Genetically encoded lipid biosensors

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ABSTRACT **Lipids convey both structural and functional properties to eukaryotic membranes. Understanding the basic lipid composition and the dynamics of these important molecules, in the context of cellular membranes, can shed light on signaling, metabolism, trafficking, and even membrane identity. The development of genetically encoded lipid biosensors has allowed for the visualization of specific lipids inside individual, living cells. However, a number of caveats and considerations have emerged with the overexpression of these biosensors. In this Technical Perspective, we provide a current list of available genetically encoded lipid biosensors, together with criteria that determine their veracity. We also provide some suggestions for the optimal utilization of these biosensors when both designing experiments and interpreting results.**

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INTRODUCTION

Lipids are central molecules in cell biology. They are the building blocks for membranes, which give structure to eukaryotic cells. Yet the diversity of lipid species is paramount, too, since different lipids convey different physical and thus functional properties on membranes. Membrane fluidity, curvature, peripheral protein recruitment, as well as membrane protein regulation are all controlled by the different lipids that form a membrane (van Meer and de Kroon, 2011). It follows that understanding the intricate molecular choreography of cells requires a detailed understanding of the dynamics and disposition of lipids in addition to the more familiar proteins.

Lipids present unique challenges for developing tools to study them. When compared with proteins (the biomolecules most familiar to a typical cell biologist), there are some parallels: Like proteins, antibodies have been developed to probe lipids, forming the basis of approaches akin to Western blot and immunofluorescence. The chemistry of lipid extraction for blotting is very different, and blotting is now largely being superseded by mass spectrometry approaches (Nguyen *et al.*, 2017). Immunofluorescence of lipids is possible, though it is even more tricky and artifact prone than it is for proteins, since lipids fix poorly and membranes must be disrupted to allow access of antibodies to internal lipids. Alternatively, akin to fluorescent protein tagging, fluorophore labeled lipids have been developed and successfully used to probe lipid distribution and traffic. However, a huge caveat to these approaches is that a significant region of the hydrophobic tail or head group is replaced with the fluor. Therefore, there is greater potential for occluding crucial interactions that drive lipid localization or function. In addition, selective delivery of the lipids to the correct cellular locale can be difficult or impossible, and the lipid may be metabolized into other species that are indistinguishable by fluorescence. Therefore, in this Perspective, we focus on the genetically encoded lipid biosensors, derived from specific lipid-binding domains. These possess the advantages of being compatible with living cells, detecting the endogenous lipids, and (when used carefully) having limited effects on lipid metabolism and distribution. For a wider primer on the full range of approaches described above, we direct the reader to an excellent recent review (Maekawa and Fairn, 2014)

CURRENT LIPID BIOSENSORS

The association of proteins with lipids is usually mediated by specialized lipid-binding domains (Maekawa and Fairn, 2014; Hammond and Balla, 2015). In addition, a number of bacterial effectors and toxins can target host cell membranes by binding to lipids (Maekawa and Fairn, 2014; Várnai *et al.*, 2017). These isolated domains, effectors, and toxins form the basis of genetically encoded lipid biosensors. They can be made as recombinant proteins and used for staining or blotting approaches. More commonly, they are expressed as fusions with fluorescent proteins to allow direct visualization of the lipids in cytosolic membrane leaflets of living cells. Table 1 lists lipid-binding domains that can be used for this purpose.

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Abbreviations used: Chol, cholesterol; DAG, diacylglycerol; EE, early endosome; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; LE, late endosome; NE, nuclear envelope; PA, phosphatidic acid; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; PI5P, phosphatidylinositol 5-phosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PM, plasma membrane; PS, phosphatidylserine; SM, sphingomyelin.

