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# Protein microarrays: potentials and limitations

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## 1. ABSTRACT

Protein microarray technology has made enormous progress in recent years. It has been successfully applied for the identification, quantification and functional analysis of proteins in basic and applied proteome research. Protein microarrays have the potential to replace singleplex analysis systems. A variety of different analytical platforms have been developed that are likely to evolve into key technologies for the characterisation of complex samples. However, in contrast to well-established DNA microarrays, the robustness and automation needs to be demonstrated before protein microarray technology will reliably be integrated into high-throughput and routine applications. In this review we will summarise the current stage of protein microarray technology. Recent applications used for the simultaneous determination of a variety of parameters from a minute amount of sample will be described and future challenges of this cutting-edge technology will be discussed.

## 2. INTRODUCTION

After the completion of the human genome sequencing project, DNA microarrays and sophisticated bioinformatics platforms enabled scientists to take a global view of biological systems. In today's 'omics' era, protein microarray technology, the basic principles of which were already described in detail by Roger Ekins in the 1980s (1), is becoming a powerful tool for analysing the expression of a large number of proteins simultaneously. The technology can also be used to screen entire genomes for proteins that interact with particular factors, catalyse particular reactions, act as substrates for protein-modifying enzymes and/or as targets of autoimmune responses. A variety of different platforms have been developed for measuring large numbers of parameters from a minute amount of sample. Protein microarrays have a huge potential to be applied in basic research, drug target/biomarker discovery and validation, drug development as well as in clinical trials and diagnostics. Especially within the growing expectations

within the field of personalised medicine, protein microarrays will be very valuable and powerful tools towards the goal to provide safer treatments tailored to individuals, and with a greater degree of success, at lower cost. In its Critical Path Initiative Fact Sheet, the FDA outlines its "goal […] to stimulate the development of powerful new scientific and technical tools -- such as proven biomarkers, innovative clinical trial designs, simulation models of physiology and disease processes, and manufacturing quality assessment methods -- capable of rapidly predicting the safety, effectiveness, and quality of new medical products" (2).

Today, protein arrays are used to identify panels of biomarkers or to screen large number of patient samples for limited sets of biomarkers (3). Biomarker testing can help to considerably reduce the time of drug development. More effective drugs as well as early detection of adverse effects can be identified earlier in the drug development process (4, 5).

Nevertheless, the avenue to success has been all but easy. Although DNA microarrays have become well-established high-throughput hybridisation systems that are able to generate large amounts of mRNA expression data within just one experiment, one has to take into account that there is no absolute correlation between mRNA levels and corresponding protein levels (6). More sophisticated and higher throughput technologies for the expression analysis of large sets of proteins are being developed to overcome the gap between genomics and proteomics. One of these technologies is protein microarray-based assays. Despite the promise they offer, protein microarrays are still associated with numerous unsolved problems, not least due to the complex nature of the proteins.

## 3. POTENTIALS AND LIMITATIONS OF PROTEIN MICROARRAYS

A microarray is a collection of miniaturised test sites that allows many tests to be carried out simultaneously in order to achieve higher throughput and speed. The most familiar format is the 2-dimensional format in which the miniaturised test sites are placed on a microscopic glass slide. However, multiplexed bead-based assay platforms, i.e. three-dimensional formats, are becoming increasingly popular because an enormous amount of quantitative information can be obtained with considerable savings in labour and sample volume (7).

