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Genetically encoded fluorescent voltage indicators: are we there yet?

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Abstract

In order to understand how brain activity produces adaptive behavior we need large-scale, high-resolution recordings of neuronal activity. Fluorescent genetically encoded voltage indicators (GEVIs) offer the potential for these recordings to be performed chronically from targeted cells in a minimally invasive manner. As the number of GEVIs successfully tested for in vivo use grows, so has the number of open questions regarding the improvements that would facilitate broad adoption of this technology that surpasses mere “proof of principle” studies. Our aim in this review is not to provide a status check of the current state of the field, as excellent publications covering this topic already exist. Here, we discuss specific questions regarding GEVI development and application that we think are crucial in achieving this goal.

Introduction

In the brain, neuronal cells interconnect via synapses to build complex structural and functional units (neuronal circuits) that underlie functions ranging from basic processes necessary for maintenance of life, to the higher cognitive abilities that define us as humans. The information flow and processing in neuronal cells is based on ion channels activity that produces electrical transients across the cell's plasma membrane. The successful monitoring on all levels, from ion channels and single cells to neuronal circuits, has been a long-standing goal of neuroscience [1]. Traditionally neuronal electrical activity is recorded using electrodes that allow for direct, high-fidelity measurement of electrical transients on all functional levels [2,3]. However, the subsequent development of optical methods based on the use of small molecule voltage dyes emerged as a less invasive alternative that offers higher spatial resolution enabling activity detection from multiple locations and from

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electrode-inaccessible structures (i.e. dendritic spines and axonal boutons) [4,5]. The most recent advance in voltage imaging has arisen from the development of genetically encoded voltage indicators (GEVIs) that were conceived to allow for genetically- targeted, cell specific recordings [6]. The GEVIs also alleviate other problems related to the use of dyes. First, genetic targeting eliminates indiscriminate labeling of all cell membranes. Specific labeling decreases background fluorescence and increases the signal-to-noise ratio (SNR). Genetically encoded indicators also have reduced cytotoxicity, and exhibit less heterogeneous labeling across cells, a problem that arises from inconsistent dye distribution.

After two decades of development, the latest generation of GEVIs is beginning to yield successful physiological experiments *in vivo* in various organisms. Promising as they are, these studies are bringing to focus the range of issues that still need to be improved or resolved before GEVIs can become part of the toolbox that allow for an “all optical” approach to study brain activity. Here, we focus on the open questions in GEVI engineering and application which make voltage imaging extremely challenging.

Challenges in developing voltage indicators

The multipoint recording problem

To be able to understand the relationship between brain activity and behavior it would be advantageous to monitor neural activity with single-neuron resolution, across large populations, in multiple brain areas, during behavior [7]. Optimally, we would like indicator(s) that allow monitoring of the full range of relevant electrical events (i.e. subthreshold and suprathreshold) from identified nerve cells [1]. Additionally, a palette of indicators with variable spectral characteristics would facilitate combinatorial use with other activity indicators or optogenetic proteins for control of neuronal activity [8–10]. Since there are no known naturally-occurring fluorescent proteins with acceptable characteristics for optical detection of membrane voltage transients (although see opsins in [11]), design of GEVIs is based on engineering of molecular chimeras between voltage sensitive and optical (i.e. fluorescent) proteins (FPs) (Figure 1). (For detailed overview of approaches in GEVI design please see recent reviews: [12–16]).

The temporal problem

Action potentials (APs), postsynaptic potentials (PSPs), subthreshold oscillations (i.e. brain rhythms) and semi-stable potential transients (e.g. up and down states) have temporal and spatial characteristics that span several orders of magnitude, from microseconds to minutes and from micrometers to millimeters, respectively. The remarkable temporal complexity of electrical transients requires indicators that respond reliably over several orders of magnitude in time [1].

