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Supporting Information

On the Mechanisms of Cyanine Fluorophore Photostablization

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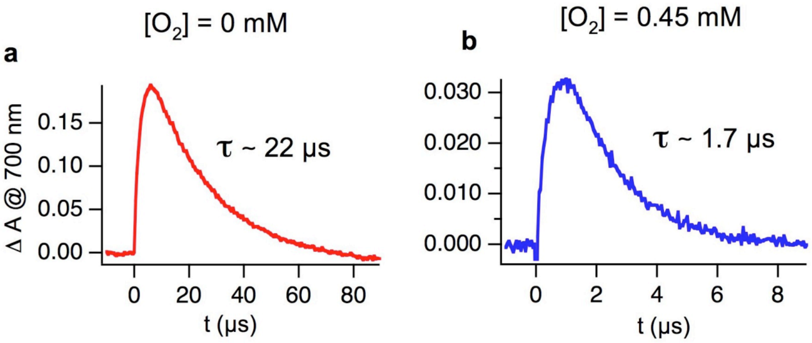

Figure S1. Transient absorption traces recorded at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of acetonitrile solutions of BP (5 mM) and Cy5 (22 μM). The solutions were purged with argon (a) or a gas mixture of 95% N_2 and 5% O_2. Optical path length = 6 mm.

$$\frac{d(\Delta A)}{dt} = -a_1e^{(-k_1 t)} + a_2e^{(-k_2 t)}$$

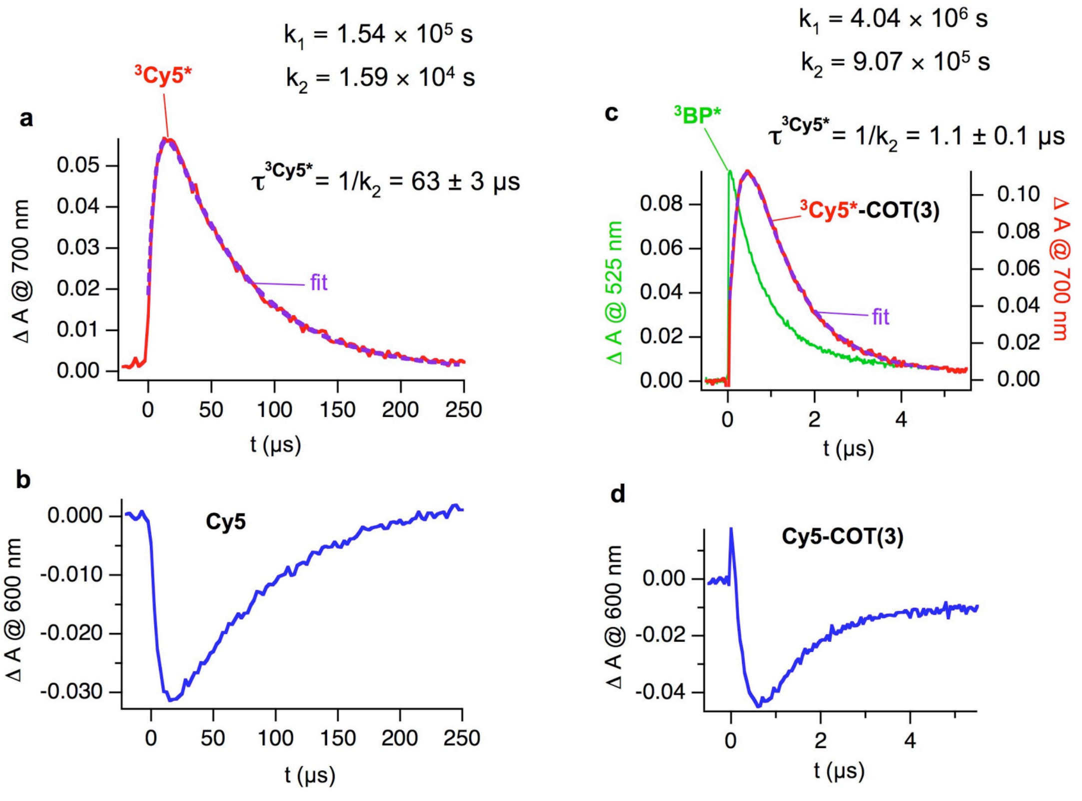

Figure S2. Transient absorption traces after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of BP (a, b: 3 mM; c, d: 10 mM) and Cy5 (a, b: 10 μM) or Cy5-COT(3) (c, d: 82 μM). Optical path length 10 mm (a, b) or 2 mm (c, d). The transients were fitted (purple line) to a biexponential function, which accounts for the growth kinetics (k_1) and decay (k_2) of Cy5 triplets.

