Supporting information for

A rationally-designed, general strategy for membrane orientation of photoinduced electron transfer-based voltage-sensitive dyes

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General method for chemical synthesis and characterization

Chemical reagents and anhydrous solvents were purchased from commercial suppliers and used without further purification. All reactions were carried out in oven-dried flasks under an inert atmosphere of N_2. Thin layer chromatography (TLC) (Silicycle, F254, 250 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 a Bruker AV-600 MHz, 150 MHz. Chemical shifts are expressed in parts per million (ppm) and are referenced to d_6-DMSO, 2.50 ppm. Coupling constants are reported as Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; sep, septet dd, doublet of doublet; ddd, doublet of double of doublet; dt, doublet of triplet; td, triplet of doublet; m, multiplet. High-resolution mass spectra (ESI EI) 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. Columns used for the analytical and semi-preparative HPLC were Phenomenex Luna C18(2) (4.6 mm I.D. × 150 mm) and Phenomenex Luna 5μm C18(2) (10 mm I.D. x 150 mm) columns with a flow rate of 1.0 and 3.0 mL/min, respectively. The mobile phase were MQ-H2O with 0.05% formic acid (eluent A) and HPLC grade acetonitrile with 0.05% formic acid (eluent B). Signals were monitored at 254 and 460 nm in 20 min with gradient 5-100% eluent B.

Spectroscopic studies

Stock solutions of VF dyes were prepared in DMSO (1.0–10 mM) and diluted with PBS (100 mM Na_2HPO_4, pH 7.4, 0.1% Triton-X). 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).

Differentiation of mDA neurons

mDA neurons were derived from H1 human embryonic stem cells (hESCs) using medium conditions adapted from previously established protocols.[1] On Day 25 of differentiation, cells were harvested from the culture platform and pipetted to generate small 50-100μm clusters, and seeded on 12mm glass coverslips coated with 20μg/ml Laminin (ThermoFischer Scientific). Cells were subsequently matured for 10 days before voltage sensitive imaging.

Voltage sensitivity in HEK cells

Functional imaging of VF dyes was performed using a 20x objective paired with image capture from the EMCCD camera at a sampling rate of 0.5 kHz. VF dyes were excited using the 488 nm LED with an

intensity of 2.5 W/cm^2. For initial voltage characterization emission was collected with the QUAD filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP).

Voltage imaging of neurons

Functional imaging of mDA cells was performed using a 20x objective paired with image capture from the ORCA-Flash4.0 camera (Hamamatsu) at a sampling rate of 100 Hz. VoltageFluor dye was excited using the 488 nm LED with an intensity of 2.5 W/cm^2. Light was collected with the QUAD filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). For image analysis, a custom Matlab routine ("SpikeMapper", which is available upon request, please email evanwmiller@berkeley.edu) was employed. In summary, a TIFF stack was imported into Matlab and the intensity values a user-designated block of pixels was extracted as an array. The intensity over time for each block was then baseline corrected by fitting to a 2nd degree polynomial. Cells were selected by drawing regions of interest around cells in a DIC image corresponding to the video, outputting the bleach-corrected trace as an Excel file and spike times (frames in which the intensity was greater than 3x the standard deviation of the overall trace) as a text file.

Determination of Photostability

HEK cells were incubated separately with ms- and ds-VF2.1.Cl and VF2.2(OMe).Cl at 1μM in HBSS at 37 degrees Celsius for 15 minutes. Data were acquired with a W-Plan-Apo 63x/1.0 objective (Zeiss) and the ORCA-Flash4.0 camera (Hamamatsu). Images were exposed for 10 milliseconds each across 50 seconds with a constant illumination of the 488 nm LED (153 W/cm^2). The excitation light was collected with the QUAD filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). The fluorescence curves were background subtracted and then normalized to the fluorescence intensity at t = 0 and averaged across five cells of each dye.

Electrophysiology

For electrophysiological experiments, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 5–8 MΩ, and were filled with an internal solution; (in mM) 115 potassium gluconate, 10 BAPTA tetrapotassium salt, 10 HEPES, 5 NaCl, 10 KCl, 2 ATP disodium salt, 0.3 GTP trisodium salt (pH 7.25, 275 mOsm).

Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with Digidata 1332A, 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 HEK 293T cells, cells were held at -60 mV and 100 ms hyper- and de-polarizing steps applied from -100 to +100 mV in 20 mV increments. For whole-cell, following membrane rupture, resting membrane potential was assessed and recorded at I = 0 and monitored during the data acquisition.

Image analysis

Analysis of voltage sensitivity in HEK cells was performed using custom Matlab routines. Briefly, a region of interest (ROI) was selected automatically based on fluorescence intensity and applied as a mask to all image frames. Fluorescence intensity values were calculated at known baseline and voltage step epochs.

Solubility of VoltageFluor dyes

Suspensions of 5 milligrams each of solid mono- and disulfo-dichlorofluoresceins in 5 mL of distilled water were created. Over the course of three days, 5 milligrams of solid material was added at a time, ensuring that no visible particulates were present in the solution prior to addition. At the end of the experiment, the suspension was filtered through filter paper and then through a 0.22-μm polytetrafluoroethylene filter into a scintillation vial. 10 μL of this filtrate was used as a stock solution to determine the concentration of

fluorescein present via UV-Vis spectrophotometry and the remainder was lyophilized and the amount of solid resulting was measured using an analytical microbalance. For VF dyes (VF2.1.Cl and VF2.2(OMe).Cl, both ms and ds versions), saturated solutions in distilled water were prepared via dilution from a 100 µM stock in DMSO for a resulting solution containing 0.1% DMSO. The solutions were allowed to sit overnight at 20 degrees Celsius and then filtered through 0.22-µm filters. The resulting filtrates were used as stock solutions to determine the amount of VF present via UV-Vis spectrophotometry.

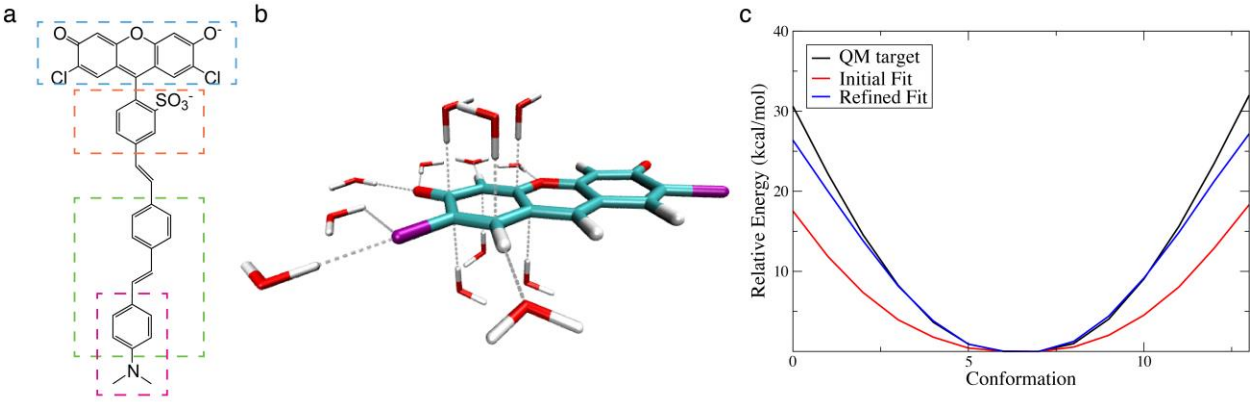
Molecular dynamics simulation parameters and protocols

The msVF and dsVF molecules were parameterized in CHARMM General force field (CGenFF).[2] The CGenFF program[2,3] was used to obtain initial parameters, which were subsequently optimized with the Force Field Toolkit (fftk)[4]and the program Gaussian 09.[5] Briefly, an entire VF molecule is divided into four parts during the parameterization (**SI Figure 1**). As benzenesulfonate and N,N-dimethylaniline were already in the CHARMM general force field, only the remaining two parts (xanthene derivative and (E)-stilbene) were submitted to the CGenFF program. Based on the resulting penalty scores, two dihedrals in (E)-stilbene were selected and optimized via fftk and Gaussian 09 following protocols outlined in ref [6]. For the -1 charged xanthene derivative, its atom typing and initial charge assignment was not based on the CGenFF program output. Instead, we first performed its geometry optimization at the MP2/6-31+G* level via Gaussian 09, which confirmed a symmetric and planar structure of the tricyclic ring, consistent with a previous density functional theory calculation.[7] We then assigned atom type and initial charges for the xanthene derivative based on the compounds phenoxazine, phenoxide and chlorobenzene from the CHARMM general force field and performed charge and dihedral optimization (**SI Figure 1**) following protocols outlined in ref.[6]. The final topology and parameter files for both VF molecules are provided in the supporting information.

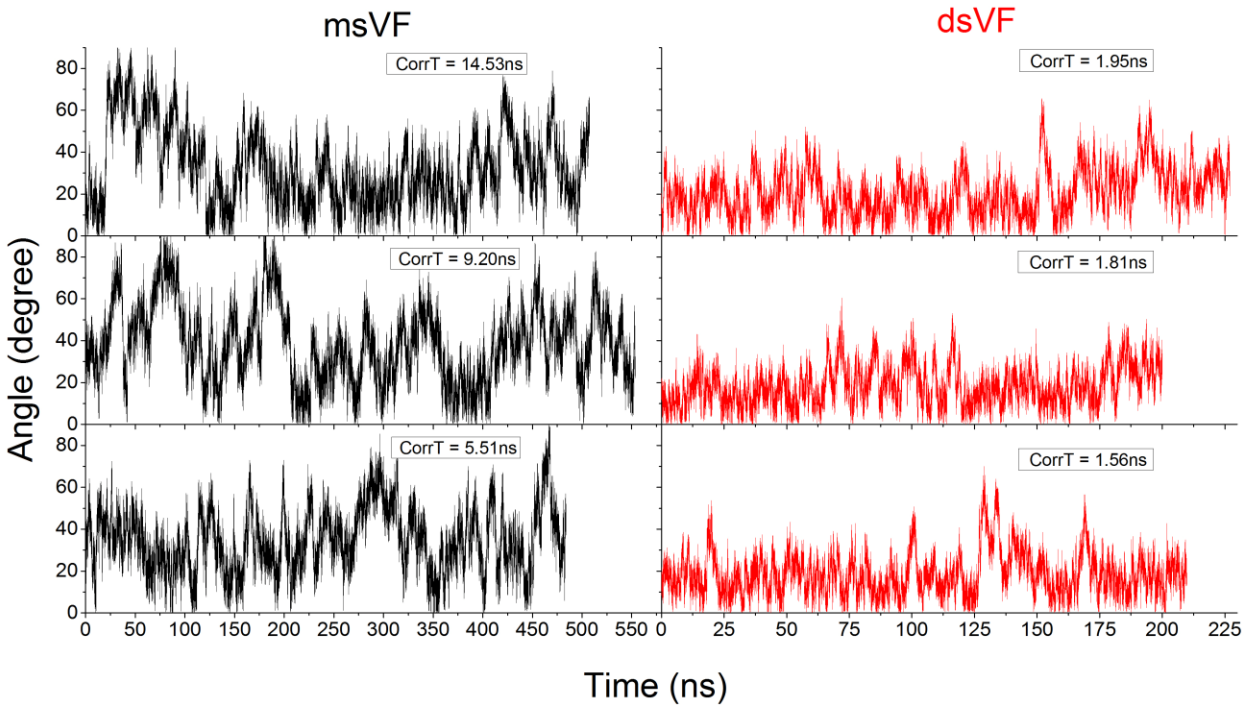
MD simulation system with either a msVF or a dsVF molecule was constructed by inserting it vertically inside the upper monolayer of a POPC bilayer, which was previously equilibrated in a 1-µs simulation[8] performed on the specialized machine Anton.[9] In each system, one lipid molecule overlapping with the inserted msVF or dsVF was removed. Using the autoionize plugin of VMD,[10] the systems were neutralized by adding sodium and chloride ions at a concentration of 0.1 mol/L. Final simulation systems contain ~46,000 atoms, with a size of approximately 75 x 75 x 73 $Å^3$. Three replicas of ~500 ns simulations were performed to examine the orientation of msVF in the POPC bilayer, while three replicas of ~200 ns simulations were performed for the dsVF molecule in POPC. The longer simulation time for the former VF molecule is chosen based on its longer correlation time shown in **SI Figure 2**.

All simulations were performed with the 2.10 release of NAMD[11] and the CHARMM36 force field for lipids[12] as well as the CGenFF force field.[6] A time step of 2 fs was adopted in all simulations, with bonds involving hydrogen atoms constrained using RATTLE[13] and water geometries maintained using SETTLE.[14] The multiple-time-stepping algorithm was used, with short-range forces calculated every step and long-range electrostatics calculated every two steps. The cutoff for short-range nonbonded interactions was set to 12 Å, with a switching distance of 10 Å. The CHARMM force switching was used for vdW forces, in order to be consistent with the CHARMM36 force field for lipids. Assuming periodic boundary conditions, the Particle Mesh Ewald (PME) method[15] with a grid density of at least $1/Å^3$ was employed for computation of long-range electrostatic forces. Langevin dynamics with a damping coefficient of 1 ps^{-1} was used to keep the temperature constant at 310 K, while a Nosé–Hoover–Langevin piston[16] was used to keep the pressure constant at 1 atm. The pressure control was performed semi-isotropically: the z axis of the simulation box, which is normal to the membrane, was allowed to fluctuate independently from the x and y axes.

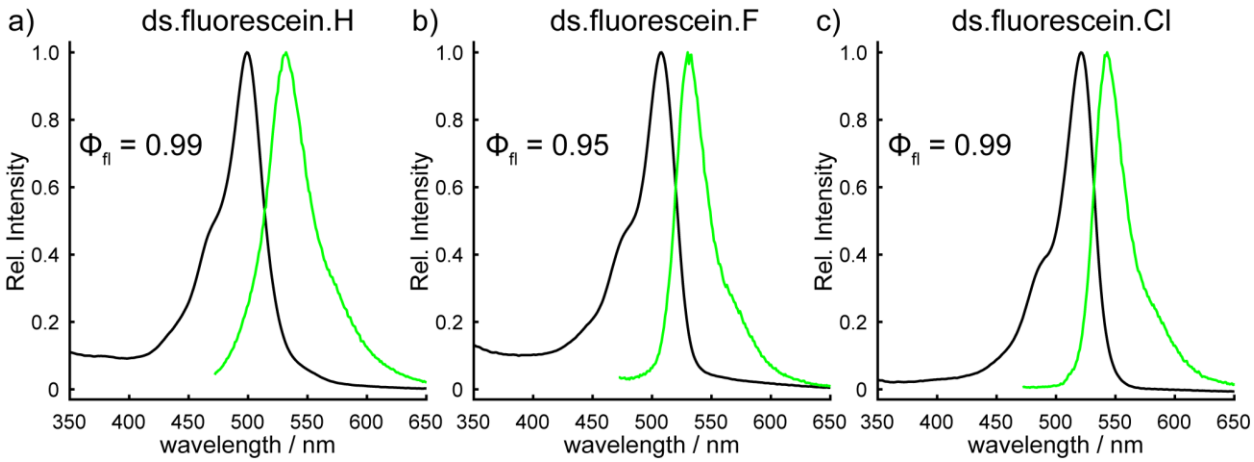
Supporting Figures



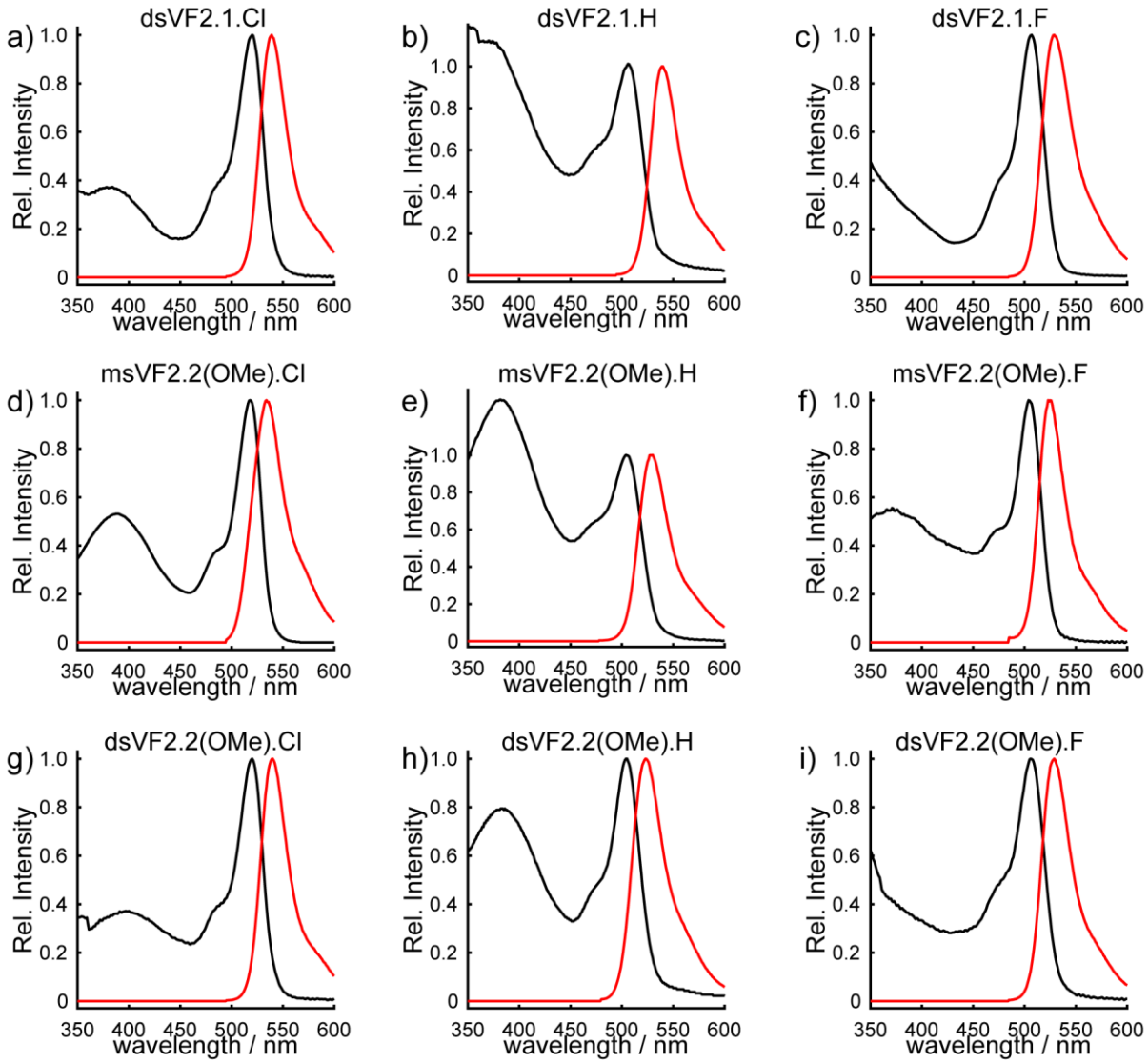
SI Figure 1. Parameterization of the msVF molecule in CHARMM general force field. (a) Division of msVF into four parts: xanthene derivative (blue), benzenesulfonate (orange), (E)-stilbene (green), and N,N-dimethylaniline (magenta). (b) Interaction energy calculation between atoms on the xanthene derivative and a TIP3p water. The calculation was performed at the MP2/6-31G* level for the two chlorine atoms (colored in purple) and at the HF/6-31G* level for the remaining ones. Due to the symmetry of the compound, calculation was only performed on half of the atoms. (c) Optimization of the dihedral OG312-CG2R61-CG2R61-CLGR1 (see topology and parameter files of msVF in the SI) through potential energy scan performed via fftk and Gaussian 09.



