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QUANTIFYING LIPID CHANGES IN VARIOUS MEMBRANE COMPARTMENTS USING LIPID BINDING PROTEIN DOMAINS

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SUMMARY

One of the largest challenges in cell biology is to map the lipid composition of the membranes of various organelles and define the exact location of processes that control the synthesis and distribution of lipids between cellular compartments. The critical role of phosphoinositides, low-abundant lipids with rapid metabolism and exceptional regulatory importance in the control of almost all aspects of cellular functions created the need for tools to visualize their localizations and dynamics at the single cell level. However, there is also an increasing need for methods to determine the cellular distribution of other lipids regulatory or structural, such as diacylglycerol, phosphatidic acid, or other phospholipids and cholesterol. This review will summarize recent advances in this research field focusing on the means by which changes can be described in more quantitative terms.

Graphical abstract

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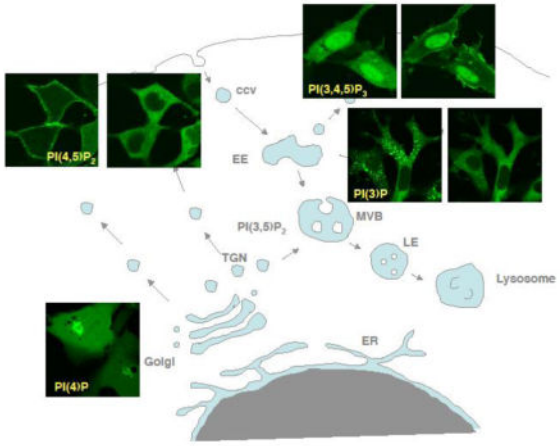

I. Introduction

The genomic era combined with GFP technology has yielded unprecedented progress in understanding the function, cellular distribution and interaction of protein signaling networks and their regulation both in normal cells and in disease states. Many proteins have been characterized at the structural level providing important clues about their functions or dysfunctions as defined by their atomic architecture. There has also been an enormous progress in analyzing the lipid composition of tissues and cells revealing a complexity that is hard to comprehend. Each major lipid class is represented with a repertoire of molecular species with various acyl side chain lengths and saturation and in addition to ester lipids, ether lipids and sphingolipids add to this enormous wealth of lipid variety. What is the significance of this range of variability when it comes to building the complex membrane architecture of eukaryotic cells? Where do enzymatic reactions that contribute to this heterogeneity take place and how the lipids move around in the cell's aqueous environment? How do cells decide whether to build membranes, store lipids or use them as energy source? These questions and many more, although not new, keep coming back to the frontiers as more and more signaling processes are recognized to require active participation of membranes not only as localization platforms but regulatory ones as well.

Most of our knowledge on lipid enzymology and cellular distribution has been based on classical membrane fractionation studies. However, now it is clear that several specific enzymatic reactions of lipid conversions are catalyzed by multiple enzymes encoded by distinct genes or by splice variants originating form a single gene. The cellular localization of the enzymes can be determined relatively easily with our modern tools, but as it turns out the lipid products of these enzymes can be rapidly transferred to other cellular compartments and this process often controls the metabolic flux through these pathways. Such transport-coupled enzymatic reactions, however, also mean that cell fractionations can lead to misleading conclusions concerning the distribution of lipids as it relates to an intact cell. We have to come to realize that we lack solid knowledge about the cellular distribution of even the most common phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) in spite of the fact that we more

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or less know where the enzymes that make these lipids are found. We have even fewer clues about the distribution of the various side-chain variants of these phospholipids or of any other lipid classes and the compartments where fatty acid remodeling takes place.

Several approaches have been used to find answers to these questions over the last 40 or so years. Fluorescent derivatives of lipids have been developed and added to the cells to follow their localization movements and metabolic fate [1, 2]. While these studies have made great contributions to our understanding how these lipids behave, there are some caveats regarding their reliability. The fluorescent tag, which is attached to the lipid headgroup or acyl side chain has a major impact on the behavior of the lipid altering its partitioning between various membranes or between the two leaflets or in the aqueous phase. Moreover, lipids added from the outside may not follow the same metabolic routes that are used by their endogenous counterparts. These caveats are being addressed by a new generation of chemical biological tools that address the means of delivery as well as the minimalization of changing the hydrophobic character of side chains [3–5].

An additional approach that has gained popularity is the use of antibodies raised against specific lipid species [6, 7]. In addition to the usual problems with specificity, the disadvantage of this approach is that it requires fixation so live cells studies cannot be performed. Still, anti phospholipid antibodies have also been providing us with very useful information. They have a great advantage over some other methods in that they show the distribution of the endogenous lipids without distortions caused by adding exogenous lipids or expressing lipid-binding domains (see below). One area of caution is the largely unknown effects of the various fixation procedures on the physicochemical states or distribution of the lipids. More and more data suggest that lipids remains mobile even when cells are fixed with conventional methods used for protein detection. Moreover, multivalent detection by antibodies can cluster the still mobile lipids creating false conclusions regarding clustering or other localization features [8–11]. Nevertheless, this approach will remain an important one especially when combined with the use of the lipid binding modules (see below) in fixed cells as “antibodies”. These latter ones have been extended for use at the EM level [12–14].

The third approach to determine where lipids are in the cell and how they change upon cellular responses was born out of the realization that many proteins contain domains for lipid recognition that can determine their cellular localization. Such lipid recognizing domains include pleckstrin homology (PH) domains, C1 or C2 domains (see below). In some cases these isolated domains fused to fluorescent proteins can specifically recognize lipid species in the membrane allowing the visualization of these lipids in live cells. The great advantage of these tools is that they can be used in intact cells allowing kinetic analysis of changes in real time. A disadvantage of this approach is that it requires the introduction of a protein that will bind to the lipids and disturb the physiology of the cells and distort the amount and distribution of the lipid in question. In addition, there is often an additional interaction (mostly with proteins) that contributes to the membrane attachment of these protein modules that complicates the interpretation of the data obtained. These caveats have received ample coverage in many reviews written by us [15–17] and others [18–21]. In spite of these limitations, this method, when used with caution, has contributed significantly to our understanding of the distribution and functions of many lipid species.

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In addition to fluorescence-based microscopy, there are additional labeling-free methods that do not require introduction of any probes to get information about lipid distribution in cells or tissues. Differential interference contrast (DIC) microscopy and phase contrast microscopy has been widely used to resolve structural details of cells or tissues. These techniques exploits differences between the refractive indices of various lipid membranes but provide no information on lipid composition. To distinguish chemically specific structures one needs to generate chemical contrast, which can be achieved by analysis of vibrational spectra of molecules. Coherent anti-Stokes Raman scattering (CARS) microscopy exploits this principle and has been particularly used for analysis of lipid distribution in cells or tissue. It is beyond the scope of this review to go to more details regarding this technique. More on these label-free methods can be found in [22].

CARS microscopy requires special instrumentation and understanding the complex analysis process, which has limited its wider appeal. Therefore, fluorescence-based microscopy methods are still more popular. Moreover, the increasing repertoire of ever improving genetically encoded fluorescent proteins is making the lipid binding probe approach more widely used. This method will, therefore, be the primary focus of this review.

II. Protein Domains that are used for imaging intracellular lipid pools

II. 1. PH domains

Several protein modules have evolved in biology that contribute to the localization of the proteins to the various membranes in eukaryotic cells. Some of these modules are well-folded structures with binding pockets that provide strong and specific interaction with the headgroups of phospholipids that face the cytosol. One of the best known and most studied of these is the pleckstrin-homology (PH) domain. PH domains have become most famous because of the phosphoinositide recognition of some of its members making them suitable to visualize inositol lipids in the cell (see Table I). Unfortunately, this has distorted views regarding the functions of PH domains in that they have been considered primarily as lipid binding domains even though often they also bind proteins and in many cases do not even bind lipids [23]. It is also important to recognize that even when PH domains do bind lipids, their lipid binding is not always responsible for membrane localization; instead, PH domains can serve as allosteric regulatory modules [24, 25]. Although PH domains are most famous for inositol lipid binding, some PH domains recognize other lipids, such as the Evectin PH domain that binds PS [26, 27]. An important point of consideration while using PH domains as lipid imaging tools is the fact that PH domains often bind proteins together with the lipids to bind to membranes. The best examples are PH domains from several lipid transfer proteins, such as CERT, OSBP and FAPP2 (FAPP1 PH behaves the same way but it is not a lipid transfer protein) that recognize Golgi PI4P together with the GTP-bound form of Arf1 [28]. This dual recognition is an important feature of the Golgi-related function of these proteins, allowing monitoring Golgi-specific changes in PI4P, but also making it difficult sometimes to distinguish between Arf1 or PI4P changes as the primary cause for altering localizations of these PH domains. Lastly, it is also important to recognize that high lipid binding specificity of PH domains is not a requisite for specific regulation by a particular lipid in the context of signaling. Even with promiscuous lipid binding of a PH domain,

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specific regulation can be achieved if a particular lipid is directly delivered to the domain by enzymes that are closely associated with the signaling complex to which the PH domain-containing protein belong. As an example, the PH domain of the yeast OSH2 protein or the OSBP proteins and their yeast homologues poorly discriminate between PI4P and $PI(4,5)P_2$ [29, 30] but because of the Arf1 requirement and the abundance of PI4P relative to $PI(4,5)P_2$ in the Golgi, the OSBP proteins are primarily controlled by PI4P at the Golgi. PH domains have been mostly used to localize phosphoinositides, and only the Evectin PH domain is used to label PS in the cell [26]. PH domains that are mostly used for imaging purposes are listed in Table I. This Table is an updated version of ones we have used over the years. Our experiences with the specific individual lipids and PH domains have been described recently [15] and will not be detailed here.

