Microfluidic Methods for Protein Microarrays

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Doctoral Thesis

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Stockholm, Sweden, 2010
Abstract

Protein microarray technology has an enormous potential for in vitro diagnostics (IVD)\(^1\). Miniaturized and parallelized immunoassays are powerful tools to measure dozens of parameters from minute amounts of sample, whilst only requiring small amounts of reagent. Protein microarrays have become well-established research tools in basic and applied research and the first diagnostic products are already released on the market. However, in order for protein microarrays to become broadly accepted tools in IVD, a number of criteria have to be fulfilled concerning robustness and automation. Robustness and automation are key demands to improve assay performance and reliability of multiplexed assays, and to minimize the time of analysis.

These key demands are addressed in this thesis and novel methods and techniques concerning assay automation, array fabrication as well as performance and detection strategies related to protein microarrays are presented and discussed. In the first paper an automated assay format, based on planar protein microarrays is described and evaluated by the detection of several auto-antibodies from human serum and by quantification of matrix metalloproteases present in plasma. Diffusion-rate limited solid phase reactions were enhanced by microagitation, using the surface acoustic wave technology, resulting in a slightly increased signal-to-noise ratio. In the second paper of the thesis, a novel multiplexed immunoassay system was developed by combining a direct immunoassay with a competitive system. This set-up allows quantification of analytes present in widely varying concentrations within a single multiplex assay. In the third paper, a new concept for sample deposition is introduced, addressing contemporary problems of contact or non-contact microarrayers in protein microarray fabrication.

In the fourth paper, a magnetic bead-based detection method for protein microarrays is described as a cost-effective alternative approach to the commonly used fluorescence-based confocal scanning systems. The magnetic bead-based detection could easily be performed by using an ordinary flatbed scanner. In addition, applying magnetic force to the magnetic bead-based detection approach enables to run the detection step more rapidly. Finally, in paper five, a microfluidic bead-based immunoassay for multiplexed detection of receptor tyrosine kinases in breast cancer tissue is presented. Since the assay is performed inside a capillary, the amounts of sample and reagent material could be reduced by a factor of 30 or more when compared with the current standard protein microarray assay.

Stockholm, 2010 - Michael Hartmann
Keywords: automation, microfluidic, microagitation, miniaturization, parallelization, protein microarray, bead-based microarray, magnetic beads, immunoassay, auto-antibodies, receptor tyrosine kinase

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ISBN 978-91-7415-761-1
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*PLoS ONE*, **5** (2010) e13125

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The contributions of the author of this thesis to these papers are:
  I. All experiments and major part of the writing
  II. All experiments and writing
  III. Part of the experiments and major part of the writing
  IV. Half of the experiments and writing
  V. Minor part of the experiments and writing
Parts of the work presented in this thesis have also been presented at conferences:

- Expanding assay dynamics: a combination of competitive and direct immunoassays for the quantification of proteins in microarray-based systems

- Protein microarrays for diagnostic assays
  M. Hartmann
  Talk given at SENSOR+TEST Conference 2009, Nürnberg, Germany, May 27, 2009
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µFBI</td>
<td>Microfluidic bead-based immunoassay</td>
</tr>
<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
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<td>Cy5</td>
<td>Cyanine 5</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>(ss)DNA</td>
<td>(Single-stranded) Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FC</td>
<td>Fluorocarbon</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGFR</td>
<td>Hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IVD</td>
<td>In vitro diagnostic</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet-derived growth factor receptor beta</td>
</tr>
<tr>
<td>(m)RNA</td>
<td>(Messenger) Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver-operating characteristics</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SAW</td>
<td>Surface acoustic wave</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>S-PE</td>
<td>Streptavidin-phycoerythrin</td>
</tr>
<tr>
<td>TCM</td>
<td>Two-compartment model</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Tyrosine kinase with immunoglobulin and EGF repeats 2</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
</tbody>
</table>
Introduction

1 Miniaturized multiplex immunoassays

1.1 Historical review

Immunoassays have been widely employed as highly sensitive biological assay formats for almost half a century\textsuperscript{2,3}. Antibody-based assays or immunoassays, respectively, are highly robust methods that can be easily standardized and automated. Nowadays there are hundreds of antibody-based assays on the diagnostic market\textsuperscript{4}. A miniaturization of immunoassays for diagnostic purposes started already in the early sixties\textsuperscript{5,6}. Feinberg and his colleagues developed a microspot-based assay that enabled them to diagnose some autoimmune diseases. Auto-antigens were immobilized in a microspot on a cellulose-coated slide and incubated with human serum. The presence of auto-antibodies in the serum led to a spontaneous precipitation of the auto-antigens in the microspot. The authors suggested that such microspot assays “would have a particular advantage for routine use on clinical specimens because it is simple, sensitive, objective, quickly carried out and read, requires but minute quantities of serum and antigen, and provides a permanent record for the “case files”. However, this microspot-based assay format did not attract many users and never achieved market acceptance.

More than two decades later, Ekins developed the ambient analyte theory, explaining the potential of miniaturized multiplexed protein assays\textsuperscript{8}. His microspot assays can be seen as the ancestor of contemporary microarrays. Ekins’s ambient analyte theory explained why high sensitivity can be achieved with miniaturized immunoassays. Also due to the fact that multiple analytes can be detected in a single experiment, Ekins was convinced that miniaturized and parallelized protein microarrays had enormous potential for diagnostic applications.
1.2 From genomics to proteomics

Despite the huge potential of protein microarrays for diagnostic applications, it was in fact the field of genomics that was the driving force for the development of microarray technology. DNA microarrays have been developed and applied to large-scale genomics research. Comprising up to tens of thousands of different oligonucleotide probes per square centimeter, DNA microarrays are perfectly suited as high-throughput hybridization systems that enable the expression analysis of the entire transcriptome in a single experiment. Nowadays, DNA microarrays are well-established, and reliable methods are available for mRNA expression profiling and single nucleotide polymorphism (SNP) analysis. In addition, diagnostic tools, such as the AmpliChip®, which was the first array-based clinical test, have been placed on the market. Despite the great potential of DNA analysis, proteins are in fact the key players in cellular processes. Cellular functions depend on protein activity, and
proteins are not only regulated by the differential expression of the underlying genes but also by posttranslational modifications. Furthermore, protein expression does not generally quantitatively correlate to mRNA expression\(^\text{13}\). Within the last decade, protein microarrays have entered the field of proteomic research\(^\text{14,15}\) demonstrating their capability for identifying and quantifying proteins as well as their suitability for studying the function of proteins from the perspective of the proteome as a whole\(^\text{16}\). Besides their application in proteome-wide research and the discovery of biochemical activities of proteins, protein microarrays are also excellent tools for quantifying subsets of proteins in complex mixtures. Here, the terminology “focused protein-profiling” or “analytical microarrays” is often used. As foreseen by Ekins, such analytical microarrays are in extensive use and are already used as diagnostic tools.

### 1.3 Tame the proteins

As mentioned above, DNA microarrays preceded the use of protein microarrays. When dealing with proteins rather than DNA, a variety of additional challenges arise due to the more complex nature of proteins. A DNA molecule is built up from four different nucleotides that all have the same hydrophilic sugar backbone. DNA is a very uniform and stable molecule due to its chemical structure and the complementary base pairing mechanism. DNA molecules exhibit a strong 1 to 1 interaction, which, under physiological conditions, is biochemically more or less inert; whereas proteins are much more instable. Proteins are assembled from 20 different amino acids that are extremely heterogeneous in size and physico-chemical properties. Amino acids can have either hydrophobic or hydrophilic side chains; some of these are positively or negatively charged and may have an acid or alkaline character. The proper function of proteins frequently depends on their tertiary and/or quaternary structures. This structure is a balanced system of electrostatic forces, hydrogen bonds, hydrophobic and van der Waals interactions. In contrast to DNA, slight changes in salt concentration or pH, the presence of oxidants or the removal of water, often irreversibly harms the structure of proteins and hence interferes with their function. Furthermore, for DNA, tailor-made capture molecules with a very high affinity can be easily predicted and generated from the primary sequence of the target DNA. This is possible due to the polymerase chain reaction (PCR) method and the possibility to chemically synthesize oligonucleotides. So far, no universal method exists to amplify proteins, nor is it possible to predict or design protein capture molecules on the basis of a “PCR equivalent” method, since protein-protein interaction is based on a broad range of the aforementioned molecular forces. In addition, posttranslational modifications such as phosphorylation, acetylation or glycosylation have to be taken into account when designing capture or binding molecules.
1.4 Binder Molecules

Antibodies are extensively used in research and diagnostic applications because of their ability to bind to target proteins with high specificity and high affinity. When protein microarrays were still in their infancy, scientists initially resorted to antibodies since the properties of these proteins were well known from immunoassays. The invention of monoclonal antibodies in 1975 represented a major step forward in the generation of almost unlimited resources of defined capture molecules. From there on, it was possible to produce pure and highly specific antibodies against almost any type of antigen. However, the generation and validation of antibodies, regardless of whether these are monoclonal or polyclonal, is a time- and cost-intensive process. In vitro strategies that enabled the generation of binding molecules were eventually developed. For instance, the phage display technology can be used to screen large synthetic libraries of antibody fragments (e.g. antigen binding fragments or single chain variable fragments) for identification of suitable binders against a target molecule of interest within a few weeks. However, additional maturation steps are required for the generation of high affinity binders that have affinities, comparable to antibodies. Another strategy for producing synthetic binders is the generation of aptamers. Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) with a specific and complex three-dimensional shape which provides optimal binding properties. Aptamers are produced using an in vitro selection and amplification technique called SELEX (Systematic Evolution of Ligands by Exponential Enrichment). Many of the selected aptamers show affinities comparable to those observed for monoclonal antibodies. Affibodies are another class of synthetic binding molecules, which are based on combinatorial protein engineering of the small and robust α-helical structure of the domains of protein A. These small binders (~ 6 kDa) are more robust than antibodies, which makes them very suitable as capture or detection agents in protein microarrays. In addition, by permutation of single amino acids in the binding domain, it is possible to improve affinity further. Despite all the advances in recombinant and scaffold-based technologies, the most advanced binding reagent project today, the “Human Protein Atlas”, uses a polyclonal antibody approach to generate binding reagents against human proteins.

