Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Neurobiology

Fluorescent sensors for neuronal signaling

Colin P O'Banion and Ryohei Yasuda

Dissecting neuronal structure and function in relation to behavior is an immense undertaking. Researchers require imaging tools to study neuronal activity and biochemical signaling *in situ* in order to study the roles of neuronal and biochemical activity in information processing. A large number of genetically encoded fluorescent biosensors have been reported in the literature over the past few years as there is a push to develop new technology in neuroscience. Here, we review the classes and characteristics of fluorescent biosensors and highlight some considerations that investigators should keep in mind when choosing their tool. In addition, we discuss recent advances in biosensor development.

Address
Max Planck Florida Institute for Neuroscience, United States

Corresponding author: Yasuda, Ryohei (ryohei.yasuda@mpfi.org)

Current Opinion in Neurobiology 2020, **63**:31–41

This review comes from a themed issue on **Cellular neuroscience**

Edited by **Thomas Schwarz** and **Holly Cline**

https://doi.org/10.1016/j.conb.2020.02.007

Introduction

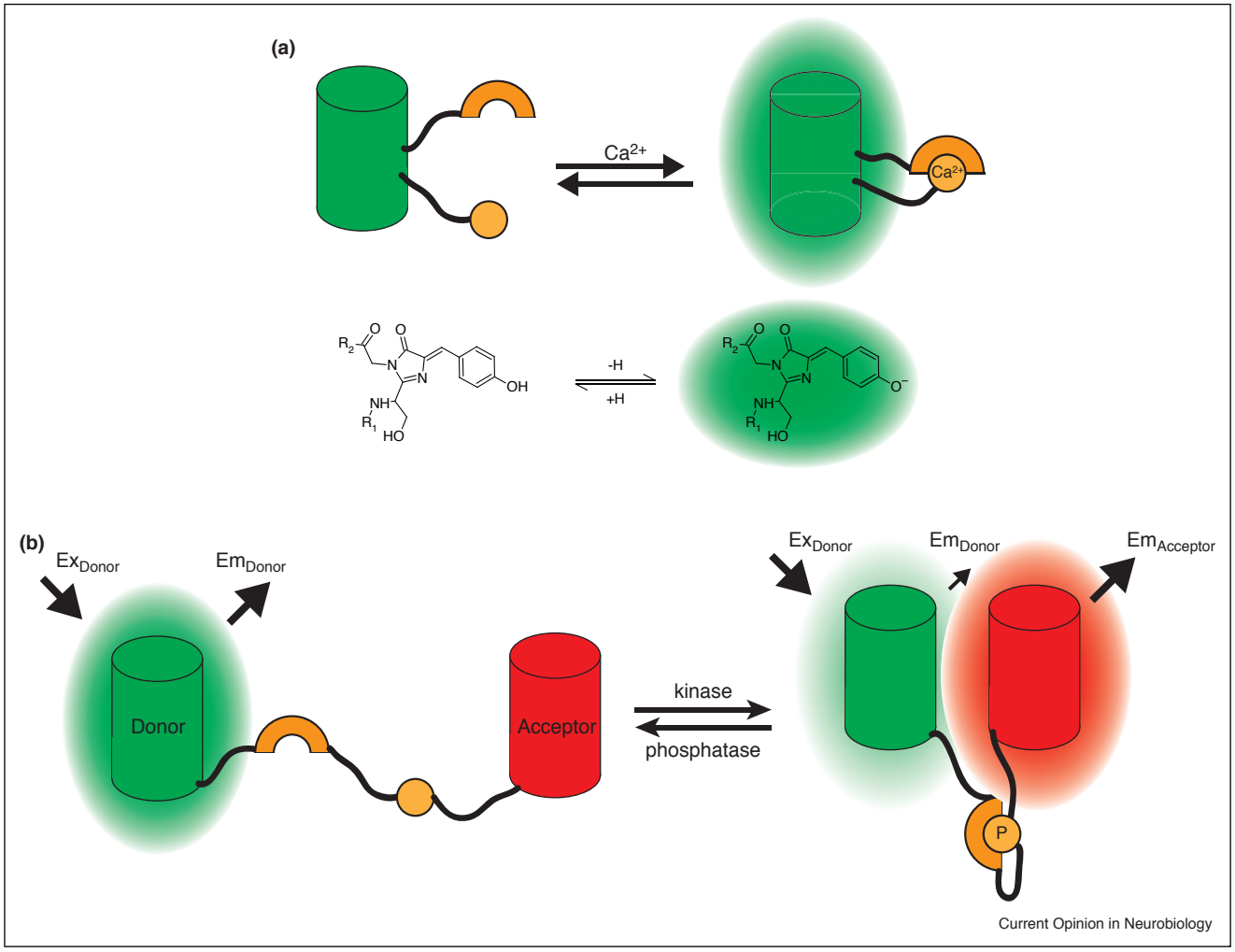
The brain is a highly complex organ composed of nearly 90 billion neurons [1], each with an estimated average of 7000 synaptic connections [2], which yields an estimated ~600 trillion synapses. These staggering numbers alone highlight the herculean feat that neuroscientists face when researching the interplay of more cellular components than the estimated 100 billion stars in our Milky Way. Ensembles of neurons make connections with each other and form circuits to process information. These circuits also interact to form larger circuits. Going down the scale, each neuron includes intracellular molecular networks that regulate synaptic function and neuronal biochemical states, downstream of neurotransmitters and neuromodulators. Interplays between intracellular signaling and synaptic function play essential roles in brain function at different time intervals, especially for the time scale longer than seconds. Therefore, when investigating neural function we need to consider that there are different spatial and temporal scales to monitor the brain. Functional imaging has become one of the major routes to studying neuronal function on different scales from subcellular studies of dendritic spine dynamics to whole cell and circuit level imaging to meso scale and, in small animals, chronic whole brain imaging. Therefore, there is a need for a variety of tools to study the different aspects of neuronal signaling and function. In this review, we will focus on genetically encoded fluorescent sensors for studying neuronal signaling. We will start by discussing the two major classes of fluorescent protein (FP)-based sensors, circularly permuted FP (cpFPs) scaffolds and FRET-based reporters. Then, we will describe recent reports of different sensors starting with those that detect ligand binding to the extracellular surface, followed by second messenger sensors and sensors for their effectors, and finishing with a brief discussion of activity indicators.

cpFP-based sensors

Fluorescent proteins, such as GFP, are composed of a rigid beta barrel with an internal, conformationally constrained helix which serves as a scaffold for the autocatalytic formation of a chromophore from three conserved amino acid residues [3]. The barrel structure of FPs protects the chromophore from environmental effects. The native N and C termini of FPs are located in relatively flexible regions of the protein and therefore, attempts to couple fusion protein conformational changes with GFP fluorescence met limited success plagued by limited dynamic range [4]. However, fusion of the native N and C termini with a short linker and installation of a new N and C terminal in a position near the chromophore (such as at residues 144 and 149 of GFP in GCaMP) results in a cpFP with conformationally dependent fluorescence intensity when coupled to sensory domains that change conformation in response to a signal (Figure 1). In the example of GCaMP above, calmodulin (CaM) and the Ca^{2+}/CaM binding peptide M13 are fused to the new C and N termini of cpGFP respectively. In the absence of Ca^{2+}, CaM and M13 do not interact and the sensor is dim, but upon binding of M13 to Ca^{2+}/CaM, fluorescence increases by several fold [5]. The binding of M13 to Ca^{2+}/CaM induces a conformational change in GFP which correspondingly changes the local chromophore environment and the pKa of a chromophore phenolate residue from neutral (non-fluorescent) to anionic (fluorescent, Figure 1) [6].

