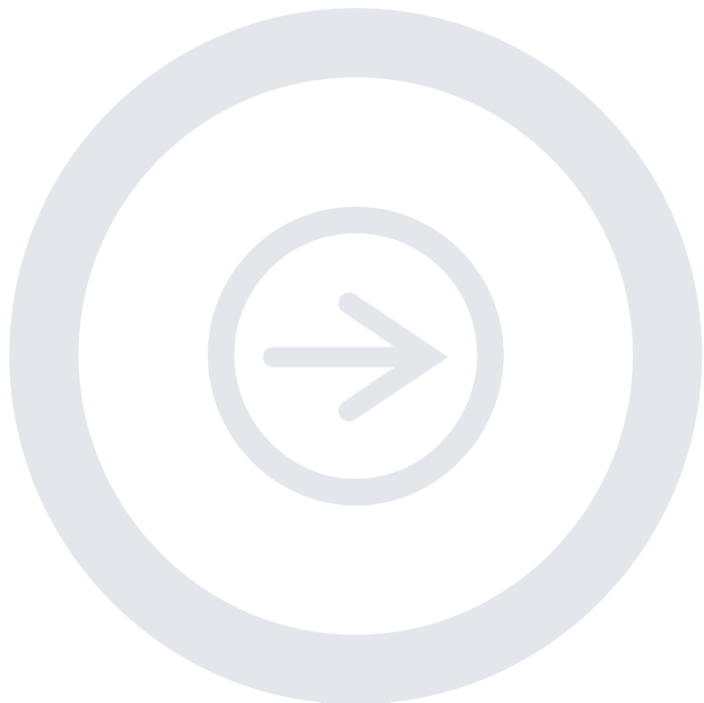
In-Cell Western™ Assay

For Assessing Response of A431 Cells to Stimulation with Epidermal Growth Factor

Developed for:

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

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.



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The most recent version of this protocol is posted at http://biosupport.licor.com

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I. Required Reagents

LI-COR® Reagents

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

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Epidermal Growth Factor (Millipore, P/N 01-107)
- 37% formaldehyde
- 10% Triton® X-100
- Nunc® 96 Microwell™ Plate (Nunc, P/N 167008)
- Primary antibodies as described below

SPECIAL NOTE: Phosphorylated-EGFR and phosphorylated-ERK are purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. ***All primary antibodies should be requalified for optimal results. Serum starvation of the cells is required to obtain maximal response.***

II. SEEDING, STIMULATION, AND DETECTION OF THE A431 CELLULAR RESPONSE TO EPIDERMAL GROWTH FACTOR

1. Allow A431 cell growth in a T75 flask in DMEM and 10% fetal calf serum (FCS; Gibco®), using standard tissue culture procedures, until cells reach 80% - 90% confluency (~1.5 x 10^7 cells).

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

3. Neutralize displaced cells with culture media and pellet by centrifugation.

4. Remove supernatant and resuspend cell pellet in remaining media by manually tapping the collection tube. Avoid vigorous pipetting or vortexing to resuspend cells in order to maintain cell integrity.

5. Dilute cells to 20 mL in complete media and count cells using a hemacytometer.

6. Dilute cells with complete media to concentration of 200,000 cells/mL.

7. Gently mix the cell suspension thoroughly.

8. Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ plate (40,000 cells plated per well).

9. Incubate cells and monitor cell density until cells are consistently confluent in each well. This should take approximately three days.

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

11. Remove complete media from the microwell plate by aspiration.

12. Replace media with 200 µL of pre-warmed, serum-free media per well, and incubate 4 to 16 hours.

13. In a separate 96-well Microwell plate, dispense 100 µL of DMEM per well.

14. Leave the first and second wells without EGF (resting cells controls). In the remaining wells, add aliquots of a solution of EGF to make serial dilutions ranging 0.2 to 100 ng/mL in the microplate. The experimental layout should look like that shown in Figure 1 (Page 7).

15. Remove starvation media from plate wells by aspiration.

16. Transfer EGF dilutions from the dilution plate into the cell-containing plate.

17. Incubate at 37°C for 7.5 minutes.

18. Prepare fresh *Fixing Solution* as follows:

1X PBS	45.0 mL
37% Formaldehyde	5.0 mL
3.7% Formaldehyde	50.0 mL

19. Remove EGF-containing media by aspiration. Immediately fix cells by addition of 150 µL of fresh *Fixing Solution* and incubate at room temperature (RT) for 20 minutes with no shaking. ***Add the solution carefully by pipetting down the side of the wells to avoid detaching the cells.***

20. 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

21. Remove the *Fixing Solution* by aspiration.

22. Wash four times with 200 µL of *Triton Washing Solution* for 5 minutes per wash to permeabilize the cells.
    NOTES:
    - *Allow each wash to shake on a plate shaker for 5 minutes at RT.*
    - *Do not allow cells/wells to become dry during washing. Add washes immediately after each other.*

23. Remove the *Triton Washing Solution* by aspiration.

24. To each well, carefully add 150 µL of LI-COR® Odyssey® Blocking Buffer (P/N 927-40000) down the side of the wells and incubate for 1.5 hours at RT with moderate shaking on a rotating platform.
    NOTES:
    - *No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding 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 for infrared fluorescent detection.*

- *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 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).*

- *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.*

25. Dilute the antibodies in LI-COR® Odyssey Blocking Buffer to give the concentrations specified below.

    Primary antibodies can be added in a variety of combinations. Generally, one antibody will be directed against the phosphorylated form of the target protein and the second antibody will be directed against the target protein, regardless of phosphorylation status. The following are suggested combinations of primary antibodies, depending on the target to be detected:

    a. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237)
       Total EGFR (Mouse; 1:500 dilution; Biosource International, P/N AHR5062)

    b. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237)
       Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647)

    c. Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383)
       Total ERK1 (Rabbit; 1:200 dilution; Santa Cruz Biotechnology, P/N SC-94)

    d. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237)
       Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383)

26. Add 50 µL of LI-COR Odyssey Blocking Buffer to one set of wells. These wells will serve as a control for any potential background due to the dye-labeled secondary antibody. See Figure 1 (Page 7) for an ex ample of the desired plate layout.

