

Mechanistic Insights on How to Avoid and Harness Cyanine Photoconversion

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A mechanistic understanding of photoconversion of fluorophores opens new doors for their applications in super-resolution microscopy.

The capture and release of light by organic molecules have proved invaluable for elucidating biological function. For example, cyanine dyes and their photochemical transformations are extensively used to create valuable tools for molecular imaging. Schnermann and co-workers have now identified the mechanism underpinning the photoconversion of cyanine dyes—a process termed “phototruncation”, which unlocks new avenues of harnessing light-induced chemical transformations into valuable optical tools for use in super-resolution imaging techniques.¹

The past decade has seen the great potential of super-resolution microscopy techniques that break the diffraction limit of light, affording imaging at the nanometer scale.² From the range of techniques that fall within super-resolution microscopy, single-molecule localization microscopy (SMLM) techniques such as photoactivation localization microscopy (PALM), direct stochastic optical reconstruction microscopy (d-STORM), and DNA points accumulation for imaging in nanoscale topography (DNA PAINT) rely on photoconversion (PALM, d-STORM) or short-lived binding (DNA PAINT) of dyes to create the transient spatial separation necessary for precise localization of fluorophores on the nanoscale.³ Photoconversion is facilitated by specialized buffer systems that include oxygen scavenging systems and nucleophiles such as thiols. Increased adoption of SMLM in biomedical imaging has created a greater demand for new fluorophores capable of photoswitching/photoconversion and a strong need to understand the photochemistry of currently used dyes. Even though photochemical reactions of dyes such as rhodamine and cyanine are central elements to SMLM, the

underlying mechanism and the chemical species resulting from light-mediated conversion have remained unknown. Through recent studies, the photochemical reactions have been identified to be predominantly of two types⁴ (Figure 1):

- (i) Light-mediated switching between a fluorescent state and a dark state, commonly termed photoswitching; and
- (ii) photoconversion of dyes to form new molecules with altered fluorescence properties, predominantly blue-shifted fluorescence.

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Initial efforts to elucidate photoswitching mechanisms of cyanines were made by Zhuang and co-workers who reported that the product of photoconversion is a cyanine-thiol adduct in which the thiol covalently attaches to the polymethine bridge, thereby disrupting the π -electron conjugation of the fluorophore.⁵ More recently, the Schnermann and Cosa groups, using transient-absorption spectroscopy and computational modeling, identified that photoswitching arises from photoinduced electron transfer from thiolate to cyanine in the triplet state, followed by intersystem crossing to the singlet state and cyanine-thiol adduct formation.⁶

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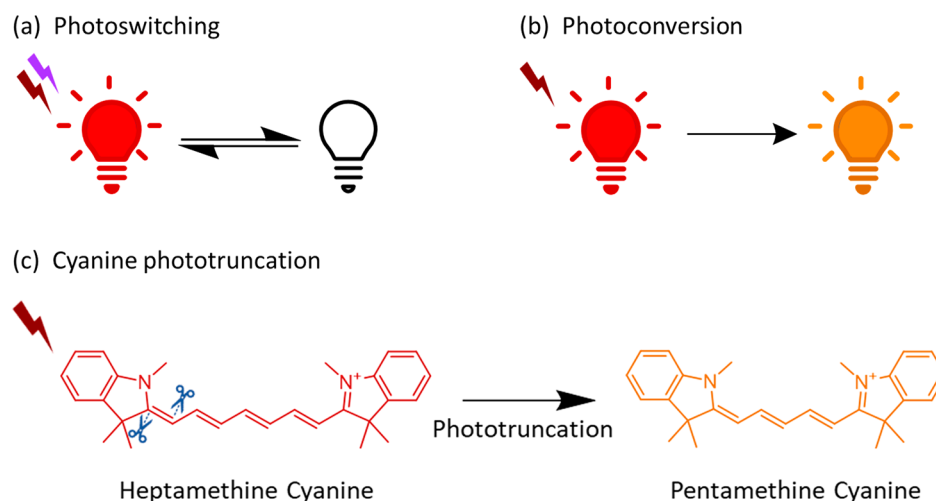


Figure 1. Schematic representation of common photochemical reactions observed in fluorescent dyes: (a) photoswitching of dyes between the emissive and nonemissive state; and (b) photoconversion of dyes, which involves the formation of new molecules with blue-shifted fluorescence emission. (c) Two-carbon phototruncation of heptamethine cyanine responsible for photoconversion to a pentamethine cyanine with blue-shifted absorbance and emission.

