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Fluorescence Spectroscopy

A Reducing and Oxidizing System Minimizes Photobleaching and Blinking of Fluorescent Dyes**

*Jan Vogelsang, Robert Kasper, Christian Steinhauer, Britta Person, Mike Heilemann, Markus Sauer, and Philip Tinnefeld**

The exquisite selectivity, sensitivity, and spatial resolution obtained with fluorescence spectroscopy and imaging have led to an ever-increasing number of applications. With the development of detectors approaching 100% quantum efficiencies and sophisticated collection optics, the bottleneck of current fluorescence microscopy is the fluorophores used, which pose severe limitations owing to photobleaching and blinking. Most of the basic dye structures that are currently used in fluorescence microscopy have been known since their use in the development of dye lasers.[1] Increasing demands posed by fluorescence microscopy and single-molecule and high-resolution applications[2,3] have spurred the development of new kinds of emitters such as semiconductor nanocrystals, silver nanoclusters, and new derivatives of fluorescent proteins.[4] In comparison, the advancement of classical organic dyes such as rhodamine or cyanine derivatives has been incremental despite some progress with regard to labeling chemistry, solubility in water, and the availability of bright and photostable near-IR dyes. Approaches for their improvement comprise increasing brightness by multichromophore systems, intramolecular triplet quenching, and decreasing the sensibility for reactions with singlet oxygen.[5] For different reasons, none of these approaches has been implemented with great success in fluorescence microscopy.

Here we present a new approach to minimize photobleaching and blinking by recovering reactive intermediates. The method is based on the removal of oxygen and quenching of triplet as well as charge-separated states by electron-transfer reactions. For this reason, a structure that contains reducing as well as oxidizing agents, that is, a reducing and oxidizing system (ROXS) is used. The success of the approach is demonstrated by single-molecule fluorescence spectroscopy of oligonucleotides labeled with different fluorophores, that is, cyanines, (carbo-)rhodamines, and oxazines, in aqueous solvents; individual fluorophores can be observed for minutes under moderate excitation with increased fluorescence brightness. Thermodynamic considerations of the underlying redox reactions support the model, yielding a comprehensive picture of blinking and photobleaching of organic fluorophores.

Typically, the photophysics of fluorophores is described by a three-state model including the ground and first excited singlet states, S_0 and S_1, respectively, and the lowest triplet state T_1. Owing to its longer lifetime, T_1 is considered to be the photochemically most active state. Quenching of T_1 by molecular oxygen, for example, can generate reactive singlet oxygen, and therefore oxygen is removed in demanding applications, for example, with the aid of an enzymatic oxygen-scavenging system.[6] The disadvantage of oxygen removal, however, is the increase of the triplet state lifetime with negative effects for the brightness of the fluorophore and increased probability for other follow-up reactions from the triplet state. Alternatively, reducing agents such as ascorbic acid (AA), *N*-propyl gallate, β-mercaptoethanol, and Trolox (TX) have been used to recover photoionized fluorophores and to remove singlet oxygen. However, the success of this strategy is strongly dependent on the fluorescent dye used, and sometimes photobleaching is even promoted.[7–9]

This ambiguous behavior is exemplified by single-molecule fluorescence transients of immobilized DNA carrying MR121 or ATTO647N in phosphate-buffered saline (PBS, pH 7.4) with and without addition of 2 mM TX (Figure 1) (see the Supporting Information for experimental details). The oxazine derivative MR121 shows stable fluorescence over many seconds and rare OFF states under ambient conditions (Figure 1a). Under identical conditions the carborhodamine derivative ATTO647N exhibits long OFF states of several hundred milliseconds (Figure 1c). Addition of TX leads to stable emission of ATTO647N (Figure 1d), whereas MR121 exhibits pronounced blinking and reduced photostability (Figure 1b). To resolve this ambiguity, we pursue the following rationale. First, oxygen must be removed owing to its dye-dependent influence on photostability and oxidizing properties. As an alternative triplet-quenching mechanism we suggest exploiting electron-transfer reactions. Triplet quenching by electron transfer, however, yields a radical anionic or cationic dye molecule (with respect to an assumed neutral ground state). Such ionized dyes can also be formed by other pathways such as photoionization, and they represent additional potentially reactive intermediates in photobleaching

