

A Cell Based Immunocytochemical Assay For Monitoring Kinase Signaling Pathways And Drug Efficacy

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ABSTRACT

Protein kinases play an important role in many disease processes, and are a primary target for drug development. Because cellular phosphorylation cascades are complex multidirectional pathways, the behavior of a drug in a biochemical enzyme assay may not accurately reflect its performance in the context of a whole cell. We have developed a near-infrared (NIR) cyto blot assay that can be used to investigate both kinase signaling and the effects of kinase inhibitors. Adherent cells were grown in either 96 or 384 well plates. Following stimulation, protein phosphorylation was detected immunohistochemically by simultaneous staining with two primary antibodies: a phospho-specific primary, and an additional normalization antibody that recognized either the target protein regardless of phosphorylation status (pan protein) or a housekeeping protein. Secondary antibodies labeled with two spectrally distinct near-infrared dyes were used for visualization. Nuclear staining with TO-PRO-3 was also used in place of the normalization antibody. Normalization for well-to-well variability was accomplished by ratiometric analysis of the two wavelengths. The near infrared cyto blot was used to analyze phosphorylation of EGFR, Akt, Stat3, MEK 1, and ERK1/2. This assay format was also able to simultaneously assess the phosphorylation of multiple signaling proteins in response to known kinase inhibitors. We observed that the IC₅₀ for the EGFR inhibitor PD168393 was similar for EGFR and Stat3 but was significantly higher for ERK1/2, a downstream modulator of EGFR function. The observation that the receptor and its effectors show different IC₅₀ values for the same inhibitory drug could be important for target selection in drug development.

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INTRODUCTION

Protein kinases have emerged as important cellular regulatory proteins in many diseases [1–6]. Protein kinases are enzymes that covalently transfer the gamma phosphate group of ATP to specific tyrosine, serine, or threonine residues in proteins, thereby changing the activity of key signaling proteins or facilitating formation of multi-enzyme complexes. Kinases directly or indirectly control most cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis, and differentiation [1, 2].

With the completion of the human genome sequence, it is estimated that there are approximately 500 protein kinases encoded within the genome [7, 8], representing approximately 1.7% of all human genes [7]. A majority of the more than 30 known tumor suppressor genes and more than 100 dominant oncogenes are protein kinases [9]. Thus protein kinases offer an abundant source of potential drug targets at which to intervene in cancer and other diseases.

While *in vitro* biochemical assays for protein phosphorylation are easily carried out, they cannot duplicate the cellular environment. Cell signaling cascades are multidirectional pathways rather than single biochemical reactions. Although a particular member of a signaling pathway may be effectively inhibited by a candidate drug, a given inhibitor may affect other kinases downstream of the desired target which can be reflected as a false positive, or the pathway itself may remain unaffected due to alternative signaling routes that bypass the targeted kinase. Thus a kinase inhibitor may not achieve the desired effect when tested in a cellular system. To more precisely assess the effects of a given compound on a kinase-mediated pathway, *in vivo* cell based assay formats can be used to validate the inhibitory effects of a drug candidate on both the

target and the targeted pathway. These methods enable assessment of entire pathways or even multiple pathways to fully evaluate the functional effects of the drug compound [10], and address cell permeability and toxicity issues as well.

Here we describe a near-infrared (NIR) immunocytochemical method for analyzing cellular signaling pathways and the effects of kinase inhibiting drugs on these pathways. The system consists of a near infrared assay chemistry and a microplate scanner that employs two NIR lasers for excitation of fluorescent signals. While the assay is based on standard immunocytochemical methods, the use of NIR fluorescence enables extremely sensitive and quantitative analysis of protein signaling pathways in cultured cells in a higher throughput manner, with reduced interference from cell, plate, and drug compound autofluorescence. The assay has broad applicability for the analysis of protein signaling pathways, cell-based determinations of IC₅₀ concentrations for lead optimization, as well as the examination of the effects of drug compounds on multiple points within one or more signaling pathways.

