Optical voltage imaging in neurons: moving from technology development to practical tool

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Abstract | A central goal in neuroscience is to determine how the brain's neuronal circuits generate perception, cognition and emotions and how these lead to appropriate behavioural actions. A methodological platform based on genetically encoded voltage indicators (GEVIs) that enables the monitoring of large-scale circuit dynamics has brought us closer to this ambitious goal. This Review provides an update on the current state of the art and the prospects of emerging optical GEVI imaging technologies.

Brain functions, observed as an animal's or human's goal-directed actions, emerge from electrical signals that are generated by individual neurons and transmitted between selected neurons organized in systems of circuits. Evolutionarily more recent ('higher') mammalian brain functions depend on the cerebral cortex, where the distributed cooperation of multiple local circuits involves millions of neurons. The large number of cortical neurons and their delicate wiring diagram (connectome) is only a relatively small part of the technological challenge involved in understanding the mechanism of information processing in the cerebral cortex[1,2]. Additional complications result from the presence of many different types of neurons, each type characterized by distinct input and output properties, and from sensitivity to neurotransmitters and neuromodulators. Whereas the functional properties of a single representative muscle cell (contraction when activated) tell us much about the function of the whole muscle (contraction when activated), brain functions are by far less deducible from the actions of a few representative neurons. The elucidation of cortical circuit mechanisms necessitates experimental approaches that give access to the electrical activities of large numbers of identified neurons (that is, of a known cell class) across large portions of cortical space with a temporal resolution consistent with the millisecond timescale of synaptic communication. One possible strategy for extracting the needed data from the brains of behaving animals is to compile information from cellular resolution recordings and brain-wide population signals to generate a comprehensive composite picture. Optical methods are perfectly set to enable such 'zoom in–zoom out' approaches[3–5].

The current 'workhorse' technology for optical imaging of neuronal activities uses microscopic and mesoscopic calcium signals to estimate action potential occurrences over a large number of neurons and large spatial scales, respectively[6–9]. However, calcium imaging can only provide limited information about natural signal processing in the nervous system. Most importantly, calcium imaging provides little or no data on the hyperpolarizing (inhibitory) and subthreshold depolarizing (excitatory) signals that occur continuously in most neurons. Adequate time resolution is needed in order to see sequential activation of the neurons recruited during specific neuronal operations. The widely employed technique of calcium imaging helps to identify the spatial distribution of recruited neurons but fails to track the fast sequential activation of neurons during formation of an assembly. Optical voltage imaging has the potential to overcome the limitations inherent to calcium imaging. Optical voltage imaging, as a tool to investigate neuronal circuits, has a history longer than that of calcium-imaging approaches, but it has suffered from a lack of suitable voltage indicators and a lack of fast multi-cell imaging technologies. The feasibility of voltage imaging of many individually resolved neurons in intact tissue has been predicted by detailed realistic simulations[4,10], but only recently have genetically encoded voltage indicators (GEVIs) and genetically targetable voltage indicators (hereon dubbed 'hybrid GEVIs') become available that have the necessary performance properties. Improved GEVIs and advances in optical imaging instrumentation have now expanded the use of voltage-imaging technologies from a small group of specialized laboratories to a broader range of brain researchers. The present Review is designed as an update and primer on new and exciting advances in GEVI imaging and as a re-evaluation of GEVI's promises and the challenges yet to be tackled.

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GEVI structures and mechanism

The generation of fluorescent GEVIs became imaginable with the discovery of green fluorescent protein (GFP) and with understanding the molecular concepts of voltage sensing in voltage-gated ion channels[11,12]. Synthetic proteins constructed by molecular fusion of fluorescent proteins (FPs) and an isolated voltage-sensing domain (VSD) provided the first series of GEVIs that exhibited robust voltage reports in mammalian cells[13]. These early GEVIs (dubbed voltage-sensitive fluorescent proteins (VSFPs)) have parented a large number of VSD-based GEVIs to date (FIG. 1). In these GEVIs, the coupling of voltage sensing with optical output is achieved either via Förster resonance energy transfer (FRET) between a pair of FPs, via sensitizing a single FP by circular permutation (cpFP) or

