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Monitoring phosphatidic acid signaling in breast cancer cells using genetically encoded biosensors

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i. Summary/Abstract

Phospholipids are important signaling molecules that regulate cell proliferation, death, migration and metabolism. Many phospholipid signaling cascades are altered in breast cancer. To understanding the functions of phospholipid signaling molecules, genetically encoded phospholipid biosensors have been developed to monitor their spatiotemporal dynamics. Compared to other phospholipids, much less is known about the subcellular production and cellular functions of phosphatidic acid (PA), partially due to the lack of a specific and sensitive PA biosensor in the past. This chapter describes the use of a newly developed PA biosensor, PASS, in two applications: regular fluorescent microscopy and fluorescence lifetime imaging microscopy-Förster/fluorescence resonance energy transfer (FLIM-FRET). These protocols can be also used with other phospholipid biosensors.

Keywords

phospholipid; phosphatidic acid; PASS; cancer; immunofluorescence; fluorescence lifetime imaging microscopy; FLIM; fluorescence resonance energy transfer; FRET

1. Introduction

Phospholipids are not only the building components of cellular membranes, they can also act as signaling molecules to regulate cell proliferation, death, migration and metabolism (1,2). Several phospholipids have been demonstrated to function as signaling molecules, *e.g.*, diacylglycerol (DG), phosphatidylinositol 4,5-bisphosphate (PI4,5P₂), phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P₃), phosphatidylinositol 3-phosphate (PI3P), and phosphatidic acid (PA) (1,2). These phospholipids specifically bind to a variety of effector proteins, and thus modulate their enzymatic activity and subcellular localization, which are critical for normal physiological processes. The alterations of phospholipid-regulated cellular processes directly contribute to human diseases including cancer (1,3,4). For example, the PI3K/PTEN pathway is one of the most mutated oncogenic pathways in breast cancer (1,5).

Cellular phospholipid levels are tightly controlled by external signals. Activation of lipid metabolizing enzymes, such as phospholipases, lipid kinases, and phosphatases at different

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times and subcellular locations, can lead to distinct signaling outcomes (2,6). Therefore, faithfully monitoring where and when signaling phospholipids are generated is critical for the understanding of their functions. Biochemical methods, such as thin-layer chromatography, high-performance liquid chromatography, and mass spectrometry (7), can only measure total cellular phospholipid levels, and cannot precisely monitor the spatiotemporal production of a particular phospholipid. To circumvent these limitations, genetically encoded phospholipid biosensors have been developed by fusing green fluorescent protein (GFP) or other fluorescent proteins to protein domains or motifs that bind to a specific phospholipid, *e.g.*, the PH domain of PLC δ for PI4,5P $_2$, the PH domain of AKT1 for PI3,4,5P $_3$, the FYVE domain of EEA1 for PI3P $_3$, and the C1 domain of PKC δ for DG (8,9). These biosensors have greatly advanced our knowledge of phospholipid signaling and function (2,8).

Compared to DG and phosphoinositides, much less is known about the subcellular production and cellular functions of PA, partially due to the lack of a specific and sensitive PA biosensor in the past. Signaling PA can be produced by multiple enzymes, including two well-known families of enzymes: phospholipase D (PLD) and DG kinase (DGK) (10–12). Several PLD and DGK family members have been demonstrated to promote cancer cell proliferation, survival, and migration (4,12–15). This chapter describes the use of a recently developed PA biosensor; PA biosensor with superior sensitivity (PASS) (16) in two applications: regular fluorescent microscopy and fluorescence lifetime imaging microscopy-Förster/fluorescence resonance energy transfer (FLIM-FRET). These protocols can be also used for other genetically encoded phospholipid biosensors.

