

I

Application of Microarray Technologies

1

Electronic Microarray Technology and Applications in Genomics and Proteomics

Ying Huang, Dalibor Hodko, Daniel Smolko, and Graham Lidgard

Nanogen Inc., 10398 Pacific Center Court, San Diego, CA 92121, USA.

Keywords: Electronic microarray/ Miniaturization/ Single nucleotide polymorphism/ Gene expression profiling/ Cell separation/ Protein kinase/ Forensic detection/ Biological warfare

Electronic microarrays that contain planar arrays of microelectrodes have been developed to provide unique features of speed, accuracy and multiplexing for genomic and proteomic applications through utilizing electric field control to facilitate analytes concentration, DNA hybridization, stringency and multiplexing. An overview of electronic microarray technology is presented followed by its variety applications in genomic research and DNA diagnostics, forensic detection, biologic warfare, and proteomics.

1.1. INTRODUCTION

DNA microarrays have provided a new and powerful tool to perform important molecular biology and clinical diagnostic assays. The basic idea behind DNA microarray technology has been to immobilize known DNA sequences referred to as probes in micrometer-sized spots on a solid surface (microarray) and specifically hybridize a complementary sequence of the analyte DNA or a target. A fluorescently labeled reporter facilitates fluorescent detection of the presence or absence of a particular target or gene in the sample. By using laser-scanning and fluorescence detection devices such as CCD cameras, different target hybridization patterns can be read on the microarray and the results quantitatively analyzed. This chapter describes a specific microarray technology where an electric field and

phenomena induced by the application of the electric field are used to direct and concentrate the DNA molecules through permeation layer [25] on the array.

Whereas, in the past, different technologies have been used to immobilize DNA probes including physical deposition [13, 40], photolithographic synthesis [Fodor et al., 1993, 7], and utilization of electric field [25]. Accordingly, several substrates have been used to generate different DNA microarrays ranging from glass slides, membrane to silicon. High density microarrays have been used to identify disease outcomes through relevant RNA expression patterns on thousands of genes [1] and for gene sequencing [33]. However, focused arrays, which often consists of 100–1,000 test sites, are better suited to detect a panel of genes for applications in point of care diagnostics, detection of infectious diseases, as well as identification of biological warfare agents. In these particular applications, speed, accuracy and multiplexing are basic requirements. Electronic microarrays, one type of the focused arrays, can meet all these requirements through utilizing electric field control to facilitate analytes concentration, DNA hybridization, stringency and multiplexing [17, 23–25, 45]. In this chapter, an overview of electronic microarray technology is presented followed by its applications in genomics and proteomics.

1.2. OVERVIEW OF ELECTRONIC MICROARRAY TECHNOLOGY

Nanogen, Inc. has developed an electronic micro-array based technology (NanoChip[®] Electronic Microarray) for manipulation, concentration and hybridization of biomolecules on the chip array (Figure 1.1). This approach extends the power of microarrays through the use of electronics by connecting each test site on the NanoChip[®] array to an electrode.

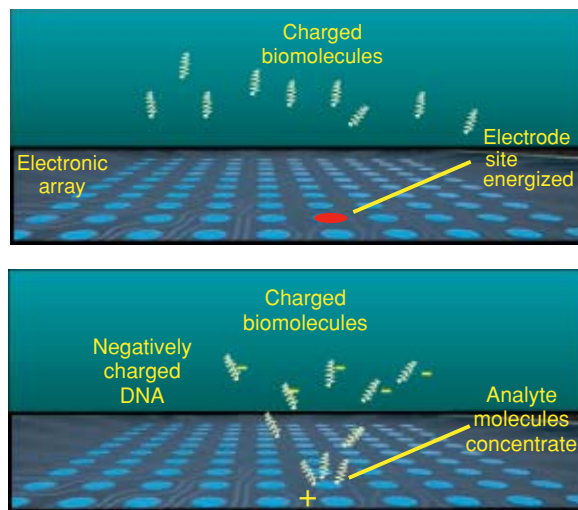


FIGURE 1.1. Nanochip[®] micro-array technology uses electronic addressing of charged biomolecules on the electrode array to separate and concentrate analyte targets. Negatively charged DNA targets and molecular probes (top) are moved to a particular site by energizing the electrodes at a reverse potential (bottom). Targeted molecules concentrate at the array site where they can be bound chemically or hybridized to a DNA probe. Fluorescent signal is obtained from the reporter probes hybridized to the target DNA and signal proportional to the concentration of analyte DNA is measured.

Most biological molecules have a natural positive or negative charge. When biological molecules are exposed to an electric field (Figure 1.1), molecules with a positive charge move to electrodes with a negative potential, and molecules with a negative charge move to electrodes with a positive potential. Current and voltages are applied to the test sites via individual electrode activation to facilitate the rapid and controlled transport of charged molecules to any test sites. Additional advantages of electrically facilitated transport include (1) the ability to produce reconfigurable electric fields on the microarray surface that allows the rapid and controlled transport of charged molecules to any test sites [22, 25]; (2) the ability to carry out site selective DNA or oligonucleotide addressing and hybridization [11]; (3) the ability to significantly increase DNA hybridization rate by concentration of target at the test sites (Kassegne et al., 2003); and (4) the ability to use electronic stringency to improve hybridization specificity (Sosnowski, et al., 1997).

1.2.1. NanoChip[®] Array and NanoChip[®] Workstation

1.2.1.1. Fabrication Electronic microarrays consist of an array of electrodes that have been fabricated on silicon with array sizes ranging from 5 to 10,000 individual electrodes or test sites [24, 25, 42]. Figure 1.2 shows a number of electronic microarrays with arrays ranging from 4 to 100 electrodes or test sites. These arrays have been designed

