



Imaging voltage and brain chemistry with genetically encoded sensors and modulators

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Abstract

Neurons and glia are functionally organized into circuits and higher-order structures that allow the precise information processing required for complex behaviors. To better understand the structure and function of the brain, we must understand synaptic connectivity, action potential generation and propagation, as well as well-orchestrated molecular signaling. Recently, dramatically improved sensors for voltage, intracellular calcium, and neurotransmitters/modulators, combined with advanced microscopy provide new opportunities for *in vivo* dissection of cellular and circuit activity in awake, behaving animals. This review focuses on the current trends in genetically encoded sensors for molecules and cellular events and their potential applicability to the study of nervous system in health and disease.

Addresses

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Introduction

Precise patterns of synaptic connectivity and transmission produce neural circuits and collectively give rise to our perception of the world. To understand neuronal system function, we need to decipher signals within individual neurons and the organization of neuronal populations into circuits and larger structures. A vast array of genetically encoded sensors has been created to monitor neurotransmission, synaptic spillover, excitable membrane potential, calcium dynamics, vesicle trafficking, receptor mobilization, and other biochemical events.

Genetically encoded sensors/modulators can be divided into three main categories: fluorescent [1–5], bioluminescent [6,7], or hybrid sensors [8,9] (Sensors consist of proteins as sensing domain and chemical fluorescent dyes as reporting domain). Fluorescent sensors couple the ligand-induced conformational changes to changes in fluorescence intensity, providing optical readouts of ligand transients. Fluorescent sensors, however, require an external, invasive light source to excite the fluorophore. This may lead to difficulty of light delivery, as well as tissue heating and bleaching of the fluorophore itself. An alternative is to use bioluminescent sensors that encode a light-emitting luciferase, so that the sensor itself is responsible for the required excitation source. Besides protein-based fluorophores, small-molecule-based dyes provide a rich resource for the fluorophore to circumvent the issues of a fluorescent protein-based indicator. Here, we review advances in the development and application of biosensors (Table 1). Such reagents can reveal the underlying molecular mechanisms of nervous system disorders and favor efforts to develop diagnostic and treatment options.

Voltage

Though genetically encoded calcium indicators are the most widely utilized for *in vivo* imaging in model systems including worm, zebrafish, fly, rodent, and recently nonhuman primate, the advance of monitoring circuitry function depends on the improvement of genetically encoded voltage sensors (GEVIs), which directly probe membrane voltage. However, voltage imaging has always been challenging largely because of the fast dynamics of action potentials that occur at the sub-millisecond timescale which in turn limits photon budget. Rigorous protein engineering efforts including rational design and large-scale screening in the past few years have led to significantly improved GEVIs in terms of intrinsic properties such as membrane localization, brightness, sensitivity and kinetics. Combined with KHz acquisition, these highly optimized sensors finally permit robust spike imaging of populational neurons in various brain regions in behaving animals [8,10–14].

The design of GEVIs is either based on a voltage-sensitive domain (VSD) from voltage-sensitive

Table 1

A summary of biophysical properties of genetically encoded indicators.

