Example Experiment

Characterizing AKT Activation in Response to PDGF-BB Treatment in NIH-3T3 Cells

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I. Background

Accurate quantification of protein expression and/or post-translational modifications is important for advancing both basic and translational research. Given the flexibility, reproducibility, and higher throughput of the In-Cell Western™ Assay, it offers a convenient alternative to Western blotting and is a powerful platform for meaningful *in situ* analyses. The In-Cell Western microplate format can be used to analyze:

- Protein phosphorylation and signaling (1 - 3)
- Off-target effects of drugs on signaling pathways (4)
- Timing and kinetics of signaling events (5 , 6)
- Quantification of viral load (7 - 11)
- Genotoxicity assays (12, 13)
- Cell proliferation and apoptosis assays (14)
- Bacterial-induced epithelial signaling (15)
- Glycoprotein analysis (16, 17)
- Library screening (18 - 20)
- Screening of monoclonal antibody clones (21)

II. Introduction

The Platelet Derived Growth Factor, PDGF, is a mitogen that regulates numerous processes in both developing and adult tissues such as, cellular proliferation, wound healing, migration, and angiogenesis (22). PDGF-BB acts by binding to the fibroblast growth factor receptor (PDGFR), thereby triggering a signal transduction cascade that initiates a variety of biochemical and molecular changes, including the phosphorylation and activation of AKT (23). Due to the multifaceted roles of the PDGFR signaling pathway, it is not surprising that the perturbation of this pathway can promote malignant behavior in cancer cells, such as increased cell survival, proliferation, migration, invasion, and angiogenesis (24). Additionally, PDGF has been linked to several other vascular and fibrosis diseases (25 - 27). Consequently, the PDGF pathway has emerged as an attractive target for pharmacological intervention and understanding how new therapies impact this multi-dimensional pathway is of great importance (28 - 30).

III. Experimental Design

In the following In-Cell Western™ Assay example, we present a protocol for monitoring AKT phosphorylation in response to stimulation of the PDGFR pathway by PDGF-BB. Changes in phospho-AKT and total-ERK protein levels in NIH-3T3 cells treated with a serial dilution of PDGF-BB are characterized through immunodetection. The information obtained from this experimental protocol could be used to identify appropriate experimental conditions to screen for and characterize compounds that modulate this branch of the PDGF signaling pathway.

IV. Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Rabbit Secondary Antibody (LI-COR P/N 925-32211 or 926-32211)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (LI-COR P/N 925-68070 or 926-68070)
- Intercept® (PBS) Blocking Buffer

> **Note:** Odyssey® Blocking Buffer was used in the original experiment and has been discontinued. Intercept Blocking Buffer is now available instead of Odyssey Blocking Buffer.

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Platelet Derived Growth Factor BB (PDGF-BB) (Millipore, P/N 01-305)
- Primary Antibodies
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100
- Black-sided 96-well or 384-well microplates with clear well bottoms

V. Plate Handling

Be extremely cautious and delicate in handling plates and pipetting to avoid detaching the cells.

NIH3T3 cells do not adhere strongly to TC-treated plates, resulting in the need for Poly-D-Lysine-coated plates in this assay. However, even with lysine-coated plates, the adherence of cells remains relatively weak compared with other cell lines.

VI. Prepare Cells

1. Allow NIH3T3 cell growth in a T75 flask using standard tissue culture procedures until ~80% confluency is achieved (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2. Remove growth media and wash cells with sterile 1X PBS (room temperature (RT)).
3. Add trypsin and incubate 3-5 minutes at 37 °C to displace cells.
4. Neutralize displaced cells with culture media and pellet by centrifugation (500 x g).
5. Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube.

> **Note:** To maintain cell integrity, do not pipet or vortex during pellet disruption.

6. Resuspend cells in 20 mL of complete media and count cells using a hemocytometer.
7. Reconstitute and dilute cells with complete media to a concentration of 75,000 cells/mL.
8. Manually mix the cell suspension thoroughly.
9. Under sterile conditions, dispense 200 µL of the cell suspension per well into a microplate (15,000 cells plated per well).
10. Incubate cells at 37 °C and monitor cell density until 70% confluency is achieved. This should take approximately about 24 hours.

