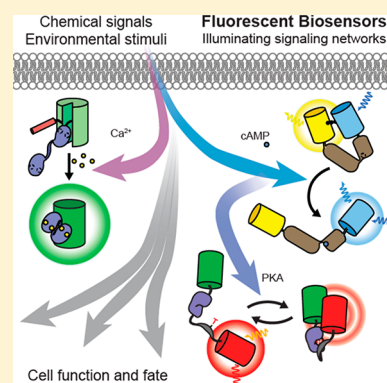


Genetically Encoded Fluorescent Biosensors Illuminate the Spatiotemporal Regulation of Signaling Networks

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Supporting Information

ABSTRACT: Cellular signaling networks are the foundation which determines the fate and function of cells as they respond to various cues and stimuli. The discovery of fluorescent proteins over 25 years ago enabled the development of a diverse array of genetically encodable fluorescent biosensors that are capable of measuring the spatiotemporal dynamics of signal transduction pathways in live cells. In an effort to encapsulate the breadth over which fluorescent biosensors have expanded, we endeavored to assemble a comprehensive list of published engineered biosensors, and we discuss many of the molecular designs utilized in their development. Then, we review how the high temporal and spatial resolution afforded by fluorescent biosensors has aided our understanding of the spatiotemporal regulation of signaling networks at the cellular and subcellular level. Finally, we highlight some emerging areas of research in both biosensor design and applications that are on the forefront of biosensor development.



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Received: May 25, 2018

Published: December 14, 2018

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1. INTRODUCTION

One of the most fundamental aspects of life is the ability of cells to respond to external cues originating from other cells or from the environment. Doing so requires cells to exist in a state of dynamic equilibrium, constantly poised to unleash cascades of chemical reactions from which all complex biological phenomena emerge. Through this network of signaling pathways, cells alter their physical state and composition such that they can appropriately determine their fate and perform the functions essential to their role within an organism. In the postgenomic era, wherein the identities of most signaling pathway components are known, elucidating the relationships among these components, and thus the dynamics and regulation of signal transduction, has become one of the foremost avenues toward furthering our understanding of both physiological and pathological cellular function. Because these signaling reactions are often discrete and transitory in nature, the development of fluorescence-based methods capable of monitoring and quantifying signaling dynamics in real time in their endogenous cellular context has proven instrumental to these efforts.

In many ways, fluorescence is an ideal tool for monitoring the behavior of signaling pathways within cells: fluorescence is both rapid, with the absorption and emission of light by a fluorescent molecule (i.e., fluorophore) occurring on the order of nanoseconds, and spatially precise, as the emitted wavelengths are smaller than many cellular structures. Thus, fluorescence-based readouts permit the high-fidelity recording of extremely rapid processes with single-cell and even subcellular spatial resolution. Combined with the development of increasingly sophisticated and sensitive microscopy hardware, fluorescence can be used to nondestructively visualize cellular processes directly in living cells, providing data with a combined temporal and spatial resolution that is not possible through traditional biochemical methods.

Although fluorescence has served as a sensitive in situ label for the better part of a century, beginning with the synthesis of fluorophore-conjugated antibodies and the advent of immunofluorescence,¹ the first bona fide fluorescent sensors capable of probing intracellular signaling dynamics emerged much later and included covalently labeled versions of endogenous proteins (i.e., “fluorescent analogs”, reviewed in ref 2) and organic indicator dyes (reviewed in ref 3). These synthetic probes enabled the first real-time measurements of dynamic changes in cellular parameters such as messenger and ion concentrations. However, this budding field was soon

revolutionized by the isolation and engineering of fluorescent proteins (FPs).^{4–6}

What began with the *Aequorea victoria* green fluorescent protein (GFP) has expanded through protein engineering and discoveries in other species to encompass a diverse assortment of FPs with unique photophysical properties (recently reviewed in ref 7). Currently, FPs span the colors of the rainbow and beyond, from the near-ultraviolet⁸ to the near-infrared,⁹ with similarly varying brightness and quantum yield.⁷ In parallel to the development of FPs, a suite of technologies have been developed for in situ labeling of recombinant proteins (e.g., FLAsH, HaloTag, SNAP-Tag) (reviewed in ref 10). These technologies, particularly the development of FPs, opened up new avenues by facilitating the construction of genetically encoded fluorescent biosensors, which can be synthesized directly within cells, expressed in specific subsets of cell populations, and easily fused with different protein tags for targeting to specific subcellular microdomains. As such, a diverse array of genetically encoded fluorescent biosensors have been engineered to monitor the dynamics of various signaling pathway components, including cell-surface receptors, intracellular messengers, and enzymatic effector proteins. Here, we first discuss the different design strategies that have been exploited to develop genetically encoded fluorescent biosensors, including some novel biosensor classes that have been developed recently. To this end, we have endeavored to assemble a comprehensive list of published fluorescent biosensors, which includes over 750 different biosensor variants, as seen in Table 1 and with more detail online at BiosensorDB.ucsd.edu. We then review how fluorescent biosensors have been utilized to examine and dissect the spatiotemporal dynamics of signal transduction and, finally, highlight new avenues of exploration with fluorescent biosensors.

2. DESIGNING GENETICALLY ENCODED BIOSENSORS

2.1. Elements of Genetically Encoded Biosensors

For the purposes of this review, we define genetically encoded fluorescent biosensors as chimeric proteins that can be expressed intracellularly and are engineered to act as sensors for monitoring signal transduction (Table 1). The basic function of any sensor is to translate information on a physical property or state of a system into a measurable readout. For fluorescent biosensors, this means converting diverse signaling activities, such as the concentration of intracellular messengers, metabolic compounds and other analytes, or the localization, conformations, and activity of signaling proteins into one of several types of fluorescence signals. Fluorescent biosensors are thus fundamentally defined by two essential components: a sensing unit which detects changes in signal transduction and a reporting unit which conveys these changes in a quantifiable form.

The sensing unit is often derived from an endogenous cellular protein that participates in the signaling pathway of interest and is thus intrinsically sensitive to the target signaling event (e.g., calmodulin to sense changes in Ca²⁺ concentrations). In other cases, isolated protein domains or peptide sequences can also be combined to confer a biosensor with the desired sensitivity. Meanwhile, the reporting unit typically consists of one or more FP variants (or fragments thereof) coupled to the sensing unit in such a way that signaling-induced changes in the state of the sensing unit alter FP

Table 1. Engineered Genetically Encoded Fluorescent Biosensors Are Organized by Their Signaling Targets^a

target	readout mech	variants
cell cycle		
cell cycle phases	Inten. (^I ★)	Fucci: Fucci(CA)2.1 ^I ★ (2017) ²²⁰ Fucci4 ^I ★ (2016) ²²¹ NIR Fucci ^I ★ (2016) ²²² NIR cell cycle reporter ^I ★ (2016) ²²³ Fucci2.1 ^I ★ (2013) ²²⁴ Fucci2 ^I ★ (2011) ²²⁵ zFucci ^I ★ (2009) ²²⁶ S/G2/M-X(NC) ^I ★ (2008) ²²⁷ Fucci ^I ★ (2008) ²²⁸
G1 phase	Trans. (^T ⇌)	G1 phase biosensor ^T ⇌ (2009) ²²⁹
S phase	Trans. (^T ⇌)	S phase biosensor ^T ⇌ (2009) ²²⁹
cell environment		
mechanical strain	FRET (^{FR} ◆)	HP35: HP35st-TS ^{FR} ◆ (2015) ²³⁰ HP35-TS ^{FR} ◆ (2015) ²³⁰
	Ex. Rat. (^{EX} ★)	PriSSM ^{EX} ★ (2008) ²³¹ stFRET: cpstFRET ^{FR} ◆ (2012) ²³² sstFRET ^{FR} ◆ (2011) ²³³ stFRET ^{FR} ◆ (2008) ²³⁴ TSMOD: TSMOD F25 ^{FR} ◆ (2016) ²³⁵ TSMOD ^{FR} ◆ (2010) ²³⁶
molecular crowding	FRET (^{FR} ◆)	crowding sensor ^{FR} ◆ (2015) ²³⁷
pH	Em. Rat. (^{EM} ★)	deGFP: deGFP family ^{EM} ★ (2002) ¹³⁴
	Ex. Rat. (^{EX} ★)	dual pH and Cl sensor: ClopHensor ^R ■ (2010) ¹⁴²
	FRET (^{FR} ◆)	E*GFP: E ¹ GFP ^R ■ (2007) ¹⁴¹ E ² GFP ^R ■ (2006) ¹³⁸
	Inten. (^I ★)	FluBpH: FluBpH 7.5 ^{FR} ◆ (2017) ²³⁸ FluBpH 6.1 ^{FR} ◆ (2017) ²³⁸ FluBpH 5.7 ^{FR} ◆ (2017) ²³⁸
	Rat. (^R ■)	mtAlpHi ^I ★ (2004) ²³⁹
		native FP pH sensitivity: mNect ^I ★ (2009) ²⁴⁰ pHVenus ^I ★ (2007) ¹²⁹ CFP-YFP pH FRET ^{FR} ◆ (2004) ²⁴¹ EYFP ^I ★ (1998) ¹²⁵ EGFP ^I ★ (1998) ¹²⁴
		pHCEC: pHCECSensor01 ^R ■ (2008) ²⁴²
		pHluorins: pHluorin2 ^{EX} ★ (2011) ²⁴³ superecliptic-pHluorin ^I ★ (2000) ¹²⁶ ratiometric-pHluorin ^{EX} ★ (1998) ¹²¹ ecliptic-pHluorin ^I ★ (1998) ¹²¹
		pHRed ^{EX} ★ (2011) ²⁴⁴
		pHTomato ^I ★ (2012) ¹²⁷
membrane voltage (V ⁺)		pHuji ^I ★ (2014) ²⁴⁵
		pHusion ^R ■ (2012) ²⁴⁶
		SypHer: SypHer3s ^{EX} ★ (2018) ⁹⁹⁶ SypHer-2 ^{EX} ★ (2015) ⁹⁹⁷ SypHer ^I ★ (2011) ²⁴⁷
		XFpH: YFpH ^{FR} ◆ (2001) ²⁴⁸ GFpH ^{FR} ◆ (2001) ²⁴⁸
	BRET (^{BR} ●)	ArcLight species variants: Human Q193 ArcLight ^I ★ (2013) ²⁴⁹ Zebrafish Q175 ArcLight ^I ★ (2013) ²⁴⁹ Chicken Q175 ArcLight ^I ★ (2013) ²⁴⁹ Frog Q174 ArcLight ^I ★ (2013) ²⁴⁹
	FRET (^{FR} ◆)	Ci-VSP based: VSD-FR189–188 ^I ★ (2017) ²⁵⁰ LOTUS-V ^{BR} ● (2017) ²⁵¹ Marina ^I ★ (2017) ²⁵² tdFlicR1 Δ110AR ^I ★ (2016) ²⁵³ FlicGR1 ^I ★ (2016) ²⁵³ ASAP 2f ^I ★ (2016) ¹⁸⁴ tdFlicR-VK-ASAP ^R ■ (2016) ²⁵³ FlicR1 ^I ★ (2016) ¹⁸⁵ Bongwoori ^I ★ (2015) ²⁵⁴ ArcLightning ^I ★ (2015) ²⁵⁵ Nabi2.213 ^{FR} ◆ (2015) ²⁵⁶ ASAP1 ^I ★ (2014) ¹⁷⁵ Chimeric VSFP-Butterfly YR ^{FR} ◆ (2014) ²⁵⁷ Chimeric VSFP-Butterfly CY ^{FR} ◆ (2014) ²⁵⁷ Mermaid2 ^{FR} ◆ (2013) ²⁵⁸ ArcLight A242 ^I ★ (2012) ²⁵⁹ Chimera Cx ^{FR} ◆ (2012) ²⁶⁰ VSFP-CR ^{FR} ◆ (2012) ²⁶¹ ElectricPk ^I ★ (2012) ¹⁸² VSFP Butterfly 1.2 ^{FR} ◆ (2012) ²⁶² VSFP2.3 ^{FR} ◆ (2010) ²⁶³ VSFP2.42 ^{FR} ◆ (2010) ²⁶⁴ VSFP2.4 ^{FR} ◆ (2009) ²⁶⁵ VSFP3.1 ^I ★ (2008) ¹⁸⁰ Mermaid ^{FR} ◆ (2008) ²⁶⁶ VSFP2.1 ^{FR} ◆ (2007) ²⁶⁷
	Inten. (^I ★)	Danio VSP based: Zahara 2 SE (227D) ^I ★ (2015) ²⁶⁸ Zahra 1 SE (227D) series ^I ★ (2015) ²⁶⁸ Zahra 2 ^{FR} ◆ (2012) ²⁶⁹ Zahra 1 ^{FR} ◆ (2012) ²⁶⁹
	Rat. (^R ■)	Kv based: FlaSH (CFP) + FlaSh (YFP) ^{FR} ◆ (2002) ²⁷⁰ FlaSh L366A ^I ★ (2002) ²⁷⁰ FlaSh IR ^I ★ (2002) ²⁷⁰ VSFP1 ^{FR} ◆ (2001) ²⁷¹ FlaSh ^I ★ (1997) ¹⁷⁶
		Na channel based: SPARC ^I ★ (2002) ¹⁷⁷
		Nematostella VSP based: Nema ^{FR} ◆ (2012) ²⁶⁹
cellular analytes		Rhodopsin based: Archon2 ^I ★ (2018) ²⁷² Archon1 ^I ★ (2018) ²⁷² VARNAM ^I ★ (2018) ⁹⁹⁸ FRET-opsin Ace1Q-mNeon ^I ★ (2015) ²⁷³ FRET-opsin Ace2N-mNeon ^I ★ (2015) ²⁷³ QuasAr2 ^I ★ (2014) ²⁷⁴ QuasAr1 ^I ★ (2014) ²⁷⁴ Archer1 ^I ★ (2014) ²⁷⁵ eFRET GEVI ^{FR} ◆ (2014) ²⁷⁶ FRET-opsin Mac-mCitrine ^I ★ (2014) ²⁷⁷ Arch-EEx variants ^I ★ (2013) ²⁷⁸ PROPS ^I ★ (2011) ²⁷⁹ Arch (D95N) ^I ★ (2011) ²⁸⁰
	Inten. (^I ★)	mOGsor ^I ★ (2014) ²⁸¹
	Inten. (^I ★)	AmTrac: AmTryoshika1;3-LS-F1381 -T78H ^R ■ (2017) ²¹⁹ AmTryoshika1;3-LS-F1381 ^R ■ (2017) ²¹⁹ AmTrac ^I ★ (2013) ²⁸²
	Rat. (^R ■)	
	FRET (^{FR} ◆)	FLIPara: FLIPara-250n ^{FR} ◆ (2008) ²⁸³
	FRET (^{FR} ◆)	FRET Arg Reporter ^{FR} ◆ (2007) ²⁸⁴
	Biolum. (^{BL} ●)	ATeam: BTeam ^{BR} ● (2016) ²⁸⁵ ATeam1.03NL ^{FR} ◆ (2013) ²⁸⁶ GO-ATeam ^{FR} ◆ (2011) ²⁸⁷ ATeam3.10 ^{FR} ◆ (2009) ²⁸⁸ ATeam1.03 ^{FR} ◆ (2009) ²⁸⁸

Table 1. continued

target	readout mech	variants
cellular analytes		
ATP (cont.)	BRET (^{BR} ●) Ex. Rat. (^{EX} ★) FRET (^{FR} ◆)	EAF-ATP ^{FR} ◆ (2013) ²⁸⁹ nanolatern ATP ^{BL} ● (2012) ²⁹⁰ QUEEN: QUEEN-2m ^{EX} ★ (2014) ²⁹¹ QUEEN-7mu ^{EX} ★ (2014) ²⁹¹ Syn-ATP ^{BL} ● (2014) ²⁹²
ATP:ADP ratio	Ex. Rat. (^{EX} ★)	Perceval: PercevalHR ^{EX} ★ (2013) ²⁹³ Perceval ^{EX} ★ (2009) ²⁹⁴
BDNF	FRET (^{FR} ◆)	BesCell ^{FR} ◆ (2008) ²⁹⁵
cAMP	Biolum. (^{BL} ●) BRET (^{BR} ●) FRET (^{FR} ◆) Inten. (^I ★) Rat. (^R ■)	cADDIs: cADDIs-green ^I ★ (2016) ¹⁹¹ Camps: Epac2-camps300-cit ^{FR} ◆ (2010) ²⁹⁶ Epac2-camps300 ^{FR} ◆ (2009) ²⁹⁷ HCN2-camps ^{FR} ◆ (2006) ²⁹⁸ Epac2-camps ^{FR} ◆ (2004) ²⁹⁹ Epac1-camps ^{FR} ◆ (2004) ²⁹⁹ PKA-camps ^{FR} ◆ (2004) ²⁹⁹ CAMEL ^{BR} ● (2007) ³⁰⁰ CUTie ^{FR} ◆ (2017) ³⁰¹ Epac-S: Epac-S H188 ^{FR} ◆ (2015) ³⁰² Epac-S H187 ^{FR} ◆ (2015) ³⁰² H74 ^{FR} ◆ (2011) ³⁰³ H96 ^{FR} ◆ (2011) ³⁰³ H90: CFP(nd)-EPAC(ΔDEP/CD)-cp173Venus(d) ^{FR} ◆ (2008) ³⁰⁴ H84: CFP(nd)-EPAC(ΔDEP/CD)-Venus(d) ^{FR} ◆ (2008) ³⁰⁴ H81: GFP(nd)-EPAC(ΔDEP)-mRFP ^{FR} ◆ (2008) ³⁰⁴ Epac1 (ΔDEP-CD) based: CEPAC* ^{FR} ◆ (2011) ³⁰⁵ CFP-Epac(ΔDEP-CD)-YFP ^{FR} ◆ (2004) ³⁰⁶ Flamindo: Pink Flamindo ^I ★ (2017) ¹⁹² Flamindo2 ^I ★ (2014) ¹⁸⁹ Flamindo ^I ★ (2013) ¹⁷¹ FPX: pPHT-PKA ^R ■ (2015) ³⁰⁷ ICUE: RAB-ICUE ^I ★ (2018) ²¹¹ ICUPID ^{FR} ◆ (2011) ³⁰⁸ ICUE-YR ^{FR} ◆ (2011) ³⁰⁸ ICUE3 ^{FR} ◆ (2009) ³⁰⁹ Rluc-EPAC-YFP ^{BR} ● (2008) ³¹⁰ ICUE2 ^{FR} ◆ (2008) ³¹¹ ICUE1 ^{FR} ◆ (2004) ³¹² mCRIS based: cit-mCNBD-cer ^{FR} ◆ (2013) ³¹³ mICNBD-FRET ^{FR} ◆ (2016) ³¹⁴ Nano-lantern cAMP: Nano-lantern cAMP1.6 ^{BL} ● (2012) ²⁹⁰ R-Flinca ^I ★ (2018) ³¹⁵ R1α #7 ^{FR} ◆ (2016) ³¹⁶ Split Luc cAMP biosensor: 22F ^{BL} ● (2011) ³¹⁷ Split Luc cAMP biosensor ^{BL} ● (2008) ³¹⁸ YFP-PKAc + CFP-PKAr: ΔPKA RIIb-CFP + PKAc-YFP ^{FR} ◆ (2006) ³¹⁹ R2-Rluc + GFP-C ^{BR} ● (2006) ³²⁰ R1-Rluc + GFP-C ^{BR} ● (2006) ³²⁰ YFP-PKAc + CFP-PKAr(R230 K) ^{FR} ◆ (2004) ³²¹ YFP-PKAc + CFP-PKAr ^{FR} ◆ (2002) ³²² PKAc-S65T + PKArII-EBFP ^{FR} ◆ (2000) ³²³
cGMP	Ex. Rat. (^{EX} ★) FRET (^{FR} ◆) Inten. (^I ★)	cGES: Cygnus ^I ★ (2010) ³²⁴ cGES-DE5 ^{FR} ◆ (2006) ³²⁵ cGKI based: cGi family ^{FR} ◆ (2007) ³²⁶ cGull: Green-cGull ^I ★ (2017) ¹⁷² CGY: CGY-del1 ^{FR} ◆ (2000) ³²⁷ cygnet: cygnet-2.1 ^{FR} ◆ (2005) ³²⁸ cygnet-2 ^{FR} ◆ (2001) ³²⁹ FlincG: H6-FGA ^M ^{FR} ◆ (2013) ³³⁰ H6-FGB ^I ★ (2013) ³³⁰ δ-FlincG ^{EX} ★ (2008) ¹⁷³
citrate	FRET (^{FR} ◆)	FLIP-Cit: FLIP-Cit-Y ^{FR} ◆ (2011) ³³¹
carbon monoxide (CO)	Inten. (^I ★)	COSer ^I ★ (2012) ³³²
glucose	FRET (^{FR} ◆)	FLIPglu: FLII ¹² Pglu-Y Series ^{FR} ◆ (2008) ³³³ FLIPglu-YΔ13 ^{FR} ◆ (2006) ³³⁴ FLII ^X Pglu-Y Series ^{FR} ◆ (2005) ³³⁵ FLIPglu-600u-Δ(X) series ^{FR} ◆ (2005) ³³⁵ FLIPglu-Y series ^{FR} ◆ (2003) ³³⁶ GIP: AcGFP1-GBPCys-mCherry ^{FR} ◆ (2010) ³³⁷ GIP C ₀ Y _i ^{FR} ◆ (2008) ³³⁸ GIP ^{FR} ◆ (2003) ³³⁹
glutamine	FRET (^{FR} ◆)	FLIPQ: FLIPQ-TV3.0 ^{FR} ◆ (2012) ³⁴⁰
heme	FRET (^{FR} ◆) Inten. (^I ★) Rat. (^R ■)	CHY: CH49Y ^{FR} ◆ (2017) ³⁴¹ CISDY: CISDY-9 ^{FR} ◆ (2015) ³⁴² Fluorescence quenching Heme: HS1 ^R ■ (2016) ³⁴³ HS1-M7A ^R ■ (2016) ³⁴³ CG6 ^I ★ (2012) ³⁴⁴
histidine	FRET (^{FR} ◆) Rat. (^R ■)	FLIP-HisJ: FLIP-cpHisJ194 ^{FR} ◆ (2009) ³⁴⁵ FHISJ ^R ■ (2017) ⁹⁹⁹
insulin	Rat. (^R ■)	RINS: RINS1 ^R ■ (2017) ³⁴⁶
lactate	FRET (^{FR} ◆)	Laonic ^{FR} ◆ (2013) ³⁴⁷
maltose	BRET (^{BR} ●) FRET (^{FR} ◆)	FLIPmal: GFP2-MBP-RLuc2 ^{BR} ● (2013) ³⁴⁸ FLIPmal-YΔ1 ^{FR} ◆ (2008) ²⁸³ FLIPmal-Y Series ^{FR} ◆ (2002) ³⁴⁹ MBP: PPYF-green ^I ★ (2011) ²⁰²

Table 1. continued

target	readout mech	variants
cellular analytes		
maltose (cont.)	Inten. ($I_{\star}$)	
NAD ⁺	FRET ($^{FR}\blacklozenge$) Inten. ($I_{\star}$)	NAD ⁺ Biosensor $I_{\star}$ (2016) ³⁵⁰ NAD-Snifit $^{FR}\blacklozenge$ (2018) ³⁵¹
NAD ⁺ /NADH ratio	Ex. Rat. ($^{EX}\star$) Inten. ($I_{\star}$) Rat. ($^R\blacksquare$)	T-Rex Based: RexYFP $I_{\star}$ (2014) ³⁵² Peredox $^R\blacksquare$ (2011) ²¹⁴ SoNar $^{EX}\star$ (2015) ²⁰⁹
NADH	Inten. ($I_{\star}$)	Frex: FrexH $I_{\star}$ (2011) ³⁵³ Frex $I_{\star}$ (2011) ³⁵³
NADP ⁺	FRET ($^{FR}\blacklozenge$)	Apollo-NADP ⁺ $^{FR}\blacklozenge$ (2016) ³⁵⁴ NADPsor $^{FR}\blacklozenge$ (2016) ³⁵⁵
NADPH	BRET ($^{BR}\bullet$) Ex. Rat. ($^{EX}\star$)	iNap: iNap3 $^{EX}\star$ (2017) ²¹⁰ iNap2 $^{EX}\star$ (2017) ²¹⁰ iNap1 $^{EX}\star$ (2017) ²¹⁰ semisynthetic BRET sensor: SNAP-P30-[cpNLuc inserted]eDHFR $^{BR}\bullet$ (2018) ³⁵⁶
NADP ⁺ :NADPH ratio	FRET ($^{FR}\blacklozenge$)	NADP-Snifit $^{FR}\blacklozenge$ (2018) ³⁵¹
O ₂	FRET ($^{FR}\blacklozenge$) Inten. ($I_{\star}$)	dUnOHR hypoxia-reoxygenation sensor $I_{\star}$ (2016) ³⁵⁷ FluBO $^{FR}\blacklozenge$ (2012) ³⁵⁸
phosphonate	Inten. ($I_{\star}$)	EcPhnD based: EcPhnD-cpGFP $I_{\star}$ (2011) ²⁰³
pyruvate	FRET ($^{FR}\blacklozenge$)	PdhR based: PYRATES $^{FR}\blacklozenge$ (2017) ³⁵⁹ Pyronic $^{FR}\blacklozenge$ (2014) ³⁶⁰
ribose	FRET ($^{FR}\blacklozenge$)	FLIPrib: FLIPrib-Y family $^{FR}\blacklozenge$ (2003) ³⁶¹
sucrose	FRET ($^{FR}\blacklozenge$)	FLIPsuc: FLIPsuc-Y family $^{FR}\blacklozenge$ (2006) ³⁶²
trehalose	FRET ($^{FR}\blacklozenge$)	FLIP: FLIPsuc90 $\mu\Delta$ 1Venus $^{FR}\blacklozenge$ (2016) ³⁶³
tryptophan	FRET ($^{FR}\blacklozenge$)	FLIPW $^{FR}\blacklozenge$ (2007) ³⁶⁴
ions		
Ca ²⁺	Biolum. ($^{BL}\bullet$) BRET ($^{BR}\bullet$) Em. Rat. ($^{EM}\star$) Ex. Rat. ($^{EX}\star$) FRET ($^{FR}\blacklozenge$) Inten. ($I_{\star}$) Rat. ($^R\blacksquare$)	Ca ²⁺ Snapshot: FLARE $I_{\star}$ (2017) ³⁶⁵ Cal-Light $I_{\star}$ (2017) ³⁶⁶ Cameleons: D3 BRET $^{BR}\bullet$ (2015) ³⁶⁷ D1GO-Cam $^{FR}\blacklozenge$ (2012) ³⁶⁸ CaYang1 $^{FR}\blacklozenge$ (2011) ³⁶⁹ CaYin1 $^{FR}\blacklozenge$ (2011) ³⁶⁹ YC-Nano $^{FR}\blacklozenge$ (2010) ³⁷⁰ D3cpv $^{FR}\blacklozenge$ (2006) ³⁷¹ D4 cPV $^{FR}\blacklozenge$ (2006) ³⁷¹ D1 $^{FR}\blacklozenge$ (2004) ³⁷² YC3.60 $^{FR}\blacklozenge$ (2004) ³⁷³ YC2.12 $^{FR}\blacklozenge$ (2002) ³⁷⁴ YC6.1 $^{FR}\blacklozenge$ (2001) ³⁷⁵ YC2.1 $^{FR}\blacklozenge$ (1999) ³⁷⁶ Split-Cameleon $^{FR}\blacklozenge$ (1997) ³⁷⁷ Cameleon 3 $^{FR}\blacklozenge$ (1997) ³⁷⁷ Camgaroo: Camgaroo2 $I_{\star}$ (2001) ³⁷⁸ Camgarool $I_{\star}$ (1999) ¹⁵² CaMPARI: CaMPARI $I_{\star}$ (2015) ³⁷⁹ CASE: Case16 $I_{\star}$ (2007) ³⁸⁰ Case12 $I_{\star}$ (2007) ³⁸⁰ CatchER $I_{\star}$ (2011) ¹⁴⁸ ddFP Ca ²⁺ sensor $I_{\star}$ (2012) ³⁸¹ ER-GCaMP: ER-GCaMP6–150 $I_{\star}$ (2017) ³⁸² ER-GCaMP6–210 $I_{\star}$ (2017) ³⁸² ER-GCaMP3–373/GCaMPer-10.19 $I_{\star}$ (2015) ³⁸³ CEPIA1er $I_{\star}$ (2014) ³⁸⁴ ER-GECO: R-CEPIA1er $I_{\star}$ (2014) ³⁸⁴ GEM-CEPIA1er $^{EM}\star$ (2014) ³⁸⁴ G-CEPIA1er $I_{\star}$ (2014) ³⁸⁴ FPX Ca ²⁺ sensors: tripartate FPX Ca ²⁺ Sensor $^R\blacksquare$ (2015) ³⁰⁷ single polypeptide FPX Ca ²⁺ sensor $^R\blacksquare$ (2015) ³⁰⁷ GAP: GAP3 $I_{\star}$ (2016) ³⁸⁵ GAP1 $I_{\star}$ (2014) ³⁸⁶ GCaMP: axon-GCaMP6 $I_{\star}$ (2018) ¹⁰⁰⁰ ncpGCaMP6s $I_{\star}$ (2018) ³⁸⁷ MatryoshCaMP6s $^R\blacksquare$ (2017) ²¹⁹ sfMatryoshCaMP6s $^R\blacksquare$ (2017) ²¹⁹ sfMatryoshCaMP6s-T78H $^R\blacksquare$ (2017) ²¹⁹ FGCaMP $^{EX}\star$ (2017) ³⁸⁸ GCaMP-R3 $^R\blacksquare$ (2017) ²¹⁵ GCaMP-R-6f $^R\blacksquare$ (2017) ²¹⁵ GCaMP-R-6s $^R\blacksquare$ (2017) ²¹⁵ GCaMP3bright $I_{\star}$ (2015) ³⁸⁹ GCaMP3fast $I_{\star}$ (2015) ³⁸⁹ GCaMP2.2Low $I_{\star}$ (2014) ³⁹⁰ GCaMP6f $I_{\star}$ (2013) ¹⁶⁴ GCaMP6s $I_{\star}$ (2013) ¹⁶⁴ GCaMP6m $I_{\star}$ (2013) ¹⁶⁴ GCaMP5G $I_{\star}$ (2012) ¹⁶³ G-CaMP 3–8 (Nakai Variants) $I_{\star}$ (2012) ³⁹¹ GCaMP-HS $I_{\star}$ (2011) ³⁹² GCaMP3 $I_{\star}$ (2009) ¹⁶² GCaMP2 $I_{\star}$ (2006) ¹⁶¹ GCaMP $I_{\star}$ (2001) ¹⁵⁸ GECO: K-GECO1 $I_{\star}$ (2018) ³⁹³ LUCI-GECO1 $^{BR}\bullet$ (2018) ³⁸⁷ jRGECO1a $I_{\star}$ (2016) ¹⁷⁰ REX-GECO $^{EX}\star$ (2014) ²⁰⁸ LAR-GECO $I_{\star}$ (2014) ³⁹⁴ GR-GECO $I_{\star}$ (2013) ³⁹⁵ R-GECO1.2 $I_{\star}$ (2013) ¹⁶⁷ O-GECO $I_{\star}$ (2013) ¹⁶⁷ CAR-GECO $I_{\star}$ (2013) ¹⁶⁷ G-GECO1 $I_{\star}$ (2011) ¹⁶⁶ B-GECO $I_{\star}$ (2011) ¹⁶⁶ G-GECO1.2 $I_{\star}$ (2011) ¹⁶⁶ R-GECO1 $I_{\star}$ (2011) ¹⁶⁶ GEM-GECO $^{EM}\star$ (2011) ¹⁶⁶ GEX-GECO $^{EX}\star$ (2011) ¹⁶⁶ G-GECO1.1 $I_{\star}$ (2011) ¹⁶⁶ nanolantern Ca ²⁺ : CeNL(Ca ²⁺) ₁₁₀ $^R\bullet$ (2018) ³⁹⁶ CeNL(Ca ²⁺) ₁₉ $^R\bullet$ (2018) ³⁹⁶ GeNL(Ca ²⁺) $^{BR}\bullet$ (2016) ³⁹⁷ ONL(Ca ²⁺) $^{BR}\bullet$ (2015) ³⁹⁸ CNL(Ca ²⁺) $^{BR}\bullet$ (2015) ³⁹⁸ nanolantern Ca ²⁺ $^{BL}\bullet$ (2012) ²⁹⁰ NTnC: YTNc $I_{\star}$ (2018) ³⁹⁹ iYTNc2 $I_{\star}$ (2018) ⁴⁰⁰ NTnC $I_{\star}$ (2016) ⁴⁰¹ pericam: ratiometric-pericam $^R\blacksquare$ (2001) ¹⁵⁶ inverse-pericam $I_{\star}$ (2001) ¹⁵⁶ flash-pericam $I_{\star}$ (2001) ¹⁵⁶

Table 1. continued

target	readout mech	variants
ions		
		RCaMP: jRCaMP1b ^{FR} (2016) ¹⁷⁰ jRCaMP1a ^{FR} (2016) ¹⁷⁰ R-CaMP2 ^{FR} (2015) ⁴⁰² RCaMP1h ^{FR} (2013) ¹⁶⁸ R-CaMP1.07 ^{FR} (2012) ⁴⁰³
		TN Ca ²⁺ Sensors: CalfluxVTN ^{FR} (2016) ⁴⁰⁴ Twitch series ^{FR} (2014) ⁴⁰⁵ RS-TN-XL ^{FR} (2013) ⁴⁰⁶ YO-TnCl.0 ^{FR} (2012) ⁴⁰⁷ TN-XXL ^{FR} (2008) ⁴⁰⁸ TN-XL ^{FR} (2006) ⁴⁰⁹ TN-humTnC ^{FR} (2004) ⁴¹⁰ TN-L15 ^{FR} (2004) ⁴¹⁰
Cd ²⁺	FRET (^{FR} ◆)	Cd-FRET: Cd-FRET2 ^{FR} (2011) ¹⁴⁶
Cu ⁺	FRET (^{FR} ◆)	yeast copper regulator based: Ace1-FRET ^{FR} (2011) ⁴¹¹ Mac1-FRET ^{FR} (2011) ⁴¹¹ Amt1-FRET ^{FR} (2010) ⁴¹²
halide ions (Cl ⁻ , I ⁻)	FRET (^{FR} ◆)	Clomeleon: SuperClomeleon ^{FR} (2013) ⁴¹³ Cl-sensor ^{FR} (2008) ¹⁴⁰ Clomeleon ^{FR} (2000) ¹³⁹
	Inten. (^I ★)	Dual pH and Cl sensor: ClopHensor ^R (2010) ¹⁴²
	Rat. (^R ■)	halide sensitive fluorescent proteins: Cl-YFP ^{FR} (2014) ¹³³ YFP(H148Q, I152L) ^{FR} (2001) ¹³² YFP(H148Q) ^{FR} (2000) ¹³⁰
Hg(II)	Inten. (^I ★)	eGFP based: eGFP205C ^{FR} (2008) ⁴¹⁴ IFP based: IFP based Hg sensor ^{FR} (2011) ⁴¹⁵
Mg ²⁺	FRET (^{FR} ◆)	MagFRET: MagFRET-1 ^{FR} (2013) ⁴¹⁶
phosphate	FRET (^{FR} ◆)	FLIPPi: FLIPPi-5μ ^{FR} (2006) ⁴¹⁷ FLIPPi-30m ^{FR} (2006) ⁴¹⁷
Zn ²⁺	BRET (^{BR} ●)	Atox1 WD4 based: BLCALWY-1 ^{BR} (2016) ⁴¹⁸ redCALWY ^{FR} (2013) ⁴¹⁹ eCALWY series ^{FR} (2009) ⁴²⁰ CALWY ^{FR} (2007) ⁴²¹ CFP-Atox1 + WD4-YFP ^{FR} (2006) ⁴²²
	FRET (^{FR} ◆)	GZnP: GZnP2 ^{FR} (2018) ⁴²³ GZnP1 ^{FR} (2016) ⁴²⁴
	Inten. (^I ★)	minimal zinc finger based: mito-ZifCY1.173 ^{FR} (2012) ⁴²⁵ His4-Zn ²⁺ sensor ^{FR} (2009) ⁴²⁶ Cys2Hys2 - Zn ²⁺ Sensor ^{FR} (2009) ⁴²⁶
		Zap1 based: ZapCV5 ^{FR} (2017) ⁴²⁷ ZapCV2 ^{FR} (2017) ⁴²⁷ ZapCY2 ^{FR} (2011) ⁴²⁸ ZapCY1 ^{FR} (2011) ⁴²⁸ ZF1/2-FRET ^{FR} (2006) ⁴²⁹
		ZinCh: BLZinCh-2 ^{BR} (2016) ⁴¹⁸ BLZinCh-3 ^{BR} (2016) ⁴¹⁸ eZinCh-2 ^{FR} (2015) ¹⁴⁷ eZinCh-1 ^{FR} (2011) ¹⁴⁶ Cly9-2His ^{FR} (2008) ⁴³⁰ ZinCh-x ^{FR} (2007) ¹⁴⁵
kinases/phosphatases		
Abl	FRET (^{FR} ◆)	Abl indicator ^{FR} (2001) ⁴³¹
Akt	Ex. Rat. (^{EX} ★)	Akind ^{FR} (2006) ⁴³²
	FLIM (^{FL} ◇)	Akt translocation Reporters: Akt-FoxO3a-KTR-EGFP ^T (2016) ¹¹⁴ FoxO1-clover ^T (2015) ¹¹³
	FRET (^{FR} ◆)	AktAR: ExRai-AktAR ^{EX} ★ (2018) ²¹¹ AktAR2 ^{FR} (2015) ⁴³³ AktAR ^{FR} (2008) ⁴³⁴
	Trans. (^T ↔)	AktUS ^{FR} (2003) ⁴³⁵ BKAR: BKAR v2 ^{FR} (2014) ⁴³⁶ BKAR ^{FR} (2005) ⁴³⁷ dual-labeled Akt: GFP-PKB-RFP ^{FR} (2007) ⁴³⁸ GFP-AKT-YFP ^{FL} ◇ (2003) ⁴³⁹ Eevee-Akt: Eevee-iAkt ^{FR} (2014) ⁴⁴⁰ Eevee-Akt ^{FR} (2011) ⁴⁴¹ ReAktion: ReAktion1 ^{FR} (2007) ⁴⁴²
AMPK	FRET (^{FR} ◆)	AMPKAR: AMPKAR-EV ^{FR} (2017) ⁴⁴³ bimABKAR ^{FR} (2015) ⁴⁴⁴ ABKAR ^{FR} (2015) ⁴⁴⁵ AMPKAR ^{FR} (2011) ⁴⁴⁶
ATM kinase	FRET (^{FR} ◆)	ATOMIC ^{FR} (2007) ⁴⁴⁷
Aurora B kinase	FRET (^{FR} ◆)	Aurora B sensor: Aurora B sensor (Chu) ^{FR} (2011) ⁴⁴⁸ Aurora B Sensor (Fuller) ^{FR} (2008) ⁴⁴⁹
Aurora kinase A	FRET (^{FR} ◆)	AURKA Biosensor ^{FR} (2016) ⁴⁵⁰
B-Raf	FRET (^{FR} ◆)	Prin-BRaf ^{FR} (2006) ⁴⁵¹
Bcr-Abl	FRET (^{FR} ◆)	Pickles: Pickles-2.3 ^{FR} (2010) ⁴⁵²
C-Raf	FRET (^{FR} ◆)	Prin-CRaf ^{FR} (2005) ⁴⁵³
CaMKII	FLIM (^{FL} ◇)	Camui: Camuiα-mRmC ^{FL} ◇ (2016) ⁴⁵⁴ ShadowG-Camuiα ^{FL} ◇ (2015) ⁴⁵⁵ Camuiα-CR ^{FR} (2012) ²⁶¹ green-Camuiα ^{FL} ◇ (2009) ⁴⁵⁶ mRFP/GFP-Camuiα ^{FL} ◇ (2008) ⁴⁵⁷ Camuiα ^{FR} (2005) ⁴⁵⁸
	FRET (^{FR} ◆)	K2α: RS-K2α ^{FR} (2013) ⁴⁰⁶ RY-K2α ^{FR} (2013) ⁴⁰⁶ YC-K2α ^{FR} (2013) ⁴⁰⁶
CaN	FRET (^{FR} ◆)	CaN activation: YC-CaN ^{FR} (2013) ⁴⁰⁶ RY-CaN ^{FR} (2013) ⁴⁰⁶ CaNAR: CaNAR2 ^{FR} (2014) ⁴⁵⁹ CaNAR1 ^{FR} (2008) ⁴⁶⁰

Table 1. continued

target	readout mech	variants
<u>kinases/phosphatases</u>		
		CaNARi ^{FR} ◆ (2014) ⁴⁵⁹ DuoCaN: UniCaN ^{FR} ◆ (2015) ⁴⁶¹ DuoCaN ^{FR} ◆ (2015) ⁴⁶¹
CDK1	FRET (^{FR} ◆)	CyclinB1-Cdk1 activity sensor: CyclinB1-Cdk1 activity sensor V2 ^{FR} ◆ (2014) ⁴³⁶ CyclinB1-Cdk1 activity sensor V1 ^{FR} ◆ (2010) ⁴⁶²
CDK2	Trans. (^T ⇌)	CDK2 translocation reporter: DHB-Ven ^T ⇌ (2013) ¹¹¹
DAPK1	FRET (^{FR} ◆)	DAPK1 ^{FR} ◆ (2011) ⁴⁶³
EGFR	FRET (^{FR} ◆)	ECaus ^{FR} ◆ (2008) ²⁹⁵ EGFR Reporter ^{FR} ◆ (2001) ⁴³¹ FLAME: PTB-EYFP, EGFR-ECFP ^{FR} ◆ (2004) ⁴⁶⁴ FLAME ^{FR} ◆ (2004) ⁴⁶⁴ Picchu-Z ^{FR} ◆ (2005) ⁴⁶⁵
ERK	BRET (^{BR} ⊙) FLIM (^{FL} ◇) FLINC (^{FC} ▲) FRET (^{FR} ◆) Inten. (^I ✱) Rat. (^R ■) Trans. (^T ⇌)	EAS: EAS3 ^{FR} ◆ (2005) ⁴⁶⁶ EKAR: VcpV FLARE EKAR ^{FR} ◆ (2018) ⁴⁶⁷ mCh-mCh FLARE EKAR ^{FR} ◆ (2018) ⁴⁶⁷ C3C3 FLARE EKAR ^{FR} ◆ (2018) ⁴⁶⁷ VV FLARE EKAR ^{FR} ◆ (2018) ⁴⁶⁷ RAB-EKAREV ^I ✱ (2018) ²¹¹ EKARET ^{FL} ◇ (2017) ⁴⁶⁸ EKAR ^{FL} ◇ (2017) ⁴⁶⁹ bimEKAR ^{FR} ◆ (2015) ⁴⁴⁴ EKAR3 ^{FR} ◆ (2015) ⁴⁷⁰ EKAREV-TVV ^{FR} ◆ (2014) ⁴⁷¹ EKAR-TVV ^{FR} ◆ (2014) ⁴⁷¹ REV ^{BR} ⊙ (2013) ⁴⁷² EKAR2G ^{FR} ◆ (2013) ⁴⁷³ EKAREV ^{FR} ◆ (2011) ⁴⁴¹ EKAR ^{FR} ◆ (2008) ⁴⁷⁴ ERK-KTR ^T ⇌ (2014) ¹¹⁶ Erkus ^{FR} ◆ (2007) ⁴⁷⁵ ERKy ^{FR} ◆ (2012) ⁴⁷⁶ FLINC-EKAR1 ^{FC} ▲ (2017) ⁴⁷⁷ FPX EKAR ^R ■ (2015) ³⁰⁷ Miu2 ^{FR} ◆ (2006) ⁴⁷⁸
FAK	FRET (^{FR} ◆)	FAK activation biosensor: CYFAK413 ^{FR} ◆ (2008) ⁴⁷⁹ FAK autophosphorylation biosensor ^{FR} ◆ (2008) ⁴⁷⁹ FAK sensor ^{FR} ◆ (2011) ⁴⁸⁰
Fus3	Trans. (^T ⇌)	Far1-SKARS ^T ⇌ (2015) ¹¹⁷
GCK	FRET (^{FR} ◆)	GCK activation biosensors: FRET-GCK (cp173-mCer3/mVen) ^{FR} ◆ (2016) ⁴⁸¹ FRET-GCK (mCer/mVen) ^{FR} ◆ (2011) ⁴⁸² Cerulean-GCK-mCit ^{FR} ◆ (2004) ¹⁸¹ FRET-GCK ^{FR} ◆ (2002) ⁴⁸³
H3-S10p	FRET (^{FR} ◆)	H3S10p biosensor ^{FR} ◆ (2018) ¹⁰⁰¹
H3-S28p	FRET (^{FR} ◆)	histone phosphorylation reporter ^{FR} ◆ (2004) ⁴⁸⁴
INSR	BRET (^{BR} ⊙) FRET (^{FR} ◆) Inten. (^I ✱)	insulin receptor activation BRET assay ^{BR} ⊙ (2001) ⁴⁸⁵ Phocus: Phocus-2pp nes ^{FR} ◆ (2002) ⁴⁸⁶ Phocus2 ^{FR} ◆ (2002) ⁴⁸⁶ Sinphos: yellow-sinphos ^I ✱ (2004) ⁴⁸⁷ green-sinphos ^I ✱ (2004) ⁴⁸⁷ cyan-sinphos ^I ✱ (2004) ⁴⁸⁷
JNK	FRET (^{FR} ◆) Trans. (^T ⇌)	dJUN-FRET ^{FR} ◆ (2008) ⁴⁸⁸ JNK-KTR ^T ⇌ (2014) ¹¹⁶ JNKAR: NIR JNKAR ^{FR} ◆ (2018) ⁴⁸⁹ bimJNKAR ^{FR} ◆ (2015) ⁴⁴⁴ JNKAR1EV ^{FR} ◆ (2011) ⁴⁴¹ JNKAR1 ^{FR} ◆ (2010) ⁴⁹⁰ JuCKY ^{FR} ◆ (2010) ⁴⁹¹
Kss1	Trans. (^T ⇌)	Ste7-SKARS ^T ⇌ (2015) ¹¹⁷
LATS	Biolum. (^{BL} ●)	LATS-BS ^{BL} ● (2018) ⁴⁹²
Lck	FRET (^{FR} ◆)	Lck activation sensor: CLckY-2 ^{FR} ◆ (2009) ⁴⁹³
MAPK/MK2	FRET (^{FR} ◆)	GMB ^{FR} ◆ (2001) ⁴⁹⁴
MARK	FRET (^{FR} ◆)	MARK sensor: MARK-AR1 ^{FR} ◆ (2011) ⁴⁹⁵
Mpk1	Trans. (^T ⇌)	Mkk2-SKARS ^T ⇌ (2015) ¹¹⁷
mTORC1	FRET (^{FR} ◆)	TORCAR ^{FR} ◆ (2015) ⁴³³

