An update on genetically encoded lipid biosensors

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ABSTRACT **Specific lipid species play central roles in cell biology. Their presence or enrichment in individual membranes can control properties or direct protein localization and/or activity. Therefore, probes to detect and observe these lipids in intact cells are essential tools in the cell biologist's freezer box. Herein, we discuss genetically encoded lipid biosensors, which can be expressed as fluorescent protein fusions to track lipids in living cells. We provide a state-of-the-art list of the most widely available and reliable biosensors and highlight new probes (circa 2018–2021). Notably, we focus on advances in biosensors for phosphatidylinositol, phosphatidic acid, and PI 3-kinase lipid products.**

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INTRODUCTION

Lipids are fundamental building blocks of cellular life. Their amphiphilic nature makes them a keystone of bilayer membranes, as simply and elegantly illustrated by the double-tailed "tadpoles" of so many BioRender cartoons. Yet this deft simplicity belies the diversity of phospholipid, sphingolipid, and sterol species that make up biological membranes. The tightly crafted recipe of these lipids, with their unique shapes and charges, endows key functional properties on membranes: fluidity, curvature, and the capacity to selectively recruit or activate proteins are all regulated by lipids (Meer *et al.*, 2008; Meer and Kroon, 2011; Balla, 2012). For this reason, cell biology demands approaches that can detect and enumerate membrane lipid compositions in their native cellular environment (Stahelin, 2009; Narwal *et al.*, 2018; Dickson and Hille, 2019; Quinville *et al.*, 2021).

This is where the genetically encoded lipid biosensors enter: these are typically lipid-binding domains from effector proteins or pathogen toxins, engineered to incorporate a tag for detection. Most conveniently, this involves fusion to a fluorescent protein for imaging in live cells. In this way, lipid biosensors can give information about the relative abundance, dynamics, and subcellular localization of lipids—in real time and in living cells. On the downside, the biosensors may be subject to biases in their localization, especially when not thoroughly characterized. We previously proposed two main criteria a biosensor should satisfy: 1) Is the biosensor selective for the lipid? This is typically determined in vitro. 2) Is the presence of the lipid both necessary and sufficient to localize the biosensor? This must be determined by modulation of lipids in the native cellular environment, and is often overlooked (Wills *et al.*, 2018). Other caveats that must be considered include limitation to the detection of lipids in the outer plasma membrane or cytosolic membrane, because limits of fluorescence microscopy make interpreting localization in organelle lumens challenging. There are also extreme challenges to calibration, generally preventing quantification in terms of absolute lipid mass or mole fraction, though there have been technical tour-de-force studies that have done so (e.g., Liu *et al.*, 2018). The strengths and weaknesses of lipid biosensors have already been explored in depth by ourselves and others (Balla *et al.*, 2000; Lemmon, 2003; Maekawa and Fairn, 2014; Hammond and Balla, 2015; Wills *et al.*, 2018). Suffice to say here, when it comes to genetically encoded lipid biosensors, a quote from Han Mi-nyeo, a character in the hit Netflix show *Squid Game*, sums it up: "I'm good at everything, except the things I can't do."

There have been many comprehensive reviews detailing currently available lipid biosensors (Stahelin, 2009; Kay *et al.*, 2012; Maekawa and Fairn, 2014; Hammond and Balla, 2015; Narwal *et al.*, 2018; Wills *et al.*, 2018). We refer the reader to these resources for a comprehensive picture. Our goal here is to summarize a few notable

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Abbreviations used: Chol, cholesterol; DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic reticulum; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; NES, nuclear export sequence; PA, phosphatidic acid; PABD, phosphatidic acid binding domain; PASS, phosphatidic acid biosensor with superior sensitivity; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphoinositide 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI5P, phosphatidylinositol 5-phosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PLD, phospholipase D; PM, plasma membrane; PS, phosphatidylserine; SM, sphingomyelin.

