

Microchip Methods in Diagnostics

Edited by
Ursula Bilitewski



Humana Press

Microchip Methods in Diagnostics

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METHODS IN MOLECULAR BIOLOGY™

Microchip Methods in Diagnostics

Edited by

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Preface

The continuously increasing degree of miniaturization of electronic circuits and the development of corresponding fabrication technologies stimulated progress also in other fields, such as analytical chemistry. The ideas of “labs-on-chips,” in which all manual procedures required to obtain an analytical result are automatically performed on a chip, were presented almost 20 years ago, and fabrication technologies for DNA chips, which allowed to obtain genetic information in a highly parallel manner, were suggested already in the beginning of the nineties. These early dreams of miniaturized highly integrated analytical devices were based on the combination of developments in very different fields. The development and industrial fabrication of integrated electronic circuits had shown that by photolithography silica and glass could be precisely structured in all three dimensions on the micrometer or even nanometer scale. In biology, amplification methods such as the polymerase chain reactions (PCR), gene sequencing technologies, and biotechnological production methods for proteins were established. In organic chemistry, methods of combinatorial solid phase synthesis were developed, which made peptides and oligonucleotides easily accessible, and analytical separation methods were developed in which columns or planar surfaces were replaced by capillaries, such as in capillary electrophoresis or gas chromatography. This was accompanied by improvements in detectors, which had to deal with lower amounts of analyte molecules, a side effect of miniaturization.

Nowadays the field of microchip methods is rather heterogeneous, and there is even no common definition for the different approaches and devices. In this book microchip methods are analytical methods, which are based on miniaturized systems. This covers not only arrays for the simultaneous determination of several analytes (DNA microarrays and protein or peptide microarrays) or the simultaneous analysis of several samples (cell arrays), but also labs-on-chips, for which phrases such as μ TAS (micro total analytical systems) or MEMS (micro electronic and mechanical systems) are also used. Whereas miniaturization of arrays involves the sizes and densities of spots, labs-on-chips are miniaturized fluidic systems and are not necessarily multidimensional.

The present book wants to illustrate the diversity of possibilities, as they are applicable now in medical diagnostics. Thus, only those approaches are included, which have reached a certain degree of maturation so that they are applicable in practice also by the nonexpert, and for some approaches the corresponding systems are even commercially available.

As mentioned earlier three types of microchips were chosen: DNA microarrays, protein microarrays, and labs-on-chips in particular related to cell analysis. There is one chapter for each of these areas as an introduction to the fundamentals of the respective technology, followed by chapters describing methods related to specific applications. However, the chosen examples are by no way comprehensive and the methods are easily applicable to other diagnostic areas. This is in particular true for the field of DNA microarrays, which is at present the dominating microchip technology allowing the analysis of gene sequences and of gene expression. There is only one chapter for each of these applications (Chap. 3 for gene expression analysis, Chap. 4 for gene sequence analysis), though there are numerous publications on different diagnostic problems using different types of arrays. However, the basic procedures are identical for all these applications, and are not

dependent on the particular diagnostic application and even not on the array platform. Moreover, the increasing experience with these arrays shows that the comparability of results among array platforms and laboratories is improved, if experimental protocols are harmonized. Thus, the presentation of different protocols would be counterproductive with respect to harmonization, if not the need for the deviations is discussed. The same idea of representative examples was followed for the choice of contributions dealing with protein arrays and fluidic or cell-based chips. However, compared with DNA microarrays the number of commercially available systems is much less, and thus, the fabrication of those systems is also included (in particular Chaps. 9 and 12, but also protein and peptide arrays in Chaps. 7 and 8).

Although the application of DNA microarrays has developed into an essential tool for biomedical research the applicability of this new technology in practical diagnostics is still debated. Thus, Chap. 2 was included to discuss the comparability of diagnoses based on microarrays and on established methods. Again leukemia profiling is to be considered just as an example.

This handbook wants to support the introduction of diagnostic methods based on microtechnologies, as miniaturization leads to a reduction of sample volumes for a single analysis or allows the parallel determination of several analytes without the need for more sample or time. Moreover, novel molecular information could be made available from patient samples, such as more comprehensive information about gene or protein expression, improving diagnostic possibilities. Whether these expected benefits for patients prove to be true can be verified only by application and validation in practice.

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Chapter 1

DNA Microarrays: An Introduction to the Technology

Ursula Bilitewski

Summary

DNA microarrays allow the comprehensive genetic analysis of an organism or a sample. They are based on probes, which are immobilized in an ordered two-dimensional pattern on substrates, such as nylon membranes or glass slides. Probes are either spotted cDNAs or oligonucleotides and are designed to be specific for an organism, a gene, a genetic variant (mutation or polymorphism), or intergenic regions. Thus, they can be used for example for genotyping, expression analysis, or studies of protein–DNA interactions, and in the biomedical field they allow the detection of pathogens, antibiotic resistances, gene mutations and polymorphisms, and pathogenic states and can guide therapy. Microarrays, which cover the whole genome of an organism, are as well available as those which are focussed on genes related to a certain diagnostic application.

Key words: Specific probes, Immobilization, Hybridization, 2D pattern, Gene expression analysis, ChIP-chip

1. Introduction

DNA arrays are two-dimensional substrates on to which different nucleic acids are immobilized as spots in an ordered pattern ([Fig. 1](#)). Each spot contains one type of nucleic acid and each nucleic acid is a polymer of nucleotides of defined length, which differs from the other nucleic acids in the array in the sequence of the bases adenine (A), cytosin (C), guanine (G), and thymine (T) ([1,2](#)). Depending on the diameter, density, and number of spots the arrays are called macro- or microarrays ([3](#)). Typical dimensions of macroarrays are up to 22-cm length per side of the substrate with up to approximately 1,200 spots per array (www.clontech.com). They are made by spotting cDNAs with

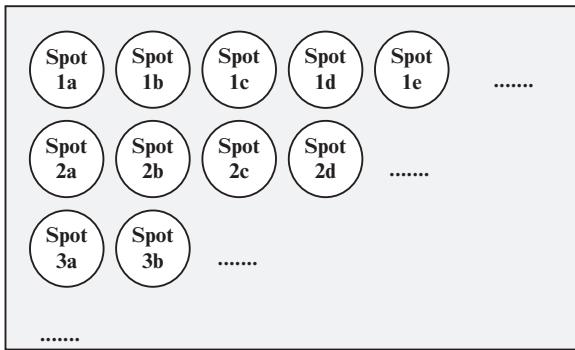


Fig. 1. Scheme of a DNA microarray. Each spot represents a specific probe, which is characterised by sequence and length and can be a chemically synthesised oligonucleotide or a cDNA resulting from PCR. The scale of arrays can be adjusted to the application, i.e. all ORFs identified in the genome of an organism can be represented in the array by specific probes to allow comprehensive gene expression analysis, or a limited number of genes is queried by probes in the array for a focussed application.

a length of 400–1,000 base pairs (bp) on positively charged nylon membranes. Microarrays typically have the size of a microscopic slide with up to approximately 100,000 spots/cm² and are made of glass substrates onto which oligonucleotides are spotted or synthesized. When similarities to electronic devices such as the high degree of miniaturization and the application of corresponding fabrication technologies are highlighted, DNA microarrays are also called DNA chips.

DNA arrays are considered to be one of the major analytical tools, with which the information from the various gene sequencing programmes can be explored. Nowadays sequences that are characteristic for specific organisms or a particular feature of an organism, or sequence variations [mutations or single nucleotide polymorphisms (SNPs)] associated with disease are known. Thus, it is possible to distinguish pathogenic from non-pathogenic microorganisms and identify features such as antibiotic resistances not only by microbiological methods but via the detection of the corresponding genes without the need to cultivate the respective organism (4). DNA samples can be analysed simultaneously for several mutations in genes, which could increase the probability for certain diseases (*see* Chap. 4) or influence the metabolism of drugs (5). Most of these data were accessible in the past using methods such as Southern blots, gel or capillary electrophoresis in combination with PCR and restriction fragment length analysis, etc. With the introduction of DNA microarrays, however, it became possible to simultaneously analyse several hundreds of genes, even all genes in an organism, i.e. the whole genome. Applications range from genotyping of organisms, gene expression analysis for example to classify patients according to the type of disease (*see* Chaps. 2 and 3) or to guide therapy, and gene–protein interaction analysis,

which is relevant for the identification of binding sites of proteins that regulate gene expression (6). Among these application areas gene expression analysis or “transcriptomics”, as it is called, when it is performed on a genome-wide scale, is the best established, whereas genome-wide analysis of interactions between proteins and DNA is a more recent extension of chip applications (7, 8). These investigations are called ChIP-chip experiments as they are based on the combination of chromatin immunoprecipitation (ChIP) and DNA chips. The following section is focussed on the technological fundamentals of DNA chips as background information for the application-oriented following chapters of this book.

2. Technology

DNA arrays are based on the specific base pairing of complementary nucleotides (A-T and C-G) leading to double-stranded sequences of nucleic acids. Unlike traditional analysis of blots the strand being specific for the gene under investigation is immobilized as capture probe and the corresponding counterpart, the target, is isolated from the organism or cell culture and present in solution. Thus, probes have to be designed that allow unambiguous identification of genes, as no separation of nucleic acids with respect to size occurs, and the target usually has to be labelled prior to detection. In macroarrays, probes are cDNAs with a length of 400–1,000 bp (e.g. www.eurogentec.com; www.clontech.com), which leads to a high specificity for the targets. In microarrays, probes are oligonucleotides with lengths <200 bp (e.g. www.eppendorf.com; www.operon.com; www.ocimumbio.com; www.agilent.com; www.affymetrix.com), which are chemically synthesized either separately or directly on the chip (www.affymetrix.com). Synthesis on the chip is possible only for rather short oligonucleotides of 25 bases. As the specificity of gene detection is reduced, when short oligonucleotides are used, on those chips the recognition of a single gene is based on the combination of several probe oligonucleotides covering an extended sequence of the target gene (*see Chaps. 2.1 and 2.2*) and control oligonucleotides with a mismatch base in the centre of the sequence. When longer probes are used (at least 50 bp), for each gene a single specific probe is designed. All probes are immobilized on the substrate in an ordered two-dimensional pattern (**Fig. 1**), and on a single slide all open reading frames (ORFs) of a genome or only a subset of genes can be represented (2).

2.1. Probe Design

The design of probes and the choice of hybridization conditions are crucial points for chip development and application and still offer room for improvements (9). Here, only some general aspects are mentioned:

The most important aspect is the specificity of the probe for the gene of interest. In gene expression analysis RNAs are isolated from the cells and labelled cDNAs are produced by reverse transcription. The whole mixture is applied to the array and allowed to hybridize (Fig. 2) (10). Thus, each probe should bind only the transcript of the respective target gene among the presence of all other transcripts. To achieve this degree of specificity with a single probe per gene a minimal length of 50 nucleotides per probe was reported (11). When shorter oligonucleotides are used, several probes should be combined for each gene. Bioinformatic tools and services are offered, which help to design probes of suitable sequences and lengths. The detection of mutations or SNPs in genes does not allow the free choice of the probe sequence,

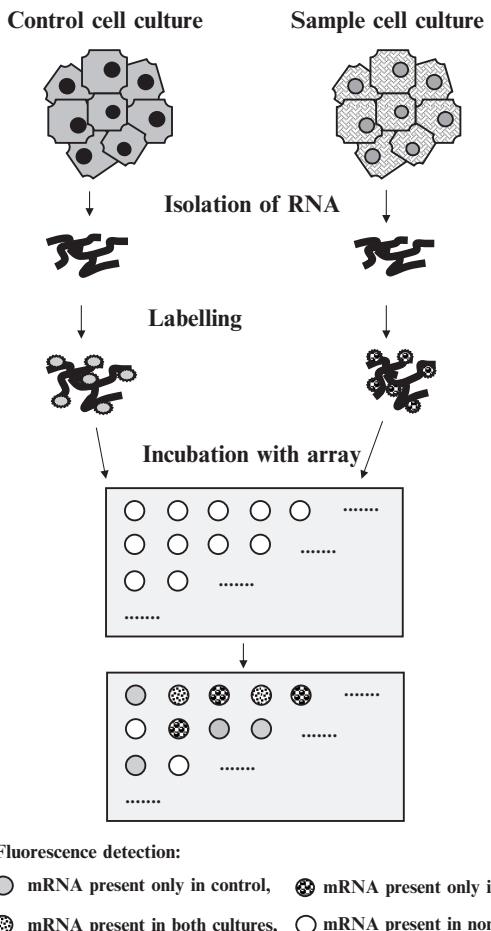


Fig. 2. Scheme of the experimental procedure.

because this is given by the sequence neighbouring the variable region in the target gene (6).

The hybridization reaction of nucleic acids is characterized by the affinity constant and by the kinetic constants of the association and dissociation reaction. The affinity between two single strands (i.e. probe and complementary sequence on the target) is influenced by the sequences of the nucleic acids and by experimental conditions. The guanine – cytosine (G – C) base pair contains three hydrogen bonds, the adenine – thymine (A – T) base pair only two, so that G – C-rich sequences are more stable than A – T-rich sequences of the same length. Moreover, as each base pair contributes to the strength of the overall binding, the affinity increases with the length of the interacting sequences, i.e. the number of matching nucleotides (12). If the sequence of matching base pairs is interrupted by a mismatch, this does not totally prevent hybridization of both strands but leads to a reduced stability (13). The degree of destabilization is dependent on the lengths of the remaining perfectly matching sequences and, thus, on the position of the mismatch. Usually double strands are less stable, when the mismatch is localized in an internal position compared with mismatches at the end of the sequence (13, 14). Thus, signal intensities increase with the length of the probe and decrease, if mismatches are present in the centre of the sequence. If, however, variations in nucleotide sequences have to be detected, such as in the analysis of SNPs, too long probes are not suitable, as it is more difficult to find hybridization conditions, which allow the distinction between completely matching sequences and single mismatches. Thus, for SNP detection another assay format was developed, called minisequencing, in which the specificity of DNA polymerases for completely matched double strands as substrates in the extension reaction of primers is utilized. The 3'-ends of corresponding probes are the nucleotides of interest, so that the primer is extended by a labelled nucleotide only in the case of a perfect match. With different probes for each SNP to be investigated, which query the different possibilities for the SNP, identification of the respective sequence is possible (15). However, there is no free choice of probe sequences; only the length of the probes is a variable, which can be used for the adjustment to experimental conditions.

Even for given nucleic acid strands the affinity between the two complementary strands can be influenced by additives to the hybridization solution (16). It was found that formamide inhibits the formation of hydrogen bonds, and thus it reduces the stability of the double-stranded helix. Moreover, nucleic acids are negatively charged due to the phosphate groups of the nucleotide backbone. Without compensation of this charge by counterions even complementary single strands are electrostatically repelled and monovalent cations (Na^+) are added to enable formation of

double strands. Another important experimental parameter is the hybridization temperature (14, 16), as double strands are separated into single strands by increasing the temperature, a reaction called “melting of DNA”. The temperature, at which 50% of the double strands are dissociated, is called the melting temperature T_m of the sequence and is used for characterization of the stability of the double strand. For oligonucleotides it can roughly be calculated from the sequence by using the approximation

$$T_m = 2^{\circ}\text{C} \times (A - T) + 4^{\circ}\text{C} \times (G - C), \quad (1.1)$$

with (A-T) being the number of A-T pairs and (G-C) the number of G-C pairs in the sequence.

For longer hybrids the approximation

$$T_m = 81.5^{\circ}\text{C} + 16.6 \log [c_{\text{Na}^+}] + 0.41(\%G - C) - 500 / n \quad (1.2)$$

is used with n being the length of the hybridising strand and c_{Na^+} the concentration of Na^+ ions.

If T_m exceeds the hybridization temperature by 10–15°C, efficient hybridization is observed. If the hybridization temperature is much lower than T_m , hybridization of strands containing mismatches or being only partly complementary will also occur.

However, the length of the probe is important not only for the specificity of the hybridization and the stability of the double strand, but also for the kinetics of the reaction. The hybridization rate is mainly determined by the access of the targets to the immobilized probes, which is influenced by the density of the immobilized probes (17), the length and complexity of the sequence, and by the diffusion rate, which depends on temperature, concentrations, and viscosity of the target solution. In longer probes secondary structures may be formed and the complexity of the sequence, i.e. the degree of non-repetitive sequences, increases; both also lead to a reduction of the hybridization rate. It was found that hybridization equilibrium is reached only after at least 24h hybridization time, even if oligonucleotide probes are used (14).

2.2. Immobilization

Probes, which are prepared by PCR or by chemical synthesis of oligonucleotides, are applied to the substrate surfaces with spotters, which allow the deposition of pL to nL volumes (10, 18). Contact spotters dip pins into the probe solution and place the adhering liquid to the substrate by touching the substrate surface. Non-contact spotting relies on the generation of drops from a capillary with piezoelectric pumps. The major disadvantage of contact spotters is the need for precise adjustment of the height of the pins so that contact forces and area are the same for all spots. This is difficult to achieve in particular, when a number of pins are combined for the simultaneous deposition of several

probes. Non-contact spotting requires a clean environment to prevent blocking of capillaries by ambient dust. In any case the low volumes rapidly evaporate, so that fast-binding reactions are a prerequisite, though evaporation times can be prolonged by additives, for example glycerol. Additionally, environmental conditions, such as temperature and humidity, should be controlled to increase the reproducibility of spot quality. Spots typically have a diameter of 100–200 µm with spacings in the same order of magnitude (200–300 µm), so that up to 244,000 features (spots) are combined on a single slide (www.agilent.com).

Nucleic acids (oligonucleotides or PCR products) are immobilized on glass slides or nylon membranes (3). However, systems based on beads or tubes are also available (www.illumina.com; www.clondiag.com).

As nucleotides are negatively charged, they interact by electrostatic attraction with positively charged surfaces as delivered by nylon membranes or glass slides pre-treated with poly-L-lysine [e.g. (10); <http://cmgm.Stanford.edu/pbrown/protocols>] or aminopropyltriethoxysilane (APTS or GAPS) [e.g. (19); www.corning.com]. If PCR products are used, they are denatured either prior (19) or after spotting. Usually, UV irradiation is recommended as an additional cross-linking step, but heating to 60 and 120°C is also possible (10). As each nucleotide contributes with an additional charge, the electrostatic forces increase with increasing length of the nucleic acids, which makes this immobilization method applicable mainly for longer probes, such as cDNAs. Zammattéo et al. (19) showed that for a 255bp capture probe the efficiency of this electrostatic attraction exceeded the efficiency of even covalent attachment.

The alternative to immobilization via physical interactions is covalent binding. This requires the availability of suitable functional groups on both the probe to be immobilized and the immobilization substrate, usually glass. Amino-functionalized nucleic acids can be coupled easily to epoxy- or aldehyde-modified glass surfaces (10). Resulting Schiff bases can be reduced by sodium borohydride. This method proved to be highly effective and specific and suitable for the application of very small volumes of liquid (19).

As solid-phase synthesis of oligonucleotides is well established, oligonucleotide capture probes can also be synthesized directly on the chip surface. This was described by Pease et al. already in 1994 (20), and later (1996) by Weiler and Hoheisel (21) and Blanchard et al. (22). The basic reaction is the reaction of phosphoramidite-activated deoxynucleosides with suitable functional groups, usually hydroxyl groups, on the glass or polypropylene surface. At Affymetrix these hydroxyl groups are generated at selected sites by illumination of the chip through an appropriate mask (1, 18 20). In a first step the solid support is derivatized with a covalent

linker molecule terminated with a special, photolabile protecting group. Illumination leads to deprotection and the formation of hydroxyl groups. In the next step the nucleoside derivative to be coupled is added, being the corresponding 3'-phosphoramidite and 5'-photoprotected. Illumination with another mask generates a different pattern of hydroxyl groups allowing each desirable sequence to be synthesized. As the number of probes in one array is limited by the physical size of the array and the achievable photolithographic resolution, approximately 750,000 oligonucleotides were synthesized on $1.28 \times 1.28 \text{ cm}^2$ chips with a spot diameter of only 5 μm (www.affymetrix.com). A sequence of 200–300 bases of the gene of interest is chosen and a number of non-overlapping 25-mer probes are designed and synthesized on the chip together with mismatch control probes containing a single base difference in the central position. This redundancy should improve accuracy and improve the signal-to-noise ratio.

2.3. Detection Principles

Hybridization of a target nucleic acid to the immobilized probe is an affinity reaction between two complementary reaction partners. Hence, this reaction was followed in real time by affinity sensor systems, such as surface plasmon resonance (SPR) devices [e.g. (13, 17, 18)], resonant mirrors (12), or grating coupler systems (23). Real-time monitoring of the hybridization allowed the analysis of the influence of probe and target length, probe and target concentration, and the position of mismatches not only on the resulting steady state signal but also on the association and dissociation rates. It was shown that the affinity constants determined by SPR correlated well with melting temperatures and that decreasing affinities due to decreasing lengths of the target influenced mainly the dissociation rate (13).

Usually the detection of mRNAs utilises specific features of nucleic acids, i.e. the possibility to synthesise a copy DNA strand (cDNA) by a reverse transcriptase reaction, which allows the integration of labelled nucleotides as components of the reaction mixture. Suitable labels are radioactive isotopes, such as ^{33}P or ^{32}P (www.clontech.com), fluorescent dyes, biotin (www.clondiag.com), amine groups, or micro- and nanoparticles (3, 10, 24). Labelling with biotin or with amine groups requires additional staining, e.g. with streptavidin conjugates or conjugates of an anti-biotin antibody with horseradish peroxidase or with gold (www.eppendorf.de) or with amino-reactive fluorescent dyes. The advantage of gold labels is the light pink colour, which appears on the arrays and which can be amplified by silver deposition from the reduction of silver ions with hydroquinone, so that successful hybridization is visible and can even be quantified by a simple flatbed scanner (24). With fluorescence detection, however, it is possible to use different dyes for the control and the sample, and combine both labelled mixtures during hybridization so that a

direct comparison of signals on the same array is possible (**Fig. 2**). As fluorescence intensities and labelling efficiencies usually are different for different labels, a dye-switch has been performed, i.e. the dye previously used to label the control has to be used to label the sample and vice versa.

Consideration of the aforementioned labels usually integrated in cDNAs (Cy3, Cy5, fluorescein, Alexa 647, phycoerythrin, biotin) shows that the most often used detectors are fluorescence detectors allowing the analysis of chip surfaces. Light sources are preferably lasers with the appropriate wavelengths. Nowadays systems are available with more than one light source, which allow the excitation of different dyes. The emitted fluorescence is captured by CCD cameras or by photomultipliers with the latter showing the higher sensitivity.

3. Data Analysis

A two-dimensional pattern of spots of different intensities results from scanning the array (**Fig. 2**). Independent of the detection principle signals are converted to electrical signals and, thus, appear in a grey scaling. Typically signals from the control and the sample are shown with different colours, which, however, are not the true colours of the labels, but are artificially chosen.

Because of the highly regular arrangement of spots, grids specifying the spot locations can be easily overlaid on the images. To quantify the signal intensity of each spot the background has to be quantified, which is usually done by measuring signals in a circle surrounding the spot. These background values are used for correction and filtering and only data that are significantly above the background are considered for further evaluation. Significant intensities are two standard deviations above the background level (10). These data are the basis for the comparison of different samples, which requires further data treatment, such as data transformation to a logarithmic scale, calculation of ratios, and normalization to account for different amounts of nucleic acids in each sample or for different labelling efficiencies. The final outcome of data analysis should be a matrix, in which for each gene (row in the matrix) a quantitative measure is given for each sample (column in the matrix) (**Fig. 3**). There are several challenges, which make a direct access to these data difficult, some of which were already mentioned (different signal intensities resulting from different labels and varying amounts of nucleic acids). In addition, a single gene may be represented by several probes; each array may be present several times on the same slide, and the experiments should be repeated to give biological replicates.

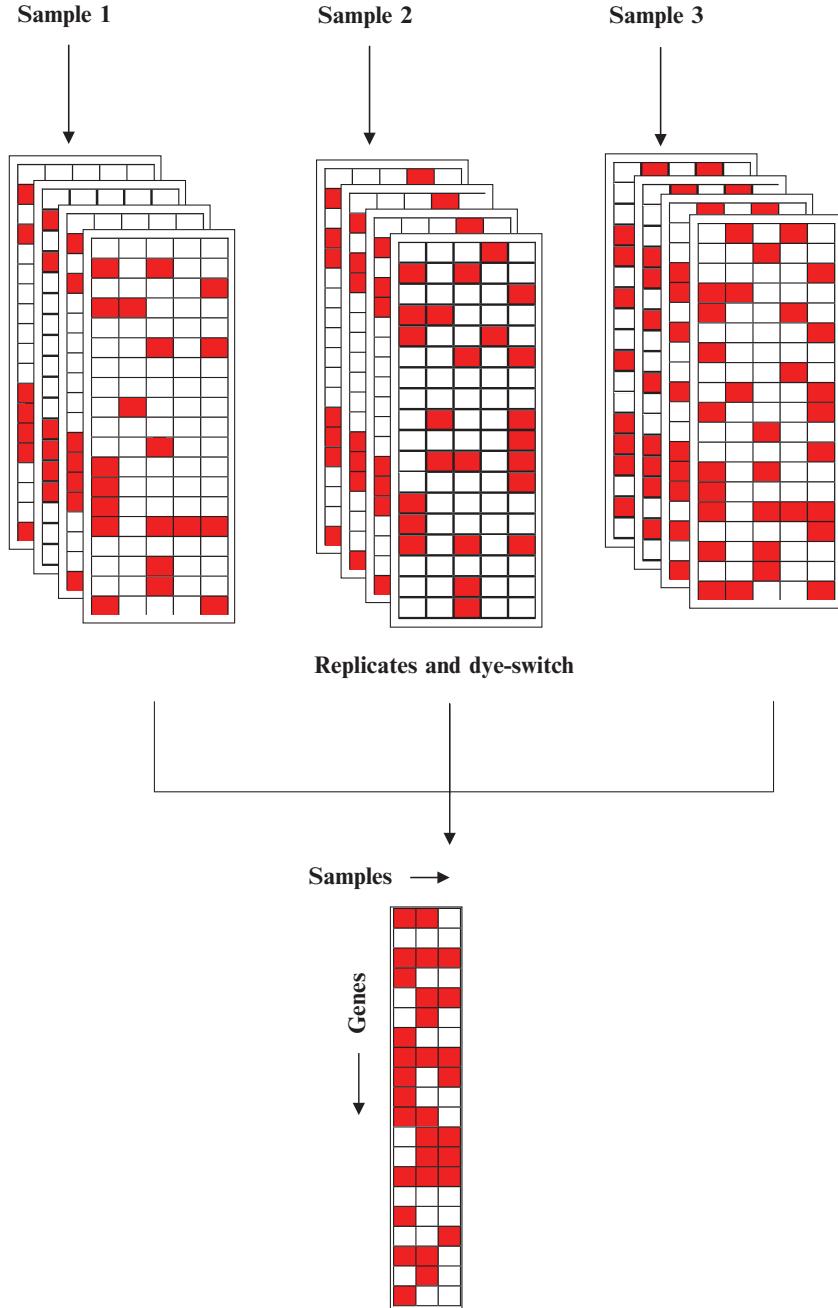


Fig. 3. Simplified scheme of data analysis. Signals are indicated just as qualitative data as “present” or “absent”, whereas in practice quantitative information can be obtained from data acquisition. From each sample several values exist for each gene, as experiments have to be repeated (replicates), a dye-switch may be necessary, or each gene is represented by more than one probe on the chip. This information has to be extracted, so that finally in each sample or experimental condition a single value can be assigned to each gene, which is represented on the chip. This results in a matrix, in which the samples are the columns and the genes are the rows. Cluster algorithms are used to cluster genes according to a similar behaviour in the different samples.

All these data have to be combined to a single value for each gene, and the procedures to achieve this goal are not standardized. Thus, according to the guidelines of Minimum Information About a Microarray Experiment (MIAME) (www.mged.org/Workgroups/MIAME/miame.html), which are proposed by the Microarray Gene Expression Database group (MGED), not only the final data, from which conclusions were drawn, are to be delivered, but also information about data processing routines and the raw data (25).

4. Examples for Applications

4.1. DNA Analysis

Microarrays are used for genotyping, as they allow the detection of specific genes or gene variants. Pathogenic bacteria (26) and fungi [(27), www.clondiag.com] can be detected via species-specific sequences within the ribosomal DNA, and for the analysis of resistances against antibiotics sequences that are specific for β -lactamase genes can be used (4). For example 27 and 28 oligonucleotides, respectively, with a length of up to 24 bp proved to be sufficiently specific and were immobilized as probes on glass slides to distinguish 12 *Candida* and *Aspergillus* species or several bacterial groups (gram-negative and gram-positive) and species (e.g. *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Staphylococcus aureus*). For pre-enrichment of organisms samples were incubated overnight (26) or for up to 72 h (filamentous fungi). After DNA isolation, PCR was performed to amplify the target region of the DNA and introduce fluorescent labels. Hundred and twelve out of 115 strains of bacteria isolated from food (26), and in 16 out of 21 clinical isolates the fungal strains (27) were correctly identified requiring just a single relatively short cultivation step.

The detection of sequence variants, such as mutations or SNPs that are causes of diseases, was described as early as 1989 (15, 28). A frequently applied format for SNP analysis is minisequencing or allele-specific primer extension, in which the 3'-end of the probe on the microarray is the variable nucleotide. The primer can only be extended with a fluorescently labelled nucleotide by a DNA polymerase, if there is a perfect match. Thus, fluorescent spots indicate the matching sequences. Alternatively, with high stringency conditions hybridization to only allele-specific probes can be achieved, so that no additional extension reaction is required. For reliable analysis sense and anti-sense probes should be used, with different probes for all four possible nucleotides at the target position within the sequence. The length of the probe, the position of the target nucleotide

(close to the centre of the probe), and hybridization conditions have to be carefully optimized, as for high-quality SNP results only perfectly matching sequences should give a hybridization signal (www.febit.eu).

4.2. mRNA Analysis

Transcriptional profiling, i.e. the detection of mRNAs that are present in the cell or tissue at the time of sampling, is one of the major application areas of DNA microarrays (3). As these data indicate, which genes are required in a given state of the cell or organism, the profiles are used to distinguish pathogenic from healthy states (e.g. cancer diagnosis) and guide therapeutic strategies, to examine the functions of genes or elucidate the mode of action of drugs (29) or toxic compounds (30, 31). Suitable microarrays can comprise oligonucleotides but also cDNAs, and may cover all ORFs identified within the genome or just a subset of ORFs.

Repetition of experiments in different laboratories, with different array platforms and even in the same laboratory usually shows more or less significant variations. These are partly due to different experimental protocols starting from sample preparation to data analysis and can be minimized by standardization of as many steps as possible (31). Other fluctuations, however, are due to biological noise, which means that the expression of some genes is inherently sensitive to already minor differences in cell treatment. These genes can only be identified by repetitive analysis and should be eliminated from the analysis of transcriptional profiles. Thus, the reliability of an expression signature is not necessarily improved when the number of genes in an array is increased.

4.3. Analysis of Protein-DNA Interaction

DNA-binding proteins perform a variety of important functions in cells, such as the regulation of gene expression. Thus, the analysis of interactions of transcription factor proteins with their respective DNA-binding sites in response to environmental stresses or associated with the progression of diseases has become an important issue, and several new techniques were developed, which allow this type of analysis on a genomic scale. Among these, ChIP-chip analysis, also called ChIP-on-chip analysis, and the DamID technologies are based on microarrays (7, 8).

ChIP-chip is the combination of chromatin immunoprecipitation with microarray detection. The cellular sample is treated with formaldehyde so that DNA-binding proteins are covalently attached to their target DNA. Shearing of the chromatin, immunoprecipitation of the protein-DNA adduct from nuclear extracts with specific antibodies, and reversal of the formaldehyde cross-links leads to an enrichment of the target DNA sequences. They are amplified, labelled with a fluorescent dye, and finally hybridized to the microarray. Comparison to a reference sample

without enrichment shows the binding regions of the protein of interest. This analysis was first applied to transcription factors of the yeast *S. cerevisiae* and showed that binding of regulatory proteins occurred even several kilobases away from the transcription start of genes. The major drawbacks of this principle are the limited availabilities of suitable antibodies and microarrays and the low expression of some transcription factors. The first ChIP-chip experiments were performed with microarrays spotted with PCR amplicons covering essentially all intergenic regions. With the utilization of microarrays with oligonucleotides designed to tile a portion of the genome, binding sites of proteins can be defined with higher resolution. However, this type of microarrays is available only to a limited degree. Low expression of transcription factors or antibodies with poor affinities results in only a poor enrichment of the target sequences. This problem is overcome to a certain extent by the DIP-chip assays and by the DamID technology. DIP-chip assays are based on purified proteins, which are in vitro incubated with genomic DNA fragments so that binding occurs. The concentration of the protein is defined and known, and as it is expressed with a suitable tag, it can easily be separated from the sample and the bound DNA sequences are analysed as described earlier. However, as this is an in vitro assay, results do not reflect a physiological state (8). DamID technologies are based on overexpression of the protein of interest as a fusion to Dam (DNA adenine methyltransferase). Dam methylates adenine in the vicinity of the protein binding site. Digestion with a methyl-specific restriction enzyme, amplification, labelling, and hybridization then highlight the target regions. However, definition of binding sites with high resolution is not possible, because methylation can extend over a few kilobases from the binding site (7).

These analyses are recent extensions of the applications of DNA microarrays, and are yet mainly used in research.

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Chapter 2

Discussion of the Applicability of Microarrays: Profiling of Leukemias

Torsten Haferlach, Ulrike Bacher, Alexander Kohlmann, and Claudia Haferlach

Summary

Leukemias are classified according to clinical, morphologic, and immunologic phenotypes, caused by specific genetic aberrations in association to distinct prognostic profiles. Usually the subtypes are defined using complementary laboratory methods, such as multiparameter flow cytometry, cytogenetics in combination with fluorescence *in situ* hybridization, and molecular methods such as the polymerase chain reaction. The genetic variations of the different subtypes lead to distinct changes also in gene expression, which is comprehensively analysed by DNA microarrays. Thus, first gene expression profiling studies showed that analysis with whole-genome DNA microarrays leads to a prediction accuracy of 95.6% with respect to the classical methods, and even allowed a further distinction of subtypes. It is expected that diagnostic strategies can be optimized with this new technology and that the understanding of the molecular pathogenesis of leukemias will be significantly improved. This could also lead to the identification of new targets for future drugs.

Key words: Gene expression profiling, ALL, AML, CLL, Classification of subtypes

1. Introduction

Acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic lymphatic leukemia (CLL) are very heterogeneous disorders composed by a variety of different subtypes. These subtypes are defined by different clinical, morphologic, and immunologic phenotypes caused by specific genetic aberrations in association to distinct prognostic profiles. Chronic myeloid leukemia (CML) is defined by the Philadelphia or *BCR-ABL*

translocation, but the progress of stages is accompanied by acquisition of additional chromosomal abnormalities (“clonal evolution”).

In AML clonal karyotype abnormalities are detected in ~55% of all cases; in ALL in ~80%. These abnormalities cover a broad spectrum of numerical changes and balanced and unbalanced translocations. Karyotype is the strongest prognostic parameter in AML and in ALL: In AML survival ranges from ~75% in the patients with the favorable reciprocal rearrangements t(8;21), t(15;17), and inv(16)/t(16;16) to <10% in complex aberrant karyotype, which is defined by the simultaneous occurrence of 3 clonal chromosomal changes. In childhood ALL the reciprocal t(12;21) rearrangement shows an excellent prognosis with >90% long-term survival following standard chemotherapy, whereas Philadelphia positive ALL with the t(9;22) shows survival of ~40% even when allogeneic stem cell transplantation is performed.

Cases with normal karyotype can in the majority of cases be further characterized and categorized by molecular investigations. The molecular mutations are likewise heterogeneous and affect genes coding for transcription factors, tyrosine kinases, protooncogenes, or tumor suppressor genes, which show specific interactions.

In the majority of CLL cases it was as well possible to determine prognostically relevant genetic aberrations, such as deletions of the 17p53 tumor suppressor gene, which are associated to an inferior outcome. In CML clonal evolution is found in ~5% of all patients at diagnosis only, but the acquisition of additional karyotype abnormalities during follow-up predicts progress and serves as a marker of progression.

This diversity of leukemia subtypes can only be mastered by a combination of complementary laboratory methods: cytomorphology/chemistry, immunophenotyping by multiparameter flow cytometry (MFC), cytogenetics in combination with diverse fluorescence in situ hybridization (FISH) techniques, and molecular methods such as the polymerase chain reaction (PCR). This interactive approach only allows an optimized risk stratification as basis for individualized therapeutic decisions. Beyond that, the interplay of these methods paved the way to targeted therapy in some leukemia subtypes, e.g., in acute promyelocytic leukemia (APL) with t(15;17)/*PML-RARA*. This AML subtype is characterized by a differentiation stop in granulopoiesis and by aberrant promyelocytes with multiple Auer rods (“faggot cells”). Therapy with all-trans retinoic acid (ATRA) eliminates the differentiation stop in granulopoiesis and can prevent the life-threatening complications that often result from coagulopathy, which is pathognomonic for this leukemia subtype.

However, all leukemia subtypes as defined by specific genetic changes show variability with respect to response to therapy, risk of relapse, and long-time survival within the different subgroups. This can again be exemplified in APL where the combination of ATRA and standard chemotherapy results in long-time survival of ~75%, removing this AML subtype from the list of hematologic malignancies requiring hematopoietic stem cell transplantation (HSCT) as first-line strategy (1). However, ~20% of patients relapse within 2 years. In the minority of cases coincident mutations in the *FLT3* class-III-receptor tyrosine kinase conferring a negative prognostic impact can be identified (2, 3), but in the majority of cases relapse remains unexplained.

Furthermore, even the combination of these extended cytogenetic and molecular analyses does not succeed to detect a disease-defining aberration in all leukemia cases. Approximately 10% of all AML cases cannot be further classified, which results in high uncertainty at diagnosis and during the course of disease, as modern treatment concepts encompass minimal residual disease (MRD) diagnostics to assess the residual leukemia cell burden. This requires the knowledge of specific genetic aberrations, which can be sensitively monitored by interphase FISH or real-time PCR.

These vacancies make more diversified classification systems based on the underlying molecular pathogenic desirable (4). In addition, the earlier summarized comprehensive diagnostic approach including broad PCR screening and the clarification of more complex chromosomal aberrations by extended FISH analyses have increased the requirements to time, costs, equipment, manpower, and experience in leukemia diagnostics considerably and enhanced the need for reference laboratories.

Gene expression profiling (GEP) on the basis of the microarray (MA) technology was found highly appropriate to improve molecular classification in leukemias. The pioneer studies of Golub et al. (5, 6) and of Moos et al. (7) who succeeded to reproduce the classification of acute leukemias in adult and infant cases according to T- or B-lymphatic lineage or AML by limited sets of discriminatory genes initiated the performance of GEP studies in hematology. Yeoh et al. included successfully diverse cytogenetic, immunologic, and molecular subtypes in GEP analyses and demonstrated that "first class prediction" was possible for any of these leukemia classification systems. This study group showed in addition the predictability of response to therapy and the individual risk of therapy induction of AML in ALL cases (8). The latter approach was beyond the possibilities of all so far established diagnostic methods. It is expected that GEP is further able to define new subclasses within apparently homogeneous leukemia subentities ("class discovery"): Alizadeh et al. succeeded to separate diffuse large cell B lymphoma (DLCL) into two different

new and prognostically relevant subtypes due to molecular B-cell characterization (9).

Thus, GEP opens up new vistas in leukemia diagnostics and therapy of hematologic malignancies. An inclusion in hematologic routine and in clinical testing will probably be realized already in a few years from now. Thus, a comparison with established standard diagnostic methods and the definition of its position in the diagnostic panel should be performed without delay (10).

2. Technical Aspects

Assays for leukemia investigation mostly use spotted arrays, which are constructed on the basis of DNA collections and allow specific gene selections. The immobilized probes on the array platform contain either cDNA, oligonucleotides, or genomic fragments. cDNA arrays contain PCR products of cDNA clones of the genes of interest, which are spotted on nitrocellulose filters or glass slides. This technique is very flexible, as the panel of gene probes on the array surface can be individually chosen. Oligonucleotide arrays are more specific than the cDNA arrays, as they can be tailored to minimize the risk of cross-hybridization and show high reproducibility (6, 11).

Frequently used chip designs are, e.g., the HG-U133 Plus 2.0 (Affymetrix) containing ~42,000 genes. Hybridization is based on ~5 µg patient's RNA, which is transcribed in cDNA and then amplified in a transcription reaction accompanied by incorporation of biotin-labelled nucleotides. Subsequently, ~10 µg biotin-labelled patient's cRNA is hybridized on the MA with the complementary gene sequences. The resulting DNA/cRNA hybrids are coloured by the fluorescence marker streptavidin-phycocerythrin, which is excitable by an argon laser. This is accompanied by negative controls and by controls with housekeeping genes (**Fig. 1**).

Analyses are mostly performed from mononuclear cells, often following Ficoll-Hypaque density gradient. They can as well be performed from whole blood or from specifically selected cells (e.g., CD34+ stem cells). As leukemia samples mostly contain >75% of malignant cells and show a homogeneous cell composition, they allow optimized conditions in the GEP analyses.

The preparation of leukemia samples by different operators and the use of different sample-handling procedures do not affect the robustness of gene expression patterns (12), which further qualifies GEP for the inclusion in hematological routine.

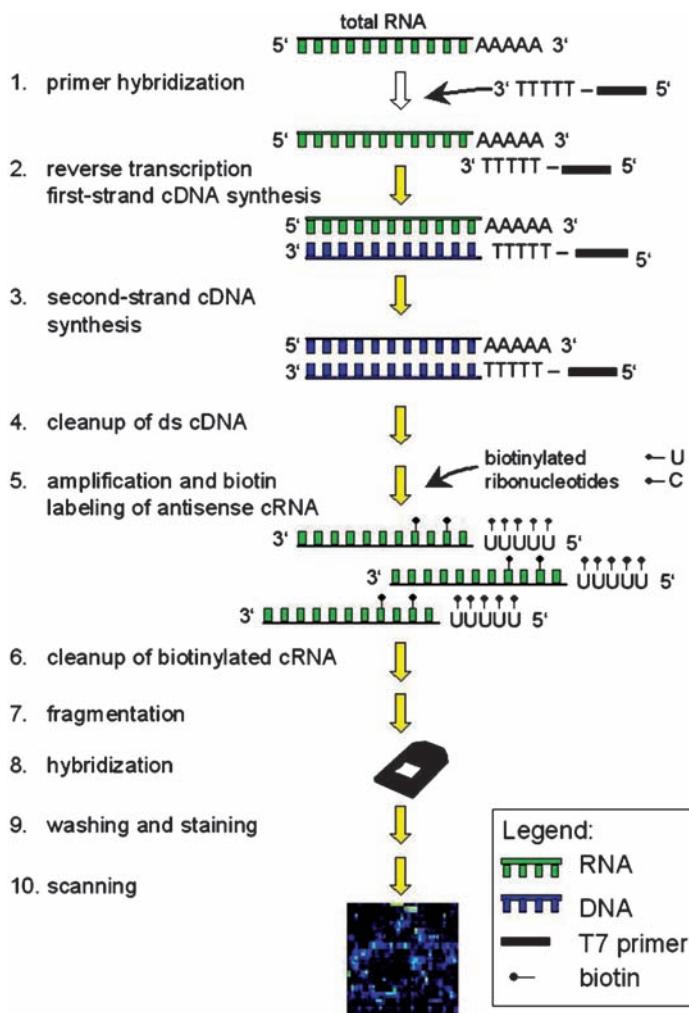


Fig. 1. Workflow for microarray experiments, as an example Affymetrix protocol for HG-U133 Plus 2.0 is given.

