An Oxygen Scavenging System for Improvement of Dye Stability in Single-Molecule Fluorescence Experiments

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ABSTRACT   The application of single-molecule fluorescence techniques to complex biological systems places demands on the performance of single fluorophores. We present an enzymatic oxygen scavenging system for improved dye stability in single-molecule experiments. We compared the previously described protocatechuic acid/protocatechuate-3,4-dioxygenase system to the currently employed glucose oxidase/catalase system. Under standardized conditions, we observed lower dissolved oxygen concentrations with the protocatechuic acid/protocatechuate-3,4-dioxygenase system. Furthermore, we observed increased initial lifetimes of single Cy3, Cy5, and Alexa488 fluorophores. We further tested the effects of chemical additives in this system. We found that biological reducing agents increase both the frequency and duration of blinking events of Cy5, an effect that scales with reducing potential. We observed increased stability of Cy3 and Alexa488 in the presence of the antioxidants ascorbic acid and n-propyl gallate. This new O_2-scavenging system should have wide application for single-molecule fluorescence experiments.

INTRODUCTION

Single-molecule fluorescence spectroscopy has proven to be a powerful tool in the study of biological systems. The observation of single molecules, which can be achieved by a variety of techniques, eliminates ensemble averaging and reveals transient or rare species in heterogeneous systems (1,2). Single-molecule spectroscopy has probed various biological phenomena, including protein and RNA folding (3–5), enzyme kinetics (6,7), and more complex systems such as the ribosome (8–10). In particular, immobilization-based techniques such as total internal reflection fluorescence microscopy (TIRF-M) have recently allowed the observation of various events on the milliseconds to seconds timescale (11–13).

Critical observation of complex multistep processes requires prolonged and stable fluorescence emission on the observation timescale (seconds to minutes). However, organic fluorophores commonly employed in single-molecule studies of biological systems display fast photobleaching, intensity fluctuations on the millisecond timescale (blinking), or both. These phenomena complicate the interpretation of fluorescence fluctuations and limit observation time (14,15). Emitting fluorophores undergo millions of cycles between ground and excited states; blinking and photobleaching are the result of excited states converting to transient or permanent dark states. Several species—including triplet states (16), conformational isomers (17), and radical ions (18,19)—have been proposed to contribute to dye instability, whether as dark states themselves or as reactive precursors to dark states.

Molecular oxygen (O_2) plays a key role in modulating dye stability. Triplet O_2 efficiently quenches dye triplet states responsible for blinking, resulting in the formation of the higher energy singlet oxygen species (20–22). Singlet O_2 reacts rapidly with exposed chemical groups in organic dyes; amino acids such as cysteine, histidine, tyrosine, and tryptophan; and guanosine in DNA (23,24). Oxidized dyes are no longer fluorescent, and oxidative damage impairs the folding and function of biomolecules. In the presence of saturating dissolved O_2 (DO), the blinking of fluorescent dyes is suppressed, but oxidative damage to dyes and biomolecules is rapid. Current approaches for improving dye and biomolecule stability typically involve the addition of an enzymatic O_2-scavenging system as well as chemical additives that ameliorate dye blinking.