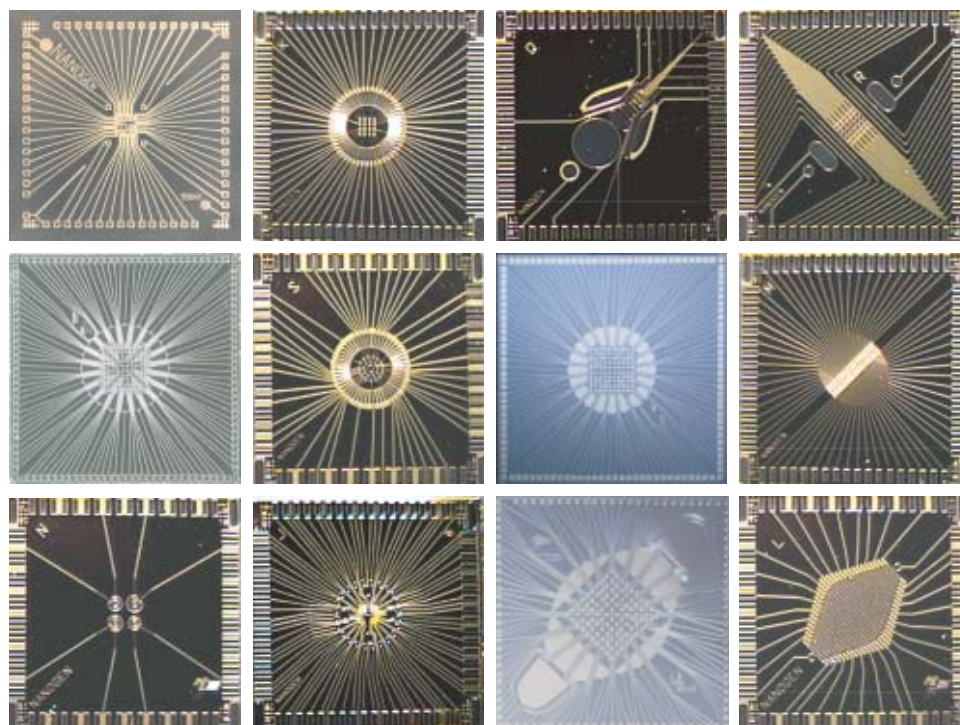


FIGURE 1.2. A series of designs of Nanogen's silicon microarray chips. Chip sizes shown range from 4–100 sites. These include different electrode geometries as well as chips designed for particles and or biomolecular microseparations (last column of chips).

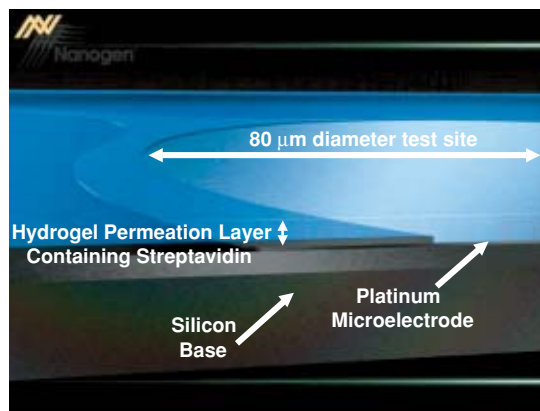


FIGURE 1.3. Cross-section of a single platinum micro-electrode pad on the NanoChip[®] microarray. A hydrogel permeation layer loaded with streptavidin covers the electrode array and serves for capturing electronically addressed molecules.

for both microassays and microseparations. The current commercialized NanoChip[®] array comprising 100 platinum microelectrodes with additional 20 outer microelectrodes acting as counter-electrodes.

The electrodes on the NanoChip[®] array are fabricated on a silicon substrate using standard photolithography and deposition processes. Each electrode is 80 μm in diameter with 200 μm center-to-center space between and is connected to the outside edge of the chip by a platinum wire trace. Figure 1.3 shows a cross section of a single electrode pad on the NanoChip[®] array. The base structure of the array consists of silicon over which an insulating layer of silicon dioxide is applied. Platinum is deposited and selectively held in place to form electrodes and accompanying electrical traces. These wire traces terminate at the edges of the chip forming electrical contact pads. Additional layers of silicon dioxide and silicon nitride are deposited to electrically insulate the platinum electrical traces, leaving the central array of 80 μm diameter microelectrodes, outer microelectrodes and contacts pads exposed. The chips are flip-chip bonded to a ceramic substrate which contains contacts to pogo-pins.

1.2.1.2. Permeation Layer Typically, on the surface of the array, a 10 μm thick hydrogel permeation layer (Figure 1.3). containing co-polymerized streptavidin is deposited by microreaction molding. This permeation layer serves two main functions [24]. First it protects the sensitive analytes from the adverse electrochemical effects at the platinum electrode surface during active operation. These electrochemical products include the generation of hydrogen ions (H^+) and oxygen at the positively biased (anode) microelectrodes and hydroxyl ions (OH^-) and hydrogen at the negatively biased electrodes (cathode), as well as various free radical entities. Secondly, the permeation layer also serves as a matrix for the attachment of biotinylated molecules e.g., analytes capture oligos, antibodies, and primers through biotin and streptavidin binding [24, 25]. Figure 1.4 is a close-up photograph of the 100-site array covered with the permeation layer.

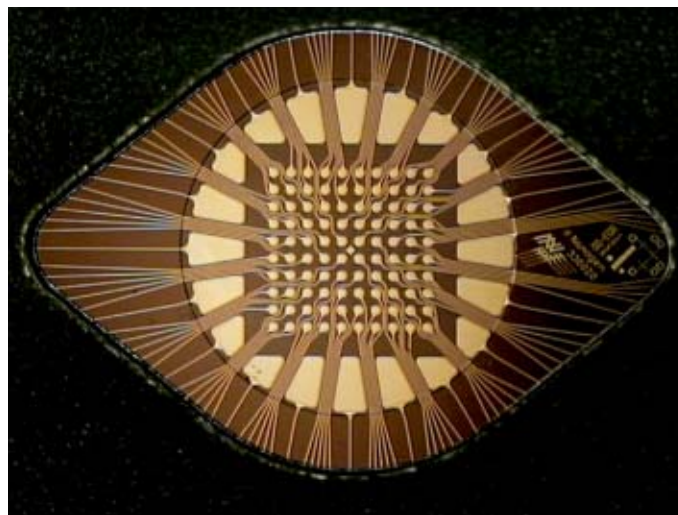


FIGURE 1.4. Photograph of the 100-site NanoChip[®] microarray. A hydrogel permeation layer covers the electrode array including working and counter electrodes. The hydrogel layer is visible as a circle surrounding counter electrodes (yellow pads).

1.2.1.3. NanoChip[®] Cartridge The 100-site array is assembled into a complete NanoChip[®] cartridge (Figure 1.5a) by ultrasonically welding two molded polymethyl methacrylate (PMMA) cartridge bodies that contain fluidic channels and inlet and outlet ports. The cartridge eliminates sample evaporation, prevents sample contamination and provides a fluidic interface to the NanoChip[®] Workstation.

1.2.1.4. NanoChip[®] Workstation The NanoChip[®] electronic microarrays are operated through a fully integrated and automated NanoChip[®] Workstation (Figure 1.5b). The system consists of three major subsystems: (1) the NanoChip[®] Loader for loading patient samples on one to four NanoChip[®] Cartridges, (2) the NanoChip[®] Reader, a highly sensitive, laser-based fluorescence scanner for detection of assay results and (3) computer hardware and software which automates import, analysis and export of sample information making data analysis simple.

1.2.2. Capabilities of the NanoChip[®] Electronic Microarrays

Using electric field, Nanochip[®] electronic microarrays have provided many unique features over other passive microarrays (Table 1.1). Electronic addressing allows users to quickly customize arrays in their own laboratory. Electronic hybridization provides an extremely accurate and specific hybridization process by creating optimal electric and pH conditions at the hybridization sites [11, 25]. Electronic stringency, in combination with thermal control, enables researchers to remove unbound and nonspecifically-bound DNA quickly and easily after hybridization at the microarrays, achieving rapid determination of single base mismatch mutations in DNA hybrids [44].



FIGURE 1.5. Photograph of the Nanochip[®] cartridge containing the electronic microarray, and b) Nanogen's Nanochip[®] Workstation which allows fully automated processing of 4 cartridges simultaneously in the loader and fluorescent detection in the reader.

