Single-molecule photophysics of dark quenchers as non-fluorescent FRET acceptors

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Dark quencher chromophores are interesting alternatives to common single-molecule FRET acceptors. Due to their short excited state lifetime, they should be less prone to complex photophysics and bleaching. We find, however, that for common enzymatic oxygen scavenging systems and photoprotection strategies – the gold standard of single-molecule measurements – the quenchers BBQ650 and BHQ-2 induce frequent blinking of the donor molecule. They switch in a photoinduced process to what we identify as a radical anion state and back. We further make use of the broad absorption spectrum for selective bleaching of the quenchers in order to photoactivate the fluorescence of initially completely quenched dye molecules. This represents a general strategy to turn fluorescent dyes into photoactivatable probes.

Introduction

One of the most widely used tools in single-molecule spectroscopy is Förster Resonance Energy Transfer (FRET), a radiationless energy transfer between a donor and an acceptor molecule. Because the degree of energy transfer is strongly distance dependent in the range of 1–10 nm, the technique is often used to measure inter- and intramolecular distances as well as to monitor conformational changes in real-time. Most commonly, the FRET-pair consists of two organic dyes like Cy3/Cy5, where the absorption spectrum of the acceptor (Cy5) significantly overlaps with the emission spectrum of the donor (Cy3). One of the biggest drawbacks is the photophysical behaviour of the acceptor molecules, because they blink (show transient transitions to dark states) and undergo photobleaching, thus limiting single-molecule FRET experiments.[1]

Over the last few years, a number of non-fluorescent FRET acceptors have become increasingly popular in single-molecule spectroscopy, as quenchers for molecular beacons[2] or for the observation of enzymatic processes[3–6] or simply for the detection of single molecules by absorption.[7] Dark FRET acceptors offer several advantages compared to conventional FRET acceptors. Their broad absorption spectrum spans a larger part of the visible spectrum, which makes them candidates for quenching of many different fluorophores simultaneously.[8] Because they do not fluoresce, the spectral range required for such a FRET-pair is much smaller than that for a conventional FRET-pair. Hence, the spectral region usually occupied by the acceptor is now free to monitor colocalization of a third label as has been exploited by the Puglisi lab to correlate ribosome subunit rotation with the arrival of several ligands.[4,5] Besides such multiplexing approaches, dark FRET acceptors are expected to exhibit less complex photophysics. This is because dark FRET acceptors have substantially reduced excited state lifetimes due to non-radiative relaxation pathways to the ground state. With a short excited state lifetime the probability for other competing relaxation pathways such as intersystem crossing, successive multi-photon processes and photochemistry is reduced.

Blinking would be particularly detrimental for the use of dark FRET acceptors in biomolecular single-molecule experiments because intensity fluctuations of the donor could be interpreted as conformational changes.[1] While in conventional single-molecule FRET measurements the detected signal of the acceptor molecule can prevent misinterpretation of the data, *e.g.* using alternating laser excitation,[9] this option does not exist for dark quenchers. From previous publications it indeed seemed that photophysics are no issue. Schwartz *et al.* did not observe significant quencher induced blinking with the Cy3/BHQ-2 pair[3] and Le Reste and co-workers only observed blinking in 4% of the traces they measured with a Cy3/Quencher/ATTO647N sandwich for the quenchers QSY7 and QSY21.[8]

In this article we show that even under common measurement conditions frequent blinking induced by the quenchers BBQ650 and BHQ-2 can occur. The blinking can be explained in the context of the ROXS concept.[10] This means that the

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FRET pair and control experiments need to be carried out carefully when dark FRET acceptors are used. On the other hand, the photophysics of these quenchers can provide new possibilities to control the fluorescence of single molecules. With an ATTO532 dye that is initially completely quenched by one of the dark FRET acceptors, we show selective bleaching of the quencher molecule using a wavelength that is redshifted with respect to the ATTO532 absorption spectrum. Hence, such dye–quencher pairs could be used as photoactivatable dyes with a high on/off contrast.

