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Visualization of RAS/MAPK signaling *in situ* by the proximity ligation assay (PLA)

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Abstract

RAS/MAPK signaling responds to diverse extracellular cues and regulates a wide array of cellular processes. Given its biological importance, abnormalities in RAS/MAPK signaling cascade have been intimately implicated in numerous human diseases, including cancer. Herein, we describe a novel methodology to study activation of this pivotal signaling. The Proximity Ligation Assay (PLA) is employed to monitor kinase-substrate interactions between MEK1 and HSF1, or MEK1 and ERK1 *in situ*.

Keywords

ERK; fluorescence imaging; HSF1; MAPK signaling; MEK; PLA

1: Introduction:

RAS/MAPK signaling is essential to biology, controlling cell growth and differentiation through a mitogen activated protein kinase cascade [1–3]. Among this cascade, MEK1 and MEK2 are MAPK kinases with dual-specificity kinases activity [2]. Phosphorylation of two serine residues at positions 217 and 221 of MEK by RAF results in activation of MEK1 and MEK2 [4]. All three RAF family members are able to phosphorylate and activate MEK [5]. Subsequently, MEK activates ERK1/2 (Extracellular signal-regulated kinases) by phosphorylating both threonine and tyrosine residues at sites Thr202/Tyr204 of ERK1 and Thr185/Tyr187 of ERK2. [6]. Upon activation, ERK1/2 can enter the nucleus to phosphorylate many downstream targets [7]. A number of transcription factors have been identified as ERKs' targets including ETS-1, c-JUN and c-MYC [8]. Moreover, ERK phosphorylates many proteins involved in cell cycle progression [9].

ERK has long been perceived as the only physiological substrate for MEK [10]. Surprisingly, we recently found that MEK signaling critically regulates the activation of heat shock factor 1 (HSF1) [11], the master regulator of the evolutionarily conserved proteotoxic stress response (PSR) [11]. To determine whether HSF1 is a previously unrecognized

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substrate for MEK, we employed PLA, an emerging technique to detect *in situ* protein-protein interactions with high specificity and sensitivity. Protein associations can be visualized in unmodified cells and tissues and be objectively quantified by PLA [12]. To detect interactions between two protein targets of interest, two primary antibodies raised in different species and recognizing the two protein targets are needed. Then PLA probes, secondary antibodies with short DNA oligonucleotide strands, will be applied to bind to the two primary antibodies. When the two protein targets of interest physically interact, resulting in PLA probes in close proximity, the oligo strands attached to the secondary antibodies can be ligated by enzymes and the ligated products can be subsequently amplified by polymerases. Following amplification, the products will be visualized through hybridization with fluorescent dye-labeled complementary oligonucleotides. The hybridized products, marking interactions between two proteins of interest, can be readily visualized as distinct bright fluorescent spots under a fluorescent microscope [13]. The localization and intensity of fluorescent spots can reveal the important spatial and quantitative information of protein associations [14]. According to our results (Figure 1), PLA signals were marginally visible but markedly intensified by heat shock in Hela cells. Of note, the PLA signals were more manifest in the nucleus than in the cytoplasm, revealing a prominently nuclear localization of MEK-HSF1 interactions. This finding strongly supports that MEK directly interacts with and activates HSF1. To further rule out the possibility that HSF1 is a substrate for ERK, the long perceived ultimate effector of the RAS/MAPK signaling cascade, we performed PLA assays for HSF1-ERK interactions. According to our results (data not shown) no apparent PLA signals denoting ERK-HSF1 interactions were detected, suggesting lack of direct associations between these two proteins. In addition, our PLA data reveal that heat shock activates the canonical RAS/MAPK signaling, showing intensified PLA signals denoting MEK-ERK interactions under heat shock (data not shown). Taken together, these experiments demonstrate both the feasibility and applicability of PLA in assessing activation of RAS/MAPK signaling.

2: Materials:

1. 60x15mm tissue culture dishes.
2. 10ml serological pipets.
3. 1× Trypsin-Versene (EDTA) mixture (Lonza Walkersville Inc. 17–161E).
4. Superfrost plus glass slides.
5. Fisherbrand microscope cover glasses.
6. SP5 confocal microscope.
7. Grace Bio-labs ProPlate tray sets.
8. Dulbecco's modified eagle's medium containing 10% fetal bovine serum, 1% sodium pyruvate and 1% streptomycin/penicillin.
9. PBS 10× to prepare PBT (PBS 1× with 0.1 % Triton X100).

10. 4 % paraformaldehyde (PFA) in PBS 1× (1 ml aliquots kept at −20 °C; aliquots prepared from PFA powder).
11. Blocking buffer: 5% normal goat serum, 0.3% Triton X-100 in PBS.
12. Primary antibodies: mouse monoclonal anti-HSF1 (E4) antibodies [15–18]; rabbit monoclonal anti-MEK1 (C-18) antibodies [19–23]; mouse monoclonal anti-MEK1/2 (D1A5) antibodies [24]; and rabbit monoclonal anti-ERK1/2 antibodies [25–27].
13. Duolink® In Situ PLA® probe anti-rabbit plus (Sigma-Aldrich DUO92002).
14. Duolink® In Situ PLA® probe anti-mouse minus (Sigma-Aldrich DUO92004).
15. Duolink® In Situ Detection Reagents Red (Sigma-Aldrich DUO92008), containing 1× T4 DNA ligase (1 unit/μl), 5×ligation buffer, 1× phi29 DNA polymerase (10 units/μl), and 5×amplification buffer.
16. Mounting medium: 20 % (v/v) PBS 1×, 80 % (v/v) glycerol.
17. Wash buffer A (0.01M Tris, 0.15M NaCl, 0.05% Tween 20, pH 7.4).
18. Wash buffer B (0.2M Tris, 0.1M NaCl, pH 7.5).
19. Hoechst 33342 solution: 10 μM Hoechst 33342 in Wash Buffer B.
20. HeLa cells expressing scramble shRNAs.
21. HeLa cells expressing MEK1/2 shRNAs.
22. Temperature-controlled cell incubator (37°C).
23. Temperature-controlled water bath for heat shock (42°C).