3. Data Analyses

Data mining in leukemia classification uses two different types of analyses ([Fig. 2](#)): Supervised analyses group the patients according to already known characteristics such as clinical parameters or survival and correlate the array results with these data. Unsupervised analyses test whether specific characteristics, e.g., distinct mutations, are also reflected at the level of gene expression signatures without predefinition of groups of interest. Acute leukemias are often analyzed by multiclass statistical analyses due to the

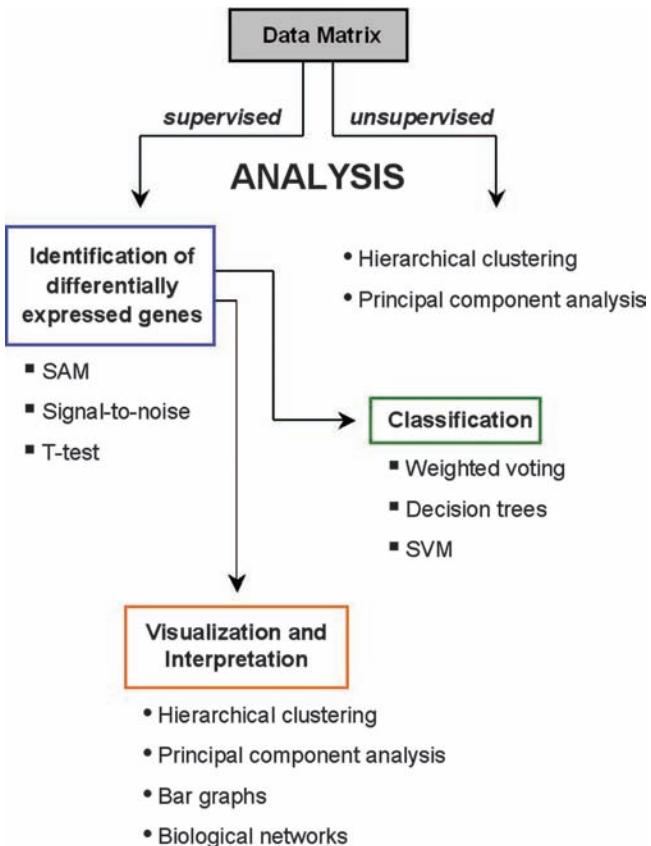


Fig. 2. Algorithm for a possible data management in gene expression profiling.

necessity of a comparison of different leukemia subclasses. Gene expression levels discriminating the known groups are selected by variants of common statistical tests. Differential gene expression is followed by different machine-learning algorithms, which are applied for an adequate classification of the samples into these known groups. Ideal class prediction is performed by dividing gene expression data into training and independent test sets.

Data are organized by hierarchical clustering according to similar signatures (Fig. 3). It leads to a reduction of data complexity and more understandable visualization. Hierarchical clustering further predicts the categorization of unknown samples and thus provides useful information about the relationship between adjacent clusters. Reduction of the multidimensional and matrix-like structured array data into a new set of variables in principal component analysis (PCA) is used to reduce the dimensionality of array data and visualize large data sets (Fig. 4).

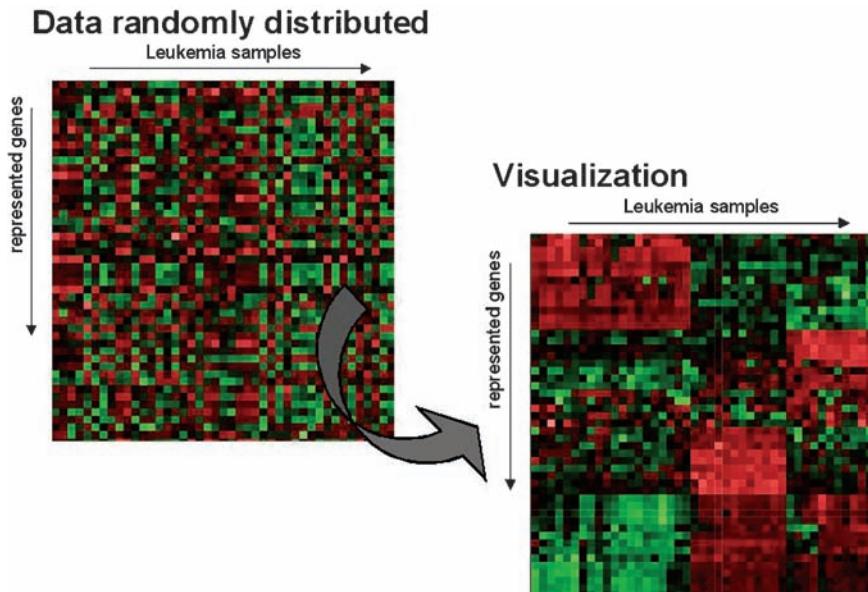


Fig. 3. Example of data visualization for different genes (*top to bottom*) and different samples (*left to right*) to detect respective subclasses and cluster of genes or biological entities of leukemias.

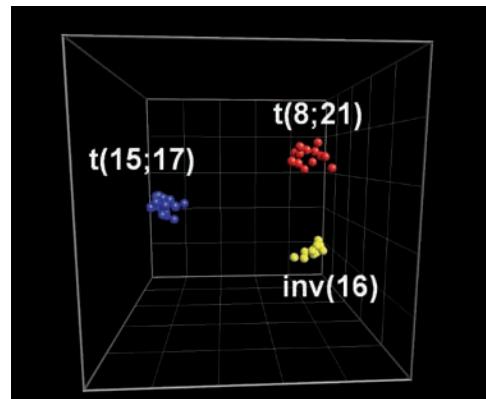


Fig. 4. Principal component analysis of three different subtypes of leukemia that cluster together due to their genetically defined biology but are clearly separated from each other (each sphere represents one individual patient).

4. Acute Myeloid Leukemia

4.1. Cytomorphology in AML

Morphology in combination with cytochemistry still plays a central role in AML. These fast available methods allow an initial classification in >80% of all AML cases and allow the discrimination

between AML and myelodysplastic syndrome (MDS). Furthermore, experience in these methods allows the guidance of more specific and labor-demanding methods, e.g., cytogenetics on PCR. The most established morphologic classification system in AML is the FAB classification of 1976 (13, 14) dividing AML in the subtypes M1–M7. This classification was revised and new aspects were added by the WHO in 2001 (15).

It was shown that the FAB subtypes M3 and M3 variant (M3v), which are the morphologic correlates to APL with t(15;17)/*PML-RARA*, M4eo as correlate to inv(16)/*CBFB-MYH11*, and M6 characterized by >50% erythropoiesis in the bone marrow, can be separated from all other FAB subtypes with 100% accuracy with a very limited number of discriminative genes (16). Thus, the FAB categories can be reproduced by GEP.

GEP is further able to reproduce both morphologic subtypes of APL/t(15;17)/*PML-RARA*, FAB M3 and M3v. M3v is characterized by bilobular cells; M3 shows the classical “faggot cells.” The M3v subtype is prognostically inferior than the M3 subtype, which can only in part be explained by a higher coincidence of internal tandem duplications of *FLT3* (*FLT3-ITDs*, *FLT3-LM*) (2, 3). GEP analyses comparing the M3 and the M3v subtypes revealed differential expression of genes coding for cell granulation and maturation. These differences in the gene signatures gave support for the different clinical and morphologic profiles (17).

4.2. MFC in AML

Immunophenotyping by MFC is essential for diagnosis and classification of the acute leukemias. This method is able to quantify and characterize blasts, to define the involved lineages, and to provide important prognostic parameters such as ZAP70 or CD38 in B-CLL. It further provides the basis for minimal residual disease diagnostics (MRD), as the individual leukemia-associated immunophenotypes (LAIP) can be determined at diagnosis and be very sensitively monitored during follow-up.

We analyzed the correlation between protein expression of cells as assessed by MFC and of gene signatures as assessed by abundance of the corresponding mRNA in GEP and found a high congruence between both methods in AML. This was especially true for antigens, which have a high value in leukemia diagnostics and classification, e.g., the myeloid antigens CD13 and CD33 or the progenitor marker CD34 (18, 19). Thus, GEP is able to reproduce the protein expression data revealed by MFC.

4.3. Cytogenetics in AML

As karyotype aberrations in AML are highly important for risk stratification and subclassification, many efforts were made in recent years to investigate the correlations to GEP analyses.

Reciprocal (balanced) interchromosomal translocations leading to aberrant gene fusions play an important role in the

acute leukemias. In AML ~25% of all adult patients show the t(8;21)/*AML1-ETO*, t(15;17)/*PML-RARA*, inv(16)/*CBF-B-MYH11*, and 11q23/*MLL* rearrangements, which were combined to the first hierarchy of the WHO classification of AML (15). They all interfere with the transcription process and induce a differentiation stop in granulopoiesis. They probably induce AML in cooperation with activating mutations such as the *FLT3-ITD* or mutations of the *NRAS* protooncogenes (20). The *MLL* rearrangements modify transcription due to their regulatory function for *HOX* gene expression and are involved in methylation and chromatin condensation. In AML approximately 65 different chromosomal regions were so far identified as fusion partners for the *MLL* genes (21). *MLL* rearrangements have a special profile as they occur frequently in therapy-related AML (t-AML) and are associated with a poor prognosis in contrast to the t(15;17), the inv(16), and the t(8;21), which all are more frequent in de novo AML and are prognostically favorable.

In contrast to the unbalanced cytogenetic aberrations where the heterogeneous pathogenetic mechanisms so far were not conclusively defined, the pathogenetic mechanisms could be clearly defined in these leukemia-specific gene fusions. This led to the postulation that AML with balanced abnormalities might represent promising candidates for MA analyses (22). Indeed, several studies confirmed univocally the clear discrimination of specific gene expression profiles of the t(15;17), inv(16), and t(8;21) rearrangements (22–24). Thirty-five genes were sufficient to predict these subtypes and to separate them from normal bone marrow with 100% accuracy (25). The expansion to 39 genes allowed in addition the separation of the 11q23/*MLL* gene rearrangements, which were characterized by upregulation of several *HOX* genes (23, 26).

Thus, GEP was in support of an own category of these recurrent reciprocal rearrangements in the WHO classification. Beyond that, GEP was proven more reliable than the cytogenetic evaluation of these balanced translocations (27) as the latter can be hampered by cryptic rearrangements or by low metaphase quality.

Ten to fifteen percent of all adult AML patients are diagnosed with complex aberrant karyotype outlined by 3 chromosomal abnormalities and by an adverse prognosis. The median overall survival is ~7 months only (28). The chromosomal aberrations cluster in loss of the chromosomes 5q14q33, 7q32q35, and 17p13. This was translated into reduced expression of genes involved in these regions as detected by GEP analyses. Complex aberrant karyotype further demonstrated a significantly higher expression of genes, which are involved in DNA repair (e.g., *RAD21*). Thus, GEP is able to further unravel the biology of AML with a complex aberrant karyotype and probably also the

reasons for chemoresistance, which is frequently observed in this AML subtype (29).

Numerical gain of chromosome 8 as sole or combined chromosomal aberration represents the most frequent trisomy in AML and is frequent in many other hematologic malignancies, too. Its contribution to tumorigenesis is so far unclear, and in the so far established classification systems this aberration does not represent an own entity. GEP analyses failed to clearly separate +8 from AML with normal karyotype in AML. Nonetheless, a gene dosage effect was clearly demonstrated since many genes coded on chromosome 8 such as *FABP5* and *PTDSS1* were expressed at higher levels in AML with +8. There was no evidence of specific regions on chromosome 8 with a significantly higher expression (30, 31). These results support the hypothesis that trisomy 8 has no disease-defining function but probably is a secondary aberration.

4.4. Molecular Genetics in AML

Forty-five percent of all AML patients show a normal karyotype and represent the largest subgroup. However, from molecular aspects this subgroup represents a complex of diverse disorders defined by different molecular mutations. The broadening of the spectrum of known molecular mutations in recent years and the improvement in PCR screening strategies allow subclassification of 80–85% of normal karyotype AML cases.

Most important are mutations affecting receptor tyrosine-III kinases such as the *FLT3* kinase in ~40% of all AML cases. They lead to increased cell proliferation and to a reduction of apoptosis. They can be represented by internal tandem duplications (*FLT3-LM*; *FLT3-ITDs*) in the juxtamembranous portion of the receptor tyrosine kinase, which are prognostically very unfavorable (32–34). Small mutations in the tyrosine kinase domain (*FLT3-TKD*) are less frequent and play a minor prognostic role.

The *FLT3-LM* showed distinct gene expression profiles in several studies (35–37). However, in the study of Schnittger et al. the expression profile of AML with *FLT3-LM* could be clearly separated from several distinct cytogenetic subgroups but not from normal karyotype AML, whereas it was possible to discriminate *FLT3-LM* positive and negative cases clearly within distinct cytomorphologic FAB subgroups. This suggests that the effects of the *FLT3-LM* depend on other genetic aberrations differently distributed in the diverse FAB subtypes (37). Lacayo et al. were able to further classify the *FLT3-LM+* patients: One subgroup showed a marked expression of the *AML2* and a low expression of the *ATRX* in association to poor outcome. Mutations in the *ATRX* gene induce changes in DNA methylation; mutations in the *AML2* or *RUNX3* gene induce alterations in transcription (36). Thus, GEP adds new aspects for a more differentiated classification also within the *FLT3-LM+* subgroup.

NPM1 mutations that affect a nucleocytoplasmic shuttle protein with involvement in a tumor-suppressor pathway represent the most frequent known molecular marker in AML. They were detected in 50% of all AML cases and in 70% of normal karyotype AML in 2005. Prognosis is favorable when the *NPM1* mutations are present as sole mutation, but deteriorated in the presence of a concomitant *FLT3-LM* (38–40). Some GEP studies succeeded to discriminate *NPM1*-mutated cases from other molecular subclasses in AML. Mutated cases showed upregulation of several genes putatively involved in the maintenance of a stem-cell phenotype, suggesting that *NPM1*-mutated cases might derive from a multipotent hematopoietic progenitor cell (41). Further, *NPM1* mutations were found in a cluster with monocytic AML (42). However, further studies are needed to evaluate the role of *NPM1* mutations in GEP, as another study was not able to clearly separate *NPM1*-mutated and unmutated cases (43).

Intragenic *MLL* abnormalities as partial tandem duplications (*MLL*-PTD) occur mainly in normal karyotype AML or in trisomy 11 and are seen in 5–10% of all AML cases contributing a gain-of-function mechanism (44). In contrast to the chimeric (intergenic) *MLL* rearrangements that are found also in ALL the *MLL*-PTD are limited to AML. The prognosis of *MLL*-PTD+ cases is equally poor as in the chimeric *MLL* rearrangements. It was demonstrated that AML with *MLL*-PTD did not cluster with chimeric *MLL* rearrangements (23). This enhances different pathogenic mechanisms in inter- and of intragenic *MLL* mutations in AML.

The studies of Valk et al. and Bullinger et al. focused also on other molecular markers in AML. They were able to separate distinct subgroups with different prognoses within AML and also within normal karyotype in GEP analyses. The clustering was determined both by karyotype aberrations [e.g., t(8;21), t(15;17), inv(16)] and by molecular mutations, e.g., of the *CEBPA* transcription factor, *MLL*-PTD, and *FLT3-LM*, or by an abnormal expression of the *EVII* oncogene. As these mutations all are important in the characterization of normal karyotype AML, these studies enhanced the perspectives of GEP for the characterization of this subgroup. These studies revealed in addition new pathogenetic aspects: Valk et al. detected only 16 distinct gene expression clusters, although the analyses included a wide spectrum of genetic abnormalities. This suggested a functional redundancy of the molecular lesions and might implicate that the number of pathways to AML might be smaller as previously thought (4, 27).

5. Acute Lymphatic Leukemia

5.1. Cytogenetics and Molecular Genetics in ALL

As in AML cytogenetic, molecular, and immunologic categories are essential for a prognostically relevant classification in ALL. Cytomorphology plays a minor role for classification than in AML, but is important for some special situations such as the detection of cases suspicious for Burkitt/B-ALL.

In childhood ALL the prognostically favorable translocation t(12;21)/*TEL-AML1* could easily be discriminated as distinct subentity by GEP analyses (7). Moderate hyperdiploidy with 47–51 chromosomes due to numerical chromosomal gains, which is also associated with favorable outcome, was as well separated from other ALL subtypes in GEP analyses (8).

Chimeric rearrangements involving the *MLL* gene on 11q23 are prognostically unfavorable in ALL (as in AML). GEP analyses showed an overlap with the gene signatures of reciprocal *MLL* gene rearrangements in AML: Both show an association to the signatures of early progenitor cells and reveal *HOX* gene dysregulation (45). However, ALL with chimeric *MLL* rearrangements can be discriminated from AML by the involvement of genes that are associated with early B-cell development. In addition, transcription follows different pathways in both lineages (46).

Another high-risk ALL group is defined by the Philadelphia rearrangement with the t(9;22)(q34;q11)/*BCR-ABL* increasing in frequency with higher age to up to 40% of all ALL cases. This ALL subtype is associated to precursor ALL and shows in many cases an aberrant myeloid coexpression as assessed by MFC. This immunologic variety corresponds to MA analyses, which failed to detect a distinct gene signature in *BCR-ABL* positive precursor ALL (8, 18, 47).

B-ALL/Burkitt-ALL with the t(8;14)/*IgH-c-MYC* shows a specific morphology with basophile vacuolated blasts and is frequently associated with a large intraabdominal tumor burden leading to vital emergencies. An immediate diagnosis is of high importance as this subtype needs intensified therapy protocols, which are able to improve prognosis significantly. With the established strategies diagnosis provides often difficulties due to the morphologic variability of the blasts and the necessity to perform cytogenetics in combination with interphase FISH probes for *cMYC* rearrangements. An additional problem is provided by Burkitt-like lymphoma, which shows overlapping features with diffuse large B-cell lymphoma (DLCL). However, B-ALL could be clearly discriminated from other ALL subtypes by GEP analyses (26); thus, future diagnostic strategies might be facilitated also in this ALL subtype.

5.2. MFC in ALL

Immunphenotyping with MFC has a central part in ALL. It classifies ALL cases according to the involved lineage (B- vs. T-) and defines the degree of maturation.

Apart from the aforementioned precursor ALL with the Philadelphia translocation t(9;22), GEP can reproduce immunologic classifications of ALL with a high prediction accuracy and is able to reproduce the diverse subtypes of B-lineage ALL (Pro-B-ALL, precursor-B-ALL, mature B-ALL) (18). T-ALL could be separated in cortical T-ALL and immature T-ALL. However, there was a large overlap between different precursor subtypes of T-ALL: Pro-T-ALL and Pre-T-ALL (18). Ferrando et al. related the distinct gene expression signatures in T-ALL to different stages of normal thymocyte development (48, 49).

6. Chronic Myeloid Leukemia

CML is defined by the t(9;22)(q34;q22)/*BCR-ABL* translocation. It is usually diagnosed in chronic phase with < 5% of blasts in combination with increased left-shifted granulopoiesis and by basophilia. There is a characteristic sequence of stages as the progress of CML is characterized by an increase of blasts leading to accelerated phase and finally to blast phase with ≥ 20% of blasts. This process of stages is accompanied by an accumulation of chromosomal aberrations.

In GEP analyses CML shows differential expression of several genes, which are involved in cell cycle regulation and in DNA repair (50, 51). Blast phase of CML is also outlined by a distinct gene signature (52).

Beyond that, GEP might influence future therapeutic strategies in CML: The tyrosine kinase inhibitor imatinib, which blocks the enzymatic action of the *BCR-ABL* fusion protein, was introduced in CML treatment few years ago. Although high molecular remission rates are achieved, some patients initially fail to respond to this treatment. Villuendas et al. determined a subset of 46 genes whose expression was associated with imatinib resistance. These genes were involved in cell adhesion – such as *TNC* and *SCAM-1*, drug metabolism – e.g., genes coding for cyclooxygenases, protein tyrosine kinases, and phosphatases (53). These results enhance further exploration of the utility of GEP analyses for diagnostic and probably also therapeutic strategies in CML.

7. Chronic Lymphatic Leukemia

B-CLL is a very heterogeneous disorder with a large variability in the clinical outcome. Spontaneous survival ranges from a few months to some decades. Chromosome banding analyses are difficult to perform due to low metaphase quality, which limits the use of classical cytogenetics in CLL. However, in recent years the panel of prognostic parameters has increased significantly, e.g., due to the establishment of interphase FISH diagnostics. Deletions in the p53 tumor suppressor gene are associated with an inferior prognosis, whereas deletions of 3q are associated with a favorable prognosis. Monoclonal immunoglobuline heavy chain gene rearrangements (IgH-rearrangements) are detectable by molecular analyses and are prognostically favorable.

GEP was able to discriminate CLL clearly from other leukemia subtypes (54, 55). A high expression of the *ZAP70* gene was found correlated to an unmutated immunoglobuline status in GEP analyses in association to an unfavorable prognosis (56). In addition some genetic subgroups of B-CLL such as deletions of 11q or 17p53 have distinct gene signatures (57). Thus, MA analyses allow also in CLL novel approaches with respect to classification and prognostic predictions and might improve efficiency and prognostic predictions in this complex disorder.

8. Perspectives

The introduction of GEP analyses in leukemia diagnostics must be prepared by large studies evaluating the position of this novel technology in leukemia diagnostics. Following this purpose the MILE study (MILE: Microarray Innovations in Leukemia) is analyzing prospectively 4,000 cases of acute leukemias and MDS in 11 centers within the European Leukemia Network (ELN, WP13) (10, 58). MA protocol, technical equipment, and arrays (Affymetrix HG-U133 Plus 2.0) are identical. This study was already able to demonstrate the high reproducibility of MA analyses even in an international multicenter research study. So far a prediction accuracy of 95.6% was achieved (59).

The high reproducibility of leukemia and lymphoma subclassification with respect to all so far established diagnostic methods – cytomorphology, histology, cytogenetics, molecular genetics, and immunophenotyping – by GEP ([Fig. 5](#)) probably allows an optimization of the diagnostic strategies and will significantly improve the understanding of molecular pathogenesis of leukemias and lymphomas (60). This novel approach will not

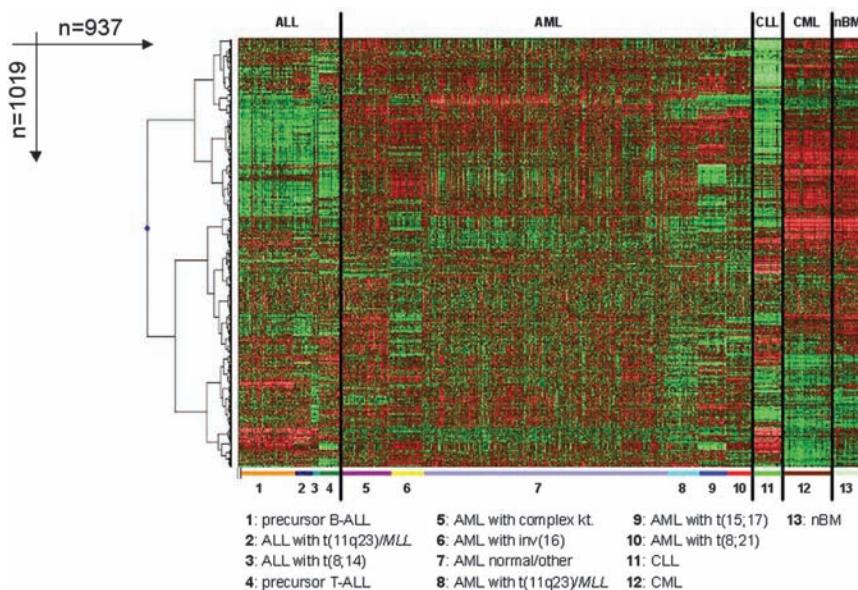


Fig. 5. Hierarchical cluster of 12 different subtypes of leukemia and healthy bone marrow that could be separated with a very high prediction accuracy of 95.1% (55).

only lead to an improved prognostication but might also facilitate the choice of parameters that might serve as basis for minimal residual disease (MRD) diagnostics. Beyond that, the inclusion of GEP into leukemia diagnostics might be helpful to identify pathogenetically essential structures and alterations as potential targets of future drugs hopefully leading to an improved management of these complex disorders. Thus, GEP will open new perspectives for diagnosis, prognostication, and individual treatment decisions in the acute and chronic leukemias and lymphomas in the near future.

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Chapter 3

Expression Profiling Using Affymetrix GeneChip Microarrays

Herbert Auer, David L. Newsom, and Karl Kornacker

Summary

The approximately 25,000 genes in mammalian genomes can be transcribed at different levels. Measurements of gene expression for ten thousands of genes in parallel give the most comprehensive picture of steady-state levels of transcripts and is used in basic and applied research. Microarrays are the most frequently used technology for genome-wide expression profiling; from the various available microarray platforms, Affymetrix GeneChips are most frequently used for expression profiling and over 3,000 scientific publications describe results of this technology. In medical research, expression profiling by microarrays holds great promises for better understanding of diseases, identification of new therapeutic targets, and subclassification of diseases to identify individualized treatment strategies.

Key words: Expression profiling, Microarrays, RNA, Differential gene expression, GeneChips

1. Introduction

High-density DNA microarrays provide an important tool to study the global patterns of gene expression. One of the earliest descriptions of today's most frequently used platform for expression profiling, Affymetrix GeneChips, is from 1996 (*1*) and many advances have been made over the last decade. The latest generations of GeneChips interrogate over 40,000 transcripts (Genome arrays), analyze individual exons of genes separately (Exon arrays), or even more advanced, the entire genome for transcriptional activity without focusing on predicted genes (tiling arrays). Genome arrays providing one measurement of transcriptional activity per gene became tools to generate data reproducible

across laboratories (2), and results from genome arrays can be reproduced by other measurements of gene expression such as quantitative PCR (3). Exon arrays and tiling arrays hold great promises, but reproducibility has not been shown yet; sample processing recommendations get modified every few months and an agreement on data analysis methods does not even appear on the horizon.

One of the best promises held by expression profiling is in the field of medicine. Physicians hope for a detailed understanding of the molecular characteristics of the specific disease of a certain patient. This knowledge would allow them to provide an individualized treatment based on the molecular profiles of the affected tissue. As an example, expression profiles could provide information on the success of leukemia treatment in children (4).

2. Materials

2.1. RNA Isolation

1. Tissue grinder “Pellet pestle” for microtubes (Kimble/Kontes, Vineland, NJ).
2. Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA).
3. Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).
4. Ninety-six percent ethanol (Fisher Scientific, Chicago, IL), 70% ethanol (see [Note 1](#)).
5. Agilent 2,100 Bioanalyzer (Agilent, Waldbronn, Germany).
6. Agilent RNA 6,000 Nano Kit (Agilent, Waldbronn, Germany).

2.2. RNA Amplification and Labeling

1. Ovation Biotin RNA Amplification System and Labeling System 2,300 (NuGEN Technologies, San Carlos, CA).
2. Eighty percent ethanol.
3. DNA Clean & Concentrator – 25 (Zymo Research, Orange, CA).
4. Dye Ex 2.0 Spin Kit (Qiagen, Valencia, CA).

2.3. Microarray Hybridization, Washing, Staining, and Scanning

1. 12× MES stock buffer: 1.22 M MES, 0.89 M Na⁺ from 61.61 g MES hydrate (4-morpholineethanesulfonic acid monohydrate) for molecular biology and 193.3 g MES sodium salt (4-morpholineethanesulfonic acid sodium salt), biotechnology performance certified (both Sigma-Aldrich) per liter. The pH should be between 6.5 and 6.7.

- Store at 4°C, shield from light, and discard if solution turns yellow.
2. Fifty milliliters 2× Hybridization buffer: mix 8.3 ml 12× MES stock buffer, 17.7 ml 5 M NaCl, 4.0 ml 0.5 M EDTA disodium salt, 0.1 ml 10% Tween-20, and 19.9 ml water. Store at 4°C and shield from light.
 3. BSA, 50 mg/ml (Invitrogen Life Technologies, Carlsbad, CA).
 4. Herring sperm DNA, 10 mg/ml (Promega, San Luis Obispo, CA).
 5. GeneChip Eukaryotic Hybridization Control Kit containing 20× eukaryotic hybridization controls and control oligo B2 (Affymetrix, Santa Clara, CA).
 6. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO).
 7. R-phycoerythrin streptavidin (Molecular Probes, Carlsbad, CA).
 8. Wash buffer A (6× SSPE, 0.01% Tween-20): mix 300 ml 20× SSPE (3 M NaCl, 0.2 M NaH₂PO₄, 0.02 M EDTA, BioWhittaker Molecular Applications/Cambrex, Rockland, MA), 1 ml 10% Tween-20, and 699 ml water per liter. Filter through a 0.2 µm filter and store at room temperature.
 9. Wash buffer B (100 mM MES, 0.1 M Na⁺, 0.01% Tween-20): mix 83.3 ml 12× MES stock buffer, 5.2 ml 5 M NaCl, 1.0 ml 10% Tween-20, and 910.5 ml water per liter. Filter through a 0.2 µm filter, store at 4°C, and shield from light.
 10. Two-hundred fifty milliliters 2× stain buffer (200 mM MES, 2 M Na⁺, 0.1% Tween-20): mix 41.7 ml 12× MES stock buffer, 92.5 ml 5 M NaCl, 2.5 ml 10% Tween-20, and 113.3 ml water. Filter through a 0.2 µm filter, store at 4°C, and shield from light.
 11. Resuspend 50 mg goat IgG, reagent grade (Sigma-Aldrich, St. Louis, MO) in 5 ml 150 mM NaCl.
 12. Anti-streptavidin antibody (goat), biotinylated (Vector Laboratories, Burlingame, CA).

2.4. Affymetrix Equipment

1. GeneChip 3,000 7G (Affymetrix, Santa Clara, CA).
2. Fluidics Station 450 (Affymetrix, Santa Clara, CA).
3. Hybridization Oven 640 (Affymetrix, Santa Clara, CA).

2.5. Microarray Quality Control and Data Analysis

1. GCQC software (<http://www.dnaarrays.org/downloads.php>).
2. ArrayAssist software (<http://www.stratagene.com/tradeshows/feature.aspx?fpId=91>).

3. Methods

Microarray expression profiling works most effectively when samples are compared with each other instead of attempting to measure gene expression in an absolute manner (5). These measurements of differential expression should represent biological differences between samples, and the influence of technical variation on results should be kept as small as possible. A plethora of factors can contribute to measurements of differential expression caused by technical variation. Some examples are different RNA isolation protocols, variable amounts of starting material, variable RNA integrity between samples, different labeling chemistries used within a group of samples, which should be compared afterwards, and different technicians processing samples. As an example, up to 75% of genes measured as differentially expressed can be false positive measurements (not representing biological differences between samples), if two samples with pronounced differences in RNA integrity are compared (6). Many factors contributing to technical variation in expression profiling are most likely unknown; therefore, within a project (i.e., a group of samples where differential expression between samples should be calculated afterwards), technical variation should be kept as small as possible. This is best achieved by parallel processing of samples by the same laboratory personnel within the shortest possible time; all chemistry and microarrays should have the same lot number and identical equipment (like hybridization oven, washing station, and scanner) should be used for all samples within a project.

In many cases only limited cell numbers are available for expression profiling. Isothermal amplification (7), the herein described method, utilizes chemistry applicable to very small numbers of cells. It can start from as low as 5 ng total RNA and therefore can be used for a broad range of projects. In many cell types, this amount of RNA can be isolated from several thousand cells. This chemistry generates cDNA as an amplification product instead of cRNA (as generated by many other chemistries used for RNA labeling). cDNA hybridization to microarrays has been shown to generate more information on differential expression than cRNA (8) and less technical variability (9).

3.1. RNA Isolation

1. Adherent cells are lysed in 100 µl lysis buffer containing β-mercaptoethanol without prior washing with PBS. Cells grown in suspension are spun down before lysis. Tissue should be snap-frozen in liquid nitrogen immediately after biopsy and stored at -80°C until lysis. For homogenization, tissue is placed in a 1.5 ml tube containing 100 µl lysis buffer and immediately homogenized using the tissue grinder until no pieces of tissue are visible anymore. For further isolation

steps see technical manual. The therein described optional step of DNase treatment is performed.

2. Use 1.25 µl of RNA for quantification by the ND-1000 spectrophotometer (*see Note 2*). The blank measurement is performed using the elution buffer. The ratio of absorption at 260/280 nm should be above 1.8.
3. Perform RNA integrity control using RNA Nanochips 6,000 according to the technical manual. This step consumes 1 µl RNA (*see Note 3*).

3.2. Amplification and Labeling

3.2.1. Thermal Cycler Programming

1. Program 1 – primer annealing: 65°C 5 min, 4°C forever.
2. Program 2 – first strand synthesis: 48°C 60 min, 70°C 15 min, 4°C forever.
3. Program 3 – second strand synthesis: 37°C 30 min, 75°C 15 min, 4°C forever.
4. Program 4 – amplification: 48°C 60 min, 95°C 5 min, 4°C forever.
5. Program 5 – fragmentation and labeling: 50°C 30 min, 4°C forever.

3.2.2. cDNA Synthesis

1. Place A3 (first strand enzyme mix) on ice and thaw A1 (first strand primer) and A2 (first strand buffer) at room temperature. Once thawed, mix A1 and A2 by vortexing, A3 by flicking the tube several times; place all reagents on ice.
2. Add 30 ng of total RNA sample to a 0.2 ml tube and add water to 5 µl. Add 2 µl of A1 to the RNA. Vortex briefly and spin down shortly.
3. Incubate 5 min at 65°C (program 1); as soon as it is completed, place samples immediately on ice (*see Note 4*).
4. Prepare a master mix of 12 µl A2 plus 1 µl A3 per sample on ice, add 13 µl of this per sample, mix by pipetting, and spin down briefly.
5. Incubate in thermal cycler using program 2 (first strand synthesis).
6. Place B2 (second strand enzyme mix) on ice and thaw B1 (second strand buffer) at room temperature. Once thawed, mix B1 by vortexing, and B2 by flicking the tube several times; place all reagents on ice.
7. Prepare a master mix of 2 µl B2 plus 18 µl B1 per sample on ice, add 20 µl of this per sample, mix by pipetting, and spin down briefly.
8. Incubate in thermal cycler using program 3 (second strand synthesis); after completion, proceed immediately with amplification.

3.2.3. Amplification

1. Place C3-C (SPIA enzyme mix) on ice and thaw C2-C (SPIA buffer) and C1 (SPIA primer) at room temperature. Once thawed, mix C2-C and C1 by vortexing, and C3-C by flicking the tube several times; place all reagents on ice. Place one additional 0.2 ml tube per sample on ice.
2. Prepare a master mix of 4 µl water, 4 µl C1, 40 µl C3-C, and 72 µl C2-C per sample; add 120 µl of this per sample (see Note 5). Mix by pipetting on ice.
3. Split reaction by removing 80 µl in prechilled 0.2 ml tube.
4. Start program 4 (amplification) at thermal cycler; once cycler reaches 48°C, place tubes in cycler. After amplification, samples can be stored at -20°C.

3.2.4. Purification of Amplified cDNA

1. Per sample, add 320 µl DNA binding buffer into a 1.5 ml tube.
2. Combine both 80 µl aliquots of each sample into one of these tubes, mix by vortexing, and spin down briefly.
3. Place one Zymo 25 column per sample into a collection tube.
4. Load the entire volume of the sample (480 µl) onto the column.
5. Centrifuge the column in the collection tube for 10 s at >10,000 × g.
6. Discard flow-through. Place the column back in the same collection tube.
7. Wash the sample by adding 200 µl of room temperature 80% ethanol. Do not use the wash buffer provided with the columns.
8. Centrifuge the column in the collection tube for 10 s at >10,000 × g.
9. Discard flow-through. Place the column back in the same collection tube.
10. Add 200 µl of room temperature 80% ethanol.
11. Centrifuge the column in the collection tube for 30 s at >10,000 × g.
12. Place the column in a clean 1.5 ml tube.
13. Add 30 µl of room temperature water to the center of each column. Let the columns stand for 1 min at room temperature.
14. Centrifuge the column in 1.5 ml tube for 30 s at >10,000 × g.
15. Discard the column; there should be approximately 30 µl of purified cDNA.
16. Mix the sample by vortexing, and then spin briefly.

17. Use 1.25 μ l of cDNA for quantification by the ND-1000 spectrophotometer. The conversion factor is 33 for single stranded DNA. The blank measurement is performed using water. The ratio of absorption at 260/280 nm should be above 1.8.
18. Analyze cDNA molecular weight distribution using RNA Nanochips 6,000 according to the technical manual. An example of molecular weight distribution is shown in **Fig. 1**.

3.2.5. Fragmentation and Labeling

1. Place F2 (fragmentation enzyme) on ice and thaw F1 (fragmentation buffer mix) at room temperature. Once thawed, mix F1 by vortexing, F2 by flicking the tube several times; place all reagents on ice.
2. Pipette 4 μ g of purified cDNA into 0.2 ml tube and fill up to 25 μ l with water.
3. Add 5 μ l F1 per sample, and mix by pipetting.
4. Add 5 μ l F2 per sample, mix by pipetting, and spin down briefly.
5. Incubate in thermal cycler using program 5 (fragmentation and labeling).
6. Thaw F3 (labeling buffer mix), F4 (biotin reagent), and F5 (stop buffer) at room temperature. Once thawed, mix by vortexing, place F4 on ice, and keep F3 and F5 at room temperature.
7. Add 5 μ l F3 per sample, and mix by pipetting.
8. Add 2.5 μ l F4 per sample, mix by vortexing, and spin down briefly.
9. Incubate in thermal cycler using program 5 (fragmentation and labeling).

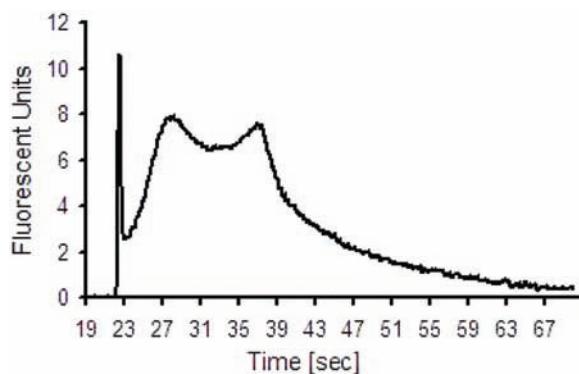


Fig. 1. Size distribution of amplified cDNA measured using the 2,100 bioanalyzer.

10. Add 7.5 μ l F5, mix by vortexing, and spin down briefly.
11. After labeling, samples can be stored at -20°C.

3.2.6. Purification of Biotin-Labeled cDNA

1. Vortex DyeEx columns for 3 s.
2. Loosen the column cap, snap off bottom closure, and place into a 2 ml collection tube.
3. Spin at $750 \times g$ (2,800 rpm in a standard benchtop microcentrifuge) for 3 min, discard flow-through, and transfer the column to a clean collection tube.
4. Apply labeled cDNA to the center of the resin surface.
5. Spin at $750 \times g$ for 3 min. Discard the column.
6. Purified labeled cDNA can be stored at -20°C.

3.3. Microarray Hybridization, Washing, Staining, and Scanning (see Note 6)

1. Heat frozen 20 \times eukaryotic hybridization controls for 5 min at 65°C.
2. Prepare hybridization cocktail using the entire volume of labeled and purified cDNA in 1.5 ml tubes following Table 1.
3. Equilibrate arrays (see Note 7) in the packing at the bottom of the hybridization oven at 45°C for at least 10 min and set two heat blocks to 99 and 45°C.
4. Heat-denature hybridization cocktail for 2 min at 99°C, and equilibrate for 5 min at 45°C.
5. Centrifuge tubes for 10 s at >10,000 $\times g$.

Table 1
Preparation of the hybridization cocktail

Component	Standard array (μ l)	Midi array (μ l) (see Note 6)
Labeled cDNA	50	34
Control oligonucleotide B2	3.7	2.5
20 \times Eukaryotic hybridization controls	11	7.5
Herring sperm DNA	2.2	1.5
Acetylated BSA	2.2	1.5
2 \times Hybridization buffer	110	75
100% DMSO	22	15
Water	19	13
Final volume	220	150

6. Place one pipette tip into one of the two septa of each array.
7. Load hybridization cocktail through second septum into the arrays.
8. Hybridize the arrays for 16–24 h in a hybridization oven at 45°C, rotating at 60 rpm.
9. Remove the hybridization cocktail from the arrays, fill the arrays with wash buffer A, and store the hybridization cocktails at –20°C. For washing and staining, use EukGE-WS2v4_450 for standard arrays and Midi_Euk-2v3 for midi arrays (see [Notes 7 and 8](#)).
10. Before scanning, control arrays for presence of air bubbles. If bubbles are present, remove them by adding wash buffer A through septum.
11. Scan arrays; CEL files are generated automatically after the scan is finished (see [Note 9](#)).

3.4. Microarray Quality Control and Data Analysis (see Note 10)

1. Copy CEL files into the folder containing the GCQC.exe file and press *enter*. Two files are generated by GCQC, GCQCL, and Raw_AFFX_PM_Intensities (see [Note 11](#)).
2. In the GCQCL file, PM95 should be >1,000, PM95/MM95 should be >4, PM95/PM50 should be >10, and MM05 should be <100 (see [Note 12](#)). Some examples of GCQC results are shown in [Table 2](#).
3. Load CEL files in ArrayAssist software. Use RMA from the options for data analysis algorithms (see [Note 13](#)).
4. Perform log transformation of expression estimates and calculate differential gene expression (see [Note 14](#)).

Table 2
Quality control parameters provided by GCQC software

Sample	MM05	MM25	MM50	MM75	MM95	PM05	PM25	PM50	PM75	PM95	PM50	PM95/ MM95
A	29	31	33	39	75	29	32	35	49	176	5.029	2.347
B	279	380	486	629	1,004	301	427	572	830	2,920	5.105	2.908
C	57	72	97	154	468	60	82	124	256	1,489	12.008	3.182
D	97	133	173	238	600	108	156	221	418	2,661	12.041	4.435

For each sample, 5th, 25th 50th, 75th, and 95th percentile of intensities for mismatch (MM) and perfect match (PM) probes are provided, respectively. Sample A shows results of a microarray where phycoerythrin-staining worked poorly; sample B shows results of a microarray with high background; sample C shows results of cRNA hybridization (instead of cDNA), resulting in lower PM95/MM95 ratios; sample D shows results of a successful microarray experiment. Problematic quality control parameters are highlighted in bold

3.4. Notes

1. All solutions used for RNA have to be RNase-free. The frequently used practice of treating water with DEPC can inhibit enzymatic reactions. Therefore, nuclease-free water provided with the labeling chemistries or nuclease-free water (Non-DEPC treated, Ambion, Austin, TX) should be used. Pipette tips and tubes must be RNase-free too. Do not perform DEPC treatment of tips and tubes but order instead specified as “RNase-free.” All buffers for washing of arrays should be prepared in water that has a resistance of $20\text{ M}\Omega/\text{cm}$ or more; buffers should be filtered through $0.2\text{ }\mu\text{m}$ filter. Water for buffers does not have to be nuclease-free. Solutions as indicated to be purchased as solutions should not be prepared from powder. The chance of making errors is too big in relation to the potential financial loss from nonusable array data.
2. Many other photometers consume big volumes of RNA for quantification. Therefore, a large portion of RNA would have to be used for quantification or the sample would be diluted such that the absorption reading is below accuracy of the photometer. For the ND-1000 spectrophotometer, $1.25\text{ }\mu\text{l}$ is sufficient; the $1\text{ }\mu\text{l}$ mentioned in Nanodrop’s manual is sometimes not sufficient for accurate readings.
3. Agilent Expert software version 1.2.3.4 or higher provides quantification of RNA integrity by the “RNA integrity number” (RIN). For cell cultures, RIN should be above 9 and for primary cells RIN should be above 7.
4. Leaving the samples in the thermal cycler until it reaches 4°C generates subsequently shorter cDNAs products.
5. The amplification enzyme should not reach temperature above 4°C before the reaction is placed in the thermal cycler. All pipetting must be performed on ice and as quickly as possible.
6. This protocol contains deviations from the protocol provided by Affymetrix. Follow the instructions as given here.
7. Standard arrays are for example 133 Plus 2.0 or 430 2.0 arrays, and midi arrays are for example 133A 2.0 or 430A 2.0 arrays.
8. For detailed instructions on array washing and staining refer to Affymetrix GeneChip Expression Analysis Technical Manual (revision 4). Streptavidin phycoerythrin is unstable and should be protected from light; a once-opened tube should not be used longer than 2 months.
9. CEL files contain information on the 75th percentile of intensities of each probe. Almost all downstream data analysis is performed using CEL files.

