

Genetically Encoded Neural Activity Indicators

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Introduction

A central goal of neuroscience is to record activity from identified populations of neurons. The injection of aequorin purified from jellyfish (Shimomura et al., 1962) into the barnacle muscle fiber (Ashley et al., 1968) heralded the beginning of protein-based indicators of neural activity. The “DNA revolution” quickly led to the cloning of the genes encoding aequorin and its partner, green fluorescent protein (GFP). Several years later, the first genetically encoded calcium indicators (GECIs), produced by the host organism, were introduced (Miyawaki et al., 1997; Persechini et al., 1997).

The parallel development of fluorescence microscopy, most notably multi-photon imaging, and its subsequent adoption for *in vivo* imaging of the nervous system (Helmchen and Denk, 2005), have spurred interest in such activity reporters. Probes remain the rate-limiting step for progress in this field: They should be bright and photostable and ideally come in multiple colors. 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, genetically specified cells in order to address questions of nervous system development and maintenance, learning, and memory. In this chapter, we review recent progress in neural activity indicator engineering and highlight important results and approaches.

Membrane Potential

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 critical. Although it is a more “fundamental” signal in neuroscience, membrane potential (“voltage”) imaging has been notoriously difficult (Peterka et al., 2011). Small-molecule probes (voltage-sensitive dyes, or VSDs) have been in use for nearly three decades (Homma et al., 2009), 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. As a result, cellular-resolution voltage imaging must be performed with both high temporal and

spatial resolution; these requirements, in turn, limit 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 rather large cellular aggregates. At the other extreme, single cells are loaded with VSDs through a patch pipette. The former method fails to take advantage of the features of the voltage signal and falls within the purview of calcium imaging (see “Calcium,” below); the latter method cannot address systems neuroscience questions.

Genetically encoded voltage indicators

The first genetically encoded voltage indicator (GEVI) was developed in 1997 (Siegel and Isacoff, 1997), 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 (Siegel and Isacoff, 1997), SPARC (Ataka and Pieribone, 2002), and VSFP1 (Sakai et al., 2001), were based on either intact voltage-gated potassium channels or their “voltage paddle” domains. Such indicators had poor membrane targeting, low SNR, slow kinetics, and caused cytotoxicity (Baker et al., 2007).

The next generation of GEVIs was based on the paddle domain of a voltage-gated phosphatase, CiVSP (Murata et al., 2005). Such probes have dramatically improved targeting and toxicity profiles and marginally improved SNR; however, they remain slow, on the order of current GECIs (Lundby et al., 2008; Tsutsui et al., 2008). Two-photon excitation of these probes also remains elusive, which limits their imaging abilities to wide-field modalities. VSFP2.3, one of the most advanced probes of this class, was expressed in mouse somatosensory cortex *in vivo*. Responses in ~1 mm² areas to single whisker deflection events were observed, but single-trial signal was low (ratio change ~0.25%; SNR ~2–3) (Akemann et al., 2010).

Another GEVI variant is the hybrid hVOS indicator (Chanda et al., 2005), which transduces the voltage-dependent migration of dipicrylamine (DPA) through the membrane leaflet to “dark FRET” (fluorescence resonance energy transfer) with a membrane-targeted GFP. DPA is extremely toxic, however, and distribution in tissue is problematic. Given these limitations, and the low SNR of the sensor, hVOS has not achieved wide usage.