The most commonly used system employs coupled glucose oxidase and catalase (GODCAT) in a buffer containing millimolar glucose (8,9,11–13,25). Blinking is controlled by the addition of low millimolar concentrations of β-mercaptoethanol (BME) (26). This system allows observation on the tens of seconds timescale and has been employed in numerous studies. The utility of this system is, however, limited by several factors. Certain in vitro conditions, such as experiments in cell extract, are incompatible with the enzymatic system. Moreover, mismatched GODCAT activities can result in a buildup of hydrogen peroxide, a reactive oxygen species that may damage fluorophores and biomolecules (Fig. 1 A) (27). Whereas it suppresses blinking on the millisecond timescale, BME induces transitions to long-lived (seconds) dark states, or slow blinking, in certain fluorophores. Rasnik et al. recently characterized this effect and that of Trolox, a water-soluble vitamin E analog that suppresses slow blinking and photobleaching,
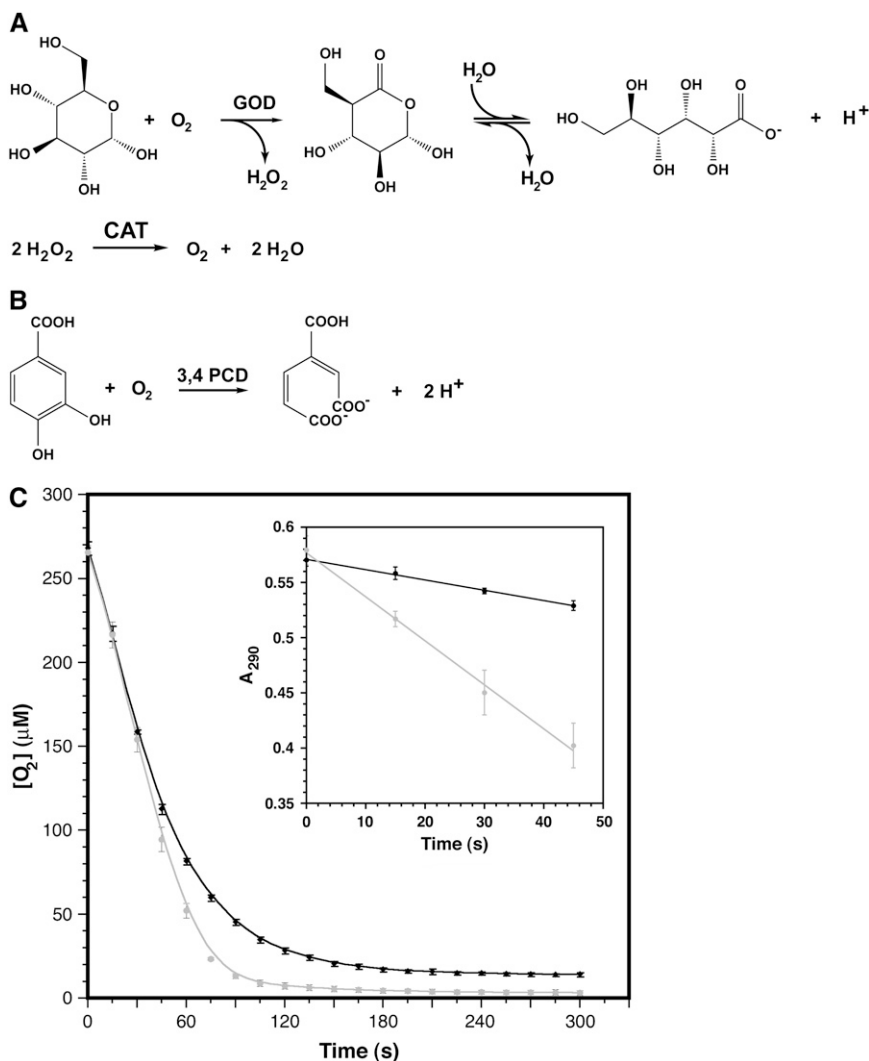
FIGURE 1 Comparison of GODCAT and PCD O_2-scavenging systems. (*A* and *B*) Schematics of chemical reactions in GODCAT and PCD systems, respectively. (*C*) Dissolved oxygen time courses for GODCAT (*solid*) and standardized PCD (*shaded*) systems. Conditions for each reaction are as follows: GODCAT ~100 nM glucose oxidase, ~1.5 μM catalase, 56 mM glucose; PCD ~50 nM PCD, 2.5 mM PCA. (*C inset*) Time courses of PCA consumption as monitored by absorbance at 290 nm. Each reaction contains 200 μM PCA and either 50 nM (*solid*) or 10 nM (*shaded*) PCD.

as compared to BME (28). Other chemical additives—including antifading agents, antioxidants, triplet state quenchers, and singlet oxygen quenchers—have been employed to improve dye stability in other systems (28–37).

Here, we present a system for improved dye stability in single-molecule experiments. We applied the protocatechuic acid (PCA)/protocatechuate-3,4-dioxygenase (PCD) O_2-scavenging system to single-molecule TIRF-M experiments. PCD is a well-characterized enzyme that has been applied to biological systems requiring anaerobic conditions (38–41). PCD is a multimeric enzyme that employs a non-heme iron center to catalyze the conversion of PCA to β-carboxy-*cis, cis*-muconic acid in one step, resulting in consumption of 1 mole of O_2 and the production of two protons per mole of PCA converted (Fig. 1 *A*). We characterized the effect of PCD-catalyzed O_2-scavenging on the stability of three dyes commonly used in single-molecule TIRF-M experiments: Cy3, Cy5, and Alexa488. To catalog the effects of additives on single-molecule TIRF-M exper-

iments, we characterized the effects of commonly used biological reducing agents, as well as other chemical additives, on the behavior of single fluorophores. The system presented here should have broad application to single-molecule experiments.

MATERIALS AND METHODS

Reagent preparation

Labeled oligonucleotides

5′-Amino, 3′-biotin functionalized oligonucleotides were synthesized by IDT (Coralville, IA) and labeled with NHS (*N*-hydroxysuccinimide) esters of Cy3, Cy5 (GE Healthcare, Piscataway, NJ), and Alexa Fluor 488 (Alexa488) (Molecular Probes, Carlsbad, CA). Labeling reactions were performed under the following conditions: 250 μM oligo, 2.5 mM dye NHS ester, 500 mM NaCl, and 100 mM KBO$_3$ pH 9. Reactions were allowed to proceed at room temperature for 1 h. Labeled oligos were subsequently polyacrylamide gel electrophoresis purified, eluted by crush and soak, ethanol precipitated, and stored at −20°C.

Enzymes and other reagents

All reagents are from Sigma (St. Louis, MO) unless otherwise noted. TP50 is 50 mM KCl, 50 mM Tris-OAc (acetoxy group) pH 7.5. Glucose oxidase from *Aspergillus niger* and catalase from bovine liver were obtained as lyophilized powders and stored as 50% glycerol stocks in TP50 at $-20°C$. The lyophilized powder of protocatechuate 3,4-dioxygenase from the *Pseudomonas* species was resuspended and stored as a 50% glycerol stock in 50 mM KCl, 1 mM EDTA, and 100 mM Tris-HCl pH 8. The concentration of each enzyme was estimated using molecular mass information provided by Sigma (glucose oxidase = 160 kDa, catalase = 250 kDa, PCD = 700 kDa). Purified PCDs from *Burkholderia cepacia* and *Pseudomonas putida* were obtained as generous gifts from D. Ballou and D. Ohlendorf, respectively, and used as supplied. 3,4-Dihydroxybenzoic acid was resuspended to 100 mM in deionized water and adjusted to pH 9 using NaOH. A total of 100 mM Trolox solution was prepared by dissolving Trolox in methanol followed by the addition of deionized water and 1 M NaOH to the appropriate volume (final concentration methanol = 10% at pH $\sim$9.5). Ascorbic acid was dissolved to 100 mM in water and brought to pH 7 with 1 M NaOH. *n*-Propyl gallate (nPG) was dissolved to 10 mM in 1% ethanol/water (v/v). 1, 4-Diazabicyclo[2.2.2]octane (DABCO) was dissolved in water and brought to pH 7.5 with 1 M HCl, final concentration 1 M. Mercaptoethylamine (MEA) was dissolved in water and brought to pH 7.5 with glacial acetic acid, final concentration 1 M. Dithiothreitol (DTT) was dissolved to 1 M in deionized water. Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride solution (.5 M in water) and BME were used as supplied.

