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Fluorogenic Probes

BODIPY–Tetrazine Derivatives as Superbright Bioorthogonal Turn-on Probes**

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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 1) be catalyst-free (to minimize toxicity and the need for multiple reaction partners), 2) have fast reaction kinetics (to allow efficient imaging and temporal resolution), and 3) be 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, nontoxic) probes with fluorescence turn-on ratios exceeding 10-fold.

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, which were chosen for their ready synthetic accessibility, are quenched with moderate efficiency, yielding turn-on ratios in 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 10^2 would be preferable for robust utility in cellular imaging applications, and a ratio of 10^3 may be necessary for low-abundance targets or super-resolution imaging.[9] Mechanistic observations have suggested that quenching in bichromophoric fluorophore–tetrazines occurs through Förster resonance energy transfer (FRET), offering an initial theoretical framework 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 interchromophore distance (varying as r^6) and upon transition-dipole alignment, which are both parameters that can be optimized.[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 (Scheme 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° rotation between the BODIPY and the phenyl ring, a conformation that prevents π-system conjugation.[12] We hypothesized 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 (Scheme 1).[13] On treatment of **1** with hydrazine, formamidine acetate, and nickel triflate (50 mol%), 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 (Scheme 1) was characteristically fast and of significantly greater magnitude than for reported Tz–fluorophore derivatives, with a turn-on ratio of several hundred fold (Figure 1c). However, it proved challenging to completely eliminate traces of bright fluorescent impurities from the reaction products. We therefore turned to

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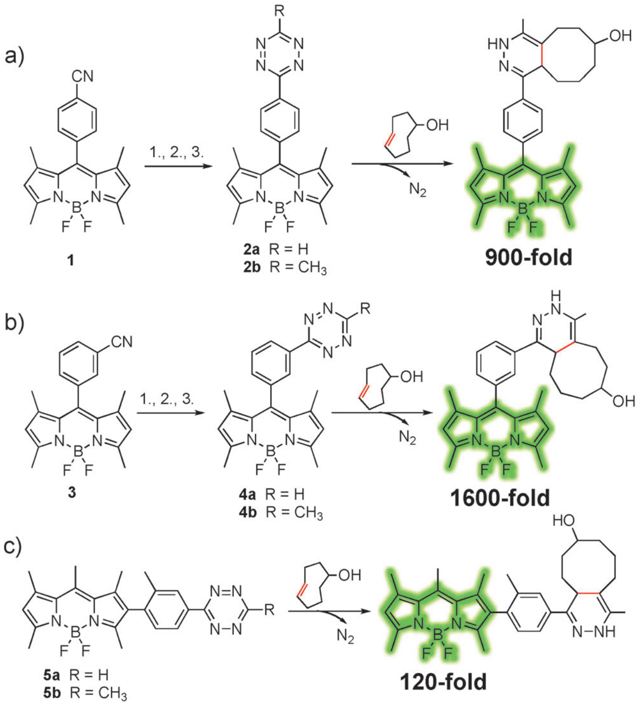

Scheme 1. a) Initial synthesis of BODIPY–*p*-Tz derivatives (**2a**, **2b**), schematic of the fluorogenic reaction with *trans*-cyclooctenol (TCO), and observed turn-on ratio in water. Reaction conditions: 1.) Zn(OTf)$_2$, NH$_2$NH$_2$, acetonitrile, 60 °C, 18 h; 2.) NaNO$_2$; 3.) HCl (1 M). b) Synthesis of BODIPY–*m*-Tz derivatives (**4a**, **4b**) and fluorogenic turn-on in water; reaction conditions as in (a). c) Equatorial BODIPY–Tz derivatives (**5a**, **5b**) and observed turn-on upon reaction with TCO in acetonitrile. In (a–c) the turn-on ratios for the methyltetrazines are given below the fluorescent product.

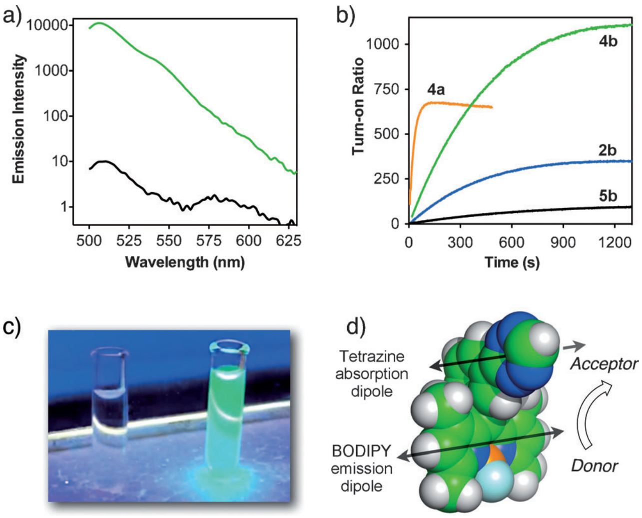

Figure 1. a) Fluorescence emission spectra for compound **4b** in acetonitrile at baseline (black) and after addition of TCO (green); excitation at 490 nm. b) Normalized fluorescence turn-on after addition of TCO (240 μM) to a solution of the indicated fluorophore (1 μM) in acetonitrile. c) Equimolar solutions of compound **2a** (left) and **2a** plus TCO (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).

