Department of Immunotechnology

Lund University

PROTEIN MICROARRAYS BASED ON SINGLE FRAMEWORK RECOMBINANT ANTIBODY FRAGMENTS (SINFABS)

CATCHER AND CARRIER – A CRUCIAL COMBINATION

Cornelia Steinhauer

ACADEMIC DISSERTATION submitted to the Technical Faculty of Lund University
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY IN ENGINEERING.

The public defense will take place in Lundmarksalen, Astronomic Institute, Sölvegatan 27, Lund, Friday, the 16th of December 2005, 13:15 p.m..

The Opponent of the Faculty is Dr. Jörg Hoheisel, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany.

Lund 2005

Contents

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numeral (I-V).

I. Steinhauer, C., Wingren, C., Malmborg Hager, A. and Borrebaeck, C. (2002) Single framework recombinant antibody fragments designed for protein chip applications, *Biotechniques, Suppl.: High-Throughput Proteomics: Protein Arrays*, 38-45.

II. Steinhauer, C., Borrebaeck, C. and Wingren, C. (2005) Long-term stability of scFv antibodies designed for antibody microarray applications on different solid supports. *Submitted for publication*

III. Wingren, C., Steinhauer, C., Ingvarsson, J., Persson, E., Larsson, K. and Borrebaeck, C. (2005) Microarrays based on affinity-tagged scFv antibodies: sensitive detection of analyte in complex proteomes, *PROTEOMICS* **5**(5), 1281-1291.

IV. Steinhauer, C., Wingren, C., He, M., Kahn, F., Taussig, M. and Borrebaeck, C. (2005) Improved affinity coupling of probes for antibody microarrays: engineering of double-$(his)_6$-tagged single framework recombinant antibody fragments. *Submitted for publication*

V. Steinhauer, C., Ressine, A., Marko-Varga, G., Laurell, T., Borrebaeck, C. and Wingren, C. (2005) Biocompatibility of surfaces for antibody microarrays: Design of macroporous silicon substrates, *Anal. Biochem* **341**(2), 204-213.

ABBREVIATIONS

2DE	2-dimensional gel electrophoresis
ADCC	antibody-dependent cell-mediated cytotoxicity
CD	cluster of differentiation
CDR	complementary determining region
CH	constant domain of the heavy chain
CL	constant domain of the light chain
CV	coefficient of variation
DC	dendritic cell
DSC	differential scanning calorimetry
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
FW	framework
HRP	horseradish peroxidase
IMAC	immobilised metal ion affinity chromatography
LC	liquid chromatography
LOD	limit of detection
MALDI	matrix assisted laser desorption ionisation
MIST	multiple spotting technique
MS	mass spectrometry
NTA	nitrilotriacetic acid
ORF	open reading frame
PEG	polyethylene glycol
RCA	rolling circle amplification
SAM	self-assembled monolayer
scFv	single-chain fragment variable
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI	surface enhanced laser desorption ionisation
sinFab	single-framework recombinant antibody fragment
SPR	surface plasmon resonance
TNF	tumor necrosis factor
TOF	time of flight
TSA	tyramide signal amplification
VDW	van der Waals
VH	variable domain of the heavy chain
VL	variable domain of the light chain

Chapter 1

INTRODUCTION

Differential expression profiling is one of the key technologies of the genomic century. The implementation of techniques for global analysis and comparison of whole genomes, such as those from diseased and normal tissue, was a tremendous step towards the understanding of biological processes in general and disease biology in particular. It was, however, not until the development of DNA microarray technology, that global gene expression profiling could be standardised and implemented into several areas of functional genome analysis. The microarray/chip format enabled a comprehensive, sensitive and fast genome analysis in a high-throughput manner (chapter 2.1).

While the human genome project revealed less than 30.000 human genes (Pennisi, 2003), the human proteome was estimated to comprise more than one million proteins of distinct structural and functional properties (Harrison et al., 2002; Laurell and Marko-Varga, 2002; Wingren and Borrebaeck, 2004). Thus, in order to get an insight into complex biological processes, genome analysis had to be complemented by technologies for comprehensive proteome analysis. Until the end of last century, differential proteome profiling was accomplished by means of 2-dimensional gel electrophoresis (2DE). However, this technology was time- and cost-intensive and very restricted in resolution (chapter 2.2). Thus, a technology had to be developed, which – in analogy to DNA microarrays – allowed for rapid global analysis of entire proteomes in a high-throughput manner.

The first protein microarrays were established in the beginning of this century. Although the early publications by Wright Jr et al. (1999), Haab et al. (2001), MacBeath and Schreiber (2000), Ge (2000) and Zhu et al. (2000) were mainly proof-of-principle studies, they already showed the great potential of this technology. Nowadays, protein microarrays are used not only in protein profiling (Wingren et al., 2003; Sreekumar et al., 2001; Miller et al., 2003; Hudelist et al., 2004) and clinical diagnostics (Belov et al., 2001, 2003), but also in the analysis of fundamental biological research (Zhu et al., 2000, 2001). Meanwhile, the multiple

applications and individual setups have led to the classification into functional and analytical protein microarrays (Phizicky et al., 2003; Zhu and Snyder, 2003; Wingren and Borrebaeck, 2004). While in functional microarrays the proteins of interest are arrayed and analysed for their biological activities, analytical microarrays utilise specific probes, such as antibodies, as catcher molecules for the screening of proteomes (chapter 2.3, Figure 1).

So far, antibody microarrays have been designed as small dedicated arrays composed of up to 400 antibodies (Hudelist et al., 2004) (chapter 2.4). Although the technology has evolved rapidly from printing and detection facilities adapted from DNA microarrays or traditional ELISAs to equipment and protocols optimised for protein microarrays, it is in many ways still in a developmental stage (chapter 3). Besides these technical challenges, major bottlenecks in the design of high-density antibody microarrays are the two key components of the system: the probe and the solid support, i.e. "catcher and carrier".

The aim of this thesis was to design and optimise the antibody microarray setup in a way that in the end allows for the establishment of high-density antibody microarrays for global proteome profiling. For this purpose both probe and solid support were characterised in detail and evaluated for their biocompatibility with each other.

The design of high-density antibody microarrays requires access to a large variety of antibody specificities, which can be expressed and purified in a high-throughput manner. Thus, we suggest recombinant antibody fragments, such as single framework recombinant antibody fragments (sinFabs) selected from the nCoDeR-library (Soderlind et al., 2000), as probes for antibody microarrays. In the scope of this thesis, sinFabs have been shown to provide the optimal design for antibody microarray probes, as they are structurally uniform, functional and stable on the chip and provide sensitive detection of analytes (chapter 4).

In **paper I,** the on-chip performance of four single chain fragments (scFvs) based on different frameworks was evaluated with regards to functional adsorption on the chip and long-term stability. The results illustrated the importance of a structurally uniform probe design: Only sinFabs could be immobilised to three different surfaces in a functional state and were stable on the chip for at least 47 days. In a more comprehensive study (**paper II**), five sinFab molecules displaying different specificities were shown to be stable on four solid supports for up to 16 months. However, this study also demonstrated an impact of both the CDR-loops as well as the structure and coupling chemistry provided by the solid support on the overall long-term performance. In **paper III**, we evaluated another key parameter of antibody microarrays, the sensitivity. Independently of the probe-format (purified or non-purified) and the analyte-format (pure or spiked into human sera), the limit of detection (LOD) for sinFab-microarrays

was in the nM to fM range. This could be further reduced to a total of 300 zeptomol, applying the multispotting technique, i.e. the analyte was being spotted on top of the arrayed probe. The possibility to array the probe in a non-purified format is another important step on the way to high-density antibody microarrays and was further examined in **paper IV**. By the introduction of a novel polyhistidine-tag, the double-$(his)_6$-tag, into the sinFab-framework, the affinity to Ni^{2+}-chelate derivatised solid supports was significantly increased and crude periplasmic preparation or expression supernatant were successfully purified on-chip. Finally, in **paper V** we studied the general performance and biocompatibility of different commercially available solid supports. Furthermore, we co-developed a novel macroporous support coated with nitrocellulose (MAP3-NC7), which was shown to provide an increased probe binding capacity with retained overall biocompatibility.

Chapter 2

MICROARRAY ANALYSIS

2.1 DNA microarray

In the late 1990s the genomic century hit its peak with several large sequencing projects and the development of global gene expression profiling techniques. While classical genetics focussed on the examination of single genes, novel technologies such as the DNA microarray enabled the analysis of several thousand genes simultaneously. Differential expression profiling, in which the mRNA profiles of diseased cells were compared with those from healthy donors, led to the identification of numerous disease specific genes and biomarkers, as reviewed in e.g. Mischel et al. (2004). Nowadays, DNA microarray is a well established technique in both fundamental and applied medical science, such as cancer research (Ek et al., 2002), but also in other biological disciplines such as microbiology (Karlsson et al., 2005) and plant research (Yazaki et al., 2004).

Two different methodologies have been established shortly after each other. In 1995, Patrick O. Brown developed the so-called cDNA microarray, in which PCR-generated cDNA-probes are robotically spotted onto solid supports, such as glass slides or nylon membranes (Schena et al., 1995). The samples to compare are labelled with two different fluorescent dyes, co-hybridised to the same array and the relative abundance of sample vs. control is measured. In 1996, Affymetrix Inc. launched the so-called oligonucleotide or GeneChip® array, in which several short oligonucleotide probes representing one gene are synthesised *in situ* by photolithography (Chee et al., 1996). The samples under comparison are labelled with the same dye and hybridised to different arrays. Thereby the absolute mRNA-content is measured, which requires careful data processing and normalisation (Liu et al., 2003).

More detailed information about the different technologies and the challenge of data analysis can be found in Leung and Cavalieri (2003) for cDNA arrays and Liu et al. (2003) for oligonucleotide arrays.

2.2 Classical proteomics

Due to differential splicing and posttranslational modifications proteomes are significantly larger and more complex than genomes (e.g. less than 30.000 human genes are estimated to code for more than a million proteins) (Pennisi, 2003; Harrison et al., 2002; Laurell and Marko-Varga, 2002; Wingren and Borrebaeck, 2004). Thus, mapping the proteome with regards to not only amino acid sequences but also tertiary structures, cellular localisations and interactions with other proteins or DNA is the major task of the 21^{st} century.

The term "proteomics" in analogy to "genomics" was first formalised by Ian Humphrey-Smith in 1995 (Wasinger et al., 1995). Already twenty years earlier one of the key technologies in proteomic research, two-dimensional gel electrophoresis (2DE), had been established (O'Farrell, 1975). 2DE separates proteins first by charge using isoelectric focussing and second by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) leading to unique pictures of the proteome. It was, however, not until in the beginning of the 90s that the combination of 2DE and mass spectrometry established global differential proteome analysis similar to differential gene expression profiling. The development of electrospray ionisation (ESI) by Fenn et al. (1989) and matrix assisted laser desorption ionisation (MALDI) by Tanaka (1988) enabled the ionisation of macromolecules such as peptides and proteins and was recognised by the Nobel Prize for Chemistry in 2002. Today, MALDI-TOF is the standard analysis for so-called "peptide-mass-fingerprinting", where a set of proteolytic (peptide) fragment masses is compared to the theoretical masses in a database. Furthermore, Tandem-MS enables the direct identification of amino acid sequences. The combination of mass spectrometry and protein microarray technology is highly desirable and will be discussed in chapter 3.3.2.

Although 2DE-MS is still a common technology for comparative proteome analysis, it is technically challenging: Firstly, the resolution of 2DE is limited by the facts, that highly basic proteins are difficult to resolve and that high-abundant proteins can mask low-abundant proteins. Furthermore, hydrophobic membrane proteins are often underrepresented due to solubility problems (Steel et al., 2005). Finally, 2DE-MS is a time and cost-intense process of two to three days per analysis. Alternative technologies, such as LC-MS/MS (Appella et al., 1995) supplement traditional 2DE-MS analysis, but the demand for comprehensive, sensitive and fast technologies for complex proteome analysis is still high.

A more detailed overview about the technologies and recent developments in the field of proteomics is given in Patterson and Aebersold (2003) and Steel et al. (2005).

2.3 Protein microarray

Following the continuous growth and demand of new technologies in the field of proteomics, the next logical step on the way to global proteome analysis was the establishment of protein microarrays in analogy to DNA microarrays. In 1999, Ciphergen Biosystems (Fremont, CA) launched the SELDI ProteinChip® System (Wright Jr et al., 1999). The ProteinChips® are available with different chromatographical properties (hydrophobic, hydrophilic, anion exchange, cation exchange and metal affinity) to purify crude samples directly on the chip. A special TOF-mass spectrometer, the surface enhanced laser desorption ionisation (SELDI) mass spectrometer, is used as read-out. With Ciphergen being one of the first protein chip companies, there are 140 companies meanwhile involved in protein arrays and related technologies (http://www.functionalgenomics.org.uk). A brief overview over the progress and challenges on the protein chip market in the beginning of 2005 is given in Sheridan (2005).

On the academic side, it was again Patrick O. Brown together with Brian B. Haab who developed one of the first proof-of-principle protein microarrays (Haab et al., 2001). In analogy to the cDNA microarray technology, they printed 115 specific antibody or antigen solutions onto derivatised microscope slides. Two complex protein samples, one serving as a standard, the other representing an experimental sample were labelled by covalent attachment of fluorescent dyes and specific antibody-antigen interactions were analysed. The relative intensity of the fluorescent signal representing the experimental sample and the reference standard then provided a measure for the abundance of each protein in the experimental sample. However, one major limitation of this setup was the fact that only 50% of the antigens and 20% of the antibodies provided specific and accurate results.

Another pioneering paper by MacBeath and Schreiber (2000) demonstrated an important proof for protein microarrays: the ability to immobilise minute amounts of native protein with retained binding capacity. The proteins to analyse were spotted in nanoliter scale, covalently attached to chemically derivatised glass slides and detected by the interaction with their fluorescently labelled binding partners. Specific interaction between protein and protein, enzyme and substrate and small molecule and protein was demonstrated. Complementing this study Ge (2000) showed, that not only protein-protein interactions, but also specific interactions of proteins with DNA, RNA and several other ligands can be analysed in the protein microarray format.

Two large-scale microarrays of the yeast proteome where published by Heng Zhu and Michael Snyder, demonstrating the feasibility of protein chips for the analysis of entire proteomes (Zhu et al., 2000, 2001). In the first paper, 119 re-

combinant yeast protein kinases were analysed on a chip with microwells. Several known and so far unknown activities were observed (Zhu et al., 2000). In the follow-up paper, 5800 recombinant yeast proteins (94% of the predicted yeast ORFs) were expressed, purified and immobilised on a Ni^{2+}-NTA slide. Novel calmodulin- and phospholipid-interacting proteins and a common potential binding motif for calmodulin-binding proteins were identified (Zhu et al., 2001). Only recently, Zhu and Snyder reported on a new large-scale protein microarray study on the yeast proteome, which revealed more than 4000 novel protein kinase interactions allowing them to map a complex signalling network within yeast cells (Service, 2005).

Since these proof-of-concept studies have been published, the concept of protein microarray technology has evolved and the number of publications in the area increased rapidly (Table 1). Nowadays protein microarrays are frequently classified into functional and analytical protein microarrays (Phizicky et al., 2003; Zhu and Snyder, 2003; Wingren and Borrebaeck, 2004).

Table 1. Number of publications until September 2005 as quoted at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

Publications including the term	**Total number of publications (no. of reviews)**
DNA microarray	14664 (1917)
Protein microarray	1086 (268)
Antibody microarray	530 (56)
Recombinant antibody microarray	56 (6)

In functional protein microarrays, such as those from MacBeath and Schreiber (2000), Ge (2000), Zhu et al. (2000), and Zhu et al. (2001), single proteins, peptides or whole proteomes are arrayed onto the chip and used to analyse specific protein activities, binding properties and post-translational modifications. Their main application is in drug and drug-target identification and in building biological networks. Further examples of functional protein microarrays were published by Lueking et al. (2003) and Michaud et al. (2003).

