We describe the design, synthesis, and application of voltage-sensitive silicon rhodamines. Based on the Berkeley Red Sensor of Transmembrane potential, or BeRST, scaffold, the new dyes possess an isomeric molecular wire for improved alignment in the plasma membrane and contain 2' carboxylic acids for ready functionalization. Conjugation with secondary amines affords tertiary amides that localize to cellular membranes and respond to voltage changes with a 24% ΔF/F per 100 mV. When combined with a flexible polyethyleneglycol (PEG) linker and a chloroalkane HaloTag ligand, the new indicators, or isoBeRST dyes, enable voltage imaging from genetically defined cells and neurons. Covalent ligation of isoBeRST to cell surface-expressed HaloTag enzymes provides up to 3-fold improved labeling over previous, rhodamine-based hybrid strategies. We show that isoBeRST-Halo hybrid indicators achieve single-trial voltage imaging of membrane potential dynamics from dissociated rat hippocampal neurons or mouse cortical neurons in brain slices. With far-red/near infrared excitation and emission, turn-on response to action potentials, effective cell labeling in thick tissue, and excellent photostability, the new isoBeRST-Halo derivatives provide an important complement to voltage imaging in neurobiology.
A silicon-rhodamine chemical-genetic hybrid for far red voltage imaging from defined neurons in brain slice

Gloria Ortiz,‡ Pei Liu,‡ Parker E. Deal,‡ Ashley K. Nensel,‡ Kayli N. Martinez,‡ Kiarash Shamardani,§ Hillel Adesnik,§† and Evan W. Miller‡§†*

Departments of ‡Chemistry and §Molecular & Cell Biology and †Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States.

Supporting Information Placeholder

ABSTRACT: We describe the design, synthesis, and application of voltage-sensitive silicon rhodamines. Based on the Berkeley Red Sensor of Transmembrane potential, or BeRST, scaffold, the new dyes possess an isomeric molecular wire for improved alignment in the plasma membrane and contain 2’ carboxylic acids for ready functionalization. Conjugation with secondary amines affords tertiary amides that localize to cellular membranes and respond to voltage changes with a 24% ∆F/F per 100 mV. When combined with a flexible polyethylene glycol (PEG) linker and a chloroalkane HaloTag ligand, the new indicators, or isoBeRST dyes, enable voltage imaging from genetically defined cells and neurons. Covalent ligation of isoBeRST to cell surface-expressed HaloTag enzymes provides up to 3-fold improved labeling over previous, rhodamine-based hybrid strategies. We show that isoBeRST-Halo hybrid indicators achieve single-trial voltage imaging of membrane potential dynamics from dissociated rat hippocampal neurons or mouse cortical neurons in brain slices. With far-red/near infrared excitation and emission, turn-on response to action potentials, effective cell labeling in thick tissue, and excellent photostability, the new isoBeRST-Halo derivatives provide an important complement to voltage imaging in neurobiology.

Voltage imaging in the central nervous system promises to transform the ways in which we observe brain systems.1–2 Recently, a number of approaches to voltage imaging have emerged, including methods that rely solely on synthetic dyes3–9 or genetically encoded proteins.10–17 Alternatively, hybrid methodologies can combine the unique properties of synthetic dyes—high molecular brightness, wide availability of colors, or fast response kinetics—with the cellular specificity of genetically encoded methods.18–22 Our group recently reported the development of a completely synthetic voltage-sensitive fluorophore, Berkeley Red Sensor of Transmembrane potential 1, or BeRST 1, a silicon-rhodamine-based indicator that we hypothesize operates via voltage-sensitive photoinduced electron transfer (PeT).24 The high sensitivity (24% ∆F/F per 100 mV), fast response kinetics, photostability, and far red/near infra-red excitation and emission profile have enabled the use of BeRST 1 in a number of voltage imaging applications.25–31

However, the use of BeRST 1 has been largely restricted to in vitro systems of homogeneous cell types. Usage in more complex settings, like thick brain tissue, remains a challenge because of a lack of methods to genetically target BeRST 1 to defined cells. Here we report two new synthetic BeRST dyes and show that this new class of indicator can be combined with a genetically-encoded protein tether to enable voltage imaging from defined cells in mouse brain slice.

To enable genetic targeting of BeRST-style dyes, we redesigned the synthesis of BeRST. We replaced the 2’-sulfonate of BeRST with a carboxylate: this allows for addition of covalent tethers and mimics our previous design success with Rhodamine-based Voltage Reporters (RhoVRs).32–33 We also used the 5’ version of molecular wire, since the 5’, or isomeric, version showed improved voltage sensitivity compared to the 4’ RhoVR.34 Additionally, the commercial availability of the precursors to the aldehyde starting material substantially simplified the synthetic route (Scheme S1). The optimized synthesis of isoBeRST-sarc 10 begins with a Heck reaction between fluorophore 134–35 and (E)-3-methoxy-N,N-dimethyl-4-(4-vinylstyrlyl)aniline16 to obtain carboxy silicon rhodamine 8 (Scheme S1). Dye 8 is coupled to sarcosine tert-butyl ester using oxaaryl chloride, followed
Table 1. Properties of isoBeRST indicators

| Compound             | \( \lambda_{\text{max}} \) / nm | \( \lambda_{\text{em}} \) / nm | \( \varepsilon \) | \( \Phi^{a,b} \) | \( \% \Delta F/F \) / 100 mV | Relative brightness\(^d\) |
|----------------------|-------------------------------|-------------------------------|-------------------|----------------------|-------------------------------|--------------------------|
| isoBeRST-pipcys 6    | 662                           | 681                           | 172,000           | 0.061                | 24 ± 1.9                      | 100%                     |
| isoBeRST-Halo 7      | 662                           | 677                           | -                 | 0.042                | 21 ± 1.2                      | 30%                      |
| isoBeRST-sarc 10     | 661                           | 681                           | 107,700           | 0.098                | 24 ± 2.6                      | -                        |

\(^a\)In PBS, pH 7.4, 0.1% SDS. \(^b\)Referenced to Cy5.5-carboxylic acid in PBS. \(^c\)Voltage-clamped HEK cells. Error is ± S.D. for n = 5-6 cells. \(^d\)In HEK cells. Error is ± S.E.M for n = 4 coverslips (>100 cells per coverslip for relative brightness).

All of the new Si-rhodamine indicators are voltage-sensitive. In human embryonic kidney (HEK) cells, targeted dyes isoBeRST-sarc 10 (Figure S1) and isoBeRST-pipcys 6 (Figure S2) localize to the plasma membrane and are voltage sensitive. IsoBeRST-pipcys 6 has a voltage sensitivity of 24±2% \( \Delta F/F \) per 100 mV (SNR = 110 ± 15), identical to BeRST 1 (24±2% \( \Delta F/F \) per 100 mV)\(^4\) and to isoBeRST-sarc 10 (Table 1). We selected isoBeRST-pipcys 6 to evaluate in neurons because of the higher yielding synthesis and stability compared to isoBeRST-sarc 10. In cultured rat hippocampal neurons, isoBeRST-pipcys 6 (500 nM) provided clear resolution of action potentials (Figure S3).

The genetically-targetable isoBeRST-Halo 7 selectively labels HEK cells expressing cell-surface HaloTag (Figure 1 and S4). We expressed HaloTag on the surface of mammalian cells using a fusion with a single-pass transmembrane domain.\(^3\) At 500 nM isoBeRST-Halo 7, cells expressing cell-surface HaloTag are approximately 14-fold brighter than un-transfected control cells (Figure S4f). At lower concentrations (50 nM), fluorescence intensity in HaloTag-expressing cells increases to approximately 30-fold over non-HaloTag expressing cells (Figure S4f). This is three times better contrast than RhoVR-Halo labeling (10-15 fold).\(^3\) Although expression levels of HaloTag vary slightly with transient transfection, a screen of isoBeRST-Halo 7 concentrations reveals that HaloTag binding sites appear to saturate at around 50 to 100 nM (Figure S4f). The drop in contrast ratio, from ~30-fold at 50 nM to about 14-fold at 500 nM comes from a small increase in background staining in control cells (an increase of about 4 percentage points, from 5% to 9%). Importantly, isoBeRST 7 (50 nM) is voltage-sensitive, with a voltage sensitivity of 20±1% \( \Delta F/F \) per 100 mV and an SNR of 42 ± 7 (Figure 1 and Table 1). IsoBeRST-Halo 7 maintains about the same voltage sensitivity as isoBeRST-pipcys 6 (500 nM), indicating that the covalently tethered dye remains properly oriented in the plasma membrane (Table 1 and Figure 1).
Figure 1. Cellular and in vitro characterization of isoBeRST-Halo 7. a) Normalized absorbance (solid line) and emission (dashed line) spectra of isoBeRST-Halo 7 in PBS, pH 7.4. b) Plot of the fractional change in fluorescence of 7 vs time for 100 ms hyper- and depolarizing steps (±100 mV in 20 mV increments) from a holding potential of -60 mV for single HEK cells under whole-cell voltage-clamp mode. c) Plot of % ΔF/F vs final membrane potential. Data are mean ± S.D. for n = 6 cells. d-g) Wide-field microscopy images of HEK cells transfected with CMV-HaloTag-pDisplay and stained with isoBeRST-Halo 7 (50 nM, 30 mins). d) DIC image of HEK cells. e) Nuclear EGFP fluorescence indicates HaloTag expression. f) isoBeRST-Halo fluorescence. g) Merge of fluorescence from EGFP (green) and isoBeRST-Halo (magenta). Scale bar is 10 μm.

