Long time scale blinking kinetics of cyanine fluorophores conjugated to DNA and its effect on Förster resonance energy transfer

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The blinking kinetics of individual Cy5 fluorophores conjugated to DNA are directly measured using single-molecule spectroscopy. Under deoxygenated aqueous conditions, Cy5 fluorescence exhibits spontaneous and reversible on/off fluctuations with a period lasting seconds. This blinking is observed when directly exciting Cy5 with 640 nm light and by Förster resonance energy transfer (FRET). We find that Cy5 blinking is influenced by the proximity of the donor, the structure of the donor, the presence of 514 nm excitation, and FRET. In the context of single-molecule FRET, blinking of the acceptor produces anticorrelated donor-acceptor intensity fluctuations, which can be difficult to discern from variations in the interdye distance. Slow blinking is, in particular, problematic because it overlaps with biologically relevant time scales. By employing an alternating 514/640 nm laser excitation scheme, we show that the dark states can be readily resolved and discriminated from FRET distance fluctuations. © *2005 American Institute of Physics*.
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I. INTRODUCTION

Single-molecule fluorescence resonance energy transfer (smFRET) has become an extremely powerful method for the investigation of different nucleic acid forms,[1–5] enzyme kinetics,[6,7] and protein folding dynamics.[8] FRET is based on the nonradiative dipole-dipole coupling between donor and acceptor fluorophores separated by a distance R.[9,10] The efficiency of energy transfer is inversely proportional to the sixth power of R/R_0, where R_0 defines the interdye distance over which the energy transfer efficiency is half of its maximum. Unlike FRET experiments in bulk, with the single-molecule approach the evolution of individual molecules can be followed over a broad range of times, from milliseconds to minutes, making it especially useful for the study of biomolecular processes that involve complicated nonequilibrium kinetics. In order to observe biomolecular kinetics over long periods of time, the molecules under study can be immobilized, but care must be taken to ensure that the dyes and the immobilization methods do not interfere with the biomolecules' normal activities.

Single-molecule spectroscopy of immobilized light-emitting molecules has revealed the existence of intermittency in diverse systems such as nanocrystals and quantum dots,[11–14] fluorescent proteins,[15] organic dyes, and conjugated polymers.[16–22] In the context of the smFRET experiment, intermittency of the acceptor fluorophore is problematic. An inactive acceptor can no longer quench the donor via FRET, and thus the emission of the donor increases. The apparent fluctuations in acceptor emission, coupled with the anticorrelated donor fluctuations, can be misinterpreted as variations in R. Slow ($\sim$1 s) blinking of the acceptor is particularly problematic, because this time scale is comparable to the time scale of many biomolecular processes one wants to characterize by smFRET. While the underlying physical mechanism responsible for fluorophore blinking is poorly understood, it is of concern due to the widespread use of organic fluorophores in single-molecule kinetics measurements.

The cyanine dye, Cy5, is a red-emitting fluorophore widely used in smFRET experiments. Recently Heilemann and coworkers have reported large anticorrelated donor-acceptor intensities even with an interdye distance well within R_0,[23] which were attributed to an inactive acceptor. However, due to the inherent background in the single-molecule experiments,[24] there remains a challenge to discern low FRET as a result of a large interdye distance increase from no FRET due to an inactive acceptor. Moreover, the FRET-induced spontaneous blinking kinetics were not characterized, and it remained unclear if Cy5 blinking behavior is directly affected by green illumination[23] (e.g., 532 nm) or if a short-range (1–2 nm) interaction with a secondary chromophore is additionally required.[25]

Here we show that under conditions that are typically employed in smFRET experiments a lone Cy5 conjugated to DNA oscillates between a fluorescent state lasting $\sim$1 s and a long dark state lasting tens of seconds. The addition of a donor results in almost an order of magnitude decrease in the dark period, but this does not involve the excitation of the donor fluorophore and FRET, and thus can be probed using the 640 nm excitation only. Moreover, without a donor present, an additional 514 nm illumination has a similar effect on Cy5 blinking as a proximal donor fluorophore. We quantified the Cy5 blinking kinetics as a function of three separate parameters: (1) the proximity of the donor to the acceptor (in the absence of FRET) and the type of the donor, (2) the presence of additional green light, and (3) excitation of the acceptor through FRET.