Enzyme kinetics

Dissolved oxygen kinetics

DO measurements were performed using an Orion 835A meter (Thermo, Waltham, MA). Probe calibration was performed according to the manufacturer's protocol before each set of experiments, using ZERO OXYGEN SOLUTION (Oakton, Vernon Hills, IL) and water-saturated air. The DO probe was inserted into a 20 mL glass scintillation vial containing 4 mL TP50 (TP50 + 1.25% glucose for glucose oxidase/catalase measurements) under constant stirring. Stock solutions of glucose oxidase, catalase, PCD, and PCA were diluted to 500 μL each and delivered by pipetting to the reaction vial. Measurements were recorded manually.

PCA kinetics

The conversion of PCA to β-carboxy-*cis*,*cis*-muconic acid was monitored by absorbance at 290 nm using a Shimadzu UV1201 ultraviolet/visible (UV/VIS) spectrophotometer (Kyoto, Japan); and 2 μl of PCA stock solution was rapidly mixed with 1 mL of dilute PCD in buffer to a final concentration of 200 μM PCA. Measurements for the determination of initial rates were recorded manually.

Single-molecule TIRF microscopy

Surface immobilization

Flow cells for single-molecule TIRF microscopy were prepared following a protocol derived from Ha et al. (42). Surfaces were incubated with 1 μM Neutravidin (Pierce, Rockford, IL) before the delivery of fluorescently labeled oligos at $\sim$10 pM, yielding hundreds of well-resolved single dyes per viewing field. Cy3 and Cy5 functionalized oligos were immobilized in the same flow cell and simultaneously imaged. Alexa488 functionalized oligos were immobilized and imaged in a separate flow cell. Unless otherwise mentioned, measurements were performed in the presence of 1 mM Trolox. All measurements were performed after the selected O_2-scavenging system had achieved steady state as determined by previously dissolved oxygen measurements.

TIRF microscope

All measurements were performed using a prism-based total internal reflection instrument built in house using a Nikon TE-2000 inverted microscope (Nikon Instruments, Melville, NY). Fluorophores were excited with laser lines at 532 nm (CrystaLaser, Reno, NV), 647 nm (CrystaLaser), and 488 nm (Coherent, Santa Clara, CA); laser intensities were $\sim$1 kW/cm^2, 400 W/cm^2, and 320 W/cm^2, respectively. Light from a $\sim$100-μm^2 viewing area was collected through a 60$\times$, 1.20 numerical aperture water-immersion objective (Nikon Instruments) and filtered using a Quad-View apparatus containing several long-pass filters—540, 630, and 720 nm—followed by band-pass filters—510/20, 575/40, and 680/50 nm (Roper Bioscience, Tucson, AZ). Unbinned fluorescent images were collected with a Cascade 512B charge-coupled device camera (Photometrics, Tucson, AZ) at 100 ms time resolution. Image acquisition and analysis was controlled with the MetaMorph software package (Molecular Devices, Sunnyvale, CA). Spot picking and fluorescence intensity trajectories were recorded postacquisition using MetaMorph. Analysis of fluorescence intensity trajectories was performed using MATLAB (The Mathworks, Natick, MA).

RESULTS

PCD reproduces oxygen scavenging by GODCAT

The GODCAT system has been employed successfully for O_2-scavenging in a variety of single-molecule studies. To establish the requisite parameters for O_2-scavenging in single-molecule systems, we performed simple kinetic assays of enzyme activity. Initial reaction rates and steady-state O_2 concentration were determined using a dissolved oxygen probe submerged in glucose-containing buffer under constant stirring; reaction progress was followed upon the addition of enzyme. At enzyme and substrate concentrations currently employed in the literature ($\sim$100 nM glucose oxidase, $\sim$1.5 μM catalase, 56 mM glucose), the GODCAT system scavenges O_2 at an initial rate of 4 μM s^{-1} and achieves a steady-state O_2 concentration of 14 $\pm$ 1 μM within $\sim$3 min (Fig. 1 *B*).

