THE JOURNAL OF PHYSICAL CHEMISTRY Letters

pubs.acs.org/JPCL

Perspective

Self-Healing Dyes—Keeping the Promise?

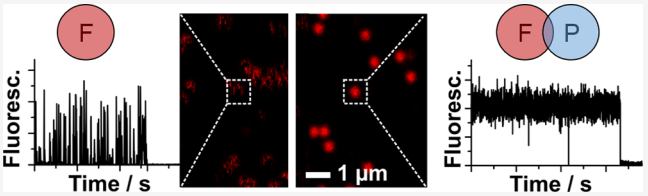
Michael Isselstein,[#] Lei Zhang,[#] Viktorija Glembockyte,[#] Oliver Brix, Gonzalo Cosa, Philip Tinnefeld, and Thorben Cordes*

Cite This: J. Phys. Chem. Lett. 2020, 11, 4462−4480

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Self-healing dyes have emerged as a new promising class of fluorescent labels. They consist of two units, a fluorescent dye and a photostabilizer. The latter heals whenever the fluorescent dye is in danger of taking a reaction pathway toward photobleaching. We describe the underlying concepts and summarize the developmental history and state-of-the-art, including latest applications in high-resolution microscopy, live-cell, and single-molecule imaging. We further discuss remaining limitations, which are (i) lower photostabilization of most self-healing dyes when compared to solution additives, (ii) limited mechanistic understanding on the influence of the biochemical environment and molecular oxygen on self-healing, and (iii) the lack of cheap and facile bioconjugation strategies. Finally, we provide ideas on how to further advance self-healing dyes, show new data on redox blinking caused by double-stranded DNA, and highlight forthcoming work on intramolecular photostabilization of fluorescent proteins.



Fluorescence microscopy, including single-molecule and super-resolution imaging, has evolved as a powerful tool to study the structure and dynamics of biological systems *in vitro* and *in vivo* down to the molecular level. A greatly improved performance of the contrast agents—fluorescent dyes and proteins—is achieved via suppression of undesired long-lived and reactive fluorophore states generated upon the continuous excitation typical of single-molecule studies.[1,2] This suppression can be realized via oxygen removal[3−5] in combination with the addition of photostabilizing compounds P *in vitro* that quench, e.g., triplet- or radical states (Figure 1a; triplet T_1).[6,7]

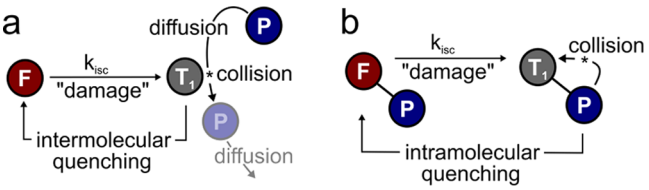


Figure 1. Schematic illustration of interactions between photostabilizers and fluorophores (where T_1 is the triplet state of a fluorophore) via (a) intermolecular and (b) intramolecular quenching processes. Reprinted from ref 14 with permission. Copyright 2019 Royal Society of Chemistry.

Here, a fluorophore F is protected against damage in an intermolecular approach (Figure 1a), where the triplet state is depleted via diffusional collisions resulting in photophysical or photochemical quenching. Photophysical triplet-state-quenchers, such as cyclooctatetraene (COT),[8−10] diphenylhexatriene,[11] or Ni^{2+} ions,[12,13] rely on energy transfer between the fluorescent dye (donor) and the quencher (acceptor).

Photochemical triplet-state quenching requires a combination of redox-active agents [e.g., Trolox (TX),[2,15] ascorbic acid (AA[3,7,16]), ferrocene,[17] nitrobenzylalcohol (NBA),[10] nitrophenylalanine (NPA),[18,19] nitrophenylacetic acid (NPAA),[18] methylviologen (MV),[7,16] Trolox-quinone[15]]. Triplet states are then quenched via photoinduced electron transfer (PET) resulting in the formation of a radical anion or cation. Simultaneous use of both reducing and oxidizing agents (termed ROXS[7]) allows the quenching of both newly formed radical intermediates and restoring the ground state of the dye via consecutive redox reactions.[7]

High concentrations of (toxic) photostabilizers are required for both photophysical and photochemical quenching, to ensure an effective depletion of reactive states. Such conditions are often incompatible with certain biological settings, such as live cell imaging, because organic (hydrophobic) compounds can perturb biological structures and/or function.[20] As illustrated in Figure 1a, intermolecular photostabilizers require direct collisions with the fluorophore, restricting their use to systems where the dyes are solvent-accessible.[14,21]

An alternative approach, which is the focus of this perspective, uses direct conjugation of photostabilizing compounds to the fluorophore, thereby creating high local concentrations of photostabilizer around the fluorophore. As

Received: December 24, 2019
Accepted: May 13, 2020
Published: May 13, 2020

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

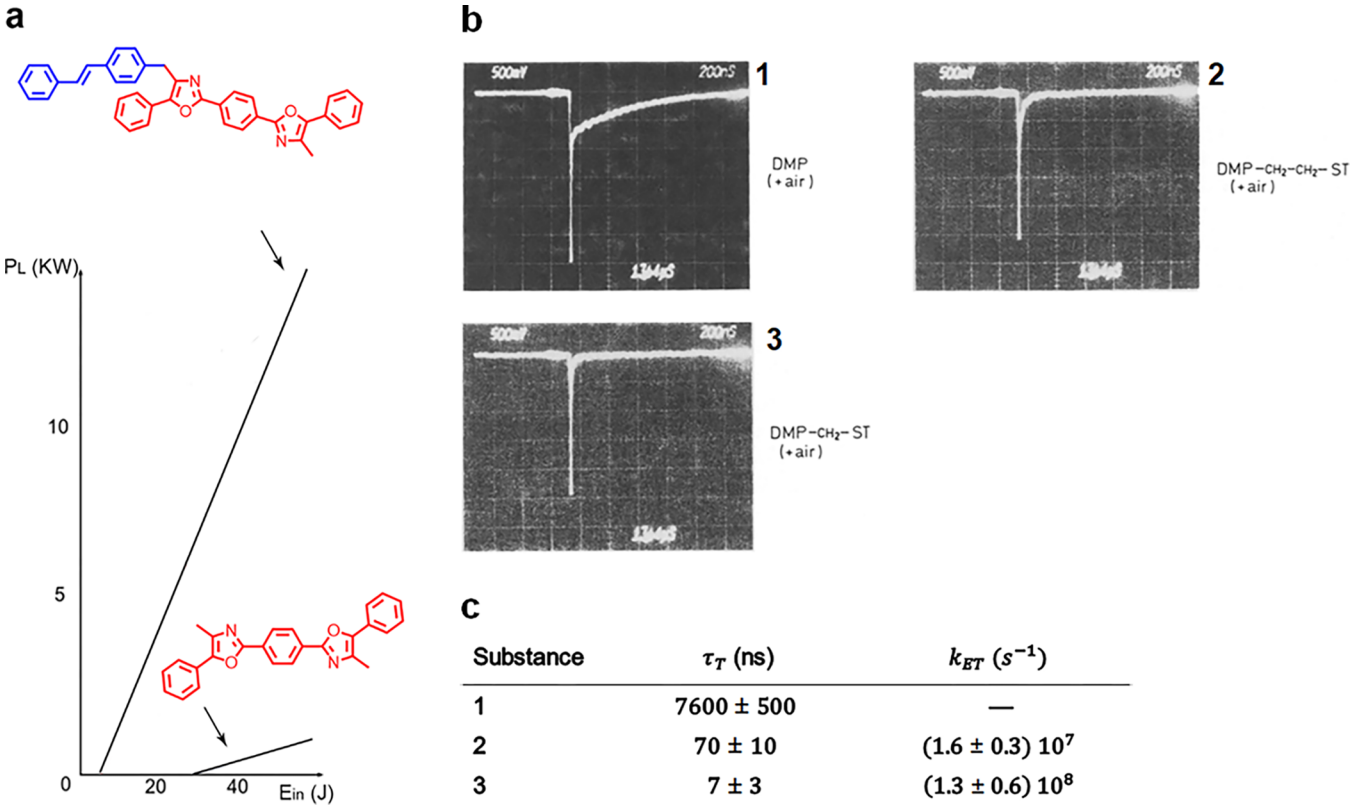


Figure 2. (a) Dye-laser output power (P_L) versus electrical input energy (E_{in}) using dimethyl-POPOP (DMP) and DMP-stilbene as laser-dye. Data are from ref 27. (b) Oscillograms of transient absorption of DMP (1) and DMP-stilbene compounds (2 and 3). All compounds were measured in dioxane solution and were air-equilibrated. Dye 2 has two CH_2 groups, whereas dye 3 has one CH_2 group between DMP and *trans*-stilbene. Oscillograms are taken from ref 36. (c) Measured triplet lifetimes τ_T and calculated TT energy-transfer rates k_{ET}. A decreasing distance between laser dye and triplet-quenching molecule results in increasing k_{ET} rates. Data are from ref 36. Copyright 1982 Springer.

illustrated in Figure 1b, this allows intramolecular quenching of triplet or radical states. Such a strategy obviates the need for complex buffer systems, rendering these dyes with intramolecular photostabilization "self-healing"[28] and thus compatible with diverse biological systems,[19,22−25] even when the fluorescent dyes are inaccessible to solution-based stabilizers.

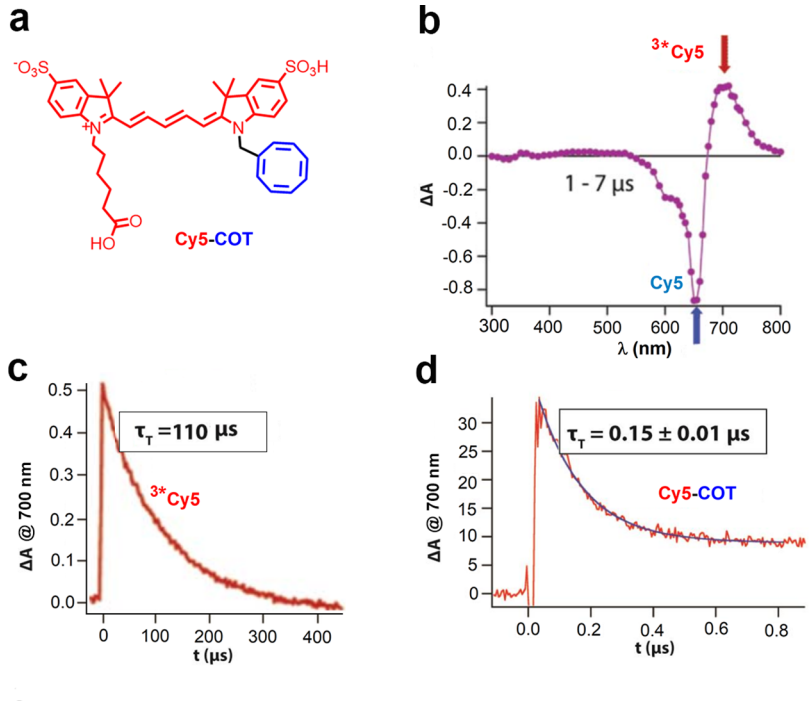
In this Perspective, we describe the concepts, history,[26,27] and state of the art of self-healing dyes[19,22−25,28−30] with a focus on the current mechanistic understanding and applications. For this, we summarize how, over the past years, the photophysical properties of self-healing dyes have been greatly enhanced;[14,18,31,32] versatile bioconjugation strategies for different fluorophore types, photostabilizers, and biomolecules were established;[19,33] important mechanistic insights were obtained;[34] and exciting applications, e.g., in super-resolution imaging,[19,35] were presented. Most importantly, however, we go beyond the current state of the art and discuss remaining limitations and challenges that have to be overcome to further improve this new class of dyes. In our view, remaining key limitations are (i) lower photostabilization efficiency of most self-healing dyes when compared to the pristine dye in combination with solution additives, (ii) limited mechanistic understanding of the influence of the biochemical environment and (iii) molecular oxygen on self-healing, and (iv) the lack of cheap and facile bioconjugation strategies. Finally, we provide new data and information on self-healing processes in double-stranded DNA with differing base sequences and preliminary data toward use of intramolecular photoprotection of fluorescent proteins.

History, Development, and State of the Art of Self-Healing Dyes. The concept of enhancing the photostability of fluorescent dyes via linking triplet state-quenchers was proposed, experimentally realized, and verified in the early 1980s.[26,27,36] Lüttke and co-workers linked 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP) and its derivatives to stilbene with the goal of quenching triplet states (Figure 2a). These self-healing POPOP−stilbene constructs showed very good performance as lasing medium in dye lasers (Figure 2a) and were the first self-healing dyes with intramolecular photostabilization.[27]

Schäfer et al. experimentally confirmed the proposed triplet-state quenching through triplet−triplet energy transfer in dimethyl-POPOP (DMP) covalently linked to *trans*-stilbene in 1982 via transient absorption spectroscopy (Figure 2b).[36] It was also possible to calculate the intramolecular triplet−triplet energy-transfer rates k_{ET} by measuring the triplet lifetimes of the DMP-triplet-quencher compounds. Furthermore, Schäfer et al. demonstrated decreasing triplet−triplet energy-transfer times with decreasing distances between laser dye and triplet-quencher (Figure 2c). With that, the groups pioneered both the concept and established the mechanistic basis for self-healing dyes.

The idea to utilize intramolecular triplet state quenching for photostabilization was revived by Blanchard in 2012,[22] by Cordes in 2013,[24] and by other groups in subsequent years.[30,37] These papers demonstrated improved photophysical properties of self-healing dyes and the possibility of using intramolecular photostabilizers for a variety of dyes from distinct structural classes.[14,19,33] A direct correlation between

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480



e

	τ_T (µs)	Counts in deoxygenated buffer ($\times 10^6$)	Counts in ambient oxygen ($\times 10^4$)
Cy5	110 ± 5	0.04 ± 0.02	0.5 ± 0.1
Cy5–COT	0.15 ± 0.01	0.19 ± 0.02	2.4 ± 0.2

Figure 3. (a) Structure of self-healing Cy5-COT. (b) Transient absorption spectra of Cy5 in deoxygenated acetonitrile solution and triplet sensitization via covalent linkage of 9-oxothioxanthene. (c) Transient absorption kinetics of Cy5 at 700 nm (corresponding to absorbance of ^{3}*Cy5) after pulsed laser excitation of Cy5 in deoxygenated acetonitrile solution. (d) Transient absorption kinetics at 700 nm after pulsed laser excitation of self-healing Cy5-COT in deoxygenated acetonitrile solution. (e) Triplet-state lifetime (τ_T) and average number of photons detected prior to fluorophore photobleaching in deoxygenated buffers as well as ambient oxygen conditions recorded for Cy5 and Cy5-COT on double-stranded DNA. Panels b–e are reprinted from ref 31 with permission. Copyright 2017 Royal Society of Chemistry.

triplet-state quenching and reduced photobleaching was observed directly in Cy5 conjugates (Figure 3a) using transient absorption spectroscopy (TAS) (Figures 2b and 3b–d).[23,31] In self-healing dyes, a much shorter triplet-state lifetime was observed, for example, for Cy5-COT conjugation (Figure 3d,e) in combination with increased photostability (Figure 3e).