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In order to characterize extensive numbers of individual molecules under well-controlled chemical conditions, we employ a high-throughput scanning confocal microscope that utilizes a custom flow chamber.[26] This apparatus allows us to maintain tightly controlled chemical conditions in the cell throughout the experiment, which typically extends over a day without loss in sample vitality. Automation of the instrument also allows us to acquire and analyze thousands of single-molecule traces, under various conditions, yielding highly robust statistics. Our data suggest that while the blinking kinetics of Cy5 coupled to DNA is a highly complex process that involves many factors, a simple experimental approach can be employed to discriminate between real FRET fluctuations (due to distance changes) and the acceptors' nonfluorescent states. We show that with an alternating green/red laser excitation scheme, dark acceptor states can be directly probed and discriminated from FRET states. This approach is similar to the alternating laser excitation method recently applied to freely diffusing molecules in a burst analysis study.[27]

II. MATERIALS AND METHODS

Single-molecule measurements are performed on fluorophores conjugated to B-DNA according to Ref. 24. The DNA constructs TMR-6-Cy5 and TMR-14-Cy5 contain a FRET pair, tetramethylrhodamine (TMR) and Cy5, that are separated by 6 and 14 nucleotides on the same strand, respectively (approximately 38 and 58 Å).[24] Cy3, another cyanine dye spectrally similar to TMR, is used for the constructs Cy3-6-Cy5 and Cy3-14-Cy5. The construct DNA-Cy5 contains a single fluorophore at the same location as the FRET-labeled molecules. The DNA molecules have a biotin moiety used to immobilize them on highly specific streptavidin-coated microfluidic flow cells. The smFRET experiments utilize an oxygen scavenging system [10 mM tris-Cl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) 2-mercaptoethanol, 4 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 0.04 mg/ml catalase], which was exchanged through the imaging cell at a constant rate of 5 μl/min from a reservoir. We found that the constant accumulation of gluconic acid (a by-product of the glucose oxidase reaction) eventually overwhelms the buffering capacity of the system, resulting in a pH drop from 8 to about 4 over a few hours. This significant pH drop can result in protein and DNA denaturation and can also affect the photophysical properties of fluorophores that are sensitive to pH. In order to circumvent this problem, the buffer was degassed by sonication prior to the addition of enzymes in order to reduce the oxygen concentration. Additionally, argon gas was trickled over the reservoir to prevent molecular oxygen from diffusing into the buffer during the course of the experiment. These modifications allowed the buffer pH to be maintained at its initial value for over 24 h.[28]

The experimental setup utilizes two laser sources: the 514 nm line of Ar^+ ion laser and a 640 nm diode laser. Acousto-optic modulators are used in experiments where an alternating red and green laser excitation scheme is employed. A counter/timer board (NI-PCI 6602) was utilized to generate the alternating laser pulses. Photon data collected by two spectrally resolved avalanche photodiodes were acquired by the same board at 20 μs time resolution using a custom computer code, thus allowing an accurate deinterleaving of the photons to the red and green excitation portions in each trace. For each experiment, approximately 1000 time traces are collected overnight. The dwell times of the Cy5 emission (t_{on}) and no Cy5 emission (t_{off}) periods of the traces were automatically identified for analysis using custom computer code. We define the off rate k_{off} as the inverse of the characteristic time over which Cy5 switches from the emitting to the nonemitting state, t_{on}. Similarly, the on rate is the inverse of the characteristic time over which Cy5 switches from the nonemitting state to the emitting state, t_{off}.

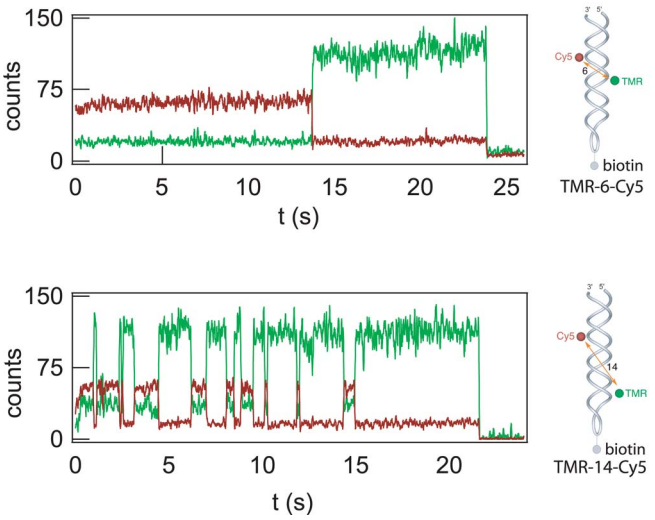

FIG. 1. Representative time traces of individual DNA molecules internally labeled with TMR and Cy5 fluorophores with an interdye separation of six nucleotides (top panel) and 14 nucleotides (bottom panel). Schematics of the constructs are given to the right of the traces. The red lines correspond to counts (per 10 ms) from the acceptor channel and the green lines from the donor channel, excited by a 514 nm laser (0.5 kW/cm^2 at sample).

