

## Measurement and Immunofluorescence of Cellular Phosphoinositides

Hiroko Hama, Javad Torabinejad, Glenn D. Prestwich,  
and Daryll B. DeWald

### Summary

Phosphoinositides are a vitally important class of intracellular-signaling molecules that regulate cellular processes, including signaling through cell-surface receptors, remodeling of the cytoskeleton, vesicle-mediated protein trafficking, and various nuclear functions. Methods for the analysis of *in vivo* phosphoinositide concentration, such as the one described in this chapter enable quantification of all phosphoinositides from a population of cells. This method involves metabolic labeling of cells with *myo*-[2-<sup>3</sup>H] inositol, followed by lipid extraction, and quantification by high-performance liquid chromatography (HPLC). It provides improved efficiency and reproducibility when analyzing yeast, plant cells, and is applicable to animal cells as well. In addition, a technique for determining the intracellular location of phosphoinositides is described. When quantification and localization techniques are used in parallel, an investigator can identify cell, and even subcellular concentration changes. The technique described in this chapter uses immunodetection with antiphosphoinositide antibodies to determine the localization and relative concentrations of phosphoinositides in fixed cells. The availability of antibodies allows an investigator to perform immunofluorescence and potentially immunoelectron microscopy of phosphoinositide localization on particular cellular, organellar, or vesicular membranes.

**Key Words:** Signal transduction; HPLC; animal; plant; yeast cells.

### 1. Introduction

The potential role of phosphoinositides as intracellular signaling molecules was first reported half a century ago by Hokin and Hokin (*1*). Since then, a great

deal has been learned about the complexity of cellular processes regulated by phosphoinositides. The growing list of phosphoinositide-regulated cellular processes includes signaling through cell-surface receptors (2–4), remodeling of the cytoskeleton (5,6), vesicle-mediated protein trafficking (7,8), and various nuclear functions (9–11). Each process can be simultaneously regulated by one or more specific phosphoinositides, owing to temporally and spatially restricted formation of phosphoinositides. In addition to phosphatidylinositol (PtdIns) itself, there are seven known phosphoinositides in eukaryotic cells with phosphate monoesters attached to the 3, 4, or 5 positions of the inositol ring of phosphatidylinositol: PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub>, PtdIns(4,5)P<sub>2</sub>, and PtdIns(3,4,5)P<sub>3</sub>. The remodeling of phosphoinositide phosphorylation in space and time is precisely coordinated via regulated actions of phosphatidylinositol and phosphoinositide kinases, phosphoinositide phosphatases, and phospholipases.

Following synthesis at a specific site of action, phosphoinositides recruit and bind effector (phosphoinositide-binding) proteins and activate a variety of downstream signaling cascades. Unique among the phosphoinositides, PtdIns(4,5)P<sub>2</sub> either binds effector proteins and/or serves as a precursor for the second messengers inositol trisphosphate and diacylglycerol. However, binding to proteins and altering their localization and/or activity appears to be the primary function of phosphoinositides. Effector protein relocalization involves specific interactions between phosphoinositides and phosphoinositide-binding domains effector-protein. Among the 10 or more recognition motifs characterized are the PH (pleckstrin homology) domains, PX (Phox homology) domains, and FYVE (Fab1p, YOTB, Vac1, EEA1) domains. These phosphoinositide-binding motifs facilitate relocalization, conformational changes, and activation or inactivation of numerous downstream-effector proteins. This is significant because hundreds of eukaryotic proteins contain motifs that are likely to confer phosphoinositide binding. For example, in the human genome, there are approx 150–200 proteins containing predicted PH domains.

A number of studies have been conducted to measure the activities of phosphatidylinositol kinases in cell extracts to demonstrate the roles of phosphoinositides in signaling pathways (reviewed in **ref. 12**). Although these studies provided evidence for involvement of phosphoinositides and phosphoinositide kinases in regulatory systems, further analyses were necessary to demonstrate *in vivo* formation of specific phosphoinositides. Methods for the analysis of *in vivo* phosphoinositide concentrations such as the one described in this chapter enable quantification of all seven phosphoinositides from a population of cells. This method involves metabolic labeling of cells with *myo*-[2-<sup>3</sup>H] inositol, followed by lipid extraction, and quantification by high-performance liquid chromatography (HPLC). The procedure described here is a modified

version of the one developed by Cantley et al. (13). It provides improved efficiency and reproducibility when analyzing yeast and plant cells (14,15) and is applicable to animal cells as well (16).

In addition to whole-cell and tissue quantification of phosphoinositides, techniques have been developed to determine the intracellular localization of phosphoinositides. When quantification and localization techniques are used in parallel, an investigator can identify whole-tissue, cell, and even subcellular concentration changes. This is valuable, because phosphoinositide synthesis or degradation usually does not occur uniformly throughout the cell. Instead, it occurs at distinct membrane sites like the Golgi or plasma membrane.

