1. Introduction

The sequencing of the human genome and other ongoing sequencing projects have accelerated the pace of gene discovery and caused the identification of thousands of new genes. However, it also entails realisation that the genome alone could not provide enough information to understand the complex cellular network on the molecular level. Although genetic information provides us with the sequence of each protein, it is currently not possible to entirely deduce its localisation, structure, modifications, interactions, activities, and, ultimately, their function from it sequence. This lack of information becomes especially obvious upon observation of a relatively closely linked relationship, the stoichiometry between RNA transcripts and their corresponding protein abundances. Although gene-protein dynamics were analysed for several tissues (1, 2), there is still no reliable correlation between gene activity and protein abundance. Besides this, protein abundances and their entirety, the proteome, are highly dynamic and therefore require tools that are amenable for describing several variables simultaneously. Up to today two-dimensional (2D) gel electrophoresis for protein separation, followed by mass spectrometry (MS) and database searches for protein identification, are the only real high-throughput techniques for the complex description of a proteome. They are especially important in the classical proteome analysis, which focuses on studying complete proteomes, e.g. from two differentially treated cell lines, and the corresponding identification of single proteins.

1.1 Established Techniques in Proteomics

1.1.1 2D-Gel Electrophoresis

2D-gel electrophoresis was already invented in 1975 by Klose and O'Farrel (3, 4) independently, who demonstrated its usefulness with the separation of *E. coli* lysates. However, it took till the mid-nineties, until the introduction of immobilised pH gradient isoelectric focussing increased reproducibility as well as resolution by an order of magnitude (5-8). Based upon this improvement, 2D-gel electrophoresis today allows separation and detection from a wide variety of sources and permits the characterisation of samples by different expression profiles (9-11). Moreover, the resolution of 2D gel electrophoresis allows separation of proteins isoforms that differ by post-translational modifications, such as glycosylation (12), deamination (13) and phosphorylation (14).

Although improvements allowed 2D-gel electrophoresis to become the major tool for proteomics, there are still problems to solve. Automation is difficult (15) and experienced scientists are required to reduce variance and maintain reproducibility, even though introduction of precast gels as well as standardised reagents, hardware and protocols have improved performance (16). Moreover, 2D-gel electrophoresis is time consuming, expensive and lacks sensitivity (17) as well as dynamic range in comparison to ELISA (18). Prefractionation (19) and the use of narrow pH gradients (20, 21) are often necessary, and hydrophobic membrane proteins (22, 23) as well as basic or high-molecular mass proteins are difficult to separate with sufficient resolution (24).

Besides 2D-gel electrophoresis, other chromatographic separation technologies, such as ion exchange liquid chromatography (LC), reverse phase LC, carrier ampholyte and affinity based separations have been applied solely and in combination to separate complex protein mixtures and allow subsequent analysis by mass spectrometry (25) or on microarrays (26). Especially the first approach can be combined with isotope-coded affinity tags (ICAT), which are based on derivatisation of cysteine residues by an isotope and allow an accurate quantification of derivatised proteins (27).

1.1.2 Mass Spectrometry

2D-gel electrophoresis is often combined with mass spectrometry for identification of protein spots. Spots are excised and in-gel digested with trypsin, before they are identified and characterised by mass fingerprinting using matrix-assisted laser desorption and ionisation – time of flight (MALDI-TOF) mass spectrometry (28-30). Mass spectrometry was, similar to 2D-gel electrophoresis, commonly used decades ago since the 1960s for mass and structure determination of volatile compounds. The problem of applying mass spectrometry to proteins was that such large and charged molecules could not be transferred into vacuum, since ionisation by electron bombardment would have caused destruction of the sample. In 1988, Karas and Hillenkamp developed the matrix-assisted laser-desorption ionisation (MALDI), in which proteins were packed into crystals of UV-light absorbing molecules (31). This matrix charged the proteins and a desorption event was initiated by the absorption of energy from a laser by the matrix crystals. The absorbed energy was converted into heat and caused a sublimation of the matrix crystal and a transition of the matrix and analyte molecules into gas-phase.

Although MALDI is the high-throughput protein identification technique of choice in proteomics, it requires large databases and computer facilities for comparison and causes destruction of protein samples. Analysis of intact proteins and non-covalently bound protein complexes therefore requires electrospray ionisation-mass spectrometry (ESI-MS), which applies a milder ionization technique (32). A spray of fine droplets, containing analyte and solvent molecules is generated upon application of a high electrical tension through a needle. This allows ESI to be applied for obtaining the primary sequence of peptides that were not previously characterized. However, this approach is very time-consuming and typically entails manual interpretation of product ion data and, if necessary, sample derivatisation with further analyses to influence and allow rationalisation of the product ion data. (33).

