Microarray Technology as a Universal Tool for High-Throughput Analysis of Biological Systems

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Abstract: Over the last years microarray technology has become one of the principal platform technologies for the high-throughput analysis of biological systems. Starting with the construction of first DNA microarrays in the 1990s, microarray technology has flourished in the last years and many different new formats have been developed. Peptide and protein microarrays are now applied for the elucidation of interaction partners, modification sites and enzyme substrates. Antibody microarrays are envisaged to be of high importance for the high-throughput determination of protein abundances in translational profiling approaches. First cell microarrays have been constructed to transform microarray technology from an *in vitro* technology to an *in vivo* functional analysis tool. All of these approaches share a common prerequisite: the solid support on which they are generated. The demands on this solid support are thereby as manifold as the applications themselves. This review is aimed to display the recent developments in surface chemistry and derivatization, and to summarize the latest developments in the different application areas of microarray technology.

Keywords: DNA microarray, protein microarray, antibody microarray, peptide microarray, cell microarray, reverse transfection, surface chemistry.

INTRODUCTION

During the last decades a progressive industrialization has changed the working principles of bio-medicinal research. Similar to the economical industrialization in the 19th century, automation and highly-parallel processing led to an increase in speed and throughput. One of the key technologies of this development, that arose as a versatile platform for a multitude of applications, is microarray technology. Due to its high flexibility and boosted by large-scale sequencing projects, microarray technology has evolved as a propitious platform for the high-throughput analysis of DNA, proteins and cells.

As one of the first applications, DNA microarray technology has already become an indispensable tool in applications such as gene expression profiling or SNP genotyping projects. In parallel, the use of microarray technology for the investigation of other molecules apart from DNA gained importance. Beside peptide microarrays, the applicability of protein and antibody microarrays has been shown, and other technologies, such as cell microarrays are rapidly gaining importance. All microarray technologies can thereby be closely linked in a consecutive workflow. While DNA microarrays are used as a first step for the identification of genes, which show a differential transcription, antibody microarrays can be applied as a second step for the validation of differential translational levels. The identified proteins can then be characterized with regard to interaction and modifications using protein microarrays, and binding epitopes and modifications sites can be mapped by peptide microarrays. The last step in the workflow is then the *in vivo* confirmation and functional evaluation of the *in vitro* obtained results on cell arrays.

All of the presented technologies share a common prerequisite, which is crucial for a successful application: A microarray support that accommodates the different molecules in a way that they display maximum functionality with minimal background binding and optimal signal-to-noise ratios. This review is aimed to highlight the latest developments of microarray supports and applications, and to point out crucial bottlenecks and future directions.

DEVELOPMENTS IN MICROARRAY SLIDE COATINGS

The immobilization of biomolecules requires a chemical functionalization of the surface in order to facilitate a stable attachment, to provide a suitable environment and a favorable presentation of the molecules for binding experiments. There are many different methods described in the literature on how to construct such a surface by applying different strategies of chemical surface modification. The resulting molecular architecture on the surface can be illustrated by means of a building block system as shown schematically in Fig. **1**.

The chemical surface coating can generally be divided into 3 main building blocks. The binding moiety can either consist of a silane for glass surfaces, of thiols for

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immobilization on gold surfaces [1], or of polyelectrolytes [2], including poly-L-lysine (PLL), which binds to glass and dielectric materials such as the oxides of titanium, tantalum, and niobium [3, 4]. The linker moiety can vary from a simple construction as a propyl chain in case of γ-aminopropylsilane, to an extended polyethylene glycol (PEG) or a complex 3D polymeric network of a hydrogel. The molecular structure of the linker and the nature of the reactive group mainly determine slide properties with respect to details of probe immobilization, such as the density of immobilized probes, their molecular arrangement (end-tethered immobilization or *via* multiple attachment sites), and the accessibility by the target [5-9]. Moreover, surface chemistry and molecular architecture may determine the chemical stability of the immobilized probe, the degree of denaturation and unspecific adsorption of probe and target. Spot morphology, i.e. the distribution of spotted probe on the surface, depends on surface chemistry as well. It is determined by the combination of interfacial free surface energy, which is a measure of the wettability, and surface tension of the spotting solution, among other factors such as probe concentration [10].

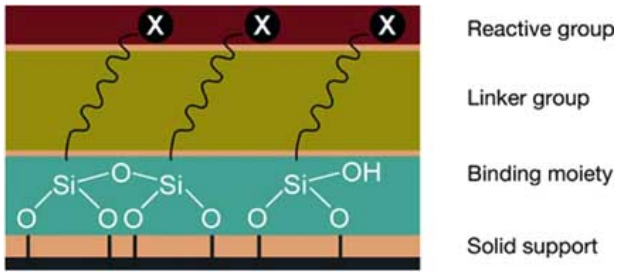

Fig. (1). Building block system illustrating schematically the structure of compounds used for coating.

Molecular Architecture

The molecular architecture of the most common types of slides is schematically shown in Fig. **2**.

A stable attachment of probe molecules is usually achieved by forming covalent bonds to the surface [11, 12]. Reactive groups for bond formation can be attached at the end of (Fig. **2A**, **B**, **D**) or distributed over the molecular scaffold of the coating (Fig. **2C**). Due to chemical crosslinking, polymers can form 3-dimensional networks such as hydrogels and membranes. Some hydrogels, nylon and nitrocellulose membranes used on microarray slide surfaces do not possess reactive groups and allow binding of large molecules *via* physical adsorption. For immobilization *via* complex formation, the nickel complex of nitrilotriacetic acid derivatives and biotin derivatized surfaces are used, reacting with 6mer histidine or avidin/streptavidin modified probes. In addition, gold surfaces can be used to directly immobilize thiol containing probe molecules without any chemical surface modification [9], permitting e.g. the attachment of 5`- thiotriphosphate modified RNA [13].

Functional groups used on commercially available slides can be electrophilic, such as aldehyde, epoxy, iso(thio) cyanate, N-hydroxysuccinimide ester (NHS), or nucleophilic, such as semicarbazide and amino. Electrophilic groups are highly reactive to amino, thiol, and hydroxy groups and can be used for a covalent immobilization of a large variety of classes of compounds including peptides, proteins, and oligonucleotides under mild coupling conditions. However, due to the reactivity of these functional groups, slide surfaces are susceptible to decay processes caused by humidity and oxygen, which is crucial when some thousand samples are to be printed. For example, the amount of oligonucleotides which bind to an epoxysilane slide is reduced by a factor of about two when the slide is kept at room temperature and 50% humidity for 24 hours. NHS ester activated surfaces are more susceptible to humidity leading to a considerable deactivation even by storing under optimal conditions [14].

New reactive groups were recently introduced for covalent immobilization of small molecules and proteins on microarray surfaces. Those include a protected isocyanate group [15], diazobenzylidene [16], azidobenzoate attached to poly-L-lysine [17], diazirin derivatives [18, 19], and azide [20].

