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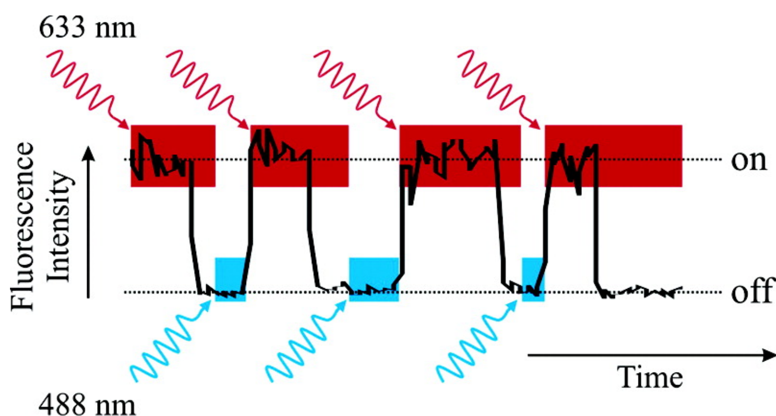

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Carbocyanine Dyes as Efficient Reversible Single-Molecule Optical Switch

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Abstract: We demonstrate that commercially available unmodified carbocyanine dyes such as Cy5 (usually excited at 633 nm) can be used as efficient reversible single-molecule optical switch, whose fluorescent state after apparent photobleaching can be restored at room temperature upon irradiation at shorter wavelengths. Ensemble photobleaching and recovery experiments of Cy5 in aqueous solution irradiating first at 633 nm, then at 337, 488, or 532 nm, demonstrate that restoration of absorption and fluorescence strongly depends on efficient oxygen removal and the addition of the triplet quencher β-mercaptoethylamine. Single-molecule fluorescence experiments show that individual immobilized Cy5 molecules can be switched optically in milliseconds by applying alternating excitation at 633 and 488 nm between a fluorescent and nonfluorescent state up to 100 times with a reliability of >90% at room temperature. Because of their intriguing performance, carbocyanine dyes volunteer as a simple alternative for ultrahigh-density optical data storage. Measurements on single donor/acceptor (tetramethylrhodamine/Cy5) labeled oligonucleotides point out that the described light-driven switching behavior imposes fundamental limitations on the use of carbocyanine dyes as energy transfer acceptors for the study of biological processes.

Introduction

Recent developments in nanotechnology and optoelectronics have focused research attention onto the possibility to use single fluorescent molecules as molecular photonic switches and optical data storage elements.[1−7] To store one bit per molecule by its fluorescence intensity in a reversible fashion, single molecules have to be switched digitally in a controlled manner by external stimuli. Usually, changes in fluorescence intensity from single molecules are attributed to quenching, stochastic intersystem crossing events to triplet states, or spectral diffusion due to fluctuations in the local environment of the chromophores. In cases where such fluctuations can be controlled, highly reproducible switching can be achieved, as was shown for light-induced frequency jumps in liquid helium temperature experiments.[8−10] The green fluorescent protein (GFP) and some derivatives constitute the first room temperature all-optical examples of chromophores that can be reversibly switched between different nonfluorescent and fluorescent states at the single-molecule level.[4,11,12] These natural photoactivatable chromophores are particularly interesting for precise photolabeling and tracking of proteins in living cells.[13]

In general, controlled on/off switching of the fluorescence of a single chromophore at room temperature affords the introduction of a controllable and highly efficient competing quenching pathway that prevents emission from the excited singlet state of the chromophore via, for example, excitation energy transfer or photoinduced electron transfer. Switching might be accomplished by light-induced deactivation of the quencher or changes in the chromophore/quencher interaction geometry.[5]

More recently, Irie et al. developed the first room temperature single-molecule photoswitch based on optical switching of the transfer efficiency in a fluorescence resonance energy transfer (FRET) pair.[6,14] They showed in a two-color experiment that a donor chromophore (bis(phenylethynyl)anthracene) connected

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to a switchable quenching unit (a diarylethene derivative) could be switched on and off by 488- and 325-nm light, respectively. UV light was used to activate the quencher (energy transfer acceptor), while 488-nm light was used for deactivation of the quenching unit and probing of the fluorescence of the donor chromophore. The use of identical wavelengths (488 nm) for probing and switching was possible because the deactivation (isomerization) is about 1000 times less efficient than the activation of the quenching unit. Thus, probing and isomerization can be controlled by changing the excitation light intensity.

