

Measuring proteins on microarrays

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A prerequisite of proteomics is the ability to quantify many selected proteins simultaneously. Immunoassays on microarrays are an attractive solution, as equipment and antibodies are available and assays are simple, scalable and reproducible. Recently, considerable progress has been made in this area as evidenced by increased sensitivity and coverage (degree of multiplexing). Routine use of antibody microarrays in research and diagnostic settings will require increased availability of binding reagents, novel signal amplification procedures, inexpensive and robust platforms for microarray production and detection, and turn-key systems for running high-throughput assays.

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Abbreviations

ELISA enzyme-linked immunosorbent assay

RCA rolling circle amplification

Introduction

Comprehensive, quantitative measurement of the components that make up a biological system is highly desirable for describing the state of that system. The development of DNA-chip technology [1] has enabled this to be done for the first time in tissues and cells at the level of RNA expression. Measuring RNA levels, however, might not give a complete or accurate description of a biological system: it is proteins that mediate nearly all cellular activities and, in fact, RNA levels often do not correlate well with protein levels or activities [2,3]. The fact that proteins also constitute the vast majority of pharmaceutical targets clearly indicates the need for high-throughput technologies for protein expression analysis, analogous to those in use for RNA profiling. Expression proteomics technologies will find broad application in determining the molecular basis for disease (functional proteomics) and the mechanistic basis for drug action and toxicity (pharmacoproteomics). They will also be used to identify and validate new biomarkers for disease diagnosis and for monitoring drug efficacy and safety.

High-throughput platforms for protein expression analysis employ either an open or closed architecture. The most common implementation of an open architecture system for proteome analysis is the combination of two-dimensional gel electrophoresis and mass spectrometry. Open architecture platforms are best suited for first pass comparisons of proteomes to identify a few, typically novel, proteins that exhibit the greatest differences in abundance. Although

this approach theoretically offers complete coverage of the proteome, in practice there are limitations with resolution, reproducibility, sensitivity and, importantly, with decoding. Significant difficulties with automation and quantitation also exist.

Ordered microarrays of proteins or protein ligands represent an attractive closed architecture platform for high-throughput analysis of proteins. Closed architecture proteomic platforms will be best suited for precise analysis of quantitative differences in abundance among known protein families and pathways. Advantages of this system are reproducibility, scalability, and precise quantitativeness. Although the specification of protein microarrays is rapidly improving, until recently they have lacked sensitivity and coverage. Both open and closed architecture expression proteomic methods are likely to flourish; for example, upon identification of differentially expressed proteins in an open system, further analyses, such as time- and dose-response, will be performed in a closed architecture system.

Several recent reviews have detailed the use of protein microarrays for applications such as screening antibody libraries and protein–protein interactions [4–7]. The focus of the present review is the development of protein microarrays for protein expression profiling and, specifically, their use in functional proteomics and pharmacoproteomics. Some of the challenges that need to be overcome in order for protein microarrays to be generally adopted in research and diagnostic fields will also be discussed.

Microarray immunoassays

The measurement of individual protein levels has traditionally been carried out using an enzyme-linked immunosorbent assay (ELISA) or western blot. ELISAs are available for the measurement of ~200 different proteins in a 96-well format. Such kits offer ease of use, adequate sensitivity, and are relatively inexpensive; however, these kits lack scalability for comprehensive protein measurement and require high sample volumes. A microarray of immobilized antibodies for multiplexed sandwich immunoassays can obviate these drawbacks (Table 1).

One of the first reports of a microarray-based immunoassay used alkaline-phosphatase-tagged read-out antibodies and a fluorescent substrate to detect eight analytes with a sensitivity of ~10 ng/ml [8]. Another early report described immunoassays performed on a polystyrene microarray and detected with dye-labeled antibodies [9]; this system achieved a detection limit of ~15 ng/ml for four different analytes measured simultaneously.