Indicator	Responsive element	Fluorophore	Ex/Em (nm)	$\Delta F/F$ (%) (<i>in vitro</i> unless otherwise noted)	On/off kinetics ^a	Notes	Ref.
Voltage							
ASAP3	VSD from <i>Gallus</i>	GFP	488/520	-10 ^{+ex vivo} (cortex Layer2/3) per AP	0.90/0.93 ms (on/off)		[10]
SomArchon	Archon1 (Archerhodopsin-3 with 13 point mutations)		637/700	-53 ^{+ex vivo} (cortex Layer2/3) per AP	0.61/1.10 ms (on/off)	NIR GEVI	[11]
Voltron	Ace2N	Janelia Fluor dyes	Depends on the dye	-6.5 ^{+ex vivo} (Layer2/3 cortex) per AP	0.64/0.78 ms (on/off)	Hybrid sensor	[8]
ASAP1-PATM	VSD (<i>Ciona intestinalis</i>)	sfGFP	490/516	-10 per 130 mV	N/A		[12]
VARNAM	<i>Acetabularia</i> opsin	mRuby3	588/592	-2 ^{+ex vivo} (Layer2/3) per AP	0.88/0.8 ms (on/off)		[13]
paQuasAr3s	Archaeorhodopsin 3		637/700	-23 ^{+ex vivo} (L2/3) per AP	0.90/0.93 ms (on/off)	NIR GEVI	[14]
Indicator	Responsive element	Fluorophore	Ex/Em (nm)	$\Delta F/F$ (%) (<i>in vitro</i> unless otherwise noted)	Kd	Notes	Ref.
Metals							
KIRIN1 (Potassium)	BON (bacterial OsmY and nodulation) and LysM (lysine motif)	mCerulean3 and cp173Venus	410/430-650	130 (FRET Ratio)	1.66 ± 0.05 mM	Utilizes FRET	[2]
KIRIN1-GR	BON (bacterial OsmY and nodulation) and LysM (lysine motif)	Clover and mRUBY2	470/490-700	20 (FRET Ratio)	2.56 ± 0.01 mM	Utilizes FRET	[2]
ZIBG1 (Zinc)	Zinc fingers for Zap1	GFP	480/520	150	2.81 μM		[15]
ZIBG2 (Zinc)	Zn hook	GFP	480/520	600	282 nM		[15]
pH/Redox							
roUnaG	UnaG	UnaG	495–530	800		Requires bilirubin as a co-factor	[16]
NeonOxIrr (H2O2)	OxyR transcription factor isolated from <i>E. coli</i>	mNeonGreen	508/520	137			[17]
Neurotransmitters							
GACH (Acetylcholine)	Muscarinic acetylcholine GPCR	GFP	480/520	90.1 ± 1.7	0.7 μM (EC50)		[18]
dLight1.1 (Dopamine)	DRD1	GFP	490/516	230 ± 9	330 nM		[19]
dLight1.2 (Dopamine)	DRD1	GFP	490/516	340 ± 20	770 nM		[19]
dLight1.3a (Dopamine)	DRD1	GFP	490/516	660 ± 30	1.6 μM		[19]
dLight1.3 b (Dopamine)	DRD1	GFP	490/516	930 ± 30	2.3 μM		[19]
	DRD4	GFP	490/516	170 ± 10	4 nM		[19]

(continued on next page)

Table 1. (continued)

