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# Unifying Mechanism for Thiol-Induced Photoswitching and Photostability of Cyanine Dyes

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fluorescence on Cy5 and Cy5B, transient-absorption spectroscopy, and DFT modeling on a range of cyanine dyes, herein we show that photoinduced electron transfer (PeT) from a thiolate to Cy in their triplet excited state and then triplet-to-singlet intersystem crossing in the nascent geminate radical pair are crucial steps. Next,



a bifurcation occurs, yielding either back electron transfer and regeneration of ground state Cy, required for photostabilization, or Cy-thiol adduct formation, necessary for super-resolution microscopy. Cy regeneration via photoinduced thiol elimination is favored by adduct absorption spectra broadening. Elimination is also shown to occur through an acid-catalyzed reaction. Overall, our work provides a roadmap for designing fluorophores, photoswitching agents, and triplet excited state quenchers for single-molecule and super-resolution imaging.

# INTRODUCTION

Single-molecule fluorescence (SMF) and related superresolution single-molecule localization microscopy  $(SMLM^{1-3})$  imaging methodologies have become transformative tools in biology, chemistry, and physics. Relying on monitoring single fluorophores over time, key to successful SMF experiments on immobilized substrates are high signal intensities and extended survival times. While conventional SMF techniques hinge on good intrinsic signal stability (e.g., single-molecule FRET and PIFE),<sup>4-6</sup> in turn, SMLM techniques require dyes to reversibly transition to dark transient states in photochemical reactions (photoswitching). Photoswitching thus allows restricting the number of active fluorophores in the illumination field in a given imaging cycle to achieve superresolution. Notably, given the opposing constraints for conventional SMF vs SMLM imaging, for extrinsic labeling, cyanine (Cy) dyes (Figure 1a and Figure S1) are *par excellence* the dyes utilized for both modalities when combined with an oxygen scavenger and the electron donor  $\beta$ -mercaptoethanol ( $\beta$ -ME).

The ability to control and optimize conventional SMF and SMLM imaging by judiciously steering the photoreaction pathway of cyanine dyes rests on the dichotomy between photostabilization vs photoswitching activities arising from  $\beta$ -ME. Missing is the molecular understanding reconciling seemingly divergent results on the photochemistry of cyanine dyes. Such a mechanism should account for the enhanced brightness and extended survival time conveyed by  $\beta$ -ME to Cy3 and Cy3B, the rigid trans-Cy3 (see Figure 1a for structures). The mechanism should also explain formation of a nonemissive photoproduct for structurally related dyes Cy5,<sup>3</sup> Cy5.5,<sup>7</sup> and Cy7,<sup>7</sup> as well as Alexa Fluor  $647^{7-10}$  and  $750^{10}$  (formally a thiolate addition to the iminium ion rendering a Cy-SR<sup>-</sup> adduct as shown by mass spectrometry studies,<sup>11</sup> Figure 1e). This mechanism should provide a rationale for restoring the emissive state through a competing photochemical<sup>8,12</sup> and thermal<sup>11</sup> pathway. The mechanism would also have to explain why the same level of reversible photoswitching in the presence of  $\beta$ -ME has not been observed with Cy3, Cy3B,<sup>13</sup> and Cy5B<sup>14</sup> (the rigid trans-Cy5).

Important information to consider in formulating a mechanism include the following: (i) The photostabilizing role of aliphatic thiols such as  $\beta$ -ME and  $\beta$ -mercaptoethylamine (MEA) results from quenching the triplet excited state of the fluorophore via photoinduced electron transfer (PeT).<sup>15</sup> Different electron donors such as Trolox,<sup>16,17</sup> n-propyl

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**Figure 1.** Structure and photoswitching mechanism of cyanine dyes. (a) Structures (see also Figure S1). (b) Mulliken charges and (c) Mulliken spin density for ground state and one-electron-reduced cyanines, respectively (exemplified with Cy5). (d) Proposed photoswitching mechanism of cyanines (Cy) in the presence of thiolates (RS<sup>-</sup>), illustrating relevant photophysical and photochemical processes leading to extended survival time. Rate constants for intersystem crossing  $k_{ISC}$ , forward photoinduced electron transfer (PeT) in the excited triplet state  $k_{PeT}^3$ , intersystem crossing in the geminate radical pair  $k_{ESC}^5$  and back electron transfer  $k_{BeT}$  are listed along a circular pathway regenerating Cy dyes and resulting in extended photostability (light gray background). A detour following ISC in the geminate radical pair in blue reflects formation of a Cy5-SR<sup>-</sup> adduct upon geminate radical combination (GRC) in competition with back electron transfer. Formation of the adduct is followed by either photoinduced or