Localization of specific phosphoinositides in living cells can be determined using fluorescence microscopy of cells transfected with gene constructs encoding phosphoinositide-binding proteins fused to the green fluorescent protein (GFP) (17). Colocalization of the GFP fusion and phosphoinositides occurs via association of the phosphoinositide-binding domain with phosphoinositides. This powerful technique has been described several times, and will not be covered here. A less commonly-used approach is the use of fluorescently labeled rhodamine to visualize PtdIns(4,5)P<sub>2</sub> in cells, which localizes intracellular and intranuclear PtdIns(4,5)P<sub>2</sub> that cannot be detected by the GFP-PH domain constructs (18). An alternative method described in this chapter involves phosphoinositide immunodetection with antiphosphoinositide antibodies (19–23) to determine the localization and relative concentrations in fixed cells. The availability of antibodies allows an investigator to perform immunofluorescence and potentially immunoelectron microscopy of phosphoinositide localization on particular cellular, organellar, or vesicular membranes.

### 1.1. Measurement of Phosphoinositides

Analysis of relative phosphoinositide concentrations using HPLC is sometimes called “headgroup analysis,” because chromatographic separation of the water-soluble moiety of phosphoinositides (glycerophosphoinositol phosphates; gPIPs) is done after removal of the hydrophobic acyl chains (deacylation). Cells are first metabolically labeled with *myo*-[2-<sup>3</sup>H] inositol for 12–24 h. Before lipid extraction, cells are treated with trichloroacetic acid (TCA) to inactivate enzymes that might degrade phosphoinositides during the extraction process. [<sup>3</sup>H]-Labeled phosphoinositides are extracted with organic solvents, and deacylated by alkaline treatment. The resulting gPIPs are separated using strong anion-exchange HPLC. Small fractions are collected and radioactivity is measured by scintillation counting. Here we present protocols for labeling tissue-culture cells (NIH3T3 fibroblasts and 3T3-L1 preadipocytes), yeast (*Saccharomyces cerevisiae*), and plant cells (*Arabidopsis thaliana* submerged and

hydroponic cultures). Lipid extracts from these cells are deacylated and analyzed by HPLC using the same procedure.

### **1.2. Immunofluorescence of Phosphoinositides**

Monoclonal antibodies (MAbs) directed against PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> have been developed, and immunofluorescence procedures using these reagents enable the detection of these phosphoinositide species in fixed mammalian cells. When combined with the cellular phosphoinositide analyses described earlier, this approach allows an investigator to visualize in a cell the precise location where modulation of phosphoinositide concentrations is occurring. For the cellular immunolocalization of phosphoinositides, the cells must first be fixed with a chemical fixative (e.g., formaldehyde) and then permeabilized with a detergent (e.g., Triton X-100). Nonspecific binding sites in the cells are then blocked by incubating the cells in a solution containing a blocking reagent such as goat serum. The cells are incubated with the primary antibody directed against PtdIns(4,5)P<sub>2</sub> (**20,21**) or PtdIns(3,4,5)P<sub>3</sub> (**22**). The cells are washed with a buffered salt solution and then incubated with a fluorophore-tagged secondary antibody. After a final washing to remove nonspecifically bound secondary antibodies, cells are visualized by epifluorescence or laser-scanning confocal microscopy.

## **2. Materials**

### **2.1. Growth Media**

1. NIH3T3 or 3T3-L1 cells are grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum.
2. A yeast nitrogen base medium containing appropriate supplements and carbon source is used for the growth of yeast cells. The "drop-out mix" (**24**) is prepared without inositol.
3. *Arabidopsis thaliana* is grown submerged in a medium composed of 0.5X Murashige and Skoog (MS) and B5 vitamins. Alternatively, plants can be grown hydroponically on the same medium excluding the vitamins.
4. Falcon 2059 tubes (Becton Dickinson Labware, Lincoln Park, NJ).
5. Disposable cell scrapers (Fisher Scientific, Pittsburgh, PA).

### **2.2. Radiochemicals**

1. *myo*-[2-<sup>3</sup>H] Inositol (10–25 Ci/mmol) (American Radiolabeled Chemicals, (ARC), St. Louis, MO; Amersham Biosciences, Piscataway, NJ, ICN Biomedical, Irvine, CA; or PerkinElmer Life Sciences, PerkinElmer, Boston, MA).
2. For HPLC standards, PtdIns(4)P [inositol-2-<sup>3</sup>H] (ARC). PtdIns(4,5)P<sub>2</sub> [inositol-2-<sup>3</sup>H] (ARC or PerkinElmer).

3. [ $^{32}\text{P}$ ] PtdIns(3)P is prepared by *in vitro* phosphorylation of PtdIns by yeast PtdIns 3-kinase with [ $\gamma$ - $^{32}\text{P}$ ] ATP, followed by separation by thin-layer chromatography (TLC) and extraction from the TLC media (15).
4. [ $^{32}\text{P}$ ] PtdIns(3,4)P<sub>2</sub> and [ $^{32}\text{P}$ ]PtdIns(3,4,5)P<sub>3</sub> are prepared by *in vitro* phosphorylation of PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>, respectively, by mammalian PI 3-kinase with [ $\gamma$ - $^{32}\text{P}$ ] ATP, followed by TLC purification.
5. PtdIns(3,5)P<sub>2</sub> [inositol-2- $^3\text{H}$ ] is produced *in vivo* by salt-stressed yeast cells (15,25).

