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Protein Microarrays – A Tool for the Post-Genomic Era

Angelika Lueking[1,2*], Zoltán Konthur[1], Holger Eickhoff[1,2], Konrad Büssow[1], Hans Lehrach[1] and Dolores J. Cahill[1,3]

[1] *Max Planck Institute of Molecular Genetics, Ihnestrasse 73, D-14195 Berlin, Germany*

[2] *Scienion, Volmerstraße 7A, 12489 Berlin, Germany*

[3] *PROT@GEN, Im Lottental 36, 44801 Bochum, Germany*

Abstract: The human genome is sequenced and the challenges of understanding the function of the newly discovered genes have been addressed. For this purpose, high-throughput technologies have been developed that allow the monitoring of gene activity at the transcriptional level by analysis of complex expression patterns of a specific tissue. Differential gene expression can be most efficiently monitored by oligonucleotide or cDNA hybridization on DNA arrays. Recently, protein arrays are emerging to follow DNA chips as a tool to profile protein products encoded by globally or differentially expressed cDNA clones. Array technology was enabled by the development of devices that could array biological samples at high density with high precision onto immobilizing surfaces, ranging from the classic microtiter plate to new chip-sized supports. In addition, the introduction of automated technology to the protein level involves the simultaneous expression of a large number of cDNA clones in an appropriate vector and expression system, allowing the specific detection and purification of all the recombinant proteins. With the ordered arrangement of recombinantly expressed proteins, a direct link to the corresponding DNA sequence information is possible and consequently, clone libraries become amenable to be integrated in a database including all steps from DNA sequencing to functional assays of the translated gene product. Here, we review the generation and application of microarray technology as a highly parallel approach to obtain more information on the regulation of proteins, their biochemical function and potential interaction partners. Already, a large variety of assays based on antibody-antigen interaction exists and in addition, the medical relevance of protein arrays will be discussed. Also, further applications such as protein-DNA, protein-RNA and protein-substrate interactions will be presented, since initial studies on immobilized proteins were reported.

Proteomics is an emerging field to profile protein repertoires. Because there is no reliable correlation between gene activity monitored by genomic studies and cellular protein abundance, application of protein arrays will link both genomics and proteomics.

INTRODUCTION

With the ever increasing pace and number of genome sequencing initiatives, substantial amounts of genetic data is accumulating every year. Unfortunately, sequence information in itself is neither sufficient to provide significant knowledge of the underlying mechanisms of life nor of the biology of organisms. It rather provides a sound basis and framework for further investigations. Meanwhile more than 200 genome sequencing projects are pursued in the public sector worldwide of which roughly 40% are completed, including the human genome [1]. After completion of this first step of genome sequencing, only some of these genes can be assigned a function with certainty, due to extensive research in the past, while for a larger part of the sequences, their function can only be

*Address correspondence to this author at the Max Planck Institute of Molecular Genetics, Ihnestraße 73, 14195 Berlin, Germany; Tel. +49 30 8413 1222; Fax. +49 30 8413 1128; E-mail: lueking@molgen.mpg.de

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proposed on the basis of sequence homologies by bioinformatics [reviewed in: 2]. However, even more than a third are completely unknown, with no suggested function [3]. Therefore, researchers of different functional genomics projects have begun to evaluate these functional predictions and to investigate these unknown genes. However, it has long been understood, that the "one gene – one protein hypothesis" is no longer valid and that the proteome, the world of proteins, is much more diverse and complex than the genome repertoire. It has been estimated that due to splicing, protein and peptide cleavage, multi-protein complex formation and post-translational modifications such as glycosylation and phosphorylation, there are about five times more different proteins in an organism than genes [4]. Analogous to genomics, the study of all protein species of an organism or a specific type of tissue, is termed proteomics.

The classical method of gene expression studies in terms of proteomics is two-dimensional gel electrophoresis (2-DE) [5,6] which is often combined with subsequent protein identification by mass-spectrometry (MS) of excised protein spots, e.g. matrix-assisted laser desorption ionisation time-of-flight analysis (MALDI-TOF) [7]. In 2-DE, proteins are separated according to their charge and mass. This technology is widely applied to the comparative study of expression patterns, e.g. of two differently specialized or diseased versus normal tissues [8] and even for the comparison of related pathogenic versus non-pathogenic organisms [9]. Unfortunately, the design of such experiments is crucial, since the protein complement detected is very sensitive to even slight changes in culture growth, extract preparation, running conditions and gel composition. This is one of the reasons why genomic, nucleic-acid-based approaches such as differential expression profiling and subtracted library generation are increasingly used for the evaluation of expression patterns even though mRNA levels are not to be set equivalent with translated protein levels [10,11]. Still, microarray based technologies in the field of functional genomics and proteomics are growing due to the versatility of applications, time and cost effectiveness, feasible automation and high degree of parallel analysis possibilities and is expanding from the DNA level onto the protein level. Several applications of protein arrays have been reported, from the simple identification of expression clones to binding studies [12] and to antibody arrays.