Currently, the greatest challenge and difficulty in assembling protein microarrays is still the provision of adequate capture molecules. These capture molecules must be able to capture a single type of protein, even if expressed at low levels, from a sample containing tens of thousands of different proteins in concentrations that differ more than seven orders of magnitude (for a review on protein microarray applications see Stoll *et al.* 2005 (8)). Whereas suitable capture agents can easily be designed for DNA microarrays, proteins are a lot more difficult to handle. DNA binds the complementary DNA targets according to the base-pairing principle, which is quite straightforward. Prediction of specific DNA capture sequences is easily possible on the basis of the primary sequence of the target DNA. Furthermore, high-throughput oligonucleotide synthesis and PCR-based approaches are excellent tools for the fast and cheap generation of DNA capture molecules. In contrast, it is virtually impossible to predict high-affinity capture agents for proteins on the basis of their amino acid backbone due to the proteins' complex tertiary structure and diverse interaction possibilities. Strong electrostatic forces, hydrogen bonds, hydrophobic van der Waals interactions often act in combination. The situation is made even more difficult by the proteins' ability to simultaneously interact with different binding partners and complex formation as well as post-translational modifications such as glycosylation or phosphorylation. Large sets of candidate capture molecules must be screened before suitable capture molecules can be assigned to their specific target molecules. All of the aforementioned features show that the idea of a cost-effective and fast high-throughput generation of highly-specific, high-affinity protein capture probes and targets is a tedious business. But considerable progress has in the meantime been made. Capture agents can be generated from a variety of sources including monoclonal and polyclonal antibodies, recombinant antibody technology (9), as well as scaffolds (10) and aptamers (11) are promising approaches to enable the high-throughout generation of appropriate content. But although these technologies have been available for many years, and it is in principle possible to generate capture molecules against each and every potential target molecule, we still see only "unicums" being developed and we still have to wait for the global content providers. Molecular imprints are an interesting alternative, generated through an extremely diverse set of building blocks (12). However, a prerequisite of all the different ways to generate appropriate capture molecules always is that the antigen of interest is available. So far, it is impossible to predict high-affinity capture molecules for proteins from their primary amino acid sequence.

### 3.1. Protein microarray formats and applications

Protein microarray-based assays can be grouped according to different formats and types of applications. Forward phase protein microarray assays are currently the most popular. In this case, arrays of well-defined capture molecules immobilised on the carrier allow the simultaneous analysis of large numbers of different parameters from a single sample. Examples of forward phase microarray assays include antibody microarrays used to identify and quantitate proteins of interest (like cell signalling molecules and biomarkers) and affinity arrays used to study the interactions between proteins and immobilised binding molecules such as proteins, peptides, low molecular weight compounds, oligosaccharides or DNA (7, 8, 13). The second type of protein microarrays is reverse phase arrays in which a multitude of different samples such as tissue or cell lysates are immobilised in a microarray format (reviewed in 14, 15). The individual microspots contain the whole proteome repertoire of the tissue or cell. Highly-specific antibodies or other single soluble probes are used to simultaneously screen these microspots for the presence or absence of distinct target

**Table 1.** Antibody microarrays used in protein expression analysis (7, 8)

| Objective of study | Reference |
|---|---|
| Detection of altered protein levels of LoVo colon carcinoma cells after ionising radiation treatment | 65 |
| Cancer-specific alterations | 66 |
| Identification of potential biomarkers in patients suffering from prostate cancer | 67 |
| Post-translational modifications | 68 |
| Protein phosphorylation | 69 |
| Analysis of the composition of the CD antigen level in leukaemia cells | 70 |
| Identification and characterisation of multiple antigen-specific T cell populations using peptide MHC complexes | 71 |
| Comparison of malignant and adjacent normal breast tissue | 72 |
| Clontech: Antibody arrays | http://www.clontech.com |
| SIGMA: Panorama antibody arrays | http://www.sigmaaldrich.com |
| Panomics: Interaction arrays[1] | http://www.panomics.com |

[1]For further information about microrarray companies and institutions please refer to www.biochipnet.com

**Table 2.** Early examples of successful multiplexed sandwich immunoassays[1]

| Study | Reference |
|---|---|
| Quantification of 150 different cytokines | 19 |
| Rolling circle technology for ultrasensitive antigen detection | 15 |
| Expression profiling of nearly 70 cytokines in patients suffering from renal cell cancer | 73 |
| Use of photoaptamers for the simultaneous measurement of multiple serum proteins | 74 |
| Multiplex antibody arrays in quantitative proteomics | 75 |
| xMap technology for measuring inflammatory markers | 76 |
| Clinical applications of multiplexed cytokine sandwich immunoassays | 77 |
| Comparison of sold-phase and bead-phases cytokine immunoassays | 22 |
| validation of a multiplex add-on assay for sepsis biomarkers | 78 |