Table 1. continued

target	readout mech	variants
kinases/phosphatases		
p38	FRET ($^{FR}\blacklozenge$) Trans. ($^T\rightleftharpoons$)	p38 activity reporter $^{FR}\blacklozenge$ (2015) ⁴⁹⁶ p38-KTR $^T\rightleftharpoons$ (2014) ¹¹⁶
PAK1	FRET ($^{FR}\blacklozenge$)	Pakabi $^{FR}\blacklozenge$ (2009) ⁴⁹⁷
PDGFR	FRET ($^{FR}\blacklozenge$)	PDGFR biosensor $^{FR}\blacklozenge$ (2017) ⁴⁹⁸
PDK1	FRET ($^{FR}\blacklozenge$)	PARE $^{FR}\blacklozenge$ (2011) ⁴⁹⁹
PKA	Biolum. ($^{BL}\bullet$) Ex. Rat. ($^{EX}\star$) FLIM ($^{FL}\diamond$) FLINC ($^{FC}\blacktriangle$) FRET ($^{FR}\blacklozenge$) Inten. (I*) Trans. ($^T\rightleftharpoons$)	AKAR: ExRai-AKAR $^{EX}\star$ (2018) ²¹¹ blueAKAR I* (2018) ²¹¹ RAB-AKARev I* (2018) ²¹¹ sapphireAKAR I* (2018) ²¹¹ VcpV FLARE AKAR $^{FR}\blacklozenge$ (2018) ⁴⁶⁷ VV FLARE AKAR $^{FR}\blacklozenge$ (2018) ⁴⁶⁷ C3C3 FLARE AKAR $^{FR}\blacklozenge$ (2018) ⁴⁶⁷ mCh-mCh FLARE AKAR $^{FR}\blacklozenge$ (2018) ⁴⁶⁷ NIR AKAR $^{FR}\blacklozenge$ (2018) ⁴⁸⁹ AKARet $^{FL}\diamond$ (2017) ⁴⁶⁸ AKAR ^{dual} $^{FL}\diamond$ (2017) ⁴⁶⁹ AKAR5 $^{FL}\diamond$ (2017) ⁵⁰⁰ AKAR4.1 $^{FR}\blacklozenge$ (2015) ⁵⁰¹ FLIM-AKAR $^{FL}\diamond$ (2014) ⁵⁰² AKAR-CR $^{FR}\blacklozenge$ (2012) ²⁶¹ bimAKAR $^{FR}\blacklozenge$ (2011) ⁵⁰³ LumAKAR $^{BL}\bullet$ (2011) ⁵⁰³ AKAR3EV $^{FR}\blacklozenge$ (2011) ⁴⁴¹ AKAR4 $^{FR}\blacklozenge$ (2011) ⁵⁰⁴ ICUPID $^{FR}\blacklozenge$ (2011) ³⁰⁸ AKAR-GR $^{FR}\blacklozenge$ (2011) ³⁰⁸ CRY-AKAR $^{FR}\blacklozenge$ (2008) ⁵⁰⁵ AKAR3 $^{FR}\blacklozenge$ (2006) ⁵⁰⁶ AKAR2 $^{FR}\blacklozenge$ (2005) ⁵⁰⁷ AKAR1 $^{FR}\blacklozenge$ (2001) ⁵⁰⁸ ART $^{FR}\blacklozenge$ (2000) ⁵⁰⁹ FLINC-AKAR1 $^{FC}\blacktriangle$ (2017) ⁴⁷⁷ PKA-KTR $^T\rightleftharpoons$ (2014) ¹¹⁶ RLuc-PCA PKA $^{BL}\bullet$ (2007) ⁵¹⁰ single color PKA sensor: GAk I* (2014) ⁵¹¹
PKC	Ex. Rat. ($^{EX}\star$) FLIM ($^{FL}\diamond$) FRET ($^{FR}\blacklozenge$) Inten. (I*)	CKAR: ExRai-CKAR $^{EX}\star$ (2018) ²¹¹ blueCKAR I* (2018) ²¹¹ sapphireCKAR I* (2018) ²¹¹ CKAR $^{FR}\blacklozenge$ (2003) ⁵¹² Eevee-PKC $^{FR}\blacklozenge$ (2011) ⁴⁴¹ IDOCKS: IDOCKS γ $^{FL}\diamond$ (2018) ⁵¹³ IDOCKS β $^{FL}\diamond$ (2018) ⁵¹³ IDOCKS α $^{FL}\diamond$ (2018) ⁵¹³ ITRACK: ITRACK γ $^{FL}\diamond$ (2018) ⁵¹³ ITRACK β $^{FL}\diamond$ (2018) ⁵¹³ ITRACK α $^{FL}\diamond$ (2018) ⁵¹³ KCP: KCAP-1 $^{FR}\blacklozenge$ (2006) ⁵¹⁴ KCP-2 $^{FR}\blacklozenge$ (2006) ⁵¹⁴ KCP-1 $^{FR}\blacklozenge$ (2004) ⁵¹⁵
PKD	FRET ($^{FR}\blacklozenge$)	DKAR $^{FR}\blacklozenge$ (2007) ⁵¹⁶ G-PKDrep: G-PKDrep live $^{FR}\blacklozenge$ (2012) ⁵¹⁷ G-PKDrep $^{FR}\blacklozenge$ (2009) ⁵¹⁸
PKM2	FRET ($^{FR}\blacklozenge$)	PKAR: PKAR2.3 $^{FR}\blacklozenge$ (2013) ⁵¹⁹
PTEN	BRET ($^{BR}\bullet$)	Rluc-PTEN-YFP $^{BR}\bullet$ (2014) ⁵²⁰
ROCK	FRET ($^{FR}\blacklozenge$)	Eevee-ROCK $^{FR}\blacklozenge$ (2017) ⁵²¹
RSK	FRET ($^{FR}\blacklozenge$)	Eevee-RSK $^{FR}\blacklozenge$ (2011) ⁴⁴¹
RTK	FRET ($^{FR}\blacklozenge$)	Picchu: PicchuEV $^{FR}\blacklozenge$ (2011) ⁴⁴¹ Picchu $^{FR}\blacklozenge$ (2001) ⁵²²
S6K	FRET ($^{FR}\blacklozenge$)	Eevee-S6K $^{FR}\blacklozenge$ (2011) ⁴⁴¹
SAP3K	FRET ($^{FR}\blacklozenge$)	SAP3K $^{FR}\blacklozenge$ (2009) ⁵²³
Src	FRET ($^{FR}\blacklozenge$)	Src Indicator: BG-Src1.0 $^{FR}\blacklozenge$ (2013) ⁵²⁴ YO-Src1.0 $^{FR}\blacklozenge$ (2012) ⁴⁰⁷ Src Reporter (ECFP/YPet) $^{FR}\blacklozenge$ (2008) ⁵²⁵ Src Reporter $^{FR}\blacklozenge$ (2005) ⁵²⁶ Src Indicator $^{FR}\blacklozenge$ (2001) ⁴³¹ Srcus $^{FR}\blacklozenge$ (2007) ⁵²⁷
ZAP-70	FRET ($^{FR}\blacklozenge$)	ROZA $^{FR}\blacklozenge$ (2008) ⁵²⁸
neurotransmitters		
5-HT	FRET ($^{FR}\blacklozenge$) Inten. (I*)	5-HT 3A CNiFER: 5-HT 3A LGIC CNiFER $^{FR}\blacklozenge$ (2011) ⁵²⁹ dLight: 5HT2A-dLight I* (2018) ²⁰⁴
ACh	FRET ($^{FR}\blacklozenge$) Inten. (I*)	ACh CNiFER: $\alpha 4\beta 2$ -nAChR LGIC CNiFER $^{FR}\blacklozenge$ (2011) ⁵²⁹ $\alpha 7$ LGIC CNiFER $^{FR}\blacklozenge$ (2011) ⁵²⁹ M1-CNiFER $^{FR}\blacklozenge$ (2010) ⁵³⁰ ACh-Snifit: ACh-Snifit ^{WA} -E $^{FR}\blacklozenge$ (2014) ⁵³¹ ACh-Snifit-E $^{FR}\blacklozenge$ (2014) ⁵³¹ ACh-Snifit-D $^{FR}\blacklozenge$ (2014) ⁵³¹ GACH: GACH2.0 I* (2018) ²⁰⁶ GACH1.0 I* (2018) ²⁰⁶
dopamine	FRET ($^{FR}\blacklozenge$) Inten. (I*)	dLight: dLight1.2 I* (2018) ²⁰⁴ Dopamine CNiFER: D2 CNiFER $^{FR}\blacklozenge$ (2014) ⁵³² GRABDA: GRAB_DA1h I* (2018) ²⁰⁵ GRAB_DA1m I* (2018) ²⁰⁵

Table 1. continued

target	readout mech	variants
neurotransmitters		
GABA	FRET ($^{FR}\blacklozenge$)	GABA-Snifit $^{FR}\blacklozenge$ (2012) ⁵³³
glutamate	FRET ($^{FR}\blacklozenge$) Inten. ($^{I}\star$)	FLIP Glt: FLIP-cpGlt1210 $^{FR}\blacklozenge$ (2009) ³⁴⁵ FLIPE: FLII ⁸¹ E-1u $^{FR}\blacklozenge$ (2005) ³³⁵ FLIPE-Y Surface series $^{FR}\blacklozenge$ (2005) ⁵³⁴ FLIPE-Y Series $^{FR}\blacklozenge$ (2005) ⁵³⁴ GluSnFR: superGluSnFR $^{FR}\blacklozenge$ (2008) ⁵³⁵ iGluSnFR: iGlu _u $^{I}\star$ (2018) ¹⁰⁰² iGlu _f $^{I}\star$ (2018) ¹⁰⁰² iGluSnFR $^{I}\star$ (2013) ¹⁷⁴ QBP based: Gln D157N reporter $^{FR}\blacklozenge$ (2010) ⁵³⁶ Snifit-iGluR5 $^{FR}\blacklozenge$ (2012) ⁵³⁷
glycine	FRET ($^{FR}\blacklozenge$)	Glycine FRET sensor: GlyFS $^{FR}\blacklozenge$ (2018) ⁵³⁸
NE	FRET ($^{FR}\blacklozenge$)	NE CNiFER: α 1A CNiFER $^{FR}\blacklozenge$ (2014) ⁵³²
phosphoinositides/lipids		
3' IP	FRET ($^{FR}\blacklozenge$) Trans. ($^{T}\rightleftharpoons$)	GFP AKT domains: GFP-Akt $^{T}\rightleftharpoons$ (1999) ⁹¹ GFP-AH $^{T}\rightleftharpoons$ (1999) ⁹¹ homo FRET mCherry-Akt-PH: mCherry-Akt-PH $^{FR}\blacklozenge$ (2015) ⁵³⁹ InPAkt $^{FR}\blacklozenge$ (2005) ⁵⁴⁰
DAG	FRET ($^{FR}\blacklozenge$) Trans. ($^{T}\rightleftharpoons$)	Cys1-GFP: C1 ₂ -GFP $^{T}\rightleftharpoons$ (1998) ⁹⁸ Cys1-GFP $^{T}\rightleftharpoons$ (1998) ⁹⁶ Daglas: Daglas-mit1 $^{FR}\blacklozenge$ (2006) ⁵⁴¹ Daglas-em1 $^{FR}\blacklozenge$ (2006) ⁵⁴¹ Daglas-pm1 $^{FR}\blacklozenge$ (2006) ⁵⁴¹ DAGR $^{FR}\blacklozenge$ (2003) ⁵¹² Digda $^{FR}\blacklozenge$ (2008) ⁵⁴²
IP ₃	BRET ($^{BR}\odot$) FRET ($^{FR}\blacklozenge$) Trans. ($^{T}\rightleftharpoons$)	FIRE: FIRE-3 $^{FR}\blacklozenge$ (2006) ⁵⁴³ FIRE-2 $^{FR}\blacklozenge$ (2006) ⁵⁴³ FIRE-1 $^{FR}\blacklozenge$ (2006) ⁵⁴³ fretino: fretino $^{BR}\odot$ (2015) ³⁶⁷ FRET InsP3 sensor $^{FR}\blacklozenge$ (2015) ³⁶⁷ fretino-2 $^{FR}\blacklozenge$ (2005) ⁵⁴⁴ GFP-PH: GFP-PHD $^{T}\rightleftharpoons$ (1999) ⁵⁴⁵ IRIS: IRIS-1 $^{FR}\blacklozenge$ (2006) ⁵⁴⁶ LIBRA: LIBRAvIII $^{FR}\blacklozenge$ (2009) ⁵⁴⁷ LIBRAvIIS $^{FR}\blacklozenge$ (2009) ⁵⁴⁷ LIBRAvII $^{FR}\blacklozenge$ (2009) ⁵⁴⁷ LIBRAvI $^{FR}\blacklozenge$ (2009) ⁵⁴⁷ LIBRA $^{FR}\blacklozenge$ (2004) ⁵⁴⁸
PA	FRET ($^{FR}\blacklozenge$)	Pii: Pii-DK $^{FR}\blacklozenge$ (2010) ⁵⁴⁹
PI(4)P	BRET ($^{BR}\odot$) FRET ($^{FR}\blacklozenge$)	BRET PI(4)P sensors: SidM-2xP4M $^{BR}\odot$ (2016) ⁵⁵⁰ OSH2-2xPH $^{BR}\odot$ (2016) ⁵⁵⁰ Pippi: Pippi-PI(4)P $^{FR}\blacklozenge$ (2008) ⁵⁴²
PI(3)P	Trans. ($^{T}\rightleftharpoons$)	GFP-FYVE: GFP-FYVE (FENS-1) $^{T}\rightleftharpoons$ (2001) ⁹⁴ GFP-Pib1p $^{T}\rightleftharpoons$ (1998) ⁹⁰ GFP-EEA1 (FYVE) $^{T}\rightleftharpoons$ (1998) ⁹⁰ GFP-PX $^{T}\rightleftharpoons$ (2001) ⁹⁴
PI(3,4)P ₂	FRET ($^{FR}\blacklozenge$)	Pippi-PI(3,4)P2 $^{FR}\blacklozenge$ (2006) ⁴³²
PI(3,5)P ₂	Trans. ($^{T}\rightleftharpoons$)	GFP-MLN1 $^{T}\rightleftharpoons$ (2013) ⁵⁵¹
PI(4,5)P ₂	FRET ($^{FR}\blacklozenge$) Rat. ($^{R}\blacksquare$) Trans. ($^{T}\rightleftharpoons$)	CAY $^{FR}\blacklozenge$ (2004) ⁵⁵² CYPHR $^{FR}\blacklozenge$ (2003) ⁵¹² FP-Tubby $^{T}\rightleftharpoons$ (2001) ⁵⁵³ FPX PIP2 sensor $^{R}\blacksquare$ (2015) ³⁰⁷ PH(PLC δ): PH(PLC δ)-CFP/YFP $^{FR}\blacklozenge$ (2001) ⁵⁵⁴ PH(PLC δ) - GFP $^{T}\rightleftharpoons$ (1998) ⁹² GFP-PH $^{T}\rightleftharpoons$ (1998) ⁵⁵⁵ Pippi-PI(4,5)P2 $^{FR}\blacklozenge$ (2008) ⁵⁴²
PIP ₃	FRET ($^{FR}\blacklozenge$) Trans. ($^{T}\rightleftharpoons$) BRET ($^{BR}\odot$)	FLIIP $^{FR}\blacklozenge$ (2003) ⁵⁵⁶ labeled PH domains: PH(PKB)-GFP $^{T}\rightleftharpoons$ (1999) ⁹³ PH(GRP1)-GFP $^{T}\rightleftharpoons$ (1999) ⁹³ PIP3 BRET sensor $^{BR}\odot$ (2012) ⁵⁵⁷
PS	Trans. ($^{T}\rightleftharpoons$)	2xPH(Evectin2) $^{T}\rightleftharpoons$ (2011) ⁵⁵⁸ cPLA2-C2: cPLA2-C2-GFP $^{T}\rightleftharpoons$ (2003) ⁹⁹ Lact-C2: mRFP - Lact-C2 $^{T}\rightleftharpoons$ (2008) ¹⁰¹ GFP - Lact-C2 $^{T}\rightleftharpoons$ (2008) ¹⁰¹ PKC-C2: PKC α -C2-EGFP $^{T}\rightleftharpoons$ (2003) ⁹⁹ C2-GFP $^{T}\rightleftharpoons$ (1998) ⁹⁸ PLC δ 1-C2: PLC δ 1-C2-EGFP $^{T}\rightleftharpoons$ (2002) ¹⁰⁰
proteases		
Atg4a	FRET ($^{FR}\blacklozenge$)	FRET-LC3B $^{FR}\blacklozenge$ (2012) ⁵⁵⁹

Table 1. continued

target	readout mech	variants
proteases		
Atg4b	FRET ($^{FR}\blacklozenge$)	FRET-GATE-16 $^{FR}\blacklozenge$ (2012) ⁵⁵⁹
caspase1	Rat. ($^R\blacksquare$)	FPX caspase 1 sensor: single polypeptide FPX caspase 1 sensor $^R\blacksquare$ (2015) ³⁰⁷
caspase3	FRET ($^{FR}\blacklozenge$)	ddFP based: single polypeptide FPX caspase 3 sensor $^R\blacksquare$ (2015) ³⁰⁷ bimolecular FPX caspase 3 reporter $^R\blacksquare$ (2015) ³⁰⁷ ddRFP-A1B1-DEVD I* (2012) ³⁸¹
	Inten. (I*)	DEVD: mCitrine-DEVD-mTFP1 $^{FR}\blacklozenge$ (2008) ⁵⁶⁰ mAmetrine-DEVD-tdTomato $^{FR}\blacklozenge$ (2008) ⁵⁶⁰ BFP-DEVD-GFP $^{FR}\blacklozenge$ (2002) ⁵⁶¹ Sensor C3 $^{FR}\blacklozenge$ (2001) ⁵⁶² CFP-DEVD-YFP $^{FR}\blacklozenge$ (2000) ⁵⁶³
	Rat. ($^R\blacksquare$)	DEVK: MiCy-DEVK-mKO $^{FR}\blacklozenge$ (2004) ⁵⁶⁴ EC-RP $^{FR}\blacklozenge$ (2008) ⁵⁶⁵
		far-red caspase sensors: mKate2-DEVD-iRFP $^{FR}\blacklozenge$ (2016) ⁵⁶⁶ Intein based: sDnaB-cpM4(DEVD) I* (2013) ⁵⁶⁷
		iProtease: iCasper I* (2015) ⁵⁶⁸ SCAT3 $^{FR}\blacklozenge$ (2003) ⁵⁶⁹ yDMQDc $^{FR}\blacklozenge$ (2006) ⁵⁷⁰
caspase8	FRET ($^{FR}\blacklozenge$)	Bid cleavage sensor $^{FR}\blacklozenge$ (2002) ⁵⁷¹
	Rat. ($^R\blacksquare$)	FPX caspase 8 sensor: single polypeptide FPX caspase 8 sensor $^R\blacksquare$ (2015) ³⁰⁷ IC-RP $^{FR}\blacklozenge$ (2008) ⁵⁶⁵
caspase9	FRET ($^{FR}\blacklozenge$)	SCAT9 $^{FR}\blacklozenge$ (2003) ⁵⁶⁹
MT1-MMP	FRET ($^{FR}\blacklozenge$)	MT1-MMP Biosensor: MT1-MMP Biosensor (mCherry/mOrange2) $^{FR}\blacklozenge$ (2010) ⁵⁷² MT1-MMP Biosensor $^{FR}\blacklozenge$ (2008) ⁵⁷³
receptors/GPCRs		
A1R	FRET ($^{FR}\blacklozenge$)	A1R SPASM: A1R - FL <i>Gas</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴ A1R - FL <i>Gai</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴
β 1-AR	Inten. (I*)	dLight: β 1AR-dLight I* (2018) ²⁰⁴
β 2-AR	BRET ($^{BR}\odot$)	BRET ² β 2-AR activation Probes: β 2AR-RLuc <i>Gas</i> -GFP10 $^{BR}\odot$ (2005) ⁵⁷⁵ β 2AR-RLuc GFP-G β 1 $^{BR}\odot$ (2005) ⁵⁷⁵ β 2AR-RLuc GFP-G γ 2 $^{BR}\odot$ (2005) ⁵⁷⁵
	FRET ($^{FR}\blacklozenge$)	dLight: β 2AR-dLight I* (2018) ²⁰⁴
	Inten. (I*)	β 2-AR SPASM: β 2-AR FL <i>Gaq</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴ β 2-AR FL <i>Gas</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴ β 2-AR- <i>Gα</i> SPASM $^{FR}\blacklozenge$ (2013) ⁵⁷⁶
	Trans. ($^T\rightleftharpoons$)	Nb80: Nb80-GFP $^T\rightleftharpoons$ (2013) ¹⁰⁹
<i>Dictyostelium</i> GPCR	FRET ($^{FR}\blacklozenge$)	labeled G proteins: G β -YFP + <i>Gα</i> 2-CFP $^{FR}\blacklozenge$ (2001) ⁵⁷⁷
DRD2	Inten. (I*)	iTango: DRD2-iTango I* (2017) ⁵⁷⁸
<i>Gai</i>	FRET ($^{FR}\blacklozenge$)	<i>Gai</i> sensor: <i>Gai</i> 1 sensor v2 $^{FR}\blacklozenge$ (2016) ⁵⁷⁹ <i>Gai</i> 2 Sensor v2 $^{FR}\blacklozenge$ (2016) ⁵⁷⁹ <i>Gai</i> 3 sensor v2 $^{FR}\blacklozenge$ (2016) ⁵⁷⁹ <i>Gai</i> 1 v1 $^{FR}\blacklozenge$ (2006) ⁵⁸⁰ <i>Gai</i> 2 v1 $^{FR}\blacklozenge$ (2006) ⁵⁸⁰ <i>Gai</i> 3 v1 $^{FR}\blacklozenge$ (2006) ⁵⁸⁰ Bunemann <i>Gai</i> -G γ 2 $^{FR}\blacklozenge$ (2003) ⁵⁸¹ Bunemann <i>Gai</i> -G β 1 $^{FR}\blacklozenge$ (2003) ⁵⁸¹
<i>Gaq</i>	FRET ($^{FR}\blacklozenge$)	<i>Gaq</i> sensor: <i>Gaq</i> sensor (v2) $^{FR}\blacklozenge$ (2011) ⁵⁸² <i>Gaq</i> Sensor (v1) $^{FR}\blacklozenge$ (2009) ⁵⁸³
<i>Gas</i>	BRET ($^{BR}\odot$)	<i>Gas</i> sensor $^{FR}\blacklozenge$ (2006) ⁵⁸⁴
	FRET ($^{FR}\blacklozenge$)	<i>Gs</i> activation BRET assay: RLucII-117- <i>Gas</i> + GFP10-G γ 1 $^{BR}\odot$ (2016) ⁵⁸⁵
	Trans. ($^T\rightleftharpoons$)	Nb37 based: Nb37-YFP $^T\rightleftharpoons$ (2017) ¹¹⁰
α 1-AR	FRET ($^{FR}\blacklozenge$)	α 1-AR SPASM: α 1-AR FL <i>Gaq</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴
α 2A-AR	BRET ($^{BR}\odot$)	dLight: α 2AR-dLight I* (2018) ²⁰⁴
	FRET ($^{FR}\blacklozenge$)	α 2-AR SPASM: α 2-AR FL <i>Gas</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴ α 2-AR FL <i>Gai</i> SPASM $^{FR}\blacklozenge$ (2017) ⁵⁷⁴
	Inten. (I*)	α 2A-AR + labeled G protein: α 2A-AR-Venus + G α_{i1} -122RLuc $^{BR}\odot$ (2006) ⁵⁸⁶ α 2A-AR-Venus + G α_{i1} -91RLuc $^{BR}\odot$ (2006) ⁵⁸⁶ α 2A-AR-Venus + RLuc-G γ 2 $^{BR}\odot$ (2006) ⁵⁸⁶ α 2A-YFP + CFP-G γ 2 $^{FR}\blacklozenge$ (2005) ⁵⁸⁷ α 2A-AR activation sensor: α 2A-AR-cam $^{FR}\blacklozenge$ (2003) ⁵⁸⁸
M1R	FRET ($^{FR}\blacklozenge$)	M1R activation sensor: M1R-EYFP-Cerulean $^{FR}\blacklozenge$ (2009) ⁵⁸³
MT2	Inten. (I*)	dLight: MT2-dLight I* (2018) ²⁰⁴
Odr-10	BRET ($^{BR}\odot$)	OGOR $^{BR}\odot$ (2011) ⁵⁸⁹

Table 1. continued

target	readout mech	variants
receptors/GPCRs		
κ -opioid	Inten. ($I^{\star}$)	dLight: KOR-dLight $I^{\star}$ (2018) ²⁰⁴
μ -opioid	Inten. ($I^{\star}$)	dLight: MOR-dLight $I^{\star}$ (2018) ²⁰⁴
Opsin	FRET (FR ◆)	Opsin SPASM: Opsin-G α SPASM FR ◆ (2013) ⁵⁷⁶
PTHR	FRET (FR ◆)	PTHR activation sensor: PTHR-cam FR ◆ (2003) ⁵⁸⁸
TrkB	FLIM (FL ◇)	TrkB-PLC interaction: TrkB sensor FL ◇ (2016) ⁵⁹⁰
VEGF	BRET (BR ●)	BRET VEGF biosensor BR ● (2016) ⁵⁹¹
redox		
H ₂ O ₂	Ex. Rat. ($EX^{\star}$) FRET (FR ◆) Inten. ($I^{\star}$)	HyPer: HyPerRed $EX^{\star}$ (2014) ⁵⁹² HyPer-3 $EX^{\star}$ (2013) ⁵⁹³ HyPer-2 $EX^{\star}$ (2011) ⁵⁹⁴ HyPer $EX^{\star}$ (2006) ⁵⁹⁵ roGFP based: roGFP2-Tsa2 Δ C _R $EX^{\star}$ (2016) ⁵⁹⁶ roGFP2-Tsa2 Δ C _R $EX^{\star}$ (2016) ⁵⁹⁶ roGFP2-GPx4 $EX^{\star}$ (2009) ⁵⁹⁷ roGFP2-Orp1 $EX^{\star}$ (2009) ⁵⁹⁷ unnatural amino acid based: UFP-Tyr66pBoPhe $I^{\star}$ (2012) ⁵⁹⁸ Yap1 based: PerFRET FR ◆ (2013) ⁵⁹⁹ OxyFRET FR ◆ (2013) ⁵⁹⁹
H ₂ S	Inten. ($I^{\star}$)	pAzF H ₂ S sensors: hsGFP $I^{\star}$ (2014) ⁶⁰⁰ cpGFP-Tyr66pAzF $I^{\star}$ (2012) ⁶⁰¹
NO	FRET (FR ◆)	FRET-MT FR ◆ (2000) ⁶⁰² sGC based: Piccell FR ◆ (2006) ⁶⁰³ NOA-1 FR ◆ (2005) ⁶⁰⁴
organic hydroperoxides	Inten. ($I^{\star}$)	OHSer $I^{\star}$ (2010) ⁶⁰⁵
ONOO [−]	Inten. ($I^{\star}$)	pnGFP $I^{\star}$ (2013) ⁶⁰⁶
redox status	Ex. Rat. ($EX^{\star}$) FRET (FR ◆) Inten. ($I^{\star}$)	HSP33: HSP-FRET FR ◆ (2006) ⁶⁰⁷ redox-sensitive linker: CY-RL7 FR ◆ (2011) ⁶⁰⁸ RedoxFluor FR ◆ (2010) ⁶⁰⁹ ECFP-RL-EYFP FR ◆ (2008) ⁶¹⁰ roGFP: roGFP1-iX $EX^{\star}$ (2008) ⁶¹¹ Grx1-roGFP2 $EX^{\star}$ (2008) ⁶¹² roGFP1-Rx family $EX^{\star}$ (2006) ⁶¹³ roGFP2 $EX^{\star}$ (2004) ¹⁴³ roGFP1 $EX^{\star}$ (2004) ¹⁴³ rxRFP: TrxRFP1 $I^{\star}$ (2017) ⁶¹⁴ rxRFP1.X sensitivity series $I^{\star}$ (2016) ⁶¹⁵ rxRFP $I^{\star}$ (2015) ⁶¹⁶ rxYFP: rxYFP-Grx1p $I^{\star}$ (2006) ⁶¹⁷ rxYFP ₂₀₂ $I^{\star}$ (2001) ⁶¹⁸
superoxide	Inten. ($I^{\star}$)	mt-cpYFP $I^{\star}$ (2008) ⁶¹⁹
small G-proteins		
Cdc42	FLIM (FL ◇) FRET (FR ◆)	(CDC42) GEF sensor FR ◆ (2003) ⁶²⁰ CDC42 biosensor FR ◆ (2014) ⁶²¹ Cdc42 FRET: Cdc42-CyRM FL ◇ (2016) ⁶²² Cdc42 FRET (mRuby2/mCherry (I202Y)) FL ◇ (2016) ⁴⁵⁴ Cdc42 FRET FL ◇ (2011) ⁶²³ CDC42 Raichu: CDC42 Raichu FR ◆ (2002) ⁶²⁴ CRIB Raichu FR ◆ (2002) ⁶²⁴ Cdc42–2G: Cdc42–2G FR ◆ (2016) ⁶²⁵ GDI Cdc42 FLARE FR ◆ (2016) ⁶²⁶
Rab5	FRET (FR ◆)	Rab5 Raichu FR ◆ (2008) ⁶²⁷
Rac1	FRET (FR ◆)	FLAIR FR ◆ (2000) ⁶²⁸ FLARE: Rac1-FLARE FR ◆ (2009) ⁶²⁹ GDI Rac1 FLARE FR ◆ (2016) ⁶²⁶ Rac1 Raichu: Rac1 Raichu EV FR ◆ (2011) ⁴⁴¹ CRIB Raichu FR ◆ (2002) ⁶²⁴ Rac1 Raichu FR ◆ (2002) ⁶²⁴ single chain Rac1 biosensors: NIR Rac1 biosensor FR ◆ (2018) ⁴⁸⁹ single chain Rac1 biosensor v2 FR ◆ (2016) ⁶³⁰ Rac1-2G FR ◆ (2015) ⁶³¹ single-chain Rac1 biosensor FR ◆ (2014) ⁶³²
Rac2	FRET (FR ◆)	single chain Rac2 biosensor FR ◆ (2016) ⁶³⁰
Rac3	FRET (FR ◆)	single chain Rac3 biosensor FR ◆ (2017) ⁶³³
Ral	FRET (FR ◆)	Raichu-Ral: Raichu-RalB FR ◆ (2004) ⁶³⁴ Raichu-RalA FR ◆ (2004) ⁶³⁴
Ran	FRET (FR ◆)	Ran FRET probes: YIC FR ◆ (2002) ⁶³⁵ YRC FR ◆ (2002) ⁶³⁵

Table 1. continued

target	readout mech	variants
small G-proteins		
Rap1	FRET ($^{FR}\blacklozenge$)	Rap1 Raichu $^{FR}\blacklozenge$ (2001) ⁶³⁶
Ras	FLIM ($^{FL}\blacklozenge$) FRET ($^{FR}\blacklozenge$)	DORA Ras $^{FR}\blacklozenge$ (2015) ⁶³⁷ FRas : ShadowY H-Ras sensor $^{FL}\blacklozenge$ (2017) ⁶³⁸ ShadowG FRas2-M $^{FL}\blacklozenge$ (2015) ⁴⁵⁵ FRas2-M $^{FL}\blacklozenge$ (2013) ⁶³⁹ FRas2-F $^{FL}\blacklozenge$ (2013) ⁶³⁹ FRas-F $^{FL}\blacklozenge$ (2006) ⁶⁴⁰ FRas $^{FL}\blacklozenge$ (2006) ⁶⁴⁰ Raichu : Ras Raichu EV $^{FR}\blacklozenge$ (2011) ⁴⁴¹ Ras Raichu $^{FR}\blacklozenge$ (2001) ⁶³⁶
RhoA	FLIM ($^{FL}\blacklozenge$) FRET ($^{FR}\blacklozenge$)	GDI FLARE : GDI-RhoA FLARE $^{FR}\blacklozenge$ (2016) ⁶²⁶ FLARE : RhoA DORA $^{FR}\blacklozenge$ (2015) ⁶⁴¹ RhoA2G $^{FR}\blacklozenge$ (2013) ⁴⁷³ bimolecular RhoA Biosensor $^{FR}\blacklozenge$ (2009) ⁶²⁹ RhoA-FLARE/ RhoA1G $^{FR}\blacklozenge$ (2006) ⁶⁴² RhoA FRET : RhoA-CyRM $^{FL}\blacklozenge$ (2016) ⁶²² RhoA FRET (mRuby2/mCherry (I202Y)) $^{FL}\blacklozenge$ (2016) ⁴⁵⁴ RhoA-FRET $^{FL}\blacklozenge$ (2011) ⁶²³ Raichu : RhoA Raichu CR $^{FR}\blacklozenge$ (2012) ²⁶¹ RBD Raichu $^{FR}\blacklozenge$ (2006) ⁶⁴³ RhoA Raichu $^{FR}\blacklozenge$ (2003) ⁶⁴⁴
RhoB	FRET ($^{FR}\blacklozenge$)	DORA RhoB : RhoB FRET sensor $^{FR}\blacklozenge$ (2016) ⁶⁴⁵
RhoC	FRET ($^{FR}\blacklozenge$)	DORA RhoC : RhoC FRET Sensor $^{FR}\blacklozenge$ (2016) ⁶⁴⁵ RhoC FLARE $^{FR}\blacklozenge$ (2013) ⁶⁴⁶
RhoQ	FRET ($^{FR}\blacklozenge$)	TC10 Raichu $^{FR}\blacklozenge$ (2006) ⁶⁴⁷
RRas	FRET ($^{FR}\blacklozenge$)	RRas Raichu $^{FR}\blacklozenge$ (2007) ⁶⁴⁸
other post-translational modifications		
β Arrestin 2	BRET ($^{BR}\bullet$)	βArrestin 2 ubiquitination biosensor $^{BR}\bullet$ (2004) ⁶⁴⁹
histone acetylation	FRET ($^{FR}\blacklozenge$)	Histac : Histac-H3K9/14 $^{FR}\blacklozenge$ (2016) ⁶⁵⁰ Histac-H4K12 $^{FR}\blacklozenge$ (2011) ⁶⁵¹ Histac-H4K5/8 $^{FR}\blacklozenge$ (2009) ⁶⁵²
K27H3 methyl-transf.	FRET ($^{FR}\blacklozenge$)	K27 reporter $^{FR}\blacklozenge$ (2004) ⁶⁵³
K9H3 methyltransf.	FRET ($^{FR}\blacklozenge$)	K9 reporter : H3K9me3 Biosensor $^{FR}\blacklozenge$ (2018) ¹⁰⁰¹ K9 reporter $^{FR}\blacklozenge$ (2004) ⁶⁵³
O-GlcNAc transferase	FRET ($^{FR}\blacklozenge$)	O-GlcNAc sensor $^{FR}\blacklozenge$ (2006) ⁶⁵⁴
ubiquitination	Inten. ($^{I}\star$)	REACH-Ubiquitin $^{I}\star$ (2006) ⁶⁵⁵
other signaling proteins		
Annexin 4	FRET ($^{FR}\blacklozenge$)	NEX4 : ORNEX4 $^{FR}\blacklozenge$ (2008) ⁶⁵⁶ CYNEX4 $^{FR}\blacklozenge$ (2006) ⁶⁵⁷
Bax	Trans. ($^{T}\rightleftharpoons$)	Bax translocation reporter $^{T}\rightleftharpoons$ (2016) ⁵⁶⁶
CaM	FRET ($^{FR}\blacklozenge$)	BSCaM : BSCaM2 $^{FR}\blacklozenge$ (1999) ⁶⁵⁸ BSCaM1 $^{FR}\blacklozenge$ (1997) ⁶⁵⁹ MLCK-FIP $^{FR}\blacklozenge$ (2002) ⁶⁶⁰
CRAC	Trans. ($^{T}\rightleftharpoons$)	PH(crac)-GFP $^{T}\rightleftharpoons$ (2002) ⁶⁶¹
CREB	FRET ($^{FR}\blacklozenge$)	ICAP $^{FR}\blacklozenge$ (2010) ⁶⁶²
MLKL	FRET ($^{FR}\blacklozenge$)	SMART : hSMART $^{FR}\blacklozenge$ (2018) ¹⁰⁰³ mSMART $^{FR}\blacklozenge$ (2018) ¹⁰⁰³
N-WASP	FRET ($^{FR}\blacklozenge$)	N-WASP BS $^{FR}\blacklozenge$ (2004) ⁶⁶³
plasma membrane ATPase	FRET ($^{FR}\blacklozenge$)	PMCA Activity Sensor : BFP-PMCA-GFP $^{FR}\blacklozenge$ (2007) ⁶⁶⁴

^aFor each signaling target, the different published biosensors are organized into families of related variants (with the family or group name shown in bold). The different read-out mechanisms utilized are shown with the different icons indicating the type of readout for a given biosensor. The different readout mechanisms shown in this table are bioluminescence intensity ($^{BL}\bullet$), BRET ($^{BR}\bullet$), intensity-based FRET ($^{FR}\blacklozenge$), FLIM-FRET ($^{FL}\blacklozenge$), FLINC ($^{FC}\blacktriangle$), intensity ($^{I}\star$), ratiometric (with a reference FP) ($^{R}\blacksquare$), excitation ratiometric ($^{EX}\star$), emission ratiometric ($^{EM}\star$), and translocation ($^{T}\rightleftharpoons$). Additional details, including biosensor components and FPs involved, etc., can be found in the supplemental materials and at BiosensorDB.ucsd.edu.

fluorescence behavior. Tinkering with the precise nature of this coupling (i.e., how the sensing unit affects fluorescence) lies at the heart of biosensor design. Indeed, as will be seen below, how FPs are incorporated into biosensors greatly influences the types of signaling activities that can be detected.

2.2. Biosensors for Monitoring Protein Behavior

2.2.1. Protein Expression, Turnover, And Localization. In keeping with the tradition of using fluorescence as a sensitive and specific *in situ* label, the simplest biosensor designs utilize FPs as passive, genetically encoded reporters for monitoring the dynamics of gene and protein expression, as well as protein turnover and localization, in living cells. Although generally involving little more than fusing an FP-coding sequence directly to a target DNA sequence, this approach is frequently modified to increase the richness of biological information that can be obtained. For example, despite serving as a ready indicator for the presence or absence of gene expression under steady-state conditions, the stability and long-lived fluorescence of FPs (discussed in ref 11) can nevertheless obscure the detection of endogenous transcriptional dynamics. One solution has been to incorporate destabilizing elements into the FP sequence.^{12–14} A variety of fluorescent “timers”, whose distinct chromophore maturation rates give rise to time-dependent changes in their fluorescence spectra, have also been developed to differentially label newly synthesized genes or proteins,^{15–19} permitting the chronological mapping of numerous dynamic processes, including transcriptional activity;^{16,20} protein trafficking, dynamics, and degradation;^{15,17} and even the intracellular replication and spread of influenza.²¹ Similarly, photoconvertible FPs, where the fluorescence emission spectra can be irreversibly converted to a different wavelength, have been utilized to quantify protein degradation in a manner that is not impacted by production of new FPs.^{22–24} Meanwhile, photoswitchable FPs²⁵ are increasingly used as genetically encoded protein tags in conjunction with live-cell super-resolution imaging techniques,^{26,27} thereby enabling the precise and dynamic visualization of protein localization in living cells.

However, the heterologous overexpression of FP-tagged chimeras can potentially disrupt endogenous cellular processes and induce artifacts related to protein localization and dynamics. As such, researchers are increasingly exploring methods for monitoring the dynamics of proteins expressed at endogenous levels from their native genomic loci. Notable among these approaches has been the recent rise of genome editing techniques, particularly CRISPR/Cas technology (reviewed in detail by refs 28–30). In general, these techniques are based on the sequence-specific introduction of DNA double-strand breaks that are subsequently repaired via nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). Furthermore, whereas NHEJ is typically error prone, HDR enables the precise, site-specific introduction of exogenous sequences, and numerous recent studies have successfully utilized CRISPR/Cas technology to incorporate FPs at endogenous loci in living cells^{31–33} and even *in vivo*.³⁴ Nevertheless, the low-efficiency of HDR-mediated genome editing poses a major obstacle to the wider adoption of endogenous protein tagging, as does the lack of HDR in nondividing cells. Mikuni et al. cleverly skirted the latter issue by targeting embryonic neuronal precursors to achieve endogenous protein tagging via HDR in the brain,³⁴ while a

number of groups have also utilized homology-independent repair pathways for efficient FP tagging in both dividing and nondividing cells.^{35,36} On the other hand, given that HDR efficiency is also influenced by insert size, Kamiyama and colleagues utilized a previously developed split-GFP, in which GFP is divided between the 10th and 11th β -strands into two spontaneously complementing fragments,³⁷ as an alternative strategy to improve tagging efficiency.³⁸ Here, the target protein is endogenously tagged with the GFP₁₁ “epitope” and becomes fluorescent upon complementation by exogenously expressed GFP_{1–10}. The insertion of tandem epitope arrays can further be used to amplify the fluorescence signal from proteins with low endogenous expression.³⁸

Recent advances in the development and application of a variety of scaffolds for generating intracellular affinity reagents (e.g., intracellular antibodies or “intrabodies”, reviewed in ref 39) offer another exciting approach for monitoring endogenous protein dynamics that serves as a potential alternative to genome editing. These include “monobodies” derived from the 10th domain of human type III fibronectin (10FnIII or Fn3), a well-characterized scaffold that is structurally similar to the immunoglobulin VH domain,^{40,41} as well as “nanobodies” derived from the heavy chain-only antibodies present in members of the *Camelidae* family (e.g., camels, llamas, alpacas) and in *Chondrichthyes* (e.g., sharks), wherein antigen recognition is mediated by a single variable domain.^{39,42} These small, soluble domains are easily expressed in living cells and, when fused to FPs, represent powerful, genetically encodable tools for the sensitive and specific visualization of endogenous cellular components such as neuronal proteins (PSD-95 and Gephyrin) at excitatory and inhibitory synapses,⁴³ endogenous actin dynamics,⁴⁴ Wnt-induced β -catenin translocation,⁴⁵ PARP1 translocation to sites of DNA damage,⁴⁶ and GTP-bound (i.e., active) H-Ras and K-Ras.⁴⁷ As with any biosensor, interactions between intrabodies and endogenous cellular components may alter or otherwise perturb normal function, and rigorous controls are therefore needed to ensure that downstream processes are not adversely affected. In addition, the heterologous nature of this approach means that care must be taken to minimize background fluorescence caused by excess, unbound probe, with many studies utilizing stable cell lines to achieve low nanobody expression,^{45,46,48,49} while Gross and colleagues notably employed a transcriptional regulation system to link nanobody expression to target protein levels.⁴³ A recently described “flashbody” design also promises to help increase signal-to-noise ratio (SNR) by coupling antigen binding to the intensity of a circularly permuted FP (cpFP; discussed further in section 2.3.3.1).⁵⁰ Thus, the legacy of fluorescence as a cell biological tool appears to have come full circle, with intrabodies essentially marking the emergence of “native” immunofluorescence.

2.2.2. Protein–Protein Interactions. Proteins rarely operate in isolation; most in fact participate in myriad transient interactions with other proteins, with many functioning as dimers or higher-order oligomers or assembling into multiprotein complexes that behave as dedicated molecular machines. Thus, in addition to marking the presence and location of proteins within a cell, direct FP tagging can also be used to monitor protein–protein interactions (PPIs) by utilizing fluorescence (or Förster) resonance energy transfer (FRET), a photophysical phenomenon wherein an excited “donor” fluorophore (e.g., an FP) nonradiatively transfers its

excited state energy to a nearby “acceptor” fluorophore via dipole–dipole interactions (reviewed extensively in refs 11 and 51), yielding an increase in “sensitized” fluorescence emission by the acceptor (i.e., acceptor emission upon donor excitation) at the expense of direct emission by the donor.

FRET is critically dependent on the proximity of the donor and acceptor fluorophores. Specifically, FRET efficiency (E) varies with the inverse sixth power of the distance, as defined by the equation

$$E = \left[1 + \left(\frac{r}{R_0} \right)^6 \right]^{-1} \quad (1)$$

where r is distance between the donor and acceptor and R_0 , known as the Förster distance, is the characteristic distance at which a given donor/acceptor pair exhibits half-maximal FRET efficiency, which is influenced by photophysical factors such as the degree of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, as well as by the spatial alignment of the donor and acceptor dipoles. This proximity dependence allows FRET to serve as an exquisite molecular ruler^{52,53} that is routinely used to monitor PPIs in living cells, including in recent studies visualizing direct interactions between rate-limiting glucose metabolic enzymes in the formation of multiprotein “glucosomes”⁵⁴ and between HIV proteins,^{55,56} as well as the formation of heteromeric potassium channels⁵⁷ and A-kinase anchoring protein (AKAP)-mediated signaling complexes.^{58–63}

In addition to FRET, PPIs can also be detected in live cells through the use of protein-fragment complementation assays (PCAs). PCAs are an adaptation of classic studies in which fragments of enzymes such as β -galactosidase were found to spontaneously associate in vitro to regenerate an intact, active enzyme,⁶⁴ with the major distinction being the use of protein fragments that do not spontaneously associate but instead can be induced to reassemble and reconstitute a functional unit when brought into close proximity by a pair of interacting proteins. Concurrent with the development of PCAs based on various enzymes (β -galactosidase, dihydrofolate reductase, β -lactamase),^{65–68} FPs were also found to withstand dissection into roughly 157- and 81-residue fragments that can reassemble to produce a fluorescent species with the aid of a protein interaction pair,^{69,70} yielding the widely used biomolecular fluorescence complementation (BiFC) assay (reviewed in refs 71, 72). A novel, trimolecular fluorescence complementation (TriFC) assay has also been reported featuring a modification of the aforementioned split-FP approach, in which both the 10th and 11th β -strands are dissected out of GFP and individually tagged to proteins of interest.⁷³ When brought into close proximity via PPIs, the two strands will reassemble with the separately expressed GFP_{1–9} sensor domain, thereby generating a fluorescent signal. This approach improves the expression and solubility of the tagged proteins by reducing the bulk of the fused FP fragments and was recently utilized to map PPIs among proteins related to frontotemporal dementia.⁷⁴

Despite numerous advances in FP engineering, the relatively slow kinetics of FP refolding and chromophore maturation, which exhibit half-times ranging from minutes to hours depending on the specific variant,⁷⁵ can limit the temporal resolution of BiFC. FP fragment complementation is also largely irreversible, such that protein interaction partners

essentially become trapped, rendering BiFC ill-suited to the study of dynamic PPIs. Nevertheless, the large dynamic range and irreversible nature of BiFC is in fact a tremendous boon for the detection of weak or transient PPIs. These properties also make BiFC a good fit for high-throughput approaches, and BiFC is widely used for PPI screening, similar to the yeast two-hybrid (Y2H) assay, having recently been used in conjunction with Y2H screening to map PPIs related to 2-component phospho-relays in rice⁷⁶ and to map PPIs in the pathogenic yeast *Candida albicans*.⁷⁷ When combined with photochromic FPs, BiFC can also be utilized to localize protein interaction pairs in super-resolution, as was done recently to map the interaction between the *E. coli* proteins MreB and EF-Tu,⁷⁸ to localize dimers of the microtubule plus-end protein EB1,⁷⁹ and to visualize HER2-HER3 and STIM1-ORAI1 interactions in living cells.⁸⁰ Although the latter study successfully visualized changes in STIM-ORAI complex formation in response to endoplasmic reticulum (ER) calcium (Ca^{2+}) depletion, FRET remains the preferred method for monitoring dynamic PPIs. For instance, Jean-Alphonse and colleagues used FRET to monitor dynamic changes in the interactions among β -arrestin (β arr) 2, G $\beta\gamma$, adenylyl cyclase (AC) 2, and the parathyroid hormone receptor (PTHr) in response to agonist stimulation,⁸¹ while a recent study by Smith et al. used FRET to monitor the interaction between the catalytic and regulatory subunits of 3',5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and argue against dissociation of the PKA holoenzyme under physiological conditions⁸² (discussed further in section 4.3).

2.3. Biosensors for Monitoring Biochemical Activity Dynamics

As discussed above, the simplest implementation of a genetically encoded fluorescent biosensor is to use a gene or protein of interest as a proxy for itself. Over the years, however, more sophisticated approaches have been devised to leverage the fact that proteins dynamically alter their behavior in response to a multitude of biochemical inputs. Thus, through the considered use of various proteins or protein modules conjugated to one or more FPs, genetically encoded fluorescent biosensors can be designed that variously change their localization, fluorescence intensity, or other spectral properties in response to, and thereby report on, fluctuations in a specific biochemical parameter within the native cellular environment. As such, it has become possible to directly visualize and probe the constant biochemical flux that defines a living cell.

2.3.1. Inducing the Translocation of a Fluorescent Protein. The distribution of proteins within cells is highly dynamic, with proteins often changing their subcellular localization in response to various biochemical signals. As such, the ability of FP tags to report on protein localization can logically be repurposed to utilize the signal-induced change in protein localization as a proxy for the signal itself. Indeed, such translocation-based biosensors were among the earliest genetically encoded probes developed to monitor dynamic biochemical activities in living cells.

This approach has classically been used to monitor the production and distribution of lipid messengers such as phosphoinositides (i.e., inositol phospholipids), which comprise a minute but critical component of cell membranes and are responsible for mediating numerous cellular processes.⁸³ Phosphoinositide signaling occurs through the direct recruit-

ment of effector proteins to the plasma membrane, which is mediated by different lipid-binding modules,^{84–86} including pleckstrin homology (PH) domains,⁸⁷ Epsin N-terminal homology domains,⁸⁸ and FYVE domains (named for the proteins Fab1p, YOPB, Vps27p, and EEA1).⁸⁹ Furthermore, because lipid-binding domains from different effectors have been shown to selectively bind specific phosphoinositides (discussed in depth in ref 86), such as PI(4,5)P₂ by the PH domain from phospholipase C (PLC)δ, PI(3,4,5)P₃ by the PH domain of Btk, or PI(4,5)P₂/PI(3,4,5)P₃ by the PH domain from protein kinase B (PKB)/Akt, a certain degree of selectivity can in theory be achieved based on the chosen binding module, though appropriate controls should nevertheless be employed given that lipid selectivity is typically assessed *in vitro*.

