

REVIEW

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The cell biologist's guide to detecting and modulating membrane phospholipids

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Molecular biology has benefited enormously from repurposed tools—many enzymes and antibodies evolved for other functions but are now essential for interrogating biological function by manipulating proteins or nucleic acids. In contrast, lipids have remained technically difficult to visualize or manipulate in cells. This review introduces tools that bring lipid biology into reach for molecular cell biologists, using familiar experimental approaches. We first describe adaptations of immunofluorescence and live-cell imaging of fluorescent molecules to track lipids. Then, we discuss tools for manipulating lipid levels, including pharmacologic inhibitors, synthetic biology platforms for inducible lipid generation or degradation, and optogenetic systems for precise temporal control. While some methods remain technically demanding, most tools are now broadly accessible. Our goal is to offer a practical framework for integrating lipid biology into mainstream cell biology experiments.

Introduction

In the 1979 novel “The Hitchhiker’s Guide to the Galaxy,” author Douglas Adams introduces the fictional Babel fish: a leech-like creature that lives in a person’s ear, feeding off brain energy of their interlocutors and excreting telepathic thoughts into the host. The effect on the host is coincidentally the ability to instantly understand anything said to them in any language; in effect, it is a universal translator. Peculiar as this anecdote seems, if we stop to think for a moment, we realize that modern biomedical science is powered by many real-life Babel fish: Cas nucleases, antibodies, fluorescent proteins, and even the humble restriction endonuclease; all of these evolved to meet specific and often esoteric biological functions. Yet with sometimes minimal development, they are utilized in our laboratories as exquisitely precise molecular tools; they fit our experiments so perfectly that they almost seem custom designed. Serendipitous tools such as these have been rocket fuel for the engine of biomedical discovery.

As spectacularly useful as these and many other reagents are, they are sadly limited to detect or manipulate only two of the fundamental building blocks of cells: proteins and nucleic acids. They rarely if ever target the third major class of biomolecule, lipids. Because lipids are not directly genetically encoded and cannot occupy the seemingly infinite chemical space of proteins, nature has far less commonly evolved orthogonal manipulations of these lipids in manners that are easily experimentally tractable. As a result, the study of lipids has remained in the realms

of traditional biochemistry approaches for much longer than the rest of cell and molecular biology. We often hear colleagues commenting how hard lipids are to work with! Lipids’ perceived intractability is a real barrier to exploring their function for many researchers because of the apparent lack of molecular tools to study them. Our goal in this review is to provide a practical overview for cell and molecular biologists to the basic suite of tools that can be used to detect and manipulate lipids.

Taking a thirty-five thousand foot view of our experiments as molecular cell biologists, we typically do three things: we detect and quantify biological molecules in cells; we take away these molecules to see what happens; and we give the molecules (back) and see what happens. There is no shortage of nucleic-acid and protein-driven approaches that we can all reel off to do this: We can use immunofluorescence (IF) to map the localization of endogenous proteins, or we can fuse genes to the proteins thereby generating fluorescent protein fusions to achieve the same effect in live cells. We can add increasingly potent and selective small molecule modulators of protein function. We can knockdown gene expression to remove a protein. We can edit genes to not just destroy expression but to modify function or add specific reporters and modifiers. We can generate novel protein chimeras to both report on and modulate specific molecular pathways. As we will explore in this review, the good news is that these approaches can be leveraged in combination with some specific lipid-focused technology. These facilitate analogous

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experiments to interrogate lipid function. Rather than being comprehensive, we aim to introduce a conceptual outline of these approaches, which we will illustrate with a few seminal examples. In this way, we want to equip the reader with a basic outline of what is now possible and inspire the reader to design their own experiments. We will organize the review around analogous approaches familiar to all molecular cell biologists: IF and cytochemistry, overexpression and fluorescent protein tagging, and knockdown/knockout experiment—just for lipids instead!

Cytochemistry and IF of lipids

IF is a tried and tested technique for localizing endogenous proteins in cells and has been a bedrock of cell biology approaches for decades. IF is also applicable to membranes and lipids, but with some pretty major pitfalls. Although antibodies for IF are typically raised against proteins, many have been raised to detect lipids; for example, Echelon Biosciences Inc. has a catalog against inositol lipids among others. In addition, as we discuss in the section on genetically encoded lipid biosensors, the genome is replete with specific lipid-binding domains that can be used as probes (Hammond and Balla, 2015). As we will see, these have also been used as recombinant “antibody-like” probes for cytochemistry experiments.

So what are the pitfalls? A great illustration is provided by the experience with one of our favorite lipids, phosphatidylinositol 4,5-bisphosphate, PI(4,5)P₂, which is especially enriched at the plasma membrane (PM) (Wills and Hammond, 2022). By the 1990s, several groups had raised high quality antibodies against this lipid and used them for IF, yielding a big surprise: the antibodies specifically stained the nucleus’ splicing factory, the so-called nuclear speckles (Boronenkov et al., 1998; Osborne et al., 2001). While this discovery fueled our understanding of a non-canonical role for extra-membrane inositol lipids in nuclear function (Osborne et al., 2001; Blind et al., 2012), it was also met with some justifiable skepticism, since the antibodies could not detect the well-established, canonical PM pool (Várnai and Balla, 2006). So what was going wrong? To answer this question, we need to think more about the process of IF.

For IF, cells are initially fixed—“gluing” the cells’ molecular constituents in place and killing the cell at the same time (these experiments are essentially postmortems). Typically, either methanol or another primary alcohol with or without acid is used to dehydrate the sample, which causes the proteins to denature and coagulate. Alternatively, aldehydes are used that cross-link the primary amines in proteins; namely, the lysine and arginine side chains and terminal amine (Melan and Sluder, 1992). Next, the membranes must be permeabilized to allow entry of water-soluble antibodies or other probes.

Herein lies the major problem: all of these procedures are fundamentally incompatible with preserving lipid localization and membrane structure. Rather than coagulating them, methanol solubilizes lipids. While aldehydes do not extract lipids, they are also a poor fixative; most lipids do not have a primary amine for cross-linking, and even the amines in phosphatidylethanolamine (PE) and phosphatidylserine (PS) are poorly cross-

linked relative to those in proteins (ROOZEMOND, 1969). Indeed, careful studies have shown that many membrane components, especially lipids, retain mobility after fixation with aldehydes (Tanaka et al., 2010). Finally, permeabilization usually involves application of a detergent that will solubilize and thereby extract the unfixed lipids, destroying most of the membrane. This explains the lack of PM staining with PI(4,5)P₂ antibodies. In these experiments, formaldehyde was used to fix the cells, which were permeabilized with Triton X-100. Hence all the PM PI(4,5)P₂ was washed away, leaving only the non-membranous, RNA- and protein-associated lipid (Osborne et al., 2001; Blind et al., 2012).

These apparently insurmountable incompatibilities have led to the exploration of more elaborate lipid- and membrane-preserving procedures. Platinum replica freeze-fracture scanning electron microscopy has been used to stunning effect (Fujita et al., 2009; Takatori et al., 2014). Here, cells are rapidly frozen to physically immobilize all molecules; next, the vitrified sample is fractured along membrane surfaces to expose the lipids, which are then cast with layers of carbon and platinum: think Han Solo at the end of “The Empire Strikes Back”. These replicas are then probed with lipid-selective proteins. The first seminal paper demonstrated clear PM labelling with PI(4,5)P₂ probes (Fujita et al., 2009), with many subsequent papers demonstrating the ultrastructural distribution of many other lipids throughout the cell, including phosphatidylcholine (PC), PS, phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) (Aktar et al., 2017; Tsuji et al., 2024; Orii et al., 2021; Cheng et al., 2014; Takatori et al., 2016). Stunning as these experiments are, they require specialist equipment and expertise that is not standard.

Another underappreciated study paved the way for more conventional cytochemistry of lipids: Watt and colleagues used cryo-sectioning of glutaraldehyde and formaldehyde-fixed cells for transmission electron microscopy, effectively bypassing the need for a permeabilization step (WATT et al., 2002). Curiously, in labelling PI(4,5)P₂ with a recombinant pleckstrin homology (PH) domain, they observed labelling on the grids beside the cells when sections were warmed to room temperature, but that labelling was retained exclusively on membranes when sections were kept ice-cold. Apparently, the poorly cross-linked membranes retain better integrity in the cold. This study inspired a subsequent paper performing more conventional IF on PI(4,5)P₂ (Hammond et al., 2006). In addition to keeping samples ice-cold, this study unconventionally used glutaraldehyde rather than formaldehyde to fix the cells because glutaraldehyde shows superior performance cross-linking amine-containing lipids (ROOZEMOND, 1969). Saponin was selected as the permeabilizing agent, since this naturally occurring detergent has been shown to make pores in membranes, rather than solubilizing them completely (Seeman et al., 1973). With this protocol, the elusive PM immunostaining of PI(4,5)P₂ could be visualized (Hammond et al., 2006).

A subsequent study expanded these methods to explore other membrane compartments, motivated by initial observations that an antibody against PI4P showed PM labelling, but not an

anticipated Golgi-associated pool (Hammond et al., 2009). Since saponin had been shown to remove ceramides from the Golgi in fixed cells (Pagano et al., 1989), a range of permeabilization, fixation, and temperature protocols were explored. This empirical approach yielded distinct procedures to label specific organelle lipid pools. For example, in contrast with the PM, Golgi and endosome membranes required less strong fixation and worked well at room temperature but were abolished by saponin (Hammond et al., 2009). We emphasize the ultimately empirical nature of this approach; there are several idiosyncrasies of these procedures that we do not understand: for example, the fact that saponin is detrimental to the Golgi, but the analogous detergent digitonin produces excellent results. The choice of buffer (Piperazine-1,4-bis(2-ethanesulfonic acid) or PIPES, pH 6.8) also seems to be important for unknown reasons. It should be stressed that although these staining procedures have been reproduced by many, variations have also produced good results for PM staining of PI(4,5)P₂ and other lipids (Laux et al., 2000; Micheva et al., 2001; Sharma et al., 2008; Yip et al., 2008). The immunostaining procedure has been demonstrated to work well with cultured neurons (Guo et al., 2022) and even tissue sections (Maib et al., 2024). Excitingly, the use of directly conjugated lipid-binding domains has been shown to produce robust, multiplexed labelling for up to seven distinct inositol lipids across cell and tissue samples using these procedures (Maib et al., 2024). Therefore, although our discussion of immunostaining development has focused on PI(4,5)P₂, these principles can be applied to many other lipids. They are a good starting point to optimize protocols for novel lipid-targeted probes.

Best practices and controls: Although our discussion of immunostaining development has focused on PI(4,5)P₂, these principles can be applied to many other lipids. They are a good starting point to optimize protocols for novel lipid-targeted probes. Essentially, an empirical approach has to be taken to identify the best protocol to detect a specific lipid in a given membrane. Past precedence in the literature can be consulted (including references in the preceding paragraphs), and care must be taken in terms of timing, fixatives, detergents, buffers, and temperature. Most labs have a “standard” IF protocol. It probably will not work to label lipids (and they do not work for all protein antigens, actually!). A great control when trying to interpret the presence or absence of a lipid from a given organelle membrane can be to assess the integrity of that bilayer after the staining protocol. This can be conveniently accomplished using non-fixable fluorescent lipid stains, such as DiC₄ for the endoplasmic reticulum (ER) and mitochondria (Hammond et al., 2009).

We want to end this section with a note of caution about interpreting the results from such fixed specimen images. Because the membranes must be disrupted to permit access of the probes, by definition, they change the ultrastructure of the membrane. Therefore, we still commend heavy skepticism of papers examining nanoscopic molecular distributions of lipids generated with these approaches (platinum replicas aside, where the molecules are near instantaneously immobilized). Indeed, several reports in the literature document artifactual clustering of inositol lipids in response to these manipulations

(Rheenen et al., 2005; Omar-Hmeadi et al., 2018). Instead, we prefer to use them as a method for *in situ* quantification of relative lipid levels in cell populations. In fact, we have previously demonstrated excellent agreement between standard biochemical measurements from extracted cells and cytochemistry staining measurements from hundreds to thousands of imaged cells (Hammond et al., 2006; Hammond et al., 2012). Fig. 1 A highlights some of the pros and cons of lipid IF.

“GFP” lipids

While IF is limited to postmortem samples, it is relatively trivial to image protein localization in living cells using genetic fusion to fluorescent proteins (and other reporters). Traditionally GFP and its variants were used, but now the choice of bright and photostable fluorescent proteins spans the rainbow into near-infrared (Hoelzel and Zhang, 2020) and has been joined by expression tags able to selectively ligate to even more photostable organic dyes (Wang et al., 2023). Modern gene editing technology enables these tags to be incorporated to endogenous loci, but classically (and conveniently) they can be introduced by simple transfection of plasmid-encoded transgenes. Alas, since lipids themselves are not directly genetically encoded, introducing fusion proteins is not quite so direct. Fortunately, there are a couple of analogous techniques that enable introduction of fluorescent tags to lipid species, so they can be imaged or interrogated with biophysical approaches; one is experimentally similar but conceptually different from GFP-fusion to proteins (as conjugation is indirect). The second follows the same principle of directly attaching a fluorophore to the molecule—but in this case, introduction of a fluorescent lipid to cells employs a quite experimentally distinct “transfection” procedure.

Genetically encoded lipid biosensors

By the mid 1990’s, a number of PH domains in proteins had been found to bind to various inositol lipid headgroups with high affinity and selectivity. This led to the ingenious idea to fuse GFP to the isolated PI(4,5)P₂-selective PH domain from phospholipase C, PLCδ1, to image the lipid’s distribution in cells. The Balla and Meyer labs published these experiments contemporaneously, revealing the biosensors lit up the PM and, moreover, would dissociate when the levels of PI(4,5)P₂ dropped during activation of PLC (Stauffer et al., 1998; Várnai and Balla, 1998). This opened the floodgates to the development of large numbers of isolated lipid-binding domains selective for various membrane-localized lipids (Yang et al., 2018; Wills et al., 2018), based on both isolated domains and bacterial toxins. Table 1 lists frequently studied lipids and some high-quality biosensors that detect them.