27. Mix the primary antibody solution thoroughly before addition to wells.

28. Remove the blocking buffer by aspiration and add 50 µL of the desired primary antibody combination to the remaining wells. The antibody solution should cover the bottom of each well.

29. Incubate with primary antibody for 2 hours with gentle shaking at RT.
    NOTES:
    - *For greatest sensitivity, continue incubation overnight at 4°C with no shaking.*
    - *To avoid the cells drying out, cover the plates if left overnight.*

30. Prepare *Tween® Washing Solution* as follows:

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

31. Wash the plate with *Tween Washing Solution* by gently adding buffer down the side of the wells to avoid detaching the cells. Use a generous amount of buffer (200 - 500 µL). Allow wash to shake gently on a rotator for 5 minutes at RT.

32. Repeat wash 4 more times.

33. Calculate the amount of secondary antibody required for the experiment. Dilute the fluorescent dye-labeled secondary antibodies in Odyssey® blocking buffer according to the following dilution factors. To lower background, add Tween® 20 to the diluted antibody to a final concentration of 0.2%.
    a. Goat anti-rabbit IRDye® 680RD (1:800 dilution; LI-COR®, P/N 926-68071)
    b. Goat anti-mouse IRDye 800CW (1:800 dilution; LI-COR, P/N 926-32210)

    Recommended dilution range is 1:200 to 1:1,200 for IRDye 800CW secondary antibodies and 1:200 to 1:800 for IRDye 680RD secondary antibodies.

    ⚠ ***Avoid prolonged exposure of the antibody vials to light.***

34. Mix the antibody solutions thoroughly, add 50 µL of the secondary antibody solution to each well and incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

35. Wash the plate with *Tween Washing Solution* (step 30) by gently adding buffer down the side of the wells to avoid detaching the cells. Use a generous amount of buffer (200 - 500 µL). Allow wash to shake gently on a rotator for 5 minutes at RT.

    ⚠ ***Protect plate from light during washing.***

36. Repeat wash 4 more times.

37. 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 up to several weeks (protected from light).

38. Before scanning, clean the bottom plate surface and the Odyssey Infrared Imager scanning bed (if applicable) with moist, lint-free paper to avoid obstructions during scanning.

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

    NOTE: All settings may require adjustment for optimal data quality (see Section III).

Instrument	**Resolution***	**Focus Offset**	**Scan Quality***	**Intensity Setting (700/800)**	**Scan Time** **Medium Quality**
Odyssey Classic	169 µm	3.0	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.0	medium-lowest	5 / 5	7 min
	169 µm	3.0	medium-lowest	AutoScan	16 min
Odyssey Sa	200 µm	3.0	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.0	medium-lowest	7 / 7	3 min

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

III. 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 as well as black-sided plates with clear bottoms. *Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.*

- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

- **Focus Offset Optimization** – If plates other than those recommended above 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	0.5, 1.0, 2.0, 3.0 & 4.0
Odyssey Sa & Aerius	1.7, 2.0, 3.0 & 4.0

  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 recommend the measured distance from the skirt to the bottom of the plate.

- All Odyssey® Imaging systems 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 Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software, then clicking Scanner Information). When using plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.