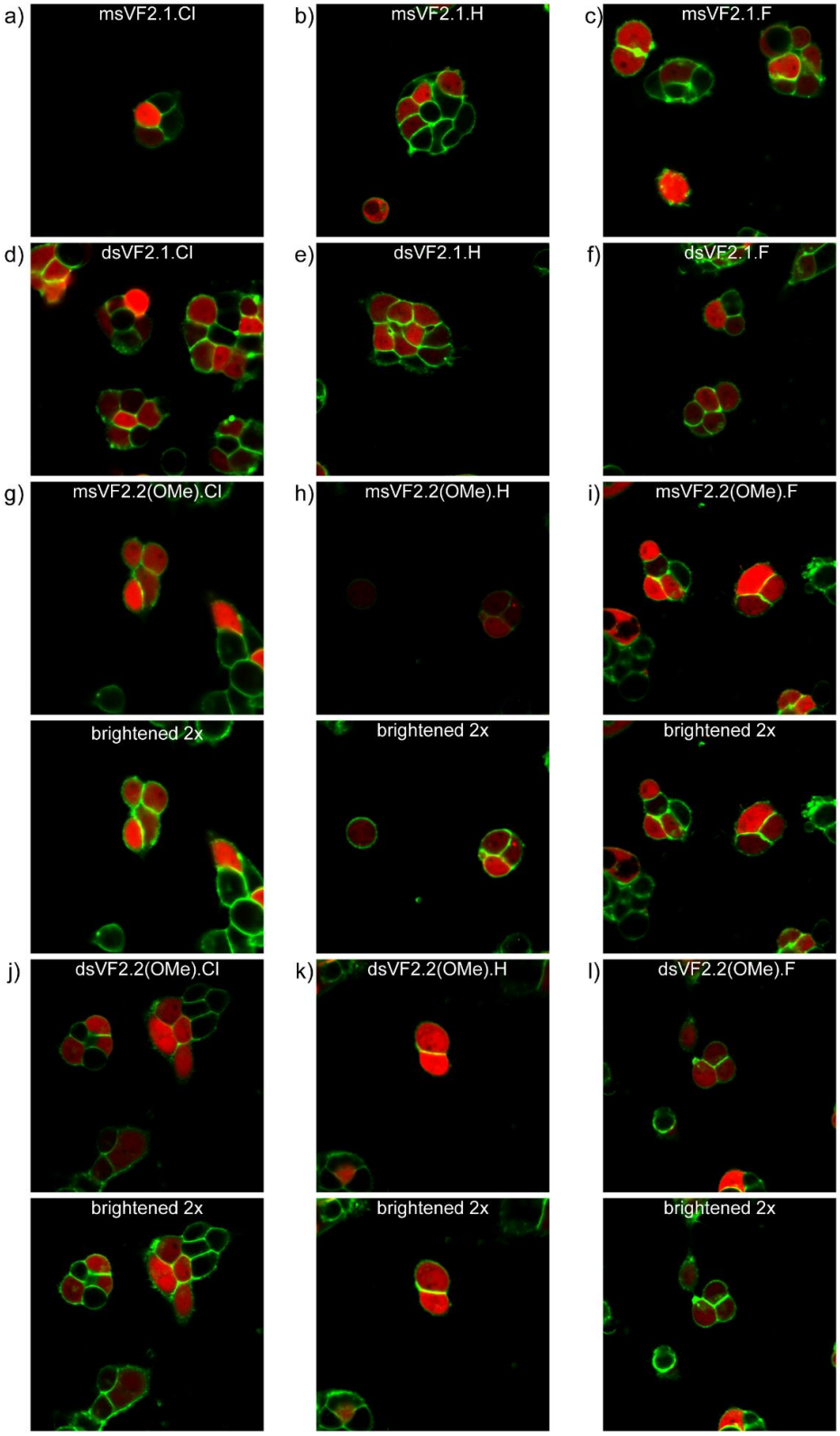
SI Figure 2. Time traces of the tilt angle measured from the three replicas of msVF (left) and dsVF (right) simulations. The correlation time (corrT) of the angle as determined from each simulation is labeled.



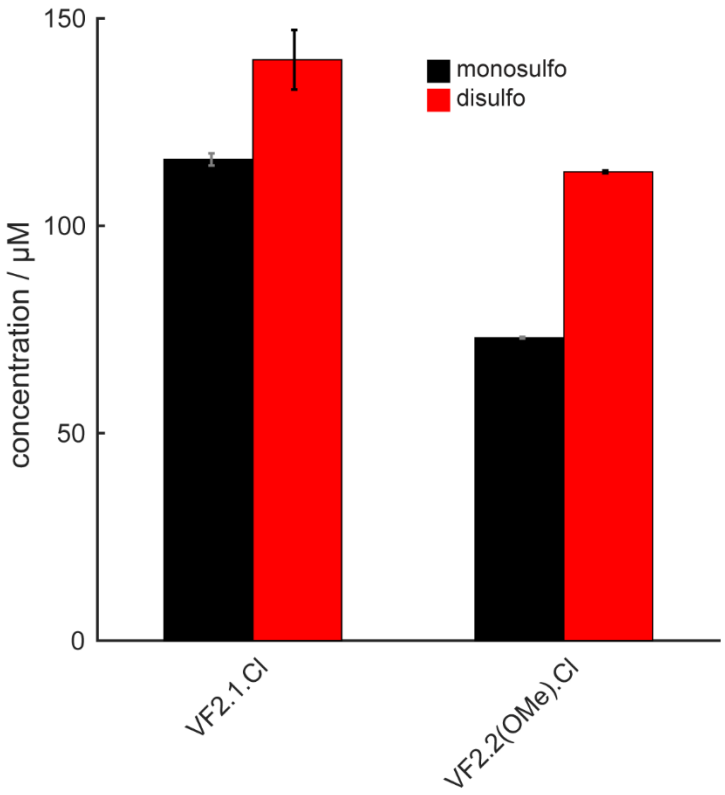
SI Figure 3. Absorption and emission spectra for disulfofluoresceins. Spectra were acquired in phosphate-buffered saline, pH 7.4 + 0.1% Triton X-100.



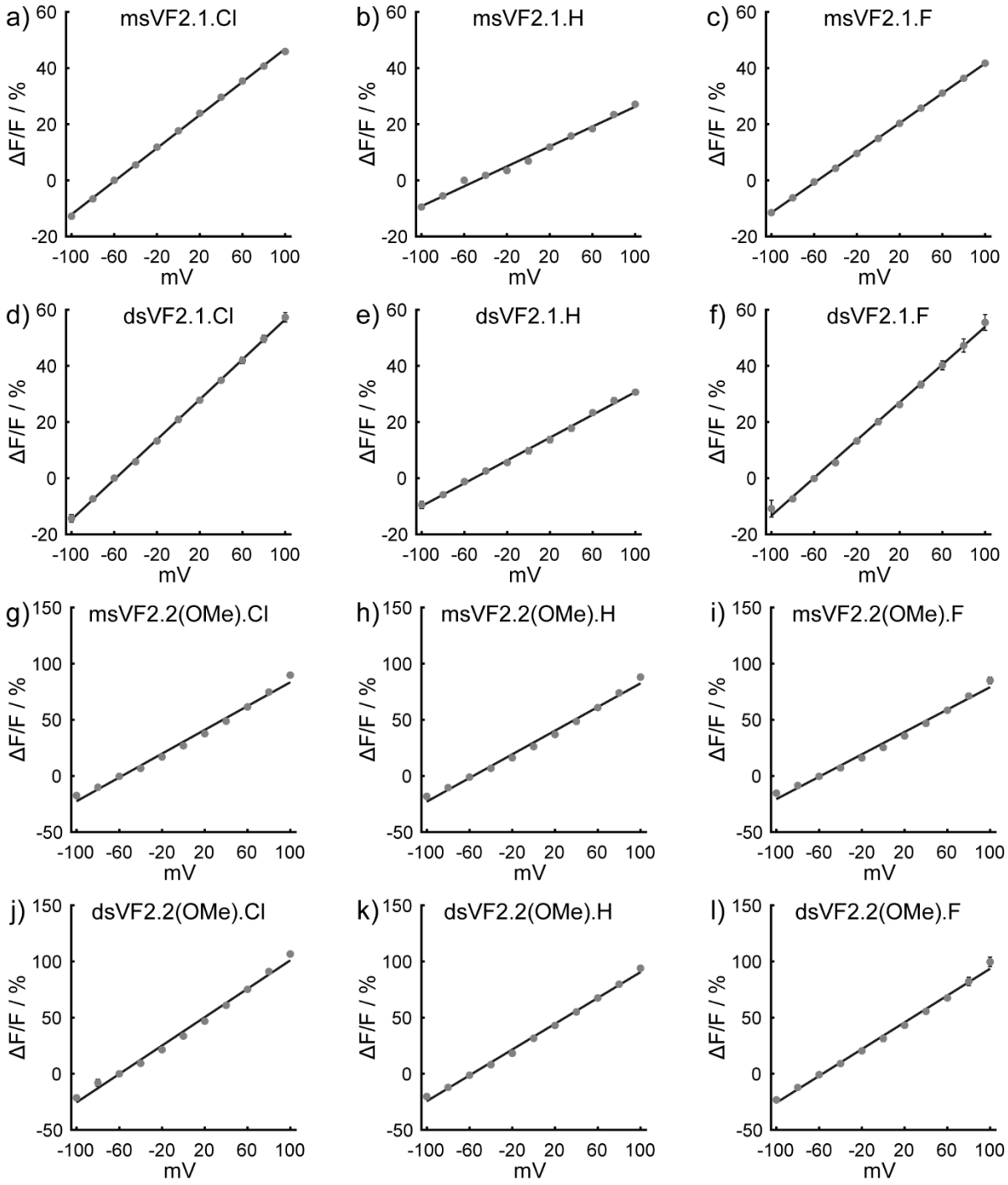
SI Figure 4. Absorption and emission spectra for msVF and dsVF dyes obtained in phosphate-buffered saline, pH 7.4 + 0.1% Triton X-100 as a surfactant.



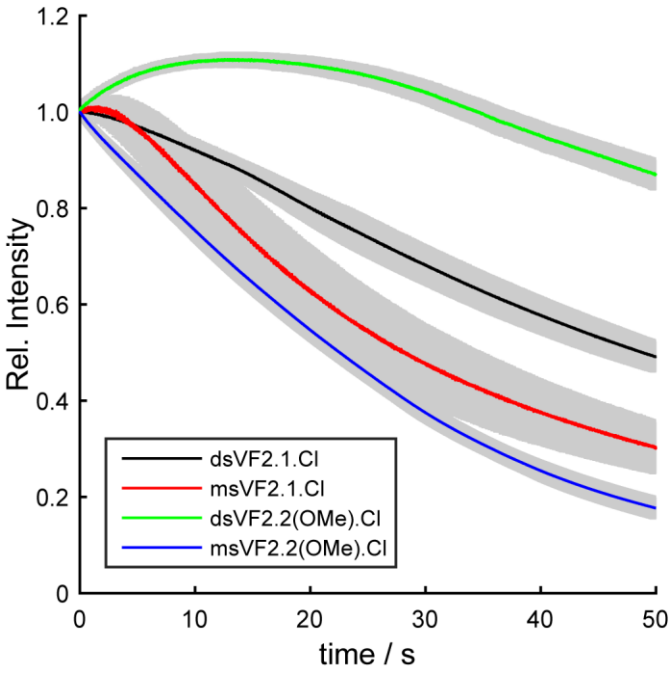
SI Figure 5. Laser-scanning confocal micrographs of HEK cells stained with the indicated msVF or dsVF dye. HEK cells also express cytosolic mCherry as marker of cytosol. The juxtaposition of red and green signals enables clear discrimination of membrane-localized staining of VF dyes.



SI Figure 6. Solubility of msVF and dsVF derivatives in distilled water with 0.1% DMSO as determined by UV/Vis spectrophotometry. Data are from three trials for each dye. Error bars are ± SEM.



SI Figure 7. Voltage sensitivity of VF dyes in HEK cells. The fractional change in fluorescence is plotted vs. final membrane potential for 100 ms hyper- and depolarizing steps (±100 mV, 20 mV increments) from a holding potential of -60 mV for a single HEK cells under whole-cell voltage-clamp mode. Data are from between 3 and 8 individual cells for each point. Error bars are ±SEM



SI Figure 8. Bleaching curves for VoltageFluor dyes in HEK cells. The fractional change in fluorescence is plotted vs. time for each of the dyes under constant 488 nm illumination. Data are from 5 individual regions of interest. Shaded areas represent ± standard deviation.

Supporting Movie Captions

Web Enhanced Object, Movie 1. Simulation trajectory of msVF in POPC bilayer. The 554-ns trajectory is shown every 0.5 ns and images are smoothed over a 10-frame window. For clarity, water molecules and ions are not shown.

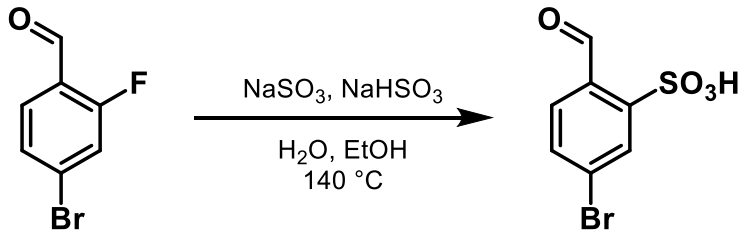
Web Enhanced Object, Movie 2. Simulation trajectory of dsVF in POPC bilayer. The 227-ns trajectory is shown every 0.5 ns and images are smoothed over a 10-frame window. For clarity, water molecules and ions are not shown.

SI Parameter file 1. CHARMM general force field topology and parameter file for msVF.

SI Parameter file 2. CHARMM general force field topology and parameter file for dsVF.

