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Genetically encoded neural activity indicators

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Recording activity from identified populations of neurons is a central goal of neuroscience. Changes in membrane depolarization, particularly action potentials, are the most important features of neural physiology to extract, although ions, neurotransmitters, neuromodulators, second messengers, and the activation state of specific proteins are also crucial. Modern fluorescence microscopy provides the basis for such activity mapping, through multi-photon imaging and other optical schemes. Probes remain the rate-limiting step for progress in this field: they should be bright and photostable, and ideally come in multiple colors. Only protein-based reagents permit chronic imaging from genetically specified cells. Here we review recent progress in the design, optimization and deployment of genetically encoded indicators for calcium ions (a proxy for action potentials), membrane potential, and neurotransmitters. We highlight seminal experiments, and present an outlook for future progress.

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Introduction

The injection of aequorin purified from jellyfish [1] into the barnacle muscle fiber [2] heralded the beginning of protein-based indicators of neural activity. The 'DNA revolution' quickly led to the cloning of the aequorin gene and its partner green fluorescent protein (GFP). Several years later, the first genetically encoded calcium indicators (GECIs), produced by the host organism, were introduced [3,4]. The parallel development of fluorescence microscopy, most notably multi-photon imaging, and its subsequent adoption for *in vivo* imaging of the nervous system [5], has spurred interest in such activity reporters. A growing number of sensors are being developed for many aspects of neural physiology, from membrane depolarization and ion flux to downstream signal transduction cascades. Critically, genetically encoded indicators facilitate targeted long-term expression with repeated observation of individual cells, to address questions of nervous system development and maintenance, learning and memory. Here we review recent progress in neural activity indicator engineering, and highlight important results and approaches.

Membrane potential

Although it is a more 'fundamental' signal in neuroscience, membrane potential ('voltage') imaging has been notoriously difficult [6]. Small molecule probes ('voltage-sensitive dyes', VSDs) have been in use for nearly three decades [7], but none has sufficient performance to achieve common usage. VSDs are typically quite hydrophobic, which makes them difficult to load; toxicity has also been a problem. The nature of the underlying voltage signal makes imaging it inherently troublesome: action potentials persist on a scale of ~1–5 ms, and are quite local. This means that cellular-resolution voltage imaging must be performed with both high temporal and spatial resolution; this in turn limits photon budget, which challenges signal-to-noise ratio (SNR). In the end, voltage imaging is commonly practiced as a 'bulk imaging' technique, to determine the relative activity of largish cellular aggregates; at the other extreme, single cells are loaded with VSDs through a patch pipette. The former fails to take advantage of the features of the voltage signal, and falls within the purview of calcium imaging (see next section). The latter cannot address systems neuroscience questions.

The first genetically encoded voltage indicator (GEVI) was developed in 1997 [8], the same year as the first GECI. In spite of this, none has yet been used for other than 'proof-of-principle' experiments, owing to poor properties. Early indicators, such as FlaSh [8], SPARC [9], and VSFP1 [10], were based either on intact voltage-gated potassium channels, or their 'voltage paddle' domains. Such indicators had poor membrane targeting, low SNR, slow kinetics, and caused cytotoxicity [11]. The next generation of GEVIs was based on the paddle domain of a voltage-gated phosphatase, CiVSP [12]. Such probes have dramatically improved targeting and toxicity profiles, and marginally improved SNR, but remain slow, on the order of current GECIs [13,14]. Two-photon excitation of these probes also remains elusive, which limits imaging to wide-field modalities. One of the most advanced probes of this class, VSFP2.3, was expressed in mouse somatosensory cortex; responses in ~1 mm^2 areas to single whisker deflection events were observed, but

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single-trial signal was low (ratio change ~0.25%; SNR ~2–3) [15•].

