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LIVE CELL IMAGING OF PHOSPHOINOSITIDES WITH EXPRESSED INOSITIDE-BINDING PROTEIN DOMAINS

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Summary

Inositol lipids and calcium signaling has been inseparable twins during the 1980s when the molecular details of phospholipase C-mediated generation of inositol 1,4,5-trisphosphate (InsP₃) and its Ca²⁺ mobilizing action were discovered. Since then, both the Ca²⁺- and inositol lipid signaling fields have hugely expanded and the tools allowing dissection of the finest details of their molecular organization also followed closely. Although phosphoinositides regulate many cell functions unrelated to Ca²⁺ signaling there are still many open questions even in the Ca²⁺ field that would benefit from single cell monitoring of PtdIns(4,5)P₂ or InsP₃ changes during agonist stimulation. This chapter is designed to provide practical guidance as well as some theoretical background on measurements of phosphoinositides in live cells using protein domain-GFP chimeras that could be also useful for people working on calcium signaling.

1. Introduction

Phosphoinositides make up only a small fraction of cellular phospholipids but their importance in cell regulation can hardly be overstated. These lipids are derived from phosphatidylinositol (PtdIns) by phosphorylation of the inositol headgroup at any but the 2 and 6 positions, giving rise to a set of regulatory lipid molecules. Phosphoinositides are produced by multiple kinases that are quite specific in their substrate preference as well as the position they will phosphorylate (1–4). The same kinase reaction is often catalyzed by several distinct enzymes that differ in their cellular location and regulation. This also means that the same lipid product can serve as a regulator in several cellular compartments as part of one of numerous unique signaling complexes. Inositol lipids are also dephosphorylated by a similar multitude of inositol lipid phosphatases that also range in their substrate and positional specificities as well as their cellular localization (5–7). Some but not all phosphoinositides are subject to hydrolysis by phospholipase C (PLC) enzymes (8) that release water-soluble inositol phosphates leaving behind diacylglycerol (DAG) in the membranes.

It was first recognized that the two product of PtdIns(4,5)P₂ hydrolysis by phosphoinositide-specific PLCs, Ins(1,4,5)P₃ and DAG serve as signaling molecules, the former by releasing Ca²⁺ from internal Ca²⁺ stores and the latter by activating a set of serine/threonine kinases now

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known as protein kinase C (9,10). However, the discovery of the PI 3-kinases (11) highlighted the importance of the membrane bound inositol lipids themselves as regulatory molecules (12). According to current ideas membrane-bound phosphoinositides usually together with the GTP-bound, active, form of small GTP binding proteins, recruit and regulate downstream protein targets (13). Several protein folding modules have been identified as phosphoinositide binding motifs, although phosphoinositide binding and regulation is often mediated by basic patches on the surface of complex structures that cannot be defined as separate folding modules once isolated from their parent structures (13).

The cellular targets and processes controlled at least in part by phosphoinositides are so numerous that it is hard to find a cellular regulatory paradigm that does not have a phosphoinositide connection. Because of this central role, phosphoinositides together with their recognition protein modules are the subject of very intensive investigations. The spatial constraints and compartmentalization of regulation by inositides demands methods that allow analysis of the spatial and temporal changes in the different phosphoinositide species. However, this is not a trivial task when it comes to rapidly changing lipids. There are antibodies that are quite specific in their recognition of phosphoinositides (14,15). However, since the sole epitope that the antibody recognizes is also the regulatory interface in the cellular context, a significant fraction of the lipid is probably engaged in any moments of time. How this pool is accessible for the antibody depends on the fixation procedure, which becomes a very critical detail that can greatly modify the results. Also, some processes are ideally followed in live cells, an application that is not possible with antibodies. Therefore, several groups have been experimenting with GFP-fused protein modules that show inositide recognition with some degree of specificity to be used as reporters of inositol lipid changes (16–19). This review will summarize some of these efforts and the experience obtained in the authors' laboratory. Since the technical details of these procedures are far less challenging than the interpretation of the data, we will concentrate on the most important questions we encounter in our discussions when evaluating of the data obtained by these techniques.

2. Description of Methods

2.1. A word of caution when using fluorescently tagged binding domains as indicators of phosphoinositides

The idea that forms the basis of the method is that if there exists a protein module that mediates a specific and direct phosphoinositide regulation to an effector protein, then this isolated module when fused to a fluorescent protein will also report on the localization and changes of the particular lipid within the cell. However, behind this simple idea there are a number of caveats that one needs to be aware of. While we may seem to be ones who promote and help propagate these methods, we have been trying to honestly caution about their pitfalls and limitations from the very beginning (20). In line with this tradition, the first and most important statement needs to be a warning concerning the overinterpretation of the results obtained by this approach. Having said that, these methods when used with caution have already provided a lot of useful information on phosphoinositide changes that would not have been possible with any currently available alternatives.

2.2. How to choose the protein modules to detect a particular lipid species?

The criteria for a good lipid probe are not dissimilar to those for any antibody. Ideally, the probe is of high specificity toward the inositide and has reasonably high affinity (K_d : ~0.5–1 μ M) with rapid association and dissociation rates. A much higher affinity (usually paired with slow dissociation) is not desirable as such probe would bind tightly to the lipid not allowing access to the enzymes that normally modify lipid levels. Although many probes have been tried and used to image a number of particular lipid species with success (see Table I), there are very

few, if any, that fulfill these simple criteria. Several methods have been used to determine the lipid binding specificity and affinity of lipid binding protein modules *in vitro*. Interestingly, these methods often yield conflicting results. Fat-blots are the most commonly used and simplest methods to assess binding specificity of a protein to inositol lipids (21,22). However, apart from the textbook cases, many protein domains show weak interactions with more than one lipid species, and some of the lipid bindings cannot be reproduced in other type of binding studies (23). This is because fat blots do not mimic the membrane environment, a limitation clearly highlighted by the extremely slow dissociation of the proteins from the lipids spotted on membranes (22). It is more reliable to measure the binding of the proteins to liposomes formed from lipid mixtures that mimic the composition of membranes (e.g. 21) or to polymerized liposomes coated with the particular inositide (24). Surface plasmon resonance (SPR) is also extensively used in determining lipid binding to proteins (e.g. 25). However, SPR can also suffer from problems depending on how the lipid or the protein is attached to the Biacore surface. These methods and their critical evaluation have been recently summarized in a comprehensive review from the Lemmon laboratory (23) and will not be detailed further. However, it is important to keep in mind that more than one of these methods should be used to determine the *in vitro* inositide binding features of protein modules.

