Spectral Identification of Specific Photophysics of Cy5 by Means of Ensemble and Single Molecule Measurements

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The triplet-state characteristics of the Cy5 molecule related to trans−cis isomerization are investigated by means of ensemble and single molecule measurements. Cy5 has been used frequently in the past 10 years in single molecule spectroscopic applications, e.g., as a probe or fluorescence resonance energy transfer acceptor in large biomolecules. However, the unknown spectral properties of the triplet state and the lack of knowledge on the photoisomerization do not allow us to interpret precisely the unexpected single molecule behaviors. This limits the application of Cy5. The laser photolysis experiments demonstrate that the trans triplet state of Cy5 absorbs about 625 nm, the cis ground state absorbs about 690 nm, and the cis triplet state also absorbs about 690 nm. In other words, the T_1-T_n absorptions largely overlap the ground-state absorptions for both trans and cis isomers, respectively. Furthermore, the observation of the cis triplet state indicates an important isomerization pathway from the *trans*-S_1 state to the *cis*-T_1 state upon excitation. The detailed spectra presented in this article let us clearly interpret the exact mechanisms responsible for several important and unexpected photophysical behaviors of single Cy5 molecules such as reverse intersystem crossing (RISC), the observation of dim states with a lower emission intensity and slightly red-shifted fluorescence, and unusual energy transfer from donor molecules to dark Cy5 molecules acting as acceptors in single molecule fluorescence resonance energy transfer (FRET) measurements. Spectral results show that the dim state in the single molecule fluorescence intensity time traces originated from *cis*-Cy5 because of a lower excitation rate, resulting from the red-shifted ground-state absorption of *cis*-Cy5 compared to that of the *trans*-Cy5.

Introduction

Cyanine dyes have been intensively studied by various photophysical and photochemical means,[1−14] owing to interest in their widespread applications such as spectral sensitizers in photography,[15,16] in biomedical application,[17−20] in nonlinear optics and laser physics,[21,22] and especially in single molecule detection.[23−30] The fluorescence and intersystem crossing properties, important for these applications, compete with the possible photoisomerization around the double bonds in the polymethine bridge, which takes place from the singlet states produced by visible excitation of the thermodynamically stable trans ground-state conformation of the cyanine dyes.[23−25,31−33]

In the past 10 years, cyanine dyes such as Cy5 have been frequently used in ultrasensitive imaging and spectroscopy to characterize local environments in large biomolecules, such as proteins or nucleic acids.[27,28,34,35] Although there has been much discussion in the literature on the mechanism of the deactivation of excited cyanine dyes,[23,24,26−30,33] the underlying mechanisms about the triplet-state transition and the trans−cis photoisomerization still remain unknown to a large extent. Recently, single molecule fluorescence experiments have revealed several important and unexpected photophysical phenomena of Cy5 related to the cis−trans isomerization and triplet-state formation,[5,23−25,27] such as reverse intersystem crossing (RISC), the observation of dim states with a lower emission intensity and slightly red-shifted fluorescence, and unusual energy transfer from donor molecules to dark Cy5 molecules acting as acceptors in single molecule FRET measurements. These unexpected phenomena oppose crucial limits for the application of Cy5.[23,28,34] On a broader level, understanding these complicated photophysical properties in detail is of considerable importance for Cy5, as it is one of a few commercially available single molecule fluorescence probes with an emission wavelength in the near-IR spectral region.

Regarding single molecule spectroscopy, in competition with intersystem crossing, trans−cis isomerization has been speculated to be involved in the on−off blinking characteristics of single Cy5 molecules, resulting in fast intensity fluctuations referred to the behavior as on/dim blinking.[23−25,27,33,35] Although the occurrence of on−off behavior in single molecule spectroscopy has attracted significant interest, and is described in the case of triplet blinking commonly by a three-state level model,[36−41] the on/dim kinetics are not yet well understood spectrally for most systems similar to the studied cyanine dye.[23−25,28,33,35] Furthermore, the unknown spectral properties of the triplet state and the lack of knowledge on the photoisomerization further complicate the analysis of the observed single molecule behavior.[5,33,42,43] Therefore, it is still under discussion whether the dim state is a consequence of the

