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Review

Protein and antibody microarray technology

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Abstract

Following the age of genomics having sequenced the human genome, interest is shifted towards the function of genes. This new age of proteomics brings about a change of methods to study the properties of gene products on a large scale. Protein separation technologies are now applied to allow high-throughput purification and characterisation of proteins. Two-dimensional-gel electrophoresis (2DE) and mass spectrometry (MS) have become widely used tools in the field of proteomics. At the same time, protein and antibody microarrays have been developed as successor of DNA microarrays to soon allow the proteome-wide screening of protein function in parallel. This review is aimed to introduce this new technology and to highlight its current prospects and limitations.

Keywords: Review; Microarray technologies; Proteomics; Proteins; Antibodies

Contents

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1. Introduction

The sequencing of the human genome and other ongoing sequencing projects have accelerated the pace of gene discovery and caused the identification of thousands of new genes. However, it also led to the realisation that the genome could not provide enough information to understand the complex cellular network. Although the genetic information provides us with the sequence information of each protein, it contains only little information about their localisation, structure, modifications, interactions, activities, and, ultimately, their function. This lack of information becomes especially obvious upon observation of a relatively closely linked relationship, the stoichiometry between the RNA transcripts their corresponding protein abundances. Although gene–protein dynamics were analysed for several tissues [1,2], there is still no reliable correlation between gene activity and protein abundance. Besides this, the protein abundances and their entirety, the proteome are highly dynamic and therefore require tools that are amenable of describing several variables simultaneously. Up to today two-dimensional-gel electrophoresis (2DE) for protein separation, followed by mass spectrometry (MS) and database searches for protein identification, are the only real high-throughput techniques for the complex description of a proteome. They are especially important in the classical proteome analysis, which focuses on studying complete proteomes, e.g. from two differentially treated cell lines, and the corresponding identification of single proteins.

1.1. 2D-gel electrophoresis

2D-gel electrophoresis was already invented in 1975 by Klose [3] and O'Farrel [4] independently, who demonstrated its usefulness with the separation of *Escherichia coli* lysates. However, it took till the mid-nineties, until the introduction of immobilised pH gradient isoelectric focussing increased the reproducibility as well as the resolution by an order of magnitude [5–8]. Based upon this improvement, 2D-gel electrophoresis today allows separation and detection from a wide variety of sources and permits the characterisation of samples by different expression profiles [9–11], Moreover, the resolution allows separation of proteins isoforms that differ by post-translational modifications, such as glycosilation [12], deamination [13] and phosphorylation [14].

Although the improvements allowed 2D-gel electrophoresis to become the major tool for proteomics, there are still problems. Even though the introduction of precast gels as well as standardised reagents, hardware and protocols have increased performance [15], automation is difficult [16], and experienced scientists are required to reduce variance and maintain reproducibility. Moreover, 2D-gel electrophoresis is time-consuming, expensive and lacks sensitivity [17] as well as dynamic range in comparison to ELISA [18]. Prefractionation [19] and the use of narrow pH gradients [20,21] are often necessary, and hydrophobic membrane proteins [22,23] as well as basic or high-molecular mass proteins are difficult to separate with sufficient resolution [24].

Besides 2D-gel electrophoresis, other chromatographic separation technologies, such as ion exchange liquid chromatography (LC), reverse phase LC, carrier ampholyte based-separations and affinity-based separations have been applied solely and in combination in order to separate complex protein mixtures and allow subsequent analysis by mass spectrometry [25] or on microarrays [26]. Especially the first approach can be combined with the isotope-coded affinity tag (ICAT), which is based on the derivatisation of cysteine residues by an isotope and allows an accurate quantification of the derivatised proteins [27].

1.2. Mass spectrometry

For identification of the spots, 2D-gel electrophoresis is often combined with mass spectrometry. Spots are excised and in-gel digested with trypsin, before they are identified and characterised by mass fingerprinting using matrix-assisted laser desorption and ionisation–time of flight (MALDI–TOF) mass spectrometry [28–30]. Mass spectrometry was, similar to 2D-gel electrophoresis, commonly used decades ago since the 1960s for the mass and structure determination of volatile compounds. The problem of applying mass spectrometry to proteins was that such large and charged molecules could not be transferred to the vacuum, since ionisation by electron bombardment would cause the destruction of the sample. In 1988, Karas and Hillenkamp developed the matrix-assisted laser desorption and ionisation, in which the proteins were packed into crystals of UV-light absorbing molecules [31]. This matrix charged the proteins and a desorption event was initiated by the absorption of energy from a laser by the matrix crystals. The absorbed energy was converted into heat and caused a sublimation of the matrix crystal and a transition of the matrix and analyte molecules into the gas-phase.

Although MALDI is the high-throughput protein identification technique of choice in proteomics, it requires large databases and computer facilities for the identification of proteins by peptide mass fingerprints. The complexity of such comparisons drastically increases if crude protein mixtures are analysed and limits the number of different proteins to be analysed simultaneously. To allow the analysis of such complex samples, prefractionation separation techniques, such as liquid chromatography [32] or isoelectric focusing [33] are required prior to mass spectrometry.

