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Review

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Genetically Engineered Fluorescent Voltage Reporters

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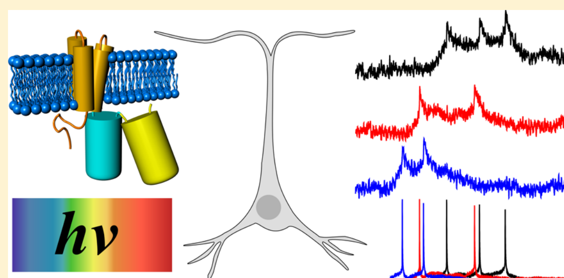
ABSTRACT: Fluorescent membrane voltage indicators that enable optical imaging of neuronal circuit operations in the living mammalian brain are powerful tools for biology and particularly neuroscience. Classical voltage-sensitive dyes, typically low molecular-weight organic compounds, have been in widespread use for decades but are limited by issues related to optical noise, the lack of generally applicable procedures that enable staining of specific cell populations, and difficulties in performing imaging experiments over days and weeks. Genetically encoded voltage indicators (GEVIs) represent a newer alternative that overcomes several of the limitations inherent to classical voltage-sensitive dyes. We critically review the fundamental concepts of this approach, the variety of available probes and their state of development.



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Elucidating the mechanisms that underlie information processing in the brain as represented by the electrical signals generated by neurons is one of the fundamental challenges of neuroscience. Computation by neuronal circuits and systems of circuits links neuronal activity with behavior.[1] These electrical signals occur as fluctuations in voltage across the outer lipid membrane of nerve cells. Individual neurons typically have branched processes (dendrites) whose electrical behavior can be described in terms of compartments that are coupled by the conductivity of the cell interior. Since this conductivity is finite, membrane voltage signals generally differ between distant compartments of the same cell, endowing dendrites with complex signal processing capabilities.[2] As neurons are electrically isolated from each other, with the exception of neurons coupled via gap junctions, their voltage signals are primarily independent of each other. However, as neurons communicate with each other via their synaptic contacts, the electrical activity of one neuron can impact on the membrane voltage of another. Synaptic interactions can cause synchronization of voltage signals over large populations of neurons.[3] Voltage signals that represent an average across a population of neurons represent activities of neuronal circuits as a whole, and such population-scale signals correlate with brain state and behavior.[3−5] Therefore, the dynamics of electrical signaling in the brain must be considered across a wide range of spatial scales, ranging from subcellular compartments to local neuronal circuits to systems of circuits.

These electrical signals can be efficiently investigated with microelectrode techniques, which can also be parallelized to monitor tens to hundreds of sites within a circuit simultaneously.[6] But given the large number of neurons within even a small volume of the brain (e.g., 10^5 neurons/mm^3 in the cortex), there is a need for tools that enable high-density and massively parallel measurement of neuronal membrane potentials, a challenge that clearly is beyond the reach of available microelectrode techniques. Optical voltage imaging, based on dyes that transduce membrane voltage into a fluorescent readout, has the potential to achieve this goal.[7] Low-molecular-weight, voltage-sensitive dyes can be used to stain single cells via intracellular injections or whole brain tissues and therefore allow optical recording of electrical signals from many cells simultaneously at single-cell resolution as well as from very large populations with high spatial resolution. Thus, voltage imaging offers the possibility of characterizing neuronal computation at any of the spatial scales described above.

Voltage-sensitive dyes that generate strong and fast optical signals are essential for the success of this approach. The magnitude of the optical response produced by a given dye is usually expressed as the change in light intensity normalized to the baseline intensity (i.e., fluorescence, F, in the case of a fluorescent dye) in response to changes in transmembrane potential (e.g., % $\Delta F/F$ per mV). Persistent efforts have led to dyes with greatly improved performance, and better dyes are still being actively developed with remarkable success in the present day.[8−10]

However, there are some fundamental limitations associated with these exogenously applied voltage-sensitive dyes.[11] Even if a dye is highly sensitive to membrane potential, in practice the optical signals are usually noisy. Noise resulting from photon statistics (shot noise) can be alleviated by sacrificing spatial or temporal resolution. However, temporal resolution cannot be reduced below the speed of the signal of interest (typically in the millisecond range), and spatial resolution for signals from

