Supporting Information for

Crowding-induced hybridization of single DNA hairpins

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Supplementary Methods

Buffers were prepared with Tris (Sigma-Aldrich), Tris-HCl (Sigma-Aldrich), NaCl (Fluka) and $MgCl_2$ (Fluka) in ultrapure water (Direct Q3, Merck Millipore). Measurement buffers were cleaned using activated charcoal. For samples under molecular crowding conditions, the measurement buffer included ethylene glycol (EG) (Spectrophotometric grade ≥99% Sigma-Aldrich) or PEG 400 (Rotipuran, Carl Roth GmbH). PEG 8000 (Sigma) was used during TIRF and ensemble measurements; the commercial PEG 8000 12%-Tris pH8, 100 mM solution (Sigma-Aldrich) was used for MFD. All MFD measurement buffers included vitamin C (Fluka,); all TIRF measurements buffer included glucose (Sigma), glucose oxidase (Sigma), glucose catalase (Sigma) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich).

DNA oligonucleotides were synthesized and labelled by Purimex GmbH (Grebenstein, Germany) using NHS-esters of Alexa488 (5'/6' mixed isomer, Invitrogen), Cy3 (GE-Healthcare) or Cy5 (GE-Healthcare). The DNA sequences for the ssDNA, DNA hairpin, and donor-only control are shown below; an illustration of the hairpin structure is presented in Figure S1.

ssDNA Sequences

S1

5'-/biotin/ TGG CGA CGG CAG CGA GGC TTT TTT TTT TTT TTT TTT TTT TTT TTX/-3'

where X= T-Cy3

A1

5'- /Cy5/GCC TCG CTG CCG TCG CCA/-3'

S2

5’ XTT TTT TTT TTT TTT TTT TTT TTTX’ 3’

where X = T-Alexa 488 and X’ = T-Cy5

Hairpin sequences

H1

5'-/biotin/ TGG CGA CGG CAG CGA GGC TTA GCG GCA AAA AAA AAA AAA AAA

AAA AAA AAA AAA AAA AGC CGCX/-3'

where X= T-Cy3

H2

5' -/TGG CGA CGG CAG CGA GGC TTA GCG GCA AAA AAA AAA AAA AAA AAA

AAA AAA AAA AAA AGC CGC X/- 3'

where X= T-Alexa488

A2

5'- / GCC TCG CXG CCG TCG CCA/-3'

where X=T-Cy5

Donor-only sequences

D1

5’CGC GCT GTC TAC CGC GTA ACC GCC TAG GCT TAC TAG TCT CTA CTC GTA

GCA AGC TCX AGC ACC GCT CGC ACG TA3’

where X= T-Alexa488

Preparation of DNA samples

Single-molecule measurements

For the hairpin, complementary strands (1:1) were annealed in buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8) by heating them to 75 °C and then they were slowly cooled overnight.

For MFD measurements, the buffer contained 20 mM Tris, 15 mM NaCl (pH 7.5). For experiments under crowding conditions, ethylene glycol or PEG 400 was added to charcoaled measurement buffer. For samples in buffer with PEG 8000, the commercial 12% PEG 8000 solution was used directly and NaCl was added to this (15 mM). Before diluting the DNA samples to the single-molecule level, vitamin C was added into the buffers (1 mM).

Single molecule TIRF experiments were made in buffer containing 20 mM Tris-HCl, 10 mM NaCl (pH 7.8) with 6% glucose (w/w), 2 mg/mL glucose oxidase, 0.08 mg/mL glucose catalase and 1 mM Trolox added to reduce the rate of blinking and photobleaching of the dyes.[1]

Ensemble measurements

Samples were annealed and measured in the same buffer as the hairpins for single-molecule measurements (20 mM Tris-HCl, 50 mM NaCl, pH 8), except for those containing Alexa488, which were annealed and measured in buffer containing 20 mM Tris, 15 mM NaCl, 10 mM $MgCl_2$ (pH 7.5).

Steady-state ensemble fluorescence spectroscopy.

Alexa488 absorption and emission spectra were acquired on Lambda 1050 (Perkin-Elmer) and Fluorolog FL3-iHR spectrometer (HORIBA Scientific) spectrometers, respectively. Cy3 and Cy5 absorption and emission spectra were acquired on Cary 60 (Agilent Technologies) and

Fluoromax (HORIBA Scientific) spectrometers, respectively. All spectra were recorded under magic angle conditions and background fluorescence from the solvent was negligible.

RatioA values[2] were calculated by measuring fluorescence emission spectra from Cy3-Cy5 labelled hairpins using excitation wavelengths of 510 nm and 610 nm, respectively. A fluorescence spectrum from the donor strand (Cy3 only) was also recorded using 510 nm excitation and subsequently normalized to the donor peak of the FRET sample. This normalized Cy3 emission was subtracted from the spectrum displaying FRET to leave only sensitized acceptor emission, which was then divided by the emission spectrum of the FRET sample recorded at 610 nm excitation to give the final ratioA values.

Time-resolved ensemble fluorescence spectroscopy.

Time-correlated single photon counting was performed using a single-photon counting controller (Fluoro3PS, HORIBA Scientific) and a photodiode detector PDM-400 (Photonic Solutions Ltd). The excitation source was a tunable supercontinuum laser SC-400P (Fianium Ltd) with instrument response of *ca.* 100 ps at a repetition rate of 1 MHz, at a wavelength of 430 nm. Decay curves were analyzed using a standard iterative reconvolution method using the DAS6 software. The best fitting was selected on the basis of the reduced chi-square statistic, χ^2. To record time-resolved anisotropy decays, parallel (I_{II}) and perpendicular ($I_{\perp}$) emission decays were recorded sequentially. To offset instrument factors, the G-factor ($G = I_{HV}/I_{HH}$) was measured during every experiment, employing horizontal polarized excitation, and applied to correct the data. Intensity decays were analyzed using DAS6; the sum ($S(t) = I_{II} + 2GI_{\perp}$) and difference ($D(t) = I_{II} - GI_{\perp}$) between polarized intensity decays were employed to generate the anisotropy curves ($R(t) = D(t)/S(t)$).

