13 Micro- and Nanoscale Technologies in High-Throughput Biomedical Experimentation

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Abstract Biological systems are highly complex. To analyze the intricate workings of biological systems in an affordable and timely manner, a number of technologies have been developed to analyze biological systems in a highly parallel manner. Of these tools, micro- and nanoscale technologies have emerged as an effective tool because they can be used to automate, miniaturize, and multiplex biochemical assays to study biological functions at the cellular and genomic level at reduced experimentation costs. Herein, we provide a broad overview of several micro- and nanoscale technologies, as well as discuss their current and future applications. Our review primarily focuses on the microarray technologies and their applications to platforms such as DNA, cell, and protein arrays. We also provide a brief description of micro- and nanopatterning of substrates and scaffolds, and their effect on stem cell differentiation and cellular co-cultures. Additionally, augmentation of the microarray technology via integration with microfluidic technologies; and the application of microarrays as biochemical detection platforms are also discussed. Wherever appropriate, current limitations, suitable alternatives, and directions for future research have also been presented.

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13.1 Introduction

Biological systems are inherently complex and regulated by simultaneous interactions between thousands of genes and genetic products in a temporally and spatially organized manner. Thus, to understand biological systems it is desirable to replicate the complexity observed within natural systems. And there is a need to development of powerful tools to undertake highly-parallel experiments to enable rapid probing and quantification of nucleic acids, proteins, molecular signals and cells (Situma et al., 2006).

Microscale technologies (which can reach nanoscale resolution) have been used in the semiconductor and microelectronics industries for nearly half a century (Franssila, 2004; Khademhosseini et al., 2006). However, their widespread use in the biomedical sciences is a recent phenomenon. Micro- and nanoscale technologies offer the promise of automating, miniaturizing, and multiplexing biochemical assays to study biological functions at the cellular and genomic level at reduced experimentation costs (Palsson and Bhatia, 2004). Biochemical and cellular microarrays, microfluidic systems, and micro- and nanoengineered biochemical detection platforms constitute some prominent examples of the application of micro- and nanoscale technologies to high-throughput experimentation. Microarrays enable the simultaneous analyses of thousands of nucleic acids, genes, proteins, and cells on a single chip. Such capacity for massively parallel biomolecular analysis is key to the high-throughput study and optimization of dynamic biological phenomena such as cell-matrix interactions. Although the exact molecular mechanisms that elicit cellular-fate processes remain imprecisely defined, microarrays can be effectively used to characterize cellular behavior in response to specific physiochemical changes and, in turn, may help elucidate the biochemical machinery of cell differentiation. Microfluidic gradient generation systems can also be used to perform high- throughput cell-based experiments. Microfluidic systems are cheap, minimize consumption of expensive reagents, and generally require low cell populations for experimentation. Microfluidic systems could potentially be used for performing rapid screening experiments, evaluating drug toxicity, and investigating optimal culture conditions for the facilitation of specific cellular-fate processes. Lastly, micro- and nanoengineered biochemical detection platforms are emerging as viable solutions for clinical, environmental, food, and chemical testing.

Libraries of molecules, candidate drugs, biomaterials, and cells will become more accessible and user-friendly in the years ahead. However, this increased availability of biological agents will be fruitless without high-throughput functional assays to verify their clinical or experimental value. Micro- and nanoscale technologies provide biomedical engineers with valuable tools for screening libraries and facilitate an unprecedented degree of control over the cellular microenvironment (Khade mhosseini et al., 2006; Palsson and Bhatia, 2004). As such, the advantages of using them for high-throughput biological experimentation to probe cellular behavior and genomic activity are manifold.

The fabrication of micro- and nanoscale devices is generally achieved using either a 'bottom-up' or 'top-down' approach. Bottom-up approaches generally involve the buildup of atoms or molecules in a controlled, thermodynamically-regulated manner to form nanostructures (Khademhosseini and Langer, 2006). An example of a bottom-up approach is atomic or molecular self-assembly. Contrastingly, top-down fabrication technologies include techniques such as photolithography, soft lithography, nanomoulding, dip-pen lithography, and micro- and nanofluidics (Khademhosseini and Langer, 2006).

Herein, we present a brief overview of the application of top-down micro- and nanoscale technologies in high-throughput biological experimentation by providing categorical examples while elaborating specific applications of each group of technologies. We initially review microarray technologies by discussing aspects such as fabrication, existing applications and emerging developments. Next, applications of microfluidic technologies in high-throughput experimentation will be presented. The automation and integration of microfluidics with other micro- and nanoscale technologies shall also be examined. Finally, novel micro- and nanoengineered detection methodologies will be introduced. We shall also discuss the current limitations of these technologies. Furthermore, directions for future research in the applications of micro- and nanoscale technologies to high-throughput biomedical experimentation will be presented (Fig. 13.1).

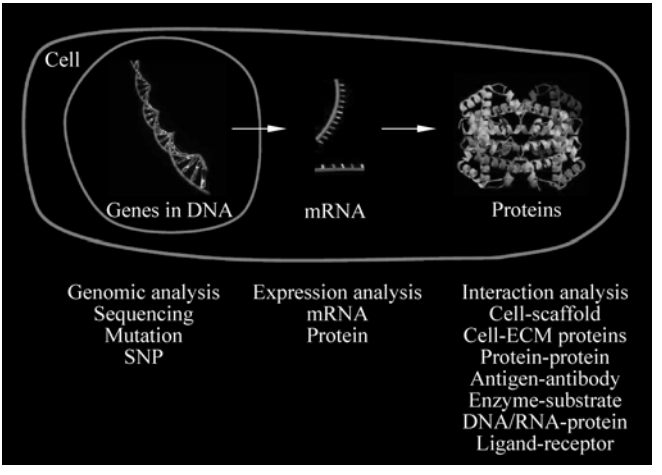

Figure 13.1 Applications of microscale and nanoscale technologies in high-throughput biological experimentation

13.2 Microarray Technologies

Microarrays tools are widely used in the analyses of biomolecules and tissues. They permit the parallel analyses of thousands of biological samples, thereby

facilitating the rapid profiling of macromolecules and their physiological and pathological changes on a genome-wide scale (Stratowa and Abseher, 2005). In 2003, the total global market for microarrays was approximately $596 million and it is expected to rise to $1 billion by 2010 (Taylor, 2005).

Microarrays were first fabricated during the late 1980s. Early microarrays were simple arrangements of peptides and oligonucleotides on glass slides (Taylor, 2005). In the years that followed, complex cDNA arrays for gene expression analyses were also developed. The subsequent emergence of proteomics has created opportunities for the development of protein and antibody-based microarrays designed to aid in identifying protein products from gene expression. More recently, advances in micro- and nanofabrication have enabled the synthesis of cellular microarrays to investigate and direct cellular fate process activities such as growth, migration, and differentiation (Table 13.1).

Table Types of microarrays and their applications (Stratowa and Abseher, 2005)

Probe	Array name	Domain	Profiling
DNA	Matrix CGH*	Genome	Genotypic
Oligonucleotide	Oligonucleotide array	Genome Transcriptome	Polymorphic Expression
CDNA	cDNA microarray	Transcriptome	Expression
Protein	Protein array Antibody array	Proteome Proteome	Expression/activity Expression
Tissue	Tissue microarray	Tissuome Organome	Histological Histological

*CGH: Comparative genomic hybridization

13.2.1 Evolution of Microarrays

A microarray generally consists of a substrate and an active layer of immobilized detection molecules. The active layer is comprised of oligonucleotides, DNA, RNA, antibodies, or other biomolecules that bind to specific analytes. In some cases, microarrays are designed with embedded optical or electrochemical transducers to detect binding events or reactions occurring between the active layer and analytes. In the absence of transducer elements within microarrays, receptor-analyte complex formation is detected via radioisotope, fluorometric or chemiluminescent scanning instruments.