In this review, we will discuss high-throughput protein expression and purification strategies, robotic production methods for protein micro-arrays and binding chemistry, their field of application in terms of medical relevance and their ability to directly bridge genomics and proteomics.

EXPRESSION AND PURIFICATION

For the generation of protein arrays, large numbers of proteins are required. High-throughput sub-cloning of open reading frames has been described, but the progress of this approach depends on the availability of sequenced genomes [13]. Also it is not always possible to determine the expressed sequence from the genome sequence due to differential splicing and post-translational processing**.** Moreover, studying tissues or complete proteomes of higher organisms remains difficult. These problems can be solved by the generation of arrayed cDNA expression libraries leading to thousands of cDNA expression products in parallel.

In our laboratory, we have developed the automated technology to express proteins from arrayed cDNA expression libraries cloned in bacterial and yeast expression vectors. Generally, these expression vectors carry an inducible promotor allowing controlled protein expression as well as sequences coding for affinity tags (e.g. His-tag, GST-tag, Strep-tag). This allows the specific detection of the recombinant fusion protein using antibodies against this epitope tag that can also be used for protein purification by immobilized metal affinity chromatography (IMAC).

Using the bacterial system, *Escherichia coli,* we have shown that a human fetal brain expression library can be screened in parallel for either DNA hybridization, protein expression and for antibody screening in a high-density array format on filter

membranes [12]. The recombinant proteins have been expressed and purified in high-throughput [14,15]. However, the expression of eukaryotic genes in this system often leads to the production of aggregated and denatured proteins, localized in inclusion bodies [16,17] and therefore functional studies are limited.

To overcome this problem, we have developed yeast expression systems in *Saccharomyces cerevisiae* and *Pichia pastoris* that allow the soluble expression of recombinant protein in high throughput [18], Holz *et al.* submitted]. In an alternative approach, all open reading frames of *S. cerevisiae* were cloned and expressed in yeast, and purified as GST fusion proteins to study biological activity [19]. Three previously unknown genes have been identified by their biochemical activity as a cyclic phospho-diesterase, an Appr-1 processing activity and a cytochrome c methyl-transferase. In a large-scale yeast two hybrid screen consisting of 5,345 full-length proteins of *S. cerevisiae* several novel protein-protein interactions were identified for previously screened proteins using randomly generated inserts, underlining the importance of full-lengths open reading frames [20]. Zhu *et al.* have created protein arrays of *S. cerevisiae* kinases [21]. In total, 119 proteins kinases were expressed, purified as GST fusion proteins, arrayed and cross-linked on a protein chip and assayed for auto-phosphorylation by treatment with radiolabelled ATP.

In less characterized systems such as tissues or complete proteomes, cDNA clones have to be made available in order to express their corresponding proteins. The use of cDNA expression libraries eliminates the need to construct individual expression clones for every protein of interest. However, since cDNA fragments contain 5´ untranslated sequences or can be fused to the vector-encoded translation initiation codon in an incorrect reading frame more than 2/3 of the clones do not express their insert [12]. To facilitate the generation of a protein expression subset, this requires the identification of non-expression clones, which can be routinely performed by the detection of the affinity tag fused to the gene product. The identified expression clones can then be rearrayed into a new subset of the expression library.

To circumvent these steps of identification and rearraying, there are several efforts to develop a system enabling the selection of cloned cDNA inserts containing open reading frames fused to the affinity tag in the right reading frame. Bacterial expression systems have been constructed on the basis of the genes coding for -galactosidase and for kanamycin resistance [22-25]. However, only small DNA fragments in the range of 100-300 bp. could be enriched using these systems. Because of the differences of the bacterial transcription/ translation machinery and the presence of polycistronic mRNA in prokaryotes, and the re-initiation of translation the expression of larger cDNA fragments leads to an increasing number of genes in incorrect reading frames. To apply a sort of screening system to large cDNA inserts we have developed an open reading frame (ORF) selecting system in the yeast *S. cerevisiae* that shows as a eukaryotic host translation initiation only at the capped 5´end of the mRNA [Holz *et al.,* submitted]. In this system, we have constructed a human fetal brain expression library enriched with 60% of expression clones when compared to approximately 14% in the *E. coli* expression systems [14].