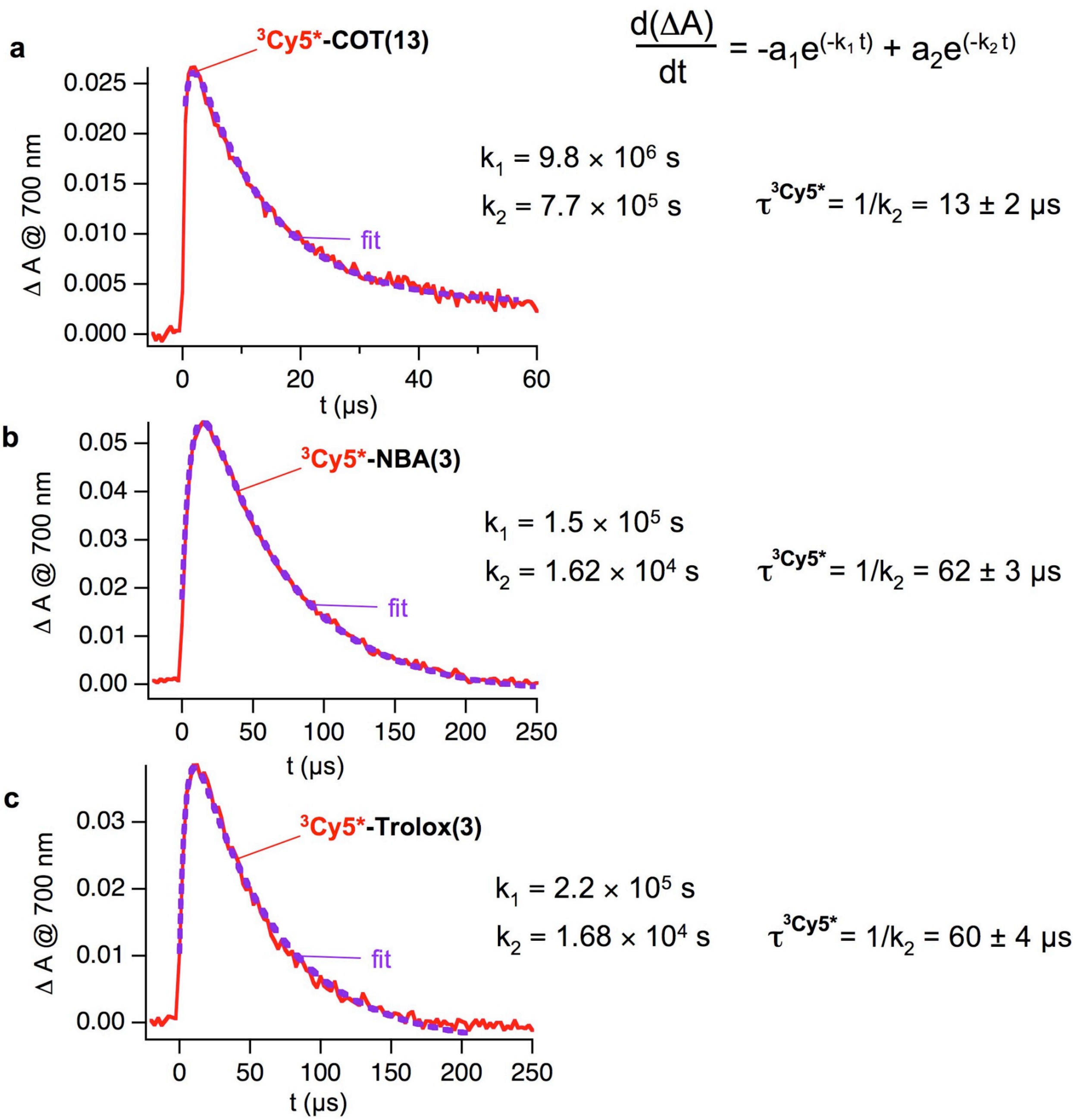

Figure S3. Transient absorption traces at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of BP (3 mM) and Cy5-COT(13), Cy5-NBA(3) and Cy5-Trolox(3) (10 ± 1 μM). Optical path length 10 mm. The transients were fitted (purple line) to a biexponential function, which accounts for the growth kinetics (k_1) and decay (k_2) of Cy5 triplets.