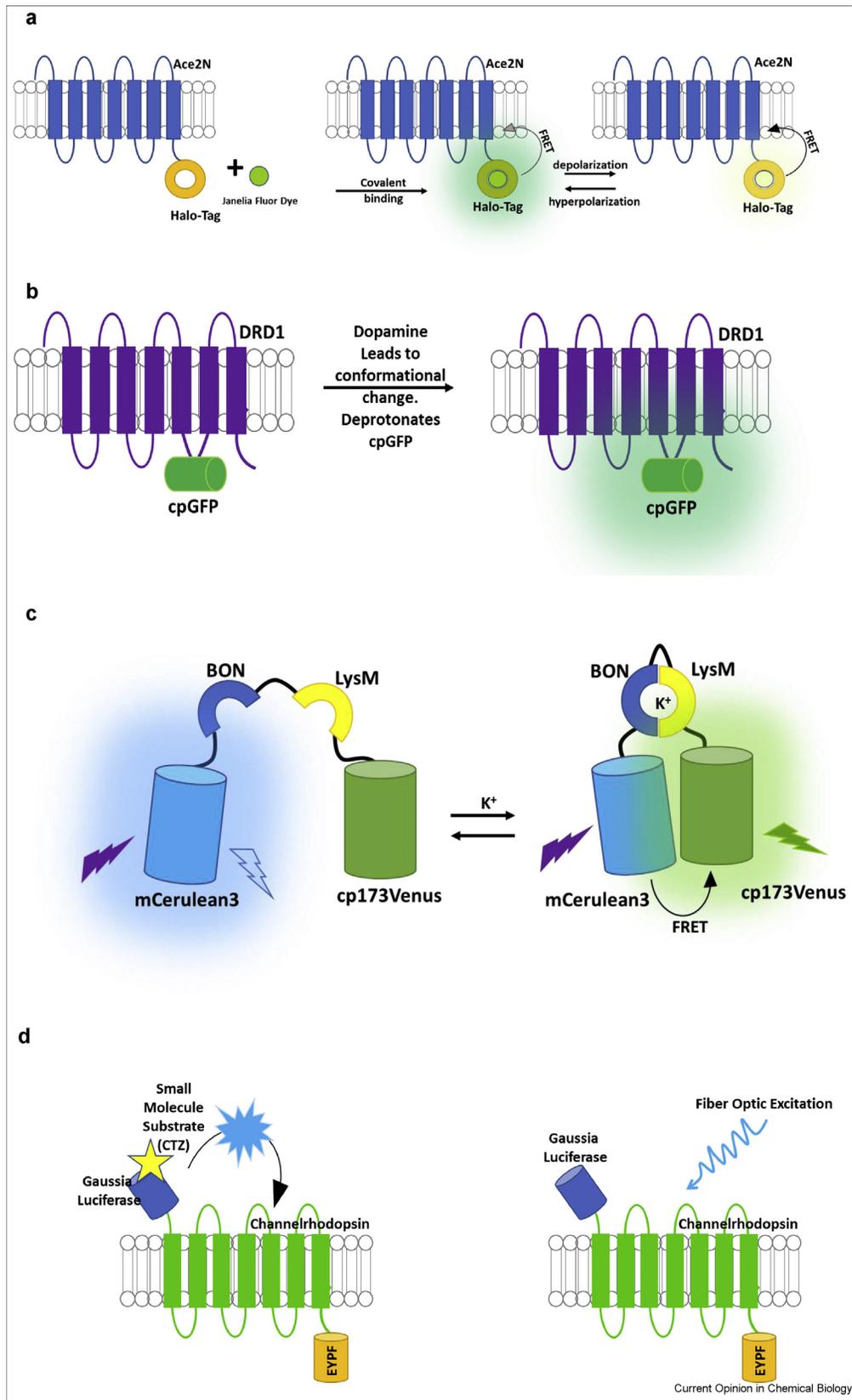
dLight1.4 (Dopamine)	GRAB _{DA1m}	DRD2	EGFP	488/520	~90	130 nM (EC50)	[20]
iGABASnFR (GABA)	GABA-binding protein Pf622 (<i>Pseudomonas fluorescens</i>)	spGFP		490/516	250	9 μ M	[21]
iGlu _u (Glutamate)	Bacterial periplasmic glutamate/aspartate-binding protein (GluBP)	EGFP		488/520	170	600 μ M	[22]
iGlu _r (Glutamate)	Bacterial periplasmic glutamate/aspartate-binding protein (GluBP)	EGFP		488/520	100	137 μ M	[22]
R-iGluSnFR1 (Glutamate)	<i>E. coli</i> protein GltI	mApple		562/588	490 \pm 20	11 μ M	[23]
GRAB _{NE1m} (Norepinephrine)	α 2AR GPCR	EGFP		488/520	230	930 nm	[24]
GRAB _{NE1h} (Norepinephrine)	α 2AR GPCR	EGFP		488/520	130	83 nm	[24]
Purinergic sensors							
iATPSnFR1.0	F ₀ F ₁ -ATPase from <i>Bacillus PS3</i>	sfGFP		488/525	100	350 μ M	[25]
iATPSnFR1.1	F ₀ F ₁ -ATPase from <i>Bacillus PS3</i>	sfGFP		488/525	90	140 μ M	[25]
ARSeNL	ATP-binding ϵ subunit of the ATP synthase from <i>Bacillus subtilis</i>	mScarlet and NanoLuc		470 nm (NanoLuc emission)/650 nm (mScarlet emission)	400%	1.1 \pm 0.2 mM	Utilizes FRET [26]
Luminopsins (LMOs)	Luciferase	Channelrhodopsin	Fluorescent protein	Coupling Efficiency (%)	Notes	Ref	
LMO1	<i>Gaussia</i> luciferase	ChR2	EYFP	0.1		[27,28]	
LMO2	<i>Gaussia</i> luciferase	VChR1	EYFP	1.2		[27,28]	
LMO3	Slow-burn <i>Gaussia</i> Luciferase	VChR1	EYFP	11		[28,29]	
SFLMO _{CS}	Slow-burn <i>Gaussia</i> luciferase	ChR2 (C128S)	EYFP	54.1%		[28]	
SFLMO _{DA}	Slow-burn <i>Gaussia</i> luciferase	ChR2 (D156A)	EYFP	60%		[28]	
SFLMO _{CS/DA}	Slow-burn <i>Gaussia</i> luciferase	ChR2 (C128S/D156A)	EYFP	66.6%		[28]	
Indicator	Responsive element	Fluorophore	Ex/Em (nm)	ΔF/F (%) (in vitro unless otherwise noted)	Kd	Notes	Ref.
Ca²⁺ XCaMP-B	CaM-M13	BFP	374/446		71 nM	Loss of function Indicator i.e. inverted response	[3]
XCaMP-Y	CaM-M13	EGFP	415/527		81 nM		[3]