In analytical protein microarrays, such as that by Haab et al. (2001), antibodies, antigens, DNA or RNA aptamers, carbohydrates or small molecules with high affinity and specificity act as catcher molecules for the proteins of interest. These arrays are mainly used for monitoring protein expression level, protein profiling and clinical diagnostics (Sreekumar et al., 2001; Huang et al., 2001; Wingren et al., 2003; Miller et al., 2003; Hudelist et al., 2004). The antibody microarrray technology will be discussed in more detail in the next chapter.

2.4 Antibody microarray

Antibody-based immunoassays have been the most commonly used diagnostic assay for decades (Borrebaeck, 2000). In antibody microarrays, the same principle is applied in a miniaturised and high-throughput manner (Figure 1). A random or specific selection of antibodies is spotted onto a solid support, each spot representing one antibody-specificity. These antibodies then act as highly specific catcher molecules for the proteins of interest and expression levels can be monitored using the analytical principles discussed in chapter 3.3. Thus enabling global expression profiling of whole proteomes, the value of antibody microarrays in cancer research, biomarker discovery, tissue profiling, etc. is increasing (Pavlickova et al., 2004; Glokler and Angenendt, 2003; Wingren and Borrebaeck, 2004) and the number of publications in the field climbed to more than 500 in the last five years (Table 1). Below, a few of the first promising examples of antibody microarray applications will be discussed.

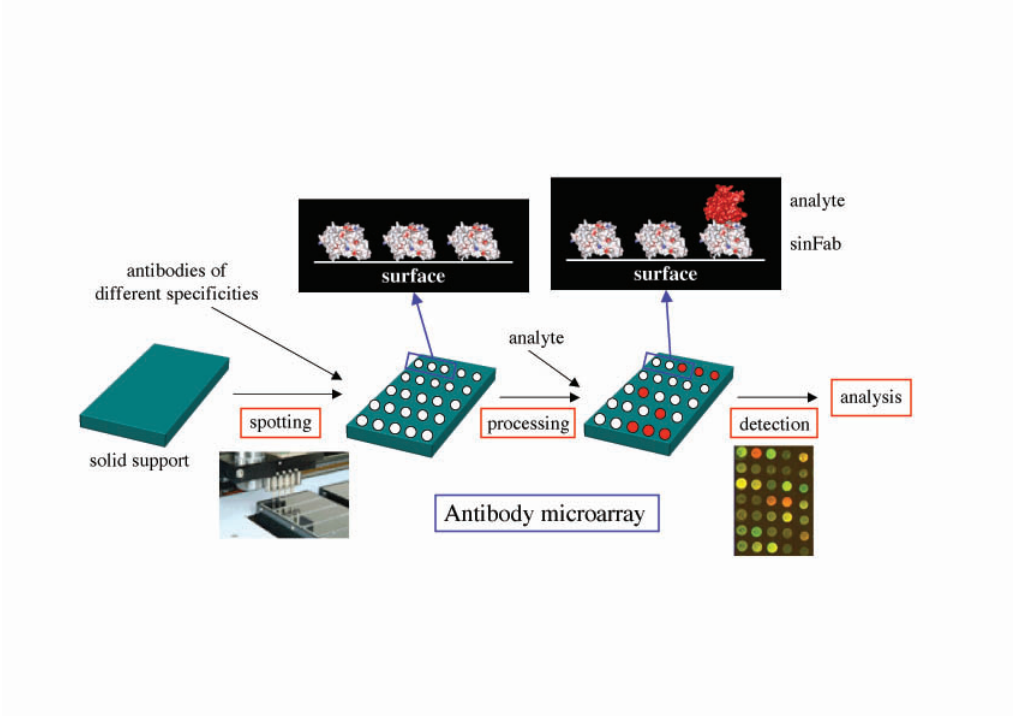

Figure 1. Antibody microarray setup.

One of the first proof-of-concept studies besides the one by Haab et al. (2001) (see chapter 2.3), has been published by Wildt et al. (2000). Comprising 18,342 bacterial clones, each expressing a different single-chain antibody variable region fragment (scFv), the authors generated the first high-density antibody microarray. Replicates of the array were screened simultaneously against 15 different

antigens and used to isolate antibodies against non-purified and complex protein-samples.

In the area of cancer research, Sreekumar et al. (2001) used an antibody microarray carrying 146 distinct antibodies to profile alterations in protein expression in colon cancer cell lysates treated with ionising radiation. Differential expression profiles with radiation-induced upregulation of several apoptotic regulators were observed. Similarly, Miller et al. (2003) developed an antibody microarray of 184 unique antibodies for the protein profiling of human prostate cancer sera. Although more than 50% of spotted antibodies lost activity on the surface, they were able to identify five potential biomarkers for human prostate cancer.

Another field of major diagnostic and therapeutic relevance is the profiling of cytokine expression levels. Huang et al. (2001) produced an antibody array for the simultaneous detection of 24 cytokines from conditioned media or patient sera. The system was based on standard sandwich ELISA technology and chemiluminescence was applied for detection. A highly sensitive antibody microarray system combining the power of sandwich ELISA and rolling-circle amplification (RCA) (described in chapter 4.3.2) has been established by Schweitzer et al. (2002). Using this system, they measured femtomolar amounts of cytokines secreted by human dendritic cells (DCs) upon lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α)-stimulation. Finally, our own group developed an antibody microarray based on sinFab-molecules to profile the cytokine-secretion of activated versus non-activated monocyte-derived human DCs. Measuring the differential intensity, a specific upregulation of four cytokines in activated DCs was observed (Wingren et al., 2003).

An antibody microarray to detect the specific binding of whole cells has been developed by Belov et al. (2001, 2003). It was composed of up to 90 different antibodies against cluster of differentiation (CD) antigens to profile normal peripheral blood leukocytes and different types of leukaemic cells. These types of cell-capture antibody microarrays use a microscope as read-out-system and do not require fluorescent labelling or other sophisticated detection systems. In addition captured cells can be further characterised with other fluorescence-labelled antibodies.

Although the demand is tremendous, only a few commercially available antibody microarrays have reached the market so far. BD Biosciences Clontech (Palo Alto, CA) launched antibody arrays containing up to 500 monoclonal antibodies immobilised on a glass slide (http://www.clontech.com). This chip allowed for the comparison of 378 differentially expressed proteins in normal and malignant breast tissue (Hudelist et al., 2004). Similarly, the AntibodyArrays^{TM} from Hypromatrix (Millbury, MA) contain hundreds of monoclonal antibodies against

proteins involved in e.g. apoptosis or signal transduction. The antibodies are immobilised on a membrane and specific interaction is detected by immunoblotting (http://www.hypromatrix.com). Both BD Biosciences Clontech and Hypromatrix antibody arrays require labelling of proteins in the biological sample. Other companies such as Zyomyx (Hayward, CA), who offered a human cytokine array using a lab-on-chip system (described in chapter 3.2), had to abandon their efforts and make room for bigger companies on the protein chip market (Sheridan, 2005). Further insight into commercially available products related to antibody microarrays is given in Pavlickova et al. (2004).

Although these early examples of antibody microarray applications are promising and show the great potential of the technology, so far they are limited in density and often further restricted by the number of antibodies that are still functional on the chip (Haab et al., 2001; Miller et al., 2003). Thus, the aim of this thesis was the evaluation and development of an antibody microarray setup, compatible with global proteome analysis. Briefly, this requires a careful and comprehensive evaluation of the technical parameters, such as printing, processing, detection and data analysis (as discussed in chapter 3), the properties of the probe (chapter 4, **paper I-IV**) and the biocompatibility with the solid support (chapter 5, **paper I-V**).

Chapter 3

PROTEIN MICROARRAY TECHNOLOGY

3.1 Spotting in pL-scale

To date, the two general approaches to microarray fabrication are contact and non-contact printing of the probes. Adapted from DNA microarrays, contact printing with pins is the most widely used microarray printing technology nowadays (MacBeath and Schreiber, 2000; Zhu et al., 2001; Ringeisen et al., 2002). Compared to non-contact arrayers, they are faster, have lower running costs and achieve a smaller spot size and higher printing density. In addition, the number of spotting pins can easily be scaled up to achieve higher-throughput (Leung et al., 2002). However, the direct contact with the surface can cause carry-over effects and cross-contamination of the spots. Further, the varying adhesion forces between probes, needles and surface can lead to the deposition of inhomogeneous amounts of proteins and thereby to fluctuations in the signal intensities and diffuse spot morphologies (Gutmann et al., 2005).

Non-contact or ink-jet printers on the other hand, have some inherent advantages over contact printers: They are more gentle for proteins, there is no risk of disrupting the target surface, the contamination potential is reduced, unused sample can be recovered and finally they are more suitable to spot on porous surfaces, such as the porous silicon structures developed in **paper V**, gel pads or nitrocellulose (Zhu and Snyder, 2003). Further, the tips can load much larger volumes for dispensing and together with a programmable printing volume and a high print speed per nozzle (Leung et al., 2002), this technology allows the simultaneous printing of several arrays in a high-throughput manner.

Based on these parameters and our particular interest in the development of porous microarray supports (**paper V**), we utilised a non-contact printer, the Biochip Arrayer 1 from Perkin Elmer (Boston, MA). This arrayer deposits

reproducible picoliter (pL) volume droplets using piezoelectric tip technology. The four dispensers are glass capillaries surrounded by a piezoelectric element collar. By the application of a voltage to the piezoelectric element, a droplet in pL scale is released (Delehanty, 2004). The computer controlled robotic system provides a high resolution and accuracy and two cameras further enable a visual dispense verification and positioning (http://las.perkinelmer.com).

Alternative printing technologies include electrospray deposition (Morozov and Morozova, 1999), dip-pen nanolithography utilising atomic force microscopy (Lee et al., 2002), a laser transfer technique (Ringeisen et al., 2002) and an affinity contact printing procedure employing a multi-use stamp (Renault et al., 2002). As an alternative to microarray printing, Niemeyer et al. (1999) developed self-addressable microarrays based on DNA-directed immobilisation (DDI). This technique uses surface immobilised oligonucleotides as capture molecules for DNA-streptavidin conjugates, which mediate the site selective immobilisation of various biotinylated proteins. Although this technology requires further optimisation with regard to the production of high density protein microarrays, it is a gentle alternative to conventional microarray printing, as the protein can be applied in large sample volumes.

3.2 Array processing

With regard to the small reaction volumes in the microarray format and the sensitivity of proteins against drying effects, the assay conditions and processing can be critical for protein microarray analysis. Several attempts are under way to create so-called lab-on-chip technologies, in which storage and reaction chambers, in the form of microwells on the chip, are connected by tubing. Those chambers and channels can be either etched (Bernard et al., 2001) or bored into the surface (McDonald et al., 2001). Products on or close to the market involve a diverse variety of supports (plastic, glass, quartz or silicon) and methods to drive the flow in the channels (mechanical pressure, vacuum pumps, inertia or electrochemical) (Mitchell, 2001). The applications for such microfluidic devices are many and experiments can range from biochemical or chemical synthetic reactions to analytical separations such as chromatography and electrophoresis (Ng and Ilag, 2003). For protein microarrays, this technique has some specific inherent advantages: Proteins tend to denature at solid-liquid and liquid-air interfaces (Mitchell, 2002). The continuous flow in a lab-on-chip device keeps both probe and analyte in solution throughout the whole assay and thereby reduces potentially denaturing effects. Further, the required sample and buffer volume is reduced significantly in the thin channels of the device and the single components on the chip can be addressed individually. Finally the lab-on-chip technology can

be automated and standardised, which is of particular importance for the microarray format as the relevance of the "human error" increases with decreasing size of an experiment (Mitchell, 2001). However, the implementation of lab-on-chip technology in protein microarrays is just at the beginning and several key challenges have still to be solved (Ng and Ilag, 2003; Mitchell, 2001).

An intermediate between microfluidic devices and flat surfaces are supports with microwells, which keep fluids in place and enable individual treatment of several microarrays on one slide. In their trend-setting study about protein kinases Zhu et al. (2000) used microwells in a disposable silicon elastomer poly(dimethylsiloxane) (PDMS) sheet, which were 1.4 mm in diameter and 300 μm in depth and could take up 300 nl. Revzin et al. (2005) developed arrays of even smaller microwells composed of polyethylene glycol (PEG) hydrogel walls and glass attachment pads 20 μm x 20 μm and 15 μm x 15 μm in size. Further modification of the wells with cell-adhesive ligands, poly-L-lysine and anti-CD5 and anti-CD19 antibodies enabled the specific capturing of whole T-lymphocytes.

Another way to keep proteins in a hydrophilic to semi-wet environment during the assay is to use three-dimensional surfaces, such as nitrocellulose and hydrogel (Zhang, 2004) or the porous structures developed in **paper V**. Although **paper I**, **II** and **V** clearly demonstrated the preserving microenvironment of these surfaces, the diffusion of antibodies into the gel-structure, as observed in **paper II**, also emphasised potential limitations of semi-wet substrates.

As the number and size of protein microarrays has increased rapidly, the automation of array processing has gained importance. To process multiple arrays in parallel and in a fully automated way, we utilised the ProteinArray Workstation from Perkin Elmer. This highly flexible system is based on novel microfluidics technology, enabling short processing times and the use of low sample volumes (http://las.perkinelmer.com). However, a major drawback of these systems is that separate arrays on one slide cannot be addressed individually. For this purpose Angenendt et al. (2003a) proposed the so-called multiple spotting technique (MIST), in which probe and analyte are spotted on top of each other. This technique does not only enable several different assays on the same slide or even within the same array, but was also shown to reduce incubation times significantly, save sample volume and to be highly sensitive. In fact, one of our early studies had already proven this technique to provide excellent sensitivities as low as 600 attomol of analyte using mass spectrometry as mode of detection (Borrebaeck et al., 2001). In **paper III** we applied the same technology and were thereby able to detect as low as 300 zeptomol analyte.

3.3 Analytical principles

Analytical principles for protein microarray analysis have to provide the possibility for large-scale and high throughput analysis, high signal-to-noise ratios, good resolution, high sensitivity and reproducible results. Based on the variety of methods and applications for protein microarray technology, selecting the most suitable detection method meeting these criteria is not straight-forward. Possible read-out systems can be divided into methods involving label-based and label-free detection.

3.3.1 Label-based detection

Most applications of functional or analytical protein microarrays have employed some type of labelling strategy; usually fluorescent (Zhu et al., 2001; Lueking et al., 2003; MacBeath and Schreiber, 2000; Robinson et al., 2002; Haab et al., 2001; Wingren et al., 2003) (**paper II-V**), colorimetric (Arenkov et al., 2000) (**paper I**) or radioactive (Ge, 2000; Zhu et al., 2000). Even though radioactive labelling, using isotopes such as ^{125}I, ^{32}P or ^{3}H, is one of the most sensitive labelling techniques, it has been sequentially replaced by other detection methods due to the high risk of contamination. Nowadays, fluorescent dyes, such as the amine-reactive Cyanine- or Alexa-dyes, are the labels of choice for high-throughput microarray applications as they are simple, safe, very sensitive and provide a high resolution (Zhu and Snyder, 2003). Especially for the simultaneous multicolour detection in analytical protein microarrays, such as antibody microarrays, bright and pH stable dyes with narrow emission and excitation spectra are optimal candidates (Angenendt, 2005). Commonly used scanners (nonconfocal or confocal) allow the application of up to four fluorophores simultaneously and permit direct comparison and relative quantification of up to four different samples (Angenendt et al., 2003a; Wingren and Borrebaeck, 2004). Alternative fluorescent protein labelling strategies involve semiconductor quantum dots, which are brighter and more photostable than organic dyes (Wu et al., 2003) and fluorophores linked to puromycin analogs, incorporated into the protein during in vitro translation (Doi et al., 2002). Further improvements to fluorescent labelling in terms of sensitivity have been made by the introduction of rolling circle amplification (RCA) (Schweitzer et al., 2002), which will be further described in chapter 4.3.2.