Since MALDI causes destruction of the protein samples, the analysis of intact proteins and non-covalently bound protein complexes requires electrospray ionisation–mass spectrometry (ESI–MS), which applies a milder ionisation technique [34]. A spray of fine droplets, containing analyte and solvent molecules is generated upon application of a high electrical tension through a needle. This allows ESI to be applied for obtaining the primary sequence of peptides that were not previously characterised. However, this ap-

Table 1
Selection of commercially available arrayers

Company	Arrayer	Mode of printing
Affymetrix	Affymetrix 427 Arrayer	Contact, pin and ring
Biorad	VersArray ChipWriter Pro systems	Contact, solid or quill pins
Biorobotics	Microgrid 2	Contact, solid or quill pins
GeneMachines	OmniGrid 300	Contact, solid or quill pins
GeneScan	TopSpot/E	Non-contact, piezo print head
Genetix	Qarray	Contact, solid or quill pins
Gesim	Nano-Plotter NP1.2	Non-contact, piezo print head
Perkin-Elmer	Spot Array 24	Contact, solid or quill pins
Perkin-Elmer	BioChip Arrayer	Non-contact, piezo print head
Schleicher & Schuell	MicroCaster	Contact, solid or quill pins
Telechem	SpotBot Personal Microarrayer	Contact, solid pins

proach is very time-consuming and typically entails manual interpretation of product ion data and, if necessary, sample derivatisation with further analyses to influence and allow rationalisation of the product ion data. [35].

In contrast to the classical proteome analysis, which mainly applies 2D-gel electrophoresis and MS to describe each measured protein independently, the functional proteomics approach focuses on the description of the cellular network and therefore requires high-throughput tools to elucidate interactions between proteins. The most promising tools to fulfil those requirements, as well as some of the classical proteome analysis, are today protein and antibody microarrays. Those consist of a large number of regularly arranged discrete spots of protein interacting elements, which are spotted on a solid support using spotting robots (Table 1, Fig. 1).

2. Classes of binders for microarrays

Basically, two formats of microarrays can be described. First, the protein chip that consists of a large diversity of proteins or the antibody chip, which has a collection of specific binder molecules, immobilised. The former can be used to screen for immune responses directed against the immobilised proteins or small peptides containing the epitopes, or for the screening of protein–enzyme interactions or protein-binder, such as DNA, RNA, proteins or

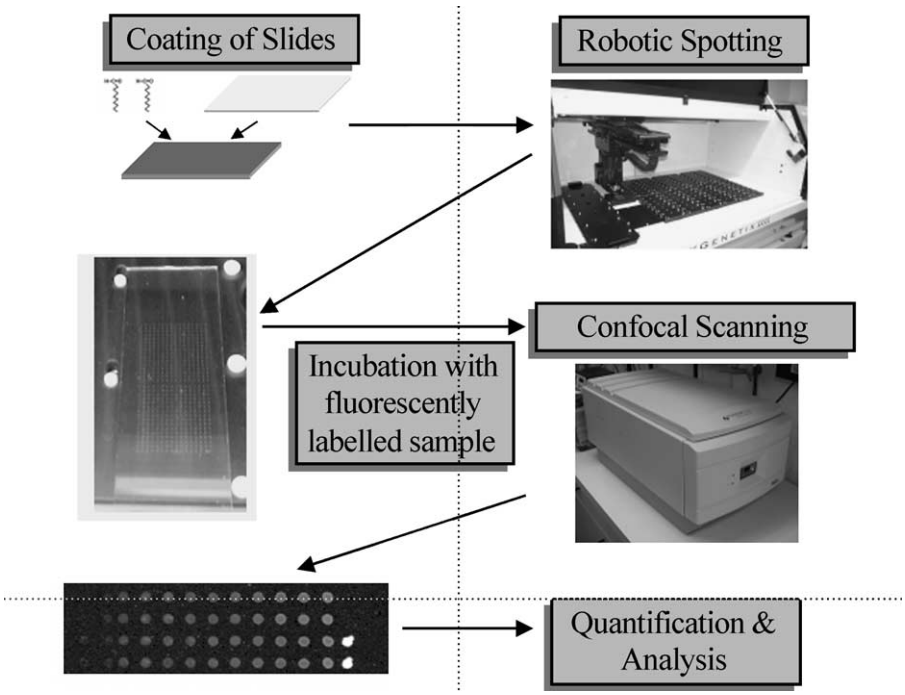

Fig. 1. Production of protein and antibody microarrays. Microarrays are generated and processed in several steps. First, either chemical groups or a gel are attached to the microscope slide to accommodate the binder. Then the binders are applied to the coated microscope slide by robotic spotting and the chip is processed. After processing the microarray is incubated with fluorescently labelled sample and the binding events are detected by confocal scanning in a laser scanner. The resulting signals are quantified and the analysis is performed.

small molecules. The latter is designed to detect specific components of complex samples and may have antibodies, antibody fragments, engineered binding proteins or even aptamers immobilised on the surface.