In recent years, there has been an explosion of biosensors based on cpFPs, including neurotransmitters and neuromodulators [7–10,11••,12••,13–18], with a wide variety of spectral variants from across the entire visible spectrum and into the near infrared (NIR) [19,20•,21–23]. A major reason for the large effort in developing cpFP-based

Figure 1



Structure of cpFP sensors and mechanism, Structure of FRET sensors and mechanisms.
(a) cpFP sensors such as GCaMP undergo a ligand-dependent conformational change that drives a change in fluorescence intensity (top). This change is mediated by the equilibrium between the non-fluorescent (neutral) and fluorescent (anionic) state of the chromophore (bottom). **(b)** FRET-based sensors undergo a molecular event which drives conformational changes that drive fluorophores to be in close proximity in space which increases FRET efficiency. AKAR is shown as an example.

biosensors is that they are relatively simple to use. As single wavelength, intensiometric sensors they can be used on the majority of microscopes without the need for specialized optics while taking up relatively small spectral space. In addition, cpFP sensors have been engineered to have high dynamic range and are smaller than FRET-based sensors which allows for easier expression across a wide variety of species. However, cpFP biosensors make the FP chromophore more sensitive to environmental changes such as pH or ionic concentrations which can change the spectral properties of the sensor in a ligand-independent manner [4]. In addition, the apparent response amplitude is dependent on sensor concentration as well as other parameters like depth within tissue [24••] resulting in semiquantitative sensing of biomolecules. Finally, extensive optimization of sensor properties such as brightness, dynamic range, and binding kinetics is generally required for each new sensor making development of new biosensors a challenging endeavor. cpFP sensors are listed in Table 1.

FRET-based sensors

Förster resonance energy transfer (FRET)-based tools are another large class of biosensors. FRET is a nonradiative energy transfer mechanism in which a blue shifted donor fluorophore transfers energy to a red shifted acceptor fluorophore exciting an electron and resulting in acceptor emission or relaxation to the ground state (Figure 1). FRET efficiency is dependent on distance between the fluorophores and generally only occurs when fluorescent

Table 1

cpFP fluorescent reporters

Sensor	Ligand	Mechanism of Action	Sensory domain	Donor	Donor Ex/Em	Acceptor	Acceptor Ex/Em	Kd/EC50 (nM)	Citation
iGluSnFR	Glu	cpFP	Glt1 *E. coli*	cpGFP	489/509*			4000	Marvin *et al.* (2013) Nat Methods
SF-iGluSnFS	Glu	cpFP	Glt1 *E. coli*	cpSFGFP	489/509*			4000	Marvin *et al.* (2018) Nat Methods
iGABASnFR	GABA	cpFP	Pf622P. fluorescence	cpGFP	489/509*			9000	Marvin *et al.* Nat Methods 2019
iATPSnFR	ATP	cpFP	Epsilon subunit F0F1-ATPase Bacillus	cpSFGFP	489/509*			50000	Lobas *et al.* Nat Commun 2019
iGlucoSnFR	Glucose	cpFP	Glucose binding protein Thermus thermophiles	cpGFP	489/509*			2.3×10^6	Keller *et al.* 2019
iGlucoSnFR-TS	Glucose	cpFP	Glucose binding protein Thermus thermophiles	cpT-Sapphire	400/510			1.8×10^6	Diaz-Garcia *et al.* J Neuroscience Research
GACh	Acetylcholine	cpFP	M3 Receptor	cpGFP	489/509*			70	Jing *et al.* Nat Biotech 2018
GRABDA1h	Dopamine	cpFP	D2 Receptor	cpGFP	489/509*			10	Sun *et al.* Cell 2018
GRABDA1m	Dopamine	cpFP	D2 Receptor	cpGFP	489/509*			130	Sun *et al.* Cell 2018
dLight1.1	Dopamine	cpFP	D1 Receptor	cpGFP	489/509*			330	Patriarchi *et al.* Science 2018
dLight1.2	Dopamine	cpFP	D1 Receptor	cpGFP	489/509*			770	Patriarchi *et al.* Science 2018
dLight1.3a	Dopamine	cpFP	D1 Receptor	cpGFP	489/509*			2300	Patriarchi et al Science 2018
dLight1.3b	Dopamine	cpFP	D1 Receptor	cpGFP	489/509*			1680	Patriarchi *et al.* Science 2018
GRABNE1h	Norepinepherine	cpFP	alpha2A Receptor	cpGFP	489/509*			93	Feng *et al.* Neuron 2019
GRABNE1m	Norepinepherine	cpFP	alpha2A Receptor	cpGFP	489/509*			1900	Feng *et al.* Neuron 2019
GCaMP7	Calcium	cpFP	CaM/M13	cpGFP	489/509			68-300	Dana *et al.*, Nat Methods 2019
GCaMPX	Calcium	cpFP	CaM/M13/IQ domain	cpGFP	489/509*			N/A	Yang *et al.* Nat Commun 2018
XCaMP-G	Calcium	cpFP	CaM/ckkap	cpGFP	487/514			<100	Inoue *et al.* Cell 2019
XCaMP-B	Calcium	cpFP	CaM/ckkap	cpBFP	374/446			<100	Inoue *et al.* Cell 2019
XCaMP-Y	Calcium	cpFP	CaM/ckkap	cpYFP	503/527			<100	Inoue et al. Cell 2019
XCaMP-R	Calcium	cpFP	CaM/ckkap	cpRFP	561/593			<100	Inoue et al. Cell 2019
jRCaMP	Calcium	cpFP	CaM/M13	cpmRuby	558/605**			214/712	Dana *et al.* eLife 2016
jRGECOP1a	Calcium	cpFP	CaM/M13	cpmApple	568/592**			148	Dana et al. eLife 2016
K-GECO1	Calcium	cpFP	CaM/ckkap	cpFusionRed	508/608**			165	Shen et al. BMC Biology
NIR-GECO1	Calcium	environmental chromophore	CaM/RS20	mIFP	678/704			215	Qian *et al.* Nat Methods 2019
Orange CaMBI	Calcium	BRET	CaM/M13	split NanoLuc	460	CyOFP	497/589		Oh *et al.* Nat Chem Biol 2019
Flamindo2	cAMP	cpFP	EPAC1 CNB	cpCitrine	504/523			3.2	Odaka *et al.* PLoS One 2014
Pink Flamindo	cAMP	cpFP	EPAC1 CNB	cpmApple	567/590			7.2	Harada *et al.* Scientific Reports 2017

Table 1 (*Continued*)

Sensor	Ligand	Mechanism of Action	Sensory domain	Donor	Donor Ex/Em	Acceptor	Acceptor Ex/Em	Kd/EC50 (nM)	Citation
RFlincA	cAMP	cpFP	PKA RI CNB	cpmApple	561/585			300	Ohta *et al.* Scientific Reports 2017
cAMPr	cAMP	cpFP	PKA RI 91-244	cpGFP	489/509*			1000	Hackley *et al.* Science Signaling 2018

* Value from Nakai 2001 Nat. Biotech.
** Values from non-cp version on fpbase.org.

molecules are less than 10 nm away from one another (Figure 1). This property makes FRET an excellent tool for monitoring protein-protein interactions and conformational changes in single polypeptides. However, FRET biosensors require the presence of two fluorescent proteins, which take up more spectral space for multiplexed imaging. In addition, ratiometric FRET measurements can be technically challenging and require specialized optics for proper image acquisition. Ratiometric FRET changes are also dependent on the relative concentrations of donor and acceptor molecules, sensitive to photobleaching, and dependent on imaging depth due to light scattering [24••]. Some of these obstacles can be overcome by the use of 2-photon (2p) fluorescence lifetime imaging (FLIM) in which the donor fluorophore's fluorescence lifetime is measured. Donor fluorescence lifetime decreases when FRET occurs. FLIM is less sensitive to photobleaching, light scattering, and concentration compared to ratiometric FRET, making it the primary method to image FRET *in vivo* [14,24••,25,26]. In addition, since donor lifetime is the only measurement in FLIM, sensors can be designed using dim acceptors with a large molar extinction coefficient but very poor quantum yield, opening spectral space for multichannel imaging. Indeed, a recent publication describes simultaneous imaging of CaMKII activity and RhoA activity in organotypic CA1 neurons using green and red FLIM sensors, respectively [27]. FRET-based sensors are listed in Table 2.