1.5 Diffusion related limitation of solid phase assays

During an immunoassay, antibodies (or other binders) are used to bind to their complementary antigen. For the detection of the antibody-antigen complex, the bound analytes have to be separated from all other molecules and subsequently visualized. Capture antibodies, immobilized on a solid phase are suited to do this in a rapid and convenient manner since bound analytes are retained at a surface, and all unbound molecules can
easily be washed away. Regardless of the assay format, e.g. microarray assays, macroscopic lateral flow tests or microtiter plate based enzyme-linked immunosorbent assays (ELISA’s), the limitations of a solid phase assay remains the same. The inherent drawback of any solid phase assay is the reduced mobility of the immobilized reaction partner. Steric hindrances may occur at the surface since the immobilized partner has fewer possibilities to orient itself for the reaction. Another scenario is that the area in the vicinity of the reaction zone depletes of analyte and therefore the binding reaction is slowed down. The question is therefore: in which way are the reaction rates of solid phase assays limited by diffusion of the analyte molecules? And what are the consequences regarding protein microarrays?

From DNA microarray assays, it is known that efficient mixing can accelerate the hybridization of immobilized oligonucleotides and soluble DNA fragments. In this way, Toegl et al.\textsuperscript{23} observed that it is possible to reach an increase of signal by a factor of 6 or to reduce assay time by a factor of 4, respectively. How can these findings be translated to protein assays? Nygren and Stenberg\textsuperscript{24} studied binding reactions at the solid phase by immobilizing bovine serum albumin (BSA) and detecting the specific binding of an antibody to the BSA by means of ellipsometry. They observed that the binding was limited by diffusion, especially at the beginning of the reaction, or when the analyte (i.e. antibody) concentration was low. However when the incubation had already progressed for some time, or when the analyte concentration was high, the reaction of analyte towards the immobilized reaction partner did not show a limitation, caused by diffusion.

For analysis of mass transport-limited biomolecular interactions, a two-compartment model (TCM) has been proposed in surface plasmon resonance-based biosensors, to describe the binding reaction as a two step process\textsuperscript{25,26}: (i) the transport of the analyte from the bulk compartment to the surface reaction area (reaction compartment) and (ii) the subsequent binding process (Figure 2). Within the TCM, the overall binding kinetics can be described as a steady-state approximation by the following nonlinear differential equation\textsuperscript{26}:

\[
\frac{dS(t)}{dt} = \frac{k_+ A_0 (S_{\text{max}} - S(t)) - k_- S(t)}{1 + k_+ (S_{\text{max}} - S(t)) / k_m}
\]

Where S(t) and S_{\text{max}} are the current and maximum signal intensities; A_0 is the initial analyte concentration; k_+ and k_- are the association and dissociation rate constants and k_m is a mass transport related constant.
Figure 2 The two-compartment model describes the kinetics of a reaction at the solid phase taking mass transport into account. According to equation (1) the overall kinetics is described as a two step process: (i) diffusion ($k_m$) and (ii) the binding, i.e. association ($k_+$) and dissociation ($k_-$) (adapted from reference\textsuperscript{26})

Kusnezow et al. adopted the TCM to protein microarrays by using human interferon-gamma as analyte and anti-INFG antibody as immobilized binder\textsuperscript{27}. They observed mass transport related limitations of the binding kinetics up to a factor of 160 especially at low analyte concentrations. Even when the fluid in the reaction compartment was stirred, the binding was still slowed down by a factor of 40. Accordingly, they observed a four- to fivefold increase in signal when the mixture reaction was stirred. Apparently, solid phase assays are diffusion-limited under certain circumstances, especially at the beginning of a binding process when reaction rates are high, but also at low concentrations of the soluble analyte, or at high concentrations of the immobilized binding partner, where a local depletion zone of analyte can occur.

1.6 “Ambient analyte” theory

In the late eighties, Ekins introduced the “ambient analyte” theory showing the feasibility of highly sensitive multi-spot and multi-analyte immunoassays\textsuperscript{8}. According to this theory, miniaturization itself leads to an increase in detection sensitivity. Considering the law of mass action for the dissociation of a complex between an antibody Ab and an antigen Ag at equilibrium state, the dissociation constant $K_D$ can be written as:

$$K_D = \frac{([Ab_0]-[AbAg])([Ag_0]-[AbAg])}{[AbAg]}$$

(2)
In the case of protein microarrays, only a minute amount of capture molecules \([\text{Ab}_0]\) is immobilized in a microspot. Therefore, according to the mass action law, only a tiny fraction of the initial amount of analyte \([\text{Ag}_0]\) is captured onto the microspot. According to Ekins, “ambient analyte” conditions prevail, when the total concentration of capture molecules \([\text{Ab}_0]\) is approximately 100-fold below the value of the dissociation constant \(K_D\). The consequence is that under ambient analyte conditions, the initial concentration of analyte \([\text{Ag}_0]\) in the sample is not changed significantly even though the capture molecule is a high affinity binder and the analyte concentration is low (Figure 3). This type of miniature immunoassay is concentration dependent: i.e. the analyte molecules captured in the spot directly reflect the analyte concentration in the sample. Due to the unaltered analyte concentration, the signal becomes independent of the sample volume. High sensitivity is achieved because the measurement always takes place at the highest analyte concentration possible. Thus, for a given analyte concentration, it is possible that the detection sensitivity in a microspot is higher than in a macrospot since the analyte is contained in a small area and only signal density (i.e. signal per area unit) not the total signal strength, is relevant for signal-to-noise ratios, defining the lower limit of detection. Saviranta et al. confirmed the “ambient analyte” theory for sandwich type protein microarrays\(^{28}\). Titration curves for 24 mouse serum proteins were obtained for assay volumes of 20, 40 and 80 \(\mu\)L and no significant depletion of analytes was observed for the different assay volumes.

**Figure 3**  
Signal density (signal/area unit) and signal (total intensity) of captured analyte (antigen) in microspots are shown for different concentrations of capture molecules (antibody). The capture molecules are immobilized with the same surface density on all spots.
The signal (total signal) increases with increasing amount of capture molecules at growing spot size. When all of the analyte molecules are captured from the sample solution, the signal reaches its maximum. The signal density increases with decreasing amount of capture molecules (decreasing spot size). However, when the capture molecule concentration is $< 0.01 K_D$, the signal density reaches a constant level. Under these ambient analyte conditions, analyte concentration in solution is only minimally altered by the amount of captured analyte in the microspot (adapted from reference 29).

1.7 Multiplex detection

Approximately 7% of the human body weight belongs to blood and roughly 45% of blood consists of cellular components. The cell-free component of blood (the plasma) contains the proteins and other low molecular weight compounds, e.g. electrolytes, glucose etc. The total protein amount in plasma is between 60 and 80 mg/mL and the plasma proteome is the most complex human-derived proteome. Therefore and due to the simple availability, plasma is perfectly suited as a source of proteins for IVD, making it clinically the most important proteome. However, the dynamic range of the plasma proteome extends over more than 10 orders of magnitude (Figure 4). Approximately two thirds of the total protein amount in plasma belongs to albumin while there are low abundant proteins, such as Interleukin 2, which is present at concentrations below 1 pg/mL.

![Figure 4](Figure 4 The abundance of proteins in a plasma sample ranges over more than 10 orders of magnitude (data from www.plasmaproteome.org))

It is a very challenging task to quantify or even detect a single species of protein in plasma among the other matrix components. First, an analytical detector must have the sensitivity in
terms of detection limit. By using mass spectrometry in combination with enrichment strategies, it is possible to detect proteins down to concentrations of a few ng/mL\textsuperscript{31,32}, while sandwich immunoassays allow a detection down to 1 pg/mL\textsuperscript{33}. A second problem arises when multiplex detection is performed and the difference of two protein concentrations is larger than the measurement range of the detector. E.g. if one would be interested to quantify the total immunoglobulin G (IgG) concentration and Interleukin 2 in one assay, a detector with a measurement range of 10 orders of magnitude would be required. Typical sandwich immunoassays as they are used in protein microarrays reveal good linearity within 2 to 3 orders of magnitude\textsuperscript{33}. This problem of measurement range for protein microarray-based assays is addressed in paper III.
2 Protein Microarrays

2.1 Planar microarrays

Protein microarrays are highly miniaturized and parallelized solid-phase assay systems that use a large number of different capture molecules immobilized in microspots (diameter < 250 µm), with a density of up to several thousand spots per square centimeter (Figure 5). Such assay systems require only tiny amounts of sample and reagent volumes. Technologies that have previously been established for DNA microarrays have now been adapted for the generation of protein arrays. Microscopy glass slides are used as solid support and countless surface chemistry strategies have been employed to firmly attach proteins onto the surface. However, regardless whether the proteins are attached to the surface by non-specific interaction or by covalent cross-linking or whether the surface is 2- or 3-dimensional, the main goal is to achieve maximum binding capacity. It is also extremely important to effectively block the remaining surface prior to the assay, since it minimizes the unspecific binding of analytes or detection molecules. As a consequence, high signal-to-noise ratios can be obtained, which leads to an improved sensitivity. Arraying techniques were also successfully adopted from DNA microarray production methods. However, great care has to be taken to keep the capture reagents in a functional state, especially with regard to the storage of protein arrays. Most of the protein microarray assays are performed manually, but automation is starting to become more common. In the same way as for DNA arrays, protein microarrays often rely on fluorescence labeling and the use of confocal microarray scanners. The images obtained are subsequently analyzed with appropriate software.