Microarray slide types can be divided according to the molecular architecture of the coating (Fig. **2**). The most simple molecular architecture consists of a silane presenting a reactive group *via* a short linker (Fig. **2A**). Aminosilane and 3-glycidoxypropyl (epoxy) silane coated slides are widely used and work reliable especially for DNA applications. Standard protocols often recommend the immobilization of cDNA by UV crosslinking or "baking" on both type of slides. However, these procedures lead to uncontrolled binding of the DNA *via* radical reactions

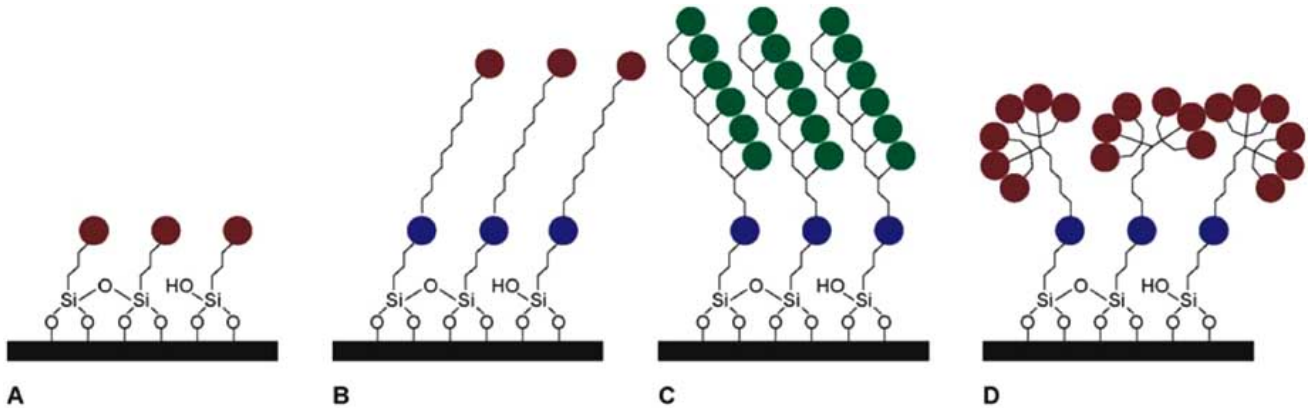

Fig. (2). Molecular architecture of chemical surface coating.

initiated by the UV light [21] and various side reactions at elevated temperatures. The shorter the length of immobilized DNA the more these effects need to be considered, since they may be of consequence for the hybridization. Our own experiments have shown that the reactivity of surface epoxy groups is sufficient for a covalent immobilization of longer oligonucleotides. No difference in hybridization signal intensity was observed between a non-modified and an aminohexyl-modified oligonucleotide of identical sequence when slides were kept in a humidity chamber at room temperature for immobilization.

New applications of slides of type **2A** include the use of aminopropyl silane coated slides for the manufacturing of membrane microarrays and their use-directly or after chemical modification with a chemolabile linker-as starting point for the preparation of microarrays for cell transfection with small molecules and peptides [22], cDNA [23], and plasmids [24].

Microarray coatings of type **2B** can be prepared using a variety of chemically different linker systems including glutaraldehyde ("aldehyde slides"), phenylendiisothio-cyanate, PEGs of different molar mass, or various heterobifunctional crosslinker [7, 22, 25-31]. To date, only a few of these slide surfaces are commercially available. Slides of type **2 B-D** are recommended for immobilization of small molecules such as short peptides, oligonucleotides, monosaccharides and other low molecular weight compounds [32], due to various distance dependant surface effects. First, there is an effect of decreased diffusion rate constant near surfaces leading to reduced rates of molecular recognition [33, 34]. Second, surface charges cause changes in properties and reactivity of immobilized molecules, leading, for example, to substantial differences in hybridization thermodynamics [35-37]. Therefore, an extended linker is indispensable in order to move the immobilized probe molecule away from the proximity of the surface to avoid these effects [5, 38-41].

On gold surfaces, self-assisted monolayers can be prepared using linker systems of type **2B**. Mrksich *et al.* presented monolayers composed of an alkanethiolate-PEG linker terminated by different coupling groups such as sulfonamide, hydroquinone, quinone, or maleimide [42]. This very flexible system was used for binding studies and to detect enzyme activity. A system presenting an acyl derivatized hydroquinone as terminal group was shown to react with cutinase leading to deacylation. After reduction to the quinone, the product was detected by MALDI-TOF mass spectrometry [43]. In a different approach, a mixture of 4 peptides was immobilized on a maleimide-terminated monolayer. The authors were able to show that the transfer of a phosphate group to kinase-specific peptide substrates with c-Src kinase can be detected by MALDI-TOF MS semi-quantitatively.

Microarray slides with a sophisticated molecular architecture of type **2 B-D** are widely used for protein and antibody as well as for small molecule applications [16, 18, 27, 29, 44, 45]. Coatings based on polymeric materials are distinguished according to their molecular structure and the number of reactive groups, which can be one, many, or no binding group(s) per polymer chain. Polymer coatings of type 2C and 2D can be chemically crosslinked resulting in a 3D network (structure not shown in Fig. **2**) [46-48]. Hydrogels based on polyacrylic acid derivatives and other gel forming compounds such as dextranes and cellulose derivatives contain a large amount of water thus providing a convenient environment with solution-like properties preserving protein function [19, 49, 50]. Moreover, the extended 3D network allows for efficient immobilization of a higher number of probe molecules compared to planar 2D surfaces [17]. Other polymer based coating materials used for protein applications include membranes made of nylon [51, 52] and nitrocellulose [53], agarose [54, 55] and polyethylene glycols. PEGs are widely used coating materials especially for surfaces of biomedical devices that resist bioadhesion [27, 30, 56]. Due to its favorable chemical and protein repelling properties, PEGs are ideal materials for the production of protein microarray slide surfaces acting as linker (Fig. **2B**) [27, 40] or as crosslinker for polymers (Fig. **2C**) [57].

Poly-L-lysine coated slides cannot be classified in one of the groups in scheme 2. PLL commonly used for microarray applications is of high molecular weight (150-300 kDa) and does not bind to the surface with the full length of its backbone. This results in a 3-dimensional architecture which supports the immobilization of probe molecules, e.g. cDNA. In contrast to the covalently bound silanes, PLL is attached to the surface by electrostatic interactions and shows a reduced stability at high ionic strength ("high salt" conditions). PLL provides a very flexible substrate for many different applications including classical cDNA applications, as well as the immobilization of oligonucleotides [58], peptides and proteins [59, 60], antigens [61], and antibodies [62, 63]. PLL slides were successfully applied as a solid support of a siRNA microarray used for cell transfection [64]. The lysine ε-amino group can chemically be modified with a linker reversing its reactivity [17]. However, insufficient stability, structural changes and aging effects [58] restrict the usefulness of PLL slides [65]. Recently, three-dimensional multilayer polyelectrolyte thin films were used as coating material [2]. The authors report on favorable properties such as an increased amount of immobilized oligonucleotide compared to conventional amino silane and aldehyde slides, a wider dynamic range, low detection limit, and a cost-effective production.

New methods of protein microarray production include a sol-gel based process [66], the spotting of a protein in a mixture with a photolinker polymer followed by immobilization *via* a UV light induced carbene reaction [19], and the preparation of a 3D matrix using a monomer with anchored protein [67]. Acrylic acid modified proteins mixed with a suitable co-monomer and a crosslinker were subjected to a radical polymerization by Mirzabekov *et al.* The reaction was performed by spotting the mixture to a silane modified surface and subsequent polymerization under UV light irradiation resulting in a gel with the protein immobilized. The properties of the resulting material and the amount of immobilized protein can be controlled by adjusting the ratio of monomers and crosslinker. This technique was also applied to immobilize DNA in a hydrogel [48]. A filtration-based microarray technique using nylon filter membranes as support material for the detection of low abundance target protein was described by Xu and Bao [68]. The incubation solution is filtered through a stack of 14

membranes thereby accelerating the binding of carcinoembryonic antigen in human plasma samples by a factor of about 10 compared with a classical microarray experiment. The authors state that this technique has the potential to be extended to other types of microarrays.

Despite the large variety of surfaces prepared to date, protein arrays are still limited in their performance. The optimal slide surface would allow proteins to be immobilized without changes in their properties retaining full activity. The best way to immobilize proteins is *via* a specific and site-directed coupling to the surface with control of orientation to avoid a blocking of the binding site. The most straightforward method consists of immobilization of the unmodified protein by adsorption or by reaction of available protein groups with corresponding electrophilic groups on the slide surface. Both methods result in a sufficiently strong binding, however, without control of the orientation of the protein active site on the surface and running the risk of a high degree of denaturation [69].

