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Protein Microarrays as Tools for Functional Proteomics: Achievements, Promises and Challenges

Smiths S Lueong, Jörg D Hoheisel and Mohamed Saiel Saeed Alhamdani*

Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany

Abstract

The quest for a better understanding of organisms, and the human body in particular, at a comprehensive level has stimulated the development of techniques and processes that permit the analysis and assessment of biomedical information at high throughput. This has had substantial impact, not only by merely gathering knowledge but also by making scientists aware of the necessity to view organisms as complex biological systems rather than an assembly of individual biochemical pathways. Proteomics is still lagging behind genomics in this holistic analysis, however, because of the much higher degree of complexity that needs to be dealt with. Protein arrays, among other techniques, offer the prospect of advancing global protein analysis, similar to the impact of arrays in genomics. In basic research, protein arrays already contributed substantially, permitting the identification of many protein interactions and providing information on expression variations and structural changes, for example. Beside the fact of high throughput, arrays require relatively small reaction volumes, which is critical in view of the lack of means for *in vitro* protein amplification and beneficial for good assay sensitivity. Applications comprise many facets, such as the search for drug targets, analysis of host-parasite interactions and, above all, biomarker discovery. Despite the achievements and promises of the technology, it is still far from being a standard approach and many technical developments are ongoing. In this review, we look at the state of protein array technology and discuss future perspectives.

Keywords: Proteomics; Protein microarray; Functional proteomics

Introduction

With the complete genome sequence of very many organisms available and an analysis of (cancer) patient genomic information on the brink of becoming routine, the next challenge will be a comprehensive investigation of the functional roles of the encoded proteins. This knowledge will be crucial for a better understanding of the very many biochemical processes that are responsible for cellular functioning. Research in this field will particularly focus on the elucidation of pathological mechanisms that underlie disease, and the identification of avenues for targeting drug activity or other medically relevant interventions. Proteins are involved in almost every biological process. As soon as the word 'protein' was coined and first reports about protein activity were published [1], the importance of protein interactions became apparent [2]. Their analysis is therefore as old as the investigation of protein activity itself and has evolved to cover different aspects, such as structure, metabolism and enzyme regulation, signal transduction, pathway analysis, cellular networks and systems biology [3-5]. Interactions between biomolecules in general and protein molecules in particular are at the center of most physiological and pathological phenomena. Intercellular as well as intracellular communication is frequently mediated by proteins, which act either as messengers themselves or participate in signalling cascades. Proteins are key players in the many cellular networks [6] that in combination form the basis of the highly dynamic and flexible but at the same time well organised system represented by a cell or organism [7]. Also for interactions between organisms, for example in cases of infectious diseases, the biological crosstalk between host and pathogen proteins is vital for both the colonization of the host but also the elimination of the pathogen. It is for the above reasons that protein interactions are a topic that is central to proteome research.

For gaining an overall view of the proteome and its activities, high throughput is essential, given the amount of information that needs to be produced, evaluated and interpreted. Owing to transcriptional variations and posttranslational modifications, the diversity of a proteome could be multiple orders of magnitude larger than the corresponding number of genes of a particular organism [8]. For the human proteome, for example, which is encoded by about 22,000 genes, estimates range from 100,000 to more than 1,000,000 protein molecules and their derivatives. This complexity poses a significant challenge to proteomics, since techniques are required that deliver a broad but at the same time detailed and quantitative view. Gel-based methods, immunoassays and mass spectrometry were the first processes toward such ends. However, although particularly mass spectrometry and related technologies have advanced enormously, there are limitations to what they can deliver [9,10]. Alternative and complementary approaches are required for acquiring a comprehensive understanding of the proteome.

In continuation of earlier developments at the level of nucleic acids, protein array formats are obvious candidates for contributing to the evaluation of protein activity and interaction. The combination of high sensitivity, essentially procedural simplicity and robustness and the capacity of multiplexing analyses without losing information about the individual molecule make protein microarrays a promising addition to the tool arsenal for proteome exploration [11-16]. An advantage

***Corresponding author:** Mohamed Saiel Saeed Alhamdani, Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany, Tel: +49-6221-422718; Fax: +49-6221-424687; E-mail: m.alhamdani@dkfz.de

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of this approach is also that there is extensive experience with high-throughput affinity assays for clinical and industrial applications, in particular the enzyme-linked immunosorbent assay (ELISA). This could simplify eventual translation into a routine setting. Protein arrays are composed of a collection of molecules that are immobilized onto a planar solid support in an organised manner so that positional information identifies each particular protein (Figure 1). They permit simultaneous access to all molecules on the surface and can therefore be used to study and compare their reactivity with different analytes in parallel [17], hence reducing processing time and cost [18]. Another advantage is the small reaction volume that is generally required. For the lack of means for *in vitro* protein amplification, protein concentration and material consumption are frequently a critical issue in proteomics. In addition, the small volume greatly enhances sensitivity [19].