Figure 5 Protein microarrays are highly miniaturized and parallelized solid-phase assay systems that use a large number of different capture molecules immobilized in microspots (diameter < 250 µm) with a density of a thousand spots per square centimeter or more.
2.2 Bead-based micro-assays

Besides planar microarrays, robust and flexible bead-based assay systems have been developed over the last decade. In planar microarrays, the position within the array allows to identify individual analytes, whereas bead-based assay systems differentiate between individual bead types either by means of an internal color code or by size (Figure 6). Such multiplex bead-based assay systems are also referred to as “bead arrays” or “liquid arrays”. The individual bead types are classified according to their internal color code or size in an instrument resembling a flow cytometer. E.g. the Luminex xMAP reader can differentiate between 100 bead types which are dyed with different amounts of two fluorophores. On each bead type, an antibody against a certain analyte is immobilized. Analytes that are captured on the bead surface are then detected using an appropriate reporter molecule. The bead suspension assays are performed using standard laboratory equipment, i.e. using microtiter plates, and can therefore easily be automated. Sensitivity and accuracy of bead-based systems are similar to what can be obtained with established ELISA systems involving planar arrays. Bead-based immunoassays proved to be as sensitive, accurate, and precise as competitive radio immunoassays or microtiter plate-based sandwich immunoassays. The Luminex xMAP technology has become the most popular bead-based platform, which allows researchers to easily setting-up multiplex assays. In addition, there are an increasing number of commercially available kits. At present, the washing steps in bead-based assays mainly involve filter plates. However, the introduction of magnetic beads contributed to further simplification of bead-based assays. Problems experienced with filter plates such as clogging, leaking or unspecific adsorption of analyte onto the large surface of filters are avoided. The integration of established magnetic bead handling technologies such as magnetic plate separators, magnetic pin heads (KingFisher, Thermo) or in-tip magnetic capture (Magnatrix™, NorDiag) represented a decisive step towards a further automation of bead-based microarrays.
Figure 6 In planar microarrays, individual capture agents are immobilized in a microarray format, containing several hundreds to several thousands of spots. The arrays are probed with sample and the analytes of interest bind to their cognate capture agent. The binding reaction is verified by a fluorescent readout. In bead-based microarrays, individual capture agents are bound to color- or size-coded microspheres. The assay can be performed with standard laboratory ware; a flow cytometer is used to detect the fluorescent label (adapted from Hartmann et al.42).

2.3 Assay formats

In general, protein array assay formats can be divided into forward-phase and reverse-phase arrays (Figure 7). Forward-phase protein microarray assays involve the immobilization of capture agents and hence enable the analysis of multiple parameters from a single sample that is incubated on the array. In reverse-phase protein microarrays, many different samples, e.g. cell or tissue lysates, are immobilized in a microarray format and are simultaneously analyzed for the presence of a single target protein using a target-specific antibody. Replicates of such protein arrays allow the analysis of hundreds of parameters from minimal
amounts of sample. The reverse-phase array format is ideally suited for looking into large sample cohorts. It enables the quantification of differentially regulated proteins in tissue of healthy or diseased individuals, treated and non-treated cells, the identification of disease-specific biomarkers or the analysis of cell signaling networks. Tissue arrays are a special type of reverse-phase microarrays and consist of tissue slices that are immobilized on a surface. These are the miniature equivalent of classical immunohistochemistry assays and enable a simultaneous analysis of large numbers of tissues with minimum reagent consumption. Reverse-phase microarrays also have an enormous potential as diagnostic tools, in particular in the identification of disease-specific biomarkers, which may be used as markers to initiate and monitor therapy. There are high expectations that biopsy analysis from individual patients may lead to therapies that are specifically tailored to individual requirements.

Figure 7  In a forward-phase array format, a large number of immobilized capture molecules (e.g. antibodies) enable the analysis of many parameters from a single sample, regardless whether the sample is directly labeled or a sandwich assay is performed. In a reverse-phase array, many samples are immobilized and a highly specific antibody is used to analyze the expression of a single parameter in the immobilized samples (adapted from Hartmann et al.).
2.4 Global and focused approaches

Currently, forward-phase assays are the most frequently used protein microarray format. Forward-phase protein microarray assays are mainly applied for antibody arrays to quantify analytes that are present in complex samples. In antibody arrays, antibodies are immobilized on a carrier at densities of a few up to several thousands of antibodies. Protein microarrays enable screening for the disease-related up- or down-regulation of proteins in patient samples\textsuperscript{46}. Similarly to DNA microarrays used for mRNA expression analysis, protein microarrays are used in dual labeling approaches to investigate relative protein abundance in two differentially labeled samples. Haab et al.\textsuperscript{46} demonstrated the potential of such protein capture arrays by analyzing 115 characterized antibody-antigen interactions. Capture antibodies and antigens were immobilized on poly-L-lysine-coated glass slides. Direct labeling approaches have already been commercialized by several companies (www.biochipnet.com).

Besides protein expression analysis, protein microarrays can also be used for functional analysis of proteins, including protein interaction with immobilized proteins or peptides, low molecular weight compounds, DNA, oligosaccharides, tissues or cells. For example, in a study on protein interaction, Zhu et al.\textsuperscript{16} arrayed 5800 recombinant yeast proteins on a microscope slide and probed this yeast proteome microarray with proteins and phospholipids to identify new interaction partners. They confirmed the already known existence of calmodulin- and phospholipid-interacting proteins and they also identified new interacting proteins.

In another approach, focused protein microarrays such as miniaturized sandwich immunoassays, either planar or bead-based, have evolved into tools that deliver abundant clinical data of high diagnostic and prognostic relevance. E.g., lysed breast tumor biopsy samples were analyzed along with normal tissue for 14 relevant marker proteins from only 50 µg of protein, using the bead-based Luminex xMAP system\textsuperscript{47}. The obtained expression profile for estrogen receptor and human epidermal growth factor receptor 2 (Her-2) exactly matched the results from immunohistochemistry. Recently, the expression of 11 soluble receptors was analyzed in samples from 36 critically ill intensive care unit patients. Hierarchical clustering analysis allowed the scientists to group the patients into a sepsis and a trauma group\textsuperscript{33}. Protein microarrays as diagnostic tools have a huge potential in the field of inflammation, especially in the field of sepsis which is often hampered by the lack of quantitative IVD tests. Due to the complex nature of sepsis, many clinical parameters ought to be monitored in parallel. Protein microarrays are perfectly suited to fulfill this requirement\textsuperscript{48}. 
2.5 Detection strategies

Captured analytes can either be visualized by directly labeling the samples (analogous to DNA microarrays) or by multiplexed sandwich immunoassays. Fluorescent labeling of a sample necessitates careful optimization in order to obtain optimum signal-to-noise ratios. Insufficient labeling results in a decrease in detection sensitivity whereas over-labeling of a sample would lead to high background signals. The sample can also be labeled with biotin and subsequently incubated with fluorescently labeled streptavidin, which was reported to improve the signal-to-noise ratio\(^49\). However, it has to be kept in mind that labeling of the target proteins may interfere with the antibody-antigen interaction as the labeling procedure could negatively affect the epitope or inactivate it. Finally, one has to be aware of the fact that proteins appear in complexes. A strong signal can thus either be derived from large amounts of captured target analytes, or, alternatively, from capturing a huge protein complex. Here, the use of a single capture reagent cannot discriminate between specific and unspecific interaction.

Specificity is greatly enhanced using matched antibody pairs in miniaturized sandwich immunoassays, in which high affinity antibodies are immobilized in an array format and capture the target analytes during incubation with the sample. Unbound molecules are washed off and the array is incubated with detection antibodies that bind to another epitope on the target analytes. In sandwich immunoassays, two separate binding reactions are employed, which leads to a considerably higher specificity than direct labeling approaches where only a single binding interaction is involved. The detection antibody can be directly labeled with a fluorescent marker; alternatively, the signal can be further enhanced by using the biotin-streptavidin reporter system. The biotin-streptavidin model is a universal reporter system that simplifies the assay procedure since all detection antibodies can be used in a biotinylated form. Despite high selectivity and detection sensitivity, it is nevertheless not possible to achieve every desirable degree of multiplexing of sandwich immunoassays due to cross reactivity arising from the increasing overall concentration of the cocktail of different detection antibodies, which increases background signals. E.g. in an 11-plex sandwich immunoassay, the detection sensitivity decreased up to a factor of 5 compared to the singleplex assays since higher background signals were observed in the multiplex format\(^33\). In all complex multiplexed sandwich immunoassays discussed so far, multiplexed sandwich immunoassays used for the detection of up to 100 analytes had to be divided into several multiplexed subsets\(^60\).
2.6 Antigen microarrays for detection of auto-antibodies

A special type of forward phase microarray is the antigen microarray for the detection of antibodies. Here, antigens are immobilized to test serum for the presence of antibodies towards these antigens, and bound antibodies are detected with a species-specific antibody. Antigen arrays are used for detection of antibodies against microbes or viruses\textsuperscript{51-53}, for allergy testing\textsuperscript{54-56} or detection of auto-antibodies that are involved in autoimmune diseases\textsuperscript{57-59}. A very early example of a multiplex assay system is the MASTpette test chamber which was developed for allergy testing in the 1980s\textsuperscript{60}. 35 allergens were immobilized on cellulose threads, which were bonded in the test chamber and incubated with patient serum. Bound IgEs were detected with enzyme-labeled anti IgE antibody using a chemiluminescent reaction.

