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Intra-molecular Triplet Energy Transfer is a General Approach to Improve Organic Fluorophore Photostability

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Bright, long-lasting and non-phototoxic organic fluorophores are essential to the continued advancement of biological imaging. Traditional approaches towards achieving photostability, such as the removal of molecular oxygen and the use of small-molecule additives in solution, suffer from potentially toxic side effects, particularly in the context of living cells. The direct conjugation of small-molecule triplet state quenchers, such as cyclooctatetraene (COT), to organic fluorophores has the potential to bypass these issues by restoring reactive fluorophore triplet states to the ground state through intra-molecular triplet energy transfer. Such methods have enabled marked improvement in cyanine fluorophore photostability spanning the visible spectrum. However, the generality of this strategy to chemically and structurally diverse fluorophore species has yet to be examined. Here, we show that the proximal linkage of COT increases the photon yield of a diverse range of organic fluorophores widely used in biological imaging applications, demonstrating that the intra-molecular triplet energy transfer mechanism is a potentially general approach for improving organic fluorophore performance and photostability.

Introduction

Single-molecule and super-resolution fluorescence imaging techniques have yielded unprecedented breakthroughs in a diverse range of biological systems and shed new light on cellular structure and organization.[1, 2] Such methods rely critically on the availability and performance of bright fluorescent probes spanning the visible and near infrared spectrum (400 – 800 nm) that exhibit high brightness and photostabilities. However, the inherent photo-chemical and photo-physical instabilities and related photo-toxicities of fluorescent species remain limiting features of most imaging applications.[3-6]

Fluorophore photo-instabilities, which manifest as intermittent dark states and irreversible photobleaching, compromise imaging duration, signal-to-noise ratios (SNR) and the spatial and temporal resolution that can be achieved.[7-9] Phototoxicity, which arises from the generation of reactive oxygen species through excitation of a fluorophore, can perturb the system under investigation.[3-5] Experimental control of these factors is particularly challenging in complex biological settings where the fluorophore's physical environment may be confined or changing over time. As a consequence of these limitations, many biological systems and questions remain beyond the reach of imaging methods that require the maximization of finite photon budgets or a minimization of time, such as single-molecule or super-resolution imaging.[6, 7, 10] Hence, a general approach to increase fluorophore photostability would enable these powerful imaging modalities to open new biological frontiers and have the potential impact of broadly advancing the boundaries of numerous fluorescence applications.

The most commonly employed strategies to improve organic fluorophore photostability require the removal of molecular oxygen.[11, 12] and the addition of small-molecule protective agents, such as cyclooctatetraene (COT),[13] nitrobenzylalcohol (NBA),[14] Trolox (TX),[15, 16] and a combination of reducing and oxidizing chemicals (ROXS).[17] While such methods have a proven capacity to increase fluorophore performance in a range of biological settings, they are restricted by their fluorophore-specific impacts, the solubility limits of extant protective agents (ca. 1 mM), and their potential to exhibit biological toxicities,[18] particularly in live-cells.[19]

It has been recently shown that cyanine-class organic fluorophores can be intra-molecularly photostabilized by the covalent attachment of a single COT, NBA, or TX molecule in proximity of the fluorogenic center.[20-22] Mechanistic studies have since revealed that Trolox and NBA, like other small-molecules employed for ROXS,[17] operate by redox chemistries on fluorophores trapped in relatively long-lived, non-fluorescent excited states.[22-25] Such mechanisms, which necessarily entail the generation of charged intermediates, are strongly dependent on the fluorophores employed and the

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environment setting. Protective agents operating *via* electron transfer also have the potential to quench singlet excited states,[26] thus reducing fluorophore brightness by reducing their effective fluorescence quantum yield. By contrast, COT operates through a triplet-triplet energy transfer mechanism that recovers the fluorophore from triplet states without charge separation.[9, 27, 28] As most organic fluorophores that absorb in the visible spectrum have triplet state energies higher than that of COT, we hypothesized that the proximal attachment of COT may provide a potentially general approach to improve fluorophore photostability. Here, we show that the proximal attachment of a single COT molecule improved the performance of a broad range of chemically distinct organic fluorophores, suggesting that intra-molecular triplet energy transfer is a potentially general strategy for improving organic fluorophore photostability.

