A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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To be cited as: *ChemBioChem* 10.1002/cbic.201700656

Link to VoR: http://dx.doi.org/10.1002/cbic.201700656

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10.1002/cbic.201700656

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FULL PAPER

Design and Synthesis of a BODIPY-Tetrazole Based "Off-On" In-Cell Fluorescent Reporter of Hydrogen Peroxide

Peng An,[a] Tracey M. Lewandowski,[a] and Qing Lin*[a]

Dedication ((optional))

Abstract: The BODIPY-linked bithiophene-tetrazoles were designed and synthesized for bioorthogonal photoclick reactions in vitro and in vivo. The reactivity of these tetrazoles toward dimethyl fumarate was found to depend on BODIPY attachment site, with the *meta*-linked BODIPY-tetrazole being the most reactive. The resulting BODIPY-pyrazolines showed drastically reduced BODIPY fluorescence. Interestingly, the BODIPY fluorescence recovered after treatment with hydrogen peroxide, which was attributed to the conversion from the pyrazoline to the pyrazole. Finally, this type of BODIPY-tetrazole "off-on" fluorescence probe was employed to detect hydrogen peroxide in HeLa cells.

Introduction

Bioorthogonal chemistry allows selective functionalization of biomolecules with biophysical and chemical probes in their native environment.[1] Due to their small size and tunable photophysical property, small-molecule fluorophores are frequently introduced into a target biomolecule via bioorthogonal reactions to visualize either a dynamic biological process or an environment change in living systems.[2] In particular, small-molecule fluorophore based sensors with fluorescent "turn-on",[3] "quenching"[4] or "color switching"[5] properties have been developed to detect the reactive oxygen and nitrogen species, metal ions and metabolites as well as the macromolecular interactions in living cells and organisms.

We recently reported *in situ* formation of fluorescent pyrazolines via a photoinduced tetrazole-alkene cycloaddition reaction ("photoclick chemistry"),[6] and showed that this reaction is capable of fluorescently labeling proteins in mammalian cells with a spatiotemporal control.[7] However, the pyrazoline is in general not a bright fluorophore because of its low to modest quantum yield and variable intensity depending on solvent polarity.[8] To overcome this limitation, we sought to couple the change of its fluorescence state with a bright fluorophore so that we can obtain fluorescent probe to monitor environmental cues in living systems. To this end, we chose BODIPY fluorophore in this study because: i) it exhibits excellent photochemical stability and high fluorescence quantum yield; ii) it can be readily synthesized from an aldehyde[9]; iii) the BODIPY fluorescence intensity is highly susceptible to the intramolecular photoinduced electron transfer (PeT) process,[10] which can be modulated by the presence of a second fluorophore such as pyrazoline.

Because the intramolecular photoinduced electron transfer efficiency depends on the distance between a donor and an acceptor,[11] we designed three BODIPY-tetrazole probes by connecting BODIPY with the bithiophene-tetrazole[12] through a benzene linker in different orientations (Chart 1) to enable light-triggered formation of the pyrazoline fluorophore via photoclick chemistry. We envisioned that the conversion of the adjacent tetrazole to pyrazoline would modulate fluorescent properties of BODIPY through intramolecular PeT process. In addition, the pyrazoline can be further oxidized to pyrazole,[13] allowing the BODIPY-pyrazoline to serve as a chemical sensor for oxidative environment in cells. Herein, we report the synthesis of a series of BODIPY-tetrazoles, and characterization of their reactivity in the photoclick chemistry and their photophysical properties before and after the reaction as well as in response to hydrogen peroxide treatment. We further show that a water-soluble *meta*-BODIPY-tetrazole could serve as an "off-on" fluorescent probe formed *in situ* at microtubules for detection of hydrogen peroxide in HeLa cells.

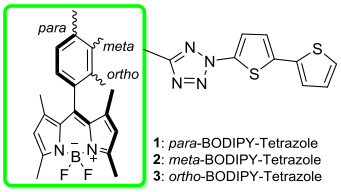

Chart 1. Structures of BODIPY-tetrazoles **1**–**3**.

Results and Discussion

Scheme 1 shows synthetic routes for three BODIPY-tetrazoles. Notably, the tetrazole and BODIPY moieties were constructed consecutively for compounds **1** and **2**, while the sequence was reversed for compound **3**. In brief, the nitriles were reacted with NaN_3 in the presence of triethylammonium chloride in toluene to afford corresponding *2H*-tetrazoles **4a** and **4b** in 98% and 91% yield, respectively. Then, tetrazoles **4a** and **4b** were treated with phenyl(bithiophen-2-yl)iodonium salt **7** in a Cu^{II}-catalyzed cross-coupling reaction to give bithiophene-tetrazole esters **5a** and **5b** in modest yields. The esters were then reduced to the alcohols with LAH followed by oxidation with PCC to generate aldehydes **6a** and **6b** in good yields. Next, the aldehydes were treated sequentially with 2,4-dimethylpyrrole in the presence of catalytic amount of trifluoroacetic acid (TFA), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), and $BF_3{\cdot}Et_2O$ and *N*,*N*-

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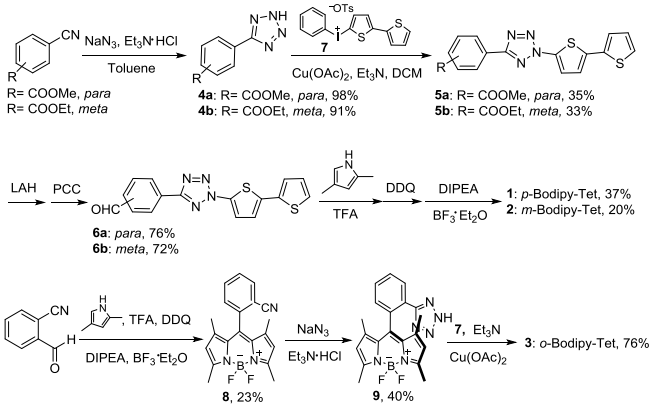
10.1002/cbic.201700656

diisopropylethylamine in one-pot to give *para*-BODIPY-tetrazole **1** and *meta*-BODIPY-tetrazole **2** in 37% and 20% yield, respectively. For *ortho*-BODIPY-tetrazole **3**, 2-formylbenzonitrile was transformed to BODIPY derivative **8** with 23% yield. Then, tetrazole **9** was synthesized using the same procedure as tetrazoles **4a**/**4b**, followed by Cu^{II}-catalyzed cross-coupling with the iodonium salt **7** to give BODIPY-tetrazole **3** in 30% yield over two steps.



