Fast, in vivo voltage imaging using a red fluorescent indicator

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Genetically encoded voltage indicators (GEVIs) are emerging optical tools for acquiring brain-wide cell-type-specific functional data at unparalleled temporal resolution. To broaden the application of GEVIs in high-speed multispectral imaging, we used a high-throughput strategy to develop voltage-activated red neuronal activity monitor (VARNAM), a fusion of the fast Archaerhodopsin opsin and the bright red fluorophore mRuby3. Imageable under the modest illumination intensities required by bright green probes (<50 mW mm−2), VARNAM is readily usable in vivo. VARNAM can be combined with blue-shifted optical tools to enable cell-type-specific all-optical electrophysiology and dual-color spike imaging in acute brain slices and live Drosophila. With enhanced sensitivity to subthreshold voltages, VARNAM resolves postsynaptic potentials in slices and cortical and hippocampal rhythms in freely behaving mice. Together, VARNAM lends a new hue to the optical toolbox, opening the door to high-speed in vivo multispectral functional imaging.

Optical approaches to monitor and control neuronal activity provide live snapshots of activity patterns in individual neurons within large ensembles. When combined with the genetic delivery of indicator and actuator proteins, they can generate functional maps of precisely identified synaptic partners.

GEVIs are proteins that exhibit fluorescence intensity changes in response to transmembrane voltage transients12. Their direct voltage-sensing capability and fast kinetics make GEVIs ideal candidates for functional neural imaging. The large voltage sensitivity of ArcLight, a prototypical GEVI consisting of the Ciona phosphatase voltage-sensing domain (GVSD) and a pH-sensitive green fluorescent protein (GFP), permits visualization of synaptic potentials, hyperpolarization and population voltages in vitro and in vivo1−6. The fast kinetics of ASAPs (τ ≈ 3 ms), another family of VSD-based GFP GEVs, enables imaging of single action potentials (APs) in fast AP trains in cultures and subcellular voltage dynamics in flies1−3. The exceptional spike detection fidelity of Ace-mNeon, a fluorescence resonance energy transfer (FRET)-opsin indicator that integrates the submillisecond kinetics of Archaerhodopsin (Archer) with the superior brightness of mNeonGreen, allows near error-free spike-timing estimations in awake animals5,6.

Despite a surge in faster and more sensitive probes7,8,11−13, contemporary GEVIs offer limited spectral diversity. In paired recordings, green fluorescent indicators cannot be effectively coupled with red-shifted channelrhodopsins, which experience substantial cross-activation under blue light3,13−15. Single-color GEVIs are also constraining for multispectral studies, such as functional mapping of distinct cell types, where it becomes necessary to combine spectrally non-overlapping voltage indicators with each other, with calcium indicators or neurotransmitter indicators3.

Existing red-shifted GEVIs provide better spectral separation for coupling with blue-shifted optical tools13,14. However, they are less optimized for in vivo applications. For instance, GEVIs consisting of stand-alone Archaerhodopsin (Arch) mutants exhibit impressive kinetics for spike detection, but, despite recent efforts to engineer brighter variants, require intense illumination (1−12 W mm−2 versus <50 mW mm−2 for GFP-based probes) to enable voltage recordings with high signal-to-noise ratios (SNRs)16−18. The CiVSD-mApple fusion protein FlieR1 is brighter, yet requires ~100 mW mm−2 in cultures and exhibits moderate sensitivity and kinetics, inadequate for spike recordings in vivo7.

Here, we developed a red GEVI, voltage-activated red neuronal activity monitor (VARNAM, which means 'hue' in Sanskrit) using a high-throughput voltage-screening approach. We identified VARNAM, an ultrafast FRET-opsin indicator based on Ace and the bright red FP mRuby3, in a voltage screen comprising ~1,056 sequence variants transfected across ~206 mammalian cells. Readily imageable under moderate intensities of 15 mW mm−2 in cultures, acute slices and live Drosophila, VARNAM reports APs with sensitivity and kinetics comparable to Ace-mNeon. VARNAM captures postsynaptic potentials in slices as well as cell-type-specific transmembrane voltage dynamics in freely behaving mice. Lastly, VARNAM enables dual-color spike imaging and all-optical electrophysiology with minimal spectral crosstalk.

Results

High-throughput screening of voltage indicators. To develop red voltage indicators, our voltage screening workflow encompasses site-directed saturation mutagenesis of the indicator sequence followed by high-throughput voltage screening13 (Fig. 1a). Our semi-automated imaging platform utilizes a single field stimulation electrode to deliver current pulses one well after another, while fluorescence time-series images are recorded using a sCMOS camera (Fig. 1b, Supplementary Fig. 1 and Supplementary Note 1).

In pilot studies, we determined that the maximum fluorescence change to baseline fluorescence (ΔF/F) obtained on the platform for preexisting GEVIs5,10,17 matched their optical responses to 120 mV depolarization in simultaneous whole-cell patch-clamp and
fluorescence imaging in HEK cells (Fig. 1c,d). Field stimulation versus patch-clamp ΔFF/F for FlicR1, ArcLight and Ace-mNeon were 6.1 ± 0.5% (mean ± s.e.m.) versus 5.2 ± 0.6%, −33.2 ± 0.8% versus −32 ± 0.9%, and −10.3 ± 0.4% versus −12.1 ± 0.7%, respectively (n = 10 and 6 cells (FlicR1), 15 cells each (ArcLight) and 16 and 7 cells (Ace-mNeon)).

