Photochemistry and Photobiology, 2014, 90: 448–454

The Contribution of Reactive Oxygen Species to the Photobleaching of Organic Fluorophores†

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Received 2 September 2013, accepted 31 October 2013, DOI: 10.1111/php.12204

ABSTRACT

Photoexcitation of fluorophores commonly used for biological imaging applications generates reactive oxygen species (ROS) which can cause bleaching of the fluorophore and damage to the biological system under investigation. In this study, we show that singlet oxygen contributes relatively little to Cy5 and ATTO 647N photobleaching at low concentrations in aqueous solution. We also show that Cy5 generates significantly less ROS when covalently linked to the protective agents, cyclooctatetraene (COT), nitrobenzyl alcohol (NBA) or Trolox. Such fluorophores exhibit enhanced photostability both in bulk solutions and in single-molecule fluorescence measurements. While the fluorophores ATTO 647N and ATTO 655 showed greater photostability than Cy5 and the protective–agent-linked Cy5 derivatives investigated here, both of ATTO 647N and ATTO 655 generated singlet oxygen and hydroxyl radicals at relatively rapid rates, suggesting that they may be substantially more phototoxic than Cy5 and its derivatives.

INTRODUCTION

Fluorescence microscopy has seen revolutionary advancements in both sensitivity and resolution over the past decade (1). Progress in this field is highlighted by recent developments in single-molecule and super-resolution fluorescence imaging techniques that have yielded new insights into a variety of biological systems in unprecedented detail (2–4). However, photoinduced degradation (photobleaching) of the fluorophores used remains a key obstacle that limits the temporal and spatial resolutions of imaging (5). Photoinduced fluorophore toxicity (phototoxicity) may also lead to unwanted perturbations to the biological system (6–8) that can obscure the signal of interest. Further advancements in fluorescence imaging would therefore be greatly aided by a deeper understanding of the mechanisms of fluorophore photobleaching and the development of fluorophores exhibiting slow photobleaching and low-toxicity.

Molecular oxygen plays a critical role in fluorophore photobleaching and phototoxicity (5,7,9,10) as it can participate in two probable reactions with an excited fluorophore. First, energy transfer can occur from a fluorophore in the triplet excited state to molecular oxygen leading to the formation of energetically excited singlet oxygen. Second, electron transfer can occur from the triplet fluorophore to molecular oxygen leading to the formation of a superoxide radical (Scheme 1) (9,11). Singlet oxygen and superoxide radical, along with other oxidizing species formed subsequently (Scheme 1), are collectively termed reactive oxygen species (ROS) which can degrade fluorophores (10–14) and damage biomolecules in various contexts (6,7,15–17). However, the role of specific ROS in fluorophore photobleaching is not well understood. In particular, while it is widely held that singlet oxygen is a direct participant in the photobleaching pathway (9–11,13,18,19), at least one early study provided evidence suggesting that it does not directly participate in the photobleaching of fluorescein isothiocyanate (FITC) (20). Here, we aim to elucidate the contribution of singlet oxygen to the photobleaching of Cy5 and ATTO 647N, two organic fluorophores commonly used in single-molecule and super-resolution measurements.

To mitigate fluorophore photobleaching and phototoxicity, molecular oxygen, normally at 0.3 mM in aqueous solution (21), can be depleted by enzymatic oxygen scavenging system (18,22). Moreover, small-molecule protective agents (PA), such as COT, nitrobenzyl alcohol (NBA) and Trolox (23,24), can be added to the imaging solution to mitigate the transient dark state caused by the triplet and radical state species (5,10). However, the use of oxygen scavenging systems and PA in solution can be incompatible with, or perturbing to, biological systems, particularly for measurements involving lipid membranes (25) or live cells.

Recently, we demonstrated that the covalent linkage of a single COT, NBA and Trolox to cyanine fluorophores substantially reduces the frequency of transient dark state and the rate of photobleaching, thereby circumventing the requirements of PA and, in some cases, of oxygen scavenger system in solution (26,27). The protective effect for Cy5-COT results from its capacity to reduce the lifetime of the triplet state by up to 50-fold through energy-transfer quenching, whereas the distinct mechanism for Cy5-NBA and Cy5-Trolox was unknown (28). Here, we show that COT-, NBA- and Trolox-linked Cy5 derivatives (Cy5-PA) (Scheme 2) exhibit reduced rates of ROS generation compared with the parent Cy5 molecule. These findings imply that PA-conjugated fluorophores may exhibit reduced phototoxicity compared with the fluorophores commonly used for fluorescence imaging, and suggest that the Cy5-NBA and Cy5-Trolox in aqueous solution are also protected by triplet state quenching.

