Multiplex approaches in protein microarray technology

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The success of genome sequencing projects has provided the basis for systematic analysis of protein function and has led to a shift from the description of single molecules to the characterization of complex samples. Such a task would not be possible without the provision of appropriate high-throughput technologies, such as protein microarray technology. In addition, the increasing number of samples necessitates the adaptation of such technologies to a multiplex format. This review will discuss protein microarray technology in the context of multiplex analysis and highlight its current prospects and limitations.

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The sequencing of the human genome has brought about a strong interest in analyzing the function of DNA-encoded information. Since a genome can express various proteomes according to localization, differentiation and development, a direct elucidation of gene function and expression rate from the DNA sequence alone is not possible. Additionally, proteomes are modified in response to biotic and abiotic environmental changes and only a fraction of genes are activated in a specific cell type at a certain time point.

In striking contrast to the estimated 22,221 genes [1], the complexity of the human proteome is expected to range from 100,000 to several million protein molecules [2]. This disparity is mainly due to post-transcriptional control of protein translations [3] and the over 200 possible post-translational modifications of proteins that exist [4]. All this shows that protein expression, folding and modification, rather than merely the genetic layout, determine the cell's phenotype. Therefore, the systematic analysis of proteins and their interaction partners by proteomic approaches will significantly contribute to our understanding of cell function and the identification of novel drug targets for therapeutic intervention [5].

Protein microarrays have become an important high-throughput tool in this field because they allow parallel, fast and easy analysis of thousands of addressable immobilized proteins for expression, modification and molecular interaction [6–9]. In several applications, the analysis of two or more parameters of immobilized proteins by multiplexing approaches is of increasing importance [10]. To define disease markers, not only the expression and amount of a protein may be of specific interest, but also its modifications; for example, determination of the phosphorylation state of tau proteins in cerebrospinal fluids may significantly improve early and differential diagnosis of Alzheimer's disease, in addition to established biologic markers such as the measurement of total tau protein concentration [11].

Currently, several multiplexing techniques have been introduced that allow analysis of different parameters or different samples in parallel. This review will discuss protein microarray technology in the context of multiplex analysis and highlight its current prospects and limitations.

Generation of protein microarrays & detection methods

Capture molecules

Protein arrays consist of a large number of regularly arranged discrete spots of capture molecules, which are transferred on a solid support using spotting robots [6]. Depending on the application, the following different capture

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molecules are currently used: purified recombinant proteins [12], antibodies [13], antibody fragments [14], antibody mimics (e.g., aptamers) [15–17], peptides [18,19] or complex protein extracts [20]. The diversity of a microarray is thereby mainly limited by the accessibility of large numbers of capture molecules and the availability of technologies for their generation and purification. Another important issue is also the functionality of the spotted molecules. While antibodies are relatively stable, membrane proteins, for example, tend to unfold and lose their functionality upon immobilization and storage of the arrays. It is therefore advisable to transfer the content to the chip in a stable format. Ramachandran and coworkers printed complementary DNAs (cDNAs), which are more stable than proteins, onto glass slides [21]. Proteins were generated just prior to the use of the arrays by cell-free transcription and translation *in situ*. The results of protein–protein interaction studies performed with these microarrays indicated that the synthesized proteins were in a functional form. It can be expected that additional methodologies applying this concept will be developed soon.

Surfaces of protein microarrays

One crucial factor in the generation of protein microarrays is the choice of the surface coating used for immobilization of the proteins. Such a surface must obey several requirements, which include the provision of optimal binding conditions and an environment that promotes functionality of the immobilized substances. While in previous years, polyvinylidene fluoride (PVDF) membranes were used for protein macro- [22,23] and microarrays [24], the demand for even higher densities, as well as the need for decreased sample consumption and quantification, led to the application of glass slides as solid supports for microarrays. The surface of the slides is thereby activated to allow attachment of the proteins in a covalent or noncovalent fashion with a maximum binding capacity. Surfaces that allow noncovalent attachment can be positively charged (e.g., poly-L-lysine and aminosilane), hydrophobic membranes (e.g., nitrocellulose), hydrophilic polyacrylamide or agarose gels. Covalent attachment applies a variety of chemically activated surfaces (e.g., aldehyde, epoxy and active esters) that are highly reactive to amino, thiol and hydroxyl groups of proteins.

Beside their mode of attachment, surface chemistries can also be differentiated according to their structure. Plain (non-gel coated) microarrays display different functional groups, such as poly-L-lysine [25,26], aldehyde [12] or epoxy moieties [27], while 3D microarrays have a gel-coated surface, such as polyacrylamide [28–30] or agarose [31]. This type of microarray also includes FAST™ (fluorescence array surface technology slide) slides from Schleicher & Schuell, which are coated with a nitrocellulose-derived polymer [27,29,32–34]. A recent addition to 3D slides are hydrogel slides composed of glycosylated amino acetate, which combine hydrophilic and hydrophobic groups and were used to monitor enzymatic activities on the chip [35]. 3D microarrays have a higher immobilization capacity and the homogenous water environment minimizes protein denaturation. This could have a positive influence on the active state of the proteins. Besides plain and 3D slide surfaces, there are also slide coatings that cannot easily be classified in either group, such as the polymeric coating introduced by Cretic and colleagues [36], since they neither display a plain functionalized glass surface nor a 3D gel. Comparisons of the different surface coatings with regard to their suitability in protein and antibody microarray technology have been performed and highlight optimal coatings for each type of application [7,27,30,37].