The state of functionality of immobilised proteins determines the usefulness of protein arrays for the appropriate applications. Protein arrays for the purpose of immunoprofiling of patient sera will not have to present native proteins and allow a simple detection by a labelled secondary species-specific antibody [36]. Furthermore, domains that readily bind to unstructured sequences present in peptides or unfolded proteins, such as PDZ, EF-hand (like calmodulin), SH3, and WW can also be applied for screening of protein or peptide arrays. However, most protein–protein interactions require a more cooperative contact from both binding partners. In this case the immobilised proteins will have to be presented in an active state.

Similar to protein arrays, nativity is also an important prerequisite for antibody arrays. Since antibodies can be considered as the active binding partner, these must retain their specific binding properties upon immobilisation. Therefore, protocols for the immobilisation, storage and assays need to be optimised. At the same time different surfaces have to be evaluated to accommodate the antibodies for the assay.

3. Sources of antibodies and proteins

Another important issue is the source of antibodies to be used on antibody microarrays. So far, monoclonal antibodies have become commercially available that are directed against thousands of different antigens. However, the costs associated with hybridoma technology and production are too high to create antibody microarrays of great diversities. Moreover the antibodies have to be purified and BSA-free to be immobilised on microarray surfaces. Therefore, phage display has become increasingly important to address this problem. Antibody fragments can be selected and produced using inexpensive media and purification methods for *E. coli* [38,39]. To further increase the properties of selected antibodies, new strategies completely working in vitro have been developed using ribosomal display [40,41] or mRNA–protein fusions [42]. At the same time other binding scaffolds than immunoglobulin domains like antibodies [43], fibronectin [44], lipocalin [45] or repeat domains [46] are explored to meet the requirements associated with antibody array technology. Finally, oligonucleotides can be selected to bind certain antigens [47]. These so-called aptamers are especially interesting because of the low production costs and the use of materials established for DNA array technology.

At the same time, proteins are also very efficiently handled and produced by cDNA expression libraries using *E. coli*. Therefore, protein expression and purification methods were developed that meet the requirements of protein and antibody microarray technology.

The discovery of immobilised metal affinity chromatography (IMAC) purification technology together with the engineering of recombinant affinity tags has led to a widely applied technology for high-throughput purification. Hochuli et al. [48] have introduced histidine affinity handles fused to either N- or C-terminus of recombinant proteins and combined these with Ni(II)-nitrilotriacetic acid (Ni-NTA) affinity media. The addition of short affinity handles like six histidines (His6) does not severely change the properties of recombinant proteins and increases the molecular weight by less than 1 kDa. Both Ni-NTA as well as the alternative Co(II)-carboxymethylated aspartic acid (TALON^{TM}, Clontech) tetradentate complex show a selective affinity towards histidine stretches and are stable enough for protein purification under strong denaturing conditions [49,50]. This is an important feature, since many eukaryotic proteins form inclusion bodies upon expression in *E. coli*, and solubilisation by denaturing chaotropes like guanidinium or urea is required for the purification. Several attempts to use IMAC for the refolding of proteins directly on the affinity resin have been successful, too [51,52]. Additionally, the inexpensive materials, the purity after a single step, and the high scalability has made the use of IMAC purification the standard procedure for high-throughput protein purification in the context of expression libraries [53].

Alternative affinity fusions have been introduced, like the strep-tagII [54], calmodulin-binding peptide [55], chitin-binding domain [56], glutathione-*S*-transferase [57], maltose-binding protein [58], and thioredoxin [59]. The latter three are used to increase solubility of protein fusions under native conditions. Some of these have been characterised more recently by Braun et al. [60] in context with high-throughput purification.

Further hosts for high-throughput protein expression like *Pichia pastoris* [61] and *Saccharomyces cerevisiae* [62] have been tested. The main advantage is a better expression yield of soluble proteins than *E. coli*. However, handling of yeast expression libraries and purification is still more sumptuous in comparison to *E. coli*.

To obtain proteins, that are toxic to their expression hosts, strategies involving in vitro transcription and translation systems have been developed [63]. Such a system would be completely independent of any host organism, needing only a cDNA with a T7 promoter as a template, which in turn can be propagated by PCR. This would allow the protein synthesis to occur on the microchips directly, relying on well-established DNA microarray technology [64].

Another generation of samples for protein microarrays works by the chromatographic separation of a crude mixture like cell culture extracts [26]. The protein extract is first separated and fractionated by anion exchange, and then by reverse phase liquid chromatography to achieve a high resolution of the proteins to be immobilised on the microarray. The special advantages of such protein samples are the correct post-translational modifications allowing further properties to be assayed in comparison to the recombinant sources.

4. Microarray formats

Today many different chip formats to study the proteome have been developed. These include macro- and microarrays, which were spotted on filters and glass slides, chips bearing microwells, microfluidic and liquid-chips, as well as alternative formats, such as SELDI and SPR.