To enable direct comparison of dye stability in the GODCAT and PCD systems, we employed this dissolved oxygen assay to standardize O_2-scavenging by PCD to that of the GODCAT system. At $\sim$50 nM PCD and 2.5 mM PCA, the PCD system reproduces the initial rate of O_2-scavenging by the GODCAT (Fig. 1 *B*). Interestingly, the PCD system proceeds to a fivefold lower steady-state O_2 concentration (3 $\pm$ 1.5 μM); this oxygen concentration is maintained, under constant stirring, for several hours with no effect on pH (data not shown). Furthermore, we observed no appreciable loss in activity for enzymes stored at $-80°C$ for 6 months and at 4°C for 5 weeks, similar to the observations of Patil et al. (data not shown) (41). Reaction progress was also monitored with a UV/VIS assay for disappearance of PCA via loss of absorbance at 290 nm. Initial rates determined using this method are in agreement with dissolved oxygen measurements (Fig. 1 *C, inset*). This complementary assay allows quick verification of PCD activity with equipment common to most labs. Application of this assay demonstrated the feasibility of using PCD purified from a variety of organisms (Supplementary Fig. 1).

Improved dye stability with the PCD system

We next compared the photophysical stability of single Cy3, Cy5, and Alexa488 fluorophores on immobilized oligonucleotides in the GODCAT and standardized PCD systems. A minimum of ~700 fluorescence intensity trajectories were initially superimposed to diagnose the bulk stability of excited fluorophores (Fig. 2 A). Individual trajectories were subsequently analyzed for each condition to determine more accurately the behavior of single fluorophores. Trajectories were parsed into three component events: initial fluorescence, blinked-off fluorescence, and blinked-on fluorescence, as defined in Fig. 2. All events limited by the completion of observation were discarded (Fig. 2 B). Normalized lifetime distributions of these component events were fit to single exponential probability distribution functions to determine mean event lifetimes (Fig. 2 C). Mean intensities were also calculated. The relative frequency, duration, and intensity of these component events determine the utility of a fluorophore in single-molecule experiments: an ideal fluorophore would display extended initial fluorescence, with little to no blinking, as well as a high signal/noise ratio.

The photostability of Cy3, Cy5, and Alexa488 in the GODCAT O_2-scavenging system in the presence of Trolox was determined using the analysis described above. Fluorescence is detected from ~90% of molecules upon initial observation of all three fluorophores. Cy3 and Cy5, the two dyes most commonly employed in single-molecule TIRF-M studies, exhibit long initial lifetimes, infrequent blinking, and average signal/noise ratio values of ~4.0 (Fig. 3 A). Alexa488, a rhodamine derivative, shows significantly shorter initial lifetimes, increased blinking, and average signal/noise ratio values of ~2.5 (Fig. 3 A).

Cy3, Cy5, and Alexa488 fluorophores all show improved stability in the standardized PCD system with Trolox. PCD improves initial lifetimes up to 140% with no effect on on-blink and off-blink lifetimes (Fig. 3 B). No significant effect on blinking frequency or signal/noise ratio was observed. Interestingly, similar results were achieved using a PCD system with a fivefold lower enzyme concentration, provided the system was allowed to achieve a steady-state level of O_2 (Fig. 3 C). UV/VIS activity measurements confirmed a fivefold decrease in the initial rate of O_2-scavenging under these conditions (Fig. 1 C, inset), suggesting that the rate of O_2-scavenging currently employed in single-molecule measurements exceeds the minimum requirements for dye stability. All subsequent measurements with PCD employed these conditions (10 nM PCD, 2.5 mM PCA, and 1 mM Trolox).

To test the origin of observed improvements in dye stability with the PCD system, we added 2.5 mM PCA to the GODCAT system. Under these conditions, any observed differences between the GODCAT and PCD systems would likely be a result of lower O_2 concentrations or reaction products of PCD. However, the addition of PCA to the GODCAT system recapitulates the improvements in Cy3 and Cy5 stability observed in the PCD system (Fig. 3 C). Improvements in Cy3 and Cy5 stability can thus be attributed to the presence of PCA. In contrast, the addition of PCA to the GODCAT system does not replicate Alexa488 initial lifetimes observed with PCD. PCA extends Alexa488 initial lifetimes to 11.0 ± 0.4 s, whereas lifetimes longer than 15 s are observed in both the standardized and dilute PCD systems (15.9 ± 0.6 s and 15.4 ± 0.4 s, respectively). Increased Alexa488 stability in the PCD system is due to PCA and other factors, potentially lower steady-state O_2 concentrations.
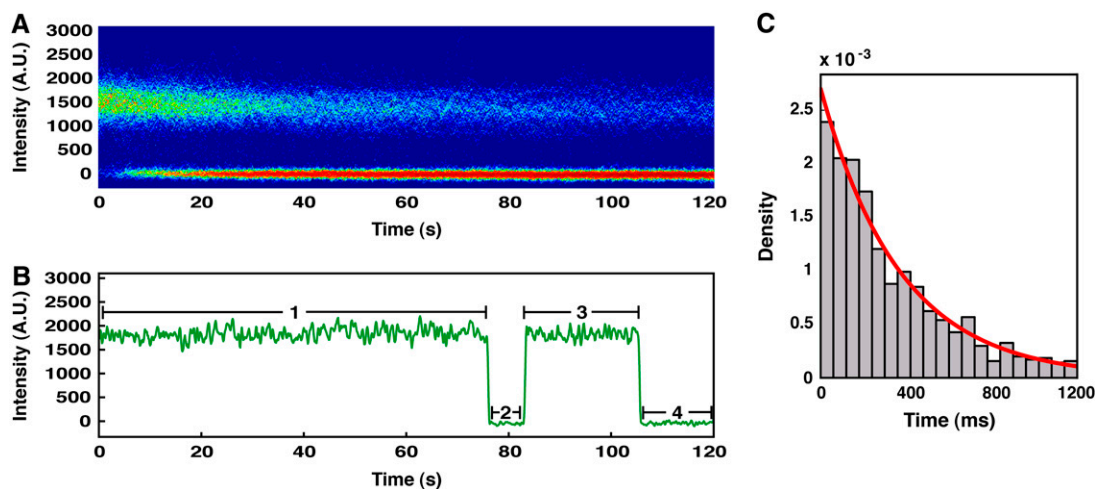

