Photoelectrochemical Synthesis of Low-Cost DNA Microarrays

by

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B.S. Chemistry
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Massachusetts Institute of Technology, 2003

Submitted to the Program in Media Arts and Sciences,
School of Architecture and Planning,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Media Arts and Sciences
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Abstract

Recent advances in de novo gene synthesis, library construction, and genomic selection for target sequencing using DNA from custom microarrays have demonstrated that microarrays can effectively be used as the world’s cheapest sources of complex oligonucleotide pools. Unfortunately, commercial custom microarrays are expensive and not easily accessible to academic researchers, and technical challenges still exist for dealing with the small amount of DNA synthesized on a chip. Genomic research would certainly benefit from the creation of cheaper custom microarrays with larger oligonucleotide concentrations per spot.

This thesis presents the development of a novel DNA microarray synthesis platform based on semiconductor photoelectrochemistry (PEC) designed with these needs in mind. An amorphous silicon photoconductor is activated by an optical projection system to create "virtual electrodes" that electrochemically generate protons in a site-selective manner, thereby cleaving acid-labile dimethoxytrityl protecting groups with the spatial selectivity that is required for in-situ DNA synthesis. This platform has the potential to be particularly low-cost since it employs standard phosphoramidite reagents, visible wavelength optics, and a cheaply microfabricated and reusable substrate. By incorporating a porous thin-film glass that dramatically increases the DNA quantity produced by over an order of magnitude per chip, this platform may also simplify the handling of DNA cleaved from chip and drive down the cost per base synthesized. The hybridization detection of single-base errors was successfully demonstrated on PEC synthesized microarrays. This thesis also reports a suite of new surface chemistries and high-resolution techniques for patterning biological molecules.

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Joseph M. Jacobson
Associate Professor, Program in Media Arts and Sciences
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Chapter 1

Introduction

1.1 Statement of purpose

Over the past two decades, DNA microarray technologies have greatly changed the landscape of genomics. A typical microarray (Figure 1) contains $10^3$-$10^5$ spots per cm$^2$ of DNA on a two-dimensional surface (e.g., microscope slide), with each spot bearing multiple copies of oligonucleotides of unique sequence (typically 16 – 60 bases long). Since such arrays can yield sequence and quantity information in a massively parallel fashion by hybridization-based methods,$^1$ microarrays quickly spurred great interest in large-scale genotyping, expression profiling,$^2$ and DNA sequencing as a potential alternative to traditional methods like Sanger sequencing.$^3, 4$ More intricate functional analyses are currently being developed,$^5$ such as chromatin immunoprecipitation on chip (ChIP-chip).

![Figure 1. Schematic of a typical microarray used in expression or transcription profiling. Hybridization assays of fluorescence-labeled target strands allows researchers to determine what genes are expressed in a cell by color, and how much it is expressed relatively to other genes by signal intensity.](image)

More recently, novel applications in multi-plexed de novo gene synthesis$^6-9$ have been developed using the complex pools/libraries that can be obtained by cleaving the
DNA from the chip surface and assembling them by PCR-mediated construction strategies (Figure 2). The ability to synthesize *de novo* genes in a cost-effective manner would greatly benefit research in genetic studies, gene therapy, protein design, synthetic biology, metabolic engineering, and codon remapping. These microarray-based gene synthesis reports are extremely exciting because they represent ~1000-fold reductions in the cost per base for the primer library, which at this time is highly limiting.

![Diagram](image)

**Figure 2.** DNA assembly from microarray-synthesized oligonucleotides by Cerrina and co-workers. The assembly procedure on the right can be repeated multiple times to make longer length constructs.

This then begs the question: Can we move from the traditional 96-well plate (3"x5", ~25 nmol well, $0.18 per base or ~$700 for a plate of 40mers) to the microarray as an “ultra-high density plate”? Just as “Lab-on-a-Chip” technology miniaturizes and reduces the cost of biomolecule fluid handing processes, this technological approach would miniaturize and reduce the cost of the biomolecule production processes. Such dramatic cost reductions would revolutionize biological research. In addition to gene synthesis, cleaved DNA pools have been used for construction of short hairpin RNA libraries, site-directed mutagenesis, and multi-plexed amplification of exons for...
targeted sequencing. Genomic selection schemes for targeted sequencing that do not cleave the DNA from the surface, but still utilize microarrays in a similar non-analytical fashion, have also been demonstrated.

Unfortunately, there are major two impediments to this paradigm shift. First, custom microarrays are not easily accessible to academic laboratories since the economics of the industry have driven the field towards standardized arrays that are mass-produced. Second, very little DNA per spot is synthesized and makes it very difficult to achieve solutions at biologically relevant concentrations after the DNA is cleaved from the chip.

The goal of this thesis is to develop a novel microarray synthesis platform that is programmable and high-resolution, and utilizes low-cost equipment and consumables. This will be accomplished using semiconductor photoelectrochemistry (PEC). Briefly, an amorphous silicon photoconductor is activated by an optical projection system to create virtual electrodes that electrochemically generate protons with spatial selectivity, thus enabling the site-selective cleavage of acid-labile protecting groups of standard synthesis reagents. Furthermore, the use of a thin-film porous glass on top of the photoconductor dramatically increases the oligonucleotide concentration per spot (i.e. amount of DNA synthesized per chip), which adds to the cost (per base) savings and addresses the issue of low DNA quantities synthesized on microarrays.

**Broader Vision**

It is our vision that by constructing such a system, researchers will have access to a technology for cheap “ultra-high density plates” of DNA in the form of a DNA microarray. Other current work in our laboratory includes microfluidic gene synthesis that also aims to address the issue of handling minute oligonucleotide quantities cleaved from chips, and enzyme-based error-correction protocols that aim to improve the fidelity
of the gene assembly process.\textsuperscript{18} It is our ultimate goal to combine these technological platforms to construct an all-integrated gene synthesizer, as described in the integration roadmap in Figure 3. Ideally, such an integrated platform would allow researchers to design DNA sequences \textit{in silico} and synthesize them on a microarray. These strands would be cleaved and assembled into larger constructs in a microfluidic environment. After error correction to improve the yield, these constructs can then be assembled into even larger scale assemblies that biologists and bio-engineers can then use in a variety of ways (clone, sequence, express, etc.).

\textbf{Figure 3.} Ideal integration gene synthesis roadmap from DNA designed \textit{in silico} to large-scale genetic assemblies, and the individual components developed by the Jacobson research group at the MIT. Image modified from original integration roadmap, courtesy of David Kong, MIT.

\textbf{1.2 The proposed system}

The photoelectrochemical (PEC) principles behind the synthesis platform are illustrated in the band and energy diagrams in Figures 4A and 4B, respectively. When an n-type semiconductor anode is illuminated with light above its bandgap energy ($E_g$), valence band electrons are photo-excited into the conduction band (Figure 4A), which
creates valence band holes that can participate in an oxidation reaction in solution and increase its reaction rate. The anodic reaction of interest here is the oxidation of hydroquinone to benzoquinone, a reaction that liberates two protons. Large levels of illumination also increase the surface potential at the semiconductor electrolyte interface (SEI) by shifting the bias potential drop across the SEI from the bulk semiconductor to the Helmholtz layer of the electrolyte (Figure 1B), in addition to creating a positive photopotential.\textsuperscript{15, 16, 19} Since the efficiency of electrochemical reactions depends on the potential energy of the surface charge carriers, the larger surface potential also contributes to an increased reaction rate.

![Figure 4. Principles of photoelectrochemical (PEC) microarray synthesis. (a) Band diagram of the PEC cell. Illuminating the semiconductor electrode photogenerates charge carriers that can participate in the oxidation of hydroquinone to liberate protons. (b) Surface potential profile of an illuminated ("light") and non-illuminated ("dark") semiconductor electrolyte interface (SEI). The "light" semiconductor electrode has a much higher surface potential at the SEI, making it a more efficient electrochemical anode.](image)

Reports in electrophoretic biomolecule manipulation\textsuperscript{20-23} and light-addressable potentiometric sensors\textsuperscript{24} have demonstrated that “virtual electrodes” can be created by illuminating a semiconductor electrode with spatially modulated light. The microarray synthesis scheme (Figure 5) reported here utilizes a digital micromirror device (DMD) to address virtual electrodes that locally PEC-generate acid from the aforementioned
hydroquinone oxidation, which is a reaction that provides a clean source of protons.\textsuperscript{25, 26} The crux of any microarray platform is the ability to perform a spatially selective addition or deprotection reaction (see Section 1.3.2). The PEC-generated protons can cleave acid-labile dimethoxytrityl (DMT) protecting groups of standard phosphoramidite synthesis reagents with the spatial selectivity required to create microarrays. For simplicity, the growing DNA strand is drawn on top of a thin-film porous glass that coats the substrate, but the reaction occurs throughout the entire film, whose large internal surface area increases the crude product yield per chip. Figure 5 also illustrates the structure of the photoconductor anode, whose fabrication and properties will be discussed in detail in Chapters 2 and 3.

\textbf{Figure 5.} Schematic of the proposed PEC synthesis platform, showing site-selective cleavage of acid-labile protecting groups by PEC-generated protons. For simplicity, the growing strand is only drawn on top of the thin-film porous glass, but the synthesis occurs throughout the film.

It is conceivable that PEC virtual electrodes could be used to not only synthesize the microarray, but also to photoelectrochemically manipulate,\textsuperscript{20-23} detect,\textsuperscript{24, 27-29} and
stimulate target molecules in solution. Because the system can also generate bases and radicals with different electrochemical solutions and bias potentials, the platform can conceivably be extended to the synthesis of other biopolymer and small molecule microarrays.

1.3 Background

1.3.1 Traditional phosphoramidite DNA synthesis

Microarray technologies are based upon the traditional DNA phosphoramidite synthesis, developed by Marvin Caruthers in the early 1980's. Briefly, it is a linear polymerization reaction that employs chemical protecting groups that ensure only one nucleotide base is added per cycle. After the nucleotide is added, an acid cleaves the protecting group to create a reactive hydroxyl group with which a subsequent protected-nucleotide can react. The automated procedure is performed on a porous glass bead that enables the use of large excesses of reagents because the purification at each step is a simple solvent wash.

The phosphoramidite synthesis cycle is shown in detail in Figure 6A. Figure 6B shows an image of an automated DNA synthesizer. First, the 5'-hydroxyl is exposed by cleaving the acid-labile dimethoxytrityl protecting group with a haloacid, typically 2-3% dichloro- or trichloroacetic acid. A DNA phosphoramidite is then added with a slightly acidic tetrazole activator that catalyzes the reaction between the exposed 5'-hydroxyl group and the incoming phosphoramidite. Since the incoming phosphoramidite is protected, only one addition may occur. The unreacted 5'-hydroxyl groups are subsequently capped with acetic anhydride and imidazole catalyst in order to prevent deletion errors and facilitate their removal/purification. Because the resultant phosphite triester is not suitably stable, the bond is oxidized with an iodine/pyridine/water mixture to create a more stable phosphate triester. This cycle is repeated until the full-length
strand is completed. Each cycle including wash steps takes approximately 5 minutes, depending on the synthesis scale (typically 20 nmol – 10 μmol).

Figure 6. (a) The standard phosphoramidite synthesis cycle. Image taken from www.trilinkbiotech.com. (b) An ABI 394 synthesizer from Applied Biosystems.

The growing strands are stripped then from the CPG ("controlled pore glass") beads with a basic solution (typically aqueous ammonium hydroxide or 0.05M potassium carbonate in methanol) that also converts the phosphate triesters into phosphates and removes the base-labile protecting groups that prevent the exocyclic amines of the nitrogenous bases (A, G, C, T) from reacting during the synthesis. The solution is then lyophilized/dried and desalted by ethanol precipitation or Sephadex filtration. The phosphoramidite synthesis has a typical step-wise yield of ∼99% and crude yield of 0.99^N \* 100%, where N is the number of bases in the strand. The most common errors are typically (in order of relative occurrence): truncation, internal deletions, and damage to bases that lead to their conversion to dA by polymerase. It should be noted that several alternative synthesis strategies exist\(^3\) (e.g. phosphotriester method), as well as variations to the phosphoramidite cycle (e.g. 3’ to 5’ reverse synthesis).
1.3.2 Microarray technologies

Eventually, automated DNA synthesizers were made parallel by Brennan and co-workers at Stanford \(^{32}\) by fanning-out the fluid lines a linear array of nozzles (originally eight) that sprayed the reagents into a plate of microwells containing CPG during the phosphoramidite addition step. The plate is filled by repeating the addition row-by-row using a robotic arm. A practical upper limit of 384 unique strands can be delivered to an end user in a 12.8 x 8.6 cm plate, although plates with up to 3072 wells exist. However, this is a far cry from the spot density of 500k spots / cm\(^2\) that can be obtained today by commercial microarray technologies. Ultimately, the multi-plexing of DNA synthesizers is limited by the cost and difficulty of valving / plumbing.

\[\text{Figure 7.} \quad \text{The Affymetrix platform uses contact lithography to achieve spatially selective removal of photocleavable protecting groups. Image taken from www.affymetrix.com}\]

The crux of \textit{in-situ} microarray technologies is the ability to remove the protecting group or add the protected monomer with two-dimensional spatial resolution in a massively parallel manner (or ultra-fast serial manner). By doing so, the same global plumbing or fluid delivery system can be used for the entire chip. This is true for any
biopolymer microarray, whether it be DNA, RNA, peptide, or carbohydrate. While working on combinatorial drug synthesis and high-throughput chip-based screening in the late 1980's, Fodor and co-workers at Affymax realized that one could create massively parallel arrays on a two-dimensional surface by employing semiconductor lithography techniques to drive photochemical reactions, namely the cleavage of photocleavable protecting groups. In a tour-de-force that made the giant leap to spot densities that we see today, they created the world's first microarray and forebear to in-situ synthesized DNA microarrays, a peptide microarray, in 1991. It was only natural that in-situ DNA synthesis would follow suit, which they accomplished in 1994 after creating the Affymax spin-off, Affymetrix (Figure 7).

To date, there exists a myriad of microarray technologies (summarized in Table 1 at the end of this chapter and a review by Gao and co-workers), each with their own benefits and shortcomings with respect to resolution, throughput, cost, or accessibility. The technologies can be informally divided into three categories that refer to the manner in which spatial selectivity is achieved during the addition or deprotection step: 1) light addressable, 2) electronically addressable, and 3) physically addressable reactions.

Light addressable reactions

The first class of approaches utilizes spatially modulated light to drive photochemical reactions. As previously mentioned, optical lithography techniques have been adopted from the semiconductor fabrication industry by Affymetrix to remove UV-photocleavable protecting groups by shining 365 nm light through a photomask. This system is parallel, high-resolution, and well-suited for production-scale manufacturing of standardized screening and expression-profiling arrays. However, this approach is incapable of producing customizable arrays because a new mask set must be created for each new array. With high-resolution photolithography masks costing ~$400 for a 4"
Micro-electromechanical devices capable of projecting "virtual masks," such as the digital micromirror devices (DMD) by Texas Instruments or zone-plates by Silicon Light Machines, are interesting alternatives to lithographic masks. The DMD (Figure 8) is a programmable array (1024 x 768, 0.7" diagonal) of 13.68 μm micro-electromechanical mirrors capable of swiveling ±10-12°. In the "on" state, light can be deflected towards a lens train and projected outwards, and in the "off" state, light is deflected away from the collection optics (typically toward a light absorber). Gray scaling is achieved by modulating the time duration the mirror is in the "on" state. The most common use of DMD's is by far in digital light projectors (DLP).

![Figure 8. (a) Principle of operation and (b) scanning electron micrograph of the digital micromirror device. Images from www.dlp.com.](image)

A more versatile platform for driving photocleavage reactions known as the "maskless array synthesizer," developed at the University of Wisconsin and commercialized by Nimblegen, utilizes a DMD as its spatially modulated light source that can be easily reconfigured to produce any customizable high-resolution microarray.
Researchers at University of Texas Southwestern also implemented this platform in conjunction with Texas Instruments, calling it the "digital optical chemistry" platform. A microarray synthesizer based on this technology has been commercialized for sale by Febit, but the system is ~$250k and the consumable costs per experiment are ~$1500 (personal communication with Febit).

Unfortunately, there are a variety of drawbacks to photocleavable chemistry. Since direct photocleavage reactions are linear with illumination intensity, any inhomogeneous optical profile will lead to a similar profile in deprotection completeness. The potential for photo-induced damage is high. The novel photocleavable reagents cost ~20X more than standard phosphoramidite reagents alone, which affects the cost per base synthesized for commercial microarray suppliers. Additionally, high-power UV-illumination sources and optics are expensive and more difficult to work with than visible wavelength optics.

Researchers at the University of Houston also employ an ultraviolet DMD to illuminate semiconductor photo-acids that deprotect standard acid-labile dimethoxytrityl-protecting groups. The technology has been commercialized by Xeotron and Atactic. This approach has major benefits in that the reagent costs are very low, and to date offers some of the best gene synthesis yields from commercial microarrays (personal communication with Peter Carr, MIT). However, because the photogenerated acids easily diffuse between spots, the platform requires the use of micro-fabricated glass structures with 200 x 90 x 15 μm wells (L x W x H) that currently limits the commercial array to 3698 spots. On the hand, the large amount of DNA synthesized per microwell has enable Gao and co-workers to synthesize the green fluorescent protein gene without PCR amplification prior to assembly.
Electronically addressable reactions

The second class of approaches involves the use of micro-fabricated devices to create individually addressable electrodes to drive electrochemical reactions. Combimatrix has developed a CMOS chip that electrochemically generate acids from the oxidation of hydroquinone to deprotect standard phosphoramidite reagents (Figure 9).25, 39 The resolution of such chips is potentially very high, depending on the fabrication design rules used to create the substrate. The synthesizer is also commercially available, but at similar costs to the Febit system. The substrate is also extremely expensive because programmable CMOS architectures typically require ∼30-35 high-resolution mask steps to fabricate them.

![Figure 9. (a) Principle of electrochemical acid generation used in a (b) Combimatrix chip. Images from Maurer, et al. 25 and www.combimatrix.com.](image)

Edwin Southern at the University of Oxford developed a similar scheme where the acids are electrochemically generated on a chip that is in close proximity to the substrate, rather than on the microarray itself.26 This has the cost benefit of switching the microfabricated chip from a consumable item to capital equipment, where one expensive chip can create large amounts of microarrays. However, the resolution is low
because of poorly controlled proton diffusion, and in fact, a true microarray has never been demonstrated with this technology.

**Physically addressable reactions**

The third class of technologies relies on physical spatially selective fluid delivery, much like the previously mentioned robotic arm-based system created by Brennan and co-workers. The significant advantage to these approaches is that it is easier to make the spatially selective reactions the phosphoramidite addition step rather than a deprotection step. This potentially lowers the reagent usage and synthesis time by 75%, thereby limiting the chemical exposure and step-wise engineering errors. The simplest technology, ink jetting / spotting is an extremely flexible platform that can easily be constructed at poor resolutions. An open-source ink-jet platform has been created by Hood and co-workers at the University of Washington. High-resolution ink jetting is certainly possible, but in order to compete with the resolution achievable by optical technologies, the use of complex chemistries (surfactants, solvent mixtures, etc.) that could hurt the synthesis yield may be required. At this time 65 μm spot sizes are the standard for commercial ink jet microarrays by Agilent.

Using robotics and high-resolution spotting pins, Brown and co-workers at Stanford University covalently immobilized cDNA libraries onto microscope slides in 1995. Today, there are many commercial manufacturers of these systems capable of easily achieving 150 μm spot sizes. Because they are designed to immobilize pre-synthesized DNA supplied in 96- or 384-well plates, this is not a cost-effective strategy for generating arrays of high complexity. However, it remains a reasonable option for generating cDNA microarrays because they are not in-situ synthesized.

Micro-contact printing, essentially a miniature stamping process, has been demonstrated by Lu and co-workers. This approach is parallel and potentially high-
resolution, but it is not a flexible platform for customizable arrays because of the need to
generate new stamps for each new array design. Furthermore, there are significant
technical challenges in stamping uniformly over large areas.

Most recently, microfluidic devices that miniaturize the entire DNA synthesizer
onto the chip have been demonstrated by Southern and co-workers,\textsuperscript{42} as well as Quake
and co-workers.\textsuperscript{43} This system is not suitable to be a standalone microarray platform
because no reasonable architecture exists that will allow for multi-plexing hundreds of
thousands of reaction chambers. However, a microarray synthesis platform with
integrated on-chip microfluidic valves could prove to be very powerful for three reasons.
First, it allows various synthesis cycle parameters to be altered in parallel (e.g. one
channel with capping and the other without capping) for side-by-side quality comparison
on one chip. Second, the chip can be divided into individually addressable gene
synthesis reactors that limit cross talk between complex pools. Third, it eliminates the
need to align and bond another microfluidic device onto a microarray.

1.3.3 Evaluation of technologies

Critical parameters

In evaluating the merits of each technology, the following parameters are critical:

- **Programmability / flexibility** – This is most relevant to custom microarrays, not
  standardized screening chips. One would like the ability to change the
  information content between substrates quite easily.

- **Hardware costs and difficulty of implementation**

- **Consumable costs including reagents and the physical substrate**

- **Spot size / resolution** – This is critical for screening applications because more
  information per microscope field-of-view can be obtained with smaller spots. It is
questionable whether applications using cleaved DNA benefit significantly by decreasing the spot size because less DNA of each sequence will be synthesized. At this moment, the microarray pool complexity is not limiting.

- **Quantitative and qualitative yield** – How much DNA with the correct sequence is synthesized per unit chip area?

It has become evident recently that the last parameter, “yield,” is an ill-defined term and requires refinement that puts it in terms of applications. For example, yield for general DNA use is determined by length since truncation errors are the dominant ones. On the other hand, yield for gene synthesis is assayed by sequencing data after assembly. By such a measure, truncated strands are barely a concern for gene synthesis because they contain less information, rather than introducing wrong information. Yield must also be viewed in terms of cost. An expensive procedure such as double coupling to limit truncation and deletion errors may not make sense for a general DNA manufacturer, but could be justified for one specializing in making DNA for gene synthesis. Cerrina and co-workers\textsuperscript{9} demonstrated the impact of such alterations that illustrate the complexity of DNA synthesis. Likewise, members of our own research group are also examining this issue (Carr \textit{et al.}, “Practical Gene Synthesis,” manuscript in preparation).

\textit{Analysis of photoelectrochemical synthesis}

For high-resolution, programmable, and low-cost microarrays, the use of a DMD to achieve a spatially selective reaction appears to be the most logical choice. Optical techniques have better resolution limits than ink-jet. The hardware costs of optical systems drop significantly if the illumination is at visible wavelengths because of the high cost of ultraviolet illumination sources and optics. The use of standard phosphoramidite reagents over novel photocleavable ones represents a dramatic decrease in consumable cost by a factor of nearly twenty. It is not entirely clear how electrochemical
systems scale in terms of resolution, but their CMOS chip costs are non-trivial. If the substrate requires any microfabrication, it is desirable that the complexity be minimal to limit the consumable cost of the substrate.

Synthesis by photoelectrochemistry theoretically combines many of these features mentioned above. The system is potentially high resolution because it is optically driven. The hardware and consumable costs are low because the system utilizes visible wavelength optics and standard reagents, instead of their ultraviolet counterparts; the photoconductor is cheaply microfabricated and reusable (a cost analysis of the hardware and consumables is provided in Appendix A). Furthermore, the thin-film porous matrix employed vastly increases the surface area of the chip, thereby increasing the amount of DNA synthesized per chip. If one requires a fixed amount of DNA of a particular sequence, this film decreases the spot redundancy required to achieve that amount and allows more unique sequences to be synthesized per chip, thereby reducing the cost per base.

1.4 Structure of the thesis

Chapter 2 will begin with background information on the behavior of semiconductor electrodes at the semiconductor-electrolyte interface (SEI). Next, the design, fabrication, and characterization of the amorphous silicon photoconductor are discussed in the context of PEC microarray synthesis (rather than traditional solid-state device behavior). Conclusions are empirically drawn about what physical semiconductor properties are required for successful PEC microarray synthesis.

Chapter 3 begins with a discussion of the design choices for the other components of the PEC platform: the composition of the deprotection solution, the fluidic cell for reagent delivery, and the spatially modulated light source. PEC analysis then verifies the proposed gain mechanisms (Figure 4 of this chapter). Next, experimental
results on site-selective detritylation using PEC-generated acid are shown, including the optimization of the process, control experiments, and proof of optical non-linearity with respect to illumination profile. Finally, PEC microarray synthesis is demonstrated by hybridization assays.

Chapter 4 covers the formation mechanism and characterization (thickness, loading density, physiochemical stability, optical transmission, and electrochemical properties) of the thin-film porous glass that coats the amorphous silicon photoconductor. It will be shown that the anisotropic geometry of the colloidal precursors contributes to many of the useful properties of the film.

