Supporting Information for:

*In Vivo Two-Photon Voltage Imaging with Sulfonated Rhodamine Dyes*

Published in *ACS Central Science*

DOI: 10.1021/acscentsci.8b00422

**General methods for chemical synthesis and characterization**

No unexpected or unusually high safety hazards were encountered. 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\textsubscript{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. NMR spectra were measured on a Bruker AV-900 MHz, 226 MHz. Chemical shifts are expressed in parts per million (ppm) and are referenced to d\textsubscript{6}-DMSO, 2.50 ppm or CDCl\textsubscript{3}, 7.26 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 preparative HPLC were Phenomenex Luna C18(2) (4.6 mm I.D. × 150 mm) and Waters XBridge 10μ C18 (19 mm I.D. x 250 mm) columns with a flow rate of 1.0 and 30.0 mL/min, respectively. The mobile phase were MQ-H\textsubscript{2}O 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 580 nm in 20 min with gradient 10-100% eluent B.

**Imaging Parameters**
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). The sCMOS was used to acquire large fields of view for recording from multiple neurons (i.e. Figure 4a-d). Two-photon imaging was performed with a Zeiss LSM 880 NLO AxioExaminer equipped with a Chameleon Ultra I laser (Coherent Inc.) Fluorescence images were acquired using a Zeiss BiG-2 GaAsP detector.

**Spectroscopic studies**

Stock solutions of VF dyes were prepared in DMSO (1.0–10 mM) and diluted with PBS (100 mM Na₂HPO₄, pH 7.4, 0.1% Triton-X) or with absolute ethanol. 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). For solubility studies, 5 mg each of SulfoRhoVR 1, RVF5, and RhoVR 1 were dissolved in 10 mL of distilled water. Over the course of seven days, and while stirring, 1 mg of each of the dyes was added daily until no more solid went into solution. The resulting suspensions were then filtered through a 0.22-micron polytetrafluoroethylene filter into a scintillation vial. The resulting filtrates were then used to determine the amount of voltage dye in solution via UV-Vis absorbance by dilution into ethanol for sulfoRhoVR 1 and PBS for RVF5 and RhoVR 1. Molar extinction coefficient values were used to determine concentration (RVF5: 83,000 cm⁻¹M⁻¹, RhoVR 1: 87,000 cm⁻¹M⁻¹).

**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 and Laboratory Animals and the Public Health Policy. Human embryonic kidney 293T (HEK) cells were passaged and plated onto 12 mm glass coverslips pre-coated with Poly-D Lysine (PDL; 1 mg/ml; Sigma-Aldrich) to provide a confluency of ~15% and 50% for electrophysiology and imaging, respectively. HEK cells were plated and maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% FBS and 1% Glutamax.

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. SulfoRhoVR dyes were excited using the 550 nm LED with an intensity of 9.7 W/cm². 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).

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. Neurons were switched to current clamp mode if they displayed series resistance in voltage clamp less than 30 megaohms. Pipette tip resistance was corrected by performing a bridge balance compensation.

To test if loading SulfoRhoVR 1 onto the membrane of neurons has any effect on action potential firing, ten 500 ms current steps were injected into neurons in increments of 0.05 pA. The action potentials for each sweep were analyzed in Clampfit 10 software (Molecular Devices) to give amplitude and kinetic data.

**Image analysis**

Analysis of voltage sensitivity in HEK cells was performed using custom Python scripts. Briefly, a region of interest (ROI) was selected 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.

**Two-Photon Excitation Cross Section Measurement**

The two-photon excitation (TPE) cross section of SulfoTMR dyes and SulfoRhoVR 1 was determined according to previously reported procedures. We first measured the fluorescence signal generated by two-photon excitation of a rhodamine b standard using a Zeiss BiG-2 GaAsP detector on a Zeiss LSM 880 NLO AxioExaminer equipped with a Chameleon Ultra I laser. We then determined the one-photon quantum yield of the dyes of interest and measured the fluorescence signal generated by two-photon excitation of the dyes of interest. We then calculated the TPE of the dyes of interest via the following equation:

\[
\sigma_{TPE, DYE} = \frac{\phi_{Rho B} \cdot \sigma_{TPE, Rho B}}{\phi_{DYE}} \cdot \frac{F_{DYE}}{F_{Rho B}}
\]

Where \(F_{DYE}\) and \(F_{Rho B}\) are the measured fluorescence signal from the dye of interest and rhodamine b, respectively. The values for \(\sigma_{TPE}\) at different wavelengths for rhodamine b were obtained from Xu and Webb.

