

Biomarker Discovery Using Arrays Printed With the BioOdyssey™ Calligrapher™ MiniArrayer

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Abstract

The use of microarrays in transcriptional profiling has provided scientists with a wealth of information. This same technology has the potential to accelerate biomarker discovery in cancer research through the examination of protein expression and posttranslational modifications in cells, tissues, or serum in a multiplexed miniature environment. Reverse-phase protein arrays (RPPAs) are prepared from tissue or cell lysates from multiple samples that are arrayed on a slide and then probed with specific antibodies. These arrays are being used to uncover biomarkers in ovarian (Wulfkuhle et al. 2003) and breast (Cowherd et al. 2004) cancers, as well as in leukemia (Tibes et al. 2006). In this poster, we demonstrate the preparation of RPPAs for biomarker discovery by the BioOdyssey Calligrapher miniaarrayer.

Introduction

Microarrays contain hundreds to thousands of small amounts (μ l to nl) of material deposited onto a surface the size of a microscope slide. The arrays can be produced mechanically by contact printers, highly precise robots with the ability to move in microns, that can create complex arrays of molecules in specific locations. Microarrays have many advantages for use in life science research, including the ability to multiplex, the conservation of samples and reagents, and the reduction of experimental time. While microarrays have traditionally been used for transcriptional profiling, the technique can also be applied to proteomic research and clinical assays, and it holds promise for the emerging field of personalized medicine, theranostics.

Protein microarrays can be designed in many different formats, but the two most common are the forward-phase array, essentially a micro ELISA, and the reverse-phase protein array (RPPA), a miniature dot blot. RPPAs can be prepared from samples dissected from patients or from cells grown in tissue culture. Following a number of incubation steps, detection of the molecule(s) of interest is performed via an antibody interaction assay. This method is currently being used in proteomic research to identify protein biomarkers involved in cancer in various tissues (Wulfkuhle et al. 2003, Cowherd et al. 2004) and to follow progress of experimental treatments in tissue culture (Tibes et al. 2006). Once experimental biomarkers are well established, they can become tools for the clinical laboratory and be used to identify disease, as well as to monitor a patient's progress during treatment.

The use of RPPAs requires a microarrayer that can precisely deposit replicate amounts of samples at discrete locations on a slide. We present data here that demonstrate the BioOdyssey Calligrapher miniaarrayer is capable of producing arrays of the high quality required for use in the discovery of disease biomarkers, and we show both tissue and cell RPPAs produced by the instrument.

Cell Line Experiments

Materials and Methods

A549 cells (American Type Culture Collection, ATCC) were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (ATCC). Approximately 2.75×10^6 cells were plated per well in 6-well microplates. The following day, growth medium was replaced with serum-free medium. After 24 hr, medium supplemented with EGF peptide (Cell Signaling Technology, Inc.) or medium alone was added to the treated and untreated cells, respectively, at various times. At each time point, the cells were washed twice with Dulbecco's phosphate buffered saline and then lysed in a 2.5% solution of 2-mercaptoethanol in extraction reagent for tissue protein (T-PER, Pierce)/2X Tris/glycine/SDS buffer (Invitrogen).

The BackTracker™ program provided with the BioOdyssey Calligrapher system was used to determine sample placement on the arrays. All samples were plated into a 384-well microplate in a 4-point, 2-fold dilution curve (neat, 1:2, 1:4, 1:8), and arrayed onto FAST nitrocellulose slides (Whatman, Inc.) using the miniaarrayer equipped with Stealth SNS15 solid pins (TeleChem International, Inc.). Samples were printed at three depositions per feature to ensure ample protein concentration.

Arrays were blocked (I-Block, Applied Biosystems) and subsequently stained for Akt (Ser⁴⁷³) and p90RSK (Ser³⁸⁰) (Cell Signaling Technology Inc.) in triplicate using a Dako autostainer with a catalyzed signal amplification system (CSA; Dako) according to manufacturers' recommendations. Negative control slides were stained with secondary antibody alone (goat anti-rabbit IgG (H+L), Vector Laboratories). Chromogenic detection was achieved with diaminobenzidine (DAB) (Dako) and arrays were imaged on a flatbed scanner (UMAX PowerLook 1120). Arrays were also stained for total protein using SYPRO Ruby protein gel stain (Invitrogen Corporation) and visualized with a Molecular Imager® PharoFX™ Plus imaging system (Bio-Rad Laboratories, Inc.).

