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Brief Technical Report

Development of Multiplexed Protein Profiling and Detection Using Near Infrared Detection of Reverse-Phase Protein Microarrays

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Abstract

Protein microarrays have been recently employed for signal pathway profiling and high-throughput protein expression analysis. Reverse-phase arrays, where the array consists of immobilized analytes and lysates has especially shown promise in low abundance analyte detection and signal pathway profiling using phospho-specific antibodies. A limitation to current reverse phase array methodology is the inability to multiplex proteomic-based endpoints as each array can only report one analyte endpoint. In this study, we report on the use of a dual dye based approach that can effectively double the number of endpoints observed per array allowing, for example, both phosphospecific and total protein levels to be measured and analyzed at once. The method utilizes antibody bound dyes that emit in the infrared spectral region as a means of sensitive and specific detection.

Key Words: Proteomics; signal profiling; networks; protein microarrays.

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Introduction

An important goal of proteomics is to characterize and develop "circuit maps" of cellular signaling pathways in normal and diseased cells. Defective, hyperactive, or dominating signal pathways may drive cancer growth, survival, invasion, and metastasis *(1–7)*. Mapping the information flow through signaling pathways in normal and cancer cells may serve as a means to identify key alterations that occur during tumor progression and provide targets for rational, molecular-targeted drug design. With the advent of molecular targeted therapeutics, the identification, characterization, and monitoring of the signaling events within actual human biopsies will be critical for patient-tailored therapy.

In the past, efforts to elucidate the activation of signaling pathway events have relied mainly on either gene microarray/gene-based analysis and bioinformatic tools to help infer coordinate upstream signaling events or two dimensional-polyacryalmide gel electrophoresis (2D-PAGE) coupled with phosphorylation detection by immunoblotting *(8–16)*. Unfortunately, coordinate gene transcription profiling cannot accurately reflect the posttranslational modifications such as protein phosphorylation that are the drivers of the cellular signaling processes. Immunoblotting techniques often require considerably greater amounts of test material than can be provided by clinical samples. To understand the state of cellular signaling, new technologies that can characterize and directly monitor the activity and protein phosphorylation of various signaling pathways in small quantities of tumor tissue would be beneficial for both the identification of important targets for therapeutics and assessing the efficacy of these therapies. Reverse-phase protein microarrays represent a technology uniquely suited to screening a broad range of pathway targets in large numbers of tumors simultaneously in a high-throughput manner *(17–23)*. Reverse-phase arrays are amenable for analysis of biopsy material and involve spotting nanoliter quantities of cellular lysates onto immobilized supports to allow for probing with specific antibodies. In past studies, analyte detection was performed by colorimetric based detection using tyramide amplification *(17–23)*. While this method remains extremely sensitive and reproducible, the end user is unable to analyze more than one analyte per array. For example, analysis of phosphorylation of extracellular signal-regulated kinase (ERK) would require two array slides, one for the phospho-specific antibody readout and one for the total ERK report. A method that could determine the levels of both from the same material spotted on the same slide could, in effect, double the number of endpoints one could analyze for a given amount of lysate. In this study, we employed a two-dye fluorescent detection system (Fig. 1) using secondary antibodies that have been labeled with fluorochromes that can be detected simultaneously at both 700 nm and 800 nm wavelengths. The choice of near infrared (IR)-based dyes for this study was based on their reported performance in Western blot applications in which highly sensitive detection was observed on a nitrocellulose substratum, the same material used for the reverse phase protein microarray studies. These IR fluorochromes are uniquely suited for use on nitrocellulose materials because background autofluorescence from the nitrocellulose and cellular material at infrared (IR) wavelengths is dramatically lower than the background at visible wavelengths.

