

Oligonucleotide and DNA Microarrays: Versatile Tools for Rapid Bacterial Diagnostics

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Abstract

The rapid and unambiguous detection and identification of microorganisms, historically a major challenge of clinical microbiology, gained additional importance in the fields of public health and biodefence. These requirements cannot be well addressed by classical culture-based approaches. Therefore, a wide range of molecular approaches has been suggested. Microarrays are molecular tools that can be used for simultaneous identification of microorganisms in clinical and environmental samples. Main advantages of microarrays are high throughput, parallelism and miniaturization of the detection system. Furthermore, they allow for both high specificity and high sensitivity of the detection.

Microarrays consist of set of probes immobilized on a solid surface. Even though the first application of the microarrays can be seen as relatively recent (Schena et al. 1995), the technology developed rapidly reaching the milestone of 5,000 published papers in 2004 (Holzman and Kolker 2004). This development encompasses both the successful transfer of various technological aspects as well as the expansion of the application scope. The most important technological elements of custom-made platforms as well as the characteristics of the commercially available formats are reviewed in this chapter. Furthermore, application potential is presented together with considerations about quality control.

1. Introduction

For almost two centuries, clinical microbiology laboratories have relied on culture methods. The essence of this discipline was to provide growth evidence of a suspected pathogen, and enable physicians to correlate this observation with a given clinical presentation. Koch's postulates, derived from his work on infectious diseases such as anthrax and tuberculosis, established a standard for causation in infectious disease. The standard was intended to convince skeptics that microbes can cause disease and to push microbiologists to use more rigorous criteria before claiming a causal relationship between a microbe and a disease (Fredericks and Relman 1996).

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Addressing Koch's postulates implies good knowledge of bacterial physiology. Current diagnostic methods have to care about dozens of living species that require various growth culture media, growth temperatures, and sometimes specific incubation atmospheres. Bacteria could therefore be considered as "challenging analytes."

Generally speaking, results generated by a clinical microbiology laboratory fall within the following categories: detection, identification (or speciation), antimicrobial susceptibility testing, and typing. Rapid reporting time is a constant requirement. Results have to be delivered to clinicians in due time, i.e., early enough to meaningfully influence patient work-up and therapy.

We have to admit that culture-based approaches have reached their golden age: their performance can no longer be significantly improved. In particular, time for bacterial growth represents a barrier that prevents more rapid, and clinically relevant, delivery of results to physicians. Simply speaking, we should bypass all culture steps that are, by essence, limited by microbial physiology. Culture-based approaches also display limitations towards groups of clinically important microorganisms (e.g., fastidious or hardly cultivable organisms), not to mention those that cannot be cultivated (e.g., *Treponema pallidum*) or that have been killed during handling (e.g., improper transport of sample containing *Neisseria meningitidis*) or by exposure to antimicrobials.

Thinking of molecular approaches, real-time PCR (qPCR) sounds rather appealing. However, due to limited multiplexing capabilities, qPCR will likely not be the appropriate molecular method to replace cultures. Miniaturization of conventional assays is a general trend in diagnostics as well as in biomedical research. Working with smaller volumes leads to reduced reagent consumption and faster reaction kinetics due to increased sample concentration. Parallel determination of numerous reactions is also highly desirable to save time and reduce costs, not to mention the possibility of large-scale comparison and intra-experimental quality control. Probes bound to a solid surface were therefore developed to allow spatial discrimination of numerous reactions performed in parallel, and culminated in the recent development of microarrays (Schena et al. 1995; Fodor et al. 1991, 1993).

Microarrays consist of an orderly arrangement of probes (oligonucleotides, DNA fragments, proteins, sugars, or lectins) attached to a solid surface. The main advantages of microarray technology are: high-throughput, parallelism, miniaturization, speed, and automation. Despite the fact that microarray analysis is a relatively novel technology, described only a decade ago (Schena et al. 1995), microarrays are now broadly applied. The scientific and technological background discussed here will be limited to DNA microarrays, excluding the new evolving field of protein microarrays and that of glycomics (Raman et al. 2005).

2. Microarray Technology

With the publication of the first microarray studies in 1995 (Schena et al. 1995; Lipshutz et al. 1995), and the milestone of nearly 5,000 published microarray papers in 2004 (Holzman and Kolker 2004), there is no doubt that this technology has rapidly spread into basic and applied research.

A microarray refers to a checkerboard-like ordering of molecules on a surface. It is the molecular equivalent of a spreadsheet, where each cell or address contains a specific probe designed to detect a given target (Southern, Mir and Shchepinov 1999). By analogy to antigen-antibody interactions on immunoarrays, DNA microarrays rely on sequence complementarity of the two strands. They put into practice the fundamentals of complementary base-pairing (hybridization) that were first described by Ed Southern (Southern 1975). In general, the strategy

of microarray hybridization is reversed to that of a standard dot-blot, leading to recurring confusion in the nomenclature. Therefore, the suggestion has been made to describe tethered nucleic acid as the probe and the free nucleic acid as the target (Phimister 1999).

Earlier studies on duplex melting and reformation, carried out on DNA solutions, have provided the basic knowledge (the reaction kinetics as well as the computational determination of the melting point as a function of nucleic acid composition and salt concentration) (SantaLucia 1998; SantaLucia, Allawi and Seneviratne 1996; Allawi and SantaLucia 1998). Much of the pioneering work can be linked to the use of nitrocellulose membranes (Gillepsie and Spiegelman 1965), dot-blot (Kafatos, Jones and Efstratiadis 1979), and Southern blots (Southern 1975). Development of cDNA or oligonucleotide arrays was made possible by combined innovations in microengineering, molecular biology (Case-Green and Southern 1994; Maskos and Southern 1993b; Mir and Southern 1999; Shchepinov, Case-Green and Southern 1997; Southern et al. 1994; Southern, Mir and Shchepinov 1999), and bioinformatics (Fodor et al. 1991). The real breakthrough in microarray technology was initiated by two key innovations: the use of nonporous solid supports (such as glass and silicon) and the development of methods for high density synthesis of oligonucleotides directly onto the microarray (Fodor et al. 1991).

Powerful fundamental or applied projects using miniaturized, high-density microarrays have been demonstrated for a broad variety of applications such as cell differentiation (Tamayo et al. 1999), whole-genome expression analysis (Gasch et al. 2000; Schena et al. 1995), cancer research (Alizadeh et al. 2000; Perou et al. 2000; Golub et al. 1999), comparative genome hybridization (CGH) (Salama et al. 2000), drug discovery (Debouck and Goodfellow 1999), vaccine development (Grifantini et al. 2002), and single nucleotide polymorphism (SNP) analysis (Fan et al. 2000).

Technology transfer to diagnostic applications is therefore very appealing (Lucchini, Thompson and Hinton 2001; Ye et al. 2001; Aitman 2001). High-throughput technologies, such as DNA microarrays, have significant potential for identifying organisms in many areas of biomedical science, including health care, biological defense, and environmental monitoring. Using data from increasing numbers of whole microbial genomes, thousands of sequences can be selected to probe numerous genes of interest in cultures, clinical specimens, environmental samples, or host tissues. Reports have already shown that oligoarray hybridization can provide bacterial detection of a conserved bacterial gene (Small et al. 2001), species identification (Wilson et al. 2002), and genotyping of bacterial pathogens, by using large sets of discriminative epidemiological markers (Gingeras et al. 1998; Yue et al. 2001; Call, Borucki and Loge 2003; Volokhov et al. 2002; Chizhikov et al. 2001). Detection of genetically-encoded virulence or antimicrobial resistance determinants (Lucchini, Thompson and Hinton 2001; Troesch et al. 1999; Chizhikov et al. 2001; Bekal et al. 2003) may also afford a major benefit for the selection of an adequate chemotherapy.

Current limitations for the routine implementation of microarrays to detect DNA signatures are, for example, their high manufacturing costs and the requirement of large amounts of nucleic acids. Availability of high amounts of nucleic acid targets requires either a large volume of bacterial culture (biological amplification), or target amplification (Mikulowska-Mennis et al. 2002; Puskas et al. 2002). Additional technical achievements in signal amplification methods (Francois et al. 2003) and novel optical techniques (Pawlak et al. 2002; Ferguson, Steemers and Walt 2000) have already improved target detection to the femtomole range.