On the other hand, single-molecule fluorescence experiments have revealed several expected and unexpected photophysical phenomena of the carbocyanine dye Cy5 such as cis−trans isomerization, off states additional to triplet formation, and complex photobleaching pathways including nonfluorescent intermediates that still absorb light in the visible range.[15−19] Here, we demonstrate that commercially available carbocyanine dyes can intriguingly act as an efficient reversible single-molecule photoswitch, whose fluorescent state after apparent photobleaching by 633-nm excitation can be restored by irradiation in the range of ∼300−532 nm. In addition to the importance of single-molecule photoswitches (e.g., for optical data storage), our data imply limitations for the use of carbocyanine dyes such as Cy5 and Alexa 647 in single pair FRET (sp-FRET) experiments.[20−27]

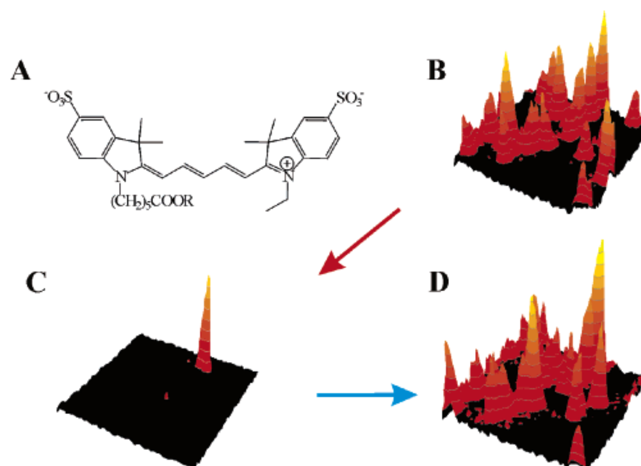

Figure 1. (A) Molecular structure of the carbocyanine dye Cy5. In aqueous solvents Cy5 exhibits an absorption and emission maximum at 649 and 670 nm, respectively. (B) Fluorescence scanning image (5 μm × 5 μm; 1-ms integration time per pixel, 50 nm/pixel) of Cy5-labeled dsDNA in deaerated PBS, pH 7.4, at an excitation intensity of 14 kW/cm^2 at 633 nm. (C) Fluorescence image (excitation at 633 nm) of the same surface area, demonstrating that most Cy5-labeled molecules are prepared in a nonfluorescent state after the first image scan. After the surface was scanned with 14 kW/cm^2 at 488 nm, the fluorescence of most Cy5-labeled conjugates is recovered in the next image scan using 633-nm excitation (D). This cycle of reversible optical switching of single Cy5 molecules can be repeated several times.

Materials and Methods

Cy5-NHS was labeled to a 20mer oligonucleotide via 5′-amino modifier C_6: 5′-ATC GTT ACC AAA GCA TCG TA-3′-biotin. For energy transfer experiments, a complementary oligonucleotide labeled with a tetramethylrhodamine (TMR) at position 10 (incorporated as TMR-labeled thymidin phosphoramidite) was used: 5′-TAC GAT GCT **T***TG GTA ACG AT-3′. After hybridization, dsDNA was immobilized on a streptavidin-coated surface as described in ref 28. Measurements were performed in standard buffer unless stated otherwise, removing oxygen by an oxygen-scavenging system: phosphate-buffered saline (PBS), pH 7.4, containing 10% (wt/vol) glucose, 12.5% (vol/vol) glycerine, 100 mM β-mercaptoethylamine (MEA), 50−100 μg/mL of glucose oxidase, 100−200 μg/mL of catalase, and 0.4−0.8 mM DTT.

Ensemble absorption and fluorescence spectra were taken on standard spectrometers using 1-mL quartz cuvettes. The fluorescence anisotropy, r, of Cy5 attached to dsDNA was calculated from the polarization of the emission components I_{VV}, I_{VH}, I_{HV}, I_{HH} (where the subscripts denote the orientation of the excitation and emission polarizers) as $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$ where $G = I_{HV}/I_{HH}$.