The major twist regarding the practical usefulness of these protein modules to see lipids in the cells is that the *in vitro* binding data do not exactly translate to cellular localization. This was prominently demonstrated in the most comprehensive and thorough analyses of PH domains, performed on the whole yeast PH domain collection (a highly recommended reading for those who plan to use PH domains) (26). In this analysis it was revealed that the *in vitro* inositide binding specificity of a PH domain is not necessarily a good predictor of whether it is suitable for detection of lipids in the cell. PH domains with broad *in vitro* inositide binding profile and relatively low affinity were found to report changes in particular inositides with unexpected specificity (26). Conversely, in spite of a specific and high affinity lipid binding *in vitro* some PH domains fail to show the location of the same lipid within the cell (27,28). The main reason for this apparent discrepancy is that recruitment of PH (or other protein) domains to cellular membranes is determined by multiple interactions, only one of which being the inositol lipid. A PH domain with a limited inositide binding specificity could still be a useful reporter of a particular inositide species. In such cases the specificity comes from the inositide kinase enzyme that is part of the signaling complex in which the inositide binding-module finds its place in a lipid dependent manner. A corollary of this notion is that PH domains are probably restricted to report on certain inositide pools that are produced in a specific molecular context that may be characteristic to the particular PH domain being used. Therefore, the lack of localization of an expressed PH-GFP molecule cannot be a proof that the particular lipid is not present within a membrane compartment.

These are general considerations that should be kept in mind. As to the specifics we refer to the known probes listed in Table I for the individual lipid classes. Among these, the proven ones are the PLC δ 1PH-GFP for PtdIns(4,5)P₂ (Fig. 1), the AktPH-GFP for PtdIns(3,4,5)P₃ (but also PtdIns(3,4)P₂), and the BtkPH-GFP or Grp1PH-GFP when PtdIns(3,4,5)P₃ detection has to be more specific. Our experience with the Grp1PH-GFP is not so positive because of its high affinity to the nucleus and its binding being also dependent on Arf6-GTP in the membrane (29). There is relatively little experience with specific detection of PtdIns(3,4)P₂ with the TAPP2PH-GFP and no domain in isolation has been proven to be a reliable signal of PtdIns(3,5)P₂. As to the monophosphorylated phosphoinositides, the FYVE domains of Hrs (used as a tandem) or EEA1 has been both successfully used to detect PtdIns3P in the endosomes. PtdIns4P, on the other hand can be detected either in the Golgi (FAPP1PH-GFP or OSBP-PH-GFP) or the plasma membrane (OSH2-PH-GFP) but no single probe can detect all of the PtdIns4P pool of a cell. There is also very limited information available on cellular PtdIns5P using the PHD domain of the nuclear ING2 protein.

2.3. Designing a probe

To create a fusion protein with enhanced GFP (EGFP) the lipid-binding domain is subcloned in either the pEGFP-N1 or the pEGFP-C1 plasmids that differ only in the position of the multiple cloning sites relative to the EGFP sequence. Since the introduction of this method, a large number of fluorescent proteins have been introduced offering a wide variety of colors and other unique features. These include photoactivation, photoswitching, color-change during maturation, pH stability, resistance to photobleaching or dimerization etc. summarized in several recent reviews (30,31). These proteins are often very useful for a particular application. However, it would be a mistake to believe that they only differ in their spectral behavior and they are biologically as “inert” as EGFP or its analogues. Our experience is that from time to time a probe behaves quite differently depending on whether an EGFP or an mRFP molecule is attached to it. Therefore, it is better to stay with a few colors that are proven to work similarly with a particular lipid binding domains than generate a whole series of colors assuming that they will behave identically within the cells. Unfortunately, the original pEGFP-N and -C series plasmids are not available anymore since Clontech became part of Takara and these companies now offer their own fluorescent proteins. The original EGFP and its color variants are now sold by Invitrogen, in a different plasmid backbone. This causes some confusion among users who just begin collecting their fluorescent proteins but also want to rely upon constructs that have been built based on the pEGFP-N and -C series plasmids.

To define the domain boundaries is an important part of the design. Often a few residues outside the strict borders of the domain as defined in the Pfam or SMART databases or based on sequence alignment can make a big difference in the ability of the construct to localize within the cell. This is because these additional flanking regions can alter the association of the domain with additional components. A good example is the FYVE domain that will only show PtdIns3P-dependent localization to the endosomes if it contains a region that is also responsible for dimerization (and perhaps serves as a partial Rab5 binding sequence), whereas the *in vitro* PtdIns3P binding does not require these additional flanking sequences (18,19). When the strictly defined minimum domain is not sufficient for membrane localization in cells, it has proven to be useful to make a tandem construct fusing two copies of the domain after one another thereby increasing the apparent affinity of the construct. This has been done with the Hrs FYVE domain of Hrs (19) as well as the PH domain of the yeast OSH2 protein (32,33).

The position of the PH domain relative to the fluorophore could also make a difference. Our practice is to place the PH domain relative to EGFP following the domains' natural location within the parent molecule (i.e., when the PH domain is in the NH₂ terminus of the molecule, we place it before EGFP in the pEGFP-N1 plasmid). Nevertheless, many PH domains would work in both locations. It is important to remember that a consensus translation start sequence with a Kozak sequence should be inserted 5' to the lipid-binding domain when it is placed at the NH₂-terminal orientation (when using pEGFP-N1), unless the natural start sequence is part of the sequence of the lipid-binding domain portion of the construct. If the lipid-binding domain is in the COOH-terminal orientation (when using the pEGFP-C1), a stop codon should be inserted before the cloning site at the 3' end. This can prevent unexpected (although unlikely) complications caused by the few unchecked amino acids that are added to the construct before the vector's own stop codon is reached. All oligonucleotide amplification steps should be performed with a high-fidelity DNA polymerase, such as Pfu.

2.4. Checking the integrity of the probe

In addition to sequencing the DNA construct, it is important to determine whether the fusion protein is intact once expressed in mammalian cells before any imaging. Often, the fusion protein is cleaved within the cell so that the fluorescence is not coming from the molecule that was intended. We recommend analyzing cell lysates by sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE). If the sample is not boiled it can be directly checked with a phosphorimager equipped with a blue laser line (in the case of GFP) as described below. Alternatively, one could use conventional Western blotting techniques using antibodies against the FP portion of the fusion protein. We find that in many cases, samples that are boiled and analyzed by Western blotting show more degradation product than those analyzed directly from the gel. Remember that anti-GFP antibodies will not recognize other fluorescent proteins isolated from other species.

