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Cy3-Cy5 Covalent Heterodimers for Single-Molecule Photoswitching

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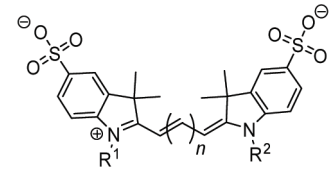
Covalent heterodimers of the Cy3 and Cy5 fluorophores have been prepared from commercially available starting materials and characterized at the single-molecule level. This system behaves as a discrete molecular photoswitch, in which photoexcitation of the Cy5 results in fluorescence emission or, with a much lower probability, causes the Cy5 to enter into a long-lived, but metastable, dark state. Photoinduced recovery of the emissive Cy5 is achieved by very low intensity excitation (5 W cm^{-2}) of the Cy3 fluorophore at a shorter wavelength. A similar system consisting of proximal, but *not covalently linked*, Cy3 and Cy5 has found application in stochastic optical reconstruction microscopy (STORM), a single-molecule localization-based technique for super-resolution imaging that requires photoswitching. The covalent Cy3-Cy5 heterodimers described herein eliminate the need for probabilistic methods of situating the Cy3 and Cy5 in close proximity to enable photoswitching. As proof of principle, these heterodimers have been applied to super-resolution imaging of the tubular stalk structures of live *Caulobacter crescentus* bacterial cells.

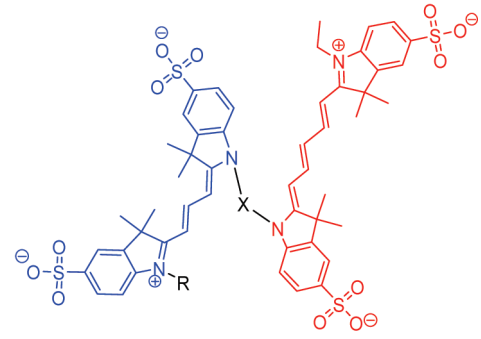
Proximal Cy3 and Cy5 fluorophores, separated by less than 3 nm, form an optical switch in the presence of a thiol and an enzymatic oxygen scavenging system.[1] Photoexcitation of the Cy5 results in fluorescence emission or, with a much lower probability, causes the Cy5 to enter into a long-lived, but metastable, dark state ("photodarkening"). Photoinduced recovery of the emissive Cy5 is achieved by excitation of the Cy3 fluorophore at a shorter wavelength. This switch operates for hundreds of cycles and has been demonstrated using other structurally analogous fluorophores.[2] Significantly, proximal, but not covalently linked, Cy3 and Cy5 has found application in stochastic optical reconstruction microscopy (STORM),[2,3] a technique for super-resolution imaging which requires single-molecule (SM) imaging and photoswitching. However, this method suffers from the drawback that complementary strands of DNA[1,2] or antibodies[2,3] must be employed to pair the fluorophores. To provide controllably linked Cy3 and Cy5, we have prepared the discrete Cy3-Cy5 covalent heterodimer **4** and its amine-reactive derivative **5** (Chart 1), and we report the photoswitching properties of the latter at the SM level in vitro and in bacteria.

Cy3-Cy5 covalent heterodimers were prepared from commercially available, reactive cyanine dyes, utilizing the coupling reaction between hydrazides and NHS esters.[4] Cy3-NHS ester **1** and Cy5-hydrazide **2** (Chart 1) were coupled in DMSO/triethylamine at 50 °C to give Cy3-Cy5 dimer **4**. Similarly, NHS ester-Cy3-Cy5 **5** was prepared from Cy3-bis(NHS ester) **3** and Cy5-hydrazide **2**. Both **4** and **5** were readily purified by column chromatography, and were isolated in yields of 76% and 24%, respectively. The structures of dimers **4** and **5** were confirmed by ESI-MS and photophysical characterization, and purity was assessed by HPLC (see Supporting Information).

CHART 1: Structures of Reactive Cyanine Dyes and Covalent Heterodimers



1: $R^1 = CH_2CH_3$; $R^2 = (CH_2)_5$(NHS ester); $n = 1$ (Cy3 core)
2: $R^1 = CH_2CH_3$; $R^2 = (CH_2)_5$(hydrazidyl); $n = 3$ (Cy5 core)
3: $R^1 = (CH_2)_5$(NHS ester); $R^2 = (CH_2)_5$(NHS ester); $n = 1$ (Cy3 core)



X = $(CH_2)_5C(O)(NH)_2C(O)(CH_2)_5$
4: R = CH_2CH_3
5: R = $(CH_2)_5$(NHS ester)

