Supporting Information for

Tracking a Molecular Motor with a Nanoscale Optical Encoder

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Supporting Methods

Buffers and Reagents. All solutions were prepared with distilled deionized water in order to minimize fluorescence background.

Protein labeling (see below) was carried out in a buffer consisting of 0.15 M sodium bicarbonate and 1 mM sodium azide, adjusted to pH 8.3 with HCl ("reaction buffer"). Labeled DnaB helicase was stored in 20% w/v glycerol, 20 mM Tris, and 500 μM EDTA, adjusted to pH 7.5 with HCl ("storage buffer").

DNA was stored in 10 mM Tris-HCl (pH 8.0) with 1 mM EDTA ("TE buffer").

The buffer used for DnaB helicase measurements ("helicase buffer") was 50 mM Tris adjusted to pH 7.9 at room temperature with HCl, 10 mM magnesium acetate, 0.1 mg/ml bovine serum albumin (Sigma-Aldrich B4287), 100 μM EDTA (Sigma-Aldrich E7889),[1] and 0.01% w/v Pluronic F127 surfactant (Sigma-Aldrich P2443).

An enzymatic oxygen-scavenging system, consisting of 35 μg/ml catalase (Sigma-Aldrich C1345), 0.2 mg/ml glucose oxidase (Sigma-Aldrich G7141), 4.5 mg/ml β-D-glucose (Sigma-Aldrich G8270), and 2 mM Trolox antioxidant (Sigma-Aldrich 238813),[2] was used during fluorescence measurements.

The flow cell passivation solution was TE buffer containing 1% w/v Pluronic F127.

DNA Sequences. All unlabeled single-stranded DNA sequences were purchased from Integrated DNA Technologies (Coralville, IA), as was the Cy5 version of the labeled "Z" oligonucleotide (see main text Fig. 1A and Supporting Table 1). The Z sequence containing Alexa Fluor 594 was purchased from IBA Biologics GmbH (St. Louis, MO).

FRET Encoder Synthesis. FRET encoders were designed to self-assemble from a set of unique single-stranded oligonucleotide components (Supporting Table 1), which were combined in TE buffer at an equal molar ratio, except for Z, which was added at 40× or 50× molar excess for the four- or five-period encoders, respectively. The component mixture was heated to 75 °C for 10 minutes and then cooled over approximately 1 hour to 37 °C. The temperature was held at 37 °C for three days to allow the encoders to fully anneal. Single-stranded nicks between oligomers were ligated with T4 DNA ligase (New England Biolabs M0202S) according to manufacturer instructions. Encoders were purified on a 1.6% w/v agarose gel and ligated to biotinylated λ phage genomic DNA molecules (New England Bio-

labs N3011S). The full-length product was finally purified with a spin column (Invitrogen K3100-01) according to manufacturer instructions. Proper self-assembly of the encoders was verified by gel electrophoresis (Supporting Fig. 1) and by photobleaching assays (main text
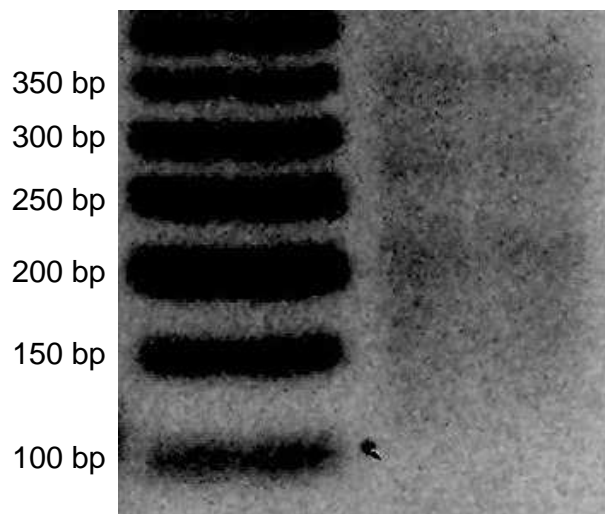
Supporting Figure 1. Gel electrophoresis separation of a 50 bp ladder (left lane, New England Biolabs N3236S) and the products of a four-period FRET encoder synthesis. The band separation in the right lane corresponds to addition of a single 69 bp encoder period. The full-length 276 bp product migrates at the same rate as a 350 bp blunt-ended DNA duplex because of the two 20-nucleotide poly(dT) tails (main text Fig. 1A). A third lane (not shown) containing the ligated FRET encoder was run on this gel, but was shielded from UV light in order to prevent DNA damage. Encoders for use in single-molecule experiments were purified by excising the area in the third lane next to the slowest band in the FRET encoder lane shown above.