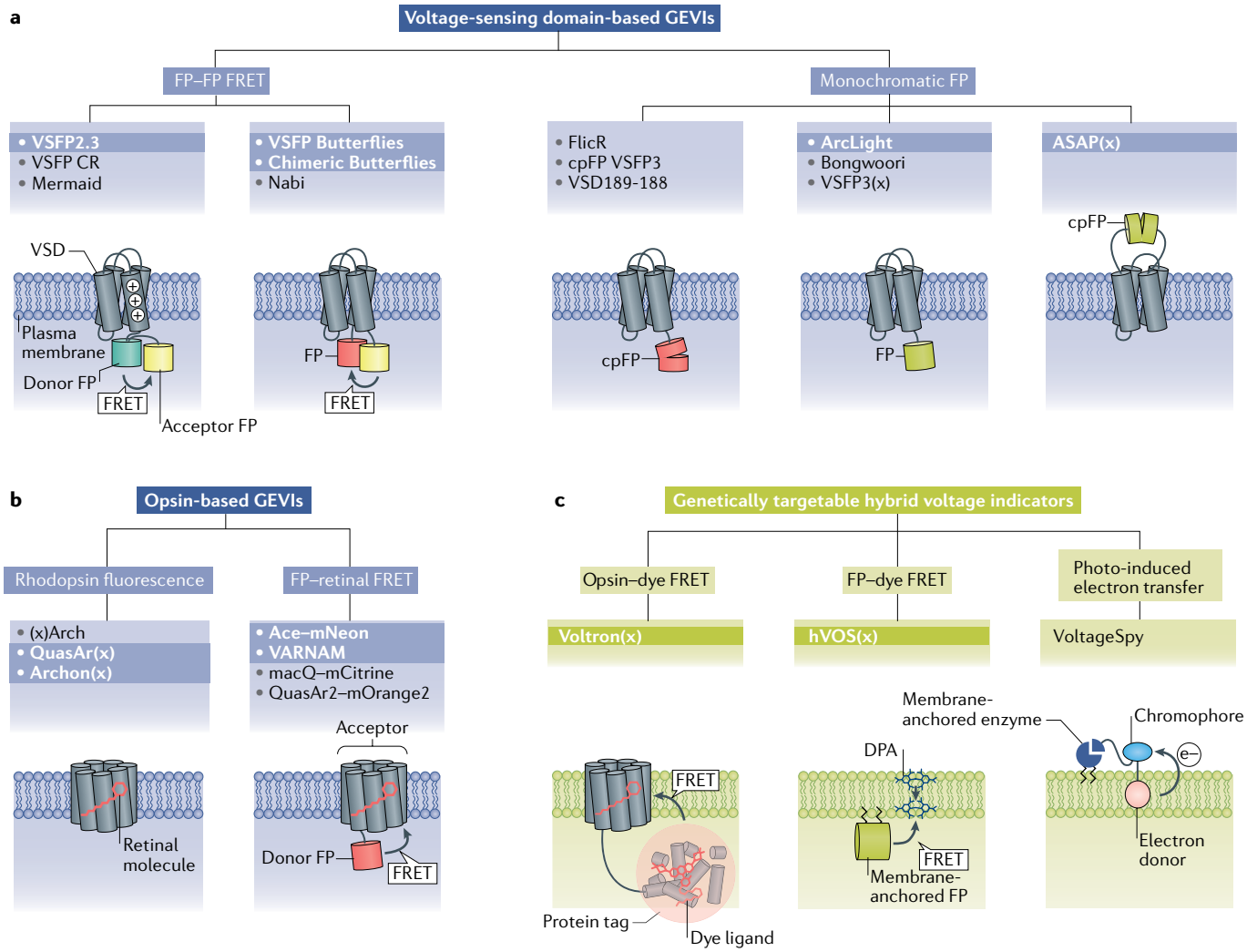

Fig. 1 | **Structural features and optical reporting mechanism of selected GEVIs and hybrid GEVIs. a** | Voltage-sensing domain (VSD)-based genetically encoded voltage indicator (GEVI) variants are fusions between a four-transmembrane segment VSD (S1–S4) and fluorescent proteins (FPs). For FP–FP Förster resonance energy transfer (FRET) indicators, the voltage fluctuations are ratiometrically reported via modulation of FRET efficacy (indicated by the curved arrows) between a pair of FPs attached in tandem to VSD S4 (such as in VSFP2.3), or attached before S1 and after S4, thus flanking the VSD (Butterflies (Korean: *Nabi*) family). Insertion of segments from ion channels with fast kinetics (such as the potassium channel Kv3.1) has been used to generate VSD chimaeras with improved kinetic properties (chimeric Butterflies). For monochromatic FP indicators, a single FP is attached, for instance, to the C-terminal end of the VSD. The FP can be in its native structure (for example, VSFP3(x), Bongwoori and ArcLight) or in a circularly permuted form (cpFP, indicated by a split in schematic structure) that is sensitive to local environment changes (for example, cpFP VSFP3 and FlicR). Alternatively, a cpFP is inserted between the S3 and S4 transmembrane segments of a VSD (ASAP family). **b** | In opsin-based GEVIs, voltage fluctuations can be reported via protonation of the opsin retinal Schiff base (rhodopsin fluorescence; Arch, QuasAr and Archon families) or via a FRET mechanism through which the opsin retinal acts as a voltage-dependent acceptor (FP–retinal FRET). **c** | Genetically targetable hybrid indicators selectively label the desired cell classes but require a synthetic counterpart that needs to be delivered to cells via an invasive experimental procedure. These indicators operate on a voltage-dependent modulation of FRET (opsin–dye FRET (Voltron variants) or FP–dye FRET (hVOS variants using dipicrylamine (DPA))). Chemical photo-induced electron transfer (PeT) dyes can be made genetically targetable via a linker that specifically binds to a genetically encoded targeting protein (VoltageSpy). The GEVIs highlighted in bold have proof-of-principle validation in vivo in the mammalian brain. Blue and green backgrounds indicate full GEVIs and hybrid GEVIs, respectively. The GEVIs named in the figure are VSFP2.3 (REF.[16]), VSFP CR[69], Mermaid[70], VSFP Butterflies[35], chimeric Butterflies[71], Nabi[72], FlicR[49], cpFP VSFP3 (REF.[73]), VSD189-188 (REF.[74]), ArcLight[75], Bongwoori[76], VSFP3(x)[77], ASAP(x)[26,78], (x)Arch[79], QuasAr(x)[38,52], Archon(x)[39], Ace–mNeon[80], VARNAM[51], macQ–mCitrine[81], QuasAr2–mOrange2 (REF.[48]), Voltron(x)[42], hVOS(x)[82,83] and VoltageSpy[84].

Box 1 | **Considerations when choosing appropriate GEVIs**

Photophysical properties

The flux of emitted photons (fluorescence, *F*) and the dynamic range of its voltage-dependent modulation are the most important physical performance parameters of genetically encoded voltage indicators (GEVIs). Photon flux can be enhanced by increasing excitation intensity at the cost of an increased bleaching rate. A useful consideration is therefore the number of photons that can be emitted until the indicator is photobleached. This quantity is indicated by the product of the fluorescence quantum yield and bleaching time constant, termed the molecular brightness, measured under the envisaged experimental conditions.