XCaMP-R	CaM-M13	mApple	561/593		97 nM		[3]
jGCaMPs	Ca68 M-M13	GFP	561/593	373 ± 11.5	68 nM		[30]
jGCaMPf	CaM-M13	GFP	495/515	266.4 ± 9.5	174 nM		[30]
jGCaMPb	CaM-M13	GFP	561/593	241.8 ± 8.3	82 nM		[30]
jGCaMPc	CaM-M13	GFP	495/515	271.8 ± 13.2	298 nM		[30]
iYtNC	TroponinC	EYFP	499/518	−96 ± 31*	295 nM (0 mM Mg ²⁺); 331 nM (1 mM Mg ²⁺)	Inverted response, *responses compared to RGECO1 [31]	[4]
YTnC	TroponinC	EYFP	499/518	1060 ± 37 (0 mM Mg ²⁺); 290 ± 23 (1 mM Mg ²⁺)	223 nM (0 mM Mg ²⁺); 410 nM (1 mM Mg ²⁺)		[32]
GLICO	CaM-M13	GFP/NanoLuc	480/520 (fluorescence mode), (470 nm in bioluminescence mode)	2000 (intensiometric fluorescence mode), 200 (ratiometric bioluminescence mode)	590 nM (Fluorescence); 230 nM (Bioluminescence)	Bimodal Indicator, can be measured in both intensiometric and ratiometric modes	[6]
ReBLICO	CaM-M13	mApple/NanoLuc	480/520 (fluorescence mode), (470 nm in bioluminescence mode)		1576 μM (Fluorescence); 1526 μM (Bioluminescence)	Red bimodal Indicator can be measured in both intensiometric and ratiometric modes. Low affinity indicator. Ideal for ER Ca ²⁺ imaging	[6]
K-GECO1	CaM-M13	eqFP578			165 nM	Red indicator	[33]
GCEPIA1-SNAP _{ER}	CaM-M13	EGFP	488/512		514 nM	ER calcium indicator. Detects the real-time ER calcium store, local calcium microdomains in live cells	[34]
GCaMP6-Xc	CaM-M13	EGFP	488/512			Stabilized GCaMP in its apo state to prevent interaction of CaM with the distal carboxyl tail of CaV1.x and CaV2.x	[35]

VSD, voltage-sensitive domain; GFP, green fluorescent protein; AP, action potential; sfGFP, super folder GFP; NIR, near infrared; GEVI, genetically encoded voltage indicator; FRET, Förster resonance energy transfer; GPCR, G protein-coupled receptor; DRDx, dopamine receptor Dx; EGFP, enhanced GFP; ChR, channelrhodopsin; VChR, Volvox ChR; CaM, calmodulin; BFP, blue fluorescent protein; ER, endoplasmic reticulum.

^a Voltage kinetics evaluated by bi-exponential fitting (in the format fast/slow), where the value in parentheses represents the % of current magnitude in the fast τ component.