**Animal procedures for imaging under anesthesia**
All experimental procedures were performed in accordance with the guidelines established by the UCSD Institutional Animal Care and Use Committee (IACUC). Adult wild-type C57Bl/6 or ICR mice (age: 3-6 months) were used for in vivo imaging experiments.

Mice were anesthetized with isoflurane (2% initially, 1-1.5% for maintenance) in 100% oxygen during surgical procedures. A cannula was inserted into the femoral artery, tracheotomy was performed, and a metal holding bar was glued to the temporal bone for immobilization of the head during imaging. An area of skull overlying whisker pad and forepaw region of the primary somatosensory cortex (SI) contralateral to the holding bar was exposed and the dura mater removed. A ~5x3 mm cranial window was kept moist with artificial cerebrospinal fluid (ACSF) containing 142 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 3.1 mM CaCl2, 1.3 mM MgCl2, pH 7.4. A drop of agarose (0.7% wt/vol, A9793, Sigma) in ACSF was applied on the brain surface, and the exposure was covered with a round glass coverslip (8 mm, WPI) cut straight on one side, and sealed with dental acrylic. A gap was left in the seal to allow insertion of recording microelectrodes and/or glass pipettes. To avoid herniation of the exposed brain due to excessive intracranial pressure, the dura mater over the IVth cerebral ventricle was punctured, thus allowing cerebrospinal fluid drainage. After the exposure was closed, the drainage hole was sealed with agarose.

After closing the exposure, mice were left to rest under 1% isoflurane for 45 min. Then, isoflurane was discontinued, and anesthesia was maintained with α-chloralose (50 mg/kg/h, C0128, Sigma or 100459, MP Biochemicals). Mice were paralyzed with pancuronium bromide (0.4 mg/kg/h, P1918, Sigma) and ventilated (~110 min⁻¹) with 100% O₂. Expired CO₂ was measured continuously using a micro-capnometer (Cl240, Columbus instruments). Heart rate, blood pressure, and body temperature were monitored continuously. α-chloralose, pancuronium, and 5% dextrose (all in saline) were supplied through the femoral line every 30 min for the duration of data acquisition. At the end of each imaging experiment, fluorescein isothiocyanate (FITC)-labeled dextran (MW=2 MDa, FD-2000S, Sigma) was injected (50-100 μL of a 5% (w/v) solution in phosphate-buffered saline) to visualize the vasculature and to control for the integrity of the capillary bed.
The stock solution of the voltage probe sRhoVR 1 (10 mM in DMSO) was diluted in ACSF yield a final probe concentration of 90-180 μM. 20% Pluronic F-127 in DMSO (P3000MP, Thermo-Fisher Scientific) was added to the solution to yield a final concentration of 0.35-0.7% Pluronic. The solution was filtered and pressure-microinjected into the cortical tissue using a glass or quartz micropipette. The microinjection pipette was guided under the glass coverslip and positioned ~ 200 μm below the cortical surface using a Luigs & Neumann translation stage (380FM-U) and manipulation equipment integrated into the 2-photon imaging system. The fluorescence of sRhoVR 1 was used to visualize the micropipette during manipulation and to provide visual feedback during pressure-microinjection into the cortical tissue. The pressure was manually adjusted to ensure visible spread of sRhoVR 1 while avoiding movement of tissue, which would indicate excessive pressure.

**Animal procedures for imaging awake mice**

For imaging in awake mice, three weeks prior to the first imaging session, mice underwent surgery for implantation of a chronic cranial window and headpost for repeated immobilization of the head. During surgery, mice were anesthetized with isoflurane (2% initially followed by 1-1.5% during surgery) in 100% oxygen; their body temperature was maintained at 37 °C. A custom-made holding bar was glued to the skull overlaying the right hemisphere. Over the Barrel cortex of the left hemisphere, a 3-mm diameter area of skull was removed and replaced by a premade glass window assembly with silicon injection port. The glass window was fixed along the perimeter with dental acrylic. Additional dental acrylic was applied around the holding bar joining to the perimeter of the window to reinforce the overall assembly. Animals received 4.8 mg/kg Dexamethasone via intraperitoneal injection 2-4 h before surgery, and 0.05 mg/kg Buprenorphine via subcutaneous injection for 1-3 days after surgery. Ibuprofen (20 mg/ml) and Sulfatrim (5 ml/250 ml) were supplied via the drinking water 1 day before until 5 days after surgery.