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detection efficiency at different fluorescence wavelengths or due to a lower excitation rate of isomerized form.[25,27] Moreover, for FRET measurements, where Cy5 is frequently used as an acceptor dye, it is unexpected that Cy5 can still efficiently accept the energy transferred from donor dyes without fluorescence output when Cy5 stays in its dark states.[28,34,44] Until now there has been no spectral evidence clarifying the possible candidates, whether the cis isomer of Cy5 or the triplet state of *trans*-Cy5 would accept the energy from the respective donor.[23,28,34,44] The unexpected loss in FRET efficiency will lead to an overestimation of the distance between the donor and acceptor.

To answer these open questions only at the single molecule level would be difficult since the single molecule properties are environmentally sensitive and differ from molecule to molecule. Therefore, the detailed spectral properties of the dye are required for revealing these unexpected photophysical and photochemical phenomena of Cy5, but they are unknown spectrally to a large extent.

In this article, we report the distinct spectral results of the triplet-state transitions of Cy5 structurally related to trans−cis isomerization by means of ensemble and single molecule measurements. The triplet-state absorption and photoisomerization of Cy5 are distinguished by using the heavy-atom effects. Our results show that the lowest cis triplet state is involved in the formation of the isomer. The triplet−triplet ($T_1 \rightarrow T_n$) absorptions largely overlap the ground-state ($S_0 \rightarrow S_1$) absorptions for both *trans*-Cy5 and *cis*-Cy5, respectively. The $T_1 \rightarrow T_n$ absorption of *trans*-Cy5 locates around 625 nm; the $T_1 \rightarrow T_n$ absorption and the ground-state absorption of *cis*-Cy5 locate around 690 nm. These results clearly interpret the unexpected phenomenon of single Cy5 in spFRET measurements. Furthermore, the spectral data show that the dim state of single Cy5 molecules originates from *cis*-Cy5 because of the lower excitation rate resulting from a red-shifted absorption of *cis*-Cy5 compared to that of *trans*-Cy5. Reverse intersystem crossing (RISC) only happens when Cy5 stays in its trans isomer because the *trans*-$T_1 \rightarrow T_n$ absorption is in resonance with the excitation wavelength.

Materials and Methods

Cy5 was purchased from Amersham Biosciences. Coverslips from Menzel-Glaser (Germany) were used for single molecule measurements. Absorption spectra were recorded with a UV−vis spectrophotometer (Model U-3010, Shimadzu). Fluorescence spectra were measured with a fluorescence spectrophotometer (F4500, Hitachi).

Laser flash photolysis experiments were carried out using an Edinburgh LP920 spectrophotometer (Edinburgh Instruments). In this setup, sample was excited using 630 nm output with pulse energies of 1.5 mJ/pulse from an OPO pumped by a Nd:YAG laser (10 Hz, 8 ns) (Continuum Surelite). All solutions were bubbled with nitrogen for about 30 min before the laser flash photolysis experiments. Data were analyzed by the online software of the LP920 spectrophotometer. The fitting quality was judged by weighted residuals and a reduced χ^2 value.

