New Molecular Scaffolds for Fluorescent Voltage Indicators

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The ability to non-invasively monitor membrane potential dynamics in excitable cells like neurons and cardiomyocytes promises to revolutionize our understanding of the physiology and pathology of the brain and heart. Here, we report the design, synthesis, and application of a new class of fluorescent voltage indicator that makes use of a fluorene-based molecular wire as a voltage sensing domain to provide fast and sensitive measurements of membrane potential in both mammalian neurons and human-derived cardiomyocytes. We show that the best of the new probes, fluorene VoltageFluor 2 (fVF 2) readily reports on action potentials in mammalian neurons, detects perturbations to cardiac action potential waveform in human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes, shows a substantial decrease in phototoxicity compared to existing molecular wire-based indicators, and can monitor cardiac action potentials for extended periods of time. Together, our results demonstrate the generalizability of a molecular wire approach to voltage sensing and highlights the utility of fVF 2 for interrogating membrane potential dynamics.

File list (2)

01 EWM fVF MS ChemRxiv.pdf (0.91 MiB) view on ChemRxiv  download file
02 EWM fVF SI ChemRxiv.pdf (6.63 MiB) view on ChemRxiv  download file
New Molecular Scaffolds for Fluorescent Voltage Indicators

Steven C. Boggess,† Shivaani S. Gandhi,‡ Brian A. Siemons,§# Nathaniel Huebsch,§#0 Kevin E. Healy,§# and Evan W. Miller‡†⊥‡.

Departments of †Chemistry, §Bioengineering, ※Materials Science & Engineering, and ‡Molecular & Cell Biology and ⊥Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States.

ABSTRACT: The ability to non-invasively monitor membrane potential dynamics in excitable cells like neurons and cardiomyocytes promises to revolutionize our understanding of the physiology and pathology of the brain and heart. Here, we report the design, synthesis, and application of a new class of fluorescent voltage indicator that makes use of a fluorene-based molecular wire as a voltage sensing domain to provide fast and sensitive measurements of membrane potential in both mammalian neurons and human-derived cardiomyocytes. We show that the best of the new probes, fluorene VoltageFluor 2 (fVF 2) readily reports on action potentials in mammalian neurons, detects perturbations to cardiac action potential waveform in human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes, shows a substantial decrease in phototoxicity compared to existing molecular wire-based indicators, and can monitor cardiac action potentials for extended periods of time. Together, our results demonstrate the generalizability of a molecular wire approach to voltage sensing and highlights the utility of fVF 2 for interrogating membrane potential dynamics.

Optical methods to measure biochemical and biophysical events in living cells provide a powerful approach to monitor cellular physiology in a non-invasive and high-throughput manner. The success of such light-based ventures depends critically on the ability to design and construct molecules that change their optical properties—e.g., for example, color, fluorescence intensity, or lifetime—in response to changes in the cellular environment.1 Fluorescence microscopy is one of the most commonly used modalities because it is operationally simple, instrumentation is widely available, and an ever-growing library of small molecule fluorescent indicators exists to probe the dynamics of a host of cellular analytes, properties, and structures.2−5 Of particular interest is the plasma membrane. Changes in the electrochemical potential across the lipid bilayer profoundly shapes cellular physiology. Excitable cells employ a consortium of ion channels both to maintain tight control over their simple potential (V_m) and to initiate and propagate rapid changes in V_m. Rapid changes in V_m such as an action potential (AP), drive the unique physiology of excitable cells like neurons and cardiomyocytes. In neurons, APs evoke the release of neurotransmitter into the synaptic cleft, and, in heart tissue, waves of APs coordinate contraction and maintain regular rhythm. Disruption of the frequency, timing, and/or shape of APs are linked to serious human diseases ranging from epilepsy to long QT syndrome. Because of the importance of V_m to both health and disease, robust methods to optically monitor membrane potential remain a critical complement to more traditional approaches. The gold-standard for measuring V_m and APs in live cells is electrophysiology: direct determination of V_m through the physical interaction between the cell of interest and an electrode. Electrophysiology is highly invasive, low throughput, and difficult to interpret in samples like cardiac tissue or cardiomyocyte monolayers, where electrical coupling between cells confounds single-cell measurement. Optical recording of membrane potential using voltage-sensitive fluorescent indicators provides an attractive alternative to probe V_m and AP dynamics in multiple cells, in monolayers, or 3D tissue.6

Recently, we initiated a program to develop a new class of voltage-sensitive fluorescent indicators that utilize photoinduced electron transfer (PeT) as a rapid trigger to sense changes in V_m.7,8 These small molecule voltage-sensitive fluorophores, or VoltageFluors (VF dyes), combine a xanthene-based dye as a fluorescent reporter and a conjugated molecular wire that localizes the indicator to the cell membrane and facilitates PeT from an electron-rich aniline donor to the fluorophore within the low dielectric environment of the lipid bilayer. All previously reported PeT-based voltage indicators utilize phenylenevinylene (PV)-based molecular wires (Scheme 1, VF2.1.Cl).9,10 attractive because of their exceptionally low electron transfer attenuation values in donor-bridge-acceptor (DBA) systems (β = 0.04 Å⁻¹).11-12 Within a PV wire framework, voltage sensitivity can be improved by altering the redox potentials of the fluorophore electron acceptor and the aniline electron donor.13 Additionally, fluorophores such as rhodamine9 and silicon-rhodamine10 can be substituted for fluorescein, after some adjustment to the identity of the aniline donor. To date, we have not explored alterations to the identity of the molecular wire component of PeT-based voltage indicators. Now, we present a new class of PeT-based voltage sensitive fluorescent indicators that use a 9,9-dimethyl-9H-fluorene monomer in place of the canonical 1,4-divinylbenzene moiety (Scheme 1). In other DBA scaffolds, 2,7-oligofluorene bridges effectively facilitate electron transfer.
Scheme 1. Synthesis of fluorene VoltageFluor dyes (fVF dyes).

Scheme 2. Synthesis of electron-rich fluorene VoltageFluor dyes

Table 1. Properties of fluorene VoltageFluor dyes (fVF dyes)

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<tbody>
<tr>
<td>fVF 0 (7)</td>
<td>-H</td>
<td>-H</td>
<td>519</td>
<td>535</td>
<td>0.77</td>
<td>-0.3 ± 0.03</td>
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<tr>
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<td>22:1</td>
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<td>fVF 2 (20)</td>
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<td>-H</td>
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<td>535</td>
<td>0.07</td>
<td>10.5 ± 0.8</td>
<td>39:1</td>
</tr>
<tr>
<td>fVF 3 (21)</td>
<td>-N(Me)[2]</td>
<td>-OMe</td>
<td>520</td>
<td>535</td>
<td>0.19</td>
<td>12.2 ± 1.6</td>
<td>13:1</td>
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</tbody>
</table>

[a] Measured in PBS + 0.1% SDS (pH = 7.2). [b] per 100 mV. Recorded in HEK 293T cells at 0.5 kHz optical sampling rate.

across large distances and demonstrate “wire-like” transport similar to PV wires.14 Because β values depend both on the identity of the wire and the donor/acceptor pair,15-16 we wanted to explore fluorene molecular wires as a platform for optical voltage sensing, demonstrating the generalizability of a PeT-based approach to voltage sensing. We now report the design, synthesis, characterization, and application of a new series of fluorene-based VoltageFluors, or fVF dyes.

The synthesis of fluorene-based voltage indicators starts with Suzuki-Miyaura cross-coupling of bromo-iodo-fluorene 1 with either boronic ester 8 or phenylboronic acid, providing monomeric fluorenes 2 and 3 as bright yellow solids (Scheme 1). Attachment to a sulfonated dichlorofluorescein was achieved by Pd-catalyzed cross-coupling of a pinacol boronic ester with the terminal aryl bromide to provide 4 and 5. Suzuki-Miyaura cross-coupling yielded voltage indicator 6 (fVF 1) and indicator 7 (fVF 0), which lacks an aniline donor (Scheme 1). For the wires with electron rich donors (12, 13; Scheme 2), transformation of the nitro group to an aniline was performed with tin(II) chloride to yield wires 14 and 15. This was followed by reductive amination of formaldehyde with NaCNBH3 to provide alkylated wires 16 and 17. These were prepared in a similar method to Scheme 1 to yield electron-rich voltage indicators 20 (fVF 2) and 21 (fVF 3).

New fVF dyes have a λmax centered around 520 nm and a second major absorption band around 340 nm arising from the fluorene molecular wire (Fig. 1, Fig. S1, Table 1). Each fVF dye has a maximum emission around 535 nm, indicating little ground state interaction between the fluorene-based molecular wire and xanthene chromophore. fVF 1, 2 and 3 have lower fluorescence quantum yields (Φfl, 0.05 to 0.19) than control indicator fVF 0 (0.77, Table 1).
Figure 1. Characterization of fluorene VoltageFluor 2 (fVF 2). a) Live cell fluorescence microscopy image of fVF 2 in HEK cells. Scale bar is 10 μm. b) Normalized absorption and emission spectra of fVF 2. Spectra were acquired in PBS (pH 7.2) +0.1% SDS. For emission scan, excitation was provided at 485 nm. c) Voltage sensitivity of fVF 2 in patch-clamped HEK cells. d) Plot of ΔF/F vs. membrane potential (in mV) for fVF 2. Red line is the line of best fit. Error bars are standard error of the mean for 8 independent determinations. e) Live-cell, wide-field fluorescence images of rat hippocampal neurons stained with 500 nM fVF 2. Scale bar is 20 μm. f) Representative ΔF/F plot of evoked neuronal activity of a single cell recorded optically with fVF 2.

To measure the voltage sensitivity of these indicators, we used whole-cell voltage-clamp electrophysiology in tandem with epifluorescence microscopy. By applying voltage steps ranging from +100 mV to -100 mV in 20 mV increments to HEK293T cells stained with fluorene voltage indicators, we observe that indicators fVF 1-3 possess moderate sensitivity to changes in Vm. Similar to PV-based molecular wire voltage indicators, fVF 1-3 become brighter in response to depolarizing (more positive) membrane potentials. The two most sensitive compounds, fVF 2 and 3, have sensitivities of 11 and 13% ΔF/F per 100 mV, respectively; however, fVF 2 has an overall signal that was much brighter in cells, resulting in better SNR (Fig. 1, Fig. S2, Table 1). fVF 1 is also very bright in cells but has low SNR due to a low sensitivity (5% ΔF/F). Somewhat surprisingly, the electron-deficient compound fVF 0 (7, Scheme 1) exhibits a small amount of voltage sensitivity, -0.3% ΔF/F per 100 mV, becoming less fluorescent in response to depolarizing potentials (Table 1, Fig. S2, Table 1), the opposite of every molecular wire indicator synthesized in our laboratory. We chose to characterize fVF 2 in subsequent experiments due to its brightness and superior SNR.

fVF 2 readily detects fast changes in membrane potential
**Figure 3. fVF 2 displays low phototoxicity in cardiomyocyte monolayers.** Fluorescence intensity vs time for a) fVF 2 and b) VF2.1.CI in monolayers of hiSPC-CMs. The raw fluorescence intensity from an entire field of view over an entire 10 s recording session is plotted vs. the total illumination time (in minutes). Individual action potential (AP) traces for c) fVF 2 and d) VF2.1.CI are indicated by red stars in panels (a) and (b). Plot of mean e) signal-to-noise ratio (SNR) f) cAPD30, and g) cAPD50 as a function of total illumination time for fVF 2 (black) and VF2.1.CI (blue). For panels e-g, mean values are determined from n = 3 independent trials, and error bars are ± standard error of the mean. Statistical tests are two-tailed, unpaired t-test for each cAPD at the indicated time vs. t = 0. ** = p<0.005, *** = p<0.001, **** = p<0.0001.

In mammalian neurons, fVF 2 gives clear membrane staining (Fig. 1e) and faithfully records evoked action potentials with an average ΔF/F of 5.1% and SNR of 21:1 (N = 54 spikes, Fig. 1f, S4). fVF 2 clearly resolves spontaneous activity in cultured rat hippocampal neurons (Fig. S5). Despite the lower nominal voltage sensitivity of fVF 2 relative to a first generation VoltageFluor dye (VF2.1.CI – 27% ΔF/F per 100 mV in HEK cells; 10% ΔF/F and SNR of 43:1 in evoked action potentials, n = 54 spikes, Fig. S4), the improved brightness of fVF 2 relative to VF2.1.CI (1.2x brighter in neurons, Table S1) makes it useful for recording action potentials in neurons. When we evaluate fVF 2 against an electrochromic VSD with a similar voltage sensitivity, di-4-ANEPPS, we observe evoked spikes with ΔF/F of -1% and SNR of 15:1. However, a 5-fold higher concentration was needed to make these recordings (Fig. S4, SI Table 1). In addition to reporting on neuronal activity, we detect no changes to neuronal membrane properties or action potential kinetics when comparing the electrophysiological parameters of neurons with or without the presence of fVF2 (Fig. S6, Table S2).

