

Review

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Protein expression profiling arrays: tools for the multiplexed high-throughput analysis of proteins

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Published: 10 June 2003

Received: 31 May 2003

Proteome Science 2003, 1:3

Accepted: 10 June 2003

This article is available from: <http://www.Proteomesci.com/content/1/1/3>

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Abstract

The completion of the human genome sequence has led to a rapid increase in genetic information. The invention of DNA microarrays, which allow for the parallel measurement of thousands of genes on the level of mRNA, has enabled scientists to take a more global view of biological systems. Protein microarrays have a big potential to increase the throughput of proteomic research. Microarrays of antibodies can simultaneously measure the concentration of a multitude of target proteins in a very short period of time. The ability of protein microarrays to increase the quantity of data points in small biological samples on the protein level will have a major impact on basic biological research as well as on the discovery of new drug targets and diagnostic markers. This review highlights the current status of protein expression profiling arrays, their development, applications and limitations.

Introduction

The analysis of the entire set of proteins of a biological system, commonly called proteomics, represents a research area that has emerged in the past decade as a largely technology-driven field [1–4]. Techniques like mass spectrometry in combination with separation tools such as two-dimensional gel electrophoresis or multidimensional liquid chromatography, allow for the parallel analysis of abundances of dozens to hundreds of proteins [5–7]. These techniques, however, are very labor intensive and require a significant amount of biological material. In particular, two-dimensional gel electrophoresis lacks the sensitivity to detect low abundance proteins.

These disadvantages of existing proteomics technologies have driven the development of novel miniaturized tools for the investigation of proteomes. An emerging technology in this field is the protein microarray [8–12]. Depend-

ing on the configuration, these arrays can measure protein expression levels, protein-protein interactions, protein-small molecule interactions as well as enzymatic activities. Protein expression profiling arrays are the most advanced in their development and therefore the major focus of this review.

The concept of protein expression profiling arrays was inspired by DNA microarrays, which enable the measurement of mRNA expression level of thousands of genes in a single experiment [13]. DNA microarrays have proven to be very powerful tools for the multiplexed comparative analysis of gene expression and led to important insights into gene expression patterns associated with disease states [14–20]. The possibility of performing similar analyses at the level of proteins – the functional products of almost all genes – is therefore very attractive.

There are, however, several reasons why DNA microarray technology cannot readily be adapted towards the development of protein microarrays. First, design and synthesis of gene-specific capture probes is straightforward since it is based on simple base-pairing rules and standard solid phase phosphoramidite chemistry, respectively. In contrast, the development of capture agents for protein arrays is far more complicated and requires significantly more time for development. Currently the preferred capture agents for protein expression profiling arrays are antibodies or antibody fragments, which have a very long and costly development time (see below). Furthermore protein expression levels span a huge range (up to 8 orders of magnitude). To avoid multiple measurements of the same sample at different dilutions, protein capture agents with different affinities have to be developed to address such dramatic differences in expression level.

Second, an appropriate surface attachment strategy has to be implemented to immobilize the protein capture agents onto the array while retaining their binding activity. Furthermore, during the dispensing and immobilization process, the proteins must remain hydrated to assure the integrity of their three-dimensional structures, an issue not relevant to the production of DNA arrays.

Third, especially for high density protein expression profiling arrays, novel detection schemes with adequate sensitivity are required to monitor the specific binding of proteins by the immobilized capture reagents on the microarray.

This review will summarize these issues and how they are addressed. Despite these limitations, impressive advances have been made towards the development of protein expression profiling arrays and several publications have been appeared over the last years showing the use and power of this technology.