Single molecule measurements were performed with a commercial time-resolved confocal fluorescence microscope (MicroTime 200, PicoQuant, Germany), in which single molecule fluorescence images were recorded by raster scanning the sample through the excitation light focus by means of a linearized $x-y-z$ piezo scanner. Samples were prepared for single molecule measurements by spin-coating a 0.1 nM Cy5 solution in ethanol onto a clean glass coverslip. Individual molecules were positioned sequentially in the laser focus to measure the fluctuations in fluorescence intensity and fluorescence lifetime with time. For the fluorescence measurements of one individual molecule with different excitation intensities, a shutter was used to block the laser beam during changing laser intensity. A PC plug-in card (TimeHarp200, PicoQuant, Berlin, Germany) allowing for TCSPC (time-correlated single photon counting) with time-tagged time-resolved (TTTR) mode was used to register the detected photon from two separated single-photon avalanche diodes (SPCM AQR-13, Perkin-Elmer, ∼60% quantum yield of detection at 670 nm, less than 150 dark counts per second). The TTTR mode allows the simultaneous registration of a detected photon on two independent time scales: the TCSPC time (∼40 ps/channel) relative to the respective excitation laser pulse and the macroscopic arrival time (with 100 ns time resolution) on a continuous time axis relative to the start of the measurement. For excitation, a single-mode continuous wave (CW) He−Ne laser at 632.8 nm and a pulsed laser diode (635 nm, 100 ps, 40 MHz) (LDH-P-635, PicoQuant, Germany) were used. The collimated laser beam after a polarization-maintaining single-mode fiber was changed from linear to circular by a polarizer and a $\lambda/4$ plate and spectrally filtered by an excitation filter (D637/10, Chroma, USA) before being directed into the inverted microscope body (Olympus IX 71). A dichroic mirror (Q655LP, Chroma, USA) reflected the laser light into a high-aperture oil immersion objective (100×, NA1.4, PlanApo, Olympus). Sample fluorescence was collected through the same objective and transmitted through the dichroic mirror. After passing one emission band-pass filter (HQ675/40, Chroma), a tube lens (200 mm focal length) focused the fluorescence onto a 100 μm pinhole for confocal imaging. The fluorescence after this pinhole was refocused onto the active area of the avalanche diode (SPAD). The instrumental response function (IRF) of the entire system was measured to be about 400 ps (fwhm). The fluorescence lifetimes were fitted by online MicroTime 200 software and FluoFit software (Version 3.1, PicoQuant, Germany). All single molecule measurements were performed in an N_2 atmosphere, allowing for both a better observation of triplet dynamics and a large increase in the photochemical stability of Cy5 molecules.[28]

Results and Discussion

Cy5 is believed to adopt an all-trans configuration in its ground state as its thermodynamically stable conformation.[23−25,31−33] Figure 1a shows the absorption and fluorescence spectra of *trans*-Cy5 solution in ethanol. There is a broad absorption ranging from 600 to 660 nm, while the prompt fluorescence peak is around 673 nm.

Figure 1b shows the transient absorption spectra of the Cy5 (1.0×10^{-4} M) solution in nitrogen-saturated ethanol after laser flash photolysis at 630 nm. The spectrum is a broad absorption ranging from 580 to 720 nm, where two main absorption bands around 625 and 690 nm are observed, corresponding to the possible T−T absorption and/or the cis isomer ground-state absorption, alternatively (see Figure 1). The time evolutions of these two transient absorption bands after the 630 nm laser flash are also shown in the inset of Figure 1b. To distinguish these two components, ethyl iodide was added to the Cy5 solution in nitrogen-saturated ethanol, where the heavy-atom effect is expected to increase the triplet-state yields.[36,38] As shown in Figure 1c, upon heavy-atom effect, the broad absorption with a maximum about 625 nm was enhanced by a factor of about 3, indicating a promoted intersystem crossing via spin−orbit coupling through heavy-atom effects. The enhanced absorption at 625 nm is, therefore, attributed to the T_1-T_n triplet-state

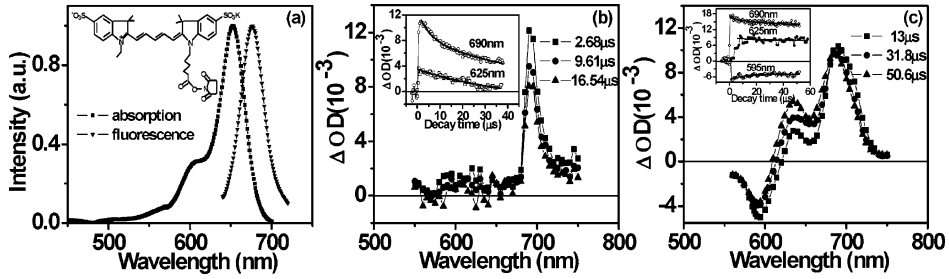

Figure 1. (a) Absorption and fluorescence spectra of Cy5 solution dissolved in ethanol (6.7×10^{-6} M). The inset shows the molecular structure of a Cy5 molecule. (b, c) Spectra observed by laser photolysis of nitrogen-saturated solutions of Cy5 (1.0×10^{-4} M) in ethanol before (b) and after (c) adding ethyl iodide. Decay dynamics at different detection wavelengths are shown in the insets, respectively. The value of χ^2 for each fitting was in the range of 1.02−1.24.