III. RESULTS

A. Blinking during FRET

Figure 1 displays typical single-molecule time traces for TMR-6-Cy5 and TMR-14-Cy5, with green laser excitation. Notice that while the TMR-6-Cy5 trace is photostable showing high FRET efficiency until the acceptor is bleached at $t \approx 13$ s, the TMR-14-Cy5 trace clearly displays anticorrelated on/off intensity fluctuations. Over 80% of ~1000 traces of the TMR-14-Cy5 exhibit multiple on/off fluctuations. In contrast, less than 10% of ~1000 traces of the TMR-6-Cy5 display these fluctuations. The distributions of the t_{on} and t_{off} acceptor periods for the two constructs are shown in Fig. 2(a). The histograms of the TMR-14-Cy5 increase for short times followed by decaying exponential tails. This is not an experimental artifact, but rather a signature of the complex kinetics observed. Fitting the tails of the distributions using monoexponential functions, we obtain the average $\bar{t}_{on}$ and $\bar{t}_{off}$ values of 0.98±0.02 and 0.97±0.03 s for TMR-14-Cy5 and an average $\bar{t}_{on}$ 4.0±0.1 s for TMR-6-Cy5. The distribution of t_{off} was not determined for TMR-6-Cy5 because multiple on/off fluctuations were rare. We also performed experiments

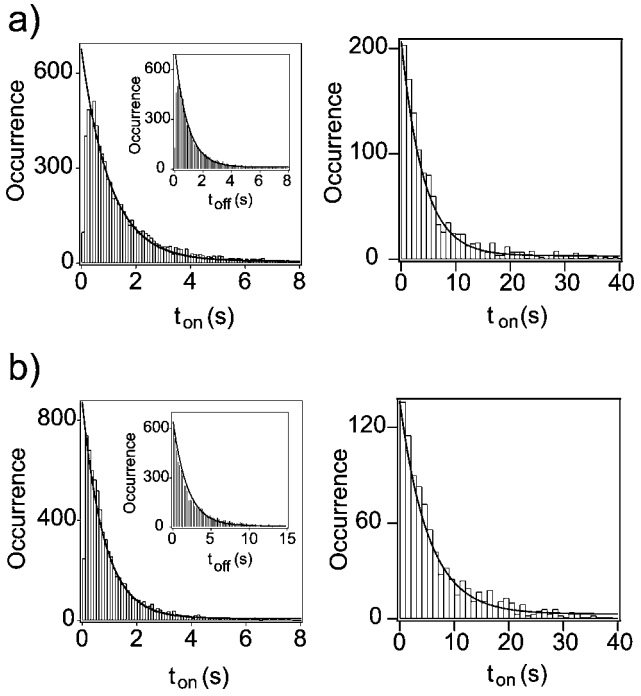

FIG. 2. a) Distributions of the low (off) and high (on) FRET dwell times obtained from single-molecule traces. The left panel corresponds to TMR-14-Cy5 and the right panel to TMR-6-Cy5. The main figures are for the distributions of t_{on} and the inset shows distributions of t_{off}. The solid lines are monoexponential fits to the data ($1/e$ values are given in the text). The distribution of t_{off} was not determined for TMR-6-Cy5 because multiple on/off fluctuations are rare. (b) Similar distributions as above for Cy3-14-Cy5 and Cy3-6-Cy5 (left and right panels, respectively).

using the Cy3 dye as the donor and obtained similar results [Fig. 2(b)]: average $\bar{t}_{on}$ and $\bar{t}_{off}$ values of 0.81 ± 0.01 and 1.91 ± 0.01 s for Cy3-14-Cy5 and average $\bar{t}_{on}$ 5.3 ± 0.2 s for Cy3-6-Cy5. The two donors used yielded similar blinking kinetics except for the characteristic $\bar{t}_{off}$, which differ by a factor of 2.