II. 2. C2 domains

C2 domains have been recognized as lipid binding modules present in a large number of proteins that interact with membranes and/or act on membrane lipids [31]. Most of these domains show Ca^{2+}-dependent lipid binding to acidic membrane lipids without a great degree of specificity. In some cases, however, the lipid-binding partner can be defined. For example, the C2 domains of PKCs bind to PS [32], the C2B domain of Synapotagmin to $PI(4,5)P_2$ [33], the C2C domain of ESyt1 to $PI(4,5)P_2$ [34]or the C2 domain of cPLA2 to Cer-1-P [35]. A few C2 domains show lipid (membrane) binding even at resting Ca^{2+} such as the C2 domain of Lactadherin to PS [36] or the C2C domains of ESyt2 and-3 to $PI(4,5)P_2$ [34]. Importantly, the presence of C2 domains does not necessarily mean lipid binding: the C2 domain of PKCdelta has been shown to bind to phosphotyrosine residues in the membrane [37]. Because of the influence of Ca^{2+} on their lipid and membrane interactions, few C2 domains have been found useful for imaging studies (see Table II).

II. 3. C1 domains

These cysteine-rich domains were first recognized in PKC as the sites of DAG binding and regulation [38]. C1 domains since have been found in a large number of proteins often in tandem and the structure of the domain from several proteins have been solved [39]. Since tumor promoting phorbol esters mimicked DAG activation on PKC enzymes and they also bound to C1 domains of certain PKCs [40] C1 domains were believed to be also phorbol ester receptors and phorbol esters as ligands for the C1 domain of PKC. Today it is clear that many C1 domains have structural features that prevent phorbol-ester binding and that phorbol-esters can also bind to non-PKC proteins [41] and even to proteins that lack C1 domains [42]. Still, several C1 domains have been used as DAG sensors fused with GFP. The first of them was introduced by the Meyer lab [43], but C1 domains from other proteins have also been used for detection of DAG [44–47]. Interestingly, these domains obtained from various proteins appear to recognize DAG in distinct membranes, some detecting DAG in the PM and Golgi, others are more sensitive to DAG in the nuclear envelope or the Golgi membranes (see Table II). It is not clear at this point what other factors are responsible for the selectivity of DAG detection by the various domains, but it is a good reminder that lack of detection does not necessarily mean lack of DAG in any membrane compartment.

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Although not C1 domains, FYVE domains have structural similarities to C1 domains [48] and they have been identified as PI3P binding modules [49, 50]. This made them widely popular as tools to visualize PI3P distribution both in yeast and metazoan cells [51]. FYVE domains have some limitations, though. Sequences adjacent to the C-terminal end of the strictly defined FYVE domain often mediate dimerization or Rab5 binding adding a bias toward Rab5 positive structures [52]. Because of this feature the 2xFYVE domain of Hrs is the most widely used and tested domain for detecting PI3P [51].

II. 4. PX domains

These domains have been identified as PI3P binding modules [53–55]. PX domains are present in a large number of proteins that regulate endosomal trafficking such as the sorting nexins [56] and also in various oxydases and some lipid modifying enzymes, such as PLD or Class II PI3Ks [57]. Some PX domains can bind other lipids such as p47(phox) which binds $PI(3,4)P_2$ and at another site can simultaneously bind PA or PS [58, 59]. PX domains also bind proteins [60], and their localization may be significantly influenced by protein-protein interactions. Because of the popularity of FYVE domains for PI3P detection, relatively few studies explored the utility and limitations of PX domains as PI3P reporters. The best of the PX domains for this purpose has proven to be the p40(phox) PH domain [54].

II. 5. Protein domains from pathogens

Many pathogenic organisms infect cells and reorganize their trafficking pathways to create an optimal milieu for their life cycle and to avoid degradation. This is achieved by the evolution of protein virulence factors that interfere with the cell's own trafficking machinery. Some of these proteins target membranes by binding to phosphoinositides or other lipids in the host cell membrane. One example of such binding is the targeting of PI4P by the virulence factors, SidC [61] and SidM [62] of the intracellular parasite, Legionella pneumophila [63]. The phosphoinositide-binding segments of these proteins have been identified and their X-ray structures solved [64–66]. The PI4P-binding modules of both SidM and SidC have been used to detect PI4P in living cells [64, 67]. In our hand, expression of the SidC PI4P domain as a GFP fusion protein causes enlargement of Rab7 positive endosomes and seem to distort trafficking more than the PI4P binding segment of SidM (Balla T, Hilbi H and Szentpetery Zs unpublished observation). Therefore, we prefer the use of the SidM P4M domain for imaging purposes in live cells [67].

Another example of a virulence factor binding to phosphoinositides is the ActA protein of Listeria Monocytogenes. This protein that initiates actin polymerization and drives the actin-based motility of the bacteria inside the cells has a binding site for $PI(4,5)P_2$ at the N-terminus [68, 69]. Pathogenic agents have a whole slew of ways to interfere with the host phosphoinositide signaling network but discussion of those is beyond the scope of this review and can be found elsewhere [70, 71].

One example that is relevant to lipid imaging is the Perfringolysin O Theta Toxin (PFO) of Clostridium perfringens. This secreted pore-forming toxin is a member of the cholesterol-dependent cytolysins. It binds to cholesterol of the extracellular leaflet of the PM followed by oligomerization eventually leading to cell lysis [72]. It has a cholesterol binding domain

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(D4) that is non-toxic and is sufficient to target the toxin to cholesterol-rich domains. This D4 domain has been used as a recombinant protein to detect cholesterol in the exofacial leaflet of the PM but a mutant form (D434S) called D4H, with increased affinity was introduced to visualize intracellular cholesterol in the form of a transfected mCherry-fused fusion protein [73]. This promising tool has to be tested in many more studies to fully understand its benefits and limitations, but it has been a great step forward in helping understand cholesterol biology at the cellular level.

Other examples of bacterial toxins that bind to specific sphingolipids, are lysenin that binds sphingomyelin [74, 75]} or the non-toxic Cholera toxin B subunit that targets the glycosphingolipid, GM1 [76] and the Shiga toxin that binds to Gb3 [77]. Since these lipids are found primarily on the external (luminal) leaflet of membranes they are not listed in Table II.

II. 6. Amphipathic helices (AHs)

One of the most exciting and least understood ways proteins are recruited to specific membrane compartments is via short amphipathic helices (AHs). These short 20–40 residue sequences are characterized by the presence of large hydrophobic residues interspersed with polar and charged residues [78, 79]. Special cases of AHs are the so-called ALPS (for Amphipathic Lipid Packing Sensor) that are able to sense lipid curvature via insertion of their hydrophobic residues into packing defects in highly curved membranes [79, 80]. Some of the AHs specifically recognize the single phospholipid coating of lipid droplets due to relative PC deficiency as exemplified by the CCTa protein [81, 82]. We still have very limited understanding of what determines the specificity of these interactions. The AH of the yeast SNARE protein, Spo20p has been introduced as a reporter for PA accumulation [83]. This reporter is useful for monitoring PA increases in the PM [84–87], but its PA detection does not seem to be ubiquitous as large PA increases based on lipid analysis seem to be undetected by this probe (Kim, YJ, Pemberton JG and Balla T unpublished observations). Whether the Spo20 probe is sensitive to the unique lipid composition of the PM or the curvature of the membrane where PA is present is not clear at this point. Nevertheless, detecting all PA pools in cells remains to be a challenge in spite of significant efforts in finding a suitable PA sensor [21, 83, 88].

II. 7. Probes to detect membrane fluidity

2-(Dimethylamino)-6-Acylnaphtalenes Fluorescent Probes have been introduced to probe membrane fluidity and disorder. In particular, particularly Laurdan, was used to study membrane lateral structure and dynamics. The fluorescent life-time of the nascent fluorescence of this compound is highly dependent on the state of the membrane (ordered or disordered phase) and its cholesterol content. [89]. A newer dye dubbed PA (for push-pull pyrene, not to be confused with the similarly abbreviated phosphatidic acid or palmitic acid) changes its emission maximum as a function of lipid order and has been shown to be suitable to monitor the liquid-ordered state of cellular membranes at much lower concentration than Laurdan [90]. More experience will be needed to fully understand the information that these reporters convey and to properly interpret the differences that they detect between various membrane compartments.

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III. Quantitative imaging

It is very satisfying to see the cellular localization and changing dynamics of specific lipids in single living cells. However, the heterogeneity of cells makes it difficult to determine the extent of changes after stimulation or other manipulations, especially when changes are relatively small and have to be compared to one another between stimuli or groups of cells treated in different ways. There are a number of ways one can quantitate changes in membrane localizations or conformational changes of reporter constructs. Some of these measurements rely upon analysis of data obtained from single cells and summarizing the results from a number of individual cells. Such analysis includes measuring membrane translocation of the probes by analyzing changes in membrane to cytosol intensity ratios from confocal images. For plasma membrane, TIRF analysis can be used to follow the intensity changes after manipulations. Fluorescence Resonance Energy Transfer (FRET) can be monitored in individual cells and summarized from many cells, or if the signal is large enough can even be followed in cell suspensions. For internal organelles especially for fast moving ones, one has to use a reference image (such as an organelle marker) and use that as a mask to monitor the intensity change only in the pixels identified by the mask. Even with these methods, sometimes small changes will be lost in the error bars unless a huge number of cells are analyzed. This problem has been overcome by the development of Bioluminescence Energy Transfer (BRET) method that can monitor membrane specific changes in probe distribution from many thousand cells using simple plate readers. We will go through these methods in the following paragraphs.