Immobilization Strategies for Different Probes

Beside the design of optimal surface coatings, the properties of the probe molecules are of high importance. Probe molecules with more than one reactive group such as peptides and saccharides may attach at multiple sites at the surface. Therefore, a carefully optimized immobilization strategy is needed. Site-specific immobilization strategies were developed in order to avoid a blocking of binding sites resulting in a loss of activity of the probe molecules. Synthetic peptides can be modified with a variety of binding moieties such as maleimide [70], a cyclopentadien conjugate [71], N-terminal cystein [72, 73], biotin [74, 75], thiol [76], and by the oxidation of N-terminal serine and threonine [74, 75] yielding a glyoxylyl group [77, 78]. These groups react with surfaces presenting thiol (maleimide, bromoacetyl), quinones (cyclopentadien), hydrazides (oxidized serine and threonine), reactive α-oxo-semicarbazone groups, cysteine (glyoxylyl), and streptavidin or neutravidin (biotin). In a different approach, Lee described the expression of peptides with a protein tag [39]. The protein is used for immobilization by adsorption, thereby acting as a linker to gain distance from the slide surface.

Due to their properties, membrane proteins must be spotted with their associated lipid phase environment. Vogel *et al.* were the first who immobilized a G protein coupled receptor in a supported lipid bilayer at a surface coated with a mixed self-assisted monolayer consisting of a biotinylated thiol and ω-hydroxy-undecanethiol [79, 80]. Streptavidin was used to bind biotinylated bovine rhodopsin receptor in a uniform orientation to the surface by homogeneous immobilization and microcontact printing. The authors were able to follow ligand binding, G protein activation, and receptor deactivation by surface plasmon resonance (SPR). Fang *et al.* investigated the structure and properties of a G protein-coupled receptor embedded in a supported lipid bilayer on a microarray slide surface [81, 82]. Lipid microspots of a mixture of vesicular solutions of lecitins and the membrane protein are stable on an aminopropyl silane coated surface. The slides were used in a ligand array to detect the binding of neurotensin by the immobilized neurotensin receptor. Karlsson *et al.* immobilized successfully a G protein-coupled receptor on a modified amphiphilic carboxylated dextrane hydrogel surface activated with NHS [50]. The experiments were performed in an SPR flow system. After immobilization of rhodopsin at the hydrogel, a mixed micelle containing detergent and lipid was used to form a lipid bilayer composed of both components. After extraction of the detergent, a lipid bilayer was formed, which reconstituted the function of the membrane protein that was investigated using surface plasmon resonance spectroscopy. Ataka *et al.* immobilized recombinant cytochrome c oxidase mixed with a detergent *via* a His-tag to a nitrilotriacetic acid modified gold surface [29]. A lipid bilayer was formed with detergent destabilized liposomes after removing the detergent using macroporous bio-beads. All reactions on the surface were followed by surface-enhanced infrared absorption spectroscopy. Electron transfer from cytochrome c to the oxidase was detected by cyclic voltammetry demonstrating the functionality of the preparation.

Peluso *et al.* compared antibody immobilization strategies on streptavidin and PLL-PEG coated surfaces with regard to surface density and binding activity [83]. Full size antibodies and Fab` fragments were randomly biotinylated, modified with biotin on carbohydrate of the Fc domain and, after mild oxidation of the antibodies, in the hinge region. SPR measurements confirmed that oriented antibodies and Fab` fragments are more densely packed and also show a up to ten times higher specific activity. A comparison of different antibody immobilization methods including DNA-directed immobilization of DNA-antibody conjugates, direct spotting to a 3D dendrimer coated slide surface, or attachment *via* streptavidin-biotin has recently been published by the group of Niemeyer [84].

Different approaches were developed in order to subsequently modify proteins and antibodies with a reactive group for specific binding to the slide surface [85-87]. Standard methods use protein surface groups, such as lysine, cysteins and (reduced) cystines [88], and (oxidized) carbohydrates [89, 90] for either a directed immobilization at the surface or to attach a linker group including heterobifunctional linker [91], acrylic acid derivatives [67], biotin [74], and phenylboronic acid [92]. These reactive groups bind to surfaces activated with electrophiles such as NHS ester and epoxy groups (lysine, cysteine and reduced cystine), aldehydes (lysine), maleimide (cysteine and reduced cystine), amino groups (oxidized carbohydrates), and salicylhydroxamic acid (phenylboronic acid). In most cases, these methods do not allow a control of the protein attachment site which may lead to immobilization at unfavorable orientation. Moreover, a non-specific introduction of a labeling group may cause a variable degree of local denaturation and sometimes a complete loss of activity [93].

Only a few methods of site-specific derivatization and immobilization are known including the expression of the protein with a suitable binding group such as a 6mer histidine or derived tags [29, 94-96], or an intein which can in a second step be exchanged by a biotin linker [97, 98]. These proteins bind to nitrilotriacetic acid [67] and streptavidin coated surfaces, respectively, which are commercially available. Besides the need of costly protein

expression the stability of the Ni-His complex is a limiting factor of the immobilization *via* a His tag. In a similar approach Hodneland *et al.* fused calmodulin to a serine esterase as a capture protein [1]. The fusion protein was immobilized at a gold surface presenting a phosphonate capture ligand *via* an alkanethiolate-ethylene glycol linker. The immobilized calmodulin was shown to bind calcineurin in an SPR experiment.

The fusion of RNA to proteins and the following hybridization to the respective complementary oligonucleotide target on a linker modified slide surface of type **2B** was described by Weng *et al.* [99]. This technique positions the RNA-protein fusion in a uniform orientation thereby avoiding the delicate contact of the proteins with the slide surface.

Staudinger ligation [100] and other highly site-specific chemical reactions can also be used for site-specific immobilization of proteins including the reaction of a ribonuclease with a phosphinothioester-derived surface [101] and an alkyl transferase fusion protein with an O^6-alkylguanidine group immobilized at the glass surface *via* a tetraethylene glycol linker [102].

Antibody immobilization strategies are mainly borrowed from affinity chromatographical methods developed in the 1970ies and 80ies [103]. Immobilization chemistry was successfully transferred to microarray applications such as the reaction with protein A and G at the Fc part of the antibody [104-106], the reaction at an oxidized carbohydrate site [83], and the use of antibody thiol groups [83, 88] for a site-directed binding to the surface. Pavlinkova *et al.* reported on a site-specific method for labeling antibodies using a photoinduced insertion of a azidoadenosine-biotin conjugate into the Fab' domain [93]. The method takes advantage of a highly conserved purine base stacking site in the Fv region of the antibody and can be used for a mild derivatization of antibodies [107]. Despite a multitude of available methods, the development of a suitable chemical system for site-directed immobilization of proteins and antibodies to microarray surfaces still remains a challenge.

DNA MICROARRAYS

DNA-microarrays have changed the way biology is being done. From initial attempts to develop a method for large-scale, low-cost sequencing of genomic DNA [108-110], the technology has diversified enormously and is now applied in nearly every area of nucleic acid analysis. Concomitantly, the kind of data produced in such studies is much different from earlier results in biology in so far as they are too complex to be analyzed and interpreted directly. Even compared to sequencing, complexity is increased for the dynamic nature of the measurements. While a second analysis of the sequence of an individual person yields the same result, a study of transcriptional activities, for example, produces different data during time or in analyses of different tissues. As a matter of fact, it is likely that such variations in the molecular "interpretation" of the basic genomic blueprint are more responsible for many differences between species than is the mere sequence variation. For this complexity, statistic and computational means are fundamental to extract useful information from microarray studies, which paved the way for the emergence of theoretical biology-termed systems biology-in a development that is very similar to the split of physics into a theoretical and an experimental branch. Also, one can perform experiments without an initial hypothesis with a very good chance of success. Since large amounts of data can be produced in an unbiased manner, new and especially unexpected results are likely to be obtained, which in turn lead to original hypotheses that then require confirmation at a more focussed level of analysis. Initially, DNA-microarrays represented technically nothing else really but a reverse Southern blot arrangement. Meanwhile, however, structure and format of the various assay types differ significantly from each other and frequently have nothing in common but the basic layout of the tool.

