**REVIEW ARTICLE**

Am J Pharmacogenomics 2001; 1 (1)
1175-2203/01/0001-0000/$22.00/0

# Protein Array Technology

## Potential Use in Medical Diagnostics

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## Abstract

The human genome is sequenced, but only a minority of genes have been assigned a function. Whole-genome expression profiling is an important tool for functional genomic studies. Automated technology allows high-throughput gene activity monitoring by analysis of complex expression patterns, resulting in fingerprints of diseased versus normal or developmentally distinct tissues. Differential gene expression can be most efficiently monitored by DNA hybridization on arrays of oligonucleotides or cDNA clones. Starting from high-density filter membranes, cDNA microarrays have recently been devised in chip format. We have shown that the same cDNA libraries can be used for high-throughput protein expression and antibody screening on high-density filters and microarrays. These libraries connect recombinant proteins to clones identified by DNA hybridization or sequencing, hence creating a direct link between gene catalogues and functional catalogues. Microarrays can now be used to go from an individual clone to a specific gene and its protein product. Clone libraries become amenable to database integration including all steps from DNA sequencing to functional assays of gene products.

### 1. Introduction

The first complete human genome sequence is now public knowledge. Automated technology, genomics databases and software tools will allow the fast and efficient identification of all estimated 100 000 human genes. The medical application of this information is expected to lead to new generations of drugs for the diagnostics and therapeutics markets. However, genes will only be useful for drug development and medical diagnostics if their functions are known. To date, genomics-based research into common diseases has led to only a few diagnostic tools and as yet no drugs. Progress has been hampered by the very nature of these diseases. Common diseases, such as cancer, are generally not caused by a single gene but by multiple genes and are further impacted by environmental influences. The contribution of a particular gene to a disease may be relatively small, and research may require much larger samples of individuals than would be required in a disease caused by a single gene. In addition, some genes can affect more than one disease. To tackle these current limitations in the medical use of genome information, ‘functional genomics’ is now unfolding as a new research and development area. The central idea is the annotation of the human genome with data that can ascribe functions to genes. New technology for the analysis of gene expression profiles of normal and diseased cells and tissues, protein structures and interactions, *in vitro* functionality and metabolic networks enables the first steps in this direction.

In general, the functional status of tissues of different developmental stages, differentiation and disease status correlates with the expression of certain sets of genes. By monitoring the amounts of transcript or protein in a cell, the expression strength of these genes can be determined and ideally, the function of the genes can be inferred by their level of expression. In recent years, technologies have become available which increase the throughput of these efforts. Most prominently, gene expression patterns are compared on the transcriptional level by DNA hybridization or by sequencing approaches. As a consequence, expressed sequence tags (ESTs) for most human genes have been found and deposited in the nucleotide databases.[1] However, only a minority of the proteins encoded by these sequences have yet been assigned a function.[2]

With the introduction of automated technologies in the field of molecular biology and especially microarray technology, genome and gene expression analysis has been accelerated enormously. Microarray technology was enabled by the development of devices that can array biological samples at high density and with high precision.[3] Oligonucleotide and cDNA microarrays have become hot commodities for researchers, representing thousands of individual genes arrayed on filter or glass slide supports.[4] To examine variation in gene expression, sets of oligonucleotides or complex probes, generated by reverse transcription of RNA from different tissues and cell-lines, are hybridized on the arrays.[5] Multiplexed genotyping by analysis of single nucleotide polymorphisms (SNPs) is performed with oligonucleotide arrays by primer extension approaches. Allele-specific arrayed oligonucleotides are extended by reverse transcription using RNA from complex mixtures as the template.[6,7] cDNA microarrays have already been used to profile human tissues like bone marrow, brain, prostate and heart[8] and complex diseases such as rheumatoid arthritis[9] and cancer.[10,11] However, DNA chip technology is still hampered by the lack of common quality standards that enable the comparison of results obtained in different laboratories and with arrays of different origin.[12,13] Nonetheless, protein chips are already emerging to follow DNA chips as tools for automated and miniaturized functional analysis.[14,15] Analogous to DNA microarrays, protein arrays offer the opportunity to screen thousands of immobilized biomolecules at a time, using steadily reduced amounts of sample (fig. 1).