Result and Discussion

Spontaneous energy transfer can occur when the excited-state energy of a protective agent is lower than that of a fluorophore.[26] COT possesses a low-energy ("relaxed") triplet state due to its potential for extensive geometry relaxation and triplet state aromaticity,[29-32] which makes it possible to quench triplet states above 0.8 eV in energy.[33] This is substantially lower than the triplet state energies of most commonly used organic fluorophores employed for biological imaging applications (ca. 1.1-2.1 eV[26]). Importantly, the energy of COT's first singlet excited state (S1, ca. 4.4 eV[34]) is markedly higher than the S1 energy of organic fluorophores (ca. 1.6-3.1 eV).[30] Thus, COT imparts little to no impact on fluorophore brightness. These characteristics render COT a promising, general triplet state quencher candidate for improving the performance of organic fluorophores commonly used in biological imaging applications. Based on this rationale, and direct evidence that COT mediates intramolecular photostabilization for certain cyanine dyes spanning the visible spectrum,[21] we examined whether COT can mediate intramolecular photostabilization of the following chemically and structurally diverse organic fluorophores: Cyanines (Cy3B, Cy5, DY654); Carbopyronines (ATTO647N); Oxazines (ATTO655); Rhodamines (TMR, ATTO565, Alexa633, Alexa568); and Fluoresceins (fluorescein) (Fig. 1).

In order to enforce the proximity of COT to each of these fluorophore species, while bypassing fluorophore-specific synthesis challenges and potentially negative impacts on fluorophore solubility, N-hydroxysuccinimide (NHS)-activated COT and fluorophore molecule were linked to the 3'- and 5'-ends, respectively, of complementary strands of a short, double-stranded DNA oligonucleotides 21-base pairs in length (Scheme 1). Thus positioned, collisional interactions are expected to occur with a high frequency. [20] The performance of each fluorophore was examined in this context at the single-molecule scale by tethering the opposite end of the DNA duplex to a passivated-quartz, microfluidic device *via* a biotin-streptavidin interaction such that fluorophore excitation and emission performance could be examined under solution conditions using a prism-based, wide-field total-internal-reflection fluorescence (TIRF) microscope.[20]

Absolute and comparative fluorophore performance evaluations were based on the number of photons detected from individual fluorophores per unit time (brightness), the variance in the fluorescence signal detected (SNR = (average of fluorescence intensity)/(standard deviation of fluorescence intensity)) as well as the total number of photon detected (photon counts) prior to photo-induced degradation and signal loss (Fig. 2). Each fluorophore was also examined in the same context without proximally attached COT and with 1 mM COT in solution. All experiments were performed in a buffered saline solution (50 mM Tris-Acetate, 50 mM KCl) supplemented with an enzymatic oxygen scavenging system (see Experimental section) to remove oxygen from the buffer.

To quantify the effectiveness of the proximal COT molecule in this context, we benchmarked its impact for specific cyanine fluorophores, where beneficial impacts have been previously documented.[20-22] As anticipated from these investigations, the photostability of both Cy5 and Cy3B were significantly improved. The Cy5 fluorophore exhibited a 50-fold increase in total photon count, a 2-fold increase in brightness, and a 2.5-fold increase in SNR. As expected, these effects were more pronounced than when 1 mM COT was present in solution (Fig. 2).[14, 21] The impact of proximally linked COT was significant, albeit less pronounced, for Cy3B (~4 fold increase in SNR and 20% increase in brightness and photon counts), whereas Cy3B was strongly photostabilized (~6-fold increase in photon counts) with 1 mM COT in solution (Fig. 2). Analogous impacts were observed for DY654, also a cyanine-class fluorophore. The reason why Cy3B and DY654 are less responsive to proximally linked COT is not presently clear. We speculate, however, that this observation may relate to differences in the probability of effective collisions between COT and the Cy3B/DY654 fluorophore when proximally conjugated.