Biophysical properties

By convention, the sensitivity of a GEVI is described as the voltage-dependent change of photon flux ($\Delta F/\Delta V$) normalized to the baseline photon flux (that is, $\Delta F/(F \cdot \Delta V)$). Some GEVIs have a nonlinear fluorescence–voltage relationship. Detection of subthreshold signals is facilitated if the sensitivity is maximal around the resting membrane potential. Sensitivity, along with molecular brightness, determines the molecular signal-to-noise ratio (SNR). The practical SNR may be lower (see BOX 2). Of some concern may be the movement of mobile charges in the GEVI molecules during voltage transients, which translates into an increased dynamic membrane capacitance[67].

Performance in intact tissue

The sensitivity of a GEVI is proportional to the fraction of the expressed GEVI localized in the plasma membrane. This fraction may be reduced after long-term expression in intact tissue, and therefore the sensitivity reported in acute transfection experiments using cell cultures may be higher than in intact tissue (that is, in a living animal or brain slices). GEVI performance may further be compromised by less favourable optical conditions in intact tissue. The phototoxicity of GEVIs is not a known issue.

The final choice of an appropriate GEVI will also depend on the availability of transgenic mouse lines or viruses capable of delivering the GEVI gene to the target cells.

via mechanisms that await full explanation[14]. A second pedigree of GEVIs is rooted in the discovery of the voltage-dependent fluorescence of some microbial opsins (FIG. 1). The optical output of these opsin-based GEVIs is fluorescence either of the opsin itself or of an attached FP that is quenched by voltage-dependent absorbance of the opsin. Although leading opsin-based GEVIs exhibit large voltage-dependent changes of fluorescence, they are relatively dim overall, thus requiring illumination at very high intensities. A second potential caveat is a residual photocurrent, even in nominally non-conducting channel mutants.

Most of the currently available GEVIs are known by their acronyms, but their structural design principles are rooted in these two distinct design scaffolds (FIG. 1). Chemigenetic or hybrid GEVIs employ genetically targetable proteins that bind compounds that either are not proteins or do not occur naturally in brain tissue (FIG. 1).

Physical characterization of GEVIs

GEVIs and hybrid GEVIs are now available to a broad range of scientists interested in neural systems and brain circuits. These prospective GEVI users are faced with a variety of indicators that have been described to date and need to make an informed decision about which indicator to use. GEVIs are available with fluorescence excitation and emission spectra encompassing the visible and near infrared spectrum. The spectra of a given GEVI are those of its constituent FPs and opsins, the former tabulated at www.fpbase.org. Since the same range of wavelengths is well covered by affordable light-emitting diodes, solid-state lasers and optical filters, spectral properties constrain the choice of appropriate GEVIs only in multicolour imaging approaches. Compared with GEVIs with shorter-wavelength spectral properties, GEVI variants that operate with longer-wavelength light (red and, in particular, near infrared) are preferable, because of reduced light scattering in brain tissue, tissue autofluorescence and absorption by haemoglobin, thereby facilitating imaging deeper in the tissue. Some, but not all, GEVIs are compatible with two-photon (2p) microscopy, in which case the 2p cross-section is a critical selection parameter. More critical than spectral properties for the choice of a suitable GEVI are a range of photophysical and biophysical properties (BOX 1). These molecular characteristics need to be considered when choosing an indicator that best meets the requirements for a specific research question and the available instrumentation. However, considering just the molecular properties is not sufficient: another essential aspect of each GEVI imaging application is its performance in the specific biological target preparation that is being studied. The most attractive option is GEVI imaging applications in intact brain tissue (brain slices, living animals), which are preparations that require GEVIs to be expressed over several weeks or months. Under these intact tissue conditions, the signal-to-noise ratio (SNR) may be considerably lower (often by a factor of 10) than in cell culture systems[15]. Moving from brain slices to living animals comes with additional complications, such as contamination of the voltage signals by haemodynamic signals and movement artefacts[16].

Delivery of GEVI genes

One of the strongest conceptual and technical advances offered by state-of-the-art voltage indicators is the ability to target them to specific cell classes. In classical mesoscopic voltage-sensitive dye imaging, it was impossible to differentiate the signal components arising from excitatory or inhibitory cells. In modern experimental designs, the use of specific regulatory sequences allows the researcher to associate indicator signals with specific cell types or neural circuits. This is of particular advantage when imaging indicators across a population of neurons without the optical resolution of single cells. A second important benefit of genetic encoding is the availability of non-invasive or minimally invasive methods for delivering genes to target cells in living animals. Applicable in vivo gene delivery methods include in utero electroporation, viral approaches and transgenesis. These approaches have already been reviewed in the context of GEVIs[3], so here only two recent advances are highlighted. The first advance has addressed the call for indicator expression at high levels but in a chosen cell type (that is, with highly selective expression). Although coarse cell classes (such as glutamatergic and GABAergic cells) are genetically well defined by the transcriptional activity of strong marker promoters (including those for calcium calmodulin kinase 2a (CaMK2a) and vesicular GABA transporter (VGAT)), the regulatory sequences that define refined subclasses are often too weak to drive sufficiently strong indicator expression. This problem has been solved by the development of two component systems, in which a strong ubiquitous promoter (for example, *TET*) drives GEVI expression (the indicator

Quenched
Submitted to a process that (reversibly) deactivates fluorescence emission.

2p cross-section
A measure describing how well a fluorescent dye is excited by light of a given intensity; similar to the one-photon absorption extinction coefficient, but two-photon absorption increases with the square of the light intensity.

Signal-to-noise ratio
(SNR). A measure that compares the level of a desired signal (for example, voltage-dependent change in fluorescence) to the level of background noise (in this case, random fluctuations of measured fluorescence). The SNR is defined as the ratio of signal power to noise power.