2. Materials

1. PASS constructs: see details in Methods.
2. Lentiviral packaging plasmids: pMDLg/pRRE, pRSV-Rev and pMD2.g (Addgene).
3. TLA-293T cells (Thermo Fisher).
4. MCF-7 cells (American Type Culture Collection, ATCC).
5. HCC1806 cells (American Type Culture Collection, ATCC).
6. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone).
7. RPMI-1640 medium supplemented with 10% FBS (Hyclone).
8. 6- and 12-well cell culture plates (Greiner Bio-One).
9. Opti-MEM medium (Thermo Fisher)
10. Lipofectamine and Plus reagent (Thermo Fisher).
11. Polypropylene falcon tubes.
12. Polybrene stock (10 mg/mL).

13. Glass coverslips, 25 mm, #1 thickness (Thermo Fisher).
14. Microscope slides (Thermo Fisher).
15. Phosphate-buffered saline (PBS) without calcium and magnesium: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 mL of distilled H₂O. Add additional distilled H₂O until the volume is 1 L. Adjust pH to 7.4 with 1 M HCl. Store at room temperature.
16. Epidermal Growth Factor (EGF, AF-100-15) from Peprotech. The stock concentration is 100 µg/mL.
17. 4 % paraformaldehyde (PFA) solution: Dissolve 0.4 g of paraformaldehyde powder in 10 mL of PBS. Heat the solution in a 60°C water bath until the powder is completely dissolved. Store in a refrigerator and use within 1 week.
18. 0.1% Triton X-100 in PBS: Dilute from 10% Triton X-100 using PBS.
19. Blocking solution: 5% normal goat serum in PBS.
20. Mounting medium: 4% *n*-Propyl gallate (w/v) in 90% glycerol (v/v) and 10% PBS. Aliquot and store in a freezer.
21. Clear nail polish.
22. Straight tip forceps.
23. 50 mM Ammonium Chloride in PBS: Dilute from 1 M NH₄Cl in PBS stock solution. For 500 mL of 1 M stock solution, dissolve 132.5 mg of NH₄Cl in PBS. Stock solution can be stored at room temperature.
24. Mowiol mounting medium: Combine 2.4 g of Mowiol 4–88, 6 g of glycerol, 6 mL of H₂O, and 12 mL of 0.2 M Tris-Cl (pH 8.5). Store aliquots in a freezer. Mowiol can be stored in a refrigerator for less than one month.
25. 35 mm glass bottom dishes, #1 thickness (MatTek).
26. 1 µM fluorescein (Sigma).

3. Methods

3.1 Regular fluorescent microscopy

The PASS biosensor was derived from the PA-binding domain of yeast Spo20 (Spo20-PABD) by fusing Spo20-PABD to the nuclear export sequence of protein kinase A alpha (PKI-alpha)(16). The exclusive cytoplasmic localization of PASS, in contrast to the nuclear localization of the original Spo20-PABD, significantly improves its sensitivity of PA binding. PASS biosensors fused with the monomeric GFP or red fluorescent protein (RFP) are cloned in either a regular mammalian expression vector or a lentiviral vector (Figure 1). The RFP-PASS-4E is a mutant version of PASS that does not bind to PA, and can be used as a control to determine whether the observed localization of the biosensor reflects lipid

binding or interaction with protein molecules, as have been used for phosphoinositide biosensors (8).

3.1.1 Transient Transfection

1. One day before transfection, seed MCF-7 or other breast cancer cells into 6-well plates (approximately 2×10^5 – 5×10^5 cells per well). For optimal transformation efficiency, cells should be about 50–60% confluent on the day of transfection. Plate cells in a 6-or 12-well plate containing coverslips if cells will be used for imaging (see Note 1).
2. On the day of transfection, add 1 μ g of plasmid DNA into a 1.5 mL microfuge tube.
3. Dilute DNA with 100 μ L of Opti-MEM and add 6 μ L of Plus reagent. Mix well and incubate for 15 minutes (see Note 2).
4. Dilute 4 μ L of Lipofectamine reagent in 100 μ L of Opti-MEM in a new microfuge tube and mix well. Add this mixture to the tube containing DNA and mix well. Incubate for 15–30 minutes.
5. During incubation, aspirate media from target cells and add 800 μ L of pre-warmed Opti-MEM to each well (see Note 3).
6. Add the DNA and Lipofectamine mixture onto the target cells. Incubate cells for 4–5 hours in a 37°C incubator with 5% CO₂.
7. Replace Opti-MEM with DMEM supplemented with 10% FBS.
8. For transient protein expression, cells can be used for image analysis 24–48 hours after transfection.