Sensors for neurotransmitters

Neurotransmitters are the chemical messengers of the brain and range from small molecules such as glutamate and GABA to neuropeptides and proteins such as BDNF. Release and binding of neurotransmitters to receptors directly modulates excitability and inhibition of action potentials as well as potentiates or suppresses processes such as synaptic plasticity. In recent years, a large number of neurotransmitter sensors have been developed. All use cpFPs to convert a ligand binding event into a change in GFP fluorescence emission. There are two main sensory scaffolds that are used: bacterial periplasmic binding proteins (PBPs) and G-protein coupled receptors (GPCRs).

PBPs are bacterial proteins that bind small molecule metabolites with a clamshell like mechanism. This conserved conformational change has been leveraged to develop a variety of small molecule neurotransmitter biosensors known as iSnFRs (intensity-based Sensing Fluorescent Reporters). iGluSnFR, a glutamate sensor, is the first in this class of sensors and most widely used as glutamate is the primary excitatory neurotransmitter in the brain. iGluSnFR is constructed from the *Escherichia coli* GltI PBP and cpGFP and expressed on the extracellular surface of the plasma membrane. iGluSnFR showed a $\Delta F/F_{max}$ of 4.5 and bound the structurally similar aspartic acid with similar affinity to glutamate but was highly selective over other amino acids, neurotransmitters and glutamate receptor pharmacological agents. iGluSnFR detects single action potentials (APs) in hippocampal organotypic preps and is responsive to subthreshold levels of glutamate uncaging. 2p *in vivo* simultaneous imaging with the red GECI RCaMP was performed in zebrafish and mice as well [7]. Furthermore, ultrafast variants of iGluSnFR have been developed to resolve high frequency (100 Hz) glutamate release in Schaffer collaterals and identification of a presynaptic mechanism for excitatory post synaptic potential (EPSP) depression during high frequency stimulation [8,28]. A new version of iGluSnFR, SF-iGluSnFR, was recently reported and has improved brightness, response amplitude, and photostability due to replacing cpEGFP with cp-SFGFP (superfolder GFP [29]). This optimized version of iGluSnFR was sensitive enough to perform single spine imaging in rodent barrel cortex and could detect orientation selectivity in individual spines of ferret visual cortex [10]. In addition, Marvin *et al.* [10] introduced a series of blue, cyan, and yellow iGluSnFR variants by replacing cpSFGFP with cp variants of Azurite, mTurquoise2, and mVenus, respectively. Yellow iGluSnFR was capable of resolving spatial and temporal diffusion of glutamate release in culture as well as individual spine orientation tuning in mouse visual cortex when used in conjunction with the recently reporter scanned line angular projection (SLAP) microscopy [30]. The design principle of iGluSnFR used for PBP-cpFP has been recently applied to develop a wide variety of neurotransmitters including GABA [18], ATP [17], and glucose [14,31].