- **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

Color images can be seen at http://biosupport.licor.com

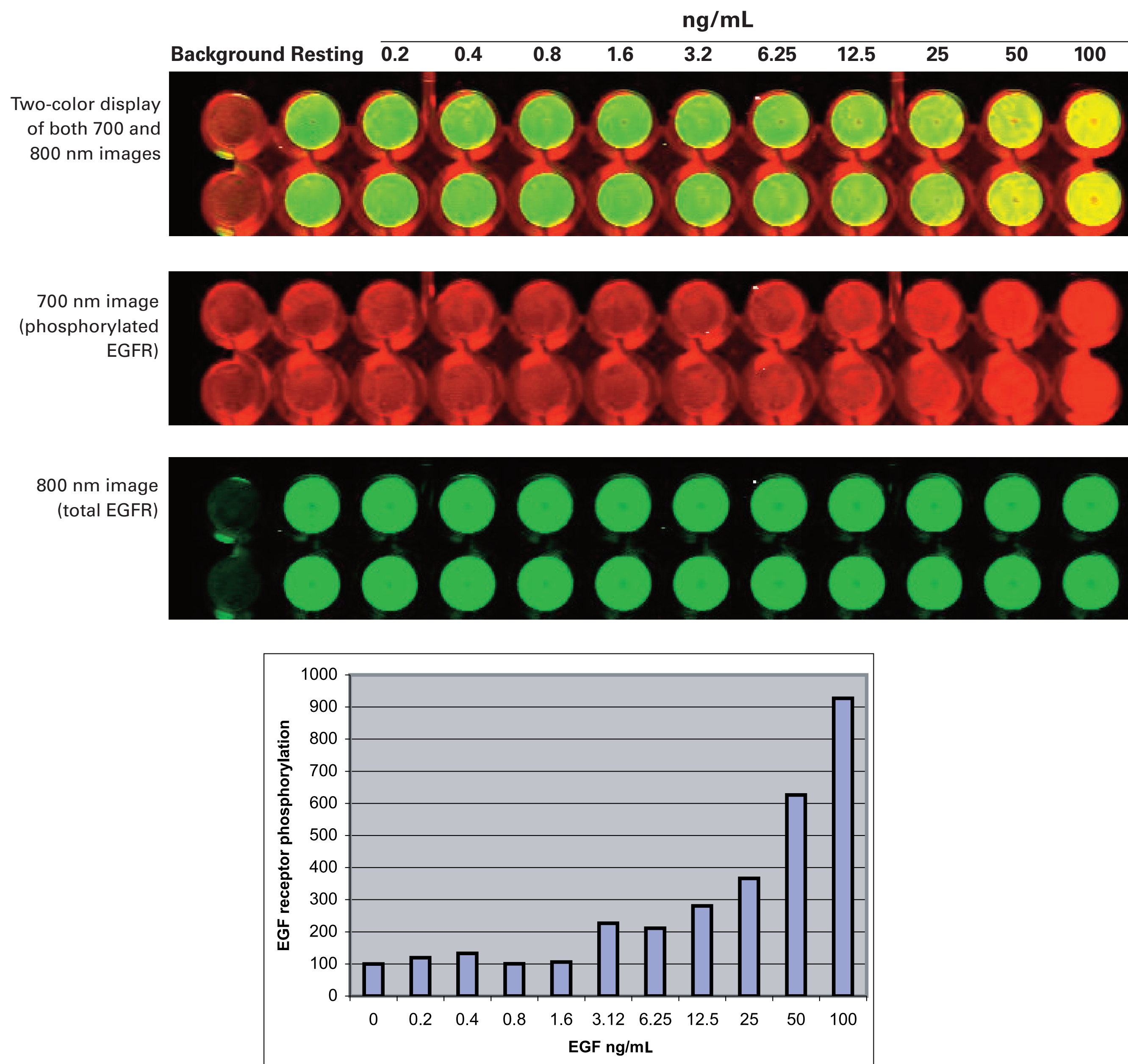


Figure 1. Dose response of A431 cells to Epidermal growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045). The image represents a 96-well two-color In-Cell Western™ assay with the 800 and 700 nm channels detecting total EGF receptor (as normalization) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of EGF receptor.

Quantitative and simultaneous measurements of ERK and phosphorylation of EGF receptor in response to EGF stimulation

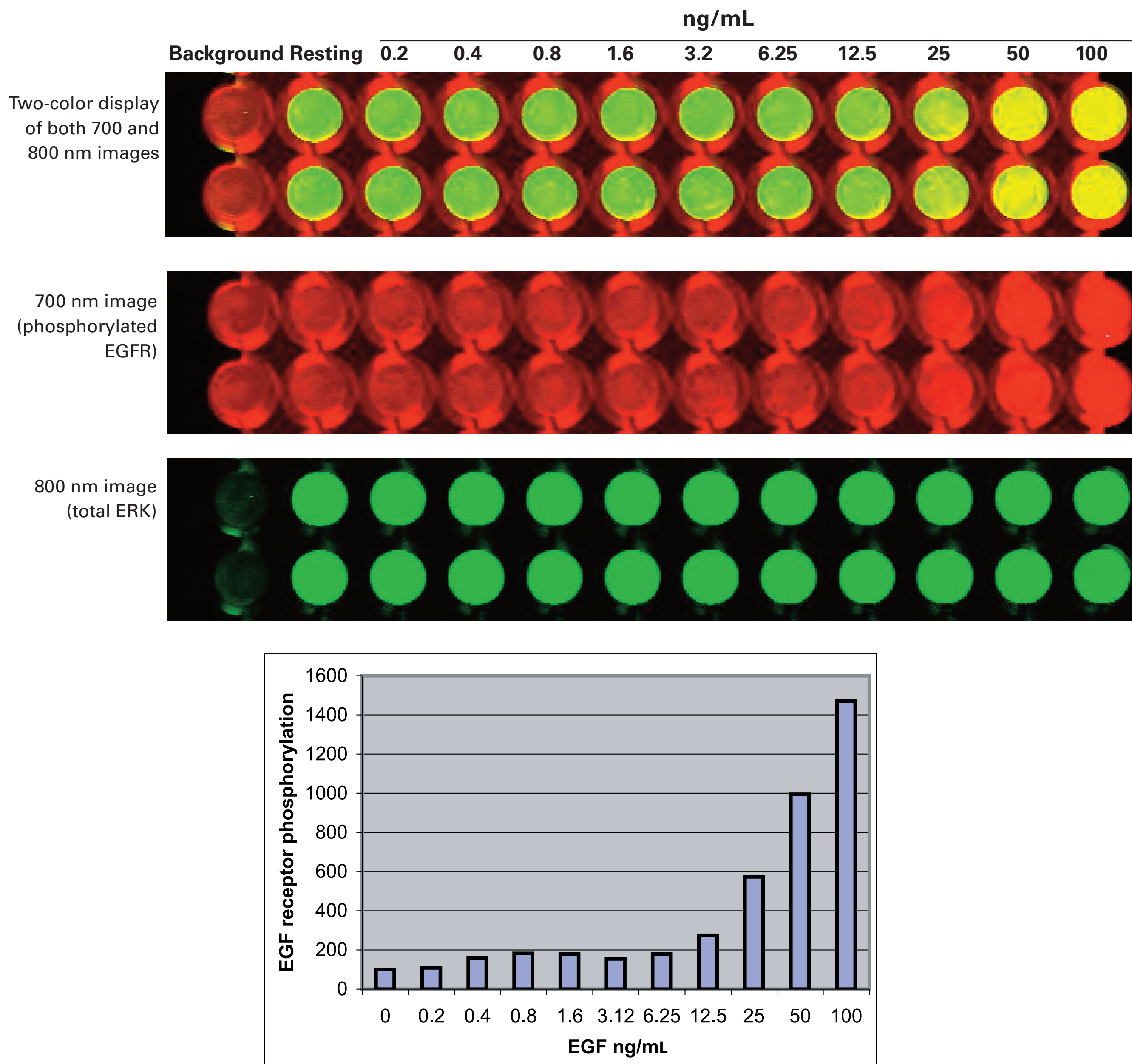


Figure 2. Dose response of A431 cells to Epidermal growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045). The image represents a 96-well two-color In-Cell Western™ assay with the 800 and 700 nm channels detecting total ERK (as normalization) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of EGF receptor.