Scheme 1. Synthesis of BODIPY-tetrazoles **1**–**3**.

With the three BODIPY-tetrazoles in hand, we first tested their reactivity toward dimethyl fumarate in the photoclick chemistry. We found that BODIPY-tetrazoles **1** and **2** gave clean conversions to pyrazolines, **1-pyr** and **2-pyr**, respectively, after 2-min photoirradiation (Figure S1),[14] with a higher yield for BODIPY-tetrazole **2** (95% under 365-nm photoirradiation and 40% under 405-nm photoirradiation based on the HPLC analysis). BODIPY-tetrazole **3** was stable under 405-nm photoirradiation and gave only trace amount of pyrazoline product under 365-nm photoirradiation, likely due to its high fluorescence quantum yield (Table 1) which turns the incident light energy to fluorescence instead of rupturing the tetrazole ring. Accordingly, we decided to focus on BODIPY-tetrazoles **1** and **2** going forward. To assess how the pyrazoline formation affects the BODIPY fluorescence, we compared the photophysical properties of BODIPY-tetrazoles to their pyrazoline counterparts (Figure 1 and Table 1). For the BODIPY-tetrazoles, two absorption bands centered at 355 and 500 nm were observed (Figures S2-S4), which we attributed to the bithiophene-tetrazole and BODIPY moiety, respectively. For the BODIPY-pyrazolines, new absorption bands at around 390 nm were observed (Figures S2-S3), consistent with the formation of the bithiophene-pyrazolines, while the BODIPY absorption bands remained the same. In fluorescence measurement, only one emission band at 509 nm was observed for both BODIPY-tetrazoles and BODIPY-pyrazolines (Figures 1b and S2–S3), indicating that the pyrazoline fluorescence is negligible. The fluorescence quantum yields of BODIPY-tetrazoles range from 14% to 50% depending on the linkage, with BODIPY-tetrazole **3** giving the highest quantum yield (Table 1). For BODIPY-pyrazolines, however, drastic reduction (~97%) in BODIPY fluorescence was observed (Figure 1b). We attribute this pyrazoline-induced fluorescence quenching to intramolecular PeT process.

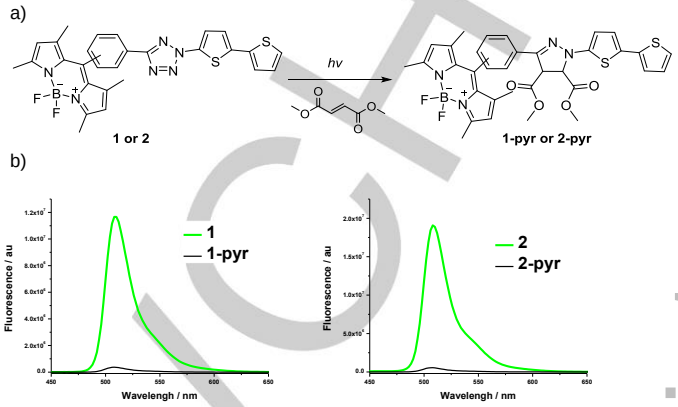


Figure 1. (**a**) Scheme for BODIPY-tetrazole reactions with dimethyl fumarate to generate a racemic mixture of BODIPY-pyrazoline, **1-pyr** or **2-pyr**, under 365 or 405 nm photoirradiation. (**b**) Fluorescence spectra of BODIPY-tetrazole **1** (left, green line) and **1-pyr** (left, black line), and BODIPY-tetrazole **2** (right, green line) and **2-pyr** (right, black line). All compounds were dissolved in PBS/ACN (1:1) to obtain a concentration of 5 μM. λ_{ex} = 405 nm.

Table 1. Photophysical Properties of BODIPY-Tetrazoles **1–3** and BODIPY-Pyrazolines, **1-pyr** and **2-pyr**[a]

Compound	λ_{abs} (nm)	λ_{em} (nm)[b]	Φ_F (%)[c]	quenching efficiency (%)[d]
1	354, 500	510	14	-
1-pyr	392, 500	509	0.08	97
2	354, 500	508	36	-
2-pyr	390, 500	508	0.11	97
3	355, 506	514	50	-

[a] Pyrazolines were dissolved in PBS/ACN (1:1) to obtain a concentration of 5 μM. [b] λ_{ex} = 405 nm. [c] Fluorescence quantum yield was determined using DAPI as a reference. [d] Quenching efficiency = 1 - fluorescence intensity of BODIPY-pyrazoline/fluorescence intensity of BODIPY-tetrazole at 509 nm.

To assess the kinetics of fluorescence quenching as a result of the cycloaddition reaction, a solution of BODIPY-tetrazole **2** and dimethyl fumarate in PBS/ACN (1:1) was photoirradiated with a handheld 365-nm UV lamp and the BODIPY fluorescence was measured over a period of 200 sec (Figure 2a).[6] We found that the BODIPY fluorescence quenching proceeded very fast, with a half-life of 53 sec (Figure 2b). As a control, irradiating the BODIPY core with the 365-nm UV lamp did not lead to any decrease in fluorescence intensity over a period of 320 sec (Figure S5). Based on the fluorescence signal decay, a second-order rate constant, k_2, was calculated to be 26 M^{-1} s^{-1} for the cycloaddition (Figure 2b), confirming that the *in situ* pyrazoline formation is indeed fast.