Assay Formats

Although all types of analyses are based on the specificity of the binding between complementary sequences, three basic chip-based formats of analysis exist as displayed in Fig. **3**. Many assays are based on a straight hybridization event of target molecules, which usually consist of a complex mixture of RNA or DNA fragments, and a set of complementary probe molecule that are attached to the solid support. Discrimination is based on the difference in the stability of full-match and mismatch duplexes. The actual binding of the target is identified *via* an appropriate label, which can be bound to the target or the probe, although the former is the currently most frequent detection mode. In some assays, such as mapping, the arrangement of target and probe is reversed for the reason of increasing the efficiency of data production. Other analysis forms require an additional reaction by which discrimination is achieved. Frequently, this reaction is performed directly on the microarray. A typical example is a primer extension reaction at the probe oligonucleotides, which act as primers for the polymerase. However, other types of (enzymatic) modification do exist. As a third option, the actual assay takes place in homogeneous solution rather than on the solid support, which can make quite a difference with respect to mass transport and kinetics, for example. Here, the microarray acts only as a tool to separate physically the molecules for performing individual measurements, although in a parallel manner.

DNA Analysis

Mapping of genomic fragments was the first large-scale analysis format performed on DNA-microarrays [111]. Hybridization of either short oligonucleotides or fragments themselves produce fingerprint information on arrayed DNA-fragments that can be used to define overlapping regions between fragments and deduce from this the overall order of the DNA pieces. By using known stretches of DNA, such as gene fragments or oligonucleotides that represent sequence motifs, initial functional information can be acquired simultaneously [112]. Ultimately, even the sequence of each fragment could be inferred from hybridization information.

Already at the level of DNA, differences in copy number occur. Deletion of a genomic region or the amplification of an allele frequency are a mechanism of regulating the interpretation of the basic genomic information encoded in the sequence. Comparative genomic hybridization (CGH)

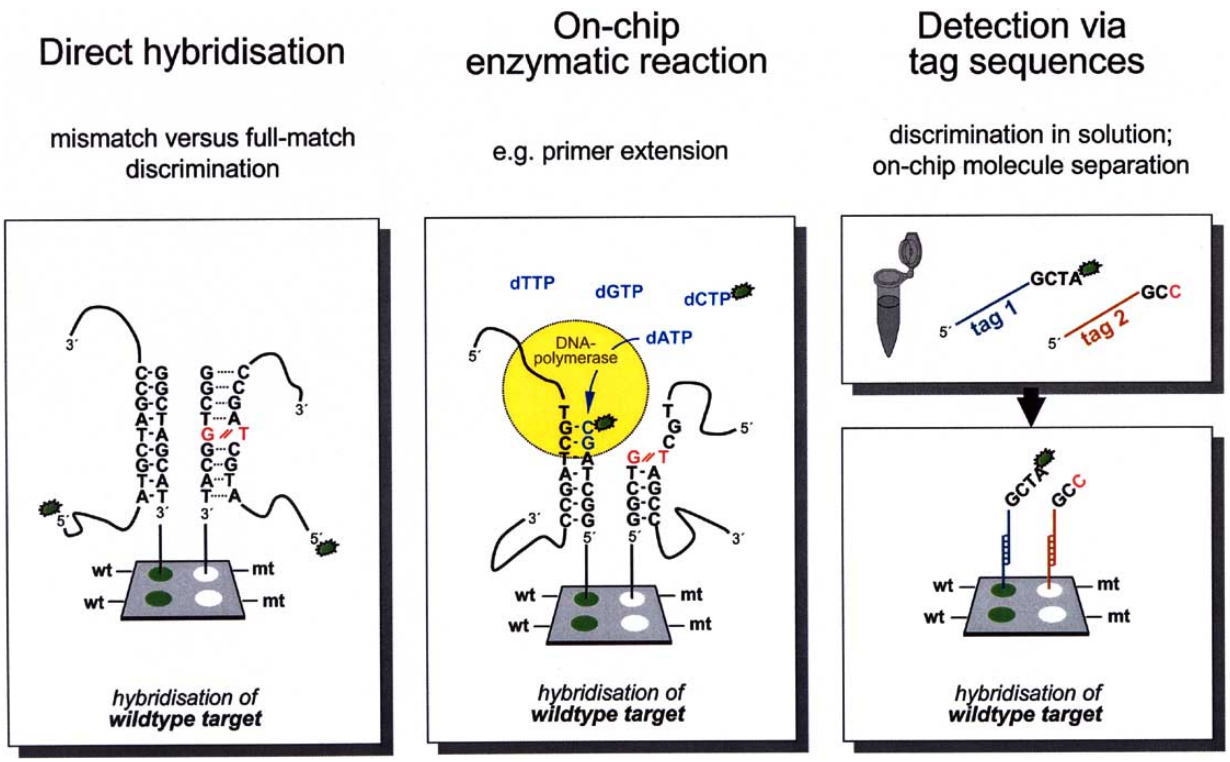

Fig. (3). Microarray-based formats of analysis.

picks up such differences by a comparative analysis of DNA preparations isolated from different cells or tissues, in a process that is similar to the analysis of RNA variations. Either DNA-target is labeled and their signal intensities on probes that represent particular genomic regions are compared. Especially in cancer, many disease-specific imbalances have been identified (e.g., [113]).

Another source for differences between individuals are sequence variations at individual base positions, named single nucleotide polymorphisms (SNPs). Such variation in DNA sequence is known to affect the activity of genes, for example, and can therefore also be responsible for disease. However, the majority of SNPs has no immediate effect on the cell. Nevertheless, their analysis can be very useful for identifying those sequence variations that actually are responsible for a particular disease. Several million SNPs are already known in the human genome and it is likely that many more will be identified the more studies will be performed. Their linear order represents a high-resolution genetic map of the human genome, which can be utilized for the localization of genes. Rearrangements that occur during meiosis allow an identification of genomic regions that are associated with particular phenotypic traits. Technically, polymorphisms are identified by their difference in duplex stability on a set of oligonucleotides that exhibit all possible sequence variations. Alternatively, discrimination is achieved by the specificity of a polymerase, incorporating the respective nucleotide at the position of interest (e.g., [114]).

Studies of epigenetic patterns in genomic DNA represent a special case of SNP typing. About 4% of all cytosines of the human genome may be methylated. It is known that the variations in the epigenetic status are a regulative factor, too [115]. By treating genomic DNA with sodium bisulfite, unmethylated cytosines are converted into uracil, while methylated cytosines remain unaffected. This conversion is nothing else but a chemically introduced polymorphism, which can be identified by usual SNP-typing processes.

In principle, SNP-typing can be extended toward an analysis of each base in a nucleic acid, returning to the origin of microarray technology [116]. As with the mere typing of few bases, the techniques are either based on direct hybridization or an enzymatic primer extension reaction. Potentially, a single chip could be sufficient to obtain all sequence information of a mammalian genome [117], although no such experiment has been performed successfully as yet.

RNA Analysis

Transcriptional profiling is the currently most widely used form of array-based analysis. Even though, standardization is not yet progressed far enough to ensure an easy comparability of data from different sources, although it can be done in principle [118]. As a matter of fact, the quality and reproducibility of sample selection and preparation has become more problematic experimentally. At the other end of the analysis process, data interpretation is the major bottleneck. Also, current techniques measure RNA levels rather than transcription rates and are thus of limited value in some instances, since the result of several regulative mechanisms-production rate, stability, inhibition and active degradation-is measured. In addition, mostly relative molecular ratios of RNA species between different tissues are being determined instead of absolute amounts. All this restricts the usability of the technique, apart from the fact that the actual degree of quantification is still inadequate.

Nevertheless, the technique has proved powerful (e.g. [119]). Since the information gained represents the dynamic nature of the RNA pool in a cell, pattern differences rather than variations of individual molecules are likely to be more informative for diagnostic and predictive purposes.

One surprising result from genomic sequencing was the fact that the human genome seems to contain far fewer genes than originally anticipated, although the difference by a factor of two or three is not that significant after all, considering the overall complexity of a human being. Also, the actual number of coding sequences is not yet certain and there are some indicators that suggest that a considerable number is still being missed. In any case, splice differences rather than the mere variation in RNA level represent an additional regulative factor by providing a mechanism to produce from one gene different RNA molecules and thereby a protein family, whose members could have distinct activities. Therefore, analyzing the RNA level of each individual exon adds another dimension of RNA complexity [120, 121].