†This paper is part of the Special Issue honoring the memory of Nicholas J. Turro.
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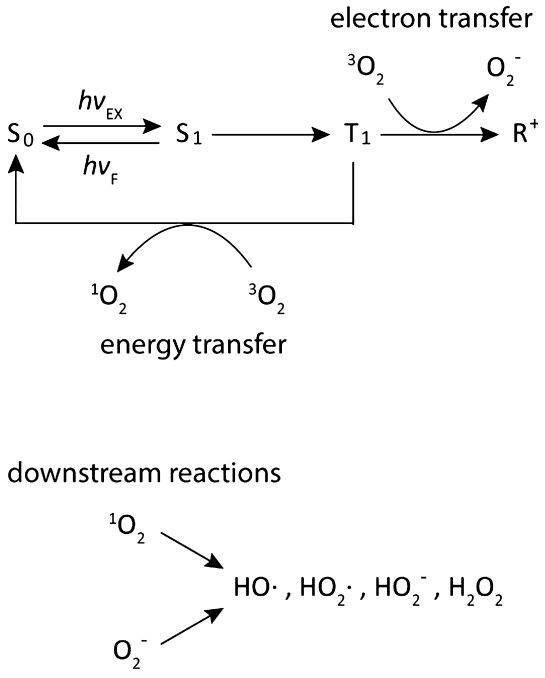
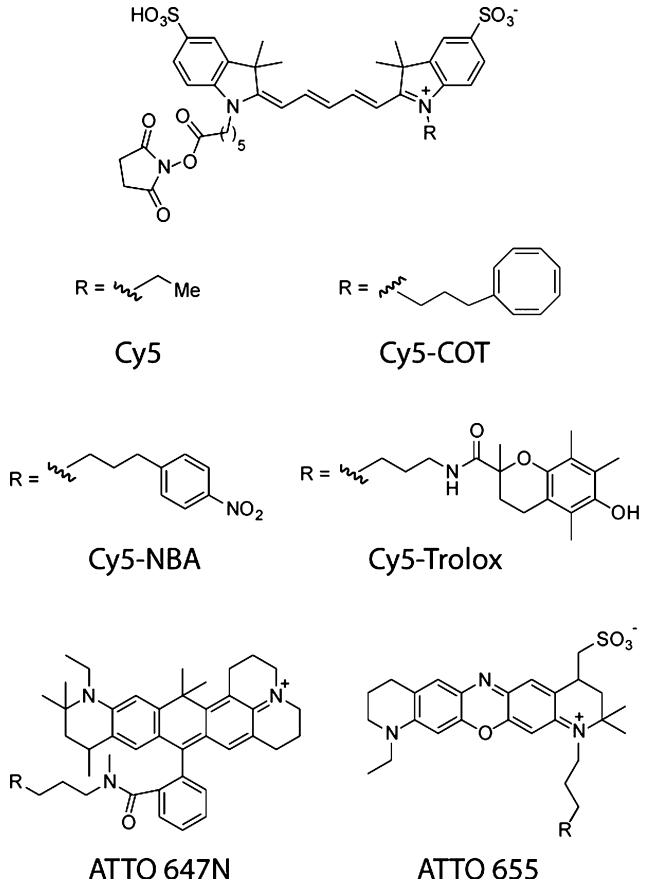


Scheme 1. Some probable steps to reactive oxygen species *via* the reactions between molecular oxygen ($^3O_2$) and the fluorophore. S_0: fluorophore in ground state; S_1: fluorophore in the singlet excited state; T_1: fluorophore in the triplet state; R^+: fluorophore in the radical cationic state.



Scheme 2. Structures of the compounds used in this study.

MATERIALS AND METHODS

Materials. N-hydroxysuccinimide (NHS) ester activated fluorophores Cy5 (GE Healthcare), ATTO 647N (ATTO-TEC GmbH) and ATTO 655 (ATTO-TEC-GmbH) were used as received. Cy5-COT, Cy5-NBA and Cy5-Trolox were synthesized as previously described (28).