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single cells cannot be reduced below the spatial dimensions of those cells. Since the largest portion of the plasma membrane surface (and hence the largest component of optical signals) is provided by neuronal processes with diameters in the range of 1 μm, sorting out signals from individual cells is difficult, if not practically impossible, using standard methods of bulk tissue staining. Moreover, even if signals from single cells could be sorted out from mixed signals, the shot noise associated with the fluorescence from other cells degrades the signal from an individual neuron. This dilemma has prompted many neuroscientists to resort to calcium imaging as an indirect or proxy indicator of electrical signaling.[12]

These issues can be overcome through the use of genetic methods that enable the targeted labeling of specific cell populations.[11] This approach requires the development of fluorescent voltage reporter proteins, also known as genetically encoded voltage indicators (GEVI), analogous to the similarly named genetically encoded calcium indicators (GECI). The genetic engineering and targeting approach has several advantages over organic dyes. For instance, staining of brain tissue with organic dyes for *in vivo* experiments usually involves invasive procedures, such as craniotomy for delivery of the dyes and organic solvents for the lipophilic organic dyes, whereas transgenic gene delivery avoids these potentially harmful procedures. Because genetic "staining" can be permanent, long-term (chronic) experiments are feasible. Genetic targeting also offers the possibility to record signals from well-defined populations. This is particularly attractive when studying genetically defined subpopulations of cells that correlate or synchronize their activities, such that the average signal from that population conveys specific functional information. For example, GEVIs can enable one to track the engagement of specific cell types during rhythmic brain activities, which have traditionally been observed using EEG or field potential recordings.[3]

Fluorescent voltage reporter proteins have been conceived and engineered over the past two decades and have evolved considerably in that time. Initial design concepts exploited voltage-dependent structural rearrangements of voltage-gated ion channels or voltage-sensor domains isolated from those proteins.[13,14] The first reported fluorescent voltage reporter protein was FlaSh, a construct that uses a nonconducting mutant of a voltage-gated potassium channel as the voltage sensor and a fluorescent protein inserted into the C-terminal region of the channel protein as a reporter.[15] A conceptually closely related prototype, SPARC, is based on the insertion of GFP into a skeletal muscle sodium channel.[16] A different design principle, for which we introduced the acronym VSFP (voltage-sensitive fluorescent protein[17]), exploits the voltage-dependent conformational changes of a voltage-sensor domain coupled to a pair of fluorescent proteins. The basic idea behind the design of VSFPs is that movement of the voltage-sensor domain will modulate the efficiency of Förster resonance energy transfer (FRET) between the two fluorescent proteins by shifting their position and orientation relative to each other. The first generation of VSFPs, VSFP1, used the voltage-sensor domain of a potassium channel subunit.[17] FlaSh, SPARC, and VSFP1 provided proof of principle for the GEVI concept, but failed in their intended application in brain tissue due to small (or practically absent) signals. However, the second generation of VSFPs, described in this review, overcame this initial disappointment.

GEVIs complement the genetically engineered light-activated ion channels and pumps that are the principal tools for optogenetic control of neuronal circuits.[18−20] This pairing of monitoring and control tools forms the basis for a comprehensive optogenetic approach to electrophysiology[21,22] or, as it also has been termed, "electrophysiology in the age of light".[23] The field of optogenetic electrophysiology is still a work in progress, but recent innovations have given cause for optimism. In this review, we will focus on the four most promising current approaches for optogenetic monitoring of electrical signaling in neuronal circuits.

DESIGNS FOR THE ENGINEERING OF GEVIS

Isolated Voltage-Sensor Domain-Based Voltage Indicators. Voltage-dependent potassium channel (Kv channel) subunits consist of a four-transmembrane-segment voltage-sensor domain (S1−S4) and two transmembrane segments (S5−S6) that form the ion channel in subunit tetramers. Since the fourth transmembrane segment (S4) of the voltage-sensor domain contains positively charged amino acids that are presumably exposed to the strong electric field within the plasma membrane, it has been questioned whether an isolated voltage-sensor domain can exist as a self-contained and functional protein in plasma membranes without structural support by the overall ion channel structure. This issue was resolved with the description of *Ciona intestinalis* voltage sensor-containing phosphatase (*Ci*-VSP),[24] which consists of a voltage-sensor domain attached to an intracellular enzyme and does not appear to be dependent on interactions with other membrane proteins. Since *Ci*-VSP naturally occurs in a monomeric configuration, whereas the obligate tetramerization of Kv channel subunits likely affects the membrane trafficking of their voltage-sensor domains, the *Ci*-VSP voltage-sensor domain has become a promising alternative scaffold for the development of GEVIs, such as the VSFPs. During the past several years, several *Ci*-VSP-based VSFP designs have been developed.[22] The first design involves a tandem of fluorescent proteins fused to the end of S4. Since this design corresponds to that of the potassium channel-based VSFP1, this family of GEVIs was named the VSFP2s. The variant VSFP2.3[25,26] was the first FRET-based GEVI to enable optical imaging of spontaneous action and synaptic potentials in neurons (Figure 1a).