Initially, the development and use of protein arrays was impeded by several factors, such as surface and immobilization chemistry, mass transport limitations [20], or even the lack of sufficiently reproducible processes for protein isolation. As a matter of fact, there is still no universal surface chemistry that is suitable for every application, for example. Nonetheless, highly functionalized and adapted surfaces are available that have been applied successfully [21,22]. Protein arrays have been used to analyse various aspects of proteomics both in basic and clinical research. For example, they contributed to the analysis of protease-associated cellular networks in malaria, the establishment of the p53/63 interactome in cancer, studies about posttranslational modifications, patient screening, kinase substrate identification, or the detection of DNA/RNA binding proteins [23-29]; protein arrays have also been successful as tools for biomarker and drug discovery, the evaluation of host-pathogen interactions and antibody selection [30-33]. However, array-based proteomics is in its early phase and still faces even basic technical challenges. Most demanding may be the problems of an intact three-dimensional structure of the arrayed proteins – preferably having available a fully functional protein – and the fact that most systems used for protein expression are lacking the capacity to account for posttranslational modifications [34]. But also less striking but nevertheless crucial aspects require further technical developments. The currently available methods for detection, for example, are very good with respect to accuracy and sensitivity – down to the level of single-molecule detection [35]. However, they could influence the assay outcome or may simply be expensive and thus an obstacle to routine application.

In the present review, we present and discuss the achievements of array-based protein analyses and particularly interaction studies, examine the challenges that the technology is still facing, and deliberate about its merits and advantages over other techniques used for functional proteomics.

Relation to Other Techniques

Traditional approaches that have been applied for the study of protein interactions include equilibrium dialysis, Western blotting, co-immunoprecipitation, phage and ribosome display, pull-down and yeast two-hybrid screens. Meanwhile, some of these methods offer the opportunity to query *in vivo* phenomena and isolate protein complexes. They require relatively large amounts of sample material, however, are labour intensive and of varying sensitivity, require technical expertise and are prone to bias [36]. Some techniques, such as equilibrium dialysis, take a long time and greatly reduce the functionality of most proteins [37]. Pull-down assays and co-immunoprecipitation, on the other hand, are highly biased toward high-affinity interactions and do not provide any information about the direct physical interaction between two protein entities [38]. Phage and ribosome display are labour intensive and require the cloning of the genes of interest in frame with surface proteins [39]. This greatly affects the folding and three-dimensional structure of the proteins, thus their functionality as well as their correct presentation on the surface coat. Most of these techniques also do not permit a multiplexed analysis and are therefore not suitable for high-throughput proteomics. Although applicable

