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# **BODIPY Fluorophores for Membrane Potential Imaging**

Jenna M. Franke,<sup>‡</sup> Benjamin K. Raliski,<sup>‡</sup> Steven C. Boggess,<sup>‡</sup> Divya V. Natesan,<sup>‡</sup> Evan T. Koretsky,<sup>‡</sup> Patrick Zhang,<sup>‡</sup> Rishikesh U. Kulkarni,<sup>‡</sup> Parker E. Deal<sup>‡</sup> and Evan W. Miller<sup>‡§†</sup>\*

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**ABSTRACT:** Fluorophores based on the BODIPY scaffold are prized for their tunable excitation and emission profiles, mild syntheses, and biological compatibility. Improving the water-solubility of BODIPY dyes remains an outstanding challenge. The development of water-soluble BODIPY dyes usually involves direct modification of the BODIPY fluorophore core with ionizable groups or substitution at the boron center. While these strategies are effective for the generation of water-soluble fluorophores, they are challenging to implement when developing BODIPY-based indicators: direct modification of BODIPY core can disrupt the electronics of the dye, complicating the design of functional indicators; and substitution at the boron center often renders the resultant BODIPY incompatible with



the chemical transformations required to generate fluorescent sensors. In this study, we show that BODIPYs bearing a sulfonated aromatic group at the *meso* position provide a general solution for water-soluble BODIPYs. We outline the route to a suite of 5 new sulfonated BODIPYs with 2,6-disubstitution patterns spanning a range of electron-donating and -withdrawing propensities. To highlight the utility of these new, sulfonated BODIPYs, we further functionalize them to access 13 new, BODIPY-based voltage-sensitive fluorophores. The most sensitive of these BODIPY VF dyes displays a 48%  $\Delta$ F/F per 100 mV in mammalian cells. Two additional BODIPY VFs show good voltage sensitivity ( $\geq$ 24%  $\Delta$ F/F) and excellent brightness in cells. These compounds can report on action potential dynamics in both mammalian neurons and human stem cell-derived cardiomyocytes. Accessing a range of substituents in the context of a water soluble BODIPY fluorophore provides opportunities to tune the electronic properties of water-soluble BODIPY dyes for functional indicators.

#### Introduction

Synthetic chemistry has long been a source of colorful compounds<sup>1-3</sup> whose ability to absorb light enable applications in far-ranging fields. Fluorescent dyes find wide-spread use in the modern research laboratory where features such as visible excitation and emission profiles, large molecular brightness values, and photostability are highly prized, along with biologicallycompatible properties like water-solubility. Since the late 19th century, xanthene dyes like fluoresceins<sup>4</sup> and rhodamines<sup>5-6</sup> offered a fertile source of inspiration as scaffolds for biologicallyuseful dyes and indicators.<sup>7-9</sup> More recently, BODIPY, or 4,4difluoro-4-bora-3a,4a,-diaza-s-indacene, (Scheme 1) dyes have emerged as a versatile complement to xanthene dyes. Owing to the relatively mild reaction conditions for the generation of BODIPY fluorophores,<sup>10</sup> a number of flexible synthetic routes afford the opportunity to install a range of substituents directly to the BODIPY core, tuning both the color and electronic properties of BODIPY dyes.

Since the initial report of BODIPY in 1968,<sup>11</sup> a proliferation of synthetic methods<sup>10, 12-13</sup> and conceptual understanding<sup>14-15</sup> enabled the application of BODIPYs as indicators for a number of important, biologically-relevant analytes and properties,<sup>16-17</sup> including pH,<sup>18-19</sup> cations like Na<sup>+</sup>,<sup>20-21</sup> K<sup>+</sup>,<sup>20, 22</sup> Mg<sup>2+</sup>,<sup>23</sup> and Ca<sup>2+</sup>;<sup>24-25</sup> transition metals,<sup>26-28</sup> reactive oxygen<sup>29</sup> and nitrogen

