“Analysis of the phosphoinositide composition of subcellular membrane fractions”

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Summary

Phosphoinositides play critical roles in the transduction of extracellular signals through the plasma membrane and also in endomembrane events important for vesicle trafficking and organelle function [1]. The response triggered by these lipids is heavily dependent on the microenvironment in which they are found. HPLC analysis of labeled phosphoinositides allows quantification of the levels of each phosphoinositide species relative to their precursor, phosphatidylinositol. When combined with subcellular fractionation techniques, this strategy allows measurement of the relative phosphoinositide composition of each membrane fraction or organelle and determination of the microenvironment in which each species is enriched. Here, we describe the steps to separate and quantify total or localized phosphoinositides from cultured cells.

1. Introduction

Phosphatidylinositol and its seven phosphorylated phosphoinositide (PI) forms are interconnected by a network of kinases and phosphatases, which act in a dynamic balance to allow for the rapid initiation and termination of the PI signal in response to extracellular and intracellular cues [2]. Given that the cellular responses to activation/inhibition of PI-kinases and phosphatases depend not only on changes in the local concentration of the PI being formed, but also on changes in the local concentration of its precursors, precise measurement of the changes in all seven PIs within the cell is critical for full understanding of the outcome. High Performance Liquid Chromatography (HPLC) analysis of metabolically labeled phosphoinositides (PIs) has been the golden standard for quantitative measurements of cellular PI composition for many years. With the advancement in our understanding of the function of PIs in cells, new methodology for measuring cellular PIs have been developed. The discovery of PI-specific probes, for example, allowed for the detection of the subcellular localization of specific PIs in real-time [3]. The use of phospho-specific antibodies against proteins that are phosphorylated in a PI-dependent manner (as for phospho-Akt) became a popular method for quickly evaluating the activity of PI-kinases (e.g. PI 3-kinase) and the potential changes in the levels of PIs in cells. More recently, the development of PI-specific antibodies together with new methodology for staining PIs *in situ* added another valuable tool in the study of these lipids [4]. However, one major shortcoming of these techniques is the potential for generating false negative results. For example, certain subpopulations of PIs may not be accessible to PI probes and antibodies, or the local concentration and/or microenvironmental factors may prevent proper detection [5,6]. HPLC analysis of PIs remains the most sensitive and the only technique that allows for a direct comparison between the levels of various PIs. However, this technique also has its own limitations. Unlike the *in situ* PI probes and antibodies, HPLC analysis of labeled PIs only gives a snapshot of the global cellular PI composition, with no spatial information. With this in mind, we developed a strategy for analyzing the subcellular PI composition, which combines two well-established techniques: subcellular fractionation of microsomal membranes and HPLC analysis of labeled PIs. Together these two techniques become a powerful tool for mapping the dynamic membrane distribution of all PIs within the cell and unraveling the coordinated function of these signaling molecules. Nonetheless, one has to keep in mind that for precise comparison of the levels of the various PI species, the metabolic labeling step needs to reach equilibrium. The subcellular fractionation methods described below have been developed by applying fundamental principles for organelle fractionation [7] and adapted from protocols used to isolate lipid binding proteins [8]. They have been optimized to reduce sample loss and minimize lipid dephosphorylation. Analysis of the subcellular distribution of PIs in HeLa and BTC6 cells using this strategy was previously reported [9]. For an overview of the procedures, refer to the workflow chart (Figure 1).

2. Materials

Cells of interest and tissue culture reagents and facilities.
Tabletop ultracentrifuge and centrifuge tubes
HPLC quaternary pump with degasser and autosampler (optional)
Strong anionic exchange columns (Agilent Zorbax SAX or Whatman Partisphere SAX)
Flow scintillation analyzer or ß-counter and scintillation fluid
Chemical fume hood
Centrifugal evaporator
Protective equipment for radioactivity and permit

3. Methods

3.1. Labeling cells with [^{3}H] inositol.
Materials:
1. Inositol-free media
2. L-glutamine 100x
3. [^{3}H] inositol
4. Treated Tissue Culture Plates, 100mm (P100)
5. Dialyzed fetal bovine serum (see Note 1)
6. Phosphate Buffered Saline (PBS)

Protocol:
For tips for avoiding contamination of the area, see note 2:
1. Seed a tissue culture plate with the cells of interest the day before the start of the labeling.
2. Rinse the cells with about 5 mL PBS and incubate them with inositol-free media for about 30 min (pre-labeling).
3. Add fresh inositol-free media (5 ml / P100) containing 1x (200 mM) L-glutamine, dialyzed serum (at the concentration in which your cells grow) and 10 µCi of [^{3}H] inositol per ml (see note 3)
4. Culture cells in labeling medium for 24-72 hrs (see note 4).
***At this point, you can proceed to extract the total phosphoinositide content (as described in steps 1 through 6 in protocol 3.2.), or use the labeled cells for subcellular fractionation (as described in protocol 3.3.).