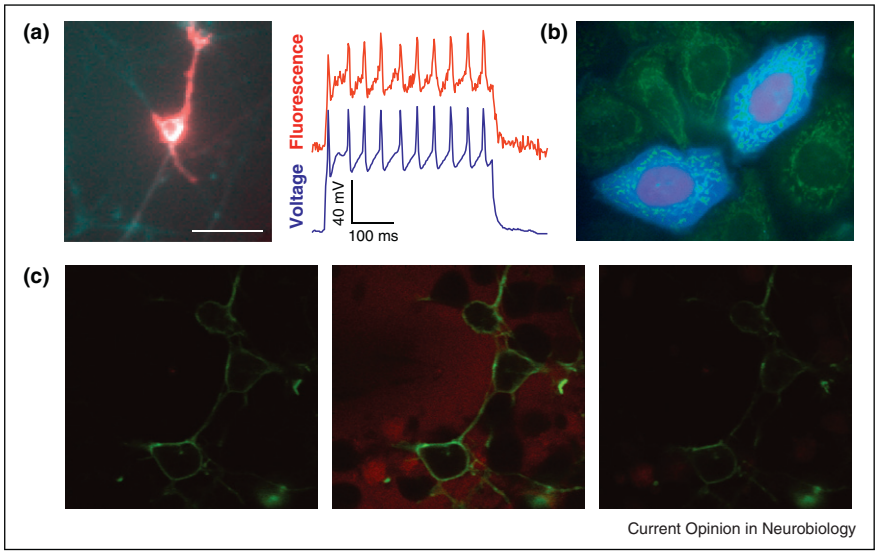
Another GEVI variant is the hybrid 'hVOS' indicator [16], which transduces the voltage-dependent migration of dipicrylamine (DPA) through the membrane leaflet to 'dark FRET' with a membrane-targeted GFP. DPA is extremely toxic, however, and distribution in tissue is problematic. Given this, and the low SNR of the sensor, hVOS has not achieved much usage.

A new class of GEVI is based instead on voltage-induced fluorescence modulation of the retinal co-factor of bacterial and archaeal rhodopsins. The first of these, PROPS [17••], harnesses local pK_a and pH modulation of the pore of a non-conducting proton pump (a mutant of green proteorhodopsin) to control protonation, and resulting fluorescence, of the retinal co-factor. Such voltage-induced proton movements are extremely fast, and the resulting sensors have submillisecond kinetics, sufficient to resolve clear blinking events in individual *Escherichia coli* bacteria [17••]. An improved version of this sensor, VIP1, based on the archaerhodopsin-3 (Arch) proton pump, robustly targets eukaryotic membranes and has been used to image single action potentials and subthreshold depolarization in cultured neurons [18••] (Figure 1a). A non-conducting Arch mutant, VIP2, had significantly slower kinetics but improved SNR. It is likely that further mutants of these rhodopsins, potentially combined with brighter retinal chromophore derivatives, may address the fundamental concern with this technology: the probes are prohibitively dim in their current form. Improved versions of these indicators may finally facilitate systems level, cellular-resolution voltage imaging.

Calcium

Action potential firing leads to Ca^{2+} influx through voltage gated calcium channels located throughout the cell [19]. Synaptic input directly gates Ca^{2+} through neurotransmitter-gated ion channels [20], primarily NMDA and nicotinic receptors. Calcium is unique among ions in terms of the magnitude of activity-induced changes [21]. Quantification of free [Ca^{2+}] changes can thus be used as a reliable proxy for neural activity, in spine, dendrite, axon, or soma. Calcium transients are significantly longer in duration than the voltage fluctuations that give rise to them – this both integrates signal and fundamentally limits the temporal resolution with which Ca^{2+} indicators may resolve neural activity. Small molecule Ca^{2+} dyes have achieved widespread use in the last three decades, and state-of-the-art indicators like Oregon Green BAPTA-1 (OGB-1) and fluo-4 have been the workhorses of *in vivo* functional imaging. These dyes are bright, photostable, high-affinity, and show very large Ca^{2+}-dependent fluorescence changes. They are, however, limited to acute imaging experiments owing to