## 2. From 2D Electrophoresis and Microtitre Plates to Microarrays of Biomolecules

Two-dimensional gel electrophoresis separates proteins according to size and charge, therefore allowing the study of cell, tissue and even whole organism proteomes.[38] Until recently however, the identification of the thousands of separated proteins

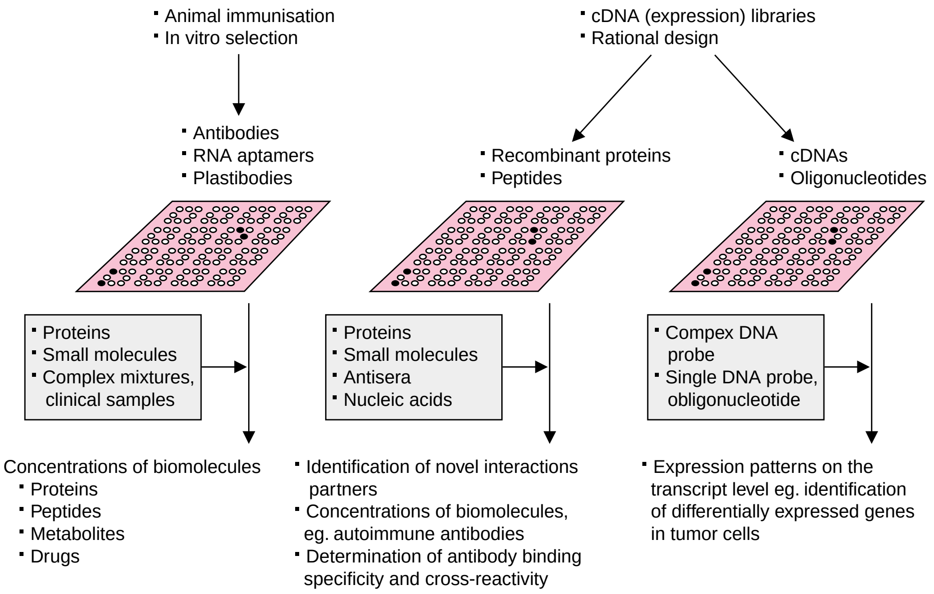

**Fig. 1**. High density biomolecule arrays. Biomolecule arrays are divided into 3 categories, antibody arrays, protein/peptide arrays and DNA arrays. DNA arrays are used to characterize cDNA libraries by DNA hybridization with single DNA probes and to determine gene expression patterns by hybridization with complex hybridization probes. [3-5,10,11,16] Multiplexed genotyping is achieved by primer extension of arrayed oligos using reverse transcription with complex RNA mixtures as the transcription templates.[6,7] The main application of arrays of antibodies, RNA aptamers or plastibodies with known binding specificities in the future will presumably be in the detection and quantification of biomolecules as proteins, peptides or chemical compounds in complex mixtures, for example, clinical samples.[17-22]
In contrast, arrays consisting of recombinant proteins or synthetic peptides are mainly used to identify and characterize interactions or biological activities of proteins with various kinds of biomolecules, for example, by screening an arrayed expression library with an antibody of unknown binding specificity.[20,23-37]

used to be a major challenge. With the introduction of new and automated mass spectrometric protein identification procedures, the high throughput identification of the separated proteins is much simplified[39] and allows to generate catalogues of expressed proteins in a cell or tissue of interest. Nevertheless, as the separated proteins are obtained in denatured form and in limited amounts, the expression of a protein of interest in recombinant form is usually required for functional characterization. The other classical array format in proteomics, the microtitre plate, is a well established and still widely used standard in medical diagnostics. To increase the number of samples and decrease reagent volume, the 96-well microtitre plate has been developed further to plates with 384 and 1536 wells, maintaining the original plate footprint. As it has already occurred in DNA analysis, the microtitre plate is now gradually being replaced by microarrays on flat surfaces such as glass slides (‘chips’) or membranes.