Lipid	Biosensor	Localization	Affinity	Lipid specific?	Cellular localization: Lipid dependent?	Cellular localization: Lipid sufficient?	References
Chol	D4-PFO + mutants	PM (exoplasmic > cytosolic	2–30 mol%	✓	✓	?	Shimada *et al.*, 2002; Maekawa and Fairn, 2015; Liu *et al.*, 2016
SM	Lysenin	PM (exoplasmic)	K_D ~ 5 nM	✓	✓	?	Yamaji *et al.*, 1998; Kiyokawa *et al.*, 2005; Abe *et al.*, 2012
PA	PASS/2xPABD (spo20p$^{51-91}$)	PM, ER	?	✗ (binds PI(4,5)P_2 weakly)	✓	?	Bohdanowicz *et al.*, 2013; Zhang *et al.*, 2014
PS	C2-lactadherin	PM, endosomes, TGN	K_D ~ 0.5 μM	✓	✓	✓	Yeung *et al.*, 2008; Maeda *et al.*, 2013; Vecchio and Stahelin, 2018
DAG	C1ab-PKCε	Golgi, ER, NE	K_D ~ 10 nM	✓	✓	?	Stahelin *et al.*, 2005; Domart *et al.*, 2012
	C1ab-PDK1	Golgi	K_i (PDBu) ~0.2 μM	✓	✓	✓	Chen *et al.*, 2008; Kim *et al.*, 2011
PI4P	N-PH-ORP5, N-PH-ORP8	PM	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	Golgi/PM	K_D ~ 2–4 μM	✗ – binds PI(4,5)P_2	✓	✗ – requires Arf1	Levine and Munro, 2002; Szentpetery *et al.*, 2010; Lenoir *et al.*, 2015
	P4M-SidM	PM, Golgi, Endosomes	K_D ~ 1 μM or ~18.2 nM FL	✓	✓	✓	Brombacher *et al.*, 2009; Schoebel *et al.*, 2010; Hammond *et al.*, 2014
	P4M-SidMx2	PM, Golgi, Endosomes	K_D < P4M-SidM	✓	✓	✓	Hammond *et al.*, 2014; Levin *et al.*, 2017
	P4C-SidC	PM, Golgi, Endosomes	K_D ~ 250 nM	✓	✓	✓	Dolinsky *et al.*, 2014; Weber *et al.*, 2014; Zewe *et al.*, 2018
PI5P	3xPHD (ING2)	PM, nucleus	?	✗ – binds PI3P	✓	✓	Gozani *et al.*, 2003; Pendaries *et al.*, 2006
PI(4,5)P_2	PH-PLCδ1	PM	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	PM	K_D ~ >PH-PLCδ1	✗ – binds to IP_3	✓	✓	Lee *et al.*, 2004; Hammond and Balla, 2015
	Tubby$_c$	PM	K_D > PH-PLCδ1	✗ – binds PI(3,4)P_2 and PI(3,4,5)P_3	✓	✓	Quinn *et al.*, 2008; Halaszovich *et al.*, 2009; Szentpetery *et al.*, 2009; Hammond and Balla, 2015
	Tubby$_c^{R332H}$	PM	K_D >Tubby	✗ – binds PI(3,4)P_2 and PI(3,4,5)P_3	✓	?	Quinn *et al.*, 2008
	ENTH/ANTH	PM	K_D ~ 5 μM	✗ – binds PI(3,4,5)P_3	✓	?	Ford *et al.*, 2001; Itoh *et al.*, 2001; Yoon *et al.*, 2011

TABLE 1: Commonly used genetically encoded biosensors and the criteria used to assess their interpretation; see the text for a discussion.

Continues

Lipid	Biosensor	Localization	Affinity	Lipid specific?	Cellular localization		References
					Lipid dependent?	Lipid sufficient?	
PI3P	FYVE-Hrsx2	EE	K_D ~ 2.5 μM	✓	✓	?	Burd and Emr, 1998; Gaullier *et al.*, 1998; Gillooly *et al.*, 2000; Sankaran *et al.*, 2001
	FYVE-EEA1	EE	K_D ~ 45 nM	✓	✓	?	Burd and Emr, 1998; Gaullier *et al.*, 1998, 2000
	PX-p40phox	EE	K_D ~ 5 μM	✓	✓	?	Bravo *et al.*, 2001; Ellson *et al.*, 2001; Kanai *et al.*, 2001
$PI(3,5)P_2$	ML1-Nx2	EE, LE, lysosomes	K_D ~ 5.6 μM	✓	✓/✗[a]	✓/✗[a]	Li *et al.*, 2013; Hammond *et al.*, 2015
PIP_3	PH-Akt	PM	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	PM	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)	PM	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
$PI(3,4)P_2$	PH-TAPP1-CT	PM	K_D ~ 80 nM	✓	✓	✓	Dowler *et al.*, 2000; Thomas *et al.*, 2001; Kimber *et al.*, 2002; Marshall *et al.*, 2002; Manna *et al.*, 2007

[a]The accuracy of this probe is disputed.