Figure 1



Three new classes of genetically encoded sensors. **(a)** Archaerhodopsin-3 (Arch)-based voltage sensor. Left, rat hippocampal neuron expressing Arch (VIP1). Time-averaged fluorescence shown in cyan, voltage-responsive pixels shown in pink. Scale bar 50 μm. Right, recorded membrane potential (blue) and deconvolved fluorescence signal (red). Spiking induced by injection of 200 pA DC. Data are single-trial recording. Panel adapted from reference 18 courtesy of Adam Cohen. **(b)** New colors of GCaMP-based calcium indicators. 3-color fluorescence imaging of HeLa cells expressing nuclear red R-GECO1, cytoplasmic blue G-GECO1, and mitochondrial green B-GECO1. Figure adapted from reference 31 courtesy of Takeharu Nagai. **(c)** IntenseGluSnFr glutamate sensor displayed on the surface of cultured hippocampal neurons. Left, resting; middle, after a 'puff' of glutamate and AlexaFluor 568; right, after washout with buffer. Figure courtesy of Jonathan Marvin.

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clearance and accumulation in high-[Ca^{2+}] organelles. Furthermore, non-specific labeling severely impairs imaging through high background and neuropil contamination; axonal or dendritic imaging is typically only possible via laborious direct loading.

Genetically encoded calcium indicators (GECIs) circumvent many of these problems. They may be targeted to specific cell populations and subcellular locales, and are amenable to stable expression over months. GECIs, however, have long lagged behind synthetic indicators in terms of SNR and action potential detection threshold, limiting their uptake. Recent advances in indicator engineering and standardized testing formats have brought the current generation of GECIs closer to the performance of dyes. The GCaMP (also 'G-CaMP') scaffold [22] has been iteratively optimized. The incorporation of GFP-stabilizing mutations produced GCaMP1.6 [23], and subsequent random mutagenesis yielded a brighter variant GCaMP2 [24]. X-ray crystal structure determination of GCaMP2 in the Ca^{2+}-bound and Ca^{2+}-free states [25,26] allowed systematic mutagenesis around the chromophore and GFP/CaM interface, giving rise to GCaMP3 [27]. Independently, incorporation of a subset of 'superfolder GFP' mutations [28] into GCaMP2 produced GCaMP-HS [29]. Targeted mutagenesis of the linkers connecting cpGFP to CaM and the M13 peptide yielded the high SNR variants Case12 and Case16 [30], and more recently, GCaMP5 (L. Looger, submitted).

Recently, the color palette of single-wavelength GECIs has been expanded [31••]. Incorporation of chromophore mutations into GCaMP3, followed by random mutagenesis, produced a blue indicator B-GECO1; using the red fluorescent protein mApple in place of GFP led to the creation of R-GECO1 [31••] (Figure 1b). Independently, incorporation of chromophore mutations or 'stacking' interactions, followed by targeted mutagenesis, yielded blue, cyan, and yellow versions of GCaMP3, and the use of a circularly permuted version of mRuby provided the basis for RCaMP (L. Looger, submitted). Intriguingly, during the development of B-GECO1 and improved versions of GCaMP3 (G-GECO1.1 and G-GECO1.2), variants were discovered with ratiometric blue/green emission or excitation (GEM-GECO1 and GEX-GECO1, respectively) [31••]. These variants may offer many of the advantages of 2-fluorescent protein FRET indicators, while maintaining a small size and potentially faster kinetics.

Several state-of-the-art FRET GECIs have attained broad usage in neuroscience. Derivatives of the original Yellow Chameleon (YC) [3], most notably YC2.6 and YC3.6 [32], have been used in a number of model systems. Computational redesign of the CaM/M13 interface produced several variants with decreased binding to wild-type CaM [33]. Of these, D3cpVenus (D3cpV) and YC3.6 are the most widely used. Recently, linker mutagenesis of YC3.6 produced a series of very high-affinity (low nM) indicators, dubbed 'Chameleon-Nano' sensors [34•]. These indicators may be useful for studying variations in basal [Ca^{2+}] levels in different cell types [34•], and could potentially improve spike detection under very sparse conditions, although these aspects have not been investigated in detail yet. Calcium buffering and potential cytotoxicity could, however, be a problem following long-term expression of such high-affinity indicators.

