Supplementary Methods

Preparation of switch-labeled DNA constructs. Biotinylated and/or amine-modified DNA oligonucleotides were purchased, PAGE purified, from Operon. Amine-modified oligos were labeled with amine reactive Cy3 or Cy5 (Amersham Bioscience) post-synthetically following the protocol provided by the manufacturer. The dye-labeled oligos were purified using reverse phase HPLC. Complementary strands of DNA were annealed to form biotinylated double-stranded DNA (dsDNA) by mixing equimolar amounts of the two complementary strands in 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, heating to 90 degrees C for two minutes, and then allowing the mixture to cool to room temperature in a heat block over a period of one hour.

Biotinylated dsDNA of varying lengths having an intra-switch distance of 135 bp were constructed by annealing complimentary oligos as described above to form three different 45 bp dsDNA segments denoted A, B, and C, followed by a ligation reaction. The oligos were designed with specific sticky ends which only permit A to ligate to B, B to ligate to C and C to ligate to A, reading in the 5' to 3' direction. One oligo (A) contained amine-reactive sites three base pairs apart on opposite strands which were specifically labeled with Cy3 and Cy5 prior to annealing to form the optical switch. Oligos B and C contain two internal biotin modifications per strand to facilitate multivalent linkage to the streptavidin surface. After annealing, the oligos were mixed at equal concentrations and ligated overnight using T4 ligase (New England Biolabs). The resulting ligation product was purified on a 1.5% agarose gel to select bands containing the desired concatamerized dsDNA length.

Preparation of switch-labeled antibodies. Goat polyclonal to mouse IgG secondary antibodies (Abcam ab6708) were labeled non-specifically with amine-reactive Cy3 and Cy5. The average dye-to-antibody ratio was characterized to be 2.2 : 1 for Cy3 and 0.1 : 1 for Cy5 using a UV-Vis spectrometer. These labeling ratios were chosen to minimize the fraction of antibodies labeled with more than one Cy5 molecule, due to the inefficient switching observed for antibodies labeled with multiple Cy5. The presence of more than one Cy3 molecule on a single antibody does not interfere with switching. With this labeling ratio, a large fraction of the secondary antibody do not carry Cy5, and are not observed in our experiments. This lower density of labels is typically not a problem for indirect immunofluorescence imaging and, in

particular, did not prevent us from resolving the circular structure of the RecA-plasmid filaments.

Preparation of RecA filaments. Biotinylated RecA was prepared by reacting purified recombinant RecA (New England Biolabs) with amine-reactive biotin-XX (Invitrogen) in 0.1 M carbonate buffer at pH 8.3. The resulting biotinylated protein was purified on a NAP-5 size exclusion column (Amersham). RecA filaments were formed on ΦXRF-II plasmid DNA (New England Biolabs) by incubating RecA (25% biotinylated : 75% unbiotinylated, concentration 80 μg / mL) with plasmid DNA (2 μg / mL) in 10 mM Tris buffer pH 7.0, 100 mM NaCl, 7 mM $MgCl_2$, and 0.8 mg/mL ATP-γ-S for 1 hour at 37 C. The resulting RecA-DNA filaments were stored at 4 C and used for imaging the same day.

Microscope slide preparation. Quartz microscope slides (G. Finkenbeiner) were cleaned using Alconox detergent, followed by sonication for 15 minutes in acetone, 1 M aqueous KOH, ethanol, 1 M aqueous KOH, sequentially. Slides being prepared for lipid bilayers were submerged into a 5% HF solution for 2 hours after the second KOH step. Finally, slides were rinsed with deionized water, and flame dried. Flow channels were prepared using two pieces of double-sided adhesive tape (3M) and covered with a No. 1.5 glass coverslip (VWR).

Oxygen scavenging system. All imaging buffers were supplemented with the oxygen scavenging system, consisting of 10 % (w/v) glucose (Sigma), 0.1% (v/v) β-mercaptoethanol (Sigma), 500 μg/mL glucose oxidase (Sigma), and 10 μg/mL catalase (Roche). The oxygen scavenging system is critical for reliable photoswitching of the fluorophores.

