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Complete List of Authors:	Gonzalez, Monica; University of California Berkeley, Chemistry Walker, Alison; University of California Berkeley, Chemistry Cao, Kevin; University of California Berkeley Lazzari-Dean, Julia; University of California San Francisco, Pharmaceutical Chemistry Settineri, Nicholas; University of California Berkeley, Chemistry Kong, Eui Ju; University of California Berkeley, Chemistry Kramer, Richard; University of California Berkeley, Department of Molecular & Cell Biology Miller, Evan; University of California Berkeley, Chemistry

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Voltage imaging with a NIR-absorbing phosphine oxide rhodamine voltage reporter

Monica A. Gonzalez,^{*,‡} Alison S. Walker,^{*,†} Kevin J. Cao,[§] Julia R. Lazzari-Dean,[‡] Nicholas S. Settineri,[‡] Eui Ju Kong,[‡] Richard H. Kramer,^{§†} and Evan W. Miller^{‡§†*}

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

ABSTRACT: Developing fluorescent dyes that emit and absorb light at wavelengths greater than 700 nm and that respond to biochemical and biophysical events in living systems remains an outstanding challenge for non-invasive optical imaging. Here, we report the design, synthesis, and application of near infrared (NIR)-absorbing and -emitting optical voltmeter based on a sulfonated, phosphine-oxide (po) rhodamine for voltage imaging in intact retinas. We find po-rhodamine based voltage reporters, or poRhoVRs, display NIR excitation and emission profiles at greater than 700 nm, show a range of voltage sensitivities (13 to 43% $\Delta F/F$ per 100 mV in HEK cells), and can be combined with existing optical sensors, like Ca^{2+} -sensitive fluorescent proteins (GCaMP), and actuators, like light-activated opsins ChannelRhodopsin-2 (ChR2). Simultaneous voltage and Ca^{2+} imaging reveals differences in activity dynamics in rat hippocampal neurons, and pairing poRhoVR with blue-light based ChR2 affords all-optical electrophysiology. In *ex vivo* retinas isolated from a mouse model of retinal degeneration, poRhoVR, together with GCaMP-based Ca^{2+} imaging and traditional multi-electrode array (MEA) recording, can provide a comprehensive physiological activity profile of neuronal activity, revealing differences in voltage and Ca^{2+} dynamics within hyperactive networks of the mouse retina. Taken together, these experiments establish that poRhoVR will open new horizons in optical interrogation of cellular and neuronal physiology in intact systems.

INTRODUCTION

Fluorescence microscopy has revolutionized the life sciences. Advances in both synthetic and genetically encoded fluorophores make fluorescence microscopy routine in laboratories across the globe. Coupled with progress in areas like super-resolution microscopy,¹ light sheet microscopy,² and tissue clearing,³⁻⁵ fluorescent dyes bring the high spatial and temporal resolution of optical microscopy to bear on samples ranging from single molecules to interacting cells.

However, non-invasive optical imaging in intact tissues and organisms remains challenging. Commonly used fluorophores, like fluorescein and rhodamine, possess excitation and emission profiles within the visible region of the spectrum and overlap extensively with endogenous chromophores, especially heme.⁶⁻⁷ Although alternative imaging modalities like magnetic resonance imaging, positron emission tomography, and photoacoustic imaging afford excellent penetration into thick tissue, these modalities require specialized instrumentation, are half-life limited, require radioactive reagents, and/or cannot access the micrometer spatial and millisecond time resolution routinely afforded by optical microscopy.⁸

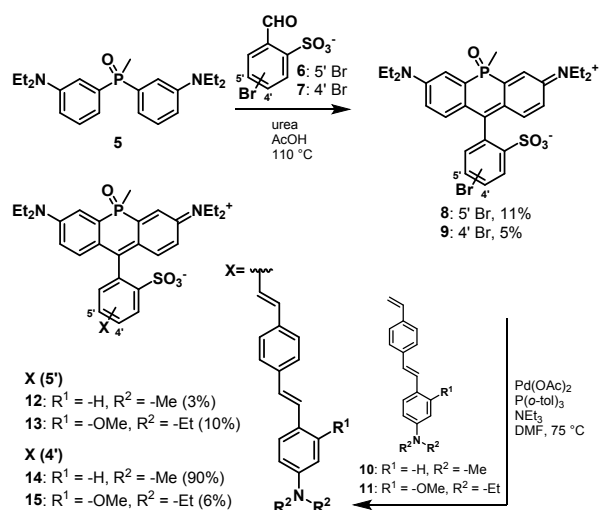
In the context of neurobiology, an outstanding challenge is tracking neuronal voltage dynamics with sub-micron spatial resolution and sub-millisecond temporal resolution within intact tissues. Although two-photon (2P) microscopy provides access to structures in deeper tissue, traditional raster-scanning 2P microscopy cannot achieve the temporal resolution required to image large numbers of neurons simultaneously.⁹

Therefore, fluorescent dyes and voltage indicators that possess near-infrared (NIR, 700 – 1000 nm)¹⁰⁻¹⁴ excitation and emission profiles are of considerable interest. First, NIR photons scatter less than visible photons in thick tissue. Second, by avoiding endogenous chromophores like heme (<650 nm) and water (>900 nm),^{6-7, 15} fluorophores that operate in the so-called NIR window gain profound advantages for tissue and *in vivo* imaging. Finally, NIR fluorophores can be more readily deployed for multiplex imaging alongside existing fluorescent labels, indicators, and actuators that use visible wavelengths of light, on account of the larger spectral separation between the two.

Despite the attraction of NIR dyes that both absorb and emit at wavelengths above 700 nm, traditionally-employed NIR dyes, such as porphyrins and phthalocyanins,¹⁰ polymethines,¹⁰ BODIPY derivatives,¹⁶⁻¹⁸ and xanthenes with extended annulation¹⁹⁻²¹ tend to suffer from high molecular weights, low water solubility, a propensity to aggregate, and chemical or photochemical instability, complicating their use in biological contexts. A number of creative approaches have recently been employed to address these concerns.^{14, 20, 22-24} In particular, the compact, fused 3-ring system of xanthene-based dyes promotes stability and decreases molecular weight. Annulation of xanthenes can push their absorbance above 700 nm,¹⁹ but at the expense of decreased water solubility. Recent efforts replace oxygen at position 10 with carbon²⁵⁻²⁷ or heteroatoms such as Si,²⁸⁻³⁰ S,³¹ and P. Indeed, recent examples of phosphinate³² and phosphine-oxide based xanthene dyes³³⁻³⁴ demonstrate this unique scaffold can access absorption profiles at or above 700 nm while maintaining a compact, three-ring structure.