Figure 1



a) Schematic representation of VOLTRON. Hybrid voltage indicator. Inverted response. The Janelia Fluor dye is conjugated to halo ligand, and hence, it covalently binds to the HaloTag **(b)** Schematic representation of dLight1.1, a GPCR-based dopamine sensor. The cpGFP is attached to the intracellular

VSD in which a circularly permuted green fluorescent protein (cpGFP) is fused between the third (S3) and fourth transmembrane domain (S4). During membrane depolarization, S4 is charged and prefers to move toward the negative side of the membrane [8,12], which leads to a decrease in fluorescent intensity of cpGFP.

The rhodopsin-based GEVIs consist of two distinct classes. One class uses increases in rhodopsin absorption and fluorescence emission driven by voltage-triggered Schiff base protonation. Archaeorhodopsin 3 (Arch) was the first rhodopsin-based GEVI that is capable of voltage imaging of mammalian neurons. In recent years, a palette of improved Arch have been developed, such as QuasAr 1 [36] to 2 [37], paQuasAr3 [14], and paQuasAr3s [14] and recent Archon [38] and SomArchon [11]. These sensors enable response times in the sub-millisecond range with large sensitivities albeit with one-photon excitation and extremely low quantum yield which is usually orders of magnitude lower than that of fluorescent proteins (FPs). To improve the brightness of rhodopsin-based GEVIs, microbial rhodopsins have been combined with FPs or self-labeling protein tag/synthetic dyes to the design of another class of GEVI, Förster resonance energy transfer (FRET)-opsin. The bright FPs or synthetic dyes serving as the donor of the FRET pair are fused near voltage-sensing rhodopsin serving as the acceptor, enabling voltage-sensitive quenching of fluorophore by the rhodopsin. Several rhodopsins have been combined with a color palette of fluorescence proteins with great kinetics and sensitivity. Most recently, a red-shifted GEVIs VARNAM has been reported [13], which displays exceptional membrane localization and photostability compared with past generation of GEVIs (In VARNAM, the voltage-sensing domain is as opsin, and the change in voltage is reported by a fluorescent protein. The VSD and the fluorescent protein communicate via FRET). However, in acute slices (*ex vivo* preparation of the brain obtained by sectioning brain tissue), VARNAM showed increased intracellular aggregation compared with Ace-mNeon [39] (uses same responsive element as VARNAM), although the sensitivity ($\Delta F/F_0 \sim 4.5\%$ per spike) is similar. Furthermore, neurons expressing VARNAM (ex/em: 588/592) showed little to no response to light of the following wavelengths: 565 nm, 505 nm, or 488 nm which makes it an ideal candidate to be used in multispectral imaging applications.

Besides FPs, small organic synthetic molecules are a rich resource for optical imaging because of their improved

photophysical properties compared with FPs. Recently, a hybrid strategy was used to design Voltron combining Ace2N (an *Acetabularia acetabulum* opsin [39]) in conjunction with Janelia Fluor dyes with high photostability and brightness (Figure 1a). These novel rhodamine dyes are attached to the HaloTag [40] ligand. The Halotag is located at the cell membrane, fused to the VSD. The HaloTag ligand bound to the rhodamine dye is thus localized to the cell membrane and binds to the HaloTag covalently. Voltron₅₂₅ can reliably report voltage changes from the single parvalbumin interneuron in the hippocampal CA1 region (The CA1 region of the hippocampus is made up of various neurons expressing various biomarkers. Parvalbumin is one such biomarker [41]) from a depth of 60 μm and detect voltage signals from pyramidal neurons (a type of unipolar neuron that serve as the primary excitation unit of the mammalian prefrontal cortex [42]) from a depth of 148 μm when the cells are loaded with 1 μM dye.

It is worth mentioning that current generation of GEVIs use special targeting motifs to improve membrane trafficking and localization. For example, ER export tag FCYENEV has been broadly used to prevent cytoplasmic aggregation of the GEVI due to improper trafficking out of the endoplasmic reticulum. Another commonly used localization motif is Kv2.1 tag from inward rectifying potassium channels, which can selectively express GEVIs only on the soma (the cell body of the neuron that contains the nucleus, connects to the dendrites and the axon [42]) [8,43] to avoid signal contamination from the neuropil (the area of the nervous system made up to axons and dendrites with a relatively low number of cell bodies [42]).