From a technological standpoint, microarrays represent an evolution in biosensors (Taylor, 2005). Conventional biosensors can only measure a single analyte at a time. In contrast, microarrays have up to 10^6 detection sites/cm^2, and can perform simultaneous, multianalyte detection (Schultz and Taylor, 2005).

Both, biosensors and microarrays utilize a biospecific surface for affinity or indirect analyte capture, coupled with a transducer or detector for qualitative or quantitative detection (Taylor, 2005; Schultz and Taylor, 2005). While biosensor detection and data processing is built into the unit, microarrays require highly sophisticated scanners for detecting and reporting capture events (Table 13.2).

Table 13.2 Comparison between construction of biosensors and microarrays (Taylor, 2005)

Component/function	Biosensors	Microarrays
Substrates	Glass, membrane, polymer	Glass, membrane, polymer
Receptors	Antibodies, other proteins, oligo-nucleotides, DNA, RNA	Antibodies, other proteins, oligo-nucleotides, DNA, RNA
Transducers	Optical, electrochemical, piezo-electric, surface acoustic wave, thermal etc.	None
Detection	Transducer output	Radioactivity, fluorescence and chemiluminescene scanning
Data processing	Integrated software	Scanner software
Detection site density	1 detection site per device	10^6 detection sites/cm^2

13.2.2 Microarray Fabrication and Applications

As shown in Fig. 13.2, microarray fabrication is typically initiated by substrate activation via surface-treatment, followed by the binding of receptor molecules to the substrate to yield an array. Receptor molecules are applied to the activated substrate by one of three major methods: (1) direct spotting, (2) in situ synthesis, and (3) polymeric embedding. Then, samples and controls are hybridized onto the array, fluorescently or radioisotopically labeled, and scanned. This is followed by data reduction and interpretation.

Generally, the desirable material properties of microarray substrates are high degree of surface smoothness, low background fluorescence, low coefficients of thermal expansion, low reflectivity, and high transmission. The most commonly used substrates for microarray fabrication include fused silica, borosilicate glass, aluminosilicates, and zinc titania (Taylor, 2005). Additionally, the successful use of porous materials such as nitrocellulose, nylon membranes, polyacrylamide gels, and block copolymers to fabricate microarrays for the study of receptor- target interactions in three-dimensions has also been reported (Timofeev et al., 1996).

The simplest approach to fabricate a microarray is the direct adsorption of receptor molecules onto a substrate. However, adsorption onto a substrate could potentially result in inactivation of the biomolecules or blocking of its active

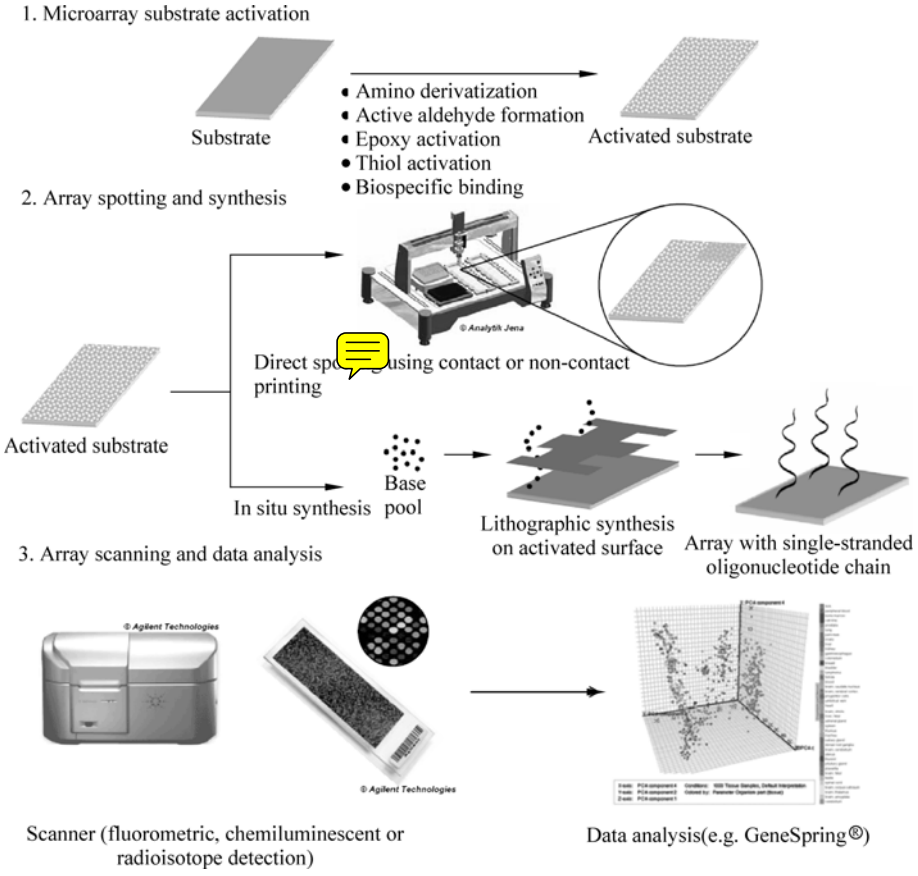

Figure 13.2 Common steps in the fabrication and use of a microarray

binding sites. To prevent inactivation and blocking, substrates are first activated via chemical modification. Some common surface modification processes include amino derivatization of available hydroxyl groups on the surface; formation of highly-reactive, free aldehyde groups; epoxy activation; thiol activation; and biospecific binding by immobilization of biotin-containing biomacromolecules using avidin (Taylor, 2005). These functional groups bind to available groups in oligonucleotides, proteins, antibodies and on the cell-surface, and, by properly orienting the receptor molecule alleviate the problems associated with the direct electrostatic conjugation of biomolecules onto substrates.

Array spotting follows surface activation. Arrays are printed using either direct spotting or in situ synthesis. Both methods are capable of applying picoliter to nanoliter volumes of DNA, RNA or protein onto a substrate. Automated robotic spotting instruments perform direct spotting. Robotic spotters use either contact printing or non-contact piezoelectric ink-jet printing for reproducible generation of arrays. Direct spotting is viable in the $10 - 10^4$ picoliter of solution per reaction center deposition range, resulting in spot diameters ranging from 50 to

1000 μm (Taylor, 2005). Direct spotting is used to generate arrays that contain 100 – 10,000 reaction centers, although arrays with as high as 50,000 reaction centers have also been synthesized (Taylor, 2005).

On the other hand, in situ synthesis is predominantly used to generate oligonucleotide, DNA and cDNA microarrays. In situ synthesis is a photolithographic technique by which oligonucleotide reaction centers can be directly synthesized on the substrate itself. In situ synthesis involves the repeated addition of amino acids and nucleotides to a growing peptide or oligonucleotide chain via a step-wise synthesis using linkers containing photochemically removable protecting groups. At each addition step, photolithographic masks are used to direct the deprotection of specific chain end-groups, while monomers containing protecting groups on one of their two linker sites are linked to the deprotected end of the growing chain. This fabrication methodology, which yields high-density arrays with specifically-known oligonucleotide sequences, was first successfully commercialized by Affymetrix. The hallmark of this process is that it permits variation in the length of oligonucleotide chains at different reaction centers. In situ synthesis produces the highest density arrays possible—with some microarrays possessing hundreds of thousands of reaction centers (Taylor, 2005). A more recent approach, called polymeric embedding, involves entrapment of receptor molecules within a multilayered polymer matrix to generate inexpensive microarrays.