Lipid	Biosensor	Affinity	Lipid specific?	Cellular localization of lipid		References
				Lipid dependent?	Lipid sufficient?	
Chol	D4-PFO + mutants	2–30 mol%	✔	✔	?	Shimada *et al.*, 2002; Maekawa and Fairn, 2015; Liu *et al.*, 2016
SM	Lysenin	K_d ~ 5 nM	✔	✔	?	Yamaji *et al.*, 1998; Kiyokawa *et al.*, 2005; Abe *et al.*, 2012
PA	NES-PABD-spo20p$^{51–91}$ (PASS)	?	✘ - binds PI(4,5)P_2 and PIP_3 weakly	✔	?	Zhang *et al.*, 2014
	NES-2xPABD-spo20p$^{51–91}$	?	✘ - suspected to bind PI(4,5)P_2 and PIP_3 weakly	✔	?	Bohdanowicz *et al.*, 2013
	α-Syn-N	K_d ~ 6.6 μM (18:1/18:1 PA)	✔	✔	?	Yamada *et al.*, 2020
PS	C2-lactadherin	K_d ~ 0.5 μM	✔	✔	✔	Yeung *et al.*, 2008; Maeda *et al.*, 2013; Vecchio and Stahelin, 2018
DAG	C1ab-PDK1	K_i (PDBu) ~ 0.2 μM	✔	✔	✔	Chen *et al.*, 2008; Kim *et al.*, 2011
	C1ab-PKCε	K_d ~ 10 nM	✔	✔	?	Stahelin *et al.*, 2005; Domart *et al.*, 2012
PI	BcPI-PLC^{H82A}	?	✘ - binds to PC	✔	✔	Pemberton *et al.*, 2020
	BcPI-PLC^{ANH}	?	✘ - binds DAG	✔	✔	Pemberton *et al.*, 2020
PI4P	P4M-SidM	K_d ~ 1 μM or ~18.2 nM FL	✔	✔	✔	Brombacher *et al.*, 2009; Schoebel *et al.*, 2010; Hammond *et al.*, 2014
	P4M-SidMx2	K_d < P4M-SidM	✔	✔	✔	Hammond *et al.*, 2014; Levin *et al.*, 2017
	P4C-SidC	K_d ~ 250 nM	✔	✔	✔	Dolinsky *et al.*, 2014; Weber *et al.*, 2014; Zewe *et al.*, 2018
	N-PH-ORP5, N-PH-ORP8	K_d ~ 5 μM for PI(4,5)P_2	✘ - binds PI4P and PI(4,5)P_2	✔	✘ - requires PI(4,5)P_2	Chung *et al.*, 2015; Ghai *et al.*, 2017; Sohn *et al.*, 2018
	PH-OSBP, PH-FAPP1	K_d ~ 250 nM	✘ - binds PI(4,5)P_2	✔	✘ - requires Arf1	Levine and Munro, 2002; Szentpetery *et al.*, 2010; Lenoir *et al.*, 2015
PI5P	3xPHD (ING2)	?	✘ - binds to PI3P	✔	✔	Gozani *et al.*, 2003; Pendaries *et al.*, 2006
PI(4,5)P_2	PH-PLCδ1	K_d ~ 2 μM	✘ - binds to IP_3~20-fold more tightly than PI(4,5)P_2	✔	✔	Garcia *et al.*, 1995; Lemmon *et al.*, 1995; Stauffer *et al.*, 1998; Várnai and Balla, 1998; Hirose *et al.*, 1999; Suh *et al.*, 2006
	PH-PLCδ4	K_d > PH-PLCδ1	✘ - binds to IP_3	✔	✔	Lee *et al.*, 2004; Hammond and Balla, 2015

TABLE 1: Current genetically encoded lipid biosensors for a variety of selective lipid species.

(*Continued*)