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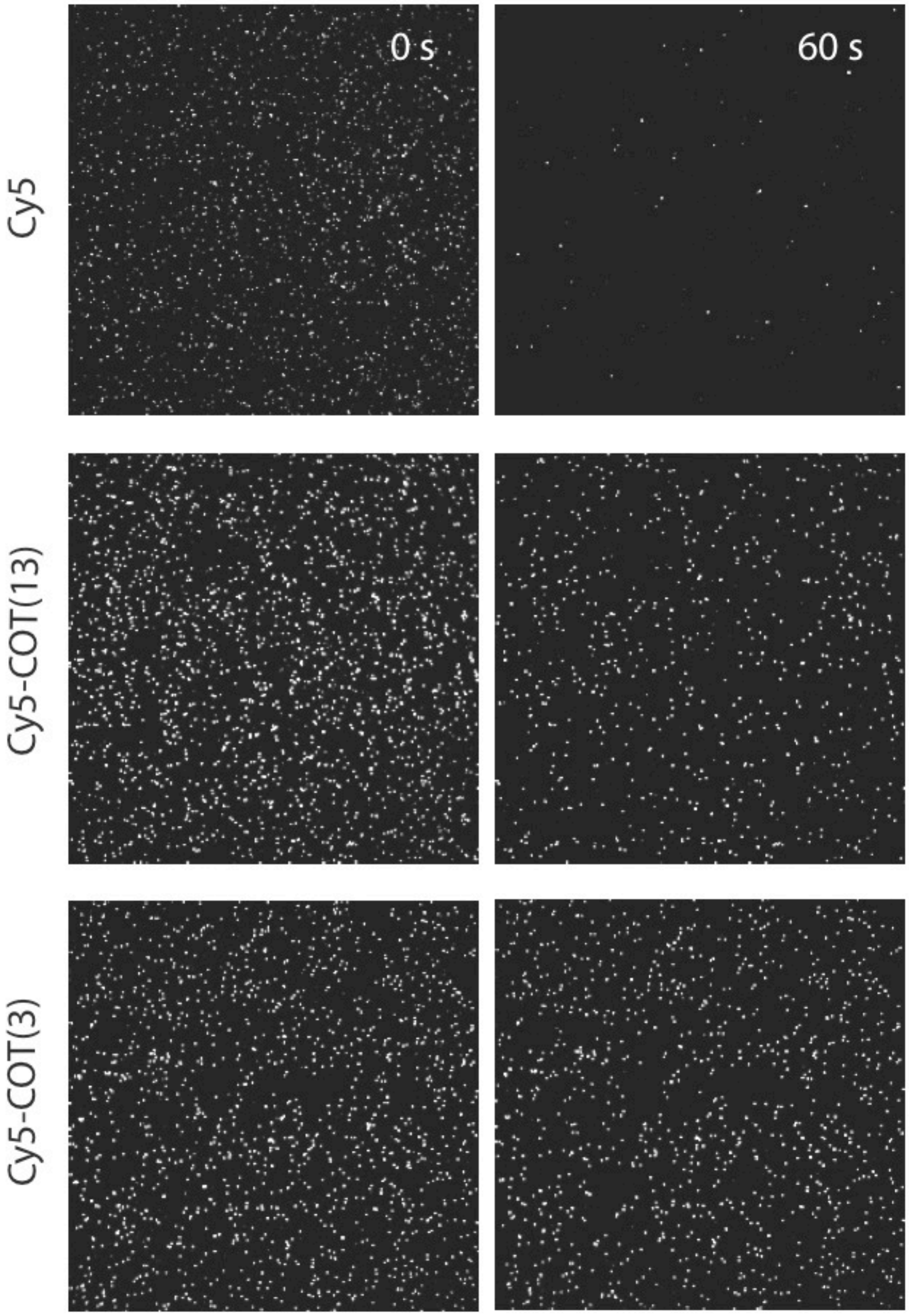

Figure S4. Single-molecule images of duplex DNA oligonucleotide labeled with Cy5, Cy5-COT(13) and Cy5-COT(3) under deoxygenated solution conditions using a total internal reflection microscope with 641 nm illumination.