Multiparameter confocal fluorescence spectroscopy.

For single-molecule measurements in solution, we used a home-built MFD setup, which is based around a confocal microscope with photon-counting detection (Becker and Hickl) and pulsed laser excitation (Picoquant), allowing the simultaneous measurement of fluorescence intensity, color, lifetime and polarization; details of the setup and procedure are found elsewhere, with the only difference that one photon-counting board was used for all four detection channels.[3] All measurements were recorded at 21±1°C. Data analysis for MFD used software written by the group of Prof. Claus Seidel (Heinrich Heine Universität, Düsseldorf).

The gray scale in Fig. 2 indicates an increasing number of single-molecule bursts (from white to black). Also shown are the corresponding 1D histograms. FRET efficiencies were measured from raw green and red signals and corrected for background (for 0% PEG 0.696 KHz green, 0.481 KHz red; for 11% ethylene glycol 0.958 KHz green, 0.488 KHz red; for 11% PEG 400 1.033 KHz green, 0.539 KHz red; and for 10% PEG 8000 1.130 KHz green, 0.649 KHz red), spectral crosstalk (3.1%), detection efficiencies (green:red = 0.3) and the fluorescence quantum yields (0.80 for donor; 0.32 for acceptor).

The DNA dilutions were prepared in Nunc Lab-Tek II chambered cover glasses, #1,5 (4 wells, Catalogue# 155382, Thermo Scientific, Massachusetts, USA).

TIRF microscopy.

The single-molecule TIRF experiments presented in this work were performed using objective-type total-internal reflection (o-TIR) wide-field microscopy, where the DNA constructs were immobilized to a PEG passivated glass coverslip via biotin-neutravidin interactions. Microfluidic flow cells containing immobilized DNA were mounted on an inverted microscope (IX71, Olympus). Excitation light (532 nm line of a Scherzo (Klastech) diode-pumped solid

state laser) was provided via the evanescent wave of a totally internally reflected beam. For proper angles of incidence, TIR takes place at the interface between the lower coverslip and the sample. The intensity of the evanescent wave decays exponentially upon penetrating the sample, with a decay length of approximately 100-200 nm. Fluorescence emission was collected using a 100 x 1.49 numerical aperture oil-immersion objective lens (Olympus) and separated from scattered excitation via a 550 nm long-pass filter and a 532 nm band-reflect dichroic mirror (Chroma Technology Corp.). Fluorescence was subsequently collimated and separated by donor and acceptor emission wavelengths with an emission splitting system (DV2 Multichannel Imaging System, Photometrics) such that spatially identical but spectrally distinct images of donor and acceptor fluorescence were acquired simultaneously on an EMCCD camera (Evolve, Photometrics). All measurements were performed at room temperature (21 ± 1^{o}C).

Recording movies

DNA hairpins were immobilized to the cover slip surface using biotin-neutravidin interactions at an approximate concentration of 10 pM. This gave approximately 200 visible spots on the donor channel. An enzymatic oxygen scavenging system and Trolox were used in combination to suppress the rate of blinking and photobleaching. The fluorescence intensities of the emission from the Cy3 (donor) and Cy5 (acceptor) dyes were detected using an EMCCD camera and the TIF movies were recorded by ImagePro-Plus 7.0 software using an exposure time of 50 ms.

Processing movies

The TIF movies were analyzed by a freely available MATLAB program called TwoTone.[4] A mapping file was made from a TIF image taken from a recording of fluorescent microspheres (Life Technologies) that emit in both the donor and acceptor channels. This enables any corrections to be made when finding spots in different channels that correspond to the same donor/acceptor pair. Each movie was loaded and intensity thresholds were set for the donor and

acceptor channels so that only the brightest 200 spots were analyzed. The intensity of each FRET pair for each frame was then recorded as an array in MATLAB.

Measuring the dwell times

The fluorescence vs. time traces were viewed using a MATLAB program written in-house. The efficiency of the FRET was calculated using the donor and acceptor intensities (I) given from the TwoTone software, using E= I(acceptor)/[I(donor) + I(acceptor)]. These files were then stitched together and the resulting .dat file was analyzed by HaMMy, which employed a hidden Markov model to identify transitions between the open and closed states.[5] The HaMMy program gave the dwell times of each transition (over 250-1000 transitions per condition), which were imported into OriginPro 9.0 and a frequency count gave a histogram that was fitted to a single exponential curve. This allowed the rate constants and standard errors of the transitions to be calculated.

Calculating ΔG^0 and $\Delta\Delta G^0$

The stitched time traces were imported into OriginPro 9.0 and a frequency count was taken to give histograms of the E values of all points from 65-219 traces. This gave two peaks in each histogram that were fitted to Gaussian curves. The area underneath each curve was recorded as A(open) and A(closed) and was used to calculate the equilibrium constant K=A(closed)/A(open). This in turn was used to find the Gibbs free energy for the closing of the hairpin, $\Delta G^0 = -RT\ln K$, and the change in Gibbs free energy of closing due to the presence of crowders $\Delta\Delta G^0 = \Delta G^0$(n% PEG)$-\Delta G^0$(0% PEG).