thermal elimination  $(k_E^{f'})$  and regeneration of Cy (dark gray circular background). The geminate radical pair in the singlet manifold is formed directly, in parallel, following PeT quenching of the excited singlet state by thiolate  $(k_{PeT}^{1})$ ; see dashed line in violet. Competing photoprocesses include, in red, the formation of the nonfluorescent *cis*-Cy construct  $(k_{ISO})$ ; in green, the oxidation via photoionization to generate Cy<sup>+•</sup> ( $h\nu$ , note that oxidation may also happen in the presence of an electron acceptor via PeT, not shown); in orange, radical escape to yield Cy<sup>-•</sup> and RS<sup>•</sup>  $(k_{esc})$ . (e) CyS structure before and after a photoinduced thiolate addition as proposed by Dempsey et al.<sup>11</sup>

gallate,<sup>18–21</sup> nitrobenzyl alcohol,<sup>22,23</sup> nitrophenylalanine,<sup>24,25</sup> nitrophenyl acetic acid,<sup>24</sup> and ascorbic acid<sup>21,26,27</sup> have also been reported as antifading agents in bulk and SMF imaging, providing optimum results when combined with an oxidizing counteragent (ROXS stabilizers).<sup>28–30</sup> (ii) It was also proposed via heavy atom induction of the triplet excited state of CyS that photoswitching arises from the triplet excited state.<sup>12</sup> (iii) The last piece of information rests on the observation that quantitative back electron transfer (BeT)<sup>31</sup> takes place when using  $\beta$ -ME, precluding the use of ROXS. We recently reported that this outcome is due to thiyl radical-assisted triplet-to-singlet intersystem crossing (ISC) in a newly formed triplet geminate radical pair (GRP).<sup>32</sup>

Herein we provide a unified mechanism on Cy-dye photochemistry that rationalizes the above SMF observations and new results we obtained utilizing single-molecule fluorescence microscopy, steady state fluorescence, transient absorption spectroscopy (laser flash photolysis, LFP), and density functional theory (DFT) modeling on various Cy dyes. Building on the thiol-mediated photostabilization mechanism of cyanines, our results show that the dark state formation initially requires PeT from a thiolate to the Cy triplet excited state, forming a triplet geminate radical pair. Efficient ISC in the latter allows for radical—radical combination to occur, before escaping from the solvent cage. Bifurcation between photostabilization and photoswitching results from competition between (i) BeT, central to enhanced signal stability and extended survival times,<sup>31</sup> and (ii) radical combination, yielding a nonemissive Cythiol adduct, accounting for photoswitching to a nonemissive state, key to super-resolution imaging.

We also show that for the Cy5-thiol adduct absorption of a red photon—a consequence of a vanishingly small yet nonzero absorption cross section—results in uncaging of the iminium ion upon thiolate  $\beta$ -elimination, and restoration of emission. Careful analysis of this, to our knowledge, previously unreported phenomena notably enables us to further observe an acidcatalyzed component in thiol elimination (see Figure 1e). Accordingly, the lifetime of the Cy-thiol adduct (dark state) may be tuned by pH. Longer *off times* are thus achieved at both higher pH and lower, red-shifted, photon fluxes.

In general, our mechanistic studies set the stage to engineer better chromophores and imaging cocktails toward improved brightness, extended survival times, signal stability, and controlled photoswitching/on—off cycling for SMF and superresolution SMLM imaging.

# RESULTS AND DISCUSSION

Dark State Genesis from the Triplet Manifold through Geminate Radical Combination. We initially monitored the intensity—time profile of Cy5 and Cy5B dyes at the singlemolecule level to study the precursors involved in photoswitching. Solutions were prepared bearing increasing concentrations of potassium iodide to catalyze Cy5 excited singlet to triplet intersystem crossing<sup>12,33</sup> via the heavy atom effect.<sup>34</sup> Studies on surface-immobilized Cy5-ssDNA and Cy5B-ssDNA (see Figure S2 for structures) were performed by total internal

