Quantitative Measurement of the Activation of Signaling Pathways Using Two-Color Infrared Fluorescent Western Blotting and Cell-Based Assays

Huaxian Chen and D. Michael Olive
LI-COR, Inc., 4308 Progressive Avenue, Lincoln, NE 68504
hchen@licor.com

Abstract

There are more than 500 protein kinases and 100 protein phosphostases encoded in the human genome. Protein phosphorylation/dephosphorylation by these kinases and phosphotases is a critical process regulating almost every signal transduction event. The study of protein phosphorylation has largely relied on conventional Western blot and enzymatic kinase assays. Since both assays can assess only one target at a time, an additional step is usually needed for data normalization.

We have developed methods employing two-color infrared fluorescent technology for the analysis of signal transduction events. With three different systems (INF-γ, ERK1/2, and Stat3), we have demonstrated the capability of two-color Western blots to simultaneously detect both the phosphorylated protein and the total protein regardless of its phosphorylation status.

Furthermore, we have extended the assessment of the phosphorylation state of the EGF receptor using simultaneous direct two-color immunostaining of stimulated and unstimulated cells. Dose-response curves demonstrated that the quantitative cell-based assay is sensitive, highly reproducible and linear over a wide dynamic range. The ability to monitor the activation of signaling pathways by in vitro and in situ assays should greatly facilitate the characterization of pharmacological inhibitors and target validation of specific molecules within the cell.

Introduction

Common visible fluorophores cannot be used effectively for direct protein detection on membranes and in plastic plates because of their high background fluorescence in the visible range. Near-infrared (IR) fluorophores (670-1100 nm) have a distinct advantage over visible dyes, in that very low background fluorescence at longer wavelengths provides an excellent signal-to-noise ratio. Furthermore, antibodies labeled with IR dyes at different wavelengths can be used for detection of multiple targets on membranes and in plates, a feature that cannot be accomplished by other technology such as ECL and radioisotopes.

LI-COR has developed an IR imaging system designed to image membranes and plates for protein application. The imager simultaneously detects two distinct wavelengths. A scanning optical assembly carries two laser diodes that generate excitation light at 680 and 780 nm, as well as two avalanche photodiodes, which detect emitted fluorescence at 720 and 820 nm. Here we demonstrate two-color infrared fluorescent technology for the analysis of signal transduction events by in vitro and in situ assays.

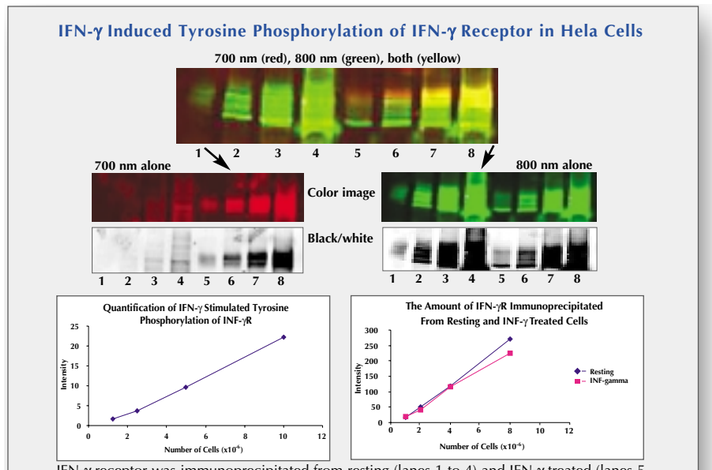
Materials & Methods

Primary antibodies against INF-γR, EGFR, ERK, Stat3 and their phosphorylated forms (4G10, phospho-EGFR, phospho-ERK, phospho-stat3) were purchased from commercial sources. Goat anti-mouse and anti-rabbit secondary antibodies were labeled with IRDye 38 (800 nm, Rockland Immunochemicals) or with Alexa Fluor® 680 (700 nm, Molecular Probes).

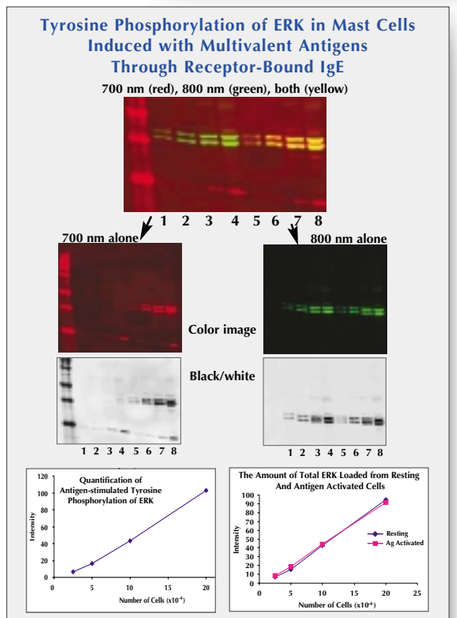
The basic procedure for two-color infrared fluorescence detection is as follows:

Mouse antibody against the phosphorylated protein
+
Rabbit antibody against the total protein regardless of its phosphorylation status
Simultaneously added to Western blots or plates
↓
Alexa Fluor® 680-labeled goat α-mouse (red)
+
IRDye 38-labeled goat α-rabbit (green)
↓
Odyssey™ Analysis