Selective photoactivation of cells has also been used to improve the signal-to-noise ratio of voltage imaging by reducing background noises from non-firing cells and neuropils. For example, paQuasAr3 and soma-targeted paQuasAr3s [14] contain a V59A mutation in the responsive element domain which makes them photoactivatable. paQuasAr3 can faithfully report voltage activity from the oriens layer in the hippocampus (20–60 μm below the hippocampal surface) and the pyramidal cell layer (up to 130 μm in depth). Photoactivatability can also be achieved by using photoactivatable fluorescent proteins, as seen with ASAP1-PATM [12]. ASAP1 [44] is a green fluorescent proteins (GFP) based GEVI that uses the voltage-sensitive phosphatase from *Ciona intestinalis* as the responsive element. In ASAP1-PATM, the original GFP

loop 3 of the DRD1 GPCR. This hijacks the GPCR cascade, and the conformational change is conferred to the cpGFP. (c) Schematic representation of KIRIN1, a FRET-based potassium indicator. In the presence of potassium, the responsive element (BON and LysM) brings the mCerulean and cpVenus together such the emission from the excited mCerulean excites the cpVenus (right). In the absence of potassium, only the mCerulean fluoresces. (d) Schematic representation of luminopsins showing both chemogenetic (left) and optogenetic activation of the channelrhodopsin. BON, bacterial OsmY and nodulation; LysM, lysin motif.

is switched out with the super folder GFP (sfGFP) and adding three mutations [45] to the sfGFP that impart photoswitchability. As incremental improvements are made to existing and future probes, GEVIs may finally facilitate systems-level, cellular-resolution voltage imaging in awake behaving mammals.

Neurotransmitters

Building on the successful development of genetically encoded calcium indicators, neurotransmitter sensors have also been developed to sense neurochemistry in the brain. The main component of these neurotransmitter indicators is their responsive element. These responsive elements are either periplasmic-binding protein (PBP) based [21–23] or based on G-protein coupled receptors [18–20,24].

PBP-based sensors works by coupling ligand-induced conformational changes to the fluorescent intensity changes of a circularly permuted Fluorescent Proteins (cpFPs) that is fused near the ligand-binding domain of a PBP. A prime example of a PBP-based neurotransmitter sensor is iGluSnFR (intensiometric glutamate-sensing fluorescent reporter) [46] and its optimized variants iGlu_f (fast) and iGlu_u (ultrafast) [22] widely used for both *in vivo* and *in vitro* glutamate imaging. iGlu_u allows for the imaging synaptic depression and glutamate clearance when 100-Hz spike trains applied to neurons *in vitro*. Currently, there are red versions of iGluSnFR also available known as R-iGluSnFR1 [23]. R-iGluSnFR1 uses mApple instead of the GFP and is available in both circularly permuted and noncircularly permuted forms. Although the fluorescence intensity decreases with the increase of glutamate concentration, R-iGluSnFR1 can reliably resolve action potential-evoked glutamate transients in hippocampal slice culture. iGABASnFR [21] is a PBP-based indicator sensing the most abundant inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). iGABASnFR works by coupling a GABA-binding *Pseudomonas fluorescens* Pf622 to a circularly permuted sfGFP. iGABASnFR offers robust signal *in vitro* in cultured neurons in response to 50 Hz field stimulation. It also allows for imaging of extracellular GABA transients from apical dendrites (a dendrite that emerges from the apex of a pyramidal neuron) of the CA1 region in acute hippocampal slices in response to electrical stimuli (*ex vivo* preparation of the brain obtained by sectioning brain tissue especially the hippocampal region). Using a similar design platform, highly sensitive sensors for maltose [47], organophosphorus [48], acetylcholine [49], glucose [50,51], nicotine [52], and, most recently, serotonin [53] have been developed.

