IOFF Generally Extends Fluorophore Longevity in the Presence of an Optical Trap

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Abstract: The combination of optical tweezers force microscopy and single molecule fluorescence has previously been complicated by trap-induced photobleaching. Recent studies have suggested that this effect is caused by a sequential absorption of photons, leading to ionization of the fluorescent singlet state. In this work, we show the range of effects of optical trapping radiation on common fluorescent dyes. Using the interlaced optical force fluorescence (IOFF) laser modulation technique, we show that the removal of simultaneous near infrared radiation dramatically reduces photobleaching effects. However, these studies show that the sequential addition of near infrared radiation in some cases extends photobleaching longevity beyond the natural intrinsic decay. We suggest a refined photoelectronic mechanism that accounts for the possibility of reverse intersystem crossing from a reactive triplet state and explains the nature of trap-induced photobleaching.

Keywords: Single-molecule fluorescence, optical tweezers, biophysics, force-fluorescence microscopy, photobleaching.

INTRODUCTION

The development of a coincident combination of single molecule force and fluorescence techniques provides an approach for measuring and controlling the molecular state of the system under study. Building on previous work with optical tweezers force microscopy and single molecule fluorescence detection [1-6] we demonstrated such a simultaneous measurement on individual DNA hairpin molecules with a generally adaptable assay that has broad applicability to a range of biomolecular systems [7, 8]. This approach, which we termed Interlaced Optical Force-Fluorescence (IOFF) spectroscopy, dramatically reduced the optical trap-induced photobleaching that previously complicated this type of measurement. While IOFF showed a 20-fold temporal improvement for the most problematic dye, Cy3, the systematic effects of trap-induced photobleaching on different fluorophores have yet to be mapped. In this work, we present such a study by testing nine of the most common single molecule dyes in a combined, coincident photobleaching arrangement subjected to our IOFF solution. Our results, suggest IOFF is generally useful and for some dyes, it features an enhanced emission phenomenon suggesting a reverse intersystem crossing mechanism.

In a combined optical force-fluorescence experiment, a large biological molecule is chemically labeled with a high intensity fluorescent label and tethered to a dielectric bead. Manipulation of the bead with an optical trap provides both nanometer level position resolution and piconewton level mechanical control over the molecular state [9, 10]. Simultaneous observation of the fluorescence dye emission yields insight into the localized effects of mechanical force, while the incorporation of more sophisticated fluorescence energy transfer arrangements can be used to give a direct view of force-induced intramolecular interactions [8, 11]. This assay geometry has been used in several different experimental arrangements, but until recently it was severely limited by the effects of trap-induced photobleaching, which reduces fluorescence emission longevity to a few seconds with commonly used fluorophores [5, 7, 12].

The electronic mechanism of trap-induced photobleaching is not yet understood, though it has similarities to the natural photodecay of fluorescent molecules. Natural photobleaching is thought to occur when an electron in an excited triplet state reacts with singlet oxygen, leading to irreversible destruction of the fluorescence pathway [13, 14]. However, an alternative mechanism includes two-photon absorption in polar solvents, which leads to the creation of a highly reactive dark state fluorophore and a solvated electron [12, 13, 15, 16]. In both cases, common methods to counteract photobleaching include deoxygenation by vacuum degassing and adding reducing agents and oxygen scavengers, such as β-mercaptoethanol and glucose oxidase [12-15, 17-20]. While these approaches greatly extend fluorescence emission times, they do not completely block the photobleaching pathway.

Even in the presence of antioxidants, simultaneous irradiation with both fluorescence excitation and optical trapping lasers dramatically increases photobleaching rates. Previous work hypothesized that the presence of a low energy, high intensity trapping laser leads to sequential photon absorption and population of a higher energy singlet state that is more prone to destructive ionization [12]. To support this hypothesis, van Dijk *et al.* further showed the effects of molecular structure on trap-induced photobleaching rates, demonstrating that Cy3 and Alexa 555 were dramatically affected by

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the presence of the trap, while tetramethylrhodamine (TMR) was minimally influenced.