3.2. Collection of total PI content:

1. Rinse the cells in 5 ml PBS. After removing the PBS, keep the plate tilted for a few seconds and remove the last drop of PBS with a P1000 pipet tip (see note 5).
2. Add 400µl of 1M HCl, then 400µl of methanol.
3. Scrape the cells and transfer to a 1.5 ml microfuge tube (see note 6) using a wide mouth P1000 pipet tip, clipped about 5 mm from the end (because the lysate will have a lot of white, insoluble material).
Optional: add carrier lipids to the tube to which the lysate is being transferred (see note 7).
4. Add 400µl chloroform, vortex very well (see note 8), centrifuge approximately 1 min in a microfuge at maximum speed (if using nanofuge, spin for at least 5 min) and collect bottom phase (organic phase) into a new microfuge tube (see note 9). The white, insoluble material should make a thin line at the interface. Avoid this material when collecting the lipids.
5. To each tube containing the organic phase, add 400 µl of a mixture of freshly prepared methanol : 0.1M EDTA pH8.0 (10:9, v:v). Vortex very well, spin and collect the bottom phase into a new microfuge tube (see note 10).
6. Evaporate the organic phase containing your lipids under a nitrogen stream and store the dried lipids at -80 ^{o}C until deacylation (protocol 3.4.1).

3.3. Subcellular fractionation:
Materials:
Cytosol buffer with phosphatase and protease inhibitors: 0.2 M sucrose; 25 mM HEPES, pH 7; 125 mM potassium acetate; 1 mM dithiothreitol; 1 mM sodium orthovanadate; 2 mg/ml sodium fluoride; 2 mg/ml β-glycerophosphate; 1 mM phenanthroline; 1 mM benzamidine and protease inhibitor cocktail (see note 11).
Sucrose solutions: using the cytosol buffer with phosphatase and protease inhibitors, make a 16% (w:vol) sucrose solution (light) and a 64% (w:vol) sucrose solution (heavy).

Protocol:
1. Prepare cytosol buffer (25 ml for four 100 mm plates)
2. Turn on the tabletop ultracentrifuge and set temp to 4^{o}C.
3. Pre-chill the rotor in cold room.
4. Start with one 100mm tissue culture dish, approximately 90-100% confluent, containing cells labeled with [^{3}H]-inositol as in protocol 3.1 (see note 12).
5. Treat cells as required for your particular experiment. At the end of the treatment, place cells on ice.
All steps below are conducted on ice with solutions at 4^{o}C.
6. Rinse plate 3 times with 5 ml PBS, removing excess in between washes (see note 13).
7. Rinse twice with cytosol buffer plus inhibitors, 2 ml per plate.

8. Remove excess buffer by tilting the plate and aspirating remaining liquid with a pipet, without drying cells (see note 14).
9. Scrape cells from plate: for optimal recovery, scrape the borders first and then tilt the plate towards you to scrape the center downward, letting the liquid flow to the bottom edge of the plate.
10. Transfer lysate to a microcentrifuge tube using a 29-gauge needle. Each plate of cells should yield about 150 μl of lysate. Pass cells through the needle about 6 to 12 times, avoiding creation of foam (see note 15).
11. The percentage of cell lysis will vary from cell line to cell line and from experiment to experiment. If you are running a non-radioactive test for protein analysis, you can check for lysis at this point by mixing 5 μl of the lysate with 5 μl of Trypan Blue reagent and visualizing on a hemocytometer with an inverted phase contrast microscope.
Optional: If lysis is less than 90%, spin the lysate in a nanofuge for 1 min, transfer the supernatant to a new microcentrifuge tube and pass the concentrated pellet through the 29-gauge needle a few more times, returning the supernatant back gradually.
12. Centrifuge the lysates at approximately 100 x g for 10 minutes at 4^{o}C to pellet the nuclear fraction and associated membranes.
13. Remove the supernatant (post-nuclear fraction) carefully with clean pipet tip and transfer to new centrifuge tube.
14. Resuspend the nuclear fraction in 200 μl of cytosol buffer.
Optional: pass the resuspended pellet through the same 29-Gauge needle 6-12 times and spin the tube at approximately 100 x g for 10 min to remove any remaining buffer. Remove supernatant and combine with the other supernatant, containing post-nuclear fraction.
15. Process the nuclear fraction by following protocol 3.3.1. and process the post-nuclear supernatant by following either protocol 3.3.2. (for separation of microsomal fraction through differential centrifugation) or protocol 3.3.3. (for separation of microsomal fractions through sucrose density gradient) (see note 16).

