Microarrays as Research Tools and Diagnostic Devices

Karin Lemuth and Steffen Rupp

Contents

Abstract Molecular diagnostics comprises a main analytical division in clinical laboratory diagnostics. The analysis of RNA or DNA helps to diagnose infectious diseases and identify genetic determined disorders or even cancer. Starting from mono-parametric tests within the last years, technologies have evolved that allow for the detection of many parameters in parallel, e.g., by using multiplex nucleic acid amplification techniques, microarrays, or next-generation sequencing technologies. The introduction of closed-tube systems as well as lab-on-a-chip devices further resulted in a higher automation degree with a reduced contamination risk. These applications complement or even stepwise replace classical methods in

K. Lemuth
Robert Bosch GmbH, Corporate Sector Research Microsystem Technologies, Robert Bosch Platz 1, 70839 Gerlingen, Germany

S. Rupp (✉)
Fraunhofer Institut für Grenzflächen- und Bioverfahrenstechnik, Nobelstr. 12, 70569 Stuttgart, Germany

Institut für Grenzflächenverfahrenstechnik und Plasmatechnologie, Universität Stuttgart, Nobelstr. 12, 70569 Stuttgart, Germany
e-mail: Steffen.Rupp@igb.fraunhofer.de

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clinical microbiology like virus cultures, resistance determination, microscopic and metabolic analyses, as well as biochemical or immunohistochemical assays. In addition, novel diagnostic markers appear, like noncoding RNAs and miRNAs providing additional room for novel biomarkers.

This article provides an overview of microarrays as diagnostics devices and research tools. Introduced in 1995 for transcription analysis, microarrays are used today to detect several different biomolecules like DNA, RNA, miRNA, and proteins among others. Mainly used in research, some microarrays also found their way to clinical diagnostics. Further, closed lab-on-a-chip devices that use DNA microarrays as detection tools are discussed, and additionally, an outlook toward applications of next-generation sequencing tools in diagnostics will be given.

Keywords DNA microarray • Lab-on-a-chip • Molecular diagnostics • Next-generation sequencing

1 Introduction

With their pioneering work, Schena and colleagues published a completely outstanding method in molecular technologies in 1995 (Schena et al. 1995). First used for multiparametric transcriptional profiling, the technology rapidly developed toward a tool for the detection of all kinds of biological targets (DNA, RNA, proteins, cells, nucleic acids, carbohydrates, etc.) or their modifications (methylation, phosphorylation, etc.) within the last 20 years. The principle is simple. The biological interaction of molecules, e.g., the interaction of complementary bases of nucleic acids, antibody–antigen interactions, or the interaction of carbohydrates with lectins, represents the basic principle of microarray technology. As an example, in the following, the basic principles of a DNA microarray is described: to detect target molecules, e.g., mRNA or genomic DNA; oligonucleotides complementary to the respective target molecule are immobilized on solid supports like glass, nylon, other polymers, or silicon in an ordered manner—the microarray. These oligonucleotides may correspond to all open reading frames of an organism to allow transcriptional profiling. From the sample of interest, e.g., RNA is extracted and labeled using nucleic acid polymerizing enzymes (e.g., reverse transcriptase, T7 polymerases to generate cDNA) and fluorescently labeled nucleotides. Hybridization to the microarray leads to a specific molecular interaction at the location where the complementary strand is immobilized. Readout using a fluorescence scanner with a photomultiplier tube or imaging with a CCD camera will give the information if the target molecule is present in the analyzed sample or not (Pollack 2009) (Fig. 1). Next to the direct labeling, also indirect labeling techniques can be used. Biotin-labeled nucleotides are incorporated during PCR und afterwards detected using streptavidin or antibody conjugates. One of the first examples was published shortly after the work by Schena et al. (1995) which started the

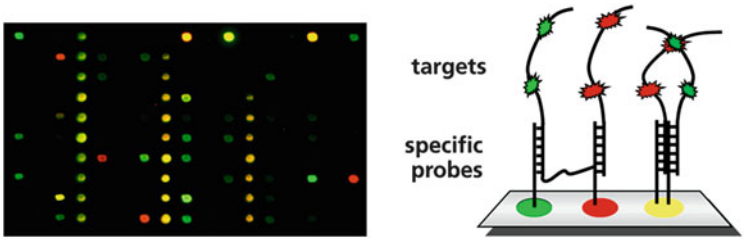

Fig. 1 Basic principle of a DNA microarray. On the *left side,* the readout of a diagnostic microarray is shown using *two-color labeling*, representing two samples to be compared, used, e.g., in transcriptional profiling. Cy3- and Cy5-labeled target cDNAs are hybridized to their specific probes on the *array* as indicated on the *left side*. The schematic overview on the *right* side shows a typical hybridization using a competitive *two-color* (Cy3 and Cy5) hybridization in transcriptional profiling experiments. Targets present only in one sample result in either *green* or *red* signals, and targets present equally in both samples result in a *yellow* signal. The ratio between the *red* and *green* signal gives the difference in mRNA levels in the different samples

commercialization of nucleic acid microarrays by Affymetrix, USA (Wodicka et al. 1997). Another example is the colorimetric Silverquant® technology used by Eppendorf for their DualChip® microarrays. Biotin-labeled nucleotides are used in the PCR reaction. Gold-coupled antibodies against biotin are added after hybridization. The colorimetric reaction is started by adding silver nitrate and a reducing agent. This leads to silver precipitation at the gold particles. Another labeling technology uses a streptavidin–horseradish peroxidase conjugate for colorimetric detection (Rubtsova et al. 2010).

