Visualizing Cellular Phosphoinositide Pools with GFP-Fused Protein-Modules

Tamas Balla[1]* and Péter Várnai[2]

(Published 26 March 2002)

[1]Unit of Molecular Signal Transduction, Endocrinology and Reproduction Research Branch, National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, MD 20892, USA. [2]Department of Physiology, Semmelweis University Medical School, Budapest, Hungary.

*Corresponding author. National Institutes of Health, Building 49, Room 6A35, 49 Convent Drive, Bethesda, MD 20892-4510, USA. Telephone, 301-496-2136; fax, 301-480-8010; e-mail, tambal@box-t.nih.gov

Abstract

Inositol phospholipids are well known for their pivotal role in calcium signaling as precursors of important second messengers generated in response to various stimuli. However, over the last 10 years, inositides have also emerged as universal signaling components present in virtually every membrane of eukaryotic cells. These lipids are locally produced and degraded by the numerous inositide kinase and phosphatase enzymes, to control the recruitment and activity of protein signaling complexes in specific membrane compartments. The spatial and temporal constraints imposed on changes in cellular inositides pose new challenges in finding experimental techniques through which such changes can be examined. Taking advantage of the protein domains selected by evolution to recognize cellular phosphoinositides, we have created fluorescent molecules by fusing these domains to the improved version of enhanced green fluorescent protein (EGFP); the distribution of these fusion proteins can be followed within live cells, thereby reporting on changes in phosphoinositides. Although this technique is one of the few that provide information on phosphoinositide dynamics in live cells with subcellular resolution and has rapidly gained popularity, it also has limitations that need to be taken into account when interpreting the data. Here, we summarize our experience in designing and using these constructs and review our position concerning the interpretation of the data obtained by this technique.

Introduction

Our desire to follow inositide changes at the single-cell level stems from the difficulty detecting the small and often rapid and transient changes in inositide levels that occur in subcellular membrane compartments during activation of any of the numerous inositide kinase enzymes. Labeling cells with myo-[^{3}H]inositol has been widely used to measure phosphoinositide changes [for example, see (*1*)], but this technique has several limitations. First, millions of cells must be labeled in order to obtain a sufficient signal and, depending on the labeling time and the metabolic turnover rate of the apparently numerous metabolically distinct inositide pools, it is not certain whether isotopic equilibrium is reached during the labeling period. Determination of the subcellular distribution of the inositides requires even more cells and lengthy

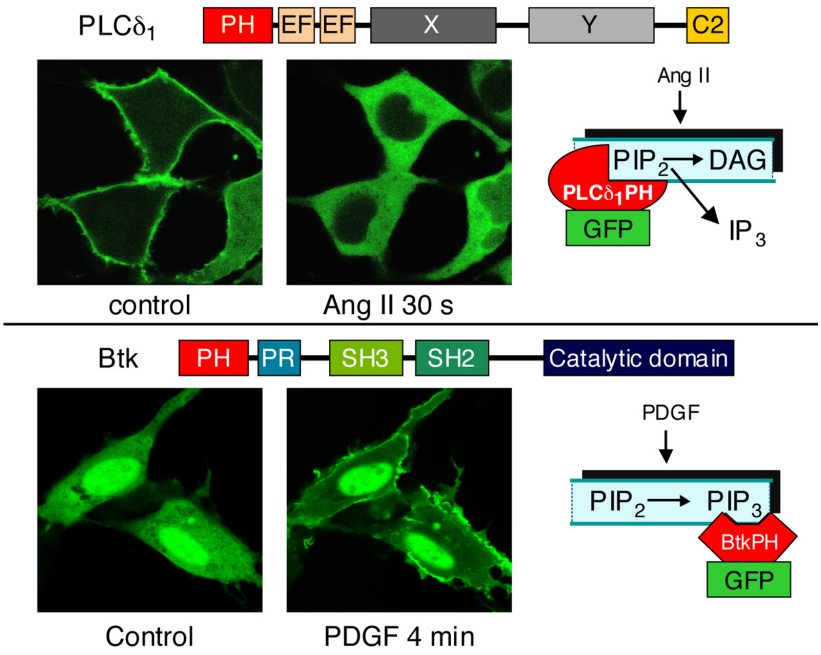

Fig. 1. The principle of visualization of phosphoinositides. The lipid-binding domain of proteins that contain a sequence motif with high enough affinity and specificity to recognize individual phosphoinositide isomers are used to create a GFP fusion protein. When the fusion protein is expressed in cells, it will follow the distribution and dynamics of the particular phosphoinositide. The PH domain of PLCδ_1 recognizes PI4,5P_2, which is most abundant in the plasma membrane and is rapidly hydrolyzed by PLC enzymes after stimulation of G protein-coupled AT_1 angiotensin II (Ang II) receptors. This is reflected in the translocation of the fluorescent probe from the membrane to the cytoplasm. In contrast, the PH domain of Btk recognizes PI3,4,5P_3, a lipid that is not present in the membranes of quiescent cells, but is formed after stimulation of most receptor tyrosine kinases, such as the platelet -derived growth factor (PDGF) receptor. Here, the initially cytoplasmic probe translocates to the plasma membrane after stimulation. DAG, diacylglycerol; Btk, Bruton's tyrosine kinase; IP_3, inositol 1,4,5-triphosphate; PIP_3, phosphatidylinositol 3-kinase; SH2, Src-homology domain 2; SH3, Src-homology domain 3; PH, pleckstrin homology; EF, calcium binding motif; X, conserved catalytic region 1 of phospholipase C enzymes; Y, conserved catalytic region 2 of phospholipase C enzymes; C2, protein kinase C homology region 2, Ca^{2+}-dependent phospholipid binding domain; PR, proline-rich region.

cell fractionation procedures, and the result may not be a reflection of what had been present in the intact cell. To rule out labeling efficiency as a variable, total cellular mass of inositides has also been measured, often in combination with quantitation of the inositide head group that is liberated from the extracted lipid species (*2*). These methods are usually very cumbersome and are also unable to resolve the small changes that occur in subcellular compartments, especially against the higher background of the unchanging inositide pools.

To measure phosphoinositide changes in single cells, we explored the possibility that protein modules with high enough affinity and specificity to bind the inositide head group of specific phosphoinositides could find these lipids within the cell. By fusing the lipid-binding protein module to a green fluorescent protein (GFP), the changes in inositol lipids can be visualized within a single cell (*3*) (Fig. 1). The initial success of these efforts has been followed by numerous requests for use of these plasmids and a wealth of questions and helpful information from their users. The following is a summary of what we hope is a useful collection of knowledge for those who may not have all the background and expertise they need to use this approach in their experimental systems. This protocol is divided into sections that reflect all the steps involved in this method, but we provide detailed procedures in only those sections covering specialized or unusual techniques. We assumed that the reader is familiar with common laboratory practices or those specific to individual needs. We would like to emphasize that these comments are intended for less experienced users and not for the experts of this research field.

Creation of the Fusion Proteins

Protein domains for detecting inositol lipids

Four years ago, the only protein domains that seemed to be useful for creating inositide-binding probes were the pleckstrin homology (PH) domains (*4*). Today the number of protein domains known to bind inositides is larger and constantly increasing. FYVE domains (based on the first letters of four proteins containing this motif) (*5*) and PX-domains (described first in NADPH oxidase subunits) (*6*) bind phosphatidylinositol 3-phosphate (PI3P) and perhaps other 3-phosphorylated inositides (*7*-*9*). The all-helix phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$)-binding domain of the AP180 adaptor protein is a recent addition to the inositide-binding domains for which structure information is available (*10*). Because of the extensive characterization of the in vitro inositide-binding specificity of a series of PH domains (*11*), and the information available regarding their use for imaging purposes, PH domains are still the favorite choice for creating GFP fusion proteins to monitor changes in inositide levels (Fig. 2). Several PH (and other) domains that have been used to detect various inositide species in live cells are listed in Table 1.