Fig. 1). The encoder structure supports *in vitro* reconstitution of DnaB-catalyzed unwinding: the helicase loads on the free 5′ tail, and the 3′ tail of the acceptor-labeled strand is forced around the periphery of the hexamer.[3]

DnaB Labeling. In order to remove Tris prior to amine-reactive labeling, 10 μL of 15 μM DnaB monomer stock solution was dialyzed against 2 L of reaction buffer using 12–14 kDa cutoff regenerated cellulose tubing (Fisher Scientific 21-152-8). 2 μL of 75 μM Alexa Fluor 488 carboxylic acid, succinimidyl ester (Invitrogen A-20000) was then added to the DnaB solution and allowed

to react for 24 hours. The completed reaction was dialyzed against 1 L of reaction buffer to remove reactive Alexa Fluor 488, followed by dialysis against 2 L of storage buffer. Recovery of protein was 50%, with at most 5% of monomers labeled, as determined by UV and visible absorbance. All steps were conducted at 4 °C. As the label is amine reactive, the dye was not site-specifically attached to the DnaB monomers.

Flow Cell Preparation. Flow cells were fabricated using two coverslips, a 25 mm fused-silica square (Esco Products R425025) and an 18 mm Pyrex square. The coverslips were bonded together with two strips of paraffin wax/polyolefin film (Parafilm M, Alcan Packaging), which formed a flow channel 1–2 mm wide with a volume of approximately 5 μL.

Prior to bonding, coverslips were sonicated in 2% v/v Micro-90 glass cleaning solution (Cole-Parmer EW-18100-01) for 10 minutes, and then in distilled deionized water for 10 minutes. They were blown dry with filtered compressed air, exposed to a hand-held corona surface treater (Electro-Technic Products BD-20AC) for 10 seconds, and vapor-coated in a sealed Petri dish with a drop of hexamethyldisilazane for 20 minutes. The Parafilm strips were placed between the coverslips and melted under pressure on a 120 °C hotplate until a reliable seal was formed.[*]

A solution containing 10 pM encoder DNA in TE buffer and 1 nM anti-digoxigenin was prepared, allowing anti-digoxigenin to bind to digoxigenin labels on the 3′ termini of the encoders (main text Fig. 1A). This solution was placed in the flow cell for 30 minutes so that anti-digoxigenin would adsorb to the fused silica surface, after which the cell was flushed with 50 μL of passivation solution. Fluid exchange was accomplished by drawing old solution out of the flow cell with absorbent paper. After 10 minutes, 2 μL of passivation solution containing 100 pM of 1 μm-diameter streptavidin-coated superparamagnetic beads (Invitrogen 650-01) were added to the flow cell. The beads were allowed to bind to the biotinylated λ DNA handles for 10 minutes, after which unbound beads were washed out of the cell with 100 μL of passivation solution.