DNA-Protein Interaction

For the transmission of the basic conceptual sequence information encoded in DNA into actual cellular activity, close interaction of proteins and DNA is required. Most prominently, the binding of the more than 2000 transcription factors in mammalian organisms are important for the regulation of gene transcription. But also other protein-DNA and protein-RNA interactions are crucial for the functioning of a cellular system. For assaying such interaction, a combination of chromatin immunoprecipitation and chip-based analysis of the DNA fragments obtained in this process (ChIP-on-chip) proves to be crucial [122, 123]. Complexes of protein and nucleic acids are crosslinked and the proteins of interest precipitated and purified with appropriate antibodies or other ligand binding molecules. Subsequently, all protein is digested and the released nucleic acid is labeled. By hybridizing these labeled fragments to an array that represents a genomic region of interest or the entire genome, the binding position of the protein that was precipitated is revealed. With this technology, another important level of cellular management is made accessible.

Another application is the *in situ*, *in vitro* transcription and translation of DNA fragments [124]. In a combinatorial approach, combining particular promoter structures and coding sequences, the interrelationship of sequence variations and protein activity could be analyzed in a degree of detail that is essential for an eventual systemic, biological description and prediction of such processes.

Production

More recently, microarrays have become a tool for molecule synthesis rather than analysis [125]. Several processes exist that allow an *in situ* synthesis of oligonucleotides. Taking advantage of the ability to produce separately, but nevertheless in a single process, many different molecules in small but sufficient quantities, microarrays have been utilized for the synthesis of complex oligonucleotide mixtures, which were then released from the chip surface and eluted for subsequent manipulations [126]. Potential applications reach from the synthesis of entire genes [127] or possibly even (small) genomes [128] to complex RNAi libraries.

DNA-microarrays have diversified a lot in recent years. As wide as the areas of application are, as wide is the degree of maturity of the different techniques. While some are at the brink of becoming routine, others are still in early phases of development, and new procedures and areas of application emerge continuously.

PEPTIDE MICROARRAYS

Even though proteins and not peptides play key roles in nearly all cellular processes, the main binding regions are in most cases only very small sequences of a few amino acids in length. Hence, in many cases synthetic peptides or peptidomimetics, which mimic the sequence of the binding site of any given protein, demonstrate the same biological activity as the protein itself, although sometimes with partial loss of activity. Therefore, arrays of synthetic peptides offer a fast and easy possibility to screen for protein interactions [129], binding regions (epitopes) [130, 131] and enzyme activities [132, 133]. Moreover, they allow the screening for inhibitors [134] and the engineering of antibodies, enzymes or inhibitors [135] with regard to modified binding sites. The field of applications is manifold and rapidly growing. In contrast to the production of complex protein arrays, synthetic peptide arrays offer several advantages. Since they are easily produced, they offer a relatively high stability, long storage time and easy handling.

Generally, peptide arrays can be produced in two different ways: by *in situ* synthesis, in which the compounds are synthesized directly onto the solid support of the array or by spotting and site specific immobilization of pre-synthesized, purified peptides on chips. Both ways have their assets and drawbacks and can be subdivided in several methods depending on the chemistry used.

In Situ Synthesis

The very first peptide microarray was produced by means of light-directed *in situ* synthesis. In 1991, Fodor and co-workers synthesized more than one thousand different peptides on a glass slide using NVOC, as a photolabile N-terminal amino acid protecting group [136]. Indeed, this approach was the precursor for the development of *in situ* synthesized DNA-microarrays by Affymetrix. However, in the field of peptide arrays light-directed *in situ* synthesis did not become well established so far, probably because of the low practicability and the need for light sources, masks and optics as well as special and therefore expensive monomers. Addressing the last issues, Gao *et al.* introduced a light-directed *in situ* synthesis based on standard *t*-BOC chemistry in a microfluidic format [137]. Here, spatially directed deprotection takes place by using light photo generated acids and mask-less projection lithography. The use of such microfluidic chips enabled moderately high densities, and required only short reaction times and "off-the-shelf chemicals".

The second technique which is used for *in situ* peptide synthesis is the SPOT method, first reported by Frank in 1992 [138]. Here, peptides are synthesized by sequential spotting of small amounts of activated amino acids onto a porous membrane. Compared to the light-directed approach,

peptide arrays resulting from the SPOT method are macroarrays with only very low densities and spot sizes of at least 2-3 mm. Nevertheless, the SPOT method became widely used, particularly because of its simplicity. The use of standard Fmoc-peptide chemistry, inexpensive equipment, flexible array formatting and the possibility to distribute the amino acids in a manual as well as automatical manner made this method easily accessible even to the low-budget non-chemist researcher [139].

With regard to miniaturization and higher spot densities, there have been two developments. On the one hand, peptides were synthesized on dissolvable cellulose membranes according to the standard SPOT method procedure. After synthesis the cellulose spots were dissolved and re-arrayed onto glass slides. In this case the peptides remain bound to the dissolved cellulose molecules, which build a three dimensional structure on the re-arrayed spots of the resulting glass-chips. On the other hand, a Bio Disc-Synthesizer was developed, aiming at a fully automated SPOT synthesis at high speed [140]. Using a drop-on-demand technology, this machine is capable of simultaneously spotting 2500 spots from up to 24 separate reagent valves onto a rotating disc as the planar array substrate in less than three minutes. A wash station is implemented allowing for multi-step combinatorial synthesis without manual attention.

A related, but yet different approach for *in situ* synthesis of complex peptide microarrays is described by Bischoff *et al.* [141]. Here, activated amino acids were distributed in a solid solvent (toner) and printed onto glass slides by means of a color laser printer (spot sizes ~ 190μm). After printing, the slides were slightly heated until the toner particles melt and the reaction takes place according to standard Fmoc-chemistry. This process may also be carried out directly on a computer chip, on which the activated monomer-toner particles are directed towards their binding positions *via* electrodes on the chip, thereby enabling spot sizes as small as 20μm.

Immobilization of Pre-Synthesized Peptides

Another route for the production of peptide arrays is the immobilization of pre-synthesized peptides onto chip surfaces. One major advantage of this approach is the possibility of using purified peptides, thereby avoiding false-positive signals caused by truncated sequences or other by-products. Due to quality control and purification, longer peptides and difficult sequences can also be used without diminishing the array quality. Since this approach is more expensive than *in situ* synthesis, this strategy is only used when relatively small numbers of peptides are needed or when large numbers of identical arrays should be produced. However, recent developments of high throughput peptide synthesis in a small scale and parallel purification methods [142-145] may decrease the costs in the near future.

For the immobilization of peptides on solid supports many strategies have been reported. In order to maintain the biological activity of the bound peptides, most groups developed site-specific coupling methods, allowing for a selective binding of the N- or C-terminal residue of the peptide to a respective surface. As pointed out in the chapter "solid supports" versatile methods exists due to different coupling chemistry. For example, Falsey *et al.* immobilized N-terminal cysteine modified peptides by means of a thiazolidine ring ligation reaction on glass slides functionalized with glyoxylic acid [72]. Uttamchandani *et al.* used a related approach where cysteine-peptides react selectively to thioester moieties. As an alternative they also used biotinylated peptides which bound selectively to avidin or streptavidin surfaces [133]. Sun et. al. describe a method where peptides were firstly ligated to an intein-generated carrier protein, which is then bound to a solid support [146]. Because the carrier protein dominates the binding of ligation products and contains one peptide reactive site, the amount of peptides arrayed to the membranes can be effectively normalized. Chemically interesting is also the work of Houseman *et al.* [42] who introduced a cyclopentadiene linker during the peptide synthesis and coupled the resulting peptides *via* a Diels-Alder reaction on functionalized glass slides.

Even though site-specific immobilization results in better array quality and should therefore clearly be preferred, early reports also demonstrate the functionality of peptide arrays, on which peptides were immobilized non-specifically *via* their nucleophilic groups (NH_2-, SH, OH-), for example to succinimidyl-activated BSA modified surfaces [45]. Quality of this array was absolutely sufficient for qualitative kinase studies with ^{33}P.