10. Report files generated by GCOS and ArrayAssist software provide information about data quality derived from scaled values and therefore are not very informative.
11. The file Raw_AFFX_PM_Intensities contains raw (nonscaled) probe intensities of probe sets whose identifiers start with AFFX. Using Excel, graphs of intensities for these probe sets can be drawn. These graphs are helpful in troubleshooting.
12. MM05 reports the 5th percentile of intensity of mismatch probes; this parameter is used to assess background signals. PM95 reports the 95th percentile of perfect match probes; this parameter reports the upper end of signal intensity. The ratio PM95/MM95 reports the signal over background ratio. The PM95/PM50 reports the slope of PM intensities at the upper end; high background compresses this ratio.
13. ArrayAssist can be downloaded from Stratagene's website for free. After a test period, the software downgrades to ArrayAssist Light, still capable of calculating RMA expression estimates. Other data analysis algorithms (GCOS, PLIER) can generate high percentages of false positive reports of differential gene expression. Any kind of software for generating expression estimates summarizes the 11 or more probes within one probe set to provide one estimate for quantification of the measured transcript.
14. When replicates of experiments have been performed, a T test for statistical significance of differential expression should be applied. $P < 0.05$ is considered as significant and corrections for multiple testing should not be applied. When only one sample has been processed per biological group, differential expression measured above twofold usually can be considered as reliable since the majority of these measurements can be confirmed by real-time PCR. If more than one probe set on the array measures a certain transcript, the probe set with the highest absolute value of differential expression should be used for further analysis.

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Chapter 4

Genotyping of Mutation in the Beta-Globin Gene Using DNA Microarrays

Martin Dufva and Lena Poulsen

Summary

Genotyping using DNA microarrays is a cost-efficient method compared with real-time PCR and DNA sequencing. Here, a DNA microarray-based method using allele-specific oligo hybridization is demonstrated. This method relies on immobilization of probes that are specific for wild-type sequences or the mutated sequences, respectively. The method makes use of agarose film-coated glass slides, unmodified DNA probes, target preparation using T7 in vitro transcription, labeling of target using biotin labels, and detection using alkaline phosphatase precipitation reaction. Visualization is performed using a desktop computer scanner. Because the biotin/strepavidin chemistry is utilized, the method described here is compatible with many different detection methods. The demonstrated colorimetric detection of mutations has an expected error frequency of about 5×10^{-7} per mutation or better. Given this low error frequency, an array diagnosing 100 different mutations would misclassify about 1 patient in 100,000.

Key words: DNA microarray, Genotyping, Mutation detection, Beta-globin, Allele-specific hybridization, Colorimetric detection

1. Introduction

DNA microarray technology is a powerful tool for genotyping as it allows analysis of thousands of mutations at the same time (1, 2). It has therefore been used for large-scale single nucleotide polymorphism (SNP) analysis (1). Because of the high parallelism, DNA microarrays are also rapid and cost-efficient substitutes for mutation detection in specific genes where ten to several hundred disease causing mutations are assayed at the same time. The most commonly employed methods for genotyping using DNA microarrays

include allele-specific oligo hybridization (ASOH) and enzyme-assisted reactions such as mini-sequencing and allele-specific primer extension. By use of these technologies, DNA microarrays have been developed to detect mutations in the beta-globin gene (3–5). Mutations in the beta-globin gene lead to beta-thalassemia, the most common genetic disorder in the world. There are a few hundred well described mutations in the beta-globin gene that give rise to thalassemia of various severities. The types of mutations are substitutions and small (few bases) insertions and deletions. Thus, the types of mutations are heterogeneous compared with SNPs.

ASOH is simple to perform, inexpensive, and scalable. Finding probes that function together is, however, difficult. The reason is that the efficiency of allele-specific hybridization depends on the type of mutation (substitution and insertion/deletion) and on the sequences surrounding the mutation (2, 6). It is presently not possible to predict probe characteristics according to thermodynamic models, which makes probe choice difficult (7). A further complication is that microarray experiments are usually performed at one condition (e.g., at one temperature for posthybridization wash). An accurate genotyping assay requires therefore extensive optimization comprising a long trial and error process. It should be noted that large single nucleotide polymorphism (SNP) arrays are less subjected to this problem given that SNPs can be chosen to avoid problematic regions that are predominantly AT- or GC-rich. In contrast, analysis of gene-specific mutations requires that the probes are located at the site of the mutation. Even though these difficulties exist, ASOH is a robust method when the proper probes are selected.

Here, we will present ASOH as a method to detect beta-thalassemic mutations using a colorimetric readout. We utilize relatively short probes that are modified with ten thymidines and ten cytidines (TC-tag) in the 5'-end of the specific part of the probe (8). The TC tag serves to link the specific part of the probe to an agarose film. The TC tag can also be employed for quality control of the fabricated DNA microarray, because all the probes contain the sequence in the 5'-end. The TC tag was selected from a subset of tags for its property to link DNA covalently to an agarose film (8). However, care should be taken to check for cross hybridization of the TC tag and the targets used prior to fabrication of large arrays. The agarose film-coated slides are cost-efficient DNA microarray substrates, particularly if the film is immobilized on ordinary glass (9, 10). Preparation of single-stranded target for hybridization on DNA microarrays is performed by T7 *in vitro* transcription (T7-IVT). The protocol comprises two main steps. In the first step a bacteriophage promoter sequence, T7, is incorporated into PCR products by PCR amplification of the fragment/s of interest, using either the sense or antisense primer with the 5' promoter sequence. With,

i.e., sense probes the antisense primer is designed with the promoter sequence upstream of the gene-specific sequence. The second step is T7-IVT, where the obtained PCR products serve as template for the enzyme T7 RNA polymerase, which specifically recognizes its promoter sequence in double-stranded DNA and makes thousands of single-stranded RNA copies (11). DNA microarray detection is usually done using fluorescence, and the genotyping example given here works excellent with fluorescent detection method as well (8). However, DNA microarray scanners are expensive to buy and in this protocol we use a BCIP/NBT alkaline phosphatase reaction to stain the DNA microarrays. The spots can subsequently be visualized using a desktop scanner (5). Colorimetric staining results in the same robust genotyping as fluorescence (5).

2. Materials

2.1. Fabrication of Slides

1. Mix 150-mL milliQ water with 1.5 g of agarose (Invitrogen, Taastrup, Denmark), and boil in a microwave oven until the agarose is completely dissolved. Finally add 0.32-g sodium (meta) periodate INaO_4 (Sigma-Aldrich, Brøndby, Denmark) and mix. The glass slides used are 26 mm \times 76 mm cut edges SuperFrost® slides (VWR International, Rødovre, Denmark).

2.2. Spotting the Slides

1. DNA probes are listed in **Table 1**. Pipette 15 μL of 100 μM probe solution in the respective wells in a 384-well microtiter plate. Spotting is performed using a Genetix Q-array spotting robot (Genetix, New Milton Hampshire, UK) with Chipmaker CMP3B pins (Telechem, Sunnyvale, CA, USA).
2. 0.1 \times Sodium saline citrate (SSC) is prepared from stock solution of 20 \times SSC (Promega, Madison, WI, USA) diluted in MilliQ or deionized water. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich) is added to a final concentration of 0.5% (w/v). A 5-L 0.1 \times SSC + 0.5% SDS solution is made by adding 25-g SDS and 25-mL 20 \times SSC. Fill up with milliQ water and mix thoroughly. A 0.1 \times SSC solution is prepared in the same manner, however, omitting SDS.

2.3. Target Preparation

1. For the PCR reaction a 100 μM stock solution of each primer BCF and T7-BCR (**Table 1**) is diluted with milliQ water to 10 μM . The 12.5 mM stock of dNTPs (Bie & Berntsen) is diluted to 1 mM. The DNA polymerase TEMPase Hot Start DNA polymerase (5 U/ μL) (Bie & Berntsen) is used with the supplied 10 \times PCR buffer (TEMPase buffer II). DNA template

Table 1
Primers and probes

BCF	AGCAGGGAGGGCAGGAGCCA	None
T7-BCR	GAAATTAATACGACTCACTATAAGGGAGAAGAGTCAGT-GCCTATCAGAAACCC	None
CD5 – CT mt	TTTTTTTTTCCCCCCCCCCC GCACCTGACTCGAGGAGAAGT	TC-tag
CD5 – CT wt	TTTTTTTTTCCCCCCCCCCC GCACCTGACTCCTGAGGAGAA	TC-tag
CD8 – AA mt	TTTTTTTTTCCCCCCCCCCC GCACCTGACTCCTGAGGAGAA	TC-tag
CD8 – AA wt	TTTTTTTTTCCCCCCCCCCC CCTGAGGAGGTCTGCCG	TC-tag
CD8-9 + G mt	TTTTTTTTTCCCCCCCCCCC CCTGAGGAGAAGTCTGCCG	TC-tag
CD8-9 + G wt	TTTTTTTTTCCCCCCCCCCC GAGGAGAAGGTCTGCCGTTAC	TC-tag
CD15 G > A mt	TTTTTTTTTCCCCCCCCCCC GAGGAGAAGTCTGCCGTTACTG	TC-tag
CD15 G>A wt	TTTTTTTTTCCCCCCCCCCC ACTGCCCTGTAGGGCAAGGT	TC-tag
CD17 A>T mt	TTTTTTTTTCCCCCCCCCCC TGCCCTGTGGGGCAAGG	TC-tag
CD17 A>T wt	TTTTTTTTTCCCCCCCCCCC CCCTGTGGGGCTAGGTGA	TC-tag
CD19 A>G mt	TTTTTTTTTCCCCCCCCCCC CCCTGTGGGGCAAGGTG	TC-tag
CD19 A>G wt	TTTTTTTTTCCCCCCCCCCC GCAAGGTGAGCGTGGATGAA	TC-tag
CD24 T>A mt	TTTTTTTTTCCCCCCCCCCC GGCAAGGTGAACGTGGATGAA	TC-tag
CD24 T>A wt	TTTTTTTTTCCCCCCCCCCC AAGTTGGAGGTGAGGCCCT	TC-tag
CD27-28 + C mt	TTTTTTTTTCCCCCCCCCCC GAAGTTGGTGGTGAGGCC	TC-tag
CD27-28 + C wt	TTTTTTTTTCCCCCCCCCCC GTGAGGCCCTGGGC	TC-tag
IVS I – 5 G>C mt	TTTTTTTTTCCCCCCCCCCC GTGAGGCCCTGGCAG	TC-tag
IVS I – 5 G>C wt	TTTTTTTTTCCCCCCCCCCC GGCAGGTTGCTATCAAGGT-TACA	TC-tag
IVS I – 6 T>C mt	TTTTTTTTTCCCCCCCCCCC GGCAGGTTGGTATCAAGGT-TACA	TC-tag
IVS I – 6 T>C wt	TTTTTTTTTCCCCCCCCCCC GGCAGGTTGGCATCAAGG	TC-tag

for the PCR reaction can be either PCR product or genomic DNA.

2. For verification of PCR products by agarose gel electrophoresis prepare a 2% (w/v) agarose gel in 0.5× Tris/borate/EDTA (TBE) buffer containing 0.5-μg ethidium bromide/mL agarose solution. Remember to wear nitril gloves as ethidium bromide is a potent mutagen. Alternatively perform separation on

an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) using a DNA 1000 LabChip kit.

3. For the T7 in vitro transcription reaction, we directly use PCR products as DNA template, without purification. The reagents include 2.5 mM of each NTP (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), which are diluted from a 100 mM stock, 1.0 mM UTP-11-biotin (PerkinElmer, Waltham, MA) diluted from a 10 mM stock, and the enzyme T7 RNA polymerase Plus (20 U/ μ L) (Applied Biosystems, Austin, TX) together with the supplied 10 \times T7 transcription buffer.
1. Hybridization buffer is easiest prepared by making a 2 \times hybridization buffer by mixing 1% w/v SDS and 10 \times SSC. Note that the salt will precipitate the SDS immediately and stay insoluble at room temperature. Prior to use, put the flask with the 2 \times hybridization buffer in the microwave oven and heat *gently* until a clear solution is obtained.
2. Five liters of the wash buffer 0.1 \times SSC + 0.1% SDS is prepared by adding 5 g of SDS and 25 mL of 20 \times SSC, filling up with milliQ water, and mixing thoroughly. One liter of the blocking solution 0.5% (w/v) bovine serum albumin [BSA (Sigma-Aldrich)] is added to 1 L of phosphate buffered saline (PBS): NaCl (8 g/L), KCl (0.2 g/L), and Na₂HPO₄ (1.15 g/L).

2.5. Staining and Detection

1. One microliter of Streptavidin-alkaline phosphatase solution (Sigma-Aldrich) is added to 10-mL PBS.
2. 5-Bromo-4-chloro-3-indolyl phosphate solution is made by dissolving a substrate tablet (Sigma-Aldrich #B-5655) into 10-mL water.

3. Methods

3.1. Fabrication of Slides

1. Place 100–150 superfrost slides on a planar surface. Pour 1-mL agarose gel onto the surface using a 1-mL pipette. It is simplest if the tip of the pipette is held approximately where the frosted area starts and a rapid ejection will push the melted hot agarose toward the area of the slides that is not frosted. Normally if done correctly, the solution will form a layer of agarose over the whole slide surface.
2. Let the agarose polymerize for 5 min and transfer the slides to a water bath of deionized water or milliQ water. When submerging the slides, tilt the slides a little and let the slide slip very gently into the water. The slides should not be placed on top of each other. Incubate for 3 h.

3. Dry the slides in air over night or in a couple of hours at 37°C. The slides should be covered with a thin film (about 8 µm) of agarose.

3.2. Spotting the Probes onto the Slides

1. Place the agarose-coated slides in the spotter. Make a plate of probes where the probes are diluted in water to 100 µM. Put the microtiter plate in the spotter and spot the slides using 70% humidity. Alternatively, spot at 10°C without humidifier (*see Note 1*). After spotting, UV crosslink the agarose slides for 4 min in a Stratalinker 2400 equipped with 254-nm bulbs (9) or similar setup.
2. Remove unbound probes by washing the slides in 0.1× SSC + 0.5% SDS. Place the slides in a Microarray Wash Station (Arrayit, Sunnyvale, CA) and put the washing station in a 400-mL beaker. Add a stirring magnet and 400 mL 0.1× SSC + 0.5% SDS. Stir vigorously using a magnetic stirrer for 10 min. Subsequently wash slides in 0.1× SSC for 10 min. Finally dry the slides by centrifuge or by gently blowing air or nitrogen over the slide.

3.3. Target Preparation

1. For a 20-µL PCR reaction, mix in a PCR tube 2 µL of 10× TEMPase buffer II, 2 µL of each primer BCF (10 µM) and T7-BCR (10 µM), 4 µL of dNTPs (each 1 mM), 1 µL of TEMPase Hot Start DNA polymerase (5 U/µL), and 0.5-µL template DNA (genomic DNA or PCR product). Finally add 11.5 µL of MilliQ water and mix gently by pipetting. The PCR cycling conditions are an initial hot-start of 15 min at 95°C followed by 35 amplification cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min and storage at 4°C until use.
2. Amplification products can be confirmed by electrophoresis on a 2–3% agarose gel. Alternatively PCR products can be separated and quantified on an Agilent Bioanalyzer 2100 using a DNA 1000 LabChip kit. Note that the T7 promotor sequence adds another 28 basepairs to the fragment.
3. The PCR products obtained are directly used as template DNA for the T7 in vitro transcription. For a final volume of 20 µL the following reagents are mixed in a PCR tube: 2 µL of 10× T7 transcription buffer, 4 µL of NTPs (2.5 mM each), 0.25 µL of UTP-11-Biotin (1.0 mM), 1 µL of T7 RNA polymerase Plus (20 U/µL), and 2 µL of template DNA. Lastly, 10.75 µL of MilliQ water are added, and the reaction is mixed by gently pipetting up and down. The T7 reaction mixture is incubated in a heat incubator or thermocycler at 37°C for 2 h. If not used immediately, the amplified RNA target can be stored at -80°C.

3.4. Hybridization and Posthybridization Wash

1. Mix target (Subheading 3.3) with hybridization buffer in a one to one mixture resulting in a final hybridization solution of 5× SSC and 0.5% SDS. Denature the target RNA molecules by heating the target solution for 2 min at 95°C. Place 20 μ L of target hybridization solution where the microarray is located on the slide. Place a 15 mm × 15 mm coverslip on the microarray avoiding air bubbles. Put the slide in a hybridization chamber with humidity and place the chamber in a heat incubator at 37°C, and hybridize for 2 h (see Note 2).
2. Following hybridization, the slides are washed as described in Subheading 3.2 in 0.1× SSC with 0.1% SDS at 35°C for 30 min (see Note 3). Next the slides are blocked with 0.5% BSA in 1× PBS and dried prior to linking of extravidin conjugates.

3.5. Staining and Detection

1. Two-hundred fifty microliters of a 1:10,000 dilution of extravidin-alkaline phosphatase (AP) in 1× PBS buffer is applied to a whole slide using an unmodified microscope glass slide for dispersion and cover. After incubation for 30 min at room temperature, the slides are washed with 0.1× SSC for 5 min and dried.
2. Five-hundred microliters of 5-bromo-4-chloro-3-indolyl phosphate solution are added to each slide. The reaction is stopped with deionized water (see Note 4). The slides are subsequently dried.
3. The slides are scanned with a HP scanjet 5470C desktop flatbed scanner at 1200 or 2400 dpi 8-bit grayscale (see Note 5). Save the figures as TIF files. A representative image is shown in Fig. 1.

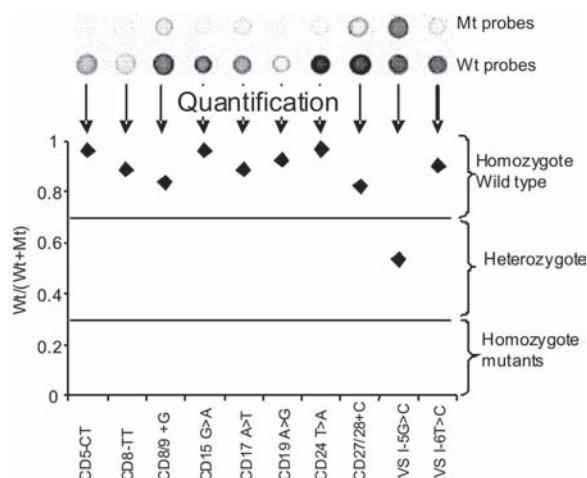


Fig. 1. The upper panel shows a scanned image of a genotyping microarray hybridized with aRNA derived from a carrier that is heterozygote in the IVS I-5 position while being homozygote wild type in all other positions. The graph shows a ratio constructed from the quantified values of the image in the upper panel.

3.6. Quantification and Data Analysis

1. Import the TIF file into a Corel Draw Photopaint and invert the picture (*see Note 6*).
2. Import the inverted picture into Scanalyze 2.5 (<http://rana.lbl.gov/EisenSoftware.htm>) and quantify the spots according to the manual.
3. Export the quantified data and import the tab delimited file into Excel.
4. Calculate a ratio as signal from a wild-type probe divided with the sum of the signal from the wild-type probe and the corresponding signal from the mutant probe (*see Note 7*).

4. Notes

1. Perfect spots are homogenous and often round if the arrays are fabricated using a spotter. Most spotting procedures require humidity to reduce the evaporation rate. Additives such as polymers and salts (phosphate buffers) can be mixed with the DNA to reduce evaporation. We have found that spotting at low temperatures results in low evaporation rates and acceptable spot morphologies even when spotting DNA without such buffers. It should be noted that other spotting schemes might be necessary if other substrates are used instead of the agarose slides (12).
2. It is very important that the array is kept wet during hybridization; otherwise, undesirable background will be obtained. There are many ways to keep the array wet and the solution is depending on the hybridization time. For short incubations like 1–2 h, the coverslip will reduce evaporation enough if the slide is placed in a humid chamber. Such can easily be constructed using a closed box with wet paper towels in the bottom and small rods atop the towels to avoid the slides being in contact with the towels. Another solution (more expensive) is to use gene frames (Abgene, Epsom, United Kingdom) where the microarray is hermetically closed into a plastic structure that is taped onto the slide. Even more expensive solutions involve dedicated hybridization machines that also provide mixing in small volumes.
3. Washing the slides in stringent condition is needed to dehybridize mismatch hybrids but not the perfect match hybrids. It is presently difficult to predict the optimal stringency, and optimal stringency must therefore be determined experimentally. The optimal stringency for the array is easiest determined by washing at different conditions. For instance the temperature

can be held constant at 30°C and the salt concentration can be varied. For a diagnostic array, the optimal stringency has to be determined for each genotype. It is not sufficient to determine the stringency for probes toward only one mutation and assume that the probes for the other mutation are working at the same condition. At least 30 optimization experiments are required for an array detecting ten different mutations because (if available) homozygote wild-type, heterozygote, and homozygote mutated must be tested for each mutation.

4. The reaction needs to be timed accurately. We use reaction times between 2 and 10 min but sometimes up to 30 min are required. The simplest way is to make the reaction in a dark box where the slides lie on a piece of white paper. From time to time, the lid of the box is lifted and when the spots are visible by the eye, the reaction can be stopped. It is important that the reaction is carried out in a dark box because unspecific reactions can otherwise occur.
5. There is no difference in signal intensity or the variance of the signal if the slides are scanned in different resolutions. However, we used 1,200 dpi or higher resolution to get more than 40 pixels per spot (a spot is about 200 µm in diameter). Dias illumination is essential for scanning in colors while having only minor effect on grayscale scanning.
6. Scanning a slide in a desktop scanner gives a “bright field” representation of the slides. This means that a signal is black (or colored) because some of the light is absorbed while no signal is represented by white in the picture because no light is absorbed. Most quantification software for quantification of microarrays is designed to handle fluorescence, which is a dark field technology where signal is light emitted in a background of no light. To use such microarray spot quantification software using images generated with bright field detection, the image must be inverted. After inverting the bright field image in a computer program, the image appears as an image generated from a dark field technology. After inverting the picture from the desktop scanner, microarray spot quantification software can be used for image analysis. Note that there are many other softwares than Corel Photopaint that can invert a photo.
7. There are many ways to evaluate a genotyping microarray. One way is to simply look at the spots (see image in [Fig. 1](#)). It is clear that IVS-I-6 is a homozygote wild type because the signal from the wild-type probe is strong while the signal from the mutant probe is weak. In contrast the signals from both the wild-type probe and the mutant probe for IVS-1-5 are equally strong indicating that the patient is heterozygote for this mutation. Often, however, statistical methods are

required to evaluate if an assay is robust or not and then the quantified spots must be converted into a ratio. The simplest ratio to construct is to divide the signal from the wild-type spot with the signal from the mutant spot. However, if the mutant signal is very small, this will have a large influence on the obtained ratio. In the special case where the signal of the mutant probe is zero, a ratio cannot be constructed. To circumvent this problem, the signal ratio of $\text{wt}/(\text{wt} + \text{mt})$ is used. Using this ratio, homozygote wild types are close to 1, heterozygotes close to 0.5, and homozygote mutants have a ratio close to 0 (**Fig. 1**).

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Chapter 5

Antibody-Based Microarrays

Christer Wingren and Carl A.K. Borrebaeck

Summary

Antibody-based microarrays are a new powerful proteomic technology that can be used to generate rapid and detailed expression profiles of defined sets of protein analytes in complex samples as well as high-resolution portraits of entire proteomes. Miniaturized micro- and nanoarrays can be printed with numerous antibodies carrying the desired specificities. Multiplexed and ultra-sensitive assays, specifically targeting several analytes in a single experiment, can be performed, while consuming only minute amounts of the sample. The array images generated can then be converted into protein expression profiles, or maps, revealing the detailed composition of the sample. This promising proteomic research tool will thus provide unique opportunities for e.g. disease proteomics, biomarker discovery, disease diagnostics, and patient stratification. This review describes the antibody-based microarray technology and applications thereof.

Key words: Antibody microarrays, Disease proteomics, Disease diagnostics, High-throughput proteomics, Nanoarrays, Oncoproteomics, Protein expression profiling, Recombinant antibodies

1. Introduction

The concept of antibody micro- and nanoarrays is based on the arraying of small amount (pL scale or less) of several individual antibodies in discrete positions (nm- to μm -sized spots) in an ordered pattern, an array, onto a solid support where they will act as specific probes, or catcher molecules (1–7) (**Fig. 1**). These miniaturized arrays (mm^2 to cm^2 range), ranging in density from a few antibodies to several thousands, are then incubated with minute amounts (μL scale) of (labeled) non-fractionated biological sample, e.g. plasma. Next, specifically bound analytes (e.g. proteins, haptens, and carbohydrates) are detected and quantified, mainly using fluorescence as mode of detection. Assay sensitivities in the

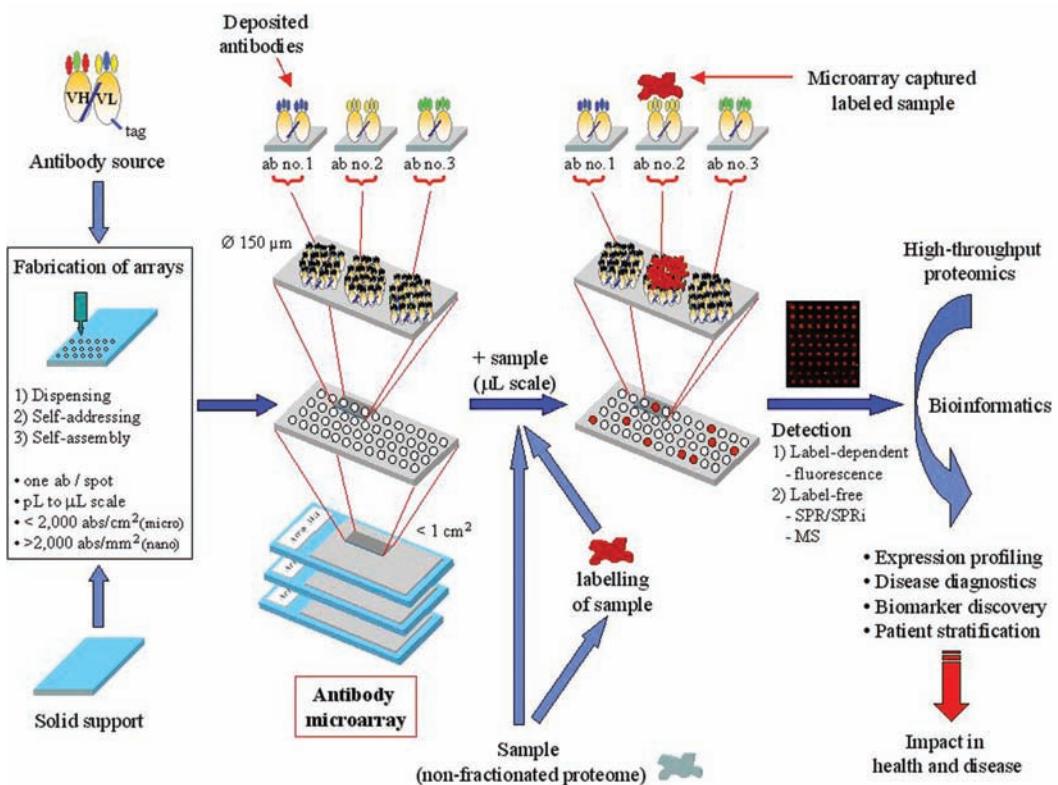


Fig. 1. Schematic of the antibody microarray setup.

pM to fM range may be obtained by adopting high-performing microarray platforms. Finally, the microarray images can be transformed into (differential) protein expression profiles, or protein atlases, revealing the composition of the sample at the molecular level.

The antibody microarray technology has made tremendous progress during the last decade (1, 3, 5–7), going from the first proof-of-concept studies (8–10) to demanding serum protein profiling applications within for example oncoproteomics (7, 11–14). This progress has been made possible by adopting a cross-disciplinary approach and simultaneously addressing the key technological issues required to design a high-performing antibody microarray platform (6, 7, 15, 16), including (a) solid support (6, 17–20), (b) content (6, 21), (c) array format (4, 7, 22), (d) array fabrication (6, 7, 23, 24), (e) sample (6, 25–28), (f) analytical principle (6, 7, 29–34), and (g) bioinformatics (6, 7).

2. Antibody-Based Microarrays: A Novel Technology for High-Throughput Proteomics

Advancing beyond the field of genomics, proteomics has become a key discipline for addressing global protein expression patterns in health and disease (1, 3, 7, 11, 35–37). Performing global proteome analysis has, however, proven to be technologically very demanding due to the complex nature of proteomes, such as human serum, composed of thousands of different proteins present at a dynamic range of at least 11 orders of magnitude (38–42). While traditional proteomic technologies are powerful and have been extensively used, they suffer from impaired resolution, selectivity, sensitivity and throughput, clearly demonstrating the need for novel complementary high-performing proteomic technologies (5, 38, 39, 41–44). In this context, antibody-based microarrays have emerged as a novel promising proteomic technology (3, 5–7, 36–38). In particular, this methodology could enable rapid, miniaturized and multiplexed assay setups to be designed, capable of targeting low-abundant protein analytes in complex, non-fractionated proteomes. In the end, integration of different data sets generated by multiple proteomic strategies and technology platforms will probably be required to generate complete atlases over the human proteomes.

The possibility to simultaneously monitor the detailed expression patterns of numerous proteins in clinical samples will open up new avenues within disease proteomics (3, 5, 7, 11, 35, 38). The long-term goal is to implement such proteomic technologies for routine use in clinical settings for diagnostics, patient stratification, evaluating drug efficacy etc., taking the first steps towards personalized medicine. In the end, this may provide novel means for early and improved disease diagnostics, surveillance, predicting relapses and disease recurrence, biomarker discovery, and identification of new improved therapeutics etc.

3. The Antibody Microarray Technology

The introduction of the antibody (protein) microarray technology immediately raised great expectations and hopes for rapid progress within biomedicine. So far, the impact of the technology within disease proteomics has been moderate (1, 3, 5, 7, 35, 36), mainly reflecting the demanding and time-consuming process of developing high-performing antibody microarray platforms (6, 7, 15, 16). However, the technology has now matured to the point where the number of applications is expected to increase significantly in the coming years.

4. Solid Support

As outlined in **Tables 1–3**, a variety of surface designs and materials has been evaluated as solid supports for antibody-based microarrays. While microarrays fabricated on planar solid supports in the format of microscope slides (12–14, 25, 27, 45) still dominate (1, 3, 6, 7, 36, 46), alternative surface architectures have been introduced, including well-based arrays (ELISA plates) (47), nanovial-based arrays (48, 49), atto-vial-based arrays (50, 51), and suspension arrays, i.e. arrays based on probe functionalized beads in solution (52–54) (**Table 1**). These various surface designs are

Table 1
General overview of surface designs of solid supports used for antibody-based microarrays

Surface designs	Stage of development	Format	Potential array density	Amount of reagent required	Compatibility with current spotters and detectors	Selected references
Planar solid supports	Well established	Microscope slide (75 mm × 25 mm)	High (< 5,000/cm ²)	Low to moderate (μL scale or larger)	High	(12–14, 25, 27, 45)
Well-based arrays	Established	ELISA plates	Moderate (<100/well)	Moderate to high	Low	(47)
Nanovial-based arrays	1st generations	1.5-μL volume vials (100–1,000 nL reaction volumes/vial)	Very high (>>5,000/cm ²)	Very low	Low to medium	(48, 49)
Atto-vial-based arrays	1st generations	Ø 200 nm to 5 μm (6 aL to 4 fL reaction volumes/vial)	Very high (< 225,000/mm ²)	Very low	Low to medium	(50, 51)
Suspension arrays	Established	Bead-based suspension arrays (functionalized beads)	Moderate (<100/tube)	Medium	Low (FACS compatible)	(52–54)
Micro/nano-fluidics	1st generations	Fluidics for reagents handling/delivery; built-in surface designs or devices added on afterwards				(22, 57–61)

Table 2
General overview of selected solid supports used for antibody-based microarrays

Surface structure	Examples of surface chemistries/ modifications	Coupling chemistry	Examples of commercially available variants	Comments	Selected references
<i>(A) Glass-based supports</i>					
Planar	Ni ²⁺ -NTA	Affinity	Xenoslide N		http://www.xenopore.com
	Streptavidin	Affinity	SuperStreptavidin		http://www.arrayit.com
	Epoxy groups	Covalently	Superepoxy2		http://www.arrayit.com
	Amine groups	Covalently			http://www.arrayit.com
	Aldehyde groups	Covalently	Superaldehyde2		http://www.arrayit.com
	Amino-PEG	Covalent			(65)
3D	Agarose-coated	Adsorption			(62)
	Nitrocellulose	Adsorption	FAST-slides	Biocompatible ^a	http://www.whatman.com
	Nitrocellulose (ultra-thin)	Adsorption	GenTel	Biocompatible	http://www.gentelbio.com
	Polyacrylamide-based gel	Covalently	Nexterion slide H	Inert ^b /sensitive ^c /biocompatible	http://www.schott.com
	Polyacrylamide-based gel	Adsorption	Hydrogel, HC hydrogels		http://perkinelmer.com , http://www.xantec.com
	Ni ²⁺ -NTA/polyacrylamid gel	Affinity	NIHC hydrogel		http://www.xantec.com
Nanovials	Glass	Adsorption			(49)
Atto-vials	Modified Zep-tomark slide	Adsorp./ aff.bind.		Inert/biocompatible	(51)
<i>(B) Plastic/polymer-based supports</i>					
Planar	Polystyrene-based	Adsorption	Protein binding	Inert/sensitive/biocompatible	http://www.nuncbrand.com
	Polystyrene-based	Adsorption	Black polymer Maxisorb	Inert/sensitive/biocompatible	http://www.nuncbrand.com

(continued)

Table 2
(continued)

Surface structure	Examples of surface chemistries/ modifications	Coupling chemistry	Examples of commercially available variants	Comments	Selected references
Wells	Polystyrene-based	Adsorption		Biocompatible	(47)
Beads	Polystyrene micro-spheres	Covalently	xMAP technology	FACS compatible	http://www.luminexcorp.com
	PMMA, PC, or Zeonex	Adsorption		3D surface generated by ion etching	(64)
<i>(C) Silicon-based supports</i>					
Planar	Silicon surface	Adsorption		Inert	(20, 28)
3D	macro- and nano-porous	Adsorption		Mass spectrometry compatible	(63)
	Nitrocellulose-coated	Adsorption		Biocompatible	(20)
Macroporous					

PMMA poly(methyl methacrylate), PC polycarbonate

^aArrayed antibodies display high functionality

^bThe substrate is inert, i.e. can be effectively blocked, meaning that low non-specific binding is observed even when targeting complex proteomes

^cHighly sensitive array setups have been designed using this substrate

associated with different possibilities and limitations with respect to specific array requirements, such as potential probe (array) density, consumption of reagents as well as compatibility with current instrumentation for array fabrication and detection (1, 3, 5–7, 55, 56). Furthermore, advanced surface designs, introducing micro- and nanofluidic architectures for reagent handling/delivery have also been fabricated (22, 57–61), adding yet another dimension of array layouts to be considered when setting up a microarray experiment (6, 7).

To date, solid supports based on predominantly glass-, plastic-, polymer- or silicon slides, often modified with one-, two- or three-dimensional structured surface modifications have been fabricated (6, 7, 17–20, 28, 47, 49, 51, 62–65) (**Table 2**). These surface materials will display a wide range of biophysical properties with respect to e.g. biocompatibility, wettability, inertness,

Table 3
General overview of coupling chemistries used for antibody-based microarrays

Coupling chemistry	Protocol ^a	Probe selectivity ^b	Probe format ^b	Probe orientation ^c	Probe binding ^d
Adsorption	+++	Non-selective	Purified	Random	Reversible
Covalent	+	Non-selective	Purified	Random	Irreversible
Affinity binding	++	Selective	Non-purified	Orientated	Reversible

^aFrom simple and rapid immobilization protocols (++) to more complicated and time-consuming protocols (+)

^bAdopting a non-selective coupling chemistry means that any irrelevant protein present in the probe mixture can be immobilized, forcing pre-purified probes to be used. In contrast, using a selective coupling chemistry means that the probes will be specifically immobilized in a one-step procedure on the chip, enabling non-purified probes to be directly applied

^cWhile the functionality of arrayed probes immobilized in any, i.e. random, orientation may result in an impaired functionality due to e.g. sterical hindrance, probes immobilized in an orientated, i.e. favorable, manner are likely to display a higher on-chip functionality^dReversibly immobilized probes may leak-off due to stringent washing protocols, whereas irreversibly (covalently) bound will not

surface chemistry, coupling chemistry, and probe binding capacity (**Tables 2 and 3**) that directly affect the performance and applicability of the setup (6, 17–19, 28, 66). The precise choice of material and surface architecture is not obvious, and is heavily dependent on the specific array and assay conditions/demands at hand. Some key factors to consider are the (a) probe source/format, (b) coupling chemistry, e.g. efficiency, capacity, selectivity, and orientation, (c) sample format, e.g. degree of complexity, and (d) analytical principle, e.g. assay sensitivity required and compatibility with the selected detection method/instrumentation (6, 17–19, 28, 66) (**Tables 1–3**).

5. Probes

Antibody-based microarrays have been fabricated using intact monoclonal and polyclonal antibodies of different classes, e.g. IgG and IgM, or fragments thereof, e.g. F(ab')₂, as well as recombinant scFv antibody fragments as probes (2, 3, 6, 7, 21, 55, 56, 67, 68) (**Fig. 2**). The dominating probe formats used so far are intact polyclonal and monoclonal antibodies, a bias mainly reflecting the availability of the various antibody formats to the broad community (1, 3, 6, 35, 36, 56, 66). However, the choice of probe format is a key parameter, as the on-chip performances of readily available off-the-shelf monoclonal and polyclonal-based

reagents have been found to vary significantly, with up to 95% displaying poor performances in some studies (3, 6, 7, 21, 69). These observations have clearly highlighted the importance of using probes that have been designed to perform well in microarray-based applications (6, 7, 21, 56). In recent years, large recombinant antibody phage display libraries, microarray adapted by design, have been introduced as an almost limitless supply of high-quality probes (6, 66, 70). These probes have been found to display high on-chip functional stability, surviving several months of storage as arrayed dehydrated probes at room temperature, providing selective and highly specific (fM range) assays (6, 15, 16, 25, 28, 71). Notably, adopting such a probe format will bypass several of the common limitations otherwise associated with the conventional format of polyclonal and monoclonal reagents, including range of specificities, availability, renewability, and on-chip performances etc. (7, 15, 16, 21) (Fig. 2).

Of note, by designing antibodies specific for short peptide motifs, present in one or several proteins, the number of antibody probes required for targeting the non-redundant proteome

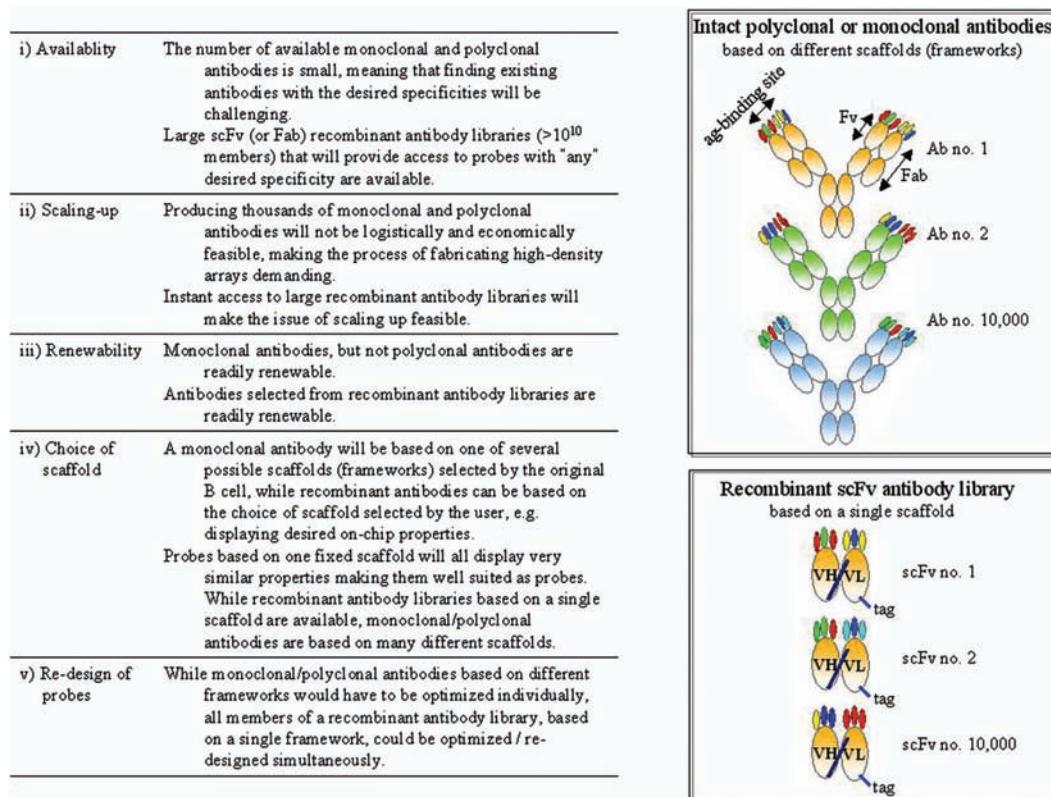


Fig. 2. Comparison of the properties displayed by monoclonal, polyclonal, and recombinant antibody probe sources intended for microarray applications.

can be significantly reduced. By developing such context-independent motif-specific (CIMS) antibodies, a novel array setup targeting trypsin digested proteomes has recently been outlined, providing a novel avenue for interfacing antibody array platforms with mass spectrometry-based read-out systems (Borrebaeck and Wingren, unpublished observations).

The issue of microarray adaptation of the probes by molecular design so that they not only display improved but also a more similar range of properties, such as on-chip stability and functionality, will become increasingly important as the demands on the assay performances continue to increase. To this end, adopting recombinant antibody libraries, based on a single framework, i.e. minimizing the molecular probe-to-probe variations to the complementarity determining regions only, i.e. the antigen-binding site, as probe source (7, 15, 16, 21, 70) is an appealing approach (**Fig. 2**). In fact, these antibody-based probes will then display as similar molecular properties as possible, thereby minimizing any effects due to probe-to-probe variations. Of note, all members of such a library can then simply be re-designed in the same manner adopting a single strategy to improve their molecular performances, considerably facilitating the continuously on-going efforts of evolving the probes even further.

6. Array Format

Three main classes of array formats can be identified based on support, density, and size (3–7, 69) (**Table 4**). First, the current antibody microarrays can be divided into two major designs based on the choice of support, featuring arrays based on solid support vs. solution-based bead arrays (**Tables 1 and 4**). The former layout is the most common and provides the option to fabricate arrays of a wide range of probe densities, which are read using mainly conventional array scanners, while the latter design, which is interfaced with a FACS instrument for detection, is limited by a multiplexity of <100 probes/assay (<http://luminexcorp.com>). Depending on the architecture of the solid support, several sub-formats can also be observed, as outlined in **Table 1**.