### 2.3. Chromatography Media

1. Silica gel 60 TLC plates (0.25 mm thick, Merck no. 5724 or equivalent) are used to purify [ $^{32}\text{P}$ ]-labeled standards prepared by *in vitro* phosphorylation.
2. Strong anion-exchange columns Partisil 5 SAX (4.6 mm  $\times$  250 mm) or Partisil 10 SAX (4.6 mm  $\times$  250 mm) (Whatman, Clifton, NJ) are used for HPLC headgroup analysis.
3. Ion-exchange columns must be fitted with guard columns (e.g., Phenomenex, SecurityGuard™, Torrance, CA, part no. KJO-4282) containing SAX inserts (Phenomenex, part no. AJO-431).

### 2.4. Immunofluorescence Reagents

1. Coverslips and slides (Fisher Scientific, or Ted Pella, Redding, CA).
2. Purified RC6F8 anti-PtdIns(3,4,5)P<sub>3</sub> IgM and 2C11 anti-PtdIns(4,5)P<sub>2</sub> IgM (Echelon Biosciences, Salt Lake City, UT).
3. High quality formaldehyde (Ted Pella, Redding, CA).
4. Fluorophore-tagged (e.g., FITC, Texas Red) antimouse IgM secondary antibodies are available (Jackson ImmunoResearch Laboratories, West Grove, PA or Rockland, Gilbertsville, PA).

### 2.5. Other Chemicals

1. All eight forms of nonradioactive phosphoinositides, with different acyl-chain lengths and a variety of reporter groups (Echelon Biosciences).
2. Additional natural and synthetic phosphoinositides (Avanti Polar Lipids, Alabaster, AL; Matreya State College, PA; and Calbiochem, San Diego, CA).
3. Organic solvents and all other chemicals can be purchased from several commercial sources (*see Subheading 4.*) for methylamine.
4. Scintillation cocktail must be miscible with aqueous solutions in order to provide consistent results when counting fractions.

### 2.6. Lipid Handling

Lipid extracts and deacylated lipids should be dried under a stream of nitrogen gas or in a Speed-Vac concentrator (Thermo Savant, Holbrook, NY). Dried

lipids should be re-suspended using an bath sonicator such as Bransonic table-top cleaners (Branson Ultrasonics, Danbury, CT). A TLC tank is used for standard preparation by *in vitro* phosphorylation.

## 2.7. HPLC

Headgroup analysis can be performed with a Beckman System Gold HPLC with 32 Karat software (Beckman Coulter, Fullerton, CA) or equivalent. The system should be equipped with pumps for dual solvent delivery, an ultraviolet (UV) detector, and a fraction collector. The fraction collector is fitted with a rack for liquid-scintillation vials. Alternatively, an on-line radioactivity detector (e.g.,  $\beta$ -RAM, INUS, Tampa, FL) can be used to detect separated gPIPs, in lieu of fraction collection and scintillation counting.

## 2.8. Microscopy

Epifluorescence or laser scanning confocal microscopes are used to examine cells labeled with fluorescently tagged antibodies. For the work presented in this chapter, cells were examined using a Nikon TE-300 inverted microscope interfaced with a Bio-Rad MRC 1024 confocal system (Bio-Rad Laboratories, Hercules, CA). Images are collected using 60X oil-immersion objective. Depending on the fluorophore, appropriate excitation wavelengths and emission filters are used.

## 3. Methods

### 3.1. Labeling and Lipid Extraction of Yeast Cells

1. A synthetic medium containing 5  $\mu\text{Ci}/\text{mL}$  of *myo*-[2- $^3\text{H}$ ] inositol is inoculated with a small amount of fresh culture to give an  $A_{600\text{nm}}$  of 0.01. A 5 mL culture is sufficient for each sample.
2. Cells are grown with shaking (200 rpm) until the  $A_{600\text{nm}}$  is 0.6–1.0, typically 14–20 h at 30°C.
3. Growth is terminated by addition of TCA (final concentration 5%), followed by incubation on ice for 1 h in polypropylene tubes (Falcon 2059). This treatment prevents degradation of lipids by lipases during manipulation of the cells (26). It is important to avoid excessive exposure to TCA (higher concentrations, higher temperatures, or prolonged periods), because lipids can be degraded by the acid (26).
4. Cells are harvested by centrifugation, and washed twice with 1 mL of  $\text{H}_2\text{O}$ .
5. The washed pellet can be stored at  $-80^\circ\text{C}$  or used directly in **step 6**.
6. This procedure relies on a solvent for extraction that is very effective for yeast cells (26) and can be used for other cells as well (15,16). The extraction solvent contains 95% ethanol/ $\text{H}_2\text{O}$ /diethyl ether/pyridine/conc.  $\text{NH}_4\text{OH}$

(15/15/5/1/0.018 v/v). This solution without H<sub>2</sub>O (called EEP solvent) can be prepared for use in a series of experiments for a few days, but long-term storage of EEP solvent is not recommended.