Using this approach, we identified a FlicR1 variant, FlicR2, with twice the sensitivity (Fig. 1e, Supplementary Fig. 2 and Supplementary Note 2). However, FlicR2 continued to exhibit dim fluorescence and impaired localization in intact tissue, unlike its predecessor17.

VARNAM, a red FRET-opsin indicator. Ace rhodopsin variants exhibit exceptional spike detection fidelity in fusion constructs with the bright green fluorophore mNeonGreen18. For high-fidelity red voltage imaging, we envisioned an indicator based on Ace and a bright red fluorophore. However, in Ace-mNeon, the voltage-dependent absorption of the chromophore retinal (FRET-acceptor) is natively blue-shifted, allowing optimal quenching of the fluorescence of mNeonGreen during depolarization. We surmised that replacing mNeonGreen with a red fluorophore might not retain the FRET efficiency nor voltage sensitivity of the parental indicator. Indeed, we found that voltage sensitivities of Ace fusion constructs with bright FPs peaked in the green and yellow wavelengths, corresponding to Ace-mNeon and Ace-mCitrine, but tapered off in the far red (Fig. 1f and Supplementary Fig. 3a).

Ace-mRuby3 exhibited a ΔFF/F of −7.9 ± 0.5% (mean ± s.e.m.; SNR = 22) and 4.8 ± 0.3% for maximum depolarization and hyperpolarization, respectively (n = 10 cells). The probe showed remarkable membrane expression in HEK cells, was substantially brighter than FlicR1 and FlicR2 and readily imageable at 15 mW mm⁻² (565 nm). In concurrent electrical and optical recordings in acute slices, Ace-mRuby3 resolved current-induced and spontaneous APs with −1.2 ± 0.2% (mean ± s.e.m.; SNR = 4;
To further enhance the sensitivity of Ace-mRuby3, we targeted factors crucial for FRET\textsuperscript{21}: the length (and composition) of the linker between the opsin and FP, and Ace’s absorption spectrum, which is modulated by residues lining the retinal-binding pocket (RBP) in prototypical opsins\textsuperscript{22–24} (Fig. 1g). In platform-based assays comprising 288 linker variants, the double insertional variant Ace-WR-mRuby3 N81S, which was about twice as sensitive as Ace-mRuby3 with unaltered resting brightness (Fig. 1h–j) and Supplementary Table). The probe followed 10 and 50 Hz voltage steps and 50 Hz simulated APs (Δ120 mV).

In HEK cells, Ace-WR-mRuby3 N81S, which we named voltage-activated red neuronal activity monitor (VARNAM), showed excellent membrane expression (Fig. 1i) and exhibited $-14.1 \pm 0.4\% \Delta F/F$ (mean ± s.e.m.; SNR = 49) to 120 mV depolarization and $9.1 \pm 0.3\% \Delta F/F$ to 80 mV hyperpolarization (n = 14 cells), comparable to those of Ace-mNeon ($-13.4 \pm 0.5\%$ (SNR = 59) and $6.1 \pm 0.5\% \Delta F/F$, respectively, n = 11 cells) (Fig. 2a,b). VARNAM’s fluorescence responses exhibited hysteresis, as noted for opsin indicators\textsuperscript{16,22}, VARNAM was significantly more sensitive to hyperpolarization than FlicR1 or FlicR2 ($\Delta F/F = -2.7 \pm 0.2\%$, n = 4 cells (FlicR1), mean ± s.e.m.) and $-4.2 \pm 0.5\%$, n = 6 cells (FlicR2), P = 0.0003 and P = 0.009, respectively, one-way ANOVA with Dunn’s multiple comparisons test) (Fig. 2b and Supplementary Fig. 2). At room temperature, the kinetics were akin to that of Ace-mNeon but substantially faster than FlicR1 or FlicR2 (Fig. 2c and Supplementary Table). The probe followed 10 and 50 Hz voltage steps and 50 Hz simulated APs, retaining 89.9% and 61.5% of its 10 Hz sensitivity in the latter protocols, respectively, whereas Ace-mNeon retained 92.3% and 65%, respectively, and FlicR2 retained 77.3% and 29%, respectively (Fig. 2d).

VARNAM showed superior photostability (time constant = 256 s) compared with Ace-mNeon, ASAP1 and ASAP2, ArcLight and FlicR1 (refs. \textsuperscript{8,13,17}) (Supplementary Fig. 7), likely ascribable to the innate photostability of mRuby3 (ref. \textsuperscript{25}).

In primary cortical neurons, VARNAM was mostly membrane-localized with some intracellular aggregation (Fig. 2e). Optical responses to current-induced APs had a mean spike amplitude of $8.4 \pm 0.3\% \Delta F/F$ (mean ± s.e.m.; SNR = 36; n = 8 neurons). VARNAM reported APs at 100 Hz, plateau potentials and spontaneous bursts (Fig. 2f–h), without perturbing AP duration or amplitude (Supplementary Fig. 8).
VARNAM reports APs and postsynaptic potentials in acute slices. We co-electroporated embryonic day 15 (E15) pups in utero with pan-neuronal expression plasmids encoding VARNAM and eGFP for longitudinal expression in intact tissue (Supplementary Fig. 4a). In fixed postnatal brain slices, VARNAM was brightly expressed at the membrane of superficial pyramidal neurons. Intracellular aggregates were minimal (Fig. 3a and Supplementary Fig. 9a). In acute slices, VARNAM resolved current-induced APs and ~113 Hz spontaneous bursts with a sensitivity comparable to that of Ace-mNeon (ΔF/F per spike of ~4.8 ± 0.3% (mean ± s.e.m.), SNR = 12, n = 7 neurons and ~4.3 ± 0.04%, SNR = 15, n = 6 neurons, respectively) and reported spontaneous APs with −2.0 ± 0.02% ΔF/F per spike (Fig. 3b–e and Supplementary Figs. 4c and 9b,c). Passive properties were unaltered by indicator expression (Supplementary Fig. 8). Further, VARNAM-positive neurons exhibited negligible steady-state photocurrents under 565 nm, as well as 505 or 455 nm
light used in multispectral imaging (Supplementary Fig. 10). While wildtype Ace carries a conserved aspartic acid at position 81, which is speculated to partake in proton transfer, substitutions obviate H⁺-pumping activity\(^2,\textsuperscript{27}\).