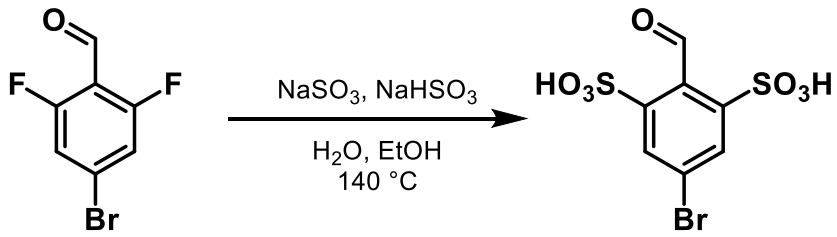
Synthetic Details

Preparation of formylbenzenesulfonic acids:



Synthesis of 5-bromo-2-formylbenzenesulfonic acid

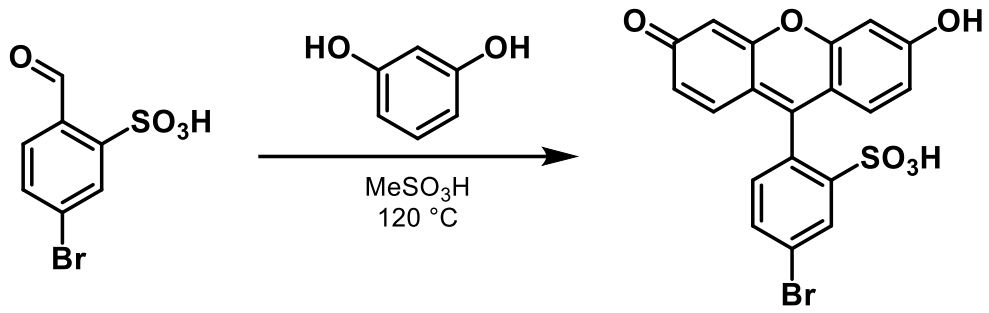
4-bromo-2-fluorobenzaldehyde (3.0 g, 14.78 mmol) was placed in a long-necked bomb flask and dissolved in a 1:1 mixture of ethanol and water. Sodium sulfite (1.49 g, 11.82 mmol) and sodium bisulfite (123 mg, 1.18 mmol) were added and the reaction was stirred for 16 hours at 140 degrees Celsius. The reaction mixture, after cooling, was poured into methanol while stirring so as to make 20% aqueous content of the whole volume. This process precipitated the inorganic salts, which were then removed by vacuum filtration. The solvent from the filtrate was removed under reduced pressure to obtain a solid residue, which was triturated with methanol/ethyl ether to produce a fluffy white solid (2.82 g, 72%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 8.09 (d, J = 1.7 Hz, 1H), 7.72 (d, J = 7.9 Hz, 1H), 7.66 (dd, J = 7.4, 1.5 Hz, 1H). ^{13}C NMR (226 MHz, DMSO) δ 192.65, 151.31, 132.34, 131.46, 129.58, 128.88, 126.97. HR-ESI-MS m/z for $C_7H_4BrO_4S^-$ calcd: 262.9092 found: 262.9017.



Synthesis of 5-bromo-2-formylbenzene-1,3-disulfonic acid

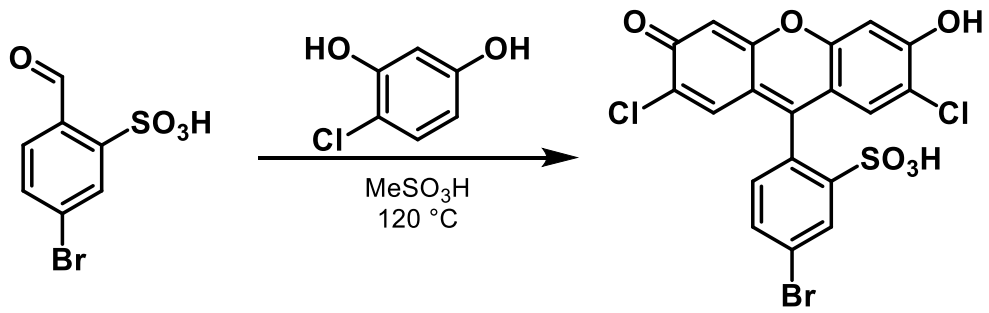
4-bromo-2,6-difluorobenzaldehyde (1.0 g, 4.52 mmol) was placed in a long-necked bomb flask and dissolved in 10 mL of a 1:1 mixture of ethanol and water. Sodium sulfite (1.14 g, 9.05 mmol) and sodium bisulfite (94 mg, 0.90 mmol) were added and the reaction was stirred for 16 hours at 140 degrees Celsius. The reaction mixture, after cooling, was poured into methanol while stirring so as to make 20% aqueous content of the whole volume. This process precipitated the inorganic salts, which were then removed by vacuum filtration. The solvent from the filtrate was removed under reduced pressure to obtain a solid residue, which was triturated with methanol/ethyl ether to produce a fluffy white solid (1.19 g, 76%), which was judged to be pure by NMR. 1H NMR (400 MHz, Methanol-d4) δ 10.62 (s, 1H), 8.12 (s, 2H). ^{13}C NMR (226 MHz, DMSO) δ 186.45, 165.53, 155.33, 130.84. HR-ESI-MS m/z for $C_7H_4BrO_7S_2^-$ calcd: 342.8660 found: 342.8596.

Preparation of fluorescein dyes:



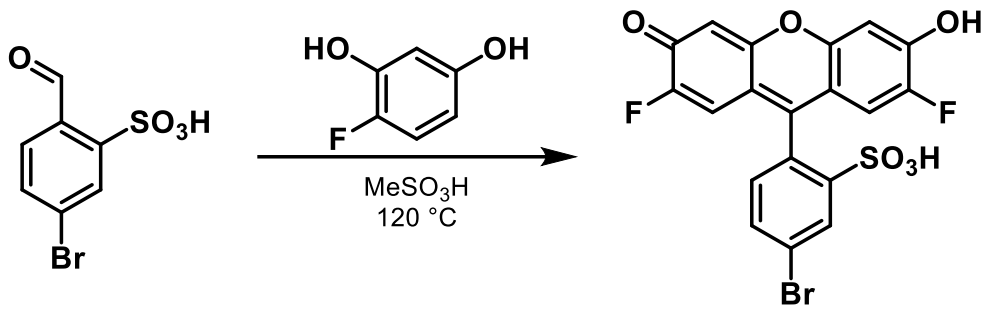
Synthesis of 5-bromosulfofluorescein

5-bromo-2-formylbenzenesulfonic acid (300 mg, 1.13 mmol) and resorcinol (249 mg, 2.26 mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was poured into 5 mL water, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (400 mg, 79%) and judged to be pure by NMR. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.07 (s, 1H), 7.83 (d, J = 6.8 Hz, 1H), 7.44 (d, J = 9.2 Hz, 2H), 7.33 (s, 2H), 7.27 (d, J = 8.3 Hz, 1H), 7.18 (d, J = 8.4 Hz, 2H). ^{13}C NMR (226 MHz, DMSO) δ 170.84, 167.13, 158.57, 158.47, 148.63, 134.74, 131.77, 130.21, 119.48, 117.07, 106.24, 102.51, 101.72. HR-ESI-MS m/z for $C_{19}H_{10}BrO_6S^-$ calcd: 444.9460 found: 444.9379.



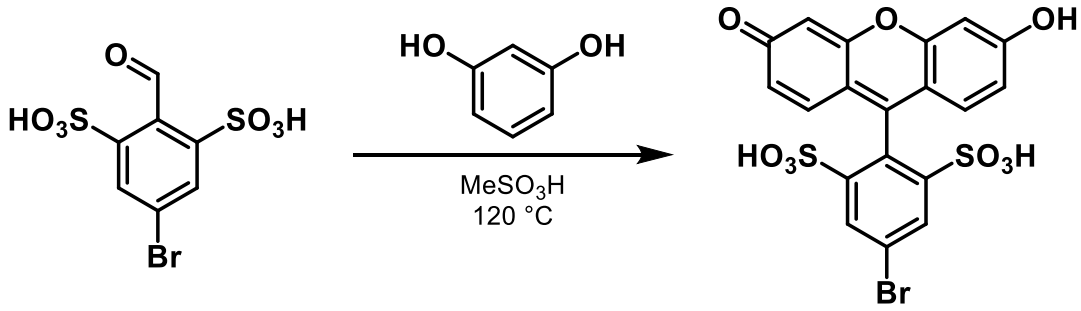
Synthesis of 5-bromo-2',7'-dichlorosulfofluorescein

5-bromo-2-formylbenzenesulfonic acid (250 mg, 0.94 mmol) and 4-chlororesorcinol (272 mg, 1.89 mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was poured into 5 mL water, resulting in precipitation of a reddish-brown solid. The solid was isolated via vacuum filtration (380 mg, 78%) and judged to be pure by NMR. 1H NMR (400 MHz, DMSO-d6) δ 8.06 (s, 1H), 7.75 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 8.1 Hz, 1H), 6.89 (s, 2H), 6.73 (s, 2H). ^{13}C NMR (226 MHz, DMSO) δ 157.13, 153.54, 131.98, 131.89, 130.20, 129.81, 129.62, 122.76, 115.78, 109.47, 107.39, 103.68, 103.32. HR-ESI-MS m/z for $C_{19}H_8BrCl_2O_6S^-$ calcd: 512.8680 found: 512.8598.



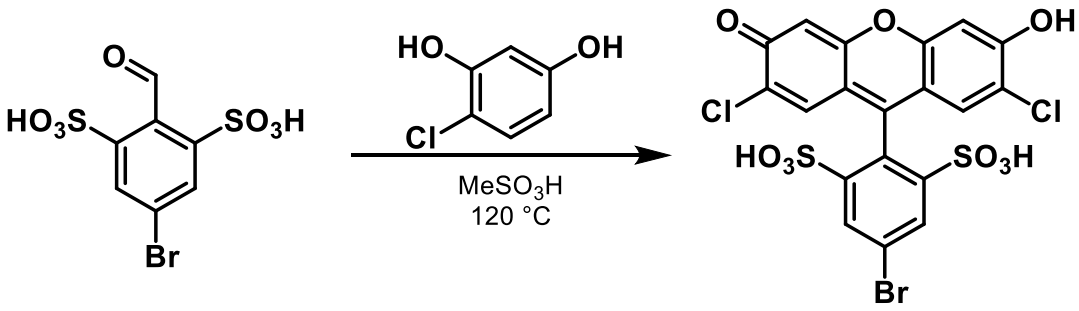
Synthesis of 5-bromo-2',7'-difluorosulfofluorescein

5-bromo-2-formylbenzenesulfonic acid (300 mg, 1.13 mmol) and 4-fluororesorcinol (290 mg, 2.26 mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was poured into 5 mL water, resulting in precipitation of a brown-black solid. The solid was isolated via vacuum filtration (463 mg, 85%) and judged to be pure by NMR. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (d, J = 2.0 Hz, 1H), 7.73 (d, J = 6.9 Hz, 1H), 7.45 (d, J = 8.2 Hz, 1H), 6.78 (d, J = 7.1 Hz, 2H), 6.58 (d, J = 11.4 Hz, 2H). ^{13}C NMR (226 MHz, DMSO) δ 153.57, 151.96, 150.85, 148.58, 132.07, 131.76, 130.29, 128.27, 122.86, 115.24, 113.54, 113.45, 104.26. HR-ESI-MS m/z for $C_{19}H_8BrF_2O_6S^-$ calcd: 480.9271 found: 480.9187.



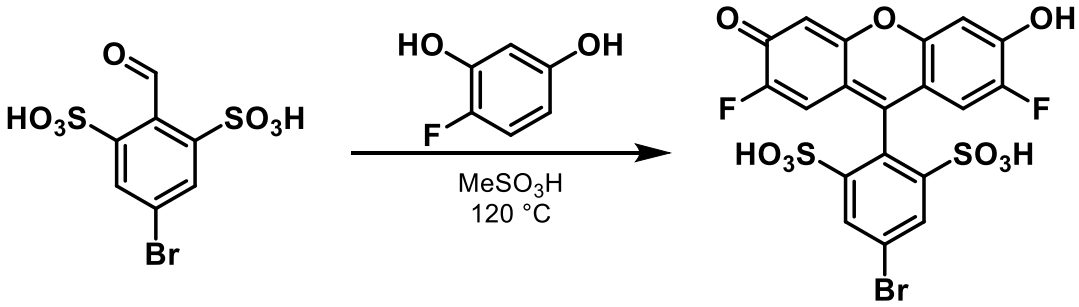
Synthesis of 5-bromodisulfofluorescein

5-bromo-2-formylbenzene-2,6-disulfonic acid (300 mg, 0.87 mmol) and resorcinol (191 mg, 1.74mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and poured into 50 mL diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (129 mg, 28%) and judged to be pure by NMR. 1H NMR (600 MHz, DMSO-d6) δ 8.11 (s, 2H), 7.36 (d, J = 9.2 Hz, 2H), 7.26 (s, 2H), 7.08 (d, J = 6.4 Hz, 2H). ^{13}C NMR (226 MHz, DMSO) δ 170.00, 157.81, 149.34, 149.32, 135.70, 133.36, 130.65, 123.69, 118.56, 118.17, 100.93. HR-ESI-MS m/z for $C_{19}H_{10}BrO_9S_2^-$ calcd: 524.9028 found: 524.8944.



Synthesis of 5-bromo-2',7'-dichlorodisulfofluorescein

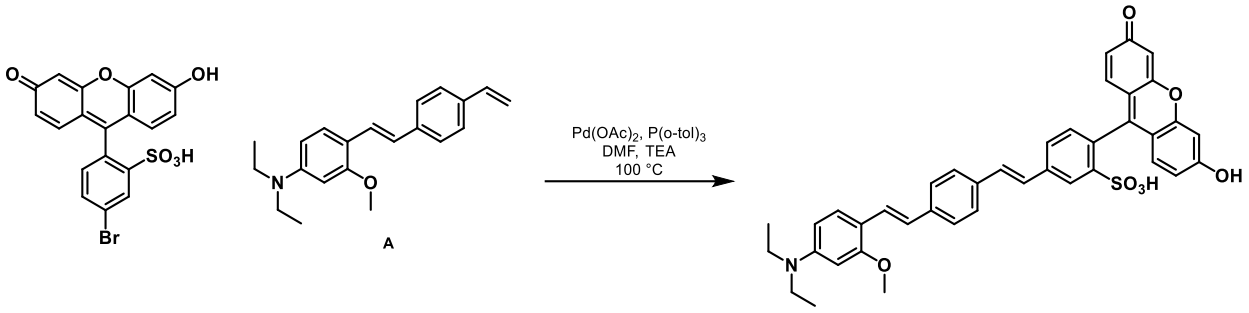
5-bromo-2-formylbenzene-2,6-disulfonic acid (250 mg, 0.72 mmol) and 4-chlororesorcinol (209 mg, 1.45 mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and poured into 50 mL diethyl ether, resulting in precipitation of a reddish-brown solid. The solid was isolated via vacuum filtration (200 mg, 46%) and judged to be pure by NMR. 1H NMR (600 MHz, DMSO-d6) δ 8.11 (s, 2H), 6.82 (s, 2H), 6.67 (s, 2H). ^{13}C NMR (226 MHz, DMSO) δ 155.04, 149.45, 131.49, 130.81, 124.17, 123.49, 122.48, 117.72, 102.47. HR-ESI-MS m/z for $C_{19}H_8BrCl_2O_9S_2^-$ calcd: 592.8248 found: 522.8157.



Synthesis of 5-bromo-2',7'-difluorodisulfofluorescein

5-bromo-2-formylbenzene-2,6-disulfonic acid (300 mg, 0.87 mmol) and 4-fluororesorcinol (222 mg, 1.74 mmol) were placed in a roundbottom flask dissolved in 3 mL of neat methanesulfonic acid and stirred for 16 hours at 120 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and poured into 50 mL diethyl ether, resulting in precipitation of a reddish-brown solid. The solid was isolated via vacuum filtration (330 mg, 67%) and judged to be pure by NMR. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.11 (s, 2H), 7.16 (d, J = 6.7 Hz, 2H), 6.84 (d, J = 11.1 Hz, 2H). ^{13}C NMR (226 MHz, DMSO) δ 161.45, 161.37, 154.30, 151.48, 150.37, 149.45, 131.27, 124.34, 123.26, 118.05, 118.01, 116.10, 116.01, 103.66. HR-ESI-MS m/z for $C_{19}H_8BrF_2O_9S_2^-$ calcd: 560.8839 found: 560.8752.

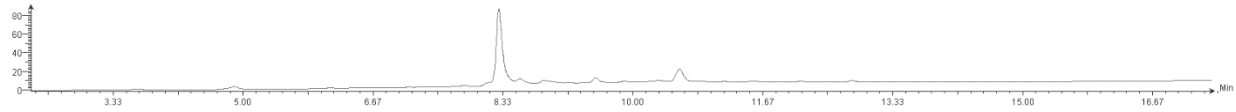
Preparation of VoltageFluor dyes:

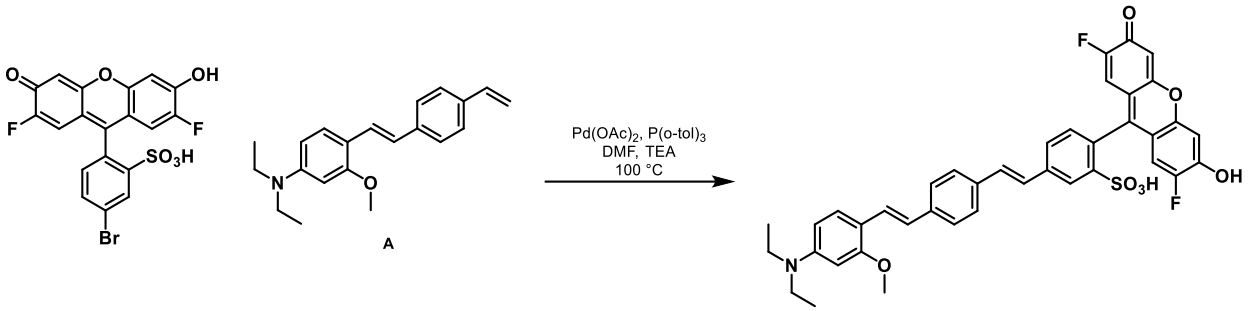


Synthesis of monosulfoVF2.2(OMe).H

5-bromosulfofluorescein (100 mg, 0.22 mmol), **A** (83 mg, 0.27 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (66 mg, 44%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.15 (s, 2H), 7.94 (s, 2H), 7.75 (d, J = 8.2 Hz, 2H), 7.64 (d, J = 8.0 Hz, 2H), 7.50 (d, J = 7.9 Hz, 4H), 7.38 (d,14.1 Hz, 2H), 7.34 (d, J = 16.5 Hz, 2H), 7.21 – 7.17 (m, 3H), 7.12 (s, 1H), 7.06 (s, 1H), 3.85 (s, 3H), 3.08 (dd, J = 7.3, 4.7 Hz, 4H), 1.16 (t, J = 7.3 Hz, 6H). HR-ESI-MS m/z for $C_{40}H_{34}NO_7S^-$ calcd: 672.2134 found: 672.1844.