Over the past years, TAS,[23,31] single-molecule total internal reflection microscopy (smTIRF),[14,18,19,22,24,28,30−33,35,38] as well as confocal microscopy[14,19,24,32,35] were important spectroscopic tools used for characterization of the photophysical properties of self-healing dyes in comparison to their nonstabilized counterparts in various buffer systems. TAS can directly trace key photochemical and photophysical intermediates and their spectra with high time resolution (Figure 3b–d); in these experiments, fluorescent dyes are analyzed freely diffusing in bulk solution at high concentrations.[39,40] smTIRF and confocal microscopy, on the other hand, enable collection of time-resolved fluorescence trajectories of individual fluorescent molecules (Figure 4a), allowing the extraction of photophysical properties of the dyes, such as their photobleaching lifetime, fluorescence intensity (brightness), total photon output before photobleaching, signal-to-noise ratio, as well as their fluorescence lifetimes in different intensity regimes at the single-molecule level.[14,22,28,30,32,35] For this, fluorophores are typically immobilized on a streptavidin-functionalized coverslip via bioconjugation with biotin-modified double-stranded DNA-scaffolds (Figure 4a), via proteins[30,41,42] or others, and imaged in the presence of an enzymatic oxygen-scavenging buffer to remove molecular oxygen.[7,15,43,44]

Established Building Blocks for Self-Healing Dyes. Over the past years, a variety of fluorophore-stabilizer conjugates have been synthesized and tested using different photostabilization agents and classes of fluorescent dyes (Figure 5).[14,18,19,22−24,31−33,45] These conjugates include rhodamines (ATTO565, Alexa555, Alexa633, Alexa568, KK114, STAR635P, TMR);[14,19,33,35] cyanines (Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Dy549, Cy7);[14,18,19,22−24,28,31−33] carbopyronines (ATTO647N);[14,19,33,35] bophy-dyes;[45] oxazines (ATTO655);[33] and fluoresceins,[33] with representative examples shown in Figure 4a. Among all the conjugates, the photostability of the fluorophore was enhanced when coupled to the photostabilizer with the exception of oxazines and fluoresceins.[33] The self-healing dye constructs included photostabilizers such as stilbene,[36] COT,[14,22,23,31,33] TX,[19,22−24,32] nitrophenyl-based stabilizers,[14,18,19,22,23,32,35] dibutulated-hydroxytoluene (BHT),[45] Ni^{2+} ions,[30] as well as a combined molecule comprising TX and NPA[32] (Figure 5b).

Three very successful combinations of dye and photostabilizer were Cy5-COT (Figure 4d),[22] Cy5-iROXS (Figure 4e),[32] and *tris*NTA-Pro_4-Alexa647 (Figure 4f).[30] The structures of COT, the iROXS compound TX-NPA, and tris-NTA

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

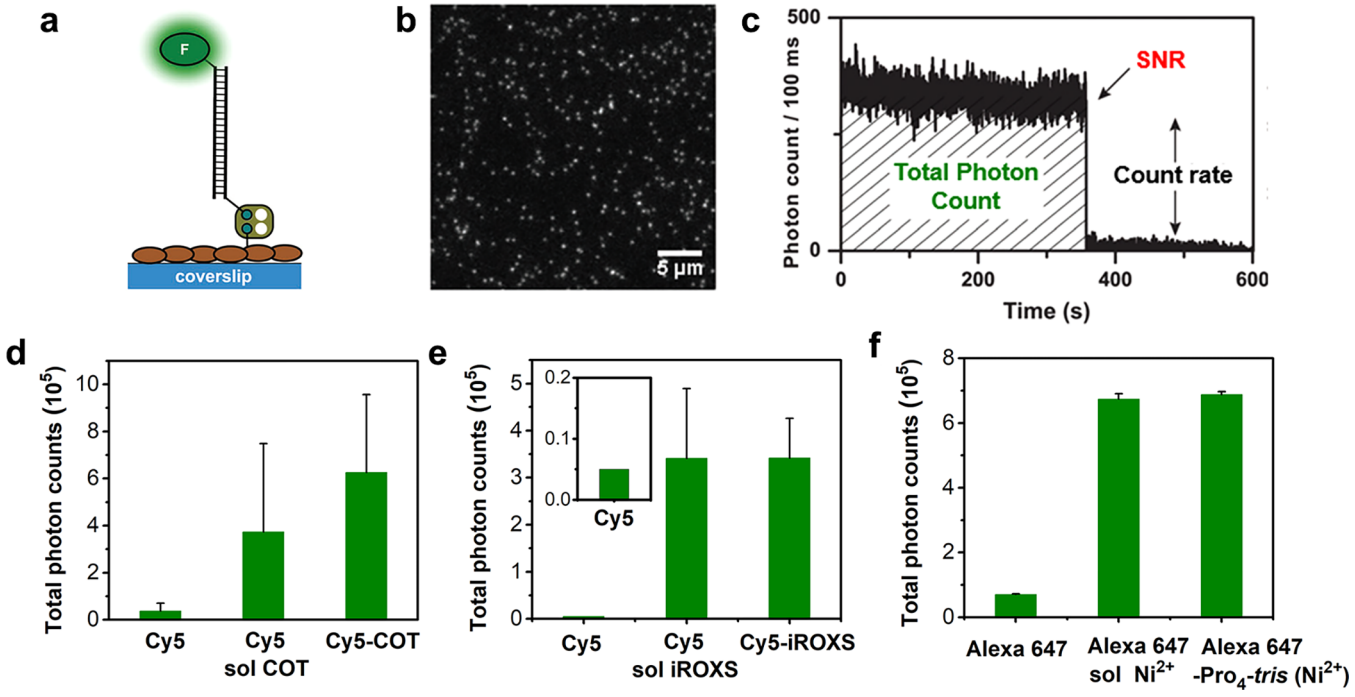


Figure 4. (a) Scheme of fluorophores immobilized on a streptavidin-functionalized coverslip via bioconjugation with biotin-modified double-stranded DNA-scaffolds. (b) Coverage of a typical sample area obtained with TIRF microscopy showing individual fluorophores. (c) Fluorescence time trace of an individual fluorophore allowing the determination of count rate, SNR ratio, and the total number of detected photons. (d–f) Total photon counts detected from individual fluorophores in deoxygenated buffer before photobleaching of (d) Cy5, Cy5 in buffer containing 1 mM COT (sol COT), and Cy5 conjugated to a single COT. Data are from ref 22. (e) Cy5, Cy5 in buffer containing 1 mM iROXS (a conjugation of NPA and TX), and Cy5 in proximity to a single iROXS molecule. Data are from ref 32. (f) Alexa 647, Alexa 647 with 0.1 mM Ni^{2+} as solution additive, and Alexa 647 with three proximal Ni^{2+} ions in a *tris*NTA moiety. Data are from ref 30.

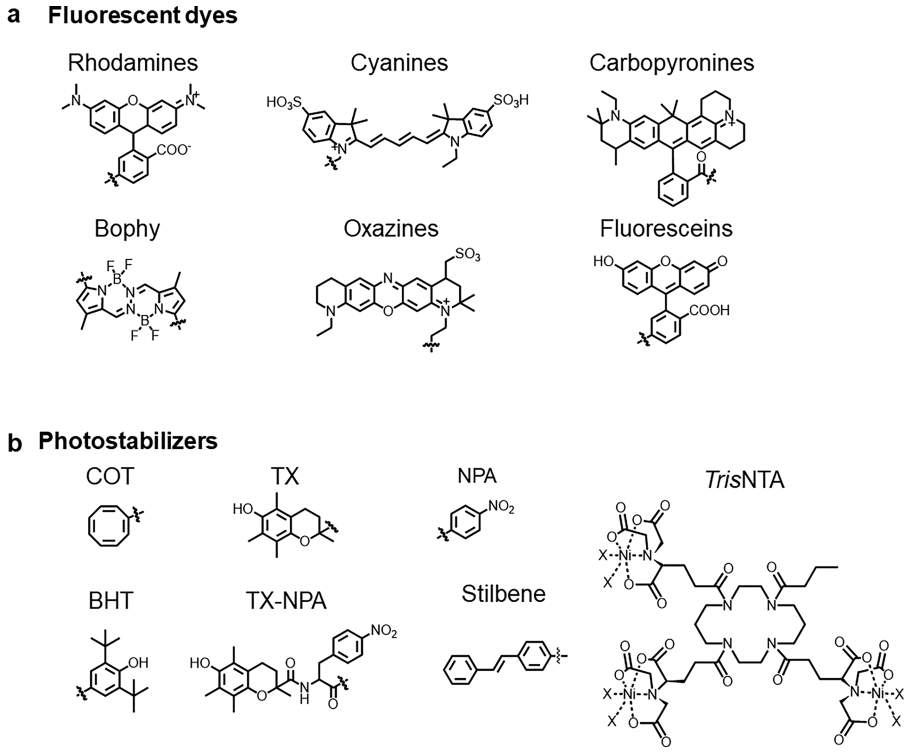


Figure 5. Structures of fluorescent dye classes (a) and photostabilizers (b) that are established building blocks of self-healing fluorophores. Displayed examples for corresponding dye classes: TMR for rhodamines; sulfo-Cy5 for cyanines; ATTO647N for carbopyronines; ATTO655 for oxazines, bophy, and fluorescein.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462–4480

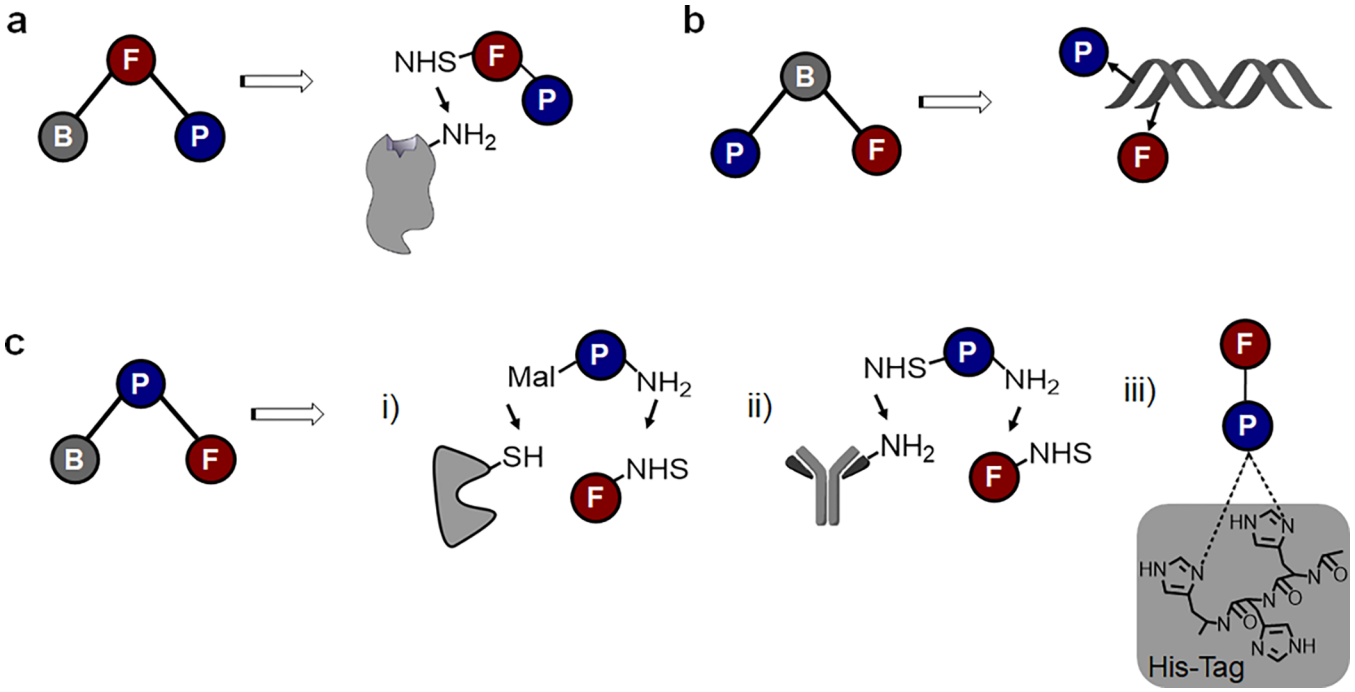


Figure 6. Conjugation approaches for self-healing dyes on biomolecular targets. (a) The dopamine receptor D2; (b) double-stranded DNA; and (c) proteins, antibodies, and affinity-tags. Biomolecular target, photostabilizer, and fluorophore moieties are denoted as B, P, and F, respectively. Please note that the chemical structures of the photostablizers in (i/ii) are no used as reactive materials in a one-pot reaction, but more complex reactions schemes are required for assembling the self-healing dye on the biological target.

are depicted in Figure 5. In these constructs the obtained total photon output of the self-healing dyes is comparable or even higher (Cy5-COT) compared to that of the solution-based photostabilization agents in buffer solution. Furthermore, all photophysical parameters of individual fluorophores (e.g., brightness and signal-to-noise ratio) were found to be improved, as indicated by the total photon count (see Figure 4, lower panel). While such a photophysical/chemical behavior is desirable, the performance of the dye−stabilizer combination shown in Figure 4 is rather the exception than what is commonly seen with most self-healing dyes.

Conjugation Strategies of Self-Healing Dyes. To date, three conceptually distinct approaches have been introduced to obtain high local concentrations of photostabilizer around the fluorophores. Life science applications require the simultaneous conjugation of a self-healing fluorophore, i.e., dye F and photostabilizer P, to a biomolecule B (Figure 6). In the seminal papers, Lüttke used conjugation of dye and photostabilizer without further tethering to any biological or chemical target. Blanchard and co-workers used *bis-N*-hydroxysuccinimidyl ester-modified cyanine fluorophores for simultaneous coupling of photostabilizer and biomolecules such as DNA, proteins, and antibodies (Figure 6a).[22,46] They also established a simpler, yet less general approach, which uses the proximity of photostabilizer and fluorophore in double-stranded DNA constructs, where covalent attachment of dyes was achieved via standard DNA-coupling chemistry and proximity between P/F results as a consequence of DNA hybridization (Figure 6b).[22,24,33] The Cordes lab later established a versatile bioconjugation approach through the use of an unnatural amino acid scaffold (Figure 6c), where the photostabilizer acts as a bridge between dye and biomolecule.[19] This scaffold allows the use of any combination of fluorophore, photostabilizer, and biological target (Figure 6c) and provided the first experimental evidence that self-healing can be applied to various dye classes. Recently, Cosa and co-workers established *tris*NTA-fluorophore complexes as parts of self-healing dyes by combining both the specific polyhistidine tag (His-Tag) and photostabilizing properties of Ni^{2+} (Figure 6c, iii).[30]

In this Perspective, we refer to a fluorophore that is covalently linked to a photostabilizer (as shown in Figure 6a,c) as "directly linked", i.e., the equivalent of a self-healing dye. The linkage of dye and stabilizer to two distinct biomolecules and self-assembly to induce proximity of fluorophore and stabilizer (as shown in Figure 6b) is termed "proximal-linkage". It is important to note that a direct linkage of self-healing dyes so far requires complex synthesis of reactive precursors for biolabeling, something that still limits the applicability of the conjugates (see discussion section).