2 DNA Microarrays as Research Tools

2.1 Microarray Development

Microarray technology has revolutionized both DNA and RNA research. In contrast to most classical biological assays, microarrays enable the parallel analysis of several tens of thousands of analytes. For the analysis of nucleic acids, the application possibilities range from a focused set of multiple transcriptional units for transcriptional profiling or genomic fragments for analysis of copy number or genetic variation up to entire genomes and beyond. The availability of complete genomic sequences was the keystone for the development and use of genome-wide technologies, like array technologies. In the past century, the sequencing of entire genomes has been a major effort both in academic and in commercial research. The first sequenced eukaryotic genome was the genome of *Saccharomyces cerevisiae*, the second completely sequenced genome at all, in 1996. Before 2,000, genome sequencing was a daunting task. In total, the number of sequenced organisms was rather small, including 38 bacteria, 1 fungus (*S. cerevisiae*), 2 invertebrates

(*Caenorhabditis elegans* and *Drosophila melanogaster*), and 1 plant (*Arabidopsis thaliana*), all with relatively small and simple genomes. Only 5 years after the first eukaryotic genome was presented, a first draft of the human genomic sequence was published (Lander et al. 2001; Venter et al. 2001). The human draft sequence, although a landmark, was still rather imperfect. It covered only 90 % of the euchromatic genome and was interrupted by 250,000 gaps. About 10 years later, the catalogue of sequenced organisms included already over 16,000 projects, including more than 10,000 microbial organisms and close to 1,700 vertebrate organisms of completed or ongoing sequencing project of organisms (status Sept 2012) (Pruitt et al. 2012). The major reason for this explosion in genome sequencing is due to the development of an additional revolutionizing technology at the beginning of the twenty-first century, generally termed next-generation sequencing (NGS) (Margulies et al. 2005) to which we refer in a later paragraph of this article. At the last NCBI update (October 2013), the number in public archives again increased to 24,788 prokaryotic registered genome projects representing 4,528 different species; 14,311 of them have assembled genomes either complete (2,670) or draft (11,641), and the remainder either do not have submitted sequence data yet or have only raw sequence reads uploaded to the Sequence Reads Archive (Kodama et al. 2012). Sequencing is not restricted to individual organisms any more but includes also microbial communities, like samples from the mid-ocean and environmental remediation sites and human samples from the gut and skin (Grice et al. 2009; Gill et al. 2006). Remarkably, partial genome sequences have even been obtained from several extinct species, including the woolly mammoth and the Neanderthal (Green et al. 2010). In addition populations of one species are sequenced, like in the 100 K Genome Project for microbial pathogens (Timme et al. 2012) and the 1,000 Genomes Project for humans (Genomes Project Consortium et al. 2012). This will enable completely new approaches to medical research and diagnostics, including the development of diagnostic microarrays.

In parallel to the completion of the first genomic sequences, DNA microarray technology has been developed as mentioned above. Pioneering experiments were focused on expression profiling using model organisms like *S. cerevisiae* to monitor changes of transcriptional activity of every known or annotated gene in a single experiment. For *S. cerevisiae*, the first genome-wide transcriptional analyses indeed appeared shortly after completion of the genomic sequence (DeRisi et al. 1997; Hauser et al. 1998; Wodicka et al. 1997). On the nucleic acid level, this set the start for genome-wide analyses of organisms based on the knowledge of their genomes. *S. cerevisiae* as one of the major model organisms was the key in developing both the biochemical and bioinformatic methods necessary for transcriptional profiling. Today, thousands of transcription profiles have been generated from almost all sequenced species. Besides the knowledge of the genome, ways to generate and analyze the data are a prerequisite for transcriptomics. An overview of data analysis methods, however, is beyond the scope of this chapter. Several books on microarray technology and data analysis have been written which introduce perfectly into these topics (Bremer et al. 2010; Dufva 2009). Microarray fabrication, which has been shortly introduced above, will be discussed in the following in more details.

2.2 DNA Microarray Fabrication

There are three main ways to manufacture DNA microarrays: light-directed synthesis (photolithography), piezoelectric ink-jet printing, and robot spotting (Hughes et al. 2001; Tan et al. 2003; Dufva 2009). For photolithography, premade masks or digital micromirror devices are used. Premade masks are used by the Affymetrix platform. For every nucleotide (nt) added to the growing strand on the solid support, a mask covering regions on the microarray where no base addition should take place is applied. Previous nucleotide blocking groups are removed by a photosensitive reaction after UV light-induced deprotection, and the extension by one further nucleotide can take place. The technology is rather expensive due to mask costs. Every array design needs its own set of masks. Usually, probes are 20–25 nt in length, and 22–40 probes per gene are synthesized. Another photolithography technology is used by NimbleGen: single-nucleotide extension is performed by light-mediated inactivation of a photolabile protective group and afterward a base addition. This is similar to the Affymetrix technology. However, NimbleGen uses micromirror devices that can lead the light needed for inactivation of protective groups to every desired spot on the microarray. Therefore, there is no need for masks anymore which makes this technology much cheaper. Another technology for making microarrays is the piezoelectric ink-jet printing technology, also called HP technology deduced from HP printing technology. The Agilent sure print technology is an example for an industrial platform. They produce cDNA or oligonucleotide microarrays. cDNA is spotted directly, oligonucleotides are synthesized base-by-base in repetitive print layers using standard phosphoramidite chemistry (https://www.chem.agilent.com/Library/technicaloverviews/Public/5988-8171en.pdf). The most widely used technology for microarray production especially in the field of research institutions and universities is the robot spotting technology: e.g., proteins, PCR products or oligonucleotides are spotted on a solid glass support using split or solid pins. Using split pins, DNA is fed in by capillary forces and deposited at defined locations on the microarray using robot technology. Usually, PCR products up to 1 kb and oligonucleotides between 20 and 100 nts in length are printed. This technology is affordable and therefore often used in individual research labs. Next to these classical technologies, also semiconductor-based systems have been developed. For example, CombiMatrix uses such a system for the production of their microarrays. Thousands of platinum microelectrodes can be addressed simultaneously to synthesize individual oligonucleotides by digitally controlled synthesis. The activation of a microelectrode leads to the production of an acid by an electrochemical reaction. This leads to the deprotection of the growing oligonucleotide strand activating it for the next synthesis step. The possibility to individually address thousands of microelectrodes in parallel allows the comfortable production of individual microarrays (Ghindilis et al. 2007). Over 12,000 oligos are synthesized in parallel as 50 mers.