In preparation for fluorescence measurements, the cell was flushed with 10 μL of oxygen scavenging sys-

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[*]After the experiments, fused-silica coverslips were recovered by soaking flow cells in hexane overnight to dissolve the Parafilm. The coverslips were then submerged for 30 minutes in a mixture of 4 parts by volume 17.8 M sulfuric acid to one part 30% w/w hydrogen peroxide to remove any organic residue.
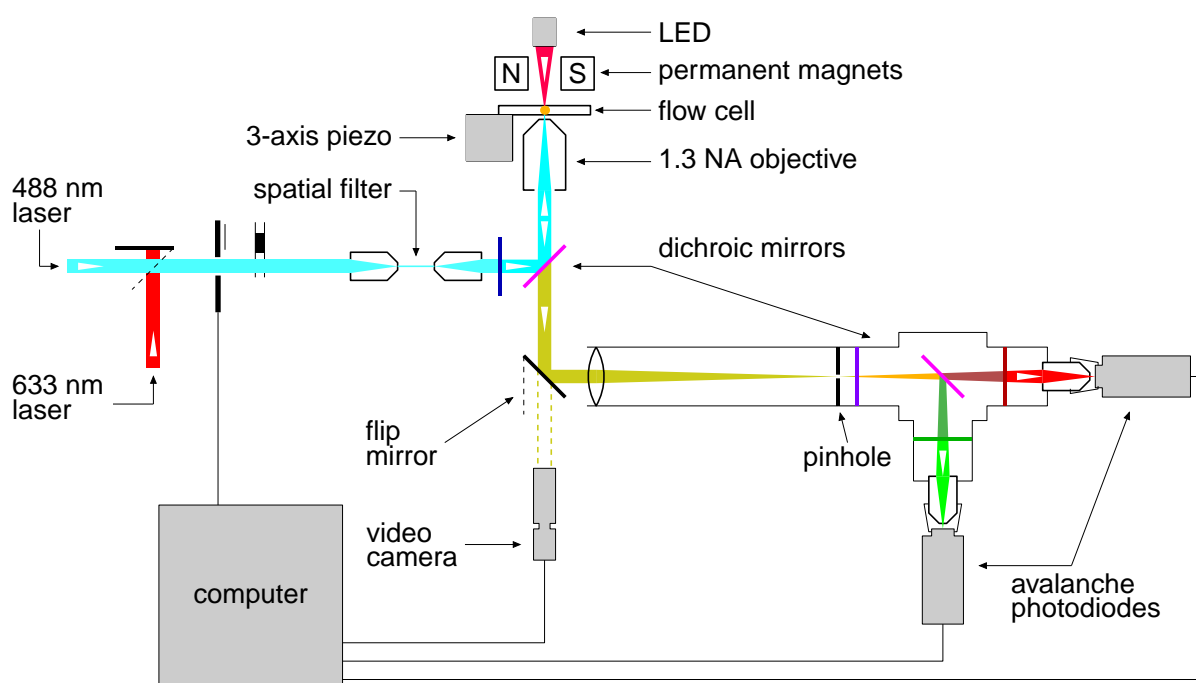
Supporting Figure 2. Custom confocal microscope and magnetic tweezer apparatus used for single-molecule fluorescence measurements.

Supporting Table 1. Single-stranded oligonucleotide sequences for FRET encoders. All except T20-a-z-b have a 5′ phosphate to facilitate ligation. Sequence A-T20 has a 3′ digoxigenin label. Two different versions of sequence Z were prepared, each internally labeled with a FRET acceptor dye between bases 12 and 13. The version used in the four-period encoder contained Alexa Fluor 594, while the five-period encoder used Cy5. When paired with the donor dye Alexa Fluor 488, Alexa Fluor 594 and Cy5 produce similar Förster radii (R_0) between 5 and 6 nm. The five-period encoder uses all sequences except COS-H. The four-period encoder uses all sequences except for COS-J, i-z-j and I-H.

