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Brief Technical Report

Application of Sector Protein Microarrays to Clinical Samples

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Abstract

Many protein functions are conferred by posttranslational modifications, which allow proteins to perform specific cellular tasks. Protein microarrays enable specific detection of posttranslational modifications not attainable by gene arrays. Reverse-phase protein microarrays have been widely adopted for use with clinical biopsy specimens because they have many advantages including highly reproducible printing of cellular lysates onto array surfaces, built-in dilution curves, and direct detection using one antibody per analyte. This results in high-sensitivity, broad dynamic range, and favorable precision. Reverse-phase arrays have been restricted to a one slide/one antibody format. Although this is suitable for analyzing treatment effects over populations of samples, it is not well suited to individual patient assessments. One means of reaching this goal is the sector array format. Through the sector array, multiple antibody probes can be multiplexed on a single slide containing replicate immobilized aliquots from one patient. Thus, on one slide, a complete set of analytes can be characterized and used to support a therapy decision. This article describes a method for constructing sector arrays and demonstrates feasibility and adequate sensitivity applied to apoptosis related pathways.

Key Words: Protein microarray; molecular profiling; individual targeted therapy; sector arrays; clinical analysis.

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Introduction

In order to fully understand a complex disease state such as cancer, both the genetic and proteomic components of the disease must be explored and correlated. The information content of the cell, or the genome, is directly responsible for the production of the cellular proteome through transcriptional and translational processes. Defects in that genomic information enable tumor cells to produce a proteomic environment in and around the tumor cell that enable the tumor to thrive *(1,2)*. Modifications in protein-based signaling pathways may lead to the hallmarks of cancer, which are exuberant growth, invasion, and metastatic spread *(3)*. Genomic studies have been propelled by advances in DNA microarray technology *(4–10)*. Although DNA microarray tools can uncover errors in the genetic code that can alter a cell's behavioral characteristics, the power of the protein microarray is its ability to directly delve into the molecular microanatomy of cellular signaling pathways, defining and assessing important regulatory nodes that contribute to pathophysiologic functional states. For example, posttranslational modification of a protein can reflect an activated signaling state, as in phosphorylation. Using protein microarrays, the presence of the phosphorlyated form of a protein vs the total, or unactivated, form of the protein can be measured *(11–13)*. In this way protein microarrays can reveal the level of activity of a given protein signaling pathway.

The idea of a physician giving a careful and thorough physical exam to an ill patient is a familiar concept. High throughput clinical proteomic microarrays aim to provide a physician with a thorough exam of a patient's intracellular molecular anatomy. Through individually assessing a patient's tumor, personalized therapy regimens may be directed towards a particular patient's molecular defect and monitoring the efficacy of a particular therapy can be envisioned *(14–18)*. Protein microarrays will also make it possible to measure signal pathway activity before, during, and after treatment *(19,20)*. The identification of key points in the signaling pathways, or nodes, would also result in the identification of drug targets *(19,20)*. Cellular lysates from tumor cells have been probed on protein microarrays with either an antibody directed against either the total or the phosphorylated forms of a particular signaling protein. The ratio of phosphorylated to total protein levels enables the level of activity for a given protein signaling network to be evaluated.

Protein microarrays consist of a substrate, such as nitrocellulose, upon which proteins have been immobilized in discrete areas, or spots *(21–24)*. The immobilized spots may contain a mixture of proteins or a homogenous protein population. Varying permutations of the concept have been used. The arrayed spots can consist of antibodies, cellular lysates, or small molecules such as nucleic acid probes, small drugs, or polypeptides *(12,23,25–32)*. The presence of a protein of interest is determined by probing the array with a tagged probe. The end result is a series of positive or negative spots, based on degree of signal present. The presence of the protein of interest is proportional to the intensity of signal *(12)*.

Two basic formats for protein microarrays are used: the forward phase array and the reverse-phase array (RPA). With the forward phase array, a bait molecule, such as an antibody, is immobilized on the array surface. Each spot will have one type of bait molecule. The surface is then incubated with a heterogenous mixture of analytes. For detection, the analyte may be labeled directly or may be detected by a second detection molecule, such as a labeled antibody. The alternative

approach is the RPA. The RPA grew out of the need for a system to assay proteins present in limited amounts of clinical material *(11)*. Using robotic arrayers, cellular lysates are deposited on a substrate such as nitrocellulose, typically with a sample size of a few hundred microns *(33)*. Through this strategy, each spot comprises an individual patient's cell lysate proteome. Thus, specimens from multiple patients can be assayed on the same chip using a single detection molecule with uniform experimental conditions. Since the sample sizes are small and many proteins of interest are in low abundance, amplified detection systems are required *(34–38)*. Borrowing from immunoassay technology, biotin-tyramide conjugates can be enzymatically generated where a ligand/biotinylated antibody complex is present (CSA kit, DakoCytmation, Inc.) *(34,39–42)*.