Following the application of receptor molecules to the surface of the substrates, samples, and control solutions are hybridized onto the active arrays. As intended, the analytes in the samples bind to the receptors. Post-hybridization, the receptor-analyte complexes are tagged with fluorescent, chemiluminescent or radioisotopic labels, and then detected and quantified using fluorometric, chemiluminescent or radioisotope scanners respectively. Fluorescent labeling is the method of choice for genomic and proteomic array analyses. Protein and immunoassay microarrays are occasionally analyzed using chemiluminescent scanners. Probe molecules are only labeled using radioactive isotopes when difficulties with conventional labeling techniques arise. Generally, proteins are labeled with ^{32}P to avoid the undirected, non-specific, multisite labeling generally observed during biotinylation (Taylor, 2005). Labels can be incorporated into the oligonucleotide chains tethered to the surface of the substrate by synthesizing the chains from labeled nucleotides or PCR primers. Some commonly used labels include the cyanine labels (Cy2, Cy3, Cy5, etc.), the Alexa fluorescent dyes (Alexa 488, 532, 546, 568, etc.), fluorescein, rhodamine 6 G and phycoerythrin.

Inside the microarray scanner, the fluorescent dyes and labels are excited with lasers or white light, and the subsequently emitted light is detected using a photomultiplier tube (PMT) or a charge-coupled device (CCD) camera. A PMT is capable of both, amplifying and measuring low levels of fluorescent emissions, whereas, CCD scanners/imagers utilize emission filters to focus the fluorescent light onto the CCD camera. Evidently, CCDs are less sensitive than PMTs, and most CCD detectors integrate emission signals over time, leading to longer scan

and analysis times. Most commercially available scanners, such as the Affymetrix GeneChip, have two lasers—at excitation wavelengths of 532 and 635 nm—and use a PMT detector to analyze microarrays. Such scanners can generate a pixel resolution between 1 – 10 nm (Taylor, 2005). More recently, Tecan and Perkin Elmer have manufactured microarray scanners that employ four lasers. The Tecan LS scanner is integrated with two PMTs, and can scan a variety of substrates, ranging from microarray glass slides to microtitreplates. Fluorometric scanners are able to detect receptor-target concentrations as low as a few attomoles (10^{-18} mol/L).

13.2.3 DNA and cDNA Microarrays

DNA and cDNA microarrays were among the first microarrays developed, and are primarily used in genomic and expression analysis, determination of the base sequence of DNA fragments, and defining genetic activity and function in samples (Hong et al., 2005). The principal objective of early experiments involving DNA and cDNA microarrays was to analyze whole and/or targeted segments of the genomes of humans, animals, plants, and microbes for drug discovery, diagnostics, and other bio-based products (Khademhosseini, 2005).

DNA and cDNA microarrays are extensively used in toxicological and pathological research. In fact, the ability to efficiently expedite biochemical assays has furthered the development of toxicogenomics—a scientific discipline that combines the emerging technologies of genomics and bioinformatics to identify and characterize biochemical mechanisms of action of known toxicants inside the bodies of animals and plants. In a toxicogenomic study, mRNA from healthy and diseased individuals are isolated, refined and then reverse-transcribed to obtain cDNA. Individual genes or genetic fragments from the DNA are analyzed for their expression levels, and differences between the healthy and diseased samples, called single nucleotide polymorphisms (SNPs) are determined. The result is collection of genes that genetically characterize a disease state. Gene family identification of this type would be unobtainable without high-throughput analysis by DNA microarrays. Affymetrix has successfully designed microarrays that host the entire genomes of humans as well as other organisms (Affymetrix, 2006). Such arrays can aid in the rapid analysis of the whole genome—subsequently enabling genome-wide SNP detection and diagnoses for diseases such as AIDS. Additionally, technologies such as Affymetrix's GeneChip HIV PRT are also able to detect known mutations in the protease and reverse transcriptase enzymes of HIV-1, facilitating the development of effective therapeutic solutions for countering AIDS (Khademhosseini, 2005; Affymetrix, 2006).

Expression arrays are also being applied to pharmacogenomics to predict how a patient might respond to a specific drug, taking into account the mRNA and proteins produced by the patient; and the potential toxicity of a drug. For example, microarrays are now used to assess drug toxicity in the liver (Taylor,

2005). The method for elucidating drug toxicity mechanisms using microarrays is very similar to the technique used for detecting SNPs. This is shown graphically in Fig. 13.3. In addition to toxicological and pathological research, DNA microarrays are also successfully being used for drug discovery applications (Khademhosseini, 2005; Gerhold et al., 2001; Modden et al., 2000), and cancer (Brem et al., 2001; Gwssman, 2001; Graveel et al., 2001; Kalma et al., 2001; Monni et al., 2001; Okabe et al., 2001) and neuroscience research (Cavallaro et al., 2001; Geschwind, 2000; Zirlinger et al., 2001). Presently, considerable research is being directed towards improving the sensitivity of DNA and cDNA microarrays. Studies have demonstrated that sensitivity can be vastly improved by engineering surfaces with nanoscale features (Hong et al., 2005; Sunkara et al., 2006).

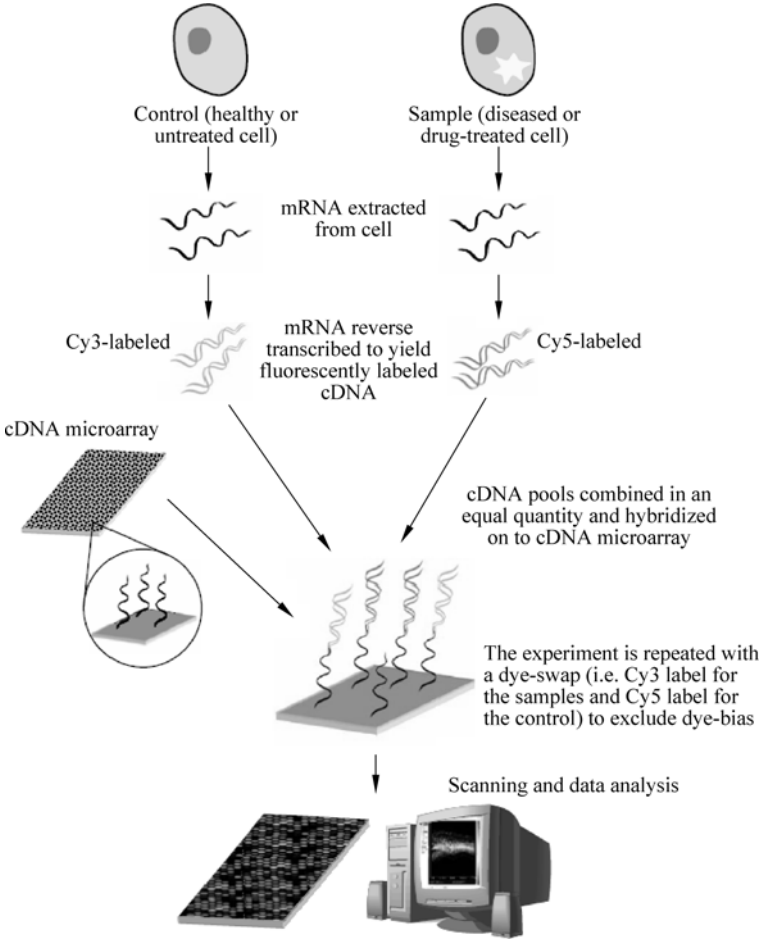

Figure 13.3 Steps for evaluating potential drug candidates and understanding the biological mechanisms of toxicants

13.2.4 Protein and Antibody-Based Microarrays

Although DNA microarrays can effectively determine RNA expression within a cell, they are inadequate for estimating levels of protein expression in cells (Khademhosseini, 2005). In 1999, nearly a decade after the fabrication of the first DNA microarrays, the need to accurately explain protein expression led to the development of protein microarrays (Haab et al., 2001). The primary goal of protein microarray technology is to determine the presence and quantify the proteins in cells or tissues. There are two types of protein microarrays: functional protein microarrays and antibody arrays. Functional protein microarrays are used to study the interaction of proteins with other molecules. On the other hand, antibody microarrays are miniaturized enzyme-linked immunosorbent assays (ELISAs), and can detect proteins with high sensitivity and selectivity (Khademhosseini, 2005).