Name	Nucleotide Sequence (5′ → 3′)
Z	GTCTTGGATCGTTCTGCATATTG
COS-H	AGGTCGCCGCCCGTGTCCATGTTCCAACTCCTTGC
COS-J	AGGTCGCCGCCCGTGTATCATTGTGGCACTCGGAG
A-T20	GATCACTGAGTACGTGTTTTTTTTTTTTTTTTTTTT
C-B	CTCGTTAGCGCGAATCGATGACCTTATTATCCACGGTAAGGAATGT
E-D	AACTTGGAAGCTTCGCGCACCAATACAGCAAGAACCGTGCGGTAAC
G-F	CGGAGCTGTGCAGGTGTCACGGCTCACCGGCTCAATCGATATCAAG
I-H	TTAACCGTATCACGCCAGGAACGGTGTCCATGTTCCAACTCCTTGC
c-z-d	GGTCATCGATTCGCGCTAACGAGCAATATGCAGAACGATCCAAGACGTTACCGCACGGTTCTTGCTGTA
e-z-f	TTGGTGCGCGAAGCTTCCAAGTTCAATATGCAGAACGATCCAAGACCTTGATATCGATTGAGCCGGTGA
g-z-h	GCCGTGACACCTGCACAGCTCCGCAATATGCAGAACGATCCAAGACGCAAGGAGTTGGAACATGGACAC
i-z-j	CGTTCCTGGCGTGATACGGTTAACAATATGCAGAACGATCCAAGACCTCCGAGTGCCACAATGATACAC
T20-a-z-b	TTTTTTTTTTTTTTTTTTTTCACGTACTCAGTGATCCAATATGCAGAACGATCCAAGACACATTCCTTACCGTGGATAATAA

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tem in helicase buffer.

Fluorescence Measurements. Fluorescence measurements were carried out using the custom-built confocal microscope[4, 5] and magnetic tweezer apparatus illustrated in Supporting Fig. 2. One of two CW laser excitation sources can be selected with a flip mirror, at 488 nm (Coherent Sapphire 488-25) or 633 nm (JDS Uniphase 1507P-1). The excitation beam is spatially filtered, after which it is brought to focus by a 1.3 numerical aperture, 100× oil-immersion microscope objective (Olympus UPLFLN 100XO2). The flow cell is mounted in a custom holder attached to a 3-axis feedback-controlled piezoelectric stage (Physik Instrumente P-611.3S). The cell is oriented so that observations are made through the fused-silica coverslip.

Fluorescence emission is collected by the excitation objective, separated from scattered laser light by a dichroic mirror (Chroma Z488/633RPC), and focused through a 100 μm-diameter pinhole. After additional laser light rejection by a long-pass filter (Omega Optical 493AELP), donor and acceptor fluorescence photons are separated by a second dichroic mirror (Omega Optical 560DCLP), bandpass filtered (Chroma Technology D525/50m and D630/60m for the donor and acceptor channels, respectively), and detected by avalanche photodiodes (PerkinElmer Optoelectronics SPCM-AQR-16). Photon arrival times are recorded by a 100 ps time digitizer (Ortec 9353) and stored on a computer.

Magnetic beads (see above) attached to sample molecules can be manipulated with a pair of NdFeB magnets (Dura Magnetics NS-505050) mounted on linear and rotary translation stages. The 3-D position of each bead can be tracked in real time at 60 Hz by imaging its interference pattern on a CCD camera (Basler scA640-70fm).

An assembled flow cell was mounted in the single-molecule instrument and prepared as described above. A field of view, typically containing on the order of 10 beads, was then located using the camera. The magnetic beads were pulled away from the surface with a force[6] of 0.5–3.0 pN, which is small enough to avoid significant alteration of protein-DNA interactions.[7]

Occasionally, beads will bind to multiple tethers, or individual tethers will adhere non-specifically to the glass surface. In order to identify potential problems of this nature, the magnetic tweezers were rotated 600 times, causing multiple and rotationally-constrained tethers to coil, thereby reducing the heights of the affected beads. At the end of this procedure, beads which were less than the expected distance from the fused-silica surface were eliminated from consideration. Several encoders under the remaining beads were located as described below and excited directly with 1 μW at 633 nm in order to verify complete assembly by stepwise photobleaching (see main text Fig. 1).