We sought to use fVF 2 for optical measurements of cardiac AP waveforms to provide a holistic assessment of drug cardiotoxicity in vitro, a major goal of the Comprehensive in vitro Proarrhythmic Assay (CiPA) initiative. We cultured hiPSC-CM monolayers and tested the ability of fVF 2 to report cardiac AP waveforms.

In spontaneously beating monolayers, fVF 2 clearly stains the sarcolemma of hiPSC-CMs and faithfully reports ventricular-like AP waveforms, showing a large increase in fluorescence just before contraction of the monolayer (Fig. 2a-e). Using methods previously described, we calculate action potential duration (APD) for each AP waveform in the fluorescence trace at 70, 50, and 10% of the maximum depolarization (APD30, APD50, APD90, respectively). To correct for APD variation arising from difference in beat rate from spontaneously beating monolayers, we used Fridericia’s formula to provide a beat-rate corrected APD (cAPD). From our optical measurements, we calculated cAPD90 values from 500-700 ms in spontaneously beating monolayers after 14 days in culture, consistent with previous reports in hiPSC-CMs.

To demonstrate the utility of fVF 2 for parsing the pharmacological effects of drug treatment on cardiomyocytes, we treated hiPSC cardiomyocytes with cisapride. Cisapride, formerly a useful gastroprotective agent, was withdrawn from the US market in 2000 due to its connection to torsades de pointes (TdP) induced by acquired long QT-syndrome caused by blockade of K\(_{\text{v}}\)11.1. Cisapride is also one of 12 training and calibration compounds used in the CiPA initiative. Observation of
cardiomyocyte monolayers treated acutely with cisapride results in several phenotypic alterations to cardiac AP, readily detectable by fVF2. At a concentration of 300 nM, we saw three different manifestations of the effect of prolonged cAPD caused by If, blockade, an extended phase 3 (Fig. 2g), a tachycardia-like train of drastically shortened APs (Fig. 2h), and the appearance of early-after depolarizations (EADs) (Fig. 2i). K11.1.1/HERG channel blockade results in action potential prolongation (Fig. 2g) which has been connected to a higher risk of TdP and higher arrhythmogenic potential. The rapid, sub-threshold spiking activity may be analogous to tachycardia, which was accompanied by a loss in monolayer automaticity (Fig. 2h). The appearance of EADs (Fig. 2i) corresponds to the observed cAPD prolongation, and significantly increase the risk of arrhythmia and TdP.

Monitoring the effect of potentially cardiotoxic drug relies on the ability to make stable, long-term recordings from cardiomyocytes. When using voltage-sensitive fluorescent indicators, this often requires careful titration of illumination intensity and indicator concentration to minimize phototoxicity. Therefore, we were pleased that fVF2 displays lower phototoxicity in hiPSC-CMs relative to VF2.1.Cl. We stained hiPSC-CM monolayers with either fVF2 or VF2.1.Cl and continuously illuminated for 10 minutes and optically recorded membrane potential dynamics every minute. Despite the initial photobleach of fVF2 (Fig. 3a, Fig. S2i), the shape of recorded action potentials (Fig. 3c), SNR (Fig. 3e), and action potential duration (Fig. 3f,g) remain relatively constant (Fig. 3c and 3d, S7). In sharp contrast, however, VF2.1.Cl had a dramatic, detrimental impact on cardiomyocyte function. Although VF2.1.Cl initially has a high SNR compared to fVF2 (Fig. 3b,e), SNR drops quickly after the first minute of illumination (Fig. 3b,e), and action potential duration (Fig. 3d) and duration (Fig. 3f,g) undergo substantial and significant changes, as early as 2 minutes into illumination (Fig. 3b,d,f,g). Even after 4 minutes, only sub-threshold activity was recorded with VF2.1.Cl and required an automated analysis script to detect these events (Fig. S7h). After 5 minutes, monolayers imaged with VF2.1.Cl cease to contract (Fig. 3d and S7). However, monolayers imaged with fVF2 continue to beat even after 10 minutes of continuous illumination (Fig. 3c and S7). Increasing the illumination intensity from 9 to 29 mW/mm² when making recordings with fVF2 did not alter cAPD (Fig. S7l). Together, these results suggest that fVF2 has lower phototoxicity than VF2.1.Cl and can be used to measure activity in cardiomyocyte monolayers for prolonged time periods.

To assess the ability of fVF2 to measure changes to cardiac electrophysiology in responds to chronic drug treatment, we calculated IC50 values for cisapride using in-well dose escalation with optical recording of cAPD. Measurements were taken after incubation with increasing cisapride concentrations in each well from 0.1 to 300 nM. Our optical measurements show an increase in cAPD90 (IC50 = 10.6 nM, 14 days in culture) up to 300 nM of cisapride (Fig. 4e,f), which is in the range of IC50 values previously measured for cisapride in other in vitro studies (SI Table 3). We also observe an increase in cAPD50 up to 100 nM, but a decrease at higher concentrations of cisapride. Similarly, measured cAPD30 seems to have little variation from DMSO vehicle control; however, a decrease is detected at higher concentrations of cisapride (Fig. 4e,f). Together, these results demonstrate the ability of fVF2 to enable the rapid assembly of dose-response data in hiPSCs using an all-optical approach.

In summary, we present the design, synthesis and application of a new class of molecular wire-based fluorescent indicators. We show, for the first time, that fluorene-based molecular wires provide a platform for PeT-based voltage sensing. In general, these fluorene-based indicators have lower nominal voltage sensitivities (ΔF/F per 100 mV of 5 to 13% in HEK cells, compared to approximately 27% for VF2.1.Cl) to changes in membrane potential, but are brighter, than their phenylene-vinylene counterparts. fVF2 exhibits adequate sensitivity and excellent brightness in cells for reporting AP waveforms in neurons and cardiomyocytes with high SNR. More importantly, fVF2 displays substantially reduced phototoxicity in cardiomyocytes relative to VF2.1.Cl, allowing for prolonged, continuous measurement of cardiomyocyte activity. Fluorene-based molecular wires may provide an attractive, general solution to the phototoxicity often associated with voltage-sensitive fluorescent indicators.

**ASSOCIATED CONTENT**

Supporting Information.
Experimental details, synthetic procedures, imaging conditions, cell culture and differentiation protocols, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
* evanmwiller@berkeley.edu

Present Addresses
1 Department of Biomedical Engineering, Washington University in St. Louis, Missouri, 63130 United States.

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REFERENCES


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VF2.1.Cl

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Supporting Information for:

New Molecular Scaffolds for Fluorescent Voltage Indicators

Steven C. Boggess,† Shivaani S. Gandhi,‡ Brian A. Siemons,§# Nathaniel Huebsch,#‡ Kevin E. Healy,#‡ and Evan W. Miller†,#,‡

Departments of †Chemistry, ‡Bioengineering, #Materials Science & Engineering, and †Molecular & Cell Biology and †Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States.

1. General method for chemical synthesis and characterization

Chemical reagents and solvents (dry) were purchased from commercial suppliers and used without further purification. References to previously synthesized compounds are provided along with characterization data. Thin layer chromatography (TLC) (Silicycle, F254, 250 μm) and preparative thin layer chromatography (PTLC) (Silicycle, F254, 1000 μm) was performed on glass backed plates pre-coated with silica gel and were visualized by fluorescence quenching under UV light. Flash column chromatography was performed on Silicycle Silica Flash F60 (230–400 Mesh) using a forced flow of air at 0.5–1.0 bar. NMR spectra were measured on Bruker AVB-400 MHz, 100 MHz, AVQ-400 MHz, 100 MHz, Bruker AV-600 MHz, 150 MHz. NMR spectra measured on Bruker AVII-900 MHz, 225 MHz, equipped with a TCI cryoprobe accessory, were performed by Dr. Jeffrey Pelton (QB3). Chemical shifts are expressed in parts per million (ppm) and are referenced to CDCl₃ (7.26 ppm, 77.0 ppm) or DMSO (2.50 ppm, 40 ppm). Coupling constants are reported as Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet. High-resolution mass spectra (HR-ESI-MS) were measured by the QB3/Chemistry mass spectrometry service at University of California, Berkeley. High performance liquid chromatography (HPLC) and low resolution ESI Mass Spectrometry were performed on an Agilent Infinity 1200 analytical instrument coupled to an Advion CMS-L ESI mass spectrometer. The column used for the analytical HPLC was Phenomenex Luna 5 μm C18(2) (4.6 mm I.D. x 75 mm) with a flow rate of 1.0 mL/min. The mobile phases were MQ-H₂O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254, 350 and 480 nm over 10 min with a gradient of 10-100% eluent B unless otherwise noted. Ultra-high performance liquid chromatography (UHPLC) for purification of final compounds was performed using a Waters Acquity Autopurification system equipped with a Waters XBridge BEH 5 μm C18 column (19 mm I.D. x 250 mm) with a flow rate of 30.0 mL/min, made available by the Catalysis Facility of Lawrence Berkeley National Laboratory (Berkeley, CA). The mobile phases were MQ-H₂O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254 and 350 nm over 20 min with a gradient of 10-100% eluent B, unless otherwise noted.

2. Spectroscopic studies

Stock solutions of Fluorene VoltageFluors were prepared in DMSO (500 μM) and diluted with PBS (10 mM KH₂PO₄, 30 mM Na₂HPO₄·7H₂O, 1.55 M NaCl, pH 7.2) solution containing 0.10% (w/w) SDS (1:1000 dilution). UV-Vis absorbance and fluorescence spectra were recorded using a Shimadzu 2501 Spectrophotometer (Shimadzu) and a Quantamaster Master 4 L-format scanning spectrofluorometer (Photon Technologies International). The fluorometer is equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples were measured in 1-cm path length quartz cuvettes (Starna Cells).

Relative quantum yields (Φₘ) were calculated by comparison to fluorescein (Φₘ = 0.93 in 0.1 M NaOH) and rhodamine 6G (Φₘ = 0.95 in ethanol) as references.[1] Briefly, stock solutions of standards were prepared in DMSO (0.25-1.25 mM) and diluted with appropriate solvent (1:1000 dilution). Absorption and emission (excitation = 485 nm) were taken at 5 concentrations. The absorption value at the excitation wavelength (485 nM) was plotted against the integration of the area of fluorescence curve (495-675 nm). For fluorescein, the integration of the area of the fluorescence curve was also taken with an excitation at 450 nm. The area from 460-675 nm and 495-675 nm was used to extrapolate the area of the fluorescence curve with an excitation at 485 nm. This ensured the full fluorescence area of fluorescein excited at 485 nm was used for Φₘ calculations. The slope of the linear best fit of the data was used to calculate the relative Φₘ by the equation \[ \Phi_{\text{rel}} = \Phi_{\text{rel}}(S_x/S_y)(\eta_y/\eta_x)^2 \], where \( S_x \) and \( S_y \) are the slopes of the reference compound and unknown, respectively, and \( \eta \) is the refractive index of the solution. This method was validated by cross-referencing the reported Φₘ values of fluorescein and rhodamine 6G to the calculated Φₘ using the one standard as a reference for the other and vice versa. Calculated Φₘ within 10% of the reported value for both standards ensured that Φₘ calculated for fluorescein VoltageFluors was reliable within 10% error.

3. Cell Culture

All animal procedures were approved by the UC Berkeley Animal Care and Use Committees and conformed to the NIH Guide for the Care and Use of Laboratory Animals and the Public Health Policy.
3a. Human embryonic kidney (HEK) 293T cells were acquired from the UC Berkeley Cell Culture Facility. Cells were passaged and plated onto 12 mm glass coverslips coated with Poly-D-Lysine (PDL; 1 mg/mL; Sigma-Aldrich) to a confluence of ~15% and 50% for electrophysiology and imaging, respectively. HEK293T cells were plated and maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), and 1% Glutamax.

3b. Rat hippocampal neurons. Hippocampi were dissected from embryonic day 18 Sprague Dawley rats (Charles River Laboratory) in cold sterile HBSS (zero Ca2+, zero Mg2+). All dissection products were supplied by Invitrogen, unless otherwise stated. Hippocampal tissue was treated with trypsin (2.5%) for 15 min at 37 °C. The tissue was triturated using fire polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS; Thermo Scientific), 2% B27, 2% 1M D-glucose (Fisher Scientific) and 1% glutamax. The dissociated cells were plated onto 12 mm diameter coverslips (Fisher Scientific) pre-treated with PDL (as above) at a density of 30-40,000 cells per coverslip in MEM supplemented media (as above). Neurons were maintained at 37 °C in a humidified incubator with 5% CO2. At 1 day in vitro (DIV) half of the MEM supplemented media was removed and replaced with Neurobasal media containing 2% B-27 supplement and 1% glutamax. Electrophysiological experiments which were performed on 12-15 DIV neurons. Unless stated otherwise, for loading of HEK cells and hippocampal neurons, Fluorene VoltageFluors were diluted in DMSO to 500 μM, and then diluted 1:1000 in HBSS. Imaging experiments were performed in HBSS.