The members of the VSFP2 family differ with regard to the fluorescent proteins used for the FRET pairing. The best-tuned versions for each variant differ only modestly in sensitivity and kinetic parameters when compared in cultured PC12 cells.[27] However, the species of fluorescent protein used has a dramatic impact on membrane-targeting efficiency and effective signal amplitude when used in live mammalian preparations.[14,21]

An alternative VSFP design, the VSFP3s (Figure 1b), uses a single fluorescent protein instead of a fluorescent protein pair.[28] VSFP3s offer the advantages of broad coverage of the color spectrum and relatively fast overall kinetics but with smaller signal amplitudes than the VSFP2s.[28] VSFP3 variants have also been designed to incorporate circularly permuted fluorescent proteins (cpFPs, Figure 1c).[29] Although calcium indicator proteins containing cpFPs have proven successful,[30,31] cpFP-based VSFPs have yet to match this success. A recently reported VSFP3 variant uses a mutated ecliptic pHluorin pH indicator, originally derived from GFP.[32] VSFP3 variants based on *Ci*-VSP homologues from other species (e.g., zebrafish) have been explored as well.[13]

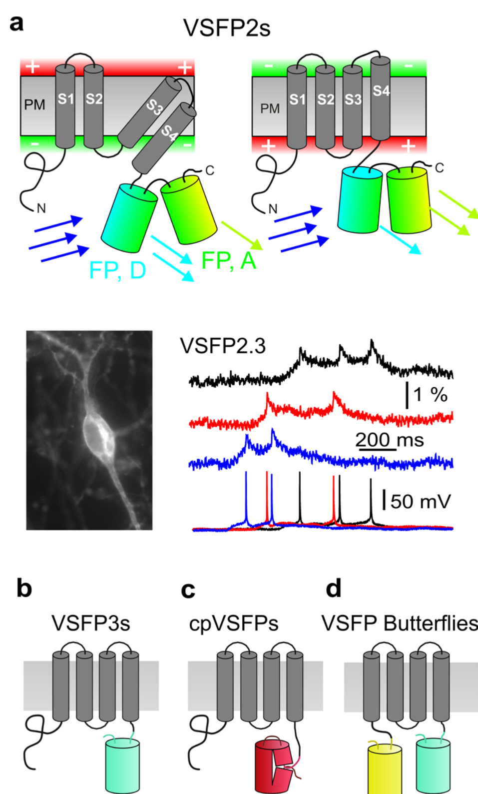

Figure 1. Designs for voltage-sensor domain-based voltage indicators: (a) Upper panel, schematic of FRET-based voltage-sensitive probes of the VSFP2 family. The voltage-sensor domain, consisting of four segments (S1−S4) crossing the plasma membrane (PM), is fused to a pair of fluorescent proteins (FP, D FRET donor; FP, A FRET acceptor). A change in membrane potential induces a rearrangement of the two fluorescent proteins that is optically reported as a change in the ratio between donor and acceptor fluorescence. Lower panels, example recording from cultured hippocampal cells showing spontaneous action potential firing. The three sweeps of optical recordings shown in black, red, and blue color correspond to the superimposed microelectrode recording traces of same color. (b) Single fluorescent protein probes of the VSFP3 family. (c) VSFPs incorporating a circularly permuted fluorescent protein. (d) FRET-based voltage sensitive probe of the VSFP-Butterfly family, where the voltage-sensor domain is sandwiched between two fluorescent proteins.

In the latest VSFP design, the voltage-sensor domain is sandwiched between two fluorescent proteins (Figure 1d). We termed this series VSFP Butterflies, and these currently represent the best-performing probes for monitoring subthreshold membrane oscillations *in vivo*. Since the sensing mechanism of VSFP involves the movement of charges, corresponding to the "gating" or "sensing" current measured from voltage-gated ion channels, concerns have been expressed that their expression adds prohibitive amounts of extra capacitance to the plasma membrane.[33] However, experimental data and detailed computer simulations[34] have revealed that the adverse effects of increased capacitance can be essentially avoided by proper indicator design (minimizing the number of sensing charges) and occur only at VSFP expression levels that exceed those used in practice.