Development of a microarray-based bacterial identification starting directly from the biological sample, without any enzymatic target amplification, is an important objective (Straub and Chandler 2003). This procedure would significantly reduce the turnaround time and overcome enzymatic-induced signal alterations or biases (Yershov et al. 1996; Bavykin et al. 2001).

3. Technical Aspects of Microarray Technology

The development of a new microarray platform requires consideration of many different features, most of them being co-dependent. Different approaches have been reported, each of them exhibiting certain advantages and limitations. A summary of the most important technological features will be presented to introduce the specific platforms in association with their intended experimental applications.

3.1. Probes

The nature of the probe used is related to the experimental question. In general a distinction can be made between genome fragments, PCR products, and oligonucleotide probes. The application potential as well as the advantages and limitations of each probe type will be described briefly.

3.1.1. Genome Fragments

The use of entire bacterial or community genomes (suitably fragmented) as probes was first employed for the reverse sample genome probing (RSGP) technique (Voordouw et al. 1991). The same principle was later applied for community genome arrays (Murray et al. 2001; Bae et al. 2005). The major problem related to such microarray platforms is the huge complexity of the system.

3.1.2. PCR Products

PCR products used as probes for microarray fabrication are mostly amplified inserts of the clone libraries. Different types of clone libraries can be used as a template for microarray fabrication (cDNA libraries, SSH libraries, shotgun libraries). Microarrays utilizing PCR products are used for gene expression analysis (Schena et al. 1995; Gill et al. 2002; Zaigler, Schuster and Soppa 2003; Stowe-Evans, Ford and Kehoe 2004; Murray et al. 2001; Lindroos et al. 2005). Furthermore, PCR products can be used as probes for functional gene arrays (Wu et al. 2001; Call et al. 2003). The inappropriate labeling of a substantial fraction of the PCR products (1 to 5 %) can lead to poorly controlled microarrays, even when originating from prestigious research centers or commercial entities (Knight 2001). The IMAGE consortium (Integrated Molecular Analysis of Genomes and their Expression) revealed that only 62 % of 1,189 cDNAs were pure and correct, after resequencing.

3.1.3. Oligonucleotide Probes

Compared to cDNA microarrays, oligoarrays provide a flexible design and are considered more reliable in terms of sensitivity and specificity (Blanchard and Friend 1999; Barczak et al. 2003; Kothapalli et al. 2002; Li, Pankratz and Johnson 2002). Differing from the previously described PCR probes, oligonucleotide probes are typically designed with a predefined specificity. The development of generic or universal microarrays will be described in a separate section (see Section 23.5.3).

Two main features influence probe specificity: probe length and the degree of conservation of the marker gene. In general, probe design is carried out *in silico* using different software tools, e.g., ARB (Ludwig et al. 2004) or OligoCheck (Charbonnier et al. 2005), and is based on a sequence database of the targeted marker gene. The extent and quality of the sequence database has a major effect on the probe quality.

The general criteria that need to be considered during probe design are: (1) the required probe specificity, and (2) the uniformity of the probe set regarding hybridization behavior. *In silico* approaches allow for partial prediction of the hybridization behavior of the designed probes. However, it has become clear that the simple notion that short oligonucleotides with a mismatch (MM) should hybridize less efficiently than perfect match (PM) probes is not always applicable (Pozhitkov et al. 2006). It has been demonstrated that the hybridization intensity of MM probes can depend on the nucleotide type (i.e., A, C, G, or T) and the position of the MM relative to the termini (Urakawa et al. 2002); and that some MM probes yield even higher signal intensities with the target than those of corresponding PM probes (Naef and Magnasco 2003). Even well-designed probes can display differences in maximal hybridization capacity of 2 orders of magnitude under different hybridization conditions (Bodrossy and Sessitsch 2004); and thus, it is difficult to find one set of conditions that is optimal for all probes on an array (Kajiyama et al. 2003; Urakawa et al. 2003). Factors affecting duplex formation on DNA microarrays include: probe density, microarray surface composition, spacer length, and the stabilities of the oligonucleotide-target duplexes, intra- and intermolecular self-structures, and RNA secondary structures (Matveeva et al. 2003; Peterson, Heaton and Georgiadis 2001; Halperin, Buhot and Zhulina 2005).

More generally, there is a lack of a simple relationship between hybridizations of probe-target duplexes as inferred from signal intensity values and *in silico* predictions based on Gibbs free energies (Pozhitkov et al. 2006). This does not apply as strictly for high-density microarrays, where the high level of redundancy accounts for the specificity of the signals; nor for long oligonucleotide microarrays, where inter-allele distinction is not required. In any case, a thorough wet-lab validation with a set of reference strains or clones is warranted, as with the implementation of any other molecular tool (Charbonnier et al. 2005).

Original approaches were recently published to address the issue of cross-hybridization while maintaining target sensitivity. Using *E. cucululi* as a biological model and conventional probe design, Rimour et al. could determine specific 50-mer oligonucleotides for only approximately 40 % of the genome. When relying on their new probe design strategy (GoArrays), based on the determination of two specific subsequences, they were able to design specific probes for each CDS of the genome (Rimour et al. 2005).

3.1.3.1. Long Oligonucleotides

The main advantages of long oligonucleotides (over 50 nucleotides in length) are high target binding capacity and irreversible hybridization kinetics. These features allow for enhanced detection sensitivity. However, the threshold for the differentiation is at 85 to 90 % sequence similarity, resulting in reduced specificity. This can be compensated by the host specificity of the targeted genes. Due to their high sensitivity, long oligonucleotide microarrays are typically used in combination with universal amplification techniques or without any amplification, allowing the researcher to target an unlimited number of different genes (Tiquia et al. 2004). Long oligonucleotide microarrays have been used for the detection of viruses (Wang et al. 2002) and pathogens (Vora et al. 2004).

3.1.3.2. Short Oligonucleotides

Short oligonucleotide (15 to 30-mer) microarrays are more precise in the detection of shorter nucleotide polymorphisms, including single nucleotide differences under optimized hybridization conditions. On the downside, short oligoarrays frequently require a larger number of probes for reliable diagnostics. Reversible hybridization kinetics and lower target binding capacity (in comparison to long oligonucleotides) are responsible for somewhat limited detection

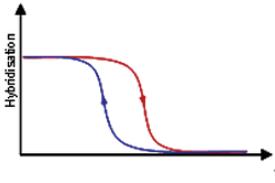
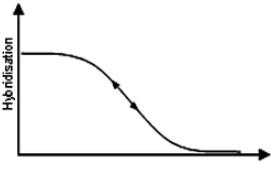
	Long	Short
Typical length	>50	15-30
Optimal hybridization conditions	1 M Na ⁺ , 65-70 °C, no formamide	1 M Na ⁺ , 40-60 °C, no formamide
Optimal wash conditions	0.015 M Na ⁺ , 65 °C	0.015 M Na ⁺ , 20-22 °C
hybridization kinetics	Irreversible	Reversible
		
Melting temperature is at which...	50% of the nucleotides are unbound (T_m^∞)	50% of the strands are single stranded (T_m)
Dissociation is ...	intramolecular process $\rightarrow T_m^\infty$ is concentration independent	intermolecular process $\rightarrow T_m$ is concentration dependent
Target used for hybridization...	...can be very complex, no specific amplification needed.	...has to be restricted to one or a few genes, PCR amplification required

Figure 23.1. Comparison of long *versus* short oligonucleotide probes.

sensitivity (Fig. 23.1). Short oligonucleotides are widely used for both environmental and diagnostic microbial diagnostic microarrays (MDMs) (Bodrossy et al. 2003; Hashsham et al. 2004; Sergeev et al. 2004; Loy et al. 2005).

3.2. Substrates for Printing

The choice of the substrate for microarray printing depends primarily on the nature of the probes. An important factor to be taken into consideration is the effect of steric hindrance on the hybridization efficiency (Bodrossy et al. 2003). This may be a considerable problem in the case of short oligonucleotide probes, and therefore such probes are generally appended to spacer molecules. Further important parameters to be considered during fabrication of the microarray are the probe concentration, spotting buffer, and surface blocking strategies. Most of these features have been discussed thoroughly in the literature (Zammatteo et al. 2000; Lindroos et al. 2001; Taylor et al. 2003; Hessner et al. 2004). The most widely used microarray format is a planar glass slide (1 × 3 in). Slides for microarray printing are usually coated with different active surfaces that facilitate deposition of nucleic acids. An overview of the more commonly used substrates and their applications will be given hereafter.