3c. Differentiation of hiPSC into cardiomyocytes and culture. hiPSCs were cultured on Matrigel (1:100 dilution; Corning)-coated 6 well-plates in E8 medium. When the cell confluency reached 80–90%, which is referred as day 0, the medium was switched to RPMI 1640 medium (Life Technologies) containing B27 minus insulin supplement (Life Technologies) and 10 μM CHIR99021 GSK3 inhibitor (Peprotech). At day 1, the medium was changed to RPMI 1640 medium containing B27 minus insulin supplement only. At day 2, medium was replaced to RPMI 1640 medium containing B27 supplement without insulin, and 5 μM IWP4 (Peprotech) for 2 days without medium change. On day 4, medium was replaced to RPMI 1640 medium containing B27 minus insulin supplement for 2 days without medium change. On day 6 and 7, medium was replaced to a serum-free medium - RPMI 1640 containing B27 with insulin supplement. After day 7, the medium was changed every other day. Confluent contracting sheets of beating cells appear between days 7 to 15 and are ready for dissociation after this time.

Confluent sheets were dissociated with 0.25% trypsin-EDTA (8-30 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated Ibidi ® 24 well-plates in RPMI 1640 medium containing B27 supplement (containing insulin). Medium was changed every 3 days until imaging. For loading hiPSC cardiomyocytes, fluorene VoltageFluors were diluted in DMSO to 1 mM, and then diluted 1:100 in RPMI 1640 with B27 supplement minus Phenol Red. Imaging experiments were performed in RPMI 1640 with B27 supplement minus Phenol Red.

4. Imaging parameters

For HEK293T cells and rat hippocampal neurons, Epifluorescence imaging was performed on an AxioExaminer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with Slidebook (v6, Intelligent Imaging Innovations). Images were acquired with either a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss) or a W-Plan-Apo 63x/1.0 water objective (63x; Zeiss). Images were focused onto either an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu) or an eVolve 128 EMCCD camera (EMCCD; Photometrix).

Inverted epifluorescence imaging of hiPSC cardiomyocytes was performed on an AxioObserver Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor), controlled with µManager (V1.4, open-source, Open Imaging).[2] Images were acquired using a Plan-Apochromat 20/0.8 air objective (20x, Zeiss). Images were focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu).

More detailed imaging information for each experimental application is expanded below.

4a. Membrane staining and photostability in HEK293T cells

HEK293T cells were incubated with a HBSS solution (Gibco) containing Fluorene VoltageFluors (500 nM) at 37°C for 20 min prior to transfer to fresh HBSS (no dye) for imaging. Microscopic images were acquired with a W-Plan-Apo 20x/1.0 water objective (Zeiss) and OrcaFlash4.0 sCMOS camera (Hamamatsu). For fluorescence images, the excitation light was delivered from a LED (20.9 mW/mm²; 100 ms exposure time) at 475/34 (bandpass) nm and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP).

For photostability experiments HEK cells were incubated separately with FVF 2 (500 nM) and VF2.1.Cl (500 nM) in HBSS at 37°C for 20 min. Data were acquired with a W-Plan-Apo 63x/1.0 objective (Zeiss) and Evolve 128 EMCCD camera (Photometrics). Images (pixel size 0.38 μm × 0.38 μm) were taken every 1 second for 2.5 minutes with constant illumination of LED (46.8 mW/mm²; 50 ms exposure time). The obtained fluorescence curves (background subtracted) were normalized with the fluorescence intensity at t = 0 and averaged (five different cells of each dye).

4b. Voltage sensitivity in HEK293T cells

Functional imaging of the fluorene VoltageFluors was performed using a 20x water immersion objective paired with image capture from the EMCCD camera at a sampling rate of 0.5 kHz. Fluorene VoltageFluors were excited using the
475 nm LED with an intensity of 20.9 mW/mm². For initial voltage characterization emission was collection with the emission filter and dichroic listed above.

4c. Evoked and spontaneous activity in rat hippocampal neurons

Extracellular field stimulation was delivered by a Grass Stimulator connected to a recording chamber containing two platinum electrodes (Warner), with triggering provided through a Digidata 1332A digitizer and pCLAMP 10 software (Molecular Devices). Action potentials were triggered by 1 ms 80 V field potentials delivered at 5 Hz. To prevent recurrent activity the HBSS bath solution was supplemented with synaptic blockers 10 µM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Santa Cruz Biotechnology) and 25 µM DL-2-Amino-5-phosphonopentanoic acid (APV; Sigma-Aldrich). Functional imaging was performed using the scMOS camera and a 20x water objective. fVF 2 and VF2.1.Cl (500 nM in HBSS) were excited with a 475/34 (bandpass) nm LED with an intensity of 6.9 mW/mm² and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP). Di-4-ANNEPS (2.5 µM in HBSS, incubated for 10 minutes at 25 °C) was excited with an LED (bandpass; 475/34 nm; 6.9 mW/mm²) and emission was collected with an emission filter (bandpass; 650/60 nm) after passing through a dichroic mirror (594 nM LP). (SI Figure S4).

Spontaneous activity from neurons (SI Figure S5) was obtained using the sCMOS camera with a 20x objective. fVF 2 was excited using the 475 nm LED with an intensity of 6.9 mW/mm² and emission was collected with the filter and dichroic listed above. Images were binned 4x4 to allow sampling rates of 0.5 kHz.

4d. Voltage recordings of hiPSC cardiomyocytes and assessment of phototoxicity

Functional recordings of fVF 2 (1µM) were performed using a 20x air objective paired with a sCMOS camera at a sampling rate of 0.2 kHz (used 4x4 binning and restricted to a 512x125 pixel frame for high-speed acquisition over long periods). FVF 2 was excited at 475/34 (bandpass) nm with an intensity of 9.0 mW/mm² and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP). Typical recordings were made for ten seconds.

Phototoxicity of VoltageFluor dyes was assessed in cardiomyocyte monolayers incubated with 1 µM of fVF 2 or VF2.1.Cl. These were exposed to constant illumination from the excitation LED (475/34; bandpass) for up to ten minutes, while typical ten second fluorescence recordings were made at the beginning of each minute. For monolayers incubated with VF2.1.Cl, recordings were halted when a loss of automaticity (spontaneous beating) was observed. (Figure 3, SI Figure S7). The phototoxicity of fVF 2 was further assessed in relation to increasing power of the excitation source. Ten second recordings were taken after illumination of the region for one minute, using different regions of the monolayer for each recording. Excitation intensity was raised from 9.0 to 28.9 mW/mm². (SI Figure S7).

5. Image analysis

Analysis of voltage sensitivity in HEK293T cells was performed using ImageJ (FIJI). Briefly, a region of interest (ROI) encompassing the cell body was selected and average fluorescence intensity was calculated for each frame. For background subtraction, a ROI encompassing a region without cells was selected and the average pixel intensity was calculated for each frame. A linear fit to the background trace was calculated and applied to the background, and this was used to subtract background signal from the fluorescence intensity trace. F/F₀ values were calculated by dividing the background subtracted trace by the median value of fluorescence when the cell is held at -60 mV. ΔF/F values were calculated by plotting the change in fluorescence (ΔF) vs the applied voltage step and finding the slope of a linear best-fit. For analysis of voltage responses in neurons, regions of interest encompassing cell bodies were drawn in ImageJ and the mean fluorescence intensity for each frame extracted. ΔF/F values were calculated by first subtracting a mean background value from all raw fluorescence frames, bypassing the noise amplification which arises from subtracting background for each frame, to give a background subtracted trace. A baseline fluorescence value is calculated either from the first several (10-20) frames of the experiment for evoked activity, or from the median for spontaneous activity, and was subtracted from each timepoint of the background subtracted trace to yield a ΔF trace. The ΔF was then divided by baseline fluorescence value to give ΔF/F traces. No averaging has been applied to any voltage traces.

Analysis of action potential (AP) data from hiPSC cardiomyocytes was performed using in-house MATLAB scripts based on previously developed software by the Efimov lab (Washington University, St. Louis, MO).[3] Scripts are available upon request. Briefly, raw OME-tiffs recorded in µManager was read directly into MATLAB for batch-processing of large datasets (>30 Gb per experiment). The mean pixel intensity of the entire image (512x125 pixels) was calculated for each frame and a mean fluorescence trace was extracted for the entire stack. Photobleach correction was performed by subtracting an asymmetric least-squares fit of the data from the mean trace.[4] No subtraction of background was possible due to staining of the entire monolayer. Individual AP events were identified through threshold detection based on a Schmidt trigger. Action potential duration (APD) values were calculated for each AP by finding the activation time (time of the maximum derivative of the AP upstroke) and the time the signal returns to 70, 50, and 10% of the maximum depolarization (APD30, APD50, APD90, respectively). APD values were corrected for variation due to spontaneous beat rate by Fridericia’s formula (SI Eq. 1). CL is the cycle length, calculated as the time period from the beginning of one beat to the beginning of the succeeding beat.
SI Equation 1:

$\text{APD}_c = \frac{\text{APD}}{\sqrt{CL}}$

Electrophysiology
For electrophysiological experiments in HEK293T cells and rat hippocampal neurons, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 5–6 MΩ, and were filled with an internal solution; 125 mM potassium gluconate, 1 mM EGTA, 10 mM HEPES, 5 mM NaCl, 10 mM KCl, 2 mM ATP disodium salt, 0.3 mM GTP trisodium salt (pH 7.25, 285 mOsm). Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with a Digidata 1440A, sampled at 50 kHz and recorded with pCLAMP 10 software (Molecular Devices) on a PC. Fast capacitance was compensated in the on-cell configuration. For all electrophysiology experiments, recordings were only pursued if series resistance in voltage clamp was less than 30 MΩ. For whole-cell, voltage clamp recordings in HEK293T cells, cells were held at -60 mV and hyper- and de- polarizing steps applied from -100 to +100 mV in 20 mV increments. For whole-cell, current clamp recordings in rat hippocampal neurons,

6. Drug-response experiments
For evaluation of cardiac action potentials in response to cisapride, cardiomyocytes were incubated with 1 µM fVF 2 in RPMI plus B27 supplement media (minus Phenol Red) for 20 minutes at 37 °C. For each well containing cardiomyocytes, baseline activity was recorded in three areas of interest (475/34 nm bandpass, emission filter and dichroic listed above; 51.4 mW/mm²). Cisapride stocks were made in DMSO (0.1-300 µM) and added as a 1:1000 dilution in a 0.1 mL addition of RPMI B27 supplement media (minus Phenol Red). An addition of 0.1% DMSO in a 0.1 mL addition of media was used as a vehicle control. Cardiomyocytes were incubated at 37 °C for 15 minutes with added drug/vehicle and imaged in three different areas of interest per well. The concentration of cisapride was raised through subsequent addition of media containing drug, and the vehicle was matched to add the same percentage of DMSO. This process was repeated for each concentration of cisapride (0.1-300 nM). Dose response curves and IC50 values were calculated in GraphPad using APD data extracted from tiff stacks using the above MATLAB scripts.

7. Synthesis
Scheme S1. Synthesis of fluorene VoltageFluor dyes (fVF dyes)
Preparation of fluorene (FL) molecular wires:

**Synthesis of 2-bromo-7-iodo-9,9-dimethyl-9H-fluorene, 1:**

2-bromo-7-iodo-9H-fluorene (5.0 g, 13.5 mmol) was stirred in DMSO with potassium hydroxide (7.6 g, 134 mmol) and potassium iodide (156 mg, 1.35 mmol) at room temperature. Methyl iodide (4.2 mL, 67.4 mmol) was added dropwise after 10 minutes. The reaction was stirred for 24 hours before pouring into 50 ml of water. An off-white precipitate was collected by filtration. Recrystallization in methanol gave 1 as long, yellow crystals (2.81 g, 52%). NMR spectra matched published values.\(^{[5]}\)

\(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 7.75 (d, \(J = 1.6\ Hz\), 1H), 7.66 (dd, \(J = 8.0, 1.6\ Hz\), 1H), 7.55 (d, \(J = 5.8\ Hz\), 1H), 7.54 (s, 1H), 7.46 (dd, \(J = 8.1, 1.7\ Hz\), 1H), 7.43 (d, \(J = 8.0\ Hz\), 1H), 1.46 (s, 6H).

\(^{13}\)C NMR (101 MHz, CDCl3) \(\delta\) 136.24, 132.14, 130.33, 126.16, 121.80, 121.52, 47.28, 26.88.