While numerous approaches to NIR-emitting fluorophores exist, there are relatively few examples of NIR-absorbing fluorescent reporters—fluorophores that sense and respond

Scheme 1. Synthesis of sulfonated phosphine oxide rhodamine voltage reporters (poRhoVRs)



reversibly to changes in a specific physiological phenomena, for example, metal ions, reactive oxygen species, or transmembrane potential. The few examples of NIR-absorbing indicators employ polymethine, rather than xanthene, scaffolds.^{4, 35-37} Therefore, we were eager to adapt phosphorous-substituted xanthenes into a fluorescent voltage-sensing scaffold. Previous work in our lab suggests that xanthene dyes with a range of bridgehead (O,³⁸ C,²⁷ Si³⁹) and terminal atoms (O^{27, 38} or substituted N³⁹⁻⁴⁰) can be employed as voltage-sensitive dyes via introduction of a lipophilic, conjugated molecular wire. We hypothesized that the installation of phenylene vinylene molecular wires into the context of a phosphorous-substituted xanthene dye would yield voltage-sensitive indicators with peak excitation above 700 nm. There are no examples of xanthene-based voltage-sensitive fluorophores with peak excitation above 700 nm, although there are a few examples of chemically-synthesized⁴¹⁻⁴⁴ and genetically encoded⁴⁵⁻⁴⁷ voltage indicators with emission maxima beyond 700 nm.

We now present the phosphine-oxide Rhodamine Voltage Reporters, or poRhoVRs, which all feature excitation and emission maxima above 700 nm, 13% to 43% $\Delta F/F$ per 100 mV voltage sensitivity, and compatibility with commonly used optical sensors and actuators, including Oregon Green BAPTA (OGB), GCaMP6, and ChannelRhodopsin-2 (ChR2). We use poRhoVR to provide the first direct voltage imaging of neuronal hyperactivity in retinas isolated from a mouse model of retinopathy.

Results

Synthesis of sulfonated phosphorous rhodamines

Although phosphorous rhodamines possess attractive optical properties³²⁻³⁴ – NIR excitation and emission, photostability,³²⁻³⁴ and good water solubility – their tendency to localize to intracellular membranes, including mitochondria³⁴ and lysosomes,⁴⁸ precludes their straightforward deployment as cytosolic indicators or voltage sensors. Water solubility and exclusion from cellular uptake can be achieved through installation of anionic groups on the exocyclic rhodamine

nitrogens;⁴⁹⁻⁵⁰ however, we recently found that sulfonation of the *meso* aromatic ring of rhodamine-type systems prevents cellular uptake of dyes.^{39, 51} We

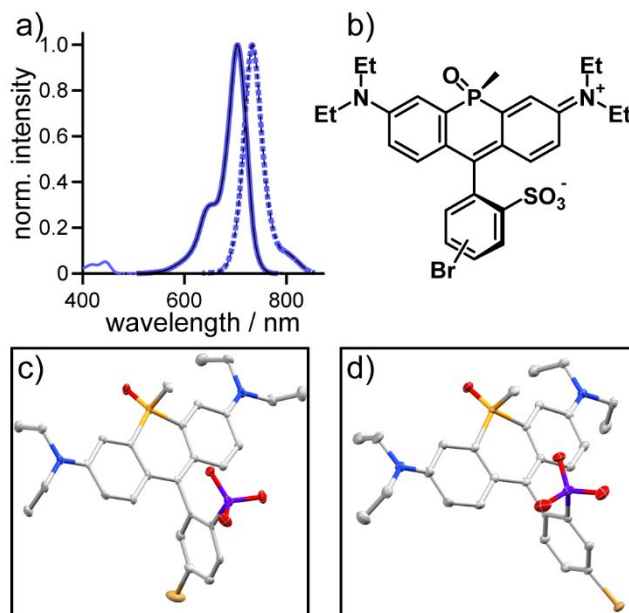


Figure 1. Characterization of sulfonated phosphine-oxide rhodamine dyes **8** and **9**. **a)** Plots of normalized absorbance (solid lines) and fluorescence intensity (dashed lines; excitation provided at 625 nm) of phosphine-oxide rhodamine **8** (thick blue lines) and **9** (black lines) in phosphate buffered saline (5 μM dye, 1% DMSO). **b)** Chemical structure of the *cis* isomers of **8** and **9**. Thermal ellipsoid plots (50%) of **c)** **8** and **d)** **9**. Hydrogen atoms, lattice solvent molecules and resolved disordered fragments have been omitted for clarity.

hypothesized that a similar strategy could provide sulfonated phosphorous rhodamines for incorporation into voltage sensing scaffolds.

Phosphine-oxide RhoVRs were accessed in 6 steps from commercially available triphenylphosphonium salts (**Scheme S1**). Anilino phosphine oxide **4** was prepared in high yield over three steps.^{33, 52} Phosphine oxide **4** was alkylated through a slightly modified procedure using ethyl iodide and potassium carbonate in dimethylformamide (DMF) to produce phosphine oxide **5** in 89% yield. The key sulfonated phosphorous rhodamine precursors with bromine substitution for installation of the phenylene vinylene molecular wire were prepared in one step by condensing bromosulfonylbenzaldehydes⁵¹ **6** or **7** with phosphine oxide **5** in the presence of urea⁵³ and glacial acetic acid (**Scheme 1**). Traditional methods for acid-catalyzed construction of rhodamines or sulfonrhodamines, for example, condensation with methane sulfonic acid⁵⁴ or propionic acid without urea^{40, 55} resulted in no reaction or were extremely low yielding.

The condensation between *m*- (**6**) or *p*-bromosulfonylbenzaldehyde (**7**) and phosphine oxide **5** produced two isomers of *m*- (**8**) and *p*- (**9**) bromosulfo-phosphine oxide rhodamines³³⁻³⁴ in which the P-methyl substituent and sulfonate exist in either a *cis* or *trans* relationship to one another (**Figure 1b**), consistent with previous reports and nomenclature³³ (**Figure S1**). For both **8** and **9**, the two isomers were separable on a basic alumina column, eluting with 0-5% methanol in dichloromethane. After this step, no further purification was

necessary for the major isomer of either **8** or **9**. Preparative HPLC was required to isolate the minor isomer. Analysis of the NMR spectra of the purified major and minor isomers of **8** and **9** revealed differences in the chemical shifts of both the phosphorous atom and the P-methyl substituents (Table S1): for the P-methyl group, $\delta = 2.00$