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A new class of GEVI is based instead on voltage-induced fluorescence modulation of the retinal cofactor of bacterial and archaeal rhodopsins. The first of these was PROPS, engineered from the prokaryotic light-driven proton-pump green proteorhodopsin, which reports membrane potential fluctuations as fluorescence transients in *Escherichia coli* (Kralj et al., 2011). PROPS harnesses local pK_a and pH modulation of the pore of a nonconducting proton pump to control the protonation, and resulting fluorescence, of the retinal cofactor. The sensor, the first of a new class, showed greater signal change and faster kinetics than current GEVIs. Such voltage-induced proton movements are extremely fast, and the resulting sensors have submillisecond kinetics, sufficient to resolve clear blinking events in individual *E. coli* bacteria (Kralj et al., 2011). An improved version of this voltage 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 mammalian neurons (Kralj et al., 2012) (Fig. 1a). A nonconducting 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 (Jaffe et al., 1992). Synaptic input directly gates Ca^{2+} through neurotransmitter-gated ion channels (Muller et al., 1991), primarily NMDA and nicotinic receptors. Calcium is unique among ions in terms of the magnitude of activity-induced changes (Hille, 1992). 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 last significantly longer 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 their clearance and accumulation in high- Ca^{2+} organelles. Furthermore, nonspecific labeling severely impairs imaging through high background and neuropil contamination; axonal or dendritic imaging is typically possible only 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, thus 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. For example, the GCaMP (also “G-CaMP”) scaffold (Nakai et al., 2001) has been iteratively optimized. The incorporation of GFP-stabilizing mutations produced GCaMP1.6 (Ohkura et al., 2005), and subsequent random mutagenesis yielded a brighter variant, GCaMP2 (Tallini et al., 2006). Further, X-ray crystal structure determination of GCaMP2 in the Ca^{2+} -bound and Ca^{2+} -free states (Wang et al., 2008; Akerboom et al., 2009) has allowed systematic mutagenesis around the chromophore and GFP/CaM interface, giving rise to GCaMP3 (Tian et al., 2009). Independently, incorporation of a subset of “superfolder GFP” mutations (Pedelacq et al., 2006) into GCaMP2 has produced “hypersensitive” GCaMP (GCaMP-HS) (Muto et al., 2011). Targeted mutagenesis of the linkers connecting cpGFP to CaM and the M13 peptide has yielded the high SNR variants Case12 and Case16 (Souslova et al., 2007), and more recently, GCaMP5 (Akerboom et al, in press).

Recently, the color palette of single-wavelength GECIs has been expanded (Zhao et al., 2011). 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 (Zhao et al., 2011) (Fig. 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 (J. Akerboom, N. Calderón, L. Tian, S. Basi, M. Prigge, J. Tolö, T. Wardill, K. Sarkisyan, J. Marvin, A. Gordus, M. Orger, R. Portugues, J.