[1]in the meantime many more have been added

**Table 3.** Bead-based systems for multiplexed ligand-binding assays

| Objective | Reference |
|---|---|
| Detection of human cytokines in a sample of stimulated peripheral blood mononuclear cells | 79 |
| Measurement of HIV-1 antigens in newborn dried blood-spot specimens | 80 |
| Detection of simulants of biological warfare agents | 81 |
| Measurement of human cytokines | 82 |
| Quantification of immune mediators | 83 |
| Binding specificities in highly multiplexed bead-based arrays | 84 |
| Detection of soluble cytokines in blister fluid | 85 |

proteins (reviewed in 14, 15). Reverse-phase arrays allow the screening of a large collection of tissue or cell lysates with a large number of patient sera or antibodies requiring only low amounts of sample.

### 3.1. Protein expression analysis using forward phase protein arrays

Protein array-based assays can be performed using a two-colour labelling approach, which has been successfully applied for mRNA expression analysis. For example, two different protein samples labelled with two different fluorophores are incubated on an antibody microarray. Bound molecules are visualised using dual wavelength fluorescence and reveal immediately the difference in target protein concentration. Table 1 shows a selection of antibody microarray experiments used for the analysis of protein expression. This direct labelling approach can, at least in principle, capture thousands of proteins when high-density antibody microarrays are used. However, as aforementioned, the lack of highly specific capture molecules and the lack of sensitivity for low-abundance proteins are still some of the major drawbacks of this approach and prevent the technology from becoming a sincere alternative to alternative methods, e.g. miniaturised and parallelised sandwich immunoassays. The growing demand in research and clinical applications for the simultaneous analysis of an increasing number of target proteins can be achieved with the development of highly sensitive miniature sandwich immunoassays (7, 16 – 23; Table 2). Apart from such planar microarrays, bead-based systems are an excellent alternative when the number of parameters that need to be determined is rather low. Such systems involve different colour- or size-coded microspheres or beads as the solid support for the capture molecules. These beads are subsequently analysed in a flow cytometer. Adequate reporter systems provide information about the amount of captured target protein. The sensitivity, reliability and accuracy of the bead-based system is similar to that of ELISAs. One hundred different colour-coded beads are commercially available from the American company Luminex (xMap technology) that have been shown their value in determining the concentration of cytokines or antibodies in biological samples for example (Table 3). Based on the Luminex technology, several companies are offering a constantly growing list of ready-to-use multiplexed sandwich immunoassays for the quantification of cytokines and call signalling molecules and the analysis of kinase activities (go to www.luminexcorp.com for more information on Luminex partners). BD Biosciences offers an alternative bead-based system that discriminates between different bead sizes and relies on two-colour detection, hence enabling the design of more complex assays.

**Table 4.** Interaction studies using protein microarrays

| Objective | Reference |
|---|---|
| Yeast proteome chip containing recombinant protein probes of 5800 ORFs | 36 |
| Specificity and cross-reactivity screening of antibodies using high-density protein microarrays | 87 |
| Glass-chip based high-density protein microarray from 2413 non-redundant purified human fusion proteins with a spot density of 1600 proteins/cm2 | 88 |
| Detection of functional differences between p53 oncogene and mutated p53 oncogene | 89 |
| Protein interaction network for ErbB receptors | 43 |
| SH3 domain protein-binding arrays | 42 |

Protein mciroarrays are an excellent tool for the diagnosis of autoimmune diseases, in which patients are screened for the presence or absence of a broad range of autoantibodies (4, 24-31). While some of the companies have even received FDA clearance for their test kits, it will most likely still take a while before such multiplexed systems will be able to enter the highly competitive diagnostics market in which immunoassays are highly automated. In addition, if only a few parameters have to be analysed from a sample, which is often the case in clinical settings, then microarrays are not the method of choice. We will see whether the growing number of diagnostic parameters and growing understanding of systems biology and cellular networks will result in a therapeutic relevance of protein microarray generated results.

