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Protein microarrays as tools for functional proteomics
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Protein microarrays present an innovative and versatile approach to study protein abundance and function at an unprecedented scale. Given the chemical and structural complexity of the proteome, the development of protein microarrays has been challenging. Despite these challenges there has been a marked increase in the use of protein microarrays to map interactions of proteins with various other molecules, and to identify potential disease biomarkers, especially in the area of cancer biology. In this review, we discuss some of the promising advances made in the development and use of protein microarrays.

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Introduction
Advances in genomics and proteomics have created a demand for miniaturized, robust platforms for the high-throughput (HT) study of proteins. Microarrays, generated by spotting biomolecules on a solid surface at high spatial density, offer these features by allowing investigators to query thousands of targets simultaneously. DNA microarrays comprising thousands of different DNA molecules or oligo sequences, for example, provide a snapshot of the transcriptional state of a biological sample [1–4]. The widespread use of this technology for monitoring gene expression has generated valuable insight into various disease states [5,6]. DNA microarrays have found particular value in analyzing clustered gene expression, revealing co-regulated gene networks; however, gene expression analysis does not readily predict protein abundance nor does it provide information about protein function [7]. Given the central role that proteins play in biology and physiology, we need better methods to study protein abundance and activity in HT. Protein-based microarrays offer one such approach.

Several properties of proteins make building protein microarrays more challenging than building their DNA counterparts. First, unlike the simple hybridization chemistry of nucleic acids, proteins demonstrate a staggering variety of chemistries, affinities and specificities. Moreover, proteins may require multimerization, partnership with other proteins or post-translational modification to demonstrate activity or binding. Second, there is no equivalent amplification process like PCR that can generate large quantities of protein. Third, expression and purification of proteins is a tedious task and does not guarantee the functional integrity of the protein. Lastly, many proteins are notoriously unstable, which raises concerns about microarray shelf life. Despite these challenges, the development of protein microarrays has begun to achieve some recent success.

Two general strategies have been pursued. The first, abundance-based microarrays, seeks to measure the abundance of specific biomolecules using analyte-specific reagents (ASRs), such as antibodies. The second, function based microarrays, examines protein function in HT by printing a collection of target proteins on the array surface and assessing their interactions and biochemical activities. Here we discuss recent advances in both types of protein microarrays.

Abundance-based microarrays
Currently, there are two types of abundance-based microarrays: capture microarrays and reverse-phase protein (RPP) blots. Capture microarrays are generated by spotting specific capture molecules (e.g. antibodies, aptamers, photoaptamers, affibodies, etc.) on the array surface to trap and assay their targets from complex mixtures. These profiling arrays are directly analogous to DNA microarrays. Typically, the capture microarray is probed with a complex sample, and then relative amounts of the targeted analytes can be determined by comparison to a reference sample [8–13]. Although sometimes referred to as an array, the RPP blot is not a true array, in that it is not an arrangement of known elements with defined content. Instead, RPP blots are produced by spotting the unknown experimental samples themselves (or a series of experimental samples), which are then probed with ASRs in a fashion that is directly analogous to nucleic acid dot blots [14,15]. Both capture microarrays and RPP blots rely heavily on the availability of well-defined and highly specific ASRs.

The best characterized and most available ASRs are currently antibodies, although recent alternatives such as aptamers (photoaptamers), affibodies, and/or ankyrin

repeat proteins have shown promise [16–18]. Given their stability, selectivity and high affinity for ligands, antibody-based microarrays will probably be both sensitive and stable during storage. However, significant challenges must be addressed for capture microarrays to become common and generally useful. First, high quality ASRs are not available for most targets. Second, most existing antibodies do not function well in the microarray format. It is estimated that only 30% of commercially available antibodies can be used qualitatively and even fewer (20%) can be used quantitatively [8]. Thus, it is not surprising that successful capture microarrays thus far have been restricted to analytes such as cytokines, where there exist many antibodies to choose from [19]. Finally, even antibodies advertised as monospecific demonstrate significant cross-reactivity on the microarray format [20]. Despite these challenges, several successful approaches for capture and detection have emerged.

Capture microarrays

Two schemes predominate for the detection of analytes by capture arrays — direct labeling and sandwich assays — each with its own advantages and disadvantages (Figure 1). In direct labeling, the entire experimental sample is subjected to a labeling procedure that modifies all analytes with some detectable marker, such as a fluorescent tag. The advantage of this approach is that it allows the simultaneous measurement of many ana-

lytes. Unfortunately, labeling methods do not uniformly or even adequately label all analytes. They may lead to higher backgrounds and the modifications may alter analyte behavior [21]. Moreover, this approach depends entirely on the absolute selectivity of each ASR, requiring exceptionally specific reagents. Any cross-reactivity will result in a false-positive signal.