The protein domains shown in Table 1 are either available or can be easily duplicated. The literature describing their behavior can be used as a reference. Further, the increasing number of domains that bind inositides offers the potential to develop new fusion proteins useful for following changes in a wide variety of intracellular lipids. However, just because a domain recognizes inositides with certain specificities in vitro does not necessarily mean that a GFP fusion protein with that domain will show the cellular distribution of the same lipid when expressed in cells. Within a cell, the probe's

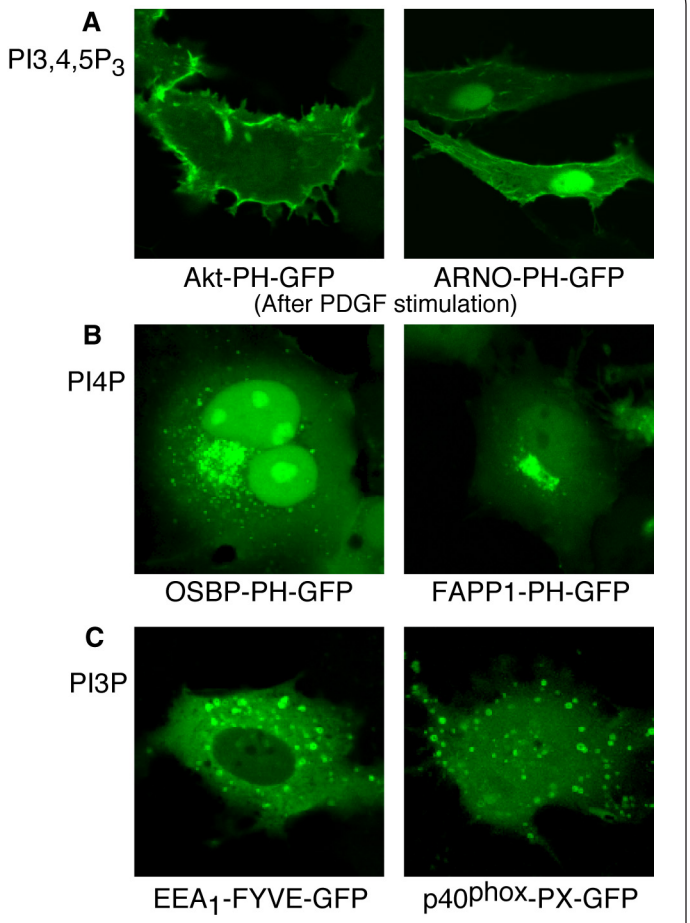

Fig. 2. Cellular localization of various inositide-binding domains fused to GFP. (**A**) The PH domains of both Akt and ARNO bind to $PI3,4,5P_3$ in vitro, and AktPH recognizes $PI3,4,5P_3$ and $PI3,4P_2$. These domains localize to the plasma membrane in cells stimulated with PDGF or other growth factors that activate PI3K. (**B**) The PH domains of oxysterol binding protein (OSBP) and phosphatidylinositol-4-phosphate adaptor protein-1 (FAPP1) recognize PI4P in in vitro binding assays. These domains primarily localize to the Golgi and to some other, yet unidentified, intracellular vesicular structures. (**C**) The PX domain of $p40^{phox}$ and the FYVE domain of EEA1, which both recognize PI3P in vitro, localize to intracellular vesicular structures corresponding to early endosomes and perhaps of other vesicular pools of the endocytic pathway. Although lipid binding is necessary for the localization of these proteins to these cellular structures, it is not certain that their localization is solely determined by their binding to the lipids.

recognition of and affinity to a particular lipid is affected by other membrane components and proteins involved with the same signaling complex in which the participation of the inositide is believed to be important. This complexity must be considered when designing a new lipid probe. Although the boundaries of new protein modules can be easily defined from alignments with their relatives already present in the Pfam conserved structure database [(*12*) (http://pfam.wustl.edu)], in many cases, the minimal domain sequence may not be sufficient for localization in cells despite their binding to lipids in vitro. Several constructs may need to be tested before making conclusions about their lipid recognition within the cell, and the potential complexity of their interactions with other players in the signaling cascade should be considered.

GFP fusion protein	Mutant	Lipid	Citation
PLCδ_1-PH-GFP	R40L	PI4,5P_2	(*23,13*)
Btk-PH-GFP	R28C	PI3,4,5P_3	(*24*)
GRP1-PH-GFP	R284C	PI3,4,5P_3	(*25*)
ARNO-PH-GFP	R279C	PI3,4,5P_3	(*26*)
Akt-PH-GFP	R25C	PI3,4,5P_3 and/or PI3,4P_2	(*27,28*)
OSBP-PH-GFP	R108E	PI4P	(*29*)
EEA1-2x-FYVE-GFP	R1375L	P3P	(*16*)
p40^{phox}PX-GFP	R58L	PI3P	(*30,31*)

Table 1. The PH domains that have been used to create fusion proteins for tracking changes in the concentrations of cellular phosphoinositides.

It is not yet clear what fraction of the inositide pools are visualized by even the well-documented constructs. This aspect of inositide recognition by the domains is still being studied, and it is quite possible that each of these modules only recognizes the lipid in a very specific molecular context and, therefore, does not image all of the target inositides in every cellular compartment (*3*). Therefore, it is imperative that the data be interpreted as an indication of inositide changes as "seen" only by the specific domain used.

For experimental controls, we recommend proteins mutated so that they do not bind lipids (Table 1). These controls help determine whether the observed localizations reflect lipid binding or interaction with other protein molecules. The mutant proteins also serve as controls for determining adequate expression levels and for monitoring toxicity and morphological changes induced by the lipid-binding protein.

Choosing a GFP-encoding plasmid

To create a fusion protein with enhanced GFP (EGFP), we usually subclone the lipid-binding domain in either the pEGFP-N1 or the pEGFP-C1 plasmids that differ only in the position of the multiple cloning site relative to the EGFP sequence. We usually place the PH domain relative to EGFP to reflect the domains' natural location within the parent molecule (that is, when the PH domain is in the NH_2 terminus of the molecule, we place it before EGFP in the pEGFP-N1 plasmid). However, our experience is that PH domains often work in both locations. Unless a specialized application is sought, such as fluorescence resonance energy transfer (FRET) or double labeling, EGFP is the best choice for simple fluorescence microscopy or confocal imaging. In addition to the original EGFP, spectrally shifted versions (EYFP and ECFP) are now available from Clontech oriented to make either an NH_2-terminal or a COOH-terminal fusion protein. Whereas ECFP-fused constructs show a significantly lower signal with the conventional 488-nm laser and filter sets optimized for EGFP work, the EYFP versions are effective in most standard applications.

Double labeling with two fusion proteins can be very useful, but it is not possible with the ECFP and EYFP pairs using a basic confocal microscope without additional laser lines and special filter sets. Although the spectral characteristics of another fluorescent protein, dsRed2, would make it a good partner to EGFP, it can form tetramers and seems to be less inert than EGFP.

The one limitation of the Clontech pEGFP plasmids designed for mammalian expression is their lack of a T7 or T3 promoter for making complementary RNA (cRNA) for microinjection studies in oocytes. This obstacle can be overcome either by subcloning the entire GFP construct into another suitable plasmid or by preparing recombinant proteins for microinjection.

A few additional issues must be considered when designing the completed DNA construct. A consensus translation start sequence with a Kozak sequence should be inserted 5′ to the lipid-binding domain when in the NH_2-terminal orientation (when us-

ing 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 one of the vector's stop codons is reached. All oligonucleotide amplification steps should be performed with a high-fidelity DNA polymerase, such as Pfu.