### 3.1. Protein interaction studies

Protein microarrays have become complementary tools for studying protein interactions *in vivo*, in particular for the analysis of protein-protein, enzyme-substrate, protein-DNA, protein-oligosaccharide and protein-drug interactions. Low and high density protein, peptide and small molecule arrays have been used to investigate the binding of small chemical ligands, proteins, DNA and RNA to their binding partners (32-37). Examples of early interaction studies using protein microarrays are listed in Table 4. Whereas protein interaction studies involving full-length proteins allow the identification of interaction partners under experimental conditions, they will not provide information about the interaction sites. This kind of information can be provided by protein domain arrays (38-43). Jones and colleagues (43) used protein microarrays comprising basically all Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains encoded in the human genome to measure the equilibrium dissociation constant of each domain for 61 peptides representing physiological sites of tyrosine phosphorylation on the four ErbB receptors. Miniature multiplexed assay systems are also suited for measuring kinase activity and specificity in a single experiment (44-46). Another promising application is carbohydrate microarrays. The interaction of proteins and carbohydrates, which are the key components for glycoproteins, glycolipids and proteoglycans, are essential for many biological processes (tissue growth and repair, cell-cell adhesion and inflammation, fertilisation, viral replication, tumour cell motility and progression, etc.). They might therefore be useful tools in the determination of different kinds of infections (47-51). Protein microarrays have also been shown to be valuable tools in drug screening processes, in which the identification of drug candidates depends on the immobilisation of small organic compounds and the subsequent screening for receptor-ligand interactions (52, 43, 53).

In recent years, label-free protein arrays have been developed which also provide kinetics information as well as enable *in situ* identification. A recent article by Yu *et al.* summarises the most important label-free detection methods such as surface plasmon resonance imaging, atomic force microscope applications, electrochemical impedance and mass spectrometry (54).

### 3.1. Reverse phase microarrays

Reverse phase microarrays have become a popular screening method because of the low amount of sample used and because they enable the more effective validation of candidate biomarkers. A large collection of tissue or cell lysates can be screened with a large number of antibodies or patient sera (55, 56). Proteins representing a millionth (1/1,000,0000) fraction within a microspot can be detected in such a complex protein mixture. Two major problems are the lack of PCR-like amplification systems for proteins for the analysis of lower abundant molecules, such as is available for DNA, and the lower sensitivity of protein microarrays. It has been shown that the separation of proteins prior to detection enhances detection sensitivity (57, 58).

Efforts are undertaken to increase protein-binding affinity by developing suitable array substrates (59). The advantages of reverse phase microarrays are that there is no need to label the sample proteins, an approach that is able to speed up the early drug profiling process. The CalLyA Cell Lysate Arrays commercialised by the company Zeptosens – a Division of Bayer AG in Switzerland are able to detect a defined set of proteins using multiplexed, direct affinity assays in a much higher throughput than is possible with traditional Western blots. Ciphergen's SELDI (surface enhanced laser desorption ionisation) approach requires a mass spectrometer for read-out (60). The SELDI technology is suitable for the rapid detection of differences in total protein content of different samples, but is has its limitations with respect to the detection of high-molecular weight proteins or membrane proteins. Although this approach is useful for the identification of unknown protein biomarkers (61), it has a lower sensitivity than sandwich immunoassays (62). Other reverse phase approaches have been put forward by Y. Wang in 2004 (63) who developed an immunostaining method that enables the simultaneous detection of a large number of different proteins in one immobilised cell type (dissociable antibody arrays). As things stand, tissue microarrays currently seem to be the most advanced reverse screening method. It has been shown that tissue microarrays enable the simultaneous screening of a large number of paraffin-embedded tissues whereas the traditional histological analysis of tissue specimens is rather slow and work-intensive (56, 64).