Materials and methods

Sample and immobilization

Double stranded DNA molecules are attached to a BSA/biotin/neutravidin surface.[11] First, single-stranded DNA (ssDNA) labelled with biotin at the 3′ end and one of the dyes Cy5 or ATTO532 is bound to the surface (5′ GTT CCC GGA TXG ACT ATA GCT TT 3′, X = dye labelled T, all DNA samples were purchased from IBA Göttingen). Then the complementary strand (5′ GCT ATA GTC AAT CCG GGA AC 3′) optionally carrying one of the quenchers BHQ-2 and BBQ650 at the 3′ end is hybridized to this strand (for quencher structures see Fig. 1a and b).

For switching experiments we use a different dye carrying strand (5′ GTX CCC GGA TTG ACT ATA GCT TT 3′, X = ATTO532-labelled T) where the dye is located on the third base to increase the quenching efficiency.

Fluorescence and absorption spectra

Absorption spectra of the quencher molecules (Fig. 1c) are recorded using an absorption spectrometer (NanoDrop 2000, ThermoScientific). Emission spectra of the dye molecules are measured using a Cary Eclipse (Agilent Technologies).

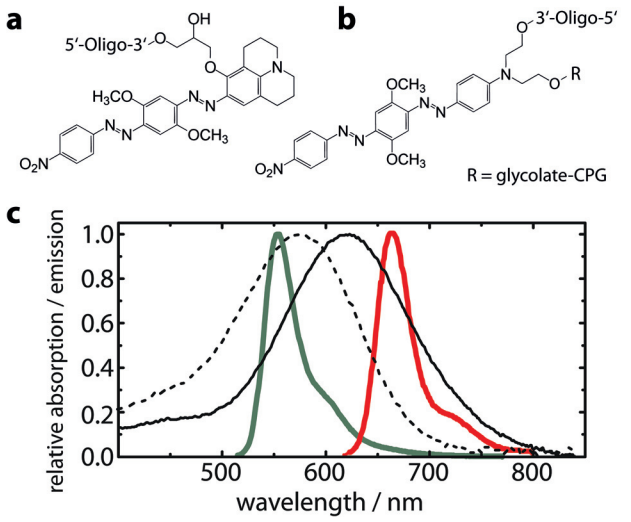

Fig. 1 (a) Chemical structure of BBQ650. (b) Structure of BHQ-2. (c) Normalized emission spectra of ATTO532 (green) and Cy5 (red) together with normalized absorption spectra of BHQ-2 (black dashed line) and BBQ650 (black solid line).

Confocal microscopes

Single-molecule measurements are carried out using two confocal microscopes. The first microscope is equipped with an 80 MHz pulsed laser for the excitation of Cy5 at 640 nm (LDH-D-C-640, Picoquant). The laser beam is coupled through the rear port of an inverted microscope (IX-71, Olympus) and focussed by the objective (UPLSAPO100XO, NA 1.40, Olympus) to a diffraction limited spot. The sample is scanned through this spot by a piezo stage (P-517.3CL, Physik Instrumente) to obtain an image of the molecules immobilized on the surface, and the same stage is used to position molecules of interest in the laser focus for time-resolved measurements. Light emitted by the dye molecules is detected through the same objective and separated from the excitation light using a dichroic mirror (Dualband z532/633, AHF). After passing through a 50 μm pinhole and spectral filters (bandpass ET 700/75 m, AHF; RazorEdge LP 647, Semrock), the emission light is focussed on the chip of an APD (τ-SPAD 100, Picoquant) and registered with a PC-card (SPC-830, Becker & Hickl) for time-correlated single photon counting. The second microscope is designed in a similar fashion, but employs an 80 MHz pulsed white light laser (SuperK Extreme EXW-12, NKT Photonics, Denmark) and an AOTF (SuperK Dual AOTF, NKT Photonics, Denmark) for selection of the excitation wavelength (532 nm for excitation of ATTO532 and 640 nm for excitation of Cy5). The beam is focused on the sample by an oil immersion objective (APON 60XOTIRF, NA 1.49, Olympus, Germany) and the resulting emission is collected by the same objective. A dichroic mirror (640dcxr, CHROMA, USA) splits the ATTO532 and Cy5 emission onto one APD (SPCM, AQR 14, Perkin Elmer, USA) for the red detection and one MPD (SPAD, PicoQuant, Germany) for the green detection. The emission is filtered in front of each detector by a longpass filter (Cy5: RazorEdge647, Semrock, USA) and a bandpass filter (Atto532: BrightLine 582/75, Semrock, USA).