Surface-immobilization of DNA and antibodies. To immobilize the DNA sample labeled with multiple switches on a quartz slide, a lipid bilayer was first formed on the slide by flowing in liposomes consisting of egg PC and 5% biotin-PE (Avanti). The liposomes were formed according to the manufacturer's instructions, extruded through a 0.05 μm filter membrane at a concentration of 5 mg lipids / mL in DI water and then mixed with a 1:1 ratio with a buffer containing 10 mM Tris pH 8.0, 100 mM NaCl immediately before use. After a 2 hour incubation the bilayer was rinsed extensively with 50 mM Tris pH 8.0, 10 mM NaCl, and the bilayer was incubated with streptavidin (0.25 mg/mL, Invitrogen) for 30 minutes. After extensive washing, the streptavidin surface was crosslinked in 4% v/v formaldehyde in PBS for 1 hour and rinsed with Tris buffer before allowing the biotinylated, switch-labeled DNA to bind. DNA was imaged

in Tris buffer (50 mM Tris-Cl pH 7.5, 10 mM NaCl) with the oxygen scavenging system described above.

To immobilized switch-labeled antibodies on a surface, quartz slides were cleaned as described and incubated for 5 minutes with mouse anti-transferrin IgG (Abcam) allowing it to bind non-specifically. The slide was then incubated with the labeled secondary antibodies in PBS buffer containing 3% bovine serum albumin (BSA) for 10 min. The buffer was replaced with 50 mM Tris, 10 mM NaCl, pH 7.5 containing the oxygen scavenging system for imaging.

Immunofluorescence imaging of RecA-dsDNA filaments. Biotinylated RecA-dsDNA filaments were attached via streptavidin linkages to a quartz slide non-specifically coated with biotinylated BSA. The surface was then washed with 50 mM Tris pH 7.0, 100 mM NaCl, 7 mM $MgCl_2$, 3% BSA w/v (block buffer) and incubated for 30 minutes to block the surface against non-specific antibody binding. The slide was then incubated in this block buffer containing monoclonal mouse antibody against RecA (Stressgen) at 2 μg/mL for 1 hour. After extensive washing with block buffer, the slide was incubated in the block buffer containing switch-labeled secondary antibody at 0.3 μg/mL for 1 hour. Finally, the sample was washed and imaged in 50 mM Tris pH 7.5, 100 mM NaCl, 7 mM $MgCl_2$, supplemented with the oxygen scavenging system as described above.

Imaging procedures. An Olympus IX71 microscope was used for single-molecule imaging with the prism-type total internal-reflection excitation scheme. Samples were excited with a 633 nm HeNe laser (Melles Griot) and a 532 nm diode-pumped Nd:YAG laser (Crystalaser). The fluorescence emission of Cy5 was collected with a N.A. 1.25 60X water immersion objective, and imaged onto an electron multiplying CCD camera (Andor Ixon DV897) after passing through a 665 nm long pass filter (Chroma). Images were recorded at a frame rate of 9.7 Hz. To track motion of the sample stage, 200 nm red fluorescent polystyrene beads (Invitrogen, F-8810) were added to the slide in Tris buffer containing 10 mM $MgCl_2$ and allowed to bind to the surface. Data was acquired using custom data acquisition software written in Labview, which enabled sequences of alternating red and green laser excitation pulses to be applied to the sample, switching the dyes on and off. Laser excitation was synchronized with the camera exposure to 1 ms accuracy.

Imaging experiments on DNA labeled with multiple switches typically consisted of 60 laser pulse cycles to activate and deactivate the switches, where each cycle lasted for 5 s so that the final image took 5 minutes to acquire. The multiple cluster configuration of the centroid position distribution is typically apparent within 2 minutes. Experiments on antibody-labeled RecA-dsDNA filaments consisted of 70 switch cycles, each lasting 10 s. The ring shape of the RecA-dsDNA filaments typically became evident within 2 minutes, although the position of every switch present in the sample had not yet been identified by this time. As the rate of switching depends linearly on the excitation intensity, we expect that the cycling time, and hence the overall imaging time, can be shortened without substantially affecting the localization accuracy by increasing the red laser intensity. Green laser intensity was chosen so that typically 1-3 switches were switched on during the green pulse and, in most cases, all activated switched were turned off during the red phase of the cycle.

Raw images of RecA-dsDNA for comparison with STORM reconstructed images were formed by taking the maximum fluorescence value recorded for each pixel throughout the imaging sequence so that each switch would be equally represented regardless of the amount of time it spent in the fluorescent or dark states.

Image analysis. Fluorescent structures (DNA or RecA filaments) in an averaged image were first isolated in 13 x 13 pixel square fitting window for data analysis. In a given window, the total fluorescence intensity was integrated in each frame to produce a fluorescence time trace (**Supplementary Fig. 3** online). Several criteria were used to ensure high accuracy localization of single switches:

(1) In each switching cycle, only regions where a single switch is on for at least three frames (0.3 seconds) were used for localization analysis (see **Supplementary Fig. 3** online).