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[*] J. Vogelsang,[+] C. Steinhauer, Dr. B. Person, Prof. Dr. P. Tinnefeld
Angewandte Physik – Biophysik, and Center for NanoScience
Ludwig-Maximilians-Universität
Amalienstrasse 54, 80799 München (Germany)
Fax: (+49) 89-2180-2050
E-mail: Philip.Tinnefeld@lmu.de

R. Kasper,[+] Dr. M. Heilemann, Prof. Dr. M. Sauer
Angew. Laserphysik and Laserspektroskopie
Universität Bielefeld
Universitätsstrasse 25, 33615 Bielefeld (Germany)

[+] These authors contributed equally to this work.

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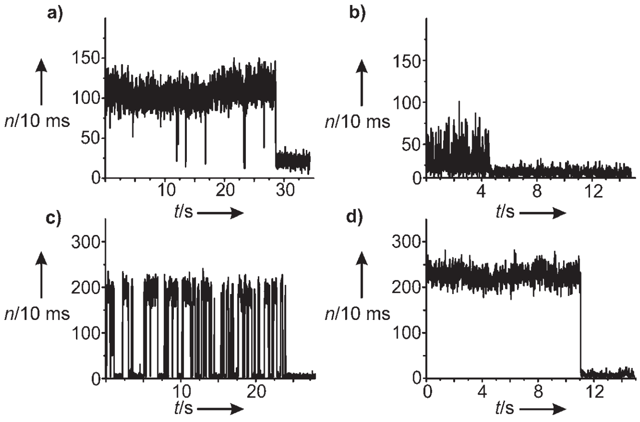

Figure 1. Single-molecule fluorescence transients of immobilized dye-labeled DNA were measured in PBS (a, c) and in PBS with 2 mM Trolox (b, d), respectively. Fluorescence transients of MR121 are shown in (a) and (b); fluorescence transients of ATTO647N are depicted in (c) and (d).

pathways.[8,10] Depending on the predominant photobleaching pathway, that is, whether for a certain fluorophore the triplet, the reduced, or the oxidized form is the more reactive intermediate towards photobleaching, redox-active agents might retard or even increase the photobleaching rate.

We introduce a universal method to improve the photostability and reduce blinking of fluorescent dyes using both a reducing and an oxidizing agent to quickly recover all triplet states and ionized states. The working principle of the developed reducing and oxidizing system (ROXS) is shown schematically in Figure 2. After intersystem crossing to T_1, the fluorophore can be reduced by the reducing agent yielding the radical anion $F^{\bullet-}$. The radical anion is then quickly reoxidized by the oxidizing agent to repopulate the singlet ground state. Alternatively, the fluorophore is oxidized from T_1 by the oxidant to form $F^{\bullet+}$ and subsequently returns to the ground state by the reductant. The fast recovery of the singlet ground state is essential to successfully compete with side reactions leading to photobleached products P. Other possible pathways can also yield radical ions as indicated in gray in Figure 2, but they are not explicitly discussed in this context.

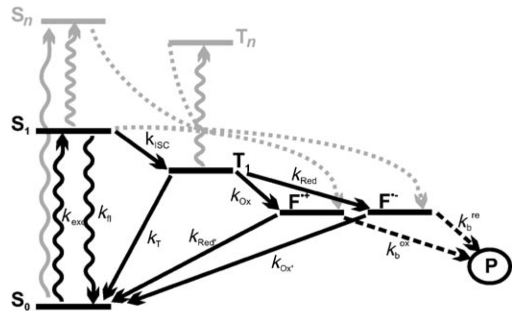

Figure 2. Photoinduced processes of common organic fluorophores. After excitation to the first excited singlet state S_1, fluorescence is emitted at rate k_{fl} (neglecting nonradiative processes). Intersystem crossing competes with fluorescence and leads to the infrequent formation of triplet states T_1 with rate constant k_{isc}. With ROXS, the triplet state is rapidly depleted by electron transfer either through oxidation by, for example, methylviologen (MV) forming a radical cation $F^{\bullet+}$ or through reduction by, for example, AA yielding a radical anion $F^{\bullet-}$. The two possible radical ions are rapidly recovered to singlet ground-state fluorophores by the respective reduction (in case of a radical cation it is reduced by AA) or oxidation (in case of a radical anion it is oxidized by MV). The rapid recovery by ROXS prevents the formation of the photobleaching product P. Higher excited states shown in gray might also be populated yielding further intermediates.[8,11–13]