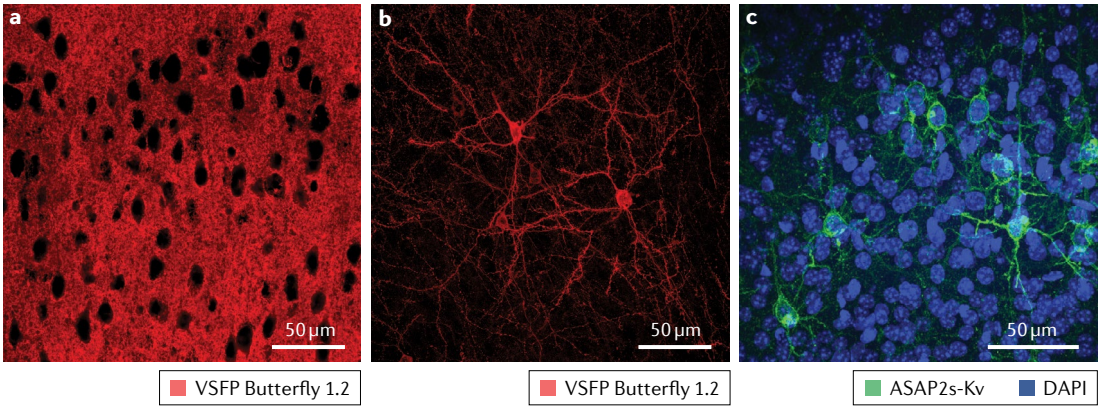

Fig. 2 | **Isolation of individual neurons for single-cell-level voltage imaging. a** | If genetically encoded voltage indicators (GEVIs) are expressed in a type of neuron that densely populates the tissue, the stained surface (plasma membrane) of their cell bodies and processes merge into a sea of fluorescence, in which individual cells can be identified only through the negative stain of their somatic cytosols. Shown is a section through the cerebral cortex of a mouse that expresses the GEVI VSFP Butterfly 1.2 (red colour) in all pyramidal cells. The dark spots are the unstained interiors of the cell bodies. **b** | Expression of the same GEVI in only a small fraction of the pyramidal cell population (sparse expression) results in a Golgi staining-like resolution of individual cells and their processes (red colour). **c** | Targeting the GEVI ASAP2s-Kv preferentially to the somatic plasma membrane decreases the fluorescence of processes, further facilitating the allocation of GEVI signals (green) to individual cells. Blue DAPI staining indicates the nuclei of all cells (both GEVI expressing and non-expressing). Panels **a** and **b** adapted from REF.[85] CC-BY-4.0, https://creativecommons.org/licenses/by/4.0/. Panel **c** adapted with permission from REF.[17], Elsevier.

expression component) but is itself under the efficient control (for example, via a transactivator) of a possibly weak but highly cell-class-specific promoter (the cell selection component). Targeting the indicator expression component to a well-selected genomic locus (for example, TIGRE, standing for 'tightly regulated') resulted in a powerful toolkit for mouse models[17,18]. The second notable promising gene delivery advance is set to overcome the need to inject a virus directly into the brain tissue. This has been achieved by developing virus capsids that cross the blood–brain barrier; hence, genes for CNS neuronal transduction can be delivered by injection into the peripheral bloodstream[19]. The intravenous (that is, systemic) viral approach will allow powerful combinations of transgenic and viral strategies for cell-type-specific and brain-wide targeting of GEVIs.

Somatic and sparse targeting

GEVI signals represent membrane voltage signals averaged across the tissue volumes from which photons are sampled for each voxel or pixel. Under conditions with sufficient signalling photons, these volumes are typically not diffraction-limited spots but volumes (defined by spatially extended indicator excitation and scattering of the emitted photons) that usually contain somata and processes from several or many neurons. Voltage signals are typically more spatially widespread than calcium signals because the surface of cells with processes is less concentrated than their cytoplasmic volume. Therefore, calcium imaging is strongly biased towards the detection of action potential at the level of individual cell bodies, but voltage signals are prone to contamination by the subthreshold synaptic potentials of neighbouring cells imaged by the same pixels of the detector. This feature of the voltage signal not only impedes the allocation of signal components to individual cells but can also worsen the SNR.

During recent years, two approaches have been explored to tackle this problem: localized GEVI expression to the soma, and sparse but strong GEVI expression (FIG. 2). In somatic expression targeting, an amino acid motif derived from proteins that are naturally concentrated at the somatic plasma membrane (for example, Kv2.1 potassium channels) is added to the GEVI protein at the DNA level[17]. This reduces the optical signals from small processes and increases the concentration of the optical signal around the cell bodies.

Although somatic targeting facilitates single-cell resolution voltage imaging both in vitro and in vivo, it compromises a feature in which voltage imaging had been superior to intracellular microelectrode recordings — namely, the ability to investigate voltage signalling in very thin processes, dendrites and axons. With this in mind, an alternative strategy to isolate single-cell-level voltage signals is sparse targeting of GEVI expression in representative neurons within a dense population of neurons of interest. Sparse expression can be achieved by lowering the probability that a gene is delivered to a given neuron (for example, by using viruses at a low titre), but this approach results in delivery of mostly single-gene copies per expressing cell, and hence can suffer from low expression levels. Sparse but strong expression has been achieved by devising two-component systems (see the 'Delivery of GEVI genes' section above) with the cell-type-selecting component being sparsely delivered (FIG. 2).

Instrumentations

The development of GEVIs and demonstration of their practical usefulness has been a driving force for recent advances in instrumentation optimized for voltage imaging. Voltage imaging typically requires the resolution of smaller fraction-intensity changes at higher sampling

Pixel
A term standing for 'picture element'; the light detected by one pixel of the detector may come from anywhere within the corresponding area in the object plane.

Bessel beam
A laser beam with a profile shaped in the form of a Bessel function that can be used to generate an axially elongated excitation volume.