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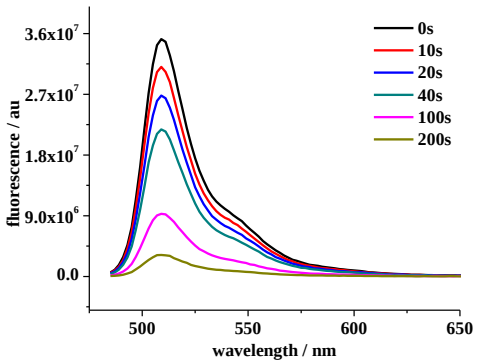
a)



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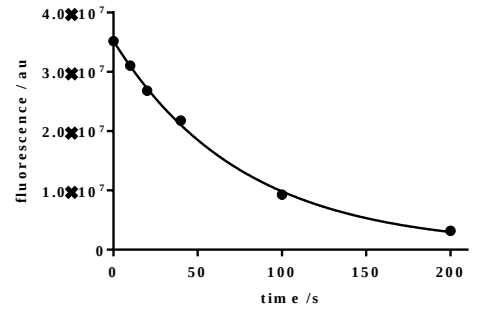
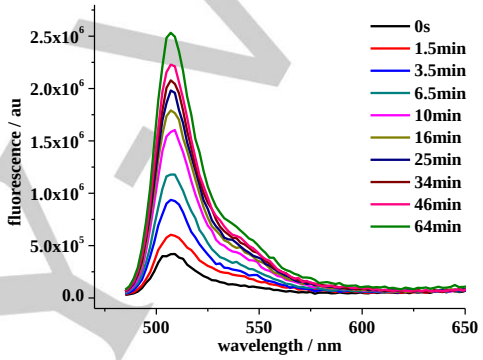


Figure 2. (**a**) Time-dependent fluorescence spectra of BODIPY-tetrazole **2** (5 μM) after photoirradiation at 365 nm in the presence of 500 μM dimethyl fumarate. (**b**) Plot of fluorescence intensity at 509 nm vs. photoirradiation time. For spectrum acquisition, λ_{ex} = 480 nm.

Since pyrazolines can be oxidized to pyrazoles,[12] we examined the possibility of turning back on BODIPY fluorescence through chemical oxidation of the adjacent pyrazoline. Accordingly, we treated **2-pyr** with numerous oxidants including phenyliodine(III) diacetate, Dess–Martin periodinate and hydrogen peroxide, and confirmed the recovery of BODIPY fluorescence (Figure S6) and the conversion of pyrazoline to pyrazole (Figures S7 and S8) under these conditions. We then incubated a solution of **2-pyr** in PBS/ACN (1:1) with 10 mM hydrogen peroxide and monitored the rate of fluorescence recovery (Figure 3a), and observed a time-dependent BODIPY fluorescence increase to a maximum of 5-fold compared to BODIPY-tetrazole **2** after about 1 h (Figure 3b). To verify that the H_2O_2-induced fluorescence turn-on is not due to BODIPY oxidation, we treated the BODIPY core (compound **8**) with 10 mM H_2O_2 and found that the BODIPY fluorescence decreased over a course of 1 h (Figure S9), indicating that the turn-on is indeed due to the conversion of pyrazoline to pyrazole.

Because hydrogen peroxide is a reactive oxygen species affecting cellular redox potential, significant efforts have been devoted to the development of small-molecule probes of cellular hydrogen peroxide in the literature.[15] Based on the observation that hydrogen peroxide can “turn-on” the BODIPY-pyrazoline fluorescence, we envisioned a fluorescence “off-on” detection system in which the BODIPY-pyrazoline sensor is generated *in situ* in cellular systems using the photoclick chemistry and subsequent oxidation of the pyrazoline to pyrazole serves as a basis for detection of hydrogen peroxide. To test this idea, we synthesized a water-soluble *meta*-BODIPY-tetrazole analog 15 (scheme 2). In brief, dimethyl 5-cyanoisophthalate was treated with NaN3 in the presence of triethylammonium chloride in toluene to give tetrazole **10** in 84% yield. Then, the Cu^{II} catalyzed cross-coupling with iodonium salt **7** afforded bithiophene-tetrazole **11** in 55% yield. Next, diester **11** was reduced to diol **12** with LAH followed by oxidation with PCC to give mono-aldehyde **13** in 35% yield over two steps. Applying the same BODIPY synthesis procedure using aldehyde **13** the starting material gave BODIPY-tetrazole **14** in 26% yield. The final water-soluble succinate derivative **15** was obtained in 70% yield after acylating BODIPY-tetrazole **14** with succinic anhydride.

a)



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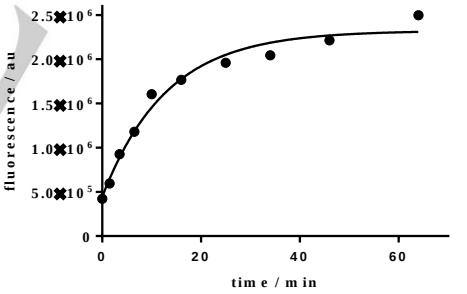

Figure 3. (**a**) Fluorescence spectra of **2-pyr** (1 μM in 1:1 PBS/acetonitrile) after treatment with 10 mM hydrogen peroxide for various times. (**b**) Plot of fluorescence intensity at 509 nm vs. incubation time. For spectrum acquisition, λ_{ex} = 480 nm.

