Supplementary Information, Shroff *et al.*

Preparation of Force Sensor - DNA Constructs

Cy3 and Cy5 dyes were covalently attached to single stranded DNA (Fidelity Systems Non-Vanilla Oligonucleotide Synthesis) to make force sensors of variable length:
FS10, 5'GGGCGGCGACCTATTT[U-Cy5]ACGGATGGGA[T-Cy3]TGATAC[Biotin]T;
FS15, 5'GGGCGGCGACCTATTT[U-Cy5]ACGGATGGGATGAGA[T-Cy3]TGATAC[Biotin]T;
FS20, 5' GGGCGGCGACCTATTT[U-Cy5]ACGGATGGGATGAGAAGGGA[T-Cy3]TGATAC [Biotin]T;
FS10-L, 5'TAGTCTCTAC[U-Cy5]ACGGATGGGA[T-Cy3]GGCCGGGTCGTAGCA.

For some experiments, the force sensors were annealed and ligated to lambda-phage dsDNA, or ligated with CircLigase and then annealed.

Ligation and Purification of Sensors

For single molecule calibration experiments, FS10, FS15, and FS20 were annealed and ligated to λ phage dsDNA. The force sensors were annealed to λ DNA (NEB, N3011L) in a 10:1 ratio. The resulting constructs were ligated with T4 DNA ligase (NEB, M020S) at 16^{o}C for two hours and annealed in a 1:10 ratio with digoxigenin labeled oligonucleotides (Qiagen, 5' AGGTCGCCGCCCAAAAAAAAAAAA[Digoxigenin]). The constructs were ligated with T4 DNA ligase as before, passed through size exclusion columns (BD Biosciences, 636079) to remove unligated force sensors, diluted to approximately 30 pM and frozen at -20^{o}C.

For the DNA loop experiments, FS10-L was circularized with CircLigase ssDNA Ligase (Epicentre, CL4115K). Reactions were carried out in 20μL volumes, combining 300 Units of CircLigase with 10pmol oligos, and incubating for 1 hour at 60^{o}C. The circular ligase was then inactivated by heating the reactions to 80^{o}C for 10 minutes. The circularized DNA migrates slower than the linear oligos on a denaturing gel; we purified the circular product from a 15% TBE-Urea gel (Biorad, 161-1117) and resuspended the product in T50 buffer (10 mM Tris, pH 8.0 and 50 mM NaCl). For some experiments, the purified circular molecules were annealed in a 1:100 ratio to another oligonucleotide, 5' GTAGAGACTATGCTACGACCCGGCC.

Bulk Experiments

Bulk FRET measurements were taken on a fluorimeter (Jobin Yvon Fluoromax-3) using a 50 μL quartz cuvette. Measurements were performed using magic angle conditions in order to correct for polarization artifacts. All spectra were corrected for wavelength-dependent differences in detector efficiency and lamp output. Two sets of emission spectra (excitation at 542 nm or 640 nm; scanning over 562-750 nm, and

660-750 nm respectively; excitation and emission slit widths set to 5 nm) were taken for each measurement. Spectra of T50 buffer were used for background correction.

Extraction of FRET Values from Bulk Data

Following Clegg (Eqn. 11b) [1], the fluorescence signal $F(\nu, \nu')$, (excitation at ν' and detection at ν), can be represented as follows:

$$F(\nu,\nu') \propto \{\epsilon^D(\nu')\Phi^A(\nu)Ed^+a^+ + \epsilon^A(\nu')\Phi^A(\nu)a^+\} + \epsilon^D(\nu')\Phi^D(\nu)d^+[(1-E)a^+ + a^-] \tag{1}$$

