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Toward better genetically encoded sensors of membrane potential

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Abstract

Genetically encoded optical sensors of cell activity are powerful tools that can be targeted to specific cell types. This is especially important in neuroscience because individual brain regions can include a multitude of different cell types. Optical imaging allows for simultaneous recording from numerous neurons or brain regions. Optical signals of membrane potential are useful because membrane potential changes are a direct sign of both synaptic and action potentials. Here we describe recent improvements in the *in vitro* and *in vivo* signal size and kinetics of genetically encoded voltage indicators (GEVIs) and discuss their relationship to alternative sensors of neural activity.

Keywords

genetically encoded voltage indicators; GEVIs; genetically encoded calcium indicators; GECIs; Ehud Isacoff; Thomas Knopfel

Optical measurement of neural activity - realizing Sherrington's dream

Optical measurements of brain activity are attractive because they allow for simultaneously monitoring of activity from many individual neurons or from many different brain regions (population signals). In 1937 Sherrington[1] imagined points of light signaling the activity of nerve cells and their connections. During sleep only a few remote parts of the brain would twinkle. But at awakening, "Swiftly the head-mass becomes an enchanted loom where millions of flashing shuttles weave a dissolving pattern." In the 80 years since this poetic

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description there have been significant advances towards making optical measurements of neural activity a reality. Here we attempt to explain why genetically encoded voltage indicators (GEVIs) (see Glossary) would be useful towards this end and describe recent progress in developing improved sensors with larger and faster signals. We also compare GEVI signals with those from genetically encoded calcium indicators (GECIs).

GEVIs have been used *in vivo* for measurement of spike activity from individual cells and for measurement of population signals. With exceptions, [2] [3, 4] most of the individual neuron recordings have been from invertebrates [5] [6] [7, 8] while population signals have been measured from mammalian preparations (e.g., [9, 10] [11] [12]). This review emphasizes (is restricted to?) recordings from mammalian preparations. For earlier reviews of GEVIs see [13, 14, 15].

Tuning optical reporters of neural activity

The membrane potential changes of neuronal cells can vary dramatically from hyperpolarizations during neuronal inhibition to depolarizations from excitatory synaptic activity to the firing of action potentials. These different kinds of membrane potential change can complicate the interpretation of GEVI optical signals from populations of neurons especially in situations where different cell types have different kinds of activity. Restricting the response of a GEVI to one type of activity (e.g., inhibition or action potentials) would facilitate the analysis of population signals. The voltage sensitivity of mosaic GEVIs (see below), with sigmoidal fluorescence vs voltage relationships, can be ‘tuned’ to respond to specific voltage ranges and thus report specific types of neuronal activity [16, 17]. In addition there are different kinetic requirements for measuring different kinds of neuron activity. Measuring synaptic signals require a high dynamic range indicator but only low (> 10 ms) temporal resolution. In contrast action potentials can be measured with lower dynamic range but fast (< 1 ms) temporal resolution.

Calcium indicators

Beginning in 2003 [18], calcium signals have been used as a surrogate for a direct measure of action potentials on the assumptions that action potentials are the only source of the measured calcium changes and that calcium changes occur during action potentials in all types of neurons. These assumptions are certainly not universally true [19, 20] but nonetheless calcium signals are widely used as indicators of spike activity. This is due to a combination of the large signal-to-noise ratios of genetically encoded calcium indicators (GECIs) and, until recently, the quite small signal-to-noise ratios of GEVIs. Furthermore, calcium changes and the resulting signals are much slower and thus are better suited to the frame rates available in 2-photon imaging. Recent reviews of calcium imaging and GECIs are available elsewhere [21, 22, 23].

Following a brief history of the development of GEVIs we describe the varieties of GEVI structures that have been explored and present results from 11 different probes including one example GEVI from each of the laboratories developing GEVIs (Table 1). Following a discussion of four characteristics of these probes we then present examples of GEVI

function in cultured HEK293 cells, cultured neurons, and both cellular and population *in vivo* measurements. We then present evidence that voltage sensitive intermolecular FP interactions underlie the fluorescence change of some mosaic GEVIs. Lastly, we compare GEVIs with GECIs.