Not only natural DNA has been used as probes or capture molecules on DNA microarray systems. In order to reduce cross-reactivity and to increase interaction with the targets in the sample applied, nonnatural variants have been used as

capture molecules, including L-DNA (Hauser et al. 2006) and PNA variants (Sforza et al. 2014). L-DNA probes have the great advantage that they interact with the exact same kinetics with its antiparallel L-DNA strand, like the D-DNA duplex. Thus, all the knowledge gained with D-DNA-microarrays can be used for the L-DNA equivalent. To bind to targets like RNA, DNA, or even peptides/epitopes or chemical molecules, the so-called ZIP-code arrays are used. The probes used in ZIP-code arrays are fusions in which the targets are bound in solution using, e.g., natural D-DNA oligomer complementary to the target DNA/RNA which is fused to an L-DNA. This L-DNA oligomer addresses an immobilized complementary L-DNA oligonucleotide on the microarray surface, which allows a literally background-free hybridization on the arrays. However, the price for synthesizing L-DNA oligomers is rather high, hampering further development. P-DNAs, synthetic nucleic acid analogues based on a pseudopeptide backbone instead of a phosphodiester backbone, show excellent sequence-specific recognition properties and are less susceptible to changes in ionic strength. Since they are not charged, repulsion between the negatively charged backbone present in DNA duplexes is missing resulting in higher affinities as shown by the higher melting curves of PNA/DNA or PNA/RNA duplexes if compared to their natural counterparts (Brandt and Hoheisel 2004; Jacob et al. 2004; Sforza et al. 2014).

Next to these planar array systems, bead-based systems have been developed like random bead arrays or suspension arrays. The beads are marked with different amounts of fluorescent dye or another barcoding technology and are individually identifiable. After coupling, e.g., oligonucleotides to such beads, a specific binding event can be detected. For example Illumina has developed a bead array technology, where they use either planar silicon slides or fiber-optic bundles. Silicon wafers or optical fibers are etched such that the individual beads can fit into the resulting three micron-sized wells. After the binding event, the beads are subsequently identified. This technology uses fluorescently labeled primers http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechBeadArray.shtml. Similar to this, suspension arrays also use optically differentiable beads that are coupled to the interesting biomolecule which can be, e.g., oligonucleotides or antibodies. The hybridization reaction with fluorescently labeled substrates takes place in solution, and a subsequent FACS (fluorescence-activated cell sorting) sorting process allows the identification of the binding process. An example is the Luminex xMAP technology (http://www.luminexcorp.com/TechnologiesScience/xMAPTechnology/).

DNA microarrays have been widely used as research tool in the lab, and according to this, a huge amount of literature has been published in this field. Microarrays addressing specific targets like proteins, carbohydrates, tissue, or cells have been developed as well, but will not be reviewed here. Many reviews exist that deal with topics like whole-genome expression analysis (over 2,200 reviews in PubMed with the search strings “microarray” and “expression analysis”), cancer research (Chibon 2013; Daigo et al. 2013; Ho et al. 2013; Sato-Otsubo et al. 2012; Tiwari 2012), molecular karyotyping (Dhillon et al. 2014), chromosomal microarray analysis (Brady and Vermeesch 2012), phylogenetics/microbiome (Nikolaki

and Tsiamis 2013), or gene regulation via ChIP-chip (Falk 2010; Powell et al. 2013). DNA microarrays have been used to study heart diseases (Phan et al. 2012), aspects in dermatology (Villasenor-Park and Ortega-Loayza 2013), or mental disorders like autism (Carter and Scherer 2013) and many others. A more detailed overview focusing on applications in clinical microbiology has been given by Miller et al. (Miller and Tang 2009). In the following section, a more detailed overview of a few selected research-driven microarrays is given.

2.3 Microarrays in Infection Biology

The past century brought the availability of vaccines and antibiotics, leading to a dramatic fall in mortalities caused by infectious diseases. Nevertheless, today again nearly 25 % of the annual deaths worldwide are directly related to pathogens (Morens et al. 2004). This can be attributed to the appearance of new diseases like HIV, SARS, West Nile Virus, or the recent Ebola outbreak, but also to an increase of resistance to antibiotics in pathogens thought to be defeated, like *Mycobacterium tuberculosis* or *Staphylococcus* and *Enterococcus* strains. In addition, the progress in medical care results in a large proportion of temporarily or lifelong immune-deficient patients and consequently in an increase in opportunistic infections often resulting in sepsis, requiring fast and accurate diagnosis to save patient lives. Approximately 1,415 species have been identified as known to be pathogenic to humans, including 538 bacteria and 307 fungi (Cleaveland et al. 2001), indicating the multiparametric problem of identification of the disease-causing pathogen.

DNA microarrays have emerged as a viable platform for the detection of pathogenic organisms. Microbial detection arrays both with regard to cost and application range are in between low cost, narrowly focused assays like multiplex PCR and the more expensive, broad-spectrum technologies like high-throughput sequencing. Pathogen detection arrays have been used primarily in a research context; however, several groups have developed arrays for clinical diagnostics, food safety testing, environmental monitoring, and biodefense. Statistical algorithms that enable data analysis and provide easily interpretable results are absolutely required for an applicable detection array.

One of the first microarrays designed for a wide range of pathogens was the ViroChip (Wang et al. 2002). An updated version was published shortly thereafter (Wang et al. 2003). This array, fabricated by robotic spotting of 70-mer oligos, contained initially 1,600 probes derived from 140 viral genomes available at that time. Since the probes were designed against conserved sequences common to a taxonomic family, it could be used to identify novel viruses within the same family. The ViroChip, therefore, could contribute to characterizing the novel coronavirus responsible for the SARS outbreak in 2003 (Ksiazek et al. 2003). Also today the ViroChip is still used in clinical studies. Its utility for detection of viruses in acute respiratory tract infections in children could be shown in a clinical study of more

than 200 children, showing superior performances with regard to sensitivity and specificity profile and expanded spectrum for detection of viruses if compared to serologic or PCR-based detection methods (Chiu et al. 2008). The latest version of the array is now based on the Agilent ink-jet platform (Chen et al. 2011).