We next tested the impact of proximally linked COT for representative fluorophores of distinct structural families. Here, we observed that the photostability of each of the fluorophores examined, with the exception of ATTO655 and fluorescein, was enhanced by a proximally linked COT molecule (Fig. 2). For ATTO647N, a fluorophore widely used for single-molecule and super-resolution measurements,[35-38] proximal COT provided a 120-fold increase in photon count, a 4-fold increase in brightness, and a 2.5-fold increase in SNR (Fig. 2 and 3). A substantial, albeit relatively modest impact, was also observed with COT in solution (Fig. 2 and 3). Analogous impacts were observed for ATTO565 (Fig. 2 and 3). The proximally linked COT molecule also improved the photostability of Alexa568, Alexa633, and TMR. Here, the impacts were, however, more modest in nature, exhibiting ~4-8 fold increases in photon counts and in SNRs. These specific fluorophores exhibited more pronounced increases in photostability when 1 mM COT was present in solution (Fig. 2).

We therefore conclude that the photostability of most organic fluorophores can be markedly improved by a single, proximally linked COT molecule and that the precise extent of the observed enhancement varies dramatically for each

fluorophore species. These fluorophore-specific distinctions may be attributed to differences in triplet energy transfer rate and/or differences in the competing photochemical processes, such as redox reactions from the triplet excited states. Under the conditions of the present experiments, redox partners include an enzymatic oxygen scavenging system as well as the oligonucleotides to which the fluorophore is attached. To investigate why COT exhibited distinct impacts on fluorophore photostabilities when proximally linked in a manner such that relatively uniform collision frequencies are enforced, the redox potentials of each fluorophore were measured using cyclic voltammetry.[39] Here, we hypothesized that a fluorophore's responsiveness to COT reflects differences in the rates of competing chemical pathways that generate radical fluorophore species which COT is unable to rescue. According to the Marcus theory, the rate of electron transfer in the normal region increases with the thermodynamic driving force of the reaction.[40] Here, the responsiveness of a fluorophore to COT is expected to be lowered when the rate of gaining or losing electrons shortens the triplet state lifetime[9, 41] such that redox reactions are faster than the rate of triplet energy transfer with COT.

The reduction potentials of the triplet state for each fluorophore were estimated using the Rehm-Weller equation: $E_{red}^{T} = eE_{red}^{0} + E_T + C$, where E_{red}^{0} is the first one-electron reduction potential of the ground state obtained by cyclic voltammetry (Fig. 4, table 1), E_T is the triplet state energy of the fluorophore obtained by phosphorescence (Fig. 5, table 1), and e is the unit charge.[26] In aqueous solutions, the coulombic attraction energy *C* can be neglected due to the high polarity of water.[26] Consistent with the notion that fast electron transfer between the triplet fluorophore and its environment competes with triplet energy transfer to COT, both fluorescein and ATTO655, the two fluorophores for which COT had a negligible impact, exhibited the highest E_{red}^{T} (Table 1). By contrast, the fluorophores improved by COT tended to exhibit lower E_{red}^{T}, consistent with the notion that a fluorophore's responsiveness to COT-mediated intra-molecular triplet state quenching is determined by the rates of competing redox reactions from the triplet state. The absence of direct correlations between E_{red}^{T} and COT responsiveness is not presently known but may relate to other factors affecting the rates of charge transfer, such as the frequency of productive collisions between the fluorophore and COT, as well as the reorganization energies in these structurally distinct fluorophores.[40]

Conclusions

The present observations demonstrate that photostabilization through intra-molecular triplet energy transfer is a potentially general approach for improving the experimental performance of organic fluorophores of various structures in biological settings. We observe, however, that the effectiveness of this approach is strongly dependent on the nature of the fluorophore. Our data suggest that competing electron transfer processes, which are highly fluorophore specific, may be the explanation for the observed differences in effectiveness. To obtain increased photostability, the relative rates of intra-molecular triplet energy transfer need to be substantially faster than the rates of electron transfer from/to the triplet state of the fluorophore. The rate of intra-molecular triplet energy transfer when sufficiently rapid has the potential to shield the fluorophore from redox damage even in the presence of highly reactive species such as molecular oxygen, which is present at nearly millimolar concentrations in aqueous solutions. These observations rationalize future efforts aimed at developing general synthetic strategies for linking COT, or other compounds capable of intra-molecular triplet energy transfer, directly to a range of organic fluorophores where their performance can be examined in diverse imaging contexts. For example, a fluorophore may be first coupled to an "adapter molecule" where a COT is covalently linked, before being labelled to the biomolecule of interest. Here it will be particularly impactful to examine whether such methods can be extended to organic fluorophore species commonly employed for live-cell imaging, such as TMR and the near-IR emitting silicon-rhodamine (SiR),[6, 42, 43] where fluorophore performance in complex cellular milieus are particularly demanding and prone to exhibit undesirable phototoxicities.