Current Mechanistic Understanding of Intramolecular Triplet-State Quenching and Photostabilization. In contrast to intermolecular approaches, intramolecular photostabilization does not rely on a large reservoir of stabilization agents (Figure 1). Instead, quenching of the triplet state via energy transfer or PET is promoted by a high local concentration of one photostabilizer molecule.[22,25,29,31] Thus, successful self-healing relies on the (chemical) stability of both fluorophore and photostabilizer under specific experimental conditions and the ability of the stabilizer to conduct many consecutive healing cycles (Figure 7).

In the case of self-healing dyes with redox-active agents such as TX or NPA (Figure 7a) the generated triplet state is quenched via collisions with the stabilizer, resulting in PET (Figure 7c).[18,22,29,34] This process is similar to the mechanism of the reducing−oxidizing photostabilization scheme (ROXS), where subsequent complementary redox reactions of solution stabilizers recover the electronic ground state of the fluorophore. While for ROXS either radical-anion or cation

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

Successful self-healing relies on the (chemical) stability of both fluorophore and photostabilizer under specific experimental conditions and the ability of the stabilizer to conduct many consecutive healing cycles.

are formed by diffusion-based collision with different photostabilizers (Figure 1a), in self-healing the same photostabilizer has to undergo two subsequent redox reactions. Initially it seemed surprising that a single stabilizer can undergo multiple rounds of photostabilization without loss of function.[22,29]

For self-healing with photochemical triplet-state quenchers, at first a charged biradical intermediate with triplet character (Figure 7a,c) is formed.[34] The formation of the biradical is solvent-dependent.[47,48] In support of this, Cy5-TX showed no triplet quenching in TAS studies in acetonitrile.[23,31] Furthermore, bulk photobleaching studies showed the solvent-dependency of the photostabilizing effect of Cy5-NBA and Cy5-TX in acetonitrile and aqueous solutions, respectively.[38] It is also important to note that, in contrast to intermolecular stabilization, the newly formed biradical persists until subsequent reverse intersystem crossing (k_{ISC}) and back electron transfer (geminate recombination, rate k_{gr}) occurs.[34,49] A process competing with photostabilization via geminate recombination is separation of the photostabilizer and fluorophore biradical, i.e., separation of the two radical centers resulting in the formation of two noncorrelated radicals with unrelated spin states (not shown in Figure 7a).[34] Successful recovery of the fluorophore singlet state via intramolecular PET quenchers thus requires an efficient intersystem crossing of the biradical intermediate with subsequent geminate recombination (Figure 7a). In PET-based self-healing dyes, the chemical nature of the photostabilizer, i.e., the redox-potential, is expected to have a large impact on the photostabilization efficiency. Single-molecule studies as well as molecular dynamic simulations suggest, however, that collision rates and the molecular interactions governed by the biochemical environment also share an important role for the self-healing process (see discussion below).[18]

In contrast to redox-active conjugates, photostabilization by physical triplet-state-quenchers such as COT and Ni^{2+} (Figure 7b) proceeds through a physical mechanism without formation of charge-separated intermediates (Figure 7d). For an efficient photostabilization via this energy transfer, the photostabilizer should have a triplet state with lower (or equal) energy compared to that of the fluorophore (Figure 7d).[50] In COT this triplet−triplet energy transfer requires a planarization of the "boat-shaped" COT, which leads to molecular relaxation and lowering of its triplet state, allowing for quenching of fluorophore triplet states with energies as low as 0.8 eV (Figure

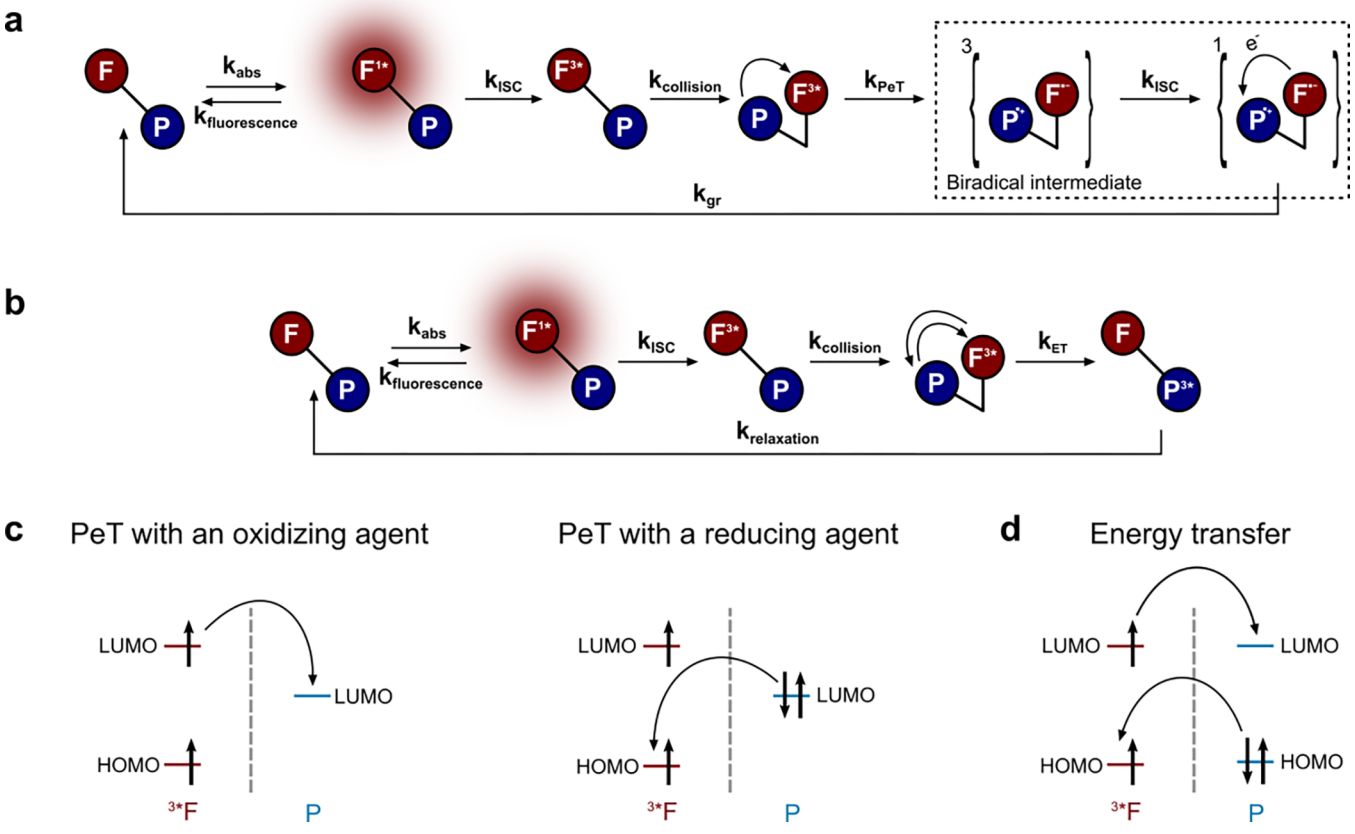


Figure 7. Photostabilization mechanisms (a and b) and energy-diagrams (c and d) of self-healing fluorophores with intramolecular PET (a and c) and triplet−triplet energy-transfer processes (b and d). k_{abs}, rate of photon absorption; $k_{fluorescence}$, rate of emitting a photon through fluorescence; k_{ISC}, intersystem crossing rate; $k_{collision}$, collision rate; k_{PeT}, rate of photoinduced electron transfer; k_{gr}, geminate recombination rate; k_{ET}, rate of energy transfer.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

7b, k_{ET}).[51] Triplet−triplet energy transfer, however, becomes inefficient for large energy gaps between the triplet states of photostabilizer (acceptor) and fluorophore (donor) explaining the poor performance of COT for certain dye classes, e.g., fluoresceins.[33] A similar behavior has been observed for Ni^{2+}, which works through a different energy-transfer mechanism.[13] Additionally, the resulting sensitized photostabilizer triplet state should be short-lived for efficient self-healing, i.e., it should be available as a triplet-acceptor as soon as possible after energy transfer. In COT, the triplet-state lifetime is on the order of ∼100 μs,[52] which allows for a fast relaxation and recovery of the photostabilizer as triplet-acceptor (Figure 7b, $k_{relaxation}$).

A final important point is that high (local) concentrations of photostabilizers can cause singlet quenching decreasing the fluorescence quantum yield of the dye.[24,30] Therefore, care has to be taken in linker design to optimize the collision rate and geometric orientation between the fluorophore and photostabilizer. The overall goal is to achieve efficient triplet quenching in combination with low singlet quenching. This should also be taken into account for physical triplet-state-quenchers, such as Ni^{2+},[12,13,30] that can potentially quench the singlet excited state by enhanced ISC and increased formation efficiency of triplet states. Again, the high-lying singlet excited state of COT minimizes singlet state quenching because of the energetic mismatch with most fluorophores.[31]

Optimization Strategies of Photostability in Self-Healing Dyes. In many cases, the photostability of self-healing dyes was found to be lower than that of the pristine dye in photostabilizing buffer.[14,19,25,33] Reasons for this can be low (chemical) stability of the photostabilizer, generation of fluorophore radicals as intermediates, the requirement of the stabilizer to efficiently undergo intersystem crossing, and a nonoptimal collision rate between healer and fluorophore that does not favor triplet- over singlet-quenching.

One key parameter for optimization of self-healing fluorophores is the collision frequency and contact time between fluorophore/photostabilizer that can be tuned via the linker length and linking chemistry on the specific biomolecule.[30,31] This was already supported by the early data of Lüttke[25,26] and Schäfer;[36] Blanchard and co-workers provided additional DNA-based ruler experiments that supported the strong distance-dependence of self-healing.[22] While this relation followed the qualitative trend expected for energy-transfer processes (the closer the better), they also demonstrated that there is an optimal linker length at short distances.[31] At short distances of course other interactions may become relevant too and might impact the final performance of the self-healing dye. In selected cases for Cy5 conjugation to COT maximized photostability also correlated with the shortening of triplet state lifetimes, and thus, photostabilization efficiency increased monotonously with decreasing linker length.[31]

The crucial role of the linker length and geometry was also used by Cosa and co-workers for optimization of self-healing Alexa647-*tris*NTA constructs.[30] Here, even the nature of the linker was crucial for minimizing singlet excited-state quenching by Ni^{2+} while maintaining an efficient quenching of the triplet state. A direct coupling of fluorophores to the *tris*NTA moiety containing three Ni^{2+} ions resulted in fluorescence quenching and only moderate improvement in photostability. Coupling to a rigid and rather long Pro_{12} linker prevented this unwanted quenching; however, it also reduced the triplet-state quenching resulting in almost no effect of *tris*NTA group on photostability of Alexa647. The best photostabilization was obtained when utilizing a rigid and short Pro_4 linker allowing for even slightly better photostabilization efficiency than solution-based photostabilization by Ni^{2+} (Figure 4f).[30] In a previous study, Wagner et al. investigated the dependence of intramolecular triplet−triplet energy-transfer rates between a triplet donor and acceptor both based on collisions and through a through-bond energy transfer.[53] At very short bond lengths of less than three carbon atoms, a through-bond energy transfer was detected. Strikingly, the corresponding energy-transfer rate was at least 1 order of magnitude higher than that of compounds with longer linker lengths, in which diffusive collisions dominate the energy-transfer rate. The information gained from this study may be helpful for the future design of self-healing dyes with physical triplet state quenchers featuring improved triplet quenching rates. The linker-length dependency of energy transfer in Wagner et al. does, however, not match data shown by Zheng et al.[31] on Cy5-COT-self-healing compounds with varying linker length, suggesting a more complex mechanism.

Another straightforward idea to improve photophysical dye properties, which was tested only recently,[14] is the combination of inter- and intramolecular approaches. The idea was to either quench reactive fluorophore states, which the intramolecular photostabilizer cannot recover, via intermolecular pathways or to support the photostabilizer in its function directly. While there is no strong evidence that photostabilizer destruction is a dominant reaction pathway,[14,54] redox-blinking (i.e., radical formation) was identified as one major problem of intramolecular stabilization with physical quenchers such as Ni^{2+} and COT. The latter manifests itself as ON- or OFF-blinks in otherwise stable single-molecule traces (see data below).[13,33]

In this study it was shown that solution photostabilizers had little to no effect on the photostability of self-healing dyes.[14] Mechanistically, this could be explained by the idea that the intramolecular photostabilizer could outcompete the intermolecular stabilizers on the basis of high local concentration.[14] The tested compounds included NPA conjugates (NPA-ATTO647N, NPA-Alexa555, NPA-Cy5) as well as proximal Cy5-COT. Compounds were benchmarked against the effects of 2 mM TX or 2 mM COT in solution, respectively. While there was an increase in count rate for NPA-Atto647N with TX in solution, as well as to NPA-Alexa555 with TX or COT in solution compared to the self-healing compound without stabilization agent, the overall trend suggested only minor positive effects of photostabilization when solution additives are present in addition to self-healing dyes.[14]

Also the use of multiple intramolecular stabilizers was tested for self-healing dyes. For this Cy5 was combined with a photostabilizer consisting of both an oxidizing and a reducing agent (iROXS), which provided two complementary redox-pathways for triplet- and radical-quenching.[32] The origin of the high photostability of proximal Cy5-iROXS (Figure 4c) was, however, not based on two synergistic stabilizers but was dominated by the linking geometry and contact rates between Cy5 and NPA in these constructs. The second stabilizer TX had little effect on the overall photostability of the dye. Smit et al. more recently tested a combination of similar photostabilizers based on nitrophenyl groups (direct linkage of NPA and proximal NPAA), which did also not result in higher

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

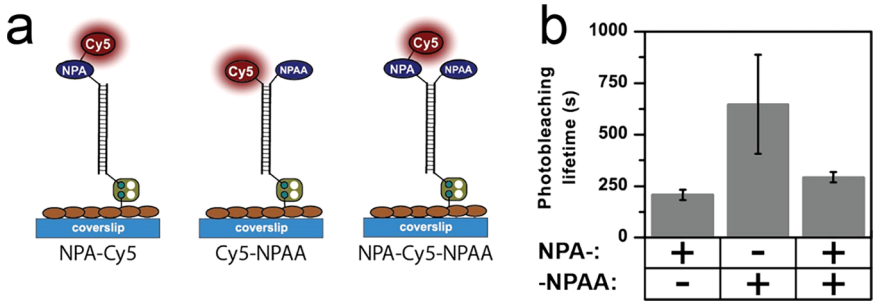


Figure 8. Comparison of Cy5 photostability with different geometries of intramolecular photostabilization by NPA and a combination of both. Reprinted from ref 14 with permission. Copyright 2019 Royal Society of Chemistry.

photostability than observed for a single photostabilizer as seen in Figure 8.[14]

Interestingly, the Blanchard lab showed an increased photostability of Cy5 upon linkage to two (instead of one) COT moieties.[31] While the average counts before photobleaching could be increased by coupling two COT molecules to Cy5, the mechanistic origin of this gain remains unclear because of potential convolution of thiol-induced photoswitching and self-healing processes in the characterization experiments of these double-COT conjugates (see next section).