> **Important:** 70% confluency is very important. 90-100% confluent cells have a higher likelihood of detachment during washing.

VII. Treat Cells

1. Warm serum-free media (DMEM, Gibco®) to 37 °C.
2. Remove complete media from plate wells by aspiration or manual displacement.
3. Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of PDGF-BB ranging from 0.4 to 200 ng/mL for activated cells. Use 100 µL of resting or activation media per well.
4. Incubate at 37 °C for 7.5 minutes.

VIII. Fix and Permeabilize Cells

Fix Cells

1. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

2. When incubation period is complete, carefully remove activation media manually or by aspiration to avoid detaching the cells.
3. Using a multi-channel pipettor, add 150 µL of fresh Fixing Solution (RT). Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow incubation on bench top for 20 minutes at RT with no shaking.

Permeabilize Cells

1. Prepare Triton® Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

2. Remove Fixing Solution to an appropriate waste container (contains formaldehyde).
3. Using a multi-channel pipettor, add 200 µL Triton Washing Solution (RT). Add the solution down the sides of the wells carefully to avoid detaching the cells.
4. Allow plate to shake on a rotator for 5 minutes at RT.
5. Repeat washing steps 4 more times, removing wash manually each time.

Important: Do not allow cells to become dry during washing. Immediately add the next wash after manual disposal.

IX. Block Cells

1. Using a multi-channel pipettor, add 150 µL of Intercept® Blocking Buffer to each well. Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells.
2. Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

X. Primary Antibodies

Dilute Primary Antibodies

1. Dilute the two primary antibodies in Intercept Blocking Buffer. Combine the following solutions for phospho-Akt target analysis, using total ERK2 for normalization:

   Phospho-Akt (Rabbit, 1:100 dilution in the combined solution; Cell Signaling Technology, P/N 9101 or 4058)

   Total ERK2 (Mouse; 1:100 dilution in the combined solution; Santa Cruz Biotechnology, P/N SC-1647)
2. Mix the primary antibody solution thoroughly before adding to wells.

Incubate with Primary Antibodies

1. Remove blocking buffer from the wells and add 50 µL of the desired primary antibodies in Intercept Blocking Buffer to cover the bottom of each well.
2. Make sure to include control wells without primary antibody to serve as a source for background well intensity (see Figure 1). Only add 50 µL of Intercept Blocking Buffer to background wells.
3. Incubate with primary antibody for 2 hours with gentle shaking at RT.

Wash

1. Prepare Tween® Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS + 0.1% Tween 20	1000 mL

2. Remove primary antibody solution.
3. Using a multi-channel pipettor, add 200 µL Tween Washing Solution (RT). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow wash to shake on plate shaker for 5 minutes at RT.
5. Repeat washing steps 4 more times.

XI. Secondary Antibodies

Dilute Secondary Antibodies

1. Dilute the fluorescently-labeled secondary antibodies in Intercept® Blocking Buffer as specified below. To lower background, add Tween 20 to the diluted antibody to a final concentration of 0.2%. Recommended dilution range is 1:200 to 1:1,200.
   Goat anti-Rabbit IRDye® 800CW (1:800 dilution in the combined solution)
   Goat anti-Mouse IRDye 680RD (1:800 dilution in the combined solution)

   > **Note:** Minimize exposure of the antibody vials to light.

2. Mix the antibody solutions and add 50 µL of the secondary antibody solution to each well.

Incubate with Secondary Antibodies

1. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

Wash

1. Remove secondary antibody solution.
2. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution at RT (see "Wash" on the previous page). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
3. Allow wash to shake on a plate shaker for 5 minutes at RT.
4. Repeat washing steps 4 more times. Protect plate from light during washing.