Lipid sensors are thus constructed by directly fusing the coding sequence of an FP to that of a full-length effector protein^{90,91} or an isolated lipid-binding module,^{90,92–94} whereby the translocation of the fluorescence signal to and/or from a membrane structure serves as a clear indicator for the visualization of phosphoinositide dynamics (Figure 1A). The application of this type of biosensor has provided numerous insights into the function and regulation of phosphoinositide signaling, particularly with respect to the spatial organization of 3'-phosphoinositides during chemotaxis and cell migration (discussed further in section 4.2.3), while also remaining a straightforward and powerful approach that continues to be utilized to study the molecular details of phosphoinositide signaling. For example, in their study investigating neutrophil migration in live zebrafish embryos, Yoo and colleagues used a translocation-based biosensor in which the PH-domain of Akt is fused to GFP to visualize plasma membrane PI(4,5)P₂/PI(3,4,5)P₃ accumulation, and thus phosphoinositide 3-kinase (PI3K) activation, at the leading edge in neutrophils migrating toward laser-induced wounds *in vivo*.⁹⁵ This approach has also been used to detect other lipids in various membrane compartments. For instance, diacylglycerol (DAG), a plasma membrane lipid product formed by the PLC-catalyzed cleavage of PI(4,5)P₂, can be detected using the C1 domain of protein kinase C (PKC),^{96–98} whereas phosphatidylserine can be recognized using C2 domains derived from either PKC,^{98,99} PLCδ,¹⁰⁰ or the milk glycoprotein lactadherin.¹⁰¹

However, the overall generalizability of this biosensor design strategy is somewhat restricted given the limited availability of endogenous binding modules that are capable of being recruited by specific intracellular targets to drive probe translocation. In this regard, nanobodies have emerged as a promising new resource for the construction of translocation-based sensors. In particular, their compactness renders nanobodies amenable to high-throughput library screening,¹⁰² thereby facilitating the rapid development of novel nanobodies, and the diversity of potential epitopes that can be recognized means that a much wider range of targets can be detected compared with more traditional translocation-based sensors. For example, Rajan et al. recently generated a nanobody to detect endogenous histone H2AX phosphorylated at serine 139 (γ-H2AX), which is frequently used as an intracellular marker of DNA double-strand breaks, and used the resulting GFP-tagged chromobody to directly visualize the endogenous formation of DNA double-strand breaks in living cells.¹⁰³ Specifically, by inducing DNA breaks using laser micro-irradiation, the authors were able to observe the translocation of the γ-H2AX chromobody to break sites. Nanobodies can

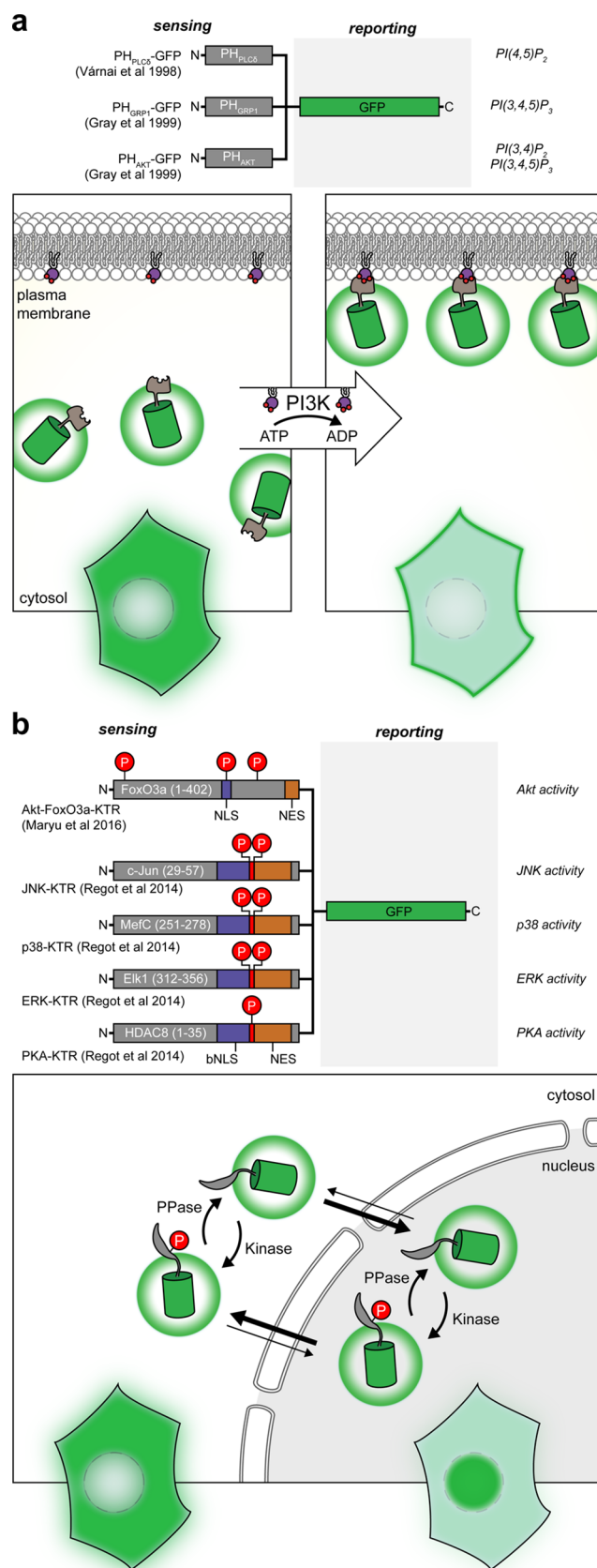


Figure 1. Translocation-based fluorescent biosensors. (A) PH domains from different proteins are fused to an FP and translocate to the plasma membrane upon the production of specific phosphoinositides.^{92,93} For example, phosphorylation of PIP₂ by PI3K to produce PIP₃ at the plasma membrane causes translocation

Figure 1. continued

of the biosensor from the cytosol to the plasma membrane. (B) Kinase translocation reporters utilize kinase-specific substrate sequences within nuclear localization sequences (NLS) and/or nuclear export sequences (NES) to promote nuclear import when dephosphorylated and nuclear export when phosphorylated.^{114,116}

also be generated that selectively recognize specific protein conformations. Indeed, several nanobodies have been developed that specifically bind to the active forms of G-protein-coupled receptor (GPCR) signaling pathways components, including the β_2 -adrenergic receptor (β_2 -AR) (Nb80¹⁰⁴ and Nb6B9¹⁰⁵), the muscarinic acetylcholine receptor (Nb9–8¹⁰⁶), the μ opioid receptor (Nb39¹⁰⁷), and the $G_{\alpha s}$ heterotrimeric G protein subunit,¹⁰⁸ some of which have already been utilized as translocation-based sensors to probe GPCR signaling in living cells (see, for example, section 4.1 below).^{109,110}

In contrast to the above examples, in which the sensing unit intrinsically relocates to the site of the target signal, a slightly different implementation of this approach involves triggering the relocation of a sensor to a predetermined subcellular site. For example, Spencer and colleagues were able to develop a biosensor that exits the nucleus in response to CDK2 activity¹¹¹ by fusing Venus (YFP) to the C-terminal domain of human DNA helicase B (HDHB), which mediates the cell cycle-dependent nuclear localization of HDHB in response to CDK2-dependent phosphorylation.¹¹² Gross and Rotwein similarly generated a translocation-based Akt kinase sensor by fusing full-length FoxO1 to the green FP Clover,¹¹³ whereas Maryu et al. utilized the central region of FoxO3a, which contains the Akt phosphorylation site and phospho-regulated nuclear localization (NLS) and export (NES) sequences, to generate their own Akt kinase sensor.¹¹⁴ Regot and colleagues have also demonstrated the generalizability of this approach by engineering a family of optimized kinase translocation reporters (KTRs) based on a minimal translocation domain that contains a kinase-specific substrate peptide fused in tandem to a bipartite NLS¹¹⁵ and an NES, such that phosphorylation inhibits the NLS and activates the NES¹¹⁶ (Figure 1B). This design was successfully applied to develop KTRs for monitoring JNK, p38, extracellular signal-regulated kinase (ERK), and PKA activity.¹¹⁶ A similar technology known as synthetic kinase activity relocation sensors (SKARS) was also reported by Durandau et al. based on the phosphorylation-dependent charge disruption of a tandem NLS sandwiched between a MAPK substrate domain and an FP.¹¹⁷

2.3.2. Directly Sensitizing the Chromophore of a Fluorescent Protein. GFP represents an ideal fluorescent tag because it requires no exogenous cofactors and instead forms its chromophore intrinsically through the autocatalytic cyclization of three consecutive amino acids (e.g., S65, Y66, and G67 in native *Aequorea victoria* GFP) encoded in its primary sequence (see ref 11 for a more detailed overview of GFP chromophore formation). The native chromophore further adopts either of two chemical states, a major “neutral” species in which the phenolic –OH of Y66 is protonated and a minor “anionic” species in which the Y66 phenolic –OH is deprotonated, which are responsible for the dual-excitation, single-emission behavior of wild-type GFP (wtGFP) via conversion of the neutral to the anionic chromophore through

excited-state proton transfer (ESPT).^{118,119} ESPT is driven by an extensive H-bond network that functions as a proton relay to abstract a high-energy proton from the excited chromophore while alternately serving to stabilize the neutral (i.e., protonated) chromophore in the ground state. As a result, wtGFP fluorescence is largely insulated from external perturbations.^{120,121} Yet many spectrum-shifting GFP mutations (e.g., S65T, Y66H, T203I),^{5,119,122,123} which disrupt the wild-type H-bond network, were shown to impart marked pH sensitivity to GFP fluorescence, responding rapidly and dynamically to intracellular pH changes and suggesting that GFP may be capable of serving as a genetically encoded pH indicator in live cells.¹²⁴

This approach represents a conceptual departure from the biosensor designs mentioned in the preceding sections, wherein the FP largely serves as a passive bystander, by instead directly integrating the FP into the detection scheme and making probe fluorescence actively responsive to the detection of the target cellular parameter (e.g., pH), analogous to the behavior of small-molecule fluorescent indicators.³ Indeed, Llopis and colleagues were able to utilize different GFP spectral variants (e.g., EGFP, ECFP, and EYFP) with differing pK_a values (6.15, 6.4, and 7.1, respectively) to visualize pH changes in HeLa cells, further taking advantage of the subcellular targetability of these genetically encoded probes to specifically monitor pH dynamics within the cytoplasm, Golgi lumen, and mitochondrial matrix.¹²⁵ Miesenböck and colleagues also set out to specifically engineer a GFP-based pH sensor to monitor synaptic vesicle exocytosis by targeting residues known to participate in the H-bond network with Y66 or to otherwise alter the spectral properties of GFP.¹²¹ The authors scanned these sites with histidines, which had the desired pK_a value to detect the pH transition upon vesicle fusion and also used random mutagenesis to ultimately obtain a “pH-sensitive fluorescent protein” (pHluorin), whose fluorescence intensity was eclipsed at low pH (i.e., “ecliptic” pHluorin). Ecliptic pHluorin is thus dark when localized to the vesicle lumen and rapidly increases in intensity upon exposure to the extracellular space, thereby enabling the visualization of individual fusion events as discrete flashes.¹²¹ Sankaranarayanan et al. subsequently generated a super-ecliptic pHluorin variant by incorporating the EGFP mutations F64L and S65T,¹²⁶ while Li and Tsien recently developed a red-fluorescent pH sensor, pHTomato, which they coimaged alongside GCaMP3 to simultaneously visualize synaptic vesicle and Ca^{2+} dynamics.¹²⁷

Of the spectral variants initially derived from GFP, YFP fluorescence intensity displays a particularly high degree of environmental sensitivity, with the numerous amino acid substitutions having introduced gaps around the chromophore and the further substitution of H148 with Gly even producing a solvent channel directly to the chromophore, which substantially increases the already high pK_a value (i.e., pH sensitivity) of YFP.¹²⁸ In fact, Tojima and colleagues utilized this H148G substitution to reintroduce pH sensitivity into Venus for use as a pH sensor.¹²⁹ YFP fluorescence is also strongly affected by halide ions, especially Cl^- and iodide, with the H148Q mutant being particularly sensitive.^{130,131} Halide ions are able to bind directly to YFP and increase the apparent pK_a of the chromophore, leading to decreased YFP intensity at a fixed pH, thus allowing Jayaraman and co-workers to use YFP(H148Q) as an intracellular Cl^- sensor.¹³⁰ Galletta and colleagues later set out to improve the halide sensitivity of

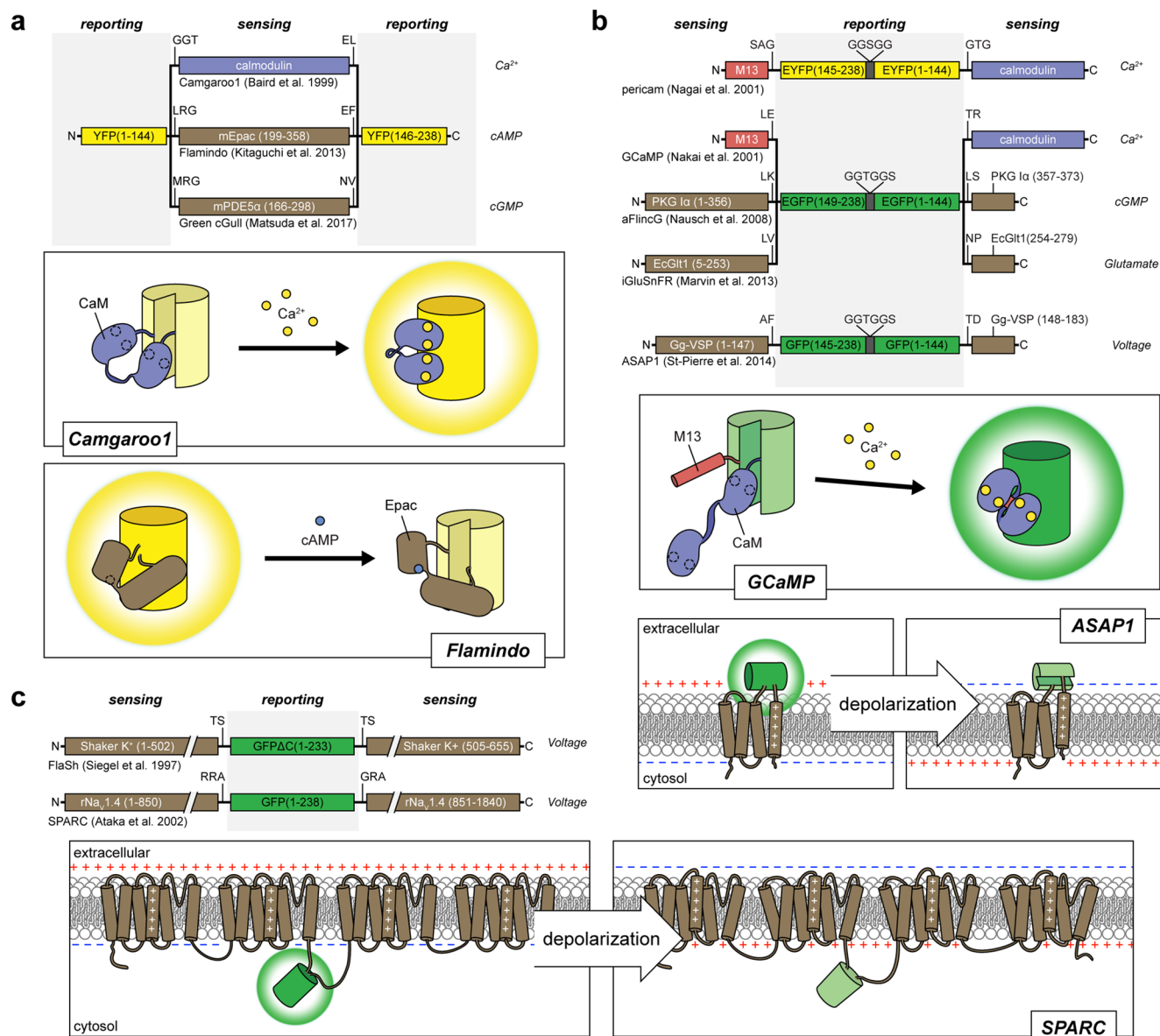


Figure 2. Single-FP fluorescent biosensor designs for cellular analytes and membrane potential. (A) Insertion of a sensing unit into an FP. Calmodulin, mEpac, and mPDE5 α undergo conformational changes in response to binding Ca^{2+} ,¹⁵² cAMP,¹⁷¹ and cGMP,¹⁷² respectively, which perturbs the chromophore and alters the fluorescence. Binding can either lead to an increase in fluorescence, as seen in the Ca^{2+} biosensor camgaroo1,¹⁵² or a decrease in fluorescence, as seen in the cAMP biosensor flamindo.¹⁷¹ (B) Sandwiching a cpFP between sensing units. Biosensors have utilized sensing units that comprise either separate receiver and switch domains^{156,158} or split proteins that reconstitute during protein folding.^{173–175} For example, GCaMP biosensors utilize separate domains of CaM and M13, where calcium binding to CaM promotes the binding of CaM to M13 and results in a conformational change that leads to an increase in GFP fluorescence. On the other hand, the membrane voltage sensor ASAP1 inserts cpGFP into the voltage-sensing domain of the chicken voltage-sensitive phosphatase Gg-VSP, which reconstitutes after folding, and depolarization leads to a conformational change in the 4th transmembrane segment that alters the fluorescence of cpGFP.¹⁷⁵ (C) Insertion of an FP into a voltage-sensitive channel. The conformational changes induced in voltage-sensitive K $^{+}$ and Na $^{+}$ channels alter the fluorescence of GFP to act as biosensors of membrane potential.^{176,177}

YFP(H148Q) via random mutagenesis, resulting in variants with enhanced affinity for either Cl^{-} (H148Q/V163S; $K_d \sim 40$ mM) or I^{-} (H148Q/I152L; $K_d \sim 2$ mM).¹³² However, the fact that YFP(H148Q) is sensitive to both pH and Cl^{-} can complicate its application as a Cl^{-} sensor. Thus, Zhong et al. also recently performed a series of mutagenic screens to generate monomeric Cl-YFP (EYFP-F46L/Q69K/H148Q/I152L/V163S/S175G/S205 V/A206 K), which binds Cl^{-} with a K_d of 14 mM and has a pK_a of only 5.9.¹³³

As part of their original screen for pH-sensitive GFP variants, Miesenböck et al. also identified a mutant whose dual

excitation peaks responded oppositely to pH, with the ratio of intensity at each excitation wavelength ultimately serving as the readout (e.g., “ratiometric” pHluorin).¹²¹ Given that fluorescence intensity in cells is often influenced by multiple factors that are unrelated to the parameter being detected (e.g., variable expression, cell shape/thickness, sample illumination, bleaching, etc.), such ratiometric sensors are often advantageous for live-cell imaging because collecting fluorescence at two wavelengths that show opposite changes in response to a cellular parameter largely cancels out these variations, while also providing more quantitative measurements.^{134,135} Thus,

Hanson and colleagues developed a family of emission-ratiometric pH sensors called “dual-emission GFPs” (deGFPs), which switch from green to blue emission with decreasing pH,¹³⁴ while Bizzarri and co-workers also found that GFP F64L/S65T/T203Y/L231H, or E²GFP,^{136,137} can serve as both an excitation- and an emission-ratiometric pH sensor.¹³⁸

To generate a ratiometric Cl[−] sensor, Kuner and Augustine utilized FRET between YFP and a tethered CFP, which is not sensitive to Cl[−] ions.¹³⁹ In the resulting sensor, Clomeleon, Cl[−] binding decreases YFP fluorescence, thereby reducing FRET and dequenching CFP emission.¹³⁹ Markova et al. used a similar approach to generate the ratiometric “Cl-sensor” but instead utilized a YFP mutant with increased Cl[−] sensitivity.¹⁴⁰ Interestingly, E²GFP, which contains the T203Y mutation found in YFPs, is also sensitive to halide ions but does not display an intrinsic ratiometric response.¹⁴¹ However, because pH and Cl[−] differentially affect the E²GFP spectrum, Arosio and colleagues were able to generate a ratiometric sensor capable of monitoring both parameters (ClopHensor) by linking E²GFP to the monomeric RFP DsRedm.¹⁴²

2.3.3. Engineered Modulation of the Photophysical Behavior of a Fluorescent Protein. The above examples represent a somewhat unique case in which the FP doubly performs as both the sensing and reporting unit. However, this design scheme is not very generalizable beyond the detection of a few select ions, as most cellular analytes and biochemical reactions do not directly affect the chromophore. Instead, a more universal strategy involves returning to the use of an extrinsic sensing unit that is engineered to couple the detection of a biochemical signal to the modulation of fluorescence behavior. As exemplified by the development of certain redox^{143,144} and metal ion sensors^{145–147} such as the ER Ca²⁺ sensor CatchER,^{148–150} the sensing unit can be as simple as a few amino acids inserted into the surface of an FP. In broader practice, however, a more sophisticated design is often required in which the sensing unit comprises a conformationally dynamic element capable of adopting either of two states (i.e., conformations) and readily switching between them in response to a specific input signal. Sensing units containing such “molecular switches” can often be generated from intrinsic conformational switches found in many native proteins or artificially engineered by linking isolated proteins or peptides in a two-component sensing unit that contains a “receiver” domain to sense the signal of interest and a “switch” domain to trigger the conformational change. Below, we describe how molecular switches can be utilized to modulate the photophysical behavior of one or more coupled FPs in order to directly visualize a vast array of biochemical and cellular parameters in living cells.

2.3.3.1. Designs Based on the Modulation of a Single Fluorescent Protein. The stereotypical β -can architecture of an FP not only protects the chromophore from the external environment but also is directly responsible for providing the internal microenvironment that supports fluorescence. Any alteration that distorts this β -can conformation can thus affect chromophore behavior, allowing conformational changes in a coupled molecular switch to directly influence FP fluorescence. One way to achieve conformational coupling is to directly insert an FP into a target protein. Because the native N- and C-termini of an FP are located in close proximity in the folded 3D structure, FP insertion typically leads to minimal perturbation of the native conformation of the target protein, while at the same time rendering the FP sensitive to the

conformation of the fused protein such that conformational changes are transmitted into the FP β -can to alter chromophore behavior. Given that the native N- and C-termini of FPs are unstructured, conformational coupling can also be enhanced through the use of circular permutation, which has long been used to study the structure and function of biological macromolecules¹⁵¹ and refers to the rearrangement of the linear sequence of a macromolecule in order to shift the positions of the native termini along the molecule surface without altering the overall 3D structure. Several FPs have been shown to tolerate circular permutation while retaining fluorescence behavior,¹⁵² with the resulting cpFPs possessing new termini located within the β -can itself; the added rigidity thus improves the transmission of conformational changes into the β -can. As such, inserting an FP into a conformational switch, or vice versa, provides a mechanism to sensitize the photophysical properties of a single FP to a given biochemical parameter (Figure 2).

2.3.3.1.1. Genetically Encoded Calcium Indicators. Advances in live-cell imaging are intimately linked with the study of Ca²⁺ signaling, which can be attributed to the long history of efforts to visualize Ca²⁺ dynamics as a proxy for neuronal activity. Indeed, the use of fluorescent indicator dyes was largely popularized by the success of Ca²⁺ indicators as live-cell probes (also discussed in section 4.2.1 below), while the discovery of GFP was itself an offshoot of work that yielded another live-cell Ca²⁺ probe, the photoprotein aequorin.¹⁵³ Thus, genetically encoded Ca²⁺ indicators (GECIs) were naturally among the earliest biosensors to be engineered and remain a major focus of biosensor development efforts to this day.

Barring the notable exception of CatchER,¹⁴⁸ which is based on an engineered Ca²⁺ binding site, the development of single-FP GECIs has universally centered around calmodulin, a ubiquitous intracellular Ca²⁺ sensor and major effector of Ca²⁺ signaling in all eukaryotes (reviewed in refs 154 and 155), which undergoes a switchlike conformational change upon Ca²⁺ binding and recognition of its target proteins. In their work developing the first cpFPs, for example, Baird and colleagues found that inserting *Xenopus laevis* calmodulin into EYFP yielded a chimeric protein that displayed an approximately 7-fold increase in fluorescence intensity in the presence of Ca²⁺ in vitro and functioned as a Ca²⁺ indicator, nicknamed “camgarool”, in living cells¹⁵² (Figure 2A). Nagai and co-workers modified this design by utilizing a bipartite molecular switch.¹⁵⁶ In this scheme, calmodulin was fused to the C-terminus of cpEYFP, while the M13 peptide, which is derived from the calmodulin-binding region of myosin light-chain kinase,¹⁵⁷ was fused to the N-terminus. Calmodulin forms a compact complex with M13 upon Ca²⁺ binding, and the M13 peptide thus acts as a switching domain to promote a Ca²⁺-dependent conformational change in the sensor (Figure 2B). These efforts ultimately yielded a family of GECIs known as “pericams”¹⁵⁶ (Table 1).

In a parallel effort, Nakai and co-workers set out to construct a GECI by first testing multiple fusion sites for linking the M13 peptide and calmodulin to the N- and C-termini of cpEGFP in a design scheme similar to that of pericams.¹⁵⁸ Notably, the two early candidates with the best responses both introduced a 5-residue gap in the β -can structure where the molecular switch is inserted. Subsequent optimization focused on the linker sequences at interdomain junctions, and the 85th variant tested, which showed the highest basal fluorescence and Ca²⁺-

dependent response, was named “GCaMP”.¹⁵⁸ Tallini et al. were able to improve GCaMP behavior by introducing several mutations previously shown to improve GFP stability (V163A, S175G)¹⁵⁹ or promote monomer formation (A206 K),¹⁶⁰ while also identifying new substitutions (D180Y, V93I) via random mutagenesis.¹⁶¹ Adding an N-terminal RSET leader sequence also helped improve thermal stability, resulting in GCaMP2. A mammalian-cell screen for point mutants with increased brightness, dynamic range, and Ca^{2+} sensitivity then identified two mutations in GFP (M153 K, T203 V) and a third in calmodulin (N60D), resulting in a third-generation sensor (GCaMP3) with 3-fold higher basal fluorescence and a 2-fold higher dynamic range. GCaMP3 could thus be used to successfully image Ca^{2+} dynamics in brain slices and in vivo.¹⁶²

Given the importance of GECIs for imaging neuronal activity, one area of focus for continued engineering efforts has been to enhance the speed and sensitivity of GCaMP responses in neurons to more accurately report the dynamics of action potentials. For instance, Akerboom and colleagues generated a panel of 12 GCaMP5 variants, 3 of which (GCaMP5A, 5G, and 5K) exhibited 2- to 3-fold better SNR and more faithfully recapitulated electrophysiological recordings of in vivo neuronal activity compared with GCaMP3.¹⁶³ Chen et al. extended this work even further by screening for optimized GCaMP5G variants directly in cultured neurons to obtain GCaMP6s, 6m, and 6f with slow, medium, and fast kinetics, respectively.¹⁶⁴ Of equal importance has been the development of a wider array of GECI color variants, especially red-shifted sensors, which are particularly desirable for in vivo imaging given the reduced phototoxicity, decreased absorption and scattering, and thus deeper tissue penetration, associated with longer-wavelength illumination. Zhao and colleagues, for example, developed a red-shifted GECI by replacing the cpEGFP domain of GCaMP3 with a circularly permuted version of the RFP mApple,¹⁶⁵ which after several rounds of directed evolution yielded “R-GECO1”.¹⁶⁶

However, mApple is known to exhibit substantial photo-switching behavior,¹⁶⁵ and subsequent studies found that this behavior is preserved in R-GECO1 and its descendants,^{167,168} which significantly increase in intensity upon illumination with blue light, thus potentially limiting their compatibility with optogenetic tools. As part of an independent effort, Akerboom and co-workers¹⁶⁸ elected to replace the cpEGFP domain of GCaMP3 with mRuby.¹⁶⁹ A dimly fluorescent “RCaMP” prototype was subjected to multiple rounds of both random and structure-guided mutagenesis to produce a series of improved variants culminating in RCaMP1h. Importantly, despite possessing a lower dynamic range, RCaMP1 did not exhibit any of the complex photophysical effects that characterize mApple-based GECIs. RCaMP1 was successfully employed for dual-color Ca^{2+} imaging along with GCaMP5G in mixed neuronal/astrocyte cultures, for in vivo imaging, and for combined optogenetics/imaging studies.¹⁶⁸ Recently, Dana and colleagues were able to further optimize these red GECIs by screening for variants directly in cultured neurons, yielding three improved probes, mRuby-based jRCaMP1a and jRCaMP1b and mApple-based jRGECO1a.¹⁷⁰ Yet despite their promise as powerful tools for interrogating neuronal function in vivo, red GECIs nevertheless continue to lag behind GCaMPs in overall dynamic range, and evidence also suggests that these probes produce multiple species in cells due to incomplete chromophore maturation.¹⁷⁰ Thus, additional

optimization remains a priority for these sensors, likely entailing re-engineering of the RFP itself.

2.3.3.1.2. Genetically Encoded Voltage Indicators. Plasma membrane voltage is a key indicator of electrical activity in neurons. Thus, in addition to GECIs for Ca^{2+} imaging, the potential to use genetically encodable probes to directly visualize changes in membrane voltage in specific neuronal subsets has also spurred the development of genetically encoded voltage indicators (GEVIs). GEVIs couple changes in membrane potential to changes in fluorescence by taking advantage of the fact that multiple cellular proteins contain a voltage-sensing domain (VSD) that enables them to sense and respond to changes in membrane potential. VSDs consist of a group of four helical segments (S1–S4) that sit in the plasma membrane, with the positively charged S4 undergoing a structural rearrangement in response to voltage changes (otherwise known as “gating”). These conformational changes can be transmitted into the barrel of a coupled FP, thereby allowing a VSD-containing protein to serve as a voltage-dependent molecular switch to control biosensor fluorescence.

Initial efforts relied on voltage-gated ion channels as the source of the VSD. For example, Siegel and Isacoff constructed the very first GEVI by inserting a C-terminally truncated form of GFP into the plasma membrane-proximal region of the cytosolic tail of the Shaker potassium channel, just after the sixth transmembrane segment of the channel¹⁷⁶ (Figure 2C). Similar to the reasoning underlying the use of cpFPs in single-FP-based sensors, the unstructured C-terminus of GFP was deleted based on the hypothesis that direct fusion of Shaker to the less flexible β -can would improve conformational coupling and voltage-dependent fluorescence changes. A W434F mutant form of Shaker, in which the ion-conducting pore is disrupted without affecting channel gating, was also used to prevent overexpression of the sensor from altering cell physiology. However, although the resulting “fluorescent Shaker” (FlaSh) protein displayed a clear decrease in GFP fluorescence upon membrane depolarization in *Xenopus* oocytes, the kinetics of the fluorescence response were ~ 30 -fold slower than the actual gating kinetics of the channel and thus ill-suited to imaging the rapid dynamics of neuronal action potentials.¹⁷⁶ Ataka and Pieribone similarly constructed a GEVI by using the μI voltage-gated sodium channel as the VSD.¹⁷⁷ In contrast to potassium channels, sodium channels function as monomers, which offers a wider selection of FP insertion sites and also eliminates the risk that the sensor will reconstitute with native channel subunits. After screening 10 different insertion sites, the authors found that inserting full-length EGFP between the second and third transmembrane regions yielded a sodium channel protein-based activity reporting construct (SPARC) (Figure 2C). Importantly, although SPARC exhibited a much smaller fluorescence change compared with FlaSh, the response kinetics were fast enough to report voltage pulses on the order of 2 ms.¹⁷⁷

Although successful as a proof of concept, GEVIs based on ion channels can be difficult to express in cells and exhibit poor membrane localization. Thus, subsequent efforts have simplified GEVI architecture by turning toward more compact VSDs, beginning with the discovery of the *Ciona intestinalis* voltage-sensitive phosphatase (CivSP).¹⁷⁸ Genomic surveys found that this sea squirt (family *Ascidiascea*) expresses a lipid phosphatase fused to a single VSD, which is homologous to those found in voltage-gated potassium channels yet functions as a monomer. As with the VSDs from voltage-gated channels,

the fourth helical segment in the VSD of CiVSP (i.e., Ci-VSD) undergoes a voltage-dependent structural rearrangement within the plasma membrane, which can be directly coupled to an FP, though this requires incorporating point mutations in the VSD to shift voltage sensitivity into the physiological range of membrane potential in neurons.¹⁷⁹ Notably, electrophysiological studies performed by Lundby and co-workers found that the Ci-VSD produces a very rapid gating current (i.e., current generated by the movement of positively charged segment 4 within the membrane) in response to membrane depolarization.¹⁸⁰ Thus, by fusing the CFP variant Cerulean¹⁸¹ directly to the C-terminus of Ci-VSD using a short linker, they were able to generate an improved voltage-sensitive fluorescent protein (VSFP) with a robust voltage-dependent fluorescence change and ~ 1 ms activation kinetics. Taking their inspiration from the development of GECIs, Barnett and colleagues similarly appended cpEGFP to the C-terminus of Ci-VSD.¹⁸² Screening through 90 constructs that varied either the fusion site between Ci-VSD and cpEGFP or the precise residues surrounding the circular permutation site yielded the sensor “ElectricPk”, which also exhibited a rapid fluorescence response capable of resolving 1 ms voltage pulses.¹⁸²

More recently, St-Pierre et al. based the design of their single-FP GEVI, “accelerated sensor of action potentials” (ASAP1), on structural studies of the gating mechanism of VSDs.¹⁷⁵ Specifically, given reports demonstrating a large conformational change within the loop connecting helical segments S3 and S4,¹⁸³ they inserted a circularly permuted superfolder GFP (cpsfGFP)³⁷ into this loop region in the chicken (*Gallus gallus*) VSD (Gg-VSD) (Figure 2B). The loop connecting S3 and S4 is shorter in Gg-VSD than in Ci-VSD, which the authors reasoned would increase conformational coupling to cpsfGFP. ASAP1 exhibited fast (~ 2 ms) kinetics, as well as a much higher dynamic range compared with previous GEVIs.¹⁷⁵ To develop an optimized GEVI that was suitable for use with 2-photon imaging of in vivo neuronal activity in the *Drosophila* visual system, Yang and colleagues generated variants of ASAP1 by mutating the junction sequence connecting S3 from Gg-VSD to cpsfGFP.¹⁸⁴ These efforts yielded the improved variant ASAP2f, which produced a larger voltage-dependent fluorescence change in neurons compared with ASAP1 while exhibiting the same fast response kinetics. Abdelfattah et al. also recently reported the development of a red-shifted GEVI with comparable performance characteristics to optimized GFP-based GEVIs.¹⁸⁵ Similar to the development of R-GECO1, the authors fused cpmApple to the S4 helix at the C-terminus of Ci-VSD and then performed directed evolution. After screening thousands of variants, they obtained “Fluorescent indicator for voltage imaging Red”, or FlicR1, which exhibited submillisecond activation and deactivation kinetics and a large dynamic range.¹⁸⁵ Overall, the development of GEVIs is still an ongoing effort, with even greater improvements in sensitivity and dynamic range hopefully to come in the near future.

2.3.3.1.3. Single Fluorescent Protein-Based Indicators for Other Cellular Analytes. The “killer app” of the molecular-switch-as-sensing-unit design is versatility: given the availability of an endogenous effector protein that undergoes a suitable conformational change, this approach can be used to engineer biosensors for monitoring virtually any cellular analyte, or in some cases, ratios of analytes (e.g., ATP/ADP and NADH/NAD⁺, discussed in ref 186), in living cells.

For instance, exchange protein regulated by cAMP (Epac), a Rap1/2 guanine nucleotide exchange factor (GEF) regulated by the ubiquitous intracellular messenger cAMP, has been shown to undergo a major conformational change in response to cAMP binding.^{187,188} Kitaguchi and colleagues were thus able to develop a yellow-fluorescent cAMP sensor by inserting the murine homologue of Epac1 (mEpac1) into Citrine.¹⁷¹ A screen of 12 variants featuring different mEpac1 fragments and linkers yielded a successful “fluorescent cAMP indicator” (Flamindo) containing just the mEpac1 cyclic nucleotide binding domain (CNBD) and exhibiting an $\sim 50\%$ cAMP-dependent intensity decrease (Figure 2A). Odaka et al. subsequently improved the response by mutating the N-terminal Citrine-mEpac1 junction, with the resulting Flamin-do2 sensor exhibiting an $\sim 75\%$ intensity decrease in response to cAMP.¹⁸⁹ Tewson and co-workers also constructed “cAMP difference detector *in situ*” (cADDiS), which shows a $\sim 35\%$ cAMP-dependent fluorescence decrease, by inserting a circularly permuted version of the bright green FP mNeon-Green¹⁹⁰ into the hinge region of Epac2.¹⁹¹ Finally, Harada et al. recently generated the red-shifted “Pink Flamindo” cAMP sensor by inserting the mEpac1 switch from Flamindo into mApple and screening multiple linker variants.¹⁹² Pink Flamindo, which exhibits a 4-fold cAMP-dependent increase in fluorescence, was successfully coimaged with a green-fluorescent GECI (G-GECO¹⁶⁶), yet it remains to be seen whether future applications of this sensor face the same photoswitching behavior as other mApple-based probes.

Likewise, 3',5'-cyclic guanosine monophosphate (cGMP) is another key intracellular messenger that regulates numerous cellular processes, especially in relation to vascular and neuronal biology.¹⁹³ As with cAMP, cGMP exerts its biological effects through the regulation of a number of protein targets, such as the cGMP-dependent protein kinase (PKG), which has been shown to undergo isoform-specific conformational changes in response to cGMP binding.^{194,195} In fact, Nausch and co-workers previously found that inserting cpEGFP into the C-terminal tail region of the tandem CNBDs isolated from PKG I was sufficient to yield a cGMP biosensor.¹⁷³ By incorporating the CNBD regions from the α , β , or δ isoforms of PKG I, they were thus able to generate a family of “fluorescent indicators of cGMP”, or FlincGs, that each displayed a range of cGMP-binding affinities while all showing fairly robust cGMP-dependent fluorescence increases in cells. More recently, Matsuda et al. developed a cGMP sensor using another cGMP-binding domain known as a GAF domain,¹⁷² which is present as an allosteric regulatory domain in many enzymes, including certain phosphodiesterase (PDE) isoforms, and is also known to undergo a conformational change upon cGMP binding.^{196,197} Similar to the design of Flamindo, the authors inserted the GAF-A domain from murine PDE5 α (mPDE5 α) into citrine and screened several mPDE5 α fragments and linker sequences to obtain the single-FP biosensor “Green cGull”, which exhibited an ~ 8 -fold cGMP-dependent fluorescence intensity increase, as well as 3 orders of magnitude higher affinity for cGMP (~ 1 μ M) compared with cAMP (~ 1 mM).¹⁷²

A number of studies have also explored the potential for bacterial periplasmic binding proteins (PBPs) to serve as scaffolds for biosensor engineering. PBPs are responsible for mediating the uptake of various nutrients and other solutes in Gram-positive bacteria such as *Escherichia coli* and are therefore capable of binding a wide range of small

molecules.^{198,199} Members of this large protein superfamily generally share a similar protein architecture comprising a pair of globular domains that are linked by a hinge region, with ligand binding inducing substantial conformational rearrangement of the globular domains.^{200,201} In one early proof-of-concept study, Marvin and colleagues utilized structural data on the *E. coli* maltose-binding protein (MBP) to identify candidate sites for the optimal insertion of cpGFP.²⁰² Four different insertion sites were tested, and after screening libraries of linker variants, a candidate construct named MBP165-cpGFP.PPYF, or “PPYF”, was obtained that displayed a ~2.5-fold increase in green fluorescence upon maltose binding. Similarly, Alicea et al. were able to generate a genetically encoded phosphonate sensor by inserting cpGFP into the *E. coli* EcPhnD protein.²⁰³ Most recently, Marvin and co-workers developed “iGluSnFR”, a single-FP sensor for monitoring the neurotransmitter glutamate that exhibits an up to 4-fold glutamate-dependent fluorescence intensity increase in cells.¹⁷⁴ iGluSnFR was constructed by inserting cpGFP into the *E. coli* PBP Glt1 (Figure 2B); in keeping with the engineering of the above maltose and phosphonate sensors, a position close to the hinge region of Glt1 was chosen as the insertion point, followed by screening to optimize the interdomain junction sequences. PBPs thus show considerable promise as biosensor scaffolds and, given the relative ease with which their ligand-binding sites can be computationally redesigned,¹⁹⁹ will potentially yield a myriad of new single-FP biosensors.

GPCRs are another promising scaffold for biosensor engineering, as illustrated by the recent development of novel single-FP biosensors for monitoring endogenous neurotransmitters such as dopamine and acetylcholine. For instance, Patriarchi and colleagues inserted the cpEGFP module from GCaMP6 into the third intracellular loop (ICL₃) of the dopamine D1 receptor (D₁R) and screened 585 linker variants to obtain the dLight1.1 sensor.²⁰⁴ Further engineering, including the use of the D₂R and D₄R, yielded a family of 5 dopamine sensors (dLight1.1–1.5) with low nanomolar to micromolar affinity. Using a similar principle, Sun et al. systematically tested various insertion site and linker sequences to introduce cpEGFP into the D₂R and then performed mutational screening to obtain a pair of dopamine biosensors, GRAB_{DAIm} and GRAB_{DAIh}, with moderate (~130 nM) and high (~10 nM) binding affinity, respectively.²⁰⁵ Jing and co-workers also succeeded in engineering a single-FP acetylcholine sensor, GACH1.0, by inserting cpEGFP into ICL₃ of the M₃ muscarinic acetylcholine receptor, after which they obtained an improved GACH2.0 sensor via linker optimization, including 723 single and 23 combinatorial mutants.²⁰⁶ Given the general applicability of these designs and the abundance of GPCRs, we can expect the development of single-FP biosensors for a variety of naturally occurring cellular analytes.

2.3.3.1.4. Engineering Single-Fluorescent-Protein Biosensors with a Ratiometric Readout. The single-wavelength, intensimetric biosensors discussed above are popular for in vivo imaging due to their large dynamic ranges and high SNR, as well as for biosensor multiplexing and all-optical (e.g., optogenetic) studies enabled by their small spectral footprint. Nevertheless, as alluded to previously, dual-wavelength ratiometric sensors are often preferable for canceling out artifacts related to probe expression and for obtaining quantitative measurements.

In some cases, ratiometric behavior can be obtained as an intrinsic property, in which the molecular switch induces a shift in the excitation or emission spectrum of a single FP. For instance, Nagai et al. were able to generate a ratiometric Ca²⁺ sensor by introducing an H203F mutation, which increases fluorescence by the protonated YFP chromophore,²⁰⁷ into the intensimetric “flash-pericam”.¹⁵⁶ Screening identified two additional mutations (F46L, H148D) that, along with linker optimization, improved the response to yield a “ratiometric-pericam” that undergoes a Ca²⁺-dependent shift from ~415 nm to ~495 nm excitation. More recently, a trio of ratiometric GECO-family Ca²⁺ indicators were also identified through screening that undergo a Ca²⁺-induced shift from blue (~450 nm) to green (~510 nm) emission upon ~400 nm excitation (“GEM-GECO”), from ~400 nm to ~488 nm excitation with ~510 nm (green) emission (“GEX-GECO”), and from ~575 nm to ~480 nm excitation with ~600 nm (red) emission (“REX-GECO”).^{166,208} Zhao and colleagues also obtained a ratiometric sensor for monitoring the intracellular NADH/NAD⁺ concentration ratio after inserting cpYFP into the interdomain loop of the *Thermus aquaticus* Rex (T-Rex) protein.²⁰⁹ This sensor, named “SoNar”, undergoes an increase in the 420/480 nm excitation ratio upon NADH binding, whereas NAD⁺ induces the opposite response. By computationally redesigning the T-Rex binding pocket in SoNar, Tao et al. were then able to generate a ratiometric sensor that directly reports NADPH concentrations, yielding a family of “iNap” sensors with a range of affinities.²¹⁰ Finally, we recently developed a novel suite of excitation ratiometric kinase activity reporters (ExRai-KARs) in which cpGFP undergoes a phosphorylation-dependent shift from ~400 nm to ~488 nm excitation with ~515 nm (green) emission.²¹¹

Alternatively, a more straightforward approach for generating ratiometric sensors is simply to add a second, spectrally distinct FP as an internal reference. Hung and colleagues, for example, also generated a ratiometric NADH/NAD⁺ redox sensor, “Peredox”, by inserting the long-Stokes-shift FP cpT-sapphire²¹² between a tandem dimer of T-Rex proteins and fusing mCherry²¹³ to the C-terminus.²¹⁴ cpT-Sapphire exhibits an increase in fluorescence intensity upon NADH binding to T-Rex, whereas mCherry fluorescence remains unaltered, thereby providing a ratiometric readout. Recently, Cho et al. used a similar approach to design ratiometric versions of GCaMP by fusing mCherry to the C-terminus of GCaMPs 3, 6s, and 6f.²¹⁵ However, they included a rigid ER/K α -helical linker^{216,217} between calmodulin and mCherry, reasoning that this 30 nm spacer would eliminate potential FRET with cpGFP. The resulting GCaMP-R3, GCaMP-R-6s, and GCaMP-R-6f sensors all displayed large emission ratio changes and enabled quantitative Ca²⁺ imaging in cells and tissues. Meanwhile, Ast and co-workers added a twist to this approach by creating a nested FP pair, in which a long-Stokes-shift OFP (LSSmOrange²¹⁸) is inserted into the cpGFP linker, to develop “GO-Matryoshka” (so named after the famous Russian dolls).²¹⁹ Importantly, the use of LSSmOrange allows both FPs to be excited at a single wavelength (~440 nm), while the lack of spectral overlap between cpGFP emission and LSSmOrange absorption prevents FRET. This approach successfully yielded the ratiometric Ca²⁺ sensor MatryoshCaMP6s (derived from GCaMP6s), as well as the ammonium sensor AmTryoshka1;3, which was constructed by inserting GO-Matryoshka into the *Arabidopsis thaliana* ammonium transporter AtAMT1;3.²¹⁹

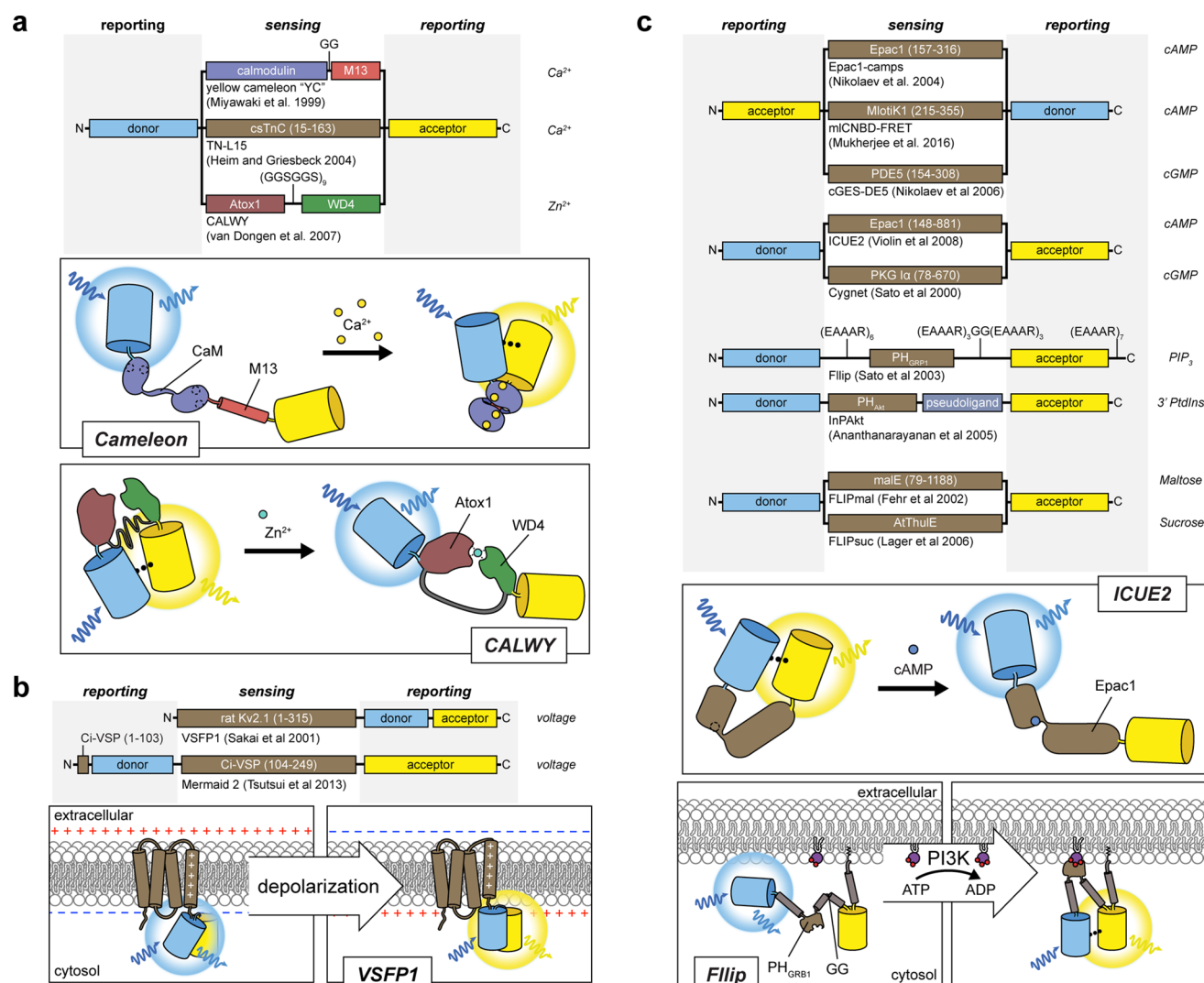


Figure 3. Designs of FRET-based reporters for ions, cellular analytes, and membrane potential. (A) FRET-based metal ion biosensors utilize receiver and switch domains that bind to each other^{376,421} or endogenous proteins that undergo a conformational change⁴¹⁰ in response to binding of metal ions. These conformational changes alter the intramolecular distance and orientation of a pair of FPs and thus changes the amount of energy transferred from the donor FP to the acceptor FP. (B) Voltage sensors that utilize a FRET-based readout often rely primarily on changing the orientation of FP dipoles.^{258,271} VSFP1 contains a CFP inserted between the 3rd and 4th transmembrane domain and a YFP fused to the C terminal tail of a truncated portion of the rat Kv2.1 channel where cell depolarization induces a conformational shift in the 4th transmembrane domain, thus changing the relative angle of the two dipoles of the FPs.²⁷¹ (C) Several biosensors for cellular analytes have utilized the design of sandwiching a conformationally switching domain between a FP FRET pair.^{299,311,314,325,327,349,362,540,556} For example, the cAMP biosensor ICUE2 utilizes the conformational change induced by cAMP binding to the CNB domain of Epac1 to increase the intramolecular distance between the donor and acceptor FPs.³¹¹ Alternatively, having both a PIP₃ binding PH domain from GRP1 and a C-terminal membrane localization sequence in combination with engineered rigid α -helical linkers yielded a chimeric protein that exhibits significant conformational changes in response to PIP₃ production, leading to changes in FRET between a FRET pair flanking the chimeric protein.⁵⁵⁶

2.3.3.2. Designs Based on the Modulation of a Pair of Fluorescent Proteins. Far from providing a mere reference signal, a second FP can be directly coupled to the sensing unit such that both FPs function as the reporting unit, with the molecular switch inserted between a pair of FPs to modulate the photophysical interaction between them. Compared with the engineered modulation of a single FP by a molecular switch, this dual-FP sandwich configuration represents a more straightforward and accessible design scheme that is less strictly reliant on extensive protein engineering and, to date, remains the most popular and generalizable approach to biosensor design. The most common implementation of this dual-FP design scheme involves sandwiching a molecular switch between two FPs that constitute a FRET pair. Recall

from section 2.2.2 that FRET efficiency not only falls off with the sixth power of the interfluorophore distance but also requires the donor and acceptor dipoles to be properly oriented relative to one another. In addition to monitoring interactions between proteins (i.e., intermolecular FRET), this proximity and orientation dependence therefore make FRET an excellent reporter of conformational changes within a protein (i.e., intramolecular FRET), allowing the straightforward coupling of a biochemical signal to a change in FRET and forming the basis for a seemingly endless array of genetically encoded FRET-based biosensors.