Lipid	Biosensor	Affinity	Lipid specific?	Cellular localization of lipid		References
				Lipid dependent?	Lipid sufficient?	
	$Tubby_c$	K_d > PH-PLCδ1	✘ - binds $PI(3,4)P_2$ and $PI(3,4,5)P_3$	✔	✔	Quinn *et al.*, 2008; Szentpetery *et al.*, 2008; Halaszovich *et al.*, 2009; Hammond and Balla, 2015
	$Tubby_c^{R332H}$	K_d > Tubby	✘ - binds $PI(3,4)P_2$ and PIP_3	✔	?	Quinn *et al.*, 2008
	ENTH/ANTH	K_d ~ 2 μM	✘ - binds to PIP_3	✔	?	Ford *et al.*, 2001; Itoh *et al.*, 2001; Yoon *et al.*, 2011
PI3P	FYVE-Hrsx2	K_d ~ 2.5 μM	✔	✔	?	Burd and Emr, 1998; Gaullier *et al.*, 1998; Gillooly *et al.*, 2000; Sankaran *et al.*, 2001
	FYVE-EEA1	K_d ~ 45 nM	✔	✔	?	Burd and Emr, 1998; Gaullier *et al.*, 1998, 2000
	PX-p40phox	K_d ~ 5 μM	✔	✔	?	Bravo *et al.*, 2001; Ellson *et al.*, 2001; Kanai *et al.*, 2001
$PI(3,5)P_2$	ML1-Nx2	K_d ~ 5.6 μM	✔	✔/✘[a]	✔/✘[a]	Li *et al.*, 2013; Hammond *et al.*, 2015
$PI(3,4)P_2$	PH-TAPP1-CT	K_d ~ 80 nM	✔	✔	✔	Dowler *et al.*, 2000; Thomas *et al.*, 2001; Kimber *et al.*, 2002; Marshall *et al.*, 2002; Manna *et al.*, 2007
	eTapp1-PH[c]	K_d ~ 80 nM	✔	✔	✔	Liu *et al.*, 2018
	TAPP1-cPHx3	K_d > 80 nM	✔	✔	✔	Goulden *et al.*, 2019
PIP_3	$PH\text{-}ARNO^{2G\text{-}I303E}x2$	K_d ~ 170 nM	✘ - binds IP_4	✔	✔	Goulden *et al.*, 2019
	eMyoX-PHx2[c]	K_d ~ 33 nM[b]	✘ - binds IP_4	✔	✔	Hokanson *et al.*, 2006; Plantard *et al.*, 2010; Lu *et al.*, 2011; Liu *et al.*, 2018
	PH-Akt	K_d ~ 590 nM	✘ - binds $PI(3,4)P_2$ and IP_4	✔	?	Frech *et al.*, 1997; Watton and Downward, 1999; Manna *et al.*, 2007
	PH-Btk	K_d ~ 80 nM	✘ - binds IP_4	✔	?	Fukuda *et al.*, 1996; Salim *et al.*, 1996; Rameh *et al.*, 1997; Kontos *et al.*, 1998; Manna *et al.*, 2007
	PH-GRP1 (2G), PH-ARNO (2G)	K_d ~ 170 nM	✘ - binds IP_4	✔	✘ - binds Arf/Arl	Klarlund *et al.*, 1997; Venkateswarlu *et al.*, 1998; Gray *et al.*, 1999; Cohen *et al.*, 2007; Hofmann *et al.*, 2007; Li *et al.*, 2007; Manna *et al.*, 2007

[a]The accuracy of this probe is disputed.
[b]The K_d value is derived from myosin-c tail and IP_4 headgroup binding.
[c]Requires chemical ligation with a solvatochromic dye for optimal performance.

TABLE 1: Current genetically encoded lipid biosensors for a variety of selective lipid species. Continued

recent advances and tools available for specific lipids. We also present an updated table (Table 1) showing some of the most widely used and (in our opinion) reliable genetically encoded lipid biosensors.

The phosphoinositide that we all forget about

There have been many iterations of biosensors for phosphoinositides, which are cardinal regulators of membrane function (Dickson and Hille, 2019). These lipids are all phosphorylated derivatives of a single parent lipid, phosphatidylinositol (PI). PI is a major lipid, approximately 10% of cellular phospholipids, with the derivatives being <1% (Vance, 2015), but a biosensor for PI itself had been lacking. As an abundant lipid, its distribution may be assumed to be ubiquitous. Yet, because PI is the key substrate for synthesis of the other phosphoinositides, its availability in given membranes is an important variable best not left to assumption.

Pemberton *et al.* recently created a PI biosensor utilizing the *Bacillus cereus* PI-specific phospholipase C (*Bc*PI-PLC). *Bc*PI-PLC was mutated in order to eliminate catalytic activity of the enzyme, yet retain the active site configuration that can accommodate the inositol headgroup (*Bc*PI-PLC^{H82A}). However, in vitro assays showed nonspecific *Bc*PI-PLC^{H82A} binding to PC-containing liposomes. To remove this nonspecific binding, two membrane-penetrating tyrosine residues were mutated to create the *Bc*PI-PLC^{ANH} probe. It should be noted, though, that neither the *Bc*PI-PLC^{H82A} nor the *Bc*PI-PLC^{ANH} probes are fully specific for PI in vitro, as the *Bc*PI-PLC^{ANH} showed enhanced binding to liposomes containing DAG and PI (Pemberton *et al.*, 2020). PI was shown to be necessary for membrane localization of both sensors, because depletion of PI by PI-PLC recruitment or *Ang*II stimulation caused a decrease in membrane localization of the biosensors. The sufficiency of PI for biosensor recruitment was demonstrated when PI levels at the plasma membrane were increased with either pseudojanin-induced degradation of PI4P and PI(4,5)P_2 to PI, or GSK-A1 inhibition of PI4KA-mediated conversion of PI to PI4P (Pemberton *et al.*, 2020). Notably, the *Bc*PI-PLC^{ANH} probe showed similar patterns of localization compared with *Bc*PI-PLC^{H82A} within cells. However, the *Bc*PI-PLC^{ANH} showed more cytosolic localization than *Bc*PI-PLC^{H82A}, indicating that *Bc*PI-PLC^{H82A} may be a higher affinity probe for PI.

