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Superbright Bioorthogonal Turn-on Probes**

Jonathan C.T. Carlson⁺,

Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114 (USA)

Labros G. Meimetis⁺,

Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114 (USA)

Scott Hilderbrand, and

Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114 (USA)

Ralph Weissleder^{*}

Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114 (USA)

Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, (USA)

Abstract

The fastest and the brightest: A new design that intimately connects tetrazine to a BODIPY fluorophore enables exceptionally efficient energy transfer and quenching. Upon reaction of the tetrazine, the brightness of the fluorophore increases more than a thousand-fold, a fluorogenic activation up to two orders of magnitude greater than previously described.

Keywords

click chemistry; fluorescent probes; fluorogenic; tetrazine; through-bond energy transfer

Visualizing biomolecular processes has been enhanced by combining fluorophores with bioorthogonal chemistry, resulting in new tools to study the complex biochemical milieu of living cells and organisms.^[1] The resulting probes have been applied to image glycosylation and phospholipid uptake^[2,3], cellular proteins^[4–6], and intracellular drug distribution^[7]. In some of these applications, the capacity of *in situ* chemical conjugation has been paired with fluorogenic turn-on, whereby fluorophore emission increases upon reaction with its bioorthogonal counterpart (“turn-on” probes).^[8] This has the very attractive feature of reducing background fluorescence when doing *in vivo* imaging, potentially allowing real time imaging, without washing or clearance steps. For optimal performance, such a fluorogenic real-time reporter should be i) catalyst-free (to minimize toxicity and need for multiple reaction partners), ii) fast (to allow efficient imaging and temporal resolution) and

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^{*}rweissleder@mgh.harvard.edu.

⁺These authors contributed equally to this work

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iii) highly fluorescent after turn-on (to maximize signal and minimize background). Existing methods that exploit azide-phosphine, azide-alkyne, or inverse electron demand Diels-Alder tetrazine cycloadditions have only partially satisfied these criteria, resulting in subsets of probes with good fluorescence turn-on ratios but slow reaction kinetics and another subset with agreeably fast kinetics but only modest turn-on. What has been missing however, are exceptionally bright, fast, and biocompatible (water soluble, cell membrane permeable, non-toxic) probes with turn-on ratios exceeding 10x.

Previously described tetrazine-based probes achieve their fluorogenic turn-on by a unique mechanism, in which the tetrazine (Tz) chromophore is both quencher and bioorthogonal reactant.^[8] In published studies, flexibly-linked Tz-fluorophore pairs—chosen for their ready synthetic accessibility—are quenched with moderate efficiency, yielding turn-on ratios on the order of 10–20 fold after reaction with dienophile targets.^[3–5,8] Although intriguing applications have been demonstrated, the limited turn-on ratios almost always result in native background during imaging applications. In our estimation, a turn-on ratio of 10² would be preferable for robust utility in cellular imaging applications, and a ratio of 10³ may be necessary for low abundance targets and super-resolution imaging.^[9] Mechanistic observations have suggested that quenching in bichromophoric fluorophore-tetrazines occurs via Förster resonance energy transfer (FRET), offering a starting point for efforts to optimize turn-on.^[8,10] Although its relatively weak visible light absorbance inherently limits the range of tetrazine as a FRET acceptor, Förster theory dictates that energy transfer efficiency will be crucially dependent upon inter-chromophore distance (varying as r^6) and upon transition dipole alignment, which are both optimizable parameters.^[11] As an alternative way of designing more efficient turn-on probes, one might consider adapting through-bond energy transfer (TBET) for fluorescence quenching. With these goals in mind, we synthesized a series of new bioorthogonal boron dipyrromethene (BODIPY)-tetrazine derivatives (Figure 1). These structures enhance spatial donor-acceptor proximity, provide predictable donor-acceptor transition dipole orientation, and afford the possibility of accessing alternate modes of fluorescence quenching.

