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PROTEIN MICROARRAY TECHNOLOGY

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TABLE OF CONTENTS

1. ABSTRACT

This review summarizes the major activities in the field of protein microarray technology. A short summary of the theoretical concepts of miniaturized ligand binding assays explains why such microspot assays represent the most sensitive approaches for capture-target assays. The main focus of this review is centered on the applications using miniaturized and parallelized protein binding assays which rely on the product formation between immobilized capture molecules and their corresponding target molecules which are present in the sample. These types of ligand binding assays are useful tools for protein identification, quantification and protein affinity studies. Protein identification and quantification assays have a great potential in the field of diagnostics and proteomics where many different protein markers which are present in complex samples have to be analyzed in parallel. Protein affinity assays can be used to analyze interactions between proteins such as antibodies, receptors or enzymes with other proteins, peptides, low molecular weight compounds, oligosaccharides or DNA. Different applications of protein microarray-based assays and their huge potential for diagnostic and proteomic approaches will be discussed.

2. INTRODUCTION

The basic principles of protein micro array assays were already described at the end of the eighties by Roger Ekins (1) for immunological microarray assays. The interest in microarray-based assays increased enormously with the development of DNA chip technology. The possibility to determine thousands of different binding events in a massively parallel fashion in a single experiment perfectly suited the needs of genomic approaches in biology. The rapid progress in whole genome sequencing e. g. (2, 3), and the increasing importance of expression studies (EST sequencing) was matched with

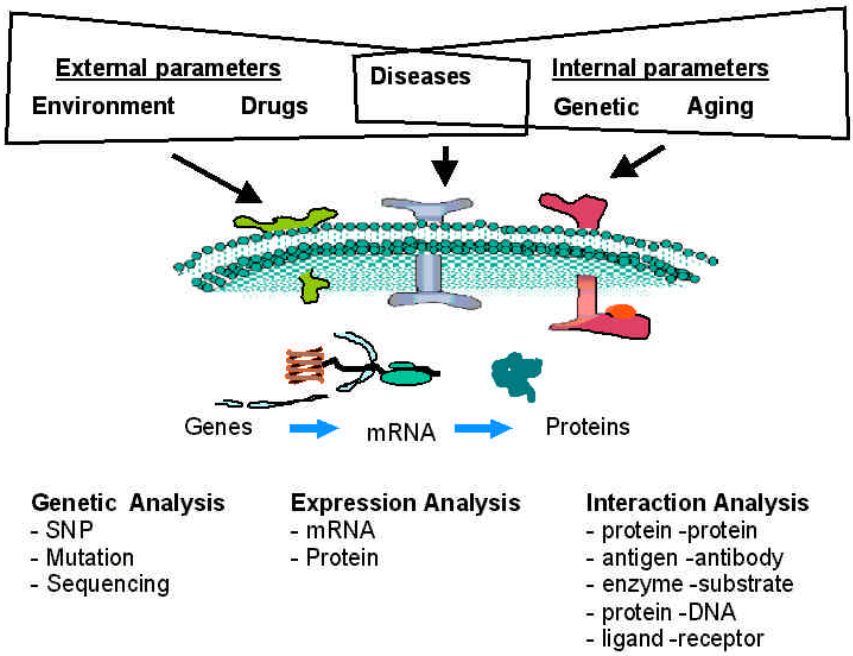

Figure 1. Different physiological states of cells correlate with different cellular protein expression and with differences in protein function – the cellular proteomes. Interactions of a cell with its environment are influenced by several parameters in parallel e.g. cell-cell and cell-matrix interactions. Cellular processes controlling and defining the physiological state of a cell regulate mRNA and protein expression, control protein modification, localization and therefore protein function. Analysis of the dynamic proteome of a cell is a very laborious process, if not impossible. Microarray technology offers new tools to monitor complex intracellular gene and protein expression and allows massive parallel protein interaction analysis. DNA-microarrays are used for genetic analysis as well as expression analysis on mRNA level. Protein microarrays are employed for protein identification and quantification as well as for protein interaction analyses.

efficient *in-vitro* techniques to synthesize specific capture molecules for ligand binding assays. Oligonucleotide synthesis and PCR amplification allows the generation of thousands of highly specific capture molecules in a very efficient way. New trends in technology, mainly in microtechnology and microfluidics, newly established detection systems and improvements in computer technology and bioinformatics have been rapidly integrated into the development of microarray based assay systems. Nowadays, DNA-microarrays, some of them built from tens of thousands of different oligonucleotide probes per square centimeter, are well-established high-throughput hybridization systems for the generation of huge sets of genomic data within a single experiment. Their application in the analysis of single nucleotide polymorphisms and in expression profiling has already changed pharmaceutical research. The use of microarrays as diagnostic tools will certainly have a great impact on medical and biological research.

As is known from gene expression studies, mRNA levels and protein expression are not necessarily correlated (4-6). Protein functionality often depends on the post-translational processing of the precursor protein. Regulation of cellular pathways are frequently regulated by specific interactions between proteins and/or by reversible covalent modifications such as phosphorylation or glycosylation. To get detailed information on a complex biological system, information on the state of many proteins is required. The analysis of the proteome of a cell, i.e. the quantification of all proteins and the determination of their post-translational modifications in dependence of cell-state and environmental influences (Figure 1), is not possible without novel experimental approaches. High throughput protein analysis methods which allow a fast, direct and quantitative detection are needed. The current state-of-the art protein analysis technologies, such as 2D-PAGE combined with mass spectrometry, have still to cope with significant drawbacks before they are able to fulfill all the needs for high-throughput proteome analyses. Therefore, enormous efforts have been undertaken to expand microarray technology beyond DNA chips and to establish protein array-based approaches for proteomics also (7-9). Protein microarrays might be the future tools for proteome analysis and individualized proteome-based diagnostics.

The skills of biologists, biochemists, chemists, material scientists, engineers, bioinformatics and physics have to be combined within multidisciplinary projects to generate protein microarrays which can be used successfully for diagnostic or proteomic applications (Figure 2). The microarray technology field is very dynamic, competitive and fast growing. Technologies discussed in this review will only represent the actual situation and give an overview of future trends and chances. The challenge has only just begun!

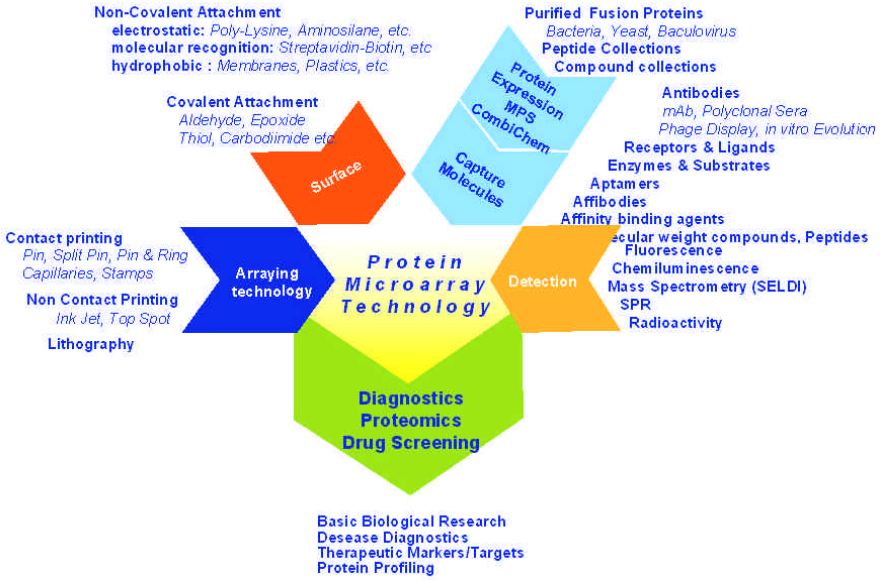

Figure 2. Different technologies must be combined to generate protein microarrays for diagnostic and proteomic applications. First, the chemistry for the protein attachment on the surface has to be optimized in order to keep the protein probes in place and in a functional state. Furthermore, unspecific binding of proteins has to be reduced. Some of the most common attachment strategies are shown. Second, the high throughput generation of proteins is a prerequisite for high-density protein arrays and for identification of optimal capture molecules. Small compounds from combinatorial organic chemistry and peptides are useful tools for identification of binders or substrate-target interactions. Third, technologies for the production of arrays have to cope with very different conditions of buffers, which are necessary for keeping proteins in an active state. Fourth, sensitive detection systems are a key technology for the detection of low frequency proteins from small sample volumes, because, to date, there is no amplification protocol for proteins available. The combination of optimized technologies from all these fields will generate reliable protein microarrays as tools for diagnostic and proteomic research. Within this field, skills in biology and bioinformatics are required to interprete the huge sets of data and to develop new assay formats and test systems.