For bleaching and restoration experiments, excitation was carried out with an argon ion laser at 488 or 514 nm, a frequency-doubled Nd:YAG laser at 532 nm, and a helium neon laser at 632.8 nm. Restoration of the fluorescent state upon excitation at around 337 nm in ensemble experiments was performed using a 100 W mercury lamp and a band-pass filter (337DF75). In ensemble experiments the laser or lamp beam was defocused to homogeneously irradiate the cuvette. To facilitate absorption measurements, oxygen was removed by bubbling the cuvette with argon for several minutes before hermetically sealing instead of using the oxygen-scavenging system. For single-molecule experiments, the laser beams were coupled into an oil immersion objective (100×, NA 1.45; Zeiss) by dichroic beam splitters. Fluorescence was collected by the same objective and spatially filtered by a 100-μm pinhole positioned in the image plane of the microscope system (Axiovert 200 M; Zeiss). The fluorescence signal was spectrally filtered using a long pass filter (500ALP) and split into two detection channels by a dichroic beam splitter 650DCLP. Finally, the signals were imaged onto the active areas of two avalanche photodiodes (APDs) (AQR-14; EG&G, Canada). In front of the "green" and "red" detector, additional band-pass filters (Omega Optical and Chroma Technologies) were used to select the desired spectral detection range. For simultaneous excitation at 488 and 633 nm, a dichroic beam splitter (500DCLP) and a band-pass filter (700DF75) in front of the long-wavelength APD were used. For excitation of the double-labeled DNA with an argon ion laser at 514 nm, another dichroic beam splitter (560DCLP) and a band-pass filter (580DF60) in front of the short-wavelength APD were used.

Results and Discussion

For single-molecule switching experiments, Cy5 (Figure 1A) was coupled covalently to double-stranded biotinylated DNA and immobilized on a glass substrate coated with BSA/biotin-streptavidin under aqueous conditions.[28] Oxygen was removed from solution using an oxygen-scavenging system,[24] and 100 mM MEA was added as triplet quencher. Fluorescence images

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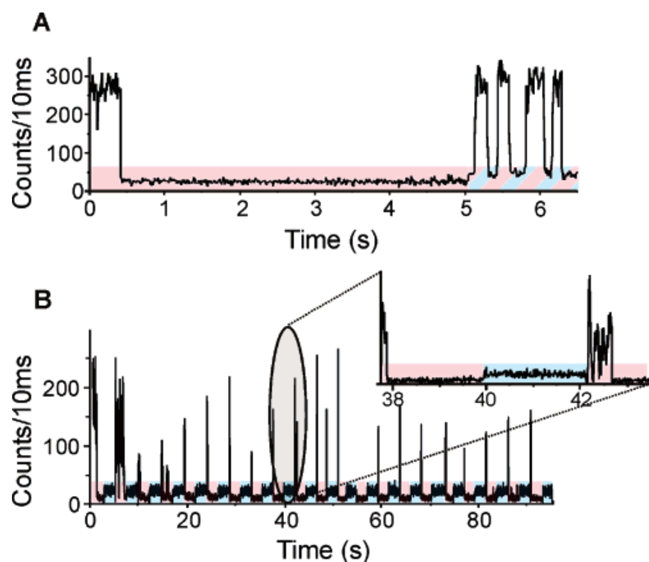

Figure 2. (A) Fluorescence trajectory of a single Cy5-labeled dsDNA molecule in deaerated PBS, pH 7.4. During the first 5 s, the sample was irradiated at 633 nm with an excitation intensity of 14 kW/cm^2. After ~500 ms, the fluorescence of the molecule disappeared and did not recover. At 5 s, the molecule was irradiated simultaneously at 488 nm with equal intensity (14 kW/cm^2). Subsequently, the fluorescence recovered and ceased in an alternating fashion. (B) Reversible optical switching of a single Cy5 molecule. The molecule was irradiated at 633 nm until fluorescence ceased (generally within 1 to 2 s) and then recovered by irradiation at 488 nm for 2.5 s. The underlayed color indicates the excitation wavelength (blue: 488 nm, red: 633 nm).

and fluorescence trajectories of single Cy5 conjugates were recorded using a confocal scanning microscope equipped with two spectrally separated detectors.[17,18] First, a fluorescence image was recorded exciting the sample at 633 nm to verify the presence of single immobilized labeled dsDNA molecules (Figure 1B). Depending on the excitation intensity, most molecules were found in a nonfluorescent state after the first image scan (Figure 1C). After the same area was scanned with 488-nm laser light under otherwise identical conditions, the fluorescent state was recovered, as shown in Figure 1D (see Supporting Information for web movie 1).