HRMS (EI+) calculated for C\(_{15}\)H\(_{12}\)BrI 397.9167; Found 397.9170.
Synthesis of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-N,N-dimethylaniline, 2:

Compound 8 (800 mg, 3.2 mmol) was combined with compound 1 (1.42 g, 3.6 mmol), 1,1'-ferrocenediybis(diphenylphosphine) (180 mg, 0.32 mmol), and Pd(dppf)Cl2 (27.8 mg, 38 µmol) in a flame-dried Schlenk flask. This was evacuated/backfilled with N2 (3x). Anhydrous dioxane (4 mL) and degassed K2CO3 (5 M, 1.6 mL) was added, the flask was sealed, and stirred at 80°C. After 18 h, the reaction was cooled and diluted with DCM. This was washed with sat. ammonium chloride (2X) and brine (1X). The organic fraction was collected, dried with MgSO4, and concentrated onto silica in vacuo. This was purified by flash chromatography (hexanes to 10% EtOAc in hexanes), affording 2 as a light yellow solid (389 mg, 30%).

1H NMR (600 MHz, Chloroform-d) δ 7.70 (d, J = 7.9 Hz, 1H), 7.61 – 7.54 (m, 5H), 7.54 (dd, J = 7.9, 1.7 Hz, 1H), 7.46 (dd, J = 8.0, 1.8 Hz, 1H), 6.86 – 6.81 (m, 2H), 3.02 (s, 6H), 1.52 (s, 6H).

13C NMR (151 MHz, CDCl3) δ 155.80, 153.80, 149.99, 140.96, 138.16, 136.08, 130.02, 129.37, 127.73, 126.04, 125.43, 121.13, 120.55, 120.45, 120.23, 112.77, 47.10, 40.56, 27.10.

HRMS (ESI) m/z: [M+H]+ calculated for C23H23BrN 392.1008; Found 392.1009.

Synthesis of 2-bromo-9,9-dimethyl-7-phenyl-9H-fluorene, 3:

Phenylboronic acid (500 mg, 4.1 mmol) was combined with compound 1 (2.88 g, 8.2 mmol) and Pd(dppf)Cl2 (150 mg, 0.21 mmol) in a flame-dried Schlenk flask. This was evacuated/backfilled with N2 (3x). Anhydrous dioxane (3 mL) and degassed K2CO3 (2 M, 300 µL) was added, the flask was sealed, and stirred at 80°C. After 18 h, the reaction was cooled and diluted with DCM. This was washed with sat. ammonium chloride (2X) and brine (1X). The organic fraction was collected, dried with MgSO4, and concentrated onto silica in vacuo. This was purified by flash chromatography (hexanes to 10% toluene in hexanes), affording 3 as a white solid (260 mg, 18%).

1H NMR (600 MHz, Chloroform-d) δ 7.75 (dd, J = 7.9 Hz, 1H), 7.69 – 7.64 (m, 2H), 7.64 (dd, J = 1.6, 0.7 Hz, 1H), 7.63 – 7.58 (m, 2H), 7.59 (s, 0H), 7.58 (d, J = 1.3 Hz, 1H), 7.51 – 7.45 (m, 3H), 7.38 (s, 1H), 1.54 (s, 6H).

13C NMR (151 MHz, CDCl3) δ 155.91, 153.88, 149.99, 140.96, 138.16, 136.08, 130.02, 129.37, 127.73, 126.04, 125.43, 121.13, 120.55, 120.45, 120.23, 112.77, 47.10, 40.56, 27.10.

HRMS (El+) calculated for C21H17Br 348.0514; Found 348.0520.

Synthesis of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-N,N-dimethylaniline, 4:
Compound 2 (150 mg, 0.38 mmol) was combined with Pd(dppf)Cl$_2$ (5.6 mg, 7.6 µmol), bis(pinacolato)diboron (146 mg, 0.573 mmol), and potassium acetate (113 mg, 1.14 mmol) in a flame dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (2 mL) was added and the flask was sealed, and stirred at 70º C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed twice with sat. ammonium chloride (2x) and brine (1x). Organics were collected, dried with MgSO$_4$, and concentrated onto silica. This was purified by flash chromatography (10-20% EtOAc in hexanes), affording 4 as a yellow solid (64 mg, 38%).

$^1$H NMR (600 MHz, Chloroform-d) δ 7.89 (t, $J$ = 0.9 Hz, 1H), 7.83 (dd, $J$ = 7.5, 1.1 Hz, 1H), 7.77 (dd, $J$ = 7.6, 0.8 Hz, 1H), 7.60 − 7.56 (m, 2H), 7.55 (dd, $J$ = 7.9, 1.7 Hz, 1H), 6.84 (d, $J$ = 8.3 Hz, 2H), 3.01 (s, 6H), 1.55 (s, 6H), 1.39 (s, 12H).

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 154.86, 152.88, 142.16, 141.01, 136.96, 133.90, 128.65, 127.77, 125.26, 120.62, 120.48, 119.15, 112.84, 112.82, 83.67, 83.46, 46.85, 40.60, 27.18, 25.00, 24.89.

HRMS (ESI) m/z: [M+H]$^+$ calculated for C$_{29}$H$_{35}$BNO$_2$ 440.2755; found: 440.2753.

Synthesis of 2-(9,9-dimethyl-7-phenyl-9H-fluoren-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 5:

Compound 13 (350 mg, 0.89 mmol) was combined with Pd(dppf)Cl$_2$ (32.6 mg, 45 µmol), bis(pinacolato)diboron (341 mg, 1.3 mmol), and potassium acetate (263 mg, 2.7 mmol) in a flame dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (3 mL) was added and the flask was sealed, and stirred at 70º C. After 18 h, the reaction was cooled, then extracted with DCM. The crude reaction was washed twice with sat. ammonium chloride (2x) and brine (1x). Organics were collected, dried with MgSO$_4$, and concentrated onto silica. This was purified by flash chromatography (hexanes to 10% EtOAc in hexanes), affording 5 as a white solid (334 mg, 85%).

$^1$H NMR (600 MHz, Chloroform-d) δ 7.92 (s, 1H), 7.85 (d, $J$ = 7.5 Hz, 1H), 7.82 (dd, $J$ = 7.8 Hz, 1H), 7.68 (d, $J$ = 9.3 Hz, 2H), 7.67 (d, $J$ = 4.3 Hz, 1H), 7.60 (dd, $J$ = 7.8, 1.6 Hz, 1H), 7.48 (td, $J$ = 7.8, 1.6 Hz, 2H), 7.40 − 7.35 (m, 1H), 1.57 (d, $J$ = 1.6 Hz, 6H), 1.40 (d, $J$ = 1.6 Hz, 12H).

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 154.91, 152.99, 141.82, 141.56, 140.91, 138.19, 133.95, 128.74, 127.21, 127.17, 126.18, 121.44, 120.69, 119.39, 83.73, 46.94, 27.14, 24.90, 24.85.

HRMS (EI+) calculated for C$_{27}$H$_{29}$BO$_2$ 396.2261; Found 396.2267.

Synthesis of N,N-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline, 8:

4-bromo-N,N-dimethylaniline (500 mg, 2.5 mmol), Pd(dppf)Cl$_2$ (36.6 mg, 50 µmol), bis(pinacolato)diboron (952 mg, 3.75 mmol), and potassium acetate (736 mg, 7.5 mmol) were combined in a flame-dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (4 mL) was added and the flask was sealed, and stirred at 80º C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed with sat. ammonium chloride (2x) and brine (1x). Organics were collected, dried with MgSO$_4$, and concentrated onto silica. This was purified by flash chromatography (hexanes to 10% EtOAc in hexanes), affording 8 as a grey powder (294 mg, 60.9%). Reported NMR spectra matched published spectra.

$^1$H NMR (400 MHz, Chloroform-d) δ 7.71 (d, $J$ = 8.7 Hz, 2H), 6.70 (d, $J$ = 8.7 Hz, 2H), 3.00 (s, 6H), 1.34 (s, 12H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 152.57, 136.17, 111.27, 83.17, 40.14, 24.88.
Synthesis of 1-bromo-2,5-dimethoxy-4-nitrobenzene, 9:
1-bromo-2,5-dimethoxybenzene (2.0 mmol, 5.0 g) was added to stirring glacial acetic acid (10 mL) at room temperature. Concentrated nitric acid (2 mL) was then added dropwise to the reaction, immediately causing the colorless solution to become yellow. This was stirred for 15 minutes before putting on ice and precipitating by addition of water. 9 was collected as a yellow precipitate by filtration (6.0 g, 100%). Reported NMR spectra matched published spectra.[8]

$^1$H NMR (400 MHz, Chloroform-d) δ 7.46 (s, 1H), 7.33 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H).

$^{13}$C NMR (101 MHz, CDCl3) δ 149.62, 147.60, 119.28, 118.58, 108.68, 57.35, 57.04.

HRMS (EI+) calculated for C$_{15}$H$_{13}$BrI 248.1816; Found 248.1813.

Synthesis of 2-(2-methoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 10:
1-bromo-2-methoxy-4-nitrobenzene (2.0 g, 8.6 mmol), Pd(dpdpf)Cl$_2$ (126 mg, 172 µmol), bis(pinacolato)diboron (3.3 g, 12.9 mmol), and potassium acetate (2.5 g, 25.9 mmol) were combined in a flame-dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (8 mL) was added and the flask was sealed, and stirred at 70° C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed twice with sat. ammonium chloride and once with brine. Organics were collected, dried with MgSO$_4$, and concentrated onto silica. This was purified by flash chromatography (5% EtOAc in hexanes to 20% EtOAc in hexanes), affording 10 as a yellow solid (1.02 g, 44%).[9]

$^1$H NMR (400 MHz, DMSO-d6) δ 7.80 (dd, J = 8.1, 1.9 Hz, 1H), 7.76 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 1.9 Hz, 1H), 3.88 (s, 3H), 1.30 (s, 12H).

$^{13}$C NMR (101 MHz, DMSO) δ 164.52, 151.03, 137.36, 115.14, 105.48, 84.33, 83.29, 56.49, 25.29.

HRMS (EI+) calculated for C$_{13}$H$_{18}$BNO$_2$ 279.1278; found 279.1277.

Synthesis of 2-(2,5-dimethoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 11:
Compound 9 (5.0 g, 19 mmol), Pd(dpdpf)Cl$_2$ (279 mg, 0.38 mmol), bis(pinacolato)diboron (7.3 g, 7.3 mmol), and potassium acetate (5.6 g, 57 mmol) were combined in a flame-dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (10 mL) was added and the flask was sealed, and stirred at 70° C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed twice with sat. ammonium chloride and once with brine. Organics were collected, dried with MgSO$_4$, and concentrated onto silica. This was purified by flash chromatography (20% EtOAc in hexanes to 30% EtOAc in hexanes), affording 11 as a yellow solid (3.68 g, 62%).[10]

$^1$H NMR (400 MHz, Chloroform-d) δ 7.37 (s, 1H), 7.32 (s, 1H), 3.95 (s, 3H), 3.83 (s, 3H), 1.37 (s, 12H).

$^{13}$C NMR (101 MHz, CDCl3) δ 157.31, 146.58, 141.09, 121.91, 107.43, 84.33, 57.19, 56.69, 25.05, 24.83.

HRMS (EI+) calculated for C$_{14}$H$_{20}$BNO$_2$ 309.1384; found 309.1388.
Synthesis of 2-bromo-7-(2-methoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 12:
Compound 10 (500 mg, 1.79 mmol) was combined with compound 1 (1.26 g, 3.58 mmol), and Pd(dppf)Cl₂ (65.5 mg, 90 µmol) in a flame-dried Schlenk flask. This was evacuated/backfilled with N₂ (3x). Anhydrous dioxane (3 mL) and degassed 2 M K₂CO₃ (1 mL) was added and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 80 °C. After 18 h, the reaction was cooled and diluted with DCM. This was washed with sat. ammonium chloride (2X) and brine (1X). The organic fraction was collected, dried with MgSO₄, and concentrated onto silica in vacuo, affording 12 as a light-yellow powder (431 mg, 57%).

¹H NMR (600 MHz, DMSO-d₆) δ 7.94 – 7.91 (m, 1H), 7.91 (d, J = 4.4 Hz, 1H), 7.88 (d, J = 2.2 Hz, 1H), 7.83 (d, J = 5.8 Hz, 1H), 7.82 (s, 1H), 7.71 (d, J = 1.6 Hz, 1H), 7.64 (d, J = 8.3 Hz, 1H), 7.55 – 7.54 (m, 1H), 7.53 (s, 1H), 3.92 (s, 3H), 1.47 (s, 6H).

¹³C NMR (151 MHz, DMSO) δ 157.04, 156.61, 153.50, 147.99, 137.90, 137.77, 137.28, 135.96, 131.68, 130.53, 129.06, 126.62, 124.20, 122.77, 121.28, 120.56, 116.30, 106.89, 56.74, 47.46, 26.90.

HRMS (EI+) calculated for C₂₂H₁₈BrNO₃ 423.0470; Found 423.0475.