Experimental

Materials. N-hydroxysuccinimide (NHS) ester-activated fluorophores were used without further purification. The structures of the fluorophores were obtained from previous publications.[44, 45] The structures of the linkers to the NHS reactive group were deduced from the molecular weight provided by the vender. 3,4-dihydroxybenzoic acid (PCA) was purchased from Sigma-Aldrich and used without further purification. 100 mM PCA solution in water was made and the pH was adjusted to 9 with potassium hydroxide. Protocatechuate 3,4-deoxygenase (PCD) was purchased from Sigma-Aldrich and purified with gel-filtration chromatography (HiLoad 16/60 Superdex 200 prep grade) to collect the fraction of 700kDa.

Generation of fluorophore- and COT-labeled DNA duplexes. The fluorophore-labelled DNA oligonucleotides were generated as previously described.[20] To label COT to DNA, NHS derivatives of COT were synthesized[20] and reacted with a complementary strand with a C6-amino thymine at the 3'end (CTGGTCATGGTCATGGTCATG/3AmMC6T/, Integrated DNA Technologies, Inc.). The two strands were hybridized and purified as previously described.[20]

Single-molecule fluorescence microscopy. Single-molecule fluorescence measurements were performed using a wide-field, prism-based total internal reflection fluorescence (TIRF) microscope as previously described.[20, 22] ATTO647N, Alexa633, DY654, ATTO655, and Cy5 were excited with a Coherent Genesis MX639-1000 STM laser (639 nm, ca. 150 W/cm^2). Alexa568, TMR, ATTO565, and Cy3B were excited with a Laser Quantum 532 Opus laser (ca. 200 W/cm^2). Fluorescein was imaged with a Laser Quantum 473 nm Ciel laser (ca. 150

W/cm^2).

Data were analyzed using automated software built in-house using Matlab (MathWorks) as previously described.[14, 20] In summary, traces were extracted from wide-field TIRF movies by finding peaks of fluorescence intensity, and were idealized and fitted to obtain the mean of total photon counts, brightness, and SNR for molecules (>500) of each movie. The statistics from 3 movies were then calculated and are reported in Fig. 2.

Phosphorescence measurements. Solutions of the fluorescence dyes in ethanol/iodomethane (2:1, v/v) in 3 mm quartz tubes (inner diameter) were frozen at 77 K in a optical liquid N_2 quartz dewar. Iodomethane was added to increase intersystem crossing into the triplet state. The frozen samples inside the quartz dewar were excited with a pulsed Spectra Physics GCR-150-30 Nd:YAG laser (532 nm, ca. 1 mJ/pulse, 7 ns pulse length) or excited with chopped (20 Hz) cw lasers at 473 nm and 633 nm. The time-resolved phosphorescence spectra at 1 ms after pulsed excitation and a gate width of 2 ms were recorded on an Acton Spectrograph (SpectraPro-2150) in conjunction with an intensified CCD detector (PI-MAX from Princeton Instruments) with fiber optics attachment.

Reduction and oxidation potential measurements. Cyclic voltammetry was performed in DMSO using glassy carbon electrodes against an Ag/AgCl reference electrode and a platinum wire as a counter electrode at a scan rate of 100 mV/s, with 0.1 M tetrabutylammonium perchlorate as the electrolyte. Prior to measurement, the solution was purged with nitrogen gas for 15 min. All electrochemical measurements were made with a Hokuto Denko HSV-100 potentiostat. To test whether solvent significantly affects the reduction/oxidation (redox) potential of fluorophores, a subset of fluorophores (Cy5, ATTO655, Alexa633, and fluorescein) was measured with water or water/DMSO mixture as solvent. No significant change of redox potential was detected.