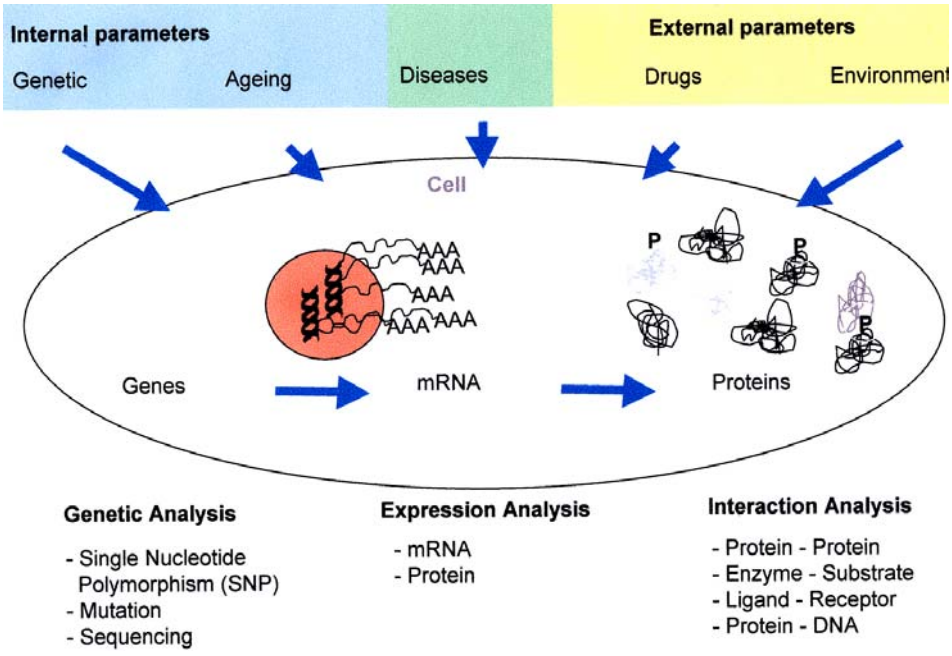


**Figure 1.** The power of genomics and proteomics: microarray applications.

### 3.4. Market perspectives of protein arrays

Although the market perspectives for protein arrays are tremendous, there are still a few limitations that make routine application difficult, e .g. for antibody arrays, using a direct labelling method. Here, all proteins of a sample are tagged either with a fluorophore or a hapten-like biotin, which can be visualised with a streptavidin-based reporter system. Following incubation on an antibody array, bound proteins can be detected on the corresponding antibody microspot. Although these arrays enable the multiplexing of hundreds of analytes, they lack sensitivity and specificity. Unspecific binding in a microspot occurs due to the high concentration of labelled sample material. In addition, the antibody array cannot discriminate whether a protein complex is a co-immune precipitate in a microspot or whether it is the pure antigen that generates the observed signal. Several companies, including Agilent Technologies, Telechem International Inc., PerkinElmer – to name just a few – sell kits (including fluorescent dyes, reagents, purification columns) for the generation of home-made antibody arrays, using a direct labelling approach. Other companies, for example Takara Clontech or Sigma, are selling ready-to-use antibody arrays together with a labelling kit for the samples.

To achieve appropriate sensitivity combined with high specificity, the user usually relies on sandwich immunoassay technology, where a second, not necessarily highly specific, antibody linked to a label is added. Miniaturised multiplexed sandwich immunoassays are quantitative, highly specific and sensitive; however, they are limited to about 30 -50 analytes per array. However, matched antibody pairs are required, which exhibit minimal cross-reactivity, a prerequisite that is not easily obtained. The higher the concentration of the detection antibody, the higher the non-specific interaction, which results in an increase in background. In practice, this limits the number of different features in a protein array, unless the number of different detection compounds can be reduced. As an example, in an array for the detection of antibodies directed against specific allergens or autoantibodies, the detection systems rely only on one type of antibody, namely anti-human-IgG or IgE antibody, respectively. Therefore, protein microarray assays cannot compete in number with one of the newer nucleic acid arrays. Nevertheless miniaturised and parallelised sandwich immunoassays are currently the most advanced assays formats among the different protein microarray applications.

Reverse phase protein microarrays are receiving increasing interest, as an alternative to replace classical Western blots and to increase throughput tremendously. To date, the major bottleneck is the validation of antibodies, which should be highly specific and should not cross-react with any other protein in the cell lysate. In a reverse phase protein array, the whole proteome is immobilised in a microspot, in which approximately 90% of all immobilised proteins represent structural proteins such as actin or tubulin. Highly specific antibodies are available, which allow the detection of a millionth fraction of a specific antigen. Therefore, each and every antibody has to be characterised to detect only a single band in a Western blot. Currently reverse phase protein arrays are used in research and service laboratories, where samples are processed in batches. While sandwich immunoassays can be sold as kits, this is still difficult with reverse phase microarrays. Their strength lies more in the service business where they are used as special tools for solving particular scientific questions.