The indicators above all use calmodulin and a binding peptide to transduce Ca^{2+} binding into fluorescence. A parallel development has employed troponin C (TnC), a component of the muscle tropomyosin complex, as the Ca^{2+} recognition element [35]. Iterative optimization, mainly creating different combinations of EF-hands and mutagenesis to reduce Mg^{2+} competition at the Ca^{2+}-binding sites, produced TN-XXL [36]. TN-XXL has been used for imaging in fly and mouse [36], and may be more 'bio-orthogonal' than CaM-based indicators.

It is likely that additional headroom remains in the various FRET GECI scaffolds. Design goals include increasing brightness and FRET in the Ca^{2+}-bound state, decreasing FRET in the Ca^{2+}-free state, improving kinetics and ameliorating Ca^{2+} buffering. The introduction of brighter or more photostable fluorescent proteins improves baseline sensor brightness and may also improve saturated FRET efficiency. Recently, an improved version of cyan fluorescent protein, dubbed '3xCFP', was incorporated with cpVenus into a FRET indicator analogous to TN-XXL, which led to improved FRET ratio change during slow calcium transients in PC12 cells [37]. Modulation of the dimerization tendency of *Aequorea* GFP variants is another approach to tune response [38]. The use of minimal Ca^{2+}-binding domains (Thestrup and Griesbeck, unpublished) might simultaneously reduce Ca^{2+} buffering and interactions with endogenous proteins, alleviating cytotoxicity concerns. Complementary to rational design strategies, improvements in donor/acceptor library construction and screening allow for higher throughput [39].

Although optimization continues, even with current performance levels GECIs have been instrumental in opening new ground for research. For instance, expression of TN-XXL in L1/2 neurons in the *Drosophila* visual system enabled imaging individual axon terminals within the medulla and resulted in the first physiological readout of these neurons that feed into motion-detection circuitry [40•,41]. The use of regenerative amplification multiphoton microscopy (RAMM) of GCaMP3 facilitated the first *in vivo* optical recording of activity from layer 5 neurons in mouse somatosensory cortex, nearly a millimeter below the pial surface [42•]. A small library of promoter and viral serotype variants of GCaMP3 allowed

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targeted imaging of visual stimulus-evoked responses from each major cell type in the mouse retina [43], rivaling the synthetic dye OGB-1. Subcellular targeting of GCaMP variants to the pre-synapse [44] or the membrane [45] increases SNR and kinetics, and has been used to record activity from specific synapses *in vivo* and to detect 'spotty calcium' activity transients in tiny astrocytic processes, respectively.

Long-term expression of genetically encoded indicators facilitates the study of learning, memory, and neural circuit evolution in an awake, behaving animal over weeks to months [27,36,46]. Viral delivery of YC3.6 into mouse somatosensory cortex allowed for fiber optic recordings of neuronal activity in freely moving mice [47•]. Hippocampal expression of GCaMP3 followed by a small cranial excavation permitted imaging navigation-dependent activity of CA1 place cells over several weeks in behaving mice [48••]. Concomitant with GECI optimization, improvements in imaging modalities, behavioral paradigms and image analysis techniques are making real the prospect of observing the contribution of specific cells and circuits to the execution of complex tasks in intact animals.

Synaptic transmission

In recent years, a variety of fluorescent indicators of synaptic transmission have been developed. In addition to measuring voltage changes or Ca^{2+} flux at either the pre-synaptic or post-synaptic membrane, covered above, it is possible to detect either the neurotransmitter molecule itself, or the H^+ ion flux associated with vesicle exocytosis/reacidification cycles. Several versions of 'synaptopHluorin' [49], based on pH-sensitive mutants of GFP, are in use, and recently a 'synaptopHluorange' sensor based on the acid-sensitive mOrange2 fluorescent protein has been published [50].