FIGURE 2 Analysis of single fluorophores. (A) Representative color overlay of all fluorophores observed in one acquisition movie. (B) Representative fluorescence versus time trace for a single dye. Initial events (1), off-blink (2), and on-blink events (3) were segregated before lifetime analysis; events limited by acquisition length (4) were discarded. (C) Initial, off-blink, and on-blink event distributions were fit, as described, to an exponential probability function to determine mean lifetimes, here Cy5 initial lifetimes with PCD.

A

	Cy3			Cy5			A488		
	Initial	On-blink	Off-blink	Initial	On-blink	Off-blink	Initial	On-blink	Off-blink
Mean Lifetime (s)	31.6 ± 1.6	10.3 ± 0.6	2.0 ± 0.2	31.1 ± 0.8	20.2 ± 1.8	24.5 ± 2.1	6.7 ± 0.3	3.3 ± 0.1	13.4 ± 0.3
Events per mol.	.88	.36	.26	.97	.08	.09	.71	2.14	2.17

B

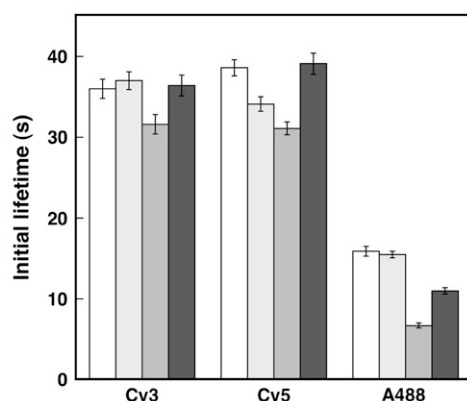|	Cy3			Cy5			A488		
	Initial	On-blink	Off-blink	Initial	On-blink	Off-blink	Initial	On-blink	Off-blink
Mean Lifetime (s)	36.0 ± 1.2	10.5 ± 0.6	21.5 ± 1.2	38.6 ± 1.0	18.9 ± 1.7	22.4 ± 2.0	15.9 ± 0.6	4.4 ± 0.1	6.8 ± 0.2
Events per mol.	.92	.38	.31	.95	.08	.12	.87	2.07	2.10

C

FIGURE 3 Characterization of dye stability in the GODCAT and PCD systems. (*A*) Table of mean lifetimes and event frequencies (per molecule) in the GODCAT system. (*B*) Table of mean lifetimes and event frequencies (per molecule) in the standardized PCD system. (*C*) Comparison of initial dye lifetimes in the standardized PCD (*open*), dilute PCD (*light shaded*), GODCAT (*shaded*), and GODCAT+PCA (*dark shaded*) systems.

These results underscore the different responses of distinct dyes to solution conditions.

Biological reducing agents adversely affect dye stability

Biological reducing agents such as BME, DTT, and TCEP are commonly used to reduce protein disulfide groups in vitro (43–46). Furthermore, BME has been shown to suppress fast (millisecond) blinking of Cy5. Consequently, the majority of current single-molecule TIRF-M experiments are performed in the presence of millimolar concentrations of BME. Recent work by Rasnik et al., however, demonstrated that BME induces slow blinking of Cy5 (28). The authors further demonstrated that L-glutathione, another thiol-containing compound, similarly suppresses fast blinking, probably by quenching triplet states, while inducing slow blinking. This suggests that thiol-containing compounds, and perhaps other biological reducing agents, adversely affect dye photophysics.

We characterized the stability of Cy3, Cy5, and Alexa488 in the presence of 10 mM BME, DTT, and TCEP. The redox potentials of BME and DTT at pH 7 are −0.26 V and −0.33 V, respectively (46,47); TCEP has been shown to reduce DTT in solution (44), and so has a more negative redox potential, though, to our knowledge, precise values have not been determined in the literature. All measurements were performed using the PCD system to scavenge O_2. Dissolved oxygen measurements showed no effect on PCD O_2-scavenging in the presence of BME, DTT, or TCEP (data not shown). As previously observed, the presence of thiol-containing compounds most drastically affects the stability of Cy5. Both BME and DTT significantly shorten the initial lifetime of Cy5, as well as increase the frequency of blinking events ~10-fold. In fact, in the presence of reducing agents, all observed fluorescence on-states—initial and blinking events—are of equal duration. Off-blink events are drastically lengthened, to 64.8 ± 2.1 s, in the presence of DTT. Additionally, both compounds reduce the signal/noise ratio for Cy5. The addition of TCEP, which contains no thiol groups, has even more striking effects (Fig. 4 *A*). Biological reducing agents, not only those containing thiol groups, cause nonideal dye behavior.