Another fast and attractive way for the production of peptide arrays circumvents covalent coupling at all. In this case peptides were microarrayed within individual nanoliter droplets of glycerol onto glass slides. Using aerosol deposition, subsequent reagents and water can be added into each reaction center without cross-contamination and the need for surface linkage. Gosalia *et al.* spotted a 722-member library of fluorogenic protease substrates of the general format Ac-Ala-X-X-(Arg/Lys)-coumarin along with fluorescent calibration standards in such a way and used this arrays for the rapid determination of protease substrate specificities with minimal sample and enzyme usage [147].

A totally different approach to fabricate peptide arrays was followed by Blake *et al.* [148]. They developed a preparative mass spectrometer for the production of protein arrays using the ion soft landing technique to collect ions on a surface after separation by mass/charge ratio. Small microarrays have been prepared by isolating and soft landing individual protein and peptide ions after electrospray ionization of mixtures. In contrast to conventional peptide arrays, which represent a "bottom-up method" this approach may allow a "top-down" analysis, thereby complementing the existing methods. In general, proteins isolated from biological samples by 2-D gel electrophoresis are fragmented and identified by mass spectrometry. Here, fragment ions of a given protein, can be collected separately on a chip and can then be further analyzed in a parallel manner regarding their biological function.

Applications

While early reports on peptide arrays dealt mainly with epitope mapping followed by enzymatic assays, the field of applications has grown rapidly during the past five years. Very small arrays of only a few tens of peptides up to complex libraries with more than a thousand compounds

were used. Depending on the application, different screening strategies can be employed, such as iterative deconvolution (with two, three or four defined positions), epitope mapping and motif scanning. A short list of these strategies with the respective literature is given by Frank [139]. Binding events or enzymatic reactions on the arrays were in most cases detected either by fluorescence, for example by means of fluorescently labeled antibodies / proteins or fluorogenic substrates exhibiting fluorescence by cleavage, or by radioactivity. However, some reports also demonstrate the usefulness of label free detection methods. Rickert *et al.* used quartz crystal microbalances (QCMs) for quantitative biosensing and characterization of protein mono- and multilayers [149]. Houseman *et al.* showed the possibility to use surface plasmon resonance (SPR) as well as fluorescence and phosphorimaging for the identification of phosphorylation on peptide arrays [42]. Real-time surface plasmon resonance imaging was also used by Wegner *et al.* who examined protein adsorption/desorption kinetics and surface enzymatic reactions on peptide microarrays [150].

In addition to the identification and characterization of binding domains, peptide arrays can also be used as a diagnostic tool to identify antibodies, enzymes or biomarkers in blood serum or cell lysates. Duburcq *et al.* described the performance of peptide and protein arrays for the simultaneous serodetection of antibodies directed against hepatitis B and C virus, human immunodeficiency virus, Epstein Barr virus and syphilis antigens [151]. The microarrays displayed high signal-to-noise ratios, sensitivities and specificities for the analysis of a collection of human sera referenced against these five pathogens. Diks *et al.* used peptide arrays consisting of 192 peptides to study the activity of all kinases of a whole cell lysate, the kinome [152]. The authors could show that lysates of cells, stimulated with kinase inhibitors, effected peptide array phosphorylation patterns consistent with the expected action of these inhibitors. This kind of array allowed for the determination of enzymatic activities of a large group of kinases, thereby offering high throughput analysis of cellular metabolism and signal transduction.

A nice example for the combination of different array types is given by Bialek *et al.* They described the development of a process for the genome wide mapping of interactions between protein domains and peptide ligands. The authors "panned" a phage library displaying protein domains from a randomly fragmented and cloned cDNA-library on an array of synthetic peptide ligands. After multiplexed affinity enrichment, peptide specific phage populations will be automatically eluted, propagated, labeled and identified by hybridization to a DNA-microarray [153].

In order to study weak or transient interactions Espanel *et al.* developed a strategy to synthesize up to four peptides on one spot by the SPOT method [154]. For a special assay they used two different peptides on one spot. In this case, one peptide can be a substrate for the enzyme, whose conversion is indicative of a transient interaction of the enzyme with the other peptide. By this, multiple interactions can theoretically be analyzed, provided that linker length and the position of the different peptides to each other allow for these interactions.

However, for the identification and characterization of weak or more complex interactions longer peptides or proteins might be the better choice. The largest peptide synthesized by the SPOT method so far is a 53mer [155], which will probably present a practical upper limit because of synthesis time, costs and decreased product purity. Toepert *et al.* generated large peptide arrays which present 38-mer peptides by a combines SPOT synthesis and chemical ligation [156]. Finally bridging the gap between synthetic peptide arrays and protein arrays, Haehnel described a combinatorial synthesis of *de novo* proteins for investigation of cofactor binding and folding [157]. However, the ability of long peptides to fold unassisted into "relevant" conformations, still remains a problem. Therefore the direct spotting of whole proteins might a better choice.

PROTEIN AND ANTIBODY MICROARRAYS

Protein and antibody microarrays are one of the latest developments in microarray technology. They consist of a multitude of proteins and antibodies that are transferred and immobilized on an activated solid support. Since proteins and antibodies are highly diverse with regard to their stability and chemical structure, this step represents a mayor challenge and requires a versatile surface activation that is capable of accommodating all proteins in a way that their native conformation with all interaction sites are maintained. In addition, elaborate handling protocols have to be created, that allow a robust and highly-specific interaction screening under conditions that are suitable for all immobilized proteins. While this task seems to be more facile for antibody microarrays, due to their relatively uniform and stable structure, studies have shown, that antibodies may require different environments to be functionally immobilized on microarrays [62, 158]. Although it is very likely that surfaces and protocols are developed that conserve functionality after immobilization for a majority of antibodies, these observations hint towards the problems that arise, when surfaces and protocols are developed for protein microarrays.

Protein chips are applied for a large variety of applications, such as screens of immune responses, the analysis of enzymatic modifications or interactions between proteins and binders, which can comprise DNA, RNA, proteins or small molecules. The requirements with regard to native conformation are thereby highly diverse and depend on the assay [159]. Most assays will require a native conformation to elucidate binding partners or functional relations. Nevertheless, some applications, such as the immunoprofiling purposes of patient sera suffering from autoimmune diseases can be performed with denatured proteins [61]. In addition, domains that readily bind to unstructured sequences present in peptides or unfolded proteins, such as PDZ, EF-hand, SH3, and WW can also be screened by protein arrays harboring denatured proteins.

Antibody chips, which harbor a large collection of specific binder molecules, such as antibodies, antibody fragments, engineered binding proteins, phage particles or even aptamers are mainly applied to specifically capture and quantify components of complex samples. Since those binders can be considered as the active binding partner, these

must retain their specific binding properties upon attachment.

Generation of Content

An important issue of protein and antibody microarrays is the lacking availability of large sets of proteins and antibodies. While such sets can relatively easy be obtained for DNA microarrays, the expression of a multitude of proteins and antibodies is rather difficult, especially since there is no facile amplification procedure analogous to PCR for proteins and antibodies. So far, a large variety of monoclonal antibodies have become commercially available that are directed against thousands of different antigens. Nevertheless, the costs associated with hybridoma technology and production are too high to create antibody microarrays of great diversities. Therefore, display technologies, like phage display or ribosome display have become increasingly important to address this problem [160].

The production of a large set of proteins normally comprises the generation of a cDNA expression library and the subsequent expression in a cellular host, such as *Escherichia coli* or *Saccharomyces cerevisiae* [161]. Alternatively, cell-free transcription and translation systems can be used, which are available from a variety of different prokaryotic and eukaryotic cells. Nevertheless, such a high-throughput generation of proteins entails massive costs and the problem of limited shelf-life of proteins. As a alternative approach to the purification and subsequent spotting of proteins was introduced by Madoz-Gurpide and colleagues, who prepared cell lysate from adenocarcinoma cell lines and fractionated the protein extract first by anion exchange and then by reverse phase liquid chromatography [162]. The obtained fraction were then characterized by mass spectrometry and immobilized on a microarray. The special advantages of such protein samples is that they have the correct post-translational modifications, which allows further properties to be assayed in comparison to proteins from recombinant sources. However, this approach suffers from the elaborate preparation and the problems associated with the storage of the samples. An even higher degree of complexity is displayed on reverse phase arrays. Those are created by the immobilization of a complex mixture, such as patient sera, and are used to compare the abundances of proteins between different samples [163].