We next measured fluorescence responses to evoked excitatory and inhibitory postsynaptic potentials (eEPSPs and eIPSPs, respectively) in layer 2/3 VARNAM-positive neurons following electrical stimulation of layer 4 afferents. VARNAM resolved eEPSPs (vertical blue lines) (0.5 ms, 0.5–1 mW mm\(^{-2}\)). Arrow in c indicates spike failure. Gray box indicates interval shown at an expanded time scale. *Blue-light artifact (1 mW mm\(^{-2}\)). d. Fluorescence responses to 400 ms blue-light illumination (blue steps) at increasing intensities (0.4–2.4 mW mm\(^{-2}\)). e. Confocal images of a fixed slice from PV-Cre\(^\text{+}\) mouse showing cell-type-specific VARNAM and CheRiff expression (n = 7 fields-of-view). f,g. Mean optical and electrical IPSPs evoked by (f) blue-light pulses (2.5 mW mm\(^{-2}\)) at depolarized (left) and resting potentials (right) and (g) longer trains of pulses at depolarized potentials. n = 15 trials, each. Shading denotes s.e.m.

**Fig. 4 | All-optical electrophysiology in acute slices.** a. Confocal images of a slice co-electroporated with CheRiff and VARNAM (n = 5 fields of view). Single and double arrows in the merged image indicate neurons expressing one or both constructs, respectively. b,c. Optical and electrical spikes induced by 10 Hz (b) and 50 Hz (c) blue-light pulses (vertical blue lines) (0.5 ms, 0.5–1 mW mm\(^{-2}\)). Arrow in c indicates spike failure. Gray box indicates interval shown at an expanded time scale. *Blue-light artifact (1 mW mm\(^{-2}\)). d. Fluorescence responses to 400 ms blue-light illumination (blue steps) at increasing intensities (0.4–2.4 mW mm\(^{-2}\)). e. Confocal images of a fixed slice from PV-Cre\(^\text{+}\) mouse showing cell-type-specific VARNAM and CheRiff expression (n = 7 fields-of-view). f,g. Mean optical and electrical IPSPs evoked by (f) blue-light pulses (2.5 mW mm\(^{-2}\)) at depolarized (left) and resting potentials (right) and (g) longer trains of pulses at depolarized potentials. n = 15 trials, each. Shading denotes s.e.m.

We further sought to optically recapitulate changes in neuronal excitability using adolescent monocular deprivation as a model for homeostatic plasticity\(^2,\textsuperscript{28–30}\). In our recordings of excitability from neurons (PV-Cre\(^\text{+}\) females), expressing Cre recombinase in parvalbumin interneurons (PV-Cre\(^\text{+}\)) and binocular deprived, the optical and electrical recordings (Fig. 4b,c). Neurons expressing only VARNAM were unresponsive to blue light stimulation. In some instances, we observed a positive blip of ~0.1% ∆F/F in our optical recordings, which coincided with blue light onset and likely arose from tissue autofluorescence\(^\text{28–31}\) (Fig. 4c and Supplementary Fig. 11c). Increasing the amplitude and duration of light pulses elicited spontaneous bursts, which were readily discernible optically and, in background-subtracted traces\(^\text{18–31}\), reliably corresponded to electrical APs (Fig. 4d).

The 561 nm light (15 mW mm\(^{-2}\)) itself produced a sustained inward current of 35.21 ± 0.9 mV (mean ± s.e.m.; n = 5 neurons) (6.4 ± 0.9 mV) in CheRiff-positive neurons. This depolarization, however, had little effect on neuronal excitability (Supplementary Fig. 11d–g) and is half of that reported for FlicR1, which is also excited at 561 nm but using 100 mW mm\(^{-2}\) (ref. \textsuperscript{19}).

We next tested VARNAM in proof-of-concept cell-type-specific all-optical experiments. We electroporated embryos carried by transgenic females, expressing Cre recombinase in parvalbumin interneurons (PV-Cre\(^\text{+}\)), with VARNAM plasmid to label superficial
pyramidal neurons and postnatally injected the pups with a floxed adeno-associated virus (AAV) encoding CheRiff-EGFP to target layer 4 PV interneurons (Fig. 4c).

In all-optical slice recordings, paired pulses of light generated ~10 mV IPSPs in VARNAM-positive neurons held at depolarized membrane potentials and produced ~0.5% ΔF/F. We did not observe IPSPs or optical signals at rest. Longer trains of light pulses reliably evoked electrical and optical IPSPs with closely matched amplitude and waveform (Fig. 4f,g). Presynaptic release and IPSP amplitude were unaffected by the imaging laser (Supplementary Fig. 11h).