The format and the preparation of protein microarrays depend on the nature of the immobilized biomolecule and its application (fig. 1). While peptide arrays are manufactured synthetically directly on the support,[23] proteins are delivered using either pin-based spotting or liquid microdispensing. To date, the most commonly arrayed proteins are antibodies, since they are robust molecules which can be easily handled and immobilized by standard procedures without loss of activity. Microarrays have been developed for highly parallel enzyme-linked immunosorbent assay (ELISA) applications[24] and could be used for parallel analysis of multiple biomolecules within a single biological sample. For such binding studies, RNA aptamers are now arising as an interesting alternative to antibodies. They are selected for specific binding properties *in vitro* (systematic evolution of ligands by exponential enrichment, SELEX[40-42]). Using an automated SELEX procedure, arrays of aptamers are being developed and can complement antibody arrays to specifically detect biomolecules in complex mixtures.[17,43] Another future binding format are specific recognition sites in polymers created by molecular imprinting using template molecules.[44,45] The goal of this approach is to obtain surfaces with moulds, so called ‘plastibodies’, which fit only certain protein shapes and therefore bind these shapes specifically. Surfaces specifically binding BSA, IgG, ribonuclease A or lysozyme from two-component mixtures have been described.[18]

## 3. Protein Resources for Arrays

For protein arrays, resources of large numbers of proteins, preferably in purified form, represent a major technical challenge. A highly parallel and automated approach to protein expression is required. While in prokaryotes genomic fragments can be directly cloned into expression vectors, this is not possible in eukaryotes due to the presence of introns in their genes. The recombinant expression of all open reading frames of the yeast *Saccharomyces cerevisiae* has recently been achieved,[46] and a nearly complete collection of yeast strains for the expression of 6144 open reading frames as fusion proteins was generated, divided in pools and screened for biological activities. Collections like this can form the basis of future protein microarrays by representing a large proportion of gene products. Using a similar expression strategy, Zhu et al.[25] have created protein arrays of *S. cerevisiae* kinases. In total, 119 protein kinases were expressed, purified as glutathione S-transferase (GST) fusion proteins, arrayed and cross-linked in a protein chip format and assayed for autophosphorylation by treatment with radiolabeled ATP. Substrate specificity was assayed with protein chips each carrying one of a set of kinase substrates. The kinases and the radiolabeled ATP were arrayed by pipetting onto the substrate coated surfaces and phosphorylation was monitored.

In less well-known systems such as human tissues, full-length cDNA clones must be isolated before protein expression can be started. High-throughput subcloning of open reading frames has been described[47] but remains a major difficulty if complete proteomes of higher organisms are to be studied. To overcome these problems, arrayed cDNA expression libraries, cloned in bacterial and yeast expression vectors, have been developed in our laboratory. These libraries are characterized by standard DNA hybridization and sequencing techniques, screened for properties of their expression products and hence, represent a source for large numbers of recombinant proteins.[26,48,49] In addition, expression libraries eliminate the need to construct individual expression systems for every protein of interest. By arraying, the expression products of complete libraries can be characterized in parallel. On the other hand, a large proportion of clones do not express their insert in a suitable form, mainly due to cDNA fragments being fused to the vector-encoded start codon in the wrong reading frame. Therefore, non-expression clones have to be identified and removed from the library. To identify expression clones, hundreds of thousands of clones are arrayed on filter membranes and protein expression is induced. By detection of a $His_6$-tag peptide fused to the protein products, desired expression clones are identified and rearrayed into a new library.

