Silicon Technology Based Module of High-Density DNA Microarray with Electro-Optic System

By

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A thesis submitted to
Hong Kong University of Science and Technology
In partial fulfilment of the requirements for
The Degree of Master in Philosophy

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DECEMBER 2001
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ACKNOWLEDGEMENTS

I would like to express my gratitude, admiration and respect to my supervisor, Professor Mansun Chan, for his guidance and help throughout my Mphil programme. This dissertation would not have been accomplished without his wisdom and knowledge in various fields, such as engineering, science, philosophy, and art. Through his intelligent talks and works, I have explored the real beauty of thinking. His enthusiasm, high standards in research were an important power for me to do better.

I also appreciated the excellent research environment created by Mr. Allen Ng in the Device Characterization Lab. Allen helped me a lot from setting up the computer and experiments, buying needed materials. His kindness and helping hand impressed me a lot. There are several people I would like to thank. Peng Huajun, a PhD candidate now, is very knowledgeable about optics. Whenever I got stuck in doing optical experiments, his would discuss with me and give me his keen insight into solving the problems. I would like to thank another highly talented labmate, Li Jiong. He is my precious dictionary on biochip and chemistry. Without his cooperation, my research would never have sound result. I would like to extend my gratitude to Shan Chao, who gave me emotional support in hard time. She is a very pretty and smart lady. My pressure from work was always blown gone by her unique sense of humor. I shall also express my special gratitude to Zhang Zhikuan, who gave me support during the two years in many aspects. His characteristic thinking would always inspired me through my life.

I appreciated the help of Maria Carles, Ralf Lenigk, Dr. I-ming Hsing and all the others in the biochip groups also. Their enthusiasm in work and kindness impress me a lot. I am thankful to all my labmates, Zhang shendong, Wang Hongmei, Singh
Jagar, Sang Lam, Xu Chen, Xue, Mei, Allan Chan and Lin Xinnan for their help and kindness. All of them are very excellent in research. I learnt so many things from them. Without them, my life would never be so exciting.

I would like to thank my parents for their pushing hand. Without them, I would never try to be better and better. The last, but not the least, one I want to extend my gratitude to is Guan Feng, who always gives me the support in every aspect.
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For the degree of
Master of Philosophy (M.Phil.)
Department of Electrical and Electronic Engineering
Hong Kong University of Science and Technology
in December, 2001

ABSTRACT

DNA microarray is an important technology for DNA analysis in various applications. The adoption of mature electronic micro-fabrication to realize fully integrated and portable DNA analyzer is of great importance to meet the ever-growing demand for on-site clinical diagnosis, high speed and throughput DNA analysis. In this work, silicon materials compatible with the biochemical process were studied to realize high density DNA microarray. Covalent immobilization DNA oligonucleotides is used to selectively attach DNA probes onto the surface of silicon dioxide. Different surface cleaning methods and concentrations of DNA oligonucleotides for immobilization and hybridization were tested so as to increase the sensitivity of the microarray. Fluorescence microscopy revealed the success of hybridising of complementary oligonucleotides labeled by FAM to the probe.
Contact angle measurement and atomic force microscopy (AFM) were used to monitor the process.

The Compatibility of silicon materials in regard to the immobilization procedures on silicon dioxide was studied. As a result of the corrosive chemical used in the process, common metals used in micro-fabrication process like aluminum were etched away. Silicon nitride, polysilicon, gold and platinum withstood the process of immobilization and no DNA was hybridized on these materials. Fluorescence intensity analysis showed that using a combination of silicon dioxide (where immobilization takes place) and silicon nitride or polysilicon (where no immobilization takes place), immobilization was confined to the desired area and thus reduce cross talk between different array elements. The fluorescence image from the scanner indicated silicon nitride with line width down to 1 μm can be used as an effective isolation material.

The compatibility of DNA microarray and electro-optic system in regard to the integration was also studied. Fluorescence detection is now the most commonly used detection method. When using this detection method, it is necessary to use on-chip color filter. Red dyed polyimide was the suitable on-chip color filter material. As color filter is used, the covalent immobilization method cannot be used. Avidin-biotin complexation was selected as the immobilization method instead. Thus the fabrication process of DNA microarray on agarose film, which can facilitate the integration, was developed.
CHAPTER 1

INTRODUCTION

Recently, a lucrative technology giant stands up in this biotechnology era, which is commonly called DNA microarray technology. A DNA microarray is a solid surface with arrays of DNA fragments at discrete addresses, where the fragments are ready for hybridization of the target DNA sample. As a high-throughput technology, DNA microarray is a powerful tool to analyze vast amount of genetic information in gene activity or expression. In order to develop an efficient and economical microarray system, all the components such as DNA types, chip fabrication, sample preparation, assay, detection and software have to be characterized in detail and then optimized. In this work, a novel method of fabricating a high-density DNA microarray facilitated by silicon technology and the process compatibility issue on integrated system of DNA microarray and electro-optic device were carefully studied.

This chapter, the background information of our study, is divided into five sections. In Section 1.1, the biologic background information is given. In Section 1.2, the focus is the application of the DNA microarray system. State-of-the-art DNA microarray technology is reviewed in Section 1.3 and this is followed by a discussion of contributions of silicon technology to the DNA microarray system in section 1.4. Finally, in Section 1.5, the thesis objective is stated.
1.1 Biological Background Information

Deoxyribonucleic acid (DNA) is widely believed to contain the biochemical codes for life. It is a macromolecule built from two strands in a double helix or twisted-ladder shape, each with a sugar-phosphate support backbone and nitrogenous base rungs [1,2], as illustrated in Figure 1-1. The nitrogenous base consists of adenine (A), cytosine (C), guanine (G), and thymine (T). The base pairs in double-stranded DNA must occur as A-to-T or C-to-G. The strands of DNA have orientation. One end of a strand is designated five prime (5') and the other three prime (3'). The two complementary strands are formed in an anti-parallel way. For example, if one strand appears as “5'-ATCGGCTTACCA-3'”, it’s complementary strand thus is “3'-TAGCCGAATGGA-5'”. With the four DNA letters as A, T, C and G, the genetic code has words to specify each 20 (known) amino acid. With a two-letter combination, only 16 words could be formed. However, a three-letter code produces 64 words. Thus, three consecutive bases, together named codon, are to be used in the assembly of amino acid chains.

There is another type of nucleic acid, RNA, which is involved as a messenger in the translation of genetic information. In the cell nucleus, DNA code is transcribed into a messenger RNA (mRNA) molecule. However, in RNA, thymine (T) is replaced with the base uracil (U). In the cytoplasm, the mRNA code is translated one codon at a time into amino acids. Translation is orchestrated at the ribosome with transfer RNA playing the role of adaptor. After the assembly of amino acid, full-length protein is produced, which carries out myriad cellular reactions constituting life.

DNA stores the genetic information needed to produce protein. However, this is not simply a one-way process. The process of producing protein is affected by the
feedback information of biomedical inhibitors and promoters. These are controlled by a regulatory protein. Also, various environmental factors have an impact on the DNA coding. The whole living system can be described as a complicated network system in which there are many unknown feedback branches. In Figure 1-2, how the DNA, together with other factors in the process, changes the protein production through the complicated feedback network is shown schematically. As the DNA sequence of the human genome has been revealed [3], researchers have started to seek the answers to what parts of the sequence actually code genes, an understanding of entire complex live activities like metabolism, which requires information about the multiple protein-DNA interactions of the cells’ regulatory mechanisms. The first key to gaining an understanding of these life mysteries is to measure the mRNA concentration of every gene. Furthermore, to fully understand about gene activities, it is necessary to test every possible gene expression under all environments. Given ten genes, there will be $2^{10}$ possible combination of genes under one specific condition. The smallest free-living organisms have about 500 genes and humans have about 120,000 genes. Thus, an economical DNA microarray technology, which can achieve high-density and provide a precise analysis is vitally important.
Figure 1-1: Schematic structure of DNA: (a) two strands of DNA in the helix or twisted-ladder shape with a sugar-phosphate support backbone and nitrogenous base rungs and (b) duplication of DNA according to the base pairing rule.

Figure 1-2: Schematic of the transcription of information from genes (DNA) to message (RNA) and the translation of mRNA to proteins. The environment and the DNA change the scheduling of protein production through a complex feedback network.
1.2 Applications of DNA Microarray

DNA microarray is a solid surface with arrays of DNA fragments at discrete addresses. The fragments are ready to hybridize the target DNA sample according to the base-pairing rule. On a typical microarray, about ten thousand genes can be analyzed. As the technology develops, it will be possible to monitor the human genes on a single chip. This will mean that simultaneous interactions between thousands of genes can be realized [4].

There are several other terminologies to describe this technology such as biochip, DNA chip, gene array etc. DNA microarray is used in this thesis and chip is used to describe the specific function.