One of the most comprehensive arrays has been described by Gardener et al. (Gardner et al. 2010), based on NimbleGen technology platform. In this report, a pan-Microbial Detection Array (MDA) to detect all known viruses (including phages), bacteria, and plasmids at that time is described including a novel statistical analysis method to identify mixtures of organisms from complex samples hybridized to the array. On this array, family specific probes were selected for all sequenced viral and bacterial complete genomes. The probes on the array were designed to tolerate sequence variations which will allow the detection of divergent species provided that some homology to sequenced organisms is given, as described for the ViroChip. Using this comprehensive chip in blinded testing on spiked samples and in clinical fecal, serum, and respiratory samples, the chip was able to correctly identify multiple species or strains as confirmed by PCR.

In addition, many different arrays focusing on defined pathogens or families of pathogens as well as resistance patterns have been described.

To detect fungal infections, especially in intensive care units in hospitals where they have emerged as a major cause of morbidity and mortality in immune-compromised patients, a diagnostic microarray for the rapid and simultaneous identification of the 12 most common pathogenic *Candida* and *Aspergillus* species has been developed. Oligonucleotide probes were designed based on sequence variations of the internal transcribed spacer (ITS) regions using a universal PCR amplifying the fungal ITS target region. The array was validated by using 21 clinical isolates as blinded samples (Leinberger et al. 2005).

Arrays for detecting resistance determinants like lactamases have been established as well. For example, Leinberger et al. described an extended-spectrum beta-lactamases detection array able to detect resistance mechanisms based on the TEM, SHV, or CTX-M type conferring resistance to beta-lactam antibiotics in gram-negative bacteria (Leinberger et al. 2010). The activity of these enzymes against beta-lactam antibiotics and their resistance against inhibitors can be influenced by genetic variation at the single-nucleotide level. The array is described to consist of 618 probes that cover mutations responsible for 156 amino acid substitutions. The validity of the DNA microarray was demonstrated with 60 blinded clinical isolates, which were collected during clinical routines. The chip was characterized successfully with regard to its resolution, phenotype–genotype correlation, and ability to resolve mixed genotypes.

Also resistance mechanisms of emerging pathogens like *Acinetobacter baumannii* can be detected using arrays. Dally et al. have developed a microarray that can be used to detect 91 target sequences associated with antibiotic resistance within 4 h from a bacterial culture (Dally et al. 2013). The array was validated with 60 multidrug-resistant strains of *A. baumannii* in a blinded, prospective study and compared to results determined by the VITEK2 system, based on phenotypic susceptibility. This array is able to detect all relevant resistance determinants of

A. baumannii in parallel. Thus, it enables fast results in order to initiate adequate anti-infective therapy for critically ill patients and can be used for epidemiologic surveillance.

2.4 Microarray-Based Gene Expression Profiling in Cancer

Transcriptional profiling rapidly expanded to research on all types of human cancers, starting already before the initial draft of the human genome was published in 2001. Breast cancer has been one of the most intensively studied human cancers, with regard to microarray-based gene expression profiling for cancer classification, prognosis, and prediction. One of the reasons for this, besides the high frequency of breast cancer in the population, is the observation that although approximately 60 % of all patients with early breast cancer receive some form of chemotherapy, only a minority will benefit from it (Schmidt et al. 2009). Therefore, reliable prognostic and predictive markers are needed to guide the selection of the most appropriate adjuvant therapies for individual patients with breast cancer. Microarrays actually contributed significantly to a change in the understanding of breast cancer as a heterogenous group of complex tumors instead of a single group. Pioneering work by Perou and colleagues rediscovered the large heterogeneity of breast cancer tumors on a molecular level (Perou et al. 2000). The demonstration that ER-positive and ER-negative tumor cells are fundamentally different in their expression profiles led to the suggestion to use microarray-based multigene prognostic classifiers, known as gene signatures or diagnostic signatures, as a prognostic/predictive marker panel for therapy decisions. In the last decade, several groups have embarked in the identification of gene signatures for breast cancer diagnostics with the goal to predict if chemotherapy can be omitted in early-stage breast cancer or select the most appropriate treatment. One of the first prognostic signatures consisted of 70 genes enabling the identification of good prognosis patients with a minimal risk of developing of metastases within the next 5 years (van 't Veer et al. 2002). This 70-gene signature has been validated in several clinical trials, in general using fresh biopsy tissue for preparation of the transcriptional profiles during the last years and is now commercially available via Agendia as MammaPrint® (using Microarrays based on Agilent technology) for guided therapy of early-stage breast cancer (Exner et al. 2014). In parallel, a gene signature consisting of 16 diagnostic genes (and 5 controls) was established, based on qRT-PCR (Oncotype DX) (Paik et al. 2004). This assay also underwent several clinical trials and was recently positively evaluated for economic impact on patient management (Nerich et al. 2014). Several other assays have been developed, which are nicely reviewed by Colombo et al. and by Zanotti et al. (Colombo et al. 2011; Zanotti et al. 2014).

In summary, this shows how within the last 15–20 years the development of microarrays enabled the successful setup of multigene assays, designed to support physicians and patients in clinical decision-making in early-stage breast cancer.