Materials and Methods

Tissue Samples

Frozen breast tumor tissues were collected at the Lombardi Cancer Institute, Georgetown

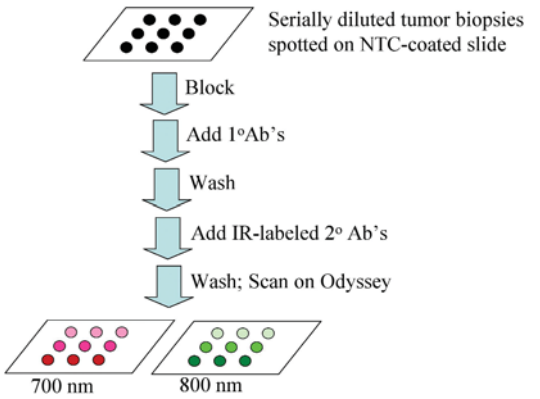


Fig. 1. Protocol for near infrared reverse phase array detection. Lysates from cell lines or patient samples procured via laser capture microdissection are arrayed in a miniature dilution curve on nitrocellulose coated glass slides. The slides are blocked, primary antibody is added and the slide is incubated for at least 30 min. The slide is then washed several times with PBS, and the IR dye-labeled secondary antibodies are added, and allowed to incubate at least 30 min. The slides are washed several times with PBS, and scanned with an image analyzer capable of detecting IR-based dyes in two channels (e.g., LI-COR Odyssey).

University, Washington DC. The tissues were anonymized and approved for use by the National Cancer Institute Office of Human Subjects Research. The histopathology of each case was confirmed by a pathologist prior to being used in these studies.

Tissue Processing and Microdissection

Eight millimeter frozen tissue sections were placed on uncoated glass slides and stored at –80°C prior to use. Immediately before dissection, the sections were thawed and fixed in 70% ethanol for 5 s, stained with hematoxylin for 8 s and dehydrated in 70, 95, and 100% ethanol for 1.5 min each, followed by xylene for 2 min, and then air-dried. The 70% ethanol and hematoxylin staining solutions were supplemented with Complete™ Mini protease inhibitor tablets (Roche Applied Science, Indianapolis, IN). Tumor epithelial cells or other relevant cell populations were microdissected with a Pixcell II Laser Capture Microdissection system (Arcturus, Mountain View, CA). Approximately 5000 LCM shots (20,000–25,000 cells) were microdissected for each case and stored on microdissection caps at –80°C until lysed.

Cell Lysis and Cellular Lysate Arraying

Microdissected cells were lysed directly from the microdissection caps into 50 µL of lysis buffer containing a 1:1 mixture of 2X Tris-Glycine SDS Sample buffer (Invitrogen Life Technologies, Carlsbad, CA) and Tissue Protein Extraction Reagent (Pierce, Rockford, IL) plus 2.5% β-mercaptoethanol for 30 min at 75°C. The samples were boiled for 8 min, centrifuged briefly and stored at 4°C. Immediately prior to arraying, lysates were loaded into a 384-well plate and serially diluted with lysis buffer into a 10-point curve ranging from undiluted to 1:256. Approximately 60 nL of each sample was spotted onto nitrocellulose-coated glass slides (Schleicher and Schuell Bioscience, Keene, NH) with a GMSE 470 microarrayer (Affymetrix, Santa Clara, CA). Spot sizes were approx 500 µm. Slides were stored desiccated at –20°C. For estimation of total protein amounts, selected arrays were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and visualized on a Fluorchem™ imaging system (Alpha Innotech, San Leandro, CA). One day prior to antibody staining, the lysate arrays were treated with Reblot™ antibody stripping solution (Chemicon, Temecula, CA) for 15 min at room temperature, washed 2 × 5 min in PBS, and then incubated overnight in blocking solution (1 g I-block [Tropix, Bedford, MA], 0.1% Tween-20 in 500 mL PBS) at 4°C with constant rocking.

Reverse Phase Array Colorimetric Detection

Blocked arrays were stained with antibodies on an automated slide stainer (Dako Cytomation, Carpinteria, CA) using the Catalyzed Signal Amplification System kit according to the manufacturer's recommendation (CSA; Dako Cytomation). Briefly, endogenous biotin was blocked for 10 min using the biotin blocking kit (Dako Cytomation), followed by application of protein block for 5 min; primary antibodies were diluted in antibody diluent and incubated on slides for 30 min and biotinylated secondary antibodies were incubated for 15 min. Signal amplification involved incubation with a streptavidin-biotin-peroxidase complex provided in the CSA kit for 15 min, and amplification reagent, (biotinyltyramide/hydrogen peroxide, streptavidin-peroxidase) for 15 min each. Development was completed using diaminobenzadine/hydrogen peroxide as the chromogen/substrate. Slides were allowed to air dry following development.