III. 1. Quantification of images obtained by confocal microscopy, Total Internal Reflection Fluorescence (TIRF) microscopy or by fluorescence resonance energy transfer (FRET)

Regardless of the method to be used for analysis, it is an important point to keep in mind that low expression levels of the sensors should be used to reduce the distorting effects of the abundance of these sensors, due to sequestration of the lipids and dominant negative effects of the lipid binding domains. The most widely used lipid probes report on lipid changes in the membrane by changing their distribution between the membrane and the cytosol. This “translocation” can be monitored in a number of ways: the original and simplest method of quantification of confocal images was based on the increase or decrease of cytoplasmic intensities after stimulation (see [136] and [86] as examples). A more accurate method was to draw a line across the cells to create a line-intensity histogram that allowed the calculation of the intensities at the membrane vs. the cytosol (Fig. 1A and [93]). This method has subjective elements as the intensity may not be uniform along the membrane and the shape of the cells can also change yielding uneven membrane distribution. A much more accurate method, which can also be applied to internal organelles is the use a membrane marker (marking the membrane of interest) and create a mask across all the images in the time series that defines this membrane compartment. The masks can be used to identify as well as to normalize the organelle-associated lipid probe (Fig 1B and [67] as an example).

PM-associated intensities can also be followed in TIRF microscopy, which detects only a narrow section of the cells within 100 nm above the coverslip that corresponds to the plasma membrane. It is also advised to use a PM marker along with the lipid-specific probe to

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normalize for changes in membrane geometry or thickness in the TIRF plane (Fig 1C). An important advantage of TIRF quantification compared to confocal microscopy is the use of sensitive CCD cameras with high dynamic range. TIRF images can be easily quantified by measuring the pixel intensity that corresponds the amount of the bound domains [137, 138]. Limitations of the TIRF approach include that it requires a dedicated microscope, and that it can be used only in plasma membrane-associated events.

A really elegant approach for quantification of membrane-associated probe amounts was the use of FRET. FRET is based on the radiation-less energy transfer between two molecules, usually between two fluorescent proteins with proper spectral features when they are in close proximity. Since such energy transfer depends on the 6^{th} power of the distance between the donor and acceptor molecules it is a widely used method to measure molecular proximity. This principle can be applied to determine the extent of the membrane localization of a lipid-binding domain, either by using a CFP (or the much better mTuorquoise [139]) – fused lipid binding domain as donor and the YFP (or the better Venus)-fused same lipid binding domain or simply a membrane targeted Venus as acceptor [140]. As the membrane association of the probe decreases (or increases) so will change the FRET signal between the two molecules. A special application of the FRET principle is the use of single molecule FRET constructs. In this case both the donor and acceptor fluorophore is fused to the same lipid sensor protein and lipid binding would change to distance and probably more importantly the dipole orientation of the two fluorophores. Such probes usually have a constitutively high FRET value that changes slightly (either decrease or increase) upon lipid binding. This change is relatively little, but can be detected with a good quality detector (see [141] for an example). FRET can be measured either by the method of sensitized emission or by fluorescence life time imaging (FLIM). The latter does not require the acceptor to be fluorescent, enough if it can steal the energy from the fluorescent donor. Description of these methods is beyond the scope of this review and can be found elsewhere [142–144]. One big advantage of the FRET method when using sensitized emission is that it does not require a confocal microscope; in fact, it works better in a wide-field fluorescent microscope [145]. FLIM, on the other hand, requires special instrumentation [143].

III. 2. Bioluminescence resonance energy transfer (BRET)

All of the methods described above require expensive microscopes and many individual cells to be analyzed and averaged to obtain reliable statistics for the changes observed. FRET measurements can be done in cell suspensions of transfected cells in a fluorescence spectrophotometer [121] or in a plate reader but it has a poor signal to noise ratio. In order to quantitate the lipid changes in cell suspension, we have recently developed a set of BRET-based biosensors allowing the measurements of various phospholipids [PI4P, $PI(4,5)P_2$, $PI(3,4,5)P_3$, or PS] in biological membranes, especially in the plasma membrane [127, 146]. The principle of how these sensors work is similar to the FRET approach: these new sensors are based on an energy transfer process with the difference that the donor energy is generated by the enzymatic effect of a luciferase enzyme on its substrate, coelenterazine (energy donor), and the acceptor is a wavelength-matched fluorescent protein, such as Venus. By targeting Venus to the PM and fusing the Luciferase to a lipid binding domain of

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our choice, BRET will depend on how much of the lipid binding domain is located at the PM in the proximity of the Venus protein (Fig. 2A). There are two important differences where BRET differs from FRET. First, BRET measurements do not require excitation of the donor, therefore, analysis of the BRET data does not require corrections for bleed-through of fluorescent proteins that makes analysis of FRET data more complicated. The rate of energy transfer in case of BRET measurements can simply be calculated as the ratio of the emissions measured at the respective wavelength of the acceptor and the donor. Another important difference between FRET and BRET is that the bioluminescence signal from a single cell is too weak to be detected so the measurements are derived from a large number of cells. Therefore, BRET measurements are performed in cell suspensions, or attached cells using sensitive plate readers. These average responses are capable of detecting small differences that are almost impossible to reliably detect with the single-cell-based methods.

The specificity of the signal related to the membrane compartment of choice (such as the PM) is provided through targeting the acceptor (Venus) protein to that particular membrane. Therefore, even if the lipid-binding domain binds to other membrane compartments, the BRET signal only derives from the membrane where Venus is targeted. Conversely, by changing the lipid-binding domain fused to Luciferase, one can monitor the changes in the various lipids in the PM [146]. With this method, it is important to keep the ratio of the Luciferase and Venus constructs in a constant value, optimally close to 1:1 [147]. Therefore, we designed these sensor sets in a single plasmid configuration where the two fusion proteins are separated by the T2A viral peptide interrupting the polypeptide chains during translation [148]. This method allows a single mRNA molecule to code for two separate proteins with a theoretical ratio of 1:1. The efficiency of the system is quite good (in the experiment shown in Fig. 2B, the Luciferase enzyme is replaced with Cerulean, another fluorescent protein that allows its detection with the anti-GFP antibody): The two separate fusion proteins are shown in comparable amounts while the amount of full length, unseparated protein is negligible.

There are a few details that are important for successful BRET measurements. The expression level of the constructs has to reach a certain level in order to mount a measurable energy transfer. This means that signal detection is not the rate limiting factor and one cannot decrease expression levels beyond a certain point even if the signal is clearly measurable. However, using the super luciferase enzyme, we found that above a certain expression level the normalized BRET changes are relatively stable at a wide range of expression levels even if the absolute signal is higher when expressions are higher. Normalization in this case means that the basal BRET signal is considered 100% and the luciferase alone (no acceptor, so no energy transfer) corresponds to 0%. The time resolution of BRET-based lipid measurements is limited by the exposure time (may need hundreds of ms), and the time required for plate movements and change emission filters. If measurements are carried out by rows using triplicates in 96 well plates, this will result in the time resolution of 4–5 points per minute. This is far from the time-resolution of either TIRF or the FRET measurements so when rapid changes are to be detected, the latter methods are still more appropriate. Nevertheless, BRET analysis has several advantages and its popularity will only increase because of its statistical robustness, lower equipment costs and time commitments.

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IV. A word of caution

It is always pointed out in our reviews that there are limitations of using lipid binding domains to follow kinetics of lipid changes in living cells. We have to express domains that will distort the amount and distribution of the lipids and which will also disturb biology. However, some of the probes successfully made it to whole organisms (from *C. elegans* to mice) at expression levels that are easily detectable without causing appreciable problems (e.g. [149–153]). Another important point to consider is the presence of lipids in multiple compartments. This is not a major problem for $PI(4,5)P_2$ or $PI(3,4,5)P_3$ both of which are primarily found in the PM, but it can be a problem with PI4P. If a lipid binding domain binds to more than one compartments (such as the PI4P detecting SidM-P4M, which finds PI4P in the PM, the Golgi and endosomes [67]), then changing PI4P in one compartment will release the probe from that compartment and due to the elevated cytoplasmic level of the probe, it will now increase its binding to other PI4P-rich compartments even if the PI4P levels of those are unchanged (see Fig. 1D). Also, the affinity or avidity of the probe (talking about tandems) will change their distribution between the various compartments. In some compartments the lipids can change easily to compensate for binding the probe whereas in others the lipid remains in limited supply and hence the probe distribution will not be the same. Since BRET measurements do not require microscopes, it is our practice (and advice) to check the movement of the probes after the various manipulations in confocal microscopes before relying on data obtained by BRET analysis. For these we also make fluorescent versions (in place of the Luciferase) of the BRET constructs to test their behavior.

V. Conclusions

This review focused on the use of lipid binding domains for detection of inositides and other lipid pools inside living cells and the question of quantification. As pointed out in the Introduction, there are alternative methods to obtain information on lipid distribution, but for living cells the use of lipid binding domains remains one of the few if not the only method to obtain information on lipid changes. The limitations of this approach are obvious. However, the amount of new knowledge that these tools helped us obtain is substantial and we do not see these tools disappear in the foreseeable future. Even with their limitations as long as we understand them we can learn more about how cells organize their lipid membranes as signaling platforms. When looking in the future we see better fluorescent proteins with higher quantum yield or improved photo-switchable features. Fluorescent tags that can be added to proteins using click chemistry such as the HALO, SNAP or CLIP tags have already been around and are used with great success [154–156] to offer an alternative to the larger fluorescent proteins. These would allow applications for superresolution imaging or moving toward high temporal resolutions. Equally important, we need to find new ways to detect lipids that so far have eluded detection such as PC, PE, PI or ceramide. The success of this approach should encourage further efforts in these directions.