The maximum length of the encoders, about 120 nm, is approximately an order of magnitude smaller than the axial extent of the excitation and detection volumes of the optical system. Proper centering of the sample therefore ensures that the rates of excitation and detection remain constant along the length of the encoder. The equal heights of the observed photobleaching steps confirm that the vertical position of the focal volume was optimized.

A solution was prepared containing 5 μL of 100 mM ATP and 95 μL of oxygen scavenging system in helicase buffer. The flow cell was flushed with approximately 10 μL of this solution, after which DnaB helicase was thawed and added to the remainder. For each batch of DnaB, the concentration was adjusted based on labeling efficiency so that there would be approximately 10 nM of labeled monomer in solution. The required hexamer concentrations were between 32 and 200 nM.

A bead was selected and illuminated from above by an LED. The confocal volume was positioned near the centroid of the resulting diffraction pattern, and focused at the cover slip surface. The LED was then turned off, the acceptor-channel avalanche photodiode was turned on, and the DnaB/ATP solution described above was introduced into the flow cell. The 633 nm laser, reduced to 1 μW with absorptive neutral density filters, was used for direct excitation of the acceptor dyes while the sample stage was scanned in an outwardly-directed spiral search pattern. When a significant signal was detected, an algorithm previously described by Ha *et al.*[8] was used to precisely center the confocal volume on the FRET encoder, after which the beam shutter was immediately closed to minimize photobleaching.

The 488 nm laser, reduced to a power between 11 and 15 μW, was selected as the excitation source, and the shutter was reopened. Fluorescence photon arrival times were recorded for approximately one minute, after which a new encoder was found and the process repeated. This was necessary because direct excitation of acceptor dyes by the 488 nm laser caused them to pho-

tobleach after a few minutes.[9] The acceptor signal from direct excitation at 488 nm is, however, negligible. Significant fluorescence in the acceptor channel indicates FRET from a nearby labeled helicase.

The event frequency was very low, and consequently hours of observation were required to collect a single complete periodic signal. Helicase concentration could not be increased, as leakage into the acceptor channel of fluorescence from donor-labeled DnaB in solution was the dominant source of background noise.

Supporting Discussion

Data Analysis. Acceptor photon arrival times were binned in 10 ms intervals to produce a fluorescence intensity time series. Periodic events with sufficient signal-to-noise ratios, such as those shown in main text Fig. 3 and Supporting Fig. 3, were readily apparent from visual inspection of the data. Once an event was located, its start time was determined by approaching in the direction of increasing time until a bin was found with a signal level at least one third of the maximum for that event. The event was then scanned from this bin in the direction of decreasing time, until a bin was found at no more than one fourth the maximum level, and that point was designated the start time. The end time was found in a similar manner, with the initial scan in the direction of decreasing time, from a point after the event. For events D and H in Supporting Fig. 3, thresholds were adjusted to compensate for large variations in peak height. Helicase translation speeds were determined as described in the main text.

Additional Events. Additional five-period encoder events are shown in Supporting Fig. 3. Direct excitation of acceptors by the 488 nm laser is infrequent, but does occur. This effect and FRET can both eventually cause photobleaching of encoder dyes. It is therefore natural to expect that some events will have fewer peaks than the number of acceptors on the encoder. Bleaching of the donor is also possible, and would result in the abrupt loss of acceptor signal. Furthermore, if the helicase encounters a nick in either DNA backbone, it will fall off of or shear the encoder, and quickly diffuse away. Supporting

Figs. 3B and 3C show examples of events in which the second of five encoder dyes appears to be missing, and the events in Supporting Figs. 3F and 3G also show only four peaks. In Supporting Fig. 3H, the signal disappears in the middle of the last peak. Shortly after that event was detected, the number of active acceptors remaining on the encoder was checked by switching to direct excitation with the 633 nm laser. The fluorescence count rate was then approximately 80% of its original value. This measurement strongly suggests that of the possible causes of an incomplete event, acceptor photobleaching is the most likely in this case.