All the above-mentioned surfaces lead to a nonorientated (i.e., random) attachment of immobilized proteins. However, an investigation into the effects of orientation of antibodies and Fab antibody fragments, performed by Peluso and colleagues [38], points towards an increased binding capacity of slide surfaces, which promotes oriented immobilization. The capture moieties that lead to oriented immobilization can be manifold and include Ni-NTA [39,40], nucleic acids [41], as well as biotin or streptavidin [42,43]. Nevertheless, this mode of immobilization either requires an intrinsic affinity tag, or a separate attachment step of the tag, which becomes cumbersome for large collections of proteins.

Spotting robots

Microarrays can be generated in many different ways. Currently, the technology for the production of protein microarrays is similar to DNA microarray technology [44]. However, optimization of the hardware is needed to generate functional protein arrays because proteins are less stable than DNA. Hence, they may lose their functional structure by mechanical forces exerted on the protein during spotting. For long spotting runs of high-content protein microarrays, the spotting robots have to be equipped with cooling capacities, which are not required for the generation of DNA microarrays.

Currently, different types of contact and noncontact arrayers are in use for the generation of protein microarrays (for an overview of commercially available arrayers, see [6,108]). Contact printing arrayers deliver subnanoliter sample volume directly to the surface using tiny pins with or without capillary slots [9]. These spotters are very robust and well suited to produce microarrays containing many different capture molecules. Disadvantages of contact printers are that they cannot align their pins to prefabricated slide structures, such as nanowells [45], and that they are not well suited to multiple component reactions, in which carry-over may be an issue [46]. Furthermore, mechanical forces during spotting may harm protein structure. Noncontact spotters use piezoelectric elements to transfer the proteins to the slide within nanoliter to picoliter droplets [9]. Since they do not touch the surface during spotting, their usage is not restricted by the surface structure. They enable the exact dosage of the spotted sample volume and are therefore more suitable for use in quantitative microarray applications than contact printers. Noncontact printers are not well suited for spotting many different capture molecules, since the spotting quality and robustness is very much dependent on the substance being spotted. Furthermore, shearing forces during drop formation may damage some samples [47].

Incubation & detection on protein microarrays

The methodology of incubation does not significantly differ between DNA and protein microarray technology. After spotting of the slide with the capture molecules, the remaining surface is blocked using a relatively inert protein and incubated with the sample. This step can be carried out either manually or in an automated fashion using hybridization stations, which are available for DNA and protein microarrays.

Labeling of the samples can be performed in two ways: the sample may be directly labeled for detection of captured sample molecules (direct labeling) or the slides may be incubated with the unlabeled sample followed by incubation with a labeled detection molecule (e.g., antibodies) that recognizes the captured sample molecules (indirect labeling). Antibodies are often used as detection molecules. While direct labeling is the most straightforward approach, it may alter the properties of the sample molecules. In contrast, indirect labeling methods rely on the generic binding properties of the sample being investigated. The binding properties are not affected, since incubation with the labeled detection molecule is performed after the binding of the sample to the immobilized capture molecules.

Labeling of the sample or detection molecules can be by fluorescence, radioactivity or chemiluminescence. The preferred method is labeling with fluorescence dyes (e.g., cyanine [Cy]3 and Cy5), which is easy to handle and extremely sensitive [13]. Detection of fluorescence labels is performed using a laser scanner with a resolution in the micrometer range. Although radioactive labeling is one of the most sensitive detection methods, it is not widely used and is restricted to modification studies, such as substrate profiling of kinases [33,45]. The main problems are associated with proper waste disposal and the risks of radioactive contamination. Furthermore, there are only a few scanner systems that support a radioactive readout at the high spatial resolution required in microarray technology. The same limitation is true for the application of chemiluminescence. Although it has been applied as a readout for microarrays and intrinsically has several advantages, such as signal amplification capabilities, it is not widely used in microarray technology [48].

Beside chemiluminescence, other methods of signal amplification have been developed, such as rolling-circle amplification (RCA) [49]. In this case, the detection antibody is tagged with a DNA primer. The antibody–DNA conjugate binds specifically to the immobilized interaction partner and a circular DNA molecule hybridizes to the complementary primer, which is extended in the presence of DNA polymerase. The resulting RCA product is then detected by hybridization with multiple fluorescent, complementary, oligonucleotide probes. The fluorescence, which can be measured at each spot, is thus directly proportional to the protein concentration in the original sample on the microarray. This method has high sensitivity, a wide dynamic range and excellent spot-to-spot reproducibility. It has been applied for the detection of low-abundance proteins, such as immunoglobulin (Ig)E antibodies in sera [50].

Since labeling of molecules can affect their functionality, label-free detection schemes are favorable. Several such detection schemes have been utilized for microarray technology, such as real-time detection by surface plasmon resonance (SPR) [51], intrinsic time-resolved ultraviolet fluorescence [52], mass spectrometry [53], ellipsometry [54] and Kelvin nanoprobe detection [55]. Although advances with regard to throughput have been achieved for SPR and mass spectrometry [51,53,56], transfering these technologies to high-throughput applications remains difficult. Furthermore, these label-free detection methods such as mass spectrometry are orders of magnitudes less sensitive than, for example, fluorescence-based detection in a sandwich assay [57].