Presently, the two major application forms of microarray technology are identification chips and expression chips [5-7]. Identification chips are used to detect novel polymorphisms in genes whose sequence is known, or mutations in genomic DNA. Expression chips are involved in monitoring the expression level of the arrayed genes in mRNA populations from living cells.

Identification chips are largely used for disease diagnosis and to find out why people vary in their susceptibility to diseases [8-10]. Each individual has many single nucleotide polymorphisms (SNP) that together create a unique DNA pattern for that person. SNPs are mutations at a single spot in a gene sequence and they are the reasons for genetic variation. As single-stranded DNA prefers to hybridize with its perfectly complementary strand, identification chips thus can discover the SNPs by analyzing the hybridization efficiency facilitated by tagged fluorescent dyes.

Just as it was possible to take a whole-organisms approach to DNA sequencing, it is now possible to consider a whole-organism approach to understanding the mechanisms that control the biochemistry in a cell, therefore, to prevent or treat
disease. Expression chip technology used to measure mRNA abundances in cells allows the monitoring of gene expression levels for tens of thousands of genes in parallel. This technology is a powerful tool especially in the field of toxicology, drug discovery [11-13]. By measuring expression responses across hundreds of different conditions or timepoints, a relatively detailed gene expression map starts to emerge. Using cluster analysis techniques, it is possible to identify genes that are consistently coexpressed under several different conditions or treatments. These sets of coexpressed genes can then be compared to existing knowledge about biochemical or signal pathways. The function of unknown genes can be hypothesized by comparing them to other genes with characterized functions, or from trends in expression profiles in general - why cell need to transcribe or silence the genes during particular treatments. The regulation of genes on the DNA level is largely guided by particular sequence features, the transcription factor binding sites, and other signals encaptured in DNA. By analyzing the regulatory regions of the DNA of the genes consistently coexpressed, the potential signals hidden in DNA can be discovered using computational analysis methods. The prerequisite for this kind of analysis is the existence of a genomic DNA sequence, knowledge about gene locations, and experimental gene expression measurements for a variety of conditions.

Figure 1-3 illustrates the process of a typical experiment in toxicology. RNA is extracted from normal cells and cells treated with a drug. Then, single-stranded complementary DNA (cDNA) copies of both mRNAs are made through a series of chemical reactions and are tagged with two florescent dyes of different colors. Then the tagged single-stranded DNA is introduced on the chip of the single-strand DNA probe with a designed sequence. The tagged DNA will bond fast to the probe with a complementary sequence of it own. When excited by a laser or by other excitation
sources, detectors can pick up the fluorescence at specific locations. Some software can analyze the location, color, and brightness of each chip. Comparing the colors found at certain points on the chips will reveal the differences in gene expression between the two cells.

Figure 1-3: Schematic process of a typical toxicology experiment.
1.3 State-of-the-Art Microarray Technologies

Twenty-seven years ago Ed Southern demonstrated the first array that labeled the nucleic acid molecules could be used to interrogate nucleic acid molecules attached to a solid support [14]. This was a great step in molecular biology and since then, some advances were made through the use of nylon filters [15]. However, the implementation of this advance was clumsy.

The field of array technology would not have been so exciting without these two innovations, which are still the two main formats in microarray technology. In 1995, at Stanford University, Pat Brown and his colleagues established the protocol for the use of non-porous solid supports, such as glass [16]. In this method, the probe cDNA is immobilized on a solid support that is chemically treated for the binding by spotting at specific location. Then the chip is exposed to a set of targets labeled by different fluorophores. The set of targets is either in a separate form or in a mixture depending on the application. After the washing off of the remaining free targets, the analysis is facilitated by the level of the fluorescence intensity. In this method, now usually assisted by printing technology, uniform and small dots of DNA probe are deposited onto the surface for the immobilization. There are two main printing technologies: contact printing and inkjets. Contact printing involves wetting a printing pin with the DNA solution and tapping it on to the microarray surface. During inkjetting uniform droplets of solution are ejected onto the substrates.

The second innovation came from Steve Fodor and his colleagues at Affymetrix, Inc [17-19] who developed a method for the high-density spatial synthesis of oligonucleotides. This group adapted photolithography, which is used in the manufacture of semiconductors to produce arrays with 400,000 distinct oligonucleotides, each in its own 20μm² patch. Glass slides are treated to be
chemically ready for binding nucleotides, and are then covered by a layer photoresist. A laser with 365nm is shone through a patterned mask onto the chip and thus causes photoresist on specific sites to break away. One type of nucleotides, either A, T, C or G, is then washed over the chip and attaches only to the areas that have been exposed. Then the process is repeated with another mask and another nucleotide. Thus, a variety of DNA sequences can be built on the chip. This photolithography method is one of the 'in situ' fabrication technologies. Inkjetting is another one. In inkjetting, solutions of nucleotides are ejected from the nozzle onto the substrate and then chemically fixed to the surface. The next set of nucleotides are jetted onto the first, and are chemically fixed to those. The process is repeated until the desired set of DNA is complete.

In Figure 1-4 the schematic mechanisms of the above two dominant methods facilitated by printing technology and photolithography are illustrated.

Other significant technology when realizes the high-speed, high density DNA microarray have been developed. In 1997, Nanogen illustrated DNA microarray system by direct electric field control. Since DNA is negatively charged in nature, the application of an electrical field can transport the molecules to selected micro locations where they concentrate over the immobilized substrate. The reversal of the field is used to selectively repulse those molecules. The electric field denaturation control allows single base pair mismatch discrimination to be carried out in less than 15 seconds.
Figure 1-4: Schematic mechanisms of DNA microarray fabrication techniques.
1.4 Contributions of Silicon Technology to DNA Microarray

With the development of integrated circuits (IC) and microelectromechanical (MEMS) systems, micro-sensor fields have been greatly enlarged. To realize a fully integrated and portable DNA analyzer is of great importance in order to meet the ever-growing demand for on-site clinical diagnosis, and high speed and high throughput DNA analysis. This can be realized by using silicon technology. The components of this system include sample preparation, DNA amplification, product separation, detection and hybridization. Through adopting silicon technology, a silicon-based reactor that can efficiently amplify DNA through a polymerase chain reaction (PCR) can be developed. With the continuing development in regard to the compatibility of various material and processes, silicon materials and image sensors can be adopted into the integrated DNA analysis system.

Facilitated by mass production in the silicon industry, the miniaturized and integrated DNA analysis system by IC and MEMS technologies can process increasingly more genetic information at a low cost. Also, a reduction in DNA sample volume and PCR reagent consumption can be realized. The typical volume used in conventional DNA analysis is in micro-liters. With this system, only nano-liter volume will be needed. Thus, the analysis cost, which is currently very expensive, can be reduced. If most of procedures were automated, the manual manipulation of samples in between different modules would be minimized, if not eliminated. Hence, the chance of contaminating a sample would be much lower and would lead to higher confidence in the analysis.

Another advantage of the approach is its ability to speed up the analysis procedure. The time needed for DNA separation in conventional gel electrophoresis
is one hour. This time could be reduced to a few minutes through the use of capillary electrophoresis in the integrated approach. Also, the DNA amplification reaction time could be greatly shortened when miniaturized silicon based reaction is adopted.

In our research, the focus was on the capability of silicon technology to fabricate a precise, finely structured, and high throughput DNA microarray system. Silicon materials used as a solid support can reduce the volume of DNA probe and facilitate the integration of a DNA microarray and a detection system. The density of DNA microarray can also be increased using silicon materials.

1.5 Thesis Objectives

In this work we studied the issues surrounding the integration of high-density DNA microarray and a detection system. The integration issues included material compatibility and process modification of the combined DNA microarray and detector. The integrated system had to have a solid surface as the platform for biochemical reaction. As requirements for later analysis, the low background noise and strong genetic signal is necessary. In Chapter 2, silicon materials used as the solid surface are discussed. Surface characterization was carried out to monitoring the process.

Silicon materials compatible with the above process are also discussed in Chapter 3. Through the use of micropatterning technology, a high density DNA microarray was achieved. A comparison of these silicon materials was also carried out.
Fluorescence is now the most commonly used detection method because of its sensitivity and simplicity in labeling DNA. This detection method and the integration approach of a DNA microarray with electro-optical devices are discussed in Chapter 4. The selection of compatible fluorophore for labeling DNA, an on-chip color filters and a laser source for this approach was analyzed. Furthermore, fabrication process of a DNA microarray on agarose film using avidin-biotin complexation as the immobilization method was also studied.