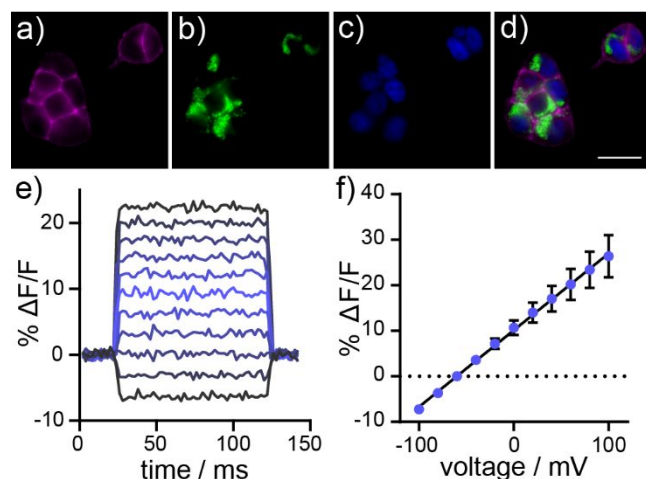


Figure 2. Cellular characterization of poRhoVR indicators in HEK cells. (a-d) Widefield, epifluorescence images of a) poRhoVR **14** (1 μ M) in HEK cells. Cells are counter-stained with b) rhodamine 123 (1 μ M) and c) Hoechst 33342 (1 μ M) to visualize mitochondria and nuclei, respectively. d) An overlay of poRhoVR **14**, rhodamine 123, and Hoechst 33342. Scale bar for (a-d) is 20 μ m. e) Plot of fractional change in fluorescence of poRhoVR **14** vs time for 40 ms hyper- and depolarizing voltage steps from a holding potential of -60 mV for a single HEK cell labeled with poRhoVR **14** (1 μ M). f) Plot of $\Delta F/F$ vs membrane potential, summarizing data from $n = 6$ individual HEK cells. Error bars are \pm S.D. If error bars are not visible, the error is smaller than the marker.

ppm (^1H , major isomer) and $\delta = 1.96$ (and Figure S1). Furthermore, x-ray crystal structures of the major isomer of both **8** and **9** reveal a *cis* relationship between the sulfonate and the methyl group on the phosphorous bridgehead atom (Figure 1c and d). Attempts to crystallize the minor isomer of **8** or **9** were unfruitful. In aqueous solution (phosphate buffered saline, 5 μ M dye), both bromo phosphine oxide rhodamine **8** – *cis* and **9** – *cis* had an absorption maximum at 704 nm, emission maxima around 728 nm (Figure 1a), and quantum yields near 20% (22% for **8** and 19% for **9**).

Synthesis of phosphorous rhodamine voltage reporters

After determining that sulfonated po-rhodamines have reasonable quantum yields and desirable NIR excitation and emission maxima above 700 nm, we hypothesized that incorporation of phenylene vinylene molecular wires should render these NIR xanthenes voltage-sensitive. We installed molecular wires **10** (dimethyl aniline) or **11** (diethyl aniline and methoxy substituent) onto the phosphine-oxide rhodamine fluorophore using a Pd-catalyzed Heck coupling to generate a total of four new phosphine oxide Rhodamine VoltageReporters (poRhoVRs, **12** – **15**, Scheme 1). For each reaction, the cross coupling was performed using the purified, major isomer (*cis*) of either **8** or **9**. However, in each case this did not prevent the formation of *cis/trans* isomers in the complete poRhoVR molecules: analytical HPLC/MS showed two peaks with the product mass corresponding to poRhoVR. As previously reported,³³ separation of the isomers is extremely

arduous but in the case of **13**, extensive column chromatography on silica gel and preparative scale HPLC on C_{18} -functionalized silica afforded small quantities of major (9 % yield) and even smaller quantities of minor isomers (1% yield). Comparison of the ^1H and ^{31}P NMR data for the isomers of **13** closely match the chemical shifts observed for the *cis* and *trans* isomers of **8** and **9**, suggesting that the major product is the *cis* isomer of **13** (Figure S1). Isomers of the other three poRhoVRs (**12**, **14**, and **15**) were not separated due to the difficulty of purification. ^{31}P showed that for poRhoVRs **12**, **14** and **15** major products isolated were *cis* (^{31}P $\delta = 20$ ppm), consistent with the results for poRhoVR **13** – *cis* (^{31}P $\delta = 20.2$ ppm).

All of the poRhoVR compounds possess excitation and emission profiles well-matched to the parent phosphine-oxide rhodamines: absorption was maximal at 700 nm, and emission peaked at around 728 nm (Table 1, Figure S3a-d).

Cellular characterization of phosphorous rhodamine voltage reporters

All four poRhoVR dyes (**12** – **15**) localize to the cellular membrane and display differing cellular brightness when applied to HEK cells under identical experimental conditions (Figure S2a-d). Although all poRhoVRs localize to the membrane, poRhoVRs **13** and **15**, with *N,N*-diethyl, methoxy-aniline substituents, are approximately 10-fold dimmer in cell membranes than poRhoVR **12** and **14** (*N,N*-dimethyl aniline) (Table 1, Figure S2e). The long wavelength excitation and emission of poRhoVRs enables multi-color imaging with organelle specific dyes. Live-cell imaging⁵⁶ of poRhoVR **14** (1 μ M, Figure 2a) in HEK cells co-stained with rhodamine 123⁵⁷ (the methyl ester of rhodamine 110),⁵⁸ which localizes to the mitochondria on account of its overall positive charge (Figure 2b) and Hoechst 33342,⁵⁹ a bisbenzimidazole dye which binds to nucleic acid, labeling the nuclei of cells, (Figure 2c) demonstrate good membrane localization for poRhoVRs along with compatibility with simultaneous, multi-color imaging (Figure 2d).

The cellular fluorescence of poRhoVRs is voltage sensitive. Simultaneous fluorescence microscopy during sequential depolarizing and hyperpolarizing steps from a holding potential of -60 mV in HEK cells under whole-cell voltage-clamp conditions reveals linear fluorescence vs. voltage relationships for poRhoVR dyes **12** – **14** (Figure 2e and f, 14; Figure S3 for all indicators). Voltage sensitivities range from 13% (poRhoVR **12**) to 43% (poRhoVR **13**) per 100 mV (Table 1). The sensitivity of poRhoVR **13**, at 43%, is nearly 2-fold the sensitivity of our most red-shifted voltage indicator to date, the Si-rhodamine based BeRST (24% $\Delta F/F$ per 100 mV)³⁹ and is comparable to the sensitivity of our previously reported, tetramethyl rhodamine based RhoVR (47% $\Delta F/F$ per 100 mV).⁴⁰ However, the low cellular brightness of poRhoVR **13** (more than 10x dimmer than poRhoVRs **12** and **14**) means that indicators like poRhoVR **14**, with intermediate voltage sensitivity (17% $\Delta F/F$ per 100 mV), but high cellular brightness (12x brighter, compared to **13**) are also attractive for monitoring voltage dynamics in living systems. This is because, in a shot-noise-limited system, SNR scales with the square root of the number of collected photons, or the cellular brightness.⁶⁰⁻⁶¹ Therefore, even indicators with only middling $\Delta F/F$ values can be quite effective detectors of rapid voltage changes.⁶²⁻⁶³ For poRhoVR indicators, it appears that the OMe-substituted compounds (**13** and **15**) show lower cellular brightness

compared to the unsubstituted aniline molecular wires of **12** and **14** (Table 1). This effect appears to be specific to poRhoVR indicators, as rhodamine-based RhoVR indicators show the highest SNR values for OMe-substituted molecular wires.⁴⁰