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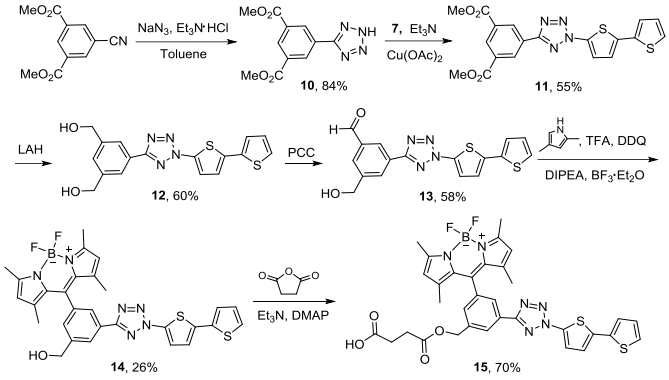
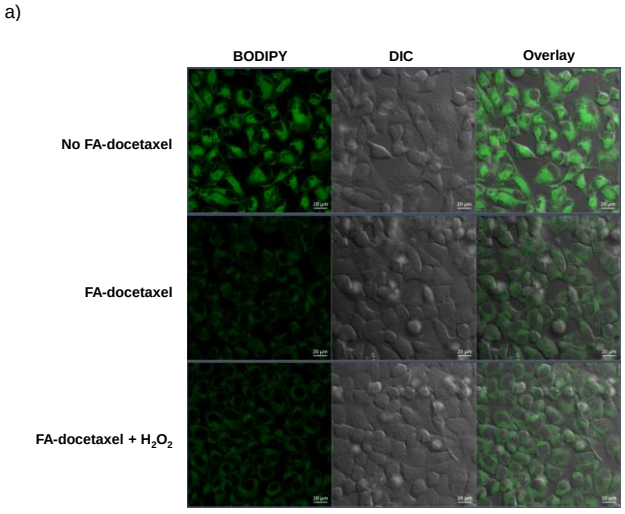


Scheme 2. Synthesis of a water-soluble BODIPY-tetrazole **15**.



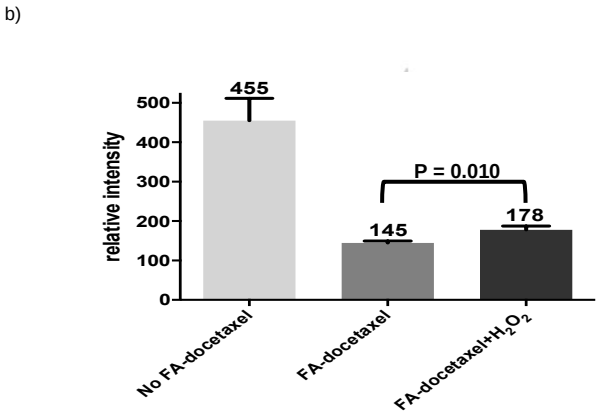


Figure 4. Confocal microscopic imaging of HeLa cells after the docetaxel-directed in situ synthesis of the BODIPY-pyrazoline probe *via* the photoclick chemistry, and the response to H_2O_2 treatment. (**a**) Top row: cells were treated with tetrazole **15** (500 nM) only prior to photoirradiation. Middle row: cells were treated with tetrazole **15** (500 nM) and FA-docetaxel (30 μM) prior to photoirradiation. Bottom row: cells were treated with tetrazole **15** (500 nM) and FA-docetaxel (30 μM) prior to photoirradiation. Afterward, cells were treated with 500 μM H_2O_2 for 1 h. A 3-min photoirradiation with a handheld 365 nm UV lamp was applied to all cells. Scale bar = 20 μm. (**b**) Quantification of fluorescence of HeLa cells. Relative fluorescence intensity was obtained by applying ImageJ program to the fluorescent images to obtain mean and SEM. Student *t*-test was performed to obtain the *P*-value.

To test whether BODIPY-tetrazole **15** allows *in situ* synthesis of BODIPY-pyrazoline probe for in-cell detection of hydrogen peroxide, we introduced the fumarate substrate into mammalian cells via a microtubule binding ligand as reported previously.[7c] In brief, we treated HeLa cells with 500 nM tetrazole **15** overnight, and afterwards washed away excess reagents with DMEM medium. The cells were incubated in OPTI-MEM medium containing 30 μM fumarate-modified docetaxel (FA-docetaxel)[7c] for 30 min at 37 °C. Excess FA-docetaxel was washed away with DMEM medium, and 365-nm photoirradiation was applied to the cells for 3 min before confocal microscopy. We observed ~66% reduction of BODIPY fluorescence for the FA-docetaxel treated cells (row 2 in Figure 4a) compared to the untreated cells (row 1 in Figure 4a), consistent with the formation of the weakly fluorescent BODIPY-pyrazoline probe (Figure 2). The DMEM medium was then removed and the cells were incubated in DMEM medium containing 500 μM hydrogen peroxide at 37 °C. After 1 h, stronger fluorescence was observed inside HeLa cells (row 3 in Figure 4a). Quantification of fluorescence intensity revealed about 23% increase after H_2O_2 treatment (Figure 4b), consistent with the H_2O_2-induced BODIPY-pyrazoline to BODIPY-pyrazole conversion (Figure 3). The magnitude of fluorescent "turn-on" is similar to the arylboronate-based H_2O_2 detection system reported in the literature.[15b]

Conclusions

In summary, we have synthesized three BODIPY-tetrazoles with *para*-, *meta*- or *ortho*-linkage between the bithiophene-tetrazole and the BODIPY fluorophore. BODIPY-tetrazole **2** displayed excellent reactivity towards dimethyl fumarate under 365 or 405 nm photoirradiation in the photoclick chemistry. Interestingly, the BODIPY fluorescence showed sharp decrease after the reaction, which can be recovered through treatment with chemical oxidants such as hydrogen peroxide. A water-soluble BODIPY-tetrazole derivative was synthesized and served as an "off-on" fluorescent probe for bioorthogonal generation of the probe and subsequent detection of hydrogen peroxide in HeLa cells.