Chapter 5 discusses many new surface science technologies that were developed during the course of this thesis work. These technologies all seek to improve the current methods used in biomedical and nanotechnology applications on surfaces (microarrays, microfluidics, hybrid bio-nanoelectronics, etc.). The technological advances include reliable silanization procedures for chemically modifying glass, silicon, and indium tin oxide, as well as new routes to patterning biomolecules at all length scales.

Chapter 6 contains concluding remarks, suggestions for future improvements to the synthesis platform, and proposals for future research directions.
## Table 1: Summary of DNA Microarray Technologies.

<table>
<thead>
<tr>
<th>Class</th>
<th>Affiliation</th>
<th>Technology</th>
<th>Resolution</th>
<th>Cost</th>
<th>Throughput/ Flexibility</th>
<th>Other factors / issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light addressable</td>
<td>Affymetrix</td>
<td>UV photocleavage using lithographic masks</td>
<td>11 μm</td>
<td>Expensive chemistry; High capital (masks and aligners)</td>
<td>Massively parallel; Low flexibility</td>
<td>UV-induced photodamage; Ideal for production, not custom scale</td>
</tr>
<tr>
<td>Light addressable</td>
<td>Affymetrix</td>
<td>UV photolithography for physical masking</td>
<td>11 μm</td>
<td>Cheap chemistry; High capital (masks and aligners)</td>
<td>Massively parallel; Low flexibility</td>
<td>Photoresist reactivity with DNA; Ideal for production, not custom scale</td>
</tr>
<tr>
<td>Light addressable</td>
<td>Nimblegen; Febit; Cerina, et al.; Garner, et al.</td>
<td>UV photocleavage using DMD</td>
<td>13.68 μm</td>
<td>Expensive chemistry; Average capital (DMD and illumination)</td>
<td>Parallel; Flexible</td>
<td>UV-induced photodamage; Febit system available in U.S.; Optically linear with beam intensity and profile</td>
</tr>
<tr>
<td>Light addressable</td>
<td>Atactic; Xeotron; Gao, et al.</td>
<td>Photoacid generation using DMD and microfabricated structures</td>
<td>200 x 90 μm</td>
<td>Cheap chemistry; Average capital (DMD and illumination); Average microfabrication costs</td>
<td>Parallel; Flexible</td>
<td>Diffusion issues; Gene synthesis without amplification demonstrated</td>
</tr>
<tr>
<td>Electronically addressable</td>
<td>Combitmatrix</td>
<td>Microfabricated electrodes to generate local acids</td>
<td>44 μm</td>
<td>Cheap chemistry; Expensive CMOS structures</td>
<td>Parallel; Flexible</td>
<td>Electrochemical damage; System available in U.S.</td>
</tr>
<tr>
<td>Electronically addressable</td>
<td>Southern, et al.</td>
<td>Microfabricated electrodes to generate acids proximal to separate substrate</td>
<td>TBD</td>
<td>Cheap chemistry; Reusable microfabricated structures</td>
<td>TBD</td>
<td>Actual microarray has not yet been demonstrated; Diffusion issues</td>
</tr>
<tr>
<td>Physically addressable</td>
<td>Agilent; Metrigen; Hood, et al.</td>
<td>Ink-jet during addition step</td>
<td>65-150 μm</td>
<td>Cheap chemistry</td>
<td>Fast serial; Flexible</td>
<td>Least chemical steps and simplest chemistry; Fundamentally lower resolution than other systems. Open source version available.</td>
</tr>
<tr>
<td>Physically addressable</td>
<td>Quake, et al.; Southern, et al.</td>
<td>Elastomer-based microfluidics</td>
<td>N/A</td>
<td>Cheap chemistry; Cheap capital</td>
<td>N/A</td>
<td>Not suited for standalone microarrays; Compatible with other microarrays</td>
</tr>
<tr>
<td>Physically addressable</td>
<td>Lu, et al.</td>
<td>Micro-contact printing during addition step</td>
<td>90 μm</td>
<td>Cheap chemistry; high capital (masks, aligners)</td>
<td>Parallel; Low flexibility</td>
<td>Difficult to maintain uniform contact over large areas</td>
</tr>
<tr>
<td>Physically addressable</td>
<td>Brown et al.</td>
<td>Spotted cDNA libraries</td>
<td>150 μm</td>
<td>Extremely expensive for de novo libraries</td>
<td>Multiplexed serial; Flexible</td>
<td>Not viable for de novo libraries; Designed for cDNA microarrays using 96 or 384-well plate formats</td>
</tr>
</tbody>
</table>

TBD - "to be determined." N/A - "not applicable"
1.5 References


Chapter 2

Substrate fabrication and analysis

This chapter discusses the design, fabrication, and evaluation of suitable substrates for the photoelectrochemical generation of acids and bases that can cleave common protecting groups employed in biomolecule microarray synthesis. Since the primary goal of this work was to synthesize DNA microarrays utilizing standard DMT-protected phosphoramidite reagents, the PEC generation of acids at positive potentials is of main interest. Several desired traits contribute to photoconductor design, such as low fabrication cost, high PEC contrast ratio, and low impedance (i.e. capable of sinking large current) to limit deprotection times. A simple amorphous-silicon Schottky-diode was ultimately chosen with such considerations in mind.

2.1 Theoretical considerations for photoelectrochemistry (PEC)

The points of focus in this discussion of semiconductor physics and photoelectrochemistry (PEC) will vary largely from those in primary areas of PEC research like fuel cells. It is geared towards the particular goal of DNA microarray synthesis, and is not meant to be a general overview of Schottky diodes, semiconductor device physics, or PEC. It is assumed that the reader is familiar with basic principles of semiconductor physics and electrochemistry, but detailed background information on Schottky diodes,\(^1\) properties of amorphous silicon,\(^2\)\(^-\)\(^4\) electrochemistry,\(^5\)\(^-\)\(^6\) and photoelectrochemistry\(^7\)\(^-\)\(^8\) can be found in the cited references [1-8].

Some terminology must be first clarified in order to disambiguate the polarity of the applied bias (Figure 1). In typical semiconductor diode analysis, an ohmic back contact (usually indium tin oxide or NiChrome) is considered ground and a top metal
contact is considered the applied bias. The polarity or forward/reverse bias orientation is then defined with respect to the doping of the semiconductor (e.g. an n-type semiconductor is forward biased when a positive voltage is applied to the top metal). Likewise, “anodic” and “cathodic” are also defined with respect to this orientation.

![Diode measurements](image1)

![PEC synthesis](image2)

(C) Electrochemical measurements

Figure 1. Electrode orientation for (a) solid-state diode analysis, (b) two-electrode fluid cell for photoelectrochemical synthesis, and (c) three-electrode fluid cell for electrochemical analysis.

On the other hand, the measurement orientation in electrochemistry is the opposite of the orientation in semiconductor diode analyses. In two-electrode measurements and electrochemical synthesis, the electrode at which the desired redox reaction occurs is biased and the ground electrode resides on the other side of the solution. The situation is further complicated in three-electrode measurements because the working electrode is held at floating ground despite being considered the applied bias electrode (see Chapter 3.2.4 for a more detailed explanation). The terms “forward” and “reverse” currents refer to voltage sweep direction, not the bias of the electrodes.

Therefore, from this point on in this work, the terms “forward/reverse bias and current” and “positive/negative photopotentials” will refer to n-type solid-state diode measurements (Figure 1A); the undoped a-Si substrate is assumed to be slightly n-type.
because defects and dangling bonds tend to be electron pairs rather than holes.\(^4\) The terms “anodic/cathodic current or bias” and “surface potential” will refer to electrochemistry.

2.1.1 Band-edge pinned semiconductor-electrolyte interfaces

The description of an ideal semiconductor-electrolyte interface (SEI) is based on a solid-state Schottky-diode (Figure 2), where the highly conductive electrolyte acts as a soft metal contact, and the Fermi level \((E_F)\) of the redox pair is analogous to the metal work function in determining the semiconductor band bending. This scenario is known as “band-edge pinning” because the energy levels at the interface are pinned and adjusting the bias potential only affects the barrier height. For the combination of n-type semiconductors and most organic redox pairs, the band bending is upwards as in a rectifying Schottky diode.

Two things occur when a semiconductor is illuminated with light whose energy is above its bandgap energy \((E_g)\). First, electron-hole pairs are photo-generated by kicking up electrons from the valence band \((E_{VB})\) to the conduction band \((E_{CB})\), leaving excess holes in the valence band. These carriers can participate in electrochemical reactions at the SEI and increase the current that passes through the cell. It is analogous to irradiating a solid-state photoconductor. Second, an internal electric field is created to oppose the migration of the charge carriers from the bulk to the surface, which is the photopotential \((V_{ph})\).\(^7\) Since the photopotential always moves towards the flatband direction of the SEI,\(^7, 9, 10\) it is positive for band-edge pinned n-type semiconductors because the bands bend upwards. For PEC photovoltaics, \(V_{ph}\) is the maximum potential energy the circuit can create when the terminals are connected, and the maximum \(V_{ph}\) is the barrier height given the Fermi levels of the semiconductor and electrolyte.
Three situations for a n-type semiconductor-electrolyte interface at equilibrium (a), under reverse bias (b), and under forward bias (c). The size of the arrows denotes the magnitudes of the current in the two (i.e. anodic and cathodic) directions.

Figure 2. Band diagrams for band-edge pinned, n-type PEC cells, based on Schottky diode models. (a) The bands bend upwards when the semiconductor comes into equilibrium with the electrolyte. (b) The current flow under different bias conditions in a PEC cell. Images taken from Bard.

Figure 3. The potential profile of the semiconductor-electrolyte interface for band-edge pinned semiconductors with very low surface state densities. It is typically assumed that electrolyte concentrations are sufficiently large to ignore the Gouy layer of the electric double layer. Image taken from Bard.

A typical metal-electrolyte interface is described as two series capacitors (Figure 3) composed of the metal and the Helmholtz electrolyte layer, where nearly the entirety of the potential drop occurs across the latter. Unlike in metals though, the space-charge regions of band-edge pinned semiconductors are large, typically 100-1000 nm,
and the surface charge density is low. The semiconductor capacitance is much lower than the Helmholtz capacitance, and thus, most of the drop occurs across the semiconductor, as given by Equation 1:

\[
\frac{\delta \Delta \phi_H}{\delta \Delta \phi_{SC}} = \frac{d_H \varepsilon_{SC}}{L_{D,SC} \varepsilon_{it}}
\]  

where, \( \Phi_H \) = drop across the Helmholtz layer, \( \Phi_{SC} \) = drop across space charge layer, \( d_H \) = Helmholtz distance, \( L_{D,SC} \) = debye length of space charge layer, \( \varepsilon_{it} \) = dielectric of Helmholtz layer, and \( \varepsilon_{SC} \) = dielectric of space charge layer. Equation 1 does not account for the photopotential. In a situation where there is an applied bias from the backside, the surface potential \( V_{surface} \) is the applied potential \( V_{applied} \) minus the drop across the bulk \( V_{bulk} \) plus the photopotential \( V_{ph} \), as shown in Equation 2.\(^7\) The potential distribution of the band-edge pinned SEI is schematically shown in Figure 3. It will be assumed that the electrolyte concentration is sufficiently large so that the Guoy layer of the electric double-layer can be ignored.\(^8\)

\[
V_{surface} = V_{applied} - V_{bulk} - V_{ph}
\]  

Band-edged pinned semiconductors are typically the most desirable because most PEC research is geared towards renewable energy. PEC cells offer an interesting alternative to photovoltaic cells because not only is the light energy converted to electrical energy, but also chemical energy. The redox molecules can be used as batteries to store the energy or create other useful molecules, most notably oxygen and hydrogen gas from water that can be used in fuel cells. However, for our purposes, band-edge pinned semiconductors are undesirable because the sensitivity to the Fermi level of the redox species greatly limits the possible combinations of semiconductors and
redox pairs. Furthermore, large biases must be applied in order to reach the desired surface potentials because most of the drop occurs across the semiconductor. If a pinhole is present anywhere in the cell, such high potentials at the exposed surface may lead to rapid chemical decomposition of the solution.

2.1.2 Fermi-pinned semiconductor electrolyte interfaces

An alternative semiconductor electrode behavior exists, in which the band-edges are unpinned; this case is known as "Fermi pinning." As shown in Figure 4, the band bending of a Fermi-pinned semiconductor is fixed at the SEI, regardless of the Fermi level of the redox species. This is in contrast to ideal semiconductors, where different redox Fermi levels adjust the band bending because the edges are pinned. The band-edge can be unpinned by the presence of surface defect states, large illumination intensities, or high doping concentrations because the surface charge density is extremely high (much like a metal) and/or the bulk semiconductor capacitance becomes comparable to the Helmholtz layer capacitance, either of which would cause a redistribution of voltage drops across the cell.\textsuperscript{7, 8, 10}

![Figure 4. Band-edge pinning versus Fermi pinning. (a) When the edges are pinned, the band bending $E_v$ changes with the Fermi level of the electrolyte. (b) When the edges are unpinned or the semiconductor is Fermi pinned, $E_v$ is fixed and the edges move relative to the Fermi level of the redox species. Image modified from Bard.\textsuperscript{11}](image-url)
Figure 5A shows a lumped circuit model of the SEI in presence of surface states. Based on this model, one can see how the relative potential drops vary with surface state density (Figure 5B) in the dark when one assumes the bulk capacitance (denoted as \( C_{sc} \) for "space charge") is relatively fixed. It can also be seen how large illumination intensities result in the redistribution of potential drops. Equation 3 gives the potential distribution in a Fermi-pinned SEI as a function of the surface state density (\( Q_{ss} \) or \( \sigma_{ss} \)). It is typically assumed that this equation holds true as \( Q_{ss} \) approaches 1E12 /cm\(^2\).

\[
\frac{\delta \Delta \phi_H}{\delta \Delta \phi_{sc}} = \frac{ed_H \sigma_{ss}}{kT \varepsilon_H} \tag{3}
\]

For PEC photovoltaics, Fermi-pinning is undesirable because it mitigates the impact of the Fermi level of redox species, but unpinning has benefits for this work because the substrate immediately becomes more versatile by allowing a variety of redox pairs to be used. In effect, Fermi pinning causes the semiconductor to behave like a metal in that the potential drops across the Helmholtz layer and the band structure is unaffected by the redox pair, and hence is sometimes called quasi-metallization. While this term gives a sense of the behavior of a Fermi-pinned semiconductor, it is somewhat of a misnomer.

Quasi-metallization is a Fermi-pinned condition that refers to degenerate semiconductors (Figure 5C) whose doping level is so high that its Fermi level overlaps with the valence or conduction for p-type and n-type semiconductors, respectively. Quasi-metallization on account of extremely large surface state densities is cause for major concern. At extremely large densities, the bands bend downwards towards the sub-gap Fermi level of the defects (Figure 5C), thereby lessening the positive \( V_{ph} \) that is desirable for anodic reactions at n-type anodes, as well as creating an accumulation region at the SEI. Furthermore, surface states decrease the photosensitivity due to their
ability to trap charges. As will be discussed throughout Chapters 2 and 3, the surface state density critically affects PEC synthesis.

![Diagram of equivalent lumped circuit model of a SEI in the presence of significant surface states.](A) Potential distribution between the space charge and Helmholtz layers of a SEI as a function of surface state density. (C) Band level diagrams of a semiconductor in its flat state and in a degenerate Fermi-pinned state. In the latter state, the n-type semiconductor bands bend downwards, creating a negative potential and accumulation region at the SEI. Images from Sato.

**Figure 5.** (a) Equivalent lumped circuit model of a SEI in the presence of significant surface states. (b) Potential distribution between the space charge and Helmholtz layers of a SEI as a function of surface state density. (c) Band level diagrams of a semiconductor in its flat state and in a degenerate Fermi-pinned state. In the latter state, the n-type semiconductor bands bend downwards, creating a negative potential and accumulation region at the SEI. Images from Sato.

The above pinning scenarios represent the two extremes of semiconductor electrode behavior. In reality, few real semiconductors are completely band-edge- or Fermi-pinned. For example, a n-type quasi-metal with downward bending bands due to high surface state densities can still be made rectifying with a large work function metal contact that forces the bands to bend upwards, as will be shown in this chapter.

### 2.2 Initial substrate design choices

Several semiconductors are commonly used in photoelectrochemistry as shown in Figure 6. Amorphous silicon (a-Si) was deemed a good choice because its narrow bandgap (1.7 eV ≈ 730 nm) meant visible light could photogenerate electron-hole pairs with relatively high efficiency. Furthermore, it can be deposited by PECVD (plasma enhanced chemical vapor deposition, also known as glow-discharge), which offers a wider film thickness range than electron beam evaporation and higher quality films than sputtering, which are methods required to deposit other materials. In order to limit
radiation-induced damage to the growing DNA strands, a backside illumination scheme was desirable, but it necessitated that a thin photoconductive film be deposited on a transparent conductor. Early synthesis attempts with doped silicon wafers failed due to insufficient illumination power to penetrate the whole substrate.

![Vacuum MrIC GeP GAs AE-1.301-]

Figure 6. Band positions of several semiconductors in contact with aqueous electrolytes. The conduction and valence band energies are in red and green, respectively. On the right are the standard potentials of several redox couples. Image taken from Gratzel.¹²

The Schottky diode design (Figure 7), which in its simplest form is merely a semiconductor in contact with a metal with a different Fermi level, was chosen for two reasons. First, it was the easiest structure to fabricate, and second, placing a metal in between the a-Si and electrolyte prevented a-Si oxidation and corrosion. In terms of the material selection for the metal exposed to solution, most common metals used in electrochemistry were ruled out for various reasons. Gold readily inter-diffuses with amorphous silicon, even at room temperature.¹³ Nickel induces crystallization and causes spiking.¹⁴ Silver oxidizes at the annealing temperatures required in the fabrication process. Platinum was ultimately chosen because of its chemical inertness.

The substrate (Figure 7) consists of a transparent ITO rear contact, PECVD deposited a-Si thin-film, and e-beam deposited Ti/Pt electrodes. Since platinum is inert and a-Si can easily be oxidized, the background areas between the pads can be
rendered non-conductive by a self-aligned oxidation process (oxygen plasma treatment and/or thermal annealing in air).\textsuperscript{15} Therefore, the substrate can be microfabricated in a single, unaligned mask step, making it very cheap to produce. The metal must be patterned because otherwise the photogenerated charge carriers would spread laterally throughout the entire metal film, and thus, all spatial selectivity would be lost; as will be discussed in Chapter 3.4, this spreading had the unanticipated benefit of making the spatial deprotection uniformity optically non-linear with respect to illumination profile. Coating the substrate uniformly with an ultra-thin titanium dioxide film was also considered for oxidation resistance. Such a film would negligibly contribute to the overall conductivity of the system and barely spread charge carriers laterally.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{The structure of the amorphous silicon photoconductor / non-rectifying Schottky diode after photoresist lift-off, porous glass film formation, and annealing. Exposed a-Si areas are thermally oxidized during the annealing step and rendered 1000-fold less conductive. (b) Optical micrograph of a substrate without the porous glass (scale bar = 400 \textmu m)}
\end{figure}

A thin-film porous glass coats the entire substrate after substrate fabrication. A porous matrix on top of the electrode is required in order to prevent electrochemical damage to the growing DNA strand. Such a layer keeps the DNA in proximity to the electrode, but spaces the majority of the DNA further than the Helmholtz distance from it. As will be shown in Chapter 4, this matrix also provides a high-loading CPG-like support that vastly increases the amount of DNA synthesized per chip.
2.3 Substrate fabrication and testing details

Different a-Si films were fabricated and tested that varied in their thickness and PECVD conditions, including the outsourcing of the film deposition. The most successful films for PEC synthesis were deposited at the Exploratory Materials Laboratory at MIT (EML) as described immediately below, and will be called "EML substrates" from here on. Substrates with outsourced PECVD-deposited films will be called "Noel substrates" (a-Si deposited by Noel Technologies).

2.3.1 Fabrication of EML substrates

Indium tin oxide-coated (ITO) glass (25 mm x 50 mm x 0.7 mm, 8-12 Ω/sq, Delta Technologies) was cleaned by ultra-sonication in 20% ethanolamine (based on the manufacturer's specifications), rinsing with water and isopropanol, and drying with nitrogen. In some cases, this procedure was preceded by ultra-sonication in hexanes and acetone, which removed most of the visible contaminants on the slides as received. Undoped amorphous silicon was PECVD deposited at 250 °C (Plasmatherm 740 RIE/PECVD); detailed conditions can be found in Appendix B. The film thickness within each PECVD run had ~10% standard deviation that was largely dependent on the location within the chamber, as measured with a Sloan Dektak III profilometer (Appendix B). EML1 and EML2 substrates had a-Si film thicknesses of 600 nm and 1 μm, respectively. These were deposited under the exact same conditions. EML3 substrates also had 1 μm thick films, but deposited under slightly different conditions. A pre-cleaned microscope coverslip (VWR) was used as a shadow mask at the edge of the slide to facilitate electrical contact to the ITO backside contact. Pinhole formation was unavoidable, presumably because of contamination in the chamber or residual particulates left after the solvent cleaning steps.
Metals pads were defined by contact lithography using either Futurrex NR9 (EML1 and EML2) or AZ Electronic Materials 4620 (EML3) photoresists. Transparency masks were obtained from PageWorks and Photoplotstore. Platinum layers (50-100 nm thick) with 10 nm-thick Ti adhesion layers were e-beam evaporated with a Sloan PAK evaporator, and then lifted off with acetone and/or NMP. Specific photoresist processing and e-beam deposition parameters can be found in Appendix B. These conditions were not found to be critical, although the NR9 photoresist did lift-off more easily. AZ4620 hardened more during metal deposition, thus requiring more aggressive lift-off conditions (typically long sonications) that could cause a-Si delamination.

The porous glass was spun coat from colloidal silica (see Chapter 4 for full details and characterization), and the substrate was annealed at 200 °C for one hour, 400 °C for 30 minutes, and then slowly cooled to room temperature. This annealing oxidizes the exposed regions in a self-aligned manner that allows for the substrate to be fabricated with one unaligned mask step, and was performed on all substrates regardless of the presence of the porous glass. In some cases, substrates were oxygen plasma cleaned for 10 minutes (Anatech, 50 sccm, 50 W) to oxidize the exposed regions and improve surface wetting prior to the porous film deposition, but this step was not necessary.

Typically, the porous glass did not coat the slides at their edges, which is common when spin-coating viscous solutions. If bare ITO was exposed, copper tape was used to soften the contact of an electrical alligator clip that can scratch the ITO film. When the porous oxide left no ITO exposed, silver paste (Acheson Colloids) was applied to the side of the slide, hardened at 150 °C for 10 minutes, and then covered with copper tape. It was surprising that the colloids could make sufficient contact to the thin ITO film between the glass and a-Si film, but the silver bus added only ~35 Ω resistance.
However, one downside to the silver bus is that the substrate is not always level when imaging.

2.3.2 Fabrication of Noel substrates

Since EML substrates were plagued by pinhole formation and a-Si delamination without slow cooling under vacuum after PECVD deposition, it was thought that improved fabrication yields were achievable if the films were formed on borofloat glass, whose thermal expansion coefficient better matches amorphous silicon’s than normal float glass, and if the PECVD step was outsourced to a third party. To that end, 1μm thick films were PECVD deposited at 250 °C by Noel Technologies on top of ITO-coated borofloat wafers (25 Ω/sq deposited by Evaporated Coatings), and diced after metal lift-off. The vendors provided pinhole free films over the entirety of the wafer, but the films were hazy, which is indicative of stressed films. Selective detritylation was eventually demonstrated (Appendix D), but the result was poor and largely unrepeatable. Noel substrates were deemed unsuitable for PEC synthesis.

2.3.3 Custom-built probe station

Diode measurements were made with a Hewlett Packard 4516A semiconductor analyzer and a custom-built probe station, since commercial probe stations cannot accommodate rear illumination schemes because the substrate backside is a wafer chuck. Both the substrate and tungsten probes (1.2 μm diameter with micro-positioner, American Probes & Technologies) could be adjusted in all axes (x, y, and z). The fiber illumination source (Leeds EKE) was coupled to a f = 25 mm lens (Edmund Optics) and provided up to 1 W/cm² white light as measured by a photodiode (Melles Griot FDS1010) or power meter (Molelectron Power Max 1500). The intensity was adjusted with neutral density filters (Melles Griot) and the control knob of the fiber source. The
spectral output was adjusted with dichroic filters from Edmund Optics (red long pass, yellow long pass, and blue short pass).

A Panasonic GP-KR222 CCD camera was coupled to a 5X Mitutoyo microscope air objective for image alignment. The imaging is in transmission mode so that the backside illumination that excites the photoconductor also serves as the light source for the camera imaging. Figure 8 shows a screenshot of a 200 μm pad being probed. The metal pads appear dark because they are mirrors that reflect the light that passes through the substrate from the backside.

Figure 8. A captured image of a 200 μm pad being probed using the custom-built probe station. The metal pads appear dark because they reflect the backside illumination, and the imaging is in transmission mode.