Table 1. Properties of po-RhoVR indicators

Compound number	isomer ^a	R ¹	R ²	$\lambda_{\text{abs}} / \text{nm}^b$	$\lambda_{\text{em}} / \text{nm}^b$	Φ^b	rel. cell brightness ^c	$\Delta F/F^d$
8	<i>meta</i>	n/a	n/a	704	728	0.22	n/a	n/a
9	<i>para</i>	n/a	n/a	704	728	0.19	n/a	n/a
12	<i>meta</i>	H	Me	705	731	0.01	15 ± 0.9	13 ± 0.3%
13	<i>meta</i>	OMe	Et	703	728	0.01	1.0 ± 0.1	43 ± 0.9%
14	<i>para</i>	H	Me	704	723	0.07	12 ± 0.1	17 ± 0.5%
15	<i>para</i>	OMe	Et	704	726	0.09	1.3 ± 0.03	31 ± 0.5%

^a relationship between fluorophore and molecular wire ^bdPBS with 0.1% DMSO ^cRelative cellular brightness, in HEK cells ^dper 100 mV, in HEK cells. Data are mean ± S.E.M. for at least 3 separate determinations.

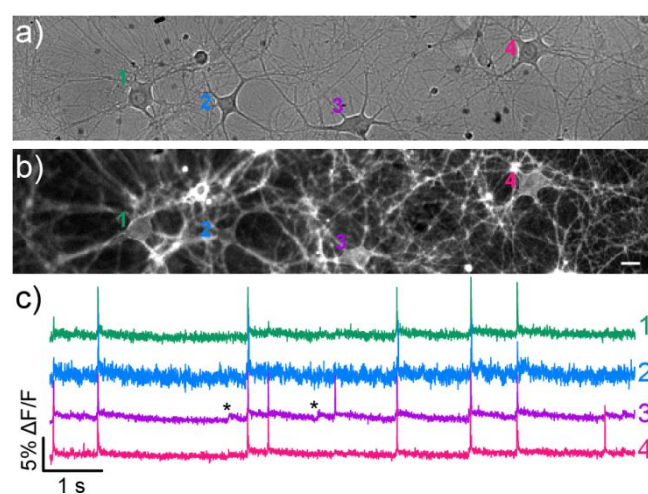


Figure 3. Voltage imaging in dissociated rat hippocampal neurons with poRhoVR **14**. Transmitted light image of neurons loaded with **a**) poRhoVR **14** (500 nM). **b**) Epifluorescence image of neurons showing poRhoVR **14** staining. Scale bars are 20 μm . **c**) Plot of fractional change in poRhoVR **14** fluorescence ($\Delta F/F$) vs time emanating from cells 1-4 in image **(b)**. Optical sampling rate is 500 Hz. Asterisks indicate subthreshold voltage changes.

could function as well as a dim, but more sensitive dye (**13**). Both poRhoVR **13** (Figure S4a-c) and **14** (Figure 3a-c) localize well to cellular membranes and readily report on spontaneously-firing action potentials in single-trial acquisitions, revealing a range of neuronal firing rates similar to previously reported ranges.⁶⁴ In some cases, poRhoVR **14** clearly visualizes sub-threshold voltage dynamics, likely post-synaptic potentials (Figure 3c, cell 3, asterisks).

Consistent with its performance in HEK cells, poRhoVR **14** was approximately 8 to 12-fold brighter than **13** under identical experimental conditions (Figure S5a). In a complementary fashion, the $\Delta F/F$ value per spike/action potential for **13** was larger than for **14** (12% vs 6%, Figure S5b) but with lower overall signal-to-noise ratio (SNR, 20:1 vs 40:1, Figure S5c). Because of the higher brightness and SNR for detecting action potentials, we used poRhoVR **14** for subsequent experiments.

We evaluated both poRhoVR **13** and **14** in cultured hippocampal neurons isolated from rat embryos. We selected **13** and **14** to investigate whether a bright, but less sensitive dye (**14**)

We next examined the photostability, internalization rate, and toxicity of poRhoVR **14**. The photostability of poRhoVR **14** is comparable to the photostability of the previously-reported BeRST **1** (Figure S6).³⁹ We also measured the persistence of poRhoVR **14** on the cell membrane in HEK cells. We examined the degree of poRhoVR **14** internalization from the membrane by measuring the colocalization between calcein-AM⁶⁵ fluorescence – a cytosolic stain that becomes fluorescent and is retained in living cells – and poRhoVR **14** (Figure S7). Increased colocalization values indicates a higher degree of internalization of the membrane-associated poRhoVR **14**. We find a low degree of colocalization between poRhoVR **14** signal and cytosolic calcein-AM after an initial 20 minutes of loading (the Pearson's correlation coefficient is 0.22 ± 0.2 , mean \pm S.E.M. for $n = 3$ coverslips) (Figure S7a). There is an increase in the degree of colocalization, to approximately 0.26, after additional observation for up to 2 hours (Figure S7), but the changes are not statistically significant ($P > 0.05$, Dunnett's multiple comparisons test, see Figure S7). Finally, we examined the toxicity of poRhoVR **14** in HEK293T cells using calcein-AM. Comparison of the colocalization of Hoechst 33342 fluorescence, which stains the nuclei of both dead and live cells, and calcein-AM fluorescence reveals no difference in colocalization, and therefore toxicity, between vehicle-treated HEK293T cells (DMSO) and up to 5 μM poRhoVR **14** (Figure S8).

poRhoVR **14** and optogenetic activator ChR2

poRhoVR **14** can be used in multicolor experiments, not only for tracking static fluorescence associated with distinct organelles (Figure 2a-d), but also in concert with commonly employed optogenetic actuators and sensors. We expressed the blue light-activated opsin, ChannelRhodopsin-2 (ChR2),⁶⁶⁻⁶⁷ fused to yellow fluorescent protein (YFP) in a subset of hippocampal neurons isolated from rat (Figure 4a and b). Bath application of poRhoVR **14** to these same neurons results in membrane-localized fluorescence (Figure 4c) that is spectrally isolated from the YFP signal (Figure 4d).