TABLE 1: Fitting Parameters of Time Evolutions of the Transient Absorption Bands

	τ (μs)	
wavelength (nm)	Cy5	Cy5 + CH_3CH_2I
625	35	45
690	5.8 (19%)	5.6 (52%)
	150 (81%)	196 (48%)

absorption of *trans*-Cy5. Compared to the absorption spectrum of *trans*-Cy5 as shown in Figure 1a, this T_1-T_n triplet-state absorption of *trans*-Cy5 about 625 nm largely overlaps the ground-state absorption spectrum (see Figure 1). Correspondingly, the absorption at 690 nm is, therefore, attributed to the absorption from *cis*-Cy5, a photoisomer of the starting material formed following the *trans*-$S_0 \rightarrow$ *trans*-S_1 excitation at 630 nm.

Table 1 lists the fitting parameters for the time evolutions of these two transient absorption bands. The decay of the 625 nm species is extracted well with a monoexponential fit with lifetimes about 35 μs in ethanol (see inset of Figure 1b) and about 45 μs in ethyl iodide doped ethanol solution (see inset of Figure 1c), respectively. The decay of the photoisomer about 690 nm can be well fitted with a double-exponential function with about 5.8 and 150 μs with the respective normalized amplitudes 0.19 and 0.81 in ethanol (see inset of Figure 1b), and about 5.6 and 196 μs with the respective normalized amplitudes 0.52 and 0.48 in ethyl iodide doped ethanol solution (see inset of Figure 1c). The increased amplitude of the fast decay component in the latter case implies that the population of the *cis*-Cy5 triplet state is also promoted upon adding ethyl iodide; we thus attribute the fast decay component with 5.8 μs to be an isomerized triplet-state (T_1-T_n) absorption (i.e., triplet-state absorption of the *cis*-Cy5). The slow decay component at 690 nm is assigned to the ground-state absorption of the photoisomer (*cis*-Cy5) of the dye formed following the *trans*-$S_0 \rightarrow$ *trans*-S_1 excitation with 630 nm laser pulse. The observation of two decay species for *cis*-Cy5 at 690 nm indicates that there are two isomerization pathways from the excited singlet state of *trans*-Cy5 up excitation. These two isomerization pathways are the following: (a) from the lowest singlet state S_1 of the *trans*-Cy5 to the ground state of *cis*-Cy5, evidenced by the *cis*-$S_0 \rightarrow S_1$ absorption; (b) from the lowest singlet state S_1 of *trans*-Cy5 via ISC to the triplet state T_1 of the *cis*-Cy5, evidenced from the *cis*-$T_1 \rightarrow T_n$ absorption. Pathway b has also been shown in our previous delayed fluorescence and phosphorescence measurements, in which we directly observed the delayed fluorescence of *trans*-Cy5 via back-isomerization from the cis triplet state (*cis*-T_1) to the trans singlet state (*trans*-S_1) of Cy5 by thermal activation.[45] Because of the higher yield of photoisomerization compared to that of the intersystem crossing from *trans*-S_1 state to *trans*-T_1 state as shown in Figure 1b, in addition to the effective back-isomerization from *cis*-T_1 state to *trans*-S_1 state,[45] the back-isomerization from *cis*-T_1 to *trans*-T_1 is also expected to be more efficient. This may lead to the slow growth of the 625 nm band in the presence of ethyl iodide (see Figure 1c). Furthermore, similar to the case of *trans*-Cy5, we find that the ground-state (S_0-S_1) and triplet-state (T_1-T_n) absorption spectra of *cis*-Cy5 also closely overlap each other. Meanwhile, as shown in Figure 1c, there is also a complete bleaching of the ground state of *trans*-Cy5 centered about 595 nm upon addition of ethyl iodide. This is related to the slow ground-state recovery from the T_1 state to the S_0 state where the *cis*-T_1 state is highly populated following the $S_0 \rightarrow S_1$ excitation in the presence of ethyl iodide and/or slow thermoinduced back-isomerization from *cis*-S_0 to *trans*-S_0.