2.4  Diode analysis of EML substrates

The PEC behavior of the various substrates differed greatly, with EML1 and EML3 substrates being the only reliable ones (see also Chapters 2.6 and 3.4). Diode analysis was performed to understand the general characteristics of the substrate (e.g. photoconductive gain, wavelength selectivity, Fermi- vs. band edge-pinning, etc.), in hopes of explaining why certain substrates succeeded and others failed.

2.4.1  Photosensitivity

Figure 9A shows a current-voltage trace (I-V) of the 1 μm thick EML3 substrates, measured on a 125 μm Ti/Pt pad with the custom-built probe station. Its shape is what one would expect of a poor n-type Schottky diode in that at large reverse biases, the
slop increases because of Schottky barrier lowering. Undoped amorphous silicon films tend to exhibit n-type behavior because unhydrogenated sites are dangling electrons rather than holes. The films were rather resilient against “light-soaking” where prolonged high-power illumination induces Wronski-Staebler defect states\(^\text{16}\) that can diminish the contrast ratio by charge trapping, as well as increase the overall conductivity by acting like n-type dopants. Spectrally selective measurements made by using dichroic filters showed that longer wavelengths contributed most to the photoconductivity, presumably because of the short penetration depth and increased thermal ionization associated with short wavelengths (blue short pass filter).

Figure 9. (a) Current voltage trace of an EML3 substrate, measured at 500 mW/cm\(^2\) on a 125 \(\mu\)m pad. The shape is of a poor Schottky diode with non-rectifying contacts, where the forward current is larger than the reverse current, and barrier lowering occurs as the reverse bias increases. (b) Photosensitivity curves of all films shown were made on substrates with Ti/Pt pads. Values are the average over a –2 V to 2 V range, and the forward : reverse current ratio is in parenthesis.

Figure 9B shows photosensitivity curves of both EML and Noel substrates. Forty measurements were made from 0.1 mW/cm\(^2\) to 1 W/cm\(^2\), except between 1-10 mW/cm\(^2\) because of the absence of an appropriate neutral density filter at the time. The systematic curvature per decade results from non-linear control of lamp intensity with the
knob, or in other words, the knob markings did not correspond exactly to the percentage output power. The resistivities are the average values over a –2 V to +2 V range, which are only approximations because the I-V traces have different slope regimes. The approximation sufficed for the purpose of gaining some understanding of the diode behavior, and the ratio of the magnitudes of the forward and reverse currents was generally fixed for each substrate. However, the ratios, which are indicated in parentheses in the graph key, varied between substrates from 1.1 (EML3) to 12.1 (EML2). Substrates with poor reverse currents (i.e. EML2) failed as suitable substrates for PEC synthesis (see Chapter 2.5.1). Measurements on substrates that were thermally oxidized prior to metal deposition were on average ~290-fold less conductive.

For Noel substrates, thicker films exhibited higher photosensitivity (larger slope) but lower overall conductivity. These trends are indicative of large surface state densities. The defect states act as n-type dopants and charge traps. Assuming a fixed thickness of the surface state region between Noel substrates, the defects contribute more to the overall behavior of the thinner films because there is relatively less defect-free bulk semiconductor. 500 nm and 750 nm thick Noel substrates were very susceptible to light soaking, which is also highly indicative of large surface state densities.

### 2.4.2 Photopotential and capacitance measurements

Photopotential measurements are a useful way to quantify band bending and to gain a rough sense of surface state density. For example, the maximum photopotential represents the flat band voltage, or energy difference between the band and the band-edge. Photopotential measurements ($V_{ph}$) taken with a high impedance multimeter (Fluke III, Figure 10) showed that EML1-3 substrates demonstrated a log-linear response with respect to illumination intensity, until maximum positive photopotentials of
+105 mV, +205 mV, and +205 mV were reached, respectively. At this saturation point, the semiconductor bands are flat. The negative photopotentials of the Noel substrates (maximum $V_{ph} = -310$ mV) indicate that the films are degenerate quasi-metals as a result of excessive surface state density, as illustrated in Figure 5C.

Figure 10. Photopotentials ($V_{ph}$) of the various substrates as a function of dose. The values change log-linearly with intensity until a saturation photopotential is reached, at which point the semiconductor bands are flat. The negative $V_{ph}$ values exhibited by Noel substrates indicate that the outsourced films are degenerate quasi-metals.

A more common, yet still simple way to study surface state density is by capacitance measurements. A general rule of thumb in PEC is that a semiconductor is 50% Fermi-pinned when the surface state density is $\sim 1E12$/cm$^2$ and 100% complete at $1E13$/cm$^2$. As a point of reference, high-quality crystalline materials typically have surface state densities of $\sim 1E10$/cm$^2$, or 0.0001% of the surface atoms. Capacitance measurements were made with a LCR meter (HP4263A) to determine the surface state density using Equation 4:

$$Q_{ss} = \left( \frac{C_{ss} \cdot V}{e} \right)$$  \hspace{1cm} (4)

where $C_{ss}$ is the surface state capacitance per unit area, $V$ is the applied voltage, and $e$ is the charge of an electron. Because of metal screening effects at the metal-semiconductor interface, capacitance measurements made on Schottky diodes provide the bulk capacitance rather than $C_{ss}$.\textsuperscript{17} However, screening effects were circumvented
by measuring $C_{ss}$ on metal-insulator-semiconductor (MIS) tunnel diodes\textsuperscript{17} that were created by thermally oxidizing the substrate prior to metal deposition. It was assumed that $C_{\text{oxide}} \gg C_{ss}$, so that $C_{ss}$ dominated the measured series capacitance $C_{\text{total}}$.

The average measured surface state density for EML substrates was $Q_{ss} = 1.9 \times 10^{11}$ /cm$^2$, and thus, the EML substrates should exhibit some degree of Fermi-pinned behavior. The bulk capacitance was also measured at various illumination intensities without the tunnel oxide. The charge density in the bulk, $Q_{\text{bulk}}$ was $1.2 \times 10^{11}$ /cm$^2$ in the dark and saturated at $7.1 \times 10^{12}$/cm$^2$ above 500 mW/cm$^2$ illumination. Given such large $Q_{\text{bulk}}$ values (equal to and exceeding the carrier density of the surface states), the substrate will be Fermi-pinned under illumination, regardless of surface state density. The surface state density for Noel substrates was $Q_{ss} = 3 \times 10^{12}$ /cm$^2$, which is nearly an order of magnitude greater than the values for the EML substrates. At these levels, complete degeneracy on account of excessive surface states (Figure 5C) is certainly a possibility. This conclusion is consistent with the negative photopotential findings (Figure 10).

2.4.3 True surface potential measurements

In order to unequivocally verify that the material was indeed Fermi-pinned and the potential drop in a PEC cell would be across the Helmholtz layer, true surface potential measurements were made by depositing very large metal pads films, applying a 0.5 V bias between the back ITO contact and an aqueous NaCl solution enclosed by an o-ring, and measuring the surface potential outside of the o-ring, as shown in Figure 11A. From Equation 2, one would expect the measured potential ($V_{\text{surface}}$) to be the applied potential ($V_{\text{applied}}$) minus the drop across the bulk ($V_{\text{bulk}}$) plus the photopotential ($V_{\text{ph}}$). The drop across the EML substrate is expected to vary with the illumination intensity because of the increased photopotential and capacitance, as well as the drop in
the semiconductor impedance, which is a factor that is taken into account with more complex SEI models than the one provided here.

(A)

Ground

V_{surface}

NaCl (aq.)

TiiPt

a-Si

ITO

V_{applied}

Illumination

(B)

Figure 11. (a) Configuration for measuring the true surface potential. (b) True surface potential measurements with a 0.5 V applied bias. (c) Calculated relative potential drops across the Helmholtz and space charge layers with a 0.5 V bias, and estimated drops with a 1.7 V bias.

Under no illumination, \( V_{surface \ dark} = 178 \) mV, meaning that the drop across the bulk semiconductor was \( V_{bulk \ dark} = 322 \) mV (65% of the applied bias). This is a reasonable value considering the measured values for \( Q_{ss} \) and \( Q_{bulk} \). This value of \( V_{surface} \) increased to a maximum of 617 mV at 1 W/cm\(^2\) illumination, which is attributable to both the positive photopotential and charge carrier generation (lower impedance, higher capacitance). These values are shown in Figure 11B. Knowing the applied potential, surface potential, and photopotential, the dependence of \( V_{bulk} \) or \( V_H \) on illumination...
intensity can be determined. The normalized values of the drop across the Helmholtz layer for EML3 substrates (\(V_{\text{H}}\)), as a function of dose, are shown in Figure 11C. These surface potential measurements clearly show that the substrates are indeed Fermi-pinned. The estimated drops with an applied bias of 1.7 V (the bias used for PEC detritylations) are also plotted in Figure 11C. The curves differ between the two voltages because photopotential is an absolute number that does not change with bias. Surface potential measurements with the PEC solution agree surprisingly well with these expected values, as will be seen in Chapter 3.

2.5 Increased rectification with large work function metals

The next step was to determine if larger positive photopotentials could be created using rectifying contacts in order to further increase the PEC gain as proposed in Figure 4B of Chapter 1. This was accomplished by eliminating the Ti adhesion layer, which has a low work function and can diminish the rectification of high work function metals. Extremely high positive photopotentials were attainable with rectifying Pd contacts (+432 mV) on EML films, but the reverse currents were over an order of magnitude lower than before because of the increased Schottky barrier. Platinum rectifying contacts gave similar values, but could not be fabricated reliably on EML substrates due to extremely poor metal adhesion. Rectifying platinum contacts could reliably be created on Noel substrates because of the inherent roughness of the films. The rectifying Noel structures had \(V_{\text{ph}}\) values of +115 mV, but also had much lower reverse bias currents.

2.6 Towards PEC synthesis: Explaining failure / predicting success

Table 1 summarizes the various amorphous silicon films and diode architectures that were analyzed in this chapter, and their suitability as substrates for PEC microarray synthesis (Chapter 3). The observed properties of substrates that failed, including low
impedance, susceptibility to light soaking, high surface state density, and poor fabrication yield, are also listed. As Table 1 shows, only EML1 and EML3 batches with Ti/Pt electrodes were suitable. The 1 μm-thick Noel substrates with Ti/Pt electrodes also worked on occasion.

Before discussing what makes a good photoconductor for PEC synthesis, let us first attempt to explain the failures. The categorical failure of highly rectified photoconductors that employed large work function metals was most likely due to the extremely low conductivity under reverse bias. The decreased current passing through the metal would lower the PEC gain over the background of oxidized amorphous silicon, as well as make the detritylation time unacceptably long.

<table>
<thead>
<tr>
<th>Metal pad</th>
<th>EML1 (600 nm)</th>
<th>EML2 (1000 nm)</th>
<th>EML3 (1000 nm)</th>
<th>Noel (500 nm)</th>
<th>Noel (750 nm)</th>
<th>Noel (1000 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti/Pt</td>
<td>Good I and LS</td>
<td>Good LS</td>
<td>LS</td>
<td>LS</td>
<td>LS</td>
<td>SS</td>
</tr>
<tr>
<td>Pt</td>
<td>F and I</td>
<td>F and I</td>
<td>F and I</td>
<td>LS</td>
<td>LS</td>
<td>SS and I</td>
</tr>
<tr>
<td>Pd</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>LS</td>
<td>LS</td>
<td>SS and I</td>
</tr>
</tbody>
</table>

Table 1. Summary of the substrate suitability for PEC microarray synthesis. Colors indicate the viability of each substrate, where green = success, yellow = moderate success, and red = failure. The abbreviations denote the presumed failure mechanism, where I = low impedance, LS = light soaking susceptibility, SS = high surface state density, F = poor fabrication yield.

The Noel substrate failure was likely due to charge traps present in the hazy films. Charge trapping from surface states may perhaps diminish conduction in PEC cells more than it does in solid-state Schottky diodes. In the PEC cell, surface states can mediate electron-hole recombination events that impede the ability of the hole to participate in the anodic reaction, and they can also lead to reverse reactions (i.e. electron injection into solution or hole injection from solution), as shown in Figure 12.
The large surface state density also led to very negative photopotentials that lower the efficiency of the anodic reaction.

\[ \text{(A)} \quad \text{Surface state-mediated electron injection from the conduction band into the electrolyte.} \]

\[ \text{(B)} \quad \text{Hole injection into the valence band by an oxidant and the injection and recombination pathways. Both surface state-mediated and depletion layer-mediated routes are shown for the recombination. Both images taken from Bard.}^8 \]

Figure 12. Undesirable reverse reactions at n-type semiconductor anodes. (a) Surface state-mediated electron injection from the conduction band into the electrolyte. (b) Hole injection into the valence band by an oxidant and the injection and recombination pathways. Both surface state-mediated and depletion layer-mediated routes are shown for the recombination. Both images taken from Bard.\(^8\)

The failure of the EML2 substrate is slightly more complicated, since the capacitance and \( V_{ph} \) measurements indicated that the surface state density was similar for all EML substrates. Furthermore, the PECVD conditions for the three films were all very similar, if not the same (EML1 and EML2). However, EML2 substrates did vary from the others in that its conductivity under reverse bias was an order of magnitude less. The EML2 substrate was also slightly susceptible to light-soaking, which can occur by many mechanisms other than the photo-generation of surface states.\(^18\) Therefore, the EML2 failure could be attributable to low impedance and/or non-surface state Wronski-Staebler defects.\(^18\)

Even though EML1 and EML2 substrates were theoretically deposited under the exact same conditions, the PlasmaTherm PECVD machine lacks any \textit{in-situ} metrology (\textit{e.g.} interferometer or quartz crystal microbalance) equipment to make real-time
adjustments; their deposition rates varied by 50%. The inconsistency in EML film quality between identical PECVD runs highlights the importance of well-characterized depositions parameters. Thus, future work will either require much better PECVD equipment, or other deposition methods that are more easily controlled.

Ultimately, based on the diode data gathered in this chapter, there appeared to be two empirical substrate requirements for PEC microarray synthesis success. First, surface states must not be abundant enough to create significant charge recombination pathways, defect-mediated electron injection mechanisms into solution, or negative photopotentials that lower the efficiency of the anode. Second, the impedance must be low under reverse bias so that the reaction proceeds quickly enough to keep deprotection times reasonable.
2.7 References


Chapter 3

Photoelectrochemistry and microarray synthesis

This chapter discusses the photoelectrochemical analysis of the substrate and PEC microarray synthesis. Even though this material is presented after the solid-state diode analysis of Chapter 2, the experiments were done concurrently, with PEC analysis and synthesis informing diode analysis, and vice-versa. Hence, some of the results presented in this chapter have already been alluded to in the previous one.

3.1 Design choices vs. other electrochemical microarray platforms

Just as there were design choices for diode fabrication, similar choices needed to be made for the composition of the electrochemically active solution. Luckily, since the substrate is largely Fermi-pinned as seen in Chapter 2, its behavior will not be significantly altered by the nature of the redox pair, electrolyte, or solution (see Figure 4 of Chapter 2), thereby allowing great flexibility in designing the solution. The hydroquinone / benzoquinone (HQ/BQ) redox couple (Figure 1A) is one of the most commonly studied reactions in organic electrochemistry, and also the redox system of choice for electrochemical DNA synthesis.\(^2\)\(^-\)\(^5\) The anodic oxidation of hydroquinone provides a “clean” source of protons, and hydroquinone is highly soluble in acetonitrile, which is the main solvent for DNA synthesis. The HQ oxidation potential in acetonitrile is +1.12 V vs. the saturated calomel electrode (SCE).\(^6\)

Acetonitrile (AcCN) is an excellent electrochemical solvent because it has a wide potential window and large electrolyte solvation capability. High concentrations (100mM) of both HQ and tetrabutyammonium hexafluorophosphate salt (NBu\(_4^+\)PF\(_6^-\)) were used to ensure fast deprotection times and that the system is never diffusion
limited under large illumination, which would otherwise diminish the contrast ratio. Acetonitrile with 100mM tetrabutylammonium salts have a limiting conductivity\(^7\) of 6.163mS, so the solution resistance will not be limiting given the fluidic dimensions.

![Chemical reaction diagrams](image)

**Figure 1.** (a) The hydroquinone/benzoquinone redox couple (HQ/BQ). (b) Anodic oxidation mechanism of HQ to BQ. (c) Cathodic reduction mechanism of BQ to HQ.

![Combimatrix synthesis scheme](image)

**Figure 2.** (a) The Combimatrix synthesis scheme. Electrochemically generated protons that diffuse between microarray spots are scavenged with a buffer and electrochemically generated bases. (b) Hybridization assays by fluorescence imaging show fuzzy edges or “halos” from the loss of proton containment when the current density is too high. (c) Buffering contains the acid within the active electrode area (yellow box). Image from McShea.\(^3\)
Other electrochemically driven microarray platforms utilize more complex deprotection cocktails (Figures 2 and 3)\textsuperscript{2-6} to limit proton diffusion between spots. Such diffusion (also observed with photo-generated acids\textsuperscript{8,9}) creates fuzzy edges or “halos”\textsuperscript{2,5} around the spots that can cause problems in fluorescence-based microarray analysis,\textsuperscript{10} and create sequence errors that are highly detrimental to applications using cleaved strands.\textsuperscript{8,11-16} The synthesis platforms developed by Combimatrix\textsuperscript{2,3} (Figure 2) and by Southern and co-workers\textsuperscript{4,5} (Figure 3) both employ quinhydrone cocktails (1:1 HQ : BQ) and pattern/place the counter electrode between the individual working electrodes to create a cloud of proton scavengers between spots (semiquinone radical from quinone reduction). The Combimatrix system also buffers their solutions to further limit diffusion between spots (Figure 2C).\textsuperscript{2} The main difference between the two platforms is that the electrodes in Southern’s system are on a different substrate than the microarray. It is instead held parallel and in close proximity to the electrode chip using a movable piston. This leads to more spatial non-uniformity in the acid exposure profile, as evident by the increased level of depurination in the pattern center (Figure 3B).

We have chosen a much simpler PEC solution that only contains the electrolyte and HQ. There were several reasons for not choosing to buffer the system or utilizing in-plane counter electrodes with a quinhydrone solution. First, solutions buffered with 1/10 molar concentration of triethylamine completely decomposed in 1 - 2 days. Such fast decomposition would affect the deprotection efficiency during the course of a several-hour long synthesis. Second, rather than increasing the micro-fabrication difficulty by patterning a counter electrode on the substrate itself, the top surface of the fluidic was used as the counter electrode, making the use of an electrochemically generated scavenger moot. Third, adjusting the current density or time can be sufficient for proton containment (Figure 2B). Fourth, it simplifies the chemistry, eliminating
possibilities like cyanoethyl deprotection by a basic semiquinone radical with long lifetimes in organic aprotic solvents like acetonitrile (Figure 1C).

![Figure 3. (a) Electrochemical synthesis scheme by Southern and co-workers. The microarray is synthesized on a different substrate as the electrode array, which is placed in close proximity by a movable piston. By using the quinhydrone redox pair and inter-digitated cathodes or counter electrodes, clouds of proton scavengers are created between the anodes or working electrodes to keep the acidic patterning area spatially confined. (b) Under this electrode orientation, the acid exposure is not spatially uniform within the patterning area, as evident by the increased depurination in the center of the stripe, as determined by fluorescence hybridization assays. Images from Southern.](image)

### 3.2 Instrumentation and Automation

There are three main components to the PEC microarray synthesis platform: the optical projection system, the electrochemical fluidic cell, and the substrate that was extensively characterized in Chapter 2. Additionally, special three-electrode fluidic cells and a digital potentiostat were constructed for electrochemical characterization.

#### 3.2.1 Optics

For customizable arrays, the best option for a spatially modulated light source is the digital micromirror device (DMD), which is used commercially by Nimblegen, Febit, Xeotron, and Atactic. The DMD is an array of microelectromechanical mirrors that can
be swiveled ±10 - 12°, which is sufficient to deflect light towards or away from a lens train. The 1024 x 768 array with 13.68 μm mirrors can be purchased for ~$10k from Texas Instruments, which includes all the electronics and software required to address individual pixels, but does not include any optics or illumination sources.

The most common use of DMD’s is to make digital light projectors (DLP), which can be purchased for under $1k. DLP’s represent low cost options for high-power, spatially modulated visible-light sources. However, there are many drawbacks to modifying a DLP to be used as a lithography system. First, the pixels flicker at a fixed rate that is synchronized to a color wheel, and thus cannot be fixed in the “on” state. Second, the projection optics design is non-telecentric to maximize contrast ratio and naturally project the image on an upward angle. Light is collected with an aspheric lens that is placed in close proximity to the DMD, and in the absence of the collection lens, light does not exit the DLP. Since this lens set expands the image, the light must be focused significantly to achieve micron-scale resolution. It is extremely difficult to place an appropriate light-collecting lens inside of the projector without blocking the illumination path. Lastly, the individual pixels cannot be programmed without custom control software.

Figure 2 shows a picture and diagram of the projection system, including all of the axes of motion and rotation for each component. The Optoma EP719 DLP projector contained two sets of lenses, a zoom assembly that includes the aspheric collection-lens, and a focusing lens assembly with a net negative focus. The first lens set could not be removed because it is required for the light to exit the DLP. The second lens set was removed in order to limit the size of the image before focusing, and a 75 mm focal length lens was press-fit into its place. The closeness to the DLP of this lens was the main contributor to the long working-distance of the projection system that accommodates the size of the fluidic.
All lenses were achromats since the light source was a white light lamp. All optical components in the train were from Thor Labs unless otherwise noted. No more than half the area of any lens was illuminated to limit spherical aberrations. The main aberration was coma, which was limited by a combination of the pinhole aperture, the lens shift of the entire train, and the tilt of the DLP. The CCD camera (Sony XC-ES50) was placed in-line with the lens cage to ensure that it was centered. An Infini-tube lens (Infinity Solutions USA) that had a much longer working-distance than a microscope objective allowed for the necessary space to accommodate the fluidic. Individual pixel control was achieved via a Python script that wrote pixel values on the desktop display, with both the LCD monitor and DLP in true XGA mode (1024 x 768).

Figure 2. (a) Diagram of the optical projection system, including the individual axes of control in parenthesis. (b) Picture of the system. (c) Captured image of a projected “virtual mask.” Scale bar = 300 μm.
The optics system generated an aberration-field 1.44 - 1.8 mm (center 300 x 300 pixels) per side with 4.8 - 6 μm pixels, depending on the zoom setting of the DLP aspheric collection lens. Figure 2C shows a video screen capture of regularly spaced 60 μm boxes. Photodiode (Melles Griot FDS1010) and power meter (Molelectron Power Max 5100) measurements showed that the system, when set to 6 μm pixel sizes, had 86.7% image uniformity, 950 : 1 contrast ratio, and 890 - 1070 mW/cm² output power. The main optical error was that for each group of pixels that represent one electrode pad, an outermost row or column would sometimes be lower in intensity (at most one row and column). This was likely a video processing error with the DLP and not attributable to the optics because no amount of lens shifting could fix it. The electrode pad size was chosen to be at least 60μm, or a 12 x 12 array of 5 μm pixels, so that this error would lead to a 16% intensity variation at worst (i.e. one row and column are completely truncated). The working distance (~5 cm) was sufficiently long to accommodate the fluidic.

3.2.2 Automation

A very simple Python script used the computer parallel port to synchronize the DLP, voltage supply (via a MOSFET switch), optical shutter (Uniblitz), and DNA synthesizer (ABI394). The DNA synthesizer can be programmed to close a relay switch that was used to trigger the detritylation events. The script also dynamically generated the virtual masks for the synthesis from text files of DNA sequences. The exposure time could be set either by the optical shutter, the display module, or both. The output current of the power supply was limited with a 50Ω resistor, and was offset by +13 mV to account for the voltage drop across the MOSFET and resistor. Source code can be found in Appendix C.
3.2.3 Fluidic cell for DNA synthesis

The fluidic adapter for the synthesizer requires a planar counter electrode to ensure field homogeneity (see Chapter 3.3). It is also desirable that the counter electrode be optically transparent to enable transmission-mode image alignment that eliminates the use of a beam-splitter. Given these considerations, ITO-coated glass was used as the counter electrode. Because the ITO counter electrode is biased negative with respect to the substrate, the irreversible ITO reduction commonly seen in LCD display technologies is not of concern.

Figure 3. (a) Diagram and (b) picture of the fluidic. (c) Captured image of the alignment between the virtual mask and metal pads. The pads appear dark because they block the light passing through the substrate. Scale bar = 100 μm

The design and optical image of the fluidic are provided in Figures 3A and 3B. Fluid thru-holes were drilled using a diamond-coated stick drill bit, and Nanoport HPLC fittings (Upchurch Scientific) were used to interface the fluidic with the DNA synthesizer.
The substrate and ITO counter electrode (Delta Technologies, 1.1 mm thick) are sandwiched between two laser-cut (Universal Laser Systems) acrylic plates with a Kalrez o-ring (McMaster-Carr) in between them. It was important that the ITO-coated glass be 1.1 mm thick in order to limit cracking under compression that often occurred with 0.7 mm thick electrodes. Acrylic was chosen because it was easier to machine (by laser cutting) than other plastics, but high-density polyethylene (HDPE) or high-density polypropylene (HDPP) is a more appropriate choice in the long run. These two polymers that compose DNA synthesizer columns strike the right balance between chemical inertness and mechanical properties. The more chemically inert Teflon is very soft and can be difficult to use as a compression plate. A laser-cut Viton gasket (Eagle Elastomer) was used to center the o-ring. Despite the optical transparency of acrylic, it acts as a diffuser, and thus, holes were cut in the beam path.