#### Scheme 1. Design of H<sub>2</sub>O-soluble BODIPYs



species,<sup>14</sup> electron transfer reactions,<sup>30</sup> and membrane viscosity.<sup>31</sup> Because of the broad tunability of BODIPY-based scaffolds, we thought these fluorophores would make an excellent choice for incorporation into a molecular wire-based, photo-induced electron transfer (PeT) membrane potential sensing framework.<sup>32</sup> Previous work in our lab showed that tuning the relative electron affinities between a fluorescein-based reporter and electronically-orthogonal phenylenevinylene molecular wire voltage-sensing domain profoundly altered the voltage sensitivities of fluorescein based dyes. However, the limited synthetic scope of sulfonated fluorescein only allowed access to a narrow range of substituents (H, F, Cl, Me).<sup>33</sup>

Here, we introduce new, water-soluble sulfonated BODIPYs with substituents ranging from highly electron donating (R = Et) to

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#### Scheme 2. Synthesis of BODIPY VoltageFluor dyes with Et, H and CN at the 2,6-positions



withdrawing (R = CN). We incorporate the new, sulfonated BODIPYs into a molecular wire voltage-sensing scaffold to provide the first examples of PeT-based voltage-sensitive BODIPYs. The most sensitive of these dyes displays a 48%  $\Delta$ F/F per 100 mV in HEK cells, and two others possess  $\geq$ 24%  $\Delta$ F/F, making them useful for voltage sensing applications in both neurons and cardiomyocytes.

#### Design of water soluble BODIPYs

We prepared a total of 13 BODIPY-based Voltage-sensitive Fluorophores, or BODIPY-VF dyes. All of the BODIPY compounds feature a common ortho-sulfonic acid substituted meso aromatic ring (8-position, Scheme 1) and substitution patterns at the 2,6 positions that include hydrogen, ethyl, carboxylate, amide, and cyano functionalities (Scheme 1). Our initial attempts to access BODIPY-based VoltageFluor indicators centered around the development of water-soluble tetramethyl, diethyl BODIPY fluorophores. Ionizable groups, such as sulfonates or carboxylates, are essential for the proper orientation of VF-type dyes in cellular membranes.<sup>34-35</sup> We first sought to introduce water-solubilizing groups centered on substitution at boron.<sup>36-38</sup> However, in our hands, these modifications proved incompatible with the Pd-catalyzed Heck coupling for installation of voltage-sensing phenylenevinylene molecular wires. Functionalization of the 2 and 6 positions of the BODIPY core offered a route to the installation of water-solubilizing groups like sulfonates<sup>39-40</sup> or carboxylates,<sup>41</sup> but direct functionalization of the BODIPY core can profoundly alter redox properties, confounding the tuning of fluorophore redox potential<sup>15, 33</sup> with installation of water solubilizing groups. One solution is to include a sulfonate on the *meso* aromatic ring (Scheme 1), which we hypothesized would improve solubility, be generalizable across a range of 2,6-substitution patterns on the BODIPY core, and aid in the proper orientation within cellular plasma membranes.

#### Synthesis of H- and Et-BODIPY VoltageFluors

Owing to the commercial availability of the 3-ethyl-2,4-dimethyl-*1H*-pyrrole precursors (kryptopyrrole), we first synthesized BODIPY **3** and **11** (**Scheme 2**) for use in subsequent coupling with phenylenevinylene molecular wires. The sulfonated benzaldehyde precursor, **9** (Scheme 2, and related *para*-isomer, **1**, Scheme 2), was completely insoluble in CH<sub>2</sub>Cl<sub>2</sub> and toluene, the most commonly used solvents for BODIPY condensations. <sup>10, 14, 20, 31, 42-45</sup> We screened polar solvents for the TFA-catalyzed condensation of aldehyde **9** (or **1**) with kryptopyrrole **2** (Scheme 2). DMF gave the best conversion to the dipyrromethane. Oxidation with DDQ to form the corresponding dipyrromethene followed by BF<sub>2</sub> chelation with boron trifluoride diethyl etherate (BF<sub>3</sub>·OEt<sub>2</sub>) in CH<sub>2</sub>Cl<sub>2</sub> solvent gave *ortho*-sulfonated BODIPY **3** (Br *para* to BODIPY, Scheme 2) in 49% yield and **11** (Br *meta* to BODIPY, Scheme 2) in 33% yield.