Events with fewer than two distinct peaks cannot be distinguished from occasional scattering caused by contaminants drifting through the laser focus.

Resolution. The observed acceptor fluorescence signal in a FRET encoder measurement is directly proportional to the energy transfer efficiency E. Owing to the form of main text Eq. 1, the position resolution varies over the course of each encoder period. From that equation, we find

$$\frac{dE}{dr} = \frac{-6r^5}{R_0^6 \left[1 + (r/R_0)^6\right]^2}. \tag{1}$$

At a signal maximum, when the donor-acceptor separation $r \approx 0$, $dE/dr \approx 0$, and no signal change results from incremental movement of the donor. On the leading and trailing edges of the signal peaks where $r = R_0$, however, $|dE/dr| = 3/2R_0$. At $r = R_0 = 5$ nm, a displacement of 1 bp (0.34 nm) will then result in a change from $E = 0.45$ to $E = 0.55$, producing a proportional increase in acceptor signal.[*]

In a single measurement of duration τ, for example one 10 ms bin in main text Fig. 3D, we collect a signal $S = EA$, where A is the maximum observed signal.[†] A is a function of laser power P and of τ. So long as P is kept below approximately 200 μW in our system, saturation of the dyes will not occur, and $A = aP\tau$, where a is a constant. We then have

$$S = EaP\tau. \tag{2}$$

For a small change Δr, the corresponding signal change

$$\Delta S \approx \frac{dS}{dr}\Delta r = \frac{dS}{dE}\frac{dE}{dr}\Delta r = aP\tau\frac{dE}{dr}\Delta r. \tag{3}$$

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[*]The $4R_0$ acceptor spacing of the present encoders prevents significant interaction between the donor and more than one acceptor at a time.