Fluorescence transients of ATTO647N recorded in aqueous buffer demonstrate the concept (Figure 3). In the absence of oxygen, dark states of ATTO647N, which are related to triplet formation and possibly subsequent ionization, have a monoexponential lifetime of $\approx 28 \pm 8$ ms (data not shown). Figure 3 a shows a fluorescence transient of ATTO647N after removal of oxygen in buffer containing 1 mM oxidizing agent 1,1′-dimethyl-4,4′-bipyridinium dichloride hydrate (methylviologen, MV). Pronounced blinking on the millisecond time

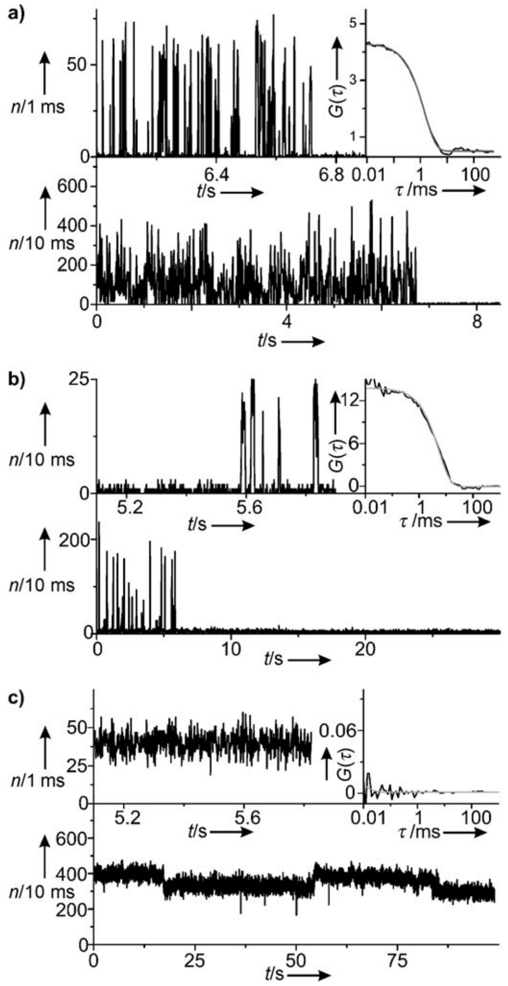

Figure 3. Fluorescence transients of ATTO647N-labeled DNA immobilized in aqueous environment under the following conditions: a) oxygen removed plus 1 mM MV, b) oxygen removed plus AA (1 mM), and c) oxygen removed plus MV (1 mM) and AA (1 mM). The bottom trace is binned in 10 ms, the left inset provides a magnified view with 1 ms resolution, and the right inset shows the second-order autocorrelation function $G(\tau)$ with monoexponential fit. Samples were excited at 635 nm with an average excitation intensity of approximately 2 kW cm^{-2}.

scale is visible in the magnified view (left inset of Figure 3 a). Autocorrelation analysis (right inset) and monoexponential fitting reveals an OFF state with a lifetime $\tau_{off} = 8 \pm 1$ ms. We ascribe this OFF state to photoinduced oxidation and the formation of a radical cation. The formation of radical cations of cyanines and rhodamines with lifetimes in the millisecond range has been known since the 1970s when they were determined by flash photolysis,[14] and has also been suspected on the level of single molecules.[15] Here, however, blinking due to reversible electron transfer is shown to result in monoexponential kinetics.[10,16] In analogy, ATTO647N exhibits similar blinking after removal of oxygen and the addition of the reducing agent AA (1 mM), likely because of the reversible formation of radical anions ($\tau_{off} = 28 \pm 7$ ms). The assignment of the OFF state to a radical ion is strongly supported by the transient in Figure 3 c, which depicts a fluorescence transient of ATTO647N after oxygen removal and addition of both AA (1 mM) and MV (1 mM). The molecule emits practically without blinking throughout the 100 s recording time.