Over the last ten years, antigen arrays with several hundreds and even up to several thousands of immobilized antigens have been used for the detection of specific auto-antibodies involved in autoimmune diseases. Joos et al.\textsuperscript{57} used complex protein microarrays to detect up to 18 different rheumatic disease-specific auto-antibodies in human sera, achieving sensitivities and specificities that were similar to established ELISA methods. This concept was further developed by implementing peptide antigens on a microarray, which allowed a detailed characterization of the patient’s autoimmune status\textsuperscript{58}. Protein microarrays are perfectly suited for such types of analysis, especially when the quantity of sample material is limited. Sharp et al.\textsuperscript{59} suggested the application of auto-antibody profiling to improve diagnosis and prediction of disease onset and severity. Protein microarrays are valuable diagnostic tools for disease monitoring and therapy since new clinical information can be gained from comprehensive auto-antibody profiling, especially when combined with other clinical parameters, e.g. cytokine levels. These studies demonstrate the potential of antigen microarrays, which can compete with ELISA tests in terms of sensitivity and robustness. Antigen microarrays are excellent tools for these type of applications and can support health personnel in diagnosis and prognosis.
Present Investigations

3 Objectives

Protein microarrays made their way into research laboratories as valuable tools, generating a maximum output of data while requiring only a minimum amount of sample and reagent material. However, to become broadly accepted in IVD, further efforts have to be spent on improving fabrication methods of microarrays, automation of assays, reducing of assay time, performance and handling, data acquisition as well as improvement of robustness and reliability.

The content of the work, performed as a basis for this thesis, is briefly displayed in the form of some pictures, shown in Figure 8. The work has been focused on the development of improved technology and methods for planar as well as bead-based protein microarrays. First a new printing technique for fabrication of microarrays has been developed (paper III) that combines the benefits of contact and non-contact arraying. In the following paper (I), the focus is put on improvements of assay performance. Here, automation of an assay for planar protein microarrays is addressed and a new mixing technology is implemented to reduce the limitations, imposed by mixing based on diffusion. Furthermore, a novel assay set-up to extend the assays dynamics has been developed (paper II), by combining a direct and a competitive assay system in order to overcome the problem of limited measurement ranges in multi-analyte assays. Finally two alternative methods for protein microarrays are presented. Magnetic beads were utilized for detection on planar microarrays to achieve an accelerated read-out, while using a simple flatbed scanner (paper IV). Finally, an integrated bead-based microarray inside a capillary is described (Paper V), which reduces the required sample and reagent volumes to more than a 30-fold.
Figure 8  Pictorial summary of the original papers, presented in this thesis
4 Semi-contact arraying (paper III)

Microarrays represent highly miniaturized and parallelized solid-phase assay systems containing different test spots with up to several thousand spots per square centimeter (Figure 5). Typical spot sizes are below 250 µm in diameter which means that sample solutions less than 1 nL have to be reliably delivered. Technologies that had previously been established for DNA microarrays have been adapted for the generation of protein arrays. Microscopic glass slides are used as solid support and numerous surface chemistry strategies have been employed to firmly attach protein samples onto the surface. Besides delivery or dispensing of samples, on-chip synthesis has been performed for fabrication of DNA microarrays. Although the on-chip synthesis of peptides with protecting group chemistry is nowadays also feasible, this technique has only played a minor role for fabrication of protein microarrays since it cannot be used for the synthesis of proteins. Also, within the reverse phase array format, complex samples instead of a defined capture molecule are immobilized, which necessitates delivering these samples in one step. Typically, protein microarrays are therefore produced by using low-volume printing techniques. There are several instruments that enable the challenging liquid handling of sub-nanoliter droplets and the accurate positioning on a microscope glass slide. The terminologies, used for such instruments are: arrayers, spotters or microarray printers and depending on the modus operandi, the instruments can be divided into two categories: contact or non-contact arrayers. Contact arrayers touch the surface during the delivery of a sample solution. Usually, solid steel pins transfer tiny volumes of capture molecules onto a surface. Split-pin arrayers comprise a cavity inside the pin and therefore have a larger sample loading capacity, allowing the preparation of up to a few hundred spots in consecutive order, before running out of material. A modification of the solid pin-based arrayer is the pin and ring concept. Here, the sample is loaded onto a ring and for deposition, a solid pin passes through the ring and takes up sample solution. This process can be repeated for producing multiple spots.

For the production of protein arrays, complex polymer substrates such as nitrocellulose-coated slides, are often used to achieve high loading capacities of proteins and to keep these proteins in their native folding state. Thus there is always a risk that the fragile polymers are damaged by abrasion when sample is delivered by contact printing. Furthermore, since proteins are prone to adsorb onto metal surfaces, a random sticking of sample to the surface of the steel pins may give rise to cross-contamination. These problems accentuate the need for non-contact dispensing techniques for fabrication of protein microarrays. The central element of a non-contact arrayer is usually a glass or plastic capillary which can be more effectively washed than metal surfaces. In addition, the use of a capillary provides a
sample reservoir which is basically only limited by the length and diameter of the capillary. This means that many more spots can be generated before running out of sample, something which clearly improves the reproducibility of the spots. For non-contact arraying, small droplets are generated by means of high-speed solenoid, piezoelectric or pressure pulse-driven dispensing technologies, as also used in office ink-jet printers. For reasons discussed above, non-contact printers are most often preferred for the production of protein microarrays, but artifacts and problems such as misplaced spots, satellite spots, and other difficulties, leading to high failure rates are frequently observed. These problems are to a large extent caused by the accumulation of proteins or salts onto the printing head nozzles. To reduce such problems, the arrays procedure is usually conducted under humid conditions in order to reduce sample evaporation. Very challenging are highly viscous sample solutions that have low surface tension and high ionic strength. These properties may lead to a rapid change of the characteristics of the solution, resulting in irregularities in droplet formation during printing. Due to this exacerbated droplet formation at the dispenser nozzle, droplet deposition can be skewed, satellite drops can be generated or the formation of droplets could be completely inhibited.

In paper III, a pressure-based droplet delivery system is described to address the aforementioned problems. A droplet of sample solution is formed with the aid of a short pressure pulse at the distal end of a capillary in close proximity to the substrate. The solution forms a liquid bridge between the capillary and the substrate surface and after retraction of the capillary, the solution is subsequently deposited on the surface (Figure 9). This deposition can regarded upon as a semi-contact arraying mode since the capillary is connected to the surface by the liquid bridge formation but it does not touch the surface. As for contact arraying, the position of the spot depends only on the spatial precision of the robotic XY-movement.

![Figure 9](image) 

**Figure 9** A sample solution (human serum albumin, 200 mg/mL) is transferred to a glass slide using a capillary. The photo series shows the deposition of a droplet: elevated capillary, printing position (10 µm above the slide surface), droplet just touching the slide surface, droplet forming on the slide surface after liquid bridge snap-off, initial position with remaining sample now withdrawn from the capillary end (adapted from Paper III).
A 30 cm capillary with an inner diameter of either 20 or 50 µm was employed to aspirate the sample solutions from a source plate. The aspiration and dispensing of fluids was triggered by applying under- or over-pressure to the capillary by means of a computer-controlled valve. The droplet formation at the capillary end and the subsequent attachment of the droplet to the surface has also been described in mathematical terms. The Z-movement of the capillary was controlled by a linear translation stage whereas the horizontal movement of the microscope glass slide relative to the capillary was controlled by a XY-table. A Visual Basic.NET-based software was used to control the robotics, to set up the layout of the protein microarrays and to calibrate the position of the capillary outlet vis-à-vis the chip and the glass slide. The sample aspiration and deposition was carried out under a cover of a water-immiscible and volatile liquid fluorocarbon (FC) to eliminate adverse effects caused by evaporation of the aqueous sample solution. Before aspiration of the next sample, the content of the capillary was emptied and thoroughly flushed with water.

The robustness of the semi-contact arraying method has been evaluated by depositing samples which usually cause severe problems or are even impossible to print using current contact or non-contact arraying techniques. A highly concentrated solution of 200 mg/mL human serum albumin could be smoothly deposited (Figure 9) which was not at all possible when using a commercial non-contact arrayer (BioChip Arrayer, PerkinElmer). In addition, a high-salt cell-lysis buffer was printed. Interestingly, the formation of salt crystals was occasionally observed at the printing nozzle when printing was performed at ambient conditions. Here, the use of the protective FC environment prevented such incidences. A successful dispensing of pure dimethyl sulfoxide (DMSO) was possible, and this offers new possibilities since this also suggests the potential of arraying chemical compound libraries.

Finally, a suspension of 5 µm polystyrene beads was deposited on a surface, showing that the system, in principle, has the ability to handle particles or cells. This should be very useful, since it has been reported that contact printers may destroy cells and that standard non-contact arrayers are not able to dispense sedimenting particles.

In order to evaluate effects of the liquid FC on the activity of immobilized capture molecules, an immunoassay and a cell-adhesion assay were performed in presence of the fluorocarbon. For the immunoassay capture antibodies against CD-30, CD-117 and CD-130, proteins were printed using the FC or at standard ambient conditions in air. Human serum was incubated on the arrays and bound proteins were detected with a mixture of fluorescently labeled detection antibodies. No significant differences were observed between the results obtained with the two different printing conditions. In another test, four different cell-adhesion molecules (Collagen I and II, Fibronectin and Poly-L-Lysine) were printed on Nitrocellulose slides under FC and in air. Since these cell adhesion molecules are very sticky and tend to aggregate on surfaces, the failure rates during arraying are normally high. The semi-
contact arrayer was able to reliably deposit sample solutions that were 5- to 20-fold more concentrated than in the case, where established protocols for non-contact arraying are used. The functionality of the arrayed cell-adhesion molecules was demonstrated by seeding human embryonic kidney cells on the array. After 2 h, the adhesion of the cells was inspected by microscopy, which revealed that cells adhered very well to the spots regardless whether the printing was carried out under FC or in air. This suggests that the structure and the activity of the capture molecules is not affected by the liquid FC. This is a very important issue, but these results were not entirely unexpected. Perfluorinated fluorocarbons, in which all hydrogen atoms are substituted by fluorine atoms, exhibit an interesting lipophobic behavior. The interactions between the C-F groups of the FC and C-H groups of other molecules are rather weak. As a consequence, it is reasonable to assume that higher-order protein structures and associated biochemical activity are preserved in the presence of perfluorocarbons.