where $\epsilon^F(\nu)$ is the molar absorption coefficient of the fluorophore F (donor (D) or acceptor (A)), at wavelength ν, $\Phi^F(\nu)$ is the quantum yield of fluorophore F at wavelength ν, E is the FRET efficiency, and f^+,f^- are the fractions of sample labeled with and without fluorophore f respectively. The signal can further be decomposed into two components, emission from the acceptor (F^A) and emission from the donor (F^D). The FRET value E can be calculated from the acceptor fluorescence emission values at $\nu = 670nm$ taken at the two excitation wavelengths ($\nu' = 542nm$ and $\nu' = 640nm$) from the following formula:

$$E = \{\frac{F^A_{em}(670,542)}{F^A_{em}(670,640)} - \frac{\epsilon^A(542)}{\epsilon^A(640)}\}\frac{\epsilon^A(640)}{\epsilon^D(542)}\frac{1}{d^+} \tag{2}$$

where we have assumed that $\epsilon^D(640) = 0$. Other ratios of extinction coefficients were obtained from excitation spectra. Donor labeling was measured to be almost complete, so we used d^+ = 1. The acceptor component of the fluorescence signal is isolated by least-squares fitting a measured donor-only curve to the region $\nu = [590, 612]$ and subtracting the fit from the total spectrum. The resulting acceptor-only curves are then least-squares fit to a measured acceptor-only curve in the region $\nu = [660, 680]$. The intensity values at $\nu = 670nm$ from the resulting fits are used in the calculation of FRET efficiencies.

Hybrid Instrument

Objective-type Total Internal Reflection Fluorescence (TIRF) [2–4] and magnetic tweezers capabilities were added to a standard Nikon TE2000-E epifluorescence microscope with a 60x TIRF objective (Nikon, 1.45 NA, oil immersion). We modeled our TIRF system after that described in Yildiz *et al*. [5]. Linearly polarized 532 nm laser light (CrystaLaser GCL-050-S, 50 mW) was attenuated 10 fold with a neutral density filter (Thorlabs, NE510B) and beam expanded 5X (Thorlabs, BE05). The light was passed through a 1", 50 cm focal-length lens and reflected from a dichroic mirror (Omega Optical, 540 DRLP) to be focused at the back focal plane of the objective. The excitation

area in the sample plane was a circle roughly 20 microns in diameter. Fluorescence light from the sample was collected through the same objective and passed through the same dichroic. Laser light was removed with two filters, an Omega Optical 532-D2C and Spectra-Physics 53683. Fluorescence light was separated into Cy3 and Cy5 channels using a DualView Image Splitter (Optical Insights) equipped with a dichroic filter (Chroma Technology, 630 dcxr) and Cy3 and Cy5 emission filters (Omegafilters, 575AF50 and 3rd Millenium 660-740 nm bandpass). Fluorescence emissions were imaged onto two halves of an electron-multiplying, back-thinned CCD (Roper Cascade 512B, 512 x 512 pixel array with 16 μm x 16 μm pixels). For all experiments, the camera was operated in 2x2 hardware binning mode (making the effective pixel size 554nm), with a Gain Multiplication Factor of 3900 and an integration time of 100 ms.

Magnetic Tweezers were added to the microscope by attaching a miniature uniaxial motorized stage (National Apertures, MM-3M-F-0.5) to the microscope. A pair of rare earth magnets was mounted to the stage and positioned directly above the sample flow cell. A Linear Variable Differential Transformer (LVDT) gauge head (Shaevitz, PCA-116-300 LVDT) was used to monitor the height of the magnets. The output of the LVDT was recorded with a computer using custom software; the same software was also used to control the motorized stage and thus the height of the magnets.