Second, based on the probe density, low- (<25 probes/array), medium- (<500 probes/array), to high-density array (<10,000 probes/array) formats/designs have been outlined (5–7, 22) (**Table 4**). Because of the spot size (100–300 μm), the array features are limited to mainly <2,000 probes/cm² (5–7, 22). To date, mainly low- to medium-density antibody arrays have been fabricated (3, 6, 7, 69). The observed limitations in array density are commonly explained by the limited access to numerous

Table 4
General overview of the main classes of antibody array formats

Main classes	Division	Features			
Support		<i>Multiplexity</i>		<i>Detection unit</i>	<i>Stage of development</i>
Solid support		Any		Conv. array scanners	Well established
Solution-based beads		<100 probes/assay		FACS	Established
Density		<i>Density</i>		<i>Stage of development</i>	
Density	Low-density	<25 probes/array		Well established	
Density	Medium-density	<500 probes/arrays		Well established	
Density	High-density	<10,000 probes/array		First generations	
Density	Mega-density	>10,000 probes/array		Proof-of-concept	
Size		<i>Spot (feature) size</i>	<i>Array density</i>	<i>Total no. of unique probes</i>	<i>Array size</i>
Size	Microarrays	100–300 µm	<2,000 probes/cm ²	<1,000 ^a	cm ² range
Size	Nanoarrays	3–1,000 nm	<1 × 10 ⁶ probes/mm ²	<10 ^b	mm ² range

^aTotal number of unique probes is yet rarely above 1,000. Values up to 10,000 or higher are anticipated

^bSince mainly proof-of-concept studies have been generated, the total number of unique probes is still low, and not representative for the inherent capacity of nanoarrays. The anticipated value is >>10,000

high-performing probes as well as the overwhelming logistics in producing and purifying (most current substrates require pre-purified probes to be applied) thousands of probes prior to dispensing them one by one (5–7). As major efforts are under way to resolve the issues of extending the array density, a fourth format, yet to be realized, has recently been outlined, namely mega-dense arrays (>10,000 probes/array) (22). Such a comprehensive array layout will be required if the technology is to be used for true global proteome analysis targeting complex proteomes, such as human serum (1, 3, 6, 7, 35, 69).

Third, the on-going work of extending the array density is accompanied by parallel efforts in reducing the array size (4). Hence, the third main class of array formats is the distinction between microarrays (cm² range) vs. nanoarrays (mm² range)

(**Table 4**). Adopting nanotechnology approaches to miniaturize the microarray format even further will ultimately enable us to fabricate truly high-/mega-dense nanoarrays composed of >50,000 probes/mm² (22, 72–74). While the development of nanoarrays is still in its infancy, proof-of-concept has been demonstrated for a range of low-density (< 10 probes/arrays) tentative array sub-formats, including planar arrays (75–77), well-based arrays (78), nanovial arrays (49, 79), atto-vial arrays (50, 51), nanowire arrays (34, 80, 81), random arrays (82), bead arrays (83), nanoparticle arrays (84), and cantilever arrays (29, 85–88). In this work, both polyclonal and monoclonal antibodies, as well as recombinant scFv antibody fragments have been used as model probes. Additional work will be required to establish the concept of antibody nanoarrays resolving many of the remaining bottlenecks concerning in particular fabrication, detection/sensitivity, and applicability (4).

7. Array Fabrication

To date, a majority of all antibody microarrays has been fabricated by dispensing the probes one-by-one in the pL scale using either non-contact or contact dispensers (1, 3, 5–7, 69). While the former printers (piezo technology) are more biocompatible and show less carry-over effects, the latter type of printers are generally faster and display a higher capacity. A variety of printers are now commercially available from a range of vendors. The probes are commonly printed in standard PBS or PBS supplemented with various additives, such as glycerol. The latter increases the viscosity of the printing buffer (required by some printers) and reduces the risk for the dispensed material to evaporate. In fact, when dispensing reagents in the pL scale, the drops evaporate a few seconds after hitting the surface, unless e.g. glycerol is added. This is a crucial feature to notice, as many proteins tend to denature and subsequently lose their activity if they are allowed to dehydrate on a solid support (6, 7, 21, 71). As outlined earlier (**Heading 5**) this issue can, however, also be resolved by using probes that have been microarray adapted by molecular design to survive such harsh conditions (5–7, 16, 21, 71, 89).

Recently, alternative ways of fabricating antibody microarrays have been outlined (**Fig. 1**), including self-addressing (6, 7, 24, 49, 90) and self-assembly (6, 7, 23, 49, 91, 92). The first generations of self-addressable probes have been designed by taking advantage of the features associated with affinity immobilization (6, 7, 24, 49, 90). Briefly, the probes are then equipped with

unique zip-code tags, e.g. DNA, and when added to the chip in bulk solution, the tag will guide them to their corresponding spot, i.e. unique address, on the chip composed of e.g. complementary DNA. To become an established procedure, the issue of functionalizing each probe with a unique zip-code tag in a simple and efficient manner remains to be resolved (7). Furthermore, the design of self-assembling antibody microarrays is another interesting way of fabricating arrays (6, 7, 23, 49, 91, 92). In this case, the protein is fabricated directly on the chip in the desired spot by cell-free protein expression. While only proof-of-concept efforts have yet been shown for antibody microarrays (6, 7, 23, 92), large-scale protein microarrays based on this technology are now already commercially available (e.g. <http://www.lumera.com>).

In contrast, no dominating technology(ies) for fabricating antibody nanoarrays is yet identified. As of today, a range of tentative methodologies for generating nanopatterns with biological function are available (74, 93), including soft-lithographic techniques, microcontact printing, nanoimprint lithography (75), nanosphere lithography, electron beam lithography (51, 77, 94), focused ion-beam lithography, conductive atomic force microscopy (95), dip-pen nanolithography (76, 96–98), and nanodispensing (50, 99, 100). While these techniques can be used to generate patterns of nanosized features, e.g. vials, or spots, the issue of functionalizing the individual features with different antibodies is a key issue that remains to be resolved. Of note, two different nanodispensers have been designed that can be used to functionalize individual nanosized features (50, 99, 100). Albeit demonstrating proof-of-principle, it can be argued whether such an approach, i.e. direct dispensing, is compatible with fabrication of high-density arrays whether working in the micro- or nanoformat, paving the way for adopting for self-addressing and self-assembling methodologies also for the fabrication of nanoarrays.

8. Sample

All proteomes generated in a soluble format, whether in a native, denatured, or digested (6, 7, 54) form, can tentatively be targeted by antibody microarrays in a crude non-fractionated format (3, 6, 7, 25, 27, 28). To date, mainly water-soluble proteins have been analysed (1, 3, 5–7, 69), but recent efforts have shown that this is true also for membrane proteins, as illustrated by profiling of the cell surface membrane proteomes (67, 101–108). This provides key advantages compared to most classical proteomic approaches, which (a) require the sample complexity to be reduced prior to

analysis, either by depletion of the most high-abundant species or pre-fractionation of the sample, and (b) are limited to analysing mainly water-soluble protein analytes (38, 39, 41, 42, 44).

Another key advantage compared to conventional proteomic methodologies is the fact that the antibody microarray technology consumes only minute amounts of the sample (sub- μ L scale). As for example, less than 1 μ L of a non-fractionated serum is sufficient to run an assay, simultaneously targeting thousands of protein analytes in a single assay (16, 25, 28). Of note, the sample consumption can be reduced another 1,000,000 times (pL scale) by spotting the sample on top of the already dispensed antibody, i.e. adopting multispotting techniques (8, 79, 109).

As for all proteomic approaches, the format of the clinical sample, e.g. serum sample vs. plasma sample, as well as how the sample has been handled and stored, e.g. frozen directly or stored at room temperature prior to freezing, are key parameters that must be optimized and standardized (6, 7, 38, 110, 111).

Interfacing the array setup with a label-dependent read-out system, the sample can be fluorescently labelled, either directly with e.g. Cy-, Alexa-, or ULS dyes, or indirectly with e.g. biotin or ULS-biotin (which is then visualized with directly labelled streptavidin). When analysing complex samples, recent work has shown biotinylation to be the preferred way of labelling the sample, in particular when targeting low-abundant protein analytes in non-fractionated proteomes (16, 27, 28). This was manifested by an increased assay sensitivity and a minimal non-specific background binding (16, 27, 28). Further, single- or dual-colour approaches can be adopted when performing differential protein expression profiling. Although a two-color approach is appealing, recent work has shown distinct discrepancies in the observed signal intensities for the two dyes in several matching pairs of dyes, with very low signal intensities obtained for the least performing dye (28). The data clearly indicated on inherent differences in labelling efficiency or “transmitted signal intensities” of the two dyes in each matching pair tested (28). Hence, the results implied that a one-color approach should be adopted to obtain high and adequate signal intensities when targeting complex samples.

9. Analytical Principle

Two main conceptual approaches have been used for reading the antibody microarrays based on adopting either label-dependent detection technologies (12–14, 25, 28, 45, 55, 112) or label-free methodologies (29, 32–34, 86) (**Table 5**). To date, the former approach relying on fluorescence is by far the most

Table 5
General overview of the analytical read-out systems used for antibody micrarray detection

Approach	Technique	Comments	References
Label-dependent	Fluorescence	Dominating technique. LOD in the sub pM to fM range	(12, 16, 27, 28)
		Various signal amplification techniques available	
	Chemiluminescences		(112)
Label-free	Electrical read-out (nanowire)	LOD in the pg/mL range has initially been reported	(34)
	Electrochemical read-out		(114)
	Light-scattering		(47)
	Mass spectrometry (MS)	Mainly single MS, but tandem MS (MS-MS) under way	
	MALDI-TOF MS	LOD in the attomole to zeptomole range	(8, 115–117)
	SELDI-TOF MS		http://www.ciphergen.com
	Microcantilever	LOD in the μM to nM range	(29, 85–88)
	Quartz crystal microbalance (QCM)	LOD in the picomole range	(31)
	Surface plasmon resonance (SPR)		(32, 118), http://www.biacore.com
	SPR imaging (SPRi)		(30, 118–120), http://www.genoptics-spr.com

frequently used analytical read-out system (6, 7, 33). A limit of detection (LOD) in the sub-pM to -fM range has been observed for antibody microarrays targeting indirectly labelled samples (biotinylated) that were visualized with fluorescently tagged streptavidin (12, 16, 27, 28). Of note, various signal amplification technologies, such as rolling-circle amplification (113) and thymidine signalling amplification kit (<http://www.perkinelmer.com>)

([com](#)) may also be used to improve the assay sensitivity even further. Adopting a sandwich approach may be another route to improve both the assay sensitivity and the specificity of the antibody microarray setup. Although appealing, such a layout will then introduce other key limitations, including the (a) logistics in generating two antibody clones per antigen, and (b) reduced multiplexity (about 50 probes per sandwich array) to maintain adequate assay features (2).

However, major efforts are under way to develop and implement label-free read-out systems to bypass the tentative limitations associated with labelling of the sample, including the risk that the immunoreactivity may be lost upon labelling (masking) the epitope and the issue of labelling a complex sample in a representative manner (5–7). To date, proof-of-concept has been outlined for a range of alternative setups, including electrical read-out (nanowires) (34), electrochemical read-out (114), light scattering (47), MALDI-TOF mass spectrometry (MS) (8, 115–117), SELDI-TOF MS (<http://www.ciphergen.com>), microcantilever (29, 85–88), quartz crystal microbalance (31), surface plasmon resonance (SPR) (32, 118) (<http://www.biacore.com>), and SPR imaging (SPRi) (30, 118–120) (<http://www.genoptics-spr.com>). Of note, these methodologies may not only allow us to detect the bound analyte, but could also provide information about the analyte, e.g. identification (MS and tandem MS) and binding characteristics (affinity) (SPR, SPRi, and QCM). These initial layouts have commonly reported LODs in the μM to nM range (4, 6, 7). Interestingly, one setup, based on electrical read-out (nanowires), has indicated sensitivities in the pg/mL range even when targeting complex samples (34). However, additional work will be required to establish whether nanowires indeed will be capable of delivering such LODs on a regular basis. Except for SPR- and SPRi-based approaches (<400 spots/array) (30, 32, 118–120), only low-density arrays have so far been designed for label-free read-out systems. Taken together, many of these label-free technologies display great promise, although outstanding issues regarding sensitivity, dynamic range, multiplexity (array density), and non-specific binding (blocking) must be resolved before they can start to seriously challenge fluorescent-based analytical read-out systems for antibody microarrays.

10. Bioinformatics

No standardized procedure(s) for antibody microarray data quantification, normalization, and analysis etc. have yet been established. In fact, validated procedures and softwares have more or

less been directly adopted from the more mature field of DNA microarrays (5–7).

Having quantified the arrays, normalization is a key step to enable data generated on different subarrays and/or chips to be compared (6, 7, 25, 121). As no equivalents to the house keeping genes commonly used for DNA microarray normalization exist, novel procedures have been outlined for antibody (protein) microarrays (7, 12, 25, 121–123). Briefly, the first generations of normalization procedures involved the use of (a) the amount of antibody spotted (123), (b) one or several reference proteins spiked in at a known concentration prior to labelling (12, 25, 121), (c) a single analyte for which the sample concentration was determined *a priori* using an alternative method, such as ELISA (25, 121), (d) an internally normalized ratio algorithm based on a dual-colour approach (122), or (e) semi-global approach (7). In the latter case, large arrays are required, and about 10% of the antibodies displaying the lowest coefficient of variation over all samples (arrays) are identified and used for normalization (Wingren and Borrebaeck, unpublished observations). Of note, the choices of approach and reference protein(s) are critical, clearly illustrated by the fact that not all single analytes tested performed well for normalization (25, 121). Future work will be required before any standardized procedure(s) can be established.

Once the data have been normalized, the bioinformatics analysis of the antibody array data, such as identification of differentially expressed proteins and tentative biomarker signatures, has been successfully performed, e.g. (12–14). In many of these endeavours, validated approaches, statistics, and softwares from the DNA microarray community have been used, including Cluster, Genespring, Principle Component Analysis, Receiver Operating Characteristics curves, Sammon maps, significance of microarray analysis, Support Vector Machine, and TreeView. The issue of handling antibody microarray data in an adequate manner is expected to mature significantly in the coming years as the technology continues to mature towards more clinical applications based on truly high-density arrays.

11. Antibody Microarray-Based Applications

A number of antibody microarray-based applications have been published, ranging from small proof-of-concept studies to semi-global proteome expression profiling efforts (**Table 6**); for more extensive reviews see (1–3, 5–7, 35–37, 69). In these endeavours, a variety of array platforms have been used, relying on both in-house designs as well as commercially available alternatives.

Table 6
Overview of antibody microarray-based applications

Area of application	Disease or biological process	References
Autoimmunity	Primary Sjögren's syndrome	(53)
	Systemic lupus erythematosus	Unpublished observations
Allergy	Cytokine profiling	(185)
Bladder proteomics	Smooth muscle hypertrophy	(151)
Cell proteomics	Amphotericin B exposure	(156)
	Blood cells	(67)
	Cell differentiation	(155)
	Chondrocytes	(152)
	Model systems	(15, 26, 107, 108, 154)
Drug abuse	Screening	(133)
Glycomics	Pancreatic cancer	(136)
Heart proteomics	–	(157)
	Myocardial infarction	(134, 135)
Hereditary disease	Cystic fibrosis	(158)
Inflammation/infection	Artherosclerosis	(159)
	Inflammatory bowel disease	(161)
	Obesity	(124)
	Rhinovirus infection	(160)
	Complement deficiency	(25)
Liver proteomics	APAP-induced liver disease	(162)
Lung proteomics	Chromium(VI) treatment	(163)
Medical microbiology	Bacterial infection	(166)
	Detection of bacteria and/or toxins	(164, 165, 167–174)
	<i>Helicobacter pylori</i> infection	(12)
	Serotyping of bacteria	(129, 130)
Neurology/psychiatry	Cerebral palsy	(176)
	Drug abuse	(178)
	Transverse myelitis	(177)
Obstetrics/gynaecology	Pre-eclampsia	(179), unpublished observations

(continued)

Table 6
(continued)

Area of application	Disease or biological process	References
Oncoproteomics	Angiogenesis	(137)
	Bladder cancer	(14)
	Breast cancer	(125, 126, 142, 143, 146, 147, 186)
	Colon cancer	(128)
	Colorectal cancer	(106)
	Gastric adenoma carcinoma	(12)
	Glioblastoma	(112)
	Hepatocellular carcinoma	(150)
	Intestinal cancer	(141)
	Leukemia	(103, 104)
	Liver cancer	(148)
	Lung cancer	(13, 138)
	Mantle-cell lymphoma	(140)
	Model system	(139)
Periodontology	Ovarian cancer	(144)
	Pancreatic cancer	(45, 187)
	Prostate cancer	(145, 149)
	Squamous cell carcinoma	(127)
	Model system	(180)
	Model system	(132)
	Lung cancer	(131)
	Post-translational modifications	(182)
	Biosynthetic pathways	(181)
Protein signalling	Proapoptotic/-survival stimuli	(183)

While the in-house designed arrays have been applied in antibody microarray studies of more exploratory nature, e.g. (12–14, 67), the commercial setups have mainly been used as one tool among many, e.g. (53, 124, 125). Further, the work has shown that in-house designed (custom made) antibody microarray platforms, in most cases, displayed improved assay performances, e.g. sensitivity

and on-chip functionality, as well as flexibility, with respect to the number of antibodies and range of specificities included.

These studies have clearly demonstrated the potential of antibody microarray as a versatile, rapid, and multiplexed proteomic technology displaying high selectivity, sensitivity, and specificity (**Table 6**). A wide range of applications have been outlined, including e.g. (a) identification of disease-associated biomarker signatures (12, 13, 126–128), (b) identification of protein signatures associated with clinical parameters, e.g. survival (14), (c) serotyping of bacteria (129, 130), (d) cell phenotyping (67, 101, 104, 105), (e) phosphoproteomics (131, 132), (f) screening of drug abuse (133), (g) detection of myocardial infarction (134, 135), and (h) carbohydrate profiling (67, 136). Although a focus towards oncoproteomics has been observed (7, 11–14, 45, 103, 104, 106, 112, 125–128, 137–150), a feature that is likely to become even more pronounced in the coming years (7, 11), the technology has also been applied within e.g. autoimmunity (53), allergy (185), bladder proteomics (151), cell proteomics (26, 67, 107, 108, 151–156), drug abuse (152), glycomics (136), heart proteomics (134, 135, 157), hereditary diseases (158), inflammation/infection (25, 124, 159–161), liver proteomics (162), lung proteomics (163), medical microbiology (12, 129, 130, 164–175), neurology/psychiatry (101, 176–178), obstetrics/gynaecology (179), periodontology (180), phosphoproteomics (131, 132), protein expression (181, 182), and signalling (183).

Reviewing these applications in detail is beyond the scope of this review. Briefly, in several studies differential protein expression profiling of complex proteomes, e.g. serum or tissue, has been performed in order to identify protein signatures associated with disease, such as bladder cancer (14), breast cancer (125, 126, 142, 143, 146, 147, 186), cerebral palsy (176), cystic fibrosis (158), gastric adenoma carcinoma (12), *Helicobacter pylori* infection (12), inflammatory bowel disease (161), lung cancer (13, 138), pancreatic cancer (45, 187), and prostate cancer (145, 149). The results have shown that disease-associated biomarker signatures could be identified that successfully can be used to distinguish between healthy controls vs. the patient group under study displaying a discriminatory power (sensitivity/specificity) of around 80–90% (11, 13, 14, 45, 143), as well as to group patients into low vs. high risk based on their survival time (14). However, in some cases, more general disease signatures, overlapping between different conditions, were observed, rather reflecting the fact that the patients were sick than being indicative of the specific disease that they were suffering from (5, 7, 11). To some extent this reflects the need of continuously developing the antibody microarray technology further. By adopting high-performing antibody microarray setups providing higher sensitivity and a wider range

of specificities, the more low-abundant space of the proteomes, anticipated to contain more tentative biomarkers, could be screened.

Another interesting area of application is the use of antibody microarrays for detection and serotyping of bacteria (129, 130, 164), bacterial proteins (e.g. toxins) (129, 130, 164, 165, 170, 173) as well as bacterial related diseases (12). Efforts have been made to design small devices, in some instances aiming for small hand-held devices that could be used for simple and rapid detection, as for example, rapid detection of toxins in clinical fluids, food, and drinking water is vital in order to expedite appropriate and immediate counter measures.

To date, membrane proteins have been difficult to address due to limitations in conventional proteomic technologies (38, 39, 41). In this context, it should be noted that membrane proteins, and in particular the plasma membrane proteome, constitute a key group of proteins being one of the most common targets for therapeutics and disease diagnostics (38, 41, 184). Of note, the first antibody microarray designs targeting (cell surface) membrane proteins and/or carbohydrates have recently been published (67, 101–108, 153). Initially, these antibody microarray setups, based on antisera, purified intact polyclonal and monoclonal antibodies, or recombinant scFv antibody fragments, have been used to perform mammalian cell phenotyping (67, 101–108, 153, Dexlin et al., unpublished observations) or serotyping of bacteria (129, 130). In the end, the technology may provide us with the means to efficiently map the changes that occur in the cell surface membrane proteome during differentiation, in response to various stimuli, and will provide an increased understanding of fundamental processes in both health and disease.

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Chapter 6

Application of Protein ArrayTubes to Bacteria, Toxin, and Biological Warfare Agent Detection

Ralf Ehricht, Karin Adelhelm, Stefan Monecke, and Birgit Huelseweh

Summary

Microarray technology enables the fast and parallel analysis of a multitude of biologically relevant parameters. Not only nucleic acid-based tests, but also peptide, antigen, and antibody assays using different formats of microarrays evolved within the last decade. They offer the possibility to measure interactions in a miniaturised, economic, automated, and qualitative or quantitative way providing insights into the cellular machinery of diverse organisms. Examples of applications in research and diagnostics are, e.g., O-typing of pathogenic *Escherichia coli*, detection of bacterial toxins and other biological warfare agents (BW agents) from a variety of different samples, screening of complex antibody libraries, and epitope mapping. Conventional O- and H-serotyping methods can now be substituted by procedures applying DNA oligonucleotide and antibody-based microarrays. For simultaneous and sensitive detection of BW agents microarray-based tests are available, which include not only relevant viruses and bacteria, but also toxins. This application is not only restricted to the security and military sector but it can also be used in the fields of medical diagnostics or public health to detect, e.g., staphylococcal enterotoxins in food or clinical samples. Furthermore, the same technology could be used to detect antibodies against enterotoxins in human sera using a competitive assay. Protein and peptide microarrays can also be used for characterisation of antibodies. On one hand, peptide microarrays allow detailed epitope mapping. On the other hand, a set of different antibodies recognising the same antigen can be spotted as a microarray and labelled as detection antibodies. This approach makes it possible to test every combination, allowing to find the optimal pair of detection/capture antibody.

Key words: Protein microarray, Antibody microarray, Peptide microarray, Bacterial toxins, Pathogenic bacteria, Biological warfare agents, Antibody screening, Epitope mapping.

1. Introduction

In nature, proteins interact with a variety of different ligands, e.g. DNA, modified and non-modified proteins, and peptides during different fundamental biological processes such as antigen–antibody

interaction or enzyme–substrate binding. Since the entire set of proteins involved in a biological machinery operates simultaneously, a massive parallel measurement is needed for the analysis and understanding of the system.

Amongst others, two-dimensional gel electrophoresis combined with mass spectrometry, multidimensional liquid chromatography, or protein microarray tests are possible analytical tools with different specificity, sensitivity, and hands-on-time to address such questions. Depending on the used format, protein or peptide microarrays offer the possibility to measure interactions in a miniaturised, economic, automated, and qualitative or quantitative way providing insights into the cellular machinery of a given organism or into the functionality of a protein type (1, 2).

The concept of protein microarrays was inspired by DNA microarrays, which enable the simultaneous detection of thousands of different genes or mRNA molecules for genotyping or expression profiling, respectively (3). It was a logical step to measure the translated and functional products of the genes, using basically the same experimental approach. However, one can not directly transfer all principles from DNA into protein microarray technology. Details such as the type of the reactive surface, spotting procedure, stability of the proteins on the microarray, and the experimental protocol adaptation have to be optimised (4, 5). In the last decade, different protein microarray formats and manufacturing procedures have been developed (6) that allow diverse applications in research and diagnostic tests.

O-typing of pathogenic *Escherichia coli*, detection of bacterial toxins and BW agents from a variety of different samples, screening of complex antibody libraries, and epitope mapping are applications that will be discussed in the present contribution.

E. coli is a commensal in the colon of animals and humans, and some of the serotypes or pathotypes are associated with diseases such as meningitis, urinary tract infection, diarrhoea, and even septicaemia. O-serotyping is an established method, which is based on variable highly immunogenic lipopolysaccharides (LPS) on the cellular surface. It has been shown previously that conventional O- and H-serotyping methods can be substituted by DNA oligonucleotide and antibody-based microarrays, which consume less time and resources (7, 8).

A further application arises from the growing threat of terroristic attacks and nations' vulnerability to BW agents including toxins, viruses, and bacteria. Here, protein microarrays might be used to analyse environmental and air samples as well as to constantly monitor food and water supplies. Additionally to a sensitive and definite identification of single agents, protein arrays with carefully selected antibodies allow the parallel detection of BW agents and can distinguish closely related pathogens (9–12).

Compared with DNA arrays, their application is not restricted to the detection of nucleic acid carrying pathogens. They can also be used to detect toxic substances such as ricin or bacterial exotoxins, e.g., staphylococcal enterotoxins in food and environmental matrices (13). Additionally, an antibody array might be used in a competitive assay to detect specific antibodies in human sera, indicating a previous exposure to a given antigen.

The most critical step in microarray assays comprises sample preparation and labelling, which is easier for protein applications than for DNA array-based tests. However, the challenge is to find the optimal combination of capture and detection antibody for a specific antigen. Because of the parallel detection of different agents that might be expected even within a single specimen, cross reactivities of the different labelled detection antibodies have to be ruled out although a high overall sensitivity of the complete test regarding each target needs to be retained. The very concept of an array offers the possibility to screen all available antibodies for a specific antigen by using them as both capture antibody to be spotted and detection antibody to be labelled. By testing every possible combination, the detection/capture antibody pairs best suited in terms of specificity and sensitivity can be easily detected. After performing these experiments, the best combination of antibodies for each distinct target can be used for the routine application of the assay, or for further optimisation.

Another experimental option is to immobilise antigens (peptides as single epitopes or even complex molecules) instead of antibodies. Microarrays with spotted peptides can so be used for detailed epitope mapping (14).

Altogether protein microarrays offer various opportunities in research and diagnostics, which might, combined with the appropriate protocols and platforms, change our understanding of the nature of biological processes, and which could help to perform diagnostic tasks in a faster, more reliable, and economic way.

2. Platforms, Microarrays, and Detection and Assay Principle

2.1. Platforms

For the techniques and protocols discussed later, a microarray platform was combined with a unique detection principle (**Fig. 1**). The ArrayTube system is a 1.5 ml reaction vial where a 3 mm × 3 mm glass microarray with a spotted area of 2.4 mm × 2.4 mm is mounted onto the bottom of a via that is compatible to standard laboratory equipment such as thermomixers (http://www.clondiag.com/products/array_tube/index.php). For automation of such tests, the ArrayStrip system

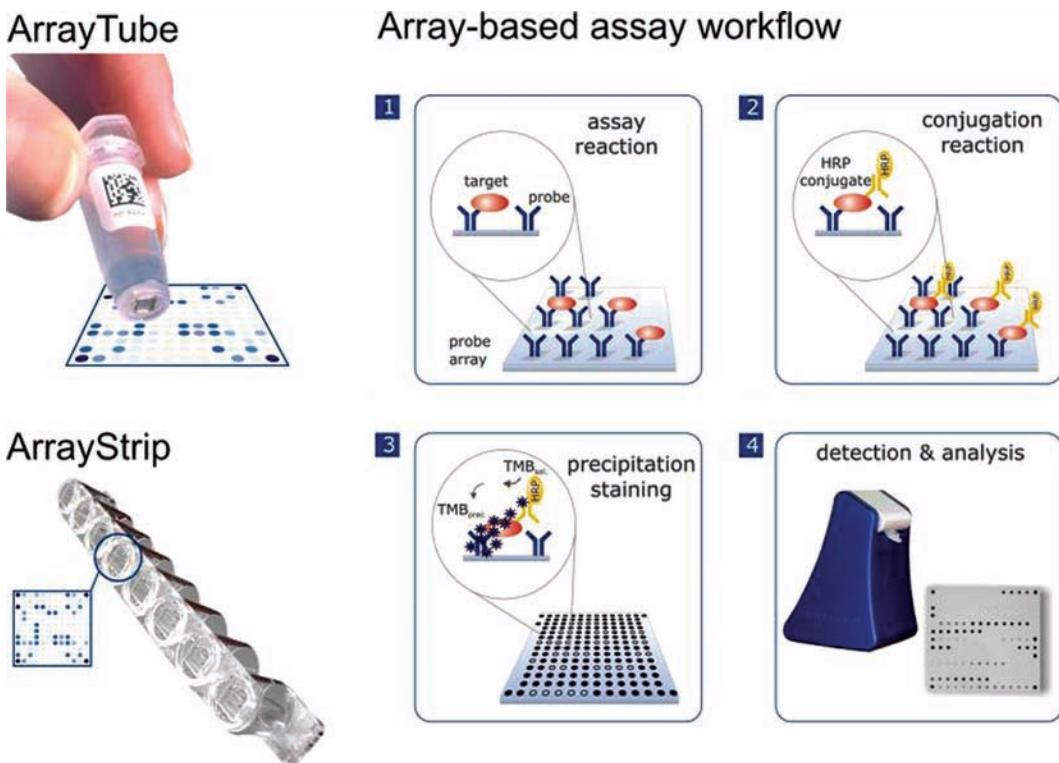


Fig. 1. Within the ArrayTube platform $3\text{ mm} \times 3\text{ mm}$ pre-spotted microarrays are mounted onto the bottom of 1.5 ml reaction vessels that are used manually with existing lab equipment. The ArrayStrip platform contains $4.2\text{ mm} \times 4.2\text{ mm}$ microarrays that can be completely processed and analysed in an automated device. In both platforms the workflow and analysis principle is the same. A directly or indirectly (via a secondary antibody) biotinylated antigen binds to a previously spotted and covalently coupled capture antibody. In a subsequent step, HRP catalyses a local TMB substrate precipitation, which can be detected simply by transmission measurement.

(http://www.clondiag.com/products/array_strip/index.php) was developed. It comprises eight microarrays of $4.2\text{ mm} \times 4.2\text{ mm}$ and a spotting area of $3.4\text{ mm} \times 3.6\text{ mm}$, which are included in one strip. Twelve strips can be combined and used as one 96-well microtiter plate.

2.2. Microarrays

Protein/peptide microarrays were produced using three dimensional epoxy-modified glass as spotting substrate. Substances were spotted without any detergents using final concentrations between 0.05 and 1 mg/ml protein or 1 and 5 mg/ml peptide (Jerini, Berlin, Germany) in $1\times$ phosphate buffered saline (PBS) containing $5\text{--}20\text{ mM}$ sucrose or trehalose. All peptides used consist of one C-terminal glycine residue and three N-terminal lysine residues combined with a trimesyl-tris(3,5-dibromo)salicylate

spacer (Jerini, Berlin, Germany). To avoid the disadvantages of piezo-based and split-pin-based spotting (e.g., undefined spotting volumes, cross contamination of substances due to incomplete washing procedures, “ghost spots” caused by dust particles, and waste of expensive spotting solutions of which always a portion remains in microtiter plates or cartridges that finally will be discarded) a newly developed contact spotting procedure was generally applied. Each substance to be spotted has its own spotting needle capillary, which is suited for both spotting and storage. Because of this approach, the complete amount of protein solution can be spotted without wasting any substance and without the possibility of cross contamination. After manufacturing, protein microarrays were sealed under argon atmosphere into nontransparent bags and stored at 4°C or even room temperature until usage.

2.3. Detection Principle

For both possible platforms shown in [Fig. 1](#), the labelling technology is based on a catalytically induced tetramethylbenzidine (TMB) precipitation, which directly correlates to the amount of target molecules binding to the probe array. Analysis of the resulting precipitation patterns is done by simple CCD-based transmission measurements within a dedicated reader.

The resulting pictures were always analysed automatically using the software Iconoclust in combination with the Partisan ArrayLIMS System Database and defined scripts (CLONDIAG, Jena, Germany) for the different microarray layouts used. Spots were detected, and mean values of the spots (MV) as well as the local background (BG) were measured. Normalised signal intensities (NSI) were calculated by the equation $NSI = 1 - MV/BG$. Using this algorithm, changes or inhomogeneities in background intensity can be eliminated resulting in better comparability between different experiments. Assay-specific threshold values for interpretation as positive/negative were defined using reference samples characterised with another method.

2.4. Assay Principles

2.4.1. Serotyping of *Escherichia coli*

O-serotyping is used routinely as a presumptive guide to distinguish between pathogenic and commensal *E. coli*. Compared with conventional serotyping assays, protein arrays can produce O-serotyping results more efficiently considering the high costs of typing sera (8). Such sera, which have been raised against *E. coli* pathogenic to both humans and animals and which are used routinely for O-serotyping, were immobilised on the surface of protein microarrays. The two basic types of the assay and three resulting images are shown in [Fig. 2](#).

2.4.2. Antibody Screening and Epitope Mapping

To screen a defined set of antibodies against an antigen for optimal specificity and sensitivity, different concentrations of the antibodies (sometimes diluted in an inert protein such as BSA) are

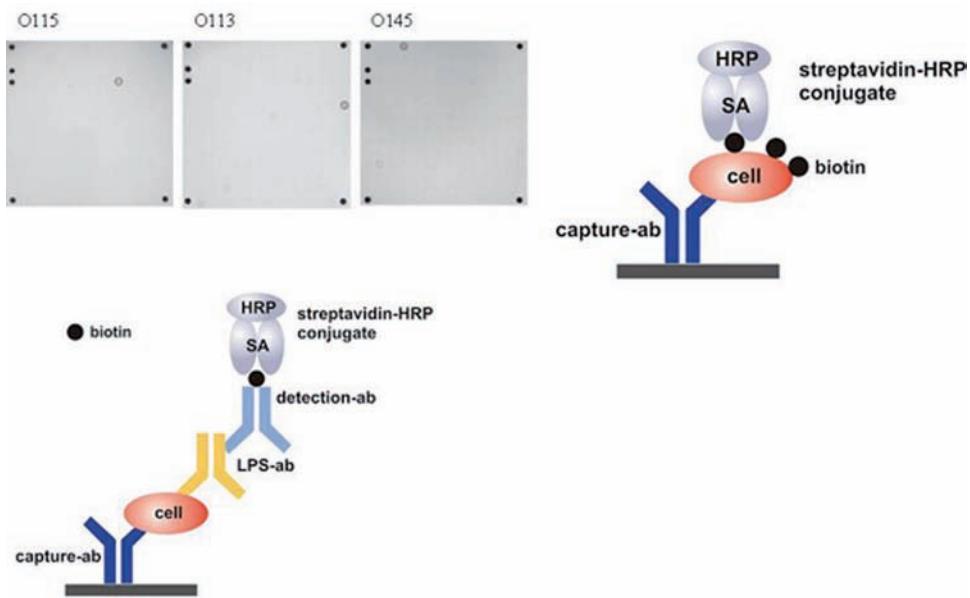


Fig. 2. Two different experimental set ups for *E. coli* O-typing are shown. The indirect labelling procedures using LPS monoclonal antibody WN1222-5 (15, 16) and biotinylated anti-mouse IgG (Sigma 8520) are described in (8). In the second labelling procedure, complete *E. coli* cells are biotinylated directly. Three pictures of processed arrays after reaction with *E. coli* O-Types 0113, 0115, and 0145 are shown. The microarray contains 176 different, single spotted LPS-specific sera in a 14×14 grid layout.

combined and immobilised on the same array. In addition, all spotted antibodies are labelled individually with, e.g., biotin or HRP. Every antibody can either be used as a covalently coupled capture antibody on the array or as a labelled detection antibody (Fig. 4). By testing the resulting microarrays with an appropriate concentration of the detection antibody and different concentrations of the antigen, the optimal antibody pair in terms of specificity and sensitivity can be found. Even an approximate antigen quantification can be performed using this approach (Fig. 4).

To characterise a specific monoclonal antibody, peptide arrays can be used for epitope mapping. For this application, a set of overlapping peptide sequences is derived from the original amino acid sequence of the given protein. These peptides are to be synthesised and subsequently immobilised as a microarray. If a labelled antibody interacts with such an array, it will bind preferentially to its epitope peptide (Fig. 3).

2.4.3. Detection of *Staphylococcal Enterotoxin B*

Staphylococcus aureus is a gram-positive bacterium, which can cause skin and soft tissue infections, pneumonia, and septicaemia. It also produces a variety of toxins, which manipulate or disrupt functions of the host immune system. Among them there are

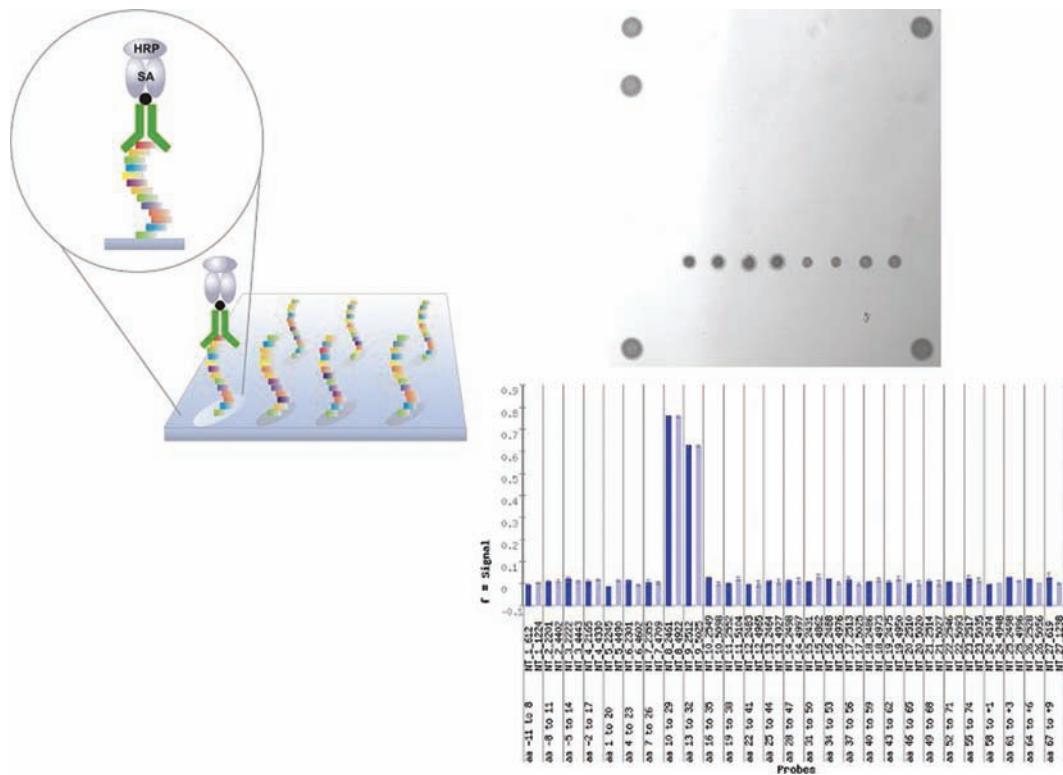


Fig. 3. A biotin-labelled antibody specific for an antigenic protein is applied within an array-based assay, where the used microarray contains multiple and overlapping peptide sequences resulting from a clustering of the antigen. In the shown example, a 76 amino acid protein is mapped, by spotting 27 overlapping peptides on a microarray. All peptides are immobilised in duplicate and in two different concentrations. The results of the interaction between the labelled antibody and all covalently attached peptides show clearly that the epitope is situated between amino acid positions 10 and 32.

so-called enterotoxins, which induce, when ingested, intense but transient vomiting, diarrhoea, and general malaise (17). These heat-stable toxins are a common cause of food poisoning as *S. aureus* is able to grow in foodstuffs after being inoculated by, e.g., infected lesions at the hands of kitchen staff. Because of their incapacitating effect, they also have been investigated as a possible agent in biological warfare (9, 11, 18).

The assay described earlier was designed to detect, beside others, one of the more common enterotoxins, enterotoxin B (*seB* or *entB*). The toxin is bound to spotted, i.e., immobilised, monoclonal antibodies or sera, and detected using a biotinylated monoclonal antibody (which yields the specificity of the assay) and a precipitation reaction as described earlier. The assay can be used either to detect enterotoxin B directly from homogenised foodstuffs or to screen staphylococcal cultures for their ability to produce the toxin (Fig. 5). An application for the detection of neutralising antibodies in human sera by competitive

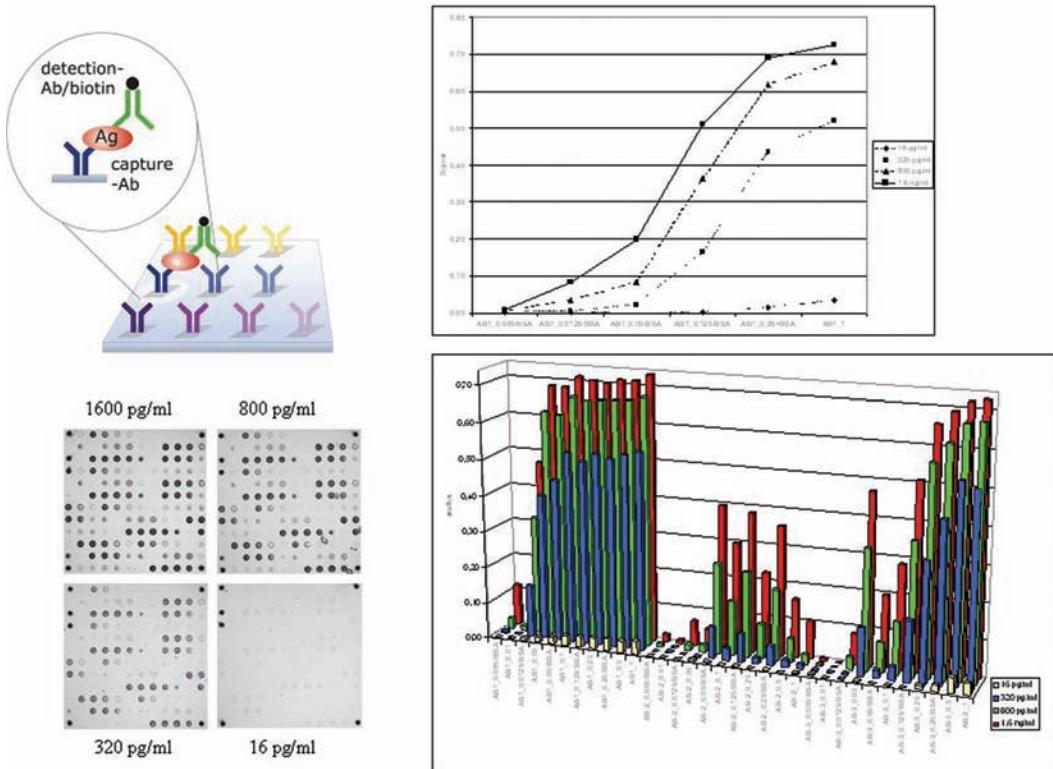


Fig. 4. Three different antibodies specific for C-reactive protein (CRP) are spotted in two different dilution series for each antibody in a 12×12 layout, four times redundant and mounted in ArrayTubes. One dilution series contains decreasing antibody concentrations whereas the other one consists of constant protein but decreasing antibody concentrations. For the additional protein component, BSA was used. As shown in the diagram, the resulting microarrays were processed with characterised human serum diluted (between 1:1,000 to 1:10⁶) in 1 \times PBS to end concentrations of CRP of 16, 320, 800, and 1,600 pg/ml. The detection is performed using a biotinylated secondary antibody (which was the same as one of the three spotted antibodies). The resulting microarray pictures show a detection limit near 16 pg/ml. Furthermore, the 3D bar plot of all clustered results of the four images enables the shown diagram with the calibration curve for rough quantification of CRP within one microarray experiment.

binding is investigated. The protocols refer to the ArrayTube system (see later).

2.4.4. Detection of Biological Warfare Agents

For the specific detection of BW agents the ArrayTube™ platform is used and diverse antibodies against such agents including toxins, viruses, and bacteria are screened (Fig. 6). Commercially available antibodies as well as monoclonal antibodies prepared from hybridoma cells (9, 21–25) and polyclonal antibodies raised according to standard procedures (26) were manufactured and spotted as described earlier. After incorporation of the microarray into the ArrayTube, the tubes were stored under argon at 4°C. For more details concerning the applied antibodies refer to (9).