Selection and evaluation of genetically encoded biosensors is something that must be considered carefully, and we have previously discussed these issues in-depth (Wills et al., 2018; Hammond and Balla, 2015). In short, to be an effective biosensor, the protein domain must have excellent selectivity for the target lipid, which should also be demonstrated to be both necessary and sufficient to localize the biosensor in cells. This is illustrated by experience with biosensors against PI4P, found in endosomes, PM, and Golgi. Many early biosensors only detected the

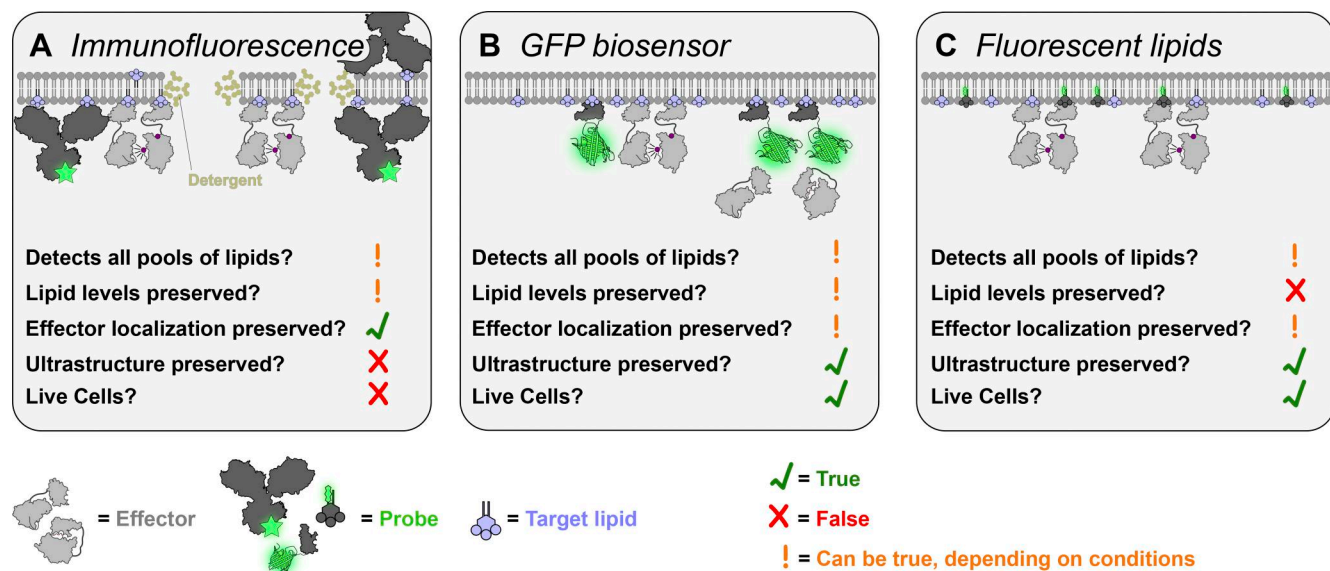


Figure 1. **Pros and cons of lipid imaging techniques.** (A–C) The three major imaging modalities for lipids are shown in A, B, and C, with the fluorescent probes in each colored green. Note the perturbation of membrane ultrastructure in A, the potential displacement of endogenous effectors in B, and recruitment of additional effectors by fluorescent lipid in C.

Golgi pool because although lipid binding was selective, the lipid was not sufficient to localize the biosensor, which often needed coincident binding of Golgi-localized Arf1 (Roy and Levine, 2004; Hammond et al., 2014).

Experimentally, using these tools is not different than transfecting cells with any other GFP-conjugated protein. However, in this case, we do not see the lipid directly; we only highlight membranes where the lipid is enriching the biosensor. Herein lie many of the caveats: Firstly, typically the biosensors are cytosolic and therefore only “see” the lipid localized to cytosolic membrane leaflets. Secondly, the lipid has to be relatively abundant compared with the biosensor; if the biosensor is in great excess, very little will be membrane localized, and there may not be sufficient contrast to see it. Thirdly, and perhaps most troublingly, by binding the lipid’s headgroup, the biosensor sequesters the lipid, thus preventing interaction with endogenous effector proteins. Because interactions between lipid and biosensor are typically very dynamic, this is not a huge problem if the lipid is highly abundant relative to the biosensor. However, if the biosensor concentration approaches both its dissociation constant and the total lipid concentration, then significant fractions of lipid can be biosensor bound, outcompeting endogenous proteins and blocking their function. For example, recent work on the low-abundance lipid phosphatidylinositol 3,4,5-trisphosphate (PIP₃) showed that biosensors bound to PIP₃ inhibit endogenous protein kinase B (AKT) activation in a dose-dependent manner. However, expressing these sensors at much lower levels mitigates this effect (Holmes et al., 2025). In light of this, more work should be done to investigate inhibitory effects of biosensors on other lipid species with higher abundances, such as the constitutive inositol lipids.

An alternative to direct competition of effector proteins is perturbation of the lipid’s homeostasis by biosensors. For

example, PI(4,5)P₂ is known to possess a homeostatic feedback mechanism by which tonic lipid levels are sensed and fed back to the synthetic machinery (Wills et al., 2023). This seems likely to explain why, upon overexpression of the PH-PLCδ1 biosensor, PI(4,5)P₂ levels actually increase (Traynor-Kaplan et al., 2017): sequestration of PI(4,5)P₂ may cause an increase in synthesis to restore levels of unbound lipid.

Lipid biosensors can have a broad range of affinities for their specific lipid, and it is often advantageous to have both a high-affinity and low-affinity option. For example, when Koh et al. (2023) characterized their cholesterol sensors, they showed that the high-affinity GRAM-W better showed cholesterol depletion, as it was already PM-localized under basal conditions, while the low-affinity GRAM-H better reported cholesterol addition due to its basal cytosolic localization. Fortunately, these biosensors were created from the same effector protein, with just a single point mutation in the lipid-binding domain (Koh et al., 2023). Another way to increase a biosensor’s affinity for its lipid is to include tandem lipid-binding domains to increase avidity, as has been done in the design of PI(3,4)P₂ biosensors (Goulden et al., 2019). It is for this reason that in Table 1 we list two biosensors for several lipids: both high-affinity and low-affinity sensors are shown when these have been characterized. Fig. 1 B shows some pros and cons of the biosensor-based approach.

A recent high-throughput Cell surface Liposome Binding (CLiB) assay has made the identification and optimization of lipid-binding domains easier. This assay mixes fluorescent liposomes containing a target lipid with yeast expressing different lipid-binding domains on the cell surface, thereby allowing for the quantification of liposome binding. The CLiB assay was used to screen mutants of the PI(3,5)P₂ biosensor SnxA that were produced through directed evolution to uncover a clone with increased affinity and specificity for PI(3,5)P₂. It also was able to

Table 1. Examples of the most common biosensors for frequently-studied lipids

Lipid	Biosensors	Affinity	<i>In vitro</i> characterization Lipid specific?	Cellular localization		References
				Lipid dependent?	Lipid sufficient?	
Chol	GRAM-H	Low	✗ also binds PS	✓	?	Koh et al. (2023), Ercan et al. (2021)
	GRAM-W	High	✗ also binds PS	✓	?	
SM	Lysenin		✓	✓	?	Abe et al. (2012), Kiyokawa et al. (2005), Yamaji et al. (1998)
PA	PASS	Low	✗ binds PI(4,5)P ₂ /PIP ₃	✓	?	Zhang et al. (2014), Weckerly et al. (2025)
	PILS-Nir1	High	✗ binds PI(4,5)P ₂	✓	✓	
PS	C2-lactadherin		✓	✓	✓	Maeda et al. (2013), Vecchio and Stahelin (2018), Yeung et al. (2008)
DAG	C1ab-Prkd1		✓	✓	✓	Chen et al. (2008), Kim et al. (2011)
PI	Bc-PI-PLC ^{ANH}		✗ also binds DAG	✓	✓	(Pemberton et al., 2020)
PI4P	P4M-SidM	Low	✓	✓	✓	Brombacher et al. (2009), Levin et al. (2017), Schoebel et al. (2010), Hammond et al. (2014)
	P4M-SidMx2	High	✓	✓	✓	
PI3P	2xFYVE-Hrs1		✓	✓	?	Gillooly et al. (2000), Hsieh et al. (2021)
PI(4,5)P ₂	Tubby _c	Low	✗ also binds PI(3,4)P ₂ /PIP ₃	✓	✓	Halaszovich et al. (2009), Quinn et al. (2008), Szentpetery et al. (2009), Hammond and Balla (2015)
	Tubby _c ^{RH}	High	✗ also binds PI(3,4)P ₂ /PIP ₃	✓	?	
PI(3,5)P ₂	2xPX-SnxA	Low	✓	✓	?	Vines et al. (2023), Pemberton et al. (2025)
	SnxA	High	✓	✓	?	
PI(3,4)P ₂	eTAPP1-cPH	Low	✓	✓	✓	Shu-Lin et al. (2018), Goulden et al. (2019)
	TAPP1-cPHx3	High	✓	✓	✓	
PIP ₃	PH-ARNO ^{2G} -I303E _{x2}	Low	✗ binds IP ₄	✓	✓	Hokanson et al. (2006), Plantard et al. (2010), Lu et al. (2011), Shu-Lin et al. (2018), Goulden et al. (2019)

When applicable, a high-affinity and low-affinity biosensors are listed. For a more comprehensive list, see (Hammond et al., 2022).

analyze the effects of various mutations in lipid-binding domains to reveal conserved features of their lipid-binding pockets. Finally, the CLiB assay was able to identify phosphatidylinositol (PI) lipid-binding nanobodies and screen them in conjunction with *in silico* design and directed evolution (Nishimura et al., 2025, Preprint). Therefore, this assay has the potential to lead to the next generation of lipid biosensors and nanobodies.

More precise quantitative measurements of lipid localization can be accomplished with genetically encoded lipid biosensors using Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET). Broadly speaking, these methods use a biosensor tagged with a donor fluorophore or luciferase and co-express an acceptor fluorophore either on another biosensor with the same specificity or tethered to a specific membrane compartment. Therefore, there is an increase in the emission ratio upon lipid binding only when lipids are enriched at the specific intracellular membrane with the acceptor. However, it should be noted that proximity-based intermolecular resonant transfer probes are very sensitive to probe density. Consequently, they work less well for the low-affinity biosensors and are more prone to the lipid-sequestration artifacts.

Intramolecular FRET biosensors use a single sensor expressing both the donor and acceptor fluorophore that is localized to a specific membrane. In this case, lipid binding induces a conformational change to increase the FRET ratio (Fabian et al., 2020; Sato et al., 2003). This intramolecular FRET design was used to create the Pippi-PI(4,5)P₂ sensor, the Pippi-PI4P sensor, and the Digda sensor for DAG. These sensors were used to collectively show an increase in lipid turnover at the leading edge of migrating cells (Nishioka et al., 2008). The same design was used for a PA biosensor, named Pii, which was used to describe an antithetical relationship between basal PA levels and the amount of PA produced by growth factor stimulation (Nishioka et al., 2010). Finally, intramolecular sensors for PI(3,4)P₂ (InPTapp) and PIP₃ (InPGRP) with added lysosomal-targeting domains recently provided novel insights into intracellular PIP₃ production by showing that lysosomal PIP₃ comes from PM PIP₃ in an endocytosis-dependent manner (Sahan et al., 2025).

In a principle similar to FRET, some biosensors utilize dimerization-dependent fluorescent proteins (ddFPs). These fluorophores will only fluoresce when two subunits dimerize, so attaching one subunit to a membrane and the other subunit to a biosensor will result in fluorescence only when the biosensor is

lipid bound at that target membrane, eliminating the majority of background fluorescence. Such ddFP biosensors were used in a flow cytometry assay to show changes in accessible cholesterol in a large population of cells, as opposed to measuring translocation of biosensors in single cells (Koh et al., 2023). Large-population measurements are also a benefit of BRET biosensors, exemplified by a recent BRET PI(3,5)P₂ biosensor used to measure lipid production at Rab5 endosomes in cells plated on 96-well plates (Pemberton et al., 2025).

As an alternative to FRET/BRET sensors, ratiometric biosensors utilize organic fluorophores that have a spectral shift when membrane bound. For example, an epsin1-based PI(4,5)P₂ sensor was able to demonstrate that PI(4,5)P₂ showed spatial heterogeneity across the PM and levels fluctuated extensively over time (Yoon et al., 2011). But, these probes are not as effective when trying to calculate mole percents of lipid species. As measured with the DAN-epsin1 PI(4,5)P₂ sensor and an NR3-eMyoxPH PIP₃ sensor, resting NIH 3T3 cells had a PIP₃/PI(4,5)P₂ ratio of 0.25 (Liu et al., 2014). However, this seems to be a large overestimation as mass spectrometry techniques utilizing methylation of the phosphate groups in PIP₃ showed that the maximal PIP₃/PI(4,5)P₂ ratio, which occurred when PTEN knockout MCF10a cells were stimulated with EGF, was only 0.015–0.02 (Clark et al., 2011). Despite this caveat when it comes to absolute quantification of lipid abundance, these ratiometric biosensors still show the expected relative changes in lipid levels, such as 5-phosphatases depleting PI(4,5)P₂, apoptosis increasing PS in the outer leaflet of the PM, or the conversion of PI(4,5)P₂ to PIP₃ when 3T3 cells are treated with insulin (Liu et al., 2014; Yoon et al., 2011).

Best practices and controls: To address the selectivity of lipid biosensors, it is best to induce lipid production within a membrane where it normally does not occur to confirm that this lipid is sufficient for biosensor membrane localization. This protocol utilizes live cells and so avoids discrepancies that often arise between *in vitro* and cellular assays. Although mutant sensors that cannot bind lipid could be a useful control, they do not clarify selectivity: they only show the binding site is necessary, not what it binds to!

As discussed, the transfection of biosensors should aim to minimize lipid sequestration or signaling perturbation. In practice, biosensors should be expressed at low levels using weak promoters or shorter transfection times. Additionally, because transfection efficiency varies among cells, biosensor quantification should be in the form of the ratio of membrane fluorescence to total or cytosolic fluorescence rather than just raw intensity values.

Membrane-targeted BRET, FRET, or ddFP sensors can reduce the cytosolic background seen with traditional biosensors, detect subtler lipid changes, and in the case of BRET and ddFP sensors, quantify lipid changes across whole populations. However, BRET/FRET sensors require careful optimization of donor and acceptor ratios, while also considering lipid concentration. Each method also has specific challenges: FRET sensors use multiple fluorophore channels, limiting the number of lipid species that can be simultaneously monitored, and it requires donor-only and acceptor-only controls to correct for cross talk. Also, for

intermolecular FRET sensors, a high density of fluorescent proteins on the membrane is required for measurable FRET, greatly increasing the propensity for lipid sequestration artifacts. For ddFP sensors, the lack of reversibility of the fluorophore reconstitution can be problematic as split GFP fragments can remain stably associated even under denaturing conditions.

Fluorescent lipid analogs

Predating genetically encoded lipid biosensors is the direct conjugation of an organic fluorophore to a lipid species, which can be added to a cell. Conceptually, this is equivalent to protein transfection to introduce extra copies of a molecule of interest, with the (often correct) assumption that the molecule recapitulates the localization and interactions of its native counterpart. Lipids that have been modified in this manner are known as fluorescent lipid analogs, with the term “analog” being used to demonstrate that they are derivatives of naturally occurring lipids and therefore not equivalent. Even different analogs of the same lipid have been shown to display vastly different biophysical characteristics (Sezgin et al., 2015), therefore warranting caution when selecting a probe. Despite this, fluorescent lipid analogs have been shown to recapitulate properties of many of their endogenous counterparts (Bernecic et al., 2019; Chen et al., 2025a).