Quantitative and simultaneous measurements of total ERK and phosphorylation of ERK in response to EGF stimulation

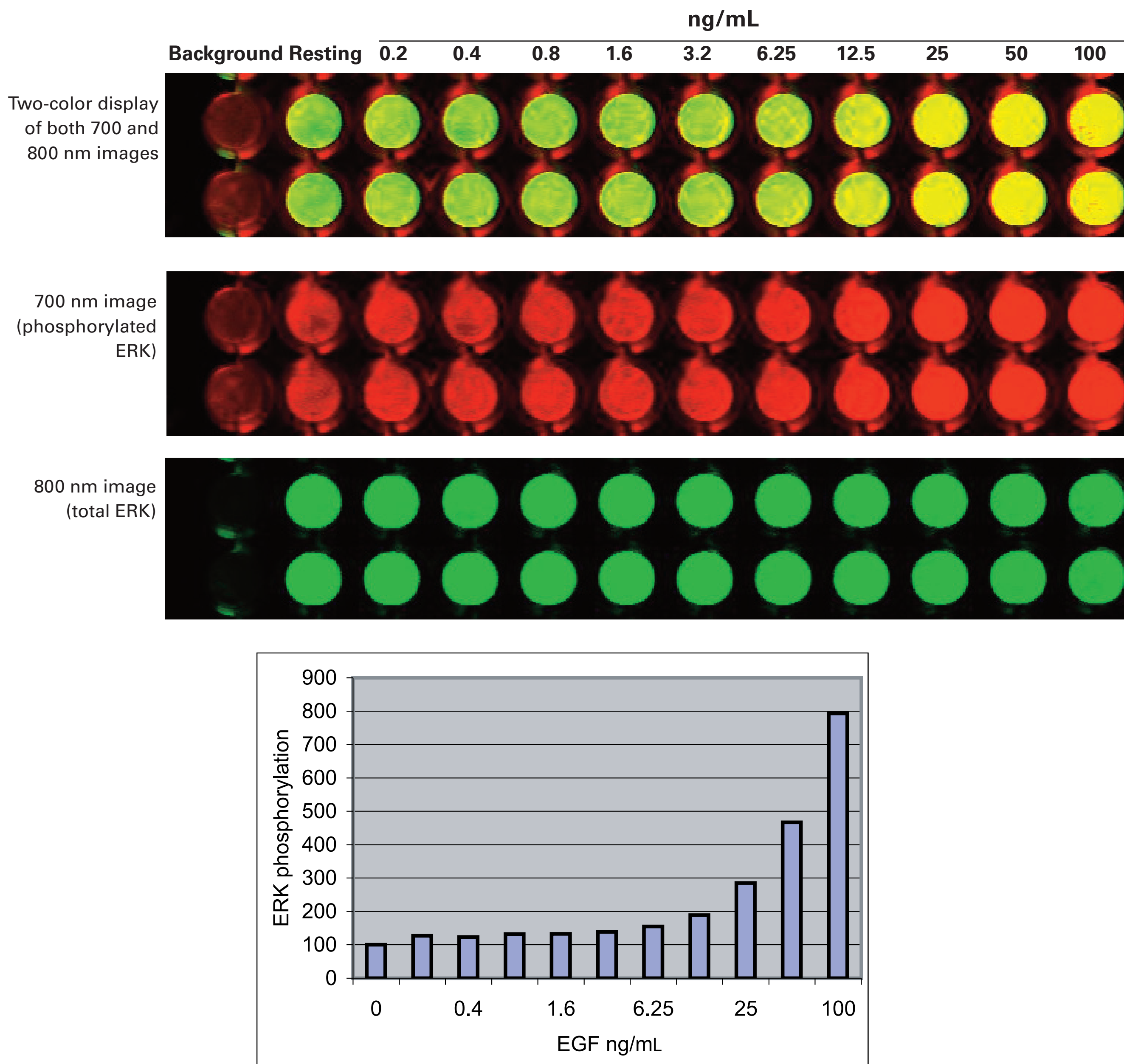


Figure 3. Dose response of A431 cells to Epidermal growth factor (EGF) as measured by specific antibody detecting phosphorylated ERK receptor (Tyr204). The image represents a 96-well two-color In-Cell Western™ assay with the 800 and 700 nm channels detecting total and phosphorylated ERK, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of ERK.

Quantitative and simultaneous measurements of EGFR and ERK phosphorylation in response to EGF stimulation