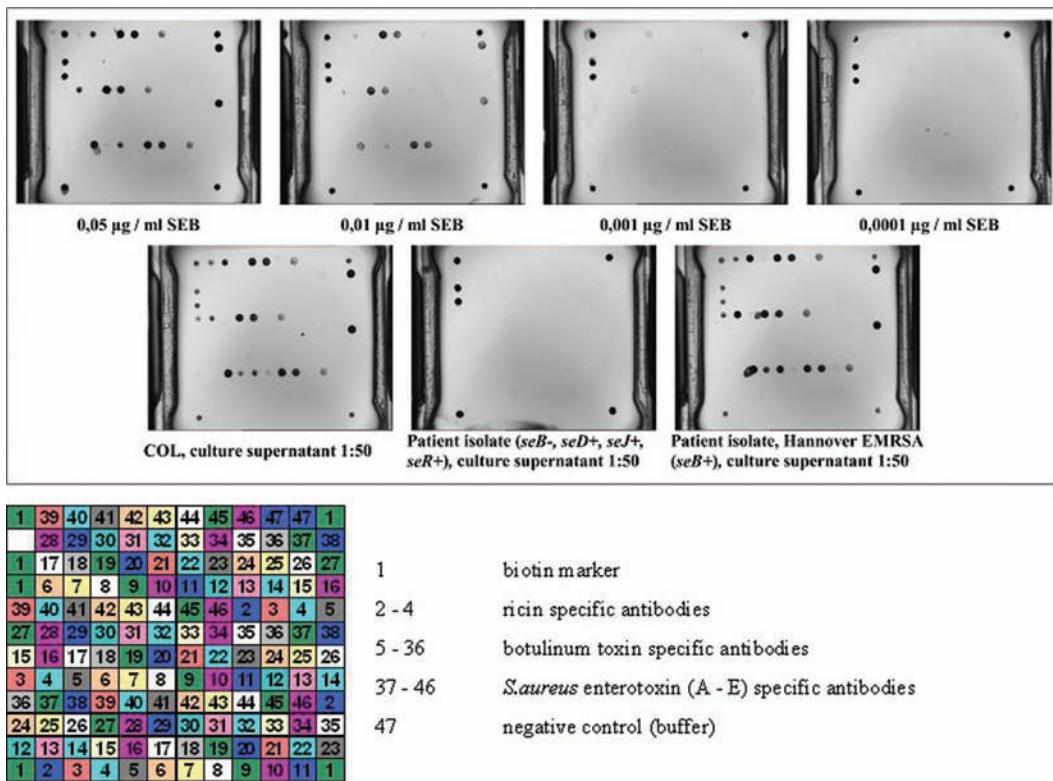


Fig. 5. The applied array contained 47 different substances spotted in a 12×12 layout in threefold redundancy. The substances contain a biotin marker as positive control, buffer spots without protein as negative control, and several antibodies in varying concentrations with different specificities for ricin, botulinum toxins, and *S. aureus* enterotoxins A, B, C, D, and E. The figure shows the detailed layout and serial dilution of recombinant *S. aureus* enterotoxin B and several culture supernatants using the array. The specificity of the assay for enterotoxin B results from the selection of the labelled detection antibody (see text).

3. Materials

3.1. O-Serotyping of *Escherichia coli*

Instruments:

Eppendorf biophotometer: Eppendorf, Hamburg, Germany.

Rotary shaker: Thermomixer comfort, Eppendorf, Hamburg, Germany.

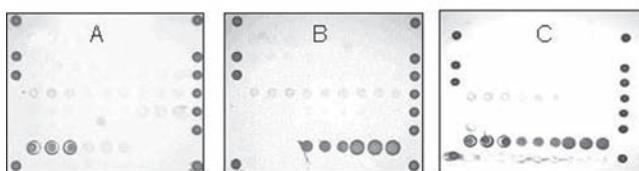
atr01 or atr03 reader: CLONDIAG, Jena, Germany.

YM-30 columns: Millipore, Schwalbach, Germany.

2	1	1	1	1	1	1	1	1	1	1	2
	3	3	3	1	1	1	10	10	10		
2	5	5	5	11	11	11	18	18	18	2	
2	8	8	8	12	12	12	14	14	14	2	
	13	13	13	6	6	6	17	17	17	2	
22	22	22	23	23	23	23	24	24	24	2	
26	26	26	25	25	25	1	1	1	1	2	
19	19	19	27	27	27	21	21	21			
2	10	10	10	1	1	1	1	1	1	1	2

Spot ID.	Specificity	Spot ID.	Specificity
1	negative control	19	sheep anti-SEB
2	positive control	21	lectin specific for ricin
		27	mouse anti-ricin
3	mouse anti-Vaccinia		Bacteria
5	mouse anti-SLEV	22	goat anti- <i>E. coli</i> O157:H7
6	mouse anti-WNV	23	mouse anti- <i>Y. pestis</i>
7	mouse anti-WNV	24	mouse anti- <i>F. tularensis</i>
8	mouse anti-Dengue	25	mouse anti- <i>B. mafiei</i>
10	mouse anti-YFV	26	mouse anti- <i>B. melitensis</i>
11	mouse anti-SLEV		
12	mouse anti-VEEV		
13	mouse anti-WNV		
14	mouse anti-VEEV		
17	mouse anti-WEEV		
18	mouse anti-SLEV		

Single and simultaneous detection of SEB and Ricin:



Simultaneous detection of SEB or Ricin in combination with other BW agents:

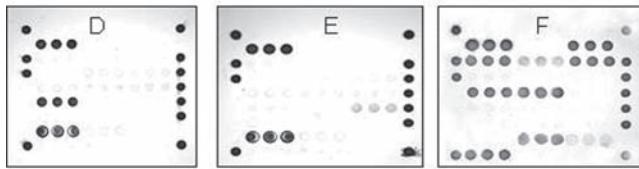


Fig. 6. Shown are representative ArrayTube results obtained for a protein chip after performing single and parallel analysis of different BW agents. From each analysis the 60th picture is presented. Concentrations of analytes in experiments A–F were as follows: A: SEB (2ng/ml), B: ricin (1ng/ml), C: SEB (5ng/ml) and ricin (2ng/ml); D: Vaccinia virus (10^5 TCID₅₀/ml), *E. coli* O157:H7 (5×10^4 cfu/ml) and SEB (2ng/ml); G: Vaccinia virus (10^5 TCID₅₀/ml), *E. coli* O157:H7 (5×10^4 cfu/ml), *Francisella tularensis* (10^7 cfu/ml) and SEB (2ng/ml); I: yellow fever virus 17 D (10^5 TCID₅₀/ml), West Nile virus NY (10^3 TCID₅₀/ml), St. Louis encephalitis virus (2×10^7 TCID₅₀/ml), Vaccinia virus (10^5 TCID₅₀/ml), and ricin (2ng/ml). The following biotinylated (*) detection antibodies were used: A: ^bantiSEB, B: bRCH1, C: ^bRCH1, ^banti-SEB1; D: ^b5B1, ^banti-*E. coli*, ^banti-SEB; E: ^b5B1, ^bantiSEB, ^bFT140/11/1/06; F: ^bG2, ^bWNV MAB8151, ^b5B1, ^bSLEV MAB 8744, ^bRCH1. For details about the applied antibodies refer to (10).

Solutions:

1. $2 \times$ TY broth: 1 l of $2 \times$ TY broth containing 16 g tryptone/peptone, 10 g bacto yeast extract (both BD, Heidelberg, Germany), 5 g NaCl.
2. 2.2 mg NHS-LC-biotin (Pierce, Boston, MA, USA) is stored at -20°C and is (always freshly) dissolved in 400 μl double-distilled (dd) water.
3. PBS-Tween wash buffer: 1 \times PBS with 0.01% Tween20.

4. PBS–Tween–FCS (*10*): 90 µl 1 × PBS–Tween plus 10 µl foetal calf serum.
5. PBS–FCS–Tween: 1 × PBS with 0.01% Tween20 and 1% FCS.
6. Anti-*E. coli* core LPS monoclonal antibody WN1222-5 (*15*, *16*): diluted to 52ng/ml in PBS–FCS–Tween.
7. Secondary biotinylated anti-mouse IgG: Sigma 8520, diluted 1:10,000 in PBS–FCS–Tween.
8. Streptavidin-poly-horseradish peroxidase (SA-poly-HRP, Pierce, Boston, MA, USA) is diluted in PBS–FCS–Tween to 100pg/µl.
9. Seramun green (Seramun, Woizig, Germany) – precipitation substrate of streptavidin-poly-horseradish peroxidase.

3.2. Antibody Screening and Epitope Mapping

Instruments

1. Photometer: Nanodrop ND1000, PeqLab, Erlangen, Germany.
2. Rotary shaker: Eppendorf, Hamburg, Germany.
3. atr03 reader in combination with the Iconoclast software package and an assay specific script: CLONDIAG, Jena, Germany.
4. Microcon YM-30 column: Millipore, Schwalbach, Germany.
5. ZEBA spin columns: Pierce, Boston, MA, USA.

Solutions

1. 2.2 mg NHS-LC-biotin (Pierce, Boston, MA, USA) is stored at -20°C and is always freshly dissolved in 400 µl dd water.
2. PBS–Triton: 1 × PBS buffer containing 0.5% Triton X100.
3. Streptavidin-buffer: 1 × PBS, 1% BSA, 0.5% Triton X100, and 3 µg/ml poly-HRP-streptavidin (Pierce, Boston, MA, USA).
4. True Blue peroxidase substrate: Medac, Hamburg, Germany.
5. PBS–FCS–Tween: 1 × PBS, 0.05% Tween, 1% FCS.
6. PBS–Tween–FCS (*10*): 90 µl 1 × PBS plus 10 µl FCS plus 0.05% Tween.
7. Streptavidin poly-HRP (Pierce, Boston, MA, USA) concentration of 0.2ng/ml in 100 µl PBS–FCS–Tween.
8. Seramun green (Seramun, Woizig, Germany) precipitating substrate.

3.3. Detection of Staphylococcal Enterotoxin B

Instruments

- Rotary shaker: Thermomixer Comfort, Eppendorf, Hamburg, Germany.
atr01 reader: CLONDIAG, Jena, Germany.

Solutions

1. Medium [Noda et al. (19)]: 25 g yeast extract, 20 g casamino acid, 20 g sodium glycerophosphate, 0.64 g $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 0.64 g citric acid, 6.25 g $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ (or alternatively, 3.65 g $\text{Na}_2\text{HPO}_4 \times 1\text{H}_2\text{O}$), 400 mg KH_2PO_4 , 20 mg $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 10 mg $\text{MnSO}_4 \times 4\text{H}_2\text{O}$, 19.8 mg sodium lactate solution (50%), and add to 1 l finale volume with water.
2. PBS: 1.2 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 4.4 g Na_2HPO_4 , and 8.2 g NaCl, as well as 1,000 ml de-ionised, sterile water. Its pH value is adjusted to 7.4 using 1 M NaOH.
3. PBS-Tween: 50 μl Tween20 in 100 ml PBS.
4. PBS-Tween-BSA: 3 g bovine serum albumin in 100 ml PBS-Tween.
5. Antibody BBC202 (Toxin Technology, biotinylated as described earlier; 1:2,000 in PBS-Tween).
6. Streptavidin-HRP: Streptavidin-HRP (Pierce, Boston, MA, USA) needs to be stored at -20°C . A stock solution of 1:100 in PBS buffer can be kept at 4°C for 5–7 days. Prior to use, this stock solution is to be diluted to 1:100 in PBS-Tween-BSA. This working solution can not be stored.
7. Seramun Green (Seramun, Woizig, Germany) – precipitating substrate.

3.4. Detection of Biological Warfare Agents*Instruments*

Horizontal tube shaker: Eppendorf, Hamburg, Germany.

atr01 reader: CLONDIAG, Jena, Germany.

PD-10 columns: GE Healthcare, Munich, Germany.

Solutions

1. All viruses used represent models for BW relevant Flavi- or Alphavirus species, and are part of the collection of the German Armed Forces Scientific Institute for Protective Technologies (WIS).
2. Bacterial strains are either part of the collection of the German Armed Forces Scientific Institute for Protective Technologies or are available from the American Type Culture Collection (ATCC).
3. Staphylococcal enterotoxins: Toxin Technology, Inc., USA.
4. Biotin-NHS ester: Vector Laboratories, Burlingame, CA, USA or Sigma-Aldrich Chemie GmbH, Munich, Germany.
5. Streptavidin-HRP conjugates: GE Healthcare, Munich, Germany.
6. PBS-Tween: 1 × PBS + 0.01% Tween20.
7. PBS-FCS-Tween: 1 × PBS + 0.1% FCS + 0.01% Tween20.

8. Streptavidin-poly-horseradish peroxidase (SA-Poly-HRP, Pierce, Boston, MA, USA): 1:10,000 dilution.
9. TMB: CLONDIAG, Jena, Germany.

4. Methods

4.1. O-Serotyping of *Escherichia coli*

4.1.1. Bacterial Culture, Antigen Preparation, and Labelling

1. Starting from a single colony on solid growth media, *E. coli* cells are grown in 2 × TY broth overnight to stationary phase at 300 rpm and 37°C.
2. OD (600 nm) of an adequate dilution (culture diluted in 2 × TY broth) is measured and calculated using the Eppendorf biophotometer according to manufacturers' instructions to compare the growth of different strains.
3. An aliquot of 2.6 ml of the overnight culture is mixed with 60 µl 37% HCHO and subsequently incubated on a rotary shaker at 65°C with 300 rpm for 10 min.
4. The cells are centrifuged at room temperature and 6,000 rpm for 10 min, and the supernatant is discarded. The cell pellet is dried and re-dissolved in 1.3 ml 1 × PBS buffer and stored at 4°C until use in the O-typing assay or direct labelling with NHS-biotin.
5. Twenty microlitres of the *E. coli* cells are mixed with 30 µl 1 × PBS and 5 µl of the freshly prepared NHS-LC-biotin solution, vortexed, sonicated for 5 min, and incubated at 21°C and 550 rpm for 45 min.
6. The biotinylated cell mixture is purified using YM-30 columns by the addition of 350 µl 1 × PBS to 50 µl sample, placed on the column, and centrifuged at 14,000 rpm for 9 min. The column is subsequently washed twice with 400 µl 1 × PBS and the flow-through is always discarded. The filter is then placed at a new vial, and 30 µl of 1 × PBS is added and the column is slightly vortexed. After this the filter is turned around, centrifuged at 1,500 rpm for 3 min, and the flow-through containing the biotinylated cells is used for further analysis.

4.1.2. Microarray-Based Analysis (see Note 2)

1. Prior to reaction with *E. coli* cells (see earlier), each ArrayTube is washed twice with 500 µl PBS-Tween wash buffer.
2. The microarray is blocked with 100 µl of freshly prepared PBS-Tween-FCS (10) for 15 min and washed with 500 µl PBS-Tween for 5 min. All the three steps are performed at 23°C and 550 rpm in the rotary shaker.

3. An aliquot of biotinylated or non-labelled *E. coli* cells [30 or 10 µl, respectively (see earlier)] is mixed with PBS–FCS–Tween to a final volume of 100 µl, added into the AT, and incubated at 23°C and 400 rpm for 15 min.
4. After removing the sample carefully the microarray is washed in PBS–Tween for 5 min.
5. If the non-biotinylated cells are used, 100 µl of anti-*E. coli* core LPS monoclonal antibody WN1222-5 is applied at 23°C and 400 rpm for 20 min. After that, the array is washed with PBS–Tween at 23°C and 400 rpm for 5 min.
6. 100 µl of a secondary biotinylated anti-mouse IgG is added and incubated at 23°C and 550 rpm for 10 min.
7. The microarray is again washed with PBS–Tween at 23°C and 550 rpm for 10 min.
8. For conjugation, 100 µl of streptavidin-poly-horseradish peroxidase (SA-poly-HRP) is added to the ArrayTube.
9. After 15- min conjugation at 30°C and 550 rpm, two washing steps using 500 µl PBS–Tween at 23°C and 550 rpm for 5 min are carried out.
10. The solution is completely removed and the microarray is stained at 25°C for 10–15 min by adding 100 µl of a precipitation substrate of streptavidin-poly-horseradish peroxidase, e.g., Seramun green, followed by the readout of the resulting picture using the atr01 or atr03 reader (CLONDIAG, Jena, Germany) according to manufacturers' instructions.
11. If the biotinylated cells were used, the incubation with primary and secondary antibody and subsequent washing steps were omitted with otherwise the same protocol.

4.2. Antibody Screening and Epitope Mapping

4.2.1. Antibody Purification and Labelling with Biotin

1. The protein concentration of the purified antibody to be labelled is determined by measuring the extinction at 280 nm. Afterwards, the concentration is adjusted to 1 mg/ml with 1 × PBS buffer.
2. To purify the antibody from interfering substances, 100 µl of the 1 mg/ml solution is mixed with 300 µl 1 × PBS buffer, added to a Microcon YM-30 column, and centrifuged at 14,000 rpm for 10 min. The flow-through is discarded, 400 µl 1 × PBS is added, and a second centrifugation step is performed at 14,000 rpm for 10 min. The filter is then placed at a new vial and the column is slightly vortexed. After this step, the filter is turned upside down and centrifuged at 1,500 rpm for 3 min. The flow-through is collected and adjusted to 100 µl with 1 × PBS.

3. 1.5 µl of NHS-LC-biotin solution is mixed with 100 µl of the purified antibody solution and stored at 21°C for 30 min. Ten microlitres 1 × PBS is added and the solution is divided into two 55 µl aliquots. To remove free biotin, these 55 µl aliquots are applied to two ZEBA spin columns and centrifuged at room temperature at 1,500 rpm for 1 min. The flow-through contains the biotinylated antibody and can be used in microarray experiments.

4.2.2. Microarray-Based Epitope Mapping

1. The ArrayTubes with spotted peptides are washed with 500 µl PBS–Triton for 2 min at 500 rpm and 37°C on a rotary shaker.
2. The biotinylated antibody is applied to the peptide microarray in a final concentration between 10 and 100 ng/ml in a streptavidin-buffer at 37°C and 800 rpm for 15 min.
3. The microarray is washed at 37°C, 800 rpm and for 1 min subsequently once with 500 µl 1 × PBS + 0.1% Triton X100 and twice with 1 × PBS.
4. After removing the wash buffer, the TMB staining is performed by adding 100 µl of True Blue peroxidase substrate, incubating at 25°C (no shaking!) for 10 min and subsequent image recording and analysis with the atr03 reader device (CLONDIAG, Jena, Germany).

4.2.3. Microarray-Based Antibody Screening (see Note 2)

1. The ArrayTubes with spotted antibodies to be screened are washed with 500 µl PBS–FCS–Tween, at 37°C and 400 rpm for 5 min using a rotary shaker.
2. A blocking step follows using 500 µl PBS–Tween–FCS (*10*) at 37°C and 500 rpm for 5 min.
3. After removal of the blocking solution a characterised human serum in a dilution range between 1:10⁶ and 1:1,000 in 1 × PBS is applied at 500 rpm and 37°C for 30 min followed by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
4. The biotinylated detection antibody is applied in a dilution between 1:500 to 1:10,000 in 100 µl PBS–FCS–Tween at 300 rpm and an incubation at 37°C for 10 min followed again by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
5. For conjugation streptavidin poly-HRP (0.2 ng/ml) is used at 400 rpm and 37°C for 10 min followed by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
6. After removal of the washing solution, 100 µl of the precipitating substrate seramun green is added and the microarrays are incubated at 25°C without shaking for 10 min. After-

wards, the resulting picture is taken and analysed using the atr03 reader in combination with the Ikonoclast Software package and an assay-specific script.

4.3. Detection of Staphylococcal Enterotoxin B

4.3.1. Bacterial Culture (see Note 1)

1. *S. aureus* is grown in liquid media based on a publication by Noda, Hirayama, Kato, and Matsuda (19).
2. One Microbank bead (Viva Diagnostika, Cologne, Germany) of a stored culture, or one inoculation loop of a fresh one, is inoculated into 1.5 ml of medium and incubated for 12 h on a shaker using test tubes with cotton plugs in order to facilitate oxygen influx. Then, cultures are transferred into Eppendorf tubes and centrifuged (7,000 rpm, 10 min) to obtain cell-free supernatants.
3. Prior to use with the antibody array, the supernatants are diluted either 1:10 or 1:50 in 1 × PBS. Applying this procedure on *seB*-positive reference strain COL (20), it is possible to yield a working concentration of approximately 0.01–0.05 µg/ml enterotoxin B.
4. To detect enterotoxin B from spiked minced meat, or from minced meat inoculated with *seB*-positive strains such as COL, an equal amount of PBS-Tween (see later) is added. After vigorous vortexing, the suspension is centrifuged (15,000 rpm, 10 min).
5. The supernatant is filtered using 0.2 µm syringe filters. The resulting clear solution is used for further experiments with the antibody array. When incubating reference strain COL with minced pork meat for 60h at 37°C, approximately 0.001 µg/ml enterotoxin B is yielded after elution, centrifuging, and filtering.

4.3.2. Array Procedure (see Note 2)

All incubation or washing steps are carried out at 25°C using a thermo shaker set at 300 rpm.

1. The ArrayTube is washed using 500 µl PBS-Tween for 5 min.
2. Unspecific binding capacities are blocked by incubation with 100 µl PBS-Tween-BSA for 5 min.
3. The culture supernatant or toxin preparation (see earlier) is added and incubated for 20 min.
4. The microarray is washed with PBS-Tween-BSA for 5 min.
5. After removal of the washing buffer, 100 µl biotinylated antibody BBC202 (1:2,000) is added and the ArrayTube is incubated for 20 min.
6. The microarray is washed again with PBS-Tween-BSA for 5 min.

7. 100 ml diluted Streptavidin-HRP (1:10,000, see earlier) is added and the ArrayTube is incubated for 20 min.
8. The microarray is washed twice (2×5 min) with PBS-Tween.
9. Hundred microlitres of a precipitating substrate for staining is added. The ArrayTube is incubated for another 10 min and images are recorded using a atr01 reader.

4.4 .Detection of Biological Warfare Agents

4.4.1. Antigen Preparation (see Note 1)

Viruses:

1. Alpha- and flaviviruses are either grown in Vero or BHK 21 cells in the biosafety level 2 and 3 facilities. Virus titres are determined by the 50% tissue culture infective dose ($TCID_{50}/ml$) method (27, 28).
2. All pathogenic viruses are inactivated prior to use. Inactivation of viruses is performed by incubation with 0.1% β -propiolactone for 1 h at 4°C and 4 h at 37°C.

Bacteria:

1. Bacterial strains are cultured according to standard cultivation procedures.
2. Inactivation of bacteria is achieved either by formaldehyde, heat incubation, or by a combination of both.

Toxins:

1. Toxins are to be handled as required by national legislation (Ordinance on safety and health protection related to work involving biological agents including toxic substances, BioStoffV).
2. Crude extracts of Ricin are prepared from castor beans (*Ricinus communis*) by aqueous extraction and ammonium sulphate precipitation (29), using a modified protocol (Binder, Central Institute of the German Armed Forces Medical Corps, Munich, 2003, personal communication).

4.4.2. Biotinylation of Detection Antibodies

1. All mono- and polyclonal antibodies applied for the specific detection of BWAs are coupled to biotin-NHS ester.
2. Incubation is performed for 2 h at room temperature according to the manufacturer's direction.
3. Unincorporated biotin is removed by gel filtration on PD-10 columns.
4. Positive detection reactions are reported by streptavidin-HRP conjugates.
5. To minimise unspecific cross reactions, each detection antibody is titrated prior to regular use.

**4.4.3. Array Analysis (see
Note 2)**

1. All incubation steps of the analysis are carried out on a horizontal tube shaker at 350 rpm and 25°C.
2. Before starting a protein array analysis all ArrayTubes are conditioned by washing them twice with 500 µl PBS-Tween for 2 min.
3. To block unspecific binding sites, the ATs are incubated in PBS-FCS-Tween + 1% fat-free milk powder or in PBS-Tween containing 1% FCS for 15 min.
4. This incubation is followed by three washing steps with 500 µl 1 × PBS-Tween for 2 min each.
5. Antigen binding is allowed to proceed in PBS-FCS-Tween for 30 min.
6. The ATs are washed three times as described earlier.
7. The incubation of ATs with specific biotinylated secondary antibodies is performed in PBS-FCS-Tween for 30 min.
8. After three washing steps as described earlier, specific binding of the secondary antibody is detected by Streptavidin-poly-horseradish peroxidase and TMB staining. SA-poly-HRP is used in a 1:10,000 dilution.
9. Online read-out of washed ATs is performed in an ArrayTube reader (atr01) for 6 min at 25°C, recording one image every 10s.
10. Data analysis is done with the manufacturers' specifications and the IconoClust™ software.

5. Notes

1. The microorganisms described herein are potentially dangerous pathogens, which need to be cultured at least under biosafety level 2 conditions. All experiments are to be performed by experienced staff in an appropriate facility.
2. Human sera and other specimens are potentially infectious because of possible contamination with HIV, HBV, etc. Thus, human specimens need to be handled using protective gloves.
3. Recombinant enterotoxins and ricin may cause pulmonary edema, as well as other severe conditions upon inhalation, ingestion, or eye/skin contact. Thus, toxins are to be handled using protective gloves, goggles, and masks. Handling of these substances might be regulated by national legislation (in Germany, "Ordinance on Safety and Health

Protection Related to Work Involving Biological Agents Including Toxic Substances, BioStoffV").

4. The ArrayTubes (ATs) are stored in a light-protective foil pouch sealed under inert gas (argon). Within these packages, the protein microarrays can be stored at 4°C or room temperature. When a pouch was opened, humidity, direct sunlight, and dust should be avoided, and the ATs should be used within the next 24h.
5. Never let the microarrays run dry during processing.
6. Before adding a new solution into ATs, always carefully remove precedent solution by using a fine pipette without touching the array at the bottom of the AT.
7. Follow standard lab safety regulations when using the ATs with any hazardous or potentially infectious material that may be required for your individual experiments.
8. Do not expose the ATs to direct sunlight.
9. Avoid scratching or touching the array surface (e.g. during pipetting steps with pipette tip).
10. Do not centrifuge the ArrayTubes.
11. Do not heat ArrayTubes over 60°C for a longer time period.
12. Avoid the deposition of dust particles and/or filaments on the outer side of the AT array. In case you detect any dust or filaments, carefully remove it with a dust-free cloth moisturised with 70% ethanol.
13. Avoid formation of air bubbles during all assay steps and remove them, if necessary, by mixing with a pipette or by short agitation.
14. Pre-warm the portion of TMB or serum green solution needed for the actual experiments to 25°C prior to use. Then, shortly (ca. 15s) centrifuge the solution at 5,000 rpm before finally adding it to the processed ATs for staining.
15. Prepare all blocking reagents such as FCS or milk powder solutions always freshly prior to use.
16. Always use minimum volumes of 90–100 µl per step within the different protocols.
17. Some batches of fetal calf serum may degrade biotin spots. Thus, it is recommended to test new brands or batches of FCS prior to use in crucial or large-scale experiments.

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Chapter 7

Detection of Known Allergen-Specific IgE Antibodies by Immunological Methods

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Summary

An increasing number of patients are suffering from allergic diseases such as rhinoconjunctivitis, atopic eczema, urticaria, anaphylaxis, and food and drug allergies. Although it is possible to measure a multitude of allergen-specific IgE antibodies by radio or enzyme immunoassays in the patients' blood, these tests are expensive, time-consuming, and usually need a rather high volume of reagent solutions (allergens and blood). Protein microarrays offer the possibility to circumvent these limitations. The described in vitro allergy testing system is based on microscopic glass slides activated with glycidyloxypropyl-trimethoxysilane. Allergen solutions (allergen extracts and/or purified allergens; approximately 10 nL) are printed on the activated glass surface with a piezoelectric spotting machine. The protein components of the allergen solutions are immobilized on the modified glass surface via hydrophobic interaction and/or covalent binding. After a blocking step, the slides are incubated with the respective diluted serum sample (approximately 25 µL serum required) and bound IgE antibodies are detected with a secondary horseradish peroxidase (HRP) labelled anti-human-IgE antibody via chemiluminescence. The measurement can be performed automatically with the so called *PASA system*. Test results are directly visualized with a CCD-camera. Analytical and clinical data have shown that the microarray-based test format offers significant advantages in time and costs compared with traditional test formats. The described allergen microarray demonstrated a sufficient qualitative reproducibility and enabled the distinction between allergic and non-allergic patients. Detection limits of 0.35 kU/L (r Bet v1), 0.16 kU/L (PLA₂), 1.9 kU/L (Der p1), and 41 kU/L (total IgE) were achieved.

Key words: Serum IgE, In vitro allergy diagnosis, Immunoassay, Recombinant allergens, Purified allergens (Der p1, Der f1, PLA₂), Allergen extracts, Chemiluminescence, Protein microarrays, Protein chips.

1. Introduction

Elevated levels of immunoglobulin E (IgE) play a key role in the development of allergic diseases. IgE was identified in the mid sixties of the last century by Ishizaka and Johansson et al.

(1, 2). After contact with the allergen the immune system starts to produce IgE antibodies against the specific allergen. These antibodies bind to receptors on the surface of mast cells. The cell degranulation is triggered by the bridging of cell-bound IgE antibodies via the specific allergen. This results in the release of chemical substances such as leucotrienes, histamine, and prostaglandins that cause smooth muscle contraction, itching, swelling, and transmucosal leakage of extracellular fluids. The most common clinical manifestations are hay fever, asthma, hives, and anaphylactical shock (3).

The majority of allergenic substances consist of water-soluble proteins with molecular masses of 5–70 kDa. Aqueous extracts are prepared by suspending the raw material (e.g. pollen, fruits, and nuts) in physiological buffer solutions while continuously shaking (4). Extracts are mixtures of different allergenic and non-allergenic proteins, and the quality (raw material used, contaminations and storage conditions) of the extract strongly influences the biological activity (5). The combination of two-dimensional gel electrophoresis together with immunoblotting techniques allowed the determination and purification of the major allergenic compounds of several allergen mixtures (6). Recombinant DNA technology offered the possibility to isolate allergen-encoding cDNAs. In eucaryotic or prokaryotic expression systems different recombinant allergenic proteins were produced (7). As a result most of the important allergens from trees, grass pollens, mites, animal epithelia, insect venoms, and foods have been cloned, sequenced, and expressed in the past (8–10). It was shown by further studies that recombinant allergens are useful for the diagnosis and therapy of allergic diseases (11).

The diagnosis of allergic disease is performed either *in vivo* or *in vitro* (12). For the *in vivo* diagnosis the patient is brought into direct contact with the allergenic substance using different techniques, e.g. prick or challenge tests. *In vivo* tests bear the risk of resulting in an anaphylactical reaction, which could be very dangerous for the patient. For that reason, *in vitro* immunoassays are a safer alternative.

Since 1967, when the first assay (RAST) for serum immunoglobulin E (IgE) and allergen-specific antibodies in serum was described (13), different immunoassay-based procedures (e.g. modified labels, surfaces) were tested (14–18). Radioactive labels were replaced by chromogenic (EIA) or fluorescent (FIA) detection labels. In clinical routine the most common immunoassay is the “CAP system-specific IgE FEIA test” (Pharmacia Upjohn, Uppsala Sweden) for total and allergen-specific IgE. The CAP system consists of a cellulose polymer densely loaded with allergen extracts or recombinant allergens (6). The results are expressed in arbitrary units (kU/L) or specific IgE classes as shown in **Table 1**.

Table 1
Specific IgE classes

Specific IgE class	Specific IgE ^a (kU/L)
0	<0.35
1	0.35–0.69
2	0.70–3.49
3	3.50–17.50
4	17.50–52.5
5	52.5–100
6	>100

^a1 IU/L is equivalent to 2.4 ng IgE/L

Other commercially available methods are based on either liquid phase inhibitor assays (e.g. ALASTAT, DPC Biermann, Los Angeles, USA) or multi-allergen coated nitrocellulose strips (e.g. IgEquick, Teomed AG, Greifensee, Switzerland; CMG Immunodot, Trimedal AG, Brütsellen, Switzerland). Certain characteristics of these assays, notably large sample volume in combination with time-consuming and intensive protocols, render them unsuitable for diagnostic tests requiring the ability to determine hundreds of allergenic substances in a timely and cost-effective manner in a single run.

Multi-allergen dipstick tests based on nitrocellulose strips were a first step of miniaturisation and cost saving, but mostly lack automation. Microdeposition technologies enabled the defined immobilization of small protein volumes in the low nanoliter range on a modified surface (19). Twenty years ago, Ekins proposed the application of the microarray technology in the field of medical diagnosis (20). However, the lack of necessary instrumentation delayed the realization of miniaturized immunoassays. Therefore, the first experimental microarray system for allergy diagnosis was not reported before 2000 (21). Recently, a fluorescence-based microarray technique was published (22).

Here, the authors describe a method for the development of chemiluminescence-based microarrays for the simultaneous detection of serum IgE antibodies to various common allergen extracts and recombinant allergens using the so-called *PASA* system (23).

2. Materials

2.1. Equipment

1. Piezoelectric spotting machine (GeSIM, Großberkmannsdorf, Germany): The spotting system consists of a three-dimensional stage, piezoelectric nanoliter pumps (standard micro pipette SPIP, manufactured by silicon etching), and a liquid handling system (syringe pumps) for priming and washing the silicon pumps. A standard 96-well microtiter plate was used as reagent reservoir for the diluted solutions.
2. Equipment for on-line measurement (*PASA system*, Fig. 1):
 - (a) Flow chamber: 4.5 cm × 5.5 cm × 2.0 cm, PEEK painted black; reaction area: 4.5 cm × 2.0 cm × 0.1 cm.
 - (b) Sealing (0.5-mm silicon).
 - (c) Flow system: air trap bubble trap (Trace-trap, Trace, Braunschweig, Germany); XL smart valve 3+ (Cavro Scientific Instruments, Sunnyvale, CA, USA); XL 3000

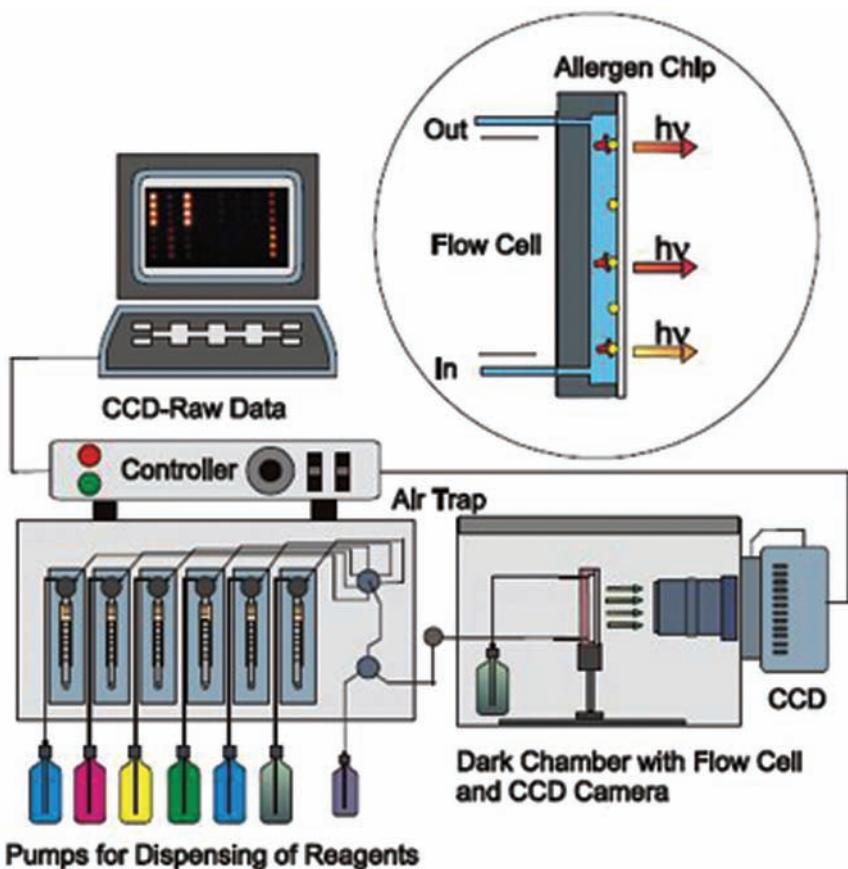


Fig. 1. General setup of the parallel-affinity-sensor-array (PASA) system including close-up of the flow cell.

modular pump (Cavro); Teflon tubing 1/16 in. × 0.25 mm (Upchurch Scientific, Postnova Analytics, Landsberg, Germany).

- (d) CCD system: Objective (50 mm, 1:1.4D, AF Nikkor, Nikon Precision Europe, Langen, Germany); Software Winview (Version 1.6.2, Spectroscopy & Imaging GmbH, Erwitte, Germany); TE/CCD – 1100PB/VISAR/1 CCD-detector: grade 1, back-illuminated, VIS-to-NIR coating; 1,100 × 300 pixels (Spectroscopy & Imaging).
- 3. Equipment for off-line measurement: Hybri-slips: 22 mm × 40 mm (Sigma-Aldrich, Deisenhofen, Germany), Hybri-well chamber slides (180–200 µL, Sigma-Aldrich).
- 4. Equipment for ELISA:
 - (a) Microtiter plate washer: Columbus washer (SLT, Crailsheim, Germany).
 - (b) Microtiter plate reader: eight-channel photometer (TR 340, SLT, Overath, Germany).

2.2. Common Reagents and Materials

1. Raw pollen material: *Alnus glutinosa* (black alder), *Betula verrucosa* (white birch), corylus, avellana (hazel), *Secale cereale* (rye), *Phleum pratense* (timothy), *Triticum aestivum* (wheat), *Ambrosia artemisiifolia elatior* (ragweed common), *Malus domestica* (apple), *Felis domesticus* (cat hair), *Canis familiaris* (dog hair), *Cladosporium herbarum* (mould), *Apis mellifera* (honey bee), from Allergon, Ängelholm, Sweden; Peanuts, unroasted (local Chinese food store, Munich, Germany).
2. Purified allergens: bee venom, phospholipase A₂ (PLA₂), casein, gliadin, and BSA (Sigma-Aldrich), affinity purified Der p1 and Der f1 (Indoor Biotechnologies, Cardiff, UK).
3. Allergen extracts: prick tests for apple, celery, cat epithelia, and mould allergens (*Aspergillus fumigatus*, *Penicillium notatum*) from Allergopharma, Reinbek, Germany.
4. Recombinant allergens: r Cor a1, r Aln g1, r Phl p1, r Api g1, r Mal d1, r Bet v2, r Alt a1 (Biomay, Linz, Austria); r Fel d1 (Indoor Biotechnologies).
5. Immunoglobulins: Myeloma-IgE and purified IgG (Calbiochem-Novabiochem, Bad Soden, Germany).
6. Anti-human-IgE antibodies: monoclonal rat-anti-human-IgE antibody (clone LO-HE-10), Technopharm, Paris, France; polyclonal affinity-purified horseradish peroxidase (HRP)-labelled goat-anti-human-IgE antibody (Dunn, KPL, Reinbek, Germany).
7. Antibodies against allergenic proteins: polyclonal rabbit-anti-Betv1 antibody and monoclonal mouse anti-Betv1 antibody from ALK-Abelló, Horsholm, Sweden. For the detection

the following HRP-labelled antibodies were used: goat-anti-rabbit-IgG (Sigma-Aldrich); goat-anti-mouse-IgG (Vector Laboratories, Burlingham, NY, USA).

8. Protein tests: Bicinchoninic acid (BCA) assay from Sigma-Aldrich; Micro-BCA assay (Pierce, Rockford, IL, USA).
9. Chemiluminescence reagent: SuperSignal ELISA Femto Maximum Sensitivity substrate (Pierce, Rockford, IL, USA); SuperSignal West Pico Chemiluminescence substrate (Pierce).
10. Human sera: 19 patients according to the reference measurement with Pharmacia CAP system (CAP-RAST-FEIA, Pharmacia Upjohn, Uppsala, Sweden) performed at the hospital "Biederstein". Munich was selected. Samples with and without specific IgE antibodies have been used for the determination.
11. Water HPLC grade: Millipore, Milli-Q Plus 185.
12. Washing solution: 60 mM KH_2PO_4 , 420 mM K_2HPO_4 , 870 mM NaCl, 0.3% Tween-20.
13. Printing carbonate buffer: 15 mM Na_2CO_3 , 25 mM NaHCO_3 , pH 9.8, 1% glycerol.
14. Antibody dilution buffer: PBS, 0.1% BSA, 0.01% Tween-20.
15. Ammonium hydrogen carbonate solution contains 0.015 M sodium azide, 20 mM EDTA, 5 mM ϵ -amino-caproic acid (EACA), pH 7.5.
16. Coupling buffer: 300 mM Na_2HPO_4 , pH 8.5, 0.5% trehalose, 1 mM EDTA.
17. Blocking solution: 1% BSA, 0.5% glycine in Tris-HCl, pH 8.3.
18. Microtitre plates (MTPs): Microlon MTPs (Greiner, Nürtingen, Germany); NHS amine – binding plates ("NOS", Corning, Cardiff, UK).
19. Slide-A-Lyzer dialysis cassettes, cut off: 10 kDa, Pierce.
20. Microscopic glass slides: Merck, Darmstadt, Germany.
21. Filter: cellulose acetate, 0.45 μm , Sartorius, Göttingen, Germany.

3. Methods

3.1. Cleaning of the Microscopic Glass Surfaces

The microscopic glass slides are cleaned using a 1:1 mixture of dry methanol and HCl (36%) for 30 min at ambient temperature, washed with water (HPLC grade), and immersed in H_2SO_4 (98%) for 30 min. The glass slides are rinsed with water, dry methanol and dried under a nitrogen stream.

3.2. Activation of Glass Surface with 3-Glycidyloxypropyltrimethoxysilane (3-GOPS)

Activation of the glass slides is achieved with a solution of 1% 3-GOPS in dry toluene for 18 h at ambient temperature. After washing steps with toluene and methanol, the glass slides are dried under a nitrogen stream and stored until use in a desiccator.

3.3. Extraction of Allergens

3.3.1. Raw Pollen Materials

1. For preparation of allergen extracts 10% raw pollen material (w/v) is extracted with either PBS buffer (pH 7.8) or 0.125 M ammonium hydrogen carbonate solution.
2. After suspending the material for 1 h, the medium is centrifuged ($14,000 \times g$, 10 min, 4°C). The supernatant is collected and the insoluble residue is extracted again for 1 h.
3. After the filtration step the supernatant is dialyzed (Slide-A-Lyzer dialysis cassettes) against water/PBS (10:1) for 24 h and then lyophilized.
4. The lyophilized samples can be stored at -20°C in small aliquots.

3.3.2. Prick Test Solutions

1. Prick test solutions are dialyzed (Slide-A-Lyzer dialysis cassettes) against water/PBS (10:1) for 24 h and then lyophilized.
2. The lyophilized samples can be stored at -20°C in small aliquots.

3.4. Preparation of Allergen Solutions

1. Ten milligrams of dry substance is dissolved in water/PBS (10:1).
2. To characterize the obtained allergen extracts the protein content is determined using the Micro-BCA assay.
 - (a) For this purpose the allergen extracts are diluted 1:100 to 1:1,000 with PBS.
 - (b) One-hundred fifty microlitres of this solution is added to a MTP.
 - (c) One-hundred fifty microlitres of a BSA solution (0.125–200 µg/L) is used for calibration.
 - (d) By addition of 150 µL of the Micro-BCA solution, a colour change to green-purple occurs.
 - (e) After an incubation time of 30 min at 36°C the purple colour is measured at 550 nm.

3.5. Printing and Immobilization of Protein Solutions

3.5.1 Preparation for Printing

1. Crude allergen extracts (total protein content 5–10 g/L, 1:40) and recombinant allergens (protein concentration 1–2 g/L, 1:100) are diluted with coupling buffer.
2. Positive controls: monoclonal anti-human-IgE antibody clone LO-HE-10 (1 g/L, 1:100 diluted with printing carbonate buffer + 0.01% Tween-20 protein grade), Myelome – IgE (1 g/L, 1:100 diluted with printing carbonate buffer), streptavidin-HRP (1:1,000 diluted with printing carbonate buffer).