Experimental Section

General reagents and instrument: Solvents and chemicals were purchased from commercial sources and used directly without further purification. Flash chromatography was performed either manually with SiliCycleP60 silica gel (40−63 μm, 60 Å) or using an automatic Yamazen AKROS flash system equipped with SiliaSep HP pre-packed columns. ^{1}H NMR spectra were recorded with Inova-300, -400 or -500 MHz spectrometers and chemical shifts were reported in ppm using either TMS

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or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75.4 MHz, and chemical shifts were reported in ppm using the deuterated solvents as internal standards ($CDCl_3$, 77.0). Absorption spectra were recorded using 1-cm quartz cuvettes on a HP-8452 Diode Array Spectrometer. Fluorescence spectra were recorded using 1-cm cuvette on Horiba FluoroMax-4 spectrofluorometerat 25 °C. The fluorescence images were acqured using Zeiss LSM-710 confocal microscope equipped with continuous laser and fluorescence lifetime (FLIM) detector.

Chemical synthesis: Methyl 4-(2-([2,2'-bithiophen]-5-yl)-2*H*-tetrazol-5-yl)benzoate (**5a**):[12b] To a solution of methyl 4-cyanobenzoate (322 mg, 2.0 mmol) in toluene (15 mL) was added NaN_3 (260 mg, 4.0 mmol), followed by triethylammonium chloride (548 mg, 4.0 mmol). The resulting mixture was stirred at 110 ºC for 24 h before cooled to room temperature. Then, 10 mL water was added to the reaction and the mixture was stirred for 10 min. The mixture was extracted with water (15 mL) 3 times and the combined aqueous phase was acidified with 3 N HCl solution. The suspension was filtered and the resulting solid was washed with water and diethyl ether to give compound **4a** as a white solid (400 mg, 98% yield): ^1H NMR ($CDCl_3$, 500 MHz) δ 8.22 (d, J = 8.0 Hz, 2H), 8.13 (d, J = 8.0 Hz, 2H), 3.94 (s, 3H). Compound **5a** was synthesized by following the reported procedure.[12b]

4-(2-([2,2'-Bithiophen]-5-yl)-2*H*-tetrazol-5-yl)benzaldehyde (**6a**): To a solution of tetrazole **5a** (130 mg, 0.35 mmol) in anhydrous THF (10 mL) was added $LiAlH_4$ (40 mg, 1.05 mmol) at 0°C, and the suspension was stirred at room temperature until thin layer chromatography (TLC) showed complete disappearance of the starting material. The reaction was quenched with 1 mL MeOH and filtered through a layer of Celite. The filtrate was collected, concentrated, and dried in vacuum. The residue was dissolved in dichloromethane (15 mL) and pyridinium chlorochromate (110 mg, 0.50 mmol) was added. The mixture was stirred at room temperature under argon overnight. The solution was concentrated and the residue was purified by silica gel flash chromatography to give the title compound as a green solid (91 mg, 76% yield): ^1H NMR ($CDCl_3$, 500 MHz) δ 10.11 (s, 1H), 8.41 (d, J = 8.5 Hz, 2H), 8.04 (d, J = 8.5 Hz, 2H), 7.63 (d, J = 4.0 Hz, 1H), 7.32 (dd, J = 5.5, 1.5 Hz, 1H), 7.27 (dd, J = 3.5, 1.0 Hz, 1H), 7.14 (d, J = 4.0 Hz, 1H), 7.08 (dd, J = 5.0, 3.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 191.6, 130.3, 128.2, 127.7, 126.0, 124.98, 122.7, 120.0.

para-BODIPY-tetrazole (**1**): To a solution of aldehyde **6a** (90 mg, 0.27 mmol) and 2,4-dimethylpyrrole (68 µL, 0.66 mmol) in DCM (20 mL) under argon was added two drops of TFA, and the mixture was stirred at room temperature until TLC showed complete disappearance of aldehyde **6a**. A solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ: 66 mg, 0.29 mmol) in DCM (10 mL) was then added, and the mixture was stirred for 30 min. Afterwards, *N*,*N*-diisopropylethylamine (555 µL, 3.2 mmol) and BF_3•OEt_2 (266 µL, 2.1 mmol) were added, and the mixture was stirred overnight. The reaction was quenched by adding 100 mL water, and the aqueous layer was extracted three times with CH_2Cl_2 (300 mL). The organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography using DCM/hexanes (1:2) as eluent to give the title compound as a red solid (55 mg, 37% yield): ^1H NMR ($CDCl_3$, 500 MHz) δ 8.39 (d, J = 5.0 Hz, 2H), 7.64 (d, J = 5.0 Hz, 1H), 7.48 (d, J = 10.0 Hz, 2H), 7.32 (d, J = 5.0 Hz, 1H), 7.28 (d, J = 5.0 Hz, 1H), 7.15 (d, J = 5.0 Hz, 1H), 7.08 (dd, J = 5.0, 6.0 Hz, 1H), 6.00 (s, J = 2H), 2.57 (s, 6H), 1.45 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.4, 155.9, 143.0, 140.5, 137.5, 136.2, 135.6, 135.5, 131.2, 128.9, 128.2, 127.8, 127.5, 125.9, 124.9, 122.6, 121.5, 121.4, 119.7, 110.0, 14.6; HR-MS (EI) calcd for $C_{28}H_{23}BF_2N_6S_2$ 556.14867 [M^+], found 556.14972.