Expression of the Fusion Proteins

Once it is decided what lipids are to be followed and what construct is to be used, the GFP fusion protein has to be introduced to the cell. The easiest method for delivery of the GFP-fusion protein into the cells is by transfection with a mammalian expression plasmid encoding the desired fusion protein. Although we describe the basic process for expressing fusion proteins by standard transfection techniques, we refer the user to the manufacturer's instructions for details about specific transfection methods. Alternative methods for delivering the construct are microinjection of cRNA or of bacterially expressed and purified fusion proteins.

Materials

Bovine serum albumin (BSA)

Buffers for cell incubations [Phosphate-buffered saline (PBS) or Modified Krebs-Ringer Medium (Recipe 2)]

$CaCl_2$

Cell culture dishes, plastic, 35 mm diameter [Falcon, 35-3001,PG Science (http://www.pgcscientifics.com) # 81-6570-01]

Coverslips, 25 mm [PG Science (http://www.pgcscientifics.com) no. 60-4884-25]

Clear nail polish

Cultured cells of individual choice

Ethanol

Fetal bovine serum (FBS)

Fluorescence mounting medium [Aqua Poly/Mount, PolySciences Inc. (http://www.polysciences.de)]

Glucose

Hepes, sodium salt (Na-Hepes)

KCl

$MgSO_4$

Na_2HPO_4

Paraformaldehyde, electron microscopy (EM) grade

Poly-lysine (Sigma-Aldrich, #P-8920)

Plasmid DNA [EGFP-N1 or EGFP-C1, Clontech (http://www.clontech.com)]

Saponin

Transfection reagents (individual choice depending on the cells in use)

Equipment

Heated stage (optional, but required for live-cell imaging) [Harvard Apparatus (http://www.harvardapparatus.com)]

Laser confocal microscope (or fluorescence microscope)

Metal chambers to hold 25-mm coverslips [Molecular Probes (http://www.molecularprobes.com), #A-7816]

Microinjection apparatus (optional)

Objective heater (optional, but recommended for live-cell imaging) [Bioptechs (http://www.bioptechs.com)]

Perfusion system (optional)

Phosphorimager with a blue laser line [STORM, Molecular Dynamics (http://www.mdyn.com)]

Recipes

Recipe 1: Poly-lysine

Prepare a 1:50 or 1:100 dilution of poly-lysine in sterile deionized water.

Note: Two concentrations are described because some cells require the higher concentrations, but others do not tolerate it well. Cells should be plated using coverslips coated at each concentration to see which works best.

Recipe 2: Modified Krebs-Ringer Medium

NaCl	120 mM
KCl	3.7 mM
Na_2HPO_4	1.2 mM
$CaCl_2$	1.2 mM
$MgSO_4$	0.7 mM
Glucose	10 mM
Na-Hepes	20 mM
BSA	0.1%

Prepare in distilled water and adjust the pH to 7.4 with HCl. The solution can be stored at 4°C for 4 to 6 weeks after sterile-filtration.

Note: Do not include BSA when applying reagents that bind to BSA, such as ionomycin or wortmannin.

Recipe 3: 2% Paraformaldehyde

Dissolve EM grade paraformaldehyde in PBS at a concentration of 2% (w/v).

Heat in a chemical hood to 60°C until fully dissolved; leave loosely covered to allow gas to escape.

Allow the solution to cool to 20°C and adjust the pH to 7.4 with NaOH.

Note: This solution should be prepared fresh each time.

Recipe 4: Blocking Solution

Prepare a 10% solution of FBS in PBS.

Note: This solution should be prepared fresh each time.

Recipe 5: Antibody Diluent

FBS	10%
Saponin	0.2%

Prepare in PBS.

Note: This solution should be prepared fresh each time.

Instructions

The following procedures describe general methods for preparing to analyze cells expressing GFP or GFP-fusion proteins by microscopic techniques.

Preparation of Poly-lysine-Coated Cover Slips

Because most plasticware used for cell culture work has high autofluorescence, we recommend that the cells be cultured and transfected on poly-lysine-coated glass cover slips so that they can be viewed in the microscope after transfection without replating. For certain cell types and transfections, it is more advantageous to transfect the cells in culture dishes and re-plate the transfected cells on the poly-lysine-coated coverslips before microscopy.

1. Rinse 25-mm coverslips with 98% ethanol in the cell culture hood and air dry.
2. Place one coverslip in each 30-mm diameter culture dish.
3. Add 1 ml of 1:50 or 1:100 dilution of Poly-lysine (Recipe 1) to cover the entire surface and allow to stand at room temperature for 1 hour.

   Note: Plate cells on coverslips coated at each concentration to determine which is best for the cells being studied.
4. Gently aspirate the solution from the coverslip and air dry before plating cells.

Transfecting Cells

Transfection protocols are available for the different reagents, and for each cell type the reagent and the procedure that gives the best result can differ considerably. Therefore, we refer the user to the manufacturer's instructions as far as the specifics for cell transfection are concerned. The optimal level of expression has to be determined for each cell type and expression construct. It is important to remember that the expressed proteins often interfere with the functions of the lipids that they recognize, and in high concentrations the lipid-binding fusion proteins are often toxic to the cells.

As an important control, we recommend that parallel cultures be transfected with a mutant version of the lipid-binding domain that does not bind lipids. For example, many constructs localize to the nucleus, but this localization is not dependent on lipid binding; thus, lipid-mediated localization can be confirmed by comparing the distribution of the native lipid-binding domain to that of the nonlipid binding mutant. We also recommend transfecting 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 to serve as controls for monitoring localization that accurately reflects lipid binding.

1. Plate ~50,000 cells directly onto the poly-lysine-coated coverslips in 2 ml of the appropriate culture medium and grow to the density best suited for transfection (usually 2 days).
2. Transfect the cells with the desired plasmid DNA construct using a method that is most appropriate for the cells.
3. Grow the cells for 24 hours to allow expression of the transfected protein.
4. Incubate cells in serum-free culture medium for 6 to 10 hours to render them quiescent before microscopy.

   Note: We do not recommend growing the cells longer than 34 hours total after transfection, because of the potential toxicity of the lipid-binding fusion protein and the ability of the expressed protein to interfere with the functions of the lipids they bind.

Confirming the Integrity of the Expressed Protein

In addition to sequencing the DNA construct before performing any transfections, it is important to determine whether the fusion protein is intact once expressed in mammalian cells. Often, the fusion protein is cleaved within the cell so that the green fluorescence is not coming from the molecule that was designed. Our experience is that free EGFP is more often present (probably as a cleavage product) when using the pEGFP-N1 plasmids than with the COOH-variant (pEGFP-C1), but in one case we found that a protein expressed in COS-7 cells was cut in half when placed COOH-terminal to EGFP. We recommend analyzing cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) either using a phosphorimager equipped with a blue laser line (described below) or by conventional Western blotting techniques using antibodies against the GFP 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. The electrophoretic mobility of the EGFP molecule is clearly different when ex-

pressed from pEGFP-C1 versus pEGFP-N1. This effect may be due to altered migration of the nondenatured protein by the extra amino acids encoded by the multiple cloning site of the pEGFP-C1 plasmid.

1. Transfect COS-7 cells and incubate for 24 hours in wells of a 24-well cell culture plate.

   Note: A sample from one well of a 24-well plate grown to 80% confluency gives sufficient signal to analyze.

