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Recent advances of protein microarrays

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Technological innovations and novel applications have greatly advanced the field of protein microarrays. Over the past two years, different types of protein microarrays have been used for serum profiling, protein abundance determinations, and identification of proteins that bind DNA or small compounds. However, considerable development is still required to ensure common quality standards and to establish large content repertoires. Here, we summarize applications available to date and discuss recent technological achievements and efforts on standardization.

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Introduction
The global concept of array technology is the simultaneous analysis of thousands of molecules for a specific property under investigation. To this end, protein arrays were initially introduced to screen cDNA libraries for clones expressing recombinant proteins in *Escherichia coli* [1]. For this purpose, thousands of different expression clones were arrayed as bacteria on large protein binding membranes and — after induction and cell lysis — the presence of recombinant proteins on the array was correlated to individual clones. Subsequently, miniaturization has led to protein microarrays that are typically constructed by spotting protein samples onto microscope slides.

Current protein microarrays come in a variety of formats. These include 'standard' protein microarrays (PMAs), which consist of purified recombinant proteins; antibody microarrays (AMAs); and reverse protein microarrays (RPMAs) generated from whole or fractionated cell lysates, as depicted in Figure 1a. Although the applications of PMAs can differ widely, the same general concept

to detect interaction partners is applied in all. Putative binding partners are incubated with the arrayed proteins and binding is detected by using a label, either covalently bound to the putative interaction partner (Figure 1b) or a secondary antibody, or by novel label-free methods detailed below.

In addition, the principle of delineating array results is the same for all PMA types; the signals — or labeled array spots — correlate the interaction to a known spot content according to the position on the array. Here, we discuss applications, technological advancements and detection systems developed in the past two years. Moreover, efforts towards standardization of protein microarray experimentation are reviewed.

Protein microarrays
The earliest application of PMAs (Figure 1, left), in 1999, was to determine antibody specificities using arrays of denatured recombinant proteins [2]. Since then, Michaud *et al.* have extended this approach to proteome-wide yeast PMAs and compared the specificity of monoclonal and polyclonal antibodies [3]. Additionally, PMAs of recombinant proteins have been used to identify potential diagnostic markers. For example, PMAs of human recombinant proteins were used to determine the humoral immune responses associated with different diseases, such as the autoimmune diseases discussed by Robinson in this issue. Also, PMAs presenting microbial and viral proteins have been introduced for the identification of new potential diagnostic markers and vaccine candidates. For example, Kreutzberger and co-workers [4] analyzed sera of meningitis patients with PMAs presenting 67 selected recombinant *Neisseria meningitides* proteins. Patient serum antibodies recognized 70% of these proteins. More than half of all patients tested had antibodies against the OpaV protein, making it a potential diagnostic marker. Another bacterial PMA, containing 149 *Yersinia pestis* proteins, was used to profile antibody responses in sera of rabbits immunized previously with live plague vaccine [5]. In total, 50 proteins triggered an immune reaction, of which eleven were identified as potential candidates for new diagnostic markers or vaccine components. Viral PMAs were used by Qiu *et al.* [6] for investigating the severe acute respiratory syndrome (SARS) and screening identified a unique immunogenic protein as a promising vaccine candidate.

As well as screening with antibodies, PMAs are used for the identification of enzyme substrates. For instance, PMAs