Abundance-based applications & multiplexing

Different abundance-based applications

Abundance-based microarrays are one type of protein microarray, in terms of application, according to a recent classification by LaBaer and Ramachandran [8]. These microarrays are mainly used for the translational profiling of crude protein samples in a semiquantitative or quantitative manner [13,58,59]. Their application in several studies revealed differences in protein composition between healthy and disease states leading to the identification of new disease markers [13,20,58,60–62]. Furthermore, abundance-based microarrays have been successfully applied to profile phosphorylation states of signaling proteins in signal transduction studies [58,63].

Two types of abundance-based microarrays have been described: capture microarrays (FIGURES 1A & 1B) and reverse-phase protein blots (FIGURE 1C) [8]. Capture arrays contain immobilized capture molecules (e.g., antibodies, antibody mimics and antigens) on the surface. They are probed with a complex protein sample (protein extract) to profile specific proteins [64]. The detection of captured proteins can be performed by direct labeling of the protein extract (FIGURE 1A) or by a subsequent incubation step with a labeled detection antibody that recognizes the captured proteins on the microarray (FIGURE 1B; sandwich immunoassay, reviewed in [65]). The second type of abundance-based microarrays, reverse-phase protein blots, are generated by spotting the complex protein samples themselves, which are then probed with labeled antibodies to profile specific proteins in these samples (FIGURE 1C) [8].

Advantages and disadvantages of all these types of abundance-based microarrays as well as their different applications have recently been described in detail [8].

Multiplexing in abundance-based applications

Multiplexing in abundance-based applications for the analysis of several samples or several parameters on the same chip is becoming increasingly important. This is due to increased sample numbers as well as the need for low sample consumption.

There are currently two main principles to perform multiplexing in abundance-based applications: the analysis of several samples on the same array by applying them to different compartments of the array (compartmentation strategy); and the

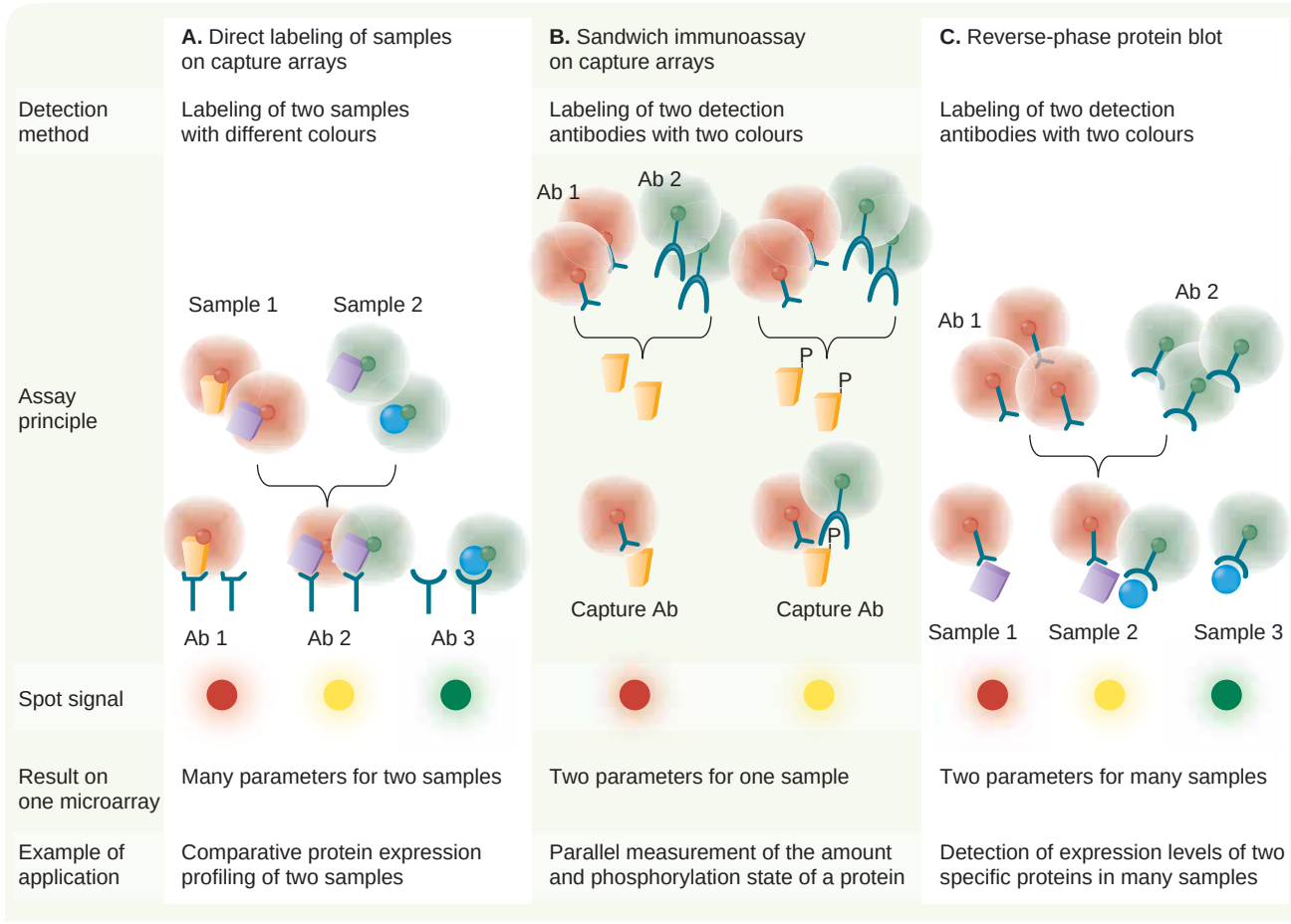

Figure 1. Multiplexing using the labeling strategy in different abundance-based microarray set-ups. (A) Capture arrays with direct labeling, (B) capture arrays with sandwich immunoassay and (C) reverse-phase protein blots. This classification is taken from LaBaer and Ramachandran [8].
Ab: Antibody.

incubation of the array with a mixture of different samples or of different detection molecules, respectively, which are labeled before application with fluorescent dyes of different colors (labeling strategy) (FIGURE 1A–C).