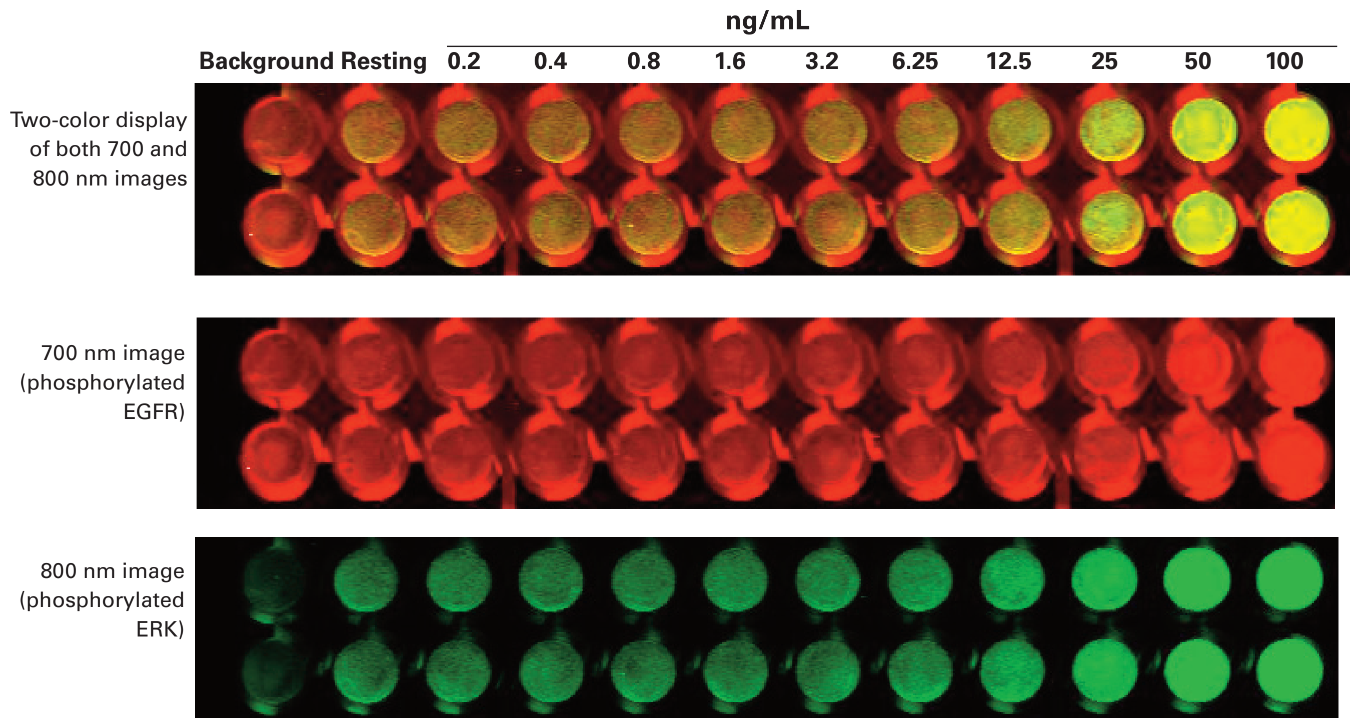
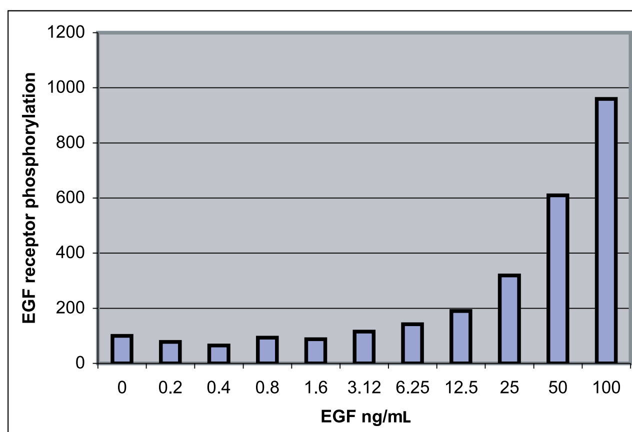
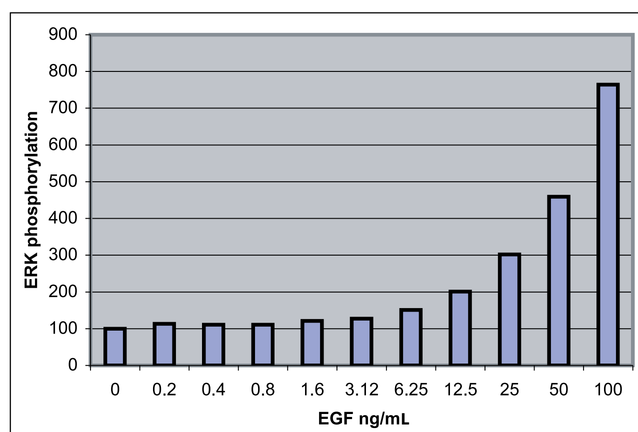


Figure 4. Dose response of A431 cells to Epidermal growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045) and phosphorylated ERK (Tyr204) simultaneously. The image represents a 96-well two-color In-Cell Western™ assay with the 800 and 700 nm channels detecting phosphorylated ERK (Tyr204) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody.





Signal specificity confirmation of In-Cell Western™ Assay using conventional Western blots

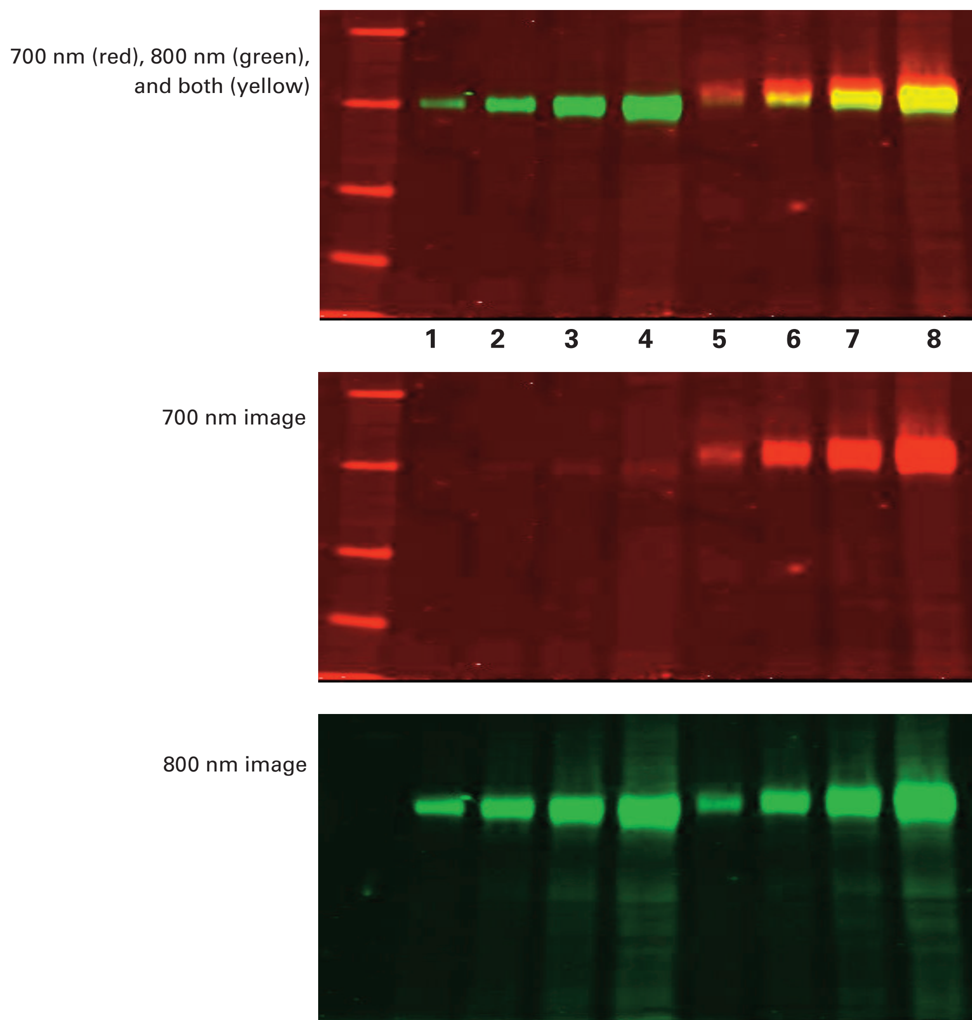
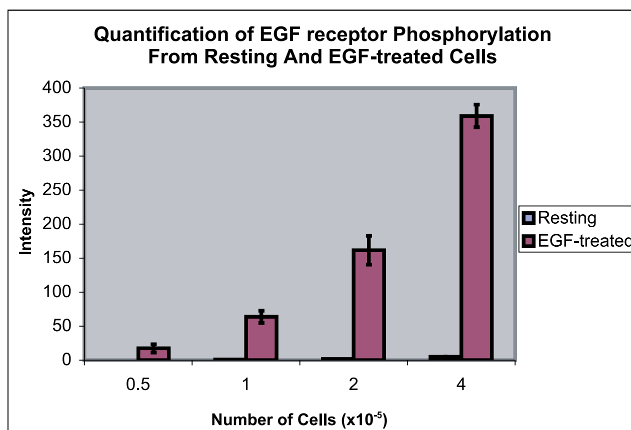
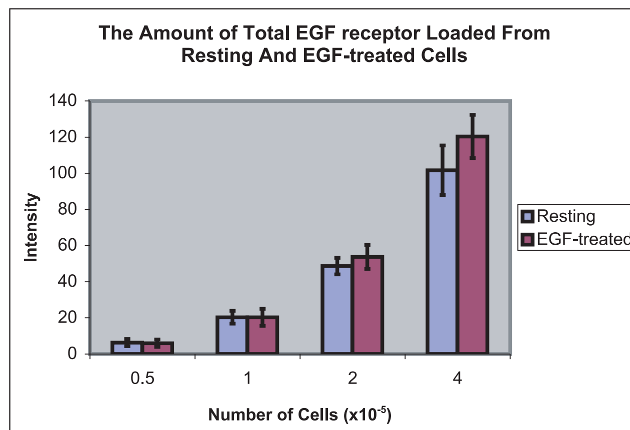