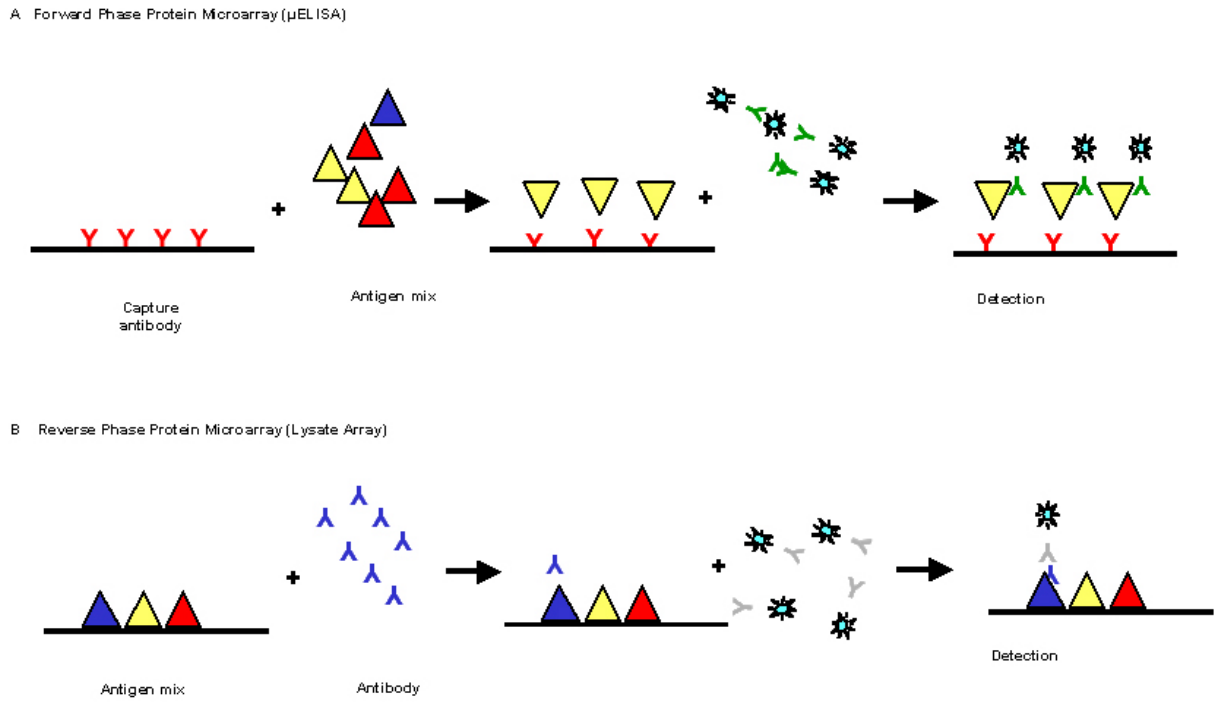


**Figure 2.** Difference between forward phase and reverse phase protein microarrays. A: Forward phase protein microarray; B: reverse phase protein microarray

## 4. SUMMARY

DNA chips are nowadays well-established research tools allowing a global view into the transcriptome. Although protein microarrays still have a while to go, there is a growing list of already available tools for basic and applied research. A constantly increasing number of publications have clearly demonstrated the extraordinary power of DNA- and protein-based miniaturised and multiplex assays. Before any of the new emerging technologies, allowing higher throughput, or higher degree of multiplexing can be applied in high-throughput screening approaches or in clinical diagnostics, it will be necessary to demonstrate appropriate precision, sensitivity and reliability in fully automated systems. At present, HT systems used by the pharmaceutical industry or in clinical diagnostics are highly automated and extremely robust. Any new assay format, any new technology will have to compete with the already existing highly developed technology - not only with respect to their performance and content, but also with respect to their costs. New instruments in combination with novel formats always involve an enormous investment before such systems will be integrated into routine.

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