The employed labels can be either attached directly to the analyte (direct labelling) or to secondary antibodies directed against the analyte itself or a common motif or tag at the analyte (indirect labelling). For functional protein microarrays, in which the proteome on the chip is probed against individual analytes, indirect labelling is often the method of choice. Zhu et al. (2001) used biotinylated probes and Cy3-labelled streptavidin as detecting agent and Lueking et al.

(2003) specific mouse antibodies, visualised by Cy3-labelled anti-mouse IgG. As an example of analytical microarrays employing indirect labelling, Robinson et al. (2002) used the constant region of human IgM and IgG as a common motif and Cy-3-conjugated anti-human IgM/IgG to profile the autoantibody response in human autoimmune disease. However, for most analytical protein microarrays, probed against whole proteomes, indirect labelling is not an option due to the missing common motif or tag. The application of sandwich assays is even less suited, since the generation of high-quality antibody pairs against a large number of analytes would be too ambitious (Wingren and Borrebaeck, 2004). On the other hand, direct attachment of dye to the analyte can alter the structure of the analyte and might interfere with the specific binding to the probe. Further, the labelling of whole proteomes is complicated by the fact that approximately 90% of proteins constitute only about 10% of the total protein mass (Miklos and Maleszka, 2001) and high-abundant proteins can mask low-abundant analytes and/or catch most of the label (Haab, 2003; Kodadek, 2001). To overcome the latter problem, it is critical to have access to a large number of high-affinity antibodies. As described in **paper III**, the cooperative binding of sinFab-clones with specificities directed against different epitopes on the same analyte increases the sensitivity of the assay significantly. Further, for the successful detection of low-abundant analytes, it might be necessary to pre-fractionate the proteome before labelling (Ingvarsson et al., in prep.).

Taken together, the application of detection methods employing labelling strategies is not always straightforward. However, it is still the most sensitive and readily-available analytical principle (Wingren and Borrebaeck, 2004), (see also chapters 3.3.2 and 4.3.2). So far, direct labelling has been the most frequently used detection method for analytical protein microarrays (Sreekumar et al., 2001; Haab et al., 2001; Miller et al., 2003; Wingren et al., 2003) and is the analytical principle for our specific antibody microarray setup.

3.3.2 Label-free detection

Although label-free detection technologies, such as surface plasmon resonance (SPR), mass spectrometry and others, are highly desirable, their specific applicability and sensitivity have so far failed to allow their common use for protein microarrays. Real-time detection by SPR-analysis (Biacore AB, Uppsala, Sweden) is a well-established optical technique based on the change in the refractive index, i.e. the angle of the reflected light, upon specific affinity interactions (Jonsson et al., 1991; Malmqvist and Karlsson, 1997). The so-called Biacore-instrument provides an automated sample injection and continuous flow system and together with the SPR-read-out in real-time, Biacore analysis is the standard technology

for kinetic-studies of receptor-ligand or antibody-antigen interactions. Only recently the first prototype implementing SPR and protein microarray technology, the FLEXCHIP kinetic analysis system, has been launched by HTS Biosystems (East Hartford, CT, USA) and is now distributed by Biacore AB. Although the technology looks promising, it still requires some optimisation and so far the achieved sensitivities in the μg/ml range are too low for the analysis of low abundant analytes in complex proteomes (Usui-Aoki et al., 2005). Other companies working on the development of SPR instruments capable of analysing several hundreds of spots simultaneously are summarised in Nedelkov and Nelson (2003).

The main detection technology to reveal the actual identity of bound analyte is mass spectrometry (Marko-Varga et al., 2004; James, 2002). In an early proof-of-concept study our group showed the applicability of MALDI-TOF MS as a read-out system for recombinant antibody microarrays on different surfaces achieving sensitivities as low as 600 amol of analyte (Borrebaeck et al., 2001). I a more general sense, one of the first protein chip companies, Ciphergen Biosystems, employed mass spectrometry as their standard read-out system (see chapter 2.3). The SELDI-technology enables the desorption and ionisation directly from the chip and data analysis is performed by a TOF-MS (Wright Jr et al., 1999) or a Tandem-MS interface (Kwapiszewska et al., 2004). However, the provided ProteinChip® Arrays have a limited number of 8 or 16 active sites and sample volumes are only in the μl to nl scale. Thus, applications of the ProteinChip® technology comprise small dedicated arrays rather than high-throughput protein microarrays (Tang et al., 2004).

Besides difficulties to optimise the chip/MS interface (Williams and Addona, 2000), a major limiting factor in the analysis of protein microarrays by mass spectrometry is the concern of blocking and sample handling. Traditional protein blockers are not compatible with mass spectrometry as they disturb the read-out of individual analytes. Alternative blocking solutions involve high amounts of detergent, which is in most cases also incompatible with mass spectrometry (Järås et al., unpublished observations). The latter is also a limitation in assay processing, as the complexity of the microarray system requires stringent washing conditions commonly involving the application of detergent (Wingren et al., unpublished observations). Finally, larger proteins must be enzymatically digested to fly in the mass spectrometer. However, proteases show a strong decrease in activity at analyte concentrations below 500 μM, a concentration rarely achieved in the microarray format (James, 2002).

Alternative label-free detection methods include intrinsic time-resolved UV fluorescence (Striebel et al., 2004), ellipsometry (Wang and Jin, 2003) and Kelvin nanoprobe detection (Cheran et al., 2004). However, neither of these technologies is currently suitable for high-throughput applications (Angenendt, 2005).

One way to address the inherent limitations of current read-out systems is to combine the different technologies, each of them providing its specific advantages. Finnskog et al. (2004) recently introduced a novel dual read-out system implementing fluorescence imaging as a fast first screening for binders and subsequent Maldi-TOF MS for protein identification. As a solid support compatible with both detection methods they used a further development of the macroporous silicon support presented in **paper V** (Ressine et al., 2003). Similar attempts have been made to combine SPR analysis and mass spectrometry (Williams and Addona, 2000; Nelson et al., 2000; Nedelkov and Nelson, 2003).

3.4 Data analysis

So far, the number of protein microarray applications and the size of the individual arrays have been fairly low and manageable. However, the amount of data expected to be generated by protein microarrays requires the development and implementation of efficient methods to process and manage the resulting data. While for DNA microarrays the discussion about a reasonable data analysis has been long and intense (Leung and Cavalieri, 2003; Liu et al., 2003), it is only in the initial phase for the field of protein microarrays.

Using direct labelling two-colour comparative fluorescence as read-out for analytical microarrays, one has to account for systematic errors such as the labelling efficiencies of the different dyes, variations in the scanner read-out and the overall quality of the array. Although the same coupling chemistry is employed, the labelling efficiency of the different dyes in particular varies significantly for both Cy- and Alexa-dyes (Ingvarsson et al., unpublished observations).

One possibility to principally address the problematic labelling is a criss-cross labelling procedure, where in a second experiment the dyes of sample and reference protein are swapped and resulting spectra are normalised against each other (Miller et al., 2003). Another common data processing procedure to account for possible systematic experimental variations is normalisation (Park et al., 2003). Normalisation of microarray data adjusts the data from each microarray to an internal or spiked-in standard (Haab et al., 2001). A major difficulty in the normalisation of data from biological samples is to find a reliable standard. On the one hand, a spiked-in reference does not correct for sources of bias occurred before the standard was introduced, e.g. during protein extraction (Hamelinck et al., 2005) and, on the other hand, internal references often show a high variability (Pavlickova et al., 2004). In a recent study, Hamelinck et al. (2005) compared seven different normalisation methods, representing major classes of normalisation for antibody microarrays, with regard to reproducibility, accuracy in comparison to known values and the integrity of overall trends in the data set. Normalisation

against an internal standard (in this case IgM for serum samples), taking the ELISA-determined concentration of the internal standard into account to exclude the factor of standard variability, was found to deliver the most accurate, reproducible and reliable data. However, although this study was a first important step in antibody microarray normalisation, it certainly requires adaptation and optimisation for each individual platform.

Data analysis and knowledge management systems for handling the generated data are currently under development. A free web-based data analysis and organising software, BioArray Software Environment (BASE), has been developed by Saal et al. (2002) and might soon be implemented for our protein microarray facility. As an alternative, standard DNA microarray systems, such as Spotfire (http://www.spotfire.com) and GeneSpring (http://www.agilent.com/chem/genespring), can be applied on antibody microarray data.

Chapter 4

THE PROBE: SINGLE FRAMEWORK RECOMBINANT ANTIBODY FRAGMENTS (SINFABS)

4.1 Antibodies in nature

Antibodies or immunoglobulins are the key effector molecules of the humoral adaptive immune system. Secreted by B-lymphocytes upon antigen challenge, they provide or mediate a variety of defence mechanisms against invading pathogens: activation of the complement system (Carroll, 2004) or cytotoxic cells (ADCC) (Sun, 2003), phagocytosis of bacteria (Swanson and Hoppe, 2004) and the direct neutralisation of toxins and viruses (Burton, 2002). Furthermore, they play an important role in the induction of allergic responses (Galli and Lantz, 1999). Membrane-bound antibodies mediate the antigen-uptake in B cells, thus connecting humoral and cellular immune response (Bernard et al., 2005).

The attractiveness of antibodies as tools in fundamental biology and especially as catcher molecules in protein microarrays lies in the high diversity of their binding region. Nature developed a sophisticated system to create antibody-molecules to almost any antigenic structure, which results in an estimated antibody repertoire of 10^{11} (Perelson and Oster, 1979). *In vitro* technologies such as directed evolution and antibody engineering enable additional specificities beyond nature (against e.g. toxic compounds or lethal pathogens) and can create diversities of up to 10^{11} to 10^{12} variants per library (Soderlind et al., 2000; Kusnezow and

Hoheisel, 2002), as described in chapter 4.2.

4.1.1 Antibody structure

Figure 2A shows the basic quaternary structure of the antibody molecule. It is Y-shaped and consists of two identical heavy chains of approximately 50 kD and two identical light chains of 25 kD. This structure is stabilised by non-covalent intra- and interchain binding forces and disulfide bridges between heavy and light chain and between the two heavy chains. Each light chain comprises two and each heavy chain four or five structurally related domains of approximately 110 amino acids each. Based on their proteolytic properties upon papain cleavage the heavy and light chains form two functional units: Two antigen-binding Fab-fragments comprised of the variable domains VH and VL and the constant domains CL and CH1 (Figure 2B) and the Fc-fragment out of the remaining heavy chain domains CH2 and CH3 (+ CH4 for IgE and IgM) mediating effector functions. In strong contrast to the variable regions, the constant regions show only a low sequence variability and can be categorised into two classes for the light chain (λ and κ) and five classes for the heavy chain (α, δ, ϵ, γ and μ). The different heavy chain classes determine the antibody-isotypes IgA, IgD, IgE, IgG and IgM, each of them conducting specific biological effector functions (Amzel and Poljak, 1979; Alzari et al., 1988).

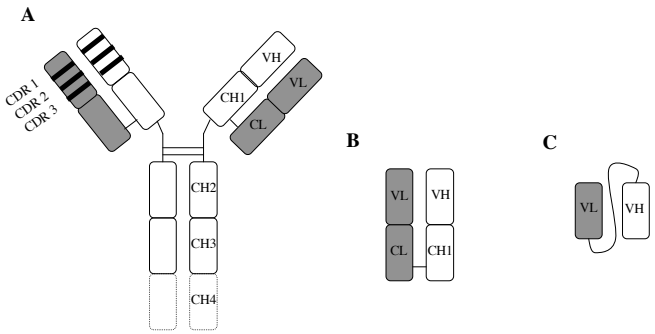

Figure 2. Schematic presentation of the antibody structure. (A) native antibody (B) Fab-fragment (C) scFv

Antibody domains adopt a specific tertiary structure known as the immunoglobulin fold, a beta-barrel made up of two antiparallel beta-sheets (Poljak et al., 1973; Schiffer et al., 1973). This structure is stabilised by mainly hydrophobic interactions and an intrachain disulfide-bridge between the sheets (Amzel and Poljak, 1979). In the constant domains the beta sheets are composed of four and three beta-strands. The variable domains, however, are made up of four and five strands with three connecting loops in each end of the beta-barrel. These so-called hypervariable loops or complementarity determining regions (CDR1-3) face the exterior of the variable domains and form the antigen-binding site. While the structurally important framework regions (FW1-4) in between the loops are more rigid and conserved, the CDRs are characterised by a high sequence variability and flexibility. Thus, the close interaction between the three CDRs from the heavy chain and the three CDRs from the light chain creates antigen-binding sites in a great variety of shapes, hydrophobicities and charges (Wu and Kabat, 1970; Stanfield and Wilson, 1994).

4.2 Recombinant single chain antibody variable region fragments (scFvs)

In contrast to Fab fragments, which can also be produced by proteolytic cleavage, single-chain antibody variable region fragments (scFvs) are recombinant antibody fragments comprised of the VH and VL domains connected by a 15 to 25 amino acid linker (Bird et al., 1988; Huston et al., 1988) (Figure 2C). The generation of combinatorial scFv-libraries has been diverse and can be categorised based on the source of antibody genes or the applied selection method. Many different methods have been described for antibody selection, including yeast, bacterial and ribosomal display as recently reviewed by Bradbury et al. (2003). Presently, the most robust, versatile and widespread antibody selection method is phage display (McCafferty et al., 1990; Barbas III et al., 1991), which is also the method of choice for the nCoDeR-library (Soderlind et al., 2000). In phage display, the genotype and phenotype of an antibody are linked together by fusion of its gene and a gene coding for a coat protein of the phage. Thereby, an antibody fusion protein is expressed on the surface of the phage, which allows for the affinity purification of scFv-coding genes by specific antibody-antigen interactions.

Based on the source of antibody genes, human scFv-libraries can be naive, semi-synthetic or fully synthetic. While naive libraries are constructed from rearranged V genes of B cells from non-immunised donors, semi-synthetic libraries are derived from un-rearranged V genes from pre-B cells or from a single antibody framework with genetically randomised CDR3s. Fully synthetic libraries have a

human framework with randomly integrated synthetic CDR cassettes (Hust and Dubel, 2004). The nCoDeR-library is a semi-synthetic human scFv-library, which has been constructed around a single master-framework using the VH3-23 and VL1-47 immunoglobulin genes. This master-framework was selected based on the excellent folding- and expression-properties, which often correlates with molecular stability (Worn and Pluckthun, 2001), and the frequent occurrence in human immune repertoires (Jirholt et al., 1998). **Paper I** and **II** further demonstrated the superior functional long-term stability on-chip of this framework compared to other frameworks. To generate a large genetic variety, *in vivo* formed proofread CDR-regions from rearranged immunoglobulin-genes of different germlines were amplified and combined randomly to create novel specificities. The resulting library contained $2x10^9$members (meanwhile updated to $2x10^{10}$) with specificities in the subnanomoler range (Soderlind et al., 2000).

4.3 Choice of probe format

In contrast to single-charged DNA, proteins are heterogeneous amphiphilic molecules with a complex on-chip behaviour. Thus, the design of antibody microarrays is much more challenging than that of DNA microarrays and requires a careful evaluation of both probe and solid support (see also chapter 5).