Figure 1
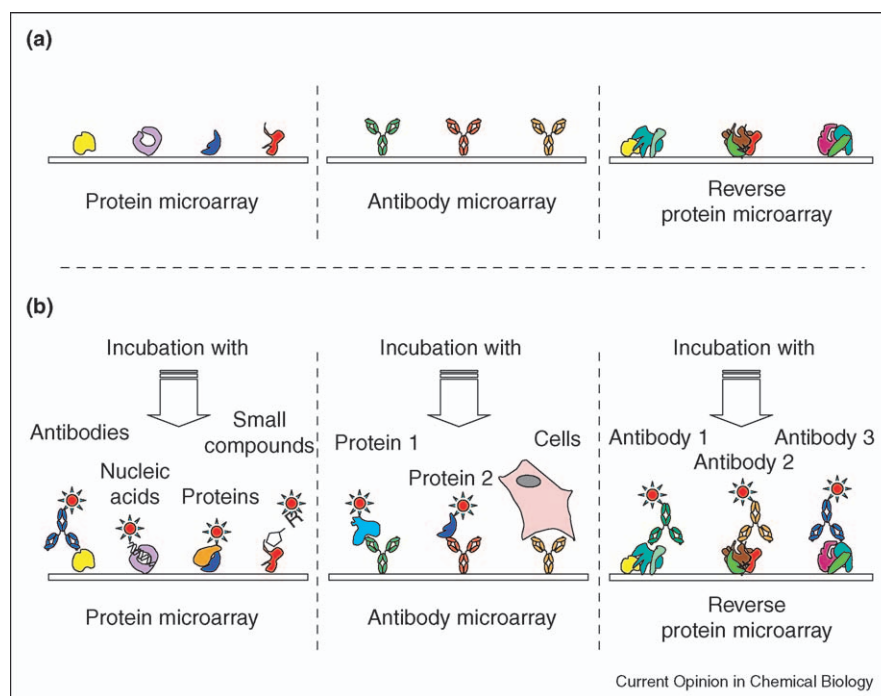
Types of protein microarrays and their possible applications. **(a)** The three most common types of arrays in use: protein microarrays (PMAs, consisting of individual recombinant proteins); antibody microarrays (AMAs, consisting of antibodies or fragments thereof); and reverse protein microarrays (RPMAs, consisting of whole or fractionated protein lysates/extracts). **(b)** Screening applications of the three array types with known or putative directly labeled interaction partners.

presenting a non-redundant set of approximately 1700 denatured *Arabidopsis thaliana* proteins were addressed with different mitogen activated protein (MAP) kinases [7•]. Besides known and suspected targets, novel unpredicted kinase substrates such as transcription factors, histones, kinases and ribosomal proteins were identified.

A recent example of protein–protein interaction screening was demonstrated by Kawahasi and colleagues using three selected pairs of model proteins known to interact [8••]. All proteins were synthesized using a wheat-germ-based cell-free protein translation system shown to be suitable for high-throughput protein expression [9]. Supplementing the translation system with Cy3-dC-puromycin yielded fluorescence-labeled proteins that could be directly used for array-based interaction screens. The authors demonstrated previously [10] the incorporation of different fluorescent dyes at the C-terminus of the proteins, providing the basis for array-based interaction screens with multiple putative interaction partners in parallel. Accordingly, we expect a rapid application of this innovative approach on a larger scale. In another instance, Letarte *et al.* studied the interaction between the human leukocyte membrane protein CD200 and its cell surface receptor hCD200R. Using a panel of point-mutated receptors, the antigenic epitopes of two mono-clonal antibodies were mapped. Subsequently, a low affinity interaction, CD200 binding to its receptor, was demonstrated [11].

One of the first studies to detect a small-molecule-dependent protein–protein interaction was conducted by Sasakura and co-workers. They successfully demonstrated the strictly cAMP-dependent interaction between an *Escherichia coli* phosphodiesterase (*Ec* DOS) and the isolated PAS domain of the enzyme [12].

Meanwhile, PMAs have also been used to analyze protein–DNA interactions regulating the coordinated expression of genes. Using a bacterial model system, the specific detection of protein–DNA interactions on protein microarrays was demonstrated to have a dynamic concentration range of four orders of magnitude. The findings were verified by electro-mobility shift assays [13]. Probing a proteome-wide yeast PMA with fluorescently labeled genomic DNA identified over 200 DNA binding proteins [14]. The results are in excellent agreement with chromatin immunoprecipitation experiments. Half of the identified proteins are known or expected to be DNA binders, whereas the remaining half are novel DNA interactors. Follow-up experiments revealed that the metabolic enzyme Arg5,6 — which is involved in

ornithine biosynthesis (a precursor to arginine) — associates with specific mitochondrial and nuclear loci *in vivo*.