Prism-TIRF microscope

We use a home-built PRISM-TIRF setup based on an Olympus IX71 to perform widefield measurements. The Cy5 fluorophore is excited at 639 nm (iBeam laser, Toptica) with 4 kW cm^{-2}. The fluorescence is collected using a 60× Olympus 1.20 N.A. water immersion objective, filtered with a 633 nm RazorEdge longpass filter (Semrock, AHF) and detected by an EMCCD camera (Andor IXon 789DU, preGain 5.1, gain 250, integration time 20 ms). The videos are analysed with custom made software based on LabVIEW (National Instruments). The molecule spots are selected by an automated spotfinder and the resulting transients are filtered using the built in cubic filter of LabVIEW 2011.

Buffers

2 mM Trolox (TX) is dissolved in 1× phosphate buffered saline (PBS) and then placed on an UV table (VWR GenoSmart) for Trolox quinone buildup (TXQ).[12] Additionally all TX/TXQ

buffers contain 1% (w/v) glucose as a fuel for the oxygen scavenging system.

The oxygen scavenging system (glucose oxidase and catalase, GOC) consists of 1 mg ml^{-1} glucose oxidase, 0.4% (v/v) catalase (50 μg ml^{-1}), 30% glycerol and 12.5 mM KCl in 50 mM TRIS, pH 7.5. For oxygen scavenging, this GOC buffer is added to the measurement buffer at a concentration of 10% (v/v).

Results and discussion

Blinking induced by dark quenchers BHQ-2 and BBQ650

We immobilize double stranded DNA molecules *via* biotin/neutravidin/biotin anchors to a BSA or PEG surface (for experimental details see the Materials and methods section). We use fluorescence lifetime imaging to first determine the fluorescence lifetime of Cy5 without quencher molecules. We record fluorescence lifetime decays of individual molecules and use a reconvolution with the instrument response function to obtain values of 1.46 ± 0.08 ns (mean ± standard deviation, number of molecules N = 113) for ssDNA and 1.18 ± 0.16 ns (N = 115) for double-stranded DNA (dsDNA) samples. Using common measurement buffers containing a mixture of glucose, glucose oxidase and catalase (GOC) for oxygen scavenging and 2 mM Trolox/Trolox quinone (TX/TXQ) for photostabilization,[12,13] we observe frequent blinking induced by the quencher molecules which has not been reported before (see Fig. 2). Considering only molecules that have a reduced fluorescence lifetime (thus indicating successful hybridization) and that do not bleach within the first two seconds of recording, the fraction of molecules that exhibit blinking is almost 100% for both quenchers. We further observe frequent quencher induced blinking with other common oxygen scavenging systems, namely the PCD/PCA system[14] and a GOC buffer additionally containing TCEP.