7. Washed yeast cells (5 mL of culture in late-log phase growth) are suspended in 0.5 mL of H<sub>2</sub>O and 0.75 mL of EEP solvent is added. Extraction is conducted at 57°C for 30 min with occasional mixing. While the mixture is still warm, cell debris is removed by centrifugation for 5 min at 5000 *g* at room temperature. The supernatant fluid is dried under a stream of N<sub>2</sub> or in a Speed-Vac concentrator with appropriate traps.
8. Extracted lipids can be stored at -80°C or immediately deacylated.

### 3.2. Labeling and Lipid Extraction of Mammalian Cells

1. For labeling NIH 3T3 fibroblasts or 3T3-L1 preadipocytes, cells should be grown to at least 60% confluency in 75 cm<sup>2</sup> flasks.
2. Cells are washed and then labeled for 36 h with *myo*-[2-<sup>3</sup>H] inositol (20 μCi/mL) in inositol-free DMEM + 10% calf serum.
3. After 24 h, the medium should be removed and replaced with a fresh *myo*-[2-<sup>3</sup>H] inositol-containing medium. At this point, if growth-factor stimulation (e.g., with PDGF to activate PI 3-kinase) is part of the experiment, cells are serum deprived for 2 h in inositol-free and serum-free DMEM containing 0.2% BSA and 10 μCi/mL *myo*-(2-<sup>3</sup>H)-inositol, followed by platelet-derived growth factor (PDGF) (50 ng/mL) stimulation and harvest.
4. For cell harvest, ice-cold TCA is added to the medium in the flasks to a final concentration of 10%. The flasks containing the 10% TCA are incubated on ice for 1 h with the cells immersed in the solution.
5. Cells are released from the flasks by gently scraping with a disposable cell scraper followed by pipeting them into 15- or 50-mL conical screw-cap centrifuge tubes.
6. Centrifuge the samples for 5 min at 5000 *g*.
7. Remove supernatant fluid and add 5 mL of a 5% TCA, 1 mM EDTA solution to the pellets.
8. After the cells are resuspended, they should be centrifuged as above and the supernatant removed. Pellets can be stored at -80°C, or lipids can be extracted immediately.
9. Lipids are extracted from the cell pellet by resuspending cells in 0.75 mL chloroform/methanol/HCl (40/80/1 v/v) and vortexing the cells vigorously every 60 s for 15 s. Cells must be kept on ice between vortexing.
10. Add 0.25 mL of chloroform and 0.45 mL of 0.1 M HCl to the cells and vortex the tube.
11. Samples are centrifuged at 5000 *g* for 2 min, and the bottom, organic layer is transferred to another tube for continued processing.

12. Ammonia (50  $\mu\text{L}$  of a 1 *M* solution) is added to the cells and the solutions in the tubes are dried in a Speed-Vac concentrator or under nitrogen. The samples can then be deacylated (*see Subheading 3.4.*) or stored at  $-80^{\circ}\text{C}$  prior to deacylation.

### 3.3. Labeling and Lipid Extraction of Plant Cells

1. Two-week-old *A. thaliana* plants grown in a liquid medium (0.5X MS basal salt mixture, pH 5.8) containing B5 vitamins are submerged in 1 mL of the same medium with reduced myo-inositol (10  $\mu\text{M}$ ) and 50  $\mu\text{Ci}/\text{mL}$  of *myo*-[2- $^3\text{H}$ ] inositol. Labeling is accomplished in 1.6-mL microcentrifuge tubes for 20 h on a gyratory shaker (80 rpm) at 22–26°C. Alternatively, seeds can be germinated and plants grown hydroponically in 0.5X MS basal salt mixture, pH 5.8. Labeling of the hydroponically grown plants is achieved by placing the roots in a 2.0 mL cup containing the 0.5X MS medium and 100  $\mu\text{Ci}/\text{mL}$  of *myo*-[2- $^3\text{H}$ ] inositol.
2. Growth of radiolabeled *A. thaliana* plants is terminated by addition of TCA (final conc. = 5%) followed by incubation on ice for 1 h.
3. Plantlets are washed 5 times with 10 mL of  $\text{H}_2\text{O}$  (room temperature) and transferred into a 5-mL Dounce tissue grinder after resuspension in 0.5 mL of  $\text{H}_2\text{O}$ , and 0.75 mL of EEP solvent. Tissues are homogenized and transferred into microcentrifuge tubes and incubated at 57°C for 30 min.
4. Cell debris is removed by centrifugation and the supernatant is dried under a stream of  $\text{N}_2$  or in a Speed-Vac concentrator.

### 3.4. Deacylation of Glycerolipids

Lipids are deacylated by the method of Serunian et al. (13) with minor modifications. All the procedures are carried out in 1.6 mL or 2-mL plastic microcentrifuge tubes.

1. Dried lipids are resuspended in 0.5 mL of methylamine reagent (42.8% of 25% methylamine, 45.7% of methanol, 11.4% of *n*-butanol) by bath sonication, incubated at 53°C for 50 min, and dried in a Speed-Vac concentrator (*see Note 1*).
2. Deacylated lipids are suspended in 0.75 mL  $\text{H}_2\text{O}$  by sonication and then extracted 3 times with 0.5 mL *n*-butanol/petroleum ether/ethyl formate (20/4/1 v/v) or until the aqueous phase is no longer cloudy. The aqueous phase is dried in a Speed-Vac concentrator and suspended in 200  $\mu\text{L}$  of  $\text{H}_2\text{O}$ .
3. A small portion (10–20  $\mu\text{L}$ ) of each sample is used to determine the radioactivity by liquid-scintillation counting. For preparation of loading samples for HPLC, standardization can be done using the [ $^3\text{H}$ ] counts, which is a close approximation of the content of PtdIns (phosphoinositides are minor

components). Alternatively, cell number, protein contents, or lipid phosphate radioactivity could be used for the same purpose.

