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REVIEW

# Protein microarrays for diagnostic assays

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**Abstract** Protein microarray technology has enormous potential for in vitro diagnostics (IVD). Miniaturized parallelized immunoassays are perfectly suited to generating a maximum of diagnostically relevant information from minute amounts of sample whilst only requiring small amounts of reagent. Protein microarrays have become well-established research tools in basic and applied research and the first products are already on the market. This article reviews the current state of protein microarrays and discusses developments and future demands relating to protein arrays in their role as multiplexed immunoassays in the field of diagnostics.

**Keywords** Protein microarrays · Multiplexed diagnostics · In-vitro diagnostics · Focused protein-profiling · Analytical microarrays

## Introduction

### Ambient analyte theory

Immunoassays have been widely employed as highly sensitive tools for almost half a century [1, 2]. Antibody-based immunoassays enable the generation of highly robust assays that can be easily standardized and automated. Nowadays there are hundreds of antibody-based assays on the diagnostic market [3]. The miniaturization of immunoassays for diagnostic purposes started way back in the early sixties [4, 5]. Feinberg and his colleagues developed a microspot-based assay that enabled them to diagnose autoimmune diseases. Auto-antigens were immobilized in a microspot and incubated with human serum. The presence of auto-antibodies in the serum led to the spontaneous precipitation of the auto-antigens in the microspot. The authors assumed that such microspot assays "would have a particular advantage for routine use on clinical specimens because it is simple, sensitive, objective, quickly carried out and read, requires but minute quantities of serum and antigen, and provides a permanent record for the case files". However, this microspot-based assay format did not have many supporters. More than two decades later, Roger Ekins came up with the ambient analyte theory on the feasibility of highly sensitive multi-spot and multi-analyte immunoassays [6]. According to Ekins' theory, miniaturization also leads to an increase in detection sensitivity[1]. According to the law of mass action, only a small amount of analyte molecules will be captured on to a spot that only contains a minute amount of immobilized capture molecules. Under ambient analyte conditions, the concentration of analyte in the sample will not change significantly even though the capture molecule is a high-affinity binder and the analyte concentration is low (Fig. 1). Therefore, this type of miniature immunoassay is concentration-dependent: the analyte molecules captured in the spot directly reflect the analyte concentration in the sample. In consequence of the unaltered analyte concentration, the signal becomes independent of the sample volume. High sensitivity

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[1] Here "detection sensitivity" is used in terms of limit of detection, i.e. the smallest amount of analyte which can be detected with acceptable statistical significance. By contrast we use the term "assay sensitivity", i.e. the smallest detectable difference in analyte concentration.

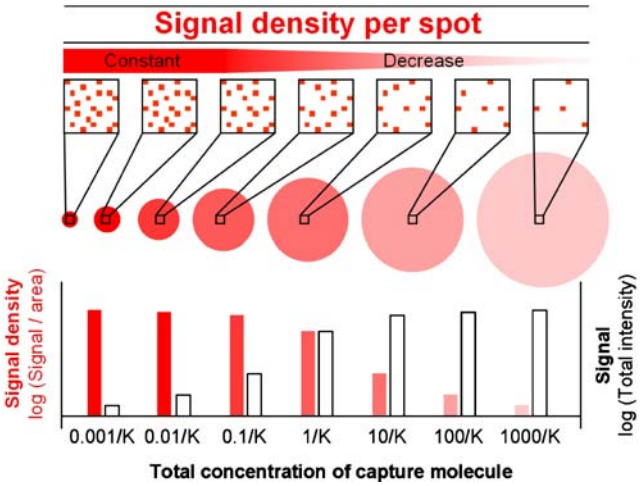


**Fig. 1** Signal and signal density in microspots. Signal density (signal/area) and signal (total intensity) of captured targets in microspots are shown for different concentrations of capture molecules. The capture molecules are immobilized with the same surface density on all spots. The signal (total signal) increases with increasing amount of capture molecules for growing spot size. When most of the targets are captured from the solution the signal reaches its maximum. By contrast, signal density (signal/area) increases with decreasing amount of capture molecules (decreasing spot size), reaching a constant level when the capture molecule concentration is $<0.1/K$ ($K$ is the association constant). Under these ambient analyte conditions, target concentration in solution is minimally altered by the amount of captured targets on the microspot. The figure was adapted from Ref. [61]

is achieved because the measurement always takes place at the highest analyte concentration possible. Thus, for a given analyte concentration, it is possible that the detection sensitivity in a microspot is higher than in a macrospot, because the analyte is contained in a small area and only signal density (i.e. signal per area) not the total sum of signal is relevant for signal-to-noise ratios defining the lower limit of detection. For this reason and the possibility of being able to detect multiple analytes in a single experiment, Ekins was convinced that miniaturized and parallelized protein microarrays had enormous potential for diagnostic applications [6].