Protein microarrays play an important role in proteomics—the analysis of total proteins expressed in a cell. Recent studies have successfully demonstrated the use of protein microarrays constructed from an entire cloned yeast proteome to study cellular structure and function within the organism (Zhu et al., 2001). Each of the nearly 5800 yeast proteins were produced, purified, and immobilized to glutaraldehyde-activated slides in duplicates. The chip was then used to investigate the specific binding of avidinfluorophone-labeled PI (a secondary messenger in transmembrane cellular communication) to the immobilized proteins. Following binding and washing, the bound PI was detected using fluorometry. Results suggested a differential binding of PI to the yeast membrane proteins—supporting the hypothesis that PI interacts with only specific membrane proteins. The study demonstrated a method for understanding a component of cell functionality based on spatial distribution of proteins in the cell and biomolecule adhesion quantified in a protein microarray. Similar studies are now being conducted to develop new drug therapies for treating dysfunctional cells and tissue; and characterizing novel functions for some well-studied proteins (Taylor, 2005; Khademhosseini, 2005; Zhu et al., 2001).

Protein microarrays are also being used to study phosphorylation mechanisms, as demonstrated by the studies aimed at understanding the binding of yeast protein kinases to large protein microarrays (Khademhosseini, 2005). Using data generated from protein-protein binding, protein-DNA binding and phosphorylation experiments, network maps containing information on intracellular interactions and regulatory pathways can been generated (Jansen et al., 2003; Luscombe et al., 2004). A better understanding of regulatory networks within the cells could potentially lead to the development of superior drug candidates (Ge, 2000; MacBeath et al., 1999).

In addition to drug discovery, protein microarrays have been used to detect and investigate autoimmune reactions in the body by incubating small volumes of the patient's serum onto antigen-immobilized arrays (Joos et al., 2000). By using a patient's serum as a test analyte, the antigen-marker complex bindings can be

quantified. The degree of formation of such complexes characterizes the autoimmune reactions of the body. This method offers a highly-parallel, high-efficiency and high-throughput disease diagnosis and detection platform. Antibody and antigen microarrays have tremendous potential in cancer research as diagnosis and characterization tools. Recent studies have demonstrated that combining multiple serum markers in a single antibody microarray radically increases the number of proteins that can be simultaneously detected and quantified (Carpelan-Holmstrom et al., 2002; Louhimo et al., 2002). Antibody microarrays have also been used to detect protein expression levels in UV-irradiated neoplasial tissue (Knezevic et al., 2001). The recently developed ProteinChip assay is based on the protein microarray technology. The ProteinChip technology has already been shown to be extremely useful in the identification and analyses of protein markers in diseased cells and tumours (Snijders et al., 2001). It is hypothesized that the integration of protein microarrays with bioinformatics would aid the rapid and accurate discrimination of cancerous cells from non-cancerous ones, thereby exhibiting utility as a high-throughput screening tool. Enzyme-based microarrays have also been used in the study of peroxidases, phosphotases, and kinases (MacBeath and Schreiber, 2000; Zhu et al., 2007). Protein and antibody-based microarrays are also used in immunoassays, profiling of protein and protein complexes (e.g. proteoglycans and proteolipids), and detection of biological warfare agents (Taylor, 2005; Timofeev et al., 1996).

Several technical challenges hinder the widespread use of protein microarrays as a disease detection tool. Firstly, protein function is strongly dependent on its three-dimensional structure. Active-site inhibition by structural deformation on account of binding to substrates is a common stumbling block for this technology. Additionally, protein-protein interactions are complicated by the onset of post-translational modifications such phosphorylation and glycosylation. Therefore, protein arrays are now being fabricated with post-translationally modified proteins or specific antibodies in order to actively sense such interactions (Khademhosseini, 2005).

Most of the current research in protein microarrays is directed at further increasing the sensitivity and throughput of these assays, and minimizing false positives (Khademhosseini, 2005). One potential method for increasing the sensitivity of the assay is to enhance the functionality of the immobilized proteins by using spacer molecules that minimize non-specific protein adsorption through surface modification (Khademhosseini, 2005). Studies using biomimetic surfaces have successfully demonstrated a 6- to 50-fold increase in assay sensitivity (Khademhosseini, 2005). Additionally, the development of biomaterials that offer improved control over the attachment and density of proteins on the substrate could alleviate some of the problems associated with protein immobilization onto the microarrays. Recent studies focusing on the use of self-assembling monolayers (Houseman et al., 2002), three-dimensional microstructures (Kang et al., 2005), and immobilized proteins specially inserted with a histidine-tag onto glass slides

containing a metal-chelating group (Zhu et al., 2001) have shown improvement in protein attachment to the microarray substrates.

13.2.5 Cell-Based Microarrays

The use of cell-based microarrays as high-throughput analytical platforms is a fairly recent phenomenon, and as such, the market for these technologies is still miniscule in comparison to the DNA and protein microarray markets. Generally, cell-based microarrays are used for biosensing and cell-screening applications, and studying the interactions between cells and biomaterials.

Characterizations of ligand-receptor interactions on cellular surfaces are of interest to a broad range of researchers in the biomedical sciences. In one application, microarrays of B cells derived from murine spleens and human blood were successfully developed (Yamamura et al., 2005). These microarrays contained nearly 2×10^5 microchambers, and used Ca^{2+} mobilization within activated cells to effectively profile ligand-receptor binding. The microchambers have been designed such that each compartment can accommodate no more than a single cell. Using fluorometry, intracellular Ca^{2+} in the B cells is quantified prior to and following incubation of characteristic antigens on the microarray. Subsequently, signal transduction in ligand-receptor pathways is elucidated by estimating the change in intracellular Ca^{2+} levels in the pre- and post-antigen binding states of the B-cells. This approach to studying ligand-receptor interactions offers significant advantages over flow cytometry, which is unable to monitor individual cells. Similar single-cell arrays have also been fabricated by using poly (dimethylsiloxane) (PDMS) (Rettig and Folch, 2005) and polystyrene (Dusseiller et al., 2005) microwells to study the cellular microenvironment in three dimensions.

Cell microarrays have proved useful for investigating stem cell biology and cellular fate processes, two areas vital to the field of tissue engineering. In one such study, a modified cell microarray was utilized to characterize the differentiation of human embryonic stem (hES) cells on different substrates (Anderson et al., 2004; Anderson et al., 2005). Therein, numerous biomaterials were directly synthesized onto a glass slide and subsequently incubated with hES cells for six days. Cells were then characterized to reveal the influence of each biomaterial on stem cell differentiation. Using a similar approach, the differentiation of hES cells cultured on different combinations of natural extracellular matrices (ECMs) was also investigated (Flaim et al., 2005). Each of these approaches enables the simultaneous screening of thousands of cell-material interactions in a relatively inexpensive, efficient manner.