The main contributions of this work are highlighted in Chapter 5, and future work is investigated.
References


CHAPTER 2

FABRICATION OF DNA MICROARRAY ON SILICON MATERIALS

In this Chapter, immobilization methods are reviewed in Section 2.1. The reasons for selecting silicon dioxide as the DNA microarray solid support and the covalent immobilization of thiol-terminated DNA oligonucleotides on self-assembled layers of (3-mercaptopropyl) trimethoxysilane (MPTS) by disulfide bond formation will be given. There are three steps in the immobilization process: surface cleaning and hydroxylation (Section 2.2.1), MPTS formation (Section 2.2.1), immobilization and hybridization (Section 2.2.2). In order to get accurate information when undertaking DNA analysis, each step was carefully studied to ensure the best possible results. The protocol of the whole procedure was established (Section 2.3) and the experimental results show that the protocol is feasible. Surface characterization methods were used to monitor the process and to provide an understanding of the interaction between the biomaterial and the solid support (Section 2.4).

2.1 Review of Immobilization Methods

DNA microarray technology is more attractive in DNA diagnostics than other conventional direct gene sequencing methods because of its simplicity [1]. The technology starts from the observation that single-stranded DNA binds strongly to nitrocellulose membranes in a way that prevents the strands from reassociating with each other, but permits hybridization to complementary RNA [2]. Since then, numerous methods for the attachment or direct synthesis of DNA to different
surfaces have been developed [3-10]. For most applications, the immobilization of a readily detectable quantity of functional DNA, the film stability and the fidelity of the immobilized sequence, are the keys to developing a DNA microarray. The three main reported successful immobilization methods are: adsorption, avidin-biotin complexation, and covalent attachment. The mechanisms of each method are illustrated in Figures 2-1, -2, and -3. The details and comparison of three methods are given in Table 2-1.

Although adsorption is the simplest method, the hybridization efficiency is low because of the use of the permeable support. The avidin-biotin complexation method can achieve high sensitivity, but it is more costly than other methods. The covalent attachment methods are currently the dominant DNA immobilization techniques because the probes used in this approach can benefit from the structural flexibility. It also allows conformational changes and increased hybridization without the leakage of the nucleic acid from the surface. From the theoretical standpoint, the kinetics of the interactions in covalent attachment are the simplest, and the molecular attachment of the short probes is the most well defined. Moreover, the impermeable and rigid substrates used in the covalent methods have a number of advantages over porous membranes. As liquid cannot penetrate the surface of the support, target nucleic acids can find immediate access to the probes without diffusion into pores. This enhances the rate of hybridization. Further, the physical rigidity permits the incorporation of automated processing into the flow cells. This is essential for high throughput analysis. The locations of the probes are better defined than they are on a flexible membrane so that microarray of smaller feature size can be realized.

A lot effort has been made on covalent immobilization of DNA probes onto various solid supports in different applications. Glass-slides, nitrocellulose-coated
microscope slides, and silicon-based DNA microarray are now widely used. Glass microarray with optical fluorescence detection, pioneered by Patrick Brown's laboratory at Stanford, is now the most popular method because an external CCD camera can be used as part of the detection system. Recently, in order to facilitate the fabrication of so-called "lab-on-a-chip" devices, there has been a surge in the study of silicon-based DNA arrays. Compared to the commonly employed microscope slides, silicon materials have reduced surface roughness and allow DNA deposition at a higher density with better uniformity, and smaller DNA spot size. Furthermore, the flat surface allows high-density DNA arrays to be analyzed with a confocal laser light sources. This is required by later integration systems.

The reported covalent methods based on silicon compatible materials are: (A) the attraction of the negatively charged DNA to a positively polarized electrode that is coated with a gel containing functional groups for subsequent cross-linking [11]; (B) self-assembled-monolayers (SAMs) based on the interaction of chlorosilanes with OH-terminated oxide surfaces [12], or the absorption of thiol on gold [13] and tantalum oxide surfaces [14]. SAM is a highly flexible approach for the creation of concentrated planes of functionality. Of the materials studied, silicon dioxide was selected as the solid support in our research. It was because its chemical property is similar to that of glass, but is more stable and pure. We believe it is a promising substitute for glass in the integrated approach of the DNA microarray and detection system. It has a few unique advantages. Firstly, DNA samples can be covalently attached onto a treated surface. Secondly, silicon dioxide is a durable material that sustains high temperatures and washes of high ionic strength. Thirdly, it is non-porous so the hybridization volume can be kept at a minimum. This enhances the
kinetics of annealing the probes to the target. Finally, as a consequence of its low fluorescence, it does not significantly contribute to background ‘noise’.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages and Disadvantages</th>
<th>Supports</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D: nucleic acid will desorb from the surface due to the hybridization conditions and results in poor hybridization efficiency</td>
<td></td>
</tr>
<tr>
<td>Avidin-biotin Complex</td>
<td>A: it provides a versatile approach in biology and biotechnology. It can be adopted in 3-D microarray to enhance the sensitivity</td>
<td>Graphite electrode surface, carbon strip electrode, platinum.</td>
</tr>
<tr>
<td></td>
<td>D: it is more costly methods than covalent attachment. The presence of large protein layer may have non-specific binding sites and compromise the sensitivity and the selectivity of certain types of sensor.</td>
<td></td>
</tr>
<tr>
<td>Covalent Attachment</td>
<td>A: the location of each spot can be well defined, high-density can be achieved. It provides a way to strip the DNA target and rehybridization.</td>
<td>Glass, carbon paste modified electrodes, gold, platinum, silica, and silicon dioxide.</td>
</tr>
<tr>
<td></td>
<td>D: the process time is comparatively long.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1: Comparison of the immobilization methods.
Figure 2-1: DNA immobilization by electrochemical adsorption. (A) DNA probes adsorption to an electrochemical transducer applying a positive potential. (B) Hybridization between the probe and the target, holding the same positive potential. (C) Transduction: (C1) Transduction using the guanine oxidation signal. (C2) Indicator preconcentration in the dsDNA at a fixed positive potential and transduction based on the electroactive hybridization indicator.
Figure 2-2: DNA immobilization involving avidin-biotin complexation. (A) Avidin adsorption onto the graphite electrode. (B) Complexation between avidin and biotinylated DNA probes. (C) Hybridization of avidin-biotin probes with DNA target. (D) Signal transduction using CPSA based on an electroactive hybridization indicator preconcentrated into the dsDNA.
Figure 2-3: Some common reactions used for the covalent binding of DNA. (A) Attachment through 5’phosphate group of ssDNA onto aminoethanethiol modified gold electrode. (B) DNA immobilization onto a mercaptosilane coating of a platinum surface via the amino groups of the bases, (NH2) ssDNA (TMSPT: 3-trimethoxysilyl-1-propanethiol; CDI: N-cyclohexyl-N’-[2-(N-methylmorpholino)-ethyl]-carbodiimide-4-toluene sulfonate). (C) Immobilization using fictionalized polypyrrole (Py: 3-acetic acid pyrrole; Py: 3-N-hydroxyphthalimide pyrrole; (NH2..DNA): an amino-substituted oligonucleotide).
2.2 Oligonucleotides Immobilization on Silicon Dioxide

We developed a method for the covalent attachment of thiol-modified DNA oligonucleotides to self-assembled thiol monolayer film. The DNA immobilization process we developed has three components: (1) surface cleaning and hydroxylation; (2) silanization; and (3) immobilization and hybridization of DNA oligonucleotides. In Figure 2-4 chemistry of the process is schematically illustrated. The immobilization process on glass has already become standard. As silicon dioxide has similar chemical property as glass, our attempts to immobilize DNA on it were focused on the selection of the surface cleaning methods and the concentration of DNA oligonucleotides for the immobilization and hybridization.

Figure 2-4: Typical immobilization process on silicon dioxide.
2.2.1 Surface Cleaning, Hydroxylation and Silanization

Silane assembles on the surface uniformly under some optimal conditions [15][16]. The covalent attachment of functional organosilanes to silicon dioxide is very critical because the uniform monolayer of silane allows the bimolecular and cross-linker to be attached to the moiety of the silane in a uniform way. This is of great importance with regard to the sensitivity and the reproducibility of the DNA microarray. However, the uniform deposition of silane monolayer upon silicon dioxide has been shown to require small amounts of water along with hydroxyl groups in an isolated or germinal configuration on the surface. To expose these groups and achieve optimal uniformity and reproducibility, the surface must be cleaned to rid it of any contaminants, and the reactive hydroxyls must be activated [17]. We summarized four main cleaning and hydroxylation methods that use various combinations of acids, bases and organic solvents at different temperatures [18][19]. The procedures of each method are listed in Table 2-2. In order to examine the effectiveness of the cleaning methods and the following silanization step, contact angle measurement was performed before and after the attachment of (3-mercaptopropyl) triethoxysilane to ensure a qualitative evaluation of the four methods.