Results

For the eight slides that were printed in the same run, inter- and intraslide variability were within acceptable limits. As shown in Figure 1, the first three dilution points (neat, 1:2, and 1:4) had excellent intraslide CVs ranging from 2.5 to 5.6% and interslide CVs ranging from 4.1 to 8.2%. The 1:8 dilution spot had higher variability due to lower protein concentration. These results illustrate the necessity of printing reverse-phase arrays in dilution curves in order to match the sample protein concentration with antibody probe affinity that is within the linear dynamic range of the assay.

Empty wells were printed on the array in quadruplicate for evaluation of carryover (Figure 2). No apparent contamination resulting from carryover of the previous samples was revealed by antibody or SYPRO Ruby staining.

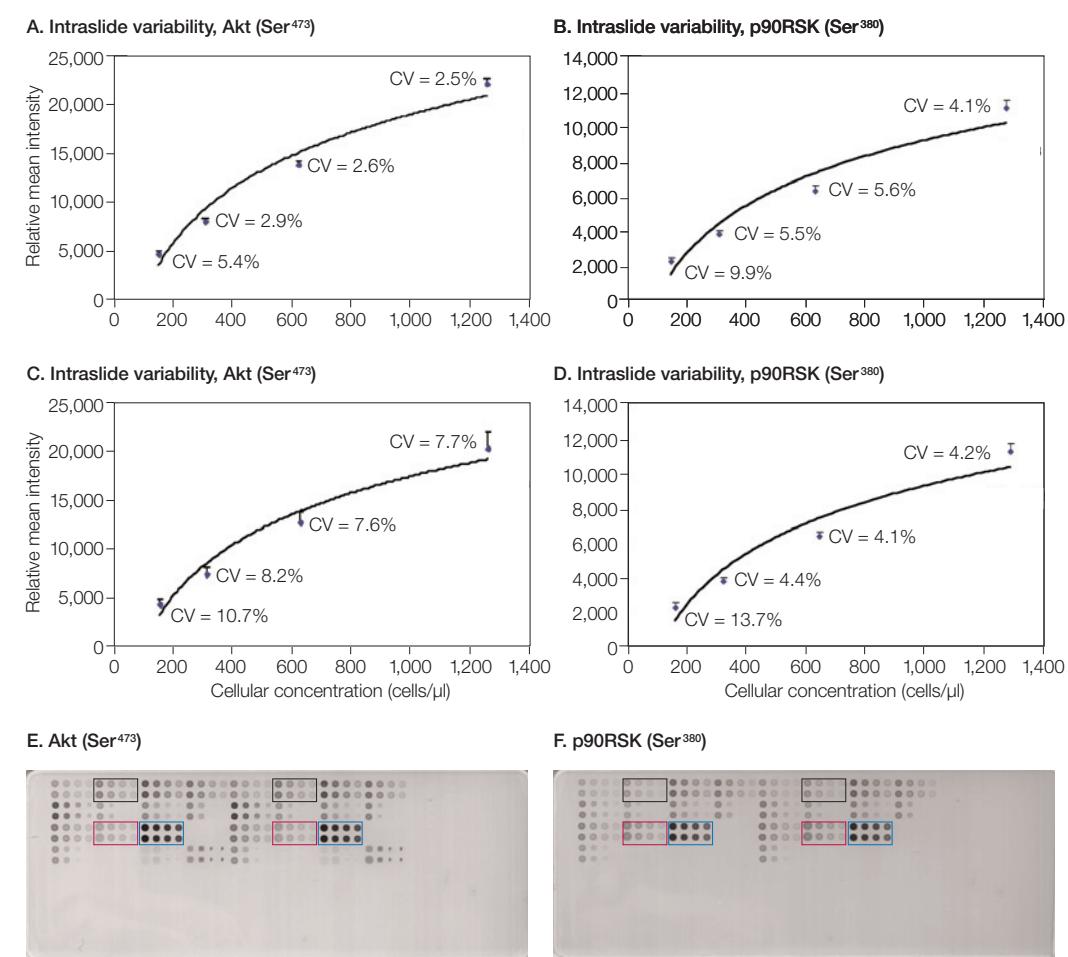


Fig. 1. Precision and linearity studies. The linear dynamic range for two different antibody probes was assessed for arrays printed with cellular concentrations of 156–1,250 cells/ μ l. **A** and **B**, Intraslide values obtained from one sample printed four times on one array. Arrays probed with **A**, Akt (Ser⁴⁷³) and **B**, p90RSK (Ser³⁸⁰). **C** and **D**, Interslide values obtained from one sample printed four times on three separate arrays. **E**, Akt (Ser⁴⁷³)-stained array; **F**, p90RSK (Ser³⁸⁰)-stained array. The dilution curve inside the black boxes represents samples whose raw background-subtracted intensities were used for analysis above. The dilution curves of A431 and A431 + EGF control samples printed on the arrays are indicated in red and blue boxes, respectively.