2. Wash the cells in 2 ml of PBS and aspirate the PBS.
3. Dissolve the cells in 100 µl of SDS-PAGE loading Laemmli buffer. Do not boil, but sonicate to disrupt the DNA.
4. Load 40 µl of the sample per lane and electrophorese through a small (10 cm) SDS-PAGE gel at an acrylamide concentration that will resolve proteins in the size range of interest.
5. Visualize the fusion protein in the gel using a phosphorimager that has a blue laser line .

   Note: Do not boil the cell sample before loading on the gel if visualizing the protein with a phosphorimager.

Immunostaining of Cells Expressing GFP Fusion Proteins

It may be necessary to confirm expression of the fusion protein, especially if the GFP signal is not bright enough. This confirmation can be accomplished by immunocytochemistry with antibodies to the GFP portion of the fusion protein. Colocalization of the GFP-fused domain with other molecular markers may also require immunostaining.

1. Rinse the transfected cells with 2 ml of PBS.
2. Add 2 ml of 2% Paraformaldehyde (Recipe 3) and incubate for 10 min at room temperature.
3. Wash the cells three times with 2 ml of PBS for 10 min each wash.
4. Wash the cells once with 2 ml of Blocking Solution (Recipe 4) to block nonspecific antibody binding. Incubate for 10 min at room temperature.
5. Add the primary antibody diluted appropriately in Antibody Diluent (Recipe 5). Incubate for 1 hour at room temperature.

   Note: 100 µl of diluted antibody should be sufficient for a 25-mm circular or 22 mm × 22 mm square coverslip, if it is inverted on a glass slide and incubated in a humidified petri dish.

6. Wash the cells three times with 2 ml of Blocking Solution (Recipe 4) for 5 min each wash.
7. Add the fluorescent secondary antibody diluted in Antibody Diluent (Recipe 5). Incubate for 1 hour at room temperature protected from light.
8. Wash the cells three times with 2 ml of PBS.
9. Air dry until the coverslips are only damp.
10. Mount the coverslips with the cells down on a glass slide using Aqua Poly/Mount.
11. Seal the coverslips on the slide with clear nail polish to prevent drying.

Observing the GFP Signal by Microscopy

Once the plasmids are completed and the fusion protein is found to be intact, transfected cells can be observed under the microscope. The first decision to be made is whether to analyze the cells live or fixed. Both choices have pros and cons. EGFP fluorescence can persist in fixed cells under proper fixation conditions described below. Fixed cells can be processed for immunostaining, which is often necessary to determine colocalization 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 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, if one can tolerate having the bulky and difficult-to-clean objective in the culture medium.

The second choice that needs to be made is whether to view the cells with a conventional fluorescence microscope or with a confocal microscope. The distribution of fluorescence and other initial experiments can be performed with a conventional fluores-

cence microscope using filters suitable for fluorescein isothiocyanate (FITC) detection (excitation at 470 to 490 nm, emission at 500 to 550 nm). It is not necessary to examine the cells immediately with a confocal microscope. In fact, it is more difficult to get a general impression of how cells expressing the construct look in a confocal microscope than by looking at them with 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. One of the greatest difficulties is recognizing patterns caused by the expressed proteins that are not obvious given the morphological variability of the cells. 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 not much 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 green color of GFP. It is important to remember that confocal microscopes record light intensity only, not colors; the "color" given is artificial. Therefore, in each case, the autofluorescence needs to be determined so that the GFP signal can be reliably used. For this, observation of a set of untransfected cells is a very useful control.

When starting to collect confocal image data, our experience is that people save hundreds of pictures, only to discover later that few of the saved data are good enough to be used. It is very useful to document the various "phenotypes" of cells that may be seen, as a function of expression levels. Often, this can be done using conventional microscopy. We suggest that only pictures of high quality be saved. Also, it is not necessary to record images of a series of z-sections through the cell, unless three-dimensional reconstruction is critical to demonstrate the important observations. When analyzing live cells, one major advantage of confocal microscopy is its ability to record time-lapsed images sequentially after a stimulus is applied to the cells. In confocal

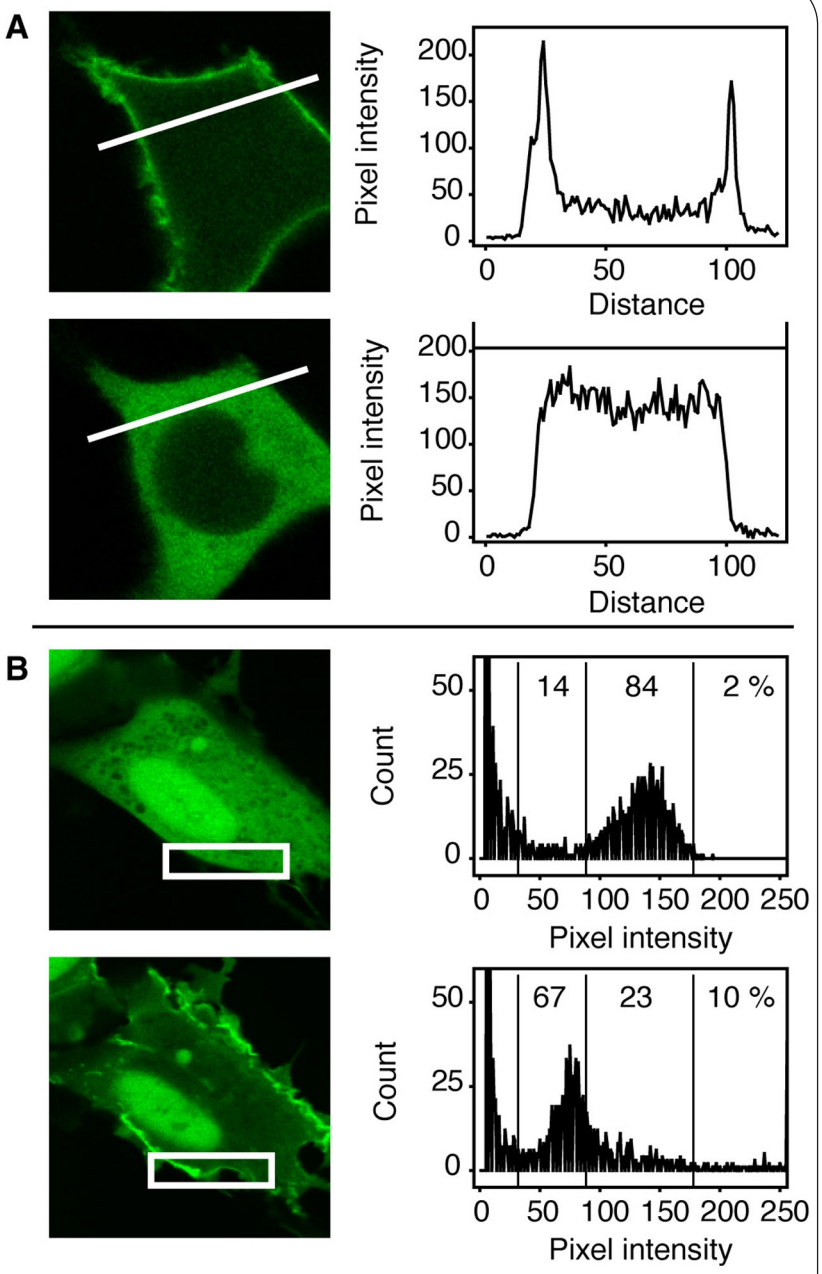

Fig. 3. Quantitation of changing fluorescence distribution in cells expressing PH domain-GFP fusion proteins. (**A**) Line intensity histograms clearly demonstrate the localization of PLCδ_1PH-GFP in the plasma membrane in quiescent cells and its translocation to the cytosol after stimulation by a Ca^{2+}-mobilizing hormone. However, the line intensity histogram represents one arbitrarily chosen cross section of the image. (**B**) Pixel intensity histograms analyze a larger area of the cell and plot the distribution of pixels as a function of their intensities. The larger percentage of high-intensity pixels (reflecting the high intensity in the membrane ruffles), together with the decrease in the medium-intensity ones (reflecting the drop in the cytosolic fluorescence), are an indication of translocation of the probe from the cytosol to the membrane in stimulated cells.

microscopes, the speed of scanning determines how fast one can record an image, but in general, the faster the scan, the poorer the quality of the individual pictures.