TABLE 1: Commonly used genetically encoded biosensors and the criteria used to assess their interpretation; see the text for a discussion. Continued

Each biosensor has a number of potential caveats and pitfalls that the user should be aware of. We think of three crucial criteria that should be considered when determining the validity of a probe: Is the probe specific for the target lipid? Is the biosensor's localization dependent on that lipid? And, if it is dependent, is the lipid alone sufficient to localize the biosensor? A previous review (Hammond and Balla, 2015) has explained each of these criteria in detail. Table 1 describes whether these criteria are met, and it is intended as a guide in selecting the most appropriate biosensors. Many are available through the plasmid sharing resource Addgene (www.addgene.org).

THINGS TO THINK ABOUT WHEN USING LIPID BIOSENSORS

Selectivity of the biosensor

A quick perusal of Table 1 reveals that relatively few probes meet all of our prescribed criteria for truly selective, unbiased lipid biosensors. However, this does not mean that they cannot be used to gather useful information about lipid localization and dynamics. However, care must be taken with the interpretation of results. This is illustrated well by the number of probes that recognize PI4P; most have additional binding sites for other ligands that bias lipid detection in compartments that also contain this ligand. They can be useful in reporting changes in PI4P levels in these individual compartments but are often blind to changes in other regions of the cell.

Sometimes it can be assumed that alternative interactions with other lipids will have minimal effects; for example, as Table 1 shows, the Tubby c-terminal domain binds to $PI(3,4,5)P_3$ and $PI(3,4)P_2$ in addition to $PI(4,5)P_2$. Nevertheless, even after stimulation, these other lipids are present at <5% of $PI(4,5)P_2$ (Stephens *et al.*, 1993) and are therefore assumed not to alter localization of the biosensor.

A long-time controversy has been whether the soluble headgroup, which are usually present in the cytosol, competes with binding to the lipid. The biggest example has been competition of PH-PLCδ1 from membrane $PI(4,5)P_2$ by the lipid's hydrolysis product, IP_3. The short answer is that both reductions in the lipid as well as increases in IP_3 that occur after phospholipase C activation can lead to dissociation of the biosensor from the membrane, and it is not possible to prescribe a priori the contribution of each (Xu *et al.*, 2003). Therefore, we urge caution when interpreting loss of localization of this biosensor after phospholipase C activation. This is a perfect example of when the Tubby c-terminal domain and its variants, which do not bind IP_3, are a better probe (Quinn *et al.*, 2008).

Inhibitory effects of the biosensor

Perhaps the greatest fear associated with the use of lipid biosensors is that they can sequester their target lipid and hence disrupt physiologic interactions with effector proteins. For this reason, articles such as this usually advise the reader to use the lowest possible

expression levels of biosensor possible (e.g., Várnai *et al.*, 2017). This is good advice, but it reminds us of Rick Sanchez, the fictitious mad scientist from the sci-fi cartoon "Rick and Morty," who once quipped: "Ok… well, sometimes science is more art than science… A lot of people don't get that." Rather than leave the reader to the art of determining appropriate expression levels, we will sketch out some slightly more quantitative considerations.

