Complete Sample Protocol Detailing the Seeding, Stimulation, and Detection of the NIH3T3 Cellular Response to Acidic Fibroblast Growth Factor (aFGF-1)

LI-COR Biosciences

Abstract

Developed for:

Aerius,
Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa
Imaging Systems

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Guidelines

Required Reagents

LI-COR Reagents

- IRDye® 800CW goat anti-mouse secondary antibodies (LI-COR P/N 925-32210 or 926-32210)
- IRDye 680RD goat anti-rabbit secondary antibodies (LI-COR P/N 925-68071 or 926-68071)
- Odyssey® Blocking Buffer (LI-COR P/N 927-40000 or 927-50000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Poly-D-Lysine coated 96-well microplate clear/black or clear (Corning P/N 354640 or 354461)
- Heparin (CALBIOCHEM®, P/N 375097)
- Acidic Fibroblast Growth Factor (Upstate Group Inc., P/N 01-116)
- Primary antibodies
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

SPECIAL NOTE: 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 to other cell lines.

Be extremely cautious and delicate in handling plates and pipetting when washing, removing, and adding solutions to avoid detaching the cells.

Experimental Considerations

Establish the specificity of the primary antibody by screening plate-like lysates through Western blotting and detection on an Odyssey instrument. If significant non-specific binding is present, choose alternative primary antibodies to avoid results with non-specific signal detection.

Proper selection of microplates can significantly affect results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear and black-sided plates with clear bottoms. **Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.**
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.
- **Focus Offset Optimization** – If plates other than those recommended are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	1.0, 1.5, 2.0, 2.5, 3.0, 3.5, & 4.0
Odyssey Sa & Aerius	1.5, 2.0, 2.5, 3.0, & 3.95

Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise ratio for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, the plate manufacturer can be consulted to obtain the measured distance from the skirt bottom to the bottom of the plate.

All Aerius and Odyssey® Imaging Systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Aerius and Odyssey Sa instrument, and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified for In-Cell Western assays, the recommended focus offset is 3.5 mm or higher.

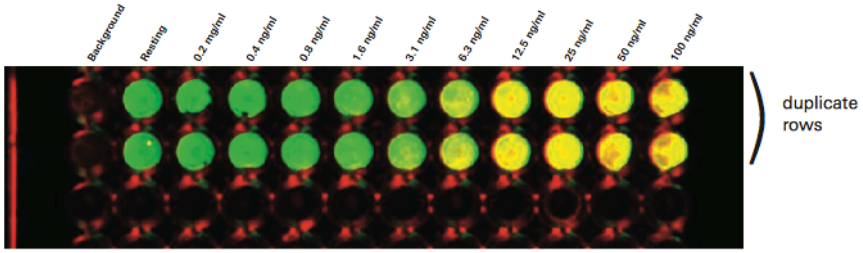
- **Intensity Setting Optimization -**

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings: Weak Signal (700/800 nm)	Intensity Settings: Saturated Signal (700/800 nm)
Odyssey Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoScan*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

**The Odyssey CLx AutoScan function alleviates the need to scan the plate at multiple intensity settings.*

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.

IV. Experimental Results



Two-color display of both 700 nm and 800 nm channels.

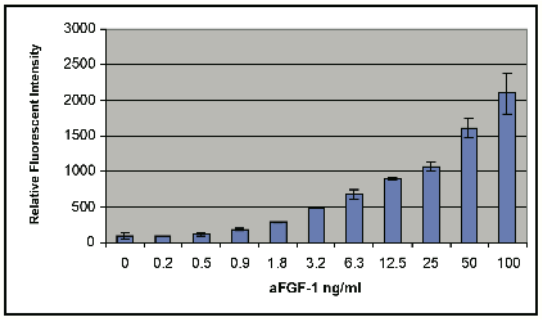


Figure 1. Dose response of NIH3T3 cells to Fibroblast growth factor (aFGF-1) as measured by specific antibody detecting, dual-phosphorylated ERK (Thr202/Tyr204). The image represents a 96-well two-color In-Cell Western with the 800 and 700 nm channels detecting total and phosphorylated ERK, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the phosphorylation percentage of ERK.

Materials

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

Odyssey Blocking Buffer (TBS) 927-50000

927-50100 by LI-COR

IRDye® 800CW Goat anti-Mouse Secondary Antibody 925-32210 926-32210 by LI-COR

IRDye 680RD Goat anti-Rabbit Secondary Antibody 925-668071 926-68071 by LI-COR

Poly-D-Lysine coated 96-well microplate clear/black 354640 by Corning

Poly-D-Lysine coated 96-well microplate clear 354461 by Corning

Protocol

Cell Preparation

Step 1.