Selection of a single Cy5 molecule in an image scan and monitoring its fluorescence with time under alternating or simultaneous 488- and 633-nm excitation enables the investigation of the switching behavior in more detail. Figure 2A shows a fluorescence intensity trajectory of a single Cy5 labeled dsDNA molecule continuously excited at 633 nm. After about 500 ms, the fluorescence ceased and did not recover for 5 s of 633-nm laser irradiation. The bright state could, however, be reproducibly recovered within a few hundred milliseconds by simultaneous irradiation at 488 nm (Figure 2A after ~5 s; see also Supporting Information for web movies 1 and 2). Subsequently, driven by simultaneous irradiation at 488 and 633 nm the molecule switches between a fluorescent and nonfluorescent state on a time scale of a few hundred milliseconds. To demonstrate the applicability of single Cy5 molecules as controllable and reversible optical switches, a single Cy5-labeled dsDNA molecule was irradiated at 633 nm until fluorescence ceased (generally within 1 to 2 s) and then recovered by irradiation at 488 nm for 2.5 s (Figure 2B). Out of 21 on/off cycles shown, the fluorescent state of Cy5 could be recovered 20 times upon irradiation at 488 nm with an excitation intensity of 14 kW/cm^2. Switching failed only once either because irradiation at 488 nm was not sufficient to recover the emissive state or because the time the molecule spent in the fluorescent state was too short to detect a sufficient number of fluorescence photons for an unequivocal discrimination against background signal.

The efficiency of Cy5 as a photoswitch strongly depends on the buffer conditions used: For best performance, oxygen has to be removed rigorously (e.g., by use of an oxygen-scavenging system) and a triplet quencher such as MEA or β-mercaptoethanol has to be added. The need for a triplet quencher also indicates that the triplet state is not involved in the formation of the nonfluorescent switchable state. Therefore, we used PBS (pH 7.4) with rigidly removed oxygen containing 100 mM MEA as standard buffer in all experiments, except stated otherwise. Under such conditions, more than 100 switching cycles could be achieved for single Cy5 molecules with a reliability of >90%. While the efficiency of switching strongly depends on switching conditions (i.e., oxygen, MEA, and irradiation wavelength), it does not require double-stranded DNA. Both biotinylated Cy5 and Cy5-labeled single-stranded DNA immobilized on BSA/biotin-streptavidin coated glass substrates could be switched as well with comparable efficiency irradiating either at 488 or at 532 nm. Under dry conditions in the presence of oxygen, however (e.g., adsorbed on bare glass surface), the fluorescent state could not be recovered. Furthermore, switching could also be observed for immobilized Alexa 647, a structural related dye,[29] which implies that the underlying mechanism constitutes a general feature of certain carbocyanine dyes.

In addition, optical switching does not require single-molecule conditions, that is, high excitation intensity. The fluorescent state can be reproducibly but only partly restored as well from an ensemble of molecules irradiating, for example, a 10^{-6} M aqueous Cy5 solution first at 647 nm and then, to restore the fluorescent state, at either 337, 488, or 532 nm (Figure 3). Most notably in the ensemble experiment, the extinction of the reversible off state shows an increased absorption around 310 nm. Although no substantial increase in extinction is observed in the range of ~450−532 nm, it is advantageous to use this wavelength range for switching as the fluorescent subpopulation of Cy 5 is not excited significantly. A small band appearing at ~500 nm after continuous bleaching is not related to the photoswitched product as the band does not vanish when Cy5 is switched on again. It is also interesting to note that switching is more efficient at the level of single molecules than at the ensemble level. In ensemble experiments on average only about 40% of the molecules can be switched on independent of the irradiation wavelength. The different switching efficiency observed at the single-molecule and ensemble levels might be caused by the different experimental conditions such as different oxygen-removing efficiencies, different excitation intensities, and/or different local environment on a surface and in solution.

To investigate the nature of the switchable state and the switching mechanism, the photophysics of Cy5 under 633-nm irradiation was compared to the photophysics of Cy5 simultaneously using 633 and 488 nm irradiation. In agreement with Widengren and Schwille, autocorrelation of fluorescence intensity trajectories of immobilized Cy5 molecules revealed two components, which we ascribe to intersystem crossing and