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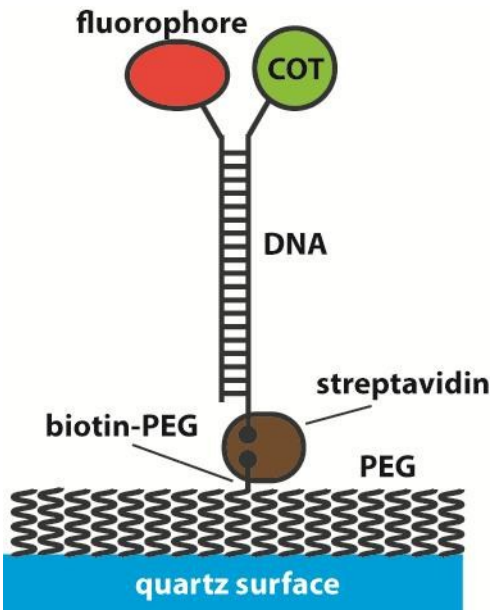

Scheme 1. Assembly of fluorophores on quartz surfaces in close proximity to COT using DNA base pairing and biotin-streptavidin binding.

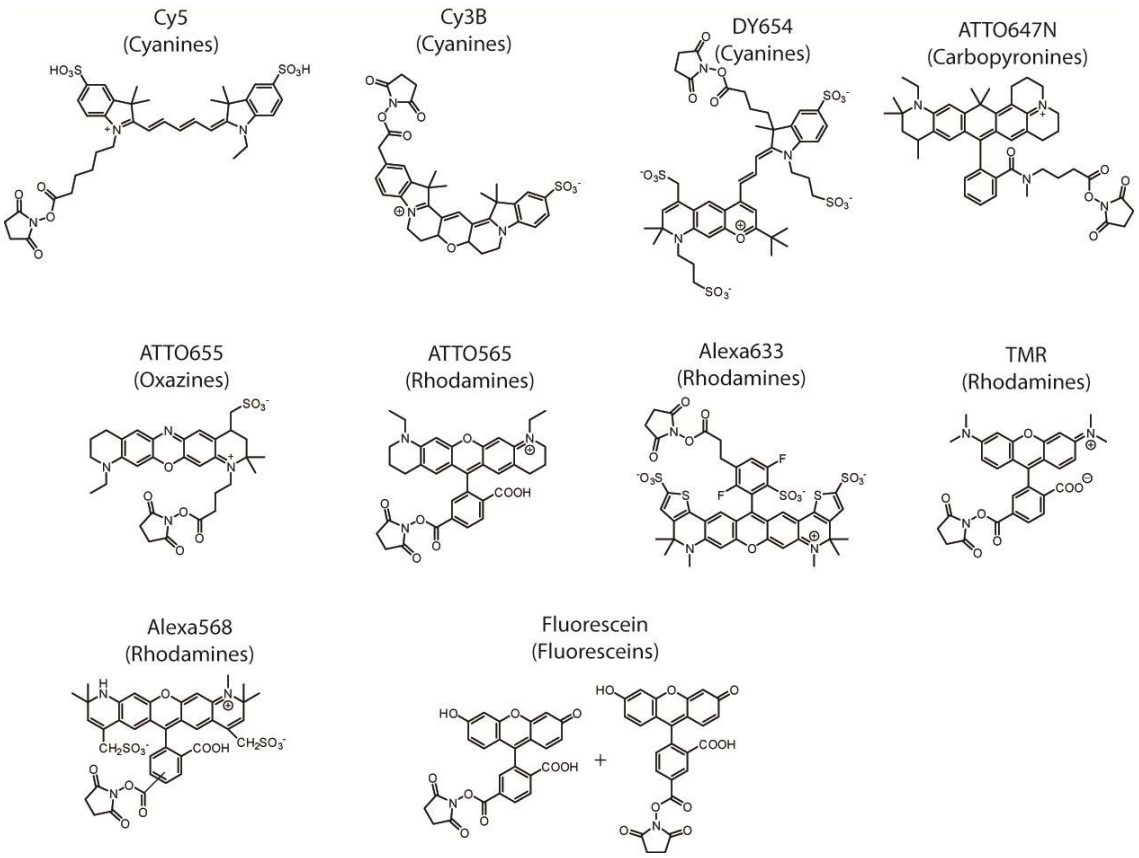

Figure 1: Chemical structures of the fluorophores used in this study.