By spectrally resolved measurements, the two intensity levels are assigned to infrequent transitions between two spectrally distinct but elsewise photophysically similar states of the fluorophore (data not shown). The impression that no further intensity fluctuations beyond shot noise occur is supported by the insets of the magnified fluorescence transient as well as by the intensity autocorrelation function, which does not exhibit a characteristic time constant down to 1 µs (Figure 3 c). As a result of reduced blinking, the fluorophore is more efficiently cycled between S_0 and S_1 which results in higher fluorescence brightness recorded from single ATTO647N molecules. The fact that the photostability increases in the presence of ROXS corroborates the idea that the OFF states are closely linked to photobleaching pathways.[10]

Similar results are obtained for other fluorophores from different classes and spectral ranges such as Cy5, Alexa647, Cy3B, and ATTO565 (see Figure S1 in the Supporting Information). These fluorophores show similar properties with blinking in the presence of only reductant or oxidant. Blinking is efficiently eliminated in the presence of reductant and oxidant.

The simultaneous application of a reductant and an oxidant might appear paradoxical or counterintuitive. We therefore discuss the thermodynamic rationale in the following. It is noteworthy that although reductant and oxidant have the tendency to donate and accept electrons, respectively, the driving force for the photoinduced electron transfer requires the energy from the absorbed photon. It can be easily seen from the redox potentials of the reductant and oxidant that they do not react in a ground-state electron-transfer reaction (see below). From a thermodynamic perspective, the energy supplied by photon absorption must be sufficient to complete the whole cycle through T_1 and a charge-separated state and back to the ground state. Absorption provides the zero–zero energy $E_{0,0}$, which corresponds to 1.88 eV in the case of Cy5. An energy loss of 280 meV results from intersystem crossing since the triplet of Cy5 is at 1.60 eV.[20] Using the Rehm–Weller equation,[21] we estimate the free enthalpy change for charge separation, $\Delta G_{cs} = e[E_{ox} - E_{red}] - E_{0,0} + C$, where E_{ox} and E_{red} are the first one-electron oxidation potential of the donor and the first one-electron reduction potential of the acceptor, respectively, obtained by cyclic voltammetry, and e is the unit charge.[13] The solvent-dependent Coulombic attraction energy C can be neglected due to the high polarity of water. If T_1 of Cy5 is reduced by AA, we obtain with E_{red} (Cy5, vs. SCE) = −0.84 V, and E_{ox} (AA, vs. SCE) = 0.06 V,[22] $\Delta G_{cs} = -0.70$ eV. Similarly, the oxidation of the Cy5 triplet state by MV is also exergonic (E_{ox}(Cy5) = 0.97 V, E_{red}(MV) = −0.45 V)[23] by $\Delta G = -0.18$ eV. These data support the idea that the triplet state of Cy5 is quenched by AA as well as by MV by means of electron transfer.

In the next step we estimate whether the ground state of Cy5 can be efficiently recovered from the charge-separated state by ROXS. To this end we compare the reduction potential of Cy5 with the reduction potential of MV and obtain the free enthalpy for charge recombination $\Delta G_{cr} = -0.39$ eV in the case of the Cy5 radical anion. Analogously comparison of the oxidation potential of Cy5 with the oxidation potential of AA yields $\Delta G_{cr} = -0.93$ eV. The fact that all calculated reaction steps exhibit exergonic free energy changes for charge separation and recombination is in accordance with the data presented in Figure 3 and Figure S1 in the Supporting Information. Similar calculations for ATTO647N also yield negative free enthalpy values for the different reaction steps ($E_{0,0} = 1.90$ eV, $E_{ox} = 1.11$ V, $E_{red} = -0.64$ V;[13] E_{T1} is assumed to be ≈ 1.6–1.7 eV). Interestingly, the same calculation yields one important positive free energy value for the oxazine derivative MR121, which has a very high-lying reduction potential of $E_{red} = -0.42$ V. Accordingly, reduced MR121 molecules might not be recovered efficiently by MV with $E_{red} = -0.45$ V. Indeed, single-molecule fluorescence transients of MR121-labeled DNA under ROXS conditions exhibit frequent blinking that is not completely suppressed by ROXS (Figure S2 in the Supporting Information). We also find that blinking of Alexa532 is reduced but not completely suppressed with strong variations from molecule to molecule (data not shown).

