

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

DNA standards

Oligonucleotides were prepared by automated synthesis (IBA GmbH, Göttingen, Germany). The sequences used are shown from 5' to 3' and the underlined T base was labeled either with donor fluorophores (Cy3, Cy3B, Atto532) at the top strand, STD45T, or acceptor fluorophores (Cy5, Alexa647, Atto647, Atto647N) at the bottom strands, STD45B series.

STD45T: TAAATCTAAAGTAACATAAGGTAACATAACGTAAGCTCATTCGCG

STD45BdT13: CGCGAATGAGCTTACGTTATGTTACCTTATGTTACTTTAGATTTA

STD45BdT28: CGCGAATGAGCTTACGTTATGTTACCTTATGTTACTTTAGATTTA

STD45BdT38: CGCGAATGAGCTTACGTTATGTTACCTTATGTTACTTTAGATTTA

Labeling was performed by IBA GmbH using the amino-C6-modifying group with excess of N-hydroxy-succinimidyl ester of the respective organic fluorophore.

Singly-labeled dsDNA standards were prepared by annealing singly-labeled STD45T (donor fluorophores) and singly-labeled STD45BdT38 (acceptor fluorophores) with unlabeled STD45dT38 and STD45T, respectively.

DNA FRET standards (low FRET DNA STD and intermediate FRET DNA STD) were prepared by annealing Cy3B-labeled STD45T with Atto647N-labeled STD45BdT13 and STD45BdT28, respectively. The high FRET DNA standards were prepared by annealing Cy3B-labeled STD45T with Atto647N or Atto647 or Cy5-labeled STD45BdT38. The oligonucleotides were annealed in a low-salt annealing buffer (20 mM Tris-HCl (pH 8.0), 10-100 mM NaCl, 1 mM EDTA) by heating to 94 °C and subsequent cooling to 4 °C over 45 min in steps of 10 °C. After annealing, the high FRET DNA standards were run on a Native gel and only doubly-labeled bands were cut out and purified.

Electrocompetent cells

ElectroMAX DH5 α -E Competent Cells (Invitrogen, Carlsbad, CA, USA), a commercially available electrocompetent *E.coli* cell line was used for electroporation. Cells were diluted 1:1 with sterile milli-Q water and stored at -80 °C. For each electroporation experiment 20 μ L of electrocompetent cells were used.

Electroporation

Up to 5 μ L of labeled dsDNAs stored in a low-salt buffer were added to 20 μ L electrocompetent cells (10 nM to 1 μ M dsDNA concentration in cuvette) and incubated 10 min on ice. The mixture of electrocompetent cells and labeled dsDNAs was transferred into a prechilled electroporation cuvette (0.1 cm gap cuvette, Bio-Rad, Hercules, CA, USA) and placed into an electroporator (MicroPulser, Bio-Rad). An electric field of 1.4 kV/cm (1.0-1.8 kV for viability and loading study) was applied for electroporation. 500 μ L of super optimal broth with catabolite repression (SOC) was added immediately after electroporation. Cells were recovered for 20 min at 37 °C. After

recovery, cells were harvested by centrifugation at 3300 g for 1 min at 4 °C and washed 5 times with 500 μ L phosphate buffered saline (PBS). Cells were resuspended in 80-150 μ L PBS and placed on 1% agarose pads before imaging. The pads themselves were made from ~250 μ L of M9 medium containing 1% (v:w) BioRad Certified Molecular Biology Agarose on a coverslip. After about 5 μ L of cells were pipetted onto the pad, another coverslip was added on top. The slide/agar/slide sandwich was inverted and placed on the microscope with the side containing the cells closest to the objective.

For viability analysis, the cells were recovered for another 60 min after washing. Cells were pipetted on agarose pads, EZ rich-defined medium containing 1% agarose (BioRad), and imaged at 37 °C over the course of up to 1.5 h.