4.1. Macro- and microarrays

Early developments of the protein microarray technology included the generation of low-density protein arrays on nitrocellulose filter membranes, such as the universal protein array system (UPA) [65]. This concept, which is based on the 96-well microtitre plate format, allows the analysis of specific protein interactions with proteins, DNA, RNA, ligands and other small chemicals. A miniaturisation of this technique was shown by Lueking et al. [66], who spotted lysates from 92 cDNA in a density of 600 spots/cm^2 and screened this array of proteins by specific antibodies. Mendoza et al. [67] employed another method of generating protein microarrays and displayed its usefulness for multiplex analysis. The proteins were printed on a glass plate containing 96 hydrophobic Teflon masks, each with a capacity of 144 elements per mask. The spotted antigens were detected by standard ELISA techniques using a CCD detector for imaging of the arrayed antigens. De Wildt et al. [68] generated one of the first high-density microarrays comprising 18,342 clones, which were screened for binders. Since this effort was not practical to be done by commercially available antibodies, they used scFv-fragments from existing libraries. The breakthrough in terms of surfaces was achieved by MacBeath and Schreiber [69], who showed the practicability of spotting proteins and antibodies onto glass slides, that were functionalised with aldehyde groups. A widespread investigation on poly-L-lysine coated microarrays was done by Haab et al. [70], who compared the performance of 115 antibody–antigen pairs. Although both types of surface chemistries proved their practicability, the provision of an ideal surface chemistry is still an issue of current research (Table 2). Angenendt et al. [71] tested eleven different surface chemistries, comprising plastic slides, derivatised glass slides and gel coated slides [72] for their use in antibody microarray technology. Based on these findings, second and third generation supports were developed, which have been recently compared in a second study [73]. Besides the provision of an optimised surface, several production parameters for the generation of antibody microarrays were evaluated by Kusnezow et al. [74]. Those included the kind and length of cross-linkers, the composition and pH of

Table 2
Table listing a collection of slide surfaces and their respective evaluation studies

Name	Surface chemistry	Supplier	Reference
Prototypes			
Dendrimer slides	Dendrimer layer with reactive epoxy groups	Chimera Biotech GmbH, Dortmund, Germany (http://www.chimera-biotec.de)	[73]
PEG-epoxy slides	PEG layer with reactive epoxy groups	Jens Sobek, Functional Genomics Centre Zurich, Zurich, Switzerland/Microsynth GmbH, Schützenstrasse 15, 9436 Balgach, Switzerland (jens.sobek@fgcz.unizh.ch)	[73]
MaxiSorb slides (transparent, black)	Polystyrene-based modified surface	Nunc A/S, Roskilde, Denmark (http://www.nalgenunc.com)	[37]
Commercially available			
Amine slides	Amine groups (extended chain length silane)	Telechem International Inc., Sunnyvale, CA, USA (http://www.arrayit.com)	[37,73,74]
Epoxy slides	Epoxy groups		
Silanated slides	Amine groups		
Aldehyde	Reactive aldehyde groups		
FAST^{TM}-slides	Nitrocellulose-based matrix	Schleicher & Schuell Biosciences Inc., Keene, NH, USA (http://www.schleicher-schuell.com)	[73,74]
HydroGel	Modified polyacrylamide gel	Perkin-Elmer, Meriden, CT, USA (http://www.packardbioscience.com)	[37]
Reflective microarray	3-Amino propyl triethoxysilane	Amersham Biosciences, Amersham, UK (http://www.apbiotech.com)	[37]
Polystyrene cell culture slide	Polystyrene	Nalge Nunc International, Naperville, IL, USA (http://www.nalgenunc.com)	[73]
Aminosilane slides	Amine groups	Sigma–Aldrich Chemie GmbH, Munich, Germany, Corning Inc., Corning, NY, USA	[74]
QMT epoxy slides	Epoxy groups	Quantifoil Micro Tools GmbH, Jena, Germany	[74]
Manually prepared slides			
Poly-L-lysine slides	Amine groups		[37,73,74]
Polyacrylamide slides	Polyacrylamide		[37]

the spotting buffer, the kind of blocking reagents as well as the antibody concentration and storage procedures.

4.2. Microwell arrays

Besides these flat microarrays, a new technology is emerging, which applies microwells. Zhu et al. [75] employed arrays of microwells to study protein kinases. The microwells, 1.4 mm in diameter and a 300 μm in depth, could carry up to 300 nl and were fabricated in a disposable silicon elastomer, poly(dimethylsiloxane) (PDMS) [76]. Microwell arrays of much small diameters of 2.5 and 5 μm were fabricated by Biran and Walt [77]. They prepared an array of microwells from etched imaging fibres and were able to accommodate and detect single living cells carrying reporter genes or fluorescent indicators. In another approach, Bernhard et al. [78] fabricated microwell arrays on the distal face of coherent fibre-optic bundles and applied them as pH-sensitive or O_2-sensitive microwell array sensors. A typical microwell was 1–14 μm deep, approximately 22 μm wide and filled partially with a 2–10 μm thick chemical sensing layer, consisting of a polymer and a dye.