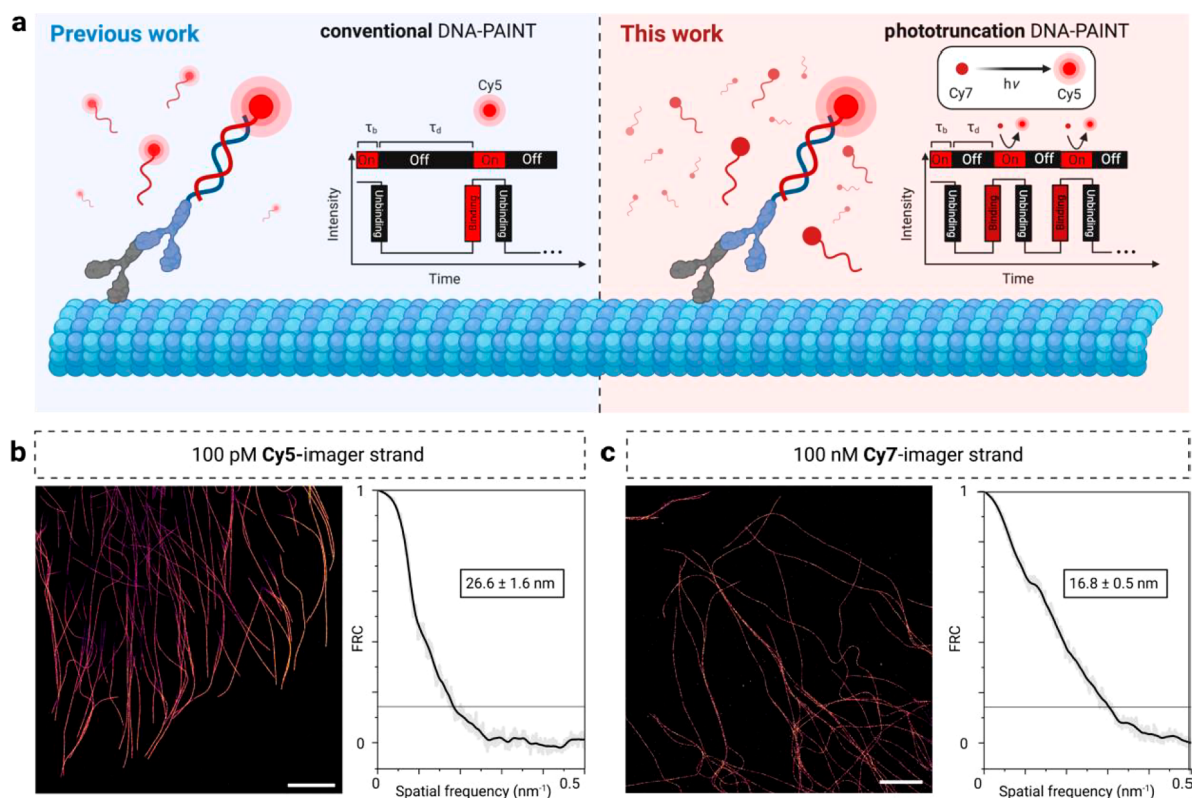


Figure 2. DNA-PAINT with Cy7 phototruncation. (a) Schematic comparison of conventional DNA PAINT with cyanine phototruncation DNA-PAINT. Super-resolution of microtubules obtained using (b) conventional DNA PAINT with Cy5-imager strands (100 pM) and (c) phototruncation DNA PAINT with Cy7-imager strands (100 nM) and a comparison of the estimated spatial resolutions. Reproduced with permission from ref 1. Copyright 2021 The Authors. Published by American Chemical Society.

Contrary to photoswitching, a desirable property for application in SMLM, photoblueing, the photoconversion of dyes to products with a hypsochromic shift in emission, is commonly viewed as a problematic artifact in multicolor SMLM experiments. For example, rhodamine-based dyes undergo stepwise photo-oxidative N-dealkylation resulting

in hypsochromic shifts of absorption and emission maxima, with a single dealkylation causing 10–15 nm shifts.⁷ Similar blue-shift artifacts have been reported for cyanine-based dyes, and a thorough understanding of the photochemical mechanisms underpinning these conversions is necessary to avoid and rationally utilize these light-mediated transformations.⁸

In their latest research published in *ACS Central Science*, Schnermann and co-workers report the first study that uncovers a two-carbon phototruncation reaction as the mechanistic basis of cyanine photoconversion (Figure 1c). Examining evidence from spectroscopy and computational studies, the authors demonstrate a singlet oxygen-initiated intramolecular rearrangement cascade that involves a zwitterionic intermediate followed by elimination of hydroperoxyethenol to form the truncated cyanine. The authors discovered that phototruncation occurred in a range of polymethine dyes commonly employed in SMLM including Cy5, Cy7, and AlexaFluor 647 and 750 variants. However, phototruncation can be inhibited by conformationally constraining the cyanines, through the incorporation of one or more rings onto the polymethine chromophore. This observation is consistent with studies from the Hell lab, which report that ring-substituted rhodamines do not undergo photo-oxidative N-dealkylation.⁹ Collectively, these discoveries inform how the biomedical imaging community can judiciously select dyes for their SMLM investigations to minimize photobleaching imaging artifacts and pave the way for rationally designing new fluorescent dye and sensor scaffolds for use in fluorescence and super-resolution imaging.

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Using a 384-well approach, the authors also examined the role of different types of nucleophiles, reactive oxygen species, solvents, pH, and irradiation power on the yield of phototruncation. These studies led to the identification of optimal conditions that improve the yield of the hepta- to pentamethine phototruncation reaction by an order of magnitude, ensuing exploitation of cyanine phototruncation for application in super-resolution imaging techniques. In optimized phototruncation conditions for Cy5 fluorophores, imaging at low irradiation intensity resulted in sparse localizations and poorly reconstructed SMLM images. While these sparsely reconstructed SMLM images can be converted into a high-quality image using deep-learning techniques such as artificial neural networks (ANNs), the more promising application

of phototruncation lies in DNA PAINT, where a high background signal and long acquisition times have been a long-standing challenge.¹⁰ In their DNA PAINT experiments, despite using 1000-fold higher concentrations, the authors successfully employed Cy7 photoconversion to eliminate the high background signal, achieving a spatial resolution similar to conventional DNA PAINT (Figure 2). Furthermore, with DNA PAINT experiments using the Cy7 phototruncation, the authors achieved approximately 2-fold faster acquisitions compared to traditional DNA PAINT protocols. These exciting results open new doors toward productive optimization of SMLM imaging by rationally steering photoconversion reactions of cyanine dyes.

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