MATERIALS AND METHODS

General Materials

Epidermal growth factor (EGF), and platelet derived growth factor (PDGF) were obtained from Upstate Biotechnology (Rochester, NY). U0126 was obtained from Promega Corporation (Madison, WI). PD168393 was obtained from Calbiochem (San Diego, CA). LY294002 and Sigma Screen Poly-D-Lysine coated 96-well microplates were obtained from Sigma Biochemicals (St. Louis, MO). Falcon 384 well plates were obtained from BD Biosciences (San Jose, CA). Odyssey® Blocking Buffer, the Odyssey Infrared Imager, and Aeries Infrared Imager were from LI-COR® Biosciences (Lincoln, NE). TO-PRO-3 was obtained from Molecular Probes, Inc. (Eugene, OR). Antibodies were obtained from the sources shown in Table 1.

Table 1
Antibody sources and concentrations used in the study

Antibody	Company	Cat. No.	Dilution Used
Mouse α -EGFR	Biosource	AHR5062	1:500
Rabbit α -phospho-EGFR	Cell Signaling Technology	2237	1:100
Rabbit α -ERK	Santa Cruz Biotechnology	SC-94	1:200
Mouse α -phospho-ERK	Santa Cruz Biotechnology	SC-7383	1:100
Rabbit α -phospho-ERK	Cell Signaling Technology	9101	1:100
Rabbit α -phospho-Stat3	CalBiochem	568389	1:40
Rabbit α -phospho-Akt	Cell Signaling Technology	9271	1:100
Goat α -mouse IRDye™ 800CW	Rockland Immunologicals	610-131-121	1:800
Goat α -rabbit IRDye™ 800CW	Rockland Immunologicals	611-131-122	1:800
Goat α -mouse Alexa Fluor 680	Molecular Probes	A-21050	1:200
Goat α -rabbit Alexa Fluor 680	Molecular Probes	A-21109	1:200

α , anti

Antibody Validation

All antibodies were tested for their performance on Western blots against a lysate of appropriately stimulated cells using infrared dye labeled secondary antibodies and either the Odyssey or Aeries instruments. The antibodies reported here all yielded single bands after Western blotting. Phospho and pan antibodies were also screened for interference by comparing the intensity of the respective bands on Western blots on which the phospho and pan proteins were detected either simultaneously or separately. None of the antibodies selected for the final assays exhibited interference.

Cell Culture

A431 and NIH3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture medium and fetal calf serum were obtained from Invitrogen-Gibco (Carlsbad, CA). Cells were maintained in DMEM supplemented with 10% fetal calf serum. For 384 well plates, cells were seeded at approximately 10^4 cells per well. For 96 well plates, cells were seeded at approximately 1.5×10^4 cells per well. For serum starvation, the media was removed by aspiration, replaced with serum free media, and incubated at 37°C for 4-16 h.

Analysis of Pathway Induction

For induction of signaling pathways, EGF or PDGF were added to a concentration of 100 ng/ml and incubated for varying time intervals to determine the optimal time for detection of phosphorylation as detected by Western blotting of stimulated cell lysates. For the near infrared cyto blot assay, cell cultures were incubated with the appropriate stimulatory substance, and incubated for the predetermined optimal time interval. After incubation, the media was discarded and the cells were fixed with either 3.7% formaldehyde in PBS or 1X Prefer (Anatech; Ann Arbor, MI) for 20 minutes. Following fixation, the fixative was discarded and the cells were permeabilized by five washes with 0.1% Triton X-100 in PBS at room temperature with gentle rocking. After the last wash, Odyssey Blocking Buffer was added and the cells incubated for 1.5 h at room temperature with gentle rocking. Blocking buffer was discarded prior to antibody staining.