Compartmentation strategy

The first approaches for the development of compartmentation methodologies were described in 1999 by Mendoza and colleagues [66]. In their work, they applied biochips, which consisted of a flat glass plate with 96 wells formed by a hydrophobic Teflon mask. Each of the 96 wells contained four identical 36-element arrays (144 elements per well) comprising eight different antigens and a marker protein. By using a detection scheme that was based on the formation of a fluorescent precipitate, they could demonstrate the multiplexing capabilities through the analysis of several antigen–antibody interactions [66].

A similar approach was performed by Tam and colleagues for the detection of eight different cytokines involved in the change of T-cells from a naive phenotype to a T-helper (Th)1/Th2 phenotype [67] and by Nielsen and coworkers to quantify the amounts and modification states of ErbB receptors in crude cell lysates [58]. In the latter work, arrays of antibodies were printed on glass slides and a bottomless 96-well plate was then attached on top of the slide to apply different samples. Schweitzer and coworkers produced a 51-feature cytokine array and applied RCA to measure secretion of proteins from human dendritic cells induced by lipopolysaccharide or tumor necrosis factor-α [49]. Shao and colleagues extended this application to detect a total of 150 cytokines simultaneously in a sandwich immunoassay format [59]. Many proteins could be detected with a sensitivity in the pg/ml range. All the studies described above used the compartmentation strategy on capture microarrays for detection and quantification of proteins from extracts.

Commercially available tools for the multiplex analysis of different samples by the compartmentation strategy are incubation chambers distributed by several companies such as Whatman Schleicher & Schuell [101] or Grace Biolabs [102]. Masks can be attached to the slides and allow the generation of incubation chambers with two to 16 wells (Whatman Schleicher & Schuell) or 16 wells (Grace Biolabs) on top of the slide. In principle, this technique can be used for all types of abundance- and function-based applications.

Labeling strategy

FIGURE 1 provides an overview of the application of the second current multiplexing strategy, the labeling strategy (reviewed in [69]), for different types of abundance-based microarrays. Dual fluorescent-labeling strategies of complex mixtures on capture arrays are increasingly used to compare protein expression of two different cell states analogous to mRNA profiling on DNA microarrays (FIGURE 1A). Skreekumar and coworkers monitored alterations of protein levels in LoVo colon carcinoma cells after ionizing radiation using antibody arrays with 146 distinct antibodies [70]. They probed the arrays with protein extracts obtained from cell cultures prior as well as after irradiation and were able to detect the up- and downregulation of distinct proteins caused by irradiation. Miller and coworkers also used the direct Cy3/Cy5 labeling strategy on antibody arrays to perform differential profiling of prostate cancer biomarkers in serum samples [71]. The authors identified five proteins (von Willebrand Factor, IgM, α_1-antichymotrypsin, Villin and IgG) that had significantly different levels between the prostate cancer samples and the controls.

Dual labeling strategies have also been applied in sandwich immunoassays on capture arrays (FIGURE 1B). For example, in the study by Nielsen and coworkers, the amount and phosphorylation state of ErbB receptors in crude cell lysates were quantified in parallel in order to monitor receptor activation [58]. The receptors were captured on the microarrays using pan-specific capture antibodies (antibodies that bind to the receptor outside of the phosphorylation site) (FIGURE 1B). The arrays were then probed with a mixture of two detection antibodies, a Cy3-labeled phosphospecific antibody to measure the phosphorylation state and a Cy5-labeled, receptor-specific antibody to determine the amount of the protein retained on the chip by the capture antibody. The ratio of Cy3 to Cy5 fluorescence allowed quantification of the fraction of receptor molecules that were phosphorylated.

Multiplexing within the individual spot using distinct labels may also be performed in reverse-phase array applications as demonstrated schematically in FIGURE 1C. Chan and coworkers studied the kinetics of mitogen-activated protein kinase (MAPK) phosphorylation during the activation of Jurkat T-cells using this strategy [63]. Lysates generated from the cells over a 30-min time course after activation were used to fabricate the reverse-phase microarrays. The arrays were incubated with a mixture of Cy3-labeled phospho-MAPK antibodies and Cy5-labeled actin antibodies. Increasing intensity of Cy3 fluorescence after cell activation indicated an increasing level of MAPK phosphorylation. The Cy3 intensity was normalized to Cy5 fluorescence, which corresponded to the level of actin in the different samples.