Ethyl 3-(2*H*-tetrazol-5-yl)benzoate (**4b**): To a solution of ethyl 3-cyanobenzoate (760 mg, 4.3 mmol) in toluene (20 mL) was added NaN_3 (423 mg, 6.5 mmol), followed by triethylammonium chloride (892 mg, 6.5 mmol). The resulting mixture was stirred at 110 °C for 24 h before cooled to room temperature. Then, 10 mL water was added to the reaction and the mixture was stirred for 10 min. The mixture was extracted with water and the combined aqueous phase was acidified with 3 N HCl. The suspension was filtered and the resulting solid was washed with water and diethyl ether to give the title compound as a white solid (862 mg, 91%), which was used directly for the next step without further purification.

Ethyl 3-(2-([2,2'-bithiophen]-5-yl)-2*H*-tetrazol-5-yl)benzoate (**5b**): To a solution of tetrazole **4b** (262 mg, 1.2 mmol) in DCM (25 mL) was added iodonium salt **7**[12a] (648 mg, 1.2 mmol), $Cu(OAc)_2$ (434 mg, 2.4 mmol) and Et_3N (836 µL, 6.0 mmol). The resulting mixture was purged with argon and sealed, and mixture was stirred at room temperature for 20 h. The resulting mixture was diluted with water (10 mL) and extracted with DCM (10 mL × 3). The organic layers were combined, washed with saturated NH_4Cl solution and brine, dried over anhydrous $MgSO_4$ and concentrated in vacuum. The residue was purified by silica gel flash chromatography to give the title compound as a white solid (150 mg, 33% yield): ^1H NMR ($CDCl_3$, 300 MHz) δ 8.89 (s, 1H), 8.43 (d, J = 9.0 Hz, 1H), 8.20 (d, J = 9.0 Hz, 1H), 7.64–7.62 (m, 2H), 7.30 (d, J = 6.0 Hz, 1H), 7.26 (s, 1H), 7.15 (d, J = 3.0 Hz, 1H), 7.10 (t, J = 6.0 Hz, 1H), 4.45 (q, J = 6.0 Hz, 2H), 1.45 (t, J = 6.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.9, 164.4, 136.2, 135.7, 135.5, 131.7, 131.5, 131.2, 129.1, 128.1, 127.1, 125.8, 124.9, 122.6, 119.7, 76.6, 61.3, 14.4; HR-MS (EI) calcd for $C_{18}H_{14}N_4O_2S_2$ 382.05582 [M^+], found 383.06410.

3-(2-([2,2'-Bithiophen]-5-yl)-2*H*-tetrazol-5-yl)benzaldehyde (**6b**): Aldehyde **6b** was synthesized as a green solid using the same procedure as **6a** with 72% yield: ^1H NMR ($CDCl_3$, 500 MHz) δ 10.15 (s, 1H), 8.74 (t, J = 1.5 Hz, 1H), 8.51 (dt, J = 7.5, 1.5 Hz, 1H), 8.04 (dt, J = 7.5, 1.5 Hz, 1H), 7.72, (t, J = 7.5 Hz, 1H), 7.64 (d, J = 4.0 Hz, 1H), 7.33, (dd, J = 5.0, 1.0 Hz, 1H), 7.28 (dd, J = 4.0, 1.0 Hz, 1H), 7.15 (d, J = 4.0 Hz, 1H), 7.09 (dd, J = 5.5, 3.5 Hz, 1H).

meta-BODIPY-tetrazole (**2**): Tetrazole **2** was synthesized as a red solid using the same procedure as tetrazole **1** with 20% yield: ^1H NMR ($CDCl_3$, 500 MHz) δ 8.38 (d, J = 5.0 Hz, 1H), 8.21 (s, 1H), 7.68 (t, J = 5.0 Hz, 1H), 7.60 (d, J = 5.0 Hz, 1H), 7.46 (d, J = 10.0 Hz, 1H), 7.32 (d, J = 5.0 Hz, 1H), 7.25 (d, J = 5.0 Hz, 1H), 7.14 (d, J = 5.0 Hz, 1H), 7.07 (t, J = 5.0 Hz, 1H), 6.01 (s, J = 2H), 2.58 (s, 6H), 1.45 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.3, 155.9, 143.0, 140.3, 136.2, 136.0, 135.6, 135.4, 131.3, 130.3, 129.9, 128.1, 127.8, 127.6, 126.8, 125.9, 124.9, 122.6, 121.4, 119.7, 14.7; HR-MS (EI) calcd for $C_{28}H_{23}BF_2N_6S_2$ 556.14867 [M^+], found 556.14850.

BODIPY-acetonitrile (**8**): Compound **8** was synthesized as a red solid using the same procedure as tetrazole **1** with 23% yield: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.84 (d, J = 3.0 Hz, 1H), 7.75 (t, J = 7.5 Hz, 1H), 7.62 (t, J = 7.5, 1.5 Hz, 1H), 7.47 (d, J = 7.5 Hz, 1H), 6.02 (s, 2H), 2.57 (s, 6H), 1.36 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.9, 142.2, 138.8, 136.0, 133.5, 129.8, 129.7, 121.9, 116.4, 113.1, 76.6, 29.7, 14.7, 14.0; HR-MS (EI) calcd for $C_{20}H_{18}BF_2N_3$ 349.15618 [M^+], found 349.15594.

BODIPY-2*H*-tetrazole (**9**): Compound **9** was synthesized as a red solid using the same procedure as **4b** with 40% yield: ^1H NMR ($CDCl_3$, 300 MHz) δ 8.42–8.39 (m, 1H), 7.71–7.69 (m, 2H), 7.45–7.42 (m, 1H), 5.99 (s, 2H), 2.55 (s, 6H), 1.34 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 157.5, 142.4, 137.5, 133.2, 132.2, 130.8, 130.5, 130.3, 129.7, 123.2, 122.3, 29.7, 14.7, 13.9; HR-MS (EI) calcd for $C_{20}H_{19}BF_2N_6$ 392.17323 [M^+], found 392.17294.