These probes revealed a surprising distribution of PI: an abundance at the endoplasmic reticulum (ER), peroxisomal, Golgi, and mitochondrial cytosolic leaflets, some on the endosomal network, but a notable absence at the plasma membrane at steady state (Pemberton *et al.*, 2020). Satisfyingly, these findings were corroborated by additional approaches, including acute activation of PI-PLC or PI4Ks to generate diacylglycerol or PI4P from PI localized in specific membranes, which could be detected with other biosensors for these lipids (Pemberton *et al.*, 2020; Zewe *et al.*, 2020), and the trafficking of exogenously applied fluorescent PI (Zewe *et al.*, 2020). Taken together, these results support a model where PI within the ER is transferred to the plasma membrane (PM) and then quickly converted into PI4P and PI(4,5)P_2 to maintain homeostasis of these crucial PM phosphoinositide species.

The phospholipid backbone: phosphatidic acid

Phosphatidic acid (PA) is a crucial lipid, being both an intermediate in more complex phospholipid biosynthesis, and a second messenger molecule in diacylglycerol kinase (DGK) and phospholipase D (PLD) signaling pathways (Thakur *et al.*, 2019). The most widely used biosensor is the phosphatidic acid biosensor with superior sensitivity (PASS) developed by Zhang *et al.* (2014). An added nuclear export sequence (NES) to the Spo20 phosphatidic acid–binding domain (PABD) prevented accumulation of PASS within the nucleus. This newly designed probe was able to show clear translocation to the PM after stimulation with phorbol-12-myristate-13-acetate, without having to overexpose images. However, the PASS did still retain some slight binding to PI(4,5)P_2 and PIP_3 within liposomes that the original Spo20 biosensor also showed (Zhang *et al.*, 2014). A higher avidity, tandem dimer has also been developed (Bohdanowicz *et al.*, 2013).

The usefulness of these PA biosensors has been recently corroborated by some new, ingenious tools, which have increased confidence in the accuracy of the Spo20-based PA lipid biosensors. An optogenetic bacterial PLD demonstrated that PA production in a variety of organelles is indeed sufficient to recruit PASS (Tei and Baskin, 2020). Additionally, click chemistry was used to label the products of PLD transphosphatidylation reactions as a proxy for PA, PLD's endogenous product. This method showed in real time that active PLDs localize to the PM, ER, and Golgi, with slight localization on endosomes, lysosomes, and the mitochondria (Liang *et al.*, 2019; Tei and Baskin, 2020).

Recent work has gone into characterizing the N-terminus of α-synuclein as a novel PA biosensor (Yamada *et al.*, 2020). Using liposomes, this construct (α-Syn-N) was shown to be selective for PA as compared with other lipids. However, it also showed higher selectivity for 18:1/18:1 PA species, which could limit its use in endogenous systems where many different acyl chains are likely to occur, and the 18:1/18:1 species is rare (Lorent *et al.*, 2020). Within Cos7 cells, the α-Syn-N biosensor was shown to be dependent on PA, as it colocalized with wild-type DGKs and PLD, but not when catalytically dead enzymes or inhibitors were used to prevent PA production. However, it is still not clear that this biosensor will be as sensitive as PASS when PA levels are modulated in a more physiological context (Yamada *et al.*, 2020). Therefore, we still recommend the more robustly characterized Spo20-based PA biosensors.

Class I PI 3-kinase products: both of them

The class I PI 3-kinase pathway is a paramount regulator of growth in metazoa; it is often activated in cancer and other diseases (Fruman *et al.*, 2017). Mechanistically, PI 3-kinase signaling operates through production of the lipid second messenger PIP_3 by 3-OH phosphorylation of PI(4,5)P_2. PIP_3 can then be converted (to varying extents) into an additional signal, PI(3,4)P_2, by 5-OH phosphatases (Malek *et al.*, 2017). Both PIP_3 and PI(3,4)P_2 interact with effector proteins, which may be selective for one or both lipids (Hawkins and Stephens, 2016). Therefore, distinguishing these two lipids, and their subcellular localizations, is vital for delineating PI3K signaling at the cellular level.

