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Double immunostaining for PIP2 and phosphorylated ERK

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Hiraku Miyagi[1,2], Michio Hiroshima[1,2,3], Yasushi Sako[1,2]

[1]Cellular Informatics Laboratory, RIKEN; [2]CREST, JST; [3]Laboratory for Cell Signaling Dynamics, RIKEN

Hiraku Miyagi
Cellular Informatics Laboratory, RIKEN

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We use this protocol and it's working

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Abstract

Immunofluorescence microscopy is used to determine molecular localization and to measure fluorescence intensity of activity in cultured cells. Reagents for fixation and/or permeabilization must be suitable for the molecule of interest. We developed a procedure to immunostain phosphatidylinositol biphosphate (PIP_2) and phosphorylated Extracellular signal-Regulated Kinase (ERK) simultaneously. PIP_2 is a phospholipid component of cell membranes. ERK is a mitogen-activated protein kinase (MAPK) that localized in the cytoplasm and nucleus after phosphorylation triggered by ligand activation.

Different compounds are used to permeabilize cells in order to stain for these two molecules. Digitonin is used for PIP_2 and Triton X-100 is used for phosphorylated ERK. However, since Triton X-100 can solubilize cell membrane lipids, it is not suitable for PIP_2 labeling. In order to confer resistance against Triton X-100, we devised a method to fix anti-PIP_2 antibody on the cell membrane antigen before permeabilization with Triton X-100.

We modified a conventional immunostaining method to include secondary fixation and permeabilization after anti-PIP_2 antibody labeling. The secondary fixation probably immobilizes antibodies bound to PIP_2 on the cell membrane, perhaps by cross-linking them to surrounding protein molecules. Whatever the mechanism, this fixation enables PIP_2-antibody complexes to remain on the membrane, despite treatment with Triton X-100, which is used to visualize phosphorylated ERK.

Since PIP_2 and ERK are major cell signaling molecules in various cellular phenomena, simultaneous detection and visualization of their phosphorylation states by this method should be useful for many studies.

Materials

MATERIALS

- Paraformaldehyde **Wako Catalog #**162-16065
- Accumax **Funakoshi Catalog #**AM105
- PIP2 Antibody (PIP2 2C11) **Santa Cruz Biotechnology Catalog #**sc-53412
- Blocking One-P **Nacalai Tesque Inc. Catalog #**05999-84
- Abberior Mount Solid without Antifade **Abberior Catalog #**MM-2011-2X15ML
- Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb **Cell Signaling Technology Catalog #**9106
- Alexa Fluor™ 647 Microscale Protein Labeling Kit **Molecular Probes Catalog #**A30009
- TRITC conjugated Anti-Mouse IgM antibody **Beckman Coulter Catalog #**731781
- DMEM **Wako Catalog #**044-29765
- Trypsin **Gibco - Thermo Fisher Scientific Catalog #**27250018
- FBS **Nichirei Biosciences Catalog #**172012-500ML
- Recombinant Murine EGF **Funakoshi Catalog #**315-09-1MG

"Rinse x1 and wash x3" indicates the following procedure :

1. Aspirate medium from the culture dish.
2. Add buffer solution (HBBS/PBS) to the dish.
3. Gently swirl the buffer in the dish.
4. Aspirate the buffer.
5. Add buffer solution (HBBS/PBS) to the dish.
6. Incubate for 5 min at room temperature (RT) on the bench.
7. Repeat steps 4-6 three times.

Sterilization of cover slips

Cover slips should be 9 × 9 mm square, 0.13 - 0.17 mm thick.

1. Soak cover slips in 100% ethanol and sterilize it by flaming.
2. Put the cover slips in a covered 24-well plate.

Humidified box

Use a plastic box with a lid.

1. Lay a paper towel in the box.
2. Soak the paper towel with distilled H_2O (dH_2O).
3. Lay Parafilm (Bemis Company) on the soaked paper towel in the box.

Preparation of 10x DPBS (Dulbecco's PBS (-))

Components

- 80 g NaCl
- 29 g Na_2HPO_4 •$12H_2O$
- 2 g KCl
- 2 g KH_2PO_4

Dissolve the above reagents in 1 L of dH_2O.