3.2.1. Slides with Poly-L-Lysine Coating

The binding of DNA fragments to poly-L-lysine involves charge interactions that can be converted to covalent bonding by baking or UV-crosslinking. Advantages of poly-L-lysine surfaces are their low background and good signal intensities. The main disadvantage is low temperature resistance that can lead to the damage of the surface during denaturation or hybridization at higher temperatures. Poly-L-lysine coated slides have been successfully used for the binding of PCR products (Schena et al. 1995; Diehl et al. 2001; Zaigler, Schuster and Soppa 2003) and short oligonucleotides (Taylor et al. 2003).

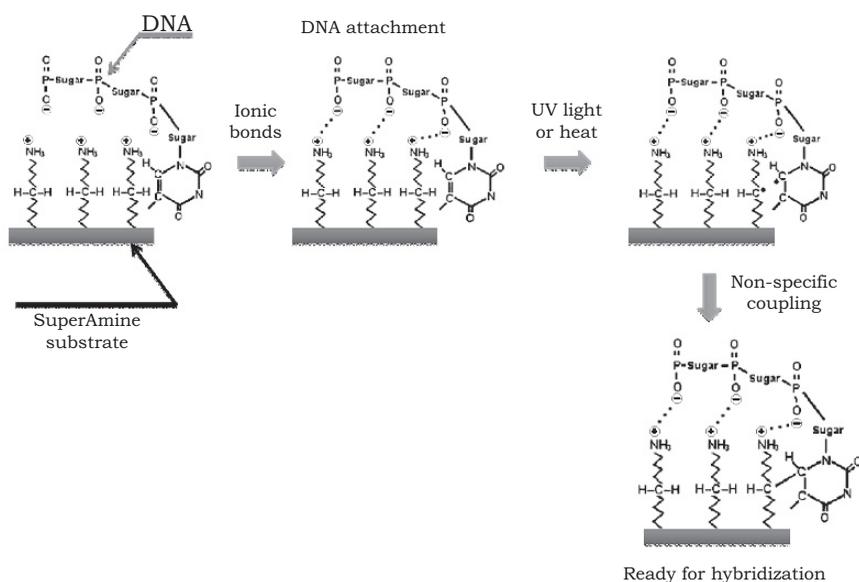


Figure 23.2. Binding chemistry of amino silane-coated slides (Reprinted with permission from TeleChem International, Inc., www.arrayit.com).

3.2.2. Slides with Amino Silane Coating

Amino silane surface chemistry allows for electrostatic interactions between the amino groups of the silane (positively charged at neutral pH) and the negatively charged phosphodiester backbone of the DNA. This interaction can be additionally stabilized by UV-crosslinking (Fig. 23.2). Amino silane coating demonstrates enhanced resistance towards high temperatures. However, somewhat higher background signals may occur when low quality coatings are used. Substrates based on amino silane surface chemistry are widely used for deposition of PCR products (Gill et al. 2002; Stowe-Evans, Ford and Kehoe 2004; Deneff et al. 2003; Rhee et al. 2004; Rudi et al. 2003; Treimo et al. 2006).

3.2.3. Slides with Aldehyde Coating

Covalent binding between aldehyde groups and DNA fragments (Fig. 23.3) can be facilitated either through a 5'-amino linker on chemically modified DNA fragments or through aromatic amines of nucleotides. Probes with the 5' amino group bind more efficiently than native DNA fragments. Furthermore, coupling via the 5' amino group is directional, allowing for the defined orientation of the probes on the microarray. In general, slides with aldehyde coating are characterized by high binding capacity and low background. Aldehyde-coated slides are mostly used for short oligonucleotide microarrays (Bodrossy et al. 2003; Loy et al. 2002).

3.2.4. Slides with Epoxy Coating

Similarly to aldehyde coated slides, epoxy coated substrates (Fig. 23.4) also allow for covalent binding utilizing amino groups of the DNA fragments. Epoxy coated slides have been used for the deposition of PCR products (Call et al. 2003), and short (Bailey et al. 2006) and long (Wang et al. 2004) oligonucleotides.

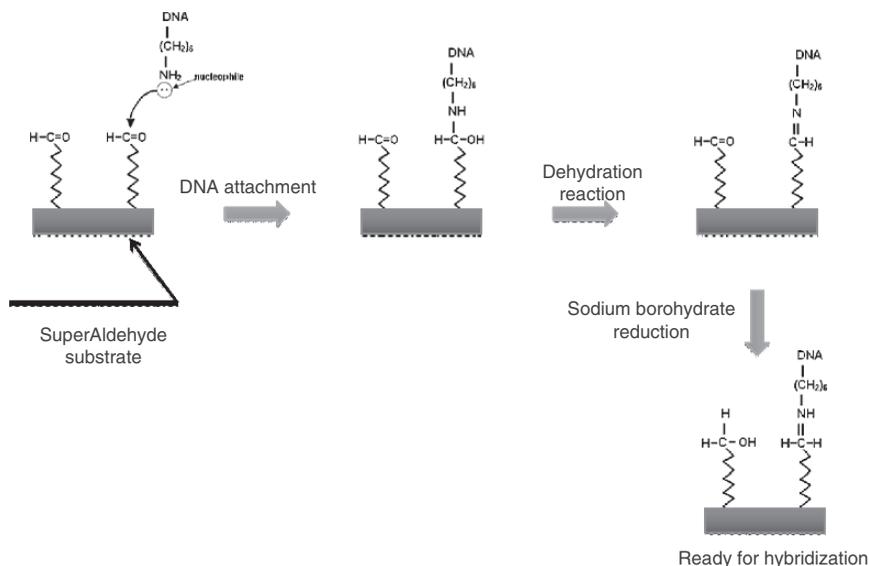


Figure 23.3. Binding chemistry of aldehyde-coated slides (Reprinted with permission from TeleChem International, Inc., www.arrayit.com).

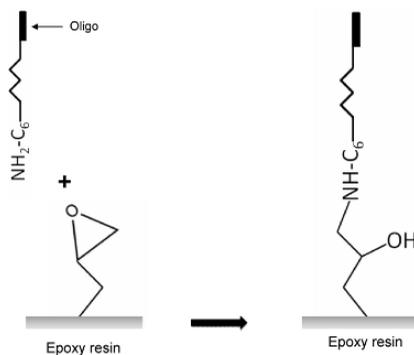


Figure 23.4. Binding chemistry of epoxy-coated slides.

3.2.5. Proprietary Surface Chemistries

Finally, it is worth mentioning that the array surface of various commercial microarrays can display very different properties, e.g., a highly hydrophobic surface on the Agilent SurePrint technology (Agilent, Palo Alto, CA) because of proprietary surface chemistry. This leads to the use of specific hybridization and washing buffers adapted to the surface chemistry.

3.2.6. Probe Spacers

To reduce the effect of steric interference (steric hindrance and surface electrostatic forces) (Vainrub and Pettitt 2002; Shchepinov, Case-Green and Southern 1997) on hybridization of targets to planar surfaces (e.g., glass and silicon), spacer molecules with a length of more than 50 \AA can be used to physically separate the probes from the microchip surface (Peplies, Glockner and Amann 2003; Shchepinov, Case-Green and Southern 1997). These are typically

C₆-C₁₂ alkane spacers and/or 5–15 thymidine or adenine residues added to the tethered end of the oligoprobe (Anthony, Brown and French 2000; Bodrossy et al. 2003; Zhang, Hurek and Reinhold-Hurek 2006; Francois et al. 2005).

3.3. Targets for Microarray Analysis

Targets used for microarray analysis are typically fluorescently labeled nucleic acid derivatives. Two basic types of targets can be distinguished: those derived from RNA, and those derived from DNA templates. The selection of nucleic acids used as a template for target preparation is primarily dependent on the experimental question. Gene expression studies use mRNA-based targets, whereas microbial diagnostic arrays employ primarily DNA-based targets, or rRNA targets that are more abundant. The parallel analysis of mRNA- and DNA-based targets provides a complex picture correlating presence and activity (Bodrossy et al. 2006). See Section 23.5 for further discussion of possible applications.