Competing Pathways: Photostabilization versus Photoswitching. Self-healing dyes are commonly characterized and used in biological imaging applications *in vitro* and *in vivo* or for single-molecule spectroscopy. In such experiments they become exposed to varying biochemical conditions, which can impact their function and photophysical properties (Figure 9). Examples of molecules that largely impact dyes are reductive agents such as ß-mercaptoethanol (BME), mercaptoethylamine (MEA), glutathione, DTT, or TCEP and molecular oxygen.[55,56] All of the latter can be present up to millimolar concentrations *in vitro* (e.g., for stabilizing proteins or components in a buffer) or are encountered naturally *in vivo*. In STORM-type super-resolution microscopy, similar compounds are even used at even higher concentrations [BME (143 mM), MEA (50 mM), and TCEP (25 mM)][57−61] to reversibly switch fluorophores between bright and dark states for single-molecule localization and by that facilitate the reconstruction of images with a resolution beyond the diffraction limit.

We recently investigated the interplay between inter- and intramolecular processes in self-healing dyes both for photostabilizing (see previous section) and photoswitching compounds.[14] It became apparent that reducing agents used for photoswitching in STORM microscopy induce fast "apparent" photobleaching already in the presence of low reducer concentrations, e.g., 0.2 mM TCEP (Figure 9a) or 5 mM MEA.[35] This effect was found to be most prominent for cyanine fluorophores. In detail, the reversible OFF-switching caused by reductive agents such as TCEP (see Cy5 in Figure 9a,b + UV) was much less efficient compared to Cy5-dyes with proximal COT (see Cy5-COT in Figure 9a,b + UV).

ATTO647N showed no significant perturbation in terms of apparent photobleaching time, whereas Alexa 555 showed an approximately 2-fold decrease in apparent photobleaching time with 200 μM TCEP in solution. Here, the effect was strongly reduced after NPA conjugation, and on-times were almost as long for the self-healing ATTO647N dye as for the parent dye with solution-based stabilization. On Cy5, however, the effect on apparent photobleaching time was a lot more prominent, as it decreased more than 10-fold under addition of 200 μM TCEP on the parent fluorophore, which mostly mitigated during solution-based or intramolecular stabilization. In our view the differing influence of TCEP is due to the fact that only cyanines with methine chains ≥5 react efficiently,[58] whereas Cy3B seems to be more susceptible to thiol-induced photoswitching or caging via sodium borohydride rather than with TCEP.[62]

We suggest that in self-healing dyes the conjugated photostabilizer efficiently competes with the photoswitching agent (in the buffer) because of its high local concentration in a "first-come first-quench" manner. This has important consequences especially for cyanine dyes. It implies that photoswitching, which is the basis for STORM super-resolution microscopy, occurs at least to some extent via excited fluorophore states, because OFF-switching was shown to be photoinduced (Figure 9). We also showed that intramolecular photostabilizers strongly influence photoswitching kinetics. Thus, the composition of photoswitching buffers has to be adapted when self-healing dyes are used in localization-based super-resolution microscopy.[35]

Additionally, photoswitching agents are problematic components of imaging buffers in characterization experiments of self-healing dyes. For example, it was previously concluded that proximal conjugation of COT to Cy5 dramatically enhances its photostability via intramolecular photostabilization with respect to Cy5 with COT as a solution additive. When arriving at this conclusion, the effect of BME, TCEP, and MEA on the photostabilization efficiency and the photoswitching behavior of Cy5 and Cy5-COT derivative was neglected. In the light of these findings, the photostabilization efficiency of self-healing dyes was often overestimated, because the pristine dye in the photostabilizing buffer showed fast apparent photobleaching (which is reversible). The positive effect of an intramolecular stabilizer on dye stability and properties persists but might be influenced slightly by the solution stabilizers. Thus, various mechanistic studies of self-healing dye in TCEP or thiol-containing buffers, especially those on cyanine fluorophores, have to be evaluated critically and possibly revisited.

To the best of our knowledge, there are to date only three published mechanistic studies on the photophysical properties of self-healing fluorophores that were conducted in the absence of any intermolecular effectors such as oxygen and reducing agents.[14,30,35] Smit and van der Velde et al. screened a variety of fluorophores directly conjugated to NPA, as well as Cy5 proximally conjugated to COT. The experiments showed a significantly higher photostability, signal stability, and bright-

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

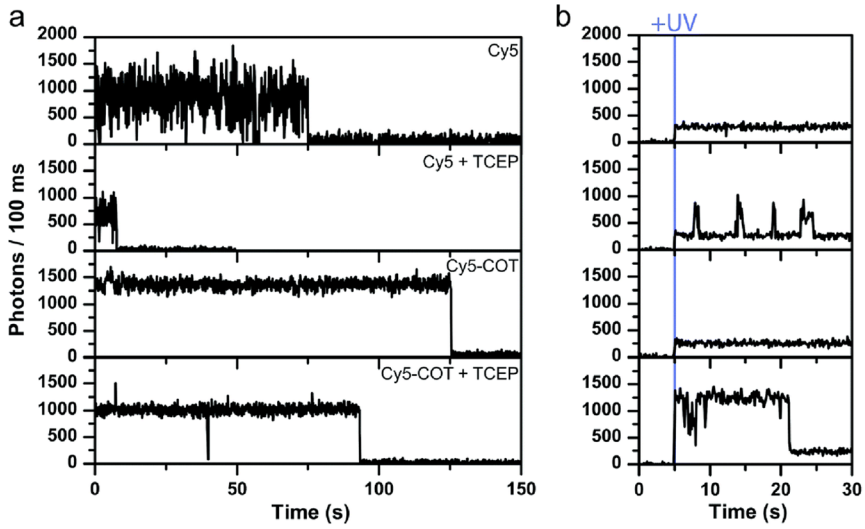


Figure 9. Single-molecule fluorescence transients before (a) and after (b) UV irradiation of parent Cy5 fluorophore as well as Cy5-COT conjugates in the presence and absence of low TCEP concentrations of 0.2 mM. Reprinted from ref 14 with permission. Copyright 2019 Royal Society of Chemistry. In the absence of TCEP (first and third panels) fluorophores photobleach under continuous red excitation (a) but cannot be reactivated (b). In the presence of TCEP, apparent photobleaching is fast (because of photoswitching) and molecules can be reactivated via UV radiation. The background increase in all parts of panel b is due to 375 nm excitation. Copyright 2019 Royal Society of Chemistry.

ness for all combinations compared to the native fluorophore. The improvement could, however, not reach that acquired from intermolecular stabilization of the native fluorophore through 2 mM TX or 2 mM COT.[14] In another study, Glembockyte et al. reported a highly photostable *tris*NTA-Alexa647 self-healing construct (Figure 5b), which showed a superior photostability when compared to intermolecular stabilization with the same stabilizer (Ni^{2+}, Figure 4f).[30]

Self-Healing Dyes and Molecular Oxygen. Oxygen-dependent processes were so far studied only to some extent in the context of self-healing dyes.[22,31,38] Most experiments were conducted in the absence of oxygen to avoid convoluted effects. Because oxygen can promote a complex network of primary and secondary photodamage pathways,[38] we will here focus on only the fundamental question of whether the intramolecular triplet state-quenchers, which are present in self-healing dyes, can "outcompete" molecular oxygen. The mechanistic idea would be to disable the action of oxygen by providing an intramolecular reaction partner—as was observed in the competition of intramolecular and intermolecular photostabilization and photoswitching, respectively (see previous sections).[14]

To date, self-healing dyes were shown to be rather ineffective in the presence of molecular oxygen. In one study, the same self-healing dye in the absence and presence of oxygen exhibited at least 1−2 orders of magnitude loss of total photon count.[22] Blanchard and co-workers had success in modifying the chemical nature of COT by electron-withdrawing groups and with that could increase the performance of self-healing cyanine and silicon-rhodamine fluorophores;[31] however, the large discrepancy between the performance of self-healing dyes with and without oxygen is not yet solved.

As intermolecular pathways compete with intramolecular processes (see previous sections), one aspect causing this poor performance of self-healing dyes might be that oxygen is always faster compared to triplet-quenching via intramolecular stabilizers. Thus, intramolecular triplet state quenching has not yet overcome the diffusional speed limit. For oxygen we consider the diffusion-limited rate of quenching by molecular oxygen to be $\sim 10^{10}$ M^{-1} s^{-1} and the concentration of molecular oxygen in solution of roughly ~0.5 mM.[63] This suggests quenching rates of $\sim 10^{6}$ s^{-1} for oxygen, which need to be overcome by a competing intramolecular photostabilizer. The upper limit of intermolecular triplet quenching in solution by stabilizers such as azobenzene[64] was experimentally determined to range from 10^{7} to 10^{9} M^{-1} s^{-1}. Assuming a similar reaction speed for COT, this suggests quenching rates between 10^{4} and 10^{6} s^{-1} for 1 mM COT solutions. Importantly, these rates are comparable to those estimated with molecular oxygen, suggesting a more complex reason than only kinetics for the experimental findings of rather unstable fluorophores in the presence of oxygen even when using COT. To quantitatively assess intramolecular effects and compare them to intermolecular effects (from oxygen), effective concentrations would have to be calculated. These could be based on molecular dynamics simulations and represent missing key pieces in our understanding of the correlation between photostability and effective local concentrations of the photostabilizer.

Impact of Biological Structures on Self-Healing Dyes. DNA has become a well-established model system for characterization of fluorophore photophysics in general, especially when working with single-molecule techniques. In self-healing dyes with proximally linked photostabilizers on a DNA scaffold, the effect of DNA itself on the photophysical properties of the dye cannot be neglected, and other less perturbing systems such as rigid poly proline motifs[14,19,22] might be preferable for future studies. DNA can influence the photophysics of dyes via a number of different mechanisms reported in the literature.[65−67] First, the DNA base guanine is an electron donor, which can quench the fluorescence of dyes with low reduction potentials, e.g., oxazines and rhodamines that become reduced easily via PET.[67−70] This can, in turn, decrease the brightness of these fluorophores and cause redox blinking when attached in close proximity to guanine or, in particular, when attached close to guanine repeat sequences.[67] That being said, the electron-donating ability of guanine could also potentially

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

influence the lifetimes of radical species of fluorophore that are formed via other pathways, e.g., photooxidation.

Another important interaction possibility of dyes with DNA is stacking of the dyes to the end-bases as well as binding to major and minor grooves of the DNA duplex,[65,66,71,72] which can influence the rate and efficiency of photostabilization in self-healing dyes. This was shown for terminal attachment of dyes in direct conjugation to a nitrophenyl-group and in proximity, which likely resulted in completely distinct photophysical properties of the dye due to different interactions with the DNA.[18]

The effect of DNA duplex on photophysical properties and photostabilization efficiency of a common cyanine dye Cy5 is illustrated in Figure 10 by yet unpublished data from the Cosa

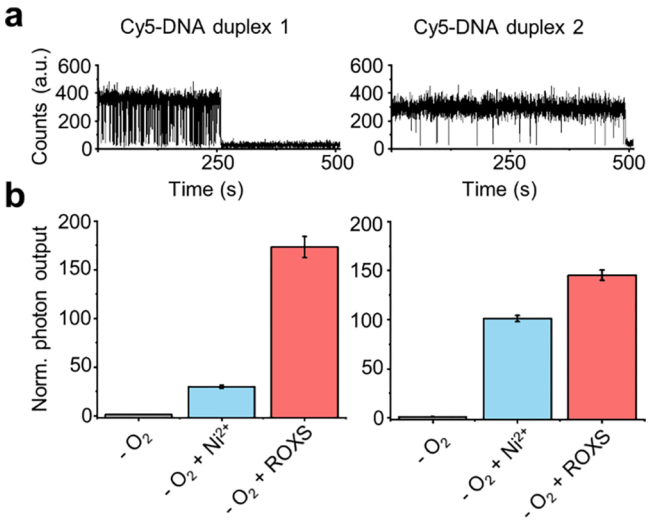


Figure 10. Effect of DNA duplex on the photophysical properties and phostabilization efficiency of Cy5. (a) Single-molecule trajectories of Cy5 when attached to two different DNA duplexes (left and right) in the presence of enzymatic oxygen scavenger and 0.4 mM Ni^{2+} acquired at 100 ms time resolution. Under these conditions the triplet state of Cy5 is efficiently quenched; therefore, short blinking events that are observed correspond to different extent of radical formation. (b) Normalized photon outputs obtained for the two duplexes in the presence of photophysical (0.4 mM Ni^{2+}) and photochemical (ROXS consisting of 1 mM AA and 1 mM MV^{2+}) photostabilization approaches. Cy5-DNA duplex 1 shows that the high extent of redox blinking is photostabilized much more efficiently with the ROXS system. In contrast, for Cy5-DNA duplex 2 almost no redox blinking is observed and both photostabilization approaches are comparable, emphasizing a crucial role of DNA environment. A full description of material and methods including additional experimental data is provided in Supplementary Note 1 and Figure S1. Data are from the Cosa lab.

lab. Here, the dye was attached to two distinct DNA duplexes (see Table S1 for ssDNA sequences) and its photostability was evaluated in the presence of photophysical (0.4 mM Ni^{2+}) and photochemical (ROXS consisting of 1 mM AA and 1 mM MV^{2+}) photostabilizers in solution. Figure 10a contains single-molecule fluorescence trajectories obtained for the duplexes in the presence of Ni^{2+}. Under these conditions, the triplet states are expected to be efficiently quenched by Ni^{2+}; therefore, any blinking that is observed in single-molecule fluorescence trajectories is attributed to the formation of long-lived radical species, which are not observed in the presence of ROXS. Cy5-DNA duplex 1 (Figure 10, left panel) displayed a large extent of redox blinking and, in turn, less efficient photostabilization by Ni^{2+} when compared to photochemical stabilization by the ROXS system capable of scavenging reactive radical intermediates (Figure 10b, left). On the other hand, the Cy5-DNA duplex 2 showed almost no redox-associated blinking and, consequently, very comparable photostabilization efficiency by Ni^{2+} and ROXS. These findings illustrate a crucial role that a DNA duplex can play on the photostabilization of dyes, suggesting either a DNA-induced radical formation (prominent in duplex 1) or possibly potential quenching of radicals induced via other pathways (e.g., photooxidation) by the DNA scaffold (prominent in duplex 2). Further mechanistic studies, e.g., of DNA sequence dependence on these processes, may help to shed light on the nature of these blinking events and the role of the DNA scaffold.