The use of two ASRs recognizing different epitopes of the same analyte, such as in a sandwich immunoassay, avoids the need for labeling and yields a highly specific signal. However, this approach is less convenient for simultaneous assessment of many analytes and requires the existence of two independent ASRs for each analyte. A useful hybrid approach is to print multiple different capture ASRs for the same analyte at different features on the array and then detect using the labeling approach. Concordant signals from the multiple ASRs confirm the specificity of the signal while still allowing simultaneous measurement of multiple analytes.

Application of capture microarrays

Haab *et al*. first demonstrated the feasibility of direct-labeling of proteins for capture arrays using well defined antibody–antigen pairs [8]. This approach was then applied by Sreekumar *et al*. to monitor changes in protein abundance in colon carcinoma cells following exposure to ionizing radiation [22]. Whole cell lysates from control

Figure 1
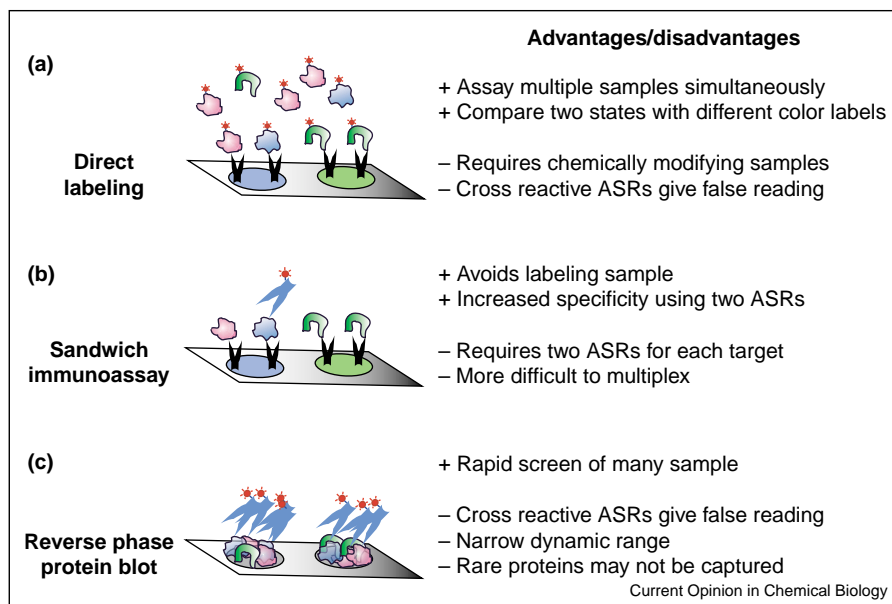
Detection methods for abundance-based protein microarrays. **(a)** Capture microarrays can be analyzed by chemically modifying the sample with fluorescent (or other readable) markers before applying the sample to the microarray. This has the advantage that all features of the microarray can be assessed simultaneously, but the limitation that any cross-reactivity in the analyte specific reagents (ASRs) used to capture the analytes will give false readings (blue feature). **(b)** Alternatively, the analytes can be detected by capture with one ASR and detection with a second specific to a different epitope in sandwich immunoassay fashion. This significantly reduces false readouts (blue feature), but can be more cumbersome to multiplex. **(c)** In the reverse-phase protein blot, the complex experimental sample itself is printed and probed with an ASR. This allows the rapid screening of many samples, but is highly subject to the specificity of the detecting ASR.

and irradiated cells were directly labeled with fluorescent dyes and used to probe a low density array of 146 antibodies to proteins involved in stress response, apoptosis and cell cycle regulation. Some of the observed changes were subsequently validated by western blot analysis. More recently, Miller et al. used a similar strategy to identify potential prostate cancer biomarkers in prostate cancer serum [23]. A capture microarray containing 184 antibodies targeting serum proteins ($\sim$22%), proteins previously detected in cancer patient sera ($\sim$7%) and intracellular proteins ($\sim$71%) was used to screen labeled sera from prostate cancer patients. From this, five proteins were able to discriminate prostate cancer serum from control. Most of these (4/5) were serum proteins that had been previously associated with prostate cancer. Two of these candidate markers also showed good correlation (r = $\sim$0.8) between protein microarray and standard ELISA. However, the microarray detection limit for prostate-specific antigen was only 200 ng/ml when serum levels ranged from 0.2 to 335 ng/ml, making this approach much less sensitive than existing methods. This highlights the need for better capture and detection reagents. Nielson et al. compared direct labeling to sandwich immunoassay for determining the abundance of EGFR and ERB2 and to monitor the kinetics of EGF-dependant phosphorylation in various human tumor cell lines [24•]. The sandwich immunoassay showed 10-fold less sensitivity but overall better reproducibility. Importantly, the transferrin receptor, a control protein, was detected by the sandwich immunoassay but not by direct labeling.