### 3.5. Headgroup Analysis

1. It is useful to include AMP, ADP, and ATP in each sample in order to monitor the column performance by UV absorption. Typically, a portion of each sample ( $1.5\text{--}2.5 \times 10^6$  cpm) is mixed with 40 nmoles each of AMP, ADP, and ATP. Glycerophosphoinositol monophosphate species [gPI(3)P, gPI(4)P, and gPI(5)P] elute between AMP and ADP, glycerophosphoinositol bisphosphate species [gPI(3,5)P<sub>2</sub>, gPI(3,4)P<sub>2</sub>, and gPI(4,5)P<sub>2</sub>] elute between ADP and ATP, and gPI(3,4,5)P<sub>3</sub> elutes after ATP.
2. Phosphoinositides are resolved with the following mobile phase of ammonium phosphate (pH 3.8; flow rate of 1 mL/min; *see Notes 2–4*).
  - a. Gradient I for separation of gPI(3)P, gPI(4)P, gPI(3,4)P<sub>2</sub>, gPI(3,5)P<sub>2</sub>, and gPI(4,5)P<sub>2</sub>.
    - 5 mL of 10 mM
    - 40 mL of a linear gradient, 10 mM to 0.7 M
    - 2 mL of a linear gradient, 0.7 to 1 M
    - 3 mL of 1 M
  - b. Gradient II for separation of gPI(3)P, gPI(4)P, gPI(3,4)P<sub>2</sub>, gPI(3,5)P<sub>2</sub>, gPI(4,5)P<sub>2</sub>, and gPI(3,4,5)P<sub>3</sub>.
    - 5 mL of 10 mM
    - 60 mL of a linear gradient, 10 mM to 0.8 M
    - 2 mL of a linear gradient, 0.8 to 1 M
    - 3 mL of 1 M

Fractions are collected every 0.3 min (0.3 mL/fraction), mixed with 2 mL of water-miscible scintillation cocktail, and counted in a liquid scintillation counter (*see Notes 5 and 6*).

### 3.6. HPLC Standards

1. [<sup>3</sup>H]-Labeled standards obtained from commercial sources are mixed with small amounts of non-radioactive carrier lipids (any phospholipids) and deacylated (*see Subheading 3.4*). In vitro phosphorylation of PtdIns, PtdIns(4)P, and PtdIns(4,5)P<sub>2</sub> is performed as previously described (27).
2. Desired substrates are sonicated in 20 mM HEPES, pH 7.5, and mixed with 60 μM ATP, 0.2 mCi/mL [ $\gamma$ -<sup>32</sup>P] ATP, 10 mM MgCl<sub>2</sub>, and appropriate enzyme sources in a total volume 50 μL. The mixture is incubated for 5 min or longer, depending on the enzyme sources, at room temperature.

3. The reaction is terminated by the addition of 80  $\mu\text{L}$  1 M HCl and the lipids are extracted with 160  $\mu\text{L}$  of chloroform/methanol (1/1). The lower organic phase is dried and re-dissolved in chloroform for TLC.
4. Entire samples are spotted onto silica gel 60 TLC plates that have been pre-treated with *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, and developed with a solvent containing 75 mL of methanol, 60 mL of chloroform, 45 mL of pyridine, 12g of boric acid, 7.5 mL of  $\text{H}_2\text{O}$ , 88 % (v/v) 3 mL of formic acid, 0.375g of 2,6-di-*tert*-butyl-4-methylphenol, and 75  $\mu\text{L}$  of ethoxyquin (28). (**Note:** It is necessary to dissolve the boric acid in water prior to the addition of organic solvents.)
5. After development, TLC plates are completely dried and exposed to X-ray films for 1–2 h at room temperature to identify the phosphoinositide spots (carried-over [ $\gamma$ - $^{32}\text{P}$ ] ATP stays at the origin).
6. The spots of desired products can be identified by comparison to non-radioactive phosphoinositides run on the same TLC system and stained by iodine vapor. The TLC plate is overlaid on the X-ray film, and the area on the TLC plate corresponding to the radioactive products is marked.
7. The silica matrices are carefully scraped off from the TLC plates and collected in 1.5-mL tubes. It is advisable to remove the silica surrounding the marked spots first, and then recover the spots.
8. Lipids are extracted from the matrices with a solvent containing chloroform/methanol/ $\text{H}_2\text{O}$  (16/16/5). The extracted phosphoinositides are mixed with carrier lipids and deacylated (*see Subheading 3.4.*).
9. The enzyme used for preparation of PtdIns(3)P is obtained by ammonium sulfate precipitation (25–30%) of yeast cytosol from a strain overexpressing the PtdIns 3-kinase Vps15p/Vps34p complex (strain TVY614 [29] carrying pJSY324.15 [30] and pPHY52 [31]).
10. Similarly, mammalian cell extracts may also be used for preparation of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. The most effective extracts are prepared from tissue-culture cells transfected with an expression vector for the p110 subunit of PI 3-kinase (32).