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**Figure 2.** Triplet state genesis of the dark state through GRC as seen via SMF and LFP studies. (a) Wide-field images vs time depicting fluorescent single molecules studied; arrows point to a single molecule switching from on to off and back to on states. (b) Intensity—time single-molecule trajectories of surface-immobilized ssDNA bearing Cy5 (top) and Cy5B (bottom) under oxygen-depleted conditions with 143 mM  $\beta$ -ME. Time intervals until photobleaching (Cy5B) and where intensity is either on or off (Cy5) are established. (c) Histograms showing number of events vs on time are next constructed to extract the average on time of Cy5 by fitting an exponential decay function (similar histograms were constructed to extract

the average number of photons and survival times). (d) Linear dependence (Pearson's r = 0.995 24) between iodide concentration  $[I^-]$  and  $v_{off}^{f}/v_{off}^{o}$  (the ratio of the switching rates in the presence and absence of iodide) as anticipated by eq 1. (e) Transient absorption spectra of Cy5 recorded in aqueous buffered solution (Tris 20 mM, 50 mM NaCl, pH 8) containing ~5  $\mu$ M Cy5, 50 mM KI, and 14.3 mM  $\beta$ -ME. (f) Temporal evolution of  $\Delta$ OD traces recorded at 490, 645, and 710 nm. (g) Quadratic power dependence ( $R^2 = 0.995$  45) for the photoinduced formation of Cy5<sup>+•</sup>. (h) Linear correlation between  $v_{off}$  vs excitation power at 23 °C (Pearson's r = 0.999 42) and 37 °C (Pearson's r = 0.996 71). (i) Average on time (green) and average number of photons per "on" event (gray) for Cy5 with  $\beta$ -ME alone or supplemented with 1 mM ascorbic acid (AA). When supplemented with 1 mM methyl viologen (MV<sup>2+</sup>), the survival time, rather than the average on time, is reported as Cy5 undergoes irreversible photobleaching. Errors displayed in plots are those retrieved from the fittings.

reflection fluorescence (TIRF) microscopy (Figure 2a).<sup>35</sup> Intensity—time trajectories were obtained under oxygendepleted conditions, via enzymatic scavenging, in the presence of 143 mM  $\beta$ -ME. The duration of the *on* and *off time* intervals for every single molecule under a given set of conditions was recorded (Figure 2b) and next combined in a histogram to establish average *on* and *off times* ( $\tau_{on}$  and  $\tau_{off}$ , respectively, Figure 2c).

Consistent with previous observations by Bates et al., we observed a linear correlation between the rate at which Cy5 reaches the off state ( $v_{off} = 1/\tau_{on}$ ) and iodide concentration (see Figure 2d and SI section iii), supporting the involvement of the triplet state in Cy5 photoswitching.<sup>12</sup> A linear correlation between the average number of photons per on interval and its average duration  $\tau_{on}$  also follows (Figure S5). Quantitative analysis involving plotting of  $v_{off}^{I'}/v_{off}^{o}$  (the ratio of the switching

rates in the presence and absence of iodide) vs  $[I^-]$  in turn yielded the value  $k_{\rm ISC}^{\rm cat}/k_{\rm ISC}^{\rm oc}$  where  $k_{\rm ISC}^{\rm cat}$  is the rate constant of catalyzed ISC of Cy5 by iodide, and  $k_{\rm ISC}^{\rm o}$  is the intrinsic intersystem crossing rate constant of Cy5; see eq 1 below, Figure 2d, and SI section v. The value of  $231 \pm 9 \, {\rm M}^{-1}$  we calculated for  $k_{\rm ISC}^{\rm cat}/k_{\rm ISC}^{\rm oc}$  is within an order of magnitude of that previously reported based on fluorescence correlation studies,  $125 \pm 65$  ${\rm M}^{-1}$  (corrected by the kinetic salt effect; see SI section v),<sup>34</sup> consistent with the proposed triplet-mediated dark state formation.

$$\frac{\tau_{\rm on}^{\rm o}}{\tau_{\rm on}^{\rm I-}} = \frac{\nu_{\rm off}^{\rm I}}{\nu_{\rm off}^{\rm o}} = 1 + \frac{k_{\rm ISC}^{\rm cat}}{k_{\rm ISC}^{\rm o}} [I^-]$$

$$\tag{1}$$

We posit that the mechanism of formation of the Cy-thiol adduct from the triplet manifold relies on radical combination within the solvent cage. Here following PeT, thiyl radical assisted

triplet-to-singlet ISC in the newly formed GRP<sup>32</sup> leads, albeit in small yields, to adduct formation. Laser flash photolysis studies conducted with Cy5 confirmed no radical escape following *PeT* between  $\beta$ -ME and Cy5, consistent with efficient ISC (Figure 2e,f).<sup>32</sup> Our transient absorption studies however failed to show the formation of a long-lived adduct in the form of a ~310 nm band. Failure to see this transient is however expected given its extremely low probability of formation. In agreement with a radical combination mechanism Mulliken spin density for oneelectron-reduced cyanines (illustrated for Cy5 in Figure 1b) points to attack at position  $\gamma$  (C<sub>3</sub>) to the nitrogen atom, as experimentally observed for addition of the alkyl thiol.<sup>11</sup>