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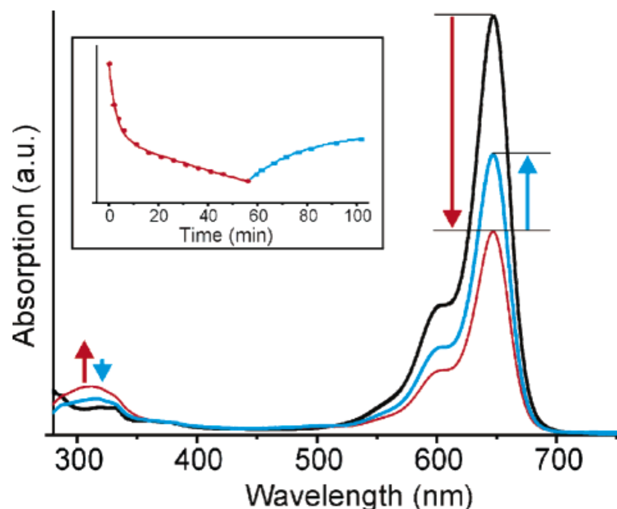

Figure 3. Ensemble switching experiment of an argon-bubbled 10^{-6} M aqueous Cy5 solution (PBS, pH 7.4, containing 100 mM MEA). The original Cy5 absorption spectrum is shown in black. After being bleached for 30 min at 647-nm (300 mW) irradiation by a defocused laser beam, the absorption decreased by ∼50% at 650 nm (red). About 40% of the absorption could be restored upon irradiation at 488 nm (300 mW) for 30 min (blue). The inset shows the temporal evolution of bleaching and absorption restoration. While the bleaching curve has to be fitted with a two-exponential model with decay times of $\tau_1 = 3.1 \pm 0.2$ min (32%) and $\tau_2 = 162 \pm 11$ min (68%), absorption recovery is well-described by a single exponential with $\tau = 23 \pm 1$ min.

cis−trans isomerization.[16] In these experiments, oxygen was removed while no triplet quencher was added to separate the time scale of triplet blinking and cis−trans isomerization. Additionally, another off state in the lower millisecond time range (2−20 ms) is visible in the fluorescence intensity trajectory (Figure 4A) as well as in the autocorrelation function of immobilized molecules (Figure 4B).

In the presence of triplet quencher (100 mM MEA) and oxygen scavenger, simultaneous irradiation at 488 and 633 nm produces similar intensity fluctuations and an additional off state with a duration of about 200 ms (Figure 4C). The additional off state in the higher millisecond time scale only appears with simultaneous 488-nm excitation. Thus, we ascribe the long off state to the photoswitched state. As these rare long off times are not well amenable to autocorrelation analysis, we used off-time histograms (Figure 4D) for closer analysis. The first bins in the histogram of Figure 4D represent predominantly the shorter millisecond off state. Longer off states show a broad distribution on time scales of up to several seconds. In contrast to the on state, which shows a simple power dependence (i.e., independent of 633-nm excitation power), on average 5000 photons could be detected during each on time, and the long off times show a weak 488-nm excitation power dependence (Figure 4D), indicating the presence of an additional intermediate that might be formed thermally. However, the broad distribution of off states and also significant differences in the time scale of this off state from molecule to molecule suggest that the local environment of the chromophore could play an important role. Although the molecules are immobilized in a way that surface interactions are minimized,[28] temporary interactions of the chromophore with the DNA and proteins used for immobilization cannot be excluded. Evidence that changes in the local environment influence the photophysics of single Cy5 molecules comes from the observation of triplet blinking