Cell-based microarrays have also been applied to guide stem cell fates in numerous ways (Chin et al., 2004). Cellular micropatterning has been used to control cell shape and cellular fate processes such as migration, proliferation, differentiation and apoptosis (Hong et al., 2005; Sunkara et al., 2006). Rapidly

screening individual cells for specific chemical stimuli is another demonstrated area of application for cell microarrays to stem cell research (Love et al., 2006). For studying chemical stimuli, individual cells are first exposed to characteristic microenvironments in inverted microchambers, following which, antibodies secreted by each cell was captured and detected.

Also printed arrays of full-length open reading frames (ORFs) of the genes in expression vectors, along with lipid transfection reagents was used for parallel transfection of hundreds of genes in a microarray format, thereby enabling the analysis of phenotypic effects of various genes (Ziauddin et al., 2001). Using similar approaches, cell microarrays could also be used to test the repression or silencing of genes in a sequence-specific manner using small, single-stranded anti-sense oligonucleotides, or small interfering RNA (Khademhosseini, 2005). Cell array systems are also being used for studying cellular behavior (Chin et al., 2004; Tourovskaia et al., 2005), profiling cellular interaction with potential drug candidates, and evaluating phenotypic changes resulting from the expression of specific proteins within cells (Khademhosseini, 2005; Khademhosseini et al., 2003; Suh et al., 2004).

Cell-based microarrays have also been used as biosensing devices (Kim et al., 2006). For example, colonies of live T cells were immobilized into functionalized patterned hydrogel microwells and then cultured with a confluent layer of antigen-capturing B cells. Samples were then first exposed to colonies of B cells, and following their capture by the B cells, peptide analyses in the samples were subsequently delivered to the T cells due to the dynamic interactions between T cells and B cells within the microwells. The T cells respond to the introduction of the peptides analyses by producing a fluorescently detectable calcium signal. Thus, by engineering T cells to detect and distinguish a suite of pathogen-derived peptides, such cell-based biosensing platforms could effectively serve as large-scale disease diagnostic tools. Current limitations of cell-based assay include the ability to test many soluble conditions simultaneously as well as maintaining proper cell function in culture. Microengineered systems can be used to solve these problems. For example, to enable the simultaneously testing of multiple chemicals whilst maintaining proper cellular phenotypes in culture microfluidic channels may be used while to enhance cell function in vitro, patterned co-cultures and multiphenotype cell arrays could be used (Khademhosseini, 2005). It is anticipated that future developments in cell-based microarrays will make this technology an invaluable resource for undertaking high-throughput functional cell-based bioassays.

13.2.6 Other Microarrays and Microarray-Based Diagnostics

Microarrays are also extensively used in tissue engineering research. Microarrays fabricated from biopolymers and proteins derived from the ECM have also been

used to test molecular libraries and the effect of extracellular processes on cellular behavior (Khademhosseini, 2005). High-throughput testing of molecular libraries has already yielded favorable candidates for inducing osteogenesis (Jansen et al., 2003) and cardiomyogenesis (Wu et al., 2002) from ES cells, as well as dedifferentiation of committed cells (Chen et al., 2004). Recently, synthetic arrays, fabricated from a host of biomaterials, were successfully used to test the interaction of stem cells with various extracellular signals, and investigate the effects of polymeric materials on the differentiation of hES cells (Anderson et al., 2004) and mesenchymal stem cells (Anderson et al., 2005). Combinatorial matrices of numerous ECM proteins were utilized to evaluate the ability of these proteins to induce hepatic differentiation from murine ES cells, and maintain function of the differentiated hepatocytes (Flaim et al., 2005).

The use of microarrays has also been extended to clinical, environmental, food, and chemical testing (Taylor, 2005). Microarrays offer many advantages such as multiplexing of assays on a single chip, increasing analytical throughout, and decreasing costs. Microarrays are now being developed to utilize existing human and veterinary diagnostic technologies. An example of this is the recently-developed AmpliChip CYP450 microarray which is able to detect genetic variations in the genes for cytochrome P450 isoenzymes 2D6 and 2C19 in a reliable and efficient manner (Taylor, 2005). Variations in the genes that encode cytochrome P450 and its expressed enzymes could potentially change the metabolic mechanisms within an individual, and consequently affect the toxicity and efficacy of specific drugs in that individual (Taylor, 2005).

Other emerging microarray technologies include patterned polysaccharide and bead arrays (Blixt et al., 2004). Polysaccharide arrays can be used to study the interaction of libraries of chemicals and/or drugs with polysaccharides. It is hoped that future developments in this technology would provide a fillip to the discovery of drugs capable of interacting with cell-surface polysaccharides, and perhaps, aid in the advancement of the nascent field of glycomics—the study of the interaction of sugars with other molecules (Khademhosseini, 2005).

Developments such as the AmpliChip microarray have led to an increased investment in microarray diagnostic technologies. It is postulated that microarrays able to diagnose infectious diseases, blood disorders, and cancer; identify blood proteins and cardiac markers; analyze miniscule concentrations of industrial effluents and chemical waste; examine the quality of food products; and detect the presence of chemical and biological warfare agents will soon be developed.

13.3 Micro- and Nanoengineering for Biomedical Experimentation

Micro- and nanoengineering can also be used to generate topographical features on cell culture substrates in order to direct cellular fate processes such as cell

growth, migration, and differentiation (Thapa et al., 2003). Such techniques could potentially be extended to high-throughput microarray technologies. Micro- and nano-textured substrates have been shown to significantly influence cell alignment (Thakar et al., 2003), adhesion (Thapa et al., 2003; Deutsch et al., 2000; Stato and Webster, 2004), gene expression (den Braber et al., 1998; van kooten et al., 1998; Walboomers et al., 1999), metabolic activity(de Oliveira and Nanci, 2004), and migration (Teixeira et al., 2003). Textured nanotopography generated by chemical etching, anodization and embedding nanoscale objects within biomaterials has also been used to increase osteoblast adhesion while decreasing the adhesion of other cell types (Price et al., 2003). Nanoparticles have also been embedded within biomaterials to reduce cell adhesion (Webster, 2001; Webster et al., 1999, 2000, 2001), and control cell-surface interactions (Stevens and George, 2005).

The successful in vitro replication of in vivo cellular fate processes is highly dependent on the control and regulation of cell-cell and cell-substrate interactions in three dimensions. Dynamic co-cultures of different cell types on specially-designed, geometrically optimized two-dimensional patterns is one alternative for achieving this objective. Substrate patterning techniques have also shown to direct cellular fate processes. For example, variations in the size and shape of adhesive protein patterns on substrates can induce varied cytoskeletal reconfigurations, and have been shown to effect cell adhesion (Miller et al., 2004), proliferation, and apoptosis (Chen et al., 1997). Human MSCs patterned on micropatterns of different shapes have been shown to differentiate to either adipocytes or osteoblasts (McBeath et al., 2004). Dynamic cell patterning has also been achieved using photocrosslinkable gels (Elbert and Hubbell, 2001; Schutt et al., 2003), thermally-responsive polymer surfaces (Okano et al., 1995), and reversibly cracked substrates (Zhu et al., 2005). Such techniques could be potentially integrated with the microarray technology to explore the two-dimensional effects of planar patterns of various adsorbed or chemically-bound molecules in a high-throughput manner. Additionally, hepatocytes and endothelial cells have been shown to produce better differentiated phenotypes when co-cultured with each other, as compared to their individual cultures owing to higher hepatocyte spheroid induction and subsequent optimum albumin secretion in the co-culture (Fukuda et al., 2005). Micropatterned co-cultures have also been successfully used to control the degree of heterotypic and homotypic cell-cell interactions, and study cell-cell interactions between hepatocytes and non-parenchymal fibroblasts (Bhatia et al., 1998a, 1998b, 1999). Micropatterned co-cultures have also been generated using thermally-responsive polymers (Hirose et al., 2000; Yamato et al., 2001), layer-by-layer deposition of ionic polymers (Khademhosseini et al., 2004), microfluidic deposition (Chiu et al., 2000), and micromoulding hydrogels (Tang et al., 2003).