2.2.1.1 Materials Preparation

The starting material was a four-inch p-type silicon wafer with (100) crystal orientation. The wafer was first cleaned using de-ionized water and then put into an oxidation furnace at a temperature of 950°C in oxygen and water ambient to grow 4000Å of silicon dioxide. The finished wafer was then cut into many 1cm×1cm pieces and these were then individually cleaned using the procedures described below.
2.2.1.2 Cleaning and Silanization

Four cleaning methods were tested for the efficacy in the preparation for silanization (Table 2-2). Eight dies went through each cleaning method. During the cleaning process, after each rinsing, the dies were dried by a nitrogen gun to avoid cross-contamination and dilution. Four dies (from each cleaning method) were then used to evaluate the efficiency of each cleaning method through contact angel measurement. Four other dies were immersed into a boiling solution composed of 30 mL of 2-propanol, 400μL MTPS, and 100μL DI water under reflux for 30 minutes. The dies were then cooled and washed by 2-propanol followed by DI water. Then contact angle measurement after the silanization was carried out on the rest of the dies to get qualitative evaluation of the surface silanization.

2.2.1.3 Contact Angle Measurement

A drop of water, or other liquids, in contact with one or several media always assumes the shape that gives the lowest total energy. If the surface is hydrophilic, for example if it has -OH or -COOH groups in the outermost layer, it is favorable for the water to spread out over the surface. If the surface is made up of hydrophobic groups, like CH₃ or CF₃, the drop assumes a shape which exposes a minimal area to the surface. The contact angle is measured by optical inspection and is used as a measure of the hydrophobicity of the surface (Figure 2-5).

In the experiment, the Ramé-Hart goniometer model # 100-00 was adopted to measure the contact angle. We deposited 20μL of DI water onto the surface of a die and the measurement was performed at room temperature with 60% relative humidity. Every value was represented in the form of the mean contact angle for four dies ± the standard error of means. Measurements were taken within 15 seconds after
formation of the sessile drop. The goniometer was not able to accurately measure contact angles below 8°.

![Figure 2-5: Contact angles at a hydrophobic and a hydrophilic surface, respectively.]

<table>
<thead>
<tr>
<th>Method</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30min in conc. H₂SO₄, Rinse in H₂O, dry by N₂, 30min in 1:1 MeOH: HCL, rinse in H₂O, dry by N₂, 30min in 100°C H₂O</td>
</tr>
<tr>
<td>2</td>
<td>2min in 1 g of potassium permanganate dissolved in 30 mL of concentrated sulfuric acid, rinse in H₂O, dry by N₂, 30min in a 1% ammonium hydroxide solution</td>
</tr>
<tr>
<td>3</td>
<td>5 min in 1:1:5 HCL:H₂O₂:(30%):H₂O at 80°C, 5min in 1:1:5 NH₄OH:H₂O₂ (30%): H₂O at 80°C, Rinse in H₂O, dry by N₂.</td>
</tr>
<tr>
<td>4</td>
<td>20 min in 1M NaOH, rinse in H₂O, dry by N₂.</td>
</tr>
</tbody>
</table>

Table 2-2: Four commonly used cleaning methods.
2.2.1.4 Results and Discussion

If the cleaning methods efficiently remove contaminants from the silicon dioxide, a hydrophilic surface can be observed and the contact angle should be small [20]. Furthermore, the exposure of the OH group also makes the surface hydrophilic. After the self-assembled silane monolayer forms on the cleaned and hydroxylated surface, a hydrophobic surface should be observed. This leads to a great increase in the contact angle. The results of our experiment are listed in Table 2-3.

After cleaning, a small contact angle on the surface below 8° was achieved through Methods 1 and 2. This illustrates the high efficiency of these two cleaning methods. The high contact angle results from using Methods 3, and 4 show that these two methods are not as efficient as Methods 1 and 2 for surface cleaning. However, after silanization, the dies prepared using Method 3 demonstrate a high contact angle (66°). It is not known if this high value is due to silane deposition or organic contaminants that were not effectively removed by the cleaning treatments. When Method 4 was used, the contact angle after silanization only increased to 32°. This indicates a poor silanization. Thus, Methods 3 and 4 are not suitable for pre-silanization cleaning. Methods 1 and 2 both display the hydrophilic characteristics consistent with clean surfaces. Further, after silane treatment, the increased contact angles are consistent with effective silane deposition. As the ammonium hydroxide involved in Method 2 has a stronger etching nature than the acid involved in Method 1, we adopted the Method 1 as the cleaning method.
<table>
<thead>
<tr>
<th>Method</th>
<th>CA before silanization</th>
<th>Variation</th>
<th>CA after silanization</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;8</td>
<td></td>
<td>58</td>
<td>±3</td>
</tr>
<tr>
<td>2</td>
<td>&lt;8</td>
<td></td>
<td>48.5</td>
<td>±5</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>±2</td>
<td>66</td>
<td>±3</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>±6</td>
<td>32</td>
<td>±2</td>
</tr>
</tbody>
</table>

Table 2-3. Contact angle on chips using different cleaning methods after each step.

2.2.2 Immobilization and Hybridization

To increase the sensitivity of the array, both the concentration of the DNA oligonucleotides used for immobilization and hybridization should be optimized. The DNA oligonucleotides used for immobilization and hybridization are both labeled with FAM. Thus, the immobilization and hybridization efficiency can be indicated by the fluorescence intensity.

Chips ready for immobilization from the above experiment, were used as the solid support. The detailed process of immobilization and hybridization is presented in Section 2.3. FAM labeled 16mer oligonucleotides (5’-FAM-CAC-AAA-ACG-GGG-GCG-G-HS-3’) used for immobilization were diluted by immobilization buffer to final concentrations of 0.1μM, 0.5 μM, 1μM, 5 μM, 100 μM, 40 μM, 80 μM, 100 μM. The fluorescence analysis was then taken using the ScanArray 5000™. The sequence for 16mer immobilization DNA oligonucleotides was 5’-HS-carbon_{18-}CAC-AAA-ACG-GGG-GCG-G-3’ and the complementary FAM labeled...
hybridization DNA oligonucleotides sequence was 5'-FAM-CCG-CCC-CCG-TTT-TGT-G-3'. After 20 μM DNA probes were immobilized, FAM labeled complementary DNA oligonucleotides with concentrations of 10nM, 50nM, 100nM, 250nM, 500nM, 750nM, 1500nM, 2000nM, 4000nM respectively, were hybridized.

The relationship of the probe concentration with fluorescence intensity is shown in Figure 2-6. It can be seen that the fluorescence intensity is increased linearly while the probe concentration is increased up to 20μM. When the probe concentration is larger than 20 μM, the immobilization efficiency is saturated. Thus, 20μM was selected as the immobilization concentration. Figure 2-7 shows the relationship of fluorescence intensity with the concentration of the DNA targets. The fluorescence intensity increases linearly while the target DNA oligonucleotides concentration is increased within the range of 0.1-1.5 μM. When the target concentration is 2 μM, fluorescence intensity drops rapidly and then gets saturated. This can be explained by the quench effect of the fluorescence groups when they come to a limited distance [21].
Figure 2-6: Relationship of fluorescence and DNA probe concentration.

Figure 2-7: Relationship of florescence and DNA target concentration.
2.3 Protocol of the Fabrication of DNA Microarray on Silicon Dioxide

2.3.1 Fabrication of Solid Support

The starting material was a 4-inch p-type silicon wafer with (100) crystal orientation. The wafer was first cleaned using de-ionized water and then put into an oxidation furnace at a temperature of 950°C in oxygen and water ambient to grow 4000Å of silicon dioxide. The finished wafer was then cut into many 1cm×1cm dies.

2.3.2 Surface Cleaning and Formation MPTS Layer

After dipping the dies into concentrated sulfuric acid for 30 minutes, the dies were rinsed in de-ionized (DI) water for five minutes. Then dies were immersed into a solution of 1:1 methanol/HCL for 30 minutes. This was followed by a rinse in DI water and blow-drying with nitrogen gas. Afterwards, the dies were immersed into a boiling solution composed of 30 mL of 2-propanol, 400μL MTPS, and 100μL DI water under reflux for 30 minutes. The dies were cooled and washed by 2-propanal followed by DI water. In order to improve the surface quality, dies were put into an oven at 115°C for 30 minutes to anneal the MPTS layer.

2.3.3 Immobilization and Hybridization

The oligonucleotides were diluted by sodium citrate buffer (SSC; 3 M NaCl, 0.3 M Na citrate-2 H2O, pH4.5) to a final concentration of 20 μM. Then 1μL diluted solution was deposited onto the surface of the dies by pipette tips. These dies were prepared using the method described before. The dies were then put into a humidified chamber at room temperature for six hours. Then dies were immersed
into a washing buffer (10 mM Tris-Hcl pH 7.5, 150mM NaCl, 0.05% Tween 20) for five minutes. This was followed by a rinse in DI water. The dies were ready for hybridization after being dried with the nitrogen gun.