Lastly, we co-electroporated VARNAM and Ace-mNeon plasmids and performed sequential imaging in the red and green channels with concurrent electrical recordings. In co-expressed neurons, both indicators resolved APs but with slightly different sensitivities (ΔF/F of −3.8 ± 0.4% (VARNAM, mean ± s.e.m.) and −2.6 ± 0.2% (Ace-mNeon) (n = 3 neurons); Supplementary Fig. 12).
Fig. 6 | Odor-evoked and dual-color voltage imaging in live flies. a, Cartoon of PPL1-α′2x2 projection (red) in a MB058B-GAL4>20xUAS-VARNAM fly. The axonal region in the mushroom body (MB) α′2x2 compartment was imaged. b, Spatial map of ΔF/F in the PPL1-α′2x2 axon (n = 6 flies). Scale bar: 10 μm. c, Optical recordings of spontaneous spiking over 10 s (left) and a 0.3 s interval shown at an expanded time scale (right). Yellow dashes indicate spike detection threshold. Green arrowheads denote identified spikes. d, Mean optical waveform of spontaneous spikes (blue trace) in c (n = 188 spikes). Light blue shading denotes s.d.

Top, example trace of evoked spiking during 5 s presentation of isoamyl-acetate (IAA). Bottom, Raster plots of 18 trials of odor-evoked spiking (n = 6 flies). f, Time-varying mean spike rates in e before and during odor presentation. Red shading denotes s.d. g, Mean spike rates before and during odor presentation (P = 0.03, Wilcoxon two-sided signed-rank test). Error bars represent s.e.m.

h, Cartoon depicting projections of PPL1-α′2x2 (red) and PPL1-γ2x1 (orange) in the VARNAM channel (upper left) and those of MBON-α2sc neuron (green) in the Ace-mNeon channel (lower left) in the dual-expression line. Right, epifluorescence images in the two channels showing regions-of-interest (boxed), comprising the axonal region of PPL1-α′2x2 (red), PPL1-γ2x1 (orange) and the dendrite of MBON-α2sc (green) (n = 2 flies). Scale bar: 10 μm. i, Simultaneous dual-color recordings of spontaneous spiking in the three neurons over 10 s (left) and a 0.3 s interval shown at expanded time scale (right). Yellow dashes indicate spike detection threshold. Arrowheads denote identified spikes. j, Left, mean spike rates of PPL1-α′2x2 and MBON-α2sc neurons during 5 s IAA presentation. Shading denotes s.e.m. Right, raster plots of 6 trials of odor-evoked spiking from the neurons. k, Time-varying single-trial spike rates in PPL1-α′2x2 and MBON-α2sc neurons in three consecutive trials in j.
and Ace-mNeon voltage imaging (7.4±2.8 s−1)10 (Supplementary Fig. 15h–m).

Finally, we applied VARNAM in proof-of-concept dual-color in vivo imaging. We expressed VARNAM in dopaminergic PPL1-α’2α2 and PPL1-γ2α1 neurons and Ace-mNeon in a MBON-α2sc neuron. The axon of the PPL1-α’2α2 neuron and the dendrite of the MBON-α2sc neuron innervate the same compartment in mushroom body lobes (Fig. 6h). Simultaneous recordings of spontaneous firing in the three neurons revealed that spike incidence was desynchronized, thus indicating minimal crosstalk between the VARNAM and Ace-mNeon channels (Fig. 6i). We next imaged odor-evoked activity in synchronically connected PPL1-α’2α2 and MBON-α2sc. The mean spike rate of the neurons exhibited a synchronized increase during odor presentation, although spiking remained desynchronized in the single trials (Fig. 6j,k).

As our dual-color setup required a beam-splitter and additional lenses, which affected overall photon efficiency, we observed lower spike detection fidelities in these experiments compared with those in single-color recordings (d’ = 4.8 for VARNAM and 5.4 for Ace-mNeon).

Discussion

Engineering red fluorescent indicators is challenging. Red fluorescent proteins are not only structurally distinct from GFPs, introducing unique problems in structure-guided evolution of fusion proteins, but are also notorious for folding poorly16,17 and exhibit inferior photophysical properties. Prototypical red calcium indicators arrived tardily and performed worse than contemporary GFP-based indicators18–20. Likewise, current red-shifted GEVIs are slow and exhibit intracellular aggregates and poor voltage sensitivities17,21,22 or are too dim and require high illumination intensities18,20.

Past hierarchical voltage engineering efforts have focused heavily on brightness-selection followed by low-throughput voltage screening of a fraction of the brightest variants17,23,24. Brightness-selection does not guarantee enhanced voltage performance but may enrich for voltage-insensitive mutations. For instance, Arch variants with large improvements in brightness show decreased voltage sensitivities and vice versa2. Hitherto, platform-based voltage testing has been low content and non-quantitative, warranting patch-clamp validation. Our one-stop screening protocol tests 100% of variants for voltage sensitivity on a semi-automated, high-throughput platform. Our one-stop screening protocol tests 100% of variants17,19,25. Brightness-selection of a fraction of the brightest variants17,25,44 or are too dim and require high illumination intensities18–20.

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Imageable under low light intensities, VARNAM, based on the brightest and highly photostable non-synthetic red fluorescent protein15, enables voltage recordings in a variety of systems, including nontransparent animals. The probe can be combined with optogenetic actuators with minimal crosstalk and GFP-based indicators under routine imaging conditions.