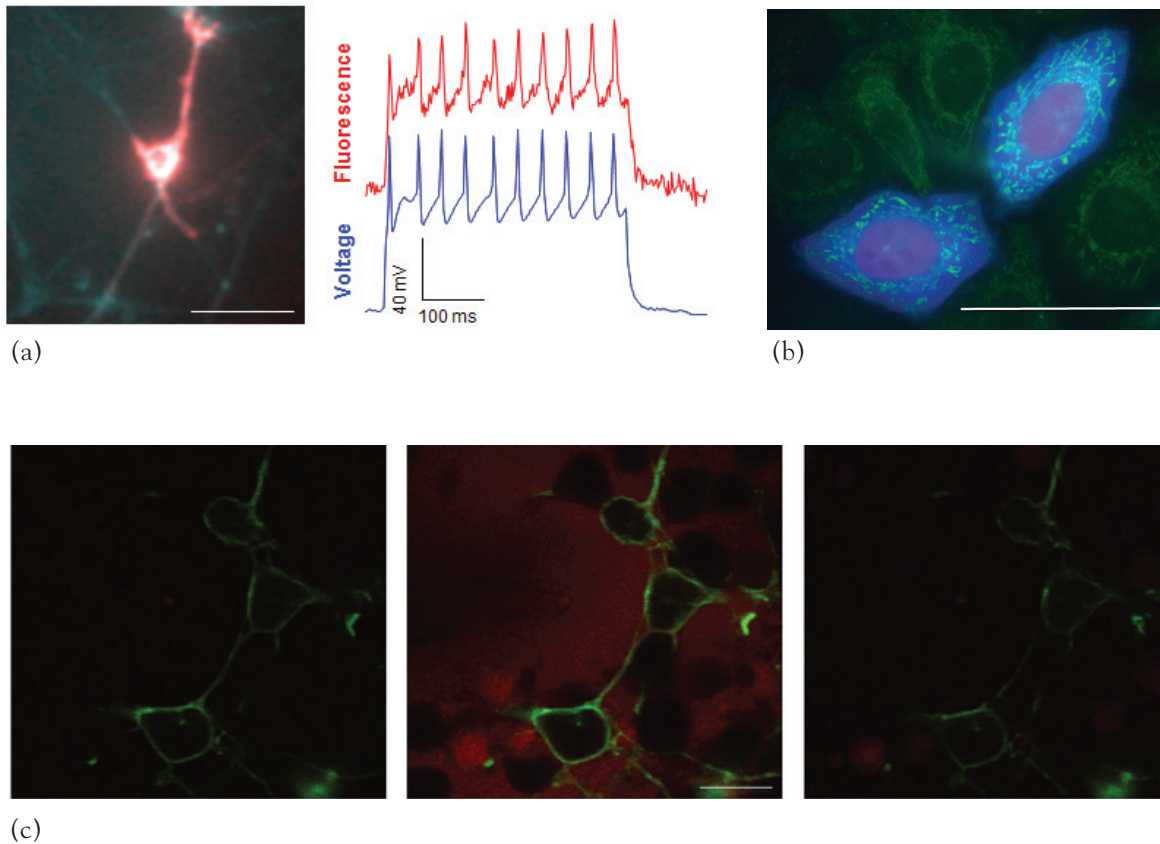


Figure 1. Three new classes of genetically encoded sensors. **a**, Arch-based voltage sensor. Left, rat hippocampal neuron expressing Arch (VIP1). Time-averaged fluorescence shown in cyan, voltage-responsive pixels shown in pink. Right, recorded membrane potential (blue) and deconvolved fluorescence signal (red). Spiking induced by injection of 200 pA DC. Data are from a single-trial recording. Panel courtesy of Adam Cohen. **b**, New colors of GCaMP-based calcium indicators. Three-color fluorescence imaging of HeLa cells expressing nuclear red R-GECO1, mitochondrial green G-GECO1, and cytoplasmic blue B-GECO1. Figure courtesy of Takeharu Nagai. **c**, iGluSnFr 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. Scale bars: **a–b**, 50 μm ; **c**, 10 μm .

Stirman, J. Macklin, C. Bargmann, B. Kimmel, V. Jayaraman, K. Svoboda, D. Kim, S. Kügler, et al., unpublished observations). 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) (Zhao et al., 2011). 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 (Nagai et al., 2004), 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 (Palmer et al., 2006). Of the YC variants,

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 range) indicators, dubbed “Chameleon-Nano” sensors (Horikawa et al., 2010). These indicators may be useful for studying variations in basal $[\text{Ca}^{2+}]$ levels in different cell types (Horikawa et al., 2010) and could potentially improve spike detection under very sparse-firing conditions, although these aspects have not yet been investigated in detail. 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 (Heim and Griesbeck, 2004). Iterative optimization, mainly

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creating different combinations of EF-hands and mutagenesis to reduce Mg^{2+} competition at the Ca^{2+} -binding sites, produced TN-XXL (Mank et al., 2008). TN-XXL has been used for imaging in fly and mouse (Mank et al., 2008) 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 improve saturated FRET efficiency. Recently, an improved version of cyan fluorescent protein, dubbed “3 × CFP,” was incorporated with cpVenus into a FRET indicator analogous to TN-XXL. This version led to improved FRET ratio change during slow calcium transients in PC12 cells (Liu et al., 2011).

Modulation of the dimerization tendency of *Aequorea* GFP variants is another approach to tune response (Kotera et al., 2010). The use of minimal Ca^{2+} -binding domains might simultaneously reduce Ca^{2+} buffering and interactions with endogenous proteins, thereby alleviating cytotoxicity concerns. Complementary to rational design strategies, improvements in donor/acceptor library construction and screening allow for higher throughput (Piljic et al., 2011).