A probe for global proteome analysis, which is comparable to state-of-the-art DNA analysis and superior to traditional proteome analysis, has to be specifically designed to fulfil most, if not all, specific requirements of the array technology, i.e. reproducibility, sensitivity and stability. First and foremost, high-density antibody microarrays require access to a high diversity of antibodies. Although there are several tens of thousands mono- and polyclonal antibodies commercially available, the available range of specificities and high production-time and costs restrict their applicability (Pavlickova et al., 2004; Kusnezow and Hoheisel, 2002; Tomlinson and Holt, 2001; Wingren and Borrebaeck, 2004). In addition, natural antibodies are large and heterogeneous molecules, which differ not only in the antigen-binding site, but also in isotype and individual amino acid sequences of the constant and variable regions (see chapter 4.1.1). Combinatorial scFv libraries, such as the nCoDeR-library (Soderlind et al., 2000), on the other hand, provide up to 10^{12} different antibodies with specificities beyond nature (Kusnezow and Hoheisel, 2002). The development of automated screening and expression procedures enables the high-throughput selection of high-affinity binders (Hallborn and Carlsson, 2002). In addition, scFvs from the nCoDeR-library (sinFabs) are specifically designed around one framework and should therefore act as structural uniform probes with a similar on-chip behaviour. As observed in **paper I**, the choice of framework is of major importance for the immobilisation of the

probe in a functional active state. While three of the frameworks under comparison did not adsorb to any of the three surfaces in a functional active state, sinFab-molecules showed superior binding properties to all of them. **Paper II** further illustrated the similar long-term on-chip performance of scFvs constructed around a single framework, while the influence of CDR-loops was found to be of minor relevance.

In the following chapters two other key features of the antibody microarray probe will be discussed: stability and sensitivity.

4.3.1 Stability

The further discussion of the stability of scFvs as probes for antibody microarrays requires a brief definition of the term "stability". In general one should distinguish between molecular and functional stability (Worn and Pluckthun, 2001). The molecular stability of a scFv is its intrinsic stability in the monomeric state and in solution. It is usually measured as a function of either thermal or denaturant (urea or GdnHCl) induced denaturation. In the determination of functional stability, on the other hand, the unfolding rates are measured directly under the conditions of interest and usually followed by some kind of functionality test. In the case of antibody microarrays, the conditions of interest are printing in pL-scale on different solid supports and the long-term on-chip stability in a dried-out state. ScFvs are not fractionated prior to dispensing, i.e. they can be mono- or dimeric. **Paper I** well documented the difference between molecular and functional stability as a correlation between the Tm-values and long-term on-chip stability could not be observed.

Functional stability of probes for antibody microarrays is important from a manufacturing and practical point of view. As discussed in chapter 3.2, proteins often denature upon contact to solid supports and under dry conditions (Mitchell, 2002; Pavlickova et al., 2003). Thus, the stability of the probe has to be monitored carefully both directly upon surface-contact and after long-term storage.

To address the issue of pH and buffer composition Kusnezow et al. (2003) tested several spotting buffers covering a pH range from 4.5 to 8.5. Surprisingly, the pH seemed to have little effect on the stability of the probe during spotting. Similarly, Gutmann et al. (2005) tested 128 spotting buffers and observed only minor differences in performance, which were mainly surface-dependent.

A common supplement to prevent proteins from potentially denaturing drying effects is glycerol (MacBeath and Schreiber, 2000). Pavlickova et al. (2003) showed the beneficial effect of 40% glycerol for the storage of antibody microarrays over a period of 28 days. However, Kusnezow et al. (2003) observed that the addition of only 5% glycerol already led to decreased signal intensities and bad

spot morphologies, an effect which progressed with increasing amounts of glycerol. Alternative additives, such as PEO (Wu and Grainger, 2004) and trehalose (Kusnezow et al., 2003) showed improved signal intensities and signal to noise ratios, but were not studied after a four week period. In our hands, commercially available stabilisers such as StabilCoat and StabilGuard (SurModics Inc., Eden Prairie, USA) led to poor signal intensities and spot morphologies and did not improve long-term stability (Steinhauer et al., unpublished observations).

Several groups studied the long-term stability of antibody microarrays: Using monoclonal antibodies, Kusnezow et al. (2003) did not observe any deterioration over an interval of two months independent of the storage conditions (dry or humid). Similarly, Angenendt et al. (2002) were able to show stable signals over a period of eight weeks for polyclonal anti-fibrinogen antibodies on different solid supports and Wu and Grainger (2004) did not see any decrease in signal intensity for several immobilised mono- and polyclonal antibodies stored at –70°C over a duration of four weeks. However, for recombinant scFv antibody fragments selected from the Human Combinatorial Antibody Library (HuCAL) (Knappik et al., 2000) Pavlickova et al. (2003) observed a significant decrease in signal intensity after only 28 days of storage under humid (approximately 60% remaining activity) and even more severe under dry conditions (less than 20% remaining activity). This study further supported the importance of a careful probe design, as scFv-molecules especially can be very fragile (Kramer et al., 2002).

In view of the production of high-density antibody microarrays, it was decided to test the performance of the selected scFv-design (sinFab molecules from the nCoDeR-library) in the simplest format using readily available printing facilities and standard buffers and allowing them to dry out immediately after spotting. Compared to three other scFv-frameworks (VH5-51/L2-23, VH5-51/KIIIb, VH3-30/KIIIa) the nCoDeR-framework (VH3-23/L1-47) showed superior on-chip stability of more than 70% remaining activity after 47 days of storage, both at 4°C and at room temperature (**paper I**). In a follow-up study sinFab-clones of various specificities spotted on different solid supports could be stored for up to sixteen months at room temperature and in a dried-out state (**paper II**). Although the long-term performance varied distinctly between the surfaces and to a minor degree also between the different clones, this study further underlined the excellent functional long-term stability of sinFab-probes on the chip.

Parameters determining scFv-stability

So far, there have been no studies about the parameters determining functional on-chip stability. Thus, attempts to explain the observed differences in long-term

stability on the chip have to start with features known to contribute to intrinsic molecular stability.

The overall molecular stability of scFv-molecules depends on the intrinsic stability of the VH and VL domains as well as the stability of the VH/VL interface with a contribution from both the framework and the CDR loops (Reiter et al., 1994; Worn and Pluckthun, 2001).

One of the most important parameters for intrinsic domain stability of both VH and VL is the intradomain disulfide bond between highly conserved cysteine residues in the framework. Tightly packed hydrophobic side chains further stabilise the structure (Amzel and Poljak, 1979).

The VH/VL interface on the other hand, is not stabilised by disulfide bonds or salt bridges *in vivo* (Reiter et al., 1994). It mainly depends on the size of buried surface area defined by hydrophobic residues and hydrogen bonds (Worn and Pluckthun, 2001). Although weak, van der Waals (VDW) interactions also contribute significantly to the interdomain stability by providing higher packing efficiencies (Jaenicke, 2000). A loss of stability in the VH/VL interface has often been suggested as the main cause of irreversible scFv inactivation, since transient opening of the interface exposes hydrophobic patches that favour aggregation (Reiter et al., 1994).

In **paper I**, the functional stability was found to correlate well with the size of buried surface area and the number of VDW interactions, thus suggesting an important role for the VH/VL interface in the long-term stability on the chip. In **paper II**, further in-depth studies of the on-chip stability of several different sinFab-clones, confirmed this strong contribution of the VH/VL-interface. However, besides VDW interactions, hydrophobic, aromatic stacking and in particular charged interactions between VH and VL were shown to play a significant role in the functional long-term stability of sinFabs.

Evolutionary and rational approaches to stabilise the scFv-format have been many (Worn and Pluckthun, 2001) and a discussion thereof would be beyond the scope of this thesis. However, stabilising mutations give an interesting insight into the relevance of the different parameters contributing to overall molecular stability. As an example, engineered disulfide bonds (Reiter et al., 1994; Young et al., 1995) have been most successful in stabilising the VH/VL interface. Further, the mutation of amino acids where the hydrophobicity is incompatible with solvent exposure (Chowdhury et al., 1998) or of hydrogen bond forming amino acids is promising (Proba et al., 1998; Woern and Plueckthun, 1998; Honegger and Pluckthun, 2001). However, despite intense studies the parameters contributing to scFv-stability are only party resolved and reliable prediction remains difficult.

4.3.2 Sensitivity

Most proteomes are a complex composition made up of high- and low-abundant proteins with the majority of physiologically relevant proteins existing only in small subpopulations, sometimes not more than a few molecules per cells (Miklos and Maleszka, 2001). Thus, the sensitivity of antibody microarrays has to be at least in the picogram (i.e. attomole) range in order to perform true proteome analysis (Kusnezow and Hoheisel, 2002). To date, most setups using fluorescent detection without signal amplification report limits of detection (LODs) in the ng/ml, i.e. nM to pM range, corresponding to total measures in the low femto- to attomole range (Miller et al., 2003; Pavlickova et al., 2003; Haab et al., 2001; Sreekumar et al., 2001; Angenendt et al., 2002, 2003a). Using custom made solid supports based on covalent coupling chemistry Kusnezow et al. (2003) were able to further reduce assay sensitivities to the low pg/ml range. Even more sensitive, our sinFab microarrays on MaxiSorp^{TM} black polymer (NUNC A/S, Roskilde, Denmark) allowed detection of analyte in the sub pg/ml range utilising biotinylated non-fractionated human whole serum (Wingren et al., in prep.).

Although mass spectrometry is usually less sensitive than fluorescence detection, our group was able to detect as low as 600 attomole of analyte using MALDI-TOF MS as mode of detection (Borrebaeck et al., 2001). An alternative, highly sensitive microarray detection system based on proprietary planar waveguide technology was developed by Zeptosens (now a division of Bayer AG Technology Services, Basel, Schweiz). Utilising the specific advantages of the evanescent field fluorescence detection, this technology provided a LOD as low as 2 pM, corresponding to 0.8 zeptomole, i.e. 500 protein molecules per spot (Pawlak et al., 2002).

Various strategies have been developed to further improve the sensitivity of antibody microarrays by signal amplification. A powerful signal-enhancement method, the rolling circle amplification (RCA), enables protein detection in the sub-pM range. RCA utilises circular DNA, covalently attached to a secondary antibody against the analyte. In an ordinary PCR reaction, implementing fluorescently labelled nucleotides, the DNA can be amplified at the antibody-antigen reaction site, resulting in a signal enhancement of more than 1000 fold (Schweitzer et al., 2000, 2002). Another protocol utilises tyramide signal amplification (TSA) and is based on detection by secondary antibodies labelled with horseradish peroxidase (HRP) (http://probes.invitrogen.com). Using TSA signal enhancement, Woodbury et al. (2002) were able to detect hepatocyte growth factor (HGF) in human serum at sub-pg/mL concentrations. However, both RCA and TSA signal amplification are based on detection by secondary antibodies. Although sandwich assays in general are known to enhance the sensitivity, the complexity of

the system makes it incompatible with the generation of high-density antibody microarrays (chapter 3.3.1) (Wingren and Borrebaeck, 2004; Pavlickova et al., 2004).

In **paper III**, we studied the sensitivity of sinFab microarrays in a direct labelling approach. Both probe and analyte were applied in a purified and non-purified manner using different solid supports based on affinity coupling of the probe. The resulting LODs were in the nM to fM range strongly depending on both the affinities of the individual clones and the chosen support and is one of the lowest reported so far. Interestingly, the complexity of the analysed proteome seemed to have no or little effect on the overall sensitivity of the system. Using the multiple spotting technique, i.e. direct deposition of analyte on top of the probe, further reduced the total amount of required analyte from 1200 attomole to 300 zeptomole (see also chapter 3.2).

In addition, cooperative binding of several sinFab-clones directed against the same analyte increased the assay sensitivity approximately 40 times (see also chapter 3.3.1). Taken together, the sensitivity of antibody microarrays was found to be a critical interplay between antibody affinities, choice of solid support and the mode of analyte deposition.

Chapter 5

THE SOLID SUPPORT

The second key-component for the production of high-density protein and antibody microarrays is the carrier or solid support. Immobilised on a surface, protein-protein interactions show considerable differences in the reaction kinetics and affinities, mainly due to lower reaction volumes and changes in the molecular configuration of the reactants (Butler, 2000; Vijayendran and Leckband, 2001). As immobilised proteins are exposed to enormous local forces, they often denature on the surface and the concentration of functional reactant is reduced (Wu and Grainger, 2004). Accordingly, the amount of functionally active immobilised sinFabs varied significantly between different surfaces, as observed in **paper I**. Thus, the careful evaluation of solid supports suitable for the immobilisation of functional proteins and biocompatible with the probe of choice is a prerequisite for successful protein microarray analysis.

The term "biocompatibility" summarises various features a "suitable" solid support has to provide: distinct and homogenous spot morphologies, high binding capacities combined with a low non-specific background (i.e. high signal to noise ratios), a wide dynamic range, good sensitivities and reproducibility. These parameters have been studied on a variety of commercially available and in-house produced solid supports in **paper V**. Further, we evaluated the long-term performance and stability of our probes on four commercially available surfaces of distinct structure and coupling chemistry in **paper II**. In **paper III** and **IV**, we studied solid supports providing the chemistry for specific affinity interactions and evaluated the possibility to couple the probe in an orientated way and purify it directly on the chip.

Substrates for protein microarrays have previously been classified according to their structure (1-, 2- and 3-dimensional) or their probe coupling chemistry (physical adsorption, covalent coupling and affinity coupling), both being equally important for surface biocompatibility (Kusnezow and Hoheisel, 2003; Wingren and Borrebaeck, 2004). As most of the 3-dimensional structures are based on adsorp-

tion, the following chapters are categorised by the different coupling chemistries with a short subsection about 3-dimensional surfaces based on adsorption (chapter 5.1.2).

5.1 Substrates based on adsorption

In antibody microarray applications physical adsorption is the simplest and most frequently used approach to immobilise probes on a solid support (Borrebaeck et al., 2001; Miller et al., 2003; Sreekumar et al., 2001; Belov et al., 2001, 2003; Haab et al., 2001; Robinson et al., 2002). It is based on several binding forces, such as hydrophobic, van der Waals (VDW), electrostatic, ionic, Lewis acid-base and entropically driven interactions (Kusnezow and Hoheisel, 2003; Blawas and Reichert, 1998), thus making it a non-selective approach with a random orientation of probe on the surface. Most soluble proteins adsorb best on non-charged surfaces at neutral pH and physiological ionic strength (Butler, 2000), but the amount of adsorbed protein in a functionally active state depends strongly on the chemical properties of both protein and substrate (**paper I, II** and **V**).

5.1.1 1- and 2-dimensional substrates

One- and 2-dimensional substrates adapted from DNA-microarrays (poly-L-lysine or silane) (Haab et al., 2001; Miller et al., 2003) (**paper V**) or standard ELISA-techniques (polystyrene plastic) (Borrebaeck et al., 2001) (**paper I**) show diminished adsorption properties due to their strong hydrophobicity. As proteins unfold to permit internal hydrophobic side chains to form hydrophobic bonds with the solid phase, physical adsorption on hydrophobic surfaces often leads to denaturation and poor shelf-life (Metzger et al., 2002; Butler, 2000). Further, hydrophobic surfaces exhibit a higher degree of non-specific binding than hydrophilic (Piehler et al., 1996, 2000) and a strong spot-to-spot and chip-to-chip variability (Angenendt et al., 2003b). In accordance to these observations, we found a significantly reduced adsorption of functionally active antibodies on hydrophobic substrates, such as polystyrene plastic (**paper I**) and silane (**paper V**), and the highest coefficient of variation (CV) (**paper V**).

Although silicon is a more hydrophilic substrate with improved adsorption properties compared to polystyrene plastic (**paper I**), plain 1-dimensional silicon still showed a rather low probe binding capacity and overall diminished biocompatibility (**paper V**). However, its unique material properties enable the production of 3-dimensional porous structures (Ressine et al., 2003), as described in **paper V**.