Covalently-tethered isoBeRST-Halo 7 visualizes voltage changes in genetically-defined neurons. Dissociated, cultured rat hippocampal neurons transfected with HaloTag under control of the synapsin promoter (to drive neuron-specific expression) were labeled with 50 nM isoBeRST-Halo. Neurons expressing HaloTag show excellent selectivity, revealing good localization of the dye to the outer membrane (Figure 2 and S5). The best contrast between HaloTag-expressing and control cells is achieved using 50 nM isoBeRST-Halo (50x brighter than untransfected cells) when compared to 100 nM isoBeRST-Halo (30x brighter than untransfected cells). High isoBeRST-Halo fluorescence correlates with high levels of HaloTag/GFP (Figure S5e-g). Using these optimized loading conditions, we demonstrated the ability to record spontaneous and evoked activity in neurons (Figure 2e and Figure S6). IsoBeRST-Halo responded to field stimulated evoked action potentials with a 10% ± 0.3% ΔF/F and SNR of 15 ± 1 (19 cells).

We next evaluated the ability of isoBeRST-Halo 7 to monitor voltage dynamics from neurons in brain slice. We introduced genes for HaloTag and a co-expression marker, blue fluorescent protein, or BFP, on separate plasmids via in utero electroporation in mouse embryos.37 We prepared tissue slices from the brains of these mice and stained the slices with isoBeRST-Halo 7 (250 to 500 nM, 15 min). Confocal fluorescence microscopy reveals localization of isoBeRST-Halo fluorescence in the cell membranes of neurons that express BFP and HaloTag (Figure 3a,b). Both cell bodies and more distal processes like axonal and dendritic membranes appear fluorescent (Figure 3a,b and Figure S7), mirroring results in dissociated rat neurons (Figure 2). Unlabeled

Figure 2. Monitoring spontaneous activity in neurons with isoBeRST-Halo 7. a-d) Wide-field microscopy images of isoBeRST-Halo in a HaloTag-expressing neuron. a) DIC image of neurons. b) Nuclear EGFP fluorescence indicates HaloTag expression. c) Merge of EGFP (green) and isoBeRST-Halo (magenta) fluorescence. d) isoBeRST-Halo fluorescence is restricted to the membrane. Scale bar is 20 μm. e) Optical recordings at 500 Hz (1.94 W/cm²) of spontaneous activity shown as ΔF/F vs time for HaloTag-expressing neurons from different coverslips labeled with 7.
Voltage imaging with isoBeRST-Halo (7) provides an important complement to voltage imaging efforts. It offers a turn-on indicator for action potentials, possesses an excitation spectrum aligned with common excitation sources, operates in the far-red / near infrared, and takes advantage of the high photostability of silicon-rhodamines.24 In the future, we will maximize expression of cell-surface HaloTag, since one limitation of the covalent tethering approach is that the stoichiometric labeling limits the number of indicators that can be added to a cell membrane. Finally, we envision that isoBeRST-Halo can pair with optically33 and enzymatically orthogonal hybrid genetic labeling strategies38-40 to provide multi-color voltage imaging in complex tissues.

Supporting Information
Supplementary data, including supporting figures, spectra, procedures, and analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

Corresponding Author
* Evan W. Miller, evanwmiller@berkeley.edu

ACKNOWLEDGMENT
Research in the Miller lab is supported by grants from the NIH (R01NS08088) and Klingenstein-Simon Foundations (40746). E.W.M. and H.A. acknowledge support from NSF Neuronx (707350). G.O. was supported by a Gilliam Research Fellowship from Howard Hughes Medical Institute. P.L. was supported by a graduate fellowship from A*STAR. K.N.M. was supported in part by a training grant from the NIH (T32GM066698). Confocal imaging experiments were performed at the CRL Molecular Imaging Center, supported by the Helen Wills Neuroscience Institute. HRMS data were collected at the QB3/Chemistry Mass Spectrometry Facility (UC Berkeley) with the assistance of Dr. Ulla N. Andersen.