Cell imaging

Single-cell fluorescence microscopy in live bacteria was performed on a customized inverted Olympus IX-71 microscope equipped with two lasers, a 637 nm diode laser (Vortran Stradus, Vortran Laser Technology, Sacramento, CA, USA) and a 532 nm Nd:YAG laser (Samba, Cobolt AB, Solna, Sweden). Laser light was combined into a single-mode optical fiber (Thorlabs, Newton, NJ, USA) and collimated before focusing on the objective. Cells were imaged using either WF illumination for loading and single-cell photobleaching studies or near-TIRF (nTIRF; also known as HILO (Tokunaga et al., *Nature Methods*, 2008, 5:159-161)) illumination for single-cell FRET studies by adjusting the position of the focused excitation light on the back focal plane of the objective. Typical excitation powers were 0.3-2 mW for WF and nTIRF illumination and exposure times ranged from 50-100 ms. Cellular fluorescence was collected through the same objective, filtered to remove excitation light through a long-pass filter (HQ545LP, Chroma, Taoyuan Hsien, Taiwan) and a notch filter (NF02-633S, Semrock, Rochester, NY, USA), and spectrally separated by a dichroic mirror (630DRLP, Omega, Brattleboro, VT, USA). Each channel was imaged onto separate halves of the chip of an electron-multiplying charge-coupled device camera (iXon+, BI-887, Andor, Belfast, UK). The illumination for brightfield images comprised a white-light lamp (IX2- ILL100, Olympus, Shinjuku, Tokyo, Japan) and condenser (IX2-LWUCD, Olympus) attached to the microscope. Movies and images were recorded using manufacturer's software or Micromanager (valelab.ucsf.edu/~MM/MMwiki/). Single-cell photobleaching measurements were obtained at 100 ms temporal resolution and 600 μ W-green and 300 μ W-red excitation powers; donor and acceptor fluorophores, respectively. Single-cell FRET measurements were carried out using an alternating laser excitation scheme¹⁸. Typical exposure times and excitation powers were 50 ms, 1 mW-green and 0.5 mW-red.

Single-cell photobleaching analysis

Cells were segmented automatically by adapting the MATLAB implementation Schnitzcells (Young et al., *Nature Protocols*, 2012, 7:80:88) for brightfield cell images. The manually adjustable cell masks were saved and were then used to extract cells' fluorescence data by calculating the total fluorescence intensity per cell area within each cell mask for each movie frame. The cell autofluorescence per cell area after photobleaching was subtracted using a custom-written Matlab script (The MathWorks, Natick, MA, USA).

Baseline-subtracted photobleaching timetraces of heavily loaded cells were fitted with a single exponential to obtain the cell-based photobleaching lifetime as a measure of fluorophore photostability.

Baseline-subtracted photobleaching timetraces of very little loaded cells, i.e. showing < 6 quantized steps were fitted with a hidden Markov model (HMM) as described in¹ to obtain the fluorophore brightness. Briefly, HMM is a stochastic model that maps measured values to unobserved (or hidden) states. Here, the time trajectories were modeled as a sequence of up to 10 hidden states (different cell intensity levels) and transitions between these states (photobleaching event). A custom-written Matlab script was run recursively; keeping only the state values for the last photobleaching step and removing this data before the next iteration step. Each iteration allowed up to 10 hidden states to be fitted. Such a method took advantage of the exponential photobleaching kinetics: the last photobleaching step was likely to last significantly longer than previous steps, and thus fitting the last step was likely to have the greatest data support. For the step size estimate of the fitted steps, the absolute difference between consecutive intensity states was calculated taking fluorophore blinking into account. Only step sizes from about 70-100 cells per sample with photobleaching timetraces with less than 6 steps recovered by the HMM algorithm were plotted in the step height histogram. The step height histogram was fitted with a single one-dimensional-Gaussian (free fit parameters: position1, width1, amplitude1) for donor fluorophores and double Gaussian (free fit parameters: position1, width1, amplitude1, amplitude2; position2 = 2 × position1, width2 = $\sqrt{2}$ × width1) for acceptor fluorophores. The single-molecule unitary intensity was given as the center of the Gaussian fitting the main peak. Small populations of multiple simultaneous bleaching events account for larger step sizes that are fitted by second Gaussians in case of acceptor fluorophores single-cell photobleaching studies.

***In vitro* single-molecules FRET analysis**

In vitro single-molecule FRET data was acquired on a confocal microscopy setup and analyzed using Matlab scripts as described in⁴. Fluorescence bursts in the donor and acceptor channel were recorded under donor or acceptor excitation (ALEX). Uncorrected FRET efficiencies, E^* , and relative probe stoichiometry, S , were calculated as follows

$$E^* = \frac{F_{DA}}{F_{DA} + F_{DD}}$$

$$S = \frac{F_{DA} + F_{DD}}{F_{DA} + F_{DD} + F_{AA}}$$

where F_{XY} represents the fluorescence of a burst arising in the Y emission channel under X excitation.

