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PERSPECTIVE

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Leveraging Baird aromaticity for advancement of bioimaging applications

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Abstract

In this perspective, we highlight the recent progress in utilizing Baird aromatic species to improve fluorophore performance in microscopy and imaging applications. We specifically focus on the origins of the use of Baird aromaticity in fluorescence applications, the development of "self-healing" fluorophores leveraging cyclooctatetraene' Baird aromaticity, and where developments need to occur to optimize this technology.

KEYWORDS

Baird aromaticity, cyclooctatetraene, fluorophores, imaging

1 | INTRODUCTION

Organic fluorophores are in widespread use across medical, biomedical, and fundamental research applications. Their utility stems from their high absorbance and emission cross sections as well as their relatively low cost. Organic fluorophores are commercially available for a wide range of excitation and emission wavelengths from UV- light to near-infrared, 350–1,100 nm.[1] Such chemically and photophysically diverse compounds as Figure 1A are commonly used as contrast agents for *in vitro* diagnostic, cell microscopy, and biomedical research applications.[4,5] However, they are also in increasing demand for advanced imaging applications such as single-molecule fluorescence (Förster) resonance energy transfer (smFRET) and super-resolution as well as in frontier medical applications such as fluorescence-guided surgeries.

Organic fluorophores are a fraction of the size of fluorescent proteins or quantum dots and are significantly more efficient at emission after the absorption of incident excitation energy (higher fluorescence quantum yield).[6,7] Traditionally, this key performance parameter is often referred to as "brightness," a measure of the number of photons generated per excitation cycle. In ensemble and single-molecule settings, however, fluorescence is typically measured under continuous-wave illumination over extended periods. Hence, in experimental settings, "brightness" refers to the number of photons collected (usually a fraction of the total number of emitted photons) over a specified integration time in which multiple cycles of fluorescence excitation and emission are measured. In "multi-turnover" settings of this kind, fluorophores are subject to distinct performance demands that are critical to consider. For instance, modern microscopy applications focused on low-abundance species require high illumination intensities and extended integration times to achieve adequate

brightness and the necessary total photon budget (average brightness x duration of fluorescence) to robustly detect molecules of interest.

An ideal organic fluorophore efficiently absorbs light to achieve singlet excited states, followed by the efficient emission of light of a longer wavelength on its return to the ground state. In this ideal case, a common imaging fluorophore of the rhodamine or cyanine class characterized by nanosecond excited state lifetimes (Figure 1A) could then, in principle, emit roughly 1 billion photons per second at maximal excitation. Even modest photon collection efficiency by a microscope would thus yield millions of photons per second. An idealized fluorophore of this kind would also continue to fluoresce with regular photon flux ad infinitum. Organic fluorophores would be extraordinarily bright and have an infinite photon budget. In reality, modern organic fluorophores yield just a small fraction of this theoretical photon yield, often limited to just a few to tens of thousands of total detected photons prior to irreversible damage, commonly referred to as photobleaching.[3]

A key source of the tremendous disparity between ideality and reality is that organic fluorophores spontaneously enter relatively long-lived and nonfluorescent triplet excited states that often react transiently or permanently with their surrounding environment.[3,8,9] Today, there is broad acknowledgement that organic fluorophore performance can be dramatically improved by efforts to engineer their physical attributes and electronic properties.[10–12] In this perspective, we focus on recent efforts to leverage Baird aromaticity to govern the brightness and total photon budget of organic fluorophores in aqueous settings under multi-turnover conditions to advance modern cellular single-molecule applications focused on relatively low-abundance species. We will specifically cover how compounds with 4nπ-electrons that demonstrate aromaticity in their lowest triplet state, defined as Baird aromaticity, allow for stabilization, robustness, and excited-state protection of fluorophores, resulting in the improvements previously described.[13] Excellent reviews on the subject of maximizing total photon budgets and challenges associated with efforts to mitigate fluorophore triplet states and downstream radical states using methods other than Baird aromaticity can be found elsewhere.[14–20]

2 | HISTORICAL CONTEXT

Early research in the "dye-laser" industry on specific organic fluorophores in highly concentrated nonaqueous solutions led to speculation that performance limitations of dye lasers were due, at least in part, to sporadic excursions of lasing fluorophores from singlet excited states (S_1) to long-lived triplet excited states (T_1) in Figure 2.[21,22]

Efforts to reduce time-averaged triplet state occupancy in these ensemble settings where dye–dye