The quencher induced blinking appears as intensity fluctuations of the Cy5 fluorescence which is proportionally correlated with fluctuations of the Cy5 fluorescence lifetime (Fig. 2b and c). For the fluorescence lifetime transients, the lifetime was determined by MLE-fitting of the lifetime decay with a single exponential for every 10 ms of the transient.[15] Blinking on the upper millisecond timescale is not observed for Cy5 alone under these buffer conditions (Fig. 2a).[10,13] During phases of higher intensities, the fluorescence lifetime obtained by reconvolution is 1.20 ± 0.14 (N = 90) for Cy5/BBQ, which matches the lifetime of Cy5 on dsDNA without a quencher. This indicates that the quencher exists in a non-absorbing state or in a spectrally shifted form with no significant overlap with the Cy5 emission spectrum. While the quencher is in an absorbing state, the fluorescence lifetime is reduced to 0.49 ± 0.08 ns (N = 93).

This corresponds to a quenching efficiency of 59 ± 8%. We calculated the distance between the molecules as 5.0 nm[8] and the Förster radius of the Cy5/BBQ650 pair from the spectra shown in Fig. 1 to be 5.5 nm. This yields an expected quenching efficiency of 63%, which is in agreement with our experimentally determined value.

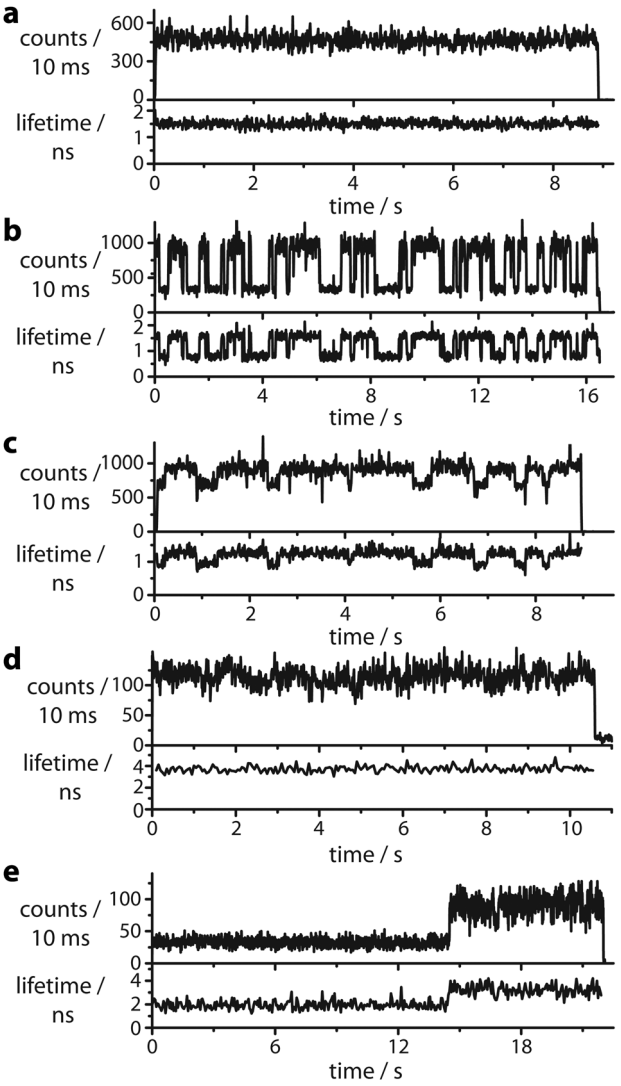

Fig. 2 (a) Top: fluorescence transient of Cy5 on dsDNA without a quencher. Bottom: corresponding fluorescence lifetime transient. Both transients display no fluctuations until the dye bleaches after 9 s. (b) Exemplary fluorescence transient of a Cy5/BBQ650 construct (top) displays frequent intensity fluctuations between two intensity levels. The transient of the fluorescence lifetime (bottom) shows analogous fluctuations. (c) The fluorescence transient of the Cy5/BHQ-2 construct (top) and the corresponding fluorescence lifetime transient (bottom) exhibit similar blinking. (d) ATTO532 on dsDNA without a quencher molecule shows no such fluctuations for the fluorescence intensity (top) and the fluorescence lifetime (bottom). (e) No blinking caused by the quencher is visible for an ATTO532/BBQ650 pair. After BBQ650 bleaches at 14 s, the lifetime (bottom) and intensity (top) of ATTO532 increase until the dye bleaches at 22 s. For all transients, a DNA construct with a medium quenching efficiency was chosen to distinguish quenched states from dark states of the donor. The fluorescence lifetime is determined for every 10 ms (Cy5) or 50 ms (ATTO532).