Recently, a new design using G-protein coupled receptors as the ligand-binding domain have been introduced to develop sensors for neuromodulators, such as dopamine, norepinephrine, and serotonin. Patriarchi

et al. [19] (Figure 1b) reported a suite of intensity-based genetically encoded indicators for dopamine, by replacing the intracellular loop 3 of various dopamine receptors with a cpGFP, which is ideal to maximize the coupling of fluorescence changes with conformational changes of the receptor upon ligand binding without interfering with membrane trafficking. The dLight1 family consists of six sensors based on three dopamine receptors (DRD1, DRD2, and DRD4) with broadly tunable affinity and dynamic range to probe dopamine transients across the pM–nM range of concentrations in various brain regions. Similar design has been used to develop sensors for norepinephrine (NE), serotonin, and endorphin peptides [19,54]. Recently, an improved NE sensor, nLight1.3 has been used to examine the distinct temporal integration of NE by astrocytic second messengers (e.g. calcium and cyclic AMP) during vigilance [54].

In addition, GRAB_{DA} [20], on the other hand, is generated based on the same principle as dLight but used dopamine receptor 2 instead of dopamine receptor 1 or 4. GACH [18] is a similar GPCR-based sensor that hijacks the intracellular loop 3 of muscarinic receptor 3 with a cpGFP. The intrinsic properties and applications of both types of GPCR-based sensors have been in depth reviewed and compared Patriarchi et al. [55], Andreoni et al. [56], and Mizuno et al. [57].

Metal ions

The current field of genetically encoded sensors is also seeing the development of various soft and heavy-metal indicators such as potassium and zinc. The role of potassium is very important for the maintenance of fluid and electrolyte balance [58]. Neuronal membrane potential equilibrium and neuronal signal transduction is mediated by potassium and sodium ions [42]. KIRIN1 is a genetically encoded potassium indicator that has two fluorescent proteins with a potassium-responsive element, bacterial OsmY and nodulation (BON) and lysin motif (LysM), in between them [2]. KIRIN1 uses the phenomenon of FRET to convey change in potassium levels (Figure 1c). In the absence of K⁺, the donor (mCerulean) fluoresces as expected. In the presence of K⁺, BON, and LysM bind to each other and pull the two fluorescent proteins close enough so that the emission from mCerulean excites Venus, the FRET acceptor. In cultured neurons, application of glutamate leads to a strong efflux of potassium from the neurons. KIRIN1 can successfully report potassium efflux in cultured neurons in response to 30s perfusion of glutamate. This results in a decrease of FRET ratio 15% (within 200s) before coming back to baseline levels in about ~400s of glutamate perfusion.

In the context of non-neuronal cells, zinc plays an important role in insulin synthesis and is released alongside insulin in the pancreatic β cells. ZIBG [15] is a

genetically encoded zinc indicator. In HEK293 cells, ZIBG showed an increase in fluorescence in response to the addition of zinc. In a mouse insulinoma cell line, MIN6, it detected glucose mediated corelease of zinc. Therefore, this makes ZIBG a *de facto* insulin sensor.

Sensors for purinergic signaling

Adenosine 5' triphosphate (ATP) is the universal cellular energy source, providing energy to perform all physiological functions. Given ATP's widespread functionality in terms of energy equilibrium [59] and cellular signaling, a reliable genetically encoded sensor is crucial. Unfortunately, previous sensors have lacked temporal precision and genetic targetability. However, new generation sensors, such as iATPSnFRs [25] and ARSeNL [26], try to mediate these issues. Both iATPSnFR and ARSeNL use periplasmic binding proteins as their responsive elements. The former is an intensimetric sensor whereas the latter is a bioluminescent ratiometric indicator that uses bioluminescent resonance energy transfer (BRET) to convey signal. Even though iATPSnFR has faster kinetics, ARSeNL has an output signal dynamic range about four times higher than that of its competitor. ARSeNL is a much less-invasive sensor as the fluorophore is excited by light produced by the bioluminescent moiety of the indicator and negates the use of an external invasive light delivery to excite the fluorophore. iATPSnFR expression in astroglia and hippocampal cultures (cultured cells comprising only of astrocytes or dissociated hippocampus plated on tissues culture dishes) displayed good membrane trafficking and a $\Delta F/F_0$ of 110% and 150%, respectively, in response to ATP. It is important to mention that iATPSnFR has great molecular specificity with negligible response to ADP. Sensors such as iATPSnFR are very useful *in vivo* for tumor detection as they secrete high levels of ATP.