Microbial Opsin-Based Voltage Indicators. The newest concept for GEVI design is based on the use of microbial opsins.[35,36] (Figure 2) These proteins bind retinal (a vitamin A-related organic chromophore) and have evolved naturally to

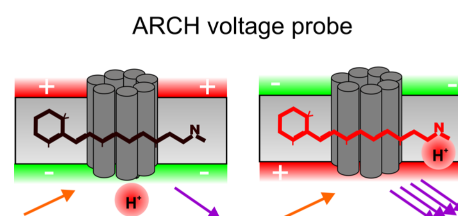

Figure 2. Cartoon of microbial rhodopsin-based voltage indicator Arch. A change in membrane potential induces increased fluorescence of the retinal molecule.

function as transducers of light into cellular signals, including changes in membrane voltage. Adam Cohen's group at Harvard found that the natural relationship between light and voltage can be reversed, so that membrane voltage changes are reported as an optical signal. The proof of principle was first demonstrated with a proteorhodopsin-based optical proton sensor (PROPS) from green light-absorbing bacteria.[35] PROPS produced signals that appeared to represent voltage fluctuations in *Escherichia coli* but did not target well to plasma membranes of eukaryotic cells. Subsequently, the researchers determined that archaerhodopsin-3 (Arch), a previously established optogenetic control tool, produces a fluorescent signal that correlates with changes in membrane voltage.[36] Their findings were cause for considerable excitement, because the change in Arch fluorescence is very fast and linear, two desirable features for a GEVI. However, since the natural function of Arch is to drive a proton current with the absorbed light energy, voltage sensing also changes voltage. This undesirable effect was fixed by a point mutation in the Arch protein that abolished its capacity to elicit light-driven currents. Unfortunately, this mutation also dramatically slowed the optical signal in response to membrane-potential changes.[36] While additional protein engineering might solve the latter issue, the most serious limitation of the Arch class of voltage probes is their very low quantum efficiency (0.001).[36] This is probably the reason why we failed to detect Arch-related fluorescence from the cortex of live mice expressing EGFP-tagged Arch (Figure 3). In comparison, fluorescence of the EGFP tag and of VSFP2.3 was readily detected and greatly exceeded autofluorescence under the same experimental conditions. The very low brightness of this prototypic GEVI could potentially be improved by developing a synthetic chromophore with higher quantum yield that, when applied to the biological system, replaces the endogenous chromophore. However, this strategy may fail due to interference with the physiological role of retinal. For example, the application of a retinal substitute could theoretically leave animals blind by disrupting the function of rhodopsins required for light transduction in the retina. Similarly, this substitution could cause other optogenetic control tools based on opsins to fail, undermining the exciting possibility of combining optogenetic monitoring with optogenetic control.

FlaSh-Type Voltage Indicators. In an attempt to overcome the limited membrane localization of FlaSh-type fluorescent protein voltage sensors, a consortium of researchers led by Lawrence Cohen[13] sought to improve plasma membrane expression of FlaSh−YFP by splitting the fluorescent protein into two nonfluorescent halves and attaching the two halves to different subunits of the Kv channel[37] (Figure 4). They screened 56 fluorescent probes (generated by coexpression of Kv subunits containing either half of the fluorescent protein), of