XII. Image

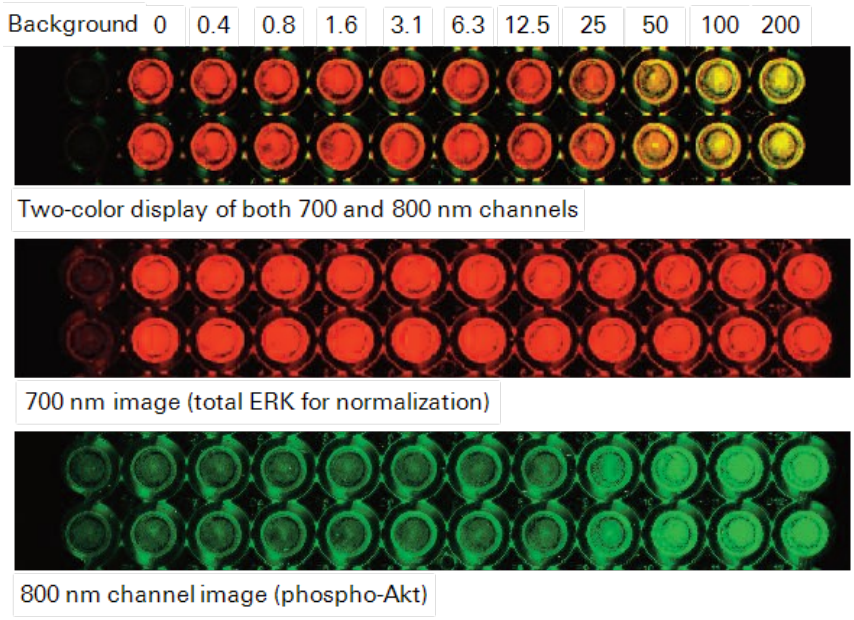
1. After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for several weeks (sealed and protected from light).
2. Before plate scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed (if applicable) with moist, lint-free tissue to avoid any obstructions during scanning.
3. Scan plate with detection in both 700 and 800 nm channels.

Suggested Scan Settings

All settings may require adjustment for optimal data quality. Higher resolutions or scan qualities can be used, but the scan time will increase.

Instrument	Resolution	Scan Quality	Intensity Setting (700 nm)	Intensity Setting (800 nm)
Odyssey Classic	169 µM	lowest	5	5
Odyssey CLx	169 µM	lowest	Auto Mode	Auto Mode
Odyssey Sa	200 µM	lowest	7	7
Aerius™ Imager	200 µM	lowest	7	7

XIII. Experimental Results



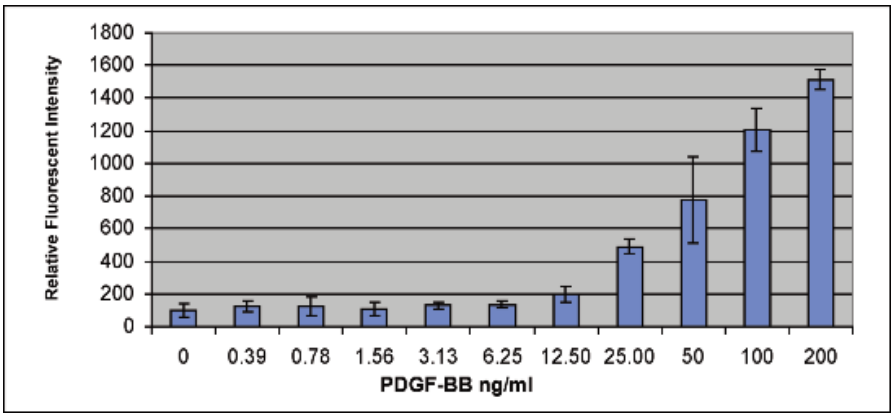


Figure 1. Dose response of NIH3T3 cells to Platelet Derived Growth Factor (PDGF-BB) as measured by specific antibody detecting phosphorylated Akt (Ser473) using total ERK2 for normalization. The image represents a 96-well two-color In-Cell Western™ Assay with the 700 and 800 channels detecting total ERK2 and phosphorylated Akt (Ser473), respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents four sets of quantitative data demonstrating AKT phosphorylation.

XIV. References

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