In this report, we explore the effects of wavelength and timing on trap-induced photobleaching across a range of fluorophores. By applying IOFF laser switching to a range of fluorescent dyes, we show that fluorophores excited at 532 nm are uniquely susceptible to trap-induced photobleaching. We also extend the study of 532 nm dyes to suggest a possible trap-induced photobleaching mechanism that agrees with the accepted mechanism of natural photobleaching, in which the absorption of a near infrared trap photon populates a reactive triplet state through intersystem crossing from the fluorescent singlet state. This mechanism is further supported by observations consistent with reverse intersystem crossing to drive fluorophores back to a bright state, seen in different switching configurations of the fluorescence excitation and trapping lasers.

MATERIALS AND METHODS

Instrumentation

In this work, we employed instrumentation developed to combine optical tweezers force microscopy and single molecule fluorescence spectroscopy in a combined, coincident arrangement. The optical layout and instrument specifications are detailed elsewhere [7]. Briefly, the optical trap is formed on a stabilized inverted microscope outfitted with a high numerical aperture objective (100X, 1.45 NA; Nikon) that tightly focuses a continuous wave 1064 nm laser (Coherent, Santa Clara, CA). Before entering the microscope, this laser is transits a pair of orthogonally oriented acousto-optic deflectors (AODs; IntraAction, Bellwood, IL), which provide fast and precise switching and steering in the specimen plane. Coincident with the trapping laser, fluorescence excitation is introduced in an objective-side total internal reflection fluorescence (TIRF) arrangement. TIRF excitation is controlled by a single independent AOD and, depending on the fluorescent dye, provided by either a 488 nm argon-ion laser (MWK Industries), a 532 nm diode laser (World Star Tech, Toronto, ON), or a 635 nm diode laser (World Star Tech). Furthermore, appropriate filter cubes consisting of a dichroic mirror and a long-pass emitter filter (Chroma, VT) are located below the objective both to spatially steer excitation light and spectrally filter fluorescence emission. Fluorescence emission is finally spatially isolated through a 300 nm pinhole that is imaged on a photon-counting silicon avalanche photodiode (SAPD, Perkin Elmer).

The temporal modulation of the trapping and excitation lasers is performed as described previously [7], but instead of a function generator, a field programmable gate array board (FPGA; Xilinx, San Jose, CA) is used to generate the modulated square waves for the two AOD controls. Our studies paralleled conditions typically used in a measurement with respect to power and timing comparing our out of phase, interlaced method vs. non interlaced conditions. We note there are many additional timing and power scenarios that would be interesting to study but are beyond the scope of this project. With custom programming of the FPGA, the frequency of both square waves is set to 50 kHz and the duty cycle of the trapping and excitation lasers is adjusted to 50% (10 μs FWHM) and 30% (6 μs FWHM) respectively. The phase delay between the two square waves is also controlled with the FPGA in order to irradiate the specimen with the trapping and excitation lasers in either an alternating out-of-phase timing or a simultaneous in-phase arrangement. In the out-of-phase (OP) condition, the phase delay was adjusted for a 2 μs dark period between the pulses, while for the in-phase (IP) condition, the phase delay was shifted by 180° to cause coincident illumination by the fluorescence and trapping pulses. The post-modulation, time-averaged power was set to 100 mW and 250 μW for the trapping and excitation lasers, respectively, in order to approximate the irradiation conditions for a typical single molecule experiment.

Sample Preparation

Carboxylated beads (100 nm, Polysciences, Inc., Warrington, PA) were covalently biotinylated and then incubated at 4°C with an excess of fluorophore-labeled streptavidin in pH 7.4 phosphate buffer. Unbound streptavidin was removed by centrifugation, and resuspended beads were diluted 1:100 and non-specifically immobilized on a glass coverslip surface. After immobilization, the phosphate buffer was exchanged with deoxygenated fluorescence buffer (20 mM Tris, pH 7.5, 6 mM NaCl, 1.7 mM $MgCl_2$, 10% glycerol, 120 nM catalase, 25 mM β-D(+) glucose, 1.8 μM glucose oxidase, 1% β-mercapto-ethanol, degassed for 30 minutes under vacuum) and flow cells were sealed with epoxy. For experiments using GFP, phosphate buffer was used in place of fluorescence buffer, as the former negatively affected longevity on this fluorophore.