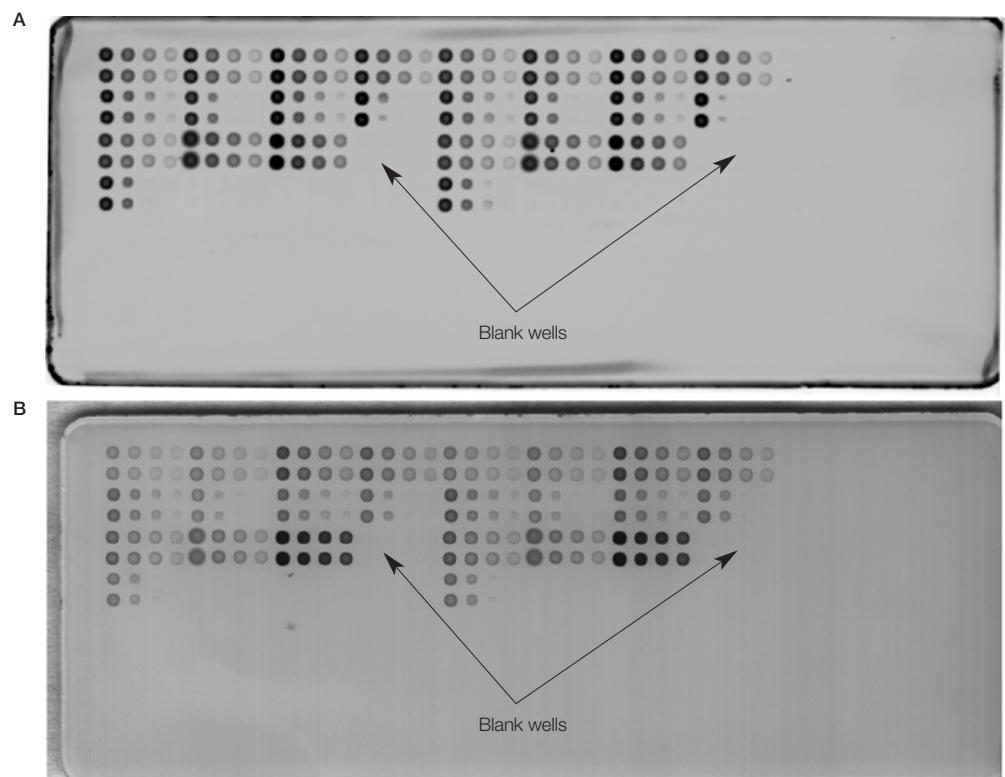


Fig. 2. Assessment of sample carryover. The blank wells represent areas on the array in which carryover was assessed by intentionally leaving microplate wells empty directly adjacent to sample-containing wells for assessment of sample carryover. Arrays stained with **A**, SYPRO Ruby and **B**, p90RSK (Ser³⁸⁰), did not show any evidence of sample carryover with fluorescence and colorimetric detection (DAB) in the indicated areas.

Tissue Extract Experiments

Materials and Methods

Six pairs of patient-matched normal and tumor tissue protein extracts from lung, breast, and colon were spotted in triplicate on specially prepared nitrocellulose-coated glass slides (Grace Bio-Labs). Lysates were spotted in modified RIPA protein extraction buffer according to the manufacturer's recommended protocols. Positive (purified IgGs) and negative (buffer only and BSA) controls were also included in the arrays. Arrays were printed with a BioOdyssey Calligrapher miniaarrayer using 160 μ m pins that delivered ~2.0 nl/spot of a 1.0 mg/ml protein solution. Sample tracking and spot placement were validated using the BackTracker program.

Slides were treated using protocols similar to those used for western blotting of proteins transferred to nitrocellulose membranes (Krajewski et al. 1996). Monoclonal and polyclonal primary antibodies raised against β -actin, tubulin (Imgenex Corp.), glyceraldehyde-3-phosphate dehydrogenase and laminin-1 (Proteus BioSciences, Inc.), human IgG, and rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) were used according to suppliers' recommendations and were detected using HRP-conjugated secondary antibodies. Color development was achieved by incubating the slides in tetramethylbenzidine (TMB) peroxidase substrate (Sigma-Aldrich Co.), and the slides were subsequently scanned using an ArrayIt SpotWare colorimetric microarray scanning system (Telechem International, Inc.).

Results

False-color images were generated to reveal differential protein content within each matched pair of samples, as shown in Figure 3. For example, in breast tumors, all normal samples displayed higher levels of a circulating protein than the tumor samples. Spot size and deposition were within acceptable limits (10% CV) as was signal-to-noise ratio for colorimetric detection of protein biomarkers using immunological detection methods. Sensitivity using TMB was in the low picogram range.