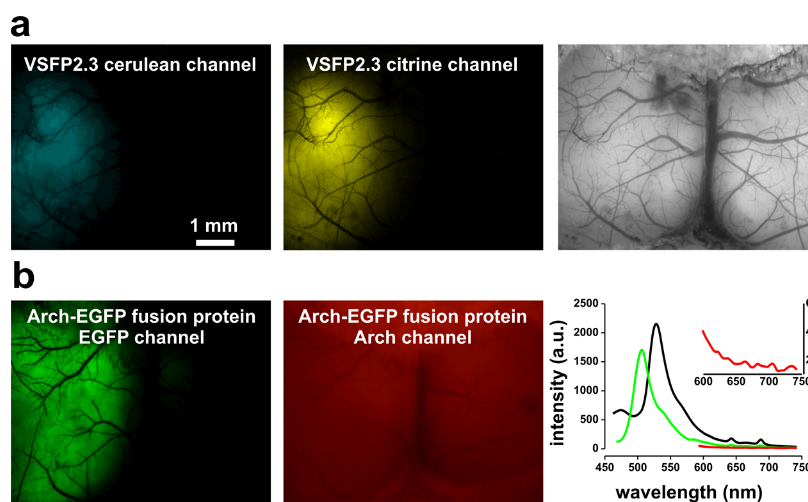

Figure 3. Evaluation of *in vivo* fluorescence output generated by Arch in comparison to VSFP2.3. Mice were electroporated *in utero* with plasmids expressing either VSFP2.3 or EGFP-tagged-Arch (kindly provided by Dr. Ed Boyden) and at adulthood prepared for imaging under a Nikon C1si/FN1 confocal microscope in spectral mode through the thinned bone overlying the somato-sensory cortex. (a) Fluorescence images obtained with a VSFP2.3-expressing mouse (444 nm excitation); from left to right, VSFP2.3 donor (cerulean) fluorescence, acceptor (citrine) fluorescence, and bright-field view of the mouse cortex *in vivo*. (b) Fluorescence images obtained from a mouse expressing an Arch-EGFP construct. EGFP fluorescence was readily detected in the green channel (middle, 488 nm excitation) while the red channel (543 nm excitation, > 600 nm emission) showed only scattered excitation light and nonspecific autofluorescence. Graph shows emission spectra obtained from the same preparation over targeted cortical areas. VSFP2.3 (black) and GFP (green) spectra were obtained with excitation at 440 nm. The red-line spectrum obtained with excitation at 543 nm is shown at expanded scale in the inset. Note that the spectrum lacks the peak expected at 687 nm for the Arch-based voltage indicator.[30]

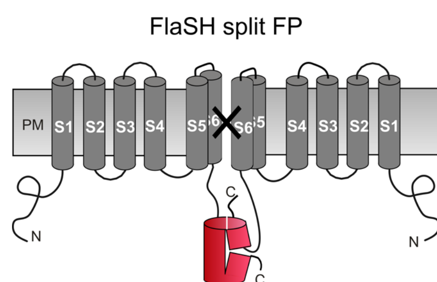

Figure 4. Schematic depiction of the most recent FlaSh-type voltage indicator. This protein incorporates fluorescent protein complementation, with subunits of the Shaker potassium channel fused to either the N- or C-terminal portion of a split fluorescent protein. Tetramerization of the Shaker subunits facilitates complementation of the two fluorescent protein portions to recover fluorescence, while misfolded subunits and monomers that do not traffic to the membrane remain uncomplemented and hence nonfluorescent. Modulation of FlaSh fluorescence is triggered by voltage-dependent rearrangement of the (nonconducting, as indicated by cross) Shaker potassium channel.

which 30 were expressed at the plasma membrane and capable of optically reporting changes in membrane potential. The largest signal from these novel FlaSh-derived sensors was −1.4% in $\Delta F/F$ for a 100 mV depolarization, with on-time constants of ∼15 ms and off-time constants of ∼200 ms. Unfortunately, this "split-can" approach did not yield probes with better performance than previously available GEVIs. In addition to their slow kinetics, FlaSh-type GEVIs involve a large number of gating charges in voltage sensing and therefore are more problematic with respect to increased membrane capacitance than probes based on single voltage-sensor domains.[34]

Hybrid Voltage Indicators. A fourth general design concept for voltage indicators is a two-component FRET-based strategy, originally developed without genetic components[38] but subsequently adapted to a genetically targetable probe by Chanda and colleagues.[33,39,40] The first component of their hybrid voltage sensor (hVOS) is a fluorescent protein with attached farnesylated and palmitoylated motifs that anchor it to the plasma membrane (Figure 5). The second component is

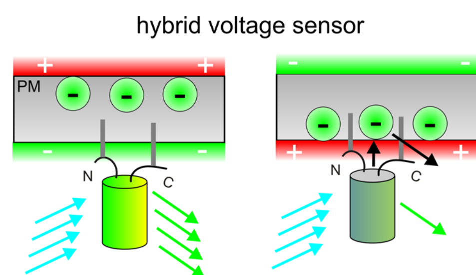

Figure 5. Schematic depiction of the hybrid voltage sensor hVOS. hVOS consists of a combination of a fluorescent protein (genetically encoded component) with dipicrylamine (DPA) (exogenous component). The fluorescent protein is anchored to the intracellular side of the plasma membrane by a prenylation motif. Positively charged DPA is partitioned in the membrane as a function of the membrane potential. With membrane depolarization, DPA moves within Förster distance of the fluorescent protein and quenches its fluorescence.