3.1.2 Generation of Lentivirus and Lentiviral Infection of Target Cells

1. 24 hours before transfection, seed TLA-293T cells in a 6-well plate (approximately 0.5×10^6 – 1×10^6 cells per well). Cells should be 40–50% confluent on the day of transfection.
2. Transfect cells using Lipofectamine and Plus reagent or other transfection reagents as described in 3.1.1. Use 1 μ g of total DNA for each well (160 ng of PASS lentiviral construct, 400 ng of pMDLg/pRRE, 200 ng of pRSV-Rev, and 240 ng of pMD2.g)(see Note 4).
3. Harvest lentivirus-containing medium and pool similar stocks if desired (see Note 5).

¹Some breast cancer cell lines, *e.g.*, HCC1806, are very difficult to transfect. If transfection efficiency is lower than 10%, image quantification will be challenging. To more efficiently deliver genes of interest, lentiviral infection or other transfection reagents can be used for these cell lines.

²Both Lipofectamine and Plus reagents tend to adhere to polypropylene microfuge tubes. When adding reagents, pipette directly into the middle of the Opti-MEM medium, avoiding the sides of the tube. Alternatively, polystyrene tubes can be used to decrease the amount of DNA-lipid complexes that bind to the side of the tubes.

³Aspirate and pipette gently when handling TLA-293T packaging cells since they detach from the plate very easily.

⁴Third-generation lentiviral packaging plasmids are described in this protocol, but the second-generation packaging plasmids can also be used for PASS in the lentiviral vector. Extra care should be taken when handling second-generation lentiviral packaging plasmids.

4. Transfer medium to a polypropylene tube, and spin at 500 g for 5 minutes to pellet remaining packaging cells.
5. Transfer viral supernatant to a polypropylene tube and discard remaining packaging cells.
6. Repeat viral harvesting after 24 hours. If virus will not be used immediately, freeze in a -80°C freezer.
7. Plate HCC1806 cells on coverslips in a 6- or 12-well plate 16–24 hours before lentiviral infection. At the time of infection, cells should be around 40–50% confluent.
8. Add 1–2 mL of viral supernatant to a single well. Add polybrene to a final concentration of 1 $\mu\text{g/mL}$ (see Note 6).
9. Remove and discard virus-containing medium and replace with fresh growth medium 8–24 hours after infection.
10. Continue to incubate HCC1806 cells for 24–48 hours to allow for expression of the sensor (see Note 7).
11. Plate cells on coverlips in a 12- or 6-well plate for imaging.
12. Cells expressing RFP-PASS can be selected with 0.1–0.2 $\mu\text{g/mL}$ puromycin to generate cells stably expressing RFP-PASS (see Note 8).

3.1.3 EGF Stimulation of Cells and Slide Preparation

1. Serum starve cells expressing PASS constructs generated from 3.1.1 or 3.1.2 from 10% FBS RPMI-1640 to 0.1% FBS RPMI-1640. Incubate cells overnight in 0.1% FBS RPMI-1640 overnight (see Note 9).
2. Stimulate cells with 100 ng/mL of EGF for 0.5–30 minutes.
3. Wash cells with PBS once or twice to remove serum and phenol red at room temperature. This step is optional (see Note 10).
4. Fix cells with 4% paraformaldehyde for 10 minutes at room temperature.

⁵BL2 safety practices should be followed when preparing and handling lentiviral particles. Personal protective clothing should be worn at all times. Use plastic pipettes in place of glass pipettes or needles. Liquid waste should be decontaminated with at least 10% bleach. Laboratory materials that come in contact with viral particles should be treated as biohazardous waste and autoclaved. Please follow all safety guidelines from your institution and from the CDC and NIH for work in a BL2 facility.