3. MICROSPOT ASSAYS – THEORETICAL CONSIDERATIONS

In his “ambient analyte theory”, Roger Ekins already described the fundamental principles of miniaturized and parallelized ligand binding assays. Based on theoretical considerations, it was demonstrated that microspot assays are more sensitive than any other type of ligand binding assay (1, 10). In a ligand binding assay, capture molecules are immobilized to a solid phase. Target molecules are extracted from the sample and bound to the immobilized capture molecules. When the concentrations of capture and target molecules and their association constant are known, the product formation of capture-target molecules can be theoretically calculated according to the mass action law. Of course, one must take into account that the mass action law is only true for product formation of binding partners which are present in solution. For a theoretical consideration of solid phase binding assays, the calculations have to be performed under the assumptions that there will be no diffusion, no mass transport limitation effects and no differences in capture molecule density which could influence the rebinding of dissociating target molecules (11). Such theoretical considerations show that the concentration of capture-target molecule complexes will increase with a larger number of capture molecules, and that the sum signal will reach a maximal value when all the target molecules have been harvested from the sample. This is illustrated in Figure 3. Capture molecules are immobilized with a constant surface density onto spots of increasing size. With increasing spot size, the total number of capture molecules present in an assay increases as well as the sum signal emitted from the spot. However, the signal density, i.e. the signal intensity per area, starts to decrease with increasing spot sizes because the number of targets in the sample is limited. The capture process leads to a significant reduction of target concentration in the solution. At the same time, the formed probe-target complexes are distributed across a larger area. As a result, the maximal signal that can be obtained from any point in a spot is lower. On the other hand a decrease in spot size will lead to a decrease in the overall signal per microspot. The signal density, however, will increase for smaller spots (Figure 3). Below a certain spot size, signal density nearly reaches its optimal value and will remain almost constant even if the spot size is reduced further. In this case, ambient analyte conditions are reached and the total number of target molecules in the sample is no longer a limiting factor. As a result, only a tiny fraction of the target molecules present in the sample solution are captured which does not change their concentrations significantly. Ambient analyte conditions are reached if less than 0.05/K of capture molecules are captured on a microspot (K is the affinity constant of the binding reaction). Ambient analyte

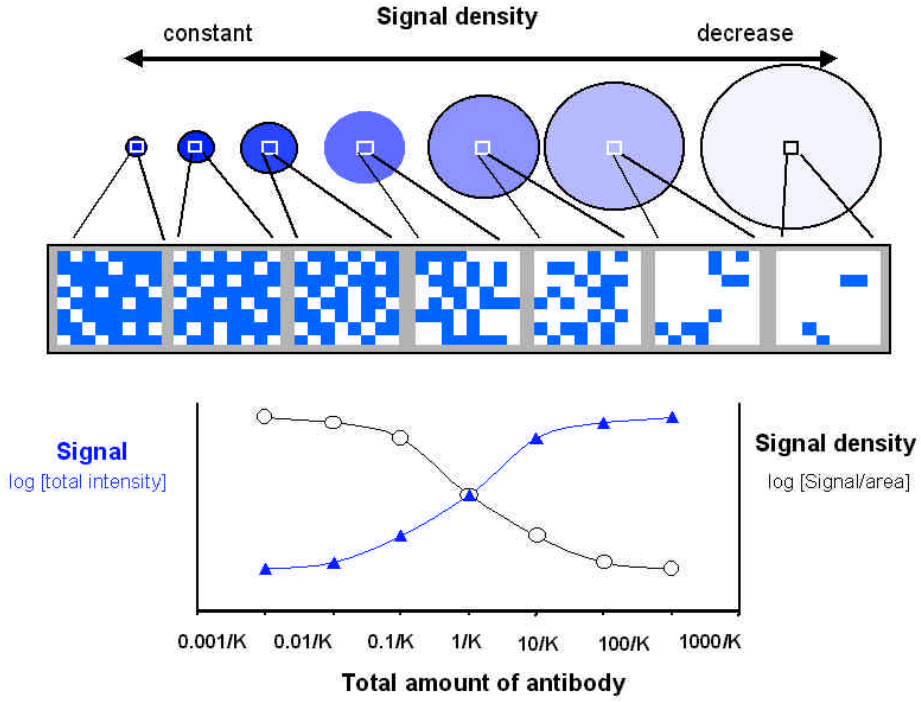

Figure 3. In miniaturized binding assays capture molecules are immobilized on the solid phase in a very small area, the microspot. Assuming constant surface density of the captured molecules, the total signal obtained from one spot will increase with increasing spot diameter, simply because more targets are captured on a larger spot area. When all targets from the solution are captured the signal reaches a maximum. In contrast, signal density increases with a decreasing quantity of capture molecules (decreasing spot size). According to the mass action law, a constant signal per area is reached, when the capture molecule concentration is less than 0.05/K (K is the affinity constant). These considerations are schematically illustrated for signals from microspots of different sizes. The capture molecules on the different microspots are immobilised with the same surface density. Signal density (signal/area, relative intensities, log scale) and signal (total intensity, log scale) are shown in correlation to the total amount of antibody in terms of K (affinity constant). Below a certain spot size, when the capture molecule concentration is less than 0.05/K ambient analyte conditions are reached and the target concentration in the solution is minimal altered by the amount of captured targets on the microspot.

conditions enable the direct measurement of the real concentrations of different target analytes in a sample, as the probe does not significantly influence the free target concentration in solution. The number of target or analyte molecules captured on the microspot directly reflects their concentration in solution. Interestingly, the concentration measurement under ambient analyte conditions makes the system independent of the actual sample volume used. The best sensitivity will be obtained under ambient analyte conditions as the binding reaction occurs at the highest possible target concentration and as the capture molecule-target complex exerts high signal density in the small area of the microspot (Figure 3). Therefore, the highest signal intensities and the optimal signal-to-noise ratio can only be achieved in minute spots of microarray based assays. The major message of the complex ambient analyte assay theory is very simple – small is better !

4. MICROARRAY TECHNOLOGY - FROM DNA TO PROTEINS

Today, biochips are widely used tools in life science research. At present mainly DNA chips or microarrays are used routinely. Protein arrays are still in the developing phase but an increase in the number of publications on protein microarrays clearly demonstrates their large potential. There are many well established technologies such as microarray surfaces, arraying and detection systems that have initially been developed for DNA chip applications. These can now be adapted to protein based microarray experiments. However, all the problems which are not yet perfectly solved in the DNA chip technology e.g. detection of low abundant signals, interpretation of spots of different quality, standardization of detection etc. have to be addressed in the protein microarray field as well. Furthermore,when switching from DNA to protein microarrays one has to keep in mind that DNA and proteins are very different types of molecules (Table 1). DNA is constituted by four different nucleotides, which generate a very uniform molecule with a well-defined structure and a hydrophilic, negatively charged backbone. In contrast, proteins are made from 20 different amino acids, thus resulting in highly diverse molecules with different abilities. Proteins can be hydrophilic or hydrophobic, acidic or basic and therefore interact with their environment via strong electrostatic forces, hydrogen-

Table 1. Comparison of DNA and proteins with respect to their use for microarray applications

Properties	DNA	Protein
Structure	Uniform, stable	Individual types, individual strability
functional state	Denatured, no loss of activity => can be stored dry	3D Structure important for activity, => keep wet all the time
Activity prediction	Based on primary nucleotide sequence	Hardly possible yet. Bioinformatics is working on prediction models based on sequence homologies, structure prediction etc.
Amplification	Established (PCR)	Not available yet
Interaction sites	1 by 1 interaction	Multiple active interaction sites

bonds or via weak Van der Waals or hydrophobic interactions. Furthermore post-translational modifications (glycosylation, acetylation, phorphorylation) have a big influence on protein interactions which makes the sequence based prediction of interactions nearly impossible. In addition, one protein can interact with very different partners (Figure 4). Therefore, different assay conditions for protein-protein, enzyme-substrate, protein-DNA, protein-oligosaccharide or protein-drug interactions must be developed.