We also considered, and ruled out, the nucleophilic attack to the Cy5 radical cation (Cy5<sup>+•</sup>) by  $\beta$ -ME in its anionic form as a possible way to generate the Cy-thiol adduct. The Cy5 radical cation could form upon the absorption of two photons (Figure 1d, green arrow; see Figure 2g): in this case, the rate  $v_{off}$  would scale with the square of the excitation intensity. In contrast, we have found that  $v_{off}$  scales linearly with the excitation intensity and is temperature independent, consistent with a one-photon process (Figure 2h, also Figures S6 and S7 and Tables S3 and S4). To rule out a monophotonic process, we generated Cy5<sup>+•</sup> photochemically in the presence of the electron acceptor methyl viologen (MV<sup>2+</sup>). Upon addition of MV<sup>2+</sup> in the presence of  $\beta$ -ME, the Cy5 dye underwent irreversible photobleaching, yielding ~4000 photons on average before degradation, less than the average  $\sim$ 5000 photons collected on the on-periods in photoswitching experiments (no MV<sup>2+</sup>, Figure 2i, also Figures S8 and S9 and Table S5). Addition of a competing triplet quencher leading to Cy-• (1 mM ascorbic acid) showed a decreasing rate for Cy5 switching off, as previously shown by Dempsey et al.<sup>11</sup> This increased the average number of photons per switching event by nearly 3-fold (~16 000 vs ~5000, Figure 2i).

Importantly, steady state fluorescence experiments and Stern–Volmer analysis show that  $\beta$ -ME quenches Cy5 fluorescence via PeT (Figure S10). The singlet excited state of Cy5 thus can also give rise directly to the dark state adduct upon PeT from the thiolate to the singlet excited state, as illustrated in Figure 1d (dashed line). This pathway does not need to invoke ISC. Accordingly, while the fleeting lifetime of the excited singlet state ( $\tau \approx 1 \times 10^{-9}$  s)<sup>36</sup> makes it an elusive intermediate to trap experimentally compared to the triplet excited state ( $\tau \approx 2 \times 10^{-4}$  s), it is generated at a much higher frequency (quantum yield for triplet generation  $\phi_{\rm ISC} \approx 10^{-3}$ ). We have estimated that, under typical SMLM experimental conditions (no oxygen,  $\beta$ -ME 143 mM, and pH = 8), the rate of thiolates yielding an adduct from the singlet excited state is on the same order of magnitude as that for the triplet (see SI, section x).

We also considered whether other species arising form the triplet manifold may give rise to long-lived nonemissive states also contributing to photoswitching. Transient absorption spectroscopy<sup>37</sup> studies on Cy5 yielded relatively short—microsecond to millisecond time scale—lifetimes for its *cis* photoisomer (*cis*-Cy5), one-electron-oxidized form (radical cation Cy5<sup>+•</sup>), and one-electron-reduced form (radical anion Cy5<sup>-•</sup>) (Figure 3 and Table 1; see also SI (section i-c) for details). The latter intermediate was of particular interest given the long lifetimes—up to several hours—reported by Sauer on the radical anions of several rhodamine dyes which are thus exploited in SMLM.<sup>38</sup>

Given their short—sub-millisecond—lifetimes and their spectral properties, the above transient species may not account



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**Figure 3.** Absorption spectra for various transient species of Cy5. Absorption spectra of Cy5 and related transient species such as *cis* photoisomer, triplet excited state, photooxidized Cy<sup>+•</sup>, photoreduced Cy<sup>-•</sup>, and the dark state of Cy5 ( $\beta$ -ME-Cy5 adduct, Cy5-SR<sup>-</sup>). The Cy5-SR<sup>-</sup> adduct was isolated upon irradiating Cy5 with a 100 mW expanded laser beam (638 nm) in a 450 mM Na<sub>2</sub>CO<sub>3</sub> (pH = 11) solution containing 430 mM  $\beta$ -ME.

Table 1. Lifetimes and Spectral Properties for the Transient Species of Cy5 and Cy5B

	$ au$ ( $\mu$ s)		$\lambda_{\max}$ (nm)	$\varepsilon \; (\mathrm{cm}^{-1} \; \mathrm{M}^{-1})$
species	Cy5	Cy5B	Cy5	Cy5
cis-Cy	196 ± 5	n/a	675	326 000
<sup>3</sup> *Cy	$209 \pm 20$	$172 \pm 25$	695	105 000
Cy5 <sup>+•</sup>	$440 \pm 10$	$206 \pm 6$	525	95 000
Cy <sup>−•</sup>	$853 \pm 7$	$1042 \pm 15$	490	82 000
Cy-SR <sup>-</sup>	>10 <sup>6</sup>	n/a	311	47 000

for the long-lived (seconds) dark state of Cy5. Along the same lines, we note that transient absorption studies with Cy5B, the locked analogue of Cy5 which is unable to undergo photoswitching,<sup>14</sup> showed transient species with lifetimes on the same order of magnitude as those of Cy5. The fact that the transient species of Cy5B show similar lifetimes to those of Cy5 and that Cy5B is unable to undergo photoswitching (see Figure 2b for a representative trajectory with  $\beta$ -ME) further support the hypothesis that none of these transient species account for the dark state.