Photobleaching Assay

On the microscope, each slide was visually scanned for immobilized beads on the coverslip surface. An individual fluorophore-coated bead was isolated and precisely centered in 3D using our detection sub-system at the predetermined location corresponding to the pinhole aperture. The bead was exposed to either IP or OP irradiation conditions and its fluorescence emission was recorded for 5 minutes. Control beads were similarly exposed to the fluorescence excitation laser with no trap, which we term NT. In each case, the fluorescence emission was recorded and averaged over 8-12 different beads. These signals were sampled on a 16-bit A/D board, acquired at 20 Hz using custom software (LabView, National Instruments), and analyzed with MATLAB (MathWorks, Natick, MA) and Origin (OriginLab Corp, North Hampton, MA).

RESULTS AND DISCUSSION

Our first experiments aimed both to quantify the natural photobleaching rate of a range of common fluorescent dyes and to relate this phenomenon to the effects of trap-induced photobleaching. Several dyes were chosen at three different wavelengths: 488 nm, 532 nm, and 635 nm, corresponding to available laser sources and commonly used excitation wavelengths. Measurements were performed on relatively small, 100 nm beads to ensure uniformity of the optical trapping and fluorescence excitation fields. In addition, construction of the assay on small beads reduced surface effects and permitted isolation of consistent quantities of dye per measurement.

In order to measure the natural photobleaching rate of each dye, NT decay curves were first measured in the absence of the trap, and integrated for comparison. These measurements, tabulated in Table **1** and shown in Fig. (**1**), give a baseline indication of the quality of each fluorescent dye. For example, the blue dyes, Alexa 488 and GFP, emitted for the least amount of time and had the smallest integrated emission. This quantity, which is an intrinsic function of the molecular structure, is an important contributor to the limited use of these dyes in single molecule experiments. For the same reason, the dyes with the largest integrated emission, Cy3, TMR, and Cy5, are the most commonly used fluorophores for single molecule studies.

With a NT baseline measure of each dye's intrinsic photostability, we tested the effects of trap-induced photobleaching with the IP modulation condition using simultaneous irradiation with fluorescence excitation and optical trapping lasers. To facilitate comparison between conditions and remove any photobleaching contribution from the natural decay rate, the IP integration of each dye was normalized to the NT integration. These results, presented in Table **1** and shown in Fig. (**2**), indicate that trap-induced photobleaching is most significant at longer wavelengths, with the greatest effects on Cy3 at 532 nm. Furthermore, trap-induced photobleaching is strongest in three of the four dyes with the longest intrinsic photobleaching rate. This correlation has important implications for the combination of single molecule fluorescence and optical tweezers force microscopy, as this technique requires long-lived fluorophores for practical applications.

Previous IOFF experiments explored the feasibility of combining these two techniques by alternating fluorescence and trapping laser with the most susceptible dyes, Cy3 and Alexa 647 in a FRET configuration [21]. This case, which we term OP, effectively removes the photodecay that is promoted by the near infrared trap. As shown in Fig. (**2**), IOFF

Table 1. The Relative Intrinsic Photobleaching of Common Single Molecule Fluorescence Dyes and the Effects of Optical Trap-induced photobleaching. Dye-coated beads were exposed to fluorescence excitation alone (NT); simultaneous, in-phase fluorescence excitation and optical trapping radiation (IP); and alternating out of phase fluorescence excitation and optical trapping radiation (OP). The most commonly used single molecule dyes, Cy3 and Cy5, are some of the most susceptible to trap-induced photobleaching, though they also show the most recovery in the OP condition