Fluorophore bleaching experiments. Fluorophores (5 μM or 26 μM for Cy5; 5 μM for Cy5-COT, Cy5-NBA and Cy5-Trolox; 10 μM for ATTO 655 and ATTO 647N) were dissolved in solvent (H_2O, D_2O, $CHCl_3$, $CDCl_3$, acetontrile or 100 mM Tris-acetate aqueous solution at pH 7.5). Samples (2 mL) were examined in a 1 cm cuvette, illuminated with a 300 W Tungsten halogen lamp, using an RG570 longpass filter to block short-wavelength light. Absorbance spectra of each fluorophore were measured using a UV–vis spectrometer (Agilent 8453). The decrease in the absorption was used to estimate the relative rate of fluorophore photobleaching over the first 2 min using a linear fit.

Singlet oxygen measurements. Singlet Oxygen Sensor Green (SOSG; Invitrogen) experiments were performed in aqueous buffer as previously described (29) in a 1 cm cuvette containing 2 mL of 100 mM Tris-acetate (pH 7.5), SOSG (2 μM) fluorophore (5 or 10 μM). Samples were illuminated for fixed periods of time with a 300 W Tungsten halogen lamp in conjugation with a RG570 longpass filter. After each photolysis period, the fluorescence of SOSG was recorded over a range of 510–630 nm using 504 nm for excitation.

Due to the incompatibility of SOSG with organic solvent, 9,10-diphenylanthracene (DPA) was used to detect singlet oxygen in acetonitrile. DPA exhibits strong absorbance at 373 and 393 nm, and forms 9,10-endoperoxide, a compound that has no absorbance above 350 nm (Figure S4a). The absorption of DPA was measured with a UV–vis spectrometer (Agilent 8453).

Single-molecule fluorescence measurements. Single-molecule fluorescence measurements were performed using a wide-field, prism-based total internal reflection fluorescence microscope as previously described (26,28).

Fluorescence quantum yield measurements. Fluorescence spectra of solutions containing 0.7 μM Cy5, Cy5-COT, Cy5-NBA or Cy5-Trolox were recorded with a spectrofluorometer (Fluorolog 3; HORIBA Jobin Yvon), using an excitation wavelength of either 649 (aqueous Tris buffer, pH 7.5) or 654 nm (acetonitrile). The fluorescence quantum yield was calculated using Cy5 in aqueous solution (ϕ = 0.20 (30)) as a standard.

Fluorescence lifetime measurements. Fluorescence lifetimes were measured by time correlated single photon counting (TC-SPC) with an OB920 spectrometer (Edinburgh Analytical Instruments) in conjunction with a pulsed diode laser emitting at 659 nm. Fluorescence decay traces were monitored at 670 nm.

RESULTS AND DISCUSSIONS

The organic fluorophores Cy5, ATTO 647N and ATTO 655 used for the present investigation represent the three main categories of fluorophore structures (cyanines for Cy5, carbopyronines for ATTO 647N, and oxazines for ATTO 655) that are widely used for single-molecule and super-resolution fluorescence imaging (31–33). Removal of molecular oxygen from the imaging solution substantially reduces the photobleaching rate of each fluorophore (20,31), implying that photobleaching involves molecular oxygen. Here, we show that the photobleaching rates of these dyes follow the order: Cy5 > ATTO 647N > ATTO 655 (Fig. 1), inversely correlating with the oxidation potential of the fluorophores ($E_{OX,Cy5}$ = 0.97 V, $E_{OX,ATTO647N}$ = 1.11 V, $E_{OX,ATTO655}$ = 1.31 V (32,34)), where an increased oxidation potential implies a decreased propensity to be oxidized. Given the distinct structures and properties of these fluorophores (Scheme 2), this inverse correlation strongly supports that photobleaching is dominated by photoinduced oxidation reactions in the presence of oxygen.