2.5 Detection of Small Noncoding RNAs and Their Precursors

Noncoding RNAs, especially microRNAs, have been described in the last decade as key developmental regulators (Bushati and Cohen 2007). Therefore, considerable efforts have been made to unravel the function of miRNAs and use them as diagnostic markers, especially in cancer (Jansson and Lund 2012; Zhang et al. 2014) but also in other diseases, including neurodegenerative diseases (Gandhi et al. 2013), cardiovascular diseases (Ellis et al. 2013), and infectious diseases (Shrivastava et al. 2013). Detection of small noncoding RNAs like microRNAs is a challenge, since they are very short in their mature form, containing neither cap nor poly (A) tails naturally and in addition to the mature form exist in a pri-form and a preform which are processed and transported out of the nucleus by a complex sequence of events as reviewed nicely by Bartel (Bartel 2004). Several systems have been described which enable microRNA detection on a microarray platform. For example, Agilent has developed such a platform including a special labeling technology which enables the detection of miRNAs for tissue or formalin-fixed biopsy material (D'Andrade and Fulmer-Smentek 2012). The profiling of different miRNA maturation levels in parallel is important for a comprehensive cancer classification (O'Hara et al. 2008). Therefore, it is desirable to distinguish between mature, pri- and preforms. Since the mature miRNA is also part of the preforms the available microarray systems in general are not able to distinguish between pre-mature and mature miRNAs without costly size exclusion or approximate data analysis. However, using a ZIP-code array system together with a defined labeling approach, a system enabling the distinction between pre-miRNAs and their mature form was described recently by Weishaupt et al. (Weishaupt et al. 2013). By labeling all forms of miRNAs with a poly(A) tail, they can distinguish between miRNA containing still a precursor sequence and a mature miRNA containing the poly(A) tail in direct proximity to the mature sequence. This is done by using primer extension reactions in combination with two distinct ZIP-code primers hybridizing to the mature miRNA targeted and the specific complementary ZIP on the array. The two ZIP-code primers are used for two distinct labeling reactions containing (1) only unlabeled dTTP together with Cy3-dUTP in the labeling reaction allowing for the detection of the mature form and (2) unlabeled dATP, dCTP, dGTP, and dTTP together with Cy3-dATP in the labeling reaction for the detection of the precursor form as shown in Fig. 2. Combining both labeling reactions on one array, we can now identify via the specific complementary ZIP code the amount of both the premature and mature miRNA. This is possible because the precursor miRNA is the only species which is able to carry labeled dATP, since the poly(A) tail directly following the mature form does not allow the integration of dATP into the mature form (for labeling reaction see Fig. 2), and the mature miRNA is the only species which is able to contain Cy3-dUTP via the poly (A) tail. The label reaction has to be performed separately to achieve this and is mixed on one array for readout. Using a set of well-defined, evaluated ZIP-code primers a quantitative readout could be demonstrated.

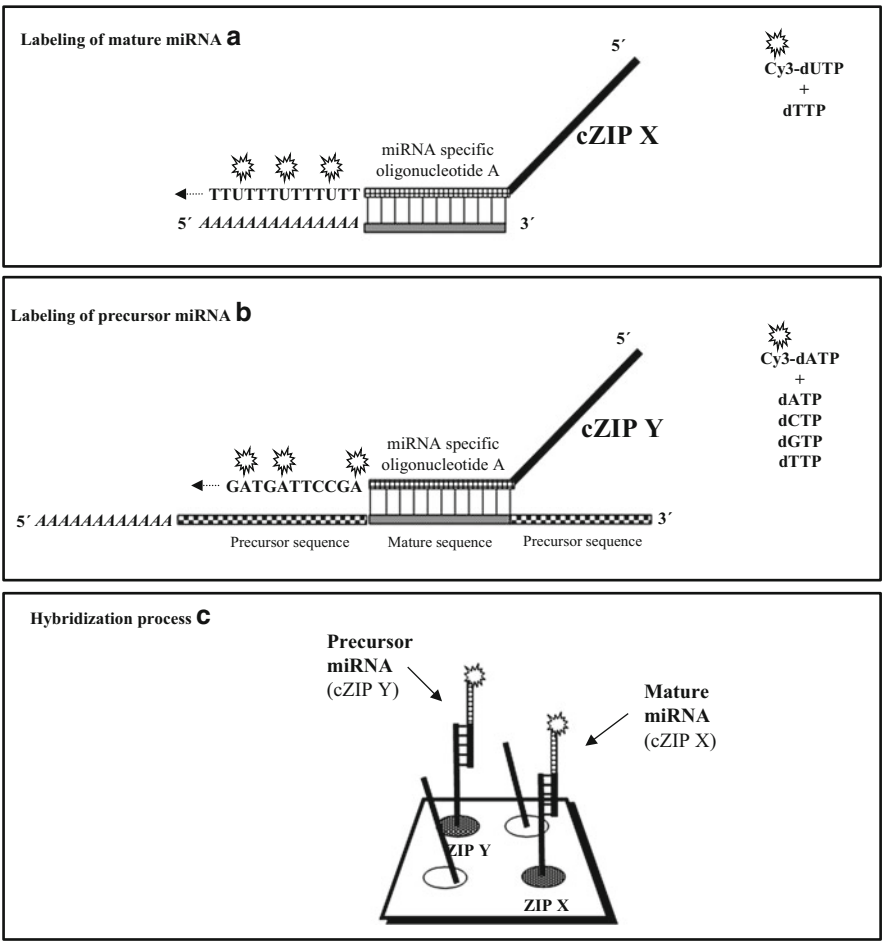

Fig. 2 Principle of the ZIP-code miRNA array for the differentiation of mature (**a**) and precursor miRNA (**b**) forms (Weishaupt et al. 2013). miRNA-specific cZIP-primer with an identical part complementary to the mature miRNA sequence and an individual cZIP-part are used in individual labeling reactions for every miRNA analyzed. Mature miRNA (**a**) is labeled with Cy3-dUTP in the presence of unlabeled dTTP; precursor miRNA is labeled with Cy3-dATP in the presence of all four unlabeled dNTPs. Differentiation takes place during the hybridization process (**c**) where the individual cZIP codes hybridize to their corresponding counterparts on the *array*

3 DNA Microarrays: Already in the Clinical Laboratory?

There are many commercially available DNA microarray platforms for research applications as introduced in Sect. 1. However, arrays that are used for diagnostic purposes in the clinics are rare. DNA microarray-based systems often face problems like reproducibility, sensitivity, ease of use, and the contamination risk.

However, there are several examples of DNA microarrays that made it into diagnostics. One example is the Amplichip®CYP450 from Roche, based on the Affymetrix system. It is the first microarray-based pharmacokinetic test cleared for clinical use. An excerpt from their homepage: "The AmpliChip CYP450 Test provides comprehensive detection of gene variations—including deletions and duplications—for the CYP2D6 and CYP2C19 genes, which play a major role in the metabolism of an estimated 25 % of all prescription drugs. It is intended to be an aid to clinicians in determining therapeutic strategy and treatment dose for therapeutics metabolized by the CYP2D6 or CYP2C19 gene product" (http://www.roche.com/products/product-details.htm?type=product&id=17).