To normalize the phosphorylation signal against total target protein, the phospho-specific and pan antibodies were diluted together in blocking buffer at the concentrations listed in Table 1, and added simultaneously to the cells. The cells were incubated with the primary antibodies overnight at 4°C. The next day, the primary antibody solutions were discarded and the plates were washed 4 times with 0.1% Tween-20 in PBS for 5 min with gentle shaking, using a generous amount of buffer. The appropriate labeled secondary antibodies were diluted in blocking buffer containing 0.2% Tween-20 to the concentrations listed in Table 1, and applied to the cells, then incubated for 60 min at room temperature. The labeled secondary antibodies were discarded and the cells washed 4 times with 0.1% Tween-20 in PBS for 5 min with gentle shaking. The plates were imaged by scanning simultaneously at 700 nm and 800 nm with an Odyssey or Aeries instrument at 169 μ m resolution, medium quality, focus offset of 3.0 mm, and intensity setting of 5 for both 700 and 800 nm channels. To normalize the phosphorylation signal against TO-PRO-3

staining, the phospho antibody was added and incubated as described previously. TO-PRO-3 diluted 1:5000 in PBS was added, along with the dye-labeled secondary antibody, and treated as previously described.

Pathway Analysis Following Treatment with Kinase-Inhibitory Drugs

For assessment of the effects of kinase inhibitory drugs on pathway function, cells were seeded into 96 or 384 well microwell plates and grown as described above. Prior to treatment with pathway-specific inducing agents, the cells were pre-treated with serial dilutions of the kinase inhibitor. Serial dilutions of PD168393, U0126, or LY294002 in DMEM were incubated with the cells for 1 to 2 h at room temperature. The cells were then induced for the pathway of interest and stained as described above.

RESULTS

Detection of Phosphorylation by Near Infrared Cyto blot Assay

The NIR cyto blot assay is a two-color fluorescent immunocytochemical microplate assay in which one fluorescent channel is used to detect the target protein while the second fluorescent channel is used to normalize for the well-to-well variations in cell numbers. The signals from the two channels are expressed as a ratio which reflects the normalized amount of target protein present in the sample.

This assay can be used to examine the conditions which lead to stimulation of phosphorylation. Once the conditions for stimulation are established, the assay can be used to examine inhibitory compounds. For these purposes, it is not necessary to achieve maximal stimulation of the pathway. Rather, sufficient increase in phosphorylation of the target marker protein must be achieved such that the effects of the inhibitor can be reliably measured. In our experience, this generally requires a minimum of a 5-fold increase in phosphorylation following stimulation. For example, A431 cells were treated with serial dilutions of EGF to stimulate phosphorylation of proteins in the signaling pathway. As shown in Figs. 1A and 1B, 100 ng of EGF gave highly efficient stimulation of ERK and EGFR phosphorylation. Under these conditions, ERK phosphorylation was increased greater than 16-fold over the resting state. Under the same conditions, EGFR autophosphorylation was increased approximately 12-fold (Figs. 1C and 1D). This level of increased phosphorylation was sufficient to reliably measure the effects of inhibitors on the pathways.

The assay was used to determine the IC_{50} of a known EGFR inhibitor, PD168393. A431 cells were treated with dilutions of PD168393 and incubated for 1 to 2 h.