### 3.7. Immunolocalization of Phosphoinositides

1. NIH3T3 cells at logarithmic stage on coverslips are serum-starved overnight and stimulated with insulin (100 ng/mL) or PDGF (50 ng/mL). Growth factor stimulation is typically done for 1–15 min.
2. Reactions are stopped by washing the cells with cold tris-buffered saline (TBS) and cells are processed for immunofluorescence.
3. Cells on glass coverslips are fixed with 2% formaldehyde (in cell media) for 20 min, and then permeabilized with 0.5% Triton X-100 in TBS for 15 min at room temperature.

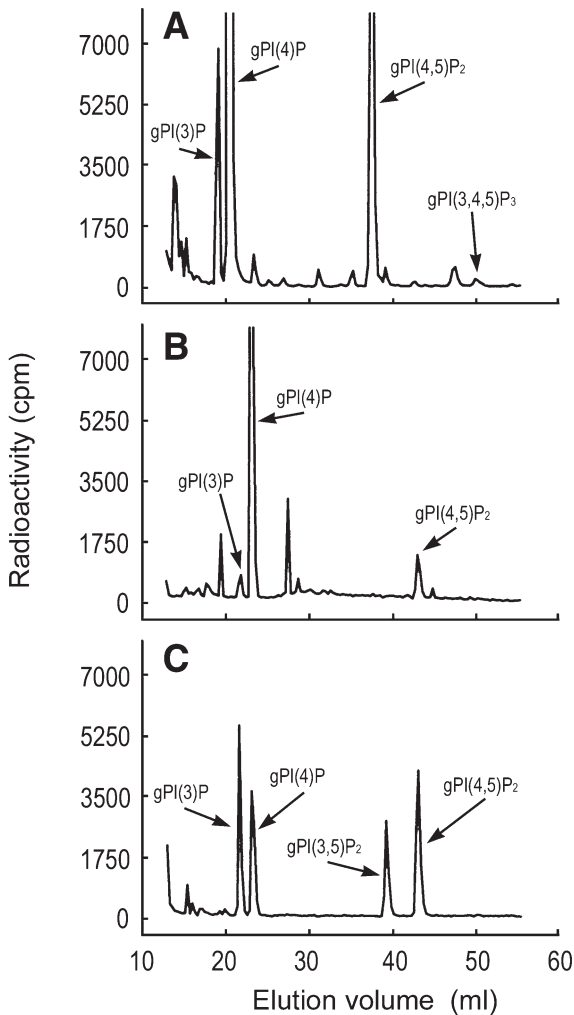


Fig. 1. HPLC chromatograms of glycerophosphoinositols from mammalian, plant, and yeast cells. The NIH3T3 fibroblast cells (A) were stimulated with platelet derived growth factor (PDGF) for 5 min prior to harvest and analysis of glycerophosphoinositols (Gradient II). Plant cells (B) and yeast cells (C) were subjected to 0.25 M NaCl for 30 min and 1.0 M NaCl shock for 20 min, respectively, prior to lipid extraction and headgroup analysis (Gradient I). Then lipids were extracted, deacylated, and glycerophosphoinositols analyzed by HPLC using a Partisil 10 SAX column and gradients described in the text. Fractions were collected, counted in a scintillation counter, and counts in each fraction were plotted. gPI(3)P is glycerophosphoinositol 3-phosphate, gPI(4)P is glycerophosphoinositol 4-phosphate, and all other glycerophosphoinositols are likewise designated.

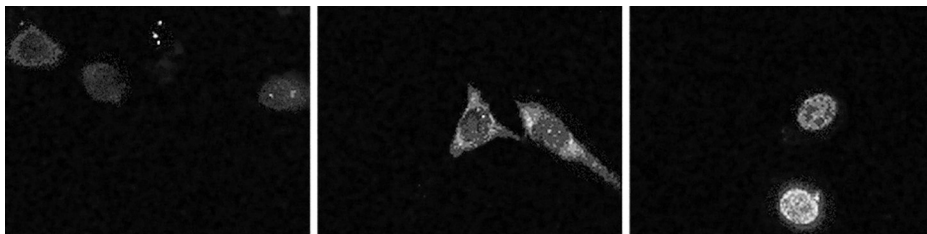


Fig. 2. Immunofluorescence detection of PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> in PDGF-stimulated 3T3 L1 preadipocytes. Cells were stimulated with PDGF (50 ng/mL) for 5 min and then prepared for immunofluorescence detection of phosphoinositides as described in the text. The control cells (**left panel**) were not incubated with antiphosphoinositide antibody. Cells that were probed with anti-PtdIns(3,4,5)P<sub>3</sub> antibody (**middle panel**) displayed primarily cytosolic and membrane staining, and those probed with anti-PtdIns(4,5)P<sub>2</sub> antibody (**right panel**) displayed primarily nuclear staining.