3.3.1. Target Amplifications and Sensitivity Issues

In general, targets used for short oligonucleotide diagnostic microarrays have been previously PCR-amplified. PCR amplification ensures enrichment of the targeted gene(s) and therefore increases the sensitivity of microarray detection, but this introduces an inherent PCR bias (Vora et al. 2004). Long oligonucleotide probes exhibit higher target binding capacity and therefore allow hybridization with highly complex target mixes (i.e., unamplified environmental DNA, a native mixture of mRNA from an organism, or the products of universal, whole genome amplification methods). The main advantage of the latter targets is that they represent the entire gene pool to be studied, without reduction of its complexity.

Thus, the potential of DNA microarray-based microbial screening and diagnostic technologies is currently limited by front-end target-specific nucleic acid detection. The presence of a ubiquitous poly-adenylated tail at the 3'-end of eukaryotic messenger RNAs offers the possibility of converting minute amounts of RNA to micrograms of labeled material, with minimal effects on the respective abundance of the mRNA mixture (Aoyagi et al. 2003; Puskas et al. 2002). Prokaryotic RNAs are not poly-adenylated and thus are more challenging to work with when starting material is scarce. In such cases, the use of generic primers able to amplify parts of the 16s rRNA gene is often required (Anthony, Brown and French 2000; Rudi et al. 2002), but the universality of such primers is questionable: false negative signals are not rare (Anthony, Brown and French 2000). Other options include intact or even degraded RNA amplification using T3-coupled random primers (Xiang et al. 2003) or a limited set of genome-derived cognate primers (Talaat et al. 2000).

The interested reader is referred to two recent publications that have analyzed and validated different target amplification strategies before array hybridization (Francois et al. 2006; Vora et al. 2004). Vora et al. investigated four front-end amplification strategies: random primed, isothermal Klenow fragment-based, Phi 29 DNA polymerase-based, and multiplex PCR. Their results underscore the feasibility of using random amplification approaches and begin to systematically address the versatility of these approaches for unbiased pathogen detection from environmental sources (Stenger et al. 2002). Francois et al. (2006) compared commercially available amplification methods, such as MessageAmp and GenomiPhi. They showed that this type of enzyme represents an interesting alternative of moderate cost for transcriptomic studies. Such amplifications permitted them to obtain significant amounts of nucleic acids, sufficient to perform microarray studies even when starting with a few tens of ng of material. Importantly, these methods showed exquisite reproducibility, even considering the data before normalization, which is the major requirement for their utilization in transcriptomic studies (Wilson et al.

2004). Finally, these nucleic amplification methods can be coupled to signal amplification; see for example Borucki et al. (2005) and/or array-based methods for improving detection sensitivity, as discussed in Section 23.3.5.

3.3.2. Labeling of the Targets

Fluorescently labeled targets are in general prepared using one of the many commercially available kits (Lynch et al. 2006) or following standardized labeling protocols (Bodrossy et al. 2003). Incorporation of the fluorescently labeled nucleotides occurs during enzymatic amplification of the nucleic acids (e.g., PCR amplification, *in vitro* transcription, reverse transcription, random DNA amplification). Alternatively, modified nucleotides (i.e., amino-allyl nucleotides) can be incorporated in the target followed by subsequent coupling with fluorescent dye esters.

3.3.3. Hybridization and Wash Conditions

Hybridization specificity is of paramount importance, especially when one has to differentiate targets from nontargets or to discriminate closely related DNA or RNA sequences that may possibly differ by only one base pair. Probes on the microarray are subjected to the same washing procedures (e.g., buffers, salt concentrations, and temperature). Strategies to overcome problems arising therefrom include the acquisition of melting curves for every individual probe (Liu, Mirzabekov and Stahl 2001); the careful design of probes with similar predicted hybridization properties (usually combined with the application of 2–3 probes per targeted group) (Bodrossy et al. 2003; Sanguin et al. 2006; Zhang, Hurek and Reinhold-Hurek 2006); the addition of tetramethylammonium chloride that equalizes the melting temperature of different probes by stabilizing the AT base pairs composition (Maskos and Southern 1993b); or the use of highly redundant probe sets with multiple probes to target each specific group of microorganisms (Wilson et al. 2002).

Secondary structure formation within the targets can reduce the binding constant of a specific probe by as much as 10^5 to 10^6 times (Lima et al. 1992), leading to an increase in false negative signals and a decrease in hybridization specificity (Armitage 2003; Southern, Mir and Shchepinov 1999). Several methods have been suggested to alleviate this problem, such as the use of helper oligonucleotides (Peplies, Glockner and Amann 2003), a two-probe proximal chaperon detection system (Small et al. 2001), an appropriate labeling method (Franke-Whittle et al. 2006), and a protocol to achieve optimized target lengths (Nguyen and Southern 2000; Southern, Mir and Shchepinov 1999; Yershov et al. 1996). Since long targets can form secondary and tertiary structures that hinder efficient probe-target duplex formation, the sizes of the target molecule and its amplicon are often reduced via chemical, enzymatic, or thermal fragmentation methods (Kelly et al. 2002; Liu, Mirzabekov and Stahl 2001; Nguyen and Southern 2000; Proudnikov and Mirzabekov 1996; Small et al. 2001; Bodrossy et al. 2003). Liu et al. have recently elegantly reviewed these issues and experimentally demonstrated that microarray hybridizations with short rRNA fragments were more dependent on target sequence than on the competition between probe-target interaction and RNA self-folding (Liu, Guo and Wu 2007).

Hybridization with short gene fragments increases the potential for the accumulation of background signal from nonspecific hybridization events. In order to circumvent this negative target effect, an alternative protocol, sequence-specific end labeling of oligonucleotides, was developed (Rudi et al. 2003; Kostic et al. 2007). In this approach, targets are complementary to oligonucleotide probes on the array, and the labeling is performed by incorporating single-labeled ddNTP in the presence of the targeted PCR product. This method ensures both high specificity and sensitivity; however, it is still affected by PCR bias.

3.4. Classical Commercially-Available Microarray Formats

All platforms share the common attribute that a sensor detects a signal from target sequences hybridized to immobilized nucleotidic probes. The intensity of this signal provides a measure of the amount of bound nucleic acid from a sample (Pozhitkov et al. 2006). Schematically, we have divided this section into discussions of spotted and in situ synthesized arrays. The next section focuses on alternative platforms that provide improved detection sensitivities.

3.4.1. Spotting Approaches

Currently, up to 50,000 gene fragments or oligonucleotides can be spotted onto a single microscope slide using robotic technology. The advantages of this technology are: flexibility in the design of the array; the relative ease of production; and its relatively low cost. Multiple identical microarrays can be robotically printed in batches of over a hundred in a single run. Most of the cost of printing such arrays is associated with the synthesis of oligonucleotide probes or primer pairs required for the amplification of the probe gene fragments (Dorrell, Hinchliffe and Wren 2005). We review here briefly various commercially-available microarray formats.

3.4.1.1. *Operon*

The Qiagen Operon format (www.operon.com) uses optimized 70-mer oligonucleotides to represent each gene in a given genome. Each 70-mer probe is designed to have optimal specificity for its target gene and is melting-temperature normalized. This approach provides a reduction in cross-hybridization and an increase in the differentiation of overlapping genes or highly homologous regions. Theoretically, mutant alleles could be detected using such oligonucleotide microarrays, owing to the shorter probe size compared to PCR product-based microarrays (Dorrell, Hinchliffe and Wren 2005).

3.4.2. In Situ Synthesis

In situ synthesis allows higher yields and lower chip-to-chip variation, as well as higher probe densities. These methods also allow the manufacture of true “random access” arrays, meaning that each oligonucleotide in any position can have any chosen sequence (Southern, Mir and Shechepinov 1999). Manufacturing techniques include photolithographic masks to control chemical activation by photodeprotection steps (Fodor et al. 1991; Lipshutz et al. 1999), ink-jet deposition (Stimpson et al. 1998; Hughes et al. 2001), and physical barriers to sequential flooding of precursors (Maskos and Southern 1993b).