In closing this section, we note that single-molecule intensity-time trajectories acquired with Cy5B in the presence of  $\beta$ -ME displayed no photoswitching as previously reported<sup>11</sup> and only a small, 1.6-fold, increase in the average number of photons before photobleaching. Thus, the average number of photons under oxygen-depleted conditions increased from  $2.1 \times$  $10^4$  photons with no  $\beta$ -ME to  $3.3 \times 10^4$  photons with  $\beta$ -ME. For comparison under similar conditions Cy3 exhibited a ~35-fold increase in photon budget upon  $\beta$ -ME addition.<sup>39</sup> This rather small effect that  $\beta$ -ME has on Cy5B is consistent with an inability to quench the triplet excited state of Cy5B by the former; see also Table 2 and discussion below. Notably, 44% of the intensity time trajectories acquired with  $\beta$ -ME revealed an intensity jump before photobleaching (Figure 2b), while the remaining trajectories showed a typical one-step photobleaching (Figure S11). We are currently studying this intensity jump phenomenon.

Photoinduced and Acid-Catalyzed Restoration of the Fluorescent State. Photoswitching of Cy5 requires fine-tuning of the rate at which Cy5 recovers ( $\nu_{on}$ ) from the newly formed

Table 2. Rate Constants for Photoinduced Electron Transfer to Cy Dyes by  $\beta$ -ME and Estimated Transition State Energies for Forward and Back Electron Transfer

dye/ $\beta$ -ME	$k_{\rm PeT} \ ({ m s}^{-1} \ { m M}^{-1})$	$\Delta E_{ m PeT}^{ m \dagger}$ (kcal/mol)	$\Delta E^{*}_{ m BeT}$ (kcal/mol)
Cy3	$3.5\pm0.3\times10^8$	9.17	4.73
Cy3B	$1.6 \pm 0.1 \times 10^{7}$	12.60	3.50
Cy5	$9.6 \pm 0.5 \times 10^{7}$	11.39	5.01
Cy5B	$1.3\pm0.4\times10^{6}$	13.90	3.93

Cy5-thiol adduct. Notably, uncaging of the thiolate moiety and restoration of the chromophore were previously reported to take place (i) through indirect illumination of a closely lying, orangeemitting fluorophore (Cy3)<sup>12</sup> and (ii) via direct illumination at relatively short wavelengths, namely, 337 nm, 405 nm, and up to 532 nm.<sup>8,9</sup> Both findings present a major conundrum as to how the Cy5-thiol adduct, reported to have an absorption peak at 310 nm,<sup>8,11</sup> may undergo photoinduced reactions at wavelengths where no absorption is detected.

We speculated that the observed photoinduced uncaging results from spectral broadening of the electronic transition of the 310 nm peaked Cy5-thiol adduct absorption.<sup>40</sup> To test our hypothesis, we calculated the rate at which Cy5 recovers ( $v_{on} =$  $1/\tau_{\rm off}$ ) at different laser excitation wavelength and power conditions by building the distribution of off times  $t_{off}$  (the times to Cy5 recovery) from single-molecule intensity-time trajectories. Here, surface-immobilized ssDNA bearing Cy5 was studied under conditions favorable to thiol adduct formation (oxygen-depleted and presence of  $\beta$ -ME). The data were acquired in a two-color scheme, exciting with the "uncaging" wavelength while simultaneously exciting at 647 nm at a constant power to record the Cy5 emission intensity over time (Figure 4a). The data revealed a linear dependence of the rate of Cy5 recovery  $v_{on}$  with laser power upon either 405, 488, or 561 nm irradiation, consistent with a one-photon process (vs a quadratic dependence that would be expected for a biphotonic process), in agreement with an electronic transition allowed by spectral broadening. Markedly shallower slopes (decreasing sensitivity to excitation power) were recorded with increasing wavelengths. Notably, even excitation at 647 nm resulted in uncaging of the Cy5-thiol adduct, although the dependence of  $v_{\rm on}$  on power was much shallower than for 561 nm (see Figure 4a and SI sections xiv and xv).