Experimentally, working with these fluorescent lipid analogs is quite distinct from using GFP-tagged proteins, mainly because the “transfection” process for introducing lipids to cells is quite distinct. Fortunately, it is usually a fairly simple procedure. Lipids can be transferred to the outer leaflet of PMs by simply incubating cells with fluorescent lipid analogs present as liposomes or micelles, or even as a complex with serum albumin (Lipsky and Pagano, 1985). From here, the analog often follows the cellular itinerary of their endogenous counterparts using the same vesicular and non-vesicular transport machinery. A recent example was shown with a PI analog. The PM contains a flippase able to translocate PI to the inner PM leaflet (Muranaka et al., 2024), from where it can be transported to the ER by nonselective transport proteins such as E-Syts (Luan et al., 2024) and onward from there. Thus, TopFluor-PI rapidly localizes to the ER, Golgi, and mitochondria (Zewe et al., 2020), a localization closely matching endogenous PI detected using PI-consuming enzyme activity or the localization of a novel genetically encoded PI biosensor (Zewe et al., 2020; Pemberton et al., 2020).

Despite these clear successes, it should also be noted that such faithful mirroring of lipid analog traffic and steady-state distribution should not be taken for granted. For example, while BODIPY-fluorescein labelled ceramide correctly accumulated in the Golgi, its BODIPY-Texas Red derivative fails to efficiently traffic out of the ER (Tóth et al., 2006). This exemplifies the importance of the choice of fluorophore and conjugation position in generating reliable fluorescent lipid analog probes. Fig. 1 C details some pros and cons of this approach.

Among a number of suitable fluorophores, BODIPY has emerged as a popular choice, owing significantly to its tunable emission spectrum, photostability, and strong hydrophobicity that keeps it buried in the bilayer (Malinin et al., 2001). Furthermore, the broad range of excitation and emission wavelengths

available are ideal for designing FRET sensors, where BODIPY can serve as a donor, acceptor, or both (Malinin et al., 2001; McIntosh et al., 2012). Several phospholipid analogs tagged with BODIPY have been created and marketed under the name TopFluor, including PE, PS, PC, PI(4,5)P₂, and PI4P, which are commercially available through Avanti Research.

A common alternative is nitrobenzoxadiazole (NBD), a fluorophore that is small, environmentally sensitive, and highly reactive toward amines and biothiols (Jiang et al., 2021). The environmentally sensitive nature of NBD has made it an ideal fluorophore for fluorescence lifetime imaging microscopy measurements to determine membrane asymmetry (Gupta et al., 2020), for serving as a FRET sensor for PC transfer to small unilamellar vesicles (Panagabko et al., 2019), and for working as a depth-dependent fluorescence quenching probe to measure membrane penetration (Ladokhin, 2014), among other applications. There are a number of limitations for NBD probes, particularly in relation to BODIPY. NBD has a lower fluorescence yield, lower photostability, and a tendency to “loop” back to the membrane surface rather than staying embedded (PAGANO and CHEN, 1998).

Pyrene is a fluorophore that has seen common usage due to unique spectral properties. Two pyrene monomers within close proximity form an excited state dimer known as an excimer, which is associated with an emission band centered around 460-nm (Bains et al., 2011). This factor, coupled with high cell permeability and brightness make it effective in studying membrane organization. Pyrene lipid probes have been used to visualize lipid order membrane variations (Niko et al., 2016), polarity mapping of organelles (Valanciunaite et al., 2020), and liquid–liquid phase separation (Hazawa et al., 2021).

While fluorescent lipid analogs have existed for half a century, their popularity has seen renewed interest due to more recently developed chemical techniques. The introduction of probes that utilize click chemistry has allowed for the tandem quantification of both lipid metabolism and localization (Haberkant and Holthuis, 2014; Jao et al., 2009). By combining bifunctional lipid probes and ultrahigh resolution mass spectrometry, lipid transport between organelles can be tracked (Iglesias-Artola et al., 2025). Click probes have additionally found use in enabling super-resolution imaging techniques, such as of the inner mitochondrial membrane using stimulated emission depletion microscopy (Zheng et al., 2023). Incorporation of alkyne-modified or azide-modified isoprenoid analogs into proteins has enabled the live-cell tracking of prenylation and subsequent identification of several prenylated proteins (Jiang et al., 2018).

The development of probes with variable fluorescence as a function of external stimuli, known as fluorogenic probes, has further opened doors for the study of lipid metabolism. These probes utilize quenching, where fluorophores exist in a low emission fluorescence state that can be amplified up to four orders of magnitude following target binding (Kozma and Kele, 2019) or cleavage of the probe. A PC probe that had been modified with bis-pyrene on the sn-2 acyl chain and lipid headgroup was developed to measure hydrolytic activity of PLA₂, with potential to create probes for other phospholipases such as PLC and

phospholipase D (PLD) (Sagar et al., 2023). To monitor lipophagy, a fluorogenic probe reliant on an electron donor–acceptor system that is specific to lipid droplets was developed (Zhang et al., 2020).

Another class of emergent tools are proximity sensors, which use enzymes to catalyze the production of reactive species near a target, with the target frequently being a protein. In one example, these sensors were applied to lipids in an organelle-selective manner to quantify PS and PE transport between organelles and were done so without introducing chemical modifications to the lipids of interest (Chen et al., 2025b). In another case, the use of fluorogenic systems coupled with the specificity of biorthogonal reactions for molecular sensing allowed for the imaging of phospholipids in living cells, as well as their transport between organelles and orientation across leaflets (Moore et al., 2024, Preprint). Another instance utilizing spatially limited biorthogonal reactions saw the dynamic tracking of PC into which azido-choline was metabolically incorporated, serving to characterize lipid transport in living cells (Tamura et al., 2020). Further analysis traced the origin of the autophagosomal membrane to the ER, supporting the results of other researchers who utilized indirect imaging methods or cell fixation.

Beyond the use of genetically encoded lipid biosensors and lipid analogs, deuterium labelling with Raman microscopy has emerged as a method that does not perturb the natural chemistry of lipid species (Uematsu and Shimizu, 2021). Raman microscopy probes the vibrational modes of molecules, revealing information about chemical structure as well as physical characteristics such as viscosity, which specifically can be derived from the gauche/trans conformational ratio. This approach can potentially discern the physical properties of lipids on a subcellular level, particularly as they pertain to Lipid Droplets (LDs).

Best practices and controls: The simplest protocol for insertion of fluorescent lipid analogs into cells can be performed by complexing the analog of interest with BSA and then incubating it in the extracellular media. Caution should be taken, however, when selecting a lipid analog for this process. For example, TopFluor PS is eagerly inserted into the PM inner leaflet from the outer leaflet through native translocases, while TopFluor PI(4,5)P₂ is apparently restricted to entry via endocytic trafficking at low volumes and does not localize to the PM inner leaflet at all (Zewe et al., 2020). Other methods include the use of fusogenic liposomes (Csiszár et al., 2010) or microinjection (Golebiewska et al., 2011). Avanti Research’s TopFluor line of phospholipid analogs are a great starting point (Table 2), and one should consult the literature to ensure which sufficiently recapitulate the behaviors of native counterparts.

Lipidated fluorophores

Fluorescent lipid analogs allow us to add fluorophores to lipids, but we can also add lipids to fluorophores to induce constitutive membrane localization for membrane imaging. This is done by creating a construct where a fluorophore is joined to a protein sequence that gets lipidated once expressed in cells. The N-terminus of the Src kinase Lyn (named Lyn11, as it is made up of the first 11 amino acids of the protein) contains a conserved Gly2 and Cys3 that get myristoylated and palmitoylated,

Table 2. Resources available for the study of the major lipid classes

Lipid		Antibodies	Lipid analogs	Small molecule modulators		Membrane editing tools		
Chol	PFO	Johnson et al. (2012)	TopFluor cholesterol	Avanti Research	X	X	X	X
SM	Lysenin	Yamaji et al. (1998)	C11 TopFluor sphingomyelin	Avanti Research	14I	Yang et al. (2025)	caCer	Facilitates conversion of ceramide to SM by SMS2. Kol et al. (2019)
CL	Antibody	Guarnieri et al. (1971), Sorce et al. (2004)	TopFluor CL	Avanti Research	X	X	X	X
BMP	Monoclonal antibody 6C4	Kobayashi et al. (1999)	X	X	PLD3, PLD4, and LPLA2 are possible future targets	Singh et al. (2024), Chen et al. (2023)	X	X
PC	X	X	TopFluor PC	Avanti Research	Geranylgeraniol and farnesol	Miquel et al. (1998)	azo-PC	Photo-switchable azobenzene-containing lipid. Pritzl et al. (2022) , Urban et al. (2018) , Pernpeintner et al. (2017) , Pritzl et al. (2025)
PE	Lantibiotic probes	Zhao (2011)	TopFluor PE	Avanti Research	X	X	X	X
PG	X	X	X	X	Alexidine dihydrochloride	Doughty-Shenton et al. (2010)	X	X
PI	Bc-PI-PLC ^{ANH}	Maib et al. (2024)	TopFluor PI	Avanti Research	X	X	X	X
PPIn	PI3P, PI4P, PI(4,5)P ₂ , PI(3,4)P ₂ , and PIP ₃ antibodies plus recombinant probes for these and PI(3,5)P ₂ .	Puri et al. (2018), Hammond et al. (2009), 2006; Posor et al. (2013), Yip et al. (2008), Maib et al. (2024)	TopFluor PI(4,5)P ₂ , TopFluor PI4P, TopFluor PI(3,5)P ₂ , TopFluor PI(3,4,5)P ₃ , and TopFluor TMR PI4P	Avanti Research	SAR088 (PI3P4K), the PITCOIN series (PI3KC2α), apilimod (PIKfyve), and recently optimized PI4KA/PI4KB inhibitors	Lo et al. (2022), Jin and Xue (2023), Burke et al. (2023), Takeuchi et al. (2025)	CRY2 and FKBP systems for many PPIn species	CRY2 is optogenetically activated. FKBP is chemically inducible. Heo et al. (2006) ; Suh et al. (2006) ; Varnai et al. (2006) ; Fill et al. (2006) ; Goulden et al. (2019) ; Idevall-Hagren et al. (2012)
PS	Annexin V and PS antibody 1H6	van Engeland et al. (1998); Mourdjeva et al. (2005)	TopFluor PS	Avanti Research	DS07551382, DS5980254, and PTDS51	Yoshihama et al. (2022); Omi et al. (2024)	FKBP-ΔPHD-ORP5 and FKBP-ΔPHD-ORP8	Facilitates PM PI4P reduction and PS increase. Chung et al. (2015) ; Chung et al. (2023)
PA	X	X	TopFluor TMR PA	Avanti Research	R59022 BMS-502 FPI	Courcelles et al. (1985); Su et al. (2009); Wichroski et al. (2023); Chupak et al. (2023)	superPLD	Optical activation to induce PA synthesis. Li et al. (2024)

This table is not an exhaustive list, rather it is widely used and/or commercially available tools. An X denotes a lack of tools.

respectively (Resh, 1994; Resh, 1999). Similarly, the C-terminus of HRAS contains a CAAX motif (Cys-aliphatic-aliphatic-any residue) where Cys186 gets isoprenylated, further processing such as cleavage of the AAX residues occurs, and then Cys residues upstream get palmitoylated (Buss and Sefton, 1986; Hancock et al., 1989; Gutierrez et al., 1989).

GFP-Lyn11 or GFP-HRAS-CAAX are commonly used as PM markers to quantify the fluorescence of lipid biosensors at the PM as compared with the rest of the cell (Wills et al., 2021). Measuring biosensor localization as a membrane to cell ratio is an important control to account for cells expressing differing amounts of the biosensors. As membrane markers, these constructs are also used to study PM morphology and thus are widely used in studies of cell migration (Bisaria et al., 2020; Gong et al., 2024) or for observing formation of membrane structures such as caveolae (Tillu et al., 2021).

Perhaps the most popular use of lipidated fluorophores is in lipid raft studies, as different lipidation patterns show different localization patterns within PM microdomains. FRET studies with fluorophores conjugated to the N-terminus of Lck, which partitions to lipid-ordered domains, and the N-terminus of Src, which localizes to disordered domains, showed that cholesterol levels regulate the size of rafts and that PI(4,5)P₂ breakdown and recovery can vary in kinetics when it occurs in raft vs non-raft domains (Myeong et al., 2021).

GFP-Lyn11 and GFP-HRAS-CAAX are also a useful way to study membrane properties as these fluorophores act as a proxy for endogenous lipid behavior. However, as the fluorophore does not recapitulate an endogenous lipid headgroup and lipidated proteins have more variability in the number of acyl chains they can carry as compared with the standard 2 acyl chains per lipid, care should be taken to not overinterpret data from GFP-Lyn11 or GFP-HRAS-CAAX. Despite these caveats, several studies are aided by the inclusion of these constructs. Lyn11 with a photoactivatable mCherry fluorophore and CAAX with a photoconvertible fluorophore were used with single-molecule microscopy techniques to track lipid diffusion within the PM (Pacheco et al., 2024; Štefl et al., 2024). Lyn11 and CAAX domains are also commonly used to anchor chemically inducible or optogenetic systems for membrane editing, which will be discussed in a later section.

“Knockdown/knockout” of lipids

Arguably the biggest catalyst of discovery in molecular cell biology over the last 2 decades has been the ease and rapidity of genetic manipulation of cells. Small interfering RNA followed by CRISPR/Cas9 technology gave us rapid knockdown of messenger RNA or disruption of chromosomal DNA loci, respectively. In both cases, proteins' expression is selectively blocked, allowing interrogation of their function. Since lipids are not directly genetically encoded, these approaches at first seem useless to the budding lipidologist. However, it is worth remembering that lipids are generally only one step removed from the central dogma: DNA makes RNA makes protein (i.e., enzyme) makes lipid (Fig. 2 A). Therefore, it can be possible to knockout a lipid by genetic depletion of the terminal enzyme involved in its

synthesis (Fig. 2 B). That said, there are many pitfalls with such an approach. Firstly, lipids may have several orthologous enzymes that synthesize them, and worse still, are usually generated through complex metabolic networks rather than simple linear pathways, making targeting of a single gene partially or even completely ineffective. Secondly, phenotypes can be hard to interpret because whereas they may be due to depletion of the target lipid, they may also be due to increases in the substrate lipid or perturbation of upstream or downstream components of the metabolic network. A striking example was experiments to acutely increase PI(4,5)P₂: when the lipid was increased by induction of its terminal enzyme, PIP5K, actin comets formed. Yet when PI(4,5)P₂ was acutely “uncaged” by release from overexpressed biosensor, ruffles formed instead (Ueno et al., 2011). The difference was in the precursor, PI4P; PIP5K depletes this lipid from the PM, whereas the biosensor leaves it unchanged. Many lipid-metabolizing enzymes also have “moonlighting” functions in addition to their catalytic activity, which can further complicate interpretation. For this reason, an essential control when knocking down a lipid-metabolizing enzyme (or any other kind) is demonstrating rescue by the catalytically active enzyme but not an inactive mutant.

In light of all of these pitfalls to traditional knockdown/knockout approaches, we will instead entertain alternative strategies to the same effect, namely pharmacologic and synthetic biology approaches.