3.3.1. Separation of membrane associated with the nucleus from the nuclear fraction:
1. Resuspend the pellet in 200 μl of cytosol buffer and go through 3 freeze-thaw cycles (using dry ice and ethanol) to break remaining unbroken cells (see note 17).
2. Spin at approximately 100 x g for 10 min in cold room. Remove supernatant (we usually save this fraction for analysis and label it Fraction X, see note 18).
3. Resuspend the pellet with 200 μl of fresh cytosol buffer + 1% Triton X-100.
4. Rock for 10 min in cold room and spin at 16,000 x g for 10 min in cold room.
5. Remove and save supernatant (this is the fraction containing the Triton-soluble membranes associated with the nucleus).

6. Resuspend the pellet in 200 µl of fresh cytosol buffer + 1% Triton X-100 and save (this is the nuclear fraction).

3.3.2. Separation of microsomal fractions from cytosol by differential centrifugation:
1. If you opted to rinse the nuclear pellet as in step 14 of section 3.3 (above), combine the supernatant for each sample into one microcentrifuge tube.
2. Using a microfuge, spin the post-nuclear supernatant at 16,000 x g for 30 minutes.
3. Collect the supernatant and transfer to a thick-wall polycarbonate ultracentrifuge tube (11 x 34 mm). The pellet generated in step 2 is the heavy microsomal fraction and should be resuspended in 200 µl of cytosol buffer containing 1% Triton X-100 with protease and phosphatase inhibitors.
4. Balance and centrifuge the supernatant at 400,000 x g for 1 hr on a tabletop ultracentrifuge with fixed angle rotor (we use Beckman TLA120.2).
5. Remove supernatant carefully and transfer to new tube (this is the cytosol fraction).
6. Add 200 µl of cytosol buffer containing 1% Triton X-100 to pellet and let it soak for 45-60 min on ice (see note 19). Resuspend by pipetting up and down and transfer to a new tube, making sure that nothing is left behind (this is the light microsomal fraction).

3.3.3. Sucrose density fractionation of the microsomal fractions:
1. Prepare light (16%) and heavy (64%) sucrose solutions in cytosol buffer with protease and phosphatase inhibitors (4 ml of each is more than sufficient for four gradients).
2. Prepare 9 microfuge tubes with mixtures of the 2 sucrose solutions, such that the first tube (solution A) has 10% of the light sucrose and 90% of the heavy sucrose solution, the second tube (solution B) has 20% of light and 80% of heavy, etc., increasing the ratio of the light to heavy solution by 10% each time until you reach 90% light and 10% heavy sucrose solution (for solution I). Mix each tube well.
3. In a thick-wall polycarbonate centrifuge tube (11 x 34 mm), carefully overlay 100 µl of each sucrose solution, starting with the heaviest mixture (solution A) and ending with the 16% sucrose solution alone, for the 10^{th} layer.
4. Layer 200-250 µl of the post-nuclear supernatant on top of the discontinuous gradient, balance and spin at 250,000 x g for 4.5 hrs on a tabletop ultracentrifuge using a swinging bucket rotor (we use TLS55).
5. Collect 6 fractions of 200 µl each, starting from the top of the tube and going down, by placing the pipet tip just below the meniscus of the gradient and aspirating slowly as you move down with the meniscus (see note 20). Fractions are labeled 1-6, 1 containing the lightest and 6 containing the heaviest organelles and cellular debris.

3.4. Lipid or protein extraction of samples from subcellular fractionation protocols (see note 21):

If performing protein analysis of subcellular fractions from unlabeled cells, add 40 µl of 6x loading buffer containing reducing agent to all fractions and proceed to western-blot analysis, using conventional methods.