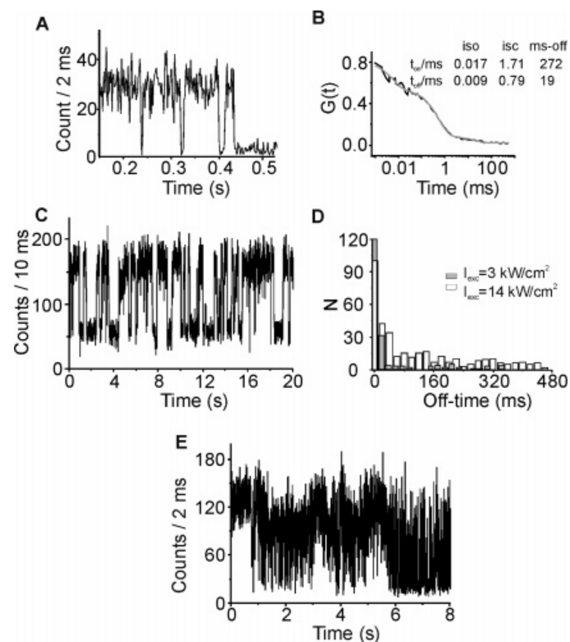

Figure 4. (A) Fluorescence intensity trajectories of single immobilized Cy5-labeled DNA molecules show additional off states with durations of several milliseconds (excitation intensity of 3 kW/cm^2 at 633 nm). Measurements were performed in the buffer system described, with oxygen scavenger but without addition of triplet quencher to separate the time scale of triplet blinking and cis−trans isomerization. (B) Autocorrelation of an intensity trajectory recorded in the absence of a triplet quencher shows off states with three distinct time scales: lower microsecond time scale for cis−trans isomerization, higher microsecond scale for triplet states at reduced oxygen concentration, and an additional off state in the millisecond range. Additionally, a three-exponential fit plus on and off times for three independent processes is given. (C) Trajectory of a single Cy5-labeled DNA molecule under simultaneous 488- and 633-nm excitation (each with 3 kW/cm^2). In these experiments, 100 mM of the triplet quencher MEA was added. (D) Off-times histogram of Cy5 trajectories as in (C). (E) In the absence of a triplet quencher and oxygen, Cy5 exhibits dynamic changes in fluorescence intensity and blinking parameters (excitation at 633 nm with 14 kW/cm^2).

fluctuations (Figure 4E). In the absence of triplet quencher, the observed changes in blinking pattern account for variations in intersystem crossing yield and triplet lifetime, which are caused by varying local oxygen concentration, different interaction geometries with DNA or protein, or other influences of the macromolecules on the chromophore.[30]

To further characterize the photophysical properties of the reversible switchable state and to evaluate the implications of photoswitching for common single-molecule FRET experiments, we attached an energy transfer donor, TMR, together with Cy5 to the dsDNA. For a separation of ∼3.4 nm (10 base pairs), strong coupling of donor and acceptor or the formation of ground-state complexes is prevented, and the donor transfers its excited-state energy via nonradiative dipole−dipole interaction efficiently to the acceptor Cy5. Therefore, the donor fluorescence can be advantageously used to report on the photophysical states of the acceptor Cy5. Figure 5A shows a fluorescence trajectory recorded from a single double-labeled DNA molecule recorded in standard buffer. During the first few seconds of the experiment, Cy5 was prepared in its nonfluorescent state by 633-nm excitation.

Upon changing the excitation wavelength from 633 to 514 nm after ∼3 s, the donor TMR shows strong emission,

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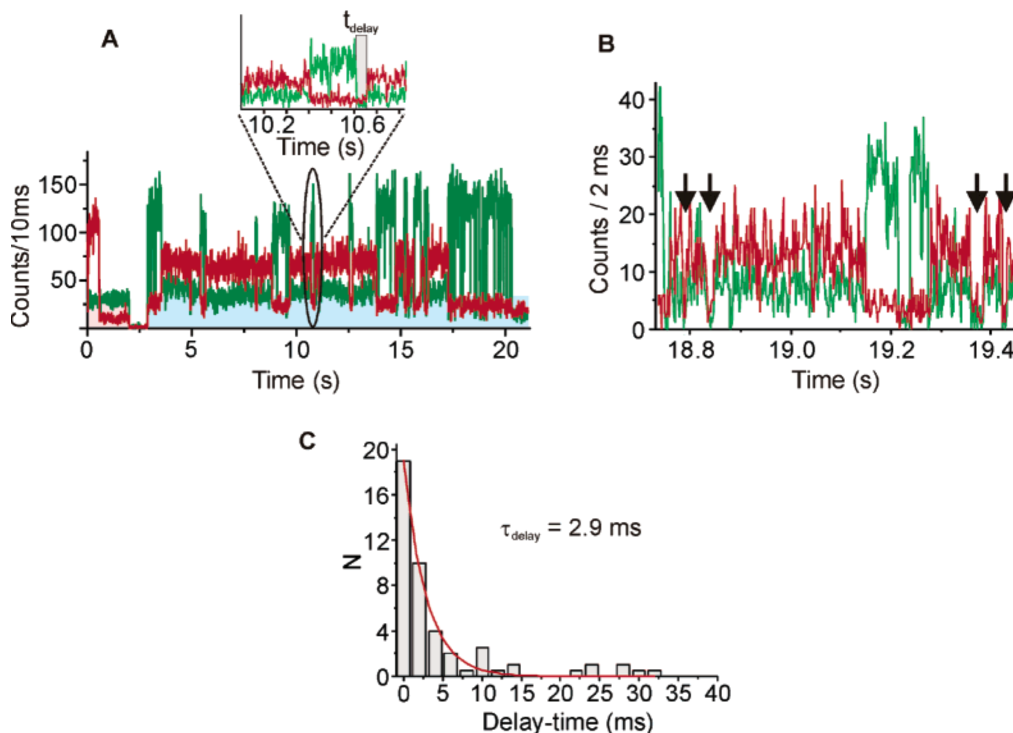