3. Method to use the lipid probes in live cells

In this chapter we describe the technical details of using these probes to monitor inositol lipids in live cells. Most of this methodology requires basic equipments, which are now widely available.

3.1. Expression of the fusion proteins in mammalian cells

The easiest method for delivery of the fluorescent reporter into the cell is by transfection with a mammalian expression plasmid. Alternative methods used for delivering the construct are microinjection of cRNA or the bacterially expressed and purified fusion proteins. However, these latter methods require more preparations and a microinjection apparatus and will not be discussed in this chapter. For most microscopy application 5×10^5 cells in 2 ml culture medium are plated onto 25 mm glass coverslips (PG Science, # 60–4884–25). The coverslips are usually precoated with Poly-lysine (Sigma, #P-8920) by preparing a 1:50 or 1:100 dilution of poly-lysine in sterile deionized water (some cells require the higher concentrations, but others do not tolerate it well) and adding 1.0 ml of the diluted poly-lysine for 1 h to the coverslips that had been rinsed with 98% ethanol and air dried. After 1 h treatment, the poly-lysine is removed and the coverslips are air-dried and are ready for plating.

Cells are grown for 1–2 days in the appropriate medium containing 10% FBS and antibiotics before transfection. The preferred transfection method varies between cell-to-cell and even among laboratories. For COS-7 and HEK293 cells we routinely use Lipofectamine 2000 (Invitrogen) or Fugene (Roche) and follow the manufacturer's recommended protocols. The time of transfection can also depend on the application. However, we do not recommend keeping the cells longer than 24 h after transfection because of the potential toxicity of the lipid-binding fusion protein due to interference with the functions of the lipids that they bind. An important control that we always recommend doing is to use a mutant version of the lipid-binding domain that does not bind lipids. Many constructs also localize to the nucleus, but this localization is not dependent on lipid binding based on the fact that the mutant versions also show nuclear localization. It is also recommended that one simultaneously transfect cells with the GFP protein alone without the lipid-binding domain. These two controls help to track phenotypic changes and potential cellular toxicity associated with overexpression of the lipid-binding domain, as well as serve to serve as controls for monitoring localization that depends on lipid binding

3.2. Confirming the integrity of the expressed protein using a phosphorimager

For this, we use cells seeded in 12 well plates (2×10^5 cells/2ml) and transfected the next day with plasmid DNA. After 24 h, cells are washed with 2 ml of PBS and dissolved in 75 μ l of SDS-PAGE loading Laemmli buffer. Cells are sonicated to disrupt the DNA but are not boiled. A 25 μ l aliquot is loaded on a small (10 cm) SDS-PAGE gel at an acrylamide concentration that will resolve proteins in the size range of interest. After running, the gel is removed from the casing and is subjected to scanning in its wet-form in a phosphorimager using the blue laser line. It is our experience that the electrophoretic mobility of the EGFP molecule (or the fusion protein) can be very different from their calculated mass. This is due to altered migration of

the non-denatured protein due to conformational effects. For accurate size analysis, it is recommended that samples are boiled and, after SDS-Page, detected with an anti-GFP antibody using standard protocols.

3.3. Analysis of transfected cells in the microscope. Fixed or live cells?

Once the plasmids are completed and the fusion protein is found to be intact, transfected cells can be observed under the microscope. Cells can be analyzed either live or fixed, and there are pros and cons for both cases. Fluorescence can persist in fixed cells under proper fixation conditions for a period of time. Fixed cells can be also processed for immunostaining with an anti-GFP antibody that is often more sensitive than the GFP fluorescence. Immunostaining can also help determine co-localization of the GFP construct with markers for which antibodies are available. Also, fixed cells can be stored and studied when convenient. However, one disadvantage of using fixed cells is that changes cannot be followed as they happen in real time in stimulated cells. Moreover, fixation and permeabilization procedures may distort cellular morphology. For example, we find that vesicular structures shrink during fixation, and long canaliculi can turn into small vesicles. The use of live cells definitely is the most reliable way of assessing undistorted morphology, but it is also the most time-demanding and least efficient in terms of data collection. For live cell imaging, it is best to use an inverted microscope. New water-immersion objectives make it possible to look at cells in upright microscopes, but it requires to that the bulky and difficult-to-clean objective be immersed in the culture medium.

3.3.1. Fixing and immunostaining of cells expressing GFP fusion proteins—Cells are rinsed with 2 ml PBS before the addition of 2 ml 2% paraformaldehyde (PFA) solution for 10 min at room temperature. This solution should be freshly made by dissolving electron microscopy (EM) grade PFA in phosphate-buffered saline (PBS) followed by heating in a chemical hood to 60 °C until fully dissolved with loose cover to allow gas to escape. After cooling to room temperature the pH is adjusted to 7.4 with NaOH. After the PFA incubation cells are washed three times with 2 ml of PBS for 10 min each. At this point the cells can be examined under the microscope for the GFP fluorescence or can be further processed for immunostaining. For this the cells are incubated for 10 min at room temperature with 2 ml of Blocking Solution (10% solution of FBS in PBS) to block nonspecific antibody binding. This is followed by addition of the primary antibody diluted appropriately in Antibody Diluent (10% FBS and 0.2% Saponin in PBS). The cells are incubated with the primary antibody for 1 hour at room temperature. (100 µl of diluted antibody should be sufficient for a 25 mm circular or 22×22 mm square coverslip, if it is inverted on a glass slide and incubated in a humidified petri dish). After the 1st antibody incubation, cells are washed three times with 2 ml of Blocking Solution for 5 min each before adding the fluorescent secondary antibody also diluted in Antibody Diluent. Incubations continue for 1 hour at room temperature protected from light. Finally the cells are washed three times with 2 ml of PBS and air dried until the coverslips are only damp. The coverslips are then mounted with the cells down on a glass slide using Aqua Poly/Mount and sealed on the slide with clear nail polish to prevent drying.