4.3. Microfluidic chips

An advanced format of the microwell chips are the microfluidic chips, which consist of microwells that are connected by tubing and allow liquid handling in a miniaturised format. Cohen et al. [79] fabricated a microchip-based enzyme assay for protein kinase A by standard photolithographic techniques. Using electroosmosis for the transport of reagents within the network of etched channels, they were able to monitor the phosphorylation of the kemptide peptide by protein kinase A. Moreover they could prepare on-chip dilutions of the reagents and measure kinetic constants of the reactions. Another enzymatic assay applying β-galactosidase and resofurin was performed by Hadd et al. [80]. Using electrokinetic flow, precise concentrations of all reagents could be mixed, and Michaelis–Menten constants could be derived with and without an inhibitor added. Although the technology is in an early stage, products using microfluidic devices are already on the market. Agilent Technologies (Palo Alto, CA, USA) offers Lab-on-a-Chip products for the analysis of DNA, RNA, proteins and cells, which are based on chips with interconnected fluid reservoirs and pathways. For cell assays, movement of cells on the chip is controlled by a pressure-driven flow, while for the analysis of RNA, DNA and proteins electrokinetic forces are applied.

4.4. Alternative formats

Besides microarrays, microwell and microfluidic chips some alternative formats have been evolved. One of them is the surface-enhanced laser/desorption ionisation (SELDI) technology, which was introduced by Hutchens and Yip in 1993 [81]. The technology combines purification of the sample by surface-enhanced affinity capture on a wide variety of affinity matrices and the identification by MS–TOF. Another technology is using surface plasmon resonance (SPR), which is a technology based on the measurement of the mass concentration of biomolecules [82]. The so-called Biacore consist of an activated gold chip onto which an interacting biomolecule is attached and a flow cell, which applies a continuous flow to the chip surface. By introduction of the sample to the flow cell, the sample flows over the interacting biomolecule attached on the gold surface and causes a change in the local concentration upon binding, which can be measured by SPR. Finally, a third alternative format, the bead-based LiquiChip has recently been introduced by Qiagen GmbH (Hilden, Germany). The technology involves the interaction of immobilised, bead-bound capture molecules with a reaction partner in solution and the subsequent detection by a reporter molecule that is specific for the analyte. For detection, two lasers are applied, one for the identification of a classification-code fluorescence on the bead and one for the identification of the reporter fluorescence. This allows the use of the LiquiChip system for multiplex assays applying bead sets with different classification-code fluorescences. More recently, our group has developed a 3D-protein chip that allows multiplexed screening on a classical microchip surface by introducing a multiple spotting technique (MIST) [83]. This technology combines advantages of microwell and microfluidic systems with the ease of handling of common protein microarrays in the slide format by immobilisation of a binder onto a surface and subsequent spotting of the second compound on the same spot, on top of the immobilised binder. Using this technique we were able to detect specific antigen–antibody interactions down to 400 zmol of analyte in a multiplex way, without the need of extra incubation time.

4.5. Microarray versus conventional separation procedures

Protein and antibody microarrays provide a complementary approach to conventional separation technologies used in proteomics. Those conventional separation technologies comprise chromatography, such as affinity chromatography as well as electrophoretic methods, such as isoelectric focussing or gel electrophoresis. For separation of complex mixtures multi-dimensional separation techniques, such as 2D-gel electrophoresis are applied to ensure successful separation [84].

Both microarrays and conventional separation techniques display distinctive advantages depending on the area of application. While conventional separation techniques allow separation of uncharacterised complex mixtures according to chemical, physical or biological properties, protein microarrays provide information only after the selection of specific antibodies or proteins for immobilisation. However, conventional separation techniques either lack the ability to quantify low concentration compounds, e.g. 2D-gel

electrophoresis [17] or are not amenable to detect biological interactions in a high-throughput fashion, e.g. affinity chromatography.

Although microarrays rely on the same principle as affinity chromatography, they have significant advantages compared to common separation technologies. Although they do only allow characterisation of complex fluids with regard to special molecules that are determined by the selection of the immobilised binders, they require only relatively low amounts of sample compared to affinity chromatography. Moreover they are able to detect even zeptomoles of compounds [83], do not require fractionation steps prior to application and are amenable to high-throughput screening.

5. Areas of application

Protein and antibody microarray are applied in a several areas of applications, mostly in diagnostics, basic proteome research and in the development of therapeutics.