***In vivo* single-cell FRET analysis**

Single-cell FRET efficiencies were obtained by measuring the variation in acceptor emission intensity on the cell-level. For low, intermediate and high FRET dsDNA Cy3B-Atto647N (**Fig. 4B**) cells were automatically segmented. Whereas for high FRET dsDNA Cy3B-Cy5/Atto647/Atto647N (**Fig. 4C**) cells were manually segmented using a custom-written Matlab script (MathWorks). Cells were segmented in the brightfield image corresponding to the red emission channel and the mask was transformed onto the green emission channel. Thus, the cell masks were used to extract cells' fluorescence data in the red and green emission channel. The fluorescence intensity was corrected for autofluorescence background. In the case of automated segmentation the average pixel intensity from non-treated cells was subtracted as a background value for each emission channel and in the case of manual segmentation the background around the

cell was locally obtained for each cell. Background subtracted fluorescence intensities were then used to calculate single-cell FRET values as follows

$$E_{\text{single cell}} = \frac{F_{\text{DA}} - BG_{\text{DA}}}{F_{\text{DA}} - BG_{\text{DA}} + F_{\text{DD}} - BG_{\text{DD}}}$$

where F_{XY} and BG_{XY} represent the fluorescence and background signal in the Y emission channel under X excitation, respectively.

Fluorescence overlay images were obtained by overlaying the donor (green) and acceptor (red) fluorescence channels colored green and red, respectively. The green fluorescence channel was transformed onto the red fluorescence channel. All transformation matrices were based on a calibration matrix generated each day where fluorescent beads were mapped from the green onto the red fluorescence channel.

SUPPLEMENTARY FIGURES

Figure captions.

Figure S1

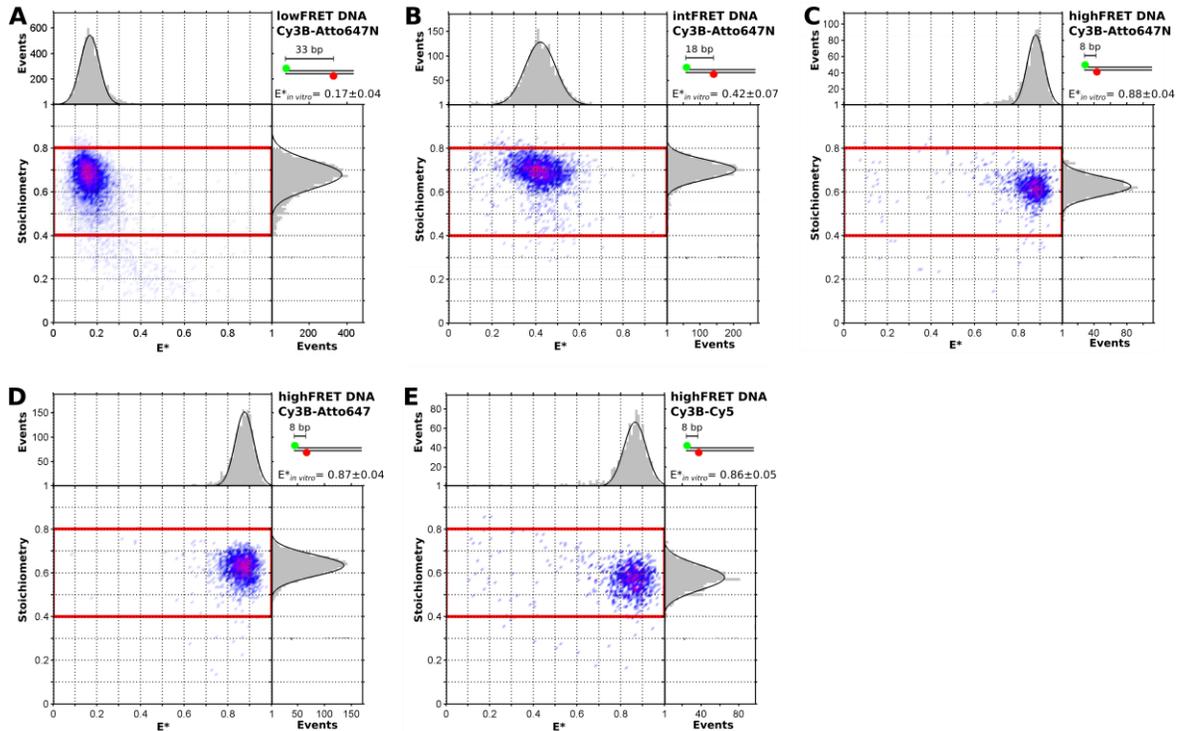
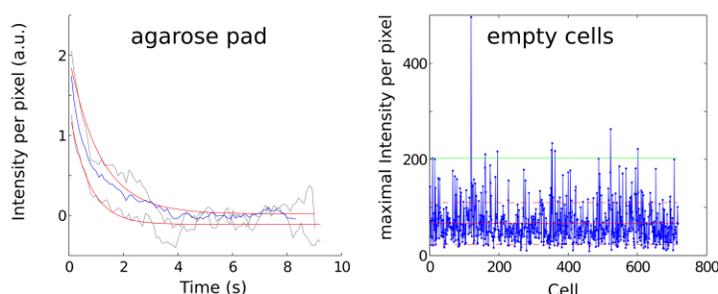