If performing PI analysis of labeled cells, proceed to steps 1 to 4, described below:

1. To each tube containing 200 µl of fractionated lysate, add 200 µl of 2M HCl, 400 µl of methanol, and 400 µl of chloroform.

Optional: add carrier lipids to the tube to which the lysate is being transferred (see note 22)

2. Vortex the tube well, and then centrifuge approximately 1 min in a microfuge at maximum speed to separate the organic and aqueous phases. Collect the bottom organic phase containing lipids into a fresh microfuge tube, as in section 3.2, step 7, for total lipids.

3. To each tube containing the organic phase, add 400 µl of a mixture of freshly prepared Methanol : 0.1M EDTA pH8.0 (10:9, v:v). Vortex very well, spin and collect the bottom phase into a new microfuge tube (see note 23).

4. Evaporate the organic phase containing your lipids under a nitrogen stream and store the dried lipids at -80 ^{o}C until deacylation (protocol 3.4.1).

3.4.1.Chemical Deacylation of the lipids:

1. Prepare fresh methylamine reagent in the chemical hood. For 10 ml (10 samples):

40% Methylamine in water	2.68 ml
H_2O	1.61 ml
Methanol	4.57 ml
n-Butanol	1.14 ml

2. Prepare a vacuum pump inlet trap by filling the bottom of the trap with 50-100 ml of H_2SO_4 and surrounding it with dry ice to freeze the acid and avoid aspiration. Connect it to the centrifugal evaporator, in between the centrifuge and the condenser.

3. Add 1ml methylamine reagent to each sample in a chemical fume hood.

4. Incubate samples at 53^{o}C for 50 min in well-sealed tubes. Cool samples to room temperature, quick spin to remove condensation, and dry them in the centrifugal evaporator. For proper set up of the centrifugal evaporator, see note 24.

5. Store dried lipids at -80 ^{o}C until fatty acid extraction (protocol 3.4.2).

3.4.2. Fatty acid extraction (see note 25):

1. Mix n-butanol/ petroleum ether/ ethyl formate at 20:4:1 (v:v:v) (we call this “fatty acid extraction mix”).

2. Label 2 sets of tubes per sample such that each set can be distinguished from each other and from the set of tubes already containing your sample.
3. Add 500 µl deionized H_2O and 500 µl fatty acid extraction mix to each deacylated, dried sample in the chemical fume hood. Vortex twice for at least 30 seconds and centrifuge for 1 minute at top speed.
4. Remove the bottom (aqueous) layer with a P200 (see note 26) and add it to the first set of tubes with 500µl fatty acid extraction mix already in it. Vortex twice for at least 30 seconds and centrifuge for 1 minute in a microfuge at top speed.
5. Extract the bottom (aqueous) layer using a P200 pipet tip and store in the empty set of labeled tubes.
6. Dry these samples by centrifugal evaporation (~2hrs) and store at -80 ^{o}C.

3.5. HPLC analysis of PIs

3.5.1. Preparing Samples for HPLC Injection:
1. Resuspend samples in 100-120 µl H_2O .
2. Add the appropriate standards to the sample (see note 27). Final volume should be about 120 µl.
3. Transfer to a microfuge spin filter and spin sample together with standards in a tabletop centrifuge, making sure to filter most of the sample volume.
4. If you have an automatic HPLC injector, transfer sample to a mini conical glass tube and insert into the HPLC sample holder, with all bubbles removed. You should have at least 110 µl.

3.5.2 Prepare the HPLC
1. For conventional method, install a 250 mm anionic exchange column. For PI-5-P separation, install two 250 mm anionic exchange columns in tandem (these must be Whatman Partisphere SAX columns).
2. Prepare 1M ammonium phosphate dibasic pH 3.8 or 6.0, for conventional or PI-5-P separation methods, respectively. Use phosphoric acid to adjust the pH.
3. Fill reservoir for pump A with HPLC grade H_2O.
4. Fill reservoir for pump B with 1M ammonium phosphate at appropriate pH.
5. Wash column as suggested by the vendor. We usually run H_2O followed by ammonium phosphate, then equilibrate with H_2O before running samples.
6. For conventional phosphoinositide separation, program the HPLC to run gradient A. For separation of PI-5-P from all other phosphoinositides, use gradient B:

Ammonium phosphate pH 3.8, gradient A:
Step 1A. H_2O 5 min

Step 2A. Ammonium phosphate gradient from 0% to 15% over 55 minutes
Step 3A. Isocratic 15% ammonium phosphate for 15 minutes
Step 4A. Ammonium phosphate gradient from 15% to 65% over 25 minutes
Step 5A. Isocratic 65% ammonium phosphate for 5 minutes
Step 6A. H_2O for 15 min

Ammonium phosphate pH 6.0 (see note 28), gradient B:
Step 1B. H_2O 5 min
Step 2B. Ammonium phosphate gradient from 0% to 1% over 5 minutes
Step 3B. Ammonium phosphate gradient from 1% to 4% over 60 minutes
Step 4B. Ammonium phosphate gradient from 4% to 15% over 5 minutes
Step 5B. Isocratic 15% ammonium phosphate for 20 minutes
Step 6B. Ammonium phosphate gradient from 15% to 65% over 25 minutes
Step 7B. Isocratic 65% ammonium phosphate for 5 minutes
Step 8B. H_2O for 15 min

3.5.3. Inject and run HPLC
1. Using an automatic or manual injector, inject 100 µl of sample into the HPLC.
2. Run ammonium phosphate gradient A or B, described above, at 1 ml/min.
3. If using an online flow scintillation analyzer, use UltimaFlo AP (Perkin Elmer) as your scintillation cocktail, running at 5ml/min. If using a 0.5 ml cell, set up detection for every 6 seconds (see note 29).
4. If using a fraction collector, collect fractions as you desire and as frequently as possible. Mix them with scintillation fluid and measure the counts in a scintillation counter. This will need to be optimized for your own system to avoid losing fractions of interest.
5. Wash column with 65% ammonium phosphate solution for 20 minutes followed by H_2O for 20 minutes.

3.5.4. Data Analysis:
Using HPLC analysis software, identify and quantify the peaks equivalent to each phosphoinositide species. Enter the values in an excel spreadsheet, correct and normalize the data according to your particular application. At this point, you may have to correct for any loss that may have occurred during the process. For example, if you only load 50% of the post-nuclear supernatant into the sucrose gradient, then the values from the sucrose gradient fractions need to be multiplied by 2. For analysis of fractionation data, we normalize the data two ways. First, we calculate the distribution of each species over the various fractions (see Figure 2, Table B). Second, we calculate the distribution of all PI species within a particular fraction (see Figure 2, Table

C). By adding the total counts from each PI species and comparing the results with the expected relative amount of each PI in samples prepared without fractionation, you can determine whether there was any loss or gain of PIs due to dephosphorylation.

Based on our previous analysis, the bulk of the PIs will fractionate with the nuclear-associated membranes (9). This fraction contains most of the endoplasmic reticulum (ER), where phosphatidylinositol is synthesized. The ER will also be present in the post-nuclear fractions and will be enriched in the heavy fractions of the density gradient (fractions 5 and 6) together with other heavy organelles, such as mitochondria and lysosomes. Plasma membrane derived microsomes will be enriched in fraction 4. Light organelles will be enriched in fractions 3 and 2 of the gradient. Fractions 1 and 2 will also contain cytosolic proteins. This pattern of distribution may vary from cell to cell.

4. Notes:

1. Dialyzed serum can be purchased or prepared in the lab using a small pore dialysis bag and performing the dialysis at 4^{o}C overnight against PBS, to remove inositol from the serum.
2. Always use proper personal protective equipment, including gloves, lab coat, and appropriate shielding for isotope in use; use pipet tips with cotton plug when pipetting radioactive material. During incubation, place cells in a labeled box or tray, with shielding if necessary. Cover benches with absorbent paper pad. Use plastic bag for disposal of all solid consumables, and add absorbent material to container for liquid waste. Label area, waste container, and incubation box with radiation tape. Always check for spills by wipe testing the area when finished. Monitor with Geiger counter when appropriate.
3. Some commercial [^{3}H]-inositol comes dissolved in ethanol. If the final concentration of ethanol in the labeling medium is likely to harm your cells, you may choose to first evaporate the ethanol in a microfuge tube and resuspend the dried inositol in inositol-free media.
4. The ideal labeling period will depend on each cell line. If the cell line grows well in the labeling medium, it should incorporate the [^{3}H]-inositol into lipids in approximately 24 to 48 hrs (equilibrium). HeLa cells label well within 48 hrs. However, slow-growing cells may need to be labeled for 72 hrs or more, assuming that they won’t die in the labeling medium. In order to optimize labeling time, perform a test labeling.
5. It is important to remove all PBS to avoid diluting the HCl in the next step.
6. Use good quality microfuge tubes. Some brands don't seal well and will leak during vortexing.
7. This is especially important if you are starting with few cells, to avoid loss of the [^{3}H]-labeled lipids. You can use crude brain phosphoinositides or a mixture of any lipids available.
8. When “vortexing” radioactive materials dissolved in organic solvents, use a paper wipe around the