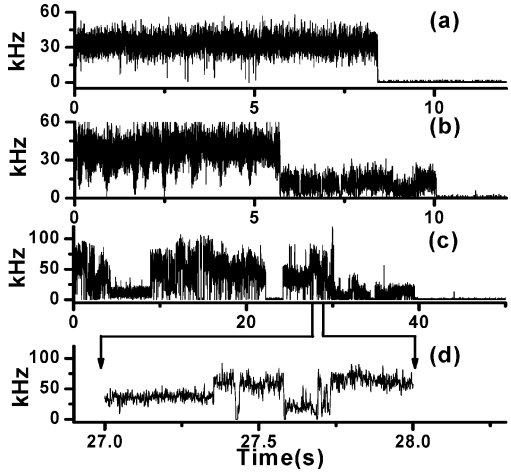

Figure 2. (a−c) Typical fluorescence intensity time traces with an integration time of 1 ms/bin from three different Cy5 molecules at an excitation intensity of about 1.5 kW/cm^2. (d) Expanded view of the fast fluctuations between the dim state and on state from (c).

Because of the flexible conformation of Cy5 in solution, it is experimentally not possible to distinguish the single molecule fluorescence fluctuation caused by the ISC and isomerization in solution because of the competition between them. To show the complexity of the unexpected photophysics of Cy5 at the single molecule level, we present three typical fluorescence intensity traces from three different Cy5 molecules on a glass surface, as shown in Figure 2. Trace a shows an example of a single Cy5 molecule emitting photons with nearly constant count rate during the on state until an irreversible photobleaching, whereas trace b shows an example of a single molecule fluorescence fluctuation that has behaviors similar to those of trace a during the first several seconds and then finally undergoes a stable dim state (about 1/3 of the count rate compared to that of the on state) before photobleaching. Trace c shows the fluorescence from a single Cy5 molecule with fast intensity fluctuations with two discrete intensity levels (on/dim

mixing): a strong emitting "on" state and a "dim" state just above the background. Compared to the rigid conformations of the molecules in traces a and b, the molecule in trace c may exist within an environment allowing conformation changes more easily, resulting in fast on/dim fluctuations. These fluctuations are correlated in time with two different correlation times, at several hundred microseconds and several tens of milliseconds, corresponding to the triplet-state lifetime and a specific conformational-state lifetime of this individual Cy5 molecule, respectively. More than 60% of the total investigated 320 Cy5 molecules show the same behaviors as in trace a, especially when excited with low intensity. The fluctuations in fluorescence intensity are typically due to intersystem crossing, where the correlation times obtained from autocorrelations are mostly fitted by good first-order kinetics. Once the molecule undergoes a dim state as shown in trace b, the molecule constantly emits a lower number of photons per time until it is photobleached. Again, the correlation times could be extracted by fitting the intensity autocorrelation function with a monoexponential decay function, indicating that the intensity fluctuations are also in the dim state typically caused by the triplet state.

In addition, it should be mentioned here that the single molecule measurements could be carried out on the dry glass surface to distinguish the fluorescence intensity fluctuations from ISC and isomerization, respectively, because the rigidity of molecule conformation can somewhat be increased when Cy5 molecule is absorbed on the dry surface. Although the interaction (mainly electron transfer) between Cy5 and glass surface may modify the radiative components of the excited-state kinetics rather than the spectral peak positions, the spectral peak positions on the surface are not necessarily directly related to the interaction between the dye and the glass surface.[46,47] In other words, there is no distinct correlation between the electron-transfer rate and the spectral peak positions. We find that the ensemble-averaged absorption spectrum of Cy5 on glass surface shows a spectral shape similar to that of Cy5 in solution with a slight red shift, which results from aggregation (see the Supporting Information). Furthermore, the two fluorescence decays from both solved Cy5 and surface-adsorbed Cy5 can be described by a monoexponential fit with decay time about 1.0 ns for solved Cy5 and about 2.0 ns for Cy5 on the glass surface. The longer fluorescence lifetime of a single Cy5 molecule about 2.0 ns results from a reduced flexibility of the molecule on glass surface in comparison to that in solution. If there is any electron transfer between Cy5 and the glass surface, the fluorescence lifetime of Cy5 on the dry glass surface should be much shorter than that in solution.[47] It is found that single Cy5 molecules adsorbed on glass surfaces show stationary transition dipoles, leading to a narrow distribution of fluorescence lifetime around 2 ns (data not shown), which are also in accordance with those results reported by refs 27 and 48. Therefore, the electron transfer between Cy5 and glass surface should play a minor role in the observed single molecule behavior. Since the spectra of both solved and surface absorbed Cy5 molecules are similar, the spectral data of Cy5 obtained in solution from laser photolysis could still be suitable for use in interpreting the underlying mechanisms of unexpected single molecule behavior.[5,33,42,43]