Fig. S1 *In vitro* measurements of DNA FRET standards using single-molecule confocal microscopy and an alternating laser excitation scheme. **A-C**. Analyzed low, intermediate and high FRET DNA standards Cy3B-Atto647N give uncorrected FRET efficiencies of 0.17 ± 0.04 , 0.42 ± 0.07 and 0.88 ± 0.04 , respectively. **D-E**. Uncorrected FRET efficiencies of 0.87 ± 0.04 and 0.85 ± 0.05 were obtained for high FRET DNA standards labeled with Cy3B-Atto647 and Cy3B-Cy5, respectively.

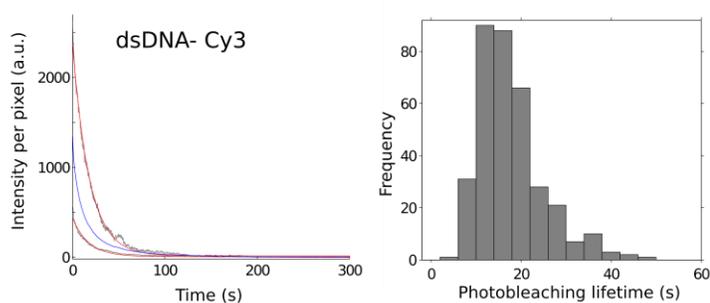
Figure S2

A Green fluorescence channel



Donor fluorophores

B



C

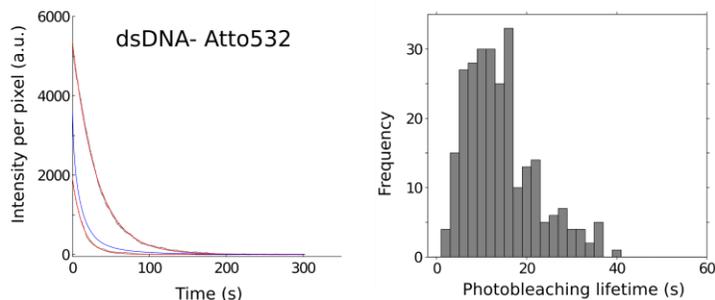
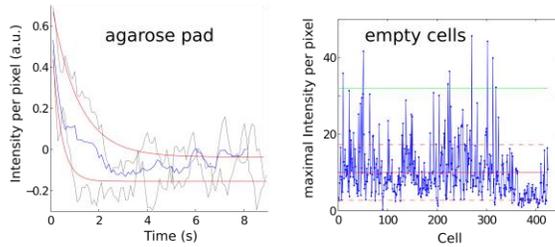


Fig. S2 Cell-based photobleaching studies of organic fluorophores in green fluorescence channel. **A.** Autofluorescence signal in green fluorescence channel. Left: Autofluorescence decay of agarose pad only; two photobleaching timetraces (gray), single-exponential fits (red) and average photobleaching timetrace (blue). The photobleaching lifetime of the agarose pad is less than 1 s. Right: Maximal normalized cell intensity of non-treated cells ('empty cells') with loading threshold of mean \pm 3 stdev of non-treated cells (green line); here about 1% of non-treated cells would be classified as loaded. **B.** and **C.** Cell-based photobleaching studies of dsDNA-Cy3 and dsDNA-Atto532, respectively. Left: Cell-based photobleaching timetraces (gray), single-exponential fits (red) and average photobleaching timetraces (blue). Right: Histogram of photobleaching lifetime obtained by single-exponential fit of cell-based photobleaching timetraces. Each data set consists of 300-600 cells. Photobleaching lifetime of (18 ± 7) s, median: 16 s, and (14 ± 8) s, median: 13 s, were obtained for Cy3 and Atto532, respectively.