GEVI history

The first human-made GEVI was a mosaic constructed by inserting a fluorescent protein (FP) into a voltage sensitive protein residing in the plasma membrane. The sensor, FlaSh, [24], was a voltage gated potassium channel with GFP inserted following the 6th transmembrane segment. Aside from its importance as a proof-of-principle, FlaSh had the useful feature of a steep, sigmoidal fluorescence vs voltage relationship that could be tuned to select for different ranges of membrane potential. However, FlaSh also had drawbacks; its signal was relatively slow ($\tau \sim 100$ msec), and small ($\Delta F/F < 5\%$). But, most importantly, FlaSh, and the analogue Flare [25], worked in frog oocytes but not at all in mammalian cells. In mammalian cells, Flare's expression, and that of two other first generation GEVIs that were also based on mammalian ion channels, was mainly intracellular [25]. This obstacle was overcome by changing the membrane resident voltage sensor to the voltage sensitive domain of the *Ciona intestinalis* voltage sensitive phosphatase [16]. The first member of this family, VFSP2.1, expressed well in the plasma membrane of mammalian cells and signaled changes in membrane potential. However, the *Ciona* voltage sensitive domain exhibits a gating current that lies far outside of the physiological voltage range [26]. The R217Q mutation shifted the voltage curve to a physiological range [16] but made the sensor slower [17].

Comparison of GEVI structures

A number of recent GEVI developments have focused on mosaic proteins using the voltage sensitive domain of the *Ciona* voltage sensitive phosphatase as well as analogues from other vertebrate species. Three varieties of mosaic GEVIs are illustrated schematically in Figure 1A. The left panel illustrates a single fluorescent protein (FP) mosaic (example, ArcLight; [27]); the middle panel a mosaic with a “butterfly” FRET pair [28] of fluorescent proteins, where the two FPs are on opposite sides of the voltage sensitive domain (example, VSFP-Butterfly 1.2 [28],) and the right panel a mosaic with a circularly permuted fluorescent protein inserted in the external loop between transmembrane segments 3 and 4 (example, ASAP1; [29]).

A second type of GEVI utilizes the voltage sensitivity of microbial rhodopsins (Figure 1B; [30]). Both the absorption and fluorescence of the microbial rhodopsins are voltage sensitive but their fluorescence quantum efficiency is ~ 100 times smaller than GFP and thus GEVIs that are composed of the single microbial rhodopsin chromophore (left panel) require high intensity illumination to achieve an adequate fluorescence signal-to-noise ratio (example, Arch; [31]).

By combining the microbial rhodopsin in a mosaic with a second, bright, fluorescent protein (Figure 1B, right panel) a voltage sensitive FRET quenching signal is obtained (example

Ace2N-4AA)-mNeon [4]). A third type of GEVI is a two component sensor that utilizes FRET quenching between a membrane bound fluorescent protein and a charged membrane resident molecule as the voltage sensor (Figure 1C; example, hVOS; [32]). The voltage sensor, dipicrylamine, in hVOS has to be externally applied by the experimenter.

Signal characteristics of 11 different GEVIs

Table 1 lists three important characteristics of 11 GEVIs from 11 different laboratories. The Table includes representatives of all six structural types illustrated in Figure 1. Because the fractional fluorescence change, F/F (first column), was measured with relatively long (~100 msec) voltage steps, these values are most informative concerning the ability of the GEVI to detect relatively slow changes in membrane potentials. (We report values of the fractional fluorescence change, F/F ; the fractional ratio change, R/R , is only applicable to sensors with two-component FRET signals).

The ability to detect fast action potentials will depend on both the F/F and the speed of the response (second column). Clearly, some sensors (e.g. Ace2N-4AA-mNeon) are fast enough to accurately follow the time course of mammalian action potentials while slower sensors (e.g. ArcLight) will have a smaller signal size for action potentials than might be expected from its F/F value.