Multiplexing using distinct labels has special demands on bioinformatic data evaluation. The color intensity for every color is affected by different labeling quality of the samples and differences in the detection efficiency for the fluorescent labels, which is, for example, influenced by the band width of the light filters and the ratio between photomultiplier gains selected for each wavelength during scanning. Furthermore, differences in the quantity of proteins in the different labeled samples are important. Therefore, the following issues need to be addressed when analyzing data: normalization of the different color intensities; and background subtraction of each color. For evaluation of antibody array data, strategies similar to the analysis of DNA microarray data may be applied [72]. Normalization may be performed via total intensity as in the antibody array study by Sreekumar and coworkers [70]. After background correction, the authors scaled the data such that the average value for all of the spots from each array was normalized to one in both the Cy3 and Cy5 channel. This kind of normalization relies on the assumption that the average spot intensity would represent unchanged protein expression. In their search for potential biomarkers in cancer patient sera, Miller and coworkers used a normalization factor that had been calculated based on both the signal intensity from control spots containing anti-IgG antibody and the serum IgG concentration measured by enzyme-linked immunosorbent assay (ELISA) [71].

Function-based applications & multiplexing

Different function-based applications

Function-based protein microarrays are another microarray type in terms of application [8]. These microarrays, in contrast to abundance-based arrays, aim at the qualitative investigation of protein interactions and modifications, and can be used to generate protein interaction maps [73]. They are created by immobilizing large numbers of purified, recombinant, preferably native proteins from a given cell, meristem or organism on a solid surface. These arrays can then be used to screen the interaction of the immobilized proteins with antibodies, proteins, DNA or small molecules [6–9]. Furthermore, they can be utilized to study the modification of the proteins by enzymes in a qualitative manner. The use of functional protein microarrays for the identification of protein–protein interactions has been demonstrated in several studies [21,39,73]. Furthermore, whole-proteome microarrays were applied to test the specificity and crossreactivity of antibodies [74]. Using protein microarrays, initial studies for the detection of protein–DNA interactions have been performed [26,34] and can be applied in future to identify and characterize nucleic acid-binding proteins, such as transcription factors. Another possibility is to detect the interaction of proteins with small molecules/ligands using protein microarrays [12,75]. Applications of protein microarrays to analyze the phosphorylation of proteins by kinases have also been reported [12,33,45].

Multiplexing in function-based applications

In function-based applications, multiplexing is commonly used to measure the abundance of capture molecules by one dye and the abundance of captured sample molecules by another [21,39]. In addition, new technologies based on microwell technology have been introduced that allow multiplexing in functional protein assays. Biran and coworkers constructed a high-density, ordered array containing thousands of microwells on optical

imaging fibers to monitor cellular responses using reporter genes or fluorescent indicators [76]. Since each fiber monitors the signals of one well, they were able to detect *in vivo* protein–protein interactions with a resolution of a single cell. Microwell technology was also used for the cell-free expression of proteins [77] and the high-throughput analysis of protein kinases [45]. The technologies described above for multiplexing in functional applications have the intrinsic disadvantage that they only allow multiplexing on the site of capture molecules or require additional machinery or nanowell plates.

Multiple spotting technology

To overcome the limitations mentioned above, the authors have developed a new technology known as multiple spotting technology (MIST) [78]. The power of this technology has been demonstrated in the following different applications: antibody–antigen assays (FIGURE 2A), screening of phage display selections (FIGURE 2B) and several enzymatic assays (FIGURE 2C).

MIST allows multiplexing not only on the level of capture molecules, but also on the sample level, hence allowing analysis of different combinations of capture molecules to sample similar to commonly used microtiter plates. The principle of MIST is based on the spotting of the sample to the same position, in which the capture molecules were fixed to the surface (FIGURE 3). By the prevention of total evaporation of the sample, for example, by hygroscopic substances, a reaction entity with a subnanoliter volume is formed, in which the reaction rapidly progresses due to limited diffusion possibilities. To demonstrate the flexibility and specificity of MIST, an immunosorbent assay was performed in which four different concentrations of protein were subjected to four different concentrations of an antibody mixture (FIGURE 2A) [78]. The study revealed the feasibility of the approach and displayed minimal crossreactivity of the antibodies (FIGURE 4).

MIST technology consumes only nanoliters of sample and capture molecules, requires only standard spotting and scanning machinery and enables assays that rely on unbound reaction partners. One example in which the latter key feature is especially beneficial is the screening of inhibitors for enzymatic reactions. In a proof-of-principle study, it was demonstrated that MIST can be applied to conduct enzymatic assays using alkaline phosphatase, horseradish peroxidase and cathepsin D (FIGURE 2C) [79]. Furthermore, it was demonstrated that it is possible to monitor the effects of inhibitors and that extremely sensitive detection of down to 35 enzyme molecules is possible. In another application, MIST was utilized for the screening of large pools of recombinant antibodies (FIGURE 2B) [80]. This was important, since with the advent of automated phage display selection platforms [81], the bottleneck was shifted further downstream to the screening of large sets of monoclonal binders obtained from the selections for affinity and specificity. Utilizing MIST, the authors' immobilized the antigen used for the selection, and transferred unpurified recombinant antibody fragments on top of the antigen. While sensitivities comparable to the commonly used ELISA were obtained, manual interaction steps were minimized and the technique was streamlined to be accessible within the automated selection procedure. The same approach of spotting the sample on top of the capture molecules was also applied in a recent publication of Wingren and coworkers, who applied unpurified single-chain Fv antibody fragments for the sensitive detection of analyte in complex proteomes [40]. Although MIST offers several advantages, it also has intrinsic drawbacks. These include the extra time required for spotting and the lack of ability to quantify proteins within dilute solutions, which limits its use in abundance-based applications. For example, measurements of proteins with nanomolar concentrations are not possible, since only approximately two molecules of protein are present in the reaction volume.