The membrane insertion problem

An additional constraint in GEVI design is their need to be inserted into the plasma membrane, a feature not necessary for genetically encoded calcium indicators (GECIs)[17]. Neuronal electrical events can only be measured within, or in a close vicinity to, the thin (~8 nm) plasma membrane [18]. This requirement for membrane localization of GEVIs has

multiple implications for both probe engineering and subsequent application. First, due to limited physical space only a small number of indicator molecules can be inserted in the membrane before the cell's integrity is irreversibly perturbed. Additionally, an excessive number of indicator molecules inserted into the membrane will alter the electrical properties of the membrane (specifically the membrane resistance and capacitance). If the indicator molecule possesses any ion conductance it can cause additional perturbation of intrinsic electrical properties of the cell. Compared to intracellularly localized calcium indicators (i.e. GCaMPs), the predicted maximum number of GEVI molecules present in the somatic membrane is several orders of magnitude lower ($\sim 10^4$ vs. $\sim 10^7$) [19], which puts a high demand on efficiency of the coupling between voltage sensing and optical output. Second, for optimal targeting to the plasma membrane, indicator proteins must fold and traffic well in neuronal cells. For fluorescent GEVIs, non-targeted intracellular fluorescence increases background and diminishes the SNR. Poor membrane localization can be an inherent property of the voltage sensing component and/or result of their artificial fusion with fluorescent proteins. For example, microbial opsins and even ion channels exhibit suboptimal membrane targeting in mammalian cells [11,20,21], while many red FPs have an innate propensity toward oligomerization that results in protein misfolding and intracellular retention [10,22]. The first generation of GEVIs exhibited poor plasma membrane localization arising from poor targeting of the voltage sensitive domain (VSD), derived from ion channels, which was further degraded by the addition of an FP [6,23–25]. Third, membrane localization of the GEVI makes mechanistic predictions based on structure (i.e. structure aided design) very challenging and limits the potential for rational indicator design [26].

“Brute force” approach in GEVI optimization

The most successful and promising probes for *in vivo* use are chimeras between a VSD isolated from voltage sensitive phosphatases [27–29] and fluorescent proteins, or fusions between opsins [21,30,31] and fluorescent proteins (Figure 1). These two design approaches use very different mechanisms for linking membrane potential fluctuations to changes in optical properties (i.e. fluorescence output). In any case, the existing hypotheses about mechanism of sensitivity in these different GEVIs are incomplete and do not provide sufficient guidance for rational optimization of indicators [26,30]. Multiple studies have demonstrated the profound and unpredictable effects on GEVI performance that can arise from discrete changes in amino acid sequence of the indicator [28,29,32–34]. The serial and individual creation and testing of reporter constructs guided by structure aided design can lead to the development of novel scaffolds. We have found that a wide range of design motifs will produce a construct that will exhibit voltage-dependent changes in fluorescence. However, the creation of probes with specific properties (i.e. positive voltage/fluorescence output slope relationship) or significant improvements in performance is unlikely to be achieved using time consuming and labor intensive patch clamp microfluorometry. As with the optimization of calcium indicators, GEVI improvements depend on our ability to implement high throughput, directed evolutionary approaches *in vitro*. Therefore the development of platforms that allow testing of a large number of mutants has led to the greatest progress towards development of optimal indicators [33,35].

Recently, several groups [9,10,30,33] have reported high throughput screening platforms for GEVI development. Firstly, the creation of highly diverse GEVI libraries relies on sequential rounds of site-directed and/or random mutagenesis made to a functional starting template. The routine nature of site-directed and random mutagenesis makes creation of these libraries relatively straightforward [10,33]. The more challenging step is automated, high fidelity, reproducible testing of mutants for voltage-linked changes in fluorescence output. Such screening needs to be performed in a cell that has plasma membrane characteristics and electrical properties similar to that of a neuron (i.e. the cell has a stable resting potential and preferably active properties that resemble action potentials). Ideally, mutagenic GEVI libraries should be screened directly in neurons which is technically challenging [33]. For example, transfection of embryonic neurons in vitro with exogenous DNA using popular lipid-based methods is inefficient, toxic, and highly variable. The use of viral particles for neuronal cell transfection is less cytotoxic and provides more uniform expression patterns [35]; however, producing viral particles for thousands of different mutants is costly, slow and labor intensive reducing throughput. An alternative is to use an immortalized cell line (i.e. HEK293, HeLa) that has been rendered excitable via the stable expression of sodium and/or potassium channels [36]. However, even this is challenging as it is difficult to achieve uniform and reliable electrical properties and consistent expression levels of GEVIs in transduced cells. Inconsistent electrical properties of these cells mandate individual mutants to be tested multiple times while directly comparing hundreds of cells in large multiple well screening platforms in order to produce reliable data [36]. However, once the process has been systemized, it is possible to achieve impressive results. The potential use of genetically encoded activators [8] to achieve more uniform cell stimulation (as opposed to electrical field stimulation) has been demonstrated [9,10], but in most cases is still limited by insufficient spectral separation between actuator and reporter, and unreliable expression levels across many separate transfections [37]. Finally, existing screening platforms do not yet allow for comprehensive screening of all relevant GEVI properties (membrane localization, brightness, photostability, voltage sensitivity and kinetics) but the ability to efficiently select for improved variants in short period of time is invaluable [9,10,30,33] and holds the greatest potential for future GEVI optimization.