5.1.2 3-dimensional substrates

3-dimensional substrates, such as membranous, gel-coated or porous surfaces, provide a 100-1000 times larger surface area than 1- or 2-dimensional surfaces, resulting in significantly increased probe binding capacities (Butler, 2000). Hydrogels and membranes are further recognised for their hydrophilic and preserving microenvironment for native proteins (Angenendt, 2005).

Adapted from traditional protein analysis, nitrocellulose is well-known as an excellent substrate for protein adsorption, providing both a preserving environment and binding capacities comparable to covalent coupling (Angenendt et al., 2003a). In our hands, nitrocellulose supports were compatible with mass spectrometric (Borrebaeck et al., 2001), colorimetric (**paper I**) as well as fluorescence detection (**paper II, III, V**) and showed excellent biocompatibility with the probe. The commercially available nitrocellulose support FAST-slide (Schleicher and Schuell, Dassel, Germany) is well-established in protein microarray analysis due to its high binding capacity (Stillman and Tonkinson, 2000; Kusnezow et al., 2003; Knezevic et al., 2001) (**paper III** and **V**). **Paper II** further demonstrated its stability and homogenous performance during long-term storage. On the other hand, FAST-slides, like most membranous surfaces, suffer from a low resolution and high background binding (Zhu and Snyder, 2003; Kusnezow and Hoheisel, 2003) (**paper III** and **V**). In a study by Knezevic et al. (2001), only 51 of 368 antibodies immobilised on FAST-slides gave a significant signal above background. In order to design nitrocellulose-based supports with a high binding capacity and at the same time low non-specific background, we evaluated different thicknesses of nitrocellulose-layers: a 0.2% nitrocellulose-solution for plain silicon and a 0.07% nitrocellulose-solution for macroporous silicon resulted in the highest signal to noise ratios in comparison to other silicon-based supports (**paper V**).

The first gel-based structures for protein microarray analysis were developed in the group of Andrei Mirzabekov (Arenkov et al., 2000). Their polyacrylamide gels, co-polymerised with chemically modified probes, have meanwhile been simplified and provide a stable and reliable support for the immobilisation by way of different coupling chemistries (covalent and affinity binding) (Rubina et al., 2003). Since then several hydrogels based on polyacrylamide (Angenendt et al., 2002; Charles et al., 2004), agarose (Afanassiev et al., 2000) or amphiphilic nanofibers (Kiyonaka et al., 2004) have been developed for protein microarray applications, utilising both physical adsorption and covalent coupling. The superiority of hydrogels against 1- and 2-dimensional substrates lies in the highly hydrophilic (i.e. semi-wet) and porous microenvironment (Zhu and Snyder, 2003), an advantage for both protein survival (Zhang, 2004) and free diffusion of reactants (Charles et al., 2004). In a direct comparison between polyacrylamide gels and

1-dimensional structures providing the same coupling chemistry, Charles et al. (2004) observed 2-3 times higher signal intensities, suggesting a higher binding capacity and better accessibility to antigenic sites on 3-dimensional slides. Similarly, Miller et al. (2003) saw a six times higher signal to noise ratio on HydroGel (Perkin–Elmer Life and Analytical Sciences, Boston, MA, USA) than on poly-L-lysine slides. In our hands, HydroGel slides showed good binding capacity and biocompatibility (**paper V**) and free diffusion of analyte yielded in significantly increased signal intensities (**paper II**). However, maximal binding capacity was only achieved after several weeks of storage and the adsorption of probe to substrate was not stable, i.e. it diffused into the gel (**paper II**).

In order to design a biocompatible substrate with a high binding capacity, we co-developed a set of 3-dimensional micro– and macroporous silicon supports with increased surface area for the immobilised probe (**paper V**). The macroporous substrate MAP3 was shown to provide the highest pore density combined with large pore openings. Thus, it was possible to further coat MAP3 with a thin layer of nitrocellulose, without restricting the access to the pores. The resulting substrate, MAP3-NC7, provided excellent sensitivities combined with high signal to noise ratios and low spot-to-spot variability.

5.2 Substrates based on covalent coupling

Substrates providing covalent coupling chemistry are usually based on glass- or gold-slides chemically modified to mediate the interaction of protein and surface. For reasons of economy, glass is still the most popular carrier, however, gold has some inherent advantages, e.g. the applicability in SPR- and MS-analysis (Zhu and Snyder, 2003). The chemistry of mediating layers is diverse, comprising a variety of self-assembled monolayers (SAM), polyethylenglycol (PEG) or dendrimers (as reviewed in Kusnezow and Hoheisel (2003)). However, to reduce the amount of non-specific adsorption, it is important to choose a highly inert surface with a regular and homogenous presentation of functional groups (Houseman and Mrksich, 2002). The latter can be aldehydes (MacBeath and Schreiber, 2000; Angenendt et al., 2002; Kusnezow et al., 2003), epoxy-groups (Angenendt et al., 2003a; Kusnezow et al., 2003; Zhu et al., 2000), or amines (Angenendt et al., 2003a, 2002; Kusnezow et al., 2003), while epoxy-groups were found to provide the highest sensitivities so far (Kusnezow et al., 2003; Seong, 2002).

Although covalent coupling has the advantage of a strong attachment combined with low background and variability (Angenendt, 2005) (**paper V**), its application in protein microarrays is often limited by the rapid evaporation of carrier solvent (Metzger et al., 2002). Remarkably, SpotOn slides (Scandinavian Micro Biodevices A/S, Copenhagen, Denmark) showed excellent biocompatibil-

ity and binding capacity even though the probes were not deposited in glycerol (**paper V**). However, in the long-term performance antibody microarrays on SpotOn slides displayed a high spot-to-spot and chip-to-chip variability (**paper II**).

5.3 Substrates based on affinity coupling

While physical adsorption and most covalent coupling techniques lead to a random immobilisation of the probe on the chip, specific affinity interactions by way of genetically engineered affinity tags or secondary labelling open the possibility for an orientated coupling. Avoiding denaturation of the probe by chemical modifications upon surface contact or the physical blockade of the antigen-binding site (Peluso et al., 2003; Butler, 2000), Peluso et al. (2003) found up to ten times increase in analyte binding capacity and remaining 90% activity upon orientated attachment of both full-size antibodies and Fab-fragments. On the other hand, orientated coupling often leads to a lower surface coverage (Butler et al., 1992; Vijayendran and Leckband, 2001) and a direct positive effect is therefore sometimes difficult to observe (Kusnezow et al., 2003).

Besides probe-orientation, affinity-based supports enable the on-chip purification and enrichment of crude probe preparations (Kusnezow and Hoheisel, 2003; Pavlickova et al., 2003). The possibility to directly apply non-purified antibody preparations is a further step in the development of high-density antibody microarrays, as the purification of several thousand antibodies is too time-consuming. However, most affinity-based supports are not inert enough and thereby suffer from a high background adsorption interfering with the specific affinity interaction (Metzger et al., 2002; Butler, 2000). **Paper III** and **IV** demonstrate the possibility for probe-orientation and on-chip purification utilising engineered affinity tags. However, they also revealed the specificity of coupling chemistry provided by the solid support to be crucial for successful on-chip purification of the probe and sensitive detection of analyte.

While orientated coupling of full-size antibodies and Fab-fragments requires chemical modification of the thiol-groups or carbohydrates (Peluso et al., 2003; Kusnezow et al., 2003), recombinant antibodies can be genetically engineered to carry N- or C-terminal affinity tags. The introduction of affinity tags is a prominent technology to facilitate recombinant antibody purification and detection and so far a wide variety of tags has been employed (as reviewed in Nilsson et al. (1997) and Terpe (2003)). The nCoDeR-design involves a C-terminal $(his)_6$- and c-myc-tag, which have been utilised for affinity-coupling in **paper III**. This proof-of-principle study demonstrated the specificity and sensitivity of antibody microarrays based on both purified and non-purified affinity-coupled

probes. **Paper IV** further describes the development of sinFab-fragments carrying a novel polyhistidine tag, the double-$(his)_6$-tag, with improved binding capacity (see chapter 5.3.1).

5.3.1 Ni^{2+}-coated substrates and polyhistidine-tags

Utilising the interaction between the imidazole ring of exposed histidines and immobilised transition metal ions (Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}), polyhistidine-tags were developed for protein purification by means of immobilised metal ion affinity chromatography (IMAC) (Porath et al., 1975; Hochuli et al., 1987). In protein microarrays, Ni^{2+}-chelate derivatised solid supports were successfully applied in a comprehensive study to analyse interactions of 5800 recombinantly expressed yeast proteins (Zhu et al., 2001). However, Zhu et al. used a Ni^{2+}-NTA support, which requires pre-purification of protein due to non-specific background binding (Xenoslide N, see **paper II, III, IV** and **V**). More sophisticated and inert supports based on Ni^{2+}-NTA modified lipid bilayers (Svedhem et al., 2003; Larsson et al., 2005), anti-tag antibody coated substrates (**paper III**) or preblocked Ni^{2+}-NTA HisSorb Strips (Qiagen, Hilden, Germany) (**paper IV)** are more promising substrates for the on-chip purification of polyhistidine tagged probes.

Previously, tags of six histidine-residues in a row were found to be the best compromise between good affinity and minor side effects (Hochuli et al., 1988). Although longer polyhistidine-tags usually provide higher affinities, they often suffer from lower expression yields, higher oligomerisation rates and a decreased stability and solubility of the protein (Mohanty and Wiener, 2004; Ramage et al., 2002). However, in **paper IV** we showed that a novel polyhistidine-tag, the double-$(his)_6$-tag (He and Taussig, 2001), led to an increased binding capacity of sinFab-molecules without observing any of these diminishing side effects. Furthermore, depending on the solid support it was possible to array non-purified sinFabs without any significant decrease in signal intensity. Biacore analysis of the binding kinetics revealed a superior off-rate of double-$(his)_6$-tagged compared to single-$(his)_6$-tagged sinFabs, i.e. affinity-coupled double-$(his)_6$-tagged sinFabs were bound tighter and were more resistant against washing effects. Thus, double-$(his)_6$-tagged sinFabs provide important prerequisites for the application in high-density antibody microarrays.

Chapter 6

CONCLUDING REMARKS AND FUTURE OUTLOOK

In the last five years antibody microarrays have been established as an important tool for focussed proteome analysis. As a fast and sensitive technology, which can be performed in a high-throughput manner, its potential for clinical diagnostics, biomarker discovery and fundamental disease characterisation is tremendous. However, so far antibody microarray applications have been mainly small dedicated arrays of less than 400 antibodies, which supplement traditional genomic and proteomic techniques (Haab et al., 2001; Sreekumar et al., 2001; Miller et al., 2003; Huang et al., 2001; Schweitzer et al., 2002; Wingren et al., 2003; Belov et al., 2001, 2003; Hudelist et al., 2004).

The aim of this thesis was to optimise the antibody microarray setup in a way, that allows for the establishment of high-density antibody microarrays for global proteome profiling. For this purpose two key components of antibody microarrays, the probe and the solid support, i.e. "catcher and carrier", were characterised in detail and evaluated with regard to functionality, long-term performance and stability, sensitivity, the possibility for on-chip purification and biocompatibility.

In **paper I**, the complexity of protein-immobilisation was illustrated by the fact that only one out of four scFv-frameworks was successfully coupled to the surface in a functionally active state: single framework recombinant antibody fragments (sinFabs) selected from the nCoDeR-library. The phenomena of antibody denaturation and/or immobilisation in a non-accessible way were observed earlier (Haab et al., 2001; Miller et al., 2003) and demonstrated the need for a careful selection of a structural uniform probe and a biocompatible solid support. SinFab-molecules not only fulfilled the fundamental requirement of on-chip functionality, but were also shown to be stable on the chip for more than a year in a dried-out state (**paper I** and **II)**. Remarkably, the applied protocol neither

involved any specific buffers or stabilisers, nor had the slides to be stored under special conditions. In order to improve the on-chip stability of sinFab-probes even further, we initiated two distinct stability-engineering projects: In collaboration with Alligator Bioscience AB (Lund, Sweden), we are planning to perform directed evolution of the nCoDeR-framework using their proprietary FIND^{TM}-technology (http://www.alligatorbioscience.se). The resulting scFv-library can then be screened for clones with improved long-term stability on the chip. In a more specific approach, single amino acids of the nCoDeR-framework, which are known to mediate molecular stability of scFvs, will be mutated and resulting variants will be studied for improved functional on-chip stability.

In **paper III,** sinFabs were shown to provide a sensitive detection in the nM to fM range. In recent studies, these LODs could be further reduced to the sub-pg/ml, i.e. low fM range, using an optimised solid support and alternative labelling technique (Wingren et al., in prep.) (see chapter 4.3.2). LODs in the fM-range are expected to be sufficient for the detection of low-abundant analytes and in fact low cytokine expression levels were successfully analysed by sinFab-microarrays (Wingren et al., 2003). However, further optimisation of the solid support, the probe-immobilisation strategy and/or proteome-labelling or the implementation of described signal amplification techniques (see chapter 4.3.2), are likely to reduce the amount of required analyte even further. Efforts to interface label-free detection, in particular mass spectrometry, are under development.

As shown in **papers III** and **IV**, affinity tags like the myc- and $(his)_6$-tag (**paper III**), or the double-$(his)_6$-tag (**paper IV**) allow an orientated coupling and on-chip purification of the probe on the surface, thus providing important prerequisites for the high-density format. Although the binding capacity of sinFab-probes was already significantly improved by the implementation of double-$(his)_6$-tags, alternative affinity tags, such as high-affinity Strep-tags, or novel specifically designed affinity tags should be evaluated in the antibody microarray-format. However, the choice of affinity tag will be significantly influenced by the availability of suitable solid supports, which require further optimisation.

The selection of a solid support with high binding capacity and good overall biocompatibility was less straightforward than the selection of a probe. Similar to earlier studies (Angenendt et al., 2002, 2003b; Kusnezow et al., 2003), a comparison of commercially available substrates based on different coupling chemistries did not reveal one support to be superior in all parameters (**paper II** and **V**). Although SpotOn- (covalent coupling) and FAST-slides (nitrocellulose; adsorption) showed extraordinary binding capacities (**paper V**), the latter suffered from a high non-specific background (**paper V**) and SpotOn slides from a high variability in the long-term performance (**paper II**). However, with the constantly growing protein microarray field and the demand for solid supports biocompati-

ble with proteins, new specifically optimised surfaces reach the market frequently. In our hands, MaxiSorp^{TM} black polymer slides (NUNC) displayed an excellent biocompatibility and increased the sensitivity of sinFab microarrays significantly (see chapter 4.3.2). Future developments regarding optimised surface chemistries will be evaluated accordingly.

The only commercially available slide providing a Ni^{2+}-chelate chemistry for the affinity coupling of polyhistidine-tagged scFvs (Xenoslide N), was significantly restricted by an additional background adsorption, affecting both the possibility for on-chip purification (**paper III** and **IV**) and the long-term performance (**paper II**). Thus, new substrates with affinity coupling chemistry are highly desirable with regard to the production of high-density antibody microarrays. These slides need to be pre-blocked, like the Ni^{2+}-NTA HisSorb Strips applied in **paper IV**, to avoid non-specific background adsorption. Recently, inert supports based on Ni^{2+}-NTA modified lipid bilayers were shown to be biocompatible with both single- and double-$(his)_6$-tagged sinFab-molecules (Svedhem et al., 2003; Larsson et al., 2005). The possibility to implement these supports into the sinFab microarray setup, using our spotting and detection facilities, will be evaluated.