Reverse-phase protein blots
The detection of signal on RPP blots relies entirely on the specificity of the ASR (Figure 1) [14,15,25•]. This is particularly important because the effective concentration of analytes within the spot is high, and will tend to facilitate spurious interactions. Nishizuka et al. used RPP blots to differentiate cancers based on protein abundance. The authors spotted lysates from the NCI-60 cell lines corresponding to breast, prostate, renal, colon and other cancers and then probed with antibodies [26•]. To address the issue of specificity, the authors first characterized by western blot over 200 antibodies, and from that analysis chose 52 antibodies that reliably detected only their cognate proteins. In addition, the cell lysates were arrayed in 10 twofold dilutions to reduce the high local effective concentration. The authors also compared protein abundance to transcript levels for 19 of the 52 proteins. Of these, the seven cell structure-related proteins displayed a better correlation (r = $\sim$0.6 to 0.9) than the other 12 proteins (r = $\sim$ −0.1 to 0.8).

Given the small surface area of microarray spots, there are a limited number of available binding sites for printed proteins. When printing a complex protein mix in this context, abundant proteins may interfere with capture of other proteins, limiting the dynamic range and preventing the assay of rare and potentially interesting proteins on the array surface. To an extent, this can be mitigated by pre-fractionating the lysate before spotting on the array [27]. Nevertheless, given these limitations, RPP blots will probably find their greatest application in screening experiments across many experimental samples for a handful of well-defined proteins, which will then require a more detailed and quantitative analysis using other methods.

The increased interest in using abundance-based array approaches to profile analytes highlights the need for high-quality ASRs. Current collections of ASRs require careful validation of their specificity using alternative techniques such as western blot analysis and functional testing in the microarray format. A central challenge facing proteomics and biology is the assembly and validation of a complete set of ASRs for the proteome.

Function-based protein microarrays
The focus of function-based microarrays is to study the biochemical properties and activities of the target proteins printed on the array. Function-based microarrays can be used to examine protein interactions with other proteins, nucleic acids, lipids, small molecules and other biomolecules [28•,29•,30–32]. In addition, function-based microarrays can be used to examine enzyme activity and substrate specificity. These microarrays are produced by printing the proteins of interest on the array using methods designed to maintain the integrity and activity of the protein, allowing hundreds to thousands of target proteins to be simultaneously screened for function [10–13,33].

The list of potential applications of such microarrays is large. A microarray of a particular class of enzymes such as kinases could be screened with a candidate inhibitor to examine binding selectivity. A candidate drug could be used to probe a broad range of enzymes to look for unintended binding targets that might suggest possible toxicities. Proteins expressed by pathogenic organisms can be screened with serum from convalescent patients to identify immunodominant antigens, leading to good vaccine candidates. Protein interaction networks, including the assembly of multiprotein complexes, can shed light on biochemical pathways and networks. Eventually, it may even be possible to use these high-density microarrays as a MALDI source for mass spectrometry, allowing users to probe complex samples for binding partners to many proteins simultaneously.

However, as with the abundance-based microarrays, there still remain challenges in building and using function-based protein microarrays. First, the notorious lability of proteins raises concerns about their stability and integrity on the microarray surface. Second, it is time consuming and costly to produce proteins of good purity and yield, and many proteins cannot be purified at all. Finally, the