3.4. Examination of the transfected cells in the microscope

With the rapid advances in microscope technology there are different ways the cells can be studied depending on the application and the purpose of the experiment. However, it is always advised that cells are first examined in a wide-field fluorescence microscope. In fact, it is more difficult to get a general impression of how cells expressing the construct look in specialized microscopes, such as a confocal microscope or TIRF, than in a fluorescence microscope. Single cells, especially COS cells, show enormous variability in their shape, size, and general appearance, and often the level of expression changes their appearance. This morphological variability of cells is the largest problem when one would like to determine the effects of the expressed proteins on cellular morphology. Conventional fluorescence microscopy is a

significantly more efficient way to browse through many cells and notice trends in cellular morphology. Moreover, many cells are flat in culture (especially COS cells), so there is a limited benefit in analyzing the cells in a confocal microscope. Confocal microscopy can be saved for recording cells and changes in fluorescence distribution once the conditions have been optimized with a fluorescence microscope. An additional advantage of viewing the cells with a fluorescence microscope is that autofluorescence often can be distinguished from the GFP signal because its color is different from the color of GFP. It is important to remember that confocal microscopes measure light intensity, but no colors; the “color” that is given is artificial. Therefore, in each case, the autofluorescence has to be determined so that the fluorescence signal can be reliably used. For this, observation of a set of untransfected cells is a very useful control.

3.4.1. What kind of cells should be selected for analysis?—Looking into the fluorescence microscope, there is a wide range of cells with varying fluorescence intensities (Fig. 2). Depending on the quality of the microscope and the intensity of the light source, sometimes only the cells with the highest expression levels are visible and these are the cells one would like avoid. It is always best to study cells in which the fluorescent signal is as low as possible but is still clearly distinguishable from the autofluorescence. High expression levels of lipid probes exert profound effects on the cells and also increase the background of signal in the cytosol as the lipid binding sites may become saturated. The best way to be certain that even the lowest level of expression is visible in the microscope is to find the autofluorescence of untransfected cells. There are no general rules for this as the sensitivities of confocal microscopes or of the cameras attached to conventional fluorescence microscopes vary from manufacturer to manufacturer. However, the following practices are recommended: 1. Observe the transfected cells (and if necessary, the control untransfected cells) with the fluorescence setting of the confocal microscope. (a separate untransfected cell control is not always necessary, because not all cells will express the fusion proteins). The untransfected cells in the population can usually be distinguished by their autofluorescence, allowing easy identification of transfected cells from the same sample. In COS-7 cells the autofluorescence (in the GFP/FITC setting) appear as small vesicular puncta most densely arranged around the nucleus probably originating from the peroxysomes). 2. Choose healthy looking cells that express protein levels just enough so that the signal can be clearly resolved from the background autofluorescence. Analysis of cells that show signs of toxic effects induced by the expression of the fusion protein should be avoided. For example, cells round up or contain large intracellular vesicles when they express high levels of the PLCd1PH-GFP construct. 3. A good quality image should be obtained with no more than 3% to 5% of the maximum laser power (assuming ~ 30 mW lasers provided with many confocal microscopes) to avoid photobleaching and damaging the cells. Higher % power may be required in the red laser as some red lasers (543 nm) have lower overall power and the red detectors are usually less sensitive.

3.4.2. Time-lapse analysis of live cells—The most satisfying and exciting aspect of inositol lipid imaging is working with live cells. However, this also is the most time consuming and technically demanding. First of all, live cell imaging requires keeping the cells at the proper temperature. Some cellular processes, such as hormone-induced PLC activation are robust enough to be recorded at 22–25 °C as shown by many studies on Ca²⁺ signaling. However, trafficking steps such as receptor endocytosis is very inefficient below 30 °C. Therefore, it is desirable to run experiments at a higher temperature. An important point to remember is that even when the medium in the observation chamber is kept at the desired temperature by a heated stage, the objective in an inverted microscope acts as a heat sink keeping cells just within the observation field at a temperature of the objective. Because lasers require proper cooling and, therefore, the microscopy rooms are usually kept on the colder side, this means that even on a heated stage the recorded cells are examined at room temperature. Therefore we

recommend the use of objective heaters (available from Bioprotechs <http://www.bioprotechs.com>). However, the heater collar does not fit all objectives, and heating may be damaging to the objective if it is warmed very fast from a cold temperature. Alternative methods of maintaining the proper temperature include perfusing the cells with a high flow of warm medium and using a hair dryer to keep the objective at the proper temperature during live cell imaging. There are complete incubator enclosures available from various companies that can keep both the temperature and CO₂ concentration of cells on the stage at the desired levels. Unfortunately, they make manipulations of the cells often difficult.

We use the metal Atto chambers from Molecular Probes (now part of Invitrogen) as holders in a heated stage and an objective heater. After securing the coverslips with the cells in the metal chamber we add 1 ml of prewarmed (37°C) Modified Krebs-Ringer solution (NaCl, 120 mM; KCl, 3.7 mM; Na₂HPO₄, 1.2 mM; CaCl₂, 1.2 mM; MgSO₄, 0.7 mM; Glucose, 10 mM; Na-Hepes, pH 7.4, 20 mM; Bovine serum albumin (BSA) 0.1%). Ideally, one should include cells transfected with lipid-binding fusion protein construct, nontransfected control cells, and cells transfected with a control construct that does not bind lipids.

Most current softwares allow recording of time-lapsed images. We usually record at every 5 s to 20 s (depending on the speed of the response), and with a scanning speed of 1.6 μ s/pixel, to capture the cellular response with proper image resolution. Remember that the higher the resolution and the more frequent the scans are the more photobleaching and phototoxicity of the cells occur. Addition of stimuli or inhibitors is another technical challenge. The ideal way to stimulate cells is with a constant perfusion system with small dead-volumes and a valve-system that can change the composition of the medium. However, our experience is that with many of the lipophilic compounds (ionomycin, thapsigargin, rapamycin etc.) it is extremely difficult to wash the system with the valves and plastic tubes clean. Washing the Atto chambers with their plastic oring is already a challenge after using these compounds. This minor detail is often overlooked producing all kind of hard to explain artifacts.

3.4.3. Quantification of the data—The most demanding part of the analysis of time-lapsed sequences is the quantification of data. When the fluorescence redistribution is obvious a series of pictures or movies might be sufficient to describe what is happening. However, when determining a dose-response effect or comparing the relative effectiveness of two stimuli, or investigating the efficacy or potency of an inhibitor, it is necessary to quantify the changes. The most common way of this analysis is to create a line-intensity histogram through a selected line spanning the image (Fig. 3). The highest intensities should not saturate the detector, which requires a fine optimization of the dynamic range before recording, especially with 8-bit systems that only have 256 levels of intensity. Saturation is less of a problem with the 12-bit systems that have 4096 levels of intensity. A simple way of quantifying the translocation of a fluorescent protein from the membrane to the cytosol is to monitor the cytosolic pixel intensity of selected cells (Fig. 3 shows an example). A more accurate but also more demanding way of quantification of the extent of membrane association of fluorescent proteins is to use fluorescence resonance energy transfer (FRET) or total internal reflection fluorescence (TIRF) microscopy. The former is able to detect the radiationless energy transfer between two appropriate fluorophore pairs when they are within an optimal distance (see below), while the latter only detects fluorescence coming from the plane of the membrane. The variability of the cell population and the requirement for analysis of a large number of cells to obtain reliable quantitative estimates of the fluorescence changes remain the most laborious part of obtaining reliable, reproducible results.