To test the involvement of singlet oxygen in fluorophore photobleaching, we examined whether fluorophore photobleaching exhibits a solvent isotope effect (9). Given that the lifetime

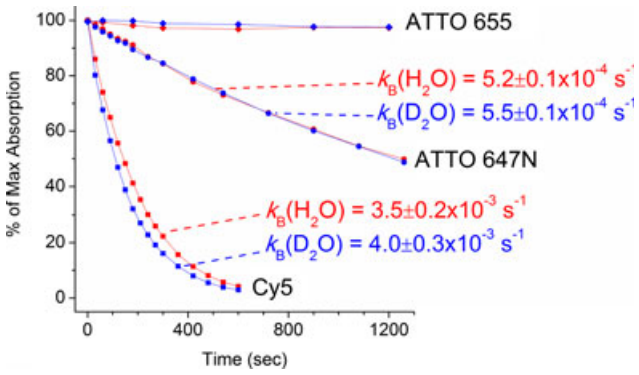


Figure 1. Photobleaching of Cy5 (5 μM), ATTO 647N (10 μM) and ATTO 655 (10 μM) in air-saturated H_2O (red) or D_2O (blue), where k_B is the photobleaching rate.

of singlet oxygen is extended by more than 20-fold in D_2O (68 μs *vs.* 3.1 μs in H_2O) (35), if fluorophore photobleaching is principally mediated by singlet oxygen, a substantial enhancement of the rate of photobleaching should be observed in D_2O (9). However, as observed previously for the organic fluorophore fluorescein isothiocyanate (FITC) (20), in an initial test the rates of Cy5 (5 μM) and ATTO 647N (10 μM) photobleaching were only slightly higher in D_2O than in H_2O (Fig. 1). In contrast, a significant solvent isotope effect was observed in aqueous conditions when the Cy5 concentration was increased five-fold to 26 μM (Fig. 2), consistent with the involvement of singlet oxygen in the photobleaching reaction. Experiments at high concentration were not possible for ATTO 647N due to aggregation of the fluorophore. The absence of a solvent isotope effect at low concentration, and its presence at high Cy5 concentration, can be explained by the fact that encounter rate between singlet oxygen and fluorophore increases with increasing fluorophore concentration. At low fluorophore concentrations, singlet oxygen decays much faster than it encounters and reacts with fluorophores. Under such conditions singlet oxygen contributes negligibly to Cy5 photobleaching compared with other photobleaching reactions (Scheme 1). At high fluorophore concentration, singlet oxygen can react with neighboring fluorophores to contribute to photobleaching to a greater extent. Consistent with the model that singlet oxygen decays too rapidly in aqueous solutions to contribute meaningfully to the bleaching of the fluorophore that

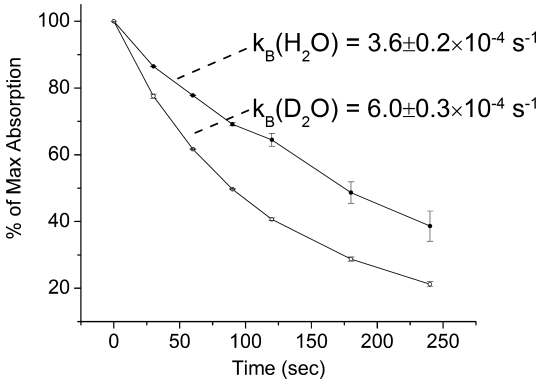


Figure 2. Photobleaching of Cy5 at 26 μM in air-saturated H_2O (solid) or D_2O (open), where k_B is the photobleaching rate.

led to its generation, a significant solvent isotope was observed for Cy5 at 5 μM in chloroform ($CHCl_3$) and deuterated chloroform ($CDCl_3$) (Figure S1), where singlet oxygen has much longer lifetime (~230 μs in $CHCl_3$ and 7 ms in $CDCl_3$ (35)) than it has in water. The significant solvent isotope effects observed in water with high Cy5 concentration, and in chloroform, demonstrate that singlet oxygen can contribute to fluorophore photobleaching under certain conditions. Consistent with previous results (20), the absence of any significant of solvent isotope effect at low fluorophore concentration in aqueous solution, suggests that singlet oxygen contributes negligibly to fluorophore photobleaching under the conditions of most biological studies at the single-molecule scale.

To examine whether the PA-conjugated fluorophores produce less singlet oxygen, we attempted to spectroscopically measure singlet oxygen *via* its phosphorescence emission band at 1270 nm (36) upon fluorophore illumination. However, under the conditions of the experiment (5 μM fluorophore at room temperature), the yield of singlet oxygen was too low to be reliably detected using this approach (data not shown). We therefore used SOSG as an indirect probe of singlet oxygen generation. SOSG itself is weakly fluorescent due to intramolecular electron-transfer quenching and becomes highly fluorescent following cycloaddition of singlet oxygen (Fig. 3a) (29).