To quantify the effect of increased photostability we carried out total internal reflection fluorescence (TIRF) microscopy of single, immobilized molecules. The parallel detection of roughly 100 molecules per movie allows rapid accumulation of bleaching statistics. In a standard protocol we applied 150 $W\,cm^{-2}$ of 647 nm or 531 nm excitation and recorded time-lapse movies of up to 20 minutes (see the Supporting Information). In most cases the bleaching curves could be approximated by a single exponential fit for short times (Figure S3a in the Supporting Information). The time constants of the fits together with the brightness of the molecules were used to determine the average number of photons detected per molecule before photobleaching. To optimize ROXS conditions we varied concentrations of reductant and oxidant for measurements of Cy5 (see Figure S3b,c in the Supporting Information) and found a stability maximum at 1 mM reductant and 1 mM oxidant. Although the stability maximum might depend on the specific photobleaching pathways of the different dyes, we used this concentration as ROXS reference. We then compared photostabilities for different fluorophores under various conditions: 1) in PBS buffer, after removal of oxygen and 2) after addition of 1 mM AA, 3) after addition of 1 mM MV, and 4) after addition of ROXS (1 mM AA and 1 mM MV). Average overall photon counts from different fluorophores are presented in Figure 4 a,b. No bar is shown when the detected number of photons is below 1000 (e.g. Cy5 in PBS).

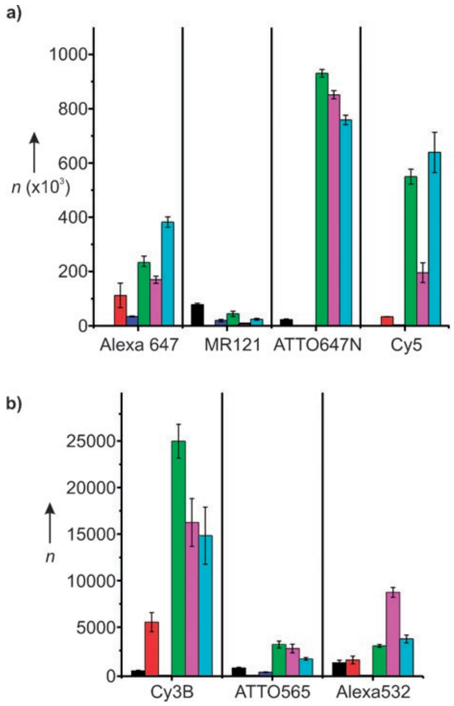

Figure 4. Average photon counts per molecule a) for the red fluorophores Alexa647, MR121, ATTO647N, and Cy5, and b) for the green/yellow fluorophores Cy3B, ATTO565, and Alexa532 were obtained from at least three photobleaching movies using objective-type total internal reflection microscopy. Measurements were carried out in PBS (black bars), oxygen-depleted PBS with 1 mM AA (red), with 1 mM MV (blue), with ROXS (1 mM AA plus 1 mM MV (green), or 2 mM TX plus 1 mM MV (cyan), or 2 mM TX (magenta)).

For all fluorophores except for the oxazine derivative MR121, we find that the photostability with ROXS is greater than that in PBS solution. Moreover, for all fluorophores the photostability is greater when both reductant (AA) and oxidant (MV) are used than when only the reductant or oxidant are used alone. In the absence of oxygen, less than 1000 photons are detected on average for single ATTO647N molecules. In the presence of 1 mM MV or 1 mM AA only, also less than 1000 photons are detected on average. However, after addition of both MV and AA a synergetic effect is observed and the photostability is increased to $(8.40\pm0.13)\times10^5$ detected photons. These data show that the photostability of fluorophores can be strongly extended using the combination of reductant and oxidant, for example, by a factor of >800 for ATTO647N. Although we found a similar effect regarding blinking reduction for shorter wavelength fluorophores such as Cy3B and ATTO565, the photostabilizing effect was not as pronounced (Figure 4 b). Thus far, the photophysical properties have been reported to be rather dominated by the dye class a fluorophore belongs to.[12,13] Using ROXS, the zero–zero energy of the lowest electronic transition seems to dominate the photostability. This could be related to the fact that higher excited electronic states become populated with decreasing excitation wavelengths, offering alternative bleaching pathways.