The FDA-cleared 70-gene MammaPrint profile commercially available from Agendia as described above is one of the examples for a transcriptional profiling array that allows predicting the risk of metastasis formation for breast cancer. Biopsy- or formalin-embedded tissue is analyzed by expression analysis. Agendia also has several other cancer tests in their portfolio, e.g., for colon cancer recurrence or mutation analysis assays for EGFR, KRAS, BRAF, and PIK3CA to select appropriate patients for therapy (http://www.agendia.com/managed-care/). The ViroChip described above is an example of a diagnostic device for respiratory diseases, which however is not commercialized as such by a company but used as a research tool. Nevertheless, several commercial products are or were available for detection of (partially) overlapping panels of respiratory viruses, based on microarrays as the identification method (Miller and Tang 2009). These products include the ResPlex II assay from Qiagen (Valencia, CA); the MultiCode-PLx RVP from EraGen Biosciences (Madison, WI); the Infiniti RVP from AutoGenomics, Inc. (Carlsbad, CA); the Ngen respiratory virus ASR assay from Nanogen (San Diego, CA); and the xTAG RVP from Luminex Molecular Diagnostics (Toronto, Canada).

Randox also offers DNA microarrays in the field of molecular diagnostics (http://www.randox.com/evidence.php). Assay kits are provided for cardiac risk prediction, familial hypercholesterolemia, KRAS/BRAF/PIK3CA assay, respiratory multiplex array, or a multiplex array for sexually transmitted diseases. A convenient-to-use array system is offered by Euroimmun Medizinische Labordiagnostika AG. The company uses the so-called BIOCHIP technology. Small DNA microarray glass plates are adhered to plastic EUROArray object slides. The hybridization is performed in a standardized manner using the TITERPLANE® technology. CE-IVD certified assays, e.g., for autoimmune diseases (e.g., detection of rheumatic disease-associated alleles of human leukocyte antigen B27 (HLA-B27) or detection of psoriasis-associated alleles of HLA-Cw6) or an assay for the detection of human papilloma virus (HPV) types, are provided (http://www.euroimmun.de/index.php?id=startseite). Greiner Bio One International AG also offers CE-IVD certified microarray test kits. Their oCheck® product line comprises DNA microarrays for the diagnostic of 24 different HPV types or 20 periodontal pathogens and even a DNA microarray for the identification of animal species for food control. An open array platform for the development of multiparametric tests for research and diagnostics is offered by Alere. The

ArrayTube system consists of a custom microarray integrated into a microreaction tube. Parallelization is allowed by the ArrayStrip system. Here, the assays are available in a microplate-compatible format. Several assay types can be implemented like nucleic acid as well as protein- and peptide-based arrays. An overview of relevant publications is given on their website (http://alere-technologies.com/en/science-technologies/publications.html).

3.1 Lab-on-a-Chip Devices: Applicable for Multiparametric Tests with Need for Fast Information

There are several manual steps in diagnostics with DNA microarrays from sample to result like nucleic acid extraction, amplification or reverse transcription, labeling, hybridization, data readout, and data analysis. Each step is a potential risk for contamination and mistakes. One possibility to overcome these limitations is automation by using so-called micro total analysis systems (μTAS) or lab-on-a-chip devices (LoC) that allow for a complete sample preparation, analysis, and readout in a single closed device. In such microfluidic devices μ-, nano-, or even picoliter volumes are transported through micrometer-sized channels and are filtered, mixed, further processed, and detected. Fluid transport is often pressure-driven but alternatively also induced by electroosmotic flow or capillary forces. Complete automation harbors several advantages like getting by with smaller sample volumes, diminished contamination risk, faster assay processing due to small diffusion ways, efficient thermodynamics, and favorable surface-to-volume ratio as well as multiplex- and high-throughput capabilities. The implementation of complete processes from sample to result in LoC devices, however, is complex. First, one has to implement single functional units that in the end have to be combined to run in concert in one complete process. Challenges next to others are the choice of the material that has to be compatible to all assay steps, the assembly process, and the modification of the biological assay for maximal compatibility. Many research institutions are working on the development of LoC devices. Reviewing these, however, is beyond the scope of this review. The focus lies on array-based LoC devices that found or may find their way into the clinical routine laboratory.

Several companies are working on LoC devices for nucleic acid analytics; some of them use DNA microarrays as detection system, like Curetis, a company in Holzgerlingen, Germany, that uses a patented special array technology in a LoC device. Cell lysis is done in a separate processing unit. All other steps (purification, amplification, and detection) are then performed in a single cartridge. The Curetis technology can be used to diagnose infections. CE-certified cartridges are offered for the detection of pathogens and resistance markers for pneumonia (Unyvero™ P50) and implant and tissue infections (Unyvero™ i60 ITI). FDA clearance is aspired. Another complete solution with array-based detection is developed by

Nanosphere. Their Verigene system allows for the simple testing of nucleic acid and protein on a single platform (http://www.nanosphere.us/technology). For the detection of DNA or RNA targets, nanoparticle probe technology is used. Automated nucleic acid extraction and PCR amplification from a clinical sample take place on the Verigene® Processor SP. Eluted nucleic acids are transferred automatically into a Verigene Test Cartridge for hybridization of target DNA to capture oligonucleotides on a microarray. Detection is realized by specific mediator oligonucleotides and gold nanoparticle probe; the signal amplification of hybridized probes is done via a silver staining process. Automated qualitative analysis of results is performed on the Verigene Reader. Diseases like bacterial infections or cardiac tests are addressed. Rheonix (http://www.rheonix.com/technology/technology-overview.php) develops the Encompass platform. Disposable cards with onboard reagents and a low-density microarray- or qPCR-based detection system are the heart of the system. The company states that every user-designed or future FDA-cleared test can be run in their card due to the high flexibility of the system. Product development programs for infectious diseases, pharmacogenomics, and environmental applications are in progress.