Flow Chambers

Circular quartz coverslips (SPI Supplies, 01019T-AB) were cleaned in a multistep process. The coverslips were immersed in Piranha solution (70% Sulfuric Acid, 30% Hydrogen Peroxide) at 60^{o}C overnight, rinsed with clean, deionized water (Barnstead, D4641), and sonicated for twenty minutes. Next, the coverslips were incubated overnight in a 2% Hellmanex II cleaning solution (Hellma, 320.001), and then rinsed and sonicated again with clean water. The coverslips were removed from the water and dried with clean nitrogen gas immediately before assembling the flow cells. A cleaned coverslip formed the bottom surface of the flow chamber, and a chamber was cut out of Nescofilm sheets (Karlan, N-0540) and placed onto the quartz surface. Glass slides (VWR Scientific, 48300-025) were cleaned overnight in 2% Hellmanex solution; a cleaned slide formed the top surface of the flow chamber. After assembly, the entire chamber was heated until the Nescofilm sheets formed a seal between the quartz and glass surfaces. The completed chambers were then exposed to intense UV light in a home-built UV oven for approximately two hours.

Surface Immobilization of Force Sensor Constructs

Biotin-BSA (Sigma, A8549) was dissolved in T50 buffer (10 mM Tris, pH 8.0 and 50 mM NaCl) to 1 mg/mL, and 100 μL was flowed into the chamber and allowed to

incubate for half an hour. The chamber was then washed with 200 μL of T50, and 100 μL of 0.2 mg/mL streptavidin (Molecular Probes, S-888) dissolved in T50 was then added to the chamber and allowed to incubate for half an hour. Next, the chamber was washed again with T50 and incubated overnight with 100 μL of Superblock (Pierce, 37515). The chamber was then washed as before with T50 and the force sensor constructs (100 μL, approximately 30pM) were flowed into the chamber and allowed to incubate for an hour. The chamber was washed again with T50, and 93 μL of magnetic bead solution was added to the chamber. The magnetic bead solution was made immediately before the experiments by combining 1 μL magnetic, anti-digoxygenin beads (Protein G magnetic beads (Dynal Biotech, 2.8 μm diameter, 100.03) functionalized with anti-dig (Roche, 1333 089) as described in the Pierce DMP instructions) with 90 μL glucose solution (0.1 g/mL glucose in T50) and 2 μL imaging solution (100 μL 2-mercaptoethanol (Sigma, 516732), 30 μL catalase (Roche, 0106810), 900 μL T50, and 2.5 mg glucose oxidase (Sigma, G2133)). The catalase and imaging enzyme solutions were filtered with a 0.2 μm filter before being flowed into the chamber. Prior to making the bead solution, beads were vortexed gently for one minute to break up bead clumps. The bead solution was then vortexed again before being flowed into the chamber. After two minutes the magnets were brought close to the chamber to prevent nonspecific sticking of beads to the chamber surface, and to stretch out the tethered bead complexes.

Single Molecule Experiments

Force sensor constructs were immobilized on the surface of home-built flow chambers using a biotin-streptavidin system. An oxygen scavenging system was employed to retard photobleaching of the sensors. For the experiments at zero force, the force sensor complexes were excited and data were taken until the sensors bleached. For the experiments where the force was ramped, magnetic beads were added to the chamber and allowed to incubate with the force sensors for two minutes. For each magnet position, 2 s of data were collected; a typical experiment involved ramping the magnet position over five positions, with each successive position increased a distance of one mm from the previous position. While the magnets were being moved, the laser light was blocked from the sample using a shutter, to prevent photobleaching of the dyes. The ramping procedure was repeated twice for each force sensor/bead complex. After ramping, the laser was left on for 30 s to bleach the dyes. Only complexes that were separated by at least one field of view were analyzed, in order to prevent bleaching of multiple sensors during one ramping experiment. FRET Efficiency values were then extracted for each magnet position.