9. When pipetting organic solvents, saturate the pipet tip first, to avoid dripping. When transferring chloroform from one tube to another, hold both tubes in one hand to shorten the distance between them and avoid sample loss and contamination of the work area.

10. At this point you can estimate the total counts obtained by measuring the cpm present in 1 µl of each sample using a scintillation counter and scintillation fluid. You should have at least 1,000,000 cpm total per sample.

11. Sodium orthovanadate, sodium fluoride and β-glycerophosphate are phosphatase inhibitors and are essential to the preservation of the phosphoinositide composition of the lysates during the fractionation. Other phosphatase inhibitors can be added to supplement these inhibitors.

12. Prior to using radioactive labeled cells, it is recommended to run through the protocol using unlabeled cells. This will allow for analysis of the distribution of organelle markers through western-blot and troubleshooting before conducting PI analysis.

13. Use plastic bag for disposal of all contaminated solid consumables, and add absorbent material to container for liquid waste.

14. Excess buffer is removed to reduce the total volume of the lysate to the minimum retained by the cell layer. The more concentrated the cells in the cytosol buffer, the higher the percentage of lysed cells after passage through the narrow needle.

15. Unlike other subcellular fractionation protocols, we avoid freezing and thawing at this stage, to avoid disrupting organelles. Passing the cells through a small needle will mechanically disrupt the cells without affecting the structure of most organelles. However, creation of foam may lead to protein denaturation and should be avoided.

16. Protocol 3.3.2 separates the microsomes based on size to isolate heavy microsomes from light microsomes and from cytosol. This is a faster fractionation protocol and suitable for quickly isolating small cytosolic vesicles such as Golgi-derived vesicles (COPI and COPII-containing vesicles) from other organelles and plasma membrane, and from nucleus and nuclear-associated membranes. Protocol 3.3.3. is more time consuming, but allows for separation of dense organelles, such as mitochondria and lysosome, from lighter organelles, such as smooth ER and Golgi. Protocol 3.3.3 also allows for separation of plasma membrane-derived microsomes from the other organelles.

17. By freezing and thawing the lysates several times, you should achieve 100% lysis. Thus, this step allows removal of any contaminating post-nuclear material from unbroken cells.

18. Fraction X may contain a mixture of organelles. It will contain, for example, the microsomes from the leftover unbroken cells. We save and analyze this fraction to ensure that no material was lost at the end of the protocol.

19. You can also freeze the resuspended pellet overnight.

20. After centrifugation, the total volume may decrease such that the last fraction may have less than 200 µl. Be sure to record the volume of the last fraction for normalization during data analysis. If you loaded more than 200 µl of lysate on top of the gradient, you should collect 250 µl (or your actual load volume) as your first fraction.

21. We recommend running the procedure for protein extraction and western-blot analysis prior to any lipid analysis to confirm the separation of various organelles using antibodies against proteins markers. We also recommend checking the density of the collected fractions using a refractometer. When performing protein extraction for western-blot and density measurements, cells should not be labeled with [^{3}H]-inositol.

22. This is especially important if you are starting with few cells, to avoid loss of the [^{3}H]-labeled lipids. You can use crude brain phosphoinositides from Sigma or a mixture of any lipids available.

23. At this point you can estimate the total counts obtained by measuring the cpm present in 1 µl of each sample using a scintillation counter and scintillation fluid.

24. Make sure a dry ice H_2SO_4 trap is properly fixed to the centrifugal evaporator, to allow circulation of the vapors from the samples into the trap, without aspiration of the acid into the pump. After 2 hrs have passed, remove the H_2SO_4 trap and continue until lipids are dry. Avoid overnight usage of the centrifugal evaporator. Monitor the increasing volume of the sulfuric acid/methylamine reagent mixture and empty when necessary to prevent contamination of your samples or aspiration by the pump. Store H_2SO_4 trap in a chemical fume hood and dispose of waste properly.