GEVI use for routine physiological studies: almost there?

Recent advances in probe performance have resulted in use of GEVIs *in situ* in physiological studies in several model organisms: fruit flies [38], worms [39], zebrafish [40] and mice [27]. Voltage imaging studies are usually performed either by focusing on the activity of single cells, or averaged activity of cell populations. Assuming optimal brightness, photostability and low cytotoxicity of the indicator, the ability of GEVIs to detect various signals (subthreshold and suprathreshold events) will depend on size, speed and linearity of voltage-dependent fluorescence intensity changes. Generally speaking, action potential detection and propagation mandates indicators with fast (submillisecond) kinetics in order to monitor activity state of simultaneously active cells within populations. In addition, practical use of GEVIs is highly dependent on the ability to target expression of the indicators and on their compatibility with available imaging modalities (single or two photon microscopy methods). Temporal resolution of single photon (wide-field) microscopy is optimal for

detection of electrical transients [41]. However, light scattering caused by brain tissue mandates the use of two photon excitation in order to achieve cellular resolution in deeper layers [42,43]. On the other hand, current frame rates available in 2-photon microscopy over large imaging areas are inadequate for recording fast voltage signals. Recent GEVI-based studies in fruit flies and mice provide insight into how these different factors play into our ability to use GEVIs to study relevant neurological processes (Table 1, Figure 2 and 3).

Of mice and flies: case studies for GEVI application

Short life cycle, genetic tractability and binary targeting system (i.e. Gal4/UAS) that allow for highly controlled expression of GEVIs in various cell types made *Drosophila melanogaster* a model of choice for functional testing of the current generation of indicators (Figure 2). The small size of the fly brain and restricted GEVI expression allows cellular resolution optical recordings with wide-field microscopy. Single cell and subcellular recordings of spontaneous spiking activity and subthreshold potentials were first demonstrated in clock neurons (LNV) in brain explants of transgenic flies expressing ArcLight [38] (Figure 2). ArcLight flies have been also successfully used for recordings of cell populations *in vitro* and *in vivo* for studying sleep [44–46], olfaction [38,47] and thermosensitivity [48].

In spite of constant progress, methods for production of transgenic mice expressing GEVIs in targeted brain areas are time consuming, expensive, and can have unpredictable outcomes [49,50]. In the majority of published studies, cortical expression of GEVIs is achieved either via use of viral vectors (i.e. adeno-associated virus, AAV) or *in utero* electroporation techniques that result in densely labeled tissue. In mice, single photon imaging methods using ArcLight have allowed for reliable detection of population electrical activity [51,52,53] in single trials (Figure 3). Similar results are seen in studies using the VSD-based probe, Butterfly 1.2 [27]. In densely labeled tissue, these GEVIs produce *in vivo* signals that are dominated by the summation of electrical transients arising from the neuropil (dendrites and axons) [27,51,52,54,55] (Figure 3).

The high voltage sensitivity of ArcLight and its broad activity profile allows for reliable, single-trial detection of various electrical transients (action potentials, subthreshold depolarizations). However, slow kinetics of the probe (~10ms) diminishes the fluorescence response for fast transients and lowers the ability to resolve high speed electrical events. More recently, the speed of recordings has been significantly improved with indicators that have faster kinetics (e.g. ASAP- family and Ace-mNeon). For example, the sub-millisecond speed of opsin-based GEVI Ace-mNeon variants allowed for high fidelity detection of evoked electrical transients in a single cell in the fly olfactory system and mouse visual system *in vivo* [31].