Fewer studies of self-healing dyes have been done on biological scaffolds such as proteins, antibodies, or "neutral" scaffolds such as polyprolines.[14,19,22,30] As for DNA, the effect of the protein environment plays an important role in the photophysics of the dye. Proteins present a challenging environment for prediction of possible interactions of the dye/photostabilizer with charged/hydrophobic residues, structural elements, flexible loops, or chemically reactive sites such as cysteines.

A first step into a systematic characterization of interactions between (self-healing) dyes and proteins was recently published.[14] Here, ATTO647N and Cy5 were not linked to a photostabilizer but conjugated to aromatic amino acids (Trp, Phe, and Tyr). Interestingly, Trp-ATTO647N and Trp-Cy5 showed barely any change in photostability or signal quality compared to the pristine dyes in the absence of oxygen and intermolecular photostabilizers. The addition of TX as intermolecular photostabilizer, i.e., buffer additive, was much less effective in the presence of covalently coupled Trp. This suggests Trp can act similarly to NPA/COT via intramolecular PET-processes as discussed above but inhibits the function of buffer additives such as TX. In essence, Trp creates a truly bad environment in that it does not stabilize fluorophores by itself, yet it does not allow solution photostabilizers such as TX to interact with the dye either. Such interaction, however, was not observed while using COT as a solution-based stabilizing agent, suggesting the more effective action of COT. Interestingly, other amino acids with lower tendency for reductive PET (phenylalanine and tyrosine) had no notable effect on the photophysics of Cy5 and ATTO647N.[14] For applications in proteins, the effect of tryptophan (and other factors) would now need to be studied in self-healing dyes to understand their full implications.

Impact of Fluorescent Labels on Biological Systems. Covalently linking a fluorophore to a photostabilizer does not only alter its photophysical behavior, but it also increases the size and molecular weight of the compound to a large extent. While an increase in label size is one possible problem, also changes of the chemical nature of the label have to be considered. The introduction of hydrophobic groups via aromatic or aliphatic rings might alter the interactions of the label with a biomacromolecule.[20]

Only a limited number of studies were done to characterize such perturbations of, e.g., paramagnetic spin-labels for EPR or fluorescent probes on biological constructs such as DNA or proteins.[73] To the best of our knowledge, there is no systematic investigation on the impact of self-healing dyes on, e.g., the biochemical behavior of proteins or DNA.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

Especially in studies with the aim of drawing conclusions about the dynamics or structure of biological systems, care has to be taken while selecting labeling position and compounds, as in most other labeling approaches. Control experiments such as K_d-estimation of labeled proteins with self-healing dyes[19] are one example for assessing the label influence. Sánchez-Rico et al. presented a comprehensive guideline for choosing fluorescent labels and labeling positions in a way that minimizes interference on protein function, which in our opinion should also be taken into account during such investigations using self-healing dyes.[73] In combination with fluorescence-independent biochemical assays to ensure functionality of the system under investigation, we expect assays with low biological interference, especially because the molecular weight and size are still much smaller than those of frequently used fluorescent proteins.

Recent Applications of Self-Healing Dyes. The self-healing approach could potentially improve the performance of fluorophores in various imaging, spectroscopy, and diagnostic applications. Recent examples of their use include high-resolution optical imaging (STED/STORM),[19,35] single-molecule FRET,[19,25,46] near-infrared photostable bioimaging,[37] superlong imaging-tracking of single individual molecules,[74] live-cell imaging using self-healing dyes for multichromophore probes,[75] and tracking of submersible nanomachines.[76]

The self-healing approach could potentially improve the performance of fluorophores in various imaging, spectroscopy, and diagnostic applications.

Cellular Imaging and High-Resolution Microscopy. In particular for cellular and super-resolution imaging, the available photon budget is important, and thus, various photostabilization buffers have been tested in the field.[77] Blanchard's group demonstrated increased photobleaching lifetime of Cy5-COT conjugates *in vivo* Chinese hamster ovary (CHO) cells compared to the parent fluorophore using fluorescence imaging (Figure 11a).[22]

Applications of self-healing dyes in super-resolution imaging and live-cell imaging were shown for stimulated emission depletion (STED) microscopy and stochastic optical reconstruction microscopy (STORM).[14,19,35] The Cordes group has successfully shown a reduced fading of fluorescence from KK114-NPA conjugates compared to the parent fluorophore as stains in the nuclear pore complex in fixed mammalian PtK2 cells in STED imaging (Figure 11b).[19] Recently, they also demonstrated a significant increase in STED resolution from ATTO647N-NPA conjugates compared to the parent fluorophore, resulting from highly improved photophysical performance providing count-rates of individual molecules of up to 1 MHz without use of plasmonic effects.[35] To date, self-healing dyes with increased photostability have been able to demonstrate their potential for STED-type microscopy, where the achievable resolution is determined by the increased number of laser excitation cycles.[54] It remains to be shown how much improvement can be made via intramolecular photostabilization in STED in comparison (or combination) with approaches such as protected STED[78] and Minflux.[79]

Applications of self-healing dyes in localization-based super-resolution microscopy (STORM-type imaging) turned out to be more complicated as discussed in previous sections. STORM demands fluorophores with on−off switching property as well as good photostability.[14] Because thiol derivatives (TCEP, BME, or MEA) are used as standard photoswitching agents to make the fluorophores cycle between the on and off states,[57] the photoswitching agents are largely altered in self-healing dyes.[35] Thus, more research efforts are required in order to lay out general design rules for self-healing dyes capable of photoswitching and to enable their widespread use in super-resolution imaging.

Single-Molecule Förster Resonance Energy Transfer. Single-molecule Förster resonance energy transfer (smFRET) is an emerging tool for probing dynamics and interactions in biological systems both *in vivo* and *in vitro*.[80] In these experiments, energy transfer is used as a molecular ruler to determine inter- and intramolecular distances with the temporal resolution of spectroscopic techniques down to nanoseconds.[80] Mapping dynamic biological processes presents high demands on the brightness, signal stability, and photobleaching lifetime of the fluorescent probes involved.[46]

The applicability of self-healing dyes for smFRET has already been demonstrated for smTIRF microscopy[25,46] and solution-based alternating laser excitation (ALEX)[81,82] in confocal microscopy.[19] Recently, Juette et al. demonstrated the applicability of self-healing dyes in two- and three-color smFRET experiments on ribosomal pretranslocation complexes.[46] Their two-color FRET experiments show the versatility of the Cy3 and Cy5-COT fluorophore pair with distinct FRET states for both high time resolution (2 ms), showing subsecond protein dynamics, as well as low time resolution (500 ms) visualizing protein dynamics that occur over tens of minutes. Furthermore, in the same study, Cy3B, Cy5-COT, and Cy7-COT were used to monitor correlated movement inside the ribosomal pretranslocation complex through three-color FRET (Figure 12). The time traces demonstrate stable fluorescence, illustrating the excellent fluorophore quality under multiwavelength excitation.[46]

For solution-based ALEX experiments,[82] a clear increase in photostability of the FRET pair Cy3B and NPA-ATTO647N compared to the nonstabilized pair Cy3B-ATTO647N was observed under high irradiation conditions[19] While the photostability for this dye pair did not meet the level of solution-based stabilization conditions, the increase in photostability was sufficient for practial use.

Solution-based photostabilization through ROXS is especially challenging for multicolor FRET applications because of differences in fluorophore redox potentials. As a general approach, self-healing could bypass this problem by using a stabilizing moiety that is optimized for each fluorophore. The physical quencher COT has shown an efficient photostabilizing effect for a wide range of fluorophores throughout the visible spectrum, allowing it to be used for a variety of fluorophore pairs.[33] However, the effect does not seem to be optimized for some selected fluorophores such as fluoresceins or specific combinations of donor−acceptor dyes required for FRET,[28,33] leaving room for the use of self-healing fluorophores with distinct and optimized fluorophore−stabilizer combinations.

Toward a Next Generation of Self-Healing Dyes. As is apparent from this Perspective, self-healing dyes have become an attractive alternative method for photostabilization since their introduction in the 1980s. Their successful applications show

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

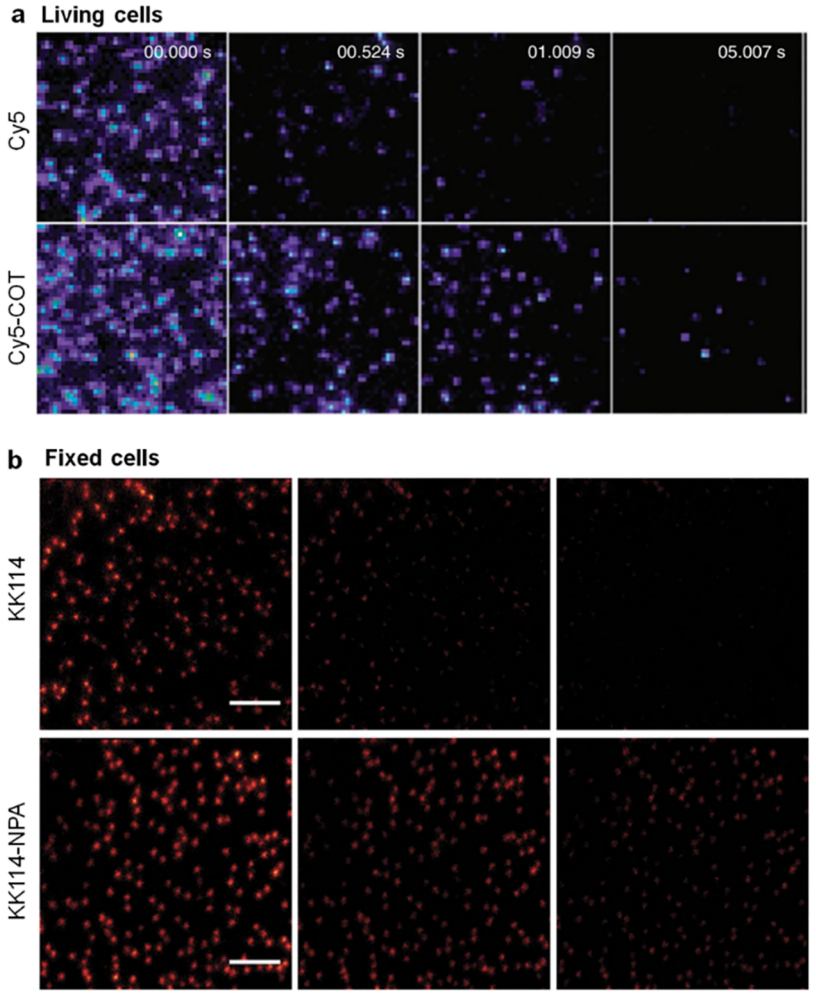


Figure 11. (a) Single-molecule total internal reflection fluorescence image sequences of living CHO cells containing dopamine D2 receptors labeled with Cy5 and Cy5-COT. Reprinted from ref 22 with permission. Copyright 2012 Nature Publishing Group. (b) Repeated scanning of the same area of KK114 and NPA–KK114 labeled fixed PtK2 cells in the STED mode. The figure was partially adapted from Van der Velde et al.[19]

possible positive impact on various research fields, yet they still have to reach their full potential. In our view, there are three major directions that require further attention to make the approach more mature and generally applicable.

New Stabilizers and Strategies to Further Improve Self-Healing. When comparing the photostability of PET-based and energy-transfer-based self-healing dyes, the latter ones often show better performance. One possible explanation for this is that in PET-based photostabilization with redox active triplet-state-quenchers the reactive triplet biradical intermediate is a key bottleneck (Figure 7). Therefore, a possible approach to improve the performance of these dyes would be to enhance the rate of ISC in this newly formed triplet biradical (i.e., reduce its lifetime). Mechanistic transient absorption studies with Cy3B have evaluated the ability of different reducing agents/triplet-state-quenchers (such as ascorbic acid, *n*-propyl gallate, Trolox, and BME) to assist in ISC in the newly formed geminate radical pair formed following PET. Studies involved comparing the extent of geminate recombination versus radical escape from the geminate radical ion pair in solution.[34] Similar mechanistic approaches could be used to screen different redox-active photostabilizers to identify promising candidates for self-healing dyes capable of enhancing the ISC rate via different interactions (e.g., spin–orbit coupling or hyperfine interactions). Given the almost quantitative geminate recombination for the thyil radical for solution-based photostabilization with BME, thiol containing antioxidants could be of potential interest. Furthermore, the lifetime of the triplet biradical intermediate has been shown to be quite sensitive to the length of the linker between the two radical centers.

Several approaches have been reported to improve photophysical properties of the fluorophores, including by protein partner,[84,85] through fluorination,[86,87] and by making dendrimers or nanoconjugates.[75,88−90] For the self-healing mechanism, the excited triplet-state quenching via Dexter-type energy transfer is considered to be more efficient for various fluorophore scaffolds, because it has little impact on singlet-state quenching. The smaller energy gap between the excited triplet state and ground state of the photostabilizer imparts the quicker rate of the triplet-state quenching via Dexter-type energy transfer. From previous research, COT has shown significant improvement in the fluorescence performance of the conjugated fluorophores. COT-based derivatives with even more extended conjugated π-system would have even lower ΔG and are predicted to largely contribute to the energy transfer for triplet-state quenching. However, these chemicals have poor solubility in water, which limits their further conjugation and application. To solve this problem, we propose utilizing host–guest chemistry.[91−93] Cucurbiturils would be promising hosts for the complexation of organic

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

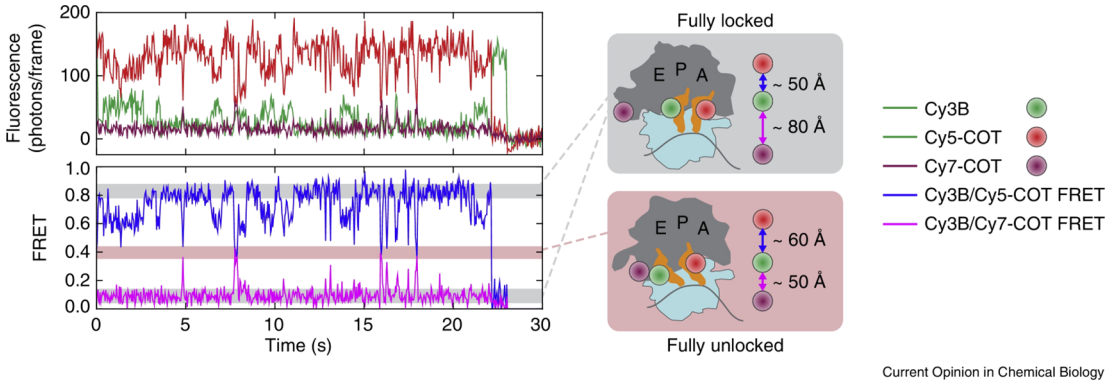


Figure 12. Multicolor smFRET with self-healing fluorophores. Ribosomal pretranslocation complexes were labeled as shown (cartoon) on tRNA molecules and ribosomal protein L1 and imaged as described previously.[83] Fluorescence (top) and FRET traces (bottom, defined here as acceptor fluorescence divided by total fluorescence) of labeled pretranslocation complex imaged at 40 ms time resolution during continuous laser excitation (120 mW, 532 nm wavelength). Reprinted from ref 46 with permission. Copyright 2014 Elsevier.

guests because of their ability to internalize alkyl chains or cyclobenzenes within its hydrophobic cavity formed by carbonyl groups.[91] As illustrated in Figure 13, cucurbituril has a large enough cavity to encapsulate photostabilizers and fluorescent dyes simultaneously and is known to improve dye brightness and photostability.[91] It is expected that such a complex of cucurbituril, photostabilizer and fluorophore can have superior photophysical properties. This idea may explore the possibilities of other currently unavailable mechanisms for novel self-healing dyes.