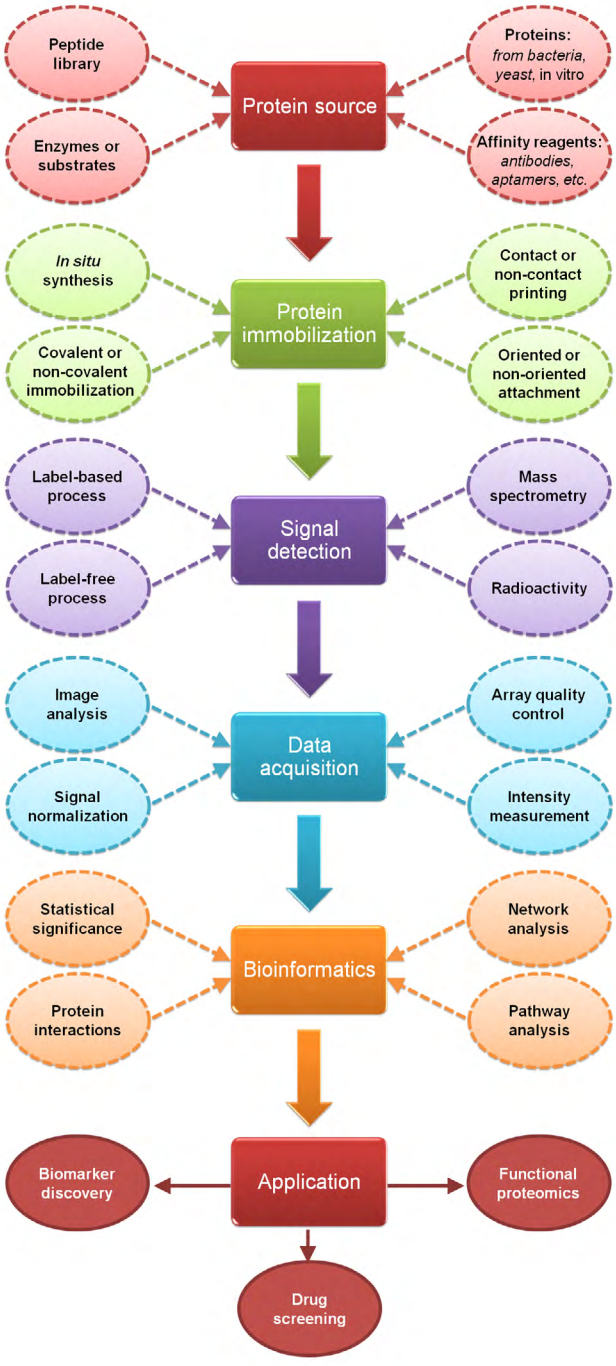

Figure 1: Schematic presentation of the various types of protein array analysis.

at high throughput, yeast two-hybrid assays have three major disadvantages: auto activation by the bait protein can lead to extremely high levels of false positives [40]; second, genes that do not localize into the yeast nucleus will lead to false negatives; and third, genes that are transcriptional repressors are not suited for this technique. Besides, two proteins may interact via an intermediary third protein and bring the DNA-binding and activating domains close to each other, resulting in transcriptional activation and hence false positives [40].

Although array-based assays do not allow an *in vivo* analysis and do not permit the isolation of protein complexes, they offer other advantages, apart from their flexibility. First, they produce immediate information about the direct contact between two proteins [14]. This is very helpful when it comes to the establishment of interaction networks and pathway analyses. Second, there is no need for the generation of bacterial or yeast strains that are expressing the recombinant protein of interest, which is cost and labour intensive. In fact, with the advent of protein *in situ* expression technologies for array production [16,41], it has become even more cost-efficient and simple to produce and use protein arrays. Screening can be done within a matter of few hours post array production, as opposed to the days or even weeks required for yeast two-hybrid and pull-down assays. Fascinating is the fact that protein arrays allow the analysis of weak and therefore transient protein interactions, a feature that is nearly impossible to achieve with classical methods [42]. Such interactions come very much into play when studying signalling pathways, for instance. Besides, with protein arrays being produced by *in situ* expression, it has now become possible to screen membrane-associated and other toxic proteins that could not be harboured in a host cell or might localise to the cell membrane in yeast two-hybrid screens [43]. It is only with the advent of protein arrays that some major biological processes are being studied in more width. For example, literature is gradually accumulating about the large-scale identification of kinase substrates or DNA/RNA binding proteins using protein array approaches [29,44].

Basic Assay Formats

As a result of their relatively high flexibility, protein arrays are versatile and the protocols can be adapted to fit to a variety of applications and technical facets (Figure 2). The platforms contribute to many aspects of understanding protein biology, help in gaining more insight into the molecular nature of some disease condition, are essential for finding novel diagnostic information and drug targets, and provide a basis for systems biology [45]. The arrays are particularly useful for analysing the interactions of proteins with other molecules, may they be proteins, lipids, DNA, RNA, drug compounds, glycans or other posttranslational modifications, and also permit the performance of enzyme kinetics studies in a high-throughput fashion [46].

Protein-protein interactions

The first array-based protein-protein interaction analysis was reported for yeast proteins in 2001, identifying binding partners of calmodulin and phosphatidylinosotides [47]. In 2006, an array of 159 human SH2 and phosphotyrosine-binding domains was used to study protein-peptide interactions involved in cell signalling. A semi-quantitative measurement of the interactions was performed and the binding kinetics was determined [48]. In 2007, a protein chip of 1,133 *Arabidopsis* proteins was applied to find binding partners of calmodulin and other calmodulin-like proteins, identifying transcription factors, such as the GRAS and AUX/IAA families, that had not been known to interact before [49]. Recently, arrays were employed to study complex networks and signalling pathways [50-54]. These studies and others clearly document the relevance of protein microarrays for proteome analysis.