Assay formats

The simplest protein array format consists of a large number of protein capture reagents bound to defined spots on a planar support material. This array is then exposed to a complex protein sample. The binding of the specific analyte proteins to the individual spots can then be monitored using different approaches (Figure 1). In cases where the analytes have been pre-labeled with a fluorescent dye, the binding can be monitored directly using a fluorescence scanner. A major limitation of this assay configuration lies in the often disappointing sensitivity, which prohibits the measurement of low abundance proteins. More often, however, the classical antibody sandwich type format is used, in which two protein binding reagents simultaneously bind to the same antigen: one antibody is immobilized onto the surface, and the other

one is fluorescently labeled or conjugated to an enzyme that can produce a fluorescent, luminescent or colored product when supplied with the appropriate substrate. The disadvantage of this sandwich assay format lies in the fact that two highly specific protein capture agents must be developed. The assay itself, however, is more reliable because direct sample labeling, which is not very consistent between samples, is not required. Furthermore this assay setup has been successfully used to monitor protein expression levels at physiologically relevant concentrations [21–23].

Capture agents for protein profiling microarrays

Current estimations assume that the human genome encodes about 30,000 – 40,000 genes. Due to splice variants on the mRNA level and a variety of post-translational modifications, the number of functionally distinct proteins is significantly higher, probably approaching one million. The development of protein microarrays for highly multiplexed protein profiling, similar to the multiplexing capabilities of DNA microarrays, would therefore require a large number of capture agents. The development of these capture agents is currently the most challenging bottleneck in protein microarray research.

Monoclonal antibodies or their antigen-binding fragments are currently the preferred choice for capture agents due to their high specificity, affinity and stability. They have been used in a variety of classical single analyte protein profiling assays such as enzyme-linked immunosorbent assays (ELISA) since the seventies. The long development times and labor-intensive nature of the process is, however, a major disadvantage [24,25].

To overcome this problem, different 'display' methods are currently being used and industrialized for the high-throughput development of protein capture agents. Phage-display libraries of antibody fragments offer the potential for antibody production at proteomic scales. These libraries can be used to isolate high-affinity binding agents against protein targets in a significantly shorter time frame than it is possible with immunization-based methods [26–30]. Furthermore, such methods can be used to create binding agents against proteins that are toxic, highly conserved or murine in origin, which are problematic for traditional mouse monoclonal antibody generation.

In order to measure picomolar concentrations of proteins, protein-binding reagents with nanomolar or sub-nanomolar affinities must be developed. Monoclonal antibodies can usually reach affinities with dissociation constants (K_d) in the lower nanomolar to picomolar range and are therefore suited for protein chip applications. The size of naïve phage display libraries is the key feature for