1.2 Microarray Formats

In contrast to classical proteome analysis, which mainly applies 2D-gel electrophoresis and MS to describe each measured protein independently, the functional proteomics focuses on the description of the cellular network and therefore requires high-throughput tools to elucidate interactions between proteins. The most promising tools to fulfil those requirements, as well as some of classical proteome analysis, are today protein and antibody microarrays. Protein and antibody microarrays consist of a large number of regularly arranged discrete spots of proteins or antibodies (Figure 1), which are spotted on a solid support using robots (Figure 2) equipped with solid steel pins (Figure 3) or piezoelectric nozzles (Figure 4).

Figure 1: Microarray with 512 spots

Figure 2: Interior of spotting robot

Figure 3: Spotting head with 16 solid steel pins

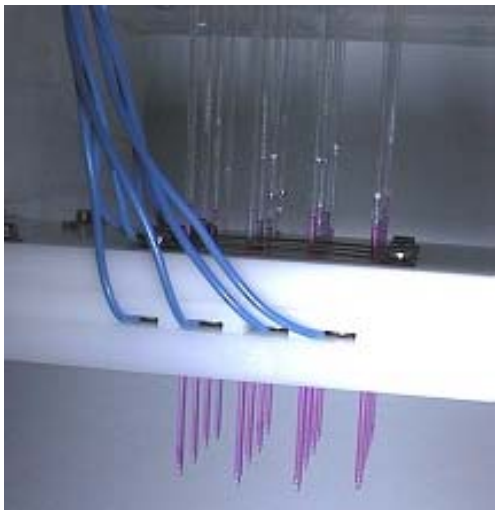

Figure 4: Spotting head with 16 piezoelectric nozzles (courtesy of Dr. L. Nyarsik, MPIMG)

The basic procedure starts with derivatisation of glass slides with appropriate coupling chemistries, which are required for the attachment of proteins. The source plate with the proteins to be immobilised as well as derivatised glass slides are then placed into a spotting robot and a routine is started in which the tip of each pin dips into protein solution and transfers solution to the surface of a deriviatised glass slide. By this means, about 0.19 nl

(100 µm-pins) or 0.6 nl (150 µm-pins) of solution is transferred (34) and spots with slightly larger diameters compared to the diameter of the tip are created. The slides are then blocked to prevent unspecific binding and fluorophore-tagged samples are applied to the microarray. After binding of the sample, the microarray is washed and read out by a confocal laser scanner (Figure 5, Figure 6), which excites the fluorophores at a wavelength near excitation maxima and measures the resulting emission.

Figure 5: Confocal laser scanner

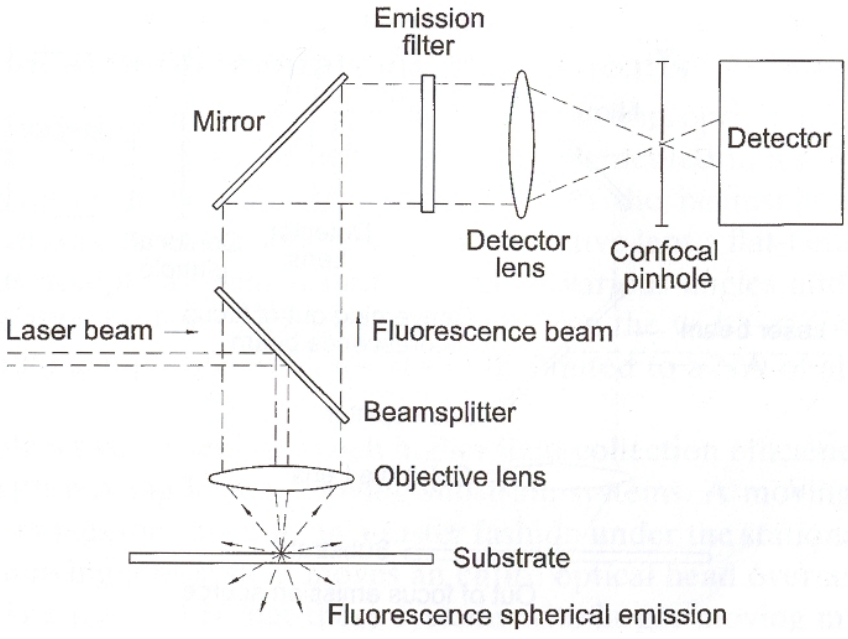

Figure 6: Principle of confocal scanner (35)

Apart from common microarrays, many different chip formats have been developed to study the proteome. These include macro- and microarrays, which were spotted on filters, chips bearing microwells, microfluidic and liquid-chips, as well as alternative formats, such as surface enhanced laser/desorption ionization (SELDI) and surface-plasmon-resonance (SPR).