The flow cell did not leak or crack under the moderate pressure from the DNA synthesizer. Point contacts (i.e. o-rings) were required for good compression sealing when using organic solvents. While planar Viton gaskets that were laser-cut into unique patterns to create good mixing and low volumes, sealed well using aqueous solutions, the elastomeric swelling in the presence of organics was sufficient to cause leakage. Figure 3C shows the alignment of a virtual mask to the microfabricated electrodes, as imaged through the entire fluidic.

3.2.4 Electrochemical cell for potentiometry

Since current passing through an electrode can polarize it and cause dramatic peak shifts in two-electrode systems, electrochemical measurements are made by three-electrode potentiometry. In potentiometric measurements, a feedback circuit fixes the bias between the working electrode of interest and a high-impedance reference electrode that draws no current. To be more specific, the working electrode in a
potentiostat (Figure 4A) is a floating ground through which current is measured via an I/E converter. The counter electrode is connected to a control amplifier that provides whatever current is needed to pin the bias between the working and reference electrodes, which is measured via an electrometer.

Figure 4. (a) Digital potentiostat that can be interfaced with a semiconductor analyzer or National Instruments Data Acquisition card (NI/DAQ). (b) Schematic of a three-electrode fluidic for electrochemical measurements, using a nickel plate as the counter electrode and platinum wire as the quasi-reference electrode (QRE) (c) Schematic of a second three-electrode fluidic design, with platinum pellets as both the QRE and counter electrode. Figures 4A and 4B from Emig. 18

Making electrochemical measurements in non-aqueous solutions is far more difficult than in water because stable reference electrodes are aqueous. The most common reference electrodes for organic solutions are either aqueous electrodes connected to the cell via a salt bridge, or a platinum or silver quasi-reference electrode (QRE), whose potential is calibrated against the ferrocene redox couple. The counter
electrode must be chemically inert and large/conductive enough so that it does not limit the current that passes through the working electrode.

A digital potentiostat (Figure 4A) was constructed to interface with the semiconductor analyzer (HP4156A). Two different three-electrode fluidics were used in this work (Figures 4B and 4C). One fluidic used a nickel plate as the counter electrode and a platinum wire as the QRE (Figure 4B), and details of its fabrication can be found elsewhere. The second fluidic (Figures 4C) used 1/8" diameter platinum pellets (99.99%, Kurt J. Lesker) that were press-fit into 10-32 HPLC fittings (Upchurch Scientific) as both the QRE and counter electrodes. After feeding the fittings through a machined HDPE plate, the pellets were electrically contacted using silver paste (Acheson Colloids) and stainless steel HPLC tubing (Upchurch Scientific), and then potted with epoxy resin. A laser-cut Viton gasket positions the ½" Kalrez o-ring (McMaster) that is sandwiched between the HDPE plate and the substrate, which is backed by a clear acrylic plate. The fiber illumination setup used for the diode analysis in Chapter 2 was also used as the illumination source for PEC experiments.

Figure 5. Cyclic voltammogram (C-V) of ferrocene using platinum foil as the counter electrode, and 1/8" diameter platinum pellets as the quasi-reference and working electrodes. The C-V was taken at a 25 mV/s scan rate, using 10 mM ferrocene in AcCN with 50 mM NBu₄⁺PF₆⁻ salt.

Figure 5 shows a C-V (scan rate = 25 mV/s) of 10 mM ferrocene in acetonitrile with 50 mM NBu₄⁺PF₆⁻ salt, that was acquired with the custom potentiostat and fluidic...
diagrammed in Figure 4C. One of the platinum pellets was used as the working electrode, and platinum foil (99.9%, Aldrich) was used as the counter electrode, since the counter electrode must be able to sink/source whatever current is needed by the working electrode (i.e. must be larger or significantly more conductive than the working electrode).

3.3 Electrochemical and PEC system characterization

3.3.1 Electric-field homogeneity in the fluidic cell

While testing the potentiostat and three-electrode fluidics, it became clear that the counter electrode must be planar in the two-electrode fluidic to ensure field homogeneity. When gold-coated glass (EMF Corporation) was used as the working electrode for extensive periods of time in the presence of 50 mM aqueous NaCl, a 1/8" diameter hole was etched down to the glass, directly beneath the counter electrode pellet (Figure 6). This meant that the electric field in the cell is highly directional, and in order to obtain uniform detritylations, the distance between any substrate spot and the counter electrode must be the same (i.e. planar counter electrode).

(A) Prolonged use of a gold working electrode in aqueous solutions chemically etched a 1/8" hole through the film directly under the 1/8" diameter counter electrode.

Figure 6. Evidence of the strong directionality of the electric field in the z-axis perpendicular to the electrode surface. (a) The fluidic cell as described in Figure 4C. (b) Prolonged use of a gold working electrode in aqueous solutions chemically etched a 1/8" hole through the film directly under the 1/8" diameter counter electrode.
3.3.2 Helmholtz distance at the semiconductor electrolyte interface

An important estimate to know is the length of the Helmholtz layer at the semiconductor electrolyte interface (SEI) as a function of electrolyte concentration. The thickness measurements give an estimate of the z-distance from the surface at which the growing strands will be damaged by direct electrochemistry of the DNA itself.\textsuperscript{19} The Helmholtz layer thickness ($d_H$) can be calculated from capacitance measurements using Equation 1:

\[ C_H = \left( \frac{\varepsilon_H \varepsilon_0}{d_H} \right), \]  \hspace{1cm} (1) 

where, $\varepsilon = $ dielectric constant.

Figure 7 shows the calculated Helmholtz distance as a function of NBu\textsubscript{4}$^+$PF\textsubscript{6} salt concentration in acetonitrile. Measurements were made between two nickel plates, one of which was the fluidic in Figure 4B. The measured Helmholtz layer thickness varies by almost three orders of magnitude over a concentration range of 5 μM - 100 mM, with an asymptotic value of $d_H \approx 0.2$ nm, which is slightly smaller than the diameter of a tetrabutylammonium ion. It is expected that only a small portion of growing strands within the porous glass will be electrochemically damaged at high salt concentrations.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure.png}
\caption{Semi-log plot of the Helmholtz distance as a function of tetrabutylammonium hexafluorophosphate electrolyte concentration in acetonitrile, as determined by capacitance measurements. The distance levels out at 0.2 nm, which is the approximate hydrodynamic radius of one salt ion.}
\end{figure}
3.3.3 Verification of proposed PEC mechanisms

As shown in Figure 4 of Chapter 1, there are two mechanisms that are responsible for the selective PEC acid generation at the illuminated virtual electrodes. First, there is the kinetic factor that more charge carriers are at the surface that can participate in the oxidation reaction. Second, there is the thermodynamic factor that the surface potential is larger when the substrate is illuminated, which increases the electrochemical efficiency of the anode. Figures 8 and 9 verify these kinetic and thermodynamic mechanisms, respectively.

Figure 8. Evidence of different photoelectrochemical kinetics. Cyclic voltammograms of 2.5 mM (a) hydroquinone and (b) ferrocene in acetonitrile with 100 mM NBu₄⁺PF₆⁻ salt were taken using the amorphous silicon photoconductor as the working electrode. The “light” and “dark” states correspond to 1 W/cm² and 1 mW/cm² white light illumination, respectively. A diffusion limited peak is clearly seen in the “light” states (red asterisk), whereas no such peak is observable in the dark (inset boxes).

Figure 8 shows cyclic voltammograms of 2.5 mM hydroquinone (Figure 8A) and ferrocene (Figure 8B) in acetonitrile (with 100 mM NBu₄⁺PF₆⁻ salt) that were taken using the amorphous silicon photoconductor as the working electrode. To emulate the optical projection system specifications (950X contrast ratio, 890 - 1070 mW/cm² output power), the “light” and “dark” states of illumination were set at 1 W/cm² and 1 mW/cm². In both
cases, the light C-Vs show mass transport-limited peaks (red asterisk), while the dark samples do not (inset boxes). These graphs clearly show that the reaction kinetics can be significantly altered by the illumination intensity.

Figure 9 plots the relative potential drops (left ordinate) across the semiconductor and Helmholtz layer at the semiconductor electrolyte interface (SEI), as a function of illumination intensity. The right ordinate of Figure 9 shows the actual measured surface potential of the metal pad at the SEI given a +1.7 V bias. Figure 11 of Chapter 2 showed a similar plot using aqueous sodium chloride as the electrolyte, with an applied bias of 0.5V. Here, the actual deprotection conditions were used to make the measurements (1.7 V bias, 100 mM hydroquinone with 100 mM salt in acetonitrile). As previously proposed, the surface potential is much higher at an illuminated SEI, and therefore, the electrochemical oxidation efficiency should increase upon illumination.

![Figure 9](image)

**Figure 9.** (Left ordinate) Relative potential drop across the semiconductor (green circle) and Helmholtz layer (blue square) at the SEI as a function of illumination intensity. (Right ordinate) The measured surface potential of the substrate at the SEI, given an applied bias of +1.7 V. The solution was composed of 100 mM HQ with 100 mM NBU4PF6 salt in acetonitrile.

3.4 Steps towards PEC synthesis: Site-selective detritylation

Now that the substrate and system components have all been studied by solid-state diode analysis, electrochemistry, and PEC, the next step was to characterize the site-selective detritylation process. Because the crux of any microarray technology is the ability to perform a spatially selective reaction, it is extremely important that the
detritylation process be well understood. Ultimately, the main difference between a
singular detritylation event and full-on microarray synthesis is the number of times that
the step is repeated. The simplest way to demonstrate site-selective cleavage is to
selectively couple a phosphoramidite dye, and then image the substrate with a
fluorescence microscope. Because the coupling/condensation reaction is the equivalent
to the one used to synthesize DNA, the experiment gives valuable information about
spatial resolution and reaction efficiency of the eventual synthesis process.

3.4.1 Materials and methods: PEC detritylation

All reagents were used as received from Aldrich unless specified otherwise. Millipore water is denoted as dH$_2$O. Prior to synthesis, substrates were silanized with
0.5% N-(3-triethoxysilylpropyl)-4-hyrdoxybutyramide (Gelest) in ethanol (v:v) for at least
2 hours at room temperature, washed with acetone, dried under nitrogen. The hydroxyl
groups were protected with 50 mM 4,4'-dimethoxytrityl chloride with one equivalent
triethylamine catalyst in anhydrous toluene or pyridine for at least 4 hours under
nitrogen, washed with acetonitrile, and dried under nitrogen. All surface
functionalizations were performed in Teflon vials from Savillex.

An ABI 394 synthesizer was used as the reagent manifold. The synthesis cycle
was based on the commercial 0.2 μmol cycle, with delivery times modified to
accommodate the extra volume of the fluidic and additional tubing. All DNA synthesis
reagents were used as received from Glen Research. "Ultramild" cyanoethyl
phosphoramidites and phenoxyacetic anhydride "Cap A" solution were used to
accommodate mild final deprotection conditions. The deblock mixture was composed of
100 mM hydroquinone and 100 mM tetrabutylammonium hexafluorophosphate salt in
anhydrous acetonitrile. Unless stated otherwise, the substrate was biased at 1.7 V.
The substrates were recovered by soaking them for at least 20 minutes in Nochromix (Godax) solutions, washing with copious amounts of dH$_2$O, and drying under nitrogen. Nochromix is a sulfuric acid-based oxidizer that obliterates organic molecules like the "piranha" cleaning solutions used in microfabrication (3:1 sulfuric acid : hydrogen peroxide). The Nochromix physically strips the silane linker molecule from the surface of the porous glass. A very small amount of exposed ITO conductivity is lost with each recovery cycle since sulfuric acid can etch ITO, but the increased resistance of the electrical contact is still negligible compared to the resistance of the amorphous silicon film. When the porous glass films became significantly damaged, they were stripped by buffered oxide etching for two minutes, washing with copious amounts of dH$_2$O, and drying under nitrogen. The ability to recover substrates is significant because they can be treated as quasi-capital whose cost can be amortized, rather than as consumables.

All fluorescence images shown were taken with a Zeiss LSM Pascal confocal microscope, operated in both confocal and bright-field fluorescence modes. In both cases, colors in the image are false-colors, not real colors imaged by a color-sensitive CCD detector. All images are confocal images, unless otherwise specified as bright-field images.

3.4.2 Initial proof of detritylation with EML substrates

Many of the earliest experiments focused on the Noel substrates, but as mentioned in Chapter 2, most of them failed completely. Nonetheless, moderate levels of PEC detritylation were possible with Noel substrates that were 1 µm thick and had Ti/Pt pads, but the results were not very reproducible. Fluorescence images of these substrates are shown in Appendix D.

The first conclusive experiment of site-selective proton generation and detritylation is shown in Figure 10. Five bright-field micrographs were stitched together.
to create the large image field. This particular substrate was an overexposed (60 seconds, 2 V) EML1 substrate with 200 µm pads, where the intended pattern was a checkerboard. The last two right columns of the checkerboard are visible, but the rest of the pattern is washed out by flow-induced "diffusion" of excess protons. It should be noted that flow-induced "diffusion" is a misnomer, and that "convection" is a more appropriate term. However, in order to be consistent with previously reported electrochemical synthesis platforms, any movement of excess protons will be called "diffusion," regardless of convective or electro-migration forces.

Figure 10. Initial success with an EML1 substrate with 200 µm pads. (a) Bright field fluorescence micrograph of an overexposed substrate (+2 V bias, 60 seconds). The intended pattern was a checkerboard, the edge two columns of which are visible on the right. The rest of the pattern is washed out by the laminar flow of excess acid, whose flow profile is given by the red arrows. The total image is stitched together from five images.

The flow in the fluidic cell is laminar and the fluid flow profile (red arrows) matches the expected profile in a circular cell with symmetric fluid input and output holes at opposite ends. The flow-induced movement of protons is primarily in one direction (right to left), and when the excess protons reach the edge of the fluidic, they travel along the edge of the o-ring.

3.4.3 Optimization of detritylation conditions

Even though EML1 and EML3 substrates both worked, their operating conditions were different. Experiments to optimize the detritylation conditions focused on EML3
substrates because more of them were available. All EML3 substrates had 125 μm Ti/Pt pads on them, separated by 75 μm (i.e. 100 μm pitch). It can be assumed that all data below refers to EML3 substrates biased at +1.7V, unless stated otherwise.

In order to determine the optimal exposure time, a “dose test” was performed on a 13 x 13 array by programming the script to turn pads “on” in 250 ms intervals (Figure 11A). Note that the dose test was performed with respect to time instead of gray scaling because gray virtual masks have lower contrast ratios (i.e. lower “on” intensity, but fixed “off” intensity). After the dose test was complete, five minutes was allowed to elapse prior to emptying the fluidic to eliminate flow-induced proton movement. Pixel intensities were determined from line sections of high-magnification confocal fluorescence images and plotted in Figure 11B. The intensities were normalized against the average values after 30 seconds, when halos from proton diffusion are clearly observed. There exists an operable time window between 20 - 25 seconds in which substrates can be fully detritylated without observable diffusion. This time can vary slightly (± 2 seconds) from substrate to substrate, or between different areas of the same substrate, depending on parameters like the amorphous silicon film thickness (~ ± 10%), the porous glass thickness uniformity, the presence of defects such as pinhole and scratches in the porous glass, and the optical field uniformity.

Why does such an operable time window exist? In electrochemistry, the preferred direction of movement of redox species away from a planar electrode follows Fick’s Laws of Diffusion (proton diffusion constant in acetonitrile, \( D = 2E-5 \text{ cm}^2/\text{s} \)). The field-induced migration of charged species is typically ignored because the high salt concentration creates a very thin Helmholtz layer beyond which the ions feel a negligible electric field. Given the electrode size and geometry, the movement is perpendicular to a planar electrode.\(^{17}\) It will be assumed that PEC-generated protons are all consumed in the detritylation reaction, or diffuse into the bulk solution in the perpendicular direction.
prior to the critical exposure time, which is reasonable assumption given that no diffusion is observable during short exposures. At the beginning of the operating window, the system begins to generate excess acids that still move perpendicularly away from the surface, rather than laterally along it towards adjacent pads. As time elapses though, the proton accumulation layer over each pad grows so thick that the diffusion profile away from the surface becomes more and more isotropic (mushroom-like to be more exact, since there is a boundary condition imposed by the surface). This eventually leads to noticeable halos.

Figure 11. Dose test to find the critical exposure times for complete detritylation and the onset of diffusion. (a) Confocal fluorescence image of the dose test. The substrate was biased at +1.7 V. Pads were exposed for different time durations by turning on DMD mirrors every 250 ms. Scale bar = 200 μm. (b) Plot of the normalized fluorescence intensity versus time showing the onset of diffusion and a suitable operating window for complete detritylation without noticeable diffusion.

It should be noted that faster detritylation rates are achievable with larger biases, but the operating window would also be smaller. Lowering the bias to slow the reaction down could be beneficial, but 1.7 V appeared to be a good operating voltage. When the massive loading density of the porous glass (see Chapter 4) is taken into account, the
20 second deprotection step is by far the shortest reported deblock time.\(^(*)\) Considering that a typical detritylation cycle consumes nearly two minutes in a five-minute standard 0.2 \(\mu\)mol scale phosphoramidite synthesis, there is no particular need at this moment to drive the PEC detritylation reaction any faster. Current deprotection times are not limiting in other platforms either.

However, the fast deprotection times are worth noting because they could one day be time-limiting in the highly probable event that microarray synthesis platforms begin to employ integrated microfluidic devices with active valves.\(^{21, 22}\) Gao and co-workers found that, in addition to providing huge cost savings by decreasing the reagent usage, the use of integrated microfluidic channels (no valves) decreased the deprotection step time thirty-fold because of improved mass transport in the system.\(^8\) It is already known that the active mixing employed in "primer scale" synthesis improves the phosphoramidite condensation yield. Fluid cycling in microfluidic devices has also been used to improve DNA hybridization rates.\(^{23}\)

\(\text{(A)}\) \(\text{(B)}\) \(\text{(C)}\)

**Figure 12.** Confocal fluorescence images of cy3 after optimization (22-second exposure, 1.7 V bias) \(\text{(a)}\) Checkerboard pattern at low magnification and \(\text{(b)}\) the same substrate at high magnification. The apparent contrast between "light" and "dark" pads varies significantly with microscope settings. Scale bar = \(\text{(a)}\) 600 \(\mu\)m and \(\text{(b)}\) 200 \(\mu\)m. \(\text{(c)}\) Fluorescence emission plot of a pattern spelling "MIT."

\(^(*)\) 1.05E-12 mole \(\cdot\) mm\(^2\) / sec; Fastest previously reported rate\(^8\) = 1.48E-13 mole \(\cdot\) mm\(^2\) / s.
Figure 12 shows confocal fluorescence images of substrates that were exposed for 22 seconds and biased at 1.7 V. Note that images of Figures 12A and 12B are from the same substrate, taken at different magnifications. Even though the commercial “auto-find” function of the microscope was used to determine the detector settings for both images, the fluorescence contrast ratio between “light” and “dark” pads is much different. Therefore, quantifications by fluorescence imaging are normalized against complete detritylations, such as the halo spots in Figure 11A, instead of being reported as absolute detector values. Figure 12C shows a fluorescence emission image (“FRE” or isometric view) of cy3-phosphoramidite patterned to spell “MIT.”

3.4.4 Proof of optical non-linearity in PEC synthesis

One incredibly useful feature of the PEC microarray synthesis platform is its non-linearity with respect to illumination profile. On the other hand photocleavable chemistries are linear with respect to both the intensity and the illumination profile (Figure 13).\textsuperscript{24} The simulated image in Figure 13A, taken from Cerrina et al.,\textsuperscript{24} shows that the beam from a single DMD mirror will have diffraction peaks at the edges, as well as a dark spot in the middle where the hinge is located. This non-uniform beam profile creates similar spatially inhomogeneous profiles in deprotection efficiency (Figures 13B and 13C), that ultimately result in sequence errors and non-uniform fluorescence signals during hybridization experiments.

The deprotection efficiency in PEC synthesis is non-linear with respect to the illumination profile. Figure 14 shows an image of cy3-phosphoramidite coupled to pre-patterned metal electrodes spelling “GeneFab.” Even though each letter in “Gene” was only partially illuminated where the boxes are in the figure, the fluorescence intensity is still uniform over the entire area of the letter because the charge carriers spread through the metal faster than they can participate in the oxidation reaction. This optical non-
linearity from charge carrier spreading greatly simplifies the requirements over the image drift, uniformity, and alignment capability of the PEC system.

Figure 13. Optical linearity using photocleavable reagents and a DMD as the spatially modulated light source. (a) The simulated intensity profile of a DMD mirror has diffraction peaks at the edge and a dark spot in the middle from the mirror hinge. (b) Hybridization to a 25-mer microarray shows that the non-uniform beam profile creates similar profiles in deprotection and synthesis efficiency. (c) Fluorescein phosphoramidite coupling after one deprotection step shows that the error per step is quite dramatic. Image taken from Cerrina et al.24

Figure 14. The optical non-linearity of the PEC synthesis platform with respect to spatial illumination profile. The white boxes indicate where a checkerboard pattern was projected onto pre-patterned metal electrodes that spell “GeneFab.” (Top) Even though only part of each letter in “Gene” was illuminated, the detritylation was uniform over the entire letter because the charge carriers spread through the metal faster than they can participate in the oxidation reaction. (Bottom) Optical micrograph of the mask, provided to aid the eye towards the faint “Fab” in the fluorescence image. Scale bar = 200 µm.
The PEC platform is also optically non-linear with respect to illumination intensity. Because electrochemical reaction efficiency depends on the potential energy of surface charge carriers, the larger surface potential at an irradiated electrode translates to a much higher Faradaic current efficiency (Equation 2). The Faradaic current efficiency is the amount of current injected into solution that actually participates in the electrochemical reaction, as opposed to non-Faradaic processes like salt adsorption or solvent polarization:

\[
\%\text{Efficiency} = 100\times\left(\frac{I_{\text{hydroquinone+salt}}}{I_{\text{salt}}}\right)
\]  

As a result, the PEC gain should be larger than the photodiode gain, which indeed turns out to be the case. Normal pulse voltammetry ("NPV," time-sampled current after a voltage step) showed that the PEC gain was 550X, whereas the photodiode gain was only 325X (Figure 9 in Chapter 2). The difference between the PEC contrast and solid-state photosensitivity is attributable largely in part to the difference in Faradaic current efficiency, which was 89% for the illuminated substrate and 54% for the dark one.

Another benefit of the metal pad is that it eliminates DNA exposure to light when the substrate is illuminated from the backside, thereby reducing the potential for UV-induced DNA damage, photo-bleaching of fluorescent dyes, cross-linking of modified bromo-dT nucleotides, etc. Furthermore, ultraviolet front-side illumination can still be used for selective DNA release\textsuperscript{25} from the surface using standard photocleavable linkers,\textsuperscript{11} regardless of the illumination wavelength during PEC synthesis (\textit{i.e.} if larger band-gap materials are used); selective release is useful in limiting cross-talk in extremely complex DNA pools.
3.4.5 Detritylation control experiments

There were two questions that had to be answered in order to conclusively say that the images above result from site-selective detritylation by PEC-generated acids. First, is the patterning actually coming from PEC-generated protons rather than direct electrochemical removal? Second, are the acids actually detritylating the surface, as opposed to changing the morphology of the surface to make it more susceptible to non-specific adsorption of dye molecules?

Figure 15 shows bright-field fluorescence images of cy3-phosphoramidite checkerboards coupled to EML1 substrates that were biased at +1.5 V and exposed for 30 seconds. Note that these EML1 exposure conditions are not optimized. To show that the patterning is attributable to PEC-generated protons, a 1/10 equivalent of triethylamine scavenger was mixed into the solution to quench some portion of the protons and slow the reaction down. One would expect the fluorescence contrast of the buffered sample to be lower than the unbuffered sample, which is clearly seen in the left (buffered) and center (unbuffered) images of Figure 15.