A Pd-catalyzed Heck coupling between BODIPY 3 and substituted styrenes 4 and 5 gave two different 2,6-diethyl, para molecular wire BODIPY VoltageFluors: EtpH (6) and EtpOMe (7) in 92 and 25% isolated yield, respectively (Scheme 2). The naming convention represents the ethyl groups at the 2,6-positions, molecular wire para from the fluorophore, and the identity of the  $R_1$  substituent. Derivatives with the molecular wire meta from the fluorophore were prepared via a similar route from BODIPY 11 (Scheme 2; EtmH 15, 26% yield, and EtmOMe 16, 29%). Tetramethyl BODIPY VoltageFluors 17-19 (R = H) were prepared first by reacting 2,4-dimethyl-*1H*-pyrrole 10 with sulfonated aldehyde 9, resulting in a 38% yield of ortho-sulfonated tetramethyl BODIPY 12. Heck coupling with substituted styrene 4, 13, or 14 then gave TMmH (17), TMmMe (18), and TMmOMe (19) in 35-62% yield after silica gel chromatography.

#### Synthesis of CN-BODIPY VoltageFluor

Access to electron-withdrawing BODIPY derivatives provide a useful counterpoint to H- and ethyl-substituted BODIPYs and may produce lower levels of reactive <sup>1</sup>O<sub>2</sub> than more electron-rich derivatives.<sup>41</sup> Synthesis of cyano VoltageFluor derivative **22** was more challenging than either H- or Et-substituted BODIPY VoltageFluors. Because of the poor nucleophilicity of 2,4-dimethyl-*1H*-pyrrole-3-carbonitrile (**20**), no reaction with sulfonated benzaldehyde **9** was observed unless heated to 60 °C. The heated condensation resulted in only an 8% isolated yield of **21**. Switching the solvent to a 2:3 DMF:CH<sub>2</sub>Cl<sub>2</sub> mixture and

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Scheme 3. Synthesis of BODIPY VoltageFluor dyes with CO<sub>2</sub>H and CONHR at the 2,6-positions.



adding an excess of TFA (100  $\mu$ L, 6 equiv.) allowed the synthesis to proceed at room temperature and increased the isolated yield to 29% (**Scheme 2**).

BODIPY 21 is less stable than BODIPYs 11 and 12, possibly due to the lower effective charges on the dipyrromethene nitrogen atoms.<sup>46</sup> When subjected to the Pd-catalyzed Heck coupling conditions that afforded previous BODIPY VF dyes, BODIPY 21 decomposed before conversion to product. Lowering the reaction temperature from 100 °C to 70 °C did not prevent decomposition. By exposing BODIPY 21 to Heck reaction conditions and systematically removing single reaction components, we determined that the presence of trimethylamine (NEt<sub>3</sub>) was initiating decomposition of BODIPY 21. Replacing NEt3 with inorganic bases (Cs<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub>) or bulky amine bases (1,8bis(dimethylamino)naphthalene) resulted in scant improvement in conversion; decomposition of 21 remained a problem. To circumvent the sensitivity of BODIPY 21, we attempted a basefree Heck coupling, relying only on the substituted aniline of styrene reactant 4 to buffer generated HBr. The resulting Heck coupling was low yielding (6%), but provided sufficient 22 to purify and characterize (Scheme 2).

Synthesis of dicarboxy- and diamido-BODIPY VoltageFluors

The 2,6-dicarboxy VoltageFluor series was synthesized via two different routes. Initially, BODIPY **32** was synthesized in a 49% yield from aldehyde **9** and 2,4-dimethylpyrrole-3-carboxylic acid **31** (Scheme 3), then subjected to the same base-free Heck coupling conditions as the BODIPY **21**, giving the 2,6dicarboxylic acid VoltageFluor, **28** in a 6% yield after preparative thin layer chromatography (pTLC). Subsequent Heck couplings with unprotected BODIPY **32** gave inconsistent results: either unmodified starting material or decomposition. We suspected the carboxylates could be chelating the palladium catalyst and decided to switch to a protecting group approach, which would likely improve the Heck coupling and allow for more facile purification of intermediates by normal phase chromatography.