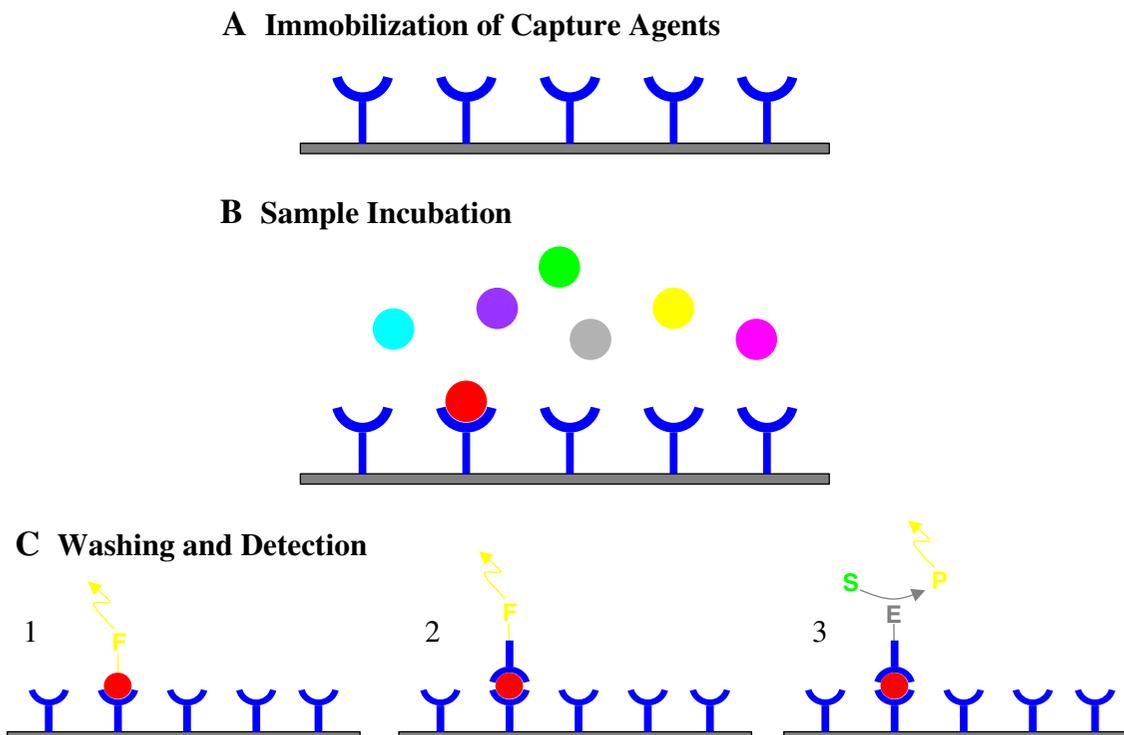


Figure 1

Basic principle of a protein expression profiling array and different detection schemes. (A) Capture agents with different analyte specificity are immobilized on a surface. (B) Incubation with protein samples leads to specific capture of target proteins in a concentration dependent manner. (C) After washing, the specifically bound target protein can be visualized by a variety of detection schemes, such as (1) direct fluorescence labeling of the analyte with a fluorophore (F) (labeled prior to the experiment), (2) detection of the target protein by a fluorescently-labeled detection antibody or (3) an enzyme-conjugated detection antibody, allowing an ELISA-based detection with a fluorescent, chemiluminescent or colorimetric readout (S = substrate, P = product).

the successful isolation of high affinity capture agents. Only very complex libraries with many billions of individual clones are suited for the isolation of high quality binding reagents. Several companies have developed highly complex libraries over the last years including Dyax, Cambridge Antibody Technology and Morphosys. The construction of high complexity phage libraries does, however, require a great deal of labor and is limited by the transformation efficiency of bacteria.

Due to the fact that the size of capture agent libraries is one of the major limitations for the development of high-affinity binding agents, additional *in vitro* evolution meth-

ods have emerged that circumvent the size-limitation of phage libraries. Ribosome display and mRNA display are completely *in vitro* methods that rely on physically linking the library proteins to their encoding mRNA sequences. Such methods have successfully been used to select high-affinity binding reagents to target proteins [31].

These library-based methods provide capture agents based on antibody fragments or other protein scaffolds. One inherent limitation of protein-based capture agents is their lack of stability during array dispensing processes and subsequent array storage. Several groups have therefore taken a completely different approach to develop

high affinity protein capture reagents for protein biochips. Aptamers are single stranded RNA or DNA molecules originating from *in vitro* selection experiments (termed SELEX: systematic evolution of ligands by exponential enrichment) with high affinities to proteins [32,33]. Aptamers against a number of proteins have been successfully selected from libraries with a complexity of over 10^{14} molecules (for review see [34]). A further development in aptamer technologies are so called photoaptamers. These molecules have an additional attribute that enhances their utility as protein capture reagents. They carry the photoactivatable crosslinking group 5'-bromodeoxyuridine, which, when activated by UV light, can cause covalent crosslinking with bound target proteins [35]. The photocrosslinking event provides a second dimension of specificity similar to the binding of a secondary detection antibody in a sandwich immunoassay. In a study by Golden et al. photoaptamers were used to crosslink low picomolar concentrations of fibroblast growth factor, in the presence of serum, with very high specificity [36].