## From genomics to proteomics

Despite protein microarrays' huge potential for diagnostic applications, it was, nevertheless, the field of genomics that made the greatest contribution to the advance of microarray technology. Following the sequencing of the entire human genome, DNA microarrays were developed and applied to large-scale genomics research. Comprising up to tens of thousands of different oligonucleotide probes per square centimeter, DNA microarrays are perfectly suited to high-throughput hybridization systems that enable the expression analysis of the entire transcriptome in a single experiment [7, 8]. Nowadays, DNA microarrays are well-established and reliable methods for mRNA expression profiling and SNP analysis. In addition, the first diagnostic assays have been placed on the market [9, 10]. Despite the huge potential of DNA, it is nevertheless proteins that are the key players in cellular processes. DNA microarrays only provide a limited amount of information on the status of cells and tissues. Cellular functions depend on protein activity, and proteins are not only regulated by the differential expression of the underlying genes but also by post-translational modifications. Furthermore, protein expression does not generally quantitatively correlate with mRNA expression [11]. Within the last decade, protein microarrays have entered the field of proteomic research [12, 13] demonstrating their huge capacity for identifying and quantifying proteins and for studying the function of proteins from the perspective of the proteome as a whole [14]. Besides their application in proteome-wide research and the determination of the biochemical activities of proteins, protein microarrays are also perfect tools for quantitating subsets of proteins in complex mixtures. This approach is known as focused protein-profiling or analytical microarrays. As correctly foreseen by Roger Ekins, multiplex immunoassays like these have been adapted to the microarray format and are about to be used in diagnostic applications. This article reviews the present state of multiplex immunoassays and discusses the obstacles that still slightly hamper their breakthrough in IVD.

## Protein microarrays

### *Principles and basics*

DNA microarrays preceded the use of protein microarrays. When proteins rather than DNA are used, a variety of additional challenges arise due to the more complex nature of proteins. A DNA molecule is built up from four different bases that all have the same hydrophilic sugar backbone. DNA is a very uniform and stable molecule due to its chemical structure and the pairing of complementary bases. DNA molecules exhibit a strong one-to-one interaction, which, under physiological conditions, is biochemically more or less inert. Proteins, which are a lot more fragile, are assembled from 20 different amino acids that are extremely heterogeneous in size and can have either hydrophobic or hydrophilic side chains. The proper functioning of proteins depends on their tertiary and quaternary structures. The foundation of these structures is a balanced system of electrostatic forces, hydrogen bonds, and hydrophobic Van der Waals interactions. In contrast with DNA, slight changes in salt concentration or pH, the presence of oxidants, or the removal of water often irreversibly harms their structure and hence interferes with their function. Furthermore, PCR and the ability to chemically synthesize oligonucleotides made a considerable contribution to the success of DNA microarray analytics. The amplification of proteins is impossible. High-

affinity and high-specificity capture molecules can be easily predicted and generated from the primary sequence of the target DNA. Protein capture molecules cannot be predicted or designed on the basis of a similarly easy principle, because the interaction underlies a broad range of the aforementioned molecular forces. In addition, post-translational modifications such as phosphorylation, acetylation, or glycosylation have to be taken into account when designing capture or binding molecules. At present, the lack of highly specific and high-affinity capture molecules is still the main limitation of protein microarrays.

Antibodies are broadly used in research and diagnostic applications because of their ability to bind to target proteins with high specificity and high affinity. When protein microarrays were still in their infancy, scientists initially resorted to antibodies since these proteins were well known from immunoassays [1]. The invention of monoclonal antibodies was a major step forward in the generation of unlimited resources of defined capture molecules. From then on, it was possible to produce pure and highly specific antibodies against almost any type of antigen [15]. However, the generation and validation of antibodies, regardless of whether they are monoclonal or polyclonal, is a time-intensive and cost-intensive process. In-vitro strategies that enabled the generation of binding molecules were eventually developed. For instance, phage-display technology can be used to screen large synthetic libraries of protein clones, within a few weeks, for detection of suitable binders against a target molecule of interest [16]. However, it also turned out that additional maturation steps were needed for generation of high-affinity binders that were similar to monoclonal or polyclonal antibodies. Another promising strategy for producing synthetic binders is the generation of aptamers. Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) with a specific and complex three-dimensional shape which causes their well-fitting binding. Aptamers are produced using an in-vitro selection and amplification technique called SELEX (systematic evolution of ligands by exponential enrichment). Many of the selected aptamers show affinities comparable with those observed for monoclonal antibodies [17]. Affibodies are another class of binding molecule. They are based on combinatorial protein engineering of the small and robust $\alpha$-helical structure of the domains of protein A [18, 19]. These synthetic binders are more robust than antibodies, which makes them perfectly suitable as capture or detection agents in protein microarrays. However, they had to prove that they had an affinity and specificity that was similar to that of antibodies. Despite all the advances in recombinant and scaffold-based technologies, the most advanced binding reagent project today, the “Human Protein Atlas”, uses a polyclonal antibody approach to generate binding reagents against human proteins [20].