After surgical implantation of the bar and 5-7 days of recovery, mice were habituated in 1 session per day to accept increasingly longer periods of head restraint under the microscope objective (up to 2 h). During head restraint, the animal was placed on a suspended bed. A drop of sweetened condensed milk...
was offered every 15 min during the fixation as reward. Animals were free to readjust their body position and displayed natural grooming behavior. A video camera (Lifecam Studio, Microsoft; IR filter removed) with an NIR longpass filter (LP920-25.5, Midwest Optical) was used for continuous observation of the mouse. Infrared illumination (M940L3-IR (940 nm) LED, Thorlabs) was invisible for the PMT photodetectors and generated no imaging artifacts. The camera frames were recorded and synchronized with 2-photon imaging during data analysis. Periods of extensive body movement (e.g., grooming behavior) were excluded during data analysis.

**Two-photon imaging**

Images were obtained using an Ultima 2-photon laser scanning microscopy system (Bruker Fluorescence Microscopy). Excitation light was delivered by an Ultra II mode-locked Ti:Sapphire femtosecond laser (Coherent) tuned to 840 nm. Green (FITC) and red (sRhoVR 1) fluorophores were imaged using a cooled GaAsP (H7422P-40, Hamamatsu) and a multialkaline PMT (H7422-01, Hamamatsu) detectors, respectively. We used a combination of Zeiss 5x (Plan-NEOFLUAR, NA=0.16) and Olympus 20x (UMPlanFL, NA=0.5) objectives for a coarse approach and fine manipulation under the glass coverslip, respectively. The laser beam diameter was adjusted to overfill the back aperture. The laser power after the objective was ~30 mW. Time-resolved imaging was performed in frame-scan mode using ~200x25 pixel ROIs (~100x20 μm) acquired at ~20 Hz, ~4 μs dwell time. Image data was analyzed with custom-written software in MATLAB (MathWorks Inc.).

**Extracellular electrophysiological recordings**

Extracellular recordings of LFP and MUA were acquired using a tungsten microelectrode (FHC, 6-8 MΩ). The recorded potential was amplified and filtered into two signals: a low-frequency part (0.1–500 Hz, sampled at 2 kHz with 16 bits) and a high-frequency part (150-5,000 Hz, sampled at 20 kHz with 12 bits). The low-frequency part is referred to as the LFP. The high-frequency part was further filtered digitally between 750 and 5,000 Hz using a zero phase-shift second-order Butterworth filter to provide the MUA.
Microelectrodes were guided under the glass coverslip and positioned in cortical layer II/III within 200 μm from the imaged ROI using Luigs & Neumann translation stage (380FM-U) and manipulation equipment integrated into the Ultima system. Simultaneous 2-photon imaging and electrophysiological recordings from exactly the same location is not possible because of the photovoltaic artifact resulting from direct exposure of the metal microelectrode to focused Ti:Sapphire laser light.

**Sensory stimulation and synchronization with data acquisition**

In experiments under anesthesia, sensory stimulation was delivered to forepaw or whisker pad contralateral to the cortical exposure through a pair of thin needles inserted under the skin using weak electrical pulses (300 μs, 1 mA). In awake mice, the sensory stimulus consisted of air puffs onto the whiskers contralateral to the cortical window. We used 100-ms puffs delivered through a plastic tube (2 mm inner diameter). The tube was positioned behind the whiskers to minimize the eye blink reflex.

Stimulation devices (A365 stimulus isolator or PV830 picopump, WPI) were triggered using a separate PC that also acquired timing signals for data acquisition (“trigger out” signals for each frame/line) and physiological readings using a National Instruments IO DAQ interface (PCI-6229) controlled by custom-written software in MATLAB. The timing of each frame/line relative to the stimulus onset was determined during data analysis based on acquired triggering signals.
Table S1. Photophysical properties of sRhoVR dyes

| Compound | $\varepsilon / \text{M}^{-1} \text{cm}^{-1} (\lambda_{\text{max}}/\text{nm})^a$ | $\Phi (\lambda_{\text{max}}/\text{nm})^a$ | $\Delta F / F (%)^b$ | SNR$^b$ |
|----------|---------------------------------|------------------|-----------------|--------|
| 5        | 69000 (552)                     | 0.52 (574)       | N/A             | N/A    |
| 6        | 88000 (553)                     | 0.57 (575)       | N/A             | N/A    |
| 7        | 75000 (548)                     | 0.29 (571)       | 3 ± 0.01%       | 6:1    |
| 8        | 83000 (548)                     | 0.24 (572)       | 24 ± 2%         | 10:1   |
| 9        | 77000 (549)                     | 0.33 (572)       | 8 ± 0.02%       | 19:1   |
| 10 (sRhoVR 1) | 60000 (547)                     | 0.30 (570)       | 44 ± 0.02%      | 91:1   |