3.4.2.1. *Affymetrix*

With Affymetrix microarrays (www.affymetrix.com), oligonucleotide probes are not deposited but directly synthesized on the surface. The company has coupled photochemical deprotection to solid-phase DNA synthesis by adapting techniques from the semiconductor industry (Pease et al. 1994; Lipshutz et al. 1999; Lockhart et al. 1996). The main advantage of this approach is a very high probe density (over 500,000 probes can be deposited on a surface of 1.6 cm²). The limitations are a high price, low flexibility, and lack of properly validated probe sets. Therefore, in order to ensure the specificity of the detection, applications of the Affymetrix platform require multiple probes to monitor a single target, relying on empirical algorithms (Brodie et al. 2006).

3.4.2.2. *NimbleGen*

Recent technical developments, such as NimbleGen's micromirror device (www.nimblegen.com), facilitate maskless photoreactive synthesis of oligonucleotide probes, and currently permit the simultaneous deposition and analysis of as many as nearly 800,000 probes on one array platform (Albert et al. 2003). Such probe density now permits detailed comparative genome hybridizations (CGH) for detecting small deletion changes in the studied genome. However, insertions of genes compared with the sequenced reference strain cannot be detected by CGH DNA microarray analyses. This problem can be alleviated by adding nonredundant amplified sequences from several closely related bacteria to the array, once new genetic information is available (Borucki et al. 2005; Borucki et al. 2003; Porwollik et al. 2003; Cassat et al. 2005).

3.4.2.3. *Agilent*

A more versatile, but still essentially mechanical, method for producing DNA arrays is to use the print heads out of commercial piezoelectric ink jet printers to deliver reagents to individual spots on the array (Southern 1989; Brennan and Heinecker 1995; Baldeschwieler, Gamble and Thierault 1995; Blanchard, Kaiser and Hood 1996). A piezoelectric ink jet head consists of a small reservoir with an inlet port and a nozzle at the other end. When a voltage is applied to the crystal, it contracts laterally, thus deflecting the diaphragm and ejecting a small drop of fluid from the nozzle. Such devices are inexpensive and can deliver drops with volumes of tens of picoliters at rates of thousands of drops per second. In conjunction with a computer-controlled XY stepping stage to position the array with respect to the ink jet nozzles, it is possible to deliver different reagents to different spots on the array. Arrays of approx 250,000 spots can be addressed in a few minutes, with each spot receiving one drop of reagent. Agilent (www.agilent.com) has developed a flexible method for microarray production, centered around an *in situ* oligonucleotide synthesis method in which the ink jet printing process is modified to accommodate the delivery of phosphoramidites to directed locations on a glass surface (Blanchard, Kaiser and Hood 1996). Achieving high density with the ink jet approach requires one more trick. Two drops of liquid applied too closely together on a surface will tend to spread into each other and mix. For 40 picoliter drops the minimal center-to-center spacing is about 600 microns. This limits the array density achievable with the ink jet method. One way around this is to engineer patterns in the surface chemistry of the array to produce spots of a relatively hydrophilic character surrounded by hydrophobic barriers [Southern (1989) PCT WO 89/10977] [Brennan (1995) US Patent 5,474,796] (Blanchard, Kaiser and Hood 1996). Design flexibility and high densities constitute the two major advantages of this technique that can generate arrays at moderate costs.

3.4.2.4. *CombiMatrix*

CombiMatrix's technology (www.combimatrix.com) is a specially modified semiconductor adapted for biological applications. These integrated circuits contain arrays of microelectrodes that are individually addressable using embedded logic circuitry on the chip. Placed in a specially designed fluidic chamber, the chip digitally directs the molecular assembly of biopolymers in response to a digital command.

Under a controlled process, each microelectrode is addressed to selectively generate chemical reagents by means of an electrochemical reaction. These chemical reagents facilitate the *in situ* synthesis of complex molecules such as DNA oligonucleotides. The parallel process drastically reduces the cost and time of synthesizing hundreds or thousands of different molecules. Currently, this technology is able to produce arrays with approximately 45,000 features.

3.5. Alternative Methods for Improving Microarray-Based Detection Sensitivity

Most microarray applications are limited by the starting amounts of the nucleic acids to be studied. In other words, detection sensitivity is a major limitation of microarray-based approaches that has to be compensated for by several enzymatic steps for target amplification and/or labeling (Call, Borucki and Loge 2003; Loge, Thompson and Call 2002; Borucki et al. 2003), as discussed in Section 23.3.3.1.

The next section illustrates various array-based methods that can also improve detection sensitivity, independently from any target or signal amplification.

3.5.1. Resonance-Light Scattering (RLS)

New optical techniques are now available for microarray detection (Ferguson, Steemers and Walt 2000; Pawlak et al. 2002) which provide sensitivities high enough to detect femtomolar amounts of targets. Francois et al. (2003) nicely illustrated the improvements in detection sensitivity that can be achieved with different optical detection methods when using direct non-enzymatic labeling of bacterial nucleic acids. Microarrays detected by resonance light scattering (www.genicon.com) offer short turn-around times and exquisite sensitivity. Interestingly, the labeling and detection schemes offer an alternative at a reasonable cost to the expensive fluorescence-based methods. The principle of RLS is the following: when a suspension of nano-sized gold or silver particles is illuminated with a fine beam of white light, the scattered light has a clear (not cloudy) color that depends on its composition and particle size. This scattered light can be used as the signal for ultrasensitive analyte detection (Pasternack and Collings 1995).

3.5.2. Planar-Waveguide Technology (PWT)

Fig. 23.5 depicts PWT-based microarrays. A 150 to 300 nm thin metallic oxide film (green) with high refractive index (e.g., Ta_2O_5 or TiO_2) is deposited on a transparent support (grey) with a lower refractive index (e.g., glass or polymer). A parallel laser light beam (red) is coupled into the wave-guiding film by a diffractive grating that is etched into the substrate. The light propagates within this film and creates a strong evanescent field perpendicular to the direction of laser propagation into the adjacent medium (Duveneck et al. 2003). The field strength decays exponentially with the distance from the waveguide surface, and its penetration depth is limited to about 400 nm (large orange arrow). This effect results in the selective excitation of fluorophore molecules located at or near the surface of the waveguide (red circles). For microarray applications, specific capture probes or recognition elements are immobilized on the waveguide surface. Upon fluorescence excitation by the evanescent field, the excitation and detection of fluorophores by a CCD camera is restricted to the sensing surface, whilst signals from unbound molecules in the bulk solution (blue) are not detected. This yields a significant increase in the signal/noise ratio compared to conventional optical detection methods (Francois et al. 2005).

3.5.3. Liquid Arrays

The Luminex (www.luminex.com) suspension array is simply a transfer of the microarray format from a glass slide to a high-throughput and efficient bead format (“suspension microarray”). With this type of assay, the DNA probes (e.g., oligonucleotides) are attached to 5.6-nm polystyrene microspheres (“beads”) containing an internal fluorescent dye. Each probe is assigned to a particular bead set containing a unique mixture of fluorescent dyes, or “spectral address.” Bead sets coupled to the probes of interest are then mixed together in the wells

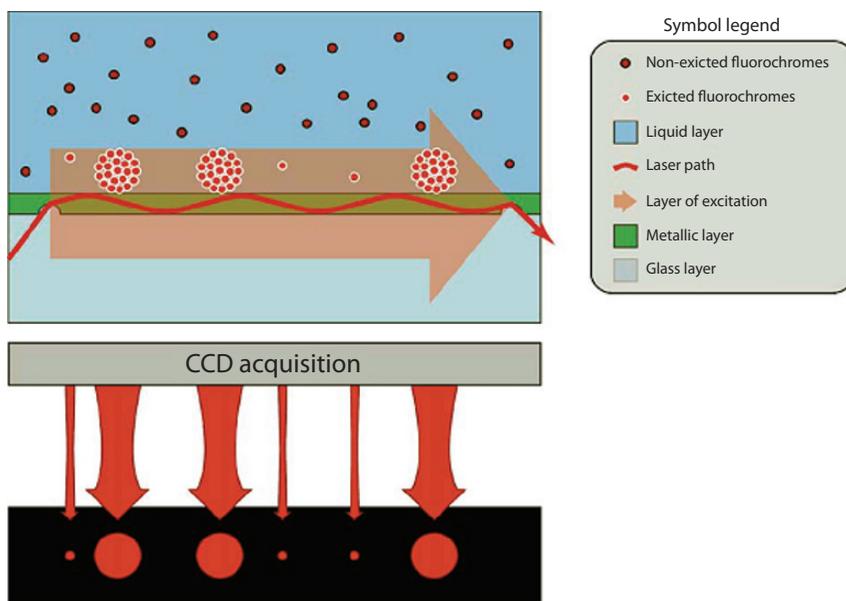


Figure 23.5. Schematic representation of PWT technology-based microarrays (reprinted from Francois et al. (2005), with permission of Elsevier).

