

Probing neuronal activities with genetically encoded optical indicators: from a historical to a forward-looking perspective

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Abstract Optical imaging has a long history in physiology and in neurophysiology in particular. Over the past 15 years or so, new methodologies have emerged that combine genetic engineering with light-based imaging methods. This merger has resulted in a tool box of genetically encoded optical indicators that enable nondestructive and long-lasting monitoring of neuronal activities at the cellular, circuit, and system level. This review describes the historical roots and fundamental concepts underlying these new approaches, evaluates current progress in this field, and concludes with a forward-looking perspective on current work and potential future developments in this field.

Keywords Optical imaging · Genetically encoded indicators · Calcium · Membrane potential

Historical roots of optical imaging in physiology and the emergence of genetically encoded indicators

Molecular probes that convert alterations in biophysical or biochemical parameters into changes in light intensity have been powerful tools in biological research for many decades. In particular, fluorescent probes that report cellular function in living single cells, tissues, and whole organisms have provided a powerful alternative to electrode-based approaches for the measurement of electrical and chemical parameters. Compared to even the finest electrodes, light-based imaging of molecular probes is less invasive, offers higher spatial resolution, and, in

some cases, may even yield better temporal resolution. Moreover, parallelized detection systems such as photodiode arrays and digital cameras allow for acquisition of biological signals as a function of space and time.

The initial concept of using optical methods to measure neuronal activities emerged in the late 1960s from the study of endogenous mechanisms underlying changes in light scattering, birefringence, and fluorescence associated with action potentials [16]. In the 1970s, tissue staining with organic dyes enabled greater modulation of measured light intensity and, thus, superior signal-to-noise ratios (SNR) [79]. This opened up the field of voltage-sensitive dye imaging, which has subsequently enabled neuroscientists to monitor changes in membrane potential from single or large numbers of neurons in a variety of preparations, including mammalian cortical brain tissue [26–28]. Another, more widely pursued way for monitoring neuronal activities has emerged from the development of organic molecules that change their fluorescence in response to variations in the concentration of intracellular calcium [29]. Such calcium indicators can, under many conditions, serve as a substitute for voltage indicators for the detection of membrane voltage depolarization, because sufficiently large voltage signals are typically associated with the opening of voltage-gated calcium channels, which results in a large and transient elevation of the intracellular calcium concentration [43, 45, 61, 78, 80]. Measurements of calcium signals have become a widely used proxy for electrical activity in studies entailing noninvasive imaging of neuronal activity, a field that has coevolved with modern imaging techniques such as confocal and two-photon (2P) microscopy [34]. Other indicators have been developed that report the concentration of different ions including sodium [44, 49], chloride [54], and pH [56], but these probes are far less commonly used as proxies for the detection of neuronal activity.

These low-molecular-weight (LMW) organic voltage and calcium dye indicators have been valuable in addressing

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many physiological questions but are limited by difficulties in labeling selected structures within a given preparation. Aqueous solutions of indicators can be injected into single cells via micropipettes, but it has proven difficult to achieve labeling of organelles (e.g., endoplasmic reticulum, mitochondria) or subcellular compartments (e.g., axons, dendrites, spines of neurons) with biochemical approaches (e.g., using membrane-permeable indicator precursors [85]). Similarly, LMW optical indicators may not be well suited for detailed analysis of brain tissues consisting of many different classes of cells due to the lack of generally applicable methods for labelling defined cell populations [42]. It is interesting to note that similar specificity and selectivity issues have arisen with traditional lesion studies but were overcome by molecular biological techniques that allowed for the introduction of lesions targeted at the molecular and cellular levels. In analogy, genetically encoded indicators of neuronal activity were conceived to overcome some of the fundamental specificity limitations of classical LMW indicators [42]. Among the earliest demonstrations of the potential of such genetically encoded indicators was the use of the bioluminescent calcium-sensitive reporter protein aequorin from the jellyfish *Aequorea victoria* [76]. Quite remarkably, the first reported use of aequorin as an optical calcium sensor protein preceded its molecular cloning [40, 73], when Ridgway and Ashley [75] optically recorded calcium transients following microinjection of aequorin into single muscle fibers of the barnacle. Unfortunately, the light output of such bioluminescent probes is rather limited and requires the application of a consumable cofactor. However, research on *A. victoria* bioluminescence ultimately led to the molecular cloning and functional expression of *A. victoria* green fluorescent protein (GFP) [13, 72]. Subsequent efforts have yielded similar fluorescent proteins derived from corals, which were then modified to obtain brighter, spectrally and optically improved variants [13, 15, 72]. These proteins generated bright fluorescence without the need for added cofactors, features that were critical to their success as components for genetically encoded indicators suitable for monitoring a large variety of biological parameters, including neuronal activity. Such genetically encoded indicators overcome many of the limitations of organic dyes; they can be specifically targeted to subcellular compartments or classes of cells, and “staining” via genetic methods is generally permanent and noninvasive or minimally invasive. This makes it straightforward to conduct repeated imaging sessions extending over periods of days and weeks during the course of normal physiological behavior. The feasibility of chronic imaging opens the window for a large variety of studies of development, plasticity, functional regeneration, and slowly developing diseases associated with abnormal wiring of neuronal circuits.