Figure 5. Simultaneous measurement of total and phosphorylated EGF receptor in resting and EGF-treated A431 cell lysates. Two-fold serials of dilutions of resting (lanes 1 to 4) and EGF-treated (lanes 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and total EGFR (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of phosphorylated EGF receptor and total ERK in resting and EGF-treated A431 cell lysates

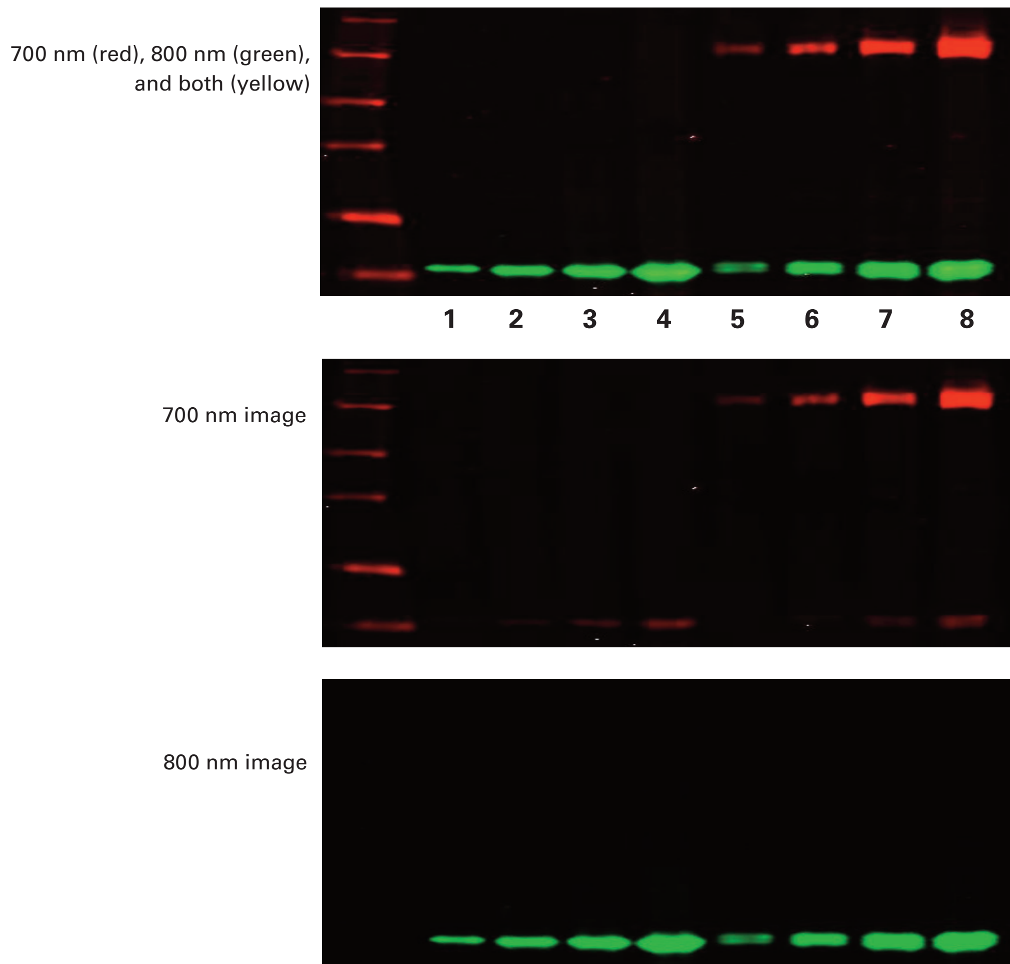
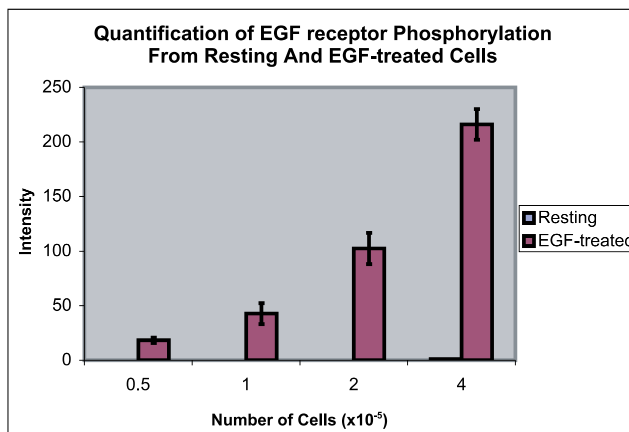
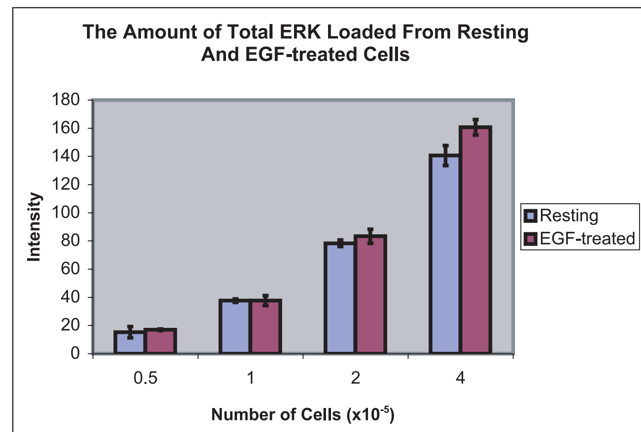