(2) The fluorescence images within these regions were fit by nonlinear least-squares regression to a continuous ellipsoidal Gaussian:

$$I(x,y) = A + I_0 e^{\left[-(x'/a)^2-(y'/b)^2\right]/2}$$

where:

$$x' = (x - x_0)\cos\theta - (y - y_0)\sin\theta$$
$$y' = (x - x_0)\sin\theta + (y - y_0)\cos\theta$$

Here, A is the background fluorescence level, I_0 is the amplitude of the peak, a and b define the widths of the Gaussian distribution along the x and y directions, x_0 and y_0 describe the center coordinates of the peak, and θ is the tilt angle of the ellipse relative to the pixel edges. Based on this fit, we compute a peak ellipticity defined as $\left|2(a-b)/(a+b)\right|$. If this ellipticity exceeded 15%, indicating poor image quality or the possible presence of multiple active switches, the region was rejected from the analysis.

(3) We calculated the total number of counts collected in the peak as $2\pi\ ab\ I_0$ and then converted this number to photoelectrons, and thus the number of photons detected, using the camera manufacturer's calibrated curve for the electron multiplication and ADC gain settings used during imaging. If the total number of photoelectrons in the peak was less than 2,000, the region is rejected due to insufficient statistics to achieve high localization accuracy.

Regions of the fluorescence traces that passed the above tests were used for the final localization analysis. The fluorescence images corresponding to these regions were subjected to a final fit using a pixelated Gaussian function to determine the centroid position. Because the CCD chip consists of square pixels of finite size, for optimum accuracy, the image is fit to:

$$I(x,y) = A + \int_{x-\delta}^{x+\delta} dX \int_{y-\delta}^{y+\delta} dY\ I_0 e^{\left[-\left(\frac{X-x_0}{a}\right)^2-\left(\frac{Y-y_0}{b}\right)^2\right]/2}$$

which, for ease of evaluation, can be re-expressed in terms of error functions as:

$$I(x,y) = A + I_0 \frac{ab\pi}{4}\left[\mathrm{erf}\left(\frac{x+\delta-x_0}{a}\right)-\mathrm{erf}\left(\frac{x-\delta-x_0}{a}\right)\right]\left[\mathrm{erf}\left(\frac{y+\delta-y_0}{b}\right)-\mathrm{erf}\left(\frac{y-\delta-y_0}{b}\right)\right]$$

where A, I_0, a, b, x_0 and y_0 are as defined previously and δ is the fixed half-width of a pixel in the object plane. The final centroid coordinates (x_0, y_0) obtained from this fit were used as one data point in the final STORM image.

To correct for mechanical drift in the microscope during imaging, the same fitting algorithm was used to automatically track the motion of several fluorescent beads in the field of view. The beads served as fiducial marks and their positions were sampled during each imaging cycle. The averaged motion of the beads was subtracted from the coordinates obtained for each single switch position, yielding a drift-corrected reconstructed image.

For quantitative analysis of images using switches equally spaced on dsDNA, the coordinates in the reconstructed image were classified using a *k*-means clustering algorithm[1], and inter-switch distances were calculated as the distance between the cluster centroids. The number of clusters input to the algorithm was chosen according to the number of photobleaching steps observed during the initial exposure to red light.

Prediction of DNA configuration. Possible configurations of a 135 bp piece of dsDNA bound to the surface were computed by Monte Carlo simulation of the DNA as a worm-like chain in the plane. According to the Watson-Crick structure of dsDNA and accounting the length of the C_6 linkers attaching the Cy5 molecules to the nucleotides we calculate an expected contour length between neighboring Cy5 molecules of 46 ± 1 nm. The DNA was treated as a series of 1 Å joints lying in the plane, each of which is deflected by a random angle selected from a Gaussian distribution chosen to give a persistence length of 50 nm[2]. The measured inter-switch spacing was compared to the distribution of end-to-end distances of the polymer in this simulation.

References

1. MacQueen , J. B. *Some Methods for classification and Analysis of Multivariate Observations, Proceedings of 5-th Berkeley Symposium on Mathematical Statistics and Probability* (Berkeley, University of California Press, 1967).

2. Bustamante, C., Marko, J. F., Siggia, E. D. & Smith, S. Entropic elasticity of lambda-phage DNA. *Science* **265**, 1599-1600 (1994).