Table 2

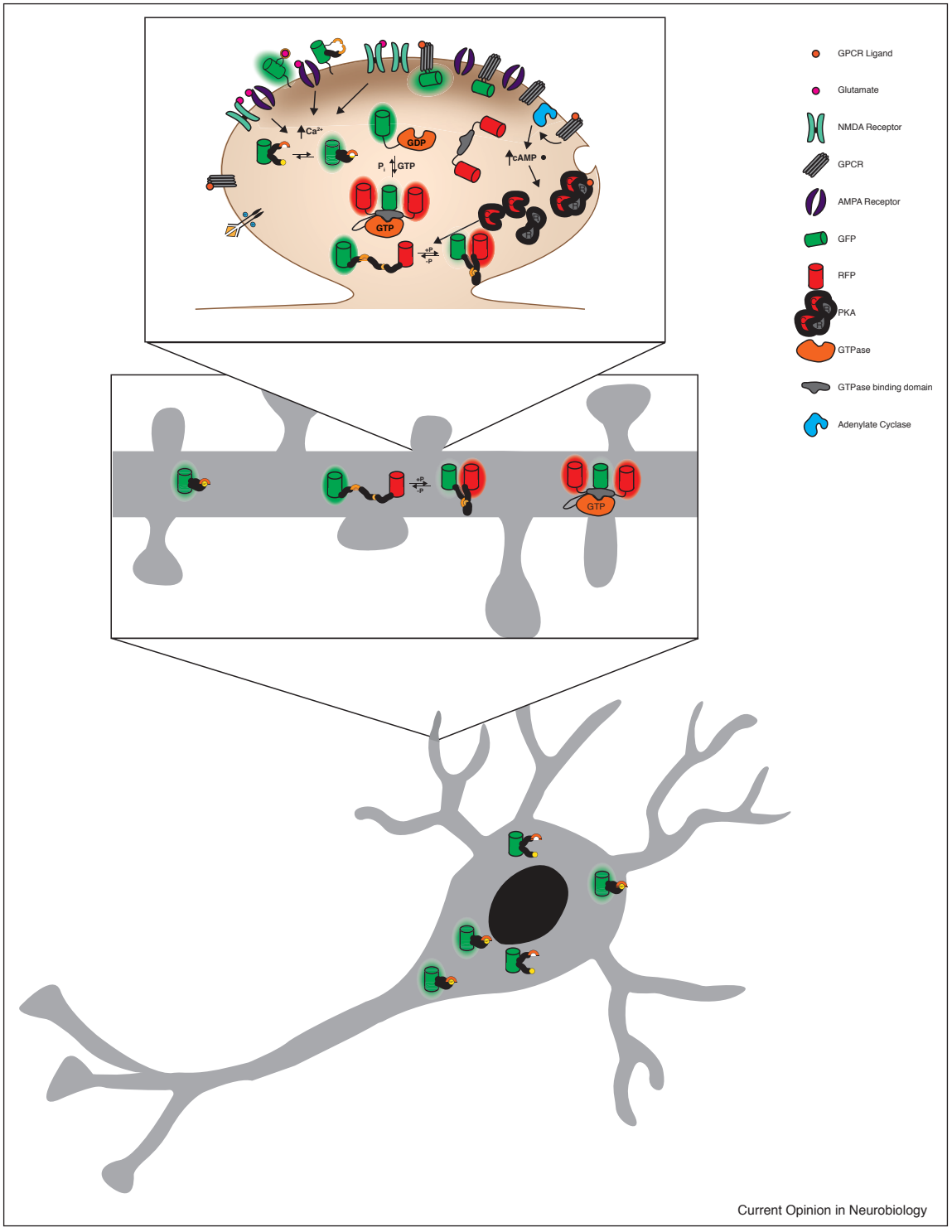
FRET fluorescent reporters

Sensor	Ligand	Mechanism of Action	Sensory domain	Donor	Donor Ex/Em	Acceptor	Acceptor Ex/Em	Citation
ICUE3	cAMP	FRET	EPAC	ECFP	434/477	cpVenus	515/527	DiPilato *et al.* Mol Biosystems 2009
H187	cAMP	FRET	EPAC	mTurquoise2	434/474	tandem cpVenus	515/527	Klarenbeek *et al.* PLoS One 2015
H189	cAMP	FLIM-FRET	EPAC	mTurquoise2	434/474	tandem cpDarkVenus	515/527	Klarenbeek *et al.* PLOS One 2015
AKAR3	phosphoThr	FRET	PKA substrate/ FHA1	ECFP	434/477	cpVenus	515/527	Allen *et al.* Biochem Biophys Res Commun 2006
AKARet	phosphoThr	FLIM-FRET	PKA substrate/ FHA1	EGFP	488/507	sREACHet	510/538	Tang and Yasuda Neuron 2017
EKARet	phosphoThr	FLIM-FRET	ERK substrate and docking/ WW domain	EGFP	488/507	sREACHet	510/538	Tang and Yasuda Neuron 2017
FLIM-AKAR	phosphoThr	FLIM-FRET	PKA substrate/ FHA1	mEGFP	488/507	cpsREACH	510/538	Chen *et al.* Frontiers in Pharmacology 2014
tAKARalpha	phosphoThr	FLIM-FRET	PKA substrate/ FHA1	mEGFP	488/507	cpsREACH	510/538	Ma *et al.* Neuron 2018
iTRACK	N/A	FLIM-FRET	PKC	mEGFP	488/507	tandem mCherry	587/610	Colgan *et al.* Nat Neurosci 2018
iDOCKS	PKC pseudosubstrate	FLIM-FRET	PKC	mEGFP	488/507	tandem mCherry	587/610	Colgan *et al.* Nat Neurosci 2018
Camui		FLIM-FRET	CaMKII alpha	mEGFP	488/507	REACh	510/538	Chang *et al.* Nature Commun 2019
CaMKIIalpha/CaM docking sensor	CaM	FLIM-FRET	CaMKII alpha	mEGFP	488/507	mCherry	587/610	Chang *et al.* Nature Commun 2019
RhoA Sensor	N/A	FLIM-FRET	RhoA/PAK-RBD	mEGFP	488/507	mCherry	587/610	Murakoshi *et al.* Nature 2011
Cdc42 Sensor	N/A	FLIM-FRET	Cdc42/PAK-RBD	mEGFP	488/507	mCherry	587/610	Murakoshi *et al.* Nature 2011
Rac1 Sensor	N/A	FLIM-FRET	Rac1/PAK-RBD	mEGFP	488/507	mCherry	587/610	Hedrick *et al.* Nature 2016

The second sensory scaffold for neurotransmitter sensors is the GPCR. One of the major benefits of using natural GPCRs as sensory modules for neurotransmitter sensors is that evolution has provided a sensor with high ligand specificity and affinity within the ideal physiological range [13]. In addition, binding of ligands to GPCRs occurs in a physiologically relevant temporal domain. GPCRs are the largest family of membrane receptors and bind peptide and protein ligands [16] extending the potential number of unique sensors far beyond those of PBPs. However, GPCRs are notorious for poor trafficking to the membrane [9], and chimeric fusions may inhibit surface expression. Therefore, significant efforts to optimize trafficking to the membrane surface are necessary. However, lessons learned from developing efficient optogenetic trafficking strategies [32] have proven useful in enhancing sensor trafficking [33]. In addition, overexpression of a GPCR may function as a strong local buffer against small concentrations of neurotransmitter. However, GPCR-based sensors show reduced coupling to downstream signaling and internalization relative to endogenous GPCRs [13,16]. GPCR-based sensors are generated by insertion of a cpGFP molecule between transmembrane helices 4 and 5, replacing intracellular loop 3 due to the ligand-dependent conserved conformational change at this position [34].

The labs of Lin Tian and Yulong Li have pioneered a series of GPCR-based sensors over the past few years (Figure 2). The Li lab's sensors are termed the GPCR-activation-based (GRAB) sensors. All of these sensors were optimized for conformational coupling of the GPCR and cpEGFP via linker modification to yield a response of at least 90% $\Delta F/F_{max}$ and were optimized for membrane trafficking via iterative site directed mutagenesis. Despite the relatively small change in fluorescent intensity upon ligand binding, the GRAB sensors are all sensitive enough to detect endogenous release of

Figure 2



Fluorescent reporters for neuronal signaling activity scale from the single spine to whole cell levels.
The spatial resolution of an experiment is defined by the question at hand. Studies can be performed at the single spine level (top) all the way through a whole neuron level (bottom) or even beyond to populations of neurons. Therefore, spatial and temporal signaling dynamics can be measured at many different scales.

neurotransmitters *in vivo*. GRAB sensors have been developed for acetylcholine (GACh) [9], norepinephrine ($GRAB_{NE}$) [15], and dopamine ($GRAB_{DA}$) [12••] using the M3R, α2AR, and D2R, respectively. Interestingly, a D1R-based dopamine sensor was also reported by the Tian lab (dLight) with similar timing to $GRAB_{DA}$ [11••]. Both dopamine sensors were developed with an almost identical approach in terms of scaffold concept, design, and optimization. dLight and $GRAB_{DA}$ variants were tuned to different affinities and dLight variants generally show a larger response amplitude. However, $GRAB_{DA}$ variants express at a brighter basal level than dLight which makes imaging more straightforward. In addition, the dLight paper showed that the intracellular loop 3 insertion of cpEGFP was generalizable across a wide variety of GPCRs including βARs, μ and κ-opioid receptors, 5HT2A receptors, and the melatonin type-2 receptor [11••]. All of the GPCR base sensors have been used for monitoring neuromodulator release *in vivo* by fiber photometry or 2p imaging [9,11••,12••,15,35–39]. It will be exciting to see what else comes from the use of GPCR-based sensors in the coming future.

Sensors of second messengers

Second messengers are intracellular molecules whose release is mediated via an extracellular signal. For example, binding of dopamine to a D1 receptor results in an increase in intracellular cAMP, the second messenger, which binds to, and activates downstream effectors such as the cAMP-dependent protein kinase (PKA) and others. Second messengers are highly regulated in space and time and play core roles in neuronal signaling. Calcium (Ca^{2+}) is, by far, the most well studied neuronal second messenger as Ca^{2+} entry into depolarized postsynaptic terminals is the major driver of action potential generation. Cyclic nucleotides, specifically cyclic adenosine monophosphate (cAMP), are also heavily studied for their roles in modulating synaptic plasticity and coupling synaptic activity with nuclear transcription. Because of their relative synaptic strength in terms of proportion of research focus, calcium and cAMP represent the major targets for sensor development in second messengers.

As stated above, Ca^{2+} signaling is the most widely studied second messenger and used extensively to image neuronal activity. Genetically encoded Ca^{2+} indicators (GECIs) are the primary method by which neuroscientists image intracellular Ca^{2+} levels. GECIs are composed of a Ca^{2+} binding domain, calmodulin (CaM) or troponin, fused to either a cpFP as an intensiometric indicator or to donor and acceptor fluorophores for FRET. An exhaustive review of GECIs is beyond the scope of this manuscript, but several significant advancements in GECI development have been reported recently [40–42].