The liquid FC was easily removed after arraying, since it does not stick to the glass slide when it is removed from the printing stage. Moreover, the boiling point of the FC is only around 100 °C, thus any traces left on the slide will quickly evaporate. In addition, the FC dissolves only 13 ppm of water by weight, which results in a negligible loss of the deposited aqueous solutions. Whereas nanoliter sized droplets evaporate within seconds at ambient conditions, these can be stored under water-saturated FC for hours. Therefore it is possible to manipulate all the sample solutions under FC and to protect them effectively from evaporation and concentration changes. Besides deposited aqueous solutions, the FC can be used to protect the surface itself from influences of ambient conditions. For protein microarrays, moisture-sensitive surfaces are often used, such as N-hydroxysuccinimide (NHS)- or Epoxy-surfaces. These are frequently employed for covalent binding of the proteins to the surface, and hydrolyze in humid ambient air. In order to evaluate the efficiency of FC in protecting NHS-activated slides from moisture, three slides were stored under different conditions 72 h prior to printing. The first slide was kept dry, representing the maximal achievable binding capacity; the second slide was stored under FC and the third one was placed in a humid chamber. After storage, fluorescently labeled bovine serum albumin (BSA) was spotted on the different slides. The printing was also carried out on a negative control slide that had previously been deactivated with ethanolamine in order to ensure that the binding is not caused by unspecific adsorption. Then the slides were blocked with BSA and washed in order to remove unbound BSA and fluorescently labeled BSA. Finally the slides were scanned by a confocal microarray scanner. The results revealed that the slides did not lose activity when stored under FC, while only 60 % of the activity remained when the slide was stored in the humid chamber (Figure 10). In conclusion, the semi-contact arraying combines advantages of contact and non-contact arraying such as
high spatial accuracy or abrasion-free sample deposition. Applying samples in this way proved to be very robust enabling the deposition of samples which cannot be handled with common contact and non-contact printing. In addition it is possible to submit and to deposit the sample under a liquid FC in order to avoid evaporation of the sample or to protect moisture-sensitive surfaces from humidity.

Figure 10 NHS-ester preactivated microarray slides were initially stored in a dry environment, under liquid FC or in a humid chamber for 72 h. After storage, Cyanine 3 (Cy3) labeled BSA (200 µg/mL) was spotted on the slides (25 replicates). An ethanolamine-deactivated slide was included as negative control. After blocking and washing, the relative amount of bound BSA was measured with a confocal laser fluorescence scanner (adapted from Paper III76).
5 Microagitation and automation for planar protein microarrays (paper I)

Protein microarrays belong to the class of solid phase assays and as already discussed above, the reaction of a soluble analyte, and a capture molecule that is immobilized in the spot of a planar protein microarray is slowed down by diffusion. Kusnezow et al. found that this limitation can be diminished by mixing the sample solution during the reaction. However, since microarrays are of a small size, mechanical stirring or shaking is not trivial. If the whole slide, on which the microarray is printed, is incubated with a single sample solution, the incubation can be performed in a macroscopic container, such as a Petri dish or something similar. Here, the sample container and the slide can easily be put on a rocking shaker for mixing. But often an application is focused only on a small and clearly defined subset of analytes which need for instance to be investigated in a diagnostic test. In such a case it is desirable to have multiple identical arrays to analyze multiple samples in parallel on the same slide. The separation of the arrays on the slide can be realized by commercially available devices. A consequence of this compartmentalization is that the incubation volumes can be as small as a few microlitres. In such a small scale, mechanical stirring is very difficult to realize, and shaking is particularly ineffective when the small amounts of liquid adhere to the walls of the compartments.

Surface acoustic waves (SAWs) can be utilized to mix microlitre-scale volumes of liquids in a contactless manner. The SAWs are generated by a piezoelectric transformation of alternating voltage in a solid-state “mixer chip”. In our work (paper I), a mixing device with multiple mixer chips capable of mixing within twelve compartments on a slide, was employed. The SAWs are propagated from the mixer chip through the slide into the sample solution where efficient mixing is initiated. The mixing device was integrated into an automated system to process assays with planar protein microarrays. All liquid handling steps, including application of sample solutions and detection reagents as well as washing steps, were done by a pipetting robot (BioRobot 3000, Qiagen). The robot is capable of aspirating and dispensing liquids down to 10 µL and can move objects in the format of the microtiter plate. Therefore it was possible to cover the reaction compartments with a lid during the incubations in order to avoid evaporation. The slides were adjusted to the microtiter plate format by using adapters (ProPlate™ multi-array chambers, Grace Bio-Labs) in order to separate arrays and to generate distinct compartments, which were accessed by the robot. After automated processing of the assay, the adapters were dismounted from the slide to perform the readout in a fluorescent confocal microarray scanner.
Two assays were used to evaluate the influence of SAW mixing. In the first assay, seven different matrix metalloproteases (MMPs) were detected in sandwich type assays. An array of capture antibodies against these MMPs was incubated with a mixture of recombinant MMPs. After a washing step, bound MMPs were detected with a mixture of biotinylated detection antibodies and after another washing step, fluorescently labeled streptavidin was applied. The assay was performed with as well as without SAW mixing. The results (Figure 11) revealed that SAW mixing leads to an increase in signal for all reactions and a maximum amplification up to a factor of 4 was observed for MMP-1. MMP-3 was only detectable when the reaction was SAW-mixed. In addition, the intra assay variance was decreased; the general relative standard deviation was lowered by a factor of 3 and even more for MMP-1, -2, -7 and -8. The results obtained confirm that SAW mixing leads to diminished limitation by diffusion and to an improved intra assay variance.

![Graph showing comparison between SAW mixed and passive incubation for MMPs](image)

**Figure 11** Simultaneous detection of different recombinant MMPs in a mixture. Assays enhanced by microfluidic mixing are compared with passive incubation. Each column represents quadruplicates (adapted from Paper I).

In a second assay, the reactions of auto-antibodies towards immobilized auto-antigens were detected. The serum of a patient suffering from an autoimmune disease was incubated on the arrays and bound auto-antibodies were detected with fluorescently labeled anti-human IgG antibody. The impact of mixing on the assay performance was investigated by analyzing a time course and a dilution series for the incubation of the patient serum (Figure 12). The
reactivity against two auto-antigens, Ro 52 and Pm-Scl, is shown. All signals showed an amplification; the maximal amplification was more than a factor of 3. Analogously to MMP detection, the intra assay variance was decreased by up to a factor of 3.

Figure 12 Serum of an autoimmune patient was applied to protein microarrays containing Ro 52 kDa and Pm-Scl antigens to detect auto-antibodies. For a time course between 0 and 30 min, serum was diluted 1:400. In addition, serum was diluted from 1:400 to 1:6400 and incubated for 30 min. The bars represent standard deviations of 28 replicates and the columns show the ratio of SAW mixed vs. passive incubation and can be interpreted as an amplification factor (adapted from Paper I).

The results of the MMP- and the autoimmune-assay are in congruence with the results, obtained by Kusnezow et al. who observed a 4- to 5-fold amplification of signal in their immunoassay (q.v. chapter 1.5). In addition, results from dilution series with the autoimmune
assay suggest that the SAW mixing is more effective when analyte concentration is low (Figure 12). This confirms the observations of Nygren and Stenberg\textsuperscript{24} that for solid phase reactions, limitation by diffusion is more pronounced when the analyte concentration is low. Nygren et al. also observed that reactions are slowed down during the start of the assay. The results from our time-frame experiments indicate that the amplification by mixing is reduced when the serum was incubated for 32 min. According to Nygren and Stenberg, the amplification should be strong at short incubation times, which cannot be seen from the data, obtained from our experiments. However, short incubation times are difficult to control and minimal variations have great influence on the results. Nygren and Stenberg used real time measurements which provides a better temporal resolution than a series of distinct end point measurements. In summary an automated assay procedure for planar protein microarrays is presented and a stirrer-less mixing technology is implemented to diminish limitations by diffusion. The observed signal amplification by mixing is in accordance with earlier reported results in the literature.
6 Expanding assay dynamics by combining a direct and a competitive assay (paper II)

The fact, that concentrations of proteins in plasma span over more than 10 orders of magnitude\textsuperscript{30}, and the problems that arise for multiplex detection, were already discussed in chapter 1.7. The simultaneous detection and quantification of analytes in a single test is not feasible if the difference of concentrations is larger than the dynamic range of the immunoassays or the measurement range of the detector. Usually, a sample is diluted such, that the analytes of interest are covered by the dynamic ranges of each immunoassay in a multiplex test. Depending on the sample dilution, some analytes may be present at concentrations below the limit of detection or, on the other hand, be still so highly concentrated that the signal is saturated. In such a case, sets of dilutions of the sample are necessary in order to allow detection of analyte or its correct quantification within the linear range. Ohmura et al.\textsuperscript{85} presented a method to extend the dynamic range for the detection of an analyte by the use of two antibodies with the same specificity but with different affinities. In this way, they were able to generate a standard curve, covering 5 orders of magnitude. Theoretically, this dynamic range could be further extended by the use of even more antibodies with different affinities. However, the measurement range of the detection system has to be wide enough as well, in order to enable the detection within one run. Basically, in the same way, two antibodies with different affinities and different specificity can be employed to detect a high- and a low abundant analyte in the same assay. This approach would certainly diminish the concentration range problem of multiplex assays, but to solve this problem completely may be difficult, since it is probably not possible to find reliable binders exhibiting essential affinities.