[†]Here we assume $E \approx 1$ at the point in the encoder cycle when the donor and acceptor are closest, and that the difference between a molecular motor step and the corresponding change in r is negligible.
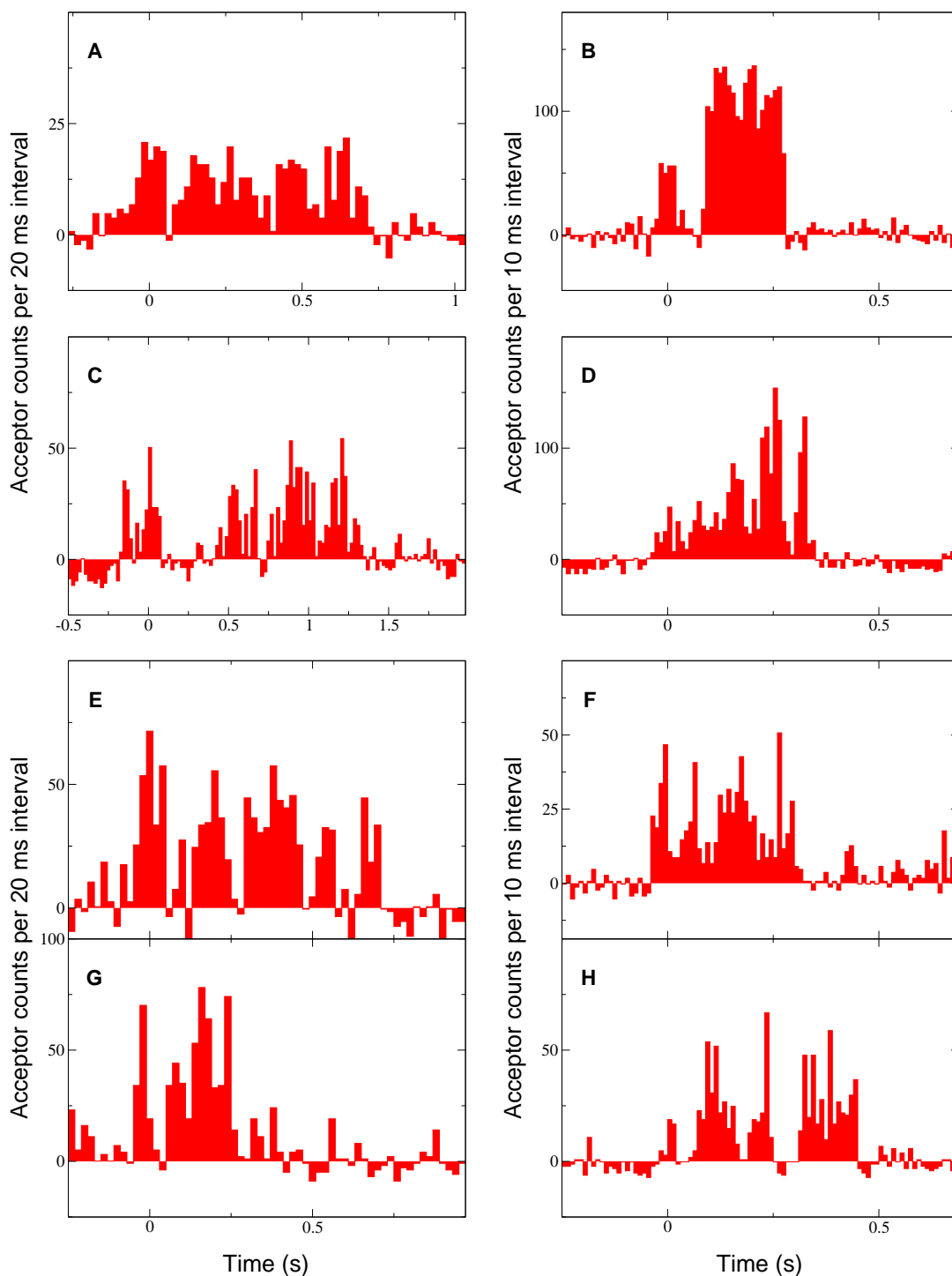
Supporting Figure 3. Additional five-period encoder events. Helicase translation speeds were determined by fitting functions of the form $A \cos^2 \left[\pi f (t - t_0) \right]$ to the data using least squares. The speeds were (**A**) 432 ± 7 bp/s, (**B**) 1060 ± 30 bp/s, (**C**) 229 ± 5 bp/s, (**D**) 850 ± 18 bp/s, (**E**) 390 ± 11 bp/s, (**F**) 700 ± 20 bp/s, (**G**) 790 ± 20 bp/s, and (**H**) 610 ± 16 bp/s. For all events except (**B**), speeds determined using power spectra were within 4% of those given here. A missing peak and poor signal contrast in (**B**) prevented reliable frequency determination using the power spectrum.

FRET encoder measurements contain both shot noise and fluorescence background. The shot noise $\sigma_s = \sqrt{S}$. As with A, the background level $B = bP\tau$, where b is constant. After background subtraction, a noise component $\sigma_B = \sqrt{B}$ remains. Since shot noise and background noise are uncorrelated, the total noise

$$n = \sqrt{\sigma_s^2 + \sigma_B^2} = \sqrt{S + B} = \sqrt{(Ea + b)\,P\tau}. \quad (4)$$

It follows that for a change in separation Δr, the signal-to-noise ratio

$$\text{SNR} \equiv \frac{|\Delta S|}{n} = \frac{aP\tau}{\sqrt{(Ea + b)\,P\tau}} \left| \frac{dE}{dr}\Delta r \right| \quad (5)$$

$$= \frac{a\sqrt{P\tau}}{\sqrt{Ea + b}} \left| \frac{dE}{dr}\Delta r \right|. \quad (6)$$