Great efforts have been placed in developing cpFP-based GECIs in recent years as they have proven to be the backbone of *in vivo* functional imaging in the CNS (Figure 2). GCaMPs are composed of CaM and the Ca^{2+}/CaM binding peptide M13 fused to cpGFP [43]. Recently, new GCaMP variants were introduced to rectify some issues that remained with previous versions. The jGCaMP7 series was described this year to have improved kinetics, brightness, and response amplitude relative to GCaMP6 [44]. GCaMPX addressed issues with the CaM sensory domain of GCaMP interfering with gating and signaling of L-type Ca^{2+} channels and nuclear accumulation/cytotoxicity. The inclusion of a competitive blocker of apoCaM from the IQ domain of neuromodulin was sufficient to inhibit interaction of apoCaM with L-type Ca^{2+} channels but not compete with M13 binding in the Ca^{2+}/CaM state. The addition of a nuclear exclusion sequence (NES) was sufficient to reduce nuclear accumulation [45]. There has also been a recent barrage of chromatic variants on the GCaMP scaffold including a number of red [19,22], NIR [21], and bioluminescent/BRET [46,47] GECIs. Bito *et al.* reported the development of XCaMPs, a multicolored suite of GECIs in which the M13 peptide of GCaMP4.1 was replaced with ckkap peptide from CaMKK-α/β which generates a hill coefficient near 1 indicating linear and not cooperative binding of Ca^{2+} driving the response. To expand the spectral and temporal pallet, the investigators made blue, green, yellow, and red variants as well as ultra-fast green variants. An impressive three color experiment in labeled PV, somatostatin, and excitatory pyramidal neurons in the mPFC with red, fast green, and blue XCaMPs, respectively. Imaging was performed in awake mice with a novel object recognition test at 380 Hz and spikes were observed in all three cell populations [20•].

cAMP is responsible for a wide variety of cellular functions through its effectors including modulation of pre and postsynaptic plasticity, growth cone dynamics, and activity-transcription coupling. cAMP signaling is highly compartmentalized. cAMP biosensors are composed of a cAMP binding domain, either the cyclic nucleotide binding domains of EPAC or PKA, fused to either a cpFP or to a donor and acceptor fluorophore for FRET. A number of cpFP cAMP sensors have been described including green [48,49], yellow [50], and red [51,52] versions. Pink Flamindo, a cpmApple-based cAMP sensor [51], and cAMPr, a cpGFP sensor [49] proved capable of imaging murine astrocytes and drosophila brains *in vivo*. However, cpFP cAMP sensors have seen limited use outside of their original publications. It will be interesting to see how these tools are used in the near future, especially given the potential for coupling red cAMP sensors with optogenetic tools such as photoactivated adenylate cyclases.

FRET-based cAMP sensors have found more applications to imaging at this point. Two groups of FRET-based

cAMP sensors are primarily used and both are based on the cyclic nucleotide binding domain of EPAC [53,54]. Klarenbeek *et al.* extensively optimized a series of cAMP sensors for FRET and FLIM using an mTurquoise2 donor and tandem cpVenus or cpdarkVenus acceptors, respectively. The use of tandem acceptor molecules enhanced FRET efficiency (up to 80% change in FRET in the FLIM version), and enhanced uniform expression in the cytoplasm. The use of mTurquoise2 as a donor served dual purposes of being brighter than other CFP variants such as Cerulean3 and also has a monoexponential lifetime decay curve, which is important for FLIM. A number of interesting studies utilize these FRET-based tools. They have been used to monitor cAMP-dependent respecification of GABAergic D neurons in *Caenorhabditis elegans* and showed phosphodiesterase (PDE) 4 regulated the rate of respecification [55]. A number of studies in culture use cAMP FRET sensors to decipher cellular mechanisms involved in neuroprotection [56] and neurotransmitter's roles in hypertension [57]. More interestingly, cAMP FRET sensors have been used to dissect the complex roles of cAMP in synaptic plasticity. For example, a study by Jayachandran *et al.* used the sensor ICUE3 to show that coronin 1, an actin binding protein, is necessary for cAMP-dependent plasticity [58]. ICUE3 proved useful in identifying the role of the N-terminus of PDE4A5 in compartmentalized signaling involved in cAMP-dependent plasticity in hippocampal cultures [59]. Finally, cAMP FRET sensors were used to define an AKAP mediated, PDE4-dependent negative feedback loop in developing hippocampal cultures that mediate axonal length [60]. While the majority of cAMP studies to date have been performed in culture, we hope that the use of cpFP and FLIM cAMP sensors will be used to image dynamic cAMP signaling *in vivo* in the near future. The development of spectrally resolvable up and downstream sensors to cAMP will pave the way for simultaneous imaging of multiple signaling pathways illuminating the spatiotemporal dynamics of a highly compartmentalized system.

Sensors for signaling downstream of second messengers

Sensors for biochemical effectors such as kinases and GTPases are primarily FRET based sensors and FLIM-FRET is the dominant imaging mode for neuroscience (FRET sensors are listed in Table 2). This is perhaps because the time scale of downstream signaling tends to be much slower, from seconds to hours, making it difficult to quantify with single-wavelength sensor in the presence of drift, bleaching and concentration/structural fluctuation. Sensor designs vary but generally are based on the catalytic induction of protein-protein interactions, which causes donor-accepter distances less than 10 nm, favoring FRET as a quantitative measure of distance. As stated in a previous section, FLIM has also been especially popular as its compatibility with 2p imaging for organotypic slices and *in vivo* [14,24••,25]. In addition, FLIM allows imaging of only the donor fluorophore in a FRET sensor which, when a dim acceptor is used, allows for multiplexed imaging of other FLIM or intensiometric reporters. Finally, FLIM is preferable for organotypic and *in vivo* imaging over ratiometric FRET because lifetime signals are largely independent of reporter concentration and light scattering due to imaging depth [61].

PKA is one of the most studied kinases in all of biology and neuroscience is no different. A-kinase activity reporter (AKAR) (Figure 2), a FRET-based sensor for PKA activation, and its variants have been widely used [62]. AKAR is made of a donor FP fused to the FHA1 phosphoSer/Thr binding domain which is connected to a PKA substrate and acceptor FP via a variable linker region. A number of PKA FLIM sensors have been reported in recent years. Sabatini and colleagues developed FLIM AKAR by replacing the CFP and YFP FRET pair of AKAR3 with the mEGFP and the non-radiative cp-sREACH (Resonance Energy-Accepting Chromoprotein [63,64]) pair, respectively[25,65]. These changes to AKAR3 were necessary to generate a FLIM probe as early generation CFPs did not have a monoexponential decay, a necessary attribute of good FLIM donors. FLIM AKAR was then used to perform an elegant study describing PKA activation via Gαq coupled mAChRs in hippocampal neurons and that PKA signaling via Gαq is conserved amongst multiple GPCRs [26]. The Sabatini group has also reported, recently, the use of FLIM AKAR in 2p FLIM fiber photometry experiments monitoring deep striatal neurons and behavior induced PKA dynamics in the nucleus accumbens [66]. FLIM AKAR was also recently targeted to multiple subcellular compartments and targeting to microtubules via a Map2c microtubule binding domain generated a version of the sensor with enhanced dynamic range and response amplitude. The study also identified major differences in basal PKA activity between neuronal subtypes and imaged dynamic PKA signaling *in vivo* in multiple brain regions in response to behavioral stimuli [24••,67]. Tang and Yasuda reported the development of PKA and ERK sensors with enhanced sensitivity for imaging spines during structural LTP 2p glutamate uncaging [68]. EKAR (ERK sensor) [69] and AKAR3 (PKA sensor) were used as scaffolds. Donor and acceptor fluorophores were replaced on each scaffold with mEGFP and sREACHet and the linker region between the phosphobinding domain and kinase substrate domain was replaced with a long flexible linker. In addition, a monomeric mutation in the acceptor fluorophore was removed and mutations to increase surface hydrophobicity were introduced to promote interactions between the donor and acceptor fluorophores thus enhancing FRET sensitivity. Indeed, 2p glutamate uncaging combined with FLIM imaging of the sensors revealed rapid activation of ERK and PKA signaling which originated in the spine and diffused about

10 μm down the adjacent dendrite indicating that kinase activity serves to propagate sLTP signals to distal cellular locations.