Consistent with our spectral broadening hypothesis, we obtained an action spectrum for the thiolate photoinduced uncaging, showing a tailing dependence on the wavenumber that can be approximated with a Gaussian function;<sup>40</sup> see Figure 4b. The action spectrum consisted of the product of  $\varepsilon_{(\lambda)}\Phi_u$  as a function of wavenumber.  $\varepsilon_{(\lambda)} \Phi_u$  was obtained from the derivative of the on rate  $v_{on}$  vs the illumination power P ( $dv_{on}/dP$ ), where  $\varepsilon_{(\lambda)}$  is the extinction coefficient (in  $M^{-1}$  cm<sup>-1</sup>) at a given wavelength  $(\lambda)$  and  $\Phi_{\rm u}$  is the quantum yield of uncaging, independent of  $\lambda$  (see SI, section xii). Related to this, the indirect uncaging of the Cy5-thiol adduct via a closely lying blue-shifted, i.e., Cy3, fluorophore would also tap on the broadened absorption spectrum via a combination of Förster and Dexter energy transfer mechanisms between the blue-shifted fluorophore and Cy5-SR<sup>-</sup>, where the blue-shifted emitter can act as an antenna to assist the recovery of the fluorescent form of Cy5, allowing to decrease the illumination intensity by over 1000-fold when Cy3 is positioned at distances closer than 1 nm.<sup>12</sup>

Whether direct or indirect, we postulate that photoinduced uncaging of the thiol involves homolytic cleavage to regenerate a



Figure 4. Single-molecule mechanistic studies on photoinduced and acid-catalyzed restoration of Cy5 fluorescence. (a) Linear correlations of  $v_{on}$  as a function of the irradiance at 647 nm (red squares, Pearson's r = 0.994 78). Additionally, a two-color scheme was used to monitor  $v_{on}$ when exciting with different "uncaging" laser wavelengths (405, 488, and 561 nm) while simultaneously exciting at 647 nm at a constant power of 298 W/cm<sup>2</sup> (Pearson's r = 0.985 63, 0.998 19, and 0.995 22, respectively, for 405, 488, and 561 nm correlations). (b) Action spectrum based on the sensitivity of  $v_{on}$  to excitation power. (c) Kinetic salt and temperature effects on the rate of elimination monitored as a function of the irradiance of a 647 nm wavelength laser (100 mM Tris, 23 °C Pearson's r = 0.97792; 100 mM Tris, 37 °C, Pearson's r =0.985 63; 20 mM Tris, 23 °C, Pearson's *r* = 0.937 42). (d) Correlation of  $log(v_{on})$  vs pH monitored at 647 nm (298 W/cm<sup>2</sup>) supports an acidcatalyzed/proton-assisted thiol elimination (Pearson's r = -0.98852). Errors displayed are those retrieved from the fittings.

geminate radical pair whose likelihood to undergo back electron transfer highly outcompetes the probability of reassociation ( $k_{\rm BeT}/k_{\rm GRC} \approx 10^3$ ; see SI section xiii for details on estimation).

Single-molecule uncaging studies on Cy5-thiol adducts as a function of power and wavelength exposed a most insightful piece of the puzzle behind Cy5 photoswitching, namely, that thiolate dissociation and restoration of Cy5 takes place in a parallel thermal (dark) reaction. This was manifested by a nonzero intercept in the linear dependence of the rate of recovery of Cy5 ( $v_{on}$ ) as a function of excitation power (at 647 nm excitation), where the intercept (rate at zero excitation power) reports the thermal rate of recovery. Comparing the intercept of  $v_{on}$  as a function of the 647 nm irradiance at both 23 and 37 °C and at pH 8.0, we found a minimal change in the intercept ( $0.010 \pm 0.001 \text{ s}^{-1} \text{ vs } 0.011 \pm 0.001 \text{ s}^{-1}$ ), indicative of a very low activation energy process for the thiol photo-uncaging (Figure 4c).

The rate of thermal uncaging, recorded as described above via single-molecule studies on the distribution of  $\tau_{off}$  values, was observed to increase with decreasing ionic strength at pH 8.0, as revealed by an increase of the intercept  $(0.020 \pm 0.002 \text{ s}^{-1})$  with no change in the slope for the correlation of  $v_{on}$  with power; see Figure 4c and SI section xvi. Given that the net charge of Cy5-SR<sup>-</sup> is negative (two sulfonate groups (-2)), the observed kinetic salt effect points to a rate-limiting step in thiolate uncaging involving a bimolecular reaction with a positive charge,

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Figure 5. Energy landscape for Cy dye photoreactions. Energy of Cy/ $\beta$ -ME pairs and the computed transition states (gray) for the photoinduced electron transfer (PeT) and back electron transfer (BeT).

which we assign to a protonation of the adduct to activate the leaving group.