Finally, one of the greatest difficulties is keeping the cell in focus after stimulation, because of shape changes that often occur in response to the stimulus (HEK 293 cells are especially lively). This change in position of the originally imaged plane can make the whole recording unsuitable. We are aware of software programs being developed to compensate for "focal-drift" due to cell movements. These new developments may be available for some confocal imaging systems soon.

The most demanding part of the analysis of time-lapsed sequences is the quantification of data. When the fluorescence redistribution is obvious, it is sufficient to document this information qualitatively as a series of pictures or movies that describe what is happening. However, when determining a dose-response relationship, 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 is to create a line-intensity histogram through a selected line spanning the image. The highest intensities should not saturate, which requires a fine optimization of the dynamic range before recording in 8-bit systems that only have 256 levels of intensity (Fig. 3). Saturation is less of a problem with the 12-bit systems that have 4096 levels of intensity. An alternative method of quantifying the results is to generate a pixel-intensity histogram over the entire area, or only a selected area, of the cells (Fig. 3). Redistribution of fluorescence is then reflected in the change of the distribution of pixel intensities. 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 largest obstacles to obtaining reliable, reproducible results.

Fixing EGFP-expressing cells

Note: This is the same fixation conditions that are used to prepare samples for immunofluorescent staining.

1. Rinse the transfected cells with 2 ml of PBS.
2. Add 2 ml of 2% paraformaldehyde (Recipe 3) and incubate for 10 min at room temperature.
3. Wash the cells 3 times with 2 ml of PBS for 10 min each wash.
4. Air dry until the coverslips are only damp.
5. Mount the coverslips with the cells down on a glass slide using fluorescence mounting medium.
6. Seal the coverslips on the slide with clear nail polish to prevent drying.
7. View the cells in the microscope or store at –20°C.

Choosing a cell

Looking into the fluorescence microscope, usually one can see cells with a wide range of fluorescence intensities. 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. As a general rule, it is best to study cells in which the GFP signal is as low as possible but is still clearly distinguishable from the autofluorescence of untransfected control cells. It is crucial to establish the proper signal-to-noise ratio. Unfortunately, there is no general recipe to follow, because the sensitivities of confocal microscopes or of the cameras attached to conventional fluorescence microscopes vary from one manufacturer to another. However, we can recommend the following steps.

1. Observe the transfected cells and, if necessary, the control nontransfected cells using the fluorescence setting of the confocal microscope fitted with filters suitable for FITC analysis (470- to 490-nm excitation and 500- to 550-nm emission).

   Note: Often, a separate nontransfected cell control is not necessary, because even in the transfected samples, not all cells express the fusion proteins. The nontransfected cells in the population can usually be distinguished by their autofluorescence, allowing easy identification of transfected and nontransfected cells from the same sample when viewed using the fluorescent setting.

2. Choose cells in which expression levels are just high enough to be resolved above the background autofluorescence, and that are not obviously unhealthy.

   Note: Analysis of cells that are unhealthy and suffering from toxicity induced by the expression of the fusion protein should be avoided. For example, cells that round up and are about to detach [a common phenomenon with cells expressing very high levels of a fusion protein consisting of the PH domain of phospholipase (PLC) and GFP (PLCδ_1PH-GFP) (13)] are not likely to behave normally. Another indication of toxicity in transfected cells is the appearance of large intracellular vesicles.

3. Record an image of the cell by confocal microscopy using 3% to 5% of the maximum laser power (to avoid photobleaching and damaging the cells) with a scan speed of about 1 s.
4. Save only images of high resolution and quality.

Note: It is not necessary to record images of a series of z-sections through the cell, unless 3D-reconstruction is critical to demonstrate the important observations.

Time-lapse analysis of live cells

An important and often unappreciated problem in live cell imaging is the proper temperature of the cells that are being observed. In an inverted microscope, even when the medium in the observation chamber is kept at the desired temperature by a heated stage, the objective acts as a heat sink, keeping the cells in the observation field at a temperature near that of the objective. Because lasers require proper cooling and, therefore, the rooms are usually kept cool, this means that even on a heated stage the recorded cells are examined at room temperature, which significantly slows many processes. Before we learned how serious this problem was, we had attributed to mere bad luck the fact that very often the only cell that showed no change after stimulation was the one we had recorded, while all others in the surroundings showed a very nice response. The best solution that we can recommend is to use an objective heater available from Bioptechs (http://www.bioptechs.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 or using a hair dryer to keep the objective at the proper temperature during live cell imaging.

1. Transfer and secure the coverslips with the cells in the metal chamber and add 1 ml of prewarmed (37°C) Modified Krebs-Ringer Solution (Recipe 2). Ideally, include cells transfected with lipid-binding fusion protein construct, nontransfected control cells, and cells transfected with a control construct that does not bind lipids.
2. Observe the cells with a confocal microscope fitted with a lens-heater collar and a heated stage, as described above. Record a set of images of the unstimulated cells.
3. Set the software to record time-lapsed images every 5 s to 20 s (depending on the speed of the response), and at a scanning speed of 1.5 s to 2.5 s, to capture the cellular response with proper image resolution.
4. Start data acquisition and add stimuli or inhibitors after the first few images have been taken. Stimuli that activate phosphatidylinositol 3-kinases (PI3Ks) or PLC should evoke redistribution of probes as their lipid-binding partners change because of the enzyme's action.
5. Quantify the results off line (that is, after completing an experiment) with either a line-intensity histogram through a selected line spanning the image or with a pixel-intensity histogram over the entire area, or only a selected area, of the cells.

Troubleshooting

No Bacterial Colonies

The pEGFP plasmids have the kanamycin selection marker, so bacteria replicating the plasmids must be grown on kanamycin-containing plates and not ampicillin-containing plates. This should be the first thought when no colonies are found after transformation of bacteria during the subcloning to create the transfection plasmid. Problems with subcloning, ligation reaction, or bacterial transformation can also result in no bacterial gowth, but discussion of these variables is beyond the scope of this article.

No Green Fluorescence

However trivial it may sound, the most common reason for not seeing fluorescence is inappropriate microscope settings. Confocal microscopes used for fluorescence imaging are fairly complicated and sometimes intimidating instruments for novices, and there are several filters and light directions that must be set properly to see the fluorescent signal. Once the cells are found with transmitted light, one has to see the blue light coming through the objective to make sure that the illumination and the light pass is properly set. It is very useful to have a slide of fixed GFP-expressing cells to use as a control.

If the microscope settings are right and there are still no GFP positive cells (yet the autofluorescence is visible with a 40× or higher objective), then the problem is with the transfection. Transfection problems can be caused by several factors, including inaccurate subcloning, impure plasmid for transfection, and inappropriate transfection conditions for the cells. Even transfected primary cultured cells should have a few positive cells. To determine whether the transfection procedure or the DNA construct is the source of the problem, cells transfected with the original pEGFP plasmid should be used as transfection control. If these cells are positive for GFP fluorescence, then the most likely source of the problem is the fusion protein construct. The fusion protein-encoding DNA construct could be defective by harboring a stop codon or a frame-shift either because of wrong design or by mutation. The fusion protein-encoding plasmids should be confirmed by DNA sequence analysis, and the expression of the full-length protein should be confirmed by SDS-PAGE, as described above.