The most convenient way of protein production for protein microarrays seems to be the production of proteins on a chip from DNA templates as shown by Ramachandran and colleagues [164]. This approach, called NAPPA (nucleic acid programmable protein array), entails the immobilization of DNA encoding the proteins on a chip and the incubation of the chip with cell-free expression mix, which produces protein, that is captures by a nearby antibody. The NAPPA approach offers several advantages, since it very cost effective due to the small consumption of reagents and it allows he production of proteins just prior to the microarray experiment, which diminishes problems associated with the storage of protein microarrays.

Applications

Although protein and antibody microarray technology is in an early stage of development, several applications in areas, such as autoantibody profiling, cancer research or signal pathway characterization highlight its potential.

In the area of autoimmune profiling, Feng and coworkers spotted 15 antigens for the investigation of rheumatoid arthritis, systemic lupus erythematosus, systemic and polymyositis sclerosis as well as Sjogren syndrome. Within their experiments, they obtained microarray results that were similar to the data obtained by conventional methods[165]. In a larger approach Robinson and colleagues [61] fabricated arrays containing 196 distinct biomolecules, comprising proteins, peptides, enzyme complexes, ribonucleoprotein complexes, DNA and post-translationally modified antigens. With such arrays, they characterized sera from eight human autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis. Quintana and coworkers applied protein microarrays consisting of 266 different antigens to confirm that the future response of mice to induced diabetes could be predicted by IgG autoantibody repertoires[166]. Using protein macroarrays, Lueking and colleagues, were able to identify alopecia areata autoantigens out of 37,200 redundant, recombinant human proteins and to validate them in a second step on protein microarrays [167].

The investigation of antiviral antibody responses to vaccine trials with a simian-human immunodeficiency virus (SHIV), a model for human immunodeficiency virus (HIV), was done by Neuman de Vegvar and colleagues [59]. They produced antigen microarrays with 430 different proteins and overlapping peptides spanning the whole SHIV proteome and identified eight immunodominant epitopes. Antibody responses of immunized rabbits to *Yersinia pestis* vaccine was performed by Li and colleagues [168]. They applied protein microarrays harboring 149 *Yersinia pestis* proteins and were able to detect 13 imunogenic proteins. In an investigation of SARS, Chen and coworkers studies the antigenicity of different regions of the SARS coronavirus by printing all of the N protein fragments [169]. After incubation with 52 sera, four important regions with possible epitopes could be identified and named. The identification of new potential diagnostic markers for *Neisseria meningitides* was done by Steller and colleagues, who cloned all known phase-variable genes for expression and spotting. They were able to express 67 of them, subjected them to 20 sera of patients and identified the OpaV protein as a highly immunogenic protein [170].

Characterization of cytokine release is another area of application for antibody microarrays. In a proof of principle study, Lin *et al.* quantified human cytokines, chemokines, growth factors, angiogenic factors and proteases in estrogen receptor positive and negative cells [171]. In a second screen they applied their antibody arrays to measure cytokine expression levels in human breast cancer cell lines and identified interleukin (IL)-8 as a key factor involved in breast cancer invasion and angiogenesis [172]. Cytokine release in primary Sjogren's syndrome was also measured by Szodoray and colleagues. They determined the level of 25 plasma cytokines and were able to elucidate different cytokine profiles between patients with or without extra-glandular manifestations [173]. Turtinen and coworkers studied the effects of different amphotericin B formulations on cytokine release from THP-1 leukemic monocytes and showed that TNF-alpha and IL-8 levels correlated well

between the antibody microarray and quantitative ELISA measurements [174].

Cancer research is currently one of the largest areas of application for protein and antibody arrays. Serum screening was performed in several studies to characterize the serum and plasma of patients suffering from diverse cancers, such as colon, lung or nasopharyngeal cancer [175-178]. All studies demonstrated the applicability of arrays to this field and led to the identification of known or new potential biomarkers. In another application, mutations and polymorphisms of p53 were functionally characterized with regard to their DNA-binding capacity on protein microarrays [179]. An antibody microarray for squamous cell carcinoma of the oral cavity was generated by Knezevic and co-workers [180], utilizing laser capture to gain total protein from specific microscopic cellular populations. Using this approach, they were able to correlate differential expression of stromal cells adjacent to the diseased epithelium to tumor progression. Commercial antibody microarrays displaying 512 antibodies were used by Ghobrial and colleagues to perform translational profiling on mantle-cell lymphoma [181]. Depending on the cut-off parameters, they identified 77 or 13 overexpressed proteins, including cell cycle regulators and chaperones. In a similar approach, Gao *et al.* applied a 84-feature antibody microarray to profile lung cancer patients [182].

Reverse phase protein arrays were applied by Paweletz *et al.* [183]. They created organ and disease specific microarrays to quantify the phosphorylated status of signal proteins and to monitor cancer progression from histologically normal prostate epithelium to prostate intraepithelial neoplasia (PIN) and invasive prostate cancer [184]. Reverse phase microarrays for the characterization of signaling pathways were also applied by Gulmann and colleagues investigating follicular lymphoma [185] and by Sheehan *et al.* analyzing metastatic ovarian carcinoma [163].

First methodologies for the analysis of membrane proteins were developed by Fang *et al.* [81], who generated membrane protein arrays for analysis of the ligand-binding properties of receptors. The microarrays consisted of an array of G protein-coupled receptors and andrenergic receptors, which were employed for the subtype-specific detection of a cognate antagonist analogue specific for beta-adrenergic receptors. Since important members of signaling pathways are membrane located, systematic analysis of membrane proteins provides a valuable task for proteomics.

Additionally, efforts were made to monitor enzymatic reactions on a chip. As a first step, kinase activity was measured quantitatively on a peptide chip [42]. Houseman *et al.* applied surface plasmon resonance, fluorescence, and phosphorimaging for the detection of phosphorylation and evaluated three inhibitors quantitatively. Zhu *et al.* [94] used protein chips bearing microwells to analyze nearly all of the protein kinases from *Saccharomyces cerevisiae*. Many novel activities were found as well as 27 protein kinases with an unexpected tyrosine kinase activity. The same group investigated protein-protein interaction in large scale by the production of the first proteome chip [94]. For the generation of the chip, 5800 open reading frames of yeast were cloned and the corresponding proteins were overexpressed in *E. coli*, purified and spotted. The resulting protein microarray was used to screen for interactions with calmodulin and phospholipids and allowed identification of binding motifs. Phosphorylation was also studied in plants by Feilner *et al.*, who identified targets of mitogen-activated protein kinases on microarrays displaying 1690 Arabidopsis proteins [186]. Jung and Stephanopoulos performed a pathway reconstruction on a microarray by immobilizing enzyme mixtures using mRNA-enzyme fusions. By this, they were able to monitor reaction products from a two- and a five step chemical reaction [187]. A miniaturization of enzymatic assays on a chip was also conducted by Dietrich *et al.*, who performed assays for alcohol dehydrogenase, pyruvate kinase and enolase in volumes of 6.3-8 nl using a fluorescent microscope equipped with a CCD camera as a readout system. The screening of chemical libraries for inhibitory effects was performed by Funeriu *et al.*, who used low-molecular-weight fluorescent affinity labels to monitor the activity of the immobilized enzymes [188].

A new approach, named multiple spotting technology (MIST) was developed by Angenendt and coworkers. This approach relies on the sequential spotting of several reaction components, such as substrate, inhibitor and enzyme on the same position of a glass slide to generate single and discrete nanoliter reaction entities on a flat glass surface. In these reaction entities enzymatic assays can be conducted that catalyze the formation of unbound reporter molecules. In a first study, enzymatic activity of enzymes such as alkaline phosphatase, ß-galactosidase or cathepsin D was detected [189]. Moreover, the applicability of MIST was shown for multiplex immunosorbent assays [190, 191] and for the screening of large populations of recombinant antibody fragments on a microarray [192].