4. After blocking with 10% goat serum in TBS, either RC6F8 MAb (anti-PtdIns(3,4,5)P<sub>3</sub> antibody) ascites (1:50 dilution) or 2C11 (anti-PtdIns(4,5)P<sub>2</sub> antibody) ascites (1:5000 dilution) is added and incubated at room temperature for 1 h.
5. After washing three times with the blocking solution, fluorophore-labeled antimouse IgM (1:2000 dilution) is added and incubated at room temperature for 1 h.
6. Cells are washed three times with deionized water and observed using a laser scanning confocal microscope or fluorescent microscope.

#### 4. Notes

1. For deacylation of lipids, we have experienced variations in efficiencies depending on the source of methylamine. It is advised to test deacylation with nonradioactive phospholipids, followed by TLC.
2. Ammonium phosphate (used for HPLC mobile phase) contains surprisingly large quantities of impurities. It is necessary to remove the impurities by filtration through nitrocellulose membranes (0.45 μm). Once prepared, the buffers are easily contaminated by molds. It is not recommended to store ammonium phosphate solutions for an extended period.
3. The samples applied onto the HPLC columns contain some H<sub>2</sub>O-insoluble materials as well as small amount of lipids that are carried over through extractions. After repeated uses, columns start to lose resolution. When the number of counts in the fractions in-between the gPI(3)P and gPI(4)P peaks

is above background (loss of baseline resolution), the guard column should be replaced with a new one.

4. A new SAX column should be conditioned first by running left-over samples or nonradioactive samples. For unknown reasons, a significant portion of sample is so tightly bound to a new column that it never elutes. We have also observed that a very small portion of bound species remains bound throughout the gradient even at 1 M ammonium phosphate. When the same gradient is run without injecting a sample, sometimes small glycerophosphoinositol peaks elute. To avoid any interference from a previous column run, we typically apply two “cleaning cycles” of 10 mL linear gradient of 10 mM to 1 M ammonium phosphate between each HPLC run.
5. Recently, a nonradioactive method was reported (33). In this method, gPIPs are separated by anion-exchange HPLC and detected by conductivity measurements. Although the apparent resolution of this procedure is not as high, it is less expensive and avoids the use of radioactive materials. In addition, a sensitive mass assay for PtdIns(3,4,5)P<sub>3</sub> has been reported, but this only works with this phosphoinositide and requires an isotope-dilution method with deacylation and analysis of the Ins(1,3,4,5)P<sub>4</sub> head group (34). A third approach is the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which has been used for the detection and quantification of phosphoinositides (35).
6. Phosphoinositides from cell extracts and in vitro enzyme-catalyzed reactions may also be analyzed by competitive displacement reactions using tagged and/or labeled PH domains as probes. Stable complexes are formed between a biotinylated target lipid and an appropriate PH domain, and phosphoinositides present in samples are detected by their ability to compete for binding to the PH domain. The complexes are detected using time-resolved FRET (36). This concept has been independently used to develop a competitive fluorescence polarization (FP)-based assay amenable to high throughput screening. The FP assay has been used to determine activity of phosphoinositide 3-kinase (PI 3-K) and the type-II SH2-domain-containing inositol 5-phosphatase (SHIP2) (37). This assay is based on the interaction of specific phosphoinositide binding proteins with fluorophore-labeled phosphoinositide and inositol phosphate tracers. The enzyme reaction products are detected by their ability to compete with the fluorescent tracers for protein binding, leading to an increase in the amount of free tracer and a decrease in polarization values. The antilipid and competitive assay methodologies offer new opportunities in detection of phosphoinositide abnormalities in cancer cells, discovery of new anticancer agents targeted at inhibition of PI 3-kinase, and targeted monitoring of the effects of these agents in vivo (38).

## Acknowledgments

The authors wish to thank the American Cancer Society and NIH (Grant NS29632 to G.D.P.) for support of work at Utah State University and the University of Utah, and Echelon Biosciences, for providing reagents in our laboratories.