Small molecule modulators: The pharmacologist's playbook

The metabolic origin of lipids does present an opportunity for their modulation: It is not necessary (or, in light of moonlighting functions, desirable) to knockout a lipid-synthesizing enzyme. Instead, inhibition of the catalytic activity is sufficient. Enter small molecule inhibitors, with an ever-expanding catalog of lipid enzyme-directed compounds (Fig. 2 C). Inhibitors of phosphoinositide synthesis have led the way here, since the terminal step of these lipids' generation involves a kinase, enabling optimization of ATP-competitive inhibitors that selectively target these enzymes. Of course, some of the same caveats as with genetic perturbations apply; namely, the presence of multiple orthologs and different synthetic pathways. That said, there are clear successes with PI kinases. For example, the lipid PI(3,5)P₂ is synthesized by a single enzyme, PIKfyve; acute inhibition of this kinase with YM201636 or apilimod causes complete depletion of the lipid in under 5 minutes (Zolov et al., 2012; Pemberton et al., 2025). Due to the inherent “druggability” of phosphoinositide kinases and the large number of disease-associated functions, preclinical and even clinical development of many compounds targeting a wide variety of kinases is now underway; we direct the reader to recent reviews for comprehensive contemporary catalogs (Vanhaesebroeck et al., 2021; Burke et al., 2023).

Discovery efforts have branched beyond phosphoinositide kinases to include phospholipases (Huang et al., 2020) and phosphatases (Suwa et al., 2009; Viernes et al., 2014; Pirruccello et al., 2014; Lim et al., 2018; Lim et al., 2024). It should be cautioned that selectivity screens and mechanism of action are less well developed for such compounds, which can exhibit confounding

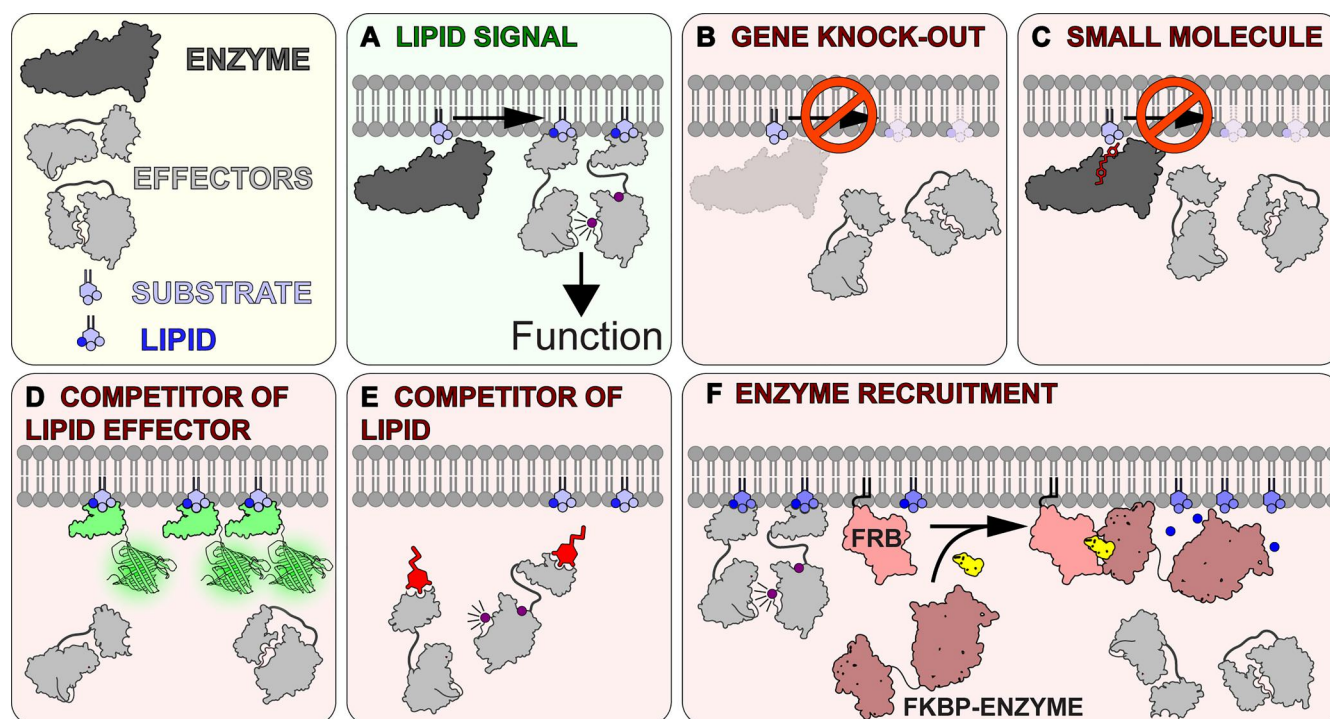


Figure 2. **Strategies for lipid knockout or knockdown.** Schematics showing the major strategies to disrupt lipids in living cells. (A–F) A shows the control condition, whereas B–F show different modes of inhibition, either targeting lipid synthesis (B and C), disrupting lipid–effector interactions (D and E), or inducing lipid degradation (F).

off-target effects (Sayed et al., 2024). Inhibitors have also been developed for the other lipid-generating enzymes, including PLD (Cho and Han, 2017; Brown et al., 2017; Noble et al., 2018) and diacylglycerol (DAG) kinases (Wichroski et al., 2023; Chupak et al., 2023). Furthermore, inhibitors for lipid synthases have also been generated, with many exciting prospects. One such example is PS, a lipid enriched at the inner leaflet of the PM. PS synthase 1 (PSS1) and PSS2 synthesize the majority of PS from PC and PE at mitochondria-ER contact sites (Gibellini and Smith, 2010; Chakrabarti, 2021; Doyle et al., 2024; Sohn et al., 2018). Recently, researchers have developed PSS1-specific inhibitors with promising results against PS-enriched cancers (Yoshihama et al., 2022; Omi et al., 2024). However, a downside to inhibiting PSS1 is that this can initiate an imbalance of lipids within the cell. Of course, a potential downside to any small molecule modulator is assuming adequate cellular permeability. Additionally, the compound has no selectivity in terms of target organelle; the enzyme and thus product lipid will be modulated in all cellular compartments where it is found.

While we normally discuss small molecules as “inhibitors” rather than “modulators” in this context, we chose the latter term for good reason: Allosteric binding of small molecules can also cause activation of enzymes. An exciting recent example was the discovery of UCL-TRO-1938, a compound that binds to the catalytic subunit of PI3K α and likely disrupts the inhibitory interface with regulatory subunits; the outcome is potent activation of the enzyme (Gong et al., 2023). Additional screens are underway to identify allosteric activators for PLC enzymes (Carr et al., 2025).

Beyond targeting enzymes, direct inhibition of the biological effects of lipids can also be accomplished. As we discussed in the section on genetically encoded lipid biosensors, overexpressed biosensors with expression levels and dissociation constants approaching the lipid levels can out-compete endogenous effector proteins for lipid binding, inhibiting them (Fig. 2 D). Indeed, soon after discovery of the PI(4,5) P_2 -selective PH domain from PLC δ 1, excess probe was used to sequester the lipid, demonstrating its requirements in endocytosis and attachment of the cortical cytoskeleton (Jost et al., 1998; Raucher et al., 2000). Such experiments can be conveniently controlled using a lipid binding-deficient mutant. In addition to biosensors, antibodies have also found utility here: for example, antibodies that can sequester PS have been identified, with several currently in clinical trials (Chang et al., 2020). Alternatively, antagonizing the lipid–effector protein complex has been attacked from the protein side: cell-permeable lipid headgroup analogs have been developed that compete for effector proteins (Fig. 2 E), effectively eliminating effector membrane recruitment and activation (Indarte et al., 2019; Mahadevan et al., 2008; Miao et al., 2010).

Best practices and controls: It could be claimed that the only specific small molecule modulators of proteins are the ones where the off-target interactions have not been discovered...yet. In short, controls for the selectivity of any small molecule modulator should be included. These can take the form of validation, where genetic ablation of the target removes the effect of the small molecule. Conversely, orthogonal approaches to manipulate the lipid should give convergent results; for example

using bio-orthogonal membrane editing approaches described in the next section. In addition, appropriate controls should be included to establish that it is indeed the intended increase or decrease of the target lipid that generates the experimental effect—and not consequential decrease or increase in an upstream substrate.

Acute lipid knockdown and membrane editing: Molecular dimerization systems

A more recent and powerful approach to knockout lipids has been the use of synthetic biology. Specifically, the engineering of catalytic domains from lipid-metabolizing enzymes to be acutely, experimentally controlled. This allows lipids to be selectively generated or degraded with exquisite temporal and spatial precision. Since these approaches acutely change lipid composition of a target organelle, they have been eloquently labelled as “membrane editing” (Tei et al., 2023). While there are a variety of enzymes that can alter levels of different lipid species throughout the cell, there are also a variety of techniques to control the localization and regulation of those enzymes. The distinction that divides these techniques into two major groups is determined by their method of activation, either through optogenetics with genetically encoded light-sensitive components or chemogenetics with proteins modulated by specific small molecules. This method of activation is a major factor in the selection of the specific techniques for experimental application, as it directly impacts the systems’ spatial control and magnitude of enzymatic activity.

In 2006, there was a flurry of development around rapamycin-induced chemical dimerization systems (Fig. 2 F). At the time there was a need for new methods to directly modify compartmental lipid organization and measure the subsequent influence on cellular signaling processes (Fili et al., 2006). The system is composed of FRB (FKBP12-binding fragment of mammalian target of rapamycin [mTOR]) and FKBP (FK506-binding protein); through the addition of rapamycin, the two units heterodimerize (Ho et al., 1996). FRB is fused to a specific membrane targeting sequence, and FKBP is fused to the enzyme of interest, allowing for the controlled translocation of the enzyme to a specific subcellular location upon heterodimerization. Several groups in late 2006 employed this technique to modify PI3P (Fili et al., 2006), PI(4,5)P₂ (Varnai et al., 2006; Suh et al., 2006; Heo et al., 2006), and PIP₃ (Heo et al., 2006) levels and, as a result, demonstrated the system’s value as a tool to investigate the regulatory roles of signaling lipids in various cellular pathways. The Inoue lab later developed an orthogonal system (Miyamoto et al., 2012). This utilized an analog of the plant hormone, gibberellin (GA₃) with an acetoxymethyl group (GA₃-AM), which induces dimerization between its receptor, gibberellin insensitive dwarf1, and the gibberellin insensitive protein. The new system functioned at a timescale of seconds and thus provided the field with a way to control two dimerizers at the same time. While chemical dimerization systems have been vital in the study of lipid function, there are two main challenges when using this approach: poor reaction reversibility and spatial control. Although the dimerization can be restricted to a single target organelle, there is not control over which organelles are

targeted in the whole cell. Thus, several optogenetic approaches have been developed to address these concerns.

The most common optogenetic system uses light-induced association between two individual units (Tischer and Weiner, 2014). The advantage to this system is rapid heterodimerization between a light-responsive protein and its effector (Tischer and Weiner, 2014). One blue light-sensitive binding pair is the cryptochrome 2 protein (CRY2) and its partner, cryptochrome-interacting basic helix-loop-helix 1 (CIB1). Upon blue light exposure, CRY2 homo-oligomerizes (Bugaj et al., 2013) and binds to CIB1 (Más et al., 2000). The dimerization process is completed within seconds of exposure (Kennedy et al., 2010) and the complex dissociates within 5 min after exposure (Tischer and Weiner, 2014). This system has been applied in the manipulation of several lipid species and their signaling pathways. This has included investigations into PIP₃ and its involvement in downstream actin nucleation and membrane ruffling (Idevall-Hagren et al., 2012), as well as direction of effector AKT recruitment and activation (Katsura et al., 2015). Outside the PI3K pathway, the light sensitive system was employed to optogenetically control PLD-driven PA synthesis (Tei and Baskin, 2020). However, while the system has demonstrated improvements in temporal and spatial resolution from chemogenetic approaches due to activation by direct illumination, the approach still maintains limitations with both slow switch-off kinetics and delayed dimer dissociation resulting from clustering in solution and on the membrane (Benedetti et al., 2018). Delayed dissociation of the system’s subunits allows for activated cytosolic units to diffuse away from the illuminated region of interest and remain assembled, making it difficult to fully restrict the spatial dynamics of the constructs.

An alternative blue light dimerization tool set is the iLID system, composed of binding partners SsrA fused to the C-terminal portion of the LOV2 domain and the cytosolic protein SspB (Benedetti et al., 2018). The LOV2 domain forms a steric cage on SsrA and is released upon blue light irradiation allowing for SsrA association with SspB (Ueda et al., 2022). This system has been used to construct PM-localized photoactivated PI3K (iSH2-SspB) and PLCβ (opto-PLCβ), providing spatiotemporal control over the enzyme’s catalytic activity and downstream signaling *in vitro* (Kim et al., 2024; Ueda et al., 2022). Opto-PLCβ was further utilized *in vivo*, driving amygdala synaptic plasticity enhancement in mice (Kim et al., 2024). The faster dissociation rate of the dimeric units provides tighter spatial confinement; however, the high basal affinity between subunits gives significant association between the SsrA-LOV2 and SspB pre-photoactivation (Benedetti et al., 2018). This drawback left a space open for development of the third common blue light-driven dimerization system termed “Magnets”. Developed from the fungal photoreceptor Vivid (VVD) (Kawano et al., 2015), both units in the system are photoreceptors, which dimerize through simultaneous activation (Benedetti et al., 2018). Magnets present high spatial resolution due to rapid dissociation of the dimeric units upon loss of light, and high avidity through generation of photoreceptor concatemers (Benedetti et al., 2018). The interface between units, Ncaps, was engineered to induce heterodimerization of positive

(pMag) and negative (nMag) magnets through electrostatic interactions following light exposure (Kawano et al., 2015). The optimized units were then used to drive PI3K to the PM and induce PIP₃ synthesis and cell migration; both quantitative and morphological effects were reversible upon loss of light (Kawano et al., 2015). Subsequent studies addressed the remaining system limitations around required conditions for suitable avidity and functional folding, resulting in “Enhanced Magnets” (Benedetti et al., 2020). The system was applied as optogenetic tethers that reconstituted organelle membrane contact sites: an ER-trans-Golgi network tether facilitated the VAP-OSBP1-driven PI4P-cholesterol exchange (Benedetti et al., 2020), an effect that was rapidly reversed upon loss of light (Kawano et al., 2015).

Best practices and controls: Overall, we see the evolution of dimerization systems over time toward optimization of spatial control through more regulated on-off assembly kinetics. The iLID and Magnet systems provide more rapid switch-off kinetics, thus restricting the enzymatic activity to the illuminated region of the cell (Benedetti et al., 2018). However, this does not cause irrelevance for the chemogenetic and CRY2-CIB1 optogenetic dimeric pairs. When considering the employment of a dimerization system, several experimental specifications must be taken into account: experiment timescale, necessary level of enzymatic activity for desired effect, and cellular or subcellular localization. In general, a longer experimental timescale coincides with extended enzyme activity; however, continuous exposure to optical stimulation leads to phototoxicity (Hallett et al., 2016). In this case, chemogenetic or CRY2/CIB1 optogenetic systems may be preferable as the pairs sustain activity longer following initial activation. Alternatively, shorter timescale experiments are better operated with the high affinity, fast output optogenetic pairs that restrict activity to the precise illuminated area of interest in the cell due to rapid disassembly following loss of direct stimulation (Hallett et al., 2016). The measurement acquired following system activation also dictates the specific approach; for example, if measuring downstream signaling that only requires control over initial lipid synthesis but with greater enzymatic output, chemogenetic or CRY2-CIB1 systems would be preferable. Finally, the different dimeric partners vary in their efficiency to recruit to intracellular compartments (Hallett et al., 2016). Therefore, iLID is superior for intracellular membrane localization as opposed to CRY2-CIBN, which is sufficient for activity at the cell surface. Alternatively, for longer timescale experiments, chemical dimerization systems allow for both extended activation and subcellular membrane enrichment. It is also important to note that with any of these experimental specifications, use of catalytically dead enzymes as negative controls is crucial to establish baseline lipid levels and the direct influence of the membrane editing system.