Benzyl ester protected pyrrole 23 is less nucleophilic than its carboxylic acid precursor. We performed the BODIPY condensation in 2:3 DMF:CH2Cl2, providing benzyl-protected BODIPY 24 in a 61% isolated yield (Scheme 3). Benzyl-protected BODIPY 24 proceeds cleanly through Heck coupling, even in the presence of NEt<sub>3</sub>. Benzyl-protected intermediates 26 and 27 were isolated in a 30 and 43% yield following column chromatography. Cleavage of the benzyl groups with Pd/C under hydrogen atmosphere also reduced one of the alkenes of the molecular wire, evidenced by a mass 2 m/z higher than the desired product (Figure S1) and increased brightness of the resulting dye. A Birkofer reduction<sup>47-48</sup> with Pd(OAc)<sub>2</sub>, Et<sub>3</sub>SiH, NEt<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> at room temperature gave the cleanest conversion to the free carboxylate product with minimal over-reduction of the alkenes of the molecular wire. BODIPY VF dyes 29 and 30 were isolated in 31 and 14% yield after pTLC.

BODIPY VoltageFluors **35** and **36** were synthesized via Heck coupling between styrenes **4** or **13** and BODIPY **34**, which was accessed in 82% yield from a HATU-mediated amide bond formation between BODIPY **32** and glycine methyl



**Figure 1.** Plots of normalized **a**) absorption and **b**) emission of sulfonated BODIPY fluorophores **3**, **11**, **12**, **32**, **34**, and **21**. Spectra were acquired in PBS pH 7.4. Dye concentration is 1  $\mu$ M.

 Table 1. Spectral properties of sulfonated BODIPYs

	R	$\lambda_{max} \ abs$	$\lambda_{max} \ em$	ε (M <sup>-1</sup> cm <sup>-1</sup> )	φn
3	Et	530	544	42000	0.72
11	Et	530	545	44000	0.70
12	Н	503	515	57000	0.99
32	CO <sub>2</sub> H	517	532	56000	0.95
34	CONHCH <sub>2</sub> CO <sub>2</sub> Me	507	519	76000	0.92
21	CN	502	517	38000	0.93

All spectral properties acquired in PBS pH 7.4

ester **33**. Like benzyl-protected BODIPY **24**, the amide-substituted BODIPY withstands the presence of NEt<sub>3</sub> in the Pd-catalyzed cross-coupling, which returns **35** and **36** in 21% and 34% isolated yields, respectively (**Scheme 3**).

Spectroscopic characterization of sulfonated BODIPYs

The absorption and the emission of BODIPY fluorophores (Figure 1, Table 1) and VoltageFluors (Figure 2a, Figure S2, Table 2) varied with the 2,6-substituents. Consistent with a Dewar formalism,<sup>49-51</sup> electron-withdrawing groups at the 2,6positions result in a hypsochromatic shift ( $\lambda_{max} = 502$  nm for BODIPY 21) and electron donating groups like Et (BODIPY 3 and 11) yield bathochromic shifts ( $\lambda_{max} = 530$  nm). Emission trends mirror the absorption profiles, with the electron-rich BODIPYs 3 and 11 emitting around 544 nm, and BODIPY 21, the most electron-poor, emitting at 517 nm. The absorption and emission profiles of the complete BODIPY VF dyes (acquired in ethanol to improve solubility of the complete BODIPY VF dyes) closely match the spectra of the parent BODIPY fluorophores, with absorption profiles centered at 502 to 528 nm and the phenylene vinylene molecular wire absorbing near 400 nm (Figure 2a, Figure S2, and Table 2). The ortho-sulfonated BODIPY fluorophores have impressive fluorescence quantum vields ( $\phi_{\rm fl}$ ) of 0.70–0.99 (**Table 1**), but after the addition of the phenylene vinylene molecular wire the quantum yields drop dramatically, supporting the presence of PeT within the compounds (Table 2). The absorption spectra of BODIPY VF dyes 17, 18, and 19 closely match the excitation spectra (Figure S3ac). The BODIPY region of the absorbance spectra of 17-19 does not change from pH 2.5 to 10 (Figure S3d-f). However, the emission of 17-19 increases up to 20-fold at pH 2.5, consistent with a PeT-based mechanism of fluorescence enhancement (Figure S3g-i).