## Planar microarrays

Protein microarrays are highly miniaturized and parallelized solid-phase assay systems that use a large number of different capture molecules immobilized in microspots (diameter <250 μm) with a density of a thousand spots, or more, per square centimeter. Such assay systems require only tiny amounts of sample and reagent volumes. Technologies that had previously been established for DNA microarrays were adapted to the generation of protein arrays. Microscopy glass slides were used as solid support and countless surface chemistry strategies employed to firmly attach proteins to the surface [21]. However, whether the proteins were attached to the surface by non-specific interaction or covalent cross-linking and whether the surface was 2 or 3-dimensional, the main goal was to achieve maximum binding capacity. However, it is also extremely important to effectively block the surface prior to the assay. Blocking is necessary because it minimizes the unspecific binding of analytes or detection molecules, and high signal-to-noise ratios can be achieved. This in turn is a prerequisite for higher sensitivity [22, 23]. Arraying techniques were successfully adopted from the DNA microarray production method [24]. However, great care has to be taken to keep the capture reagents in a functional state, especially relating to the storage of protein arrays. At present, most protein array assays are performed manually, but automation is starting to become more current. In the same way as for DNA arrays, protein microarrays also often rely on fluorescence labeling and the use of microarray scanners. The images obtained are subsequently analyzed with appropriate software. More information about the supply of microarray products can be found at http://www.biochipnet.com.

## Bead-based microarrays

Besides planar microarrays, robust and flexible bead-based assay systems have been developed over the last few years [25]. In planar microarrays, the position within the array enables an analyte to be identified. Bead-based assay systems rely on the use of different bead types that can be distinguished either by a color code, size, or shape (Fig. 2). Such bead-based assay systems are also referred to as bead arrays or liquid arrays. The individual bead types are classified according to their internal color code or size in an instrument resembling a flow cytometer. Analytes that are captured on the bead surface are detected by the appropriate reporter molecules. The bead suspension assays are performed using standard laboratory ware (e.g., microtiter plates). Bead-based assays can easily be automated with liquid handling solutions on the market (Qiagen, Perkin-Elmer, Stratec, Biorad). Sensitivity and accuracy of bead-

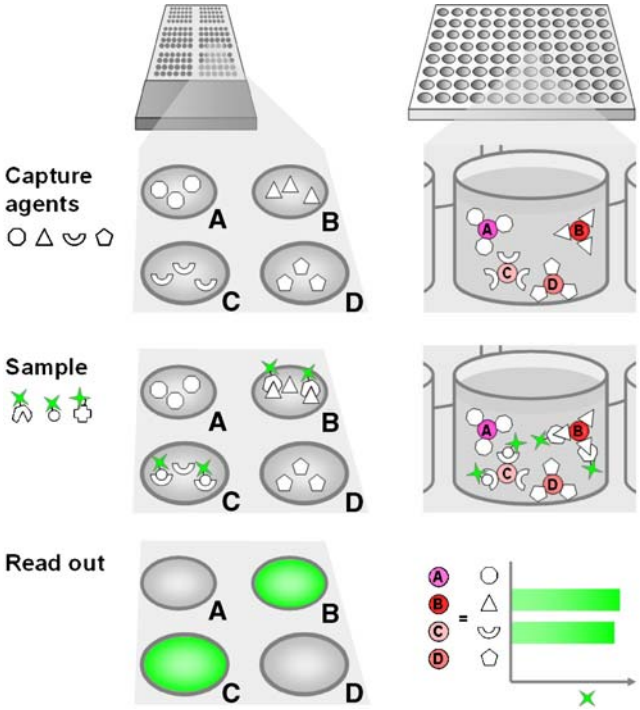


**Fig. 2** Planar and bead-based microarrays. In planar microarrays, individual capture agents are immobilized in a microarray format containing between several hundreds to several thousands of spots. The arrays are probed with sample and the analytes of interest bind to their cognate capture agent. The binding reaction is verified by a fluorescence read out. In bead-based microarrays, individual capture agents are bound to color-coded or size-coded microspheres. The assay can be performed with standard laboratory ware; a flow cytometer is used to detect the fluorescent label

based systems are similar to those obtained with established ELISA systems involving planar arrays. Some bead-based immunoassays proved to be as sensitive, accurate, and precise as competitive radio immunoassays [26]. Nowadays, the most popular platform is Luminex's xMAP technology. xMAP differentiates 100 different color-coded beads and allows researchers to easily set-up multiplex assays with small numbers of analytes; alternatively, they can choose from the increasing number of commercially available kits (http://www.luminexcorp.com). The BD FACSArray Bioanalyzer (http://www.bdbiosciences.com) or standard FACS instruments are able to classify the different bead types by their size and can use two or more different excitation lasers for multiplexed detection. This allows the design of more complex assays. At present, the washing steps in bead-based assays mainly involve filter plates. However, the implementation of magnetic beads will further simplify automation; problems experienced with filter plates such as clogging, leaking, or unspecific adsorption of analyte on to the large surface of filters will be avoided. The integration of established magnetic bead-handling technologies such as magnetic plate separators [27], magnetic pin heads (www.thermo.com/kingfisher), or in-tip magnetic capture (http://www.magbio.com) are a decisive step towards the further automation of bead arrays.