the nonfluorescent synthetic compound dipicrylamine (DPA), which serves as a voltage-sensing FRET acceptor (quencher). Since DPA is lipophilic but negatively charged, it distributes in the membrane in a voltage-dependent fashion. When the membrane is depolarized, it translocates to the inner layer of the membrane, within Förster distance of the fluorescent protein, quenching its fluorescence. Unfortunately, DPA increases the membrane capacitance, so care must be taken to ensure that the concentrations used do not disrupt the native physiological responses.[33,34]

Another notable "hybrid" strategy, proposed by Fromherz and colleagues, entails activating an organic voltage-sensitive dye via an enzyme that may be genetically targeted to specific cell populations.[41,42] This strategy is analogous to the widely used and highly successful acetyl ester-modified calcium indicators and could be generalized and applied to new improved organic dyes.[8]

The general concern related to these hybrid strategies is overcoming difficulties in the selective application of an exogenous lipophilic compound to neuronal membranes in intact tissue.

Benchmarking of Voltage Reporters. A crucial aspect in the development of molecular tools is the evaluation and benchmarking of their performance. Like the pipeline in drug discovery and development, which traditionally starts with simple and efficient biochemical assays and ends with time-consuming and costly clinical trials, GEVIs are usually evaluated under conditions of increasing complexity.

At the level of least complexity, photophysical aspects of the chromophores are in the spotlight. Relevant parameters are those that characterize the chromophores's ability to emit a large number of photons and hence yield favorable photon statistics (i.e., low shot noise). This constraint is best quantified as the ratio of the quantum yield (QY) of fluorescence to the QY of bleaching. This parameter quantifies the number of photons that can be sampled before the dye bleaches. The shot noise-limited signal-to-noise ratio (SNR) of fluorescence measurements at the molecular level can therefore be described as

$$\mathrm{SNR} \approx \Delta F/F \times \sqrt{(\text{QY of fluorescence}/\text{QY of bleaching})}$$

where $\Delta F/F$ is the baseline-normalized change in fluorescence intensity.[11] In the case of ratiometric (e.g., FRET-based) probes, where two fluorescence emission channels show opposite intensity changes, $\Delta F/F$ can be substituted by the relative change in the ratio (R) between the two channels ($\Delta R/R$). At the level of molecular probe design, the performance of FRET-based probes depends on the orientation and distance of the chromophores and the change of these parameters with voltage. A useful parameter to specify the efficiency of energy transfer between two given chromophores is the so-called Förster distance, corresponding to the distance where transfer occurs with 50% efficiency. It should be noted that even within the limitations of classical Förster theory, maximizing baseline efficiency of a FRET-based indicator by no means implies maximal indicator sensitivity or maximal signal-to-noise ratio. For instance, the SNR for FRET-based probes is optimal with balanced intensities in donor and acceptor channels.

The next level of complexity occurs at the biophysical level and comes into play when a voltage probe is expressed in cells where it needs to integrate into the plasma membrane. There are some indications that differences in the composition of plasma membranes between cell types may affect a given probe's biophysical characteristics. For instance, we have noted that the activation curve measured for VSFP2.3 slightly differs between PC12 cells and cortical pyramidal cells.[26] The fraction of fluorescence emitted from probes that are inserted into the plasma membrane is also an important factor affecting performance. Untargeted probe molecules that contribute fluorescence but no signal reduce the apparent fluorescence response, $\Delta F/F$, and add shot noise. Because of this caveat, several otherwise promising probes have failed in more advanced applications.

At the biophysical level, the signal-to-noise ratio expands to

$$\mathrm{SNR} \approx \Delta F/F \times \sqrt{(\text{QY of fluorescence}/\text{QY of bleaching})} \times \text{fraction of membrane-targeted probes}$$

where "fraction of membrane-targeted probes" refers to the number of membrane-targeted probe molecules relative to all probe molecules contributing to the observed fluorescence. Autofluorescence reduces this fraction of signal-carrying fluorescence and contributes photon noise. In the formula above, autofluorescence above negligible levels has the same effect as fluorescence from nontargeted indicator molecules. The highest level of complexity occurs in neurophysiological experiments conducted on intact brain tissue, either in brain slices or in living animals. While low light absorption (extinction coefficient of the chromophore) and low quantum yield (i.e., of the donor chromophore in FRET-based sensors) can often be compensated through the use of high-intensity excitation light at the biophysical level, experiments in intact brain tissue may need to account for the limited light tolerance of the preparation. In this experimental setting, "bright" probes are also required to overwhelm brain tissue autofluorescence.