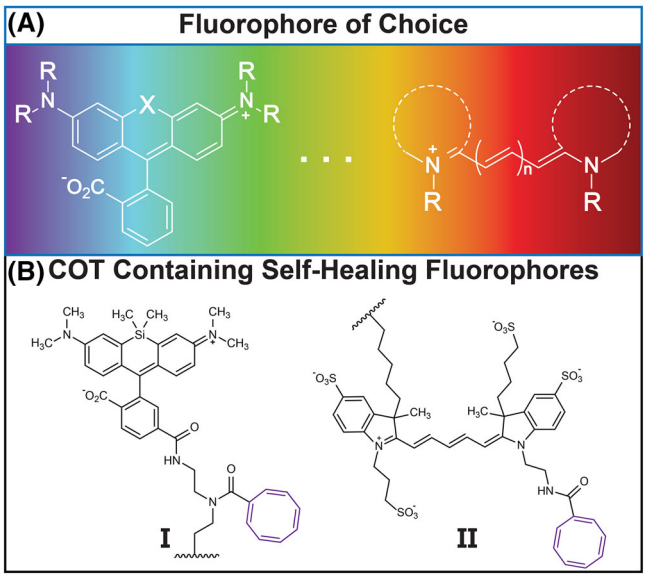

FIGURE 1 (A) Structures of rhodamine and cyanine fluorophores – two of the many molecular cores commonly utilized for bioimaging applications. (B) Published examples of COT (purple) containing self-healing fluorophores with improved performance, including rhodamine-based **(I)**[2] and cyanine-based **(II)**[3]

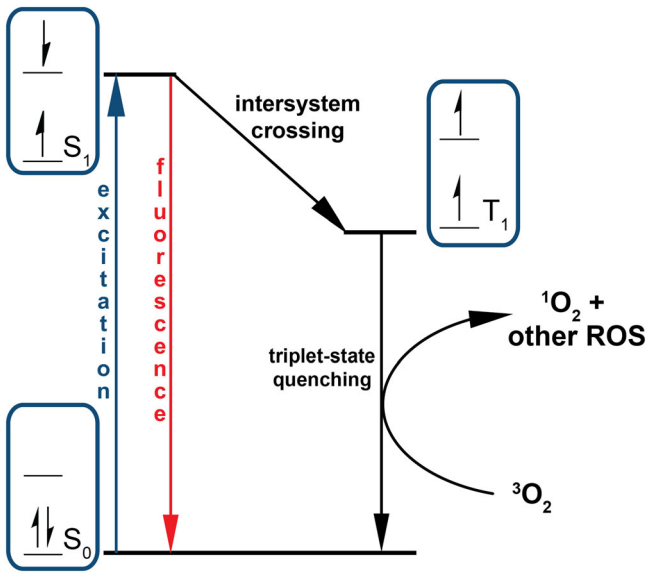

FIGURE 2 A simplified Jablonski diagram schematizing excitation (blue), fluorescence (red), intersystem crossing, and triplet-state quenching, including their electronic spin states, S_0, S_1, and T_1, that occur upon fluorophore photoexcitation. Triplet-state quenching can include reactions with molecular oxygen ($^3O_2$) to generate singlet oxygen ($^1O_2$) and other reactive oxygen species

interactions are robust include the mixing of laser-dye organic fluorophores with solution additives as well as covalent modification with specific compounds.[23–25] While the aggregation propensities of organic fluorophores, particularly at elevated concentrations, can exert potentially profound impacts on their performance characteristics, these early findings, while perceived as promising, could not differentiate intra or intermolecular triplet state quenching mechanisms. The potential for T_1 excursions to impact fluorophore performance can be profound. T_1 excited states are hundreds of thousands of times longer lived than singlet excited states and are highly prone to reactions with solvent, biological surroundings, and molecular oxygen ($^3O_2$) (Figure 2). In standing biological settings, $^3O_2$ is at near-millimolar concentrations.[26] In this concentration regime, diffusional collision of $^3O_2$ with fluorophores occurs hundreds of thousands of times per second. Ion collision of $^3O_2$ with a fluorophore in the T_1 excited state can spontaneously generate highly reactive singlet molecular oxygen ($^1O_2$) and other reactive oxygen species (ROS).[10,27–30] Such byproducts are damaging to molecules in immediate proximity of the fluorophore, the broader cellular environment on the whole, and the fluorophore itself, often leading to fluorophore photobleaching.[31,32] In addition to severely limiting the time scale of imaging, ROS generation has the potential to fundamentally compromise the nature of the collected data and its interpretation.