Early prototypes and proof of principle for genetically encoded indicators of neuronal activity

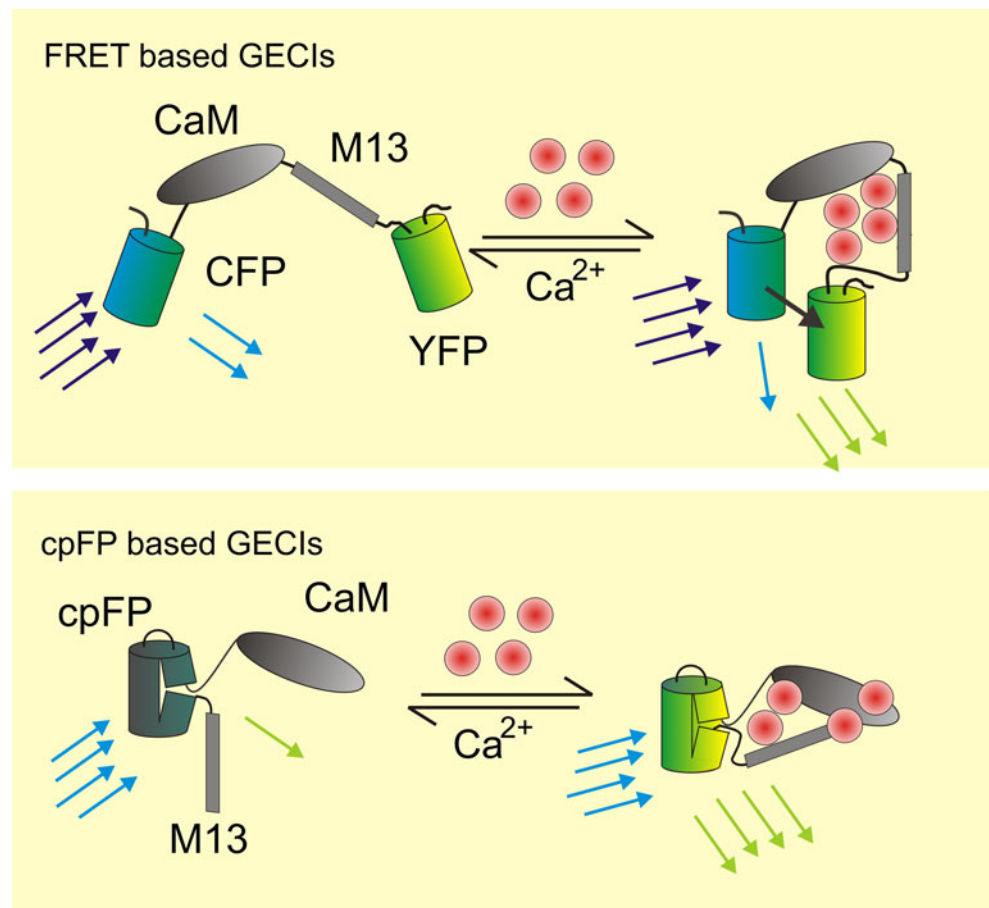
Calcium indicators

The first prototypical fluorescent protein-based indicators for calcium were described in 1997 [59, 77]. The principal design idea followed that of organic fluorescent calcium indicators, wherein a calcium-binding molecule was combined with a bright fluorescent dye in a manner that results in a change in fluorescence output upon binding of calcium (Fig. 1). For the organic dye Fura-2, these components were BAPTA and a stilbene chromophore [29], while the first genetically encoded calcium indicators (GECIs) used the calcium-binding calmodulin-M13 complex and fluorescent proteins as building blocks [59, 77]. Troponin C was introduced a few years later as an alternative calcium-binding protein suitable for the generation of GECIs [33, 52]. The calcium binding-induced conformational state transition is coupled to a conformation-sensitive fluorescent protein-based reporter component. In the first GECIs, reporting was achieved via modulation of Förster resonance energy transfer (FRET) efficiency between a fluorophore pair arising from calcium binding-induced structural changes [59, 77]. Only a few years later, circularly permuted fluorescent proteins (cpFPs) were established as an alternative reporting mechanism [6], leading to the now widely used GCaMP family of GECIs [64, 84] (Fig. 1). Under baseline conditions, cpFPs are highly susceptible to fluorophore quenching as a consequence of small conformational changes around their interface with an attached calcium-binding protein. In the most widely used GCaMP-type configuration, elevated calcium concentrations increases the quantum yield of fluorescence, observed as a large increase in fluorescence [64, 84]. Current efforts in GECI development have focused on tuning for specific applications and optical properties, such as calcium binding constant [37], subcellular targeting [22, 39], and color [90].