It is a well-known fact that cellular phenotypic expression and stem cell fates are affected by both, interactions with other cells and surfaces, and by the diffusional limitations introduced within cellular microenvironment by physically confining cells and cell aggregates within defined spaces and architectures.

Microwells and microplates have been previously used to generate cell arrays consisting of many different cell types to study the effects of culturing and co-culturing cell types within confined spaces (Khademhosseini et al., 2005). This physical confinement of cells and other biochemical entities such liposomes within microwells has also been effectively used in high-throughput microarray applications (Thakar et al., 2003; Kalyankar et al., 2006). Alternatives for alleviating this problem include passively depositing cells at controlled densities (Bratten et al., 1998; Inoue et al., 2001; Khademhosseini et al., 2004; Maher et al., 1999; Prace et al., 1989; You et al., 1997), or docking cells within the microwells using microfluidic channels (Khademhosseini et al., 2005). The advantage of the later approach is that multiple cell types can be docked within the microwell lanes in a high-throughput manner. This technique could also be extended to align a set of microfluidic channels parallel to the original lanes of microwells in order to enable simultaneous combinatorial deposition of different cell types, analogous to DNA and protein microarrays (Kanda et al., 2004; Situma et al., 2005).

Additionally, the sensitivity of microarrays can also be increased by using nanoporous silica nanotubes (Kang et al., 2005; Wu et al., 2004), microporous silicon (Ressine et al., 2003), and beds of microbeads (Sato et al., 2001) to immobilize analytes from the samples. Non-adhesive poly(ethylene glycol) (PEG) microwells have also been successfully used to induce the formation of aggregates of non-anchorage-dependent cells such as ES cells (Khademhosseini et al., 2004; Karp et al., Submitted). Evidently, the use of micro- and nanoengineering approaches to selectively adhere and culture cells presents several advantages over techniques such as hanging drop and suspension culture methods.

13.4 Microfluidics

Microfluidic systems are structures designed for the manipulation of fluids in features with micro- nanometer dimensions. Microfluidic devices can be fabricated with astonishing complexity (Thorsen et al., 2002) and are apt for high-throughput experimentation. In typical microarray applications, the interrogation of low analyte concentrations relies on probe saturation, which can take hours. In contrast, microfluidic channels have demonstrably reduced mass transport times on microarrays by enabling a continuous delivery of targets (Hashimoto et al., 2005; Pappaert et al., 2003) at variable shears (Noerholm et al., 2004; Pappaert et al., 2003) and velocities (Vanderhoeven et al., 2004). Their size of operation also speeds up conventional experimental techniques like electrophoresis (Chung et al., 2003) while using a much smaller volume of reagents and biological materials than traditionally required. The potential increase in speed and the lower quantity of reagents and samples used for experimentation translates to commensurate savings in cost, making high-throughput experimentation more practical (Fig. 13.4).

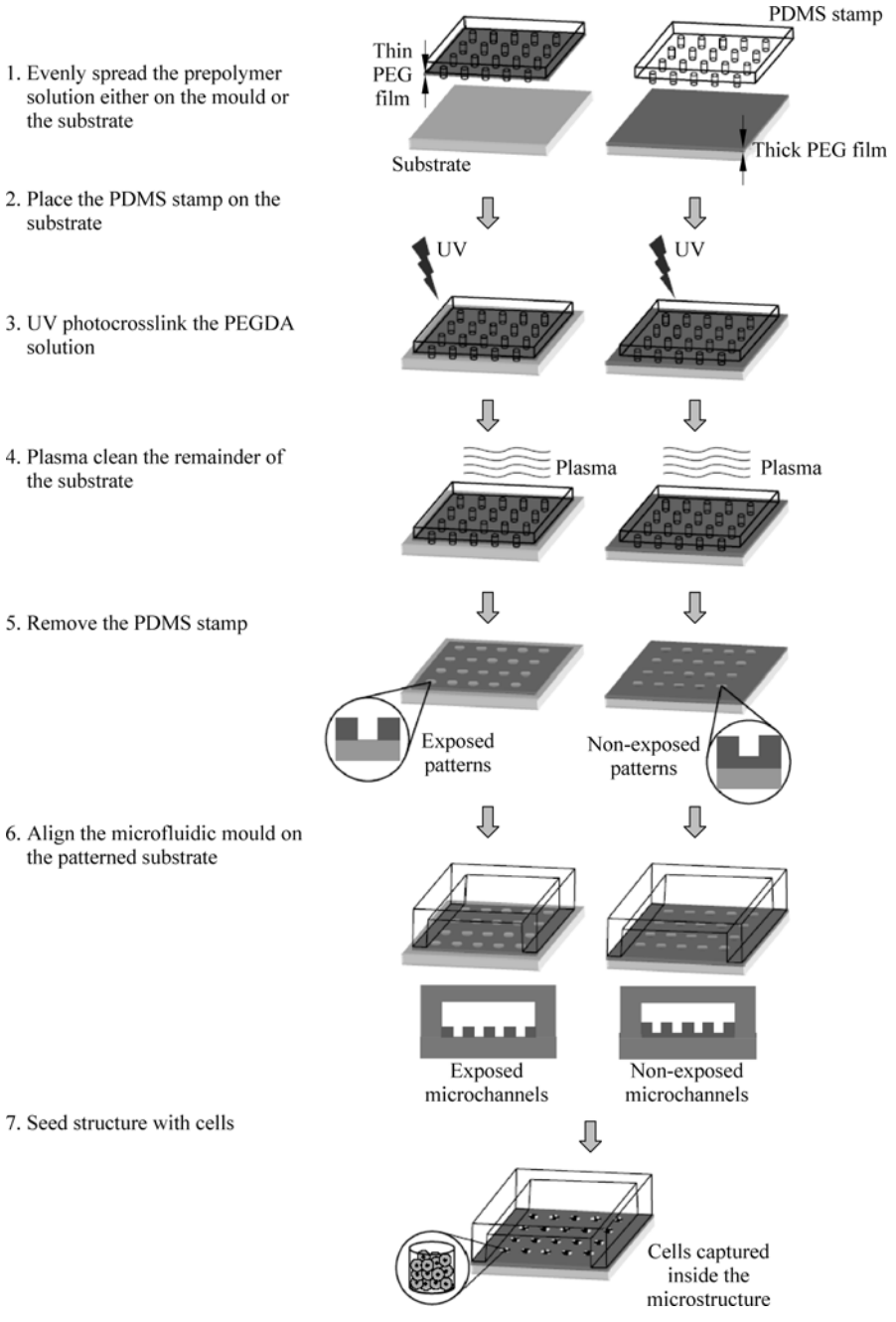

Figure 13.4 Schematic for fabrication of exposed and non-exposed microstructures inside microchannels. The final device combines the variability of a microarray assay with the efficient analyte solution delivery provided by the microfluidic channel