3. Negative controls: human IgG (10 g/L, 1:1,000 diluted with printing carbonate buffer).

3.5.2. Printing and Immobilization

The printing solutions (antibodies/allergens) are prepared just before the start of the print run and transferred into a 96-well microtitre plate (Microlon). The dispensing is done with a piezoelectric spotting system; five droplets; spot diameter approximately 200 µm, $U = 60$ V, $f = 300$ Hz. The coating is performed for 18 h at 4°C.

3.6. Determination of Immobilized Allergens

To specify whether a specific allergen is successfully immobilized on the chip surface, the protein could be detected with an allergen-specific antibody. For the detection of the major allergenic compound of Birch pollen (*Betula verrucosa*) the following procedure was successfully tested:

1. Preparation for printing: Birch pollen extracts (total protein content 5–10 g/L, 1:40) and recombinant r Bet v1 (protein concentration 1 g/L, 1:100) were diluted with coupling buffer.
2. Wash with washing solution.
3. Blocking of free surface groups: 1% BSA in PBS; 1 h at ambient temperature.
4. Incubation with diluted monoclonal mouse-anti-Bet v1 antibody (1:500 in antibody dilution buffer); 1 h at ambient temperature. Alternatively the detection could be done with the polyclonal rabbit-anti-Bet v1 antibody.
5. Incubation with diluted HRP-labelled polyclonal goat-anti-mouse-IgG antibody (1:1,000 in antibody dilution buffer), 30 min at ambient temperature. Alternatively the incubation could be done with the HRP-labelled polyclonal goat-anti-rabbit-IgG antibody.
6. Interpretation of test results: A positive signal demonstrates that the respective allergenic compound was successfully immobilized on the activated glass surface. Signal intensities for the recombinant protein are higher than the ones for the birch pollen extract. This clearly indicates that in the case of the pollen extract the available binding sites are also occupied by other proteins of the extract.

3.7. Reference

Measurement: Determination of Allergen-Specific IgE on MTPs

1. Coating with allergen solution: recombinant allergens/allergen extracts (1:400/1:100 with PBS, pH 7.8 for Microlon MTPs and 1:400 with Na₂HPO₄, pH 8.5 for “NOS” MTPs), 100 µL, 18 h at ambient temperature.
2. Washing with washing solution: this step is repeated after each reaction and is not mentioned further.

3. Blocking of free surface groups: 1% BSA (*see Note 1*) in PBS (Microlon MTP) or blocking solution (“NOS” MTP), 300 µL, 2 h at ambient temperature.
4. Incubation with diluted human serum samples: 1:8 in antibody dilution buffer, 50 µL, 1 h at ambient temperature.
5. Detection with anti-human-IgE antibody: 1:5,000 diluted HRP-labelled anti-human-IgE antibody (polyclonal, goat-anti-human, KPL), 100 µL, 45 min at ambient temperature.
6. Measurement: The colour development (25-mL citrate buffer, 300 µL of 1% TMB solution in methanol, 100 µL 1% H₂O₂ solution) is stopped after 10 min with 5% H₂SO₄ solution. The absorbance is read at 450 nm with an eight-channel photometer.

3.8. Determination of Total and Allergen-Specific IgE on GOPS Slides (*see Note 2*)

1. *Washing step* with washing solution and HPLC grade water. This washing step is repeated after each reaction step and will not be mentioned further.
2. *Blocking of free surface groups* with blocking solution for 2 h at ambient temperature.
3. Incubation with human serum samples:
 - (a) Positive controls: serum samples tested positive against specific allergens using the reference measurement (Pharmacia CAP system) are diluted 1:8 in 200-µL antibody dilution buffer and incubated in chamber slides (200 µL, Sigma; *see Note 3*) for 30 min at 36°C. The incubation with the serum sample could also be done automatically in the PASA system. For this purpose a volume of 500-µL diluted serum sample (1:8 diluted in antibody dilution buffer) is incubated for 30 min at ambient temperature.
 - (b) Negative controls: serum samples tested negative for allergen-specific IgE antibodies with the reference method (Pharmacia CAP system) or heat-denatured serum samples (serum samples treated for 2 h at 56°C; *see Note 4*) are used.
4. *Detection with anti-human-IgE antibody*: the antibody is diluted 1:500 in antibody dilution buffer; 250 µl are applied with a cover slip and incubated for 30 min at ambient temperature.
5. *Detection of bound IgE antibodies* in the flow cell of the PASA system: 500-µl chemiluminescence substrate is pumped into the flow cell.
6. *Evaluation of test results*: Light emission is accumulated for 60 s using a 2 × 2 binning. Data evaluation is performed with the software “Imagetool” (version 2.0, University of Texas health Science Center, San Antonio, TX, USA). A trimmed mean

(25%) in combination with a winsorized standard deviation is used to calculate robust statistical parameters (24). Using this method the standard error of the mean for the streptavidin-HRP signals on a single chip surface should be between 2.6 and 7.6%. In previous studies between 17 GOPS slides the standard error was 19.7% (mean) or 7.9% (trimmed mean, winsorized sample mean). Some outliers could explain these results.

7. *Calibration of the test results:* As internal standard, the intensities detected for the single allergen-specific IgE antibodies are standardized against the signal mean intensity obtained for immobilized myeloma-IgE.
8. *Example for the interpretation of test results:* The results obtained with the microarray format were compared with the reference method done with the Pharmacia CAP system. As seen in **Fig. 2** each serum sample showed its own signal pattern.
 - (a) Negative controls (serum samples without allergen-specific IgE antibodies): regarding the negative controls, no false positive signals for a single allergen have been obtained. The positive control (Myeloma-IgE) was consistently shown, but there was a variation in signal intensity present for the different microarrays tested. To be able to compare results of different microarrays, the signal intensity should be normalized against a test-independent standard (e.g. immobilized streptavidin-HRP). Nevertheless, it was possible to distinguish between patients with and without specific IgE.
 - (b) Positive results for allergen extracts: in case of allergen extracts, the immobilization of the main allergenic proteins could be difficult due to an unfavourable ratio of allergenic to non-allergenic proteins. In general, it is nearly impossible to predict which protein of the protein mixture will be immobilized on the chip surface. Positive signals in case of allergen extracts could be caused either by specific reaction between the allergen and specific IgE or by a “cross-reaction” of human IgE with other components of the allergen extract (e.g. lectins) (25). Homologous sequences of the major allergenic protein compounds [e.g. between Bet v1 (birch), Api g1 (celery), mal d1 (apple), Pru a1 (plum)] are also a reason for the cross-reactivity between birch, legumes, and fruits (26).
 - (c) Positive results for recombinant allergens: in case of recombinant allergens, a defined single protein is immobilized on the surface. Positive results are therefore directly related to the used allergen. Screening tests with recombinant allergens (**Fig. 2**) showed that recombinant or purified allergens such as r Bet v1 (birch), r Bet v2

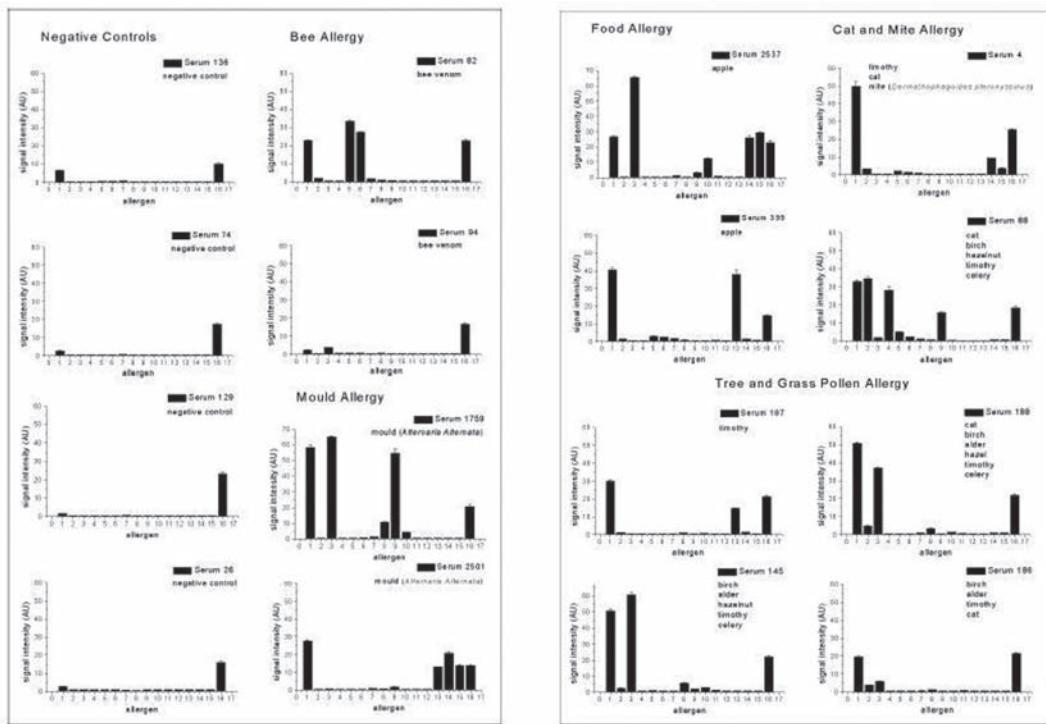


Fig. 2. Screening of single serum samples based on recombinant and purified allergens immobilized on GOPS-modified glass slides. Immobilized recombinant/purified allergens: 1 – anti-human IgE LO-HE-10, 2 – r Fel d1, 3 – r Bet v1, 4 – r Bet v2, 5 – PLA₂, 6 – bee venom, 7 – IgG, 8 – r Aln g1, 9 – r Phl p1, 10 – r Cor a1, 11 – r Api g1, 12 – r Mal d1, 13 – r Alt a1, 14 – Der p1, 15 – Der f1, 16 – streptavidin-HRP.

(birch), r Phl p1 (timothy grass), r Aln g1 (alder), r Fel d1 (cat epithelia), Der p1 (mite), Der f1 (mite), r Alt a1 (mould), and PLA₂ (bee venom) could be used for the screening of allergen-specific IgE. Only weak signals were obtained for r Api g1 (celery), r Mal d1 (apple, *see Note 5*), and r Cor a1 (hazelnut).

- (d) Missing signals for allergen extracts and recombinant allergens: working with recombinant proteins, a lacking spot on the multi-allergen chip in the test could have different reasons: an insufficient immobilisation of the corresponding protein on the chip surface, sterical hindrance, a partial or complete denaturation of the immobilized protein, or the lack of allergen-specific IgE in the patient's serum sample. Another possible reason for false negative test results is that using MTPs or GOPS-modified glass surfaces, allergens can not be immobilized as densely as on the cellulose polymer surface of the Pharmacia CAP test (*see Note 6*).

(e) Missing signals for immobilized food allergens: Direct ingestion of food allergens also stimulates the production of allergen-specific IgG antibodies. Concentration of allergen-specific IgG can be 10,000 times higher than the allergen-specific IgE level. Weak IgE signals could be caused by the competition between allergen-specific IgG and IgE for the limited binding sites. Therefore, allergens immobilized on ELISA MTPs or on microarrays may be blocked by specific IgG leading to an underestimation of IgE.

As shown in **Fig. 3** patients tested positive against birch pollen showed positive signals for the birch pollen extract and either r Bet v1 or r Bet v2 in the microarray format. A positive signal for

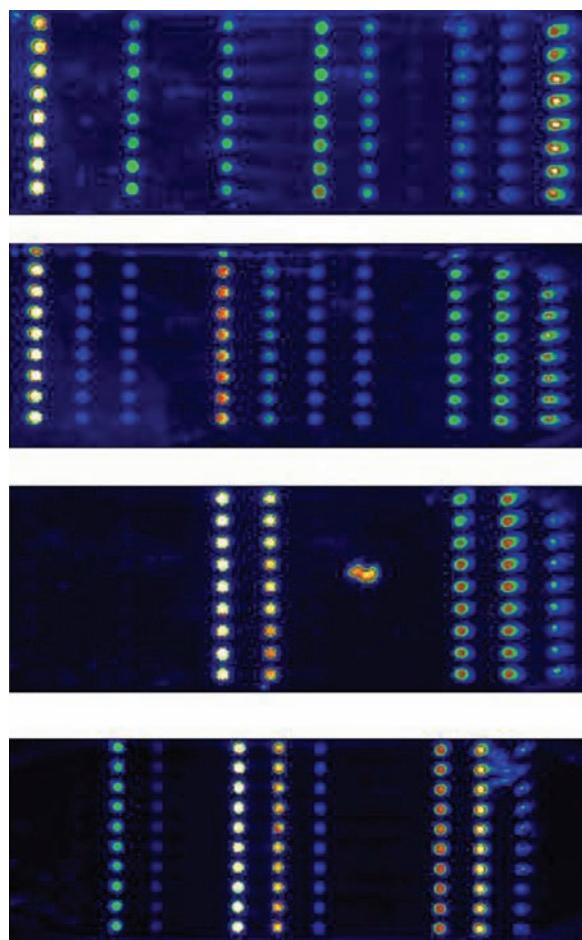


Fig. 3. Screening of serum samples of tree and grass pollen allergic patients using allergen extracts and recombinant allergens on the same chip surface. Immobilized recombinant and allergen extracts: 1 – r Bet v1, 2 – r Bet v2, 3 – birch extract, 4 – buffer, 5 – timothy extract, 6 – r Phl p1, 7 – alder extract, 8 – r Aln g1, 9 – r Mal d1, 10 – rye extract, 11 – wheat extract, 12 – myeloma-IgE.

the allergen extract (alder, timothy) correlated in most cases with a positive signal for the respective recombinant allergen (r Aln g1, r Phl p1). For some serum samples only positive signals for the allergen extracts were obtained, indicating that these patients have no allergen-specific IgE antibodies against the main allergenic proteins of the extract. The use of recombinant and purified allergens in combination with allergen extracts on the same microarray surface seems to be a good compromise to cover all possible cases.

3.9. Estimation of Allergen-Specific IgE Detection Limits on GOPS-Modified Slides

3.9.1. Test Performance

1. For the estimation of the detection limits for allergen-specific IgE antibodies, the corresponding proteins (e.g. r Bet v1, PLA₂, Der f1, LO-HE 10) are immobilized on different GOPS glass slides as described in **Subheading 3.3**.
2. A serum sample with a known specific IgE concentration against the corresponding allergen extract, e.g. birch pollen positive serum sample in case of r Bet v1, is diluted 1:1 with a heat-denatured (2 h, 56°C; *see Note 4*) blank serum sample.
3. The obtained serum samples of different IgE concentrations are diluted 1:4 in antibody dilution buffer.
4. Incubation with serum sample and HRP-labelled anti-human-IgE antibody is performed as already described in **Subheading 3.4**.
5. After a washing step the slide is placed in the flow cell of the *PASA system* (**Fig. 1**), and 500 µL of chemiluminescence substrate is filled into the flow cell.
6. Light emission is accumulated for 90 s using a 2 × 2 binning.

3.9.2. Interpretation of Test Results

Table 2 shows the detection limits of IgE against r bet v1, Der p1, Dre f1, PLA₂, and total IgE. With the microarray format it is possible to distinguish between specific IgE class 0 (0.35 kU/L) and 1 (0.69 kU/L) for r bet v1, PLA₂, and Der p1. With the detection limit of Der f1 specific IgE, class 1 and 2 could be separated. In some cases, e.g. r Phl p1, sensitivity improvements may be necessary to reach lower specific IgE classes (*see Notes 7 and 8*).

3.10. Determination of Total and Allergen-Specific IgE and IgG on GOPS Slides

3.10.1. Test Performance

1. Crude allergen extracts and recombinant allergens are immobilized on the modified glass surface as described in **Subheading 3.3**.
2. The measurement of allergen-specific IgE antibodies is performed according to **Subheading 3.4**. Serum samples could be diluted 1:500 for the determination of allergen-specific IgG antibodies (a dilution of 1:8 was applied for the allergen-specific IgE test).
3. For the detection of allergen-specific IgG antibodies an HRP-labelled rabbit-anti-human-IgG antibody (Sigma-Aldrich,

Table 2
Detection limits for specific IgE using recombinant/purified allergens on a GOPS-modified glass surface

Allergen	Serum sample	IgE ($\mu\text{g}/\text{L}$)	LOD ($\mu\text{g}/\text{L}$) ($3s_{\bar{x}}$ -method)
R bet v1	96	38.0	0.35
PLA ₂	82	54.6	0.16
Bee venom	82	54.6	1.03
Mellitin	82	54.6	4.80
Der p1	4	240	1.85
Der f1	4	240	4.62

The detection limit was determined by dilution of serum samples of known specific IgE class against the specified allergen extract (e.g. birch pollen positive serum sample in case of r Bet v1) with an IgE heat-denatured (2 h, 56°C) serum sample of a patient without specific IgE

1:1,000 in PBS/0.5% BSA/0.5% Tween-20) was used (30 min, ambient temperature).

3.10.2. Elimination of IgG Antibodies from Human Serum Samples

1. “Protein G” coated agarose beads (40 μL) are mixed with 20- μL human serum and 80- μL PBS buffer using a 1.5-mL Eppendorf tube.
2. As a control 20 μL of the same serum is diluted with 120- μL PBS and treated similar to the “agarose bead suspension”.
3. The content is mixed thoroughly with a vortexer and slightly mixed for 2 h at ambient temperature on a microtitre plate shaker.
4. The agarose beads are removed by centrifugation (Ultrafree C-Caps, 17,000 g, 5 min, 4°C).
5. The remaining serum solution is transferred into a new Eppendorf tube and diluted with 800- μL PBS.

3.10.3. Interpretation of Results

The treatment with *Protein G*-coated agarose beads influences the intensities for specific IgE antibodies only slightly. In opposition to this, the signals obtained for allergen-specific IgG antibodies could be reduced. For routine clinical testing, the elimination of IgG antibodies from human serum samples is too time and cost intensive. For this purpose, the IgE and IgG-specific antibodies should be measured with different microarrays. The clinical symptoms could thereafter be evaluated based on both test results.

4. Notes

1. It has to be considered that the patient could also be allergic against the blocking reagent (e.g. cow's milk casein – casein as blocking reagent should be avoided, sea block blocking reagent should be avoided for patients with a sea food allergy).
2. The test results of GOPS slides seemed to be closer to the results obtained with the hydrophobic Microlon MTPs than to the hydrophilic NHS activated MTPs, indicating that an adsorptive binding mechanism on the hydrophobic GOPS slide is predominant in relation to the covalent attachment of the protein's amino groups to the epoxy groups of the surface.
3. If a colorimetric detection of HRP is used, the measurement could also be performed off-line using chamber slides and coverslips in combination with a commercially available scanner.
4. Heating of blood samples over 56°C over a longer time period leads to the specific destruction of immunoglobulin IgE.
5. Some recombinant proteins, e.g. r Mal d1 (apple, 17 kDa, Bet v1 homologue) could neither be recognized on hydrophobic Microlon MTPs nor on GOPS-modified chips. Commercially available recombinant proteins such as r Mal d1 seem to be less reactive than its natural counterpart (27).
6. Summarizing all results for the samples tested so far, it was evident that for the microarray-based system false negative signals are more common than false positive ones.
7. For serum samples with higher IgE concentrations (>specific IgE class 3) a saturation of the chip surface could be reached. Appropriate dilution of serum samples could solve this problem.
8. For the simultaneous measurement of the total IgE and allergen-specific IgE (saturation level reached after different time points) the accumulation time of the chemiluminescence signal could be varied.

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Chapter 8

Peptide Microarrays for Serum Antibody Diagnostics

Heiko Andresen and Frank F. Bier

Summary

Peptide microarrays are useful instruments for miniaturized high-throughput, high-content immunoassays. The substitution of linear epitopes of the protein antigen with short, synthetic peptides is virtually a straightforward approach to capture antigen-specific antibodies from serum samples; however, both the biologically active surface display of peptides and the establishment of a solidly performing peptide microarray immunoassay are often troublesome in detail. The following protocols aim to provide facilitated access to the production of a robust peptide microarray platform and an optimized analytical processing of peptide microarrays in serological diagnosis. The functional surface display is accomplished by site-specific immobilization of peptide probes in a two-step procedure first by coupling of biotinylated peptides to hydrazide-modified streptavidin and then utilizing a subsequent chemoselective reaction between the hydrazide linkers of the streptavidin and an aldehyde-coated substrate for microstructured surface immobilization of the probe complexes. The serological assay is based on the specific capture of primary antibodies in a sandwich complex between the surface-immobilized peptides and a fluorescently labelled secondary antibody. A proposal for the diagnostic evaluation of fluorescence data obtained with the peptide microarray is also made.

Key words: Antibody, Epitope, Immunoassay, Serum analysis, Epitope mapping, Peptide microarray, Site-specific immobilization

1. Introduction

The analysis of antibodies in human serum is an established technique in the laboratory diagnosis of infectious diseases, autoimmunity, and allergy. Immunoassay technologies are generally based on immobilized antigens that serve to capture antigen-specific antibodies from serum samples. Assay formats that are routinely used for serological diagnosis include the enzyme-linked immunoassay, immunoblotting, complement fixation, and

immunofluorescence tests. The drawbacks of these conventional serologic assays are a lack of multiparameter analysis and the inability to differentiate the fine specificity of heterogeneous antibody populations.

Peptide arrays displaying biologically active small synthetic peptides in either low, medium, or high-density formats represent an attractive technology to probe complex samples for the presence of antibody analytes. Like oligonucleotides for DNA microarrays, well-established peptide chemistry and fully automated synthesis of custom-designed libraries with simultaneous ease of purification and quality analysis make peptides an economically superior alternative to native or recombinant protein antigens. Synthetic peptides are thereby chemically and physically resistant and allow for the development of relatively robust assay formats. A particular immunologic advantage of peptide arrays is their unique capacity to break down the heterogeneous B-cell response into almost monoclonal antibody specificities and to differentiate subtle changes in antibody abundance and specificity.

The technological concept of peptide array immunoassays is based on the substitution of the specific antibody recognition sites on the protein antigen, the epitopes, with short overlapping synthetic peptides, typically consisting of 10–15 amino acids. Such short sequences are rarely capable of intramolecular interaction and usually do appear in a linear rather than a three-dimensional structure. Synthetic peptides are therefore essentially restricted to the mimicking of continuous epitopes, and their use is limited when complex three-dimensional structures are necessary for a given biological function (1).

The immobilization and functional surface display of synthetic peptides in microspot immunoassays require conditions that differ from those in conventional assay systems. The factors that determine the analytical performance of peptide microarray immunoassays are (a) site-specific immobilization of peptide probes (b) high local probe density, and (c) unrestrained probe accessibility. The site-specific immobilization avoids chemical modification of functional amino acid side chains that are involved in antibody binding, and ensures surface presentation of peptides in a predefined orientation. The beneficial effect of a high probe density is the formation of multivalent antibody:peptide complexes in the microspot and the frequent rebinding of antibodies after complex dissociation. The result is a high apparent affinity of serum antibodies that correlates with a low detection limit. In addition, the sensitivity of fluorescence microarray scanners also depends on the fluorophore density rather than on the total fluorophore amount per microspot. Antibody capture with surface displayed peptide probes is critically affected by the accessibility of key residues of the peptide ligands (2). To avoid steric hindrance during immunocomplex formation and to minimize mass transport

limitation, spacer molecules of sufficient length and flexibility have to be incorporated into the peptide probe design.

The protocols described here were originally developed for antibody diagnostics and characterization; however, the peptide microarray platform may also be adopted to a variety of other assay types, e.g., the analysis of enzyme substrate specificity (3, 4).

2. Materials

2.1. Peptides

1. Peptide probes with spacer and terminal biotin linker (**Fig. 1**). Prepare 2 mM stock solutions in DMSO (see **Note 1**).

2.2. Preparation of Streptavidin-Hydrazide (SAHz) Conjugates

1. Streptavidin (Pierce, Rockford, IL, USA).
2. Adipic acid dihydrazide (Fluka GmbH, Seelze, Germany).
3. EDC (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).
4. Phosphate buffered saline (PBS): 0.1 M phosphate buffer with 0.15 M NaCl, pH 6.0.
5. NAP™-5 (GE Healthcare Life Sciences, Munich, Germany).
6. Elution buffer: 10 mM phosphate buffer.
7. Biotin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Prepare a 2 mM solution in DMSO.
8. Centrifugal ultrafiltration devices for protein concentration, e.g., iCon 20K MWCO (Pierce, Rockford, IL, USA) or Ultra-free 30K MWCO (Millipore GmbH, Schwalbach, Germany).

2.3. Microarray Printing and Processing

1. Spotting buffer: 0.1 M sodium acetate pH 5.0 with 20% (v/v) glycerol.

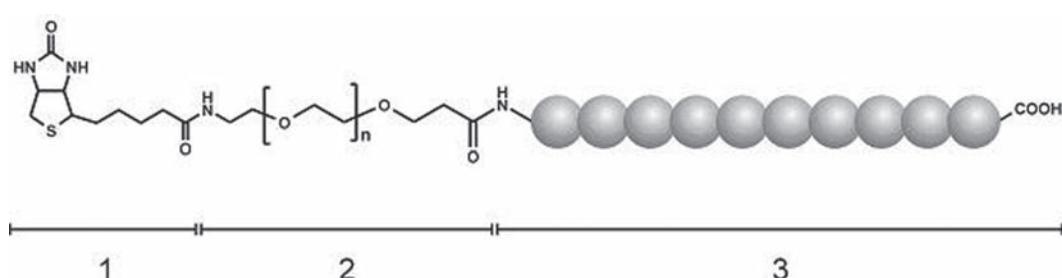


Fig. 1. Recommended design for peptide probes in microarray applications. The probe molecule consists of three functional units. 1 – linker moiety (biotin), 2 – spacer molecule (ideally a polyethylene glycol spacer with $n > 3$ units), and 3 – amino acid sequence.

2. Microscope glass slides with aldehyde surface coating (Schott Nexterion® AG, Jena, Germany).
3. Blocking Buffer: 1 M Tris–HCl pH 8.5, 0.5% (v/v) Nonidet P40, 5% (w/v) nonfat dry milk (*see Note 2*).
4. Fifty millimolar sodium hydrogen carbonate pH 9.6 with 20% (v/v) glycerol.
5. Protein A (Pierce, Rockford, IL, USA).
6. Petri dishes with 145 mm diameter (Greiner Bio-One GmbH, Frickenhausen, Germany), parafilm, and filter paper.
7. QuadriPERM® four-compartment cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany).

2.4 .Serum Analysis

1. Incubation buffer: CrossDown buffer (AppliChem, Darmstadt, Germany), 5% (w/v) nonfat dry milk.
2. Washing buffer: 12 mM PBS pH 7.4 with 0.15 M NaCl, 0.5% (v/v) Nonidet P40.
3. Secondary antibody: goat anti-human IgG/IgA/IgM antiserum conjugated to Cy5™ (Jackson Immuno Research, Cambridgeshire, GB).
4. LifterSlips™ (Erie Scientific Company, Portsmouth, NH, USA) (*see Note 3*).
5. Super Pap Pen (Electron Microscopy Sciences, Hatfield, PA, USA).

3. Methods

The immobilization of peptidic antigens on planar surfaces mediated by the carrier protein streptavidin has important advantages for both the production and the performance of peptide microarrays. The immobilization of peptides on prefabricated streptavidin surfaces, however, is unexpectedly challenging with nanoliter volumes of peptide solutions, e.g., the spotting buffer has to be optimized for individual physicochemical properties of peptides, and steric interference on the surface can drastically reduce the coupling efficiency. In contrast, the solution phase precoupling of biotinylated peptides to streptavidin is highly efficient. It is also particularly advantageous, since the peptides contribute to less than 10% of the mass of peptide:streptavidin complexes, and therefore, the physicochemical properties of any peptide are leveled by those of the streptavidin. This simplifies the handling of entire peptide libraries and allows for standardized spotting protocols (5). Antibody binding to the peptide probes is further

enhanced by the carrier protein creating an exposed and extended three-dimensional topology of the peptide presenting surface.

Peptide:streptavidin complexes can be immobilized by a variety of bioconjugate chemistries and on a variety of activated surfaces. The rationale to use a chemoselective reaction between the protein and the surface, however, is that crude reaction mixtures can be used in both the peptide-streptavidin coupling and the complex immobilization, since the sequential bioorthogonal reactions (1 – biotin–avidin affinity, 2 – hydrazide–aldehyde condensation) inherently act as purification steps. For instance, an excess of peptides used for the preparation of P-SAHz does not interfere with the subsequent immobilization reaction of the complexes when the mixture is applied to the activated microarray surface. In addition, the bioorthogonal coupling permits the use of unprotected peptide ligands yet avoids the risk of chemical modification of functional amino acid side chains crucial for a given biological function. The chemoselective reaction between hydrazide moieties of the modified streptavidin and an aldehyde-coated surface at acidic pH has proven particularly suitable for this purpose: (a) aldehyde-coated microarray substrates are readily available, (b) the surfaces are conveniently hydrophilic, which enhances contact between the protein and the surface and simultaneously prevents unspecific hydrophobic interactions, (c) as comparatively weak electrophiles, aldehydes have a longer half-life compared with strong electrophiles such as epoxides and succinimidyl esters, making them suitable for long spotting runs and prolonged coupling reactions (6).

Patient serum is a highly complex biological specimen, and serological analysis is particularly susceptible to unspecific reactions of serum components with the analytical matrix. The situation is further complicated by the fact that sera often exhibit very individual predispositions for such unspecific reactivity due to endogenous factors. Within a variety of possible mechanisms that can cause unspecific signals (background) in immunoassays, the major effects observed in peptide microarray analysis usually result from two types of reactions, known as *unspecific binding* and the more complex *matrix effects* (7). Unspecific binding is the result of a relatively weak physical interaction between serum antibodies or secondary antibodies, respectively, and components of the analytical platform, e.g., surfaces or biomolecules. Such interactions are most efficiently prevented by a physical blocking of the platform with inert proteins prior to analysis and thorough washing steps. In contrast, matrix effects are often considerably stronger and share characteristics of a specific rather than an unspecific interaction, although they are weakly defined in detail. As a consequence, physical blocking reduces the background caused by matrix effects only proportionally to the reduction of the specific binding of serum antibodies to their cognate

antigens. To obtain an adequate suppression of matrix effects, the chemical composition of the incubation buffer has to be adjusted to the specific needs of the immunoassay type. Novel, tailored incubation buffers for serum analysis have recently become commercially available to serve this purpose. Taken together, it is important to recognize that the proportion of the unspecific signal rather than the signal obtained from the specific antibody binding finally determines the sensitivity of the peptide microarray immunoassay.

3.1. Preparation of Streptavidin-Hydrazide (SAHz) Conjugates

1. Reconstitute 2 mg of streptavidin lyophilisate in 1 mL 0.1 M PBS pH 6.0 to obtain a final protein concentration of 2 mg/mL (*see Note 4*).
2. Per milliliter of streptavidin solution, add 32 mg adipic acid dihydrazide and mix until it is completely dissolved.
3. Weight 32 mg EDC per milliliter of streptavidin solution into a fresh reaction tube. Add the mixture of streptavidin and adipic acid dihydrazide to the EDC and vortex immediately until a homogeneous solution is obtained.
4. Allow the conjugation reaction to proceed over night at +4°C.
5. Equilibrate a NAP™-5 column with three column volumes of 10 mM phosphate buffer (*see Note 5*).
6. Apply 0.5 mL of the reaction solution to the column and wait until the solution has completely penetrated into the gel bed.
7. Elute the protein with 1 mL elution buffer. Collect the eluate as ca. 100–200 µL fractions and determine the protein concentration in the eluates by photometric measurement at 280 nm (accomplished almost without loss of material in a NanoDrop photometer). A sufficiently precise approximation for the protein concentration can be calculated with $c = A_{280} \times 0.32 \text{ mg/mL}$.
8. Regenerate the column with four column volumes elution buffer.
9. Repeat steps 6 and 7.
10. Blend the eluates that contain the protein and adjust the protein concentration to 2 mg/mL with elution buffer. If the concentration is lower than 2 mg/mL, then carefully concentrate the solution using the centrifugal ultrafiltration devices. Keep SAHz at +4°C for a short term or at -20°C for long-term storage.

3.2. Preparation of Peptide:SAHz Complexes (P-SAHZ)

1. For each peptide probe, prepare an aliquot of 20 µL SAHz (ca. 0.75 nmol) in a fresh reaction tube (*see Note 6*).

2. Add 3.75 µL of the respective 2 mM peptide solution to the SAHz, corresponding to a tenfold molar excess compared with the protein. Mix thoroughly and let the reaction proceed overnight at +4°C. To prepare an adequate specificity control, add 3.75 µL biotin solution instead of peptide solution to an aliquot of 20 µL SAHz.
3. P-SAHZ complexes can be stored at +4°C prior to use or at -20°C for several months.

3.3. Microarray Printing and Processing

1. Design a layout for the peptide microarray (see [Note 7](#)). Consider about 10% of all spots for the specificity control that should be randomly distributed across the subarray. Reserve the spots in the four corners of each subarray for the incubation controls ([Fig. 2](#)).
2. Dilute P-SAHZ complexes and the specificity control to a final concentration of 0.15 mg/mL in a spotting buffer. Take into account that the SAHz concentration changed from 2 to 1.68 mg/mL due to the addition of peptide solution (note that mass change of the complexes due to the addition of peptides is ignored). Dilute the incubation control (Protein A) to a concentration of 0.075 mg/mL in 50 mM NaHCO₃ pH 9.6. The appropriate volume of spotting solution needed depends on the specifications of the microarrayer in use. If the spotting solutions have to be provided in a microtiter plate for the spotting run, it is important to use polypropylene microtiter plates to avoid the adsorption of P-SAHZ to the plate walls.
3. Mark the aldehyde slides with a diamond scriber and place them dust free into the arrayer. Avoid contacting the microarray surface. Set up the arrayer and print the slides.

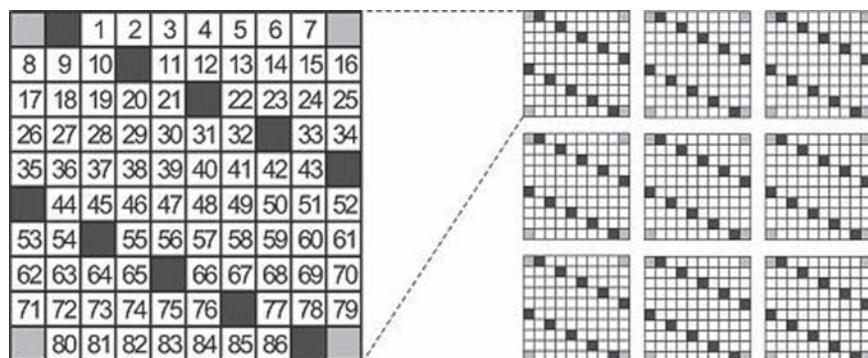


Fig. 2. Schematic of an appropriate peptide microarray layout for serum analysis. Each subarray (left panel) contains the incubation control in the four corners (light gray) and multiple repeats of the specificity control that are uniformly distributed across the area (dark gray). Numbers indicate spots containing a specific peptide. The microarray consists of several identical subarrays (right panel).

4. Put a piece of filter paper into each Petri dish and moisturize the paper with 130 µL deionized water (dH₂O). Place four microarray slides into each dish and seal the chamber with parafilm. Incubate the slides at least over night at +4°C. In this state, the slides can be stored for several weeks at +4°C (*see Note 8*).
5. Prior to use, freeze the slides for approximately 1 h at -80°C. Provide a slim 150 mL beaker with blocking buffer. Take each slide separately from the freezer and rapidly dip it into the blocking buffer. Proceed with rapid dipping for about 1 min (*see Note 9*). Afterward, place the slides printed side up in separate chambers of a quadriPERM cell culture plate and fill the chambers with 10 mL blocking buffer. Maintain 2 h of blocking at room temperature with good shaking.
6. Rinse the slides with running dH₂O and dry in a stream of nitrogen.

3.4. Serum Analysis

1. Prepare the incubation buffer by dissolving 5% (w/v) non-fat dry milk (50 mg per milliliter of buffer) in CrossDown Buffer.
2. Dilute the serum samples 1:40 in the incubation buffer. To estimate the necessary analysis volume, consider the injection volume required for the specific type of LifterSlips and include a surplus of ca. 10–20%.
3. If the slides comprise more than one array, use a Super Pap Pen or an equivalent liquid blocker to separate the areas with hydrophobic boundaries, which prevent cross-contamination of different samples. Cover the printed microarrays with LifterSlips. Place the pipette tip directly at one of the open sides of the cover glass so that the capillary force starts to absorb fluid from the tip. Then start to inject the sample in a slow, continuous action until the chamber is completely filled.
4. Prepare a moist chamber by wetting the filter paper in a Petri dish with ca. 1 mL dH₂O and carefully place the slides into the chamber (no sealing necessary). Incubate for 2 h at room temperature (*see Note 10*).
5. Remove the LifterSlips and the bulk of incubation solution by rinsing the slides with running dH₂O. Place the slides in separate chambers of a quadriPERM cell culture plate and fill the chambers with 10 mL washing buffer. Wash the slides with good shaking for 5 min. Repeat this washing step three more times using fresh washing buffer after each step. Rinse the slides with dH₂O and dry in a stream of nitrogen.
6. Dilute the secondary antibody to a concentration of 10 µg/mL in incubation buffer. Preestimate the total volume needed as described in **step 2**.

7. Cover the microarrays with LifterSlips and inject the antibody solution as described in **step 3**. Incubate in a humid chamber for 1 h in the dark.
8. Wash and dry the slides as described in **step 5**. Prevent lengthy light exposure.
9. Scan the slides with an appropriate microarray scanner in the Cy5-channel according to the manufacturers' instructions. Extract the fluorescence data with the analysis software of choice.

**3.5. Data Evaluation
(see Fig. 8.3)**

1. Work with the median value of the relative fluorescence units (RFU) of microarray spots. The median better compensates for minor artifacts and inhomogeneities in the spot than the mean value
2. For each subarray n , calculate the arithmetic mean of the median fluorescence intensities of all spots containing the specificity control ($\text{RFU}_{\text{SC},n}$). The specificity control represents the background fluorescence (see **Note 11**).
3. For each subarray n , calculate the fluorescence contrast between any spot containing a specific peptide i ($\text{RFU}_{i,n}$) and the mean fluorescence of the specificity control ($\text{RFU}_{\text{SC},n}$) according to **Eq. 8.1** (see **Note 12**):

$$\text{Contrast } i,n = \frac{\text{RFU}_{i,n} - \text{RFU}_{\text{SC},n}}{\text{RFU}_{i,n} + \text{RFU}_{\text{SC},n}}. \quad (8.1)$$

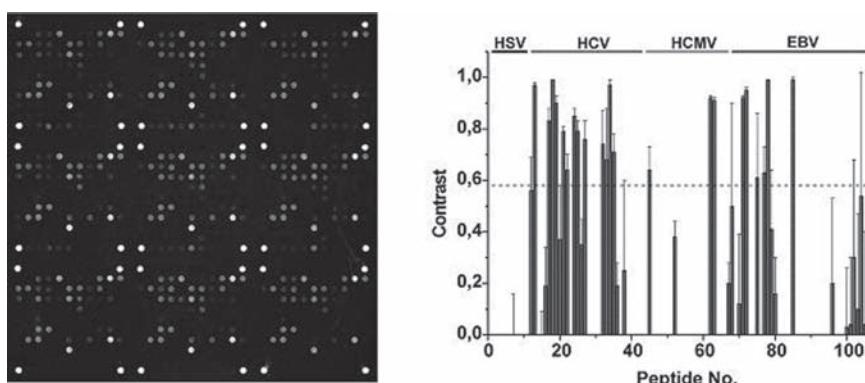


Fig. 3. Exemplar of the serological multiparameter diagnosis of four different virus infections with a peptide microarray. *Left panel:* fluorescence image of the peptide microarray after serum analysis in gray scale. The array consists of nine identical 11×11 subarrays and was printed with the noncontact piezo dispensing roboter sciFLEXARRAYER (Scienion AG, Berlin, Germany). *Right panel:* Diagnostic evaluation of the fluorescence data using the contrast value. Each column in the diagram represents the arithmetic mean of the contrast values of a specific peptide. The dashed line indicates the threshold for a positive antibody detection.

4. Through the subarrays 1 to n , calculate the arithmetic mean and the standard deviation of all contrast values 1 to n of a specific peptide i .
5. Define the threshold for a positive contrast value with **Eq. 8.2**, wherein $\overline{\text{RFU}_{\text{SC}}}$ and SD_{SC} are the mean and the standard deviation of all spots containing the specificity control through the entire microarray (*see Fig. 3* and **Note 13**):

$$\text{LOD} = \frac{(\overline{\text{RFU}_{\text{SC}}} + 3 \times \text{SD}_{\text{SC}}) - \overline{\text{RFU}_{\text{SC}}}}{(\overline{\text{RFU}_{\text{SC}}} + 3 \times \text{SD}_{\text{SC}}) + \overline{\text{RFU}_{\text{SC}}}}. \quad (8.2)$$

6. An antibody analysis is judged positive, if the mean of the contrast value (**step 4**) is above the calculated threshold and there is no overlap between its standard deviation and the threshold (*see Note 14*).

4. Notes

1. Peptide libraries can be ordered from any specialized provider. It is imperative to include a spacer molecule into the peptide probe design between the amino acid sequence and the terminal linker moiety during synthesis. The amino-PEG₁₁ propionic acid of Polypure AS (Oslo, Norway) is highly recommendable as a spacer molecule, and it is conveniently available as monodisperse building blocks for Fmoc- or Boc-chemistry, respectively. A terminal biotin linker is required for the coupling of the peptide probes to the carrier protein streptavidin.
2. In any sense, nonfat dry milk is the most efficient blocking reagent for peptide microarray immunoassays. The blocking buffer works best when the milk powder is freshly added; however, aliquots can also be frozen at -20°C. The milk powder is easily homogenized in the buffer when the solution is warmed to 60°C and ultrasound is applied.
3. LifterSlips come in different sizes, geometries, and injection volumes. It is counterproductive to dictate a specific format. The choice should rather be based on the design and dimensions of the microarray planned, i.e., the number of probes and the specifications of the microarray printer.
4. The following protocol has been adopted from (8, 9) and assumes a streptavidin solution of 1 mL. A scale up can be easily made, if the amount of other reagents is changed proportionally.

5. Gel chromatography is fast and efficient in quantitatively separating SAHz from the excess of adipic hydrazide; however, other methods may be suited as well, e.g., extensive dialysis against phosphate buffer.
6. Smaller or larger volumes may also be used, if the peptide solution is changed accordingly.
7. Against the background of analysis artifacts due to local inhomogeneities, it is advantageous to compose an array that consists of a number of identical subarrays in which each subarray contains one spot of a specific peptide and several controls. Design of square or rectangular array geometries simplifies gridding and spot finding during automated array analysis.
8. Both glycerol and DMSO are strongly hygroscopic. If too much dH₂O is applied to wet the filter paper, the spots may run into each other!
9. This treatment is a trick to avoid comet effects and smearing of spots on the surface, which is a particular problem of protein solutions on microarray substrates. The faster the dipping of the frozen slides, the better the microspot morphology!
10. The incubation time may be extended, e.g., to over night procedures at +4°C; however, with this particular peptide surface display the specific antibody binding reaches equilibrium state at the latest after 4–6 h. In contrast, the unspecific binding to P-SAHz is a nonsaturable reaction, which is why after an incubation time of 4–6 h the signal-to-background ratio may decrease again.
11. Streptavidin (or SAHz) is the major target of unspecific binding and matrix effects in the peptide microarray immunoassay and is therefore referred to as background.
12. The “contrast” is a common characteristic in optical measurement. The contrast value describes the differentiability of an optical signal from the background and takes values from 0 (not differentiable) to 1 (fully differentiable). The contrast value is highly useful for the evaluation of microarray fluorescence images, particularly for diagnostic purposes, since this value represents per se a normalization of broadly distributed data sets (i.e., signal-to-background ratios) and dramatically reduces the complexity of results in a sense that the information is reduced to the important basics as “strongly positive,” “positive,” “indeterminate,” and “not positive.” However, it is not suitable for quantitative comparisons.
13. The contrast is useless without a suitably defined threshold value. Since any serum has an individual predisposition for unspecific reactivity, the threshold value has to be dynamically

defined with characteristics following from each individual analysis. This is best accomplished by considering the intensity and the variation of the proportion of unspecific reactivity toward SAHz.