REFERENCES
1. Peterka, D. S.; Takahashi, H.; Yuste, R., Imaging Voltage in Neurons. Neuron 2011, 69 (1), 9-21.
2. Scanziani, M.; Häusser, M., Electrophysiology in the age of light. Nature 2009, 461 (7266), 930-939.
3. Liu, P.; Miller, E. W., Electrophysiology, Unplugged: Imaging Membrane Potential with Fluorescent Indicators. Accounts of Chemical Research 2020, 53 (1), 1-10.
4. Sayresmith, N. A.; Saminathan, A.; Sailer, J. K.; Patberg, S. M.; Sandor, K.; Krishnan, Y.; Walter, M. G., Photostable Voltage-Sensitive Dyes Based on Simple, Solvatochromic, Asymmetric Thiazolothiazoles. Journal of the American Chemical Society 2019, 141 (47), 18790-18790.
5. Reeve, J. E.; Corbett, A. D.; Boczarow, I.; Kaluza, W.; Barford, W.; Bayley, H.; Wilson, T.; Anderson, H. L., Porphyrins for Probing Electrical Potential Across Lipid Bilayer Membranes by Second Harmonic Generation. Angewandte Chemie International Edition 2013, 52 (34), 9044-9048.
6. Rowland, C. E.; Susumu, K.; Stewart, M. H.; Oh, E.; Mäkinen, A. J.; O’Shaughnessy, T. J.; Kusho, G.; Wolak, M. A.; Erickson, J. S.; L. Efros, A.; Huston, A. L.; Delehanty, J. B., Electric Field Modulation of Semiconductor Quantum Dot Photoluminescence: Insights Into the Design of Robust Voltage-Sensitive Cellular Imaging Probes. Nano Letters 2015, 15 (10), 6848-6854.
7. Yan, P.; Acker, C. D.; Zhou, W. L.; Lee, P.; Bollensdorff, C.; Negrea, A.; Lotti, J.; Sacconi, L.; Antic, S. D.; Kohl, P.; Mansvelder, H. D.; Pavone, F. S.; Loew, L. M., Palette of fluorinated voltage-sensitive hemicyanine dyes. Proceedings of the National Academy of Sciences of the United States of America 2012, 109 (50), 20443-8.
8. Miller, E. W.; Lin, J. Y.; Frady, E. P.; Steinbach, P. A.; Kristan, W. B.; Tsien, R. Y., Optically monitoring voltage in neurons by photo-
induced electron transfer through molecular wires. Proceedings of the National Academy of Sciences 2012, 109 (6), 2114-2119.
9. Treger, J. S.; Priest, M. F.; Iezzi, R.; Bezanilla, F., Real-time imaging of electrical signals with an infrared FDA-approved dye. Biophysical journal 2014, 107 (6), L99-12.
10. Piatekiewicz, K. D.; Jung, E. E.; Straub, C.; Linghu, C.; Park, D.; Suk, H. J.; Hochbaum, D. R.; Goodwin, D.; Pneumatikakis, E.; Pak, N.; Kawashima, T.; Yang, C. T.; Rhodes, J. L.; Shemesh, O.; Asano, S.; Yoon, Y. G.; Freifeld, L.; Saulnier, J. L.; Riegler, C.; Engert, F.; Hughes, T.; Drozhiev, M.; Szabo, B.; Ahrens, M. B.; Flavell, S. W.; Sabatini, B. L.; Boyden, E. S., A robotic multidimensional directed evolution approach applied to fluorescent voltage reporters. Nature chemical biology 2018, 14 (4), 352-360.
11. Hochbaum, D. R.; Zhao, Y.; Farhi, S. L.; Klapoetke, N.; Werley, C. A.; Kapoor, V.; Zou, P.; Kralli, J. M.; Maclaurin, D.; Smeder-Margulis, N.; Saulnier, J. L.; Bouling, G. L.; Straub, C.; Cho, Y. K.; Melkonian, M.; Wong, G. K.; Harrison, D. J.; Murthy, V. N.; Sabatini, B. L.; Boyden, E. S.; Campbell, R. E.; Cohen, A. E., All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. Nature methods 2014, 11 (8), 825-33.
12. Kammann, M.; Vasan, G.; Huang; C.; Haziza, S.; Li, J. Z.; Inan, H.; Schnitzer, M. J.; Pierbire, V. A., Fast, in vivo voltage imaging with a red fluorescent indicator. Nature methods 2018, 15 (12), 1108-1116.
13. Abdelfattah, A. S.; Farhi, S. L.; Zhao, Y.; Brinks, D.; Zou, P.; Ruangkittisasuk, A.; Platsia, J.; Pierbire, V. A.; Ballanyi, K.; Cohen, A. E.; Campbell, R. E., A Bright and Fast Red Fluorescent Protein Voltage Indicator That Reports Neuronal Activity in Organotypic Brain Slices. The Journal of neuroscience : official journal of the Society for Neuroscience 2016, 36 (8), 2458-72.
14. Jin, L.; Han, Z.; Platsia, J.; Woottorton, Julian R. A.; Cohen, Lawrence B.; Pierbire, Vincent A.; Single Action Potentials and Subthreshold Electrical Events Imagined in Neurons with a Fluorescent Protein Voltage Probe. Neuron 2012, 75 (6), 779-785.
15. Jin, L.; Huang, Z.; Ginebaugh, S. P.; Araneda, R. C.; Kristan, W. B.; Kubiak, C. P.; Miller, E. W.; Abdullatif, S. H.; Miller, E. W., Bioorthogonal double fluorogenic siloxane Rhodamine Voltage Reporters for High Speed Functional Imaging in derived cardiomyocytes. Journal of the American Chemical Society 2015, 137 (92), 14374-14377.
16. Kozma, E.; Estrada Girona, G.; Paci, G.; Lemke, E. A.; Kele, P., Bioorthogonal double-fluoroionogenic siliconrhodamine probes for intracellular super-resolution microscopy. Chemical Communications 2017, 53, 6696-6699.
17. Woodford, C. R.; Frady, E. P.; Smith, R. S.; Morey, B.; Canzi, G.; Palida, S. F.; Araneda, R. C.; Kubiak, C. P.; Miller, E. W.; Tsien, R. Y., Improved PeT Molecules for Optically Sensing Voltage in Neurons. Journal of the American Chemical Society 2015, 137 (5), 1817-1824.
18. Tabata, H.; Nakajima, K., Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. Neuron 2001, 31 (3), 865-872.
19. Liu, P.; Grenier, V.; Hong, W.; Muller, V. R.; Schreiter, E. R., Bright and photoactivatable chemogenetic indicators for extended in vivo voltage imaging. Science (New York, N.Y.) 2019, 365 (6454), 699-704.
20. Huang, Y.-L.; Walker, A. S.; Miller, E. W., A Photostable Silicon Rhodamine Platform for Optical Voltage Sensing. Journal of the American Chemical Society 2015, 137 (33), 10767-10776.
21. Ginebaugh, S. P.; Cypbers, E. D.; Lanka, V.; Ortiz, G.; Miller, E. W.; Laghaei, R.; Meriney, S. D., The Frog Motor Nerve Terminal Has Very Brief Action Potentials and Three Electrical Regions Predicted to Differentially Control Transmitter Release. Journal of Neuroscience 2020, 40 (8), 3594-3596.
22. Klimas, A.; Ortiz, G.; Boggess, S. C.; Miller, E. W.; Entcheva, E., Multimodal on-axis platform for all-optical electrophysiology with near-infrared probes in human stem-cell-derived cardiomyocytes. Progress in Biophysics & Molecular Biology 2020, 154, 62-70.
23. McNamara, H. M.; Dodson, S.; Huang, Y. L.; Miller, E. W.; Sandstedt, B.; Cohen, A. E., Geometry-Dependent Arrhythmias in Electrically Excitable Tissues. Cell Systems 2018, 7 (4), 359-75.
24. McNamara, H. M.; Salegane, R.; Al Tanoury, Z.; Xu, H. T.; Begum, S.; Ortiz, G.; Pourquie, O.; Cohen, A. E., Bioelectrical domain walls in homogeneous tissues. Nature Physics 2020, 16 (3), 357-75.
Authors are required to submit a graphic entry for the Table of Contents (TOC) that, in conjunction with the manuscript title, should give the reader a representative idea of one of the following: A key structure, reaction, equation, concept, or theorem, etc., that is discussed in the manuscript. Consult the journal’s Instructions for Authors for TOC graphic specifications.

A silicon-rhodamine chemical-genetic hybrid for far red voltage imaging from defined neurons in brain slice
Supporting Information for

A silicon-rhodamine chemical-genetic hybrid for far red voltage imaging from defined neurons in brain slice
Gloria Ortiz,‡ Pei Liu,‡ Parker E. Deal,‡ Ashley K. Nensel,‡ Kayli N. Martinez,‡ Kiarash Shamardani,§ Hillel Adesnik,§† and Evan W. Miller†‡†
Departments of ‡Chemistry and §Molecular & Cell Biology and †Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States.
DOI: [place-holder]

Table of Contents
Chemical Synthesis and characterization ........................................................................................................... 3
Spectroscopic Studies ........................................................................................................................................... 3
Cell culture .......................................................................................................................................................... 3
  HEK cell culture ............................................................................................................................................. 3
  Primary neuronal culture and transfection ..................................................................................................... 4
  In utero electroporation ................................................................................................................................ 4
  Acute brain slice preparation ......................................................................................................................... 4
Epifluorescence microscopy ................................................................................................................................ 4
Brain slice staining, imaging, and electrophysiology ....................................................................................... 5
Image analysis .................................................................................................................................................... 5
Electrophysiology and Imaging in HEK cells and primary cultured neurons ..................................................... 6
DNA constructs .................................................................................................................................................. 6
  IgK .................................................................................................................................................................... 6
  HaloTag ............................................................................................................................................................ 6
  HA ................................................................................................................................................................. 7
  pDisplay ........................................................................................................................................................ 7
  IRES .............................................................................................................................................................. 7
  Nuclear Localization Sequence ...................................................................................................................... 7
  EGFP ............................................................................................................................................................. 7
  WPRE ............................................................................................................................................................ 8
  CMV promoter ............................................................................................................................................. 8
  Synapsin Promoter ....................................................................................................................................... 8
Synthetic Procedures ........................................................................................................................................... 9
  N-(10-(5-bromo-2-(4-(tert-butoxycarbonyl)piperazine-1-carbonyl)phenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (2): ............................................. 9
N-(10-(5-bromo-2-(piperazine-1-carbonyl)phenyl)-7-(dimethylamino)-5,5-dimethylbenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (3):.................................................................................. 9
(R)-3-(4-(4-bromo-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)benzoyl)piperazin-1-yl)-2-((tert-butoxycarbonyl)amino)-3-oxopropane-1-sulfonate (4):.............................. 9
2-((tert-butoxycarbonyl)amino)-3-(4-(4-(E)-4-(dimethylamino)-2-methoxystyrlyl)styryl)-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)benzoyl)piperazin-1-yl)-3-oxopropane-1-sulfonate (5): .................................................................................................................. 10
isoBeRST-pipcys (6): ........................................................................................................... 10
isoBeRST-Halo (7): .................................................................................................................. 11
4-((E)-4-((E)-4-(dimethylamino)-2-methoxystyrlyl)styryl)-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)benzoate (8): .................................................................................................................. 11
N-(10-((2-(tert-butoxy)-2-oxoethyl)(methyl)carbamoyl)-5-((E)-4-((E)-4-(dimethylamino)-2-methoxystyrlyl)styryl)phenyl)-7-(dimethylamino)-5,5-dimethylbenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (9):.................................................................................................................. 12
isoBeRST-sarcosine (10): ........................................................................................................ 12