CELL-BASED MICROARRAYS

DNA microarrays have greatly increased the rate with which genes that are involved in certain cellular processes can be discovered. However, functional analyses of candidate genes are necessary to confirm such discoveries. They give important information about a gene´s role in cellular processes and pathways, and its potential suitability as a drug target. Cell-based assays constitute a tool for functional validation of a gene originally found with a genomic or proteomic screening approach, and to identify gene products with the desired properties [193]. However, these assays have been the slow and rate limiting step in the characterization process, due to the presence of only few high-throughput technologies for the analysis of gene function in cultured cells or tissues. Existing cell microarray technologies involve overexpression of cDNAs, loss-of-function techniques such as RNA interference (RNAi), or compound screening approaches.

Ziauddin and Sabatini [23] were the first to provide a tool with which gene products could be analyzed functionally in a high-throughput manner by gene expression in a microarray format. Their well-less screening approach was based on the localized transfection of mammalian cells growing as a monolayer on a glass-coverslide printed with 192 different cDNA expression constructs. The different DNAs were printed at defined locations, so that groups of cells growing on a certain spot were transfected with the corresponding DNA, and could be distinguished spatially

from surrounding cells. The authors called this new method "reverse transfection", since the order of addition of DNA to adherent cells was reversed compared to conventional transfection methods. They were able to demonstrate the power of this approach by identifying proteins involved in tyrosine kinase signaling, apoptosis, and cell adhesion, using microscopic readouts.

Since these cell-based microarrays can be printed with the same robotic microarrayers as conventional DNA chips, the authors proposed that in the future up to 10 000 genes could be expressed on a single microscope slide, and the phenotypic effects assayed with microscopic techniques. The advantages of this method lie in the rapid functional validation of candidate genes, and in the relatively long half-life of the printed arrays. Upon printing, these microarrays can be stored for months, ready to use for cell seeding. Limitations are constituted by the transfectability of cells, and the generation of cDNA expression constructs, but mammalian full-length cDNA libraries are being constructed [194].

The reverse transfection approach was also used in loss-of-function experiments by Mousses *et al.* [64] who arrayed 100 to 700 μm spots of synthetic small interfering RNAs (siRNAs) on glass slides for the parallel knockdown of different genes. The authors showed in pilot experiments with HeLa cells that this method is suitable to efficiently knock down genes in a time-and dose-dependent manner. Even though screening is possible in 384-well plates, the well-less format for high-throughput RNAi screening in mammalian cells is especially cost-effective since synthetic siRNAs are extremely expensive, and as few as 2.5 ng of each oligonucleotide is sufficient to mediated RNAi in the microarray format.

The high-throughput approach developed by Ziauddin and Sabatini, even though suitable for a variety of adherent cells, does not work for non-adherent cells, since differently transfected cells cannot be distinguished spatially. This problem was recently addressed by Kato *et al.* [195] who developed a microarray especially suitable for non-adherent cells. They coated standard glass coverslides with a cell membrane anchoring reagent that strongly attached the cells to the glass surface. The authors showed that DNAs printed on defined spots on the coated microarray were taken up by the anchored cells. This approach worked in gain-of-function experiments with cDNA expression vectors, as well as in loss of function experiments using small hairpin RNA (shRNA) expression vectors that mediate RNAi in mammalian cells.

In addition to cell-based microarrays that are to screen printed cDNAs or shRNAs, microarrays have been generated that can be used for the screening of small molecules. For drug discovery efforts, Bailey *et al.* [196] developed a small-molecule microarray format that is compatible with chemical libraries. This format is based on glass coverslides coated with a biodegradable polymer, which provides a diffusion barrier for small molecules impregnated as spots in the coating. The molecules diffuse slowly through the coating to effect cells growing on the spot. Prior to seeding, the cells are treated with siRNAs, in order to test the effect of the compounds on the siRNA-mediated phenotype. In total, the authors tested 980 siRNA/compound combinations. The method is limited by the printing density and needs further optimization to increase the printing density while preventing one spot from affecting cells growing on a nearby spot.

Current cell-based microarrays rely on microscopic readouts, even though many well-based assays involve enzymatic activity. Enzymatic readouts such as the measurement of light emission caused by the conversion of a substrate by a luciferase enzyme have become extremely important for studying signaling pathways. Luciferase reporter assays can be used to identify new signaling pathway components on a genome-wide scale. Since today a genome-wide RNAi screen can be performed in less than one day in a 384-well plate format [197, 198], there is no urgency for the downscaling to a well-less microarray format. The downscaling process would be also limited by the number of cells required to distinguish the enzymatic signal from the background noise. Increasing the number of wells or spots would reduce the number of cells per well or spot. Considering the transfection efficiency which can be less than one percent in primary cells, the spots must be large enough to host a sufficiently high number of transfected cells to give a statistically relevant result. This is true for enzymatic readouts as well as for the analysis of microscopic readouts, and constitutes one of the major limitations of cell-based microarray technology.

Another limitation that has to be considered is the more complex analysis of high content images produced with microscopic readouts. While enzymatic assays in well-plate formats produce numbers that can be analyzed easily with standard statistics, microscopic images harbor a variety of parameters. Thus, large-scale microscopic readouts require the storage and analysis of hundreds of gigabytes, and it is therefore of great importance to develop automated image analysis tools. Mousses and coworkers generated an automated digital image acquisition system together with algorithms for quantitative image analysis with which they stored and analyzed their RNAi microarray images. Even though suitable for the analysis of assays that rely on the increase or reduction of fluorescence, high throughput analysis systems have to be developed that can be used to evaluate assays that depend on readouts other than fluorescence.

Even though cell-based assays can provide important information on a gene's role in certain cellular processes, it is necessary to confirm these findings with retests and *in vivo* experiments. A possibility to confirm for example the potential role of a gene in cancer is subsequent tissue microarray analysis. Tissue microarrays are glass coverslides on which tissue sections are arrayed [199]. Up to 1000 tissue sections can be prepared from paraffin embedded or frozen tissue and arrayed on a single microscope slide [200]. Overexpression or underrepresentation of a candidate gene in different tumor tissue sections can be detected using histochemical or immunohistochemical staining, or in situ hybridization techniques. Nishizuka *et al.* [201] integrated genomic, proteomic, and tissue array profiling to find diagnostic markers that can be used to distinguish colon and ovarian adenocarcinomas. Tissue microarray technology is therefore a useful tool to confirm candidate genes found in genomic or proteomic screens, to test potential therapeutic

targets, and to translate conclusions drawn from cell-line based assays to human cancer.

The key advantage of cell-based microarrays for genome-wide screening is that they permit miniaturization beyond what is possible with well-based cell assays. Downscaling of well-plate formats to microarrays reduces the amount of cells, reagents, and therefore screening costs. Cell microarrays can be used to identify new genes with certain cellular functions or they can be applied to confirm candidate genes originally found with other approaches. Mainly associated with microscopic readouts, cell-based microarrays produce a large amount of data that need to be stored and analyzed. Thus, automated image analysis tools need to be developed. Another challenge is to develop methods that increase the transfection efficiencies of cultured cells, so that the number of spots on one slide can be increased and primary cells can be used. The potential of cell-based microarray technology has clearly been demonstrated, and current approaches focus on preparing this technology for genome-wide applications [202]. It will soon be possible to conduct first screens on a genome-wide scale in the well-less cell-based microarray format.

In summary, microarray technology has proceeded from its DNA based origin to a wide variety of different formats, including peptide, protein and cell microarrays. The different formats are thereby highly integrative and cooperative. Yet, microarray formats, such as protein and antibody microarrays are in an early phase of development and still lack the complexity and comprehensiveness of current DNA microarrays. Reasons for this arise from the intrinsic complexity of proteins, which challenge the high-throughput production and functional immobilization. The success of such microarray technologies will therefore be highly depended on the development of fast and especially cost-effective methods for the generation of content and the fabrication of surface coatings that are not just suitable for highly efficient immobilisation, but also allow a selective immobilization of proteins in a way that their three dimensional structure is retained.

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