$^a$ 100% ethanol. $^b$ per 100 mV in voltage-clamped HEK cells.
Scheme S1. Synthesis of sulfonated Rhodamine Voltage Reporters (sRhoVRs)

Scheme S1: Synthesis of sulfonated Rhodamine Voltage Reporters (sRhoVRs)

1. \[ \text{Conjugation} \text{ of } \text{Phenol} \rightarrow \text{Phenol} \text{ with } \text{Rhodamine} \]

2. \[ \text{Sulfonation} \text{ of } \text{Phenol} \rightarrow \text{Sulfonic Acid} \]

3. \[ \text{Amination} \text{ of } \text{Sulfonic Acid} \rightarrow \text{Aminated Sulfonic Acid} \]

4. \[ \text{Sulfonation} \text{ of } \text{Aminated Sulfonic Acid} \rightarrow \text{Sulfonated Rhodamine} \]

5. \[ \text{Reduction} \text{ of } \text{Sulfonated Rhodamine} \rightarrow \text{Reduced Sulfonated Rhodamine} \]

6. \[ \text{Amination} \text{ of } \text{Reduced Sulfonated Rhodamine} \rightarrow \text{Aminated Reduced Sulfonated Rhodamine} \]

7. \[ \text{Sulfonation} \text{ of } \text{Aminated Reduced Sulfonated Rhodamine} \rightarrow \text{Sulfonated Aminated Reduced Sulfonated Rhodamine} \]

8. \[ \text{Amination} \text{ of } \text{Sulfonated Aminated Reduced Sulfonated Rhodamine} \rightarrow \text{Sulfonated Aminated Aminated Reduced Sulfonated Rhodamine} \]

9. \[ \text{Sulfonation} \text{ of } \text{Sulfonated Aminated Aminated Reduced Sulfonated Rhodamine} \rightarrow \text{Sulfonated Aminated Aminated Sulfonated Aminated Reduced Sulfonated Rhodamine} \]

10. \[ \text{Amination} \text{ of } \text{Sulfonated Aminated Aminated Sulfonated Aminated Reduced Sulfonated Rhodamine} \rightarrow \text{Sulfonated Aminated Aminated Aminated Sulfonated Aminated Reduced Sulfonated Rhodamine} \]

Page S10
Synthetic Details

Preparation of sulfobenzaldehydes:

\[
\text{Synthesis of } 5\text{-bromo-2-sulfobenzaldehyde, 4}
\]

5-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 48 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 (3.9 g, 99%).

\[^{1}\text{H NMR} \ (900 \text{ MHz, DMSO}-d_6) \ \delta \ 10.80 \text{ (s, 1H)}, \ 7.83 - 7.82 \text{ (m, 2H)}, \ 7.76 \text{ (d, } J = 8.1 \text{ Hz, 1H)}.\]

\[^{13}\text{C NMR} \ (226 \text{ MHz, DMSO}-d_6) \ \delta \ 192.31, \ 148.83, \ 135.64, \ 134.14, \ 129.29, \ 128.91, \ 122.43.\]

\[\text{HR-ESI-MS} \ m/z \text{ for } C_7H_4BrO_4S^- \text{ calculated: } 263.9092 \text{ found: } 262.9021 \text{ (M-H)}.\]
Preparation of Sulfonated TMR dyes:

\[ \begin{array}{c}
\begin{array}{c}
\text{Br} \\
\text{O=S=O} \\
\text{SO}_2 \text{H}
\end{array} \\
\begin{array}{c}
\text{OH} \\
\text{N}
\end{array}
\end{array} \xrightarrow{\text{MeSO}_2 \text{H}} \begin{array}{c}
\begin{array}{c}
\text{Br} \\
\text{O=S=O} \\
\text{SO}_2 \text{H}
\end{array} \\
\begin{array}{c}
\text{N} \\
\text{O} \\
\text{N}
\end{array}
\end{array} \]

Synthesis of \( N\)-(9-(4-bromo-2-sulfophenyl)-6-(dimethylamino)-3\(H\)-xanthen-3-ylidene)-\(N\)-methylmethanaminium (paraTMR, 5)