Interestingly, these effects scale with reducing potential. The initial lifetime of Cy5 is shortened ~4-fold, 9-fold, and 100-fold by BME, DTT, and TCEP, respectively (Fig. 4 *A*). On-blink lifetimes decrease as a function of reducing potential. Conversely, off-blink lifetimes increase in the presence of all reducing agents. However, reducing agents do not shorten on-blink events as compared to initial events, whereas off-blink events are drastically lengthened as a function of reducing power; normalization to initial lifetimes reveals these trends (Fig. 4 *B*). The signal/noise ratio is decreased to 3.8, 3.3, and 2.2 in the presence of BME, DTT, and TCEP, respectively (Fig. 4 *C*).

Biological reducing agents show mixed effects on the stability of Cy3 and Alexa488 (Supplementary Figs. 2 and 3). Both BME and DTT increase initial lifetimes of Cy3,

A

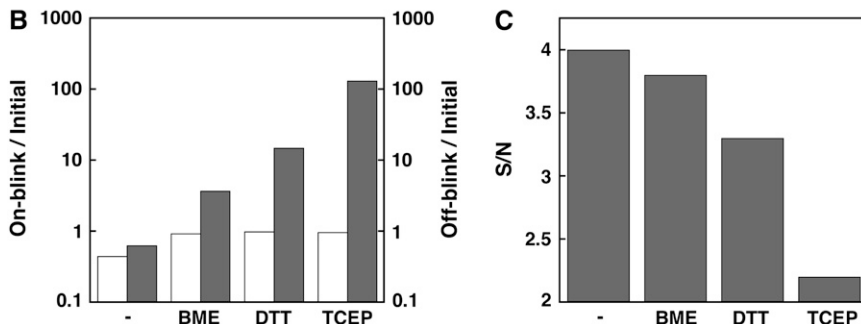|	No reductant		BME		DTT		TCEP	
	Events per mol.	Lifetime (s)	Events per mol.	Lifetime (s)	Events per mol.	Lifetime (s)	Events per mol.	Lifetime (s)
Initial	.96	34.1 ± 0.9	.91	10.6 ± 0.4	.88	4.3 ± 0.1	.51	0.4 ± 0.1
On-blink	.11	15.1 ± 1.2	1.02	9.8 ± 0.3	.79	4.3 ± 0.1	1.02	0.4 ± 0.1
Off-blink	.12	21.4 ± 1.6	1.04	39.2 ± 1.2	.80	64.8 ± 2.1	1.02	48.2 ± 1.9

FIGURE 4 Cy5 dye stability in the presence of biological reducing agents. (*A*) Table of event frequencies and lifetimes for initial, on-blink, and off-blink events (per molecule) in the absence or presence of 10 mM BME, DTT, and TCEP. Lifetime distributions are fit as described in Materials and Methods. (*B*) Bar plot of the ratios of on-blink/initial lifetimes (*open*) and off-blink/initial lifetimes (*shaded*) for Cy5 in the presence of reducing agents. (*C*) Bar plot of the signal/noise ratio for Cy5, determined on a per molecule basis.

whereas TCEP decreases initial lifetimes. As with Cy5, all compounds decrease the Cy3 signal/noise ratio as a function of reducing power. BME shows little effect on Alexa488, save for a 50% increase in the frequency of blinking events. DTT, however, drastically diminishes the signal/noise ratio for Alexa488, making an accurate determination of dye behavior impossible. The addition of TCEP hinders almost all aspects of Alexa488 fluorescence: initial lifetimes are shortened, blinking events are more frequent, and the signal/noise ratio is decreased.

As a group, biological reducing agents—not only those containing thiol groups—adversely affect many aspects of dye stability. These effects are most drastic for Cy5 and correlate with reducing power, not chemical functionality.

The effect of other chemical additives on dye stability

Many prior studies have identified chemical additives that function to improve dye photostability under certain conditions (28–37). These studies cover a broad range of methodologies; concise characterization of the effects of chemical additives in single-molecule TIRF-M experiments is needed. To this end, we characterized the effects of four previously described chemical additives in the PCD system: MEA, DABCO, ascorbic acid, and nPG. All additives, with the exception of nPG, were used at 10 mM. Due to limited solubility in aqueous solutions, nPG was used at 100 μM. These molecules were chosen because they possess a variety of chemical functionalities (Fig. 5 *A*) and have been reported to improve dye stability by acting as either singlet oxygen quenchers, triplet state quenchers, or antioxidants. Dissolved oxygen measurements detected no effect on PCD O_2-scavenging in the presence of each additive (data not shown).

However, we observed a variety of effects on dye stability in the presence of these additives (Supplementary Tables 1–3 and Fig. 4), as described below.

MEA

Fluorescence correlation spectroscopy measurements performed by Widengren et al. demonstrated MEA to effectively quench the triplet state of Rhodamine 6G (29). However, MEA contains a thiol group and thus might be expected to behave similarly to biological reducing agents such as BME. We observed with MEA many of the deleterious effects on Cy5 stability associated with the presence of biological reducing agents (Fig. 5 *B*). Most notably, MEA shortens initial and on-blink lifetimes (5.3 ± 0.2 s and 4.5 ± 0.1 s, respectively), lengthens off-blink lifetimes to 30.6 ± 0.5 s, increases the frequency of blinking events 20-fold (2.4 events per molecule), and decreases the signal/noise ratio by 25%. The ratios of off-blink lifetimes to initial lifetimes, as well as on-blink to initial lifetimes for Cy5 in the presence of MEA, closely resemble those with BME. Like BME and DTT, MEA improves the initial lifetimes of Cy3 fluorophores (40.1 ± 1.6 s) but decreases the signal/noise ratio by 20%. No significant changes to the stability or the signal/noise ratio of Alexa488 were observed in the presence of MEA.