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References

1. Lipsky NG, Pagano RE. Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. Proc Natl Acad Sci U S A. 1983; 80:2608–2612. [PubMed: 6573674]
2. Salman M, Pagano RE. Use of a fluorescent analog of CDP-DAG in human skin fibroblasts: characterization of metabolism, distribution, and application to studies of phosphatidylinositol turnover. J Lipid Res. 1997; 38:482–490. [PubMed: 9101429]
3. Nadler A, Yushchenko DA, Muller R, Stein F, Feng S, Mulle C, Carta M, Schultz C. Exclusive photorelease of signalling lipids at the plasma membrane. Nat Commun. 2015; 6:10056. [PubMed: 26686736]
4. Frank JA, Yushchenko DA, Hodson DJ, Lipstein N, Nagpal J, Rutter GA, Rhee JS, Gottschalk A, Brose N, Schultz C, Trauner D. Photoswitchable diacylglycerols enable optical control of protein kinase C. Nat Chem Biol. 2016; 12:755–762. [PubMed: 27454932]
5. Reversi A, Loeser E, Subramanian D, Schultz C, De Renzis S. Plasma membrane phosphoinositide balance regulates cell shape during Drosophila embryo morphogenesis. J Cell Biol. 2014; 205:395–408. [PubMed: 24798734]
6. Miyazawa A, Umeda M, Horikoshi T, Yanagisawa K, Yoshioka T, Inoue K. Production and characterization of monoclonal antibodies that bind to phosphatidylinositol 4,5-bisphosphate. Mol Immunol. 1988; 25:1025–1031. [PubMed: 2851103]
7. Fukami K, Matsuoka K, Nakanishi O, Yamakawa A, Kawai S, Takenawa T. Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. Proc Natl Acad Sci U S A. 1988; 85:9057–9061. [PubMed: 2848254]
8. James DJ, Khodthong C, Kowalchyk JA, Martin TF. Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion. J Cell Biol. 2008; 182:355–366. [PubMed: 18644890]
9. van Rheenen J, Achame EM, Janssen H, Calafat J, Jalink K. PIP2 signaling in lipid domains: a critical re-evaluation. Embo J. 2005; 24:1664–1673. [PubMed: 15861130]
10. Wang J, Richards DA. Segregation of PIP2 and PIP3 into distinct nanoscale regions within the plasma membrane. Biology open. 2012; 1:857–862. [PubMed: 23213479]
11. Ji C, Zhang Y, Xu P, Xu T, Lou X. Nanoscale Landscape of Phosphoinositides Revealed by Specific Pleckstrin Homology (PH) Domains Using Single-molecule Superresolution Imaging in the Plasma Membrane. J Biol Chem. 2015; 290:26978–26993. [PubMed: 26396197]
12. Watt SA, Kular G, Fleming IN, Downes CP, Lucocq JM. Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1. Biochem J. 2002; 363:657–666. [PubMed: 11964166]
13. Fujita A, Cheng J, Tauchi-Sato K, Takenawa T, Fujimoto T. A distinct pool of phosphatidylinositol 4,5-bisphosphate in caveolae revealed by a nanoscale labeling technique. Proc Natl Acad Sci U S A. 2009; 106:9256–9261. [PubMed: 19470488]
14. Takatori S, Tatematsu T, Cheng J, Matsumoto J, Akano T, Fujimoto T. Phosphatidylinositol 3,5-bisphosphate-rich membrane domains in endosomes and lysosomes. Traffic. 2015
15. Hammond GR, Balla T. Polyphosphoinositide binding domains: Key to inositol lipid biology. Biochim Biophys Acta. 2015; 1851:746–758. [PubMed: 25732852]
16. Balla T, Varnai P. Visualization of cellular phosphoinositide pools with GFP-fused protein-domains. Curr Protoc Cell Biol. 2009; Chapter 24(Unit 24):24.
17. Varnai P, Balla T. Visualization and manipulation of phosphoinositide dynamics in live cells using engineered protein domains. Pflugers Arch. 2007
18. Maekawa M, Fairn GD. Molecular probes to visualize the location, organization and dynamics of lipids. J Cell Sci. 2014; 127:4801–4812. [PubMed: 25179600]
19. Idevall-Hagren O, De Camilli P. Detection and manipulation of phosphoinositides. Biochim Biophys Acta. 2015; 1851:736–745. [PubMed: 25514766]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

20. Halet G. Imaging phosphoinositide dynamics using GFP-tagged protein domains. Biol Cell. 2005; 97:501–518. [PubMed: 15966865]
21. Platre MP, Jaillais Y. Guidelines for the Use of Protein Domains in Acidic Phospholipid Imaging. Methods Mol Biol. 2016; 1376:175–194. [PubMed: 26552684]
22. Zumbusch A, Langbein W, Borri P. Nonlinear vibrational microscopy applied to lipid biology. Prog Lipid Res. 2013; 52:615–632. [PubMed: 24051337]
23. Lemmon MA. Pleckstrin homology domains: not just for phosphoinositides. Biochem Soc Trans. 2004; 32:707–711. [PubMed: 15493994]
24. Jian X, Gruschus JM, Sztul E, Randazzo PA. The pleckstrin homology (PH) domain of the Arf exchange factor Brag2 is an allosteric binding site. J Biol Chem. 2012; 287:24273–24283. [PubMed: 22613714]
25. Roy NS, Yohe ME, Randazzo PA, Gruschus JM. Allosteric properties of PH domains in Arf regulatory proteins. Cellular logistics. 2016; 6:e1181700. [PubMed: 27294009]
26. Uchida Y, Hasegawa J, Chinnapen D, Inoue T, Okazaki S, Kato R, Wakatsuki S, Misaki R, Koike M, Uchiyama Y, Iemura S, Natsume T, Kuwahara R, Nakagawa T, Nishikawa K, Mukai K, Miyoshi E, Taniguchi N, Sheff D, Lencer WI, Taguchi T, Arai H. Intracellular phosphatidylserine is essential for retrograde membrane traffic through endosomes. Proc Natl Acad Sci U S A. 2011; 108:15846–15851. [PubMed: 21911378]
27. Chung J, Torta F, Masai K, Lucast L, Czapla H, Tanner LB, Narayanaswamy P, Wenk MR, Nakatsu F, De Camilli P. INTRACELLULAR TRANSPORT. PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. Science. 2015; 349:428–432. [PubMed: 26206935]
28. Levine TP, Munro S. Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. Curr Biol. 2002; 12:695–704. [PubMed: 12007412]
29. Levine TP, Munro S. The pleckstrin-homology domain of oxysterol-binding protein recognizes a determinant specific to Golgi membranes. Curr Biol. 1998; 8:729–739. [PubMed: 9651677]
30. Roy A, Levine TP. Multiple pools of phosphatidylinositol 4-phosphate detected using the pleckstrin homology domain of Osh2p. J Biol Chem. 2004; 279:44683–44689. [PubMed: 15271978]
31. Corbalan-Garcia S, Gomez-Fernandez JC. Signaling through C2 domains: more than one lipid target. Biochim Biophys Acta. 2014; 1838:1536–1547. [PubMed: 24440424]
32. Corbalan-Garcia S, Rodriguez-Alfaro JA, Gomez-Fernandez JC. Determination of the calcium-binding sites of the C2 domain of protein kinase Calpha that are critical for its translocation to the plasma membrane. Biochem J. 1999; 337(Pt 3):513–521. [PubMed: 9895296]
33. van den Bogaart G, Meyenberg K, Diederichsen U, Jahn R. Phosphatidylinositol 4,5-bisphosphate increases the Ca2+ affinity of synaptotagmin-1 40-fold. J Biol Chem. 2012
34. Giordano F, Saheki Y, Idevall-Hagren O, Colombo SF, Pirruccello M, Milosevic I, Gracheva EO, Bagriantsev SN, Borgese N, De Camilli P. PI(4,5)P(2)-dependent and Ca(2+)-regulated ER-PM interactions mediated by the extended synaptotagmins. Cell. 2013; 153:1494–1509. [PubMed: 23791178]
35. Ward KE, Bhardwaj N, Vora M, Chalfant CE, Lu H, Stahelin RV. The molecular basis of ceramide-1-phosphate recognition by C2 domains. J Lipid Res. 2013; 54:636–648. [PubMed: 23277511]
36. Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S. Membrane phosphatidylserine regulates surface charge and protein localization. Science. 2008; 319:210–213. [PubMed: 18187657]
37. Benes CH, Wu N, Elia AE, Dharia T, Cantley LC, Soltoff SP. The C2 domain of PKCdelta is a phosphotyrosine binding domain. Cell. 2005; 121:271–280. [PubMed: 15851033]
38. Newton AC. Protein kinase C. Seeing two domains. Curr Biol. 1995; 5:973–976. [PubMed: 8542286]
39. Colon-Gonzalez F, Kazanietz MG. C1 domains exposed: from diacylglycerol binding to protein-protein interactions. Biochim Biophys Acta. 2006; 1761:827–837. [PubMed: 16861033]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