4-bromo-2-sulfobenzaldehyde (300 mg, 1.13 mmol) and 3-(dimethylamino)phenol (310 mg, 2.26 mmol) were placed in a roundbottom flask, dissolved in 3 mL of neat methanesulfonic acid, and stirred for 16 hours at 150 degrees Celsius. After cooling, the reaction mixture was diluted with 30mL water and basified with 10 mL 5M KOH. Products were extracted with ten 50 mL portions of 90:10 DCM:IPA, after which the organic layers were dried over sodium sulfate before being concentrated via rotary evaporation. The resulting dark purple oil was dissolved in 3-5 mL of DCM and then poured into an excess of diethyl ether, precipitating the product. The deep purple solid (201 mg, 35%) was collected via vacuum filtration.

\(^1\)H NMR (900 MHz, CDCl\(_3/\)MeOD) \( \delta \) 8.43 – 8.42 (m, 1H), 7.61 (dd, \( J = 8.0, 2.0 \) Hz, 1H), 7.21 (d, \( J = 9.4 \) Hz, 2H), 6.92 (d, \( J = 8.0 \) Hz, 1H), 6.80 (dd, \( J = 9.4, 2.4 \) Hz, 2H), 6.65 (d, \( J = 2.4 \) Hz, 2H), 3.19 (s, 12H).

\(^1\)C NMR (226 MHz, CDCl\(_3/\)MeOD) \( \delta \) 157.80, 157.33, 132.96, 132.48, 132.10, 132.05, 131.87, 130.63, 128.76, 128.70, 128.39, 124.21, 114.69, 113.71, 96.10, 40.76.

HR-ESI-MS m/z for \( C_{23}H_{22}BrN_2O_4S^+ \) calculated: 523.0298 found: 523.0305 (M+Na).

LR-ESI-MS m/z for \( C_{23}H_{22}BrN_2O_4S^+ \) calculated: 501.05 found: 500.84 (M)
Synthesis of \( N-(9-(5\text{-bromo-2-sulfophenyl})-6\text{-}(dimethylamino)-3\text{H-xanthen-3-ylidene})-N\text{-methylmethanaminium (metaTMR, 6)} \)

5-bromo-2-sulfobenzaldehyde (300 mg, 1.13 mmol) and 3-(dimethylamino)phenol (310 mg, 2.26 mmol) were placed in a roundbottom flask, dissolved in 3 mL of neat methanesulfonic acid, and stirred for 16 hours at 150 degrees Celsius. After cooling, the reaction mixture was diluted with 30mL water and basified with 10 mL 5M KOH. Products were extracted with ten 50 mL portions of 90:10 DCM:IPA, after which the organic layers were dried over sodium sulfate before being concentrated via rotary evaporation. The resulting dark purple oil was dissolved in 3-5 mL of DCM and then poured into an excess of diethyl ether, precipitating the product. The deep purple solid (250 mg, 44%) was collected via vacuum filtration.

\(^1\)H NMR (900 MHz, CDCl\(_3\)) \( \delta 8.27 (d, J = 8.6 \text{ Hz}, 1 \text{H}), 7.76 (d, J = 8.6, 1.9 \text{ Hz}, 1 \text{H}), 7.32 (d, J = 9.4 \text{ Hz}, 2 \text{H}), 7.24 (s, 1 \text{H}), 6.80 (d, J = 9.4, 2.3 \text{ Hz}, 2 \text{H}), 6.67 – 6.63 (m, 2 \text{H}), 3.21 (s, 12 \text{H}). \)

\(^{13}\)C NMR (226 MHz, CDCl\(_3\)) \( \delta 159.33, 157.83, 157.33, 145.49, 133.27, 133.09, 131.44, 131.42, 131.09, 123.21, 114.75, 113.77, 96.28, 40.99. \)

HR-ESI-MS m/z for C\(_{23}\)H\(_{21}\)BrN\(_2\)O\(_4\)SNa\(^+\) calculated: 523.0298 found: 523.0309 (M+Na).