of a 96-well microtiter plate, allowing many different probes to be analyzed simultaneously. Target DNA molecules are labeled with a different and spectrally distinct fluorescent dye and hybridized to the probes on the beads. Beads with the hybridized targets are then separated and quantified using a two-laser flow cytometer. The unique internal color of the bead is read by one laser and serves to identify which probe is present on the bead. The second laser measures the fluorescent signal of the reporter dye present on the labeled target DNA and allows one to assess the strength of the hybridization between the target DNA and the probe. Because this technology allows up to 100 different probes to be analyzed in a single well of a 96-well plate, it promises to make microarray subtyping faster and less expensive. The established suspension array protocol requires that relatively short PCR products be used as targets (Dunbar et al. 2003).

Microsphere-based fiber-optic arrays (www.illumina.com) provide many advantages over other array-based methods (Ahn, La and Forney 2006): higher sensor-packing density, smaller assay sample volumes, increased array reusability, flexible array design, and reduced false positives and false negatives (Epstein et al. 2003b). Previous work has demonstrated that the microsphere-based fiber-optic array can detect as few as 600 target DNA molecules and is sensitive enough to discriminate a single-base mismatch from a perfect match (Epstein et al. 2003a; Epstein and Walt 2003).

Finally, other bead-based arrays have been suggested for high-throughput sequencing approaches (Brenner et al. 2000). Such approaches are discussed under Section 23.5.4.

3.5.4. Three-Dimensional Microarray Formats

Three-dimensional microarray formats offer the option of recording hybridization and dissociation events in real time. This enables rapid establishment of the melting curves for all probes on the microarray, facilitating the development of validated probe sets. Three-dimensional microarray systems include gel-pads (Guschin et al. 1997; Pozhitkov et al. 2005;

Liu, Mirzabekov and Stahl 2001), flow-through systems such as PamGene (www.pamgene.com) (Wu et al. 2004) or MetriGenix (www.metrigenix.com) (Kessler et al. 2004).

3.6. Marker Genes Used on Microbial Diagnostic Microarrays (MDMs)

Microarrays employing long oligonucleotide probes or gene fragments can target an unlimited number of different genes (see Section 23.3.1.3.1). Short oligoarrays, on the other hand, depend on PCR amplification to reduce the target complexity to a level compatible with the sensitivity of the probes (see Section 23.3.1.3.2). They are thus usually limited to a small number of marker genes (typically between one and ten). Marker genes used for phylogenetic analysis and the development of short oligo-MDMs need to fulfill several criteria: (1) widespread distribution throughout the targeted organism group, (2) a high degree of conservation allowing for universal PCR amplification, (3) the existence of variable regions allowing for the design of discriminating probes, and (4) no (or low rate of) horizontal gene transfer.

The most commonly employed phylogenetic marker for the detection of microorganisms is the 16S rRNA gene. Ribosomal RNAs (rRNA) are particularly suitable for species identification procedures because they occur universally, contain conserved as well as divergent regions, and are highly abundant in cells. A further advantage of the 16S rRNA gene consists in the availability of large sequence and probe databases (<http://www.arb-home.de>, <http://rdp.cme.msu.edu>, <http://greengenes.lbl.gov>, <http://www.microbial-ecology.net/probebase>). Technical challenges faced by short oligo-MDMs are related to detection sensitivity and hybridization specificity. The former usually refers to the minimum amount of target that can be reproducibly detected by individual probes in a given complex sample (see also Section 23.3.3.1); and the latter refers to the ability of the DNA microarray technique to differentiate targets from nontargets or to discriminate closely related DNA or RNA sequences that may possibly differ by only one base pair (Liu, Mirzabekov and Stahl 2001; Urakawa et al. 2002; Liu, Guo and Wu 2007). See Section 23.3.3.3 for further details. The main limitation of the 16S rRNA gene lies in its extremely high degree of conservation. In many cases (a notable example being various genera of *Enterobacteriaceae*) it is not possible to design even species-specific probes based on it.

Therefore, a range of alternative phylogenetic and functional marker genes has been suggested (Santos and Ochman 2004; Loy and Bodrossy 2006). These include the 23S rRNA gene, the rRNA intragenic spacer region, so-called house-keeping genes (e.g., *gyrB*, *rpoB*, *recA*, *atpD*, *groEL*), virulence genes, antibiotic resistance genes, and functional genes (e.g., *pmoA*, *amoA*, *nifH*, *nirK*, *nirS*). Many of these have been successfully applied on various microarray platforms (Wu et al. 2001; Bodrossy et al. 2003; Kakinuma, Fukushima and Kawaguchi 2003; Taroncher-Oldenburg et al. 2003). The major limitation of these “alternative” marker genes is the limited organism coverage of published sequence databases.

4. Analysis and QC Aspects

Each step of microarray experiments needs to be optimized and validated, from the array design and manufacture to data collection and analysis. Among the critical technical parameters that need to be controlled are the microarray surface chemistry, the probe sequence, the probe deposition process, and the hybridization conditions. The MicroArray Quality Control (MAQC) Consortium, an unprecedented, community-wide effort, spearheaded by FDA scientists, recently addressed experimentally the key issues surrounding the reliability of DNA microarray data. They assessed the performance of seven microarray platforms in profiling the expression of two commercially available RNA sample types. The results were compared not only at different locations and between different microarray formats, but also in relation to three more traditional

quantitative gene expression assays. MAQC's main conclusions confirm that, with careful experimental design and appropriate data transformation and analysis, microarray data can indeed be reproducible and comparable among different formats and laboratories, irrespective of sample labeling format. The data also demonstrate that fold change results from microarray experiments correlate closely with results from assays like quantitative reverse transcription PCR (Shi et al. 2006).

Most diagnostic microarray datasets can achieve optimal classification with no more than 5–50 discriminative genes (Bo and Jonassen 2002; Li 2005). This opens new possibilities for the design of small diagnostic microarrays used for gene expression-based diagnosis. Array-to-array normalization is crucial for microarray analysis (Yang and Speed 2002; Kroll and Wolf 2002; Smyth and Speed 2003). Various methods for normalization have been suggested. One approach is to determine a set of invariant genes for normalization (Schadt et al. 2001; Tseng et al. 2001). Another approach recommends replicating genes on the array and using this within-array replication for normalization (Fan et al. 2005; Fan et al. 2004). Standard normalization protocols rely on the assumption that the majority of genes on the microarray are not differentially expressed between samples (Yang 2002). Jaeger et al. suggest including additional normalization genes on the small diagnostic microarrays, and they propose two strategies for selecting them from genome-wide microarray studies. The first is a data driven univariate selection of normalization genes. The second is multivariate and based on finding a balanced diagnostic signature (Jaeger and Spang 2006).

When shifting from expression arrays to detection/identification arrays, QC issues persist but they definitely require other validation approaches. These microarrays are typically hybridized with a single target (i.e., one-color hybridization). Signals on short oligonucleotide arrays are then usually normalized against positive controls. These positive controls are designed for conserved regions of the targeted gene, for the PCR primers used to amplify the targeted gene, or against exogenous spiked DNA. Long oligonucleotide arrays can be normalized against general probes, targeting conserved regions of the universal genes present in all bacteria, i.e., universal 16S probe(s) or other housekeeping genes. Normalized signals are compared to arbitrary threshold values, and the targeted microbes are rated as present or absent. For low density short oligonucleotide arrays, the threshold values are ideally individual values, reflecting the hybridization potential of the individual probes. For long oligoarrays and high density arrays with a highly redundant set of short oligos, it is possible to devise universal rules for calling a signal or a set of signals present or absent.