Excitation Wavelength (nm)	Fluorophore	Modulation Condition	Integrated Fluorescence Emission	Normalized Integrated Emission
488	GFP	NT	8.392	1.000
		IP	10.815	1.289
		OP	14.309	1.705
	Alexa 488	NT	15.798	1.000
		IP	15.748	0.997
		OP	21.534	1.363
	Fluorescein	NT	31.182	1.000
		IP	33.328	1.069
		OP	32.933	1.056
	Alexa 532	NT	32.949	1.000
		IP	18.539	0.563
		OP	62.405	1.894
532	Alexa 555	NT	30.226	1.000
		IP	5.485	0.181
		OP	17.208	0.569
	Cy3	NT	38.687	1.000
		IP	2.568	0.066
		OP	42.742	1.105
	TMR	NT	67.724	1.000
		IP	29.713	0.439
		OP	57.916	0.855
635	Cy5	NT	43.911	1.000
		IP	6.958	0.158
		OP	57.182	1.302
	Alexa 647	NT	53.991	1.000
		IP	6.275	0.116
		OP	30.590	0.567

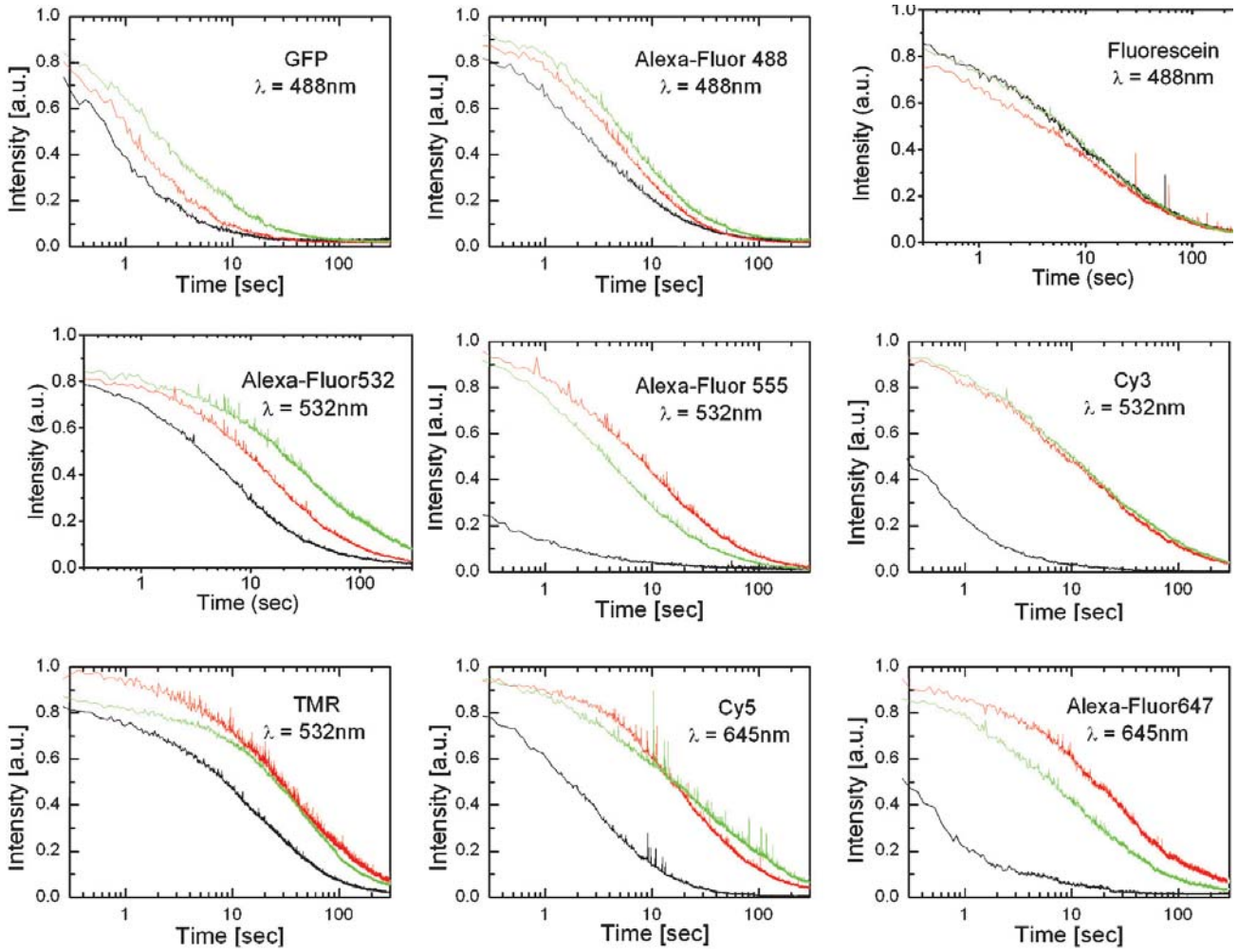

Fig. (1). Photodecay curves for intrinsic photobleaching rate (red), in-phase photobleaching (IP, black), and out-of-phase recovery (OP, green). Each curve was integrated for comparison of natural photobleaching, and integrations were normalized to NT results for quantification of the effects of trap-induced photobleaching. In most cases, OP removes the effects of optical trap radiation, though the cases where OP improves even NT give insight into the electronic mechanism.

actually extends the photobleaching rate beyond that of the NT condition for several dyes, including Cy3 and Cy5, as well as Alexa 488, Alexa 532, and GFP. These improvements, which range from 2-17 times the IP fluorescence decay, are coincidentally most pronounced in Cy3 and Cy5, the most commonly used single molecule fluorophores.