methods used to attach proteins to the array surface may affect the behavior of the proteins. Despite these challenges, there has been some success in building and using function-based protein microarrays. Here we describe two different approaches, protein spotting microarrays and self-assembling microarrays, and their recent advances.
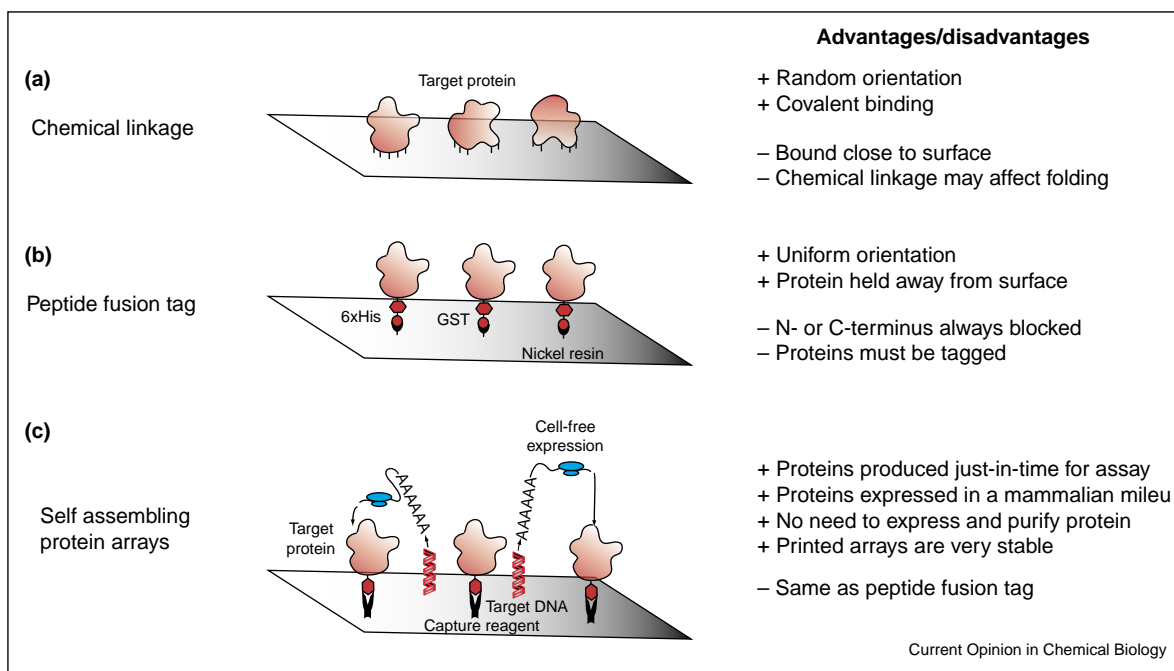
Protein spotting microarrays

Protein spotting microarrays begin by expressing and purifying the various target proteins, which are then spotted on the microarray surface. In general, two schemes to affix proteins to the surface of protein spotting microarrays are employed: chemical linkage and peptide fusion tags (Figure 2). In the chemical linkage format, proteins are attached to surfaces that display functionalized groups such as aldehydes, activated esters, or epoxy residues. The advantage of this approach is that the proteins are immobilized in random orientations using their primary amine, carboxyl or thiol moieties. In this scenario, many different domains of the protein are displayed, which increases the likelihood that all domains will be exposed to some extent. However, because this method maintains the proteins close to the array surface, the linkage chemistries may affect protein folding and surface accessibility. By spotting purified proteins in a

microarray format, MacBeath and Schreiber demonstrated the feasibility of chemically linked function-based arrays [28]. A limited number of purified proteins was immobilized on an aldehyde-treated glass slide and probed with different biomolecules. They demonstrated that their proteins maintained functional integrity upon immobilization as they were able to recapitulate specific interactions among proteins and small molecules as well as perform enzyme–substrate tests. They further demonstrated that this could be done at the expense of very little sample and at high density.

Peptide fusion tags can be appended to the amino or carboxyl terminus of the coding sequences for the target proteins. The resulting chimeric protein can be captured and immobilized via the peptide tag. This efficient capture method has the advantage that proteins are uniformly oriented at a distance from the array surface. However, activities that require a free amino or carboxyl terminus may be adversely affected depending on the location of the tag. The power of this method was demonstrated by Snyder and co-workers using the 6xHis affinity tag to immobilize sufficient quantities (10–950 fg per feature) of the yeast proteome ($\sim$5800 ORFs) on nickel-coated glass slides for screening experiments. Their functional tests

Figure 2

Advantages/disadvantages

(a)
Chemical linkage
Target protein

+ Random orientation
+ Covalent binding

– Bound close to surface
– Chemical linkage may affect folding

(b)
Peptide fusion tag
6xHis GST
Nickel resin

+ Uniform orientation
+ Protein held away from surface

– N- or C-terminus always blocked
– Proteins must be tagged

(c)
Self assembling protein arrays
Cell-free expression
AAAAAA AAAAAA
Target protein
Target DNA
Capture reagent

+ Proteins produced just-in-time for assay
+ Proteins expressed in a mammalian mileu
+ No need to express and purify protein
+ Printed arrays are very stable