**Figure 15.** Control experiments proving that cy3-phosphoramidite is covalently coupled to free 5'-OH groups that were deblocked by the PEC generation of protons. (Left) The exposure was performed in the presence of a buffer / proton scavenger that slows down the detritylation rate and diminishes the fluorescence contrast versus the unbuffered sample (Center). This experiment shows that the selective cy3-coupling results from PEC acid generation. (Right) An unbuffered sample was treated with capping solution to render the exposed hydroxyls unreactive, and then globally detritylated with standard deblock solution. The inverse image after cy3-coupling proves that the PEC generated acids actually detritylated the 5'-OH group, as opposed to making the porous glass more susceptible to non-specific adsorption. Scale bar = 400 μm.
Now that PEC acid generation has been proven, the possibility that the acid is actually detritylating the surface, rather than making it more susceptible to dye adsorption, must also be confirmed. If the surface molecules were actually being detritylated, then the exposed hydroxyls should be available for some covalent reaction other than phosphoramidite coupling. To demonstrate that this is indeed the case, the surface was exposed to the phosphoramidite capping mixture (acetic anhydride and catalyst) to render the exposed hydroxyls inert. The surface was then globally detritylated with the commercial deblock mixture (trichloroacetic acid), and cy3-phosphoramidite was coupled to the surface. This control experiment should have resulted in the inverse of the expected pattern, which is clearly seen in Figure 15.

3.5 PEC microarray synthesis

3.5.1 Materials and methods: Microarray synthesis

The substrate functionalization and synthesis procedures were largely the same as the PEC detritylation procedures described in Chapter 3.4.1. Prior to synthesis, four bases were coupled to the surface using the standard 0.2 μmol synthesis cycle (with trichloroacetic acid) to space the hybridization region away from the surface. The substrate was biased at +1.7 V and irradiated for 22 seconds at 1 W/cm². In order to limit delamination of the porous glass thin-film, the synthesis was paused after every four steps for several minutes. Following synthesis, the substrates were soaked in 50 mM potassium carbonate in methanol for 6 hours to remove the base-labile protecting groups on the phosphates and exocyclic amines, washed with methanol and acetonitrile, and then dried under nitrogen.

Cy3-labeled and (fluorescein) FAM-labeled hybridization oligonucleotides were received HPLC-purified from Integrated DNA Technologies. All buffers used were from Invitrogen. Prior to hybridization assays, microarrays were soaked in 1X SSC buffer with
0.1% SDS surfactant. Target sequences (1 μM in 2X SSC with 0.01% SDS) were hybridized to the microarray in a humidity chamber for 2 hours at room temperature. The substrates were washed with 1X SSC with 0.1% SDS, 0.5X SSC, 0.1X SSC, and then dried under nitrogen.

3.5.2 Hybridization assays of PEC microarray synthesis

The primary assay for successful microarray synthesis is the detection of sequence errors, such as mutations (wrong base), insertions (extra base), and deletions (missing base), by hybridization of fluorescent dye-labeled target strands. To test the ability to detect mutations, a microarray was synthesized with two different probe strand sequences:

5’- (TCCAGNNCGGTC)-3’, where NN = AT or TA.

The middle two base positions contain the exact same match/mismatch combination to a complementary pair of targets that were hybridized simultaneously to the microarray:

5’- (GACGCTACTGGA)TTAC - FAM -3'
5’- (GACGCATCTGGA)TTAC - cy3 -3’.

Each spot is perfectly complementary to one target strand in solution, and contains both A-A and T-T mismatches to the other at the exact same base positions. The desired fluorescence output of the cy3 channel is shown in Figure 16A.

A hybridization test such as this one ensures that the discrimination is dependent on neither sequence nor position. Figures 16B and 16C show fluorescence images of the substrate after simultaneous hybridization with both target strands. While some defects in the microarray are present, most notably because of defects in the porous glass film, this experiment clearly demonstrates that a PEC microarray synthesis platform has successfully been created. It should be noted that the bright areas within each spot (seen in Figure 16C) are caused by non-uniformities in the porous glass film.
morphology, which was verified by control experiments on the same chip that coupled cy3-phosphoramidite to halo-acid treated substrates (i.e. no PEC steps).

Figure 16. Hybridization assay of successful DNA microarray synthesis. Two different 12mers probe strands were synthesized with differences in their middle two bases only (AT or TA). Each probe strand has one perfectly matched target strand, and contains both A-A and T-T mismatches with the second probe strand (and vice-versa). (a) Expected cy3 channel output from a hybridization assay using two dye-labeled target strands (cy3 and FAM). The inverse of the image is expected in the FAM channel. (b) Low- and (c) high-magnification fluorescence micrographs after simultaneous hybridization with both targets. Channel assignments: cy3 = orange, FAM = green. Scale bar = (b) 600 μm and (c) 200 μm.

Figure 17 shows a checkerboard pattern of a single-base deletion error (dG) in the middle of a 16mer, including a line section of the fluorescence intensity. While this demonstrates that single-base errors (for probe strands of typical length) are detectable by hybridization to the PEC synthesized microarray, it is unclear why the contrast is not better. In addition to poor step-wise yield, one possibility may be very high levels of non-specific adsorption to the porous glass film. Hybridization experiments that did not use the tiny amount of surfactant in the mixture routinely showed large levels of non-specific
adsorption. As will be shown in Chapter 4, the porous glass film has a larger relative amount of surface area without full-length DNA strands than does a bare glass substrate typically used in microarray experiments.

![Image](image_url)

**Figure 17.** Checkerboard pattern and fluorescence intensity line section of a single-base dG deletion error in the middle of a 16mer.

### 3.5.3 Initial yield analysis

In order to estimate the step-wise chemical yield, the relative fluorescence signal intensity after N-coupling steps was determined by methods similar to ones reported by Nuwaysir *et al.* and McGall *et al.* Briefly, a 10 x 10 array was PEC-synthesized, where the column index corresponded to the oligonucleotide length (*i.e.* 5-mers were synthesized in column 5). After the 10 PEC synthesis steps, the substrate was treated with standard halo-acid deblock mixture, and then cy3-phosphoramidite was coupled to the surface. The signal is normalized against pads outside of the patterning area that did not participate in any PEC steps. Figure 18 shows the relative signal intensity and calculated step-wise yield.

The step-wise yield increases over the first 5 steps, finally leveling out at \( \sim 93\% \). The progressive yield increase over the first few coupling steps is consistent with both previous reports, and in all likelihood it is a trend that is observable with all DNA synthesis technologies. The DNA strand being synthesized requires more space than a
DMT-protected hydroxyl group on the surface. There are proportionally more DMT-protected hydroxyls not being coupled to (and then subsequently capped) in the early steps, than there are in the later steps. The asymptotic 93% step-wise yield is within the range of values from both previous reports (96% by Nuwaysir, et al. and 92 - 94% by McGall, et al.). These numbers vary significantly with reagent lifetime, though. Coupling efficiencies of 95% were observed with new reagents, whereas the efficiency dropped to 88% with reagents that were 2 days old.

Figure 18. Relative fluorescence intensity and step-wise yield. After PEC synthesizing an array with strands 1-10 nucleotides in length, the strands were globally deblocked with trichloroacetic acid, and then cy3-phosphoramidite was coupled to the entire surface. The step-wise yield increase over the first few addition steps is commonly observed in microarray synthesis.

As previously stated in Chapter 1.3.3, chemical synthesis yield is a complex and ill-defined term. “Yield” must be interpreted with respect to the assay and application. For example, the performed experiment essentially assumes 100% deprotection efficiency, and measures the DNA condensation efficiency (i.e. the coupling cycle and reagent delivery) and the damage to the 5'-OH during the PEC deprotection step (although comparisons by Cerrina and co-workers between the error rates obtained by this experiment and sequencing results showed that it provides a reasonable estimate of the DNA quality). Ultimately, the 93% step-wise yield is only a measure of the PEC synthesis platform’s efficiency at this moment.
3.6 References


Chapter 4

Characterization of the thin-film porous glass

The porous oxide that coats the substrates is a necessity to space the growing strands beyond the Helmholtz distance from the electrode surface, in order to prevent electrochemical damage.\(^1\) However, the exciting aspect of it is the massive increase in surface area that increases the oligonucleotide concentration per spot accordingly. For ultra-high density plate applications,\(^2\) this is a significant advance because given the minute amounts of unique DNA synthesized on a microarray makes it very difficult to obtain biologically relevant concentrations that can be easily handled. For example, proper gene assembly in a microfluidic environment requires a threshold concentration of \(\sim 5\) nM of each unique sequence once the oligonucleotides are cleaved from the surface. Increasing the loading decreases the spot redundancy required to achieve necessary concentrations and effectively increases spot density. Furthermore, it reduces the need for post-cleavage amplification procedures, which reduces the experimental cost and time, as well as the potential errors introduced by polymerase.

4.1 One-step film preparation

The formation conditions for traditional controlled pore glasses (CPG) are incompatible with thin-film chip technology, and alternatives like macroporous sol-gels\(^9\),\(^10\) and chemically etched nanostructures\(^11\),\(^12\) can be cumbersome to prepare. The porous glass reported here is formed in a one-step procedure, using a commercially available colloidal silica precursor as received or “off-the-shelf.” The precursor (Snowtex UP or OUP) was easily spun-coat on a variety of substrates (glass, silicon, thermal oxide on silicon, and Au or Al metal-coated slides), and then annealed at 400-420 °C with a
laboratory hotplate. Simple solvent washes were sufficient cleaning for most surfaces, although a brief O₂ plasma-cleaning step (Anatech, 100 mTorr, 50 sccm, 50 W) was sometimes employed to improve surface wetting. Films 0.6 - 1.4 μm in thickness were attained after a single coating step using undiluted solution (Figure 1A), as determined with a Sloan Dektak III profilometer.

![Graphs of film thickness versus spin-speed and volume percent Snowtex in ethanol.](image)

**Figure 1.** (a) Film thickness versus spin-speed curves of undiluted Snowtex solutions. (b) Film thickness of samples diluted in ethanol, spun at 2500 rpm.

Samples down to 70 nm thick can be obtained by diluting with ethanol, as determined by AFM of HF-etched structures (Figure 1B). To create step edges for measurement, the annealed films on silicon wafers (Wafernet) were first spun-coat with NR9-1000PY photoresist (Futurrex) for 40 seconds at 2000 rpm, and pre-baked for 5 minutes at 150 °C. The photoresist was exposed for 15 seconds with an ultraviolet flood lamp (Intelli-Ray 400), and then post-baked for 5 minutes at 100 °C. The exposed resist was developed for at least 10 seconds with RD6 developer, washed with dH₂O (Millipore), and dried under nitrogen. After the patterned films were buffered oxide etched (BOE, Aldrich) for up to two minutes, washed with dH₂O, and dried under nitrogen, the photoresist was removed by soaking in acetone.
It should be noted that other films have been utilized to increase the hybridization sensitivity of microarrays,\textsuperscript{12-14} including films created from different Snowtex colloidal particles. However, in our hands, the colloidal\textsuperscript{13} and chemically etched\textsuperscript{12} films were not as robust, and/or did not permit as much electrochemical current density as the film reported here.\textsuperscript{15} As will be discussed later, it is believed that the anisotropic geometry of the colloidal particles used in this work is responsible for the increased stability and apparent porosity. Attempts to recreate the porous reaction layers reported by Dill, \textit{et al.}\textsuperscript{14} were not possible due to lack of available information.

4.2 Surface loading density

UV-Vis spectroscopy was used to quantify the surface loading capacity of the film. Microscope slides (Electron Microscopy Sciences) were coated as described above. The substrates were amino-silanized with 1\% 3-aminopropyltriethoxysilane (APTES, Aldrich) in acidic methanol (5\% 1 mM aqueous acetic acid) for 30 minutes, washed with acetone, and dried under nitrogen. The amine was reacted with 10 mM DMT-dT-succinate (Monomer Sciences) and 50 mM N-N' dicyclohexylcarbodi-imide catalyst (DCC, Avocado Organics) in anhydrous dichloromethane for six hours under nitrogen, washed with acetone, and dried under nitrogen. Dimethoxytrityl (DMT) loading was quantified by treating the substrate with commercial deblock solution (3\% trichloroacetic acid in dichloromethane, Glen Research), and quantifying the supernatant using a HP8452A spectrophotometer. The area density was defined with respect to chip patterning area, not the internal surface area; the volume density or concentration was defined with respect to film volume (assuming 840 nm thick films), not the solution volume. These experiments were also performed on bare glass and with commercial CPG from Glen Research (Table 1). CPG had an experimental DMT concentration (trityl loading per volume glass) of 13.2 ± 0.3 mM, which is in good agreement with the 11.9
mM theoretical concentration based on the manufacturer's specifications (0.2 µmol scale, 25-40 µmol/g, 0.365 g/mL) and verifies the accuracy of the cleavage assay.

Table 1. Summary of cleavage experiments for 840 nm thick films. The area density is defined with respect to the chip patterning area (not the internal surface area), and the concentration is defined with respect to the volume of the solid support (not the solution volume).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dimethoxytrityl cation</th>
<th>40-mer (0.2µmol cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Area density (molecule/cm²)</td>
<td>Conc. (mM)</td>
</tr>
<tr>
<td>Flat glass (standard grade)</td>
<td>1.8 ± 0.1 × 10¹⁴</td>
<td>N/A</td>
</tr>
<tr>
<td>Porous glass (840nm thick)</td>
<td>1.3 ± 0.2 × 10¹⁵</td>
<td>25.0 ± 2.6</td>
</tr>
<tr>
<td>Controlled pore glass (0.2µmol)</td>
<td>N/A</td>
<td>13.2 ± 0.3</td>
</tr>
</tbody>
</table>

Figure 2. Fluidic cell for DNA synthesis using an unmodified 0.2 µmol phosphoramidite coupling cycle used to characterize the synthesis capacity of porous glass-coated substrates.

The DNA synthesis capacity was determined by synthesizing a 40mer with equal base content using the commercial ABI394 synthesis cycle (0.2 µmol scale) with “Sterling” phosphoramidite reagents (Glen Research). A milled-Teflon fluidic with the same dead fluid volume as a CPG synthesis column was used to adapt the synthesizer for synthesis on chips (Figure 2). Unpurified/crude samples were dried with a speed-vac after the final alkaline deblock (30% aqueous ammonium hydroxide, 8 hours, 55 °C), and then resuspended in dH₂O. Desalted samples were passed through a Sephadex G25
spin-column, and then re-suspended in dH₂O. Optical density measurements were made using a Nanodrop spectrophotometer. The data is also shown in Table 1.

The trityl concentration within the porous glass was an astounding 25 ± 2 mM, which correlates to an area loading density of 1.3 ± 0.2 x 10¹⁶ molecules/cm², or nearly two orders of magnitude greater than the loading of non-porous bare glass. The volumetric 40-mer DNA density within the porous glass was 4.7 ± 0.4 mM after desalting, which is nearly identical to the only other known colloid-based thin-film support reported by Frank and co-workers (120 pmol/cm² + 0.3 pm = 4 mM)¹³. Given this concentration, 15X more DNA is synthesized on a coated chip per micron film thickness than on a bare glass surface. In all likelihood, this relative increase is an underestimate because the float glass slides that were used were much rougher than standard microarray slides (i.e. more surface area than normal).

![Figure 3](image)

**Figure 3.** Fluorescence micrograph of cy-3 labeled 30-mer oligonucleotide hybridized to its surface-immobilized complement. The dark sections are wells that are chemically etched down to the bare glass surface, and thus the bright regions represent the relative increase in signal from the porous glass over the signal from a typical microarray on bare glass. Scale bar = 100 µm

To put this loading increase into perspective, holes were BOE-etched through the oxide down to the glass substrate. The substrate was silanized with 1% glycidoxypropyltrimethoxysilane (GPS) in acetone for 30 minutes, and then an amine-terminated 30-mer (Integrated DNA Technologies) was immobilized onto the epoxide-terminated surface overnight (1 µM in bicarbonate buffer). Figure 3 shows a confocal fluorescence image (Zeiss LSM Pascal) of the substrate after the cy3-labeled complement was hybridized to
the substrate. The fluorescence signal represents the relative increase over the amount of fluorescence intensity typically seen on a DNA chip, as the dark regions are actually monolayers of Cy3-labeled DNA.

Figure 4. Denaturing gel electrophoresis verifies that DNA of the correct length was synthesized on the porous glass films. All DNA was synthesized using the 0.2 μmol cycle of an ABI384 DNA synthesizer using the standard acidic deblock mixture. “CPG” denotes controlled pore glass.

In order to verify DNA of the appropriate length was synthesized in the porous glass matrix, denaturing electrophoresis gels (15% TBE-Urea, Invitrogen) were run against a 10 bp ladder (Invitrogen) in TBE buffer for 60 minutes at 180 V, and stained for 30 minutes with SYBR-Gold (Invitrogen) nucleic acid stain. Attempts were made to normalize the DNA concentration per lane by Nanodrop readings. The image was taken with a Polaroid GelCam. As can be seen in Figure 4, each sample shows a predominate band that is the desired 40-mer.

4.3 Physical properties and compatibility with other microarray platforms

As previously mentioned many times in this thesis, the porous glass films were physically and chemically robust. They easily survived mechanical “tape tests,” often stripping the adhesive from the tape presumably because of Velcro-like effects. Their stability towards prolonged exposure to concentrated sulfuric acid-based oxidizing solutions and strongly alkaline final-deblock mixtures permitted substrate reuse. Trityl
loading density experiments performed after these caustic treatments showed no more than 10% losses from the freshly prepared films. It should be noted, though, that the porous glass was still susceptible to scratches like all thin-films. Furthermore, portions directly on top of pads were susceptible to “lift-off” over the course of long and continuous exposures, and hence microarray syntheses were usually interrupted after every 4 base additions as a preventative measure.

Figure 5. Properties of an 840 nm-thick porous glass film. (a) Optical transmission curve. The dotted lines in the inset image delineate the edges of a coated microscope slide. (b) Cyclic voltammograms of 2.5 mM ferrocene in acetonitrile with 100 mM salt, using coated and uncoated gold working electrodes. The small difference in current density between the two curves indicates large porosity throughout the entire film.

The film is optically transparent, with better than 98% transmission from 350 - 700 nm (Figure 5A), as measured with a HP8452A spectrophotometer. Obviously, optical transparency at visible wavelengths is necessary for microarrays to allow fluorescence measurements. The transparency at ultraviolet wavelengths makes the film compatible with other optically driven microarray platforms\textsuperscript{16} that utilize near-UV illumination sources. Figure 5B shows cyclic voltammograms (C-V) of 2.5 mM ferrocene in acetonitrile with 100 mM tetrabutylammonium salt, that were taken using coated and
uncoated gold working electrodes (EMF Corporation, see Chapter 3 for testing details).
The large porosity throughout the entirety of the film is evident by the small difference in
current density between the uncoated and coated electrodes. Thus, the film is also
compatible with other electrochemical microarray synthesis platforms.\textsuperscript{16} It may also
prove useful for other applications that require a porous thin-film matrix, such as
electrophoretic manipulation of DNA,\textsuperscript{1} electrochemical bio-sensing,\textsuperscript{17} and colorimetric
gas sensing.\textsuperscript{18} The film’s chemical stability towards oxidizers is particularly attractive for
costly sensing devices that cannot be reused by stripping the film entirely, such as ones
with sealed fluidics and packaging.

4.4 Porous glass morphology and explanation of properties

The colloidal silica surface as a function of temperature. TG = Thermal gravimetric
analysis. DTA = Differential thermal analysis. Above 400 °C, surface silanol groups can cross-
link colloids in intimate contact by a condensation reaction. The particulate morphology of the
film is not expected to change until it is heated to the glass transition temperature of 800 °C.

The Snowtex UP and OUP precursors are aqueous solutions of sodium
stabilized, wire-like anisotropic particles with 9 - 15 nm x 40 - 300 nm dimensions.
Although the dimensions are larger than those typically associated with melting point
suppression,\textsuperscript{19} dehydration of the surface silanol groups above 400 °C can lead to
covalent bond formation between particles in intimate contact via silanol condensation
reactions (Figure 6). It is believed that the porous glass owes its porosity and stability to the anisotropic geometry of the colloids.

Figure 7. Porous glass film metrology by (a) AFM and (b,c) SEM. The films are disordered, with average pore sizes of 15 - 85 nm. The tilted SEM image (c) shows that the film disorder creates large pockets and channels, as well as bridge-like structures that leave much of the electrode physically untouched by the insulating particles. Scale bars = (a) 1.5 μm scan size, 15 nm vertical, (b, c) 1 μm.

Figure 7 shows atomic force microscopy (AFM) and scanning electron microscopy (SEM) images of the porous glass. The pores were typically ~15 - 85 nm in diameter. The film morphology is amorphous or disordered, thus giving rise to the large pockets and channels that are observable in the tilted SEM image (dark areas in Figure 7C). The disorder also creates many bridge-like structures that leave large portions of the electrode physically untouched by the electrically insulating particles (red box in Figure 7C). These large pockets and bridges are likely what permit significant Faradaic
current densities with coated electrodes. Another consequence of the wire-like geometry and disorder is that the film is mesh-like, and therefore contains many contact points between particles that lead to large degrees of cross-linking and film stability.

On the other hand, the films reported by Frank and co-workers,\textsuperscript{13} which were formed under nearly the same conditions with spherical colloids from the same manufacturer, are very ordered and essentially multi-layers of close-packed (CP) spheres. While the CP films were annealed at 350 °C, which is below the silanol condensation temperature and annealing temperature used to form the films reported here, it is expected that the differences in particle geometry contribute most to the increased film stability. The particles in CP films would have very few intimate contact points between them, and as a consequence, the film would be far less cross-linked and stable than the disordered ones composed of anisotropic particles. Furthermore, ordered CP films would be much less porous for geometric reasons and lack bridge-like structures (\textit{i.e.} less available electrode surface area), thus explaining the low Faradaic current densities observed with electrodes coated with these CP films.
4.5 References


Chapter 5

Advances in surface science

This chapter details the myriad of novel surfaces, surface chemistries, and biomolecule patterning techniques that were developed throughout the course of this thesis work (in order of appearance):

- **Perfecting imperfect monolayers** - A post-silanization cleaning method for reliably creating robust siloxane monolayers when modifying glass and silicon.
- **Methods for forming self-assembled monolayers on indium tin oxide.**
- **Critical energy electron beam lithography (CEEBL)** – A method to directly pattern insulators by electron beam lithography without the use of charge dissipation layers to limit pattern distortion.
- **A simple and general strategy for nanoscale biomolecule patterning on glass and silicon by creating binary chemical patterns of practically any biologically relevant terminal functionalities.**
- **Ultra-flat gold surfaces by template stripping methods.**
- **Micro-contact printing thiols on glass and silicon substrates coated with a molecularly flat adhesion layer.**
- **Patterning DNA tiles into arbitrary two-dimensional shapes.**

While colloid and surface science is by no means inherently more difficult than any other science, it often troubles researchers across all fields. The common theme among all the methods presented in this chapter is that they are simple technological solutions to needlessly difficult problems, whose solutions to date are often quite complicated or expensive to implement.
5.1 Materials and Methods

Unless stated otherwise:

Organic synthesis reagents were used as received from Aldrich. Chemical surface modification reactions were performed in Teflon vials from Savillex. DNA was synthesized and purified (as appropriate based on the manufacturer's specifications) by Integrated DNA Technologies. N-type silicon wafers (including thermal oxide-coated ones) were obtained from Wafernet. Glass slides and coverslips were obtained from Electron Microscopy Sciences and VWR, respectively. Metal pellets for thermal evaporation were obtained from Kurt J. Lesker. Indium tin oxide (ITO) coated slides were obtained from Delta Technologies.

Fluorescence images were captured with a Zeiss LSM Pascal. Scanning electron microscopy (SEM) imaging and electron beam lithography (EBL) was performed on a FEI XL30 environmental SEM (operated in standard high-vacuum mode) equipped with a Nabity NPGS lithography package. Atomic force microscopy (AFM) images were taken with a Digital Instruments Dimension 3000.

5.2 Perfecting imperfect siloxane "monolayers" by CO$_2$ treatment

All microarrays reported to date utilize a surface silane linker to space the growing strand from the surface of the chip. However, the microarray field lacks reliable methods for uniform silanizations, despite its significant impact on microarray hybridization data. Microarray papers often depict the linker molecules as self-assembled monolayers for conceptual simplicity, but in reality, that is seldom the case. Without precise control over water content, pH, concentration, temperature, humidity, and surface cleanliness, it is nearly impossible to prevent spurious multi-layer formation. This unreliability in such a critical process can be quite detrimental to the quality of the microarray. Because multi-layers have decreased physio-chemical stability,
at elevated temperatures in aqueous solutions, the surface immobilized probe strands can be unintentionally cleaved from the surface. Furthermore, the ability to perform good surface silanizations is crucial to other technological areas, such as microfluidics (e.g. polyethylene glycol coatings for anti-fouling) and nanoscale electronics.

Figure 1. Scheme of TEOS-HBA siloxane multi-layer removal by CO₂ treatment. The underlying base-layer is highly cross-linked after annealing. (a) Multi-layers form by adsorption, alkyl chain intercalation, and covalent bonds (Si-O-Si o Si-O-C) with various degrees of cross-linking. (b) Adsorbed, intercalated, and poorly cross-linked molecules are hydrolyzed and partially removed by soaking in distilled water. (c) CO₂ efficiently removes all remaining hydrolyzed silane molecules.