Peptide-protein interactions

Peptide microarrays are actually a rather old feature and were already produced at the very beginning of the microarray era [55]. For the limited length of peptides and the well-established synthesis chemistry, peptide arrays could be produced by chemical synthesis with relative ease. Since the initial reports, several processes were established to produce highly complex arrays. Using an adaptation of laser printer processes, peptide microarrays were successfully produced by repeatedly depositing small droplets of amino acids at particular array positions, for example [56]. Utilising an array design that presents a tiling path of overlapping peptide sequences, the epitope of an tetanus-specific antibody was mapped with this technique recently [57]. The highest resolution and complexity was achieved with arrays produced by light-directed synthesis [58], which also proved their worth by defining the binding sites of particular antibodies, for instance.

Identification of lipid-binding proteins

Apart from protein-protein interactions, arrays were used to study interactions with lipids. The significance of understanding such interaction has a great impact on elucidating the role of lipids, such as steroid hormones, in regulating gene expression, and also on deciphering the mechanism of membrane transport as mediated by lipid-binding proteins found in the cell membrane. Aided by a protein array of 5,800 proteins, several lipid-binding proteins have been identified in yeast [47]. About 35% of the proteins found to

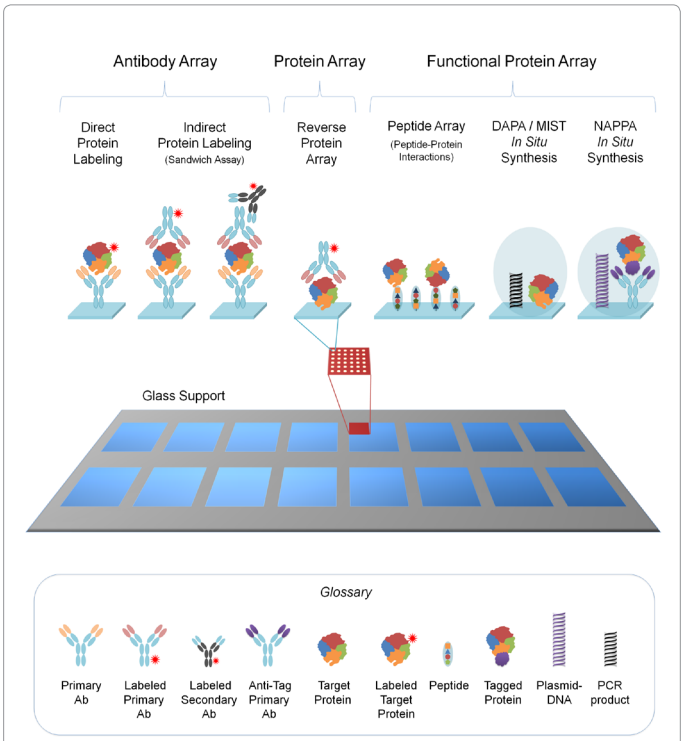

Figure 2: Schematic diagram illustrating the basic flow of a protein array analysis, presenting variations that could be adapted according to the specific kind of analysis pursued.

bind phosphoinositides had been uncharacterized previously. Of the 98 known proteins found in the analysis, only 45 had been known to be membrane-bound or to contain a membrane-spanning region. Peripherally located proteins as well as integral membrane proteins were identified, such as the GPI anchor proteins Tos6p, Sps2p and prenylated protein Gpa2p.

Characterization of protein-DNA interactions

DNA binding proteins represent a large and diverse group of molecules such as transcription factors, nuclease, polymerase, and histones. These proteins modulate and control a broad range of cellular and molecular functions owing to their affinity and interaction with DNA sequences. The nature and extent of protein-DNA interaction is vital information for understanding the governing forces behind DNA transcription and translation and DNA damage and repair. Several studies focussed on a high-throughput characterisation of protein-DNA interactions. For yeast, for example, 200 proteins with DNA binding properties were found, of which more than 50% had not been predicted to exhibit such an activity. In depth studies of one of the identified proteins revealed its involvement in arginine biosynthesis and binding to particular DNA motifs that are associated with specific nuclear and mitochondria loci *in vivo* [59]. Utilising a microarray containing 282 yeast transcription factors, their interactions with 75 conserved DNA motifs was studied, yielding more than 200 interactions, of which more than 60% had neither been predicted nor known. Some of these interactions were shown to be involved in stress response and oxidative phosphorylation [60]. In another study, a screen was performed on 4,191 full-length human proteins with 400 predicted and 60 known DNA motifs, resulting in a total of 17,718 interactions [61]. Consensus sites for 200 transcription factors were determined, which was more than the overall number of reported consensus sites in humans at the time [62].