It is important to compare the reduction of blinking and photobleaching with the most successful and empirically found formulas used in single-molecule spectroscopy. Rasnik et al. reported that TX increased photostability while also quenching dark states; this distinguishes its properties from other reductants such as AA or propyl gallate.[9] We carried out concentration-dependent measurements comparing blinking in the presence of TX, AA, and ROXS (Figure 5).

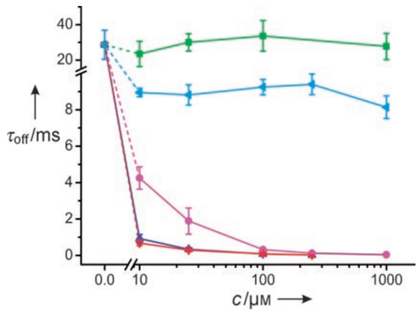

Figure 5. Dependency of the OFF-state lifetime τ_{off} of ATTO647N on the concentration of the respective electron-transfer quencher in oxygen-depleted solutions. AA (green), MV (light blue), TX (magenta), AA plus MV (blue), and TX plus MV (red) were used as triplet quenchers via electron transfer.

To avoid interference with OFF times due to *cis–trans* isomerization, we used the carborhodamine ATTO647N for these measurements. As shown in Figure 5, different concentrations of only AA or only MV have no significant influence on the OFF times of ATTO647N, as expected from the ROXS model (Figure 2). When ROXS is used, the duration of the OFF times strikingly decreases already at 10 μM concentration and no OFF state down to 1 μs is found for millimolar concentrations of ROXS. These data further support the model that radical ions are formed, which are rapidly recycled in the presence of both reductant and oxidant. It is generally accepted that TX also quenches triplet states by electron transfer (see e.g. Ref. [22]). Interestingly, TX is also capable of reducing the OFF times of ATTO647N (see Figure 5) and other fluorophores investigated. The efficiency of this surprising feature of reducing OFF times is, however, a factor of 4–6 lower for TX relative to ROXS. In other words, about five times higher concentrations of TX are required relative to the concentration of ROXS to achieve the same effect of blinking suppression. Since the rate of dark-state quenching of TX plus MV (red data points in Figure 5) is higher than those of TX only or AA plus MV (in fact it is the sum of the latter two rates), we speculate that the mechanism of dark-state quenching by TX is comparable to that by ROXS. In accordance, TX also increases the photostability of most fluorophores (Figure 4, magenta bars), and a combination with MV is also successful in some cases (Figure 4, cyan bars).

For applications of the ROXS concept, it can be advantageous to use the combination of AA and MV instead of TX since the antiblinking effect is obtained at lower concentrations, higher photostability is achieved for some fluorophores, and both AA and MV exhibit higher solubility in aqueous solvents. In this context, it is important that the ingredients, especially the toxic oxidant MV, do not interfere with biomolecular functionality. As in any high-quality experiment the addition of ROXS requires rigorous controls for each specific application. The functioning of the surface immobilization, successful enzymatic oxygen removal, and studies of the dynamics of Holliday junctions,[24] which is independent of MV concentration (see Figure S4 in the Supporting Information), however, indicate that many biological functions are not influenced by the presence of ROXS at low millimolar concentrations.

Photobleaching represents one of the main limitations of modern fluorescence spectroscopy and imaging techniques. The novel ROXS strategy exhibits distinct advantages: The photostability of organic fluorescent dyes from different classes can be improved substantially in aqueous surrounding. Simultaneously blinking is dramatically reduced, enabling fluorescence spectroscopy and imaging applications at higher fluorescence count rates over extended periods of time. The underlying unifying concept represents a paradigm change in photobleaching prevention and a step away from the purely empirical search for antifading formulas. The model to deplete triplet states and quickly recover oxidized and reduced states is supported by single-molecule fluorescence spectroscopy as well as by thermodynamic considerations. Since ROXS takes into account several photobleaching pathways at a single blow, the method is generally applicable and might find widespread implementation in modern fluorescence imaging and spectroscopy techniques, for example, for various high-resolution imaging approaches.[3]

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