Thus, a simple and reliable silanization protocol was sought out. It was shown that spurious multi-layers could be removed from films with sufficient cross-linking densities in the initial base layer by undergoing a post-silanization hydrolysis step followed by a CO₂ “snow” cleaning. The method is based on a previous report in which undesired multi-layers were hydrolyzed and removed by soaking for 24 hours in water (to our knowledge, the only other reported post-formation “cleaning” method). The authors found that multi-layers were more susceptible to hydrolysis than the initial siloxane layer because of greater cross-linking in the base layer. This is consistent with
the observation that multi-layers are less resilient to physical abrasion than monolayers and exhibit decreasing order with increasing numbers of layers. The procedure is schematically shown in Figure 1 for N-(3-Triethoxysilylpropyl)-4-hydroxybutyramide (TEOS-HBA, Gelest), a useful silane linker for DNA arrays. Adsorbed and covalently bound multi-layers sit atop a highly cross-linked base monolayer (Figure 1A). After hydrolyzing the disordered multi-layer islands (Figure 1B), CO\textsubscript{2} snow treatment (Applied Surface Technologies) removes the remaining adsorbed and hydrolyzed molecules more effectively than common solvents (Figure 1C).

In CO\textsubscript{2} snow cleaning, CO\textsubscript{2} gas and “snow,” small dry ice and liquid CO\textsubscript{2} particles, are directed towards a surface. The momentum transfer from the ice particles disrupts adsorbed surface particulates. Liquid CO\textsubscript{2} particles solvate small organics like hydrocarbons and silicones, and capture them as the liquid CO\textsubscript{2} solidifies into dry ice. The high-velocity gas stream then removes the particulates and the captured organics. However, CO\textsubscript{2} snow has been shown not to damage siloxane self-assembled monolayers (SAMs). Given that it would not destroy highly cross-linked monolayers despite its effectiveness in removing adsorbed silicones, we presumed that CO\textsubscript{2} would be an excellent solvent for multi-layers composed of loosely bound and hydrolyzed silane molecules.

A general procedure involved first soaking the substrate in a semi-dilute silane solution (0.5 - 1% v:v) in an appropriate solvent. Occasionally, acidic or basic catalysts were used to promote silanol-intermediate formation from trialkoxysilanes. After briefly curing the samples (room temperature for long chain alkyl trichlorosilanes, 150 – 200 °C for others), the samples were soaked in dH\textsubscript{2}O (Millipore), and then CO\textsubscript{2} snow cleaned. Isopropanol was used to remove any dry ice that formed during CO\textsubscript{2} snow treatment. The effectiveness of this procedure is demonstrated in Figure 2, which shows an AFM (Digital Instruments Multi-mode) image of an octadecyltrichlorosilane (OTS) monolayer.
on silicon. The post-cleaned sample had an ellipsometric film thickness of 2.5 ± 0.1 nm (Sentech SE700) and root-mean squared (RMS) roughness of 0.157 nm, which are consistent with expected values for OTS monolayers. The advancing water contact angle for the hydrophobic coating was 110 ± 2°, with a very low hysteresis value of 6 ± 3°, as measured with a custom-built goniometer, which indicates that the film was highly uniform and defect free. Thus, an imperfect film could essentially be perfected to yield atomically flat self-assembled monolayers.

**Figure 2.** Tapping-mode AFM images of OTS films. (a) Film before CO2 treatment. Multi-layers islands appear to have formed by diffusion-limited aggregation. The largest island in the scan area measured 5 - 6 additional layers thick. The majority of the multi-layers are one or more additional layers thick. (b) Film after CO2 treatment. The ellipsometric film thickness was 2.5 ± 0.1 nm, and the RMS roughness was 0.157 nm.

Infrared spectroscopic analysis provided further evidence that the films were highly ordered after CO2 snow treatment. One of the hallmarks of self-assembly is that energy minimization leads to structural order. Just because a film’s average thickness is equal to that of a monolayer, it does not mean that the film is a self-assembled monolayer. Figure 3A shows a Fourier transform infrared spectra (FTIR, Digilab…
Excalibur FTS3000) of TEOS-HBA in chloroform, in which the amide I peak appears at 1656 cm\(^{-1}\). The amide I peak is red-shifted to 1648 cm\(^{-1}\) in the attenuated total reflection spectrum (ATR-FTIR) of the monolayer film (Figure 3B), which was taken on Nicolet Nexus FTIR using a fixed 65° angle GATR accessory\(^7\) (Harrick Scientific). This red-shift results from hydrogen bonding within the monolayer that stabilizes the film, and is indicative of the energy minimization\(^8\) required for true self-assembly.

![Figure 3](image)

**Figure 3.** Infrared spectroscopy spectra of TEOS-HBA. (a) FTIR analysis of the silane molecule in chloroform, and (b) ATR-FTIR analysis of a monolayer film on silicon with native oxide (SiO\(_2\)/Si). The amide peak is red-shifted in the monolayer spectra (1648 from 1656 cm\(^{-1}\)), indicating the presence of hydrogen bonding within the film.

Table 1 at the end of this chapter provides a suite of protocols for modifying glass and silicon with chemical terminal groups commonly needed in microarrays, microfluidics, and bio-nanotechnology. It also includes the compatibility of the formation conditions with polymethyl(methacrylate) (PMMA, Microchem) and Novolak resists (Shipley 1805 and AZ Electronic Materials 4620) that are used in electron beam and optical lithography, respectively. This compatibility information is particularly helpful when the goal is to pattern chemical functionalities on a surface. This suite will later be
used to create practically any binary chemical pattern (e.g. arrays of biotin with PEG background for streptavidin binding) using standard lithographic techniques.

5.3 Studies in siloxane monolayer formation on indium tin oxide

The silanization protocols in Table 1 can be adapted to coat indium tin oxide (ITO) if trialkoxysilanes are used exclusively in lieu of trichlorosilanes to prevent ITO etching, and the soaking times are increased. When the fluoroalkane trichlorosilane, (Tridecafluoro-1,1,2,2- tetrahydrooctyl)trichlorosilane (FOTS), was deposited in perfluoromethyldecalin, the ITO layer was etched, and the etch rate was dependent on time and concentration. It was thought that the HCl liberated during formation of the silanol intermediate from the trichlorosilane instigated an auto-catalytic etching process. When the silanol forms on the surface prior to film cross-linking by condensation, the HCl etches the ITO film and removes the bound silane. A fresh ITO surface is exposed, and this process repeats until the ITO is completely etched. Indium chloride (InCl₃) and stannic chloride (SnCl₂) are common organometallic reagents that are highly soluble in water and most organic solvents, making ITO highly susceptible to HCl etching. The enthalpy of formation for HCl etching of indium oxide (ITO is primarily indium oxide) is ΔH ~ 800 kJ/mol, making the etching reaction highly favorable.

\[
\text{In}_2\text{O}_3 + 6\text{HCl} \rightarrow 2\text{InCl}_3 + 3\text{H}_2\text{O}
\]

It has been reported that trichlorosilanes can be deposited onto ITO using a (7:3) heptane : carbon tetrachloride solution. Silanizations in that solvent mixture did exhibit much slower etch rates, presumably because of chloride ion solvation by carbon tetrachloride that diminishes its reactivity at the surface. However, solvent tailoring is a sub-optimal answer to the etching problem. Vapor deposition did not etch the substrate,
presumably because the HCl is quickly carried away from the surface under vacuum. However, vapor deposition is a highly unrepeatable process without the use of complex chemical vapor deposition equipment.

Therefore, trialkoxysilanes were used to silanize the surface in lieu of trichlorosilanes. Much longer deposition times were required because trialkoxysilanes are less reactive than their halogenated counterparts. Full monolayer coverage on silicon was achieved after soaking for 6 hours in a 1% FOTS solution in perfluoromethyldecalin, and no increase in thickness (1.1 ± 0.1 nm) was noticed even after soaking for 12 hours. Similar etching issues were observed with OTS on ITO substrates, but changes to trialkoxysilanes also solved the problem. As a side note, the ability to modify ITO substrates without etching should be very useful to display technologies that use ITO as a transparent electrode material.9

5.4 Critical energy electron beam lithography 10

Electron beam lithography (EBL) is the best way to prototype high-resolution features,11, 12 but surface charging effects that can cause pattern distortions have historically limited its use in patterning electrical insulators like glass. This is a serious impediment for biological applications because glass is the surface of choice, especially in fluorescence-based studies because semiconductors and metals quench the signal. Several methods have been employed to address this issue, most notably the use of thin metallic charge dissipation layers that must be etched after patterning.13 Environmental SEM (ESEM) that can operate in the presence of a small amount of water vapor can also eliminate charging,14 but it requires a sophisticated and costly gas-delivery system. Transparent conductors like ITO can be used,15 but the multiple indices of refraction can complicate fluorescence imaging in our experience, and ITO chemical functionalization is not as robust as it is on glass.
Critical Energy Electron Beam Lithography (CEEBL)\textsuperscript{10} was developed as a means of e-beam patterning sub-micron features directly on glass. In CEEBL, the lithography system is operated at the critical energy ($E_2$) of the resist-on-insulator (Figure 4A), which is the energy at which there exists a dynamic charge balance between the injected and kicked-out electrons that leaves the surface charge neutral. Thus, insulators can be e-beam patterned without distortions created by surface charging effects that deflect the beam (Figure 4B).

![Figure 4: Principles of Critical Energy Electron Beam Lithography (CEEBL). (a) Total electron yield ($\sigma$) vs. beam energy for a typical resist. The bulk insulating material is positive charged when $\sigma > 1$ and negatively charged when $\sigma < 1$. The charge buildup is zero at the critical energy or crossover voltage ($E_1$, $E_2$), where $\sigma$ is unity. (b) Schematic of the beam-induced surface charging at different patterning energies. Note that $E_1$ (typically tens of eV) is ignored because it is well below the operating regimes of electron beam lithography.](image)

![Figure 5: Pattern distortion in electron beam lithography on insulators as a result of surface charging, and its elimination by CEEBL. (a) Design of the desired pattern. SEM images of 5nm thick Au electrodes on glass coverslips after acetone lift-off of PMMA that was patterned at (b) 1.3 keV ($E_2$) and (c) 5 keV. Charge induced pattern distortions are prominent at 5 keV (circled). Scale bar = 10 $\mu$m.](image)
Figure 5 demonstrates how surface charging causes pattern distortion in electron beam lithography by deflecting the beam, and how CEEBL effectively circumvents the issue. Figure 5A shows a desired electrode pattern to be patterned directly onto glass using 65 nm thick PMMA resist. Figures 5B and 5C show SEM images of 5 nm thick thermally evaporated gold electrodes (Edwards FL400) after PMMA liftoff in acetone. The charge induced line mismatches that are prominent at 5 keV (circled in Figure 5C) are non-existent when the substrate is patterned at $E_2$ (Figure 5B).

![Image]

**Figure 6.** Beam deflection at various voltages. Two parallel single-pass reference lines were first patterned with a 1μm gap, followed by charge pads written at 30 μC/cm$^2$. Finally, a third single-pass line was patterned between the pad and the reference line. 5 nm thick gold was thermally evaporated after developing the PMMA and imaged under SEM to determine the line deflection. The deflection was virtually eliminated at $E_2 = 1.3$ keV, whereas the beam was largely deflected due to positive surface charging below $E_2$ and negative charging above it.

In order to quantify the effectiveness of CEEBL, the beam deflection at various voltages was measured by a method previously reported by Craighead and co-workers. Briefly, two parallel single-pass reference lines were first patterned with a 1 μm gap, followed by large charge pads written at 30 μC/cm$^2$. Finally, a third single-pass line was patterned between the pad and the reference line. 5 nm thick gold was thermally evaporated after developing the PMMA and imaged by SEM to determine the line deflection. As can be seen in Figure 6, the deflection is practically eliminated by
CEEBL, whereas the beam was largely deflected below $E_2$ because of positive accumulated surface charge, and negative charging above $E_2$.

CEEBL is extremely rapid because the low-voltage beam interacts with a large volume element of the resist; for example, 65 nm thick PMMA was fully exposed at a dose of 10 $\mu$C/cm$^2$ at 1.3 keV, which is over an order of magnitude faster than in typical high-voltage (30 keV) lithography. The technique has been used to also successfully pattern NEB-31 (Sumitomo) and HSQ (Dow Corning) resists. The main drawback of CEEBL at this point is that the fundamental resolution limit is worse than it is with high-voltage EBL, which is a drawback common to all low-voltage techniques because of lateral scattering.

5.5 Simple and general strategy for nanoscale biomolecule patterning

The ability to pattern biomolecules on surfaces at the nanoscale is of great utility for applications in tissue engineering,$^{17}$ cell mechanics,$^{18}$ and hybrid bio-nanoelectronics,$^{19}$ to name a few. There have been several e-beam lithographic schemes reported in the literature for patterning biomolecules or creating binary chemical patterns for their selective immobilization (e.g. biotin arrays with PEG backgrounds for streptavidin immobilization), but none of these approaches is truly general and simple. DNA and proteins have been used as positive e-beam resists,$^{11,20}$ where the beam exposure renders them biologically inactive, but these processes are poorly understood. Furthermore, it is unlikely that unexposed biomolecules of all types will survive such processing. Functional biopolymers have also been patterned by using the e-beam to selectively cross-link the monomers,$^{12}$ but this scheme also lacks generality because the polymer choices are limited. Binary chemical patterning schemes have been demonstrated, but they require costly deposition and etching equipment in order to
circumvent silanization issues in the presence of lithographic resists,\textsuperscript{15, 19, 21-24} namely their solvent compatibility and preserving image polarity.

Surface silanization schemes inherently require that the terminal group of the first silane be chemically inert to prevent the subsequent silane from reacting with the terminus of the first, which would otherwise destroy the pattern. This imposes undesirable design constraints because PMMA, by far the most common e-beam resist, is a positive resist. Since e-beam lithography is serial, the image polarity cannot be simply reversed to accommodate this need, and negative resists often do not lift-off cleanly, leaving “scum”. The simple chemical strategy proposed here utilizes resist-compatible silanizations and protecting group chemistry to circumvent these issues, offering a facile route to generating nanoscale chemical patterns on glass or silicon.

Figure 7. High-resolution binary chemical patterning on glass. (a) General chemical functionalization scheme using PMMA resist. (b) Fluorescence micrograph of cy3-DNA-amine covalently bound via a disothiocyanate cross-linker. The background is passivated by FOTS. (c) Fluorescence micrograph of AlexaFluor546 streptavidin bound to surface immobilized biotin-NHS. The background is passivated with m-PEG-amine (MW = 5000) grafted to a surface-immobilized epoxide. Dimensions (spot diameter x pitch) = (b) 0.5 x 4 μm and (c) 1 x 4 μm. Scale bar = 10 μm.
The chemical functionalization scheme is shown in Figure 7A. The exposed glass regions are silanized for 5 minutes in 0.05% 3-aminopropyltriethoxysilane (APTES) in water, which is a PMMA non-solvent. The amine is protected by soaking the substrate for 30 minutes in 50 mM fmoc chloroformate or t-boc anhydride in carbon tetrachloride, which is another PMMA non-solvent. These protecting groups were chosen for their common use in peptide synthesis and safe deprotection conditions. After PMMA lift-off in acetone, any subsequent silane can be deposited (see Table 1) because the amine is stably protected. The key to this strategy that makes it general is the basic understanding that once the amine is deprotected, there exists a vast suite of amine-reactive molecules and labeling reagents to easily convert it into virtually any other terminal functionality useful for biochips. For example, it could be linked to biotin to immobilize streptavidin, or converted to amine-reactive electrophile using a bi-functional cross-linker to immobilize DNA. It can also be converted to another useful chemical group like an alkyne for “click” reactions (e.g. via coupling with undecynoic acid), or used to graft polymers.

In order to demonstrate the effectiveness of the proposed scheme, fluorescent biomolecules were immobilized on high-resolution binary chemical patterns that were created as proposed in Figure 7A on CEEBL-patterned substrates. Figure 7B shows a bright-field fluorescence image of 0.5 μm x 4 μm array (diameter x pitch) of cy3-labled DNA that is covalently bound to the surface via a bi-functional phenylene-diisothiocyanate (PDC) linker; the background is passivated with FOTS. In this sample, the base-labile fmoc group was used. Figure 7C shows a 1 μm x 4 μm array of AlexaFluor-546 streptavidin (Invitrogen) bound to a biotin-ylated surface. The background is passivated with surface grafted m-PEG-amine (MW = 5000, Nektar)

(*) The data reported in this section uses thermally oxidized silicon wafers (1μm oxide) in lieu of glass. The wafers are atomically flat, making them better AFM substrates.
Therapeutics) via an epoxide linkage (glycidoxypropyl-trimethoxysilane, GPS). Here, the acid-labile t-boc group was used to eliminate the possibility of fmoc deprotection by the terminal amine of the PEG compound. The t-boc protecting group is sufficiently stable towards the mild acidity of the silane reagents. Clearly, this chemical strategy is an effective means to create high-resolution patterns for immobilizing biological molecules, using only a SEM.

Figure 8. High fidelity chemical image transfer. (a) AFM of PMMA patterned on glass by CEEBL. (b) LFM of the same substrate after binary chemical functionalization with APTES (lines) and FOTS shows no measurable feature size degradation. Dimensions (line x spacing) = 0.2 x 2 μm. Horizontal scale bar = 1 μm.

Because of the resolution limit of confocal microscopy, the fluorescence images do not conclusively prove high feature size fidelity. Thus, to prove that the chemical processing did not degrade the resist, 200 nm lines spaced by 2 μm were first patterned in PMMA, as shown in the contact mode AFM image in Figure 8A. Then, binary chemical patterns of APTES and FOTS were created and imaged by fluid-phase lateral force microscopy (LFM, under 1 mM HCl), as shown in Figure 8B. No feature size degradation is observable, thereby proving the resolution fidelity despite the solvent-based processing in the presence of a polymeric resist.

Even though this chemical functionalization strategy tackles the problem of nanoscale biomolecule patterning on glass and silicon, the combination of resist-
compatible silanizations and protecting group chemistry (Table 1) still eliminates all unnecessary vacuum processing regardless of size scale, since the proposed chemistries are all also compatible with Novolak resins used in optical lithography. It should be noted that in optical lithography, where the image and/or mask polarity can easily be adjusted, simpler binary chemical patterning routes do exist (*i.e.* inert silane can be deposited first). However, in the case where both terminal groups are reactive (*e.g.* arrays of amines with a background of thiols), protecting group chemistry must be employed to preserve the pattern. Therefore, the scheme proposed in Figure 7A is a truly general binary chemical patterning scheme.

5.6 Ultra-flat gold surfaces by template-stripping

Self-assembled monolayers can only be as flat as the surface under them. While most applications of patterning molecules on gold surfaces do not require atomically flat surfaces, ultra-flat gold is an important surface for AFM studies and alkanethiol SAM-based nanotechnologies.\(^1\) One method for preparing ultra-flat gold is template-stripping,\(^3\) in which a thin-layer is evaporated onto an atomically flat surface (*e.g.* polished silicon wafers, highly ordered pyrolytic graphite, or freshly cleaved mica) and then transferred to another surface. The exposed gold surface after stripping is ultra-flat because it was originally the surface layer of atoms in intimate contact with the atomically flat surface. Template stripping is perhaps the most ideal preparative method because it allows a “fresh” surface to be exposed on demand.

A simple diffusion-bonding technique that requires only a machine-shop vise was developed to prepare ultra-flat template stripped gold (TSG). After compressing gold-on-glass with gold-on-mica, the thin layer of gold is stripped from the mica and transferred to the glass microscope slide by thermo-compression bonding (Figure 9). Since no polymeric adhesives are used in the process to fasten the layers, the
substrates have solvent and thermal compatibilities similar to normal gold-on-glass substrates. Current gold thermo-compression\textsuperscript{31} and diffusion-bonding methods\textsuperscript{32} used in the semiconductor fabrication industry are unsuitable for a normal laboratory environment. The key to this new method was the use of an aluminum foil stack to distribute the pressure uniformly across the substrate. Polymeric gaskets cannot handle the elevated temperature and pressure (300 °C, 3500 – 4000 psi) required for thermo-compression bonding. Metal foils were sufficiently malleable to even the pressure distribution at elevated temperatures by compaction, which is the densification of the metal foil layers by the removal of the voids and ridges found on the foil surfaces.

![Image of bonding stack](attachment:image.png)

**Figure 9.** Scheme for template stripped gold made by diffusion bonding. (a) The stack is compressed in a vise for 2 hours at 300 °C. (b) After removing the stack from the assembly, the mica is stripped by soaking in water or by prying with tweezers, transferring an ultraflat surface to the gold-on-glass.

Figure 10 shows AFM images of TSG substrates after gold-diffusion bonding at 300 °C for 2 hours. Step edges that arise due to strain relief through slip along the (100) glide planes\textsuperscript{33} intersect to form triangular features that indicate that the exposed surfaces are the (111) lattice plane. The RMS roughness was 0.2 - 0.5 nm over a 1 μm scan area, and uniform surfaces up to 1 cm\textsuperscript{2} in size were easily prepared by this method. It should be noted that the diffusion-bonding method reported here was primarily developed by David Mosley. My major contribution to the work was to study
the effect of metal composition and thickness on the uniformity of the diffusion bonding. These studies led to explanation that compaction of the metal foil, and not the thermal expansion coefficient alone, are responsible for the even pressure distribution.

![AFM images](image)

Figure 10. AFM images of a TSG substrate made by gold diffusion bonding at 300 °C for 2 hrs. Large areas of the gold surface consist of aligned step edges, some of which intersect to form triangular features. The step edge features, which are from 1 - 3 atoms high, are due to glide along the (100) planes in the Au lattice. Z-height scale = 5 nm.

5.7 Micro-contact printing on glass

Microcontact printing (μCP) or soft lithography is a powerful and simple way to pattern SAMs and biological molecules. The most common schemes involve printing alkanethiols on gold with polydimethylsiloxane stamps (PDMS). Long chain thiols "ink" PDMS very well, are soluble in polar solvents like ethanol and dimethylformamide that do not swell or deform the stamp, and have very little reactivity towards PDMS. On the other hand, microcontact printing silanes on glass, which is by far a more useful surface for biological experiments, is not quite as easy a task. The silane reagents can react with the stamp, and also react violently with air, which is problematic because the reaction time to form siloxane SAMs on oxide surfaces by μCP is significantly longer than it is for alkanethiols on gold. As a result, the process must be performed in an inert atmosphere, and pressure must be applied to the stamp in order to
obtain high quality siloxane films by soft lithography. Difficulties also arise when microcontact printing aqueous biomolecules because of the hydrophobicity and fouling properties of PDMS.

Figure 11. Surface-patterning of perfluoro-dodecylthiol (PDT) and immobilization of DNA.

Ideally, one would have the ability to microcontact print onto glass with the ease of alkanethiol printing on gold surfaces. It was thought that one could print thiols on a glass surface covered with a thiol-reactive, epoxy-terminated monolayer as an adhesion layer, rather than print a silane molecule directly. The difficulty of pCP silanes on glass is circumvented and replaced by the ease of pCP thiols with PDMS. Since the reaction occurs so quickly (10 seconds), biomolecules with terminal primary amines or thiol groups can then be back-filled into the exposed regions before the epoxy groups are rendered inert by reacting with water in the air. Likewise, other thiols can also be backfilled using any solvent of choice. The scheme is shown in Figure 11, where a passivating perfluoro-dodecylthiol (PDT) is printed, followed by backfilling with a nucleophile-terminated DNA.

In a typical experiment, an epoxide monolayer was formed by soaking the substrate in 0.5% GPS acetone, washing with acetone and briefly with IPA, and then drying under nitrogen. The substrate was then CO₂ snow cleaned without the hydrolysis step of soaking in dH₂O. A PDMS stamp was impregnated with thiols by the "inkpad"
method,\textsuperscript{36} in which the contoured stamp is placed in conformal contact for 40 seconds with a flat PDMS slab or inkpad that is saturated with the 0.1 mM inking solution (inkpad is dried under nitrogen immediately prior to stamp inking). After stamping the epoxide coated surface for 10 seconds, the substrate is immersed in the backfilling solution; in the cases that nucleophile-terminated biomolecules were to be back-filled, the fluid was placed under a coverslip or loaded into a Hybri-well / Secure-seal chamber (Invitrogen).

![Figure 12](image)

**Figure 12.** SEM images of masters made of NEB-31 that were e-beam patterned on gold. Images courtesy of David Mosley, MIT. Scale bars (a) = 5 µm, (b) = 10 µm.

Figures 12A and 12B show SEM images of high-resolution molds or masters for PDMS stamps that were created by electron-beam lithography of NEB-31 negative photoresist on gold-coated slides (see Mosley\textsuperscript{37} for fabrication details of both the master and PDMS stamp). After microcontact printing the inert PDT (Fluorous Technologies) and backfilling with labeled DNA, the fluorescence signal should be the same as the features on the master, both of which are the negative relief of the PDMS stamp (with respect to image polarity, not height).