The four- to five-fold difference in the average $\bar{t}_{on}$ values of TMR-6-Cy5 versus TMR-14-Cy5 (as well as Cy3-6-Cy5 versus Cy3-14-Cy5) measured over thousands of individual molecules indicate that the striking difference in the FRET kinetics is highly characteristic. Given that the molecules only differ by the donor position with respect to the acceptor, it could be presumed that these fluctuations are due to large distance and/or orientation fluctuations between the FRET pair in the Donor-14-Cy5 cases, which would not exist with the Donor-6-Cy5 cases. In particular, it could be thought that because the donor-acceptor separation of Donor-14-Cy5 is close to R_0 (65 Å for TMR-Cy5 and 54 Å for Cy3-Cy5 as determined from the spectral overlap in bulk),[29] small distance fluctuations would translate into large FRET efficiency variations. This hypothesis can be tested by directly exciting Cy5 with a red laser. If distance fluctuations are responsible for the apparent low FRET periods of the trace, then one would expect that the red-excited Cy5 emission would not be affected and that it would exhibit steady emission until Cy5 bleaches. To perform this experiment, the red and green lasers were alternated at a rate of 10 Hz. This rate is ten times faster than the typical intensity fluctuations of the trace shown in Fig. 1. Data collected in this manner can be deinterleaved into two time traces: one for the green-excited intervals and the other for the red-excited intervals, as displayed in Fig. 3. Comparing the red versus green excitations, it is evident that the fluorescence of Cy5 due to direct red excitation is perfectly correlated with the sensitized Cy5 emission via FRET. Thus, Cy5 can undergo a transition to a reversible inactive dark state that is orders of magnitude longer than previously reported,[30] in some cases lasting many seconds. The anticorrelated behavior of the donor and acceptor fluorescence further indicates that this Cy5 dark state does not participate in FRET. Hence, the anticorrelated donor-acceptor fluorescence is entirely photophysical in origin.

Referring back to Fig. 1, we note that the fluctuations in the Cy5 emission do not require the red laser excitation to drive the molecule to the dark state and that the molecule steadily oscillates between the two states. To better understand the nature of the cyanine blinking, several contributions must be considered and separately quantified: (1) the influence of energy transfer to Cy5 blinking kinetics, discussed above; (2) the direct influence of the green laser excitation on Cy5 stability; and (3) the proximity of the donor to the acceptor. In order to address these questions, we first study the effect of the green laser by using a dual (simultaneous, not alternating) red+green illumination scheme on a singly-labeled Cy5 construct. To address the influence of donor proximity on the blinking rates of Cy5, FRET pairs differing in the donor fluorophore and donor-acceptor distances are excited by the red laser only.

B. The effect of green excitation on blinking

The effect of the additional green light on the photophysical stability of Cy5 is determined using the singly-

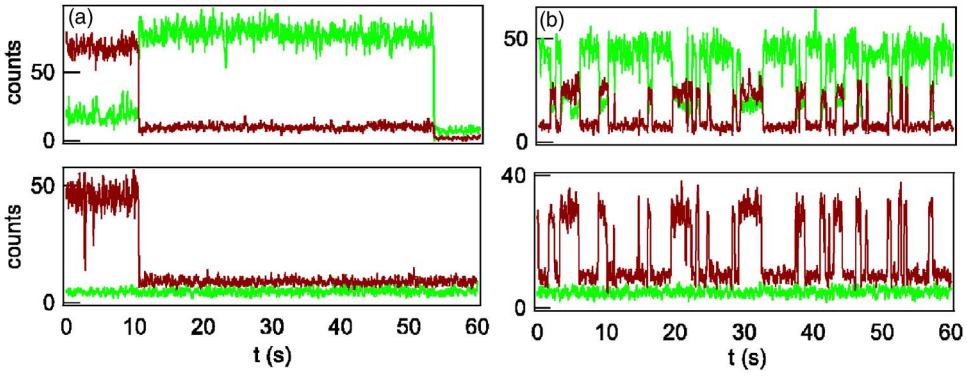

FIG. 3. Representative time traces of individual DNA molecules internally labeled with TMR and Cy5 fluorophores excited using alternating green and red lasers (0.5 and 0.13 kW/cm^2, respectively). (a) TMR-6-Cy5 during green (top panel) and red excitations (bottom panel). (b) TMR-14-Cy5 during green (top panel) and red excitations (bottom panel). Note the perfect correlation between the acceptor emissions during green and red excitations.