The various VSFP2 probes differ in specific properties that determine their performance in specific applications. For instance, the fluorescence−voltage relationship of VSFP2 matches a Boltzmann type of activation curve. Accordingly, sensitivity is maximal in the center of the dynamic range ($V_{1/2}$ value).[43] This voltage should match the absolute values of the voltage fluctuations of interest. Thus, if fluctuations around resting membrane potential are of primary concern, a VSFP variant with a $V_{1/2}$ close to resting membrane potential would be expected to provide the largest signal.

Signals recorded from populations of neurons (e.g., *in vivo*) are generally much smaller than signals from isolated cells. This is because only a fraction of these neurons, even within a genetically defined population, will be responsive, at least to physiological stimuli. Since fluorescence from nonresponsive neurons still contributes photon noise, the SNR for the *in vivo* situation can be formulated as

$$\mathrm{SNR} \approx \Delta F/F \times \sqrt{(\text{QY of fluorescence}/\text{QY of bleaching})} \times \text{fraction of membrane-targeted probes} \times \text{fraction of recruited cells}$$

where "fraction of recruited cells" refers to the number of GEVI-expressing cells that respond to a stimulus over the total number of GEVI-expressing cells that contribute to measured fluorescence (with the simplifying assumption that each cell contributes fluorescence equally). Moreover, voltage imaging *in vivo* typically represents mostly subthreshold activity, at least in the cortex, with only a minority of cells firing action potentials. For this reason, optical signals imaged *in vivo* are much slower and smaller than expected in comparison to the signals observed in single neurons during generation of action potentials.

Movement and hemodynamic artifacts introduce additional complications to *in vivo* experiments. Because dual-channel ratiometric measurements principally allow compensation for motion-induced artifacts and tissue hemodynamic signaling,

Table 1. Comparison of Various Benchmarking Parameters for Published GEVIs

	VSFPs	microbial opsins	"split can Venus"-FlaSh-type[37]	genetically encoded hybrids
fluorescence quantum yield	~0.8[44]	~0.001[35,36]	~0.5	~0.5[39,40]
photostability relative to EGFP	>1 when using newest generation FPs[44]	0.25 (Arch, photocurrent generating form) 0.1 (Arch D95N, salient variant)	0.25	>1 when using newest generation FPs[44]
maximal sensitivity, %$\Delta F/F$ or $\Delta R/R$ per 100 mV (slope at $V_{1/2}$ for nonlinear probes)	~20% $\Delta R/R$ (at $V_{1/2}$)	~100% $\Delta F/F$	−1.4% $\Delta F/F$	26% $\Delta F/F$[39]
fast (slow) component of response to depolarization (voltage step from −70 to 0 mV)	~2 ms (~50 ms) [fastest variants]	~0.5 ms [Arch] > 100 ms [Arch D95N]	~15 ms	~0.5 ms
fast (slow) component of response to repolarization (voltage step from 0 mV to −70 mV)	<10 ms (~100 ms) [fastest variants]	~0.5 ms [Arch] ~0.5 ms (41 ms) [Arch D95N]	~200 ms	~0.5 ms
dual emission ratiometric?	yes [FRET variants]	no	no	no
mobile charges/fluorescence emitter	~3	1 (?)	~12	100 (?)
targeting efficiency	>50%	>50%	low (<50%?)	>50%
concerns related to additional capacitance	minimal	not investigated, probably minimal	not investigated, probably modest	yes, concentration dependent
toxicity?	no	no	no	unknown

ratiometric probes are generally preferable over single-channel probes for *in vivo* recordings.

Finally, any thorough evaluation of probes needs to consider their possible side effects. No direct toxic effects have been described for protein-only sensors, but phototoxicity might be an issue for some fluorescent protein species. Another potentially serious issue is the possible increase in dynamic membrane capacitance expected following the introduction of mobile charges into the membrane. The extent of this effect is dependent on the number of charges per probe molecule that translocate across the membrane's electric field upon change in membrane voltage.[34]

Table 1 summarizes the key features of the best-performing representatives for each of the above-described GEVI designs.