Light sheet illumination
A method in which a thin slice (usually from a few hundred nanometres to a few micrometres) of a sample is illuminated. Compared with conventional epifluorescence microscopy, light sheet illumination produces reduced out-of-focus background fluorescence.

rates than calcium imaging. The necessary SNR is challenging to achieve and requires optical instruments that are optimized to generate and sample as many photons per time bin as possible (see BOX 2). Point-scanning approaches (sampling from one point at a time) are therefore suboptimal for voltage imaging of multiple cells. Note that the required high photon fluxes make bleaching of the chromophores the most important limiting factor. Because of photobleaching, the acquisition of maximal activity information from as many neurons as possible demands that any fluorescence excitation of the cells of interest located outside the sampling spatial and temporal bins needs to be avoided. Recent instruments optimized for voltage imaging use volume scanning, including the Bessel beam, light sheet illumination[20,21] and emerging light field deconvolution approaches[22,23]. Other optical approaches that are well suited for voltage imaging are deep one-photon imaging using gradient-index (GRIN)[24] lenses and fibre optics, to physically provide access to deeper regions with high photon yield[24,25].

Use of GEVIs to study brain function

In this section, we emphasize the potentials of GEVI imaging by highlighting examples of approaches for which the features of GEVIs are instrumental. Although GEVIs have been successfully used in flies and fishes[26–28], we focus on experimental conditions that are, at least in principle, applicable in the awake behaving mouse cortex. More comprehensive reviews of GEVI imaging experiments in various brain structures and species are found elsewhere[29–33]. The following subheadings cover the spatial scales at which voltage-imaging experiments are mostly employed (FIG. 3).

Macroscopic and mesoscopic GEVI imaging. Any modern description of cortical functions probably includes the integration of sensory information across different modalities and the association of external information with memory, leading to the generation of an action. These functions are accomplished by local and distributed (cortex-wide) computations conducted using both rapid and slow changes in neuronal membrane voltage. To understand distributed neuronal processing, large portions of cortical space need to be accessed with high spatiotemporal resolution. Simultaneous access to multiple regions of mouse cortex at high spatial (micrometres) and temporal (milliseconds) resolution is currently achievable by only one experimental method: macroscopic voltage imaging (FIG. 4), in which photodetectors capture image series of the fluorescent cortex of a living animal (FIG. 4a). Classical studies were conducted on this premise using voltage-sensitive dye imaging (VSDI)[31,33,34]. In common with classical VSDI, GEVI macroscopic epifluorescence imaging is limited to superficial tissue. However, GEVI imaging now offers three significant advantages over the classical VSDI imaging paradigms: (1) selective functional imaging of a specific cell population of interest (for example, layer 2/3 pyramidal cells or specific subclasses of interneurons); (2) transgenic indicator expression that does not require craniotomies or duratomies and that facilitates minimally invasive voltage imaging through a thinned skull; and (3) repeated imaging sessions in awake mice over the time course of an experiment lasting days or weeks. These refined methods now allow cell-class-specific cortical representation maps to be generated from the awake mouse cortex. This approach was pioneered in studies that mapped the voltage responses to sensory stimulation of cortical layer 2/3 pyramidal cells[16,35–37] (FIG. 3a). These studies revealed slow hyperpolarizing and fast (30-ms) depolarizing cortical population voltage responses, highlighting the above advances of voltage imaging over calcium imaging. Dual-emission GEVIs are advantageous for these type of experiments because they allow efficient correction for optical signal components resulting from heartbeat-associated blood volume changes[35]. However, strategies to correct monochromatic green GEVI have also been developed[25]. New GEVIs that fluoresce in the near infrared spectrum will help to reduce this type of 'haemodynamic' confounding

Box 2 | Spatiotemporal resolution in fluorescence imaging

The fundamental limits of the spatiotemporal resolution of optical imaging are defined by diffraction and the quantal nature of light. The practical limit of spatial resolution for voltage imaging in brain tissue, however, is set not by diffraction but by tissue absorption and scattering. Using state-of-the-art instrumentation, the fundamental limit of temporal resolution — shot noise caused by the quantal nature of light — is routinely reached. The number of photons (light quanta, n) detected during a sampling interval across a sampling space obeys a Poisson distribution, and hence, the noise on a signal of n photons is $\sqrt{n}$. The signal-to-noise ratio (SNR ~ $n/\sqrt{n}$) increases with $\sqrt{n}$; therefore, the time-resolved imaging of small structures requires a high fluorescence intensity. In GEVI imaging, the maximum practical photon flux is usually limited by the photobleaching time constant (τ_b) — that is, the duration of the experiment until $1/e$ of the indicator is bleached. The requirement of large photon fluxes for a large SNR can be quantified by the following formula (a detailed derivation can be found in REF.[4]):

$$\mathrm{SNR} \leq \frac{\Delta F}{F}\sqrt{n_F\left(1-f_B\right)}\sqrt{f_c f_{em}\, q_D}\sqrt{\frac{q_{em}\,\Delta t}{q_b\,\tau_b}},$$

where $\Delta F/F$ denotes the fractional fluorescence change of the GEVI induced by the voltage signal of interest, Δt the sampling interval, f_C the fraction of emission light collected by the objective, f_{em} the fraction of the emission spectrum transmitted to the detector, f_B the fraction of non-signalling background fluorescence, q_D the detector quantum yield, q_{em} the quantum yield of fluorescence, and q_b the quantum yield of photobleaching.

This formula predicts that single-cell-level voltage signals at a 1-ms time resolution can be recorded with SNR >10 if the practical $\Delta F/F$ is >5% and τ_b is >10 min (REF.[4]). The effective $\Delta F/F$ is the value effectively measured under the relevant experimental conditions (that is, with F including non-signalling background). This estimation has been validated by the performance of recent GEVIs[15,68]. The equation highlights several additional points: (1) SNR declines with increasing non-signalling background fluorescence. This explains the lower SNR obtained in intact tissue than in 2D non-overlapping cell culture systems. (2) GEVI signals that slightly outlast the signal of interest may be detected at lower temporal resolution and, hence, a better SNR than indicators that faithfully report the shape of fast signals such as action potentials. (3) The dependence of photon flux on the sampling area (or volume) links the spatial and temporal resolutions of voltage-imaging methods. Sampling over large areas (where pixels represent large areas), as in mesoscopic imaging, can achieve a better SNR than single-cell-level imaging if the non-responsive fluorescent component does not increase more than proportionally with the increase in field of view. If only a small fraction of the neurons within an imaged population are responsive (for example, recruited by the stimulus), shot noise generated by the non-responsive cells decreases the SNR of the signal of interest. (4) The oft-reported baseline noise of >1% with sampling intervals of 1 ms (REF.[15]) indicates that, commonly, >10,000 photons are sampled per Δt, quantifying the quest for bright GEVIs and high-intensity illumination.