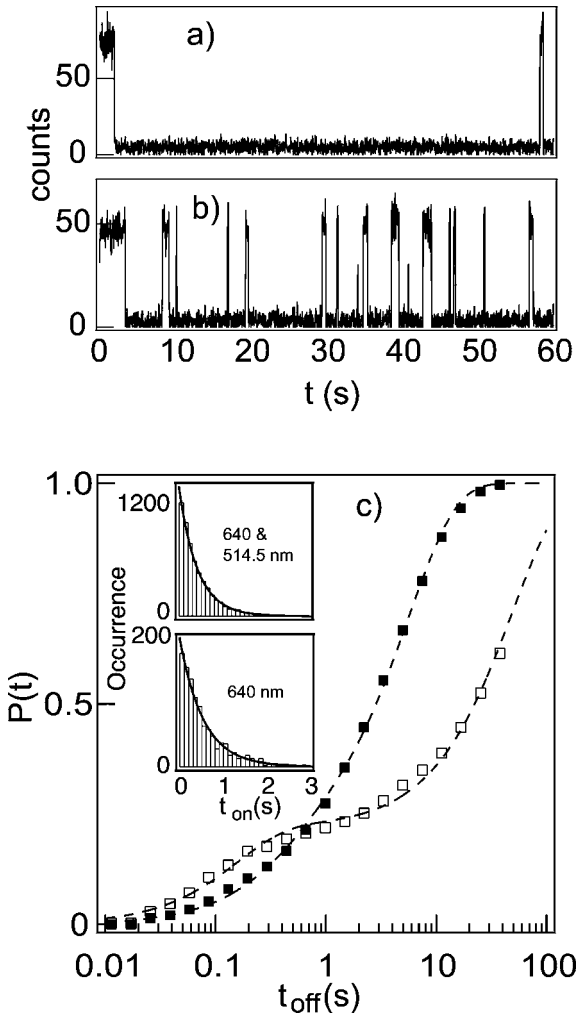

FIG. 4. The influence of green laser excitation on acceptor oscillations for the DNA-Cy5 construct. Typical time trace of the acceptor channel using (a) 640 nm and (b) 640+514 nm excitations. (c) Distributions of t_{off} and t_{on} for the DNA-Cy5 construct, excited by red (open symbols) or red+green lasers (filled symbols). A cumulative probability distribution $P(t)$ is used to determine the on rates. $P(t)$ is approximated by double-exponential fits (dotted lines), yielding on rates of 0.213 and 0.026 s^{-1} for the 640+514 nm excitation (filled symbols) and 640 nm excitation (open symbols), respectively. The insets are monoexponential distributions of the on times with the indicated excitation scheme yielding $k_{off} \sim 2\ s^{-1}$ in both cases.

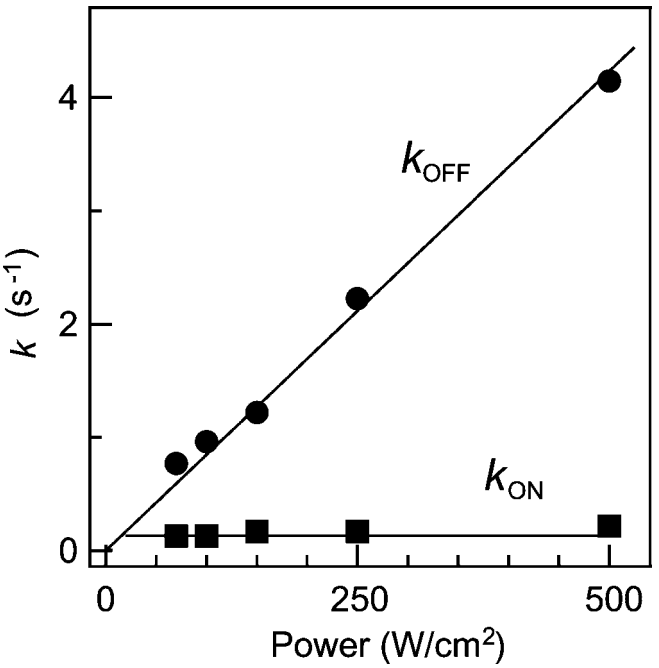

FIG. 5. Intensity dependence of the blinking rate constants of TMR-6-Cy5 molecules excited with the red laser. Each data point in this plot was obtained from the distribution of over 500 single molecules as explained in Fig. 4. The off rate is linearly dependent on the 640 nm excitation power, whereas the on rate is power independent.

labeled DNA-Cy5 construct. A typical trace for this molecule is shown in Fig. 4 with red excitation only and with red +green excitations. The differences are apparent: the additional green laser excitation induces faster fluctuations in the Cy5 emission than the red-only excitation. To quantify this behavior we collected large data sets of single-molecule traces as explained above. Because of the long t_{off} periods, often approaching the 60 s acquisition time used in our experiments, a cumulative distribution function $P(t)$ is used to characterize the dark-state duration instead of the direct distribution of the off times. Figure 4(c) displays $P(t)$ of several thousand t_{off} segments obtained from this analysis, using either red laser excitation (0.25 kW/cm^2)) or dual red+green lasers (0.25 and 0.5 kW/cm^2, respectively). For both illumination schemes, the data fits well to a sum of two exponential functions: $P(t)=1-(a_1e^{-t/\tau_1}+a_2e^{-t/\tau_2})$, where $a_1+a_2=1$, yielding average time scales (amplitudes): $\tau_1=0.16\pm0.02$ s(0.22), $\tau_2=49.9\pm2.0$ s(0.78) for the red laser excitation and $\tau_1=0.40\pm0.05$ s(0.17), $\tau_2=5.58\pm0.14$ s(0.83) for the dual red+green illumination. We define $k_{on}\equiv(a_1\tau_1+a_2\tau_2)^{-1}$ as the characteristic rate in which Cy5 recovers from its dark state, yielding $k_{on}=0.026\ s^{-1}$ for the red excitation, and $k_{on}=0.213\ s^{-1}$ for the dual red+green illumination. Thus, the addition of the green laser excitation increases k_{on} by a factor of 8.