The absorption and fluorescence emission spectra of **4** and **5** are shown in Figure 1. The Cy3:Cy5 peak absorption ratios for **4** and **5** are 0.8:1 and 0.7:1, respectively, whereas the monomeric Cy3:Cy5 molar absorptivity ratio is 0.6:1,[5] indicating a slight perturbation in the relative oscillator strengths of the Cy3 and Cy5 dyes in the dimer. As shown in Figure 1, 516 nm excitation of the Cy3 component in either **4** or **5** produced considerable Cy5 fluorescence emission at 663 nm due to Förster resonance energy transfer (FRET), indicating close proximity of the fluorophores. As expected, the fluorescence lifetime for the Cy3

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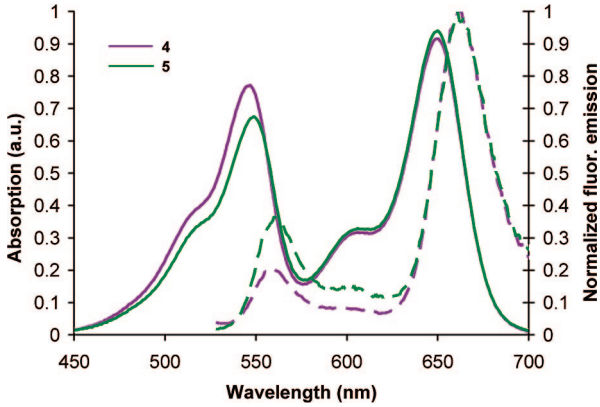

Figure 1. Absorption (solid) and fluorescence emission (dashed, λ_{ex} = 516 nm) spectra of Cy3-Cy5 covalent heterodimers **4** and **5** (in water; 3.7 μM for absorption; 37 nM for fluorescence) before photodarkening.

donor in **4** (0.15 ± 0.03 ns) was significantly shorter than the lifetime of monomeric Cy3 (0.254 ± 0.007 ns), whose measured value reproduces literature.[6] These lifetime measurements yield a FRET efficiency for **4** of 0.41 (see Supporting Information).

To study the SM photoswitching behavior, **5** was first covalently attached to an aminosilanane-terminated glass coverslip. For optimal photoswitch stability, a ratio of 1:20 reactive primary amine to unreactive tertiary amine was employed on the surface. Fluorescence time traces for representative SM photoswitches are shown in Figure 2 (see Supporting Information for movie). The sample was imaged continuously with 633 nm excitation and reactivated every 5 s with a 300 ms pulse of 532 nm light; the position of each reactivation pulse is denoted by a red dashed line. The requirement for thiol to achieve switching was met using sodium 2-mercaptoethanesulfonate as a nonvolatile substitute for the more commonly utilized, but malodorous, β-mercaptoethanol[1−3] or mercaptoethanolamine;[7] this substitution had no effect on the photoswitching properties.

The SM photoswitching properties of **5** are characterized by controllable reactivation, varying "on" times, and occasional spontaneous recovery from a long-lived dark state (a subset of data is shown in Figure 2). With only 16 exceptions out of 307 cases (e.g., Figure 2, panel E, 96 and 136 s), all switches in the "off" state that had not yet undergone irreversible photobleaching transitioned to the "on" state during reactivation, corresponding to a 95% reactivation efficiency. In about 15% of 360 reactivation attempts, switches were still in the "on" state at the time of reactivation. The switches pumped most strongly as a result of their location near the center of the 633 nm wide-field illumination region spent a shorter amount of time in the "on" state (e.g., switch C, 32% of time in "on" state) than those pumped only weakly (e.g., switch F, 62% of time in "on" state). This intensity dependence has been observed with similar systems[1,8] and is consistent with a photodarkening pathway that proceeds through the reaction of thiol with excited-state Cy5. It is interesting to note that spontaneous recovery from an "off" state is sometimes observed (e.g., panel B, 50 s, 90 s, and 110 s; panel C, 94 s; panel D, 25 s; panel E, 70 s).

It should be noted that Heilemann and co-workers[7] achieved efficient (>90%) photoinduced recovery of Cy5 in the absence of proximal Cy3 by strong excitation with 488 nm light, but the intensity required was 600× higher than that employed in this work and other studies.[1−3] Such a high intensity (3 kW cm^{-2}) at a wavelength that is known to strongly excite essential cellular components, such as flavins,[9] is likely to be incompatible with cellular imaging.