Initially, singlet oxygen and photobleaching resulting Cy5 illumination (>570 nm) were tracked by monitoring fluorophore absorption and SOSG emission spectra as a function of illumination time (647 and 528 nm, respectively) (Fig. 3b,d). As expected, the observed increase in SOSG fluorescence emission paralleled the decrease in Cy5 absorption. The increase in SOSG fluorescence was confirmed to arise from its specific reaction with singlet oxygen by performing the same measurement in 90% deuterated water, where the lifetime of singlet oxygen is significantly extended (see discussion above). In contrast to the negligible solvent isotope effect observed for photobleaching of Cy5 at low concentrations (Fig. 1), the rate of SOSG fluorescence increase was enhanced by approximately eight-fold in solution in 90% D_2O (Figure S2).

In parallel, the rates of singlet oxygen generation were also measured for Cy5-COT, Cy5-NBA, Cy5-Trolox, ATTO 655 and ATTO 647N. As observed for the experiment of Cy5, SOSG fluorescence increased upon illumination of each fluorophore (Fig. 3c,e). All three PA-conjugated fluorophores exhibited lower rates of singlet oxygen generation, which paralleled their increased photostability compared with Cy5. Specifically, Cy5-Trolox, Cy5-COT and Cy5-NBA exhibited 2.5-fold, 5-fold and 20-fold reductions in the rate of singlet oxygen generation, respectively (Fig. 3c; Table 1). In contrast, ATTO 655 exhibited rates of singlet oxygen generation that were similar to those observed for Cy5, whereas ATTO 647N generated singlet oxygen at a rate that was approximately five-fold greater than Cy5 (Fig. 3c). These data imply that Cy5-PA fluorophores may be significantly less phototoxic than Cy5, ATTO 647N and ATTO 655 in biological contexts.

To further explore the generation of ROS, we examined the rates of hydroxyl radical (HO·) formation using the probe Aminophenyl fluorescein (APF) (37). Upon reaction with hydroxyl radicals, APF converts to fluorescein, a highly fluorescent species (37) (Figure S3a). Aqueous solutions containing APF and individual fluorophores showed marked increases in fluorescein fluorescence (λ_{max} = 518 nm) as a function of illu-