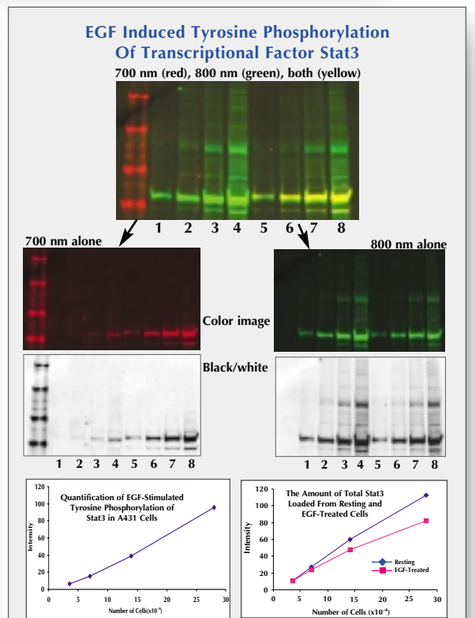
Two-Color Western Blots



IFN-γ receptor was immunoprecipitated from resting (lanes 1 to 4) and IFN-γ treated (lanes 5 to 8) cells. Two-fold serial dilutions of the immunoprecipitated proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and detected simultaneously with mouse anti-phosphotyrosine (visible in red) and rabbit anti-IFN-γ receptor (visible in green). The yellow color in the top panel indicates the presence of both dyes in a given pixel.



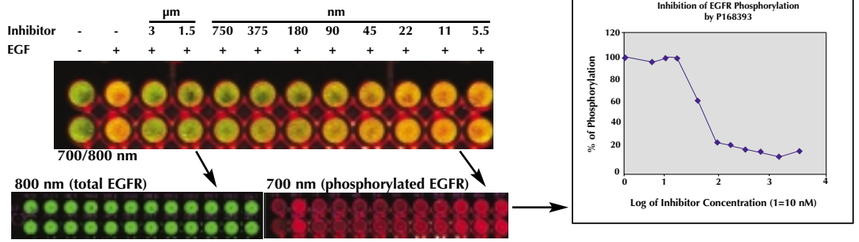
Mast cells sensitized with anti-DNP IgE were reacted with DNP_{38}-albumin for 5 minutes. Two-fold serial dilutions of total cellular lysate from resting (lanes 1 to 4) and activated (lanes 5 to 8) cells were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and detected simultaneously with mouse antibodies recognizing only tyrosine-phosphorylated ERK (visible in red), and rabbit antibodies recognizing ERK regardless of its phosphorylation status (visible in green).



A431 cells were stimulated with EGF for 10 minutes. Two-fold serial dilutions of total cellular lysate from resting (lanes 1 to 4) and activated (lanes 5 to 8) cells were separated in SDS-PAGE, transferred onto nitrocellulose membrane, and detected simultaneously with mouse antibodies recognizing only tyrosine-phosphorylated Stat3 (visible in red), and rabbit antibodies recognizing Stat3 regardless of its phosphorylation status (visible in green).

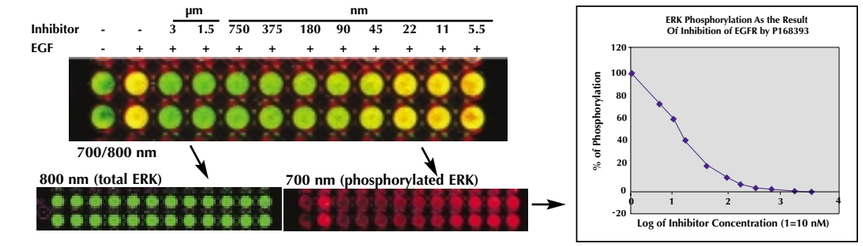
Two-Color Cell Based Assays

Quantitative and simultaneous measurements of EGFR and phosphorylation of EGFR in response to EGF in the presence of EGFR inhibitor



A431 cells were seeded in a 96-well plate and grown to be confluent. The cells were serum-starved overnight. Two-fold serial dilutions of the EGFR inhibitor, PD168393, were added to wells 3 through 12, and were incubated at 37°C for 1 hour. The cells in wells 2 to 12 were stimulated with 100 ng/ml of EGF for 15 minutes. After stimulation, the cells were fixed with 4% formaldehyde/PBS for 20 minutes at room temperature and washed three times with 0.1% Triton X-100/PBS. After blocking, the total EFGR (visible in green) and phosphorylated EGFR (visible in red) were revealed by incubation with rabbit α-EGFR and mouse α-phosphorylated EGFR, followed by incubation with Alexa Fluor® 680-labeled goat α-mouse and IRDye 800-labeled goat α-rabbit antibodies.

Quantitative and simultaneous measurements of total ERK and phosphorylation of ERK in response to EGF in the presence of EGFR inhibitor



A431 cells were treated as described above. The total ERK (visible in green) and phosphorylated EGFR (visible in red) were revealed by incubation with rabbit α-ERK and mouse α-phosphorylated ERK, followed by incubation with Alexa Fluor® 680-labeled goat α-mouse and IRDye 800-labeled goat α-rabbit antibodies.

Conclusions

- Two-color Western blot and in situ assays allow two antigens to be analyzed simultaneously on the same membrane or in the same well, a feature that is critical for drug screening, yet which cannot be offered by other technologies such as ECL and radioisotopes.
- Quantitative measurements are linear over a wide range and the fluorescent signals are directly proportional to the amount of antigens present on membranes and in wells without the influence of exposure time and stripping as in ECL. Infrared fluorescent analysis offers an excellent technique enabling the large-scale analysis of signal transduction, including drug screening.

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Phone 800-645-4267
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