Most biosensors in Table 1 bind with a 1:1 stoichiometry and with high affinity. In this case, it is permissible to estimate the concentration of lipids as if they were dissolved in the three-dimensional volume of the cell (though this does not apply to tandem arrays of lipid binding domains with more than one binding site, which are much more sensitive to the high local concentration of lipid on the two-dimensional membrane surface). Estimates of this three-dimensional-equivalent concentration in a typical mammalian cell vary from ~1 mM for bulk lipids like phosphatidylserine (PS) or phosphatidylcholine (PC), down to ~10 µM for $PI(4,5)P_2$ or 100 nM for $PI(3,4,5)P_3$ (Stephens *et al.*, 1993; McLaughlin *et al.*, 2002). The fraction of total expressed biosensor that binds to lipid ($f_{biosnesor}$) is related to the binding site's dissociation constant (K_D) by the following relationship:

$$f_{biosensor} = \frac{[Lipid]}{K_D + [Lipid]}$$

From this, we can see that a biosensor must have a K_D similar to or substantially lower than that of the effective lipid concentration for appreciable localization when expressed in cells. This is either known to be the case or inferred from the localization of the biosensors listed in Table 1.

Given these dissociation constants, it follows that if the concentration of biosensor in cells approaches or exceeds the concentration of lipid, a significant fraction of the lipid will be sequestered. This may begin to displace endogenous proteins and interactions, causing inhibitory effects. How likely is this? Typical yields for protein expression in mammalian systems reveal an upper limit of ~100 µM for overexpressed protein in the cytosol (estimated as 1 mg/ml culture volume, 50-kDa protein, 2.5×10^6 cells/ml, 15-pl cell volume), whereas expression of lipid biosensors in cells has been found in the range of 1–10 µM (Xu *et al.*, 2003). So, sequestering of significant fractions is unlikely for bulk lipids like PS or cholesterol, whereas it is a real concern with less abundant lipids, that is, the phosphoinositides. Indeed, dominant negative effects have been observed with the $PI(4,5)P_2$ biosensor PH-PLCδ1 (Várnai and Balla, 1998; Holz *et al.*, 2000). However, such dominant negative effects have been rare, and it appears that cells compensate by synthesizing more $PI(4,5)P_2$ in response to biosensor expression (Traynor-Kaplan *et al.*, 2017), likely keeping the free lipid concentration constant. This may explain how it is possible to use the $PI(3,4,5)P_3$ biosensors listed in Table 1, since these have $K_D \approx [PI(3,4,5)P_3] \approx 100$ nM, and likely sequester the majority of synthesized lipid. By contrast, we estimate that HeLa cells contain ~500 nM of endogenous effector protein from published proteomic data (Hein *et al.*, 2015).

Simple sequestering of the lipid is not the only potential concern when using biosensors. Several of the lipid binding domains listed in Table 1 also have protein ligands, which may be present at much lower effective cytosolic concentrations (Hein *et al.*, 2015), and therefore be much more susceptible to sequestration. Furthermore, the accumulation of high densities of lipid binding proteins on membranes can have other deleterious effects due to protein crowding, such as membrane deformation (Stachowiak *et al.*, 2012). For this reason, it is still always advisable to keep biosensor expression levels as low as practicable for imaging purposes. However, with modern instruments, ~1 µM is adequate for high-quality images and should not cause deleterious effects for most lipids.

The treachery of images

Belgian surrealist painter René Magritte famously produced a painting of a pipe with an accompanying legend, "Ceci n'est pas une pipe" ("This is not a pipe"); this illustrated the fact that the painting is merely a representation of an object, distinct from the real thing. Similarly, it is important to bear in mind that the biosensor is not the same as the lipid it is being used to detect. First, biosensors are in equilibrium with the free pool of lipid and will not interact with lipids bound to effector proteins; similarly, effector proteins cannot bind to biosensor-bound lipids. Given that lipid–biosensor complexes can diffuse several microns in the plane of the membrane (Hammond *et al.*, 2009), biosensors often cannot detect local enrichment of lipids associated with specific protein complexes, unless the biosensor has a low affinity (and hence rapid dissociation rate) or is specifically targeted to that protein complex via a secondary, low-affinity interaction (Trexler *et al.*, 2016; He *et al.*, 2017). That said, most complexes of biosensor or effector protein last only a few seconds—so effector-bound, free, and biosensor-bound lipids rapidly equilibrate with each other.