25. We are interested in the PI head group only. This step is to clean the sample from the fatty acid chains and from any remaining lipids and thus avoid clogging the HPLC column.

26. Using a P200 gives better control and prevents sample loss and contamination of work area. Do not use P1000 pipet tips, they are too large for 1.5 ml microcentrifuge tubes containing 1 ml sample and the mixture will spill over the tube.

27. PI-4-P and PI-4,5-P_2 peaks are easy to identify, as they are the most abundant PIs in cells. Therefore, we don't need standards for these lipids. We use [^{32}P] labeled standards to identify the peaks corresponding to the less abundant PIs. For example, we prepare [^{32}P]-labeled PI-3-P, PI-3,4-P_2, PI-3,5-P_2 and PI-3,4,5-P_3 using baculovirus-expressed PI3-kinase and the appropriate precursor as substrate.

28. We found that using ammonium phosphate pH 6.0 makes the PI-4-P and PI-5-P peaks narrower and helps avoid overlapping bases. However, with this method we sometimes loose good separation of PI-3,4-P_2 from PI-4,5-P_2.

29. In order to cut on the volume of scintillation fluid used per sample, we often opt to start detection after 45 minutes into the gradient, just before PI-3-P elutes. This can only be done if you opt to use the total counts, rather than the PI counts, for normalization.

References:

1. Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443 (7112):651-657
2. De Matteis MA, Godi A (2004) PI-loting membrane traffic. Nature cell biology 6 (6):487-492
3. Downes CP, Gray A, Lucocq JM (2005) Probing phosphoinositide functions in signaling and membrane trafficking. Trends Cell Biol 15 (5):259-268
4. Hammond GR, Schiavo G, Irvine RF (2009) Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P(2). The Biochemical journal 422 (1):23-35
5. Balla T (2005) Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J Cell Sci 118 (Pt 10):2093-2104
6. Carlton JG, Cullen PJ (2005) Coincidence detection in phosphoinositide signaling. Trends Cell Biol 15 (10):540-547
7. Graham JMH, J.A. (1997) Membrane Analysis. The Introduction to Biotechniques. Bios Scientific Publishers, New York
8. Patki V, Virbasius J, Lane WS, Toh BH, Shpetner HS, Corvera S (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. Proceedings of the National Academy of Sciences of the United States of America 94 (14):7326-7330
9. Sarkes D, Rameh LE (2010) A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. The Biochemical journal 428 (3):375-384

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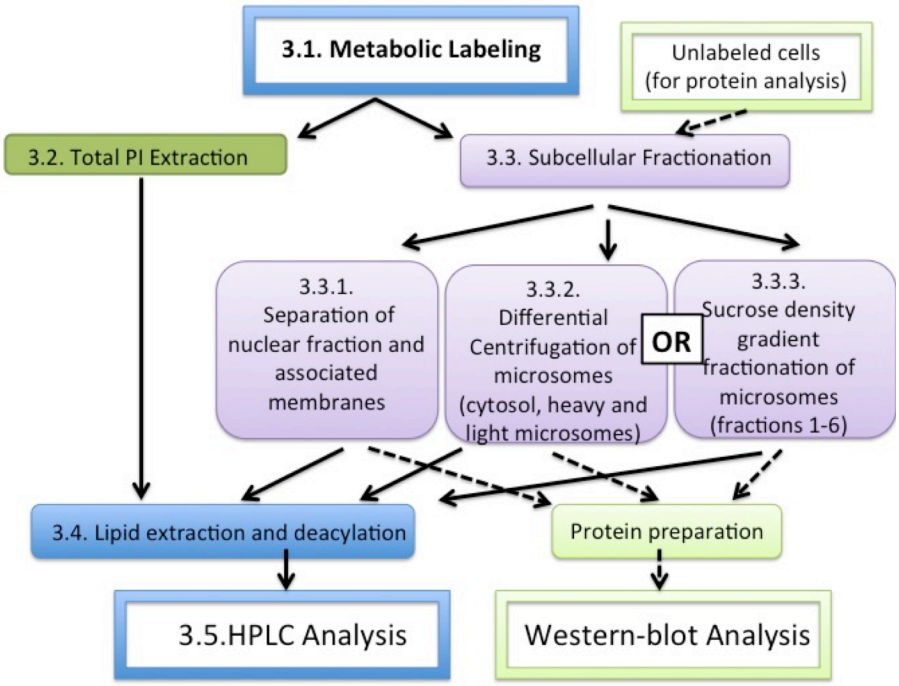