The translational potential of clinical protein microarrays for patient care is immense, but thoughtful design of the array infrastructure is required to generate the maximal amount of information from limited clinical materials. Conventional RPAs have been probed with a single detection molecule because nitrocellulose cannot be stripped and reprobed *(43)*. Sector arrays consist of multiple discrete areas of substrate on an array surface, hence the term sectors, wherein each of the sectors can be simultaneously probed with a different antibody. This significantly amplifies the information that can be extracted from a given microarray surface, a valuable attribute in the move towards high throughput profiling of patient specimens.

One approach for sector arrays is to generate an insert that is placed over the array, providing a reservoir for each of the sectors. Each one of the sectors is then incubated with a different antibody. After this incubation, the reservoir insert is removed and the array is processed as it would be in a conventional array. Herein we expand upon the RPA concept by describing the status of our on-going technical development of a sector array designed for translational clinical protein microarrays.

Materials and Methods

Whole Cell Lysates

Whole cell lysates were prepared from Jurkat cells (clone E6-1, ATCC, Manassas VA), monoclonal activating anti-Fas antibody treated Jurkat cells and epidermal growth factor (EGF) treated HeLa cells (ATCC). Briefly, Jurkat cells were grown to log phase in RPMI 1640 media (Invitrogen, Carlsbad, CA) with 10% heat inactivated fetal bovine serum (FBS) and 1 m*M* L-glutamine. Apoptosis was induced in the anti-Fas treated Jurkat cell culture by adding 0.5 ug/mL activating anti-Fas (Upstate, Waltham, MA) to 1 mL of media containing 3×10^6 cells. Cells were incubated at 37°C in 5% CO_2 for 6 h.

Activation of the EGFR pathway was stimulated in the HeLa cell line by addition of 100 ng/mL EGF (Cell Signaling, Beverly, MA) to the culture media. HeLa cells were grown to 60–80% confluency in RPMI-1640 media with 10% FBS. Media was removed and replaced with serum-free media (RPMI-1640 serum free). Cells were incubated overnight at 37°C in 5% CO_2. Cells were incubated for 10 min with EGF prepared in RPMI 1640 serum free media. The monolayer was washed twice with media. Cells were scraped from the culture dish, transferred to a flask, and washed with additional media and pelleted at 298*g* for 10 minutes at 4°C.

Jurkat, anti-Fas treated Jurkat and EGF treated HeLa cells were harvested by pelleting the cells at 1000 rpm for 10 min at 4°C. The cells were washed twice in ice cold phosphate buffered saline (PBS). Whole cell lysates were prepared by lysing the cell pellet in a 2% solu-

tion of 2-mercaptoethanol (Sigma, St. Louis, MO) in 2X Tris-glycine SDS loading buffer (Invitrogen, Carlsbad, CA) and T-PER™ (Pierce, Rockford, IL). The lysate was vortexed thoroughly and centrifuged at 4°C for 20 min at 3313*g*. The supernatant was stored frozen at –20°C.

Reverse Phase Protein Microarrays

Reverse phase protein microarrays were printed with whole cell protein lysates as described by Paweletz et al (Oncogene 2001) *(12)*. Briefly, the lysates were printed on glass backed nitrocellulose sector array slides and single pad FAST Slides (Schleicher & Schuell Bioscience, Keene, NH) using a GMS 417 arrayer (Affymetrix, Santa Clara, CA) equipped with 500 um pins. The sector array slides consisted of 4 nitrocellulose pads in a 20 × 6 mm format or 4 pads in a 6 × 6 mm format. The FAST slide format was a 20 × 50 mm single pad. Each lysate was printed in a dilution curve representing neat, 1:2, 1:4, 1:8, 1:16 and negative control dilutions *(11)*. Each spot was printed with approx 1.5 nL of lysate/spot. The slides were either stored with desiccant (Drierite, W.A. Hammond, Xenia, OH) at –20°C or immediately processed for immunostaining.