Identification of RNA-binding proteins

RNA-binding proteins are key regulators of posttranscriptional gene expression control [63], which in turn influences very many cellular processes, such as cell proliferation, differentiation, invasion, metastasis, angiogenesis and apoptosis [64]. Many RNA-binding proteins have been directly linked to cancer development and progression [65,66]. Identification of RNA-binding proteins is therefore a promising avenue for both basic and biomedical research. Previously, the identification of protein-RNA interactions relied mostly on electrophoretic mobility shift assays. However, these assays have limited throughput and are biased against transient interactions, since dissociation occurs early during electrophoresis for weak interactions. By means of applying protein arrays, comprehensive data sets are starting to be accumulated. In recent years, several studies investigated the RNA-binding properties of proteins, especially in yeast. A proteome-wide search in yeast revealed unexpected RNA-binding proteins [67] and some 200 RNA-binding proteins were identified in another study [29]. Interestingly, more than 50% of the proteins were enzymes involved in intermediary metabolism. This indicates a possibly dual activity of some metabolic enzymes, which may also have regulatory activities [29]. A screen with human proteins identified 137 protein-RNA interactions for 10 coding and non-coding RNAs [68].

Analysis of Posttranslational Modifications

The analysis of protein posttranslational modifications is one of the most challenging issues in proteomics [69]. The question of which proteins are likely to undergo posttranslational modifications and at exactly which position of a protein the modifications will occur are an area of research that could not be dealt with easily using conventional proteomic methods. With the introduction of protein arrays, it is now possible to screen hundreds to thousands of proteins for potential posttranslational modifications and characterize the exact site of modification.

Protein glycosylation

Glycosylation is one of the most general modifications that occur to membrane proteins and ensure the proper localisation of a protein as well as its folding and stability in a cell membrane. In addition, the glycosyl moiety of most heteroproteins serves in cell-to-cell adhesion and is frequently present in surface markers and antigens. Protein glycosylation was studied in yeast using lectin protein arrays; a total of 534 glycosylated proteins was identified [70]. While 406 of the proteins had been known to be glycosylated, this fact was new for a still rather large fraction

Protein ubiquitination

In most eukaryotes, ubiquitination is another prominent posttranslational modification and involved in several cellular processes, such as proteostasis, organelle biogenesis, immune response and cell cycle control [71]. A protein microarray based approach used HECT domain E3 ligase and protein Rsp5 associated with E1 and E2 enzymes to determine protein substrates for ubiquitination. About 90 novel substrates were identified, and some of them validated *in vivo* to be Rsp5 substrates [72].

Protein acetylation

It is now well established that histone acetylation plays a crucial role in the regulation of chromatin structure and hence gene expression. The reactions are mainly catalyzed by histone acetyltransferases and histone deacetylases. These enzymes are probably not only involved in histone acetylation and deacetylation, but might target other non-histone proteins as well. A typical example is the yeast Esa1 catalytic subunit of the NuA4 histone acetylase that acetylases histone H4, which is the only essential histone acetyltransferese in yeast. Without other essential acetyltransferases known to date, it is likely that this enzyme has several targets. Lin et al. [73] developed a protocol for performing acetylation reactions on yeast protein arrays in an attempt to identify non-histone substrates of the NuA4 complex [74]. In this study, 91 substrates could be identified. *In vivo* validation of 20 selected candidates defined several Esa1 substrates. Recently, Lu et al. [75] undertook the characterization of yet another non-histone substrate (Sip2), one of three regulatory beta subunits of the yeast orthologue of the AMP-activated protein kinase. In this study, the authors were able to show that intrinsic aging stress, which is signalled by the Sip2-Snf1 acetylation, is a second TORC1-dependent pathway that regulates Sch9 activity and hence life span.

Protein nitrosylation

Protein S-nitosylation is an enzyme-independent biochemical reaction, which is very important for some proteins. It was possible to identify many cysteine-rich alpha helices and potential acceptors after treatment of arrayed proteins with S-nitrosothiol, an NO^+ donor in nitrosylation reactions [76].