The chemical used for hybridization was FAM labeled oligonucleotides targets diluted by hybridization buffer to a final concentration of 1.5 μM. The hybridization buffer consisted of 6xSSC pH 7, 0.2% sodium dodecyl sulfate (SDS). 2 μL diluted target samples were dropped onto the middle of the dies. Dies were covered with a cleaned cover glass to ensure the uniformity of the hybridization. The dies were then incubated in a humidified chamber. The chamber was then put into a water bath at 42°C for two hours. After cleaning the chips with the above-mentioned cleaning solution and DI water, the chips were blown dry in nitrogen, and were ready for detection and analysis. The flow chat of the entire process is given in Figure 2-8.

2.3.4 Data Analysis after Hybridization

The sequence of the DNA oligonucleotides used for immobilization in the experiment was 5'-HS-carbon18-CAC-AAA-ACG-GGG-GCG-G-3' and the complementary FAM labeled hybridization DNA oligonucleotides sequence was 5'-FAM-CCG-CCC-CCG-TTT-TGT-G-3'. The DNA oligonucleotides with single base mismatch mutation had a sequence 5'-FAM-CCG-CCC-ΔCG-TTT-TGT-G-3'. The non-complementary FAM labeled hybridization DNA oligonucleotides sequence was 5'-FAM-TGA-TGC-GAC-AAA-AGC-G-3'.

The hybridization results were analyzed by microscope with a helium light source. The light goes through a band pass filter and excites the optical labels. Fluorescence images are observed when the hybridization process is successful. Figure 2-9 shows that using this protocol DNA microarray can be fabricated and
used in DNA diagnosis. Strong fluorescence light can be observed when complementary oligonucleotides are hybridized, as shown in Figure 2-9 (a). Much weaker fluorescence is observed when oligonucleotides with single base mismatch mutations are hybridized, as shown in Figure 2-9 (b). In Figure 2-9 (c), there is no fluorescence observed when noncomplementary oligonucleotides are hybridized. Thus, the DNA microarray on silicon dioxide was successfully fabricated and can be used in DNA analysis.
Figure 2-8: Flow chart of DNA microarray process
Figure 2-9: Fluorescence image taken by microscope: (a) Hybridization of Complementary DNA oligonucleotides, (b) Hybridization of DNA oligonucleotides with single base mismatch mutations and (c) Hybridization of noncomplementary DNA oligonucleotides.
2.4 Surface Characterization

The DNA microarray relies on the interaction of DNA used for immobilization and the free (target) DNA in a solution. This interaction is strongly influenced by the conformation and distribution of the immobilized DNA and is subject to a variety of experimental factors. Hence, it is very important to monitor the whole immobilization process. Various methods of surface characterization provide ways to monitor the whole immobilization process. In Section 2.4.1, surface characterization methods and the preparation for the materials are introduced. The results and discussions are given in Section 2.4.2.

2.4.1. Methods and Materials

The process of DNA microarray includes two major steps: Preparation for solid support ready for the attachment of the DNA probe, and the immobilization and hybridization procedure.

Surface preparation changes the wetting property of the surface greatly from hydrophilic to hydrophobic. Contact angle measurement provides a relatively quick and simple means of assessing the cleaning of the silicon dioxide surface. While not as quantitative as X-ray photoelectron spectroscopy (XPS), the method does allow for general comparisons and can provide a rapid qualitative test for the presence of silanes on the surface. In order to monitor the hydroxylation and the formation of the MPTS layer, the Ramé-Hart goniometer model # 100-00 can be used to measure the contact angle. 20μL de-ionized H₂O is deposited onto the surface of chip and measured at room temperature with 60% relative humidity. The result was presented in Section 2.2.

Silicon Technology Based Module of High Density DNA Microarray with Electro-Optic System
After immobilization, it is necessary to determine whether or not the DNA probes are immobilized on the surface successfully, and how evenly the probes are distributed on the surface. Although fluorescence images taken by microscope can show whether or not the hybridization has been achieved, quantitative surface characterization is required to understand the behavior of the hybridization on silicon oxide. Thus, the atomic force microscopy is used to monitor the immobilization and hybridization procedures.

The atomic force microscopy is one of the scanned-proximity probe microscopes. The ability of the AFM to image at atomic resolution, combined with its ability to image a wide variety of samples under a wide variety of conditions, has created a great deal of interest in applying it to the study of biological structures. Images have appeared in the literature showing DNA, single proteins, structures such as gap junctions, and living cells [22]. Unfortunately, AFM cannot image all samples at atomic resolution. The end radii of available tip confines atomic resolution to flat, periodic samples such as graphite. In addition, because biological structures are soft, the tip-sample interaction tends to distort or destroy them.

The tips used in AFM are about 20 nm in diameter. Measuring any samples with a diameter of below 20 nm will cause severe tip effect and artifacts. Oligonucleotides usually involved in the immobilization are around several nanometers. Thus, the precise measurement of surface property by AFM requires nanoparticle DNA labels to amplify the surface topology. Colloidal metal particles with diameters between 10 and 60 nm have already been applied in AFM for topological labeling of DNA [23] and as a calibration standard [24]. In our research, DNA probes and complementary DNA targets labeled by gold nanoparticles were adopted to enhance the surface topology. Silver enhancement has been used to visualize protein-gold nanoparticles.
in histochemical electron microscopy studies [25]. To facilitate visualization of nanoparticle labels hybridized to the array surface, we used this signal amplification method in which silver ions were reduced by hydroquinone to silver metal at the surfaces of the gold nano-particles. This process can increase the scanned intensity.

The detailed procedures are as follows: (a) Labeling DNA with gold: monomaleimido nanogold™ reagent (Nanoprobes, Inc) was dissolved in 0.02 ml isopropanol, then diluted to 0.2 ml using de-ionized water. 2 OD of thiol-labeled oligonucleotides were added. They were first dissolved in 0.2 ml buffer (0.02 M sodium phosphate buffer with 150 mM sodium chloride and 1 mM EDTA, pH 6.5) in the gold solution, and kept for two hours at room temperature. Purification of the gold-labeled oligonucleotides was done with NAP-5™ Column. Then the solution was diluted using 1 M NaCl with 10 mM phosphate to a concentration of 1 µM. (b) The immobilization and hybridization of the gold-labeled oligonucleotides was conducted following the protocol presented in Section 2.3. (c) Amplification with silver stain: LI SILVER™ silver enhancement (Nanoprobes) was used to amplify the size of the gold by combining two components together. It was applied onto the dies for 5 minutes at room temperature. Then it was washed using water.

By using the tapping mode with a resolution of 512 pixels per line, results were analyzed using Digital Instrument software.

2.4.2. Results and Discussion

After the MPTS formation, the AFM data (Figure 2-10) indicates that formation of the MPTS generates a uniform surface with a 1.13nm mean roughness. The average grain size of the surface is 39nm. After complementary DNA oligonucleotides are hybridized to the surface and the silver stain is completed, the
average grain size changes from 33nm to 88nm while roughness changes from 1.13nm to 10.88nm (Figure 2-11). Figure 2-12 shows the condition of the surface on which non-complementary DNA is hybridized and on which silver stain is also done. This data shows that the grain size is 55nm and that the roughness is 6.539nm. The non-complementary DNA oligonucleotides will not bond to the probes. So, after the wash, there are no nanogold particles on the surface. As the silver stain rate is largely enhanced when silver meets gold, smaller grain size can be seen on the surface where noncomplementary DNA oligonucleotides are hybridized. The clear difference in the grain size between the hybridization of complementary DNA target and the hybridization of noncomplementary DNA target demonstrates that AFM can be used to monitor the immobilization and hybridization.
Figure 2-10: Atomic force microscopy on silicon dioxide surface after MPTS formation.
Figure 2-11: Atomic force microscopy on silicon dioxide surface after complementary DNA oligonucleotides were hybridized and silver stain is done.
Figure 2-12: Atomic force microscopy on silicon dioxide surface after non-complementary DNA oligonucleotides are hybridized and the silver stain is done.
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[8] T. Livache, A. Roget, E. Dejean, C. Barthet, G. Bidan, and R. Teoule, "Preparation of a DNA matrix via an electrochemically directed copolymerization of


CHAPTER 3

SILICON TECHNOLOGY BASED MODULE OF HIGH-DENSITY DNA MICROARRAY

After describing the fabrication protocol of DNA microarray on silicon dioxide in Chapter 2, this chapter discusses the technology to reduce array feature size and increase array density. There are a variety of microarray technologies being used, and most of them can be classified into one of three dominant technologies. The advantages and disadvantages of the three technologies are reviewed in Section 3.1. A micropatterning technique was developed to shrink the feature size of DNA microarray on silicon dioxide. As a first step, silicon materials compatible to the fabrication process were studied. These are described in Section 3.2. The minimum array features achieved by micropatterning are then discussed in Section 3.3.