14. This is of course a fairly generalized but empirically supported declaration of a positive analysis result. Each application may require further modifications to obtain a reasonable relation between the sensitivity and the specificity of the analysis.

Acknowledgments

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Chapter 9

Microchips for Cell-Based Assays

Martin Dufva

Summary

Analysis of cells is a cornerstone in biomedical research. Traditional methods for cell culture and tissue analysis can be replaced by various microchips as discussed in this chapter. A tissue array is an example of microchip that provides higher throughput of tissue analysis. Other microchips provide completely new functionalities for the researcher. One such example that will be discussed is cell culture chips. The cell culture vessel will be much more competent for studies of cell and will enable real-time monitoring of cell behavior and gene expression at a single cell level, have possibilities for studying chemotaxis and shear stress phenomena in a controlled manner, provide least tissue-like culture conditions by providing micro 3D structures, and provide arrays of integrated detectors. Tissue arrays and the simplest forms of cell culture chips are commercialized today, and we may expect a large activity in this field in the future.

Key words: Tissue arrays, Cell culture chips

1. Introduction

Studies of cells in cultures have been done for more than 50 years. Culture of cells is traditionally done in batch culture where cells are seeded in flasks containing cell culture medium. The salinity, pH, and temperatures in the flasks are maintained in special incubators providing heat, humidity, and CO₂ control. Microtiter plates were later introduced so that throughput could be increased in biomedical research based on cell cultures. The obvious advantages with a microtiter plate are that it provides 96 (or 384) different cell cultures in a relatively small area; the volumes for each culture are relatively small leading to considerable saving of growth factors, cytokines, and other additives used for cell research, and a robotic system can be used for simplifying addition of soluble factors. However, microtiter plates are still only

providing possibilities for batch culturing and require incubators for cell maintenance. During the last decade, however, microchips for cell culture have been developed that provide a range of novel possibilities in terms of cell culture and manipulation. The strengths of these chips are that they allow experiments that are very difficult to perform in a batch culture. These novel types of experiment are possible because of the special properties of liquids when flow streams are miniaturized.

2. Effect of Miniaturization on Flow Systems

The one most notable difference between microflow systems and macroflow systems is that in microflow systems, two streams of water-based liquids do not mix to a large extent. The reason is that aqueous solutions in microsystems behave as if the aqueous solution were as thick as honey. Because of this behavior it is impossible to get turbulence in microsystems (think how difficult it would be to get turbulence in liquids as thick as honey). Two streams entering into the same channels can therefore only mix by diffusion, which is a relatively slow process. In contrast, on a macroscale, two fluids would mix fairly rapidly because of turbulence in the flow (compare how rapid mixing is obtained using a vortex). The nonturbulent flow and thereby slow mixing in microsystems can be both an advantage and a disadvantage. It is a clear disadvantage when rapid mixing is desired but because the dimensions of microsystems are usually small, mixing can be quite rapid if the flow is sufficiently slow and the molecules sufficiently small (e.g., fluorescent dyes) (**Fig. 1a**). However, mixing of proteins and DNA molecules (i.e., large molecules) by diffusion is a relatively slow process and would require very long channels (**Fig. 1b**). For example, a protein diffuses 80 μm in about 5

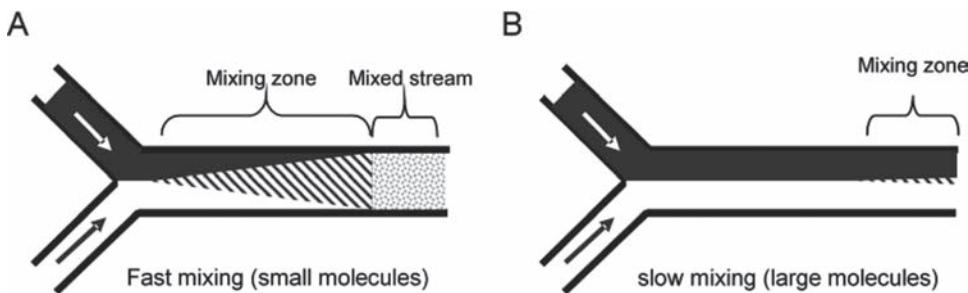


Fig. 1. Mixing in Microsystems. (a) Rapid diffusion of small molecules (drugs, ions gases) leads to rapid mixing of the microfluidics stream. (b) In contrast, larger molecules (DNA protein) do not efficiently mix between the two streams because of slow diffusion rate.

min. A protein solution introduced side by side with a buffer in a channel that is $160\text{ }\mu\text{m} \times 80\text{ }\mu\text{m}$ broad and high, respectively, at a flow rate of $10\text{ }\mu\text{L/min}$, would require a channel that is about 4 m in length before the two solutions are mixed completely. In such cases, mixing is needed to reduce channel length. Mixing can easily be obtained by patterning one side of the channel with mixer structures that resemble herring bones (1). The slow mixing can, however, also be an advantage when it is desired to precisely control patterning of surfaces using flow (see later for examples).

3. Cell Culture Chips

Cell culture chips have been used for a variety of applications such as single cell observations over long periods of time (2–4), study of wound healing processes (5), cell–cell interactions (6), differentiation (7), myocyte synchronization (8), chemotaxis (9), electrical characterization (10,11), cell stress levels (12), and online monitoring of gene expression (13). More advanced chambers have interconnected chambers each holding different cell lines representing the different organs and their interconnective metabolism (14), and these cell culture systems are used for determining the toxicological and pharmacological profiles of chemicals and pharmaceuticals. For a comprehensive review of applications of cell culture chips, see (15). Culturing cells in microfluidic devices gives a range of new possibilities to study cells that are difficult to study using standard cell culture techniques:

1. Single cells can be captured and studied individually (2–4,16,17). One commercial solution is shown in **Fig. 2c** in which in the bottom of the cell culture chamber an array of small wells is micromachined. Each well can hold one cell only upon seeding and the wall makes it difficult for cells to migrate or be disturbed during staining procedures. It is therefore possible to study cell behavior in single cell level.
2. Cells can be studied in real time (see also **Fig. 3d, e**) (18, 19). Real-time monitoring of cells can be performed by placing the cell culture flask under a microscope. However, only short time observation can be done because heat, humidity, and CO_2 are not usually provided. The microscope can be modified with a hood that functions as an incubator. Cells can be cultured in microtiter plates or flasks in such modified systems for days or until the medium has to be changed. There is a risk that the position of the microtiter plate or flask is disturbed

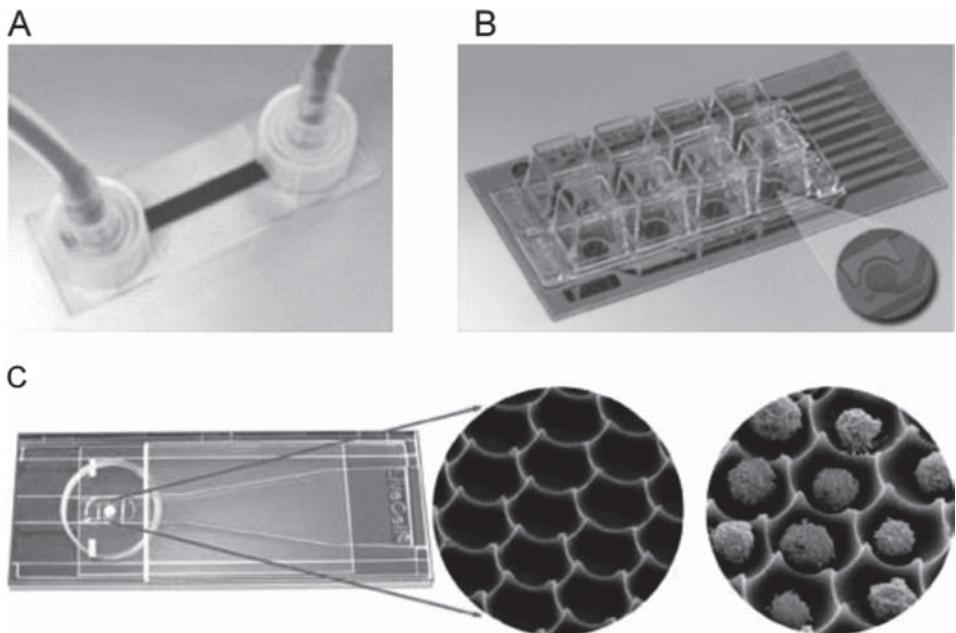


Fig. 2. Commercial cell culture chips. (a) flowthrough chip from Ibidi (www.ibidi.de). (b) Microwell with integrated electrodes for impedance measurements (www.ibidi.de). (c) LiveCell Array™ from NUNC where the bottom of the microscope slides is microstructured into small wells that accept only one cell. The chip enables single cell analysis.

during medium change, and it can therefore be very difficult to study long-term effects of cells at a single cell level without flow through cell culture chips. Perfusion chips (Fig. 2a) can be employed instead, if longer incubation is needed to capture a response in real time. The chip displayed in Fig. 2a needs, however, a microscope with environmental control to sustain long-term growth. Microchips can, however, be modified with a transparent resistive film that can provide heat (see Fig. 3 for an example of a chip). Because microchips as those described in Fig. 3 are often continuously perfused with fresh medium, there is no need for controlling the CO₂ in the environment. Thus, such microchips can be used in microscopes lacking an environmental hood. Microchips for cell culturing can furthermore be constructed to have better optical properties compared with microtiter plates and flasks.

3. Fluidics controlled flow allows the performance of co-cultures. Traditionally, untreated cells (control) and treated cells (test) are contained in different flasks. Because the cultures are not 100% similar between controls and test cultures, response to a test may be difficult to find or is overexaggerated. The need for different cultures for the control and the treated cells can be circumvented using microfluidics where different parts of the same culture (bed of cells) can be treated with different solutions (17).

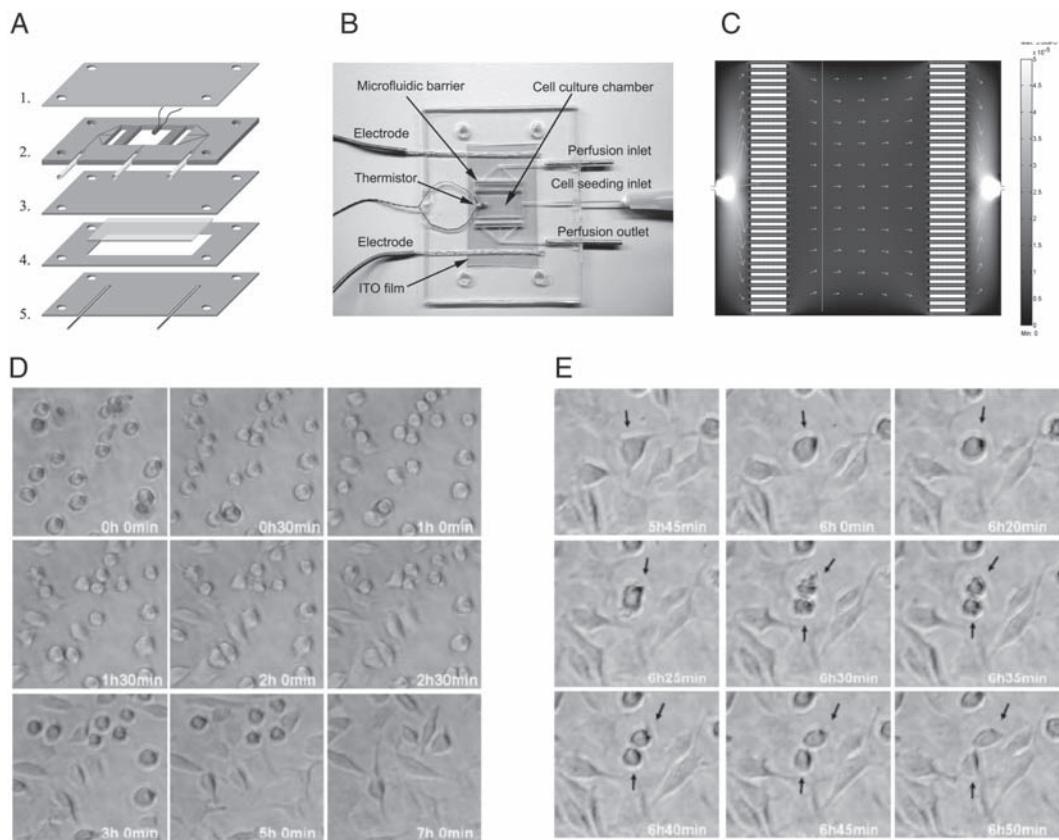


Fig. 3 Polymeric cell culture chip and filming of cells. **(a)** Parts and assembly of a polymeric cell culture chip shown in **(b)**. **(c)** Simulation of nutrient distribution within the chip. Note that the distribution is quite even in the chip despite the squared form and large dimension of the chamber of 1 cm^2 . Pictures **(a–c)** were reproduced from (19) **(d)**. Pictures of HeLa cells taken at different time intervals after plating within the cell culture chamber. **(e)** Time-laps study of cell division.

4. Complex temporal and spatial concentration gradients can be obtained using microfluidics (20) allowing experiments on cells that are impossible to perform using standard methods. Simple gradients such as a gradient from a high to low concentration can also be made using macroscale technologies or simple microfluidics using for instance the chips from Ibidi (**Fig. 2a**). In the simple chips by Ibidi, the gradient is created by diffusion of substances from one inlet to the other inlet. By coupling a microfluidics network to a cell culture chip it is possible to create stable continuous chemical gradients over the bed of cell during perfusion of the cell chamber (18). Such solutions allow long-time cultures of cells in a gradient of factors. More complicated gradients can furthermore be generated using microfluidics network. Li Jeon et al. for instance demonstrated that using microfluidics, a *periodic* soluble gradient of IL-8 over an area could be achieved. The

periodic gradient was used to study the migration pattern of neutrophiles in real time (9). Gradients of factors can also be immobilized onto the surface of the cell culture device prior to seeding of cells. An immobilized gradient of laminin could for instance be used to study axonal outgrowth of neurons, and it was demonstrated that axonal growth was directed toward the higher laminin densities on the surface (21). Another impressive example of benefits of microfluidics controlled flows was demonstrated by Lucchetta et al. who used microfluidics to perfuse a single *Drosophila* embryo with cold and warm medium and studied patterning of molecular markers such as Even-skipped on the embryo by time-laps microscopy, an experiment that is very difficult to perform with standard cell culture techniques. The result showed that patterning of Even-skipped could be disturbed by exposing the two different parts of the embryo to two different temperatures (22,23). We can expect much more complicated experiments in the future that employ microfluidic controlled flows.

5. Microfabrication can provide microscale scaffold to promote cell growth and resemble tissue (24–28). Such scaffolds are yet to be demonstrated in microtiter plates of cell culture flasks.
6. Shear stress studies can be performed because flow in shallow channels will reproducibly create shear stress.
7. Integration of other detection systems is possible using micromachined cell culture chips. A commercial example is integration of small electrodes in the bottom of a microwell (**Fig. 2b**). The electrodes can be used to study the impedance in the cell culture area. Impedance can be used to study cell growth and changes in adhesion of the cells to the surface. Impedance measurements are typically done in real time and there are no requirements that the cells are labeled. We can expect many other different sensors and actuators to be integrated into micromachined chips.

With these examples it is clear that the future cell culture chip will not only provide the basic functions for cell maintenance as microtiter wells do but also provide arrays of different detectors for studying cell responses to stimuli that are provided by combinatorial microfluidics and other means.

So why is not everyone using cell culture on-chip and all the benefits that are associated with them? First, the availability of cell culture chips is limited. The commercial cell culture chips are relatively simple and if other designs and function are required, the cell chips must be fabricated in house. Moreover, many cell culture chips are made using clean room facilities (the same laboratories as for making CPUs from, e.g., Intel), which is a very expensive technology. Soft lithography of PDMS (29) and microfabrication of PMMA [poly(methacrylate)]

(30) are inexpensive alternatives to clean room fabricated devices but these technologies are seldom implemented in biomedical laboratories. Thus, the chips are limited to very few who have access to clean room facilities, microfabrication technologies, and knowledge of microfluidics. Cell culture chips are surprisingly easy to fabricate even for biomedical researchers. As mentioned earlier soft lithography and microfabrication of polymers are inexpensive and can be performed in a biomedical laboratory. Investments in machinery cost in the range of \$30,000 for a laser to burn structures into PMMA, while spinners used for soft lithography cost much less. Pumps can be bought commercially and the simple electronics needed for on-chip heat control can be reproduced by an electronics workshop. Using relatively low technology fabrication solutions (compared to clean room facilities), it is possible to build chips for on-line monitoring of cells [Fig. 3; see also references (19,31)].

The other reason that may explain the lack of use is that the chips are not validated extensively. Usually, only a few biocompatibility parameters are tested such as adhesion, growth, and viability. It is thus not clear at present if the cell culture conditions provided on the chips are identical to the conditions that are desired, such as the condition in a cell culture flask. One dissimilarity between microchips and cell culture flasks is that microchips usually utilize perfusion to feed the cells (16,19,24,32–34). In perfusion systems, the medium is always replenished, which suggests that cell growth would be better than in a standard cell culture flask. However, this is not necessarily the case, because growth factors excreted from the cells will also be washed away from the cells in perfusion systems. In some cases this can be circumvented with perfusion systems where the medium exchange is periodic. This means that the flow is stopped at time intervals to allow accumulation of growth factors. Apart from medium perfusion, cell culture chips usually are quite shallow where the height of the chamber can be as low as about 50 μm . These shallow cell culture chambers induce shear stress on the cells upon perfusion. Furthermore, oxygen or other growth factors in the medium are depleted rapidly despite the continuous flow because the volume is very small in these chips. In fact a 1- cm^2 chamber that is 50- μm high has a volume of about 5 μL . The solution is to use chambers that are higher, and it has been shown that the growth rate of the cells can be twofold higher in chambers that are 2-mm high instead of 250 μm (35). At 2-mm height of the chambers, the cell growth rate is similar to the growth rate observed in cell culture flasks. A drawback with cell microchips is that the chip is fabricated in materials other than polystyrene that often is used in microtiter plates and cell culture flasks. Materials that are used are, for instance, glass, PDMS, and acrylic glass. These new surfaces have unknown effects on the cell during culture. One way to

rapidly determine effects on cells is to use gene expression profiling of cells grown on different surfaces. In a recent study, it was demonstrated that cells grown on PMMA and on modified SU8 closely resembles the gene expression of cells in cell culture flask (36).

A fully validated microchip for cell culture is shown in [Fig. 3](#). The chip is made of PMMA, which by itself does not change gene expression in HeLa cells (36). It is, however, possible that just by culturing the cells in a chamber can induce changes in the cells. However, using gene expression profiling, it was shown that cells grown in that chamber showed similar expression pattern of more than 44,000 genes as cells grown in cell culture flasks (31). Thus, the described cell culture chip can be viewed as a cell culture flask with the exception that the chip is perfused and can sustain cell growth for a week in a microscope that is not environmentally controlled.

4. Tissue Microarrays

Tissue microarray is a development from histological methods where, traditionally, one sample is immobilized per slide. However, the throughput of traditional method is fairly low and a way to increase throughput is to create tissue arrays consisting of up to hundreds of small samples of different tissues immobilized on a microscope slide. Such slides are tremendously powerful for analysis of expression pattern in tumors because relatively few slides can hold information from thousands of patients. In 1 day a cohort of patients could be investigated for expression of a particular marker. In comparison, using one slide per sample would require thousands of incubations, one for each patient investigated, which is labor intensive and expensive in terms of antibodies. In contrast to DNA or protein microarray, a spot in a tissue array consists of many different cells organized into a tissue. Thus, analysis of the tissue array is not simply looking on a fluorescent signal as with DNA and protein microarray. Instead, a morphological evaluation of each spot is needed, which requires that each spot is investigated manually.

5. Conclusions

It is clear that miniaturized cell analysis systems such as cell culture systems and tissue arrays will change cell and molecular biology. As opposed to cell culture chips, tissue arrays are well established

and easily adapted in existing laboratories and research. The reason is that tissue arrays are simply a higher throughput format of existing methods, while cell culture chips require heavy investment in instruments and knowledge to perform cell culture experiments in a new way.

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Chapter 10

Bio-Cell Chip Fabrication and Applications

Honggu Chun, Dong Soon Lee, and Hee Chan Kim

Summary

Bio-cell chips are microarrays, which are composed of collections of cell spots attached to the surface. They hold intact cells and therefore enable the study of gene–gene interactions and gene–protein interactions in a cell with three-dimensional positional information. The authors developed a 16 × 6 array bio-cell chip comprising a 1-mm-thick perforated polydimethylsiloxane (PDMS) layer on lattice-patterned 25 mm × 75 mm glass slide. The perforations in the PDMS layer formed cylindrical wells of volume ~1.7 µL, which were used to seed cells. The authors constructed bio-cell chips using mononuclear cells from bone marrow specimens and subjected them to fluorescent *in situ* hybridization (FISH). Bio-cell chip technology is compatible with standard clinical diagnosis protocols, requires smaller samples, provides results quickly, and is highly cost-effective. In addition, bio-cell chips can be used as a platform for distributing real samples for research purposes. These features make it a potential tool for basic research and for clinical diagnosis.

Key words: Bio-cell chip, Cell chip, Bio chip, Cell array, FISH

1. Introduction

DNA, RNA, and protein microarray technologies have greatly aided functional genomic studies (1, 2). A large number of genes or proteins can be arrayed on a small chip and assayed simultaneously, and therefore, these technologies allow efficient profiling of gene expressions and protein activities. However, while extracting DNA, RNA, and protein samples from cells, three-dimensional positional information is lost, and thus, these technologies provide an incomplete picture of gene–gene and gene–protein interactions. For this reason, new analytic technologies aimed at determining the levels, activities, regulations, and interactions of genes and proteins in intact cells have been

sought in an attempt to further our understanding of the molecular basis of functional genomics. Ziauddin and Sabatini developed a microarray for cell culture expressing defined cDNA (3), and Xu developed arrayed nanocraters for cell culture (4).

One of the major problems encountered during research involving cell specimens is availability. Cell specimens obtainable from single patients are limited and cannot be amplified easily. Furthermore, the friability of cells makes them difficult to store and distribute.

As a solution to these problems, the authors developed a bio-cell chip based on microarray technology (5). Cell specimens obtained from patients were treated to preserve cell morphologies, proteins, and genes, and then controlled minuscule numbers of cells were arrayed and immobilized on lattice-patterned glass slides. Each lattice element was indexed to allow visual identification.

The devised bio-cell chips allow a large number of different cell specimens to be assayed simultaneously under identical condition, and therefore, they facilitate quantitative analysis and comparisons of signal levels among samples. Also, these chips reduce reagent costs and assay times. Moreover, the arrayed configuration and the indexing system enable automated data analysis with image processing. Therefore, bio-cell chips facilitate mass screening and considerably reduce the costs and times involved, and they are applicable to blood, urine, sputum, and mucus. Furthermore, the devised bio-cell chips will allow researchers to easily store and distribute cells.

One major challenge presented by conventional microarray technology concerns the immobilization of many types of samples without cross contamination. Since the dimensions of cells ($\sim\mu\text{m}$) are threefold larger than those of proteins or genes ($\sim\text{nm}$), the sizes of arrayed spots, including cell specimens and fixing agent, are much larger than those of DNA or protein microarrays, which limits the total number of spots that can be located on a bio-cell chip. To solve these problems, we used a perforated PDMS layer to form separate cylindrical wells for cell seeding. We fabricated a bio-cell chip and seeded it with mononuclear cells collected from a bone marrow specimen, and then subjected it to a high-throughput FISH study to investigate the role of the *p16* gene in leukemia (5). Mononuclear cell specimens stored in Carnoy solution were dispensed into separated PDMS wells and air-dried at room temperature. The PDMS layer was peeled off after samples had completely dried, and FISH was performed.

Tissue arrays share the advantages of bio-cell chips, but the fabrication procedure, which involves formalin fixation and paraffin embedding, damages certain antigens (6,7), whereas during the course of this study we did not observe any noticeable damage or modification to cell morphology, genes, or proteins on the devised bio-cell chip.

Herein, we present an introduction to the fabrication of the bio-cell chip and describe its use for FISH studies.

2. Materials

2.1. Lattice and Index Patterning on Glass

Slide

1. Glass slide: Corning 2947 (75 mm × 25 mm × 1 mm) (Corning, NY).
2. Surface cleaning solution: piranha solution [H_2SO_4 (J.T. Baker, Phillipsburg, NJ): H_2O_2 (30%, J.T. Baker) = 3:1].
3. Cleaning solution: acetone (CMOS grade, J.T. Baker), methanol (CMOS grade, J.T. Baker), and deionized (DI) water (NANOpure Diamond, Barnstead, Dubuque, IA).
4. Adhesion layer between glass slide and the photoresist: hexamethyldisilazane (HMDS, Clariant, Switzerland).
5. Photoresist (PR): AZ5214-E (Clariant). Store it in a dark place at 10°C.
6. Spincoater (Won corporation, Korea).
7. Hotplate (PMC Dataplate 732, Barnstead).
8. UV exposure & Aligner (MDE-4000, Midas, Korea).
9. Mask for lattice and indices: custom-made Cr mask on quartz plate.
10. Developer: AZ300MIF (Clariant).
11. Sputter (ATST-SPT-0403C, Atechsystem, Korea) with titanium (Ti) and platinum (Pt) target.

2.2. Perforated PDMS layer

1. Glass slide: micro glass slide (75 mm × 50 mm × 1.3 mm, Matsumani, Japan).
2. Surface cleaning solution: same as in item 2 in **Subheading 2.1**.
3. Cleaning solution: same as in item 3 in **Subheading 2.1**.
4. Norland UV adhesive 68 (Norland, Cranbury, NJ). Store it in dark place.
5. Mask for master mold: custom-made Cr mask on quartz plate.
6. UV exposure & aligner: same as in item 8 in **Subheading 2.1**.
7. PDMS (Sylgard 184, Dow Corning, Midland, MI).
8. Oven (OV47350, Thermolyne, Barnstead).

2.3. Cell Fixation

1. Prepare leukocyte separating solution: 5 mL of dextran (T70, MW = 70,000 Da) (Sigma, St. Louis, MO) at 10% (w/v in normal saline).
2. Prepare RBC hemolysis solution: 0.84% (w/v in distilled water) ammonium chloride (NH_4Cl) (Sigma) at 4°C.
3. Leukocyte counting chamber: Neubauer improved bright-line chamber (Marienfeld, Germany).
4. Prepare fixative solution [methanol (Sigma):glacial acetic acid (Fisher Scientific, Fair Lawn, NJ) = 3:1]. These were mixed just before use.

2.4. Fluorescent In-Situ Hybridization (FISH)

1. Prepare 2× SSC solution ($\text{pH } 7.0 \pm 0.2$) containing 300 mM sodium chloride and 30 mM sodium citrate (Vysis, Downers Grove, IL).
2. Wash solution: 2× SSC plus 0.1% (w/v) NP-40 ($\text{pH } 7.0 \pm 0.2$; Vysis).
3. 70, 85, and 100% ethanol at room temperature. Make 70 and 85% ethanol by diluting 100% ethanol with distilled water just before use.
4. Denaturating solution: 70% formamide in 2× SSC at 75°C (Invitrogen).
5. 70, 85, and 100% ethanol at -20°C.
6. Probe for FISH: LSI p16/CEP 9 dual-color probe (Vysis), which is a mixture of LSI p16 probe labeled with Spectrum Orange and CEP 9 probe labeled with Spectrum Green.
7. Probe mixture: hybridization buffer 7 μL + distilled water 2 μL + probe 1 μL .
8. Cover glass: Corning 2940-245 (24 mm × 50 mm × 0.15 mm) (Corning).
9. Rubber cement: Fixogum (MP Biomedicals, Irvine, CA).
10. Prepare 50% formamide/2× SSC solution.
11. Counterstain: DAPI (4'-6'-diamine-2-phenylindole dihydrochloride) II (125 ng/mL in antifade mounting solution) (Vysis).
12. Olympus fluorescence microscope (Olympus America, Melville, NY) attached to a computer-based imaging system (Quips XL Genetics Workstation; Vysis) equipped with a triple-bandpass filter for DAPI, FITC, and Texas Red.

3. Methods

Fabrication procedure of the bio-cell chip comprises three parts: patterning the glass slide, making a perforated PDMS layer, and immobilizing cell specimens. A protocol for applying FISH to fabricated bio-cell chips is described.

3.1. Lattice and Index Patterning on Glass slide

Glass slides were patterned with a lattice and indices using photolithography and thin-film metal deposition techniques (**Fig. 1**). The lattice pattern separates cell specimens visually and the index identifies each sample location. These features facilitate automatic image processing and manual microscopic examinations.

1. Clean Corning 2947 glass slide in piranha solution for 1 h (*see Note 1*).
2. Wash the glass slide with DI water, and then clean twice with acetone, methanol, and DI water, respectively.
3. Dry the glass slide with N₂.
4. Dehydrate the cleaned glass slide on a 150°C hotplate for 10 min, and then cool down to room temperature for 2 min (*see Note 2*).
5. Place the glass slide on a spin coater. Pour HMDS over the entire glass slide. Increase the spin speed from 0 to 500 rpm over 7.5 s, and then increase to 4,000 rpm immediately and maintain for 30 s.
6. Pour photoresist AZ5214-E over the entire HMDS-coated glass slide. Increase spin speed from 0 to 500 rpm over 7.5 s, and then increase to 4,000 rpm immediately and maintain for 30 s.
7. Perform soft baking of the PR on a hot plate at 100°C for 1 min. Then cool down to room temperature for 2 min.
8. Place a patterned mask in a UV exposure system, align the glass slide under the mask, and expose to UV (365 nm) at 16 mW/cm² for 4.5 s.
9. Perform image reversal baking on a 122°C hotplate for 2 min, and then cool down to room temperature for 2 min (*see Note 3*).
10. Place the glass slide in a UV exposure system without a mask. Expose to UV (365 nm) at 16 mW/cm² for 18 s over the entire glass slide area (*see Note 4*).
11. Develop the PR with AZ300MIF for 45 s and then wash the glass slide with DI water (*see Note 5*).
12. Perform hard-baking of the PR on a hot plate at 105°C for 1 min.

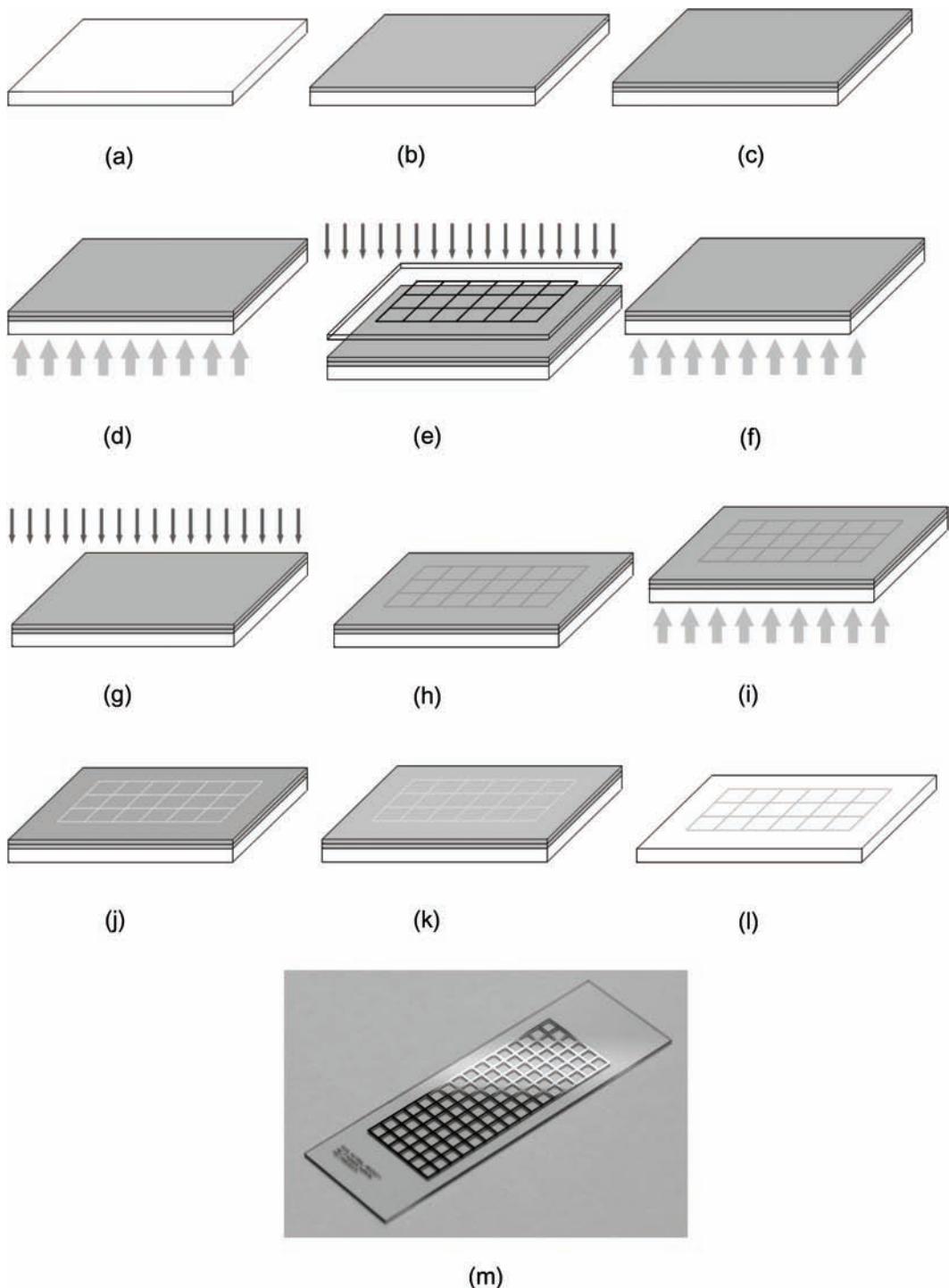


Fig. 1. Lattice and index patterning of glass slide. a: Glass cleaning. b: Spin coating with HMDS. c: Spin coating with photo resist AZ5214-E. d: Soft baking. e: UV exposure with a patterned mask in position. f: Image reversal baking. g: Flood exposure of UV light without a mask. h: Developing. i: Hard baking. j: Ti sputtering. k: Pt sputtering. l: PR Lift-off. m: Fabricated glass slide with indices and lattice pattern.

13. Plate the glass slide with Ti at 20-nm thickness, and then plate with Pt at 100 nm using a sputter system (*see Note 6*).
14. Lift-off PR residue with acetone, and then clean twice with acetone, methanol, and DI water, respectively (*see Note 7*).

3.2. Perforated PDMS Layer

The perforated PDMS layer was used to form separate cylindrical wells that allow cell seeding without cross contamination. A mold for the perforated PDMS layer was fabricated by photolithography (**Fig. 2**).

1. Clean micro glass slide (Matsumani) in piranha solution for 1 h (*see Note 1*).
2. Wash the glass slide with DI water, and then clean twice with acetone, methanol, and DI water, respectively.
3. Dry the glass slide with N₂.
4. Dehydrate the cleaned glass slide on a 150°C hotplate for 10 min and then cool to room temperature for 2 min.
5. Pour UV optical adhesive NOA 68 over the entire glass slide to a thickness of 1 mm (*see Note 8*).
6. Place a patterned mask in the UV exposure system, and align the glass slide under this mask. Expose with UV (365 nm) at 16 mW/cm² for 150 s to harden the UV optical adhesive (*see Note 9*).
7. Wash out unset residual adhesive with acetone (*see Note 10*).
8. Bake the glass slide in an oven at 120°C for 1 h, and then cool to room temperature for 5 min. The fabricated structure is then used as a master mold for the perforated PDMS layer (*see Note 11*).
9. Mix PDMS base liquid with curing agent at a ratio of 10:1 by weight. After thoroughly mixing the liquids, the mixture was degassed in a vacuum chamber.
10. Pour the PDMS mixture onto the master mold. Place the mold in a vacuum chamber and degas (*see Note 12*).
11. Place the mold in an oven and cure the PDMS mixture at 60°C for 1 h.
12. After 1 h, peel the PDMS replica off from the mold and attach it to the lattice-patterned glass slide (*see Note 13*).

3.3. Cell Fixation

3.3.1. Leukocyte Separation

Mononuclear cells obtained from bone marrow specimens were arrayed and immobilized on a bio-cell chip (**Fig. 3**).

1. Add 5 mL of dextran (T70; MW = 70,000 Da) at a concentration of 10% (w/v in normal saline) to 10 mL of EDTA-treated whole blood sample. Incubate it at 37°C for 60 min.

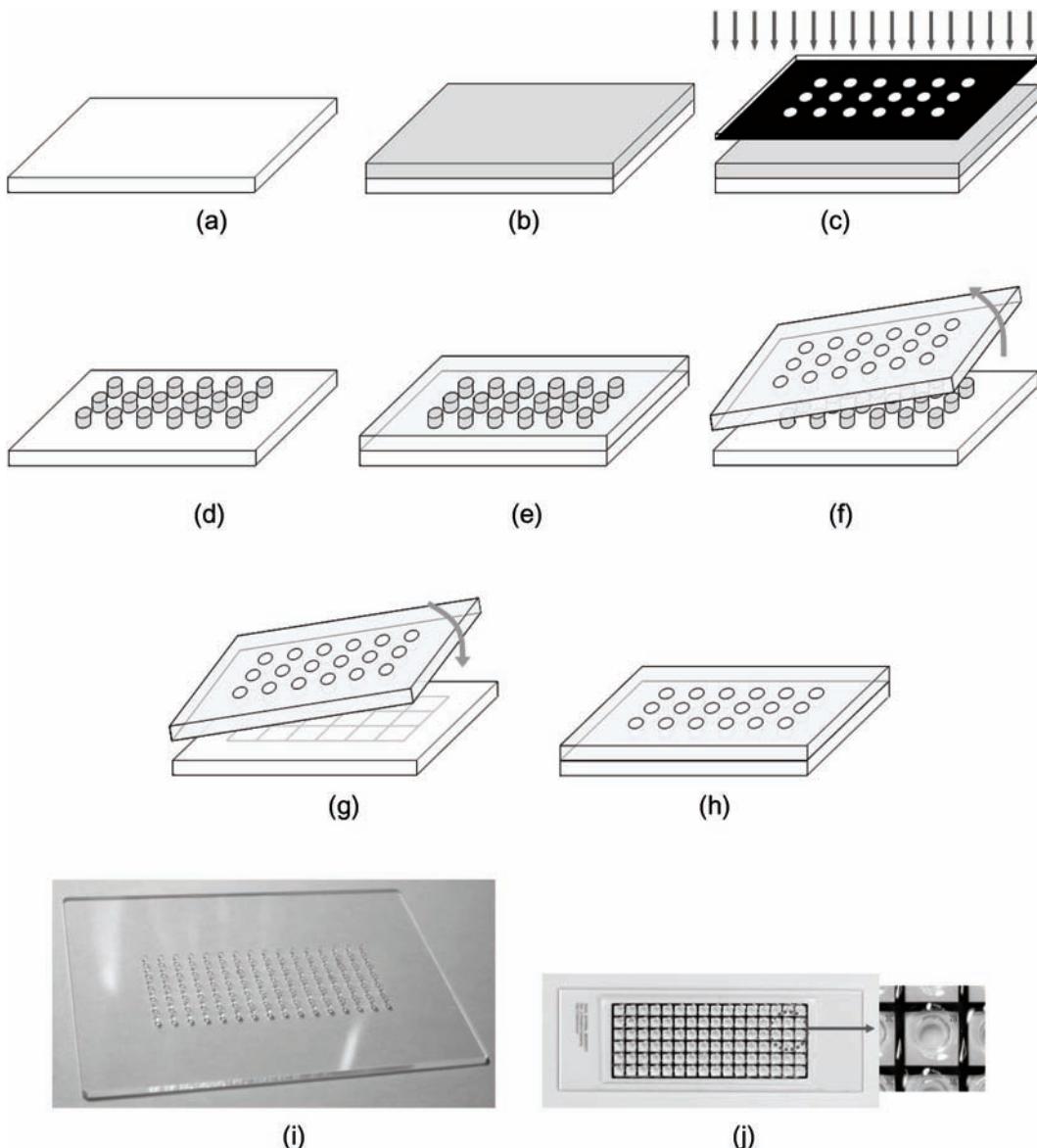


Fig. 2. Fabricating the perforated PDMS layer and combining it with a lattice patterned glass slide. a: Glass cleaning. b: Pouring UV adhesive NOA 68. c: UV light exposure through a patterned mask. d: Washing with acetone. e: Pouring the PDMS mixture over the master mold and curing. f: Peeling off the perforated PDMS layer. g: Aligning and attaching the perforated PDMS layer onto the lattice patterned glass slide. h: Complete bio-cell chip. i: Fabricated master mold for the perforated PDMS layer. 96 circular posts (6×16) of 1 mm in height and 1.5 mm in diameter were placed on a glass slide at a distance of 3 mm from each other. j: Fabricated bio-cell chip. Enlarged picture showing a single cylindrical well aligned within a lattice. The cell's index is shown in the upper-right hand corner of the lattice.

2. Collect the upper layer containing the mixed leukocyte fraction, and transfer it to a centrifuge tube.
3. Centrifuge the tube at $800 \times g$ for 25 min at room temperature.

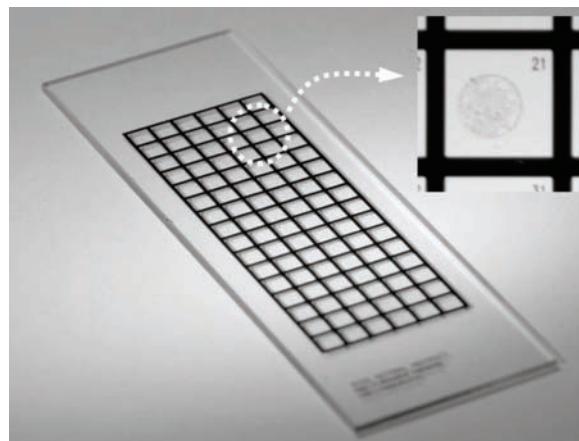


Fig. 3. Bio-cell chip mounted with mononuclear cells. The enlarged picture shows a single array element and its index.

4. Collect the separated leukocyte bands with a pipette, aseptically. Transfer them to a sterile centrifuge tube.
5. Wash and centrifuge the cells three times at $1,500 \times g$ for 10 min in 0.84% (w/v) ammonium chloride solution at 4°C to hemolyze cells.
6. Determine the concentration of the leukocyte suspension using a Neubauer improved bright-line chamber.

3.3.2. Cell Fixation

1. Add and mix 1 mL of fixative to the leukocyte suspension, leave at room temperature for 5 min, and centrifuge at 1,000 rpm for 8 min.
2. Pour off the supernatant and repeat **step 3.3.2.1** until the supernatant is clear.
3. Swab the inner wall of a conical tube containing the pellet and dilute it with a fixative solution to a concentration of 1,000 cells/ μL .
4. Dispense 1 μL of mononuclear cell samples into each PDMS well.
5. Dry the cell samples in air at room temperature.
6. After the cell samples have been completely dried, peel off the PDMS perforated layer.
7. Store the bio-cell chip in a refrigerator at -20°C .

3.4. Fluorescent In-Situ Hybridization

To demonstrate the use of the devised bio-cell chip for FISH studies (**Fig. 4**), we describe a FISH protocol for the *p16* gene.