⁶Polybrene is a polycation that reduces charge repulsion between the virus and the cell membrane. The optimal final concentration of polybrene can be determined with titration experiments, but is usually between 1–12 $\mu\text{g/mL}$. Overexposure to polybrene (>24 hours) can be toxic to cells. Certain cell types may be more sensitive to polybrene than the others. Other transduction reagents may be considered for these cell types, such as Retronectin (Clontech).

⁷The fluorescent signal of GFP and RFP may be observed 24 hours after infection; however, it may be very weak. The maximal fluorescent signal should be observed 48–72 hours after infection.

⁸Different cell lines have different sensitivities to puromycin. Additionally, the effective concentration of puromycin from different companies can vary. To induce efficient cell death of non-infected cells while reducing undesired stress responses, it is necessary to perform a titration experiment for puromycin concentrations when new cell lines are used.

⁹Medium containing 0.1% FBS can significantly reduce the basal growth factor signaling. Medium without FBS may cause unnecessary stress responses for some cells.

¹⁰Although PBS washes can remove fluorescent background, it may also alter intracellular signaling. To faithfully monitor changes in PA levels, it is preferred to fix cells directly without PBS washes.

5. Wash fixed cells with PBS for 5 minutes, repeat 2 more 3 times.
6. Pipette 3–5 μ L of anti-fade mounting medium per slide (See Note 11).
7. Using forceps, gently place the sides of coverslips on a Kimwipe to remove excess liquid.
8. Mount coverslips with cell side down onto the mounting medium.
9. Aspirate excess mounting medium and allow it to air dry before sealing the coverslips with clear nail polish.
10. Store slides in a refrigerator protected from light.
11. Examine the slides under a regular fluorescent microscope or a laser scanning confocal microscope. A typical image of GFP-PASS before and after EGF stimulation is shown in Figure 2.

3.1.4 Data Quantification and Analysis—Activation of many cell surface receptors changes the level of several signaling phospholipids including PA on the plasma membrane by activating their metabolizing enzymes (1,10). The plasma membrane translocation of PASS biosensor from cytoplasm can be used to examine the increase of PA production. One of the most common ways to evaluate the plasma membrane localization of a phospholipid biosensor is to calculate the ratio of plasma membrane to cytoplasmic intensity using a line-intensity histogram from a selected line spanning the cell with ImageJ or Fiji (8).

1. Open the selected image in ImageJ or Fiji.
2. Using the Line Tool, draw a line through the cell, including both the plasma membrane and cytoplasm (see Note 12).
3. Save a version of this image with an arrow delineating where the line was drawn for data measurements.
4. Pixel intensity along the drawn line can be plotted using “Plot Profile” under the Analyze menu. Pixel distance along the line is plotted on the X-axis while pixel intensity is plotted on the Y-axis. These data can be saved using List or Copy and analyzed with other software such as Excel or R as desired. The Live option can be selected while drawing lines in different sections of a particular cell before choosing a final cross-section.
5. The histogram generated will resemble peaks with valleys. In the case where the PASS sensor is localized to the plasma membrane, there will be 2 peaks with a valley in between. The peak values will be used as plasma membrane intensity values. The cytoplasmic intensity value will be the average of the valley values. Background pixel intensity should be subtracted from average plasma membrane and cytoplasmic values.

¹¹It is important to choose a correct mounting medium for certain fluorophores. Many commercial anti-fade mounting media do not work well for far-red fluorophores. n-Propyl gallate is a very general anti-fade mounting medium that is suitable for most commonly used fluorophores.

¹²At least 3 line-intensity histograms should be generated for a single cell to account for variation among cells.

6. To determine the change of plasma membrane localized PA, the ratio of plasma membrane pixel intensity to cytoplasmic pixel intensity can be calculated. A higher plasma membrane/cytoplasmic pixel intensity ratio can be interpreted as increased PA production on the plasma membrane. The plasma membrane/cytoplasmic pixel intensity ratio will remain unchanged if there is no increase of PA on the plasma membrane.