Protein kinase C (PKC) activity is also required for synaptic plasticity. Colgan et al. recently describe two FLIM sensors for classical PKC isozymes [70••]. One sensor (isozyme specific translocation of C kinase: iTRACK) is based on the well characterized translocation of PKC to the plasma membrane upon activation where an eGFP tagged PKC FRETs with a plasma membrane localized mCherry. The other (isozyme specific docking of C kinase substrate: iDOCKS) measures PKC binding to a pseudosubstrate as substrate binding only occurs upon activation. Using these tools and 2p glutamate uncaging in slices the investigators showed that PKC alpha is required for sLTP in a NMDAR-dependent and TrkB-dependent mechanism. FLIM sensors and 2p glutamate uncaging have also been extensively applied to studying CaMKII signaling in CA1 hippocampal neurons in organotypic slices. For example, CaMKII was shown to be a leaky biochemical integrator of Ca^{2+} signaling and autophosphorylation at Thr289 is required for spine plasticity using a FLIM version of Camui [71]. A FLIM sensor that reports CaMKIIα association with CaM was used in conjunction with FLIM Camui to show that the fraction of CaMKII bound to Ca/CaM is constant in response to trains of 2p glutamate uncaging stimuli. However, the FLIM Camui sensor showed stepwise activation of CaMKII indicating that the majority of CaMKII activity during spine plasticity is autonomous of CaM binding [72]. A series of studies from our group investigating the roles of Rho family GTPases in sLTP used FLIM sensors composed of mEGFP-GTPase fusions as FRET donors and mCherry flanked GTPase binding domains as acceptors [73,74]. Sensors were developed for RhoA, Rac1, and Cdc42 and imaged in conjunction with 2p glutamate uncaging in organotypic hippocampal slices. The studies found that activation or all three GTPases in a spine predicts the occurrence of sLTP and that Cdc42 and Rac1 activation was dependent on postsynaptic, autocrine BDNF signaling. In addition, RhoA and Rac1 activity diffused to neighboring spines, priming them for plasticity.

Concluding remarks and future directions

Recent years have produced a wealth of fluorescent biosensors for use in studying neuronal signaling. In addition to those discussed above, other types of sensors including Ca^{2+} integrators [75,76] and activity-transcription coupling indicators [77] have emerged as new modalities for monitoring neuronal activity. The number of tools being generated is constantly expanding and therefore researchers must be considerate of the different properties of each and select a given tool because it is the best fit for their experiment.

It is especially exciting to see the emergence of red and far-red/NIR genetically encoded sensors [19,20•,21,22,51,52]. This red spectral shift to opens up the possibility for simultaneous and near-simultaneous imaging of multiple biochemical pathways using a combination of sensors. Indeed, a number of studies highlighted in this manuscript have already used multiplexed sensors [7,20•,27,77]. Improvements in optical hardware and software will also enhance our ability to image many pathways in parallel thus granting researchers the ability to probe biology with greater spatial and temporal resolution.

Conflict of interest statement

RY is a founder of Florida Lifetime Imaging LLC, a company that helps researchers to setup fluorescence lifetime imaging microscopy.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Herculano-Houzel S: **The human brain in numbers: a linearly scaled-up primate brain**. *Front Hum Neurosci* 2009, **3**.
2. Drachman DA: **Do we have brain to spare?** *Neurology* 2005, **64**:2004-2005.
3. Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ: **Crystal structure of the aequorea victoria green fluorescent protein**. *Science* 1996, **273**:1392-1395.
4. Kostyuk AIDA, Kotova DA, Belousov VV, Bilan DS: **Circularly permuted fluorescent protein-based indicators: history, principles, and classification**. *Int J Mol Sci* 2019, **20**.
5. Akerboom J, Rivera JD, Guilbe MM, Malave EC, Hernandez HH, Tian L, Hires SA, Marvin JS, Looger LL, Schreiter ER: **Crystal structures of the gcamp calcium sensor reveal the mechanism of fluorescence signal change and aid rational design**. *J Biol Chem* 2009, **284**:6455-6464.
6. Barnett LM, Hughes TE, Drobizhev M: **Deciphering the molecular mechanism responsible for gcamp6m's Ca2+-dependent change in fluorescence**. *PLoS One* 2017, **12**:e0170934.
7. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen TW, Bargmann CI *et al.*: **An optimized fluorescent probe for visualizing glutamate neurotransmission**. *Nat Methods* 2013, **10**:162-170.
8. Helassa N, Durst CD, Coates C, Kerruth S, Arif U, Schulze C, Wiegert JS, Geeves M, Oertner TG, Torok K: **Ultrafast glutamate sensors resolve high-frequency release at schaffer collateral synapses**. *Proc Natl Acad Sci U S A* 2018, **115**:5594-5599.
9. Jing M, Zhang P, Wang G, Feng J, Mesik L, Zeng J, Jiang H, Wang S, Looby JC, Guagliardo NA *et al.*: **A genetically encoded fluorescent acetylcholine indicator for in vitro and in vivo studies**. *Nat Biotechnol* 2018, **36**:726-737.
10. Marvin JS, Scholl B, Wilson DE, Podgorski K, Kazemipour A, Muller JA, Schoch S, Quiroz FJU, Rebola N, Bao H *et al.*: **Stability, affinity, and chromatic variants of the glutamate sensor iglusnfr**. *Nat Methods* 2018, **15**:936-939.
11. •• Patriarchi T, Cho JR, Merten K, Howe MW, Marley A, Xiong WH, Folk RW, Broussard GJ, Liang R, Jang MJ *et al.*: **Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors**. *Science* 2018, **360**

Patriarchi *et al.* describe the generation of dLight GPCR-based sensors for imaging dopamine signaling *in vivo*. A cpGFP was inserted into the third intracellular loop of D1R and optimized for response to dopamine. Imaging was performed across multiple species highlighting the potential generalization of this tool in studying neuromodulation. See also Sun *et al.* (2018).

12. •• Sun F, Zeng J, Jing M, Zhou J, Feng J, Owen SF, Luo Y, Li F, Wang H, Yamaguchi T *et al.*: **A genetically encoded fluorescent sensor enables rapid and specific detection of dopamine in flies, fish, and mice**. *Cell* 2018, **174**:481-496.e19

This paper describes the development of GRABDA sensors for imaging dopamine signaling across a wide range of species in vivo. GRABDA sensors are similar to dLight sensors in the general architecture but use the D2R as a signaling domain. Also see Patriarchi et al. (2018).

13. Wang H, Jing M, Li Y: **Lighting up the brain: genetically encoded fluorescent sensors for imaging neurotransmitters and neuromodulators**. *Curr Opin Neurobiol* 2018, **50**:171-178.

14. Diaz-Garcia CM, Lahmann C, Martinez-Francois JR, Li B, Koveal D, Nathwani N, Rahman M, Keller JP, Marvin JS, Looger LL, Yellen G: **Quantitative in vivo imaging of neuronal glucose concentrations with a genetically encoded fluorescence lifetime sensor**. *J Neurosci Res* 2019, **97**:946-960.

15. Feng J, Zhang C, Lischinsky JE, Jing M, Zhou J, Wang H, Zhang Y, Dong A, Wu Z, Wu H *et al.*: **A genetically encoded fluorescent sensor for rapid and specific in vivo detection of norepinephrine**. *Neuron* 2019, **102**:745-761.e8.

16. Jing M, Zhang Y, Wang H, Li Y: **G-protein-coupled receptor-based sensors for imaging neurochemicals with high sensitivity and specificity**. *J Neurochem* 2019, **151**:279-288.

17. Lobas MA, Tao R, Nagai J, Kronschlager MT, Borden PM, Marvin JS, Looger LL, Khakh BS: **A genetically encoded single-wavelength sensor for imaging cytosolic and cell surface atp**. *Nat Commun* 2019, **10**:711.

18. Marvin JS, Shimoda Y, Magloire V, Leite M, Kawashima T, Jensen TP, Kolb I, Knott EL, Novak O, Podgorski K *et al.*: **A genetically encoded fluorescent sensor for in vivo imaging of gaba**. *Nat Methods* 2019, **16**:763-770.