1.2.2.1. Assay Formats on NanoChip[®] Electronic Microarrays The open architecture of electronic microarray enables flexibility in assay design. Since each individual electrode can be selectively activated, different assays can be generated depending on different analytes to be addressed (Figure 1.6). For example, a dot blot assay [19] is conducted when biotinylated PCR amplicon is addressed to the selected electrodes and remained embedded through interaction with strepavidin in the permeation layer. The DNA at each electrode is then hybridized to mixtures of allelespecific oligonucleotides (discriminators) and fluorescently labeled oligonucleotides. The thermal or electronic stringency is used to discriminate

TABLE 1.1. Comparison between NanoChip[®] microarray active hybridization technology and passive hybridization technologies.

	Hybridization time	Concentration of targets	Concentration factor at a site	Stringency control
NanoChip [®] active hybridization	10–100 seconds	Directed and localized at the array sites; Ability to control individual sites	> 1000 times	Electronic, Thermal, Chemical
Passive hybridization	1–2 hours	Non-directed; Sites cannot be controlled independently	Low, diffusion dependent	Thermal, Chemical

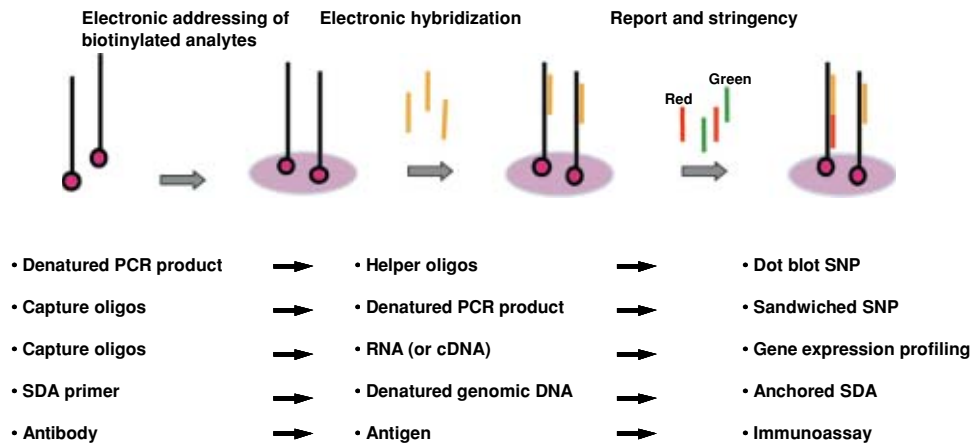


FIGURE 1.6. The open platform of the electronic microarray enables flexibility in assay design. Depending on the nature of the biotinylated analytes (denatured PCR products, capture oligos, SDA primers or antibody) in electronic addressing and on the targets (helper oligos, denatured PCR products, RNA, denatured genomic DNA or antigen), different assays (dot blot SNP, sandwiched SNP, gene expression profiling, anchored SDA or immunoassay) can be performed on the electronic microarrays.

the SNP. In this assay format, multiple samples can be analyzed at different electrodes on a single array.

A “sandwich” assay is created when biotinylated capture probes are addressed to selective electrodes. The capture probes can be sequence specific oligos or antibodies. The embedded capture probes are then electronically hybridized to the targets. Depending on the specific targets (PCR amplicons, or RNA, or antigens), the assays can be SNP analysis, gene expression profiling, or immunoassay.

Most recently, a special assay, anchored strand displacement amplification (aSDA), which integrates the amplification and discrimination, is demonstrated on electronic microarrays [12, 29, 50, 51]. In this assay format, biotinylated SDA primers were addressed and anchored on selective electrodes. These anchored primers were then electronically hybridized to the denatured genomic DNA. Target DNA was amplified over the electrodes when an enzyme mix containing restriction endonuclease and DNA polymerase and dNTPs was introduced to the array. After amplification the final discrimination was determined using same base-stacking principle [37]. Using aSDA, multiple genes from one sample or one gene from multiple samples can be simultaneously amplified and detected on a single electronic microarray.

1.2.2.2. Electronic Multiplexing By electronically controlling each test site, the electronic microarrays also provide a platform to perform different types of multiplexed assays:

- (1) single array multiplexing where multiple genes from one sample can be analyzed;
- (2) single array multiplexing where multiple samples with one gene of interest can be analyzed;
- (3) single array multiplexing where multiple samples with multiple genes of interest can be analyzed;

- (4) single site multiplexing where several targets are discriminated on the same site using different fluorescent probes;
- (5) single site multiplexing where several targets are addressed, different discriminator oligonucleotides hybridized and reporter addressed—this method allows the use of a universal reporter for different targets.

The ability to electronically control individual test sites permits biochemically unrelated molecules to be used simultaneously on the same microchip. In contrast, sites on a conventional DNA array cannot be controlled separately, and all process steps must be performed on an entire array. Nanogen's electronic microarray technology delivers increased versatility over such conventional methods. This is particularly important in applications such as biological warfare and infectious disease detection since the accurate identification of biological agents requires determination of two or more (often five) characteristic genes of a particular agent.

1.3. APPLICATIONS

1.3.1. *Single Nucleotide Polymorphisms (SNPs)—Based Diagnostics*

Given the advances in genomic studies, more and more single nucleotide polymorphisms (SNPs) are found to be contributory factors for human disease and can be used as genetic markers for molecular diagnostics. The speed, accuracy and flexibility provided by electronic microarrays have received great interest from clinical diagnostic researchers for a variety of genotyping applications (Table 1.2). Using the Nanochip[®] system, researchers at American Medical Laboratories analyzed 635 clinical samples for the G1691A mutation on the factor V Leiden, associated with thrombosis with 100% accurate in characterizing wild-type, heterozygous, and homozygous samples [15]. Researchers at ARUP Laboratories have evaluated 3 thrombosis associated SNPs, factor V (Leiden), factor II (prothrombin), and methylenetetrahydrofolate reductase (MTHFR), on 225 samples with 100% accuracy [14]. Schrijver et al at Stanford University Medical Center found that the SNP analysis based on Nanogen electronic microarray for factor V (Leiden) and factor II (prothrombin) on 800 samples were comparable with other SNP analysis methods such as restriction enzyme digestion (RFLP) and the Roche LightCycler [41]. Researchers at Children's National Medical Center, Washington DC, genotyped 8 common MeCP2 mutations associated to Rett syndrome on 362 samples with 100% specificity [47]. Using electronic microarray, researchers at Mayo Clinic Cancer Center have developed genotyping assays for 5 different cytokine polymorphisms [43]. Moreover, 8 SNPs distributed within a highly variable region of the *polC* gene from six isolates of *Staphylococcus aureus* were analyzed on electronic microarrays [9].

1.3.2. *Forensic Detection*

Short tandem repeats (STRs) represent another type of polymorphism with important applications in forensic DNA identification. In 1990, the FBI created a combined DNA index system (CODIS), which consist of 13 polymorphic STR loci, to provide a database of forensic DNA profile for nearly all forensic laboratories in the United States [5, 6]. The

TABLE 1.2. Examples of Nanochip® technology application in genomics.