With rapid response kinetics, VARNAM finds applications in spike-timing estimations, studies of fast-spiking cell types and as a diagnostic tool for epilepsy. VARNAM may be useful for investigating temporal aspects of voltage dynamics in neuronal ensembles together with fast green indicators. Further, its increased response to hyperpolarization makes the sensor conducive to studying the role of inhibition in sensory processing downstream of GABAergic neurons41.

It remains unclear, however, whether opsin-based GEVIs are suitable for two-photon microscopy6. While background can be minimized by genetic targeting, TEMPO may represent a useful modality for tracking oscillatory dynamics in identified cell populations. Here, multispectral imaging might be achieved by recruiting near-infrared fluorophores to the reference channel. Nevertheless, with high-throughput approaches, it might be possible to generate photocycle mutants of opsin-GEVIs and select for increased compatibility with multiphoton modalities66.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41592-018-0188-7.

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Author contributions


Competing interests

The authors declare no competing interests.

Additional information

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mice (P18–P30) were transcardially perfused with ice-cold choline-artificial cerebrospinal fluid (choline-ACSF) containing 110 mM choline, 25 mM NaHCO₃, 1.25 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 20 mM glucose, 11.6 mM sodium ascorbate, 3.1 mM sodium pyruvate. The mice were decapitated, and acute visual cortical slices (300 µm) were prepared using a vibratome (Leica) and mounted on glass microscope slides using ProlongGold (Invitrogen). Confocal images were obtained using Zeiss LSM 710 under 63× water-immersion objective lens (60× for inhibitory responses. Synaptic blockers (100 µM CPP, 10 µM NBQX, 20 µM gabazine) were added to measurements of spontaneous activity (Fig. 3c), modified ACSF containing 3.5 mM KCl, 0.5 mM MgCl₂ and 1 mM CaCl₂ was used. In Fig. 3g, subthreshold synaptic responses were obtained by extracellular stimulation in layer 4 using a bipolar tungsten electrode (Warner Instruments). A train of pulses (0.1–3 ms/0.5–5 ms) was applied using an A385 stimulus isolator (World Precision Instruments) to evoke monosynaptic responses. The interstimulus and intertrial intervals were 400 ms and 30 s, respectively. The ACSF composition for electrical and optical stimulation experiments was adjusted to 2 mM CaCl₂ and 2 mM MgCl₂. A depolarizing current was applied via Multiclamp to hold the membrane potential from resting (−60 mV) to −50 mV. Electrical recordings were performed using a MultiClamp amplifier controlled by pClamp10 software. In the voltage clamp mode (HEK cells), voltage steps were obtained using a custom-written software in LabView. All-optical electrophysiology. In Fig. 4, blue light pulses from a 455 nm LED were combined with continuous illumination from a 561 nm laser using a 485 LP dichroic mirror to simultaneously activate and record from neurons expressing ChRRiff. Electrical recordings were obtained using an Olympus upright microscope. Cells were visualized using a NA 0.40 or 1.0 NA 60× water-immersion objective lens using differential interference contrast or epifluorescence. For ArcLight and Ace-SEP227D, we used a 488 nm laser (CrystaLaser) and a GFP filter set (472/30 nm excitation filter, 495 nm dichroic mirror and 520/35 nm emission filter) (Semrock). Ace-mNeon and Ace-mCitrine were imaged using a 565 nm light emitting diode (LED) (Thorlabs) and a filter set comprising 509/22 nm excitation filter, 526 nm dichroic mirror and 544/24 nm emission filter (Semrock). The light source for Ace-mRuby3 and VARMN was a 561 nm laser (CrystaLaser) or a 565 nm LED (Thorlabs). FlirR1 and FlirR2 were imaged using the 561 nm laser. Both red probes required a TRITC filter set (560/40 nm excitation filter, 585 nm dichroic mirror and 630/75 nm emission filter) (Semrock). Ace-mCard2 was illuminated using a 638 nm laser (CrystaLaser) and a filter set consisting of 620/60 nm excitation filter, 650 nm dichroic mirror and 700/75 nm emission filter (Semrock). The illumination intensity at the sample plane was 15–20 W mm⁻², except for FlirR1 and FlirR2 (500 mW mm⁻²) and Ace-mCard2 (600 mW mm⁻²). For VARMN imaging using the 561 nm laser, we introduced a round variable neutral density filter (Thorlabs) to reduce the power to ~15 mW mm⁻².