Primary and secondary antibodies and dilutions used in these studies were: rabbit anti-extracellular signal-regulated kinase (ERK)1/2 1:200 (Cell Signaling Technology); rabbit anti-phosphoERK1/2 T202/Y204 1:1000 (Cell Signaling Technology); biotinlyated goat anti-rabbit IgG (H+L) 1:5000 (Vector Laboratories, Burlingame, CA); and biotinylated rabbit anti-mouse IgG 1:10 (Dako Cytomation).

Reverse Phase Array Infrared Detection

Primary and secondary antibodies were obtained from commercial sources and were used at the working dilutions listed below. Rabbit anti-ERK 1/2 IgG (Cell Signaling Technology) and mouse anti-phospho ERK 1/2 IgG (Santa Cruz, Inc.) were used at a 1:100 dilution and 1:333 dilution respectively. Rabbit anti-phospho-PKC-α IgG and mouse anti-PKC-α (Upstate Biotech, Lake Placid, NY) were both used at a 1:250 dilution. All primary antibodies were diluted in I-Block supplemented with 0.1% Tween-20. IRDye™ 800CW-labeled goat anti-rabbit IgG and goat anti-mouse IgG (Rockland Immunolochemicals, Gilbertsville, PA) were used at a 1:5000 dilution. Alexa Fluor® 680 goat anti-mouse IgG and anti-rabbit IgG (Molecular Probes, Eugene OR) were used at a 1:5000 dilution). Secondary antibodies were diluted in I-Block supplemented with 0.02% sodium dodecyl sulfate (SDS) and 0.2% Tween-20. The slides were incubated with primary antibodies for 30–120 min, washed twice for 5 min each with TBS-T wash buffer (DAKO Cytomation) and then incubated with infrared dye labeled secondary antibodies for 30 min. The arrays were washed twice for 5 min each with TBS-T wash buffer, air dried and scanned on an Odyssey™ Infrared Imager (LI-COR Biosciences, Lincoln, NE) at 42 μm resolution. The images were analyzed using Odyssey software. Background was subtracted from the intensity values before the values were plotted as a graph.

To assess the potential for antibody interference when two antibodies are employed simultaneously on the same array and spot, A431 cells were treated with 100ng/mL EGF (Upstate Biotech) for 7.5 min, and then lysed in Laemmli's buffer. NIH3T3 cells were treated with 50ng/mL PDGF for 7.5 min, and then lysed in Laemmli's buffer. Approximately 0.5 to 1 μg of protein was loaded per well onto a polyacrylamide gel. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane (Osmonics MSI NitroBind™, Fisher Scientific, Pittsburgh, PA). Membranes were blocked in Odyssey blocker for 1 h. Primary antibodies were diluted in Odyssey blocker supplemented with 0.1% Tween-20. The blocked membranes were incubated with primary antibodies specific for either the pan-protein, the phosphorylated protein, or both