Preparation of 1x HBSS (Hanks' Balanced Salt Solution)

Components

- 0.14 g $CaCl_2$
- 0.4 g KCl
- 0.06 g KH_2PO_4
- 0.1 g $MgCl_2$ • $6H_2O$
- 0.1 g $MgSO_4$ • $7H_2O$
- 8.0 g NaCl
- 0.35 g $NaHCO_3$
- 0.048 g Na_2HPO_4

Dissolve the above reagents in 1 L of dH_2O.

The recipe above excludes D-glucose although the original recipe for HBSS contains D-glucose.

Preparation of 100 mM sodium orthovanadate (Na_3VO_4)

- Dissolve 0.184 g Na_3VO_4 in 10 mL of dH_2O without heating.
- Store at 4° C until the day of experiments.

Preparation of a 3% PFA solution

1. This solution should be prepared before the day of the experiment.
2. Put 0.9 g paraformaldehyde (PFA) into a disposable 50 mL conical tube.
3. Add 25 mL of dH_2O.
4. Heat the PFA in the tube at 60° C for 10 min while gently swirling the tube in a water bath.
5. Add 30 µL of 1 M NaOH
6. Dissolve the PFA by returning the tube to water bath for 10 min with gentle swirling.
7. After 10 min, add 3 mL of 10x DPBS buffer.
8. Put the tube of PFA solution on ice and cool it to RT.
9. Add dH_2O to bring the volume to approximately 30 mL using the gradations printed on the tube.
10. Use pH test paper to confirm that the pH is in the range of 7-8.
11. Store the PFA solution at -20° C until the day of the experiment.
12. On the day of fixation, add 300 µL of 100 mM Na_3VO_4 and 1.26 mg of NaF to 30 mL of 3% PFA solution.

Preparation a 50 µg/mL digitonin solution

1. Prepare this solution on the day of the experiment.
2. Use 30 mL of 3% PFA solution containing Na_3VO_4 and NaF, as described above.

3. Split the 30 mL of PFA solution into two unequal aliquots of 1 mL and 29 mL.
4. Dissolve 1.5 mg digitonin in the 1 mL of 3% PFA solution.
5. Add the 1 mL of the digitonin/PFA solution to the 29 mL of 3% PFA solution.
6. Mix the combined solution well.

Preparation of 10% of Triton X-100 solution

1. Prepare a 10% Triton X-100 solution with DPBS.
2. Store at 4° C until the day of fixation.

Preparation of 0.01% Triton X-100 solution

1. Prepare this solution on the day of the experiment.
2. Use 30 mL of 3% PFA solution containing Na_3VO_4 and NaF, as described above.
3. Add 30 µL of 10% Triton X-100 to the 30 mL of 3% PFA solution.

Labeling anti-ppERK antibodies with fluorescent dye

- An Alexa Fluor Microscale protein labeling kit is used, following the manufacturer's instructions.
- Use Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse monoclonal antibody solution (Cell Signaling Technology) without BSA or preservatives.

Subculturing of cells

- HeLa cells are subcultured in 60-mm culture dishes every 3 days to 4 days.
- Culture medium is DMEM with 10% FBS, but without antibiotics.

Procedure for subculturing

1. Remove the culture medium from the dish.
2. Add 3 mL of DPBS/0.5 mM EDTA to the dish.
3. Remove the DPBS from the dish.
4. Add 300 µL of 0.25 % Trypsin / 1 mM EDTA.
5. Incubate 10 min at 37° C in an incubator with 5% CO_2.
6. Suspend cells with 1 mL of DPBS/0.5 mM EDTA.
7. Transfer 0.2 mL of cell suspension into a 60-mm dish containing fresh culture medium.

Cell culture

1 **Cultivation of cells on cover slips**

See "Materials" to sterilize cover slips.

1. Wash cells in a 60-mm dish with 3 mL of DPBS.
2. Add 300 µL of Accumax (Funakoshi) to the dish.
3. Incubate 10 min at 37° C in an incubator with 5% CO_2.
4. Suspend cells in 0.9 mL of DPBS containing 0.5 mM EDTA.
5. Transfer this suspension to a 1.5 mL tube and count the number of cells using a hemocytometer.
6. Dilute the cells to 2.5 × 10^4cells/mL in DMEM.
7. Place a sterilized cover slip in each well of a covered 24-well plate as needed. Then transfer the diluted cell suspension to each of those wells (400 µL/well).
8. Leave the plate on the bench for 10 min.
9. Place the plate in an incubator at 37° C with 5% CO_2 and cultivate for 12 hr.
10. In order to starve the cells, replace the medium with 600 µL/well of culture medium without serum.