While these results show the effectiveness of IOFF for relieving trap-induced photobleaching, they also provide a novel perspective of its photoelectronic mechanism. If IOFF was simply removing the additional photodestructive state, the OP condition would be expected to return the integrated emission to that of the NT condition. Several dyes, however, show greater integrated emission for the OP than the NT state. We further explored this effect by manipulating buffer components and dynamically switching between IOFF conditions to show consistency with a proposed mechanism for trap-induced photobleaching.

Beginning with the OP condition, we explored the importance of oxygen in the fluorescence buffer. As shown for Cy3 in Fig. (**3a**), the switching between OP and NT conditions in presence of an oxygen scavenger system results in an increase in fluorescence emission when the trap is introduced. However, when oxygen is reintroduced to the system, as in Fig. (**3b**), the photobleaching rate is increased and the OP improvement is lost. This effect, which was observed for all tested 532 nm dyes, suggests that photobleaching is dependent on reaction with oxygen but not on two-photon absorption, where one photon is 1064 nm and the second 532 nm, which is impossible in the OP arrangement. Though prone to ionization, the singlet state is not reactive, suggesting instead that it is likely that the population of a triplet state is critical to the photobleaching mechanism.

The role of the triplet state is further clarified by switching between the IP and NT conditions, shown for Cy3 in Fig. (**3c**). In this transition, a slow fluorescence recovery is observed in the absence of the near infrared trapping laser. If trap-induced photobleaching is due to the sequential absorption of photons, the intensity transition between IP and NT conditions would happen on a timescale comparable to the nanoseconds lifetime of the fluorescent state. Instead, this transition happens over several seconds, which is more char-