1.2.1 Macro- and Microarrays

Early developments of protein microarray technology included the generation of low-density protein arrays on nitrocellulose filter membranes, such as the universal protein array system (36). This concept, which is based on the 96-well microtitre plate format, allows the analysis of specific protein interactions with proteins, DNA, RNA, ligands and other small chemicals. A miniaturisation of this technique was shown by Lueking *et al.* (37), who spotted lysates from 92 human cDNA expression clones in a density of 600 spots/cm^2 to screen this array of proteins by specific antibodies. Mendoza *et al.* (38) employed another method of generating protein microarrays and displayed its usefulness for multiplex analysis. Proteins were printed on a glass plate containing 96 hydrophobic Teflon masks, each with a capacity of 144 elements per mask. The spotted antigens were detected by standard ELISA techniques using a CCD detector for imaging of the arrayed antigens. De Wildt *et al.* (39) generated one of the first high-density microarrays comprising 18342 clones, which were screened for binders from different sources. Since this effort was not practical to be done by commercially available antibodies, he used scFv-fragments from existing libraries. The breakthrough in terms of surfaces was achieved by MacBeath and Schreiber (40), who showed the practicability of spotting proteins and antibodies onto glass slides, that were functionalised with aldehyde groups. A widespread investigation on poly-L-lysine coated microarrays was done by Haab *et al.* (41), who compared performance of 115 antibody-antigen pairs. Although both types of surface chemistries proved their practicability, the provision of an ideal surface chemistry is still an issue of current research. Angenendt *et al.* (42) tested eleven different surface chemistries, comprising plastic slides, derivatised glass slides and gel coated slides (43) for their use in antibody microarray technology. Kiyonaka *et al.* presented a novel semi-wet peptide/protein microarray using a supramolecular hydrogel composed of glycosylated amino acetate (44). Besides the development of an optimised surface, several production parameters for the generation of antibody microarrays were evaluated (45, 46). Those included the kind and length of cross-linkers, composition and pH of the spotting buffer, the kind of blocking reagents as well as antibody concentration and storage procedures.

1.2.2 Microwell and Nanowell Arrays

Besides these flat microarrays, a new technology is emerging, which applies microwells and nanowells (Figure 7).

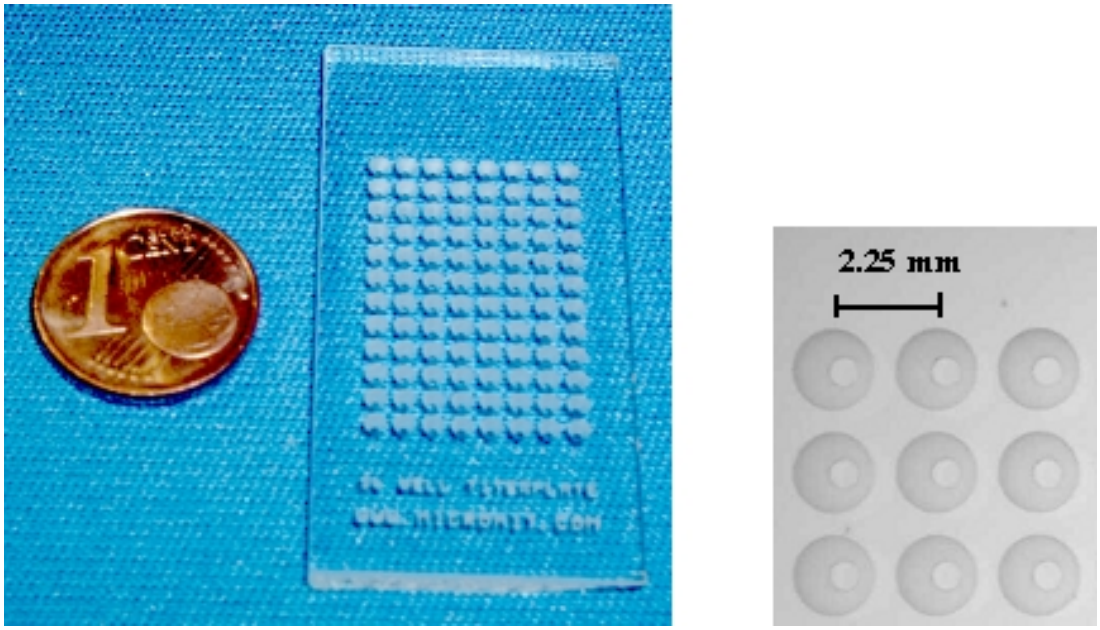

Figure 7: Left: Photograph of the nanowell plate comprising 12x8 wells with a well volume of 1.5 µl. Right: Enlarged photograph of the nanowells with a spacing of 2.25 mm between each well