Synthesis of 2-bromo-7-(2,5-dimethoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 13:
Compound 11 (1.50 g, 4.85 mmol) was combined with compound 1 (2.13 g, 5.33 mmol), 1,1′-ferrocenediylibis(diphenylphosphine) (269 mg, 0.485 mmol), and Pd(dppf)Cl₂ (177 mg, 0.243 mmol) in a flame-dried Schlenk flask. This was evacuated/backfilled with N₂ (3x). Anhydrous dioxane (6 mL) and degassed 5 M K₂CO₃ (1.9 mL) was added and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 80 °C. After 18 h, the reaction was cooled and diluted with DCM. This was washed with sat. ammonium chloride (2X) and brine (1X). The organic fraction was collected, dried with MgSO₄, and concentrated onto silica in vacuo. This was purified by flash chromatography (1-10% EtOAc in hexanes), affording 13 as a light-yellow powder (431 mg, 57%).

¹H NMR (600 MHz, Chloroform-d) δ 7.76 (d, J = 7.8 Hz, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 1.3 Hz, 1H), 7.58 (s, 1H), 7.57 (d, J = 1.2 Hz, 1H), 7.52 – 7.47 (m, 2H), 7.12 (s, 1H), 3.99 (s, 3H), 3.85 (s, 3H), 1.53 (s, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 155.94, 153.20, 149.84, 147.76, 138.41, 137.85, 137.66, 135.71, 130.25, 128.51, 126.21, 123.67, 121.59, 121.51, 119.85, 116.79, 108.79, 57.24, 56.43, 47.23, 26.98.

HRMS (EI+) calculated for C₂₃H₂₀BrNO₄ 453.0576; Found 453.0582.

Synthesis of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxylaniline, 14:
Compound 12 (500 mg, 1.18 mmol) and tin(II) chloride dihydrate (1.3 g, 5.89 mmol) was suspended in 5 ml ethanol, then set to stir at reflux for 90 minutes. The reaction was allowed to cool and was then then precipitated using sat. NaHCO₃ solution. This was stirred for 30 min before filtering and the filtrate was collected and concentrated to dryness. This was resuspended in EtOAc, then washed once with saturated NaHCO₃ and twice with Brine. The organics were collected, dried with MgSO₄, and concentrated to give 14 as a fine yellow powder. (436 mg, 93%). This was used without further purification.

1H NMR (600 MHz, DMSO-δ₆) δ 7.76 (d, J = 1.9 Hz, 1H), 7.73 (s, 1H), 7.73 (d, J = 15.2 Hz, 1H), 7.52 (d, J = 1.5 Hz, 1H), 7.49 (dd, J = 8.0, 1.8 Hz, 1H), 7.38 (dd, J = 7.9, 1.6 Hz, 1H), 7.02 (d, J = 8.2 Hz, 1H), 6.32 (d, J = 2.0 Hz, 1H), 6.25 (dd, J = 8.1, 2.1 Hz, 1H), 5.33 (s, 2H), 3.68 (s, 3H), 2.94 (s, 6H), 1.44 (s, 6H).

13C NMR (151 MHz, DMSO) δ 155.90, 152.93, 151.15, 141.57, 138.49, 138.27, 136.49, 136.17, 129.98, 128.39, 126.44, 123.42, 122.10, 120.29, 120.05, 106.99, 98.12, 55.50, 47.20, 27.09.

HRMS (ESI) m/z: [M+H]+ calculated for C23H21BrNO 394.0801; Found 394.0805.

Synthesis of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxyaniline, 15:
Compound 13 (400 mg, 0.734 mmol) and tin(II) chloride dihydrate (828 mg, 3.67 mmol) was suspended in 2 ml ethanol, then set to stir at reflux for 30 minutes. The reaction was allowed to cool and was then then precipitated using sat. NaHCO₃ solution. This was stirred for 30 min before filtering and the filtrate was collected and concentrated to dryness. This was resuspended in EtOAc, then washed once with saturated NaHCO₃ and twice with brine. The organics were collected, dried with MgSO₄, and concentrated to give 15 as a fine yellow powder. (241 mg, 77%). This was used without further purification.

1H NMR (600 MHz, Chloroform-d) δ 7.69 (d, J = 7.8 Hz, 1H), 7.57 (t, J = 8.4 Hz, 2H), 7.56 (d, J = 8.9 Hz, 1H), 7.50 (dd, J = 7.9, 1.5 Hz, 1H), 7.45 (dd, J = 8.0, 1.8 Hz, 1H), 6.86 (s, 1H), 6.46 (s, 1H), 3.98 – 3.89 (m, 2H), 3.87 (s, 3H), 3.75 (s, 3H), 1.51 (s, 6H).

13C NMR (151 MHz, CDCl3) δ 155.90, 152.93, 151.15, 141.57, 138.49, 138.27, 136.49, 136.17, 129.98, 128.39, 126.04, 123.62, 121.17, 120.58, 120.20, 119.52, 113.77, 100.35, 56.37, 56.34, 47.08, 27.05.

HRMS (ESI) m/z: [M+H]+ calculated for C23H23BrNO 424.0886; Found: 424.0886.

Synthesis of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 16:
Compound 14 (360 mg, 0.913 mmol) was dissolved in 2 ml acetonitrile, then 37% formaldehyde solution (4.57 mmol, 330 µL) was added. This was set to stir at 0°C, then NaCNBH₃ (172 mg, 2.74 mmol) was added slowly. Stil at 0°C, glacial AcOH was used to adjust the solution to a pH between 5 and 6. The reaction was allowed to warm to room temperature and stir overnight. The reaction was poured over ice and then extracted into DCM. This was washed with 2 M KOH and brine, extracting into DCM three times. This was then dried with MgSO₄ and concentrated to give 16 as a yellow powder (318 mg, 74%). This was used without further purification.

1H NMR (600 MHz, DMSO-δ₆) δ 7.78 – 7.71 (m, 3H), 7.55 (d, J = 1.6 Hz, 1H), 7.49 (dd, J = 8.0, 1.9 Hz, 1H), 7.41 (dd, J = 7.9, 1.6 Hz, 1H), 7.17 (d, J = 9.1 Hz, 1H), 6.38 (d, J = 1.2 Hz, 1H), 6.38 (d, J = 3.5 Hz, 1H), 3.77 (s, 3H), 2.94 (s, 6H), 1.44 (s, 6H).

13C NMR (151 MHz, DMSO) δ 157.46, 156.36, 153.18, 151.75, 138.92, 138.40, 135.33, 131.13, 130.33, 128.50, 126.45, 123.49, 122.15, 120.37, 120.13, 118.45, 105.42, 96.91, 55.75, 47.22, 40.61, 27.09.
Synthesis of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 17:
Compound 15 (125 mg, 0.297 mmol) was suspended in 3 ml glacial acetic acid, then paraformaldehyde (45.3 mg, 1.51 mmol) was added while stirring. After stirring for 15 minutes at room temperature, NaCNBH₃ (47.4 mg, 0.755 mmol) was added portion-wise and the reaction was stirred overnight. The reaction was neutralized with 2M KOH, then extracted into EtOAc. This was washed with 2 M KOH and brine before collecting drying with MgSO₄ and concentrating to give 17 as a yellow solid (67 mg, 98%). This was used without further purification.

¹H NMR (600 MHz, Chloroform-d) δ 7.71 (d, J = 7.8 Hz, 1H), 7.59 (s, 1H), 7.58 (d, J = 6.0 Hz, 1H), 7.56 (d, J = 1.8 Hz, 1H), 7.52 (dd, J = 7.8, 1.6 Hz, 1H), 7.46 (dd, J = 8.0, 1.8 Hz, 1H), 6.90 (s, 1H), 6.66 (s, 1H), 3.90 (s, 3H), 3.79 (s, 3H), 2.87 (s, 6H), 1.51 (s, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 155.90, 152.98, 150.68, 146.60, 142.50, 138.17, 136.54, 130.02, 129.30, 128.36, 127.99, 126.06, 123.65, 121.23, 120.72, 119.58, 114.19, 103.96, 56.68, 56.01, 47.10, 43.20, 27.04.

HRMS (ESI) m/z: [M+H]⁺ calculated for C₂₅H₂₉BrNO 422.1114; Found 422.1121.

Synthesis of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 18:
Compound 16 (230 mg, 0.55 mmol) was combined with Pd(dppf)Cl₂ (20 mg, 27 µmol), bis(pinacolato)diboron (166 mg, 0.65 mmol), and potassium acetate (160 mg, 1.64 mmol) in a flame dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (2.5 mL) was added and the flask, was sealed, and stirred at 70º C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed twice with sat. ammonium chloride (2x) and brine (1x). Organics were collected, dried with MgSO₄, and concentrated onto silica. This was purified by flash chromatography (5-20% EtOAc in hexanes), affording 18 as a yellow solid (128 mg, 50%).

¹H NMR (600 MHz, Chloroform-d) δ 7.89 (s, 1H), 7.82 (dd, J = 7.5, 1.1 Hz, 1H), 7.75 (d, J = 7.9 Hz, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.59 (s, 1H), 7.53 (dd, J = 7.9, 1.5 Hz, 1H), 7.30 (d, J = 8.4 Hz, 1H), 6.45 (dd, J = 8.5, 2.4 Hz, 1H), 6.38 (s, 1H), 3.85 (s, 3H), 3.03 (s, 6H), 1.39 (s, 12H), 1.27 (s, 6H).

¹³C NMR (151 MHz, CDC13) δ 157.37, 153.96, 152.96, 151.36, 146.60, 142.50, 138.17, 136.54, 130.02, 129.30, 128.36, 127.99, 126.06, 123.65, 121.23, 120.72, 119.58, 114.19, 103.96, 56.68, 56.01, 47.10, 43.20, 27.04.

HRMS (ESI) m/z: [M+H]⁺ calculated for C₃₀H₃₇BNO₃ 470.2861; Found 470.2864
Synthesis of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 19:

Compound 17 (65 mg, 0.143 mmol) was combined with Pd(dppf)Cl₂ (5.2 mg, 7.2 µmol), bis(pinacolato)diboron (55 mg, 0.215 mmol), and potassium acetate (42 mg, 0.429 mmol) in a flame dried Schlenk flask. The flask was evacuated/backfilled with nitrogen (3x). Anhydrous dioxane (2 mL) was added and the flask, was sealed, and stirred at 70º C. After 18 h, the reaction was cooled, then extracted with DCM. This was washed twice with sat. ammonium chloride (2x) and brine (1x). Organics were collected, dried with MgSO₄, and concentrated onto silica. This was purified by flash chromatography (20-30% EtOAc in hexanes), affording 19 as a yellow solid (58 mg, 81%).

¹H NMR (600 MHz, Chloroform-d) δ 7.89 (d, J = 1.1 Hz, 1H), 7.83 (dd, J = 7.5, 1.0 Hz, 1H), 7.77 (d, J = 7.8 Hz, 1H), 7.74 (d, J = 1.6 Hz, 1H), 7.62 (d, J = 7.8, 1.6 Hz, 1H), 6.92 (s, 1H), 6.67 (s, 1H), 3.91 (s, 3H), 3.79 (s, 3H), 2.87 (s, 6H), 1.39 (s, 12H), 1.27 (s, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 154.04, 152.98, 150.68, 146.61, 142.44, 142.17, 138.16, 137.40, 133.86, 128.66, 128.15, 123.94, 123.67, 119.97, 119.25, 114.18, 104.00, 83.69, 56.71, 55.99, 46.85, 43.22, 27.11, 24.99, 24.89.

HRMS (ESI) [M+H]⁺ calculated for C₃₁H₃₉BNO₄⁺ 500.2967; found 500.2971

Synthesis of FL-based voltage indicators:

Synthesis of 6 (fVF 1):

Compound 4 (63.9 mg, 0.145 mmol) was combined with 5-bromo-(2',7'-dichloro-sulfofluoresccin) (50 mg, 0.097 mmol) and Pd(dppf)Cl₂ (3.6 mg, 4.85 µmol) in a flame-dried screw-cap vial with a PTFE/silicon septum. This was evacuated/backfilled with N₂ (3x). Anhydrous DMF (500 µL) and degassed K₂CO₃ (5M, 50 µL) was added, and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 80º C. After 18 h, the reaction was cooled and concentrated to dryness in vacuo. This was taken up in a minimal amount of DCM/MeOH, and then precipitated into diethyl ether. The precipitate was collected by vacuum filtration to obtain a reddish brown solid. A small sample was purified by preparative-HPLC (Water/MeCN ± 0.05% TFA) to afford pure 6 as a reddish dust (5.8 mg, 8.0% yield).