3.2 FLIM-FRET

Membranes are comprised of subdomains with different physical and signaling properties. The proper localization of signaling molecules in membrane domains such as lipid rafts determines the activity of many signaling pathways, and disruption of lipid rafts or lipid raft localization of signaling molecules has been implicated in human diseases (17,18). Thus, to understand how PA regulates cell functions, it is important to know where PA is generated. The precise site of lipid signaling events can be measured by the production of a specific lipid in close proximity of a membrane marker, *e.g.*, the lipid raft marker tH (the C-terminal tail of H-Ras) or the non-lipid raft marker tK (the C-terminal tail of K-Ras) (19), using FLIM-FRET(20,21). FRET is a distance dependent non-radiative transfer of energy from a donor molecule in an excited state to a nearby acceptor molecule in the ground state (within 10 nm). In a FLIM setup, FRET is measured as a decrease in fluorescence lifetime of the donor fluorophore (22). Some advantages of FLIM-FRET are that FLIM-FRET measurements are more robust and quantitative compared to other methods to measure FRET efficiency and are independent of the expression levels of donors and acceptors (22).

To efficiently express donor-acceptor pairs in breast cancer cells, we have generated lentiviral constructs expressing donor alone (GFP-tK or GFP-tH), or bicistronic constructs expressing donor and acceptor simultaneously (GFP-tK and RFP-PASS, or GFP-tH and RFP-PASS) (Figure 3).

3.2.1 Lentiviral Infection and Slide Preparation

1. Infect HCC1806 cells with lentivirus carrying the GFP donor, or the GFP donor plus RFP acceptor as described in 3.1.2.
2. Continue to incubate cells for 48 hours to allow for expression of the sensor.
3. Switch culture medium from 10% FBS RPMI-1640 to 0.1% FBS RPMI-1640. Incubate cells expressing PASS constructs in 0.1% FBS RPMI-1640 overnight (see Note 9).
4. Stimulate cells with 100 ng/mL EGF (0–30 minutes).
5. Fix cells with 1 mL per well of 4% paraformaldehyde for 10 minutes at room temperature in the dark.
6. Wash cells twice with PBS, each time for 5 minutes.
7. Add 1 mL of 50 mM Ammonium Chloride per well to quench free aldehyde groups that can produce nonspecific fluorescent background.

8. Incubate for 10 minutes at room temperature in the dark.
9. Wash four times with PBS, each time for 5 minutes.
10. Rinse once with ddH₂O.
11. Pipette 3–5 μ L of Mowiol mounting medium per slide. Avoid creating bubbles.
12. Using forceps, gently place one side of the coverslips on a Kimwipe to remove excess liquid.
13. Mount coverslips with cell side down on the Mowiol drops. Do not press down on the coverslips.
14. Dry slides in a 37°C incubator for one hour or at room temperature overnight. Protect from light.
15. Store slides in a refrigerator protected from light.

3.2.2 Data Acquisition and Analysis

1. Choose the 497 nm light emitting diode (LED) bulb.
2. Select the GFP filter.
3. Turn on the power strip for the computer, LIFA signal generator, and Nikon TiE widefield microscope. In this protocol, the Lambert Instruments FLIM Attachment is installed on a Nikon TiE widefield microscope.
4. Start LIFA-FLIM software.
5. Turn on LED DC and adjust the LED beam to the center (see Note 13).
6. Select the 60x oil objective. Using a transfer pipette, place a drop of 1 μ M Fluorescein on the 35 mm MatTek glass bottom dish. This will be used as a lifetime reference standard, which has a lifetime of 4.0 ns (see Note 14).
7. Set exposure time at 100–150 ms, MCP at 670–700 V, frequency at 40 MHz, and phase at 330 (see Notes 15 and 16). Set up your working directory where your files will be saved.
8. Turn on LED DC, adjust focus on bright view through eyepiece or camera, select 3–4 regions of interest (ROIs), activate the FLIM button, and adjust MCP/exposure so that intensity is just below maximal (around 60,000 ADU in Statistics tab).