The previously described protein filter technology was further developed to increase spot intensity as well as the density of the arrayed proteins. Liquid bacterial expression cultures were automatically arrayed using a pin-based device mounted to a flat-bed gridding robot [14]. For this purpose, 96 proteins of the human fetal brain library hEx1 were expressed in liquid cultures, and the crude lysates were spotted onto a polyvinyl diflouride (PVDF) membrane. Recent developments include the use of purified proteins in appropriate solutions combined with either high-speed picoliter dispensing (ink-jetting) or spotting devices and placed onto new surfaces on a microscope slide scale.

ROBOTIC PRODUCTION OF PROTEIN ARRAYS AND BINDING CHEMISTRY

A very crucial part in protein array technology is played by the deposition of the proteins on a suitable surface. The character of the surface charge, viscosity, pore size, pH, binding capacity, unspecific protein binding etc plays an essential role for the generation of protein arrays that contain the proteins in a biologically active shape and form.

The density of protein molecules immobilized on the support is mainly determined by the surface structure. Protein immobilization on a flat, two-dimensional surface was achieved by either covalent coupling to a cross-linker attached to a surface [26-28] or non-covalent interaction to an immobilized biomolecule like biotin [29]. Recently, an oriented immobilization of either small molecules or proteins on chlorinated glass slides have been described [30]. The presence of chlorine on the surface of the glass slides results in the specific capture of alcohol groups. By attaching alcohol groups to small molecules or proteins like biotin or digoxigenin an orientated immobilization has been detected. In initial screens a specific protein-protein interaction has been identified.

A flat, two-dimensional surface offers less binding capacity than the three-dimensional structure of a filter membrane or a polyacrylamide gel layer [31]. The gel pads are separated by a hydrophobic glass surface and provide a native, aqueous environment and can accommodate proteins of up to 400,000 Daltons in size. Enzymatic activity of several enzymes like horseradish peroxidase, alkaline phosphatase and -D-glucuronidase has been detected in these hydrogel pads. Pre-structured surfaces consisting of hydrophilic spots on hydrophobic surfaces have also been reported for protein arraying [32]. The hydrophobic surface prevents the aqueous drops applied to the hydrophilic spots from mixing and creates wall minimized reaction vessels, where the interactions can be monitored in solution. In combination with state of the art micro-fabrication procedures, pre-structured surfaces allow the introduction of three-dimensional microstructures on a chip, offering a number of additional options for experimentation like on-line monitoring of the interaction kinetics. In addition, microfluidic devices can be equipped with channels for transporting reagents to immobilized target molecules [33].

Our laboratory mainly uses solid pins routinely for spotting nanoliter volumes of proteins. Alternative deposition methods utilize either split pin-based spotting or micro-dispensing devices. In contrast to other techniques, solid pins are less sensitive to variation of sample viscosity than slit pins or micro-dispensing systems and are much easier to clean to prevent any cross contaminations. Compared to the pin and ring technology used by MacBeath and Schreiber to produce a microarray of 10,800 spots of two distinct proteins (protein G and an FKBP12 binding domain), this system enables one to array 2500 different proteins in less than 4 hours [34]. As an alternative to modified glass surfaces, gel immobilization matrices show high binding capacities and provide the proteins with a nearly native environment, due to a content of approximately 97% water in the immobilization area. This is a key feature in protein array research and is important for the reactivity and specificity of the arrayed proteins. The technique of immobilization is substantial, both for effective concentration and orientation of immobilized proteins or antibodies on the surface.

APPLICATIONS

Microarrays allow fast and parallel data analysis, miniaturization and automation. Hence, a number of new applications have become available, which could not be performed with earlier technology (Fig. **1**). At the moment, detection of immobilized antigens with antibodies is still the most common application. In general, antibodies are stable, easily modifiable molecules, which recognize protein epitopes and bind their target with high affinity and specificity. As shown by Lueking *et al.*, [34] the binding specificity of selected monoclonal antibodies (anti-HSP90 , anti- -tubulin) were determined following incubation on protein microarrays consisting of 96 proteins of the hEx1 library. These proteins had