Weak Fluorescence

If there are positive cells but their fluorescence is weak, the microscope may not be set properly. Cells transfected with EGFP alone can serve as a control for this. If these EGFP-expressing cells are bright, but the fusion protein-expressing cells are weakly fluorescent, then there may be problems inherent to the fusion protein. Our experience is that the larger the protein fused to GFP, the weaker its fluorescence may be. This could be related to difficulty in the folding of GFP as part of a larger protein. With the protein constructs listed (Table 1), low levels of fluorescence are usually caused by transfection problems, which are revealed by few cells showing fluorescence rather than by many cells showing weak fluorescence.

Mislocalization of the Protein Construct

Appearance of large "aggregates" in the cytoplasm

The localization of the described lipid-binding domains (Table 1) to specific inositides in various cellular compartments is well documented. Here, we discuss localization issues that are not related to interactions with phospholipids and are considered to be technical problems rather than new scientific "discoveries." High concentrations of the fusion protein are usually responsible for these problems. Some constructs may have folding problems, which can result in the formation of large fluorescent "aggregates" that are stuck in various intracellular membranes, mostly in the Golgi. We experienced such problems with the EBFP-fused PLCδ_1PH and with some chimeric constructs, such as one constructed from the PH domains of PLCδ_1 and p130, which appeared to have folding problems. A fraction of the expressed protein still appeared to fold properly and function as expected (Fig. 4). It is important to recognize this aggregation artifact and not to mistake it for true localization. One can try to reduce this problem by expressing less of the fusion protein and by lowering the temperature by 5° to 7°C during transfection and culture. However, we did not have great success with these manipulations once the construct showed this aggregation behavior.

No localization is observed on the membrane where the lipid is expected to be

If GFP fluorescence is observed, but is not associated with the membrane, the first test should be to confirm that the expression construct contains the sequence for the lipid-binding domain. Preparations of pEGFP plasmid DNAs often contain a ~500-bp fragment that is not the result of digestion by restriction enzymes. It is usually faint but can be mistaken for an insert when confirming the DNA construct by restriction enzyme digestion analysis. This is especially problematic because many PH domains are encoded by DNA sequences of ~500 bp.

An alternative explanation may be poor signal-to-noise ratio resulting from the low concentration of the lipid produced. The amount of lipid that is produced and is available for binding by a fluorescent probe is limited. Therefore, in a cell that expresses a high concentration of the fusion protein, the membrane-bound fraction may not be distinguishable from the high cytosolic intensity of unlocalized fusion protein. This is another reason to study cells that express low concentrations of the fusion protein.

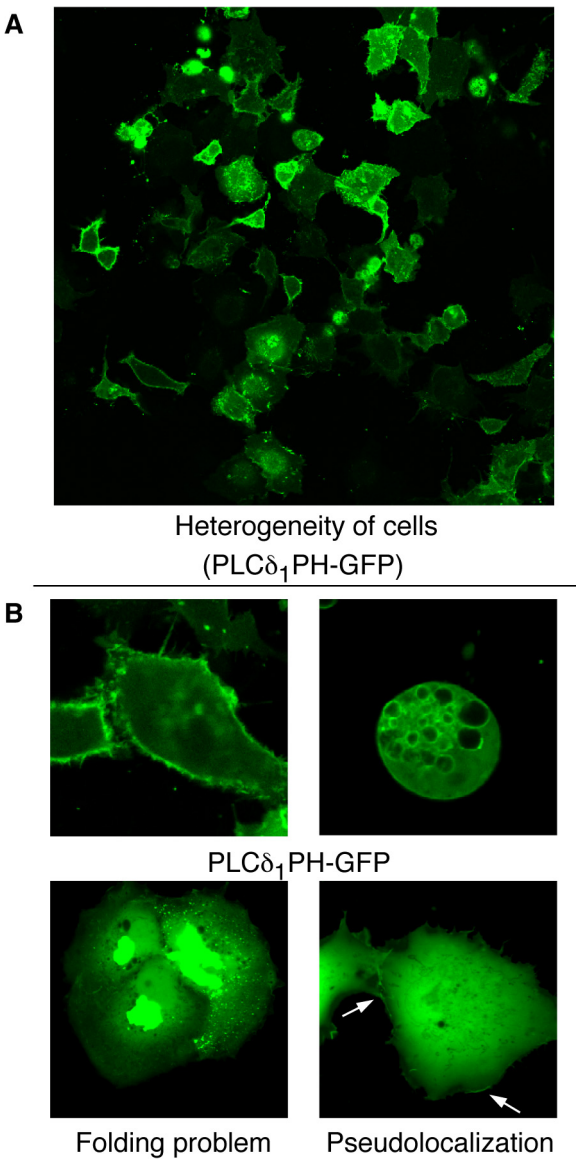

Fig. 4. Heterogeneity of cells expressing PLCδ_1PH-GFP, and artifacts in the localization of fluorescent protein domains. (**A**) The heterogeneous morphology of COS-7 cells expressing the PLCδ_1PH-GFP construct. Cells expressing high concentrations of the protein detach, become round, and develop multiple intracellular vesicles. These cells are usually above the focal plane of the flat cells that are still attached. (**B**) A cell expressing a GFP fusion protein that displays a folding problem. The protein is highly enriched in the Golgi, but also shows some cytoplasmic aggregates. Cells that express only GFP can also show some membrane ruffling that can be mistaken for membrane localization, a phenomenon called pseudolocalization (arrows).

When $PI4,5P_2$ concentrations were monitored with $PLC\delta_1$PH-GFP, we did not observe this sort of saturation, probably because of the compensatory increase in the amount of $PI4,5P_2$ that is produced in cells expressing larger amounts of this lipid-binding protein. Compensatory increases are less likely to occur with lipids that are only formed in response to stimulation, such as phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$).

Clear plasma membrane localization in cells that are very flat, such as COS cells, can also be difficult to see. For example, even in the case of the $PLC\delta_1$PH-GFP, which undoubtedly binds to $PI4,5P_2$ in the plasma membrane, it is not so easy in a very flat cell to recognize that the construct is membrane-bound even in a confocal microscope. Comparing the $PLC\delta_1$PH-GFP-expressing cells with cells expressing only GFP can also help to recognize membrane localization.

A further observation of practical importance is that live cells lose localization of the $PLC\delta_1$PH-GFP (and perhaps of other domains of less robust localization) if they are kept at room temperature for more than 15 min to 20 min. The reason for this has not been explored in detail, but it may be that lipid synthesis is slowed because of either adenosine triphosphate (ATP) depletion or the physicochemical properties of the membrane change. Importantly, warming the cells to 37°C does not correct the situation within 30 min. Thus, live cell imaging should be performed using a temperature-controlled microscope system.

Nuclear localization

All of the domains listed in Table 1 can penetrate the nuclear pore and enter the nucleus, although their movement in and out is clearly limited. More importantly, although many of them (Grp1-PH, ARNO-PH, Btk-PH, OSBP-PH) show very prominent nuclear localization, this localization is also observed with mutants that do not bind the lipids and, therefore, does not reflect lipid binding. It is very likely that the numerous basic residues characteristic of these domains are responsible for their nuclear localization; this is supported by the fact that algorithms based on the primary sequences of these proteins accurately predict their nuclear localization. However, if the protein binds to a lipid that is already present and abundant during expression, such as $PI4,5P_2$, the protein will bind the lipid and will not appear in the nucleus, provided that the amount of the expressed protein does not saturate the lipid available for binding. Thus, choosing cells in which the expression levels are low also can decrease localization to the nucleus.