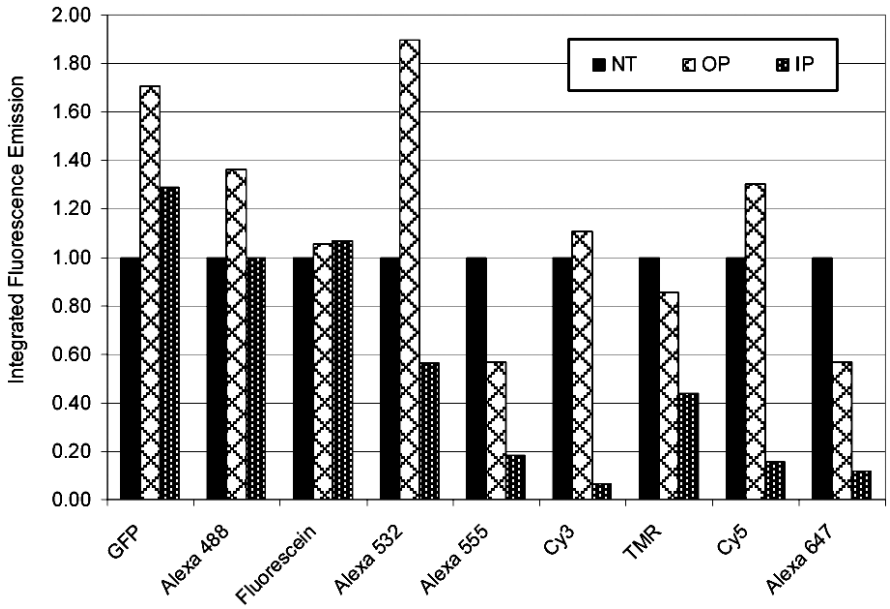

Fig. (2). Comparison of the integrated photobleaching for commonly used fluorescent dyes. Integrated emission was normalized to the natural emission (NT) to compare the effects of OP and IP radiation conditions on the different fluorophores.