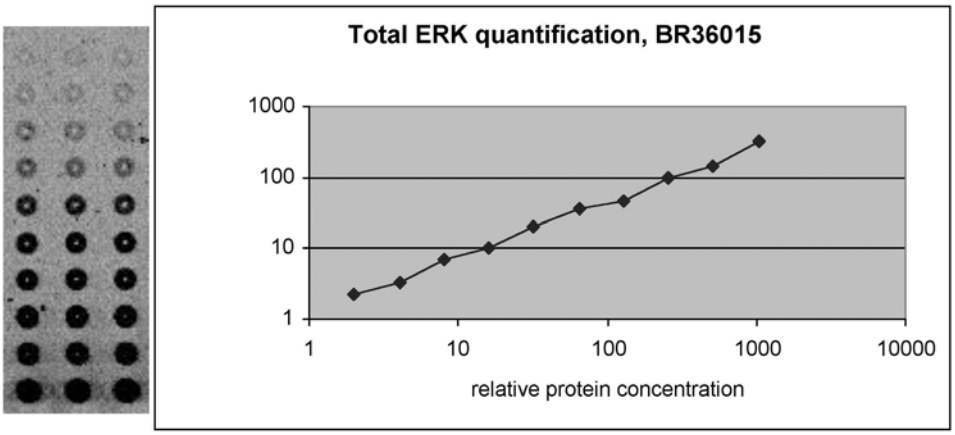


Fig. 2. Reproducibility of ERK kinase protein detection using infrared dye detection. A lysate, prepared from cells obtained from a single human breast cancer specimen (BR36015), was arrayed in triplicate in a stepwise series of twofold dilutions and stained with rabbit anti-ERK followed by detection with IRDye800CW-labeled goat anti-rabbit. Assessment of linearity and reproducibility of the average of the three rows is shown on the right.

for 1 h followed by three washes for 5 min each in PBS-T (PBS plus 0.5% Tween-20). Anti-mouse and anti-rabbit secondary antibodies diluted in Odyssey blocker and labeled with either Alexa Fluor680 or IRDye800CW were added and the membranes were incubated for 30 min. Following three washes for 5 min each with PBS-T, the membranes were scanned on an Odyssey™ Infrared Imager at a resolution of 169 μm.

Results

Reproducibility of the IR Detection Method

To assess reproducibility of the IR detection method, 40,000 cells from a breast cancer patient tumor specimen were procured via LCM, lysed and analyzed in triplicate for total ERK levels using biotinylated rabbit anti-ERK followed by detection with IRDye™ 800CW-labeled goat anti-rabbit IgG (Fig. 2). ERK protein was detected over a broad dynamic linear range in all triplicate runs. Error bars for the standard deviations at each dilution are shown, but were too small to be seen on this scale. Coefficients of variance (CVs) for each dilution were less than 7%, indicating that the IR based method achieved very good reproducibility across a broad linear detection range.

Because the absolute concentration of the target proteins in the tumor samples is unknown, we compared the sensitivity of the IR detection method to the CSA method by dilution analysis. On this basis, the sensitivity of the IR detection was comparable or even slightly better to than that achieved by colorimetric based detection (Fig. 3).

Multiplexing Signaling Endpoints From the Same Array

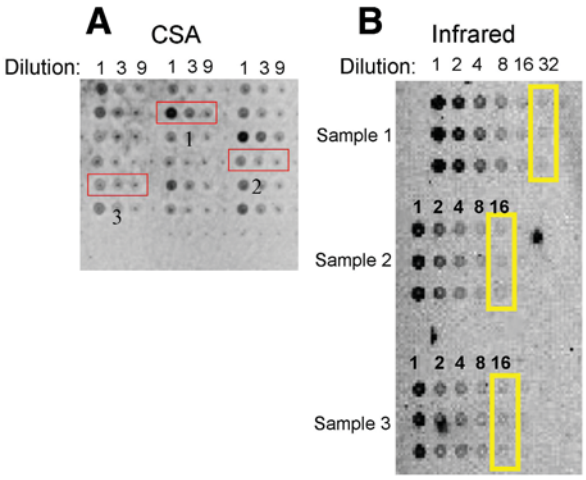
The ability to detect both the non-phosphorylated and the phosphorylated forms of the same protein using the same array was determined using a lysate study set prepared from human breast cancer epithelium obtained from three different subjects and a control cell line lysate (Jurkat T cells). One of the patients was previously known to have low levels of phosphorylated ERK compared to the other two cases within this study set. The samples