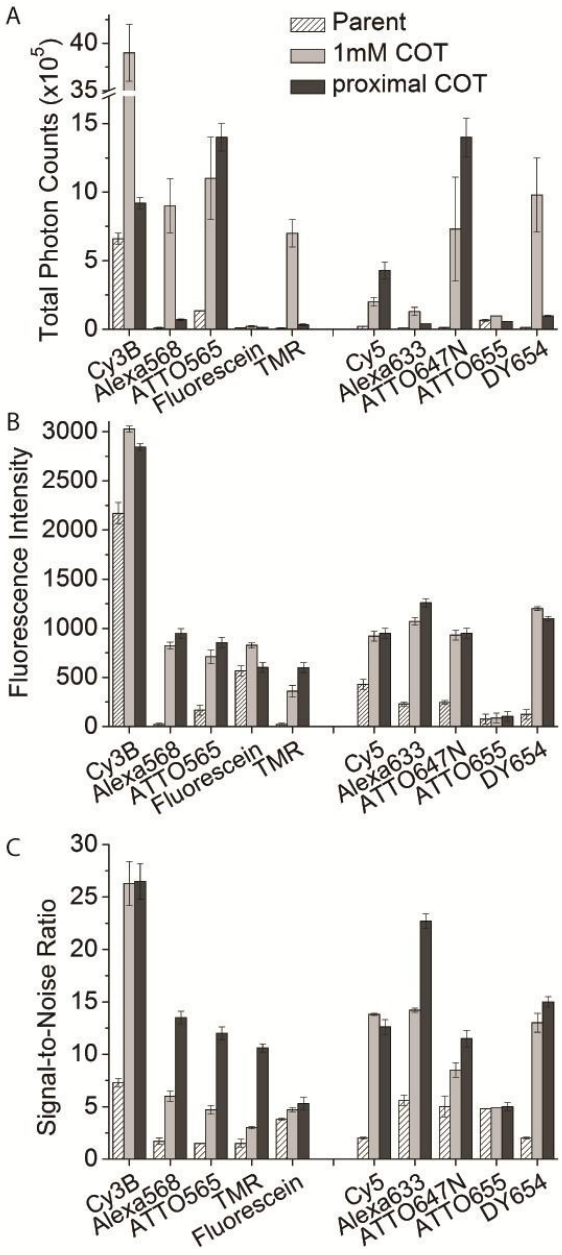

Figure 2. (A) Total photon counts, (B) number of photons detected from individual fluorophores per unit time (Brightness), and (C) signal-to-noise ratio of different fluorophores in deoxygenated buffer (hatch bars), in deoxygenated buffer with 1mM COT (grey bars), or in deoxygenated buffer with a single proximal COT (black bars). Error bars indicate standard deviation of three measurements.