3.1 Miniaturization of DNA Microarray

As in silicon technology, the shrinking of the feature size is an important trend in DNA microarray technology. In the post-genome era, identifying the biological function of hundreds of thousands of discovered genes is the main challenge. To analyze such large amount of information necessitates an increase in array density. The study of all human or other organisms' genes onto a single chip at one time requires more densely packed arrays so as to avoid chip-to-chip variation. And more statistical data is needed to reduce both the false negative and false positive results [1]. Another driving factor for the shrinkage is cost. Gordon Moore observed that the number of transistors per semiconductor chip doubles about every 18-24 months. Moore's Law also fits the microarray technology which uses photolithography.
developed by Affymetrix. In 1998, one Affymetrix array contained less than 1000 genes; by 2000, it boasted 12000 [2, 3]. Figure 3-1 shows the trends regarding the cost and density versus the year.

Although this photolithography technology offers the highest density of probes per unit area of any techniques in use, it has several disadvantages. In photolithography technology, the probe length is limited only to 25 mer nucleotides due to the coupling efficiency. When this method is used to identify a single gene, several patches are needed. Furthermore, to fabricate a new array using photolithography technology, a new set of mask has to be made and the cost is greatly increased.

Developed by Nanogen, the technology used to manipulate DNA on a silicon chip with electric fields produces a chip of silicon embedded with one-hundred 80 \( \mu \text{m} \) platinum pads, spaced about 200 \( \mu \text{m} \) apart [4,5]. Each pad can have a voltage of \(-1.3\) to \(2.0\) \( \text{V} \) applied to it through an external control circuit. Although this technology does speed up the hybridization reaction by a factor of 1000 by pooling DNA onto electrically charged sites, its density is limited by the diffusion effect of each spot, and the number of spot that can be analyzed is limited to hundreds to achieve high-speed analysis.

Using glass array facilitated by printing technology pioneered by the Patrick Brown laboratory, the probe can be 500 to 5000 bases long [6,7]. As this technology synthesizes DNA oligonucleotides off chip or use cDNA copies, it is the most flexible technology to use in applications. However, due to limitations in printing technology, the minimum spot size is now 100\( \mu \text{m} \) in diameter and the minimum spacing between two neighbor spots is 110 \( \mu \text{m} \). This value is ten times the spot spacing in Affymetrix, which is now 10 \( \mu \text{m} \). Figure 3-2 shows a typical microarray
using this technology. Each dot is 100 μm in diameter. In order to increase the density of this technology, we used the micropatterning technology. Micropatterning in silicon technology has achieved a minimum line width to 0.18μm. However, to realize shrinkage using micropatterning, two key issues must be taken into account. These two issues are the immobilization method which can be used successfully on silicon material, and silicon materials which are compatible with the immobilization process and can be used for isolation. It is important that the DNA probes demonstrate a high selectivity to the support materials and not to the insulating material. As discussed in Chapter 2, we developed microarray on a silicon dioxide surface rather than on a glass slide. Materials compatible with this process are discussed in the next section.

Figure 3-1: Moore’s Law for gene chips.
3.2 Compatibility Silicon Materials

As the process of immobilization on silicon dioxide involves the use of some chemicals with a strong etching nature such as concentrated $\text{H}_2\text{SO}_4$ and HCL, there is a need to test whether or not the common silicon materials used in the microfabrication process can tolerate the process. We tested the five commonly used silicon materials aluminum, gold, platinum, silicon nitride and polysilicon. The compatible materials should not only be able to tolerate the immobilization process, but must also be able to be used as the insulating materials so that DNA probes are much more selective to silicon dioxide than the insulating layers. Thus, after
hybridization, the fluorescence on the materials able to tolerate the process were analyzed and compared to the fluorescence on silicon dioxide to see if they could be used as the insulating materials in micropatterning.

3.2.1 Experiment

A 4000 Å thick layer of silicon dioxide was grown in an oxidation furnace at 950°C on a 4-inch p-type silicon wafer. Commonly used silicon materials as Al, Gold, platinum, silicon nitride and poly silicon were deposited to 4000 Å on five silicon wafers, respectively. These wafers were all etched to form a 1cm×1cm square exposed silicon dioxide layer in the middle. Thus, a 4000 Å step was formed. Then all wafers went through the process of DNA microarray. The thickness of the step was measured after each of the three critical steps of the microarray fabrication process using a-stepper. A clear picture of which material could tolerate the procedure was revealed by the step thickness. After hybridization, the wafers were placed in the scanner for fluorescence analysis. The fluorescence intensity on these materials indicated if there was any binding of DNA on the material. Further, the fluorescence intensity on the middle square of silicon dioxide was also measured and compared to the fluorescence intensity on the tested materials. This was done to determine the selectivity of the DNA probes to silicon dioxide and the tested materials.

All of the oligonucleotides used in the experiment were purchased from Synthetic Genetics (San Diego, CA). The sequence for the 16mer immobilization DNA oligonucleotides was 5'-HS-carbon18-CAC-AAA-ACG-GGG-GCG-G-3' and the complementary FAM labeled hybridization DNA oligonucleotides sequence was 5'-FAM-CCG-CCC-CCG-TTT-TGT-G-3'.
3.2.2 Results and Discussion

The thickness of each material on the silicon dioxide after every important step is listed in Table 3-1. After the surface cleaning and hydroxylation step, only Al is etched entirely. Other materials such as gold, platinum, silicon nitride and polysilicon remains unchanged during the process. This result is reasonable because all the chemicals with strong oxidizing properties were used in the surface cleaning and hydroxylation step. The etching rates of the HCL and concentrated H_2SO_4 to gold, platinum, silicon nitride and polysilicon are very low. Thus, despite the process time, these four materials could tolerate the fabrication process.

<table>
<thead>
<tr>
<th>Material</th>
<th>Al</th>
<th>Au</th>
<th>Pt</th>
<th>Si_3N_4</th>
<th>Poly-Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original thickness (Å)</td>
<td>4012</td>
<td>3897</td>
<td>3750</td>
<td>4116</td>
<td>3570</td>
</tr>
<tr>
<td>After surface cleaning</td>
<td>0</td>
<td>3889</td>
<td>3745</td>
<td>4112</td>
<td>3561</td>
</tr>
<tr>
<td>and hydroxylation (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After MPTS formation (Å)</td>
<td>0</td>
<td>3892</td>
<td>3745</td>
<td>4110</td>
<td>3563</td>
</tr>
<tr>
<td>After hybridization (Å)</td>
<td>0</td>
<td>3894</td>
<td>3743</td>
<td>4115</td>
<td>3566</td>
</tr>
</tbody>
</table>

Table 3-1: Thickness of Al, Au, Pt, Si3N4, Poly-Si after every step in DNA microarray process.

After hybridization, the dies with gold, platinum, silicon nitride and polysilicon were placed in the scanner to analyze the fluorescence intensity on the isolation materials. The fluorescence pictures of the chips are shown in Figure 3-3 (a), (b), (c),...
(d). The fluorescence intensities on gold, platinum, silicon nitride and polysilicon are 617, 653, 951 and 700. The difference in fluorescence intensity of the four materials is in the range of chip-to-chip variations. The low fluorescence intensity on these materials demonstrates that non-specific DNA bonding on these materials is quite low. As Figure 3-4 shows, a big contrast of fluorescence intensity between the testing materials and silicon dioxide can be observed. The ratio of fluorescence intensity on silicon dioxide to that on gold, platinum, silicon nitride and polysilicon is 35.8, 30.9, 25.11 and 28.4 respectively. Compared with glass slides, on which the signal to background noise ratio is usually around 25, these four materials have a sound isolating effect. Thus, gold, platinum, silicon nitride and polysilicon can all be used as insulting materials. As lift-off technology has to be used to etch gold and platinum and these two materials are relatively expensive, we only selected silicon nitride and polysilicon as the insulting materials in micropatterning to shrink the array feature.
Figure 3-3: Fluorescence image from scanner on which, a silicon square is in the middle and testing materials on the outside. (a) Au is used as the insulating material, (b) Pt is used as the insulating material, (c) Si3N4 is used as the insulating material, and (d) Poly-Si is used as the insulating materials.

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Figure 3-4: Comparison of fluorescence intensity between silicon dioxide and Au, Pt, Si3N4 and Poly-Si.

3.3 Miniaturization of Array Features

As the materials to be used for the micropatterning had been selected, the tasks left were to test to which degree micropatterning could help in shrinking the array feature sizes, which included the minimum size of silicon dioxide as the immobilization platform and the minimum size of the effective isolation line between every array element. By patterning the isolation materials on silicon dioxide, the immobilization platform can be shaped and sized. Moreover, isolation line itself can be patterned to a designated size and shape. In this section, the smallest feature size of silicon dioxide as the immobilization platform is discussed. This is followed by a discussion of the size of the effective isolation line.
3.3.1 Experiment

Using Cadence, a layout was drawn (Figure 3-5), and thus was used to fabricate one mask for patterning the silicon nitride and polysilicon. 4000 Å silicon dioxide was grown on silicon wafer followed by the LPCVD deposition of 2000 Å thick silicon nitride. Following the standard photolithography, silicon nitride was etched at the selected area by plasma etching. Then the wafer was cut into 1 cm × 1 cm dies. On each chip, there are eight 3 by 3 arrays with different isolation widths of 1 µm, 2 µm, 5 µm, 10 µm, 25 µm, 50 µm, 75 µm and 100 µm. Each array was composed of nine 50 µm × 50 µm platforms of silicon dioxide with silicon nitride as the isolation material between the array elements. There were also round silicon dioxide platforms with diameters of 1 µm, 5 µm, 10 µm, 25 µm, 50 µm, 75 µm and 100 µm.