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Ortho-BODIPY-tetrazole (**3**): Tetrazole **3** was synthesized as a red solid using the same procedure as **5b** with 76% yield: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.47 (dd, *J* = 7.0, 1.0 Hz, 1H), 7.66 (m, 2H), 7.46 (dd, *J* = 7.0, 2.0 Hz, 1H), 7.39 (d, *J* = 4.0 Hz, 1H), 7.27 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.24 (dd, *J* = 4.0, 1.0 Hz, 1H), 7.04 (m, 2H), 5.92 (s, *J* = 2H, 2H), 2.58 (s, 6H), 1.35 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 163.2, 155.4, 142.4, 136.1, 135.2, 134.0, 131.1, 129.7, 129.5, 128.1, 125.6, 124.7, 122.6, 121.0, 119.5, 14.7, 14.0; HR-MS (EI) calcd for $C_{28}H_{23}BF_2N_6S_2$ 556.14867 [M^+], found 556.14923.

para-BODIPY-pyrazoline (**1-pyr**): To a solution of tetrazole **1** (20 mg, 0.036 mmol) in acetonitrile (20 mL) was added dimethyl fumarate (52 mg, 0.36 mmol), and the mixture was exposed to 365 nm photoirradiation with stirring at room temperature. When TLC showed complete disappearance of the starting material, the solution was concentrated and the residue was purified by silica gel flash chromatography using 30-50% DCM/hexanes as eluent to give the title compound as a red solid (20 mg, 83%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.90 (d, *J* = 10.0 Hz, 2H), 7.33 (d, *J* = 10.0 Hz, 2 H), 7.14 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.05 (d, *J* = 2.5 Hz, 1H), 6.98 (dd, *J* = 5.0, 3.5 Hz, 1H), 6.91 (d, *J* = 5.0 Hz, 1H), 6.16 (d, *J* = 5.0 Hz, 1H), 5.99 (s, *J* = 2H), 5.14 (d, *J* = 5.0 Hz, 1H), 4.75 (d, *J* = 5.0 Hz, 1H), 3.85 (s, 3H), 3.73 (s, 3H), 2.56 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.1, 168.2, 155.8, 147.8, 144.2, 142.9, 140.8, 137.9, 136.0, 131.2, 131.0, 128.4, 127.7, 127.3, 126.5, 123.2, 122.7, 122.3, 121.4, 105.9, 68.1, 56.2, 53.3, 53.2, 14.6.

meta-BODIPY-pyrazoline (**2-pyr**): A similar procedure was performed except reaction time was about 5 h to afford the title compound as a red solid with 85% yield: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.95 (dd, *J* = 10.0, 4.0 Hz, 1H), 7.63 (t, *J* = 1.5 Hz, 1H), 7.53 (t, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 7.5 Hz, 1H), 7.12 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.05 (dd, *J* = 3.5, 1.0 Hz, 1H), 6.97 (dd, *J* = 5.0, 3.5 Hz, 1H), 6.89 (d, *J* = 4.0 Hz, 1H), 6.14 (d, *J* = 4.0 Hz, 1H), 6.00 (s, 2H), 5.10 (d, *J* = 6.0 Hz, 1H), 4.69 (d, *J* = 6.0 Hz, 1H), 3.83 (s, 3H), 3.67 (s, 3H), 2.57 (s, 6H), 1.42 (s, 6H).

Dimethyl 5-(2*H*-tetrazol-5-yl)isophthalate (**10**): Tetrazole **10** was synthesized as a white solid using the same procedure as **4b** with 84% yield: ^{1}H NMR (MeOD-d_4, 500 MHz) δ 8.92 (d, *J* = 2.0 Hz, 2H), 8.77 (t, *J* = 2.0, 1H), 4.01, (s, 6H).

Dimethyl 5-(2-([2,2'-bithiophen]-5-yl)-2*H*-tetrazol-5-yl)isophthalate (**11**): Tetrazole **11** was synthesized as a white solid using the same procedure as **5b** with 55% yield: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.07 (d, *J* = 2.0 Hz, 2H), 8.82 (t, *J* = 7.5, 1.5 Hz, 1H), 7.66 (d, *J* = 4.0 Hz, 1H), 7.33 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.28 (dd, *J* = 3.5, 1.0 Hz, 1H), 7.16 (d, *J* = 4.0 Hz, 1H), 7.09, (dd, *J* = 5.0, 3.5 Hz, 1H), 4.01 (s, 6H).

(5-(2-([2,2'-Bithiophen]-5-yl)-2*H*-tetrazol-5-yl)-1,3-phenylene)dimethanol (**12**): To a solution of tetrazole **11** (85 mg, 0.20 mmol) in anhydrous THF was added $LiAlH_4$ (38 mg, 1.0 mmol) at 0 °C. The suspension was stirred at room temperature until TLC showed complete disappearance of the starting material. The reaction was quenched by adding MeOH (1 mL) and concentrated in vacuum. The residue was purified by silica gel flash chromatography to give the titled compound (42 mg, 60%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.18 (d, *J* = 17.0 Hz, 2H), 7.62 (dd, *J* = 4.0, 2.5 Hz, 1H), 7.55 (d, *J* = 17.0 Hz, 1H), 7.33, (d, *J* = 5.0 Hz, 1H), 7.26 (d, *J* = 4.0 Hz, 1H), 7.14 (d, *J* = 4.0 Hz, 1H), 7.08, (dd, *J* = 5.0, 3.5 Hz, 1H), 4.83 (d, *J* = 5.5 Hz, 4H).