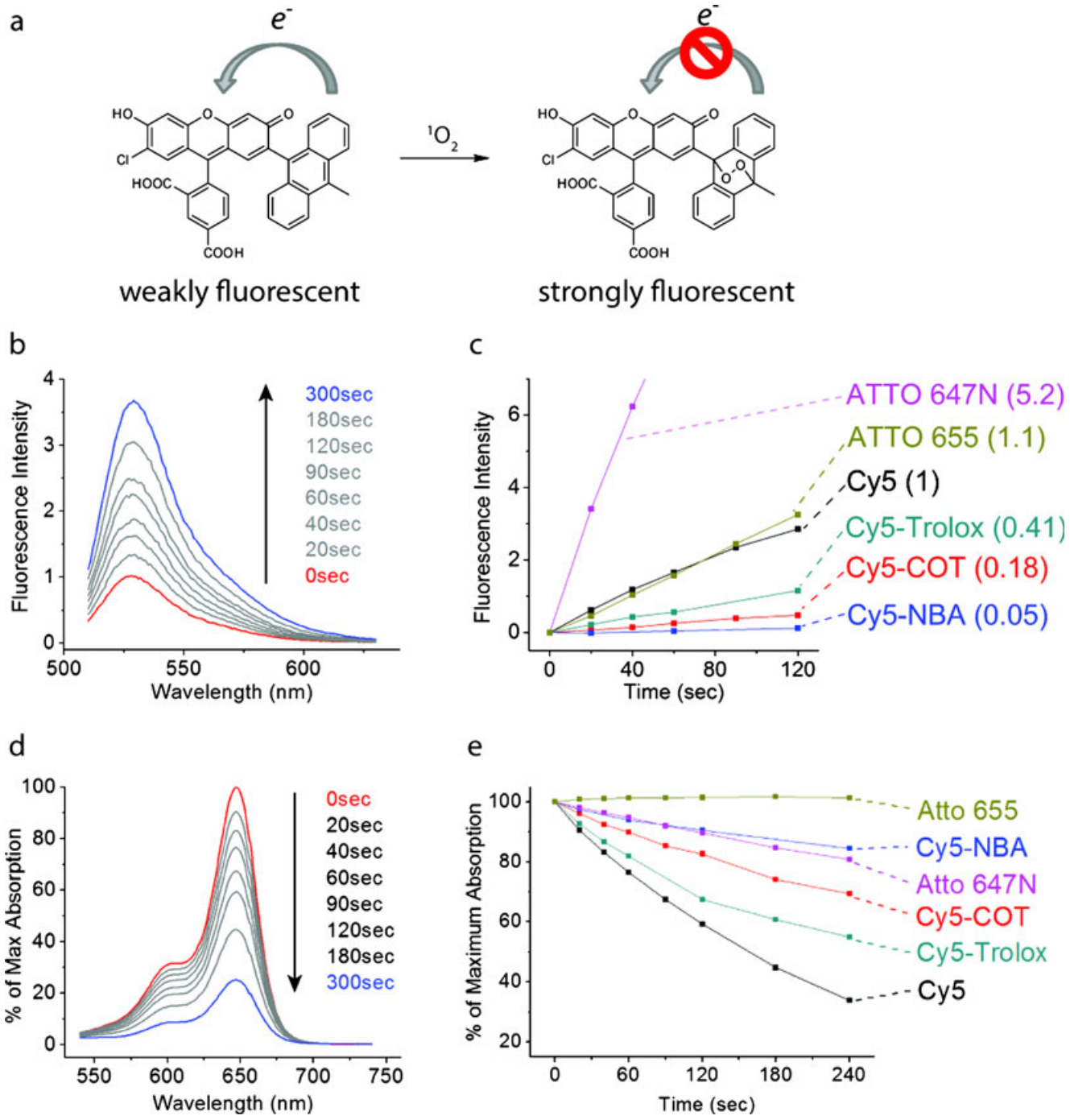


Figure 3. (a) The mechanism of singlet oxygen ($^1O_2$) induced fluorescence of Singlet Oxygen Sensor Green (SOSG). (b) The emission spectra of SOSG upon illumination of Cy5. (c) $^1O_2$ generation, reported by the increase in SOSG fluorescence, for distinct fluorophores at different illumination time. The relative rates of $^1O_2$ generation compared with the Cy5 fluorophore are shown in parentheses. (d) The absorption spectrum of Cy5 observed at different illumination time. (e) The decrease in Cy5, Cy5-COT, Cy5-NBA, Cy5-Trolox, ATTO 647N and ATTO 655 absorption at 647 nm as a function of illumination time. Experiments were performed in 100 mM Tris-acetate-buffered aqueous solution, pH 7.5.

mination time (Figure S3b). Consistent with their reduced rates of singlet oxygen generation, the PA-conjugated fluorophores (Cy5-COT, Cy5-NBA and Cy5-Trolox) exhibited significantly reduced rates of hydroxyl radical generation (Figure S3c). These data provide supporting evidence that the Cy5-PA fluorophores may be less phototoxic than the Cy5, ATTO 647N and ATTO 655.

To directly examine the performance of fluorophores in single-molecule fluorescence measurement in ambient oxygen conditions, each fluorophore was conjugated to a double-stranded DNA oligonucleotide and imaged under a total internal reflection fluorescence microscope (26). Visual inspection of fluorescence traces from individual molecules revealed that most of ATTO 647N and ATTO 655 molecules showed substantial tran-

Table 1. A summary of the data. 100 mM Tris-acetate buffer (pH = 7.5) was used in all experiments in aqueous buffer. All of the rates were normalized to the corresponding numbers for Cy5.

Solvent		Cy5	Cy5-COT	Cy5-NBA	Cy5-Trolox
Aqueous	Relative rate of single-molecule photobleaching	1 ± 0.2	0.29 ± 0.02	0.27 ± 0.02	0.42 ± 0.04
	Relative rate of ensemble photobleaching	1 ± 0.2	0.45 ± 0.03	0.24 ± 0.04	0.78 ± 0.06
	Relative rate of $^1O_2$ generation	1 ± 0.05	0.18 ± 0.01	0.05 ± 0.01	0.41 ± 0.01
	Relative rate of hydroxyl radical generation	1 ± 0.03	0.43 ± 0.01	0.235 ± 0.004	0.367 ± 0.009
	Fluorescence lifetime (ns)	0.92 ± 0.02	0.95 ± 0.02	0.59 ± 0.02	0.76 ± 0.03
	Fluorescence quantum yield	0.20 (30)	0.20	0.07	0.11
Acetonitrile	Relative rate of ensemble photobleaching	1 ± 0.08	0.50 ± 0.01	1.0 ± 0.1	0.9 ± 0.1
	Relative rate of $^1O_2$ generation	1 ± 0.08	0.57 ± 0.06	0.92 ± 0.06	0.86 ± 0.06
	Fluorescence lifetime (ns)	1.11 ± 0.03	1.18 ± 0.05	0.93 ± 0.04	1.02 ± 0.03
	Fluorescence quantum yield	0.22	0.22	0.18	0.19
	Triplet state lifetime (μs)	63 ± 3	1.1 ± 0.1	62 ± 3	60 ± 4