40. Zhang G, Kazainetz MG, Blumberg PM, Hurley JH. Crystal structure of the cys2 activator-binding domain of protein kinase Cdelta in complex with phorbol ester. Cell. 1995; 81:917–924. [PubMed: 7781068]
41. Rambaratsingh RA, Stone JC, Blumberg PM, Lorenzo PS. RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes. J Biol Chem. 2003; 278:52792–52801. [PubMed: 14532295]
42. Wakabayashi S, Nakamura TY, Kobayashi S, Hisamitsu T. Novel phorbol ester-binding motif mediates hormonal activation of Na+/H+ exchanger. J Biol Chem. 2010; 285:26652–26661. [PubMed: 20551318]
43. Oancea E, Teruel MN, Quest AFG, Meyer T. Green fluorescent protein (GFP)-tagged cystein-rich domains from protein kinase C as a fluorescent indicators for diacylglycerol signaling in living cells. J Cell Biol. 1998; 140:485–498. [PubMed: 9456311]
44. Domart MC, Hobday TM, Peddie CJ, Chung GH, Wang A, Yeh K, Jethwa N, Zhang Q, Wakelam MJ, Woscholski R, Byrne RD, Collinson LM, Poccia DL, Larijani B. Acute manipulation of diacylglycerol reveals roles in nuclear envelope assembly & endoplasmic reticulum morphology. PLoS One. 2012; 7:e51150. [PubMed: 23227247]
45. Stahelin RV, Digman MA, Medkova M, Ananthanarayanan B, Melowic HR, Rafter JD, Cho W. Diacylglycerol-induced membrane targeting and activation of protein kinase Cepsilon: mechanistic differences between protein kinases Cdelta and Cepsilon. J Biol Chem. 2005; 280:19784–19793. [PubMed: 15769752]
46. Litvak V, Dahan N, Ramachandran S, Sabanay H, Lev S. Maintenance of the diacylglycerol level in the Golgi apparatus by the Nir2 protein is critical for Golgi secretory function. Nat Cell Biol. 2005; 7:225–234. [PubMed: 15723057]
47. Kim YJ, Guzman-Hernandez ML, Balla T. A highly dynamic ER-derived phosphatidylinositol-synthesizing organelle supplies phosphoinositides to cellular membranes. Dev Cell. 2011; 21:813–824. [PubMed: 22075145]
48. Misra S, Hurley JH. Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. Cell. 1999; 97:657–666. [PubMed: 10367894]
49. Gaullier JM, Simonsen A, D'Arrigo A, Bremnes B, Stenmark H, Aasland R. FYVE fingers bind PtdIns(3)P. Nature. 1998; 394:432–433. [PubMed: 9697764]
50. Burd CG, Emr SD. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol Cell. 1998; 2:157–162. [PubMed: 9702203]
51. Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaullier LM, Parton GP, Stenmark H. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J. 2000; 19:4577–4588. [PubMed: 10970851]
52. Lawe DC, Patki V, Heller-Harrison R, Lambright D, Corvera S. The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. J Biol Chem. 2000; 275:3699–3705. [PubMed: 10652369]
53. Kanai F, Liu H, Field SJ, Akbary H, Matsuo T, Brown GE, Cantley LC, Yaffe MB. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. Nat Cell Biol. 2001; 3:675–678. [PubMed: 11433300]
54. Ellson CD, Gobert-Gosse S, Anderson KE, Davidson K, Erdjument-Bromage H, Tempst P, Thuring JW, Cooper MA, Lim ZY, Holmes AB, Gaffney PRJ, Coadwell J, Chilvers ER, Hawkins PT, Stephens LR. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40phox. Nat Cell Biol. 2001; 3:679–682. [PubMed: 11433301]
55. Bravo J, Karathanassis D, Pacold CM, Pacold ME, Ellson CD, Anderson KE, Butler PJG, Lavenir I, Perisisc O, Hawkins PT, Stephens L, Williams RL. The crystal structure of the PX domain from p40phox bound to phosphatidylinositol 3-phosphate. Mol Cell. 2001; 8:829–839. [PubMed: 11684018]
56. Teasdale RD, Collins BM. Insights into the PX (phox-homology) domain and SNX (sorting nexin) protein families: structures, functions and roles in disease. Biochem J. 2012; 441:39–59. [PubMed: 22168438]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

57. Seet LF, Hong W. The Phox (PX) domain proteins and membrane traffic. Biochim Biophys Acta. 2006; 1761:878–896. [PubMed: 16782399]
58. Stahelin RV, Burian A, Bruzik KS, Murray D, Cho W. Membrane binding mechanisms of the PX domains of NADPH oxidase p40phox and p47phox. J Biol Chem. 2003; 278:14469–14479. [PubMed: 12556460]
59. Karathanassis D, Stahelin RV, Bravo J, Perisic O, Pacold CM, Cho W, Williams RL. Binding of the PX domain of p47phox to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. EMBO J. 2002; 21:5057–5068. [PubMed: 12356722]
60. Vollert CS, Uetz P. The PX domain protein interaction network in yeast. Mol Cell Proteomics. 2004; 3:1053–1064. [PubMed: 15263065]
61. Ragaz C, Pietsch H, Urwyler S, Tiaden A, Weber SS, Hilbi H. The Legionella pneumophila phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. Cell Microbiol. 2008; 10:2416–2433. [PubMed: 18673369]
62. Brombacher E, Urwyler S, Ragaz C, Weber SS, Kami K, Overduin M, Hilbi H. Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of Legionella pneumophila. J Biol Chem. 2009; 284:4846–4856. [PubMed: 19095644]
63. Hilbi H, Weber S, Finsel I. Anchors for effectors: subversion of phosphoinositide lipids by legionella. Front Microbiol. 2011; 2:91. [PubMed: 21833330]
64. Luo X, Wasilko DJ, Liu Y, Sun J, Wu X, Luo ZQ, Mao Y. Structure of the Legionella Virulence Factor, SidC Reveals a Unique PI(4)P-Specific Binding Domain Essential for Its Targeting to the Bacterial Phagosome. PLoS Pathog. 2015; 11:e1004965. [PubMed: 26067986]
65. Suh HY, Lee DW, Lee KH, Ku B, Choi SJ, Woo JS, Kim YG, Oh BH. Structural insights into the dual nucleotide exchange and GDI displacement activity of SidM/DrrA. EMBO J. 2010; 29:496–504. [PubMed: 19942850]
66. Zhu Y, Hu L, Zhou Y, Yao Q, Liu L, Shao F. Structural mechanism of host Rab1 activation by the bifunctional Legionella type IV effector SidM/DrrA. Proc Natl Acad Sci U S A. 2010; 107:4699–4704. [PubMed: 20176951]
67. Hammond GR, Machner MP, Balla T. A novel probe for phosphatidylinositol 4-phosphate reveals multiple pools beyond the Golgi. J Cell Biol. 2014; 205:113–126. [PubMed: 24711504]
68. Cicchetti G, Maurer P, Wagener P, Kocks C. Actin and phosphoinositide binding by the ActA protein of the bacterial pathogen Listeria monocytogenes. J Biol Chem. 1999; 274:33616–33626. [PubMed: 10559250]
69. Steffen P, Schafer DA, David V, Gouin E, Cooper JA, Cossart P. Listeria monocytogenes ActA protein interacts with phosphatidylinositol 4,5-bisphosphate in vitro. Cell motility and the cytoskeleton. 2000; 45:58–66. [PubMed: 10618167]
70. Cossart P, Roy CR. Manipulation of host membrane machinery by bacterial pathogens. Curr Opin Cell Biol. 2010; 22:547–554. [PubMed: 20542678]
71. Payrastre B, Gaits-Iacovoni F, Sansonetti P, Tronchere H. Phosphoinositides and cellular pathogens. Subcell Biochem. 2012; 59:363–388. [PubMed: 22374097]
72. Maekawa M, Yang Y, Fairn GD. Perfringolysin O Theta Toxin as a Tool to Monitor the Distribution and Inhomogeneity of Cholesterol in Cellular Membranes. Toxins. 2016; 8
73. Maekawa M, Fairn GD. Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. J Cell Sci. 2015; 128:1422–1433. [PubMed: 25663704]
74. Yachi R, Uchida Y, Balakrishna BH, Anderluh G, Kobayashi T, Taguchi T, Arai H. Subcellular localization of sphingomyelin revealed by two toxin-based probes in mammalian cells. Genes Cells. 2012; 17:720–727. [PubMed: 22747662]
75. Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda M. Lysenin, a novel sphingomyelin-specific binding protein. J Biol Chem. 1998; 273:5300–5306. [PubMed: 9478988]
76. Orlandi PA, Fishman PH. Orientation of cholera toxin bound to target cells. J Biol Chem. 1993; 268:17038–17044. [PubMed: 8349592]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

77. Sandvig K, Olsnes S, Brown JE, Petersen OW, van Deurs B. Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from Shigella dysenteriae 1. J Cell Biol. 1989; 108:1331–1343. [PubMed: 2564398]

78. Drin G, Antonny B. Amphipathic helices and membrane curvature. FEBS Lett. 2010; 584:1840–1847. [PubMed: 19837069]

79. Vanni S, Vamparys L, Gautier R, Drin G, Etchebest C, Fuchs PF, Antonny B. Amphipathic lipid packing sensor motifs: probing bilayer defects with hydrophobic residues. Biophys J. 2013; 104:575–584. [PubMed: 23442908]

80. Jackson CL, Walch L, Verbavatz JM. Lipids and Their Trafficking: An Integral Part of Cellular Organization. Dev Cell. 2016; 39:139–153. [PubMed: 27780039]

81. Kory N, Farese RV Jr, Walther TC. Targeting Fat: Mechanisms of Protein Localization to Lipid Droplets. Trends Cell Biol. 2016; 26:535–546. [PubMed: 26995697]

82. Cornell RB, Ridgway ND. CTP:phosphocholine cytidylyltransferase: Function, regulation, and structure of an amphitropic enzyme required for membrane biogenesis. Prog Lipid Res. 2015; 59:147–171. [PubMed: 26165797]

83. Kassas N, Tryoen-Toth P, Corrotte M, Thahouly T, Bader MF, Grant NJ, Vitale N. Genetically encoded probes for phosphatidic acid. Methods Cell Biol. 2012; 108:445–459. [PubMed: 22325614]

84. Zeniou-Meyer M, Zabari N, Ashery U, Chasserot-Golaz S, Haeberle AM, Demais V, Bailly Y, Gottfried I, Nakanishi H, Neiman AM, Du G, Frohman MA, Bader MF, Vitale N. Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. J Biol Chem. 2007; 282:21746–21757. [PubMed: 17540765]

85. Zhang F, Wang Z, Lu M, Yonekubo Y, Liang X, Zhang Y, Wu P, Zhou Y, Grinstein S, Hancock JF, Du G. Temporal production of the signaling lipid phosphatidic acid by phospholipase D2 determines the output of extracellular signal-regulated kinase signaling in cancer cells. Mol Cell Biol. 2014; 34:84–95. [PubMed: 24164897]