2.3.3.2.1. FRET Sensors for Monitoring Cellular Analytes. Much like engineered single-FP biosensors, FRET-based sensors are often built by incorporating a sensing unit that

undergoes an engineered or intrinsic conformational change in response to the binding of an intracellular messenger or other small molecule. As detailed below, this approach has yielded numerous FRET-based sensors engineered to monitor the dynamics of metal ions, cyclic nucleotides, phospholipids, and various other analytes within living cells.

2.3.3.2.1.1. FRET-Based Indicators of Metal Ion Concentrations. The very first GECIs were also among the first ever genetically encoded FRET-based biosensors. Specifically, Miyawaki and colleagues generated the sensor “cameleon” by sandwiching a Ca^{2+} -sensitive molecular switch, composed of calmodulin linked to the M13 peptide via a diglycine linker, between the FRET donor BFP and the FRET acceptor GFP.³⁷⁷ In a design that would later inspire additional classes of FRET-based biosensors (see below), the reversible binding of Ca^{2+} causes the sensing unit to switch between an extended conformation and a more compact form in which calmodulin essentially engulfs the M13 peptide, thereby altering the relative proximity, and thus the efficiency of FRET, between BFP and GFP. As discussed above, changes in FRET cause inverse changes in the intensity of the acceptor and donor fluorophores, thereby altering the ratio of fluorescence emission and providing a dynamic, quantitative readout of intracellular Ca^{2+} elevations. However, although cameleon performed well as a Ca^{2+} indicator in vitro, the low brightness of the BFP donor fluorophore rendered cameleon less effective when expressed in mammalian cells. Substitution of CFP and YFP in place of the BFP/GFP FRET pair was therefore performed to yield “yellow-cameleon”, or “YC” (Figure 3A), which exhibited higher brightness in mammalian cells and was also less affected by cellular autofluorescence.³⁷⁷

One of the primary motivations for developing cameleons, as well as all subsequent GECIs, was to enable the direct visualization of highly localized Ca^{2+} signaling events via subcellular targeting (discussed further in section 4.2.1). However, early efforts to investigate local Ca^{2+} elevations in neurons revealed that YC suffers a considerable reduction in Ca^{2+} sensitivity when targeted to the plasma membrane (discussed in refs 352 and 382). This effect was attributed to intramolecular interactions between the sensor and endogenous calmodulin, which is present at high concentrations in the vicinity of the plasma membrane due to its role as a binding partner for Ca^{2+} channels.^{665,666} Indeed, a key consideration in biosensor design is the potential for the sensing unit, which is derived from cellular proteins, to interact with endogenous components within cells, which can both perturb biological functions and also disrupt biosensor responses. Thus, to develop a sensor that was less likely to experience these effects, Heim and Griesbeck replaced the calmodulin/M13 molecular switch with one derived from troponin C (TnC), a muscle-specific Ca^{2+} sensor that plays a far less central role in intracellular Ca^{2+} signaling, yielding the sensors TN-L15, based on chicken skeletal muscle TnC, and TN-humTnC, based on human cardiac TnC.⁴¹⁰ Alternatively, Palmer and colleagues opted to retain the original YC design scheme and instead computationally re-engineered the complementary “bumps” and “holes” that make up the calmodulin-M13 binding interface, yielding a panel of designed, or “D”-series, YCs that are largely orthogonal to their endogenous intracellular counterparts, albeit with a notable reduction in affinity.³⁷¹ Importantly, both approaches produced FRET-based GECIs capable of reporting local Ca^{2+} dynamics at the plasma membrane.^{371,410}

GECIs are highly desirable tools for monitoring neuronal activity, and FRET-based GECIs are no exception. Thus, considerable efforts have been devoted to enhancing the speed and sensitivity of FRET-based GECIs to reliably measure physiological Ca^{2+} dynamics in living neurons. As alluded to in section 2.3.3.1.1, the Ca^{2+} -binding affinity of GECIs can be tuned within a fairly broad range by mutating the two Ca^{2+} -binding “EF-hand” motifs located in both of the globular domains of calmodulin,¹⁵⁵ and this approach has successfully yielded YC variants with Ca^{2+} -binding affinities spanning several orders of magnitude.³⁷⁷ In addition, the original calmodulin-M13 switch design used in these probes is based on in vitro studies showing that a hybrid protein in which calmodulin and M13 are fused by a diglycine linker undergoes a Ca^{2+} -dependent switch from an extended “dumbbell” shape to a more compact, globular form.⁶⁶⁷ Because the binding of Ca^{2+} -bound calmodulin ($\text{Ca}^{2+}/\text{CaM}$) to target peptides is known to slow the dissociation of Ca^{2+} from calmodulin, thereby increasing apparent Ca^{2+} -binding affinity,⁶⁶⁸ shifting the conformational equilibrium toward the compact form is expected to increase the Ca^{2+} -binding affinity of the hybrid complex, and indeed, the incorporation of a 5-residue linker was found to increase the K_d compared with the original diglycine.⁶⁶⁹ Following this logic, Horikawa and colleagues were therefore able to generate YC variants with enhanced Ca^{2+} -binding affinities by lengthening the calmodulin-M13 spacer from the original 2 to between 3 and 5 residues, yielding “YC-Nano” sensors with dissociation constants as low as 15 nM, which were sensitive enough to visualize spontaneous in vivo neuronal activity in zebrafish embryos.³⁷⁰

Meanwhile, parallel efforts have yielded improved TnC-based GECIs with very fast response kinetics for neuronal imaging. Like calmodulin, TnC is also a “dumbbell”-shaped molecule that contains a pair of EF-hand motifs in each of its globular “head” domains.⁶⁷⁰ Whereas the N-terminal regions specifically bind Ca^{2+} , the C-terminal domains are only partially selective and are capable of binding Mg^{2+} , which also results in more complex Ca^{2+} -binding kinetics.^{671,672} However, by introducing mutations based on the Ca^{2+} -binding sites of calmodulin into EF-hands III and IV within the C-terminal domain of TnC, Mank and colleagues were able to eliminate Mg^{2+} binding to the TN-L15 sensor and obtain an improved variant, TN-XL, that exhibited very fast Ca^{2+} binding kinetics, with an off-rate of ~ 140 ms.⁴⁰⁹ However, these mutations somewhat decreased the affinity of TN-XL for Ca^{2+} compared with TN-L15 (~ 2 μM vs ~ 750 nM), potentially reducing its effectiveness in measuring small neuronal Ca^{2+} transients. These authors were subsequently able to boost the sensitivity of this probe by replacing N-terminal EF-hands I and II with a duplicate copy of domains III and IV to generate TN-XXL ($K_d \sim 800$ nM).⁴⁰⁸ More recently, Thestrup et al. set out to minimize the structure of TnC-based Ca^{2+} sensors by reducing the number of Ca^{2+} -binding sites in the molecular switch, reasoning that fewer binding sites would yield a sensor with a simpler, more linear response behavior.⁴⁰⁵ Although initially unsuccessful in obtaining a high-affinity, single-EF-hand derivative of the chicken TnC used in previous TN sensors, the authors were ultimately able to construct a high-affinity Ca^{2+} sensor by sandwiching a single functional EF-hand from the oyster toadfish *Opsanus tau* between CFP and YFP. The resulting “Twitch” sensors retained the rapid kinetics of the previous TN-XL/XXL sensors while exhibiting dissociation constants as low as 150 nM.⁴⁰⁵

In addition to Ca^{2+} , other metal ions also play critical roles in cells, especially transition metal ions, which often function as essential cofactors in enzyme catalysis. Because these ions, like Ca^{2+} , are often toxic, cells have evolved numerous pathways to tightly regulate their intracellular accumulation,⁶⁷³ and components of these pathways have proven useful in the development of biosensors to monitor the dynamics of transition metal ion homeostasis in living cells. For example, van Dongen and colleagues set out to generate a copper biosensor based on the Cu(I)-induced dimerization between Atox1, a copper chaperone, and WD4, the fourth copper-binding domain of the “Wilson’s disease” protein (aka ATP7B), a copper-transporting ATPase.⁴²² However, although this strategy did indeed yield a Cu(I)-induced change in intermolecular FRET between CFP-Atox1 and W4-YFP, the response was disrupted by the presence of free thiol groups (e.g., DTT), which disrupted the Cu(I)-mediated bridging of Cys residues within these two metallothioneins. Instead, this effort serendipitously yielded the first FRET-based Zn^{2+} sensor, as these two domains also exhibited a Zn^{2+} -induced FRET change that was insensitive to free thiols.⁴²² van Dongen et al. subsequently refined this design by incorporating a flexible linker between the metal-binding domains, yielding the CFP-Atox1-Linker-WD4-YFP, or CALWY, sensor (Figure 3A).⁴²² Using a strategy analogous to that described above for Cameleons, Vinkenborg and colleagues then systematically modified the length of this linker to engineer a suite of CALWY sensors with a range of Zn^{2+} -binding affinities.⁴²⁰ Qiao and co-workers were also able to construct a Zn^{2+} biosensor using the Zn^{2+} -finger (ZF) 1 and 2 domains of the yeast Zap1 transcription factor.⁴²⁹ Zn^{2+} binding causes these domains to adopt a canonical ZF fold structure and form an interdomain complex, and sandwiching this molecular switch between CFP and YFP generated the ZF1/2 sensor. This design was further optimized by Qin and co-workers to yield ZapCY1 and 2,⁴²⁸ while Dittmer et al. similarly utilized a single canonical Cys₂His₂ ZF domain from the Zif268 transcription factor, which also adopts a folded structure upon Zn^{2+} binding, to generate a more compact FRET-based Zn^{2+} biosensor.⁴²⁶ Carter and colleagues also recently compared the performance of several Zn^{2+} sensors, providing a roadmap for obtaining quantitative measurements with minimal perturbation,⁶⁷⁴ which is crucial given the much smaller labile pools of intracellular Zn^{2+} and other metal ions versus Ca^{2+} .

2.3.3.2.1.2. FRET-Based Voltage Indicators. The earliest FRET-based GEVIs, like their single-FP counterparts, were developed by utilizing full-length voltage-gated ion channels to provide the molecular switch in the sensing unit. For example, Sakai and colleagues constructed the original, FRET-based VSFP using the Kv2.1 voltage-gated potassium channel.²⁷¹ To convert the channel into a biosensor, the authors joined CFP and YFP via a single-amino-acid linker and fused the resulting “CYFP” reporting unit after the S4 helix of a C-terminally truncated channel mutant. In this design, the voltage-induced rotation of the S4 helix is expected to alter the relative orientation of CFP with respect to YFP, thereby yielding a change in FRET (Figure 3B), and indeed, the resulting construct exhibited a nearly linear relationship between CFP-sensitized YFP emission (i.e., FRET) and membrane potential.²⁷¹ Dimitrov et al. subsequently developed an improved FRET-based VSFP biosensor family by replacing the Kv2.1 sensing unit with Ci-VSD, which they coupled to a similar tandem CFP-YFP reporting unit fused to the C-

terminus of the S4 helix.²⁶⁷ Progressively lengthening the spacer between S4 and CFP yielded four variants, VSFP2A-D, with longer linkers producing larger FRET changes. However, the voltage sensitivity of all four variants was well outside the range of physiological membrane potentials observed in mammalian cells, and an R217Q mutation was therefore incorporated into the VSD to produce a sensor (VSFP2.1) with a more physiological response range.²⁶⁷ This same design has been carried forward in subsequent FRET-based GEVIs, such as VSFP2.3,¹⁸⁰ VSFP2.4,²⁶⁵ and “Mermaid”.²⁶⁶ However, more recent probe designs have reconfigured the coupling between the sensing and reporting units by sandwiching the VSD between a FRET pair (Figure 3B), including VSFP-Butterfly,^{257,262} “Mermaid2”,²⁵⁸ and the recent Nabi sensors.²⁵⁶

2.3.3.2.1.3. FRET-Based Sensors for Monitoring Cyclic Nucleotide Dynamics. Most FRET-based cAMP biosensors utilize the intrinsic, cAMP-induced conformational change within either Epac1 or Epac2 as the basis of their sensing unit. For example, Nikolaev and colleagues generated Epac2-camps (“Epac2-based cAMP sensor”) by fusing YFP and CFP to the N- and C-termini of a minimal fragment spanning the second CNBD (CNBD-B) of Epac2.²⁹⁹ Epac1-camps was similarly constructed using the solitary cAMP-binding domain of Epac1, which corresponds to CNBD-B from Epac2. Parallel work by DiPilato and co-workers alternatively utilized full-length Epac1 sandwiched between CFP and YFP to generate the “indicator of cAMP using Epac”, or ICUE,³¹² though subsequent versions of ICUE have utilized an N-terminally truncated form of Epac1.^{309,311} (Figure 3C). However, despite the prominence of Epac-based designs, various FRET-based cAMP sensors have also been generated using cAMP-binding domains from other proteins. In fact, the very first genetically encoded FRET-based cAMP sensor, PKA-GFP, was based on the cAMP-induced dissociation of EGFP- and EBFP-tagged PKA catalytic (C) and regulatory (R) subunits,³²³ a design that was based on a previous fluorescent analog² of PKA, called FICRhR, featuring fluorescein and rhodamine conjugated to purified PKA C and R.⁶⁷⁵ Surdo and colleagues also recently generated a FRET-based cAMP sensor using the CNBD-B domain of the type II PKA regulatory subunit (RII) by fusing CFP to the C-terminus and inserting YFP into a flexible loop, yielding a “cAMP universal tag for imaging experiments” (CUTie).³⁰¹ Meanwhile, Mukherjee et al. were able to construct a FRET-based cAMP sensor with nanomolar sensitivity by sandwiching the CNBD from a bacterial cyclic-nucleotide-gated potassium channel⁶⁷⁶ between CFP and YFP.³¹⁴

Several different binding domains have also been used as sensing units to construct FRET-based cGMP reporters. Initial designs utilized nearly full-length forms of PKG I α , which undergoes a cGMP-dependent conformational change to relieve autoinhibition.¹⁹⁴ For example, both the CGY (“cyan-G kinase-yellow”)³²⁷ and cygnet (“cyclic GMP indicator using energy transfer”)³²⁹ sensors were constructed by sandwiching N-terminally truncated PKG I α between CFP and YFP. Because PKG I α functions as a dimer, these N-terminal truncations were used to remove the dimerization sequences and thus avoid intermolecular FRET between individual sensors. Notably, whereas CGY sensors retain the N-terminal autoinhibitory domain and thus preserve the native regulation of PKG I α ,³²⁷ cygnet features a larger N-terminal deletion that also removes this domain, necessitating the incorporation of a Thr516Ala substitution to prevent the sensor from being

constitutively active.³²⁹ However, finding that these early designs exhibited slow response kinetics, Nikolaev et al. sought to improve the temporal resolution by engineering simplified molecular switches based on isolated cGMP-binding domains, including the CNBD-B domain of PKG I, the GAF-A domain of PDE2A, and the GAF-B domain of PDE5³²⁵. Of these, the “cGMP energy transfer sensor” based on PDE5 (cGES-DES), showed the best performance, including a large, rapid FRET change capable of reporting cGMP pulses. In contrast, Russwurm and co-workers observed slow in vitro response kinetics when they constructed a FRET sensor incorporating both the GAF-A and B domains of PDE5 and instead engineered a rapidly responding cGMP indicator (cGi) based on CNBD-A and B of PKG.³²⁶

2.3.3.2.1.4. FRET-Based Sensors for Lipid Messengers. As described in section 2.3.1, lipid-based messengers were a major focus of early biosensor development, yielding translocation-based fluorescent biosensors for detecting multiple lipid species. However, despite their continued utility, these sensors possess certain limitations, for instance, in their ability to probe lipid production in different subcellular compartments, as their reliance on translocation as a reporting mechanism is incompatible with subcellular targeting. Meanwhile, the development of FRET-based biosensors has provided an alternative reporting strategy that can be combined with targeting to specific subcellular sites.

Like translocation-based fluorescent probes, FRET-based lipid sensors utilize endogenously derived lipid-binding domains. The most commonly used design was originally reported by Sato and colleagues, who generated the FRET-based PI(3,4,5)P₃ sensor Fflip (“fluorescent indicator for lipid second messenger”) using three rigid α -helical linkers⁶⁷⁷ to position the PH-domain of Grp1 (PH_{Grp1}) between CFP and YFP and also anchor the probe to a target membrane.⁵⁵⁶ A key aspect of this design is the presence of a diglycine “hinge” within the linker connecting PH_{Grp1} and YFP; thus, in the absence of PI(3,4,5)P₃, the rigid linkers keep CFP and YFP separated, whereas PH_{Grp1} binding to PI(3,4,5)P₃ causes CFP to “flip” outward and into closer proximity to YFP (Figure 3C). Swapping out the lipid-binding domain has allowed this design to be adapted to monitor other lipid messengers, including PI(4,5)P₂ (PH_{PLC δ}),⁵⁴² PI(4)P (PH_{FAPP1}),⁵⁴² phosphatidic acid (PH_{SOS1}, Dock2 C-terminal domain),⁵⁴⁹ and DAG (PKC β C1 domain).^{541,542} Alternatively, Ananthanarayanan et al. constructed a FRET-based PI(3,4,5)P₃/PI(3,4)P₂ sensor by tethering PH_{Akt} to a negatively charged pseudoligand sequence,⁵⁴⁰ based on evidence that PH_{Akt} recognizes head groups via a basic binding pocket.⁶⁷⁸ Sandwiching this switch between CFP and YFP yielded InPAkt (“indicator for phosphoinositides based on Akt”), wherein binding of PH_{Akt} to PI(3,4,5)P₃/PI(3,4)P₂ displaces the pseudoligand and induces a FRET change, similar to the design ofameleon.

2.3.3.2.1.5. FRET-Based Sensors for Tracking Other Cellular Analytes. Numerous FRET-based biosensors have also been developed to illuminate the spatiotemporal dynamics of several additional analytes that are critical for cellular function. As discussed previously for engineered single-FP sensors (see section 2.3.3.1.3), the designs of these probes often take advantage of the diverse molecular machinery present in bacterial cells, whose genomes encode a variety of proteins capable of binding intracellular and extracellular analytes with high affinity and specificity. For example, Zhao

and colleagues recently developed a FRET-based NADP⁺ sensor (NADP_{sr}) by sandwiching *E. coli* ketopantoate reductase, which undergoes a large conformational change due to the binding of NADP⁺ between its N- and C-terminal domains,^{679,680} between CFP and YFP.³⁵⁵ San Martín and co-workers also recently developed FRET-based sensors for lactate (“lactate optical nano indicator from CECs [Centro de Estudios Científicos]”; Laconic) and pyruvate (pyronic) by similarly sandwiching the *E. coli* transcription factors LldR and PdhR, respectively, between a FRET pair.^{347,360} Notably, bacterial PBPs have proven to be a particularly rich resource for the development of genetically encoded fluorescent biosensors, given their broad target repertoire and well-characterized conformational changes (see section 2.3.3.1.3). Both the FLIPE (“fluorescent indicator protein for glutamate”)⁵³⁴ and GluSnFR (“glutamate sensing fluorescent reporter”)^{535,681} sensors, for instance, are based on the *E. coli* ybeJ/GltI protein, which is predicted to switch between open and closed conformations in response to the binding of glutamate. Meanwhile, PBPs have also yielded several FRET-based sensors that have been used to monitor the concentrations of various sugars, including maltose,³⁴⁹ glucose,³³⁶ ribose,³⁶¹ sucrose,³⁶² and more recently, trehalose.³⁶³ Recently, semisynthetic fluorescent biosensors, where proteins containing self-labeling domains (e.g., Halo Tag, SNAP-Tag) can be covalently conjugated in situ with synthetic fluorophores (reviewed in ref 10), have become a new avenue for development for genetically targetable probes of analytes. This biosensor design has been utilized to create a family of SNIFIT biosensors (SNAP-tag-based indicators with a fluorescent intramolecular tether) which include probes for GABA,⁵³³ glutamate,⁵³⁷ acetylcholine,⁵³¹ NADP⁺,³⁵¹ and NAD⁺.³⁵¹

2.3.3.2.2. FRET Sensors for Monitoring Enzyme Activation/Activity. Along with sensing the dynamics of second messengers, metabolites, and other analytes, conformational changes in protein-based molecular switches can also be used to report on the biochemical functions of proteins themselves. Indeed, the sensitivity of FRET to intramolecular conformational changes has enabled the construction of genetically encoded biosensors capable of monitoring not only the activation of proteins in response to specific signaling events but also the endogenous catalytic activity of many key signaling enzymes directly within living cells. Sensors belonging to the former category, much like early FP-based sensor designs (see section 2.2), typically utilize a full-length protein of interest to serve as a proxy for its own behavior (e.g., activation), whereas sensors in the latter category generally make use of a surrogate substrate that can be catalytically modified by an enzyme of interest as part of an engineered molecular switch. FRET-based biosensors have thus opened a window onto the biochemical behavior of proteins in their native context that extends far beyond the ability to simply track protein expression, localization, and interactions.

2.3.3.2.2.1. FRET-Based Enzyme Activation Sensors. Many proteins undergo conformational changes in response to various signaling events (e.g., messenger binding), and while this phenomenon has proven extremely useful as a proxy for monitoring the dynamics of upstream signaling processes (e.g., messenger production), these same conformational changes can also be used to directly probe the activation dynamics of signaling proteins themselves. Often, this approach involves the heterologous overexpression of the protein of interest fused

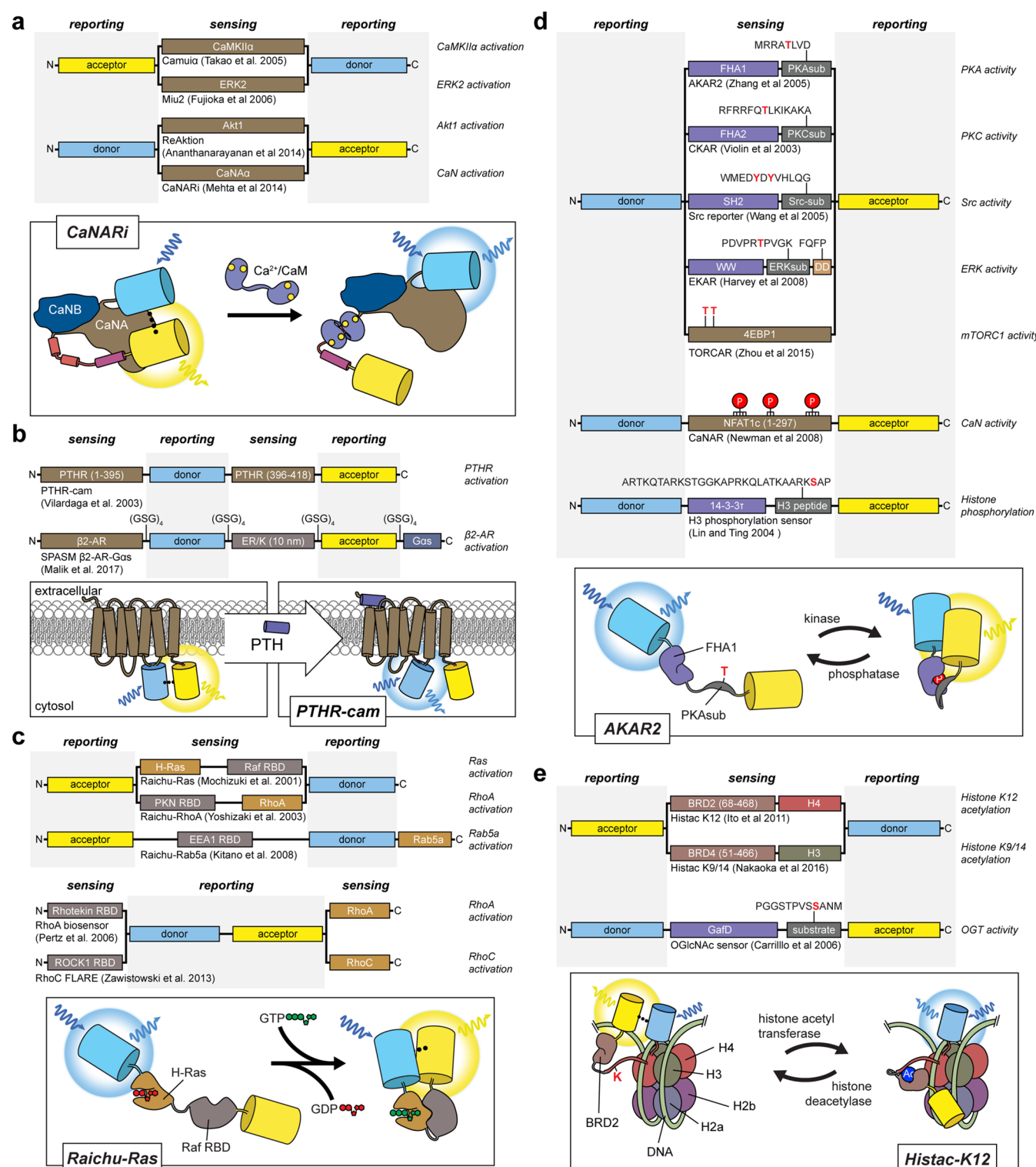


Figure 4. FRET-based biosensor designs for signaling proteins. (A) Biosensors for signaling enzyme activation consist of whole or truncated portions of the enzyme sandwiched between an FP FRET pair.^{458,459,478,540} For example, the conformational change in the phosphatase CaNAα upon activation by Ca²⁺-bound CaM increases the distance between the two FPs in CaNARI.⁴⁵⁹ (B) The insertion of FPs into a receptor⁵⁸⁸ or between a GPCR and Gαs⁵⁷⁴ have been utilized to create FRET-based biosensors of receptor activation. (C) Biosensors for small G-protein activation utilize G-protein binding domains (e.g., Raf RBD, PKN RBD, EEA1 RBD), which bind to specific G-proteins upon activation to create a conformational change.^{627,636,642,644,646} (D) FRET-based biosensors for kinase activity consist of either an endogenous kinase substrate⁴³³ or a kinase substrate sequence paired with a phosphoamino acid binding domain^{474,484,507,512,526} sandwiched between two FPs, which undergo a conformational change upon phosphorylation. Conversely, the phosphatase biosensor CaNAR uses a fragment of NFAT1c, which is phosphorylated at basal levels and exhibits a conformational shift upon dephosphorylation by CaN.⁴⁶⁰ (E) Similarly, biosensors for other PTMs use substrates paired with protein domains that recognize the modified substrates. The Histac biosensors contain a full-length histone, H3 or H4, and a

Figure 4. continued

fragment of a bromodomain (BRD)-containing protein that binds the acetylated substrate.^{650,651} Likewise, the OGT activity sensor is comprised of the O-GlcNAc binding GafD protein from *E. coli* which binds the O-GlcNAcylated substrate.⁶⁵⁴

at its N- and C-terminus to a donor and acceptor FP, such that the activation-induced conformational change will alter FRET between the FP pair. This design has been applied to generate activation indicators for a variety of different enzymes, including a number of protein kinases. For example, to study the spatiotemporal regulation of Ca^{2+} /CaM-dependent kinase II (CaMKII), a key signaling enzyme involved in learning and memory, Takao and colleagues appended YFP and CFP to the N- and C-termini of full-length rat CaMKII α to generate Camui α .⁴⁵⁸ The binding of Ca^{2+} /CaM switches CaMKII from a closed, autoinhibited state to an open, active conformation, thus inducing a FRET decrease in Camui α . Similar sensors have also been developed to probe Erk2 activation during MAPK signaling,⁴⁷⁸ the regulation of PKB/Akt activation by phosphoinositides and phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation,⁴⁴² and the activation of PDK1 itself.⁴⁹⁹ Recently, the activation of the Ca^{2+} /CaM-dependent protein phosphatase calcineurin (CaN) was similarly studied by sandwiching the full-length CaN catalytic subunit between CFP and YFP⁴⁵⁹ (Figure 4A).

Vilardaga and colleagues also previously utilized this biosensor strategy to investigate the activation kinetics of the α_{2A} adrenergic receptor ($\alpha_{2A}\text{AR}$) and PTHR, members of two distinct classes of the GPCR superfamily of seven-transmembrane cell-surface receptors.⁵⁸⁸ To detect the conformational changes that occur during GPCR activation, the authors fused YFP to the C-terminus and inserted CFP within the third intracellular loop of both the $\alpha_{2A}\text{AR}$ and the PTHR, as the activation of these GPCRs has been shown to include shifts in the relative position of the sixth transmembrane helix,⁶⁸² which flanks the third intracellular loop (Figure 4B). In fact, several FRET-based sensor designs have been employed to study the activation of GPCRs, which comprise the largest family of signaling proteins and are responsible for mediating the activation of numerous signaling pathways in response to diverse extracellular cues. Activated GPCRs function as GEFs for heterotrimeric G proteins, leading to the dissociation of the GTP-bound α subunit ($G\alpha$) from the $G\beta\gamma$ -subunit dimer and downstream signaling. Thus, early studies utilized the decrease in intermolecular FRET between FP-labeled G protein subunits as a proxy for GPCR activation,⁵⁷⁷ an approach that continues to be used.⁶⁸³ Meanwhile, others have similarly monitored the association of FP-labeled receptor and the $G\alpha\beta\gamma$ heterotrimer (e.g., ref 587 and also section 2.3.3.3). Recently, Malik et al. adapted this approach to devise a unimolecular GPCR activation sensor in which YFP and CFP, separated by a 10 nm ER/K linker (see section 2.3.3.1.4), are sandwiched between a full-length GPCR and a high-affinity binding peptide derived from a specific $G\alpha$ subunit.⁵⁷⁶ An updated version of this design incorporates a full-length $G\alpha$ subunit and permits association with endogenous $G\beta\gamma$.⁵⁷⁴

Monomeric G proteins represent another large superfamily of signaling proteins that are crucial regulators of multiple cellular processes, and several FRET-based biosensors have been developed to track the activation dynamics of these small GTPases in living cells. In general, the design of these probes utilizes the intramolecular binding between a full-length GTPase and a tethered binding domain from an effector

protein to drive a change in FRET between an FP pair, analogous to the design of cameleon. For example, Mochizuki and colleagues constructed the Raichu ("Ras and interacting protein chimeric unit") sensor by tethering full-length H-Ras to the Ras-binding domain (RBD) from Raf and sandwiching the resulting molecular switch between YFP and CFP (Figure 4C).⁶³⁶ This modular architecture has proven to be highly generalizable, and substituting H-Ras/Raf-RBD with other cognate binding pairs has yielded a family of Raichu sensors for Rap1,⁶³⁶ Rac,⁶²⁴ Cdc42,⁶²⁴ RhoA,⁶⁴⁴ RalA,⁶³⁴ TC10/RhoQ,⁶⁴⁷ and R-Ras⁶⁴⁸ (Table 1). Notably, while these biosensors play an important role in exploring the regulation of GTPases by GEFs and GTPase-activating proteins (GAPs), they cannot be used to study the specific regulation of Rho-family GTPases by guanosine dissociation inhibitors (GDIs) due to the masking of the GTPase C-terminus,⁶⁸⁴ leading to the development of sensors with reconfigured architectures. For instance, when constructing Raichu-Rab5, Kitano et al. kept the RBD between YFP and CFP but moved full-length Rab5a to the biosensor C-terminus.⁶²⁷ Others have opted to completely invert the design by sandwiching a FRET pair between the GTPase and RBD.^{641,642,646} Finally, some designs incorporate only the RBD, eschewing the inclusion of a full-length GTPase component in order to monitor endogenous GTPase activation.^{624,644,663}

2.3.3.2.2. FRET-Based Enzyme Activity Reporters. Proteins must rapidly and continuously adapt their functions to suit the ever-changing needs of a highly dynamic cellular environment. Covalently altering protein sequences by introducing post-translational modifications allows cells to dynamically modulate virtually all aspects of protein function and greatly expand the functional diversity of the cellular proteome, and FRET-based biosensors have proven particularly apt for visualizing the activities of the enzymes responsible for catalyzing the myriad post-translational modifications taking place within cells. These biosensors are based on the incorporation of a target protein sequence into the sensing unit, which therefore functions as a surrogate substrate for a given enzyme activity. Molecular switching behavior is conferred either by utilizing a protein domain that intrinsically alters the biosensor conformation when modified by the target activity or by tethering a minimal substrate sequence (i.e., receiver domain) to a separate module that recognizes and binds the modified substrate (i.e., switch domain) to drive an activity-induced conformational change. Importantly, biosensors for monitoring enzyme activity are subject to enzymatic amplification, in that a single enzyme is able to react with multiple biosensors, which increases probe sensitivity due to the exponential relationship between the biosensor response and the number of active enzymes. This property, combined with the targetability of these genetically encoded probes to specific subcellular regions, allows FRET-based enzyme activity sensors to sensitively report on the spatiotemporal dynamics of endogenous enzyme activities within the context of their native regulatory environment in living cells.

2.3.3.2.2.1. FRET-Based Sensors for Monitoring Protease Activity. What can be considered the first genetically encoded

FRET-based biosensors were generated by sandwiching an oligopeptide containing a protease cleavage site between GFP and BFP, whereby catalytic cleavage of the protease substrate liberates the two FPs and produces a dramatic decrease in intramolecular FRET.^{685,686} Although these initial constructs were built largely as a proof of concept to explore and characterize the occurrence of FRET between different FP color variants, the site-specific cleavage of proteins by various proteases is in fact an essential component of numerous physiological, and pathological, processes, and this simple probe design has thus frequently been replicated to study the dynamics of a number of important cellular proteases. Cysteine-aspartyl proteases (caspases), for example, are key regulators of apoptotic cell death pathways, and several groups have studied the dynamics of both effector and initiator caspases by sandwiching the cleavage sites for caspase-3 (Casp3) (e.g., “DMQD”⁵⁷⁰ or, more commonly, “DEVD”^{560–565,569}), as well as Casp8 (“IETD”)⁵⁶⁵ and Casp9 (“LEHD”)⁵⁶⁹ between a FRET FP pair. Ouyang and colleagues have similarly visualized the activity of membrane type 1 matrix metalloproteinase (MT1-MMP), a membrane-tethered extracellular protease that is upregulated in invading cancer cells.^{572,573} Alternatively, Li et al. utilized full-length versions of the LC3B or GATE-16 proteins sandwiched between CFP and YFP to track the activity of Atg4, a key protease involved in regulating autophagy.⁵⁵⁹

2.3.3.2.2.2. FRET-Based Sensors for Monitoring the Dynamics of Protein Phosphorylation. The reversible phosphorylation of proteins on Ser, Thr, or Tyr residues is arguably the most important post-translational modification involved in regulating protein function in cells and also one of the most abundant, with ~40% of the total human proteome estimated to be phosphorylated by protein kinases at some point in time.⁶⁸⁷ FRET-based genetically encoded kinase activity reporters (KARs) were designed based on the fact that cells contain multiple protein modules, known as phosphoamino acid binding domains (PAABDs), that are capable of specifically recognizing and binding phosphorylated residues within target proteins.^{688–690} Thus, by following the architecture established withameleon, Zhang and colleagues engineered a bipartite kinase-inducible molecular switch in which a consensus phosphorylation sequence for PKA (LRRASLP, based on the so-called “kemptide” PKA substrate⁶⁹¹) was tethered by a flexible linker to the PAABD 14-3-3 τ .⁵⁰⁸ In the resulting first-generation “A-kinase activity reporter” (AKAR1),⁵⁰⁸ PKA-mediated phosphorylation increased the intramolecular binding between 14-3-3 and the substrate, thereby inducing a conformational rearrangement and altering the FRET between a flanking CFP and YFP pair.⁵⁰⁸ However, the high affinity of the 14-3-3 domain rendered AKAR1 insensitive to cellular phosphatases, and the largely irreversible FRET response was thus unable to capture the full temporal dynamics of PKA activity. Replacing 14-3-3 τ with the lower-affinity forkhead-associated 1 (FHA1) domain, in conjunction with a modified PKA substrate (LRRATLVD) designed to better fit the preferred recognition sequence for FHA1,⁶⁹² therefore yielded a second-generation AKAR (AKAR2), as well as subsequent variants (AKAR3⁵⁰⁶ and AKAR4⁵⁰⁴), with a fully reversible FRET response (Figure 4D).⁵⁰⁷

Much like the aforementioned Raichu GTPase sensor (section 2.3.3.2.2.1), the modular architecture of AKAR proved to be highly generalizable and has yielded a diverse

family of FRET-based sensors for monitoring the activity of a multitude of different protein kinases that are too numerous to mention (see Table 1). Biosensor specificity is, of course, determined by the incorporation of a substrate sequence that will be recognized and phosphorylated by the kinase of interest but not by other kinases, while in some cases, additional sequence elements are included to enhance specificity, such as docking sequences for MAPKs.^{474,475,490,496} Yet identifying a suitable, kinase-specific phosphorylation sequence can be a difficult task that often requires considerable trial and error, though more systematic approaches may help speed up this process, especially for kinases with poorly defined or unknown substrate preferences. For example, Tsou et al. utilized a positional scanning peptide library screen⁶⁹³ when designing a substrate sequence for their AMP-dependent kinase activity reporter (AMPKAR), although multiple candidate sequences were still tested.⁴⁴⁶ More recently, when designing a FRET-based KAR for Rho-associated protein kinase (ROCK), Li and colleagues initially trialed several previously characterized substrate sequences before turning to a newly developed kinase-interacting substrate screening (KISS) approach,⁶⁹⁴ in which cell lysates are run through kinase-coated beads to enrich interacting substrates, followed by tandem mass spectrometry to deduce the consensus phosphorylation sequence.⁵²¹ Nevertheless, efforts to engineer a molecular switch can sometimes yield no successful candidates, in which case intrinsic molecular switches can also be used, as in the case of Zhou and co-workers, who utilized the native phosphorylation-induced conformational change in full-length 4EBP1 as the basis for their mTOR complex 1 activity reporter (TORCAR).⁴³³

It is important to remember that phosphorylation is a highly dynamic and reversible PTM where the addition of phosphate groups by kinases is constantly opposed by protein phosphatases which are responsible for catalyzing their removal. While cells typically contain a far smaller variety of protein phosphatases (e.g., mammalian genomes encode roughly 1/5th the number of phosphatases as kinases⁶⁹⁵), these enzymes are no less important in regulating protein function. Nevertheless, visualizing protein phosphatase activity in living cells has proven to be a far greater challenge compared with monitoring protein kinase activity. For one thing, these enzymes have differing requirements for substrate recognition: kinase specificity is defined by the residues that surround the target phosphorylation site, while phosphatases are far more promiscuous in this regard (a fact related to their smaller numbers), with specificity instead being governed by conserved docking motifs within substrate proteins⁶⁹⁶ and coordination by regulatory domains.⁶⁹⁷ The chief obstacle, however, is the fact that, unlike KARs, which become phosphorylated, a phosphatase sensor must already be phosphorylated, such that it can respond to being dephosphorylated, and constructing a constitutively “dephosphorylation-competent” molecular switch remains something of a conundrum. Thus far, the only design to successfully overcome this problem is that of the FRET-based CaN activity reporter (CaNAR),^{459,460} whose intrinsic molecular switch features an endogenous CaN substrate (nuclear factor of activated T-cells, NFAT) that is basally hyperphosphorylated by multiple kinases.⁶⁹⁸ Meanwhile, in the absence of a more generalizable design, the development of genetically encoded phosphatase sensors continues to lag far behind that of kinase sensors.

2.3.3.2.2.3. FRET-Based Sensors for Monitoring Other Post-Translational Modifications. Cells call upon an extensive repertoire of post-translational modifications to regulate protein function. This is perhaps best embodied by histone proteins, which comprise the core structural scaffold in chromatin and undergo a particularly high degree of post-translational modifications along their N-terminal tails. These modifications provide recognition sites for various chromatin-binding proteins and also control access to DNA by the replication and transcriptional machinery, and understanding the dynamics of histone modification is therefore critical for unraveling the mechanisms of epigenetic regulation. Thus, Lin and Ting constructed a sensor for visualizing the phosphorylation of histone H3 at Ser28, which is catalyzed by numerous kinases,⁶⁹⁹ by engineering a molecular switch composed of the 30 N-terminal amino acids of histone H3 tethered to 14-3-3 τ and sandwiched between CFP and YFP.⁴⁸⁴ Histones are also frequently acylated and methylated on lysine residues, and Lin et al. were able to generate a FRET-based biosensor for monitoring the methylation of histone H3 on lysine 9 or 27 by similarly using short peptides spanning these regions.⁶⁵³ However, more recent efforts have opted for designs that incorporate full-length histones, rather than just minimal peptides, including sensors developed to monitor the acetylation dynamics of both histone H3⁶⁵⁰ and histone H4.^{651,652} Again, these sensors feature engineered molecular switches wherein the specific histone is coupled to an appropriate binding domain to drive a conformational change (Figure 4E). As a critical component of these biosensors, chromodomains and bromodomains are compact protein modules that specifically recognize methylated⁷⁰⁰ and acetylated⁷⁰¹ lysines, respectively. Thus, Lin and co-workers utilized chromodomains from HP1 polycomb proteins to recognize methylated histone H3,⁶⁵³ while Nakaoka et al. used the bromodomain from the BRD4 protein to construct a histone acetylation (Histac) sensor for histone H3 acetylation.⁶⁵⁰ In a newly engineered biosensor of histone H3 trimethylation on lysine 9, a chromodomain of HP1 was again utilized for recognizing methylated histone H3 and a full-length histone H3 was incorporated into the biosensor for high specificity,¹⁰⁰¹ similar to the H3 acetylation sensor Histac.⁶⁵⁰

Meanwhile, a unique form of glycosylation, involving the specific addition of O-linked N-acetylglucosamine (O-GlcNAc) to Ser or Thr residues in proteins throughout the cell, has emerged over the last several years as a major post-translational modification that is implicated in multiple physiological and pathological processes (recently reviewed in ref 702). In contrast to many established post-translational modifications, O-GlcNAcylation is exclusively regulated by a single pair of enzymes, namely, O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, and O-GlcNAcase, which catalyzes its removal. Furthermore, the reciprocal nature of O-GlcNAcylation and phosphorylation, which often compete for the same sites on many proteins, with O-GlcNAcylation able to block protein phosphorylation, suggests a potentially important role for the dynamic interplay between these modifications in the regulation of intracellular signaling. Thus, to track the spatiotemporal dynamics of O-GlcNAcylation in living cells, Carrillo and co-workers set out to engineer an O-GlcNAc-responsive molecular switch and generate a FRET-based O-GlcNAc sensor.⁶⁵⁴ As the receiver domain, they utilized the OGT substrate sequence from casein kinase II, while the *E. coli* GafD protein, a monomeric lectin (i.e.,

carbohydrate-binding protein) that specifically recognizes O-GlcNAc, was selected as the switching domain. Again, the selection of both a specific substrate sequence and an appropriate recognition domain is essential for designing an activity-induced molecular switch. Though currently limited to empirical trial and error, it is hoped that the application of more high-throughput methods, such as molecular evolution, will propel the wider development of FRET-based enzyme activity biosensors.

2.3.3.2.2.3. FRET-Based Sensors for Measuring Mechanical Forces in Cells. Cells are physical entities that engage in a variety of mechanical processes, both extrinsic (e.g., cell–cell/cell–environment interactions) and intrinsic (e.g., chromosome segregation, cytokinesis). These processes generate mechanical forces that provide information on the physical state of the cell and its environment, sending signals to the intracellular biochemical machinery and regulating cell physiology and behavior. Indeed, mechanosensitive signaling is fundamental to virtually all aspects of biology across evolution.⁷⁰³ Elucidating the mechanisms by which cells sense and interpret stimuli from mechanical forces (i.e., mechanotransduction) is thus essential to our understanding of physiology and disease. Notably, the past decade has seen the development of several genetically encoded FRET-based biosensors capable of performing in situ measurements of the piconewton (pN)-scale mechanical forces experienced by individual molecules inside living cells. In general, these sensors make use of a tension-sensing module composed of an FP pair tethered to either side of a mechanosensitive element.^{230,233–236} This linker adopts a compact conformation at rest but becomes elongated under strain, thereby increasing the interfluorophore distance and causing FRET to vary as a function of mechanical tension across the module, which can be inserted within a protein of interest to visualize mechanical forces. For example, Grashoff et al. constructed a tension-sensing module using a molecular nanospring composed of 8 tandem 5-amino-acid repeats from the spider silk protein flagelliform,⁷⁰⁴ which they sandwiched between a FRET pair and inserted between the head and tail domains of the vinculin protein to measure tension across focal adhesions.²³⁶

Importantly, given the aforementioned mathematical relationship between distance and FRET efficiency (eq 1, section 2.2.2), these mechanosensing probes can be calibrated in vitro (e.g., using single-molecule force spectroscopy⁷⁰⁵) to convert quantitative FRET measurements into absolute pN force values. Performing such quantitative force measurements requires the mechanosensitive domain to fulfill certain requirements (reviewed in ref 706), such as relaxation upon the removal of force (i.e., reversibility) and a lack of hysteresis between elongation and relaxation. Furthermore, the identity of the mechanosensitive domain also determines the range of forces that can be detected. Thus, whereas the 40-amino-acid elastic domain used by Grashoff et al. was sensitive between 1 and 6 pN of force,²³⁶ Austen and co-workers utilized variants of the 35-amino-acid α -helical vilin headpiece peptide, which undergoes an unfolding transition in response to mechanical tension, to sense forces ranging from 7 to 10 pN.²³⁰ More recently, Brenner and colleagues found that a shorter flagelliform peptide comprising only 5 repeats showed a much broader sensitive range, spanning 2–11 pN.²³⁵ Nevertheless, these probes remain limited to monitoring intracellular forces within a relatively narrow window, while their FRET responses also exhibit somewhat poor dynamic ranges.

Meanwhile, rather than relying on modulating the distance between a pair of FPs connected by an elastic linker, Meng and colleagues developed an alternative force sensor design that produces large FRET changes by altering the relative orientation between a pair of linked FPs whose dipoles are (almost) perfectly aligned at rest.²³² Such continued innovation will be crucial for expanding the utility of FRET-based force biosensors.