Figure S3

A Red fluorescence channel



Acceptor fluorophores

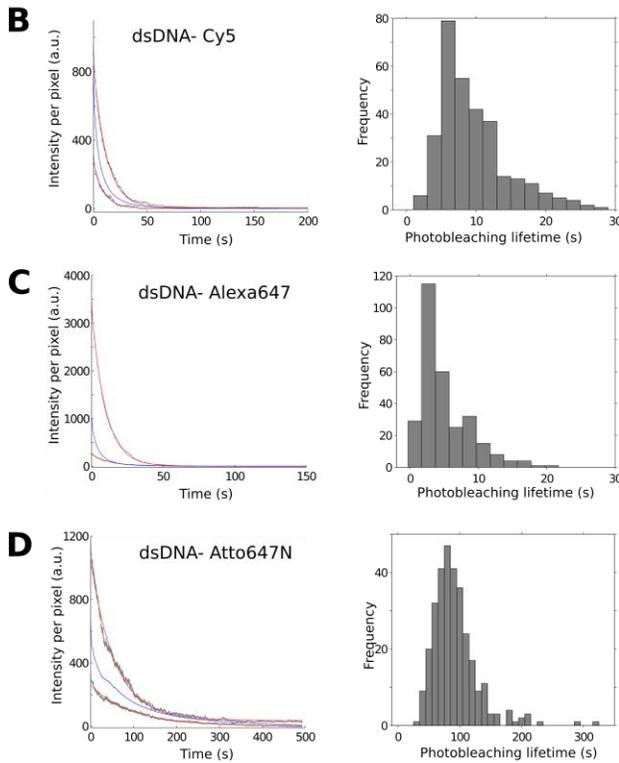


Fig. S3 Cell-based photobleaching studies of organic fluorophores in red fluorescence channel. **A**. Autofluorescence signal in red fluorescence channel. Left: Autofluorescence decay of agarose pad only; two photobleaching timetraces (gray), single-exponential fits (red) and average photobleaching timetrace (blue). The photobleaching lifetime of the agarose pad is about 0.5 s. Right: Maximal normalized cell intensity of non-treated cells ('empty cells') with loading threshold of $\text{mean} \pm 3\text{stdev}$ of non-treated cells (green line); here less than 2% of non-treated cells would be classified as loaded. **B**, **C** and **D**. Cell-based photobleaching studies of dsDNA-Cy5, dsDNA-Alexa647 and dsDNA-Atto647N, respectively. Left: Cell-based photobleaching timetraces (gray), single-exponential fits (red) and average photobleaching timetraces (blue). Right: Histogram of photobleaching lifetime obtained by single-exponential fit of cell-based photobleaching timetraces. Each data set consists of 250-450 cells. Photobleaching lifetime of (10 ± 5) s, median: 8 s, (6 ± 4) s, median: 5 s, and (92 ± 37) s, median: 86 s, were obtained for Cy5, Alexa647 and Atto647N, respectively.

