cell. C) Schematic of entire apparatus.

Figure S2. Brownian noise in transversal bead position (i.e., perpendicular to the flow) for a ‘sticky’ bead and for a ‘non-sticky’ one. As is clearly visible for the lower trace, the variance decreases as the DNA shortens during the digestion process, in accordance with the linear dependence of the variance of the Brownian motion on the tether length. Sticky behavior of the beads is clearly recognizable as a large, sudden decrease of the amplitudes (top trace, see text for details).

Figure S3. Free energy diagram for the melting and hydrolysis of a base, defining the symbols used in the text.

Figure S4 A) Average of experimentally observed rate traces $\Delta k/k$ along the DNA of four full-length trajectories (from Figure 3C of this report). B) Histogram of the average digestion rates of the first 5,000 nucleotides for 44 λ exonuclease molecules. Comparison with the averaged digestion rate (Figure S4A) illustrates the correlation of dissociation probability with digestion rate.

Movie S1. Multiplexed display of movement of tethered beads as a result of enzymatic conversion of their DNA tether from double-stranded to single-stranded DNA. Of the
five beads shown, one shows enzymatic activity directly after introducing the enzyme (start of movie). After ~ 600 seconds (one-third of the movie), the enzymatic activity terminates due to dissociation of the λ exonuclease from the DNA. At this point, enzyme is again introduced into the flow cell, after which three beads show movement. Only every 20th acquired frame is shown. The image size corresponds to ~ 40 x ~ 30 µm^2; the total time displayed in the movie is 2000 seconds.

References

Figure S1

A

λ-phage DNA (48,502 basepairs) ~16 μm length

biotin

oligo 1

oligo 2

oligo 3

digoxigenin

B

C

flow cell with DNA

syringe pump

545 NA

enzyme

dichroic

545 DCLP

intensified

CCD camera

DPSS laser 532 nm CW
Figure S2
Figure S3
Figure S4

![Graph A](image1)

Rate change Δk/k

![Graph B](image2)

# of occurrences

Template position (nt)