¹H NMR (900 MHz, DMSO-d6) δ 8.31 (d, J = 2.0 Hz, 1H), 8.00 (d, J = 1.7 Hz, 1H), 7.98 (d, J = 7.7 Hz, 1H), 7.95 (dd, J = 7.7, 2.0 Hz, 1H), 7.90 (d, J = 7.8 Hz, 1H), 7.81 (d, J = 1.7 Hz, 1H), 7.80 (dd, J = 7.8, 1.7 Hz, 1H), 7.63 (d, J = 8.2 Hz, 2H), 7.61 (dd, J = 7.7, 1.7 Hz, 1H), 7.38 (d, J = 7.6 Hz, 1H), 7.25 – 7.07 (m, 2H), 7.02 (s, 2H), 6.84 (d, J = 8.1 Hz, 2H), 2.97 (s, 6H), 1.59 (s, 6H).

¹³C NMR (226 MHz, DMSO) δ 157.99, 157.86, 157.72, 157.59, 154.57, 154.52, 151.88, 149.80, 147.47, 141.48, 139.91, 138.60, 137.68, 135.95, 130.36, 129.79, 128.89, 128.02, 127.64, 127.27, 126.01, 125.40, 124.67, 121.22, 120.75, 120.62, 119.90, 119.25, 117.93, 116.60, 115.28, 112.68, 79.11, 78.97, 78.82, 46.78, 29.00, 26.93.

HRMS (ESI) m/z: [M-H]⁻ calculated for C₄₂H₃₉Cl₂N₀₆ 746.1176; Found 746.1172
Analytical HPLC retention time: 5.42 min (10-90% MeCN in water for 7.5 min, 90-100% MeCN in water for 2.5 min, 0.05% trifluoroacetic acid as an additive).

Synthesis of 7 (fVF 0):
Compound 5 (46.1 mg, 0.116 mmol) was combined with 5-bromo-(2',7'-dichloro-sulfofluorescien) (50 mg, 0.097 mmol) and Pd(dppf)Cl$_2$ (3.6 mg, 4.85 µmol) in a flame-dried screw-cap vial with a PTFE/silicon septum. This was evacuated/backfilled with N$_2$ (3x). Anhydrous DMF (500 µL) and degassed K$_2$CO$_3$ (5M, 50 µL) was added, and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 80º C. After 18 h, the reaction was cooled and concentrated to dryness in vacuo. This was taken up in a minimal amount of DCM/MeOH, and then precipitated into diethyl ether. The precipitate was collected by vacuum filtration to obtain a red solid. A small sample was purified by preparative-HPLC (Water/MeCN + 0.05% TFA) to afford pure 7 as a brown dust (15.9 mg, 23% yield).

$^1$H NMR (900 MHz, DMSO-d$_6$) δ 8.31 (d, J = 2.0 Hz, 1H), 8.02 (dd, J = 4.7, 3.0 Hz, 2H), 7.97 (d, J = 7.8 Hz, 1H), 7.95 (dd, J = 7.7, 2.0 Hz, 1H), 7.90 (d, J = 1.7 Hz, 1H), 7.81 (dd, J = 7.7, 1.7 Hz, 1H), 7.78 – 7.75 (m, 2H), 7.68 (dd, J = 7.7, 1.7 Hz, 1H), 7.49 (t, J = 7.6 Hz, 2H), 7.38 (t, J = 7.9 Hz, 2H), 7.02 (s, 2H), 6.77 (s, 2H), 1.60 (s, 6H).

$^{13}$C NMR (226 MHz, DMSO) δ 158.11, 157.96, 157.81, 157.66, 154.55, 154.48, 151.67, 147.28, 141.28, 140.31, 139.50, 138.12, 137.97, 137.25, 130.22, 129.62, 128.75, 127.17, 126.77, 125.93, 125.74, 125.31, 121.16, 121.07, 120.81, 120.72, 117.01, 115.71, 103.24, 103.15, 48.42, 46.74, 39.77, 39.67, 26.67.

HRMS (ESI) m/z: [M+H]$^+$ calculated for C$_{40}$H$_{27}$Cl$_2$O$_6$S: 705.0900; Found 705.090

Analytical HPLC retention time: 9.26 min (30-80% MeCN in water for 7.5 min, 80-100% MeCN in water for 2.5 min, 0.05% trifluoroacetic acid as an additive).

Synthesis of 20 (fVF 2):
Compound 18 (54.6 mg, 0.116 mmol) was combined with 5-bromo-(2',7'-dichloro-sulfofluorescien) (50 mg, 0.097 mmol) and Pd(dppf)Cl$_2$ (3.6 mg, 4.85 µmol) in a flame-dried screw-cap vial with a PTFE/silicon septum. This was evacuated/backfilled with N$_2$ (3x). Anhydrous DMF (500 µL) and degassed K$_2$CO$_3$ (5M, 50 µL) was added, and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 80º C. After 18 h, the reaction was cooled and concentrated to dryness in vacuo. This was taken up in a minimal amount of DCM/MeOH, and then precipitated into diethyl ether. The precipitate was collected by vacuum filtration to obtain a reddish brown solid. A small sample was purified by preparative-HPLC (Water/MeCN + 0.05% TFA) to afford pure 20 as an orange dust (6.8 mg, 9.0% yield).

**For NMR and analytical HPLC characterization, precipitate from ether was used. Material purified by HPLC was used for spectroscopic experiments and testing in cells.**
$^1$H NMR (900 MHz, DMSO-d$_6$) δ 8.31 (s, 1H), 7.98 (s, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.86 (dd, J = 37.1, 7.7 Hz, 2H), 7.78 (d, J = 7.6 Hz, 1H), 7.62 (s, 1H), 7.46 (d, J = 7.6 Hz, 1H), 7.23 (dd, J = 19.5, 7.9 Hz, 2H), 6.79 (s, 2H), 6.41 (s, 2H), 6.17 (d, J = 6.8 Hz, 2H), 3.81 (s, 3H), 2.97 (s, 3H), 1.56 (s, 6H).

$^{13}$C NMR (226 MHz, DMSO) δ 173.31, 173.27, 157.04, 156.43, 154.46, 153.43, 152.09, 151.28, 147.28, 140.66, 138.57, 138.19, 137.88, 135.57, 130.73, 130.67, 129.39, 128.61, 128.02, 126.86, 126.61, 126.55, 125.89, 125.56, 123.12, 121.15, 120.51, 119.69, 118.13, 108.99, 108.95, 105.00, 102.68, 102.64, 96.47, 64.90, 55.33, 46.64, 40.20, 26.98, 15.16.

HRMS (ESI) m/z: [M+H]$^+$ calculated for C$_{43}$H$_{34}$Cl$_2$NO$_7$S 778.1428; Found 778.1436.

Analytical HPLC retention time: 5.45 min (10-90% MeCN in water for 7.5 min, 90-100% MeCN in water for 2.5 min, 0.05% trifluoroacetic acid as an additive).

### Synthesis of 21 (iVF 3):

Compound 19 (20.0 mg, 0.040 mmol) was combined with 5-bromo-(2',7'-dichloro-sulfofluorescien) (17.2 mg, 0.033 mmol) and Pd(dpdpf)Cl$_2$ (1.2 mg, 1.7 µmol) in a flame-dried screw-cap vial with a PTFE/silicon septum. This was evacuated/backfilled with N$_2$ (3x). Anhydrous DMF (500 µL) and degassed K$_2$CO$_3$ (5M, 16 µL) was added, and was subjected to three freeze-pump-thaw cycles. The flask was sealed and stirred at 60º C. After 18 h, the reaction was cooled and concentrated to dryness in vacuo. This was taken up in a minimal amount of DCM/MeOH, and then precipitated into diethyl ether. The precipitate was collected by vacuum filtration to obtain a dark red solid. This was further purified by preparative-HPLC (Water/MeCN + 0.05% TFA) to afford pure 21 as an orange dust (5.6 mg, 21% yield).

$^1$H NMR (900 MHz, DMSO-d$_6$) δ 8.31 (s, 1H), 8.02 (s, 1H), 7.96 (d, J = 7.1 Hz, 1H), 7.92 (d, J = 7.8 Hz, 1H), 7.70 (s, 1H), 7.81 (d, J = 7.6 Hz, 1H), 7.52 (d, J = 7.6 Hz, 1H), 7.38 (d, J = 7.6 Hz, 1H), 7.25 (t, J = 7.4 Hz, 1H), 7.17 (d, J = 7.5 Hz, 1H), 7.14 (t, J = 7.3 Hz, 1H), 7.02 (s, 2H), 6.79 (s, 1H), 3.89 (s, 3H), 3.79 (s, 3H), 2.94 (s, 6H), 1.58 (s, 6H).

$^{13}$C NMR (226 MHz, DMSO) δ 154.60, 153.53, 151.83, 150.47, 149.66, 147.49, 145.47, 141.42, 138.46, 137.93, 137.32, 130.35, 129.77, 129.47, 128.87, 128.29, 128.18, 128.00, 127.68, 127.24, 126.37, 126.18, 126.03, 125.42, 125.29, 123.62, 123.56, 121.31, 121.27, 120.80, 119.91, 115.86, 115.84, 115.13, 108.40, 103.35, 103.31, 56.40, 46.74, 45.66, 26.89, 21.02.

HRMS (ESI) m/z: [M-H]$^-$ calculated for C$_{44}$H$_{34}$Cl$_2$NO$_8$S 806.1388; Found 806.1382.

Analytical HPLC retention time: 4.99 min (10-90% MeCN in water for 7.5 min, 90-100% MeCN in water for 2.5 min, 0.05% trifluoroacetic acid as an additive).
Figure S1. Normalized absorption and emission profiles of 7 (fVF 0) (a), 6 (fVF 1) (b), 20 (fVF 2) (c), and 21 (fVF 3) (d). All spectra were acquired at a dye concentration of 1 µM in PBS (pH 7.2) with 0.1% SDS.

Figure S2. Staining and photostability of fluorene-wire voltage indicators. Widefield microscopy DIC and fluorescence images of 7 (a,e), 6 (b,f), fVF 2 (20, c, g), and 21 (d, h), loaded at 500 nM in HEK292T cells for 20 minutes. 10 µm scale bar. Image acquisition and analysis parameters were identical to permit comparison of cellular localization and brightness. (i) Relative photostability of fVF 2 (black) and VF2.1.Cl (green). Error bars are ± SEM for 5 cells. fVF 2 exhibits a shorter decay half-life (11.6 ± 0.3 sec) and time constant for the fluorescence decay ($\tau_{Fl} = 16.7 \pm 0.39$ sec) than VF2.1.Cl (31.53 ± 0.5 sec, $\tau_{Fl} = 45.5 \pm 0.8$ sec).
Figure S3. Voltage sensitivity of fluorene VoltageFluors in HEK293T cells. fVF 0 (a,e); fVF 1 (b,f); fVF 2 (c,g); fVF 3 (d,h). (upper row) Plot of fractional change in fluorescence vs time for 100 ms voltage steps ranging from -100 to +100 mV in 20 mV increments from a -60 mV holding potential. (lower row) Linear plot of fractional change in fluorescence vs membrane potential (mV), summarizing data from multiple cells (g, n = 4; f, n = 7; g, n = 8; h, n = 6). Error bars are SEM.
Figure S4. Live-cell wide-field fluorescence images of 10-14 DIV rat hippocampal neurons stained with (a) fVF 2 (c) VF2.1.Cl and (e) di-4-ANEPPS at concentrations of 500 nM, 500 nM, and 2.5 µM, respectively. Scale bar is 20 µm. (d-f) Representative ΔF/F plots of evoked neuronal activity recorded optically with either (b) FVF 2, (d) VF2.1.Cl, or (f) di-4-ANEPPS.

Figure S5. Optically recorded spontaneous activity in 14-19 DIV rat hippocampal neurons stained with 500 nM fVF 2. (DIC, a) Neurons 1 through 3 gave optical ΔF/F traces (c) recorded in the fluorescence channel (b). Scale bar is 50 µm. Optical traces (c) are plots of raw fluorescence values from single recordings and are neither filtered nor corrected for photo-bleach.
Figure S6. Comparison of action potential kinetics recorded in rat hippocampal neurons 10-14 DIV using whole-cell current clamp. Measurements in cells without incubation with fVF 2 (no fill, n = 4 cells) were not statistically significant (t-test, p > 0.05) from measurements made in cells incubated with 500 nM fVF 2 (green, n = 5 cells). (c) Comparison of brightness in neurons loaded with 500 nM of dye for 20 minutes. fVF2 (n = 19 cells) is approximately 1.2 times brighter than VF2.1.Cl (n = 14 cells), and statistically significant (students t-test, p < 0.0001 = ****)
Figure S7. Phototoxicity of fVF 2 and VF2.1.Cl in hiPSC cardiomyocyte monolayers. (a-c) fVF 2 (black traces) shows a quick initial photobleach, but activity is still recorded after ten minutes of illumination (g). (d-f) VF2.1.Cl (blue traces) has better SNR initially but exhibits phototoxicity after just two minutes of illumination (h). Plots of BPM and APDc vs time (i-j) and illumination intensity (k-l) show fVF 2 has negligible phototoxicity with prolonged illumination and increased intensity. Quantification is found in SI Table 2.
**SI Table 1: Voltage Dye characteristics in neurons**

<table>
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<tr>
<th>Dye</th>
<th>SNR</th>
<th>ΔF/F</th>
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<tr>
<td>fVF 2[^a^]</td>
<td>21:1</td>
<td>5.2%</td>
<td>1.2</td>
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<tr>
<td>VF2.1.Cl[^a^]</td>
<td>43:1</td>
<td>10.7%</td>
<td>1</td>
</tr>
<tr>
<td>di-4-ANNEPS[^b^]</td>
<td>15:1</td>
<td>-1.0%</td>
<td>3.7</td>
</tr>
</tbody>
</table>

[^a^] Dye loaded at 0.5 µM, excited at 475 nm and emission collected with a 540 nm bandpass filter.
[^b^] Loaded at 2.5 µM, excited at 475 nm and emission collected with a 650 nm bandpass filter.