An alternative approach to improve the photophysical properties of dyes is to enhance their excitation and radiative rate via the help of plasmonic nanostructures, such as noble metal nanoparticles.[94−97] The increased radiative rate of dyes in the vicinity of plasmonic nanostructures and, in particular, in the plasmonic hotspots, reduces the time spent in the excited state and, hence, increases the total number of fluorescence photons. One of the main difficulties in utilizing plasmonics for this purpose is the challenge of achieving a precise and stoichiometric control of the distance between the fluorophore and the plasmonic nanostructure. Nevertheless, in recent years the DNA origami technique[97] has been elegantly exploited to circumvent this challenge. For example, up to 30-fold increase in photon output has been reported for dyes placed in the vicinity of nanoparticles.[97,98] The combination of these two approaches, i.e., plasmonics and photostabilization via a self-healing approach, may prove extremely beneficial when maximizing the photon count rates that can be achieved from a single fluorescent dye while enabling the study of the conformational changes of biomolecules on the time scales previously unattainable by typical single-molecule fluorescence experiments.

Alternative Fluorophores. When it comes to designing improved and even more robust self-healing fluorophores, one could also envision testing fluorophores that possess superior photophysical properties, such as low intersystem crossing yield or optimized fluorescence quantum yield (azetidine dyes developed by the Lavis group[99]). One could also combine the self-healing approach with other photostabilization strategies. For example, fluorophore scaffold modifications with electron-withdrawing substituents (such as fluorine) have been shown to improve the photostability of certain cyanine, rhodamine, as well as coumarine dyes because of the reduced susceptibility of fluorophores to reaction with singlet oxygen.[84−87,100−102] Another type of dye instability that is not discussed as often is spectral instability, i.e., photoswitching to dim or spectrally shifted states.[98] An approach to overcome this spectral instability in the rhodamine class of dyes by covalent modification of their scaffold has been recently proposed by the Hell group[103] and could be of

The combination of two approaches, i.e., plasmonics and photostabilization via a self-healing approach, may prove extremely beneficial when maximizing the photon count rates that can be achieved from a single fluorescent dye while enabling the study of the conformational changes of biomolecules on the time scales previously unattainable by typical single-molecule fluorescence experiments.

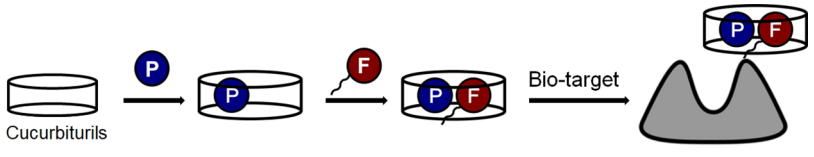


Figure 13. Illustration scheme of host−guest complexation of cucurbiturils with fluorophores and photostabilizers and their further conjugation to biomolecules.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

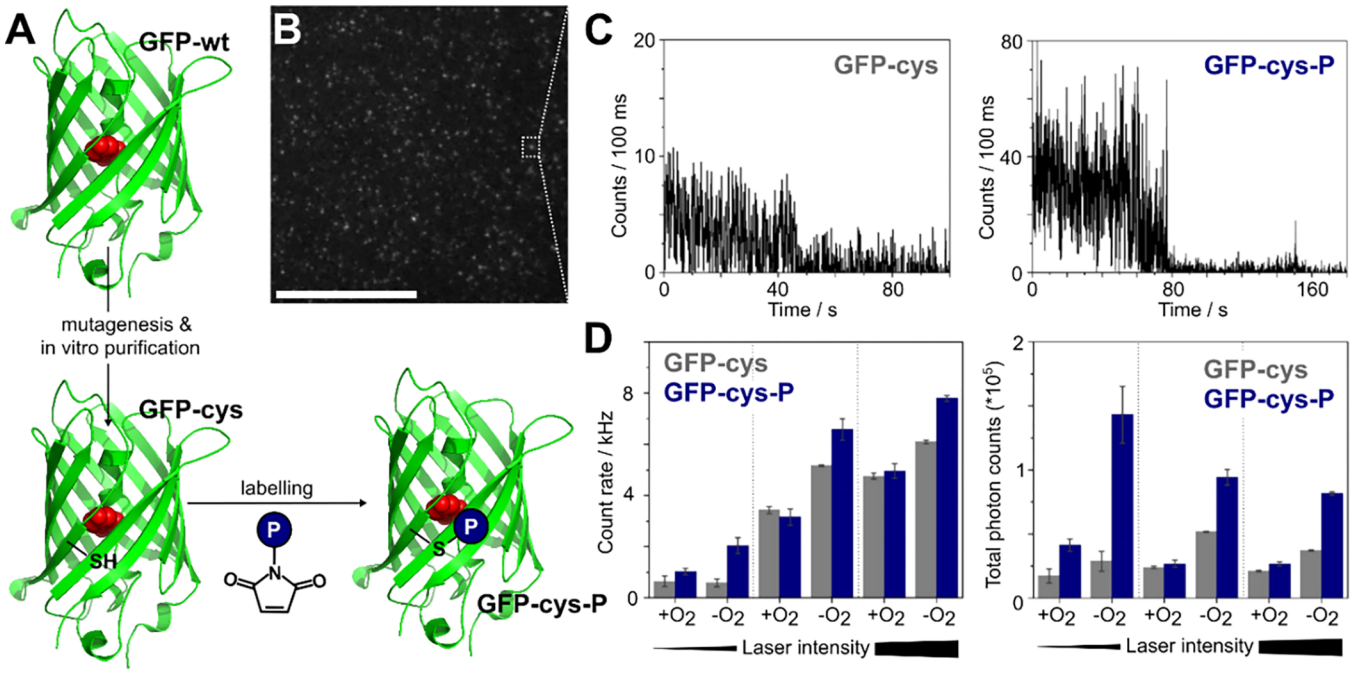


Figure 14. (A) Experimental strategy for the development of fluorescent proteins with covalently linked photostabilizers outside the ß-barrel via cysteine labeling using reactive photostabilizer derivatives P. (B–D) Fluorescence characterization of individual GFPs: (B) image with 10 μm scale bar and (C) single-molecule time traces of individual GFP. (D) Quantitative photophysical parameters of GFP in the presence and absence of covalently linked azobenzene; further parameters investigated were laser intensity (0.4, 2.0, and 3.2 kW/cm^{-2}) and the presence and absence of molecular oxygen. Data are from ref 104.

potential interest when designing even more optimized self-healing dyes for single-molecule fluorescence experiments.

Another exciting question would be whether intramolecular photostabilization can be used for fluorescent proteins (FP). A major hurdle to characterize FPs is a more complex photophysical behavior, where not only the properties of the chromophore but also other factors such as the protein barrel and specific environment will play a huge role for the observed properties. Often complex photochemical reaction pathways exist that might not render the triplet state of FPs the main problem in their photophysics. To test intramolecular photostabilization on FPs, one fundamental problem relates to how the photostabilizer could be introduced to the ß-barrel of the protein (Figure 14A). While certain unnatural amino acids exist that provide a nitrophenyl group, the experimental realization of such a strategy will present different challenges (protein expression levels, correct folding, etc.).

The Cordes lab has recently taken first steps toward testing GFP-variants containing covalently linked photostabilizers. For this, a GFP double cysteine mutant was labeled and analyzed with photostabilizer moieties in the vicinity of the chromophore, yet with no direct contact between the two (Figure 14A). This protein was taken as a basis for external labeling of the GFP barrel with photostabilizer-maleimides of TX, NPA, COT, and azobenzene.[104]

In this recent study, it could be shown that a mutant of α-GFP, mutant GFP-QC2 (C48S, A206C, L221C), features substantial increases in photostability (Figure 14C) upon conjugation of azobenzene to the two cysteines. Count rate and total detected photon numbers were derived from TIRF movies of individual GFP molecules, using methods as reported previously, and showed that azobenzene can improve these properties up to 5-fold at intermediate laser excitation power, yet mostly in the absence of oxygen (Figure 14D). The effects were also not related to a change in redox state of the two close-by cysteines (A206C, L221C) because labeling with other photostabilizer-maleimides did not have such an impact (Figure S2), and the GFP variant's fluorescence quantum yield was insensitive to the presence of reducing agents. The mechanistic basis of the improvement could not yet be related directly to triplet-state quenching; however, the results support the idea that also FPs might be within the scope of self-healing dye technology. This can even be done by rational choice of triplet-state quenchers based on recent reports on the triplet properties of FPs.[105]

Alternative Conjugation Strategies. A remaining fundamental hurdle is to synthesize and characterize self-healing dyes with appropriate reactive sites for various biomolecules while keeping the stabilizer at the right distance. Blanchard and co-workers sell a selected number of self-healing cyanine dyes (www.lumidynetechnologies.com). There is, however, no commercial solution which is generally applicable to all dyes and may allow commercial dyes to be upgraded to a self-healing dye. As shown in Figure 6, self-healing dyes are always premodified with the photostabilizer to allow biolabeling. Although the Cordes lab introduced a general approach using unnatural amino acid-based photostabilizers to generate self-healing constructs, these are not commercially available yet, require a demanding synthesis procedure, and consume large amounts of expensive commercial dyes during preparation. Even the proximity of photostabilizers to the fluorophores through DNA hybridization can reduce the chemical synthesis to some extent, but this has very limited applicability. We think one key step for advancement of self-healing dyes is an easy, universal, and modular approach that transforms normal dyes into self-healing dyes once they bind the biotarget. The procedure should be based on established linking chemistry (mostly click chemistry) and should be integrated into existing

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462–4480

protocols, thus requiring only commercially available fluorophores.

In conclusion, this Perspective summarized the current state of the art of self-healing dyes as a new promising class of fluorescent labels. We provided details on their historical development, highlighted the established building blocks of self-healing dyes and mechanistic understanding, and compared the performance to other photostabilization approaches. Besides a description of their most recent applications in high-resolution, live-cell and single-molecule imaging, we also detailed remaining key limitations: (i) How could mechanistic understanding further advance photostabilization efficiency of self-healing dyes, especially accounting for different biochemical environments and the presence of molecular oxygen? (ii) What requirements do we have in terms of bioconjugation for self-healing dyes? Finally, we discussed new data that contributed toward solving these problems showing fluorophore redox-blinking in double-stranded DNA with differing base sequences and summarized a recently published study toward the use of intramolecular photostabilization for fluorescent proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.9b03833.

Materials and methods and additional data for Figures 13 and 14 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Thorben Cordes − *Physical and Synthetic Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, 82152 Planegg-Martinsried, Germany;* orcid.org/0000-0002-8598-5499; Email: cordes@bio.lmu.de

Authors

Michael Isselstein − *Physical and Synthetic Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, 82152 Planegg-Martinsried, Germany*

Lei Zhang − *Physical and Synthetic Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, 82152 Planegg-Martinsried, Germany*

Viktorija Glembockyte − *Department of Chemistry and Center for NanoScience, Ludwig-Maximilians-Universität München, E 81377 München, Germany; Department of Chemistry and Quebec Centre for Applied Materials (QCAM), McGill University, H3A 0B8 Montreal, Quebec, Canada;* orcid.org/0000-0003-2531-6506

Oliver Brix − *Physical and Synthetic Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, 82152 Planegg-Martinsried, Germany*

Gonzalo Cosa − *Department of Chemistry and Quebec Centre for Applied Materials (QCAM), McGill University, H3A 0B8 Montreal, Quebec, Canada;* orcid.org/0000-0003-0064-1345

Philip Tinnefeld − *Department of Chemistry and Center for NanoScience, Ludwig-Maximilians-Universität München, E 81377 München, Germany;* orcid.org/0000-0003-4290-7770

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jpclett.9b03833

Author Contributions

#M.I., L.Z., and V.G. contributed equally to this work.

Notes

The authors declare no competing financial interest.

Biographies

Michael Isselstein studied physics (B.Sc. and M.Sc.) at the Ludwig-Maximilians-Universität Münich (Germany) with a focus on biophysics and microscopy. He has been working as a Ph.D. student in the lab of Thorben Cordes since 2017. His research is focused on developing novel fluorescent probes and methods for single-molecule studies of membrane transporters.

Lei Zhang is a Humboldt Research Fellow at Ludwig-Maximilians-Universität München (Germany). She studied chemistry at Nanjing Normal University (China) and obtained a M.S. degree in chemistry from Xiamen University (China) in 2012 and a Ph.D. degree in Chemistry from Nanjing University (China) in 2015. Her current research interests are the development of novel fluorescent probes and biophysical assays.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

Viktorija Glembockyte is currently a Humboldt Research Fellow at Ludwig-Maximilians-Universität München (Germany). She studied Chemistry at Jacobs University Bremen (Germany) and obtained a Ph.D. in chemistry from McGill University (Canada) in 2017. During her Ph.D. studies she investigated photophysical properties of fluorophores used for single-molecule fluorescence imaging applications and developed strategies to improve the photostability of fluorophores. In her current research she combines the advantages of DNA nanotechnology and single-molecule fluorescence imaging for the development of diagnostic tools and tunable biosensors.

Oliver Brix is a Ph.D. student within the research group "Physical and Synthetic Biology" at Ludwig-Maximilians-Universität München (Germany). He studied physics at the LMU Munich and the University of Cambridge in the UK. Currently, his main research interests are the advancement of fluorophore photostability and characterizing photodamage in DNA.

Gonzalo Cosa received his Licenciate in chemistry from Universidad Nacional de Rio Cuarto, Argentina, in 1996. He went on to pursue a Ph.D. at the University of Ottawa. He was a postdoctoral fellow at the University of Texas at Austin. In 2005, he joined the Department of Chemistry at McGill University. His current research centers on designing, preparing, and utilizing smart fluorescent probes for live cell-imaging and on applying state-of-the-art single-molecule fluorescence methodologies to study protein/DNA/lipid interactions.