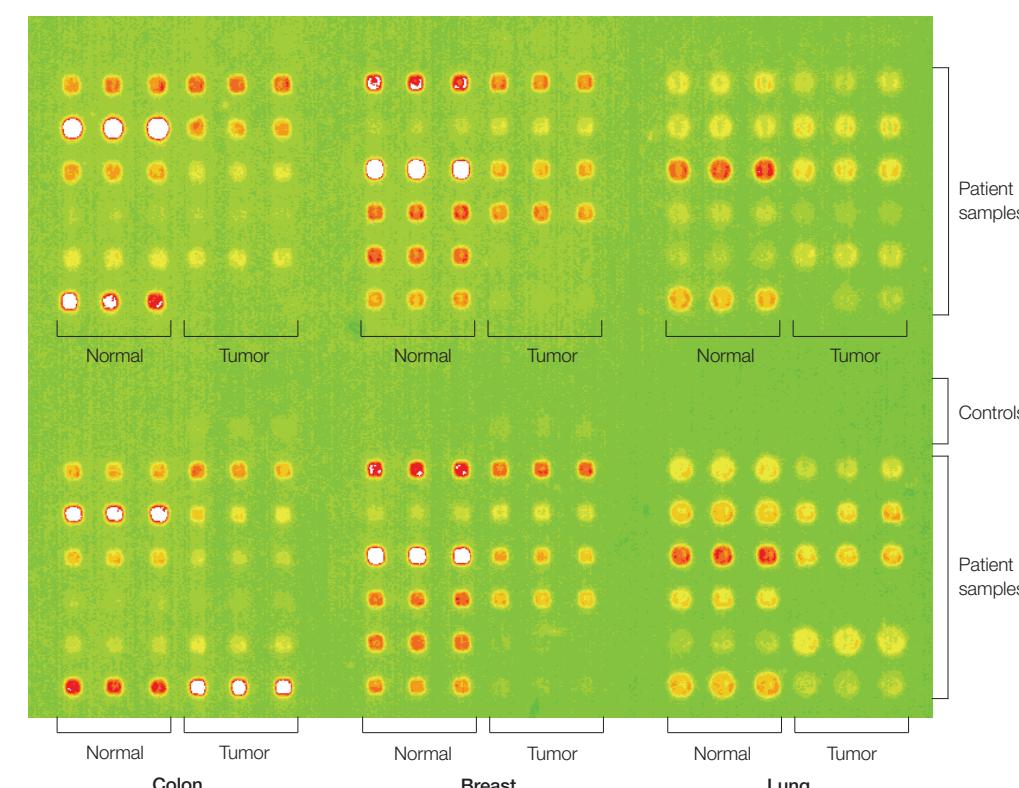


Fig. 3. False-color image of protein levels in a microarray of human clinical specimens. Normal and tumor lysates from six patients were arrayed and probed with an antibody to a circulating protein thought to correlate with differences in vascularization. Each row in a grid represents samples from a single patient. The first three spots in a row are from normal cells and the last three are from tumor cells. Upper and lower grids are duplicates of the same patient samples. The top two rows of the bottom grid consist of negative control spots to assess background noise of the assay.

Conclusions

The results presented here demonstrate the ability of the BioOdyssey Calligrapher miniaarrayer to accurately print precise amounts of material.

- Intraslide CVs were excellent, 2.5–5.6% (Figure 1)
- Interslide CVs were low, 4.1–8.2% (Figure 1)
- Higher variability was observed at lower protein concentration, illustrating the need to print RPPAs in dilution curves to match the sample protein concentration with antibody probe affinity that is within the linear dynamic range of the assay
- A stringent wash cycle used when printing lysates adequately eliminated carryover contamination (Figure 2)
- The technique was easily transferred to tissue lysates (Figure 3), and thus can be used for biomarker discovery
- BackTracker software facilitates design of arrays and placement of samples within the source plate

These evaluations confirm that the BioOdyssey Calligrapher miniaarrayer is technically effective for printing RPPAs for use in the initial qualification studies needed to develop diagnostic assays. Based on these precision and carryover experiments, the BioOdyssey Calligrapher miniaarrayer meets the technical quality characteristics that are desirable for printing small sample sets of RPPAs.

Cowherd SM et al., Clin Breast Cancer 5, 385–392 (2004)
Krajewski S et al., Anal Biochem 236, 221–228 (1996)
Tibes R et al., Mol Cancer Ther 5, 2512–2521 (2006)
Wulfkuhle JD et al., Proteomics 3, 2085–2090 (2003)

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