In anticipation that Cy3-Cy5 covalent heterodimers will eventually replace more cumbersome methods for achieving Cy3/Cy5 proximity in the super-resolution imaging of biological systems,[2,3] bovine serum albumin (BSA) was sparsely labeled with **5** (**5**:BSA = 0.015:1) using reactivity of the NHS ester with the accessible lysine residues of BSA. The labeled BSA was immobilized by incubation on a glass coverslip that had been spin-coated with an adhesion layer of pristine BSA. A representative time trace and corresponding fluorescence images of the Cy3-Cy5-labeled BSA are shown in Figure 3. Though **5** blinked to a greater extent when covalently attached to the BSA (see Figure 3A and movie in Supporting Information), switching was similarly achieved. To overcome the lack of specificity in protein labeling afforded by NHS esters, the Cy3-Cy5 heterodimer **5** is a good candidate for combination with targeting

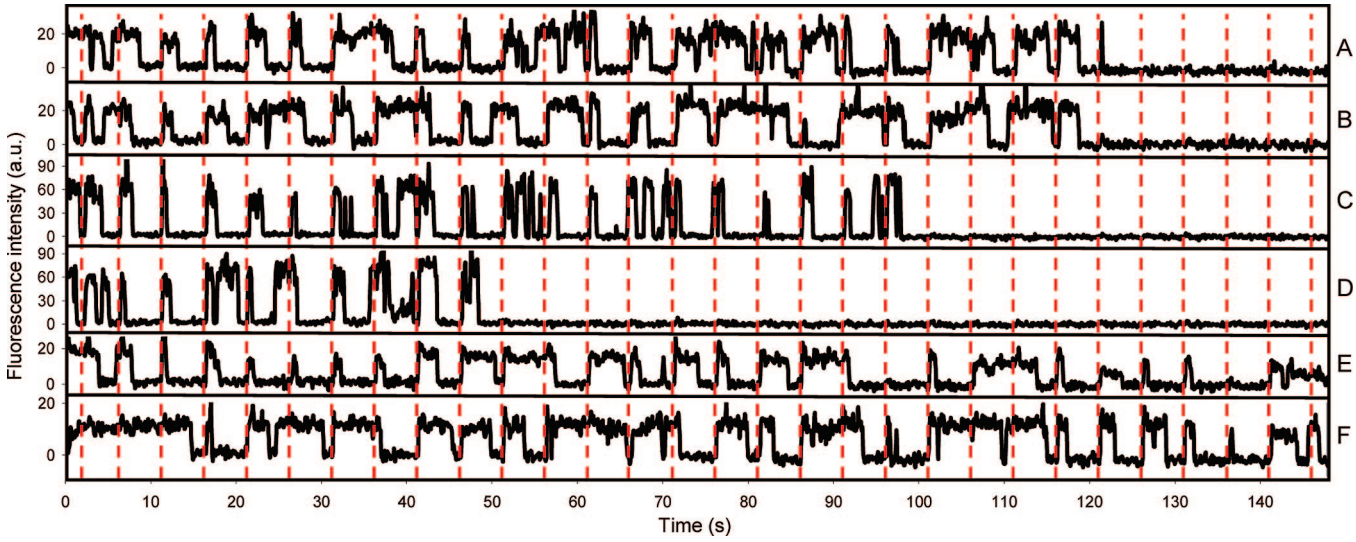

Figure 2. Representative SM fluorescence time traces of **5** covalently bound to an aminosilane-terminated glass coverslip. The molecules were imaged continuously beginning at t = 0 s with a red laser (633 nm, 150 W cm^{-2}) and reactivated every 5 s with a 300 ms green pulse (532 nm, 5 W cm^{-2}); a red dashed line shows the moment of each reactivation pulse. The imaging buffer consists of 50 mM Tris-HCl (pH 7.5), 1% (w/v) sodium 2-mercaptoethanesulfonate, and an oxygen scavenging system comprising 438 μg/mL glucose oxidase, 49.4 μg/mL catalase, and 10% (w/v) glucose.