Although optimization continues, even with current performance levels, GECIs have been instrumental in opening new ground for research, for example:

- 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 (Reiff et al., 2010; Clark et al., 2011);
- The use of regenerative amplification multi-photon microscopy (RAMM) of GCaMP3 facilitated the first *in vivo* optical recording of activity from layer V neurons in mouse somatosensory cortex, nearly a millimeter below the pial surface (Mittmann et al., 2011);
- A small library of promoter and viral serotype variants of GCaMP3 allowed targeted imaging of visual stimulus-evoked responses from each major cell type in the mouse retina (Borghuis et al., 2011), rivaling the synthetic dye OGB-1;

- Subcellular targeting of GCaMP variants to the presynapse (Dreosti et al., 2009) or the membrane (Shigetomi et al., 2010) increased 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 facilitated the study of learning, memory, and neural circuit evolution in an awake, behaving animal over weeks to months (Mank et al., 2008; Tian et al., 2009; Andermann et al., 2010). Viral delivery of the FRET calcium biosensor YC3.6 into mouse somatosensory cortex allowed for fiber optic recordings of neuronal activity in freely moving mice (Lutcke et al., 2010);
- Hippocampal expression of GCaMP3 followed by a small, chronic cranial excavation window permitted imaging navigation-dependent activity of CA1 place cells over several weeks in mice performing spatial tasks in a virtual reality setting (Dombeck et al., 2010); and
- A transgenic reporter mouse (“Ai38”) expressing GCaMP3 in a Cre-dependent manner facilitated very long-term imaging studies and developmental experiments previously inaccessible by viral injection (Zariwala et al., 2012).

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 presynaptic or postsynaptic membrane (see 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 “synaptotHluorin” (Miesenböck et al., 1998), based on pH-sensitive mutants of GFP, are in use, and recently, findings on a “synaptotHluorange” sensor based on the acid-sensitive mOrange2 fluorescent protein have been published (Li et al., 2011). A red pH indicator, “SypHTomato,” has also been published (Li and Tsien, 2012).

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 *Escherichia coli* has been employed as the recognition element in three separate genetically encoded fluorescent glutamate reporters. Developed independently, FLIP-E (Okumoto et al., 2005) and GluSnFr (Tsien, 2005), with GltI sandwiched between ECFP and EYFP, were used to image glutamate release from cultured neurons. An optimized version of GluSnFr, dubbed “SuperGluSnFr” (Hires et al., 2008), was used to quantitate several aspects of glutamate spillover in neuronal culture. Recently, a single-wavelength indicator (“iGluSnFr”) was developed from GltI and circularly permuted GFP. iGluSnFr had sufficient SNR and photostability to permit two-photon imaging in mouse retinal explant and mouse motor cortex *in vivo*. Expressed under both neural-specific and glial-specific promoters, iGluSnFr revealed the kinetics of glutamate release from neurons and uptake by glia (Fig. 1c); fluorescence events consistent with local vesicle release events were observed in several systems (J. Marvin, B. Borghuis, L. Tian, J. Cichon, T.-W. Chen, J. Akerboom, A. Gordus, C. Bargmann, S. Renninger, M. Orger, E. Schreiter, S. Hires, J. Demb, W. Gan, L. Looger, unpublished observations).

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 ACh receptor and the TN-XXL calcium indicator (Nguyen et al., 2010). 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 (Nguyen et al., 2010). Replacement of the M1 receptor with a variety of Cys-loop receptors produced hybrid sensors for serotonin, among others (Yamauchi et al., 2011). These hybrid cell-based sensors offer the advantage of modular design and exploit signal amplification through coupling to calcium, although their application is limited to volume transmitter fluctuations. Furthermore, the kinetics of response through a multicomponent signaling cascade is too slow to address single-synapse responses. Bacteria express periplasmic binding proteins for GABA, ACh, glycine, and a number of other relevant molecules; it is likely that sensors based on these molecules will produce the most direct readouts.

Conclusions and Outlook

A great deal of progress has been made in the last several years toward building up 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;
- Several improvements to high-throughput indicator screening methodologies have lowered 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; and
- Systematization of indicator validation schemes, such as simultaneous electrophysiology/imaging in acute brain slice, retinal explant, and canonical *in vivo* sensory assays (e.g., olfaction and primary visual responses), have made it easier to compare indicators and predict success in a new experiment.

Independently, advances on the imaging front have proceeded apace:

- Improvements have been made to imaging modalities, such as digital light sheet microscopy (Keller et al., 2008) and RAMM;
- Faster and more sensitive cameras (e.g., scientific complementary metal oxide semiconductor [sCMOS] and electron-multiplying charge-coupled device [EMCCD]) and other photon detectors have been developed;
- New lasers (e.g., solid-state, CW fiber) and other excitation sources have come on the market;
- Improved software to drive imaging experiments (e.g., ScanImage [Shepherd et al., 2003], <http://www.neuroptikon.org/projects/display/ephus/ScanImage>) and commercial multi-photon solutions (e.g., Prairie Technologies) have become available;
- Improved transgenesis 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) are in use; and

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- Image analysis and machine learning methods have progressed.