Due to the complementary nature of the DNA, the capture probes which are immobilized for DNA microarrays can easily be predicted on the basis of the sequence of interest. These capture DNA molecules can easily be generated using well established technologies like the PCR amplification of defined cDNAs or standard oligonucleotide synthesis. Alternatively, oligonucleotides can be directly synthesized on the solid support (12, 13). The analysis of RNA or DNA targets involves the extraction from the cell, labeling and hybridization to their immobilized complementary capture probes (Figure 5a). Finally, the captured targets are measured, quantified and the resulting data are analyzed with adequate software tools. DNA chip technology allows the analysis of a large number of targets in parallel measurements on the basis of minute amounts of sample. It is furthermore possible to perform comparative analyses of two different samples within a single array. The targets within two samples are labeled with two different fluorophores; equal sample amounts are mixed and simultaneously hybridized to the same microarray. The ratio of the two differentially labeled target signals on each microspot is a direct measure of whether the targets are present in different or similar concentrations (Figure 5a). In the protein world, it is so far difficult if not even impossible to predict protein capture molecules from the primary amino acid sequence. This is due to the very diverse and individual molecular structures, which define the biological activity of each protein. In addition, proteins cannot be amplified via PCR. Technologies for high throughput protein production and purification are necessary to generate protein microarrays and large numbers of highly specific capture molecules. On the other hand, a considerable collection of protein binding molecules (e.g. antibodies) is already available. In analogy with DNA chip technology, Brian Haab and colleagues (14) adapted the dual color labeling procedure to antibody-antigen microarrays (Figure 5a). To prove their concept of differential protein analysis, they used a set of 110 defined antigen and antibody pairs to create microarray-based immunoassays. Antibodies or antigens were immobilized on the surface and the corresponding targets were fluorescently labeled in complex solutions such as serum, for example. One sample that was spiked with antigens was labeled with cy5; a second sample containing a different concentration of the antigens was labeled with cy3-fluorophores. Subsequently, the two samples were mixed and simultaneously incubated on the same microarray. The dual color detection system, which is well established for DNA microarrays immediately revealed the different concentrations of the captured targets. Depending on the individual affinities of the antibodies, antigens were detectable in pM concentrations. However, proteins are often assembled to multiprotein- complexes. A strong signal on a microspot can therefore result from a large amount of target, but also from the capture of a huge labeled complex. The results of Haab *et al.* could, however, demonstrate that, in principle, the comparative methods established for oligonucleotide microarrays can be directly transferred to protein microarrays.

Improvements must be made on retaining immobilized capture proteins in a functional state. Oligonucleotides or DNA probes have to be denatured before they can efficiently bind to their counterparts. In contrast, the stabilization of the 3D-structure of of proteins on surfaces is a very crucial aspect in protein microarray technology. New or improved surface chemistries, in combination with stabilizing agents are needed. Arraying technologies must be capable of dealing with liquids containing different stabilizing agents, that reveal enormous differences in physical properties, e.g. viscosity or wettability. Furthermore, the sensitivity of the detection systems has to be increased, especially when searching for low abundant proteins. As mentioned before, protein amplification is so far impossible. Finally, different assay formats and assay conditions must be developed for the analysis of the different types of protein interactions (Figure 4) within a biological system. Certain applications of protein microarrays, especially in the field of diagnostics, will require only low density microarrays. The main challenge for microarrays used for diagnostics is the generation of highly reproducable quantitative data (Figure 5b). Therefore high quality surfaces and capture molecules have to be combined with reproducable manufacturing processes and strategies for the use of internal standards and controls. In the field of PROTEOME analysis, however, there is an urgent demand for high density arrays

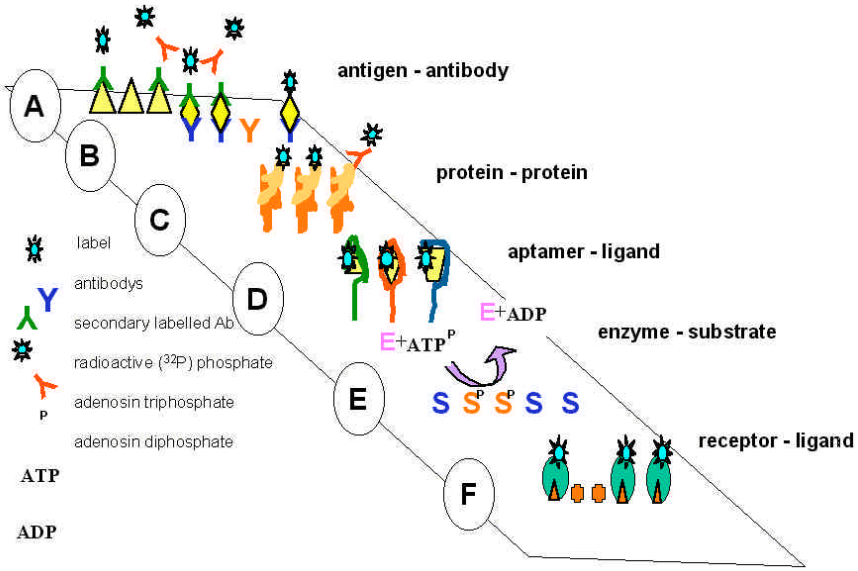

Figure 4. Different classes of molecules can act as capture molecules in microarray assays. Each specific interaction of a distinct pair of biomolecules can be used to design an interaction assay. The illustration shows examples of different capture molecules and some of their typical applications for protein microarray assays. Antigen-antibody interactions, where either the antigen (A) or a specific antibody (B) is immobilized are well known in the field of diagnostics. Detection is performed on the basis of the labeled analyte (antigen or antibody) itself or by labeled secondary antibodies. Specific protein-protein interactions (C) on protein microarrays can be measured directly using labeled analyte protein or by detection of captured protein using a secondary antibody. Synthetic oligonucleotides or peptides, termed aptamers (D), selected from random combinatorial synthetic libraries for high affinity and selectivity to their target molecules, are useful molecules for the design of capture microarrays. Arrays built from DNA fragments or oligonucleotides can be used for the determination of specific DNA-protein interactions as well. Enzymatic processing of immobilized substrates on microarrays can also be monitored in a microarray format (E). Immobilization of receptor ligands (e.g. oligosaccharides, hormones, etc.) can be used for receptor binding studies (F).

with thousands of specific capture molecules (e.g. antibodies) for the parallel identification of large sets of different target proteins within complex biological samples.

Despite to the needs for technology improvement, the generation of protein microarrays can be performed nowadays based on commercially available products and technology platforms which have been developed and commercialized for DNA chips within the last few years. More than a hundred companies on the market offer products for the microarray community (Source: www.biochipnet.com). These technical devices can be used for both, the generation of DNA and protein arrays**.**

5. TOOLS FOR THE GENERATION OF PROTEIN ARRAYS

5.1. Surface chemistry and probe attachment

Proteins can be immobilized using non-covalent surface interactions with hydrophobic (nitrocellulose, polystyrene) or positively charged (poly-Lysine, aminosilane) surfaces. These materials are well known from established protein assays such as ELISA or Western blotting. Membranes possess a large surface area and have high protein binding capacity but suffer from potential background problems due to autofluorescence and unspecific protein binding. To counteract this problem, glass slides can be prepared as ultraflat devices with a minimal degree of autofluorescence. However, glass surfaces show poor protein binding capacity and therefore surface modifications are necessary to optimize protein binding. Besides the established surface treatments involving aminosilane, poly-lysine, or aldehyde surface activation (15) in DNA microarrays, sophisticated surface chemistries are being developed by many companies and research groups to meet the specific needs for immobilizing and stabilizing proteins on microarrays. (e.g. Zeptosens AG, www.zeptosens.com, Witterswill, Switzerland, Zyomix Inc. www.zyomyx.com, Hayward CA, USA, etc.). Zyomix Inc. is establishing appropriate surface chemistry that enables the attachment of proteins in a functional state and a defined orientation with a minimum of unspecific protein binding. Furthermore, hydrogel modifications (16) can be used to prevent the immobilized proteins from drying out. Such surfaces are well known from SPR assays (17) and are also used for DNA chips (e.g. Packard BioScience, www.packardbioscience.com, Meriden, CT, USA).

Technologies derived from semiconductor productions are used to produce microfluidic systems for protein assays. Such systems are developments towards micro total analysis systems (micro-TAS) in which the large analytical lab systems are to be transferred into portable pocket devices combining sample pre-treatment