Moreover, as mentioned above, trans−cis isomerization may lead to the dim states during single molecule experiments.[23,25,27] The spectral properties of these dim states are not known yet. Although in most cases the dim state exhibits a red-shifted emission as mentioned by many authors,[25,27] the shift in emission should not have an influence on the single molecule emission rate, whereas, the shifted absorption has a direct influence on the observed emission rate because of the different excitation rate. From Figure 1, since *cis*-Cy5 has a main ground-state absorption band about 690 nm, it is obvious that the total fluorescence intensity will be lower once the molecule stays in the cis isomer, resulting in the dim fluorescence state appearing in the single molecule emission intensity trajectory (see Figure 2b). The main reason for the decrease in the total fluorescence intensity of *cis*-Cy5 is due to a lower excitation rate at the used excitation wavelength at 635 nm (please see the Supporting Information for details) for single molecule measurements. The *trans*-Cy5 shows a higher fluorescence intensity in its "on" states since the ground-state absorption of *trans*-Cy5 is right around the excitation wavelength of 635 nm.

Meanwhile, laser photolysis shows that the ground-state (S_0-S_1) and the triplet-state (T_1-T_n) absorption spectra of *trans*-Cy5 closely overlap each other, as shown in Figure 1. This spectral result can help us interpret the unexpected photophysical phenomena of single Cy5 molecules.[5,23] Typically, for single molecule FRET measurements as mentioned in the Introduction, Cy5 is frequently used as an acceptor dye.[5,28,34,44] It is unclear why and how Cy5 can still accept the energy transferred from the donor dyes without fluorescence output when Cy5 stays in its dark states.[5,28,34,44] It is still unknown whether the cis state of Cy5 or the triplet state of *trans*-Cy5 would accept the energy from the donor.[5,23,28,34,44] Since the fluorescence spectra of those donors (such as Cy3, TMR, and R6G) mainly overlap the triplet−triplet absorption of *trans*-Cy5 rather than the S_0-S_1 absorption of *cis*-Cy5,[5,26,28,34,35,49,50] Cy5 still works efficiently as an acceptor but without fluorescence output when Cy5 enters its trans triplet state because of the efficient T_1-T_n resonance absorption with the fluorescence of the donor dyes.[28]

Furthermore, we present direct spectral evidence (see Figure 1) that the excitation wavelength used for single molecule measurements is just in resonance with both the triplet-state (T_1-T_n) absorption and ground-state absorption of *trans*-Cy5. As a consequence, the triplet state can be excited efficiently from *trans*-T_1 to *trans*-T_n with a single excitation wavelength at higher power. An additional process, which results in a shortening of off times, increase of total average photon counts and increase of on times during single molecule measurements, must be considered during the analysis of the single molecule fluorescence intensity traces with single wavelength excitation at higher excitation intensity. This additional photoinduced channel (e.g., $T_1 \rightarrow T_n \rightarrow S_1 \rightarrow S_0$) referred to as reverse intersystem crossing (RISC) has been described before with both two-color excitation in a similar system and one-color excitation in Cy5 under ambient conditions.[26,28,33] As mentioned above, the deactivation processes of Cy5 are complicated. To follow the RISC in the ensemble case is difficult because of the low quantum yields of intersystem crossing and high quantum yields of trans−cis isomerization observed in the laser photolysis experiments following the *trans*-S_0-S_1 excitation as shown in Figure 1b. In this case, to determine this RISC experimentally at single molecule levels with a single excitation wavelength, we must choose those Cy5 molecules that are stable enough in their "on" state (e.g., trans isomer alone) for a sufficiently long time, allowing for sequential investigations at several different excited powers. Since the "on" state is only from single *trans*-Cy5 molecules, the power dependence of excited-state processes and on−off dynamics of single *trans*-Cy5 under single excitation wavelength will be much more simple than those of multistate (on/dim mixing) cases during single molecule measurements.