DABCO

DABCO has been employed as an antifading agent in fluorescence microscopy, as well as a stabilizer of dye lasers, presumably through triplet state and singlet oxygen quenching (Fig. 5 *B*) (34,36,37,48). However, we observed few to no effects on dye stability in the presence of this additive. Initial, on-blink, and off-blink lifetimes of all three fluorophores are largely unaffected. A small increase in the fre-
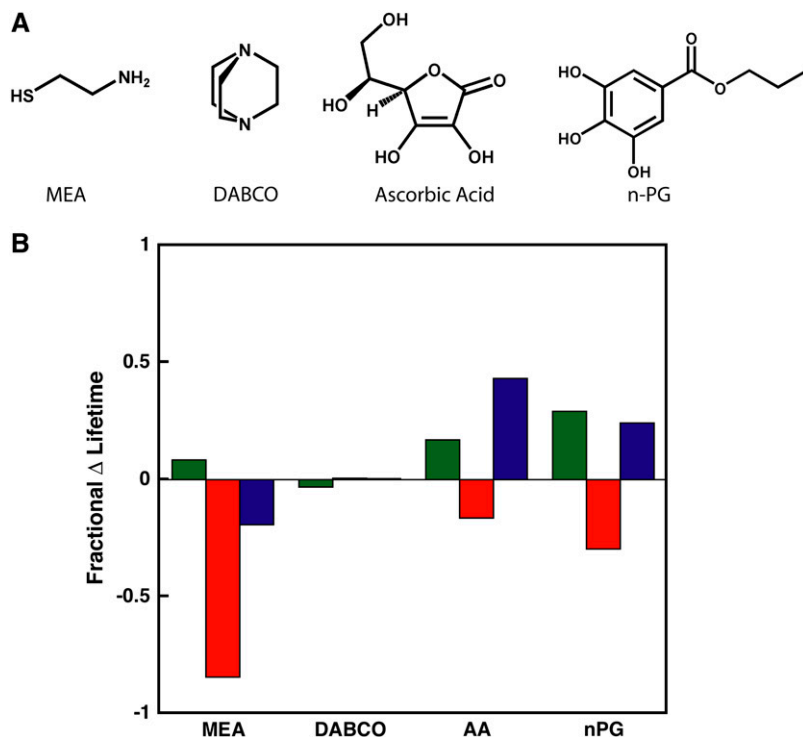
FIGURE 5 Effects of additives on initial lifetimes of Cy3, Cy5, and Alexa488. (*A*) Chemical structures of additives: MEA, DABCO, ascorbic acid, and nPG. (*B*) Bar plot of the fractional change in initial lifetimes of Cy3 (*green*), Cy5 (*red*), and Alexa488 (*blue*) in the PCD system with respective additives.

quency of blinking events was observed in all cases. No effect on the signal/noise ratio was observed, with the exception of a 13% decrease for Cy5.

Ascorbic acid

Ascorbic acid is an antioxidant that has been used to retard photobleaching of excited dyes by quenching radical species (29,32). We observed increased initial dye lifetimes for both Cy3 (43.3 ± 1.2 s) and Alexa488 (22.2 ± 0.7 s) in the presence of ascorbic acid (Fig. 5 *B*). Cy5 initial lifetimes are slightly decreased (28.5 ± 0.7 s). The frequency and lifetimes of blinking events are unaffected, as is the signal/noise ratio. Rasnik et al. similarly observed no effect on blinking of Cy5 in the presence of ascorbic acid (28). The authors do not, however, report any effects on initial lifetimes of Cy5 or other fluorophores.

nPG

Like ascorbic acid, the antioxidant properties of nPG have been employed to slow photobleaching in fluorescence microscopy applications (31,33,35,49). We observed effects similar to those of ascorbic acid in the presence of nPG (Fig. 5 *B*). Both Cy3 and Alexa488 initial lifetimes increased (47.8 ± 1.3 s and 19.3 ± 0.7 s, respectively), whereas Cy5 initial lifetimes decreased (24.1 ± 0.5 s). As with ascorbic acid, blinking frequency was unaffected. No effect on the signal/noise ratio was observed.

In sum, we observed a variety of effects on dye stability in the presence of various previously described chemical ad-

ditives. MEA, a thiol-containing compound, shows similar effects to dye behavior as the biological reducing agents BME and DTT. In contrast, DABCO was not observed to have any substantial effects on the stability of single fluorophores. Both ascorbic acid and nPG—the two compounds with antioxidant properties—increase the stability of Cy3 and Alexa488 but slightly decrease the stability of Cy5.

DISCUSSION

The GODCAT system is employed for the maintenance of anaerobic conditions in a variety of applications. However, this system has several limitations. The precise tuning of the GODCAT reactions is required to prevent the buildup of reactive oxygen species. Both enzymes employ highly colored cofactors; glucose oxidase employs flavin adenine dinucleotide, whereas catalase uses a heme group. Catalase is inhibited by a variety of molecules, including BME, DTT, and ascorbic acid (50–52). Lastly, the GODCAT system is incompatible with measurements performed in cell extract.