Allow NIH3T3 (ATCC; CRL-1555) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®)

Cell Preparation

Step 2.

Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.

Cell Preparation

Step 3.

Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).

Cell Preparation

Step 4.

Remove supernatant and disrupt the cell pellet manually by hand tapping the collection tube.
Do not pipet or vortex during pellet disruption to maintain cell integrity.

Cell Preparation

Step 5.

Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.

AMOUNT

20 ml Additional info:

complete media

Cell Preparation

Step 6.

Reconstitute cells and dilute in 40 mL of complete media so that 75,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = 40 mL).

AMOUNT

40 ml Additional info:
complete media

Cell Preparation

Step 7.

Manually mix the cell suspension thoroughly.

Cell Preparation

Step 8.

Under sterile conditions, dispense 200 µL of the cell suspension per well into a SIGMA-Screen™ Poly-D-Lysine 96-well microplate (15,000 cells plated per well).

AMOUNT

200 µl Additional info: cell
suspension per well

Cell Preparation

Step 9.

Incubate cells and monitor cell density until 70% confluency is achieved (about 24 hours).

DURATION

24:00:00

NOTES

James Thornton Jr 14 Dec 2016

70% confluency is very important. 90 to 100% confluent cells are certain to detach during washing.

Cell Treatment

Step 10.

Warm serum-free media (DMEM; Gibco) to 37°C.

TEMPERATURE

37 °C Additional info:
warming

Cell Treatment

Step 11.
Carefully remove media and inhibitor from plate wells by aspiration or manual displacement.

Cell Treatment

Step 12.

- Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of aFGF-1 ranging from 0.2 to 100 ng/mL, combined with 10 µg/mL heparin for activated cells.
- Use 100 µL of resting/activation media per well.

Cell Treatment

Step 13.
Allow incubation at 37°C for 7.5 minutes.

TEMPERATURE

37 °C Additional info:
Incubation temperature

DURATION

00:07:30

Fixing/Permeabilizing Cells

Step 14.
Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. Detailed in steps 15-17.

Fixing/Permeabilizing Cells

Step 15.
Prepare fresh Fixing Solution as follows:

Prepare fresh *Fixing Solution* as follows:

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

Fixing/Permeabilizing Cells

Step 16.
Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.**

AMOUNT

150 µl Additional info:
Fixing Solution

Fixing/Permeabilizing Cells

Step 17.

Allow incubation on bench top for 20 minutes at RT with no shaking.

DURATION

00:20:00

Fixing/Permeabilizing Cells

Step 18.

Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. Detailed in steps 19-35.

NOTES

James Thornton Jr 05 Jan 2017

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

Fixing/Permeabilizing Cells

Step 19.

Prepare Triton Washing Solution as follows:

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

Fixing/Permeabilizing Cells

Step 20.

Remove Fixing Solution to an appropriate waste container (contains formaldehyde).

Fixing/Permeabilizing Cells

Step 21.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 1/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 22.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 23.

Remove wash manually. (wash 1/5)

Fixing/Permeabilizing Cells

Step 24.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 25.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 26.

Remove wash manually. (wash 2/5)

Fixing/Permeabilizing Cells

Step 27.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 28.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 29.

Remove wash manually. (wash 3/5)

Fixing/Permeabilizing Cells

Step 30.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 31.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 32.

Remove wash manually. (wash 4/5)

Fixing/Permeabilizing Cells

Step 33.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 34.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 35.
Remove wash manually. (wash 5/5)

Blocking Cells

Step 36.
Using a multi-channel pipettor, block cells/wells by adding 150µL of Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

AMOUNT

150 µl Additional info:
Odyssey Blocking Buffer

NOTES

James Thornton Jr 14 Dec 2016

No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific banding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution.

James Thornton Jr 14 Dec 2016

Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4 °C, so diluted antibodies cannot be kept and reused for more than a few days. If using casein, a 0.1% solution in 0.2 x PBS buffer is recommended (Hammersten-grade casein is not required).

James Thornton Jr 14 Dec 2016

Blocking solutions containing BSA can be used, but in some cases may cause high membrane background. BSA-containing blockers are not generally recommended and should be used only when the primary antibody requires BSA as blocker.

Blocking Cells

Step 37.
Allow blocking for 90 minutes at RT with moderate shaking on a rotator.