¹³The LED should be allowed to warm up for at least 30 minutes before imaging slides to avoid light fluctuations while acquiring data.

¹⁴A new reference measurement should be determined for every experiment.

¹⁵Since phase and modulation lifetimes are dependent on the excitation power level used, any changes to the power level will render any data generated unusable for comparative analysis. Do not change LED power, MCP voltage, filter sets, or the objective between measurements.

¹⁶Phase settings will vary depending on the FLIM software used for analysis. It is highly recommended to test the FLIM setup and software with a mock experiment before conducting any important experiments.

9. Select Idle, record Reference, and save it.
10. Set up and focus on the sample, making sure all parameters are the same as used to record reference fluorescence lifetime, activate FLIM, and adjust exposure time so that intensity is around 55,000–60,000 ADU. Do not change MCP voltage.
11. Record the FLIM analysis of the samples (see Note 17). Select ROIs for the cells in the Camera tab, select Lifetime tab and Statistics tab, and export all statistics data. Save images for both Camera and Lifetime views (see Note 18).
12. Open exported statistics data saved in Excel format and compare lifetime (phase) values (τ) between the cells expressing donor only (GFP) and donor + acceptor (GFP + RFP).

A minimum of 30 ROIs measured per sample group is recommended for statistical analysis of a single experiment. Fluorescence lifetimes for one sample group can be averaged and compared to other sample groups for significant differences using ANOVA. Decreases in GFP fluorescence lifetime in the GFP + RFP group versus the GFP only group reflect FRET. Alternatively, decreases in GFP fluorescence lifetime in the GFP + RFP group after EGF stimulation can be also used to reflect FRET. Using the fluorescent protein-fused biosensors and FLIM-FRET, PA is found to be produced in a membrane microdomain labeled by tK in EGF-stimulated HCC1806 breast cancer cells (Figure 4).

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¹⁷These files can be opened for image analysis later.

¹⁸Lifetime images are pseudocolored based on the timescale range chosen. The scale can be adjusted if needed to include the maximum and minimum fluorescence lifetimes measured.

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Figure 1. Schematic maps of GFP- and RFP-PASS plasmids

GFP-PASS and RFP-PASS are cloned in a regular mammalian expression vector derived from pEGFP-C1 (Clontech), or in the lentiviral expression vectors pCDH-CMV-MCS or pCDH-CMV-MCS-EF1-Puro (System Biosciences). 4E is a PA-binding deficient mutant. GFP-2xPASS contains a tandem PASS. The plasmids, maps, and sequences will be distributed upon request.

Figure 2. Plasma membrane production of PA in EGF-stimulated HCC1806 breast cancer cells
HCC1806 cells were infected with lentivirus carrying GFP-PASS. Two days after infection, cells were serum starved overnight, then stimulated with or without 100 ng/mL EGF for 3 minutes. Images were captured using a Nikon A1 laser scanning confocal microscope.

Figure 3. Schematic maps of lentiviral expression plasmids for FLIM-FRET experiments

These plasmids contain either the donor alone (GFP-tH or GFP-tK), or both donor and acceptor in the same vector (RFP-PASS and GFP-tH, or RFP-PASS and GFP-tK). The use of a modified IRES (internal ribosome entry site), IRES2, in these constructs allows expression of both donor and acceptor in all infected cells, leading to more consistent results. The plasmids, maps, and sequences will be distributed upon request.

Figure 4. Spatiotemporal production of PA in a membrane microdomain labeled by tK in EGF-stimulated HCC1806 breast cancer cells

(A) Changes in fluorescence lifetime in cells expressing GFP-tK alone or GFP-tK + RFP-PASS. Cells were serum starved overnight, then stimulated with 100 ng/mL of EGF for the indicated time. Cells were imaged in the frequency domain using a wide-field FLIM-FRET microscope. A minimum of 50 cells was quantified per experiment. N=3. *, $p < 0.05$; **, $p < 0.01$ versus 0 minutes (no EGF stimulation). (B) Representative fluorescence lifetime images.