5.1. Diagnostics

In diagnostics, protein and antibody microarrays are applied for the detection of antigens and antibodies in blood samples as well as in the profiling of sera to discover new disease markers. Although it is too early to apply protein and antibody microarrays outside the laboratory, interesting proof-of-principle experiments have been made, to display the potential of this technique for example in serum profiling. Joos et al. [85] generated protein microarrays by immobilisation of 18 known autoantigens, which are commonly used as biological markers for autoimmune diseases, such as autoimmuno thyroiditis or Sjögren's syndrome. The screening of 25 sera from autoimmune patients revealed specific and very sensitive detection of autoantibodies and showed that down to 40 fg of a known protein standard could be detected with little or no cross-reactivity to non-specific proteins. In a larger approach Robinson et al. [36] fabricated arrays containing 196 distinct biomolecules, comprising proteins, peptides, enzyme complexes, ribonucleoprotein complexes, DNA and post-translationally modified antigens. By this, they were able to characterise sera from eight human autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis. In the area of cancer research, Sreekumar et al. [86] prepared one of the currently largest antibody microarray carrying 146 distinct antibodies. With this antibody microarray, they were able to monitor alterations of protein levels in LoVo colon carcinoma cells that were treated with ionizing radiation. Differential expression profiles with radiation-induced up-regulation of apoptotic regulators including p53, DNA fragmentation factor 40 and 45 as well as tumour necrosis factor-related ligand were observed. Hiller et al. [87] applied microarray technology to develop a miniaturised allergy test containing 94 purified allergen molecules from a variety of common allergy sources, such as plants, animals, fungi, and insects as well as a variety of foods. Screening with 20 patient sera revealed comparable results to techniques commonly applied in various established IgE detection systems. In another study with a similar focus, Kim et al. [88] used purified dermatophagoides pteronyssinus (Dp), egg white, milk, soybean and wheat as allergens to measure Dp-specific IgE levels in sera.

Besides the profiling of sera and blood, a novel area to apply microarray science in diagnostics is emerging: the use of arrays in the monitoring of the environment and food. Although no high-density chips have been applied so far, Samsonova et al. [89] employed the Biacore sensor to measure the antiparasitic agent ivermectin and were able to detect down to 19.1 ng/g in bovine liver.

5.2. Proteomics

Although first experiments were made to apply protein and antibody arrays in diagnostics, the major application area is basic proteome research. Even though the early stage of this technology and the lack of mature products on the markets cause both areas to overlap to a great extent, the focus of basic proteome research is rather target identification and discovery, functional analysis of proteins and cellular expression profiling than characterisation. However, the close relationship between both areas is displayed in the study of Huang et al. [90], describing the production of an antibody array for the simultaneous assay of 24 cytokines from two different sources: cultured media or patient sera. The system was based on the standard sandwich ELISA technology and chemiluminescence was applied for detection.

An interesting approach to create organ and disease-specific microarrays for the use in basic proteome research as well as in diagnostics was performed by Paweletz et al. [91]. They generated a reverse phase protein array by immobilisation of the whole repertoire of patient proteins and were able to quantify the phosphorylated status of signal proteins. They were able to monitor cancer progression from histologically normal prostate epithelium to prostate intraepithelial neoplasia (PIN) and invasive prostate cancer by increased phosphorylation of Akt, the suppression of apoptosis pathways, and the decreased phosphorylation of ERK. A specific antibody microarray for the squamous cell carcinoma of the oral cavity was generated by Knezevic et al. [92], utilising laser capture to gain total protein from specific microscopic cellular populations. Using this approach, they were able to correlate differential expression of stromal cells adjacent to the diseased epithelium to tumour progression.

First methodologies for the study of membrane proteins were developed by Fang et al. [93], which generated membrane protein arrays for the analysis of the ligand-binding properties of receptors. The microarrays consisted of an array of G protein-coupled receptors (GPCRs) and were capable to demonstrate the specific binding of their respective targets. Moreover, the subtype-specific detection of a cognate antagonist analogue specific for β-adrenergic receptors was

displayed using an array with different subtypes of adrenergic receptors. Since important members of the signalling pathways are membrane located, the systematic analysis of membrane proteins provides a valuable task for proteomics.

Additionally, efforts were made to monitor enzymatic reactions on-chip. As a first step, kinase activity was measured quantitatively on a peptide chip [94]. Houseman et al. applied surface plasmon resonance, fluorescence, and phosphorimaging for the detection of the phosphorylation and evaluated three inhibitors quantitatively. Zhu et al. [75] used protein chips bearing microwells to analyse nearly all of the protein kinases from *S. cerevisiae*. Many novel activities were found as well as 27 protein kinases with an unexpected tyrosine kinase activity. The analysis of protein interactions and enzymatic activities were both performed on microarrays as well. The same group investigated protein–protein interaction in large scale by the production of the first proteome chip [95]. For the generation of the chip, the 5800 open reading frames of yeast were cloned and the corresponding proteins were overexpressed in *E. coli*, purified and spotted. The resulting protein microarray was used to screen for interactions with calmodulin and phospholipids and allowed the identification of binding motifs.