True localization versus "pseudolocalization"

True localization has to be confirmed by several criteria. The localization of the fusion protein should follow the lipid changes that are evoked by physiological or pharmacological means. For example, PI3K inhibitors, such as wortmannin, prevent the formation of 3-phosphorylated lipid products, so treatment of the transfected cells with these inhibitors should eliminate localization of fluorescent probes that recognize 3-phosphorylated lipids.

Another important control, designed to determine whether localization of the fusion protein reflects true changes in lipid concentration, is a mutant version of the fusion protein in which the lipid-binding domain is disrupted. Cells transfected with these mutant proteins should not localize to the same compartments as the wild-type lipid-binding fusion proteins.

However, even with these criteria satisfied, a phenomenon that we call "pseudolocalization" can occur. In very flat cells that express only GFP, which shows no true localization, the edge of the cell or some membrane ruffles can often appear as a high-intensity area because of the cross sections in the imaged plane. Membrane ruffling is a common response of cells to stimuli that alter inositol phospholipids and their concentrations; it is regulated through PI3K-dependent pathways and can be often reversed with PI3K inhibitors. These effects can give the impression that the construct localizes to membrane ruffles in stimulated cells even when it does not actually bind to $PI3,4,5P_3$. Comparing the fusion protein-expressing cells with control cells expressing only GFP is essential to resolving true versus pseudolocalization. A useful trick is to monitor changes in cytoplasmic intensity. In the case of true localization, cytoplasmic fluorescence decreases when the protein redistributes to the membrane. This decrease never happens with pseudolocalization. The decrease in the cytoplasmic fluorescence can be judged best by observing an increase in contrast between the cytoplasm and the nucleus, which appears brighter as the cytoplasmic fluorescence decreases.

Translocation is caused by "displacement" rather than lipid changes

The $PLC\delta_1$PH and some other PH domains can bind the soluble inositol phosphate counterpart of the inositol lipids with comparable or even higher apparent affinity than the lipid itself. This can complicate interpretations, because the increase in the level of inositol 1,4,5-trisphosphate (IP_3) may be as important in "displacing" the $PLC\delta_1$PH-GFP from the membrane lipid $PI4,5P_2$, as the decrease in the membrane lipid after PLC activation (*14*). Our data suggest that although IP_3 can indeed cause translocation of $PLC\delta_1$PH-GFP when present at very high concentrations, it is probably not a major component of the translocation response under physiological stimulatory conditions; it is the decrease of $PI4,5P_2$ that is more important in the release of the fluorescent protein from the membrane (*15*). Nevertheless, this possibility needs to be considered in the interpretation of the results obtained by these probes.

Related Techniques

Transfection Versus Microinjection

Expression of the lipid-binding domains inevitably interferes with the function of these lipids and can alter several cellular functions. Thus, cells must be studied soon after transfection, and only cells that express the probe at a low level should be used. Theoretically, however, it is even more advantageous to inject purified recombinant proteins into cells, so that they will report on lipid concentrations without the problems caused by the chronic presence of the lipid-binding protein that occur in transfected cells. Production of just the protein domains (not fused to EGFP) and labeling them with fluorophores that have higher quantum yield than EGFP would make the labeled domains even more sensitive lipid probes. However, microinjection is a method that requires great expertise and experience, and many cells do not tolerate it well. Moreover, fluorescent conjugation chemistry is not trivial, and it is significantly easier to prepare the EGFP-fused versions of the proteins. We have created EGFP fusion proteins from some of the PH-domains for bacterial expression and found that they can be produced and purified from bacteria with relative ease. Our best results were obtained when we used pET plasmids (Novagen) with the His6 tag placed at the COOH-terminal side of EGFP (that is, when the PH domain is at the NH_2 terminus of EGFP). Having the His6 tag NH_2-terminal to the PH domain increased expression, but slightly altered the affinity of the domain to lipids. Induction at room temperature with moderate IPTG concentrations (30 μM to 100 μM) can reduce proteolysis and increase the fraction of soluble protein. Free EGFP that contaminates the preparations can be removed easily by passing the preparation through a small (1 ml) heparin column (Amersham-Pharmacia), which binds the fusion protein but not free EGFP. The fusion protein can then be eluted with a NaCl gradient. Unfortunately, this cannot be applied to dsRed, which also binds to the heparin column in its free form. After concentrating the protein, the purified protein can be stored at −20°C, but during storage, especially if kept at 4°C, the protein can form aggregates that have to be removed before use (for example, by centrifugation at 200,000*g* for 30 min at 4°C).

The possibility has also been raised that the EGFP fusion protein could be used like an antibody, added in high enough concentrations to fixed and permeabilized cells to label the inositides (*16*). We do not have experience with such an application; however, we find that PH domains dissociate very rapidly from their lipid binding sites, so we predict that they would be removed during extended washing steps. This latter alternative will require characterization and refinement to judge whether it is a better approach for following cellular inositides than expression of the EGFP fusion protein.

Alternative or Supplemental Fluorescence Techniques

The value of the described basic measurements of fluorescence-labeled inositide-binding proteins can be greatly increased by combining with other techniques that either are specific to confocal microscopy or can be performed with conventional fluorescence microscopy.

Double labeling

Colocalization of the lipids with membrane markers or certain proteins is often an important goal of the study. This can be easily achieved in fixed cells by using the GFP-fused domain in the green channel and an antibody-based immunocytochemical procedure with any red-emitting fluorescent secondary antibody. Most confocal and fluorescence microscopes would be suitable for such samples. The issue is more complicated if live cells are to be studied. The EYFP and ECFP versions of GFP cannot be used for this purpose, unless the fluorescence microscope is custom-fitted with the filters that are required to detect these separately, without considerable cross talk between the channels. Confocal microscopes can only do this with additional lasers that excite ECFP in the low 400-nm range and are not usually part of the standard package. The red-emitter dsRed, and its improved version dsRed2, are better choices to complement EGFP, but in our experience they still suffer from some limitations that make them suboptimal for such use. A more detailed discussion of this topic is found in (*17*).

Fluorescence recovery after photobleaching (FRAP)

FRAP allows the analysis of the speeds and routes by which fluorescent molecules move into areas of the cell where the fluorescence had been eliminated by photobleaching with high laser power. This technique is valuable for following fluorescent proteins that are integrated in membranes (*18*). Because of the great speed of diffusion and the rapid on and off rates of inositide-protein interactions, one needs very fast data collection (usually covering only a line or segment) to provide accurate diffusion estimates for these molecules [see (*15*) for an example].

Fluorescence resonance energy transfer (FRET)

Analysis of molecular proximity on a scale that is beyond the resolution of the light microscope is possible with the use of two fluorophores that are matched in their spectral characteristics. In this case, the high-energy state of the excited donor molecule (the partner with the lower excitation wavelength) can be “stolen” by the acceptor molecule by a radiation-less energy transfer if

the two molecules are within a certain distance (~10 nm). In practical terms, fluorophores can yield information about their proximity if the researcher uses excitation with a wavelength that excites only the donor molecules (not the acceptors), and monitors the emission of the donor as well as the acceptor. When the two molecules are within "FRET-distance," an increase in the intensity at the emission wavelength that is characteristic of the acceptor, and a decrease in the intensity at the emission wavelength of the donor, can be observed. Analysis of this energy transfer requires a specialized microscope with the proper excitation capabilities and detectors that analyze the two emission wavelengths simultaneously. However, this application is common (*19*), although it is not without certain caveats (*20*). An example of using FRET to monitor PLC activity using the PH domain of PLCδ_1PH was published recently (*15*). An alternative and perhaps more reliable way of analyzing FRET is based on fluorescence lifetime imaging (FLIM). Because in reality it is not possible to excite only the donor fluorophore during excitation, it is more difficult to estimate FRET just by analyzing emission intensities. FLIM takes advantage of the fact that the lifetime of combined fluorescence increases when there is energy transfer between the donor and acceptor fluorophores. Therefore, microscopes that are able to analyze FLIM have been also introduced for the analysis FRET (*21*).