Two recent reports described ELISA-type antibody microarrays with sensitivities superior to those described

above, although the degree of multiplexing remained limited. In one study, 3×3 microarrays of monoclonal antibodies specific for seven different cytokines were spotted in the bottom of the wells of 96-well plates [10[•]]. Captured cytokines were detected by measurement of chemiluminescence generated by biotinylated secondary antibodies and streptavidin–horseradish peroxidase. Performance was much improved (sensitivity was 1–10 pg/ml, the range of concentrations that could be measured accurately [i.e. dynamic range] was ~2 logs and the variability of the assay [coefficient of variation] was ~10%). It was sufficient to detect biologically relevant changes in cytokine abundance, as evidenced by differences detected in supernatants from a monocyte cell line treated with an anti-inflammatory agent and from lectin-stimulated peripheral blood mononuclear cells and suitable controls. A drawback to this method, however, was the mode of signal amplification: although chemiluminescence provided sensitivity, it limited the dynamic range of the assay and, more importantly, the spotting density of different capture antibodies.

The second study described microarrays of antibodies for three analytes printed in 96-well plates [11[•]]. Primary antibodies, an enzyme-linked secondary antibody, and a fluorescence-generating substrate were used for detection. Sensitivity was good (10 pg/ml), but the dynamic range was less than 2 logs. Measurements of one analyte, prostate-specific antigen, in 14 serum samples correlated reasonably well with a conventional ELISA, suggesting that the microarray assay might be suitable for clinical measurements.

The most highly multiplexed antibody microarray described to date utilized a different approach, wherein a sample containing a mixture of analytes was covalently labeled with a fluorescent dye and then mixed with a control or 'reference' sample that had been labeled with a second dye [12^{••}]. The differentially labeled protein solutions were mixed and incubated on the microarray so that the fluorescence ratio at each spot corresponded to the concentration ratio of each protein in the two samples. Using mixtures of purified analytes, 60% of the 115 microarray immunoassays detected an analyte at a concentration of 1.6 µg/ml. For a few analytes, sensitivity was 1 ng/ml or better. Higher background (and reduced sensitivity) was obtained for analytes measured in serum. This approach, however, holds promise for substantially increasing the number of specific proteins that can be detected on a microarray, as it only requires one antibody per analyte. It seems likely that a signal amplification procedure will be necessary to achieve biologically relevant sensitivities for most proteins on such microarrays.

Rolling circle amplification (RCA) [13] has been reported to permit significant increases in sensitivity for multiplexed assays on antibody microarrays [14]. In the adaptation of RCA used for protein signal amplification, the 5' end of an oligonucleotide primer is attached to an antibody. In the presence of a DNA circle, DNA polymerase and nucleotides,

Table 1**Comparison of RCA-enabled protein chip with ELISA.**

Feature	Protein chip	ELISA
Sample type	Cell culture, serum, plasma	Cell culture, serum, plasma
Sample required per assay	~10 µl	50 µl
Number of results per assay	100–1000	1
Reproducibility (CV)	<10%	~5%
Sensitivity	1–50 pg/ml*	1–50 pg/ml
Dynamic range	3 logs	2 logs
Specificity	High	High
Assay time	4 h	3 h

*With signal amplification (e.g. RCA). CV, coefficient of variation.

rolling circle replication generates a concatamer of circle DNA sequence copies that remain attached to the antibody. The concatamer is then detected by the hybridization of fluorescent, complementary oligonucleotide probes. When utilized on microarrays of printed antibodies, RCA permitted detection of protein analytes with zeptomole sensitivity and a dynamic range of 4 logs. This represented a 100–1000 fold improvement over detection strategies using fluorescently labeled antibodies or streptavidin [14].