In paper II, a method is presented which does not extend the dynamic range, but rather shifts the detection range of a certain analyte, and thus, the detection windows of several analytes in a multiplex assay set-up can be synchronized. This is accomplished by combining a non-competitive or direct assay with a competitive assay in a multiplex set-up. Thus, the detection range of the competitive assay can be shifted by the concentration of the employed competitor (Figure 13). The method was implemented in a planar microarray-based autoimmune assay to detect auto-antibodies against 13 auto-antigens and to measure the concentration of total IgG concentration in human serum. The serum was diluted 200-fold, a dilution which is commonly used for the detection of auto-antibodies in order to minimize false-positive results and to maintain an adequate assay response\textsuperscript{86}. The serum concentration of IgG is usually above 10 mg/mL (Figure 4). Thus, for the quantification of
human IgG in a sandwich type assay set-up, approximately a 100,000-fold dilution of serum would be necessary to match the linear range. In our work, a fluorescently labeled competitor, Cy3-human IgG, was added to the serum and then applied onto the microarray, which contained immobilized auto-antigens and anti-human IgG antibody (Figure 13). If auto-antibodies are present in the serum, then these will bind to the respective auto-antigen. Human IgG and the competitor are captured on the anti-human IgG antibody spots. Finally, fluorescently labeled detection antibody, Cyanine 5 (Cy5)-anti human IgG, is applied onto the array. This antibody is labeled with a different fluorophore than the fluorophore of the competitor. Therefore, in the first channel (Cy3), the signal of the competitor is recorded and in the second channel (Cy5), signals from bound auto-antibodies are measured.

![Diagram](image)

**Figure 13** (A) Serum of an autoimmune patient and fluorescently labeled (Cy3) competitor human IgG (green) are applied onto a planar microarray, containing different auto-antigens “Ag.” (B) Auto-antibodies bind specifically to the corresponding auto-antigens while human IgG and competitor are captured on anti-human IgG spots “YYY”. Then, anti-human IgG detection antibody (red), which is labeled with a second fluorophore (Cy5), is added. (C) Red fluorescence signal is generated on the antigen spots (Ag), whereas both red and green fluorescence signals are generated on the anti-human IgG spots (YYY). (D) Dose response curves: for the competitive detection of human IgG, the concentration of competitor can be adjusted to the human IgG concentration (green curves) while the detection of auto-antibodies is simultaneously performed in a direct assay set-up (red curve)( adapted from Paper II87).
Ten serum samples were analyzed for the presence of auto-antibodies and the concentration of IgG was determined (Figure 14). The patient sera had previously been analyzed for auto-antibodies against most of the auto-antigens by means of the established Varelisa assay (Phadia). The auto-antibody patterns were in good concordance, and additional auto-antibody reactivity was observed in the microarray experiments for some of the serum samples, which had been tested only for a subset of auto-antigens in the Varelisa assay. In addition, the titers of total human IgG were measured with the competitive assay. The results for the combination assay allows referencing of antibody titers to the total IgG concentration - an assay feature that provides extra clinical data to an auto-antibody profile. Hamelinck et al.\textsuperscript{88} showed that correcting antibody array expression data with IgM concentrations of samples is an effective normalization strategy. It is evident that it should be possible to employ any other protein instead of IgG (IgM) in a combined competitive microELISA.

![Figure 14](image-url)

\textbf{Figure 14}  \(\text{(A)}\) S1–S7 represent serum samples from patients with known autoimmune diseases; samples S8 –S10 are from healthy individuals. Serum samples were diluted 200-fold for analysis. \(\text{(B)}\) Total human IgG as measured with the combined competitive assay. TG, thyroglobulin; TPO, thyroid peroxidase; GAD, glutamic acid decarboxylase; CENP-B,
centromere antigen B; dsDNA, double-stranded DNA; La, Sjogren syndrome antigen B; RNP A, ribonucleoprotein A; Ro52, Sjogren syndrome antigen A 52; Ro60, Sjogren syndrome antigen A 60 (adapted from Paper I87).

As in the case of classical competitive assays, it is possible to shift the measurement range by varying the amount of added competitor. This feature was investigated with a dilution series of human IgG and different added amounts of competitor (Cy3-human IgG). Finally, bound IgG was detected with Cy5-anti human IgG and thus two signals were actually generated on the anti-human IgG spots. Figure 15 displays the results for the Cy5-signal, which reflects human IgG and competitor and the Cy3-signal of the competitor alone. In addition, the ratio of Cy5- and Cy3-signals of each spot was calculated. The Cy5-signal approaches saturation for IgG concentrations above 1 µg/mL. As already mentioned above, in a non-competitive assay the serum must be diluted 100,000-fold in order to bring the IgG concentration below this saturation level. The addition of the competitor pushes the detection range (Cy3-signal) towards higher concentrations and can be adjusted to the human IgG level. Thus, combining competitive and direct immunoassays creates a tool which allows the quantification of analytes that differ in concentration by several orders of magnitude.

A convenient side effect when normalizing the Cy3-signal for each spot with the Cy5-/Cy3-signal ratio is the back conversion from an inverted competitive calibration curve to a conventional ascending curve (Figure 15). In addition, a lower intra-array variation is obtained at the same time because in principle, two independent assay signals are used to calculate one concentration. Therefore, this calculation can be regarded upon as an internal spot-normalization strategy. Spot-to-spot variations caused by the unequal distribution of capture molecules in the microspots still represent a problem. Reproducibility problems often occur when the spot morphology is irregular and such problems can be caused by high protein concentrations, viscous buffers, and high salt or detergent concentrations. Inadequate blocking procedures and poor mixing of the reaction mixture also contribute to irregularities in signals generated from microarray spots23. Olle et al.89 presented an internal spot-normalizing strategy that uses an antibody directed against the capture molecule which generates a second signal. In our present study, the competitor signal depends on the amount of active (non-denatured) capture antibodies in the spot, and the sum of the signals from the competitor and the human IgG analyte is also dependent on active capture antibodies. Therefore, this type of normalization not only takes the presence of a capture molecule into account but also considers whether the capture molecule is still able to bind to an analyte molecule or not. The multiplex detection of autoantibodies with miniaturized immunoassays that give diagnostically relevant information are demanding, and although several interesting approaches have recently been developed90,91, standardization and validation are still required before such assays can be used in clinical diagnostics92.
Figure 15 The detection window was adjusted by incubating protein microarrays containing antihuman IgG antibody spots with calibrator solutions containing human IgG analyte and different concentrations of Cy3-labeled IgG competitor (concentrations in µg/mL indicated by the boxed values). The fluorescence measured in the Cy5 channel corresponds to the sandwich immunoassay, the fluorescence measured in the Cy3 channel represents the competitive immunoassay, and the Cy5/Cy3 ratio of signal intensities represents the combined competitive immunoassay. The values represent the mean and ratio of 14 replicate spots, and the vertical bars indicate the CVs of the replicates (adapted from Paper II).

In conclusion, we have presented a multiplex assay set-up that allows the non-competitive detection of auto-antibodies and the quantification of the total IgG level at approximately 3 orders of magnitude above the titers of single auto-antibodies. It should also be feasible to use more competitors than one simultaneously, where low abundant proteins are quantified by sensitive non-competitive sandwich assays while high abundant proteins can be quantified with sufficient sensitivity in a competitive way. The concentration of every competitor can be adjusted individually to the required detection range, considering the intrinsic lower sensitivity of competitive assay systems. It has already been shown by Barry et al. that such multiplexed competitive antibody arrays are capable of determining absolute protein concentrations instead of relative protein abundance.
7 Microfluidic assisted assay system

7.1 Magnetic bead-based detection for planar protein microarrays (paper IV)

Protein microarray technologies rely on immobilization of analyte molecules on a solid phase and the analytes are subsequently detected by labeled detection molecules. Zhu and Snyder\(^34\) have published an overview over the detection methods that have been used for protein microarrays so far, including chemiluminescence detection and radio isotope-labeled probes, which have been adopted from established immunoassays. Currently the most common detection methods are based on fluorescence detection, since this is very sensitive, safe, and simple. However, fluorescence detection requires special confocal microarray scanners that enable the discrete excitation and measuring of fluorophores at specific wavelengths. Therefore Lebrun and coworkers\(^95\) developed a colorimetric read-out system, used in standard ELISA on protein microarrays, which enabled a cost-efficient detection of several autoantibodies in patient sera using commercial flatbed scanners. Morozov and coworkers\(^96\) demonstrated the implementation of magnetic beads as detection media for protein microarrays in combination with on-chip electrophoresis. It was further shown for the identification of viral pathogens\(^97\) and in the genotyping of SNP variations\(^98\) that the use of magnetic beads can reduce assay time and simplify the readout from DNA microarrays. In conjunction with PCR-based target amplification, magnetic bead detection appeared to offer similar performance as compared to bead-based microarrays and electrophoresis techniques in terms of specificity and sensitivity\(^97\).