HPLC: 254 nm/Ref 700 nm

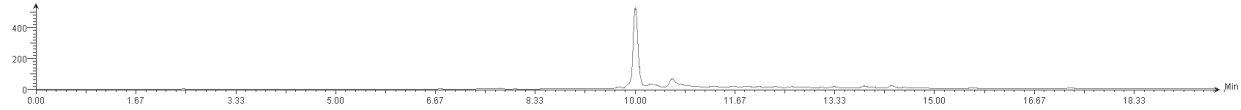


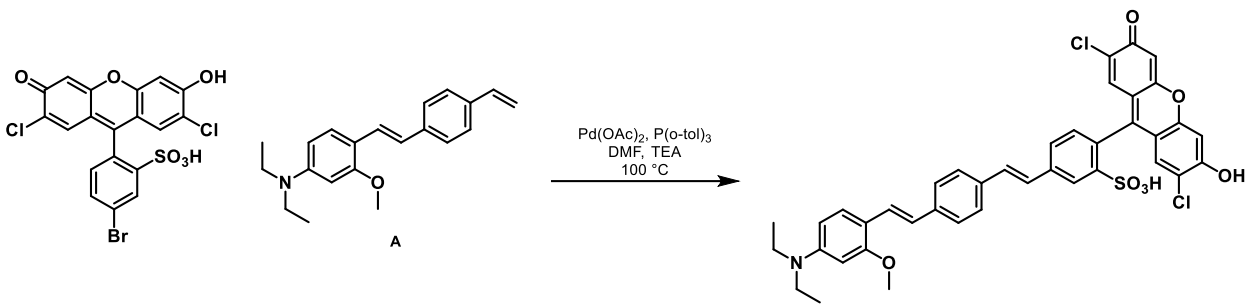


Synthesis of monosulfoVF2.2(OMe).F

5-bromo-2',7'-difluorosulfofluorescein (100 mg, 0.21 mmol), **A** (83 mg, 0.25 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (70 mg, 52%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.16 (s, 2H), 7.94 (s, 1H), 7.77 (d, *J* = 9.1 Hz, 2H), 7.72 (s, 1H), 7.69 (s, 1H), 7.65 (d, *J* = 8.1 Hz, 2H), 7.49 (m, 2H), 7.41 (d, *J* = 17.9 Hz, 1H), 7.39 – 7.32 (m, 4H), 7.21 (d, *J* = 7.8 Hz, 1H), 6.61 (d, *J* = 11.4 Hz, 2H), 3.85 (s, 3H), 3.09 (p, 4.9 Hz, 4H), 1.11 (t, *J* = 6.5 Hz, 6H). ^{13}C NMR (226 MHz, DMSO) δ 157.99, 157.71, 157.57, 138.31, 129.92, 127.91, 127.54, 127.19, 126.38, 125.92, 125.38, 117.55, 116.23, 114.91, 104.19, 55.25, 45.72, 41.34. HR-ESI-MS m/z for $C_{40}H_{32}F_2NO_7S^-$ calcd: 708.1946 found: 708.1898.

HPLC: 254 nm/Ref 700 nm

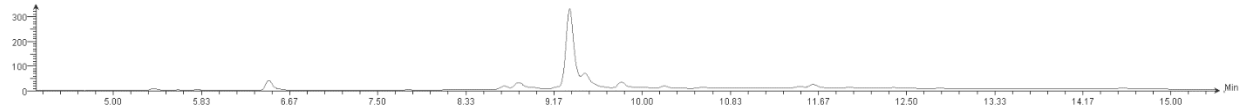


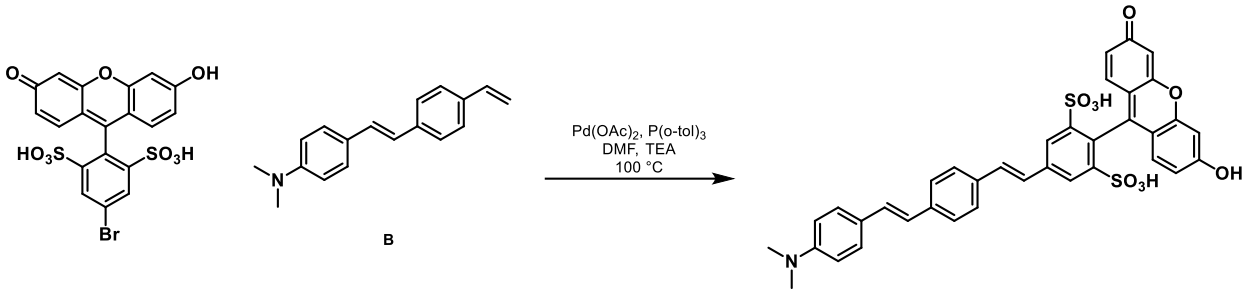


Synthesis of monosulfoVF2.2(OMe).Cl

5-bromo-2',7'-dichlorosulfofluorescein (100 mg, 0.19 mmol), **A** (71 mg, 0.23 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 3 mL dichloromethane and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (55 mg, 46%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.22 (s, 2H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 16.3 Hz, 1H), 7.45 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 16.3 Hz, 2H), 7.20 (d, *J* = 16.2 Hz, 1H), 7.15 (s, 1H), 7.07 (m, 1H), 7.00 (d, *J* = 16.5 Hz, 2H), 6.73 (d, *J* = 8.4 Hz, 3H), 3.44 (s, 3H), 3.08 (dd, *J* = 7.2, 4.9 Hz, 4H), 1.16 (t, *J* = 7.3 Hz, 6H). ^{13}C NMR (226 MHz, DMSO) δ 157.78, 157.64, 157.51, 149.97, 147.92, 127.61, 127.33, 126.16, 125.61, 118.75, 117.84, 116.52, 115.19, 112.28, 112.25, 100.92, 53.33, 45.70. HR-ESI-MS m/z for $C_{40}H_{32}Cl_2NO_7S^-$ calcd: 740.1355 found: 740.1322.

HPLC: 254 nm/Ref 700 nm

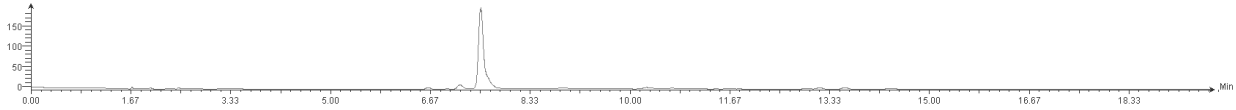


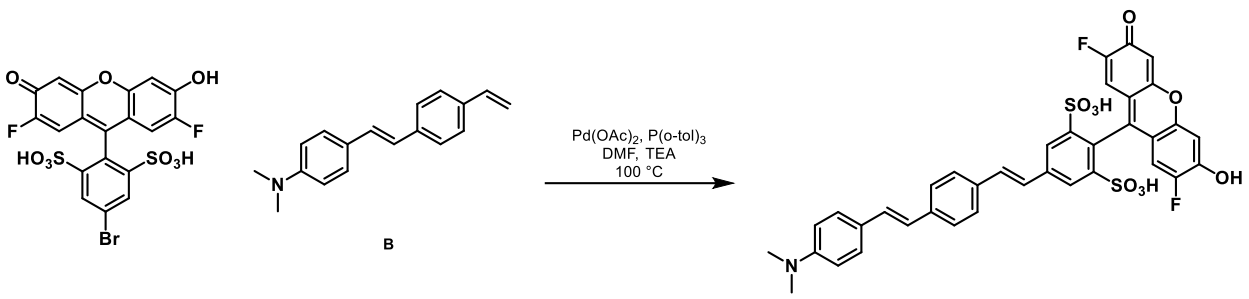


Synthesis of disulfoVF2.1.H

5-bromodisulfofluorescein (100 mg, 0.19 mmol), **B** (56 mg, 0.22 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (37 mg, 28%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.16 (s, 2H), 8.12 (s, 2H), 7.78 (d, J = 7.9 Hz, 2H), 7.64 (d, J = 7.8 Hz, 2H), 7.54 (d, J = 7.6 2H), 7.50 (d, J = 7.9 Hz, 2H), 7.40 (d, J = 11.5 Hz, 1H), 7.34 (d, J = 16.5 Hz, 1H), 7.24 (d, J = 7.7 Hz, 1H), 7.15 (s, 1H), 7.09 (s, 2H), 7.04 (s, 2H), 2.88 (s, 6H). HR-ESI-MS m/z for $C_{37}H_{28}NO_9S_2^-$ calcd: 694.1284 found: 694.1188.

HPLC: 254 nm/Ref 700 nm

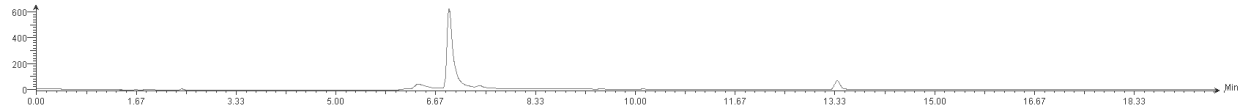


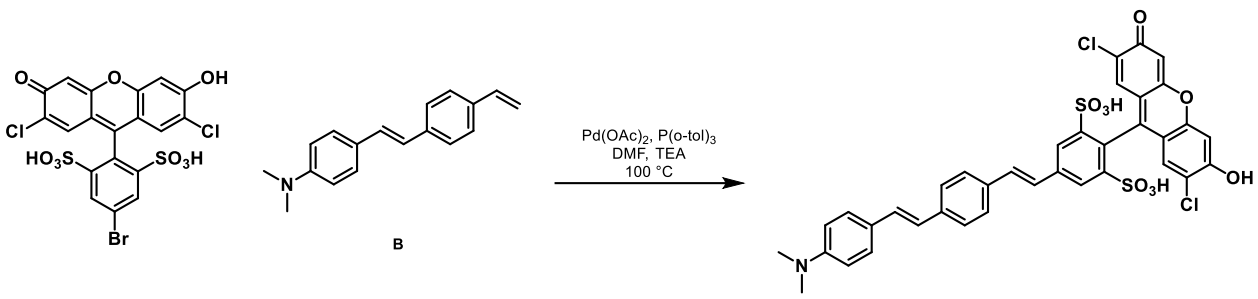


Synthesis of disulfoVF2.1.F

5-bromo-2',7'-difluorodisulfofluorescein (100 mg, 0.19 mmol), **B** (53 mg, 0.21 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (30 mg, 23%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 7.73 (s, 2H), 7.70 (d, J = 8.4 Hz, 2H), 7.56 (s, 2H), 7.53 – 7.49 (m, 1H), 7.45 (d, J = 9.0 Hz, 4H), 7.30 (d, J = 16.5 Hz, 1H), 7.19 (d, J = 18.2 Hz, 1H), 7.00 (d, J = 15.8 Hz, 1H), 6.98 – 6.93 (m, 1H), 6.75 – 6.69 (m, 3H), 2.93 (s, 6H). HR-ESI-MS m/z for $C_{37}H_{26}F_2NO_9S_2^-$ calcd: 730.1905 found: 730.1798.

HPLC: 254 nm/Ref 700 nm

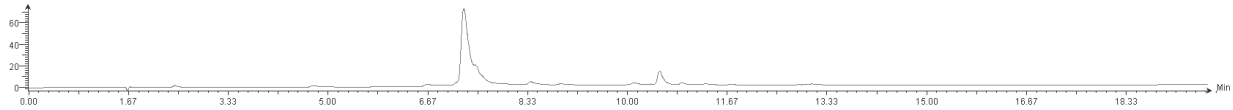


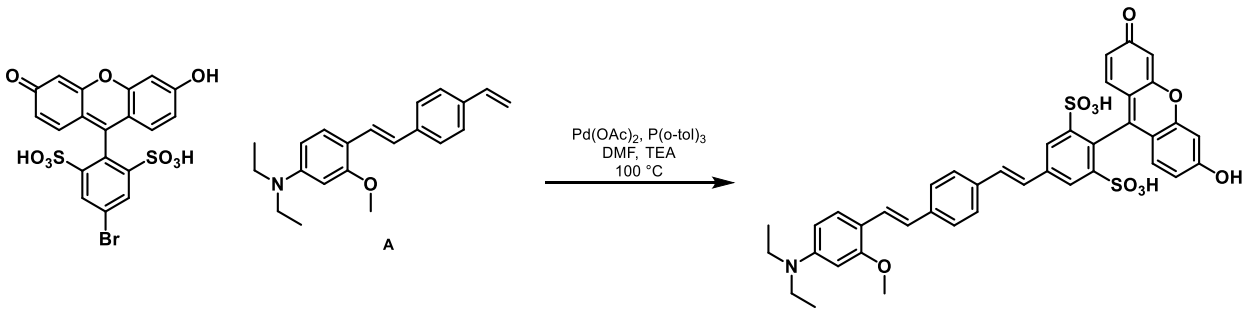


Synthesis of disulfoVF2.1.Cl

5-bromo-2',7'-dichlorodisulfofluorescein (100 mg, 0.17 mmol), **B** (50 mg, 0.20 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (48 mg, 38%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.35 (s, 2H), 8.03 (s, 2H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.68 (d, *J* = 8.2 Hz, 2H), 7.57 (d, *J* = 8.6 Hz, 2H), 7.54 (d, *J* = 16.2 Hz, 1H), 7.46 (d, *J* = 16.4 Hz, 1H), 7.29 (d, *J* = 16.3 Hz, 1H), 7.11 (d, *J* = 16.2 Hz, 1H), 7.01 (s, 2H), 6.84 (d, *J* = 8.7 Hz, 2H), 3.04 (s, 6H). ^{13}C NMR (226 MHz, $CDCl_3$) δ 162.99, 152.00, 150.42, 147.99, 138.21, 136.12, 135.25, 130.63, 129.63, 129.41, 128.04, 126.61, 126.21, 125.23, 124.46, 123.48, 112.62, 41.83. HR-ESI-MS m/z for $C_{37}H_{26}F_2NO_9S_2^-$ calcd: 762.0504 found: 762.0468.

HPLC: 254 nm/Ref 700 nm

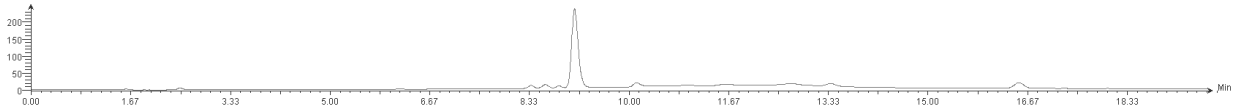


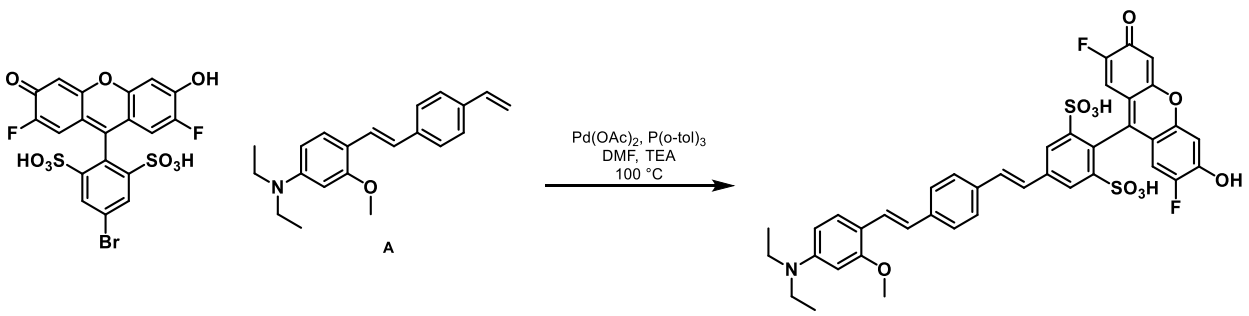


Synthesis of disulfoVF2.2(OMe).H

5-bromodisulfofluorescein (100 mg, 0.19 mmol), **A** (69 mg, 0.23 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (52 mg, 37%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.19 (s, 1H), 7.67 (d, *J* = 7.9 Hz, 2H), 7.60 – 7.56 (m, 1H), 7.54 – 7.50 (m, 1H), 7.47 (d, *J* = 7.5 Hz, 1H), 7.46 (s, 1H), 7.44 (s, 1H), 7.34 – 7.29 (m, 3H), 7.23 (d, *J* = 7.8 Hz, 1H), 7.13 (s, 1H), 6.97 (s, 3H), 6.84 (d, *J* = 9.2 Hz, 1H), 6.52 (s, 2H), 3.82 (s, 3H), 3.06 (dd, *J* = 13.6, 6.3 Hz, 4H), 1.15 – 1.11 (m, 6H). HR-ESI-MS m/z for $C_{40}H_{34}NO_{10}S_2^-$ calcd: 752.1702 found: 752.1684.