Acute lipid knockdown and membrane editing: Photocaging systems

While dimerization systems are the most common optogenetic tools used for membrane lipid manipulation, methods involving light-sensitive allosteric enzyme activation and lipid alteration are also possible. Coumarins are a class of light-sensitive

fluorescent probes capable of “caging” other proteins and then being photolyzed upon illumination (Luo et al., 2014). To utilize this caging system, the target protein is mutated to incorporate a site-specific unnatural amino acid (Luo et al., 2014). Orthogonal translational machinery facilitates the addition of the coumarin-caged lysine at the identified site, thus incorporating a bulky coumarin group that blocks the site and is released through photolysis by 405-nm light (Luo et al., 2014). The system was effectively employed to incorporate a hydroxycoumarin-cage block at the active site of the LepB protein, which when released upon 405-nm light illumination, generated wild-type LepB PI3P 4-OH kinase activity (Goulden et al., 2019). Subsequently, the system was used to rapidly activate a bacterial inositol lipid “isomerase,” SopB (Walpole et al., 2022). A similar approach was applied to PA-synthesizing PLD. Superactive PLDs (superPLDs) were originally driven to the organelle membrane through the CRY2-CIBN system but were found to maintain background activity without optical stimulation due to spontaneous engagement with the membrane, thus making full spatiotemporal control of enzymatic activity impossible (Tei et al., 2023; Li et al., 2024). Therefore, to regain direct control over the PLDs’ activation, a blue light-sensitive LOV2 domain was incorporated into the flexible loop of the superPLD, providing control over protein activation as a photo switch (Li et al., 2024).

Best practices and controls: Light-sensitive allosteric enzyme activation and lipid alteration are particularly useful when enzyme activity is too high even in a cytosolic state, necessitating a “caged” approach and precluding the simple use of dimerization for membrane recruitment. To validate the catalytic activity contributed by these light-sensitive allosteric enzyme systems, negative controls include negating specific components of the system’s assembly, as well as expressing catalytically altered enzymatic variants and measuring the comparative change in lipid production.

Acute lipid knockdown and membrane editing: Caged lipids

A major concern when employing techniques dependent on genetic manipulation is limitations in cell lines that are difficult to transfect (Tei and Baskin, 2022). To address this, photosensitive lipids have become an alternative membrane editing technique (Morstein et al., 2021). One approach for photosensitization has been through caging the lipids themselves, rather than the enzymes that make them. Multifunctional sphingosine, DAG, and fatty acid lipid derivatives contained two photo-reactive groups, one photocleavable caging group to control biological activity and metabolic turnover, and another for functionalization by a click handle (Höglinger et al., 2017). Apart from these caged lipid compounds, “photoswitchable” lipids were developed, which can undergo a switch between the acyl chain azobenzene group’s *trans* and *cis* forms, providing a reversible activation mechanism by light directed isomerization (Frank et al., 2016). Photo-switchable analogs of DAG (PhoDAG) (Frank et al., 2016), PA (AzoPA) (Tei et al., 2021), the sphingolipid precursors ceramides (caCers) (Kol et al., 2019), and lysophosphatidic acid (AzoLPA) (Morstein et al., 2020) are a few examples of lipid species where this approach has recently been applied. The first successful application of this system involved a

synthetic PI3P analog, which provided direct evidence for PI3P as a sufficient signal for EEA1-dependent endosomal fusion (Subramanian et al., 2010). This approach has since been expanded to other phosphoinositide species and refined to address issues of uptake, metabolism, and spatial control, representing a powerful alternative to genetic or pharmacological perturbation of lipid enzymes (Schultz, 2023).

Best practices and controls: Because genetic approaches for the knockout/-in of lipids are not always sufficient to keep up with the dynamic and rapid processes of lipid metabolism and transport, caged lipids present an alternative acute editing system. Proper controls used to distinguish specific from nonspecific effects by the light-directed lipids mirrors that previously outlined for the fluorescent lipid analogs, including enantiomers and/or unrelated lipid analogs, as well as the employment of nonirradiated samples.

Perspective

Perhaps the most important point we want to stress is that most of the approaches we have described in this review use cell and molecular techniques that you likely already know. For the most part, they modify techniques such as cytochemistry or transfection with some lipid-specific reagents and idiosyncrasies. The advent of synthetic biology also leverages various optogenetic and chemogenetic modules with lipid-directed enzymes; these can be “mixed and matched” with standard molecular cloning approaches. Sometimes, fusing two or more things together makes something far more functional than they could ever be when used alone. Other times, of course, they will unleash death and destruction on the cell. But that is why we publish controls.

Even better is the fact that the required reagents are also easily obtained. Lipid-directed antibodies and binding proteins are commercially available. Echelon Biosciences Inc. sells many, though like any antibody, seeking published precedence in the literature for successful use is key. Likewise, fluorescent lipid analogs are also available from Echelon, with an even larger library of fluors and lipids from Avanti Polar Lipids Inc. The genetically encoded biosensors have been developed by academic labs and are freely available. Most are deposited in the public repository, addgene.org. The same is true for many of the optogenetic and chemogenetic tools for modulating lipid levels. The bottom line is, you can order most of these reagents today (we detail many in Table 2); you could be doing experiments by next week. We hope you get great results, and we cannot wait to read your paper. Just please remember to cite this review.

Acknowledgments

Methods to modulate and detect membrane lipids are legion; we apologize to the many colleagues whose work we simply did not have space to discuss.

The work was supported by the National Institutes of Health grants R35GM119412 (G.R.V. Hammond) and 1F31HL170755-01 (C.C. Weckerly).

Author contributions: Michael Worcester: writing—original draft, review, and editing. Morgan M.C. Ricci: conceptualization

and writing—original draft, review, and editing. Claire C. Weckerly: writing—original draft, review, and editing. Jesus G. Calixto: writing—original draft, review, and editing. Gerald R.V. Hammond: conceptualization and writing—original draft, review, and editing.

Disclosures: The authors declare no competing interests exist.

Submitted: 8 August 2025

Revised: 23 October 2025

Accepted: 11 November 2025

References

- Abe, M., A. Makino, F. Hullin-Matsuda, K. Kamijo, Y. Ohno-Iwashita, K. Hanada, H. Mizuno, A. Miyawaki, and T. Kobayashi. 2012. A role for sphingomyelin-rich lipid domains in the accumulation of phosphatidylinositol-4,5-Bisphosphate to the cleavage furrow during cytokinesis. *Mol. Cell. Biol.* 32:1396–1407. <https://doi.org/10.1128/mcb.06113-11>
- Aktar, S., S. Takatori, T. Tsuji, M. Orii, Y. Ohsaki, J. Cheng, and T. Fujimoto. 2017. A new electron microscopic method to observe the distribution of phosphatidylinositol 3,4-bisphosphate. *Acta Histochem. Cytoc.* 50: 141–147. <https://doi.org/10.1267/ahc.17025>
- Bains, G., A.B. Patel, and V. Narayanaswami. 2011. Pyrene: A probe to study protein conformation and conformational changes. *Molecules.* 16: 7909–7935. <https://doi.org/10.3390/molecules16097909>
- Benedetti, L., A.E.S. Barentine, M. Messa, H. Wheeler, J. Bewersdorf, and P. De Camilli. 2018. Light-activated protein interaction with high spatial subcellular confinement. *Proc. Natl. Acad. Sci. USA.* 115:E2238–E2245. <https://doi.org/10.1073/pnas.1713845115>
- Benedetti, L., J.S. Marvin, H. Falahati, A. Guillén-Samander, L.L. Looger, and P. De Camilli. 2020. Optimized vivid-derived magnets photodimerizers for subcellular optogenetics in mammalian cells. *Elife.* 9:e63230. <https://doi.org/10.7554/elife.63230>
- Bernecic, N.C., M. Zhang, B.M. Gadella, J.F.H.M. Brouwers, J.W.A. Jansen, G.J.A. Arkesteijn, S.P. de Graaf, and T. Leahy. 2019. BODIPY-cholesterol can be reliably used to monitor cholesterol efflux from capacitating mammalian spermatozoa. *Sci. Rep.* 9:9804. <https://doi.org/10.1038/s41598-019-45831-7>
- Bisaria, A., A. Hayer, D. Garbett, D. Cohen, and T. Meyer. 2020. Membrane-proximal F-actin restricts local membrane protrusions and directs cell migration. *Science.* 368:1205–1210. <https://doi.org/10.1126/science.aay7794>
- Blind, R.D., M. Suzawa, and H.A. Ingraham. 2012. Direct modification and activation of a nuclear receptor-PIP₂ complex by the inositol lipid kinase IPMK. *Sci. Signal.* 5:ra44. <https://doi.org/10.1126/scisignal.2003111>
- Boronenkov, I.V., J.C. Loijens, M. Umeda, and R.A. Anderson. 1998. Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing Pre-mRNA processing factors. *Mol. Biol. Cell.* 9: 3547–3560. <https://doi.org/10.1091/mbc.9.12.3547>
- Brombacher, E., S. Urwyler, C. Ragaz, S.S. Weber, K. Kami, M. Overduin, and H. Hilbi. 2009. Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of Legionella pneumophila. *J. Biol. Chem.* 284:4846–4856. <https://doi.org/10.1074/jbc.M807505200>
- Brown, H.A., P.G. Thomas, and C.W. Lindsley. 2017. Targeting phospholipase D in cancer, infection and neurodegenerative disorders. *Nat. Rev. Drug Discov.* 16:351–367. <https://doi.org/10.1038/nrd.2016.252>
- Bugaj, L.J., A.T. Choksi, C.K. Mesuda, R.S. Kane, and D.V. Schaffer. 2013. Optogenetic protein clustering and signaling activation in mammalian cells. *Nat. Methods.* 10:249–252. <https://doi.org/10.1038/nmeth.2360>
- Burke, J.E., J. Triscott, B.M. Emerling, and G.R.V. Hammond. 2023. Beyond PI3Ks: Targeting phosphoinositide kinases in disease. *Nat. Rev. Drug Discov.* 22:357–386. <https://doi.org/10.1038/s41573-022-00582-5>
- Buss, J.E., and B.M. Sefton. 1986. Direct identification of palmitic acid as the lipid attached to p21 ras. *Mol. Cell. Biol.* 6:116–122. <https://doi.org/10.1128/mcb.6.1.116-122.1986>
- Carr, A.J., N. Hajicek, A.P. Tsai, P.P. Acharya, P.B. Hardy, E. Meyer, T. Wyss-Coray, K.H. Pearce, J. Sondek, and Q. Zhang. 2025. A high-throughput assay platform to discover small molecule activators of