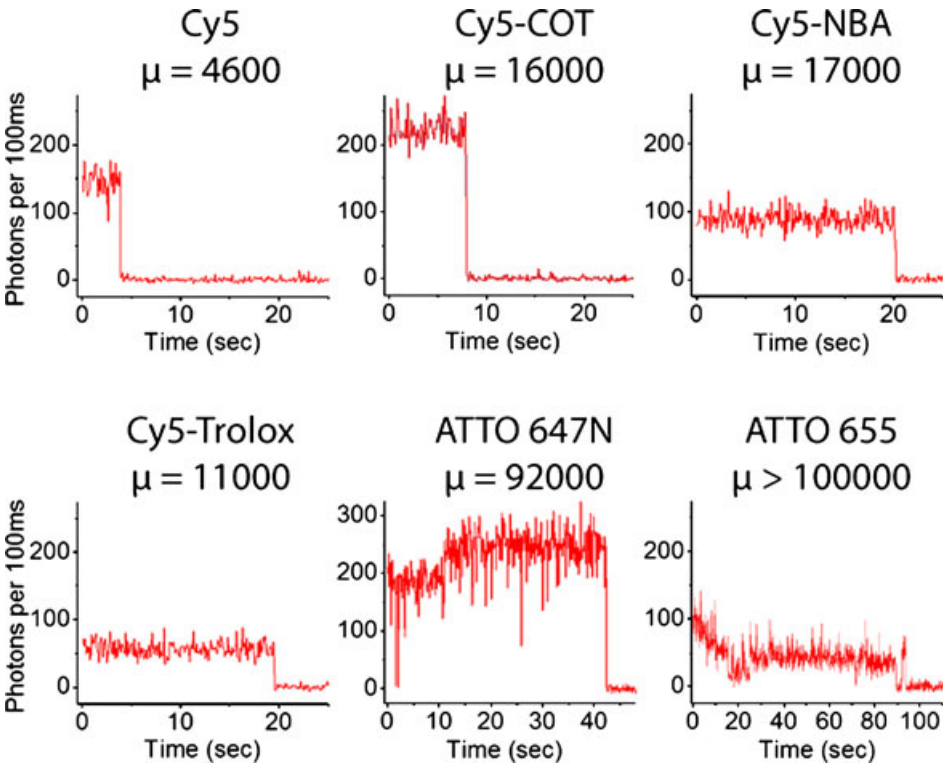


Figure 4. Representative single-molecule fluorescence traces for Cy5, Cy5-COT, Cy5-NBA, Cy5-Trolox, ATTO 647N and ATTO 655 covalently linked to DNA oligonucleotides and imaged in air-saturated aqueous solution using a total internal reflection microscope under continuous laser excitation (641 nm). μ is the average number of detected photons before fluorophore photobleaching.

sient dark states and intensity fluctuations, whereas Cy5, Cy5-COT, Cy5-NBA and Cy5-Trolox rarely blinked or fluctuated (Fig. 4). By tracking the fluorescence of many single molecules (>1000) over time (Fig. 4), the fluorescence intensity and the photobleaching rates were quantified. To compare each fluorophore's photobleaching rate, μ (the average numbers of photons detected from individual fluorophore prior to photobleaching) was calculated. As anticipated from prior investigations (26), the Cy5-COT, Cy5-NBA and Cy5-Trolox fluorophores exhibited significantly reduced rates of photobleaching (Table 1), emitting up to four-fold more photons than Cy5 prior to photobleaching (Fig. 4). As previously reported (26), the COT-conjugated fluorophore displayed a roughly 30% increase in brightness relative to Cy5 (Fig. 4). In contrast, both Cy5-NBA and Cy5-Trolox conjugates investigated here were substantially dimmer than Cy5 (~30% and 50%, respectively; Fig. 4), likely due to the electron transfer between the PA and Cy5 singlet excited state. The slow-photobleaching, nonblinking and nonfluctuating properties of the Cy5 derivatives investigated here render them suitable for single-molecule quantitative measurements, such as fluorescence resonance energy transfer, anisotropy, and stoichiometry. In contrast, the fluctuating emission behaviors of ATTO 647N and ATTO 655 make them more suitable for single-molecule tracking.