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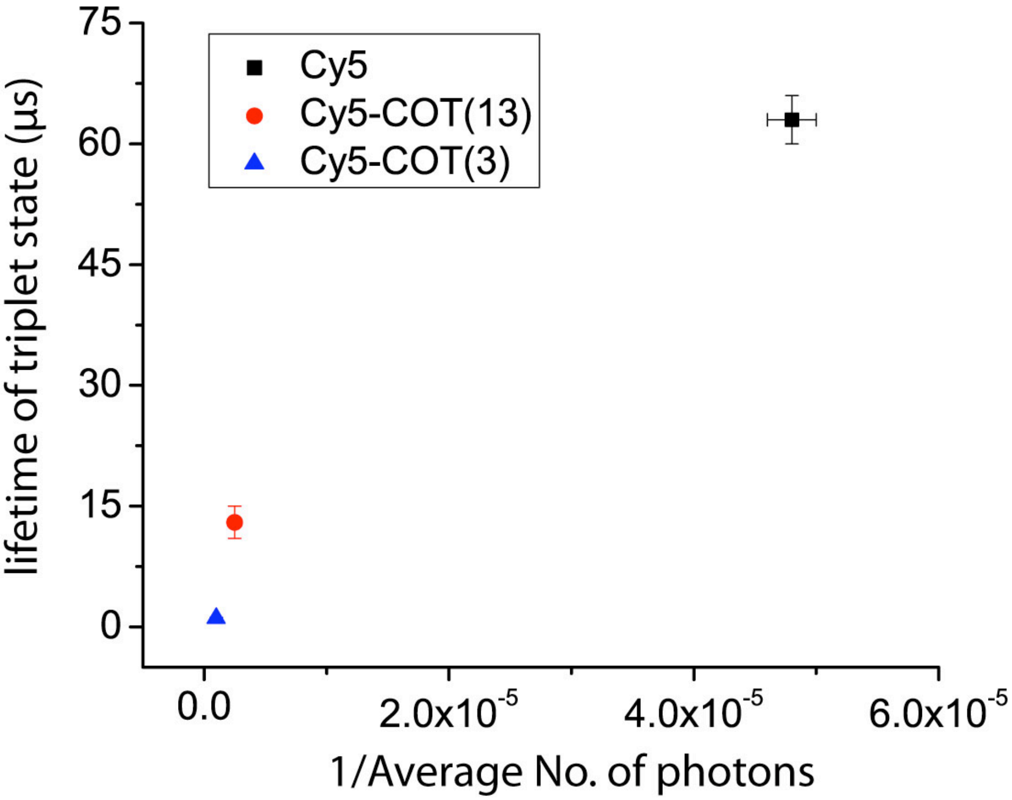

Figure S5. Correlation between the triplet state lifetime of Cy5 and the inverse average number of photons detected before photobleaching or blinking in single-molecule fluorescence measurements using a total internal reflection microscope with 641 nm laser illumination.