Neuromodulation tools

Traditionally, neuromodulation has been done either electrophysiologically with a patch pipet or multielectrode arrays [60]. But these traditional methods are quite invasive and require costly equipment and skilled technicians.

Optogenetics [61], by the use of light-sensitive ion-conducting channelrhodopsins and pumps, has propelled neuromodulation into our grasp. Channelrhodopsins and pumps are light-sensitive membrane-bound proteins that allow unidirectional passage of ions when they are activated by a physical light of a specific wavelength. Therefore, when they are expressed in specific neuronal populations, these channelrhodopsins and pumps can either excite or inhibit said population when a specific wavelength of light excites them. In most cases, the light delivery is via an optical fiber through an optical cannula right above the region of

interest. Most of these channelrhodopsins are blue light (~ 470 nm)—sensitive.

Several studies have recently pointed out that blue light by itself can negatively affect neurons [62–64]. Therefore, sensors are neuromodulators that use blue light for excitation have this undesirable effect. This has led to the emergence of bioluminescent genetically encoded sensors. As previously mentioned, bioluminescent proteins or luciferases are enzymes that can oxidize small-molecule substrates and produce light of a specific wavelength.

Recently, we have seen incorporation of these luciferases in genetically encoded sensors, especially Ca^{2+} indicators. These sensors have a luciferase which can emit its own light by oxidizing its substrate [6,7] in conjunction with a fluorescent protein. Because the light required is supplied by the bioluminescent protein itself, we can eliminate the need for an external invasive light delivery for fluorophore excitation.

A similar approach has also been seen in neuromodulation. Luminopsins [27,28,65–70] are channelrhodopsins or pumps that have a luciferase tethered to their N-terminus. In addition, they are equipped with a fluorescent protein on the C-terminus that serves as a marker of expression (Figure 1d). Application of the small-molecule substrate (coelenterazine) allows the luciferase to oxidize it and produce light, which activates the channelrhodopsin (Keep in mind that the channelrhodopsin can also be modulated by an optic fiber allowing for both optogenetic and chemogenetic control). The optical fiber is highly temporally precise but does not modulate all the neurons expressing the luminopsin, whereas, the systemic application of the small-molecule substrate, coelenterazine (intravenous or intraperitoneal) brings about modulation of all the neurons that express the luminopsin. These small-molecule substrates can cross the blood–brain barrier and have little to no off-target effects [68]. Luminopsins have not only allowed for both optogenetic and chemogenetic control on neurons in a temporally precise manner [27–29,65] but have also been shown to dampen the severity of epileptic seizures [71]. Others have shown that neural stem cells expressing luminopsins, when expressed in a parkinsonian mouse model, alleviates many of the motor issues associated with Parkinson's disease when excited daily (chemogenetically) [67]. Daily stimulation also showed better incorporation of the stem cells.

Conclusion

Protein engineering efforts will continue to improve the intrinsic properties of genetically encoded indicators permitting *in vivo* imaging with high signal to noise ratio (SNR). The hybrid sensors combine the advantage of

both world, genetic specificity/control offered by genetically encoded sensors and greater photophysical properties of synthetic dyes. However, dyes with low toxicity and better cellular permeability or bio-distribution can be better optimized. Finally, there is an emergence of bioluminescent indicators. Although both genetically encoded and genetically encoded hybrid indicators suffer from photobleaching and tissue heating because of the application of external invasive light for fluorophore activation, bioluminescent indicators genetically encode the light source required for fluorophore excitation. However, proper and effective small-molecule substrate delivery is required for optimal functionality of bioluminescent sensors. Further development and refinement of these sensors are required to unravel all the mysteries of the brain.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: L.T. is a co-founder of Seven Biosciences.

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- * of special interest
- ** of outstanding interest

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