The emergence of microfluidics has been facilitated by soft lithographic (Whitesides et al., 2001; Xia and Whitesides, 1998) techniques developed by the semiconductor/microelectronics and micro-electro-mechanical systems (MEMS) industries. In soft lithography, elastomeric materials are cast and molded upon patterned planar surfaces generated through photolithography. The resolution of molded patterns generated in soft lithography can range from tens of nanometers to hundreds of micrometers. This large range of resolution allows for a variety of feature sizes and shapes within the same microfluidic device. The variety of scales and functionality that can be incorporated into a single system makes microfluidics a powerful tool for the creation of automated, miniature biological experimentation devices. In a recently developed automated nucleic acid purification system (Hong et al., 2004), a small volume of cells was automatically isolated and lysed, and its DNA/mRNA purified and recovered with no pre- or post-sample treatments, all within a single microfluidics chip. Such devices cut down substantially on the intensive labor traditionally required to carry out these standard biochemical analysis techniques. In the nucleic acid purification system mentioned above, approximately 0.4 nL of cell solution with reagent volumes of the same order of magnitude were used. The efficient use of expensive reagents and scarce samples could enable the cost effective, rapid analysis of small, cell populations. Even single-cell analysis becomes feasible on the volume scales of microfluidic devices. The automation potential has also been extended to perform parallel PCR (Auroux et al., 2004) and RT-PCR (Marcus et al., 2006) reactions as well as bioassays of human physiological fluids such as blood, serum, plasma, urine, and saliva (Srinivasan et al., 2004). One example of parallel analysis in microfluidics is the fully automatic parallel screening of 32 high-affinity protein ligands synthesized through Click chemistry (Wang et al., 2006). High-throughput ELISA in which duplicate assays for 5 different proteins were performed on 10 unique samples (Kartalov et al., 2006) have furthermore been demonstrated.

In addition to providing an automated platform for performing established biochemical and molecular biology techniques and improving microarray throughput and efficiency, unique microscale fluid flow phenomena may be exploited to answer questions in fundamental biology in a high-throughput manner. Laminar flows found within microscale fluidic devices provide a unique tool for tuning the spatiotemporal cellular microenvironment to study the dynamic properties of biological systems in a high-throughput manner. In laminar flow regimes, convective mixing does not occur and transport between adjacent laminar streams is dominated by diffusive flux. This property has been harnessed to control the spatial positioning of soluble factors relative to cells (Takayama et al., 2001), pattern cells (Takayama et al., 2001), and etch microenvironments (Takayama et al., 2001). Additionally, diffusive flux properties have been exploited to generate well-defined gradients of soluble factors (Jeon et al., 2000), conjugated proteins (Takayama et al., 2001), and material crosslinking densities (Burdick et al., 2004). Such precise control of solution and, in turn, material properties provide

opportunities to study fundamental biological questions regarding differential temperature or chemical treatment (Kartalov et al., 2006). Microfluidic gradient generation has been applied to investigations of cell migration chemotaxis (Jeon et al., 2002; Hatch et al., 2001), axon extension (Dertinger et al., 2002; Taylor et al., 2005), and neural stem cell differentiation (Chung et al., 2006). Though these works were designed to establish basic principles and did not rely upon high-throughput methodologies, their eventual application to high-throughput systems is anticipated.

While the advantages of high-throughput microfluidic experimentation are widely noted, the practical problems of interfacing existing macroscale instrumentation with microfluidic devices has been an important obstacle in establishing microfluidic technology as a part of 'standard lab equipment' or for use within commercial devices. In particular, the precise actuation of nanoliter volumes of fluid flow is extremely challenging. Another challenge, the molecular detection of low concentrations within microfluidic channels, is discussed in Section 13.5. Though a number of solutions have been proposed, no standard methodology for engaging high-throughput applications exists. Previously, flow through arrays of microfluidic networks has been controlled using syringe pumps (Hatch et al., 2001), gas-generation based pumps (Hong et al., 2003; Munyan et al., 2003), evaporation-based pumps (Walker and Beebe, 2002), gravity driven pumps (Cho et al., 2003), acoustic pumps (Nguyen and white, 1999), thermopneumatic pumps (Handique et al., 2001), and electrokinetic flow (Broyles et al., 2003; Emrich et al., 2002). Fluid actuation through these devices typically requires an external feeding tube and individual devices have not been shown to be ideal for the independent actuation of many pumps at once. Therefore, their practicality for potential high-throughput applications and complex microfluidic device actuation (such as the previously described nucleic acid purification systems that require the independent pumping of 10 or more fluid streams) is unclear.

For potential high-throughput applications, the pneumatic/hydraulic pressure-driven suite of pumps and valves developed by Fluidigm is promising (Unger et al., 2000). In this approach, pressurizable 'valve channels' aligned above or below reagent- or analyte-containing 'flow channels' are pressurized or depressurized to compress and close or decompress and open the flow channels. Sequential compression and decompression may be used to induce peristaltic pumping. An important advantage of this approach is that pressure-driven valve channels are capable of extending across multiple flow channels thereby potentially enabling massive combinatorial actuation of fluid flow within many parallel microchannels. Although its sole high-throughput application has been in ELISA systems (Kartalov et al., 2006), this actuation approach has been used extensively to drive the previously mentioned nucleic acid purification (Hong et al., 2004), PCR (Auroux et al., 2004), and RT-PCR (Marcus et al., 2006) systems.

An alternative pumping/valving system that uses electronically-actuated pins of refreshable Braille systems similarly pumps through peristaltic action (Gu et

al., 2004; Song et al., 2005). Specifically, Braille pins aligned along microfluidic channels can be programmed to push out and close elastomeric microchannels to either hold a channel shut (valve) or to push fluid along via peristaltic sequential depression. This approach is advantageous in that each pin may be independently computer controlled thereby enabling simultaneous actuation of each pumping/ valving mechanism independently. Braille pin actuation offers an advantage over pneumatic/hydraulic pressurized valve channels by eliminating the necessity for pressurizable tubing since actuation occurs 'on the chip'. An important consideration for high-throughput applications is that in contrast to pneumatic/hydraulic systems, Braille-based systems are not suitable for the combinatorial actuation of fluid flow within many parallel microchannels.

Microfluidic systems capable of combinatorial mixing (Neils et al., 2004) are highly scalable since numerous processes may be upscaled to run in parallel. The improvement in efficiency of microarray systems through the integration of microfluidics has been demonstrated, making improvements in microfluidic device design highly pertinent to microarray applications. Though many of the previously described applications of microfluidics were not performed in a high-throughput manner, the massive arraying of these processes is the predicted evolution of microfluidic technology.

13.5 Other Micro- and Nanoscale Technologies for Biological and Chemical Detection

In addition, microarray and microfluidic devices, a variety of novel micro and nanoengineered structures have recently been developed to augment existing methodologies for chemical and biological detection. Chemical and biological species have traditionally been detected through the use of fluorescently-tagged markers. Typically, a bound marker fluoresces at a well-defined spectrum of visible light under excitation with ultraviolet (UV) light. In high-throughput applications, traditional colorimetric assays suffer drawbacks such as rapid bleaching, narrow absorption spectrums, wide and asymmetric emission spectrums, and low detection sensitivity. The materialization of a suite of new nanomaterials such as quantum dots and nanowires hold promise for circumventing some of these problems.