Polysilicon was also deposited on the wafer and patterned as stated above. Then chips went through the whole immobilization and hybridization process mentioned in Chapter 2. After the hybridization, fluorescence image were taken using the scanner and were used to study the minimum size of the immobilization platform and the effective isolation line width.

![Figure 3-5: Layout for patterning silicon nitride and polysilicon on silicon dioxide.](image)
3.3.2 Results and Discussion

After the patterned chips went through the immobilization and hybridization procedure, they were analyzed using a scanner. The observed images are shown in Figure 3-6 and Figure 3-7. Figure 3-6 shows the chip with silicon nitride as the insulating material and Figure 3-7 shows the chip with polysilicon as the insulating material. It should be noted that the color of the images does not indicate the spectral response of the label, but the intensity of the fluorescence intensity. As shown in the figure, the attachment of matched oligonucleotides is very effective on silicon dioxide. No bonding of DNA on silicon nitride and polysilicon is observed. This gives a very high contrast at the boundary of the material. We believe that DNA probes are very selective to silicon dioxide ad not selective to silicon nitride and polysilicon. Therefore, silicon nitride and polysilicon can be used to form an effective isolation material. As shown in Figure 3-6 and Figure 3-7, silicon nitride and polysilicon as narrow as 1μm can be used to isolate neighboring cells in a DNA microarray. Isolation at 1μm and below using nitride and poly is still possible, but was not observed in our experiment because the scanner used has a minimum resolution limit of 5μm. This also explains the unclear image for 1μm and 2μm nitride and poly line isolation in Figure 3-6 and Figure 3-7.

The fluorescence image taken by the scanner of silicon dioxide of different sizes is shown in Figure 3-8. A sharp contrast between the silicon dioxide and nitride can be observed on the silicon dioxide platform of 200μm, 100μm, 50μm, and 10μm in diameter. Figure 3-9 shows that the difference in intensity between the silicon dioxide platforms of the above sizes. No obvious difference in intensity can be observed between the platform of larger sizes and smaller sizes. The platforms with
the smaller feature of 5 \( \mu m \) and 1 \( \mu m \) cannot be distinguished from the background. Thus, by using the present scanner technology, the minimum detectable feature size, without a trade-off in sensitivity, is 10 \( \mu m \). As a result, a significantly higher density of microarray can be formed. This reduces the array spot size from 100 \( \mu m \) to 10 \( \mu m \), and the isolation width from 10 \( \mu m \) to 1 \( \mu m \).

![Figure 3-6: Fluorescence images showing the isolation effect of silicon nitride. (From left to right, the width of silicon nitride is 1 \( \mu m \), 2 \( \mu m \), 5 \( \mu m \), 10 \( \mu m \), 25 \( \mu m \), 50 \( \mu m \), 75 \( \mu m \) and 100 \( \mu m \).)](image-url)

![Figure 3-7: Fluorescence images showing the isolation effect of polysilicon. (From left to right, the width of silicon nitride is 1 \( \mu m \), 2 \( \mu m \), 5 \( \mu m \), 10 \( \mu m \), 25 \( \mu m \), 50 \( \mu m \), 75 \( \mu m \) and 100 \( \mu m \).)](image-url)
Figure 3-8: Fluorescence images on silicon dioxide with different size. (From the left to right, the diameter of silicon dioxide is 200μm, 100μm, 50μm, and 10μm).

Figure 3-9: Comparison of fluorescence intensity between silicon dioxide with different sizes.
References


CHAPTER 4

DNA Microarray on Electro-optic System

The integration of a detection system on the DNA microarray is an important topic in the field of biochip applications. This chapter discusses the efforts to integrate an electro-optic detection system onto a silicon substrate. In Section 4.1, the reasons to integrate DNA microarray with an electro-optic system are given. The two most important issues for on chip optical detections, which are (1) the selection of an on-chip color filter and (2) the DNA microarray on agarose film, are studied in Sections 4.2 and 4.3 respectively.

4.1 Integration Approach of DNA Microarray and Electro-optic system

There are several detection methods such as fluorescence, radioisotope, mass spectrometry [1], chemiluminescence [2-4] and electrochemiluminescence [5] available for DNA microarray. However, fluorescence is the most common detection choice for the chip-based DNA analysis system because it is the most sensitive method and introducing fluorescence label to DNA is relatively easy.

To date, the fluorescence detection of biochip micro-arrays has often been accomplished by confocal laser scanning fluorescence microscopy. Figure 4-1 shows a ScanArray 5000™ from Packard bioscience. In this approach, images of the whole DNA microarray are acquired by sequentially scanning either the laser or the specimen (or both) using a fast mechanical scanning system. Because of the use of complex optical settings, the mechanical scanning system and the laser system, the scanner is of desktop size and costs around millions of dollars. The structure of the scanner is shown in Figure 4-2.
In order to find a more effective and economical alternative to the current system, there is now an intensification of work in relation to the lab-on-a-chip project. Such work includes the integration of an optical sensor with a DNA microarray system. The integrated system not only greatly reduces the cost, but also is portable. Figure 4-3 illustrates the schematic structure of the integrated system. By putting an optical sensor directly under every element of the DNA microarray, the optical setting and mechanical parts of the scanner that controls the x-y plane movement can be eliminated.

However, to achieve this approach two main issues had to be considered: the selection of appropriate material for the use as the on-chip color filter and an immobilization method which ensured that the chip could be reused in order to reduce the cost. The fluorescence detection method uses a laser as the excitation light source. Thus, for an optical sensor to be able to detect the fluorescence from a strong background laser light, an on-chip color filter was necessary. As the immobilization and hybridization process takes place on the color filter, the color filter need to be stable in regard to chemicals, heat and light. Moreover, in order to reduce the cost, a new immobilization method had to be developed so that DNA microarray could be
established on and removed from the color filter without damaging it and the devices underneath. In this chapter, we will focus on the selection of the appropriate on-chip color filter and the corresponding fluorescence dyes. We will also discuss the development of the immobilization method on agarose film that allows a reusable DNA microarray.

Figure 4-2: Schematic structure of scanner by using confocal laser technique.
4.2 On-chip Color Filter

In a fluorescence detection system, integrated or not, there are four essential elements: (1) an excitation source, (2) a fluorophore, (3) wavelength filters to isolate emission photons from excitation photons and (4) a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, the compatibility of these four elements is essential in order to optimize the fluorescence detection. A feasible combination of the first three elements for the integrated system is presented now.

Fluorescence detection sensitivity is severely compromised by background signals, which may originate from endogenous sample constituents (referred to as autofluorescence). However, signal distortion caused by the auto fluorescence of cells, tissues, and biological fluids is most readily minimized by using probes that can be excited at wavelength longer than 500nm. Because of this, and because of the comparatively low cost and the ease of fabrication, Texas Red was selected as the fluorophore for our research. The excitation and emission spectrum is shown in
Figure 4-4. The peak excitation wavelength is 598nm and the peak emission wavelength is 618nm.

![Excitation and emission spectrum of Texas Red](image)

Figure 4-4: Excitation and emission spectrum of Texas Red.

As a requirement of the integration, the fabrication process of the DNA microarray had to be performed on an on-chip color filter. The on-chip color filter had to be stable in regard to chemicals, heat and light. The physical properties of the dyed polyimide color filter that has a high contrast ratio could mostly satisfy the requirement.

A dyed polyimide filter developed by Brewer Science Inc. was investigated. This color filter employs polyimide as the substrate and is called dyed polyimide. Both the heat stability and chemical durability of dyed polyimide are excellent. The dyed polyimide has a heat stability of up to 280°C and cannot be attacked by most chemicals once it is cured. As Texas Red was selected as the fluorophore, red dyed polyimide was used as the interfacing material between the DNA microarray and the electro-optic system.
The red polyimide color filter was deposited on a 5x5cm quartz dice. After spinning the color filter at a 1000rpm spin rate for 1.5 minutes, the color filter was 11.5nm thick. A UV/VIS Spectrophotometer (Philips's model PU8700) was used to scan the spectrum of the color filter, Figure 4-5. From the spectrum, it can be seen that there is a sharp transition at the wavelength of 600nm. In this regard, the emission fluorescence from Texas Red is mostly transmitted.