Voltage indicators

The first prototypical genetically encoded voltage indicator (GEVI) was reported in 1997 [83], the same year as the first GECIs, although getting this approach to work in mammalian cells took another 10 years. Unsurprisingly, the earliest GEVI design was based on the model of previous, non-genetic approaches. These strategies involved the use of non-protein fluorescent dye labels to track conformational changes in ion channels, an approach that has been termed voltage-clamp fluorometry [12, 53]. The first reported fluorescent protein-based voltage reporter, FlaSh, was a nonconducting mutant of the *Drosophila* Shaker voltage-gated potassium channel with a fluorescent protein inserted into

Fig. 1 Designs for genetically encoded calcium indicators. *Top panel* Förster resonance energy transfer (FRET)-based calcium indicators often use cyan (CFP) and yellow (YFP) fluorescent proteins as donor and acceptors. Excitation of CFP with 440-nm light (blue arrows) results in cyan (cyan arrows) and yellow FRET-based fluorescence (yellow arrows). Binding of Ca^{2+} (red circles) to a calcium-binding domain (illustrated is the calmodulin CaM-M13 complex) increases the efficacy of FRET between cyan and yellow fluorescent proteins and thus increases the fluorescence ratio (R) between YFP and CFP. *Bottom panel* Single fluorescent protein indicators of the GCaMP type incorporate a circularly-permuted fluorescent protein (cpFP). Binding of Ca^{2+} to CaM-M13 increases the quantum yield of cpFP fluorescence



the C-terminal region [83] (Fig. 2). FlaSh fluorescence tracks the slow voltage-dependent inactivation of the channel [83]. Unfortunately, FlaSh-type voltage indicators were found to be nonresponsive [8] or to exhibit very modest fidelity [1] when used in neurons. The likely reason for this poor performance is a limited membrane trafficking of these indicators in neurons, which, in turn, causes excessive optical noise and low effective sensitivity [42]. In an attempt to overcome this issue, a consortium of researchers led by Lawrence Cohen [9] sought to improve visible 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 potassium channel [41]. The basic idea behind this approach is that untargeted FlaSh subunits will not fluoresce as they fail to properly assemble at the membrane. The group screened 56 combinations of Kv subunits containing either half of the fluorescent protein, of which 30 were expressed at the plasma membrane and capable of producing an optical signal when the membrane potential was changed by a voltage-clamp command pulse. The largest signal from these FlaSh-derived sensors was $-1.4\% \Delta F/F$ for a 100-mV depolarization, with on- and off-time constants of ~ 15 and ~ 200 ms, respectively. 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 mobile charges and therefore are more problematic with respect to increased membrane capacitance than probes based on single voltage-sensor domains [2]. A conceptually closely related prototype, sodium channel protein-based activity reporting construct (SPARC), is based on the insertion of GFP into a skeletal muscle sodium channel [5]. Like, FlaSh-type voltage indicators, SPARC was found to be nonresponsive when expressed in mammalian cells [8].

Another GEVI design envisaged at the same time is based on an isolated voltage-sensing domain, with fluorescent proteins providing a direct readout of voltage changes [82]. This class of GEVIs has been given the acronym VSFP (voltage-sensitive fluorescent protein, Fig. 2). The voltage-sensing domain for the first series of VSFPs (VSFP1) [82] was derived from a potassium channel (Kv2.1), but, like FlaSh and SPARC, this engineered protein was not efficiently targeted to the plasma membrane, resulting in excessive background fluorescence and consequently a poor SNR [8]. Thus, all three of the earliest prototypical GEVIs (VSFP1, FlaSh, and SPARC) essentially failed to perform adequately in biological applications [8, 69].