3.4.3.1. FRET measurements: It is not easy to make quantitative assessment of the membrane localization of fluorescent probes. In fact, some “localization” is a reflection of change in membrane volume or change of membrane shape as opposed to real change in recruitment.

Therefore, several attempts were made to utilize the FRET principle to obtain a signal based upon the binding of an inositide binding domain to membranes. The simplest method we used was to co-express the CFP- and YFP-tagged versions of the same PH domain (e.g. PLC δ 1-PH). CFP and YFP are the most widely used pairs of fluorophores that when come within FRET distance (<8 nm) will show energy transfer from CFP to YFP. This will cause a decrease in CFP emission (475 nm) and increased YFP emission (525 nm) when only CFP excitation (430 nm) is applied. The efficiency of the energy transfer can be numerically calculated after making all necessary corrections (such as bleed through of the CFP and YFP signals into the other pair's emission channels). However, if the two wavelengths show opposite changes, the simple fluorescence ratio of 525/475 can be used (Fig. 3B). When the two fluorophores are bound to the lipids at the membrane via the attached PH domains there is an efficient energy transfer. However, upon PLC activation the molecules leave the membrane and the FRET signal decreases (34). This method is quite sensitive and even small PLC activation can be detected and quantified. It can also be used in individual cells or in cell suspensions (35). A disadvantage of this method is that at low expression levels (which would be desirable to minimize the ill effects of the presence of the probes) the FRET efficiency is not very high and even at high probe concentration there is low FRET signal if the density of the lipids is below a certain level.

In contrast to the above examples using two separate molecules that show intermolecular FRET, a few attempts have been made to generate inositol lipid probes based on intramolecular FRET. In this case, both fluorophores are attached to the same inositide-recognizing domain. Lipid binding then induces an alteration in the distance (or more likely the dipole orientation) of the fluorophores and, hence, a change in the FRET signal. A probe based on the Grp1-PH domain was targeted to different membranes for PtdIns(3,4,5) P_3 detection (36) and a similar principle was utilized to generate FRET probes for monitoring Ins P_3 changes in the cytoplasm (37,38). The challenge in this type of molecular engineering is to make sure that the conformational change upon lipid binding is sufficiently big to change the FRET signal. Construction of a useful probe requires lots of experimentation with the domains themselves as well as with the linkers to connect the fluorophores. In a recent study, the AktPH domain was used to detect PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 changes using a unique molecular design. The conformational change between the lipid-bound and unbound stages was achieved by inserting a negatively charged “pseudoligand” in the probe that binds to the PH domain (presumably to the lipid binding site) when lipids are not present. Binding of the appropriate lipids abolishes this intramolecular interaction amplifying the conformational change and generating a larger change in the FRET signal (39). These single molecule FRET probes do not require coexpression, their readout does not depend on their expression level (once above reliable detection limits) or on lipid density in the membrane. It is expected that more efforts will be made to generate similar probes for the detection of lipid production in the various cellular compartments.

More detailed technical and theoretical background on FRET measurements either with sensitized emission or with fluorescence lifetime imaging microscopy (FLIM), including corrections for bleed-through and uneven illumination, can be found in very comprehensive publications elsewhere (40–42).

3.4.3.2. Quantification of membrane association with TIRF analysis: TIRF (total internal reflection fluorescence) analysis has also been used to monitor plasma membrane association of inositol lipid binding domains (43). This technique detects fluorescence originating only from the thin membrane area of the cell attached to the coverslips making it a suitable method of detecting membrane-associated fluorescence (Fig. 3C). One caveat of this method is that anything that changes the footprint of the cell would cause a change in fluorescence intensity unrelated to the actual amount of fluorescent molecules at the membrane. For this reason, for

most accurate results it is desirable to use a fluorescent membrane marker and relate all fluorescence change to this reference signal.

4. Critical evaluation of results

Expression of these lipid binding protein modules has several effects on the biology of the cell, as they generate a dynamic pool of the lipid by both sequestering and protecting it from effectors and the phosphatase or phospholipase enzymes (see Fig. 4 for an example). In addition, the ability of many of the lipid binding modules to bind proteins in addition to the lipids is a major point of consideration. The less phosphate an inositide headgroup has the more likely that the localization of its binding protein also depends on other interactions mostly by small GTP binding proteins. For example, the FAPP1- and OSBP-PH-GFP fusion proteins are kept at the Golgi membrane by the combined presence of PtdIns4P and the GTP-bound form of Arf1 (44). Therefore, changing the Arf1-GTP concentration will lead to release of the construct from the membrane without any changes in the inositol lipid. Conversely, one cannot recruit more FAPP1-PH to the Golgi membrane by producing extra PtdIns4P without additional Arf1-GTP molecules. These examples show that changes in PH-GFP membrane attachment needs to be treated with caution as an indicator of inositide lipid change.

Another and even more controversial question is whether soluble inositol phosphates can compete with the membrane inositides for binding the PH-domain GFP fusion protein. This is a highly debated issue to the extent that some groups use the changes in PLC δ 1-PH-GFP localization as a faithful index of InsP₃ changes rather than lipid changes within the cell (45). We devoted ample discussion to this topic in a recent review (#3893) that will not be repeated here. The bottom line is that inositol phosphates can compete to the lipid binding of the PH-domain constructs and their effects on changing localization could be quite significant under certain circumstances and cannot be ignored. At the same time, it is equally misleading to treat the PLC δ 1PH-GFP translocation response as an index of InsP₃ change, as pointed out by simultaneous measurements of InsP₃ and PLC δ 1PH-GFP translocation (38). It would be highly desirable to obtain other binding domains of PtdIns(4,5)P₂ and compare their behavior with that of the widely used PLC δ 1-PH construct especially their sensitivity to InsP₃-induced displacement. Unfortunately, such studies would require significant commitment and are not enthusiastically supported by granting agencies.