Regardless of the type of capture agent, high specificity and affinity are crucial. Due to the multiplexed nature of microarray assays, high specificity is absolutely required to avoid cross-reactivity. Extensive cross-reactivity scans between binding agents to be used together on a microarray must be performed as part of the screening process to identify suitable reagents. Another important criterion is the affinity of the capture agents. Protein microarrays are especially attractive for protein expression profiling of low abundance proteins that cannot be visualized by 2D-PAGE techniques. The capture agents should have affinities not more than 2 orders of magnitude higher than the biological concentrations of the target proteins for reliable detection. Since some important proteins are present at low picomolar concentrations in serum, for example, antibodies with sub-nanomolar affinities should be used.

In order to create these protein capture reagents, a target protein must be synthesized and purified. Several groups have started to develop methods for rapid parallel expression and purification of proteins. The most widely used system is based on expression in *E. coli*, but a large number of human proteins expressed in this system are misfolded and insoluble. These misfolded proteins are often not useful for the development of antibodies that recognize the native form of a protein with high affinity. The refolding of proteins from inclusion bodies is difficult to perform in high throughput. For most human proteins, it will therefore be necessary to access alternative expression systems that rely on mammalian or insect cells. Automation of some of these eukaryotic expression systems is currently being explored [37].

Immobilization of capture agents for protein microarrays: where chemistry meets biology

The nature of the surface substrate and attachment strategy is one of the major factors for determining the quality of data obtained during protein microarray experiments. For optimal sensitivity and reproducibility, the activity of the immobilized capture agent has to be retained and non-specific binding of proteins to the surface must be minimized.

A wide variety of surface substrates and attachment chemistries have been evaluated for the immobilization of capture agents on protein microarrays. They fall into two basic categories. The simplest way to immobilize proteins on a solid support relies on non-covalent interactions. These immobilizations can be either based on hydrophobic or van der Waals interactions, hydrogen bonding or electrostatic forces. Examples of electrostatic immobilization include the use of materials such as nitrocellulose and poly-lysine- or aminopropyl silane-coated glass slides [38]. Protein microarrays were also fabricated by means of physical adsorption onto plastic surfaces of 96-well plates [21]. A big advantage of these immobilization concepts is their ease of use. Usually no protein modification is needed prior to printing onto the surface. The disadvantage is that proteins often get denatured on these fairly undefined surfaces due multiple uncontrolled interactions between the protein and the surface material.

Physical adsorption of proteins onto surfaces can also lead to problems with protein desorption during the assay, which can lead to signal loss. It is therefore more desirable to attach the protein capture molecule covalently and in a controlled way onto the surface. An example of covalent attachment of proteins to the surface has been described by MacBeath and Schreiber [39]. However in this case the immobilization was random, which can lead to a decreased sensitivity compared to an oriented immobilization. In the ideal setup, a single covalent bond would mediate the attachment. Due to the very high affinity of streptavidin to biotin, the immobilization of biotinylated proteins onto streptavidin surfaces can be considered quasi covalent. Using this strategy, Peluso et al. [40] were able to demonstrate that an oriented single site attachment of an antibody fragment leads to an increase in sensitivity over random attachment in a microarray assay.

The fact that a variety of different surface substrates have been used with success might indicate that the immobilization strategy is not the most critical parameter in the production of protein microarrays. The data quality with respect to parameters like signal-to-noise ratio and reproducibility, however, is influenced by the attachment strategy and processing of the arrays. Different surface

substrates, for example, require different blocking strategies to gain optimal data quality. MacBeath and Schreiber used BSA-coated slides to reduce non-specific binding [39]. A more sophisticated approach was taken by Ruiz-Taylor et al. [41,42]. They engineered surfaces to avoid non-specific protein adsorption using poly (ethylene glycol) derivatives as coatings. Protein microarrays based on this method of attachment of capture molecules onto otherwise protein-resistant surfaces have shown to be of very high quality [40].

Detection strategies for multiplexed protein microarray applications

The preferred method for detecting binding events on a protein microarray relies on fluorescence. As described above, there are two ways to incorporate fluorophores into an assay: (1) direct fluorescent labeling of the protein sample, and (2) sandwich immunoassays with labeled detection antibodies. The use of a miniaturized sandwich assay also allows for incorporation of enzymes which then can be used for signal amplification. For example Huang [43] has shown the simultaneous detection of different cytokines from conditioned media and patient sera using an array-based enzyme-linked immunosorbent assay in combination with enhanced chemiluminescence (ECL).