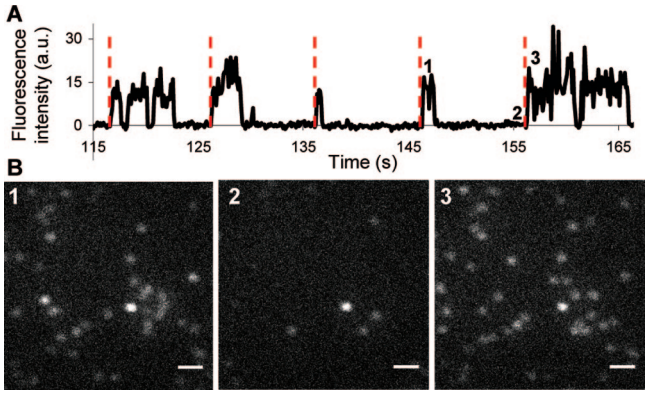

Figure 3. (A) Representative SM fluorescence time trace of **5**-labeled BSA showing reactivation numbers 12−16, denoted by the red dashed lines. (B) Fluorescence images at times 1−3 corresponding to the positions labeled in panel A. Conditions as in Figure 2; scale bar, 1 μm. Full experimental details are provided in the Supporting Information.

schemes such as O^6-alkylguanine-DNA alkyltransferase (hAGT) fusion[10] (i.e., SnapTag) or haloalkane dehalogenase fusion[11] (i.e., HaloTag).

Nonspecific protein labeling of surface lysines with the Cy3-Cy5-NHS ester **5** provides the opportunity to label cell surfaces in a straightforward way. This molecule was applied to a study of the short, thin stalks of cells of the bacterium *Caulobacter crescentus*. A concentrated suspension of these cells was incubated with Cy3-Cy5-NHS ester **5** in a growth medium to nonspecifically label the cell surface. The cells were then washed to remove unbound **5**, concentrated, resuspended in a growth medium containing a thiol source and an oxygen scavenger, and transferred to an agarose pad containing 1% β-mercaptoethanol. Super-resolution fluorescence images of the Cy3-Cy5-coated *Caulobacter* stalks were obtained by the STORM technique with 30.3 nm resolution as follows.[2,3] After the Cy5 emitters were bleached with 633 nm excitation, the sample was reactivated with 532 nm light at 10 W cm^{-2}, a dosage chosen such that the subset of activated molecules was sparse enough for each single Cy3-Cy5 dimer to be localized by point-spread-function fitting. No Cy5 emission arising from FRET was detected when **5** was excited with 10 W cm^{-2} of 532 nm light. The activated molecules were imaged over 100 100-ms frames with 633 nm excitation at 400 W cm^{-2} until photobleaching, and this cycle of reactivation/imaging was repeated 20 times to construct a super-resolution fluorescence image of the stalk from the localization of the single molecules. Emission from the cell body is not displayed, as it contains no super-resolution information. Following epifluorescence imaging, a corresponding white-light image of the cell sample was obtained in the same configuration. The cells continued to divide after the imaging sequence. The STORM image of stalks in Figure 4 is shown superimposed on a white-light image of the cells showing that the stalks are situated at the cell poles with varying length. Because the stalk elongates with every generation,[12] the distribution of stalk lengths in Figure 4 is expected for the unsynchronized population examined in the present work. The white-light image does not itself allow for visualization of the stalks.

In summary, covalent heterodimers of Cy3 and Cy5 have been prepared from commercially available starting materials and characterized at the SM level for the first time. The covalent linkage eliminates the need for probabilistic methods of situating the Cy3 and Cy5 in close proximity to enable photoswitching during SM localization-based super-resolution imaging. As a proof of principle, super-resolution images of the stalks of *C. crescentus* cells were obtained. Future work will be directed toward improving the behavior of these switches by optimizing the linker length between the Cy3 and Cy5, and investigating heterodimers based upon other dyes in the CyDye and Alexa-Fluor series.

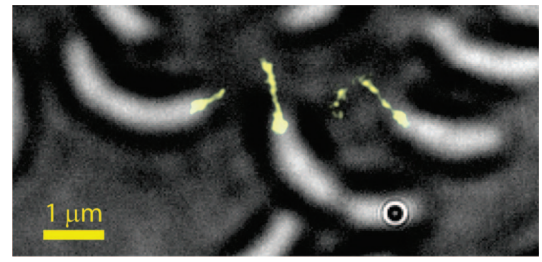

Figure 4. Super-resolution fluorescence image of *C. crescentus* stalks (yellow) with 30.3 nm resolution superimposed on a white-light image of the cells. As discussed in the Supporting Information, the *Caulobacter* cells were incubated in 4 μM of Cy3-Cy5-NHS ester for 1 h and then washed five times before imaging to remove free fluorophores. The data were acquired over 2048 100-ms imaging frames with 633 nm excitation at 400 W cm^{-2}. After initial imaging and photobleaching of the Cy3-Cy5 dimers, the molecules were reactivated every 10 s for 0.1 s with 532 nm light at 10 W cm^{-2}.

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Supporting Information Available: Experimental procedures, mass spectra, spectroscopic data, and SM photoswitching movies for **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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