Consistent with an acid-catalyzed restoration of the emissive state (Figure 4d and SI section xvii), we found a linear relationship between  $log(v_{on})$  vs pH. Here, a slope of 1 additionally reports on the molecularity of the chemical reaction. Notably, the acid-catalyzed uncaging observation we report herein reconciles the ability to isolate the thiol adduct at high pH for characterization (e.g., pH 11 in our studies and 10.5 in product studies previously reported<sup>11</sup>), with the inability to do so when experiments are conducted at low pH. Thus not only is a high pH required to increase the thiolate concentration, the rate of PeT, and of the ensuing adduct formation ( $\beta$ -ME with a  $pK_a$  of 9.6,<sup>41</sup> typically requires much higher concentrations than MEA,<sup>13</sup> with a  $pK_a$  of 8.6,<sup>42</sup> to achieve similar levels of photostabilization and photoswitching), but it additionally results in a slower rate of thiol thermal uncaging. Based on the linear correlation of pH vs  $log(v_{on})$  one may estimate that while the uncaging rate is  $\sim 0.1 \text{ M s}^{-1}$  at pH 8, where most studies are reported, it is roughly 2.5 orders of magnitude slower at 2.5 pH units higher, where product studies were reported.<sup>11</sup> The data underscore the tremendous sensitivity to pH and highlight opportunities for switching, for the Cy5-thiol adduct.

Importantly, when performing single-molecule analyses on  $v_{opt}$  we have found that the distributions of off times are better adjusted through a biexponential function, uncovering an additional dark state in a minor proportion (see Figure S12, section xiv). We propose that a GRC can alternatively proceed through the C1 of Cy5. The electron spin density for photoreduced Cy5 at this position is in fact not significantly lower than that at  $C_3$  (see Figure 1c). Ground state nucleophilic attacks on cyanines have been reported to proceed via the most electrophilic C<sub>1</sub><sup>43,44</sup> (see Figure 1b for Mulliken charges in the ground state). Adducts at this position show a red-shifted ( $\sim$ 370 nm) absorption spectrum (see Figure S21 in section xviii). Consistent with the hypothesis of an adduct with a red-shifted absorbing spectrum, we have found that the  $v_{on}$  of the minor component is  $\sim 10 \times$  more sensitive to the 647 nm light when compared to the dark state in major proportion. Regardless, note that for most analyses reported in this work we have used the major component of the distribution.

**Energy Landscape from DFT Calculations.** The mechanistic insight gained through our SMF and LFP experimental work with Cy5 and Cy5B illustrates key elementary reactions at play in Cy brightness, enhanced photon budget, signal stability, and photoswitching. Brightness is largely dictated by the ability or lack thereof to undergo relaxation in the excited state and *trans*—*cis* photoinduced isomerization (a mechanism available for Cy3 and Cy5 but not for Cy3B and Cy5B). On the other hand, for enhanced photon budget, signal stability, and photoswitching, ISC followed by PeT with thiolate is of paramount importance. Here, escape from the newly formed GRP is considered negligible based on rapid ISC in the GRP, and two-photon ionization or photoinduced oxidation from the triplet excited state are also considered very minor pathways, albeit accounting ultimately for the fluorophore photobleaching with exceedingly low probabilities. Accordingly, the fates for all cyanines will be dictated by whether they may effectively undergo PeT with thiolates and, if so, whether:

- i. rapid regeneration of the ground state upon BeT takes place to continue the excitation/emission cycle yielding stable signals and enhanced photon budget (Figure 1d, light gray background);
- ii. GRC outcompetes, bringing the Cy dye to a nonemissive thiol adduct wherefrom it may be regenerated either photochemically or thermally (Figure 1d, dark gray background).

DFT modeling on the different stages of relevance, namely, PeT, BeT, and elimination (E), shows that in all cases the reactions are energetically favorable (Figure 5, Table 2, and Figure S3; see also SI, sections i-d and xix). The different behaviors observed for the cyanines are thus dictated by the kinetics of the competing pathways, through their activation energies.

We computed the activation energies for PeT and BeT from DFT-determined energy values, within the theoretical framework of Marcus theory of electron transfer (see Table 2 and SI, sections i-d and xix). Our calculations show that the activation energies for PeT follow the trend Cy3 < Cy5 < Cy3B < Cy5B, consistent with the electron transfer rate constants obtained via transient absorption studies (see Figure 5, Table 2, and Figure S22).