A second key point is that the biosensor is expressed in the cell independently of the presence of its lipid target; the lipid only influences the localization of the biosensor. Therefore, changes in local concentrations of the lipid cause changes in biosensor localization and not overall changes in its expression level or total fluorescence—this key point is occasionally lost in the literature. For the purposes of quantification, it is necessary to keep this concept of localization and measure fluorescence intensity changes relative to the membrane compartment of interest. This can be accomplished in a number of ways; for example, by generating a mask corresponding to specific organelles via expression of secondary markers and measuring intensity in the masked region relative to the rest of the cell, by selectively imaging the plasma membrane via total internal reflection fluorescence microscopy (TIRFM), or by using resonant energy transfer approaches to detect biosensor accumulated at a specific membrane. Several recent articles have described these approaches in detail (Hammond and Balla, 2015; Várnai *et al.*, 2017)

We illustrate the need to be mindful when interpreting localization in Figure 1. The figure shows cells expressing a high-affinity PI4P probe, GFP-P4C, before and after a treatment. We visualize an increase in fluorescence on endosomes posttreatment. This observation could be the result of an increase in PI4P on endosomes or a loss of lipid where the probe was initially bound. The change in Figure 1 is actually the result of a PI4P phosphatase being recruited to the plasma membrane, leading to degradation of this lipid and loss of P4C localization there. The P4C relocalized to organelles where PI4P was still present, which is observed as an increase in fluorescence at these membranes.

Go low or go high?

The most striking fluorescence images of cell structure are produced when there is the highest contrast between the cellular structure and the rest of the cell. For lipid biosensors, this will correspond to a high fraction of biosensor bound to membrane, that is, a high-affinity (or low K_D). High-affinity probes can be made by tandem dimers or trimers of lower-affinity domains, with a resulting multiplicative increase in affinity—though this approach has pros and cons. For example, a tandem dimer of the P4M PI4P probe allows enhanced detection of

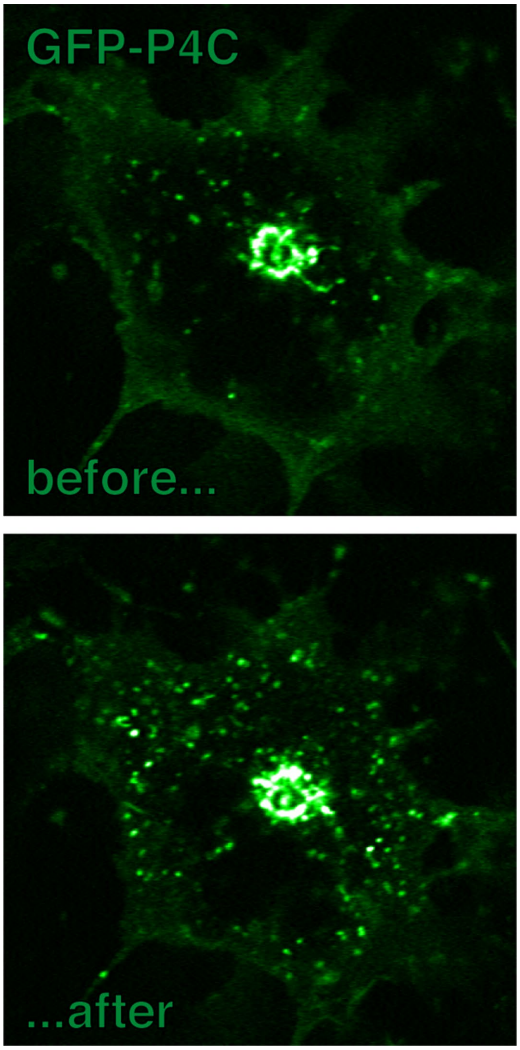

FIGURE 1: Ceci n'est pas une PIP ("This is not a PIP"). This COS-7 cell is expressing GFP-P4C, a PI4P biosensor. The images show the cell before and after a treatment that induces relocalization of the probe. What do you suppose this treatment was? Read the text for the answer.