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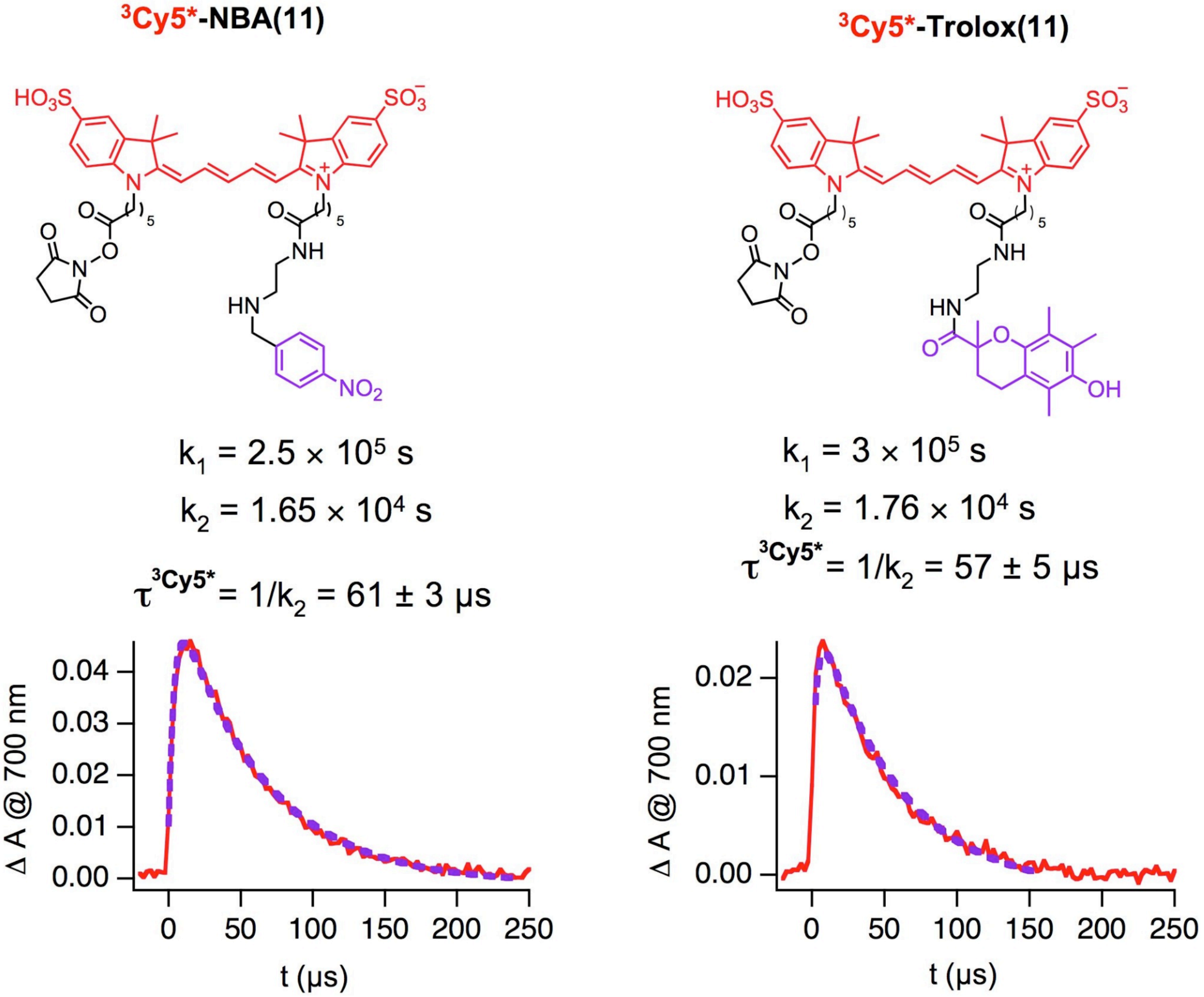

Figure S6. Cy5 triplet absorption traces recorded at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of BP (3 mM) and Cy5 derivatives (10 ± 1 μM). The transients were fitted (purple line) to a biexponential function, which accounts for the growth kinetics (k_1) and decay (k_2) of Cy5 triplets.

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Materials

Cy5-COT(13)[1-(7-((2-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propanamido)ethyl)amino)-6-oxoheptyl)-2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate], **Cy5-NBA(11)** [2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-((2-((4-nitrobenzyl)amino)ethyl)amino)-6-oxohexyl)-3H-indol-1-ium-5-sulfonate] and **Cy5-Trolox(11)** [2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(6-((2-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate] were synthesized as previously described.[S1]

Cy5-COT(3) [1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate], **Cy5-NBA(3)** [2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-(4-nitrophenyl)propyl)-3H-indol-1-ium-5-sulfonate] and **Cy5-Trolox(3)** [2-((1E,3E,5E)-5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(3-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)propyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate] were synthesized from an indole building block 2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (**1**), prepared using Fisher's condition.[S2] Coupling of **1** with 6-bromo-hexanoic acid proceeded smoothly in a sealed tube to provide 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium-5-

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sulfonate (**A)** as one of the precursors to Cy5 dyes. The same condition also worked well to attach the indole precursor with three different bromo-propyl protective agents, respectively, to give precursors: 1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (**Indole-COT**), 2,3,3-trimethyl-1-(3-(4-nitrophenyl)propyl)-3H-indol-1-ium-5-sulfonate (**Indole-NBA**); , 1-(3-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)propyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (**Indole-Trolox**). Addition of Indole-COT, Indole-NBA, or Indole-TX with **A** to malonaldehydedianilide hydrochloride in a sequential order, followed by converting the carboxylic acid end to a *N*-hydroxysuccinimide ester group, gave **Cy5-COT(3)**, **Cy5-NBA(3)**and **Cy5-Trolox(3)**, respectively.