Unlike the t_{off} distributions, the distributions of t_{on} can be well approximated by monoexponential functions [Fig. 4(c), insets] with average $\bar{t}_{on}$ values of 0.55 ± 0.02 and 0.42 ± 0.01 s for the red and dual red+green illuminations, respectively. Thus the green laser does not strongly affect the off rates: in both cases $k_{off}\approx 2\ s^{-1}$. We postulate that the off rate of Cy5 is driven by the intensity of the red laser, while the on rate is most likely related to internal conversion from a long-lived dark state to the ground state, which depends on the green illumination, as recently observed.[31] To verify this hypothesis we repeated our single-molecule experiments and measured the distributions of t_{on} and t_{off} at different red laser intensities. Our results are summarized in Fig. 5. We find that k_{off} is linearly dependent on the red excitation power, whereas k_{on} is power independent, supporting our hypothesis.

C. The effect of donor proximity on Cy5 stability

Next, we studied the influence of donor proximity on the Cy5 blinking kinetics. In these experiments we use only red excitation (direct acceptor excitation and no donor excitation) in order to separate out the donors contribution to the Cy5 emission from the stabilizing effect of the green light. We perform experiments similar to those described in Fig. 3,

placing the donor either 6 or 14 nucleotides away from Cy5. Figures 6(a) and 6(b) display two characteristic traces of red-laser-excited TMR-14-Cy5 and TMR-6-Cy5. In comparison to the Cy5-DNA molecules discussed earlier, there is pronounced Cy5 stabilization with the TMR-6-Cy5 construct where the donor is positioned ~4 nm away from the acceptor. We quantify this by the cumulative distributions of t_{off}, shown in Fig. 6(c). The distributions are well approximated as before by double exponential functions, yielding average time scales (amplitudes) $\tau_1=0.28\pm0.04$ s(0.19) and $\tau_2=5.2\pm0.2$ s(0.81) for TMR-6-Cy5 and $\tau_1=0.18\pm0.02$ s(0.20) and $\tau_2=34.5\pm1.2$ s(0.80) for TMR-14-Cy5. The characteristic on rates are 0.234 and 0.037 s^{-1} for TMR-6-Cy5 and TMR-14-Cy5, respectively. Recalling that the on rate for DNA-Cy5 is 0.026 s^{-1}, a 14-nucleotide interdye spacing yields a mild 42% increase in k_{on}. In contrast, an interdye spacing of six nucleotides increases k_{on} by ninefold. Interestingly, substituting Cy3 for TMR changes the Cy5 blinking kinetics [Fig. 6(d)]. For Cy3-6-Cy5 the average time scales (amplitudes) are $\tau_1=3.5\pm0.5$ s(0.33) and $\tau_2=35.9\pm3.1$ s(0.67), and for Cy3-14-Cy5 the average time scales (amplitudes) are $\tau_1=0.13\pm0.02$ s(0.12) and $\tau_2=51.1\pm1.4$ s(0.88). These time scales yield characteristic on rates of 0.040 and 0.022 s^{-1} for Cy3-6-Cy5 and Cy3-14-Cy5, respectively. The six-nucleotide interdye spacing results in only a modest 54% increase in k_{on}, and placing Cy3 14 nucleotides away from Cy5 has no significant effect on Cy5 blinking as compared to the DNA-Cy5 construct. Thus, Cy5 recovery from the dark state is very sensitive to the donor fluorophore type.

The distributions of t_{on} in the above experiments can be well approximated by monoexponential functions (Fig. 6, insets). The average $\bar{t}_{on}$ values of TMR-6-Cy5 and TMR-14-Cy5 were 0.44 ± 0.01 and 0.49 ± 0.01 s, and the average $\bar{t}_{on}$ values of Cy3-6-Cy5 and Cy3-14-Cy5 were 0.49 ± 0.04 and 0.44 ± 0.02 s. All these constructs yield $k_{off}\approx2$ s^{-1}. Thus, our data clearly indicate that while k_{on} strongly depends on the interdye distance, k_{off} is insensitive to the presence of a donor fluorophore. A summary of the on and off rates obtained in our experiments for the different DNA constructs and different laser excitations is presented in Table I.