#### Cellular performance of BODIPY VF Dyes

All BODIPY VF dyes localize to cell membranes (**Figure 2b**, **Figure S4a,b-S9a,b**) and display different cellular brightness (**Table 2, Figure S10a**). Despite having the highest  $\phi_{fl}$ , **6** was one of the dimmest dyes in cells (relative brightness in cells = 0.4, compared to 19), likely due to its poor solubility in aqueous buffer even in the presence of detergent (**Table 2**). On the other hand, BODIPY VF dyes **28** – **30** possessed the largest cellular brightness (relative brightness up to 12x, **Table 2**, **Figure S10a**). We speculate that the increased anionic character of BODIPY VFs **28** – **30**, with three negative charges, improves the water solubility of the dyes, enabling more efficient delivery to cellular membranes.

After confirming BODIPY VF dyes localize to the cell membrane, we next investigated their voltage sensitivity using whole



**Figure 2.** Spectroscopic, cellular, and functional characterization of BODIPY VF **19**. **a)** Plot of normalized absorbance and emission intensity for BODIPY VF **19** (1  $\mu$ M, ethanol). Excitation is provided at 470 nm. **b)** Widefield fluorescence micrograph of HEK cells stained with BODIPY VF **19** (1  $\mu$ M). **c)** Plot of fractional change in fluorescence of BODIPY VF **19** ( $\Delta$ F/F) vs. time for 100 ms hyper- and depolarizing steps ( $\pm$ 100 mV in 20 mV increments) from a holding potential of -60 mV in a single HEK cell under whole-cell voltage-clamp mode. **d)** Plot of fractional change in fluorescence ( $\Delta$ F/F) vs final membrane potential. Data represent the mean  $\Delta$ F/F,  $\pm$ S.E.M. for n = 3 separate cells. Grey line is the line of best fit.

cell voltage-clamp electrophysiology in tandem with epifluorescence microscopy (**Figure 2c,d Figure S4c,d-S9c,d**). We stepped the membrane potential of a single HEK cell stained with 2  $\mu$ M BODIPY VF from a holding potential of -60 mV to ±100 mV while recording dye fluorescence intensity. BODIPY VF dyes **6**, **7**, **15**, and **16** demonstrate little to no voltage sensitivity. BODIPY VF dyes **6** and **7**, with a *para* molecular wire configuration, show no voltage sensitivity (**Figure S4c**), while BODIPY VF dyes **15** and EtmOMe **16** display modest voltage sensitivities of 1.5 and 5 %  $\Delta$ F/F per 100 mV (**Figure S5c,d** and **Table 2**).

We hypothesized replacing the 2,6-diethyl BODIPY with progressively more electron-poor BODIPYs would increase PeT and therefore increase %  $\Delta$ F/F. Gratifyingly, we see a 67% increase in voltage sensitivity from 16 to 17, from 1.5 to 2.5 %  $\Delta$ F/F (**Figure S6c,d** and **Table 2**). Strengthening the electron-donating ability of the aniline by addition of a methyl or methoxy group increased the voltage-sensitivity to 6.2 % for 18 and 33 %  $\Delta$ F/F for 19 (**Figure 2**).

More electron-deficient BODIPY VF 22 displayed extremely low cellular brightness (**Table 2, Figure S7b, S10a**) and required increasing both illumination intensity and camera exposure time in order to obtain a reasonable estimate of its voltage sensitivity, which was low: 3.8 %  $\Delta$ F/F per 100 mV (**Table 2**, **Figure S7c,d**). While BODIPY VF 22 was slightly more voltage sensitive than its analogous precursors, 15 and 17, its extremely low cellular brightness prohibited further use as a voltage-sensitive dye in cells.