Zhu *et al.* (47) employed arrays of microwells to study protein kinases. The microwells, 1.4 mm in diameter and a 300 µm in depth, could carry up to 300 nl and were fabricated in a disposable silicon elastomer, poly(dimethylsiloxane) (PDMS) (48). Nanowell arrays of much smaller diameters between 2.5 µm and 5 µm were fabricated by Biran and Walt (49). They prepared an array of nanowells from etched imaging fibres and were able to accommodate and detect single living cells carrying reporter genes or fluorescent indicators. In another approach, Bernhard and co-workers (50) fabricated nanowell arrays on the distal face of coherent fibre-optic bundles and applied them as pH-sensitive or O_2-sensitive microwell array sensors. A typical microwell was 1 to 14 µm deep, approximately 22 µm wide and filled partially with a 2 to 10 µm thick chemical sensing layer, consisting of a polymer and a dye.

1.2.3 Microfluidic Chips

An advanced format of microwell chips are microfluidic chips, which consist of microwells that are connected by tubing and allow liquid handling in a miniaturised format. Cohen *et al.* (51) fabricated a microchip-based enzyme assay for protein kinase A by standard

photolithographic techniques. Using electro-osmosis for the transport of reagents within the network of etched channels, they were able to monitor phosphorylation of the kemptide peptide by protein kinase A. Moreover, they could prepare on-chip dilutions of reagents and measure kinetic constants of the reactions. Another enzymatic assay applying β-galactosidase and resofurin was performed by Hadd *et al.* (52). Using electrokinetic flow, precise concentrations of all reagents could be mixed, and Michaelis-Menten constants could be derived with and without inhibitor. Although the technology is in an early stage, products using microfluidic devices are already on the market. Agilent Technologies offers products in a “Lab-on-a-Chip”-series for analysis of DNA, RNA, proteins and cells, which are based on chips with interconnected fluid reservoirs and pathways. For cell assays, movement of cells on the chip is controlled by a pressure-driven flow, while for analysis of RNA, DNA and proteins electrokinetic forces are applied.

1.2.4 Alternative Formats

Besides microarrays, microwell and microfluidic chips some alternative formats have been evolved. One of them is the SELDI technology, which was introduced by Hutchens and Yip in 1993 (53). The technology combines purification of the sample by surface-enhanced affinity capture on a wide variety of affinity matrices and subsequent identification by mass spectrometry (54, 55). Another technology is surface plasmon resonance (SPR), which is a technology based on the measurement of the mass concentration of biomolecules (56). One system called Biacore consists of an activated gold chip onto which an interacting biomolecule and a flow cell is attached, which applies a continuous flow to the chip surface. By introduction of the sample to the flow cell, the sample floats over the interacting biomolecule attached on the gold surface and causes a change in the local concentration upon binding, which can be measured by SPR. Finally, a third alternative format, the bead-based LiquiChip has recently been introduced by Qiagen GmbH. The system is based on xMAP technology, which applies two fluorescent dyes for specific labelling of beads. After the interaction of immobilised, bead-bound capture molecules with a fluorescently labelled reaction partner in solution, two lasers are applied, one for identification of the bead and one for reporter fluorescence. Only if fluorescent signals are detected on both channels, an interaction event is recorded, which allows differentiation from unbound reporter molecules. Labelling of beads with different ratio of fluorescent dyes allows the use of the LiquiChip system for multiplex assays with up to 100 different interaction partners.

1.3 Areas of Application

Protein and antibody microarray are applied in several areas of applications, mostly in diagnostics, basic proteome research and in the development of therapeutics.