86. Kim YJ, Guzman-Hernandez ML, Wisniewski E, Balla T. Phosphatidylinositol-Phosphatidic Acid Exchange by Nir2 at ER-PM Contact Sites Maintains Phosphoinositide Signaling Competence. Dev Cell. 2015; 33:549–561. [PubMed: 26028218]

87. Bohdanowicz M, Schlam D, Hermansson M, Rizzuti D, Fairn GD, Ueyama T, Somerharju P, Du G, Grinstein S. Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes. Mol Biol Cell. 2013; 24:1700–1712. S1701–1707. [PubMed: 23576545]

88. Putta P, Rankenberg J, Korver RA, van Wijk R, Munnik T, Testerink C, Kooijman EE. Phosphatidic acid binding proteins display differential binding as a function of membrane curvature stress and chemical properties. Biochim Biophys Acta. 2016; 1858:2709–2716. [PubMed: 27480805]

89. Bagatolli LA. Monitoring Membrane Hydration with 2-(Dimethylamino)-6-Acylnaphtalenes Fluorescent Probes. Subcell Biochem. 2015; 71:105–125. [PubMed: 26438263]

90. Niko Y, Didier P, Mely Y, Konishi G, Klymchenko AS. Bright and photostable push-pull pyrene dye visualizes lipid order variation between plasma and intracellular membranes. Sci Rep. 2016; 6:18870. [PubMed: 26750324]

91. Lemmon MA, Ferguson KM, O'Brian R, Sigler PB, Schlessinger J. Specific and high-affinity binding of inositol phosphates to an isolated plekstrin homology domain. Proc Natl Acad Sci U S A. 1995; 92:10472–10476. [PubMed: 7479822]

92. Stauffer TP, Ahn S, Meyer T. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr Biol. 1998; 8:343–346. [PubMed: 9512420]

93. Várnai P, Balla T. Visualization of phosphoinositides that bind pleckstrin homology domains: calcium-and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol. 1998; 143:501–510. [PubMed: 9786958]

94. Santagata S, Boggon TJ, Baird CL, Gomez CA, Zhao J, Shan WS, Myszka DG, Shapiro L. G-protein signaling through tubby proteins. Science. 2001; 292:2041–2050. [PubMed: 11375483]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

95. Field SJ, Madson N, Kerr ML, Galbraith KA, Kennedy CE, Tahiliani M, Wilkins A, Cantley LC. PtdIns(4,5)P2 functions at the cleavage furrow during cytokinesis. Curr Biol. 2005; 15:1407–1412. [PubMed: 16085494]

96. Lee SB, Varnai P, Balla A, Jalink K, Rhee SG, Balla T. The pleckstrin homology domain of phosphoinositide-specific phospholipase Cdelta4 is not a critical determinant of the membrane localization of the enzyme. J Biol Chem. 2004; 279:24362–24371. [PubMed: 15037625]

97. Rameh LE, Arvidsson A, Carraway KL III, Couvillon AD, Rathbun G, Crompton A, VanRentherghem B, Czech MP, Ravichandran KS, Burakoff SJ, Wang DS, Chen CS, Cantley LC. A comparative analysis of the phosphoinositide binding specificity of plekstrin homology domains. J Biol Chem. 1997; 272:22059–22066. [PubMed: 9268346]

98. Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A, Czech MP. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing plekstrin and Sec7 homology domains. Science. 1997; 275:1927–1930. [PubMed: 9072969]

99. Klarlund JK, Tsiaras W, Holik JJ, Chawla A, Czech MP. Distinct polyphosphoinositide binding selectivities for pleckstrin homology domains of GRP1-like proteins based on diglycine versus triglycine motifs. J Biol Chem. 2000; 275:32816–32821. [PubMed: 10913124]

100. Venkateswarlu K, Gunn-Moore F, Oatey PB, Tavare JM, Cullen PJ. Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. Biochem J. 1998; 335(Pt 1):139–146. [PubMed: 9742223]

101. Venkateswarlu K, Oatey PB, Tavare JM, Cullen PJ. Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. Curr Biol. 1998; 8:463–466. [PubMed: 9550703]

102. Nagel W, Schilcher P, Zeitlmann L, Kolanus W. The PH domain and the polybasic c domain of cytohesin-1 cooperate specifically in plasma membrane-association and cellular function. Mol Biol Cell. 1998; 9:1981–1994. [PubMed: 9693361]

103. Venkateswarlu K, Gunn-Moore F, Tavare JM, Cullen PJ. EGF-and NGF-stimulated translocation of cytohesin-1 to the plasma membrane of PC12 cells requires PI 3-kinase activation and a functional cytohesin-1 PH domain. J Cell Sci. 1999; 112:1957–1965. [PubMed: 10341214]

104. Salim K, Bottomley MJ, Querfurth E, Zvelebil MJ, Gout I, Scaife R, Margolis RL, Gigg R, Smith CIE, Driscoll PC, Waterfield MD, Panayotou G. Distinct specificity in the recognition of phosphoinositides by the plekstrin homology domains of dynamin and Bruton's tyrosine kinase. EMBO J. 1996; 15:6241–6250. [PubMed: 8947047]

105. Varnai P, Rother KI, Balla T. Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. J Biol Chem. 1999; 274:10983–10989. [PubMed: 10196179]

106. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt protooncogene product by PI3,4P2. Science. 1997; 275:665–668. [PubMed: 9005852]

107. Watton J, Downward J. Akt/PKB localisation and 3' phosphoinositide generation at sites of epithelial cell-matrix and cell-cell interaction. Curr Biol. 1999; 9:433–436. [PubMed: 10226029]

108. Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science. 2000; 287:1037–1040. [PubMed: 10669415]

109. Kontos CD, Stauffer TP, Yang WP, York JD, Huang L, Blanar MA, Meyer T, Peters KG. Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. Mol Cell Biol. 1998; 18:4131–4140. [PubMed: 9632797]

110. Komander D, Fairservice A, Deak M, Kular GS, Prescott AR, Peter Downes C, Safrany ST, Alessi DR, van Aalten DM. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. Embo J. 2004; 23:3918–3928. [PubMed: 15457207]

111. Huang YE, Iijima M, Parent CA, Funamoto S, Firtel RA, Devreotes P. Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. Mol Biol Cell. 2003; 14:1913–1922. [PubMed: 12802064]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

112. Dormann D, Weijer G, Parent CA, Devreotes PN, Weijer CJ. Visualizing PI3 kinase-mediated cell-cell signaling during Dictyostelium development. Curr Biol. 2002; 12:1178–1188. [PubMed: 12176327]
113. Dowler S, Currie RA, Campbell DG, Deak M, Kular G, Downes CP, Alessi DR. Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. Biochem J. 2000; 351:19–31. [PubMed: 11001876]
114. Kimber WA, Trinkle-Mulcahy L, Cheung PC, Deak M, Marsden LJ, Kieloch A, Watt S, Javier RT, Gray A, Downes CP, Lucocq JM, Alessi DR. Evidence that the tandem-pleckstrin-homology-domain-containing protein TAPP1 interacts with Ptd(3,4)P2 and the multi-PDZ-domain-containing protein MUPP1 in vivo. Biochem J. 2002; 361:525–536. [PubMed: 11802782]
115. Friant S, Pecheur EI, Eugster A, Michel F, Lefkir Y, Nourrisson D, Letourneur F. Ent3p Is a PtdIns(3,5)P2 effector required for protein sorting to the multivesicular body. Dev Cell. 2003; 5:499–511. [PubMed: 12967568]
116. Dove SK, Piper RC, McEwen RK, Yu JW, King MC, Hughes DC, Thuring J, Holmes AB, Cooke FT, Michell RH, Parker PJ, Lemmon MA. Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. Embo J. 2004; 23:1922–1933. [PubMed: 15103325]
117. Han BK, Emr SD. Phosphoinositide [PI(3,5)P2] lipid-dependent regulation of the general transcriptional regulator Tup1. Genes Dev. 2011; 25:984–995. [PubMed: 21536737]
118. Li X, Wang X, Zhang X, Zhao M, Tsang WL, Zhang Y, Yau RG, Weisman LS, Xu H. Genetically encoded fluorescent probe to visualize intracellular phosphatidylinositol 3,5-bisphosphate localization and dynamics. Proc Natl Acad Sci U S A. 2013; 110:21165–21170. [PubMed: 24324172]
119. Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature. 1998; 394:494–498. [PubMed: 9697774]
120. Yu JW, Mendrola JM, Audhya A, Singh S, Keleti D, DeWald DB, Murray D, Emr SD, Lemmon MA. Genome-wide analysis of membrane targeting by S. cerevisiae pleckstrin homology domains. Mol Cell. 2004; 13:677–688. [PubMed: 15023338]
121. Balla A, Tuymetova G, Tsiomenko A, Varnai P, Balla T. A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: studies with the PH domains of the oxysterol binding protein and FAPP1. Mol Biol Cell. 2005; 16:1282–1295. [PubMed: 15635101]
122. Godi A, Di Campi A, Konstantakopoulos A, Di Tullio G, Alessi DR, Kular GS, Daniele T, Marra P, Lucocq JM, De Matteis MA. FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat Cell Biol. 2004; 6:393–404. [PubMed: 15107860]
123. Gozani O, Karuman P, Jones DR, Ivanov D, Cha J, Logovskoy AA, Baird CL, Zhu H, Field SJ, Lessnick SL, Villasenov J, Mehrotra B, Chen J, Rao VR, Brugge JS, Ferguson CG, Payrastre B, Myszka DG, Cantley LC, Wagner G, Divecha N, Prestwich GD, Yuan J. The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell. 2003; 114:99–111. [PubMed: 12859901]
124. Pendaries C, Tronchere H, Arbibe L, Mounier J, Gozani O, Cantley L, Fry MJ, Gaits-Iacovoni F, Sansonetti PJ, Payrastre B. PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. Embo J. 2006; 25:1024–1034. [PubMed: 16482216]
125. Michell RH, Heath VL, Lemmon MA, Dove SK. Phosphatidylinositol 3,5-bisphosphate: metabolism and cellular functions. Trends Biochem Sci. 2005; 31:52–63. [PubMed: 16364647]
126. Hammond GR, Takasuga S, Sasaki T, Balla T. The ML1Nx2 Phosphatidylinositol 3,5-Bisphosphate Probe Shows Poor Selectivity in Cells. PLoS One. 2015; 10:e0139957. [PubMed: 26460749]
127. Sohn M, Ivanova P, Brown HA, Toth DJ, Varnai P, Kim YJ, Balla T. Lenz-Majewski mutations in PTDSS1 affect phosphatidylinositol 4-phosphate metabolism at ER-PM and ER-Golgi junctions. Proc Natl Acad Sci U S A. 2016; 113:4314–4319. [PubMed: 27044099]
128. Nakanishi H, de los Santos P, Neiman AM. Positive and negative regulation of a SNARE protein by control of intracellular localization. Mol Biol Cell. 2004; 15:1802–1815. [PubMed: 14742704]