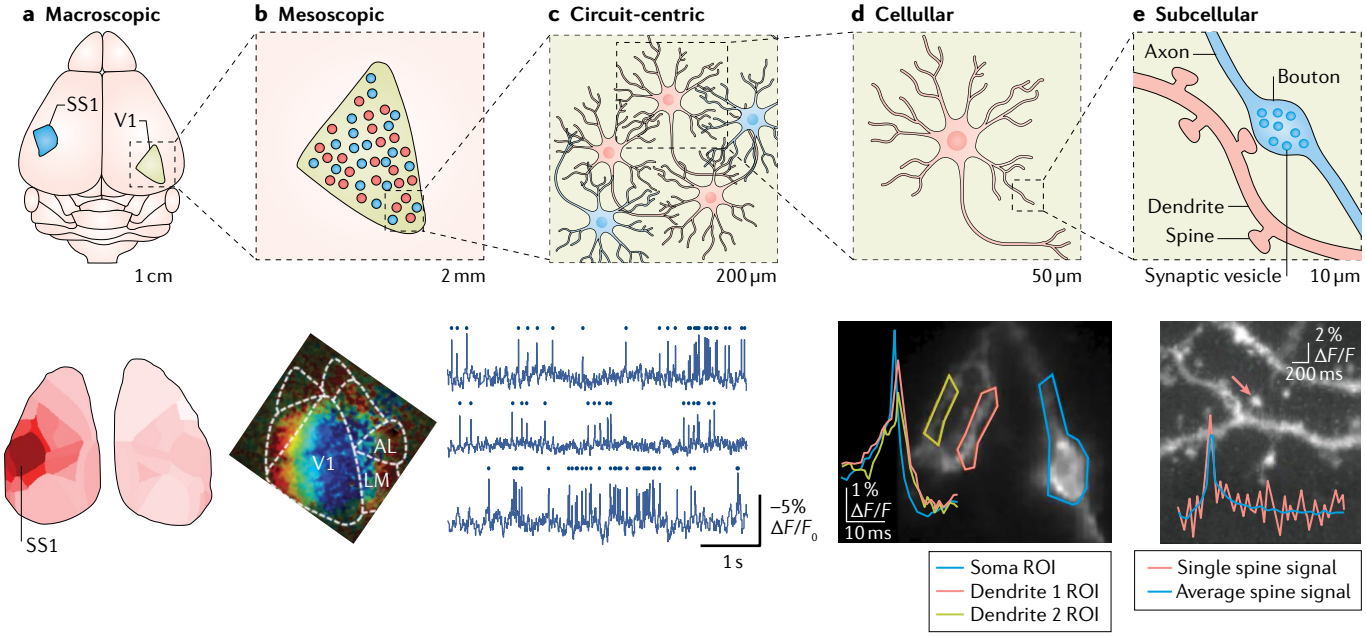

Fig. 3 | **Spatial scales and level of analysis.** One advantage of genetically encoded voltage indicator (GEVI) imaging is that it can cover multiple spatial scales (resolution and coverage), ranging from the whole brain to dendritic spines. **a** | Macroscopic population voltage imaging with a field of view of 1 cm can be performed in living mice during, for example, the activation of mouse barrel cortices (SS1; blue area in the top image) upon whisker stimulation, or visual cortex (V1; green area) upon visual stimulation. The activation of the cortex can be visualized by epifluorescence GEVI imaging (red shading in the bottom image indicates population voltage changes upon whisker stimulation). Although excitation is most intense in the barrel cortex, voltage imaging reveals the cortex-wide spread of neuronal excitation and inhibition[37]. **b** | With increased magnification, mesoscopic voltage imaging resolves finer functional organization, such as the contributions of distinct cell types (blue and red circles in the top image) and retinotopic maps (an example is shown in the lower image[36]). **c** | Circuit-centric approaches further zoom in to resolve single cells, allowing researchers to interrogate the interaction between cells within a neuronal circuit. The example shown is Voltron$_{525}$-ST imaging of interneurons in the mouse primary visual cortex[42]. Each trace shows the voltage activity of a different cell, and the black dots indicate the detected action potentials (APs). **d**,**e** | Voltage imaging at cellular resolution facilitates the study of dendritic voltage signalling and subcellular structures (for example, spines and boutons). Shown is an example of a backpropagated AP (part **d**, lower panel), as indicated by the delay between the AP waveforms measured in dendrites versus soma (using the GEVI Quasar3)[38], and an example of voltage signals from a single dendritic spine and the average over several spines, made using the GEVI Archon1 (part **e**, lower panel)[39]. AL, anterolateral area; LM, lateromedial area; ROI, region of interest. Part **a** adapted from REF.[37] CC-BY-4.0, https://creativecommons.org/licenses/by/4.0/. Part **b** adapted from REF.[36] CC-BY-4.0, https://creativecommons.org/licenses/by/4.0/. Part **c** adapted with permission from REF.[42], AAAS. Part **d** adapted from REF.[38], Springer Nature Limited. Part **e** adapted from REF.[39], Springer Nature Limited.

signal component[38,39]. Mesoscopic voltage imaging has facilitated the analysis of cortical circuit dynamics in the framework of complex self-organizing systems, providing a link between biomarkers for neuropsychiatric circuit diseases developed in clinical imaging and basic neurophysiology[40,41]. Using mildly magnifying optics, the functional organization of specific cortical areas is captured in a mesoscopic epifluorescence imaging configuration (FIG. 3b).