Relevance	Test	Ref
Cystic Fibrosis	CFTR	http://www.nanogen.com/products/cystic_fibrosis.htm
Thrombosis	Factor V Leiden	[41]
	Factor II (prothrombin)	[15]
	MTHFR	[14]
	Factor V/Prothrombin	http://www.nanogen.com/products/Factor_vII.htm
Hereditary Hemochromatosis	HFE	http://www.nanogen.com/products/HH.htm
Alzheimer's Disease	ApoE	http://www.nanogen.com/products/apoe.htm http://biz.yahoo.com/prnews/040106/latu005_1.html
β -thalassemia	Factor VII	[39]
Asthma and chronic obstructive pulmonary disease	B(2)-adrenergic receptor	[53]
Ulcerative colitis	N-acetyltransferase 1 (NAT 1)	[38]
	N-acetyltransferase 2 (NAT 2)	
Cancer	p53	[3]
Rett syndrome	MeCP2	[47]
Immunologic defect	Mannose binding protein (MBP)	[19]
Parkinson's disease	CYP1A2/CYP2E1	[10]
Cytokine	Tumor necrosis factor- α (TNF- α)	[43]
	IL-4	
	Interferon- γ (CA)n repeats	
	IL-1 RN VNTR	
	CCR5	
Bacterial ID	<i>Staphylococcus aureus</i> pol C	[9]
	<i>Escherichia coli</i> gyrA	[50]
	<i>Salmonella</i> gyrA	
	<i>Campylobacter</i> gyrA	
	<i>E. coli</i> parC	
	<i>Staphylococcus</i> mecA	

typical STR loci are selected groups of four nucleotide repeats that are represented in the human population by 4-15 alleles distinguished by a different number of repeat units [8]. Unlike SNPs, STRs are more difficult to analyze by conversional passive hybridization techniques [20, 34]. However, electronic hybridization techniques have been proven to overcome these problems and allow STR analysis to be performed on electronic microarray in a rapid and high fidelity fashion [37]. Multiplex hybridization analysis of three STR loci (CSF1PO, TH01 and TPOX) was achieved for 12 individuals, 100% concordant with genotyping results of an accredited forensic laboratory.

Given the recent discovery of abundance SNPs and the ease of automation and miniaturization of detection techniques, SNP assays have started to be implemented in DNA forensic analysis. According to Chakraborty *et al.* [6], somewhere in the range of 30-60 SNP loci would be needed to equal the power of the 13 STR loci with regard to genotypic match probability and/or paternity exclusion. The flexibility of electronic microarray will permit accelerated development of SNPs for DNA forensic analysis by allowing an easy

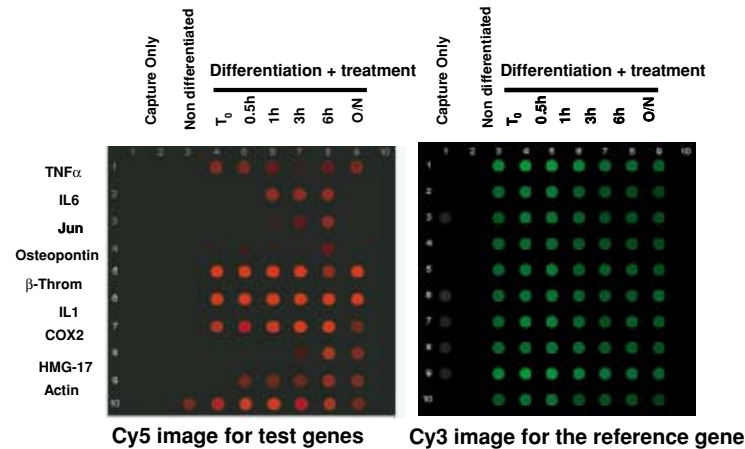


FIGURE 1.7. Multiplexed gene expression profiling on the electronic microarray. Ten target genes plus the β -la reference gene from the U937 cell lines at various time points after differentiation and LPS treatment. The genes were electronically hybridized to target-specific capture probes and to the β -la capture probe. The left panel represents the Cy5 fluorescent signals from the 10 target genes, whereas the right panel shows the Cy3 signal corresponding to the β -la reference gene. Each target gene exhibits a distinct expression pattern over the time course of LPS treatment, while the β -la reference gene remains relatively unchanged. Reprinted with permission from Weidenhhammer et al. Copyright [49] American Association for Clinical Chemistry.

method of adapting new loci. Currently, supported by government funding, assays are under development to detect 35 SNPs and 6 STRs on a single electronic microarray.

1.3.3. Gene Expression Profiling

Another array-based method in genomic studies is to simultaneously monitor global gene expression profiling of cells under a condition of interest and to identify a set of gene markers for specific disease [1]. Towards this trend, a multiplex, targeted gene expression profiling method has been developed using electronic field-facilitated hybridization on Nanochip[®] electronic microarray [49]. In this method, target mRNA generated from T7 RNA polymerase-mediated amplification were detected by hybridization to sequence-specific capture oligonucleotides on electronic microarray. The expression of a model set of 10 target genes in the U937 cell line was analyzed during lipopolysaccharide-mediated differentiation with 2-fold changes in concentration and 64-fold range of concentration (Figure 1.7). This electronic array based expression analyzing method allows simultaneous assessment of target concentrations from multiple sample sources.

1.3.4. Cell Separation

In addition to dc current, ac voltage can also be applied to the electronic microarray in certain ways to create a dielectrophoretic (DEP) force applicable to analytes such as cells. Depending on the frequency of the ac voltage and the dielectric properties of the cells, the DEP forces can be either positive (moving cells towards electrodes) or negative (moving

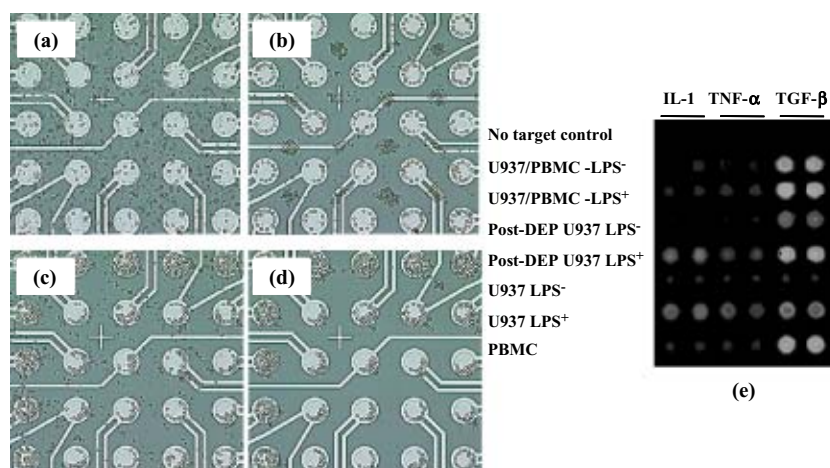


FIGURE 1.8. The procedure of DEP separation for U937 and PBMC mixture and gene expression profiling of three genes for U937 cells before and after DEP separation on electronic microarrays. (a) Mixture is introduced to the microarray. (b) U937 cells are separated from PBMC on array by dielectrophoresis 5 min after an ac voltage is applied. U937 cells are collected on the electrodes and PBMC are accumulated at the space between the electrodes. (c) Buffer is introduced from the reservoir to the array by fluid flow while the voltage is kept on. PBMC are carried away with the fluid stream. (d) PBMC are washed off from the array and U937 cells are retained on the electrodes after 10 min of washing. (e) Fluorescent image of three genes expression profiling before and after DEP separation. The specific signals for IL-1, TNF- α , and TGF- β from different samples after electronic hybridization are indicated. Reprinted with permission from Huang et al. Copyright (2002) American Chemical Society.