Direct indicators of neurotransmitters or neuromodulators are preferable, to improve SNR and kinetics, and to reduce signal confounds. The bacterial periplasmic glutamate-binding protein GltI from *E. coli* has been employed as the recognition element in three separate genetically encoded fluorescent glutamate reporters. Developed independently, FLIP-E [51] and GluSnFr [52], with GltI sandwiched between ECFP and EYFP, were used to image glutamate release from cultured neurons. An optimized version of GluSnFr, dubbed 'SuperGluSnFr' [53], was used to quantitate several aspects of glutamate spillover in neuronal culture. Recently, a single-wavelength indicator ('IntenseGluSnFr') was developed from GltI and circularly permuted GFP. IntenseGluSnFr had sufficient SNR and photostability to permit 2-photon imaging in mouse retinal explant and mouse visual cortex *in vivo*. Expressed under both neural-specific and glial-specific promoters, IntenseGluSnFr revealed kinetics of glutamate release from neurons and uptake by glia (Figure 1c); fluorescence events consistent with local vesicle release events were observed in several systems (L. Looger, submitted).

Neurotransmitter binding may also be coupled to other optical observables. A hybrid sensor (a 'CNiFER') for acetylcholine (Ach) was created from a HEK293 cell line stably expressing both the muscarinic M1 acetylcholine receptor and the TN-XXL calcium indicator [54•]. Exposure to Ach gives rise to Ca^{2+} flux through G-protein-gated channels, which produces a TN-XXL signal. These stable cells were injected into cortex of adult rats; injection of atypical neuroleptic drugs produced altered CNiFER signals, and allowed imaging of the effect of the molecules on cholinergic signaling [54•]. Replacement of the M1 receptor with a variety of Cys-loop receptors produced hybrid sensors for serotonin, among others [55]. These hybrid cell-based sensors offer the advantage of modular design and exploit amplification of signal through coupling to calcium, although their application is limited to volume transmitter fluctuations. Furthermore, the kinetics of response through a multi-component signaling cascade is too slow to address single-synapse responses. Bacteria express periplasmic binding proteins for GABA, acetylcholine, glycine, and a number of other relevant molecules; it is likely that sensors based on these molecules will produce the most direct read-outs.

Conclusions and outlook

A great deal of progress has been made in the last several years towards a usable set of genetically encoded neural activity indicators. The determination of the high-resolution crystal structure of several sensors has revealed atomic detail of mechanisms, enabling more systematic protein engineering efforts. In parallel, several improvements to high-throughput indicator screening methodologies lower the barriers to sensor design and optimization. A steady flow of improved fluorescent proteins has supplied the material for better indicators. Several new sensor classes have expanded the set of tools available to apply to new engineering targets. Systematization of indicator validation schemes, such as simultaneous electrophysiology/imaging in acute brain slice, retinal explant, and canonical *in vivo* sensory assays such as olfaction and primary visual responses, have made it easier to compare indicators and predict success in a new experiment. Independently, improvements in imaging modalities such as digital light sheet microscopy [56] and RAMM, faster and more sensitive cameras (e.g. sCMOS) and other photon detectors, new lasers (e.g. solid-state, CW fiber) and other excitation sources, improved software to drive imaging experiments (e.g. ScanImage [57]; http://www.neuroptikon.org/projects/display/ephus/ScanImage) and commercial multi-photon solutions (e.g. Prairie Technologies), improved transgenesis

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methods (e.g. sparse GAL4 expression lines in several organisms, Cre-dependent and Tet-dependent mouse lines, zinc finger nucleases to create knock-ins in organisms lacking defined integration loci), and advances in image analysis and machine learning methods, all contribute to the steady advance of the Age of Light.

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