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	Name	R	$R_1$	isomer	$\lambda_{max}abs^a$	$\lambda_{max}em^a$	$\phi_{\rm fl}{}^a$	% $\Delta F/F^{b,c}$	Cell brightness <sup>c,d</sup>
6	EtpH	Et	Н	para	528	541	0.14	0	$0.43\pm0.02^{\rm f}$
7	EtpOMe	Et	OMe	para	527	541	0.07	0	$0.76\pm0.03^{\rm f}$
15	Et <i>m</i> H	Et	Н	meta	528	541	0.15	$1.8\pm0.1$	$4.4\pm0.3^{\rm f}$
16	Et <i>m</i> OMe	Et	OMe	meta	527	541	0.05	$5.4\pm0.6$	$0.60\pm0.03^{\rm f}$
17	TMmH	Н	Н	meta	503	518	0.11	$2.5\pm0.1$	$0.62\pm0.08$
18	TM <i>m</i> Me	Н	Me	meta	504	517	0.07	$6.2\pm0.4$	$1.5\pm0.2$
19	TMmOMe	Н	OMe	meta	504	512	0.05	$33\pm0.7$	$1.0\pm0.1$
28	carboxy <i>m</i> H	СООН	Н	meta	503	516	0.07	$4.4\pm0.2$	$12\pm2$
29	carboxy <i>m</i> Me	СООН	Me	meta	503	517	0.03	$9.9 \pm 0.4$	$5.1\pm0.9$
30	carboxymOMe	СООН	OMe	meta	509	522	0.06	$24\pm0.5$	$7.1\pm0.8$
35	amide <i>m</i> H	CONHCH <sub>2</sub> CO <sub>2</sub> Me	Н	meta	508	521	0.06	$48\pm2$	$1.0\pm0.1$
36	amide <i>m</i> Me	CONHCH <sub>2</sub> CO <sub>2</sub> Me	Me	meta	509	521	0.03	$5.1\pm0.4$	$1.0\pm0.1$
22	cyano <i>m</i> H	CN	Н	meta	502	519	0.08	3.8 <sup>e</sup>	$0.34\pm0.002$

**a** Determined in ethanol. **b** Per 100 mV depolarization. **c** Determined in HEK cells. **d** Relative to **19**, set to a relative brightness of 1.0. **e** Increased exposure time and light intensity required to make measurement. **f** Pluronic F-127 (0.01%) used during loading.



**Figure 3.** Voltage imaging in mammalian neurons with BODIPY VF **30**. **a**) Transmitted light and **b**) widefield epifluorescence image of cultured rat hippocampal neurons stained with 500 nM BODIPY VF **30**. Scale bar is 20  $\mu$ m. **c**) Widefield epifluorescence image of neurons stained with 1  $\mu$ M BODIPY VF **30** and imaged at 500 Hz. Image is a single frame from this high-speed acquisition. Scale bar is 20  $\mu$ m. **d**) Plot of fractional change in fluorescence ( $\Delta$ F/F) for the cells identified in panel (c) or for the entire field of view (FOV).

We then evaluated the 2,6-dicarboxy and diamido BODIPY series, hoping to find an electronic "sweet spot" between the tetramethyl and cyano series. The BODIPY VF dyes 28, 29, and **30** had voltage sensitivities of 4.4%, 9.9%, and 24%  $\Delta$ F/F per 100 mV, respectively (Figure S8c,d and Table 2). While dicarboxy BODIPY VF dyes display a similar range of voltage sensitivities to their tetramethyl precursors, the most striking quality of the dicarboxy BODIPY VF dyes was their cellular brightness-they were 5-12x brighter compared to the cellular fluorescence intensity of 19 (Table 2, all cellular brightness values normalized to 19). The in vitro fluorescence quantum yields of the carboxy BODIPY VF dyes are slightly lower than the tetramethyl BODIPY VF dyes, so this increase in brightness is likely due to improved hydrophilicity and cell loading efficiency. We found that amide-substituted BODIPY VF 35 (Figure S9c,d) possesses voltage sensitivity 10x greater than the corresponding 28, with a fractional sensitivity of 48%  $\Delta F/F$  per 100 mV in HEK cells (compared to 4.4% for 28). Introduction of a more electron-rich molecular wire (methyl substitution) results in a loss of voltage sensitivity for 36, which displays only nominal voltage sensitivity (5.1%  $\Delta$ F/F per 100 mV).