cells towards spaces) resulting in special separation of different types of cells on electronic microarrays [8, 26]. Using DEP, U937 and PBMC were separated on the array [22]. The separation procedure is illustrated in Figure 1.8. After the mixture was introduced to the array, the flow was stopped (Figure 1.8a). Five minutes after an ac voltage of 500 kHz, 7 Vpp (volts, peak to peak) was applied to the array, U937 cells were separated from PBMC on chip (Figure 1.8b). Then by introducing the fluidic flow of 40 μ l/min and keeping on the voltage, only PBMC were washed away with the buffer (Figure 1.8c). Ten minutes after washing, only U937 cells were remained on the electrodes by positive dielectrophoresis (Figure 1.8d). These U937 cells could then be released from the electrodes by fluidic flow and subsequently collected for gene expression analysis if the applied voltage was turned off.

RNA from DEP separated U937 cells was extracted, and the expression levels of IL-1, TNF- α , and TGF- β were quantitatively monitored on a 10 \times 10 microelectronic chip array using a targeted gene expression profiling assay. The gene expression levels of IL-1, TNF- α , and TGF- β for LPS treated or untreated U937 cells were compared with those of U937 and PBMC mixture on a 10 \times 10 array before and after DEP separation (Figure 1.8e). As demonstrated in Figure 1.8e, upon LPS treatment, U937 cells exhibited a significant increase in expression levels of IL-1, TNF- α , and TGF- β . After mixing U937 cells with PBMC at 1:5 ratio (U937 cells to PBMC), the expression patterns of the three genes had changed. In the mixed sample, the LPS induction of IL-1 and TNF- α expression could not be detected, and the induction of TGF- β was decreased. This reduction of the TGF- β

expression level in the mixed samples was most likely due to the high expression level of TGF- β gene in PBMC. Apparently, in heterogeneous samples, the gene expression levels in a cell population of interest are not simply diluted by unrelated cells but are masked by the expression patterns of the contaminating cell populations. Notably, in the DEP separated U937 cells, the LPS induction of IL-1, TNF- α , and TGF- β expression was observed. This result indicates that DEP separation can improve the accuracy of gene expression profiling by purifying out cells of interest.

1.3.5. Electronic Immunoassays

The flexibility of the microarray platform in terms of the ability to electrophoretically transport charged molecules to any site on the planar surface of the array has been exploited for developing electronic immunoassays. The following is an example of an electric field-driven immunoassay developed for two biological toxins—Staphylococcal enterotoxin B (SEB) and Cholera toxin B (CTB) for biological warfare applications [16]. A 25-site electronic microarray (Figure 1.9) was transformed into an immunoassay array by electronically biasing electrodes at user defined microlocation to direct the transport, concentration, and binding of biotinylated monoclonal capture antibodies specific for SEB and CTB to streptavidin in the hydrogel layer. The detection of fl-SEB and fl-CTB were accomplished in only 6 minutes, including 1-minute electronic addressing step to bind fl-SEB and fl-CTB, followed by a 5-minute washing step to reduce nonspecific binding. More noticeably, electronic addressing permitted the detection of CTB down to concentrations of 18 nM. No fluorescence

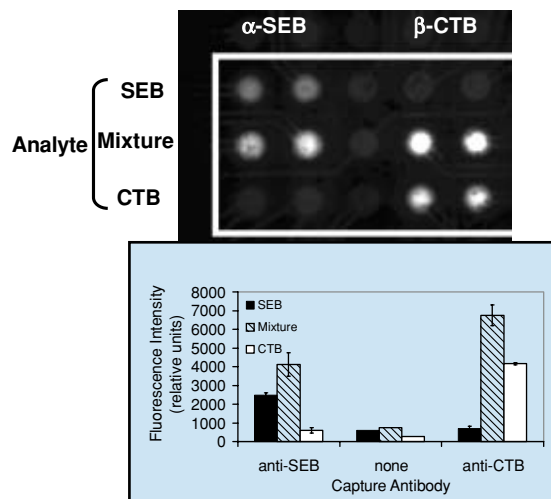


FIGURE 1.9. Electronic assay for fl-SEB and fl-CTB on the 10,000 site CMOS array. Biotinylated anti-SEB capture antibody was electronically addressed to the microlocations in the two columns on the left side of the image. Biotinylated anti-CTB capture antibody was electronically addressed to each of the microlocations in the two columns on the right side of the image. The column of microlocations in the center was left free. A mixture of fl-SEB and fl-CTB (20 nM in 50 mM histidine, pH 7.5) was applied to the chip and electronically addressed to all 15 microlocations of the chip. After washing, the fluorescence intensity at each microlocation was measured.

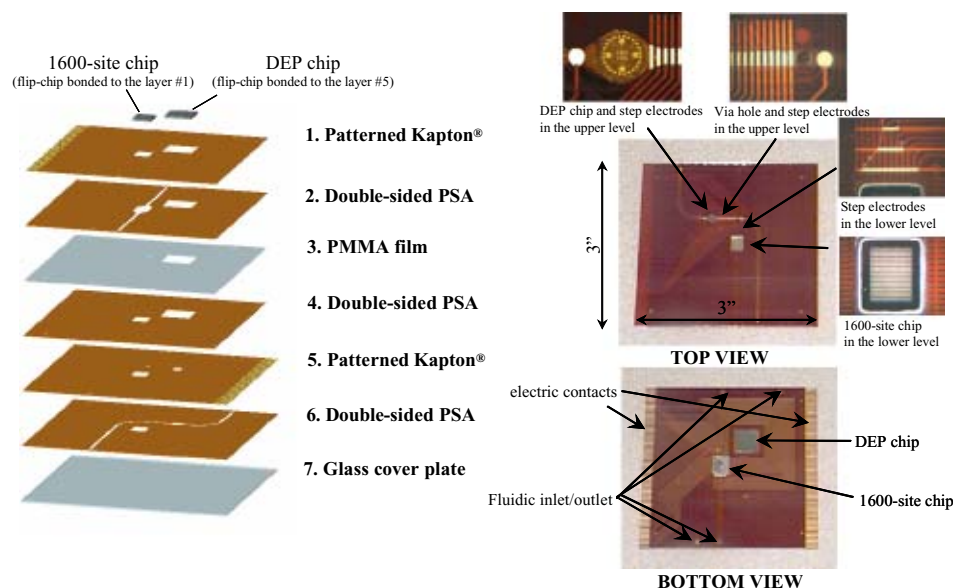


FIGURE 1.10. The stacked microlaboratory : (left) Fabrication of the stacked structure; (right) Photographs of the completed stacked structure showing the top and the bottom views.

attributable to antigen binding was observed at any other location on the chip other than the sites addressed with the appropriate capture antibody. It was possible to detect both toxins from a mixture in a single electronic addressing step (Figure 1.10). The ability to perform a rapid, electric field-mediated immunoassay for multiple analytes provides an advantage over existing approaches in terms of sample volume, speed and system complexity.