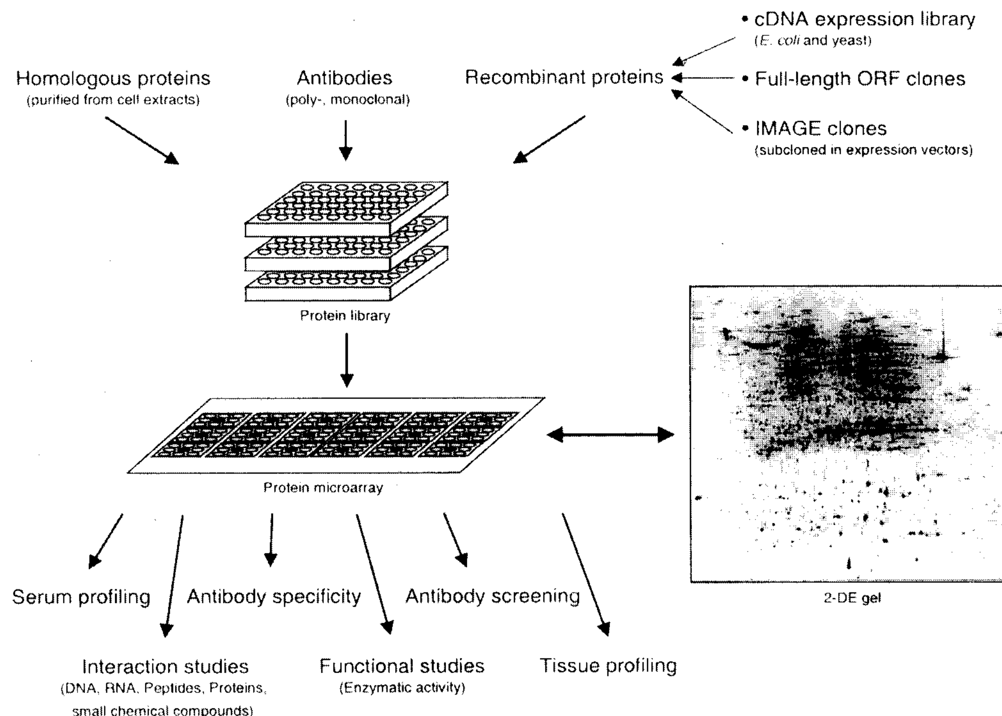

Fig. (1). Generation and application of protein microarrays.

been expressed in liquid bacterial cultures, and the cross-reactivity of these antibodies to other proteins was shown [14]. This is not surprising, as antibodies are not usually tested against whole libraries of proteins. However, in immunohisto-chemical or physiological studies against whole cells or tissue extracts such cross-reactivity of antibodies can lead to false interpretations.

When antibodies are used extensively as diagnostic tools, the characterization of their binding specificity is of prime importance. Antibody-antigen interactions can be exploited in medically relevant concepts such as autoimmune diseases. Screening protein arrays with sera from autoimmune patients would not only allow the identification of potentially new auto-antigens, but also the diagnosis and sub-typing of the autoimmune disease based on the presence of specific auto-antibodies, hence profiling the antibody repertoire of patients with autoimmune disease.

Until now, patient sera were hybridized to uncharacterized gt11 phage library plaque-lift filters to identify auto-antigens, or to tissue extracts separated by 1D or 2D gel electrophoresis [35,36]. But further characterization of the identified antigens may be problematic. It is very labor intensive and, although mass spectrometric identification of separated proteins has improved greatly, characterization often requires expensive protein sequencing which in turn needs high quantities of purified protein. Nevertheless, McCurdy *et al.* were successful in attributing a novel function to a protein, which in turn is a potential therapeutic target [37]. Utilizing microarray technology for the profiling of such auto-antibodies would greatly simplify this process, since protein arrays contain large number of proteins the sequence of which can be determined as they come from an ordered recombinant source. Moreover, using protein arrays for diagnostic purposes would minimize variations occurring in natural tissue extracts, therefore increasing reproducibility. Once disease-specific antigens are known, it is possible to create small, easy to evaluate diagnostic protein arrays. Joos *et al.* modified the classical HEp-2-test using a protein array with 20 different antigens and several control proteins spotted in various dilutions. Using minute quantities of patient sera (1µl /sample), 35 clinically characterized patients

have been analyzed and their disease state was confirmed [38].

Antibody-based immunoassays are a commonly used type of diagnostic assay and can be used for quantification of the corresponding antigen, as well as for bioassays using whole cells or tissue extracts. Previously, the enzymatic activity of various immobilized antibodies like horseradish peroxidase, alkaline phosphatase and protein kinase have been shown [31,34]. Antibody-microarrays allow the simultaneous detection and quantification of cognate antigens present in complex protein mixes at concentrations as low as in complex physiological samples like blood or urine [39]. Surface enhanced laser desorption-ionization (SELDI, Ciphergen Biosystems) protein chips coated either with antibodies or charged or hydrophobic groups for protein desorption followed by MALDI-TOF mass-spectrometry on the chip were used to identify the captured interaction partner from complex probes [40-42]. Alternatively to the antibodies of immunized animals phage display antibody libraries have been developed for the in vitro selection of antibodies [reviewed in: 43]. For this purpose, recombinant human immunoglobulin gene libraries are cloned in phagemid vectors and expressed on the surface of bacteriophage [reviewed in: 44]. Recently, antibody fragments from a phage display antibody library were used to screen for new specific antigens on protein arrays of the human fetal brain cDNA library [12,45]. From 12 randomly selected antibody fragments on an array of 27,000 expression clones, four novel and specific antigen-antibody pairs were identified.