#### Functional Imaging in Neurons and Cardiomyocytes

We evaluated the ability of BODIPY VF dyes to report on voltage dynamics in electrically excitable cells: mammalian

neurons and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Three BODIPY VoltageFluors stood out as good candidates for functional imaging: **19** and **35** because of their high  $\Delta F/F$  (33 and 48%, respectively), and **30** because of its combination of cellular brightness (7x brighter than **19** and **35**) and good sensitivity (24%  $\Delta F/F$ ).

We evaluated the photostability of these BODIPY VF dyes in HEK cells. Based on previous reports,<sup>41</sup> we predicted photostability would decrease from 35 > 19 > 30. Indeed, we find BODIPY VF 35 to be the most photostable in HEK cells, maintaining near 100% fluorescence after 6 min of constant illumination, although with some photobrightening (Figure S10b). BODIPY VF 19 displays photostability comparable to fluorescein-based VF2.1.Cl<sup>32</sup> (Figure S10b). Carboxy-substituted BODIPY 30 bleaches the most rapidly, dropping to about 20% of original fluorescence values after 2 minutes (Figure S10b). However, because of the high starting brightness of BODIPY VF 30, this indicator retains its utility for functional voltage imaging.

In cultured rat hippocampal neurons stained with BODIPY VFs, both **19** and **35** were too dim to capture evoked neuronal action potentials with sufficient signal-to-noise. BODIPY VF **30** displayed bright, membrane-localized staining in neurons isolated from rat hippocampi (**Figure 3a** and **b**). BODIPY VF



**Figure 4.** Voltage imaging in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with BODIPY VF **19**. **a**) Widefield, epifluorescence micrograph of hiPSC-CMs stained with 500 nM BODIPY VF **19**. Scale bar is 50  $\mu$ m. **b**) Single frame of a movie collected at 200 Hz for functional imaging of hiPSC-CM spontaneous action potentials. Scale bar is 50  $\mu$ m. **c**) Trace of mean pixel intensity (arbitrary fluorescence units, a.u.) from full region of interest (ROI) in panel (b) plotted vs time during 10 second acquisition, corrected for photobleach. **d**) Averaged action potential trace (black) from three 10 second recordings from 3 separate ROIs over individual AP events from each recording (blue).

**30** responded to electrically-evoked neuronal action potentials (**Figure 3c** and **d**), which could be detected when analyzing single cell regions of interest (ROIs) or when viewing the entire field of neurons.

We also evaluated the performance of BODIPY VF dyes **19**, **30**, and **35** in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). All three BODIPY VFs stain the membranes of hiPSC-CMs (**Figure 4a**, **Figure S11**). Highspeed fluorescence microscopy (200 Hz frame rate) demonstrates that all three BODIPY VFs can report on spontaneouslygenerated cardiac action potentials in hiPSC-CMs (**Figure 4** and **Figure S11**) during 10 second bouts of imaging.

We found BODIPY VF **19** was the best suited voltage reporter because of its robust 33%  $\Delta$ F/F and good signal-to noise (**Figure 4c**). Additionally, under longer-term imaging (60 sec of continuous imaging), **19** displays the least phototoxic effects among the BODIPY's tested under constant illumination. Under these conditions, the photostability of BODIPY' VF **19** was comparable to VF2.1.Cl,<sup>32</sup> although with lower signal to noise than either VF2.1.Cl or fVF 2<sup>52</sup> (**Figure S12**). Like VF2.1.Cl, BODIPY' VF **19** also slightly alters the shape and magnitude of cardiac action potentials under prolonged illumination (**Figure S12**). These effects are not seen during shorter imaging sessions (**Figure 4d**).