Our protein filter array technology was further developed to increase spot density and to facilitate the arraying of purified proteins. Lueking et al.[27] have used automated arraying from liquid expression cultures using either a new pin-based or a high-speed picoliter dispensing (inkjetting) device mounted onto a flat-bed gridding robot. For this purpose, 96 proteins of the human foetal brain cDNA library hEx1[48] were expressed in liquid bacterial cultures, and solutions were spotted onto polyvinylidene difluoride (PVDF) filters, either as crude lysates or after purification by Ni-NTA immobilized metal affinity chromatography (IMAC). 4800 samples were placed onto polyacrylamide-coated microscopic slides and simultaneously screened, using a hybridization automat, applying minimal amounts of reagents (less than 100 μl antibody solution; Lueking, [unpublished observations]). Sharp and well-localized signals allowed the detection of 250 attomol or 10 pg of a spotted test protein (GAPDH, glyceraldehyde-3-phosphate dehydrogenase, Swiss-Prot P04406).

To achieve standardized microarrays carrying thousands of verified recombinant proteins, high-throughput methods for protein expression and purification are required, as well as a pipeline for the identification and verification of expression products. By combining protein expression and purification in array (microtitre plate) format with high-throughput protein mass determination by mass-spectrometry, large numbers of library clones have been identified and their expression products characterized.[48,50] This approach can be used to both identify unknown clones from expression libraries and to verify expression products generated at high-throughput.

## 4. Planar Immobilization of Proteins

Proteins are delivered onto solid supports by either pin-based spotting or microdispensing devices. The technique of immobilization is important both for effective concentration and orientation of immobilized proteins or antibodies on the surface. A variety of methods have been reported, including the adsorption to charged or hydrophobic surfaces, covalent cross-linking or specific binding via tags (e.g. nickel chelating or streptavidin coated surfaces for Plasmon Resonance measurements, Biacore AB, Uppsala, Sweden).

Flat pins are routinely used for spotting of nanoliter volumes of proteins, as flat pins are less sensitive to variation of sample viscosity than slit pins or microdispensing systems.[27] A slit pin arraying device was used by MacBeath and Schreiber[28] to produce a microarray of 10,800 spots of 2 distinct proteins (protein G and an FKBP12 binding domain), which were then specifically detected with fluorescently labeled IgG and FKBP12, respectively. As an alternative to metal pins, Martin et al.[19] reported a hydrogel 'stamper' for disposition of sub-monolayers of antibodies. They were immobilized on an aminosilyated surface and retained their binding activity. In addition, other approaches to immobilise proteins like BSA, avidin or monoclonal antibodies have been reported using either photolithography of silane monolayers[20] or gold,[29,51] combining microwells with microsphere sensors[52] or inkjetting onto polystyrene film.[53] Protein immobilization on flat surfaces was achieved by either covalent coupling to a crosslinker attached to the surface,[24,20,29] or noncovalent interaction to an immobilized biomolecule.

The biotin/avidin system is the most frequently used noncovalent immobilization system, due to the extraordinary high affinity of the biotin-avidin interaction. Since avidin (or streptavidin) homotetramers bind up to 4 biotin molecules, they can be used to link biotinylated macromolecules to a surface that was chemically coated with biotin.[51] Proteins are either biotinylated *in vitro* using commonly available reagents, or *in vivo* by expression of proteins fused to a peptide biotinylation signal.[54]

The density of protein molecules immobilized on the support is mainly determined by the surface structure. A flat, 2-dimensional surface offers less binding capacity than the 3-dimensional structure of a filter membrane or a polyacrylamide gel layer. Mirzabekov and co-workers produced 3-dimensional polyacrylamide gel pad microarrays providing an immobilization capacity more than 100 times greater than that of 2-dimensional glass supports, thus increasing the sensitivity of measurements considerably.[30] The gel pads are separated by a hydrophobic glass surface and provide a native, aqueous environment and can accommodate proteins of up to 400 kiloDalton in size.[31] Enzymatic activity of several enzymes like horseradish peroxidase, alkaline phosphatase and β-D-glucuronidase has been detected in these hydrogel pads. Prestructured surfaces consisting of hydrophilic spots on hydrophobic surfaces have also been reported for protein arraying.[32] The hydrophobic surface prevents the aqueous drops applied to the hydrophilic spots from mixing.