## Assay formats

Besides protein expression analysis, protein microarrays can also be used for the functional analysis of proteins, including protein interaction involving immobilized proteins or peptides, low molecular weight compounds, DNA, oligosaccharides, tissues, or cells. In general, protein array assay formats can be divided into forward-phase and reversed-phase arrays (Fig. 3). Forward-phase protein microarray assays involve the immobilization of capture agents and hence enable the analysis of multiple parameters from a single sample that is incubated on the array. In reversed-phase protein microarrays, many different samples (cell or tissue lysates) are immobilized in a microarray format and are simultaneously analyzed for the presence of a single target protein using a target-specific antibody. Replicates of such protein arrays allow the analysis of hundreds of parameters from minimal amounts of sample. The reversed-phase array format is ideally suited to looking into large sample cohorts. It enables the detection of differentially regulated proteins in healthy or diseased tissue and treated and untreated cells, the identification of disease-specific biomarkers, or the analysis of cell signaling networks [28]. Tissue arrays are a special type of reversed-phase microarray and consist of tissue slices that are immobilized on a surface. They are the miniature equivalent of classical immunohistochemistry assays and enable

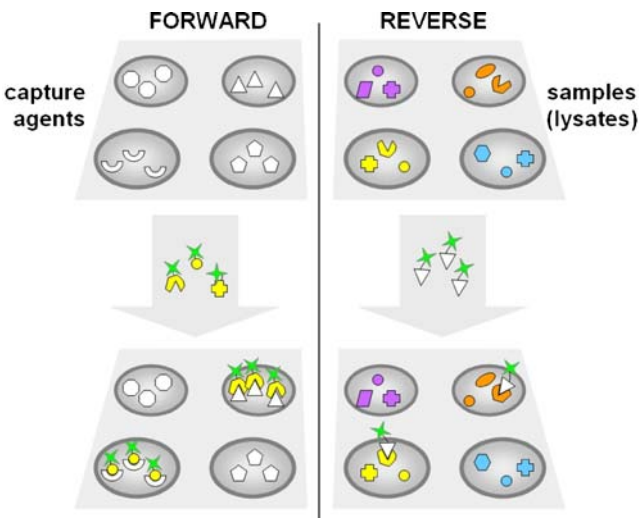


**Fig. 3** Forward and reversed-phase array format. In a forward-phase array format, a large number of immobilized capture molecules enable the analysis of many parameters from a single sample, regardless whether the sample is directly labeled or a sandwich assay is performed. In a reversed-phase array, many samples are immobilized and a highly specific antibody used to analyze the expression of a single parameter in the immobilized samples

the simultaneous analysis of large numbers of tissues with minimum reagent consumption [29].

Currently, forward-phase assays are the most frequently used protein microarray format. Protein arrays are used for detailed expression analysis and for functional protein studies. Forward-phase protein microarray assays are mainly applied for antibody arrays to quantify dozens of analytes that are present in complex samples. In antibody arrays, antibodies are immobilized on a carrier at densities of a few up to several thousand antibodies. Captured analytes can either be visualized by directly labeling the samples (analogous to DNA microarrays) or by multiplexed sandwich immunoassays. Protein microarrays enable screening for disease-related up or down-regulation of proteins in patient samples [30]. Fluorescence labeling of a sample necessitates careful optimization in order to obtain optimum signal-to-noise ratios. Insufficient labeling procedures result in a decrease in detection sensitivity whereas over-labeling of a sample will cause high background signals. The sample can also be labeled with biotin and subsequently incubated with fluorescence-labeled streptavidin; this might improve signal-to-noise ratios [31]. However, it has to be kept in mind that the labeling of the target proteins may interfere with the antibody-antigen interaction, because the labeling procedure could negatively affect the epitope and destroy it. Finally, one has to be aware that proteins appear in complexes. A strong signal can thus either be derived from large amounts of target analytes captured or, alternatively, from capturing a huge protein complex. The use of a single capture reagent cannot discriminate between a specific and an unspecific binding event. Specificity is greatly enhanced by using matched antibody pairs in miniaturized sandwich immunoassays in which high-affinity antibodies are immobilized in an array format and capture the target analytes during incubation with the sample. Unbound molecules are washed off and the array is incubated with detection antibodies that bind to another epitope on the target analytes. In sandwich immunoassays, two separate binding reactions are employed; this is substantially more specific than direct labeling approaches. The detection antibody can be directly labeled with fluorescence; alternatively, the signal can be further enhanced when using the biotin-streptavidin reporter system. The biotin-streptavidin model is a universal reporter system that simplifies the assay procedure, because all detection antibodies can be used in a biotinylated form. Despite high selectivity and detection sensitivity, it is, nevertheless, not possible to achieve unlimited multiplexing of sandwich immunoassays due to cross reactivity arising from the increasing overall concentration of the cocktail of different detection antibodies, which increases background signals above a certain threshold. In all complex multiplexed sandwich immunoassays discussed so far, multiplexed sandwich immunoassays used for the detection of up to 100 analytes had to be divided into several multiplexed assays [32]. In an 11-plex sandwich immunoassay, the detection sensitivity decreased by a factor of 1.7—up to 5 compared with the single-plex assays, because higher background signals were observed in the multiplex format [33].