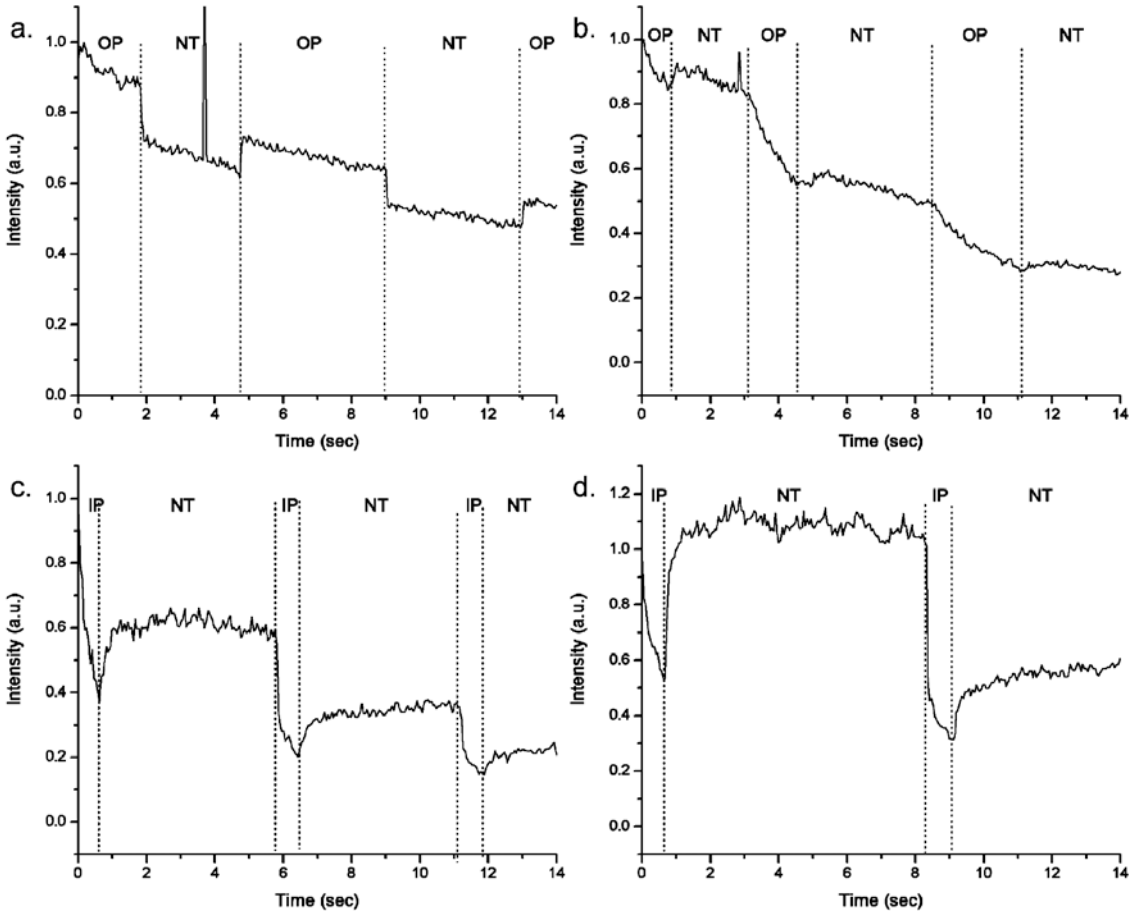

Fig. (3). Fluorescence recovery upon switching between IOFF and trapping conditions: **(a)** OP and NT switching in the presence of oxygen scavenger system; **(b)** OP and NT switching without oxygen scavengers; **(c)** IP and NT switching in the presence of oxygen scavenger system; **(d)** IP and NT switching without oxygen scavengers. The importance of the slowly decaying triplet state is evidenced by the slow recoveries in **(c)** and **(d)**, while the increased intensity in **(a)** indicates the role of reverse intersystem crossing, in which the fluorescent singlet state is repopulated from the dark triplet state by low energy trapping photons.

acteristic of phosphorescence, accessible through population of the triplet state. This rate of transition, found in all tested 532 nm dyes, suggests that trap-induced photobleaching may be due to population of a dark triplet state. This spin state transition, which is classically forbidden, is enhanced by near infrared radiation resulting in a high population of phosphorescent states. When oxygen is returned to the system, as shown for Cy3 in Fig. (**3d**), this recovery is reduced, as the detrimental triplet state reaction with dissolved oxygen increases in likelihood.

These results help clarify the mechanism of both natural and trap-induced photobleaching. Unlike previous hypotheses that such phenomena are irreversibly destructive, our results show that trap-induced photobleaching is a reversible mechanism that results from the population of a dark triplet state. When oxygen is incorporated in surrounding medium, this triplet state can irreversibly react to destroy the photoelectric pathway, though the removal of oxygen helps increase the probability of repopulation of the fluorescent singlet state. This scenario allows a refinement of the photoelectric mechanism. Relating our IOFF results into this mechanism, IP radiation populates the slowly decaying triplet state. Once populated, this state can phosphorescently decay or return to the fluorescent singlet state through reverse intersystem crossing [22]. While it is possible that some triplet state population decays through phosphorescence and is then re-excited by the fluorescence laser, the rapid recovery under OP conditions suggests that the trapping laser actively promotes intersystem crossing.

This system has several different implications for the design of fluorescence experiments, both at the bulk and single molecule levels, where reduction of photodecay is critical. As is common practice, the removal of dissolved oxygen is important for the prevention of triplet state reaction. However, near infrared radiation in an OP configuration can be used to prolong fluorescence emission timescales and improve experimental design. This approach, which was initially designed to facilitate a combination of optical trapping and single molecule fluorescence, can be used in a range of experiments to improve fluorescence emission.

CONCLUSION

In this work, we quantified the effects of trap-induced photobleaching on a range of commonly used fluorescent dyes and related this phenomenon back to the intrinsic rate of fluorescence decay. We further demonstrate an improvement in fluorescence emission that can be realized with the IOFF laser manipulation technique. The use of this approach, in which optical trapping and fluorescence excitation lasers are alternately modulated, can improve fluorescence emission properties, in some cases beyond the intrinsic decay rate. This approach can also be used to bring a new perspective to the photodecay mechanism that characterizes both natural and trap-induced photobleaching. Our results, realized by switching between alternating and simultaneous trap and fluorescence illumination and combined with different oxygenation conditions, suggests that trap-induced photobleaching may be due to the reversible population of a dark triplet state. In the presence of oxygen, this triplet state can irreversibly react to destroy the electronic structure, while in the absence of oxygen, this state can return to the fluorescent singlet state through intersystem crossing. In the presence of a lower energy trapping laser, such intersystem crossing can be induced, resulting in a higher than normal population of the fluorescent singlet state. When applied across a range of fluorescent dyes, this approach can be used with varying degrees of effectiveness to increase fluorescence longevity, but greater electronic structure information can aid in the best choice of excitation and enhancement wavelengths.

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