Figures and Schemes ................................................................................................................. 13

Scheme S1. Synthesis of isoBeRST-sarcosine ......................................................................... 13
Figure S1. ................................................................................................................................. 14
Figure S2. ................................................................................................................................. 14
Figure S3. ................................................................................................................................. 15
Figure S4. ................................................................................................................................. 16
Figure S5. ................................................................................................................................. 17
Figure S6. ................................................................................................................................. 18
Figure S7. ................................................................................................................................. 19

Spectra of Compounds ............................................................................................................. 20

1H NMR of compound 2 ........................................................................................................ 20
LCMS of compound 2 ............................................................................................................ 20
1H NMR of compound 3 ........................................................................................................ 21
LCMS of compound 3 ............................................................................................................ 21
1H NMR of compound 4 ........................................................................................................ 22
LCMS of compound 4 ............................................................................................................ 22
1H NMR of compound 5 ........................................................................................................ 23
LCMS of compound 5 ............................................................................................................ 24
LCMS of compound 6 ............................................................................................................ 25
LCMS of compound 7 ............................................................................................................ 26
1H NMR of compound 8 ....................................................................................................... 27
Chemical Synthesis and characterization

Chemical reagents and solvents (dry) were purchased from commercial suppliers and used without further purification. Acid-dPEG$_{25}$-NHS ester was purchased from Quanta Biodesign. Compounds 1$^1$, (E)-3-methoxy-$N,N$-dimethyl-4-(4-vinylstyryl)aniline$^2$, and HaloTag-amine$^3$ were prepared according to the literature procedures. All reactions were carried out in flame-dried flasks sealed with septa and conducted under a nitrogen atmosphere. Thin layer chromatography (TLC) (silica gel, F254, 250 $\mu$m) was performed on precoated TLC glass plates 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 recorded on a Bruker AVB-400 MHz and a Bruker AV-600 MHz spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) and are referenced to CDCl$_3$ (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 (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 Adion CMS-L ESI mass spectrometer. The column used for the analytical HPLC was Phenomenex Luna 5 $\mu$m C18(2) (4.6 mm I.D. × 150 mm) with a flow rate of 1.0 mL/min. Semi-preparative HPLC was performed on a Perkin Elmer Series 200 HPLC using a Phenomenex Luna 5 $\mu$m C18(2) (150 x 10 mm) column with a flow rate of 5.0 mL/min. In all cases, the mobile phases were MQ-H$_2$O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade MeCN with 0.05% trifluoroacetic acid (eluent B). For analytical HPLC, signals were monitored at 254, 380, and 650 nm over 10 min, with a gradient of 10 to 100% eluent B for 6 min, then held at 100% B for 4 min. For semi-preparative HPLC, signals were monitored at 254 over 20 min with a gradient of 10 to 100% eluent B.

Spectroscopic Studies

UV-Vis absorbance and fluorescence spectra were recorded using a 2501 Spectrophotometer (Shimadzu) and a Quantamaster Master 4 L-format scanning spectrophuorometer (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).

The maximum absorption wavelength ($\lambda_{\text{max}}$), maximum emission wavelength ($\lambda_{\text{em}}$), and extinction coefficient ($\varepsilon$) were taken in PBS (100 mM Na$_2$HPO$_4$, 7H$_2$O, 150 mM NaCl, pH 7.4) solution containing 0.10 % (w/w) SDS using stock solutions of isoBeRSTs in DMSO (0.5–1 mM); the reported value for $\varepsilon$ is an average ($n = 3$).

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 and Laboratory Animals and the Public Health Policy.

HEK cell culture
Human embryonic kidney 293T (HEK) cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS; Thermo Scientific) and 1% GlutaMax (Invitrogen) at 37 °C in a humidified incubator with 5% CO₂. Cells were passaged and plated in DMEM (as above) at a density of 750,000 cells per well in a 6-well plate. Transfection of plasmids was carried out using Lipofectamine 3000 (Invitrogen) ~18-24 h after plating. The cells were split again 48 h after transfection and plated onto 12 mm glass coverslips pre-coated with Poly-D-Lysine (PDL; 1 mg/ml; Sigma-Aldrich) at a density of 75,000 cells per coverslip in DMEM supplemented with 1 g/L D-glucose, 10% FBS and 1% GlutaMax. Imaging was performed 12-18 h after plating.

Primary neuronal culture and transfection

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold, sterile HBSS (zero Ca²⁺, zero Mg²⁺, phenol red). 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 fireshape polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% FBS, 2% B-27, 2% 1M dextrose (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 25-30,000 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. Transfection of plasmids was carried out using Lipofectamine 3000 (without P3000 reagent) at 6-7 DIV. Imaging was performed on mature neurons 13-16 DIV.

Unless stated otherwise, for loading of HEK cells and hippocampal neurons, DMSO stock solutions of isoBeRSTs (1 mM) were diluted directly into HBSS to working concentrations. For HEK cells and neurons, the typical working concentration was 500 nM for untargeted isoBeRSTs and 50 nM for isoBeRST-Halo. HEK cells were incubated for 30 mins with isoBeRSTs at 37 °C before exchanging dye/HBSS for HBSS without any dye. Neurons were treated identically, unless specified. All imaging was performed in HBSS at room temperature.

In utero electroporation

Pregnant mice at E15-16 were anaesthetized with 2.0% isoflurane, the abdomen was cleaned with 70% ethanol and swabbed with iodine, and a small vertical incision was made in the skin and abdominal wall and 8–12 embryos gently exposed. Each embryo was injected with 0.5–1 μl of DNA solution and 0.05% Fast Green dye. We used a pressure-controlled beveled glass pipette (Drummond, Custom Microbeveller) for injection. After each injection, the embryos were moistened with saline and voltage steps via tweezer electrodes (BTX, 5 mm round, platinum, BTX electroporator) were applied with the positive electrode placed over the visual cortex and the negative electrode placed under the head of the embryo. Voltage was 40 V for 5 pulses at 1 Hz, each pulse lasting 50 ms. The embryos were returned to the abdomen, which was sutured, followed by suturing of the skin. The procedure typically lasted under 30 min.

Acute brain slice preparation

Mice were deeply anaesthetized with isoflurane and quickly decapitated. After removing the scalp and skull, ice-cold artificial cerebrospinal fluid with sucrose (ACSF-sucrose) cutting solution (in mM: NaCl, 83; KCl, 2.5; MgSO₄, 3.3; NaH₂PO₄, 1; NaHCO₃, 26.2; D-glucose, 22; sucrose, 72; and CaCl₂, 0.5) was applied to the brain. BFP fluorescence was checked with a hand-held laser before the brain was taken out. The brain was cut into 300 μm thick slices with a DTK-1000 slicer in ice-cold ACSF-sucrose cutting solution. The cut slices were incubated in sucrose cutting solution, bubbled with 95% O₂ and 5% CO₂, first at 31 °C for about 30 min and then at room temperature until further use.

Epifluorescence microscopy
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 a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss). Images were focused onto either an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu) or an eVolve 128 EMCCD camera (EMCCD; Photometrix). For isoBeRST images, the excitation light was delivered from a LED (6.72 W/cm²; 20 ms exposure time) at 631/28 (bandpass) nm and emission was collected with a quadruple emission 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 EGFP images, the excitation light was delivered from a LED (5.77 W/cm²; 20 ms exposure time) at 475/34 nm and emission was collected with a quadruple emission 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).

**Brain slice staining, imaging, and electrophysiology**

For bath application of the dye and cell staining, a slice was transferred to a 35 mm dish with 3mL ACSF-sucrose cutting solution (total volume) bubbled with 95% O₂ and 5% CO₂ to which dye stock solution was added (250 nM final concentration). The slice was incubated with the dye at room temperature for 15 min with carbogen. For functional imaging, the slice was transferred to a fresh dish with ACSF recording solution (in mM: NaCl, 119; KCl, 2.5; MgSO₄, 1.3; NaH₂PO₄, 1.3; NaHCO₃, 26; D-glucose, 20 and CaCl₂, 2.5) using a plastic Pasteur pipette and washing is not necessary. A small harp or staple is used to press down the slice to minimize disturbance on the slice during perfusion and to make sure slice is flat for even illumination.