HPLC: 254 nm/Ref 700 nm

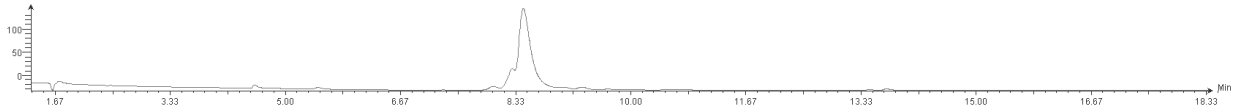


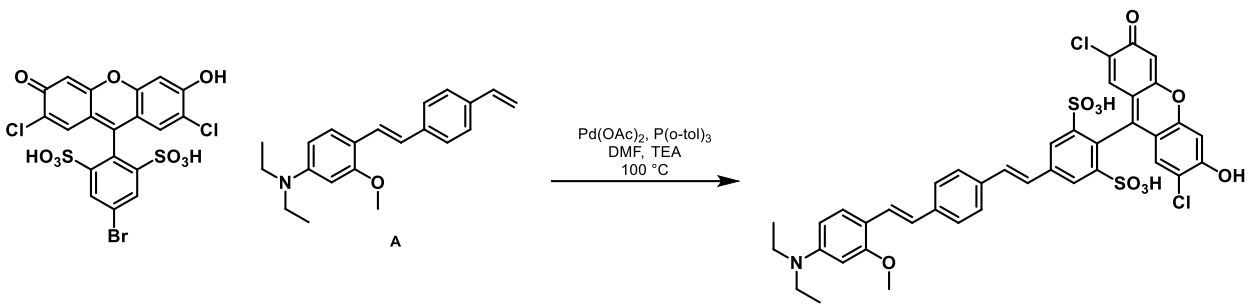


Synthesis of disulfoVF2.2(OMe).F

5-bromo-2'7'-difluorodisulfofluorescein (100 mg, 0.18 mmol), **A** (65 mg, 0.21 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (79 mg, 56%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.22 (s, 2H), 8.17 (s, 1H), 8.12 (s, 1H), 7.68 (d, J = 7.8 Hz, 2H), 7.50 – 7.47 (m, 2H), 7.47 – 7.40 (m, 1H), 7.34 (d, J = 16.4 Hz, 1H), 7.29 (d, J = 16.6 Hz, 1H), 7.09 (m, 1H), 7.03 – 6.95 (m, 1H), 6.30 (s, 1H), 6.22 (s, 1H), 3.85 (s, 3H), 3.09 (dd, J = 7.3, 5.0 Hz, 4H), 1.17 (t, J = 7.3 Hz, 6H). HR-ESI-MS m/z for $C_{40}H_{32}F_2NO_{10}S_2^-$ calcd: 788.1514 found: 788.1394.

HPLC: 254 nm/Ref 700 nm

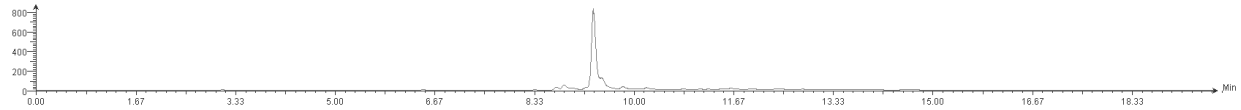




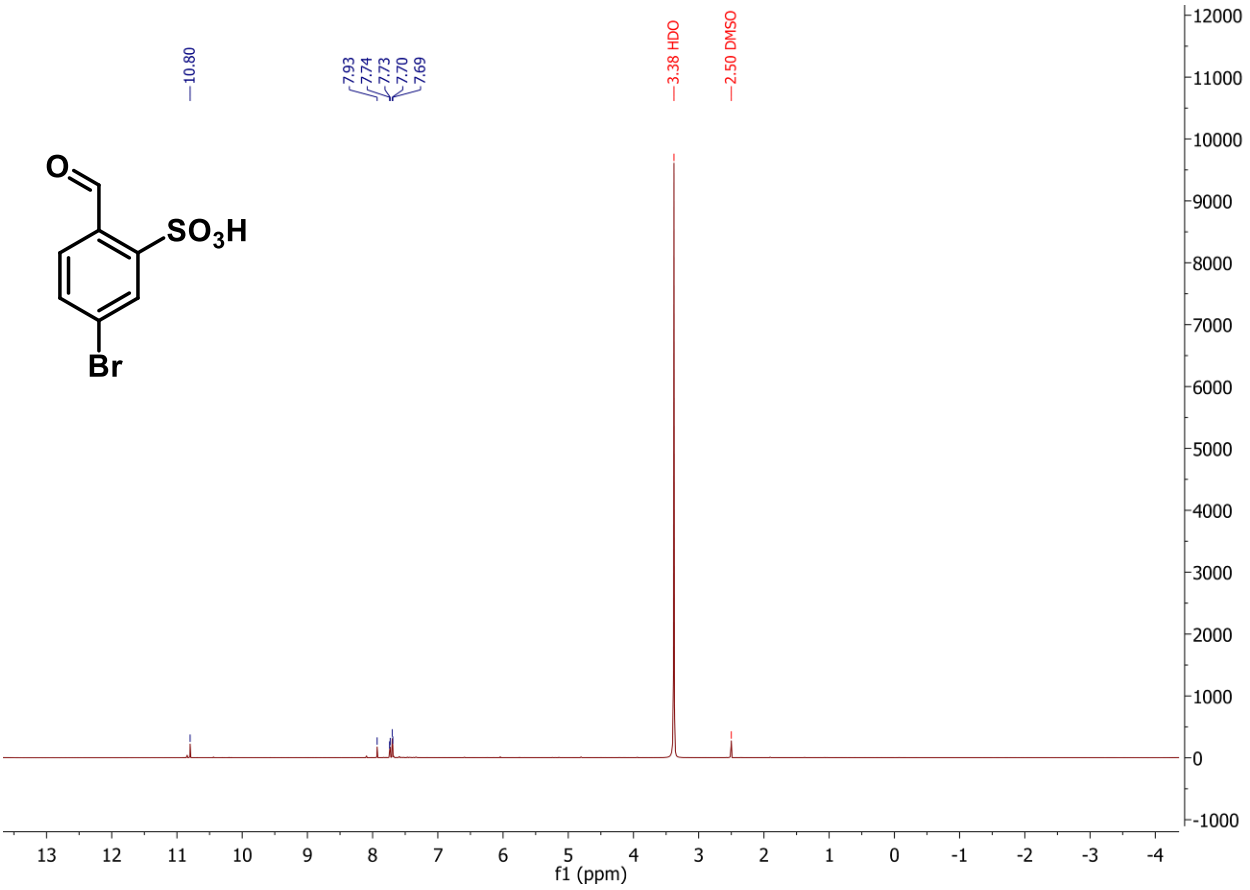
Synthesis of disulfoVF2.2(OMe).Cl

5-bromo-2'7'-dichlorodisulfofluorescein (100 mg, 0.18 mmol), **A** (65 mg, 0.21 mmol), palladium acetate (1 mg, 0.045 mmol), and P(*o*-tol)$_3$ (2.7 mg, 0.09 mmol) were placed in an oven-dried Schlenk flask. The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous Et_3N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 100 degrees Celsius. After cooling, the reaction mixture was diluted with 10 mL methanol and filtered through celite, which was then washed with methanol. The solvent was removed from the filtrate via rotary evaporation and the resulting residue was dissolved in 5 mL dichloromethane. The mixture was then poured into diethyl ether, resulting in precipitation of a brown solid. The solid was isolated via vacuum filtration (79 mg, 56%). A small amount of material was purified via RP-HPLC for further characterization. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.95 (d, J = 8.1 Hz, 2H), 7.93 – 7.89 (m, 2H), 7.69 (d, J = 16.4 Hz, 2H), 7.43 (d, J = 16.4 Hz, 2H), 7.11 (s, 2H), 7.05 (s, 1H), 7.00 (s, 1H), 6.85 (s, 1H), 6.75 (s, 2H), 6.61 (s, 1H), 3.83 (s, 3H), 3.08 – 3.03 (m, 4H), 1.13 (t, J = 7.4 Hz, 6H). ^{13}C NMR (226 MHz, DMSO) δ 192.94, 162.75, 158.44, 148.62, 143.30, 137.41, 135.74, 131.61, 131.36, 130.43, 130.16, 130.09, 129.37, 127.92, 126.74, 118.54, 118.04, 117.62, 116.31, 115.00, 102.93, 46.17, 41.80, 36.22, 34.80, 31.21. HR-ESI-MS m/z for $C_{40}H_{32}Cl_2NO_{10}S_2^-$ calcd: 820.0923 found: 819.9684.

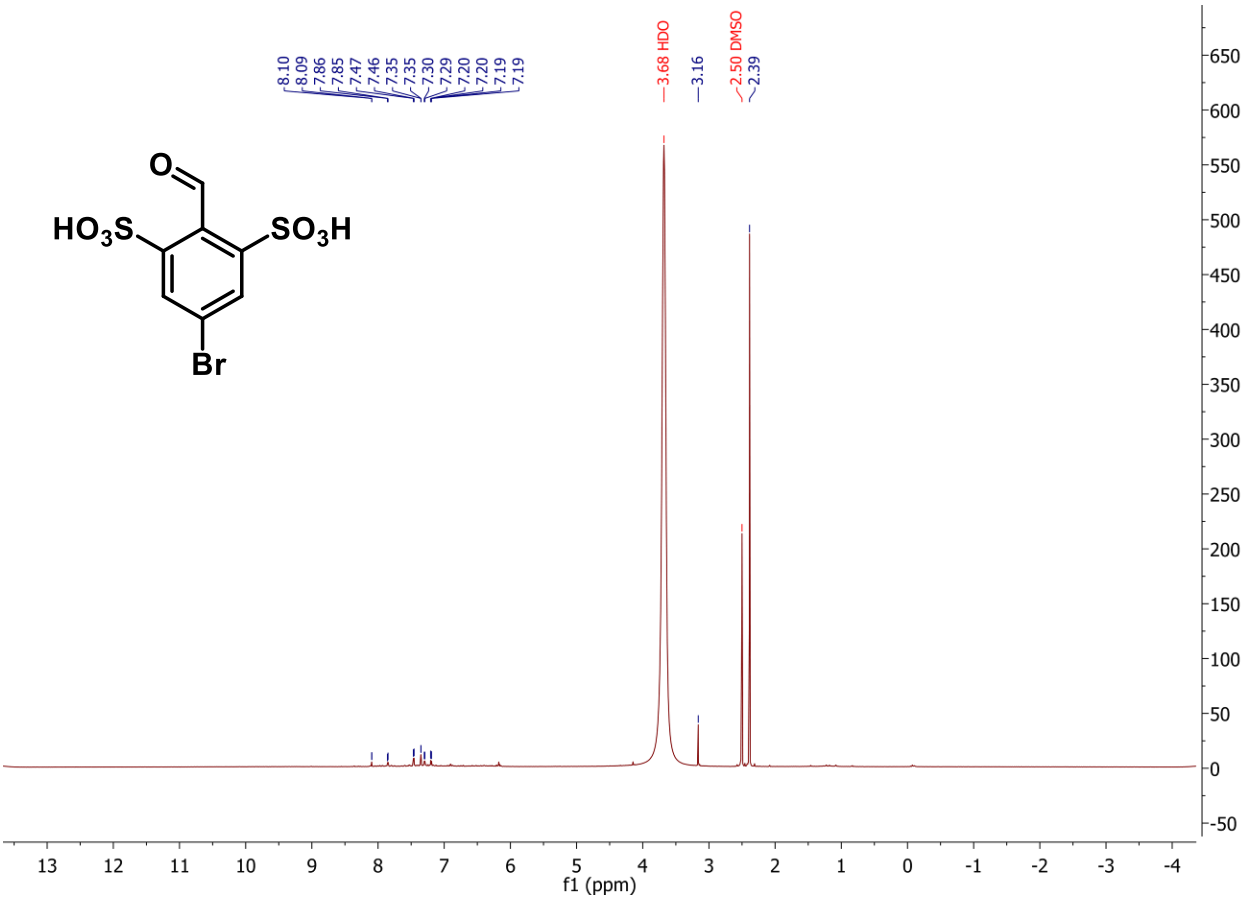
HPLC: 254 nm/Ref 700 nm



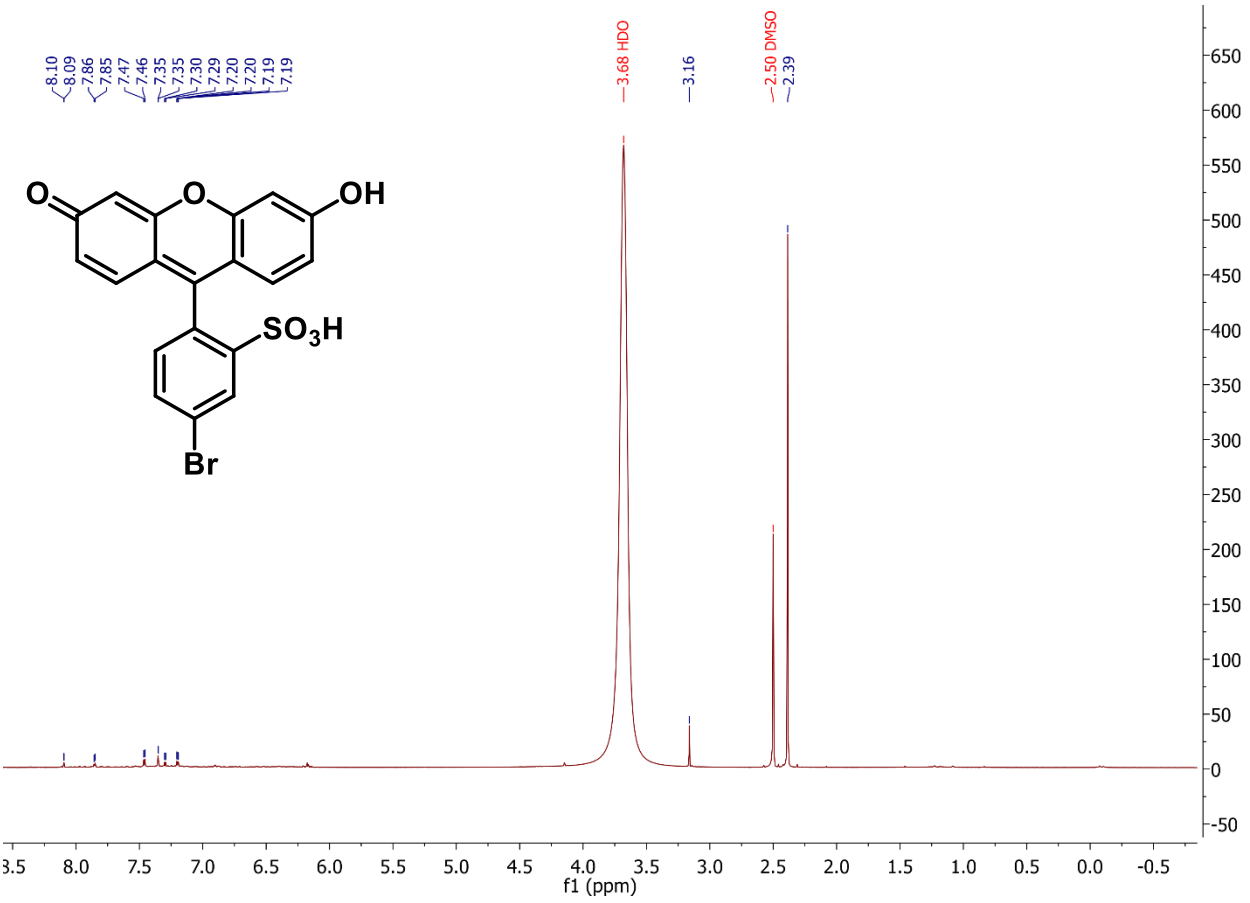
Spectra



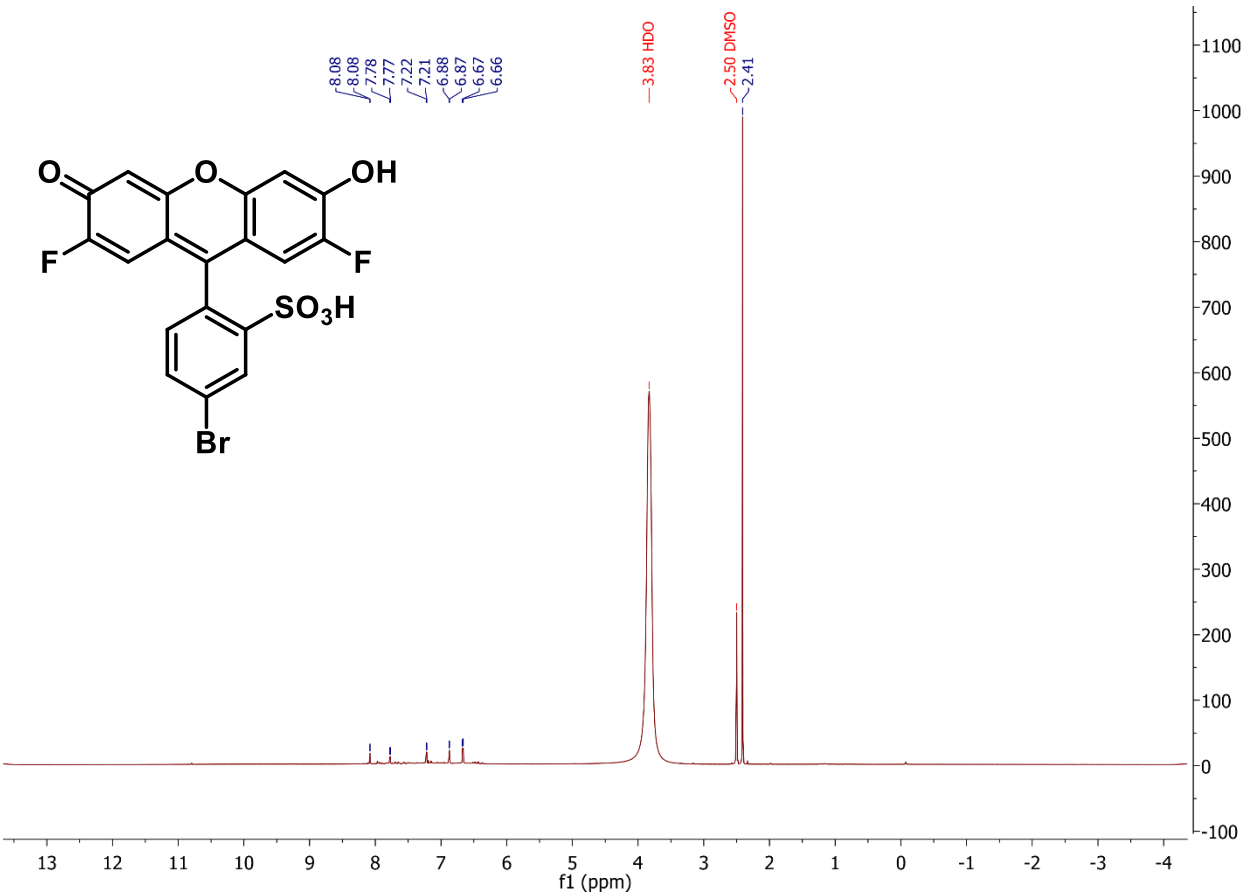
SI Spectrum 1. ^{1}H NMR of 4-bromo-2-sulfobenzaldehyde