## Discussion

We designed and synthesized 5 new sulfonated BODIPY dyes with variable 2,6-subsitution patterns. These sulfonated fluorophores represent a generalizable solution to improving BODIPY water solubility while simultaneously avoiding modification to the boron center or fluorophore core. We incorporated these fluorophores into 13 new BODIPY voltage-sensitive fluorophores for evaluation in live-cell imaging, but the tunable, water-soluble BODIPYs presented here may have applications beyond voltage sensing. BODIPY VF 35 is the most sensitive BODIPY-based voltage indicator to date,53-54 but its low cellular brightness precludes its ready adoption for functional imaging in electrically excitable cells like neurons or cardiomyocytes. Two other indicators developed in this study, BODIPY VF 19, with its slightly lower sensitivity (33%  $\Delta$ F/F per 100 mv), but good brightness, and BODIPY VF 30, which retains good voltage sensitivity (24%  $\Delta$ F/F per 100 mV) and exceptional brightness (~7x brighter than 19 or 35) are better suited for functional

imaging in cardiomyocytes or neurons, where they can each report on action potential dynamics in single trials.

The voltage sensitivity of the BODIPY VF dyes correlates with the electron-withdrawing character of the 2,6-substitution pattern in the BODIPY fluorophore. More electron-withdrawing substituents increase voltage sensitivity in the order of -Et <-H<-CO<sub>2</sub>H<-CONHR>-CN. The extremely electron-withdrawing character of nitrile substitution makes for a poorly sensitive BODIPY VF. We find that calculated values of HOMO energies (Figure S13) for the BODIPY fluorophores-lacking the molecular wire-correlate extremely well with either meta or para Hammett constants ( $\sigma_m$  or  $\sigma_p$ ),<sup>55</sup> validating the use of tabulated Hammett constants for analysis of the relative electron density of a particular BODIPY fluorophore (Figure S14a and **b**). Correlation between calculated HOMO energies and  $\sigma_m$ or  $\sigma_p$  values is best when evaluating neutral BODIPYs (Et, H, CONHR, or CN), with correlation coefficients ( $\mathbb{R}^2$ ) >0.99 for both  $\sigma_m$  and  $\sigma_p$  compared to HOMO. If carboxy-substituted BODIPYs are included, the correlation (R<sup>2</sup>) between HOMO level and Hammett parameter drops to 0.92 ( $\sigma_m$ ) and 0.78 ( $\sigma_p$ ) (Figure S14a and b).

The average  $\Delta F/F$  for a class of BODIPY fluorophore (R = Et, H, CO<sub>2</sub>H, CONHR, or CN) displays a parabolic relationship with calculated HOMO energy levels (Figure S14c), with maximum voltage sensitivity at around -4.75 eV (or  $\sigma = 0.2 - 0.4$ ). BODIPY VF dyes that have very large and negative  $\Delta G_{PeT}$ ,<sup>14</sup> either by a combination of electron deficient fluorophores (R =CN) with mildly donating anilines (R = H) as in the case of BODIPY VF 22, or by with moderately withdrawing fluorophores (R = CONHR) with electron-rich anilines (R = Me) in the case of BODIPY 35, will have low voltage sensitivity. These results suggest that 35 occupies a "sweet spot" of PeT to optimize the voltage sensitivity for BODIPY VoltageFluors, and any further lowering of the fluorophore HOMO (such as amide BODIPY to cyano BODIPY) or raising the HOMO of the aniline PeT donor (unsubstituted aniline to methyl-substituted aniline) is detrimental to the voltage sensitivity.

Despite its impressive 48%  $\Delta$ F/F of BODIPY **35** in HEK cells, its low cellular brightness (12x less bright than dicarboxy BODIPY VFs) precludes its direct use in functional imaging. The low cellular brightness likely results from poor solubility of the dye, because the highly charged dicarboxy BODIPY VFs

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displayed greater cellular brightness. The structure of the diamido BODIPY VFs lend themselves to introduction of additional water-solubilizing groups, without significantly perturbing the electronics of the 2,6-diamide substitution pattern. Experiments are underway to expand the utility of amide-substituted BODIPYs in the context of voltage imaging and beyond.

## ASSOCIATED CONTENT

**Supporting Information**. Support figures, spectra, synthetic methods and imaging details. This material is available free of charge via the Internet at http://pubs.acs.org.

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