Figure 13A shows a bright-field fluorescence image of double-stranded DNA (dsDNA) that is bound to the surface via a 5'-terminal amine on one strand, and is cy3 dye-labeled on the 5'-end of the complement. Figures 13B and 13C show fluorescence
images of streptavidin-coated Fluospheres (40 nm diameter polystyrene beads loaded with fluorescein, Invitrogen) bound to biotinylated surface-immobilized dsDNA. The patterns in Figures 13 correspond to the masters/molds shown in Figure 12.

Figure 13. Microcontact printing thiols on glass and silicon coated with an epoxide monolayer as an adhesion layer. (a) Bright-field fluorescence image of double-stranded DNA with a 5' terminal amine on one strand, and 5'-terminal cy3 dye on the other. (b,c) Streptavidin-coated Fluospheres bound to a surface immobilized biotin-ylated duplex. The background was passivated with perfluoro-dodecylthiol for both images. PDMS stamps made from the masters in Figures 12A and 12B were used to create (a) and (b,c), respectively. Scale bar (a) = 5 μm, (b,c) = 10 μm.

To the best of our knowledge, such an approach is the first of its kind for μCP on glass and silicon. Huck and co-workers have reported a similar "adhesion layer" approach for μCP polyethylene-imine polymers on gold. In a later report, they showed that the reactions between microcontact-printed and surface-immobilized molecules proceed quite quickly, by forming amide bonds for peptide synthesis without the use of a catalyst. Such an increase in reaction kinetics is crucial to the success of the μCP
scheme reported here because it limits the epoxide's exposure to air, thus making the scheme more practical than others that require inert atmospheres.

5.7.1 Towards self-replicating two-dimensional polymers

The creation of two-dimensional polymers as "molecular objects" has been an area of interest in polymer chemistry and nanoscience for over a decade because of their potential use in "bottom up" approaches to fabricating functional devices. Self-assembling DNA nanostructures are ideal candidates for molecular building blocks because of the large amount of sequence-encoded information that be used to direct not only their own assembly, but also to template the assembly of other structures onto them, such as DNA-capped nanoparticles. DNA assembly is an incredibly rich field, but unfortunately most of the architectures result in trivial shapes or periodic patterns. Given a limited number of inputs, it is very difficult to break symmetry in self-assembly.

Several strategies to break symmetry such algorithmic assembly and DNA origami have been reported, but all the complexity and symmetry-breaking is at the nanoscale. Ideally, one would also have the ability to shape the structures at the microscale, just as macroscale polymers can be molded and formed. One route to breaking symmetry in two-dimensions at the microscale is programmed assembly using surface templates. Patterned surfaces can physically define boundaries to act as molds that impose non-trivial geometric constraints on self-assembly. This is a design strategy commonly employed in nature during biomineralization processes in which protein-based scaffolds are first constructed and then filled with inorganic material.

The general scheme for patterning DNA tiles is shown in Figure 14. A base monolayer of "anchor strands" is first patterned on a surface. This base layer templates the formation of an adlayer on top of it by hybridization to linker strands that point orthogonally out of the plane of the two-dimensional polymer. The portions of the
adlayer that are not hybridized to an anchor strand are melted off. The patterned adlayer is ligated or polymerized (e.g. psoralen cross-linking), and then released from the surface (e.g. enzymatic cleavage). In theory, the released structure can also serve as a template for a subsequent cycle of pattern formation, thereby providing a route to generate large quantities of lithographically defined patterns by self-replication (Figure 15). These patterns could then be converted into functional materials using DNA-coated nanoparticles or electroless plating techniques.

**Figure 14.** Scheme for patterning DNA tiles into arbitrary two-dimensional shapes.

**Figure 15.** Scheme for self-replicating two-dimensional DNA tiles.
Figure 16 shows the structure of the DNA tile set, which has been modified from the double crossover design reported by Kiehl and co-workers,\textsuperscript{43} where a single-strand region that is complementary to the anchor strand sticks out orthogonally from the plane of the sheet. The orthogonal strand contains a 4-nucleotide single-strand gap to act as a hinge that accounts for variations in surface roughness. The anchoring system is a two-component system, where one strand is covalently bound to the surface and the other acts as a linker to the tile. The purpose of the two-component strategy is to allow the tiles to potentially be cleaved from the surface without altering the length or composition of the anchor strands. Therefore, the patterned surface, in theory, can be used as a reusable master. This particular design can be cleaved by uracil-DNA-glycosylase.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure16.png}
\caption{Structure of an individual DNA tile.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure17.png}
\caption{Melt curve of a pre-assembled sheet of tiles measured by fluorescence resonance energy transfer (FRET). Note that this curve measures the sheet melting temperature, which is representative of the inter-tile interaction, not the intra-tile melting.}
\end{figure}
Figure 17 shows a “melt curve” of a pre-assembled sheet of tiles, measured by fluorescence spectroscopy (Jovin-Yvon Spex Fluoromax3) using a dye-quencher pair. Note that if the structure in Figure 16 is considered an individual tile with four “sticky arms,” then the experiment monitors the inter-tile interaction, not the individual intra-tile melting. The melting temperature was $T_m = 44^\circ C$, which is significantly lower than the sheet/linker or anchor/linker melting temperature of $T_m = 56^\circ C$, as designed.

Thermally oxidized silicon substrates were patterned with PDT as described in the previous section, using the stamp shown in Figure 12A. After backfilling with the thiolated anchor/linker duplex (1 µM in 0.1 M bicarbonate buffer with 50 mM NaCl), excess DNA was removed by successfully washing the substrate with 0.5X SSC with 0.1% SDS, 0.01X SSC, and then dH$_2$O. Pre-assembled sheets of tiles (1 µM of each strand in TAE-Mg$^{2+}$ buffer) were hybridized to the surface overnight in a Secure-seal chamber (Invitrogen), and then washed as described above. The immobilized sheets were annealed at $37^\circ C$ for 15 minutes, and washed as described above.

Figure 18A shows a fluorescence micrograph of a post-annealed substrate, in which one of the DNA strands was labeled with cy3 as shown in Figure 16. While this demonstrates that DNA nanostructures could successfully be patterned at the microscale, it was odd that pre- and post-annealed fluorescence images were nearly identical. AFM images of pre-annealed substrates (Figure 18B) showed that the portions that should have spread beyond the edges of the pattern folded over themselves to remain within the patterning boundaries, presumably because of hydrophobic interactions with the PDT-passivated background. However, the “blobs” at the edges were removed during the annealing step (Figure 18C).

It is unclear whether the structures are “single-crystalline” (i.e. fully contiguous). Hairpins in this design should be spaced by ~16 nm, but they have not been resolved by AFM to this point. AFM imaging in this system is extremely difficult because the multi-
layered structure is far more complex than typical imaging of adsorbed DNA sheets on mica, and the resolution of the available AFM scanners was largely limiting. Successful ligation and cleavage reactions from the patterned surface have yet to be verified. Released structures could not be conclusively found by fluorescence microscopy after depositing the supernatant onto mica, although this does not automatically mean that process itself was unsuccessful. For example, the sheets could have folded over upon themselves, creating indistinguishable and trivial objects. Nevertheless, the surface patterning result alone offers extremely promising evidence that the proposed route could help solve the “chip-to-world” problem when using DNA nanotechnologies to build functional devices (e.g. positioning lithographic shadow masks⁴⁵).

![Image](image.png)

**Figure 18.** Confocal fluorescence micrograph of post-annealed cy3-labeled tiles (scale = 5 \(\mu\)m). (b) AFM of a pre-annealed square pattern. Two of the edges contain large “blobs” which are presumably DNA tiles folded over themselves to avoid hydrophobic interactions with the background. (c) AFM of a post-annealed “L” pattern. (b,c) AFM scan size = 4 \(\mu\)m and vertical scale bar = 8 nm.
Table 1. Silanization recipes for chemically modifying glass and silicon with biologically relevant terminal functionalities

<table>
<thead>
<tr>
<th>Terminal Group</th>
<th>Silane / Compound</th>
<th>Usage</th>
<th>Formation Conditions (sequential solvent washes)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorinated alkane</td>
<td>FOTS</td>
<td>DNA passivation Hydrophobic coating Chemically inert surface</td>
<td>0.5% PFD, 30 min. (hexanes)</td>
<td>PMMA and Novolak compatible.</td>
</tr>
<tr>
<td>Alkane</td>
<td>OTS</td>
<td>DNA passivation Protein / cell adhesion Hydrophobic coating</td>
<td>0.5% CCl₄, 30 min. under N₂ (hexanes)</td>
<td>PMMA compatible. Novolak compatible with hexadecane solvent.</td>
</tr>
<tr>
<td>PEG</td>
<td>(a) m-PEG-silane (b) m-PEG-amine grafted to epoxide</td>
<td>Protein / cell anti-fouling Hydrophilic coating</td>
<td>(a) 0.5% THF, 30 min. (THF, dH₂O) (b) 10mM dH₂O grafted on GPS (dH₂O)</td>
<td>(a) Aqueous reactions possible. PEG degrades resists. (b) Grafting density dependent on soaking time</td>
</tr>
<tr>
<td>Amine</td>
<td>APTES</td>
<td>Cationic DNA adhesion Nucleophile</td>
<td>(a) 0.05% dH₂O, 10 min. (IPA) (b) 0.5 % acidic MeOH, 30 min. (acetone, IPA)</td>
<td>(a) PMMA and Novolak compatible. (b) CO₂ snow clean for monolayer</td>
</tr>
<tr>
<td>Epoxide</td>
<td>GPS</td>
<td>Amine- and thiol- reactive</td>
<td>1% acetone, 30 min. (acetone)</td>
<td>Usable as adhesion layer for microcontact printing thiols</td>
</tr>
<tr>
<td>Carbamate</td>
<td>(a) fmoc-Cl (b) t-boc anhydride</td>
<td>Amine protecting group</td>
<td>(a, b) 50 mM CCl₄, 30 min. (hexanes)</td>
<td>(a, b) PMMA compatible. Novolak resists may require cyclohexane as the solvent.</td>
</tr>
<tr>
<td>Isothiocyanate</td>
<td>PDC</td>
<td>Convert amine to amine-and thiol-reactive surface</td>
<td>50 mM 10:90 pyridine: DMF, 10 min. (DMF)</td>
<td>Aqueous reactions possible</td>
</tr>
<tr>
<td>Biotin</td>
<td>Biotin-NHS</td>
<td>Streptavidin binding</td>
<td>10 mM in DMF, 10 min. (DMF, dH₂O)</td>
<td>Aqueous reactions possible</td>
</tr>
</tbody>
</table>

List of Abbreviations: FOTS = (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, PFD = perfluoromethyldecyl, OTS = Octadecyltrichlorosilane, m-PEG-silane = methoxy-(polyethyleneoxy)₆-9-trichlorosilane, APTES = 3-(aminopropyl)triethoxysilane, GPS = 3-(glycidoxypropyl)trimethoxysilane, PDC = 1,4-phenylisothiocyanate, THF = tetrahydrofuran, dH₂O = Millipore water, DMF = dimethylformamide, IPA = isopropanol, acidic MeOH = 95% methanol : 5% 1mM aqueous acetic acid, NHS = N-hydroxyl succinimide, fmoc = fluorenylmethoxy carbonyl, t-boc = tert-butyloxy carbonyl.
5.8 References


Chapter 6

Concluding remarks

6.1 Summary of results

Chapter 1 introduced the concept of DNA microarrays as “ultra-high density plates” (UHDP), as a paradigm shift that would diminish the cost of genomic research by drastically lowering the cost per base and the time required to synthesize complex DNA pools. An integration roadmap for de novo gene synthesis was presented, in which these complex pools are cleaved from DNA microarrays and assembled into genes in a microfluidic environment. A novel DNA microarray synthesis platform based on semiconductor photoelectrochemistry (PEC) was then proposed.

Chapter 2 began with a discussion on the behavior of semiconductor electrodes at the semiconductor-electrolyte interface (SEI). The amorphous silicon photoconductor was cheaply microfabricated in one unaligned mask step, which was made possible by a self-aligned oxidation procedure that leveraged the chemical inertness of the platinum pads. After significant solid-state and PEC analysis, the substrate was determined to be “Fermi-pinned” at the SEI, a case in which the semiconductor band bending is unaffected by the Fermi energy of the electrolytic redox pair. The photosensitivity and photopotential were linear and log-linear, respectively, with the illumination intensity. Two empirical requirements for the success of the PEC synthesis platform (Chapter 3) were determined. First, the film resistivity when illuminated must be relatively low ($R < \sim 10^5 \Omega \cdot cm$) at the applied bias, and second, the surface defect density must not be large enough to impede the PEC reaction by creating barriers to hole-injection into solution.

Chapter 3 began with a detailed description of microarray platforms that rely on electrochemically generated acids, and why scavengers employed in these systems to
limit proton diffusion were eliminated from the PEC deprotection cocktail. The PEC platform was constructed using a digital micromirror device (DMD) as a spatially modulated light source / optical projection system. It was particularly low-cost compared to other optical projection systems because it used visible optics instead of more costly ultraviolet ones, which was possible because amorphous silicon can be photo-excited at all visible wavelengths. PEC analysis verified the gain mechanisms for increased proton generation upon illumination as proposed in Chapter 1.

Site-selective PEC deprotection was studied by fluorescence imaging of cy3-phosphoramidite coupled to the surface. Control experiments proved that the mechanism of PEC deprotection was indeed the acid-catalyzed cleavage of dimethoxytrityl (DMT) groups by PEC-generated protons. The PEC system boasts the fastest deprotection times reported to date by over an order of magnitude, as well as optical non-linearity with respect to illumination profile that relaxes the needs for stringent substrate alignment. Successful microarray synthesis was demonstrated by first synthesizing a pair of probes with symmetric mutations (AT or TA in the middle two base positions of a 12mer), and then simultaneously hybridizing the complementary set of dye-labeled target strands; each target had one perfect match and one mismatch probe (both mismatch combinations contained A-A and T-T mismatches). Single-base deletion errors in a microarray of 16mers were also detected by hybridization assays. An initial estimate of the step-wise synthesis yield was 93%, but this value varied with the lifetime of the phosphoramidite reagents.

Chapter 4 covered the preparation and characterization of the thin-film porous glass that coats the substrate. The film was easily prepared in a single spin-coating step with tunable thickness from 70 nm – 1.4 μm. It was optically transparent with better than 98% transmission across the UV-Vis spectrum, and sufficiently porous for electrochemical sensing and PEC synthesis. The chemical stability towards
concentrated acids and bases allowed the substrates to be reused, which amortizes the already low microfabrication cost. Most importantly, the large internal surface area of the film drastically increased the synthesis capacity per unit chip area by 15X per micron film thickness, which effectively lowers the cost per base synthesized on microarrays by eliminating the spot redundancy required to obtain biologically relevant solution concentrations of cleaved DNA. Film metrology by atomic force and scanning electron microscopy showed that the film owed its chemical stability and large porosity to the anisotropic geometry of the colloidal precursors.

Chapter 5 discusses the many advances in surface science that were developed in parallel to the PEC microarray synthesis platform.

(1) In order to improve the fidelity of surface silanization procedures used to chemically modify silicon and glass, a post-formation cleaning procedure by CO2 snow treatment selectively removed spurious multi-layers, thereby allowing atomically flat self-assembled monolayers (SAMs) to be recovered from imperfect films.

(2) Studies in surface silanizations on indium tin oxide (ITO) showed that trialkoxysilanes should be used because the silanol-intermediate formation creates benign alcohols, as opposed to trichlorosilanes that etch ITO by liberating HCl. This finding should improve the quality of modifying ITO surfaces for electrochemical sensors and display technologies.

(3) A suite of silanization conditions, many of which were compatible with lithographic resists, was presented that encompassed most of the useful terminal functionalities for biological research on surfaces, including long-chain alkanes, fluoroalkanes, polyethylene glycol, primary amines, and reactive electrophiles.

(4) Resist compatible surface silanizations (Item 3) and protecting group chemistry were combined to create a simple and general strategy for patterning
biomolecules and creating binary chemical patterns at all length scales. Nanoscale arrays of DNA and streptavidin were generated by this strategy.

(5) A new technique to directly pattern electrical insulators at the nanoscale by electron beam lithography, called Critical Energy Electron Beam Lithography (CEEBL), minimized surface charging and the resultant pattern distortion from charge-induced beam deflection. This new technique does away with all expensive microfabrication tools and processing steps typically required to dissipate charge when e-beam patterning insulators.

(6) A new thermo-compression technique allowed “fresh” ultra-flat gold surfaces to be prepared on demand by template stripping. A thin film of gold-on-mica was transferred by diffusion bonding to a gold-on-glass slide; since the freshly exposed surface was in intimate contact with atomically flat mica, it is ultra-flat. The ultra-flat surfaces have the same chemical stability as normal gold-on-glass because diffusion bonding eliminates all polymeric fasteners.

(7) Alkanethiols were microcontact printed on glass and silicon using an epoxide adhesion layer. The technique replaces the difficulty of printing silanes with the simplicity of a well-characterized process of printing thiols. Because the printing step was so quick, complex DNA patterns were created by backfilling the printed substrate before the epoxide molecules reacted with air.

(8) A scheme for creating self-replicating two-dimensional polymers was proposed. As a first step towards realizing the system, DNA nanostructures were patterned at the micron-scale by hybridizing a pre-assembled sheet to a patterned surface, and then annealing off the edges outside of the patterning boundaries. This hybridization-based immobilization scheme may solve the “chip-to-world” problem for building devices out of DNA nanotechnologies.
6.2 Conclusions

While biomolecule manipulation, sensing, and stimulation based on photoelectrochemistry (PEC) have already been reported in the literature, this thesis clearly demonstrates that PEC is also a powerful tool in chemical synthesis. The PEC microarray synthesis is perhaps the cheapest high-resolution platform that can be constructed. Between the use of visible wavelength optics and standard DMT-protected reagents, it is cheaper than other optical platforms; by virtually addressing the substrate, it is also cheaper than electrochemical platforms. The large concentration of DNA synthesized per spot possibly represents further savings in price per base.

The platform holds great promise because many logical extensions to the system, many of which are discussed at the end of this chapter, have yet to be explored. For example, considering that electrochemistry is capable of generating acids, bases, and radicals, in addition to changing the oxidation state of metal ions, the PEC platform is a highly general combinatorial chemistry platform. While this thesis has focused on DNA microarrays based on our interests in gene synthesis, the principles of combinatorial DNA, RNA, peptide, carbohydrate, and small molecule synthesis are all the same. Can a truly general synthesis machine be constructed? To take things one step further, could a fully integrated synthesis, manipulation, sensing, and stimulation system be constructed, all of which would be based on PEC?

Moving forward, the main issue that must be resolved for the PEC platform is yield. The initial step-wise yield tests and hybridization assays were promising for a first report. More optimization studied by simple, fast, and cheap fluorescence-based experiments is required in the near term, but ultimately, the DNA must be cleaved and sequenced in order to gauge the long-term prospects for the PEC platform as an ultra-high density plate. Some suggestions and considerations for system optimization are provided in the next section, most of which focus on substrate microfabrication because
the photoconductor is the one truly unique component of the PEC platform. As mentioned before, the notion of “yield” must be defined with respect to application, and as such, the optimization conditions will vary significantly between applications.

The PEC system was designed with applications like gene synthesis in mind, and the early experimental results reported here indicate that the platform is far better suited for such purposes than hybridization assays for several reasons. While the porous glass offers significant benefits for non-analytical applications, it increases background fluorescence for hybridization assays. Proton diffusion is possibly less of a concern for gene synthesis than it is for hybridization analysis because one does not need to quantify fluorescence on-chip in gene synthesis and other non-analytical applications. Deprotection speed is not an issue for the PEC platform, as it already bests all other platforms by over an order of magnitude. Given the speed of the system and its low costs, the outlook is bright for a photoelectrochemical DNA microarray synthesis platform to become a viable source of ultra-high density plates.

6.3 Suggestions for improvement

This section offers some suggestions for improvement, or rather avenues of study that could potentially lead to improvements, since much is still unknown.

6.3.1 Semiconductor film

Changes to the photoconductor must strike a balance between improved performance and the increased cost that may be incurred. As the substrate design increases in complexity, so does the cost of fabrication. The Schottky diode configuration used in this thesis could be quickly and cheaply produced, but the yield was low because of the poor reliability of the amorphous silicon deposition. The structure could be fabricated even more quickly and cheaply if titanium dioxide (TiO₂) or
zinc oxide (ZnO) were used instead. Oxidation would no longer be a concern, and thus the platinum pads could be eliminated entirely, if one is willing to accept the loss of optical non-linearity. On the other hand, their bandgaps ($E_g = 3.2\text{eV}$) are in the ultraviolet, and thus, such a switch would increase the cost of the projection system. A compromise between the oxidation issue and hardware cost can be met by depositing a very thin semiconductor oxide on top of a small-bandgap semiconductor that can be excited at visible wavelengths, like amorphous silicon, cadmium selenide, or gallium arsenide.

TiO$_2$ and ZnO films could easily be formed by a variety of solution-based processes like nanoparticle deposition and sintering,$^1$ sol-gel methods,$^2$ and solvothermal synthesis.$^3$ These approaches could drastically decrease the processing costs, as can be seen in photovoltaics research.$^4$ The general use of nanostructured materials is interesting because they have many physical properties that can be exploited. Their light absorption efficiencies are quite high. Given their large surface area, they could act as both the photoactive material and the high-loading solid support (given a long enough linker molecule to prevent direct electrochemical damage). The large surface area also enables the energy levels of nanostructured materials to be modified by varying the electrolyte composition,$^5$ although this feature could also have negative effects. It is conceivable that no external bias would be required, since photovoltages as high as $+0.9\text{V}$ have been reported in the literature for nanoparticulate TiO$_2$ films.$^4$ On the other hand, many nanostructured materials are plagued by oxidation and large defect densities, and steps must be taken to ensure that the photo-generated carriers participate in the electrochemical reaction of interest, rather than recombining to emit light.
6.3.2 Photoconductor design

Another possible low-cost structure would be an “n-i-p” (n-type – insulator – p-type) diode. Even though it has more material layers than a Schottky diode, it could still be fabricated with the same number of mask steps because no patterning would be required to make the n-i-p psandwich. These structures have much larger photosensitivities than Schottky diodes, although they produce smaller current densities. The use of an in-plane counter electrode similar to the one used in the Combimatrix system would increase the flexibility in fluidic design by eliminating the ITO counter electrode from the cell. If a semiconductor oxide like TiO$_2$ or ZnO were used as the photoactive film or protective coating, a substrate with an in-plane counter electrode could be fabricated with no alignment steps (Figure 1).

![Figure 1. Self-aligned fabrication of semiconductor oxide photoconductor with an in-plane counter electrode that is electrically isolated from the anode.](image)

While increasing the number of alignment steps in any fabrication process is undesirable, fiducial markers that are etched on the backside of the substrate should be considered. This would increase the flexibility in fluidic design by eliminating the need for an optical transmission window in the fluidic for alignment; a beam-splitter could be used instead. These alignment marks would also be useful if the substrate were to be integrated with a microfluidic after synthesis, or handled by an automated robotic arm that relies on machine vision.
6.3.3 Enclosed low-volume fluidic

The incorporation of an enclosed low-volume fluidic has many downsides. It increases the cost of the substrate fabrication, reduces the number of spots by taking up space on the chip, and complicates fluorescence imaging using high-resolution objectives with short working distances that are often only the thickness of a coverslip. However, for microarrays used as ultra-high density plates, the benefits likely outweigh the downsides. Gao and co-workers have shown that a hermetically sealed fluidic improves the step-wise synthesis yield by improving mass transport and decreasing the exposure to moisture in the air. An enclosed fluidic drastically decreases the reagent consumption, which in addition to the cost savings, also probably improves the chemical synthesis yield because it is easier to purge small fluid volumes and containers of air and water than larger ones. Multiple reaction chambers like the ones used in the Febit platform, allows several different experiments to be run simultaneously.

![Optical micrograph of a PDMS gene synthesis microfluidic device sealed to a microscope slide coated with the thin-film porous glass. The channels are filled with dye-colored water for visualization purposes. Image courtesy of David Kong, MIT.](image)

The incorporation of active microfluidic valves would be a powerful innovation because it would allow a single chip to be divided into separate regions whose syntheses are optimized for completely different applications of interest (e.g. expression profiling and gene synthesis). Figure 2 shows an optical image of a PDMS microfluidic device that is bonded to a microscope slide coated with the porous glass reported in this
thesis. The channels are filled with dye-colored water for visualization purposes to show that the device is leak-free. This particular prototype is quite simple, but it does contain valving; the red channels to the right/left that open/close the blue channels that they intersect. This device was designed as a gene assembly device that would be sealed after microarray synthesis, but could be used during the synthesis process if it were composed of organic solvent-resistant elastomers.\textsuperscript{10}

6.3.4 Thin-film porous glass

There are three issues with the thin-film porous glass that must be resolved, the first of which is the presence of defects like pinholes and scratches. Fortunately, these defects can easily be avoided by taking more precautions during film preparation (e.g. colloid size filtration to prevent pinholes) and handling (e.g. soft Teflon tweezers to prevent scratching). The second concern is the film “lift-off” directly over the pad that was occasionally observed, presumably because the colloidal silica interface with the platinum electrode was ruptured. The use of a dilute sol-gel binder would likely improve the adhesion between them.