2.3.3.2.3. Strategies for Optimizing FRET-Based Biosensor Responses. Dynamic range plays a key role in determining the signal strength and detection limit of a genetically encoded fluorescent biosensor. In the case of FRET-based biosensors, this property refers to the degree of difference between the lowest FRET signal in the “off” state and the highest FRET signal in the “on” state; the larger this range, the more likely a small change in the parameter of interest will yield a robustly detectable FRET response. A substantial proportion of biosensor development is thus devoted to optimizing dynamic range, for which two major strategies include minimizing basal FRET in the “open” biosensor conformation and maximizing FRET efficiency in the “closed” conformation. The first strategy essentially entails increasing the separation between the donor and acceptor FPs and is largely accessible only to biosensors that feature an engineered, bipartite molecular switch rather than an intrinsic conformational change. For example, many such FRET-based biosensors feature a short linker between the receiver and switch domains (e.g., *cameleon*,³⁷⁷ *AKAR*,⁵⁰⁸ and *Raichu*⁶³⁶). However, in an effort to develop an optimized backbone for constructing FRET-based biosensors, Komatsu and colleagues found that progressively increasing linker length using repeating (SAGG) units yielded proportional decreases in basal FRET signals.⁴⁴¹ This work yielded the “extension for enhanced visualization by evading extraFRET” (Eevee) system and the corresponding “EV linker”, along with a number of enhanced FRET-based biosensors.⁴⁴¹

Conversely, FRET efficiency can be increased by minimizing the interfluorophore distance in the closed sensor conformation. Although this conformation is expected to bring the donor and acceptor FPs into close proximity, conformational dynamics within the sensor may cause this distance to fluctuate. Thus, allowing the FPs to associate may help ensure they achieve the closest possible approach. In fact, Vinkenberg et al.⁷⁰⁷ suggested this effect as the mechanism underlying the enhanced FRET behavior of the molecularly evolved CyPet/YPet pair⁷⁰⁸ and later applied this principle to improve the dynamic range of the CALWY Zn²⁺ sensor.⁴²⁰ Given that CALWY exhibits a FRET decrease upon Zn²⁺ binding, FP dimerization thus helps stabilize the high-FRET “off” state but, importantly, does not affect the conformational switch or the resulting FRET change. Meanwhile, this approach may conceivably pose a problem in sensor designs where the conformational change brings the donor and acceptor closer together and thus increases FRET, as FP dimerization may yield spurious FRET increases, though previous work has suggested otherwise.^{304,709} Nevertheless, a more straightforward approach is simply to use better FPs. Recall from [section 2.2.2](#) that R_0 , the Förster distance, is influenced by the photophysical properties of the donor and acceptor. These include the extinction coefficient (i.e., how efficiently photons are absorbed) and quantum yield (i.e., how efficiently photons are re-emitted), and selecting a donor and acceptor pair that optimizes these parameters can greatly improve FRET, and

thus dynamic range. FRET can also be enhanced through the incorporation of tandem acceptor FPs,³⁰⁴ as demonstrated recently by Klarenbeek et al. in the development of fourth-generation FRET-based cAMP sensors with dramatically improved dynamic ranges.³⁰²

The relative orientation of the donor and acceptor FPs also contributes to the FRET efficiency, as the donor and acceptor dipoles must be properly aligned for maximal energy transfer to occur. Although discussed only in the context of single-FP biosensors thus far, circular permutation can also be used to shift the angle at which an FP is fused to the sensing unit in a FRET-based biosensor. The resulting alterations to the relative orientation of the FP and its chromophore can have a significant impact on FRET efficiency by improving dipole–dipole alignment, which has been frequently used to increase biosensor dynamic range.^{287,309,373,425,506} Using truncated FPs (i.e., deleting the flexible N- or C-terminal regions) can also alter the orientation of the chromophore with respect to the final biosensor structure, as can modifying the linkers connecting the FPs to the sensing unit. However, absent detailed structural information, anticipating how specific changes will affect chromophore orientation is difficult, if not impossible, meaning that various incremental changes must be tested through trial and error. Furthermore, optimization typically involves pursuing multiple strategies and, given the above maze of options, may require testing dozens or even hundreds of candidates. More high-throughput approaches are thus sorely needed. To this end, Belal and colleagues developed a bacterial colony screening system for optimizing FRET-based KARs in which both kinase and sensor candidate are inducibly expressed from a single plasmid,⁴³⁶ a potentially important advance given that enzyme activity sensors are left out of current bacterial screening approaches.

2.3.3.2.4. Biosensors Based on Modulating Fluorescent Protein Dimerization. While controlling FP proximity to modulate FRET remains arguably the most popular reporting strategy for genetically encoded fluorescent biosensors, other designs have also emerged in recent years that utilize alternative fluorescent readouts based on FP proximity (also see [section 2.4.3](#) below). For example, whereas most FP-based applications favor the use of engineered monomeric variants to avoid artifacts caused by the native propensity of FPs to self-associate, Alford and colleagues have conversely sought to take advantage of the obligate oligomerization of RFPs, which stabilizes the chromophore and thus enhances brightness,^{160,710,711} in an effort to develop a novel biosensor reporting strategy based on modulating dimerization-dependent increases in RFP intensity.³⁸¹ Starting with a weakly fluorescent, monomeric form of tdTomato,²¹³ designated “A”, they performed extensive molecular evolution to identify a “dark” interacting partner, designated “B” and also derived from tdTomato, that would rescue the fluorescence of A upon heterodimer formation. This approach ultimately yielded a dimerization-dependent RFP (ddRFP) that exhibited an ~10-fold intensity increase upon dimer formation, which enabled the straightforward construction of dual-FP, single-color Ca²⁺, and protease activity sensors ([Figure 5A](#)).³⁸¹ Subsequent engineering of this initial ddFP also succeeded in producing ddGFP and ddYFP variants.⁷¹² Meanwhile, Ding et al. recently observed that the B subunits, which contain no chromophore,³⁸¹ were interchangeable between ddRFP-A (RA) and ddGFP-A (GA).³⁰⁷ This observation spurred the development of yet another novel biosensor readout based on so-called

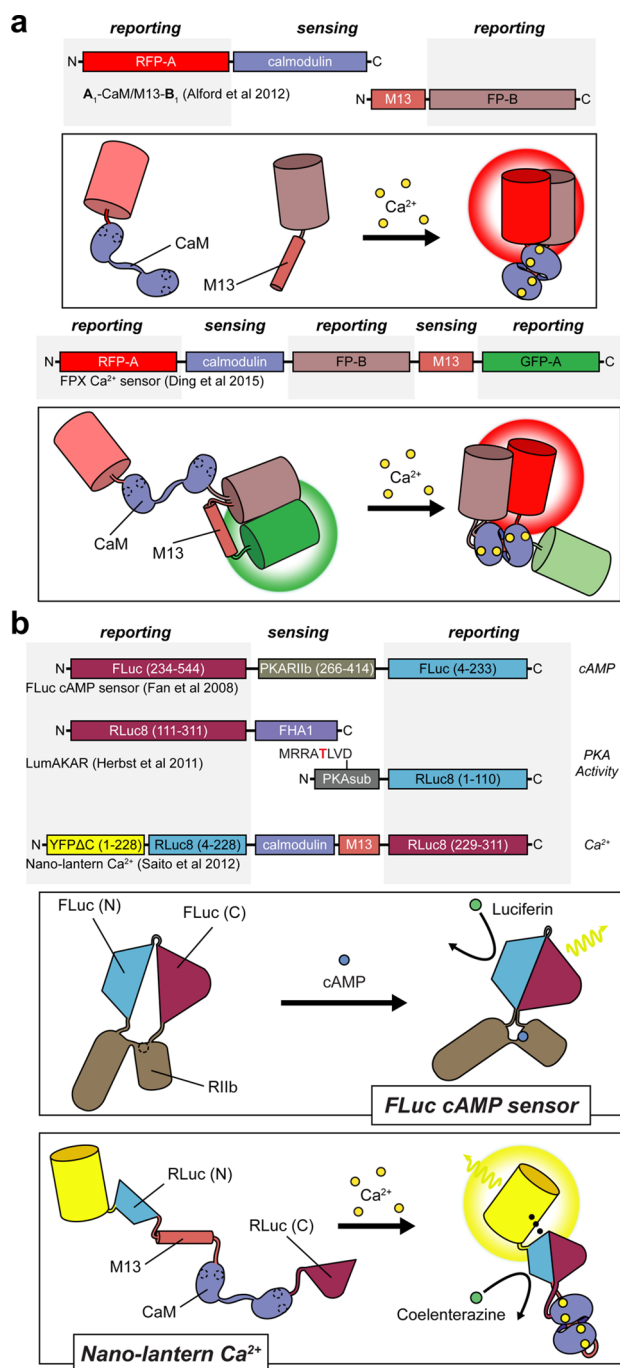


Figure 5. ddFP- and luminescence-based biosensors. (A) Bimolecular (top) or unimolecular (bottom) Ca²⁺ biosensor designs based on ddFPs.^{307,381} The dimerization of the dim ddFP partner, FP-B, with either RFP-A or GFP-A increases their fluorescence, and this dimerization is modulated by the Ca²⁺-dependent binding of calmodulin to M13. (B) Modulating the structure of split luciferases by conformational switches has been utilized in cAMP, PKA, and Ca²⁺ biosensors.^{290,318,503} In the FLuc cAMP sensor, the conformational switch induced by binding of cAMP to PKA RIIβ allows the firefly luciferase (FLuc) to properly form, thus allowing the enzyme to catalyze the degradation of luciferin and emit photons.³¹⁸ Similarly, the Nano-lantern Ca²⁺ biosensor undergoes a Ca²⁺-dependent reconstitution of renilla luciferase (RLuc), which is then capable of BRET with the adjacent, brighter YFP.²⁹⁰

fluorescent protein exchange (FPX). Specifically, these biosensors utilize a molecular switch that is designed to

exchange GA-B dimer formation for RA-B dimer formation in response to a biochemical signal, yielding reciprocal (i.e., ratiometric) changes in ddGFP and ddRFP intensity³⁰⁷ (Figure 5A). One potentially significant advantage of FPX is its apparent insensitivity to linker length/composition, suggesting a far more straightforward path for biosensor design than that for FRET-based sensors.

2.3.3.3. Biosensor Designs Incorporating Luminescent Proteins. Bioluminescence, wherein light is generated through an enzyme-catalyzed chemical reaction, represents an alternative biosensing strategy that has in some ways been overshadowed by the popularity of FP technology. Yet bioluminescence offers a number of advantages that support its application in areas where the use of fluorescence-based biosensors continues to pose a challenge (e.g., in vivo). Specifically, the wavelengths used to excite most genetically encoded fluorescent biosensors often cause problems related to autofluorescence, fluorophore bleaching, phototoxicity, and poor tissue penetration due to scattering/absorption within the specimen. In contrast, bioluminescence relies on a broad class of luminescent proteins, known as luciferases, that emit light via the oxidation of a luciferin substrate (e.g., coelenterazine or similar molecules) and therefore require no external illumination. Thus, luminescence-based readouts have the potential to yield reduced background and enhanced contrast and signal-to-noise ratios and may also lower some of the barriers to in vivo imaging.

Luciferases are present throughout the natural world, most notably in a veritable menagerie of marine organisms.⁷¹³ Among the most commonly used luciferase variants are those derived from the sea pansy *Renilla reniformis* (Renilla luciferase, RLuc) and the North American firefly *Photinus pyralis* (firefly luciferase, FLuc), which have been used to construct genetically encoded biosensors that mirror many of the reporting unit configurations found in FP-base probes. For example, based on structural data indicating that FLuc comprises two globular domains that close in a hingelike motion upon substrate binding,⁷¹⁴ Fan and colleagues generated a circularly permuted FLuc in which the native N- and C-termini were relocated to opposite sides of this hinge, such that the insertion of a sensing unit would render FLuc activity sensitive to various biochemical signals.³¹⁸ Bridging this gap using either a short “DEVG” peptide linker or the CNBD-B domain from PKA RIIβ thus yielded luminescent indicators for caspase-3 activity or cAMP accumulation, respectively³¹⁸ (Figure 5B). Luciferases are also compatible with fragment complementation, offering a sensitive and dynamic readout that contrasts with the irreversible complementation of FP fragments. Indeed, Stefan et al. previously used this approach to monitor PKA activation in living cells by tagging the regulatory and catalytic subunits of PKA with complementary RLuc fragments.⁵¹⁰ Herbst and colleagues similarly used RLuc fragment complementation to generate luminescent KARS in which the PAABD and substrate peptide are expressed as two separate polypeptides, fused to complementary RLuc fragments, which reassociate upon phosphorylation.⁵⁰³ This approach successfully yielded luminescent KARs for monitoring PKA and PKC activity (Figure 5B).

Much like the excited state of a FP chromophore, the high-energy reaction product of luciferin oxidation is also capable of nonradiative energy transfer to a nearby acceptor. Thus, a number of genetically encoded biosensors utilize biolumines-

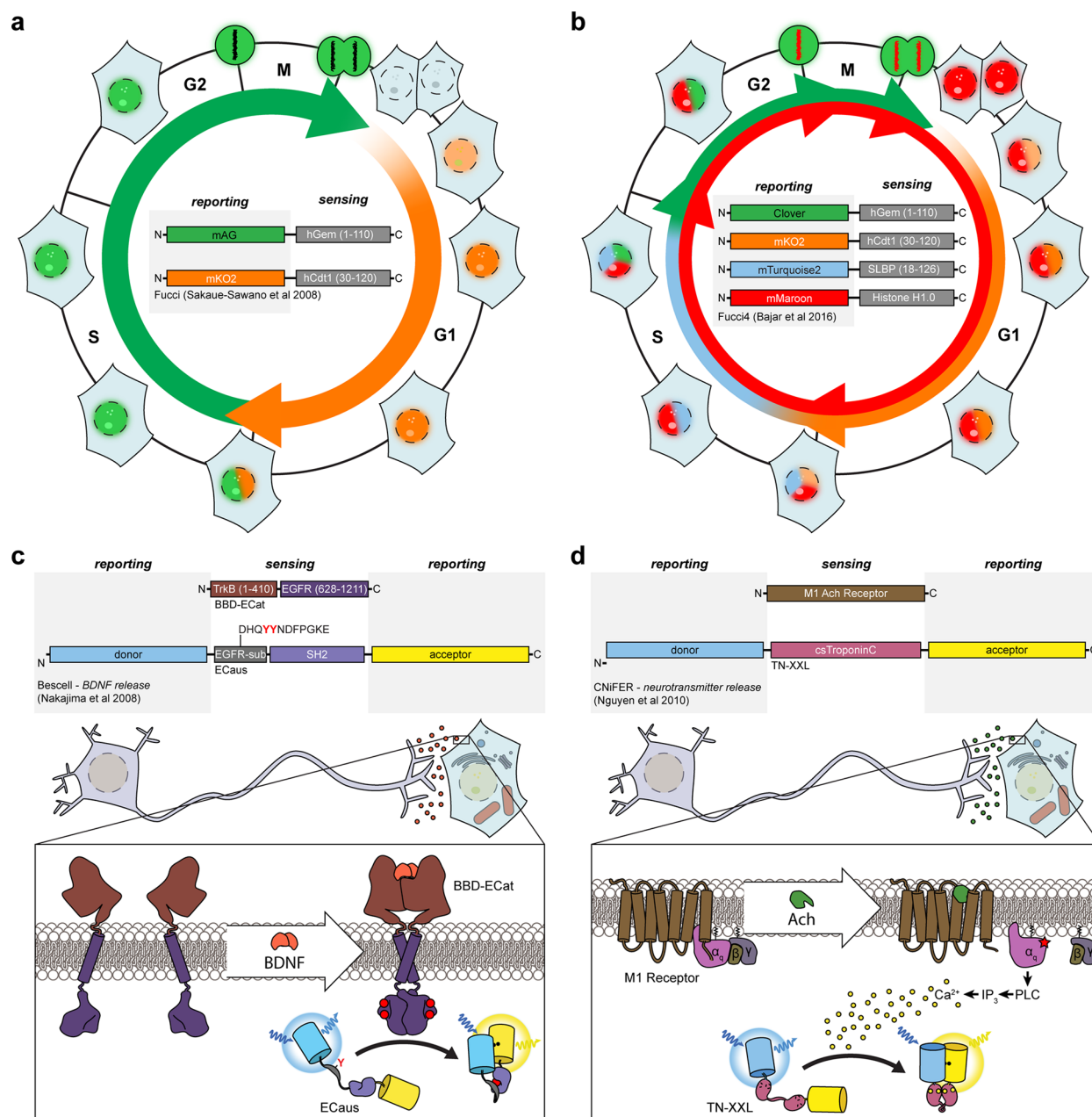


Figure 6. Coupled reporter systems. (A and B) Biosensors reporting the cell cycle phase of cells utilize fluorescent proteins fused to protein fragments that are selectively degraded during specific phases of the cell cycle.^{221,228} In the Fucci system, Gem is degraded during the G1 and late M phases and, conversely, Cdt1 is degraded in S and G2 phases.²²⁸ The improved Fucci4 adds the condensation of chromatin around Histone H1 to report the M phase, as well as SLBP, which is degraded after S phase, in addition to labeled Cdt1 and Gem from Fucci.²²¹ (C) The chimeric receptor BBD-ECat, which consists of an extracellular TrkB domain, which dimerizes upon binding BDNF, and an intracellular EGFR domain, is coupled with an EGFR activity reporter ECaus to act as a reporter for BDNF release by neurons.²⁹⁵ (D) Similarly, the expression of the M1 receptor and the Ca^{2+} biosensor TN-XXL⁴⁰⁸ in a cocultured reporter cell uses the endogenous coupling of the GPCR M1R to $\text{G}\alpha_q$, which, upon receptor stimulation, leads to an increase in intracellular Ca^{2+} to report the presence of a neurotransmitter, acetylcholine (ACh).⁵³⁰

cence resonance energy transfer (BRET) as the reporting mechanism, which often requires little more than replacing the donor FP in an existing FRET-based biosensor design with a luciferase. For example, Gulyás and co-workers constructed a BRET-based Ca^{2+} sensor by incorporating an enhanced version of RLuc⁷¹⁵ in place of CFP in the D3 variant of YC.³⁶⁷ Over the years, this strategy has similarly yielded BRET-based sensors for monitoring cAMP accumulation,^{300,310} ERK activity,⁴⁷² ATP concentration,²⁸⁵ membrane voltage,²⁵¹ and ubiquitination⁶⁴⁹ (Table 1). BRET-based biosensor assays are also widely used to study the dynamics

of GPCR signaling.⁷¹⁶ Galés et al., for instance, monitored intramolecular BRET between RLuc-tagged GPCRs and GFP-tagged G proteins to probe GPCR activation,⁵⁷⁵ as well as the conformational dynamics of preassembled receptor-G protein complexes,⁵⁸⁶ and Thomsen et al. recently used a similar approach to probe the assembly of GPCR signaling components on the endosomal surface (also discussed in section 4.1).⁵⁸⁵ Meanwhile, Dacres and colleagues used intramolecular BRET between a C-terminal RLuc and GFP inserted within the third intracellular loop to directly monitor GPCR activation,⁵⁸⁹ similar to a previous FRET-based

design.⁵⁸⁸ Notably, the authors argued that BRET offered a more sensitive readout of GCPR activation compared with FRET.⁵⁸⁹

Nevertheless, luciferase- and BRET-based sensors have historically underperformed compared with fluorescent biosensors in terms of overall intensity,²⁹⁰ despite the fact that BRET can significantly enhance the light output of luciferase (e.g., up to 6-fold higher quantum yield in vitro for RLuc paired with GFP vs RLuc alone^{717,718}). Poor energy transfer efficiency in most BRET-based probes is one likely explanation for this deficiency. Thus, Saito and colleagues set out to address this problem by developing the novel luminescent protein Nano-lantern.²⁹⁰ Nano-lantern comprises an optimized RLuc fused directly to a bright YFP variant (Venus³⁷⁴) and is designed in such a way as to maximize intramolecular BRET. Indeed, at roughly 10 times the brightness of RLuc alone, Nano-lantern was suitable for both live-cell and in vivo imaging. Furthermore, Nano-lantern facilitated the construction of novel luminescence-based biosensors in which a molecular switch is used to modulate Nano-lantern intensity through RLuc fragment complementation, as opposed to direct modulation of BRET efficiency, yielding sensors for Ca²⁺, cAMP, and ATP (Figure 5B).²⁹⁰ In addition, while some luciferase color variants have been generated through protein engineering,^{719–722} the color spectrum of Nano-lantern can be readily altered by incorporating different FPs as the BRET acceptor.^{397,398} Notably, Suzuki et al. recently developed 5 color variants of a brighter, enhanced Nano-lantern³⁹⁷ that also incorporates the highly optimized NanoLuc variant of *Oplophorus gracilirostris* luciferase.⁷²³ Although they have yet to be widely adopted in biosensor design, these and other bright luminescent proteins, such as the recently reported Antares⁷²⁴ and Akaluc,⁷²⁵ may be poised to fulfill the promise of bioluminescence-based in vivo imaging of dynamic cellular processes.

2.3.4. Coupled Reporter Systems. Although genetically encoded biosensors are primarily designed to monitor the dynamics of discrete biochemical events, designs that couple the actions of related biochemical processes can provide spatiotemporal information on complex cellular phenomena, thus greatly expanding the utility of these molecular tools. For example, the reciprocal oscillations of the APC^{Cdh1} and SCF^{Skp2} E3 ubiquitin ligase complexes mark different cell cycle phases,⁷²⁶ and Sakaue-Sawano et al. previously applied this knowledge to generate a “fluorescent ubiquitin-based cell cycle indicator” (Fucci) based on the APC^{Cdh1} and SCF^{Skp2} substrates Geminin and Cdt1, which accumulate in G1 and S/G2/M, respectively.²²⁸ Thus, fusing Cdt1 or Geminin fragments to monomeric Kusabira Orange 2 (mKO2) or monomeric Azami Green (mAG) converted the reciprocal nuclear accumulation of orange and green fluorescence into a high-contrast indicator of cell cycle dynamics powerful enough to enable the in vivo monitoring of cell cycle progression.^{226,228,727} (Figure 6A). Fucci has also enabled detailed examinations of the relationships between the cell cycle and processes such as transcriptional activity⁷²⁸ and mitochondrial dynamics.⁷²⁹ Meanwhile, several modifications and improvements to Fucci have also been reported to further extend its utility. For instance, Sakaue-Sawano et al. improved the color contrast of Fucci by replacing mKO2 and mAG with mCherry and mVenus, yielding Fucci2²²⁵, and more recently developed an alternative indicator system, Fucci(CA), that utilizes changes in the activities of the CUL4^{Dp1} and APC^{Cdh1} E3

ligases to indicate cell cycle transitions during interphase (i.e., G1, S, and G2).²²⁰ Notably, Bajar and colleagues also recently reported the construction of Fucci4, which utilizes the periodic degradation of 4 spectrally distinct FP-tagged proteins to monitor each individual phase of the cell cycle²²¹ (Figure 6B).

Several cell-based indicator systems have also been developed that couple the detection of signaling molecules secreted by one cell to a FRET-based biosensor response in a second cell, thereby providing spatiotemporal information on the dynamics of intercellular rather than intracellular signaling, particularly among neurons. For example, Sato et al.⁶⁰³ developed a reporter system for detecting secreted nitric oxide (NO) based on PK15 cells that stably express the cGMP sensor CGY.³²⁷ Importantly, PK15 cells endogenously express soluble guanylate cyclase (sGC), which selectively generates cGMP in response to NO binding.⁷³⁰ Thus, sGC couples NO detection to the CGY FRET response, with the resulting Piccel (“PK15 indicator cell”) system able to detect picomolar NO release by cocultured hippocampal neurons.⁶⁰³ Using a similar principle, Nakajima and co-workers also developed a “BDNF sensor cell” (Bescell) indicator system that couples BDNF binding by a chimeric receptor tyrosine kinase (BDNF-binding domain fused to EGFR kinase domain) with a FRET-based EGFR activity reporter²⁹⁵ (Figure 6C). Likewise, taking advantage of GPCR coupling to Gαq-containing G proteins, which activate PLC to trigger IP₃ production and ER Ca²⁺ release, Nguyen and colleagues developed a similar approach to visualize endogenous neurotransmitter release using “cell-based neurotransmitter fluorescent engineered reporters” (CNiFERs).⁵³⁰ This system, which utilizes HEK293 cells stably expressing a given metabotropic neurotransmitter receptor along with the high-performance GECI TN-XXL,⁴⁰⁸ has been used to image the in vivo dynamics of cholinergic, dopaminergic, and noradrenergic signaling in mouse brains^{530,532} (Figure 6D). Thus, the modularity of these designs holds the potential to illuminate the spatiotemporal dynamics of a broad range of intercellular signals.

2.4. New Biosensor Classes

Years of tireless innovation have spawned a verdant landscape of genetically encoded fluorescent biosensors capable of visualizing the spatiotemporal dynamics of a diverse and expansive, though by no means exhaustive, array of biochemical, biophysical, and cellular phenomena. Meanwhile, new biosensor designs continue to emerge, often based on novel reporting strategies or biosensor configurations, that not only fill gaps in the existing toolkit but also open up entirely new avenues for exploring previously unanswerable biological questions, thus promising to redefine the reach of these powerful molecular tools.

2.4.1. Sensors Based on Infrared Fluorescent Proteins. Decades of engineering have yielded GFP-like FPs in nearly every color of the rainbow.⁷ Notably absent, however, have been variants that excite and emit between 650 and 900 nm (i.e., the so-called near-infrared [NIR] window⁷³¹), wavelengths that are preferable for both cellular and whole-body in vivo fluorescence imaging for the reasons enumerated above (e.g., autofluorescence, phototoxicity, absorbance, and scattering). Yet multiple light-absorbing proteins are present in nature that derive their chromophores from the covalent incorporation of endogenous cellular cofactors,⁷³² and several bacterial phytochromes (BphPs), which utilize the heme catabolic intermediate biliverdin (BV) as their chromophore,

have been demonstrated to emit photons in the 600 to 700 nm range.^{733–735} BphPs are multidomain photoreceptors that transduce light into signaling activity,⁷³⁶ and Shu and colleagues were able to successfully engineer the first infrared fluorescent protein (IFP) based on a pared-down version of the *Deinococcus radiodurans* BphP containing just the chromophore-binding GAF and PAS domains.⁷³⁷

Intriguingly, the unique topology and chromophore-binding interactions of IFP are enabling the development of novel biosensors strategies that are not feasible using GFP-like FPs. For example, although numerous genetically encoded fluorescent protease sensors have been developed over the years (see sections 2.3.3.2.2.1 and 2.3.3.2.4), no approach has succeeded in producing a high-contrast, fluorogenic biosensor, wherein the cleaved sensor gains fluorescence. Thus, To and colleagues set out to use IFP as an alternative scaffold for constructing such a reporter.⁵⁶⁸ In BphP, and thus IFP, chromophore formation occurs through the insertion of BV into a binding pocket within the GAF domain, followed by the autocatalytic formation of a thioether bond with a conserved cysteine residue.^{736,738} Noting the proximity of this residue to the BV binding pocket in IFP, To et al. devised a way to control IFP chromophore formation by modulating the distance between the binding pocket and catalytic cysteine within a circularly permuted IFP (cpIFP).⁵⁶⁸ The original N- and C-termini of IFP were truncated and tethered using a short linker containing a protease cleavage site to physically constrain the catalytic cysteine such that chromophore formation only occurs following cleavage of the linker (Figure 7). Meanwhile, to ensure that cpIFP remained intact following

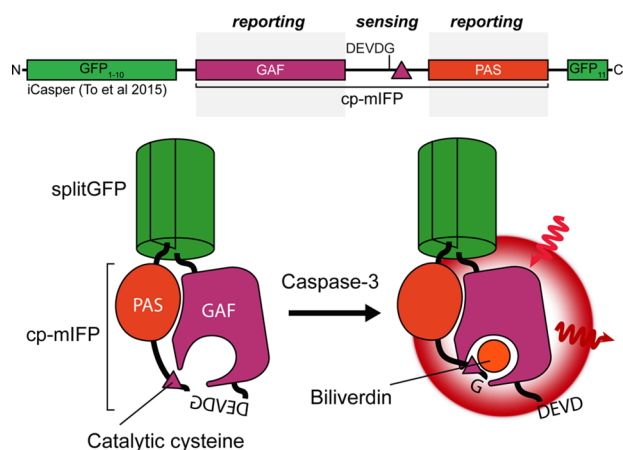


Figure 7. Infrared FP-based caspase-3 reporter, iCasper. The introduction of the caspase-3 cleavage sequence into the circularly permuted mIFP prevents the incorporation of BV into the GAF domain, but cleavage by caspase-3 liberates the catalytic cysteine to promote the incorporation of BV and the formation of the chromophore.⁵⁶⁸

cleavage, splitGFP was added to the new termini introduced between the PAS and GAF domains. This approach yielded a fluorogenic infrared fluorescent caspase reporter (iCasper) for monitoring caspase-3 activity, which allowed the authors to highlight apoptotic cells during morphogenesis and tumor development in *Drosophila*.⁵⁶⁸

One potential concern regarding the wider use of BphP-derived IFPs is their somewhat poor quantum yield and low brightness, which may be further exacerbated by low BV concentrations in certain organisms and cell types.^{739,740}

Nevertheless, the development of IFP has ignited a flurry of efforts to develop other alternative FPs, some of which display comparable brightness to that of EGFP.²²² These advances promise to yield additional novel biosensor designs beyond those currently possible.

2.4.2. Biochemical Activity Integrators As in Vivo Snapshot Reporters. The idea of a “snapshot” reporter was originally conceived as a way to get around the daunting challenge of performing three-dimensional whole-brain imaging of neuronal activity in order to delineate causal relationships between the firing of specific neuronal subsets and specific behaviors or stimuli (i.e., brain activity mapping).⁷⁴¹ Thus, instead of continually reporting the dynamics of neuronal activity (e.g., Ca^{2+} elevations), the snapshot reporter integrates, or “memorizes”, neuronal activity over an arbitrarily defined period of time by generating a stable signal only in the presence of both activity (e.g., Ca^{2+}) and an external trigger such as light, thereby enabling detailed, retrospective analyses of neuronal firing patterns.

Recently, Fosque and colleagues were able to develop one such snapshot reporter using the single-FP Ca^{2+} indicator GCaMP as a template.³⁷⁹ Specifically, based on a previous report detailing the construction of GR-GECO, a single-FP GECI whose fluorescence emission switches color from green to red upon illumination with 400 nm light,³⁹⁵ they set out to similarly engineer a photoconvertible GECI, albeit one whose photoconversion efficiency was modulated by the Ca^{2+} -dependent molecular switch, such that the change in emission wavelength occurs only in response to both Ca^{2+} and 400 nm light. This was achieved by incorporating a circularly permuted variant of the green-to-red photoconvertible FP mEos2⁷⁴² into the GCaMP backbone in place of cpEGFP, after which molecular evolution was performed to generate CaMPARI, a calcium-modulated photoactivatable ratiometric integrator³⁷⁹ (Figure 8). During 400 nm illumination, the green fluorescent signal from CaMPARI rapidly changes color to red in proportion to the concentration of Ca^{2+} ; thus, the ratio of red-to-green fluorescence intensity provides a permanent snapshot of neuronal activity during the recording period established by the length of illumination.³⁷⁹ Zolnik and co-workers also recently demonstrated that the photoconversion rate of CaMPARI can be adjusted by modulating the intensity of 400 nm illumination, thereby tuning the sensitivity of CaMPARI to different thresholds of neuronal activity.⁷⁴³ Importantly, CaMPARI also behaves as a traditional single-FP GECI, with the intensities of both the green- and red-fluorescent states decreasing strongly in response to Ca^{2+} ,³⁷⁹ thereby providing a crucial internal control for any possible effect that the photoconversion light itself may have on the system under investigation.

Alternatively, a pair of studies^{365,366} published within the past year have reported the development of snapshot reporters that hew more closely to the original design first articulated by Roger Tsien.⁷⁴¹ In this scheme, the extrinsic (e.g., light) and intrinsic (e.g., Ca^{2+}) signals are integrated to drive a transcriptional reporter that permanently labels active neurons with high spatiotemporal resolution for post hoc analysis. Thus, although differing somewhat in their particulars, both the Cal-Light system designed by Lee and colleagues³⁶⁶ and the “fast light- and activity-regulated expression” (FLARE) system devised by Wang and co-workers³⁶⁵ feature a three-component, dual-molecular-switch design that functions as an “AND gate” to control the light- and Ca^{2+} -dependent release of

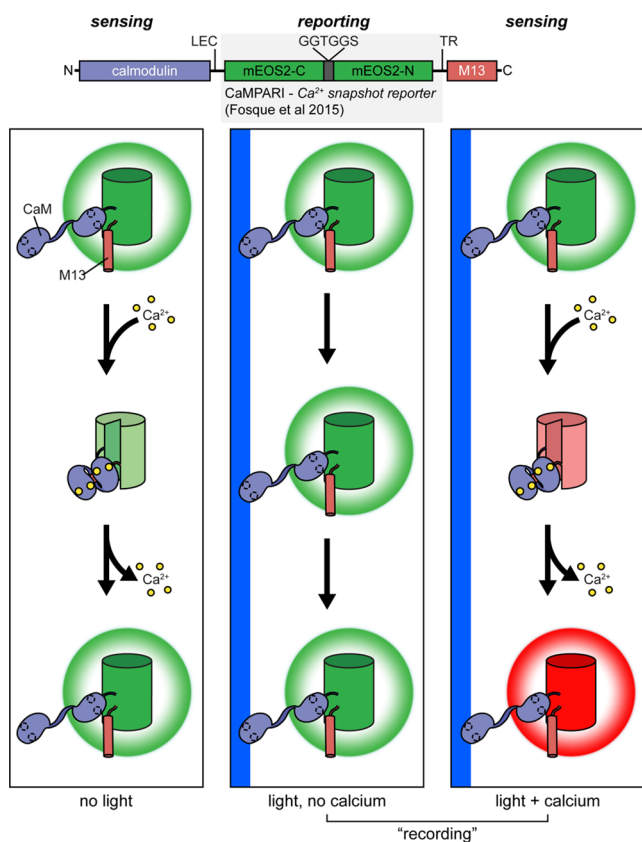


Figure 8. Photoconversion-based snapshot reporter of Ca^{2+} . CaMPARI acts similarly to other green single color Ca^{2+} biosensors where the green fluorescence intensity is dependent on the Ca^{2+} concentration (left), except it can also “record” the presence of high Ca^{2+} during a snapshot in time. When the CaMPARI biosensor is illuminated with blue/violet light and the intracellular Ca^{2+} is high, the biosensor will irreversibly convert to a red (right) Ca^{2+} biosensor.³⁷⁹

a membrane-tethered transcription factor, namely, the tetracycline-inducible transcriptional activator (tTA) (Figure 9). First, these designs utilize a light-dependent molecular switch composed of the light-oxygen-voltage (LOV) domain and C-terminal $\text{J}\alpha$ -helical extension ($\text{J}\alpha$) from *Avena sativa* phototropin, wherein blue-light absorption by the flavin-containing LOV domain causes the C-terminal $\text{J}\alpha$ helix to unwind,⁷⁴⁴ which can be used to unmask a specific peptide sequence in response illumination,⁷⁴⁵ in this case a cleavage site for the tobacco etch virus protease (TEVp). Meanwhile, the second, Ca^{2+} -dependent molecular switch utilizes calmodulin binding to the M13 peptide to modulate cleavage of the TEVp substrate, either by controlling the fragment complementation of N- and C-terminal portions of TEVp³⁶⁶ or by simply recruiting TEVp to the cleavage site.³⁶⁵ Hence, only the combined presence of both light (to define the recording window) and Ca^{2+} (in the form of neuronal activity) will permit cleavage of the TEVp substrate and release of the tethered tTA domain to drive the expression of a reporter construct in the nucleus (Figure 9).

Much like CaMPARI, both of these methods enabled active neurons to be specifically and selectively labeled with a fluorescent signal (i.e., FP expression) within the span of the illumination window.^{365,366} Importantly, however, a key advantage of these transcription-based readouts is that any

transgene can be placed under the control of the snapshot reporter, thereby facilitating precise functional manipulation of labeled neurons. For instance, the coexpression of a channelrhodopsin was used to permit the targeted inhibition³⁶⁶ or reactivation³⁶⁵ of neurons that had been fluorescently labeled by the snapshot reporter, an ability that is crucial for identifying those neurons whose firing bears a true causal link with a given behavioral response.⁷⁴¹ Furthermore, Lee and colleagues also developed a similar system for identifying and manipulating neurons that respond to specific neuromodulators by replacing the calmodulin-M13 switch with one based on GPCR- β arr binding,⁵⁷⁸ highlighting the ability of these modular designs to be reconfigured as snapshot reporters for other biochemical events, with the potential for numerous applications beyond neurobiology.

2.4.3. Fluctuation-Based Biosensors. The biochemical pathways that mediate intracellular signaling are increasingly understood to operate within an exquisitely controlled spatial framework, or activity architecture, that is established through various modes of compartmentalization (discussed in section 4). Because much of this compartmentalization is ultimately governed on a molecular scale, such as through the assembly of multiprotein signalosomes, genetically encoded fluorescent biosensors capable of visualizing and localizing biochemical activities on a comparable spatial scale are extremely desirable. That being said, a number of super-resolution imaging modalities have been developed that rely on FP photochromism, or the ability of certain FPs to stochastically and reversibly switch between a bright, fluorescent state and a dark, nonfluorescent state under specific illumination conditions (reviewed in ref 25). FP-based biosensors engineered to modulate this photochromic behavior in response to a biochemical input thus represent one potential strategy for imaging the precise locations of biochemical activities.

Photochromism is thought to involve the disruption of molecular contacts between the FP chromophore and the surrounding β -can, leading to increased conformational flexibility and decreased fluorescence emission.²⁵ In addition to exhibiting controlled on/off switching, photochromic FPs also display stochastic intensity fluctuations (i.e., blinking), and Mo et al. recently found that close proximity between the photochromic green FP Dronpa⁷⁴⁶ and the red FP TagRFP-T¹⁶⁵ induced significant fluctuations in TagRFP-T fluorescence intensity.⁴⁷⁷ These intensity fluctuations, which were specifically induced by Dronpa proximity, did not require the Dronpa chromophore but were dependent on surface residues in the Dronpa β -can, suggesting that physical contact with Dronpa deforms the TagRFP-T β -can and directly increases its intrinsic²⁰⁷ blinking behavior. Further analysis revealed that this phenomenon, termed “fluorescence fluctuation increase by contact” (FLINC), was sensitive to the intermolecular distance between Dronpa and TagRFP-T within the range of 5–6 nm, thus lending itself to the construction of a novel class of proximity-based biosensors compatible with super-resolution imaging.⁴⁷⁷ Using FRET-based biosensors as a template, Mo and colleagues substituted the FRET FP pair in AKAR3EV⁴⁴¹ with Dronpa and TagRFP-T (Figure 10). When combined with the fluctuation-based super-resolution imaging approach pcSOFI,²⁷ the resulting FLINC-AKAR1 sensor was able to map dynamic changes in PKA activity with nanometer precision, facilitating the detailed dissection of PKA compartmentalization⁴⁷⁷ (see additional discussion in section

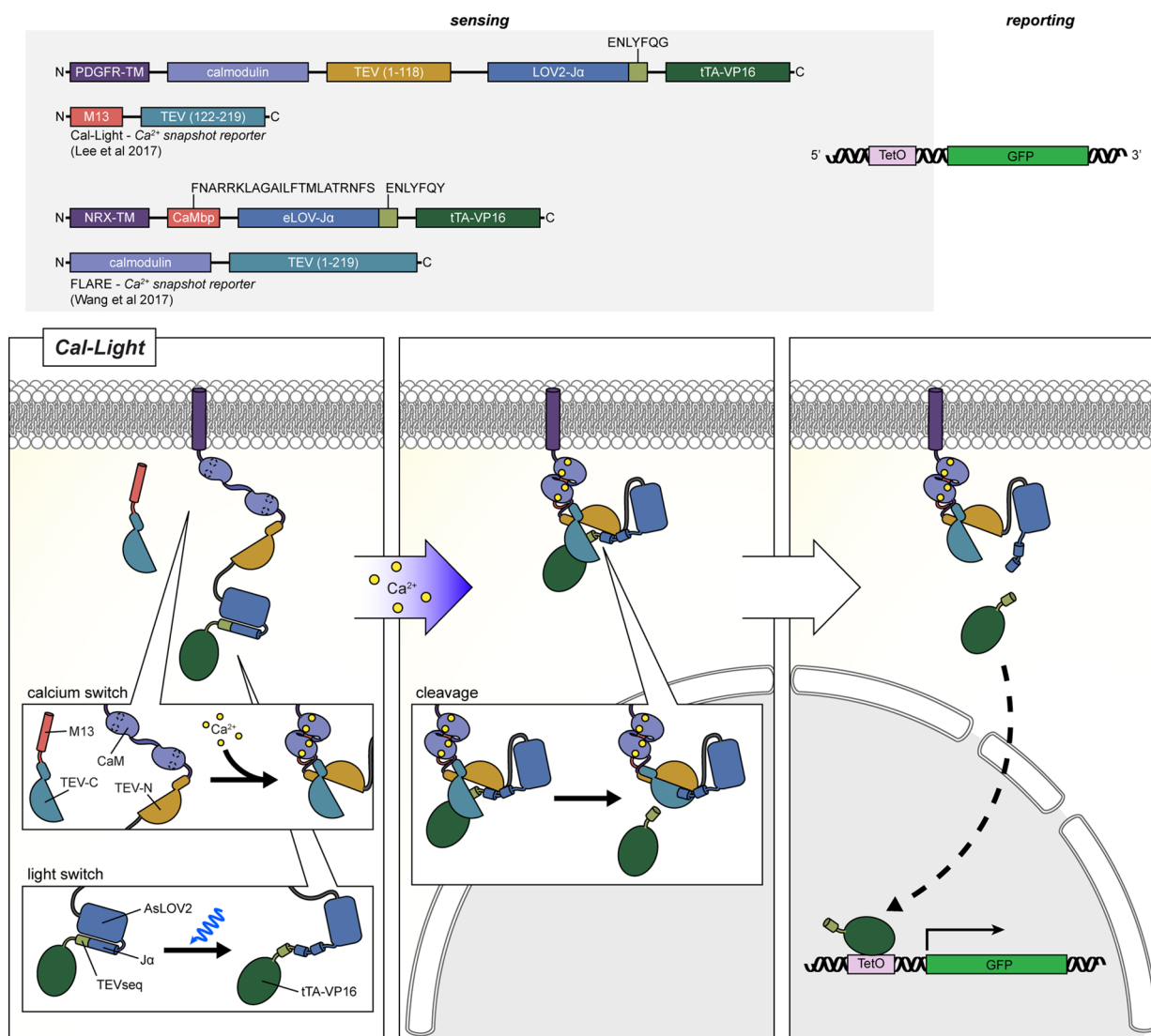


Figure 9. Transcription-based snapshot reporters. The Cal-Light and FLARE Ca^{2+} biosensors create an “AND gate” design by combining the optogenetically controlled AsLOV2 domain with a Ca^{2+} switch controlled split-TEV protease, which leads to the cleavage of the tTA-VP16 transcriptional activator only in the presence of high Ca^{2+} and blue light.^{365,366} The cleavage of the transcriptional activator will then turn on the expression of a reporter gene such as GFP.

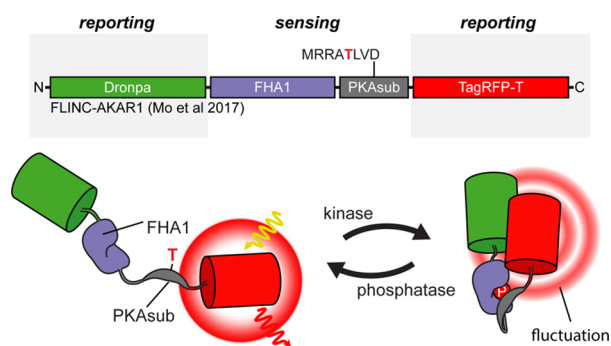


Figure 10. Fluctuation-based PKA biosensor FLINC-AKARI. Fluorescence of TagRFP-T is modulated by its interaction with Dronpa through a process termed FLINC, such that when in close proximity to Dronpa, TagRFP-T exhibits increased fluorescence fluctuation. This enables this biosensor to report PKA activity at a subdiffraction spatial resolution⁴⁷⁷ through the use of the super-resolution technique pcSOFI.²⁷

4.3.1). These initial studies hint at the potentially transformative impact of this nascent biosensing strategy.

Genetically encoded fluorescent biosensors have evolved into a remarkably diverse set of molecular tools capable of probing a broad range of cellular states and biochemical processes. In the following sections, we delve further into the application of genetically encoded fluorescent biosensors by focusing our attention on how this technology has been leveraged to extract detailed information on the temporal and spatial regulation of the intracellular biochemical machinery and thereby elucidate the molecular logic underlying signal transduction networks.

3. OBTAINING TEMPORAL INFORMATION FROM BIOSENSORS

The ability of cells to dynamically respond to a vast array of environmental changes and modes of cellular communication has arisen from the evolution of a highly connected network of fairly simple chemical reactions.⁷⁴⁷ Characterizing the dynamics of these biochemical pathways is therefore essential

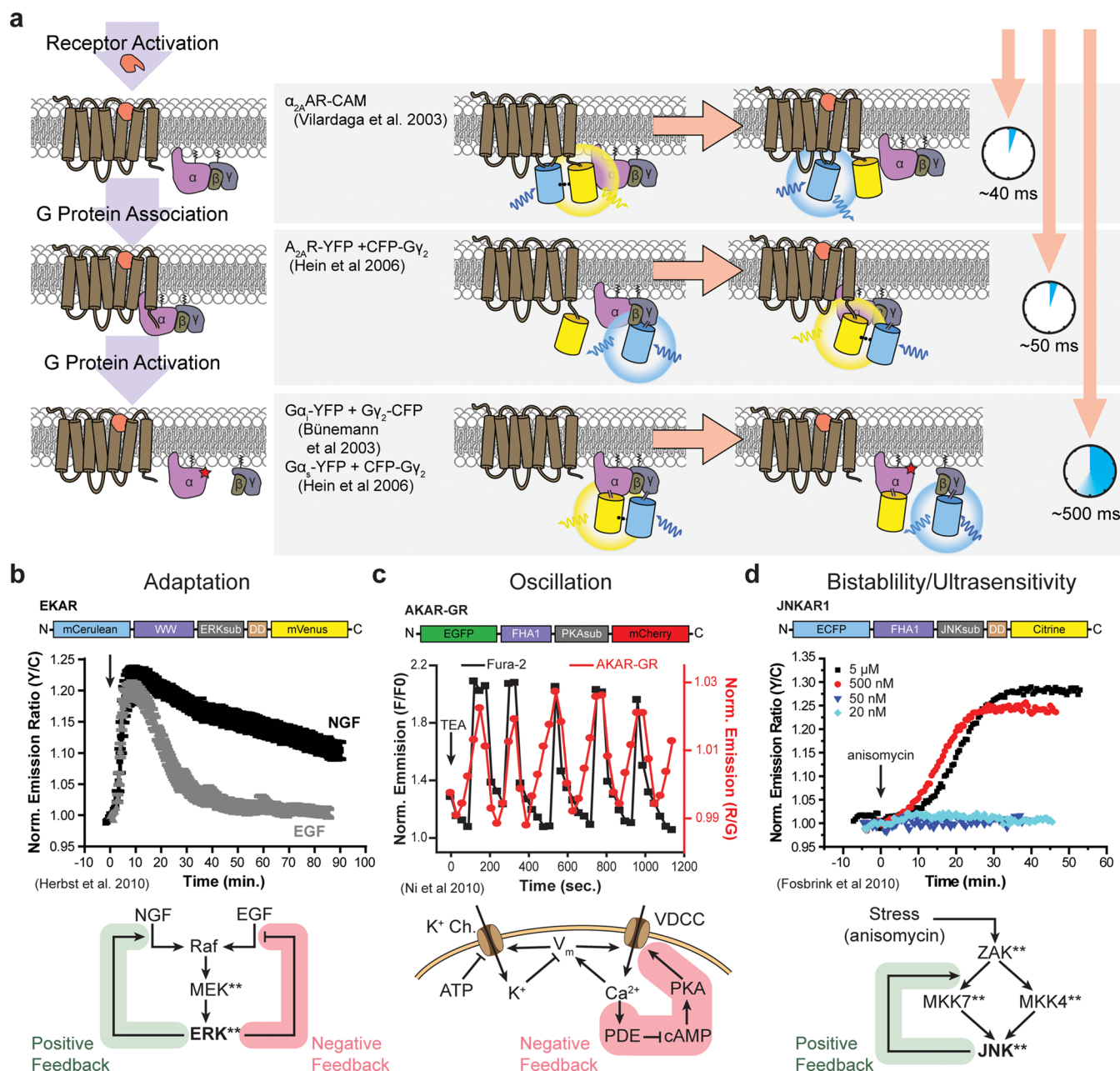


Figure 11. Temporal dynamics quantified by fluorescent biosensors. (A) The kinetics of GPCR signaling. First, the receptor undergoes a conformational change in response to ligand binding, which was observed to occur with a time constant on the order of 40 ms in the α_2 -Adrenergic Receptor using the biosensor α_2 -AR-CAM.⁵⁸⁸ Next, the heterotrimeric G protein associates with the activated receptor. A_{2A} Adenosine Receptor, $A_{2A}R$, fused with YFP at the C-terminal tail and CFP-labeled $G_{\gamma 2}$ exhibited an increase in FRET upon stimulation with adenosine with a time constant of approximately 50 ms.⁵⁸⁴ Finally, the receptor stimulates the exchange of GTP for GDP on the G_{α} subunit, thus activating G_{α} and promoting the dissociation of G_{α} from $G_{\beta\gamma}$. Biosensors consisting of a CFP-labeled G_{γ} subunit and either $G_{\alpha i}$ ⁵⁸¹ or $G_{\alpha s}$ ⁵⁸⁴ fused to YFP showed activation time constants on the order of 500 ms. (B) Adaptation is the return of a signaling pathway toward its previous state while under continued stimulation. PC12 cells expressing the ERK biosensor EKAR show an adaptive response to EGF stimulation (gray lines) but a much more sustained response to NGF stimulation (black lines), adapted with permission from ref 754. Copyright 2011 American Society for Microbiology. Negative feedback by ERK onto Raf activation is hypothesized to lead to a transient response, whereas positive feedback in the NGF signaling pathway is hypothesized to lead to bistability.⁷⁵⁵ (C) Oscillations are the regular or semiregular cycling between activity/concentration states, as can be seen in the TEA-induced Ca^{2+} and PKA activity oscillations in MIN6 cells. These oscillations were observed through the simultaneous measurement of Ca^{2+} concentration with the Ca^{2+} dye Fura-2 (black line) and a Green/Red variant of AKAR, GR-AKAR, (red line) at the single-cell level in MIN6 cells.³⁰⁸ While the inhibition of K^+ channels by ATP and the interplay between voltage and Ca^{2+} are the primary driver of the Ca^{2+} oscillations, the negative feedback of Ca^{2+} through cAMP and PKA is hypothesized to strengthen these oscillations and tune their frequencies. Adapted with permission from ref 308. Copyright 2011 Springer Nature. (D) Bistability and ultrasensitivity describe phenomena wherein a signaling pathway is insensitive to stimulation below a certain threshold dose but responds in a switchlike fashion to superthreshold stimuli. HeLa cells expressing the JNK biosensor JNKAR1 did not respond to anisomycin at concentrations of 20 (cyan $\blacklozenge$), or 50 (blue $\blacktriangledown$) nM, but exhibited a strong response to 500 nM (red $\bullet$) and 5 μ M (black $\blacksquare$). Adapted with permission from ref 490. Copyright 2010 National Academy

Figure 11. continued

of Sciences. In addition to the multistep activation along the JNK signaling pathway, positive feedback by JNK onto the activation of upstream regulator MKK7 is hypothesized to contribute to the ultrasensitive nature of activation by anisomycin.⁷⁵⁶

for determining how signaling networks are organized and understanding how cells process external stimuli and make decisions about their fate. Traditionally, quantifying the kinetics of signaling reactions has relied on the use of various *in vitro* methods such as Western blotting or enzymatic assays. Although these methods remain important tools for studying intracellular signaling, genetically encoded fluorescent biosensors offer a number of advantages that make them instrumental for elucidating the temporal dynamics of signaling networks. In particular, because they are genetically encoded, these biosensors can be used to quantitatively track the kinetics of signaling molecules within the native cellular environment. Furthermore, thanks to the rapid, nanosecond time scale of fluorescence, the relatively high brightness of FPs, and the sensitivity of modern digital imaging equipment, the signals from fluorescent biosensors can be monitored with a much higher temporal resolution than is generally achievable through other *in vitro* methods. As such, fluorescent biosensors are powerful tools for studying the dynamics of signaling networks across a wide range of time scales. Below, we discuss the types of temporal dynamics that have been observed in cellular signaling and how biosensors have been instrumental in studying these dynamics.