Benzophenone (BP) from Aldrich was recrystallized from cyclohexane and ethanol. Acetonitrile (Aldrich) was used as received.

Laser Flash Photolysis measurement for the triplet state of the fluorophores

Laser flash photolysis experiments employed the pulses from a Spectra-Physics GCR 150-30 from a Nd:YAG laser (355 nm, ~ 5 mJ/pulse, 5 ns) and a computer-controlled system, which has been described previously.[S3] Acetonitrile solutions containing the Cy5 derivatives and BP were prepared and deoxygenated by argon purging. The concentrations of the Cy5 derivatives and BP were selected for optimum signal kinetics to achieve efficient triplet energy transfer from BP triplets to Cy5, but minimize self-quenching of Cy5 triplets by Cy5 ground state molecules. To accommodate the different concentrations, quartz cells of different optical

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path length and different experimental geometry were selected (10 x 10 mm and 6 x 4 mm in right angel pump/probe geometry; 2 x 10 mm in front face pump/probe geometry).

Single-molecule fluorescence imaging

All single-molecule measurements were performed using a laboratory built, prism-based total internal reflection fluorescence (TIRF) apparatus as previously described[S4] at specified illumination intensities in T50 buffer (10 mM Tris-acetate (pH 7.5) and 50 mM KCl), containing 5 mM β-mercaptoethanol, 1 mM 3,4-dihydroxybenzoic acid (PCA) and 50 nM protocatechuate 3,4-deoxygenase (PCD) (Sigma-Aldrich). Biotinylated-DNA molecules were immobilized via a biotin-streptavidin interaction within microfluidic channels constructed on quartz slides.[S4] Fluorescence from surface-immobilized molecules, illuminated via the evanescent wave generated by total internal reflection of a 641 nm (Coherent) laser source, was collected using a 1.27 numerical aperture (NA), 60× water-immersion objective (Nikon) and imaged onto a Cascade Evolve 512 electron-multiplying charge-coupled device (EMCCD) camera (Photometrics). Data were acquired using Metamorph software (Universal Imaging Corporation) collecting at a frame rate of 10 s^{-1}.

The photophysical properties of fluorophores were investigated using automated software built in-house using Matlab (MathWorks) as previously describes.[S4] Traces were extracted from wide-field TIRF movies by finding peaks of fluorescence intensity at least 8 standard deviations (s.d.) above background noise and summing the intensity of 4 total pixels encompassing each peak. Neighboring peaks closer than 3 pixels were removed.

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To reduce analytical error, traces were only used for analysis if they passed the following criteria: signal-background noise ratio >8, single-step photobleaching and background noise levels within 2 s.d. from the mean. To extract kinetic parameters of blinking and photobleaching, fluorescence traces were idealized using the SKM algorithm and a 3-state model with one fluorescent (t_{on}) state, a transient dark state (blinking) and a permanent dark state (photobleaching). t_{on} was calculated by fitting the cumulative distribution of the duration of each "on" state to a single exponential function. Photon counts were calculated by multiplying t_{on} with photons detected per seconds.

References:

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(S2) Robinson, B. The Fisher Indole Synthesis. *Chem. Rev.* **1963,** *63*, 373-401.

(S3) Yagci, Y.; Jockusch, S.; Turro, N. J. Mechanism of Photoinduced Step Polymerization of Thiophene by Onium Salts: Reactions of Phenyliodinium and Diphenylsulfinium Radical Cations with Thiophene. *Macromolecules* **2007,** *40*, 4481-4485.

(S4) Dave, R.; Terry, D. S.; Munro, J. B.; Blanchard, S. C. Mitigating Unwanted Photophysical Processes for Improved Single-Molecule Fluorescence Imaging. *Biophys. J.* **2009,** *96*, 2371-2381.