Figure 5. (A,B) Fluorescence trajectories of donor−acceptor (TMR−Cy5)-labeled dsDNA (10 bp donor−acceptor distance, corresponding to ~3.4 nm) in standard buffer. (A) After preparation of Cy5 in its nonemissive state (irradiation at 633 nm for 2 s), the molecule was excited at 514 (14 kW/cm^2) starting at 3 s to probe donor emission. Appearance of the donor emission demonstrates that the nonfluorescent state does not quench donor emission. After a few hundred milliseconds, the fluorescent state of the acceptor recovers, thus quenching the donor emission via resonance energy transfer. As expected from the other experiments, Cy5 undergoes several additional transitions to nonfluorescent states during the experiment. The underlayed color indicates the excitation wavelength (light blue: 514 nm, red: 633 nm). The expanded view of a switching event uncovers the presence of an additional nonfluorescent state of Cy5, which efficiently quenches the donor emission (indicated by τ_{delay}). (B) The arrows indicate Cy5 off states in the lower millisecond time scale that quench TMR more efficiently than the fluorescent state. (C) Lifetime histogram of τ-delay.

demonstrating that the absorption of the nonfluorescent state of Cy5 is not in resonance with TMR emission (i.e., it does not absorb in the spectral range of the donor emission between 570 and 650 nm). Hence, no energy transfer occurs. A few hundred milliseconds later, however, the fluorescent state is recovered by 514-nm excitation and energy transfer takes place with high efficiency until Cy5 enters again the nonfluorescent state. Subsequently, Cy5 switches between the nonfluorescent and fluorescent states.

Closer examination of the switching events shown in Figure 4A reveals that TMR is nonfluorescent about to the recovery of the fluorescent Cy5 state. Although this off state, which is denoted τ_{delay}, covers the same time scale as frequent donor blinking (see Figures 4B and 5A), statistical analysis suggests that the probability of Cy5 recovery is much higher when TMR is not emitting. We therefore assume that τ_{delay} is due to quenching by the nearby Cy5 residing in an additional off state. The distribution of τ_{delay} is exponential with a decay time of 2.9 ms (Figure 4C). Because of the 3.4-nm separation, quenching of TMR by Cy5 in this nonfluorescent state occurs most likely via resonance energy transfer. This implies that the absorption of the state is in resonance with the emission of the donor. Furthermore, the efficiency of energy transfer from TMR to Cy5 in the quenching state appears to be more efficient than the standard energy transfer from TMR to Cy5 in its fluorescent state (see donor intensity in Figure 5B or in the expanded view of Figure 5A). Therefore, we conclude that the short-lived nonfluorescent state of Cy5 exhibits a slightly higher absorption cross section at shorter wavelengths. It is also possible that this off state is related to the millisecond off state of Cy5 discussed above (Figure 4A). As shown in Figure 5B, this off state is also capable of completely quenching the donor emission. The fact that it exhibits a similar lifetime and similar acceptor properties could mean that these two off states represent the same intermediate.

Overall, this study indicates that there are several nonfluorescent states involved in the switching cycle. The oxygen and triplet dependence of the switching performance propose that the triplet state is not involved in the formation of the switchable states but, rather, competes with it. After formation of the first photoswitched product there seems to be a cascade of events to restore the fluorescent state. From the off times and the FRET trajectories, we suggest one photoactive intermediate and two further intermediates, one with a duration in the 200-ms range and another with a lifetime of a few milliseconds. The 200-ms state does not function as FRET acceptor, whereas the state with a lifetime of a few milliseconds is able to absorb the donor energy more efficient than fluorescent Cy5. Furthermore, this short-lived nonfluorescent state exhibits similar properties as an off state also found for Cy5 using 633-nm excitation only and might therefore represent the same state.