Compared to ATTO532 on dsDNA without a quencher molecule (Fig. 2d), the addition of BBQ650 results in intensity and lifetime quenching (Fig. 2e). Interestingly, we observe no quencher induced blinking when ATTO532 is the donor molecule, but only bleaching of the quencher in some cases (Fig. 2e).

This is in accordance with previously published results, where combinations of the spectrally similar dye Cy3 and BHQ-2 only occasionally display quencher induced blinking.[3–6] Spectral jumps of the quencher's absorption spectrum that have a similar spectral overlap with the emission spectrum of the green dye could explain the different behaviour depending on the donor dye. Since many ATTO532 molecules exhibit blinking and intensity jumps under the tested buffer conditions already in the absence of a quencher, minor quencher induced blinking events and intensity changes are easily missed.

Mechanism of quencher induced blinking

In order to investigate the origin of the blinking we carry out intensity dependent measurements. For a Cy5/BBQ650 pair in a PCD/PCA buffer containing 2 mM Trolox, we adjust the intensity of the excitation laser to values between 3 and 21 μW. Under constant buffer conditions, the number of photons detected before the quencher enters a dark state or returns to an active state is independent of the laser intensity (data not shown). This indicates that both processes are photoinduced and do not rely on multi-photon processes.

We further use a buffer containing a 2 mM TX/TXQ mixture and enzymatic oxygen scavenging (GOC) to stabilize Cy5 and add 0.5–2.0 mM ascorbic acid (AA) and methyl viologen (MV), respectively (Fig. 3c). The fluorescence transients are fitted with a two state Hidden Markov Model (HaMMy[16]), and the dwell times of the respective states (illustrated in Fig. 3a and b) are histogrammed and fitted with a monoexponential decay. An increase of the reductant AA leads to a faster deactivation of the quencher and a higher concentration of the oxidant MV reduces the time before reactivation.

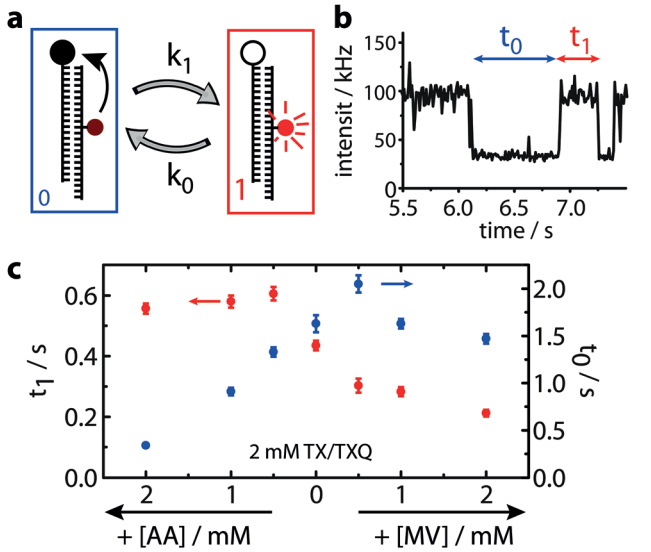

Fig. 3 (a) The quencher fluctuates between an absorbing state (0, black circle) and a non-absorbing state (1, open circle). In state 0, the fluorescence of the dye is reduced due to energy transfer to the quencher, and in state 1, the quenching is inhibited. Transitions between the two states occur at rates k_1 and k_0. (b) Magnified view of Fig. 2b. The two states of the quencher correspond to two different intensity levels with lifetimes $t_1 = 1/k_0$ and $t_0 = 1/k_1$. (c) With increasing the reductant concentration (AA, left half), the time t_0 during which the quencher is active decreases, while the time before reactivation of the quencher, t_1, shows no significant change. With increasing concentration of the oxidant MV, mainly t_1 is reduced. All measurements were carried out in the presence of 2 mM TX/TXQ for Cy5 photostabilization.