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129. Nishioka T, Frohman MA, Matsuda M, Kiyokawa E. Heterogeneity of phosphatidic acid levels and distribution at the plasma membrane in living cells as visualized by a Foster resonance energy transfer (FRET) biosensor. J Biol Chem. 2010; 285:35979–35987. [PubMed: 20826779]
130. Sharkey NA, Leach KL, Blumberg PM. Competitive inhibition by diacylglycerol of specific phorbol ester binding. Proc Natl Acad Sci U S A. 1984; 81:607–610. [PubMed: 6320198]
131. Burns DJ, Bell RM. Protein kinase C contains two phorbol ester binding domains. J Biol Chem. 1991; 266:18330–18338. [PubMed: 1917958]
132. Dries DR, Gallegos LL, Newton AC. A single residue in the C1 domain sensitizes novel protein kinase C isoforms to cellular diacylglycerol production. J Biol Chem. 2007; 282:826–830. [PubMed: 17071619]
133. Meyer T, Oancea E. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell. 1998; 95:307–318. [PubMed: 9814702]
134. Lehel C, Olah Z, Jakab G, Anderson WB. Protein kinase C epsilon is localized to the Golgi via its zinc-finger domain and modulates Golgi function. Proc Natl Acad Sci U S A. 1995; 92:1406–1410. [PubMed: 7877991]
135. Schultz A, Ling M, Larsson C. Identification of an amino acid residue in the protein kinase C C1b domain crucial for its localization to the Golgi network. J Biol Chem. 2004; 279:31750–31760. [PubMed: 15145947]
136. Suh BC, Hille B. Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. Neuron. 2002; 35:507–520. [PubMed: 12165472]
137. Dickson EJ, Jensen JB, Vivas O, Kruse M, Traynor-Kaplan AE, Hille B. Dynamic formation of ER-PM junctions presents a lipid phosphatase to regulate phosphoinositides. J Cell Biol. 2016; 213:33–48. [PubMed: 27044890]
138. Wuttke A, Idevall-Hagren O, Tengholm A. Imaging phosphoinositide dynamics in living cells. Methods Mol Biol. 2010; 645:219–235. [PubMed: 20645191]
139. Goedhart J, von Stetten D, Noirclerc-Savoye M, Lelimousin M, Joosen L, Hink MA, van Weeren L, Gadella TW Jr, Royant A. Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. Nat Commun. 2012; 3:751. [PubMed: 22434194]
140. van Der Wal J, Habets R, Varnai P, Balla T, Jalink K. Monitoring Phospholipase C activation kinetics in live cells by FRET. J Biol Chem. 2001; 276:15337–15344. [PubMed: 11152673]
141. Nishioka T, Aoki K, Hikake K, Yoshizaki H, Kiyokawa E, Matsuda M. Rapid turnover rate of phosphoinositides at the front of migrating MDCK cells. Mol Biol Cell. 2008; 19:4213–4223. [PubMed: 18685081]
142. van Munster EB, Gadella TW. Fluorescence lifetime imaging microscopy (FLIM). Adv Biochem Eng Biotechnol. 2005; 95:143–175. [PubMed: 16080268]
143. Raspe M, Kedziora KM, van den Broek B, Zhao Q, de Jong S, Herz J, Mastop M, Goedhart J, Gadella TW, Young IT, Jalink K. siFLIM: single-image frequency-domain FLIM provides fast and photon-efficient lifetime data. Nat Methods. 2016; 13:501–504. [PubMed: 27088314]
144. Kedziora KM, Jalink K. Fluorescence resonance energy transfer microscopy (FRET). Methods Mol Biol. 2015; 1251:67–82. [PubMed: 25391795]
145. Du M, Zhang L, Xie S, Chen T. Wide-field microscopic FRET imaging using simultaneous spectral unmixing of excitation and emission spectra. Opt Express. 2016; 24:16037–16051. [PubMed: 27410873]
146. Toth JT, Gulyas G, Toth DJ, Balla A, Hammond GR, Hunyady L, Balla T, Varnai P. BRET-monitoring of the dynamic changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4P increase upon EGF and M3 receptor activation. Biochim Biophys Acta. 2016; 1861:177–187. [PubMed: 26692031]
147. Szalai B, Hoffmann P, Prokop S, Erdelyi L, Varnai P, Hunyady L. Improved methodical approach for quantitative BRET analysis of G Protein Coupled Receptor dimerization. PLoS One. 2014; 9:e109503. [PubMed: 25329164]
148. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol. 2004; 22:589–594. [PubMed: 15064769]

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149. Nishio M, Watanabe KI, Sasaki J, Taya C, Takasuga S, Iizuka R, Balla T, Yamazaki M, Watanabe H, Itoh R, Kuroda S, Horie Y, Forster I, Mak TW, Yonekawa H, Penninger JM, Kanaho Y, Suzuki A, Sasaki T. Control of cell polarity and motility by the PtdIns(3,4,5)P(3) phosphatase SHIP1. Nat Cell Biol. 2007; 9:36–44. [PubMed: 17173042]

150. Wong R, Hadjiyanni I, Wei HC, Polevoy G, McBride R, Sem KP, Brill JA. PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes. Curr Biol. 2005; 15:1401–1406. [PubMed: 16085493]

151. Yadav S, Garner K, Georgiev P, Li M, Gomez-Espinosa E, Panda A, Mathre S, Okkenhaug H, Cockcroft S, Raghu P. RDGBalpha, a PtdIns-PtdOH transfer protein, regulates G-protein-coupled PtdIns(4,5)P2 signalling during Drosophila phototransduction. J Cell Sci. 2015; 128:3330–3344. [PubMed: 26203165]

152. Pan W, Pham VN, Stratman AN, Castranova D, Kamei M, Kidd KR, Lo BD, Shaw KM, Torres-Vazquez J, Mikelis CM, Gutkind JS, Davis GE, Weinstein BM. CDP-diacylglycerol synthetase-controlled phosphoinositide availability limits VEGFA signaling and vascular morphogenesis. Blood. 2012; 120:489–498. [PubMed: 22649102]

153. Ziel JW, Hagedorn EJ, Audhya A, Sherwood DR. UNC-6 (netrin) orients the invasive membrane of the anchor cell in C. elegans. Nat Cell Biol. 2009; 11:183–189. [PubMed: 19098902]

154. Hussain AF, Amoury M, Barth S. SNAP-tag technology: a powerful tool for site specific conjugation of therapeutic and imaging agents. Curr Pharm Des. 2013; 19:5437–5442. [PubMed: 23431986]

155. Horisawa K. Specific and quantitative labeling of biomolecules using click chemistry. Front Physiol. 2014; 5:457. [PubMed: 25505421]

156. Sunbul M, Nacheva L, Jaschke A. Proximity-Induced Covalent Labeling of Proteins with a Reactive Fluorophore-Binding Peptide Tag. Bioconjug Chem. 2015; 26:1466–1469. [PubMed: 26086394]

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Highlights

- Exact lipid distribution between various membranes is largely unknown
- Many proteins contain lipid binding modules with some specificity
- Some of these modules can be used to detect lipid distribution in live cells
- Quantification of lipid changes using these tools is increasingly desirable

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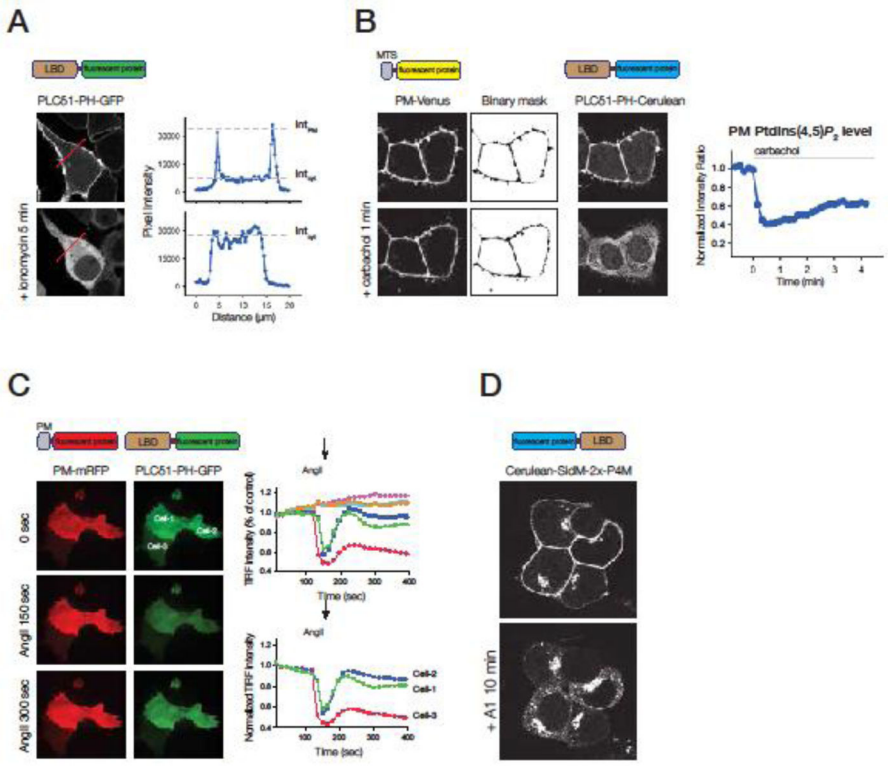

Fig 1. Methods of quantification when monitoring inositol lipids in the plasma membrane by various lipid binding domains (LBD)

(A) Various lipid binding domains (LBD) tagged with fluorescent proteins are used to follow the membrane localization of inositol lipids using confocal microscopy. Upon ionomycin treatment (causing a cytoplasmic Ca^{2+} increase) the plasma membrane localization of the $PI(4,5)P_2$ specific GFP-tagged PH domain of $PLC\delta_1$ decreases in the COS-7 cells shown on the left. The translocation of the fluorescent probe can be quantified by measuring the plasma membrane and cytoplasmic pixel intensity in a line-intensity histogram (right graphs).