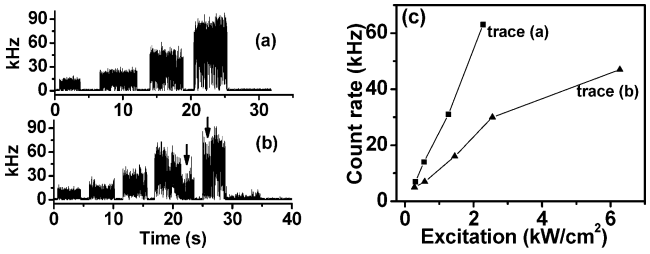

Figure 3. Fluorescence intensity time courses with 1 ms/bin from two individual molecules excited with several laser intensities (left); detected count rate as a function of the excitation intensity of the two molecules (right).

TABLE 2: Photophysical Parameters (On Time τ_{on}, Off Time τ_{off}, Detected Count Rate I_{det}, and Triplet State Transition Yield Y_{isc}) of an Individual Cy5 Molecule Excited under Different Intensities with CW Laser (632.8 nm)[a]

I_{ex} (W/cm^2)	τ_{on} (ms)	τ_{off} (ms)	I_{det} (count/ms)	Y_{isc}
277	0.056[b]	0.73	5	0.025
575	0.11	0.58	7	0.025
1453	0.20	0.30	16	0.017
2550	0.73	0.23	30	6.25×10^{-3}
6270	1.30	0.12	47	1.85×10^{-3}

[a] The bin time was set to 100 μs for the data analysis. [b] This value is smaller than the bin time; it is expected to have a large error.

In an attempt to determine how triplet−triplet transition and trans−cis photoisomerization affect single molecule properties, we undertook an investigation of the fluorescence intensity fluctuation of single Cy5 molecules at different excitation intensities since the power dependence of the on−off kinetics is still a point of discussion in the literature.[33,51,52] Fortunately, some molecules on the dried glass surface in N_2-saturated environment are stable enough to allow for recording several data sets at different excitation intensities before photobleaching. Figure 3 shows two typical intensity time traces of individual Cy5 molecules with increasing excitation intensities. The resulting emission rate dependences are shown in Figure 3c. These molecules are stable enough to stay in the on states before photobleaching. It is also noted that the dim state occurs preferentially at stronger excitation intensities as indicated by arrows in Figure 3b, implying that the molecules on a glass surface may exist with more rigid conformation compared to those in solution.[53] Unlike conventional single molecular statistical analysis, the excitation intensity dependent measurement with just "on"-state molecules under the same conditions for each individual molecule ensures that the parameters extracted for the rate constants are reliable because we do not have to average over several individual molecules. This measurement can also provide us with a better estimate of the pumping intensity, and ensures that the excitation is far from saturating. In general, for a specified molecule with kinetic processes described by a three-level model (ground state, emitting state, dark state), the change of excitation intensity should not influence the extracted photophysical rate constants. Therefore, with increasing excitation power the on times should decrease due to a light-driven transition from the emitting into the dark state whereas the off time should stay constant. Table 2 lists the parameters extracted from a typical single Cy5 molecule at different excitation intensities. In contrast to the three-level-model prediction, we note that the on time increases and the off time decreases with increasing excitation intensity (more than 90% from 80 different individual molecules show the same behavior), indicating more additional photoinduced processes are involved. A shortening of the triplet lifetime τ_{off} with increasing excitation intensity indicates photoinduced reverse intersystem crossing (RISC) from T_n to S_n followed by relaxation to the S_1 state.[28,33] The lifetime of T_n is about 200 fs, and the time of the transition from T_n via S_n ($n \geq 1$) to S_0 is less than 10 ns.[30,33,52,54,55] This photoinduced reverse intersystem crossing from T_n to S_n could only happen efficiently in the *trans*-Cy5 molecule upon laser excitation at higher intensity because the *trans*-T_1-T_n absorption largely overlaps the *trans*-S_0-S_1 absorption, and is also well in resonance with the single excitation wavelength used for single molecule excitation. A similar spectral overlap was also observed from a series of multichromophoric dendrimers, which leads to complicated single molecule behaviors, such as S_1-S_1 annihilation, S_1-T_1 annihilation, and photoinduced RISC.[56,57] The fact that those rigid *trans*-Cy5 molecules studied for power-dependent single molecule measurements (for example, see Figure 3a,b) are of remarkable photostability because of the accelerated RISC results in the increase of on time at high excitation power.[51] Finally, it should be mentioned here that we cannot reasonably obtain the laser intensity dependent on/off dynamics from those "on/dim mixing" intensity time traces with results similar to the case of pure "on"-state molecules (*trans*-Cy5) as shown in Figure 2c, because of the low quantum yields for intersystem crossing and high quantum yields for trans−cis isomerization following the *trans*-S_0-S_1 excitation demonstrated by laser photolysis experiments as shown in Figure 1b.