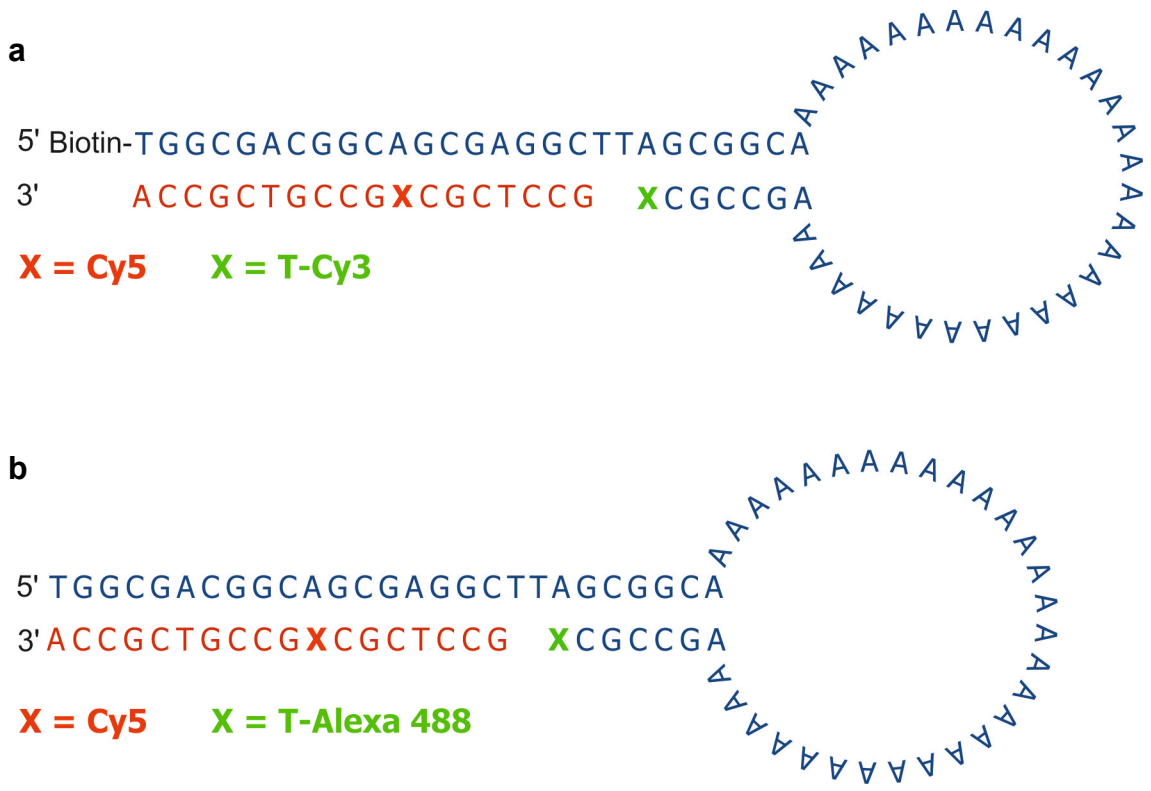

Figure S1. a) Hairpin structure (strands H1 and A2) used for TIRF experiments; b) hairpin structure (strand H2 and A2) used for MFD experiments.