- the phospholipase PLC- γ 2 to treat Alzheimer's disease. *J. Biol. Chem.* 301:108356. <https://doi.org/10.1016/j.jbc.2025.108356>
- Chakrabarti, A. 2021. Phospholipid asymmetry in biological membranes: Is the role of phosphatidylethanolamine underappreciated? *J. Membr. Biol.* 254:127–132. <https://doi.org/10.1007/s00232-020-00163-w>
- Chang, W., H. Fa, D. Xiao, and J. Wang. 2020. Targeting phosphatidylserine for cancer therapy: Prospects and challenges. *Theranostics*. 10: 9214–9229. <https://doi.org/10.7150/thno.45125>
- Chen, J., A. Cazenave-Gassiot, Y. Xu, P. Piroli, R. Hwang, L. DeFreitas, R.B. Chan, G. Di Paolo, R. Nandakumar, M.R. Wenk, and C. Marquer. 2023. Lysosomal phospholipase A2 contributes to the biosynthesis of the atypical late endosome lipid bis(monoacylglycerol)phosphate. *Commun. Biol.* 6:210. <https://doi.org/10.1038/s42003-023-04573-z>
- Chen, J., F. Deng, J. Li, and Q.J. Wang. 2008. Selective binding of phorbol esters and diacylglycerol by individual C1 domains of the PKD family. *Biochem. J.* 411:333–342. <https://doi.org/10.1042/bj20071334>
- Chen, P.-H.B., X.-L. Li, and J.M. Baskin. 2025a. Synthetic lipid biology. *Chem. Rev.* 125:2502–2560. <https://doi.org/10.1021/acs.chemrev.4c00761>
- Chen, X., R. He, H. Xiong, R. Wang, Y. Yin, Y. Chen, and Z.-J. Zhu. 2025b. Quantitative profiling of lipid transport between organelles enabled by subcellular photocatalytic labelling. *Nat. Chem.* 17:1534–1545. <https://doi.org/10.1038/s41557-025-01886-w>
- Cheng, J., A. Fujita, H. Yamamoto, T. Tatematsu, S. Kakuta, K. Obara, Y. Ohsumi, and T. Fujimoto. 2014. Yeast and mammalian autophagosomes exhibit distinct phosphatidylinositol 3-phosphate asymmetries. *Nat. Commun.* 5:3207. <https://doi.org/10.1038/ncomms4207>
- Cho, J.H., and J.-S. Han. 2017. Phospholipase D and its essential role in cancer. *Mol. Cells*. 40:805–813. <https://doi.org/10.14348/molcells.2017.0241>
- Chung, J., F. Torta, K. Masai, L. Lucast, H. Czaplá, L.B. Tanner, P. Narayanaswamy, M.R. Wenk, F. Nakatsu, and P. De Camilli. 2015. INTRACELLULAR TRANSPORT. PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. *Science*. 349: 428–432. <https://doi.org/10.1126/science.aab1370>
- Chung, W.Y., M. Ahuja, B.A. McNally, S.R. Leibow, H.K.E. Ohman, A. Movahed Abtahi, and S. Muallem. 2023. PtdSer as a signaling lipid determined by privileged localization of ORP5 and ORP8 at ER/PM junctional foci to determine PM and ER PtdSer/PI(4)P ratio and cell function. *Proc. Natl. Acad. Sci. USA*. 120:e2301410120. <https://doi.org/10.1073/pnas.2301410120>
- Chupak, L., M. Wichroski, X. Zheng, M. Ding, S. Martin, C. Allard, J. Shi, R. Gentles, N.A. Meanwell, J. Fang, et al. 2023. Discovery of potent, dual-inhibitors of diacylglycerol kinases α and ζ guided by phenotypic optimization. *ACS Med. Chem. Lett.* 14:929–935. <https://doi.org/10.1021/acsmchemlett.3c00063>
- Clark, J., K.E. Anderson, V. Juvin, T.S. Smith, F. Karpe, M.J.O. Wakelam, L.R. Stephens, and P.T. Hawkins. 2011. Quantification of PtdInsP3 molecular species in cells and tissues by mass spectrometry. *Nat. Methods*. 8: 267–272. <https://doi.org/10.1038/nmeth.1564>
- Courcelles, D.C.de C.de, P. Roevens, and H.V. Belle. 1985. R 59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J. Biol. Chem.* 260: 15762–15770.
- Csiszár, A., N. Hersch, S. Dieluweit, R. Biehl, R. Merkel, and B. Hoffmann. 2010. Novel fusogenic liposomes for fluorescent cell labeling and membrane modification. *Bioconjug. Chem.* 21:537–543. <https://doi.org/10.1021/bc900470y>
- Doughty-Shenton, D., J.D. Joseph, J. Zhang, D.J. Pagliarini, Y. Kim, D. Lu, J.E. Dixon, and P.J. Casey. 2010. Pharmacological targeting of the mitochondrial phosphatase PTPMT1. *J. Pharmacol. Exp. Ther.* 333:584–592. <https://doi.org/10.1124/jpet.109.163329>
- Doyle, C.P., A. Rectenwald, L. Timple, and G.R.V. Hammond. 2024. Orthogonal targeting of SAC1 to mitochondria implicates ORP2 as a major player in PM PI4P turnover. *Contact*. 7:25152564241229272. <https://doi.org/10.1177/25152564241229272>
- van Engeland, M., L.J.W. Nieland, F.C.S. Ramaekers, B. Schutte, and C.P.M. Reutelingsperger. 1998. Annexin V-Affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*. 31:1–9. [https://doi.org/10.1002/\(sici\)1097-0320\(19980101\)31:1<1::aid-cyto1>3.0.co;2-r](https://doi.org/10.1002/(sici)1097-0320(19980101)31:1<1::aid-cyto1>3.0.co;2-r)
- Ercan, B., T. Naito, D.H.Z. Koh, D. Dharmawan, and Y. Saheki. 2021. Molecular basis of accessible plasma membrane cholesterol recognition by the GRAM domain of GRAMD1b. *EMBO J.* 40:e106524. <https://doi.org/10.15252/embj.2020106524>
- Fabian, H., L. Simin, C. Mingyuan, P. Lutz, M. Sohum, and Z. Jin. 2020. Fluorescent biosensors for multiplexed imaging of phosphoinositide dynamics. *ACS Chem. Biol.* 15:33–38. <https://doi.org/10.1021/acscchembio.9b00691>
- Fili, N., V. Calleja, R. Woscholski, P.J. Parker, and B. Larijani. 2006. Compartmental signal modulation: Endosomal phosphatidylinositol 3-phosphate controls endosome morphology and selective cargo sorting. *Proc. Natl. Acad. Sci. USA*. 103:15473–15478. <https://doi.org/10.1073/pnas.0607040103>
- Frank, J.A., D.A. Yushchenko, D.J. Hodson, N. Lipstein, J. Nagpal, G.A. Rutter, J.-S. Rhee, A. Gottschalk, N. Brose, C. Schultz, and D. Trauner. 2016. Photoswitchable diacylglycerols enable optical control of protein kinase C. *Nat. Chem. Biol.* 12:755–762. <https://doi.org/10.1038/nchembio.2141>
- Fujita, A., J. Cheng, K. Tauchi-Sato, T. Takenawa, and T. Fujimoto. 2009. A distinct pool of phosphatidylinositol 4,5-bisphosphate in caveolae revealed by a nanoscale labeling technique. *Proc. Natl. Acad. Sci. USA*. 106: 9256–9261. <https://doi.org/10.1073/pnas.0900216106>
- Gibellini, F., and T.K. Smith. 2010. The Kennedy pathway—De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life*. 62: 414–428. <https://doi.org/10.1002/iub.337>
- Gillooly, D.J., I.C. Morrow, M. Lindsay, R. Gould, N.J. Bryant, J.M. Gaullier, R.G. Parton, and H. Stenmark. 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *Embo J.* 19:4577–4588. <https://doi.org/10.1093/emboj/19.17.4577>
- Golebiewska, U., J.G. Kay, T. Masters, S. Grinstein, W. Im, R.W. Pastor, S. Scarlata, and S. McLaughlin. 2011. Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages. *Mol. Biol. Cell*. 22:3498–3507. <https://doi.org/10.1091/mbc.e11-02-0114>
- Gong, B., J.D. Johnston, A. Thiemicke, A. de Marco, and T. Meyer. 2024. Endoplasmic reticulum-plasma membrane contact gradients direct cell migration. *Nature*. 631:415–423. <https://doi.org/10.1038/s41586-024-07527-5>
- Gong, G.Q., B. Bilanges, B. Allsop, G.R. Masson, V. Robertson, T. Askwith, S. Oxenford, R.R. Madsen, S.E. Conduit, D. Bellini, et al. 2023. A small-molecule PI3K α activator for cardioprotection and neuroregeneration. *Nature*. 618:159–168. <https://doi.org/10.1038/s41586-023-05972-2>
- Goulden, B.D., J. Pacheco, A. Dull, J.P. Zewe, A. Deiters, and G.R.V. Hammond. 2019. A high-avidity biosensor reveals plasma membrane PI(3,4)P2 is predominantly a class I PI3K signaling product. *J. Cell Biol.* 218: 1066–1079. <https://doi.org/10.1083/jcb.201809026>
- Guarnieri, M., B. Stechmiller, and A.L. Lehninger. 1971. Use of an antibody to study the location of cardiolipin in mitochondrial membranes. *J. Biol. Chem.* 246:7526–7532. [https://doi.org/10.1016/s0021-9258\(19\)45807-9](https://doi.org/10.1016/s0021-9258(19)45807-9)
- Guo, Z., C. Tong, Y. Yang, and J.-J. Liu. 2022. Immunofluorescence staining of phosphoinositides in primary mouse hippocampal neurons in dissociated culture. *STAR Protoc.* 3:101549. <https://doi.org/10.1016/j.xpro.2022.101549>
- Gupta, A., T. Korte, A. Herrmann, and T. Wohland. 2020. Plasma membrane asymmetry of lipid organization: Fluorescence lifetime microscopy and correlation spectroscopy analysis[S]. *J. Lipid Res.* 61:252–266. <https://doi.org/10.1194/jlr.d119000364>
- Gutierrez, L., A.I. Magee, C.J. Marshall, and J.F. Hancock. 1989. Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.* 8:1093–1098. <https://doi.org/10.1002/j.1460-2075.1989.tb03478.x>
- Haberkant, P., and J.C.M. Holthuis. 2014. Fat & fabulous: Bifunctional lipids in the spotlight. *Biochim. Biophys. Acta*. 1841:1022–1030. <https://doi.org/10.1016/j.bbalip.2014.01.003>
- Halaszovich, C.R., D.N. Schreiber, and D. Oliver. 2009. Ci-VSP is a depolarization-activated phosphatidylinositol-4,5-bisphosphate and Phosphatidylinositol-3,4,5-trisphosphate 5'-Phosphatase. *J. Biol. Chem.* 284:2106–2113. <https://doi.org/10.1074/jbc.m803543200>
- Hallett, R.A., S.P. Zimmerman, H. Yumerefendi, J.E. Bear, and B. Kuhlman. 2016. Correlating in vitro and in vivo activities of light-inducible dimers: A cellular optogenetics guide. *ACS Synth. Biol.* 5:53–64. <https://doi.org/10.1021/acssynbio.5b00119>
- Hammond, G.R.V., and T. Balla. 2015. Polyphosphoinositide binding domains: Key to inositol lipid biology. *Biochim. Biophys. Acta*. 1851:746–758. <https://doi.org/10.1016/j.bbalip.2015.02.013>
- Hammond, G.R.V., S.K. Dove, A. Nicol, J.A. Pinxteren, D. Zicha, and G. Schiavo. 2006. Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells. *J. Cell Sci.* 119:2084–2094. <https://doi.org/10.1242/jcs.02912>
- Hammond, G.R.V., M.J. Fischer, K.E. Anderson, J. Holdich, A. Koteci, T. Balla, and R.F. Irvine. 2012. PI4P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity. *Science*. 337:727–730. <https://doi.org/10.1126/science.1222483>

- Hammond, G.R.V., M.P. Machner, and T. Balla. 2014. A novel probe for phosphatidylinositol 4-phosphate reveals multiple pools beyond the Golgi. *J. Cell Biol.* 205:113–126. <https://doi.org/10.1083/jcb.201312072>
- Hammond, G.R.V., M.M.C. Ricci, C.C. Weckerly, and R.C. Wills. 2022. An update on genetically encoded lipid biosensors. *Mol. Biol. Cell.* 33:tp2. <https://doi.org/10.1091/mbc.e21-07-0363>
- Hammond, G.R.V., G. Schiavo, and R.F. Irvine. 2009. Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P₂. *Biochem. J.* 422:23–35. <https://doi.org/10.1042/bj20090428>
- Hancock, J.F., A.I. Magee, J.E. Childs, and C.J. Marshall. 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell.* 57:1167–1177. [https://doi.org/10.1016/0092-8674\(89\)90054-8](https://doi.org/10.1016/0092-8674(89)90054-8)
- Hazawa, M., S. Amemori, Y. Nishiyama, Y. Iga, Y. Iwashima, A. Kobayashi, H. Nagatani, M. Mizuno, K. Takahashi, and R.W. Wong. 2021. A light-switching pyrene probe to detect phase-separated biomolecules. *iScience.* 24:102865. <https://doi.org/10.1016/j.isci.2021.102865>
- Heo, W.D., T. Inoue, W.S. Park, M.L. Kim, B.O. Park, T.J. Wandless, and T. Meyer. 2006. PI(3,4,5)P₃ and PI(4,5)P₂ lipids target proteins with polybasic clusters to the plasma membrane. *Science.* 314:1458–1461. <https://doi.org/10.1126/science.1134389>
- Ho, S.N., S.R. Biggar, D.M. Spencer, S.L. Schreiber, and G.R. Crabtree. 1996. Dimeric ligands define a role for transcriptional activation domains in reinitiation. *Nature.* 382:822–826. <https://doi.org/10.1038/382822a0>
- Hoelzel, C.A., and X. Zhang. 2020. Visualizing and manipulating biological processes by using HaloTag and SNAP-Tag technologies. *ChemBiochem.* 21:1935–1946. <https://doi.org/10.1002/cbic.202000003>
- Höglinger, D., A. Nadler, P. Haberkant, J. Kirkpatrick, M. Schifferer, F. Stein, S. Hauke, F.D. Porter, and C. Schultz. 2017. Trifunctional lipid probes for comprehensive studies of single lipid species in living cells. *Proc. Natl. Acad. Sci. USA.* 114:1566–1571. <https://doi.org/10.1073/pnas.1611096114>
- Hokanson, D.E., J.M. Laakso, T. Lin, D. Sept, and E.M. Ostap. 2006. Myoic binds phosphoinositides through a putative pleckstrin homology domain. *MBoC.* 17:4856–4865. <https://doi.org/10.1091/mbc.e06-05-0449>
- Holmes, V.L., M.M.C. Ricci, C.C. Weckerly, M. Worcester, and G.R.V. Hammond. 2025. Single-molecule lipid biosensors mitigate inhibition of endogenous effector proteins. *J. Cell Biol.* 224:e202412026. <https://doi.org/10.1083/jcb.202412026>
- Hsieh, T.-S., V.A. Lopez, M.H. Black, A. Osinski, K. Pawłowski, D.R. Tomchick, J. Liou, and V.S. Tagliabracci. 2021. Dynamic remodeling of host membranes by self-organizing bacterial effectors. *Science.* 372:935–941. <https://doi.org/10.1126/science.aay8118>
- Huang, W., A.J. Carr, N. Hajicek, M. Sokolovski, E. Siraliev-Perez, P.B. Hardy, K.H. Pearce, J.S. Sondek, and Q. Zhang. 2020. A high-throughput assay to identify allosteric inhibitors of the PLC- γ isozymes operating at membranes. *Biochemistry.* 59:4029–4038. <https://doi.org/10.1021/acs.biochem.0c00511>
- Idevall-Hagren, O., E.J. Dickson, B. Hille, D.K. Toomre, and P. De Camilli. 2012. Optogenetic control of phosphoinositide metabolism. *Proc. Natl. Acad. Sci. USA.* 109:E2316–E2323. <https://doi.org/10.1073/pnas.1211305109>
- Iglesias-Artola, J.M., K. Böhlig, K. Schuhmann, K.C. Cook, H.M. Lennartz, M. Schuhmacher, P. Barahatjan, C.J. López, R. Šachl, V. Garikapati, et al. 2025. Quantitative imaging of lipid transport in mammalian cells. *Nature*:1–9. <https://doi.org/10.1038/s41586-025-09432-x>
- Indarte, M., R. Puentes, M. Maruggi, N.T. Ihle, G. Grandjean, M. Scott, Z. Ahmed, E.J. Meuliet, S. Zhang, R. Lemos, et al. 2019. An inhibitor of the pleckstrin homology domain of CNK1 selectively blocks the growth of mutant KRAS cells and tumors. *Cancer Res.* 79:3100–3111. <https://doi.org/10.1158/0008-5472.can-18-2372>
- Jao, C.Y., M. Roth, R. Welti, and A. Salic. 2009. Metabolic labeling and direct imaging of choline phospholipids in vivo. *Proc. Natl. Acad. Sci. USA.* 106:15332–15337. <https://doi.org/10.1073/pnas.0907864106>
- Jiang, C., H. Huang, X. Kang, L. Yang, Z. Xi, H. Sun, M.D. Pluth, and L. Yi. 2021. NBD-based synthetic probes for sensing small molecules and proteins: Design, sensing mechanisms and biological applications. *Chem. Soc. Rev.* 50:7436–7495. <https://doi.org/10.1039/d0cs01096k>
- Jiang, H., X. Zhang, X. Chen, P. Aramsangtienchai, Z. Tong, and H. Lin. 2018. Protein lipidation: Occurrence, mechanisms, biological functions, and enabling technologies. *Chem. Rev.* 118:919–988. <https://doi.org/10.1021/acs.chemrev.6b00750>
- Jin, Y., and J. Xue. 2023. Lipid kinases PIP5Ks and PIP4Ks: Potential drug targets for breast cancer. *Front. Oncol.* 13:1323897. <https://doi.org/10.3389/fonc.2023.1323897>
- Johnson, B.B., P.C. Moe, D. Wang, K. Rossi, B.L. Trigatti, and A.P. Heuck. 2012. Modifications in perfringolysin O domain 4 alter the cholesterol concentration threshold required for binding. *Biochemistry.* 51:3373–3382. <https://doi.org/10.1021/bi3003132>
- Jost, M., F. Simpson, J.M. Kavran, M.A. Lemmon, and S.L. Schmid. 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Curr. Biol.* 8:1399–1402. [https://doi.org/10.1016/s0960-9822\(98\)00022-0](https://doi.org/10.1016/s0960-9822(98)00022-0)
- Katsura, Y., H. Kubota, K. Kunida, A. Kanno, S. Kuroda, and T. Ozawa. 2015. An optogenetic system for interrogating the temporal dynamics of Akt. *Sci. Rep.* 5:14589. <https://doi.org/10.1038/srep14589>
- Kawano, F., H. Suzuki, A. Furuya, and M. Sato. 2015. Engineered pairs of distinct photoswitches for optogenetic control of cellular proteins. *Nat. Commun.* 6:6256. <https://doi.org/10.1038/ncomms7256>
- Kennedy, M.J., R.M. Hughes, L.A. Peteya, J.W. Schwartz, M.D. Ehlers, and C.L. Tucker. 2010. Rapid blue-light-mediated induction of protein interactions in living cells. *Nat. Methods.* 7:973–975. <https://doi.org/10.1038/nmeth.1524>
- Kim, Y.J., M.L. Guzman-Hernandez, and T. Balla. 2011. A highly dynamic ER-derived phosphatidylinositol-synthesizing organelle supplies phosphoinositides to cellular membranes. *Dev. Cell.* 21:813–824. <https://doi.org/10.1016/j.devcel.2011.09.005>
- Kim, Y.-J., S. Tohyama, T. Nagashima, M. Nagase, Y. Hida, S. Hamada, A.M. Watabe, and T. Ohtsuka. 2024. A light-controlled phospholipase C for imaging of lipid dynamics and controlling neural plasticity. *Cell Chem. Biol.* 31:1336–1348.e7. <https://doi.org/10.1016/j.chembiol.2024.03.001>
- Kiyokawa, E., T. Baba, N. Otsuka, A. Makino, S. Ohno, and T. Kobayashi. 2005. Spatial and functional heterogeneity of sphingolipid-rich membrane domains*. *J. Biol. Chem.* 280:24072–24084. <https://doi.org/10.1074/jbc.m502244200>
- Kobayashi, T., M.H. Beuchat, M. Lindsay, S. Frias, R.D. Palmiter, H. Sakuraba, R.G. Parton, and J. Gruenberg. 1999. Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol.* 1:113–118. <https://doi.org/10.1038/10084>
- Koh, D.H.Z., T. Naito, M. Na, Y.J. Yeap, P. Rozario, F.L. Zhong, K.-L. Lim, and Y. Saheki. 2023. Visualization of accessible cholesterol using a GRAM domain-based biosensor. *Nat. Commun.* 14:6773. <https://doi.org/10.1038/s41467-023-42498-7>
- Kol, M., B. Williams, H. Toombs-Ruane, H.G. Franquelim, S. Korneev, C. Schroeder, P. Schwill, D. Trauner, J.C. Holthuis, and J.A. Frank. 2019. Optical manipulation of sphingolipid biosynthesis using photo-switchable ceramides. *Elife.* 8:e43230. <https://doi.org/10.7554/elife.43230>
- Kozma, E., and P. Kele. 2019. Fluorogenic probes for super-resolution microscopy. *Org. Biomol. Chem.* 17:215–233. <https://doi.org/10.1039/c8ob02711k>
- Ladokhin, A.S. 2014. Measuring membrane penetration with depth-dependent fluorescence quenching: Distribution analysis is coming of age. *Biochim. Biophys. Acta.* 1838:2289–2295. <https://doi.org/10.1016/j.bbame.2014.02.019>
- Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. Gap43, marcks, and Cap23 modulate PI(4,5)P₂ at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* 149:1455–1472. <https://doi.org/10.1083/jcb.149.7.1455>
- Levin, R., G.R.V. Hammond, T. Balla, P. De Camilli, G.D. Fairn, and S. Grinstein. 2017. Multiphasic dynamics of phosphatidylinositol 4-phosphate during phagocytosis. *Mol. Biol. Cell.* 28:128–140. <https://doi.org/10.1091/mbc.e16-06-0451>
- Li, X.-L., R. Tei, M. Uematsu, and J.M. Baskin. 2024. Ultralow background membrane editors for spatiotemporal control of phosphatidic acid metabolism and signaling. *ACS Cent. Sci.* 10:543–554. <https://doi.org/10.1021/acscentsci.3c01105>
- Lim, J.W., S.K. Kim, S.Y. Choi, D.H. Kim, C.G. Gadhe, H.N. Lee, H.-J. Kim, J. Kim, S.J. Cho, H. Hwang, et al. 2018. Identification of crizotinib derivatives as potent SHIP2 inhibitors for the treatment of Alzheimer's disease. *Eur. J. Med. Chem.* 157:405–422. <https://doi.org/10.1016/j.ejmech.2018.07.071>
- Lim, P.C., B.K. Yap, Y.J. Tay, N.A. Hanapi, S.R. Yusof, and C.-Y. Lee. 2024. Discovery of aurones bearing two amine functionalities as SHIP2 inhibitors with insulin-sensitizing effect in rat myotubes. *RSC Med. Chem.* 15:2179–2195. <https://doi.org/10.1039/d3md00360d>
- Lipsky, N.G., and R.E. Pagano. 1985. A vital stain for the golgi apparatus. *Science.* 228:745–747. <https://doi.org/10.1126/science.2581316>
- Liu, S.-L., R. Sheng, M.J. O'Connor, Y. Cui, Y. Yoon, S. Kurilova, D. Lee, and W. Cho. 2014. Simultaneous in situ quantification of two cellular lipid pools using orthogonal fluorescent sensors. *Angew. Chem. Int. Ed.* 53:14387–14391. <https://doi.org/10.1002/anie.201408153>