All of the above have contributed to the steady advance of the Age of Light.

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References

- Akemann W, Mutoh H, Perron A, Rossier J, Knopfel T (2010) Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. *Nat Methods* 7:643–649.
- Akerboom J, Rivera JDV, Guilbe MMR, Malave ECA, Hernandez HH, Tian L, Hires SA, Marvin JS, Looger LL, Schreier ER (2009) Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. *J Biol Chem* 284:6455–6464.
- Akerboom J, Chen T-W, Wardill TJ, Tian L, Marvin JS, Mutlu S, Calderón NC, Esposti F, Borghuis BG, Sun XR, Gordus A, Orger MB, Portugues R, Engert F, Macklin JJ, Filosa A, Aggarwal A, Kerr R, Takagi R, Kracun S, et al. Optimization of a GCaMP calcium indicator for neural activity imaging. *J Neurosci*, in press.
- Andermann ML, Kerlin AM, Reid RC (2010) Chronic cellular imaging of mouse visual cortex during operant behavior and passive viewing. *Front Cell Neurosci* 4:3.
- Ashley CC, Ridgway EB. Simultaneous recording of membrane potential calcium transient and tension in single muscle fibres (1968) *Nature* 219:1168–1169.
- Ataka K, Pieribone VA (2002) A genetically targetable fluorescent probe of channel gating with rapid kinetics. *Biophys J* 82:509–516.
- Baker BJ, Lee H, Pieribone VA, Cohen LB, Isacoff EY, Knopfel T, Kosmidis EK (2007) Three fluorescent protein voltage sensors exhibit low plasma membrane expression in mammalian cells. *J Neurosci Methods* 161:32–38.
- Borghuis BG, Tian L, Xu Y, Nikonov SS, Vardi N, Zemelman BV, Looger LL (2011) Imaging light responses of targeted neuron populations in the rodent retina. *J Neurosci* 31:2855–2867.
- Chanda B, Blunck R, Faria LC, Schweizer FE, Mody I, Bezaniilla F (2005) A hybrid approach to measuring electrical activity in genetically specified neurons. *Nat Neurosci* 8:1619–1626.
- Clark DA, Bursztyn L, Horowitz MA, Schnitzer MJ, Clandinin TR (2011) Defining the computational structure of the motion detector in *Drosophila*. *Neuron* 70:1165–1177.
- Dombeck DA, Harvey CD, Tian L, Looger LL, Tank DW (2010) Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. *Nat Neurosci* 13:1433–1440.
- Dreosti E, Odermatt B, Dorostkar MM, Lagnado L (2009) A genetically encoded reporter of synaptic activity *in vivo*. *Nat Methods* 6:883–889.
- Heim N, Griesbeck O (2004) Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein. *J Biol Chem* 279:14280–14286.
- Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. *Nat Methods* 2:932–940.
- Hille B (1992) Ionic channels of excitable membranes. 2nd ed. Sunderland, MA: Sinauer.
- Hires SA, Zhu YL, Tsien RY (2008) Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters. *Proc Natl Acad Sci USA* 105:4411–4416.
- Homma R, Baker BJ, Jin L, Garaschuk O, Konnerth A, Cohen LB, Zecevic D (2009) Wide-field and two-photon imaging of brain activity with voltage- and calcium-sensitive dyes. *Philos Trans R Soc Lond B Biol Sci* 364:2453–2467.
- Horikawa K, Yamada Y, Matsuda T, Kobayashi K, Hashimoto M, Matsu-ura T, Miyawaki A, Michikawa T, Mikoshiba K, Nagai T (2010) Spontaneous network activity visualized by ultrasensitive Ca²⁺ indicators, yellow Cameleon-Nano. *Nat Methods* 7:729–732.
- Jaffe DB, Johnston D, Lasserross N, Lisman JE, Miyakawa H, Ross WN (1992) The spread of Na⁺ spikes determines the pattern of dendritic Ca²⁺ entry into hippocampal-neurons. *Nature* 357:244–246.
- Keller PJ, Schmidt AD, Wittbrodt J, Stelzer EHK (2008) Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322:1065–1069.