Immunostaining

2 **Activation of cells with EGF**

1. Remove 300 µL of medium from each well, leaving 300 µL/well.
2. Add 100 µL of EGF solution (400 ng/mL in DMEM, final concentration is 100 ng/mL) or add medium without ligand.
3. Incubate for 5 min at RT.

3 **First fixation and permeabilization**

1. Replace the medium with 400 µL of 3% PFA/NaF/Na_3VO_4.
2. Incubate for 10 min at RT.
3. Rinse x1 and Wash x3 with HBSS (see Materials)
4. Replace HBSS with 400 µL of 50 ug/mL digitonin/3% PFA/NaF/Na_3VO_4.
5. Incubate for 10 min at RT.
6. Rinse x1 and Wash x3 with HBSS.

4 **First blocking**

1. Remove the buffer solution from the 24-well dish.
2. Block cells for 30 min at RT with 300 µL of Blocking One-P (Nacalai).

5 **Labeling with anti-PIP2 antibody**

1. Transfer cover slips from the 24-well plate to the humidified box, placing them on the parafilm. Excess solution on cover slips must be blotted before placing them on the parafilm.

2. Put 40 µL of anti-PIP_2 antibody solution in blocking One-P (1:600) on each cover slip.
3. Incubate for 30 min at RT.
4. Remove the antibody solution from cover slips in the humidified box and then immediately transfer them to wells of a covered 24-well plate to prevent drying.
5. Rinse x1 and Wash x3 with HBSS.

6 **Second fixation and permeabilization**

1. Replace the medium with 400 µL of 3% PFA/NaF/Na_3VO_4.
2. Incubate for 10 min at RT.
3. Rinse x1 and Wash x3 with HBSS (see Materials).
4. Replace HBSS with 400 µL of 0.01% Triton X-100/3% PFA/NaF/Na_3VO_4.
5. Incubate for 10 min at RT.
6. Rinse x1 and Wash x3 with HBSS.

7 **Second blocking**

1. Discard buffer from the 24-well dish.
2. Block cells for 30 min at RT with 300 µL of Blocking One-P (Nacalai).

8 **Labeling with TRITC-conjugated antibody**

1. Transfer cover slips from the 24-well plate to the humidified box. Excess solution on cover slips must be blotted before placing on the parafilm.
2. Put 40 µL of TRITC-conjugated anti-mouse IgM antibody solution (1:2400) in Blocking One-P on each cover slip.
3. Incubate for 30 min at RT.
4. Remove the antibody solution from each cover slip in the humidified box and then immediately transfer them to wells of the covered 24-well plate to prevent drying.
5. Rinse x1 and Wash x3 with HBSS.

9 **Labeling with fluorescent dye-conjugated ppERK antibodies**

1. Transfer cover slips from the 24-well plate to the humified box. Excess solution on cover slips must be blotted before placing them on the parafilm.
2. Put 40 µL of Alexa647-conjugated anti-ppERK solution (1:300) on each cover slip.
3. Incubate for 30 min at RT.
4. Remove the antibody solution from the cover slips and then immediately transfer them to a covered 24-well plate to prevent drying.
5. Rinse x1 and Wash x3 with HBSS.

10 **Mounting**

1. Mount cover slips on glass slides with 4 uL of Abberior mount solid.
2. Store at 4° C in the dark overnight.

Observation and Results

11 **Observation and measurement of intensity**

- Fluorescent images of cells are captured using a fluorescence microscope. We use an Olympus IX-81 with an sCMOS camera, ORCA Flash4.0 (Hamamatsu) and a 20x objective lens, UPLSAPO 20x (Olympus). The microscope is controlled using Metamolph (Molecular Devices).
- Filter sets used to capture fluorescent signals are as follows:
  U-MRFPHQ (Olympus) for TRITC
  U-M41008HQ (Olympus) for Alexa647
- Fluorescence intensity is measured using ImageJ software (NIH).
- An example of a fluorescent image is shown in Fig. 1 and distribution of cellular fluorescence intensities is shown in Fig. 2.