Similar to the screening for protein–protein interactions, microarrays were used for the detection of protein–antibody interactions. Holt et al. [96] used a large protein microarray consisting of 27648 human fetal brain proteins immobilised on a PVDF membrane to screen 12 antibody fragments for specificity. Four highly specific antibody–antigen pairs were identified, including three antibodies that bound proteins of unknown function. The first plant protein microarray was generated by Kersten et al. [97], who immobilised 95 recombinant proteins from *Arabidopsis thaliana* on slides coated with a nitrocellulose-based polymer (FAST^{TM}-slides) or polyacrylamide. Both types of surfaces were then used for the characterisation of antibody specificity.

There are great chances for protein and antibody microarrays to acquire a third major area of application alongside proteome research and diagnostics, which is therapeutics.

5.3. Therapeutics

Although no real applications of arrays in this area have been shown, the development of new therapeutics requires high-throughput tools for target validation, pre-clinical and clinical trials as well as for the screening of compounds and capture agents for desired drug activity, specificity, and selectivity. However, further efforts have to be made to ensure reliability of protein and antibody microarrays, to meet the special demands of clinical trials.

6. Detection technologies

Detection of molecular interactions can be achieved either directly using labelled binding molecules or indirectly without modification of the binder (Fig. 2). Direct labelling can mainly be performed in two ways: radioactively, using ^{125}I or ^{3}H or fluorescently using Cyanine, Alexa or Oyster dyes. Although radioactive labelling is one of the most sensitive labelling procedures, it has become a trend over the last decades to sequentially replace radioactive labelling with other detection methods. Reasons are mainly the risks of radioactive contamination, problems associated with proper

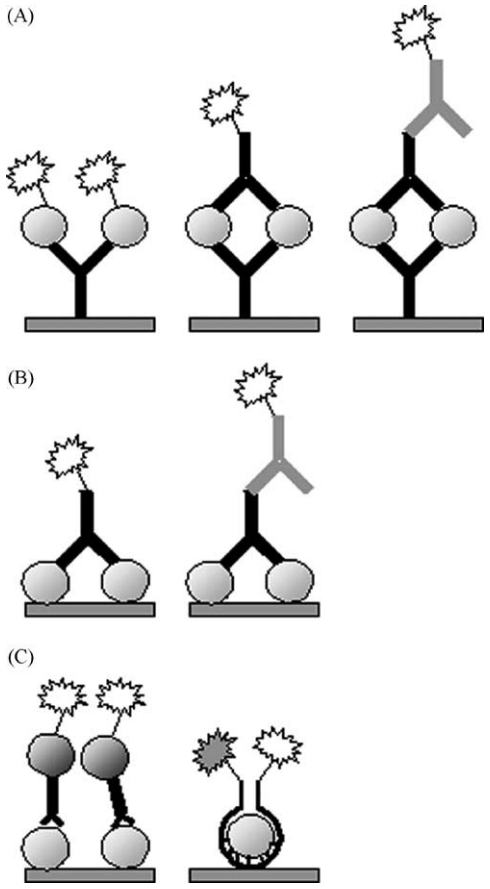

Fig. 2. Detection strategies. Binding events can be detected in several ways, depending on the scope of the experiment and the samples. (A) For antibody microarrays mainly three different strategies can be applied. The direct labelling (left) attaches the fluorescent marker directly to the protein of interest, while both indirect labelling strategies apply separate antibodies for detection. This can be done by direct labelling of the primary antibody (middle) or by the sequential incubation with an unlabelled primary antibody and with a labelled secondary antibody, which is host specific for the primary antibody. (B) For protein microarrays, the sample can be labelled directly (left) or an indirect detection can be performed by a labelled secondary antibody, that is host specific for the antibody from which the sample is applied (right). (C) Besides traditional protein and antibody microarrays new formats can be applied. These include the immobilisation of the detection of proteins by the sequential incubation with specific scFv-fragments, followed by the incubation with labelled detector molecule, such as protein L (left) as well as the immobilisation of aptamers to specifically bind proteins from the sample (right). The detection can be performed indirectly, without the labelling of the sample, by attachment of two fluorescent dyes suitable for FRET or quenching. The binding event causes the proximity of both dyes and a change in fluorescence can be detected.

waste disposal, and most of all in the area of microarrays the incompatibility with high-throughput screening.

More recently, fluorescent dyes have become the method of choice for labelling and detection of molecules in the microarray format due to the development of bright and pH stable dyes with narrow emission and excitation spectra. Commonly used scanners allow the application of up to four fluorophores simultaneously and permit the direct comparison and relative quantification of four different samples. Further improvements to the fluorescent detection in terms of sensitivity has been made by the introduction of rolling circle amplification (RCA) [98], and by the recent development of quantum dots with superior photostability compared to organic dyes [99].

Indirect detection of molecular interactions without labelling has the advantage not to interfere with the interaction as changing the properties of the analyte by labelling. Commonly used biomolecules for indirect labelling include species-specific labelled secondary antibodies for the detection of primary antibodies or labelled protein L for the detection of scFvs. It is also possible to employ a sandwich assay by having one specific antibody immobilised and add another specific antibody in solution to capture and detect the antigen. However, this approach limits the diversity of such antibody microarrays as a multitude of detection antibodies in solution cause an increasing background [100].