While the porosity of the film is sufficient for synthesizing short hybridization probes, the pore size must increase in order to synthesize longer strands useful for gene assembly (typically 60 bases long or greater). Normal controlled-pore glass (CPG) syntheses require 100 nm pore sizes for efficient synthesis of DNA greater than 60 bases long. The use of larger colloidal precursors and/or surfactants may create larger interstices. Other approaches to creating large pore matrices should also be considered, such as chemical or anodic etching, which offered promising albeit insufficient results initially,\textsuperscript{11} or the further miniaturization of plate technology (i.e. CPG beads in wells) as currently used in high-throughput pyro-sequencing.\textsuperscript{12}
6.3.5 Hardware

The first and foremost hardware improvement required is the construction of a new digital micromirror device-based (DMD) optical projection system. Because light reflected off of a DMD is fairly well collimated within several inches of the device, a commercially available lens relay (e.g. matched achromatic pairs) or simple Galilean telescope architecture should suffice as the focusing optics. These options are simpler and cheaper to construct than Offner relays using front-surface mirrors, and offer more control over the magnification. High-power light-emitting diode arrays are good low-cost options for both broadband and monochromatic visible wavelength illumination sources. The degree of alignment required for the next generation of hardware will largely depend on whether the substrate includes metal pads or not (i.e. optical non-linearity). A custom reagent manifold should be built in order to free oneself of the constraints of the commercial synthesizer, and minimize the footpath of the system.

6.3.6 Chemistry and synthesis cycle

A path towards optimizing microarray synthesis for gene synthesis and other cleavage applications has been laid out by Cerrina and co-workers, as well as Gao and co-workers. It is unclear whether adding buffers and scavengers to the deprotection cocktail is worth the complication and reagent cost. The synthesizer should be enclosed in an environment purged of air, such as a glove box (or nitrogen dry box with dessicant as a cheaper option), because the reagents are all moisture sensitive. Likewise, the use of molecular sieves in solution should further keep reagents dry.

Changing the phosphoramidite reagents has not been reported in the microarray literature as a route towards improving yields, but should be considered. One possible change to the microarray chemistry is the use of phosphoramidites whose 5'-OH groups are deprotected using peroxy-anions, as reported by Caruthers and co-workers. The
reported peroxy-anion deprotection also oxidizes the amidite, thereby eliminating the oxidation step from the synthesis cycle. This synthesis procedure reduces depurination errors because the deprotection step is basic, but the solution is buffered such that both the succinimidyld linkage to the surface and cyanoethyl protecting groups on the phosphite triesters are left in tact. Because the exocyclic amines are protected with acid-labile DMT groups and the cyanoethyl groups are far more labile than the succinimidyld linker, the microarray could be used for hybridization experiments before cleaving the standard succinimidyld linkers to release the strands into solution. Photoelectrochemistry could be used to created peroxide anions by reducing peroxides and hydroperoxides.\textsuperscript{17, 18}

6.4 Future applications

This section does not discuss applications of microarrays in general, but rather discusses a few future applications that the photoelectrochemical synthesis platform well lends itself to. It should be noted that the photoconductor as it is currently designed might not necessarily accommodate all of these applications.

6.4.1 Biomolecule sensing, manipulation, and stimulation

Several reports in the literature have demonstrated that photoelectrochemistry is a powerful tool in biological research. Light-addressable sensing applications can take on many configurations. DNA hybridization could be monitored by binding a horse radish peroxidase (HRP)\textsuperscript{19} - streptavidin conjugate to a biotinylated target, and then using the virtual electrode to measure the electrochemical activity of the HRP at each spot. Label-free detection has also been demonstrated, where hybridization or adsorption to a semiconductor electrode alters its photocurrent,\textsuperscript{20, 21} Dye-sensitized schemes have been reported in which a fluorescent dye injects electrons into the
semiconductor;\textsuperscript{22, 23} this approach allows the use of multiple colors of activation since the dye is excited, not the semiconductor with broadband absorption. These label-free and dye-sensitized mechanisms are not possible by normal electrochemical sensing using metal electrodes.

Opto-electrowetting and electrophoretic manipulation are other common PEC applications\textsuperscript{24-27} that can potentially be performed with this platform. This feature could be useful for increasing the hybridization rates by actively directing molecular transport towards the microarray spots, and also for improving the stringency of the target-probe interaction.\textsuperscript{27, 28} In addition to manipulation and sensing, PEC has been used to stimulate neurons in electrophysiological studies,\textsuperscript{29, 30} the ability to inject current or apply a voltage bias may be a useful tool in biophysical studies. PEC has an advantage over standard electrochemistry in that the sensing, manipulation, and stimulation can be spatially continuous rather than pixilated or discrete. There are gap regions that a metal electrode cannot address whereas an unpatterned photoconductor can be addressed everywhere along the substrate if the spatially modulated light source permits it.

It should be noted that many of these PEC applications work best when the materials are layered nanostructures because of their large surface area and the ability to tune the film properties by stacking materials.\textsuperscript{21, 23, 29} Therefore, solution-based processing may ultimately be the desired fabrication route of choice considering the additional benefit of decreasing the substrate production costs.

6.4.2 "Leapfrog" gene synthesis on microarrays

One example of an application for an integrated microarray synthesis and manipulation platform is a step-wise gene assembly scheme, which we have termed "Leapfrog" gene synthesis (Figure 3). The desired gene sequence is parsed into short fragments that are synthesized on a microarray in an overlapping manner. The first spot
contains segments A' + B' (synthesized 3' → 5' in the figure); the adjacent second spot contains segments B' + C', and so on. Primer A is hybridized to the first spot, and extended by polymerase. The newly formed A + B strand is released from the substrate and hybridized to the second spot. The extension – release – hybridization cycle is repeated until the full-length fragment is obtained.

Figure 3. "Leapfrog" gene synthesis. The desired gene sequence is parsed into short fragments that are synthesized on a microarray in an overlapping manner. Primer A is hybridized to the first spot, and extended by polymerase. The newly formed A + B strand is released from the substrate and hybridized to the second spot. The extension – release – hybridization cycle is repeated until the full-length single-strand product is obtained, which can then be PCR amplified.

The scheme has been termed "Leapfrog" because the growing strand essentially "leaps" between adjacent spots during each cycle. The directed hybridization / leaping
step is when the ability to PEC-manipulate biomolecules becomes especially important, because in the absence of directed transport or selective hybridization, most of the strands will be lost to bulk solution by isotropic diffusion. This type of PEC-enabled synthesis scheme allows single-strand DNA to be synthesized and released into solution without its complement, which is not possible by typical polymerase- and ligase-mediated assembly schemes that result in double-stranded DNA. The ability to produce long single-strands is useful for “DNA origami” and virus synthesis/studies. The scheme also enables some error-checking since the assembly reaction can conceivably be monitored real-time by fluorescence or photoelectrochemical sensing.

6.4.3 General synthesis platform

The principles behind DNA microarray synthesis are essentially the same for any in-situ combinatorial synthesis. In fact, DNA microarray technologies have been used to synthesize other bio-polymer arrays, most commonly peptide microarrays. Electrochemistry is capable of driving many reactions useful for combinatorial chemistry: direct electrochemical removal, the generation of acids and bases, and changing the oxidation state of metal compounds. An interesting class of chemical compounds to attempt to synthesize with the PEC platform would be “click” compounds. “Click chemistry” is a nature-inspired philosophy proposed by Sharpless and co-workers, in which small subunits are “clicked” together using a simplified set of reactions, much like bio-polymer synthesis.

6.5 Final remarks

Throughout the course of this thesis work, I have been fortunate to work on many great research projects that all have sought to answer good questions. Is there a new fabrication paradigm better than the existing one? Is there a simple and elegant
solution to a needlessly difficult problem? What technological innovations exist at the nexus of chemistry, physics, materials science, biology, and engineering? The thesis began with a proposed paradigm shift: using microarrays as ultra-high density plates to reduce the costs of genomics research. Whether it would be feasible was a largely unanswered question at the onset of this thesis work, but the numerous exciting reports since then on gene synthesis, library construction, and genomic selection have proven that the notion is both realistic and powerful.
6.6 References


Appendix A: Cost analysis

Consumables

The substrates are treated as consumables, but in reality they are quasi-capital costs because they are highly reusable. The fabrication costs reported below are for EML substrates and are unrealistically high because of the small chamber size of the PECVD and e-beam tools. Chemical costs reflect the usage at the concentrations reported in this thesis for a chip that is synthesizing 20mers based on a 0.2 μmol cycle volume. Prices do not reflect the academic discounts from Glen Research and other vendors for synthesis reagents. Reagent costs will scale with fluidic volume, and thus could be drastically reduced by bonded microfabricated structures. Costs are considered negligible if they are under $0.10.

<table>
<thead>
<tr>
<th>Step</th>
<th>Item</th>
<th>Price per chip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabrication</td>
<td>ITO (25mm x 50mm x 0.7mm) – purchased surplus from Delta</td>
<td>$3.25</td>
</tr>
<tr>
<td></td>
<td>PECVD (8 hours, including pre- and post-cleaning)</td>
<td>$14.93</td>
</tr>
<tr>
<td></td>
<td>Photoresist processing</td>
<td>$1.87</td>
</tr>
<tr>
<td></td>
<td>Titanium / platinum e-beam deposition</td>
<td>$21</td>
</tr>
<tr>
<td></td>
<td>Snowtex for porous oxide</td>
<td>Negligible</td>
</tr>
<tr>
<td>Modification</td>
<td>Silane and ethanol solvent</td>
<td>Negligible</td>
</tr>
<tr>
<td></td>
<td>Dimethoxy-trityl chloride</td>
<td>$0.36</td>
</tr>
<tr>
<td></td>
<td>Triethylamine</td>
<td>Negligible</td>
</tr>
<tr>
<td></td>
<td>Anhydrous pyridine solvent for tritylation</td>
<td>$0.56</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Phosphoramidites</td>
<td>$36.70</td>
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<td></td>
<td>Other phosphoramidite reagents</td>
<td>$46.00</td>
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<tr>
<td>Total</td>
<td></td>
<td>$124.67</td>
</tr>
</tbody>
</table>

Hardware

All prices are list prices found online for new items. Items that were actually purchased are in gray. The hardware cost number is the additional cost to convert a commercial synthesizer (with a computer) into a micorarray synthesis platform for this particular embodiment. Future systems that employ the DMD chipset from Texas Instruments and automated stage movement will have much different components.
Small items like screws, BNC cables, etc. are not included. Costs are considered negligible if they are under $0.10.

<table>
<thead>
<tr>
<th>Sub-system</th>
<th>Item</th>
<th>Use</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens train</td>
<td>Optoma EP719</td>
<td>DMD / DLP</td>
<td>$679</td>
</tr>
<tr>
<td></td>
<td>Large triangle brackets (2)</td>
<td>Mount DLP</td>
<td>$46</td>
</tr>
<tr>
<td>Breadboard</td>
<td></td>
<td></td>
<td>$985</td>
</tr>
<tr>
<td>Lab jack</td>
<td></td>
<td>Lens train height shift</td>
<td>$375</td>
</tr>
<tr>
<td>1&quot; posts (2)</td>
<td></td>
<td>Mount lens train</td>
<td>$43</td>
</tr>
<tr>
<td>Optics track</td>
<td></td>
<td>Mount lens train</td>
<td>$229.33</td>
</tr>
<tr>
<td>Micrometer</td>
<td></td>
<td>y-axis lens shift</td>
<td>$261.75</td>
</tr>
<tr>
<td>Rotation stages (2)</td>
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<td>DLP tilt and lens train angular shift</td>
<td>$614</td>
</tr>
<tr>
<td>Cage rods (6')</td>
<td></td>
<td></td>
<td>$59.70</td>
</tr>
<tr>
<td>2&quot; lens tube and cage plates (2)</td>
<td></td>
<td></td>
<td>$117.60</td>
</tr>
<tr>
<td>1&quot; lens tube</td>
<td></td>
<td></td>
<td>$12.80</td>
</tr>
<tr>
<td>2&quot; to 1&quot; cage adapters</td>
<td></td>
<td>For 1&quot; lens holder, iris, and camera</td>
<td>$72</td>
</tr>
<tr>
<td>Fine z-translation mounts (2)</td>
<td></td>
<td>1&quot; lens and camera fine focusing</td>
<td>$344</td>
</tr>
<tr>
<td>2&quot; achromats (2)</td>
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<td>Collection and relay lenses</td>
<td>$238</td>
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<td>1&quot; achromat</td>
<td></td>
<td>Focusing lens</td>
<td>$75</td>
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<tr>
<td>Iris</td>
<td></td>
<td>Limit coma</td>
<td>$57</td>
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<tr>
<td>ND 3.0 filter</td>
<td></td>
<td>Lower light intensity to camera</td>
<td>$52</td>
</tr>
<tr>
<td>TV (Sony PVM-1390)</td>
<td></td>
<td>Alignment</td>
<td>$900</td>
</tr>
<tr>
<td>CCD (Sony XC-ES50)</td>
<td></td>
<td>Alignment</td>
<td>$633</td>
</tr>
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<td>CCD power supply</td>
<td></td>
<td></td>
<td>$255</td>
</tr>
<tr>
<td>Uniblitz shutter and drive unit</td>
<td></td>
<td>Baffle light</td>
<td>$2125</td>
</tr>
<tr>
<td>Infinitube and objectives</td>
<td></td>
<td>Long working distance imaging</td>
<td>$1185</td>
</tr>
<tr>
<td>Automation</td>
<td>Mosfet switch</td>
<td>Regulate power supply to fluidic</td>
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</tr>
<tr>
<td></td>
<td>Python software</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Fluidic</td>
<td>Power supply (Agilent 3631A)</td>
<td>Apply bias</td>
<td>$985</td>
</tr>
<tr>
<td></td>
<td>1.1mm thick ITO (25 x 50mm)</td>
<td>Counter electrode</td>
<td>$10</td>
</tr>
<tr>
<td></td>
<td>Nanoport HPLC fittings (2)</td>
<td>Adapter to synthesizer</td>
<td>$35.70</td>
</tr>
<tr>
<td></td>
<td>Kalrez o-ring</td>
<td></td>
<td>$23.80</td>
</tr>
<tr>
<td>Acrylic</td>
<td></td>
<td>Compression to seal fluidic</td>
<td>$5</td>
</tr>
<tr>
<td>14mil Viton sheet</td>
<td></td>
<td>Spacer to position o-ring</td>
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</tr>
<tr>
<td>Micrometers (3)</td>
<td></td>
<td>Translational alignment</td>
<td>$785.25</td>
</tr>
<tr>
<td>Small rotation stages (2)</td>
<td></td>
<td>Rotational alignment</td>
<td>$176</td>
</tr>
<tr>
<td>Large post and mount</td>
<td></td>
<td>Coarse fluidic positioning</td>
<td>$148</td>
</tr>
<tr>
<td>Substrate</td>
<td>Lithographic mask</td>
<td></td>
<td>$115.50</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>$11,543.43</strong></td>
</tr>
</tbody>
</table>
Appendix B: Fabrication conditions for EML substrates

Table 1. Critical PECVD parameters (250°C) for EML1 and EML2 substrates.

<table>
<thead>
<tr>
<th>Step</th>
<th>Gas</th>
<th>Time</th>
<th>Flow rate (sccm)</th>
<th>Pressure (mtorr)</th>
<th>Power (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean</td>
<td>CF₄/O₂</td>
<td>30 min</td>
<td>150</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td>Defluorination</td>
<td>N₂</td>
<td>3 min</td>
<td>150</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td>Pre deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>10 min</td>
<td>250</td>
<td>700</td>
<td>25</td>
</tr>
<tr>
<td>Substrate insertion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-situ clean</td>
<td>O₂</td>
<td>3 min</td>
<td>25</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>a-Si deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>180 min</td>
<td>250</td>
<td>700</td>
<td>25</td>
</tr>
<tr>
<td>Anneal</td>
<td>N₂</td>
<td>1 hour</td>
<td>0</td>
<td>220</td>
<td>0</td>
</tr>
<tr>
<td>Overnight cool</td>
<td>N₂</td>
<td>8 hours</td>
<td>0</td>
<td>220</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Critical PECVD parameters for EML3 substrates

<table>
<thead>
<tr>
<th>Step</th>
<th>Gas</th>
<th>Time</th>
<th>Flow rate (sccm)</th>
<th>Pressure (mtorr)</th>
<th>Power (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean</td>
<td>CF₄/O₂</td>
<td>60 min</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Defluorination</td>
<td>N₂</td>
<td>10 min</td>
<td>150</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td>Pre deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>20 min</td>
<td>250</td>
<td>900</td>
<td>30</td>
</tr>
<tr>
<td>Substrate insertion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-situ clean</td>
<td>O₂</td>
<td>3 min</td>
<td>25</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>a-Si deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>40 min</td>
<td>250</td>
<td>700</td>
<td>25</td>
</tr>
<tr>
<td>a-Si deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>100 min</td>
<td>250</td>
<td>900</td>
<td>30</td>
</tr>
<tr>
<td>a-Si deposition</td>
<td>5% SiH₄ / 95% He</td>
<td>40 min</td>
<td>250</td>
<td>700</td>
<td>25</td>
</tr>
<tr>
<td>Anneal</td>
<td>N₂</td>
<td>1 hour</td>
<td>0</td>
<td>220</td>
<td>0</td>
</tr>
<tr>
<td>Overnight cool</td>
<td>N₂</td>
<td>8 hours</td>
<td>0</td>
<td>220</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure A1. EML2 film thickness distribution depending on substrate position in chamber as measured by a Dektak profilometer.
**Patterned and metal deposition**

EML3 substrates were patterned using AZ4620 photoresist, and lifted-off by sonication in acetone/NMP. For specific details, see: [Emig, C., M. Eng Thesis, MIT, 2006]. For all other substrates, pads were defined as follows. Prior to patterning, substrates were sonicated in hexanes, acetone, and IPA (1 minute each), and dried under nitrogen. 1 μm thick NR9-1000PY photoresist (Futurrex) was processed according to the manufacturer’s specification using 6 minute bake times to account for the low thermal coefficient of glass. The resist was exposed through chrome-on-glass contact photomasks (Photoplot Store) with a UV floodlamp (Intelli-Ray 400). Metal pads (10 nm / 50 nm Ti/Pt) were e-beam evaporated with a Sloan PAK evaporator (base pressure < 3e-6 torr, 1-2 Å/s rate) and then lifted off with acetone. The substrates were sonicated in acetone for 5 seconds to remove any residual resist, and then washed with IPA to eliminate solvent streaks, and dried under nitrogen.

**Appendix C: Automation software**

This automation program is admittedly very crude / inefficient, and does not contain a graphical user interface. Python was chosen as the scripting language for the simple ability to access both the parallel port and write direct pixel values onto a desktop display. This was my first attempt at automation programming and coding in Python. Manu Prakash provided tremendous help in debugging it.

Future software should be written in Labview for several reasons. First, the commercial Texas Instruments DMD chipset is driven by a NI/DAQ card. Labview also contains several modules for image analysis and automated stage movement for alignment. Labview is also the most common platform in microfluidics for driving solenoid valves, which are the principle component in a DNA synthesizer if one were to
construct a synthesizer from scratch, either out of concerns over cost or control of system functions.

This software creates and displays virtual masks from text files of sequences. This version assumes the use of a shutter to block illumination during the non-detritylation steps. The first base must already be on the substrate and the end detrityl should be on.

import sys, os, pygame, pygame.surfarray, Numeric, parallel
pygame.init()

# SETUP PARMS FOR RUNNING PROGRAM - ONLY THESE SHOULD CHANGE
pygame.display.set_caption("Dose test - Press ESC to exit")
display_size = (800,650) # Display size in pixels
canvas = pygame.image.load("600_black.bmp") # Total image that gets displayed
canvas_size = 600 # Total canvas size in pixels in one dimension
blank = pygame.image.load("600_black.bmp") # Blanking image (i.e. all black canvas)
box_size = 20 # number of pixels per pad in one dimension (Electrode size / DLP pixel size)

file = open('Checkerboard_2.txt', 'r')
repeat_on = True # Concatenate text file if less sequences than spots (True = Yes, False = No)
oligo_length = 2
steps_total = 8 # Should match sequencer steps and <= 4*oligo_length
exposure_time = 21000 # (ms) Must be synthesizer wait > parm > Uniblitz.

# SETUP PARALLEL
# We're only using Pins 2-4 and Pin 2 is always off: "00000xx0"
# Using logic 0 to trigger b/c open circuit pin 13 (in) sees logic 1
# Uniblitz settings - FRONT: EXP=01, N.C., Post / REAR: A-D high, E low
# P3 = bias, P4 = shutter
# None P3 P4 P34
# 0000 0010 0100 0110
# 0x00 0x02 0x04 0x06
p = parallel.Parallel()
p.setData(0x00)

# SETUP IMAGE DISPLAY
os.environ["SDL_VIDEO_WINDOW_POS"] = 'center'
screen = pygame.display.set_mode(display_size)
centered_image = ((display_size[0] - canvas_size)/2, (display_size[1] - canvas_size)/2)
canvas_array = pygame.surfarray.array3d(blank)
### READ INPUT FILES - Creates 1D array of sequences without seq number. Indexed to zero.

```python
curr_file = file.readlines()
L = []  # Empty list of strings
curr_line = 0
read_done = False

while not read_done:
    curr_string = curr_file[curr_line]
    line_done = False
    while not line_done:
        if curr_string == "EOF":  
            read_done = True
            line_done = True
        elif curr_string[0] == ":
            line_done = True
            curr_line = curr_line + 1
        elif True:
            line_done = True
            L.append(curr_string[5:])
            curr_line = curr_line + 1

sequences_total = len(L)  # So pixel writing loop knows when to stop

if repeat_on:
    z = 0
    total_boxes = (canvas_size/(2 * box_size))**2
    while z < (total_boxes/sequences_total):
        L.extend(temp_L)
        z = z + 1
    sequences_total = len(L)
```

Print "file read okay"

### MAIN PROGRAM

```python
n = 0  # row index
m = 0  # column index
nm_limit = canvas_size/(2 * box_size)  # Max numbers of electrodes in one dimension
position = 0  # N-position of the nucleotide minus 1, b/c arrays index at 0
curr_mask = 0  # Index of DMD masks, current step
nucleotide_ID = "A"
x = 0  # Counter for loops
y = 0  # Counter for loops

synthesis_done = False
is_on = False

while not synthesis_done:
    for event in pygame.event.get():  
        if event.type == pygame.KEYDOWN and event.key == pygame.K_ESCAPE:
            pygame.display.quit()
sys.exit()

##### WHEN DETRITYLATION CYCLE STARTS

if p.getlnSelected() == 0:
    pygame.time.wait(100) # Buffer when manually testing trigger
if _is_on == False:
    _is_on = True

print curr_mask # For debugging

##### GENERATE DMD MASKS - Writes pixels in array that can be blitted on screen

canvas_array = pygame.surfarray.array3d(blank)
seq_num = 0 # Sequence/Electrode number, starting at 0

while (seq_num < sequences_total) and (seq_num < nm_limit**2):
    if (position <= oligo_length) and ((L[seq_num][position] == "X") or (L[seq_num][position] == 
                                    nucleotide_ID)):
        m = seq_num/nm_limit # Provides row index of sequence
        n = seq_num - (m*nm_limit) # Provides column index of sequence
        while y < box_size:
            while x < box_size:
                canvas_array[(box_size*2*n)+x,(box_size*2*m)+y] = (255,255,255)
            x = x + 1
            x = 0
            y = y + 1
            y = 0
            seq_num = seq_num + 1
        else:
            seq_num = seq_num + 1

pygame.surfarray.blit_array(canvas, canvas_array)
screen.blit(canvas, centered_image)
pygame.display.flip()
pygame.time.wait(exposure_time)

if nucleotide_ID == "A": nucleotide_ID = "G"
elif nucleotide_ID == "G": nucleotide_ID = "C"
elif nucleotide_ID == "C": nucleotide_ID = "T"
elif nucleotide_ID == "T":
    nucleotide_ID = "A"
    position = position + 1

p.setData(0x06) # Trigger shutter and voltage supply
pygame.time.wait(exposure_time)
p.setData(0x00)

##### WHEN CYCLE DONE

if p.getlnSelected() == 1:
    pygame.time.wait(100) # Buffer when manually testing
if _is_on == True:
    _is_on = False
    curr_mask = curr_mask + 1
if curr_mask >= steps_total: synthesis_done = True

pygame.quit()
Appendix D: Detritylation with Noel substrates

These bright-field fluorescence images of patterned cy3-phosphoramidite were taken after exposing the substrates for 2 minutes at 2 V with 100 mM hydroquinone and 100 mM tetrabutylammonium hexafluorophosphate in acetonitrile. The metal pads are 60 µm in size. The image on the right was supposed to be a checkerboard on a double-pitch, but the uniformity was extremely poor.

Appendix E: Funding and resource acknowledgements

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