An array based on the 96-well microtitre plate footprint was developed by Mendoza at al.,[24] consisting of 96 6x6 microarrays printed with a pressure-controlled multi-capillary device. In combination with a custom scanning charge-coupled device (CCD) detector, the microarrays are used for multiplexed ELISAs. 20 samples plus controls are subjected in parallel to 96 different ELISAs by this system.

Recently, a high-precision sub-microliter liquid dispensing system has been developed for the preparation of hanging drop arrays for protein crystallization. These arrays consist of 2μl to 100nl drops and are used to screen for suitable buffer and salt conditions for protein crystallization.[55]

## 5. Microfluidic Devices

Planar microarrays are a robust and easy-to-handle format for the miniaturized immobilization of large numbers of analytes. Nonetheless, the introduction of 3-dimensional microstructures on a chip offers a number of additional options for experimentation (for a recent review see Sanders & Manz[56]). Such microfluidic devices are equipped with channels for transporting reagents to immobilized target molecules. For example, an assay for Protein Kinase A was developed on a microfluidic chip,[33] where all necessary reagents were placed in cavities on the chip and delivered to the reaction chamber through micro-channels. Microfluidic chips do certainly offer specific advantages over planar microarrays but due to their complex production procedures, their development and applications are still at an early stage.

## 6. Detection of Molecular Interactions on Microarrays

On DNA microarrays, hybridization events are detected using fluorescently or radioactively labeled probe molecules.[16] A corresponding approach for the detection of protein-protein, protein-DNA and protein-small molecule interactions has been reported. The 'universal protein array system' (UPA), consists of filter membrane arrays of purified proteins.[34] Specific binding properties of the immobilized proteins on the low-density UPA arrays were demonstrated with various radiolabeled protein, DNA, RNA and small molecule ligands. By washing the membrane under different salt conditions, high-affinity protein-protein interactions could be distinguished.

In addition to fluorescent dyes and radioisotopes, a wide range of detection options exists for protein and antibody arrays (reviewed in Rogers[57]). Unlabeled ligands can be identified indirectly by using a secondary antibody (sandwich assay). As an alternative to these noncompetitive formats, various competitive assays, relying on competition of the ligand with labeled tracers, are in use. Tracers are either detected directly on the chip or unbound tracer molecules are measured in solution. Protein chips for direct measurement of protein mass by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry have been described.[32,58] Also reported were surface enhanced laser desorption-ionization (SELDI, Ciphergen Biosystems) protein chips coated with either antibodies[21] or charged or hydrophobic groups[59] for protein adsorption, followed by MALDI-TOF mass-spectrometry on the chip to directly identify the captured polypeptides. Another method for the direct detection of unlabeled ligand binding employs surface plasmon resonance (SPR).[60] Using online detection in flow cells, this technology allows the determination of binding rates and dissociation constants. In addition to antibodies, SPR detection of protein-protein interaction on microarrays has been described.[35]

## 7. Living Protein Arrays

In contrast to oligonucleotide or peptide arrays, proteins cannot be synthesized directly on the support, but usually are gridded out of microtitre plates. Alternatively, cell arrays have been reported in which proteins of interest are produced by induction in bacterial or yeast expression hosts.[26] Recently, a comprehensive analysis of protein-protein interactions in *S. cerevisiae* was undertaken by yeast 2-hybrid screens in array format.[15,36] So called 'living arrays' were constructed consisting of a nearly complete set of yeast open reading frames cloned as fusions with the Gal4 activation domain. This clone set was co-transformed with a set of putative interaction partners cloned as fusions to the Gal4 DNA binding domain and subsequently arrayed on filter membranes. Protein-protein interaction was detected by arraying of the co-transformed clone set on selective media. By screening 5345 yeast open reading frame-Gal4 activation domain fusions with 195 Gal4 DNA binding domain fusions, 957 putative interactions, involving 1004 yeast proteins, were identified.