In addition, several other factors affect the signal-to-noise ratio of the measurements. One is the brightness of the fluorophore. In the GEVIs under consideration the brightness is largely determined by the fluorescence quantum yield (third column). The single chromophore microbial rhodopsin GEVIs have quantum yields ~100 fold smaller than other GEVIs. In principle, the reduced signal-to-noise ratio resulting from the lower quantum yield can be compensated for by a 100 fold increase in illumination intensity. However, the higher illumination intensity will result in an increased intrinsic fluorescence and heating in *in vivo* measurements in mammalian brains. Thus far, these single chromophore microbial rhodopsin GEVIs have not been used to measure activity in mammalian preparations

Lastly, membrane expression, a poorly quantified parameter, makes an important contribution to both the F/F and the signal-to-noise ratio. All of the images of GEVI expression that we are aware of have substantial intracellular expression. The first generation GEVIs expressed well in frog oocytes but not in mammalian cells [25]. Mermaid expressed well in HEK293 cells but not in neurons [33, 34]. The linker length in the Ace2N-mNeon series had to be adjusted to optimize expression in culture, in slice, and *in vivo* [4]. The factors controlling GEVI membrane expression in different cell types remain poorly understood.

The fourth and fifth columns of Table 1 indicate whether action potential signals have been measured in cultured neurons and whether activity signals have been measured *in vivo*.

FRET-based GEVIs that are ratiometric (i.e. with two similarly bright FPs) have the advantage that the ratio removes common mode noise due to movement, e.g., respiration and blood flow. However, the fluorescent intensities of the donor and acceptor FPs are often different, which means the shot noise of the ratio will be higher than that of either individual

signal [35, 36]. FRET quenching microbial rhodopsin signals are not ratiometric because there is only a single optical signal (from the fluorescent donor).

Examples of GEVI signals in cultured HEK293 cells, in cultured neurons, and *in vivo*

GEVI signals in HEK293 cells: example: ArcLight, a single FP mosaic protein [27]

Serendipity resulted in ArcLight, a mosaic of the *Ciona* voltage sensitive domain and the GFP derivative, super ecliptic pHluorin [37] A227D. Figure 2A shows the location of A227. The black trace in Figure 2B shows the response to a 100 mV depolarization in a HEK293 cell using the native super ecliptic pHluorin. The red trace is the response with the spontaneously occurring A227D mutation. This mutation increased the signal size by a factor of 15. The signal size was increased by another factor of 2 by shortening the linker between S4 and the fluorescent protein resulting in a F/F of ~40% per 100 mV (Figure 2C). This F/F was five times larger than the previous largest [33, 38] GEVI signal.

Figure 2D is a plot of the ArcLight fluorescence signal vs membrane potential. The $V_{1/2}$ for the fluorescence change is -20 mV. Thus ArcLight will be maximally sensitive to membrane potentials that occur during action potentials but will still have some sensitivity to changes near the resting potential. Other mutations to the *Ciona* voltage sensitive domain could make the GEVI more sensitive to synaptic potentials or to inhibition ([17]).

ArcLight's kinetics are not fast; the response to a step change in membrane potential is best fit by two components (see Figure 2B,C) with time constants of ~10 and ~50 msec at 33°C. As a result, ArcLight signals during action potentials will not be as large as those found in response to longer voltage clamp steps [27].

GEVI spike signals in cultured neurons: example QuasAr2; a single chromophore microbial rhodopsin GEVI [39]

The kinetics of QuasAr2 are substantially faster than those of ArcLight. The QuasAr2 signal is also best fit by two exponentials with a fast time constant of 0.3 msec and a slow time constant of 3 msec at 34°C. Because of its faster time constants, the QuasAr2 action potential signals are expected to more closely follow the time course of the action potential and the size of the action potential signal is expected to be closer to the size expected from 100 msec voltage clamp steps. These expectations are confirmed by the QuasAr2 signal shown in Figure 3.