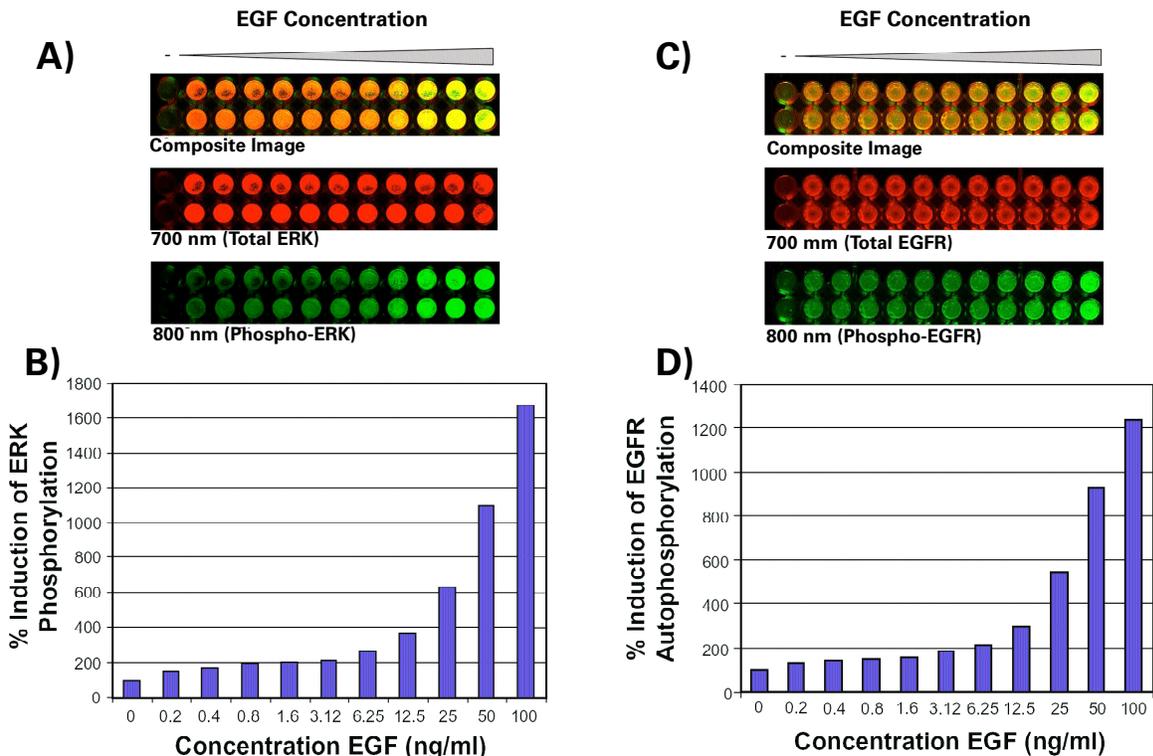


Fig. 1. Phosphorylation of ERK and EGFR in response to pathway stimulation. A431 cells were stimulated and treated as described in Materials and Methods.

- A) Detection of ERK phosphorylation. (Top) Composite image showing the fluorescence of the microplate in both the 700 nm and 800 nm detection channels. Duplicate rows are shown. (Middle) Detection of total ERK protein regardless of phosphorylation status (pan protein). (Bottom) Detection of increasing amounts of phospho-ERK as a function of increasing amounts of EGF stimulation.
- B) Quantification of the duplicate fluorescence results in A.
- C) Detection of EGFR autophosphorylation, showing composite image (top), total EGFR (middle), and increasing amounts of EGFR autophosphorylation (bottom).
- D) Quantification of the duplicate fluorescence results in C.