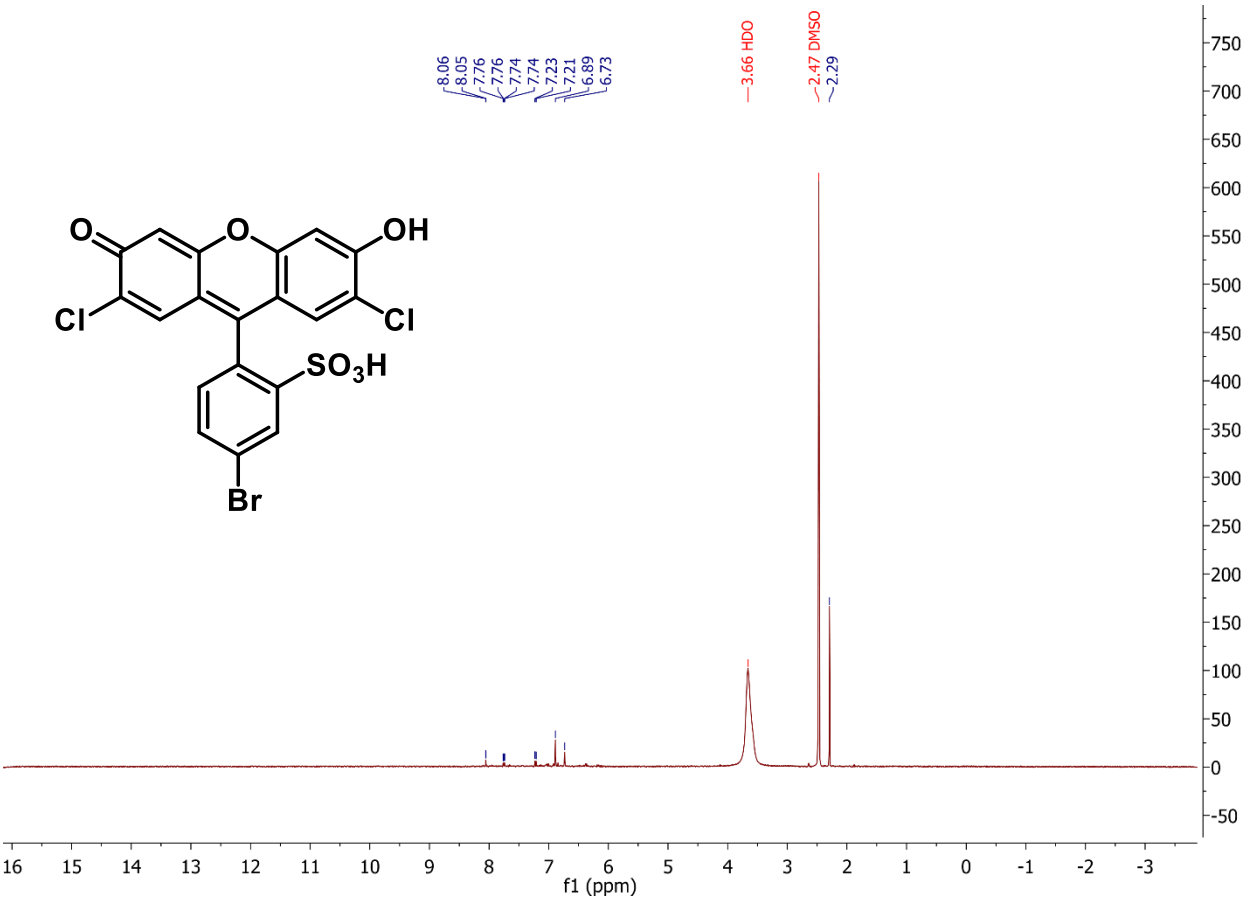
SI Spectrum 2. ^{1}H NMR of 4-bromo-2,6-disulfobenzaldehyde



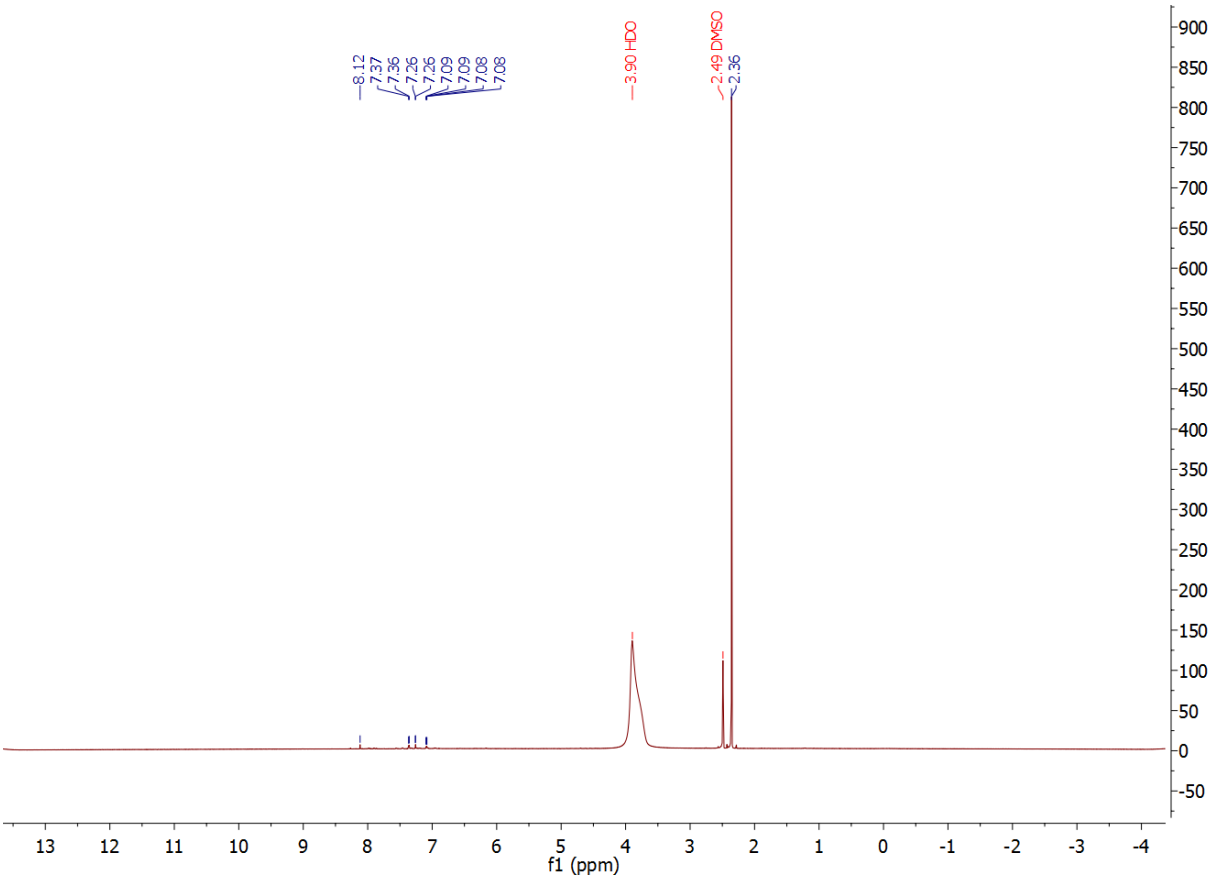
SI Spectrum 3. ^{1}H NMR of 5-bromosulfofluorescein



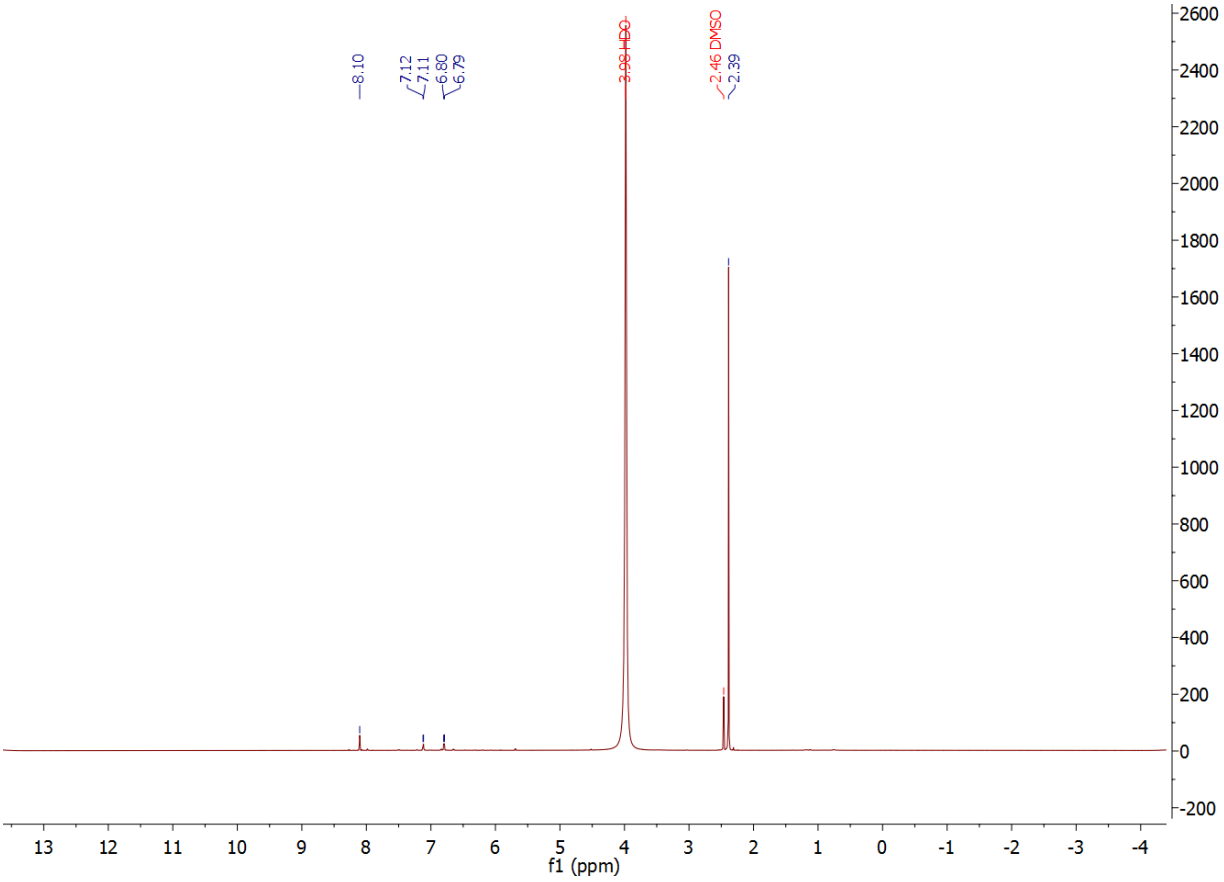
SI Spectrum 4. ^{1}H NMR of 5-bromo-2',7'-difluorosulfofluorescein



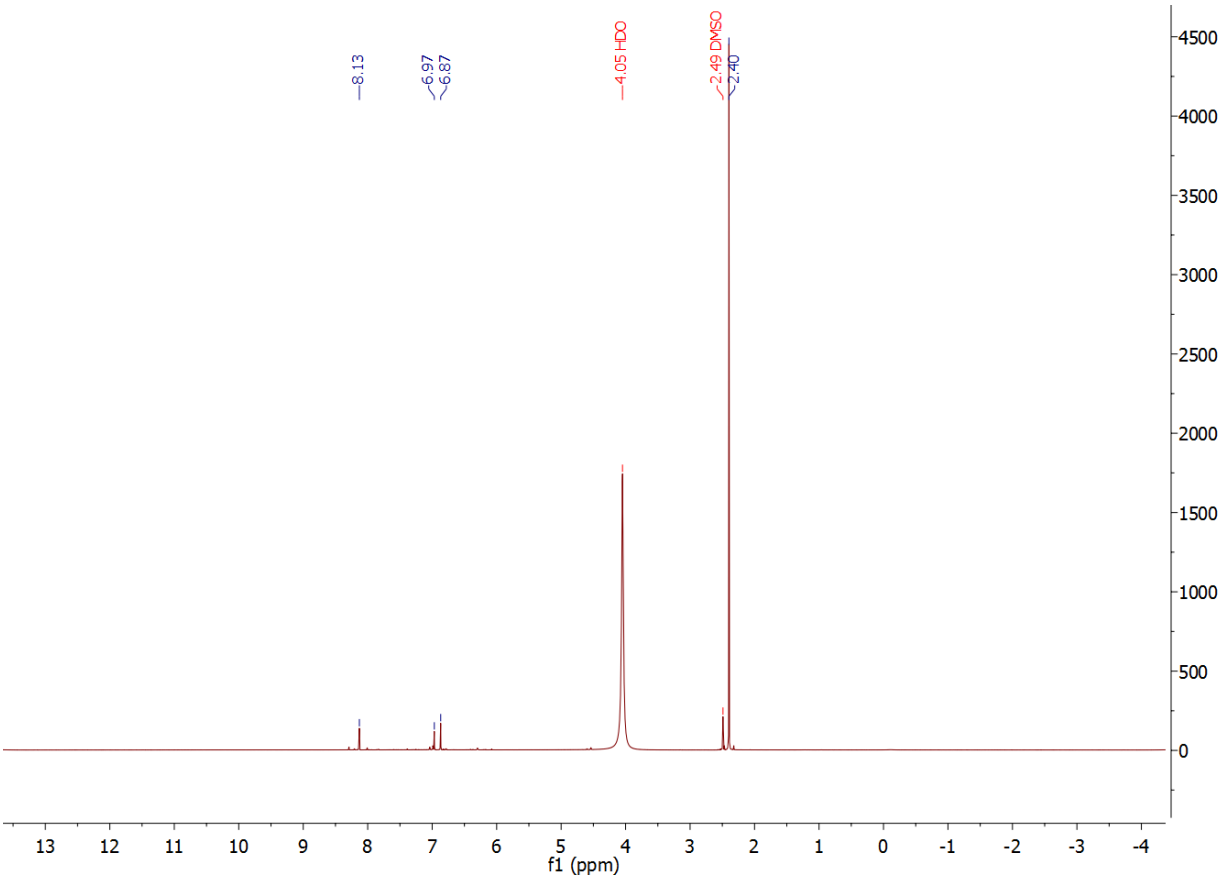
SI Spectrum 5. ^{1}H NMR of 5-bromo-2',7'-dichlorosulfofluorescein