Slide Preparation for Immunostaining

The microarray slides were prepared for immunostaining by washing with 1X Reblot mild solution (Chemicon, Temecula, CA) for 15 min, rinsing twice with PBS without calcium or magnesium for 5 min each and blocking with I-block blocking solution (Applied Biosystems, Foster City, CA) for a minimum of 1 h. After blocking, the slides were placed on an automated slide stainer (Autostainer DakoCytomation, Carpinteria, CA) and blocked with 3% hydrogen peroxide, avidin, biotin, and protein block per manufacturer's instructions. The slides were allowed to air dry at room temperature.

Immunostaining

For the sector arrays an adhesive backed silicon reservoir was placed on the sector array slides exposing only the nitrocellulose pads. Each slide was rehydrated with primary antibody diluted in Antibody Diluent (DakoCytomation) and incubated at room temperature for 30 min. Polyclonal primary antibodies were used at the following concentrations: ERK 1:1000 (Cell Signaling, Beverly, MA), Caspase 3 1:1000 (Cell Signaling) and phospho AKT 1:250 (Cell Signaling). The negative control slide was rehydrated with antibody diluent.

Following incubation, the primary antibody or diluent was decanted, the slide was washed briefly with tris-buffered saline with tween (TBST DakoCytomation), the sector reservoir was removed, and the slide was placed back on the Autostainer. The automated CSA staining protocol (DakoCytomation) was resumed with a 3 min TBST buffer wash, followed by incubation with the secondary antibody, goat anti-rabbit IgG H+L (1:5000) (Vector Labs, Burlingame, CA). Subsequent protein detection was via a horseradish peroxidase mediated biotinyl tyramide amplification reaction per manufacturer's instructions.

Results

Protein microarrays can be formatted to have discrete areas of substrate in sectors. The benefit of this type of array is that multiple sectors containing replicate patient samples can be probed with different antibodies at the same time. This allows more information to be gleaned from a single microarray surface, which is an important goal in translating proteomic arrays into clinical settings. Commercially available sector chambers produced by Schleicher and Schuell Bioscience (Keene, NH) or Grace Bio-Labs (Bend, OR) have nitrocellulose sector pads that measure 6 × 6 mm in size. An adhesive-backed silicon sheet with

holes that correspond to the sector pads is applied to the glass array slide to provide a reservoir. The reservoir allows antibody preparations to be incubated within a given sector. After the antibody incubation is complete, the antibody solution is decanted, the sector slide is washed briefly, and the reservoir is removed. The array can then be probed with a secondary antibody, presuming that all antibodies used in the primary incubation step are of the same species and isotype, at the same time using a conventional staining apparatus. Although the commercially available multichamber formats are effective in providing reservoirs for multiple different antibody incubation conditions on the same slide, the size of the sector is not ideally suited for clinical proteomic analyses.

In assessments of tissue lysates from tumor cells, it is essential to have at least six dilutions of a given lysate. These dilutions provide an adequate protein concentration range for assaying low and high abundance cellular proteins. Further, it is important to compare within the same sector a reference lysate with the patient's tumor lysate because subtle variations in the experimental conditions may exist in any given sector. Inclusion of a reference lysate permits comparison of the patient sample to a defined material, enabling a quality control evaluation of the printing and immunostaining processes. The 6 × 6 mm multi-format slides that are commercially available do not allow this type of comparison using an Affymetrix GMS 417 arrayer (Fig. 1). Accordingly, we are in the process of developing sector arrays in a format that is more applicable to patient clinical biopsy material. We are particularly interested in finding an effective sector format for use with clinical specimens procured from our on-going clinical trials.

To create a translational proteomics-compatible sector array, sectors were sculpted from the surface of a nitrocellulose coated S & S FAST slide by scraping with a single edge razor blade. The sectors were designed as four distinct 20 × 6 mm nitrocellulose pads. Silicon films with adhesive backing were modified by cutting rectangular holes that served as reservoirs for the 20 × 6 mm sector arrays. The adhesive helped in forming a hydrophobic barrier that prevented leakage or contamination from one sector to another. Notches were cut into the reservoir at the top of the slide so the sector array would be compatible with the DakoCytomation Autostainer slide rack (Fig. 2).