The frustrations associated with the development of first-generation GEVIs were alleviated by the identification of a promising new basis for a voltage-sensor scaffold, based on

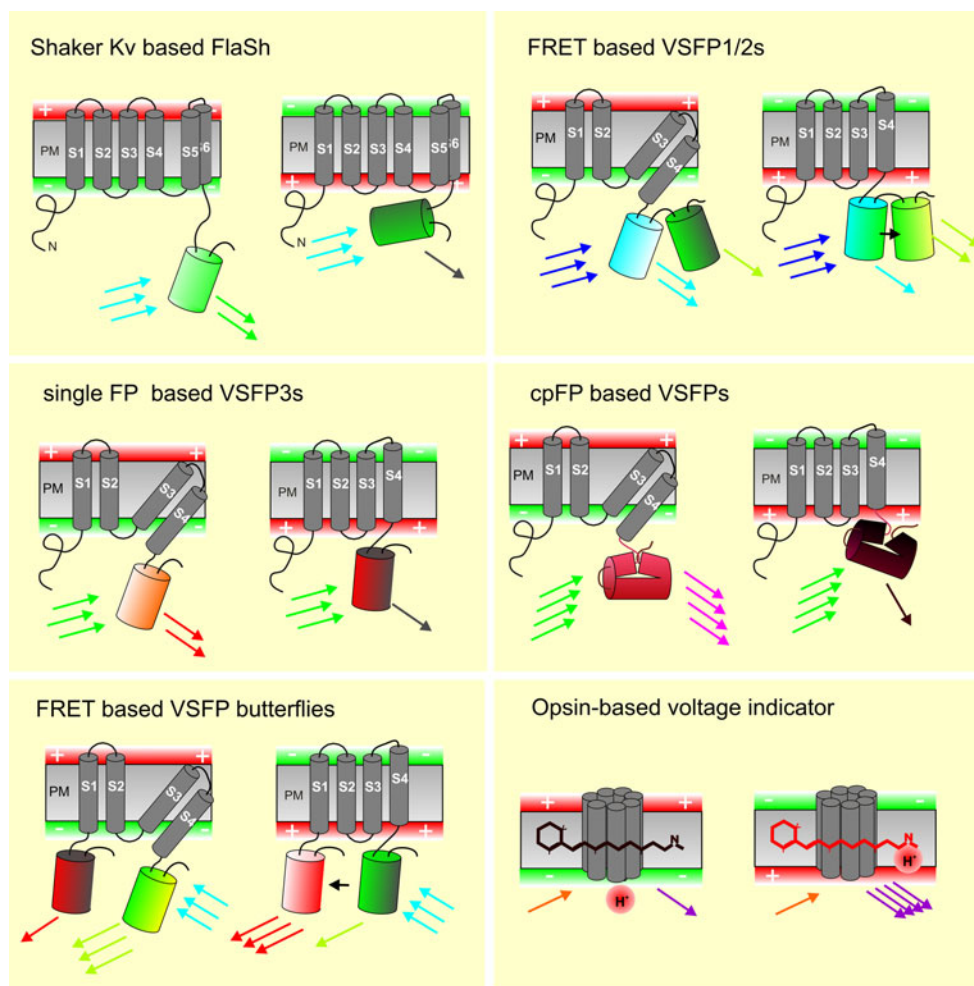


Fig. 2 Designs for genetically encoded voltage indicators. *Top left panel* FlaSh-type voltage indicator. A fluorescent protein is fused into the C-terminal portion of a Shaker potassium channel subunit. Tetramers of subunits form a channel structure which is made non-conducting by a point mutation. Modulation of FlaSh fluorescence is triggered by voltage-dependent rearrangements, probably corresponding to channel C-type inactivation. *Top right panel* FRET-based voltage-sensitive probes of the VSFP1/2 type. 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. *Middle left panel* Single fluorescent protein probes of the VSFP3 family. *Middle right panel* VSFPs incorporating a circularly permuted fluorescent protein. *Bottom left panel* FRET-based voltage-sensitive probe of the VSFP-Butterfly family, where the voltage-sensor domain is sandwiched between two fluorescent proteins. *Bottom right panel* Microbial rhodopsin-based voltage indicator Arch. A change in membrane potential induces increased fluorescence of the retinal molecule

the sea squirt *Ciona intestinalis* voltage sensor-containing phosphatase (Ci-VSP) [60]. The advantage of this voltage-sensing protein is that it provides a monomeric, self-contained voltage-sensing domain, in contrast to potassium channel subunits that evolved to form tetramers, and VSFPs based on the Ci-VSP voltage-sensing domain were efficiently targeted to the plasma membrane of mammalian cells, including neurons [19, 32].

During the past several years, a number of Ci-VSP-based VSFP designs have been developed [68]. The first involved a tandem FRET pair of fluorescent proteins fused to the end of the S4 transmembrane segment of Ci-VSP. Since this

design resembles the potassium channel-based VSFP1 indicator, this family of GEVIs was named the VSFP2s [19, 32]. The improved variant VSFP2.3 [51, 63] was the first FRET-based GEVI to enable optical imaging of spontaneous action and synaptic potentials in neurons [4].