Applying the same yeast proteome-wide PMA, the protein targets of two small molecules known to influence the target of rapamycin (TOR) pathway were determined [15]. For each molecule multiple protein interaction partners were found. In future, similar experiments will help decipher the mechanism of small-molecule-derived pharmaceuticals used in modern medicine.

To overcome the on-demand availability of purified proteins, a major bottleneck of PMAs, Ramachandran *et al*. [16**] printed cDNAs in direct vicinity to capture antibodies against a GST-fusion tag. After adding a cell-free expression system, GST-fusion proteins were generated directly on the microarray and immediately retained by the adjacent anti-GST antibodies. The resulting microarrays were quality controlled by verifying the content of protein spots with protein-specific antibodies. Subsequently, the pair-wise interactions among 29 human DNA replication proteins were demonstrated.

Antibody microarrays

Antibody microarrays (AMAs, Figure 1, middle) have great potential in many fields, as broad as commercial diagnostics, assessment of environmental pollution and quality control in, for example, the food industry, or simply as comprehensive research tools. Their use ultimately holds the promise of complementing RNA expression profiling of stable interactions or dynamic processes at the protein level. However, despite the growing number of successful applications of AMAs, their use is currently limited to specific investigations definable with a relatively small set of antibodies. This is partially due to limited availability of well-characterized antibodies, as well as technical challenges reported elsewhere [17].

A recent successful application of AMAs is the work of Koga and colleagues, who studied tissue-specific expression of proteins using 382 antibodies generated against mouse KIAA (Kazusa DNA Research Institute and 'AA' reference characters) proteins. Protein abundance was assessed by label-free and real-time signal detection using surface plasmon resonance (SPR) technology in a biosensor (FLEXCHIP^TM, Biacore AB, Uppsala, Sweden). AMA-derived protein abundance was compared to mRNA abundance and a positive correlation was found for most gene products. For some, a negative correlation was determined and attributed to pronounced differences in tissue-specific RNA and protein stability [18].

To date, label-free detection on PMAs has not been widely applied and, currently, the sandwich immunoassay is the most reliable microarray-based quantification system of protein content in use [19]. However, the detection of each analyte requires two specific antibodies binding to different regions of the analyte. A third detection antibody is commonly applied to avoid potential loss in specificity and sensitivity of the detection antibody as a result of the labeling reaction. Using a third antibody, however, can result in increased unspecific signals due to recognition of the conserved regions of both the capture and the detection antibody. To overcome this obstacle, Song *et al*. [20*] removed the Fc part of the tumor-marker capture antibodies by pepsin digestion and arrayed the antigen-binding fragments $F(ab')_2$ for six different tumor markers. Subsequently, calibration curves were derived using pure and serum diluted tumor markers. Finally, the authors demonstrated that the readout obtained with their $F(ab')_2$ microarray correlates well ($R^2 = 0,92$) with those obtained with standard immunoassays for 31 human serum samples.

A third example of an innovative use of AMAs is shown by Ko and colleagues. They applied living rat neural stem cells (NSC) onto a microarray of 15 surface-marker-specific antibodies. The presence of these markers in a heterogeneous neurosphere-forming cell population was investigated [21]. Additional *in situ* cultivation and subsequent immunostaining of array-bound cells allowed the assessment of the proliferation capability of NSC.

Reverse protein microarrays

In contrast to the types of protein microarrays described above, RPMAs (Figure 1, right) are based on the regular arrangement of complex, non-purified — sometimes fractionated — protein mixtures, usually derived from cell or tissue lysates. They can provide access to post-translationally modified proteins that are, so far, not accessible with high-throughput methods. By arraying lysates from different cell lines and/or biopsies on the same support, the relative abundance of different proteins in the mixtures can be determined, provided that highly specific detection reagents are available [22–24]. Recent applications [25–28] have focused on the analysis of cancer specimens using highly specific antibodies for different, partly post-translationally modified members of signaling cascades.