In the course of selecting a design for these cassettes, we noted that a crystal structure of *p*-cyanophenyl-BODIPY **1** exhibited a 90 degree rotation between the BODIPY and phenyl ring, a conformation which prevents pi-system conjugation.^[12] We speculated that a tetrazine attached to this phenyl ring would thus be spectrally decoupled from the fluorophore and capable of efficient FRET-based quenching. We therefore utilized recently-reported conditions for transition metal catalyzed tetrazine formation to directly install tetrazines on nitrile-derivatized BODIPY scaffolds (Figure 1).^[13] On treatment of **1** with hydrazine, formamidine acetate, and 50 mol% nickel triflate, with DMF as a cosolvent, the major product observed was the dipyrromethene core of **1**. When zinc triflate was used, however, we observed formation of the desired H-tetrazine **2a**. The fluorescence enhancement observed on reaction of **2a** with *trans*-cyclooctenol (TCO) in water (schematized in Figure 1) was characteristically fast and of significantly greater magnitude than previously reported Tz-fluorophore derivatives, with a turn-on ratio of several hundred fold (Figure 2c). However, it proved somewhat challenging to completely eliminate trace bright impurities from the reaction products. We therefore turned to the synthesis of methyltetrazine **2b**, speculating that enhanced Tz stability would facilitate purification and precise quantification.^[14] With 50 mol% zinc triflate, hydrazine, and acetonitrile at 60°C, we observed formation of both **2a** and **2b** in approximately equal quantities (Figure 1a). H-tetrazine formation was unexpected, as it has not been previously reported under these reaction conditions, but is likely derived from the DMF cosolvent. In sharp contrast to the parent compound **1**, both **2a** and **2b** are modestly soluble in aqueous solution and almost completely non-fluorescent. The methyltetrazine derivative **2b** could be isolated with a clean fluorescence baseline for accurate quantification (supporting information). Reaction of **2b**

with TCO in acetonitrile and in water allowed us to measure turn-on ratios of 340 and 900-fold, respectively (Table 1).

To explore configurational effects on turn-on efficiency, we moved the position of the tetrazine from *para*- to *meta*- on the phenyl ring. Structural modeling estimated that this one-bond shift reduces the inter-chromophore distance (center to center) from 8.4 Å to 7 Å. Condensation of dimethylpyrrole with 3-formylbenzonitrile, followed by routine oxidation with DDQ, deprotonation with Hünig's base, and boron complexation afforded the *m*-cyanophenyl-BODIPY **3** (supporting information). From this precursor, meta-Tz derivatives **4a** and **4b** were synthesized via the same reaction conditions as for **2**. Impressively, these compounds exhibit even greater fluorogenic turn on, reaching 1100-fold in acetonitrile and 1600-fold in water for **4b** (Table 1). Figure 2a plots the normalized fluorescence emission spectrum of **4b** in acetonitrile before and after reaction with TCO; a logarithmic scale is necessary to visualize both spectra simultaneously. The fluorescence time-courses for representative compounds in acetonitrile appear in Figure 2b.

The exceptional fluorogenic turn-on of **2** and **4**—as much as 100-fold greater than flexibly linked fluorophore-Tz conjugates—suggested that FRET may not be the sole quenching mechanism for these compounds. Burgess, Topp, and co-workers observed efficient through-bond energy transfer in cassettes that linked anthracene to BODIPY in a similar scaffold.^[15] Coumarin-BODIPY and quinoline-BODIPY derivatives that exploit TBET have also been reported.^[16] In each of these cases, excitation energy transfers from the attached donor chromophore into the BODIPY acceptor, whereas TBET in the reverse direction is the potential mechanism here. Redox-based quenching, such as via photoinduced-electron transfer (PET) from the excited BODIPY to the relatively electron-poor tetrazine ring, was judged unlikely, as the fluorescence emission intensity of **4b** was found to be independent of solvent polarity (supporting information).^[17]

The architectural hallmark of TBET is a conjugated, but not coplanar, pi-system, in which steric factors enforce a twist in the inter-chromophore linkage.^[18] A three-dimensional structure of compound **4a** (Figure 2b) illustrates these characteristic traits. Also depicted in the figure are the known orientation of the BODIPY transition dipole, and the likely orientation of the tetrazine $S_0 \rightarrow S_1$ ($n \rightarrow \pi^*$) absorption dipole, which is perpendicular to the plane of the Tz ring, as inferred from the known $S_0 \rightarrow S_1$ dipole of s-tetrazine.^[19,20] Limited experimental data on the conformational behavior of aryl-tetrazines indicate a preference for coplanarity, as depicted, which brings the transition dipoles into parallel alignment.^[19] Several practical features distinguish TBET from FRET, including insensitivity to spectral overlap (allowing donor chromophores to excite significantly red-shifted acceptors), decreased dependence on donor acceptor dipole alignment, and energy transfer kinetics (TBET is substantially faster, due to the lack of a strict orientational requirement).^[18] In lieu of ultrafast spectroscopy studies, we elected to perturb the donor-acceptor geometry of the BODIPY-Tz derivatives and test the effect on fluorescence quenching.