Figure 6. Two-fold serial dilutions of resting (lanes 1 to 4) and EGF-treated (lanes 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and total ERK (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of total and phosphorylated ERK in resting and EGF-treated A431 cell lysates

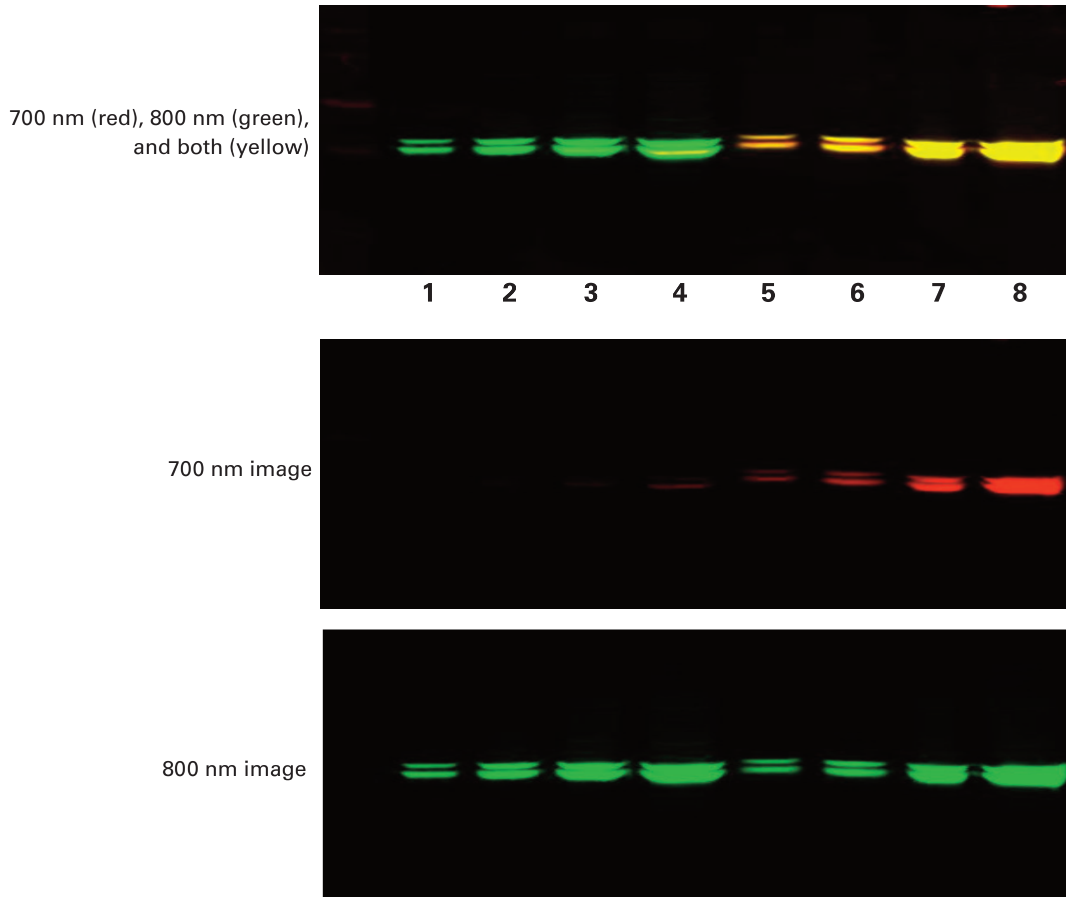
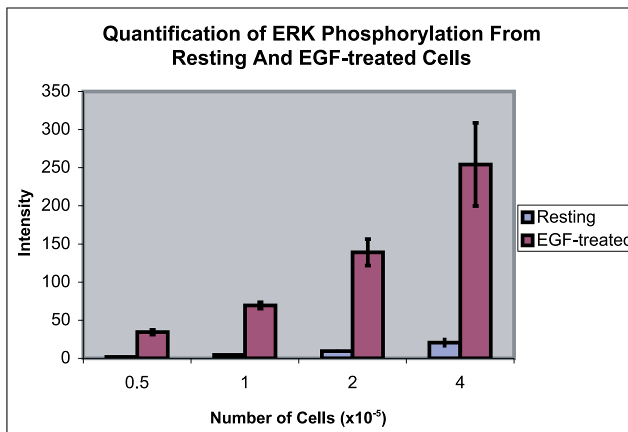
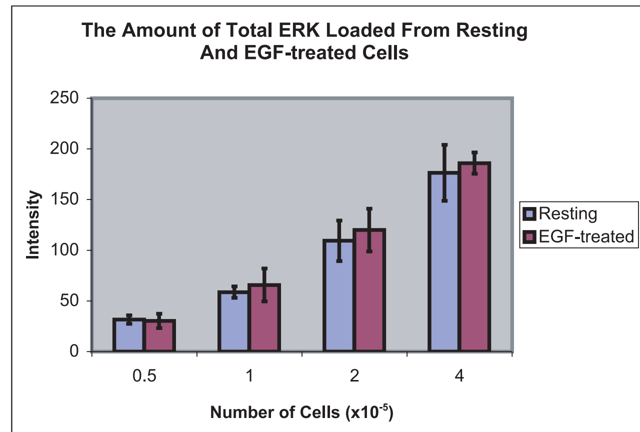