Figure S4

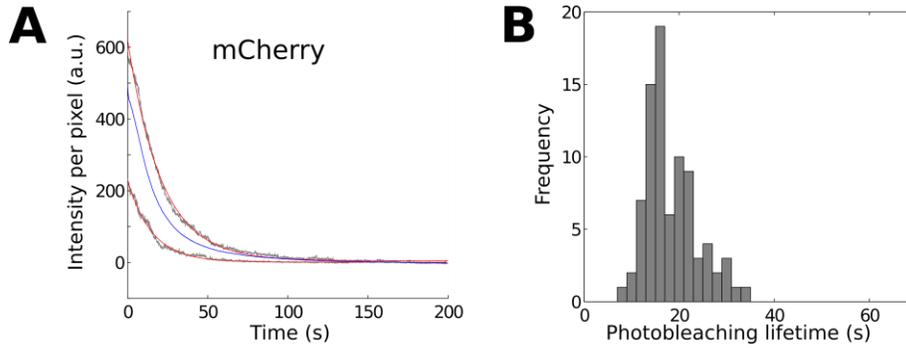


Fig. S4 Cell-based photobleaching studies of electroporated fluorescent protein, mCherry, into *E.coli* in green fluorescence channel. Left: Cell-based photobleaching timetraces (gray), single-exponential fits (red) and average photobleaching timetraces (blue). Right: Histogram of photobleaching lifetime obtained by single-exponential fit of cell-based photobleaching timetraces. The data set consists of about 100 cells. The photobleaching lifetime of mCherry is (18 ± 5) s, median: 17 s, at this illumination conditions.

Figure S5

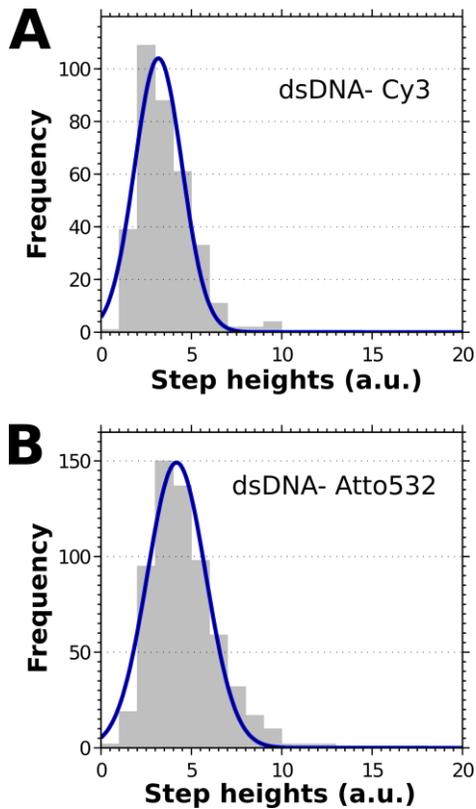


Fig. S5 Characterization of organic fluorophore brightness in green fluorescence channel. **A-B.** Histograms of single photobleaching step heights obtained from single-cell photobleaching studies of dsDNA-Cy3 and dsDNA-Atto532, respectively. Single Gaussian fits are centered at a unitary single-molecule fluorescence intensity of (3.2 ± 1.3) a.u. and (4.2 ± 1.6) a.u. corresponding to (2800 ± 1100) photon counts per second and (3600 ± 1400) photon counts per second for dsDNA-Cy3 and dsDNA-Atto532, respectively.