3-(2-([2,2'-Bithiophen]-5-yl)-2*H*-tetrazol-5-yl)-5-(hydroxymethyl)benzaldehyde (**13**): To a solution of tetrazole **12** (40 mg, 0.11 mmol) in DCM (10 mL) was added pyridinium chlorochromate (23 mg, 0.11 mmol). The mixture was stirred at room temperature under argon overnight. The reaction mixture was then concentrated and the residue was purified by silica gel flash chromatography to give the title compound as a yellow solid (23 mg, 58% yield): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.14 (s, 1H), 8.64 (s, 1H), 8.51 (s, 1H), 8.05 (s, 1H), 7.64 (d, *J* = 4.0 Hz, 1H), 7.32 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.27 (dd, *J* = 3.5, 1.0 Hz, 1H), 7.14, (d, *J* = 4.0 Hz, 1H), 7.08, (dd, *J* = 5.0, 1.5 Hz, 1H), 4.91 (d, *J* = 5.0 Hz, 2H).

meta-BODIPY-tetrazole-OH (**14**): Compound **14** was synthesized as a red solid using the same procedure as **1** with 26% yield: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.38 (s, 1H), 8.13 (s, 1H), 7.60 (d, *J* = 4.5 Hz, 1H), 7.49 (s, 1H), 7.31 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.25 (dd, *J* = 3.5, 1.0 Hz, 1H), 7.13 (d, *J* = 4.0 Hz, 1H), 7.07 (dd, *J* = 4.0, 1.5 Hz, 1H), 6.00 (s, 2H), 4.89 (s, 2H), 2.57 (s, 6H), 1.45, (s, 6H).

meta-BODIPY-tetrazole-COOH (**15**): To a solution of **14** (15 mg, 0.04 mmol) in dioxane was added succinic anhydride (8.0 mg, 0.014 mmol), 4-dimethylaminopyridine (6.8 mg, 0.056 mmol), Et_3N (19 µL, 0.14 mmol) and DMAP (0.83 mg, 0.007 mmol), and the mixture was stirred at room temperature until TLC showed complete disappearance of the starting material. The solvent was evaporated and 3 N HCl was added to adjust pH to 6.0. The solution was diluted by adding 5 mL water and extracted with DCM (5 mL × 3). The organic layer was separated, dried over anhydrous $MgSO_4$, and concentrated in vacuum. The residue was purified by silica gel flash chromatography using EtOAc/MeOH (5:1) as eluent to give the title compound as a red solid (6.6 mg, 70% yield): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.34 (s, 1H), 8.15 (s, 1H), 7.60 (d, *J* = 4.0 Hz, 1H), 7.45 (s, 1H), 7.31 (dd, *J* = 4.0, 1.0 Hz, 1H), 7.25 (dd, *J* = 3.5, 1.0 Hz, 1H), 7.13 (d, *J* = 4.0 Hz, 1H), 7.07 (dd, *J* = 3.5, 1.5 Hz, 1H), 6.00 (s, 2H), 3.71 (s, 2H), 2.71 (m, 4H), 2.58 (s, 6H), 1.43, (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 176.2, 171.7, 164.0, 156.1, 142.9, 139.7, 138.4, 136.4, 136.3, 135.6, 135.3, 131.2, 129.6, 128.2, 128.1, 126.7, 126.5, 125.9, 124.9, 122.6, 121.6, 119.9, 65.5, 28.9, 28.7, 14.8, 14.6; MS (ESI) calcd for $C_{33}H_{29}BF_2N_6O_4S_2$ 685.2 [M - H]$^-$, found 685.1.

Fluorescence microscopy: HeLa cells were allowed to grow to 50% confluency on 35-mm glass-bottom tissue culture plates in 2 mL DMEM medium supplemented with 10% FBS in a humidified 37 °C, 5% CO_2 incubator. The cells were treated with DMEM medium (supplemented with 10% FBS) containing 500 nM tetrazole **15** for 14 h followed by DMEM washing, and then the medium was switched to OPTI-MEM containing 30 µM FA-docetaxel. The treatment was continued at 37 °C for 30 min. Then excess FA-docetaxel was washed away using DMEM medium. The light triggered photoclick reaction inside HeLa cells was carried out using 365 nm UV lamp for 3 min prior to confocal microscopy. The control cells were treated the same way except that no FA-docetaxel was added. The fluorescently labeled HeLa cells were treated with DMEM medium containing 500 µM H_2O_2 in a humidified 37 °C, 5% CO_2 incubator for 1 h prior to confocal microscopy. Confocal images were acquired using Zeiss LSM 710 equipped with PlanApochromat 40×/1.3 Oil DIC M27 objective. The excitation and emission lengths for BODIPY fluorophore are ex 488 nm/em 493−598 nm.

Acknowledgements

We gratefully acknowledge the NIH (GM085092) for financial support, and Mr. Alan Siegel at SUNY Buffalo North Campus Imaging Facility for assistance with confocal microscopy.

Keywords: Bioorthogonal • fluorescent probes • tetrazole • BODIPY• hydrogen peroxide

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10.1002/cbic.201700656

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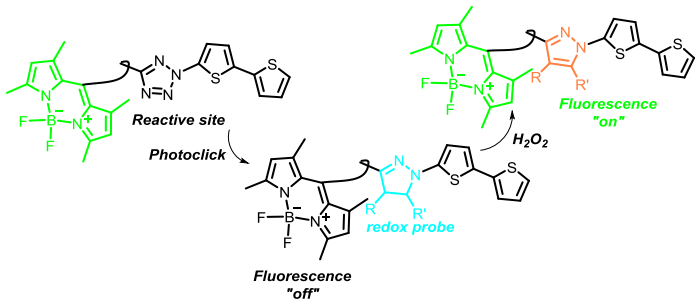

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Design and Synthesis of a BODIPY-Tetrazole Based "Off-On" In-Cell Fluorescent Reporter of Hydrogen Peroxide

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