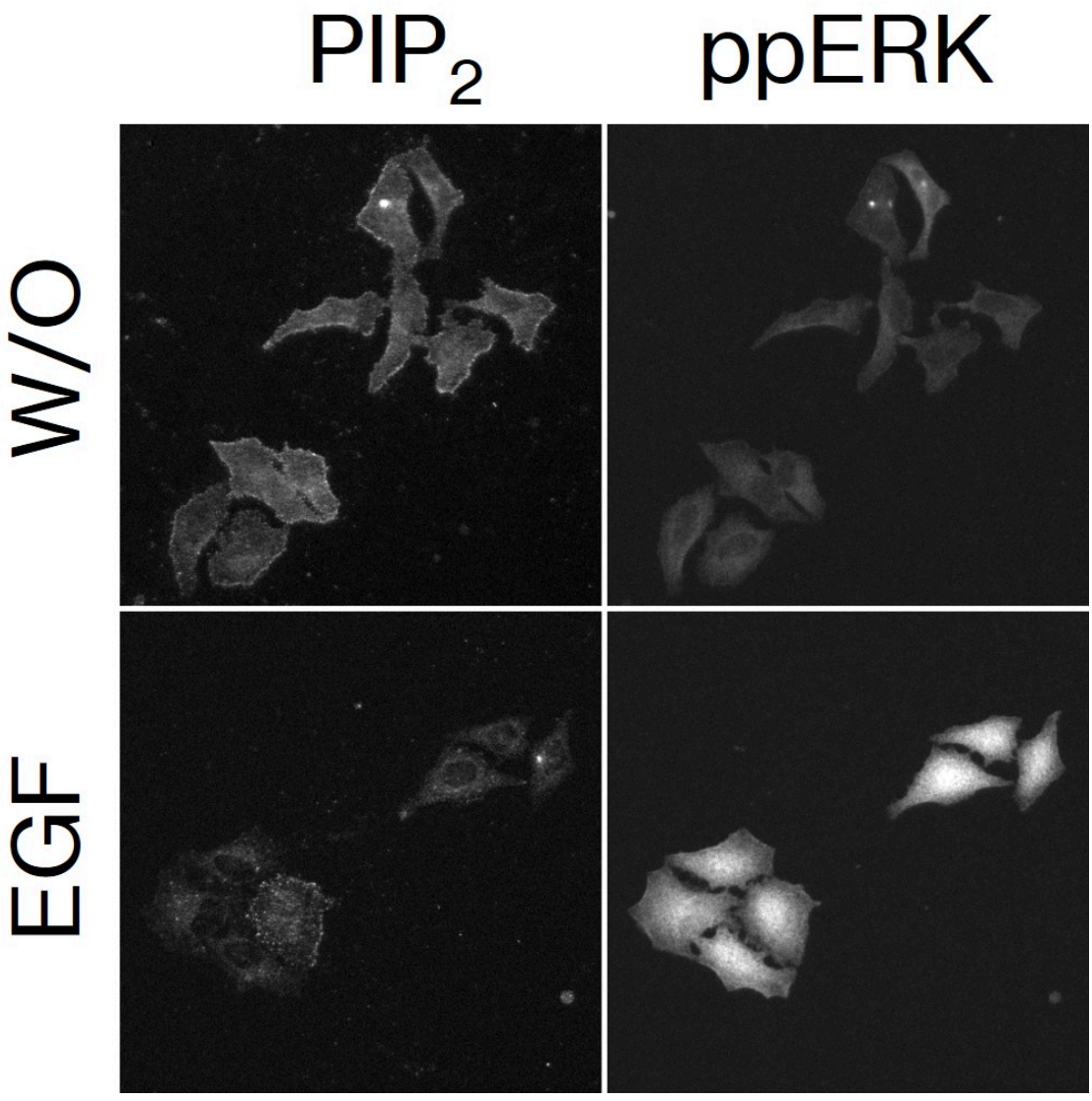

Figure 1. HeLa cells were stimulated with EGF and then labeled with anti-PIP_2antibody and anti-ppERK antibody as described above.