The members of the VSFP2 family differ with regard to the fluorescent proteins used. While the best-tuned versions for each color variant differ only modestly in sensitivity and kinetic parameters when compared in cultured PC12 cells [63], the species of fluorescent proteins used has a dramatic impact on membrane-targeting efficiency and effective signal amplitude when used in mammalian neurons [62, 68].

Other classes of VSFPs have been developed that use a single fluorescent protein instead of a fluorescent protein pair. This family of VSFP3 [70] voltage indicators contains members that span the fluorescent protein color spectrum, from cyan to far red. VSFP3 variants have also been designed to incorporate cpFPs [24], but these have yet to match the success achieved with cpFP-based calcium indicator proteins [6, 64, 84]. VSFP3 variants based on ci-VSP homologues from other species (e.g., zebrafish) have been explored as well, but these did not lead to improved indicators [9].

In the latest VSFP design, the voltage-sensor domain is sandwiched between two fluorescent proteins. We termed this family VSFP Butterflies, and these currently represent the best-performing probes for monitoring subthreshold membrane oscillations in vivo [3].

The newest concept for GEVI design is based on the use of microbial opsins [47, 48] (Fig. 2). These proteins bind retinal, a vitamin A-related organic chromophore, and have evolved naturally to 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 [48]. 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 [14], produces a fluorescent signal that correlates with changes in membrane voltage [47]. Their findings were a cause for considerable excitement, as 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 down the optical signal in response to membrane-potential changes [47]. While additional protein engineering might solve this issue, the most serious limitation of the opsin-based class of voltage probes is the very low fluorescence quantum efficiency of retinal (0.001) [22]. This is probably why Arch-related fluorescence remained virtually undetectable when EGFP-tagged Arch was expressed in the cortex of live mice (Mutoh et al. unpublished), while fluorescence of the EGFP tag and of VSFP2.3 was readily detected in control mice 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 also carries the potential for interfering with the physiological role of the retinal. For example, a synthetic retinal substitute could theoretically leave animals blind by disrupting the function of rhodopsins required for light transduction in the retina. Similarly, this substitution could hinder the performance of other optogenetic control tools based on opsins, undermining the exciting possibility of combining optogenetic monitoring and control in the same model system. A timeline of development of GEVIs along with some of their key specifications is given in Table 1.

Reporters of synaptic release

As an alternative to measuring the electrical activity of cells directly with GEVI or indirectly with GECIs, interactions between neurons may also be monitored by indicators of synaptic activity. Again, such strategies are rooted in approaches developed with organic dyes—in this case, using styryl dye FM1-43 to label synaptic vesicles and then monitoring the depletion of labeled vesicles during synaptic activity [81]. The genetically encoded conceptual descendants of this method are the synaptolucins and synaptopHluorin [57, 58], optical indicators of vesicle release and recycling. SynaptopHluorin consists of a pH-sensitive form of GFP fused to the luminal side of a vesicle-associated membrane protein (VAMP). When exposed to the acidic pH within neurotransmitter vesicles, synaptopHluorin is nonfluorescent. Following vesicular release, synaptopHluorin is exposed to the neutral extracellular space and produces increased fluorescence output. Following endocytosis, vesicles become re-acidified, and the cycle can start again. Notably, synaptopHluorin was the first genetically encoded indicator of neuronal activity to be successfully used in living animals for the imaging of neuronal activities induced by a natural stimulus [11]. Part of this early success resulted from the wise choice of a biological system (olfactory nerve synapses) where macroscopic imaging and genetic targeting of specific cell classes had already been established [10, 87]. Very recently, syPHTomato, a reporter of activity-dependent exocytosis with fluorescence emission in the red spectral band, was presented [50]. When expressed with the GFP-based indicator GCaMP3 in the same neuron, syPHTomato enabled concomitant imaging of neurotransmitter release and presynaptic Ca^{2+} transients at single nerve terminals. This study also provided a nice proof of principle for an all-optogenetic approach to electrophysiology [50, 62] by coupling expression of syPHTomato and GFP-based probes with distinct variants of channel rhodopsin.