The most promising development is the introduction of aptamers, consisting of oligonucleotides as binding molecules. Since the aptamers are non-proteinaceous, a protein-specific dye can be used to cross-link and stain the bound protein after the incubation [101]. Another possibility that does not involve the previous staining of the analyte involves the binding detection via molecular aptamer beacons. The binding event can be detected either by direct quenching between fluorophore and quencher, or by fluorescence resonance energy transfer (FRET) between donor and acceptor [102,103]. As the aptamers undergo structural changes upon ligand binding, the ends of the oligonucleotide change their relative position. Two different dyes affixed to each end may change their relative position either increasing or decreasing fluorescence. Another method for the direct and real-time detection of biomolecule interaction is surface plasmon resonance. Although this technique was originally invented to evaluate the binding coefficients of a given interaction, advances were made to increase the number of simultaneously detected nucleic acid hybridisations with SPR [104]. Nevertheless, it remains to be shown that this technology can be adapted to the scale already employed in conventional microarray technology.

Similar problems are encountered by matrix-assisted laser desorption and ionisation. Small arrays can be analysed using mass spectrometry [105]. Analysis of samples is still performed sequentially and cannot be done in parallel. Therefore, the complexity of an array determines the time of evaluation. At the same time the computational power needed for the identification still exceeds the time required for the measurement itself.

7. Current limitations

7.1. Source of proteins and antibodies

A major problem of antibody microarrays is not to acquire great quantities, but a large diversity of antibodies. Although there are many monoclonal and polyclonal antibodies commercially available, it is not feasible to buy several hundreds of these to produce an antibody microarray, not only due to lacking functionality on-chip, but mainly due to the costs. Similar is true for protein microarray technology, in which, despite the great efforts to develop automated protein expression and purification facilities, the provision of a large set of proteins still represents one of the most challenging tasks. To solve this problem for antibody microarrays, several attempts have been made to replace expensive antibody production in hybridomas by the production of scFvs of Fabs by phage display [106] or ribosomal display [41]. Other solutions include the application of aptamers from nucleic acid libraries [107] for the specific detection of proteins.

7.2. Antibody performance on microarrays

Previous studies have shown that antibodies display a widely varying performance on microarrays, with no activity, decreased specificity or a lowered affinity [70,71]. Although antibody performance can be increased by optimising the surface and applying indirect immobilisation strategies [74,108], it would be advantageous, if the suppliers of antibodies could include information regarding the suitability of their product for immobilisation on microarrays. This could be done in a similar manner as currently provided for the application in immunoblotting, indirect ELISA or dot blot.

The provision of technical solutions for lacking functionality is also a prerequisite for the application of scFvs derived from phage display libraries, since they are prone to unfold upon immobilisation. Since Fab fragments are often found to be more stable than scFv [109], it remains to be demonstrated that these are more amenable for microarray technology.

7.3. Labelling of proteins for antibody microarrays

The labelling of proteins is still a bottleneck of antibody microarray technology, although many techniques for the direct and indirect labelling of protein mixtures are available. Reasons for this are not so much the absence of applicable coupling chemistries, but the diversity and differing quantities of available amino acids, that act as targets of those coupling chemistries. This diversity prevents direct coupling methods from homogeneous labelling of complex protein mixtures in defined stoichiometry, which does not allow

absolute quantification of proteins in complex samples. Solutions for this are only available for the measurement of recombinantly produced proteins, which can be engineered to allow indirect labelling by affinity tags or fusions with fluorescent reporter molecules [110]. However, both approaches cause changes in structure and morphology, which hold the risk of changing the binding properties of the protein. Therefore, the development of label-free detection methods that are amenable to high-throughput is essential.

7.4. Surfaces and hardware

Since most of the hardware equipment was adopted from DNA microarray science, such as the microscope slide format and its surface chemistry, fluorescent detection, the spotting devices and scanners, many of these will have to be optimised to meet the different requirements of antibody and protein microarrays. Those requirements include the development of a surface that avoids the denaturing contact with the surface by the introduction of linkers or activated layers. Another important feature is the provision of an environment, which prevents dehydration of immobilised proteins and antibodies. One solution may be the introduction of microwells, which reduce evaporation. The same holds true for the microfluidic chips, which prevent dehydration due to their closed architecture. However, both formats would necessitate the alignment of the handling robots with the surface grid or the provision of additional hardware for control of the system.

8. Conclusions

Although protein and antibody microarray technology is still in its early stages, rapid progress has been made. Up to today, proof-of-principle experiments as well as large scale investigations have successfully applied microarrays with a diversity ranging from a few to several ten thousands different binders. However, it will be interesting to see whether microarray technology will be able to solve current deficiencies by the incorporation of existing methodologies. Most important issues are still detection of interactions and the stability of immobilised proteins. Nevertheless, current progress indicates the potential of protein and antibody microarray technology on research and calls for a close watch on future developments.

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