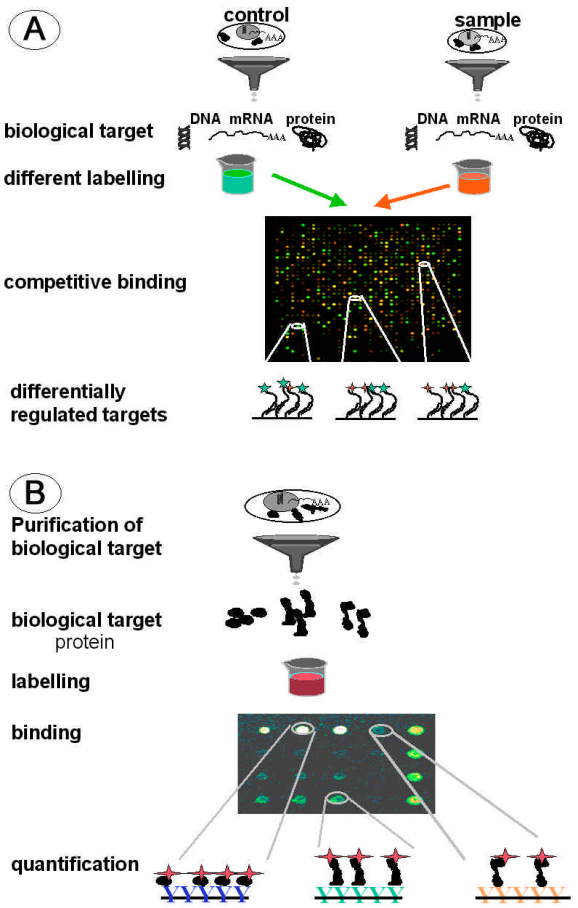

Figure 5. Comparative and quantitative proteome analysis. A. Straightforward strategies to analyse a network of interacting proteins are subtractive or comparative methods. Equivalents of the proteome (e.g. mRNA, proteins or DNA) of phenotypically different sources (e.g. individual cells or tissue samples) are isolated and differentially labeled, e.g. with two distinct colors. The two samples are mixed and incubated on a microarray. Targets will bind to their immobilized capture molecules. Bound target molecules from the two distinct samples are visualized with a microarray scanner detecting the signal intensities of each distinct color of each microspot. For example, if the expression level of a given protein derived from the sample that was labeled red is higher than in the control, the spot will exhibit a stronger signal for the red channel. A lower amount of a given protein will result in a green spot. If the expression level of a distinct protein remains unchanged the respective spot is stained in an intermediate color. B. Different fluorescently-labeled proteins can be detected and quantified in parallel using microarray-based assays. Specific capture molecules immobilised in a microarray format bind their respective target proteins present in the surrounding solution. Signal intensity correlates with the amount of captured target. Various kinds of control spots can be included within each microarray such as positive and negative control spots and/or internal calibration spots. This allows accurate signal quantification.

(filtration, reactions, separation) and detection. Capillary chip based analysis systems, which provide the sizing and concentration of DNA fragments and proteins, have been commercialized by Caliper Technologies Corp. (www.calipertech.com, Mountain View, CA, USA) and Agilent Technologies (www.agilent.com, Palo Alto, CA, USA). The technology for complex proteomic analyses on microfluidic chip systems is still in its infancy. (18).

The non-covalent attachment of protein capture molecules might be to weak to prevent the loss of capture molecules during the assay procedure. Therefore, the need for covalent, spatially defined protein immobilization procedures might increase in future. So far, the covalent attachment of proteins on microarrays is usually performed using a variety of chemically activated surfaces (e.g. aldehyde, epoxy, active esters (19-21)). Specific bimolecular interactions (e.g. streptavidine – biotin (22-24), his-tag – nickel-chelates (25) or the formation of stable complexes between phenylboronic acid labeled analytes and salicylhydroxamic modified surfaces (Versalinx™ technology, Prolinx Inc., www.prolinxinc.com, Bothell, WA, USA) have also been used successfully to immobilize capture molecules on surfaces. The future will show which surface modifications will have proven advantages over all the other surface technologies for distinct protein microarray applications.

5.2. Arraying devices

The deposition of microspots on chip surfaces is either done with contact printing arrayers equipped with tiny needles which place sub nanoliter sample volumes directly on the surface or alternatively by the use of non-contact deposition technologies which employ capillaries or ink jet technology to deposit nanoliter to picoliter droplets onto the surface. TopSpot™ (www.imit.de, Villingen-Schwenningen, Germany), a microstructured device where arrays of tiny capillaries (up to 96) are directly linked to distinct macroscopic fluid reservoirs was presented recently (26). A piezo actuator hits the back of the microcapillary device, thus generating a steep air pressure ramp to the open upper side of the liquid reservoirs. This air pressure change results in parallel dispensing of microdroplets featuring volumes down to 1 nl from each capillary tip. The TopSpot™ device will be a very useful and fast way to produce large quantities of identical DNA or protein arrays at low costs. Approximately 20 000 droplets can be generated from 20 µl of sample solution in each reservoir at equal quality.

Furthermore, micropatterned protein arrays were produced by photolithographic methods (27), scanning probe based lithography (28) or soft lithography technologies like microcontact printing (µCP) (29-31, 32). µCP allowed the printing of delicate biomolecules together with a tiny hydrogel spot onto surfaces (33, 34). Electrospray deposition (ESD) of protein solutions on slightly conductive surfaces (35) might be another future possibility to handle delicate proteins.

5.3. Detection on microarrays

A number of different detection technologies have been discussed and employed for microarray

experiments. So far, the detection of captured targets is mainly performed by fluorescence using CCD-cameras or laser scanners with confocal detection optics. A very sensitive alternative to confocal optics with regard to signal intensity, linearity, signal-to-noise ratio and background is the application of planar waveguide excitation devices combined with CCD cameras or photomultipliers as detectors (36-38). Zeptosens AG (www.zeptosens.com, Witterswil, CH) has developed the Zepto™ READER which is based on this technology. Capture molecules are immobilized in a microarray format on a thin (100-200 nm) film (planar wave guide) which consists of a high-refractive index material (e.g. Ta_2O_5) deposited on a transparent support. A laser beam is optically coupled via diffractive grading into the planar waveguide. The light is propagated in the thin layer and creates a strong, surface confined evanescent electromagnetic field. The penetration depth of this evanescent field into the adjacent medium is limited to about 200 nm. Thus, only surface confined fluorophores are excited and emit fluorescent light. Fluorophores in the bulk medium are not excited and therefore not detectable. A CCD-camera is used to detect fluorescent light with high spatial resolution. Parallel excitation and parallel detection of binding events on different spots is performed in a highly selective and sensitive way, even in solution. The application of rolling circle DNA amplification (RCA) for ultrasensitive fluorescence based antigen detection is a promising high end detection technology for diagnostic microarrays also (39). A reporter antibody conjugated to an oligonucleotide binds to the analyte captured on the microspot of a microarray. In a second step a DNA circle hybridizes to a complementary sequence in the antibody bound oligonucleotide. After washing away non hybridized cDNA and excess reagents the DNA tag is amplified by RCA. The amplified product is labeled *in situ* by hybridization with fluor-labeled oligonucleotides. Therefore the oligonucleotide tag carries several fluorophores which results in a high fluorescence yield. An approximately 3 log increase in sensitivity (down to 300 zeptomoles) was reported for immunoRCA compared to nonamplified signal detection approaches of prostate specific antigen (PSA) (39). In principle single binding events on microspots should be detectable using immuno RCA. An excellent spot-to-spot reproducibility and a dynamic range between 6 to 7 logs was estimated based on proof of principle experiments on microarrays.

Improved fluorescent labels like luminescent quantum dots have been developed recently which may turn out to become important tools for multicolor detection on protein arrays in the near future. These nanocristals are of interest, because their fluorescence emisson wavelength can be continuously tuned by changing the particle size. In addition, quantum dots have narrow, symmetrical emission peaks and are highly stable against photobleaching (40, 41). Radioactivity (42), chemiluminescence (43), mass spectrometry using surface enhanced laser desorption ionization (SELDI) (44) or label-free surface plasmon resonance (SPR) detection systems (45, 46) were also used for biomolecular interaction analyses on surfaces. Additionally, SPR based detection can be directly combined with mass spectrometric identification of captured molecules (47). Electrochemical detection (46, 48) or quartz crystal microbalance (QCM) detection systems (20, 46, 49, 50) which have been established for biosensor applications, might be useful alternatives for microarray assays. They are, however, so far not very common for microarray applications.

5.4. Recombinant protein expression

Microarrays of recombinant proteins will be employed for high-throughput screening of the different kinds of biochemical activities and posttranslational modifications. Furthermore large sets of thousands of different peptides or proteins - ideally containing all DNA coded proteins of a cell or tissue - are needed to determine the optimal highly selective capture molecules for the construction of high-density capture arrays for proteomics. Therefore recombinant sources are required to generate large amounts of natively expressed proteins which are soluble, easy to purify and biologically active. Enormous efforts are underway to develop miniaturized expression systems for high-throughput generation of recombinant proteins. Miniaturized expression systems for the massive parallel generation of recombinant proteins in *E. coli*, in the baculovirus system, and in yeast have already been described (25, 51-53). High density fusion protein microarrays as well as high density single chain antibody library arrays have already been generated and used for antibody specificity screening (54-56).