In the scope of **paper V,** we designed a novel solid support based on macroporous silicon coated with nitrocellulose (MAP3-NC7), which provided excellent probe binding capacities combined with a low background adsorption and overall good biocompatibility (**paper V**). However, this support still has to be evaluated with regard to chip-to-chip reproducibility and long-term performance. Further developments of MAP3-NC7 will also include the attempt to implement affinity coupling chemistry.

Taken together, in the scope of this thesis, sinFab-molecules selected from the nCoDeR-library have proved to be excellent probes for high-density antibody microarrays, as they are uniform in structure, functional and stable on the chip and provide a sensitive detection of analyte. Furthermore, they can be coupled to the support in an orientated and non-purified way utilising genetically engineered affinity tags. Several commercially available solid supports have been evaluated with regard to biocompatibility with high-density antibody microarrays, i.e. spot morphology, signal to noise ratio, dynamic range, sensitivity, reproducibility, long-term performance and stability. Additionally, a range of silicon-based supports has been co-developed and resulted in the design of a novel solid support, MAP3-NC7, with excellent biocompatibility and high binding capacity. The results presented in this thesis now serve as a solid foundation for several on-going and future sinFab-microarray applications under development in our group. These include the screening of healthy and diseased clinical samples (i.e. serum and tissue) with a main focus on different forms of cancer (e.g. breast cancer, pancreatic cancer, gastric adenoma carcinoma) and allergy.

POPULAR SCIENTIFIC SUMMARY

"DNA microarrays or chips" are well-established within several areas of biological research and clinical diagnostics. They can for example be used to compare expressed genes in a genome of a healthy tissue with those in the genome of a diseased tissue in a miniaturised way. Thus, important insights into the character of the disease can be gained and help significantly in the development of therapeutic strategies and potential drugs.

Genes contain all the important information for a cell to function but the actual effector molecules in the cells are proteins. Thus, to get a comprehensive picture of the disease it has to be studied on the protein-level as well. Unfortunately the development of a technology similar to DNA microarrays, i.e. protein microarrays, is not straightforward, as proteins are in contrast to DNA large and heterogenous molecules, that are difficult to immobilise on the chip. To avoid the inherent problems with the immobilisation of thousands to millions of different proteins, we suggest the use of antibodies as structurally uniform catcher molecules for the protein analytes. Antibodies are important components of the immune system as they are able to bind and disarm a tremendous variety of potentially harmful pathogens. In antibody microarrays these highly specific molecules are spotted to the chip in an array-format with each spot representing one antigen-specificity, i.e. one protein-analyte. In analogy to DNA microarrays the resulting pictures of present and absent proteins in the individual proteome (of e.g. healthy and diseased tissue) can easily be compared and used as a foundation for further in depth studies.

The aim of this thesis was to design an antibody microarray setup comparable to state-of-the-art DNA microarrays. For this purpose, I focussed my work on the two key components of an antibody microarray: the antibody-probe and the solid support, i.e. "catcher and carrier". Instead of natural antibodies, we chose genetically engineered antibodies, called single framework recombinant antibody fragments (sinFabs), as probes, as they are more structurally uniform, faster to produce and readily available in many different specificities. The studies presented in the scope of this thesis revealed sinFabs to be excellent probes for antibody microarrays: In contrast to other antibody-molecules, they could be immobilised to the chip in a way, that they were still able to "catch" the analyte. This was also possible, when the analyte was presented in a complex mixture like blood serum, where it was only present in small amounts. Furthermore, sinFab-microarrays could be stored for over a year without any loss in functionality. Thus, antibody microarrays based on sinFab-probes can be produced in industrial scale and stored until further use or during shipping.

The choice of solid support, on the other hand, turned out to be more difficult. Although several commercially available and in-house designed surfaces have been studied for their compatibility with the antibody microarray setup, no support was superior in all relevant parameters. However, important insight has been gained into the relevance of certain surface-parameters, such as the structure (1-, 2- or 3-dimensional) or the coupling chemistry (adsorption, covalent coupling or affinity coupling). Moreover, a novel 3-dimensional support was designed, which provided a large surface area for the coupling of probe and thus resulted in a very sensitive detection of analyte.

Using the right combination of specifically modified sinFabs and corresponding surface further opened the possibility to spot the antibodies directly after the production in bacteria. As the normal process of antibody-purification after production is very time-consuming, this finding was another important step in the design of large antibody microarrays.

POPULÄRVETENSKAPLIG SAMMANFATTNING

"DNA-mikromatriser eller genchips" är idag väletablerade verktyg inom biologisk forskning och klinisk diagnostik. Genmikromatriser kan exempelvis användas för att i mikroskala studera genetiska skillnader mellan sjuka och friska vävnader. Informationen som då erhålls kan ge värdefulla upplysningar om sjukdomens natur och användas vid utveckling av nya läkemedel.

Generna innehåller all information som cellerna behöver för att fungera, men proteinerna är de verkliga effektormolekylerna som utför aktiviteterna. För att få en komplett bild av en sjukdom måste följaktligen, förutom generna, även proteinerna studeras. I motsats till utvecklingen av DNA-mikromatriser, är teknologiutvecklingen av proteinmikromatriser inte lika enkel. En förklaring till detta är att proteiner, till skillnad från gener, är betydligt mer komplexa och heterogena molekyler, vilket bland annat gör dem svårare att immobilisera på en chipyta så att de fortfarande är aktiva. Vi har valt att använda oss av antikroppar på våra mikromatriser. Antikroppar är en viktig beståndsdel i vårt immunförsvar och de har en unik förmåga att specifikt binda till skadliga ämnen som sedan kan oskadliggöras. Det är just denna förmåga att specifikt kunna binda till en analyt som vi använder oss av. Vid tillverkningen av en antikroppsbaserad mikromatris sätts antikropparna ut en och en i ett bestämt mönster, en så kallad matris, på en fast yta. I varje punkt finns det sålunda en antikropp som kan binda till en unik analyt. Genom att detektera till vilka antikroppar som provets olika beståndsdelar binder till kan man bestämma proteinsammansättningen i ett prov. I likhet med DNA-mikromatriserna, kan antikroppsmatriserna användas till att identificera upp- och nedreglerade proteiner i exempelvis sjuka respektive friska prov/vävnader. De differentiellt uttryckta proteinerna som upptäcks kan sedan användas för att studera biologin bakom sjukdomen, ställa diagnos av sjukdomen samt för att utveckla nya terapeutiska läkemedel.

Målet med min avhandling har varit att utveckla en teknologiplattform baserad på antikroppsmikromatriser som är jämförbar med dagens DNA-mikromatriser. Arbetet har fokuserats på två av nyckelkomponenterna, nämligen antikroppen som fångarmolekyl och chipytan, d.v.s. fångare och bärare. Istället för att använda naturligt förekommande antikroppar så har vi valt att använda oss av genetiskt modificerade antikroppar, så kallade single framework recombinant antibody fragments (sinFabs). Fördelen med dessa är att de är mer strukturellt homogena, enklare och snabbare att producera och dessutom finns de redan idag tillgängliga med i stort sett alla möjliga bindningsspecificiteter. Studierna presenterade i denna avhandling visade att sinFabs är utmärkt lämpade som fångarmolekyler på mikromatriserna. Till skillnad från många av de naturligt

förekommande antikropparna kunde sinFab-antikropparna immobiliseras på chipytan utan att förlora sin aktivitet och inbindningsförmåga. SinFab-antikropparna uppvisade hög specificitet och känslighet vilket gjorde det möjligt att även analysera komplexa prover, d.v.s. prov bestående av tusentals olika analyter (t.ex. ett blodprov), och där mängden av de analyter man letade efter var väldigt låga. SinFabs-matriserna kunde dessutom lagras i över ett år utan att funktionaliteten hos antikropparna försämrades. I förlängningen innebär denna imponerande stabilitet att antikroppsmatriser baserade på sinFab-molekyler skulle kunna produceras i stor industriell skala och därefter förvaras under lång tid i väntan på att användas.

Utvärdering och val av chipyta var däremot mer problematiskt. Trots att flertalet kommersiella och egenutvecklade ytor utvärderades som bärare för antikroppsmatriserna, var det ingen yta som var överlägsen de andra. Däremot identifierades flertalet viktiga ytegenskaper som påverkade sinFab-antikropparna egenskaper, t.ex. en chipytans struktur (1-, 2- eller 3-dimensional) och vilken kemi som används för att koppla antikropparna till ytan (adsorption, kovalent koppling eller affinitets koppling). Under arbetet tillverkades dessutom en helt ny 3-dimensionell yta. Det faktum att ytan utgjordes av en 3-dimensionell struktur medförde att många fler antikroppar per ytenhet kunde bindas (än om en vanlig plan yta hade använts), vilket i slutänden resulterade i väldigt känslig analyser.

Normalt så måste antikropparna renas upp innan de kan immobiliseras på en chipyta. Detta är en tidskrävande och kostsam process som gör det svårt att skala upp storleken på mikromatriserna. Möjligheten att just kunna skala upp sina mikromatriser är vital i det fortsatta utvecklingsarbetet av mikromatristeknologin. I mitt avhandlingsarbete kunde jag också visa att man genom att kombinera modificerade sinFab-antikroppar med en matchande yta så kunde antikropparna tillsättas direkt efter att ha producerats i bakterier utan att först behöva renas upp. Ur en teknologisk synvinkel var detta ett stort steg framåt i det utmanande och viktiga arbetet med att skala upp antikroppsmikromatriserna.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

"DNA Microarrays oder Chips" sind ein fester Bestandteil der biologischen Forschung und klinischen Diagnostik. Sie bieten u.a. die Möglichkeit, die expremierten Gene eines Genoms von gesundem und erkranktem Gewebe im Miniatur-Format (Chip) zu vergleichen. Auf diesem Wege können wichtige Informationen über die Krankheit gewonnen werden, die bei der Entwicklung von therapeutischen Strategien oder Medikamenten eine bedeutende Rolle spielen.

Obwohl die Gene alle notwendigen Informationen für die Funktion einer Zelle enthalten, sind die tatsächlichen Effektor-Moleküle einer Zelle Proteine. Daher muss die Krankheit auch auf Protein-Level studiert werden, um ein umfassendes Gesamtbild zu erhalten. Die Entwicklung einer Technologie, die vergleichbar ist zu DNA Microarrays, sprich "Protein Microarrays", ist jedoch nicht einfach, da Proteine im Gegensatz zu DNA große und verschiedenartige Moleküle sind, die sich nur schwer auf einem Chip immobilisieren lassen. Um die Problematik, mehrere Tausend bis Millionen verschiedener Proteine immobilisieren zu müssen, zu vermeiden, empfehlen wir den Einsatz von Antikörpern, die als strukturell einheitliche Fänger-("catcher-")Moleküle für die Protein-Analyten dienen. Antikörper sind wichtige Komponenten des Immunsystems, da sie in der Lage sind, eine enorme Vielfalt von potentiellen Pathogenen zu binden und unschädlich zu machen. Für Antikörper Microarrays werden diese hoch-spezifischen Moleküle in Form eines Arrays so auf dem Chip platziert, daß jeder Punkt einer Antigen-Spezifität, d.h. einem Protein-Analyten, entspricht. In Analogie zu DNA Microarrays können die so entstehenden Bilder von ab- und anwesenden Proteinen in einem bestimmten Proteom (z.B. das eines gesunden und erkrankten Gewebes) leicht verglichen werden und als Ausgangspunkt für intensivere Studien dienen.

Das Ziel dieser Arbeit war es, ein Antikörper Microarray Setup zu entwickeln, das mit modernen DNA Microarrays vergleichbar ist. Dazu habe ich mich in meiner Arbeit auf die beiden Schlüsselkomponenten eines Antikörper Microarrays, die Antikörper-Sonde und die Träger-Oberfläche, d.h. "catcher und carrier", konzentriert. Anstelle von natürlichen Antikörpern benutzen wir gentechnisch hergestellte Antikörper, sogenannte "single framework antibody fragments (sinFabs)", als Sonde, da diese strukturell einheitlicher und schneller zu produzieren sind und zusätzlich bereits in vielen verschiedenen Spezifitäten vorhanden sind. Die verschiedenen Studien dieser Doktorarbeit belegten, daß es sich bei sinFabs um ausgezeichnete Sonden für Antikörper Microarrays handelt, die im Gegensatz zu anderen Antikörper-Molekülen problemlos so auf dem Chip immobilisiert werden konnten, daß sie noch in der Lage waren den Analyten zu "fangen". Dies war auch dann möglich, wenn der Analyt in einer komplizierten Mixtur, wie z.B. Blutserum, vorlag und darin nur in geringen Mengen vorhanden war. Weiterhin

konnten sinFab Microarrays länger als ein Jahr gelagert werden ohne an Qualität zu verlieren, was vor allem für die industrielle Produktion mit anschließender Lagerung oder Transport von Bedeutung ist.

Weniger eindeutig war die Wahl des Trägers. Obwohl zahlreiche handelsübliche und selbst-entworfene Oberflächen auf ihre Kompatibilität mit Antikörper Microarrays hin untersucht wurden, konnte kein Träger gefunden werden, der den anderen in allen relevanten Eigenschaften überlegen gewesen wäre. Nichts desto trotz lieferten diese Studien einen interessanten Einblick in die Relevanz verschiedener Oberflächen-Parameter, wie Struktur (1-, 2- oder 3-dimensional) oder die zugrundeliegende Bindungs-Chemie (kovalente Bindung, Adsorption oder Affinitätsbindung). Weiterhin wurde eine neue 3-dimensionale Oberflächenstruktur entwickelt, deren vergrösserte Oberfläche mehr Antikörper- Sonden binden konnte und somit eine sensitivere Detektion des Analyten erlaubte.

Die richtige Kombination von modifizierten Antikörpern und geeigneter Oberfläche, ermöglichte zusätzlich die direkte Anwendung der Antikörper nach Produktion in Bakterien. Da der Prozeß der Antikörper-Aufreinigung nach der Produktion sehr aufwendig ist, erspart diese neue Technologie wertvolle Zeit und Arbeitsaufwand in Anbetracht der Größe der zu entwikkelnden Antikörper Microarrays.

ACKNOWLEDGEMENTS

Numerous people contributed to the success of this work and helped me through a challenging, but instructive and fun time in Sweden. For an inspiring and friendly work environment, I would like to express my gratitude to the whole Department of Immunotechnology with a few people in particular:

First and foremost, I would like to thank my two supervisors, Carl and Christer. Carl - for committing one of your most fascinating projects to me and for all your enthusiasm and encouragement during the years. Christer - for all your help, your open-door policy and many vivid and stimulating discussions. I learned a lot from both of you and I appreciate your enthusiasm about science in general and my project in particular.

Mats – for additional practical and theoretical help and stimulating discussions.

Marianne – for all your help with the jungle of Swedish bureaucracy and your great patience in answering stupid questions.

Anabel (and Phil) – for critical proof-reading and spicy international lunches.

The proteinchip-group and all its previous and current members – I strongly believe in a good teamwork being the way to success and hope this group will continue the way we started. For this, I would like to thank Johan I. in particular. It was a pleasure and fun working with you and I deeply appreciate all the hours and days you spent with our favourite machine trying to fix it. Also I would like to thank Jacob for his curiosity and for teaching me how to teach.

On a personal basis, I would like to thank everyone at the Department for excellent "Torsdagsbullerna och Onsdagsfikorna", nice "vårutflykterna" and "retreats" and fun "parties". I missed out on too many of these occasions and always regretted to live "abroad". For trying to integrate me into Swedish society anyway and for sharing my way to the PhD all along, I would like to thank Lavinia and Helena in particular. Lavinia – for your fruitless trials to teach me Swedish, for all your help especially in the beginning and for deep talks and nice chats. My dear roomie Helena – for being such a great listener, for always having a smile on your face and for keeping up the good mood in our little cubicle. I will miss our chats and scientific discussions and in particular your positive attitude. Finally, I would like to thank Eva for bringing some Spanish touch into my life and for being the way you are. It is good to have a Spanish/English enclave in the Swedish outback.