The most popular biosensor for PI3K signaling is the lipid-binding pleckstrin homology (PH) domain from its most famous effector, Akt. Although often mistaken for a PIP_3-biosensor, this domain actually binds to both PIP_3 and PI(3,4)P_2 (Manna *et al.*, 2007; Ebner *et al.*, 2017; Liu *et al.*, 2018; Goulden *et al.*, 2019). It is worth noting that the isolated PH domain, from all three isoforms of Akt(1–3), actually exhibits a preference for PI(3,4)P_2, although this preference only holds true for Akt2 in the context of the full-length protein (Liu *et al.*, 2018). Therefore, the Akt PH domain-based biosensors can be fine indicators of PI3K activity, but they report the convolution of PIP_3 and PI(3,4)P_2 signals.

Our lab has recently published a highly selective and sensitive PI(3,4)P_2 biosensor, cPHx3, made of a tandem trimer of the C-terminal PH (cPH) domain from tandem PH-domain–containing protein 1 (TAPP1) fused to a NES and a fluorescent protein tag (Goulden *et al.*, 2019). The improved sensitivity for PI(3,4)P_2, derived from the high avidity of the tandem trimers, was evident when we detected the

lipid's synthesis after insulin stimulation, which had not previously been evident with lipid biosensors or many biochemical approaches (Goulden *et al.*, 2019). Through an assortment of orthogonal manipulations in cells, we were also able to demonstrate that $PI(3,4)P_2$ was both necessary and sufficient to drive cPHx3 localization in cells.

As an alternative to tandem arrays, Liu and colleagues improved the membrane binding of a single cPH domain by mutating a methionine to a membrane-penetrating tryptophan residue. This would undoubtedly improve the binding of a fluorescent protein conjugate. However, cysteine residues were also removed or inserted to produce a single site for chemical ligation of a solvatochromic dye, generating eTAPP1-cPH (Liu *et al.*, 2018). This solvatochromic dye exhibits a spectral shift when inserted into the hydrophobic bilayer, permitting ratiometric imaging of the probe's membrane association. When calibrated against known mole fractions of $PI(3,4)P_2$ in liposomes, precise quantification of lipid concentration was realized (Liu *et al.*, 2018). Therefore, precise spatiotemporal detection of $PI(3,4)P_2$ is now possible, which when combined with recent advances in mass spectrometry detection of this lipid (Malek *et al.*, 2017), will usher in a new era of understanding of this enigmatic lipid's role in PI3K singling.

It is important to note that single PH domains from TAPP1 had previously been used as highly selective (but less sensitive) $PI(3,4)P_2$ biosensors—but these came in two forms: one corresponding to the isolated cPH domain, and a second that includes the entire C-terminus of the protein. This C-terminal region contains a clathrin-binding domain, which biases the localization of the probe (Goulden *et al.*, 2019). Therefore, it is critical to work with domains restricted to the isolated PH domains.

We also took advantage of the highly PIP_3-selective 2G splice variant of the ARNO (also known as cytohesin-2) PH domain to make a high avidity, tandem dimer probe for this lipid (Cronin *et al.*, 2004). We engineered an I303E mutation into each domain to disrupt a secondary binding site for Arl-family GTPases; this biosensor showed excellent selectivity for PIP_3 in cells (Goulden *et al.*, 2019). Liu *et al.* also engineered optimized membrane binding and solvatochromic dye–conjugated derivatives of the PIP_3-selective MyosinX tandem PH domains, eMyoX-PH (Liu *et al.*, 2018). Thus, there are now highly sensitive and selective PIP_3 biosensors to accompany $PI(3,4)P_2$ biosensors. These are included in Table 1. As noted in the table, a potential caveat to these sensors is their binding to soluble inositol tetrakisphosphate (IP_4), the cognate headgroup of PIP_3. This could potentially limit membrane translocation when PLC-mediated IP_4 production is triggered in conjunction with PI3K. This is expected to be a more minor caveat for the dimeric probes, where local concentration of the lipid on the membrane will favor high avidity binding to the tandem PH domains.

CONCLUSION

Genetically encoded lipid biosensors continue to be a powerful and convenient tool to study lipid dynamics and function in cell biology. Here, we have focused on a brief refresher of the principles, and highlighted some of the newest biosensors that have appeared in the last 3 years. Given the recent trend from the last 3 years in biosensor development, it seems certain that new and improved probes are on the horizon, so we encourage the reader to continue keeping an eye open for the latest developments!

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