**SI Table 2. Action potential kinetics in neurons incubated with fVF 2[^a^]**

<table>
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<tr>
<th>fVF 2</th>
<th>Half-width (ms)</th>
<th>τ_rise (ms)</th>
<th>τ_decay (ms)</th>
<th>Amplitude (mV)</th>
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<td>(-)</td>
<td>1.7 ± 0.1</td>
<td>0.71 ± 0.03</td>
<td>1.6 ± 0.6</td>
<td>101 ± 7</td>
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<tr>
<td>(+)</td>
<td>1.5 ± 0.3</td>
<td>0.61 ± 0.17</td>
<td>1.5 ± 0.3</td>
<td>101 ± 10</td>
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</tbody>
</table>

[^a^] Loaded with 500 nM fVF 2 in HBSS for 20 minutes. Average values presented for each metric with standard deviation. n = 4 cells without (-) fVF 2, n = 5 cells with (+) fVF 2.

**SI Table 3. Measurements of cisapride cardiotoxicity**

<table>
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<tr>
<th>Model</th>
<th>Measurement</th>
<th>APD30 IC_{50}</th>
<th>APD50 IC_{50}</th>
<th>APD90 IC_{50}</th>
<th>hERG IC_{50}</th>
<th>CaV1.2 IC_{50}</th>
<th>NaV1.5 IC_{50}</th>
<th>+20% APD90</th>
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<tbody>
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<td>6.4 nM</td>
<td>10.6 nM</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- 14 Days[^b^]</td>
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<tr>
<td>iPSC Monolayer</td>
<td>optical</td>
<td>-</td>
<td>11.4 nM</td>
<td>53.4 nM</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>- 19 Days[^k^]</td>
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<tr>
<td>HEK293[^h^]</td>
<td>patch-clamp</td>
<td>-</td>
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<tr>
<td>HEK293[^i^]</td>
<td>patch-clamp</td>
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<td>-</td>
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<td>11.8 µM</td>
<td>337 µM</td>
<td>-</td>
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<tr>
<td>iPSC monolayer[^d^]</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>10 nM</td>
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<tr>
<td>Guinea Pig cardiomyocytes[^e^]</td>
<td>patch-clamp</td>
<td>-</td>
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<td>-</td>
<td>15 nM</td>
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</table>

[^a^] From this work. Monolayers with tested after either 14 or 19 days in culture. [^b^] [^k^] [^h^] [^i^] [^d^] [^e^] [^10^] [^11^] [^12^] [^13^]
$^1$H spectrum of 2-bromo-7-iodo-9,9-dimethyl-9H-fluorene, 1:

$^1$H NMR (400 MHz, Chloroform-d) δ 7.75 (d, J = 1.6 Hz, 1H), 7.66 (dd, J = 8.0, 1.6 Hz, 1H), 7.55 (d, J = 5.8 Hz, 1H), 7.54 (s, 1H), 7.46 (dd, J = 8.1, 1.7 Hz, 1H), 7.43 (d, J = 8.0 Hz, 1H), 1.66 (s, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 136.24, 132.14, 130.33, 126.16, 121.80, 121.52, 47.28, 26.88.
$^{1}H$ spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-N,N-dimethylaniline, 2:

$^{1}H$ NMR (600 MHz, Chloroform-d) $\delta$ 7.70 (d, $J = 7.9$ Hz, 1H), 7.61 – 7.54 (m, 5H), 7.54 (dd, $J = 7.9$, 1.7 Hz, 1H), 7.46 (dd, $J = 8.0$, 1.8 Hz, 1H), 6.86 – 6.81 (m, 2H), 3.02 (s, 6H), 1.52 (s, 6H).

$^{13}C$ spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-N,N-dimethylaniline, 2:

$^{13}C$ NMR (151 MHz, CDCl$_3$) $\delta$ 155.80, 153.80, 149.99, 140.96, 138.16, 136.08, 130.02, 129.37, 127.73, 126.04, 125.43, 121.13, 120.55, 120.45, 120.23, 112.77, 47.10, 40.56, 27.10.
$^1$H spectrum of 2-bromo-9,9-dimethyl-7-phenyl-9H-fluorene, 3:

$^1$H NMR (600 MHz, Chloroform-d) δ 7.75 (dd, $J = 7.9$, 0.6 Hz, 1H), 7.69 – 7.64 (m, 2H), 7.64 (dd, $J = 1.6$, 0.7 Hz, 1H), 7.63 – 7.58 (m, 2H), 7.59 (s, 6H), 7.58 (d, $J = 1.3$ Hz, 1H), 7.51 – 7.45 (m, 3H), 7.38 (s, 1H), 1.54 (s, 6H).

$^{13}$C spectrum of 2-bromo-9,9-dimethyl-7-phenyl-9H-fluorene, 3:

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 155.91, 153.88, 141.41, 140.89, 137.88, 137.36, 130.16, 128.80, 127.28, 127.20, 126.00, 126.16, 121.44, 121.41, 121.05, 120.34, 47.21, 27.09.
$^1$H spectrum 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-N,N-dimethylaniline, 4:

$^1$H NMR (600 MHz, Chloroform-d) δ 7.99 (s, 1H), 7.83 (d, $J = 7.5$, 1H), 7.77 (d, $J = 7.8$, 1H), 7.73 (d, $J = 7.6$, 0.8 Hz, 1H), 7.63 - 7.60 (m, 1H), 7.60 - 7.56 (m, 2H), 7.55 (dd, $J = 7.9$, 1.7 Hz, 1H), 6.84 (d, $J = 8.3$, 2H), 3.01 (s, 6H), 1.55 (s, 6H), 1.39 (s, 12H).

$^{13}$C spectrum 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-N,N-dimethylaniline, 4:

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 154.86, 152.88, 142.16, 141.01, 136.96, 133.90, 128.65, 127.77, 125.26, 120.62, 120.48, 119.15, 112.84, 112.82, 83.67, 83.46, 46.85, 40.00, 27.18, 25.00, 24.89.
$^1$H spectrum of 2-(9,9-dimethyl-7-phenyl-9H-fluoren-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 5:

$^1$H NMR (600 MHz, Chloroform-d) δ 7.92 (s, 1H), 7.85 (d, $J = 7.5$ Hz, 1H), 7.82 (dd, $J = 7.8$, 1.5 Hz, 1H), 7.77 (dd, $J = 7.6$, 1.4 Hz, 1H), 7.68 (d, $J = 9.3$ Hz, 2H), 7.67 (d, $J = 4.3$ Hz, 1H), 7.60 (dd, $J = 7.8$, 1.6 Hz, 1H), 7.48 (d, $J = 7.8$, 1.8 Hz, 2H), 7.40 – 7.35 (m, 1H), 1.57 (d, $J = 1.6$ Hz, 6H), 1.40 (d, $J = 1.6$ Hz, 12H).

$^{13}$C spectrum of 2-(9,9-dimethyl-7-phenyl-9H-fluoren-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 5:

$^{13}$C NMR (151 MHz, CDCl₃) δ 154.91, 152.99, 141.82, 141.56, 140.91, 138.19, 133.95, 128.74, 127.21, 127.17, 126.18, 121.44, 120.69, 119.39, 83.73, 46.94, 27.14, 24.90, 24.85.
\(^1\)H spectrum of N,N-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline, 8

\(^1\)H NMR (400 MHz, Chloroform-\(d\)) δ 7.71 (d, \(J = 8.7\) Hz, 2H), 6.70 (d, \(J = 8.7\) Hz, 2H), 3.00 (s, 6H), 1.34 (s, 12H).

\(^1^3\)C spectrum of N,N-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline, 8:

\(^1^3\)C NMR (101 MHz, CDCl\(_3\)) δ 152.57, 136.17, 111.27, 83.17, 40.14, 24.88.
$^1$H spectrum of 1-bromo-2,5-dimethoxy-4-nitrobenzene, 9:

$^1$H NMR (400 MHz, Chloroform-δ) $\delta$ 7.46 (s, 1H), 7.33 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H).

$^{13}$C spectrum of 1-bromo-2,5-dimethoxy-4-nitrobenzene, 9:

$^{13}$C NMR (101 MHz, CDCl₃) $\delta$ 169.62, 147.60, 119.28, 118.58, 108.68, 57.35, 57.64.
$^1$H spectrum of 2-(2-methoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 10:

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 7.80 (dd, J = 8.1, 1.9 Hz, 1H), 7.76 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 1.9 Hz, 1H), 3.88 (s, 3H), 1.30 (s, 12H).

$^{13}$C spectrum of 2-(2-methoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 10:

$^{13}$C NMR (101 MHz, DMSO) δ 164.52, 151.03, 137.36, 115.14, 105.48, 83.29, 56.49, 25.29.
**$^1$H spectrum of 2-(2,5-dimethoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 11:**

$^1$H NMR (400 MHz, Chloroform-d) δ 7.37 (s, 1H), 7.32 (s, 1H), 3.95 (s, 3H), 3.83 (s, 3H), 1.37 (s, 12H).

![NMR spectrum of 2-(2,5-dimethoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane](image)

**$^{13}$C spectrum of 2-(2,5-dimethoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, 11:**

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 157.31, 146.58, 141.09, 121.91, 107.43, 84.33, 57.19, 56.09, 25.05, 24.83.

![NMR spectrum of 2-(2,5-dimethoxy-4-nitrophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane](image)
$^1$H spectrum of 2-bromo-7-(2-methoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 12:

$^1$H NMR (600 MHz, DMSO-d$_6$) δ 7.94 – 7.91 (m, 1H), 7.91 (dd, $J$ = 4.4 Hz, 1H), 7.88 (dd, $J$ = 2.2 Hz, 1H), 7.83 (dd, $J$ = 5.8 Hz, 1H), 7.82 (s, 1H), 7.71 (dd, $J$ = 1.6 Hz, 1H), 7.64 (dd, $J$ = 8.3 Hz, 1H), 7.55 – 7.54 (m, 1H), 7.53 (s, 1H), 3.92 (s, 3H), 1.47 (s, 6H).

$^{13}$C spectrum of 2-bromo-7-(2-methoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 12:

$^{13}$C NMR (151 MHz, DMSO) δ 157.04, 156.61, 153.50, 147.99, 137.90, 137.77, 137.28, 135.96, 131.68, 130.53, 129.06, 126.62, 124.20, 122.77, 121.28, 120.56, 116.30, 106.89, 56.74, 47.86, 26.98.
$^1$H spectrum of 2-bromo-7-(2,5-dimethoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 13:

$^1$H NMR (600 MHz, Chloroform-d) δ 7.76 (d, J = 7.8 Hz, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 1.3 Hz, 1H), 7.58 (s, 1H), 7.57 (d, J = 1.2 Hz, 1H), 7.52 – 7.47 (m, 2H), 7.12 (s, 1H), 3.99 (s, 3H), 3.85 (s, 3H), 1.53 (s, 6H).

$^{13}$C spectrum of 2-bromo-7-(2,5-dimethoxy-4-nitrophenyl)-9,9-dimethyl-9H-fluorene, 13:

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 155.94, 152.20, 149.84, 147.76, 138.41, 137.85, 137.66, 137.55, 135.71, 130.25, 128.51, 126.21, 123.67, 121.59, 121.51, 119.85, 116.67, 108.79, 57.24, 36.43, 47.23, 26.96.
$^1$H spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxyaniline, 14:

$^1$H NMR (600 MHz, DMSO-d$_6$) δ 7.76 (d, $J$ = 1.9 Hz, 1H), 7.73 (s, 1H), 7.73 (d, $J$ = 1.5 Hz, 1H), 7.52 (d, $J$ = 1.5 Hz, 1H), 7.49 (dd, $J$ = 8.0, 1.8 Hz, 1H), 7.38 (dd, $J$ = 7.9, 1.6 Hz, 1H), 7.02 (d, $J$ = 8.2 Hz, 1H), 6.32 (dd, $J$ = 2.0 Hz, 1H), 6.25 (dd, $J$ = 8.1, 2.1 Hz, 1H), 5.33 (s, 2H), 3.68 (s, 3H), 1.43 (s, 6H).