5. Concluding remarks

Live cell imaging of phosphoinositides is a very useful method that should complement the molecular toolbox of scientists who study Ca²⁺-phosphoinositide signaling. However, it should be remembered that depending on the molecule used for imaging purposes one might obtain information only on a fraction of the inositide pool and some changes in probe distribution may not be caused by lipids but by competition with soluble inositol phosphates or changing interactions with protein binding components. Therefore, it is imperative that the results are evaluated in comparison with findings with other methods. PtdIns(4,5)P₂ imaging and quantification with PLC δ 1PH domain is probably the most important application for people working on the Ca²⁺ signaling field. The dependence of a large portion of PLC activation and hence Ins(1,4,5)P₃ generation on the cytosolic Ca²⁺ increase and the dependence of the Ca²⁺ increase on Ins(1,4,5)P₃ levels make the PLC δ 1PH localization changes very intricately linked to Ins(1,4,5)P₃ and the Ca²⁺ changes. Until we find a probe that binds PtdIns(4,5)P₂ without being influenced by Ins(1,4,5)P₃ (if such a probe can exist at all) we will always have to deal with the competition issue and if possible also have to measure Ins(1,4,5)P₃ changes simultaneously (38). We are hopeful that research in this area will continue to flourish and newer and newer tools will help us further explore the intricate details of how PtdIns(4,5)P₂ can be a PLC substrate and a regulator of so many membrane processes at the same time.

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References

1. Fruman DA, Meyers RE, Cantley LC. *Annu Rev Biochem* 1998;67:481–507. [PubMed: 9759495]
2. Doughman RL, Firestone AJ, Anderson RA. *J Membr Biol* 2003;194:77–89. [PubMed: 14502432]
3. Foster FM, Traer CJ, Abraham SM, Fry MJ. *J Cell Sci* 2003;116:3037–40. [PubMed: 12829733]
4. Balla A, Balla T. *Trends Cell Biol* 2006;16:351–61. [PubMed: 16793271]
5. Astle MV, Seaton G, Davies EM, Fedele CG, Rahman P, Arsala L, Mitchell CA. *IUBMB Life* 2006;58:451–6. [PubMed: 16916781]
6. Taylor GS, Dixon JE. *Methods Enzymol* 2003;366:43–56. [PubMed: 14674238]
7. Majerus PW, Kisseleva MV, Norris FA. *J Biol Chem* 1999;274:10669–72. [PubMed: 10196133]
8. Rhee SG. *Annu Rev Biochem* 2001;70:281–312. [PubMed: 11395409]
9. Berridge MJ. *Biochem J* 1984;220:345–60. [PubMed: 6146314]
10. Nishizuka Y. *Nature* 1984;308:693–98. [PubMed: 6232463]
11. Whitman M, Downes CP, Keeler M, Keller T, Cantley L. *Nature* 1988;332:644–46. [PubMed: 2833705]
12. Toker A, Cantley LC. *Nature* 1997;387:673–76. [PubMed: 9192891]
13. Lemmon MA. *Nat Rev Mol Cell Biol* 2008;9:99–111. [PubMed: 18216767]
14. Hammond GR, Dove SK, Nicol A, Pinxteren JA, Zicha D, Schiavo G. *J Cell Sci* 2006;119:2084–94. [PubMed: 16687737]
15. Thomas CL, Steel J, Prestwich GD, Schiavo G. *Biochem Soc Trans* 1999;27:648–52. [PubMed: 10917659]
16. Stauffer TP, Ahn S, Meyer T. *Curr Biol* 1998;8:343–46. [PubMed: 9512420]
17. Várnai P, Balla T. *J Cell Biol* 1998;143:501–10. [PubMed: 9786958]
18. Burd CG, Emr SD. *Mol Cell* 1998;2:157–62. [PubMed: 9702203]
19. Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaullier LM, Parton GP, Stenmark H. *EMBO J* 2000;19:4577–88. [PubMed: 10970851]
20. Balla T, Bondeva T, Várnai P. *Trends Pharmacol Sci* 2000;21:238–41. [PubMed: 10871889]
21. Kavran JM, Klein DE, Lee A, Falasca M, Isakoff SJ, Skolnik EY, Lemmon MA. *J Biol Chem* 1998;273:30497–508. [PubMed: 9804818]
22. Dowler S, Currie RA, Campbell DG, Deak M, Kular G, Downes CP, Alessi DR. *Biochem J* 2000;351:19–31. [PubMed: 11001876]
23. Narayan K, Lemmon MA. *Methods* 2006;39:122–33. [PubMed: 16829131]
24. Ferguson CG, James RD, Bigman CS, Shepard DA, Abdiche Y, Katsamba PS, Myszkowski DG, Prestwich GD. *Bioconjug Chem* 2005;16:1475–83. [PubMed: 16287244]
25. Komander D, Fairservice A, Deak M, Kular GS, Prescott AR, Peter Downes C, Safrany ST, Alessi DR, van Aalten DM. *Embo J* 2004;23:3918–28. [PubMed: 15457207]
26. Yu JW, Mendrola JM, Audhya A, Singh S, Keleti D, DeWald DB, Murray D, Emr SD, Lemmon MA. *Mol Cell* 2004;13:677–88. [PubMed: 15023338]
27. Várnai P, Lin X, Lee SB, Tuymetova G, Bondeva T, Spat A, Rhee SG, Hajnoczky G, Balla T. *J Biol Chem* 2002;277:27412–22. [PubMed: 12019260]
28. Lee SB, Várnai P, Balla A, Jalink K, Rhee SG, Balla T. *J Biol Chem* 2004;279:24362–71. [PubMed: 15037625]
29. Cohen LA, Honda A, Várnai P, Brown FB, Balla T, Donaldson JG. *Mol Biol Cell*. 2007in press
30. Shaner NC, Steinbach PA, Tsien RY. *Nat Methods* 2005;2:905–9. [PubMed: 16299475]