Sarkes and Rameh, Figure 1

TABLE A	PI-3-P	PI-4-P	PI-5-P	PI-3,5-P2	PI-3,4-P2	PI-4,5-P2	PIP3	total PIPs	PtdIns	total
Fx 1	1	2	3	4	5	6	7	28	100	128
Fx 2	2	3	4	5	6	7	8	35	101	136
Fx 3	3	4	5	6	7	8	9	42	102	144
Fx4	4	5	6	7	8	9	10	49	103	152
Fx5	5	6	7	8	9	10	11	56	104	160
Fx 6	6	7	8	9	10	11	12	63	105	168
Triton	7	8	9	10	11	12	13	70	106	176
Nuclear	8	9	10	11	12	13	14	77	107	184
Fx X	9	10	11	12	13	14	15	84	108	192
total	45	54	63	72	81	90	99	504	936	1440

TABLE B	PI-3-P	PI-4-P	PI-5-P	PI-3,5-P2	PI-3,4-P2	PI-4,5-P2	PIP3	total PIPs	PtdIns	total
Fx 1	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.28	1	1.28
Fx 2	0.01980198	0.02970297	0.03960396	0.04950495	0.05940594	0.06930693	0.07920792	0.34653465	1	1.34653465
Fx 3	0.02941176	0.03921569	0.04901961	0.05882353	0.06862745	0.07843137	0.08823529	0.41176471	1	1.41176471
Fx4	0.03883495	0.04854369	0.05825243	0.06796117	0.0776699	0.08737864	0.09708738	0.47572816	1	1.47572816
Fx5	0.04807692	0.05769231	0.06730769	0.07692308	0.08653846	0.09615385	0.10576923	0.53846154	1	1.53846154
Fx 6	0.05714286	0.06666667	0.07619048	0.08571429	0.0952381	0.1047619	0.11428571	0.6	1	1.6
Triton	0.06603774	0.0754717	0.08490566	0.09433962	0.10377358	0.11320755	0.12264151	0.66037736	1	1.66037736
Nuclear	0.07476636	0.08411215	0.09345794	0.10280374	0.11214953	0.12149533	0.13084112	0.71962617	1	1.71962617
Fx X	0.08333333	0.09259259	0.10185185	0.11111111	0.12037037	0.12962963	0.13888889	0.77777778	1	1.77777778
total	0.04807692	0.05769231	0.06730769	0.07692308	0.08653846	0.09615385	0.10576923	0.53846154	1	1.53846154

TABLE C	PI-3-P	PI-4-P	PI-5-P	PI-3,5-P2	PI-3,4-P2	PI-4,5-P2	PIP3	total PIPs	PtdIns	total
Fx 1	0.02222222	0.03703704	0.04761905	0.05555556	0.0617284	0.06666667	0.07070707	0.05555556	0.10683761	0.08888889
Fx 2	0.04444444	0.05555556	0.06349206	0.06944444	0.07407407	0.07777778	0.08080808	0.06944444	0.10790598	0.09444444
Fx 3	0.06666667	0.07407407	0.07936508	0.08333333	0.08641975	0.08888889	0.09090909	0.08333333	0.10897436	0.1
Fx4	0.08888889	0.09259259	0.0952381	0.09722222	0.09876543	0.1	0.1010101	0.09722222	0.11004274	0.10555556
Fx5	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111	0.11111111
Fx 6	0.13333333	0.12962963	0.12698413	0.125	0.12345679	0.12222222	0.12121212	0.125	0.11217949	0.11666667
Triton	0.15555556	0.14814815	0.14285714	0.13888889	0.13580247	0.13333333	0.13131313	0.13888889	0.11324786	0.12222222
Nuclear	0.17777778	0.16666667	0.15873016	0.15277778	0.14814815	0.14444444	0.14141414	0.15277778	0.11431624	0.12777778
Fx X	0.2	0.18518519	0.17460317	0.16666667	0.16049383	0.15555556	0.15151515	0.16666667	0.11538462	0.13333333
total	1	1	1	1	1	1	1	1	1	1

Sarkes and Rameh, Figure 2