Recently, Patil et al. presented PCD as an alternative O_2-scavenging system to GODCAT. The PCD system presented here has since been employed in numerous published studies to control or minimize solution O_2 concentrations (53). PCD is a single enzyme system possessing none of the aforementioned limitations. PCD is a multimeric metalloenzyme for which subunit stoichiometry varies from organism to organism. Catalysis is achieved by a nonheme iron center and does not require additional redox cofactors. The catalytic mechanism of oxidation is well understood and does not

involve the production of reactive species that could interfere with dye photophysics or biomolecular function. PCD from various *Pseudomonas* species is commercially available and stable under standard storage conditions for months. Patil et al. demonstrated that PCD efficiently scavenges O_2 in a variety of buffers of pH 6–9. Activity is reported between 4°C and 35°C over a period of 15 months, which is supported by our own observations. We have also shown that different PCD enzymes can be tuned to achieve similar rates of O_2-scavenging, thus providing flexibility in the choice of PCD for a given system.

We matched the initial O_2-scavenging rate of PCD to that of the GODCAT system. Under these conditions, PCD achieves lower steady-state O_2 concentrations than does GODCAT. This may occur due to the increased contribution of the catalase reaction with rising H_2O_2 concentrations. Fivefold dilution of the PCD system results in a corresponding decrease in the initial rate of O_2-scavenging. Nonetheless, this diluted system still achieves similar steady-state O_2 concentrations. Thus, the PCD system represents an improvement over the GODCAT system for applications that require low O_2 concentrations, such as the preservation of biomolecules from oxidative damage.

PCD significantly improves the stability of Cy3, Cy5, and Alexa488 in single-molecule TIRF-M experiments. Under GODCAT-standardized conditions, PCD extends initial dye lifetimes up to 140% with no deleterious effects. This improvement is conserved with fivefold dilute PCD, provided the system is permitted to achieve equilibrium. This suggests that the rate of O_2-scavenging under currently employed conditions exceeds any reoxygenation in our flow cells.

Increased dye stability with PCD is due, in part, to the PCA cofactor. Inclusion of PCA in the GODCAT system recapitulates the improvements in Cy3 and Cy5 dye stability observed with PCD. Notably, Jonavic et al. and Huang et al. have characterized the antioxidant properties of PCA and its ethyl ester, ethyl protocatechuate (54,55). Additionally, PCA shares chemical similarities with Trolox and nPG, both of which improve dye stability in fluorescence applications. PCA, then, serves to stabilize excited fluorophores. PCA cannot, however, substitute for Trolox as a dye-stabilizing agent; the removal of Trolox from the PCD system results in increased blinking, as described by Rasnik et al. (data not shown) (28). Interestingly, PCA is not solely responsible for improved Alexa488 stability in the PCD system. Others factors, such as reaction products of PCD or lower steady-state O_2 levels, likely contribute to improved Alexa488 stability. This highlights the unique responses of different fluorophores to environmental conditions.

Both Trolox and PCA significantly improve the stability of emitting fluorophores in single-molecule TIRF-M experiments. These improvements, however, are achieved in the absence of biological reducing agents. In fact, most in vitro studies of biological systems are performed in buffers containing reducing agents such as BME. Recent work suggests that thiol-containing reducing agents cause slow blinking of Cy5 on a timescale that limits the observation of biological systems.

In an effort to identify reducing agents compatible with single-molecule TIRF-M experiments, we compared the effect on Cy5 stability of DTT, a thiol-containing reductant, and TCEP, an alkyl phosphine, to that of BME. However, we observed increased blinking frequency when 10 mM BME, DTT, or TCEP was included in the PCD/Trolox system. The effect of biological reducing agents such as BME, then, is not limited to those containing thiol moieties. We also observe decreased initial lifetimes, increased off-blink lifetimes, and a decreased signal/noise ratio in the presence of reducing agents, an effect that scales strongly with reducing power. Cy5 photochemistry is populated by various emitting and dark states (17,28,56–61), whose identification is beyond the scope of this study. More detailed photophysical analysis of reductant effects on Cy5 emission is required.

Where possible, we suggest the exclusion of biological reducing agents from single-molecule TIRF-M experiments with Cy5. If the storage of biomolecules under reducing conditions is necessary, reductants can be removed before experimentation; in vitro measurements performed on the minutes timescale can successfully be performed in the absence of reducing agents.

To provide a practical catalog of additive effects specific to single-molecule TIRF-M experiments, we tested the effects on dye stability of four reputed antifading agents: MEA, DABCO, nPG, and ascorbic acid. MEA has previously been described as a triplet state quencher. We observe a profile of effects similar to that of BME and DTT. DABCO, a proposed singlet oxygen and triplet state quencher, had little effect on dye stability. Although DABCO has been claimed to retard fading in fluorescence microscopy experiments, its efficacy has been questioned (34,35). In contrast to MEA and DABCO, nPG and ascorbic acid showed marked improvements to dye (specifically Cy3 and Alexa488) stability. Both molecules are well-known antioxidants and may improve dye stability through interactions with dark radical ion species proposed to precede photobleaching. It is of note that 100 μM nPG improves dye stability to the same degree as 10 mM ascorbic acid. Neither ascorbic acid nor nPG improve the stability of Cy5, potentially because their redox potentials are not tuned for the quenching of Cy5 radical species.

The preliminary results presented here underscore the interplay of dye ground and excited state redox chemistry and photophysics. Improved understanding of this complex physical landscape would allow the rational pairing of fluorophores and antioxidant additives.

SUPPLEMENTARY MATERIAL

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