A major advantage of RPMAs is the requirement of minute amounts (5000 cells/10 μl [25] or 10 μl of 16.6 ng/μl of a model protein [29]) of protein extracts for the generation of tens of microarrays, which, in contrast to gel electrophoretic applications, can be analyzed in a highly automated fashion. In addition, multiple replica and dilution series can be included on the microarray, increasing the robustness of protein quantification over a wide range of concentrations [25,29].

A good example of RPMA with fractionated samples was presented by Nam *et al*. [30] arraying protein fractions from the LoVo colon cancer cell line. One of the protein fractions gave a positive signal with most of the sera derived from colon cancer patients, but not from control

sera. Subsequent mass spectrometric analysis of the fraction identified the C-terminus of the ubiquitinylated hydrolase L3 as the target.

Furthermore, Janzi *et al.* spotted total sera of more than 2000 patients on one microarray for quantitative screening for IgA deficiency [31•]. Ultimately aiming at an early detection of immunodeficiency in newborns, the authors were able to detect less than 1 μg IgA per ml serum.

Chan and colleagues applied whole-cell lysate RPMAs of Jurkat T-Cells to monitor the dynamics of site-specific phosphorylation of signaling molecules [32••]. Before analysing the signaling cascades, the authors determined the dynamic range of their approach to be approximately four orders of magnitude, with a detection limit of one protein in 10^5 to 10^6 lysate proteins. Subsequently, the kinetics of the phosphorylation of phospholipase C (PLC) γ1 in Jurkat cells activated through CD3 and CD28 receptors were analyzed. Taking the total content of phospholipase C into consideration, the authors determined the relative phosphorylation level of PLCγ1 to be rapidly up-regulated within the first 2.5 min of stimulation with CD3 cross-linking. The CD3-dependent up-regulation diminished to baseline by 10 min. CD28-dependent stimulation resulted in a less pronounced, but more prolonged phosphorylation of PLCγ1. In addition, downstream signaling pathways were delineated.

Increasing sensitivity for detection

A variety of novel methods for increasing sensitivity have been developed to detect low abundance proteins, and the reported sensitivities are summarized in Table 1.

For example, Angenendt and colleagues developed a sub-nanoliter enzymatic assay system on standard microarrays allowing the detection of the enzymatic activity of 35 molecules on individual spots [33•].

In recent AMA applications, the signal detection is often carried out by direct (multicolor) labeling with rolling-circle amplification (RCA) [34]. The system requires the direct conjugation of the proteins to be analyzed with a label such as biotin or digoxigenin. An antibody conjugated to a primer detects the label. After hybridization of a circular DNA molecule, the primer is extended 'endlessly' by a polymerase. Subsequently, specific Cy3- and/or Cy5-labeled oligonucleotides complementary for the elongated DNA fragment are hybridized, producing specific signal amplification. The major advantage of RCA is the superior sensitivity and reproducibility; up to 30-fold increase in signal intensity has been reported [35]. Gao *et al.* successfully applied the RCA detection to an AMA consisting of 84 distinct antibodies specific to serum proteins. Comparing the protein expression profile of 24 lung cancer patients to equal numbers of healthy and chronic obstructive pulmonary disease patients, several proteins were identified as more abundant in lung cancer [36].

The detection sensitivity of PMAs can be further increased with the introduction of encapsulated semiconductor nanocrystals commonly referred to as quantum dots (QDs) [37]. This novel class of fluorescence probe is available for many different wavelengths with high extinction coefficients and quantum yields [38]. QDs are expected to be inert for environmental factors (e.g. ozone) that deteriorate fluorescent dyes [39] and have a great potential to be applied for multiplexed highly parallel analysis of many different samples on a single microarray. Multiplexing strategies were reviewed recently [40].

Immobilization strategies

Many different surfaces for the generation of PMAs have been described and were discussed extensively [17,41]. Besides tethering the proteins to the surface by adhesion

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Table 1

Application and detection limits[a].