Even though the fluorescence quantum efficiency of QuasAr2 and other single chromophore microbial rhodopsins GEVIs is ~100 times smaller than that of ArcLight, the signal-to-noise ratio for the action potential recording in Figure 3 is large. This was achieved by using a high intensity incident light, 200Wcm^{-2} , ~100 times brighter than that used for GFP based GEVIs. The dim fluorescence of single chromophore microbial rhodopsins is likely to limit their use *in vivo* mammalian preparations. QuasAr2, like the other microbial rhodopsin signals, has a linear fluorescence vs membrane potential relationship [31, 39] and thus they cannot be tuned to detect specific kinds of potential changes in a population measurement.

Individual cell GEVI signals in vivo: example Ace2N-4AA-mNeon, a microbial rhodopsin FRET quenching GEVI. [4]

Measuring GEVI spike signals from individual neurons in the *in vivo* mammalian brain has been challenging because light scattering blurs the image of individual cells in a wide-field measurement and 2-photon imaging of appropriate 2-dimensional frames is too slow. By combining a very fast GEVI together with sparse expression, spike signals from individual neurons were measured from cells in the upper 150 μm of mouse cortex (Figure 4). Phototoxicity and photobleaching were minimal allowing for long recording times. [4]

Population GEVI signals in vivo: example ArcLight, a single FP mosaic protein [12]

AAV1 transduction was used to express ArcLight in the mouse olfactory bulb [12]. In the outer layers of the bulb the expression was specific for mitral/tufted neurons. Figure 5A illustrates a single trial, single glomerulus mitral/tufted response to an odorant stimulus that lasted for four inspirations. During each breath the ArcLight signal increased and then returned toward the baseline. This measurement reflects the population average of the optical signals from ~50 mitral/tufted cell dendrites in a glomerulus and is the expected result from action potential recordings from mitral/tufted cells which spike only during the inspiration phase [40]. Repeated trials gave similar results (Figure 5B) indicating that phototoxicity and photobleaching were minimal.

Choosing a GEVI for *in vivo* measurements

Not enough information is available to allow a reliable recommendation. Although Table I includes 11 different GEVIs, only four of the 11 have been tested in *in vivo* mammalian measurements. Because ArcLight has a signal size that is much larger than VSFP-Butterfly 1.2, but has similar kinetics and quantum yield (Table 1), one might expect that ArcLight's *in vivo* signal would be larger than that of VSFP-Butterfly 1.2. And, indeed, ArcLight's signal is larger. ArcLight has a large signal-to-noise ratio in individual trials (Figure 5B) while 10–20 trials have most often been averaged for VSFP-Butterfly 1.2 signals [9, 28, 41]. However, this *in vivo* comparison of ArcLight and VSFP-Butterfly 1.2 was not direct. The transformation method (virus vs electroporation), the cell type, the brain region, and the physiological stimulus were different. It is possible that VSFP-Butterfly 1.2 has better expression and membrane targeting than ArcLight and thus ArcLight and VSFP-Butterfly 1.2 could be equivalent *in vivo*. Clearly, comparisons of different GEVI's in the same experimental preparation are needed.

Mechanisms underlying a single FP mosaic GEVI signal

The development of better GEVIs may be facilitated by determining the mechanism(s) that couple changes in membrane potential to changes in fluorescence. FRET-based constructs are superficially easy to understand: movements of the voltage sensing domain change the distance and/or orientation of the donor and acceptor chromophores resulting in a change in FRET. The fluorescence changes of probes with circularly permuted FPs such as ASAP-1 [29] and Electric-PK [52] are also theoretically straightforward (movement of S4 alters the β -barrel structure). However, an explanation for the optical signal from mosaic GEVIs which

consist of a single FP residing in the cytoplasm is not obvious. How does the movement of the S4 transmembrane domain cause a fluorescent signal from a cytoplasmic FP? The results presented below suggests a surprising hypothesis.