Protein phosphorylation

In cellular metabolism and signal transduction, protein

phosphorylation and dephosphorylation play a major role as they can switch a protein from its active conformation into an inactive form and *vice versa*. For its regulative importance, protein phosphorylation is a posttranslational modification that has been studied extensively by array-based approaches. Methods have been developed for the global identification of kinase substrates on proteome chips [32,44,54]. Moreover, some studies went further and aimed at analysing enzyme kinetics using protein kinases and ^{32}P-ATP. Such studies have resulted in a rather precise determination of kinetic parameters [28,77]. Further advances in the technology required for the detection of phosphorylated proteins may help fostering the use of protein arrays in this analysis area; developments are still frustrated by detection limitations.

Protein Arrays in Biomedical Research

Biomarker discovery and functional analyses

Beyond basic research, protein arrays have also been used for the discovery of marker molecules that could be applied for disease diagnosis and prognosis, including cancer, inflammatory bowel disease, hepatitis or viral infection [78-83]. Also, antigens of several human autoimmune diseases were found, including the diseases systemic lupus erythematous and rheumatoid arthritis [30]. For understanding pathogenic mechanisms, functional aspects are critical. To such ends, the elucidation of disease-related protein interactions provides valuable information. Protein arrays were successful to establish the p53/63 interactome [25], for example. It is now possible to quantify protein interactions in a high-throughput format [84]. This is very important, as it permits to understand the relative affinities and hence strengths with which individual proteins bind to each other. A particularly fascinating application is the study of host-pathogen interactions. This approach has actually helped to understand, how some pathogens succeed in establishing infection of their host and survive the immune system reaction. Using proteome microarrays, in-depth information has been acquired about the underlying mechanisms of viral and parasitic infections, such as influenza and malaria [27,33,85-87].

Drug discovery

In the field of drug discovery, arrays were used for target identification and validation [88,89]. Huang et al. [88] were first to describe the use of protein microarrays for small molecule screens. They looked for molecular targets of rapamycin inhibitors and identified novel members of the TOR signalling pathway in this study. [88]. Since it is possible to measure the interactions of proteins and small molecules, drug off-target effects can be determined and better understood.

Current Technical Status and Challenges

Despite many technological advances in recent years, there are still technical issues that need to be addressed in order to broaden the width of application of protein arrays and to improve measurement accuracy further. Protein expression and purification, protein immobilisation, orientation, structure and functionality, as well as the modes of detection are three important points in this respect.

Protein expression and purification

Compared to nucleic acids, it is much more difficult chemically to synthesize proteins that are fully functional. Protein folding and in many cases relevant posttranslational modifications are crucial but not easily achieved. Even the basic yield and cost of peptide synthesis and ligation is prohibitive at current. In addition, much knowledge is still missing about the modifications required to make a protein functional. Therefore, the production of protein arrays is dependent on protein expression that is based on cellular components rather than chemical synthesis, and fitting processes for purification. There are two main alternatives: proteins are expressed in a cell or *in vitro* by taking advantage of the cellular transcription and translation machinery.

Several methods have been developed for high-throughput protein expression in *E. coli* and yeast [90,91]. Subsequent purification is frequently achieved by conventional processes, which make protein isolation cumbersome and costly. Alternatively, the open reading frames are cloned into sequences that encode for peptide or protein tags, which allow a relatively simple purification [47]. This fusion has several advantages, including a high degree of proper protein folding and thus functions and controlled orientation during the attachment onto the array surface. Arrays made of some 4,000 proteins with a 6x-His fusion were produced this way, for example [92]. A major difficulty of this approach is the fact that the genes must be cloned into expression vectors. Due to differences between individual proteins, it is also often very difficult to establish optimal expression conditions for all genes of interest. More so, some proteins are toxic to the host cell and cannot be expressed; proteins with signal sequences might be secreted or integrated into membranes and therefore impossible to purify.

As an alternative, several groups exploited cell-free expression systems for high-throughput protein synthesis. Both prokaryotic and eukaryotic systems are available for cell-free protein expression from DNA templates generated as PCR products, such as wheat germ extracts [93], rabbit reticulocytes [94] and S30 *E. coli* systems [95]. *In vitro* synthesis reduces the workload substantially, as cloning steps are avoided and reaction volumes can be adapted [96]. An example of such an approach is the cell-free expression of 14,000 human proteins in a 96-well plate format [11,12]. Printing protein arrays from such a resource has been reported by several groups [15,85].