An RCA-enabled microarray consisting of antibodies to 75 different cytokines was recently constructed (B Schweitzer *et al.*, unpublished data); this microarray demonstrated a similar sensitivity and dynamic range to that discussed above. The microarray has been used to study time courses of cytokine secretion from human dendritic cells after treatment with lipopolysaccharide or tumor necrosis factor- α . Not only were known cytokine inductions recapitulated with this microarray, but several novel cytokine inductions were discovered and confirmed by ELISA; for example, the chemokine I-309 was shown for the first time to be secreted by dendritic cells. Furthermore, the reproducibility of cytokine measurements on this microarray was sufficient to allow comparison with standard curves and quantitation of absolute cytokine levels, enabling certain biological correlates of the observed changes in cytokine levels to be predicted. This study also examined time courses of secretion both of cytokines and of the soluble forms of their specific receptors and antagonists. As cytokines, like many proteins, act in cocktails to elicit biological effects that can be different from those induced in isolation, measuring global patterns of cytokine expression with microarrays is more likely to yield biologically relevant information than assays of single cytokines. This study was the first to show that practical proteomics studies were possible on antibody microarrays and, indeed, can yield biological information beyond that obtained with alternative methods.

RCA-enabled microarrays have also been used to measure cytokines in serum samples without loss of sensitivity or

Figure 1

Microarray assay of a human serum sample. A 15 μ L sample of human serum was incubated for 30 min on a microarray with 75 different anticytokine antibodies printed in quadruplicate. Following washing and incubation with a mixture of secondary antibodies to each cytokine, detection was carried out using RCA. The fluorescent image

was obtained using GenePix software on an Axon Microarray Scanner. The enlarged image shown represents one-eighth of the data acquired from a 1' $\times$ 3' microscope slide. Fluorescent intensities are represented in pseudocolor, with lowest intensities in blue and highest intensities in white.

increase in interference (Figure 1). These microarrays should therefore be useful in studies of basic immunology, infection, autoimmunity, immunodeficiency, and inflammation. RCA appears to provide useful sensitivity gains on antibody microarrays without the density limitation inherent to chemiluminescence or optical immunoassays.

Protein microarrays for diagnostics

In addition to developments in the use of microarrays of capture antibodies to measure protein levels, the past year has also seen progress with microarrays printed with antigens that are used for the detection of circulating antibodies in clinical specimens. For example, RCA-enabled microarrays of allergens have been used in two clinical trials for the detection of serum allergen-specific IgE. In the first, an RCA-enabled microarray provided results that were in excellent agreement with skin-prick testing [15]. In the second, the clinical performance of an RCA-enabled microarray was compared with three commercially available conventional allergen-specific IgE assays [16•]; 44 serum samples were screened for IgE specific to four allergens. All of the comparisons produced correlation coefficients above 0.9, thus validating the use of the RCA-enabled microarray immunoassay for the detection of allergen-specific IgE with clinical specificity and sensitivity comparable to commercial assays.

Joos and coworkers [17•] have described a microarray-based immunoassay for quantifying autoantibodies to 18 autoantigens in patient sera. Microarrays consisting of serial dilutions of the various antigens were printed on nitrocellulose or glass