the synthesis of methyltetrazine **2b**, speculating that enhanced Tz stability would facilitate purification and precise quantification.[14] With zinc triflate (50 mol %), hydrazine, and acetonitrile at 60 °C, we observed formation of both **2a** and **2b** in approximately equal quantities (Scheme 1 a). H-tetrazine formation was unexpected, since it has not been 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 nonfluorescent. The methyltetrazine derivative **2b** could be isolated free of any bright fluorescent impurities (see the 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).

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[c]	0.22	ND[c]	120-fold

[a] Quantum yield for the dihydropyridazine product after complete reaction of the indicated compound with TCO; fluorescein in NaOH (0.1 M, pH 13, $\Phi = 0.925$) was used as the standard. [b] Increase in peak fluorescence intensity at reaction completion; for experiments in water, BODIPY-Tz (400 nM), and TCO (1 μM) were used. [c] Compound **5b** is insufficiently soluble in water for this determination.

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 interchromophore distance (center to center) from 8.4 to 7 Å. Condensation of dimethylpyrrole with 3-formylbenzonitrile, followed by routine oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), deprotonation with Hünig's base, and boron complexation afforded the *m*-cyanophenyl-BODIPY **3** (see the Supporting Information). From this precursor, *meta*-Tz derivatives **4a** and **4b** were synthesized by using the same reaction conditions as for **2**. Impressively, these compounds exhibit even greater fluorogenic turn-on ratios, reaching 1100-fold in acetonitrile and 1600-fold in water for **4b** (Table 1). In Figure 1 a plots of the normalized fluorescence emission spectrum of **4b** in acetonitrile before and after reaction with TCO are shown; a logarithmic scale is necessary to visualize both spectra simultaneously. The fluorescence time courses for representative compounds in acetonitrile appear in Figure 1 b.

The exceptional fluorogenic turn-on ratios of **2** and **4**—as much as 100-fold greater than of 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 through photoinduced electron transfer (PET) from the excited BODIPY to the relatively electron-poor tetrazine ring, was judged unlikely, because the fluorescence emission intensity of **4b** was found to be independent of solvent polarity (see the Supporting Information).[17]

The architectural hallmark of TBET is a conjugated, but not coplanar, π system, in which steric factors enforce a twist in the interchromophore linkage.[18] A three-dimensional structure of compound **4a** (Figure 1d) 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, owing to the lack of a strict orientational requirement).[18] Instead of ultrafast spectroscopy studies, we chose 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] (see the 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 nonconjugated anthracene–porphyrin cassettes.[22] Upon reaction of **5b** with TCO in acetonitrile, we observed a turn-on ratio of 120-fold. 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 2). 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.

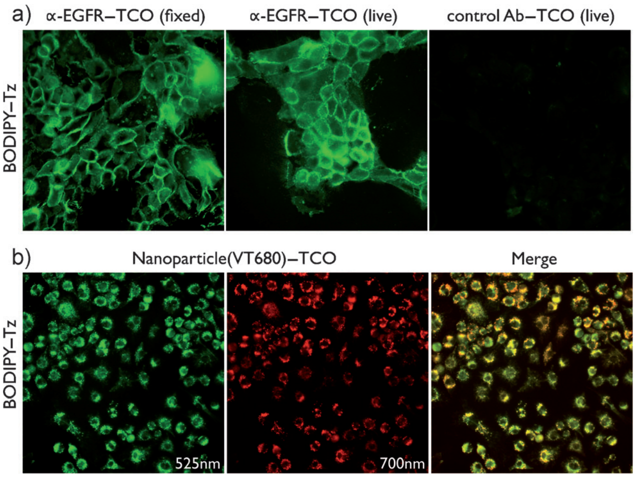

Figure 2. Biological application of activatable BODIPY. a) Fluorogenic imaging of EGFR expression on both fixed and live A-431 cells. Cells were incubated with TCO-conjugated monoclonal antibodies,[23] washed, and then imaged immediately after the addition of BODIPY–Tz (100 nM) in phosphate-buffered saline (PBS; see the 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 BODIPY–Tz (100 nM), demonstrating co-localization.

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 pretargeting, and perhaps in vivo imaging, that is, applications where washing steps are difficult to achieve. Another unique application of the described materials may be for superresolution 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 reported.

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