Another forward-phase assay type uses recombinantly expressed proteins that are immobilized as capture molecules in a microspot. For example, in a study on protein interaction, Zhu et al. arrayed 5,800 recombinant yeast proteins on a microscope slide and probed this yeast proteome microarray with proteins and phospholipids to identify new interaction partners [14]. They confirmed the already known existence of calmodulin-interacting and phospholipid-interacting proteins and identified new interacting proteins. These types of protein microarray can also be used as antigen arrays to study the cross reactivity of antibodies or to screen for autoantibodies against unknown types of antigen. They represent a special forward-phase assay type, because the immobilized antigens are not real capture agents; instead the antibodies under analysis bind to the immobilized antigens. Antigen microarrays are easy to establish, because only one species-specific detection antibody is needed to identify bound antibodies in the microspots. Antigen microarrays are ideally suited to the multiparametric challenges of autoimmune and allergy diagnostics. Several autoimmune and allergy microarray assays are already commercially available and will be discussed in more detail in the next section.

## Other analytical tools

Apart from the above mentioned classical microarray methods, other technologies are employed to analyze protein interactions in a multiplex fashion. Very interesting are label-free detection methods, because there is no need for extra detection agents. For example, Quadraspec's spinning disc interferometry (SDI) technology enables detection of up to 128 unique analytes in 264 samples per disk. They set up a veterinary diagnostic test for canine heartworm (www.quadraspec.com). Analytik Jena combined reflectometric interference spectroscopy (RIfS) with biochip technology in their BIAffinity (www.analytik-jena.de). Maven Biotechnologies' (www.mavenbiotech.com) LFIRE is an imaging system based on total internal reflection ellipsometry that measures molecular binding reactions in a microarray. According to the company, LFIRE has been validated with protein microarray densities of 2,500 spots $cm^{-2}$ and is capable of detecting molecules as small as 150 Daltons. Finally, well established surface plasmon resonance (SPR) technology has been adopted to microarrays: in research applications, the Biacore Flexchip

has been used to detect up to 400 reactions in parallel [34, 35]. Apart from optical devices there are also electrochemical platforms, for example the GRAVI-Chip from DiagnoSwiss that uses electrochemical read out of enzymatic reactions to detect a current that is proportionate to the analyte concentration. Basically, many label-free technologies allow real-time detection of analytes and, therefore, the analysis of kinetic parameters of analytes could give additional diagnostic information. However, label-free technologies have to prove to be reliable enough to enter the market of multiplexed applications.

This has already been proven by the Triage system (www.biosite.com) and the VIDAS platform (www.biomerieux-usa.com). Both are micro-fluidic and integrated devices based on fluorescent read out that offer a set of multiplex assays for detection of proteins related to cardiac diseases (Table 1). The development of technology is also driven by reducing costs for diagnostics. For example, common recordable compact disks as molecular screening surfaces and a standard optical CD/DVD drive as detector, have been reported [36, 37]. Alpha-fetoprotein has been detected in a buffer environment by enzyme or gold nanoparticle-labeled antibodies that were used as tracers, forming a precipitate on the sensing disk surface. Such low-cost devices could also contribute to a better acceptance of microarrays in IvD.

## Protein microarrays for diagnostics

### *Analytical protein microarrays for detection of antibodies*

A very early example of a multiplex assay system is the MASTpette test chamber which was developed for multiparametric allergy testing in the 1980s [38]. Allergen-coated cellulose threads were bonded in the test chamber and incubated with patient serum. Bound IgEs were detected with enzyme-labeled anti IgE antibody using a chemiluminescent reaction. Today, IVD MASTpette test chamber-based test systems use as little as 230 μL for testing 20 allergen reactions in one assay (www.invernessmedical.de). The multiplex detection of allergens was transferred to protein microarrays, which has led to further miniaturization and parallelization. One of the first protein microarrays for detection of IgE used crude allergen extracts that were immobilized in microspots on modified glass slides. This approach involved rolling circle amplification in order to achieve sufficient sensitivity [39]. Hiller et al. [40] used 94 purified allergen molecules representing the most common allergen sources to generate an allergen array to screen for specific IgEs from minimal amounts of plasma samples. Purified allergens enabled the scientists to detect bound IgEs without needing to amplify them. Nowadays, protein microarrays enable the detection of allergen-specific IgE reactivity