Next to DNA microarray-based detection in LoC devices one can find quantitative real-time PCR, pH-mediated detection, or NGS technologies as readout strategy. Cepheid (http://www.cepheid.com/us/) has developed an integrated benchtop analyzer (GeneXpert) for the detection of several pathogens and resistance markers. They have a microfluidic cartridge where ultrasonic lysis and multiplex rapid real-time PCR technique are realized. The company has CE-certified cartridges for the detection of, e.g., *Clostridium difficile* (*C. difficile*), methicillin-resistant *Staphylococcus aureus* (MRSA), or norovirus and FDA-approved tests for, e. g., MRSA or tuberculosis. Focus Diagnostics (http://www.focusdx.com/3m-integrated-cycler/ud-intl) developed the 3M™ Integrated Cycler for a disk-based qPCR-based analytical system. 96 samples can be run in parallel. Assays are offered for the detection of viral-caused infections like dengue fever, influenza, and infectious mononucleosis (Epstein–Barr virus) or for the detection of bacteria like *Bordetella* species or *C. difficile*. Idaho Technologies [now BioFire (Biomerieux)] develops a product series based on the FilmArray® (Poritz et al. 2011). This novel diagnostic platform combines automated sample preparation, nucleic acid extraction, and PCR-based detection of multiple targets from a single unprocessed sample in 1 h. It combines nesting and multiplexing of the PCR (referred to here as nested multiplex or "nmPCR") together with DNA melting curve analysis to detect and distinguish multiple pathogens simultaneously. The FilmArray and the FilmArray Respiratory Panel (RP) pouch have since received FDA clearance for use as an in vitro diagnostic (IVD) device. Advanced Liquid Logic (http://www.liquid-logic.com/technology) which was acquired by Illumina in July 2013 has a digital microfluidic technology that is based on the use of electrowetting to precisely manipulate droplets on a surface. For electrowetting, a voltage is applied between a droplet and an insulated electrode that can cause the droplet to spread on the surface and allows the precise manipulation of droplets within a sealed microfluidic LoC. Illumina is mainly interested to deliver the simplest and most efficient sample-to-answer next-generation

sequencing (NGS) workflow (press release). DNA Electronics (http://dnae.co.uk/technology/overview/) develops electronic-based microchip solutions for DNA and RNA detection. An ion-sensitive field effect transistor (ISFET) is used for genotyping or semiconductor-based sequencing. Whenever DNA or RNA is amplified, a H+ ion is released that can be detected by the ISFET. Therefore, specific hybridization events can be detected. Addressed healthcare applications are in the field of personalized medicine and infection screening. Another sensor technology is developed by GeneFluidics. The technology enables quantification of nucleic acids and proteins on a single platform with electrochemical detection. High sensitivity even in unamplified, unpurified biological samples is advertised. Results are delivered within 1 h. Sensor surfaces are functionalized by antibodies or DNA. After the binding event and washing steps, a secondary antibody with HRP (reporter enzyme binding) is applied. Signals occurring during enzymatic conversion of HRP substrate are proportional to the analyte concentration (http://www.genefluidics.com/technology). HandyLab, acquired by BD in 2009, developed disposable cartridges with onboard dry reagents and patented real-time microfluidic PCR technology. Cartridges could be run in a benchtop instrument that integrated heating, mechanical valves for fluid control, and fluorescent detection. FDA-approved tests were available for MRSA, *C. difficile*, and group B *Streptococci*. These tests and one further test (vancomycin-resistant *Enterococci*) were approved for use in Europe. Another interesting and completely different system is the lab-in-a-tube system (Liat™ system) by IQuum (http://www.iquum.com/products/technology.shtml). All assay reagents are prepacked in tube segments separated by peelable seals in the Liat. The Liat is compressed by sample processing actuators of the Liat analyzer so that reagents are selectively released from tube segments, the sample is moved from one segment to another, and reaction conditions are controlled. You can start with a variety of sample matrices, including whole blood, plasma, urine, and swab samples. All required assay processes, including reagent preparation, nucleic acid purification, amplification, and real-time detection, are performed by the analyzer in 20 min–1 h, depending on the assay. Rapid PCR amplification and real-time detection are integrated. Products are the Liat™ Influenza A/2009 H1N1 Assay (research use only), a fully automated sample-to-result detection of viral RNA in 26 min that received FDA Emergency Use Authorization in 2009 (since expired), and the Liat™ Influenza A/B Assay (IVD product). Assays for HIV, CMV, influenza subtypes, and dengue are in the product pipeline.

4 Next-Generation Sequencing: The End of the DNA Microarray Era?

After 30 years of Sanger sequencing, within 3 years between 2005 and 2007, three different novel commercially available sequencing approaches have been introduced: the pyrosequencing technology by 454 Life Sciences, later acquired by

Roche, the ligation-based short-read sequencing technology by Applied Biosystems (SOLiD), and the Solexa short-read sequencing system that uses bridge amplification and sequencing by reversible dye terminators, later acquired by Illumina. The discussion of these impressive technological approaches summed up as next-generation sequencing (NGS) approaches is not in the scope of this review. A nice overview is given, e. g., in (Voelkerding et al. 2009). Since then, several other high-throughput sequencing technologies have been developed. One example is the semiconductor-based nonoptical sequencing system by Ion Torrent/Life Technologies. A pH-shift occurring during incorporation of desoxy-nucleotides in the growing strand is monitored by an ion-sensitive field effect transistor (Rothberg et al. 2011). Today, even single molecule sequencing is possible (Pacific Biosciences; http://www.pacificbiosciences.com/), and technologies are being developed that could allow the sequencing of whole genomes through nanopores within the shortest time (announced e. g. by Oxford Nanopore Technologies; https://www.nanoporetech.com/).