Confocal imaging was performed with a Zeiss LSM 880 NLO AxioExaminer equipped with a Diode 405 nm laser line, Argon 458, 488, and 514 laser lines, a DPSS 561 nm laser line, a HeNe 633 laser line and and a BiG-2 detector with a 690+ dichroic. Images were acquired using a W-Plan-Apo 20x/1.0 water objective and a Zeiss Airyscan detector.

For whole-cell, current clamp in slices, slices were transferred to the microscope perfusion chamber with ACSF recording solution. Patch pipettes were loaded with internal solution (as stated above) and had resistances of ~5 MOhm. A Multiclamp 700B amplifier (Molecular Devices) was used to amplify the signal, which is filtered at 2 kHz and digitized at 20 kHz (National Instruments). Samples were illuminated with spectra-X Light engine LED light (Lumencor). Images were acquired with a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss) at a sampling rate of 1 kHz on a Zyla 4.2 sCMOS camera (Andor). A custom written MATLAB (Mathworks) script was used to control experiments.

**Image analysis**

For image intensity measurements, regions of interest were drawn around cells or neuronal cell bodies and the mean fluorescence was calculated in ImageJ (FIJI, NIH). Background fluorescence was subtracted by measuring the fluorescence where no cells grew. The fold turn-on was calculated by taking the ratio of transfected cells fluorescence and untransfected cells fluorescence, both background subtracted.

Analysis of voltage sensitivity in HEK cells was performed using ImageJ (FIJI). 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. For analysis of voltage responses in neurons, regions of interest encompassing cell bodies (all of approximately the same size) 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, to give a background subtracted trace (bkgsub). A baseline fluorescence value (Fbase) is calculated from the median of all the frames, and subtracted from each
timepoint of the bkgsub trace to yield a ΔF trace. The ΔF was then divided by Fbase to give ΔF/F traces. No
averaging has been applied to any voltage traces.

**Electrophysiology and Imaging in HEK cells and primary cultured neurons**

For electrophysiological experiments, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-
10), with a resistance of 4–6 MΩ, and were filled with an internal solution; 115 mM potassium gluconate, 10 mM
BAPTA tetrapotassium salt, 10 mM HEPES, 5 mM NaCl, 10 mM KCl, 2 mM ATP disodium salt, 0.3 mM 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 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 HEK 293T cells, cells were held at -60
mV and hyper- and depolarizing steps applied from -100 to +100 mV in 20 mV increments.

Extracellular field stimulation was delivered by a SD9 Grass Stimulator connected to a recording chamber
containing two platinum electrodes (Warner), with triggering provided through the same Digidata 1332A digitizer
and pCLAMP 9 software (Molecular Devices) that ran the electrophysiology. Action potentials were triggered by
1 ms 60 V field potentials delivered at 5 Hz. To prevent recurrent activity, the HBBS 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). For both evoked action potentials and spontaneous activity, images were binned 4x4 to allow
sampling rates of 0.5 kHz and 2500 frames (5 s) were acquired for each recording. For spontaneous and evoked
activity recordings of isoBeRST, the excitation light was delivered from a LED (1.94 W/cm²) at 631/28 (bandpass)
nm and emission was collected with a quadruple emission 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).

**DNA constructs**

To express the HaloTag protein on the cell surface, an IgK leader sequence was fused to the N-terminal and a
transmembrane domain (pDisplay) was added to the C-terminal of the HaloTag sequence. For the purpose of
immunostaining, an HA tag was inserted. Mammalian expression vector pcDNA3 with either a cytomegalovirus
(CMV) promoter or human synapsin promoter (Syn) was used for protein expression in HEK cells and cultured
neurons, respectively. To increase expression in neurons, a regulatory element from the woodchuck hepatitis virus
(WPRE) was used. In some constructs, nuclear-targeted EGFP was inserted down stream of HaloTag, separated by
an internal ribosome entry site (IRES) sequence, in order to track the expression of HaloTag in live cells. The cloned
constructs were verified by sequencing. All the constructs were prepared using Qiagen Maxiprep kit, except those
with CMV promoter. The following sequences were used (5’ to 3’):

**IgK**

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCAGGTGTCCACTGGTAC

**HaloTag**

GCAGAAATCGGTACTGCTGCTCTGGGTTCAGGTGTCCACTGGTAC

ACGTCCGATTTGTGGTCCGGGCGATGCCACCCCTGTGCTGCTTCTGACGGTAAACCGACCTCTCCTCCTA

CGTGTCGGGCACATCATTCCCGCACTGTTGCACCCGACCACCATCGCTGCGTATGGTCACTGCGT
ATGGGCAAAATCCGACAAACCAGACCTGGGTTATTTCTTCGACGA
CCACGTCCGCTTCATGGATGCCT
TCATCGAAGCCCTGGGTCTGGAAGAGGTCGTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTT
CCACTGGGCCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCTATC
CCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCAGGCCTTCCGCACCACCGACGTC
GGCCGCAAGCTGATCATCGAAGCCCTGGGTTATTTTTATGCAGGTTACGCTCGTGCTCCGCCTGGCC
CGCTAGTACTGCTGAGTAGTACACCTAGGCGATGCTGGTGCCACACTCCTTGCCCTTTAAGGTGGTGGTG
TCTCAGCCATCCTGGGCTGCTGTACTGAAGTCGAGATGGACCATTACCGCGAGCCGTTCCTGAATCCTG
CTGGCCAAAAGCCTGCCTAACTGCAAGGCTGTGGACATCGGC
CCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGAGCGAGATCGCGCGCTGGCTG
TCGACGCTCAGATTTTCCCGGC

HA
TATCCATATGATGTTCCAGATTATGCT

pDisplay
GCTGTGGGCCCGAGCACAGCAGGAGGTCTGTCCTGGGCACACTCCTTTGCTCTTATTTAAGGTGGTGGTG
TCTCAGGCAATCCTGGGCTGCTGTACTGAAGTCGAGATGGACCATTACCGCGAGCCGTTCCTGAATCCTG
CTGGCCAAAAGCCTGCCTAACTGCAAGGCTGTGGACATCGGC
CCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGAGCGAGATCGCGCGCTGGCTG
TCGACGCTCAGATTTTCCCGGC

IRES
GCCCTCCTCTCCTCCCCCCCCCTAAAGTTACTGCGCGCAAGCGCCTTGGAATAGGCGCGGATGTGCGT
TGCTCATATGGTATTTTCACCATATTGCGCTTTTGGCAATGTAGGATTGGGCGGCAACCTGGGCCCTG
TCTTTTTGGAGAGCATTCTCTCCCTTCCCTCTCGCAGATAAGGATCGCTGTTGGAATGTC
GTGAAGGAGAACGTTTCCTCTGGGAAAGCTTTGAGAAAGCAACGCTTGTAGCGACCTTTGCGCAG
CAGCGGAAACCCCATCTGGCCAGACAGTGCTGCTTCGCGGCCAAAAAGCCACGTGTATAAGATACCT
GCAAAGGCGCGCAACAACCCCATGCGACAGTGGGATAGTTGGAAGACTCAAATGGGCT
TCCTCAAGGCTATTACAAAGGCGCAGGAGGCTGCAAGGACCCGACCATTCGGATTAGATGCTGAT
CTGGGGGCTGGTGACATTTGCTTTAACATGTTGTTATGCGAGGTATAAAAAACGCTTAGGCCCCCGA
ACCACGGGAGCTGTTTTTCTTCTTGGAAAAACAGCATGATAATATGGGCCACA

Nuclear Localization Sequence
ATGTTGCCCCAAAGAGAGGCAGGAAGAGCTGTCGAGCAAGGGGGAGGAGGACCAACATGGCCCATCATCAA
GGAGTTTCATGCGCTTCAAGGTGAC