Consideration of these issues initiated decades of research toward improving fluorophore performance to mitigate the detrimental effects of organic fluorophore triplet state excursions. Initial efforts focused on reducing the concentration of molecular oxygen in solution to manage excitation-induced organic fluorophore phototoxicity and to increase the overall duration of fluorescence.[33,34] Strikingly, however, the absence of molecular oxygen exacerbated the duration of fluorophore triplet states, giving rise to exaggerated photophysical phenomena, including spurious redox chemistries.[33–35] Such reactions give rise to large fluctuations in fluorophore emission and "blinking" that collectively reduce fluorophore brightness.[36] Depending on the experimental setting and application, fluorophore blinking phenomena may range from a potential source of artifacts that can be taken into account to nearly complete excitation induced loss of fluorescence.[3]

The significance of this obstacle to imaging advancements was enough to initiate the search for triplet-state quencher (TSQ) molecules that could be added to solution in order to minimize triplet states and downstream redox reactions. Resourceful researchers later leveraged organic fluorophore blinking for stochastic optical reconstruction microscopy (STORM)-based super-resolution imaging.[37,38]

Motivated by the need to improve the regularity and duration of photon emissions from organic fluorophores to enable a range of biomedical imaging applications, a diverse suite of chemical solution additives has now been identified that mitigate organic fluorophore triplet states. These "protective agents" (PAs) include, but are not limited to, β-mercaptoethanol (BME), ascorbic acid, 4-nitrobenzyl alcohol (NBA), tris-Ni (NTA), and Trolox.[33–35,39–41] During this search, 1,3,5,7-cyclooctatetraene (COT) was also discovered as a solution additive.[35] Through these investigations, PAs were shown to operate through collision-based mechanisms.[35] Poor aqueous solubility and adverse effects on lipid bilayer properties were also demonstrated to be significant limitations for their practical use.[42]

3 | INTRAMOLECULAR TRIPLET STATE QUENCHING

To alleviate these drawbacks, we turned to intramolecular chemistry. By covalently attaching individual PA molecules to the cyanine-class fluorophore, Cy5, the effective concentration of the PA increased, enhancing collision frequency and quenching capabilities with the fluorophore.[43] Remarkably, these investigations demonstrated that a single PA was capable of dramatically enhancing Cy5 brightness and photostability, giving rise to orders of magnitude increases in total photon budget compared to the parent fluorophore. The observed extent of enhancement was greater than what could be achieved with saturating amounts of PA in solution. The effective rate of triplet state quenching was sufficiently rapid to afford performance enhancements in fully oxygenated aqueous buffers, suggesting a collision frequency significantly greater than the diffusional collision of molecular oxygen with the fluorophore (ca. 10^6 s^{-1}).[43] Hence, under continuous illumination conditions, one individual Trolox, NBA, or COT molecule can undergo repeated triplet state quenching cycles, punctuated by spontaneous returns to ground state configurations.[43–45] Notably, these impacts were shown to extend to a range of cyanine-class fluorophores spanning the visible spectrum.[46]

These preliminary insights prompted in-depth mechanistic investigations, revealing that NBA and Trolox return fluorophore triplet and radical states to the ground state via reduction/oxidation-based mechanisms.[47] By contrast, COT was shown to follow a nondestructive TSQ mechanism referred to as Dexter triplet–triplet energy transfer (TET), which is largely environment-independent.[48] Redox-active compounds are highly solution dependent and potentially destructive to the fluorophore as well as the surrounding environment. Our team

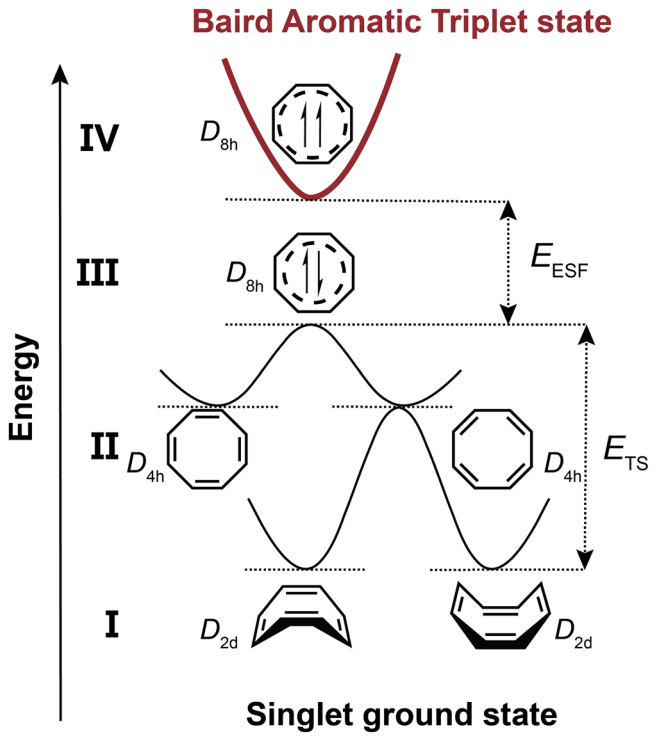

FIGURE 3 Schematic of the COT in ground states (**I**), thermally accessible excited states with bond-shifted near-planar geometries (**II**) that are conducive to triplet energy transfer (TET) mechanisms that give rise to Baird aromatic triplet states **(IV)**. E_{ESF} corresponds to the electron spin flip energy associated with TET