5. Applications of Microarray Technology in Microbial Diagnostics

Microarray technology offers a great potential for answering many different experimental questions. The nature of the experimental question at hand is the main issue that has to be taken into consideration when developing a new microarray platform. Depending on this question, there is an initial decision on the nature of the probes and on the method for target labeling. This subsequently influences the selection of substrates and hybridization strategies. Some of the most common experimental questions for which microarray technology is used will be reviewed here.

5.1. Gene Expression Studies

The most widely used application of DNA microarrays is the study of transcriptional responses. Consequently, targets are derived from mRNA. Initial studies were limited to an organism or tissue of interest and provided insights into particular aspects of the organism's

physiology (Schena et al. 1995; Gill et al. 2002; Zaigler, Schuster and Soppa 2003; Stowe-Evans, Ford and Kehoe 2004). For these studies, probes representing the genetic profile of the organism or tissue of interest were used (e.g., clone libraries containing either gDNA or cDNA fragments). Recent developments of microarray technology enabled the environmental monitoring of gene expression. Even though these studies are still limited to a few genes of interest, they can provide valuable information regarding the functionality of the whole microbial community (Zhou and Thompson 2002).

More recently, and benefiting from advanced target amplification methods (see Section 23.3.3.1), Garzoni et al. (2007) could monitor genome-wide bacterial transcription changes after *S. aureus* was ingested by nonprofessional phagocyte cells. Similar approaches (Lucchini et al. 2005), sometimes coupled to proteomics (Scherl et al. 2006), have proven instrumental for deciphering genes and/or pathways involved in physiologic adaptation—including the establishment of chronic infections or the development of antimicrobial resistance mechanisms. These techniques are valuable for developing targeted diagnostic tools and discovering focused therapeutic interventions.

Finally, Relman et al. proposed an original diagnostic approach focusing exclusively on the host innate response. Instead of identifying the pathogen itself, the authors aim at detecting pathogens through the elicited immune response, by studying the gene expression profiles of circulating monocytes (Boldrick et al. 2002). This approach, however, remains plagued by challenges and complexities that have yet to be adequately addressed. The rapidly changing nature over time of acute infectious diseases in a host, and the genetic diversity of microbial pathogens present unique problems for the design and interpretation of functional-genomic studies in this field. In addition, there are the more common problems related to heterogeneity within clinical samples, the complex, nonstandardized confounding variables associated with human subjects, and the complexities posed by the analysis and validation of highly parallel data (Liu et al. 2006).

5.2. Comparative Genome Hybridizations (CGH)

Traditional phylogenetic classification of bacteria to study evolutionary relatedness is based on the characterization of a limited number of genes, rRNA, or signature sequences. However, owing to the acquisition of DNA through lateral gene transfer, the differences between closely related bacterial strains can be vast (Dorrell, Hinchliffe and Wren 2005). By contrast, whole-genome sequencing comparisons allow a multitude of genes to be compared. Unfortunately, whole-genome sequencing is currently too expensive to allow the comparison of a large number of isolates of a species in a high-throughput scenario, as the global surveillance of infectious diseases requires. Therefore, since microbial genotyping is increasingly being used to track infectious diseases as they spread in human populations, another usage of microarrays has emerged. Comparative genome hybridization (CGH) permits assessing the genetic similarities and differences between closely related organisms. This approach is an adaptation of array methods used in gene expression studies, but applied to total genomic DNA (Murray et al. 2001; Lindroos et al. 2005). CGH enables a “bird’s-eye view” of all the genes absent or present in a given genome compared to the reference genome on the microarray. Whole-genome comparisons typically identify sets of “core genes,” which are shared by all strains in a species; and “accessory genes,” which are present in one or more strains in a species, and often result from gene acquisition. It is these differences that can often be used to identify genes and/or genetic islands related to “gain-of-function traits” in pathogenic strains (Dorrell, Hinchliffe and Wren 2005).

CGH approaches can be applied to further characterize strains and to identify novel marker genes and chromosomal regions specific for given groups of isolates, thus providing

better discrimination and additional information compared to classical genotyping methods (Koessler et al. 2006). However, ambiguities in the interpretation of the ratios of hybridization and cross-hybridization to paralogous genes remain important limitations of the technique (van Bakel and Holstege 2004). Solid statistical criteria for the absence or presence of ORFs are still lacking as a result of the diversity of the microarray design approaches, affecting the meta-analysis of the data obtained by different investigators (Joyce et al. 2002).

Garaizar et al. (2006) concluded that the construction or purchase of DNA microarrays and the performance of strain-to-strain hybridization experiments are still prohibitively expensive for routine application. The future use of arrays in epidemiology is likely to depend on the development of more cost-effective protocols, more robust and simplified formats, and the adequate evaluation of their performance (efficacy) and convenience (efficiency), compared with other genotyping methods. Indeed, more focused assays are finding broad application in routine bacterial epidemiology. Using commercially-available low-density microarrays fitted within microtubes (www.clondiag.com), papers have reported the development and validation of assays for bacterial genotyping and virulence gene detection (Korczak 2005) as well as for extensive detection of antimicrobial resistance determinants in Gram-positive bacteria (Perreten et al. 2005). Different low-density array formats permitted the recent release of the first commercially available microarray-based assay for the genotyping of human papilloma viruses, HPV (www.genomica.es) (www.greinerbioone.com).

5.3. Generic or Universal Microarrays

Combinatorial strategies refer to methods developed to make microarrays containing all sequences of a given length (also referred to as “generic arrays”). Combinatorial arrays have been promoted mostly to study large-scale hybridization behavior (Southern et al. 1994) or for solid-state nucleic acid sequencing (Drmanac and Drmanac 1999; Drmanac et al. 1993; Drmanac et al. 1998; Strezoska et al. 1991; Macevicz 1991; Lipshutz et al. 1999).

Generic arrays have been proposed as an inexpensive alternative to sequencing. Using all possible combinations of an *n*-mer allows “walking” at every position along a nucleotide sequence. This approach is currently limited by the complexity of the algorithm required to generate contigs (conversion of a listing of hybridized *n*-mers into a meaningful sequence). Also, if the sequence undergoing analysis contains a repeat region (the same sequence appearing more than once within the target molecule), the reconstruction diagram will have to display a corresponding number of branching points, leading to an ambiguous sequence. This type of array is the only one capable of detecting sequences that are lacking in large electronic libraries. In contrast, dedicated arrays are used for repetitive sequencing (resequencing) of the same target for detection of nucleotide polymorphisms or functional mutations (see Section 23.5.4).

Universal arrays refer to strategies that can provide target identification without any a priori sequence knowledge. This approach has been named the Non-Cognate Hybridization System (NCHS) (Schrenzel and Hibbs 2003). By synthesizing all probes of a given length that can be generated by a combination of four nucleotides, microarrays could detect any single organism. Unfortunately, for generating realistic probe lengths (i.e., permitting unique sequence identification as well as adequate hybridization behavior), the total number of permutations of the four nucleotides would yield very large numbers of possible probes (i.e., $4^{13} = 67,108,864$ probes for 13-mers nucleotides). The synthesis of such large microarrays is currently technically impossible. Furthermore, bioinformatics tools that would be necessary for the microarray analysis of billions of probes are not yet available. Thus, reducing the probe set and the complexity of the analytical approach is warranted. Random reduction of the probe set to a smaller number of probes might result in potentially missing sequences characteristic for given

pathogens. An alternative would be the pruning of low informative probes, i.e., probes that have no targets in a collection of selected micro-organisms. However, this approach yields ultimately to another cognate design, with the inherent limitations described above. Another way to reduce the probe set is to consider permutations of only two nucleotides. This divides the probe set by $2^{(n-2)}$ where n is the length of the probes. This microarray design is truly noncognate and guarantees that no organism is a priori favored or missed. By analogy, Roth et al. have designed a universal microarray system combined with an enzymatic manipulation step that is capable of generating expression profiles from any organism without requiring a priori sequence-specific knowledge of transcript sequences (Roth et al. 1998).