- Lo, W.-T., H. Belabed, M. Küçükdisli, J. Metag, Y. Roske, P. Prokofeva, Y. Ohashi, A. Horatscheck, D. Cirillo, M. Krauss, et al. 2022. Development of selective inhibitors of phosphatidylinositol 3-kinase C2a. *Nat. Chem. Biol.* 19:1-10. <https://doi.org/10.1038/s41589-022-01118-z>
- Lu, Q., J. Yu, J. Yan, Z. Wei, and M. Zhang. 2011. Structural basis of the myosin X PH1N-PH2-PH1C tandem as a specific and acute cellular PI(3,4,5)P3 sensor. *Mol. Biol. Cell.* 22:4268-4278. <https://doi.org/10.1091/mbc.e11-04-0354>
- Luan, L., D. Liang, D.-C. Chiu, R. Tei, and J.M. Baskin. 2024. Imaging inter-organelle phospholipid transport by extended synaptotagmins using bioorthogonally tagged lipids. *ACS Chem. Biol.* 19:1683-1694. <https://doi.org/10.1021/acscchembio.4c00345>
- Luo, J., R. Uprety, Y. Naro, C. Chou, D.P. Nguyen, J.W. Chin, and A. Deiters. 2014. Genetically encoded optochemical probes for simultaneous fluorescence reporting and light activation of protein function with two-photon excitation. *J. Am. Chem. Soc.* 136:15551-15558. <https://doi.org/10.1021/ja5055862>
- Maeda, K., K. Anand, A. Chiapparino, A. Kumar, M. Poletto, M. Kaksonen, and A.C. Gavin. 2013. Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature*. 501:257-261. <https://doi.org/10.1038/nature12430>
- Mahadevan, D., G. Powis, E.A. Mash, B. George, V.M. Gokhale, S. Zhang, K. Shakalya, L. Du-Cuny, M. Berggren, M.A. Ali, et al. 2008. Discovery of a novel class of AKT pleckstrin homology domain inhibitors. *Mol. Cancer Ther.* 7:2621-2632. <https://doi.org/10.1158/1535-7163.mct-07-2276>
- Maib, H., P. Adarska, R. Hunton, J.H. Vines, D. Strutt, F. Bottanelli, and D.H. Murray. 2024. Recombinant biosensors for multiplex and super-resolution imaging of phosphoinositides. *J. Cell Biol.* 223:e202310095. <https://doi.org/10.1083/jcb.202310095>
- Malinin, V.S., M.E. Haque, and B.R. Lentz. 2001. The rate of lipid transfer during fusion depends on the structure of fluorescent lipid probes: A new chain-labeled lipid transfer probe pair $\dagger$. *Biochemistry*. 40: 8292-8299. <https://doi.org/10.1021/bi010570r>
- Más, P., P.F. Devlin, S. Panda, and S.A. Kay. 2000. Functional interaction of phytochrome B and cryptochrome 2. *Nature*. 408:207-211. <https://doi.org/10.1038/35041583>
- McIntosh, A.L., S. Senthivayagam, K.C. Moon, S. Gupta, J.S. Lwande, C.C. Murphy, S.M. Storey, and B.P. Atshaves. 2012. Direct interaction of Plin2 with lipids on the surface of lipid droplets: A live cell FRET analysis. *Am. J. Physiol. Cell Physiol.* 303:C728-C742. <https://doi.org/10.1152/ajpcell.00448.2011>
- Melan, M.A., and G. Sluder. 1992. Redistribution and differential extraction of soluble proteins in permeabilized cultured cells. Implications for immunofluorescence microscopy. *J. Cell Sci.* 101:731-743. <https://doi.org/10.1242/jcs.101.4.731>
- Miao, B., I. Skidan, J. Yang, A. Lugovskoy, M. Reibarkh, K. Long, T. Brazell, K.A. Durugkar, J. Maki, C.V. Ramana, et al. 2010. Small molecule inhibition of phosphatidylinositol-3,4,5-triphosphate (PIP3) binding to pleckstrin homology domains. *Proc. Natl. Acad. Sci. USA*. 107: 20126-20131. <https://doi.org/10.1073/pnas.1004522107>
- Micheva, K.D., R.W. Holz, and S.J. Smith. 2001. Regulation of presynaptic phosphatidylinositol 4,5-bisphosphate by neuronal activity. *J. Cell Biol.* 154:355-368. <https://doi.org/10.1083/jcb.200102098>
- Miquel, K., A. Pradines, F. Tercé, S. Selmi, and G. Favre. 1998. Competitive inhibition of choline phosphotransferase by geranylgeraniol and farnesol inhibits phosphatidylcholine synthesis and induces apoptosis in human lung adenocarcinoma A549 cells*. *J. Biol. Chem.* 273:26179-26186. <https://doi.org/10.1074/jbc.273.40.26179>
- Miyamoto, T., R. DeRose, A. Suarez, T. Ueno, M. Chen, T.-p. Sun, M.J. Wolfgang, C. Mukherjee, D.J. Meyers, and T. Inoue. 2012. Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. *Nat. Chem. Biol.* 8:465-470. <https://doi.org/10.1038/nchembio.922>
- Moore, W.M., R.J. Brea, C. Knittel, E. Wrightsman, B. Hui, J. Lou, C.F. Ancas, M.D. Best, N.K. Devaraj, and I. Budin. 2024. Subcellular imaging of lipids and sugars using genetically encoded proximity sensors. *bioRxiv*. <https://doi.org/10.1101/2024.05.01.592120> (Preprint posted May 03, 2024).
- Morstein, J., M.A. Dacheux, D.D. Norman, A. Shemet, P.C. Donthamsetti, M. Citir, J.A. Frank, C. Schultz, E.Y. Isacoff, A.L. Parrill, et al. 2020. Optical control of lysophosphatidic acid signaling. *J. Am. Chem. Soc.* 142: 10612-10616. <https://doi.org/10.1021/jacs.0c02154>
- Morstein, J., A.C. Impastato, and D. Trauner. 2021. Photoswitchable lipids. *ChemBioChem*. 22:73-83. <https://doi.org/10.1002/cbic.202000449>
- Mourdjeva, M., D. Kyurkchiev, A. Mandinova, I. Altankova, I. Kehayov, and S. Kyurkchiev. 2005. Dynamics of membrane translocation of phosphatidylserine during apoptosis detected by a monoclonal antibody. *Apoptosis*. 10:209-217. <https://doi.org/10.1007/s10495-005-6076-5>
- Muranaka, Y., R. Shigetomi, Y. Iwasaki, A. Hamamoto, K. Nakayama, H. Takatsu, and H.-W. Shin. 2024. Novel phosphatidylinositol flippases contribute to phosphoinositide homeostasis in the plasma membrane. *Biochem. J.* 481:1187-1202. <https://doi.org/10.1042/bcj20240223>
- Myeong, J., C.-G. Park, B.-C. Suh, and B. Hille. 2021. Compartmentalization of phosphatidylinositol 4,5-bisphosphate metabolism into plasma membrane liquid-ordered/raft domains. *Proc. Natl. Acad. Sci. USA*. 118: e2025343118. <https://doi.org/10.1073/pnas.2025343118>
- Niko, Y., P. Didier, Y. Mely, G.-i. Konishi, and A.S. Klymchenko. 2016. Bright and photostable push-pull pyrene dye visualizes lipid order variation between plasma and intracellular membranes. *Sci. Rep.* 6:18870. <https://doi.org/10.1038/srep18870>
- Nishimura, T., K. Tsuboyama, Y. Nakagaki, E. Yamamoto, and N. Mizushima. 2025. Rational engineering of lipid-binding probes via high-throughput protein-lipid interaction screening. *bioRxiv*. <https://doi.org/10.1101/2025.01.05.627504> (Preprint posted January 05, 2025).
- Nishioka, T., K. Aoki, K. Hikake, H. Yoshizaki, E. Kiyokawa, and M. Matsuda. 2008. Rapid turnover rate of phosphoinositides at the front of migrating MDCK cells. *Mol. Biol. Cell.* 19:4213-4223. <https://doi.org/10.1091/mbc.e08-03-0315>
- Nishioka, T., M.A. Frohman, M. Matsuda, and E. Kiyokawa. 2010. Heterogeneity of phosphatidic acid levels and distribution at the plasma membrane in living cells as visualized by a Förster resonance energy transfer (FRET) biosensor. *J. Biol. Chem.* 285:35979-35987. <https://doi.org/10.1074/jbc.m110.153007>
- Noble, A.R., N.J. Maitland, D.M. Berney, and M.G. Rumsby. 2018. Phospholipase D inhibitors reduce human prostate cancer cell proliferation and colony formation. *Br. J. Cancer*. 118:189-199. <https://doi.org/10.1038/bjc.2017391>
- Omar-Hmeadi, M., N.R. Gandasi, and S. Barg. 2018. PtdIns(4,5)P2 is not required for secretory granule docking. *Traffic*. 19:436-445. <https://doi.org/10.1111/tra.12562>
- Omi, J., T. Kato, Y. Yoshihama, K. Sawada, N. Kono, and J. Aoki. 2024. Phosphatidylserine synthesis controls oncogenic B cell receptor signaling in B cell lymphoma. *J. Cell Biol.* 223:e202212074. <https://doi.org/10.1083/jcb.202212074>
- Orii, M., T. Tsuji, Y. Ogasawara, and T. Fujimoto. 2021. Transmembrane phospholipid translocation mediated by Atg9 is involved in autophagosome formation. *J. Cell Biol.* 220:e202009194. <https://doi.org/10.1083/jcb.202009194>
- Osborne, S.L., C.L. Thomas, S. Gschmeissner, and G. Schiavo. 2001. Nuclear PtdIns(4,5)P2 assembles in a mitotically regulated particle involved in pre-mRNA splicing. *J. Cell Sci.* 114:2501-2511. <https://doi.org/10.1242/jcs.114.13.2501>
- Pacheco, J., K.A. Peña, S. Savransky, A. Gidon, G.R.V. Hammond, J. Janetzko, and J.-P. Vilardaga. 2024. Fast-diffusing receptor collisions with slow-diffusing peptide ligand assemble the ternary parathyroid hormone-GPCR-arrestin complex. *Nat. Commun.* 15:10499. <https://doi.org/10.1038/s41467-024-54772-3>
- Pagano, R.E., and C.S. Chen. 1998. Use of BODIPY-labeled sphingolipids to study membrane traffic along the endocytic pathway. *Ann. N. Y. Acad. Sci.* 845:152-160. <https://doi.org/10.1111/j.1749-6632.1998.tb09668.x>
- Pagano, R.E., M.A. Sepanski, and O.C. Martin. 1989. Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: Interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy. *J. Cell Biol.* 109:2067-2079. <https://doi.org/10.1083/jcb.109.5.2067>
- Panagabko, C., M. Baptist, and J. Atkinson. 2019. In vitro lipid transfer assays of phosphatidylinositol transfer proteins provide insight into the in vivo mechanism of ligand transfer. *Biochim. Biophys. Acta Biomembr.* 1861: 619-630. <https://doi.org/10.1016/j.bbamem.2018.12.003>
- Pemberton, J.G., I. Barlow-Busch, M.L. Jenkins, M.A.H. Parson, F. Saranyai, S.N. Bektas, Y.J. Kim, J.E. Heuser, J.E. Burke, and T. Balla. 2025. An advanced toolset to manipulate and monitor subcellular phosphatidylinositol 3,5-bisphosphate. *J. Cell Biol.* 224:e202408158. <https://doi.org/10.1083/jcb.202408158>
- Pemberton, J.G., Y.J. Kim, J. Humpolickova, A. Eisenreichova, N. Sengupta, D.J. Toth, E. Boura, and T. Balla. 2020. Defining the subcellular distribution and metabolic channeling of phosphatidylinositol. *J. Cell Biol.* 219: e201906130. <https://doi.org/10.1083/jcb.201906130>
- Pernpeintner, C., J.A. Frank, P. Urban, C.R. Roeske, S.D. Pritzl, D. Trauner, and T. Lohmüller. 2017. Light-controlled membrane mechanics and