Fluorescence time series images (400 Hz–5 kHz) were acquired using a NeuroCCD camera, controlled by a NeuroPlex software (RedShirtImaging, GA) or an ORCA Flash4.0 sCMOS camera (Fig. 3c), controlled using a custom-written software in LabView. Image analysis and statistics. In Fig. 6 and 7, all images were analyzed and processed for use in MATLAB. For ΔF/ΔF₀ computations, we extracted the fluorescence response traces from the time-series images and ranked each pixel by its SNR, where the noise was determined as the root mean square of baseline fluorescence fluctuation. The mean ΔF/ΔF₀ of pixels with the top 25% SNRs was computed to derive the average time-dependent fluorescence response for a given cell. For extracting fluorescence responses from subcellular compartments, we derived the ΔF/ΔF₀ from all pixels within a select ROI, representing the soma or process, without pixel ranking. All fluorescence response traces were baseline-corrected by fitting a double exponential function to account for photobleaching. AP peaks were identified using the findpeaks function in MATLAB and then applying a threshold of three times the s.d. (3 s.d.) of baseline fluorescence.
The identified peaks were used as a reference to temporally align the waveforms and averaged to obtain the mean fluorescence response and mean AP waveform. Traces acquired from cultures and acute slices are unfiltered, single-trial recordings. Multiple trials were averaged for subthreshold responses in Figs. 5, 7 and S5. To determine probe kinetics, we fitted the first 50 ms of depolarizing and repolarizing step responses, acquired from HEK cells in voltage clamp, with a double exponential equation as described previously. Data were plotted using GraphPad Prism v7.0 and statistical analysis were performed using built-in tests. Nonparametric tests were performed throughout except for the monocular deprivation experiment, where data were expected to be normally distributed. We performed a two-tailed Mann–Whitney U test for comparisons between two groups and a Kruskal–Wallis test followed by Dunn’s multiple comparisons correction for multiple groups. For monocular deprivation, statistical comparisons were made using two-way ANOVA and Bonferroni’s multiple comparisons test. Probabilities of the null hypothesis of the mean fluorescence response and mean AP waveform were compared. We obtained an average spike template for each neuron's voltage trace by extracting the voltage signal over a window of ten frames centered around the peaks and averaging across them. We obtained the matched filter outputs by taking the convolution of the original traces with their respective time-reversed spike templates (Supplementary Fig. 15). To analyze spike waveforms, we determined the mean waveform by averaging across all spikes within each trial. We then performed a spline interpolation (10 μs intervals) of the mean waveform, and from that, determined the spike amplitude and FWHM.

To compute spike detection fidelity $d’$, we used a signal detection framework that takes into account both the duration and intensity of fluorescence waveforms in multiple trials of recordings. We estimated the probabilities of the null hypothesis of the optical signal value as the s.d. of the fluctuation in baseline fluorescence. We then computed $d’$ using empirically determined, mean optical spike waveforms (see equations S1–S4 in ref. 1).

**Fly stocks.** The FlyLight Project Team at Janelia Research Campus kindly provided MB058B-GAL4, MB296B-GAL4 and MB800C-GAL4 split-GAL4 stocks. We used R55D11-GAL4 and R82C10-LexA flies from the Bloomington Stock Center. We raised flies on standard cornmeal agar media under a 12 h light/dark cycle at 25 °C and 50% humidity.

To create 20 × UAS-VARNAM flies, the mCD8-GFP cassette in the plasmid pFRC7-20XUAS-IYS-VARNAM plasmid, we inserted it at the phiC31 docking site at pU2 or su(Hw)attP5 using a commercial transformation service (BestGene Inc). To create 13 × LexAap-VARNAM flies, we replaced the myr::GFP sequence with the VARNAM gene in pFRC19-13 × LexAap-2IYS-myr::GFP (Addgene #26224). We then introduced the sequence at the phiC31 docking site at attP40 or VK00027.

For dual-color imaging, we combined R28C10-Lea-13 and 13 × LexAop-VARNAM flies to generate the R28C10-Lea-13 × LexAop-VARNAM line, and MB800C-GAL4 and 20 × UAS-Ace2N-mNeon flies to generate the 20 × UAS-Ace2N- mNeon;MB800C-GAL4 line. We then crossed the flies lines to generate R28C10-Lea-13 × LexAop-VARNAM flies. We collected adult female flies (2 d old) after eclosion and raised them on artificial hemolymph containing: 3 mM KCl, 10 mM glucose, 110 mM NaCl, 1 mM NaH2PO4, 1 mM MgCl2 and 2 mM CaCl2.

**Odor delivery and voltage-imaging in flies.** A custom-built olfactometer (CFM14L05 and ADAF1, Thorlabs), we connected this patch cord to a fiber optic ferrule, which was implanted in the mouse brain. The total power delivered to the brain was 25–200 μW to maximize the emission signal from the voltage sensor and the reference channel. Fluorescence emissions from the brain passed through a dual-edge dichroic mirror and were split into red and green components using a single-edge dichroic mirror (FF493/574-Di01, FF564-Di01, Semrock). The specimen was illuminated using the 560 nm wavelength module of a solid-state light source (Spectra X, Lumencor) at an intensity of 5–7 mW at the specimen plane. Images were acquired at 500 Hz using a scientific-grade camera with 2 × 2 pixel binning. For dual-color imaging, the microscope was equipped with a dual emission image splitter (TwinCam, CAIRN) to split the two channels onto two scientific-grade CMOS cameras. In the image splitter, we used a 560 nm dichroic mirror (Chroma) and a 630/50 nm emission filter (Semrock). We illuminated the sample using the 488 nm and 550 nm wavelength modules of a solid-state light source (Spectra X, Lumencor) delivering 10–15 mW optical power at the specimen plane.

To analyze the voltage traces, raw videos were corrected for any brain movement using the Turboreg algorithm for image registration (http://bigwww. epfl.ch/thevenaz/turboreg/). Pixels with top 10% mean fluorescence intensity were selected, and their union was defined as the region-of-interest (ROI) over which we computed spatially averaged, time-dependent changes in relative fluorescence intensity $\Delta F/\Delta F_0$, where $F_0$ was the mean fluorescence in the ROI averaged over the entire video. Responses were corrected for photobleaching by fitting a double exponential function to the mean fluorescence trace $F_t$ and then normalizing $F_t$ by the fitted double exponential trace. To identify individual spikes, the $\Delta F/\Delta F_0$ trace was high-pass filtered by subtracting a median-filtered (80 ms window) version of the trace. Spikes were identified as local peaks with amplitudes >3 s.d. of the mean baseline fluorescence. The approximate firing rate was determined by sliding a rectangular window along the spike train with $\Delta t = 100$ ms. We used matched-filtering from each laser of the fluorescence traces in Fig. 5c, d. Here, we used an average spike template for each neuron's voltage trace by extracting the voltage signal over a window of ten frames centered around the peaks and averaging across them. We obtained the matched filter outputs by taking the convolution of the original traces with their respective time-reversed spike templates (Supplementary Fig. 15). To analyze spike waveforms, we determined the mean waveform by averaging across all spikes within each trial. We then performed a spline interpolation (10 μs intervals) of the mean waveform, and from that, determined the spike amplitude and FWHM.