When $r = R_0$, $E = 0.5$, and

$$\left| \frac{dE}{dr}\Delta r \right| = \frac{3}{2} \frac{|\Delta r|}{R_0}. \quad (7)$$

We then have[*]

$$\text{SNR}_{r=R_0} = \frac{3a}{2\sqrt{a/2 + b}} \sqrt{P\tau} \frac{|\Delta r|}{R_0}. \quad (8)$$

In main text Fig. 3D, the background level $B = 16$ counts in $\tau = 10$ ms, and the maximum signal $A \approx 33$ counts. The laser power $P = 11$ μW. Thus we find $a = 3.0 \times 10^8$ W$^{-1}\cdot$s^{-1}, and $b = 1.5 \times 10^8$ W$^{-1}\cdot$s^{-1}. From Eq. 8,

$$\text{SNR}_{r=R_0} = 2.6 \times 10^4 \sqrt{P\tau} \frac{|\Delta r|}{R_0}, \quad (9)$$

with P in watts and τ in seconds. During that event, the helicase was moving 4.5 bp (1.5 nm) in 10 ms, so from Eq. 9 we expect $\text{SNR}_{r=R_0} \approx 2.6$ in a single bin. For $\Delta r = 1$ bp (0.34 nm) and $R_0 = 5$ nm, we would have

$$\text{SNR}_{r=R_0}(1 \text{ bp}) = 1800 \sqrt{P\tau}. \quad (10)$$

As discussed in the main text, a significant increase in P should be possible in future experiments. Further gains might be realized with near-infrared acceptor dyes, which would have increased spectral separation from the donor. This would reduce background noise from donor photons leaking into the acceptor channel. Since R_0 depends on the spectral overlap of donor emission and acceptor absorption, it too would be reduced, increasing the SNR.

Supporting References

[1] Kaplan, D. L. & O'Donnell, M. DnaB drives DNA branch migration and dislodges proteins while encircling two DNA strands. *Mol. Cell* **10**, 647–657 (2002).

[2] Rasnik, I., McKinney, S. A. & Ha, T. Nonblinking and long-lasting single-molecule fluorescence imaging. *Nature Methods* **3**, 891–893 (2006).

[3] LeBowitz, J. H. & McMacken, R. The Escherichia-coli DnaB replication protein is a DNA helicase. *J. Biol. Chem.* **261**, 4738–4748 (1986).

[4] Schuler, B., Lipman, E. A. & Eaton, W. A. Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy. *Nature* **419**, 743–747 (2002).

[5] Schuler, B., Lipman, E. A., Steinbach, P. J., Kumke, M. & Eaton, W. A. Polyproline and the "spectroscopic ruler" revisited with single-molecule fluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 2754–2759 (2005).

[6] Gosse, C. & Croquette, V. Magnetic tweezers: Micromanipulation and force measurement at the molecular level. *Biophys. J.* **82**, 3314–3329 (2002).

[7] Bustamante, C., Smith, S. B., Liphardt, J. & Smith, D. Single-molecule studies of DNA mechanics. *Curr. Opin. Struct. Biol.* **10**, 279–285 (2000).

[8] Ha, T., Chemla, D. S., Enderle, T. & Weiss, S. Single molecule spectroscopy with automated positioning. *Appl. Phys. Lett.* **70**, 782–784 (1997).

[9] Kong, X. X., Nir, E., Hamadani, K. & Weiss, S. Photobleaching pathways in single-molecule FRET experiments. *J. Am. Chem. Soc.* **129**, 4643–4654 (2007).

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[*]Neither $\left| \frac{dE}{dr} \right|$ nor the SNR given in Eq. 6 peak exactly at $r = R_0$. For the purposes of this calculation, however, the difference between the peak value and $\text{SNR}_{r=R_0}$ is negligible. For our parameters, the peak SNR occurs at $0.988R_0$, and its value is 0.13% larger than $\text{SNR}_{r=R_0}$.