1.3.6. Miniaturization of Electronic Microarray Technology and Applications

DNA microarray technology provides a highly specific analytical response that enables the identification of a particular sequence within the DNA target molecules. Using these characteristic sequences the identification of biological agents, including biological warfare agents and infectious disease pathogens can be performed with the highest specificity and accuracy. It is of emergent interest to make portable instruments capable of performing rapid DNA analysis in the field or in hospitals. Such trends call for the development of the so-called point-of-care or field-portable instruments which could be used immediately at the patient bed or by first responders in the case of biological attacks. The requirements for such instrumentation are very stringent including high sensitivity and specificity, simultaneous detection of multiple targets, automated operation, and ease of use. Encountering various types of samples in the field, there is an urgent need to integrate the sample preparation processes with the detection and create simple to operate field portable sample-to-answer instruments [2]. Using the basic principles of the electronic separation and addressing of the targets we have developed a number of different approaches to miniaturize detection

systems as well as the integration of sample preparation with detection on the electronic microarray.

1.3.6.1. Stacked Microlaboratory A sample-to-answer prototype instrument was developed to perform pathogen isolation [28], DNA hybridization and protein immunoassays from mixed samples [52]. At the core of this instrument is a small two-level-stacked microlaboratory that is $76 \times 76 \text{ mm}^2$ and 2.77 mm thick. The stacked microfluidic structure was constructed from a set of laminated flexible substrates with fluidic cutouts, pressure sensitive adhesive layers, electrode arrays, and two Si chips. The completed stacked structure has the DEP chip in the upper level chamber for dielectrophoretic collection of bioparticles and the 1600-site chip in the lower level for performing automated electric-field-driven assays (Figure 1.10). Various automated assays have been demonstrated on this instrument. *E. coli* bacteria and Alexa-labeled protein toxin SEB were detected by electronic immunoassay. The identification of SLT1 gene from *E. coli* was accomplished in 2.5 hours starting from a dielectrophoretic concentration of intact *E. coli* bacteria and finishing with an electronic DNA hybridization assay [52].

1.3.6.2. Miniaturized Electronic Microarray System Rapid identification of pathogens or biological agents, including biological warfare agents, would ideally incorporate small automated portable systems. Supported through Department of Defense funding, a simple miniaturized system for the detection of DNA targets from pathogenic microorganisms based on the electronic microarray technology was developed. The first generation of the instrument is an automated portable DNA analysis system designed to operate with the Nanogen's 400-site NanoChip[®] array and cartridge. The 400-site array (Figure 1.11) is a

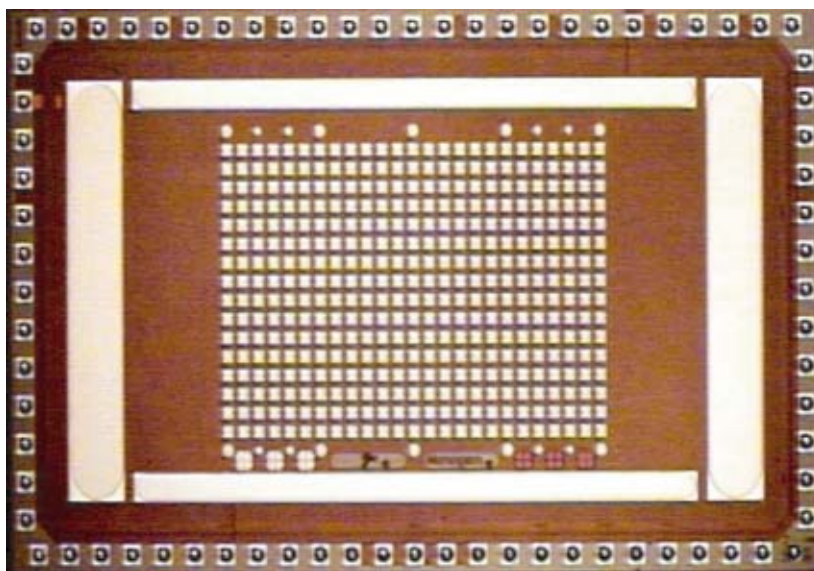


FIGURE 1.11. Photograph of a CMOS 400-site chip. Counter electrodes surround the central 400-site working electrode array.



FIGURE 1.12. Photograph of the electronic microarray system with the laptop used to operate the instrument and perform data storage and processing. The arrow indicates a port for the 400-site cartridge introduction.

second generation CMOS chip developed at Nanogen [46]. The advantages of the CMOS chip over the first generation design include simple control circuitry, improved electrode current and voltage control, four times as many assay sites and on-chip temperature sensing. The CMOS chip has an array of 16×25 (400-sites); each electrode being $50 \mu\text{m}$ in diameter with a $150\text{-}\mu\text{m}$ center-to-center distance. Voltage on each electrode can be individually controlled and measured. Furthermore, a temperature sensor is built into the chip's CMOS circuitry.

A photograph of the Nanogen's portable electronic microarray detection instrument is shown in Figure 1.12. It is a compact, fully enclosed instrument featuring automated DNA sample and reagent delivery to the chip, temperature control of the chip and the fluidic cell, electronic control of the chip array, and optical detection system. Any standard laptop or desktop computer can be used to operate and control the system. The overall dimensions of the system are (not including laptop) ca. 14 inch \times 12 inch by 6 inch. The total weight including the laptop which provide automated control of the system and data acquisition, ca. 30 lbs.

The portable instrument and electronic microarray platform allows development of a number of assays which can support PCR amplification of the target DNA from the sample, as well as solution strand displacement amplification (SDA) and anchored SDA. Anchored SDA provides an extremely convenient way to integrate the sample preparation steps. In this arrangement, the DNA amplification step and fluorescent detection steps are performed on the microarray chip. Highly sensitive assays were developed for detection of typical biological warfare agents such as anthrax and vaccinia. A number of other assays for viral and bacterial DNA identification, e.g., including *Staphylococcus enterotoxin a* (SEA) and b (SEB), *Yersinia pestis* (plague), *E. Coli*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, and others are being optimized for sensitivity using the same technology. Table 1.3 shows results of a sensitivity study performed for determination of *Bacillus anthracis*

TABLE 1.3. Results of a sensitivity study for *Bacillus anthracis*, Vollum, CapB gene*.