To compute spike detection fidelity $d’$, we used a signal detection framework that takes into account both the duration and intensity of fluorescence waveforms in multiple trials of recordings. We estimated the probabilities of the null hypothesis of the optical signal value as the s.d. of the fluctuation in baseline fluorescence. We then computed $d’$ using empirically determined, mean optical spike waveforms (see equations S1–S4 in ref. 1).

**Intracranial injections and optical fiber implantation for TEMPO.** All animal procedures were approved by Stanford University IACUC. Thy1-YFP transgenic mice were obtained from Jackson Laboratories. Male mice were used for consistency. Mice were housed under normal light cycle conditions and genotyped using tail clips. We packaged and determined the concentrations of AAV2/5::CFLP2::VARNAM (6 × 1012 GC ml$^{-1}$) and AAV2/5::CFLP2::VARNAM (6 × 1012 GC ml$^{-1}$) and injected into the cerebral cortex: –1 mm anterioposterior (AP), –2 mm mediolateral (ML), 2 mm dorsoventral (DV); hippocampus: –1 mm AP, –1.5 mm ML, –1.2 mm DV.

–2 to 4 weeks after viral injection, mice were implanted with a multimode optical fiber cannula (CFM14L02, Thorlabs; 0.39 NA, 400 μm diameter, 12 mm long. For hippocampal implant, the overlying cortex was aspirated to prevent tissue compression and the fiber was placed –100 μm dorsal to the viral injection site. The fiber-optic implants were secured to the skull using dental cement (C&B-Metabond, Parkell) and dental acrylic (Hygenic, Colteane). Recordings were obtained –1 to 2 weeks after implantation of the optical fiber.

For EEG recordings, we inserted a low impedance thread screw electrode, SS lead wire, Teflon-coated (Neurok 4000–120), into the cranium, above frontal (+1 mm AP, +2.5 mm ML, 0 mm DV), parietal (+3 mm AP, +2 mm ML, 0 mm DV) and cerebellar cortices (+6 mm, 0 mm ML, 0 mm DV).

**TEMPO instrumentation.** The optical system had two continuous wave laser light sources (488 nm and 561 nm wavelengths; 488-15LS-FP and 561-50LS OBIS Lasers, Coherent). The light from the 561 nm laser was coupled into a single mode fiber (460HP, Thorlabs) using a five-axis translation stage (561D, Newport). The 488 nm laser had its own fiber-optic pigtail provided by the manufacturer. The light exiting each single mode fiber was collimated using an aspheric lens (PAX-X-7, A, Thorlabs). We used 50/50 beam splitters (CM1-RS013, Thorlabs) to direct 50% of the laser light through each channel to the fiber-optic pigtail to a photodiode (PDA100, Thorlabs) for continuous monitoring of laser power. The other half of the light from the two beams was combined using a dichroic mirror (FF511-D1, Semrock). The resulting collinear laser beams were reflected off a dual-edge dichroic mirror (FF498/581-D1, Semrock) and focused onto a multimode fiber–optic patch cord (2 m long, 400 μm diameter core, 0.99 NA; FT400EMT, Thorlabs). Using a ceramic mating sleeve (CFM14L05 and ADAF1, Thorlabs), we connected this patch cord to a fiber optic ferrule, which was implanted in the mouse brain. The total power delivered to the brain was 25–200 μW to maximize the emission signal from the voltage sensor and the reference channel. Fluorescence emissions from the brain passed through a dual-edge dichroic mirror and were split into red and green components using a single-edge dichroic mirror (FF593/574-D1, Semrock). The emissions of each color channel (FF01-537/26, FF01-630/92, Semrock) were filtered and focused onto two variable gain photoreceivers (OE-200-Si, Femto). To gain in sensitivity, we used lock-in amplification techniques. We amplitude-modulated the 488 nm laser at 2.1 kHz (SR810, Stanford Research Systems) and the 561 nm laser at 6.3 kHz (SR850 Stanford Research Systems). This approach removed any crosstalk between the emission bands of the two FPs. Reference signal and voltage signal were demodulated with their respective lock-in. 561 nm laser fluctuation was demodulated using a third lock-in amplifier (SR830, Stanford Research Systems). To read out laser and fluorescence intensities, the three lock-ins were set up as follows: low-pass filter for demodulation, 3 ms time constant, 2 kHz lock-in, and 2 kHz sampling. These filter settings limit the measurement bandwidth to ~30 Hz. Because voltage signals fluctuation is weak, the DC-corrected signals were expanded tenfold to benefit from the dynamic range of the lock-in and therefore gain in bit-depth. After lock-in amplification, all signals were digitized at 16-bit resolution and sampled at 2 kHz using a National Instruments card (NI PCIe-6320), well above the Nyquist rate of the experiment.
In vivo electrophysiological recordings using TEMPO. EEG signals were recorded from a low-impedance thread screw electrode, SS lead wire, Teflon-coated (Neurotek #000-120), implanted in the mouse cranium. LFPs were recorded using a 50 μm diameter tungsten wire coated with polyimide insulation (California Fine Wire). To sample electrophysiological signals at a depth corresponding to the zone of TEMPO’s greatest sensitivity, the LFP wires were positioned 50–150 μm from the tip of the fiber optic ferrule in tissue, the fiber and wires were enclosed inside a 24-gauge (0.559 mm) polyimide tube and secured in place using cyanoacrylate. The EEG and LFP electrodes were grounded to the skull screw over the cerebellar cortex. The signal-bearing wires were soldered to a connector board (EIB8, Neuralynx) using manufacturer gold pin and the signals pre-amplified using a head stage (HS-8, Neuralynx). The electrophysiological signals were amplified using an acquisition board (ERP-27, Neuralynx) and an eight channels analog amplifier (Lynx-8, Neuralynx). We applied a digital bandpass filter to the data using a second-order Butterworth filter; the filter’s high-frequency 3 dB cutoff frequency was 50 Hz and the low-frequency 3 dB cutoff, 0.1–1 Hz.