Figure 7. Two-fold serial dilutions of resting (lanes 1 to 4) and EGF-treated lanes 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated ERK (700 nm, red) and total ERK (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of phosphorylated EGF receptor and ERK in resting and EGF-treated A431 cell lysates

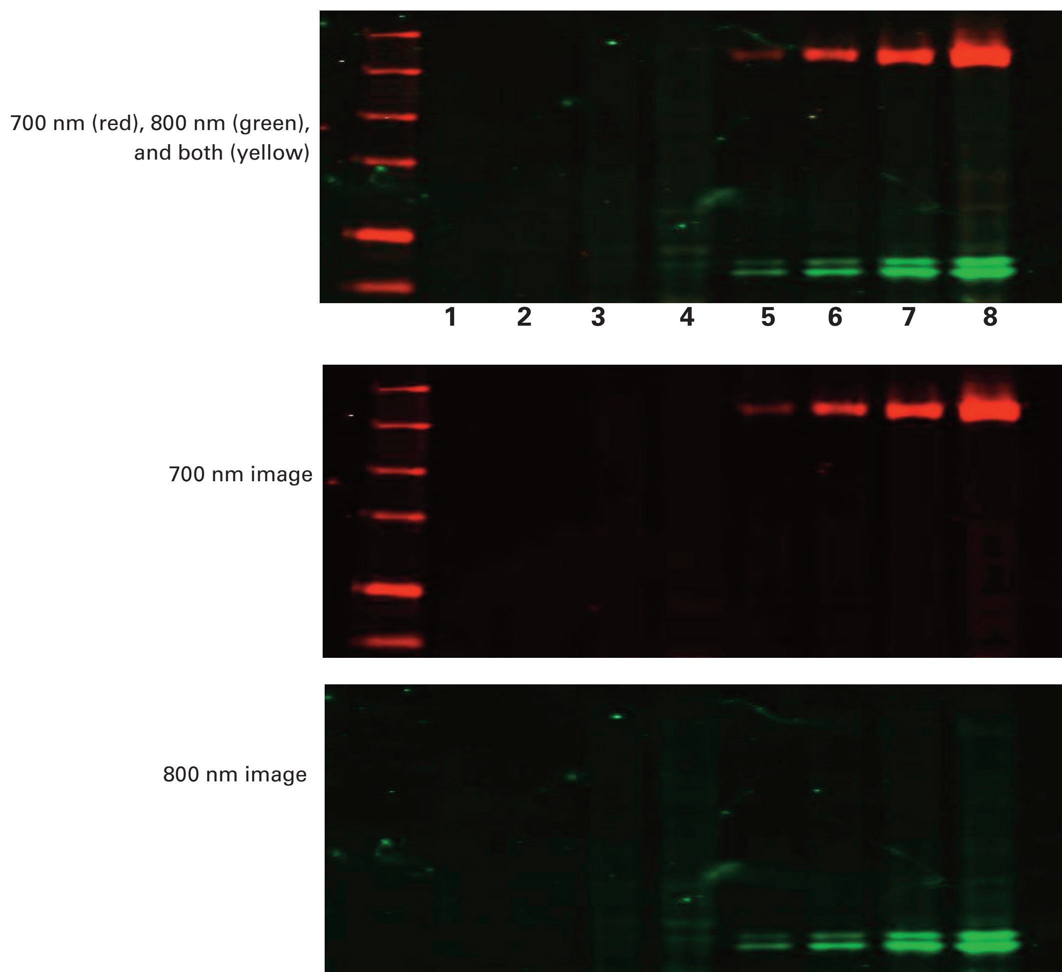
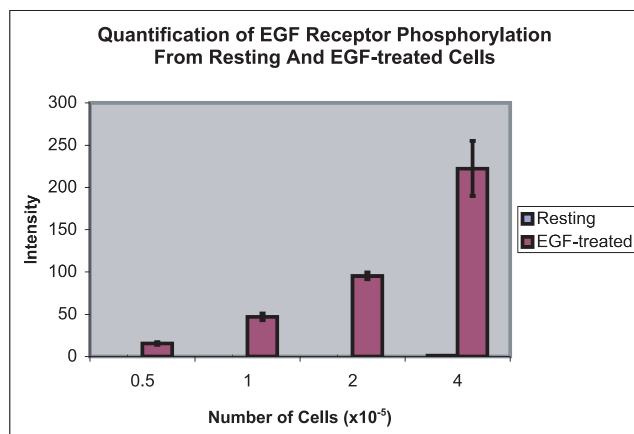
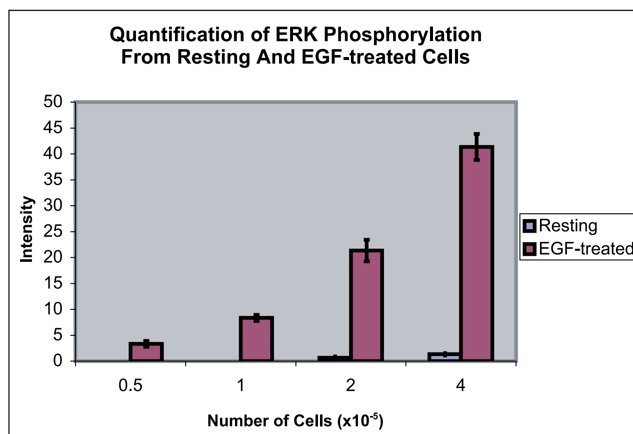


Figure 8. Two-fold serial dilutions of resting (lanes 1 to 4) and EGF-treated (lanes 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and phosphorylated ERK (800 nm, green) in these lysates were simultaneously assessed.





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Odyssey Infrared Imaging Systems and IRDye dye reagents are covered by U.S. patents, foreign equivalents, and patents pending.

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