One approach for the generation of high-density protein arrays from cDNA libraries is based on recombinant protein expression in *Escherichia coli* (54). *E. coli* colonies from a human fetal brain cDNA expression library were arrayed in microtiter plates and subsequently gridded onto PVDF membranes to produce high density colony protein filters. Expression of the recombinant fusion proteins was induced *in situ*. The filters were processed on pre-soaked blotting paper and the putative expression clones were identified using an antibody against the epitope (RGS-His6) fused to the recombinant proteins. This approach was extended by Lueking *et al.* (55) who spotted high-density microarrays of purified proteins from liquid expression cultures on PVDF filters using a transfer stamp mounted on a flat-bed spotting robot. 4800 protein samples were placed onto a microscopic slide and screened for human proteins GAPDH, HSP90-alpha and alpha-tubulin using monoclonal antibodies. 250 attomoles or 10 pg of a spotted test protein (GADPH) could be detected by a specific antibody. Cross-reactivities of some antibodies with unrelated proteins implied the use of high-density protein microarrays for antibody specificity screening against whole libraries of proteins. Based on these experiments, a multistep approach for highly efficient high throughput protein expression screening of a human cDNA libraries was developed (57). First, high-density protein filters were screened for putative expression clones by identification of the fused tag epitope. Only positive expression clones were subsequently re-arrayed into new sublibraries. Expression products of some randomly chosen positive clones were identified by means of SDS-PAGE, affinity purification and MALDI-TOF-MS. 66% of these clones contained inserts in the correct reading frame,

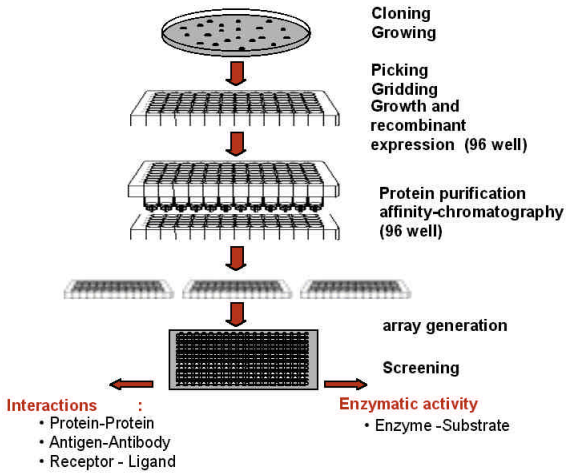

Figure 6. Scheme of a high throughput recombinant protein expression and purification system. Genes of interest are cloned into an expression vector fused to a tag suitable for protein purification (e.g. 6xHis, GST, etc.). The recombinant clones are picked and gridded into adressable microtiter plates. For protein expression, these recombinant strains are grown in microtiter plates and expression is induced. The recombinant fusion proteins generated therby are purified via affinity-chromatography (e.g. Ni-NTA, GST, etc.) in the same 96 well microtiter plate format. Purified proteins can directly be employed for the generation of high-density protein arrays, which can be used for protein interaction analysis in order to analyze enzyme activities.

whereas 64% of these inserts comprised the complete coding sequence of a human protein. Finally, functional screening was performed in the microtiter plates containing positive clones. A spectrophotometric enzyme assay was established to detect clones expressing the test protein (GAPDH).

Since cDNA libraries are usually highly redundant, a large number of clones express identical proteins. A major improvement towards efficient high throughput expression of recombinant proteins is the generation of so called Unigene-Uniprotein sets (51). The first Unigene-Uniprotein set was generated by oligonucleotide fingerprinting of the gridded cDNA library. Clones showing identical sequences were clustered in the second step and finally only one clone from each cluster was chosen and re-arrayed in order to generate the non-redundant Unigene-Uniprotein set. Such non-redundant expression libraries will be very valuable tools for the generation of high-density protein arrays in order to generate and characterize specific antibodies. Although *E. coli* is a well characterized and widely used expression system, it has some disadvantages. These include the lack of post-translational modifications and the formation of inclusion bodies. In order to overcome the limitations arising from prokaryotic expression, eukaryotic expression systems like baculovirus and yeast systems were used. Albala *et al.* (7, 52) have optimized the baculovirus system for the expression and purification of large numbers of proteins encoded by defined cDNA clones obtained from the IMAGE collection. A PCR-based method was used to amplify the cDNAs of interest. The PCR products were purified and incorporated into recombinant baculoviruses. Expression of the epitope-tagged proteins was performed in an insect cell culture system designed for the 96-well microtiter plate format. The recombinant proteins were purified by affinity chromatography, again in a 96 well microtiter plate. Thus, the starting cDNAs, the baculovirus expression system and the purification of the proteins maintained the same addressable format throughout the production scheme and could be easily subjected to high throughput structural or functional analysis (Figure 6).

Zhu *et al.* (42) established an array based high-throughput analysis system to determine the biochemical activities of 119 of the 122 known or predicted protein kinases from *Saccharomyces cerevisiae*. The entire coding regions of each of the 122 predicted protein kinases were cloned into a yeast expression vector and fused to glutathione S-transferase (GST). GST-affinity purification using glutathione agarose beads in a 96-well format was applied to purify the overexpressed proteins. Purified fusion proteins were subsequently transferred into microwells of a chip containing potential immobilized kinase substrates. These protein chips were used for simultaneous analysis of kinase activity. This approach was further improved by the same group (25). They generated a yeast proteome microarray containing approximately 80% of all yeast proteins. 5800 open reading frames (ORFs) were cloned in a yeast expression vector where all the ORFs were fused to a GST-HisX6 tag. Overexpressed recombinant fusion proteins were purified from yeast extracts in a one-step procedure using the GST tag again. The purified yeast proteins were spotted either on aldehyde-treated glass slides or nickel-coated slides. These high-density protein microarrays were screened for a number of biochemical activities involving protein-protein, protein-lipid and protein-drug interactions.

The pooling strategy is an interesting alternative to study protein interactions. (53, 58). In this approach, an array of 6144 yeast strains was constructed, each containing a different yeast ORF fused to GST. The strains were grown in 64 pools containing 96 different fusion proteins. All 96 GST-ORFs from one pool were isolated using glutathione agarose affinity chromatography. Each pool of recombinant proteins generated thereby was assayed for different activities such as e.g. tRNA splicing, tRNA ligase activity and 2' phosphotransferase activity. Active pools were then deconvoluted to identify the source strain expressing the protein responsible for the activity. The pooling strategy is a very fast and efficient approach for searching activities that occur infrequently (e.g. specific enzymatic activity), thus leading to a restricted number of positive pools. The pooling strategy could also be employed in microarray technology either by spotting arrays of pooled proteins or *vice versa* by screening protein arrays with protein pools to identify specific binding molecules.

Recent developments led to new formats of protein arrays which are based on *in vitro* expression

techniques. He *et al.* (59) developed a new protein array production procedure termed "PISA" (protein in situ array). PISA is designed to generate protein arrays directly from PCR-generated DNA via cell-free protein synthesis and simultaneous *in situ* immobilization of the generated proteins on a surface. PCR-products coding for his-tagged proteins or domains serve as templates for *in vitro* synthesis of recombinant protein. The primers for the PCR are designed according to information retrieved from DNA databases. Coupled transcription and translation is carried out directly on surfaces (e.g. Ni-NTA), capturing the his-tagged proteins as soon as they are synthesized. PISA allows the one-step-generation of arrays of soluble, functional proteins, including toxic proteins and further permits processing of a variety of post-translational modifications. The PISA-method can be adapted to high throughput syntheses of proteins or domains based on genome information alone and will be particularly useful where cloned material is not available.

Strategies using ribosome trap methods contribute a mentionable improvement to the rapid and versatile selection of peptides and proteins with desired properties generated from both natural and synthetic libraries. The PROfusion™ technology of Phylos Inc. (www.phylos.com, Lexington, MA, USA) is an example of such a very skilled *in vitro*-expression and selection method. The basic unit of the PROfusion™ system is a fusion molecule, which consists of a protein linked to its own messenger RNA via puromycin that is covalently attached to the 3' end of the mRNA, using a synthetic linker (60, 61). Starting with synthetic, semi-synthetic, and/or cellular DNA, Phylos has created libraries of up to 100 trillion proteins using the PRO Fusion™ technology and such libraries provide powerful resources to isolate appropriate capture proteins.

5.5. Capture molecules

Large numbers of highly specific capture molecules showing a high affinity to their target molecules are the main prerequisite for the identification and quantification or proteins with array based proteomic approaches. Future developments in protein microarray based assays and biosensor technology will require new custom designed capture molecules. The different types of capture molecules are summarized in Figure 4. Despite to the different types of protein capture molecules other types of substances can be useful for the development of protein arrays or the screening for protein function. DNA-protein interactions can be analyzed on DNA or oligonucleotide arrays. Peptide arrays, built from large sets of individual peptides or peptide libraries can be useful tools for screening for unknown enzymatic activities or for selection of antibodies (62-64). Recently it was shown, that arrays of small organic molecules synthesized by established solid phase combinatorial chemistry can be used for high-throughput screening for interactions between organic compounds and proteins (65). This could be an interesting future technology for high-throughput drug compound screening in pharmaceutical industry.