In paper IV, the use of magnetic beads is described to test human serum for reactivity towards 12 auto-antigens in 21 serum samples derived from patients suffering from autoimmune diseases and 3 samples from healthy donors. The serum samples are applied onto an auto-antigen microarray in order to detect the individual response patterns of disease specific auto-antibodies. After serum incubation, anti human-IgG antibodies labeled to magnetic beads (1 µm) are applied onto the microarray in order to detect bound auto-antibodies. This detection step was accelerated by use of magnetic force, directing the magnetic beads to the surface and performing detection within less than a minute (Figure 16 A). After binding of the magnetic beads, a magnetic force was also applied from above to remove unbound beads. Bound magnetic beads that are aggregated in a microspot can be observed by naked eye. Thus a simple commercial flatbed scanner can be used for an image acquisition and obtained images were analyzed with standard microarray analysis software.
(Figure 16 B & C). The magnetic bead-based detection set-up is compared to the assay performance of an established fluorescence-based detection protocol with particular emphasis on speed, sensitivity, and specificity. Until the detection was performed, the assay protocol of fluorescence-based detection was the same as for the magnetic bead-based detection procedure. Fluorescently labeled anti human-IgG antibodies are applied to the microarrays and incubated for 45 min. Finally, a confocal microarray scanner is used for image acquisition.

Relative fluorescent intensity signals obtained from patient sera were normalized to those signals, obtained from sera of healthy individuals. For instance, a signal of 20 represents a 20-fold higher signal in a patient serum than the corresponding average signal from healthy donors. First, the correlation of both detection methods was determined by plotting the normalized signals of all 21 autoimmune patient sera of fluorescence-based detection over those obtained from magnetic bead-based detection (Figure 17 A), which resulted in a reasonable correlation ($R^2 = 0.49$). The dynamic range was found to be larger for fluorescence-based detection (> 100) than for magnetic bead-based detection (< 40). This relatively small dynamic range can be explained by the maximum number of the few thousands of magnetic beads that can be immobilized in a microspot and the minimum number of roughly 100 beads that are necessary to obtain sufficient opacity for the detection.
by the flat bed scanner. For fluorescence-based detection, a threshold of 9 was introduced (horizontal line in Figure 17 A); normalized signals above that threshold were defined as positive autoimmune answers. In addition, appropriate thresholds were chosen for magnetic bead-based detection and used to assess the specificity and sensitivity based on the results from the fluorescence detection which served as reference. These thresholds are illustrated as vertical lines (Figure 17 A) and were used as classifiers for receiver-operating characteristics (ROC) analysis, described below. The upper right quadrant in Figure 17, which is defined by the horizontal and vertical lines, represents the signals that were positive in both detection methods which is also referred to as true positive rate or sensitivity. The specificity or true negative rate is represented by the lower left quadrant. In the remaining quadrants are those signals that are either falsely positive (lower right) or falsely negative (upper left) classified. Choosing a lower threshold for the magnetic bead-based detection improves the sensitivity; but at the same time, specificity is deteriorated, and the number of false positive classified signals increases.

![Figure 17](image)

(A) Signals of auto-antibody detection from patient sera using fluorescence-based detection were plotted against those obtained from magnetic bead-based detection. The vertical orange lines represent the indicated thresholds (classifiers) used for ROC analysis while the horizontal black line indicates a threshold that had been introduced to assign results as positive or negative for fluorescence-based detection. (B) ROC analysis. The true positive rate was plotted against the false positive rate. Three examples for sensitivity and specificity at different classifiers (1.5, 2.4 and 4.5) for magnetic bead-based detection are shown. Furthermore, the area under the curve was determined as a criterion for the discrimination of positive and negative signals (adapted from Paper IV).

In a ROC analysis, the area under the curve describes the probability that the magnetic bead-based detection will classify a randomly chosen positive test result higher than a
randomly chosen negative test result\textsuperscript{100}. The ROC analysis based on the dataset of fluorescence detection, revealed an area under the curve of 98\% (Figure 17 B), which indicates a high degree of concordance between the two detection methods. When a classifier of 1.5 was chosen, 98\% of all positive results were correctly classified as truly positive. In addition, 91\% of all negative results were correctly classified as truly negative. With a classifier value to 4.5, the sensitivity decreased to 81\% but the specificity improved to 99\%. The dichotomous comparison of both detection methods by the ROC analysis revealed a high concordance.

The detection of auto-antibodies in patient sera was one of the earliest applications of protein microarray assays\textsuperscript{57}. Besides clinical demands for multiplex detection in order to confirm the diagnosis of the type of rheumatic disease, the implementation of auto-antibody detection to microarrays was facilitated, since only one species-specific detection antibody is necessary. In addition, autoantibodies are present in sufficiently high concentrations and thus do not require ultra-sensitive detection techniques. For the identification of autoantibodies involved in autoimmune, but also allergy and infectious diseases, a semi-quantitative detection or even just detecting the presence of antibodies is often sufficient. An assay with limited dynamic range and sensitivity may therefore be sufficient for a qualitative analysis of the presence or absence of antibodies. In the magnetic bead-based detection method presented in paper IV, the sensitivity was not looked upon as a limiting factor. Basically the application of protein microarrays as diagnostic tools has a huge potential, but there is a need for additional technical solutions that simplify the procedure, decrease assay times, and reduce costs\textsuperscript{101}. The magnetic bead-based detection method addresses these demands. The overall assay time could be reduced by approximately one half and a simple flat bead scanner was used for the detection. This might encourage the use of protein microarrays in low resource environments where the costs of a confocal microarray scanner present a hurdle for microarray applications.

7.2 Microfluidic bead-based immunoassay (μFBI) with minimum amounts of sample (paper V)

7.2.1 Immunoassays in a capillary

Bead-based immunoassays are usually performed in a well of a microtiter plate. When using the Luminex xMAP technology, approximately 2000 beads are suspended in a typical volume of 100 \(\mu\)L. Assuming an equal distribution of the beads in the whole volume, the mean distance between two beads will be ca 400 \(\mu\)m. This means that an analyte molecule has to travel a distance of up to 200 \(\mu\)m to meet the closest binding partner that is immobilized on a
bead. Even mixing can not fully overcome this limitation. As already discussed in chapter 1.5, solid phase assays are mass transport-limited, even when the reaction mixture is stirred.

In paper V, a microfluidic system is described that allows performing a bead-based immunoassay in a capillary. Here, the beads are arrested inside the capillary by means of a filter, and analyte-containing sample solution is pumped through the capillary, thus passing the beads (Figure 18). The inner diameter of the capillary was 100 µm. Assuming that there are 1000 beads per assay, and assuming an evenly and densely distributed package of the beads (Luminex beads, 5.6 µm in diameter) on the filter, then 3 layers of approximately 300 beads per layer can be formed. In such a bed of beads, the immobilized capture molecules are in very close vicinity to each other, and consequently, each soluble analyte molecule passes potential binding partners at very short distances. Compared to the scenario of a bead-based assay in a microtiter plate as described above, the medium distance between an analyte molecule and an immobilized capture molecule is approximately two orders of magnitude lower when the assay is performed in the capillary. The flow rate in the capillary was around 0.1 µL/min which corresponds to a flow speed of 200 µm/s, thus providing a quasi-constant analyte concentration. Therefore in such an experimental setup, the reaction rate should be at maximum, since analyte concentration is always at its highest and diffusion distances are very short. As reported in the literature, similar experimental setups have been discussed and the terminology “specific interface” has been used, which is defined by the ratio of reaction surface to the volume of the reaction compartment.

![Figure 18](image_url)  
**Figure 18**  
Schematic illustration of a µFBl. The assay is performed inside a capillary by controlling solution flow in or out with a syringe pump. A filter is used to keep the antibody-coated beads at the site to capture analytes and reagents for detection. After incubation, the beads are pumped out of the capillary and transferred to a Luminex-100 instrument for a fluorescent signal readout (adapted from Paper V).
Hayes et al.\textsuperscript{103} described a bead-based assay in a capillary for the detection of fluorescein isothiocyanate (FITC). Anti-FITC antibodies were immobilized on magnetic beads which were retained in a capillary (50 µm) by magnetic force. When a FITC solution was pumped through the capillary the fluorophores were captured by the antibodies on the beads and finally, the fluorescence of bound FITC was measured by a charge-coupled device (CCD) camera. Kinetic studies with a FITC concentration of 50 µg/mL revealed that 90% of the maximum obtainable signal was reached already after 3 min. The limit of detection was approximately 5 µg/mL. Moreover, in a second assay setup, Hayes et al. detected IL-5 in a sandwich-type assay. Here, they incubated both IL-5 solution and a secondary detection antibody for 30 min and observed a limit of detection of about 10 ng/mL.

However, it remains unclear how such a capillary flow system behaves when biological samples are utilized. Such samples are very complex and often contain insoluble particles that might lead to clogging of microfluidic devices. In addition, it is often observed that sensitivity of analyte detection when dealing with complex sample solutions is lower than in a well-defined matrix, that contains only the analyte dissolved in a physiological buffer. In complex samples, the analyte represents only a tiny fraction and might be shielded by other components. Moreover, such components may contribute to unspecific interactions with the detection system and contribute to an elevated background levels. Finally, the method as presented by Hayes et al. can not be applied to multiplex detection.

Our microfluidic bead-based immunoassay was used for performing multiplexed sandwich immunoassays in a capillary. The set-up allowed the parallel detection of several parameters. The immunoassay was used to analyze the expression of seven receptor tyrosine kinases (RTKs) and their degree in tyrosine phosphorylation in breast cancer tissue lysates and in normal tissue lysates. Comparison of this microfluidic immunoassay with a standard bead-based assay revealed the same protein expression data, while a reduction of sample material by a factor of 100 and a reduction of reagents by a factor of 30 was achieved with the capillary method.

### 7.2.2 Capillary-based immunoassay for detection of receptor tyrosine kinases

The early diagnosis of cancer, or to be more precise, the detection of molecular events in the manifestation of a tumor, plays a key role in strategies for a successful treatment of cancer. However the small tumor sizes at early stages of cancer is a problem since only limited amount of material can be obtained by means of fine needle biopsies or microdissected tissue sections\textsuperscript{104-106}. The identification of the changes of protein expression in such a small sample is particularly challenging, because only a limited number of assays can be performed using conventional approaches. It is obvious that the full potential of genomic and
proteomic technologies will only be realized if these can be applied to minute amounts of biological material\textsuperscript{107-109}.