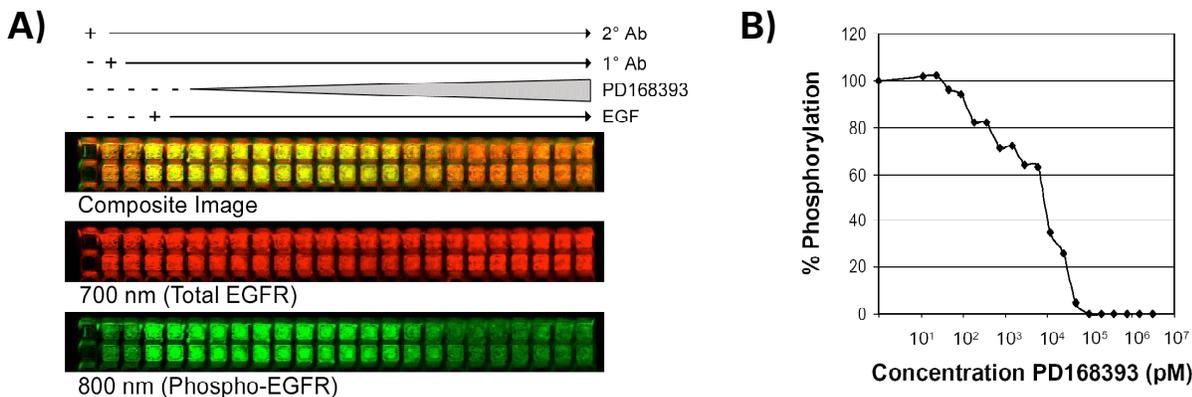


Fig. 2. IC₅₀ determination for inhibition of EGFR autophosphorylation by PD168393.

- A) Experimental plate layout and fluorescent images of the results. (Top) Composite view of both the 700 and 800 nm channels. (Center) Detection of total EGFR for the purposes of normalization. (Bottom) Decreasing amounts of phospho-EGFR as a function of increasing the concentration of PD168393. Samples were run in duplicate.
 - B) IC₅₀ plot.
- Abbreviations: Ab, antibody.

The drug was removed and 100 ng of EGF added to each well. After 7.5 min, the cells were washed and fixed. EGFR autophosphorylation was quantified using phospho-specific and pan antibodies.

Fig. 2A shows the image of a portion of the 384 well plate. The top panel is a composite image of the fluorescence from both the 700 nm and 800 nm channels. The center panel represents detection of total EGFR with the pan antibody. Amounts of total EGFR are comparable across the wells. The bottom panel shows the effects of increasing concentrations of PD168393 on phosphorylation of EGFR. At high concentrations of drug, EGFR phosphorylation is completely inhibited. The inhibition of EGFR autophosphorylation was proportional to the concentration of PD168393. Fig. 2B illustrates the IC_{50} curve plotted from the data in Fig. 2A and yielded an IC_{50} of 8 nM. Previously, Fry et. al [11] reported that PD168393 has an IC_{50} of 4.3 nM when assayed in A431 cells. Thus the NIR cytotblot format agrees well with the previously reported value.

Pathway Analysis by NIR Cytoblot

LY294002, an inhibitor of PI3K, was assessed for its effect on the PI3K/Akt and Ras/Raf/MEK/ERK pathways. LY294002 has been widely used to block PDGF-induced phosphorylation of Akt; however, LY294002 should be considerably less potent in preventing ERK phosphorylation in response to PDGF. NIH3T3 cells were treated with LY294002, followed by stimulation with PDGF. As expected, the two pathways responded very differently to LY294002 treatment (Fig. 3). In the NIR cytotblot, PI3K/Akt was effectively inhibited with an IC_{50} of 2.34 μ M of drug, which is similar to the IC_{50} of 1.40 μ M