Because super ecliptic pHluorin is pH sensitive, a possible mechanism for the fluorescence change involves pH. Only super ecliptic pHluorin was able to give large optical signals when the A227D mutation was present. The A227D mutant does not improve the optical signal for other FPs such as Clover, eGFP, or YFP [53]. However, large signals from eGFP were obtained by introducing the A227D mutation along with three other mutations found in super ecliptic pHluorin [53]. These mutations converted eGFP into a pH-sensitive FP suggesting that these GEVIs might be measuring pH. However, a single mutation that increased the pH sensitivity did not result in a large signal [53]. Furthermore, Kang and Baker [54] measured a voltage-dependent optical signal and a pH-dependent signal simultaneously using the voltage-sensing domain of a voltage-gated proton channel fused to super ecliptic pHluorin A227D. A 200 mV depolarization resulted in the activation of the voltage-gated proton current which slowly decreased the internal pH of the cell resulting in a slow increase in fluorescence. A 100 mV depolarization did not activate the proton channel and only a voltage-dependent change in fluorescence was observed. Increasing the buffering capacity of the internal patch solution inhibited the pH-dependent fluorescence change but had no effect on the voltage-dependent signal. These observations strengthen the conclusion that ArcLight and related analogues are not sensing membrane potential via a change in pH.

Hints for a different mechanism were first suggested during the development of ArcLight. Surprisingly, A227D resides on the outside of the β -barrel structure suggesting that this mutation does not directly interact with the chromophore. Second, another hint to potential mechanism was given by the fact that the signal could be improved by optimizing the length of the amino acid linker between the cytoplasmic FP and the S4 transmembrane domain ([27, 53]. Both the linker length and the amino acid composition at the fusion site of the FP and the linker affected the optical signal, suggesting that the orientation of the FP was important [51]. These results led to the hypothesis that the super ecliptic pHluorin in ArcLight is interacting with the FP from an adjacent ArcLight protein. The movement of S4 then alters that interaction resulting in the change in fluorescence.

There are three mutations to eGFP that favor the monomeric version of the FP, A206K, L221K and F223R [55]. The GEVI TM [17], an analogue of ArcLight, with 206A-221L-223F mutations which favor dimerization has the largest signal (Figure 5, orange trace). Individual mutations favoring the monomer at the three sites reduced the signal; double mutations cause a further reduction (Figure 5). These data strongly support the hypothesis that movement of the S4 domain alters the dimerization interaction thus resulting in the change in fluorescence.

Concluding Remarks

Developing improved GEVIs is both unpredictable and challenging yet success seems likely. Several results (e.g. Figures 2 and 6, [16]) have shown that changing individual amino acids in either the voltage sensitive domain or in the fluorescent protein of a mosaic GEVI can

have dramatic effects on performance. Similarly, the location of the FP in the voltage sensitive domain also has dramatic effects on GEVI function [29, 58]. Improving GEVIs involves investigations in a large multi-parameter space. Furthermore, it is not often that a planned mutation in a GEVI has the planned result. The situation is further complicated by the fact that many different characteristics of the GEVI are important for its functionality. As a result high-throughput methods for improving GEVIs have thus far concentrated on individual characteristics (e.g. brightness [39]).

At the same time, the opportunities for improving GEVIs are also very large because investigation of the multi-parameter space is only just beginning and, as Figure 1 and Table 1 indicate, many approaches are under active investigation. In addition, Kang et al [59] provide evidence that an unanticipated dimerization mechanism is responsible for some single FP mosaic GEVI signals. This finding is likely to guide further attempts at improvement of mosaic GEVIs.

Future development is needed in two new directions. First, to provide better GEVIs with red signals so that simultaneous measurements could be made from two cell types or simultaneous stimulation and recordings could be carried out in the same brain region. Second, to target GEVIs to specific regions of the neuron so that the optical signal could be specific for nerve terminals, or dendrites, or the soma. This would improve the signal-to-noise ratio in many kinds of measurements.

Thus far GEVI publications have emphasized improvements in sensor characteristics and proof of principle measurements in *in vivo* preparations. We are optimistic that the future will include the use of these improved GEVIs to obtain new insights into brain function.