Conclusions

We report here the detailed spectral data of Cy5, where the trans triplet state absorbs about 625 nm, the cis ground state absorbs about 690 nm, and the cis triplet state also absorbs around 690 nm. It is evidenced that the triplet-state T−T absorptions largely overlap ground-state absorptions for both trans and cis isomers. The cis triplet state T−T absorption was observed, indicating the presence of a lowest cis triplet state as an isomerization intermediate upon excitation. These results from both ensemble and single molecule measurements show the complexity of the cyanine dyes for the performance of the cyanine dyes in all applications, especially in single molecule detection. The detailed spectral results allow us to reveal those unexpected single molecule behaviors of Cy5 during single molecule measurements. Typically, in the fluorescence time traces of single emitters we can distinguish between fast on−off intensity fluctuations due to singlet−triplet transitions and slower on/dim fluctuations due to a cis−trans isomerization; in single molecule FRET measurements, we can clearly interpret the unexpected phenomenon of single Cy5, e.g., why Cy5 can still accept the energy transferred from a donor when Cy5 was in dark states. We also conclude that the RISC could only happen in the trans isomer rather than in the cis isomer since the excited wavelength used for single molecule measurements is in resonance with the ground-state absorption and triplet-state absorption of the trans isomer.

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Supporting Information Available: Parameters for the detected count rate (I_{det}), average on time and dim time, on-

state average counts (N_{on}), off-state yield (Φ_{off}), and triplet state transition yield (Y_{isc}) of single Cy5 molecules, scheme of the relevant five-level model and transitions for single *trans*-Cy5 molecule excited under high intensity, weighted residuals for the fitting quality of decay, absorption spectra of both solved and surface absorbed Cy5 molecules and the experimental result of singlet oxygen phosphorescence from an air-saturated Cy5−ethnaol solution, as well as fluorescence spectrum of donor TMR molecule and absorption spectrum of acceptor Cy5 molecule. This material is available free of charge via the Internet at http://pubs.acs.org.

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(53) Strictly speaking, there is still a small possibility for the molecule to jump to the dim state during the long "on" state. The dim state (*cis*-Cy5) occurs in solution more frequently than on the dry glass surface. The on ↔ dim transition is a statistical process that happens on average after a certain number of photocycles. More on ↔ dim transitions will be expected with increasing excitation intensity because of the thermoinduced back-isomerization from cis to trans conformations; the saturated counts at higher excitation power as shown in Figure 3b may probably be due to more on ↔ dim transitions before photobleaching. From the laser photolysis results, the RISC could only happen in the trans isomer rather than in the cis isomer since the excited wavelength used for single molecule measurements is in resonance with the ground-state absorption and triplet state absorption of trans isomer. Therefore, to determine the RISC at single molecule levels, we must choose those single molecules on the glass with the more stable "on" state.

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