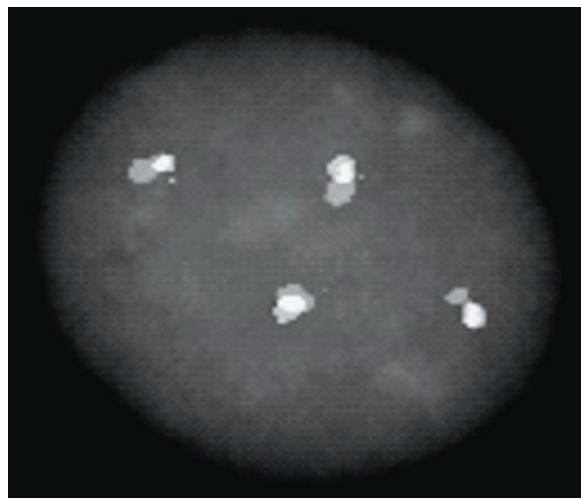


Fig. 4. Sample image of an MLL FISH test. This interphase nucleus shows four copies of the *MLL* gene. The centromeric and telomeric probes for MLL produce an orange and a green signal, respectively. Background chromatin is colored blue. A normal interphase cell shows four co-localized (fusion, F) signals denoting two normal 11 chromosomes (2F). If an MLL translocation is present in the interphase cell, separated orange (O) and green (G) signals from translocated chromosome 11 are observed along with a fusion signal from normal chromosome 11 (1F1O1G).

3.4.1 Slide Pretreatment

1. Dip a prepared bio-cell chip in 2× SSC/0.1% NP-40 solution and maintain in a 37°C water bath for 30 min. This step prevents chromosome expansion, and therefore provides good stain quality.
2. Dehydrate the sample by dipping the bio-cell chip in a graded ethanol series (70, 85, and 100%) for 1 min/stage at room temperature.
3. Dry the sample in air.

3.4.2. Slide Denaturation

1. Dip the bio-cell chip in a denaturating solution (70% formamide/2× SSC 50 mL) at 72°C for 5 min without agitation.
2. Dehydrate the sample by dipping the chip in a graded ethanol series (70, 85, and 100%) for 1 min/stage at -20°C (*see Note 14*).
3. Dry the sample with air.

3.4.3. Probe Denaturation

1. Fill a microcentrifuge tube with 10 µL of the probe mixture and wrap the tube with aluminum foil. Spin down for 2–3 min and denature the probe at 75°C in a water bath for 5 min.
2. Leave the tube in an ice box for 5 min (this prevents the probe from hybridizing to form dsDNA).

3.4.4. Hybridization

1. Drop the probe mixture on the bio-cell chip and cover it with a Corning 2940-245 cover glass. Then seal the edges of the cover glass with rubber cement.
2. Hybridize the sample with probe mixture in a 37°C humidified chamber for 18–20 h.

3.4.5. Posthybridization Wash (Conducted in a Dark Room)

1. Remove the cover glass.
2. Wash the bio-cell chip with 46°C 50% formamide/2× SSC solution twice for 10 min. Wash the bio-cell chip with 46°C 2× SSC for 10 min and then with 46°C 0.1% NP-40/2× SSC for 5 min.

3.4.6. Counterstain

After washing, counterstain chromosomes with DAPI. Drop 10 μL of DAPI II onto the bio-cell chip, and then cover the chip with a Corning 2940-245 cover glass. This counterstain procedure is required to prevent cell specimens from drying.

3.4.7. Image Analysis

Two-hundred interphase cell nuclei were analyzed per sample for reliability. Myeloid/lymphoid leukemia (MLL) FISH images were interpreted as follows. The hybridization of MLL probes with DNA in normal interphase cells results in superimposed orange and green signals, which yield two fusion signals from two 11 chromosomes. However, if an *MLL* gene translocation is present in the interphase cell, one fusion signal splits to yield separate orange and green signals (**Fig. 4**).

4. Notes

1. Safety Precautions: Piranha solution is a powerful oxidizer and reacts violently with organic materials or solvents, and thus, should be handled with extreme care.
2. All procedures described must be performed in clean room at 25°C and 30% RH. If the RH is higher than 30%, then **step 3.1.5** should be conducted immediately after **step 3.1.4**.
3. Steps **3.1.8–3.1.11** describe the image reversal technique using AZ5214-E (see datasheet for more information). AZ-214-E is a positive photoresist (the unmasked area is developed) with a positive 75–85° slope. However, it is usually used in image reversal mode to obtain a negative pattern of a mask with a negative profile. This negative profile is proper for the lift-off technique in thin-film metal deposition (**Fig. 5**). **Step 9** in **Subheading 3.1** activates the crosslinking agent of the photoresist only in area unmasked during **step**

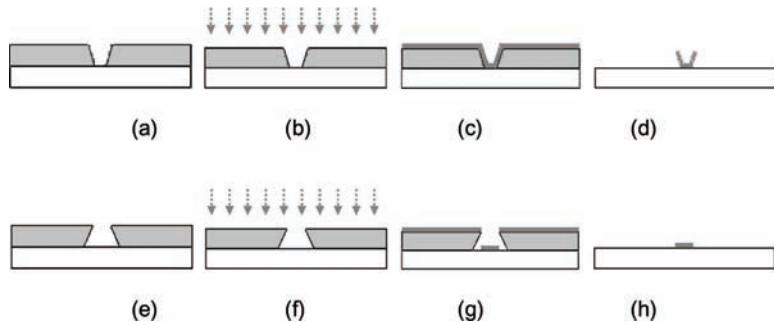


Fig. 5. Plating metal on a surface using the lift-off technique. a: PR is coated on the surface with a positive profile. b: Metal is sputtered, and c: after sputtering, all metal layers are connected. d: Lift off the PR layer with acetone. The edges of the pattern are not well defined. e: PR is coated on the surface with a negative profile. f: Metal is sputtered, and g: is disconnected at pattern edges. h: Lift off the PR layer with acetone. The edges of the pattern are then well defined.

8 in **Subheading 3.1**. The crosslinking process makes the photoresist insensitive to UV light exposure and insoluble in developer, which results in a negative profile. The image reversal baking temperature is the most critical of the photolithography procedures. Optimal temperature may differ from the value given, as it is dependent on room temperature and humidity. However, once optimized, it should be controlled within $\pm 1^{\circ}\text{C}$.

4. Flood exposure makes the area not exposed during **step 3.1.8** soluble in the developer, which results in a negative profile.
5. Developing times can vary with room temperature. To determine an optimal time, a light-interference pattern in the developing area can be used. As developing continues, the thickness of the photoresist is reduced. Therefore, if the developing area is observed obliquely, light-interference patterns change during development. When development has been completed, the light-interference pattern disappears, and the time of this disappearance represents the optimal development time.
6. Parameters for plating Ti and Pt differ for every sputter. Please refer to the manufacturer's instructions.
7. If the photoresist is successfully patterned with image reversal, lift-off takes place immediately with a sharp pattern. If not, repeat the previous steps using different parameters as follows. If the masked pattern is erased during this procedure, development was incomplete and the development time should be increased. If the masked pattern is still erased

after increasing the development time, the image reversal baking temperature was too high. If metal has sputtered into the unmasked region, the image reversal baking temperature was too low or the exposure time at **step 8** in **Subheading 3.1** was either too long or too short. If the unmasked pattern is erased and masked pattern is not clear then repeat the process using a different exposure time in **step 8** in **Subheading 3.1**.

8. A jig can be used to get a well-defined thickness of the UV optical adhesive (**Fig. 6a, b**).
9. To obtain a sharp profile, the mask should be placed as close as possible to the UV optical adhesive. Polyvinyl chloride thin film can be placed between the mask and the UV optical adhesive to prevent the mask sticking. However, ensure that no air bubbles are present between the polyvinyl chloride film and the mask and UV optical adhesive (**Fig. 6c**).
10. The unmasked area in **step 6** in **Subheading 3.2** is hardened by UV exposure, but it is not completely hardened. Therefore, if a mold is washed with acetone for a prolonged period, the hardened UV optical adhesive in unmasked areas is dissolved. Thus, wash molds with acetone for just long enough to remove unhardened UV optical adhesive.
11. Slow ramped increases and decreases in temperature are preferred to minimize stress between glass slide and UV optical adhesive. This improves adhesion.
12. Be sure that the PDMS mixture is not spread over posts.
13. The PDMS layer is flexible and elastic. Therefore, it can be difficult to align every well of PDMS layer within the lattice pattern. If this is the case try to align it in the reverse manner, and place the PDMS layer on a clean surface, align the lattice patterned glass slide over it, and then attach them.
14. This dehydration step should be performed at low temperature (normally -20°C) to prevent ssDNA from hybridizing to form dsDNA.

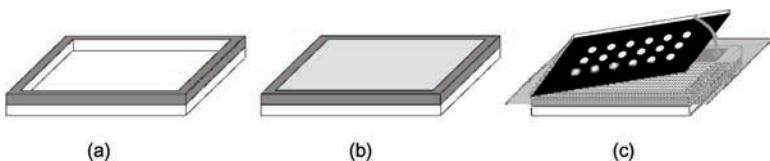


Fig. 6. A jig and thin polyvinyl chloride film can be used to produce more uniform thickness of master molds with a sharper profile. **a:** A 1-mm thick Teflon jig is placed over the glass slide. **b:** UV optical adhesive is poured in the jig, and **c:** thin polyvinyl chloride film is placed over the UV optical adhesive. The mask is then placed over the film.

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Chapter 11

Microchip Capillary Electrophoresis

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Summary

Microchip capillary electrophoresis (MCE) is gaining popularity due to the developments of simple microfabrication methods under nonstringent laboratory conditions. Moreover, the low material and production costs of polymer-based microchips have further stimulated advances in the applications of MCE in various fields, including clinical analysis, drug screening, biomarker identification, and biosensing. In this chapter, a simple and robust protocol for fabrication of microchips for lab-on-chip testing and microchip electrophoresis is described. The microchips are hybrid poly(dimethylsiloxane) (PDMS)/glass microchips, which are produced by a combination of photolithography and micromolding processes. This type of microchip has been used in a wide range of analyses.

Key words: Microchip capillary electrophoresis, Photolithography, PDMS, Replication, Microchip conditioning

1. Introduction

Since the microchip concept was developed by Manz and coworkers in the early 1990s, it had drawn considerable attention from researchers worldwide. This is witnessed by the growing number of applications for microchips in many areas of analytical science (e.g., sample preparation, detection, separation modes) (1, 2) as well as in clinical analysis (e.g., polymerase chain reaction, immunoassay, clinical point-of-care testing, clinical diagnosis, cell handling, and analysis) (3–6). The utility of microchip CE has led to the development of commercial systems such as the Agilent 2100 Bioanalyzer and Bio-Rad Laboratories Experion (7, 8) for the analysis and quantification of DNA, RNA, and proteins. These systems employ dedicated, commercially available glass-based

microchips, achieving high separation efficiency and rapid analysis time. However, these systems are not suitable for general purpose microchip capillary electrophoresis (MCE) applications due to their relatively high cost, limited types of microchip available, and difficulty in varying the microchannel design/structure.

In recent years, polymer-based microchips have gained in popularity due to several inherent advantages. Fabrication requires relatively simple infrastructure and technologies (eliminating the need for sophisticated clean room facilities and equipment). In addition, one can modify the design of the microchannel structures on these polymeric substrates with ease, and it normally costs relatively little to obtain a sizable quantity through the use of various microfabrication techniques such as injection molding (9), hot embossing (10), and imprinting (11). Several polymers, including poly(dimethylsiloxane) (PDMS), poly(methyl methacrylate) (PMMA), polycarbonate (PC), polyethylene terephthalate (PET), and polyester have all been used in various MCE applications (12). Among them, PDMS is the most commonly used polymer due to the well-established microfabrication techniques developed for this material (13, 14). Moreover, the high flexibility of this elastomeric substrate allows incorporation of other microstructural designs, such as mechanical valves for flow control. With good optical transparency and gas permeability, PDMS has been shown to have a wide range of applications and capabilities.

However, it should be noted that although microfabrication of PDMS is relatively easy, separation performance of PDMS microchips is often lower than that of glass microchips. An alternative approach based on the combination of PDMS with glass to make hybrid PDMS/glass devices has been developed (15, 16). Typically a hybrid microchip consists of two components: (a) a PDMS slab with separation channels fabricated into the surface of the slab, and (b) a cover made from a glass slide, with holes drilled through the slide to serve as buffer and sample reservoirs if necessary. By using this approach, simple fabrication processes developed for polymeric devices can be employed, and electrophoretic separations with relatively high efficiency can still be achieved.

In this chapter, the protocol for microfabrication of hybrid PDMS/glass-based microchips is described. The fabrication protocol requires no expensive infrastructure or equipment, and hence can be performed under normal laboratory conditions. In addition, the protocol for pretreatment of the microchip devices is also described so as to allow MCE analysis to be performed with high accuracy and good reproducibility.

2. Materials

2.1. Fabrication of PDMS-Based Microchip

1. A 200-mm diameter circular silicon wafer ($725 \pm 25 \mu\text{m}$ thick; orientation 100) (Wacker Silitronic, Singapore).
2. Deionized water with a resistivity of $\geq 18 \text{ M}\Omega/\text{cm}$ obtained from a ultrapure water purification system (e.g., NANOpure, Barnstead, IA, USA).
3. Piranha solution (3:1, 95–97% H_2SO_4 :35% H_2O_2). Make it fresh. Beware, this is a very strong oxidizing agent, so it must be handled with extreme precaution. Keep organic compounds away from it.
4. SU-8 50 (Microchem Corp, Newton MA, USA). Beware, this reagent is light and temperature sensitive.
5. XP SU-8 developer (propylene glycol methyl ether acetate, Microchem Corp, Newton MA, USA).
6. UV exposure machine, with wavelength range of 250–350 nm (Speedplate 650, MC10, Marchetti). Beware, chronic UV exposure may lead to skin cancer and eye cataract. Leave the room when the UV is switched on.
7. PDMS kit containing PDMS elastomer and curing agent (Sylgard 184, Dow Corning, Wiesbaden, Germany).
8. Glass microscope slides (75 mm \times 25 mm \times 1 mm thick) (Fischer Scientific, USA).

2.2. Microchip Capillary Electrophoresis

1. Polyethersulfone (PES) Minisart® filter (0.2- μm pore size, Sartorius Singapore).
2. Vacuum pump (VP0125, MEDO Inc., IL, USA).
3. Platinum wires (99%, 0.5-mm thick, 1–1.5-cm long, Aldrich).
4. Four channels high-voltage power supply (MCP-468, CE Resources, Singapore) with data acquisition and control software (MCE, CE Resources, Singapore).

3. Methods

3.1. Fabrication of PDMS-Based Microchip

3.1.1. Designing and Printing of Microchip Layout on Mask

The photolithography masks are designed using standard computer-aided design software, e.g., AutoCAD, and subsequently transferred onto a transparency film using a commercial 8,000-dpi high-resolution printer. The channel network is represented by micrometer-wide transparent lines on a black background as seen in **Fig. 1**.



Fig. 1. Schematic depicting a typical microchip design layout with a double-T intersection on the photolithography mask. Figure is not to scale. *BR* buffer reservoir, *BW* buffer waste reservoir, *SR* sample reservoir, *SW* sample waste reservoir

The microchannel layout of the PDMS microchip consists of a sample channel intersecting the separation channel. The intersection is a double-T. The distance from BR, SR, and SW to the intersection must be equivalent. The length of the separation channel can be designed to fit experimental requirements.

3.1.2. Preparation of Silicon Wafer Mold

The surface of the silicon (Si) wafer has to be free from particles and organic contaminants and this can be achieved with wet chemical cleaning. This helps to ensure that the photoresist will be uniformly coated and will adhere well onto the Si surface. In addition, the microchannel wall structures formed on the Si wafer will not be distorted or broken at the end of the fabrication process (*see Note 1*).

1. Cut the wafer into 75 mm × 25 mm pieces with a diamond cutter (*see Note 2*).
2. Rinse the wafer with acetone followed by deionized (DI) water.
3. Immerse the wafer in the hot Piranha solution for 30 min.
4. Rinse the wafer under running tap water followed by copious amount of DI water (*see Note 3*).
5. Blow-dry the wafer with N₂.
6. Dehydrate the wafer by baking it at 100°C in a clean oven for 1 h and allow it to cool to room temperature before carrying out the next step.

3.1.3. Photolithography – Pattern Transfer onto Wafer Mold

Generally, the transfer of the microchannel patterns onto the wafer involves six main steps: spin-coating, “pre-bake,” ultraviolet (UV) exposure, postexposure bake, developing, and hard-bake. SU-8 50 is often known as the negative-based photoresist as it gives an inverted microchannel structure on the Si wafer surface. The inverted microchannel structure, as seen in **Fig. 2**, arises from the polymerization of the epoxy resin in the SU-8 negative photoresist after UV exposure and a “pre-bake” step. This will cause it to be resistant to its developer while the unexposed photoresist will be washed away.

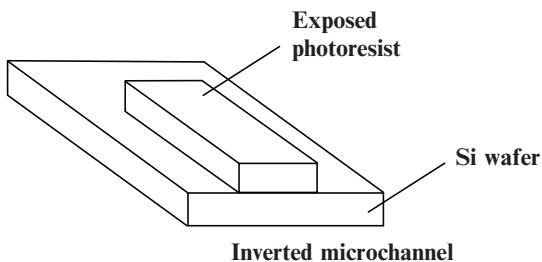


Fig. 2. Schematic of the layout of microchannel on Si wafer mold. (a) Inverted microchannel fabricated from negative-based SU-8 photoresist

1. Spin-coating of photoresist: Spin-coating on the wafer ensures that the photoresist will be evenly spread on its surface. This aids in the formation of well-defined microchannel structures. In addition, the depth of the microchannel is dependent on the spin duration and speed which in turn determines the thickness of photoresist applied.
 - (a) Drip the viscous photoresist, with a glass dropper onto the wafer before spreading it evenly with the long tip of the dropper. Care should be taken not to scratch the wafer with the tip of the dropper.
 - (b) Place the wafer at the centre of the spinner and spin-coat it at 1,500 rpm for 60 s with an accelerating ramp of 100 rpm.
2. Prebaking of coated wafer: The solvent will be evaporated off in this step, thus densifying the photoresist on the surface of the wafer.
 - (a) Place the coated wafer into a conventional oven set at 95°C for 30 min.
 - (b) Allow the wafer to cool to room temperature before the next step.
3. Ultraviolet exposure: The photolithography mask controls the UV exposure on the photoresist-covered wafer, thus allowing the transfer of microchannel structures onto the wafer.
 - (a) Place the negative photolithography mask over the coated wafer and manually align the wafer to the former with the aid of a pair of forceps (*see Note 4*).
 - (b) Preprogram the UV exposure machine to 2-min exposure.
 - (c) Start UV exposure (*see Note 5*).
4. Postexposure baking of exposed wafer: Post-exposure bake enables the exposed photoresist to cross-link, thereby enabling it to be more resistant to the developer in the subsequent step.

- (a) Place the coated wafer into a conventional oven set at 95°C for 30 min.
 - (b) Allow the wafer to cool to room temperature before the next step.
5. Developing of exposed wafer: The SU-8 developer will remove the unexposed photoresist, leaving behind the cross-linked photoresist that forms inverted microchannel structures on the wafer. At this point, the imprinted microchannels will be clearly visible.
 - (a) Immerse the exposed wafer completely in an undiluted XP SU-8 developer in a beaker.
 - (b) Shake the wafer vigorously in the developer for at least 1 min or until the unexposed photoresist, as indicated by white spots on the wafer, is completely washed off.
 - (c) Rinse the wafer thoroughly with isopropanol. If white spots are still observed on the wafer, repeat **steps 1–3** again.
 - (d) Shake off excess isopropanol on the wafer or allow the isopropanol to evaporate at room temperature before the following step.
 6. Hard-bake of imprinted wafer: The hard-bake step further helps to strengthen the structure of the cross-linked photoresist while drying the wafer after developing.
 - (a) Place the coated wafer into a conventional oven set at 150°C overnight.
 - (b) Allow the wafer to cool to room temperature before the next step.

3.1.4. PDMS Replication of Si Wafer Mold via Casting

After the fabrication of wafer mold, the PDMS microchip can be replicated with ease by a simple molding/casting procedure.

1. Mix well the PDMS elastomer and curing agent in the ratio of 10:1 (v/v) in a beaker (*see Note 6*).
2. Degas the mixture via sonication in a cold water bath for 15 min.
3. Pour the PDMS mixture onto the wafer in a polypropylene box.
4. Allow it to stand for at least 1 h or until no bubbles are observed on the PDMS.
5. Cure the PDMS/wafer in a conventional oven at 75°C for 2 h.
6. Peel off the PDMS microchip from the wafer mold with a sharp knife.
7. Create reservoirs on the PDMS microchip with a 5-mm circular punch (*see Note 7*).

3.1.5. Adhering PDMS-Based Microchip to Glass Substrate

The PDMS has self-adhesive property, thus allowing reversible sealing with most materials like glass and polymers. The sealing will be improved if the surfaces of both the PDMS and sealing substrate are free of particles.

1. Rinse the PDMS microchip with ethanol followed by running DI water.
2. Dry the PDMS with N₂ gas.
3. Immerse the glass slide in fresh piranha solution for 30 min.
4. Rinse the slide with running DI water before drying with N₂ gas.
5. Clamp the glass slide to the PDMS microchip before placing in the conventional oven set at 100°C for 1 h to improve the adhesion of PDMS to the glass slide.

3.2. Pretreatment of Microchip for Microchip Electrophoresis

Because of the simplicity of the microfabrication of PDMS-based microchips, there have emerged a wide range of applications in biomolecular analysis, cell analysis (cytometry), sensing (bio and chemical), PCR, and screening (*see Table 1*).

However, before any type of microchip is used, a pretreatment of the microchip is often necessary so as to ensure accuracy and reproducibility. The following procedures are general guidelines for most microchip separation applications. **Figure 3** shows a pictorial description of the procedures.

1. Wet the microchannels with methanol to improve the wettability of the hydrophobic PDMS.

Table 1
Applications of PDMS-based microchips

Applications	References
Biomolecular analysis	(17)
	(18)
	(19)
	(20)
Cell analysis	(21)
	(22)
	(23)
Sensing	(24)
	(25)
Polymerase chain reaction (PCR) and screening	(26)
	(27)
Flow cytometry	(28)

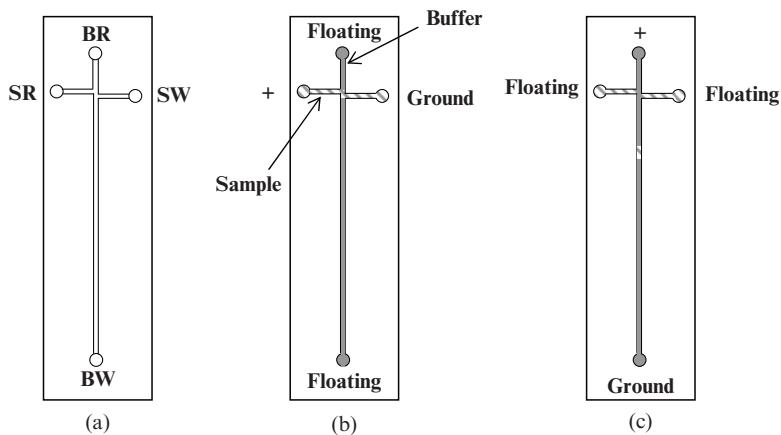


Fig. 3. Schematic of the microchip capillary electrophoresis at different stages: (a) Before filling, (b) sample injection, and (c) start of analysis

2. Flush microchannels with filtered DI water followed by running buffer.
3. Remove the washings before filling up the microchannels and reservoirs with fresh running buffer.
4. Flush the microchannels with copious amount of water at the end of the day to prevent crystallization within the microchannels.

4. Notes

1. Ensure that the clean wafer is kept in a clean plastic Petri dish that is covered at all times so as to keep out dust particles.
2. The Si wafers should be handled with a pair of latex powder-free gloves throughout the fabrication of the microchip. This is to ensure that grease on the hands will not further contaminate the Si wafer surface.
3. Hold onto a corner of the wafer with a pair of clean forceps when rinsing the wafer. This helps to minimize contact with the clean wafer. The wafer surface is checked for organic spots or evaporating water stains via reflection under white light. If the wafer is unclean, steps 3 and 4 of Subheading 3.1.2 should be repeated.
4. Check the wafer surface carefully for particles before placing the photolithography mask over it. This is to prevent the particles from disrupting the formation of the microchannel walls.

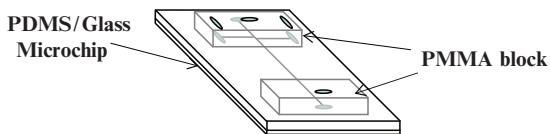


Fig. 4. Schematic showing the microchip with extended reservoirs using PMMA blocks. Reservoir wells are drilled through the PMMA block. Figure is not to scale

5. Place the coated wafer at the centre of the UV exposure machine (where the UV lamp is positioned) as the vacuum hold on the wafer as well as the UV beam intensity is the strongest.
6. The amount of PDMS mixture used will affect the thickness of the microchip cast. A thick PDMS-cast microchip may reduce the heat dissipation efficiency when voltage is applied to it during separation/analysis. A 27.5-mL PDMS mixture is estimated to fill up a 150 mm × 95 mm polypropylene box containing four adjacent Si wafer molds to give a 3-mm thick PDMS microchip.
7. The 5-mm-wide reservoirs, with a depth of 3 mm, can only hold approximately 60 mL of solution. With the heat dissipation from the electrodes when the voltage is applied, the volume of the solution may not be sufficient for a run. In addition, the placement of 1–1.5-cm-long platinum electrode in the reservoirs may not be well secured in a shallow reservoir. Thus, the depth of the reservoirs can be extended by placing a PMMA block, of 1–1.5-cm thick with drilled reservoir wells, over it as seen in Fig. 4. The PMMA block is secured on the microchip with uncured PDMS as an adhesive. Subsequently, heat the microchip in an oven at 75°C for 2 h.

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Chapter 12

Detection of Enteropathogenic *Escherichia coli* by Microchip Capillary Electrophoresis

Wai S. Law, Sam F.Y. Li, and Larry J. Kricka

Summary

There is always a need to detect the presence of microorganisms, either as contaminants in food and pharmaceutical industries or bioindicators for disease diagnosis. Hence, it is important to develop efficient, rapid, and simple methods to detect microorganisms. Traditional culturing method is unsatisfactory due to its long incubation time. Molecular methods, although capable of providing a high degree of specificity, are not always useful in providing quick tests of presence or absence of microorganisms. Microchip electrophoresis has been recently employed to address problems associated with the detection of microorganisms due to its high versatility, selectivity, sensitivity, and short analysis times. In this work, the potential of PDMS-based microchip electrophoresis in the identification and characterization of microorganism was evaluated. *Enteropathogenic E. coli* (EPEC) was selected as the model microorganism. To obtain repeatable separations, sample pretreatment was found to be essential. Microchip electrophoresis with laser-induced fluorescence detection could potentially revolutionize certain aspects of microbiology involving diagnosis, profiling of pathogens, environmental analysis, and many others areas of study.

Key words: PDMS, Microchip electrophoresis, Microfluidic devices, Laser-induced fluorescence detection, Microorganism analysis, *Enteropathogenic E. coli*, SYTO 13,

1. Introduction

Microorganisms such as bacteria, viruses, and fungi have a great impact on human daily life. On one hand, *enterohemorrhagic Escherichia coli*, such as strain O157:H7, can easily contaminate beef and milk and is the pathogen most frequently linked to capillary thrombosis, diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome by producing Shiga-like toxins (1); on the other hand, microbes are ubiquitous and essential components of the earth's ecosystem, inherent in the human intestinal flora and

nutrition supplements. Also, it is known that benign microorganisms can act as inhibitors in retarding the growth of unwanted species such as *Lactobacillus acidophilus* that produce organic acids and bacteriocin in human gut to reduce the attachment of *Helicobacter pylori*(2).

Direct inoculation method is more currently used in the detection and monitoring of microorganisms. Briefly, a pure sample is placed into a sterile growth medium and allowed to incubate for a couple of days or weeks. The metabolic activity (substrate utilization) is then examined using calorimetry, fluorescence, or turbidity (3). Although this type of culture method can provide accurate information about the presence of bacteria, it is often time consuming. Moreover, it does not necessarily track the gene of interest. Furthermore, isolation and culture of microorganisms from samples are difficult if the microorganism is easily overgrown by other bacteria in the sample or if it has entered a viable but non-culturable state (4–6). Besides, there are several indirect methods to identify the presence of a bacteria in a biological system, for example, a potent toxin in the biological fluid, called verotoxin (VT, Shiga-like toxin), could be used to determine the presence of *E. coli* O157:H7 (7,8), and the manifestation of tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β , and IL-6 in cerebrospinal fluid (CSF) and blood indicates the existence of *E. Coli* (9–11).

Recently, molecular-based assays, for instance, hybridization (12, 13), amplification (14, 15), and immunoassay (16–18) have been investigated. Hybridization methods are based on the ability of a complementary base sequences to bond with each other to form a hybrid. Upon bonding, if the unknown microorganism (target) is the same as the known source microorganism (probe), the fluorescent microorganism could be microscopically visualized. Although this method is fast and highly specific, low sensitivity and the need for cultivation significantly diminish the potential advantages. Amplification methods, such as polymerase chain reaction (PCR), involve the extraction of a small amount of complementary hybrid from a target microorganism to amplify it to a detectable level. Tedious sample preparation and DNA isolation prior to PCR analysis prove to be cumbersome, and the researchers/operators must also be trained to interpret the results. Immunoassay or enzyme-linked immunosorbent assays (ELISA) are the most frequently used. This method depends on the binding specificity of an antibody to an antigen usually displayed on the surface of a microorganism or to a secreted toxin. These techniques are less time consuming and provide considerably more accurate genetic information of the microorganisms. However, they are very complex to perform and yield presumptive results, which must be further verified by biochemical and serological testing of isolates. A study on the comparison of these methods with the conventional culturing methodology has been conducted(19).

Due to its very low sample volume requirements, capillary electrophoresis (CE) has been adopted as a new approach to address problems associated with microbial detection and identification methods (20, 21). This method relies on the fact that microbes are charged and thus move in a direct-current electric field. Microbes obtain surface charge via deprotonation or protonation of surface molecules and adsorption of ions from the surrounding solutions. Earlier works were performed by Hjerten (22), Grossman and Soana (23) with the tobacco mosaic virus whereas Kendler and co workers (24–26) had carried out extensive studies on the rhinovirus. Ebersole and McCormick were the first to report on the separation of 5 types bacteria using CE (27). Despite of the use of long capillary was applied in this study, the peaks obtained were poorly resolved. This studies was further improved with CIEF with the polymer as additives in the funning buffer, sharp and reproducible peaks could be obtained (28–34).

In 1990s, Manz and co-workers published the microchip capillary electrophoresis conceptual μTAS paper (35). Since then, the development of the microchip CE (MCE) has been a focus of research has been reviewed extensively. This technique has appeared as a result of the marriage of the ability of conventional CE to analyse ultra small volumes (in nL scale) and microfabrication techniques perfected in the semiconductor industry to produce microstructures in silicon. The potential advantages of MCE include miniaturization, instrumentation integration, reduced reagent consumption, lower costs and requires no moving parts generally fulfills the main goals of many chemical analysis methods. Although the early primary focus in the application field of microchip CE was on DNA nucleotide and restricted DNA fragment analysis, and polymerase chain reaction (36–38) and ligase chain reaction chamber (39, 40), MCE is rapidly adapted to many biological, pharmaceutical and clinical applications (41–43).

In this chapter, we present a microchip electrophoresis-based method in the identification of the *enteropathogenic Escherichia coli* (EPEC). Microfabrication protocol, sample pretreatment steps, and detection system for the analysis of *enteropathogenic Escherichia coli* will be discussed in detail (see [Notes 1–4](#)).

2. Materials

2.1. Microfabrication and Molding

1. Silicon wafer, orientation 100 (Wacker Siltronic, Singapore).
2. Negative photolithography masks were designed using standard computer-aided design software and subsequently transferred onto a transparency film using a commercial high-resolution printer with a resolution of 8,000 dpi.

3. Freshly prepared piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2 = 3:1$) (see Note 5).
4. Negative photoresist, SU8-50 and developer, propylene glycol methyl ether acetate (Microchem Corp, Newton, MA). Light and temperature sensitive, stable at 4°C for up to a year.
5. PDMS base and curing agent (Sylgard 184, Dow Corning, Wiesbaden, Germany). Stored at room temperature.
6. Methanol.
7. Isopropyl alcohol.

2.2. Cell Culture

1. Bacteria: *Enteropathogenic E. coli* (EPEC) (see Note 6).
2. Cell media: Lennox Luria-Bertani (LB) broth, 20 g/L (Becton, Dickinson and Company, Sparks, MD, USA).

2.3. Sample Pretreatment and Labeling of EPEC

1. Stock working buffer (5×): 0.455 M Tris base, 0.445 M boric acid, and 0.01 M EDTA. Prepare working buffer (0.2×) by dilution of 1 part of stock working buffer with 24 parts of DI water.
2. Labeling agent: 1 mM SYTO 13 green fluorescent nucleic acid stain (Molecular Probes, Eugene, OR, USA). Store the vials of dyes frozen at -20°C, upright and protected from light (see Notes 7 and 8).

2.4. Microchip Electrophoresis System with Laser-Induced Fluorescence Detection

1. Epi-fluorescence microscope (DMLS, Leica microsystem, Germany).
2. Filter cube (I3, Leica microsystem, Wetzlar Germany) contains a band-pass excitation filter (450–490 nm), and a dichroic mirror reflects light under 510 nm and a suppression filter at 515 nm.
3. Microscope objective (10×, Leica microsystem, Wetzlar, Germany).
4. DPSS blue laser, 500 mW (Changchun New Industries Optoelectronics Technology Co. Ltd., ChangChun, China).
5. Photomultiplier tube (H5784-02, Hamamatsu K.K., Japan).
6. Four channels high-voltage power supply and control software, MCE (MCP-468, CE Resources Pte Ltd., Singapore).
7. Vacuum pump (VP0125, MEDO Inc., IL, USA).
8. Data acquisition software CSW software (Data Apex, Czech Republic).
9. Stock running buffer (5×): Prepare 5× stock solution with 0.455 M Tris base, 0.445 M boric acid, and 0.01 M EDTA, 0.5% PEO ($M_r = 600,000$). Prepare working buffer (0.25×) by dilution of 0.5 part of stock working buffer with 9.5 parts of DI water.

3. Methods

3.1. Microchip Design

Figure 1 shows the microchip design used for the experiments outlined subsequently. PDMS microchips with double-T injection intersection were used in this experiment. The separation channel was 50 mm in length (40 mm from injection intersection to the detection point). The length from either the sample reservoir or the sample waste reservoir to the injection intersection was fixed at 10 mm. All channels were 25- μm deep and 100- μm wide. Platinum electrodes were inserted in the reservoirs, providing electrical contact from the power supply to the electrolyte solutions. During the sample injection, the voltages of the respective reservoirs were electronically controlled using MCE software to conduct pinched injection.

3.2. Microchip Fabrication

3.2.1. Photolithography and Molding

1. The silicon wafers were thoroughly cleaned by immersing in piranha solution for 30 min.
2. The wafer was then rinsed in deionized water, blown dry with nitrogen gas, and oven-dried at 100°C.
3. A negative photoresist was spun on the surface of the wafer using a spin-coater at $94.5 \times g$ for 60 s with an accelerating ramp of $0.42 \times g$.
4. After a 15-min “prebake” in a convection oven at 95°C, the photoresist layer was exposed to UV light for 45 s through the photolithography mask.
5. A 30-min “postexposure bake” was carried out in the same oven at 95°C.
6. The wafer was developed for 30 s in glass Petri dish containing the developer, and the unexposed photoresist was cleaned by 2-propanol.
7. The “hard bake” was carried out by heating the wafers at 150°C overnight.

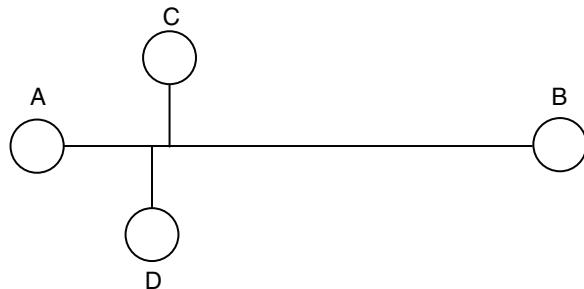


Fig. 1. Schematic of the microchip CE for LIF. A–D represents buffer, buffer waste, sample waste, and sample reservoir, respectively

8. The PDMS elastomer and curing agent were thoroughly mixed in a 10:1 ratio and degassed.
9. The mixture was then poured onto the microfabricated silicon wafer followed by curing for at least 2 h at 70°C.
10. The cured PDMS was separated from the mold, and reservoirs were made at the end of each channel using a 5-mm circular punch.
11. The PDMS slab gel with the imprinted microchannel was then put onto clean glass to form revisable bonding.

3.3. Cell Culture

1. EPEC colonies were transferred from the LB agar plate into LB broth.
2. The culture was maintained at 37°C overnight.
3. The cells should be collected when the cell concentration reaches 10^8 /mL.
4. Cell concentrations were determined from the optical density of the solution at 600 nm with a UV-visible spectrophotometer (see Note 9).

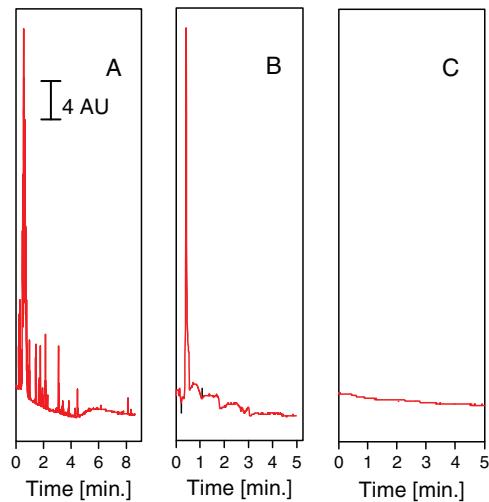
3.4. Sample Preparation and Staining

1. One milliliter solution of EPEC was pelleted by centrifugation at 5,000 rpm for 3 min (see Note 10).
2. The supernatant was decanted and 1 mL freshly diluted running buffer was added (see Subheading 2.3 and Note 11).
3. The sample solution was vortexed for 1 min followed by centrifugation.
4. Steps 2 and 3 were repeated twice and finally the bacteria samples were suspended in 1 mL diluted running buffer (see Note 10).
5. One microliter of SYTO 13 (1 mM) was mixed with diluted bacteria sample solution (see Notes 8 and 9).
6. The mixture was then vortexed gently and kept in the dark for at least 25 min.
7. The fluorescence intensity was found to be correlated with the staining time. After 25-min incubation in the dark, the fluorescence tends to level off.
8. Sample pretreatment steps, albeit labor-intensive processes, without properly controlled and optimized procedures can lead to irreproducible separations or complicate electropherograms with multiple peaks (see Fig. 2; Notes 12 and 13).

3.5. Standard Electrophoresis Procedure

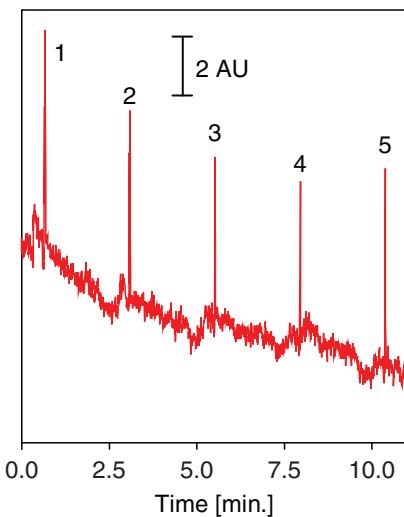
1. Fill the buffer, sample, and sample waste reservoir with DI water. Flush through the microchip by applying a vacuum at the buffer waste reservoir.

Fig. 2. Effect of sample pretreatment on electrophoretic behavior of EPEC. The separation conditions were identical. Buffer: 25 mM TBE, with 0.0125% PEO, pH 8.4, separation voltage 1.5 kV. Injection: electrokinetic injection at 1 kV for 6 s. A: sample without proper pretreatment (lysed sample), B: sample with pretreatment as described in text, C: blank buffer without sample.



2. Displace the water from the reservoirs with the running buffer (*see Subheading 2.4*). Flush through the microchip for 10 min.
3. Remove running buffer from the sample reservoir and load the EPEC sample.
4. A platinum wire was placed in each of the reservoirs to minimize the problems of formation of air bubbles (*see Note 14*).
5. The sample was injected by applying a potential of 0.5 KV for 15 s between the sample reservoir (reservoir D) and the grounded sample waste reservoir (reservoir C) whereas buffer and buffer waste reservoir (reservoir A and B) remained floated.
6. The separation process was carried out by applying 2-kV separation potential to the running buffer reservoir (reservoir A) with the buffer waste reservoir grounded (reservoir B) and all other reservoirs floated (*see Notes 15–20*).
7. Microchips were flushed with running buffer between runs to ensure reproducibility and to maintain a stable baseline (*see Fig. 3*).
8. The current during the separation was monitored during runs in order to confirm that no bubble was formed.
9. All CE experiments were performed at a room temperature of $25 \pm 1^\circ\text{C}$.
10. Data were acquired and analyzed with a CSW software.

Fig. 3. The reproducibility of bacterial analysis by PME-laser-induced fluorescence system was evaluated for five consecutive electrophoresis runs, each corresponding to injection of a solution containing 100×10^4 EPEC cells/mL. Running buffer: 25 mM TBE, 0.025% PEO, pH 8.4. Separation voltage: 2 kV, injection: electrokinetic at 0.5 kV for 15 s.



4. Notes

1. A majority of the microorganisms and materials used for the experiments described earlier fall into the biosafety level 1 category, and standard microbiological practices should be employed in their use.
2. Extra precautions should be used when handling sharps or needles contaminated by the microorganisms.
3. One should be familiar with the Material Safety Data Sheet (MSDS) of each chemical. Proper personal protective gear and gloves should be worn.
4. The high-voltage power supply should be handled with extreme care to avoid electrical shock. Hence, it is highly recommended to set up an interlock system in the experimental setup to prevent operators from electrical shocks.
5. Piranha solution is a very strong oxidizing agent, so it must be handled with extreme precaution.
6. *Enteropathogenic Escherichia coli* or microorganisms are generally amphoteric; hence, they have a net negative charge at high pH and a net positive charge at low pH.
7. Before opening the vials, allow the vials to warm to room temperature and then briefly centrifuge to allow the DMSO solution settle to the bottom of the vial. Before refreezing, seal all vials tightly. The stock solutions are stable for at least a year when stored properly.

8. Use plastic tubes when diluting any SYTO stain, as the diluted stain will readily adhere to glass.
9. All cultures used were prepared freshly daily in order to prevent the problems of cell aggregation and lysis. Prior to injection, the microbe samples were vortexed for 1 min.
10. Adherent cells in culture may be stained in situ on coverslips. Cells in suspension should be pelleted by centrifugation and resuspended in buffered salt solution or water.
11. In SYTO labeling process, the best results are obtained in buffers that do not contain phosphate.
12. Sample pretreatment steps and separation conditions must be carefully controlled and optimized. In doing so, the microorganism remains intact aiding in obtaining repeatability and high-sensitivity separation efficiency.
13. Microorganisms are highly sensitive to the environment (pH, ionic strength, presence of O₂, etc.) and can lyse under moderate to harsh conditions.
14. Electrolysis tends to occur on the surface of the electrode. Hence, a relatively long platinum wire is desirable to minimize the bubble formation problems. Besides, the long electrode could also assure that electrical potential is the same among the different separation channels.
15. Aggregation of bacteria or microbes can cause changes in both surface charges and diffusional properties. It is therefore possible to have multiple peaks in electropherograms of a single species of bacteria.
16. Microorganism can secrete substances such as proteins, poly-nucleotides, cell fragments, and some small molecules that result in unwanted multiple peaks, lysis, or altered mobilities.
17. A higher buffer concentration is preferable as it provides a more stable current, and better peak shape was obtained in addition to enhanced reproducibility.
18. Diluted polymer added into running buffer is used (a) to diminish the EOF, (b) focus microorganism into narrow zone under an applied electric field, and (c) increase the migration time of the microorganism.
19. It was reported that in order to obtain high separation efficiency, three experimental conditions are necessary: (a) a dilute polymer must be added as additives into running buffer, (b) a direct current electrical filed, and (c) the separation must be conducted under counter-EOF.
20. Each microorganism appears to have optimal separation and detection experiment conditions (e.g., buffer pH and strength, type and concentration of polymer, etc.) when it is effectively focused through the detection window.

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