The combined use of ChR and poRhoVR allows dissection of functional connectivity across a large number of neurons. Optical stimulation of cell 1 (475 nm, 5 ms, 1.92 mW/mm²) results in ChR2-evoked action potentials optically recorded at cell 1 (Figure 4e and f). Cells 4 and 5 appear monosynaptically

coupled to ChR2-positive cell 1 with action potential latencies of $6.7 \text{ ms} \pm 1.5 \text{ ms}$ (S.D. $n = 60$ pairs of spikes) and $5.6 \text{ ms} \pm 1.3 \text{ ms}$ (S.D., $n = 20$ out of 60 spikes), respectively. However,

the data reveals differences in the relative strengths of these connections with cell 1 triggering firing in cell 4 for 100% of action potentials, and only 33% for cell 5. Interestingly, bursts

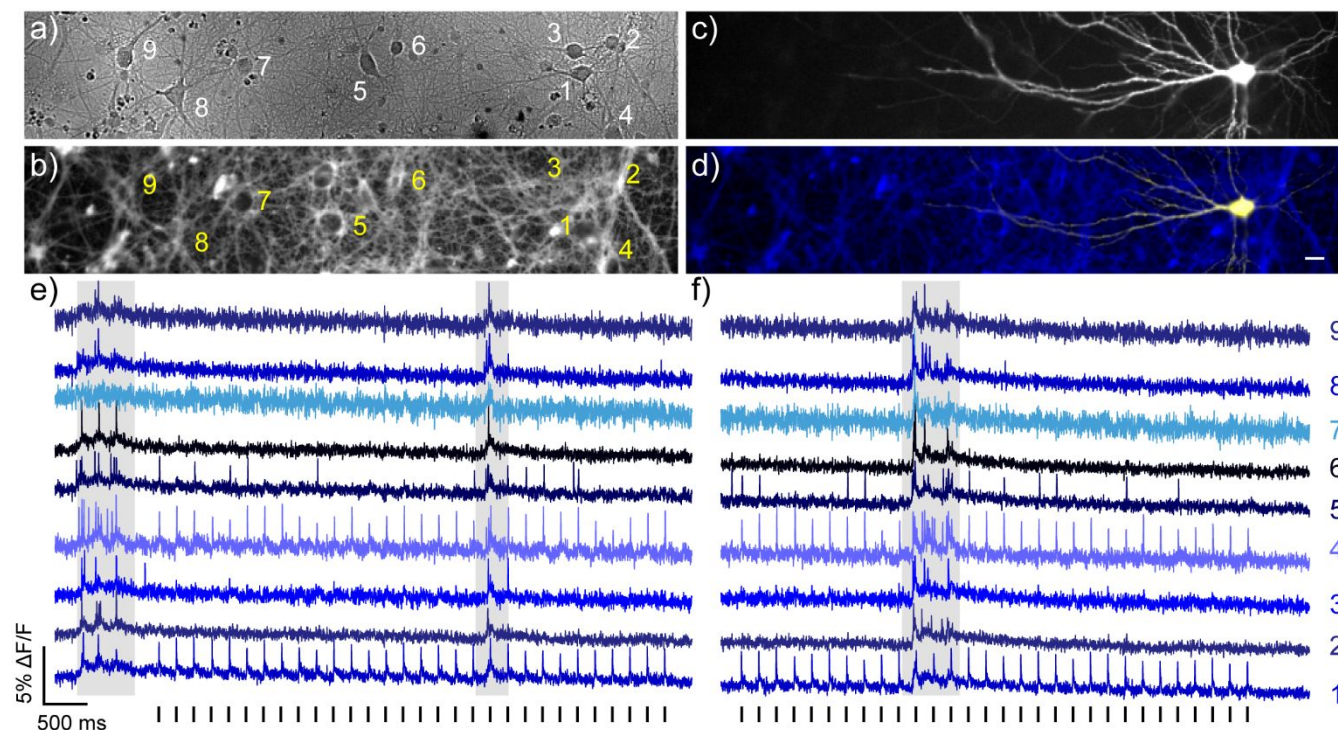


Figure 4. All-optical electrophysiology using poRhoVR 14 and ChR2. **a)** Transmitted light image of dissociated rat hippocampal neurons stained with poRhoVR 14 (500 nM). Scale bar is 20 μm . **b)** Epifluorescence image of neurons stained with poRhoVR 14. **c)** Epifluorescence image of neuron displaying YFP marker of ChR2 expression. **d)** Composite image depicting poRhoVR 14 labeling and ChR2-YFP expression. **e)** Recording of $\Delta F/F$ from the cell bodies of neurons indicated in panels **a-d**. **f)** Plot of $\Delta F/F$ from the cell bodies of neurons indicated in panels **a-d**, recorded 90 seconds after the data shown in panel **e**. Optical sampling rate was 500 Hz. The entire field was stimulated optically with flashes of cyan light (475 nm, 5 ms, 1.92 mW/mm^2) as indicated by the vertical bars below the blue optical recording in panels **e** and **f**. Optical voltage recordings are single trials.

of spontaneous activity indicate strong recurrent connectivity between all neurons (**Figure 4e and f**, grey shaded areas), but firing initiated in the ChR2-expressing neuron (cell 1, **Figure 4a-d**) activates only a subset of these neurons (**Figure 4e and f**): notably, cell 4, nearly 50 μm away from cell 1, and cell 5, over 120 μm distant from ChR2-expressing cell 1.

The combination of poRhoVR and ChR2 enables interrogation of sub-threshold potentials. In a few cases, during cyan light stimulation, we observe slower, sub-threshold potentials in cells up to 400 microns away from the ChR2-expressing neuron (**Figure S9d and e**, cells 2 – 4, asterisks), highlighting the ability of poRhoVR 14 to monitor sub-threshold voltage changes (**Figure 3a-c**). The use of cyan light to stimulate ChR2 does not cross-excite poRhoVR 14, as indicated by the lack of stimulus artifact in the ChR2-negative cells in the same field of view (**Figure 4d and e**, **Figure S9**). Together, these experiments establish poRhoVR 14 as a powerful complement for all-optical electrophysiology utilizing NIR absorbing indicators.

Two color imaging with poRhoVR 14 and Ca^{2+} indicators

In addition to deployment alongside light-activated actuators, poRhoVR can be used with optical indicators. Fluorescent sensors for Ca^{2+} are among the most widely used optical sensors. Despite some three decades since the initial reports of fluorescent indicators for this critical intracellular messenger,⁶⁸⁻

⁶⁹ most Ca^{2+} indicators utilize excitation and emission profiles firmly centered in the blue/green region of the visible spectrum (for example, Oregon Green BAPTA, OGB,⁷⁰ and the GCaMP family of genetically encoded indicators).⁷¹ Although promising new Ca^{2+} indicators, both synthetic⁷²⁻⁷⁵ and genetically encoded,⁷⁶⁻⁷⁹ possess red-shifted excitation and emission spectra, circularly-permuted (cp) GFP-based indicators, like the GCaMP family,⁷¹ dominate the landscape of functional imaging.⁸⁰⁻⁸¹ Therefore, fluorescent voltage indicators with orthogonal wavelengths are required.