$^{13}$C spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxyaniline, 14:

$^{13}$C NMR (151 MHz, DMSO-d$_6$) δ 157.66, 156.35, 153.13, 139.27, 138.45, 135.10, 131.27, 130.31, 128.45, 126.44, 123.42, 122.10, 120.42, 120.05, 106.99, 98.12, 55.50, 47.20, 40.46, 27.09.
$^{1}H$ spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxyaniline, 15:

$^{1}H$ NMR (600 MHz, Chloroform-d) δ 7.69 (d, J = 7.8 Hz, 1H), 7.57 (t, J = 8.4 Hz, 2H), 7.56 (d, J = 8.9 Hz, 1H), 7.50 (dd, J = 7.9, 1.5 Hz, 1H), 7.45 (dd, J = 8.0, 1.8 Hz, 1H), 6.86 (s, 1H), 6.46 (s, 1H), 3.98 – 3.89 (m, 2H), 3.87 (s, 3H), 3.75 (s, 3H), 1.51 (s, 6H).

$^{13}C$ spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxyaniline, 15:

$^{13}C$ NMR (151 MHz, CDCl$_3$) δ 155.90, 152.93, 151.15, 141.57, 138.49, 138.27, 136.89, 136.17, 129.98, 128.39, 126.04, 123.62, 121.17, 120.58, 120.20, 119.52, 113.77, 110.35, 56.37, 56.34, 47.08, 27.05.
\textsuperscript{1}H spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 16:

\textsuperscript{1}H NMR (600 MHz, DMSO-\textit{d}_6): \delta 7.78 – 7.71 (m, 3H), 7.55 (d, \textit{J} = 1.6 Hz, 1H), 7.49 (dd, \textit{J} = 8.0, 1.9 Hz, 1H), 7.41 (dd, \textit{J} = 7.9, 1.6 Hz, 1H), 7.17 (d, \textit{J} = 9.1 Hz, 1H), 6.38 (d, \textit{J} = 1.2 Hz, 1H), 6.38 (d, \textit{J} = 3.5 Hz, 1H), 3.77 (s, 3H), 2.94 (s, 6H), 1.44 (s, 6H).

\textsuperscript{13}C spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 16:

\textsuperscript{13}C NMR (151 MHz, DMSO-\textit{d}_6): \delta 157.46, 156.36, 153.18, 151.75, 138.92, 138.40, 135.33, 131.13, 130.33, 128.50, 126.45, 123.49, 122.15, 120.37, 120.13, 118.45, 105.42, 96.91, 55.75, 47.22, 40.61, 27.09.
\(^1\)H spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 17:

\(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) 7.71 (d, \(J = 7.8\) Hz, 1H), 7.59 (s, 1H), 7.58 (d, \(J = 6.0\) Hz, 1H), 7.56 (d, \(J = 1.8\) Hz, 1H), 7.52 (dd, \(J = 7.8, 1.6\) Hz, 1H), 7.46 (dd, \(J = 8.0, 1.8\) Hz, 1H), 6.90 (s, 1H), 6.66 (s, 1H), 3.90 (s, 3H), 3.79 (s, 3H), 2.87 (s, 6H), 1.51 (s, 6H).

\(^1\)H spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 17:

\(^{13}\)C spectrum of 4-(7-bromo-9,9-dimethyl-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 17:

\(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 133.90, 152.98, 150.68, 146.80, 142.50, 138.17, 136.54, 130.02, 129.30, 128.36, 127.99, 126.06, 123.65, 121.23, 120.72, 119.58, 114.19, 105.96, 56.68, 56.01, 47.70, 43.20, 27.04.

\(^1\)H spectrum of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 18:
$^{13}$C spectrum of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-3-methoxy-N,N-dimethylaniline, 18:

$^{13}$C NMR (151 MHz, CDCl$_3$) & 157.37, 153.96, 152.96, 151.36, 142.42, 138.54, 136.77, 133.83, 131.27, 128.61, 128.15, 123.54, 119.81, 119.14, 105.18, 96.58, 83.64, 83.46, 55.50, 46.79, 40.66, 27.13, 25.00, 24.90.

$^1$H spectrum of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 19:
$^{13}$C NMR (151 MHz, CDCl$_3$) δ 154.04, 152.98, 150.68, 146.61, 142.44, 142.17, 138.16, 137.40, 133.86, 128.66, 128.15, 123.94, 123.67, 119.97, 119.25, 114.18, 104.00, 93.69, 86.71, 55.99, 46.85, 43.22, 27.11, 24.99, 24.99.

$^{13}$C NMR (600 MHz, Chloroform-d) δ 7.89 (d, $J = 1.1$ Hz, 1H), 7.83 (dd, $J = 7.5, 1.0$ Hz, 1H), 7.77 (dd, $J = 7.8$ Hz, 1H), 7.74 (d, $J = 7.5$ Hz, 1H), 7.62 (d, $J = 1.6$ Hz, 1H), 7.52 (dd, $J = 7.8$, 1.6 Hz, 1H), 6.92 (s, 1H), 6.67 (s, 1H), 3.91 (s, 3H), 3.79 (s, 3H), 2.87 (s, 6H), 1.39 (s, 12H), 1.27 (s, 6H).

$^{13}$C spectrum of 4-(9,9-dimethyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9H-fluoren-2-yl)-2,5-dimethoxy-N,N-dimethylaniline, 19:
$^1$H spectrum of 6 (fVF 1):

$^1$H NMR (900 MHz, DMSO-d$_6$): 8.87 (d, $J = 2.0$ Hz, 1H), 8.00 (d, $J = 7.7$ Hz, 1H), 7.98 (d, $J = 2.0$ Hz, 1H), 7.95 (dd, $J = 7.7, 7.8$ Hz, 1H), 7.90 (d, $J = 7.8$ Hz, 1H), 7.81 (d, $J = 1.7$ Hz, 1H), 7.80 (dd, $J = 7.8, 1.7$ Hz, 1H), 7.65 (d, $J = 8.2$ Hz, 2H), 7.61 (dd, $J = 7.7, 1.7$ Hz, 1H), 7.38 (d, $J = 7.6$ Hz, 1H), 7.25 – 7.07 (m, 2H), 7.02 (s, 2H), 6.84 (d, $J = 8.1$ Hz, 2H), 2.97 (s, 6H), 1.59 (s, 6H).

$^{13}$C spectrum of 6 (fVF 1):

$^{13}$C NMR (226 MHz, DMSO): 157.99, 157.86, 157.77, 157.59, 154.57, 154.52, 151.88, 149.88, 147.47, 141.48, 139.91, 138.60, 137.68, 135.95, 130.36, 129.79, 128.89, 128.02, 127.64, 127.27, 126.61, 125.40, 124.67, 121.22, 120.75, 120.62, 119.90, 119.25, 117.93, 116.60, 115.28, 112.68, 79.11, 78.97, 78.82, 46.78, 29.09, 26.93.
**$^1$H spectrum of 7 (fVF 0):**

$^1$H NMR (900 MHz, DMSO-d$_6$) δ 8.31 (d, $J$ = 2.0 Hz, 1H), 8.02 (d, $J$ = 4.7, 3.0 Hz, 2H), 7.97 (d, $J$ = 7.8 Hz, 1H), 7.95 (dd, $J$ = 7.7, 2.0 Hz, 1H), 7.90 (d, $J$ = 1.7 Hz, 1H), 7.81 (d, $J$ = 7.7, 1.7 Hz, 1H), 7.78 – 7.75 (m, 2H), 7.68 (d, $J$ = 7.7, 1.7 Hz, 1H), 7.49 (t, $J$ = 7.6 Hz, 2H), 7.38 (s, $J$ = 7.9 Hz, 2H), 7.02 (s, 2H), 6.77 (s, 2H), 1.60 (s, 6H).

**$^{13}$C spectrum of 7 (fVF 0):**

$^{13}$C NMR (226 MHz, DMSO-d$_6$) δ 158.11, 157.96, 157.81, 157.66, 154.55, 154.48, 151.67, 147.28, 141.28, 140.31, 139.90, 138.82, 137.97, 137.25, 130.22, 129.62, 128.75, 127.57, 127.17, 126.67, 125.93, 125.74, 125.31, 121.16, 121.07, 120.81, 120.72, 117.01, 115.71, 113.24, 103.15, 48.42, 46.74, 39.77, 39.67, 26.67.
$^1$H spectrum of 20 (fVF 2):

$^1$H NMR (800 MHz, DMSO-d$_6$) δ 8.31 (s, 1H), 7.98 (s, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.86 (dd, J = 7.7 Hz, 2H), 7.78 (d, J = 7.6 Hz, 1H), 7.62 (s, 1H), 7.46 (d, J = 7.6 Hz, 1H), 7.23 (dd, J = 19.5, 7.9 Hz, 1H), 6.79 (s, 1H), 6.41 (s, 1H), 6.17 (d, J = 6.8 Hz, 1H), 3.81 (s, 1H), 2.97 (s, 1H), 1.56 (s, 6H).

$^{13}$C spectrum of 20 (fVF 2):

$^{13}$C NMR (226 MHz, DMSO) δ 173.31, 173.27, 157.04, 156.43, 154.46, 153.43, 152.09, 151.28, 147.28, 140.66, 138.57, 138.19, 137.88, 135.57, 130.73, 130.67, 129.39, 128.64, 128.02, 126.86, 126.61, 126.55, 125.89, 125.56, 123.12, 121.15, 120.51, 119.69, 118.13, 108.99, 108.95, 105.00, 102.68, 102.64, 96.47, 64.90, 55.33, 46.64, 40.20, 26.98, 15.16.
$^1$H spectrum of 21 (fVF 3):

$^1$H NMR (900 MHz, DMSO-d$_6$) δ 8.31 (s, 1H), 8.02 (s, 1H), 8.01 (d, $J = 7.1$ Hz, 1H), 7.86 (s, 1H), 7.92 (d, $J = 7.8$ Hz, 1H), 7.70 (s, 1H), 7.83 (d, $J = 7.6$ Hz, 1H), 7.52 (d, $J = 7.6$ Hz, 1H), 7.38 (d, $J = 7.6$ Hz, 1H), 7.25 (d, $J = 7.4$ Hz, 1H), 7.17 (d, $J = 7.5$ Hz, 1H), 7.14 (d, $J = 7.3$ Hz, 1H), 7.02 (s, 2H), 6.79 (s, 1H), 3.89 (s, 3H), 3.79 (s, 3H), 2.94 (s, 6H), 1.55 (s, 6H).

$^{13}$C spectrum of 21 (fVF 3):

$^{13}$C NMR (226 MHz, DMSO) δ 154.60, 153.53, 151.83, 150.47, 149.66, 147.49, 145.47, 141.42, 138.66, 137.93, 137.32, 130.35, 129.77, 129.47, 128.87, 128.29, 128.18, 128.00, 127.68, 127.24, 126.37, 126.18, 126.03, 125.42, 125.29, 125.62, 123.56, 121.31, 121.27, 120.80, 119.91, 115.86, 115.84, 115.83, 108.40, 103.35, 103.31, 56.40, 46.74, 45.66, 26.89, 21.02.
LC-MS of 6 (fVF 1):

Intensity DAQ: Signal B, 254 nm/44 nm Ref 700 nm/8= 50 nm
FVF1-10-100.datx 2018.06.11 19:57:53 ;

Intensity DAQ: Signal E, 480 nm/44 nm
FVF1-10-100.datx 2018.06.11 19:57:53 ;

Intensity DAQ: Signal E, 5.26 - 5.67 (63 scans)
FVF1-10-100.datx 2018.06.11 19:57:53 ;
ESI+

\([\text{M+H+ACN}]^{2+}\)
\([\text{M+2H}]^{2+}\)
\([\text{M+H}]^{+} = 748.1\)
LC-MS of 7 (fVF 0):

DAD: Signal B, 254 nm/B=4 nm Ref 700 nm/B=50 nm
PVFO_30-80.datx 2018.06.11 20:38:24 ;

DAD: Signal C, 450 nm/B=4 nm Ref 700 nm/B=50 nm
PVFO_30-80.datx 2018.06.11 20:38:24 ;

Spectrum RT 9.17 - 9.63 (69 scans)
PVFO_30-80.datx 2018.06.11 20:38:24 ;
ESI +

[M+H]^+
LC-MS of 20 (fVF 2):

DAD: Signal B, 254 nm B=4 nm Ref 760 nm B=50 nm
FVF2_10-100.datx 2018.06.11 19:10:45

DAD: Signal E, 480 nm B=4 nm
FVF2_10-100.datx 2018.06.11 19:10:45

Spectrum RT 5.37 - 5.87 (45 scans)
PVF2_10-100.datx 2018.06.11 19:10:45
ESI +

\[ [M+H+ACN]^2+ \]
\[ [M+2H]^2+ \]
\[ [M+H]^+ \]
8. References