Finally, one should mention here “multipurpose” arrays that contain probes for detecting a series of molecular barcodes. These molecular barcodes can be used as tags in various experimental conditions, for probing different targets under various experimental formats. These multipurpose arrays serve only to quantitatively detect the presence of barcoded probes, whose specificity is determined by the user. Molecular inversion probe (MIP) technology was initially developed for the detection of single nucleotide polymorphisms (SNPs) in human genes (Hardenbol et al. 2003). MIP technology has been shown to work well for multiplexing, i.e., massive parallel processing (12,000 MIPs in the same reaction tube) (Hardenbol et al. 2005). The power and versatility of MIP technology makes it perfectly suited for the identification and quantification of microbes. MIP’s high sensitivity and specificity in detecting large numbers of SNPs (Hardenbol et al. 2003; Hardenbol et al. 2005) should allow one to harness this technology to detect a large number of pathogens and to identify multiple infections in an individual sample. A molecular inversion probe is comprised of genomic recognition sequences, common amplification sequences, and a molecular barcode for each genotype assigned to a specific gene. This probe is a linear oligonucleotide with target-complementary sequences at the ends and a noncomplementary linking segment in between (Thiyagarajan et al. 2006).

5.4. Microarrays for Sequence Analysis

Microarray technology has been used successfully for sequence analysis. These applications are technologically very diverse and will not be discussed in detail here. Usually, on-chip sequence analysis involves fabrication of high-density short oligonucleotide microarrays. These arrays contain either all possible oligonucleotides of a given length (Bains and Smith 1988) or they display a range of oligonucleotides covering a DNA sequence of interest, employing the so-called “tiling strategy” (Hacia 1999). Sequence analysis is performed by comparison of the hybridization patterns of the reference vs. the test sample. Such microarrays have been used for sequencing (Yershov et al. 1996; Hacia 1999), for the detection of single nucleotide polymorphisms (Hacia 1999; Lindroos et al. 2001), and for the analysis of secondary structures (Sohail, Akhtar and Southern 1999). Alternative detection methods have been suggested, such as using a labeled common oligonucleotide primer that is extended to the site of the match or mismatch (Lodes et al. 2006). Publications have already started addressing the issue of automated interpretation of resequencing on microarrays (Malanoski et al. 1995).

Recent advances in high-density oligonucleotide arrays have enabled the development of high-throughput resequencing techniques. Resequencing arrays are designed to cover the entire genome by overlapping oligonucleotides. Multiple versions of each oligonucleotide are spotted on the array to represent the four possible base combinations (A, T, G, and C) for each nucleotide position. To date, this technique has been applied to *Bacillus anthracis*, with 56 strains being resequenced using a custom-designed resequencing array (Zwick et al. 2005). The same technique has also been used to track the evolution of the severe acute respiratory syndrome coronavirus (Wong et al. 2004). Conversely, similar tiling-based resequencing has been employed for *Staphylococcus* speciation (Couzinet et al. 2005a), genotyping (van

Leeuwen et al. 2003), and the detection of mutations conferring resistance to quinolone antimicrobials (Couzinet et al. 2005b).

Finally, other bead-based arrays have been suggested for high-throughput sequencing-based approaches (Brenner et al. 2000). The latter approach resulted in a powerful high-throughput sequencing platform (Illumina-Solexa) that currently competes against the pyrosequencing method described by Margulies et al. (Margulies et al. 2005). One can certainly conclude that advances in sequencing technologies currently allow the complete decoding of an entire microbial genome in a few hours (Margulies et al. 2005), clearly out-competing arrays for the detection of SNPs, albeit at a substantially higher cost. In any event, more accessible, technically robust, and, above all, cheaper formats are needed before the broad application of any of these technologies in a clinical and epidemiological surveillance setting.

5.5. Microbial Diagnostic Microarrays

Microbial diagnostic microarrays (MDMs) are used for the simultaneous identification of microorganisms in clinical or environmental samples. Probes used for MDMs are usually oligonucleotides designed to be specific for a given strain, subspecies, species, genus, or higher taxon. Classification and nomenclature of MDMs throughout the literature is not unanimous. According to their intended use, environmental MDMs (Bodrossy et al. 2003; Loy et al. 2005) and detection/identification MDMs can be distinguished (Hashsham et al. 2004; Sergeev et al. 2004). The main difference between these two MDM types relies in their detection requirements.

Environmental MDMs are generally used to assess the whole microbial community structure or a subset of the microbial community in a particular environment. Therefore, reliable parallel detection of many different microorganisms and the potential for some level of quantification are required. Detection/identification MDMs are primarily used in clinical (medical, veterinary), food, and biodefense microbiology. For this purpose, highly sensitive and specific detection of a few microorganisms in a complex community is required. According to the nature of the marker gene, one can further distinguish phylogenetic (Rudi et al. 2003; Loy et al. 2005; Brodie et al. 2006) and functional MDMs, also referred to as functional gene arrays (Wu et al. 2001; Bodrossy et al. 2003; Taroncher-Oldenburg et al. 2003).

There are two concepts for the sensitivity of MDMs, both of them potentially posing a bottleneck to the detection of the targeted microbes. Absolute sensitivity refers to the amount of target DNA or the number of target microbes required for successful detection. Absolute sensitivity reflects the hybridization capacity and detection sensitivity of the microarray platform used. Relative sensitivity, on the other hand, refers to the ratio of the targeted microbe within the entire microbial community analyzed. It is primarily due to low level nonspecific background signal accumulation and to the fact that the amount of target DNA applicable in microarray hybridizations is limited.

A promising approach to increase the sensitivity of a microarray assay is tyramide signal amplification (TSA) (Denef et al. 2003). Upon hybridization, this method relies on enzymatic amplification of the signal by employing the horseradish peroxidase-mediated deposition of fluorochrome-labeled tyramides at the location of the probe. The relative sensitivity can be improved by limiting the labeling to very short, specific regions (Kostic et al. 2007; Ballmer et al. 2007; Rudi et al. 2003). High density microarrays employing multiple perfect match/mismatch probe sets for each targeted microbe also enable a significant improvement in relative sensitivity (Brodie et al. 2006). A novel method to analyze microarray data holds promise for a significant improvement in terms of the relative sensitivity of MDMs (Marcelino et al. 2006).

The ultimate specificity of microarray technology depends on the discrimination between a fully complementary target and a nontarget differing in only one single nucleotide. Various enzyme-assisted hybridization strategies, also used in single nucleotide polymorphism and

resequencing assays (Ericsson et al. 2003; Lindroos et al. 2002), are being applied because of their promise in strongly discriminating single mismatches located near the 3' end of microarray probes (Rudi et al. 2003; Cassat et al. 2005; Halperin, Buhot and Zhulina 2005; Lipshutz et al. 1995; Maskos and Southern 1993a; Porwollik et al. 2003; Proudnikov and Mirzabekov 1996).

Isotope microarrays represent further development of the traditional phylogenetic MDMs, enabling linking phylogeny (community structure) to function. This approach employs "double-labeled" targets, where the first radioactive labeling is substrate-mediated, and the second labeling is performed according to standard microarray protocols (Adamczyk et al. 2003).

6. Conclusions

The development of microarray-based bacterial identification systems starting directly from the biological sample, without any enzymatic target amplification, would be most welcome (Straub and Chandler 2003). During a single hybridization, arrays can integrate probes that provide microbial identification and also enclose large sets of discriminative epidemiological markers (Call et al. 2003; Gingeras et al. 1998; van Leeuwen et al. 2003; Volokhov et al. 2002; Yue et al. 2001), or contain probes to detect virulence or antimicrobial resistance determinants (Bekal et al. 2003; Chizhikov et al. 2001; Couzinet et al. 2005b; Korczak et al. 2005; Troesch et al. 1999). The high parallelism of microarray systems appears particularly adapted for this, provided the systems' design includes the following: (1) targeted universal gene(s), (2) simplified coupling and labeling protocols, (3) exquisite sensitivity, and (4) an adapted analysis strategy.

Clinical bacteriology is witnessing a revolution. We are shifting away from Koch's postulates that required evidence of bacterial growth to the routine use of advanced molecular biology tools. Revolutions bring important changes in the way we think. The extended use of molecular biology will help discover new species that could not be cultivated. Quantitative aspects of bacterial loads, easily addressed on petri dishes, will need to be transposed and validated using molecular biology tools. This revolution should now be supported also by a better appreciation of the remarkable diversity of the bacterial world; the understanding that adequate taxonomy is needed; and the knowledge that host-pathogen interactions will have to be addressed perhaps more straightforward without last part.

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