Sample	Number positives/number replicates				
	Chip 1	Chip 2	Chip 3	Chip 4	Total
1 fg/ μ l	1/10	1/10	1/10	1/10	4/40
10 fg/ μ l	8/10	7/10	7/10	8/10	30/40
100 fg/ μ l	10/10	10/10	10/10	10/10	40/40

* Study performed as an independent evaluation of the method by, Midwest Research Institute, Florida.

Vollum DNA. A PCR amplification protocol was developed and CapB gene was used as a confirmation gene. Table 1.3 demonstrates that 100 % correct calls were made for 100 fg/ μ l DNA and 75% for 10 fg/ μ l DNA. These values correspond to ca. 170 and 17 copies of anthrax DNA. The achieved sensitivity is well within the requirements for field detection of biological warfare agents. Similar sensitivity has been achieved for other biowarfare agents.

1.3.7. Applications in Proteomics

After the completion of the human genome project, the attention of the scientific community has turned toward the gene products within the cell and tissue matrix, namely proteins. The field of proteomics is an evolving area, which may shed light on the proteins associated with diseases and tumors. Protein kinases are of particular interest because they have been shown to be key regulators of many cell functions and have been one of the main targets in drug industry. Several high-throughput screening (HTS) kinase assays have been developed based on either antibodies or radioactivity for detection. Recently, an electronic, fluorescent assay for kinases, phosphatases and proteases has been developed for the serine/threonine kinase PKA [30]. This ElectroCaptureTM PKA assay combines electric field separation of a substrate and a reaction product of different net charge and subsequent capture of the reaction product on a capture matrix (Figure 1.13). The Lissamine-rhodamine labeled Kempide peptide substrate contains a (+1) charge. Upon phosphorylation, the

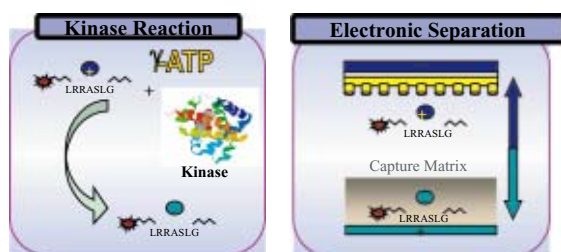


FIGURE 1.13. The ElectroCaptureTM Assay. The fluorescently labelled substrate contains a (+1) charge. Upon phosphorylation, the peptide substrate undergoes a charge inversion from a (+1) charge to a (-1) charge on the product of the reaction. When an electric field is applied, the unphosphorylated peptide substrate (+1) migrates towards the negative electrode and the product (-1) migrates towards the positive electrode. The (-1) charged phosphorylated peptides migrate towards the (+) electrode from the solution, through the diffusion barrier and bind to the membrane.

peptide substrate undergoes a charge inversion from a (+1) to a (−1) charge on the product of the reaction. When an electric field is applied, the positively charged unphosphorylated peptide substrate migrates towards the negative electrode and the negatively charged phosphorylated peptides product migrates towards the positive electrode from the solution, through the diffusion barrier and bind to the membrane. After the electrophoretic separation, the amount of Lissamine-rhodamine labeled phosphorylated peptides product can be quantitated, e.g., using a Tecan Ultra 384 fluorescence reader. The ElectroCapture™ PKA assay was validated with both known PKA inhibitors and with library compounds. The pK_i results obtained in the ElectroCapture™ assay were comparable to those generated in our current radioactive Filter Binding assay and antibody-based competitive Fluorescence Polarization (FP) PKA assay formats [30].

1.4. SUMMARY AND OUTLOOK

Future applications of focused arrays will involve improvements in speed, sample preparation and systems integration. This evolution in technology will allow the user to process a variety of samples in the disposable cartridge or directly on the embedded array. As we have demonstrated in this review, complete sample to answer systems based on site-specific electrophoresis, dielectrophoresis, DNA amplification and detection can be optimized, miniaturized and integrated into a complete sample-to-answer system. The usage of integrated and portable biological detection systems is expected to increase several fold in the next few years including point of care diagnostic applications for genotyping, pharmacogenomics, proteomics, detection of infectious agents as well as identification of biological warfare agents. The electronic microarray technology offering high speed of hybridization, target concentration and multiplexing has all the advantages to be one of highly competitive technologies in future miniaturized instruments for molecular and clinical diagnostics.

REFERENCES

- [1] A.A. Alzadeh, M.B. Elsen, R.E. Davis, C. Ma, I.S. Lossos, and A. Rosenwald et al. *Nature*, 403:503, 2000.
- [2] R. Anderson, X. Su, G. Bogdan, and J. Fenton. *Nucleic Acids Res.*, 28:12, 2000.
- [3] H.A. Behrens, M. Pignot, N. Windhab, and A. Kappel. *Nucleic Acids Res.*, 30:e64, 2002.
- [4] J. Boguslavsky. Lab-on-a-Chip: Easier, Faster, Smaller, Drug Discovery & Development, July/August, p. 32, 2001.
- [5] L. Carey and L. Mitnik. *Electrophoresis*, 23:1386, 2002.
- [6] R. Chakraborty, D.N. Stivers, B. Su, Y. Zhong, and B. Budowle. *Electrophoresis*, 20:1682, 1999.
- [7] M. Chee, R. Yang, E. Hubbell, A. Berno, X.C. Huang et al. *Science*, 274:610, 1996.
- [8] J. Cheng, E.L. Sheldon, L. Wu, A. Uribe, L.O. Gerrue, J. Carrino, M.J. Heller, and J.P. O'Connell. *Nat. Biotech.*, 16:541, 1998.
- [9] K.L. Cooper and R.V. Goering. *J. Mol. Diagn.*, 5:28, 2003.
- [10] B. Dukek and D.J. O'Kane. *IVD Technol.*, 47:Jan/Feb, 2004.
- [11] C.F. Edman, D.E. Raymond, D.J. Wu, E. Tu, R.G. Sosnowski, W.F. Butler, M. Nerenberg, and M.J. Heller. *Nucleic Acids Res.*, 25:4907, 1997.
- [12] C.F. Edman, P. Mehta, R. Press, C.A. Spargo, G.T. Walker, and M. Nerenberg. *J. Invest. Med.*, 48:93, 2000.