Compounds **5a** and **5b** were synthesized starting from 2-iodo-pentamethyl BODIPY^[21] (supporting information). An *ortho*-methyl group was added to enhance twist (i.e. minimize conjugation) of the phenyl linker. In these derivatives, the tetrazine and BODIPY transition dipoles are perpendicular irrespective of intramolecular bond rotation, a configuration in which Förster theory predicts FRET efficiency to be near zero.^[11] Elegant confirmation of this prediction has been made with non-conjugated anthracene-porphyrin cassettes.^[22] Upon reaction of **5b** with TCO in acetonitrile, we observed a 120-fold turn-on, corresponding to >99% baseline quenching. This result stands in sharp contrast to both the Förster theory prediction and the exceptionally efficient transfer observed for **2** and **4**. It is, however, consistent with the results observed for BODIPY-anthracene TBET cassettes, in which

efficient energy transfer was observed in the perpendicular dipole configuration, but with slower kinetics than when the transition dipoles were aligned head to tail.^[15] We anticipate that the tetrazine-TBET quenching mechanism can be extended to other fluorophores and other tetrazines, and that consideration of transition dipole orientation will be critical to the design of optimized turn-on probes.

Having advanced our understanding of the quenching mechanism, we sought to validate the utility of fluorogenic BODIPY-tetrazines for biological imaging (Figure 3). Both extracellular and intracellular TCO-labeled targets were readily visualized, with excellent signal intensity, very low background, and with no washing steps required after addition of the dye solution.

In summary, we report the synthesis and characterization of extraordinarily efficient bioorthogonal turn-on probes. We expect that these materials will find applications for live cell imaging, in pre-targeting, and perhaps in vivo imaging, i.e. applications where washing steps are difficult to achieve. Another unique application of the described materials may be for super-resolution microscopy,^[24] to achieve new applications, faster reconstruction, or to address existing problems where fluorochrome crowding limits reconstructions.^[25] Finally we anticipate that the described principles will find applications in the design of blue- and yellow/orange fluorochromes to widen the palette of available colors for in vivo imaging. Irrespective of the specific design, the described turn-on probes already have activation ratios that are up to two orders of magnitude higher than those previously reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Initial synthesis of BODIPY-*p*-Tz derivatives (**2a,2b**), schematic of fluorogenic reaction with *trans*-cyclooctenol (TCO), and observed turn-on in water. Reaction conditions: i = Zn(OTf)₂, NH₂NH₂, acetonitrile, 60°C, 18hrs; ii = NaNO₂; iii = HCl (1M). b) Synthesis of BODIPY-*m*-Tz derivatives (**4a,4b**) and fluorogenic turn-on in water; reaction conditions as in part a). c) Equatorial BODIPY-Tz derivatives (**5a,5b**) and observed turn-on upon reaction with TCO in acetonitrile.

Figure 2.

a) Fluorescence emission spectra for compound **4b** in acetonitrile at baseline (black) and after addition of TCO (green); excitation at 490nm. b) Normalized fluorescence turn-on after addition of TCO (240uM) to a solution of the indicated fluorophore (1uM) in acetonitrile. c) Equimolar solutions of compound **2a** (at left) and **2a** plus TCO (at right) under excitation by a handheld UV lamp. d) 3D model of compound **4a**, illustrating a twisted phenyl linker between the BODIPY and tetrazine chromophores and the orientation of the donor and acceptor transition dipoles (gray arrows).

Figure 3.

Biological application of activatable BODIPY. a) Fluorogenic imaging of EGFR expression on both fixed and live A-431 cells. Cells were incubated with TCO-conjugated monoclonal antibodies^[23], washed, and then imaged immediately after the addition of 100nM BODIPY-Tz in PBS [see supporting information]. b) Fluorogenic live-cell imaging of nanoparticles internalized by RAW 264.7 cells. The nanoparticles are labeled with both TCO and with the near-infrared dye VT680^[23], and were imaged in two channels after addition of 100nM BODIPY-Tz, demonstrating co-localization.

Table 1

Quantum Yield and Fluorogenic Activation

Probe	Φ w/ TCO in water ^[a]	Φ w/ TCO in MeCN ^[a]	Fluorescence increase in water ^[b]	Fluorescence increase in MeCN ^[b]
2b	0.80	0.23	900-fold	340-fold
4b	0.73	0.58	1600-fold	1100-fold
5b	ND ^[d]	0.22	ND ^[d]	120-fold

^[a] Quantum yield for dihydropyridazine product; fluorescein in 0.1M NaOH (pH 13, Φ =0.925) was used as the standard.

^[b] Increase in peak fluorescence intensity at reaction completion; for experiments in water, 400nM BODIPY-Tz, and 1 μ M TCO were used.

^[c] Compound **5b** is insufficiently soluble in water for this determination.