Nearly every DNA-based array platform has been mirrored to sequencing-based technological procedures. With the enormous high-throughput capabilities of NGS technology, whole-genome approaches if affordable can be performed, including transcription analysis [RNAseq, reviewed in (Mutz et al. 2013)], targeted genomic or even whole-genome re-sequencing (Ng et al. 2009, 2010; Tsuji 2010), comparison of genomes, mapping of DNA-binding proteins and chromatin analysis, epigenetics [reviewed in (Capell and Berger 2013; Mensaert et al. 2014)], methylation analysis [reviewed in (Olkhov-Mitsel and Bapat 2012)], or meta-genomics [reviewed in (Cox et al. 2013; Kim et al. 2013; Miller et al. 2013)].

Interestingly, a combination of microarrays and next-generation sequencing has been established. In order to focus on the most relevant part of the human genome for SNP detection, exome sequencing can be performed instead of using genotyping arrays. In exome sequencing, all coding sequences of the human genome are sequenced to identify variants between individuals. Approximately 180,000 exons comprising 30 million base pairs are reported which constitute about 1 % of the human genome (Directors ABo 2012). Focusing on mutations in the exons which are much more likely to cause an effect, the sequencing effort can be reduced by two orders of magnitude, if the exons can be enriched efficiently. Several enrichment methods are described (Lin et al. 2012). One of them uses DNA microarrays containing all complementary sequences of exons. For example, Nimble Gene developed a so-called Sequence Capture Human Exome 2.1 M Array to enrich all ~180,000 coding exons (Choi et al. 2009). Exome sequencing can be used as an approach to identify unknown causal genes for rare Mendelian disorders (Ng et al. 2009, 2010). The advantage of the sequencing technology is that it does not require any preexisting knowledge—one gets every mutation present—and not only the SNPs represented on the array. The challenge in this case is the specific sequence enrichment and to generate the know-how about the effect of the individual SNPs detected or their combination on human well-being. The first company making exome sequencing available for diagnostic purposes is Ambry. They introduced the Clinical Diagnostic Exome™. The company also offers more

than 300 highly specialized genetic tests (http://www.ambrygen.com/clinical-diagnostics).

Focusing on single genes, in diagnostics today, Sanger sequencing is used for the identification of rare variants. NGS has the capacity to replace Sanger sequencing by targeted genomic NGS (Sikkema-Raddatz et al. 2013). Using this approach, only subsets of genes that are known to be important and involved in a special disease are sequenced. Sikkema-Raddatz et al. constructed a targeted enrichment kit that includes 48 genes associated with hereditary cardiomyopathies and demonstrated that targeted NGS of a disease-specific subset of genes is equal to the quality of Sanger sequencing using the Illumina MiSeq sequencer (Sikkema-Raddatz et al. 2013). They state that it can therefore be reliably implemented as a stand-alone diagnostic test. *Illumina* offers the MiSeqDx Cystic Fibrosis (CF) 139-Variant Assay that detects 139 clinically relevant *CFTR* (*CF-transmembrane conductance regulator*) variants and the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay that accurately captures all variants in the protein-coding regions and intron/exon boundaries of *CFTR* (Grosu et al. 2014). These are the first FDA-cleared NGS-based assays and kits for in vitro diagnostic use on the MiSeqDx instrument (http://www.illumina.com/clinical/diagnostics.ilmn).

Currently, NGS technologies are getting cheaper and easier to handle and also might be the molecular assay of choice in the future in the clinic. Clinical diagnostics requires easy-to-handle experimental procedures, robustness, accuracy, sensitivity, and comparable or lower costs compared to existing diagnostic approaches. Although, recent developments in NGS have led to small instruments that allow flexible throughput and short run times, current drawbacks of NGS approaches are their high investment and running cost, elaborate library preparation, and complex data analysis. Detached from these shortcomings, with the possibilities of NGS, novel diagnostic scales are thinkable not only for diagnostics of cancer, mitochondrial disorders, or hereditary diseases where the assay time is in general not critical but also for assays in clinical microbiology like the detection of MRSA or sepsis causative organisms. Within the scientific community, workshops and symposiums are held everywhere dealing with the topic how NGS can be validly used for clinical diagnostics. The FDA also looks deeper into this subject and performs public workshops that focus on the evaluation of the use of NGS technologies for clinical diagnostics (e.g., Ultra High Throughput Sequencing for Clinical Diagnostic Applications—Approaches to Assess Analytical Validity: Report from the Public Meeting (June 23, 2011), http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm284442.htm and Public Workshop—Advancing Regulatory Science for High Throughput Sequencing Devices for Microbial Identification and Detection of Antimicrobial Resistance Markers, April 1, 2014, http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm386967.htm). These activities indicate a strong competition of this new technology in the field of diagnostics.

5 Conclusions and Outlook

Microarrays have been developed and are used to detect or analyze a wide range of target molecules. A great portfolio of excellent research has been done in the field not only dealing with the molecular interaction but also with data analysis or automation of assay steps. Although developed nearly 20 years ago, the technology until now didn't really find its way into routine clinical laboratory diagnostics. Only a few arrays are used for such applications as described above. As the main reasons for this, the high complexity of the technology, missing robustness, accuracy, and sensitivity are discussed. Microarray procedures require many complex, often manual, steps that are prone to errors and need skilled staff and expensive equipment for processing. One way to overcome these shortcomings might be automation of the process steps using LoC devices that allow for a convenient and reproducible processing of patient samples with minimized risk for handling errors or contamination. Diagnostic assays beginning from sample preparation up to and including data readout and processing can be easily performed using such systems. This might be the most promising path for microarrays to find their way into clinical laboratory diagnostics at a larger scale.

Nevertheless, NGS has a high potential to revolutionize the diagnostic market within the next decades. As an open technology, it clearly competes with array-based systems where you only get what you screen for. The cheaper and the more comfortable the sequencing systems get, including data storage, analysis, and interpretation, the sooner they will replace array-based systems. However, for the detection of exactly defined genetic disorders, in clinical microbiology, and for the detection of a limited set of molecular markers, e.g., on a transcriptional level for cancer diagnostics, array technology still will for the foreseeable future be a valuable diagnostic tool to aid in therapy.

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