EGFP
AGCAAGGGCGAGAGCTGCTTACCCGGGGGTGGTGGTGGCCTACCTTGGAGCTGGAAGCGCGAGCGAGCTAAAC
GGCCACAAGTTGCCAGCTGGCCCGCGAGGGCGGAGGGCGAGGTCCACCTACGCAAGCTGACCTGGAAG
TTGCATCTGCACACCGGCAAGGCTGCCCGCTGCCCCCTCGGCCCCACCTCTCGTAACCCCGCTACGGCG
TCGAGTGCTTTGACGGCGCTACCCGACCAATGAGAAGCAGACAGCTTCTTCAAATGCGGCCGACGCAG
AGGCTACGTCCAGAGGCGCACCATTTTCTTTCAAGGACAGCAGGCAACTACAAGACCCGCGCGAGAGG
GAAAGGCTAGGAGGGCGACACCTGGTGAACCGCATCGAGCTGGATAGGGGACATCGACTTTAACAGAAGAGG
CAACATCCTGGGGCCACAAAGCTGGAATCAACTACAACAGCCCAACGTTCTATATATGTGGCCGACAA

S7
GCAGAAGAAGGACGCAATCAGGAACGTCTAAGATCGCCACAAACATCGAGACGGCAGCGTGCAGCT
CGCCGACCACTACCAGGAAACACCAGACCATCGGCAAGGCCCCTTGCTGCTGCCGACAAACCCTA
CCTGAGCACCAGTCGCCCTAGAGAAGACCCCAAGAAGCCGCGATCACATGTTCCTGCTGGA
GTTCGTGACGCGCCGCGGATCACAATCGCGATCGAGCTGATAAGTAA

WPRE
GCTTATCGATAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTG
CTCTTCTTACGCTATGTGGAATACGCCTTTAAAATGCTTCTATCGATATCGCTATGAGGAGTTGTG
GACGGCCGAGGTGTCGTGTTTGTGCTACGCAAGCCGACATCCGTTGCTGGTGGGAATAT
CATCGTCTCTTCCCTGCGTCGTGCTGGTGTGGCACACCTCGATATCGCTCTTCTGCTAC
CTGCCCTTCGGCCACTAATCCGACGCGACCTCTCTCTCCGCGGCGCTGCTGCGCGCTCTCC
GCGTCTCTCGCTCCCTCCGACTGAGTGATCGGATCTCCCTTGGGCGGCTCCTCCGGCGATCGATA

CMV promoter
GACATTGGATTATTGACTGTTTATTTAATAGTAATCAATTACGGGGTCATTAGTCTAGTCAAGTCCAT
GGAGTTGCCGTTGATACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCTATTGAGTCTTATGGA
CTATTTTGCGCTAATAATGACGTATATGAGATCAAGTTGCTGCTGGCCTGAGCGCC

Synapsin Promoter
GTGTCTAGACTGCAAGGCGCCCTGCGATGATGCAATAAGTTTGGTGGTTTAGACCAGAGATGAGGC
TGAGGAGGTCAGCCCTCAGCAACGGACCACCACCACCACCTGGAGCAACGCCAACCCTGCTGCGCTG
CGGCGGCGGCCGCTCGTCCAGTCTGCGGTGGGCAGCGGAGGAGTCGTGTCG
TGCTGAGAGCGCAGTCGAGA
Synthetic Procedures

\[ \text{N-(10-(5-bromo-2-(4-(tert-butoxycarbonyl)piperazine-1-carbonyl)phenyl)-7-(dimethylamino)-5,5-dimethylldibenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (2):} \]

To a solution of silicon rhodamine 1\(^1\) (110 mg, 0.217 mmol, 1 eq) in CH\(_2\)Cl\(_2\) (5.9 mL), oxalyl chloride (24.4 μL, 0.260 mmol, 1.2 eq) was added and stirred for 30 min. Triethylamine (7.68 µL, 0.056 mmol, 2 eq) and 1-Boc-Piperzine (31 mg, 0.168 mmol, 6 eq) were added in succession. The resulting mixture was stirred at room temperature for 4 h. Dilute HCl (~ 0.1 M) was added and the organics extracted with CH\(_2\)Cl\(_2\) (3x). The combined organic extracts were dried with anhydrous sodium sulfate, filtered, and concentrated in vacuo. The crude residue was purified by preparative TLC to afford the chloride salt of 2 (90 mg, 55%) as a dark blue solid.

1H NMR (400 MHz, CD\(_3\)OD) δ 7.86 (d, \(J = 8.2\) Hz, 1H), 7.61 (s, 1H), 7.50 (d, \(J = 8.3\) Hz, 1H), 7.37 (s, 2H), 7.17 (d, \(J = 9.5\) Hz, 2H), 6.84 (d, \(J = 9.7\) Hz, 2H), 3.35 – 3.01 (m, 8H), 1.44 (s, 9H), 0.67 (s, 3H), 0.52 (s, 3H); HRMS (ESI) calcd for C\(_{35}\)H\(_{44}\)BrN\(_4\)O\(_3\)Si [M]+ 675.2361, found 675.2349; LRMS (ESI) calcd for C\(_{35}\)H\(_{44}\)BrN\(_4\)O\(_3\)Si [M]+ 675.2, found 675.3.

\[ \text{N-(10-(5-bromo-2-(piperazine-1-carbonyl)phenyl)-7-(dimethylamino)-5,5-dimethylldibenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (3):} \]

To 2 (48 mg, 0.12 mmol) in CH\(_2\)Cl\(_2\) (1.5 mL) was added trifluoroacetic acid (1.5 mL). The reaction was stirred at room temperature for 1 h, then the solvent removed under a stream of nitrogen. Toluene was added and the reaction mixture was then concentrated in vacuo (1x). Methanol was added and the reaction mixture was concentrated in vacuo (3x). It was further dried under high vacuum to afford the TFA salt of 3 (52 mg, 91%) as a dark blue solid. 1H NMR (400 MHz, CD\(_3\)OD) δ 7.87 (d, \(J = 8.1\) Hz, 1H), 7.62 (s, 1H), 7.50 (d, \(J = 8.3\) Hz, 1H), 7.37 (d, \(J = 2.6\) Hz, 2H), 7.17 (d, \(J = 9.6\) Hz, 2H), 6.84 (dd, \(J = 9.6, 2.5\) Hz, 2H), 3.54 – 3.21 (m, 4H), 2.70 (bs, 2H), 2.54 (bs, 2H), 0.66 (s, 3H), 0.54 (s, 3H); HRMS (ESI) calcd for C\(_{30}\)H\(_{36}\)BrN\(_4\)OSi [M]+ 575.1836, found 575.1840; LRMS (ESI) calcd for C\(_{30}\)H\(_{36}\)BrN\(_4\)OSi [M]+ 575.2, found 575.2.

(R)-3-(4-(4-bromo-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)benzoyl)piperazin-1-yl)-2-((tert-butoxycarbonyl)amino)-3-oxopropane-1-sulfonate (4):

To 3 (67 mg, 0.083 mmol, 1 equiv), Boc-L-cysteic acid (33 mg, 0.12 mmol, 1.5 equiv), and HATU (47 mg, 0.12 mmol, 1.5 equiv) was added anhydrous DMF (2.2 mL) under nitrogen. Anhydrous diisopropylethylamine (54 μL, 0.31 mmol, 2.5 equiv) was added and the reaction stirred overnight at room temperature. The solvent was evaporated
under reduced pressure, the mixture diluted with CH₂Cl₂, and dilute HCl (~ 0.1 M) was added. The organics were extracted with CH₂Cl₂ (3x). The combined organic extracts were dried with anhydrous sodium sulfate, filtered, and concentrated in vacuo. The crude residue was purified by preparative TLC to yield 4 (56 mg, 82%) as a dark blue solid.

1H NMR (600 MHz, CDCl₃) δ 7.70 (dd, J = 8.2, 1.9 Hz, 1H), 7.45 (d, J = 1.9 Hz, 1H), 7.28 (d, J = 8.3 Hz, 1H), 7.16 – 6.95 (m, 4H), 6.85 (d, J = 9.4 Hz, 1H), 6.62 (d, J = 8.9 Hz, 1H), 5.30 (s, 1H), 4.92 (s, 1H), 3.56 – 3.20 (m, 20H), 3.08 (dd, J = 13.1, 9.3 Hz, 1H), 3.01 – 2.95 (m, 1H), 2.32 (s, 1H); HRMS (ESI) calcd for C₃₈H₄₈BrN₅NaO₇SSi [M+Na]⁺ 848.2119, found 848.2131; LRMS (ESI) calcd for C₃₈H₄₉BrN₅O₇SSi [M+H]⁺ 826.2, found 826.3.