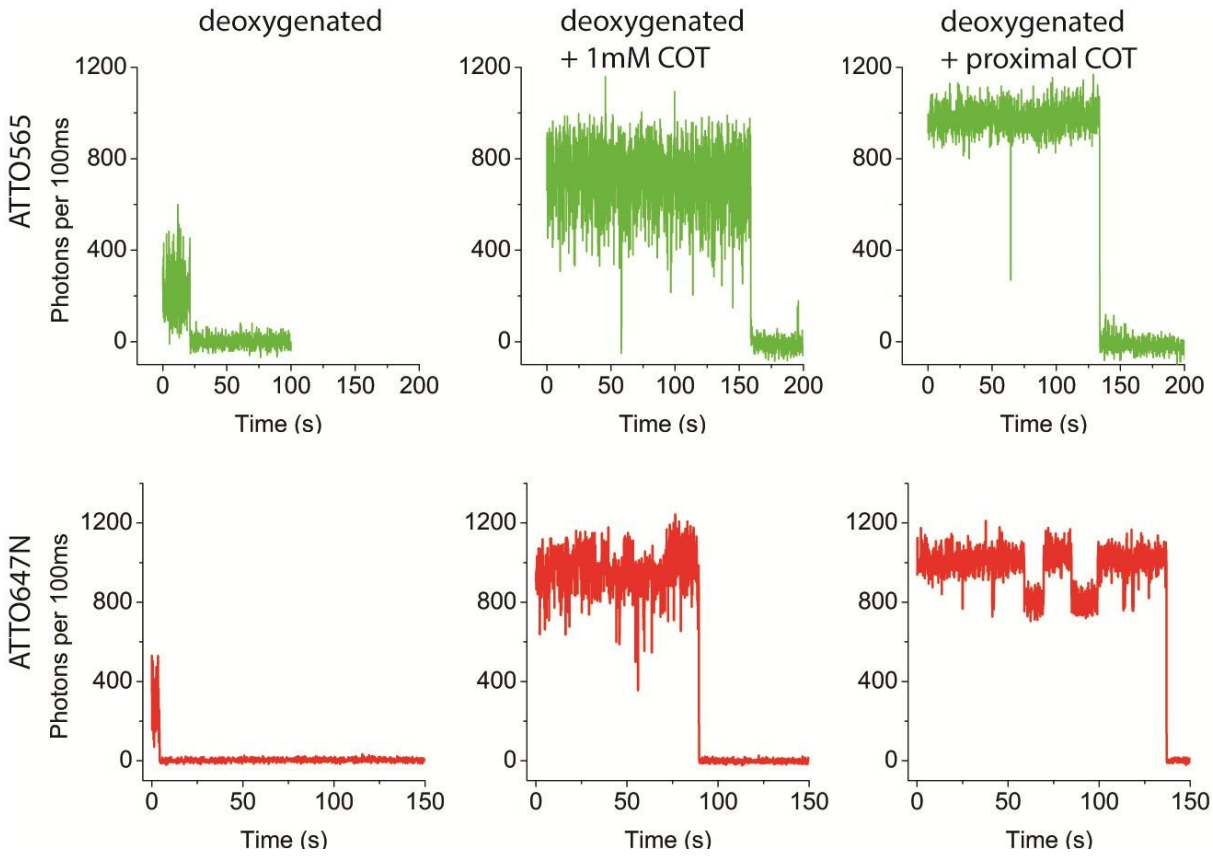

Figure 3. Representative single-molecule fluorescence traces for ATTO565 and ATTO647N in deoxygenated buffer, in deoxygenated buffer with 1mM COT, or in deoxygenated buffer with proximal COT.

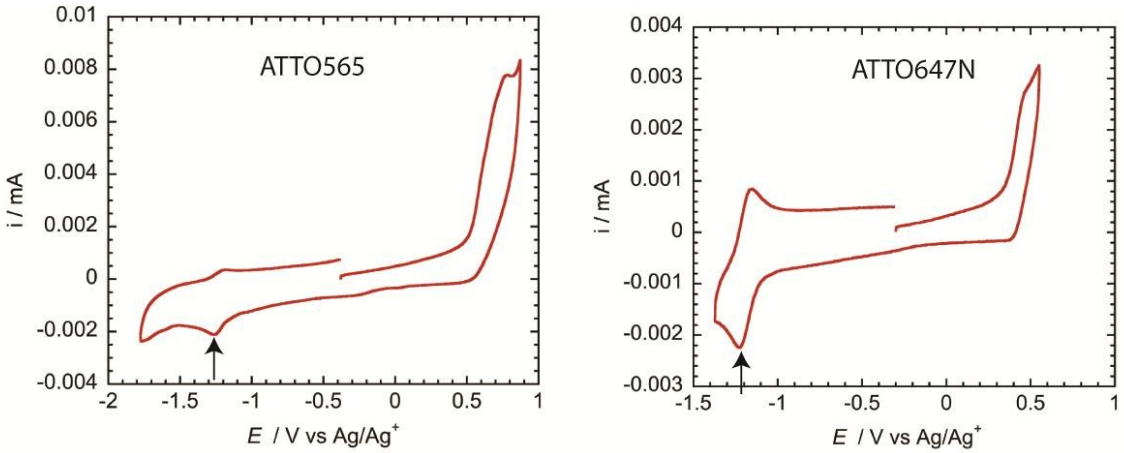

Figure 4: Representative cyclic voltammograms for ATTO565 (left) and ATTO647N (right). The reduction peak potentials are indicated by the arrows.