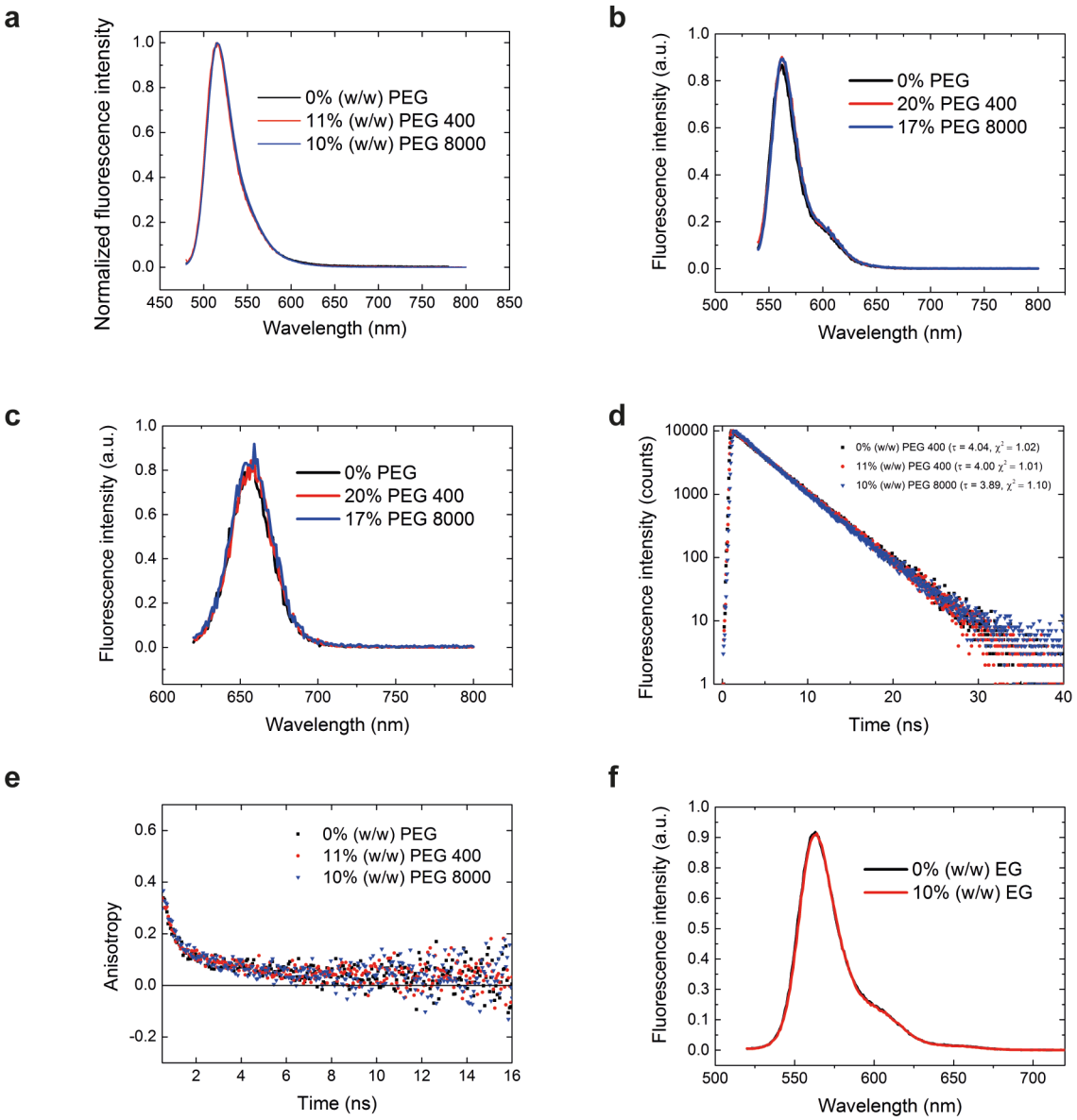

Figure S2. a) Normalized emission spectra of 400 nM Alexa488 on ssDNA (Strand D1) in PEG 400. The excitation wavelength was 430 ± 2nm. b, c) Emission spectra of 200 nM ssDNA labeled with Cy3 (b, Strand S1) and 150 nM Cy5 (c, Strand A1) without or with 20% (w/w) PEG 400 or 17% (w/w) PEG 8000. Fluorescence intensities are corrected for dilution using the absorption spectrum of each sample. The Cy3 and Cy5 were directly excited at 510 nm and 610 nm, respectively. d) Ensemble lifetime of 400 nM Alexa488-labelled ssDNA (Strand D1) in PEG 400 and PEG 8000. Intensity decays of Alexa 488 were recorded under magic angle conditions, using an excitation wavelength of 430 nm. e) Ensemble time-resolved anisotropy of 400 nM Alexa488-labelled ssDNA (Strand D1) in PEG 400 and PEG 8000. f) Emission spectrum of 150 nM hairpin sample (strands H1 and A2) in buffer with and without 10% (w/w) ethylene glycol (EG). The excitation wavelength was 510 nm.

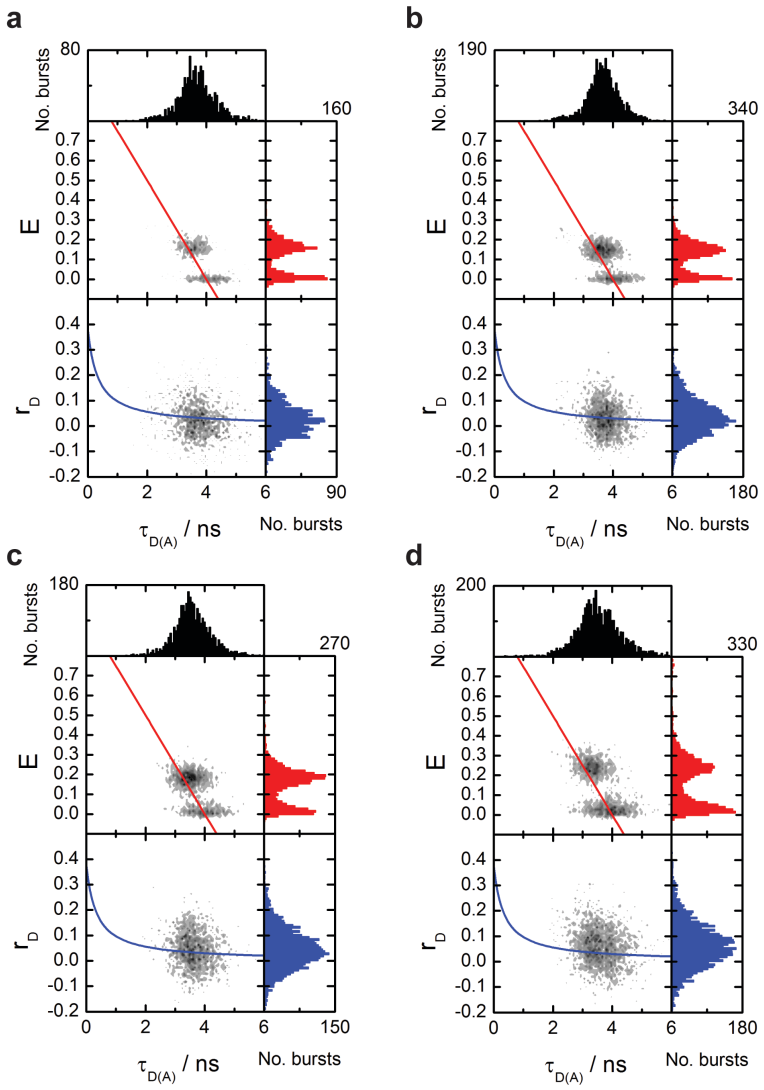

Figure S3. Multiparameter detection of ssDNA (S2) under molecular crowding conditions: 2D burst-frequency histograms of FRET efficiency (E) or donor anisotropy (r_D) versus donor lifetime ($\tau_{D(A)}$) for a doubly-labelled ssDNA (S2) in buffer only (a) or in buffer containing 11% (w/w) EG (b), 11% (w/w) PEG 400 (d) or 10% (w/w) PEG 8000 (d). The gray scale indicates an increasing number of single-molecule bursts (from white to black). Also shown are the corresponding 1D histograms. FRET efficiencies were measured from raw green and red signals and corrected for background (for 0% PEG 0.696 KHz green, 0.481 KHz red; for 11% EG 1.051 KHz green, 0.643 KHz red; for 11% PEG 400 1.101 KHz green, 0.635 KHz red; and for 10% PEG 8000 1.104 KHz green, 0.682 KHz red), spectral crosstalk (3.1%), detection efficiencies (green:red = 0.3) and the fluorescence quantum yields (0.80 for donor; 0.32 for acceptor). The red overlaid line is the theoretical FRET relationship, $E = 1 - (\tau_{D(A)}/\tau_D)$, , with $\tau_D = 4.0$ ns. The blue overlaid line is the Perrin equation, $r_D = r_0/(1 + \tau_{D(A)}/\rho_D)$, with mean rotational correlation time $\rho_D = 0.35$ ns and fundamental anisotropy $r_0 = 0.375$.