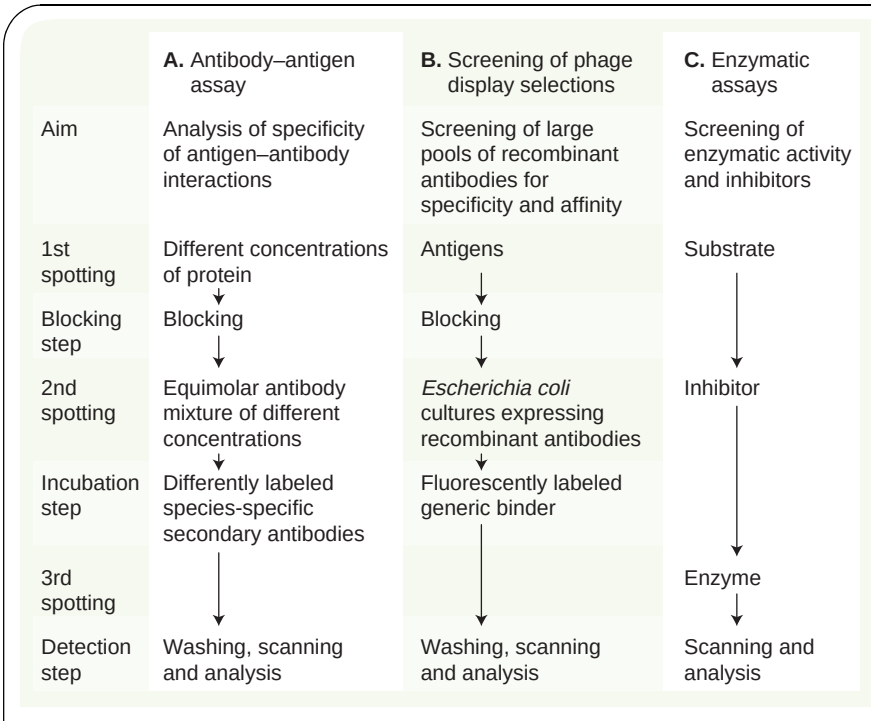

Figure 2. Workflow of the MIST. The diagram demonstrates the workflow for three applications of MIST: (A) antibody–antigen assay [78]; (B) screening of phage display selections [80]; and (C) enzymatic assays [79].
MIST: Multiple spotting technique.

Bead-based systems for multiplexing

Apart from planar microarrays, bead-based systems provide an excellent alternative for measuring a low number of samples in a multiplexed fashion in solution. This technology uses different color- or size-coded beads, which serve as solid supports on

which different capture molecules are immobilized. Flow cytometric systems enable the simultaneous discrimination of bead types and the quantification of captured sample molecules with suitable reporter dyes.

xMAP® technology from Luminex Corp. allows multiplex analysis of samples for abundance- and function-based approaches [103]. The beads used are color coded by different ratios of two fluorescent dyes. During readout, the system records the fluorescence reporter molecule that monitors a binding event as well as the color code of the bead for identification of the capture molecule. This combination allows multiplex analysis of up to 100 different species in a liquid environment without any washing steps [82–84]. Luminex bead-based xMAP technology has also been used in the Bio-Plex™ system from Bio-Rad Laboratories [104], as well as the LiquiChip™ system from Qiagen [105] (for commercially available techniques, see [10]). FACSarray™ from BD Bioscinenses is another commercially available bead-based technology, and may be used in combination with a 72-plex flexible bead set [106]. Although bead-based systems have limitations in the achievable degree of multiplexing, these systems are much further advanced in automation. Throughput of low-density applications is no longer an issue. Since no protein spotting is needed, it is easier to keep immobilized proteins in a functional state using this technology than by applying microarray technology.