IV. DISCUSSION AND CONCLUSIONS

To summarize our results, we have characterized the reversible blinking of Cy5 dyes coupled to DNA using single-molecule spectroscopy. We first demonstrated that the on/off blinking of Cy5 in our FRET experiments (green excitation only) is not due to distance fluctuations between the donor and acceptor, but are photophysical in origin. Noting that the FRET experiment mixes several possible factors that can affect Cy5 stability, namely, donor proximity, green light coupling with acceptor, and energy transfer, we investigated the contributions of these factors separately. We find that a lone Cy5 (coupled to DNA) blinks when excited with red light over a wide range of intensities ($50-500$ W/cm^2). This blinking is characterized by a small $k_{on}\sim0.02$ s^{-1} (long dark states), which can only be quantified by prolonged observations of individual immobilized single molecules and with

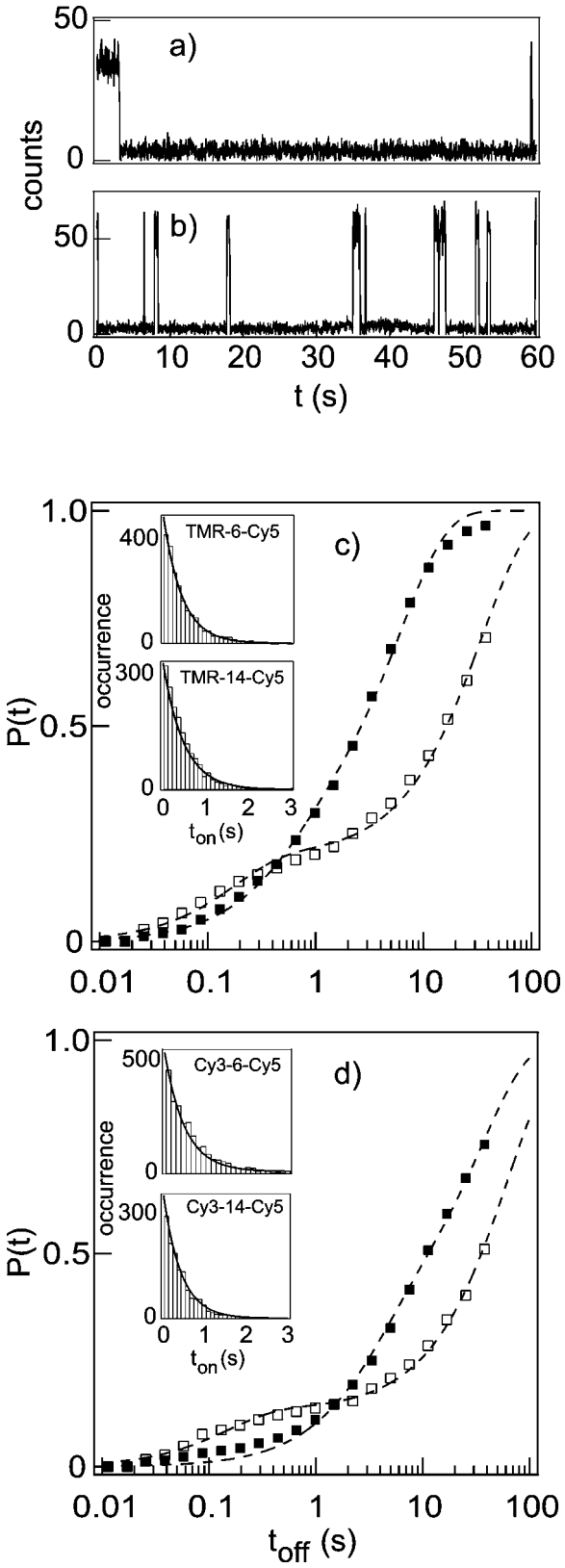

FIG. 6. Influence of donor proximity on the blinking times of Cy5 using 640 nm excitation (0.25 kW/cm^2). Typical time trace of the acceptor channel for (a) TMR-14-Cy5 and for (b) TMR-6-Cy5. The cumulative probability distribution $P(t)$ is used to determine the characteristic dwell times of the Cy5 dark states for the TMR/Cy5 constructs (c) and the Cy3/Cy5 constructs (d). The dotted lines are double-exponential fits to $P(t)$ for Donor-6-Cy5 (filled symbols) and Donor-14-Cy5 (open symbols); the average time scales and amplitudes are given in the text. The insets are distributions of the Cy5 on times yielding $k_{off}\approx2$ s^{-1} in all cases.