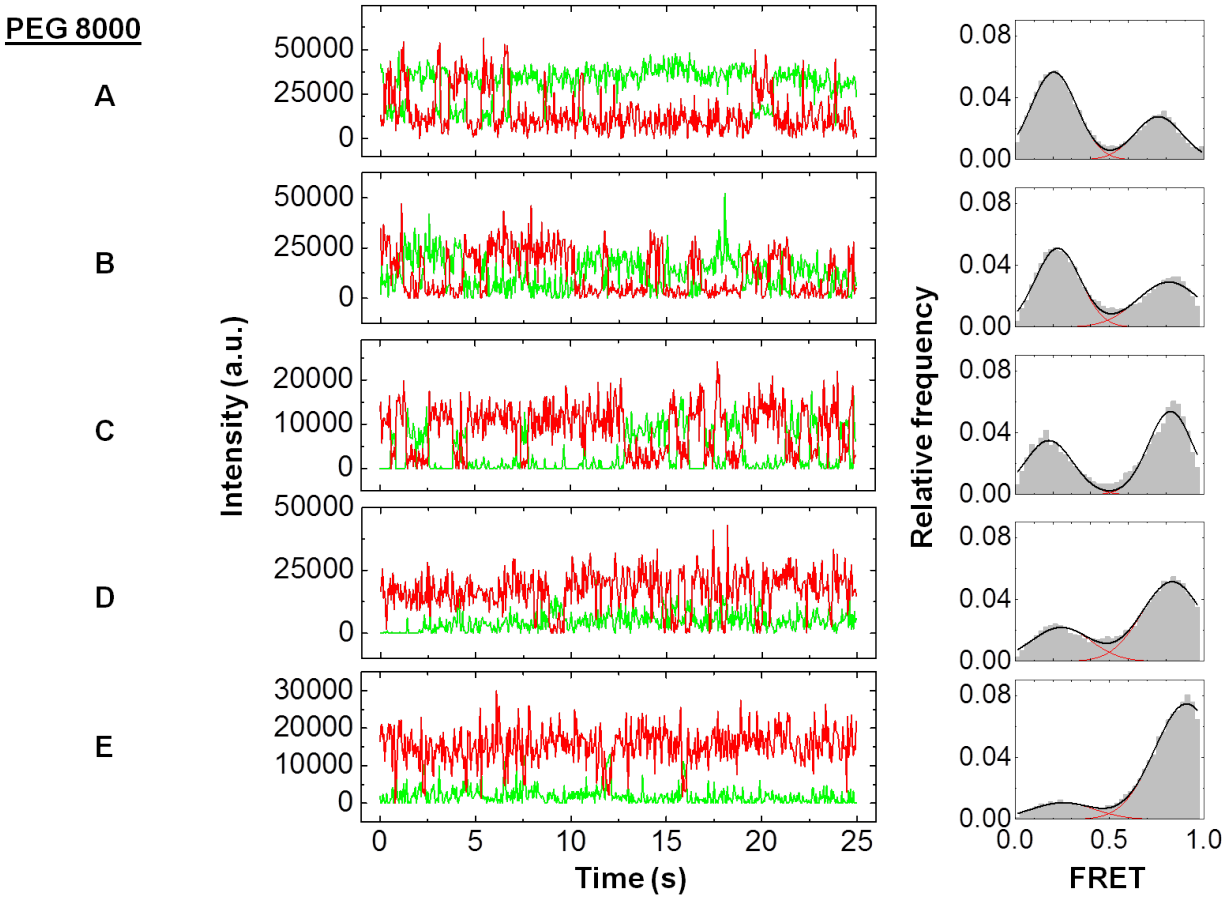

Figure S4. Representative single molecule traces from immobilized hairpins labeled with Cy3 (green) and Cy5 (red,) in the presence of increasing concentrations of PEG 8000 (a-e represent 0%, 2%, 5%, 7% and 9% (w/w) PEG 8000 respectively.) The histograms to the right of each trace represent the FRET values from 65-115 molecules in each concentration of PEG 8000 showing only two states representing the open and closed conformations of the hairpin. At low concentrations of PEG 8000, the hairpin was more likely to be found adopting an open concentration; however as the concentration of PEG 8000 was increased, the closed conformation became more favored.