PM and endosomal pools of PI4P; conversely, the increased affinity can lead to greater sequestration of lipid and even distort organelles at higher expression levels (Hammond *et al.*, 2014).

High-affinity probes come with another setback: if the majority of biosensor is already bound to lipid, then it will not detect increases in that lipid. Figure 2 shows a single (P4Mx1) or tandem (P4Mx2) PI4P probe after the recruitment of a PI(4,5)P_2 phosphatase, which elevates PI4P in the plasma membrane. We can observe an increase of the P4Mx1 probe at the membrane after the recruitment of the phosphatase, while little translocation of P4Mx2 is observed—which could be misinterpreted as a lack of lipid production. Indeed, a little trumpeted fact about the popular PI(4,5)P_2 probe, PH-PLCδ1, is that it is poorly able to detect increases in plasma membrane PI(4,5)P_2 (Suh *et al.*, 2006)

Lower-affinity probes produce much less striking images—but can be much more informative regarding changes in lipid concentrations. They are also much less prone to the inhibitory effects discussed above, and their shorter lifetime in complex with lipids can allow them to report on local lipid accumulations missed by

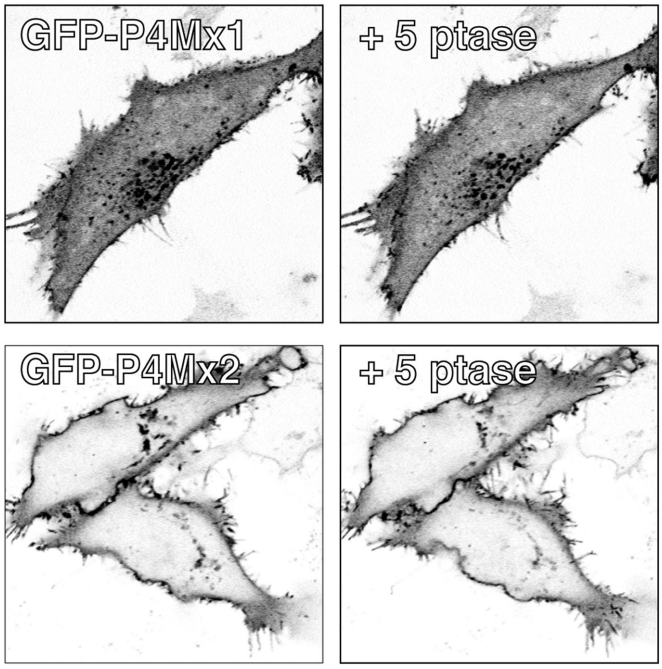

FIGURE 2: Go high or go low (affinity). These HeLa cells are expressing either a monomeric (top) or a dimeric (bottom) version of GFP-P4M, a PI4P biosensor. The images show the cells before and after activation of a 5-phosphatase that increases PI4P in the plasma membrane. The lower affinity biosensor is able to detect small changes in PI4P synthesis and relocalize to the PM (note how the cytoplasm looks dimmer in the "after" image), whereas the higher-affinity probe is already saturated at the plasma membrane.

higher-affinity domains (Kabachinski *et al.*, 2014; Trexler *et al.*, 2016). However, the low affinity can also make it hard to quantify decreases in lipid concentration in a particular membrane, for example, the drop in PI(4,5)P_2 levels after PLC activation. For these reasons, in our lab we often employ both low- and high-affinity biosensors, when they are available, for a given lipid species.

CONCLUSION

The aim of this Perspective was to provide a current (perhaps fleetingly so) overview of the available genetically encoded lipid biosensors. As we have discussed, these tools may have some limitations, but when controlled, quantified, and interpreted properly, these probes provide invaluable information regarding lipid dynamics. There are a large number of probes available for a wide selection of lipids. However, some lipids still lack high-quality biosensors or any biosensor at all (e.g., PI). The area of biosensor development is a quickly moving field with continuous advancements; new lipid-binding domains are continually being recognized or modified to improve their efficacy. Therefore, we expect the current list (Table 1) to become rapidly outdated. However, the principles and pitfalls we have outlined will be as applicable for future generations of lipid biosensors as they are for the current suite.

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