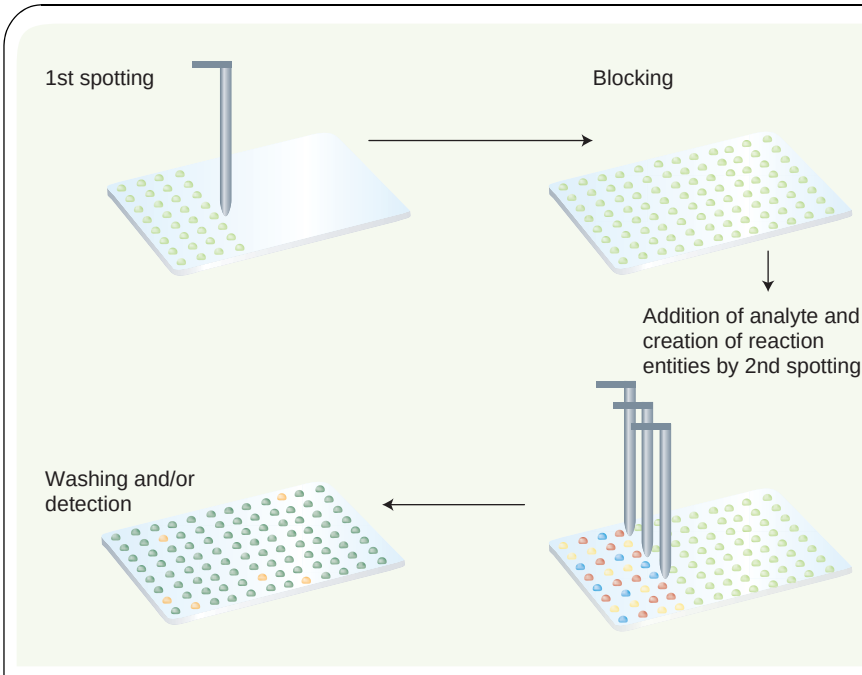

Figure 3. Principle of MIST. The principle of MIST is based on the spotting of the sample to the same position on the microarray in which the capture molecules were fixed to the surface. By the prevention of total evaporation of the sample, for example, by hygroscopic substances, a reaction entity with a subnanoliter volume is formed, in which the reaction rapidly progresses due to limited diffusion possibilities. Beside the two-step spotting of capture molecules and samples, additional spotting steps can be performed in other set-ups, such as the subsequent spotting of fluorogenic substrate, inhibitor and enzyme in enzymatic screening assays.
MIST: Multiple spotting technique.

Conclusions

Protein and antibody microarray technologies are becoming propitious tools for the high-throughput analysis of protein abundances, modifications and interactions. They can be divided into abundance- and function-based approaches in terms of their applications. Abundance-based microarrays are aimed at the description of relative protein abundances and are analogous to the DNA arrays commonly used in expression profiling projects. Comparison of different states of biologic samples by multiplexing approaches is increasingly important in order to discover disease markers and new targets for therapies. Functional applications, in contrast, are intended to identify potential interaction partners and modifications of proteins to decipher the complex cellular network. Since a single protein within this network interacts with many different interaction partners in a highly dynamic manner, it is crucial to examine as many interactions as possible simultaneously. This can be achieved by applying multiplex technologies, which are capable of analyzing different properties of the same protein in a parallel fashion. Although much effort has been invested in the optimization of such technologies, they are at an early stage of development. Nevertheless, it can be expected that further advances will enable the use of such technologies to obtain a more comprehensive view of biologic systems in the future.

Expert commentary & five-year view

Protein and antibody microarray technology is a rapidly evolving field, driven by the urgent need for high-throughput methods to functionally characterize proteins. While many proof-of-principle studies have shown potential areas of application, only a few large-scale investigative studies have been performed. One reason for this is the lack of availability of large sets of expression clones. For protein microarrays, this problem becombes even more severe upon consideration of the numerous post-translational modifications that are introduced by the different expression hosts and that are required to obtain a comprehensive view of cellular organization. Although the first steps have been undertaken to solve this problem using cell-free transcription and translation systems of varying origins, further optimization will be required to adapt such systems to a high-density microarray format.

A central challenge of antibody microarrays in abundance-based applications will be the assembly and validation of a complete set of antibodies, antibody fragments or antibody mimics for the human proteome. Although several companies are offering the first antibody microarrays for different applications,