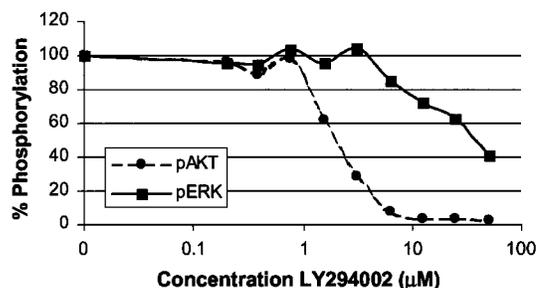
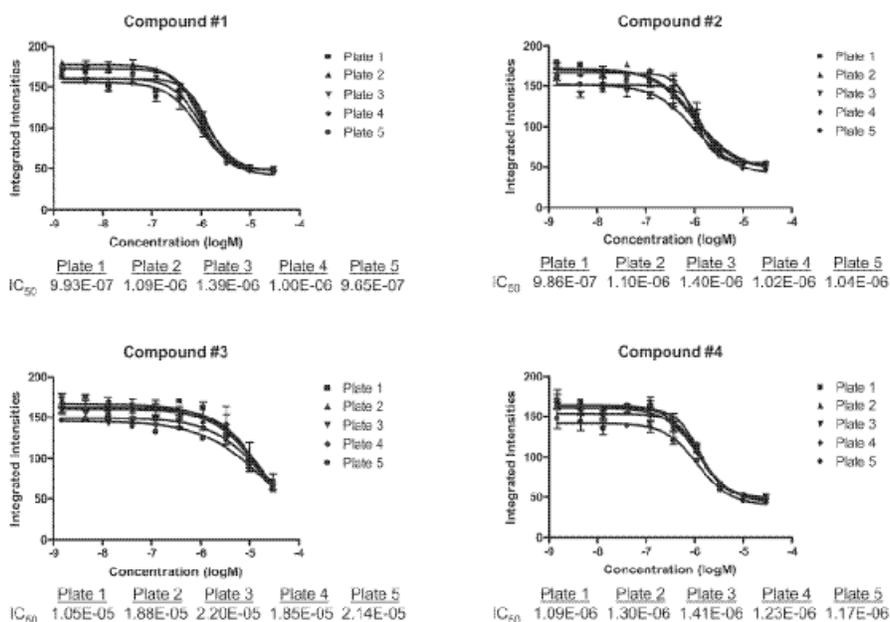


Fig. 3. Comparison of the effects of LY294002 on Akt and ERK phosphorylation. The IC_{50} curves were determined on a single microwell plate as described in Materials and Methods. Samples were run in duplicate.

previously reported by Vlahos et. al [12]. At this concentration, ERK phosphorylation was unaffected, with an IC_{50} of greater than 50 μ M, which also agrees with the results reported by Vlahos et. al [12]. This demonstrates that the NIR cytotblot assay can effectively differentiate drug effects on different pathways.

To examine the reproducibility of the NIR cytotblot format for IC_{50} determination, four experimental inhibitors that prevent PDGF-induced Akt phosphorylation were examined. For each compound, eight replicates of an IC_{50} assay were performed on a 96-well plate. The integrated fluorescence intensity was averaged across the eight replicates for each compound concentration. The experiment was repeated five times for each compound. The data are shown in Fig. 4. Each compound

Fig. 4. Reproducibility of the NIR cytotblot assay. A 96-well plate format was used to determine IC_{50} values for four experimental compounds that each inhibit PDGF-induced phosphorylation of Akt in NIH3T3 cells. At each drug concentration, the integrated fluorescence intensity for phospho-Akt staining was determined. For each data point, eight replicates were performed; the mean and standard deviation are shown. Each experiment was repeated five times (Plates 1-5) and IC_{50} values for each plate are shown.



gave IC_{50} values that were indistinguishable across the five replicate plates. The IC_{50} values for compounds 1, 2, and 4 were indistinguishable from IC_{50} values determined by a filtration binding kinase phosphorylation assay (data not shown). Compound 3 was 100-fold higher in the NIR cytotblot, possibly due to the inability of the drug to penetrate the cell.

The pathway analysis capability of the NIR-cytoblot assay was further tested by examining the effect of the MEK inhibitor U0126 on the EGFR-induced Stat3 and Ras/Raf/MEK/ERK pathways. A431 cells grown in a 96 well plate were treated with serial dilutions of U0126 and stimulated with EGF. Under EGF stimulation in the absence of U0126, increased phosphorylation of EGFR, Stat3, and ERK is expected, while in the presence of U0126, only inhibition of ERK phosphorylation is expected. As shown in Fig. 5A, in the absence of U0126, phosphorylation of EGFR, Stat3, and ERK was significantly stimulated by EGF treatment. Furthermore, as expected, only ERK phosphorylation showed the inhibitory effects of the drug with a measured IC_{50} of approximately 10 μ M in the in vivo NIR cytotblot format. This value is indistinguishable from the reported IC_{50} [13].