Figure S6

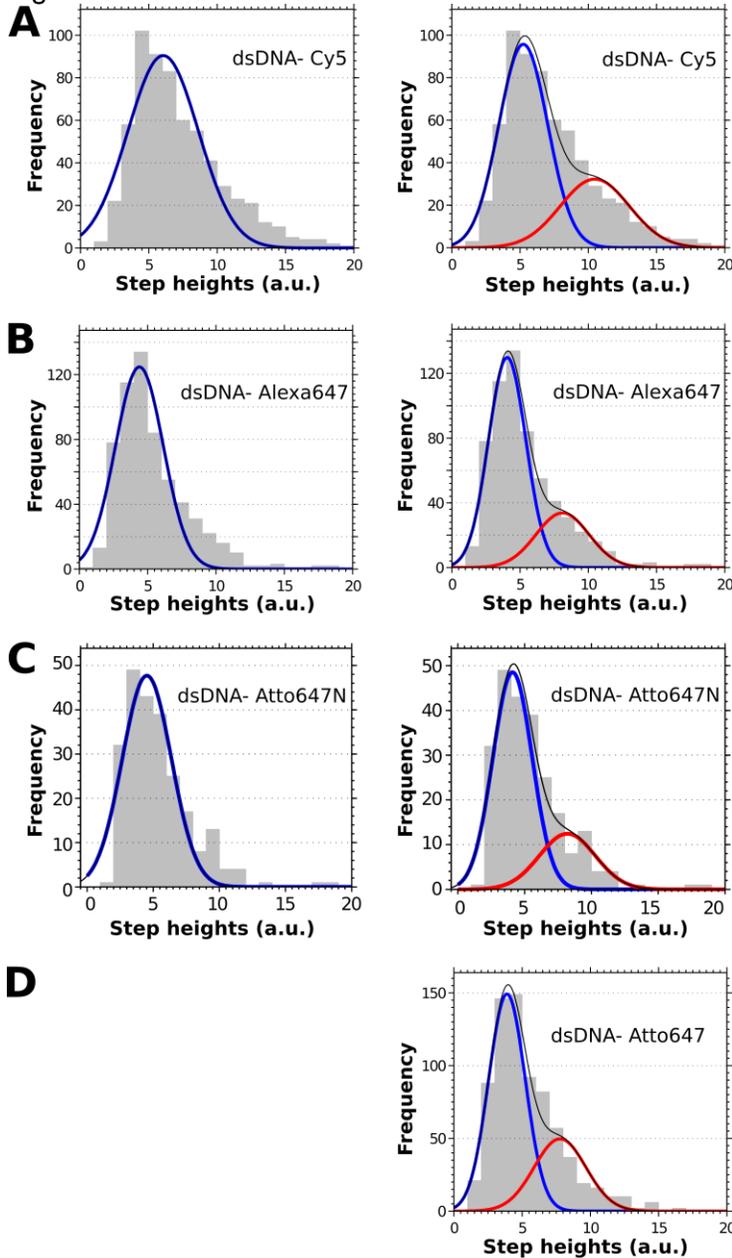


Fig. S6 Characterization of organic fluorophore brightness in red fluorescence channel. **A-D.** Histograms of single photobleaching step heights obtained from single-cell photobleaching studies of dsDNA-Cy5, dsDNA-Alexa647, dsDNA-Atto647N and dsDNA-Atto647, respectively. Single Gaussian fits not always resemble step height distribution accurately. Two Gaussians are fitted to the step height distribution to include double-steps (quasi simultaneous photobleaching of two fluorophores) due to short photobleaching lifetime of acceptor fluorophores into analysis; Gaussians are centered at x_0 and $2x_0$ with x_0 the unitary single-molecule fluorescence intensity. The following unitary single-molecule fluorescence intensities were obtained. dsDNA-Cy5: (5.2 ± 1.8) a.u., (4500 ± 1500) ph/s. dsDNA-Alexa647: (4.1 ± 1.4) a.u., (3500 ± 1200) ph/s. dsDNA-Atto647N: (4.1 ± 1.5) a.u., (3500 ± 1300) ph/s. dsDNA-Atto647: (3.9 ± 1.3) a.u., (3400 ± 1100) ph/s.

Figure S7

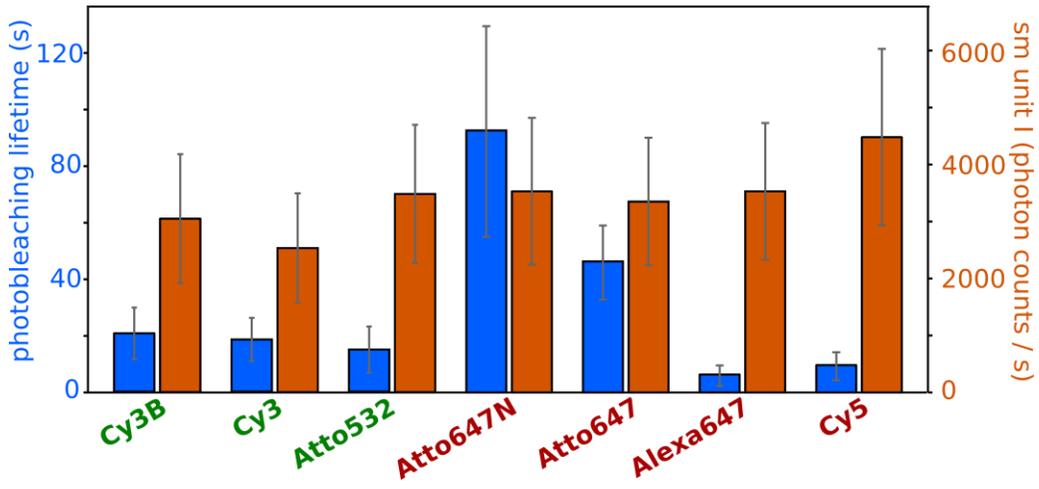


Fig. S7 Comparison of photostability and brightness for 3 donor fluorophores (Cy3B, Cy3 and Atto532) and 4 acceptor fluorophores (Atto647N, Atto647, Alexa647 and Cy5).