TABLE I. Summary of Cy5 blinking rate constants (s^{-1}).

	Excitation (nm)			
	640		514	
	k_{off}	k_{on}	k_{off}	k_{on}
DNA-Cy5	1.82	0.026	2.40[a]	0.213[a]
TMR-6-Cy5	2.27	0.234	0.250	ND[b]
TMR-14-Cy5	2.04	0.037	1.02	1.03
Cy3-6-Cy5	2.04	0.040	0.189	ND[b]
Cy3-14-Cy5	2.27	0.022	1.23	0.524

[a]These rates are for the dual 640+514 nm illumination scheme.
[b]ND, not determined.

large data sets. This might be the reason why irreversible photobleaching of Cy5 has not been previously distinguished from long-lived dark states. Unlike previous studies that focused on light-induced recovery from a dark state (using green light), we show that long time scale oscillations are natural to the Cy5 fluorophore when immersed in an oxygen-depleted aqueous environment. We observe that even with 640 nm excitation (i.e., no FRET interaction) the presence of TMR (or Cy3) nonetheless helps the Cy5 to recover from the dark state, increasing k_{on} by a factor of 9 with an interdye distance of 38 Å. This enhanced recovery is dependent on the interdye distance and also on the type of "donor" fluorophore.

Moreover, comparing DNA-Cy5 with TMR-14-Cy5, we find that FRET excitation (514 nm) results in a 40-fold increase of the on rate, which cannot be explained by simply combining the 42% enhancement due to the donor proximity and the eight-fold green laser enhancement. Most interestingly, we note that t_{on} is insensitive to the presence of a donor with direct red excitation, but when exciting Cy5 via FRET, t_{on} strongly depends on the donor position. For example, when the interdye distance is increased from 6 to 14 nucleotides, we observe a four- to five-fold increase in k_{off} using either TMR or Cy3 as the energy-transfer donor.

The role of molecular oxygen in fluorophore stability has been extensively studied in the past decade. In the absence of molecular oxygen long triplet states have been shown to persist.[22] In particular, it is well established that the triplet-state lifetime of cyanine dyes is very sensitive to oxygen concentration and can be extended from about 50 μs when exposed to air to about 100 ms when embedded in a polymer film.[17,21,31–33] Once in the triplet state, photoinduced reverse intersystem crossing can occur,[31] explaining the enhancement in k_{on} with the addition of green illumination. This observation is in agreement with the recent experiments of Heilemann *et al.*[23] However, the multisecond lifetime of the dark states that we observed are unlikely to be a result of exceedingly long triplet states because the system contains a triplet quencher, 2-mercaptoethanol. Another mechanism to produce an inactive fluorophore is the formation of a radical specie.[34] In particular, our results are in agreement with the postulation that the long Cy5 dark state involves an excited-state transition to the triplet state that ultimately leads to the formation of a long-lived radical, which was recently observed for rhodamine 6G dyes embedded in an organic matrix.[35] This mechanism is supported by the linear dependence of the Cy5 off rate on laser power (Fig. 5). Our data also show that decreasing the donor-acceptor interdye distance results in a stronger k_{on} (shorter dark periods), which can be interpreted as enhanced stability of Cy5. Furthermore, comparing different donor types, we find that the stabilizing effect is stronger for the TMR/Cy5 pair as compared with the Cy3/Cy5 pair. Because this effect is seen even when the donor and acceptor are positioned at distances comparable to or larger than their Förster radii, a direct perturbation of the electronic states of Cy5 by the donor is unlikely. A detailed model describing donor-acceptor interactions that explains this behavior requires further experiments, such as direct probing of the fluorophores' lifetimes and spectra.

Our experiments emphasize the complications that cyanine blinking introduces to the interpretation of single-molecule FRET data. We showed that TMR/Cy5 and Cy3/Cy5 FRET pairs conjugated to DNA molecules can spontaneously oscillate between high-acceptor and no-acceptor emissions and that the period of these oscillations is ~1 s when the donor and acceptor separation is ~6 nm (Donor-14-Cy5). With the addition of an alternating green/red laser excitation, however, acceptor dark states can be readily discerned from true low-FRET states. Our experiments illustrate the power of single-molecule spectroscopy in revealing the photophysical properties of fluorophores with time scales ranging from milliseconds to tens of seconds.

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