The remaining problem was the selection of the light source. Although the Texas Red has the peak absorption wavelength at 598nm, red polyimide can only block 30% at this wavelength. As the intensity of the excitation light is usually about 100 times the intensity of the fluorescence, a laser source of 598nm was not feasible. It was decided that a laser source of 543nm was the compatible excitation light source for this approach. The transmission ratio at this wavelength, 1.6%, means the color filter can block the excitation light efficiently. At this wavelength, the absorption ratio of Texas Red is still 30% of the maximum value. At this absorption ratio, the fluorophore Texas Red can still be excited efficiently.

By combining the use of Texas Red as the fluorophore, red dyed polyimide as the on-chip color filter, and a laser source of 543nm, an integrated fluorescence detection system can be realized.
Figure 4-5: The transmission spectrum of color filter before the deposition of agarose film and after the removal of agarose film.
4.3 Fabrication of DNA Microarray on Electro-optic System

After the selection of the on-chip color filter, DNA microarray fabrication process had to be developed on a color filter. As the dyed polyimide is an organic film, the covalent immobilization method could not be used. An immobilization method which could use the membrane film as the solid support, had to be adopted. For the integrated approach of DNA microarray, the optical settings were eliminated. To enhance the sensitivity of the whole system, the capacity for immobilization had to be increased. Avidin-biotin complexation provides a 3-D structure to meet this requirement. Avidin and streptavidin are large tetrameric proteins incorporating four identical binding sites. Biotin is a small molecule that attaches with very high affinity to the avidin or streptavidin binding sites (Ka=1e15 M⁻¹). This value is nearly equal to that of a covalent bond. Because of this strong interaction, the complex formation is nearly unaffected by extreme values of PH or temperatures, organic solvents, and denaturing agent.

To develop this method, a support material had to be selected. Agarose is a widely used support material in molecular biology [6] and it is well known that agarose can provide a support for hybridization reactions [7,8]. The activated agarose film can be prepared in any laboratory without special equipment. Moreover, the nature of the agarose film allows to use any type of spotting technology to deposit the desired molecules. The agarose film also has a low fluorescence background and can be used for hybridization with fluorescent dyes. Another important feature of the agarose film is that it can be melted at a low temperature of around 100°C and, thus, the immobilized probe and hybridized target can be removed with the agarose film. This
is very important in that the integrated system can be reused without damaging the electro-optic devices underneath.

Combining the agarose film and avidin-biotin complexation, the fabrication process of DNA microarray on electro-optic system was established.

4.3.1 Experiment

Deposition of the on-chip color filter: Red polyimide color filter was deposited on a 5x5cm quartz dice. After spinning the color filter at a 1000rpm spin rate for 1.5 minutes, the color filter was 11.5nm.

Deposition and activation of agarose film: A 1% agarose solution in purified water was prepared. After being heated to 70°C for two minutes, a 2 ml agarose solution was deposited in the middle of the quartz dice with a color filter. After spinning for one minute at 1000rpm, the dice was left in the air to gel for around 1 minute. Then it was dried in a dryer for 30 minutes. The dried dice could be stored or used immediately for immobilization of oligonucleotides after activation of the agarose. For the activation we used a 20 mM solution of NaIO₄. The dice was submerged in a small bath containing the NaIO₄ solution and incubated for 30 min at room temperature. It was then washed in distilled water and dried. 1mg/ml of streptavidin was then deposited on the surface. The dice was then kept in moisture for 1 hour at 37°C. We then rinsed the dice one time with water and baked the dice for 30 minutes at 37°C.

Immobilization: We spotted, by hand, a 0.1ul 20uM biotin labeled DNA probe in cysteine on the surface. In this method printing can be also used to deposit the DNA probe on the specific place. Afterwards, the dice was kept at room temperature for 1 hour. Then we washed the dice with water for three times, each time for 10 minutes.
Hybridization: The 1uM fluorescent labeled complementary DNA target was hybridized to the dice for 2 hours at 42°C. The dice was then washed with 0.1*SSC for 5 minutes and washed with water for 1 minute and blown dry with a nitrogen gun. The sequence of DNA used in this experiment was the same as the one used in experiments discussed in Chapter 2.

4.3.2 Results and Discussion

Figure 4-6 shows the fluorescence image taken after the immobilization and hybridization process. A clear fluorescence image is observed when complementary DNA oligonucleotides are hybridized to the DNA probe and there is no fluorescence detected when the non-complementary DNA oligonucleotides are hybridized. Furthermore, it can be seen that agarose film does not interfere with fluorescence detection and, thus, the fabrication process discussed above is successful for the development of an integrated DNA analysis system.

Figure 4-6: Fluorescence image from scanner: (a) hybridization of complementary DNA oligonucleotides, and (b) hybridization of non-complementary DNA oligonucleotides.
References


CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In this thesis, the state-of-the-art DNA microarray technology was reviewed. From our research work, a number of silicon-based technology modules for high density DNA microarray was proposed and experimentally demonstrated. The first step to develop a high-density DNA microarray is DNA attachment and immobilization. In current technology, such attachment and immobilization was done on a solid glass substrate. In our work, silicon dioxide was chosen as the solid support because it has similar chemical properties to glass, but more stable and pure. Among the available immobilization methods, covalent attachment was selected to bond the DNA molecule to the silicon chip.

The method to improve the immobilization and hybridization process of DNA strands on silicon dioxide was discussed. Contact angle measurement showed that using MeOH: HCL and H2SO4 for the cleaning method was the most efficient. This cleaning method led to the high silanization of the surface also. Concentrations of immobilization and hybridization DNA oligonucleotides were chosen of 20μM and 1.6 μM respectively. Using these concentrations, the efficiency of immobilization and hybridization were the highest with the use of the least possible amount of DNA oligonucleotides. Thus, the protocol of fabricating DNA microarray system on silicon dioxide was established. The fluorescence image shows obvious differences in intensity between the hybridization of the complementary DNA, the hybridization of the non-complementary DNA, and the hybridization of the complementary DNA.
with single base mismatch. Using surface characterization methods such as contact angle measurement and AFM, the process can be well monitored and ensure the best immobilization quality. The technique of colloidal gold labeling the DNA oligonucleotides and, later, the silver stain amplified the DNA probe information and therefore, through the use of AFM, the hybridization can be illustrated.

Measurement of the thickness revealed that gold, platinum, silicon nitride and polysilicon could tolerate the cleaning and silanization step. The big contrast in fluorescence intensity on silicon dioxide and the above materials revealed that these materials are compatible with the immobilization and hybridizations process on silicon dioxide. Thus, they can be used as passivation layers in later integrated approaches of the DNA microarray and detection system. As the chemical nature of these four materials and silicon dioxide are different, they can be used to shrink the size of the silicon dioxide platform and to insulate each array element. As shown by the fluorescence images, the effective isolation width of silicon nitride and polysilicon can reach 1µm, and the size of silicon dioxide platform can be shaped to as small as 10µm in diameter. With this method, the density of the DNA microarray was greatly increased.

The process of the fabricating DNA microarray on electro-optic system was also discussed. The integrated approach provides a more effective and economical alternative to the current system by integrating the optical sensor with DNA microarray. Dyed polyimide was selected as the on-chip color filter because it was stable in regard to heat, chemicals and light. The transmission spectrum of the dyed polyimide measured before agarose film deposition and after the removal of agarose film, illustrates that dyed polyimide is suitable for use as an interfacing materials between microarray and detection system. Moreover, the process of fabricate DNA
microarray on color filter coated by agarose film was also feasible. The pictures show a clear fluorescence image of hybridization of complementary DNA target, and no fluorescence is observed of the hybridization of noncomplementary DNA target. Using this process, the DNA microarray can be removed with the removal of the agarose film at 100°C, at which the color filter and devices underneath will not be damaged. Therefore, this process can be used in the integrated approach.

5.2 Future Work

Our research involved the study of the silicon technology-based module of DNA microarray and the process integrating a DNA microarray with detection system. Although the immobilization of DNA oligonucleotides on silicon dioxide was realized, the chip-to-chip variation of the fluorescence intensity has yet to be reduced. The difference in the fluorescence intensity of the hybridization of complementary target and of the target with single base mismatch mutation should be enlarged. Further modification must be done to the process in order to develop a DNA microarray which can give a more precise analysis.

The immobilization procedure on agarose film was also achieved. However, due to the diffusion effect of the DNA probes in agarose film, the location and shape of each spot were not well defined and, thus, the density of the microarray on agarose film was limited. Methods to minimize the dispersion of the sample should be developed in order to increase the density of the DNA microarray of the integrated approach. It is time to consider the development of a more sensitive electron-optic device for the detection of this integrated system.
Appendix A

Publications


Silicon Technology Based Module of High Density DNA Microarray with Electro-Optic System