(B) Co-expression of a membrane-targeted fluorescent protein (MTS-fluorescent protein), such as a PM-targeted Venus (PM-Venus) allows the generation of binary masks that can be used to identify the membrane of interest during the whole measurement. The sum of the pixel intensity values of the probe defined by the binary mask gives information about the membrane bound fraction of the probe fluorescence. Images show that the localization of PM-Venus (targeted by the Lyn N-terminal 10 amino acids) is not affected by the addition of carbachol in HEK 293T cells expressing M3 muscarinic receptors. In contrast, the PH domain of $PLC\delta_1$ translocates from the PM to the cytoplasm. The process can be quantified by measuring the PM fraction of the $PLC\delta_1$-PH-Cerulean. Normalizing these values with the PM-Venus signal can be used to factor out changes of the fluorescence due to changing membrane geometry during a time-course (right graph).

(C) TIRF analysis can be used to identify the part of the PM that is attached to the tissue culture dish. Quantification of the PM-bound LBD that is proportional to the level of inositol lipids can be carried out by calculating the total pixel intensity of the cell's footprint during a time-lapse recording. Co-expression of a PM-targeted fluorescent protein can be used to

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factor out intensity changes due to cell movements or changing membrane deformities. A limitation of this method that there is a signal even if the LBD is fully released from the membrane to the cytosol. This can be taken as the minimum value if one calibrates each trace at the end of experiment by a stimulus that causes complete translocation.
(D) Redistribution of an LBD-fluorescent lipid sensor upon elimination of one lipid pool. In this confocal experiment specific depletion of the PM PI4P by the treatment with a PI4KA inhibitor (A1) resulted in the disappearance of the PM-bound fluorescence, causing an increase in the Golgi- and endosome-associated signals in HEK 293T cells. PI4P was followed by expression of the Cerulean-SidM-2xP4M biosensor capable to bind PI4P both in the PM, Golgi and endosomes.

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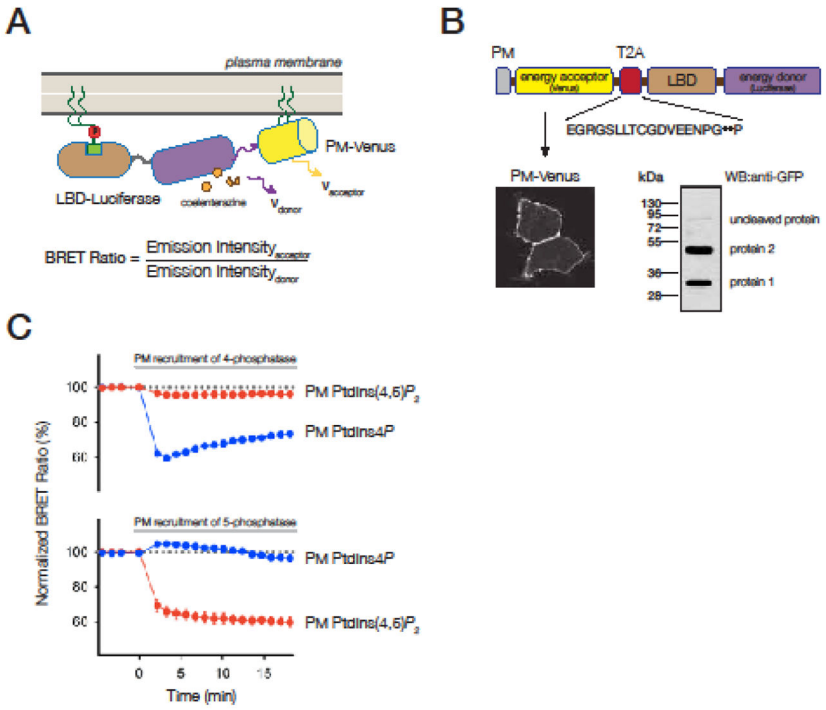

Fig 2. Quantitative measurement of inositol lipid changes using bioluminescence resonance energy transfer (BRET)

(A) Membrane localization of the LBD can be measured in cells by calculating the energy transfer between the luciferase fused to the LBD and Venus targeted to the membrane. Membrane recruitment of the probes increases, while cytoplasmic translocation of the probes decreases the BRET ratio values.

(B) Design of the BRET-based biosensors. PM-targeted Venus and the luciferase-tagged LBD (PH domain of the PLCδ_1) are linked by the viral T2A protein sequence resulting in the separate expression of the two proteins. Only a small fraction of the total expressed proteins remained uncleaved as shown by the Western blot analysis of the proteins. In this experiment luciferase enzyme was replaced by Cerulean that allowed the detection of the proteins with anti GFP antibody. The confocal image shows the plasma membrane localization of Venus.

(C) HEK 293T cells were transfected with the plasmid DNA coding for components of the BRET based PI4P and PI(4,5)P_2 biosensors (PM-Venus-T2A-Luciferase-2xSidM-P4M and PM-Venus-T2A-PLCδ_1-PH-Luciferase, respectively). Cells were also co-transfected with plasmids encoding the rapamycin-inducible plasma membrane PI4P and PI(4,5)P_2 depletion system. As shown in the graphs, the BRET biosensors can detect the changes in the respective lipid pools. Note that in case of the 5-phosphatase recruitment even the small increase of the PI4P can be detected (lower graph blue curve).

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Table I

Visualization of phosphoinositides by protein-domain GFP chimeras in live cells.

Lipid	Protein domain	Refs for in vitro	Live cell localization	Reference
PtdIns(4,5)P_2	PLCδ1-PH	[91]	PM	[92, 93]
	Tubby domain	[94]	PM, cleavage furrow	[94][95]
	PLCδ_4-PH	[96]	PM	[96]
PtdIns(3,4,5)P_3	GRP1-PH	[97, 98]	PM	[99, 100]
	ARNO-PH	[99]	PM	[101]
	Cytohesin-1-PH	[99]	PM	[102, 103]
	Btk-PH	[97, 104]	PM	[105]
PtdIns(3,4,5)P_3 /PtdIns(3,4)P_2	Akt-PH	[106]	PM	[107–109]
	PDK1	[110]	PM	[110]
	CRAC	[111]	*Dictyostelium* PM	[112]
PtdIns(3,4)P_2	TAPP1-PH	[113]	PM	[114]
PtdIns(3,5)P_2	Ent3p-ENTH *	[115]	yeast pre-vacuole	[115]
	Svp1p	[116]	yeast vacuole	[116]
	Tup1 *	[117]	yeast vacuolar-endosomal compartment, nucleus	[117]
	Cti6 *	[117]	yeast nucleus	[117]
	ML1N-2x **	[118]	late endosomes,lysosomes yeast plasma membrane	[118]
PtdIns3*P*	FYVE (Hrs, EEA1) (Vps27)	[50, 119]	early endosome	[51]
			yeast vacuole	[50]
	P40phox-PX	[53, 54]	early endosome	[54]
PtdIns4*P*	OSH2-PH ***	[120]	PM	[30, 120]
	OSBP-PH	[29, 113]	Golgi, PM	[28, 29, 121]
	FAPP1-PH	[113]	Golgi, PM	[28, 121, 122]
	SidM (P4M)	[62, 67]	PM, Golgi, endosomes	[67]
	SidC	[62, 64]	PM, Golgi, endosomes	[64]
PtdIns5*P*	3xPHD (ING2)	[123]	nucleus ?, PM	[123, 124]

* It is controversial whether these domains can be used for imaging purposes and their binding to PtdIns(3,5)*P2* has also been questioned [125].

** The ML1N2x domain has been found a questionable probe for PI(3,5)P2 upon thorough interrogation [126]

*** The OSH2-PH shows little discrimination between PtdIns4*P* and PtdIns(4,5)*P2* based on *in vitro* binding [120] and it is still not certain whether it actually reports on both of these molecules in some proportions.

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Table II

Visualization of other lipids by protein-domain GFP chimeras in live cells.

Lipid	Protein domain	Refs in vitro	Live cell localization	Reference
PtdSer	LacC2	[36]	PM, endosomes	[36, 127]
	Evectin-PH2x	[26]	PM, recycling endosomes	[26, 27]
PtdOH*	Spo20-PABD	[128]	PM	[83–85]
	DOCK2-PABD	[129]	PM	[129]
DAG	C1 domains	[130–132]	PM	[43, 133]
			nuclear membrane	[44, 45]
			Golgi	[134, 135]
Cholesterol	PFO D4H	[73]	PM	[73]

- There is significant uncertainty whether the PtdOH probes detect all lipid pools inside the cell.
- The Spo20 probe appears to be useful for PM PtdOH detection in mammalian cells but do not necessarily see PtdOH pools in other compartments.