Clearly surpassing the capacity of previous approaches, genetic targeting of voltage indicators now enables mesoscopic imaging at single-cell resolution[42] (FIG. 3d). Instrumental to the latter technological advance are chronic mouse preparations that provide optical access to an estimated 1 million individual neurons across the dorsal surface of neocortex[43] and a combination of sparse and somatic targeting of GEVIs (FIG. 2). This new mesoscopic single-cell-level approach provides data equivalent to intracellular microelectrode recordings (yet still noisier) from many representative cells that are individually resolved, instead of an average signal over many cells for each pixel, as in traditional macroscopic and mesoscopic voltage imaging.

Light field deconvolution
A technique for high-speed volumetric imaging. Using an array of lenses, the object is imaged at different angles, providing 3D information about a sample. The 3D structure is reconstructed by mathematical operations termed deconvolution. This technique allows for imaging in three dimensions simultaneously with a 2D detector.

Imaging of local population signals. Circuit-centric approaches are used to address circuit-level functions. The traditional workhorse for these approaches is a local field potential (LFP) obtained with microelectrodes that are inserted into brain tissue and that report current flowing in the extracellular space. These measurements are often considered as a proxy for the local population voltage signals but have several limitations, including unknown localization of the neuronal membranes that pass the measured currents (the source of the current can be far away from the position of the electrode) and blindness to cellular diversity[44,45]. Voltage imaging can overcome these limitations. GEVI signals acquired via an objective from brain tissue surfaces, head-mounted miniscopes (FIG. 4b) or optical light guides inserted like electrodes into the tissue ('fibre fluorimetry'; FIG. 4c) represent the responses of a chosen genetically targeted neuronal population that is spatially restricted by the optical focus (FIG. 4c). Fibre or GRIN lens-based signal acquisition systems allow for recording from deeper brain regions and overcome the limitation of traditional mesoscopic imaging approaches to surface recordings. Moreover, whereas

the LFP represents the temporal derivative of the local membrane potentials — hence filtering out the slower components — GEVI signals also report slow shifts in the membrane potential[25,46].

Single-cell resolution GEVI imaging. Fundamental insights from neuronal computations at the level of single cells had previously been obtained using heroic approaches such as patch clamp recordings from up to 12 neurons in brain slices[47]. Up-scaling the multi-patch approach by increasing the number of patched neurons and by employing this method in the awake cortex is arguably not feasible. GEVI imaging, in combination with optogenetic stimulations (dubbed as all-optical electrophysiology), is a technology that can achieve quasi-intracellular electrophysiology from a large number of neurons — orders of magnitude larger than in the in vivo multi-patch approach. All-optical electrophysiology using GEVIs is ready to begin addressing neurophysiological questions[38,48–52].

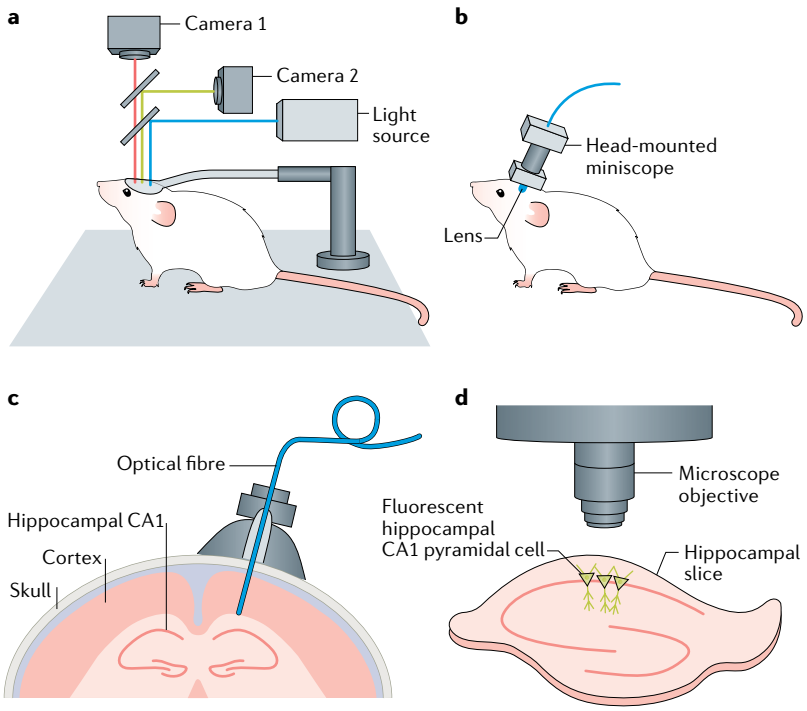

Fig. 4 | **Experimental setup configurations for different levels of GEVI imaging, from behavioural to cellular-level analysis. a** | The head-fixed experimental setup achieves macroscopic voltage imaging of animals during performance of a behavioural task. The tasks (not shown) include reporting different visual, auditory or tactile signals with motor actions such as licking a spout. **b** | A head-mounted miniscope configuration allows mesoscopic and circuit-centric voltage imaging of defined regions through a gradient-index (GRIN) lens in a freely moving animal. The GRIN lens can relay optical signals from deeper brain regions to the fluorescence imaging system within the miniscope. **c** | Fibre fluorimetry can be used in freely moving animals to report voltage signals averaged across the neighbourhood of the fibre tip. In contrast to local field potential recordings, these signals are selective for the cell type genetically targeted with a genetically encoded voltage indicator (GEVI). **d** | Higher-magnification functional imaging setups allow voltage monitoring at the cellular and subcellular levels, either in isolated brain tissue (shown is an acute hippocampal slice) or in vivo (not shown). A high numerical aperture objective is required in order to sample a sufficient number of photons from small volumes.