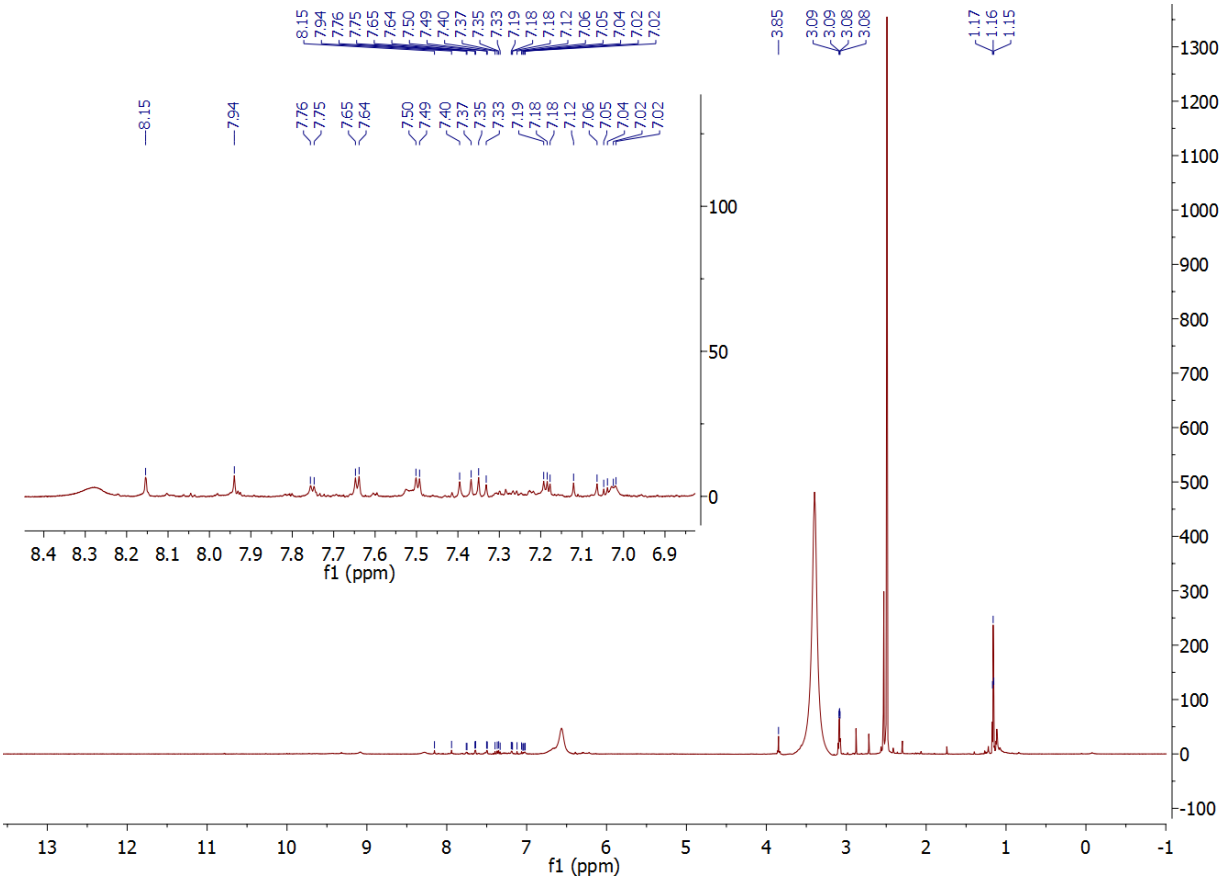
SI Spectrum 6. ^{1}H NMR of 5-bromodisulfofluorescein



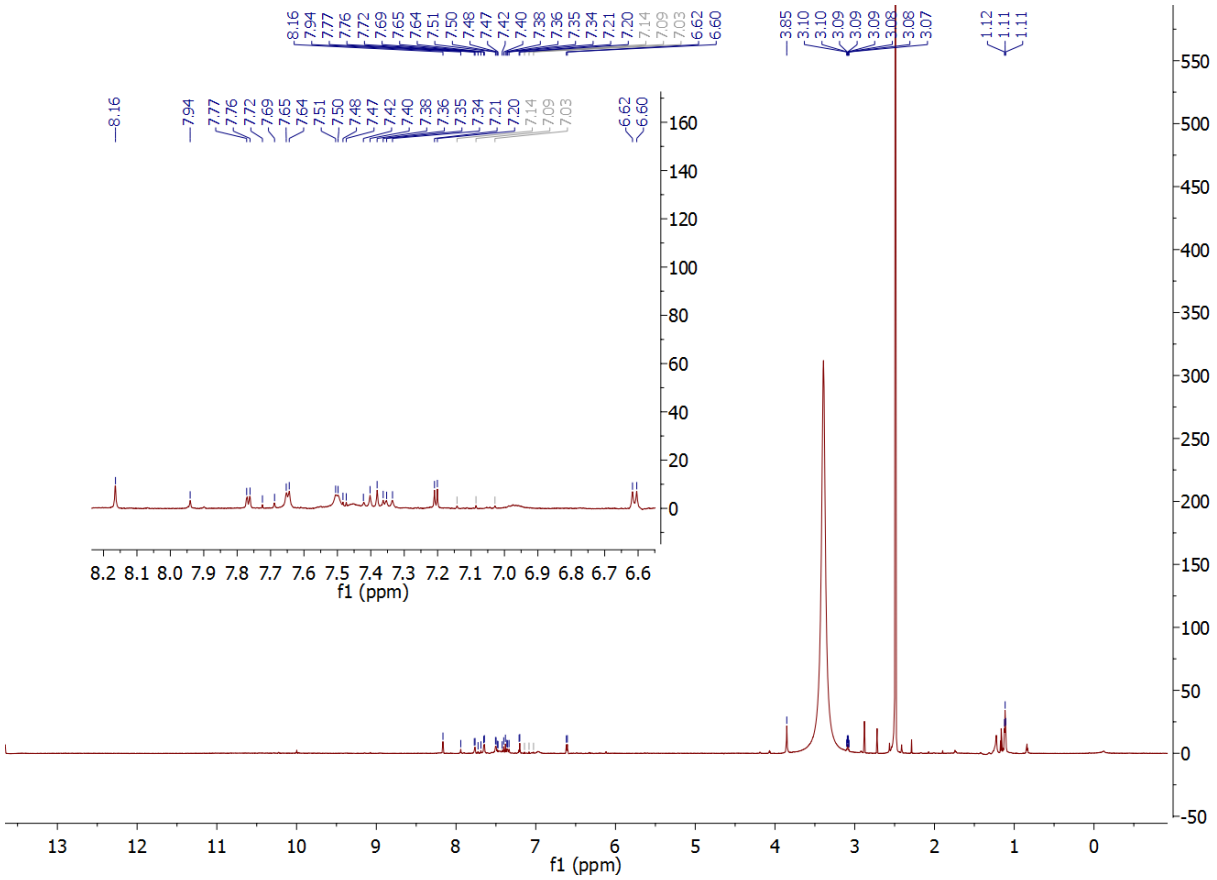
SI Spectrum 7. ^{1}H NMR of 5-bromo-2',7'-difluorodisulfofluorescein



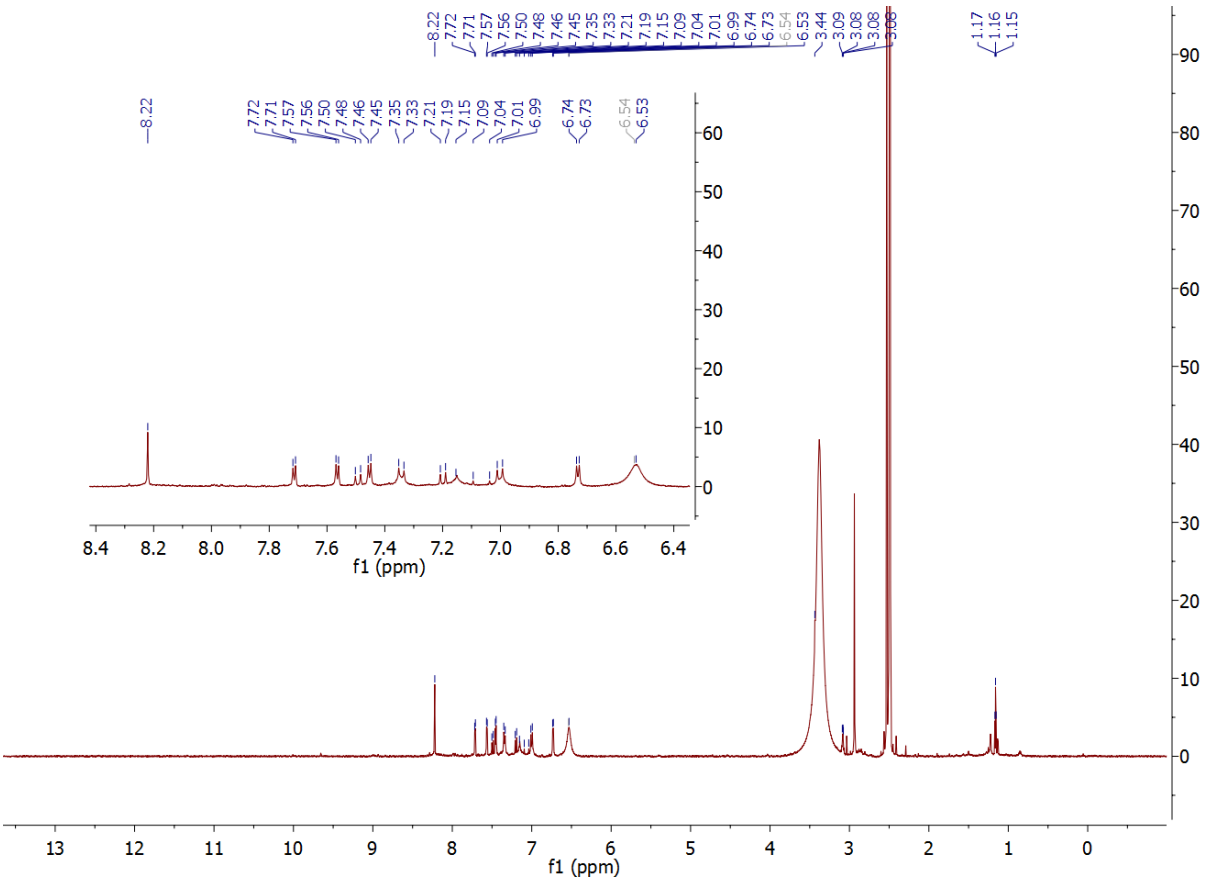
SI Spectrum 8. ^{1}H NMR of 5-bromo-2',7'-dichlorodisulfofluorescein



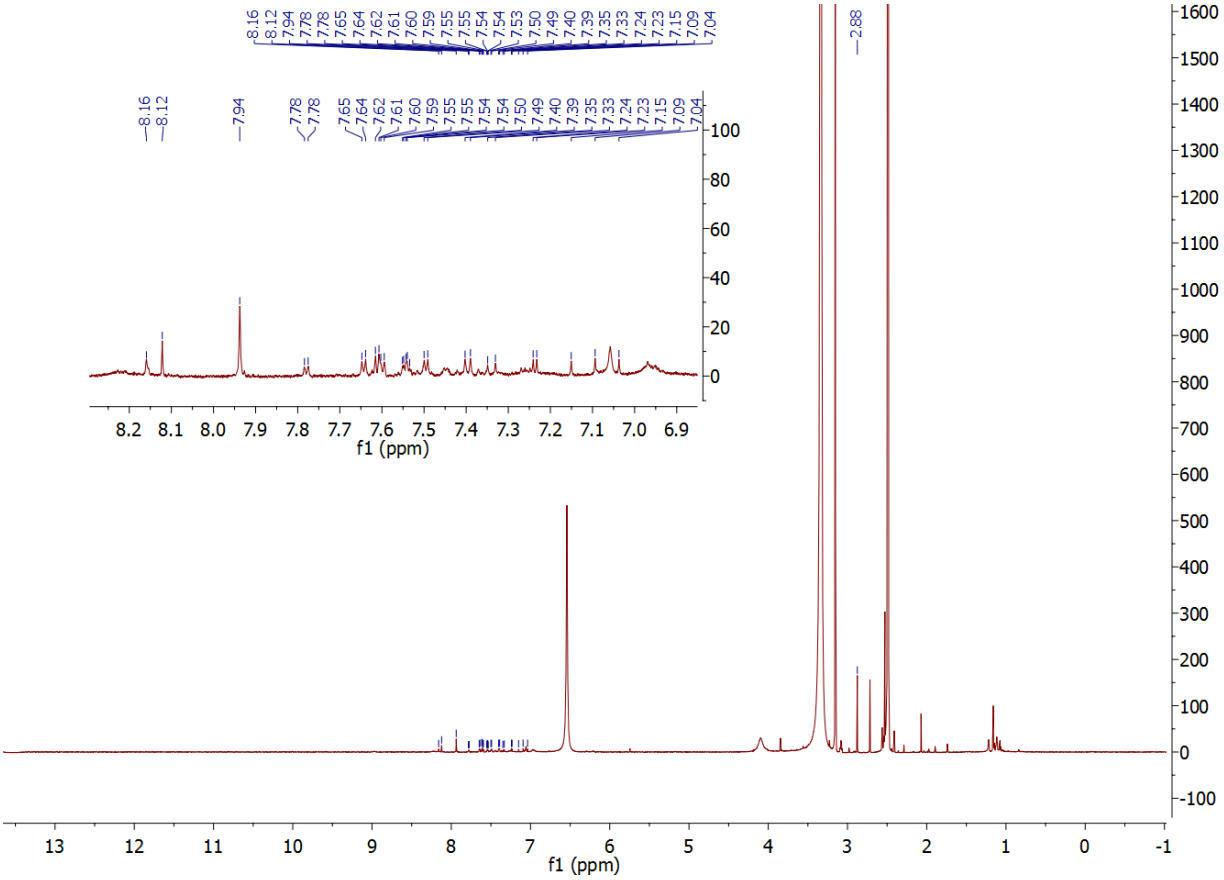
SI Spectrum 9. ^{1}H NMR of msVF2.2(OMe).H



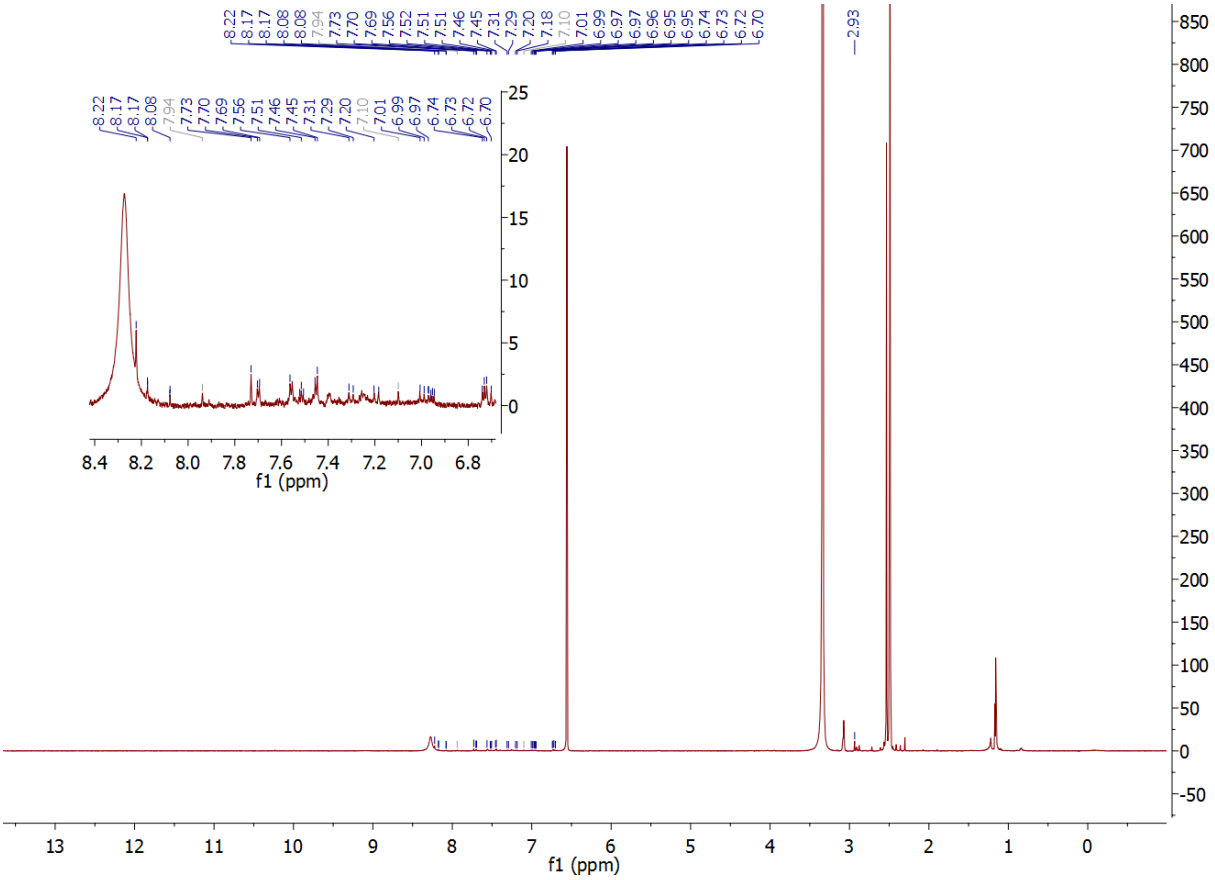
SI Spectrum 10. ^{1}H NMR of msVF2.2(OMe).F