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Glossary

FP	a fluorescent protein (e.g. Green Fluorescent Protein (GFP))
FRET	fluorescence resonance energy transfer. Transfer of energy from one FP to a second, neighboring, FP
GEVI	genetically encoded voltage indicator
GECI	genetically encoded calcium indicator
S1–S4	numbering of transmembrane helices in the voltage sensitive domain of the <i>Ciona</i> phosphatase
super ecliptic pHluorin	A pH sensitive variation of GFP

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Box 1

Comparison of GEVIs and GECIs as indicators of neural activity

Population signals

Paired recordings of ArcLight and GCaMP3 in the same preparation in response to one and two inspirations of ethyl tiglate are shown in Figure 5C. A similar comparison of ArcLight and GCaMP6f is shown in Figure 5D. The longer odor presentations resulted in breath coupled responses that were very obvious with ArcLight, but relatively small with both GCaMPs. These are population signals; both ArcLight and the GCaMPs are in the glomerular dendrites of ~50 mitral/tufted cells which innervate each glomerulus. Clearly, ArcLight had a smaller F/F and signal-to-noise ratio than the GCaMPs. However, ArcLight had a faster onset, rise time, and decay than the GCaMPs and could track the odor-evoked signal across successive inspirations of an odor [12]. In contrast, the GCaMP measurements were saturating integrals of the odor-evoked neural activity. GEVI signals (VSFP-Butterfly 1.2) and GECI signals (GCaMP3) in visual cortex were compared in response to visual stimuli [41]. In some circumstances (brief stimuli) the GECI had a much larger signal-to-noise ratio while for steady state stimuli the GECI and GEVI signal-to-noise ratios were similar.

Cellular signals

Figure 4 shows that the fast GEVI, Ace2N-4AA-mNeon, can track individual spikes with excellent timing resolution and can do so at spike rates much higher than possible using GECIs.

Because action potentials sometimes cause large influxes of calcium mediated by voltage-gated calcium channels [42] [43], calcium dyes and GECIs are commonly used as surrogates for voltage dyes and GEVIs in detecting action potentials in individual mammalian neurons. The calcium sensors have the important advantage of a large signal-to-noise ratio. However, voltage-gated calcium channels are not the only source of calcium entry in a cell, and thus calcium signals do not always reflect spike activity. For example, subthreshold depolarizations elicited clear calcium transients in mitral cells [19]. Calcium influx can also occur through ligand-gated receptors [44] [23] and can be further modulated by release from internal stores of calcium [45, 46, 47]. The fact that voltage-gated calcium channels only open at a certain voltage creates a threshold effect. In addition, calcium extrusion rates and buffering rates differ from cell to cell. Also many GECIs have further non-linearities through a Hill coefficient that is as great as 3 [22]. Finally, action potentials generate little or no calcium influx in some cell types [20].

Calcium dynamics are also slow in contrast with voltage changes which blurs the relationship between the optical signal and spike rates except at very low rates [43, 48, 49]. Optical measurements from GECIs can be further confounded by the kinetics of the protein sensor [12, 50]. GECIs are also likely to miss hyperpolarizations in neurons with low rates of spontaneous spiking, whereas some GEVIs can be tuned to respond differentially to hyperpolarization or depolarization [17, 51].

ArcLight's ability to report odor-evoked activity in single trials and Ace2N-4AA-mNeon ability to follow individual spikes in individual cortical neurons demonstrates that GEVIs can be a useful tool for *in vivo* measurements. That said, GEVIs and GECIs are complementary tools, where the appropriate use depends on whether the improved temporal resolution and membrane potential sensitivity of GEVIs is more important than the improved signal-to-noise ratio and calcium sensitivity of the GCaMPs.

Box 2

Reporting important characteristics for GEVIs

A substantial list of characteristics determine the utility of a GEVI for monitoring brain activity. We discuss how well each of these characteristics are described in the literature and make suggestions about improving the description. Signal size ($\Delta F/F$ per 100 mV), kinetics (ms), and $V_{1/2}$ of fluorescence vs voltage (for GEVIs with sigmoidal responses) are routinely reported. The characteristics below are often reported but usually only qualitatively.