Cellular resolution recordings with VSD-based GEVIs (Butterfly 1.2. and ArcLight) in mice have been demonstrated with the use of two photon excitation [53,56]. However, the slow speed of the indicators lowers the signal size so multi-trial averaging or reduction in number of lines scanned (to achieve higher frame rates) was needed to produce adequate signal-to-noise ratio. Two photon microscopy has been used more successfully in flies (Figure 2). The

recordings from L2 neurons in the visual system under two photon excitation were central to demonstrate the performance of two new GEVIs: ASAP2f and ASAP2s (Figure 2) [57,58]. In the latter study, testing of various GEVIs confirmed ArcLight functionality for two photon imaging but also the lack of signal in flies expressing opsin-FP based probes (MacQ-mCitrine and Ace2N-2AA-mNeon). It appears the complex photophysics of known opsins is not compatible with multiphoton excitation. These results highlight the inconsistency between GEVI performance under single and two photon excitation and emphasize the need for GEVI development efforts that are specifically focused on 2-photon imaging optimized probes [59].

Conclusion

Optical methods based on the use of GEVIs offer the promise of less invasive, better targeted, and greater multisite monitoring of neuronal activity compared to traditional electrode- and dye-based methods. Compared to calcium indicators, fluorescence voltage indicators provide signals which are richer in information, more temporally relevant to neuronal signaling and offer a more direct measure of neuronal electrical activity. For the first time, a new generation of GEVIs allows us to record neuronal electrical activity with cellular resolution and with increased fidelity [28,29,31]. However, broad application in physiological studies is still illusive. GEVIs that show large and fast voltage sensitivity, are bright and photostable and that perform under single and two photon excitation have yet to be developed. An ever growing list of novel molecular components (i.e. voltage-sensitive domains, opsins and fluorescent proteins) combined in multiple successful scaffolds with nearly infinite possible mutations make the design space for creating probes enormous. While it is possible to create pioneer or template indicators using traditional molecular biology methods, the identification of radically improved indicators is likely to be achieved via high throughput library creation and screening led by in depth empirical insight.

In addition, discrepancies that can be seen in performance of indicators in different organisms [38] or imaging modalities [58], point out that each new paradigm will require validation of performance and characterization of the fluorescent signal in the cell type and condition under study. The efforts to improve GEVIs need to be accompanied with optimization of application methods (e.g. transgenesis, use of different viral vectors and serotypes) that would increase our ability to control expression patterns of the probe. The last decade in which we witnessed steady increase in the number of new and improved GEVIs that are more practically useful is encouraging for future advances in the field.

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Highlights

- The new, faster generation of GEVIs facilitate more precise neuronal activity recordings.
- GEVI optimization is a “work in progress”.
- GEVIs adoption for use in various model organisms and across imaging modalities is ongoing but faces significant challenges.

Figure 1.

Design of genetically-encoded voltage sensors (GEVIs) that were successfully used for studying electrical brain activity in fruit fly and/or mouse. Left side panels in all are schematic representation of GEVI design. Right side panels in all are examples of electrical and optical traces simultaneously recorded from mammalian neurons *in vitro* expressing respective GEVI. Optical traces in all are corrected for photobleaching. In A., B., and C. GEVIs based on voltage-sensitive domain derived from voltage-phosphatase and fluorescent protein(s). In these GEVIs conformational change in voltage sensitive domain causes change in fluorescence intensity of FP(s), (a) ArcLight, based on a fusion of *Ciona intestinalis* VSD and super ecliptic pHluorin GFP (227D) has been used for recording electrical activity in flies and mice. Example traces modified from [28**]. (b) VSFP-Butterfly 1.2, based on a fusion of *Ciona intestinalis* VSD and FRET pair of fluorescent proteins mCitrine/mKate2 has been used for recording electrical activity in mice. Example traces modified from [27] (c) ASAP1/ASAP2f/ASAP2s, several variants of probe with same general design based on a fusion of *Gallus gallus* VSD and circularly permuted superfolder GFP have been used for recording electrical activity in flies. Example traces modified from [29**] (d) Ace2N-mNeon, based on a fusion of *Acetabularia acetabulum* rhodopsin and GFP mNeon-Green has been used for recording of activity in flies and mice. In this probe voltage dependent change in photophysical state of opsin causes quenching of fluorescence in spectrally compatible FP, Example traces modified from [31**]

Figure 2.