We performed two-color, simultaneous voltage and Ca^{2+} imaging in the same cells using poRhoVR 14 and the synthetic Ca^{2+} indicator, OGB (**Figure 5a-c**). We treated hippocampal neurons with both poRhoVR 14 (500 nM) and OGB (1 μM) simultaneously and imaged using an image-splitting device to project two emission wavelengths onto the same camera chip. Under these conditions, we observe clear membrane-associated fluorescence for poRhoVR 14 (**Figure 5a**) and cytosolic fluorescence for OGB (**Figure 5b**). We established that no cross-excitation exists under these conditions (**Figure S10**). Using field-stimulation electrodes, we evoked a series of 10 action potentials, across a range of frequencies, and simultaneously recorded voltage (**Figure 5c**, magenta traces) and Ca^{2+} (**Figure 5c**, green traces) dynamics.

Both poRhoVR 14 and OGB clearly resolve single action potentials when activity is evoked at rates of either 5 or 10 Hz.

poRhoVR **14** clearly resolves action potentials at firing rates of 20 and 30 Hz (**Figure 5c**). OGB, despite its fast Ca^{2+} response kinetics (<5 ms to action potential peak)⁷¹ compared to GCaMP6f (~ 45 ms to peak)⁷¹ and other genetically encoded

indicators,⁸²⁻⁸⁴ fails to accurately report individual action potential-evoked Ca^{2+} transients at firing frequencies higher than 10 Hz (**Figure 5c**). Neurons in the brain and retina can fire action

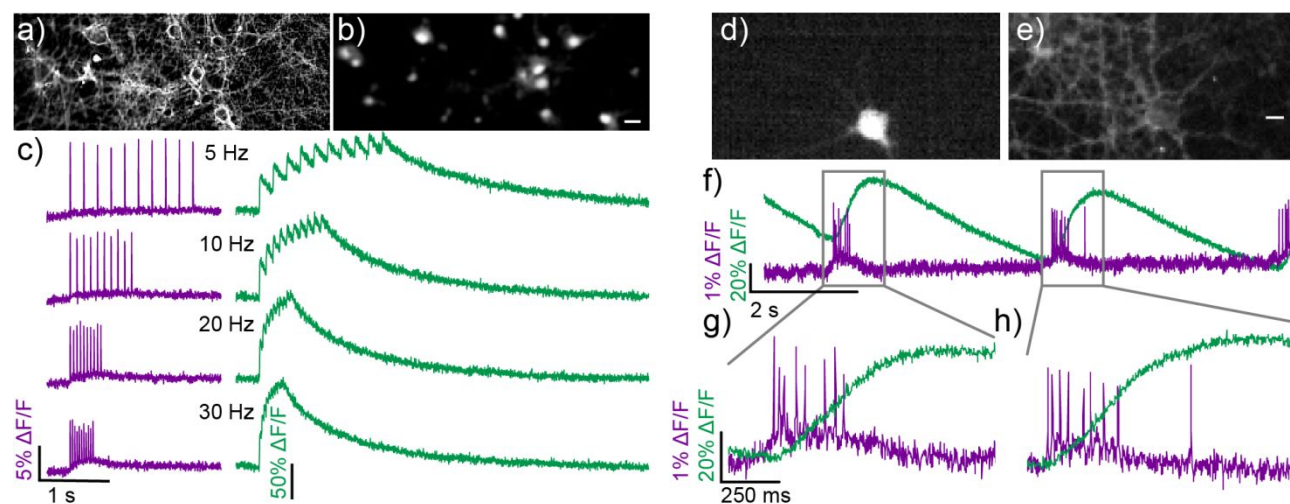


Figure 5. Simultaneous voltage and calcium imaging with poRhoVR **14**. Epifluorescence image of neurons stained with both **a**) poRhoVR **14** (1 μM) and **b**) Oregon Green BAPTA 1 AM (OGB, 1 μM). **c**) Plots of $\Delta F/F$ for voltage (poRhoVR **14**, purple) and Ca^{2+} transients (OGB, green) in response to field stimulation driven at 5, 10, 20, and 30 Hz. **d**) Epifluorescence image of a neuron transfected with GCaMP6s. **e**) This same GCaMP6s (+) neuron is also stained with poRhoVR **14** and imaged simultaneously. Scale bar is 10 μm . **f**) Simultaneously recorded traces of voltage and calcium activity from neuron in panels **d** and **e**. Activity was evoked using field stimulation at a rate of 16 Hz. **g**) The insets show that the onset and decay of voltage signals imaged with poRhoVR **14** precede that of the calcium signal visualized from the same cell with GCaMP6s.

potentials at rates up to several hundred Hz, for example in interneurons of the hippocampus,⁸⁵ Purkinje cells of the cerebellum,⁸⁶ and ganglion cells of the retina,⁸⁷ emphasizing the need for indicators with fast response kinetics.

Ca^{2+} indicators are often characterized against varying numbers of action potentials arriving at a constant frequency. However, neural information is often encoded in the form of spike rates. Therefore, resolution of individual spikes and firing frequency is critical for understanding the underlying physiology of the system under observation. Even with very fast OGB, estimating spike frequency using Ca^{2+} imaging traces alone was unsuccessful. Neither peak Ca^{2+} $\Delta F/F$ (**Figure S11a**) nor integrated area under the curve (**Figure S11b**) were able to resolve differences at 5, 10, 20, or 30 Hz firing rates. In contrast, the optically recorded voltage transients revealed by poRhoVR **14** clearly discriminates between firing frequencies of 5, 10, 20, and 30 Hz (**Figure S11c**).

Simultaneous voltage and Ca^{2+} imaging in the same cells can also be achieved alongside genetically encoded indicators, like GCaMP6. We again stained neurons with poRhoVR **14** (1 μM). This time, a subset of hippocampal neurons expressed GCaMP6s. Again, poRhoVR localizes to membranes (**Figure 5d**), while GCaMP6s fluorescence appears cytosolic (**Figure 5e**). Simultaneous voltage and Ca^{2+} imaging of spontaneous activity in hippocampal neurons reveals fast-spiking bursts resolved in voltage (**Figure 5f-h**, magenta trace), followed by slower, sustained increases in GCaMP6-associated fluorescence (**Figure 5f-h**, green trace). Notably, voltage imaging with poRhoVR **14** exhibits sufficiently high temporal resolution to distinguish individual action potentials in spike volleys (**Figure 5g**, 8 spikes; **Figure 5h**, 9 spikes), while Ca^{2+} imaging does not. Together, these experiments establish the utility of poRhoVR dyes for monitoring fast spiking in neurons

alongside commonly used synthetic and genetically-encoded Ca^{2+} indicators and emphasizes the care needed when interpreting Ca^{2+} imaging data.