## 8. Applications of Protein Arrays

A large variety of assays have been adapted to utilize protein microarrays. Currently however, the detection of immobilized antigens with antibodies is still the most common application (fig. 1). Protein and antibody arrays have been used for the selection and characterization of novel antibodies from phage display libraries and for the identification of antigens (e.g. involved in autoimmune diseases).

Phage display antibody libraries have been developed for the *in vitro* selection of antibodies as an alternative to animal immunization (reviewed in Holt et al.[61] and Hoogenboom et al.[62]). For this purpose, recombinant immunoglobulin gene libraries are cloned in phagemid vectors and antibody fragments are displayed as fusion proteins on the surface of bacteriophage (reviewed in Collins[63]). Recently, protein arrays of our cDNA expression library hEx1[48] were used to identify antigens recognized by randomly selected antibody fragments from a phage display antibody library.[37] By screening 12 different antibody fragments on an array of 27 000 expression clones, 4 novel and highly specific antigen-antibody pairs were detected. In a related approach, antibody arrays were used for the identification of specific antibody-producing bacteria.[22] For this purpose, bacteria containing phagemid selected from a phage antibody library by *in vitro* panning on chosen antigens were arrayed on filter membranes. After cell growth, antibody production was induced and specifically binding antibodies were captured and identified on a second, antigen coated membrane. By screening 18 342 antibody clones at a time, highly specific antibodies were selected after just one round of panning.

In autoimmune disorders, self-reacting antibodies (i.e. produced against the organism's own proteins and epitopes) play an important role in the clinical manifestation of the diseases. Therefore, profiling the antibody repertoire of patients with autoimmune disease is believed to be medically relevant and informative. Characterization of autoimmune patient sera on protein chips would allow the diagnosis of autoimmune diseases based upon the presence of specific autoantibodies. For the identification of antigens recognized by autoantibodies, sera from patients with autoimmune disease have been hybridized to uncharacterized λgt11 cDNA phage libraries or to tissue extracts separated by 1D or 2D gel electrophoresis.[64,65] The subsequent characterization of the identified antigens is labor intensive, also requiring expensive sequencing of the identified proteins. Such characterization resulted in attribution of novel functions to these proteins and in some cases, suggested their potential as therapeutic targets.[66] To simplify the characterization of autoantibodies, serum can be applied to protein arrays containing large numbers of recombinant proteins of known identity. Moreover, using protein arrays will overcome the problems associated with protein level variation in natural tissue extracts and hence increase reproducibility. The use of protein chips allows for the determination of the binding profile of autoimmune antibodies of each patient and for each disease. Once disease-specific antigens are known, it is possible to create a diagnostic protein array. Recently, Joos et al.[67] reported a micro-array based test for the parallel detection of autoantobodies in human sera.[67] In contrast to the standard test involving time consuming tissue or cell culture immunofluorescence, only minimal amounts of sera (1μl per sample) are required. 35 clinically characterized patients were assayed on protein arrays with 20 different antigens and several control proteins spotted in various dilutions to confirm and analyze their diseases.

As shown by Lueking et al.,[27] apparently specific monoclonal antibodies (α-HSP90, α-β-tubulin) showed considerable cross-reactivity with other proteins following incubation on protein microarrays, consisting of 96 in liquid bacterial cultures expressed proteins of the hEx1 library. In a way, this is not surprizing, as antibodies are not usually tested against whole libraries of proteins. However, in immunohistochemical or physiological studies against whole cells or tissue extracts, this cross-reactivity of antibodies can lead to false interpretations. Therefore, the characterization of the binding specificity of antibodies used extensively in diagnostic tools, is of prime importance.

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