Brightness

The brightness of an FP is the product of two factors, the extinction coefficient (ϵ) and the quantum yield (ϕ). Quantum yield is simply the fraction of absorbed photons that lead to emitted fluorescence, while the extinction coefficient describes how strongly the chromophore absorbs light. For example, the fluorescent protein Clover has an extinction coefficient of $111,000 \text{ M}^{-1} \text{ cm}^{-1}$ and quantum yield of 0.76 [56] making it one of the brightest fluorescent proteins. Brightness could be routinely reported.

Expression level

A measurement of the average total fluorescence of a cell is an indication of expression level. For comparison purposes, this measurement should be corrected for brightness. Because the measurement of fluorescence will depend on characteristics of the measuring apparatus, an absolute value of expression level will be difficult to determine. However, it would be easy to compare the expression level of a new GEVI with that of an already established GEVI.

Plasma membrane localization

All of the published images of GEVI expression have substantial intracellular expression. This intracellular expression will reduce the $\Delta F/F$ and the signal-to-noise ratio. Two different methods have been used to measure plasma membrane localization. First, Figure 3 of Baker et al [25] illustrates a measurement of plasma membrane expression using confocal microscopy. The GEVI fluorescence is compared to an externally added dye which binds to but does not cross the plasma membrane. Second, Bedbrook et al [57] used an extracellular tag in a SpyTag/SpyCatcher system to measure the plasma membrane localization of channelrhodopsin. This scheme might also work for GEVIs.

Signal-to-noise ratio

This critical GEVI characteristic is not easily absolutely quantified because of its dependence on the preparation and the measurement apparatus. But a comparison with a previously established GEVI would be relatively easy and very useful.

Steepness of fluorescence vs voltage

(for GEVIs with sigmoidal responses). This characteristic will determine how well a GEVI could distinguish between action potentials, excitatory synaptic potentials, and inhibitory synaptic potentials in a population signal. Fluorescence vs voltage curves are

typically fit with the Boltzmann equation, $Y = (A_1 - A_2) / (1 + e^{(V - V_{1/2})/dx}) + A_2$, where A_1 is the minimum value, and A_2 is the maximum value and dx is the slope (steepness) of the Boltzmann fit. The slope should be routinely reported.

***In vivo* characterization**

In vivo measurement of these characteristics is clearly important. First, because the *in vivo* result may be different from the *in vitro* result and second, because *in vivo* characterization will inform scientists who are deciding which, if any, GEVI to use. *In vivo* measurement of some characteristics will be difficult because they require simultaneous fluorescence and patch clamp measurements. But comparisons of different GEVIs in the same preparation would be very useful.

Trends Box

- Recently developed genetically encoded voltage sensors (GEVIs) have larger and faster fluorescence signals.
- The new GEVIs result in improved signal-to-noise ratios in *in vivo* measurements in the mammalian brain.
- In some instances GEVI signals are substantially faster and more informative than measurements with genetically encoded calcium sensors (GECIs).

Outstanding Questions

- Some GEVIs in Table 1 have relatively large signals while others have relatively fast signals. A GEVI with a signal that was both large and fast would increase the quality and the kinds of measurement that were possible.
- What is the mechanism responsible for the GEVI fluorescence change? The results presented in Figure 6 suggest that voltage affects the equilibrium between monomers and dimers of that GEVI. Will this conclusion be true of other mosaic GEVIs? Will this information lead to the development of better GEVIs?
- Can red shifted GEVIs with large signals be developed? Red sensors would allow simultaneous recordings from two cells types. Red sensors also have the advantage of reduced scattering of red wavelengths by the brain.
- Can GEVIs be targeted to different sub-cellular locations? Specific targeting would improve the signal-to-noise ratio and avoid some types of ambiguity in interpreting population measurements.

Schematic structures of three types of GEVIs

(A) Type 1:

voltage sensitive phosphatase based mosaic sensors

single FP

butterfly FRET pair

circularly permuted

(B) Type 2:

microbial rhodopsin based sensors

single chromophore

FRET quenching

(C) Type 3:

dual component sensors

FRET quenching

Figure 1.