Voltage and Ca^{2+} imaging and electrode recording in a mouse model of retina degeneration

The NIR (>700 nm) excitation and emission spectra of poRhoVR dyes, along with their good voltage sensitivity, compatibility with commonly used optogenetic sensors and actuators, and ready uptake into cell membranes (**Figure S12**) makes poRhoVR **14** a promising candidate for mapping voltage dynamics in intact neural tissue like retinas. The retina is a highly organized and accessible outpost of the central nervous system. Light responses initiated in rods and cones are synaptically transmitted to bipolar cells, which activate retinal ganglion cells (RGCs). RGCs generate the action potentials that carry visual information to the brain. In normally functioning retinas, the intrinsic light sensitivity of photoreceptors in rods and cones complicates optical imaging of both voltage and Ca^{2+} transients in RGCs, because visible light (or high intensity two photon excitation) used to excite the indicators triggers physiological responses.⁸⁸ We applied poRhoVR **14** to investigate membrane potential dynamics in retinas from a mouse model of retina degeneration.

In particular, retinas from *rd1* mice are an attractive model system in which functional imaging can be applied to explore mechanisms occurring in inherited visual disorders, including the degenerative disorder retinitis pigmentosa (RP).⁸⁹ Lacking a functional β subunit of rod cGMP phosphodiesterase (βPDE),⁹⁰ *rd1* mice suffer rapid loss of rod cells, followed by a delayed loss of cone cells. As a result, these mice lack rods and cones and are therefore blind, yet still retain functional RGCs in the ganglion cell layer (GCL). Therefore, apart from the rare

melanopsin-expressing, intrinsically sensitive RGC (<2% of the RGCs) the surviving RGCs in *rd1* retinas have no light response and continue to receive synaptic input from bipolar cells. RGCs in *rd1* mice develop hyperactivity, firing spontaneous bursts of

spikes in darkness. This phenomenon has been observed only through electrophysiological recordings that sample only a fraction of cells, or by Ca^{2+} imaging with GCaMP6f expressed in RGCs, which is indirect and has low temporal resolution.^{87, 91}

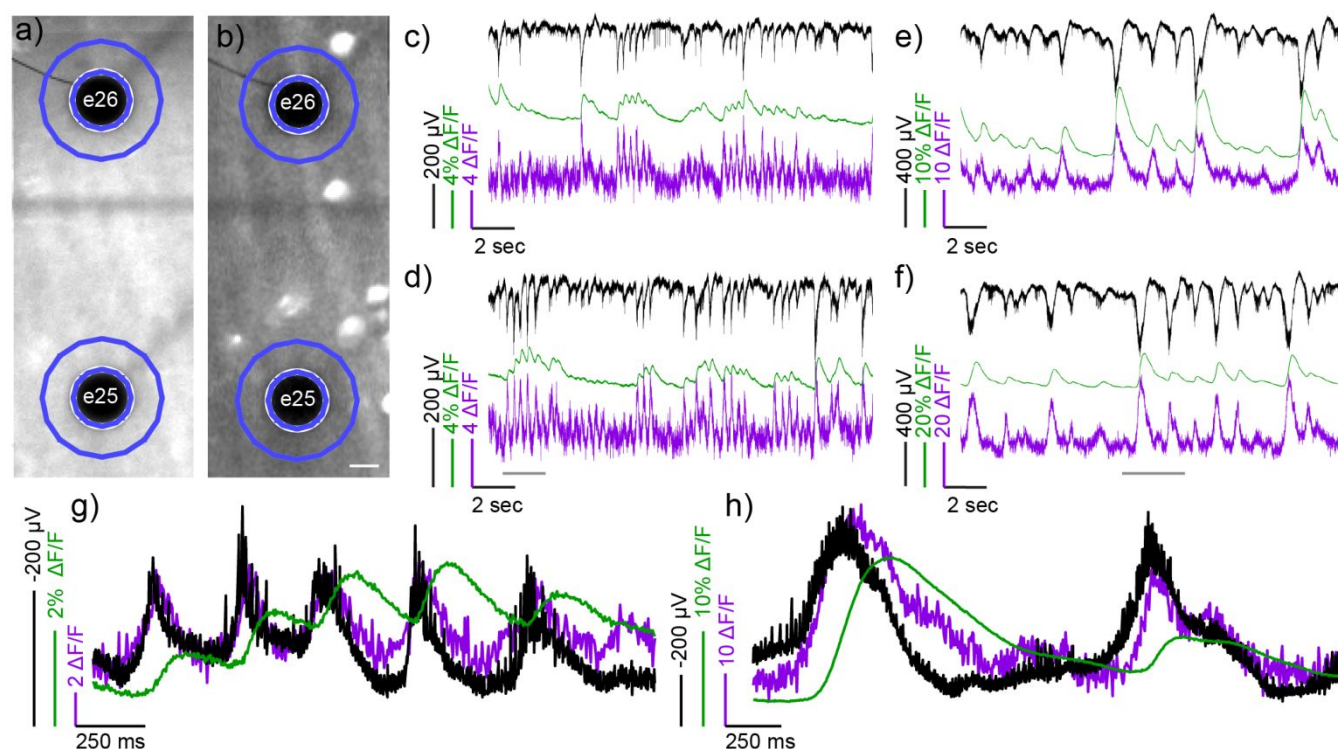


Figure 6. Simultaneous mapping of electrical and Ca^{2+} activity using poRhoVR, GCaMP6f and multi-electrode arrays (MEA) in *ex vivo* retinas from *rd1* mice. Widefield fluorescence micrographs of retina stained with **a)** poRhoVR **14**; the retinal ganglion cells (RGCs) express **b)** GCaMP6f. The black dots are MEA electrodes, labeled numerically, underneath the retina. Scale bar is 20 μm . Recordings **c-f** depict MEA, GCaMP6f, and poRhoVR **14** signals vs. time. Traces are as follows: raw MEA electrical signal (black), bleach corrected poRhoVR $\Delta\text{F}/\text{F}$ (arbitrary units) (magenta), and GCaMP6f $\Delta\text{F}/\text{F}$ (%) (green). Optical signals are from the regions of interest (ROIs) indicated in blue in panels **a** and **b**. Panels **c** and **d** depict the spontaneous activity in the retina prior to addition of synaptic blockers. Panels **e** and **f** show MEA, GCaMP6f, and poRhoVR **14** signals 15 min. after the addition of synaptic blockers. Panels **c** and **e** correspond to signals associated with electrode 26 (e26), and panels **d** and **f** correspond to signals associated with electrode 25 (e25). Panels **g** and **h** show zoomed-in regions of e25, from the time period indicated by a grey bar in panel **d** and **f**, respectively. In panels **g** and **h**, the MEA signal (black) is inverted to facilitate comparison with optical voltage recordings with poRhoVR (magenta).