DURATION

01:30:00

Primary Antibodies

Step 38.
Add the two primary antibodies to a tube containing Odyssey Blocking Buffer. Combine the solutions defined below for phospho-ERK target analysis, using total ERK2 for normalization:

- Phospho-ERK (Rabbit; 1:100 dilution; Cell Signaling Technology P/N 9101)
- Total ERK2 (Mouse; 1:100 dilution; Santa Cruz Biotechnology P/N SC-1647)

Primary Antibodies

Step 39.

Mix the primary antibody solution well before adding to wells.

Primary Antibodies

Step 40.

Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well.

AMOUNT

50 µl Additional info:
primary antibody or
antibodies

Primary Antibodies

Step 41.

Make sure to include control wells without primary antibody to serve as a source for back- ground well intensity. Add 50 µL of Odyssey Blocking Buffer to control wells only.

AMOUNT

50 µl Additional info:
Odyssey Blocking Buffer
to control wells

Primary Antibodies

Step 42.

Incubate with primary antibody overnight with gentle shaking at RT.

Primary Antibodies

Step 43.

Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. Detailed in steps 44-59.

Primary Antibodies

Step 44.

Prepare Tween Washing Solution as follows:

Prepare *Tween Washing Solution* as follows:

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

Primary Antibodies

Step 45.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add the**

solution down the sides of the wells carefully to avoid detaching the cells from the well bottom. (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 46.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Primary Antibodies

Step 47.

Remove wash manually.(wash 1/5)

Primary Antibodies

Step 48.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 49.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Primary Antibodies

Step 50.

Remove wash manually.(wash 2/5)

Primary Antibodies

Step 51.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 52.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Primary Antibodies

Step 53.

Remove wash manually.(wash 3/5)

Primary Antibodies

Step 54.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 55.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Primary Antibodies

Step 56.

Remove wash manually.(wash 4/5)

Primary Antibodies

Step 57.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 58.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Primary Antibodies

Step 59.

Remove wash manually.(wash 5/5)

Secondary Antibodies

Step 60.

Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer as specified below. To lower background, add Tween 20 to the diluted antibody to a final concentration of 0.2%.

- Goat anti-rabbit IRDye® 680RD (1:800 dilution)
- Goat anti-mouse IRDye 800CW (1:800 dilution)

NOTES

James Thornton Jr 14 Dec 2016

Recommended dilution range is 1:200 to 1:1,200.

James Thornton Jr 14 Dec 2016

Avoid prolonged exposure of the antibody vials to light.

Secondary Antibodies

Step 61.

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

AMOUNT

50 µl Additional info:
secondary antibody
solution

Secondary Antibodies

Step 62.

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

DURATION

01:00:00

Secondary Antibodies

Step 63.

Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a

generous amount of buffer. Detailed in steps 64 - 77.

NOTES

James Thornton Jr 05 Jan 2017

Protect plate from light during washing.

Secondary Antibodies

Step 64.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 44). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 65.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Secondary Antibodies

Step 66.

Remove wash manually.(wash 1/5)

Secondary Antibodies

Step 67.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 44). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 68.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Secondary Antibodies

Step 69.
Remove wash manually.(wash 2/5)

Secondary Antibodies

Step 70.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 44). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 71.
Allow wash to shake on a rotator for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Secondary Antibodies

Step 72.
Remove wash manually.(wash 3/5)

Secondary Antibodies

Step 73.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 44). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 74.
Allow wash to shake on a rotator for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Secondary Antibodies

Step 75.
Remove wash manually.(wash 4/5)

Secondary Antibodies

Step 76.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 44). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 77.

Allow wash to shake on a rotator for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Secondary Antibodies

Step 78.

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.

Imaging

Step 79.

For best results, scan plate immediately; plates may also be stored at 4 °C for up to several weeks (protected from light).

TEMPERATURE

4 °C Additional info:
Storage temperature

Imaging

Step 80.

Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed with moist, lint-free paper to avoid obstructions during scanning.

Imaging

Step 81.

Scan plate with detection in both 700 and 800 nm channels using an Odyssey or Aerius System described following:

Instrument	Resolution*	Focus Offset	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 µm	3.5	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.5	medium-lowest	5 / 5	7 min
	169 µm	3.5	medium-lowest	AutoScan	16 min
Odyssey Sa	200 µm	3.5	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.5	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

NOTES

James Thornton Jr 11 Jan 2017

NOTE: All settings may require adjustment for optimal data quality.

Warnings

See SDS for safety warnings and hazards.