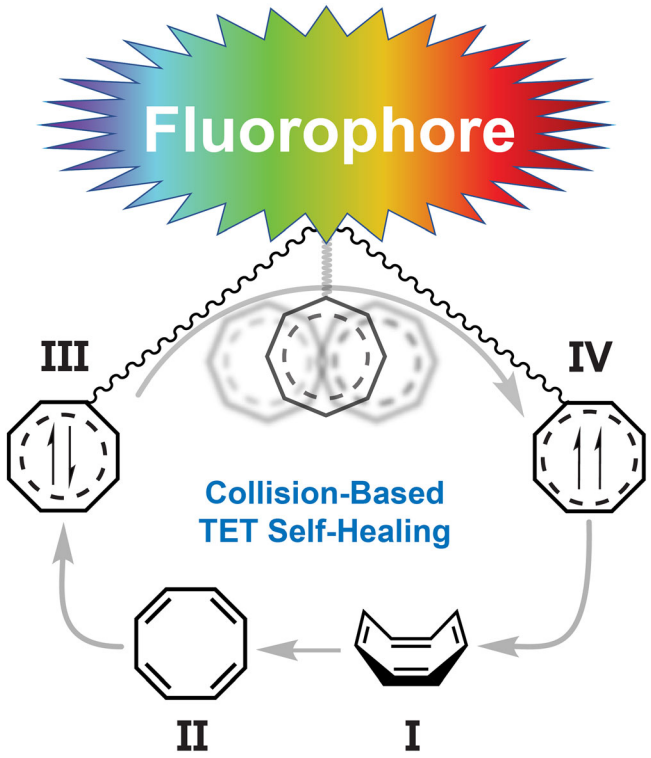

FIGURE 4 Schematic of COT-mediated, intramolecular triplet-state quenching of organic fluorophores. Collision of accessible excited singlet-state COT (antiparallel electronic spin state; **III**) and a triplet-state dye (top) leads to triplet–triplet energy transfer (TET), returning the fluorophore to the ground state and shifting COT to the Baird aromatic triplet state (parallel electronic spin state; **IV**). COT must then relax back down to the singlet ground state conformations (bottom; **I** and **II**) to enable multi-turnover, self-healing processes

therefore suspended pursuit of redox-based intramolecular triplet state quenching strategies while others continued efforts to optimize solution redox-based approaches and intramolecular redox strategies.[39,40,44,49–53]

4 | ADVANCES IN COT-MEDIATED TRIPLET STATE QUENCHING MECHANISMS

COT exhibits Baird aromaticity through excitation of a singlet transition state, making it an ideal candidate for TET (Figure 3).[13,54] When proximally linked to an organic fluorophore, via any variety of covalent or noncovalent bonds, for instance, a single COT molecule can thus undergo repetitive cycles of thermally driven excitation and relaxation cycles to intramolecularly quench fluorophores as they enter T_1 excited states (Figure 4). This COT-mediated intramolecular quenching mechanism has shown promise for enhancing the performance of chemically diverse organic fluorophore classes commonly used in the biological sciences, including xanthene-, oxazine-, and carbopyronine-class molecules.[55,56] Intramolecular triplet state quenching strategies of this kind, colloquially referred to as "self-healing dyes",[44,45] (examples of which can be seen in Figure 1B), have been employed in a multitude of applications to overcome a broad range of imaging limitations.

Our group and others have utilized "self-healing" organic fluorophores in a diverse array of smFRET experiments and settings, uncovering new information on the function and regulation of complex biological systems.[57–66] Self-healing donor- and acceptor- organic fluorophores have also been used for smFRET investigations that enabled demonstration that both the position and conformation of an individual molecule can be visualized on the surface of a live cell in oxygenated aqueous solution in the absence of exogenous TSQ additives.[3] Other groups have recently demonstrated that covalent attachment of COT to mitochondrial probes and voltage-sensitive fluorescent reporters can give rise to more robust measurements of cellular respiration and neural activity, including examples of non-cyanine-based fluorophores.