were blinded prior to analysis. Each lysate was arrayed in triplicate (Fig. 4) and the analytes were detected using anti-phospho ERK IgG (mouse) and anti-total ERK IgG (rabbit) followed by Alexa Fluor 680 goat anti-mouse IgG and IRDye800CW goat anti-rabbit IgG on the same array. The overlaid images showing both the phospho and total levels of ERK are shown on the left with the individual readouts from each separate channel shown on the right. As expected, cases 1 and 2 revealed much higher levels of phosphorylated ERK compared to case 3. Total ERK levels contained in each of the three cases were found to be nearly equivalent and reflected the equivalent total protein levels in each lysate. This result indicates the feasibility of assessing both phospho and total protein levels of important signal pathway mediators such as ERK kinase from clinical material on a single array and within the same spot. Interference experiments performed by Western blotting of lysates and comparison of signal outcomes where either both total and phospho were measured together, or individually, indicated that neither primary was interfering with the ability of the other to recognize the denatured epitope on the membrane (data not shown).

Discussion

As the drug discovery field focuses on the development of molecular-targeted therapeutics, the activity status of pathways and phosphorylated levels of kinases and kinase substrate targets of interest in individual tumors will become a major focal point of analysis. Gene microarray transcription profiling alone cannot accurately recapitulate the states of these pathways and posttranslational modification events, and will be greatly complemented by proteomic analysis of the phosphorylation states of cellular circuitry. The detection of subtle changes in the activity of various signaling pathways in normal and tumor tissue in a patient is not only valuable scientifically for the study of disease progression, but will be essential for appropriate treatment selection and monitoring treatment efficacy in the future. This is especially so now, more than ever, because the drug development pipeline is heavily skewed towards signal pathway modulators and kinase inhibitors *(24–31)*.

Reverse-phase protein microarray technology provides a means to detect, in a highly multiplexed way, the phosphorylation-mediated changes that are the direct targets for therapy. The reverse-phase protein array has shown the unique ability to analyze signaling pathways using small numbers of human tissue cells microdissected from biopsy specimens *(17–23)*. A comparison of this method with tissue arrays *(32)* or antibody arrays *(33–36)* reveals several unique advantages: (1) the reverse-phase array employs denatured lysates so that the antigen retrieval issues, currently a large limitation for tissue arrays, are significantly reduced; (2) because each lysate sample is arrayed in a miniature dilution curve, a means of providing an internal standard curve to assess linear dynamic range is directly ascertained, thus generating direct quantitative measurements; and (3) the arrays do not require direct tagging of the protein for detection, as would be required for antibody or forward phase *(23)* arrays, yielding a dramatic improvement in reproducibility, sensitivity and robustness of the assay over other techniques. An additional new benefit now highlighted in this study is that with a two-channel near IR-based system, we have the ability to effectively double the number of endpoints one could analyze with a fixed number of arrays. Because clinical material is always limiting and the number of signaling endpoints quite large, any inability to multiplex the process can have substantial practical impact.



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Method	Sample	Dilution
CSA	1	1/9
	2	1/9
	3	1/9
Odyssey	1	1/32
	2	1/16
	3	1/16

Fig. 3. Sensitivity of infrared detection on reverse phase arrays. **(A)** Multiple breast tumor samples were spotted onto nitrocellulose-coated slides along with 1:3 and 1:9 serial dilutions of each sample and subjected to detection of PKC-α using the CSA technique. Samples 1, 2, and 3 are noted. **(B)** In parallel, serial twofold dilutions of three of the breast tumor samples, labeled samples 1, 2, and 3, were subjected to analysis of PKC-α using infrared detection. **(C)** Tabulation of the detection endpoints of each technique for samples 1, 2, and 3.