1.3.1 Diagnostics

In diagnostics, protein and antibody microarrays are used for detection of antigens and antibodies in blood samples as well as in profiling of sera to discover new disease markers. Although it is too early to apply protein and antibody microarrays commercially outside the laboratory, interesting proof-of-principle experiments have been made to display the potential of this technique for example in serum profiling. Joos *et al.* (57) generated protein microarrays by immobilisation of 18 known autoantigens, which are commonly used as biological markers for autoimmune diseases, such as Autoimmune Thyroiditis or Sjögren's Syndrome. The screening of 25 sera from autoimmune patients revealed specific and very sensitive detection of autoantibodies and showed that down to 40 fg of a known protein standard could be detected with little or no cross-reactivity. In a larger approach Robinson and colleagues (58) fabricated arrays containing 196 distinct biomolecules, comprising proteins, peptides, enzyme complexes, ribonucleoprotein complexes, DNA and post-translationally modified antigens. By this, they were able to characterise sera from eight human autoimmune diseases, including Systemic Lupus Erythematosus and Rheumatoid Arthritis. In the area of cancer research, Sreekumar *et al.* (59) prepared one of the currently largest antibody microarray carrying 146 distinct antibodies. With this antibody microarray, they were able to monitor alterations of protein levels in LoVo colon carcinoma cells that were treated with ionising radiation. Differential expression profiles with radiation-induced up-regulation of apoptotic regulators including p53, DNA fragmentation factor 40 and 45 as well as tumour necrosis factor-related ligand were observed. Hiller and colleagues (60) applied microarray technology to develop a miniaturized allergy test containing 94 purified allergen molecules from a variety of common allergy sources, such as plants, animals, fungi, and insects as well as a variety of foods. Screening with 20 patient sera revealed comparable results to commonly applied techniques in various established IgE detection systems. In another study with a similar focus, Kim and co-workers (61) used purified *dermatophagoides pteronyssinus* (Dp), egg white, milk, soybean and wheat as allergens to measure Dp-specific IgE levels in sera.

Besides the profiling of sera and blood, a novel area of application for microarrays in diagnostics is emerging: the use of arrays in the monitoring of environment and food. Although no high-density chips have been applied so far, Samsonova *et al.* (62) employed the Biacore sensor to measure the antiparasitic agent and were able to detect down to 19.1 ng ivermectin per gram bovine liver.

1.3.2 Proteomics

While first experiments were made to apply protein and antibody arrays in diagnostics, the major application area is basic proteome research. Even though the early stage of this technology and the lack of mature products on the markets cause both areas to overlap to a great extent, the focus of basic proteome research is rather target identification and discovery, functional analysis of proteins and cellular expression profiling than protein characterisation. However, the close relationship between both areas is displayed in the study of Huang *et al.* (63), who described the production of an antibody array for the simultaneous assay of 24 cytokines from two different sources: cultured media or patient sera. The system was based on standard sandwich ELISA technology and chemiluminescence was applied for detection.

An interesting approach to create organ and disease specific microarrays for the use in basic proteome research and diagnostics was performed by Paweletz *et al.* (64). They generated a reverse phase protein array by immobilisation of the whole repertoire of patient proteins and were able to quantify the phosphorylated status of signal proteins. Furthermore, they could monitor cancer progression from histologically normal prostate epithelium to prostate intraepithelial neoplasia and invasive prostate cancer by increased phosphorylation of Akt, suppression of apoptosis pathways, and decreased phosphorylation of ERK. Nishizuka et al. applied reverse-phase protein lysate microarrays for the profiling of 60 human cancer cell lines (65). As a result, they were able to find two promising pathological markers for distinguishing colon from ovarian adenocarcinomas A specific antibody microarray for squamous cell carcinoma of the oral cavity was generated by Knezevic and co-workers (66), utilising laser capture to gain total protein from specific microscopic cellular populations. Using this approach, they were able to correlate differential expression of stromal cells adjacent to diseased epithelium to tumour progression. Nielsen at al. applied antibody microarrays to for monitoring of the activation, uptake, and signalling of ErbB receptor tyrosine kinases in human tumour cell lines. In this experiments the antibody microarrays

were created within the wells of a microtiter plate to minimised consumption of the samples (67).

Early methodologies for the study of membrane proteins were developed by Fang *et al.* (68), who generated membrane protein arrays for analysis of the ligand-binding properties of receptors. The microarrays consisted of an array of G protein-coupled receptors and were capable to demonstrate specific binding of their respective targets. Moreover, subtype-specific detection of a cognate antagonist analogue specific for beta-adrenergic receptors was displayed using an array with different subtypes of adrenergic receptors. Since important members of the signalling pathways are membrane located, systematic analysis of membrane proteins provides a valuable task for proteomics.