A recent cutting-edge example of powerful advances in GEVI imaging experiments acquired elegant recordings of subthreshold and action potential activities of up to 13 hippocampal neurons in the hippocampus of living mice[53]. The data obtained with such experiments not only allow the reconstruction of synaptic interactions within local circuits ('circuit diagram') but, owing to the excellent time resolution of the voltage-imaging method, also allow analyses of the computational features of the imaged circuits[53].

Subcellular voltage imaging. Understanding brain functions would be incomplete without studying electrical signalling and signal processing at the subcellular level. The 'gold standard' in this field is dendritic and axonal patch clamp recordings[54]. The limitation is invasiveness when investigating small structures such as thin dendrites, dendritic spines and axons, when electrode-induced disturbance of the ionic gradients and biophysical membrane properties would be of substantial concern, or when patch-clamping the same cell with two electrodes would be required. For example, backpropagation of action potentials from the soma to the dendrites of cortical pyramidal cells has been well described in brain slice preparations using dual-patch clamp recordings and VSDI[54,55]. Recent GEVI imaging experiments have confirmed the backpropagation of action potentials in living mice[38] (FIG. 3d). Another longstanding neuroscience issue that has been resistant to microelectrode-based electrophysiology is the functional significance of the shape of dendritic spines. GEVI imaging along with advanced data analysis methodologies is now in place to address this issue (FIG. 3e). The third cutting-edge GEVI imaging application example at the subcellular scale is optical recordings to explore the plasticity of electrical signalling in axons and synaptic terminals[56].

Concluding remarks

Recent advances in GEVI-based voltage imaging give a strong fresh momentum to the longstanding efforts to move this approach to a stage at which a broad range of laboratories are enabled to address timely circuit-level neuroscience research questions. However, this Review would be incomplete without mentioning some remaining challenges. One of the most pressing tasks is to address concerns among investigators interested in using GEVIs for their research who find that some indicators do not live up to the performance claimed by their inventors. This issue can only be resolved by stringent independent cross-validation of the most promising GEVIs. Voltage imaging remains methodologically more challenging than calcium imaging. For instance, no widely distributed off-the-shelf equipment is currently optimized for GEVI imaging. Researchers who are driven by the exciting prospects of recent advances in GEVI imaging are advised to seek training in equipment tuning and data interpretation at one of the labs that has actively contributed to the development of this technique. As techniques evolve, we expect that the following four fields will be set to take advantage of GEVI-based imaging approaches.

Dendritic plateau potentials. Dendrites of cortical pyramidal cells can generate depolarizations that outlast a triggering excitatory synaptic input, due to the recruitment of voltage-dependent calcium channels and NMDA receptors[57]. Calcium-imaging studies have associated dendritic plateau potentials with calcium signals, but their exact relation to dendritic voltage waveforms is unknown. Dendritic plateau potentials occur in vivo[58–60] but have been studied mainly in brain slices. Voltage characterization of dendritic plateau potentials in vivo is necessary to understand their functional significance. GEVI imaging could be instrumental in this task.

'Up' and 'down' states. During sleep and quiescence, the cortex and thalamus express patterned spontaneous activity in the form of synchronous network state transitions every second (1 Hz). Throughout this so-called slow oscillation, cortical and thalamic neurons fluctuate between periods of intense synaptic activity ('up' states) and almost complete silence ('down' states)[61]. Up states are important for memory consolidation[62]. Up states may also represent particular neuronal states during which neurons are more responsive to oncoming synaptic inputs[57]. There are many open questions regarding the sustained depolarized states that occur in cortical neurons of living animals — in particular, the mechanisms by which up states initiate and terminate, as well as the functional role of the rhythmic activity cycles in minimally conscious states (for example, drowsiness), waking behaviour and sensory processing[63]. Given the advances in GEVI imaging methods in both in vivo and in vitro preparations, the time is ripe to take stock of our current understanding of slow oscillations and pave the way for future investigations of its mechanisms and functions.

Excitation–inhibition balance. Theory and cellular physiology suggest that synaptic excitation and inhibition are in balance at a slower timescale but are dynamically out of balance at a fast timescale. Neuronal circuits are characterized by spontaneous ('ongoing') activity. The balance at a slow timescale is required in order to prevent the build-up of recurrent excitation from becoming explosive, in the form of seizures, as well as to prevent recurrent inhibition from silencing the network. At a faster timescale, short-lasting dominance of an excitatory mechanism followed by inhibitory dominance is the basis of rhythmic activities (including the up states mentioned above) and fast network response times. Imaging two GEVIs that exhibit non-overlapping excitation and/or emission spectra and that are targeted to excitatory and inhibitory neurons, respectively, will be a perfect approach to investigate the dynamics of this balance.

Short-range and long-range connectivity. Human brain-imaging studies suggest that major neuropsychiatric spectrum disorders are associated with changes in both short-range and long-range connectivity within and between neuronal circuits. For instance, autistic traits correlate with long-range connectivity diminution and enhanced local connectivity. Psychotic traits (lack of empathy or increased risk taking) correlate with hyper-connectivity in some subnetworks and hypoconnectivity in others[64–66]. These disorders are therefore increasingly being conceptualized as developmental (structural) or functional alterations of brain connectivity. Perturbation experiments that capitalize on animal models and the combination of voltage indicators with optogenetic actuators are now in reach of testing these concepts.

In conclusion, GEVI imaging and advanced imaging instrumentation enrich the toolbox of optical methods in neuroscience and are now set to be used towards better understanding of the dynamics and mechanisms of neuronal circuits. These investigations are likely to be important not only to approaching the basic neuroscience goals stated in the introduction but also to moving on to elucidate the neuronal circuit mechanisms of neuropsychiatric diseases.

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