31. Giepmans BN, Adams SR, Ellisman MH, Tsien RY. *Science* 2006;312:217–24. [PubMed: 16614209]
32. Roy A, Levine TP. *J Biol Chem* 2004;279:44683–89. [PubMed: 15271978]
33. Balla A, Ju Kim Y, Varnai P, Szentpetery Z, Knight Z, Shokat KM, Balla T. *Mol Biol Cell*. 2007
34. van Der Wal J, Habets R, Varnai P, Balla T, Jalink K. *J Biol Chem* 2001;276:15337–44. [PubMed: 11152673]
35. Balla A, Tuymetova G, Tsiomenko A, Varnai P, Balla T. *Mol Biol Cell* 2005;16:1282–95. [PubMed: 15635101]
36. Sato M, Ueda Y, Takagi T, Umezawa Y. *Nat Cell Biol* 2003;5:1016–22. [PubMed: 14528311]
37. Tanimura A, Nezu A, Morita T, Turner RJ, Tojyo Y. *J Biol Chem* 2004;279:38095–98. [PubMed: 15272011]
38. Matsu-ura T, Michikawa T, Inoue T, Miyawaki A, Yoshida M, Mikoshiba K. *J Cell Biol* 2006;173:755–65. [PubMed: 16754959]
39. Ananthanarayanan B, Ni Q, Zhang J. *Proc Natl Acad Sci U S A* 2005;102:15081–6. [PubMed: 16214892]
40. van Rheenen J, Langeslag M, Jalink K. *Biophys J* 2004;86:2517–29. [PubMed: 15041688]
41. Sekar RB, Periasamy A. *J Cell Biol* 2003;160:629–33. [PubMed: 12615908]
42. Thaler C, Koushik SV, Blank PS, Vogel SS. *Biophys J* 2005;89:2736–49. [PubMed: 16040744]
43. Tengholm A, Teruel MN, Meyer T. *Sci STKE* 2003;2003:PL4. [PubMed: 12582202]
44. Levine TP, Munro S. *Curr Biol* 2002;12:695–704. [PubMed: 12007412]
45. Nash MS, Young KW, Willars GB, Challiss RA, Nahorski SR. *Biochem J* 2001;356:137–42. [PubMed: 11336645]
46. Varnai P, Thyagarajan B, Rohacs T, Balla T. *J Cell Biol* 2006;175:377–82. [PubMed: 17088424]
47. Suh BC, Inoue T, Meyer T, Hille B. *Science* 2006;314:1454–7. [PubMed: 16990515]
48. Lemmon MA, Ferguson KM, O'Brian R, Sigler PB, Schlessinger J. *Proc Natl Acad Sci USA* 1995;92:10472–76. [PubMed: 7479822]
49. Santagata S, Boggon TJ, Baird CL, Gomez CA, Zhao J, Shan WS, Myszkowski DG, Shapiro L. *Science* 2001;292:2041–50. [PubMed: 11375483]
50. Field SJ, Madson N, Kerr ML, Galbraith KA, Kennedy CE, Tahiliani M, Wilkins A, Cantley LC. *Curr Biol* 2005;15:1407–12. [PubMed: 16085494]
51. Rameh LE, Arvidsson A, Carraway KL III, Couvillon AD, Rathbun G, Crompton A, VanRenterghem B, Czech MP, Ravichandran KS, Burakoff SJ, Wang DS, Chen CS, Cantley LC. *J Biol Chem* 1997;272:22059–66. [PubMed: 9268346]
52. Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A, Czech MP. *Science* 1997;275:1927–30. [PubMed: 9072969]
53. Klarlund JK, Tsiaras W, Holik JJ, Chawla A, Czech MP. *J Biol Chem* 2000;275:32816–21. [PubMed: 10913124]
54. Venkateswarlu K, Gunn-Moore F, Oatey PB, Tavaré JM, Cullen PJ. *Biochem J* 1998;335 (Pt 1):139–46. [PubMed: 9742223]
55. Venkateswarlu K, Oatey PB, Tavaré JM, Cullen PJ. *Curr Biol* 1998;8:463–66. [PubMed: 9550703]
56. Nagel W, Schilcher P, Zeitlmann L, Kolanus W. *Mol Biol Cell* 1998;9:1981–94. [PubMed: 9693361]
57. Venkateswarlu K, Gunn-Moore F, Tavaré JM, Cullen PJ. *J Cell Sci* 1999;112:1957–65. [PubMed: 10341214]
58. Salim K, Bottomley MJ, Querfurth E, Zvelebil MJ, Gout I, Scaife R, Margolis RL, Gigg R, Smith CIE, Driscoll PC, Waterfield MD, Panayotou G. *EMBO J* 1996;15:6241–50. [PubMed: 8947047]
59. Varnai P, Rother KI, Balla T. *J Biol Chem* 1999;274:10983–89. [PubMed: 10196179]
60. Franke TF, Kaplan DR, Cantley LC, Toker A. *Science* 1997;275:665–68. [PubMed: 9005852]
61. Watton J, Downward J. *Curr Biol* 1999;9:433–36. [PubMed: 10226029]
62. Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. *Science* 2000;287:1037–40. [PubMed: 10669415]
63. Huang YE, Iijima M, Parent CA, Funamoto S, Firtel RA, Devreotes P. *Mol Biol Cell* 2003;14:1913–22. [PubMed: 12802064]

64. Dormann D, Weijer G, Parent CA, Devreotes PN, Weijer CJ. *Curr Biol* 2002;12:1178–88. [PubMed: 12176327]
65. 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. *Biochem J* 2002;361:525–36. [PubMed: 11802782]
66. Friant S, Pecheur EI, Eugster A, Michel F, Lefkir Y, Nourrisson D, Letourneur F. *Dev Cell* 2003;5:499–511. [PubMed: 12967568]
67. Dove SK, Piper RC, McEwen RK, Yu JW, King MC, Hughes DC, Thuring J, Holmes AB, Cooke FT, Michell RH, Parker PJ, Lemmon MA. *Embo J* 2004;23:1922–33. [PubMed: 15103325]
68. Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H. *Nature* 1998;394:494–98. [PubMed: 9697774]
69. Kanai F, Liu H, Field SJ, Akbary H, Matsuo T, Brown GE, Cantley LC, Yaffe MB. *Nat Cell Biol* 2001;3:675–78. [PubMed: 11433300]
70. 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. *Nat Cell Biol* 2001;3:679–82. [PubMed: 11433301]
71. Levine TP, Munro S. *Curr Biol* 1998;8:729–39. [PubMed: 9651677]
72. Godi A, Di Campi A, Konstantakopoulos A, Di Tullio G, Alessi DR, Kular GS, Daniele T, Marra P, Lucocq JM, De Matteis MA. *Nat Cell Biol* 2004;6:393–404. [PubMed: 15107860]
73. 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, Payrastra B, Myszkowski DG, Cantley LC, Wagner G, Divecha N, Prestwich GD, Yuan J. *Cell* 2003;114:99–111. [PubMed: 12859901]
74. Pendaries C, Tronchere H, Arbibe L, Mounier J, Gozani O, Cantley L, Fry MJ, Gaits-Iacovoni F, Sansonetti PJ, Payrastra B. *Embo J* 2006;25:1024–34. [PubMed: 16482216]
75. Michell RH, Heath VL, Lemmon MA, Dove SK. *Trends Biochem Sci* 2005;31:52–63. [PubMed: 16364647]

Figure 1.