**Table 1** Commercial multiplexed and protein-based diagnostics

| Indication | Target | Vendor | Platform | FDA cleared |
|---|---|---|---|---|
| Allergies | Antibodies | VBC-Genomics (www.vbc-genomics.at) | Planar array | − |
| Allergies, celiac disease | Antibodies | INOVA Diagnostics (www.inovadx.com) | Luminex | + |
| Allergies, common | Antibodies | ImmuneTech (www.immunetech.com) | Luminex | + |
| Allergies, indoor allergens | Antibodies | INDOOR Biotechnologies (www.inbio.com) | Luminex | − |
| Autoimmune | Antibodies | BioArray Solutions (www.bioarrays.com) | Bead array | + |
| Autoimmune | Antibodies | Biomedical Diagnostics (www.bmd-net.com) | Luminex | + |
| Autoimmune | Antibodies | INOVA Diagnostics (www.inovadx.com) | Luminex | + |
| Autoimmune | Antibodies | Zeus Scientific (www.zeusscientific.com) | Luminex | + |
| Autoimmune | Antibodies | Bio-Rad Laboratories (www.bio-rad.com) | Luminex | + |
| Autoimmune | Antibodies | Whatman (www.whatman.com) | Planar array | − |
| Cancer | Proteins | RBM (www.rulesbasedmedicine.com) | Luminex | − |
| Cardiac, heart failure | Proteins | Biomérieux (www.biomerieux-diagnostics.com) | VIDAS | + |
| Cardiac, myocardial infarction | Proteins | Biosite (www.biosite.com) | Triage system | − |
| Cardiac, shortness of breath | Proteins | Biosite (www.biosite.com) | Triage system | − |
| Infectious disease | Antibodies | Bio-Rad Laboratories (www.bio-rad.com) | Luminex | + |
| Infectious disease, Epstein-Barr | Antibodies | Zeus Scientific (www.zeusscientific.com) | Luminex | − |
| Infectious disease, FSME, Borrelia | Antibodies | Multimetrix (www.multimetrix.com) | Luminex | − |
| Infectious disease, Herpes virus | Antibodies | Focus Diagnostics (www.focusdx.com) | Luminex | + |
| Multiple immunoassays | Proteins | Randox (www.randox.com) | Evidence | + |
| Neurologic, Alzheimer's disease | Proteins | Innogenetics (www.innogenetics.com) | Luminex | − |
| Typing, HLA | Antibodies | Tepnel (www.tepnel.com) | Luminex | + |

Genetic tests still dominate multiplexed diagnostics, but proteins are catching up. So far, predominantly antibodies have been detected in serum from patients suffering from allergies, autoimmune, or infectious diseases. Technically, the Luminex platform is the prevailing technology for multiplexed protein diagnostics (Source: Multiplexed Diagnostics 2008; www.SelectBiosciences.com)

with the same sensitivity and specificity as the diagnostic technologies that are currently used on a routine basis [41].

Over the last ten years, antigen arrays with several hundred, and even up to several thousand, immobilized antigens have been used for detection of specific autoantibodies involved in autoimmune diseases. Joos et al. [42] used complex protein microarrays to detect up to 18 different rheumatic disease-specific autoantibodies in human sera, achieving sensitivities and specificities that were similar to established ELISA methods. This concept was further developed by implementing peptide antigens on a microarray, which allowed the detailed characterization of patients' autoimmune statuses [43]. Protein microarrays are perfectly suited to such types of analysis, especially when the quantity of sample material is limited. Sharp et al. [44] suggested the application of autoantibody profiling to improve diagnosis and prediction of disease onset and severity. Protein microarrays are valuable diagnostic tools for disease monitoring and therapy, because new clinical information can be gained from comprehensive autoantibody profiling, especially when combined with other clinical parameters, e.g. cytokine levels.

Arrays of viral and microbial antigens are the third type of antigen array. Mezzasoma et al. [45] successfully demonstrated the detection of antibodies directed against *Toxoplasma gondii*, rubella virus, cytomegalovirus, and herpes simplex virus types 1 and 2 (ToRCH antigens) in serum samples. In a more comprehensive study, Waterboer et al. [46] used bead-based microarrays to analyze 756 sera for the presence of antibodies against 27 antigens derived from human papillomaviruses. Recombinant GST-tagged fusion proteins were bound to glutathione beads and incubated with patient sera. Bound antibodies were visualized using anti-human IgG-specific detection. The authors were able to correlate the protein array results with those from ELISA-based methods. However, multiplexed serology also enabled them to effectively detect weak antibody responses. Gray et al. [47] used protein arrays containing immobilized malaria antigens to profile serum from malaria patients and found a correlation of the results with resistance to malaria. The authors analyzed combinations of reactivity to different antigens instead of individual reactivity and were able to group the patients according to their increased development of clinical immunity using hierarchical clustering. These studies demonstrated the potential of antigen microarrays, which can compete with ELISA tests in respect of sensitivity and robustness. Antigen microarrays are perfect tools for these types of application and support health personnel in diagnosis and prognosis.