- [13] M. Eggers, M. Hogan, R.K. Reich, J.B. Lamture, D. Ehrlich et al. *Biotechniques*, 17:516, 1994.
- [14] M. Erali, B. Schmidt, E. Lyon, and C. Wittwer. *Clin. Chem.*, 49:732, 2003.
- [15] J.G. Evans and C. Lee-Tataseo. *Clin. Chem.*, 48:1406, 2002.
- [16] K.L. Ewalt, R.W. Haigis, R. Rooney, D. Ackley, and M. Krihak. *Anal. Biochem.*, 289:162, 2001.
- [17] L. Feng and M. Nerenberg. *Gene. Ther. Mol. Biol.*, 4:183, 1999.
- [18] S.P. Fodor, R.P. Rava, X.C. Huang, A.C. Pease, C.P. Holmes, and C.L. Adams. *Nature*, 251:767, 1991.
- [19] P.N. Gilles, D.J. Wu, C.B. Foster, P.J. Dillon, and S.J. Chanock. *Nat. Biotechnol.*, 17:365, 1999.
- [20] J.G. Hacia. *Nat. Biotechnol.*, 21(suppl):42, 1999.
- [21] M.J. Heller. *IEEE Eng. Med. Biol.*, 100: March/April, 1996.
- [22] M.J. Heller. *Annu. Rev. Biomed. Eng.*, 4:129, 2002.
- [23] M.J. Heller, E. Tu, R. Martinsons, R.R. Anderson, C. Gurtner, A.H. Forster, and R. Sosnowski. In M.J. Heller and A. Guttman (ed.), *Integrated Microfabricated Biodevices*. Marcel Dekker, New York, p. 223, 2002.
- [24] M. Heller, A.H. Forster, and E. Tu. *Electrophoresis*, 21:157, 2000.
- [25] M.J. Heller, E. Tu. U.S. Patent # 5,605,662 Nanogen, Inc., San Diego, CA, 1997.
- [26] Y. Huang, K.L. Ewalt, M. Tirado, R. Haigis, A. Forster, D. Ackley, M.J. Heller, J.P. O'Connell, and M. Krihak. *Anal. Chem.*, 73:1549, 2001.
- [27] Y. Huang, S. Joo, M. Duhon, M. Heller, B. Wallace, and X. Xu. *Anal. Chem.*, 74:3362, 2002.
- [28] Y. Huang, J.M. Yang, P.J. Hopkins, S. Kassegne, M. Tirado, A.H. Forster, and H. Reese. *Biomed. Microdev.*, 3:217, 2003.
- [29] Y. Huang, J. Shirajian, A. Schroder, Z. Yao, T. Summers, D. Hodko, and R. Sosnowski. *Electrophoresis*, 25:3106, 2004.
- [30] K. Huss, R.M. Campbell, S. Miick, S. Jalali, D. Thomas, and M. Jimenez. *9th Annual SBC Conference*, Poster 1025, Portland, OR, 2003.
- [31] S.K. Kassegne, H. Reese, D. Hodko, J.M. Yang, K. Sarkar, D. Smolko, P. Swanson, D.E. Raymond, M.J. Heller, and M.J. Madou. *Sens. Actu. B*, 94:81, 2003.
- [32] K. Liszewski. Broader Uses for Microfluidics Technologies, *Genet. Eng. News*, vol. 23, no 9, p. 40, 2003.
- [33] M. McCormick. *Genet. Eng. News*, vol. 23, no. 15, p. 34, 2003.
- [34] C.E. Pearson, Y.H. Wang, J.D. Griffith, and R.R. Sinden. *Nucleic Acids Res.*, vol. 26, 816, 1988.
- [35] E.S. Pollak, L. Feng, H. Ahadian, and P. Fortina. *Ital. Heart J.*, 2:568, 2001.
- [36] S. Raddatz, J. Mueller-Ibeler, J. Kluge, L. Wass, G. Burdinski, J.R. Haven, T.J. Onofrey, D. Wang, and M. Schweitzer. *Nucleic Acids Res.*, 30:4793, 2002.
- [37] R. Radtkey, L. Feng, M. Muralhidar, M. Duhon, D. Canter, D. DiPierro, S. Fallon, E. Tu, K. McElfresh, M. Nerenberg, and R. Sosnowski. *Nucleic Acids Res.*, 28:e17, 2000.
- [38] E. Ricart, W.R. Taylor, E.V. Loftus, D. O'Kane, R.M., Weinshilboum, W.J. Tremaine, W.S. Harmsen, A.R. Zinsmeister, and W.J. Sandborn. *Am. J. Gastroenterol.*, 97:1763, 2002.
- [39] R. Santacrose, A. ratti, F. Caroli, B. Foglieni, A. Ferraris et al. *Clin. Chem.*, 48:2124, 2002.
- [40] M. Schena, D. Shalon, R.W. Davis, and P.O. Brown. *Science*, 270:467, 1995.
- [41] I. Schrijver, M.J. Lay, and J.L. Zehnder. *Am. J. Clin. Pathol.* 119:490, 2003.
- [42] E. Sheldon, et al. In electronic sample handling. In S.A. Minden and L.M. Savage (ed.), *Diagnostic Gene Detection & Quantification Technologies for Infectious Agents and Human Genetic Diseases*, IBC Library Series, pp. 225–238, 1997.
- [43] Y.R. Sohni, J.R. Cerhan, and D. O'Kane. *Hum. Immunol.* 64:2003.
- [44] R. Sosnowski, E. Tu, W.F. Butler, J. O'Connell, and M. J. Heller. *Proc. Natl. Acad. Sci. USA*, vol. 94, p. 1119, 1997.
- [45] R. Sosnowski, M.J. Heller, E. Tu, A.H. Forster, and R. Radtkey. *Psychiatr. Genet.*, 12:181, 2002.
- [46] P. Swanson, R. Gelbart, A.E. Atlas, L. Yang, T. Grogan, W.F. Butler, D.E. Ackley, and E. Sheldon. *Sens. Actu. B.*, 64:22, 2000.
- [47] W.A. Thistlethwaite, L.M. Moses, K.C. Hoffbuhr, J.M. Devaney, and E.P. Hoffman. *J. Mol. Diagn.*, 5:121, 2003.
- [48] V.W. Weedn and J.W. Hicks. *Natl. Inst. Justices J.*, 234:16, 1997.
- [49] E.M. Weidenhammer, B.F. Kahl, L. Wang, L. Wang, M. Duhon, J.A. Jackson, M. Slater, and X. Xu. *Clin. Chem.*, 48:1873, 2002.
- [50] L. Westin, X. Xu, C. Miller, L. Wang, C.F. Edman, and M. Nerenberg. *Nat. Biotechnol.*, 18:199, 2000.
- [51] L. Westin, C. Miller, D. Vollmer, D. Canter, R. Radtkey, M. Nerenberg, and J.P. O'Connell. *J. Clin. Microbiol.*, 39:1097, 2001.

- [52] J.M. Yang, J. Bell, Y. Huang, M. Tirado, T. Thomas, A.H. Forster, R.W. Haigis, P.D. Swanson, R.B. Wallace, B. Martinsons, and M. Krihak. *Biosen. Bioelect.*, 17:605, 2002.
- [53] N. Yoshida, Y. Nishimaki, M. Sugiyama, T. Abe, T. Tatsumi, A. Tanoue, A. Hirasawa, and G. Tsujimoto. *J. Hum. Genet.*, 47:500, 2002.
- [54] K. Zimmermann, T. Eiter, and F. Scheifflinger. *J. Microbio. Methods*, 55:471, 2003.

BioMEMS and Biomedical Nanotechnology

Volume II: Micro/Nano Technologies for Genomics and
Proteomics

Editor-in-chief: Ferrari, M. - Ozkan, M.; Heller, M. (Eds.)

2007, XXIV, 540 p., Hardcover

ISBN: 978-0-387-25564-4