Name	Immobilized partner	Screening partner	Applications	Examples for sensitivity
PMA	Proteins	Proteins Antibodies DNA Chemicals	Functional characterization of proteins Target identification of interaction partners	63 amol/spot [48] < 0.8 Cy3 molecules μm^{-2} [49] 60 ymol [33•] 10–50 amol [11] 100 amol/spot [59]
AMA	Antibodies Binders	Purified proteins Complex mixtures	Antibody characterization Protein abundance quantification	400 zmol [60] < 12 μg antigen/l serum [20•]
RPMA	Fractionated proteins Complex protein mixtures (e.g. cell extracts)	Single antibodies Complex mixture (sera)	Monitoring changes in PTM upon initiation of cellular processes Serum profiling Identification of serum disease marker	<1 μg IgA/ml serum [31•] 1 protein in 10^5 to 10^6 lysate proteins [32••]

[a] Selected applications of protein microarrays (PMAs), antibody microarrays (AMAs) and reverse protein microarrays (RPMAs) are listed. The immobilized (spotted) partner could interact with different screening partners applied to the array for multiple applications. Exemplary detection limits, as stated by the authors, are given.

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or covalent attachment in a non-oriented fashion, recent developments for the directed immobilization of proteins are emerging. These efforts are addressing challenges such as loss of enzymatic activity due to unfavorable orientation of the immobilized enzyme [42]. To overcome this obstacle Ofir *et al.* attached proteins via a cellulose binding protein to cellulose-coated microarrays [43]. A related approach is linking of proteins to DNA coated microarrays via the GAL4 DNA binding domain [44] or oligonucleotide duplexes [45]. Additionally, for the directed immobilization of antibodies S-layers – self-assembling structures resembling bacterial cell walls – can be used. They are generated by recrystallizing the B-domain of protein A or analogue domains like the synthetic Z-domain on supports precoated with secondary cell wall polymer. Both domains are capturing the FC part of immunoglobulins in a directed fashion [46•].

Despite the development of many different surfaces in the last five years, notably only few systematic investigations have been conducted and yet, no universal surface ideal for all applications could be identified [47–49]. Hence, careful comparison of data derived form different surfaces and cross-technological platforms with suitable standards is still an issue.

Standardization
In microarray experiments, there are a multitude of different factors crucial for the quality and reliability of the final readout of each experiment [50•]. With respect to the limited level of consistency between different commercial DNA microarray platforms for expression profiling [51,52] common standards for protein microarrays should be agreed upon soon. Currently, several pilot studies have been carried out by the International Human Proteome Organization (HUPO: http://www.hupo.org) and the HUPO standardization initiative proposed standards for data exchange (MIAPE) [53,54]. Additionally, standards for proteomics are under discussion in different national initiatives, such as the quality control management of the German National Genome Research Network (http://www.ngfn.de) and the US National Institute of Health Roadmap: 'Standards in Proteomics' (http://nihroadmap.nih.gov/buildingblocks/proteomics/).

With respect to the divergent groups of protein microarrays, individual standards for each type of PMA and their applications are reasonable. All standards should contain detailed information, such as the aim of the array based experiment, the experimental design, the content and the design of the array as well as the quality control of the content, and controls included in the array. In addition, information (origin, isolation, labeling) on the sample incubated with the array, the procedures and parameters of incubation and subsequent downstream processing, image acquisition and quantification are needed. Ideally, an extended minimum amount of information — similar to the MIAME standards [55] proposed for DNA microarrays should be established. For the area of antibody microarrays, normalization procedures for comparative abundance analysis have already been adapted from the cDNA microarray field [50•]. Despite the significant challenge associated with this task, the work of Haab *et al.* [56] shows that applying common standards yields equivalent results from different laboratories.

Conclusion
The past two years have seen impressive progress in PMA technology. Protein and antibody microarray technology has taken further important steps towards diagnostics and biomarker discovery [57]. Whole proteome microarrays enable unbiased experiments that can reveal unforeseen biological activities of unknown but also well-characterized proteins [14]. In addition, proteome-wide PMAs have identified interacting partners missed in large-scale yeast-two-hybrid screens [58].

The field of RPMAs opened up possibilities to gain in-depth insights into cellular processes and provides access to post-translationally modified proteins.

In future, standardization efforts will be indispensable to compare PMA results obtained in independent laboratories.

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