- shape transitions of photoswitchable lipid vesicles. *Langmuir*. 33: 4083–4089. <https://doi.org/10.1021/acs.langmuir.7b01020>
- Pirruccello, M., R. Nandez, O. Idevall-Hagren, A. Alcazar-Roman, L. Abriola, S.A. Berwick, L. Lucast, D. Morel, and P. De Camilli. 2014. Identification of inhibitors of Inositol 5-phosphatases through multiple screening strategies. *ACS Chem. Biol.* 9:1359–1368. <https://doi.org/10.1021/cb500161z>
- Plantard, L., A. Arjonen, J.G. Lock, G. Nurani, J. Ivaska, and S. Strömlblad. 2010. PtdIns(3,4,5)P₃ is a regulator of myosin-X localization and filopodia formation. *J. Cell Sci.* 123:3525–3534. <https://doi.org/10.1242/jcs.069609>
- Posor, Y., M. Eichhorn-Gruenig, D. Puchkov, J. Schöneberg, A. Ullrich, A. Lampe, R. Müller, S. Zarbakhsh, F. Gulluni, E. Hirsch, et al. 2013. Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. *Nature*. 499:233–237. <https://doi.org/10.1038/nature12360>
- Pritzl, S.D., D.B. Konrad, M.F. Ober, A.F. Richter, J.A. Frank, B. Nickel, D. Trauner, and T. Lohmüller. 2022. Optical membrane control with red light enabled by red-shifted photolipids. *Langmuir*. 38:385–393. <https://doi.org/10.1021/acs.langmuir.1c02745>
- Pritzl, S.D., J. Morstein, N.A. Pritzl, J. Lipfert, T. Lohmüller, and D.H. Trauner. 2025. Photoswitchable phospholipids for the optical control of membrane processes, protein function, and drug delivery. *Commun. Mater.* 6: 59. <https://doi.org/10.1038/s43246-025-00773-8>
- Puri, C., M. Vicinanza, A. Ashkenazi, M.J. Gratian, Q. Zhang, C.F. Bento, M. Renna, F.M. Menzies, and D.C. Rubinstein. 2018. The RAB11A-positive compartment is a primary platform for autophagosome assembly mediated by WIPI2 recognition of PI3P-RAB11A. *Dev. Cell.* 45:114–131.e8. <https://doi.org/10.1016/j.devcel.2018.03.008>
- Quinn, K.V., P. Behe, and A. Tinker. 2008. Monitoring changes in membrane phosphatidylinositol 4,5-bisphosphate in living cells using a domain from the transcription factor tubby. *J. Physiol.* 586:2855–2871. <https://doi.org/10.1113/jphysiol.2008.153791>
- Raucher, D., T. Stauffer, W. Chen, K. Shen, S. Guo, J.D. York, M.P. Sheetz, and T. Meyer. 2000. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell*. 100:221–228. [https://doi.org/10.1016/S0092-8674\(00\)81560-3](https://doi.org/10.1016/S0092-8674(00)81560-3)
- Resh, M.D. 1994. Myristylation and palmitoylation of Src family members: The fats of the matter. *Cell*. 76:411–413. [https://doi.org/10.1016/0092-8674\(94\)90104-x](https://doi.org/10.1016/0092-8674(94)90104-x)
- Resh, M.D. 1999. Fatty acylation of proteins: New insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta*. 1451:1–16. [https://doi.org/10.1016/S0167-4889\(99\)00075-0](https://doi.org/10.1016/S0167-4889(99)00075-0)
- van Rheenen, J., E.M. Achame, H. Janssen, J. Calafat, and K. Jalink. 2005. PIP2 signaling in lipid domains: A critical re-evaluation. *EMBO J.* 24: 1664–1673. <https://doi.org/10.1038/sj.emboj.7600655>
- Roosmond, R.C. 1969. The effect of fixation with formaldehyde and glutaraldehyde on the composition of phospholipids extractable from rat hypothalamus. *J. Histochem. Cytochem.* 17:482–486. <https://doi.org/10.1177/17.7.482>
- Roy, A., and T.P. Levine. 2004. Multiple pools of phosphatidylinositol 4-Phosphate detected using the pleckstrin homology domain of Osh2p*. *J. Biol. Chem.* 279:44683–44689. <https://doi.org/10.1074/jbc.M401583200>
- Sagar, R., J. Lou, and M.D. Best. 2023. Development of a bis-pyrene phospholipid probe for fluorometric detection of phospholipase A2 inhibition. *Bioorg. Med. Chem.* 87:117301. <https://doi.org/10.1016/j.bmc.2023.117301>
- Sahan, A.Z., M. Chen, Q. Su, Q. Li, D. Wang, and J. Zhang. 2025. Lysosomal PIP3 revealed by genetically encoded lipid biosensors. *Proc. Natl. Acad. Sci. USA*. 122:e2426929122. <https://doi.org/10.1073/pnas.2426929122>
- Sato, M., Y. Ueda, T. Takagi, and Y. Umezawa. 2003. Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* 5: 1016–1022. <https://doi.org/10.1038/ncb1054>
- El Sayed, A., N. Gomes, A. Czerwińska, and A. Azzi. 2024. Drugs targeting SHIP2 demonstrate potent antiproliferative effects irrespective of SHIP2 inhibition. *Life Sci.* 357:123101. <https://doi.org/10.1016/j.lfs.2024.123101>
- Schoebel, S., W. Blankenfeldt, R.S. Goody, and A. Itzen. 2010. High-affinity binding of phosphatidylinositol 4-phosphate by Legionella pneumophila DrrA. *Embo Rep.* 11:598–604. <https://doi.org/10.1038/embor.2010.97>
- Schultz, C. 2023. Chemical tools for lipid cell biology. *Acc. Chem. Res.* 56: 1168–1177. <https://doi.org/10.1021/acs.accounts.2c00851>
- Seeman, P., D. Cheng, and G.H. Iles. 1973. Structure of membrane holes in osmotic and saponin hemolysis. *J. Cell Biol.* 56:519–527. <https://doi.org/10.1083/jcb.56.2.519>
- Sezgin, E., F.B. Can, F. Schneider, M.P. Clausen, S. Galiani, T.A. Stanly, D. Waithe, A. Colaco, A. Honigsmann, D. Wüstner, et al. 2016. A comparative study on fluorescent cholesterol analogs as versatile cellular reporters. *J. Lipid Res.* 57:299–309. <https://doi.org/10.1194/jlr.M065326>
- Sharma, V.P., V. DesMarais, C. Sumners, G. Shaw, and A. Narang. 2008. Immunostaining evidence for PI(4,5)P2 localization at the leading edge of chemoattractant-stimulated HL-60 cells. *J. Leukoc. Biol.* 84:440–447. <https://doi.org/10.1189/jlb.0907636>
- Shu-Lin, L., W. Zhi-Gang, H. Yusi, X. Yao, S. Indira, G. Sukhamoy, Z. Xin, S. Yoonjung, M. Jung-Hyun, G. Liang-Wei, et al. 2018. Quantitative lipid imaging reveals a new signaling function of phosphatidylinositol-3,4-bisphosphate: Isoform- and site-specific activation of Akt. *Mol. Cell.* 71: 1092–1104.e5. <https://doi.org/10.1016/j.molcel.2018.07.035>
- Singh, S., U. Dransfeld, Y. Ambaw, J. Lopez-Scarim, R.V. Farese, and T.C. Walther. 2024. PLD3 and PLD4 synthesize S,S-BMP, a key phospholipid enabling lipid degradation in lysosomes. *bioRxiv*. <https://doi.org/10.1101/2024.03.21.586175> (Preprint posted March 21, 2024).
- Sohn, M., M. Korzeniowski, J.P. Zewe, R.C. Wills, G.R.V. Hammond, J. Humpolickova, L. Vrzal, D. Chalupska, V. Veverka, G.D. Fairn, et al. 2018. PI(4,5)P2 controls plasma membrane PI4P and PS levels via ORP5/8 recruitment to ER-PM contact sites. *J. Cell Biol.* 217:1797–1813. <https://doi.org/10.1083/jcb.201710095>
- Sorice, M., A. Circella, I.M. Cristea, T. Garofalo, L. Di Renzo, C. Alessandri, G. Valesini, and M.D. Esposti. 2004. Cardiolipin and its metabolites move from mitochondria to other cellular membranes during death receptor-mediated apoptosis. *Cell Death Differ.* 11:1133–1145. <https://doi.org/10.1038/sj.cdd.4401457>
- Stauffer, T.P., S. Ahn, and T. Meyer. 1998. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* 8:343–346. [https://doi.org/10.1016/S0960-9822\(98\)70135-6](https://doi.org/10.1016/S0960-9822(98)70135-6)
- Štefl, M., M. Takamiya, V. Middel, M. Tekpinar, K. Nienhaus, T. Beil, S. Rastegar, U. Strähle, and G.U. Nienhaus. 2024. Caveolae disassemble upon membrane lesioning and foster cell survival. *iScience*. 27:108849. <https://doi.org/10.1016/j.isci.2024.108849>
- Su, W., O. Yeku, S. Olepu, A. Genna, J.-S. Park, H. Ren, G. Du, M.H. Gelb, A.J. Morris, and M.A. Frohman. 2009. 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Mol. Pharmacol.* 75:437–446. <https://doi.org/10.1124/mol.108.053298>
- Subramanian, D., V. Laketa, R. Müller, C. Tischer, S. Zarbakhsh, R. Pepperkok, and C. Schultz. 2010. Activation of membrane-permeant caged PtdIns(3)P induces endosomal fusion in cells. *Nat. Chem. Biol.* 6:324–326. <https://doi.org/10.1038/nchembio.348>
- Suh, B.-C., T. Inoue, T. Meyer, and B. Hille. 2006. Rapid chemically induced changes of PtdIns(4,5)P2 Gate KCNQ Ion channels. *Science*. 314: 1454–1457. <https://doi.org/10.1126/science.1131163>
- Suwa, A., T. Yamamoto, A. Sawada, K. Minoura, N. Hosogai, A. Tahara, T. Kurama, T. Shimokawa, and I. Aramori. 2009. Discovery and functional characterization of a novel small molecule inhibitor of the intracellular phosphatase, SHIP2. *Br. J. Pharmacol.* 158:879–887. <https://doi.org/10.1111/j.1476-5381.2009.00358.x>
- Szentpetery, Z., A. Balla, Y.J. Kim, M.A. Lemmon, and T. Balla. 2009. Live cell imaging with protein domains capable of recognizing phosphatidylinositol 4,5-bisphosphate: a comparative study. *BMC Cell Biol.* 10:67. <https://doi.org/10.1186/1471-2121-10-67>
- Takatori, S., R. Mesman, and T. Fujimoto. 2014. Microscopic methods to observe the distribution of lipids in the cellular membrane. *Biochemistry*. 53:639–653. <https://doi.org/10.1021/bi401598v>
- Takatori, S., T. Tatematsu, J. Cheng, J. Matsumoto, T. Akano, and T. Fujimoto. 2016. Phosphatidylinositol 3,5-bisphosphate-rich membrane domains in endosomes and lysosomes. *Traffic*. 17:154–167. <https://doi.org/10.1111/tra.12346>
- Takeuchi, K., L. Nagase, S. Kageyama, H. Kanoh, M. Oshima, A. Ogawa-Iio, Y. Ikeda, Y. Fujii, S. Kondo, N. Osaka, et al. 2025. PI5P4K inhibitors: Promising opportunities and challenges. *FEBS J.* 292:4446–4476. <https://doi.org/10.1111/febs.17393>
- Tamura, T., A. Fujisawa, M. Tsuchiya, Y. Shen, K. Nagao, S. Kawano, Y. Tamura, T. Endo, M. Umeda, and I. Hamachi. 2020. Organelle membrane-specific chemical labeling and dynamic imaging in living cells. *Nat. Chem. Biol.* 16:1361–1367. <https://doi.org/10.1038/s41589-020-00651-z>

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- signaling lipid phosphatidic acid by phospholipase D2 determines the output of extracellular signal-regulated kinase signaling in cancer cells. *Mol. Cell. Biol.* 34:84–95. <https://doi.org/10.1128/mcb.00987-13>
- Zhang, X., L. Yuan, J. Jiang, J. Hu, A. du Rietz, H. Cao, R. Zhang, X. Tian, F. Zhang, Y. Ma, et al. 2020. Light-up lipid droplets dynamic behaviors using a red-emitting fluorogenic probe. *Anal. Chem.* 92:3613–3619. <https://doi.org/10.1021/acs.analchem.9b04410>
- Zhao, M. 2011. Lantibiotics as probes for phosphatidylethanolamine. *Amino Acids.* 41:1071–1079. <https://doi.org/10.1007/s00726-009-0386-9>
- Zheng, S., N. Dadina, D. Mozumdar, L. Lesiak, K.N. Martinez, E.W. Miller, and A. Schepartz. 2024. Long-term super-resolution inner mitochondrial membrane imaging with a lipid probe. *Nat. Chem. Biol.* 20:83–92. <https://doi.org/10.1038/s41589-023-01450-y>
- Zolov, S.N., D. Bridges, Y. Zhang, W.-W. Lee, E. Riehle, R. Verma, G.M. Lenk, K. Converso-Baran, T. Weide, R.L. Albin, et al. 2012. In vivo, Pikfyve generates PI(3,5)P₂, which serves as both a signaling lipid and the major precursor for PI5P. *Proc. Natl. Acad. Sci.* 109:17472–17477. <https://doi.org/10.1073/pnas.1203106109>