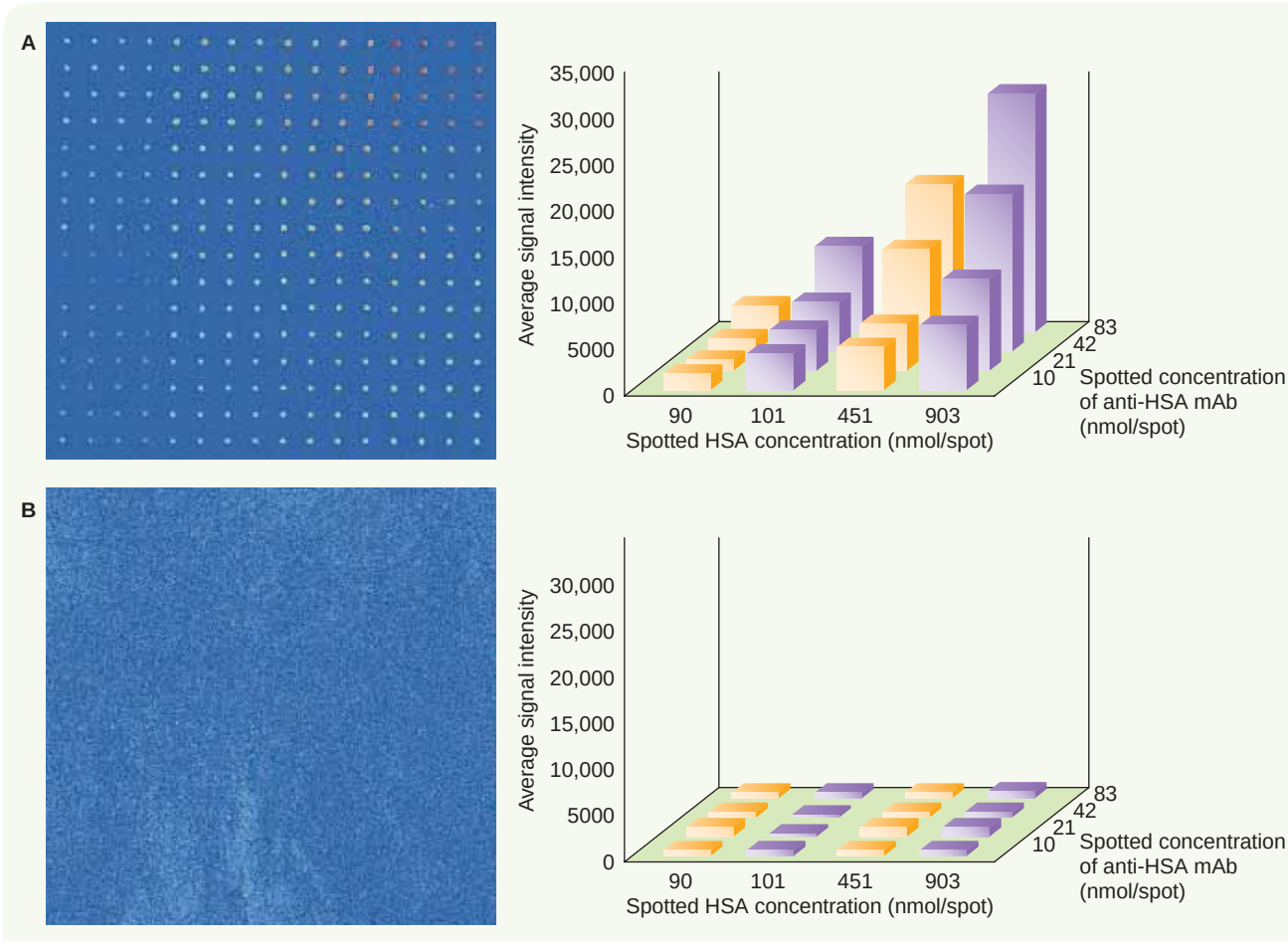

Figure 4. Example of a multiplex chip generated by MIST. To demonstrate the multiplexing capabilities, four different concentrations of HSA were spotted in columns of four from the highest concentration at the right to the lowest concentration at the left. In a second spotting run, an equimolar mixture of mouse anti-HSA antibodies and rabbit antifibrinogen antibodies was applied to the very same spotting positions with the highest concentration in the top four rows and the lowest concentration in the bottom four rows. Detection was performed by differently labeled species-specific secondary antibodies. **(A)** Scanning at the respective wavelength for bound anti-HSA antibodies (Cy5-labeled antimouse antibody). **(B)** Scanning at the respective wavelength for bound antifibrinogen antibodies (Cy3-labeled antirabbit antibody). HSA and antibody concentrations are indicated on the y- and x-axes of the diagrams, respectively, and the signal intensities obtained are shown on the z-axis. Signal intensities are illustrated in the same spatial arrangement as on the chip. Reprinted with permission from [78], © 2003 American Chemical Society.
Cy: Cyanine; HSA: Human serum albumin; mAb: Monoclonal antibody; MIST: Multiple spotting technology.

their widespread use is currently hindered by their price. Thus, display technologies that allow the cost-effective generation and production of antibodies will gain more importance. However, most of the large-scale-generated recombinant capture molecules exhibit moderate affinities and require a further maturation process to be adequate surrogates for *in vivo*-generated antibodies. For that reason, the application of recombinant binders on large antibody microarrays still requires improvements and rigorous quality assessment with regard to sensitivity and specificity [74,85], especially when highly complex solutions are used as samples. Within the next 5 years, it is expected that current display technologies for the high-throughput production of antibodies or antibody mimics will be automated. This progress will be accompanied by a further improvement of methods for analyzing the specificity of the generated antibodies in an effective manner.

Besides this content problem, several technical limitations such as the preservation of protein functionality after immobilization as well as the provision of the required absolute and relative sensitivity hinder the development. Another problem that will be rather difficult to solve is the large dynamic range of protein abundance, which can be as large as 10^{12} in human plasma [86]. This problem can be tackled by reducing sample complexity prior to microarray analysis. However, fractionation is a complex task for quantitative applications.

Another aspect of protein microarrays is the type of measurements that will be made. Although several studies show that quantitative measurements are possible, it can be expected that the majority of investigations, especially those with large sets of different binders, will be based on qualitative or semiquantitative measurements. This is due to the efforts that coincide with the establishment and calibration of the chip, and the required low

variations within the microarray. In addition, qualitative or semi-quantitative measurements are sufficient for many applications such as biomarker discovery and interaction screenings. The results obtained from such measurements can then be used in a second step for an in-depth investigation in a low-throughput fashion.

In addition, the development of new fluorescent dyes, such as quantum dots, might enhance the multiplexing capacities of microarrays in combination with suitable scanner systems [87]. Such advances will be especially important for abundance-based approaches, in which the sample volumes cannot be decreased due to the absolute limits of detection, and therefore, multiplexing by compartmentation is limited. In function-based applications, it is expected that the degree of multiplexing will be achieved by the compartmentation approach, rather than through a labeling approach, since the concentration of the interaction partners are generally not the limiting factor.

Key issues

- Multiplexing is required to gain a more global view of complex biologic networks.
- Different multiplex approaches have been developed for abundance- as well as function-based protein microarray applications.
- The main principles to perform multiplexing in abundance-based applications are currently different compartmentation strategies as well as labeling strategies.
- For functional applications, multiple spotting technology (MIST) is a promising compartmentation technique that allows multiplexing at the sample level as well as at the capture molecule level.
- Bead-based systems are an alternative to microarrays for measuring low numbers of samples in a multiplexed fashion in solution.
- The provision of large sets of expression clones as well as of high specific antibodies or antibody mimics remains a prerequisite for the development of large protein microarrays in multiplexing applications.

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