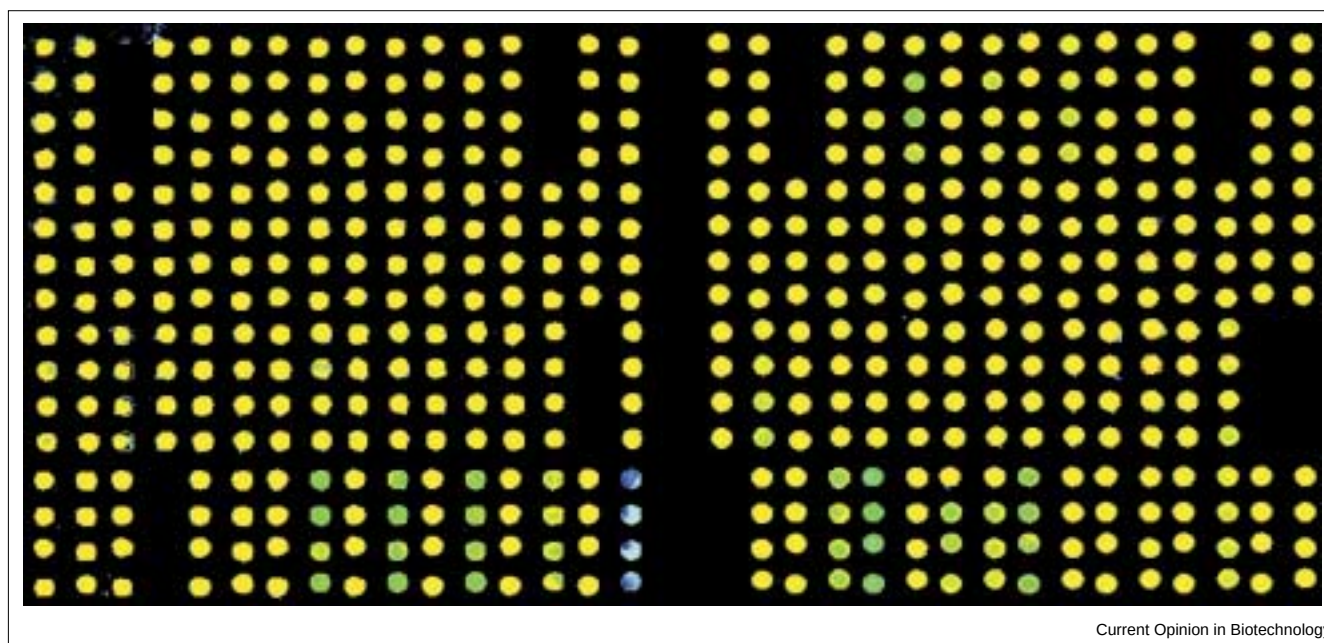
slides, thus allowing determination of the autoantibody titer. Detection used an antihuman secondary antibody and chemiluminescence. Specific detection of autoantibodies to 10 of the autoantigens was demonstrated using sera from patients with autoimmune disease. Sensitivity of autoantibody detection for five of the autoantigens tested was similar to conventional ELISA assays.

General use protein arrays

There have been several examples of the use of protein microarrays for purposes other than protein expression profiling or antibody detection. For example, protein microarrays on filters have been used to measure binding specificities of a protein expression library [18,19,20•] and to examine DNA-, RNA-, and protein-binding targets [21]. Proteins covalently attached to glass slides have also been used to detect protein–protein interactions, enzymatic targets, and protein–small-molecule interactions [22••]. For the most part, the experiments described in these reports were proof-of-concept studies with model systems.

Snyder and coworkers [23••] have recently published two notable protein microarray studies that indicated the value of protein microarrays for pure discovery. In the first report, 119 yeast protein kinases were arrayed in microwells and examined for kinase activity with 17 substrates. In the second report, proteins from 5800 yeast open reading frames were printed on glass slides and screened for calmodulin-binding activity and binding of specific phospholipids [24••]. These studies demonstrated for the first time that protein

Figure 2



Antibody arrays printed with piezoelectric spotter. A 20 μ l aliquot of Cy5-labeled goat antimouse antibody (10ng/ μ l) was added to each subarray of a microarray slide for 30 min and incubated at 37°C. After

washing (once with phosphate-buffered saline [PBS] and 0.5% Brij-35 for 2 min, and twice in PBS for 1 min), microarrays were dried by spinning and scanned on a microarray scanner.

microarrays are useful for high-throughput discovery of novel protein biochemical activity.

Protein profiling on microarrays – challenges and opportunities

Content

Bottlenecks to the development of highly multiplexed immunoassays on microarrays include the requirement for specific affinity ligands for each analyte. Although monoclonal/polyclonal pairs are more readily available than matched monoclonal antibody pairs, the use of polyclonal antibodies may lead to high background and reduced specificity and sensitivity. It is likely that *in vitro* selection of antibodies using phage- [25], ribosome- [26•] or mRNA-display technologies [27] or the use of engineered binding molecules (e.g. affibodies [28]) will have increasingly important roles in generating specific affinity ligands for analytes for which antibodies are unavailable.