One known state of Cy5 that likely exhibits an R_0 value larger than that in Cy5 in the fluorescent state is the *cis*-conformation of Cy5.[16,18] Back-isomerization from the nonfluorescent cis to the fluorescent trans conformation also occurs photoinduced. Because of the low rates for thermal relaxation of the photoinduced cis state, photostationary equilibrium is established between the two isomeric forms already at low excitation

intensities. That is, under laser irradiation a single Cy5 molecule stays in its nonfluorescent cis state about 50% of the time. trans−cis and back-isomerization generally occur on the microsecond time scale for Cy5 in solution (Figure 4B).[16] On the other hand, it can be anticipated that Cy5 molecules in free solution behave different from those attached to biomolecules and immobilized onto solid supports. If Cy5 is attached to DNA or proteins the conformational flexibility of the linker used enables occasional sticking of the dye on the DNA or protein.[31−33] The steady-state fluorescence anisotropy of Cy5 attached to dsDNA was determined to be $r = 0.24$ in ensemble measurements (i.e., it cannot be regarded as free rotor). Thus, the cis state could be stabilized for micro- to milliseconds. Accordingly, the off state with a duration of a few milliseconds that functions as an efficient FRET acceptor could be associated with a stabilized *cis*-Cy5 adsorbed to surrounding macromolecules. The duration of the off state would consequently represent the time of the sticking event. Even though the triplet state is not involved in the formation of the nonfluorescent state(s), it can still play a role in the restoration of the fluorescent state. For example, intersystem crossing of the nonfluorescent state into the triplet state and subsequent conversion to the triplet state of fluorescent Cy5 followed by intersystem crossing to the singlet ground state might also contribute to the switching mechanism.

We do not yet know the exact mechanism responsible for reversible switching. The complexity of the carbocyanines' photophysics and the multitude of possible interactions with the molecule's nanoenvironment does not allow us to precisely pinpoint the course of events. The possibility of several cis−trans isomerizations of the polymethine bridges also supplies many degrees of freedom for photochemical pathways. The fact that the ensemble spectra do not yield a well-defined absorption band of the photoswitched state also indicates a direction for future work on the way of the revelation of the underlying mechanism. At this point, we can only speculate whether redox reactions with potent electron donors, for example, from the biomolecule (DNA, BSA, streptavidin) or the triplet quencher MEA, or intramolecular ring closure reactions as found for structurally related merocyanines to form spiropyrans are the underlying processes.[34,35]

Conclusion

Thus far, conventional chromophores embedded in ordered matrixes at liquid helium temperature could be optically switched between states with different absorption frequency in a highly reproducible fashion.[8−10] At room temperature, GFP and its derivatives represent the only unmodified natural chromophores that can be reversibly switched between a nonfluorescent and fluorescent state.[4,8,12,13] The synthetic approach (i.e., the energy transfer based photoswitch developed by Irie et al.) can be switched on by irradiating with visible light for an average of 10 s after preparation in the nonfluorescent state by ultraviolet light.[6,14] The data presented here demonstrate for the first time that a commercially available standard chromophore without introduction of an additional switching unit can function as an efficient reversible single-molecule optical switch at room temperature with comparably amazing performance: it can be switched in milliseconds and exhibits a high reproducibility and reliability at the single-molecule level. The fact that, independent of the excitation intensity, on average 5000 photons can be detected for each Cy5 molecule before entering the nonfluorescent state enables probing and switching of individual molecules using the same wavelength of 633 nm but different excitation intensities. Certainly, the performance of Cy5 as an all-optical photoswitch can be further improved to enable, for example, switching under dry conditions or embedded in matrixes once the underlying switching mechanism is thoroughly clarified. Nevertheless, the presented photoswitch possesses all fundamental requirements to be potentially useful for ultrahigh-density optical data storage or reversible switching of photonic wires.[36]

In addition, our data demonstrate that the optical switching behavior of carbocyanine dyes, such as Cy5 and Alexa 647, imposes fundamental limitations for their use as energy transfer acceptors in single-molecule experiments. Even though our data and proposed model suggest that the efficiency of switching is correlated with the excitation conditions, the removal of oxygen, and the presence of relatively high concentrations of triplet quencher, it seriously renders the interpretation of energy transfer fluctuations in single-molecule studies more complicated. In this context, alternating laser excitation methods that probe independently the donor and acceptor photophysical state at the single-molecule level and at various time scales (from nanosecond to millisecond) can help to distinguish the different sources of FRET fluctuations.[37]

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Supporting Information Available: Two movies of TIR measurements showing optical switching of individual Alexa 647 molecules attached to the surface (AVI). This material is available free of charge via the Internet at http://pubs.acs.org.

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