The choice of the detection strategy is partially determined by the application. A direct labeling of the protein sample can be applied to the analysis of cell lysates or purified protein samples. Miller et al. used a direct Cy3/Cy5 labeling strategy to perform differential profiling of prostate cancer biomarkers in serum samples [44]. They were able to identify five proteins that had significantly different expression levels between prostate cancer samples and normal controls. For quantitative studies with a limited number of specificities on a chip, however, a sandwich immunoassay format is preferred. Medium density arrays of antibodies against cytokines and other medically important proteins have been developed [21–23,45].

The fact that multiplexed protein microarray assays are performed on a flat surface adds certain restrictions to an ELISA-based assay setup. The resulting enzyme product must be either an insoluble precipitate or attached to a certain component of the microarray spot. Wiese et al. [23] performed a cytokine profiling microarray assay with an alkaline phosphatase-conjugated detection antibody, which generated a fluorescent precipitate. Another highly sensitive ELISA-based detection strategy has been described which uses rolling circle amplification (RCA) as a detection strategy [46,47]. This on-chip signal amplification strategy was used to perform very sensitive assays for highly multiplexed cytokine profiling and detection of allergen-specific IgE's in serum samples [48,49]. Further

improvement in sensitivity involves the application of fluorescent labels in combination with waveguide technology. Rowe-Taitt et al. [50] have applied this technology to detect clinical analytes and biohazardous agents in complex samples at physiologically relevant concentrations. Thin film waveguides generated from a high-refractive material such as Ta₂O₅ have been successfully used by Duveneck et al. [51].

Protein microarrays: Beyond protein profiling

Although the major use of protein microarrays is currently in the field of expression profiling, several applications of functional arrays and protein-protein interaction arrays have been described. Zhu et al. [52] cloned and arrayed into nanowells 119 protein kinases from yeast. They then assayed their activity using 17 different substrates in the presence of radiolabeled ATP. Following the incorporation of radioactive phosphate, they were able to identify the substrate preferences of most of these kinases. The same later undertook the heroic effort to clone and express nearly all of the 5,800 yeast open reading frames [53]. An array of these yeast proteins was created and probed for binding to calmodulin and certain lipids. They were able to identify 6 known calmodulin-binding proteins and several lipid-binding proteins. Once this approach is transferred to human proteins, a variety of novel protein-protein as well as protein-small molecule interactions will be discovered.

Another emerging microarray format consists of peptide arrays for the profiling of protein activity. An array of peptides on a gold surface was developed to monitor c-Src kinase activity. Phosphorylation of the immobilized peptide substrates was shown using radioactivity, fluorescence or surface plasmon resonance as detection [54]. Another group has demonstrated the use of fluorescently labeled phospho-specific antibodies to detect the phosphorylation event on immobilized peptide substrates [55].

Small-molecule microarrays have also been developed to detect the binding of proteins to an array of immobilized compounds [56,57]. These new microarray platforms will prove invaluable to basic biological research and have the potential to accelerate the pace of discovery of drug targets as well as lead compounds.

Conclusions

Since the initial conception of microspot assays [58], a variety of advances have been demonstrated for this platform to improve the multiplexing capabilities, reproducibility and sensitivity. In particular, the ongoing development of new techniques for the high-throughput production of protein capture reagents represents a key to the success of multiplexed protein microarrays assays.

This technology platform, however, is still not at a stage where it could be compared with the commercial success of DNA microarrays. A few companies, such as Zyomyx, CIPHERgen Pierce, Zeptosens, and BD Clontech have commercialized protein microarrays, but their applicability and competitive advantage over other, more macroscopic protein profiling platforms still needs to be proven.

Acknowledgement

We would like to thank David Wilson for the critical reading of the manuscript.

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