Among other novel label-based biological and chemical detection schemes such as resin (Fenniri et al., 2001) or metallic barcoding (Nicewarner-Pena et al., 2004) and porous silicon photonic crystals (Cunin et al., 2002), the unique properties of quantom dots have enabled its quick emergence as a promising alternative to traditional fluorescent markers for high-throughput applications. These 10 – 20 nm fluorophores are composed of semiconductor nanocrystals that operate in a 'quantum confinement' size regime and allow for high resolution

localization of proteins and cellular structures (Bruchez Jr. et al., 1998; Chan and Nie, 1998). The spectral emission properties of quantum dots can be finely tuned to emit light ranging from blue (450 nm) to near-infrared (900) wavelengths at distinct and reproducible levels of intensity (Hotz, 2005). Furthermore, they exhibit a wide absorption band and a narrow (30 nm) and symmetric emission spectrum. These broad-absorption/narrow-emission properties make quantum dots especially suitable for high-throughput chemical and biological assays; quantum dots of many different colors and intensities may be simultaneously excited using a single light source, allowing for 'multi-plexed' labeling and detection (Mattheakis et al., 2004; Han et al., 2001). In addition, whereas many traditional fluorescent markers quickly quench or bleed within seconds, quantum dots exhibit high photostability and may be continuously tracked for minutes or iteratively imaged over many hours. Quantum dots have already been used to image proteins (Olivos et al., 2003) and analyze cell receptor dynamics (Knezevic et al., 2001) such as signal transduction (Lidke et al., 2004; Rosenthal et al., 2002; Vu et al., 2005) and diffusion (Dahan et al., 2003) and their interface with high-throughput microfluidic and microarray devices is anticipated.

Alternatively, label-free nanotechnologies that may be useful for high-throughput applications are also being developed for detection. Over 30 years ago, modified planar semiconductors were shown to be capable of detecting the presence of biological and chemical species (Bergveld, 1972). In this approach, specific molecular receptors replaced the gate oxide of a field effect transistor (FET) resulting in charge accumulation/depletion upon analyte binding. The subsequent change in conductance of the material was monitored electrically. Recently, FET silicon nanowires were employed in an identical manner and the nanoscale size of the devices was shown to be advantageous in that the large surface area-to-volume ratio of the nanowires allowed charge accumulation in the entire bulk of the conducting material (rather than in a thin layer of much larger macroscale materials) and resulted in extremely high detection sensitivity. Proof-of-concept nanowire sensors have been deployed to detect changes in ion concentration (Cui et al., 2003) as well as the presence of viruses (Cheng et al., 2006), ATP binding inhibitors (Wang et al., 2005), and cancer markers (Zheng et al., 2005). The hybridization of a nanowire and application to pH detection is shown in Fig. 13.5. While these nanowire sensors are still at an early stage of development, their size and sensitivity make them potentially suitable for interfacing with microarray and microfluidic high-throughput applications.

Silicon cantilevers are another label-free detection approach (Ziegler, 2004) that operate at the micron scale and which may be integrated into high-throughput devices. These cantilever beams detect molecules through the formation of surface stresses generated by the molecular adsorption of a specific ligand to the cantilever. Cantilever biosensing uses existing atomic force microscopy (AFM) technology to measure forces between cantilever and sample surfaces. Binding events stress the cantilever, causing it to bend and transduce this flexion for

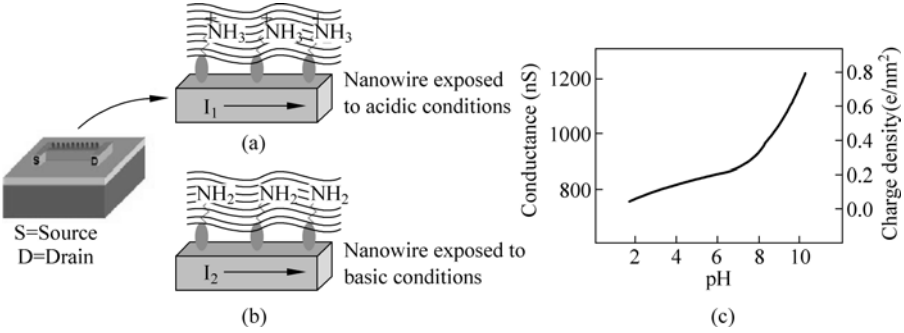

Figure 13.5 Application of a silicon nanowire in pH sensing. The silicon nanowire has been surface-treated to form pH-sensitive amino functional groups. The hybridized nanowire, when exposed to acidic solutions (a), assumes a net positive surface charge by formation of ammonium radicals. Conversely, when exposed to a basic solution (b), the ammonium groups deprotonate to form neutral surfaces. Variation in surface charge produces a change in conductance of the nanowire, which in turn is correlated to the pH of the solution (c)

detection. Cantilever bending may occur in response to one of three mechanisms: surface stress, mass loading, and temperature change. Surface stresses may be caused by the highly specific binding of analytes of interest to a single side of the cantilever. In contrast, the loading of a larger mass induces cantilever oscillation, which is transduced and can be detected. Rapid temperature shifts may also induce flexion of the cantilever. These specific detection mechanisms can be controlled through modulation of the cantilever material. Silicon, silicon nitride, and silicon oxide are currently most used in micro- and nano-scale cantilevers (Lavrik et al., 2004). Cantilever binding specificity is modulated through surface coating of the cantilever with the highly specific 'receptors' also used in microarray assays, including self-assembled monolayers, peptide sequences, DNA probes, or antibodies. Once a flexion mode/receptor combination has been characterized for a particular analyte, the bending caused by analyte binding is transductively measured and correlated with an analyte concentration. The transduction of cantilever bending can be detected through a number of techniques such as electric piezoresistive readouts, optical laser detection, piezoelectricity or capacitance changes. The piezoresistive method is compatible with cantilevers of a few hundred nanometers in length (Lavrik et al., 2004). Here, the resistance of silicon cantilevers doped in specific regions reflects the degree of deformation. Alternatively, optical beam deflection methods, though not amenable for nanometer-scale cantilevers offer high readout efficiencies. Piezoelectric methods use piezoelectric materials such as zinc oxide coated onto the surface of the cantilever; the bending of the material generates a voltage, which can then be measured. Lastly, the capacitance method detects the capacitance between the cantilever beam and a substrate. The movement of the cantilever closer to the surface increases the capacitance while movement away decreases the capacitance, which can then be recorded

and correlated to binding events. The small gap between cantilever and surface required for this latter method is an engineering challenge and therefore a disadvantage of this method.

13.6 Conclusions

Micro- and nanoscale technologies automate, miniaturize, and multiplex biochemical assays to study biological functions at the cellular and genomic level at reduced experimentation costs and in a high-throughput manner. Of these, the microarray technology is extensively used in the analysis, detection, and diagnostics of analytes—from whole cells and DNA, to environmental pollutants. Other microscale technologies such as microfluidic gradient generation systems successfully augment the applicability and functionality of the microarray platform. Presently, microarrays are moderately complex to use, and costly to manufacture. In the future, as this technology develops further, it is expected that microarrays will be routinely used, not only in the study of genes, genomes, and comparative genome hybridization, but also in nearly every single area necessitating analysis and detection of analytes. Emerging disciplines such as pharmacogenomics, toxicogenomics, proteomics, as well as traditional fields such as clinical diagnostics, drug discovery, food and environmental analysis, and chemical and biological warfare defense will witness extensive use of the microarray technology. Microarrays have also spurred the development of inventive analytical devices and technologies such as MEMS and lab-on-a-chip, and accordingly, have revolutionized the amplification, separation, analysis, and detection of DNA via integration with microfluidics. Additionally, since microarrays and microfluidic technologies can perform experiments with higher sensitivity using less reagents, they present significant opportunities to increase the throughput and efficiency of DNA, RNA, oligonucleotides, proteins, polysaccharides, as well as cell-based assays. These technologies have also been applied in numerous diagnostic applications. A majority of the current research into micro- and nanoscale technologies focuses on increasing the sensitivity and speed of assays based on these technologies, while minimizing their size and cost. The union of microarrays and microfluidics promises previously unmatched throughput for diagnostics and screening applications.

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