In a complementary approach, antibody arrays were used for the identification of specific antibody-producing bacteria [46]. For this purpose, a single round of phage selection on the chosen antigens was performed followed by the antibody array screening. By screening 18,342 bacterial antibody clones in parallel, highly specific antibodies were selected after just one round of panning.

In addition to antibody-antigen interaction studies, there are increasing demands to analyze additional molecular interactions. Using a tomato cDNA expression library cloned in a lambda phage, new viroid-RNA binding proteins were identified [47]. By screening a Jurkat cDNA library with DNA probes containing specific binding sites several transcription factors have been identified [48]. In a more systematic approach Ge employed a low-density UPA (Universal Protein Array) system for studying interactions with protein, DNA, RNA and small chemical ligand probes. Human p52 was probed against 48 purified proteins spotted onto nitrocellulose membrane and by washing the membrane with different salt conditions, high-affinity protein-protein interaction could be distinguished [49]. In a recent approach the interaction of a restriction enzyme with double stranded DNA was monitored on a micron-sized monolayer surface using atomic force microscopy [50].

The identification of a protein as a potential interaction partner or as a drug target requires often the further structural analysis such as crystallization or NMR studies. Meanwhile, structural genomics aims at the automation of three-dimensional structure determination of proteins. [reviewed in: 51, 52]. Large numbers of proteins in high amount and purity are a major requirement for Structural Genomics projects. Protein array technology enables researchers to characterize thousands of recombinant proteins in parallel prior to scale-up of expression followed by crystallization. Often proteins of interest cannot be easily expressed in sufficient yield, purity and solubility. Waldo *et al.* reported the generation of libraries of mutants of proteins with unsatisfactory expression properties [53]. The mutants were fused to green fluorescent protein (GFP). On a protein array format, GFP fusion proteins with good solubility were detected due to their strong fluorescence.

PROTEIN ARRAYS TO BRIDGE GENOMICS AND PROTEOMICS

Currently, in proteomics individual proteins are identified in sequence databases using mass spectrometrically determined peptide maps,

sequence tags, or fragment-ion fingerprints of individual proteolytic cleavage products. This approach is widely used to identify proteins separated in 2-DE gels. Due to sequencing errors in the existing DNA databases and the redundancy of the genetic code leading to incorrect conversion of the amino acid, the identification of the protein is partly limited.

To overcome these limitations and to improve the identification of proteins, we have proposed [54] and shown [Schmidt *et al.*, submitted] a new concept, which also can be seen as another application for the recombinant clones used to generate a protein array, and to link current genomics (DNA microarray) and proteomics (2-dimensional gel) fields. This approach involves the characterization of each protein by a minimal set of structural information generated by mass spectrometry, previously termed "Minimal Protein Identifier" (MPI) [55] which is stored in a database.

We have shown that MPIs can be generated from any sources of protein, such as recombinantly expressed proteins and from homologous proteins separated in 1- or 2-dimensional electrophoresis. This method can be facilitated if MPIs are generated form a large number of arrayed proteins, such as from a UNIclone set which provides a permanent source for the generation of protein and cDNA microarrays enabling the direct linking of protein spots on 2-dimensional gels with the corresponding sequence, and the gene cloned in the correct reading frame in an expression vector.

The success of this approach requires not only the generation of a database of MPIs, but in addition, the integration of a number of established technologies, such as high-performance micro-arraying, large-scale protein expression and high-resolution 2-DE. Improvements would be made by developing new technologies, such as higher throughput or full automation of 2-DE, gel imaging, spot recognition, spot-excision followed by protein proteolysis, sample purification and mass spectrometric analysis and software for data acquisition and processing.

FUTURE

Protein arrays are emerging as new tools in the armoury of functional genomics and proteomics. Current applications of protein arrays include the determination of antibody binding specificity, serum profiling and antibody screening. In future, protein arrays may have much broader applications, for example, antibodies can be arrayed and used to profile protein expression in diseased and normal tissue analogous to differential hybridizations. Furthermore, future applications will include the determination of protein-protein interaction partners and biological activities.

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