5.5.1. Antibodies

Antibodies are highly specific targeting agents and very valuable tools for *in vitro* and *in vivo* diagnostic applications. With the advent of monoclonal antibody technology, the utilization of antibodies as essential tools dramatically in almost every field of biological sciences has increased enormously (66). Monoclonal antibodies have become key components in a vast array of clinical laboratory diagnostic tests. But antibodies whether polyclonal or monoclonal have some disadvantages in terms of generation, cost, and overall applications. The continuous culture of hybridoma cells that produce monoclonal antibodies offers the potential of an unlimited supply of reagent, when compared with the rather limited supply of polyclonal antibodys. The feature of a continuous supply enables the standardization of both reagent and assay technique, but the production of monoclonal antibodies is often time consuming, laborious and therefore expensive. To overcome these limitations, some molecular biological approaches such as the phage display or ribosomal display technique and the development of alternative types of binders, i.e. aptamers, have been started. Phage antibody-display has shown to be a very effective tool for the fast generation of antibodies. The development of large primary antibody libraries and, even more, the development of entirely synthetic combinatorial antibody libraries (67, 68) - enables the selection of antibodies against nearly any target in a relatively short time (i.e. weeks). Many of these antibodies exhibit affinities with a K_D in the nM range. Maturation strategies allow the improvement of this affinity to a K_D in the pM range.

Phage-antibody display involves the fusion of an antibody gene to the gene for a phage coat protein. The antibodies encoded by this gene are displayed on the phage surfaces. From large repertoires of antibody variable region (V) genes, displayed on phage particles, a specific antibody population can be selected by multiple rounds of affinity purification on the desired target antigens. Different phage- and phagemid vectors systems have been used to display the antibodies. E.g., antibody fragments have been displayed on the surface of filamentous phage fused to the product of gene VIII (69). However, by far the most successful fusion partner to date is g3p, the product of the gene gII1. Three to five copies of g3p exist on each phage particle. Antibody fragments can be displayed, apparently equally well, either fused to the amino-terminus of the mature protein or at the amino-terminus of a truncated g3p, lacking the first two amino-terminal domains (reviewed in (70)). It has to be taken into account that most antibody fragments which are diplayed on phagemid particles are monovalent (71). The valency of displayed antibodies can be increased either by using a phage vector or a helper phage with the gII gene deleted. This results in a g3p-protein population that consists almost completely of g3p-antibody fusion proteins (72), which can later be propagated with high titres (73). Antibody fragments can be displayed as single-chain variable region fragments (scFv) and as Fab fragments. Displaying scFv, the heavy chain and light chain variable regions are fused by a linker and the carboxyl-terminus of the scFv polypeptide is fused to g3p (74). scFv fragments are encoded by relatively short genes, which leads to libraries that are genetically stable and easy to handle. scFvs form predominantly monomers, but sometimes they can also form dimers and trimers with

higher molecular weight. This can complicate the selection and characterization. But such diabodies may be bivalent molecules with enhanced avidity (75). Fab fragments are displayed by fusing either the light or the heavy chain carboxyl-terminus to g3p. The partner chain is then expressed unfused and forms an intact Fab after secretion into the periplasmic space (76). Fabs usually show a lower degree of dimerization than scFvs. This makes them easier to characterize. Their larger genes, however, may make Fab libraries less stable.

Primary phage libraries are divided into two groups based on the different sources of the antibody genes. First, 'natural' or rearranged antibody V-gene repertoires derived from human or animal donors - here the immune system (B-cells) is used to create the diversity of the V-genes - and second, synthetic antibody V-gene repertoires which are constructed *in vitro*. For natural phage libraries either immunized (77, 78) or „naive" (79) donors can be utilized. Repertoires from immune donors phage-antibody libraries made from donors naturally mounting an immune response can generate antibodies with high affinity and specificity. Immune libraries are highly biased towards V-genes that encode antibodies against the immunogen, so that relatively small (10^5 clones) libraries can be successfully selected. In addition, many of the genes will encode affinity matured antibodies, thus increasing the number of high affinity antibodies represented in the library. The drawbacks concern the little control over the immune response and the existing tolerance mechanisms against self antigens. The major advantage of repertoires from non-immunized donors over libraries from immunized donors is that a single library of sufficient largeness and diversity can be used for all antigens with no limitation concerning self-immunogenic, non-immunogenic and toxic antigens. Selection from large primary libraries generally ends up in many different antibody populations with subnanomolar to submicromolar affinities (80, 81). For most purposes the affinities of the isolated antibodies will be well suited, however, for specific applications there may exist the need to improve these primary lineages. The improvement of this antibody lineages can be achieved by the construction, selection and screening of secondary phage display libraries, a process analogous to 'affinity maturation'.

Secondary repertoires are essentially synthetic libraries based on a lead or candidate antibody that was identified selecting a primary library. Criteria such as potency, affinity, cross-reactivity, expression level, germline homology etc. have to be considered. Potential target sequences have been selected from libraries of mutants diversified at heavy and/or light chain CDR3 and higher affinity binders have been generated (68, 82). Saturation mutagenesis in combination with affinity selection of CDR3 of heavy and light chain (82) – they structurally comprise the antigen binding site – can lead to antibodies with dissociation constants below 100 pM. Further sequence analysis of human antibodies emerging in the primary and secondary immune responses also suggests other key residues for potential affinity maturation (83). Using randomized codons a complete library with only six codons would theoretically have to contain 2.5×10^9 clones (69). Variant libraries would have to be even lager to ensure complete representation. Therefore, several strategies have been developed to identify residues with functional rather than structural roles as promising targets for codon randomization (68, 82).

Since large and highly diverse repertoires have to be engaged to perform successful antibody phage display there is a need for appropriate selection strategies and for efficient screening technology. For most applications, the selection can be performed effectively by panning phage on antigen-coated plastic ware. Limitations for such an approach are the need of purified antigen, the detection of native protein antigen, the identification of high affinity clones despite avidity effects and discrimination between clones of similar affinity. Affinity chromatographic methods (i.e. biotin-streptavidin system) in combination with sequential elutions have been used to separate antibody populations with different affinities (84). Depletion and/or subtraction methods have been used to deplete antibodies against non-target antigens and to select antibodies against complex antigens (15, 84). It is very often possible and desirable to select multiple different antibodies to the same target which bind via different epitopes. The demands on antibody screening increase in terms of quality (e.g. functional screening) and quantity (high throughput). The appropriate system to identify antibodies with the optimal characteristics has not only to be fast, robust and sensitive but also amenable to automation and miniaturization. With respect to these demands, micro array technology on solid or fluid phase is very promising. Protein or peptide microarrays combining the inherent possibility to perform multiplexed assays with minimal sample consumption at enhanced sensitivity meet these requests for antibody screening and validation perfectly. Furthermore, antibody engineering allows the manipulation of genes encoding antibodies so that the antigen binding domain can even be expressed intracellularly. These intracellular antibodies are termed 'intrabodies' and demonstrate new roads to therapeutic abilities and screening strategies taking place directly in the living cell (85, 86).

5.5.2. Aptamers – an alternative class of binders

The development of a specific methodology made it possible to isolate oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such oligonucleotides derived from an *in vitro* evolution process called SELEX (systematic evolution of ligands by exponential enrichment) are referred to as „aptamers". They have the potential to build up a class of molecules that reveal antibodies in both therapeutic and diagnostic applications. Although they are molecularly different from antibodies, they mimic the molecular recognition properties of antibodies in a variety of diagnostic formats. The demand for diagnostic assays is increasing and aptamers could potentially fulfill molecular recognition needs in those assays. Compared with the skilled antibody technology, aptamer research is still at the beginning but it is progressing at a fast pace. Aptamers may play a key role

either in conjunction with, or in place of antibodies. Originally, aptamers have been evolved to bind proteins associated with distinct disease states. This led to the development of many powerful antagonists of such proteins. If aptamers contain modified nucleoside triphosphates (e.g. PNA (87) or LNA (88)) their resistance to nucleases is normally enhanced and can be kept for longer time periods in the circulation of animal disease models, especially when conjugated to vehicles of higher molecular weight. In such approaches, the aptamers can inhibit physiological functions of their target proteins.

Aptamers immobilized in a microarray format on solid surfaces can be used as diagnostic tools. They will become rapidly available because the SELEX protocol has been successfully automated (89, 90). The use of photo-cross-linkable aptamers will allow the covalent attachment of aptamers to their cognate proteins. This allows rigid wash procedures prior to detection and therefore ratios lowers the unspecific assay background and enhances signal-to-noise ratios. Finally, protein staining with any reagent, which distinguishes functional groups of amino acids from those of nucleic acids will give a direct readout of proteins on the solid support.

In the meantime, not only oligonucleotides, PNAs or LNAs but also short peptides which are generated by mRNA display technology (91), are called aptamers. The mRNA display *in vitro* selection technique allows the identification of peptide aptamers to specific target proteins. Polypeptide libraries with a complexity of 10^{13} random peptides can be generated. Aptamers with dissociation constants as low as 5 nM have been isolated from such peptide libraries. Peptide aptamer affinities are therefore comparable to those found for monoclonal antibody-antigen complexes without sophisticated engineered scaffolds. Given a sufficient length and diversity, high-affinity peptide aptamers can be obtained directly from random nonconstrained peptide libraries (92). In principle every protein or peptide showing a selective, specific high affinity binding to a substrate can be used as a template for recombinant production of capture molecules. The only prerequisite is, that mutations in the binding site do not affect protein stability to much. Based on these prerequisites other protein templates were used for the recombinant production of capture molecules. Examples for such molecules are the so called receptins (93). Receptins represent binding molecules of microbial origin including a number of key microbial proteins involved in host-parasite interactions and in virulence. Common features of these proteins are their specific binding properties for mammalian proteins. Well known binder molecules engineered based on this protein group are the so called affibodies. Affibodies are selected from naive or constructed combinatorial libraries derived from the *alpha*-helical receptor domain of protein A of *Staphylococcus aureus* (94-97). Such affibody libraries can also be subjected to affinity maturation procedures and multimerization to generate binders with high affinity.