Experimental values and theoretical estimates for  $k_{PeT}$  are consistent with the high photon budget of Cy3 in the presence of  $\beta$ -ME and the lower one found for Cy3B, Cy5, and Cy5B.<sup>14,39</sup> In turn, the low  $k_{PeT}$  value found for <sup>3</sup>\*Cy5B/ $\beta$ -ME explains both the nonblinking property of Cy5B reported herein and elsewhere<sup>14</sup> and its low photostability, as both processes require the initial formation of a GRP. Related to this, we note that working at concentrations lower than those reported herein would be detrimental to photon budget, survival time, and photoswitching arising from a reduced rate of quenching of the Cy dyes in their triplet excited state.

As to why Cy3B and Cy3 do not show thiol adducts/ photoswitching to the level displayed by Cy5 given that they readily undergo PeT, we posit that the answer lies in a combination of competition between BeT and GRC. However, we notice short-lived dark states when exciting Cy3 with  $\beta$ -ME that are absent in, for example, studies conducted with Trolox and may well point to an elusive Cy3-thiol adduct.

Geminate-radical combinations typically proceed with a low energetic barrier, which is expected to be larger for a rigidified structure (Cy3B and Cy5B) as compared to a more flexible structure (Cy3 and the less sterically hindered Cy5, respectively), as steric clashing may result upon addition and  $sp^2$  to  $sp^3$  hybridization change. The formation of the adduct will be modulated by the activation energy of the BeT process acting as a manifold. A higher activation energy for BeT will favor the GRC process, with the thiol acting as a photoswitching agent. In turn, a lower BeT activation energy will favor the restoration of the cyanine to its singlet ground state, enhancing overall fluorophore survival time and photon budget. The combination of these two effects enables not only the photoswitching but also the enhanced total photon budget required for super-resolution imaging.

# CONCLUSIONS

Herein, we provide the principles that dictate the photoswitching susceptibility of cyanines in the presence of thiols and their close relationship to the mechanism governing the photostabilizing properties of thiols. The detailed understanding of the thiolate-induced photoswitching of cyanine dyes provides a roadmap for the design of fluorophores, photoswitching agents, and triplet quenchers with desired characteristics and a set of guidelines toward a better photochemical control over currently used Cy/thiol pairs, including photoswitching rates and enhanced photon budgets.

Thiolates are required in all instances given the thiyl radical's ability to rapidly undergo spin flipping via spin-orbit coupling. Thiolates characterized by lower oxidation potential than serendipitously discovered  $\beta$ -ME are required to accelerate PeT and ensure a rapid exit along the photostabilizing cycle in Figure 1d if the triplet state is reached. This is particularly critical for Cy5B, which holds the promise to be a highly bright, stablesignal, red-emissive fluorophore for SMF in the presence of an efficient photostabilizing compound. In addition to lower oxidation potentials, BeT, rather than GRC with the thiyl radical is desired if extended survival times and stable signals are needed for conventional SMF such as single-molecule FRET,<sup>6,16</sup> protein/nucleic acid-induced fluorescence enhancement (PIFE<sup>4,45</sup> and NAIFE,<sup>46,47</sup> respectively), or in PIFE-FRET combination.<sup>5,48,49</sup> This may be achieved by increasing the steric hindrance of the thiolate-fluorophore pair.

A thiyl radical able to undergo GRC would be sought after if photoswitching toward super-resolution SMLM is desired. In the latter context, we note that given the pH dependence in elimination of thiolate from the Cy-SR<sup>-</sup> adduct, imaging under varying pH conditions such as those encountered in cellular systems may render critically different levels of turnovers and on/off duration periods based on the organelle where photoswitching is taking place. Importantly, photoswitching may be sensitive to different protein environments surrounding a Cy dye, as proximal basic residues may catalyze elimination. Also, electron donors such as tyrosine or cysteine residues may affect the triplet lifetimes and their availability toward adduct formation. The diversity of possibilities for thiolate entrapment and uncaging offers both opportunities and challenges toward optimizing the off time duration toward SMLM and the SMF performance of Cy fluorophores in the presence of thiols. More generally, it highlights the tremendous potential toward steering the photochemistry of cyanine dyes, and their iminium ion moiety, via PeT toward activation of nucleophilic attack in catalysis<sup>50,51</sup> and toward photoinduced and thermal uncaging in biology. We thus note that catalyzed ISC in Cy dyes via a heavy atom effect either intermolecularly or intramolecularly may open an avenue toward their facile integration in new applications that require efficient photochemical yields.

# ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c03786.

Additional results, materials and methods information, details of procedures, and modeling (PDF)

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# Notes

The authors declare no competing financial interest.

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