APPLICATIONS

In this review, we have focused on the motivation for developing GEVIs, their molecular design principles, and their functional characterization. For completeness, we will briefly look at some unique existing or emerging applications of GEVIs.

Neuroscience. GEVIs are being developed with the vision of directly monitoring the operation of neuronal circuit dynamics with features that overcome some of the limitations of organic dyes. At present, only VSFPs have been shown to allow imaging of neuronal activity in intact brain tissue, while other probe designs have been validated only in cultured cells. Recently, we also established that VSFPs can report rhythmic synaptic activities from large populations of genetically defined neurons in living mice. This feature is crucial for the goal of dissecting the circuit elements that govern coordinated neuronal activities associated with brain functions, such as sensory−motor integration, perception, cognition, and memory consolidation.[3]

Cardiac Physiology. Voltage imaging is also a valuable technique for cardiac physiology,[45] wherein VSFP-type voltage sensors have successfully been used to record mammalian cardiac action potentials at the level of intact hearts[46] and isolated myocytes.[46,47] Recently, the first mouse model with a GEVI (VSFP2.3) expressed exclusively in its cardiomyocytes became available.[46] We anticipate that longitudinal *in vitro* and *in vivo* studies on the electrophysiological properties of cells, tissue, and organs will greatly benefit from the availability of the stable optical cardiograms that can be readily and conveniently recorded using this model.

Drug Screening. Voltage-gated channels are potential drug targets for an increasing number of disease indications. High-throughput cellular screens for compounds that modulate ion channel activities could benefit from GEVIs that, for instance, could be coexpressed with a target channel in a stable cell line. Surprisingly, there are no published reports to date on the use of GEVIs for high-throughput drug screening. Since membrane potential is also a crucial cellular parameter in nonexcitable cells, it is imaginable that voltage imaging may play a role in drug discovery for a large number of diseases, including cancer and neuronal degeneration, and may even help in the development of related stem cell therapies.

OUTLOOK

All GEVIs published to date have response time constants greater than 1 ms (with the exception of unmodified Arch, which generates a photocurrent) and can therefore only report fast action potentials with an attenuated SNR.[26,36] Sufficiently bright and side-effect-free GEVIs that respond to both depolarization and repolarization with effective time constants of 1 ms or lower remain to be presented. It should be emphasized, however, that a reasonable SNR at high temporal resolution (as required to resolve action potentials) can only be achieved if the indicator can deliver both large photon fluxes (i.e., is bright and photostable) and high sensitivity.[34] In addition to satisfying these challenging indicator specifications, multisite optical action potential recordings in intact tissue (either living animals or brain slices) will require optical instrumentation that is far beyond what is currently available "off the shelf".

Further development will clearly be required to achieve optical imaging of action potential patterns from large number of cells with millisecond-scale time resolution in behaving mammals. However, we would like to emphasize that traditional voltage-sensitive dye (i.e., low-molecular-weight organic compounds) have provided important results by imaging the dynamics of large neuronal circuits without resolving single action potentials. Classical examples of these experiments include studies where the surfaces of cortical structures were stained with voltage indicators and imaged at the "mesoscopic" scale (pixelation at the scale of tens of micrometers, with fields of view at the millimeter scale).[7]

Because the frequency power spectrum of volume-averaged voltage signals from cortical tissue is dominated by frequencies below 50 Hz in this type of experimental setting,[7] these can be reasonably accurately reported by currently available GEVIs.[26] Indeed, we believe that the current generation of VSFPs is sufficiently advanced to expand mesoscopic-level circuit dynamics approaches into domains where the function of defined cell classes is the central biological question. Because only VSFPs have so far been shown to provide readily detectable signals in living mammals,[21] they are currently the GEVI class of choice. Future efforts should greatly benefit from further enhancements in the sensitivity and brightness of GEVIs to improve the SNR of voltage imaging in biologically relevant settings.

In conclusion, the field of GEVI development offers superb and pressing challenges for the chemical neuroscience community, giving researchers opportunities to develop novel ways to optically monitor membrane voltage, achieve improvements in voltage imaging using established GEVI designs or, more ambitiously, pursue the goal of imaging action potential patterns from large numbers of neurons at sufficiently high resolution to deduce the neuronal computations that underlie complex behaviors.

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Author Contributions

H.M. provided example data shown in Figures 1 and 3. H.M., W.A., and T.K. all contributed to drafting and revising this article.

Notes

The authors declare no competing financial interest.

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