EGFR has significant involvement in many cancers and, as a result, is a prime target for kinase inhibiting anti-cancer drugs. Anti-EGFR drug candidates are most often screened by comparing the IC_{50} values for the various EGFR inhibiting drug candidates. However, EGFR can signal through several pathways, including PI3K/Akt, JAK/Stat and Ras/Raf/MEK/ERK. The effectors of EGFR function are, in fact, downstream signaling molecules such as Stat3 and ERK. Because pathways can intersect at several points downstream of EGFR, it is important to assess multiple points in a signaling pathway in order to ascertain that the EGFR-targeted drug is effective in shutting down the actual effectors of the targeted pathway. This is especially true if more than one defect is responsible for the abnormal cellular phenotype. That the receptor and its effectors show similar IC_{50} values is critical for target selection. If, for example, in an experimental tumor model, an EGFR inhibitor does not effectively shut down Stat3 or ERK signaling at the same IC_{50} , a combination of pathway inhibitors may be necessary to correct the abnormal signaling.

To assess whether or not the NIR-cytoblot assay could identify differences in the inhibition of a receptor tyrosine kinase and a later effector protein in the same

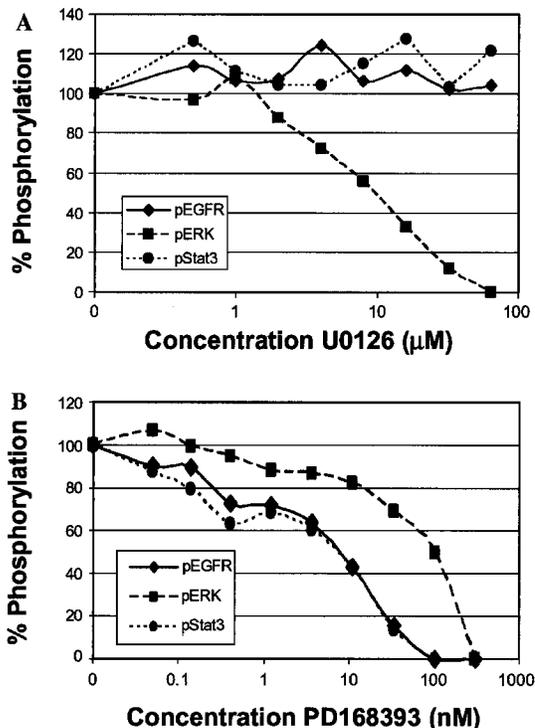


Fig. 5.

- A) Comparison of the effects of U0126 on EGFR, Stat3, and ERK phosphorylation. The IC_{50} curves were determined on a single microwell plate as described in Materials and Methods. Samples were run in duplicate.
- B) Comparison of the effects of PD168393 on EGFR, Stat3, and ERK phosphorylation. The IC_{50} curves were determined on a single microwell plate as described in Materials and Methods. Samples were run in duplicate.

pathway, we treated A431 cells with serial dilutions of the EGFR inhibitor PD168393, followed by stimulation of the cells with EGF. We then compared the IC_{50} of PD168393 on EGFR, Stat3, and ERK. The IC_{50} values of PD168393 for EGFR autophosphorylation and Stat3 phosphorylation were equivalent at approximately 11.1 nM (Fig. 5B). In contrast, the IC_{50} of PD168393 for ERK phosphorylation under these conditions was almost 10-fold higher (approximately 100 nM). Although ERK phosphorylation appeared to be stimulated to the same extent as EGFR, the inhibitory effect of the drug on ERK was significantly different than that seen for EGFR and Stat3 in this cell model.