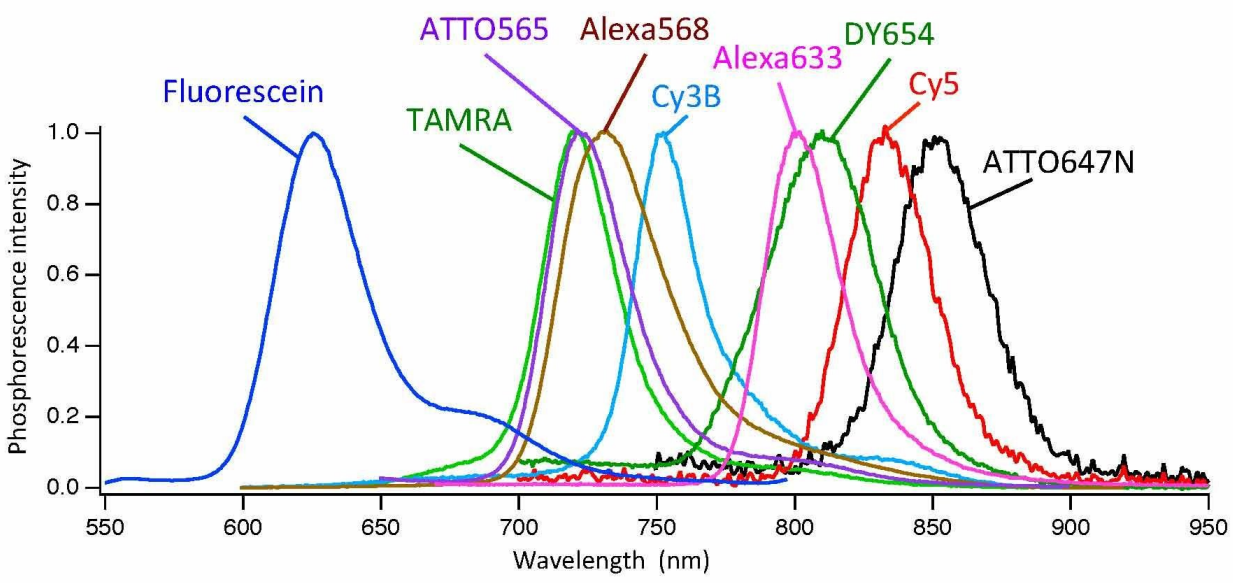

Figure 5: Phosphorescence spectra of the fluorophores in ethanol/iodomethane glass (2:1, v/v) at 77 K with excitation at 473 nm (fluorescein), 532 nm (TAMRA, ATTO565, Alexa568, Cy3B) or 633 (Alexa633, DY654, Cy5, ATTO647N). The wavelengths of each phosphorescence maxima were converted to the triplet state energy (E_T) of the fluorophores.

Fluorophore	TMR	ATTO647N	Cy5	Cy3B	Alexa633	ATTO565	Fluore-scein	ATTO655
E_T (eV)	1.72	1.46	1.48	1.66	1.55	1.72	1.98	1.6*
E^0_{red} (V vs Ag/Ag+)	-1.58	-1.23	-1.24	-1.40	-1.175	-1.25	-1.40	-0.635
E^T_{red} (eV)	0.14	0.23	0.24	0.26	0.38	0.47	0.58	1.0

Table 1. Triplet state energy, reduction potential from the ground state, and reduction potential from the triplet state for the fluorophores. DY654 and Alexa568 do not show measurable reduction peak in cyclic voltammetry measurement.

* phosphorescence of ATTO655 was not detectable. The E_T of ATTO655 is estimated to be 0.2 eV lower than the energy of the first singlet excited state (1.81 eV)

Linkage of a triplet state acceptor proximal to a fluorophore improves its photostability for biological imaging. This approach is general to fluorophores in different structural categories and colors.