A vial was charged with 4 (47.0 mg, 56.9 μmol, 1.0 eq), (E)-3-methoxy-N,N-dimethyl-4-(4-vinylstyril)aniline (17.5 mg, 62.9 μmol, 1.1 eq), Pd(OAc)₂ (6.4 mg, 28.5 μmol, 0.5 eq), and P(o-tol)₃ (17.3 mg, 56.9 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.68 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et₃N (0.24 mL) was added and the reaction was sealed and stirred at 75 °C for 2 h. The reaction was cooled, diluted with MeOH, filtered, and concentrated in vacuo. The crude residue was purified by preparative TLC (8% MeOH/CH₂Cl₂) to afford 5 (10 mg, 17%) as a green solid.

1H NMR (400 MHz, CDCl₃) δ 7.72 (dd, J = 8.0, 1.7 Hz, 1H), 7.57 – 7.41 (m, 8H), 7.30 – 7.11 (m, 5H), 7.06 (s, 1H), 6.98 (d, J = 16.4 Hz, 1H), 6.89 (d, J = 10.2 Hz, 1H), 6.67 (d, J = 9.8 Hz, 1H), 6.39 (dd, J = 8.7, 2.4 Hz, 1H), 6.26 (d, J = 2.4 Hz, 1H), 5.37 (s, 1H), 5.00 (s, 1H), 3.94 (s, 3H), 3.59 – 3.29 (m, 20H), 3.14 (m, 1H), 3.05 (s, 6H), 2.09 (s, 1H), 1.42 (s, 9H), 0.61 (s, 3H), 0.55 (s, 3H); HRMS (ESI) calcd for C₅₇H₆₈N₆O₈SSi [M+H]⁺ 1025.4661, found 1025.4659; LRMS (ESI) calcd for C₅₇H₆₈N₆O₈SSi [M+H]⁺ 1025.5, found 1025.5.

isoBeRST-pipcys (6):

Trifluoroacetic acid (0.5 mL) was added to a solution of 6 (6.0 mg, 5.8 μmol) in CH₂Cl₂ (0.5 mL). The reaction was stirred at room temperature for 30 mins, then the solvent removed under a stream of nitrogen. Toluene was added
and the reaction mixture was concentrated *in vacuo* (1x). Methanol was added and the reaction mixture was concentrated *in vacuo* (3x). It was further dried under high vacuum to afford the TFA salt 6 (6.1 mg, quant) as a green solid. LRMS (ESI) calcd for C$_{32}$H$_{61}$N$_6$O$_6$SSi [M+H]$^+$ 925.4, found 925.3.

isoBeRST-Halo (7):
A vial was charged with 6 (6.0 mg, 5.8 μmol, 1.0 eq) and Acid-dPEG$_{25}$-NHS ester (7.7 mg, 5.8 μmol, 1.0 eq). Anhydrous DMF (0.6 mL) and anhydrous diisopropylethylamine (27 μL, 40 μmol, 6.9 eq) were added and the vial flushed with nitrogen. The reaction was stirred at room temperature for 7 h. Upon completion by LCMS, HaloTag-Amine$^1$ (5.8 mg, 25 μmol, 4.2 eq) and HATU (2.7 mg, 7.0 μmol, 1.2 eq) were added and the reaction stirred for 18 h. The reaction was then diluted with MeCN (1.0 mL). The diluted reaction was purified by semi-preparative HPLC to give isoBeRST-Halo 7 (1.5 mg, 11%) as a green solid. HRMS (ESI) calcd for C$_{116}$H$_{187}$ClKN$_7$O$_{35}$SSi [M+H+K]$^{2+}$ 1185.5875, found 1185.5829. LRMS (ESI) calcd for C$_{116}$H$_{187}$ClKN$_7$O$_{35}$SSi [M+3H]$^{3+}$ 778.1, found 778.3.

4-((E)-4-((E)-4-(dimethylamino)-2-methoxystyryl)styryl)-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)benzoate (8):
A vial was charged with 1$^1$ (20 mg, 39 μmol, 1 eq), (E)-3-methoxy-N,N-dimethyl-4-(4-vinlystyrhil)aniline$^2$ (11 mg, 39 μmol, 1 eq), Pd(OAc)$_2$ (4.4 mg, 22 μmol, 0.55 eq), and P(o-tol)$_3$ (12 mg, 39 μmol, 1 eq). The vial was evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.43 mL) was added and the mixture was evacuated/backfilled with nitrogen (3x). Anhydrous Et$_3$N (0.21 mL) was added and the vial was sealed and stirred overnight at 80 °C. The crude residue was purified by preparative TLC (30% EtOAc/hexanes) to afford 8 (13 mg, 47%) as a dark orange-red solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.97 (d, $J = 8.0$ Hz, 1H), 7.71 (d, $J = 8.1$ Hz, 1H), 7.55 – 7.42 (m, 6H), 7.24 – 7.09 (m, 3H), 7.05 – 6.95 (m, 3H), 6.92 (d, $J = 8.9$ Hz, 2H), 6.63 (d, $J = 8.4$ Hz, 2H), 6.40 (d, $J = 8.6$ Hz, 1H), 6.27 (s, 1H), 3.94 (s, 3H), 3.05 (s, 3H), 3.02 (s, 12H), 0.73 (s, 3H), 0.66 (s, 3H); HRMS (ESI) calcd for C$_{45}$H$_{48}$N$_3$O$_5$Si [M+H]$^+$ 706.3459, found 706.3450. LRMS (ESI) calcd for C$_{45}$H$_{48}$N$_3$O$_5$Si [M+H]$^+$ 706.3, found 706.3.

S11
To a solution of 8 (69 mg, 0.098 mmol, 1 eq) in CH₂Cl₂ (3.5 mL), oxalyl chloride (9.9 uL, 0.117 mmol, 1.2 eq) was added and stirred for 30 min under nitrogen. Anhydrous Et₃N (27 µL, 0.196 mmol, 2 eq) then a solution of sarcosine tert-butyl ester hydrochloride (31 mg, 0.168 mmol, 6 eq) and Et₃N (81 µL, 0.586 mmol, 6 eq) were added in succession. The resulting mixture was stirred at room temperature for 2 h. Dilute HCl (~ 0.1 M) was added and the organics extracted with CH₂Cl₂ (3x). The combined organic extracts were dried with anhydrous sodium sulfate, filtered, and concentrated in vacuo. The crude residue was purified by preparative TLC to afford chloride salt of 9 (17 mg, 20%) as a dark green solid. HRMS (ESI) calcd for C₅₂H₆₁N₄O₄Si [M]+ 833.4457, found 833.4465. LRMS (ESI) calcd for C₅₂H₆₁N₄O₄Si [M]+ 833.4, found 832.8.

isoBeRST-sarcosine (10):
To 9 (17 mg, 0.02 mmol) in CH₂Cl₂ (2.0 mL) was added trifluoroacetic acid (1.5 mL). The reaction was stirred at room temperature for 3 h, then the solvent removed under a stream of nitrogen. Toluene was added and the reaction mixture was then concentrated in vacuo (1x). Methanol was added and the reaction mixture was concentrated in vacuo (3x). It was then dissolved in 1:6 DMSO/McCN and purified by preparative HPLC to afford the TFA salt 10 (5.0 mg, 28%) as a green solid. ¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, J = 8.1 Hz, 1H), 7.64 (dd, J = 8.0, 1.7 Hz, 1H), 7.54 – 7.44 (m, 7H), 7.36 – 7.33 (m, 2H), 7.20 – 7.05 (m, 4H), 7.01 – 6.95 (m, 1H), 6.80 (dd, J = 9.6, 2.9 Hz, 2H), 6.39 (dd, J = 8.7, 2.4 Hz, 1H), 6.26 (d, J = 2.4 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 2H) 3.35 (s, 12H), 3.05 (s, 6H), 2.90 (s, 3H), 0.60 (s, 3H), 0.55 (s, 3H). HRMS (ESI) calcd for C₄₈H₅₃N₄O₄Si [M+H]+ 777.3831, found 777.3839. LRMS (ESI) calcd for C₄₈H₅₃N₄O₄Si [M+H]+ 777.4, found 777.3.
Figures and Schemes

Scheme S1. Synthesis of isoBeRST-sarcosine
Figure S1. Cellular and in vitro characterization of isoBeRST-sarc 10. a) Normalized absorbance (solid line) and emission (dashed line) spectra of isoBeRST-sarc in PBS, pH 7.4, 0.1% SDS. b) HEK cells stained with 500 nM isoBeRST-sarc. Scale bar is 10 μm. c) Plot of the fractional change in fluorescence of 10 vs time for 80 ms hyper- and depolarizing steps (-100 to +80 mV in 20 mV increments) from a holding potential of -60 mV for single HEK cells under whole-cell voltage-clamp mode. d) Plot of % ΔF/F vs final membrane potential summarizing data from five separate cells, revealing a voltage sensitivity of 24% ΔF/F per 100 mV. Error bars are ±S.D.