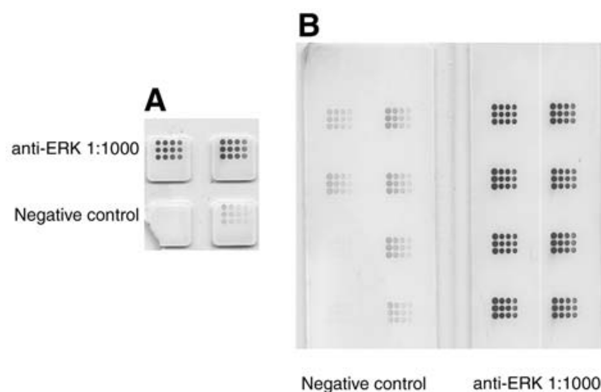

Fig. 1. Multipad slide format compared to single pad nitrocellulose slide. EGF treated HeLa cells and anti-Fas treated Jurkat cells were printed with a GMS 417 pin and ring arrayer in a mini-dilution curve in triplicate on sector arrays **(A)** and single pad S&S FAST slides **(B)**. EGF-treated HeLa cells were printed on the right side, and anti-Fas treated Jurkat cells on the left side. Sectors were probed with polyclonal anti-ERK antibody or antibody diluent. The 6 × 6 mm nitrocellulose pad does not allow array printing in a 6 point dilution curve, or inclusion of a reference lysate within the same nitrocellulose pad as compared to a 20 × 6 mm sector.

To test the format in a reverse phase array that would simulate a clinical trial specimen, a single slide was arrayed with sectors, each containing six concentrations in triplicate of either untreated Jurkat cells or Jurkat cells treated with anti-Fas to induce apoptosis. Fol-

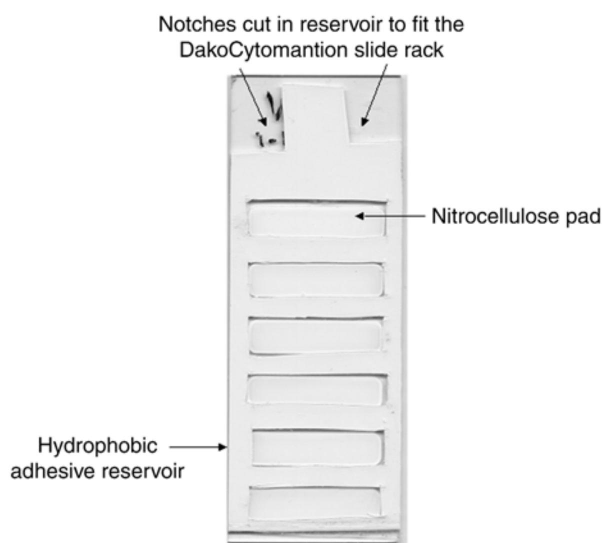

Fig. 2. Sector array with reservoir. Compatability with automated staining systems requires that the reservoir must be easily removed after incubation with the primary antibody. Incorporation of a reference lysate in each sector and triplicate sample printing necessitates a sector design in a rectangular format rather than the commonly available square format. The 20 × 6 mm pad format permits patient samples and reference lysate sample to be printed in the same area with exposure to identical experimental conditions.