### *Antibody and reversed-phase microarrays*

Similarly to DNA microarrays used for mRNA expression analysis, protein-capture arrays are used in dual labeling approaches to investigate relative protein abundance in two differentially labeled samples. Haab et al. [30] demonstrated the potential of such protein capture arrays by analyzing 115 characterized antibody-antigen interactions. Capture antibodies were immobilized on poly-L-lysine-coated glass slides. Patient samples were labeled with Cy5 or Cy3 dye. Defined mixtures containing Cy5-labeled antigens at different concentrations were incubated with Cy3-labeled control antigens. It was possible to achieve a correct linear relationship between antigen concentration and assay signal in about 20% of all interactions observed. Another 30% of the interactions reflected the antigen concentrations. Direct labeling approaches have already been commercialized by several companies (www.biochipnet.com). It must however be noted that sandwich immunoassays achieve higher specificities and usually higher detection and assay sensitivities than direct labeling approaches.

Focused protein microarrays such as miniaturized sandwich immunoassays, either planar or bead-based, have evolved into tools that deliver many clinical data of high diagnostic and prognostic value. For example, lyzed breast tumor biopsies samples were analyzed along with normal tissue for 14 relevant marker proteins from only 50 μg protein using the bead-based Luminex xMAP system [48]. The expression profile obtained for estrogen receptor and Her-2 (human epidermal growth factor receptor 2) exactly matched the results of the immunohistochemistry tests that are normally used for this type of application. Recently, the expression of 11 soluble receptors was analyzed in patient samples from 36 critically ill intensive care unit patients. Hierarchical clustering analysis allowed the scientists to group the patients into a sepsis and a trauma group [33]. The field of inflammation holds huge potential for protein microarrays as diagnostic tools, especially in the field of sepsis, which is often hampered by the lack of quantitative IVD tests. Due to the complex nature of sepsis, many clinical parameters ought to be monitored in parallel. Protein microarrays are perfectly suited to fulfill this requirement [49].

Reversed-phase microarrays also have enormous potential as diagnostic tools, in particular in the identification of disease-specific biomarkers, which may be used as markers to initiate and monitor therapy. There are high expectations that biopsy analysis from individual patients may lead to therapies that are specifically tailored to individual requirements [50].

## Commercial protein microarrays

A variety of analytical protein microarrays have been developed for different platforms and are entering the diagnostic market (Table 1). The first planar and bead-based multiplex immunoassays have been cleared by the US FDA or have been CE-marked for use in the EU. These

protein arrays are produced by the manufacturers whilst the assay and data analysis is performed by the customers. The AtheNA Multi-Lyte test system (Zeus Scientific, Raritan, USA) [51] and the BioPlex 2200 ANA screen (Bio-Rad, Hercules, USA) [52] are designed for autoimmune testing and are based on Luminex's xMAP technology. These multiplexed immunoassays can be used to screen samples for the presence of multiple autoantibodies involved in rheumatic diseases such as systemic lupus erythematosus (SLE), mixed connective tissue disease, Sjögren's syndrome, scleroderma (systemic sclerosis), polymyositis, and CREST syndrome. The AtheNA Multi-Lyte test system uses intra-well calibration technology involving internal standards for analyzing patient serum characteristics. The measured assay signals are corrected to compensate assay drift and to allow patient-specific calibration. A positive result is achieved for each individual analyte test when the assay signal is above a specific upper threshold. The result is defined as negative when the assay signal is below a defined threshold. Signals between the lower and upper thresholds are marked as questionable for this specific analyte. The BioPlex 2200 ANA Screen system uses pattern-recognizing medical decision support software that associates test results with predefined patterns that have been correlated with autoimmune diseases. The *k*-nearest algorithm is used to check for greatest concordance with 11 reference samples from a database containing data sets generated from more than 1,400 patient samples.

CombiChip Autoimmune 1.0 from Whatman (Springfield Mill, UK) is a planar microarray assay for autoimmune diagnostics. This protein microarray uses 14 different autoantigens that are immobilized on nitrocellulose-coated slides within 16 identical subarrays. Using this autoimmune microarray, 16 patient samples per slide can be processed using multi-channel pipettes in the same way as conventional ELISA testing using microtiter plates. However, imaging and image analysis has to be done manually using common microarray scanners and image analysis software. The CombiChip Autoimmune is CE-marked and sold in the EU; in the USA, the software is available for research use only.

Randox's Laboratories (Crumlin, UK) have developed Evidence, an automated biochip system enabling the analysis of miniaturized and parallelized immunoassays in a macroarray format containing 25 features and using chemiluminescence-based read out [53]. Several multiplexed immunoassays panels have been developed, including fertility, cardiac disease, tumors, cytokines and growth factors, cell adhesion molecules, thyroid function, and drug residues panels. The drugs abuse array has already received FDA clearance and other assays are currently being evaluated, thus making the Evidence biochip analyzer a pioneer in multiplexed immunoassays for clinical diagnostics.