- Kotera I, Iwasaki T, Imamura H, Noji H, Nagai T (2010) Reversible dimerization of *Aequorea victoria* fluorescent proteins increases the dynamic range of FRET-based indicators. *ACS Chem Biol* 5:215–222.
- Kralj JM, Hochbaum DR, Douglass AD, Cohen AE (2011) Electrical spiking in *Escherichia coli* probed with a fluorescent voltage-indicating protein. *Science* 333:345–348.
- Kralj JM, Douglass AD, Hochbaum DR, Maclaurin D, Cohen AE (2012) Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. *Nat Methods* 9:90–95.
- Li H, Foss SM, Dobry YL, Park CK, Hires SA, Shaner NC, Tsien RY, Osborne LC, Voglmaier SM (2011) Concurrent imaging of synaptic vesicle recycling and calcium dynamics. *Front Mol Neurosci* 4:34.
- Li Y, Tsien RW (2012) pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity. *Nat Neurosci* 15:1047–1053.
- Liu S, He J, Jin HL, Yang F, Lu JL, Yang J (2011) Enhanced dynamic range in a genetically encoded Ca^{2+} sensor. *Biochem Biophys Res Commun* 412:155–159.
- Lundby A, Mutoh H, Dimitrov D, Akemann W, Knopfel T (2008) Engineering of a genetically encodable fluorescent voltage sensor exploiting fast Ci-VSP voltage-sensing movements. *PLoS One* 3(6):e2514.
- Lutcke H, Murayama M, Hahn T, Margolis DJ, Astori S, Borgloh SMZ, Gobel W, Yang Y, Tang WN, Kugler S, Sprengel R, Nagai T, Miyawaki A, Larkum ME, Helmchen F, Hasan MT (2010) Optical recording of neuronal activity with a genetically-encoded calcium indicator in anesthetized and freely moving mice. *Front Neural Circuits* 4:9.
- Mank M, Santos AF, Drenth S, Mrcic-Flogel TD, Hofer SB, Stein V, Hendel T, Reiff DF, Levelt C, Borst A, Bonhoeffer T, Hubener M, Griesbeck O (2008) A genetically encoded calcium indicator for chronic *in vivo* two-photon imaging. *Nat Methods* 5:805–811.
- Miesenböck G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192–195.
- Mittmann W, Wallace DJ, Czubyko U, Herb JT, Schaefer AT, Looger LL, Denk W, Kerr JND (2011) Two-photon calcium imaging of evoked activity from L5 somatosensory neurons *in vivo*. *Nat Neurosci* 14:1089–1093.
- Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388:882–887.
- Muller W, Connor JA (1991) Dendritic spines as individual neuronal compartments for synaptic Ca^{2+} responses. *Nature* 354:73–76.
- Murata Y, Iwasaki H, Sasaki M, Inaba K, Okamura Y (2005) Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature* 435:1239–1243.
- Muto A, Ohkura M, Kotani T, Higashijima S, Nakai J, Kawakami K (2011) Genetic visualization with an improved GCaMP calcium indicator reveals spatiotemporal activation of the spinal motor neurons in zebrafish. *Proc Natl Acad Sci USA* 108:5425–5430.
- Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci USA* 101:10554–10559.
- Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nat Biotechnol* 19:137–141.
- Nguyen QT, Schroeder LE, Mank M, Muller A, Taylor P, Griesbeck O, Kleinfeld D (2010) An *in vivo* biosensor for neurotransmitter release and *in situ* receptor activity. *Nat Neurosci* 13:127–132.
- Ohkura M, Matsuzaki M, Kasai H, Imoto K, Nakai J (2005) Genetically encoded bright Ca^{2+} probe applicable for dynamic Ca^{2+} imaging of dendritic spines. *Anal Chem* 77:5861–5869.
- Okumoto S, Looger LL, Micheva KD, Reimer RJ, Smith SJ, Frommer WB (2005) Detection of glutamate release from neurons by genetically encoded surface-displayed FRET nanosensors. *Proc Natl Acad Sci USA* 102:8740–8745.
- Palmer AE, Giacomello M, Kortemme T, Hires SA, Lev-Ram V, Baker D, Tsien RY (2006) Ca^{2+} indicators based on computationally redesigned calmodulin-peptide pairs. *Chem Biol* 13:521–530.