– Same as peptide fusion tag

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Assembly methods used to produce function-based protein microarrays. **(a)** Expressed and purified proteins can be affixed directly to the surface of a chemically activated matrix. By this method, native protein can be used and the proteins will tend to position in random orientations, such that on average, each surface is likely to be exposed to the interacting sample. However, the close attachment to the surface may limit the overall solvent exposure of the protein and the chemical linkage may affect protein folding. Fusion peptide tags added at the N- or C-terminus affix the protein through an affinity capture reagent. Proteins are produced either by **(b)** separate expression and purification or **(c)** by simultaneous expression and capture of the protein on the array surface. The use of fusion tags allows the protein to be held at a distance from the matrix, exposing more overall surface area to solvent, but sterically blocking either the N- or C-terminus and requiring the addition of fusion tags to all target proteins.

screened for calmodulin and lipid-binding proteins, leading to several novel calmodulin-binding proteins and defining a consensus calmodulin binding motif. They also identified 150 lipid binding proteins in the yeast proteome of which 52 were previously uncharacterized [30].

Despite these successes, it is not always feasible to produce and purify the many proteins needed for protein spotting microarrays. Purification of mammalian proteins from *Escherichia coli* has proven to be especially difficult. Similar attempts using yeast expression systems have been even less successful [34,35].

One approach to circumvent this problem is to focus on purifying closely related proteins using the same protocol. Recently, Boutell *et al*. have produced a function-based microarray of allelic variants of one protein, p53 [32]. Here, ~45 variants were expressed in bacteria and then biotinylated. A lysate was then prepared and spotted onto a streptavidin-coated membrane. By focusing on a single protein and using a high-affinity binding linkage, this approach obviated the issues attendant to developing customized purification protocols for many different proteins, each with its own chemistry. This array was used to screen the p53 variants for their abilities to bind to a promoter element with a known p53 binding site (GADD45), to a protein known to interact with p53 (MDM2), and to be phosphorylated by casein kinase II. All variants interacted with MDM2, most were phosphorylated by CKII, and the most varied effects were observed in their ability to bind GADD45.

Alternatively, it may be simpler to study protein domains rather than the full-length proteins. Domains are usually smaller and easier to purify. Espejo *et al*. expressed and purified 212 domains of which 145 represented SH3, SH2, PDZ and several other domains [31]. The purified domains were microarrayed onto a nitrocellulose slide and screened with labeled peptides. The peptides bound in the predicted pattern, demonstrating that immobilized protein domains can be stable and accessible.

Self-assembling protein microarrays
The difficulty in obtaining purified proteins has no doubt limited the number of studies using functional protein microarrays. A completely different strategy avoids the need to purify proteins in advance and instead relies on the production of proteins on the microarray surface using a cell-free transcription/translation reagent. The recent development of a self-assembling protein microarray, called nucleic acid programmable protein array (NAPPA) is based on this principle [29•]. In this case, full length cDNA molecules — not purified proteins — are immobilized on a microarray surface and expressed *in situ* using a mammalian cell-free expression system (rabbit reticulocyte lysate). A fusion tag present on the protein is recog-

nized by a capture molecule arrayed (along with the cDNA) on the chip surface. This capture reaction then immobilizes the protein on the surface in a microarrayed format. This approach obviates the need to express, purify and store the proteins. As the proteins are freshly synthesized just-in-time for assaying, there is less concern about protein stability. This approach produces a sizable amount of protein per feature (270–2700 pg), averaging about 10 fmols. The microarrays are stable dry at room temperature until they are activated to make protein.

This approach has been optimized for the detection of protein–protein interactions. A further advantage of NAPPA is that the both the target and query proteins can be co-expressed, eliminating the need for any purified proteins. In a protein interaction mapping experiment among 30 human DNA replication proteins, 85% of the previously biochemically verified interactions were recapitulated. This study also demonstrated the feasibility of using this approach to simultaneously express multiple proteins to build multiprotein complexes as well as map binding domains among interacting partners.

Though the NAPPA approach addresses many concerns of the protein-spotting microarrays, this approach is still somewhat inchoate. NAPPA's robustness, reproducibility, and utility as a protein microarray remain to be determined as it gains more widespread use.

Comments
A growing enthusiasm in the area of protein microarrays has already inspired innovative approaches and applications to proteomics. To further advance this area there needs to be continued efforts in the area of protein production, surface chemistries, detection schemes and alternative platforms. These efforts must emphasize the need to develop and use high-quality reagents, and robust platforms to minimize the occurrence of false positives and negatives. Although these *in vitro* approaches are a step removed from cells and organisms, they are highly complementary to *in vivo* approaches.

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