Recording sessions and analysis of TEMPO recordings. All optical recordings were performed during the mouse’s light cycle. KX anesthesia was dissolved in PBS (10 mg/ml ketamine; 1 mg/ml xylazine) and injected peritoneally at 100 mg/kg and 10 mg/kg, respectively. Recordings began just after anesthesia administration to optically track the appearance of the up- and down-state activity in the EEG recordings. The experiments involving rest and locomotion were performed in a circular arena (31 cm diameter, 31 cm height) with clear acrylic walls and a stainless-steel floor. Video recordings were used to distinguish between behavioral states of locomotion and rest. The spectrograms and one-sided power spectral densities of optical and electrophysiological data were calculated using Welch’s method to reduce windowing artifacts. Coherence and cross-correlation were assessed using built-in MATLAB functions. Filtering and data analysis were performed using custom software written in MATLAB. All traces were filtered using a zero-phase sixth-order bandpass Butterworth filter (delta rhythm: 1–5 Hz; theta rhythm: 5–10 Hz). The voltage trace, laser fluctuations and hemodynamic artifacts were unmixed using linear regression. To quantify hemodynamic artifact, we injected three mice each with an identical volume of VARNAM or Ace-mNeon AAV in the cortex. The recordings were performed under identical conditions. The integrated signal was computed in a 1–30 Hz filtered trace and normalized to 100%. The fraction of normalized integrated signal was assessed in delta (1–4 Hz), theta (6–10 Hz), hemo (11–14 Hz), gamma (25–50 Hz) bands.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. Custom software written in LabView for high-throughput data acquisition can be obtained from the authors upon request. Custom-written software for high-throughput data analysis can be found at https://www.dropbox.com/sh/ain2by9whtp5bza/AACvcTaaE-wvUAHP9YFXTyaadl=0.

Data availability
The DNA sequence for VARNAM is available in GenBank under submission number MH763646. CMV-VARNAM (#115552) and pAAV-Syn-VARNAM (#115554) will be available from Addgene once the paper is published. Fly stocks and viruses will be provided by the authors upon reasonable request.

References
Life Sciences Reporting Summary

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Experimental design

1. Sample size
   Describe how sample size was determined. No statistical methods were used to determine sample size. Sample sizes were typical for probe development studies (eg. Hochbaum et al, Nat Methods, 2014; Gong et al, Science, 2015; Abdelfattah et al, J Neurosci, 2016)

2. Data exclusions
   Describe any data exclusions. No data were excluded from the analyses.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings. Attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups. Randomization was not applicable as there was no sample allocation into groups. Experiments involved characterization of individual voltage probes.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Blinding was used where relevant using a transfection marker.

   Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   □✓ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □✓ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □✓ A statement indicating how many times each experiment was replicated
   □✓ The statistical test(s) used and whether they are one- or two-sided
     Only common tests should be described solely by name; describe more complex techniques in the Methods section.
   □✓ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □✓ Test values indicating whether an effect is present
     Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
   □✓ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □✓ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

   See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

LabView 2016 and MATLAB 2015 versions were used to analyze data from the high-throughput platform and optical recordings in patch clamp and TEMPO experiments, respectively. Custom-written LabView code is available on Dropbox (link provided in manuscript). NeuroPlex (Redshirt Imaging) and pClamp10 were used for data acquisition for imaging and electrophysiology, respectively.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

Plasmids generated in this work will be available from commercial sources (Addgene) prior to publication. Viruses and fly stocks will be provided by authors upon request.

No antibodies were used in this study.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HEK293 cells (CRL-1573) and excitable HEK cells (CRL-3269) were obtained from ATCC (Manassas, VA).

b. Describe the method of cell line authentication used.

Authenticated by ATCC using STR analysis

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were tested negative for mycoplasma contamination

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

Yes; our cell lines were acquired from ATCC, which regularly performs STR analysis to avoid distribution of misidentified lines. In our experiments, HEK293 cells served solely as a system for expression and characterization of voltage indicators rather than the subject of investigation.

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

CS7BL/6, CD-1 wildtype, PV-Cre and Thy1-YFP transgenic mice were used. Female mice were used for in utero electroporation, male mice were used for in vivo imaging. In all other experiments, mice were used without regard to sex. The mice aged between 3 weeks and 4 months (the exact ages are mentioned in the manuscript text). In experiments involving flies, we used adult female transgenic flies (2 days old).

The study did not involve human research participants.