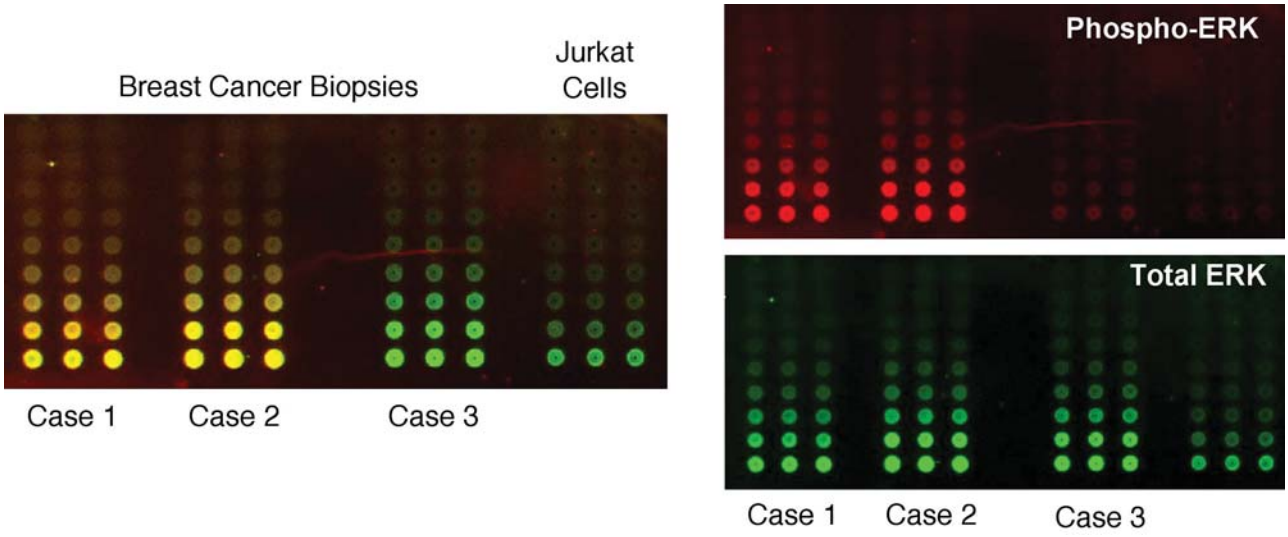

Fig. 4. Multiplexed detection of phosphorylated and total ERK protein levels in breast cancer tissue cells. Tissue cells from three cases of human breast cancer were obtained and lysates prepared from these and from a cell line control prepared from Jurkat T cells. The array was probed simultaneously for phospho and total ERK levels using phospho-ERK (mouse) and total ERK (rabbit) antibodies and then Alexa Fluor680 goat anti-mouse and IIRDye800CW goat anti-rabbit antibodies on the same array. The overlaid images of both the phospho and total levels of ERK are shown on the left, with the individual readouts from each channel shown on the right (phospho-ERK: top right; total ERK: bottom right).

The current liability with a colorimetric detection system is that only one analyte per slide can be evaluated. The dual IR dye method appears reproducible, is relatively simple, and can be performed with commercially available reagents. A limitation, however, to the IR dye-labeled secondary antibody approach is that a dual-endpoint analysis with anti-pan and anti-phospho protein antibodies is only currently achievable when sets of antibodies derived from two different species are available for a given analyte. Alternatively, if a matching pan-antibody is not available, the samples can be normalized against a housekeeping protein. Further assessments to determine any sensitivity limitations of IR-based detection need to be performed on larger study sets to evaluate the potential for optimization for this type of detection method.

Today, micro, and even nano-proteomic technologies are being developed such that the field of clinical proteomics will have direct bedside impact. It is possible that the physician will soon use these different proteomic tools at multiple steps within disease management and therapeutic intervention. In the future, a clinician may routinely employ technologies to map the state of molecular networks within biopsy samples. Functional maps of the state of key pathways within a patient's tumor cells will become the starting point for personalized therapy. Under this scenario, therapy can be tailored to the individual tumor's molecular defect. Moreover, it should be feasible to administer combination therapy targeting multiple interdependent points along a pathogenic pathway, or targeting separate pathways *(37–38)*.

On-going clinical trials at the National Cancer Institute have currently incorporated reverse phase microarray technology for evaluation of the sample procurement and processing technologies, and to elucidate hypotheses concerning treatment effects on cell signaling networks. As more cell signaling information is gleaned from protein microarrays, we envision an enhanced ability to develop/identify new combinatorial therapies or targeted therapies. The ability to determine and understand treatment effectiveness, via protein microarrays, which can analyze both the drug target and the entire pathway network, may lead to better patient management and better clinical outcomes.

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