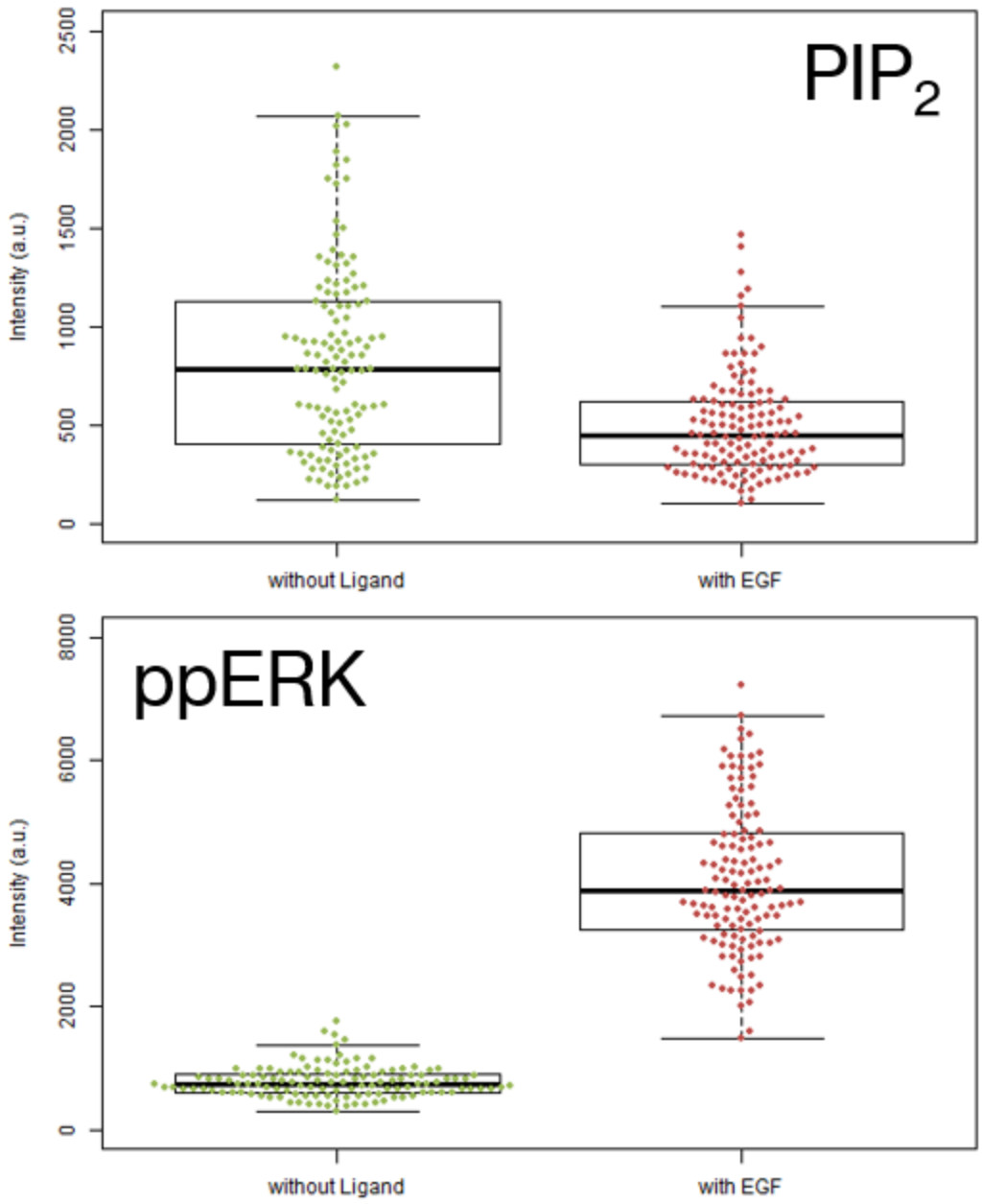

Figure 2. Fluorescence intensities of anti-PIP_2 antibody-labeled cells decreased significantly ($p = 3.43 \times 10^{-8}$) after ligand treatment. In contrast, the ppERK signal was significantly increased ($p = 1.67 \times 10^{-61}$) after ligand treatment.