LR-ESI-MS m/z for C\(_{23}\)H\(_{22}\)BrN\(_2\)O\(_4\)S\(^+\) calculated: 501.05 found: 500.85 (M)
Preparation of SulfoRhoVR dyes:

![Diagram of SulfoRhoVR dye synthesis]

**Synthesis of paraSulfoRhoVR, Compound 7**

paraTMR (100 mg, 0.19 mmol), B (60 mg, 0.24 mmol), palladium acetate (2 mg, 0.008 mmol), and P(o-tol)_3 (5.4 mg, 0.018 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 110 degrees Celsius. After cooling, the reaction mixture was diluted with 10 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 crude solid was isolated via vacuum filtration (70 mg, 52%, 85% pure by HPLC). A small amount of material was purified via RP-HPLC for further characterization (13 mg, 10%).

**^1H NMR** (900 MHz, CDCl_3/MeOD) δ 8.25 (s, 1H), 7.54 (d, J = 7.4 Hz, 1H), 7.40 (d, J = 7.9 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 7.33 (d, J = 8.3 Hz, 2H), 7.16 (d, J = 9.3 Hz, 2H), 7.08 (d, J = 16.3 Hz, 2H), 6.96 (dd, J = 18.4, 12.1 Hz, 3H), 6.83 (d, J = 16.2 Hz, 2H), 6.79 (d, J = 8.1 Hz, 2H), 6.74 – 6.71 (m, 1H), 6.60 (d, J = 2.2 Hz, 1H), 3.18 (s, 12H), 3.11 (s, 6H).

**^13C NMR** (226 MHz, CDCl_3/MeOD) δ 158.09, 157.62, 139.88, 138.20, 135.75, 133.24, 132.75, 132.25, 131.35, 130.26, 130.14, 129.04, 128.99, 128.79, 128.07, 127.45, 126.83, 126.40, 115.06, 113.87, 96.22, 46.67, 40.80.
HR-ESI-MS m/z for C₄₁H₄₀N₃O₄NaS⁺ calculated: 692.2553 found: 692.2558 (M+Na).

LR-ESI-MS m/z for C₄₁H₄₀N₃O₄S⁺ calculated: 670.27 found: 670.23.

Synthesis of paraSulfoRhoVR_OMe, Compound 8

paraTMR (100 mg, 0.20 mmol), A (73 mg, 0.24 mmol), palladium acetate (2 mg, 0.008 mmol), and P(o-tol)₃ (5.4 mg, 0.018 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 Et₃N (1 mL) were added via syringe and the reaction was stirred for 16 hours at 110 degrees Celsius. After cooling, the reaction mixture was diluted with 10 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 violet solid. The crude solid was isolated via vacuum filtration (80 mg, 55%, 50% pure by HPLC). A small amount of material was purified via RP-HPLC for further characterization (8 mg, 6%).

¹H NMR (900 MHz, CDCl₃/MeOD) δ 8.37 (s, 1H), 7.61 (d, J = 8.2 Hz, 1H), 7.59 – 7.57 (m, 1H), 7.56 (d, J = 9.4 Hz, 1H), 7.53 (dd, J = 12.1, 7.1 Hz, 1H), 7.48 (d, J = 4.0 Hz, 3H), 7.36 (d, J = 16.5 Hz, 1H), 7.25 (d, J = 9.6 Hz, 3H), 7.16 (d, J = 16.4 Hz, 1H), 7.06 (d, J = 16.5 Hz, 1H), 7.02 (d, J = 7.7 Hz, 1H), 6.85 (d, J = 8.2 Hz, 1H), 6.79 (dd, J = 9.5, 2.4 Hz, 1H), 6.64 (d, J = 2.4 Hz, 2H), 3.87 (s, 3H), 3.27 (m, 4H) 3.18 (s, 12H), 1.10 (t, J = 7.2 Hz, 6H).
$^{13}$C NMR (226 MHz, CDCl$_3$/MeOD) $\delta$ 160.67, 158.08, 157.83, 157.33, 145.55, 139.45, 137.43, 136.34, 133.13, 132.38, 132.05, 130.89, 129.69, 128.76, 128.70, 128.19, 127.84, 127.25, 127.16, 126.74, 126.36, 122.08, 114.87, 113.62, 95.97, 56.11, 40.67, 34.68, 29.70.

**HR-ESI-MS** m/z for C$_{44}$H$_{46}$N$_3$O$_5$S$^+$ calculated: 728.3153 found: 728.3156.

**LR-ESI-MS** m/z for C$_{44}$H$_{46}$N$_3$O$_5$S$^+$ calculated: 728.32 found: 727.98.
Synthesis of metaSulfoRhoVR, Compound 9

isoTMR (100 mg, 0.19 mmol), B (60 mg, 0.24 mmol), palladium acetate (2 mg, 0.008 mmol), and P(o-tol)₃ (5.4 mg, 0.018 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 Et₃N (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 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 crude solid was isolated via vacuum filtration (83 mg, 62%, 70% pure by HPLC). A small amount of material was purified via RP-HPLC for further characterization (9 mg, 7%).