## Problems and requirements

Protein microarrays are well-established analytical tools in basic and applied research, which is a sign of the capabilities and power of these assay systems. Prior to their implementation in routine clinical diagnostics, protein microarray-based results have to demonstrate clinical relevance in the initiation or changing of therapy. Medical demand, combined with an overall cost reduction, must become the driving force behind protein arrays gaining a substantial share in the IVDs market. Although protein microarrays have huge diagnostic potential, they are nevertheless still far from being widely used in IVDs. Several regulatory hurdles have to be cleared, for example, the IVD tests have to fulfill the specifications of the 98/97/EC directive of the European Parliament and of the European Council of 22/12/98. In the USA, the Food and Drug Administration (FDA) decides on the approval of IVDs for human application. Before approval is granted, the tests have to provide valid results, and proof that the results have a positive therapeutic outcome. In order to evaluate the reliability and reproducibility of DNA microarrays for gene expression analysis, the FDA's National Center for Toxicological Research (NCTR) set up the MicroArray Quality-Control (MAQC) project that involves both academic and commercial partners. (http://www.fda.gov/nctr/science/centers/toxicoinformatics/maqc). In the first phase, more than 1,300 microarray experiments were performed on different platforms at different test sites with the aim of showing that a consensus in data analysis will make a considerable contribution to reproducible lists of differentially expressed genes [54]. In the next phase, the tests are further validated in terms of clinical applicability. Special focus is put on data analysis using different algorithms in order to obtain predictive signatures and classifiers. However, DNA analysis is unable to provide data of clinical relevance in the same way as protein analysis. Only proteins, and hence the proteome, are able to reflect the physiological state of a cell. DNA analysis enables the diagnosis of a person's genetic predisposition to a specific disease, but it will be not possible to predict the onset of disease. Although analytical protein microarrays are able to analyze fairly focused sets of analytes, a variety of problems have to be solved prior to the broad application of multiplexed protein microarrays on the IVD market. To ensure the high quality of microarrays, much more effort has to be made than with singleplex measurements. A variety of controls have already been implemented in protein microarrays, such as replicate spots, marker spots for orientation, negative control spots to check for unspecific binding, application of assay buffer to test for cross reactivity between capture molecules and detection

antibodies, internal spot normalization [55, 56] and different normalization strategies [57]. However, quality control in terms of IVD testing means the routine analysis of reference sample material of low, medium and high concentration [58]. Only this will ensure that the test is effective at any analyte concentration and is able to detect an assay drift. At present, no information exists on the required complexity of such a reference sample and how stability can be guaranteed. The generation of defined mixtures of control analytes and the solubility of these molecules at high concentrations might cause problems that have not so far broadly investigated. Putting such controls in place will make it easier for the assay to be used for clinical analysis. However, additional issues will have to be addressed, for example the interpretation of assay results in cases when some of the controls fail. Can we regard the partial results gained from microarray with valid controls as sufficiently reliable or do these results have to be rejected, also? Another important issue is multiple subarrays within one slide. What happens if one of these subarrays fails? How can one tell whether the whole array is affected? How should failures within replicate spots be dealt with? Replicate spots are a compromise arising from the limited space within an array. Roche's new microarray platform, Impact, employs up to 20 replicate spots [59] and bead-based systems analyze from fifty to one hundred beads [60]. In the case of a multiplexed diagnostic assay for the analysis of dozens of individual parameters being established and a few or even just one single test being changed, will a whole new approval for IVD be required?

In addition, multiplexed assays generate huge data sets, which require appropriate data analysis. The question will be how to draw conclusions from patterns rather than looking into single interactions. The MicroArray Quality Control project (see above) was introduced to provide the necessary information for inter-platform and inter-laboratory concordance of DNA microarray experiments [54]. The BioPlex 2200 ANA screen system uses a pattern-recognition algorithm for analysis of their multiplexed protein assays [52]. However, there will be no general approach on how to apply such algorithms to generate diagnostically relevant data sets.

Besides the regulatory aspects, social and ethical issues also need to be considered. Should a customer be allowed to order only part of the data generated with a multiplexed assay and therefore pay less than the price of the full multiplexed panel? Can the manufacturer or the performer sell only parts of the microarray results, for example by using different software settings? What happens to unrequested data sets? What happens if the unrequested data sets are of diagnostic relevance that would lead to a different diagnosis? All these questions have to be carefully addressed and solved by the regulatory offices and by the diagnostic companies before protein microarrays are placed on the IVD market.

Protein microarrays assays will have to be automated before entering the IVD market. Automation increases assay performance, robustness, and reliability of multiplexed assays. However, such automated multiplex platforms have to compete with the well-established clinical analyzers that currently dominate the diagnostic market. These systems can easily increase throughput, e.g. measuring five parameters from the same sample in a sequential mode. Therefore, as long as sample material is not limited, or multiplexing does not exceed more than five parameters, the diagnostic companies are hesitant to make huge investments in order to change the assay format. In the field of autoimmune disease diagnostics, multiplexed assays are currently entering the diagnostic market, and sets of tumor marker panels may in future also be applied to monitor therapy. It can be safely assumed that protein microarrays will find their place in the IVD market in areas in which sample volume is limited and where they deliver therapeutically relevant data sets.

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