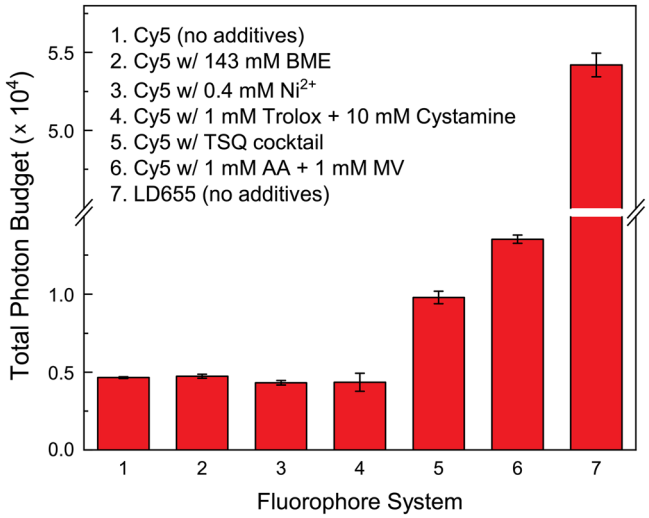

FIGURE 5 Comparison of total photon budgets for Cy5 TIRF experiments in oxygenated conditions including ambient and solution additives compared to a commercially available self-healing Cy5 derivative (LD655) in ambient conditions. TSQ cocktail contains 1 mM each of COT, Trolox, and NBA. Ascorbic acid and methyl viologen have been abbreviated as AA and MV, respectively

5 | FUTURE OUTLOOK AND CONCLUSIONS

Strategies developed over the past decade have maximized COT's performance as an intramolecular TSQ. In so doing, collaborative efforts have led to marked increases in the brightness and photostability of chemically diverse organic fluorophores and demonstrated that these impacts reduce the rates of ROS generation.[27] Such investigations led to the general conclusion that closer proximity between the TSQ and the fluorophore can improve performance even further.[43,55] Insights from these investigations also led to optimizations of the position of PA functionalization, the distance between fluorophore and TSQ, and the electronic properties of the COT moiety itself. Through these initiatives, robust fluorophore performance enhancements have been demonstrated for cyanine-class fluorophores spanning the visible spectrum, ranging from one to two orders of magnitude in diverse experimental settings, including those using solutions containing ambient levels of molecular oxygen in the absence of potentially toxic, or biologically compromising solution additives[3,10,42] Figure 5.

To further maximize the potential and utility of self-healing organic fluorophores, our team and others continue to pursue a deeper understanding of the COT-mediated triplet state quenching mechanism. Our initial findings on this front have revealed that COT itself can be engineered to tune its potential as an intramolecular TSQ for distinct cyanine-class organic fluorophores. Computational results showed that electron-withdrawing groups and increased sterics on COT can increase the energy gap to the Baird aromatic state and that such impacts correlate with improved performance for specific fluorophore species.[3] These findings have led to the global conclusion that the energy of Baird aromatic triplet states of the intramolecularly linked TSQ species may need to be optimally tuned to match the triplet state energy of the fluorophore to which it is attached. It has also been proposed that organic fluorophore performance may be enhanced under conditions of continuous illumination by reducing the lifetime of the TSQ Baird aromatic triplet state to enable repetitive TET cycles.[3]

These discoveries argue for the expansion of organic fluorophore engineering efforts to advance the frontiers and potential of the intramolecular triplet state-quenching ("self-healing") mechanism. A deeper understanding of the collision-driven TET between the spontaneously accessible singlet excited states of COT and the T_1 state of the fluorophore should inform us on how to enhance the efficiency of this process. For instance, investigations into decreasing COT's relaxation time from triplet to singlet states may lead to increased TET frequency over a given time window. Such optimizations are expected to simultaneously increase fluorophore brightness and total photon budget while reducing ROS generation. They are also expected to prolong the duration of TSQ operation, which appears to eventually fail in multi-turnover, continuous illumination conditions as a consequence of organic fluorophore excitation and/or its own reaction with the environment.[3]

New functionalization strategies to tune COT electronically and sterically are expected to be important for this frontier. Exploration of other potential TET-based TSQs would also further advance the field of "self-healing" fluorophores. Such pursuits will necessarily be accompanied by synthetic, photophysical, and quantum mechanical investigations to tune their potential as experimentally viable TSQs. Efforts of this kind hold the promise of further advancing the next generation of "self-healing" organic fluorophores, broadening their scope of impact in existing biomedical fields as well as opening new imaging frontiers that have yet to be explored.

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CONFLICTS OF INTEREST

Scott C. Blanchard and Roger B. Altman have an equity interest in Lumidyne Technologies.

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