19. Dana H, Mohar B, Sun Y, Narayan S, Gordus A, Hasseman JP, Tsegaye G, Holt GT, Hu A, Walpita D *et al.*: **Sensitive red protein calcium indicators for imaging neural activity**. *eLife* 2016, **5**.

20. • Inoue M, Takeuchi A, Manita S, Horigane SI, Sakamoto M, Kawakami R, Yamaguchi K, Otomo K, Yokoyama H, Kim R *et al.*: **Rational engineering of xcamps, a multicolor geci suite for in vivo imaging of complex brain circuit dynamics**. *Cell* 2019, **177**:1346-1360.e24

This paper describes the design, construction, optimzation, characterization, and validation of a suite of multi-color calcium indicators. The investigators report impressive multi-channel imaging experiments and the sensors show impressive performance.

21. Qian Y, Piatkevich KD, Mc Larney B, Abdelfattah AS, Mehta S, Murdock MH, Gottschalk S, Molina RS, Zhang W, Chen Y *et al.*: **A genetically encoded near-infrared fluorescent calcium ion indicator**. *Nat Methods* 2019, **16**:171-174.

22. Shen Y, Dana H, Abdelfattah AS, Patel R, Shea J, Molina RS, Rawal B, Rancic V, Chang YF, Wu L *et al.*: **A genetically encoded Ca(2+) indicator based on circularly permutated sea anemone red fluorescent protein eqfp578**. *BMC Biol* 2018, **16**:9.

23. Inoue Masatoshi, Takeuchi Atsuya, Manita Satoshi, Horigane Shin-ichiro, Sakamoto Masayuki, Kawakami Ryosuke, Yamaguchi Kazushi, Otomo Kouhei, Yokoyama Hiroyuki, Kim Ryang *et al.*: **Rational engineering of xcamps, a multicolor geci suite for in vivo imaging of complex brain circuit dynamics**. *Cell* 2019, **177**:1346-1360.e24.

24. •• Ma L, Jongbloets BC, Xiong WH, Melander JB, Qin M, Lameyer TJ, Harrison MF, Zemelman BV, Mao T, Zhong H: **A highly sensitive a-kinase activity reporter for imaging neuromodulatory events in awake mice**. *Neuron* 2018, **99**:665-679.e5

This manuscript reports an improved PKA FLIM sensor. The investigators characterize the sensor in hippocampal organotypic slices before using it to image behavior-dependent changes in PKA activity in awake mice. This is the first report describing imaging of PKA activity *in vivo*.

25. Chen Y, Saulnier JL, Yellen G, Sabatini BL: **A pka activity sensor for quantitative analysis of endogenous gpcr signaling via 2-photon fret-flim imaging**. *Front Pharmacol* 2014, **5**.

26. Chen Y, Granger AJ, Tran T, Saulnier JL, Kirkwood A, Sabatini BL: **Endogenous galphaq-coupled neuromodulator receptors activate protein kinase a**. *Neuron* 2017, **96**:1070-1083.e5.

27. Laviv T, Kim BB, Chu J, Lam AJ, Lin MZ, Yasuda R: **Simultaneous dual-color fluorescence lifetime imaging with novel red-shifted fluorescent proteins**. *Nat Methods* 2016, **13**:989-992.

28. Durst CD, Wiegert JS, Helassa N, Kerruth S, Coates C, Schulze C, Geeves MA, Torok K, Oertner TG: **High-speed imaging of glutamate release with genetically encoded sensors**. *Nat Protoc* 2019, **14**:1401-1424.

29. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS: **Engineering and characterization of a superfolder green fluorescent protein**. *Nat Biotechnol* 2006, **24**:79-88.

30. Kazemipour A, Novak O, Flickinger D, Marvin JS, Abdelfattah AS, King J, Borden PM, Kim JJ, Al-Abdullatif SH, Deal PE *et al.*: **Kilohertz frame-rate two-photon tomography**. *Nat Methods* 2019, **16**:778-786.

31. Keller JP, Marvin JS, Lacin H, Lemon WC, Shea J, Kim S, Lee RT, Koyama M, Keller PJ, Looger LL: *In vivo glucose imaging in multiple model organisms with an engineered single-wavelength sensor*. 2019.

32. Rost BR, Schneider-Warme F, Schmitz D, Hegemann P: **Optogenetic tools for subcellular applications in neuroscience**. *Neuron* 2017, **96**:572-603.

33. Wu Z, Feng J, Jing M, Li Y, Luo Q, Ding J, Fu L: **G protein-assisted optimization of gpcr-activation based (grab) sensors**. *Neural Imaging Sens* 2019, **2019**.

34. Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM *et al.*: **Activation and allosteric modulation of a muscarinic acetylcholine receptor**. *Nature* 2013, **504**:101-106.

35. Corre J, van Zessen R, Loureiro M, Patriarchi T, Tian L, Pascoli V, Luscher C: **Dopamine neurons projecting to medial shell of the nucleus accumbens drive heroin reinforcement**. *eLife* 2018, **7**.

36. Dong H, Wang J, Yang YF, Shen Y, Qu WM, Huang ZL: **Dorsal striatum dopamine levels fluctuate across the sleep-wake cycle and respond to salient stimuli in mice**. *Front Neurosci* 2019, **13**.

37. de Jong JW, Afjei SA, Pollak Dorocic I, Peck JR, Liu C, Kim CK, Tian L, Deisseroth K, Lammel S: **A neural circuit mechanism for encoding aversive stimuli in the mesolimbic dopamine system**. *Neuron* 2019, **101**:133-151 e7.

38. Mohebi A, Pettibone JR, Hamid AA, Wong JT, Vinson LT, Patriarchi T, Tian L, Kennedy RT, Berke JD: **Dissociable dopamine dynamics for learning and motivation**. *Nature* 2019, **570**:65-70.

39. Tanaka M, Sun F, Li Y, Mooney R: **A mesocortical dopamine circuit enables the cultural transmission of vocal behaviour**. *Nature* 2018, **563**:117-120.

40. Lin MZ, Schnitzer MJ: **Genetically encoded indicators of neuronal activity**. *Nat Neurosci* 2016, **19**:1142-1153.

41. Mank M, Griesbeck O: **Genetically encoded calcium indicators**. *Chem Rev* 2008, **108**:1550-1564.

42. Broussard GJ, Liang R, Tian L: **Monitoring activity in neural circuits with genetically encoded indicators**. *Front Mol Neurosci* 2014, **7**.

43. Nakai J, Ohkura M, Imoto K: **A high signal-to-noise ca(2+) probe composed of a single green fluorescent protein**. *Nat Biotechnol* 2001, **19**:137-141.

44. Dana H, Sun Y, Mohar B, Hulse BK, Kerlin AM, Hasseman JP, Tsegaye G, Tsang A, Wong A, Patel R *et al.*: **High-performance calcium sensors for imaging activity in neuronal populations and microcompartments**. *Nat Methods* 2019, **16**:649-657.

45. Yang Y, Liu N, He Y, Liu Y, Ge L, Zou L, Song S, Xiong W, Liu X: **Improved calcium sensor gcamp-x overcomes the calcium channel perturbations induced by the calmodulin in gcamp**. *Nat Commun* 2018, **9**:1504.

46. Yang J, Cumberbatch D, Centanni S, Shi S-q, Winder D, Webb D, Johnson CH: **Coupling optogenetic stimulation with nanoluc-based luminescence (bret) ca++ sensing**. *Nat Commun* 2016, **7**:13268.

47. Suzuki K, Kimura T, Shinoda H, Bai G, Daniels MJ, Arai Y, Nakano M, Nagai T: **Five colour variants of bright luminescent protein for real-time multicolour bioimaging**. *Nat Commun* 2016, **7**:13718.