Life is nothing without friends, especially when they are so far away. For keeping our friendships alive and making us feel home wherever we are, I would like to thank Andreas, Doreen, Gunhild, Hauke, Jürgen, Maja, Mark, Tine and Thomas.

Mein besonderer Dank gilt meinen Eltern – dafür daß Ihr “meine Füße auf weiten Raum gestellt habt” und mich habt ziehen lassen. Ohne Euren Rückhalt und Eure 100%ige Unterstützung, wäre ich heute nicht da, wo ich jetzt bin.

Last but not least the two men and loves of my life, Helge and Matti. Helge - for your encouragement during all those years, for making me laugh and most importantly for sharing my ideas of life. Matti - for being such a satisfied and happy child and for opening a whole new world to me. The two of you put my life into perspective and make it brighter every day.

REFERENCES

Afanassiev, V., Hanemann, V., and Wolfl, S. (2000) Preparation of DNA and protein micro arrays on glass slides coated with an agarose film, *Nucl. Acids Res.* **28**(12), e66–e66

Alzari, P. M., Lascombe, M. B., and Poljak, R. J. (1988) Three-dimensional structure of antibodies, *Annu Rev Immunol.* **6**, 555–580

Amzel, L. and Poljak, R. (1979) Three-dimensional structure of immunoglobulins, *Annu Rev Biochem.* **48**, 961–997

Angenendt, P. (2005) Progress in protein and antibody microarray technology, *Drug Discov Today.* **10**(7), 503–11

Angenendt, P., Glokler, J., Konthur, Z., Lehrach, H., and Cahill, D. (2003a) 3D protein microarrays: performing multiplex immunoassays on a single chip, *Analytical Chemistry* **75**(17), 4368–4372

Angenendt, P., Glokler, J., Murphy, D., Lehrach, H., and Cahill, D. (2002) Toward optimized antibody microarrays: a comparison of current microarray support materials, *Analytical Biochemistry* **309**(2), 253–260

Angenendt, P., Glokler, J., and Sobek, J. (2003b) Next generation of protein microarray support materials:: Evaluation for protein and antibody microarray applications, *Journal of Chromatography A* **1009**(1-2), 97–105

Appella, E., Padlan, E., and Hunt, D. (1995) Analysis of the structure of naturally processed peptides bound by class I and class II major histocompatibility complex molecules, *EXS* **73**, 105–19

Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S., Chupeeva, V., and Mirzabekov, A. (2000) Protein microchips: use for immunoassay and enzymatic reactions, *Analytical Biochemistry* **278**(2), 123–131

Barbas III, C., Kang, A., Lerner, R., and Benkovic, S. (1991) Assembly of combinatorial antibody libraries on phage surfaces: The gene III site, *PNAS* **88**(18), 7978–7982

Belov, L., de la Vega, O., dos Remedios, C., Mulligan, S., and Christopherson, R. (2001) Immunophenotyping of leukemias using a cluster of differentiation antibody microarray, *Cancer Res* **61**(11), 4483–4489

Belov, L., Huang, P., Barber, N., Mulligan, S., and Christopherson, R. (2003) Identification of repertoires of surface antigens on leukemias using an antibody microarray, *PROTEOMICS* **3**(11), 2147–2154

Bernard, A., Coitot, S., Bremont, A., and Bernard, G. (2005) T and B cell cooperation: a dance of life and death, *Transplantation.* **79**(3 Suppl), S8–S11

Bernard, A., Michel, B., and Delamarche, E. (2001) Micromosaic immunoassays, *Analytical Chemistry* **73**(1), 8–12

Bird, R., Hardman, K., Jacobson, J., Johnson, S., Kaufman, B., Lee, S., Lee, T., Pope, S., Riordan, G., and Whitlow, M. (1988) Single-chain antigen-binding proteins, *Science* **242**(4877), 423–6

Blawas, A. and Reichert, W. (1998) Protein patterning, *Biomaterials* **19**(7-9), 595–609

Borrebaeck, C. (2000) Antibodies in diagnostics - from immunoassays to protein chips, *Immunology Today* **21**(8), 379–382

Borrebaeck, C., Ekstrom, S., Hager, A., Nilsson, J., Laurell, T., and Marko-Varga, G. (2001) Protein chips based on recombinant antibody fragments: a highly sensitive approach as detected by mass spectrometry, *Biotechniques* **30**(5), 1126–30, 1132

Bradbury, A., Velappan, N., Verzillo, V., Ovecka, M., Chasteen, L., Sblattero, D., Marzari, R., Lou, J., Siegel, R., and Pavlik, P. (2003) Antibodies in proteomics I: generating antibodies, *Trends in Biotechnology* **21**(6), 275–281

Burton, D. (2002) Antibodies, viruses and vaccines, *Nat Rev Immunol.* **2**(9), 706–13

Butler, J. (2000) Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays, *Methods* **22**(1), 4–23

Butler, J., Ni, L., Nessler, R., Joshi, K., Suter, M., Rosenberg, B., Chang, J., Brown, W., and Cantarero, L. (1992) The physical and functional behavior of capture antibodies adsorbed on polystyrene, *J Immunol Methods.* **150**(1-2), 77–90

Carroll, M. (2004) The complement system in regulation of adaptive immunity, *Nat Immunol.* **5**(10), 981–6

Charles, P., Taitt, C., Goldman, E., Rangasammy, J., and Stenger, D. (2004) Immobilization strategy and characterization of hydrogel-based thin films for interrogation of ligand binding with staphylococcal enterotoxin B (SEB) in a protein microarray format, *Langmuir.* **20**(1), 270–2

Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X., Stern, D., Winkler, J., Lockhart, D., Morris, M., and Fodor, S. (1996) Accessing genetic information with high-density DNA arrays, *Science* **274**(5287), 610–614

Cheran, L., Chacko, M., Zhang, M., and Thompson, M. (2004) Protein microarray scanning in label-free format by Kelvin nanoprobe, *Analyst* **129**(2), 161–8

Chowdhury, P., Vasmatzis, G., Beers, R., Lee, B., and Pastan, I. (1998) Improved stability and yield of a Fv-toxin fusion protein by computer design and protein engineering of the Fv, *Journal of Molecular Biology* **281**(5), 917–928

Delehanty, J. (2004) Printing functional protein microarrays using piezoelectric capillaries, *Methods Mol Biol.* **264**, 135–43

Doi, N., Takashima, H., Kinjo, M., Sakata, K., Kawahashi, Y., Oishi, Y., Oyama, R., Miyamoto-Sato, E., Sawasaki, T., Endo, Y., and Yanagawa, H. (2002) Novel fluorescence labeling and high-throughput assay technologies for in vitro analysis of protein interactions, *Genome Res.* **12**(3), 487–492

Ek, S., Hogerkorp, C., Dictor, M., Ehinger, M., and Borrebaeck, C. (2002) Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells, *Cancer Res* **62**(15), 4398–4405

Fenn, J., Mann, M., Meng, C., Wong, S., and Whitehouse (1989) Electrospray ionization for mass spectrometry of large biomolecules, *Science* **246**(4926), 64–71

Finnskog, D., Ressine, A., Laurell, T., and Marko-Varga, G. (2004) Integrated protein microchip assay with dual fluorescent- and MALDI read-out, *J Proteome Res.* **3**(5), 988–94

Galli, S. and Lantz, C. (1999) Allergy, *W. E. Paul, ed. Fundamental Immunology 1137. Lippincott-Raven Press, Philadelphia.*

Ge, H. (2000) UPA, a universal protein array system for quantitative detection of protein-protein, protein-DNA, protein-RNA and protein-ligand interactions, *Nucl. Acids Res.* **28**(2), e3–e3

Glokler, J. and Angenendt, P. (2003) Protein and antibody microarray technology, *Journal of Chromatography B* **797**(1-2), 229–240

Gutmann, O., Kuehlewein, R., Reinbold, S., Niekrawietz, R., Steinert, C., de Heij, B., Zengerle, R., and Daub, M. (2005) Fast and reliable protein microarray production by a new drop-in-drop technique, *Lab Chip* **5**(6), 675–81

Haab, B. (2003) Methods and applications of antibody microarrays in cancer research, *PROTEOMICS* **3**(11), 2116–2122

Haab, B., Dunham, M., and Brown, P. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions, *Genome Biology* **1**(6), 1.1–1.22

Hallborn, J. and Carlsson, R. (2002) Automated screening procedure for high-throughput generation of antibody fragments, *Biotechniques, Suppl.: High-Throughput Proteomics: Protein Arrays* , 30–7

Hamelinck, D., Zhou, H., Li, L., Verweij, C., Dillon, D., Feng, Z., Costa, J., and Haab, B. (2005) Optimized normalization for antibody microarrays and application to serum-protein profiling, *Mol Cell Proteomics* , M400180–MCP200

Harrison, P., Kumar, A., Lang, N., Snyder, M., and Gerstein, M. (2002) A question of size: the eukaryotic proteome and the problems in defining it, *Nucl. Acids Res.* **30**(5), 1083–1090

He, M. and Taussig, M. J. (2001) Single step generation of protein arrays from DNA by cell-free expression and in situ immobilisation (PISA method), *Nucl. Acids Res.* **29**(15), e73–e73

Hochuli, E., Bannwarth, H., Dobeli, H., Gentz, R., and Stuber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent, *Bio/Technology* **6**, 1321–25

Hochuli, E., Dobeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues, *Journal of Chromatography* **411**, 177–84

Honegger, A. and Pluckthun, A. (2001) The influence of the buried glutamine or glutamate residue in position 6 on the structure of immunoglobulin variable domains, *Journal of Molecular Biology* **309**(3), 687–699

Houseman, B. T. and Mrksich, M. (2002) Towards quantitative assays with peptide chips: a surface engineering approach, *Trends in Biotechnology* **20**(7), 279–281

Huang, R.-P., Huang, R., Fan, Y., and Lin, Y. (2001) Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system, *Analytical Biochemistry* **294**(1), 55–62

Hudelist, G., Pacher-Zavisin, M., Singer, C., Holper, T., Kubista, E., Schreiber, M., Manavi, M., Bilban, M., and Czerwenka, K. (2004) Use of high-throughput protein array for profiling of differentially expressed proteins in normal and malignant breast tissue, *Breast Cancer Research and Treatment* **86**(3), 283–293

Hust, M. and Dubel, S. (2004) Mating antibody phage display with proteomics, *Trends in Biotechnology* **22**(1), 8–14

Huston, J., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M., Ridge, R., Bruccoleri, R., Haber, E., Crea, R., and Oppermann, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli, *PNAS* **85**(16), 5879–5883

Jaenicke, R. (2000) Stability and stabilization of globular proteins in solution, *Journal of Biotechnology* **79**(3), 193–203

James, P. (2002) Chips for proteomics: a new tool or just hype?, *Biotechniques, Suppl.: High-Throughput Proteomics: Protein Arrays* , 4–10, 12–3

Jirholt, P., Ohlin, M., Borrebaeck, C. A. K., and Soederlind, E. (1998) Exploiting sequence space: shuffling in vivo formed complementarity determining regions into a master framework, *Gene* **215**(2), 471–476

Jonsson, U., Fagerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., and Ronnberg, I. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology, *Biotechniques* **11**(5), 620–7

Karlsson, F., Malmborg-Hager, A., Albrekt, A., and Borrebaeck, C. (2005) Genome-wide comparison of phage M13 infected vs. uninfected Escherichia coli, *Can J Microbiol* **51**(1), 29–35

Kiyonaka, S., Sada, K., Yoshimura, I., Shinkai, S., Kato, N., and Hamachi, I. (2004) Semi-wet peptide/protein array using supramolecular hydrogel, *Nat Mater.* **3**(1), 58–64

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A., and Virnekas, B. (2000) Fully synthetic Human Combinatorial Antibody Libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides, *Journal of Molecular Biology* **296**(1), 57–86

Knezevic, V., Leethanakul, C., Bichsel, V. E., Worth, J. M., Prabhu, V. V., Gutkind, J. S., Liotta, L. A., Munson, P. J., Petricoin III, E. F., and Krizman, D. B. (2001) Proteomic profiling of the cancer microenvironment by antibody arrays, *PROTEOMICS* **1**(10), 1271–1278

Kodadek, T. (2001) Protein microarrays: prospects and problems, *Chemistry and Biology* **8**(2), 105–115

Kramer, K., Fiedler, M., Skerra, A., and Hock, B. (2002) A generic strategy for subcloning antibody variable regions from the scFv phage display vector pCANTAB 5 E into pASK85 permits the economical production of F(ab) fragments and leads to improved recombinant immunoglobulin stability, *Biosensors and Bioelectronics* **17**(4), 305–313

Kusnezow, W. and Hoheisel, J. (2002) Antibody microarrays: promises and problems, *Biotechniques, Suppl.: High-Throughput Proteomics: Protein Arrays* , 14–23

Kusnezow, W. and Hoheisel, J. (2003) Solid supports for microarray immunoassays, *Journal of Molecular Recognition* **16**(4), 165–176

Kusnezow, W., Jacob, A., Walijew, A., Diehl, F., and Hoheisel, J. (2003) Antibody microarrays: An evaluation of production parameters, *PROTEOMICS* **3**(3), 254–264

Kwapiszewska, G., Meyer, M., Bogumil, R., Bohle, R., Seeger, W., Weissmann, N., and Fink, L. (2004) Identification of proteins in laser-microdissected small cell numbers by SELDI-TOF and Tandem MS, *BMC Biotechnol.* **4**(1), 30

Larsson, C., Bramfeldt, H., Wingren, C., Borrebaeck, C., and Hook, F. (2005) Gravimetric antigen detection utilizing antibody-modified lipid bilayers, *Analytical Biochemistry* **345**(1), 72–80

Laurell, T. and Marko-Varga, G. (2002) Miniaturisation is mandatory unravelling the human proteome, *PROTEOMICS* **2**(4), 345–351

Lee, K.-B., Park, S.-J., Mirkin, C., Smith, J., and Mrksich, M. (2002) Protein nanoarrays generated by dip-pen nanolithography, *Science* **295**(5560), 1702–1705

Leung, Y. and Cavalieri, D. (2003) Fundamentals of cDNA microarray data analysis, *Trends in Genetics* **19**(11), 649–59

Leung, Y., Pang, C., and Browne, K. (2002) Guide to microarray hardware - a researcher perspective, *http://www.images.technologynetworks.net/resources/comptab.asp*

Liu, G., Loraine, A., Shigeta, R., Cline, M., Cheng, J., Valmeekam, V., Sun, S., Kulp, D., and Siani-Rose, M. (2003) NetAffx: Affymetrix probesets and annotations, *Nucl. Acids Res.* **31**(1), 82–86

Lueking, A., Possling, A., Huber, O., Beveridge, A., Horn, M., Eickhoff, H., Schuchardt, J., Lehrach, H., and Cahill, D. (2003) A nonredundant human protein chip for antibody screening and serum profiling, *Mol Cell Proteomics* **2**(12), 1342–1349

MacBeath, G. and Schreiber, S. (2000) Printing proteins as microarrays for high-throughput function determination, *Science* **289**(5485), 1760–1763

Malmqvist, M. and Karlsson, R. (1997) Biomolecular interaction analysis: affinity biosensor technologies for functional analysis of proteins, *Current Opinion in Chemical Biology* **1**(3), 378–383

Marko-Varga, G., Nilsson, J., and Laurell, T. (2004) New directions of miniaturization within the biomarker research area, *Electrophoresis* **25**(21-22), 3479–91