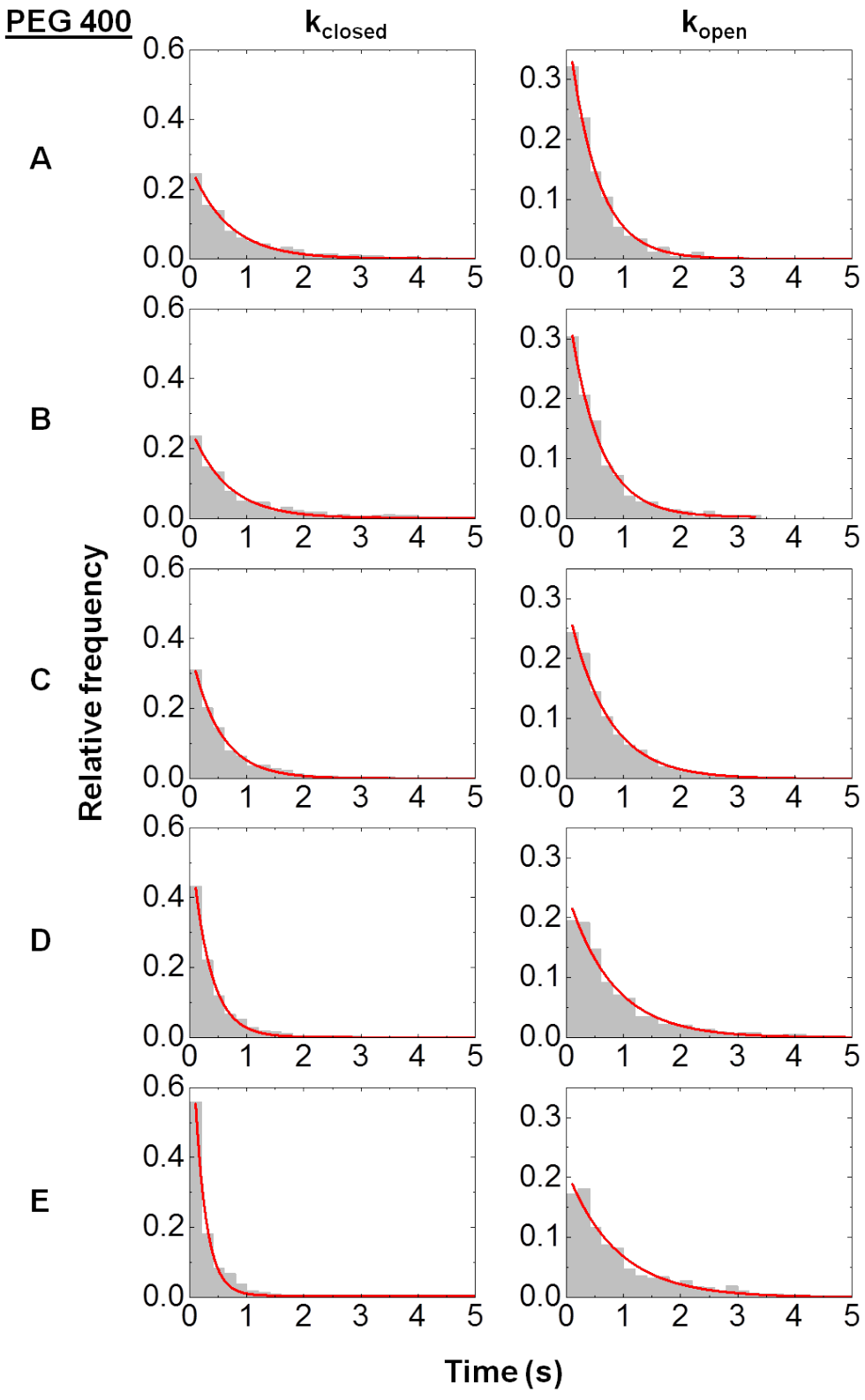

Figure S5. Histograms of binned dwell times of the open and closed states of the hairpin immobilized on to PEG surface were fitted to a single exponential and used to calculate the opening (k_{open}) and closing (k_{closed}) of the hairpin in increasing concentrations of PEG 400. (a-e represent 0%, 2.5%, 5%, 7.5% and 10% (w/w) PEG 400 respectively.)