Philip Tinnefeld has been a Professor of Physical Chemistry at Ludwig-Maximilians-University since 2017. He studied chemistry in Münster and Heidelberg and received his Ph.D. from the University of Heidelberg in 2002. After postdoctoral work with research stays at UCLA (United States) and Leuven (Belgium) and habilitation in physics at Bielefeld University, he became associate professor of biophysics at Ludwig-Maximilians-Universität Munich. In 2010, he was appointed full professor of biophysical chemistry at Braunschweig University of Technology. Philip Tinnefeld's research is inspired by our emerging abilities to study and build matter bottom-up, starting from single molecules. He has contributed to breakthroughs of single-molecule superresolution microscopy, and he combined optical single-molecule detection with DNA nanotechnology for self-assembled, functional devices including energy-transfer switches, calibration nanorulers, nanoadapters, fluorescence signal amplifiers, and molecular force clamps. Dr. Tinnefeld has authored more than 160 publications and patents. He was initiator of GATTAquant GmbH, the first company commercializing DNA origami applications.

Thorben Cordes is currently a Professor for "Physical and Synthetic Biology" at Ludwig-Maximilians-Universität München (Germany). He studied chemistry at TU Braunschweig (Germany) and obtained a Ph.D. in Physics from the Ludwig-Maximilians-Universität München (Germany) in 2008. As a postdoctoral researcher he worked in Munich (Germany) and Oxford (UK), where he applied single-molecule and super-resolution fluorescence microscopy techniques to various biological questions. From 2011 to 2017 he was a tenure-track Assistant Professor and later tenured Associate Professor at the Zernike Institute for Advanced Materials at the University of Groningen (The Netherlands). His research interests are structure–function relationships and molecular mechanisms of membrane transporters and the development of novel photophysical assays as well as fluorescent probes.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

■ ACKNOWLEDGMENTS

This work was financed by Deutsche Forschungsgemeinschaft (SFB863, project A13; cluster of excellence CiPSM, LMUexcellent), an ERC Starting Grant (No. 638536 − SM-IMPORT to T.C.) and by the Center of Nanoscience Munich (CENS). P.T. gratefully acknowledges financial support from the DFG (excellence cluster e-conversion), BMBF (Grant SIBOF, 03VP03891), and the European Union's Horizon 2020 research and innovation program under grant agreement No. 737089 (Chipscope). G.C. is grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Foundation for Innovation (CFI) for funding. L.Z. and V.G. thank the Alexander von Humboldt foundation for postdoctoral research fellowships. T.C. thanks J. S. Cordes for his patience while writing this manuscript, and J. H. Smit for useful comments.

■ REFERENCES

(1) Minoshima, M.; Kikuchi, K. Photostable and photoswitching fluorescent dyes for super-resolution imaging. *JBIC, J. Biol. Inorg. Chem.* **2017**, *22*, 639−652.
(2) Rasnik, I.; McKinney, S. A.; Ha, T. Nonblinking and long-lasting single-molecule fluorescence imaging. *Nat. Methods* **2006**, *3*, 891−893.
(3) Aitken, C. E.; Marshall, R. A.; Puglisi, J. D. An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. *Biophys. J.* **2008**, *94*, 1826−1835.
(4) Benesch, R. E.; Benesch, R. Enzymatic removal of oxygen for polarography and related methods. *Science* **1953**, *118*, 447−448.
(5) Swoboda, M.; Henig, J.; Cheng, H.-M.; Brugger, D.; Haltrich, D.; Plumeré, N.; Schlierf, M. Enzymatic Oxygen Scavenging for Photostability without pH Drop in Single-Molecule Experiments. *ACS Nano* **2012**, *6*, 6364−6369.
(6) Ha, T.; Tinnefeld, P. Photophysics of fluorescent probes for single-molecule biophysics and super-resolution imaging. *Annu. Rev. Phys. Chem.* **2012**, *63*, 595−617.
(7) Vogelsang, J.; Kasper, R.; Steinhauer, C.; Person, B.; Heilemann, M.; Sauer, M.; Tinnefeld, P. A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes. *Angew. Chem., Int. Ed.* **2008**, *47*, 5465−5469.
(8) Targowski, P.; Ziętek, B.; Bączyński, A. Luminescence Quenching of Rhodamines by Cyclooctatetraene. *Z. Naturforsch., A: Phys. Sci.* **1987**, *42*, 1009.
(9) Heupel, M.; Gregor, I.; Becker, S.; Thiel, E. Photophysical and photochemical properties of electronically excited fluorescent dyes: A new type of time-resolved laser-scanning spectroscopy. *Int. J. Photoenergy* **1999**, *1*, 165−172.
(10) Dave, R.; Terry, D. S.; Munro, J. B.; Blanchard, S. C. Mitigating unwanted photophysical processes for improved single-molecule fluorescence imaging. *Biophys. J.* **2009**, *96*, 2371−2381.
(11) Pfiffi, D.; Bier, B. A.; Marian, C. M.; Schaper, K.; Seidel, C. A. M. Diphenylhexatrienes as Photoprotective Agents for Ultrasensitive Fluorescence Detection. *J. Phys. Chem. A* **2010**, *114*, 4099−4108.
(12) Glembockyte, V.; Lincoln, R.; Cosa, G. Cy3 photoprotection mediated by Ni^{2+} for extended single-molecule imaging: old tricks for new techniques. *J. Am. Chem. Soc.* **2015**, *137*, 1116−1122.
(13) Glembockyte, V.; Lin, J.; Cosa, G. Improving the Photostability of Red- and Green-Emissive Single-Molecule Fluorophores via Ni(2+) Mediated Excited Triplet-State Quenching. *J. Phys. Chem. B* **2016**, *120*, 11923−11929.
(14) Smit, J. H.; van der Velde, J. H. M.; Huang, J.; Trauschke, V.; Henrikus, S. S.; Chen, S.; Eleftheriadis, N.; Warszawik, E. M.; Herrmann, A.; Cordes, T. On the impact of competing intra- and intermolecular triplet-state quenching on photobleaching and photoswitching kinetics of organic fluorophores. *Phys. Chem. Chem. Phys.* **2019**, *21*, 3721−3733.
(15) Cordes, T.; Vogelsang, J.; Tinnefeld, P. On the Mechanism of Trolox as Antiblinking and Antibleaching Reagent. *J. Am. Chem. Soc.* **2009**, *131*, 5018−5019.
(16) Vogelsang, J.; Cordes, T.; Forthmann, C.; Steinhauer, C.; Tinnefeld, P. Controlling the fluorescence of ordinary oxazine dyes for single-molecule switching and superresolution microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 8107−8112.
(17) Cordes, T.; Vogelsang, J.; Steinhauer, C.; Stein, I. H.; Forthmann, C.; Gietl, A.; Schmied, J. J.; Acuna, G. P.; Laurien, S.; Lalkens, B.; et al. Far-Field Nanoscopy with Conventional Fluorophores: Photostability, Photophysics, and Transient Binding. In *Far-Field Optical Nanoscopy*, Tinnefeld, P., Eggeling, C., Hell, S. W., Eds. Springer: Berlin, 2015; pp 215−242.
(18) van der Velde, J. H.; Uusitalo, J. J.; Ugen, L. J.; Warszawik, E. M.; Herrmann, A.; Marrink, S. J.; Cordes, T. Intramolecular photostabilization via triplet-state quenching: Design principles to make organic fluorophores "self-healing. *Faraday Discuss.* **2015**, *184*, 221−235.
(19) van der Velde, J. H.; Oelerich, J.; Huang, J.; Smit, J. H.; Aminian Jazi, A.; Galiani, S.; Kolmakov, K.; Gouridis, G.; Eggeling, C.; Herrmann, A.; et al. A simple and versatile design concept for fluorophore derivatives with intramolecular photostabilization. *Nat. Commun.* **2016**, *7*, 10144.
(20) Alejo, J. L.; Blanchard, S. C.; Andersen, O. S. Small-Molecule Photostabilizing Agents are Modifiers of Lipid Bilayer Properties. *Biophys. J.* **2013**, *104*, 2410−2418.
(21) Cordes, T.; Santoso, Y.; Tomescu, A. I.; Gryte, K.; Hwang, L. C.; Camara, B.; Wigneshweraraj, S.; Kapanidis, A. N. Sensing DNA opening in transcription using quenchable Forster resonance energy transfer. *Biochemistry* **2010**, *49*, 9171−9180.
(22) Altman, R. B.; Terry, D. S.; Zhou, Z.; Zheng, Q.; Geggier, P.; Kolster, R. A.; Zhao, Y.; Javitch, J. A.; Warren, J. D.; Blanchard, S. C. Cyanine fluorophore derivatives with enhanced photostability. *Nat. Methods* **2012**, *9*, 68−71.
(23) Zheng, Q.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Warren, J. D.; Turro, N. J.; Blanchard, S. C. On the Mechanisms of Cyanine Fluorophore Photostabilization. *J. Phys. Chem. Lett.* **2012**, *3*, 2200−2203.
(24) van der Velde, J. H.; Ploetz, E.; Hiermaier, M.; Oelerich, J.; de Vries, J. W.; Roelfes, G.; Cordes, T. Mechanism of intramolecular photostabilization in self-healing cyanine fluorophores. *ChemPhysChem* **2013**, *14*, 4084−4093.
(25) Zheng, Q.; Juette, M. F.; Jockusch, S.; Wasserman, M. R.; Zhou, Z.; Altman, R. B.; Blanchard, S. C. Ultra-stable organic fluorophores for single-molecule research. *Chem. Soc. Rev.* **2014**, *43*, 1044−1056.
(26) Liphardt, B.; Liphardt, B.; Lüttke, W. Laser dyes III: Concepts to increase the photostability of laser dyes. *Opt. Commun.* **1983**, *48*, 129−133.
(27) Liphardt, B.; Liphardt, B.; Lüttke, W. Laser dyes with intramolecular triplet quenching. *Opt. Commun.* **1981**, *38*, 207−210.
(28) Altman, R. B.; Zheng, Q.; Zhou, Z.; Terry, D. S.; Warren, J. D.; Blanchard, S. C. Enhanced photostability of cyanine fluorophores across the visible spectrum. *Nat. Methods* **2012**, *9*, 428−429.
(29) Tinnefeld, P.; Cordes, T. Self-healing" dyes: Intramolecular stabilization of organic fluorophores. *Nat. Methods* **2012**, *9*, 426−427 (author reply, pp 427−428).
(30) Glembockyte, V.; Wieneke, R.; Gatterdam, K.; Gidi, Y.; Tampe, R.; Cosa, G. *Tris-N*-Nitrilotriacetic Acid Fluorophore as a Self-Healing Dye for Single-Molecule Fluorescence Imaging. *J. Am. Chem. Soc.* **2018**, *140*, 11006−11012.
(31) Zheng, Q.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Zhao, H.; Asher, W.; Holsey, M.; Mathiasen, S.; Geggier, P.; Javitch, J. A.; et al. Electronic tuning of self-healing fluorophores for live-cell and single-molecule imaging. *Chem. Sci.* **2017**, *8*, 755−762.
(32) van der Velde, J. H.; Oelerich, J.; Huang, J.; Smit, J. H.; Hiermaier, M.; Ploetz, E.; Herrmann, A.; Roelfes, G.; Cordes, T. The Power of Two: Covalent Coupling of Photostabilizers for Fluorescence Applications. *J. Phys. Chem. Lett.* **2014**, *5*, 3792−3798.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