In paper V, a µFBI for multiplexed detection of proteins by using a capillary to handle minute amounts of liquids, is presented (Figure 18). By running the assay in the capillary, only 200 ng of tissue lysate, present in 1 µl sample volume, is required. In addition, only 1 µl detection antibody solution and 1 µl of reporter molecule streptavidin-phycoerythrin (S-PE) are needed for detection of bound analytes. Compared to a standard bead-based assay, which is run in a microtiter plate, this means a 100-fold reduction of sample material and a 30-fold reduction of detection reagents. In the described setup, two capillaries could be utilized in parallel by employing a syringe pump. After the incubations were performed, the beads were pumped out of the capillaries into a microtiter plate and transferred to a Luminex-100 instrument for readout. The performance of the µFBI is demonstrated by analyzing the expression of seven RTKs in lysates from breast cancer tissue and normal tissue within a single assay. RTKs play a key role in signal transduction cascades and are responsible for directing an external signal into a cell causing a cellular response\textsuperscript{110,111}. Once activated by external ligands, RTKs undergo autophosphorylation and trigger pathways that lead to expression of genes. Bead-based immunoassays are perfectly suited to profile such proteins in a multiplex way in terms of their abundance and phosphorylation in tissues or cell cultures\textsuperscript{112,113}.

![Graphs showing total protein and phosphotyrosine-specific expression of seven RTKs](image-url)

**Figure 19** Total protein expression (left) and phosphotyrosine-specific expression (right) of seven RTKs in normal (grey) and breast cancer (blue) tissue as mean fluorescence

\[\text{MFI [AU]}\]
intensity (MFI). For the µFBI, (upper set of graphs) 1000 beads per analyte were incubated with 1 µL lyzed tissue sample, 1 µL of detection antibodies mix and 1 µL of S-PE. For the "normal" assay (the two lower graphs) the amounts were 2000 beads, 100 µL sample, 30 µL antibody mixture and 30 µL S-PE. Concentrations and incubation times were the same for both assays. The read-out was done with a Luminex-100 instrument (adapted from Paper V).

A WideScreen™ RTK Assay Kit (Merck) for the expression analysis of seven RTKs was used to evaluate the µFBI concept. The RTK panel includes epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1R), hepatocyte growth factor receptor (HGFR), platelet-derived growth factor receptor beta (PDGFRβ), human epidermal growth factor receptor 2 (HER-2), vascular endothelial growth factor receptor 2 (VEGFR2) and tyrosine kinase with immunoglobulin and EGF repeats 2 (Tie-2). The capture antibody-coated beads were incubated for 60 min. with the tissue lysates, another 60 min. with biotinylated detection antibody mixture and finally 45 min. with the reporter molecule S-PE (1 µL each). In parallel, a standard bead-based assay was performed according to the manufacturer’s instruction. Signal intensities representing the RTK concentrations and their degree in Tyrosine phosphorylation correlated very well between the µFBI and the standard bead-based assay (Figure 19). Both methods revealed that the total amount of HER-2 and its degree of Tyrosine phosphorylation were significantly higher in breast cancer tissue compared to normal tissue. However, the µFBI only required 200 ng of tissue lysates, compared to the 20 µg of tissue lysates used in the standard bead-based assay. For the detection antibody mixture and the reporter solution S-PE, the µFBI required 30-fold less material accordingly.

In summary the presented µFBI can be carried out in a very small and defined zone whilst requiring only tiny amounts of sample material and detection reagents. Therefore, these devices are valuable tools when the sample material is limited. In addition, only very small quantities of precious detection reagents are used, which leads to substantial savings, since antibodies and fluorophores contribute significantly to the overall cost of a diagnostic test. It should be possible to increase the throughput of the µFBI technique by using a larger number (e.g. 96) capillaries in parallel, as described recently for a CE based separation of proteins. Moreover, the microfluidic setup of the µFBI approach could be combined with an appropriate flow cytometer, as demonstrated by Kim et al., to obtain an integrated read-out system.
Future Developments

Protein microarrays are now well-established analytical tools in basic and applied research, which is a sign of the capabilities and power of these assay systems. Prior to the implementation in routine clinical diagnostics, protein microarray-based results have to demonstrate clinical relevance in the initiation or changing of therapy. Medical demand, combined with an overall cost reduction, must become the driving force behind protein microarrays in order to be able to gain a substantial share of the IVD market. Although protein microarrays have a huge diagnostic potential, these systems are still far from being generally used in IVD. Regulatory hurdles have to be cleared and a variety of problems have to be solved prior to a broad application of multiplexed protein microarrays on the IVD market.

To ensure the necessary high quality of microarrays, much more efforts have to be made than with simplex measurements. Several quality control measures have already been taken in protein microarrays, including replicate spots, marker spots for orientation, negative control spots to check for unspecific binding, application of assay buffer to test for cross reactivity between capture molecules and detection antibodies, internal spot normalization and different normalization strategies. However, quality control in terms of IVD testing necessitates a routine analysis of reference sample material of low, medium and high concentration. Only this will ensure that the test is effective at any analyte concentration and is able to detect an assay drift. At present, no information exists on the required complexity of such a reference sample and how stability can be guaranteed. The generation of defined mixtures of control analytes and the solubility of these molecules at high concentrations might cause problems that have not yet been investigated in detail. Putting such controls in place will make it easier for the assay to be used for clinical analysis. However, additional issues will have to be addressed, for example the interpretation of assay results in cases where some of the controls fail. Can we regard only partially correct results gained from a microarray with valid controls as sufficiently reliable or do these results have to be rejected as well? Another important issue is multiple subarrays within one slide. What happens if one of the sub-arrays fails? How can one tell whether the whole array is affected? How should failures within replicate spots be dealt with? Replicate spots are a compromise arising from the limited space within an array. Roche’s new microarray platform, IMPACT, employs up to 20 replicate spots and bead-based systems analyze from fifty to one hundred beads. In the case of a multiplexed diagnostic assay, where dozens of individual parameters are measured, and where a few or even just one single test is changed, will this require a whole new approval for IVD?
In addition, multiplexed assays generate huge data sets, which require appropriate data analysis. The question will be how to draw conclusions from patterns rather than looking at single interactions. Attempts have been made to provide the necessary information for inter-platform and inter-laboratory concordance with DNA microarray experiments. The BioPlex 2200 ANA (antinuclear antibody) Screen system uses a pattern recognition algorithm for the analysis of their multiplexed protein assays. However, there is no general approach on how to apply such algorithms to generate diagnostically relevant data sets.

Besides the regulatory aspects, social and ethical issues also need to be considered. Should a customer be allowed to order only part of the data generated with a multiplexed assay and therefore pay less than the price of the full multiplexed panel? Can the manufacturer or the performer sell only parts of the microarray results for example by using different software settings? What happens with the "non-used" (non-requested) data sets? If the non-requested data sets are of diagnostic relevance, would this lead to a different diagnosis? All these questions have to be carefully addressed and solved by the regulatory offices and by the diagnostic companies before protein microarrays can be placed on the IVD market.

Protein microarray-based assays will have to be further automated before entering the IVD market. Automation increases assay performance, robustness and reliability. However, such automated multiplex platforms have to compete with the well-established clinical analyzers that currently dominate the diagnostic market. These systems can easily increase their throughput, e.g. by measuring five parameters from the same sample in a sequential mode. Therefore, as long as sample material is not limited, or multiplexing does not exceed more than five parameters, the diagnostic companies are hesitant to make huge investments for changing the assay format. However, in the field of autoimmune disease diagnostics, multiplexed assays are currently entering the diagnostic market, and sets of tumor marker panels may in the future also be applied to monitor therapy. It can therefore be safely assumed that protein microarrays will find their place in the IVD market in areas where sample volume is limited and where these systems can deliver therapeutically relevant data sets.
Acknowledgements

First of all I would like to express my utmost gratitude to Thomas and Johan who gave me the opportunity to do this work. Over all the years Thomas gave me a lot of advice and assistance also beyond the daily work of a doctoral student. Johan affiliated me in an uncomplicated way in his department at KTH and assisted me on my way to this dissertation. I would like to thank you both for a great supervision!

I thank Markus and Dieter for endless discussions and ideas that contributed substantially to the publications that served as a basis for this work. Joe, thanks for all the years at the NMI, for great support and accommodation whenever I popped up in Stockholm; it’s been always a great pleasure! Hi Olli, thanks a lot for accompanying me so long; for great discussions; U-boat-projects etc. Hello Berthold, solid rock in the lab! Thanks for being there, fatherly advice and discussing the real problems. Pope and Krane, thanks for your support and the great times at the NMI. Xiaobo, it was quite cool to set up the µFBI with you and thanks for a great job. I would like to thank the girls at the NMI, Nicole, Anette, Moni, Conni and Sarah for your great support through all the years. Another thanks goes to Ira, Helga, Frau Franck, Maggi, Frau Roscher, Nadja, Claudia and Matthias for making me feel at home at the NMI. It was really a pleasure to work with you all the years at the NMI.

Thanks a lot, Jesper, for doing our joint project, for great discussions and a successful work. I would like to thank Johan S. and Marten for great assistance in the arrayer project. Thanks for welcoming me at your place.

Meinen Eltern, Danke für eure großartige Unterstützung, ohne die meine Studien und diese Arbeit nicht möglich gewesen wären. Danke.

Ich danke meiner Tochter, die ohne es zu wissen, mich so unheimlich motiviert hat. Ich danke meiner Frau, für ihre unermüdliche Geduld. Es ist schön, dass es euch gibt.
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