Single Molecule Data Analysis

Pixels displaying single molecule FRET were identified with custom software written in MATLAB. First, each frame was divided into two regions: the area on the CCD corresponding to the Cy5 emissions, and the area corresponding to Cy3 emissions. The intensity values in the Cy5 region were then summed over all frames. We chose to search the acceptor Cy5 pixels first because any bright pixels in this region usually corresponded to FRET. For the molecules where handles were attached, the intensity from a single dye molecule was usually spread out over several pixels, so we developed a method to find groups of pixels that contained fluorescence intensity from the same dye. Pixels with intensity values less than a user-defined stringency were rejected (typically pixels with intensities less than 5 standard deviations from the mean pixel intensity). In this way, the vast majority of pixels were automatically rejected. A 3x3 pixel grid was selected around each of the remaining pixels, and MATLAB's corrcoef function was used compute the correlation between the center pixel and the surrounding pixels in the grid. Only those pixels that were correlated with a p value less than 0.05 were retained; the intensities from these pixels were summed to create larger superpixels. The superpixels were then paired with the corresponding superpixels on the Cy3 channel and the resulting pixel pairs were checked for correlation of overall intensity with changing force. For the molecules without handles, the fluorescence was typically localized to a single pixel; for these molecules we did not incorporate the correlation scheme and simply paired single pixels on the Cy5 channel with single pixels on the Cy3 channel.

For the zero force experiments, only pixel pairs that bleached in a single step in at least one dye were retained. For the force ramping experiments, we retained only pixel pairs with clear anti-correlation between Cy3 and Cy5 signals. After Cy5 dye bleaching, the background signal on the Cy5 channel was averaged and then subtracted from the fluorescence signal on both channels. The intensities for each channel were then averaged over each magnet position (typically 2 s intervals). The FRET efficiency E = $I_A/(I_A + \gamma I_D)$ was computed for each magnet position, where I_A is the background corrected, averaged acceptor intensity, I_D is the background corrected, averaged donor intensity, and γ is a factor that corrects for differences in detector efficiency and quantum yields for the Cy3 and Cy5 channels [6]. We calculated γ to be 2.35 by determining $\Delta I_A/\Delta I_D$, the ratio of the intensity changes of acceptor dye to donor dye after acceptor dye photobleaching, from 26 bleach events at zero force and averaging the results. Magnet positions were converted into forces using the equipartion theorem, as described elsewhere [7, 8]. The data analysis program TableCurve was used to fit the resulting E vs. f plots with 3 parameter exponentials.

Distribution Functions for Loop Predictions

The statistical behavior of a flexible chain with N Kuhn segments of length b ($= 2l_p$, where l_p is the persistence length) is governed by the end-to-end distribution function [9]

$$G_0(\vec{R}; N) = \left(\frac{2\pi N b^2}{3}\right)^{-3/2} \exp\left(-\frac{3R^2}{2Nb^2}\right), \tag{3}$$

which gives the probability that the vector joining the two chain ends is $\vec{R}$ (note, $R = |\vec{R}|$). The free energy of a chain with ends separated by a distance R is found as $-k_B T \log G_0(\vec{R}; N)$, which results in a quadratic free energy versus the end separation or a linear entropic spring. The statistical behavior of a flexible ring polymer is constructed by joining the individual segments together into a ring; each chain segment contributes a statistical contribution given by Eq. 3. The probability that two segments of a ring with $N_A + N_B$ total Kuhn segments of length b (N_A is the length on one section of the ring and N_B is the length on the other) are separated by the vector $\vec{R}$ is given by

$$G_R(\vec{R}; N_A, N_B) = \left[\frac{2\pi N_A N_B b^2}{3(N_A + N_B)}\right]^{-3/2} \exp\left[-\frac{3(N_A + N_B)R^2}{2N_A N_B b^2}\right], \tag{4}$$

where $R = |\vec{R}|$. The free energy found from Eq. 4 suggests that two points on a ring of length $N_A + N_B$ are acted on by two entropic springs, one of length N_A and the other of length N_B; therefore, two points on a ring are drawn together when compared to a free chain of equal length as either of the adjoining segments of the ring.

The statistical distribution functions given by Eqs. 3 and 4 are used to predict the FRET efficiency of the first two constructs in our experiments.

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