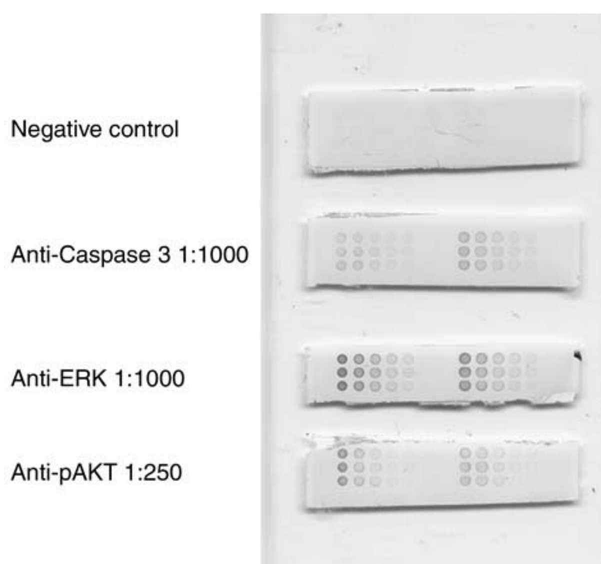

Fig. 3. Example of stained sector array. Jurkat cell lysates (left side) and anti-Fas treated Jurkat cell lysates were immobilized on each sector in a dilution curve representing neat, 1:2, 1:4, 1:8, 1:16 and negative control dilutions. Each sample was printed in triplicate with a GMS 417 pin and ring arrayer. Following pretreatment and blocking, a sector reservoir was applied and each nitrocellulose sector pad was reconstituted with either antibody diluent (negative control), polyclonal anti-caspase 3, polyclonal anti-ERK or polyclonal anti-phosphoAKT. After incubation with primary antibody, the slide was washed with TBST, the sector reservoir was removed and immunostaining on the DakoCytomation Autostainer was resumed. Detection was via horseradish mediated deposition of biotinyl tyramide and detection with DAB (diaminobenzidine). Each sector showed a distinctive staining pattern, indicating that no leaking occurred during primary antibody incubation.

lowing blocking of the nitrocellulose, a reservoir, as described above, was applied to the glass surface. The first sector was incubated only with antibody diluent, serving as a negative control. The second sector was probed with a polyclonal anti-caspase 3 antibody, the third sector was probed with polyclonal anti-ERK and the fourth sector was probed with a polyclonal anti-pAKT antibody (Fig. 3). Following incubation with the primary antibody, the array was washed, and the reservoir was removed. The sector array was probed with a biotinylated secondary antibody using an automated staining platform (Autostainer). This was followed by detection using a catalyzed signal amplification detection method (CSA kit, DakoCytomation). The experimental results indicate that the reservoirs maintained the sector integrity. Each sector showed a distinctive staining pattern, indicating that no leaking occurred during primary antibody incubation. With each probed signal protein, distinctive staining patterns were observed indicating differential proteomic responses to anti-Fas mediated signals (Fig. 3) *(13)*. The negative control shows no aberrant staining.

Thus, a sector array using a 20 × 6 format can be created that maintains the integrity of each of the sectors.

Discussion

One of the most attractive aspects of protein microarrays is its applicability to patient specimens, including biopsies and body fluids *(12,13,44,45)*. Previously, RPAs have been used to profile protein networks in tissue and serum samples *(45,46)*. Clinical trials at the National Cancer Institute are currently beginning to integrate this technology into patient specimen processing. This direct interface with a patient's molecular information will potentially alter diagnosis and treatment. Early uses of the technology involve characterizing signaling pathways in conventionally characterized disease states. As experience is developed, it is hoped that new treatment groups emerge that rely more on defined signaling pathway aberrations than on histopathologic appearance. Moreover, a defined signaling aberration can serve as a departure point for drug discovery and as a classifier of prognosis. Because multiple points within a signaling pathway can be discerned, combinatorial therapies can be devised that aim to knock out key signaling nodes that sustain and provide a growth advantage to the tumor cell *(19)*.

RPAs in a sector-type format significantly enhance the concept of high-throughput protein microarrays and individualized therapy. Through the sector array, the diagnostic power of a single slide is amplified. Probing the same patient sample in a sector array format with different antibodies provides insight into levels of signaling activity at different key points in molecular pathways. Further, direct labeling of proteins using agents such as colloidal gold allow total protein levels to be directly measured on the same slide in which antibody probing is performed.

Since a key goal of clinical proteomics is the integration of these technologies directly in patient care settings, sector arrays will necessarily undergo improvements to enhance ease and reproducibility in use. An additional concept that can be applied to sector arrays concerns delivery of the detecting antibody to the sector array surface. Although delivery of antibody suspended in an aqueous solution was used in the present system, other delivery vehicles are possible. For one, antibodies can be freeze-dried and integrated into a water soluble, polymeric film for long-term storage. These films could be cut to fit into a sector array format and applied in the clinical laboratory for rapid profiling of patient material.

Sector protein microarrays are in a state of rapid development. Formulating a sector array for fluid based staining systems presents several design challenges. The initial challenge is the selection of an appropriate hydrophobic, inert material for the reservoir. To be compatible with automated staining systems, the reservoir must be low-profile, removable, and impervious to leaks. The second challenge is designing sectors compatible with existing array and detection technologies. The 20 × 6 mm six pad sector design and the concept of a tab on the reservoir are developments that are meeting these challenges. The next step is the commercial production of sector arrays and reservoirs for individual clinical sample protein microarrays.

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