Figure S2. Cellular and in vitro characterization of isoBeRST-pipcys 6. a) Normalized absorbance (solid line) and emission (dashed line) spectra of isoBeRST-pipcys in PBS, pH 7.4, 0.1% SDS. b) HEK cells stained with 500 nM isoBeRST-pipcys. Scale bar is 10 μm. c) Plot of the fractional change in fluorescence of 6 vs time for 80 ms hyper- and depolarizing steps (-100 to +80 mV in 20 mV increments) from a holding potential of -60 mV for single HEK cells under whole-cell voltage-clamp mode. d) Plot of % ΔF/F vs final membrane potential summarizing data from five separate cells, revealing a voltage sensitivity of 24% ΔF/F per 100 mV. Error bars are ±S.D.
**Figure S3.** Voltage imaging of spontaneous neuronal activity with isoBeRST-pipcys. a) Wide-field fluorescence and b) differential interference contrast (DIC) images of cultured rat hippocampal neurons stained with isoBeRST-pipcys (6, 500 nM, 30 mins. Scale bar is 20 μm. c) Optical traces of spontaneous activity of the neurons in panels a-b) recorded at 500 Hz and shown as ΔF/F vs time.
**Figure S4.** Loading condition screen of isoBeRST-Halo in HEK cells. a-d) Wide-field microscopy images of HEK cells transfected with CMV-HaloTag-pDisplay and stained with isoBeRST-Halo (50 nM, 30 mins). a) DIC image of HEK cells. b) Nuclear EGFP fluorescence indicates HaloTag expression. c) Membrane associated isoBeRST-Halo fluorescence. d) Merge of fluorescence from EGFP (green) and isoBeRST-Halo (magenta), demonstrating the selective labeling of HaloTag-expressing cells with 7. Scale bar is 10 μm. e) Plot of isoBeRST-Halo fluorescence in HaloTag (+, transfected) and HaloTag(-, untransfected) HEK cells for isoBeRST-Halo at different concentrations. f) Plot of selectivity between transfected and untransfected HaloTag HEK cells labeled with isoBeRST-Halo. Data are mean fluorescence ± SEM from HaloTag-expressing and non-expressing HEK cells labeled with isoBeRST-Halo from 3 different coverslips and at least n = 6 fields of view, comprising 20 to 50 cells each.
Figure S5. Quantification of the selective labeling in HaloTag-expressing neurons with isoBeRST-Halo at varying concentrations of 7. a-d) Wide-field microscopy images of isoBeRST-Halo labeling in a HaloTag-expressing neuron. a) DIC image of neurons. b) Nuclear EGFP fluorescence indicates HaloTag expression. c) isoBeRST-Halo fluorescence is restricted to the membrane. d) Merge of EGFP (green) and isoBeRST-Halo (magenta) fluorescence, demonstrating selective labeling of HaloTag-expressing neuron with 7. Scale bar is 20 μm. e) Plot of isoBeRST-Halo fluorescence in HaloTag (+, transfected) and HaloTag(-, untransfected) neurons for isoBeRST-Halo at different concentrations. f) Plot of selectivity between transfected and untransfected HaloTag neurons labeled with isoBeRST-Halo. Data are mean fluorescence ± SEM from HaloTag-expressing and non-expressing neurons labeled with isoBeRST-Halo from 4 different coverslips and at least n = 5 fields of view, comprising 1 to 4 cells each. g) Plot of 7 fluorescence vs nuclear EGFP fluorescence from HaloTag-expressing neurons at 50 nM or 500 nM 7. Each point represents an individual neuron.
Figure S6. Field stimulation of neurons expressing HaloTag and stained with isoBeRST-Halo. a-d) Wide-field microscopy images of neurons sparsely transfected with HaloTag-pDisplay and stained with 50 nM isoBeRST-Halo. 7. a) EGFP fluorescence image of neuron expressing HaloTag. b) isoBeRST-Halo fluorescence image. c) Merge of EGFP (green) and isoBeRST-Halo (magenta) fluorescence, demonstrating selective labeling of HaloTag-expressing neuron with 7. d) DIC image of neurons. Scale bar is 20 μm. d) Field stimulation-evoked action potentials shown as ΔF/F vs time of HaloTag-expressing neurons stained with 50 nM isoBeRST-Halo. Voltage recording labeled trace 3 was acquired from the labeled neuron in panels a-d). Images were acquired at 500 Hz and represent single-trial acquisitions.
Figure S7. One-photon confocal microscopy imaging of isoBeRST-Halo in mouse brain slice expressing HaloTag-pDisplay and pCAG-BFP following in utero electroporation. Z-stack imaging was conducted from surface of the slice to deeper into the tissue and the depth relevant to the surface was indicated in each image. a) Membrane-associated isoBeRST-Halo (7) fluorescence was observed in BFP-positive cells with clear visualization of neuronal processes and subcellular structures, including dendritic spines. b) BFP was used for screening positive pups and expressing slices. Scale bar is 20 μm. Insets labeled “-35 μm” and “-62 μm” are overlays of the isoBeRST-Halo (7) fluorescence (magenta) and BFP fluorescence (blue) from the respective boxed areas in panels (a) and (b).
Spectra of Compounds

$^1H$ NMR of compound 2

LCMS of compound 2

Intensity

DAD: Signal G, 650 nm/4 nm
pipurified.datx 2018.05.23 17:43:29:

Intensity

Spectrum RT 5.45 - 5.63 (23 scans) - Background Subtracted 0.01 - 5.21
pipurified.datx 2018.05.23 17:43:29:

Intensity

ESI + Max: 1.5E7
$^1$H NMR of compound 3

LCMS of compound 3
$^1$H NMR of compound 4

LCMS of compound 4
$^1$H NMR of compound 5
LCMS of compound 5

Intensity

DAD: Signal B, 254 nm/B:4 nm Ref 700 nm/B:50 nm
check.dat 2019.12.10 14:19:04

100.0%

Intensity

DAD: Signal C, 380 nm/B:4 nm Ref 700 nm/B:50 nm
check.dat 2019.12.10 14:19:04

100.0%

Intensity

DAD: Signal G, 650 nm/B:4 nm
check.dat 2019.12.10 14:19:04

100.0%

Intensity

Spectrum RT 5.99 - 6.17 (23 scans) - Background Subtracted 0.22 - 5.74
check.dat 2019.12.10 14:19:04

Intensity

ESI + Max: 1.2E6

%
LCMS of compound 6
LCMS of compound 7
$^1$H NMR of compound 8
LCMS of compound 8
LCMS of compound 9
$^1\text{H NMR of compound 10}$
LCMS of compound 10
References

(1) Kozma, E.; Estrada Girona, G.; Paci, G.; Lemke, E. A.; Kele, P. Bioorthogonal Double-Fluorogenic Siliconrhodamine Probes for Intracellular Super-Resolution Microscopy. *Chem. Commun.* **2017**, *53*(50), 6696–6699. https://doi.org/10.1039/c7cc02212c.

(2) Woodford, C. R.; Frady, E. P.; Smith, R. S.; Morey, B.; Canzi, G.; Palida, S. F.; Araneda, R. C.; Kristan, W. B.; Kubiak, C. P.; Miller, E. W.; et al. Improved PeT Molecules for Optically Sensing Voltage in Neurons. *J. Am. Chem. Soc.* **2015**, *137*(5), 1817–1824. https://doi.org/10.1021/ja510602z.

(3) Neklesa, T. K.; Tae, H. S.; Schneekloth, A. R.; Stulberg, M. J.; Corson, T. W.; Sundberg, T. B.; Raina, K.; Holley, S. A.; Crews, C. M. Small-Molecule Hydrophobic Tagging-Induced Degradation of HaloTag Fusion Proteins. *Nat. Chem. Biol.* **2011**, *7*(8), 538–543. https://doi.org/10.1038/nchembio.597.