NOTES

- Pédélecq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol* 24:79–88.
- Persechini A, Lynch JA, Romoser VA (1997) Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+} . *Cell Calcium* 22:209–216.
- Peterka DS, Takahashi H, Yuste R (2011) Imaging voltage in neurons. *Neuron* 69:9–21.
- Piljic A, de Diego I, Wilmanns M, Schultz C (2011) Rapid development of genetically encoded FRET reporters. *ACS Chem Biol* 6:685–691.
- Reiff DF, Plett J, Mank M, Griesbeck O, Borst A (2010) Visualizing retinotopic half-wave rectified input to the motion detection circuitry of *Drosophila*. *Nat Neurosci* 13:973–978.
- Sakai R, Repunte-Canonigo V, Raj CD, Knopfel T (2001) Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. *Euro J Neurosci* 13:2314–2318.
- Shepherd GMG, Pologruto TA, Svoboda K (2003) Circuit analysis of experience-dependent plasticity in the developing rat barrel cortex. *Neuron* 38:277–289.
- Shigetomi E, Kracun S, Sofroniew MV, Khakh BS (2010) A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. *Nat Neurosci* 13:759–766.
- Shimomura O, Johnson FH, Saiga Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from luminous hydromedusan, *Aequorea*. *J Cell and Comp Physiol* 59:223–239.
- Siegel MS, Isacoff EY (1997) A genetically encoded optical probe of membrane voltage. *Neuron* 19:735–741.
- Souslova EA, Belousov VV, Lock JG, Stromblad S, Kasparov S, Bolshakov AP, Pinelis VG, Labas YA, Lukyanov S, Mayr LM, Chudakov DM (2007) Single fluorescent protein-based Ca^{2+} sensors with increased dynamic range. *BMC Biotechnol* 7:37.
- Tallini YN, Ohkura M, Choi BR, Ji GJ, Imoto K, Doran R, Lee J, Plan P, Wilson J, Xin HB, Sanbe A, Gulick J, Mathai J, Robbins J, Salama G, Nakai J, Kotlikoff MI (2006) Imaging cellular signals in the heart *in vivo*: Cardiac expression of the high-signal Ca^{2+} indicator GCaMP2. *Proc Natl Acad Sci USA* 103:4753–4758.
- Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, Petreanu L, Akerboom J, McKinney SA, Schreiter ER, Bargmann CI, Jayaraman V, Svoboda K, Looger LL (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods* 6:875–881.
- Tsien RY (2005) Building and breeding molecules to spy on cells and tumors. *FEBS Lett* 579:927–932.
- Tsutsui H, Karasawa S, Okamura Y, Miyawaki A (2008) Improving membrane voltage measurements using FRET with new fluorescent proteins. *Nat Methods* 5:683–685.
- Wang Q, Shui B, Kotlikoff MI, Sonderrmann H (2008) Structural basis for calcium sensing by GCaMP2. *Structure* 16:1817–1827.
- Yamauchi JG, Nemecz A, Quoc TN, Muller A, Schroeder LE, Talley TT, Lindstrom J, Kleinfeld D, Taylor P (2011) Characterizing ligand-gated ion channel receptors with genetically encoded Ca^{++} sensors. *PLoS One* 6(1):e16519.
- Zariwala HA, Borghuis BG, Hoogland TM, Madisen L, Tian L, De Zeeuw CI, Zeng H, Looger LL, Svoboda K, Chen TW (2012) A Cre-dependent GCaMP3 reporter mouse for neuronal imaging *in vivo*. *J Neurosci* 32:3131–3141.
- Zhao Y, Araki S, Wu J, Teramoto T, Chang Y-F, Nakano M, Abdelfattah AS, Fujiwara M, Ishihara T, Nagai T, Campbell RE (2011) An expanded palette of genetically encoded Ca^{2+} indicators. *Science* 333:1888–1891.