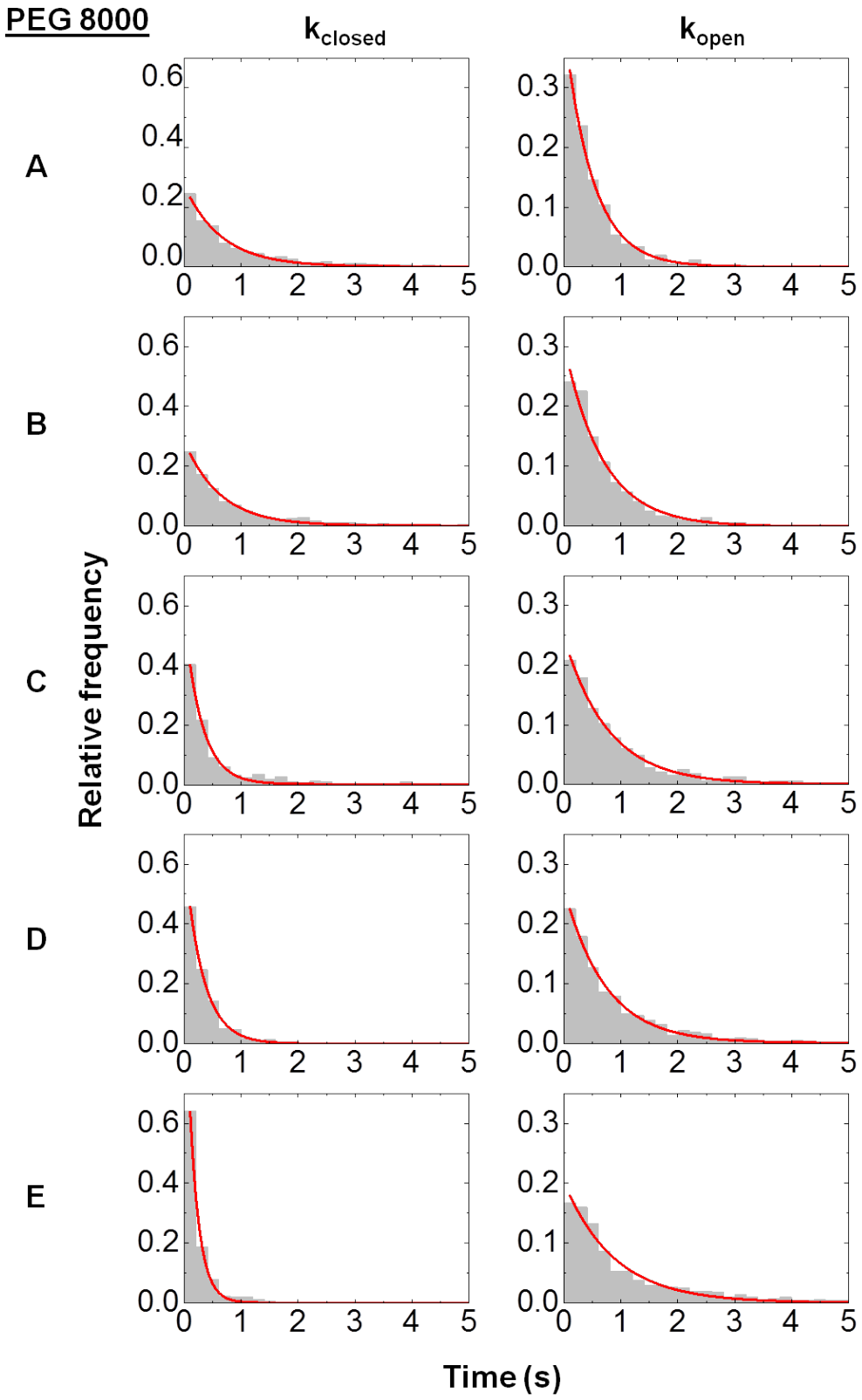

Figure S6. Histograms of binned dwell times of the open and closed states of the hairpin immobilized on to PEG surface were fitted to a single exponential and used to calculate the opening (k_{open}) and closing (k_{closed}) of the hairpin in increasing concentrations of PEG 8000. (a-e represent 0%, 2%, 5%, 7% and 9% (w/w) PEG 8000 respectively.)

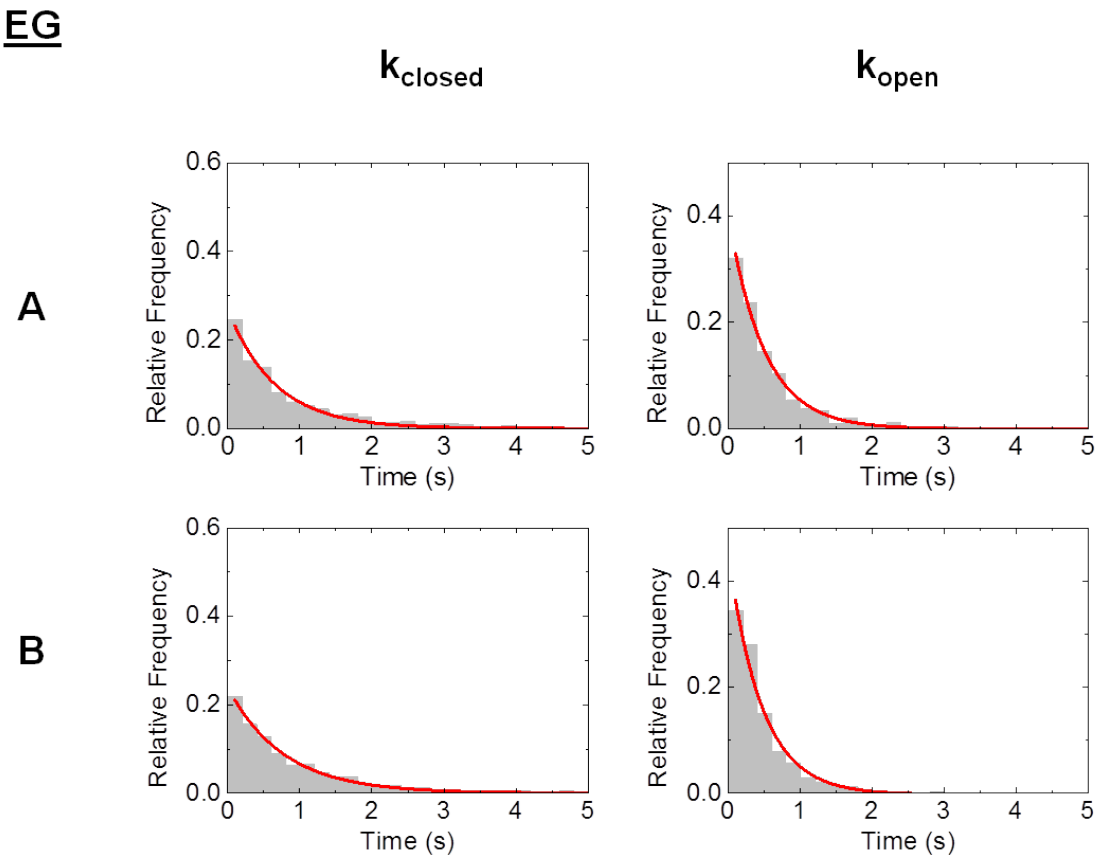

Figure S7. Histograms of binned dwell times of the open and closed states of the hairpin immobilized on to PEG surface were fitted to a single exponential and used to calculate the opening (k_{open}) and closing (k_{closed}) of the hairpin in a) 0% and b) 10% (w/w) EG.