Table 1 Genetically encoded voltage indicator development time line and basic specifications

Year	Name	Source of voltage-sensing domain	Expression system for functional characterization	Fluorescent proteins(s)/peak emission wavelength(s) (nm)	Sensitivity (at xx mV) ^a	Response time constant on ^b (ms)	Response time constant off ^b (ms)	Reference
1997	FlaSh	Shaker potassium channel	Xenopus Oocyte	GFP/505	$\Delta F/F$: 5.1 %/100 mV	23	105	[83]
2001	VSP1	Kv2.1	HEK cells	CFP/477, YFP/529	$\Delta F/F$: 1.8 %/100 mV	<1	<1	[82]
2002	SPARC	Voltage-gated sodium channel	Xenopus oocyte	GFP/505	$\Delta F/F$: 0.5 %/100 mV	~1	~1	[5]
2007	VSP2.1	ci-VSP	PC12 cells, neurons	Cerulean/477, citrine/529	$\Delta R/R$: 6.8 %/100 mV, at -70 mV	15	75	[19]
2008	VSP3.1	ci-VSP	PC12 cells	Cerulean/475	$\Delta F/F$: 2.2 % (at -43 mV)	1.8	105	[51, 70]
2008	VSP2.3	ci-VSP	PC12 cells, cultured neurons, mouse in vivo	Cerulean/477, citrine/529	$\Delta R/R$: 22 %/100 mV, at -50 mV	30, ~2	~80	[51, 63]
2008	VSP2-Mermaid	ci-VSP	Xenopus oocyte, NT cells, primary cultures of brain cells	mUKG/490, mKOw/560	$\Delta R/R$: 21 %/100 mV, at -43 mV	30, ~2	~80	[63, 86]
2009	VSP2.4		PC12 cells	Citrine/529, mKate2/633	$\Delta R/R$: 20.5 %/100 mV, at -54 mV	30, ~2	~80	[63]
2009	VSP3-x	ci-VSP	Cultured neurons	Citrine/529, mOrange/562, TagRFP/584, mKate2/633	$\Delta F/F$: 2.5–5 %/100 mV	2–100	~100	[70]
2010	VSP2.42		Neurons in culture, brain slices and in vivo	Citrine /529, mKate2/633	$\Delta R/R$: 20.5 % at -50 mV	80, 2	~80	[4]
2011	Split-Venus	Shaker Kv	HEK293 or NIE115 neuroblastoma cells	splitVenus/528	1.4 %/100 mV	~15	~200	[41]
2011	Arch(D95N)	Microbial opsin	Neurons	Retinal/687	~60 %/100 mV	41	41	[47]
2012	Zahra and Zahra 2	Voltage sensitive phosphatases of Nematostella and Danio	HEK293 cells	centulean/477, citrine/530	$\Delta F/F$: 0.2 %/100 mV	~5	~5	[7]
2012	VSP2-Butterfly 1.2	ci-VSP	Neurons in culture, brain slices and in vivo	Citrine/529, mKate2/633	$\Delta R/R$: 22.2 % (at -79 mV)	10, 2	~80	[3]

^aThe sensitivity is given either as change of fluorescence relative to baseline ($\Delta F/F$; monochromatic indicators) or change of fluorescence ratio ($\Delta R/R$; ratiometric indicators) measured in cultured cells. The sensitivity of most GVEIs is voltage-dependent. If data are available, the sensitivity is given at the membrane voltage of half maximal response. Data are preferentially from experiments where different indicators were compared side-by-side

^bMeasured with short lasting and large amplitude depolarizing voltage steps (<10 ms); for kinetics that are best described with two time constants, they are given in order of their weight

Indicators for neurotransmitters

A fourth way to optically detect neuronal activity that has seen limited use but should be mentioned for the sake of completeness is to employ an indicator that reports the presence of a neurotransmitter. This idea motivated the generation of genetically encoded indicators for glutamate, the major excitatory neurotransmitter in the mammalian brain [36, 55, 65]. A study where a glutamate indicator protein was applied in brain slices showed the feasibility of imaging extracellular glutamate concentration and suggested that this approach may provide a unique assay for network activity that complements techniques such as voltage-sensitive dyes and multi-electrode arrays [23]. Although interesting, in particular, if expanded to monitor different neurotransmitter systems, this approach is not being aggressively developed at present.

Genetically encoded versus LMW organic indicators

The development of genetically encoded indicators has been motivated by the quest to monitor biological signals from specific cellular compartments and organelles, to target specific cell classes in tissues composed of heterogeneous cell populations and to facilitate experimental approaches that require long-term imaging over multiple sessions (“chronic experiments”) [42, 52, 84]. Experiments that do not require the targeting capabilities of genetically encoded indicators or imaging over extended time periods can be performed either with LMW organic indicators or genetically encoded probes. For this latter class of experiments, the methodology of choice is driven by considerations of side effects and SNR issues.