3: Methods:

1. Culture HeLa cells expressing scramble or MEK1/2 shRNAs in 15-mm tissue culture dishes and incubate cells in temperature-controlled cell incubator at 37°C (See Note 1).
2. Put a Superfrost plus glass slide under Grace Bio-labs ProPlate tray set to form a 16-well tissue culture chamber.
3. Wash HeLa cells expressing scramble or MEK1/2 shRNAs with PBS once, and add 1ml Trypsin-Versene mixtures to each culture dish (See Note 2).
4. Add 1ml Dulbecco's modified eagle's medium to each dish and resuspend cells, then transfer 200ul of cell mixture (2000 cells) to each well of the tissue culture chamber made in 3.2.
5. Incubate the tissue culture chamber in temperature-controlled cell incubator at 37°C overnight.
6. Keep the tissue culture chamber for the control group in temperature-controlled cell incubator at 37°C and put the tissue culture chamber for the heat-shock group in temperature-controlled water bath at 42°C for 30 min.

7. Then fix the cells in tissue culture chambers for both the control and heat-shock groups with 4 % paraformaldehyde (PFA) in PBS 1× for 15 min at room temperature (RT) (See Note 3).
8. Tissue culture chambers for both control and heat shock groups are blocked with the blocking buffer for 60 min.
9. Next, fixed cells are incubated with a pair of rabbit and mouse primary antibodies, 1:200 diluted in the blocking buffer, overnight at 4°C (See Note 4).
10. Wash the wells with Wash Buffer A for 5 min twice.
11. Dilute the two PLA probes 1:5 in Blocking Buffer and add 40 µl solution, containing 8 µl PLA probe anti-rabbit plus, 8 µl PLA probe anti-mouse minus and 24 µl H₂O, to each well of tissue culture chambers.
12. Incubate tissue culture chambers in temperature-controlled cell incubator at 37°C for 1 h.
13. Repeat step 10.
14. Add 40 µl ligation solution containing 1 µl T4 DNA ligase, 8 µl 5×ligation buffer, and 31 µl H₂O to each well of tissue culture chambers.
15. Incubate the tissue culture chambers in temperature-controlled cell incubator at 37°C for 30 min.
16. Wash wells with Wash Buffer A for 2 min twice.
17. Add 40 µl amplification solution containing 0.5 µl phi29 DNA polymerase, 8 µl 5×amplification buffer, and 31.5 µl H₂O to each well of tissue culture chambers.
18. Incubate the tissue culture chambers in temperature-controlled cell incubator at 37°C for 100 min.
19. Wash wells with Wash Buffer B for 10 min twice.
20. Wash wells with 0.01x Wash Buffer B for 1min.
21. Add 40 µl Hoechst 33342 solution containing 0.02 µl Hoechst 33342 (20 mM) and 39.98 µl Wash Buffer B to each well of tissue culture chambers (See Note 5).
22. Incubate the tissue culture chambers at RT for 5 min.
23. Wash wells with Wash Buffer B for 10 min (See Note 6).
24. Disassemble the Grace Bio-labs ProPlate tray set from Superfrost plus glass slides.
25. Put Fisherbrand microscope cover glasses with mounting medium on the Superfrost plus glass slides.
26. Examine and capture fluorescent images of slides made in step 25 under a SP5 confocal microscope (See Note 7).

4: Notes:

1. In addition to live cells, paraffin-embedded samples also can be used to study MEK-ERK Signaling by PLA assay. In this case, incomplete removal of paraffin would generate high background.
2. Use of healthy cells for PLA is highly desirable. In addition, it is recommended to capture images of areas with dispersed cells, rather than areas with clustered cells.
3. Ensuring good humidity during all incubation steps is necessary. Never let slides dry out after washes and before addition of reagents.
4. It is important to use specific primary antibodies in the PLA procedure. Non-specific binding of two primary antibodies to the same location will create non-specific PLA signals. Thus, it is recommended to test the specificity of primary anti-MEK, anti-HSF1, and anti-ERK1/2 antibodies in HeLa cells expressing control shRNA and HeLa cells expressing *MEK*-, *HSF1*-, or *ERK*-targeting shRNAs.
5. It is important to minimize the concentration and incubation time of Hoechst 33342 solution. Indeed, excess of staining with Hoechst 33342 would result dark blue nuclei, which would interfere with normal nuclear PLA signals.
6. Keeping precise washing time for each washing step is crucial. It is necessary to avoid excess washing with Wash Buffer A, since it would diminish PLA signals. Washing with 0.01 Wash Buffer B is also a key step, since Wash Buffer B could reduce non-amplification dependent background caused by highly fluorescent particles including dusts, salts or fixative precipitates.
7. The fluorophore in the Amplification Red has an excitation wavelength of 594 nm and an emission wavelength of 624 nm and the fluorescence can be detected using the same filter for Texas Red.

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Figure 1. Heat shock induces MEK-HSF1 interactions.

Following heat shock at 42°C for 30 min, physical interactions between endogenous MEK1 and HSF1 proteins were detected by PLA in HeLa cells using a rabbit anti-MEK1 antibody and a mouse anti-HSF1 antibody. The images show merged PLA signals (red dots) and Hoechst 33342 staining of nuclei (blue). Scale bars: 50 μm for the low magnification (LM); 10 μm for the high magnification (HM).