¹H NMR (900 MHz, CDCl₃/MeOD) δ 8.11 (d, J = 8.3 Hz, 2H), 7.66 (d, J = 9.6 Hz, 2H), 7.37 – 7.33 (m, 5H), 7.22 (d, J = 9.5 Hz, 2H), 7.14 (s, 1H), 7.04 (d, J = 16.2 Hz, 2H), 6.98 (d, J = 16.2 Hz, 1H), 6.96 (d, J = 16.2 Hz, 1H), 6.83 (d, J = 16.2 Hz, 1H), 6.78 (dt, J = 8.2, 4.1 Hz, 3H), 6.64 (d, J = 2.3 Hz, 1H), 3.24 (s, 12H), 3.16 (s, 6H).

¹³C NMR (226 MHz, CDCl₃/MeOD) δ 157.68, 157.20, 138.88, 137.94, 134.96, 132.88, 131.10, 129.91, 128.85, 128.63, 127.62, 127.47, 126.93, 126.81, 126.36, 125.54, 124.86, 114.57, 114.00, 113.54, 95.79, 41.38, 40.45.

HR-ESI-MS m/z for C₄₁H₄₀N₅O₄S⁺ calculated: 670.2734 found: 670.2738.

LR-ESI-MS m/z for C₄₁H₄₀N₅O₄S⁺ calculated: 670.27 found: 670.19.
Synthesis of metaSulfoRhoVR_OMe, sRhoVR 1, Compound 10

isoTMR (100 mg, 0.20 mmol), A (73 mg, 0.24 mmol), palladium acetate (2 mg, 0.008 mmol), and P(o-tol)_3 (5.4 mg, 0.018 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 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 violet solid. The crude solid was isolated via vacuum filtration (82 mg, 56%, 65% pure by HPLC). A small amount of material was purified via RP-HPLC for further characterization (12 mg, 8%).

^1H NMR (900 MHz, CDCl_3) δ 8.19 (d, J = 9.4 Hz, 1H), 8.02 (s, 1H), 7.69 (d, J = 8.3 Hz, 1H), 7.38 (t, J = 10.2 Hz, 2H), 7.35 (d, J = 7.4 Hz, 3H), 7.31 – 7.29 (m, 2H), 7.15 (s, 1H), 7.06 (d, J = 16.2 Hz, 1H), 6.99 (d, J = 16.2 Hz, 1H), 6.84 (d, J = 16.4 Hz, 1H), 6.82 – 6.80 (m, 2H), 6.66 (d, J = 2.3 Hz, 2H), 6.27 (s, 1H), 6.17 (s, 1H), 3.80 (s, 3H), 3.32 (q, J = 8.3, 7.6 Hz, 4H), 3.19 (s, 12H), 1.12 (t, J = 7.0 Hz, 6H).

^13C NMR (226 MHz, CDCl_3/MeOD) δ 166.12, 160.64, 158.33, 157.74, 157.21, 143.75, 138.70, 134.40, 134.11, 133.14, 132.23, 131.94, 131.90, 131.07, 129.85, 129.42, 129.08, 128.62, 128.57, 127.49, 127.41, 126.87, 126.61, 126.23, 125.28, 124.01, 114.73, 113.57, 95.82, 67.78, 55.38, 40.57, 29.59.

HR-ESI-MS m/z for C_{44}H_{46}N_{3}O_{5}S^+ calculated: 728.3153 found: 728.3175.

LR-ESI-MS m/z for C_{44}H_{46}N_{3}O_{5}S^+ calculated: 728.32 found: 728.05.
**SI Figures:**