DISCUSSION

While biochemical assays for kinase activity are routinely used as a primary screen, they cannot duplicate the cellular environment. Cell based assays may more accurately reflect the status of multi-branched signaling pathways involved in disease. The use of NIR fluorophores enables sensitive detection of phosphorylation of receptor tyrosine kinases and intracellular signaling proteins. The two-color ratiometric approach results in precise, quantitative analysis of cell signaling with good reproducibility. In addition, the use of NIR detection should avoid the problem of compound interference, which has been reported for other methods of kinase analysis [15–17]. The value of using a cell-based approach for evaluation of kinase inhibitors was demonstrated by the different observed effects of the EGFR inhibitor PD168393 on phosphorylation of EGFR, Stat3, and ERK. The IC_{50} of PD168393 was approximately 10-fold higher for ERK than for EGFR and Stat3. There may be several explanations for this. The activity of the Ras/Raf/MEK/ERK pathway is affected by several other signaling pathways such that ERK phosphorylation levels, while decreased by inhibition of EGFR, may be maintained at a higher intracellular level. Alternatively, the enzymatic activity of the Ras/Raf/MEK/ERK proteins may amplify the signal from EGFR stimulation to higher levels than those of the JAK/Stat pathway. Regardless of the mechanism, the results emphasize the advantage of a cell based assay for assessing the effects of kinase inhibitors over an in vitro assay.

Several parameters are important to optimize for the NIR cyto blot assay. Antibody specificity is critical and all antibodies used for the assay should yield single bands on a Western blot of a cell lysate. Furthermore, if a combination of phospho-specific and pan antibodies to the same protein are used, they must be examined for interference when used together. An alternative approach that is especially useful if the phospho-specific and pan antibodies interfere is to use a phospho-specific antibody and the DNA stain TO-PRO-3. This simplifies assay optimization, and the DNA stain is less costly than an additional primary antibody. Induction of phosphorylation in a given pathway must also be optimized for the amount of inducer and the time at which maximal phosphorylation can be detected.

The NIR-cyto blot assay can be used to study pathways following stimulation of cellular receptors or to assess the inhibitory effects of candidate kinase inhibitory drugs. Thus far, we have used the assay on a variety of adherent cell lines such as HEK293, CHO, NIH3T3, HeLa, and A431 to monitor and quantify phosphorylation of markers including EGFR, Akt, ERK, Stat3, MEK1, and p53. We have also used the NIR-cyto blot assay format for detection of caspase-3 cleavage in an apoptosis assay. Because of the wide applicability of the assay format, the NIR-cyto blot can be used to assess whether different cell types utilize signaling pathways differently in response to the same stimulus. Wong [14] recently reported the use of this assay format, which he referred to as an in-cell Western, for monitoring phosphorylation of ERK in response to activation of dopamine D2 and D3 receptors. ERK phosphorylation is elevated by agonist-bound $G_{i/o}$ - and G_q -coupled G-protein coupled receptors (GPCR) which represent more than 75% of the members of the GPCR superfamily [14, 18]. This makes phosphorylation of ERK an attractive marker for monitoring ligand-induced activation of GPCRs. Besides functional K_i determinations for antagonists, the assay was used to detect the potency, efficacy, and selectivity of both partial and full agonists. The phospho-ERK assay was very sensitive and allowed activation of the D2 and D3 receptors to be effectively monitored in a situation where agonist-induced Ca^{2+} mobilization could not be detected by FLIPR.

Other traditional protein assay methods such as Western blotting are cumbersome and labor intensive. High content methods employ very expensive and complex instrumentation. As an alternative to costly high content methods, a simple, automated cell based technique such as the NIR cyto blot assay may offer a practical alternative. A 96- or 384-well microwell plate format can accommodate multiple pathway markers or drugs on a single plate. While these methods do not visualize individual cells, the level of quantification and flexibility may satisfy research needs in a more cost-effective manner. The challenge of quantitative pathway analysis is quite daunting, but examining a kinase within its cellular context should give a more complete picture of the signaling processes and the cellular response to inhibitory compounds.

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