A modification of the above is a process termed protein *in situ* arrays (PISA), which combines *in vitro* protein expression, purification and placing on the array surface [16]. Genes are arrayed on a surface and transcription and translation takes place *in situ*. Several format variants exist, in particular the DNA array to protein array (DAPA) process, the nucleic acid programmable protein array (NAPPA) format, production by multiple *in situ* spotting technique (MIST), and the *in situ* puromycin capture from mRNA arrays [41,97-100]. These techniques significantly facilitate the production of protein arrays, increase throughput and minimize cost. However, production of long proteins at full-length may sometimes be problematic. Also, the proteins at each spot may not be pure and lack posttranslational modifications. The latter could be addressed at least in part by the utilisation of eukaryotic expression systems. At present, however, their relatively low protein yield makes them inadequate for high-throughput applications. As *E. coli* strains were optimized so as to express relatively large amounts of human proteins [101], future modifications may permit similar improvements of the eukaryotic systems, too.

Similar to the spotting of proteins expressed separately, *in situ* synthesised proteins may not resemble the appropriate three-dimensional structure and thus lack functionality. This could result in false-negative interaction results and therefore erroneous conclusions. The open and flexible nature of cell-free expression systems, however, allows supplementing reactions with additives for improved synthesis

results. Lipids and other membrane components have been used in cell-free systems, for example, to achieve synthesis of functional membrane proteins [102]. Sticky proteins, which would not encounter each other in a cell under normal circumstances, but may do so on a protein chip, could lead to false-positive signals. Measuring on- and off-rates in comparison to well characterized control interactions could help minimizing the number of false results, although it is practically impossible to eliminate them entirely. Generating arrays containing proteins whose activities could directly be queried, such as kinase substrates, RNA-binding proteins or other receptors, could permit an estimate of the actual percentage of functional proteins present on an array. This would provide only an overall figure, however, and no information about individual proteins, apart from those whose structures were actually analysed.

Protein immobilisation

In most reports to date, protein immobilisation relied on unspecific capture or crosslinking using naturally occurring reactive groups of amino acid side chains [103]. For several types of analysis, lack of orientation may actually be advantageous, since all sides of a molecule are thereby available to binding of another molecule. For other application, orientation may be preferential. For an oriented attachment, frequently affinity capture is being used. During or past expression, proteins are being fused to affinity tag molecules such as biotin, histidine or glutathione S-transferase (GST). The tags have the added advantage of permitting purification before or during immobilisation. However, the strength of the interaction between the fusion tag and its receptor on the surface may not be sufficient to withstand highly stringent washing steps; the required coatings of the array surface also generate background signal. Additionally, a chemical ligation of biotin to some amino acid residues in proteins, such as lysine, could interfere with protein structure and activity. Fusion to GST necessitates cloning steps again; and in some cases, GST interfered with protein folding [104]. For antibody attachment, protein A and G coated surfaces have been reported [105] but better results were actually achieved with epoxysilane surfaces [106]. Thioredoxin, maltose-binding protein, and chitin-binding proteins have been engineered for the immobilisation of fusion proteins, and DNA-directed immobilisation was developed. However, the shelf life of these types of arrays is rather short and the immobilised proteins are less stable, because these reactions are all reversible [107-109]. Site-specific chemical ligation is another alternative for protein immobilisation [110]. In most cases, however, the reactive group has to be incorporated into the protein during synthesis and needs to be highly chemo-selective under physiological conditions, which is difficult to achieve.

Signal detection

Signal detection is still a concern, in particular in view of routine application. Labelling process and label-free detection strategies both have advantages and drawbacks. The detection of fluorophors is well established with sensitivities in the fM range or down to the detection of single-molecule binding events [35]. Labelling with the rather large fluorescent dyes can affect protein structures and particularly binding affinities to other molecules. Radioisotope labelling could circumvent this but is difficult in terms of handling and inappropriate for most settings. Nevertheless, as of now, it is the only possible process for some analyses such as phosphorylation and acetylation assays [75]. For fluorescence-based detection, it is possible to further enhance detection sensitivity with the application of rolling cycle amplification, during which labelled nucleotides are incorporated [111]. As an alternative to fluorescent dyes, quantum dot labelling has been successfully applied [112,113]. To avoid direct labelling of the analysed sample, indirect procedures are employed, utilising labelled antibodies for detection. This method does not allow quantification, however, and requires the availability of protein-specific antibodies.