The potential side effects of calcium indicators are related to their binding of calcium in order to generate a signal. The consequences of calcium buffering, such as adverse effects on synaptic plasticity, integration of synaptic inputs, and long-term survival of expressing neurons, depend on the indicator concentration and the kinetics and cell type-specific strength of endogenous calcium buffers [30, 35, 67]. This issue was recently addressed for GCaMP3, with the demonstration that expression of this GECI in CA1 pyramidal neurons does not cause detectable deficits in hippocampal long-term potentiation at concentrations typically used *in vivo* to image neuronal activities using 2P excitation [38]. Other forms of synaptic plasticity were not tested, but, from experiments using LMW calcium indicators, one would expect that the concentrations of GECIs generally achieved with viral transfection protocols (<50 μM) are within the physiologically tolerable range for most cell types and many forms of plasticity [38]. There were concerns that calmodulin (incorporated into various GECIs, including the cameleons and GCaMPs) might

interact with natural calmodulin-dependent signaling pathways [33, 66]. However, there is no direct proof that these potential interactions have physiological consequences in neurons. GCaMPs are suspected to promote breakdown of the nuclear barrier against diffusion of proteins [84] and cause cardiomegaly when expressed in cardiomyocytes [46], but it is not clear whether these potential side effects are GCaMP-specific, caused by recombinant calmodulin, or a more general feature of GECIs.

Expression of GEVIs with sensing mechanisms that involve the movement of charges can add electrical capacitance to membranes [2]. The importance of this effect depends on the specific class of GEVIs and their expression level. However, experimental data and detailed computer simulations [2] have revealed that the adverse effects of increased capacitance can essentially be avoided by proper indicator design and minimizing the number of mobile charges. Experimental data obtained with *ci*-VSP-based VSFPs have shown that there are no significant differences between expressing and nonexpressing neurons under experimental conditions in which VSFPs provide a useful readout of neuronal activities [4]. Furthermore, for the GCaMP class of GECIs and the VSFP2 class of GEVIs, it has been demonstrated that indicator-expressing mice survive and, in the case of transgenic expression, propagate with no apparent deficits [4, 18, 89].

When comparing studies performed with LMW calcium indicators or current versions of GECIs under otherwise comparable conditions, LMW calcium indicators appear to offer a larger SNR [89]. This difference can be at least partially explained by the indicator concentration, which is usually lower with GECIs, and differences in K_d and forward rate constants [35, 38, 89]. Optical recordings obtained with voltage indicators usually have a much lower SNR than corresponding measurements with calcium indicators. However, this is the case for both LMW voltage-sensitive dyes and GEVIs. LMW voltage-sensitive dyes and GEVIs generate comparable signals with comparable SNR in mesoscopic recordings of cortical circuit dynamics (Mutoh et al. in preparation).

Choosing the most appropriate genetically encoded indicator

Optical imaging methods may be employed for neuroscience studies at the synaptic, cellular, local circuit and intact system/*in vivo* level and the phenomena being studied can occur at time scales ranging from milliseconds to several days or longer. While optical imaging is well suited to cover these multiple spatial and temporal scales, there is neither a specific indicator nor a configuration of optical instrumentation that serves as an all-purpose solution. Accordingly, a given class of genetically encoded indicators will be appropriate for some but not all experimental approaches.

At the level of synaptic terminals, indicators of vesicle recycling are powerful tools to study the release probability of transmitters. Due to its red-shifted spectrum, the recently derived synaptopHluorin-like indicator sypHTomato [50] can be combined with green fluorescent reporters and actuators with that are activated with blue light. If this red-shifted emission spectrum is not necessary, there is a green “sister” indicator protein that has the advantage of enabling the use of standard optical filter combinations for GFP [50].

Surprisingly few studies have used genetically encoded indicators to study neurophysiological issues at the level of cultured cells [71, 74]. One possible reason for this is that optical approaches are typically combined with patch-clamp recordings, which provide not only high-quality recordings of voltage transients and the means to manipulate membrane voltage but also a convenient way to load cells with LMW organic indicators. Judging from the recent literature, the GCaMPs are the most established GECIs for use at the single-cell level, with GCaMP3 the most widely used representative [84]. VSFPs have been shown to allow imaging of voltage transients from processes of cultured neurons [4, 70]. However, this potential has not yet been exploited in studies that go beyond proof of principle.