Considering the high concentrations of reductant and oxidant involved, it may first appear surprising that the blinking occurs on such a slow timescale. For most organic dyes, both the reduction and oxidation steps occur with higher rate constants (for example, no blinking of Cy5 is observed because the reduction and re-oxidation processes are too fast to be visible). In a first approximation, the reduction frequency is related to the fraction of time the chromophore spends in an excited state such as the first excited singlet or triplet state. Because the dark FRET quenchers have substantially shorter excited state lifetimes, triplet transitions are also less likely, so that the fraction of time in an excited state is substantially reduced, which is in accordance with the observed long absorbing times before entering a non-absorbing state.

The fact that the non-absorbing states that we ascribed to the radical anion are also long is in accordance with a high reduction potential of the dark FRET quenchers. Dyes from the oxazine class (*e.g.* ATTO655, ATTO680 and ATTO700) with high reduction potentials show similarly long radical anion lifetimes because the oxidant is mild and only re-oxidizes slowly.[10,18,19]

High contrast switching

In recent years, the significance of blinking and switching for super-resolution microscopy has been realized. In the following we study whether the combination of a dye with a dark FRET quencher could be used as efficient super-resolution dyes with blinking and photoactivation functionality.[18,20,21] Besides the blinking and photoactivation kinetics the contrast between the on- and the off-state of the dye is another key parameter.[18,22] To test the applicability of quencher bleaching to single-molecule activation, we design a construct where an ATTO532 dye is in very close proximity (3.2 nm) to the quencher BBQ650. Because ATTO532 does not absorb above 600 nm, but the quencher still does (Fig. 1c), we can selectively bleach the quencher with 640 nm excitation without harming the donor molecule. Fig. 4 shows a number of 20 × 20 μm^2 scans of ATTO532/BBQ650 immobilized on surfaces. In the top row, individual molecules are visible on a dense (Fig. 4a–c) surface due to incomplete hybridization of the probe.

By bleaching the quencher with 640 nm excitation, the position of the laser focus on the sample can "write" active ATTO532 molecules on the surface. Fig. 4d–f shows the same