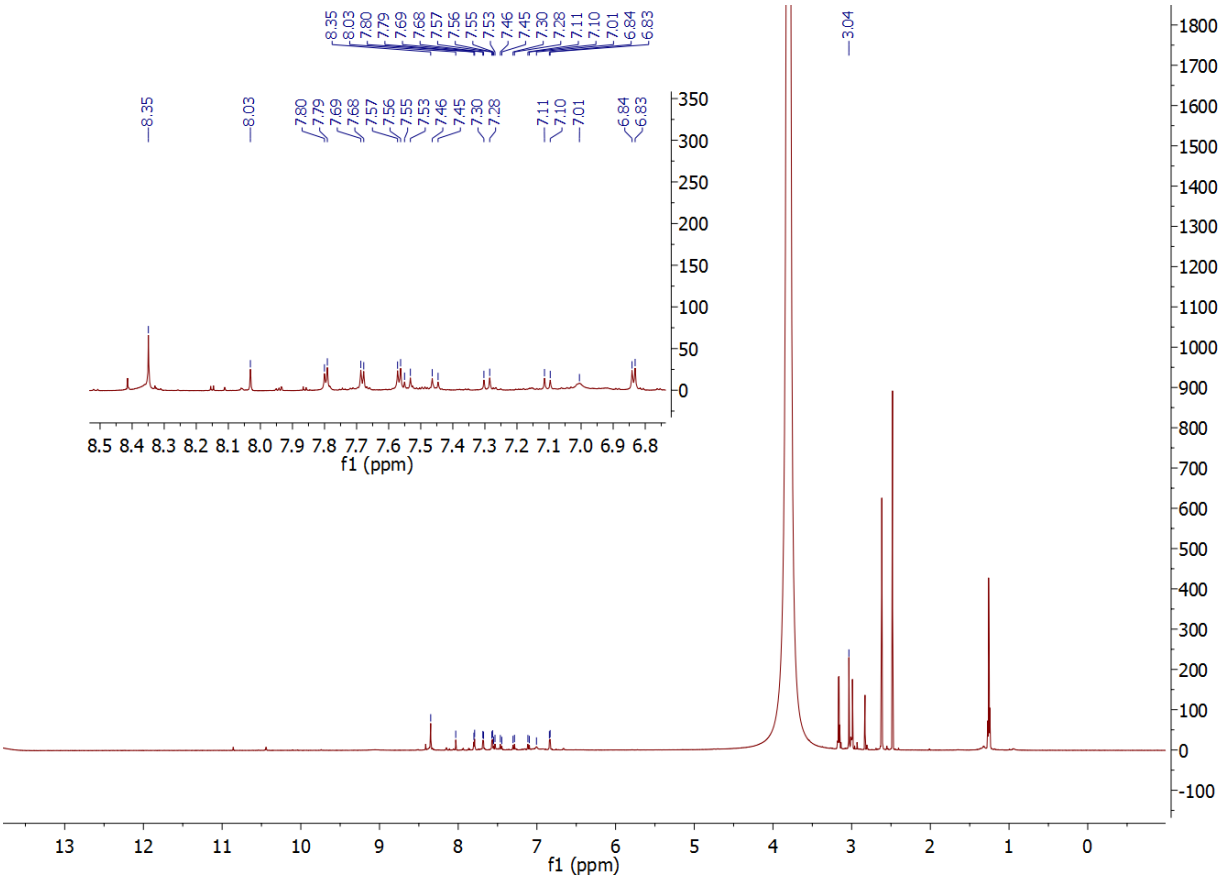
SI Spectrum 11. ^{1}H NMR of msVF2.2(OMe).Cl



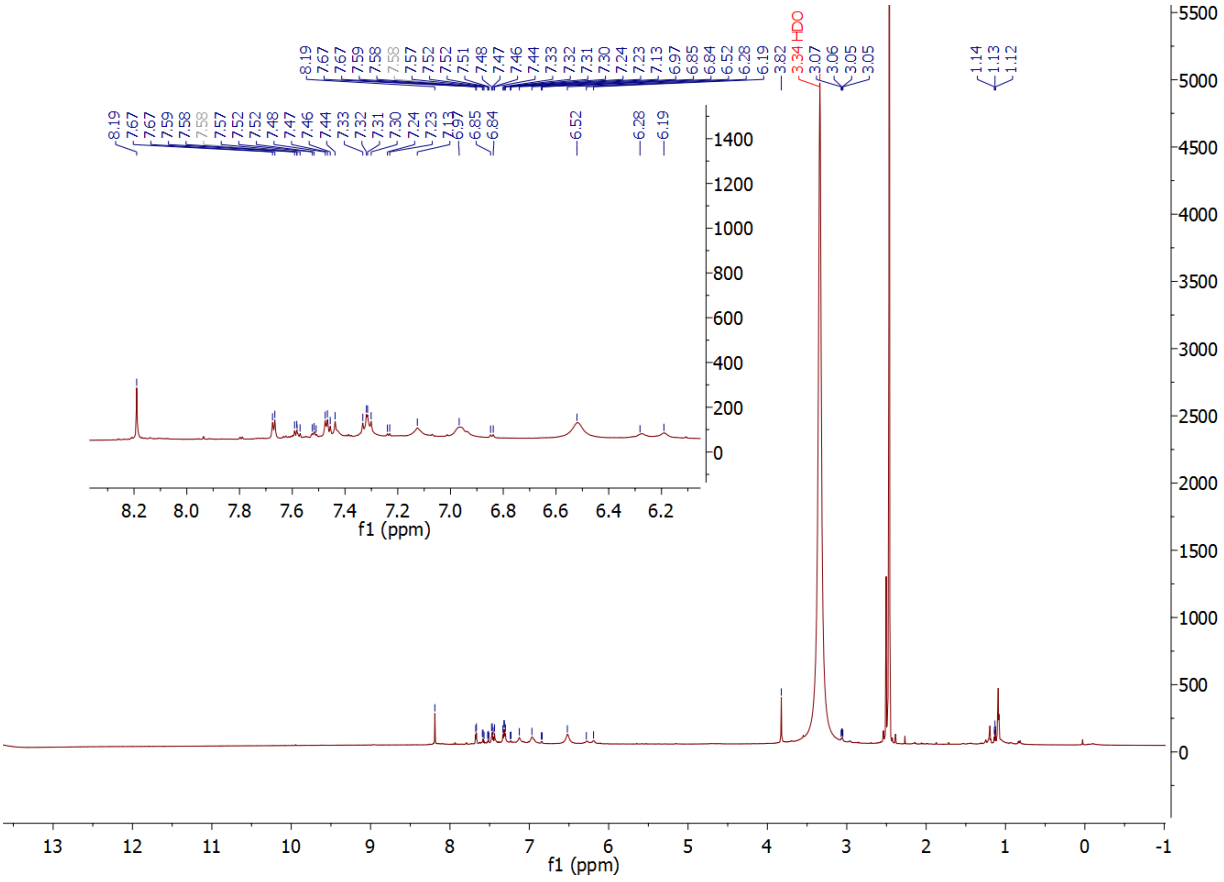
SI Spectrum 12. ^{1}H NMR of dsVF2.1.H



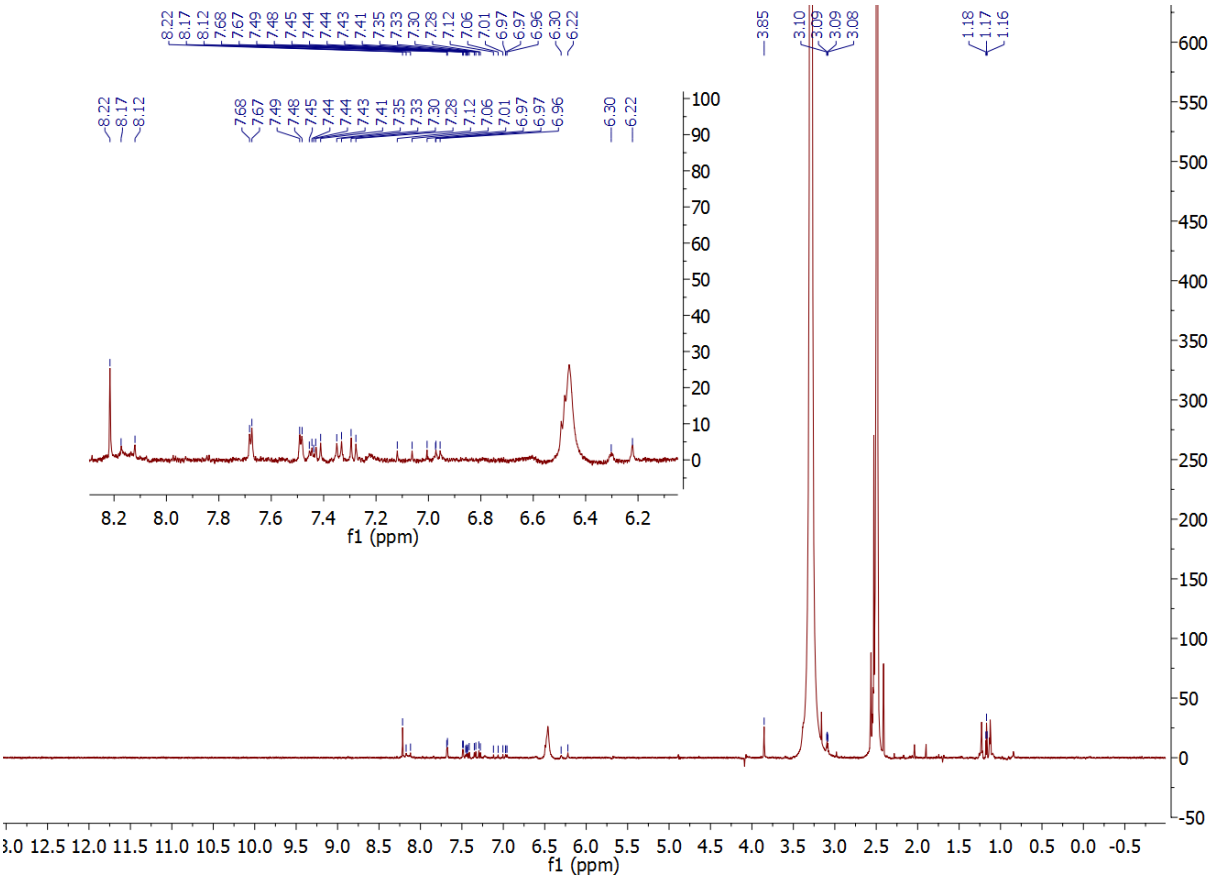
SI Spectrum 13. ^{1}H NMR of dsVF2.1.F



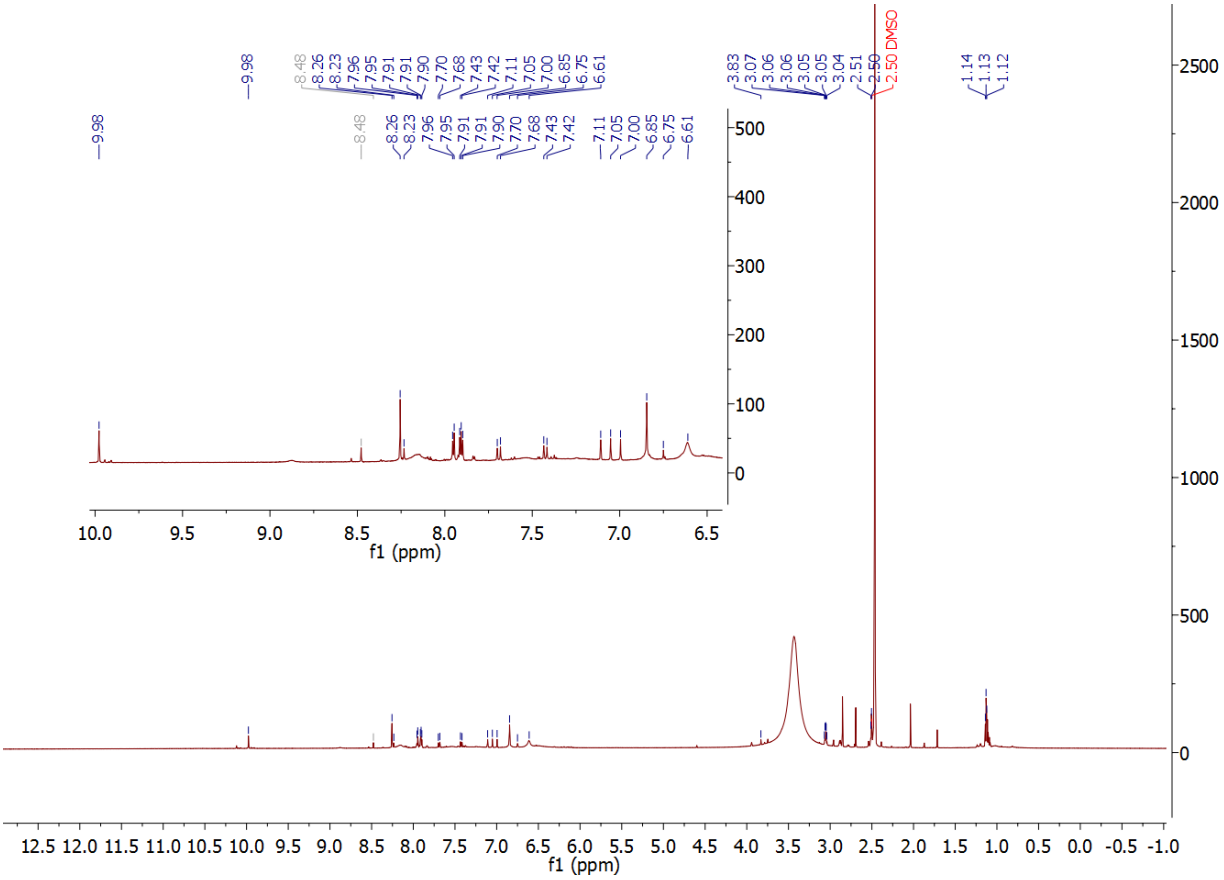
SI Spectrum 14. ^{1}H NMR of dsVF2.1.Cl



SI Spectrum 15. ^{1}H NMR of dsVF2.2(OMe).H



SI Spectrum 16. ^{1}H NMR of dsVF2.2(OMe).F



SI Spectrum 17. ^{1}H NMR of dsVF2.2(OMe).Cl

Supporting Information References

(1) Kriks, S.; Shim, J. W.; Piao, J. H.; Ganat, Y. M.; Wakeman, D. R.; Xie, Z.; Carrillo-Reid, L.; Auyeung, G.; Antonacci, C.; Buch, A.; Yang, L. C.; Beal, M. F.; Surmeier, D. J.; Kordower, J. H.; Tabar, V.; Studer, L. *Nature* **2011**, *480*, 547.

(2) Vanommeslaeghe, K.; MacKerell, A. D. *J Chem Inf Model* **2012**, *52*, 3144.

(3) Vanommeslaeghe, K.; Raman, E. P.; MacKerell, A. D. *J Chem Inf Model* **2012**, *52*, 3155.

(4) Mayne, C. G.; Saam, J.; Schulten, K.; Tajkhorshid, E.; Gumbart, J. C. *J Comput Chem* **2013**, *34*, 2757.

(5) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J.; Gaussian, Inc.: Wallingford, CT, USA, 2009.

(6) Vanommeslaeghe, K.; Hatcher, E.; Acharya, C.; Kundu, S.; Zhong, S.; Shim, J.; Darian, E.; Guvench, O.; Lopes, P.; Vorobyov, I.; MacKerell, A. D. *J Comput Chem* **2010**, *31*, 671.

(7) Batistela, V. R.; Cedran, J. D.; de Oliveira, H. P. M.; Scarminio, I. S.; Ueno, L. T.; Machado, A. E. H.; Hioka, N. *Dyes Pigments* **2010**, *86*, 15.

(8) Hong, C.; Tieleman, D. P.; Wang, Y. *Langmuir* **2014**, *30*, 11993.

(9) Shaw, D. E.; Deneroff, M. M.; Dror, R. O.; Kuskin, J. S.; Larson, R. H.; Salmon, J. K.; Young, C.; Batson, B.; Bowers, K. J.; Chao, J. C.; Eastwood, M. P.; Gagliardo, J.; Grossman, J. P.; Ho, C. R.; Ierardi, D. J.; Kolossvary, I.; Klepeis, J. L.; Layman, T.; McLeavey, C.; Moraes, M. A.; Mueller, R.; Priest, E. C.; Shan, Y. B.; Spengler, J.; Theobald, M.; Towles, B.; Wang, S. C. *Conf Proc Int Symp C* **2007**, 1.

(10) Humphrey, W.; Dalke, A.; Schulten, K. *J Mol Graph* **1996**, *14*, 33.

(11) Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. *J Comput Chem* **2005**, *26*, 1781.

(12) Klauda, J. B.; Venable, R. M.; Freites, J. A.; O'Connor, J. W.; Tobias, D. J.; Mondragon-Ramirez, C.; Vorobyov, I.; MacKerell, A. D.; Pastor, R. W. *J Phys Chem B* **2010**, *114*, 7830.

(13) Andersen, H. C. *J Comput Phys* **1983**, *52*, 24.

(14) Miyamoto, S.; Kollman, P. A. *J Comput Chem* **1992**, *13*, 952.

(15) Darden, T.; York, D.; Pedersen, L. *J Chem Phys* **1993**, *98*, 10089.

(16) Feller, S. E.; Zhang, Y. H.; Pastor, R. W.; Brooks, B. R. *J Chem Phys* **1995**, *103*, 4613.