(33) Zheng, Q.; Jockusch, S.; Rodriguez-Calero, G. G.; Zhou, Z.; Zhao, H.; Altman, R. B.; Abruna, H. D.; Blanchard, S. C. Intramolecular triplet energy transfer is a general approach to improve organic fluorophore photostability. *Photochem. Photobiol. Sci.* **2016**, *15*, 196−203.
(34) Glembockyte, V.; Cosa, G. Redox-Based Photostabilizing Agents in Fluorescence Imaging: The Hidden Role of Intersystem Crossing in Geminate Radical Ion Pairs. *J. Am. Chem. Soc.* **2017**, *139*, 13227−13233.
(35) van der Velde, J. H. M.; Smit, J. H.; Hebisch, E.; Punter, M.; Cordes, T. Self-healing dyes for super-resolution fluorescence microscopy. *J. Phys. D: Appl. Phys.* **2019**, *52*, 034001.
(36) Schäfer, F. P.; Zhang, F.-G.; Jethwa, J. Intramolecular TT-energy transfer in bifluorophoric laser dyes. *Appl. Phys. B: Photophys. Laser Chem.* **1982**, *28*, 37−41.
(37) Li, T.; Liu, L.; Jing, T.; Ruan, Z.; Yuan, P.; Yan, L. Self-Healing Organic Fluorophore of Cyanine-Conjugated Amphiphilic Polypeptide for Near-Infrared Photostable Bioimaging. *ACS Appl. Mater. Interfaces* **2018**, *10*, 14517−14530.
(38) Zheng, Q.; Jockusch, S.; Zhou, Z.; Blanchard, S. C. The Contribution of Reactive Oxygen Species to the Photobleaching of Organic Fluorophores. *Photochem. Photobiol.* **2014**, *90*, 448−454.
(39) Stolow, A.; Bragg, A. E.; Neumark, D. M. Femtosecond Time-Resolved Photoelectron Spectroscopy. *Chem. Rev.* **2004**, *104*, 1719−1758.
(40) Burda, C.; El-Sayed, M. A. High-density femtosecond transient absorption spectroscopy of semiconductor nanoparticles. A tool to investigate surface quality. *Pure Appl. Chem.* **2000**, *72*, 165−177.
(41) de Boer, M.; Gouridis, G.; Vietrov, R.; Begg, S. L.; Schuurman-Wolters, G. K.; Husada, F.; Eleftheriadis, N.; Poolman, B.; McDevitt, C. A.; Cordes, T. Conformational and dynamic plasticity in substrate-binding proteins underlies selective transport in ABC importers. *eLife* **2019**, *8*, No. e44652.
(42) Gouridis, G.; Schuurman-Wolters, G. K.; Ploetz, E.; Husada, F.; Vietrov, R.; de Boer, M.; Cordes, T.; Poolman, B. Conformational dynamics in substrate-binding domains influences transport in the ABC importer GlnPQ. *Nat. Struct. Mol. Biol.* **2015**, *22*, 57−64.
(43) Roy, R.; Hohng, S.; Ha, T. A practical guide to single-molecule FRET. *Nat. Methods* **2008**, *5*, 507−516.
(44) Wennmalm, S.; Edman, L.; Rigler, R. Conformational fluctuations in single DNA molecules. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 10641−10646.
(45) Sirbu, D.; Woodford, O. J.; Benniston, A. C.; Harriman, A. Photocatalysis and self-catalyzed photobleaching with covalently-linked chromophore-quencher conjugates built around BOPHY. *Photochem. Photobiol. Sci.* **2018**, *17*, 750−762.
(46) Juette, M. F.; Terry, D. S.; Wasserman, M. R.; Zhou, Z.; Altman, R. B.; Zheng, Q.; Blanchard, S. C. The bright future of single-molecule fluorescence imaging. *Curr. Opin. Chem. Biol.* **2014**, *20*, 103−111.
(47) Weller, A. Photoinduced Electron Transfer in Solution: Exciplex and Radical Ion Pair Formation Free Enthalpies and their Solvent Dependence. *Z. Phys. Chem.* **1982**, *133*, 93.
(48) Chandler, D. Electron transfer in water and other polar environments, how it happens. In *Classical and Quantum Dynamics in Condensed Phase Simulations*; Berne, B. J., Ciccotti, G., Coker, D. F., Eds.; World Scientific: Singapore, 1998; pp 25−49.
(49) Holzmeister, P.; Gietl, A.; Tinnefeld, P. Geminate Recombination as a Photoprotection Mechanism for Fluorescent Dyes. *Angew. Chem., Int. Ed.* **2014**, *53*, 5685−5688.
(50) Turro, N. J. *Modern Molecular Photochemistry*; University Science Books: Mill Valley, CA, 1991.
(51) Frutos, L.-M.; Castaño, O.; Merchán, M. Theoretical Determination of the Singlet - Singlet and Singlet - Triplet Electronic Spectra, Lowest Ionization Potentials, and Electron Affinity of Cyclooctatetraene. *J. Phys. Chem. A* **2003**, *107*, 5472−5478.
(52) Frutos, L. M.; Castano, O.; Andrés, J. L.; Merchán, M.; Acuna, A. U. A theory of nonvertical triplet energy transfer in terms of accurate potential energy surfaces: The transfer reaction from π,π^* triplet donors to 1,3,5,7-cyclooctatetraene. *J. Chem. Phys.* **2004**, *120*, 1208−1216.
(53) Wagner, P. J.; Klán, P. Intramolecular Triplet Energy Transfer in Flexible Molecules: Electronic, Dynamic, and Structural Aspects. *J. Am. Chem. Soc.* **1999**, *121*, 9626−9635.
(54) Hell, S. W.; Sahl, S. J.; Bates, M.; Zhuang, X.; Heintzmann, R.; Booth, M. J.; Bewersdorf, J.; Shtengel, G.; Hess, H.; Tinnefeld, P.; et al. The 2015 super-resolution microscopy roadmap. *J. Phys. D: Appl. Phys.* **2015**, *48*, 443001.
(55) Heilemann, M.; Margeat, E.; Kasper, R.; Sauer, M.; Tinnefeld, P. Carbocyanine Dyes as Efficient Reversible Single-Molecule Optical Switch. *J. Am. Chem. Soc.* **2005**, *127*, 3801−3806.
(56) Bates, M.; Blosser, T. R.; Zhuang, X. Short-range spectroscopic ruler based on a single-molecule optical switch. *Phys. Rev. Lett.* **2005**, *94*, 108101.
(57) van de Linde, S.; Löschberger, A.; Klein, T.; Heidbreder, M.; Wolter, S.; Heilemann, M.; Sauer, M. Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nat. Protoc.* **2011**, *6*, 991−1009.
(58) Vaughan, J. C.; Dempsey, G. T.; Sun, E.; Zhuang, X. Phosphine Quenching of Cyanine Dyes as a Versatile Tool for Fluorescence Microscopy. *J. Am. Chem. Soc.* **2013**, *135*, 1197−1200.
(59) Heilemann, M.; van de Linde, S.; Mukherjee, A.; Sauer, M. Super-Resolution Imaging with Small Organic Fluorophores. *Angew. Chem., Int. Ed.* **2009**, *48*, 6903−6908.
(60) Rust, M. J.; Bates, M.; Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **2006**, *3*, 793−795.
(61) Bates, M.; Blosser, T. R.; Zhuang, X. Short-range spectroscopic ruler based on a single-molecule optical switch. *Phys. Rev. Lett.* **2005**, *94*, 108101.
(62) Dempsey, G. T.; Vaughan, J. C.; Chen, K. H.; Bates, M.; Zhuang, X. Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging. *Nat. Methods* **2011**, *8*, 1027−1036.
(63) Lakowicz, J. R.; Weber, G. Quenching of fluorescence by oxygen. Probe for structural fluctuations in macromolecules. *Biochemistry* **1973**, *12*, 4161−4170.
(64) Monti, S.; Gardini, E.; Bortolus, P.; Amouyal, E. The triplet state of azobenzene. *Chem. Phys. Lett.* **1981**, *77*, 115−119.
(65) Levitus, M.; Ranjit, S. Cyanine dyes in biophysical research: the photophysics of polymethine fluorescent dyes in biomolecular environments. *Q. Rev. Biophys.* **2011**, *44*, 123−151.
(66) Sanborn, M. E.; Connolly, B. K.; Gurunathan, K.; Levitus, M. Fluorescence Properties and Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA. *J. Phys. Chem. B* **2007**, *111*, 11064−11074.
(67) Doose, S.; Neuweiler, H.; Sauer, M. Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules. *ChemPhysChem* **2009**, *10*, 1389−1398.
(68) Seidel, C. A. M.; Schulz, A.; Sauer, M. H. M. Nucleobase-Specific Quenching of Fluorescent Dyes. 1. Nucleobase One-Electron Redox Potentials and Their Correlation with Static and Dynamic Quenching Efficiencies. *J. Phys. Chem.* **1996**, *100*, 5541−5553.
(69) Heinlein, T.; Knemeyer, J.-P.; Piestert, O.; Sauer, M. Photoinduced electron transfer between fluorescent dyes and guanosine residues in DNA-hairpins. *J. Phys. Chem. B* **2003**, *107*, 7957−7964.
(70) Vogelsang, J.; Cordes, T.; Tinnefeld, P. Single-molecule photophysics of oxazines on DNA and its application in a FRET switch. *Photochem. Photobiol. Sci.* **2009**, *8*, 486−496.
(71) Spiriti, J.; Binder, J. K.; Levitus, M.; van der Vaart, A. Cy3-DNA stacking interactions strongly depend on the identity of the terminal basepair. *Biophys. J.* **2011**, *100*, 1049−1057.
(72) Ranjit, S.; Levitus, M. Probing the interaction between fluorophores and DNA nucleotides by fluorescence correlation spectroscopy and fluorescence quenching. *Photochem. Photobiol.* **2012**, *88*, 782−791.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480

(73) Sánchez-Rico, C.; Voith von Voithenberg, L.; Warner, L.; Lamb, D. C.; Sattler, M. Effects of Fluorophore Attachment on Protein Conformation and Dynamics Studied by spFRET and NMR Spectroscopy. *Chem. - Eur. J.* **2017**, *23*, 14267−14277.
(74) Tsunoyama, T. A.; Watanabe, Y.; Goto, J.; Naito, K.; Kasai, R. S.; Suzuki, K. G. N.; Fujiwara, T. K.; Kusumi, A. Super-long single-molecule tracking reveals dynamic-anchorage-induced integrin function. *Nat. Chem. Biol.* **2018**, *14*, 497−506.
(75) Reilly, D. T.; Kim, S. H.; Katzenellenbogen, J. A.; Schroeder, C. M. Fluorescent Nanoconjugate Derivatives with Enhanced Photostability for Single Molecule Imaging. *Anal. Chem.* **2015**, *87*, 11048−11057.
(76) García-López, V.; Jeffet, J.; Kuwahara, S.; Martí, A.; Ebenstein, Y.; Tour, J. Synthesis and Photostability of Unimolecular Submersible Nanomachines: Toward Single-Molecule Tracking in Solution. *Org. Lett.* **2016**, *18*, 2343−2346.
(77) Cordes, T.; Maiser, A.; Steinhauer, C.; Schermelleh, L.; Tinnefeld, P. Mechanisms and advancement of antifading agents for fluorescence microscopy and single-molecule spectroscopy. *Phys. Chem. Chem. Phys.* **2011**, *13*, 6699−6709.
(78) Danzl, J. G.; Sidenstein, S. C.; Gregor, C.; Urban, N. T.; Ilgen, P.; Jakobs, S.; Hell, S. W. Coordinate-targeted fluorescence nanoscopy with multiple off states. *Nat. Photonics* **2016**, *10*, 122−128.
(79) Balzarotti, F.; Eilers, Y.; Gwosch, K. C.; Gynnå, A. H.; Westphal, V.; Stefani, F. D.; Elf, J.; Hell, S. W. Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science* **2017**, *355*, 606−612.
(80) Lerner, E.; Cordes, T.; Ingargiola, A.; Alhadid, Y.; Chung, S.; Michalet, X.; Weiss, S. Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer. *Science* **2018**, *359*, No. eaan1133.
(81) Hohlbein, J.; Craggs, T. D.; Cordes, T. Alternating-laser excitation: single-molecule FRET and beyond. *Chem. Soc. Rev.* **2014**, *43*, 1156−1171.
(82) Kapanidis, A.; Majumdar, D.; Heilemann, M.; Nir, E.; Weiss, S. Alternating Laser Excitation for Solution-Based Single-Molecule FRET. *Cold Spring Harb. Protocols.* **2015**, *2015*, pdb.top086405.
(83) Munro, J. B.; Altman, R. B.; Tung, C.-S.; Cate, J. H. D.; Sanbonmatsu, K. Y.; Blanchard, S. C. Spontaneous formation of the unlocked state of the ribosome is a multistep process. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (2), 709−714.
(84) Shank, N. I.; Zanotti, K. J.; Lanni, F.; Berget, P. B.; Armitage, B. A. Enhanced Photostability of Genetically Encodable Fluoromodules Based on Fluorogenic Cyanine Dyes and a Promiscuous Protein Partner. *J. Am. Chem. Soc.* **2009**, *131*, 12960−12969.
(85) Shank, N. I.; Pham, H. H.; Waggoner, A. S.; Armitage, B. A. Twisted Cyanines: A Non-Planar Fluorogenic Dye with Superior Photostability and Its Use in a Protein-Based Fluoromodule. *J. Am. Chem. Soc.* **2013**, *135*, 242−251.
(86) Renikuntla, B. R.; Rose, H. C.; Eldo, J.; Waggoner, A. S.; Armitage, B. A. Improved Photostability and Fluorescence Properties through Polyfluorination of a Cyanine Dye. *Org. Lett.* **2004**, *6*, 909−912.
(87) Funabiki, K.; Yagi, K.; Ueta, M.; Nakajima, M.; Horiuchi, M.; Kubota, Y.; Mastui, M. Rational Molecular Design and Synthesis of Highly Thermo- and Photostable Near-Infrared-Absorbing Heptamethine Cyanine Dyes with the Use of Fluorine Atoms. *Chem. - Eur. J.* **2016**, *22*, 12282−12285.
(88) Yang, S. K.; Shi, X.; Park, S.; Ha, T.; Zimmerman, S. C. A dendritic single-molecule fluorescent probe that is monovalent, photostable and minimally blinking. *Nat. Chem.* **2013**, *5*, 692−697.
(89) Yang, S. K.; Shi, X.; Park, S.; Doganay, S.; Ha, T.; Zimmerman, S. C. Monovalent, Clickable, Uncharged, Water-Soluble Perylenediimide-Cored Dendrimers for Target-Specific Fluorescent Biolabeling. *J. Am. Chem. Soc.* **2011**, *133*, 9964−9967.
(90) Redy-Keisar, O.; Huth, K.; Vogel, U.; Lepenies, B.; Seeberger, P. H.; Haag, R.; Shabat, D. Enhancement of fluorescent properties of near-infrared dyes using clickable oligoglycerol dendrons. *Org. Biomol. Chem.* **2015**, *13*, 4727−4732.
(91) Mohanty, J.; Nau, W. M. Ultrastable Rhodamine with Cucurbituril. *Angew. Chem., Int. Ed.* **2005**, *44*, 3750−3754.
(92) Guether, R.; Reddington, M. V. Photostable Cyanine Dye β-Cyclodextrin Conjugates. *Tetrahedron Lett.* **1997**, *38*, 6167−6170.
(93) Yau, C. M.; Pascu, S. I.; Odom, S. A.; Warren, J. E.; Klotz, E. J.; Frampton, M. J.; Williams, C. C.; Coropceanu, V.; Kuimova, M. K.; Phillips, D.; et al. Stabilisation of a heptamethine cyanine dye by rotaxane encapsulation. *Chem. Commun.* **2008**, 2897−2899.
(94) Sokolov, K.; Chumanov, G.; Cotton, T. M. Enhancement of Molecular Fluorescence Near the Surface of Colloidal Metal Films. *Anal. Chem.* **1998**, *70*, 3898−3905.
(95) Kawawaki, T.; Takahashi, Y.; Tatsuma, T. Enhancement of Dye-Sensitized Photocurrents by Gold Nanoparticles: Effects of Plasmon Coupling. *J. Phys. Chem. C* **2013**, *117*, 5901−5907.
(96) Zijlstra, P.; Orrit, M. Single metal nanoparticles: optical detection, spectroscopy and applications. *Rep. Prog. Phys.* **2011**, *74*, 106401.
(97) Acuna, G. P.; Moller, F. M.; Holzmeister, P.; Beater, S.; Lalkens, B.; Tinnefeld, P. Fluorescence enhancement at docking sites of DNA-directed self-assembled nanoantennas. *Science* **2012**, *338*, 506−510.
(98) Grabenhorst, L.; Trofymchuk, K.; Steiner, F.; Glembockyte, V.; Tinnefeld, P. Fluorophore photostability and saturation in the hotspot of DNA origami nanoantennas. *Methods Appl. Fluoresc.* **2020**, *8*, 024003.
(99) Grimm, J. B.; Muthusamy, A. K.; Liang, Y.; Brown, T. A.; Lemon, W. C.; Patel, R.; Lu, R.; Macklin, J. J.; Keller, P. J.; Ji, N.; et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat. Methods* **2017**, *14*, 987−994.
(100) Sun, W.-C.; Gee, K. R.; Haugland, R. P. Synthesis of novel fluorinated coumarins: Excellent UV-light excitable fluorescent dyes. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3107−3110.
(101) Samanta, A.; Vendrell, M.; Das, R.; Chang, Y.-T. Development of photostable near-infrared cyanine dyes. *Chem. Commun.* **2010**, *46*, 7406−7408.
(102) Su, D.; Teoh, C. L.; Samanta, A.; Kang, N.-Y.; Park, S.-J.; Chang, Y.-T. The development of a highly photostable and chemically stable zwitterionic near-infrared dye for imaging applications. *Chem. Commun.* **2015**, *51*, 3989−3992.
(103) Butkevich, A. N.; Bossi, M. L.; Lukinavičius, G. v.; Hell, S. W. Triarylmethane Fluorophores Resistant to Oxidative Photobluing. *J. Am. Chem. Soc.* **2019**, *141*, 981−989.
(104) Hendrikus, S.; Tassis, K.; Zhang, L.; van der Velde, J.; Gebhardt, C.; Herrmann, A.; Jung, G.; Cordes, T. Characterization of fluorescent proteins with intramolecular photostabilization. *bio Rxiv* **2020**, DOI: 10.1101/2020.03.07.980722.
(105) Byrdin, M.; Duan, C.; Bourgeois, D.; Brettel, K. A Long-Lived Triplet State Is the Entrance Gateway to Oxidative Photochemistry in Green Fluorescent Proteins. *J. Am. Chem. Soc.* **2018**, *140*, 2897−2905.

https://dx.doi.org/10.1021/acs.jpclett.9b03833
J. Phys. Chem. Lett. 2020, 11, 4462−4480