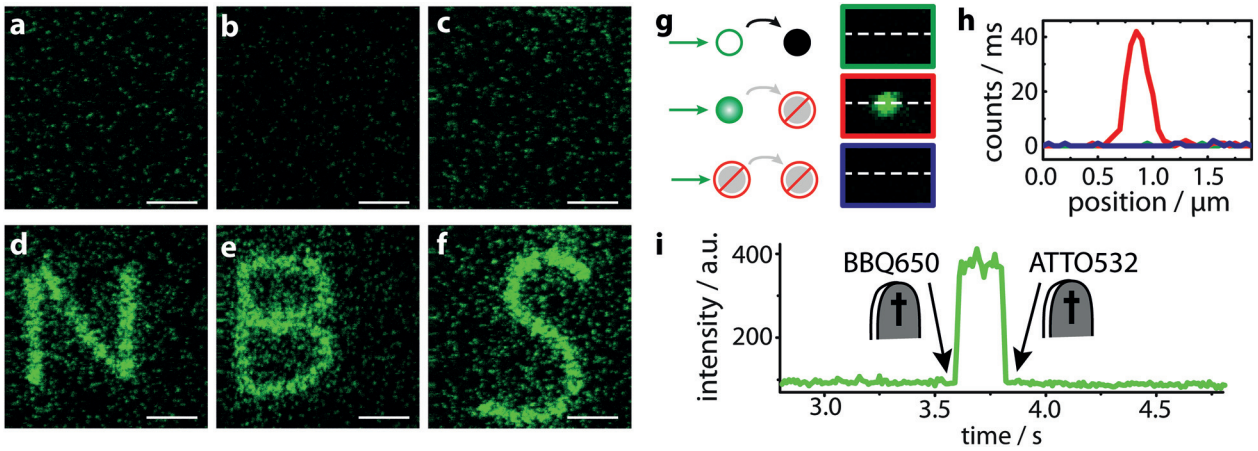

Fig. 4 (a)–(c) Scans of surfaces covered densely with ATTO532/BBQ650 pairs. The remaining fluorescence spots are dye molecules without a quencher due to incomplete hybridization (20 × 20 µm^2, scan with 1 µW (532 nm) at 0.02 ms nm^{-1}, color scale: 0–50 photons per pixel, scale bars: 5 µm). (d)–(f) The same surface areas as in (a)–(c) but after quencher molecules are bleached with 640 nm illumination (80 µW) at defined positions. This enables us, for example, to write the abbreviation of our group NanoBioSciences. (g) Switching of a single molecule reveals the high contrast between the dye with the active quencher (top, green), the dye after the quencher is bleached (center, red) and the background signal (bottom, blue). (h) Cross sections of the images in (g) (dashed white lines). (i) Representative fluorescence transient under 532 nm illumination. The dye is activated at 3.6 s and bleaches at 3.8 s. From 83 of these transients we obtain a contrast of 100 : 1.

surface areas as in Fig. 4a–c but after the writing process. The high on/off contrast for the individual molecules is visualized in Fig. 4g, where no fluorescence is detected while the quencher is still active. Cross sections through the spot (Fig. 4h) confirm that the remaining fluorescence in the presence of the quencher cannot be distinguished from the background level in the confocal scan.

We use prism-type TIRF microscopy to obtain fluorescence transients of the consecutive quencher and donor bleaching for 83 molecules (Fig. 4i) in PBS with 2.5 mM DDT. From these data we determine the quenching efficiency to be 99.0 ± 0.6% for the dye attached to the third base. This high on/off contrast for blinking and/or activation is an option for super-resolution applications and for intrinsically resolution enhancing probes.[22]

Conclusions

Dark FRET acceptors are interesting and promising additions to the single-molecule spectroscopy toolbox. Their applicability however strongly depends on the absence of strong photophysical effects because the quencher status cannot be directly observed as a control.

In this article, we have shown that frequent blinking caused by the dark acceptors BBQ650 and BHQ-2 occurs under measurement conditions commonly used in single-molecule fluorescence measurements when Cy5 was used as a donor. With different oxygen scavenging systems and the widely used photostabilizer TX, the quenchers repeatedly visit non-absorbing dark states which results in the recovery of the donor fluorescence intensity.

By varying the composition of reductants and oxidants (AA/MV) in the buffer we observed changes in the dwell times before a transition to or from a non-absorbing state occurred. Therefore, the dark FRET acceptor quenching is well explained by the ROXS concept and identifies the non-absorbing state as a radical anion. The interesting difference compared to organic fluorophores is that the dark quenchers require many more absorption/emission cycles before entering a non-absorbing state than an ordinary fluorophore which is related to their short excited state lifetime.

With a dye positioned in close proximity to the quencher, 99% quenching of the fluorescence was achieved. This high contrast between the two states opens up a variety of possible applications for such dye/quencher pairs. Selective bleaching of the dark quencher molecules recovers the fluorescence of the donor at defined positions. The high contrast for switching also introduces these pseudo photoactivatable dyes as possible alternatives for superresolution imaging as any dye can be made photoactivatable in combination with a dark quencher.

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