Imaging PtdIns(4,5)P₂ with the PH domain of PLC δ 1. (A) The domain structure of the PLC δ 1 molecule. The N-terminal PH domain has been shown to serve as a specific high affinity binding module for PtdIns(4,5)P₂. (B) Expression of the PLC δ 1PH-GFP recognizes PtdIns(4,5)P₂ of the plasma membrane and follows its changes after PLC activation or dephosphorylation by an inositol 5-phosphatase enzyme. (C) An example of plasma membrane to cytosol translocation of the PLC δ 1PH-GFP in HEK293 cells after stimulation with angiotensin II (AngII). (D) The plasma membrane localization of the PLC δ 1PH-GFP protein is more difficult to judge in a flat COS-7 cell (control), but the difference is more striking when the PtdIns(4,5)P₂ is eliminated (by ionomycin treatment (iono) that increases

intracellular Ca^{2+} and activates PLC) or after overexpression of a type-IV 5-phosphatase (5-ptase) when the construct is found in the cytosol.

Figure 2. Selection of cells expressing moderate levels of PLCδ1PH-GFP. Panels a-c show the same imaging field with increasing gain of HEK293 cells transfected with PLCδ1PH-GFP for 24 h. Note that the cells we would select for imaging (shown enlarged in **d**) are not even visible when the high expressing cells are looked at (in **a**). This would be the signal seen in a less-sensitive microscope.

Figure 3.

Quantification methods to assess changes in plasma membrane localization of PLC δ 1PH-GFP. (A) Changes in membrane localization after angiotensin II (AngII) stimulation as assessed by confocal imaging of a HEK293 cell shown on the left. The right panel shows the line-intensity histogram corresponding to the white line placed on the cell at time 0. The left lower panel shows the changing intensity values corresponding to the membranes (upper trace) and cytosol (lower trace) as a function of time and the ratio formed from the two (lower right panel). (B) The kinetics of FRET changes after AngII stimulation and ionomycin treatment (high Ca^{2+}) in HEK293 cells co-expressing the CFP- and YFP-fused versions of the PLC δ 1PH domain. The cells were illuminated with 430 nm and recorded at 525 nm and 485 nm. The FRET ratio

is expressed as arbitrary units. Means $\pm$ S.E.M. of 5 cells shown. (C) Changes in TIRF signal in HEK293 cells after rapamycin-induced recruitment of a mRFP-FKBP12-5-phosphatase domain construct to the plasma membrane (shown as an increase in the upper trace) and simultaneous decrease in the localization of the PLC δ 1PH-GFP (lower trace). In this case the cells were transfected with the plasma membrane targeted FRB fragment fused to CFP (not shown), the cytosolic mRFP-FKBP12-5-phosphatase domain and the PLC δ 1PH-GFP constructs. The phosphatase is less active against the membrane PtdIns(4,5)P₂ when in the cytosol but becomes active after the addition of rapamycin that causes the dimerization of FRB and FKBP and hence recruits the phosphatase to the membrane. See (46,47) for more details on this method.

Figure 4.

The effect of PLC δ 1PH-mRFP expression on cytosolic Ca^{2+} increases evoked by stimulation of endogenous P2Y purinergic receptors by ATP. Cells were transfected either with PLC δ 1PH-mRFP or p130PH-mRFP, another PH domain with similar Ins(1,4,5) P_3 binding affinity but no binding to $\text{PtdIns}(4,5)\text{P}_2$ in the cells. The Ca^{2+} concentration was monitored by Fura-2, and ATP (50 μM) was added at the indicated time. The calcium responses of untransfected cells were compared to those expressing similar amounts of the PH-domain constructs. The expression level was assessed by comparing the red fluorescence intensities of the cells at the end of the experiment, when ionomycin was added to eliminate $\text{PtdIns}(4,5)\text{P}_2$ making the PLC δ 1PH domain also cytoplasmic. Note that the averaged Ca^{2+} response is inhibited by both the PLC δ 1PH (red) and the p130PH (blue) domains compared to untransfected controls (black). The very strong InsP_3 buffering effect of p130PH causes an almost complete elimination of the synchronized Ca^{2+} signal, whereas the effect of PLC δ 1PH is significantly smaller causing only a slight delay and decrease in the amplitude of the Ca^{2+} rise. Clearly, the effect of the PLC δ 1PH domain is not caused primarily by InsP_3 buffering.

Table I
Phosphoinositide binding modules in use for imaging purposes in live cells.

Lipid	Protein domain	Refs for in vitro	Live cell localization	Reference
PtdIns(4,5)P₂	PLCδ ₁ -PH	(48)	plasma membrane	(16,17)
	Tubby domain	(49)	plasma membrane	(49)
			+ cleavage furrow	(50)
PtdIns(3,4,5)P₃	GRP1-PH	(51,52)	plasma membrane	(53,54)
	ARNO-PH	(53)	plasma membrane	(55)
	Cytohesin-1-PH	(53)	plasma membrane	(56,57)
	Btk-PH	(51,58)	plasma membrane	(59)
PtdIns(3,4,5)P₃/PtdIns(3,4)P₂	Akt-PH	(60)	plasma membrane	(61,62)
	PDK1	(25)	plasma membrane	(25)
	CRAC	(63)	<i>Dictyostelium</i>	
			plasma membrane	(64)
PtdIns(3,4)P₂	TAPP1-PH	(22)	plasma membrane	(65)
PtdIns(3,5)P₂	Ent3p-ENTH [*]	(66)	yeast pre-vacuole	(66)
	Svp1p [*]	(67)	yeast vacuole	(67)
PtdIns3P	FYVE (Hrs, EEA1)	(18,68)	early endosome	(19)
	(Vps27)		yeast vacuole	(18)
	P40phox-PX	(69,70)	early endosome	(70)
PtdIns4P	OSH2-2xPH ^{**}	(26)	plasma membrane	(26,32,33)
	OSBP-PH	(22,71)	Golgi	(44,71)
			+ plasma membrane	(35)
	FAPP1-PH	(22)	Golgi	(44,72)
			+ plasma membrane	(35)
PtdIns5P	3xPHD (ING2)	(73)	nucleus ?	
			plasma membrane	(73,74)

* The usefulness of these domains for imaging purposes is questionable (see 75).

** The OSH2-PH shows little discrimination between PtdIns4P and PtdIns(4,5)P₂ based on *in vitro* binding (26) and it is still not certain whether it actually reports on both of these molecules in some proportions.