Schematic structures of three types of GEVIs. **(A)**. Mosaic sensors combining the voltage sensitive domain of a voltage sensitive phosphatase and a fluorescent protein. **(B)**. Sensors based on the voltage sensitivity of a microbial rhodopsin. **(C)**. Sensors that are a combination of two separate molecules. Figure 1B was modified from [62]). Figure 1C was modified from [32]

Characterization of the GEVI, ArcLight

(A) super ecliptic pHluorin

(B) modifying position 227

(C) effect of linker length

(D) fluorescence vs potential

Figure 2.

Characterization of ArcLight, a single FP mosaic sensor. **(A)**. The location of the outward facing A227 amino acid. **(B)**. The A227D mutation increased the signal size by a factor of 15. **(C)**. A further increase in signal was found if the linker between the S4 segment and the FP was shortened. **(D)**. The fluorescence change vs membrane potential was sigmoidal with a $V_{1/2}$ of -20 mV. Modified from [27].

Action potential recording in a hippocampal neuron using QuasAR2.

Figure 3.

A simultaneous optical and electrode measurement of an action potential in a cultured hippocampal neuron using QuasAR2, a single chromophore microbial rhodopsin GEVI. Modified from [62].

Figure 4.

In vivo individual neuron spikes measured in a layer 2/3 visual cortical neuron of an awake mouse using Ace2N-4AA-mNeon, a microbial rhodopsin FRET quenching GEVI. The spike signals are riding on top of longer lasting depolarizations. Modified from [4].

In vivo population measurements; comparing GEVIs and GECIs
(A) single trial odorant response (B) seven repeated trials

Figure 5.

(A,B) *In vivo* population measurements using ArcLight, a single FP mosaic GEVI. The mitral/tufted cells were transduced by infection with an AAV1 virus containing ArcLight DNA. (A). A single trial, single glomerulus measurement of the response to the odorant ethyl tiglate presented during four breaths. (B). The overlay of the responses to six repeated trials. There was little change from trial to trial. (C). Comparing ArcLight and GCaMP3 signals from opposite olfactory bulbs in the same preparation in response to odorant presentations lasting one and two breaths. (D). Comparing ArcLight and GCaMP6f. Modified from [12].

Figure 6. The effect of monomeric mutations at the dimerization site of a single FP mosaic GEVI. **A.** The GEVI TM with 206A-221L-223F which favor dimerization has the largest signal (orange). Individual mutations favoring the monomer at the three sites reduced the signal; double mutations cause a further reduction. **B.** A schematic model of a TM dimer. Modified from [59].

Table 1

Properties of selected GEVIs

	F/F for a 100 mV depolarization (HEK293 cells)	Fast ON tau (100 mV depolarization (ms)	Approximate quantum yield	Single trial signals in neurons	Single trial <i>in vivo</i> signals	Comments	References
<i>mosaic single FP GEVIs</i>							
ArcLight	40%	10	1	yes	yes		[5, 12, 27]
Bongwoori	20%	8	1	yes	not tested		[17]
ASAP1	17%	2	1	yes	not tested		[29]
ArcLighting	10%	10	1	not tested	not tested	faster OFF	[60]
<i>mosaic butterfly GEVIs</i>							
Nab12.213	11%	3	1	yes	not tested	PC12 cells	[58]
VSP-Butterfly 1.2	4%	2	1	not tested	yes		[28]
Mermaid2	20%	2	1	yes	yes		[10]
<i>microbial-rhodopsin GEVIs</i>						linear F vs V	
Archer1	80%	2	0.01	yes	yes	cultured neurons; <i>C. elegans</i>	[6]
QuasAr2	90%	1	0.01	yes	not tested		[39]
Ace2N-4AA-mNeon	8%	0.4	1	yes	yes	mosaic	[4]
<i>two component GEVIs</i>							
hVOS	30%	0.5	1	yes	not tested	PC12 cells; linear F vs V	[32, 61]