3.1. Kinetics of Individual Reactions

Signaling networks are principally driven by changes in the concentrations of signaling molecules and the reaction rates of signaling enzymes. The most foundational understanding of temporal dynamics can be achieved by identifying the activation and catalysis rates for signal transduction. Historically, these have been studied in purified systems in an *in vitro* setting. While these assays are able to provide detailed and accurate measurements of the reaction rates of a specific signaling enzyme, they lack the ability to evaluate the dynamics and kinetics of all of the preceding steps that lead to the activation of that enzyme and any other regulatory mechanisms occurring within the cell. Genetically encoded fluorescent biosensors are able to quantify the kinetics of signal transduction in real time in a living cell. Quantifying these kinetics is the first step to understanding how the connected enzymatic reactions within a network can create the highly dynamic and complex signaling responses seen in nature.

One example of fluorescent biosensors being used to study the activation of signaling proteins has been in the study of GPCR activation kinetics. GPCRs are one of the most drug-targeted protein families,⁷⁴⁸ therefore quantifying the kinetics of their activation is of interest for both understanding their physiological function and evaluating the effects of different drugs. GPCRs consist of a seven-transmembrane receptor that couples to a heterotrimeric G protein comprising α , β , and γ subunits.⁷⁴⁹ Ligand binding by the receptor causes a conformational change that promotes the activation of the $G\alpha$ subunit, which is primarily responsible for downstream signaling, and is canonically considered to dissociate from the $G\beta\gamma$ subunits.⁷⁴⁹ There have been several fluorescent probes developed to quantify the kinetics of different aspects of GPCR activation (Figure 11A). First, the activation of the receptor was quantified by inserting CFP and YFP into the third

intracellular loop and intracellular tail, respectively, of the PTHR and α_{2A} AR.⁵⁸⁸ These biosensors showed that the kinetics of the conformational change within the receptor could vary significantly, where PTHR is activated much more slowly than α_{2A} AR, with time constants of 1 s and less than 40 ms, respectively.⁵⁸⁸ After this conformational change occurs, the receptor can then couple with its cognate heterotrimeric G proteins. Quantification of the kinetics up through this step was done by labeling $G\gamma$ with CFP and inserting YFP into the intracellular tail of the adenosine receptor A_{2A} ($A_{2A}R$) and β_1 -adrenergic receptor (β_1 -AR).⁵⁸⁴ This association between the receptor and G proteins occurred rapidly where these biosensors both exhibited a time constant near 50 ms.⁵⁸⁴ Finally, this association of the heterotrimeric G protein with the receptor stimulates the exchange of GDP for GTP on the $G\alpha$ subunit and dissociation of $G\alpha$ from the $G\beta\gamma$ subunits. Biosensors to study this final step were developed by fusing a $G\alpha$ subunit and a $G\beta$ or γ subunit with YFP and CFP, respectively.^{581,584} These studies showed that the activation of the $G\alpha$ subunit was most likely to be rate limiting, where $G\alpha_{i1}$ showed a $t_{1/2}$ of approximately 700 ms in response to an α_{2A} adrenergic agonist⁵⁸¹ and $G\alpha_s$ exhibited a time constant near 500 ms in response to $A_{2A}R$ and β_1 -AR agonists.⁵⁸⁴ The responses from these biosensors were measured at frequencies on the order of 20–75 Hz, which was essential to be able to quantify the kinetics of these rapid inter- and intramolecular changes. Given that $G\alpha$, $G\beta$, and $G\gamma$ comprise 23, 5, and 12 different isoforms, respectively, exhaustively testing all of these combinations may be infeasible, but genetically encoded fluorescent biosensors make it possible to test specific combinations.⁵⁸⁰ For example, Gibson and Gilman examined the differences between $G\alpha_i$ isoforms 1, 2, and 3 and $G\beta$ isoforms 1, 2, and 4, showing that $G\alpha_{i1}$ exhibited the greatest FRET response to α_2 -adrenergic receptor stimulation when coupled with $G\beta_1$ or $G\beta_4$ subunits but not $G\beta_2$.⁵⁸⁰ This type of work can provide evidence of subunit isoform preference or specificity and help understand how cell-type-specific isoform expression can affect the response of GPCRs.⁷⁵⁰ Similarly, the use of receptor-specific agonists/antagonists with these biosensors can reveal differences in the strength and rate of activation of different G protein isoforms for each receptor.^{579,582,584} Since GPCRs are the first step in many signaling pathways, understanding the kinetics of their activation can help identify rate-limiting steps in a signaling pathway and evaluate potential mechanisms of pharmacological perturbation. These biosensors exemplify the benefits of using genetically encoded fluorescent proteins to study the kinetics of activation because they are able to examine the dynamics of specific proteins within the cellular context at a high temporal resolution.

In addition to being able to examine the activation of proteins and protein complexes, many biosensors have been developed to evaluate the kinetics of signaling enzyme catalysis. Downstream of the $G\alpha_s$ - and $G\alpha_i$ -mediated regulation of cAMP production is the prototypical kinase PKA. PKA is a tetramer consisting of two regulatory subunits and two catalytic subunits, where binding of cAMP to the regulatory subunits unleashes the catalytic subunit from

inhibition by the regulatory subunit. The temporal dynamics of PKA have been the focus of many studies due to its importance in several physiological processes and its utilization of scaffold proteins to coordinate spatiotemporal dynamics.^{751,752} The development of AKARs (section 2.3.3.2.2.2) enabled more detailed and specific studies of these signaling dynamics.⁵⁰⁸ First, AKAR1 was able to show the different temporal kinetics of stimulating PKA activation either directly through the activation of ACs by forskolin or indirectly through the activation of the G_{α_s} linked β_2 -adrenergic receptor agonist isoproterenol. The temporal resolution of this early biosensor was a great improvement over what was possible through previous *in vitro* kinetic assays. Furthermore, biosensors have played an important role in evaluating the effects of signalosomes, coordinated complexes of interconnected signaling proteins, on enzyme kinetics. For example, A-kinase anchoring proteins (AKAPs), a family of scaffold proteins that bind PKA, coordinate the interaction of PKA with downstream substrates and upstream regulators of PKA activity, thus affecting both PKA activity and the kinetics of phosphorylation by PKA.⁷⁵³ This effect was directly observed by genetically fusing a PKA regulatory subunit directly to AKAR1, thus mimicking a scaffolded enzyme–substrate complex, which resulted in a nearly 2-fold faster response.⁵⁰⁸ In addition to modulating the temporal dynamics of signal transduction, scaffold proteins control the spatial architecture of signal transduction by anchoring signaling proteins to specific subcellular microdomains. The effects of scaffold proteins on both spatial and temporal dynamics, and the role of biosensors in studying them is discussed further in section 4.3. The capability of fluorescent biosensors to measure specific enzymatic activities within the cellular environment at a high temporal resolution has made them powerful tools for quantifying the kinetics of signaling protein activation and enzyme catalysis.

3.2. Higher-Order Signaling Dynamics

The balance between second messenger production and degradation and signaling enzyme activation and inactivation within signaling networks enable and define the complex dynamics observed within cells. The highly connected nature of signaling networks means that a very diverse array of dynamics can be observed, but these dynamics can be understood in terms of a small set of basic building blocks of signaling dynamics that are defined by how the network is connected.⁷⁵⁷ Furthermore, the organization of the network connections can be grouped into similar topologies, which are called signaling motifs.⁷⁵⁸ Here, we will briefly introduce some of the more common signaling dynamics observed and some network motifs that lead to them, but there are several detailed reviews discussing the details of signaling network topology and their effects on signaling dynamics.^{757–759}

3.2.1. Adaptive Responses. One common signaling dynamic observed is what is referred to as adaptation or a transient response. Adaptation can be defined as the resetting of a cellular signaling level to its previous state after stimulation⁷⁶⁰ (Figure 11B). This type of signaling dynamic can be important for cells to be able to respond to changes in stimulation levels instead of the absolute level of the signal. Previous work has identified that negative feedback loops, where the activation of a downstream signaling enzyme will lead to the inhibition of one or more of its upstream regulators, and incoherent feed forward loops, where an upstream

signaling enzyme activates two different pathways that converge on a downstream effector but have opposing effects,⁷⁶⁰ are key motifs involved in signaling adaptation. While these network motifs are needed for transient signaling dynamics, their existence alone does not guarantee that adaptive signaling will occur; in fact, negative feedback can lead to oscillatory dynamics instead.

Adaptation is by definition a short-lived event which has been found to be susceptible to cell-to-cell variability; therefore, fluorescent biosensors have been critical to observing and understanding these transient dynamics. While these biosensors measure the kinetics of individual reactions or second messenger concentrations, the ability to observe how a given signaling network regulates signaling dynamics over time can provide insights into how the networks architecture can shape the observed response. For example, genetically encoded fluorescent biosensors have been instrumental in identifying how different stimuli can lead to either sustained or adaptive dynamics of extracellular-signal regulated kinase (ERK) signaling. These temporal dynamics are functionally important because the duration of ERK activity has been shown to regulate different cell-fate outcomes in the neuroendocrine PC12 cell line.^{496,755} Earlier *in vitro* methods measuring ERK activity in response to EGF or NGF had observed that stimulation with EGF resulted in transient ERK activity, whereas NGF stimulation induced a sustained response.^{761,762} These differences in ERK temporal dynamics also correlated with the fact that EGF promoted proliferation while NGF led to PC12 cell differentiation.^{761,762} The desire to obtain a more detailed temporal understanding motivated the development of fluorescent biosensors of both ERK activation (e.g., Mui2)⁴⁷⁸ and ERK activity, such as EKAR and subsequent improved variants⁴⁷⁴ (Table 1). When directly comparing the dynamics of ERK activity in response to EGF and NGF stimulation in PC12 cells, Herbst and colleagues were able to use EKAR to accurately calculate a $t_{1/2}$ for ERK phosphorylation reversal that not only agreed with previous studies (Figure 11B) but also revealed that PKA activity modulated the adaptive signaling dynamics of ERK.⁵⁰³ Later work by Ryu et al., using an improved ERK biosensor, EKAR2G,⁴⁷³ examined the cell-to-cell variability of ERK dynamics in response to EGF and NGF.⁷⁵⁵ They observed that while the population averages showed a more transient response to EGF than with NGF, there was significant variability between cells. This variability convolutes what uniquely regulates these two signaling cascades, but they found that using a short pulse of growth factor stimulation, instead of sustained stimulation, yielded more uniform responses for both EGF and NGF. Using this experimental design, they observed that the EKAR2G response to low-dose NGF stimulation exhibited similar transient responses as low-dose EGF stimulation. But at high doses of NGF, a subset of cells exhibited a sustained response that was not observed with high dose EGF stimulation, suggesting that the NGF signaling pathway may exhibit bistability, wherein high doses of NGF lead to a switchlike, instead of adaptive, behavior (bistability discussed more in section 3.2.3). This evaluation of cell-to-cell variability is only possible through the use of fluorescent biosensors because these quantitative measurements of signal transduction can be performed at the single-cell level. Furthermore, this detailed analysis was coupled with a computational model of ERK signaling, which demonstrated that repeated pulses of either EGF or NGF could be used to approximate either a transient or

sustained response with much less variability across the population. Experimental evaluation of this predicted effect found that pulse frequencies of growth factor stimulation that create a more “sustained-like” ERK response led to more differentiation of PC12 cells, more so for NGF but also observed with EGF. This agreed with earlier work that found that both continuous and pulsatile activation of ERK, using an optogenetic stimulation independent of growth factor, increased PC12 differentiation.⁷⁶³ More work will still be needed to identify how these cells are able to integrate the temporal differences between a transient and sustained response into cell fate and gene expression profiles. Additionally, the complexity of these dynamics and the high temporal resolution of fluorescent biosensors has made ERK signaling a prime candidate for interrogation using computational models, which is discussed in section 5.2.1.

3.2.2. Oscillations. Oscillatory signaling dynamics can be defined as a regular or semiregular variation in the signaling response (Figure 11C). Oscillations are physiologically critical for several functions such as heart rhythm, insulin secretion, and cell cycle timing.⁷⁶⁴ For oscillations to arise, the signaling network must have both negative feedback and delay in the negative feedback.⁷⁶⁵ One of the most common oscillatory dynamics observed has been in intracellular Ca^{2+} concentrations. These oscillations are driven by the ion channels that form a complex network of feedback mechanisms that can experience a delay due to their dependence on membrane potential or ion concentration.⁷⁶⁶ While these dynamics generally fall under electrophysiological signaling, enzymes and second messengers can be both required for oscillations and regulate the rate of oscillation.^{308,767} But oscillatory dynamics are not exclusive to Ca^{2+} signaling. For example, nuclear localization of the transcriptional regulator NF- κ B has been shown to oscillate due to negative feedback caused by NF- κ B promoting the expression of its inhibitor, I κ B.⁷⁶⁸ In this system, the time required for both the nuclear translocation of NF- κ B and its induction of I κ B expression causes a delay between NF- κ B activation and feedback inhibition, leading to the oscillatory behavior. Understanding the different mechanisms that can lead to and modulate oscillatory dynamics has been of intense interest for several fields.

Oscillatory signaling dynamics are complex and may be difficult to observe without the use of fluorescent biosensors due to the temporal and single-cell resolution that may be required. For example, cardiac Ca^{2+} oscillations that regulate the rhythmic contraction of the heart are moderately fast (1–10 Hz depending on species) and homogeneous across a population when under pacing.⁷⁶⁹ Other physiological systems that exhibit spontaneous or asynchronous Ca^{2+} oscillations across a cell population rely more heavily on single-cell measurements of dynamics.^{770,771} While fluorescent dyes such as Fura-2 are commonly used to study Ca^{2+} dynamics, GECIs can offer selectivity at the cellular and subcellular level that is generally not possible using cell-permeable synthetic dyes. For example, GECIs have been expressed in specific neuronal subtypes (e.g., pyramidal neurons)¹⁶² through the use of cell-specific promoters and have been targeted to specific microdomains through the use of targeting motifs.⁴¹⁰

One particularly interesting system that relies on oscillatory signaling is the pulsatile release of insulin by pancreatic β -cells in response to glucose stimulation. Early studies using Ca^{2+} indicator dyes revealed that the elevation of glucose led to slow Ca^{2+} oscillations, with insulin release occurring in a similarly

oscillatory fashion.⁷⁷² The high temporal resolution of fluorescent biosensors makes it possible to study the kinetics of this oscillatory behavior, such as the frequency and magnitude of the oscillations.⁷⁷² These tools helped determine that the primary mechanism responsible for triggering these Ca^{2+} oscillations is the increase in the ATP/ADP ratio, due to glycolysis. This increase in ATP in turn inhibits ATP-sensitive potassium channels, leading to oscillatory cellular depolarization and Ca^{2+} influx.⁷⁷³ While Ca^{2+} oscillations are considered the master regulator of pulsatile insulin secretion, fluorescent biosensors for ATP, glycolysis, cAMP, and PKA have shown that these signaling pathways also oscillate in β -cells and can modulate Ca^{2+} dynamics.^{308,519,774,775} Furthermore, multiplexed imaging using fluorescent biosensors has made it possible to observe how oscillations in different signaling molecules are temporally related and helps elucidate the crosstalk between pathways (multiplexing discussed further in section 6.2). Temporal differences between two oscillating signals are described by their phase shift, which is the temporal shift between the waveforms of the two signals. Signals that increase and decrease at the same time are “in phase”, whereas signals that oscillate at the same time but in opposite directions are “out of phase”. In the MIN6 β -cell line, simultaneous measurement of Ca^{2+} oscillations with PKA activity or cAMP concentrations using fluorescent biosensors showed that PKA and cAMP oscillate out of phase with Ca^{2+} ^{308,775} (Figure 11C). Similarly, simultaneous measurement of ATP and Ca^{2+} also showed out-of-phase oscillations.⁷⁷⁴ Both of these findings are interesting because both ATP and PKA have been shown to play a role in the formation of Ca^{2+} oscillations, but these regulatory mechanisms are not static. Furthermore, simultaneous measurement with high temporal resolution allows the identification of which signaling mechanism happens first, providing insights into possible causal relationships that lead to the oscillations. For example, upon glucose stimulation, ATP increases before Ca^{2+} , suggesting that the increase in ATP causes the closure of the K_{ATP} channel and subsequently leads to cellular depolarization.⁷⁷⁴ Meanwhile, cross-correlation analysis of the subsequent ATP oscillations showed that Ca^{2+} began to increase before ATP levels began to drop, indicating that while the increase in ATP may be responsible for initiating Ca^{2+} oscillations, the subsequent ATP oscillations may in fact be driven by Ca^{2+} oscillations.⁷⁷⁴ Similar analyses have been performed with faster oscillations (on the order of seconds) in other cell types. For example, the IP_3 indicator IRIS1 showed that IP_3 increases prior to Ca^{2+} depolarization caused by glutamate stimulation of metabotropic glutamate receptor 5a (mGluR5a) in HeLa cells expressing exogenous mGluR5a.⁵⁴⁶ These studies highlight the importance of single-cell and high temporal resolution afforded by fluorescent biosensors in decoding the mechanisms and regulation of oscillatory signaling dynamics.

3.2.3. Bistability and Ultrasensitivity. The final types of signaling dynamics that we will discuss are the related dynamics of bistability and ultrasensitivity. Bistability generally describes a system that primarily exists in either of two almost discrete states, creating an “all-or-nothing” response to stimulation. Ultrasensitivity, on the other hand, refers to a signaling network that exhibits a “switchlike” response, where low levels of stimulation do not illicit a response, but after a certain threshold, the signaling response is very strong. Usually, this is observed as a dose response curve that has a hill coefficient greater than 1.⁷⁷⁶ Ultrasensitivity in signaling

networks can be important for signaling pathways that may be critical for a response to stimuli that is resistant to noise, such as proliferation or apoptosis. While bistability is a broader term and usually requires ultrasensitivity in the signaling network,⁷⁷⁷ for the purposes of this review, these phenomena will be discussed interchangeably. There are several signaling network factors that can lead to ultrasensitivity, which can make it difficult to identify precisely which elements of a signaling network are responsible.⁷⁷⁸ Two common mechanisms that can lead to the switchlike behavior of an ultrasensitive response are positive feedback, in which a downstream enzyme promotes the activation of its upstream regulators, and multistep activation, wherein an enzyme requires activation via two or more independent steps.^{776–780} One example of an ultrasensitive signaling pathway is MAPK signaling, which contains positive feedback loops and involves a multistep activation process that requires phosphorylation at two separate sites.^{781,782} The ultrasensitive nature of some MAPK signaling pathways is critical to ensuring that commitment to cell fate decisions or apoptotic programs is not only resistant to errant initiation by noise but also robustly induced when required.^{781,783} Again, the signaling network topology alone is not sufficient to identify ultrasensitivity a priori,⁷⁷⁸ but the ability to study these dynamics at the single cell-level using fluorescent biosensors makes it possible to dissect the key factors that regulate these dynamics.

JNK is a MAPK that is activated by cytokines and environmental stressors and regulates apoptosis.⁷⁸⁴ Early work in *Xenopus* oocytes had shown, using a very labor-intensive process, that the JNK response to stimulation was a bistable system that exhibited all-or-none responses at the cellular level but appeared to produce a graded response at the population level due to cell-to-cell variability.⁷⁸⁵ The development of a genetically encoded biosensor for monitoring JNK activity dynamics, JNKAR1, greatly improved the ability to study these heterogeneous cell responses in many different cell types.⁴⁹⁰ For example, exposing JNKAR1-expressing HeLa cells to a range of concentrations of the fungal antibiotic anisomycin, which induces ribotoxic stress and leads to the activation of JNK, revealed that cells either maximally responded to superthreshold concentrations or failed to respond at all to subthreshold concentrations.⁴⁹⁰ (Figure 11D). These data showed that the JNK response to anisomycin has a hill coefficient greater than 9 and thus represents an ultrasensitive response, which conceptually makes sense for a cellular response that regulates apoptosis.⁴⁹⁰ Similar to the regulation of apoptosis by JNK, the regulation of cell division by Aurora B kinase has also been shown to exhibit bistability.⁷⁸⁶ Aurora B kinase activity has been shown to be critical for progression through cytokinesis and exhibits highly dynamic localization throughout cell division.⁷⁸⁷ Intriguingly, multiplexed imaging of an Aurora B kinase biosensor targeted to chromatin along with mCherry-labeled Aurora B kinase showed that during anaphase, Aurora B kinase was highly concentrated at centromeres, whereas the area of high Aurora B kinase phosphorylation extended much farther.⁷⁸⁶ Using in vitro experiments and computational models, Zaytsev et al. determined that this larger area of influence was due to bistability in the activation of Aurora B kinase. This bistability arises due to Aurora B kinase activation occurring mainly through trans-phosphorylation by other Aurora B kinase molecules, while deactivation by dephosphorylation occurs slowly due to a relatively low concentration of phosphatases.

Therefore, the relocalization of Aurora B kinase at the centromere allows efficient activation due to the high concentration of Aurora B kinase, which remain active longer even when they diffuse away from the centromere, leading to a larger area of phosphorylation. Finally, recently published work by Yapo and colleagues observed, using AKAR2,⁵⁰⁷ that the nuclear PKA response in striatal neurons stimulated with a dopamine-like agonist exhibited an ultrasensitive response.⁷⁸⁸ They developed a computational model to examine potential mechanisms that may underlie this ultrasensitivity which suggested two possible feed-forward loops could be responsible: PKA activity promotes its translocation into the nucleus and PKA phosphorylation induced inhibition of nuclear phosphatases. All of these studies rely on the power of fluorescent biosensors to quantify signaling dynamics at the single-cell level in order to evaluate the bistability or ultrasensitivity of a signaling pathway that may have otherwise been hidden in population averages.

3.3. Importance of Single-Cell Readouts

Because fluorescent biosensors are able to quantify signaling dynamics in both time and space, they are able to resolve the dynamic behaviors of individual cells. This property is integral for studying dynamics that are heterogeneous across cell populations and thus difficult to detect using population-based methods such as Western blotting. For example, the MIN6 oscillations discussed earlier are largely asynchronous, and the ability to simultaneously measure Ca^{2+} and the kinetics of other signaling proteins at the single-cell level using fluorescent biosensors was essential for understanding their dynamics.^{308,519,774,775} Indeed, single-cell resolution is often essential for revealing the true dynamics of a signaling pathway that would otherwise be obscured by cell-to-cell variability at the population level, such as ultrasensitive signaling.^{490,786,789} Meanwhile, single-cell studies can also reveal distinct behaviors among cell subsets, such as in recent work by Aoki et al., who used the FRET-based biosensor EKAREV to reveal the presence of stochastic pulses of ERK activity within individual cells, which were found to have a paracrine activating effect on neighboring cells.⁷⁹⁰ Even in cases such as studies of the cell cycle, where it is possible to synchronize cells to the same point in the cell cycle, the rate at which each cell progresses through the cycle will nevertheless exhibit significant cell-to-cell variability, and quantification of signaling kinetics at the single-cell level may still be required. Therefore, a genetically encodable biosensor was used to measure Cyclin B1-CDK1 activity through the cell cycle and elucidate cell-to-cell variation during this process.^{436,462} Studying these processes at the single-cell level thus enables a greater understanding of signaling networks than can be achieved through more traditional means.

3.4. How Fast Can We Go?

In most of the examples discussed thus far, a temporal resolution on the order of seconds was adequate to resolve the dynamics of interest. However, numerous signaling processes are known to exhibit very rapid kinetics, such as GPCR activation^{579,582,584} and neuronal depolarization spikes,^{164,273,402} and a much finer time resolution is thus required in order to faithfully capture the dynamics of these processes. For example, the biosensors developed to study GPCR activation rates, Ca^{2+} dynamics, and membrane potential are able to achieve temporal resolutions on the order of 100 ms, 1.5 ms, and <1 ms, respectively.^{273,402,579}

When trying to push fluorescent biosensors to their temporal limit, there are three main factors to consider: the time it takes to collect enough photons, the time it takes for the signal to alter the sensing unit, and the time it takes for the readout mechanism to respond.

The number of photons emitted by a fluorophore is a function of both the molecular brightness, which is a measure of how readily an excitation photon is absorbed and how efficiently that absorbed photon is converted to an emitted photon, and the excitation light intensity.⁷⁹¹ Although photon gathering is not the limiting factor in most cases when the fluorescent biosensors are overexpressed, in some cases use of the newest and brightest FP variants (e.g., mTurquoise2, mNeonGreen, mScarlet) could increase the signal and enhance temporal resolution.^{579,792,793} A complicating factor is photostability. If the fluorophore is particularly susceptible to light-induced damage, photon gathering could become a limiting factor which in turn limits the temporal resolution when a significant number of fluorophores are lost due to photobleaching. For instance, photostability limited the temporal resolution of the recently developed FLINC super-resolution biosensors where the continuous excitation required for super-resolution imaging causes reversible photobleaching.⁴⁷⁷ Thus, to continue measuring biosensor activity over time, periods of darkness were included between acquisition periods to allow the FPs to recover.

Another consideration for increasing temporal resolution is how quickly the biosensor responds to signaling changes. The use of GECIs as tools to study Ca^{2+} dynamics across a diverse array of biological settings has led to several different designs and variants that have been optimized toward specific research goals, such as high Ca^{2+} sensitivity to observe small changes in Ca^{2+} or fast biosensor responses to Ca^{2+} changes to observe individual action potentials (see section 2.3.3.1.1). While it would be ideal to have a GECI that is both highly sensitive and responds quickly, it has been observed several times that there exists a trade-off between having fast Ca^{2+} binding kinetics and having a high biosensor sensitivity.^{164,389,794} For example, among the recently published GCaMP variants, the fast variants GCaMP6f and GCaMP3fast had the higher dissociation constants, 375 nM and 2800 nM, respectively, whereas the slower but brighter GCaMP6s and GCaMP3bright had K_d values of 144 nM and 930 nM, respectively.^{164,389} Through a detailed analysis of the GCaMP kinetics, Helassa et al. observed the binding of the third and fourth Ca^{2+} to CaM to be much slower than the binding of the first two Ca^{2+} ions, and in their GCaMP3fast variant, where the third EF hand of CaM was mutated to disable its Ca^{2+} binding, the kinetics of binding the third (and final) Ca^{2+} were much faster.³⁸⁹ Interestingly, their kinetic analysis showed that the conversion of the calmodulin-peptide bound sensor from dark to fluorescent was one of the slower steps in the activation process. Similar kinetic analysis of the FRET- and troponin-C-based Ca^{2+} biosensor TN-L15, which only binds one Ca^{2+} ion, found that an intermediate between the calcium binding and high FRET state is required, suggesting that the conformational change in response to Ca^{2+} binding plays a significant role in the biosensor kinetics.⁷⁹⁵ These kinetics are important considerations for these engineered biosensors as they can affect biosensor design and could be optimized. But for biosensors studying activation using native protein fusions, such as the biosensors of GPCR activation that are comprised of a fluorescently labeled $G\alpha_i$ and $G\beta$ or $G\gamma$, the kinetics of the

conformational change upon activation are precisely what is being measured.⁵⁷⁹

Finally, the time it takes to couple the change in the reporting unit to a change in the readout method can impact the kinetics of fluorescent biosensors. Most unimolecular biosensors are based on an intramolecular conformational change, which has a time-scale ranging from nanoseconds to milliseconds.⁷⁹⁶ Bimolecular biosensors that fully dissociate can be slower than unimolecular biosensors due to diffusion, which can add delays on the order of milliseconds to seconds, but it is still possible to have fast bimolecular biosensors, as the bimolecular GPCR activation biosensors have been used at 100 ms temporal resolution.^{579,797} Translocation-based biosensors, such as the KTRs,¹¹⁶ will be affected by the barriers of diffusion and efficiency of nuclear import and export systems because the readout involves translocation from the nucleus to the cytosol. This is not to say, however, that these types of biosensors are less useful, because they can be used to study signal transduction that occurs on slower time scales (e.g., minutes).

4. OBTAINING SPATIAL INFORMATION FROM BIOSENSORS

Along with providing a detailed view of the kinetics of intracellular signaling, genetically encoded fluorescent biosensors have been essential in dissecting the organization of signaling pathways at the spatial level. Yet in contrast to the obvious role of temporal dynamics in biochemical reaction networks, early frameworks of intracellular signal transduction had no inkling of spatial regulation. The molecular components of signaling pathways were instead thought to be freely diffusible and to move unhindered throughout the cytoplasm (or plasma membrane, in the case of receptors), which logically entails the random, homogeneous distribution and uniform, nonselective activation of signaling molecules within the intracellular space. However, early studies of cAMP/PKA signaling in isolated rat hearts found that only $\beta_2\text{AR}$ stimulation, and not prostaglandin E_1 (PGE_1) stimulation, was able to affect cardiac contractility and alter glycogen metabolism, despite both stimuli eliciting similar elevations in cAMP accumulation and PKA activity.^{798,799} Studies had also revealed that a substantial portion of predominantly type II PKA holoenzyme was associated with the particulate fraction in heart lysates,⁸⁰⁰ and subsequent work found that $\beta_2\text{AR}$ stimulation was able to activate this particulate PKA fraction, whereas PGE_1 stimulation was not.^{801,802} These results were incompatible with a simple model of freely diffusing messenger and enzyme and suggested that some other process must underlie the ability of hormones to selectively affect specific targets within the same cell.

As a potential solution, the idea of compartmentalized signaling, which postulates that signaling molecules are not randomly distributed within cells but are instead sequestered within different spatial compartments, was proposed to account for these findings (reviewed in ref 803). However, despite early evidence hinting at compartmentalization, the precise nature of signaling compartments and their underlying molecular mechanisms remained a mystery until the development of advanced optical tools, such as genetically encoded fluorescent biosensors. These tools enabled the nondestructive observation of signaling events within living cells, which preserves the native molecular environment. In addition, fluorescence imaging offers submicron spatial resolution,

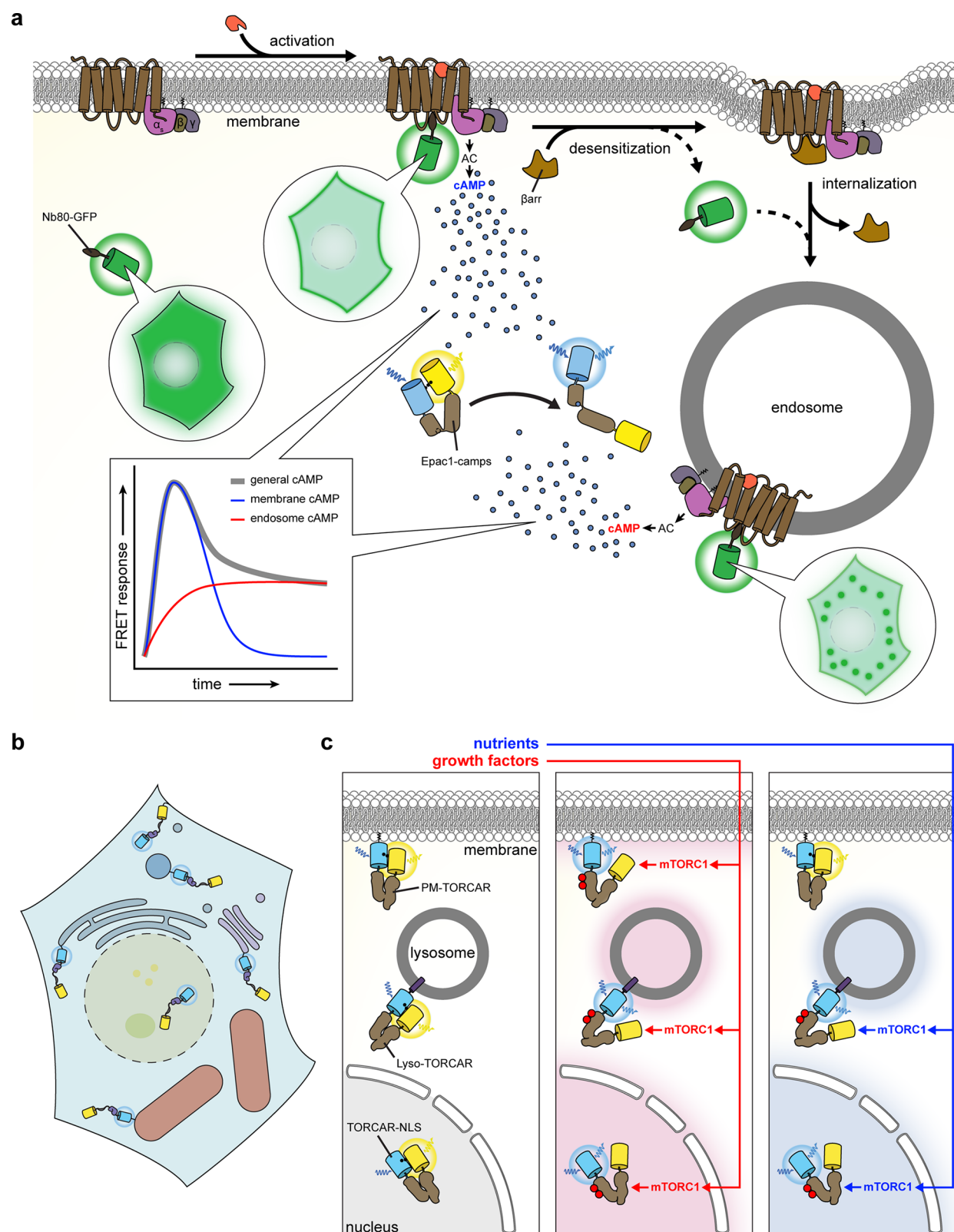


Figure 12. Probing spatial compartmentalization with fluorescent biosensors. (A) GPCRs are activated at the plasma membrane but can continue to stimulate downstream signaling after internalization. Activation of β_2 AR at the plasma membrane by an extracellular ligand leads to the production of cAMP. This activation of β_2 AR can be monitored both directly, by GFP labeled nanobody Nb80 which binds the active conformation of β_2 AR,¹⁰⁹ and indirectly, through biosensors of cAMP concentration such as Epac1-camps.⁸¹² The activation of GPCRs is reversed by the binding of β -arrestin (β arr) which attenuates downstream signaling and promotes receptor trafficking to clathrin-coated pits for endocytosis. After internalization, β arr can dissociate from the receptor and some GPCRs have been shown to then continue downstream signaling from the endosome, as shown by Nb80-GFP translocation to the endosomes¹⁰⁹ and monitoring of cAMP production after perturbing internalization or blocking the membrane pool of the receptors by using membrane-impermeable antagonists.⁵⁸⁵ (B) The fusion of targeting domains to biosensors

Figure 12. continued

enables the measurement of the signaling dynamics in several different subcellular microdomains by promoting the trafficking and localization of the biosensor to these regions. (C) For example, Zhou et al. targeted TORCAR to the plasma membrane (PM-TORCAR), lysosome (Lys-TORCAR), and nucleus (TORCAR-NLS) to examine the mTORC1 activity in each of these microdomains.⁴³³ With these targeted biosensors, it was shown that growth factor stimulation leads to mTORC1 activation in all three microdomains, whereas stimulation with nutrients activated mTORC1 at the lysosome and nucleus but not at the plasma membrane.⁴³³

which in some cases can enable the direct visualization of distinct spatial signaling domains, while further spatial selectivity can be achieved by targeting genetically encoded biosensors to specific subcellular locations, either through the incorporation of various targeting motifs or through direct fusion to a protein of interest. Thus, genetically encoded biosensors are well-suited for directly unraveling the molecular basis of compartmentalized signaling.

Key to achieving compartmentalized signaling is controlling diffusion, which is a major obstacle to signaling specificity. For example, given measured rates of molecular diffusion within the cytosol, signaling molecules are in theory capable of traversing the entire length of a cell on the order of seconds.^{804,805} Thus, any messenger produced or enzyme activated within one subcellular region would not remain there for long, thereby blurring out otherwise local signaling events. Conversely, because diffusion scales with the square-root of time, long-distance signaling processes, such as axonal transmission, would occur extremely slowly if left to diffusion alone. In addition, signaling molecules must efficiently engage in productive interactions and reactions to ensure accurate information flow. Yet cells contain hundreds of different signaling molecules, which are estimated to account for up to 10% of total cellular proteins,^{804,806} and signaling pathways typically involve multiple steps and comprise numerous components. Thus, selectivity becomes increasingly unfeasible if signaling molecules are left free to diffuse and interact randomly. Cells must therefore accomplish three things to lay the foundation for compartmentalized signaling: limit diffusion to preserve local signaling events, augment diffusion to ensure rapid, efficient long-distance signaling, and selectively promote useful interactions while preventing unwanted ones. In the following section, we will discuss how biosensor-based imaging approaches have shed light on the major mechanisms utilized by cells to regulate the diffusion of signaling molecules: (1) physical separation by membranes, (2) confined activation/deactivation by pathway regulators, and (3) association with macromolecular complexes. Through these processes, cells establish the boundaries that define signaling compartments and organize signaling pathways into various spatially regulated domains.

4.1. Membrane-Associated Signaling Compartments

4.1.1. Partitioning by Membranes. Membranes exist to divide: the plasma membrane divides cells from their external environment, while intracellular membranes further divide cells into distinct, functionally specialized structures (i.e., organelles). Thus, cellular membranes provide ready-made platforms for compartmentalizing the actions of signaling molecules, and genetically encoded biosensors have yielded numerous important insights into the specific local signaling environments associated with membrane-bound compartments.

A number of studies over the past several years, for instance, have called into question the established model of GPCR signaling. In particular, so-called “canonical” GPCR signaling,

in which receptors regulate the production of second messengers such as cAMP through coupling to heterotrimeric G proteins, has long been thought to occur exclusively at the plasma membrane and terminate when receptors are bound by β arr and undergo clathrin-mediated endocytosis. Internalized receptors are subsequently thought to engage only in “non-canonical” signaling, such as β arr-dependent MAPK signaling.^{807–809} However, along with recent evidence challenging the role of β arr in noncanonical GPCR signaling,⁸¹⁰ several biosensors-based imaging studies have revealed that internalized GPCRs remain active and continue to stimulate cAMP production, directly contradicting the classical model of canonical GPCR signaling (Figure 12A).

In an early study using primary thyroid cells derived from transgenic mice expressing Epac1-camps,²⁹⁹ Calebiro et al. set out to investigate cAMP signaling by the thyroid stimulating hormone receptor (TSHR) and found that cAMP production became increasingly sustained in cells treated with longer pulses of TSH.⁸¹¹ This transition to sustained cAMP signaling paralleled TSHR internalization, suggesting that these kinetic differences were linked to different spatial compartments. Indeed, colocalization studies confirmed the presence of the cAMP signaling apparatus (e.g., *Gas*, ACs) on the same intracellular membrane compartments as internalized TSHR. Furthermore, disrupting receptor internalization led to the loss of sustained cAMP signaling in response to longer TSH pulses, indicating that internalized TSHR continues to promote cAMP signaling from intracellular compartments (e.g., endosomes).⁸¹¹ A parallel study by Ferrandon and colleagues reported similar findings regarding PTHR signaling in HEK293 cells, suggesting the possibility of a more general phenomenon.⁸¹² Here, the authors used GPCR activation sensors in addition to the Epac1-camps FRET sensor and observed that stimulating cells with PTH1-34 led to prolonged receptor activation and cAMP production. As above, this sustained cAMP production was observed to coincide with the internalization of GFP-tagged PTHR and was blocked by disrupting PTHR internalization.⁸¹²

On the basis of these initial reports, Irannejad and colleagues then sought to use a different approach to directly probe the activation state of internalized GPCRs. In this case, the authors transfected HEK293 cells with a GFP-labeled nanobody (Nb80)¹⁰⁴ that specifically recognizes the active conformation of the β_2 AR.¹⁰⁹ As expected, the initially cytosolic fluorescence signal from Nb80 was observed to translocate to the plasma membrane upon β_2 AR stimulation using isoproterenol.¹⁰⁹ However, Nb80 was subsequently observed to relocate to intracellular puncta containing β_2 AR, indicating the presence of active receptor on the endosomal surface (Figure 12A). In contrast, a recent study by Thomsen and colleagues using biosensors to monitor both cAMP production and GPCR activation found that the vasopressin type 2 receptor (V_2 R), but not the β_2 AR, induced sustained cAMP signaling due to persistent activation of internalized receptors.⁵⁸⁵ V_2 R, a class B GPCR,^{813,814} strongly associates with β arr, and studies of a

hybrid receptor in which the β_2 AR C-terminal tail was replaced with that of V_2 R (i.e., β_2V_2 R) have revealed β arr to adopt a so-called “tail” conformation wherein only the receptor tail is bound by β arr, leaving the transmembrane receptor core exposed.⁸¹⁵ Indeed, using both colocalization and BRET-based PPI assays, Thomsen et al. were able to demonstrate the formation of an internalized GPCR/G protein/ β arr “megaplex” by both β_2V_2 R and V_2 R, suggesting a potential molecular mechanism for persistent signaling by internalized GPCRs.⁵⁸⁵

These studies establish a new conceptual framework in which internalized GPCRs are a source of sustained cAMP production within the cytosol, raising the question as to the functional significance of such internal signaling. One intriguing possibility is that cAMP generated at endosomes specifically activates PKA within intracellular compartments, such as the nucleus. Indeed, PKA is known to regulate a number of processes in the nucleus, including gene expression^{816,817} and RNA splicing,^{818–820} and although the cAMP-induced dissociation and diffusion of free PKA C subunit is classically viewed as the sole source of nuclear PKA activity,^{816,817,821,822} a growing body of evidence indicates the existence of a resident pool of PKA holoenzyme within the nucleus.^{823–828} Consistent with this model, Jean-Alphonse et al. used nuclear-targeted Epac1-camps and AKAR3 to reveal that endosomal cAMP production via synergistic β_2 AR/PTHR activation led not only to higher nuclear cAMP compared with either stimulus alone but also to the rapid induction of nuclear PKA activity by a nuclear-resident pool of PKA holoenzyme.⁸¹ These results also confirm an earlier study in which nuclear-targeted cAMP and PKA sensors, combined with local manipulation of cAMP production by targeted soluble adenylyl cyclase, demonstrated that cAMP produced within the cytosol or nucleus could specifically and rapidly activate a nuclear pool of PKA, whereas cAMP generated at the plasma membrane could not.⁸²⁷ β_2 AR/PTHR-induced endosomal cAMP production was also required to induce CREB phosphorylation and to promote mineralization in ROS17/2.8 cells, thus underscoring the functional importance of internal cAMP production.⁸¹ Similarly, Godbole and colleagues, using cAMP and PKA biosensors targeted to either the plasma or Golgi membrane, found that TSHR internalization led to Golgi-localized cAMP/PKA signaling and was important for inducing CREB phosphorylation.¹¹⁰

As alluded to above, this ability to selectively target genetically encoded fluorescent biosensors to different subcellular compartments is instrumental in directly revealing the distinct signaling activities associated with intracellular membranes and in elucidating the mechanisms that shape these unique spatial signaling domains (Figure 12B). In a classic example, Gallegos et al. used targeted versions of CKAR to monitor local PKC activity at the plasma membrane, mitochondrial membrane, Golgi membrane, nucleus, and cytoplasm.⁸²⁹ Stimulation with phorbol ester, a DAG mimic that directly activates PKC, revealed that each of these subcellular regions was characterized by distinct PKC activity profiles, which were found to be caused by differences in basal PKC activity or the action of local phosphatases.⁸²⁹ Interestingly, the strongest PKC responses were observed on the Golgi membrane, which also displayed sustained PKC activity in response to Gq stimulation, in contrast to transient plasma membrane and cytosolic PKC activity. Further investigation using a translocation-based DAG reporter demonstrated that this sustained activity was directly

correlated with sustained DAG accumulation in the Golgi membrane,⁸²⁹ and a follow-up study using targeted FRET sensors for both PKC and PKD revealed that prolonged DAG production on the Golgi surface is mediated by Ca^{2+} .⁸³⁰

Similarly, a recent study using CaNAR targeted to different subcellular compartments revealed spatial differences in the regulation of CaN activity in response to membrane depolarization-induced cytosolic Ca^{2+} oscillations in pancreatic β -cells.⁴⁵⁹ In particular, cytosolic CaN activity displayed an integrating response to cytosolic Ca^{2+} oscillations, wherein each Ca^{2+} peak produced a steplike increase in CaN activity, while CaN activity located at the ER surface displayed an oscillatory pattern, rising and falling in tandem with cytosolic Ca^{2+} concentrations. Ultimately, a detailed mechanistic investigation aided by additional targeted sensors found that these spatial differences in CaN activity were the result of differential subcellular access to free Ca^{2+} /CaM,⁴⁵⁹ which, despite its reputation as a ubiquitous signaling effector, has been shown to be a somewhat limited resource inside cells.^{668,831} This study benefited from the application of a second-generation CaNAR variant with an improved dynamic range, which is a critical determinant of probe sensitivity. Despite its clear usefulness in probing compartmentalized signaling, subcellular targeting can sometimes negatively impact the dynamic range of a biosensor, and continued improvements to increase dynamic range are therefore essential for detecting local signaling activity and revealing the spatial regulation of signaling pathways by subcellular compartments. For instance, Miyamoto and colleagues recently took advantage of an improved FRET-based AMPK sensor to investigate subcellular AMPK activity.⁸³² Interestingly, while these authors detected AMPK activity throughout the cell, AMPK activity at different subcellular compartments was found to be associated with distinct isoforms of the AMPK catalytic subunit.⁸³² The use of a bimolecular FRET-based AMPK sensor to increase sensitivity at the plasma membrane also allowed Depry et al. to characterize the bidirectional regulation of membrane-localized AMPK activity by PKA.⁴⁴⁴ Meanwhile, the cAMP sensor CUTie was designed for the express purpose of preserving dynamic range upon subcellular targeting in order to study localized signaling events.³⁰¹

The ongoing development of genetically encoded fluorescent biosensors for detecting new signaling activities also continues to expand our ability to study the spatial regulation of signaling by intracellular compartments. For example, the serine/threonine protein kinase mTORC1 serves as a critical signaling hub overseeing the regulation of cellular metabolism and energy status in response to various extracellular cues.⁸³³ Although mTORC1 has previously been reported to associate with numerous intracellular compartments,⁸³⁴ the precise role of spatial compartmentalization in regulating mTORC1 signaling is unclear. To begin addressing this question, Zhou and colleagues recently developed a first-generation FRET-based reporter for monitoring mTORC1 kinase activity in living cells.⁴³³ This probe successfully detected local mTORC1 activity when targeted to different intracellular compartments, including the nucleus, which has been somewhat controversial as a site of mTORC1 activity. The authors also found that while growth factor stimulation elicited mTORC1 activity throughout the cell, amino acid (e.g., nutrient) stimulation appeared to selectively promote lysosomal and nuclear mTORC1 signaling⁴³³ (Figure 12C). This more confined activity profile may indicate that the proposed role of nutrient

sensing in establishing baseline mTORC1 activity⁸³³ is achieved through spatial compartmentalization of the mTORC1 signaling machinery, thus providing important insights into the regulation of mTORC1 signaling by membrane compartments.