Microarray production

To carry out reproducible and reliable immunoassays on a microarray, it is necessary to immobilize antibodies in a way that results in efficient deposition of functional protein. The printing of antibody microarrays has been reported on a variety of surfaces and immobilization chemistries. The surfaces employed include polyvinylidene difluoride [29], nitrocellulose [20], agarose [30], and polyacrylamide gel pads [31]. Glass slides activated with aldehyde [22], polylysine [17] or a homo-functional cross-linker [8] have also been described. In our experience, antibody arrays produced using a piezoelectric

microarraying robot on silane-coated glass slides have shown good reproducibility from day to day, efficient immobilization of antibodies, and low background when used in conjunction with fluorescence detection (Figure 2). Dispensing antibodies on an acrylamide hydrogel (3D-Link™, SurModics) produces microarray slides with similar characteristics (Bechtel *et al.*, unpublished data). Production of high-quality microarrays of antibodies requires careful preparation of samples and slides before printing, control of printing conditions (such as humidity, temperature, dust levels, and pin washing), and implementation of stringent quality control processes.

Samples

In several of the antibody microarray studies described above, assay sensitivities and specificities were assessed using purified analytes diluted in a buffer matrix. For general adoption, antibody microarrays must be compatible with all common sample types, including culture supernatants, cell extracts, and bodily fluids such as serum, plasma, urine, and cerebral spinal fluid. Some studies cited herein (e.g. [12••]) have reported reduced sensitivity and higher backgrounds with serum samples. There have been no reports to date of using cell extracts on protein microarrays. It has been our experience that significant effort is required to develop blocking and other surface passivation procedures to deal with assay interference produced by such samples.

Detection/signal amplification

Important features for sensitive and specific detection of proteins on microarrays include a high signal-to-noise ratio,

compatibility with a multiplex format, and low instrumentation costs. Radioactivity, chemiluminescence and fluorescence, the most widely reported detection strategies on microarrays, share many of these features. The use of radioactivity, however, presents safety concerns and may require long exposure times. Chemiluminescence, although highly sensitive, has drawbacks in terms of dynamic range and compatibility with multiplexing [10•]. Signal detection on microarrays using radioactivity and chemiluminescence can only be performed once, whereas fluorescence-based microarrays can be archived for future imaging. Fluorescence detection on microarrays, however, does require a signal amplification technology, like RCA, to provide sufficient sensitivity for most applications. An alternative approach for protein detection was recently reported that used optically coated, highly reflective, silicon wafers for multiplexed detection of three cytokines [32••]. Cytokines bound to capture antibodies passively absorbed to the surface of the wafer were detected with sensitivities ranging from 4–400 pg/ml using secondary antibodies conjugated to horseradish peroxidase, which catalyzes the formation of a thin film on the surface in the presence of a precipitating substrate. The film changes the color of the microarray by altering the interference pattern of reflected light. Qualitative results from this assay can be determined visually or quantitative results can be obtained using a CCD (charge-coupled device) camera. Although this format is relatively simple and inexpensive and appears to have adequate sensitivity and reproducibility for protein detection in clinical samples, it has limited practical multiplexing ability as substrate precipitation and color change is not always localized to the correct spot.

Conclusions

In the past year, the use of protein microarrays has gone from simple proof-of-principle experiments using a few, purified analytes to functional proteomics studies using real samples to measure many proteins simultaneously. The utility of such arrays in diagnostics and proteomics is no longer speculative. The near future should see the adoption of protein microarrays by biotechnology and pharmaceutical companies in place of, or complementary to, open-architecture platforms for drug target and biomarker discovery, diagnostics development, and toxicology programs.

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