Monitoring Phosphoinositides by Immunocytochemistry

An alternative method for visualizing phosphoinositides in single cells is by conventional immunocytochemistry using antibodies to the individual phosphoinositide species. Such antibodies have been developed in several laboratories and some are commercially available. Although we have not used these antibodies to immunolabel inositides, many investigators communicated to us their difficulties in obtaining clear results with them. In addition, the usual specificity issue must be considered; fixation and permeabilization procedures appear to be critical in preserving the lipids yet allowing their accessibility to the antibodies. A nice example comparing pictures obtained by PLCδ_1PH-GFP and an antibody to PI4,5P_2 is provided in (*22*).

Concluding Remarks

Monitoring phosphoinositides with GFP-lipid binding domain fusion proteins allows analysis of the true dynamics of cellular phosphoinositides with subcellular resolution. Although this approach is an important step toward understanding inositide-based regulatory processes, it has limitations that should be always kept in mind. Like many similar techniques, this one introduces distortions because binding of the probe impairs the ability of the lipids to perform their normal regulatory functions, and it also may sequester lipids into certain compartments. On the other hand, because it relies on the same interactions that govern how inositides affect their molecular targets, understanding not only the values but the limitations of these measurements will certainly enrich our knowledge about the molecular details of inositol lipid-protein interactions.

References

1. S. B. Shears, in *Signalling by Inositides: A Practical Approach*, S. B. Shears, Ed. (Oxford University Press, New York, 1997), pp. 33-53.
2. R. A. J. Challiss, in *Signalling by Inositides: A Practical Approach*, S. B. Shears, Ed. (Oxford University Press, New York, 1997), pp. 151-165.
3. T. Balla, T. Bondeva, P. Várnai, How accurately can we image inositol lipids in live cells? *Trends Pharmacol. Sci.* **21**, 238-241 (2000).
4. M. A. Lemmon, M. Falasca, K. M. Ferguson, J. Schlessinger, Regulatory recruitment of signalling molecules to the cell membrane by plekstrin-homology domains. *Trends Cell Biol.* **7**, 237-242 (1997).
5. H. Stenmark, R. Aasland, B. H. Toh, A. D'Arrigo, Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.* **271**, 24048-24054 (1996).
6. C. P. Ponting, Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains? *Protein Sci.* **5**, 2353-2357 (1996).
7. S. Misra, J. H. Hurley, Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. *Cell* **97**, 657-666 (1999).
8. T. G. Kutateladze, K. D. Ogburn, W. T. Watson, T. deBeer, S. D. Emr, C. G. Burd, M. Overduin, Phosphatidylinositol 3-phosphate recognition by the FYVE domain. *Mol. Cell* **3**, 805-811 (1999).
9. T. K. Sato, M. Overduin, S. D. Emr, Location, location, location: membrane targeting directed by PX domains. *Science* **294**, 1881-1885 (2001).
10. Y. Mao, J. Chen, J. A. Maynard, B. Zhang, F. A. Quiocho, A novel all helix fold of the AP180 amino-terminal domain for phosphoinositide binding and clathrin assembly in synaptic vesicle endocytosis. *Cell* **104**, 433-440 (2001).
11. S. Dowler, R. A. Currie, D. G. Campbell, M. Deak, G. Kular, C. P. Downes, D. R. Alessi, Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem. J.* **351**, 19-31 (2000).
12. A. Bateman, E. Birney, R. Durbin, S. R. Eddy, K. L. Howe, E. L. Sonnhammer, The Pfam protein families database. *Nucleic Acids Res.* **28**, 263-266 (2000).
13. P. Várnai, T. Balla, Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[^{3}H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* **143**, 501-510 (1998).
14. K. Hirose, S. Kadowaki, M. Tanabe, H. Takeshima, M. Iino, Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science* **284,** 1527-1530 (1999).
15. J. van Der Wal, R. Habets, P. Várnai, T. Balla, K. Jalink, Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. *J. Biol. Chem.* **276**, 15337-15344 (2001).
16. D. J. Gillooly, I. C. Morrow, M. Lindsay, R. Gould, N. J. Bryant, J.-M. Gaullier, R. G. Parton, H. Stenmark, Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* **19**, 4577-4588 (2000).
17. J. Ellenberg, J. Lippincott-Schwartz, J. F. Presley, Dual-colour imaging with GFP variants. *Trends Cell Biol.* **9**, 52-56 (1999).
18. J. Lippincott-Schwartz, J. F. Presley, K. J. Zaal, K. Hirschberg, C. D. Miller, J. Ellenberg, Monitoring the dynamics and mobility of membrane proteins tagged with green fluorescent protein. *Methods Cell Biol.* **58**, 261-281 (1999).
19. R. M. Siegel, F. K. Chan, D. A. Zacharias, R. Swofford, K. L. Holmes, R. Y. Tsien, M. J. Lenardo, Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescent protein. *Science's STKE* (2000), http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2000/38/pl1
20. D. A. Zacharias, Sticky caveats in an otherwise glowing report: oligomerizing fluorescent proteins and their use in cell biology. *Science's STKE* (2002), in press.
21. A. G. Harpur, F. S. Wouters, P. I. Bastiaens, Imaging FRET between spectrally similar GFP molecules in single cells. *Nature Biotechnol.* **19**, 167-169 (2001).
22. M. Matsuda, H. F. Paterson, R. Rodriguez, A. C. Fensome, M. V. Ellis, K. Swann, M. Katan, Real time fluorescence imaging of PLCγ translocation and its interac-

tion with the epidermal growth factor receptor. *J. Cell Biol.* **153**, 599-612 (2001).
23. T. P. Stauffer, S. Ahn, T. Meyer, Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P_2 concentration monitored in living cells. *Curr. Biol.* **8**, 343-346 (1998).
24. P. Varnai, K. I. Rother, T. Balla, Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. *J. Biol. Chem.* **274**, 10983-10989 (1999).
25. K. Venkateswarlu, F. Gunn-Moore, J. M. Tavare, P. J. Cullen, 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.* **112**, 1957-1965 (1999).
26. K. Venkateswarlu, P. B. Oatey, J. M. Tavare, P. J. Cullen, Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr. Biol.* **8**, 463-466 (1998).
27. G. Servant, O. D. Weiner, P. Herzmark, T. Balla, J. W. Sedat, H. R. Bourne, Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**, 1037-1040 (2000).
28. J. Watton, J. Downward, Akt/PKB localisation and 3' phosphoinositide generation at sites of epithelial cell-matrix and cell-cell interaction. *Curr. Biol.* **9**, 433-436 (1999).
29. T. P. Levine, S. Munro, The pleckstrin-homology domain of oxysterol-binding protein recognizes a determinant specific to Golgi membranes. *Curr. Biol.* **8**, 729-739 (1998).
30. F. Kanai, H. Liu, S. J. Field, H. Akbary, T. Matsuo, G. E. Brown, L. C. Cantley, M. B. Yaffe, The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nature Cell Biol.* **3**, 675-678 (2001).
31. C. D. Ellson, S. Gobert-Gosse, K. E. Anderson, K. Davidson, H. Erdjument-Bromage, P. Tempst, J. W. Thuring, M. A. Cooper, Z-Y. Lim, A. B. Holmes, P. R. J. Gaffney, J. Coadwell, E. R. Chilvers, P. T. Hawkins, L. R. Stephens, PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of $p40^{phox}$. *Nature Cell Biol.* **3**, 679-682 (2001).