## References

1. Hokin, M. R. and Hokin, L. E. (1953) Enzyme secretion and the incorporation of P-32 into phospholipids of pancreas slices. *J. Biol. Chem.* **203**, 967–977.
2. Carpenter, C. L. and Cantley, L. C. (1996) Phosphoinositide kinases. *Curr. Opin. Cell Biol.* **8**, 153–158.
3. Stoyanov, B., Volinia, S., Hanck, T., et al. (1995) Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science* **269**, 690–693.
4. Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., et al. (1997) The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* **89**, 105–114.
5. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514.
6. Yin, H. L. and Janmey, P. A. (2003) Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761–789.
7. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) Phosphoinositides as regulators in membrane traffic. *Science* **271**, 1533–1539.
8. Czech, M. P. (2003) Dynamics of phosphoinositides in membrane retrieval and insertion. *Annu. Rev. Physiol.* **65**, 791–815
9. Irvine, R. F. (2002) Nuclear lipid signaling. *Sci. STKE* 2002, RE13.
10. Irvine, R. F. (2003) Nuclear lipid signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 349–361.
11. Martelli, A. M., Tabellini, G., Borgatti, P., et al. (2003) Nuclear lipids: new functions for old molecules? *J. Cell Biochem.* **88**, 455–461.
12. Toker, A. (2002) Phosphoinositides and signal transduction. *Cell Mol. Life Sci* **59**, 761–779.
13. Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) Identification and quantification of polyphosphoinositides produced in response to platelet-derived growth factor stimulation. *Methods Enzymol.* **198**, 78–87.
14. Stock, S. D., Hama, H., DeWald, D. B., and Takemoto, J. Y. (1999) SEC14-dependent secretion in *Saccharomyces cerevisiae*. Nondependence on sphingolipid synthesis-coupled diacylglycerol production. *J. Biol. Chem.* **274**, 12979–12983.
15. Hama, H., Takemoto, J. Y., and DeWald, D. B. (2000) Analysis of phosphoinositides in protein trafficking. *Methods* **20**, 465–473.
16. DeWald, D. B. (2003) Measurements of cellular phosphoinositide levels in 3T3-L1 adipocytes. *Methods Mol. Med.* **83**, 145–154.
17. Balla, T., Bondeva, T., and Varnai, P. (2000) How accurately can we image inositol lipids in living cells? *Trends Pharmacol Sci.* **21**, 238–241.
18. Holz, R. W., Hlubek, M. D., Sorensen, S. D., et al. (2000) A pleckstrin homology domain specific for phosphatidylinositol 4, 5- bisphosphate (PtdIns-4,5-P2) and

- fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P<sub>2</sub> as being important in exocytosis. *J. Biol. Chem.* **275**, 17,878–17,885.
19. Fukami, K., Matsuoka, K., Nakanishi, O., et al. (1988) Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. *Proc. Natl. Acad. Sci. USA* **85**, 9057–9061.
  20. Thomas, C. L., Steel, J., Prestwich, G. D., and Schiavo, G. (1999) Generation of phosphatidylinositol-specific antibodies and their characterization. *Biochem. Soc. Trans.* **27**, 648–652.
  21. Osborne, S. L., Thomas, C. L., Gschmeissner, S., and Schiavo, G. (2001) Nuclear PtdIns(4,5)P<sub>2</sub> assembles in a mitotically regulated particle involved in pre-mRNA splicing. *J. Cell Sci.* **114**, 2501–2511.
  22. Chen, R., Kang, V. H., Chen, J., et al. (2002) A monoclonal antibody to visualize PtdIns(3,4,5)P<sub>3</sub> in cells. *J. Histochem. Cytochem.* **50**, 697–708.
  23. Niswender, K. D., Gallis, B., Blevins, J. E., et al. (2003) Immunocytochemical detection of phosphatidylinositol 3-kinase activation by insulin and leptin. *J. Histochem. Cytochem.* **51**, 275–283.
  24. Burke, D., Dawson, D., and Stearns, T. (2000) *Methods in Yeast Genetics 2000: A Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
  25. Dove, S. K., Cooke, F. T., Douglas, M. R., et al. (1997) Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* **390**, 187–192.
  26. Hanson, B. A. and Lester, R. L. (1980) The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*. *J. Lipid Res.* **21**, 309–315.
  27. Stack, J. H., DeWald, D. B., Takegawa, K., and Emr, S. D. (1995) Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast. *J. Cell Biol.* **129**, 321–334.
  28. Walsh, J. P., Caldwell, K. K., and Majerus, P. W. (1991) Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. *Proc. Natl. Acad. Sci. USA* **88**, 9184–9187.
  29. Vida, T. A. and Emr, S. D. (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128**, 779–792.
  30. Stack, J. H., Herman, P. K., Schu, P. V., and Emr, S. D. (1993) A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *EMBO J.* **12**, 2195–2204.
  31. Herman, P. K. and Emr, S. D. (1990) Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **10**, 6742–6754.
  32. Katagiri, H., Asano, T., Ishihara, H., et al. (1996) Overexpression of catalytic subunit *p110alpha* of phosphatidylinositol 3-kinase increases glucose transport activity with translocation of glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* **271**, 16,987–16,990.

33. Nasuhoglu, C., Feng, S., Mao, J., et al. (2002) Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal. Biochem.* **301**, 243–254.
34. van der Kaay, J., Batty, I. H., Cross, D. A., et al. (1997) A novel, rapid, and highly sensitive mass assay for phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) and its application to measure insulin-stimulated PtdIns(3,4,5)P<sub>3</sub> production in rat skeletal muscle in vivo. *J. Biol. Chem.* **272**, 5477–5481.
35. Muller, M., Schiller, J., Petkovic, M., et al. (2001) Limits for the detection of (poly-)phosphoinositides by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Chem. Phys. Lipids* **110**, 151–164.
36. Gray, A., Olsson, H., Batty, I. H., et al. (2003) Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts. *Anal. Biochem.* **313**, 234–245.
37. Drees, B. E., Weipert, A., Hudson, H., et al. (2003) Competitive fluorescence polarization assays for the detection of phosphoinositide kinase and phosphatase activity. *Comb. Chem. High Throughput Screen* **6**, 321–330.
38. Prestwich, G. D., Chen, R., Feng, L., et al. (2002) *In situ* detection of phospholipid and phosphoinositide metabolism. *Adv. Enzyme Regul.* **42**, 19–38.