All label-dependent strategies have the big disadvantage that they are not really suited for a real-time investigation of the binding reactions [14]. Imaging surface plasmon resonance [114], reflectometric interference spectroscopy and ellipsometry [115] are examples for label-free detection procedures that have been applied to protein arrays [116]. By measuring the optical dielectric response on a thin film, these methods can detect changes in the physicochemical properties of the film, such as mass density and thickness, during a biochemical reaction and hence provide real-time information about a binding event [117-121]. Essentially, these methods are extremely sensitive, with a detection limit of 10 to 20 fg and a time resolution of about 20 μsec. However, they require highly sophisticated equipment and array surfaces and may not be competitive for routine applications.

MALDI-MS has recently been applied to antibody microarrays for analyses of protein glycosylation, depositing the matrix onto the antibody spots subsequent to protein binding and on-chip digestion [122,123]. The establishment of mass spectrometry as a label-free detection method would greatly advance the analysis process from the mere detection of a binding event by simultaneously characterising the binding partner and its posttranslational modifications.

Data Analysis

Several algorithms and bioinformatic resources exist for the analysis of data resulting from protein array experiments; many of them originate from research in the field of DNA arrays [124]. Usually, analysis starts with spot finding, which in most cases is achieved with the help of commercial software that is part of the relevant detection system. Subsequent steps are background correction and data normalization [125], before statistical analyses are performed. The software package Chipster is one of the systems that is commonly used to such ends, featuring many relevant processes [126]. However, also tools that were originally developed for other purposes, such as flow cytometry based proteomics [127], can be adapted and utilized more widely, while other software packages provide instruments useful mainly for a specific application, such as reverse phase array analysis [128]. Next to experimental measures to reduce the degree of false results, computational approaches have been developed to assess their impact [129]. Estimates can be based on a comparison to RNA expression profiles or paralogous proteins [130], or rely on an approximation and partitioning of empirical p-value distribution [131], for instance (Table 1).

Concluding Remarks

As an obvious extension of the DNA-microarrays, protein array technology has developed rapidly during recent years and had already considerable impact. However, there are important differences between DNA and protein arrays, so that the analogy is limited and a direct comparison inappropriate despite the apparent similarity in format. As opposed to the DNA arrays, rather different protein array formats exist. This is not merely a result of different technical possibilities to produce them but more a result of the significantly larger biological complexity of the protein world compared to that of the nucleic acids. While the

Table 1: Applications of protein arrays.

Application	Type of Assay	Sample / Co-Factor	Array Platform	Reference
RNA-binding proteins	Protein-RNA interaction	Fluorescently labelled total or mRNA	Yeast proteome array	[67]
DNA-binding proteins	Protein-DNA interactions	Fluorescently labelled DNA oligonucleotides	Human protein microarray	[134]
Kinase substrate identification	Protein phosphorylation	Radioactive ATP	Yeast protein microarray	[28]
Protein kinase activity	Enzyme kinetics	Radioactive ATP	Peptide microarray	[77]
Protein-protein interaction quantification	Protein-protein interactions	Labelled peptides	Protein domain microarray	[84]
Posttranslational modification	Protein sumoylation	Antibodies	Protein microarray	[24]
Interactome analysis	Protein-protein interaction	Antibodies	Human protein array	[6]
Host-pathogen interactions	Protein-protein interaction	Antibodies	Human protein microarray	[27]
Biomarker discovery	Protein-protein interactions	Antibodies	*E. coli* proteome chip	[79]
Drug discovery	Protein-small molecule interaction	Labelled ligands	Yeast proteome	[88]

latter consist of essentially five nucleotides, proteins are assembled of at least 20 amino acids. Also, protein structures are enormously versatile, while all nucleic acids form essentially a single molecular structure irrespective of their sequence. Technically, the chemical synthesis became the procedure of choice for the production of DNA arrays, since near quantitative yields were achieved [132] and basically all assay formats could be served. For proteins, however, neither chemical array synthesis is feasible at a realistic scale – apart from peptide microarrays with limits to their utility – nor does a process exist that is equivalent to PCR, which is required for many DNA/RNA assay formats. Nevertheless, for some protein array applications, quality measures have been reported that meet or even exceed the requirements defined for clinical applications of DNA-microarrays [133]. Despite the still existent hurdles toward an overall performance that is similar to the accuracy levels achieved with DNA microarrays, the technology has the potential to become a reliable and essential tool for proteomic studies, in particular for an analysis of protein interactions.

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