The precise mechanisms underlying hyperactivity are not understood, and voltage imaging could reveal where in the retinal circuitry this activity originates and how it propagates from neuron to neuron.

To explore the interplay between neuronal voltage and Ca^{2+} signaling in this model of retinopathy, we prepared *ex vivo*, flat-mount retinas from *rd1* mice that express GCaMP6f in retinal ganglion cells (RGCs). Bath application of poRhoVR **14** (5 μM , in oxygenated ACSF) results in diffuse poRhoVR **14** staining throughout the tissue, as assessed by widefield fluorescence microscopy (**Figure 6a**, **Figure S14d-e**). In retinas from *rd1* mice, we observed spontaneous oscillations of poRhoVR **14** fluorescence (**Figure 6d-h**, magenta traces). To confirm that these changes in poRhoVR **14** dynamics correspond to voltage changes in RGCs, we paired voltage imaging with multi-electrode array (MEA) recordings of extracellular potential.

The poRhoVR-stained retinas were mounted on a 64-channel MEA to simultaneously record transmembrane potential (poRhoVR **14**), extracellular potential (MEA), and Ca^{2+} transients (GCaMP6f) in many RGCs. Simultaneous transmembrane voltage imaging and MEA recording of

extracellular potentials (black traces) confirms that poRhoVR **14** (magenta traces) accurately reports changes in transmembrane potential in RGCs of the retina (**Figure 6c-f**). The time courses of the MEA signals (black) and poRhoVR **14** signals (magenta) match well, establishing that poRhoVR **14** senses voltage changes in mouse retina. The signs of the MEA and poRhoVR **14** signals are inversely related (**Figure 6c-f**): poRhoVR **14** measures transmembrane potential, while MEA records extracellular potentials. A zoomed-in view of poRhoVR **14** and MEA signals (**Figure 6g and h**), where the black MEA signals have been inverted to enable better comparisons, show excellent correspondence between the optically recorded transmembrane potential measured with poRhoVR **14** (magenta) and the MEA recording (black).

Voltage imaging reveals spontaneous membrane potential depolarizations that appear clustered in bursts throughout the imaging session (**Figure 6c** and **d**, magenta). These optically-recorded oscillatory bursts are, to our knowledge, the first direct imaging of membrane potential dynamics in *rd1* retinas, and are consistent with previous MEA recordings in *rd1* retinas.⁹²⁻⁹³ GCaMP6f recordings from the same areas revealed slower, Ca^{2+}

transients that were delayed relative to increases in the transmembrane potential measured by poRhoVR **14** fluorescence (**Figure 6c** and **d**, green). This lag between Ca^{2+} and voltage is similar to our observations of simultaneous Ca^{2+} / voltage imaging in hippocampal neurons (**Figure 5d-h**) and provides the first direct and simultaneous observation of voltage and Ca^{2+} in the mouse retina. We observe no evidence of oxidative photobleaching⁹⁴ of po-rhodamines under these imaging conditions (**Figure S13**).

Voltage imaging with poRhoVR **14** enables dissection of the temporal evolution of neuronal activity in *rd1* retina. Synaptic isolation of RGCs via blockade of all major forms of excitatory and inhibitory synaptic transmission results in evolution of activity from short, unsynchronized firing patterns (**Figure 6c, d, and g**) to synchronous and sustained firing (**Figure 6e, f, and h**), consistent with the idea that the driver of this behavior is presynaptic.⁹² Voltage imaging with poRhoVR **14** also allows investigation of the spatial differences in voltage dynamics with the retina. We recorded fluorescence from a region of interest (ROI) composed of a ring with a width approximately matching that of an RGC (~10 μm) around the MEA electrode (**Figure 6a** and **b**). This enables a direct comparison to extracellular potentials recorded by the MEA (**Figure 6c** and **d**, black). For the two electrodes visible in the recording image (**Figure 6a** and **b**), the MEA recording is closely matched by the poRhoVR **14** dynamics (**Figure 6c** and **d**, black vs. magenta). The use of poRhoVR **14** allows examination of the spatial relationships of voltage changes at locations distant from electrodes (**Figure S14**). Imaging of poRhoVR **14** signals recorded in ROIs in between electrodes reveal a unique activity profile that is not fully recapitulated by ROIs near the respective electrodes (**Figure S14a-b**), highlighting the ability of poRhoVR to map the spatial extent of voltage dynamics in retina. This methodology could prove a promising starting point for mapping the dendritic fields of RGCs by probing where voltage dynamics become convoluted with additional signals.

Conclusion

In summary, we present the design, synthesis, and application of phosphine-oxide rhodamines for voltage imaging. These new poRhoVRs have excitation and emission profiles above 700 nm and possess good voltage sensitivity. Their compatibility with other optical sensors and actuators makes them a powerful complement to existing approaches to dissect neuronal activity. We show that poRhoVR **14** can report on spontaneous action potentials in rat hippocampal neurons and enables all-optical electrophysiological manipulations with ChR2. Simultaneous voltage imaging with poRhoVR **14** alongside either fast synthetic Ca^{2+} indicators or genetically encoded Ca^{2+} indicators reveals the difficulty of relying solely on Ca^{2+} imaging to interpret underlying neuronal activity. Furthermore, poRhoVR **14** can be deployed in intact retinas alongside multi-electrode arrays and Ca^{2+} imaging to record from many RGCs at once in a mouse model of retinal degeneration, providing a first direct visualization of voltage dynamics alongside simultaneous Ca^{2+} imaging in the retina.

In the future, we envision combining poRhoVR with chemical-genetic hybrid methods to enable cell type-specific labelling in intact tissues.⁹⁵⁻⁹⁶ Beyond the chemical sensing presented in this manuscript, innovations in microscopy will be needed to achieve the kilohertz framerates, with micrometer resolution, across large, 3D volumes regions of the central nervous system that will be required for visualizing patterns of

activity in arrays of neurons *in vivo*.⁹⁷ Promising advances microscopy,⁹ including light sheet and swept-field microscopy,⁹⁸ redesigned optical paths,⁹⁹ holographic imaging,¹⁰⁰⁻¹⁰¹ and compressive sensing¹⁰² hint at possible avenues towards the use of poRhoVR for *in vivo* brain imaging.

ASSOCIATED CONTENT

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

AUTHOR INFORMATION

Corresponding Author

* evanwmiller@berkeley.edu

** These authors contributed equally.

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