Examples of genetically encoded voltage sensors (GEVIs) successfully used for recording neuronal activity in fruit fly (*Drosophila melanogaster*). Sophisticated genetic toolbox and tractability that allow for targeted expression made fruit fly a model of choice for functional testing of current generation of indicators. **(a)** Expression of GEVI ArcLight in fruit fly allows for recording of spontaneous electrical activity (subthreshold and action potentials) in intact neuronal circuits using single-photon excitation. Synchronous membrane activity was recorded in somata of multiple neurons (C1–3) and one neurite (N1) of wild-type clock neurons (ILNVs). Color coding of ROIs corresponds to the optical traces, The simultaneous whole-cell patch-clamp recording of the cell in the red ROI shown in black. Images were recorded at 500 Hz using 488 nm 50 mW laser with $\sim 5 \text{ W/cm}^2$ light power at the preparation. Scale bar is 10 μm , Taken from [38*], **(b)** Two-photon exciton was used in comparative study of several GEVIs expressed in L2 neurons in *Drosophila* visual system. Flies expressing VSD-based GEVIs (ASAP1, ASAP2s and ArcLight), opsin-based GEVIs (MacQ-mCitrine) and GECI GCaMP6f were tested with visual stimulus alternating between 300-ms dark (black bar) and light (white bar) flashes. Top: mean response across multiple cells (n = 44 cells/3 flies for ASAP1, 111 cells/5 flies for ASAP2s, 65 cells/5 flies for ArcLight, 64 cells/3 flies for MacQ-mCitrine, 23 cells/4 flies for Ace2N-2AA-mNeon, and 232 cells/10 flies for GCaMP6f). For each cell recordings from 100 trials were averaged. Bottom: 5 examples of single-trial responses from single L2 cell (in grey). Colored trace is mean response over 100 trials from the same cell. Cells were imaged at a frame rate of 38.9 Hz. Modified from [58**].

Figure 3.

Examples of genetically encoded voltage sensors (GEVIs) successfully used for recording neuronal activity in mice in vivo. (a) Single- (left) and two-photon (right) microscopy was used for recording of cell populations responses in vivo in mouse olfactory bulb using GEVI ArcLight. In all, responses are evoked with ethyl tiglate. For wide-field imaging preparation was illuminated with $485 \pm 25\text{ nm}$ light from either tungsten halogen lamp or a 150 W Xenon arc lamp. Images were recorded at 125 Hz frame rate. Single and averaged traces recorded with wide-field microscopy are unfiltered. In averaged trace single-trial traces were aligned to the first sniff of odorant. The optical traces using 2-photon microscopy are recorded with 920 nm laser light using either 8 Hz or 100 Hz sampling rate as indicated in the figure. In all, traces were filtered with Gaussian low-pass filter at 2 or 4 Hz. Modified from [53]. (b) Sub-millisecond speed of opsin-FP GEVI Ace2N-4AA-mNeon allows for electrical transients (subthreshold and action potentials) recordings in layer 2/3 visual cortical neurons of awake mice. Example optical traces were recorded from a cortical V1→LM neuron responding to visual drifting gratings. Orientations and motion directions are marked above each trace. The images were recorded at 1 kHz using illumination intensity of 20 mW/mm^2 . Modified from [58**].

Performance of genetically-encoded voltage sensors (GEVIs) used for studying electrical brain activity in fruit fly and/or mouse. Values for size and kinetics of voltage-dependent fluorescence change were recorded in response to 100 mV (−70 to +30 mV) depolarization step in immortalized cell line (either HEK293 or PC12) transiently expressing various GEVIs. Values are as reported in original publications.

Table 1

GEVI	Signal amplitude (% F/F)	Kinetics (ms, % fast component)		Model organism	Microscopy		Reference
		T_{on}	T_{off}		1P	2P	
ArcLight Q239	−35	9, 50%	17, 79%	fruit fly, worm, mouse	+	+	28
Butterfly 1.2	−5 (Citrine) +3 (mKate2)	1, 41%	90, 100%	mouse	+	+	27
Ace2N-mNeon	−18	0.36, 74%	0.42, 64%	fruit fly, mouse	+	−	31
ASAP1	−23	2.9, 74%	2.3, 63%	fruit fly	+	+	29
ASAP2f	−22	2.8, 81%	2.4, 71%	fruit fly	+	+	57
ASAP2s	−38	5.2, 56%	24, 49%	fruit fly	+	+	58