48. Kitaguchi T, Oya M, Wada Y, Tsuboi T, Miyawaki A: **Extracellular calcium influx activates adenylate cyclase 1 and potentiates insulin secretion in min6 cells**. *Biochem J* 2013, **450**:365-373.

49. Hackley CR, Mazzoni EO, Blau J: **Campr: a single-wavelength fluorescent sensor for cyclic amp**. *Sci Signal* 2018, **11**.

50. Odaka H, Arai S, Inoue T, Kitaguchi T: **Genetically-encoded yellow fluorescent camp indicator with an expanded dynamic range for dual-color imaging**. *PLoS One* 2014, **9**:e100252.

51. Harada K, Ito M, Wang X, Tanaka M, Wongso D, Konno A, Hirai H, Hirase H, Tsuboi T, Kitaguchi T: **Red fluorescent protein-based camp indicator applicable to optogenetics and in vivo imaging**. *Sci Rep* 2017, **7**:7351.

52. Ohta Y, Furuta T, Nagai T, Horikawa K: **Red fluorescent camp indicator with increased affinity and expanded dynamic range**. *Sci Rep* 2018, **8**:1866.

53. DiPilato LM, Zhang J: **The role of membrane microdomains in shaping beta2-adrenergic receptor-mediated camp dynamics**. *Mol Biosyst* 2009, **5**:832-837.

54. Klarenbeek J, Goedhart J, van Batenburg A, Groenewald D, Jalink K: **Fourth-generation epac-based fret sensors for camp feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for flim, for ratiometry and with high affinity**. *PLoS One* 2015, **10**:e0122513.

55. Yu B, Wang X, Wei S, Fu T, Dzakah EE, Waqas A, Walthall WW, Shan G: **Convergent transcriptional programs regulate camp levels in *C. elegans* gabaergic motor neurons**. *Dev Cell* 2017, **43**:212-226 e7.

56. Liu B, Mosienko V, Vaccari Cardoso B, Prokudina D, Huentelman M, Teschemacher AG, Kasparov S: **Glio- and neuro-protection by prosaposin is mediated by orphan g-protein coupled receptors gpr37l1 and gpr37**. *Glia* 2018, **66**:2414-2426.

57. Bardsley EN, Davis H, Buckler KJ, Paterson DJ: **Neurotransmitter switching coupled to beta-adrenergic signaling in sympathetic neurons in prehypertensive states**. *Hypertension* 2018, **71**:1226-1238.

58. Jayachandran R, Liu X, Bosedasgupta S, Muller P, Zhang CL, Moshous D, Studer V, Schneider J, Genoud C, Fossoud C *et al.*: **Coronin 1 regulates cognition and behavior through modulation of camp/protein kinase a signaling**. *PLoS Biol* 2014, **12**:e1001820.

59. Havekes R, Park AJ, Tolentino RE, Bruinenberg VM, Tudor JC, Lee Y, Hansen RT, Guercio LA, Linton E, Neves-Zaph SR *et al.*: **Compartmentalized pde4a5 signaling impairs hippocampal synaptic plasticity and long-term memory**. *J Neurosci* 2016, **36**:8936-8946.

60. Gorshkov K, Mehta S, Ramamurthy S, Ronnett GV, Zhou FQ, Zhang J: **Akap-mediated feedback control of camp gradients in developing hippocampal neurons**. *Nat Chem Biol* 2017, **13**:425-431.

61. Yasuda R: **Imaging spatiotemporal dynamics of neuronal signaling using fluorescence resonance energy transfer and fluorescence lifetime imaging microscopy**. *Curr Opin Neurobiol* 2006, **16**:551-561.

62. Zhang J, Ma Y, Taylor SS, Tsien RY: **Genetically encoded reporters of protein kinase a activity reveal impact of substrate tethering**. *Proc Natl Acad Sci U S A* 2001, **98**:14997-15002.

63. Murakoshi H, Lee SJ, Yasuda R: **Highly sensitive and quantitative fret-flim imaging in single dendritic spines using improved non-radiative yfp**. *Brain Cell Biol* 2008, **36**:31-42.

64. Ganesan S, Ameer-Beg SM, Ng TT, Vojnovic B, Wouters FS: **A dark yellow fluorescent protein (yfp)-based resonance energy-accepting chromoprotein (reach) for forster resonance energy transfer with gfp**. *Proc Natl Acad Sci U S A* 2006, **103**:4089-4094.

65. Allen MD, Zhang J: **Subcellular dynamics of protein kinase a activity visualized by fret-based reporters**. *Biochem Biophys Res Commun* 2006, **348**:716-721.

66. Lee SJ, Chen Y, Lodder B, Sabatini BL: **Monitoring behaviorally induced biochemical changes using fluorescence lifetime photometry**. *Front Neurosci* 2019, **13**.

67. Jongbloets BC, Ma L, Mao T, Zhong H: **Visualizing protein kinase a activity in head-fixed behaving mice using in vivo two-photon fluorescence lifetime imaging microscopy**. *J Vis Exp* 2019, **148**.

68. Tang S, Yasuda R: **Imaging erk and pka activation in single dendritic spines during structural plasticity**. *Neuron* 2017, **93**:1315-1324.e3.

69. Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K: **A genetically encoded fluorescent sensor of erk activity**. *Proc Natl Acad Sci U S A* 2008, **105**:19264-19269.

70. •• Colgan LA, Hu M, Misler JA, Parra-Bueno P, Moran CM, Leitges M, Yasuda R: **Pkcalpha integrates spatiotemporally distinct ca(2+) and autocrine bdnf signaling to facilitate synaptic plasticity**. *Nat Neurosci* 2018, **21**:1027-1037

This paper describes the development and use of substrate binding (iDOCKS) and translocation (iTRACK), based FRET sensors for PKC. PKCalpha was rapidly and robustly activated in dendritic spines during sLTP and was NMDAR and BDNF-TrkB dependent. They hypothesize that PKCalpha acts to integrate multiple signals to enable complex cellular computation.

71. Chang JY, Parra-Bueno P, Laviv T, Szatmari EM, Lee SR, Yasuda R: **Camkii autophosphorylation is necessary for optimal integration of Ca(2+) signals during ltp induction, but not maintenance**. *Neuron* 2017, **94**:800-808.e4.

72. Chang JY, Nakahata Y, Hayano Y, Yasuda R: **Mechanisms of ca(2+)/calmodulin-dependent kinase ii activation in single dendritic spines**. *Nat Commun* 2019, **10**:2784.

73. Hedrick NG, Harward SC, Hall CE, Murakoshi H, McNamara JO, Yasuda R: **Rho gtpase complementation underlies bdnf-dependent homo- and heterosynaptic plasticity**. *Nature* 2016, **538**:104-108.

74. Murakoshi H, Wang H, Yasuda R: **Local, persistent activation of rho gtpases during plasticity of single dendritic spines**. *Nature* 2011, **472**:100-104.

75. Moeyaert B, Holt G, Madangopal R, Perez-Alvarez A, Fearey BC, Trojanowski NF, Ledderose J, Zolnik TA, Das A, Patel D *et al.*: **Improved methods for marking active neuron populations**. *Nat Commun* 2018, **9**:4440.

76. Perez-Alvarez A, Fearey BC, O'Toole RJ, Yang W, Arganda-Carreras I, Lamothe-Molina PJ, Moeyaert B, Mohr MA, Panzera LC, Schulze C, Schreiter ER et al. (2019).

77. Laviv T, Scholl B, Parra-Bueno P, Foote B, Zhang C, Yan L, Hayano Y, Fitzpatrick D, Chu J, Yasuda R: **In vivo imaging of the coupling between neuronal and CREB activity in the mouse brain**. *Neuron* 2019, **105**:799-812.e5.