6. APPLICATIONS OF PROTEIN MICROARRAYS

6.1. Miniaturized and parallelized immunoassays for diagnostics

Miniaturized and parallelized immunoassays are of general interest for all diagnostic applications, where several parameters in an individual sample have to be determined simultaneously from a limited amount of material. Besides microarray based systems, bead-based assays can be used for such diagnostic applications, especially when the number of parameters of interest is comparably low.

Mendoza *et al.* (98) described a microarray based approach capable to perform high-throughput, enzyme-linked immunosorbent assays. This system consists of an optically flat glass plate with 96 wells separated by a Teflon mask. Within each well more than a hundred capture molecules are immobilized in a microarray format. Sample incubation and washing steps can be performed with an automated liquid pipettor. The microarrays are quantitatively imaged with a scanning charge-coupled device detector. With marker antigens the feasibility of multiplex detection of arrayed antigens in a high-throughput fashion was demonstrated. Other microarray based approaches have been published by Silzel *et al.* (99) who demonstrated that multiple IgG subclasses detection can be performed using microarray technology. Arenkov *et al.* (16) performed microarray sandwich immunoassays and direct antigen of antibody detection experiments using a modified polyacrylamide gel as a substrate for the immobilization of capture molecules. In our laboratory, we used microarray technology to screen for autoantibodies present in patient sera. Eighteen different autoantigens, commonly used as diagnostic markers for autoimmune diseases like systemic rheumatic diseases, were immobilized together with control proteins in a microarray format. Arrays were incubated with patient sera and bound autoantibodies were detected by a peroxidase labeled secondary antibody and a chemiluminescent reaction monitored by a CCD camera. The microarray based assay allowed the parallel identification of different types of autoantibodies present in the sample. From less than one µl of a patient serum the autoantibody titers were determined with high accuracy (43). Only minimal unspecific binding and no cross-reactivity to non-specific proteins could be observed. Sandwich immunoassays were also miniaturized and parallelized and performed in a microarray format. This was recently demonstrated by the parallel determination of 24 different cytokines from conditioned media and patient sera with high specificity and sensitivity (100).

Interesting high-sensitivity detection methods for microarray based assays are based on wave guide technology. Rowe *et al.* (101, 102) developed a fluorescence-based array immunosensor for the simultaneous detection of clinical analytes using a sandwich format. Biotinylated capture antibodies were immobilized on avidin-coated waveguides using a flow chamber module system. This procedure created six vertically oriented stripes of capture molecules on the surface of the waveguide. Samples of interest were incubated on the waveguide to allow binding of the targets to their capture molecules. Captured targets were visualized with appropriate fluorescently labeled detection molecules. This array immunosensor was effective for detection and measurement of targets like bacteria and protein toxins at

physiologically relevant concentrations in a variety of biological samples. A dramatic increase in sensitivity and an adaptation to standard microarray technology was achieved with the development of planar waveguide detection (103). Based on this technology it was possible to detect interleukin-6 at concentrations less than 40 fM. As only surface bound fluorophores are excited no background signals from fluorophores in the bulk are detected. Thus the technique allows online detection of binding reactions. Furthermore no washing steps are necessary to remove non-bound labelled molecules prior to detection. The planar waveguide system is an attractive choice to perform highly sensitive, miniaturized and parallelized immunoassays (104).

As an alternative to planar microarrays, bead based assays in combination with flow cytometry have been developed to perform multiparametric immunoassays. In bead based assay systems the capture molecules must be immobilized on addressable microspheres. Each capture molecule for each individual immunoassay is coupled to a distinct type of microsphere and the immunoassay reaction takes place on the surface of the microspheres. Dyed microspheres with discrete fluorescence intensities are functionalized and loaded separately with their appropriate capture molecules. The different bead sets carrying different capture probes can be pooled as necessary to generate custom bead arrays. Bead arrays are then incubated with the sample in a single reaction vessel to perform the immunoassay. Product formation of the targets with their immobilized capture molecules is detected with a fluorescence based reporter system. Targets can either be labeled directly by a fluorogen or detected by a second fluorescently labeled capture molecule. The signal intensities derived from captured targets are measured in a flow cytometer. The flow cytometer first identifies each microsphere by its individual color code. Second the amount of captured targets on each individual bead is measured by the second color fluorescence specific for the bound target.. This allows multiplexed quantitation of multiple targets from a single sample within the same experiment. Sensitivity, reliability and accuracy are compared to standard microtiter ELISA procedures. With bead based immunoassay systems cytokines can be simultaneously quantified from biological samples (105, 106). With color coded microspheres it is possible to perform simultaneously up to a hundred different types of assays so far (LabMAP system, Laboratory Muliple Analyte Profiling, www.luminexcorp.com, Luminex, Austin, TX, USA). One big advantage of bead based systems is the individual coupling of the capture molecule to distinct microspheres. Each individual coupling can be perfectly analyzed and optimized. Furthermore only quality controlled batches with defined capture loading will be used for multiparameter immunoassays. If additional parameters have to be included into the assay, only the new types of loaded beads have to be added to the bead array used for the assay.

Planar microarrays or bead based multiplexed arrays are both very well suited for multiplexed immunoassays. Accurate quantification and control of assay performance and reproducibility can be achieved by including several positive and negative controls and/or internal calibration standards (Figure 5b). Thus microarray or bead based assay have enormous potential to become robust and reliable diagnostic assays.

6.2. Protein microarrays for proteomics

A proteome represents the physiological state of a cell on the protein level. The analysis of such protein patterns and their correlation with intracellular or extracellular parameters (Figure 1) is one of the major challenges for protein chemists today. 2D-PAGE combined with mass spectrometry is the most common technology used for proteome analyses to date. Due to the inadequacies of these technologies, alternative analytical methods are needed to allow for an accurate analysis of a proteome.

Microarrays, which are well established for RNA expression or single nucleotide polymorphism analysis, may also be suitable for protein analysis. Different molecules binding or interacting with proteins can be immobilized in a microarray format, and complex interaction analysis studies can subsequently be performed (Figure 4). Microrrays of highly specific capture molecules might be used for direct protein identification and quantification out of complex protein mixtures isolated from cells or tissue samples. The principle of differential display analysis of proteins from different sources on such microarrays (Figure 5a) was recently shown (14). The results suggest that protein microarrays are a convenient tool to characterize patterns of variation in hundreds of thousands of different proteins. The generation and characterization of highly specific antibodies for high density antibody arrays, the main prerequisite for direct differential protein display analysis, is a big challenge, so far. Modern technologies for the generation of large capture molecule libraries (section 5.5.) combined with methods for the generation of highly diverse non-redundant expression libraries, as the so-called Unigene-Uniprotein sets (51), for antibody screening, will be valuable tools to pass this challenge.

An alternative approach for chip-based proteome analysis is the SELDI (surface enhanced laser desorption and ionisation) technology, which utilizes mass spectrometry as a read out system to analyze differential protein expression on spot arrays (104, 107). Cell extracts derived from different sources are incubated on different spots of the same adsorptive surface chemistry (e.g. cation/anion exchange material, hydrophobic surfaces). After washing away unbound proteins, the whole variety of non-specifically captured target proteins can be analyzed by SELDI mass spectroscopy. The mass spectrum shows the different molecular weights of the captured proteins. The comparison of two MS data sets generated from two different samples immediately identifies the differentially expressed proteins. In some cases the differentially displayed proteins can be identified immediately by their molecular weights, but usually these proteins have to be enriched by affinity chromatography and identified by methods known from protein analysis (e.g. Edman sequencing, Western blot, digest mass fingerprinting)

(108). This sophisticated expensive technology is an easy to handle tool for fast screening for differences in total protein content. As the detector sensitivity of time of flight mass analyzers is decreasing with increasing molecular weights, SELDI can be a valuable supply to 2D-PAGE technology, which usually has difficulties in the detection of small proteins and peptides. But the identification of the individual protein differences might be a laborious process. Furthermore, sensitivity is lower than with fluorescence-labeled captured targets on antibody microarrays.