McCafferty, J., Griffiths, A., Winter, G., and Chiswell, D. (1990) Phage antibodies: filamentous phage displaying antibody variable domains, *Nature* **348**(6301), 552–4

McDonald, J., Metallo, S., and Whitesides, G. (2001) Fabrication of a configurable, single-use microfluidic device, *Analytical Chemistry* **73**(23), 5645–5650

Metzger, S., Lochhead, M., and Grainger, D. (2002) Improving performance in protein-based microarray, *IVD Technol., Category: New Technologies* , 39

Michaud, G., Salcius, M., Zhou, F., Bangham, R., Bonin, J., Guo, H., Snyder, M., Predki, P., and Schweitzer, B. (2003) Analyzing antibody specificity with whole proteome microarrays, *Nat Biotechnol.* **21**(12), 1509–12

Miklos, G. and Maleszka, R. (2001) Protein functions and biological contexts, *PROTEOMICS* **1**(2), 169–178

Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R., Burke, J., Butler, E. B., Teh, B. S., and Haab, B. B. (2003) Antibody microarray profiling of human prostate cancer sera: Antibody screening and identification of potential biomarkers, *PROTEOMICS* **3**(1), 56–63

Mischel, P., Cloughesy, T., and Nelson, S. (2004) DNA-microarray analysis of brain cancer: molecular classification for therapy., *Nat Rev Neurosci* **5**(10), 782–92

Mitchell, P. (2001) Microfluidics–downsizing large-scale biology, *Nat Biotechnol.* **19**(8), 717–21

Mitchell, P. (2002) A perspective on protein microarrays, *Nat Biotechnol.* **20**(3), 225–9

Mohanty, A. K. and Wiener, M. C. (2004) Membrane protein expression and production: effects of polyhistidine tag length and position, *Protein Expression and Purification* **33**(2), 311–325

Morozov, V. and Morozova, T. (1999) Electrospray deposition as a method for mass fabrication of mono- and multicomponent microarrays of biological and biologically active substances, *Analytical Chemistry* **71**(15), 3110–3117

Nedelkov, D. and Nelson, R. (2003) Surface plasmon resonance mass spectrometry: recent progress and outlooks, *Trends in Biotechnology* **21**(7), 301–305

Nelson, R., Nedelkov, D., and Tubbs, K. (2000) Biosensor chip mass spectrometry: A chip-based proteomics approach, *Electrophoresis* **21**(6), 1155–1163

Ng, J. and Ilag, L. (2003) Biochips beyond DNA: technologies and applications, *Biotechnol Annu Rev.* **9**, 1–149

Niemeyer, C., Boldt, L., Ceyhan, B., and Blohm, D. (1999) DNA-Directed Immobilization: efficient, reversible, and site-selective surface binding of proteins by means of covalent DNA-Streptavidin conjugates, *Analytical Biochemistry* **268**(1), 54–63

Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., and Nygren, P.-a. (1997) Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins, *Protein Expression and Purification* **11**(1), 1–16

O'Farrell, P. (1975) High resolution two-dimensional electrophoresis of proteins, *J. Biol. Chem.* **250**(10), 4007–4021

Park, T., Yi, S.-G., Kang, S.-H., Lee, S.-Y., Lee, Y.-S., and Simon, R. (2003) Evaluation of normalization methods for microarray data, *BMC Bioinformatics* **4**(1), 33

Patterson, S. and Aebersold, R. (2003) Proteomics: the first decade and beyond, *Nat Genet.* **33**(Suppl.), 311–23

Pavlickova, P., Knappik, A., Kambhampati, D., Ortigao, F., and Hug, H. (2003) Microarray of recombinant antibodies using a streptavidin sensor surface self-assembled onto a gold layer, *Biotechniques.* **33**(1), 124–30

Pavlickova, P., Schneider, E., and Hug, H. (2004) Advances in recombinant antibody microarrays, *Clinica Chimica Acta* **343**(1-2), 17–35

Pawlak, M., Schick, E., Bopp, M. A., Schneider, M. J., Oroszlan, P., and Ehrat, M. (2002) Zeptosens' protein microarrays: A novel high performance microarray platform for low abundance protein analysis, *PROTEOMICS* **2**(4), 383–393

Peluso, P., Wilson, D., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K., Tolani, N., Phelan, M., Witte, K., Jung, L., Wagner, P., and Nock, S. (2003) Optimizing antibody immobilization strategies for the construction of protein microarrays., *Analytical Biochemistry* **312**(2), 113–24

Pennisi, E. (2003) HUMAN GENOME: A low number wins the GeneSweep pool, *Science* **300**(5625), 1484b–

Perelson, A. and Oster, G. (1979) Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination, *J Theor Biol.* **81**(4), 645–70

Phizicky, E., Bastiaens, P., Zhu, H., Snyder, M., and Fields, S. (2003) Protein analysis on a proteomic scale, *Nature* **422**(6928), 208–15

Piehler, J., Brecht, A., Geckeler, K., and Gauglitz, G. (1996) Surface modification for direct immunoprobes, *Biosensors and Bioelectronics* **11**(6-7), 579–590

Piehler, J., Brecht, A., Valiokas, R., Liedberg, B., and Gauglitz, G. (2000) A high-density poly(ethylene glycol) polymer brush for immobilization on glass-type surfaces, *Biosensors and Bioelectronics* **15**(9-10), 473–481

Poljak, R., Amzel, L., Avey, H., Chen, B., Phizackerley, R., and Saul, F. (1973) Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2,8-A resolution, *PNAS* **70**(12), 3305–10

Porath, J., Carlsson, J., Olsson, I., and Belfrage, G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation, *Nature* **258**(5536), 598–9

Proba, K., Worn, A., Honegger, A., and Pluckthun, A. (1998) Antibody scFv fragments without disulfide bonds made by molecular evolution, *Journal of Molecular Biology* **275**(2), 245–253

Ramage, P., Hemmig, R., Mathis, B., Cowan-Jacob, S., Rondeau, J., Kallen, J., Blommers, M., Zurini, M., and Rudisser, S. (2002) Snags with tags: Some observations made with (His)6-tagged proteins., *Life Science News, Amersham Bioscience* , 1–4

Reiter, Y., Brinkmann, U., Kreitman, R., and Jung, S.-H. (1994) Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions, *Biochemistry (Easton)* **33**(18), 5451

Renault, J. P., Bernard, A., Juncker, D., Michel, B., Bosshard, H. R., and Delamarche, E. (2002) Fabricating microarrays of functional proteins using affinity contact printing, *Angewandte Chemie International Edition* **41**(13), 2320–2323

Ressine, A., Ekstrom, S., Marko-Varga, G., and Laurell, T. (2003) Macro-/nanoporous silicon as a support for high-performance protein microarrays, *Analytical Chemistry* **75**(24), 6968–6974

Revzin, A., Sekine, K., Sin, A., Tompkins, R., and Toner, M. (2005) Development of a microfabricated cytometry platform for characterization and sorting of individual leukocytes, *Lab Chip.* **5**(1), 30–7

Ringeisen, B., Wu, P., Kim, H., Pique, A., Auyeung, R., Young, H., Chrisey, D., and Krizman, D. (2002) Picoliter-scale protein microarrays by laser direct write, *Biotechnology progress* **18**(5), 1126–1129

Robinson, W., DiGennaro, C., Hueber, W., Haab, B., Kamachi, M., Dean, E., Fournel, S., Fong, D., Genovese, M., de Vegvar, H., Skriner, K., Hirschberg, D., Morris, R., Muller, S., Pruijn, G., van Venrooij, W., Smolen, J., Brown, P., Steinman, L., and Utz, P. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses., *Nat Med.* **8**(3), 295–301

Rubina, A., Dementieva, E., Stomakhin, A., Darii, E., Pankov, S., Barsky, V., Ivanov, S., Konovalova, E., and Mirzabekov, A. (2003) Hydrogel-based protein microchips: manufacturing, properties, and applications, *Biotechniques.* **34**(5), 1008–14, 1016–20, 1022

Saal, L., Troein, C., Vallon-Christersson, J., Gruvberger, S., Borg, A., and Peterson, C. (2002) BioArray Software Environment (BASE): a platform for comprehensive management and analysis of microarray data, *Genome Biology* **3**(8), SOFTWARE0003

Schena, M., Shalon, D., Davis, R., and Brown, P. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science* **270**(5235), 467–470

Schiffer, M., Girling, R., Ely, K., and Edmundson, A. (1973) Structure of a lambda-type Bence-Jones protein at 3.5-A resolution, *Biochemistry* **12**(23), 4620–31

Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V., Christiansen, J., Velleca, M., and Kingsmore, S. (2002) Multiplexed protein profiling on microarrays by rolling-circle amplification, *Nat Biotechnol.* **20**(4), 359–65

Schweitzer, B., Wiltshire, S., Lambert, J., O'Malley, S., Kukanskis, K., Zhu, Z., Kingsmore, S., Lizardi, P., and Ward, D. (2000) Immunoassays with Rolling Circle DNA Amplification: A versatile platform for ultrasensitive antigen detection, *PNAS* **97**(18), 10113–10119

Seong, S. (2002) Microimmunoassay using a protein chip: optimizing conditions for protein immobilization, *Clin Diagn Lab Immunol.* **9**(4), 927–30

Service, R. (2005) Protein chips map yeast kinase network, *Science* **307**(5717), 1854–5

Sheridan, C. (2005) Protein chip companies turn to biomarkers, *Nat Biotechnol.* **23**(1), 3–4

Soderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R., and Borrebaeck, C. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries, *Nat Biotechnol.* **18**(8), 852–6

Sreekumar, A., Nyati, M., Varambally, S., Barrette, T., Ghosh, D., Lawrence, T., and Chinnaiyan, A. (2001) Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins, *Cancer Res* **61**(20), 7585–7593

Stanfield, R. and Wilson, I. (1994) Antigen-induced conformational changes in antibodies: a problem for structural prediction and design, *Trends in Biotechnology* **12**(7), 275–9

Steel, L. F., Haab, B. B., and Hanash, S. M. (2005) Methods of comparative proteomic profiling for disease diagnostics, *Journal of Chromatography B* **815**(1-2), 275–284

Stillman, B. and Tonkinson, J. (2000) FAST slides: a novel surface for microarrays, *Biotechniques* **29**(3), 630–5

Striebel, H.-M., Schellenberg, P., Grigaravicius, P., and Greulich, K. (2004) Readout of protein microarrays using intrinsic time resolved UV fluorescence for label-free detection, *PROTEOMICS* **4**(6), 1703–1711

Sun, P. (2003) Structure and function of natural-killer-cell receptors, *Immunol Res.* **27**(2-3), 539–48

Svedhem, S., Pfeiffer, I., Larsson, C., Wingren, C., Borrebaeck, C., and Hook, F. (2003) Patterns of DNA-labeled and scFv-antibody-carrying lipid vesicles directed by material-specific immobilization of DNA and supported lipid bilayer formation on an Au/SiO2 template, *ChemBioChem* **4**(4), 339–343

Swanson, J. and Hoppe, A. (2004) The coordination of signaling during Fc receptor-mediated phagocytosis, *J Leukoc Biol* **76**(6), 1093–1103

Tanaka, K. (1988) Protein and polymer analysis up to m/z 100.000 by laser-ionization TOF-MS, *Rapid Commun. Mass Spectrom.* **2**, 151–53

Tang, N., Tornatore, P., and Weinberger, S. (2004) Current developments in SELDI affinity technology, *Mass Spectrom Rev.* **23**(1), 34–44

Terpe, K. (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems, *Applied Microbiology and Biotechnology* **60**(5), 523–533

Tomlinson, I. and Holt, L. (2001) Protein profiling comes of age, *Genome Biology* **2**, 1004.1–1004.3

Usui-Aoki, K., Shimada, K., Nagano, M., Kawai, M., and Koga, H. (2005) A novel approach to protein expression profiling using antibody microarrays combined with surface plasmon resonance technology, *PROTEOMICS* **4**, Epub ahead of print

Vijayendran, R. A. and Leckband, D. E. (2001) A quantitative assessment of heterogeneity for surface-immobilized proteins, *Analytical Chemistry* **73**(3), 471–480

Wang, Z. H. and Jin, G. (2003) A label-free multisensing immunosensor based on imaging ellipsometry, *Analytical Chemistry* **75**(22), 6119–6123

Wasinger, V., Cordwell, S., Cerpa-Poljak, A., Yan, J., Gooley, A., Wilkins, M., Duncan, M., Harris, R., Williams, K., and Humphery-Smith, I. (1995) Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium, *Electrophoresis* **16**(7), 1090–4

Wildt, R., Mundy, C., Gorick, B., and Tomlinson, I. (2000) Antibody arrays for high-throughput screening of antibodyantigen interactions, *Nat Biotechnol.* **18**(9), 989 – 994

Williams, C. and Addona, T. (2000) The integration of SPR biosensors with mass spectrometry: possible applications for proteome analysis, *Trends in Biotechnology* **18**(2), 45–48

Wingren, C. and Borrebaeck, C. (2004) High-throughput proteomics using antibody microarrays, *Expert Rev Proteomics.* **1**(3), 355–64

Wingren, C., Ingvarsson, J., Lindstedt, M., and Borrebaeck, C. (2003) Recombinant antibody microarrays–a viable option?, *Nat Biotechnol.* **21**(3), 223

Woern, A. and Plueckthun, A. (1998) Mutual stabilization of V L and V H in single-chain antibody fragments, investigated with mutants engineered for stability, *Biochemistry (Easton)* **37**(38), 13120–13127

Woodbury, R., Varnum, S., and Zangar, R. (2002) Elevated HGF levels in sera from breast cancer patients detected using a protein microarray ELISA, *J Proteome Res.* **1**(3), 233–7

Worn, A. and Pluckthun, A. (2001) Stability engineering of antibody single-chain Fv fragments, *Journal of Molecular Biology* **305**(5), 989–1010

Wright Jr, G., Cazares, L., Leung, S., Nasim, S., Adam, B., Yip, T., Schellhammer, P., Gong, L., and Vlahou, A. (1999) Proteinchip(R) surface enhanced laser desorption/ionization (SELDI) mass spectrometry: a novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures, *Prostate Cancer Prostatic Dis.* **2**(5/6), 264–276

Wu, P. and Grainger, D. (2004) Toward immobilized antibody microarray optimization: print buffer and storage condition comparisons on performance, *Biomed Sci Instrum.* **40**, 243–8

Wu, T. and Kabat, E. (1970) An Analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complimentarity, *J. Exp. Med.* **132**(2), 211–250

Wu, X., Liu, H., Liu, J., Haley, K., Treadway, J., Larson, J., Ge, N., Peale, F., and Bruchez, M. (2003) Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots, *Nat Biotechnol.* **21**(1), 41–6

Yazaki, J., Kojima, K., Suzuki, K., Kishimoto, N., and Kikuchi, S. (2004) The Rice PIPELINE: a unification tool for plant functional genomics, *Nucl. Acids Res.* **32**(90001), D383–387

Young, N., MacKenzie, C., Narang, S., Oomen, R., and Baenziger, J. (1995) Thermal stabilization of a single-chain fv antibody fragment by introduction of a disulphide bond, *FEBS Letters* **377**(2), 135–139

Zhang, S. (2004) Hydrogels: Wet or let die, *Nat Mater.* **3**(1), 7–8

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips, *Science* **293**(5537), 2101–2105

Zhu, H., Klemic, J., Chang, S., Bertone, P., Casamayor, A., Klemic, K., Smith, D., Gerstein, M., Reed, M., and Snyder, M. (2000) Analysis of yeast protein kinases using protein chips, *Nat Genet.* **26**(3), 283–9

Zhu, H. and Snyder, M. (2003) Protein chip technology, *Current Opinion in Chemical Biology* **7**(1), 55–63

PAPERS I-V