Additionally, efforts were made to monitor enzymatic reactions on-chip. As a first step, kinase activity was measured quantitatively on a peptide chip (69). Houseman *et al.* applied surface plasmon resonance, fluorescence, and phosphorimaging for detection of phosphorylation and quantitative evaluation of three inhibitors. Zhu *et al.* (47) used microwell chips to analyse nearly all protein kinases from *Saccharomyces cerevisiae*. Many novel activities were found as well as 27 protein kinases with an unexpected tyrosine kinase activity. The same group investigated protein-protein interaction in large scale by the production of the first proteome chip (70). For the generation of the chip, 5800 open reading frames of yeast were cloned and the corresponding proteins were overexpressed in *E. coli*, purified and spotted. The resulting protein microarray was used to screen for interactions with calmodulin and phospholipids and allowed identification of binding motifs.

Similar to the screening for protein-protein interactions, microarrays have been used for the detection of protein-antibody interactions. Holt *et al.* (71) applied a large protein microarray consisting of 27648 human fetal brain proteins immobilised on a PVDF membrane to screen 12 antibody fragments for specificity. Four highly specific antibody-antigen pairs were identified, including three antibodies that bound proteins of unknown function. The first plant protein microarray was generated by Kersten *et al.* (72), who immobilised 95 recombinant proteins from *Arabidopsis thaliana* on slides coated with a nitrocellulose based polymer (FAST^{TM}-slides) or polyacrylamide. Both types of surfaces were then used for characterisation of antibody specificity.

There are great chances for protein and antibody microarrays to acquire a third major area of application alongside proteome research and diagnostics, which is therapeutics.

1.3.3 Therapeutics

Although no real applications of arrays in this area have been shown, the development of new therapeutics requires high-throughput tools for target validation, pre-clinical and clinical trials as well as for the screening of compounds and capture agents for desired drug activity, specificity, and selectivity. However, further efforts have to be made to ensure reliability of protein and antibody microarrays, to accomplish the special demands and regulatory restrictions of clinical trials.

1.4 Binders for Microarrays

Basically, two formats of microarrays can be described. First, the protein chip that consists of a large diversity of proteins or, second, the antibody chip, which harbours a collection of specific binder molecules. The former can be used for several applications, which are mainly the screening for immune responses directed against the immobilised proteins and small peptides containing epitopes, or the screening of protein-enzyme and protein-binder interactions, such as DNA, RNA, proteins or small molecules. Antibody microarrays are designed for protein expression profiling or the detection and quantification of specific proteins within complex samples and may have antibodies, antibody fragments, engineered binding proteins or even aptamers immobilised on the surface.

1.4.1 Sources of Antibodies

For antibody arrays, most monoclonal antibodies are obtained from commercial sources. Hereby, one important prerequisite is that the antibodies have to be purified and BSA-free to be immobilised on microarray surfaces. So far, monoclonal antibodies have become commercially available that are directed against thousands of different antigens. However, the costs associated with hybridoma technology and production are too high to create antibody microarrays of great diversities, which led to the development of alternatives to hybridoma technology using immortalised B-cells (73). Another alternative is phage display, which is applied for the selection of recombinant antibody fragments (74). After selection, antibody fragments can then be produced using inexpensive media and purification methods for *Escherichia coli* (75, 76). To further increase the properties of selected antibodies, new

strategies completely working *in vitro* have been developed using ribosomal display (77, 78) or mRNA-protein fusions (79).

1.4.2 Sources of Proteins

At the same time, proteins are also very efficiently handled and produced by cDNA expression libraries using *E. coli*. Therefore, protein expression and purification methods were developed that meet the requirements of protein and antibody microarray technology.

The discovery of immobilised metal affinity chromatography (IMAC) purification technology together with the engineering of recombinant affinity tags has led to a widely applied technology for high-throughput purification. Hochuli *et al.* (80) have introduced histidine affinity handles fused to either N- or C-terminus of recombinant proteins and combined these with Ni(II)-nitrilotriacetic acid (Ni-NTA) affinity media. The addition of short affinity handles like six histidines does not severely change the properties of recombinant proteins and increases molecular weight by less than 1 kDa. Both Ni-NTA as well as the alternative Co(II)-carboxymethylated aspartic acid tetradentate complex show a selective affinity towards histidine stretches and are stable enough for protein purification under strong denaturing conditions (81, 82). This is an important feature, since many eukaryotic proteins form inclusion bodies upon expression in *E. coli*, and solubilisation by denaturing chaotropes like guanidinium or urea is required for purification. Several attempts to use IMAC for refolding of proteins directly on affinity resins have also been successful (83, 84). Additionally, inexpensive materials, the purity after a single step, and high scalability led to the use of IMAC purification as the standard procedure for high-throughput protein purification in the context of expression libraries (85).