4.1.2. Partitioning within Membranes. Cells are also thought to achieve an additional layer of spatial compartmentalization within the plasma membrane via the selective partitioning of various signaling proteins into membrane microdomains, nanometer-scale subdivisions of the plasma membrane that are composed of distinct subsets of lipids and other membrane constituents.⁸³⁵ In particular, cholesterol- and sphingolipid-enriched “lipid rafts” are strongly implicated in organizing membrane proteins into signaling platforms.⁸³⁶ Yet because their small size places them below the diffraction limit of optical microscopy, membrane microdomains are difficult to directly observe and characterize in living cells, rendering their very existence and function the subject of ongoing debate.^{835,837,838} As such, genetically encoded fluorescent biosensors have provided a crucial window into the key biological roles of these subdomains. For example, through the use of a membrane-targeted cAMP biosensor, DiPilato and colleagues observed that disrupting lipid rafts via cholesterol depletion led to enhanced β_2 AR-induced cAMP production,³⁰⁹ while Depry et al. observed a similar effect of raft disruption on PKA activity,⁵⁰⁴ suggesting a negative effect of raft localization on the cAMP/PKA signaling machinery. Even further insights can be gained by virtue of the fact that biosensors can be targeted not just to the plasma membrane generally but also to specific membrane microdomains.¹⁶⁰ Thus, by comparing the responses of raft- and nonraft-targeted AKAR4, Depry and colleagues found that although the β_2 AR-stimulated PKA response in lipid rafts was lower, lipid rafts displayed significantly higher resting PKA activity compared with the bulk plasma membrane.⁵⁰⁴

By similarly comparing the responses of other genetically encoded fluorescent biosensors targeted to different membrane microdomains, researchers have further been able to illuminate key molecular differences that give rise to the distinct signaling behaviors observed in these subcompartments. For example, in a series of studies using genetically encoded FRET-based biosensors to study the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, Gao and co-workers demonstrated that platelet-derived growth factor (PDGF) stimulation leads to higher Akt activity in lipid rafts due to the increased localization and activation of the upstream kinase PDK1 within rafts compared with the bulk plasma membrane, whereas the negative regulator PTEN was preferentially located outside rafts.^{434,499} The activity of focal adhesion kinase (FAK) was also shown to be higher in lipid rafts compared with nonraft regions in response to either PDGF stimulation or cell adhesion.⁴⁸⁰ Interestingly, Seong et al. found that PDGF-induced raft FAK activity was regulated by Src, which was also reported to be more active in lipid rafts,⁸³⁹ whereas adhesion-induced FAK activation in lipid rafts was conversely required to promote Src activity.⁴⁸⁰ This difference in the relationship between raft-localized FAK and Src activity in response to PDGF or cell adhesion signaling may be related to recent observations that PDGFR activity is strongly inhibited by integrin-mediated cell tension sensing within lipid rafts, while bulk plasma membrane PDGFR activity was unaffected.⁴⁹⁸ The studious application of genetically encoded biosensors has thus allowed researchers to cut through the

debate and establish the role of membrane microdomains as spatial organizing centers for intracellular signaling pathways.

4.2. Compartmentation by the Activity of Pathway Regulators

Signaling pathways are composed of numerous molecular players that are affected by a number of regulatory processes: negative regulators function to attenuate, antagonize, or otherwise repress signaling within a pathway, while positive regulators serve to prolong or enhance signaling activities. The regulatory connections operating within a signaling pathway can also serve as feedback mechanisms, through which a signaling pathway can dynamically shape its own activity and give rise to various complex features⁸⁴⁰ (see section 3.1). When combined with the local activation of signaling enzymes anchored to cellular structures such as membranes, the action of pathway regulators can significantly influence the scope of a signaling event, yielding spatially confined signaling domains of varying sizes.

4.2.1. Calcium Signaling. Coupled with advances in optical microscopy and digital image processing, some of the earliest direct studies of spatially regulated Ca^{2+} signals were propelled by the development of small-molecule fluorescent indicators,³ which were much easier to work with and load into living cells compared with prior approaches and enabled the visualization of Ca^{2+} signals with high sensitivity and spatiotemporal precision. For example, Sawyer and colleagues used neutrophils loaded with the fluorescent Ca^{2+} indicator quin2⁸⁴¹ to monitor Ca^{2+} signals in cells undergoing phagocytosis and found that, while Ca^{2+} levels appeared to be uniform throughout the cytosol in unstimulated cells, neutrophils that were in the process of migrating toward or engulfing a target showed dramatically higher Ca^{2+} levels in the extending lamellipodia compared with trailing regions of the cell.⁸⁴² Brundage et al. similarly performed time-lapse imaging in chemotaxing eosinophils loaded with another Ca^{2+} indicator, Fura-2,¹³⁵ and found that Ca^{2+} was highest at the rear of cells that were steadily migrating in a single direction, while a localized burst of Ca^{2+} at the rear of the cell was observed to precede cell turning.⁸⁴³ Meanwhile, in their work with giant squid axons, Smith and colleagues were able to use fura-2 to visualize the formation of sharp Ca^{2+} gradients in presynaptic terminals in response to trains of presynaptic action potentials.⁸⁴⁴

To this day, fluorescent Ca^{2+} indicators remain among the most popular and widely used tools for visualizing and investigating spatially compartmentalized Ca^{2+} signals. For example, in their study investigating the coordination of growth cone and cell body motility during neuronal migration, Guan and colleagues loaded cultured rat cerebellar granule cells with fluo-4⁸⁴⁵ to examine the role of Ca^{2+} in cells responding to the guidance factor Slit-2.⁸⁴⁶ Ca^{2+} is known to be a key regulator of both neuronal migration and growth cone dynamics,^{847–849} and Slit-2 has previously been shown to act via cytosolic Ca^{2+} elevations to exert a repulsive effect on migrating neurons.^{850,851} In this instance, application of an extracellular gradient of Slit-2 at the front of the advancing growth cone was observed to reverse the direction of granule cell migration in conjunction with the rapid induction of a Ca^{2+} wave that initiated within the growth cone and traveled quickly along the leading neurite to reach the soma.⁸⁴⁶ Yet the continued popularity enjoyed by fluorescent Ca^{2+} indicators notwithstanding, genetically encoded Ca^{2+} indicators (GECIs)

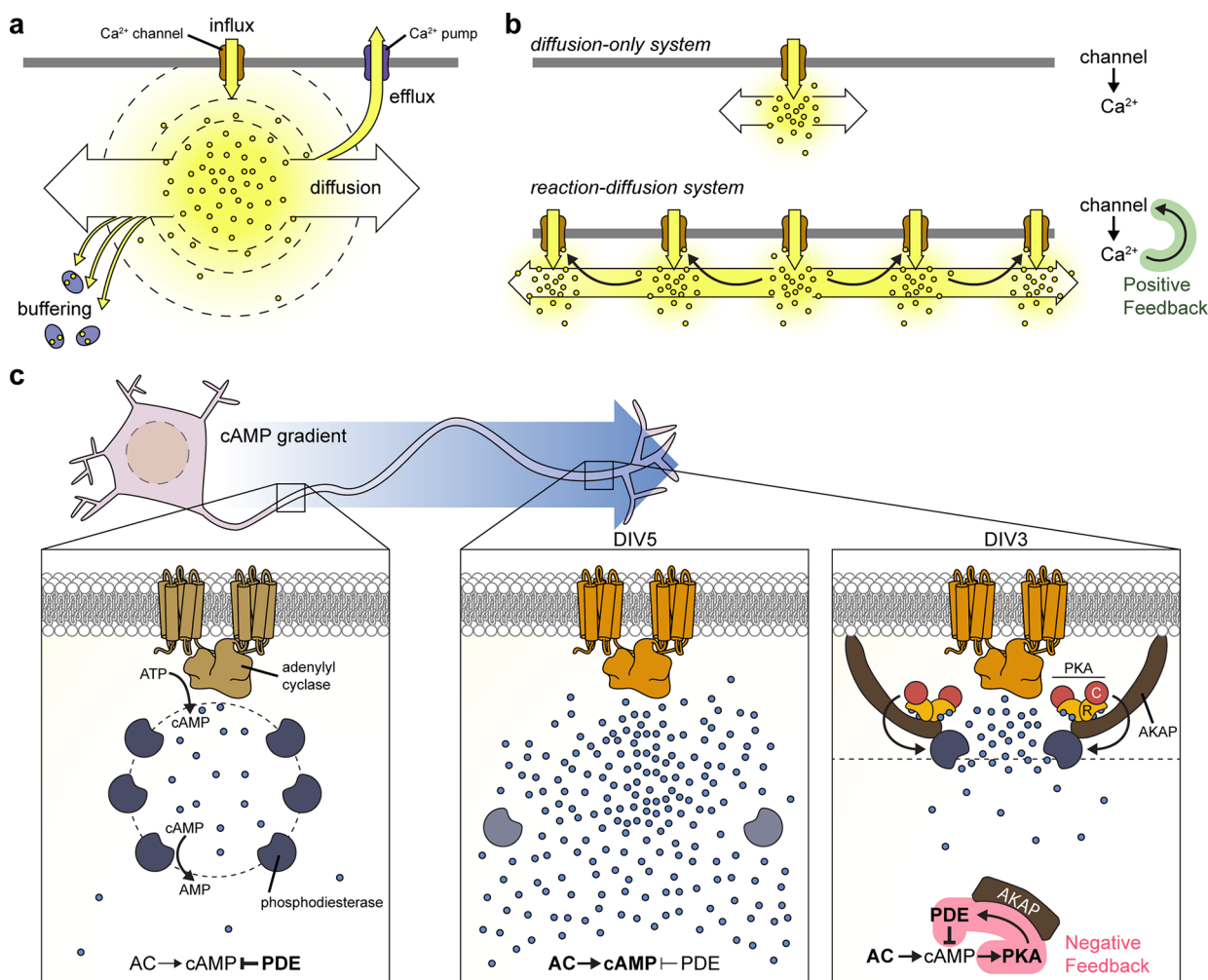


Figure 13. Spatial regulation of diffusible signaling molecules. (A) Spatial Ca^{2+} gradients are primarily shaped by 4 effects: the rate of Ca^{2+} influx into the cytosol from the extracellular environment and intracellular stores, the efflux of Ca^{2+} out of the cytosol by Ca^{2+} pumps, Ca^{2+} binding proteins which buffer the changes in the free Ca^{2+} concentration and diffusion of Ca^{2+} through the cytosol. (B) While the effect of Ca^{2+} influx by a single channel may be limited by the rate of Ca^{2+} flux through the channel and the rate of diffusion (top), Ca^{2+} -induced Ca^{2+} release (CICR), where an increase in Ca^{2+} can stimulate the opening of neighboring Ca^{2+} channels, creates a positive feedback loop that can create a wave of Ca^{2+} release that spreads faster than possible by diffusion alone (bottom). (C) The spatial arrangement of adenylyl cyclases (ACs), which produce cAMP, and phosphodiesterases (PDEs), which degrade cAMP, regulate the formation of gradients of the diffusible messenger cAMP. For example, rat hippocampal neurons at 5 days in vitro (DIV5), when the axons have started to become more thoroughly established, have been observed to exhibit an axon-directed gradient of cAMP accumulation in response to forskolin stimulation of ACs.⁸⁷² Conversely, at an earlier time point of 3 days in vitro (DIV3), cAMP production was observed to be much more limited in response to forskolin, which was hypothesized to be due to negative feedback mediated by PKA and PDE, which are localized to distal regions of an axon by an A-Kinase Anchoring Protein (AKAP).⁸⁷²

have also been widely adopted to visualize intracellular Ca^{2+} signals. Recent work by Huang et al., for instance, used both fluo-3 and the high-affinity FRET-based Ca^{2+} sensor YC-Nano 50³⁷⁰ to monitor Ca^{2+} gradients and uncover the role of mechanosensitive Ca^{2+} channels in regulating cell polarity.⁸⁵² GECIs are also essential for labeling cell populations in vivo, as was done recently by Vargas and colleagues, who expressed a modified form of GCaMP³⁹² in zebrafish somatosensory neurons and observed the passage of two distinct Ca^{2+} waves through neurons undergoing axonal degeneration.⁸⁵³

These spatially diverse Ca^{2+} signaling events are governed by various regulatory processes that work in concert to control the persistence and diffusion of Ca^{2+} fluxes within the cytosol (Figure 13A). Elevations in cytosolic Ca^{2+} concentrations are initiated via Ca^{2+} influx across the plasma membrane and release from intracellular stores, and Ca^{2+} waves and gradients often originate from highly localized Ca^{2+} elevations. Such

“elementary” Ca^{2+} release events, which assume a variety of aliases in the literature,^{854,855} involve Ca^{2+} release by individual or clusters of channels and can often be directly visualized using diffusible Ca^{2+} indicator dyes or GECIs. Early work by Bootman and colleagues, for instance, revealed the induction of Ca^{2+} waves from individual Ca^{2+} “puffs” in response to histamine stimulation in fluo-3-loaded HeLa cells.⁸⁵⁶ More recent work by Wei and co-workers also observed discrete Ca^{2+} “flickers” in migrating human embryonic lung fibroblasts loaded with fluo-4.⁸⁵⁷ These transient bursts of Ca^{2+} , which were associated with both mechanosensitive TRPM7 channels in the plasma membrane and IP_3 receptors (IP_3Rs) on the ER surface, were only present in migrating cells and were largely concentrated within the lamellipodium at the leading edge, helping to control cell turning.⁸⁵⁷ Similarly, Sonkusare et al. were able to observe Ca^{2+} “sparklets” in the vascular endothelial cells of arteries isolated from GCaMP2-transgenic

mice.⁸⁵⁸ These discrete Ca^{2+} release events were associated with the opening of individual plasma membrane TRPV4 channels and were important for the activation of potassium channels to regulate vascular tone.⁸⁵⁸

More precise monitoring of these Ca^{2+} channel microdomains can also be achieved by targeting GECIs directly to sites of Ca^{2+} entry. For example, Tay et al. were able to measure Ca^{2+} flux immediately in the vicinity of the $\text{Ca}_v2.2$ voltage-gated Ca^{2+} channel by using both the FRET-based Ca^{2+} sensor TN-XL⁴⁰⁹ fused to the C-terminus of the channel catalytic subunit and total internal reflection fluorescence (TIRF) microscopy to quantify biosensor dynamics exclusively at the plasma membrane.⁸⁵⁹ Because Ca^{2+} levels near channel openings are anticipated to greatly exceed those generally observed in the cytosol,⁸⁶⁰ it is important to tune the sensitivity of the sensor to match the concentration range being studied and to avoid unwanted probe saturation. Thus, Despa and colleagues developed a modified GCaMP2.2 with a reduced Ca^{2+} -binding affinity (GCaMP2.2Low), which they then used to monitor Ca^{2+} levels near individual ryanodine receptors (RyRs) in the junctional clefts of adult rat cardiomyocytes.³⁹⁰ Using this approach, the authors were able to observe larger and faster Ca^{2+} elevations in the channel microdomain compared with the bulk cytosol during cardiac contraction.³⁹⁰ Targeted sensors can further be used to probe connections between Ca^{2+} channel microdomains and specific Ca^{2+} effectors. For instance, Ca^{2+} -sensitive ACs are known to be selectively activated by capacitative Ca^{2+} entry (CCE, or store-operated Ca^{2+} entry [SOCE]) versus other forms of Ca^{2+} influx, suggesting close association with specific Ca^{2+} channels. Indeed, by targeting GCaMP to AC8, Willoughby et al. observed much stronger CCE-included Ca^{2+} elevations in the vicinity of AC8 compared with elsewhere in the cell, consistent with the localization of AC8 within a specific Ca^{2+} channel microdomain.⁸⁶¹

Such discrete Ca^{2+} release events are capable of generating large-scale spatial signaling domains by virtue of the fact that Ca^{2+} can stimulate its own release via positive feedback on Ca^{2+} channels such as IP_3 Rs and RyRs on the ER surface, resulting in Ca^{2+} -induced Ca^{2+} release (CICR) or “regenerative” fluxes.^{854,862} For example, Bootman and colleagues were able to block the conversion of puffs into waves by disrupting the positive feedback required for CICR.⁸⁵⁶ The rapid Ca^{2+} wave observed by Guan et al. in response to an applied Slit-2 gradient was similarly blocked by disrupting CIRC via the inhibition of RyR activation.⁸⁴⁶ Local Ca^{2+} elevations are thereby able to propagate rapidly across long distances because Ca^{2+} need only diffuse the short distance to a neighboring channel to elicit additional Ca^{2+} release, and so forth, rather than having to directly diffuse across an entire cell. Such combined reaction-diffusion systems^{805,863} effectively convert the cytoplasm into an “excitable medium”^{805,862} capable of spreading Ca^{2+} signals much more rapidly than diffusion alone (Figure 13B). Together with intercell contacts such as gap junctions, this process even allows Ca^{2+} waves to spread rapidly across groups of cells, as documented by Chifflet and colleagues, who observed Ca^{2+} waves that traversed across several cells within a monolayer in response to wounding and during regeneration in fluo-4-loaded epithelial cells.⁸⁶⁴ Similarly, Sieger and co-workers used GCaMP-expressing transgenic zebrafish to study the molecular mechanisms guiding microglia toward the sites of neuronal injury in the brain and found that targeted neuronal ablation induced a Ca^{2+}

wave capable of traversing the entire brain in a matter of seconds.⁸⁶⁵

Ultimately, the overall persistence of a Ca^{2+} signal is determined by the balance between Ca^{2+} influx and efflux mechanisms, as cytosolic Ca^{2+} elevations are constantly being antagonized by the steady action of pumps and transporters that work to remove Ca^{2+} from the cytosol. Yet in addition to control by influx and efflux mechanisms, cytosolic Ca^{2+} is also heavily buffered by its binding to cytosolic proteins such as parvalbumin, calretinin, and calbindins.⁸⁶⁶ Buffering plays an essential role in controlling the diffusional spread of Ca^{2+} within the cytosol and has been shown to exert considerable influence on the spatial confines of Ca^{2+} signals^{866–869} (Figure 13A). For example, Wang and colleagues visualized the effect of buffering on Ca^{2+} waves by loading astrocytes with fluo-3⁸⁷⁰ and treating them with exogenously applied Ca^{2+} chelators, finding that both high-affinity Ca^{2+} chelators and high concentrations of low-affinity chelators significantly attenuated the initiation and propagation of Ca^{2+} waves.⁸⁶⁷ Dargan and Parker also observed that the binding kinetics of cytosolic Ca^{2+} buffers have a significant impact on Ca^{2+} induced by photoreleased (i.e., UV uncaged) IP_3 by modulating feedback between and within IP_3 R clusters, which underlies regenerative Ca^{2+} release (i.e., CICR), with “slow” buffers promoting more local signals by disrupting feedback between clusters and “fast” buffers promoting global signals by disrupting feedback within clusters while serving as a Ca^{2+} shuttle to facilitate communication between clusters.⁸⁷¹

These studies suggest that the expression of different buffer pools with distinct kinetics may in fact serve to insulate spatially distinct Ca^{2+} signals in the same cell, such as in the case of Wei and colleagues, whose Ca^{2+} flickers were observed to coexist alongside an oppositely oriented global Ca^{2+} gradient.⁸⁵⁷ Thus, by effectively controlling the persistence and diffusion of cytosolic Ca^{2+} elevations, cells are able to generate highly specific and precisely tuned spatial signals. It should be noted, however, that fluorescent Ca^{2+} indicators, which are themselves typically derived from the chemical backbones of Ca^{2+} chelators,³ are also capable of buffering and disrupting Ca^{2+} waves when loaded at sufficiently high concentrations,⁸⁶⁷ thereby highlighting the delicate balancing act researchers must perform to investigate spatial regulation in signaling.

4.2.2. cAMP Signaling. cAMP is another ubiquitous and diffusible intracellular messenger that, like Ca^{2+} , is responsible for controlling a wide array of cellular processes and is capable of forming diverse spatial signaling domains. In one of the earliest examples of the direct visualization of spatially compartmentalized cAMP signaling in living cells, Bacskaï and colleagues utilized FICRhr⁶⁷⁵ to monitor cAMP levels in sensory neurons of the marine snail *Aplysia*.⁸⁷³ Confocal imaging of cultured *Aplysia* neurons injected with this probe revealed that bath application of serotonin induced a spatial gradient of elevated cAMP concentrations, with cAMP accumulation rising with increasing distance from the cell body and reaching its highest levels in neuronal processes.

In time, the practical advantages of genetically encoded fluorescent biosensors (e.g., more easily constructed, manipulated, and introduced into cells) would outstrip the utility of fluorescent analogues and also lead to more frequent observations of compartmentalized cAMP signaling. For example, in their study reporting the development of a panel of unimolecular FRET-based cAMP sensors, Nikolaev et al.

observed the induction of a cAMP wave that gradually traveled the length of the cell in response to local β_2 AR stimulation in hippocampal neurons expressing Epac1-camps.²⁹⁹ Subsequent work by this same group also revealed the formation of differential cAMP gradients in cells upon local stimulation with either β_1 AR or β_2 AR agonists.²⁹⁸ Specifically, in adult murine cardiomyocytes derived from transgenic mice expressing another FRET-based cAMP sensor, HCN-camps, local β_1 AR stimulation produced a large, shallow gradient of cAMP accumulation that spread far into the cell, whereas local β_2 AR stimulation prompted the formation of a much steeper cAMP gradient that was almost completely confined to the region nearest the stimulation site.²⁹⁸ Similarly, Lim and co-workers were able to observe a front-to-back cAMP gradient in migrating CHO cells expressing the cAMP sensor ICUE2,³¹¹ consistent with the key role played by cAMP signaling in cell polarization.⁸⁷⁴ Furthermore, a recent study by Gorshkov and colleagues reported the formation of a developmentally timed spatial cAMP gradient in polarizing rat hippocampal neurons transfected with ICUE3.⁸⁷² In particular, whereas bath application of forskolin induced a gradient of cAMP accumulation that was oriented toward the developing axon in neurons grown for 5 days in vitro (DIV5), no such gradient was observed in less developed DIV3 neurons.⁸⁷²

Although the compartmentalization of cAMP signaling is less well-understood than that of Ca^{2+} , substantial progress has been made over the past 15 years in unraveling the molecular mechanisms governing these spatial domains of cAMP, based on the use of fluorescent biosensors to carefully dissect the contributions of the various processes controlling cAMP persistence (e.g., synthesis and degradation) and diffusion. For instance, cAMP synthesis is typically initiated at the plasma membrane by the GPCR-induced activation of transmembrane ACs, and local cAMP production has previously been implicated as one mechanism capable of generating spatially confined domains of elevated cAMP.⁸⁷⁵ The differential regulation of AC isoforms by various signaling pathways^{876,877} may in fact yield discrete subcellular cAMP elevations in response to specific inputs, with ACs capable of producing local microdomains of high cAMP concentration that are analogous to the elementary release events and Ca^{2+} microdomains produced by the opening of small numbers of Ca^{2+} channels. Indeed, Wachten and colleagues were able to demonstrate this phenomenon directly by expressing variants of the Epac2-camps sensor, which were targeted to the cytosol or plasma membrane or directly fused to AC8, in GH_3B_6 pituitary cells.⁸⁷⁸ Specifically, treating cells with the hormone vasoactive intestinal peptide, which stimulates AC activity via G_αs -coupled GPCR signaling, strongly increased both global and plasma membrane-adjacent cAMP levels while having minimal effect on cAMP levels in the vicinity of AC8. Conversely, stimulation with thyrotropin-releasing hormone, which promotes Ca^{2+} release via G_αq -coupled signaling, selectively increased cAMP levels near AC8 while actually decreasing cAMP levels globally.⁸⁷⁸

cAMP signaling terminates with the hydrolysis of cAMP by cyclic nucleotide phosphodiesterases (PDEs), which thereby mediate the persistence of cAMP in cells. Yet the cAMP-degrading activity of PDEs has also come to be regarded as a major mechanism by which cells limit cAMP diffusion.^{879,880} For example, PDE activity was shown to be essential for promoting the aforementioned cAMP gradient in polarizing DIV5 hippocampal neurons, whereas negative feedback

signaling through axonal PDE activity actually suppressed the formation of a similar cAMP gradient in DIV3 neurons⁸⁷² (Figure 13C). PDE activity was also found to be involved in producing the differential β_1 AR- and β_2 AR-induced cAMP gradients observed by Nikolaev et al.²⁹⁸ Meanwhile, Maiellaro et al. found that PDE activity was essential for the formation of discrete cAMP gradients within single synaptic boutons in locally stimulated *Drosophila* sensory neurons.⁸⁸¹ Similarly, a prior study by Herbst and colleagues demonstrated that PDE3 activity restricted growth factor-induced cAMP accumulation to the plasma membrane in PC12 cells.⁷⁵⁴ Thus, PDEs can be seen to create spatial barriers that constrain the exit of cAMP from within a microdomain, preventing more global accumulation. However, PDEs can inversely restrict the entry of cAMP into subcellular regions, as observed by Monterisi et al.,⁸⁸³ who found that mitochondrially localized PDE2A2 blocked global cAMP signals from activating an outer mitochondrial membrane-targeted version⁸⁸² of the cAMP sensor H90. In fact, in an experiment mirroring studies designed to monitor microdomains of cAMP accumulation, Lohse and co-workers were unable to detect any measurable cAMP accumulation using Epac1-camps fused directly to PDE4A1,⁸⁸⁴ raising the possibility that PDEs are capable of generating negative cAMP microdomains, or “holes”.

However, our understanding of the molecular pathways regulating cAMP diffusion is complicated by the diffusion of cAMP itself. For example, by using FICRhR to measure the cytosolic diffusion of microinjected cAMP in *Aplysia* sensory neurons, Bacsikai and colleagues calculated an apparent diffusion coefficient for cAMP of approximately $780 \mu\text{m}^2/\text{s}$.⁸⁷³ Nikolaev et al. employed a similar approach by measuring the cytoplasmic velocity of the cAMP wave generated in response to local β_2 AR stimulation in Epac1-camps-expressing hippocampal neurons, arriving at an apparent diffusion coefficient of approximately $480 \mu\text{m}^2/\text{s}$,²⁹⁹ while Chen et al. were also able to estimate a cAMP diffusion rate of approximately $270 \mu\text{m}^2/\text{s}$ in frog olfactory cilia based on electrophysiological measurements.⁸⁸⁵ These values closely resemble those measured for cAMP diffusing freely in solution,^{886–888} and the idea of rapidly diffusing cAMP has proven difficult to reconcile with the very idea of compartmentalization, let alone the dominant role attributed to PDE activity in forming cAMP compartments. Indeed, mathematical modeling by Lohse et al. based on known catalytic rates indicated that PDEs should be incapable of producing cAMP microdomains with diffusion coefficients of this magnitude, despite their own observations to the contrary⁸⁸⁴ (for more on computational modeling and fluorescent biosensors, see section 5). Although some modeling studies have emphasized the importance of PDEs in compartmentalizing cAMP signaling,⁸⁸⁹ still more indicate that PDE activity is insufficient without additional constraints on cAMP diffusion.^{884,890,891} Indeed, recent work has provided evidence that cAMP diffusion is in fact slower than the aforementioned estimates and that some additional processes are responsible for compartmentalizing cAMP signals.

Several additional mechanisms have been proposed to participate in the spatial compartmentalization of cAMP signaling alongside local cAMP production and degradation (reviewed in ref 892). Bacsikai et al., for instance, initially articulated the concept of cell shape as a regulator of cAMP compartmentation based on their studies in *Aplysia* sensory neurons,⁸⁷³ and Neves et al. recently provided a direct

demonstration of this process through a combination of live-cell imaging of FRET-based biosensors and mathematical simulations, showing that fine cellular structures (e.g., dendrites) with confined geometries and high surface-to-volume ratios favored cAMP production by transmembrane ACs over cAMP degradation by cytosolic PDEs, thus promoting cAMP accumulation.⁸⁹³ The internal geometry of the cell has also previously been suggested to create physical barriers that restrict cAMP diffusion,⁸⁷⁵ and in a recent study, Richards et al. used the FRET-based cAMP sensor H187³⁰² to reveal that cAMP diffusion rates are slower in adult cardiomyocytes compared with neonatal cardiomyocytes, which contain fewer and less-ordered mitochondria compared with adult myocytes and thus present fewer physical barriers to diffusion within the cytoplasm.⁸⁹⁴ Meanwhile, Agarwal and colleagues used correlation spectroscopy analysis to measure the diffusion of fluorescently labeled cAMP in living cells and found that binding to PKA R subunits had a significant influence on cAMP diffusion,⁸⁹⁵ highlighting a role for cAMP buffering similar to that of Ca^{2+} buffering. Notably, both groups reported diffusion coefficients ($32 \mu\text{m}^2/\text{s}$ ⁸⁹⁴ and $5 \mu\text{m}^2/\text{s}$ ⁸⁹⁵) that are dramatically lower than previous estimates and are much more compatible with existing models of cAMP compartmentalization.

The work by Agarwal and colleagues⁸⁹⁵ is consistent with previous reports in which cAMP binding by PKA was shown to be important for cAMP gradient formation,^{890,896,897} as well as with much earlier indications that a substantial amount of total basal cAMP is bound to PKA in cells.^{800,898} Yet although the idea of cAMP buffering as a heretofore under-appreciated mechanism regulating cAMP diffusion and compartmentalization holds some appeal, particularly given the parallels to Ca^{2+} buffering, the importance of buffering in cAMP compartmentalization remains unclear. For example, Richards et al. observed little role for buffering in determining cAMP diffusion rates, as saturating intracellular cAMP binding sites did not affect their estimated diffusion coefficients,⁸⁹⁴ though Agarwal et al. conversely observed that intracellular geometry did not significantly affect their measurements of cAMP diffusion.⁸⁹⁵ Furthermore, despite its high abundance and affinity for cAMP, the reported buffering capacity of PKA is quite low compared with Ca^{2+} buffers,⁸⁹² and cells would likely have to express additional, potentially uncharacterized cAMP-binding proteins to achieve sufficient buffering. Some of these questions may potentially be resolved through the use of molecular tools to perturb cAMP buffering, and to this end, Lefkimmatis et al. previously developed a genetically encoded cAMP “sponge” that can be introduced into living cells as an exogenous cAMP buffer.⁸⁹⁹ Combining the cAMP sponge with fluorescent cAMP biosensors should enable direct visualization of the effect of different buffering rates on spatial cAMP signaling, similar to previous studies of Ca^{2+} buffering.⁸⁶⁷ Nevertheless, no single process likely plays a dominant role in cAMP compartmentalization,⁸⁹⁰ and researchers will continue teasing apart the individual contributions of the various molecular mechanisms responsible for directing the formation of compartmentalized signaling domains.

4.2.3. Other Signaling Molecules. Chemotaxing cells are capable of undergoing rapid polarization even in the presence of very shallow chemoattractant gradients,⁹⁰⁰ implying the amplification of extrinsic spatial differences by the compartmentalization of the intracellular signal transduction machinery, and our understanding of spatial signaling in chemotaxis

has greatly benefitted from the use of genetically encoded fluorescent biosensors. For instance, early studies using translocation-based sensors and GFP-tagged fusion proteins revealed the formation of a sharp gradient of 3' phosphoinositides [3'-PIs, e.g., $\text{PI}(3,4,5)\text{P}_3$, $\text{PI}(3,4)\text{P}_2$] toward the leading edge of the plasma membrane in chemotaxing cells.^{901–904} Gradient formation was found to be partially regulated by the inverse localization of PI3K and PTEN, which produce and degrade 3'-PIs, respectively, along the front and back of polarized cells,^{905,906} and to be potentially reinforced by negative feedback between these two enzymes. PI3K signaling also activates a number of small GTPases, including Ras, Rho, Rac, and Cdc42, which in turn stimulate PI3K activity, resulting in a positive feedback loop that further sharpens and amplifies the leading edge 3'-PI gradient.^{907–909}

The use of FRET-based biosensors has similarly revealed gradients of active Cdc42 and Rac at the leading edge in migrating cells,^{624,910} whereas Rho activity is concentrated toward the rear of the cell.^{910,911} These gradients have been shown to be mediated by multiple positive and negative feedback loops, including positive feedback with PI3K^{907–909} and mutual antagonism between Rho and Rac activity,⁹¹² as well as through self-organization via cytoskeletal rearrangements.⁹¹¹ Hodgson and colleagues also recently used FRET-based sensors to visualize an inverse spatial relationship between active Cdc42 and Cdc42 that was bound and inhibited by GDIs, revealing another mechanism of spatial control in this pathway.⁶²⁶ Interestingly, positive and negative feedback within this network also allow Cdc42 to behave as an excitable medium,⁹¹³ which Yang et al. recently visualized in the form of waves of Cdc42 activation that preceded spontaneous symmetry breaking in unpolarized neutrophils.⁹¹⁰

Spatial signaling by the small GTPase Ran has also been investigated using genetically encoded biosensors. The biological functions of Ran GTPase, which regulates multiple processes related to nuclear function,⁹¹⁴ are largely attributed to the predicted formation of a gradient of GTP-bound (i.e., active) Ran emanating from within the nucleus, due to the anchoring of its GEF, RCC1, to chromatin and the localization of its GAP, RanGAP1, in the cytoplasm. Using FRET-based biosensors that detect endogenous GTP-bound Ran, Kaláb and colleagues were able to confirm the existence of a RanGTP gradient in both *Xenopus* oocyte extracts and mitotic HeLa cells,^{635,915} as well as the importance of this gradient in regulating spindle assembly. Lee et al. were further able to combine FRET-based biosensor imaging of RanGTP with a temperature-sensitive RCC1 mutant to observe the loss of the RanGTP gradient surrounding metaphase chromosomes upon acute RCC1 inhibition.⁹¹⁶ This study also identified a role for the RanGTP gradient in regulating kinetochore attachment through Aurora B kinase,⁹¹⁶ whose spatial signaling during anaphase of the cell cycle had previously been observed by Fuller and colleagues.⁴⁴⁹ Specifically, the use of a chromosomally targeted FRET-based sensor revealed a gradient of Aurora B kinase activity that was shaped by a positive feedback loop between Aurora B activation and spindle microtubules to communicate the location of the spindle midzone.⁴⁴⁹ Employing a similar imaging approach, Liu and co-workers also visualized an Aurora B activity gradient that was essential for sensing chromosome biorientation.⁹¹⁷ Thus, along with Ca^{2+} and cAMP, studies of numerous signaling pathways have benefited from the use of genetically encoded fluorescent biosensors, which have illuminated the widespread formation

of signaling gradients and other dynamic spatial domains of signaling activity within cells.

4.3. Assembly of Macromolecular Signaling Complexes

Given the broad substrate specificities of most signaling enzymes and the sheer number of permutations that can be generated by the intracellular signaling machinery, diffusion alone is clearly insufficient to guarantee that useful interactions will occur among the correct molecular players once a pathway becomes activated. Instead, cells frequently utilize PPIs to channel signaling activities along specified routes by directly recruiting signaling molecules into productive complexes near their sites of action, thereby ensuring the efficiency and specificity of signal transduction.⁹¹⁸ One mechanism by which this is achieved is through the use of multivalent scaffolds, which are defined as proteins capable of binding two or more signaling enzymes and tethering them to specific subcellular locations while also coordinating their signaling activities.⁹¹⁹

4.3.1. Scaffolding by A-Kinase Anchoring Proteins.

AKAPs represent the most well-characterized family of multivalent scaffold proteins in the literature. The various members of this family, comprising more than 50 different proteins with orthologues in numerous species, show no overall structural similarity but nevertheless share common features.^{752,920} In particular, all AKAPs contain a short amphipathic helix that binds to the R subunit dimer of the PKA holoenzyme; while the majority of AKAPs specifically recognize the type II R subunit (RII), some type I (RI)-specific, and dual-specificity AKAPs have also been identified. Furthermore, all AKAPs contain targeting sequences that mediate their distinct subcellular localizations, enabling the direct recruitment of PKA to specific compartments throughout the cell.^{752,920} As such, AKAPs play a central role in establishing subcellular domains of PKA activity. For example, Terrin and co-workers used centrosome-targeted AKAR3 to observe that AKAP9/AKAP450 is responsible for creating a domain of increased PKA activity in this region.⁹²¹ Wang et al. similarly used cytosol- and membrane-targeted AKAR4 to observe the complete inhibition of kinase activity associated with an AKAP-anchored subpool of PKA at the plasma membrane in cells treated with a cell-permeable peptide that disrupts AKAP-RII binding.⁹²² Meanwhile, Schott and colleagues recently performed a study using AN3 CA cancer cells, which do not express AKAP12/gravin, along with subcellularly targeted AKAR3, to reveal that re-expressing wild-type gravin increased membrane-associated PKA activity while decreasing cytosolic PKA activity in response to either β_2 AR stimulation or direct AC activation using forskolin.⁹²³ This effect was abolished by the Ca^{2+} -induced intracellular redistribution of gravin and was absent in cells expressing a gravin mutant lacking the RII-binding site, highlighting the importance of anchoring and localization for PKA compartmentalization by AKAPs.

AKAP anchoring has also been demonstrated to yield more complex spatial patterns of PKA signaling. For example, PKA plays an important role in cell migration by phosphorylating the cytoplasmic domain of $\alpha 4$ integrin, which also functions as an atypical, RI-specific AKAP.⁹²⁴ Lim et al. therefore used plasma membrane-targeted AKAR3 to investigate the spatial regulation of PKA activity during cell migration and observed the formation of a leading-edge gradient of PKA activity in migrating cells.⁸⁷⁴ This gradient was dependent on anchoring of PKA-RI by $\alpha 4$ integrin, as the mislocalization of type I PKA

selectively abolished the PKA activity gradient at the leading edge. Gorshkov and colleagues similarly observed the formation of a PKA activity gradient in polarizing DIV5 hippocampal neurons expressing diffusible AKAR4.⁸⁷² This activity gradient paralleled the axon-directed cAMP gradient also seen in these cells and was abolished by the disruption of AKAP-PKA anchoring. Interestingly, AKAP disruption induced a cAMP gradient in DIV3 cells, suggesting that AKAP anchoring mediates the negative feedback circuit that suppresses the cAMP gradient at this developmental stage.⁸⁷² (Figure 13C). Meanwhile, Mo and colleagues were recently able to obtain an even more detailed view of PKA compartmentalization by imaging the novel super-resolution PKA activity biosensor FLINC-AKAR, which revealed the plasma membrane to be decorated with discrete PKA activity microdomains of ~ 350 nm in diameter following PKA stimulation.⁴⁷⁷ Interestingly, these high-activity puncta were observed to colocalize with clusters of plasma membrane AKAP5 (AKAP79/150) and also disappeared following treatment with a cell-permeable AKAP-disruptor peptide,⁹²² suggesting the principal involvement of AKAP anchoring and demonstrating the precision with which AKAPs regulate spatial PKA signaling.

Yet such fine compartmentalization is in apparent conflict with the classical model of a dissociating and diffusing PKA C subunit, raising questions about other mechanisms that may play a role in restricting PKA diffusion, though opinions on this topic diverge considerably. In particular, because the cAMP-triggered dissociation of the PKA C and R subunits is based on early *in vitro* experiments,^{925–929} some groups have argued that active C subunits do not in fact dissociate from R subunits under physiological conditions. Indeed, by imaging fluorescent protein-tagged PKA C and R in living cells, Martin et al. previously reported that AKAP-anchored type I PKA holoenzyme fails to dissociate in response to cAMP alone.⁹³⁰ Furthermore, by combining live-cell imaging with biochemical and genetic approaches, Smith and colleagues recently suggested that type II PKA also does not dissociate from AKAP complexes in response to physiological stimuli and instead favored a model in which intrinsic disorder within the RII dimer allows the bound, active PKA C subunit to phosphorylate targets within a defined radius.⁹³¹ However, recent chemical cross-linking studies performed by Walker-Gray and colleagues call these results into question and appear to indicate that the PKA C subunit is capable of dissociating from RII dimers in cells.⁹³² Additional findings by these authors support a previously reported model of RII isoform-specific membrane binding via the C subunit myristoyl group,⁹³³ which restricts C subunit diffusion to within the plane of the membrane and allows for more efficient recapture by RII.⁹³² Work by Mo et al. further suggests a mechanism of C subunit recapture and retention, wherein AKAP clustering selectively increases the local concentration of PKA R subunits to further enhance C subunit recapture and spatially confine PKA activity.⁴⁷⁷

Each AKAP family member is also capable of binding a diverse array of signaling proteins in addition to PKA,^{752,920} allowing individual AKAPs to assemble distinct signaling machines, or macromolecular “signalosomes”, with the potential to produce unique local signaling environments. For example, by using a modified form of AKAR2 containing PKA- and PDE4D3-binding domains to mimic the recruitment of these enzymes by mA-KAP, Dodge-Kafka et al. were able to

recapitulate the attenuation of local PKA activity by tethered PDE4D3 within the mAKAP complex.⁹³⁴ However, this effect was reversed by the activation of ERK5, which directly phosphorylates and inhibits PDE4D3 and was shown to also be recruited to the mAKAP complex. PDE4D3 itself was further shown to recruit Epac1, which in turn inhibited ERK5 via the activation of Rap1 GTPase,⁹³⁴ thus illustrating the ability of AKAPs to assemble tightly integrated signaling machines to provide context-dependent modulation of local PKA activity. Work by Willoughby and colleagues also revealed that AKAP5 directly interacts with and modulates the Ca^{2+} sensitivity of AC8, thereby generating a subpool of locally regulated AC8 with distinct activation properties.⁵⁹ Hoshi and co-workers similarly investigated the effect of AKAP anchoring on the PKC-mediated inhibition of the KCNQ2 subunit of the M-type potassium channel.⁹³⁵ Fusing the FRET-based PKC activity reporter CKAR directly to AKAP5 revealed that AKAP anchored PKC activity was accelerated compared with PKC activity measured at the general plasma membrane. Moreover, simultaneous FRET and M-current recordings revealed that AKAP-accelerated PKC activity was perfectly timed to match the kinetics of M-current inhibition,⁹³⁵ demonstrating the ability of AKAPs to coordinate enzyme activity for more efficient downstream signaling. Subsequent work by Greenwald and colleagues also revealed that this coordination by AKAPs can both accelerate and amplify anchored enzyme activity.⁹³⁶

4.3.2. Molecular Scaffolds in Other Signaling Pathways. However, the importance of scaffolding is by no means limited to the PKA signaling pathway.^{918,937} For example, macromolecular complexes have been shown to play a major role in regulating MAP kinase signaling,^{938,939} and Matsunaga-Udagawa et al. previously used a FRET-based biosensor designed to monitor the binding of Ras and Raf-1 to investigate the role of the Shoc2 scaffold protein in regulating Ras/Raf/MEK/ERK pathway activity.⁹⁴⁰ Knocking down Shoc2 led to significantly slower binding between Ras and Raf-1, thereby reducing EGF-induced MEK and ERK phosphorylation by approximately half. Computational modeling further confirmed that Shoc2-binding was required to accelerate Ras/Raf binding and promote downstream pathway activation.⁹⁴⁰ Yoshiki and colleagues similarly used a plasma membrane-targeted FRET-based Raf-1 biosensor, Prin-Raf1,⁴⁵¹ to reveal a role for Shoc2 in the Ca^{2+} -dependent regulation of Raf-1 activation.⁹⁴¹ As above, knocking down Shoc2 decreased plasma membrane recruitment and activation of Raf-1 in response to EGF or following coactivation using an engineered RasGEF and Ca^{2+} treatment. Through this line of study, the authors were able to demonstrate that calmodulin mediates Ca^{2+} -dependent Raf-1 activation by controlling Ras binding to Shoc2.⁹⁴¹

More recently, Tobias and co-workers set out to investigate the regulation of the atypical PKC isozyme PKC ξ through the use of CKAR-fusion constructs and isoform-selective PKC inhibitors, wherein they were able to determine that binding by the scaffold proteins p62 and Par6 is responsible for both modulating PKC ξ activity and also controlling its localization within the cell.⁹⁴² Kroon et al. also recently used a FRET-based sensor to examine local Rac1 activity in epithelial cells exposed to laminar flow and observed persistent Rac activation in the downstream regions of cells exposed to long-term flow stress.⁹⁴³ The authors found that the RhoGEF Trio was required to maintain localized Rac activation but by

functioning as a scaffold protein rather than through the direct activation of Rac. Scaffold proteins are thus deeply integrated into the intracellular signaling machinery, and genetically encoded fluorescent biosensors continue to play a unique and crucial role in our efforts to unravel the spatial regulation of signaling activities by molecular scaffolds.

5. COMPUTATIONAL MODELS AND FLUORESCENT BIOSENSORS

As illustrated by the preceding sections, the organization of signaling networks in both space and time can have a level of complexity that makes it difficult to intuitively deduce or infer the composition of a signaling network and the identities of key regulators. In these cases, computational models can be developed to better understand the underlying signaling mechanisms. Computational models are an important tool for quantitatively evaluating hypotheses and making predictions.⁹⁴⁴ Fluorescent biosensors aid the development of computational models by providing a source of temporally and spatially precise measurements of signaling dynamics. Conversely, biosensors are useful tools for the experimental validation of computational predictions. In this section, we will discuss the ways in which quantitative, biosensor-based imaging and mechanistic modeling can be combined to provide new insights into signal transduction.

5.1. Computational Modeling Background

Computational models of cell signaling can be developed to quantitatively evaluate hypotheses, assist in the interpretation of experimental data, or explore conditions that may be infeasible to test experimentally. Here, we will focus on mechanistic models of dynamic cellular signaling, which are based on specific chemical reactions and approximations from first principles.⁷⁴⁷ The development of computational models generally requires the iterative process of constructing a model based on a hypothesized pathway structure, generating model predictions, comparing these predictions with experimental data, and then refining the hypothesized model structure to more accurately represent experimental data⁹⁴⁵ (Figure 14A). Through this process, biological insights can be obtained from both model failures, which highlight hypothesized network architectures that are unable to capture the signaling dynamics, and successful models, which can be used to dissect the underlying signaling mechanics and generate novel hypotheses. As there are many reviews that focus on the more detailed aspects of computational modeling,^{747,945–948} here we will provide only a brief description of the types of computational models commonly used and how fluorescent biosensor data are integrated into models.