The most direct approach towards an understanding of gene function and regulation is to analyze the biochemical activities of the individual proteins (58, 109, 110). Systematically probing for biochemical activities of every protein is another possible strategy for proteome analysis. An alternative way is to produce protein products in a high throughput manner and to analyze their functions in parallel using protein microarrays. Such microarrays could provide data on direct nucleic acid-protein, protein – protein, ligand –receptor or enzyme-substrate interactions, which are required for an interpretation of protein networks within biological systems. The variety of different microarray-based assays useful for proteome analyses is impressively illustrated by recent publications where the analysis of such interactions is discussed (15, 16, 42, 43, 65, 111, 112).

Studies on **DNA-protein interactions** in a microarray format were performed by Bulyk *et al* (111) who created microarrays of double-stranded oligonucleotides. Initially, high-density microarrays of single-stranded oligonucleotides primers were produced using Affymetrix technology. Subsequently, these single-stranded oligonucleotide microarrays were converted into double-stranded oligonucleotide (dsDNA) microarrays by an enzymatic primer extension reaction. Double-stranded oligonucleotide arrays were then incubated with restriction enzymes cleaving specifically distinct dsDNA sequences. No DNA-cleavage occurred, when the dsDNA was enzymatically methylated prior to the incubation with the specific restriction enzymes. This example showed that dsDNA arrays can be further modified biochemically in order to study specific DNA-protein interactions. In general DNA-protein interaction assays could be useful for the characterization and identification of DNA-binding proteins, such as e.g. transcription factors. Heng Zhu and coworkers (25) overexpressed and purified the corresponding proteins of 5800 cloned open reading frames of yeast as GST/HisX6- fusion products. All these proteins were printed onto nickel-coated slides at high spatial density. These proteome chips were used to analyze protein-nucleic acid interactions and several other types of activities.

Enzyme-substrate arrays have been described for different kinds of enzymes such as e.g. restriction enzymes, peroxidase, phosphatase and protein kinases (16, 42, 111, 112). In a proof of concept experiment MacBeath and Schreiber (15) immobilized three different kinase substrates in a microarray onto a planar glass surface. Identical microarrays were incubated individually with one specific kinase together with radioactively-labeled ATP. Each substrate was phosphorylated only by its specific kinase. In a more advanced approach, Zhu *et al.* (42) analyzed the activities of 119 of the 122 known or predicted protein kinases from *saccheromyces cerevisiae* for 17 different substrates. They used microwell plates with substrates covalently linked to individual microwells. The overexpressed, purified kinases were subsequently incubated on these microwell arrays along with radioactively labeled ATP. After finishing the reaction, kinases and the non-incorporated radioacitve ATP were washed away and the arrays were analyzed for phosphorylated substrates by a phosphoimager. Using this approach, novel activities of individual kinases were identified. Sequence comparison of enzymes, which phosphorylate tyrosine residues, revealed that they often share common amino acid residues around their catalytic region.

In the field of **protein-protein interaction assays** dot-blot filter arrays were used to screen for specific interactions of immobilized proteins with other proteins. Filters were prepared from highly purified fully-active recombinant proteins. Used filters were recycled after the assays and were re-used many times with different targets. This so-called universal protein array (UPA) system is a very effective method of screening protein interactions at low cost. Specific protein-protein interactions were detected between a radioactively-labeled human p52 GST fusion protein and immobilized capture proteins like nucleoline or a serine-argenine protein fraction isolated from HeLa cells (112). In addition interactions of DNA, RNA, or low molecular weight ligands with the immobilized molecules were also detected. In principle, the UPA arrays could easily be miniaturized. Proteome chips generated from recombinant protein probes of all 5800 open reading frames of yeast were tested by probing for protein-protein interactions, protein-lipid interactions and protein-nucleic acid interactions (25). To test for protein-protein interactions, the yeast proteome was probed with biotinylated calmodulin. Many known CamKinases (113) and calcineurins (114)) were identified. Additionally, 33 new potential binding partners of calmodulin were found which have a potential binding motif. Furthermore, Zhu *et al.* (25) presented for the first time a genome-wide analysis of proteins interacting with phospholipids. Six types of liposomes of different composition were used to identify a total of 150 different protein targets including integral membrane proteins, peripherally-associated proteins and many others. Many of the uncharacterized proteins are predicted to be membrane associated, indicating that they preferentially bind specific phospholipids *in vivo*. This study clearly demonstrated the advantage of a proteome chip approach. An entire proteome can be prepared and directly screened *in vitro* for a wide variety of activities including protein-drug interactions and protein-lipid interactions, which might not be accessible by other approaches. Preparation of protein arrays of 10-100,000 proteins for global high throughput proteome analysis in humans and other eucaryotes is feasible using similar procedures.

6.3. Protein microarrays – promising tools for drug screening

Data from genomic or proteomic analyses can also be used for screening for new drugs in the pharmaceutical industry. Microarrays of immobilized proteins and of small organic compounds might be powerful tools for future high-throughput drug screening technologies. For **receptor-ligand assays** small organic molecules produced by combinatorial solid phase chemistry were immobilized in a microarray format. Single resin beads from combinatorial synthesis were placed in 96-well plates and the organic molecules were chemically released from the beads. The organic molecules were diluted, spotted and covalently attached on derivatized glass slides. These microarrays, produced by small molecule printing technology, were incubated with fluorescence labeled target proteins in order to identify new ligands (65). This technology enables parallel high-throughput screening for ligand-receptor interactions but requires only very small quantities of the sample, which could improve screening for active substances in the pharmaceutical industry.

The identification of inhibitors of enzymatic reactions is another field of drug screening. Arenkov *et al.* (16) presented data that were obtained by using microarrays of gel-immobilized compounds on a chip (MAGIChip). They immobilized a small set of active enzymes at different concentrations within pads of a three dimensional hydrophilic gel matrix. The chips were covered with enzyme substrates. After enzymatic cleavage of these substrates insoluble precipitates were generated only within such tiny pads containing active enzymes. By addition of enzyme inhibitors the enzyme activity was blocked and no precipitates were detected. As the screening for enzyme activity and enzyme inhibitors can be performed in parallel, such assays could be useful for high throughput screening for enzyme inhibitors.

7. CONCLUSIONS AND PERSPECTIVES

Though the principles of protein microarray technology were described and established years ago, it is only beginning to show its full potential. The number of publications dealing with protein microarray technology is increasing very fast. Several useful applications for protein microarrays were shown with different kinds of experiments. So far, real life applications for protein microarrays were developed mainly for diagnostics. Improvements in the generation of large sets of recombinant proteins and in high throughput generation of capture molecules will further increase the interest in this field. The growing demand for alternative tools for proteome analysis supplementing 2D-PAGE and mass spectrometry will promote the development of high-density capture molecule arrays. Proteomic research, high-throughput drug compound screening and diagnostic applications will be the major fields addressed by protein microarray technologies.

In medical research, protein microarrays will accelerate immune diagnostics significantly by analyzing in parallel all relevant diagnostic parameters of interest. The reduction of sample volume is of great importance also, in particular for those applications, where only minimal amounts of samples are available. One example might be the analysis of multiple tumor markers from a minute amount of biopsy material. Furthermore, new possibilities for patient monitoring during disease treatment and therapy will be developed based on this emerging technology. Microarray-based technology beyond DNA chips will accelerate basic research in the area of protein-protein interactions and will allow protein profiling from limited numbers of proteins up to high density array-based proteomic approaches. Protein and peptide arrays will be useful tools for the analysis of enzyme-substrate specificities and for the measurement of enzyme activities on different kinds of substrates in a highly parallel fashion.

The whole field of protein microarray technology shows dynamic growth driven by the increasing genomic information and the growing interest in proteome analyses. The multidisciplinary collaboration of scientists from different fields such as e.g. biology, biochemistry, material sciences or bioinformatics is a prerequisite for the development of robust, reliable protein microarrays for future applications. Protein microarray technology is just leaving its infancy.

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Abreviations: CCD: charge coupled device, cDNA: complementary DNA, µCP: micro contact printing, ELISA: Enzyme linked immunosorbent assay, Fab: fragment antigen binding, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, GST: glutathion S-transferase, HSP90-alpha: heat shock protein 90 alpha, IMAGE: Integrated Molecular Analysis of Genomes and Their Expression, LNA: locked nucleic acids, MAGIChip: microarrays of gel-immobilized compounds on a chip, MALDI-TOF-MS: Matrix assisted laser desorption ionization - time of flight - mass spectrometry, NTA: nitrilo triacetic acid, ORF: open reading frame, PCR: polymerase chain reaction, PDMS: poly (dimethyl siloxane), PISA: protein in situ array, PNA: peptide nucleic acids, PVDF: poly (vinylidene difluoride), QCM: quarz crystal microbalance, RCA: rolling circle amplification, scFv: single chain variable region fragment (of an antibody), SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis, SELDI : surface enhanced laser desorption and ionisation, SELEX: systematic evolution of ligands by exponential enrichment, SPR : surface plasmon resonance, Micro-TAS: micro total analysis system

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