When using optical indicators for monitoring neuronal activity at the level of intact local circuits in brain slices or in vivo, calcium indicators are far more widely used than voltage indicators. The reason for this is practical: optically recorded calcium signals usually have a much better SNR than voltage signals. This is due to the often larger changes in fluorescence intensity associated with biological calcium signals compared to voltage signals, as well as the larger number of indicator molecules that can be accommodated in the cytosol (calcium imaging) versus the plasma membrane (voltage imaging) [42]. Efforts to image large (<1,000) populations of cells at the single-cell level using two-photon microscopy in vivo almost always rely on the use of calcium indicators as a surrogate for voltage indicators, mainly because of SNR issues but perhaps also that laser-scanning approaches are limited by low frame rates. Moreover, calcium indicators are the obvious choice if calcium is the primary parameter of interest, as in experiments monitoring calcium spikes in dendrites of neurons and calcium transients in synaptic terminals [25]. The drawbacks of calcium indicators are the limited temporal precision with which action potential information is reported and blindness to voltage signals that are not associated with large changes in Ca^{2+} (e.g., inhibitory and sub-threshold excitatory synaptic potentials).

Voltage imaging is traditionally preferred over calcium imaging for studies of cortical circuit dynamics at the mesoscopic level, which cover millimeters of cortical space and very large (>1,000) populations of neurons under conditions where responses from single cells are not resolved (“population imaging”) [27]. GEVIs such as the VSFP2s provide a SNR

comparable to organic voltage-sensitive dyes [4], while allowing for cell type-specific labeling and chronic experiments. Based on these features, they should perform better than LMW dyes in most population imaging experiments.

State of the art GECIs

Parallel efforts by several groups have given rise to a set of well-performing GECIs. There are only a few, mostly incomplete, efforts to rigorously and directly compare GECIs under specific experimental conditions [84, 88]. But even from this limited amount of data, it is clear that under different conditions, different GECI variants will perform best. In practice, the choice of an appropriate GECI will also depend on the available instrumentation and animals or viruses with specific indicator genes. The basic, distinctive physiochemical features among GECIs are the photophysical properties of the fluorescent proteins used and the effective calcium-binding kinetics [20, 35, 84, 90]. A high affinity for calcium (low K_d value) is favorable for smaller calcium signals (e.g., those associated with single narrow action potentials), but a low K_d value also results in indicator saturation during high-frequency bursts of action potentials and causes higher baseline fluorescence. Far red- or infrared-shifted probes would be preferable in view of the fact that tissue autofluorescence peaks at greenish wavelengths, but the photophysical properties (i.e., fluorescence quantum yield and stability) are usually inferior in red-shifted FPs as compared to greenish variants [90]. Moreover 2P excitation has not been established for probes with fluorescence emission in the far red and infrared portion of the spectrum. The most widely used GECI, which is most likely also the best performing current variant under a variety of experimental conditions, is GCaMP3.

State of the art GEVIs

At present, the best performing GEVIs are from the VSFP class. The microbial opsin-based voltage indicators have not yet proven useful under biologically interesting experimental conditions, but, in principle, they could be very promising if the issue of their low brightness could be solved so that the high $\Delta F/F$ value would translate into a large SNR. With respect to VSFPs and other GEVIs for which the response to voltage changes is not quasi-instantaneous, it is important to note that the $\Delta F/F$ for fast signals strongly depends not only the steady-state value of their fluorescence–voltage relationship but also on their kinetic properties (i.e., activation/deactivation kinetics and $V_{1/2}$ value). Therefore, VSFPs tuned for fast kinetics at resting membrane potential are preferable [3]. For 2P excitation, VSFPs based on the citrine/mKate2 FRET pair fluorescent proteins are preferable (Akemann et al., unpublished observations). 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 [47]. 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 identified. It should be emphasized, however, that a reasonable SNR at high temporal resolution (as is 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 [42]. In addition to satisfying these challenging 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.” At present, the best performing VSFP variants under a variety of experimental conditions are the VSFP Butterflies [3].

Perspective

Since the proof of principle-level demonstration of the first genetically encoded optical indicators about 15 years ago, their use in neuroscience has coevolved with the development of optical imaging technologies towards the goal of in vivo monitoring of neuronal representations. At present, the most convincingly demonstrated advantage of genetically encoded indicators relates to the feasibility of in vivo optical imaging experiments over repeated sessions spanning days or even months [42, 52]. Such chronic imaging strategies have led to several important advances during the last few years [21, 31, 38], and this trend will likely accelerate in the future. The second inherent and unique advantage of genetically encoded indicators—namely, the ability to target specific cell populations—has not yet been fully exploited. We expect that cell class-specific imaging will be widely employed once transgenic animal models [89] and cell type-specific viral gene delivery strategies are available for a larger set of genetically encoded indicators. We expect that optogenetic imaging approaches will also be extended to multimodal strategies using indicators with fluorescence in different bands of the color spectrum. The combination of genetically encoded tools to monitor neuronal activities with matching tools to control neuronal activities [17] will enable a complete optogenetic approach that complements traditional electrophysiology with measurement (e.g., voltage recoding) and control (e.g., current injection) at the same time.

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