Alternative affinity fusions have been introduced, like the strep-tagII (86), calmodulin-binding peptide (87), chitin-binding domain (88), glutathione S-transferase (89), maltose-binding protein (90), and thioredoxin (91). The latter three are used to increase solubility of protein fusions under native conditions. Some of these have been characterised more recently by Braun *et al*. (92) in context with high-throughput purification. Just more recently, Lamla and Erdmann have selected a new streptavidin binding peptide, called Nano-tag, which displayed improved affinity compared to the strep-tag (93).

Further hosts for high-throughput protein expression like *Pichia pastoris* (94) and *Saccharomyces cerevisiae* (95) have also been tested. The main advantage is a better

expression yield of soluble proteins in comparison to *E. coli* and the purification of proteins with post-translational modifications. However, handling of yeast expression libraries and purification is still more sumptuous in comparison to *E. coli*.

To obtain proteins, that are toxic to their expression hosts, strategies involving cell-free transcription and translation systems have been developed (96). Such systems are completely independent of any host organism, needing only a cDNA with a T7 promoter as a template, which in turn can be propagated by PCR. This allows protein synthesis to occur on microchips directly, relying on well-established DNA microarray technology (97).

Another generation of samples for protein microarrays works by chromatographic separation of a crude mixture like cell culture extracts (26). A Protein extract is first separated and fractionated by anion exchange, and then by reverse phase liquid chromatography to achieve a high resolution of proteins to be immobilised on the microarray. The special advantages of such protein samples are the correct post-translational modifications allowing further properties to be assayed in comparison to recombinant sources.

1.4.3 Alternative Binders

At the same time other binding scaffolds than immunoglobulin domains like affibodies (98), fibronectin (99), lipocalin (100) or repeat domains (101) are explored to meet the requirements associated with antibody array technology. Finally, oligonucleotides can be selected to bind certain antigens (102). These so-called aptamers are especially interesting because of low production costs and the use of materials established for DNA array technology. A special derivative of aptamers, are hereby photoaptamers, which carry a photo-crosslinking reactive functionality to covalently bind the antigen (103-105).

1.4.4 Native or Denatured Conformation

The state of functionality of immobilised proteins determines the usefulness of protein arrays for appropriate applications. Protein arrays for the immunoprofiling of autoimmune sera do not need to present native proteins and allow a simple detection by a labelled secondary species-specific antibody (58). Furthermore, domains that readily bind to unstructured sequences present in peptides or unfolded proteins, such as PDZ, EF-hand, SH3, and WW can also be applied for screening of protein or peptide arrays. However, most protein-protein

interactions require a more cooperative contact from both binding partners, so that the immobilised proteins have to be presented in an active state.

Similar to protein arrays, activity is also an important prerequisite for antibody arrays. Since antibodies can be considered as the active binding partner, these must retain their specific binding properties upon immobilisation. Therefore, protocols for immobilisation, storage and handling as well as the surface coatings need to be optimised in order to retain native conformation of antibodies.

1.5 Detection Technologies

Detection of molecular interactions can be achieved either by using directly labelled binding molecules or indirectly without modification of the binder (Figure 8). Direct labelling can mainly be performed in two ways: radioactively, using ^{125}I or ^{3}H or fluorescently using Cyanine, Alexa or Oyster dyes. Although radioactive labelling is one of the most sensitive procedures, it has become a trend over the last decades to sequentially replace radioactive labelling with other detection methods. Reasons are mainly the risks of radioactive contamination, problems associated with proper waste disposal, and most important incompatibility with high-throughput screening.

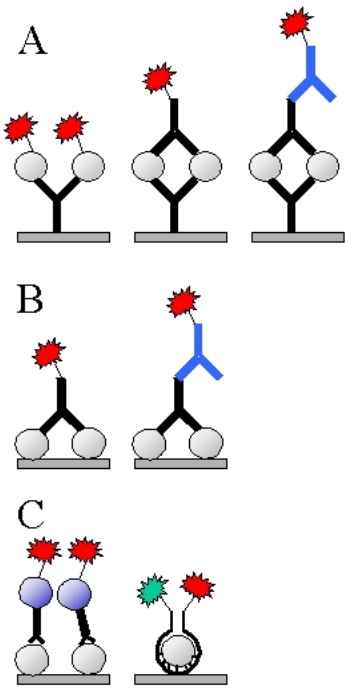

Figure 8: Labelling strategies. A displays direct and indirect labelling for antibody microarrays. B shows indirect labelling applied with protein microarrays, while C display the detection of scFv's by labelled protein L and the indirect detection of protein via labelled aptamers and fluorescence resonance energy transfer

More recently, fluorescent dyes have become the method of choice for labelling and detection of molecules in the microarray format due to the development of bright and pH stable dyes with narrow emission and excitation spectra. Commonly used scanners allow the simultaneous application of up to four fluorophores and permit direct comparison and relative quantification of four different samples. Further improvements to fluorescent detection in terms of sensitivity have been made by the introduction of rolling circle amplification (RCA) (106, 107), and by the recent development of quantum dots with superior photostability compared to organic dyes (108).