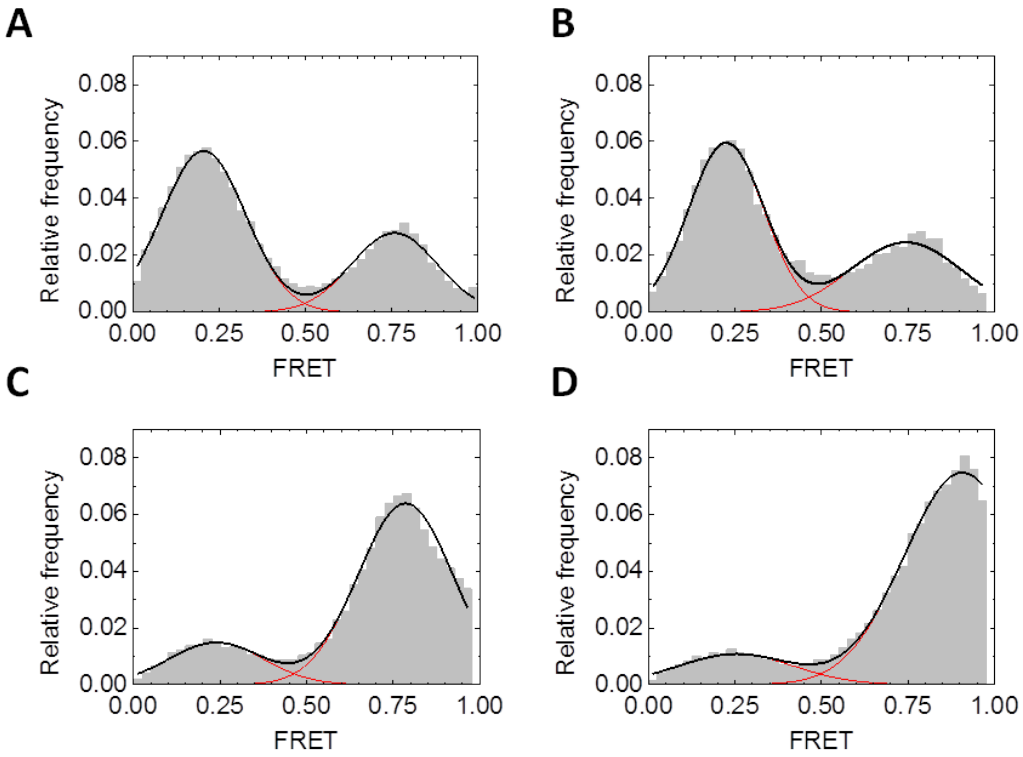

Figure S8. Histograms of FRET efficiency representing 112 and 123 single molecule traces from immobilized hairpins in a) buffer only and b) 10% (w/w) EG. The histograms from hairpins in buffer that contained c) 10% (w/w) PEG 400 and d) 9% (w/w) PEG 8000 are also shown again for comparison.

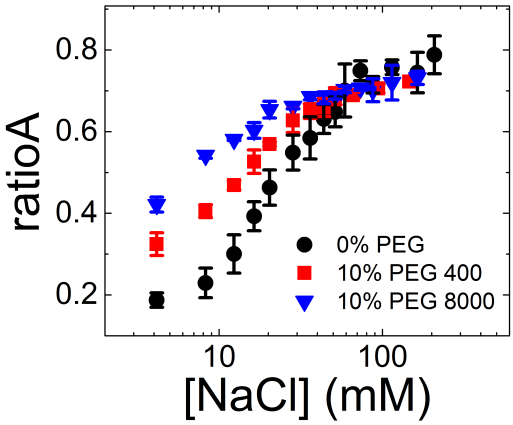

Figure S9. Ensemble fluorescence experiments were used to measure the ratioA[2] values in order to report on the changes of the FRET efficiency of Cy3-Cy5 labeled hairpin used in the TIRF experiments at increasing NaCl concentrations, in 0% PEG, 10% (w/w) PEG 400 and 10% (w/w) PEG 8000. 150 nM hairpin was excited at 510 nm and 610 nm to excite the donor and acceptor dyes respectively. The experiment was completed in triplicate and the mean values are plotted with the standard deviation shown as error bars.

Solution	**Low FRET (open) lifetime / ns**	**High FRET (closed) lifetime / ns**	**E (closed)**	**R_{DA} (closed) / Å**
Buffer only	3.68±0.01	1.91±0.01	0.52	51
10% (w/w) EG	3.71±0.01	2.01±0.02	0.49	52
10% (w/w) PEG 400	3.76±0.02	2.07±0.02	0.53	51
10% (w/w) PEG 8000	3.66±0.02	1.97±0.01	0.53	51

Table S1. Lifetimes and calculated FRET distance for diffusing hairpins. Extracted from data in Figure 2. Estimated standard deviation for R_{DA} is ca. 3Å.

Solution	Donor lifetime / ns	FRET lifetime / ns	R_{DA} / Å
Buffer only	4.07±0.02	3.52±0.01	71
10% (w/w) EG	3.96±0.02	3.54±0.01	73
10% (w/w) PEG 400	3.95±0.01	3.46±0.01	72
10% (w/w) PEG 8000	3.91±0.02	3.35±0.01	68

Table S2. Lifetimes and calculated FRET distance for polyTssDNA (S2). Extracted from data in Figure S3. Estimated standard deviation for R_{DA} is ca. 3Å.

[PEG 400] (% w/w)	k_{closed} (s^{-1})	k_{open} (s^{-1})
0	1.52±0.03	2.01±0.05
2.5	1.58±0.06	1.90±0.09
5	1.98±0.03	1.47±0.03
7.5	3.08±0.05	1.26±0.03
10	5.02±0.02	1.14±0.03

Table S3. Rate constants for the opening (k_{open}) and closing (k_{closed}) of the hairpin in PEG 400 calculated from the single exponential fits from the histograms of the dwell times in Figure S5.

[PEG 8000] (% w/w)	k_{closed} (s^{-1})	k_{open} (s^{-1})
0	1.52±0.03	2.01±0.05
2	1.59±0.03	1.47±0.07
5	1.95±0.12	1.35±0.10
7	3.13±0.05	1.37±0.04
9	5.81±0.08	1.13±0.02

Table S4. Rate constants for the opening (k_{open}) and closing (k_{closed}) of the hairpin in PEG 8000 calculated from the single exponential fits from the histograms of the dwell times in Figure S6.

[EG] (% w/w)	k_{closed} (s^{-1})	k_{open} (s^{-1})
0	1.52±0.03	2.01±0.05
10	1.29±0.02	2.16±0.01

Table S5. Rate constants for the opening (k_{open}) and closing (k_{closed}) of the hairpin in the absence and presence of 10% (w/w) EG calculated from the single exponential fits from the histograms of the dwell times in Figure S7.

References

(1) Rasnik, I.; McKinney, S. A.; Ha, T. *Nat. Methods* **2006**, *3*, 891.
(2) Clegg, R. M.; *Methods Enzymol.* **1992**, *211*, 353.
(3) Sabir, T.; Schröder, G. F.; Toulmin, A.; McGlynn, P.; Magennis, S. W. *J. Am. Chem. Soc.* **2011**, *133*, 1188.
(4) Holden, S. J.; Uphoff, S.; Hohlbein, J.; Yadin, D.; Le Reste, L.; Britton, O. J.; Kapanidis, A. N. *Biophys. J.* **2010**, *99*, 3102.
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