*Figure S1.* Absorption (UV/vis) and fluorescence emission spectra of compounds a) 5, b) 6, c) 7, d) 8, e) 9, and f) 10. Black lines indicate the UV/vis absorption spectra. Red lines indicate the fluorescence emission spectra. Spectra were acquired in ethanol. For fluorescence emission spectra, excitation was provided at 10 nm longer wavelength than the respective absorbance maximum.
Figure S2. Confocal imaging of sRhoVR dyes in HEK cells. HEK cells were stained with 200 nM in HBSS sRhoVR derivative 7 (a), 8 (b), 9 (c), or 10 (d, also known as sRhoVR 1) for 15 minutes at 37 °C. Cells were transferred to fresh HBSS (without any dye) and imaged via confocal laser scanning microscopy. All imaging parameters are identical. Histogram display values are identical; panels a' and b' have been brightened 5x to indicate the staining patterns of compounds 7 and 8. Scale bar is 20 μm. The fluorescence intensity of the membrane staining is quantified in panel e). Values represent the mean fluorescence of membrane ROIs containing HEK cells stained with compounds 7-10. n = 15. Error bars are ± standard deviation.
Figure S3. Voltage sensitivity of compounds 7 to 9, determined by fluorescence microscopy and electrophysiology. Average voltage sensitivity plots of percentage change in fluorescence vs. membrane potential values from whole-cell voltage-clamp electrophysiology of 5 HEK cells each for dyes 7 (a), 8 (b), and 9 (c). Error bars indicate standard error of the mean for \(n=5\) different cells. Summaries reveal average voltage sensitivities of 3%, 24%, and 8%, respectively. Representative traces of fractional change in fluorescence vs. time from whole-cell voltage-clamp electrophysiology for compounds 7 (d), 8 (e), and 9 (f).
Figure S4. Comparison of several action potential kinetic parameters with and without the presence of SulfoRhoVR 1. Rat hippocampal neurons were subjected to patch clamp electrophysiology under current clamp mode in whole-cell configuration, with or without the presence of SulfoRhoVR 1. Trains of action potentials were evoked (10 AP, 20 Hz) via current injection. Values for a) time to peak potential, b) action potential half-width, c) rise time constant, d) rise time from 10% to 90% of peak height, e) decay time from 90% to 10% of peak, and f) capacitance are displayed as mean values from n = 7 cells for each condition with each cell’s average action potential determined from 10 action potentials each. Error bars are ±SEM and all p values are > 0.05 (Student’s t-test, two tailed).
Figure S5. Two-photon absorption cross sections of a) sulfoTMR dyes 5 and b) 6, c) sRhoVR 1 (10), and d) RVF5. SulfoTMR spectra are taken in 100% ethanol, while RVF5 and sRhoVR 1 are in PBS (pH 7.4). Panel e) directly compares the spectral of sRhoVR 1 and RVF5.
Figure S6. Quadratic Dependence of SulfoRhoVR 1 2P Brightness on Laser Power. Plot of fluorescence intensity vs. percentage laser power reveals a quadratic dependence of fluorescence on laser power for SulfoRhoVR 1. Line of best fit via least squares regression is $y = 1.19x^2 - 14.78x + 59.73$. $R^2 = 0.987$. Error bars are ±standard deviation for $n = 3$ experiments.
Figure S7. 2P imaging of spontaneous activity in cultured mouse neurons with SulfoRhoVR 1. (a) Sample still frame from spontaneous activity imaging. (b-c) Robust spontaneous spiking is observed via 2P imaging of SulfoRhoVR 1. Addition of TTX (1 µM) abolishes activity. Traces are single trials and are not filtered.
Figure S8. Characterization of sRhoVR in mouse brains and rat neurons. a) Zoomed-in image of sRhoVR staining in mouse cortex, approximately 200 μm below the surface of the brain, as in Figure 4c and 4d in the main text. Scale bar is 40 μm. b) Plot of relative fluorescence intensity (F/F_{max}) for RVF5 (black trace) and sRhoVR (red trace) in mouse cortex, approximately 200 μm below the surface of the brain. The thick line is the average fluorescence intensity for n = 15 (RVF5, black) or 9 (sRhoVR) different imaging trials. The shaded area indicates the upper and lower bound of the standard deviation. Illumination intensity at 840 nm was 30 mW for RVF5 and 31 mW for sRhoVR. c) Plot of relative fluorescence intensity (F/F_{max}) for RVF5 (black trace) and sRhoVR (red trace) in cultured rat hippocampal neurons under identical two-photon illumination. The thick line is the average fluorescence intensity for n = 3 different neurons. The shaded area indicates the upper and lower bound of the standard deviation.

References

[1] Kulkarni, R. U., Kramer, D. J., Pourmandi, N., Karbasi, K., Bateup, H. S., and Miller, E. W. (2017) Voltage-sensitive rhodol with enhanced two-photon brightness, Proc Natl Acad Sci U S A 114, 2813-2818.

[2] Xu, C., and Webb, W. W. (1996) Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm, J Opt Soc Am B 13, 481-491.