Indirect detection of molecular interactions without labelling is advantageous, since it does not to interfere with the interaction as changing the properties of the analyte by labelling. Commonly used biomolecules for indirect labelling include species-specific secondary antibody-conjugates for detection of primary antibodies, or labelled protein L for detection of scFv's. It is also possible to employ a sandwich assay by having one specific antibody immobilised and adding another specific antibody in solution to capture and detect the antigen. However, this approach limits the diversity of such antibody microarrays as a multitude of detection antibodies in solution cause an increasing background.

One of the most promising developments in this area is the introduction of aptamers, consisting of oligonucleotides as binding molecules. Those as well as photoaptamers, can be used to crosslink and stain bound protein using protein-specific dye after incubation, since they are non-proteinaceous (109). Another possibility that does not involve previous staining of analyte involves binding and detection via molecular aptamer beacons. The binding event can be detected either by direct quenching between fluorophore and quencher, or by fluorescence resonance energy transfer (FRET) between donor and acceptor (110, 111). As aptamers undergo structural changes upon ligand binding, the ends of the oligonucleotide may change their relative position and cause increasing or decreasing fluorescence by two different dyes affixed to each end. Another method for direct and real-time detection of biomolecule interaction is SPR. Although this technique was originally invented to evaluate binding coefficients of a given interaction, advances were made to increase the number of simultaneously detected nucleic acid hybridisations with SPR (112). Nevertheless, it remains to be shown that this technology can be adapted to the scale already employed in conventional microarray technology.

Similar problems are encountered by matrix-assisted laser desorption/ionisation. Small arrays can be analysed using mass spectrometry (113). However, analysis of samples is still performed sequentially and cannot be done in parallel. Therefore, the complexity of an array determines the time of evaluation. At the same time the computational power needed for identification still exceeds the time required for the measurement itself.

1.6 Current Limitations of Protein and Antibody Microarrays

1.6.1 Source of Proteins and Antibodies

A major problem of antibody microarrays is not to acquire great quantities, but a large diversity of antibodies. Although there are many monoclonal and polyclonal antibodies commercially available, it is not feasible to buy several hundreds of these to produce an antibody microarray. This is not only due to lacking functionality on chip (41, 42), but mainly due to costs. To solve this problem of antibody microarrays, several attempts have been made to replace expensive antibody production in hybridomas by the production of scFv's or Fabs by phage display (114) or ribosomal display (78). Other solutions include the application of aptamers from nucleic acid libraries (115) for the specific detection of proteins. The same holds true for protein microarray technology, in which, despite great efforts to develop automated protein expression and purification facilities, the provision of a large set of proteins still represents one of the most challenging tasks.

1.6.2 Labelling of Proteins for Antibody Microarrays

The labelling of proteins is still a bottleneck of antibody microarray technology, although many techniques for direct and indirect labelling of protein mixtures are available. Reasons for this are not so much the absence of applicable coupling chemistries, but the diversity and differing quantities of available amino acids, that act as targets of those coupling chemistries. This diversity does not allow homogeneous labelling of complex protein mixtures in a defined stoichiometry and prohibits absolute quantification of proteins in complex samples. Currently, solutions for this are only available for the measurement of recombinantly produced proteins, which can be engineered to allow indirect labelling by affinity tags or fusions with fluorescent reporter molecules (116). However, both approaches may cause changes in structure and morphology, which hold the risk of changing the binding properties of the protein. Therefore, the development of label-free detection methods that are amenable to high-throughput is essential.

1.6.3 Surfaces and Hardware

Since most of the hardware equipment was adopted from DNA microarray technology, such as microscope slide format, surface chemistry, fluorescent detection, spotting devices and scanners, many of these will have to be optimised to meet the different requirements of antibody and protein microarrays. Those requirements include the development of a support material that avoids denaturing contact with the surface by the introduction of linkers or activated layers. Another important feature is the provision of an environment, which prevents dehydration of immobilised proteins and antibodies. One solution may be the introduction of microwells, which reduce evaporation. The same holds true for microfluidic chips, which prevent dehydration due to their closed architecture. However, both formats would necessitate alignment of the handling robots with the surface grid or provision of additional hardware for control of the system.