In previous studies (28), we showed that Cy5-COT, but not Cy5-NBA and Cy5-Trolox, exhibits shortened triplet state lifetime compared with Cy5 (Table 1). However, our previous measurements were exclusively performed in acetonitrile. Here, we

have shown that each of the Cy5-PA fluorophores exhibit reductions in photobleaching rates and in ROS generation, implying that in aqueous solution Cy5-COT, Cy5-NBA and Cy5-Trolox possess substantially shorter triplet state lifetime than Cy5. These data suggest that the effects of NBA and Trolox are strongly solvent-dependent: in aqueous solution they effectively quench the triplet state of Cy5, likely through an electron-transfer mechanism (38,39), whereas in acetonitrile they do not quench the triplet state due to the solvent-dependent nature of the electron transfer where radical states form (9). The rate of electron transfer is known to be affected by solvent polarity (40). In contrast, COT can quench the Cy5 triplet state in both solvents because of the solvent-independent nature of triplet–triplet energy transfer (9,28). This mechanism is supported by a further comparison of fluorophore properties in aqueous solution and in acetonitrile: the fluorescent lifetime, the fluorescent quantum yield, the photobleaching rate and the singlet oxygen generation rate for Cy5-NBA and Cy5-Trolox were strongly solvent-dependent, whereas the properties for Cy5 and Cy5-COT were only weakly solvent-dependent (Table 1; Figure S4).

CONCLUSION

The results presented suggest that the contribution of singlet oxygen to fluorophore photobleaching is negligible at low fluorophore concentrations (<5 μM) in aqueous buffers. Singlet oxygen does, however, play a significant role under solvent where its lifetime is significantly extended (*e.g.* chloroform) or at higher fluorophore concentrations (>20 μM) where the collision frequency with neighboring fluorophore molecules is greatly increased. These observations explain discrepancies in the literature regarding the role of singlet oxygen in fluorophore photobleaching (13,20) and suggest that singlet oxygen is unlikely to contribute to fluorophore photobleaching under most *in vitro* single-molecule imaging conditions. Cy5 derivatives covalently linked to a protective agent exhibit enhanced photostability as a direct consequence of distinct mechanisms of triplet state quenching that result in lower rates of singlet oxygen and hydroxyl radical generation. Correspondingly, these fluorophores may also be less perturbing and exhibit reduced phototoxicity when used for live cell imaging applications.

Acknowledgements—This work was supported by the National Institutes of Health (GM098859-01A1 to SCB) and the National Science Foundation (CHE 11-11392 to SJ). We are grateful to Roger B. Altman and Daniel S. Terry for critical discussions and comments on the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photobleaching rates of Cy5 in air-saturated $CHCl_3$ and $CDCl_3$, where k_B is the photobleaching rate.

Figure S2. Relative rate of singlet oxygen generation, in H2O solution and in 90% D2O solution, reported by the increase in SOSG fluorescence integrated from 518 to 538 nm. Experiments were performed with 0.7 μM Cy5 in 100 mM Tris-acetate-buffered solution (pH 7.5).

Figure S3. (a) The mechanism of hydroxyl radical detection with APF. (b) The emission spectra of APF at different time points of the photoillumination of Cy5. (c) The generation of hydroxyl radical, reported by the increase in APF fluorescence, upon illumination of Cy5, Cy5-COT, Cy5-NBA, Cy5-Trolox, ATTO 655 and ATTO 647N. In the parenthesis are the relative rates of hydroxyl radical generation.

Figure S4. (a) The reaction of $^1O_2$ with 9,10-diphenylanthracene. (b) The spectrum of 9,10-diphenylanthracene absorption during photobleaching of Cy5 (5 μM) in acetonitrile. (c) In the parentheses are the relative rates of singlet oxygen generation reported by the decrease in absorption at 393 nm.

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