There are several types of computational models, and the type utilized is dependent on the system and hypothesis being tested. When evaluating the temporal dynamics of signaling, the most commonly used models are based on ordinary differential equations, or ODEs. Biochemical ODE models are based on differential equations that approximate the change in the concentrations or amounts of signaling molecules (often referred to as model species) over time, where reaction kinetics equations approximate the production and destruction of signaling molecules, as well as changes in state, such as enzyme activation or post-translational modification (Figure 14B). In most cell signaling ODE models, the reaction rate equations are based on mass action kinetics derived from first-principles, but in some cases, empirical reaction rate equations are used,

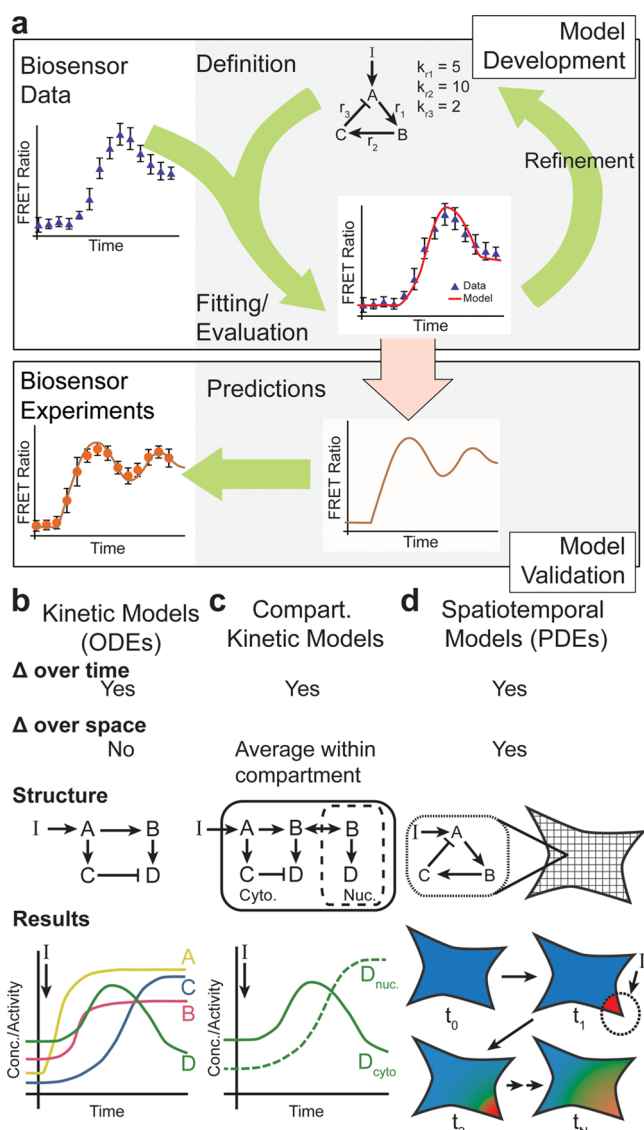


Figure 14. Integrated approach combining biosensor imaging and computational modeling. (A) Computational model development utilizes an iterative approach where the hypothesized structure of the signaling network is implemented into a computational model (top). The results of the computational model are then compared with experimental biosensor data, which serves to approximate unknown model parameters (model fitting) and identify aspects of the experimental data that the model is not capturing. This comparison is then used to refine the hypothesized model structure. This process is iterated until the model adequately reflects the experimental data. This model is then, in turn, capable of generating previously untested conditions (e.g., different stimulation, inhibition of a signaling enzyme) to generate new hypotheses (bottom). Biosensors then serve as a powerful tool to validate these model predictions. (B and C) Kinetic computational models simulate changes in the activity and concentration of different signaling reactants as defined by the hypothesized connections within the signaling networks. These models do not directly approximate changes in space (B) but subcellular compartments can be defined where specific model species can exchange between compartments (C). (D) Spatiotemporal models simulate the changes in signal transduction across both space and time; therefore, the model outputs the model species concentration or activity as it varies across the defined geometry and through time.

especially for components such as gated ion channels. Given an initial state of the signaling molecule concentrations (initial conditions), solutions to this system of equations are numerically approximated over small time increments to simulate changes in signaling dynamics. These ODE models are useful for studying the dynamics of a signaling compartment on average and therefore do not provide spatial information, but subcellular compartments can be defined in these models to evaluate the effect of spatial segregation on the overall signaling dynamics (Figure 14C). Additionally, ODE models are deterministic, in that for a given set of equations and initial conditions, the same solution will be reached every time, though in systems with very small volumes or numbers of reactants, stochastic methods may be necessary.⁹⁴⁹

When computational models need to consider dynamics in both space and time, partial differential equations (PDEs) are the most common modeling technique and can capture both reaction dynamics and diffusion. PDEs consist of equations that describe the rate of change in the concentrations of molecular species over time, similar to ODEs, while also approximating how these changes occur spatially due to both reactions and diffusion (Figure 14D). In some simplified cases, it is possible to solve these equations analytically,⁸⁸⁴ but the reactions and geometries involved typically require the use of numerical approximation, where the spatial geometry is broken up into small subregions and, by taking small steps forward in time from an initial condition, the solution to the PDE is approximated across both space and time. This added complexity requires that in addition to specifying the initial conditions of the molecular species concentration over the whole spatial area, boundary conditions must be applied to define what happens to diffusing species at the edges of the defined geometry. When the geometries studied are cells, the boundaries are most often cell membranes, with the assumption of no diffusion (zero flux) occurring across the membrane, but it is also possible to use other boundary conditions such as a constant concentration or periodic boundary condition. Furthermore, PDE models can examine the temporal dynamics of spatial gradients in two or three dimensions in biologically relevant geometries such as a given cell shape, which can be integral to study the effects of cell shape and allow for comparison of the spatial changes in biosensor data. Again, in cases of small reaction volumes or low reactant numbers, it may be necessary to utilize a stochastic model architecture to evaluate hypotheses about the spatiotemporal dynamics and the effects that stochasticity may have on these systems.^{950–952}

Finally, data such as signaling dynamics measured by using biosensor-based imaging are integrated into these models as constraints to approximate unknown parameter values and to validate model predictions. One important consideration when comparing experimental data with computational modeling results is that one needs to consider how to reconcile what the experimental measurement means in terms of model outputs. For example, when integrating Ca^{2+} biosensor dynamics, one must consider that the readout is a change in fluorescence or FRET ratio and that each biosensor has a different sensitivity to Ca^{2+} . Some studies using fluorescent biosensors utilize calibrated biosensor responses based on known standards to relate the biosensor output to quantitative concentrations.^{408,953} While this is possible for biosensors that measure second messenger concentrations, calibration can be time-consuming, and for biosensors that measure enzymatic activity,

this calibration is not as straightforward given the dependence on enzyme and substrate (biosensor) concentrations and activity levels. Instead, a more common approach is to include the biosensor in the model directly, which requires adding more reactions and species to the model. Furthermore, it is often beneficial for model fitting to experimentally include positive and negative control stimuli to normalize the biosensor response to its dynamic range.⁹⁵⁴

5.2. Examples of the Integrated Approach

5.2.1. Analysis of Temporal Dynamics. A combination of computational modeling and fluorescent biosensor-based imaging can be used to study how the kinetics and organization of individual reactions shape the dynamics of signal transduction. Depending on the scope and scale of the study, this approach can be informative for both small and large signaling networks. For example, the PKC biosensor CKAR⁵¹² was used in conjunction with computational modeling to evaluate how enzyme–substrate tethering of scaffold proteins alters the kinetics of phosphorylation by kinases.⁹³⁶ Biosensor imaging showed that scaffold tethering accelerated and amplified phosphorylation by PKC, and the computational model used these data to quantitatively evaluate a proposed kinetic mechanism to explain the effects of scaffold tethering. This new mechanistic model was able to fit well to the experimental data and was used to predict that the scaffolded enzymatic reaction would be insulated from inhibition from certain types of inhibitors. In turn, the fluorescent biosensors were then used to validate this model prediction by showing that, indeed, AKAP7 tethering insulated phosphorylation by PKC from inhibition by substrate-competitive inhibitors. This example highlights how fluorescent biosensors can provide the data necessary to study the kinetics of individual reactions in computational models and validate the predictions that arise from these models. Similarly, the high temporal resolution afforded by fluorescent biosensors can be indispensable for computational models used to study signal propagation through a network in response to a given stimulus.

While the signaling network motifs described in [section 3.2](#), such as negative feedback, are known to lead to dynamics such as adaptation and oscillations, many signaling networks contain several different feedback and feedforward loops which can make it difficult to ascertain which aspects of the network are most important. As discussed earlier, ERK can exhibit a transient response to some stimuli, which requires at least one negative feedback mechanism, and fluorescent biosensors have been combined with computational models to understand how these dynamics arise. When the first fluorescent biosensor for ERK activation, Miu2, was developed, the authors built a computational model that aimed to capture the responses of ERK to EGF stimulation.⁴⁷⁸ Within this model, they observed that the magnitude of the ERK response was highly sensitive to the concentration of Raf *in silico*. This was experimentally validated by knocking down c-Raf and observing that MEK and ERK phosphorylation in response to EGF stimulation decreases with c-Raf knockdown. While computational models of ERK activity had been developed previously, the improved temporal resolution of the fluorescent biosensor compared to Western blots improved the accuracy of the model. It should be noted that computational models may not perfectly capture all the observed dynamics, but these discrepancies can provide important clues to identify less understood components of a signaling pathway. For example, Ahmed and colleagues turned

to computational models to understand why ERK nuclear localization in response to PDGF stimulation exhibited a much stronger adaptive response than ERK nuclear activity, which is also a slow process and lags the peak in nuclear translocation.⁹⁵⁵ They used EKAR⁴⁷⁴ and mCherry-labeled ERK2 to simultaneously measure both subcellular ERK activity and ERK shuttling between the cytoplasm and nucleus in response to stimulation. They hypothesized that the competitive interactions between ERK and its different substrates may be responsible for this discrepancy between activation and translocation of ERK and its kinase activity. To test this hypothesis, they developed a computational model that explicitly models the association, phosphorylation, and dissociation of ERK substrates, instead of the commonly used Michaelis–Menten enzyme kinetics approximation. The simulations from this new model matched their imaging data, suggesting that the competitive interactions between ERK and its many substrate proteins may limit the amount of ERK available to phosphorylate EKAR in the nucleus, an effect commonly referred to as buffering. Furthermore, this model suggested that most of the nuclear ERK is bound by substrates and also protected from dephosphorylation during the initial phase of the response, which was validated experimentally. These examples highlight the back-and-forth nature of computational model-based examination of fluorescent biosensor dynamics and experimental evaluation of model predictions and how this work can provide insights into the critical mechanisms that underlie the dynamics of cellular signal transduction ([Figure 14A](#)).

5.2.2. Evaluation of Spatial Compartmentalization.

The spatial segregation of cellular signaling components into microdomains has been identified as an essential aspect in shaping signaling dynamics, as discussed in [section 4](#), and the combination of fluorescent biosensors and computational models has been useful in examining how this compartmentalization affects signal transduction. Computational models studying spatial organization of signaling generally come in two forms, compartmentalized kinetic models and models that explicitly consider the spatial gradients of signaling molecules. For the purposes of this review, compartmentalized models are generally the kinetic ODE models discussed before but contain separated compartments that have their own composition and concentration of signaling molecules and indirectly account for diffusion by incorporating equations to approximate exchange of signaling molecules between compartments. The compartments can be defined to represent organelles, such as the nucleus and mitochondria, subcellular locations, like the plasma membrane or cytoplasm, and signaling microdomains, such as scaffold proteins like AKAPs. Accounting for this spatial organization within models can improve their accuracy and help answer questions about the role of this complex organization, but these models do not explicitly approximate spatial changes in signaling activity; for this, models that account for changes in concentration over both time and space, such as PDE models, are required. Because PDE models can incorporate factors such as cell shape and diffusion coefficients, these models are useful for examining how different factors can lead to and affect the spatial heterogeneity of signaling molecules. Genetically encoded fluorescent biosensors have been critical for providing data on signaling dynamics within microdomains and spatial gradients of signaling activities ([section 4](#)), and these unique data sets have been instrumental in informing and validating spatiotemporal computational

models. As discussed in section 4.2.2, the spatiotemporal regulation of the cAMP/PKA signaling pathway is intriguing because the tethering of PKA by AKAP scaffolds creates unique signaling microdomains in different locations throughout the cell, whereas its activator cAMP is a diffusible second messenger. This discrepancy had led to two lines of questioning that have benefited from the combination of fluorescent biosensors and computational modeling: (1) how do AKAP-mediated PKA microdomains shape signaling dynamics and (2) what regulates the spatial organization of cAMP concentrations to allow compartmentalized activation by cAMP?

Compartmentalized kinetic models of cAMP/PKA signaling have been utilized to evaluate how the spatial organization of this signaling pathway enables the coordination of several different, sometimes contradictory, phenotypic outcomes. In cardiomyocytes, stimulation of the β -adrenergic receptor as part of the fight-or-flight response leads to the activation of PKA and the phosphorylation of several downstream targets to increase cardiac output.⁹⁵⁶ The diverse array of PKA substrates contributes to several facets of excitation-contraction coupling (ECC), which can sometimes have competing effects on overall cardiac function.⁷⁶⁷ To help comprehend how the different microdomains of cAMP and PKA signaling each contribute to the overall signaling outcome, several computational models have been developed.⁹⁴⁵ For example, recent work by Surdo et al. used both targeted fluorescent biosensors and computational modeling to study how cAMP compartmentalization differs between microdomains and how these differences impact cardiac function.³⁰¹ Their work focused on three PKA microdomains: AKAP79, which coordinates PKA regulation of cAMP production by ACs and Ca^{2+} influx through the L-type voltage-gated Ca^{2+} channel (LTCC); AKAP18 δ , which localizes PKA to the sarcoplasmic reticulum (SR) to promote the phosphorylation of phospholamban (PLB) to increase the rate of Ca^{2+} reuptake into the SR; and the myofilament-localized troponin complex, where phosphorylation of the TpnI subunit by PKA reduces its affinity for Ca^{2+} . While PKA activity within the AKAP79 microdomain and phosphorylation of PLB at AKAP18 δ increases cardiac Ca^{2+} amplitude and contraction, phosphorylation of TpnI has the opposite effects of reducing myofilament Ca^{2+} sensitivity and limiting contraction. By targeting their cAMP biosensor, CUTie, to these different microdomains, Surdo et al. observed that the cAMP response to β -adrenergic stimulation was dampened in the TpnI microdomain compared with the other two microdomains. In order to evaluate how this difference affects cardiac ECC, they expanded a previous computational model to evaluate how this regulation may optimize the trade-offs of TpnI phosphorylation. They used this model to predict how decreased cAMP may affect the regulation of other myofilament proteins, namely, myosin binding protein C (MyBPC) and titin, by PKA. Interestingly, their model revealed that reducing PKA phosphorylation of TpnI without reducing the phosphorylation of MyBPC and titin resulted in increased myocyte contraction compared with uniform cAMP stimulation across all microdomains. This suggested that this regulation may be specific to TpnI, and indeed, this was experimentally validated by Western blots showing that in response to an intermediate dose of isoproterenol, TpnI phosphorylation was reduced but MyBPC phosphorylation was not. This study shows how the targetability of fluorescent biosensors can provide quantitative data within specific

compartments, which in turn can yield more detailed and accurate computational models.

On the other hand, the transport and diffusion of signaling molecules throughout the cell can lead to a spatial heterogeneity that is necessary for many critical cellular functions such as migration and cell division.⁹⁵⁷ Fluorescent biosensors are capable of measuring the dynamics of these signaling gradients, and computational models that study the explicit spatial dynamics of signal transduction have helped identify what factors are critical in shaping these complex phenomena. In contrast to the aforementioned compartmentalized models of cAMP/PKA signaling that examined the consequences of cAMP diffusion and PKA activity, several studies have utilized fluorescent biosensors and spatial computational models to understand how cAMP, as a small diffusible second messenger, can be compartmentalized (reviewed in ref 892). There are 6 (nonexclusive) proposed mechanisms for the compartmentalization of cAMP: localized production by AC, localized degradation by PDEs, physical barriers, cAMP buffering, cell shape, and cAMP export.⁸⁹² The ability to control and perturb these mechanisms within computational models has been indispensable in testing these hypotheses, but these models are built on and validated by the experimental evidence afforded by fluorescent biosensors. For example, the role of cell shape in the formation of cAMP gradients in neurons was studied through the development of a spatial computational model along with the cAMP biosensor Epac1-camps.⁸⁹³ When neurons expressing Epac1-camps²⁹⁹ were stimulated with a β -adrenergic agonist, cAMP accumulated in dendrites with little change in the cell body,⁸⁹³ even though β -adrenergic receptors have been shown to localize to both the dendrites and cell body in vivo.^{958,959} This fluorescent biosensor data was used to constrain and test model parameters within a defined cellular geometry that mimics the experimentally observed cell.⁸⁹³ Then, to evaluate the role of cell shape, this parametrized model was tested in an idealized geometry where cellular characteristics that define the shape of a neuron, such as axon diameter, were systematically varied and the effects on cAMP gradients were evaluated. In their model, Neves et al. found that cAMP gradients only formed in axons with smaller diameters, whereas downstream targets such as PKA and MAPK were able to form gradients in larger-diameter axons due to slower diffusion. Later work by Li et al. used a similar approach to examine how the branching of neuronal dendrites affects the transduction of cAMP signaling from the dendrites to the nucleus.⁹⁶⁰ Their model examined how small, localized cAMP production in dendrites, as would occur through confined activation of the dopamine receptor, would be translated to nuclear signaling by the downstream activation of PKA and its substrate DARPP-32. As the location of cAMP release was moved down the dendrite and further away from the nucleus, one would also expect the extent of nuclear PKA activity to decrease monotonically. Surprisingly, the model predicted that the extent of nuclear signaling was higher when cAMP was released in a mid-dendrite location than if cAMP was released in the larger trunks of the dendrite, nearer the cell body. Within their computational model, they were able to vary several dendrite shape parameters, such as dendrite diameter and branching, to test different mechanisms for this observed phenomenon. This analysis led them to postulate that larger-volume dendrites caused the cAMP to become diluted faster, whereas intermediate-size dendrites kept cAMP more concentrated but did not constrain the

diffusion of downstream signaling proteins as much as the more distal and smaller dendrite branches. Finally, these computational model predictions were validated using the PKA biosensor AKAR3,⁵⁰⁶ where localized uncaging of cAMP in the medial regions of neuronal dendrites showed a greater nuclear biosensor response compared with cAMP uncaging in the proximal and distal regions of the dendrites. These two examples highlight the effects that cell shape can play in the distribution of cAMP signaling throughout the cell.

While these studies show that cell shape can affect the rate of diffusion, even more fundamentally, the diffusion coefficient of cAMP itself has been questioned both experimentally and computationally.^{884,891,895} For example, using the PKA activity reporter AKAR2,⁵⁰⁷ Saucerman et al. observed that the activation of PKA in the cytosol was slower than expected when cAMP production was stimulated at the plasma membrane compared with cAMP uncaging in the cytosol.⁸⁹⁶ To evaluate the underlying cause of this temporal delay in PKA activation, they developed a computational model that included both cAMP buffering by binding to PKA and compartmentalized PDE activity.⁸⁹⁶ In their model, they found that a much slower cAMP diffusion rate was required when cAMP production was stimulated at the plasma membrane than when cAMP is directly uncaged in the cytosol, suggesting the possible existence of a barrier to cAMP diffusion between the plasma membrane and cytosol.⁸⁹⁶ Similar differences between submembrane and cytosolic cAMP were observed using the cAMP biosensor H30 (aka CFP-Epac(δ DEP-CD)-YFP³⁰⁶) in HEK293 cells, but these differences were hypothesized to arise from compartmentalized PDE activity rather than a diffusional barrier.⁹⁶¹ A stochastic spatial model constructed by Oliveria and colleagues was developed to examine these two hypotheses and revealed that PDE compartmentalization, as opposed to impeded cAMP diffusion, was the key mechanism for cAMP compartmentalization in HEK293 cells.⁸⁸⁹ Nevertheless, the rates of intracellular cAMP diffusion remain a highly debated area of research, and spatiotemporal dynamic data derived from fluorescent biosensors will be critical in building and testing the spatial signaling models needed to evaluate which mechanisms are most important for establishing signaling gradients.

5.2.3. Analyses of Cellular Heterogeneity. By virtue of their ability to reveal single-cell dynamics across a population of cells, fluorescent biosensors can provide information on how communication between cells can affect signal transduction. For example, stochastic pulses of ERK activity were observed in cells expressing the ERK biosensor EKAREV,⁴⁴¹ and the frequency of these pulses of activity depended on the cell density and would propagate to neighboring cells.⁷⁹⁰ Furthermore, Aoki et al. observed that while basal ERK activity decreased with increasing cell density, the frequency of the stochastic ERK pulses did not monotonically decrease with increasing density. Rather, the frequency exhibited a bell-shaped curve with respect to density, with fewer ERK pulses at both high and low cell densities. Additionally, this biphasic dependence on cell density was correlated with the cell proliferation rate, suggesting that the ERK pulses may act as a rheostat to regulate cell number. In order to evaluate how biological noise, feedback loops and paracrine signaling shape the development of these pulses of high ERK activity, Aoki and colleagues developed a multicellular computational model of ERK activity. In this model, Raf, the upstream activator of ERK, becomes activated through canonical stimulation, noise,

or paracrine signaling, with both positive and negative feedback loops regulating Raf activity. The model parameters were adjusted to recapitulate the shape and frequency of the experimental biosensor data at different cell densities, after which the simulations suggested that noise in the ERK signaling pathway increased with cell density. This model predicted that only about 20% of the ERK activity pulses can be attributed to paracrine propagation, whereas most of the ERK pulses are due to the noise in the system. Indeed, post hoc analysis of the experimental data showed similar fractions of the cell-to-cell propagation-driven ERK activity pulses as predicted. This model suggests that while paracrine signaling has some effects on stochastic ERK pulses, the frequency of these pulses is primarily driven by some yet unidentified cell density-dependent regulation of the ERK signaling pathway.

In another study of the paracrine signaling of ERK, Handy et al. utilized both fluorescent biosensors and a mathematical model to examine how communication between neighboring cells affects the sensing and generation of chemotactic gradients in wound healing.⁹⁶² Cells that are damaged can either actively or passively release ATP from their internal stores, which can activate purinergic receptors and act as a damage-associated molecular pattern (DAMP).⁹⁶³ In the epithelial cells used in this study, ATP activation of the P2Y receptor results in a Ca^{2+} transient and the release of EGF, which in turn activates EGFR and ERK in a paracrine fashion.⁹⁶² While the Ca^{2+} response to ATP was variable, the variability of the ERK response was observed to decrease as cells clustered together more densely. Thus, Handy et al. hypothesized that optimal paracrine signaling can lead to optimal localized population averaging, which can provide adequate reduction in variability and increase the SNR of the wound response. To examine this quantitatively, they developed a computational model that incorporated the secretion, diffusion, and integration of paracrine signaling to examine the effects of these processes on the variability of the response. When considering a wound assay and the importance of gradient formation for directional guidance, their model predicted that if the effect area of a paracrine signaling ligand were too large, it would reduce the signaling fidelity and thus reduce the SNR by decreasing the signal. The model predicted that an effect distance of approximately 100 μm would maximize the SNR for this system by limiting the noise of cell-to-cell variability while still allowing for enough of a spatial gradient to maintain a strong signal. Indeed, when Handy and colleagues experimentally evaluated this model hypothesis using a novel microfluidic device, the results indicated that on average the extracellular ligand has a proximal effect distance of approximately 100 μm . As we continue to better understand the mechanics underlying cellular signaling dynamics, we will continue to see that the use of fluorescent biosensors may require evaluating how individual cells interact within a population of cells, and computational models will play a key role in helping us understand these interactions.

5.2.4. Information Theory. While cellular communication is often thought to act like a volume knob on a speaker, where turning the knob up or down directly results in an increase or decrease in volume, cellular signaling has to compete with a high level of biological variability and noise. Recently, work aiming to understand how biological signaling networks can faithfully process communication in noisy environments via imprecise signaling by chemical reactions has begun to be addressed through fluorescent biosensors and mathematical

analyses. One framework that is used to address this question is Shannon information theory, which is a mathematical field of study originally developed to study communication over man-made channels but has been extended to study biological communication.⁹⁶⁴ In information theory, information is conceptually defined as the ability to distinctly identify the state of an input or signal for a given output or response, and vice versa.⁹⁶⁴ In biological terms, this usually entails studying how many distinct levels of stimulus concentrations (e.g., receptor agonists) can be conveyed through a cellular signaling network in order to yield distinct responses (e.g., different gene expression profiles).⁹⁶⁴ One early example of information theoretic analysis of mammalian signaling was performed by examining the TNF/NF- κ B signaling pathway using immunofluorescent labeling of NF- κ B and GFP reporter genes.⁹⁶⁵ Interestingly, this work estimated that the TNF/NF- κ B signaling pathway is at most capable of distinguishing only two TNF concentrations.⁹⁶⁵ Fluorescent biosensors were used to extend this analysis beyond measurements at a single time point to time-course studies. Selimkhanov and colleagues used EKAREV⁴⁴¹ targeted to the nucleus to quantify the dynamics of MCF10a cells responding to an array of EGF concentrations to test the hypothesis that temporal dynamics may be able to convey more information.⁹⁶⁶ Through the use of automated image acquisition and analysis, they were able to quantify the ERK biosensor dynamics in over 825000 individual cells and showed that when temporal dynamics are considered, instead of a single time point, this signaling network is capable of encoding more information from an information theory standpoint.⁹⁶⁶ A similar analysis of Ca²⁺ responses to ATP stimulation and the NF- κ B nuclear translocation response to LPS stimulation revealed that including the temporal dynamics for these signaling pathways similarly increased the information carrying capacity. These studies suggest that cells may be able to discern more distinct levels of stimulation if the temporal dynamics are integrated into the downstream signaling outcome. As fluorescent biosensors continue to improve and expand to new signaling domains, these types of analyses will be fundamental in understanding how cells have optimized their ability to function and communicate in a noisy world.

6. PUSHING THE FIELD FORWARD

While genetically encoded biosensors have proven incredibly useful for studying signal transduction, there are several areas in which biosensor-based imaging is highly desirable yet remains a largely unmet need. Below, we highlight several of these areas and discuss both the current obstacles and the ways in which biosensor designs can be and are being adapted to meet these needs and enable more diverse applications.

6.1. Improved Resolution

As discussed in section 4.3, signaling activities are often organized into discrete microdomains or nanodomains. The submicroscopic size of these functional domains makes them difficult to characterize through biosensor imaging based on current diffraction-limited methods. Nevertheless, a number of super-resolution imaging methods have been developed in recent years (reviewed in refs 967 and 968) that enable virtually diffraction-unlimited imaging of subcellular structures (i.e., “fluorescent nanoscopy”). Thus, one strategy for achieving the direct visualization of signaling activity microdomains is to combine existing fluorescent biosensors with a compatible super-resolution imaging modality.

Existing biosensor designs are perhaps most compatible with super-resolution imaging methods that utilize patterned illumination, such as stimulated emission depletion (STED)⁹⁶⁹ or structured illumination microscopy (SIM).⁹⁷⁰ For example, STED involves exciting a diffraction-limited spot while using a second laser to deplete emission within an Airy ring shape to ultimately produce a subdiffraction-sized spot and allow fluorescence imaging below the diffraction limit.⁹⁶⁹ Because this method primarily requires a highly photostable fluorophore, STED-based biosensor imaging is fairly straightforward and has been used, for example, with the intensity-based H₂O₂ biosensor HyPer2.⁹⁷¹ The cpYFP in HyPer2 was found to be more photostable than TagYFP, EYFP, or citrine, thus making it a good candidate for STED imaging. In contrast to the single molecule localization-based super-resolution methods [e.g., stochastic optical reconstruction microscopy (STORM)⁹⁷² and photoactivation localization microscopy (PALM)],⁹⁷³ STED is a super-resolution technique that maintains the fluorescence intensity information in the final image, which can be used as the biosensor readout. With this improved spatial resolution, Mishina and colleagues observed H₂O₂ microdomains that were as small as 100–200 nm across.⁹⁷¹ Similarly, a recently developed pH biosensor, SRpHi, which is composed of an FP and an organic fluorescent dye that have differing pH sensitivities, was used in conjunction with two-color STED to perform ratiometric super-resolution imaging.⁹⁷⁴ This biosensor was used to study the pH of endosomes, as many structures in the endocytic pathway are <250 nm in size, making it difficult to distinguish closely packed structures. While SRpHi is not a fully genetically encoded fluorescent biosensor, given its requirement for a covalently linked fluorescent dye, the ratiometric nature of the biosensor helps reduce concentration artifacts, and its cellular delivery is facilitated by the endocytosis process itself.

On the other hand, several new biosensors have been developed that are explicitly designed for super-resolution imaging. As alluded to previously (see sections 2.2.2 and 2.4.3), these biosensor designs largely utilize photoswitchable FPs in conjunction with localization-based super-resolution imaging methods such as PALM⁹⁷³ or stochastic optical fluctuation imaging (SOFI).^{27,975} In particular, a few BiFC-based strategies have been used to visualize PPIs in super-resolution, including BiFC-PALM based on split versions of either the photoactivatable FP PA-mCherry⁹⁷⁶ or the green-to-red photoconvertible FP mEos3.2,⁷⁸ as well as reconstituted fluorescence-based SOFI (refSOFI), which utilizes the photo-switchable FP Dronpa.⁸⁰ However, BiFC offers only a limited ability to track dynamics processes, for instance, due to the relatively slow kinetics of FP reconstitution. RefSOFI may be advantageous in this regard, however, as fluctuations are only observed when overall FP intensity is dim, meaning that super-resolution information can be obtained without having to wait for complete FP maturation.⁸⁰

Nevertheless, because BiFC is fundamentally irreversible, other designs are needed to fully capture dynamic biochemical processes in super-resolution, such as the recently developed FLINC-based biosensors, which utilize a molecular switch to control FP proximity and reversibly modulate fluorescence fluctuations⁴⁷⁷ (discussed in section 2.4.3). Yet while FLINC is indeed capable of capturing biochemical activity dynamics, such as changes in PKA activity, the temporal resolution of this method is still somewhat constrained. Specifically, each super-resolution image in a time-course is itself derived from a series

of fluctuation images collected under high-intensity illumination, and the need to collect enough information to construct super-resolution images at each time point must be balanced with the need to minimize photobleaching across the experimental time-course. In the current implementation, this balance is achieved by incorporating periods of recovery between sets of imaging frames.⁴⁷⁷ However, improvements in imaging technology and FP engineering will undoubtedly yield increases in temporal resolution, while new biosensor designs will continue to emerge that offer additional strategies for performing dynamic, super-resolution activity mapping.

6.2. Multiplexed Biosensor Imaging

Signaling networks are complex and feature a high degree of connectivity, often integrating several different signaling pathways. The ability to track multiple signaling activities within the same reference frame would therefore be tremendously useful for unraveling this complex web. Multiplexing refers to the ability to send or receive several messages simultaneously, which for fluorescent biosensors entails the ability to measure multiple biosensor readouts with respect to the same frame of reference. In general, this means simultaneously measuring the responses from two or more biosensors within the same cell, though in so-called “computational multiplexing”, measurements obtained from different biosensors expressed in separate cells can be linked through a common fiduciary marker, such as the movement of the plasma membrane.⁹⁷⁷ Nevertheless, the most significant physical constraint on multiplexing remains the limited spectral space available for imaging multiple biosensors in the same cell.^{977–979} Thus, a considerable segment of biosensor development is devoted to reducing the spectral space occupied by fluorescent biosensors.

One strategy to save spectral space for potential multiplexing applications is to expand the engineering of single-color, intensity-based fluorescent biosensors to monitor signaling activities outside the well-developed areas of Ca^{2+} and voltage biosensors, as exemplified by the recent development of single-color sensors for NADH,^{350,352,353} cAMP,^{189,192,211} and certain neurotransmitters^{174,204–206} and kinases^{116,211} (Table 1). Meanwhile, alternative methods are also being devised to convert dual-wavelength FRET-based biosensors into single-color reporters. First, biosensors have been developed to take advantage of donor fluorescence quenching resonance energy transfer (FqRET), which specifically measures the decrease in donor fluorescence that occurs as a result of energy transfer (Figure 15A). These sensors exploit the fact that the acceptor need not be an FP, as any light-absorbing protein can serve as a FRET acceptor provided it satisfies the conditions for energy transfer. Thus, Ganesan et al. were able to develop a ubiquitination biosensor in which GFP was paired with a YFP-based “dark” acceptor, resonance energy-accepting chromoprotein (REACH), wherein FqRET allows what would normally be a dual-wavelength FRET probe to provide a single-color readout.⁶⁵⁵ The use of this dark acceptor-based FqRET has since been expanded to other signaling pathways and colors, including a blue cGMP biosensor,³²⁴ red and green CaMKII biosensors,^{454,456} and red Cdc42 and RhoA biosensors.⁴⁵⁴ Finally, single-color biosensors based on ddFPs (see section 2.3.3.2.4) offer another potential avenue for multiplexed imaging.^{211,381} In fact, we recently achieved 6-parameter multiplexed imaging of kinase and second

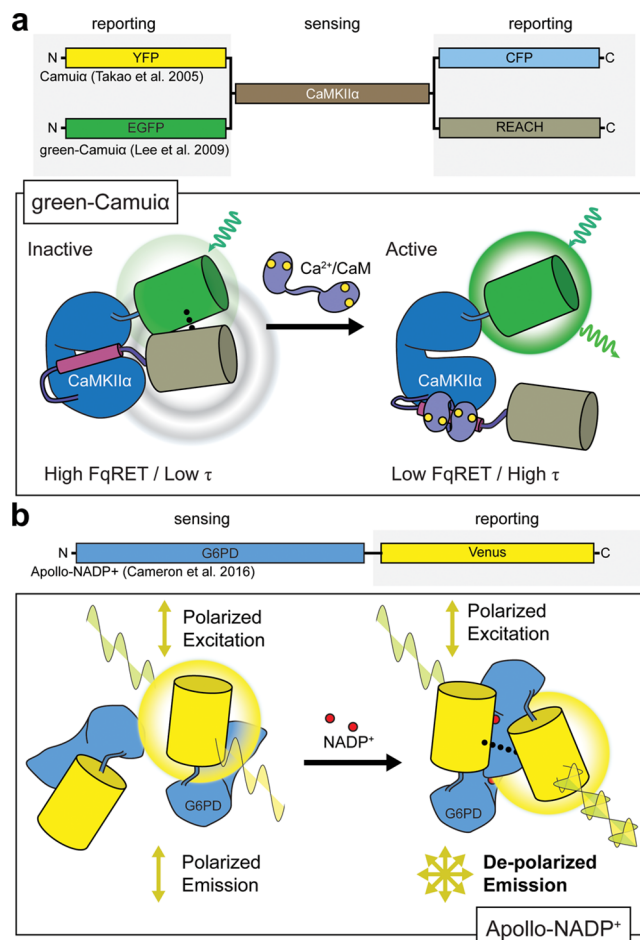


Figure 15. Single-color FRET-based methods. (A) Fluorescence quenching resonance energy transfer (FqRET)-based CaMKIIα biosensor green-Camuia.⁴⁵⁶ In the inactive state, the energy from excited EGFP is nonradiatively transferred to the dark acceptor REACH, which then dissipates that energy without emitting a photon. Upon activation, the conformational change moves REACH away from the EGFP, leading to increased EGFP emission. FqRET can also be quantified by fluorescence lifetime imaging (FLIM), where the lifetime, τ , is low in the high-FqRET state and vice versa. (B) Homo-FRET-based NADP⁺ biosensor Apollo-NADP⁺.³⁵⁴ Upon binding NADP⁺, G6PD dimerizes and thus allow FRET between the two FPs. Fluorescence polarization microscopy can be used where a polarized excitation source will only excite FP in the appropriate dipole orientation, resulting in the emitted photon also being polarized. Conversely, when FRET occurs between these two FPs, the emitted photons exhibit a mix of polarizations and thus decrease the polarized fluorescence signal.

messenger dynamics using a combination of cpFP- and ddFP-based biosensors.²¹¹

Similarly, homo-FRET, in which excited-state energy is transferred between two fluorophores with similar or identical spectra rather than from a shorter-wavelength fluorophore to a longer-wavelength fluorophore, can also be used to generate single-color biosensors.⁵³⁹ Notably, because the donor and acceptor can no longer be distinguished by their emission wavelengths, visualizing the responses from homo-FRET-based biosensors requires measuring changes in fluorescence anisotropy, wherein the loss of polarized fluorescence emission indicates an increase in energy transfer⁹⁸⁰ (Figure 15B). Two such homo-FRET biosensors, namely, Apollo-NADP⁺³⁵⁴ and

mCherry-Akt-PH,⁵³⁹ have been successfully multiplexed with biosensors for H_2O_2 and Ca^{2+} , respectively. Additionally, a suite of homo-FRET-based biosensors for PKA, PKC, ERK, Myosin Light Chain Kinase, and cAMP were recently published that utilize fluorescence anisotropy microscopy to achieve multiplexing while maintaining a ratiometric read-out.⁴⁶⁷ Thus, as the arsenal of fluorescent probes continues to expand, fluorescent biosensors will increasingly be developed with multiplexing in mind.

6.3. Applications to More Complex Biological Systems

While a majority of the studies discussed in this review have utilized genetically encoded fluorescent biosensors in more in vitro settings, the application of fluorescent biosensors to visualize cellular processes in vivo is becoming increasingly important. In vivo biosensor imaging can provide deeper insights into the behavior of signaling pathways under physiologically relevant conditions,⁹⁸¹ can be used to study responses to more complex stimuli,¹⁶⁴ and can be used to correlate signaling dynamics with organ physiology measurements or organismal behaviors.⁹⁸² However, in vivo biosensor imaging faces a number of challenges related to autofluorescence from endogenous cellular components, as well as absorption and scattering of the excitation and emission light.⁹⁸³

As described in section 2.4.1, mammalian cells and tissues exhibit much less absorption and scattering of light in the NIR region of the spectrum,⁷³¹ and several FPs that are excited and emit in this spectral range have in fact been developed to take advantage of this phenomenon.^{9,984} Fluorescent biosensors that utilize these NIR FPs have thus far been developed for monitoring caspase activity⁵⁶⁸ and cell cycle progression.^{222,223} For example, the infrared caspase 3 reporter iCasper (Figure 7 and section 2.4.1) was used to quantify apoptosis in vivo throughout *Drosophila* development and show that apoptotic cells were spatiotemporally correlated with certain developmental steps.⁵⁶⁸ NIR FPs also enable the development of far-red-shifted FRET sensors; FRET between the far-red FP mKate2 and the NIR FP iRFP has been successfully utilized in the development of a proof-of-concept caspase-3 biosensor.⁵⁶⁶ Furthermore, recently published work by Shcherbakova and colleagues showed that miRFP670 and miRFP720 are a compatible NIR FRET pair and was utilized to create NIR variants of biosensors for Rac1, PKA, and JNK, which can be multiplexed with common CFP-YFP FRET biosensors.⁴⁸⁹

Pending the more widespread development of NIR fluorescent biosensors, a more general approach to achieving higher-quality biosensor imaging in tissues has been to use multiphoton imaging, which is based on the absorption of long-wavelength photons that reach the fluorophore simultaneously (e.g., within less than a femtosecond), thereby triggering excitation and normal fluorescence behavior.^{985,986} Multiphoton imaging reduces background fluorescence due to scattering through the use of longer-wavelength illumination, as well as by reducing the imaging volume of excited fluorophores, thereby facilitating in vivo imaging (for more background on multiphoton imaging see ref 987). For example, by using two-photon excitation to image GCaMP6 in mouse visual cortex V1 neurons in vivo, Chen and colleagues were able to record Ca^{2+} transients in the brains of mice presented with different visual stimuli.¹⁶⁴ While the use of two-photon imaging with this and other single-color, intensity-based fluorescent biosensors, such as the glutamate

biosensor iGluSnFr,⁹⁸⁸ has been fairly straightforward, utilizing multiphoton imaging in conjunction with FRET-based biosensors may require some additional biosensor optimization. For example, in order to develop a two-photon-compatible version of AKAR, Tao and colleagues replaced the Cerulean in AKAR4 with mTurquoise to improve the two-photon excitation profile.⁵⁰¹ Another strategy to facilitate in vivo imaging of genetically encoded biosensors may be to forego the use of excitation light altogether by using luminescent proteins such as the Nano-lantern chimera developed by Saito et al. (discussed in Section 2.3.3.3), which has been shown to be compatible with whole-body in vivo imaging in mice.²⁹⁰ Furthermore, the development of enhanced Nano-lantern color variants containing red-shifted FPs is expected to yield further improvements through increased tissue penetration by the emitted light.³⁹⁷ Recently, Iwano and colleagues used directed evolution of FLuc to generate a luciferase, Akaluc, that utilizes the infrared substrate AkaLumine-HCl. The Akaluc and AkaLumine-HCl pair offers a number of exciting features, including greater catalytic activity and expression levels than the parental pair, excellent bioavailability, and low toxicity.⁷²⁵ These improvements, as well as the infrared emission, made it possible to detect single cells in deep tissues of living mice and to measure long-term bioluminescence in living marmosets. In the future, integration of Akaluc into a biosensor should enable the in vivo quantification of signaling dynamics in animals while they are free to complete complex tasks.

Finally, genetically encoded fluorescent biosensors may potentially be combined with photoacoustic imaging, in which an excited fluorophore does not emit a photon but instead undergoes thermoelastic dissipation, which can induce an ultrasonic pressure wave.⁹⁸⁹ Although photoacoustic imaging has a lower spatial resolution than fluorescence imaging, it can enable much deeper tissue penetration.⁹⁹⁰ Several FPs have already been utilized in photoacoustic imaging due to their known absorption spectrum, but FPs with low quantum yields tend to work much better because energy that is released as a photon cannot be dissipated thermoelastically.⁹⁹⁰ Interestingly, Li et al. discovered that FRET can be utilized for photoacoustic imaging when an FP such as EGFP is coupled with the photoacoustic-optimized dark fluorescent protein tdUltramarine2, as shown through a proof-of-concept protease biosensor.⁹⁹¹ While genetically encoded photoacoustic biosensors for signal transduction have not been demonstrated in vivo, these new types of biosensors could enable even deeper in vivo imaging in the near future.

6.4. Translational Applications of Genetically Encodable Fluorescent Biosensors

As fluorescent biosensors have both expanded in diversity and increased in sensitivity, researchers in both academia and industry have begun to utilize genetically encodable biosensors in high-throughput screens and clinical assays. The strengths of fluorescent biosensors, namely, their ability to quantify live-cell kinetics and their subcellular targetability, make them desirable tools for high-throughput screening, but several obstacles must often be overcome to obtain efficient and selective high-throughput assays (reviewed in ref 992). Among the most important requirements is a high SNR. Fortunately, improvements in both biosensor design and FP characteristics over several generations of development can often yield biosensors with sufficient SNR for high-throughput screens. For example,

Allen and colleagues demonstrated in a fluorescent plate-reader-based assay that while the Z' factor of the second-generation PKA biosensor AKAR2⁵⁰⁷ was less than the industry standard of 0.5, the third-generation AKAR3⁵⁰⁶ biosensor had a Z' factor of 0.84.⁹⁹³

More recently, Zhao et al. developed a NAD^+/NADH biosensor, SoNAR, and used it to screen for metabolism-based antitumor compounds.²⁰⁹ By screening several compound libraries against a lung cancer cell line expressing SoNAR, they found that, surprisingly, a reported AKT inhibitor KP372-1 caused the greatest increase in the NAD^+/NADP ratio and was selectively toxic to cancer cell lines. To further examine the mechanism by which KP372-1 altered metabolism, this group used the fluorescent biosensors Hyper⁵⁹⁵ and roGFP1¹⁴³ to measure H_2O_2 and disulfide redox state, respectively, and found that KP372-1 potentially induced oxidative stress.²⁰⁹ Additional work suggested that the NAD(P)H -dependent reactive oxygen species generating enzyme NQO1 modulated both the oxidative stress and cell toxicity stimulated by KP372-1. On the other hand, the growing number of fluorescent biosensors is beginning to enable a systems-level view of signaling by directly examining the responses from several different biosensors to a given compound. Taking advantage of this, Kuchenov et al. developed a FRET-based multiparameter imaging platform where an array of fluorescent biosensors is reverse-transfected into adherent cells in a spatially defined manner.⁹⁹⁴ These examples are indicative of the expanding role of fluorescent biosensors in drug discovery through both the identification of lead compounds and the characterization of hits after their identification.

Finally, as both fluorescent biosensors and the corresponding data acquisition become more robust, the translation of their use into the clinical setting may become an area of increasing interest. Mizutani and colleagues reported a proof-of-concept approach to personalized medicine in which they expressed their improved Bcr-Abl biosensor, pickles2.3, in cancer cells derived from chronic myeloid leukemia patients.⁴⁵² Using 293F cells expressing a wild-type Bcr-Abl fusion protein or mutants that are resistant to the first-line therapy imatinib, they showed that the biosensor readout decreased in response to imatinib treatment in wild-type Bcr-Abl cells but not in mutant-expressing cells. Furthermore, mixing these two cell types together at different ratios revealed that the percentage of single-cell biosensor responses that were above an observed threshold was related to the proportion of resistant mutant Bcr-Abl cells within the mixture. Using this model readout, they demonstrated a method of testing different combinations of cotherapy to identify the appropriate combination to catch both the majority of imatinib-sensitive cells and the smaller resistant population. While this assay would be too labor intensive to be practically applied in the clinic, advances in biosensor measurement throughput could one day make fluorescent biosensor applications in patient cells feasible.

Alternatively, biosensors that have been developed and utilized in living cells have the potential to be engineered into “cell-free” assays for simple and rapid quantification. For example, Yu et al. recently extended the SNIFIT biosensor design to generate a BRET-based biosensor for NADPH that is still functional when lyophilized onto test paper.³⁵⁶ This assay was then applied to quantify phenylalanine levels in phenylketonuria patient blood samples where an engineered enzyme catalyzes the conversion of phenylalanine and NADP^+ to phenylpyruvate and NADPH, thus allowing the quantification

of NADPH as a proxy for phenylalanine levels. In this assay, the biosensor, enzyme, and luciferase substrate are all lyophilized onto the test paper, with the BRET readout being measured with a digital camera after the patient blood sample is applied to the paper. Furthermore, this coupling of NADP^+ -dependent enzymatic oxidation to an optical readout for NADPH may be readily extended to other clinically important targets. As these innovations continue to advance, there will most assuredly come a time when optical biosensors play a crucial role in both medical diagnostics and personalized medicine.

7. CONCLUSION

The late Roger Tsien, one of the great pioneers of fluorescent proteins, once said “our work is often described as building and training molecular spies, molecules that will enter a cell or organism and report back to us what the conditions are”.⁹⁹⁵ Genetically encoded fluorescent biosensors have truly become these powerful molecular spies that illuminate the spatiotemporal dynamics of cellular communication and decision making. As the development of fluorescent biosensors has progressed, we have seen both improvements in sensitivity and an expansion of the breadth of signaling pathways that can be studied (Table 1). Here, we have discussed the different categories of biosensor designs utilized to quantify signaling dynamics, including several recently emerging classes of biosensors based on infrared fluorescent proteins, biosensors that act as signal integrators, and biosensors that utilize fluorescence fluctuations for super-resolution activity mapping (section 2). The dynamic nature and fluorescent readout that underlie these designs have enabled the quantification of the kinetics of signal transduction as it occurs throughout the cell. These *in situ* measurements have transformed our understanding of signaling dynamics ranging from individual reactions to evaluating the origins and regulation of complex signaling phenomena (section 3). Furthermore, the ability to observe spatial changes in biosensor signals, either through direct spatial quantification or the use of subcellular targeting, has not only confirmed the existence of spatially compartmentalized signaling domains but also provided the means with which to probe the mechanisms that give rise to this intricate spatial organization (section 4). While the multifaceted nature of signal transduction can make it difficult to directly infer the critical regulators that underlie the observations made using fluorescent biosensors, computational models have been a useful companion to biosensor-based studies, as computational models benefit from the high temporal and spatial resolution of fluorescent biosensors and can in turn quantitatively evaluate potential hypotheses and probe aspects of biology that are not directly measurable experimentally (section 5). Additionally, the adaptable nature of fluorescent biosensors has made them ideal for validating a number of computational model predictions. Finally, continuing advances are enabling fluorescent biosensor applications beyond the diffraction limit, biosensor multiplexing for simultaneous observation of multiple signaling pathways, and the development of biosensors that are more suitable for *in vivo* imaging (Section 6). These advances in genetically encoded fluorescent biosensor design will continue to push the boundaries, both spatial and temporal, of the study of signaling dynamics, with the dual goals of expanding our understanding of the native function of signaling networks and translating these tools into the

discovery and evaluation of novel therapeutic avenues for a myriad of diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrev.8b00333.

Comprehensive table containing details about the published biosensors from Table 1, including the sensing unit, FPs used, and readout method (Table S1) (XLSX)

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Notes

The authors declare no competing financial interest.

Biographies

Eric C. Greenwald attended the University of Colorado, where he received a B.S. in Chemical and Biological Engineering, a B.S. in Applied Mathematics, and an M.S. in Chemical Engineering. He then pursued a Ph.D. in Biomedical Engineering at the University of Virginia. Under the guidance of Dr. Jeff Saucerman, Eric examined how the coordination of signaling proteins by scaffold proteins changes the underlying kinetic mechanism of signal transduction between a tethered enzyme and substrate. Following graduation, Eric joined Jin Zhang's lab at the University of California, San Diego, where he is developing tools to increase the throughput of fluorescent biosensor data acquisition.

Sohum Mehta received a B.S. in Biology, with a minor in Fine Arts, from George Washington University before going on to pursue graduate studies at Johns Hopkins University, where he received a Ph.D. in Biology. For his thesis work, Sohum studied calcineurin signaling in the yeast *Saccharomyces cerevisiae*, performing structure–function analyses of the evolutionarily conserved regulator of calcineurin (RCaN) protein family. Sohum joined Jin Zhang's lab as a postdoctoral fellow at the Johns Hopkins University School of Medicine and continues to serve as a senior researcher in the lab at its new home at the University of California, San Diego, where his work includes developing novel genetically encoded tools for the sensitive and multiplexed visualization of intracellular signaling.

Jin Zhang attended Tsinghua University for her undergraduate studies and pursued her graduate studies in Chemistry at the University of Chicago. After completing her postdoctoral work at the University of California, San Diego, she joined the faculty of Johns Hopkins University School of Medicine in 2003. She was promoted to Professor of Pharmacology, Neuroscience and Oncology in 2013. In 2015, she moved back to University of California, San Diego, and is currently a member of the Moores Cancer Center and a Professor in Departments of Pharmacology, Bioengineering, and Chemistry and Biochemistry at UC San Diego. Research in her lab focuses on developing enabling technologies to probe the active molecules in their native environment and characterizing how these active molecules change in diseases including cancer. Professor Zhang has

received many awards including the National Institutes of Health (NIH) Director's Pioneer Award (2009), the John J. Abel Award in Pharmacology from American Society for Pharmacology and Experimental Therapeutics (ASPET) (2012), the Pfizer Award in Enzyme Chemistry from American Chemical Society (2012), and National Institute of Cancer Outstanding Investigator Award (2015). She was elected as a Fellow of the American Association for the Advancement of Science (AAAS) in 2014. She serves on the editorial advisory board of *Cell Chemical Biology* and is the past-chair of Molecular Pharmacology Division of ASPET and Secretary/Treasurer-Elect of ASPET.

ACKNOWLEDGMENTS

The authors would like to Jason Zhang, Xin Zhou, Jeremiah Keyes, Brian Tenner, Arielle Yoo, Chris Booth, and Yanghao Zhong for their help in compiling the database of fluorescent biosensors. Work in this lab is funded by the NIH (F32 GM120798 to E.G. and R35 CA197622, R01 DK073368, R01 GM111665, and R01 MH111516 to J.Z.) and DOD (AFOSR FA9550-18-1-0051 to J.Z.)

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